MOLECULAR MECHANISMS OF SEX-BASED DIFFERENCES IN METABOLISM

MOLECULAR MECHANISMS OF SEX-BASED DIFFERENCES IN SUBSTRATE UTILIZATION DURING ENDURANCE EXERCISE IN HUMAN SKELETAL MUSCLE

By

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ABSTRACT

Introduction: A higher rate of fat and lower rates of carbohydrate and protein oxidation are often observed during endurance exercise in women vs. men at the same relative workload. 17β-estradiol (E2) supplementation increases the oxidation of lipid, and reduces the oxidation of carbohydrate and amino acids in men. Women have a higher area percentage (%) of type I skeletal muscle fibers as compared with men. Consequently, we hypothesized that (i) sex, menstrual cycle phase and E2 would influence basal and exercise induced mRNA content for genes involved in substrate metabolism in human skeletal muscle; and (ii) sex would influence the basal mRNA content for genes involved in the determination of muscle fiber type. **Methods:** For studies concerning substrate selective utilization, 12 men and 12 women had muscle biopsies taken from their vastus lateralis before and after a 90 min bout of cycle exercise at 65 % VO_{2peak}. In a repeated measures design, men were randomly allocated to both E2 and placebo. Women were studied in the mid-follicular (FOL) and mid-luteal (LUT) phases (~ 2 wk apart). For studies concerning muscle fiber determination, a total of 24 women and 22 men had muscle biopsies taken from their vastus lateralis at rest. Twelve of the women were studied in mid-LUT, and 12 were studied in both the mid-FOL and mid-LUT, phases of the menstrual cycle. mRNA content was determined using TaqMan® real-time RT-PCR. Muscle fiber composition in 12 men and 12 women was determined from ATPase staining. **Results:** Exercise increased ($P < 0.05$) the mRNA content of peroxisome proliferator activated receptor gamma coactivator-1 alpha (PGC-1α) (men, LUT), cytosol fatty acid binding protein (FABPc) (men, FOL, LUT), carntine palmitoyltransferase I (CPTI) (men, LUT), long chain acyl-CoA dehydrogenase (LCAD)

(LUT), hexokinase II (HKII), asparatate aminotransferase (AST) and glucose transporter 4 (GLUT4) (men and women). Women had a higher ($P \leq 0.05$) mRNA content of membrane bound fatty acid transport protein 1 (FATm), FABPc, sterol regulatory element - binding protein 1c (SREBP-1c) and mitochondrial glycerol phosphate acyltransferase (mtGPAT) (FOL, LUT), peroxisome proliferator activated receptor alpha (PPARα) (FOL), CPTI (FOL), LCAD (FOL), trifunctional protein alpha subunit (TFPα) (LUT), HKII (FOL) and branched-chain 2-oxo acid dehydrogenase kinas (BCOADK) (FOL, LUT) pre- and post- exercise, peroxisome proliferator activated receptor delta (PPARδ) (FOL) and GLUT4 (FOL) at rest, and PPARα (LUT) and LCAD (LUT) after exercise, and lower mRNA content for phosphofructokinase (PFK) (LUT) pre- and post-exercise, than men. FOL women had a higher ($P < 0.05$) PPARδ, LCAD and glycogen phosphorylase and lower glycogenin mRNA content pre- and post- exercise, and tended to have higher GLUT4 mRNA content ($P = 0.056$) at rest, vs. LUT women. Post-exercise FABPc mRNA content increased to a greater extent in LUT vs. FOL phase ($P = 0.0001$). E2 increased mRNA content of PPARα, CPTI, SREBP-1c, mtGPAT, LCAD, TFP-α, GLUT4 and GS-1 ($P < 0.05$) in men at rest and during exercise, and PPARδ at rest ($P < 0.05$). E2 attenuated the exercise induced increase in PGC-1α mRNA content ($P < 0.05$). Women had lower mean area of individual fibers for type IIa and type IIx, and a higher area % of type I fibers and lower area % of type II fibers vs. men ($P < 0.05$). Women had higher mRNA content for myosin heavy chain I (MHCI) (FOL, LUT, $P \leq 0.05$) and PPARδ (FOL, $P < 0.05$), and a similar mRNA content of myosin heavy chain IIa (MHCIIa), myosin heavy chain IIx (MHCIIx), peroxisome proliferator activated receptor gamma coactivator-1 alpha (PGC-1α), myostatin and PPARδ (LUT), vs. men at

rest. FOL women had a higher mRNA content of PPARδ than LUT women (P = 0.0007). Menstrual cycle phase did not significantly alter the mRNA content of MHCI, PGC-1α or myostatin. **Conclusions and Significance**: We have comprehensively evaluated the effect of sex, acute endurance exercise, menstrual cycle and E2 on the content of mRNA species involved in substrate turnover in human skeletal muscle and found these changes directionally support a higher fat oxidation and lower protein oxidation in women vs. men. We have also evaluated the sex influences on the mRNA content of the genes involved in muscle fiber determination and found the change in PPARδ mRNA content supports a greater area % of type I fibers in women than men.

Table1. Summary of changes in mRNA expression of genes involved in subtrate metabolism

Exercise Post vs. Pre		**Sex** Female vs. Male		**Menstrual phase** FOL vs. LUT		**Estrogen** E2 vs. Placebo	
Gene	mRNA	Gene	mRNA	Gene	mRNA	Gene	mRNA
PGC-1α	M, LUT↑	PPARα	FOL>M; LUT>M(*)	PPARδ	FOL>LUT	PGC-1α	↓
FABPc	M, FOL, LUT↑	PPARδ	FOL>M at rest	LCAD	FOL>LUT	PPARα	↑
CPTI	M, LUT↑	FATm	F>M	FABPc	LUT>FOL (*)	PPARδ	↑/at rest
LCAD	LUT↑	FABPc	F>M	GLUT4	FOL>LUT Trend/at rest	CPTI	↑
GLUT4	M/F↑	CPTI	FOL>M	Gp	FOL>LUT	LCAD	↑
HK II	M/F↑	LCAD	FOL>M; LUT>M(*)	Glycogenin	LUT>FOL	TFP-α	↑
AST	M/F↑	TFPα	LUT>M			SREBP-1c	↑
		SREBP-1c	F>M			mtGPAT	↑
		mtGPAT	F>M			GLUT4	↑
		GLUT4	FOL>M at rest			GS-1	↑
		HKII	FOL>M				
		PFK	F<M				
		BCOADK	F>M				

Note:↑, increase ($P < 0.05$); >, higher ($P < 0.05$); <, lower ($P < 0.05$);↓, attenuate ($P < 0.05$);*, only after exercise; trend ($0.05 < P < 0.08$); M, men; F, both FOL and LUT; FOL, follicular women; LUT, luteal women.

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ABBREVIATIONS

ADP - adenosine diphosphate

AMP - adenosine monophosphate

AMPK - AMP-activated protein kinase

AST - asparatate aminotransferase

ATP - adenosine triphosphate

β-HAD - β-hydroxyacyl-CoA dehydrogenase

β2-M - β2- microglobulin

BCOAD - branched-chain 2-oxo acid dehydrogenase

BCOADK - branched-chain 2-oxo acid dehydrogenase kinase

3BHQ-1 - 3 - black hole quencher-1

3BHQ-2 - 3 - black hole quencher-2

BMI - body mass index

Ca^{2+}- calcium ion

CaMKIV - calcium/calmodulin-dependent protein kinase IV

CaMK - calcium/calmodulin-dependent protein kinase

CAT - acyl - carnitine translocase

cDNA - complementary deoxyribonucleic acid

CHO - carbohydrate

COX I – cytochrome c oxidase subunit I

COX II – cytochrome c oxidase subunit II

COX IV – cytochrome c oxidase subunit IV

CPTI - carntine palmitoyltransferase I

CPTII - carnitine palmityltransferase II

Depc - diethylpyrocarbonate

DNA - deoxyribonucleic acid

E2 - 17β- estradiol

ERα - estrogen receptor alpha

ERβ - estrogen receptor beta

ERγ - estrogen receptor gamma

ETC - electron transport chain

F-1,6-P- fructose - 1,6 - bisphosphate

F-6-P - fructose - 6 - phosphate;

FABPc - cytosol fatty acid binding protein

FABPpm - plasm membrane fatty acid binding protein

FAM - 6 karboxyfluorescein

FAT/CD36 - Fatty acid translocase

FATm - membrane bound fatty acid transport protein 1

FFM - fat free mass

FFA - free fatty acid

FOL - mid-follicular phase

GLUT4 - glucose transporter 4

GP - glycogen phosphorylase

GS-1- glycogen synthase 1

GSK3α - glycogen synthase kinase 3α

HEX - hexachloro-6-carboxy-fluorescein

HKII - hexokinase II

HSL - hormone sensitive lipase

IMCL - intramyocellular lipid

IMTG - intramuscular triglycerides

LCAD - long chain acyl-CoA dehydrogenase

LPL - lipoprotein lipase

LT - lactate threshold

LUT - mid-luteal phase

mATPase - myofibrillar adenosine triphosphatase

MEF1 - mitochondrial translation elongation factor G

MCK - muscle creatine kinase

MHC - myosin heavy chain

MHCI - myosin heavy chain I

MHCIIa - myosin heavy chain IIa

MHCIIx - myosin heavy chain IIx

mtGPAT - mitochondrial glycerol phosphate acyltransferase

NRF-1 - nuclear respiratory factor-1

OVX - ovarectomized

PDK4 - pyruvate dehydrogenase kinase 4

PFK - phosphofructokinase

PGC-1α - peroxisome proliferator activated receptor gamma coactivator-1 alpha

PI3 - phosphatidylinositol 3

Pi - phosphate group

PL - placebo

PPAR - peroxisome proliferator activated receptor

PPARγ - peroxisome proliferator activated receptor gamma

PPARα - peroxisome proliferator activated receptor alpha

PPARδ - peroxisome proliferator activated receptor delta

Ra - rate of appearance

Rd - rate of disappearance

RER - respiratory exchange ratio

RNA - ribonucleic acid

RNase - ribonucleic acid hydrolyase

rRNA - ribosomal ribonucleic acid

RT-PCR - reverse transcriptase-polymerase chain reaction

SREBP-1c - sterol regulatory element - binding protein 1c

SREBP-2 - sterol regulatory element - binding protein 2

TAMRA - 6-carboxy-tetramethyl-rhodamine

TCA cycle - tricarboxylic acid cycle

TET- tetrachloro - 6 - carboxy - fluorescein

Tfam - mitochondrial transcription factor A

TFPα - trifunctional protein alpha subunit

TG - triglyceride

UCP3 - uncoupling protein 3

UV – ultra-violet light

VLCAD - very long chain acyl - CoA dehydrogenase

LCAD - long chain acyl - CoA dehydrogenase

VO_2max - maxium volume of oxygen consumption

STATEMENT OF CONTRIBUTION

Manuscript 1: The principal investigator of this study was Mark A Tarnopolsky. All data were collected and analyzed at McMaster University, in the laboratories of Mark A Tarnopolsky. Study conception and design was done by Mark A Tarnopolsky and Mazen Hamadeh, and the ethics proposal was written by Mazen J Hamadeh. The subjects were recruited and coordinated by Mazen J Hamadeh. The data collection was coordinated by Mazen J Hamadeh. The planning/design of the specific mRNAs to be analyzed was determined by Minghua Fu and Mark A Tarnopolsky. RNA extraction, RT-PCR primer design and real-time RT-PCR were performed by Minghua Fu. RT-PCR data collection, statistical analysis and interpretation were performed by Minghua Fu. The entire manuscript was written and prepared by Minghua Fu, with help from Mark A Tarnopolsky and Mazen J Hamadeh.

Manuscript 2: Entirely as above.

Manuscript 3: Entirely as above.

Manuscript 4: The principal investigator of this study was Mark A Tarnopolsky. All data were collected and analyzed at McMaster University, in the laboratories of Mark A Tarnopolsky. Study conception and design were done by Mark A Tarnopolsky and Stuart M. Phillips, and the ethics proposal was written by Nobu Yasuda, Elisa Glover, and Mazen J Hamadeh. The subjects were recruited and coordinated by Nobu Yasuda, Elisa Glover, and Mazen J Hamadeh. The data were collected by Nobu Yasuda, Elisa Glover,

Minghua Fu, Mazen J Hamadeh and Mark A Tarnopolsky. RNA extraction and real-time RT-PCR were performed by Minghua Fu. ATPase staining was performed by Michaela Devires. RT-PCR data collection, statistical analysis and interpretation were performed by Minghua Fu. The entire manuscript was written and prepared by Minghua Fu, with help from Mark A Tarnopolsky.

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CHAPTER1: GENERAL LITERATURE REVIEW

(1.0) INTRODUCTION

There are sex-based differences in metabolism that are revealed with exercise characterized by women having a higher rate of fat and lower rates of carbohydrate and amino acid oxidation as compared with men (Braun & Horton, 2001; Devries et al., 2006a; Friedlander et al., 1998; Horton et al., 1998; Knechtle et al., 2004; McKenzie et al., 2000; Phillips et al., 1993; Riddell et al., 2003; Roepstorff et al., 2006c; Roepstorff, Vistisen & Kiens, 2005b; Tarnopolsky et al., 1990; Tarnopolsky & Ruby, 2001). Differences in muscle fiber composition and sex hormones have been suggested as contributors to the sex –based differences. To elucidate the role of 17β-estradiol in the sex differences in substrate metabolism, a wide range of studies have been conducted by altering the concentration of estrogen in the blood by ovariectomization in animals and supplementation of 17β-estradiol to animals or human subjects (Carter et al., 2001a; Devries et al., 2005; Gorski et al., 1976; Hamadeh, Devries & Tarnopolsky, 2005; Hatta et al., 1988; Kendrick & Ellis, 1991; Kendrick et al., 1987; Rooney et al., 1993). There are also several studies comparing fuel metabolism between the menstrual cycle phases as the concentration of estrogen fluctuates during the menstrual cycle (Bailey, Zacher & Mittleman, 2000; Horton et al., 1998). Sex differences in muscle fiber composition have also been studied in human subjects (Brooke & Engel, 1969a; Esbjornsson-Liljedahl et al., 1999; Esbjornsson et al., 1993; Hards et al., 1990; Howel, 1994; Mannion et al., 1997; Miller et al., 1993; Roepstorff et al., 2006c;

Simoneau & Bouchard, 1989; Simoneau et al., 1985; Staron et al., 2000) and animals (Fox et al., 2003; Rosen et al., 2004).

Most of the studies that have evaluated the mechanisms behind sex difference in substrate utilization during exercise have been at the substrate and enzyme activity level. A few studies have measured and reported sex differences at the mRNA level for genes involved in fat metabolism (Binnert et al., 2000; Kiens et al., 2004; Roepstorff et al., 2005a), yet a wide variety of other important proteins involved in substrate selection have not been evaluated. To date, there is very little information regarding the molecular basis of sex difference in muscle fiber composition, menstrual cycle phase effect on substrate utilization or role of estrogen in mediating selective substrate utilization in men at mRNA level. This overall theme of the current thesis was to further elucidate the molecular mechanisms of these physiological processes at the level of mRNA content.

(1.1) Sex-based differences in substrate metabolism

(1.1.1) Evaluation of the indirect colorimetric studies

Indirect calorimetric studies of sex differences in substrate metabolism during exercise at the same relative workload in women and men have shown fairly consistent results with most studies showing that women oxidize more lipid and less carbohydrate (CHO) than men (Devries et al., 2006a; Friedlander et al., 1998; Horton et al., 1998; Knechtle et al., 2004; McKenzie et al., 2000; Phillips et al., 1993; Riddell et al., 2003; Roepstorff et al., 2006c; Tarnopolsky et al., 1990; Tarnopolsky et al., 2006). A few studies (Goedecke et al., 2000; Otis et al., 1997; Roepstorff et al., 2002) reported no sex differences in substrate oxidation between men and women during exercise. Possible

reasons for the discrepancy include a lack of standardization over the phase of the menstrual cycle in which subjects were tested, training history, matching based upon maximal oxygen consumption (VO_2max)/ fat free mass (FFM), lactate threshold (LT) (which appear similar when sexes are matched for training history and VO_2max/FFM) (Phillips et al., 1993; Ruby, Coggan & Zderic, 2002), exercise intensity, and subject nutritional state (Binnert et al., 2000; Goedecke et al., 2000; Tarnopolsky & Ruby, 2001).

In order to detect the sex-based differences, one strategy is to match subjects using the aforementioned criteria and also to select an exercise intensity that provides for a fairly balanced substrate selection and is < LT (Tarnopolsky & Ruby, 2001). In the past two decades, there have been a series of reports that examined substrate metabolism during endurance exercise in males and females where careful consideration was given to the aforementioned matching parameters (Devries et al., 2006b; Friedlander et al., 1998; Horton et al., 1998; Phillips et al., 1993; Roepstorff et al., 2006c; Tarnopolsky et al., 1990). These studies consistently demonstrated that the respiratory exchange ratio was lower for women than men during submaximal endurance exercise (Devries et al., 2006b; Friedlander et al., 1998; Horton et al., 1998; Knechtle et al., 2004; Riddell et al., 2003; Roepstorff et al., 2006c; Tarnopolsky et al., 1990). Longitudinal studies ensure that the training exposure in terms of intensity, mode and duration are identical. To date, there have been several studies that have taken this approach and they consistently showed that women have a lower respiratory exchange ratio during submaximal endurance exercise as compared to men (Carter, Rennie & Tarnopolsky, 2001b; Friedlander et al., 1998; McKenzie et al., 2000). When several exercise sex-based comparative studies were combined as a "meta-analysis", containing 135 females and 162 males, whole-body RER values were still shown to be

lower for females as compared to males during endurance exercise at the same intensity (female RER= 0.869, male RER= 0.900) (Tarnopolsky & Saris, 2001). In a study with 157 men and 143 women, it was found women oxidized more fat than men and showed lower RER during exercise (Venables, Achten & Jeukendrup, 2005). Therefore, the balance of data strongly supports a proportionally 100% higher lipid and 33% lower CHO utilization during endurance exercise in women as compared with men (Tarnopolsky et al., 1990).

(1.1.2) Lipid metabolism

The lower RER in women during endurance exercise indicates a greater lipid utilization (Tarnopolsky & Saris, 2001). Animal studies have implicated the sex-based differences in fat metabolism with female rats showing higher adipose and muscle LPL activity and availability of plasma and muscle fatty acids, as compared with male rats (Ellis et al., 1994; Kendrick & Ellis, 1991; Rooney et al., 1993). Stable isotope infusion studies showed that women had a higher rate of appearance of glycerol during submaximal exercise as compared to men when cycling at 60% of their VO_2 peak (Carter et al., 2001b), suggesting that women have greater lipolysis compared with men. Sympathetic activity (as indicated by circulating epinephrine and norepinephrine concentrations) is higher during exercise at approximately 50% VO2 max in men than in women (Davis et al., 2000). *In situ* microdialysis studies showed that β-adrenergically mediated lipolysis increased and α_2-mediated antilipolysis decreased in abdominal subcutaneous tissue more in women as compared with men (Hellstrom, Blaak & Hagstrom-Toft, 1996), indicating a potential mechanism behind the higher lipolytic response in women during exercise (Davis et al.,

2000). Mittendorfer *et al.*(2002) found that adipose tissue lipolysis during moderate-intensity exercise was higher in women than in men, with greater plasma fatty acid availability leading to a greater rate of FFA uptake and oxidation in women than in men (Mittendorfer et al., 2002). It has also been suggested that a higher IMTG utilization may contribute to the higher fat oxidation seen in women during exercise (Tarnopolsky, 2000). It was reported that there was a higher IMTG content in women with men and women matched for lifestyle habits and insulin sensitivity (Perseghin et al., 2001). A higher content of intramuscular triglycerides (IMTG) was observed and correlated with greater IMTG utilization in women than men (Roepstorff et al., 2006a; Steffensen et al., 2002). Electron microscopy study demonstrated that women had higher mean lipid area density, more IMTG droplets and higher IMTG (Tarnopolsky et al., 2006).

In summary, the balance of the data indicate that women have a higher lipid oxidation during endurance exercise that appears to be predominantly related to a higher IMTG content and utilization and to a greater availability of plasma free fatty acids due to a higher lipolysis in adipose tissues in women.

(1.1.3) Carbohydrate metabolism

The lower RER in women during endurance exercise indicates a lower carbohydrate utilization (Tarnopolsky & Saris, 2001). Animal studies have supported the aforementioned sex differences in CHO metabolism with female rats showing less glycogen depletion and CHO oxidation in skeletal muscle and liver, as compared with male rats (Ellis et al., 1994; Kendrick & Ellis, 1991; Rooney et al., 1993). Furthermore, oophorectomy of female rats reduced CHO oxidation during endurance exercise (Kendrick

et al., 1987). Studies with human subjects showed that during endurance exercise, females use less muscle glycogen. It was reported that vastus lateralis glycogen depletion was less for women who had run for 15.5 km on a trendmill at 65% VO_2 peak as compared with men (Tarnopolsky et al., 1990). Using isotopic tracer methods, calculated glycogen utilization was reported to be lower in women as compared to men during 90 min of endurance exercise at 65% of VO_2max (Carter et al., 2001b). Some studies found that men had higher glucose Ra (Devries et al., 2006b), Rd (Devries et al., 2006b; Friedlander et al., 1998) and metabolic clearance rate (Carter et al., 2001b; Devries et al., 2006b) during exercise as compared with women. Davis *et al.* (2000) used a radioactive glucose tracer method and demonstrated that men and women showed similar rates of glucose appearance, yet total carbohydrate oxidation, and calculated muscle glycogen utilization was greater for the men than the women (Davis et al., 2000). At very high levels of VO_2max, Marliss *et al.* (2000) found that women appeared to have a lower rate of glucose disappearance, leading to a greater postexercise hyperglycemia and hyperinsulinemia. Romijn and colleagues (2000) calculated glycogen use at 65% VO_2max from rate of glucose Rd and total carbohydrate oxidation, and found that muscle glycogen represented 34% of total energy for women and 41% for men, though the difference was not significant. However, the comparison in the latter study used data in women compared to retrospective data collected in men from a study reported eight years prior. Biochemical analysis of muscle glycogen showed that no sex difference in muscle glycogen content at rest, while it depleted 25% more in men than women after exercise (Tarnopolsky et al., 1990).

Given the data discussed previously, showing a greater reliance on lipid oxidation in females versus males, this must mean that women also use less carbohydrate. Accordingly,

the data reported to date from both animal studies and human subject studies, it is apparent that during endurance exercise, females use less muscle glycogen than males. Moreover, the contribution of liver-derived glucose to oxidation appears also to be less in females than in males.

(1.1.4) Protein metabolism

Sex differences in protein metabolism have been found in humans at rest (Chevalier et al., 2005) and during endurance exercise (Braun & Horton, 2001) with women oxidizing less leucine than men both at rest (Boirie et al., 2001; McKenzie et al., 2000; Volpi et al., 1998) and during exercise (Lamont, McCullough & Kalhan, 2001; McKenzie et al., 2000; Phillips et al., 1993), probably due to a lower skeletal muscle BCOAD activation at rest (McKenzie et al., 2000). A sparing of muscle glycogen in women occurred in conjunction with a lack of an increase in urinary urea nitrogen excretion collected on an exercise vs. rest day, suggesting lower amino acid oxidation (Riddell et al., 2003; Tarnopolsky et al., 1990). Sex differences in β-adrenergic-receptor activity in response to exercise led to a higher availability of free fatty acid in women vs. men (Lamont, McCullough & Kalhan, 2003) and attenuated the rise in leucine oxidation during endurance exercise in women (Lamont et al., 2001; Lamont et al., 2003).

In summary, women have lower leucine oxidation than men during endurance exercise (McKenzie et al., 2000; Phillips et al., 1993; Riddell et al., 2003).

(1.2) Influence of menstrual cycle phase on substrate utilization

Studies concerning the influence of menstrual cycle phase on substrate utilization during endurance exercise have shown more variable results (Calloway & Kurzer, 1982; Devries et al., 2006b; Garrel et al., 1985; Hackney, McCracken-Compton & Ainsworth, 1994; Horton et al., 2002; Hrboticky, Leiter & Anderson, 1989; Kanaley et al., 1992; Kriengsinyos et al., 2004; Lariviere, Moussalli & Garrel, 1994; Wenz et al., 1997; Zderic, Coggan & Ruby, 2001). Some have found no effect of menstrual cycle on substrate metabolism during endurance exercise (Casazza et al., 2004; Garrel et al., 1985; Horton et al., 2002; Jacobs et al., 2005; Kanaley et al., 1992), while others reported that lipid oxidation was higher (Hackney et al., 1994; Wenz et al., 1997; Zderic et al., 2001), CHO oxidation was lower during low- to moderate- intensity endurance exercise (Hackney et al., 1994; Wenz et al., 1997; Zderic et al., 2001). In agreement with these observations that there were menstrual cycle phase differences in lipid and CHO oxidation (Hackney et al., 1994; Wenz et al., 1997; Zderic et al., 2001), LUT women had a lower plasma glucose turnover (Devries et al., 2006b; Rose & Richter, 2005), and lower pro-glycogen, macro-glycogen and total glycogen utilization during exercise (Devries et al., 2006b), as compared with FOL women. Protein turnover was consistently reported to be higher (Calloway & Kurzer, 1982; Garrel et al., 1985; Kriengsinyos et al., 2004; Lariviere et al., 1994; Moller et al., 1996) for women in the LUT vs. the FOL phase of the menstrual cycle, with LUT women having a lower plasma amino acid concentration (Kriengsinyos et al., 2004; Moller et al., 1996), a higher requirement of lysine (Kriengsinyos et al., 2004), a higher turnover of leucine (Lariviere et al., 1994), tryptophan (Hrboticky et al., 1989) and phenylalanine (Kriengsinyos et al., 2004), and a higher nitrogen excretion (Calloway & Kurzer, 1982), as compared with FOL women.

In summary, during endurance exercise, women oxidize more protein during LUT phase than FOL phase.

(1.3) Influence of muscle fiber composition on substrate utilization

Myofibrillar adenosine triphosphatase (mATPase)–based histochemical analysis has been used to identify muscle fiber types (Brooke & Kaiser, 1970). For most human muscles, mATPase-based fiber types correlate with the myosin heavy chain (MHC) content and each histochemically identified fiber has a specific MHC profile (Schiaffino & Reggiani, 1996; Staron, 1997). In human *vastus lateralis*, generally three muscle fibers, type I, type IIa and type IIx, can be identified with this method (Brooke & Kaiser, 1970; Carter et al., 2001c; Yasuda et al., 2005). The mitochondria content and oxidative capacity are the highest in type I, lower in type IIa, and lowest in type IIx fibers (Prince et al., 1981). The inverse is observed for the glycolytic capacity of these fibers (Prince et al., 1981; Schiaffino & Reggiani, 1996). It has been suggested that sex differences in muscle fiber composition be a factor contributing to the sex differences in substrate utilization during exercise (Carter et al., 2001c).

Sex differences in muscle fibers have been studied in both animals (Fox et al., 2003; Rosen et al., 2004) and humans (Brooke & Engel, 1969a; Esbjornsson-Liljedahl et al., 1999; Esbjornsson et al., 1993; Hards et al., 1990; Howel, 1994; Mannion et al., 1997; Miller et al., 1993; Simoneau & Bouchard, 1989; Simoneau et al., 1985; Staron et al., 2000). In rats (Fox et al., 2003; Rosen et al., 2004), sex differences were not found in the fiber type composition of the soleus and tibialis anterior muscles, but in the size of individual skeletal fibers, with muscle fiber type I area larger in females than in males. In

green anole lizards (Anolis carolinensis) ceratohyoideus (Rosen et al., 2004), males had a greater percentage of tonic fibers than females, whereas females had a greater percentage of slow–oxidative fibers than males. In humans, a sex difference in muscle fiber composition has been found in different skeletal muscles, such as, erector spinae (Mannion et al., 1997), internal intercostal, external intercostal, and latissimus dorsi muscles (Hards et al., 1990), biceps brachii (Miller et al., 1993), vastus medialis (Howel, 1994), vastus lateralis (Brooke & Engel, 1969a; Carter et al., 2001c; Esbjornsson-Liljedahl et al., 1999; Esbjornsson et al., 1993; Roepstorff et al., 2006c). Although the results of studies regarding sex differences in the number of muscle fibers of different types are controversial (Brooke & Engel, 1969a; Costill et al., 1976; Esbjornsson-Liljedahl et al., 1999; Simoneau & Bouchard, 1989; Simoneau et al., 1985; Staron et al., 2000), women generally appear to have a greater relative type I fiber area, a smaller relative area of type II fiber,s and a greater % area of type I fibers (Carter et al., 2001c; Esbjornsson-Liljedahl et al., 1999; Esbjornsson et al., 1993; Simoneau et al., 1985; Yasuda et al., 2005). The sex difference in muscle fiber type composition may be a factor contributing the sex differences in substrate utilization during exercise. A higher relative type I fiber proportion (Brooke & Engel, 1969a; Roepstorff et al., 2006b; Simoneau & Bouchard, 1989; Staron et al., 2000), larger type I fiber area (Carter et al., 2001b; Esbjornsson et al., 1993; Simoneau et al., 1985; Yasuda et al., 2005) , and higher area % of type I fibers in women as compared with men (Carter et al., 2001b; Esbjornsson-Liljedahl et al., 1999; Esbjornsson et al., 1993; Roepstorff et al., 2006b; Simoneau et al., 1985; Yasuda et al., 2005) may provide some explanation for the higher lipid oxidation in the skeletal muscle of females as compared with males because type I fibers have a 3-fold higher intra-muscular triglyceride content as compared with type II

fibers (Essen et al., 1975). Type I fibers also have higher β-oxidation, TCA cycle and ETC enzyme activity (Essen et al., 1975; Schantz & Henriksson, 1987).

(1.4) Influence of estrogen on substrate utilization

Because the ovarian hormones (progesterone and estrogen) are present 6-40 fold higher for estrogen and 1-30 fold higher for progesterone in women than men, most research to date has centered on characterization of the role of estrogen in mediating the sex difference in selective substrate utilization during exercise. Estrogen may play its role directly via nuclear interaction and gene expression, or nonnuclear effects on intracellular signaling (Cavailles, 2002). Alternatively, there may be indirect effects of estrogen via interactions with other endocrine hormones (Braun & Horton, 2001). Estrogen is a collective term for a group of 18-carbon steroid hormones. The most biologically active estrogen is 17β-estrdiol (E2). To elucidate the role of estrogen in the sex differences in substrate utilization, a wide range of studies have been conducted with animal models (Gorski et al., 1976; Hatta et al., 1988; Kendrick & Ellis, 1991; Kendrick et al., 1987; Rooney et al., 1993) and human subjects (Carter et al., 2001a; Devries et al., 2005; Hamadeh et al., 2005; Ruby et al., 1997) through alteration of E2 plasma concentration by ovariectomization in animals and E2 supplementation in animals or human subjects.

(1.4.1) Animal studies

The metabolic effects of estrogen have been fairly well studied using animal models. The administration of the female sex hormone, 17β-estradiol (E2), to male

(Kendrick & Ellis, 1991) or ovarectomized (OVX) female (Kendrick et al., 1987) rats resulted in lower skeletal muscle and hepatic net glycogen utilization during endurance exercise. It was also found that E2 administration attenuated the early glycogen depletion in heart, liver and skeletal muscle during endurance exercise in male rodents (Rooney et al., 1993). Gorski *et al.* (1976) reported that female rats treated with E2 had a smaller decrease in blood glucose after 5 hours of exercise compared with male rats or ovariectomized females. Kendrick *et al.* (1991) measured plasma free fatty acid (FFA) concentrations and found them to be elevated with E2 treatment (Ellis et al., 1994; Gorski et al., 1976; Kendrick & Ellis, 1991). Another study found that eight days of E2 administration to OVX rats resulted in a significant attenuation of $^{14}CO_2$ evolution from labeled glucose during endurance exercise (Hatta et al., 1988).

The effects of E2 upon lipid oxidation have also been explored in rodents treated with E2 (Ellis et al., 1994; Hatta et al., 1988). Hatta and colleagues (1988) treated female OVX rats with E2 and found a significantly greater $^{14}CO_2$ evolution from ^{14}C-palmitate during exercise. Ellis *et al.* (1994) studied the effect of E2 on fat oxidation by administration of E2 to male rats and found an increased basal plasma glycerol and FFA and a lower TG concentration, which corresponded to a decrease in adipocyte-and an increase in muscle- and heart- LPL activity. The ratio of muscle/adipocyte LPL activity was 4.65 for the E2 group and 1.15 for the placebo group, which was interpreted to indicate an E2 mediated mechanism for increasing distribution of plasma TG-derived FFA towards heart and skeletal muscle from subcutaneous triglyceride stores (Ellis et al., 1994). This group also reported that the skeletal muscle LPL activity and IMTG were greater before

exercise, and use greater, in E2 treated animals (Ellis et al., 1994). Thus, E2 can increase the availability of FFA and increase the oxidation of lipid during exercise in rats.

Studies in animals have generally concluded that E2 increases lipid oxidation and decreases carbohydrate oxidation (Kendrick et al., 1987; Rooney et al., 1993), resulting in improved exercise endurance (Kendrick et al., 1987; Rooney et al., 1993), sparing of muscle and liver glycogen during exercise (Kendrick et al., 1987; Rooney et al., 1993), increased FFA availability for oxidation (Kendrick et al., 1987; Rooney et al., 1993), elevated IMTG content (Ellis et al., 1994), and decreased lactate concentration (Kendrick et al., 1987).

(1.4.2) Human studies

The effects of estrogen on substrate metabolism have also been studied in humans through administration of 17β-estradiol. The administration of E2 to amenorrheic women (Ruby et al., 1997) and men (Carter et al., 2001a; Devries et al., 2005; Hamadeh et al., 2005) was reported to decrease the RER during exercise (Carter et al., 2001a; Devries et al., 2005; Hamadeh et al., 2005; Ruby et al., 1997). E2 reduced whole body carbohydrate oxidation, leucine oxidation, and increased lipid oxidation at rest and during exercise in men (Hamadeh et al., 2005). E2 supplementation lowered resting pro-glycogen and total glycogen concentration (Devries et al., 2005), but did not attenuate skeletal muscle glycogen utilization during exercise in men (Carter et al., 2001a; Devries et al., 2005; Tarnopolsky et al., 2001). E2 supplementation altered fuel selection in human by increasing plasma FFA concentration (Ruby et al., 1997), reducing glucose Ra (primarily

liver glucose production) (Carter et al., 2001a; Devries et al., 2005; Ruby et al., 1997) , Rd (primarily muscle glucose uptake) and metabolic clearance rate (Carter et al., 2001a; Devries et al., 2005).

In general, results from studies looking at the metabolic effects of E2 administration upon substrate selection during exercise showing a higher fat and lower carbohydrate oxidation suggest that some of the sex differences in substrate selection are hormonally mediated and not solely due to a higher proportion of type I muscle fibers (Roepstorff et al., 2006c) or other factors such as training differences (Ruby & Robergs, 1994) or body fat content between men and women favoring higher fat oxidation (Blaak, 2001).

(1.4.3) Balanced effect of estrogen and progesterone

The physiological effects of E2 appear to be modulated by the concurrent presence of progesterone. Estrogen and progesterone may interactively play some roles in mediating the sex-based differences in substrate utilization during exercise.

Studies with rats have shown that, at rest, progesterone administration increases liver glycogen content and suppresses hepatic gluconeogenesis (Matute & Kalkhoff, 1973). These effects are enhanced by concurrent E2 administration (Matute & Kalkhoff, 1973), suggesting that the two hormones work additively to decrease carbohydrate utilization. Ovariectomization removes both E2 and progesterone from rats. E2 supplementation studies (Gorski et al., 1976; Kendrick & Ellis, 1991; Kendrick et al., 1987; Rooney et al., 1993) showed that E2 reduced carbohydrate oxidation, suggesting that the removal of ovaries (E2) should increase the glucose transport. However, Hansen *et al.* (1980) reported that glucose transport after electrical stimulation of muscle in ovariectomized rats was

reduced relative to controls. These contradictory experimental results indicate that progesterone may play an antagonistic role in regulating the glucose uptake by muscle. Indeed, E2 administration in ovariectomized rats restored the glucose uptake by muscle, while progesterone administration reduced the protein content of GLUT4 and impaired the glucose uptake by muscle (Campbell & Febbraio, 2001; Campbell & Febbraio, 2002).

At rest, the rate of fatty acid synthesis was lower in E2-treated rats compared with progesterone-treated rats (Hansen et al., 1980). In agreement with these results, Campbell and Febbraio (2001) reported that estrogen supplementation increased activity of CPT I and β-3-hydroxyacyl-CoA dehydrogenase (β-HAD) in rats (both key enzymes in the fatty acid oxidation pathway). The effect was reversed with concurrent progesterone administration, implying that there is an antagonistic relationship between estrogen and progesterone in regulating fat metabolism (Campbell & Febbraio, 2001). Other studies suggest that progesterone works synergistically with estrogen to decrease utilization and increase storage of carbohydrate (Matute & Kalkhoff, 1973).

Though some studies have found no effect of menstrual cycle on substrate metabolism during endurance exercise (Bailey et al., 2000; Garrel et al., 1985; Horton et al., 2002; Kanaley et al., 1992), others reported that lipid oxidation was higher (Hackney et al., 1994; Wenz et al., 1997; Zderic et al., 2001), CHO oxidation was lower during low- to moderate-intensity endurance exercise (Hackney et al., 1994; Horton et al., 1998; Wenz et al., 1997; Zderic et al., 2001), and protein turnover was consistently reported to be higher (Calloway & Kurzer, 1982; Garrel et al., 1985; Hrboticky et al., 1989; Kriengsinyos et al., 2004; Lariviere et al., 1994; Moller et al., 1996) in the LUT phase of the menstrual cycle, as

compared with FOL women, suggesting that estrogen and progesterone may antagonistically influence substrate oxidation in human. Elkind-Hirsch *et al.*(1993) supplemented young women with premature ovarian failure with estrogen and a combination of estrogen and progesterone, and found there was decreased insulin sensitivity after the estrogen and progesterone treatment. This finding supports the concept that progesterone reduces insulin-mediated glucose uptake. D'Eon *et al.* (2002) studied the combined effect of estrogen and progesterone on carbohydrate metabolism during exercise in eight eumenorrheic women with E2 or combination of E2 and progesterone supplementation. They found that compared with baseline control group and the group supplemented with both estrogen and progesterone, total carbohydrate oxidation was lower in the group supplemented with estrogen only. Glucose R(d) tended to be lower in the group supplemented with both estrogen and progesterone and the group with estrogen relative to the baseline control. Muscle glycogen utilization was lower in the group with estrogen than baseline control or the group supplemented with both estrogen and progesterone. Plasma free fatty acids (FFA) were inversely related to muscle glycogen utilization. The data suggest that estrogen lowers total carbohydrate oxidation by reducing muscle glycogen utilization and glucose R(d). Progesterone increases muscle glycogen utilization but not glucose R(d). The opposing actions of E2 and Progesterone on muscle glycogen utilization may be mediated by their impact on FFA availability or vice versa. The combined effect of estrogen and progesterone was still a lower carbohydrate oxidation although the two hormones showed opposing actions on glucose R(d), muscle glycogen utilization and free fatty acids in blood (D'Eon et al., 2002). When myocardial fatty acid metabolism in postmenopausal women receiving estrogen, estrogen plus progesterone, or

no hormone replacement was compared with age-matched men, it was found that myocardial fatty acid utilization was higher in women taking estrogen when compared with men and trended to be higher when compared with women not receiving hormonal therapy but was not different from that of women taking estrogen plus progesterone, suggesting that estrogen and progesterone play an antagonistic roles in myocardial fatty acid oxidation in human (Herrero et al., 2005).

In conclusion, estrogen and progesterone may additively, antagonistically and synergistically influence fat and CHO oxidation in rats and antagonistically in human.

(1.4.4) Effects of estrogen through interaction with epinephrine

Researchers have found that men have significantly higher epinephrine levels during exercise as compared with women (Carter et al., 2001b; Friedlander et al., 1998; Horton et al., 1998; Tarnopolsky et al., 1990). A significant reduction in epinephrine levels late in exercise was observed with estrogen treatment in amenorrheic women (Ruby et al., 1997), implying that estrogen may play a role in the neuroendocrine response to exercise. Epinephrine stimulates lipolysis in adipose tissue and thus increases arterial FFA levels. It was found that glycerol release, an indicator of adipose tissue lipolysis, in response to infused epinephrine was greater in women than in men (Flechtner-Mors et al., 1999), suggesting that women have a greater sensitivity to epinephrine's lipolytic effects.

(1.5) Molecular mechanisms of physiological processes involved in sex-based differences in substrate utilization during exercise

Molecular approaches have been used to study the mechanisms of physiological processes in skeletal muscle during exercise at trancriptome level. It has been demonstrated that changes in the expression of genes during exercise may be the molecular mechanisms of the physiological processes associated with exercise. The molecular mechanism(s) of sex difference in muscle fiber composition and menstrual cycle phase differences in substrate utilization have not been elucidated. No studies regarding the molecular mechanism(s) by which estrogen influences substrate selection during exercise in human skeletal muscle have been completed. The reported studies regarding the molecular mechanisms of sex difference in substrate metabolism during exercise mostly focused on enzyme activity, and only a few mRNA species have been evaluated (Binnert et al., 2000; Kiens et al., 2004; Roepstorff et al., 2005a). There is still much work required to comprehensively elucidate the molecular mechanisms of sex difference in substrate utilization during exercise in human at the transcriptome level.

(1.5.1) Exercise stimulated substrate utilization

There have been many studies evaluating the effect of exercise *per se* on the mRNA expression of the genes involved in CHO, fat, and amino acids metabolism in skeletal muscle. A single bout of exercise in human subjects is capable of activating the expression of many diverse groups of genes (Cameron-Smith, 2002), which may be the underlying mechanism(s) for exercise stimulated increases in oxidation of fat, carbohydrate and amino aids (Russell et al., 2005; Saltin & Pilegaard, 2002).

Exercise influences the expression of the genes involved in transcriptional regulation of substrate metabolism (Table 2). Peroxisome proliferator activated receptor

gamma coactivator-1 alpha (PGC-1α) is a transcriptional co-activator that binds to peroxisome proliferator activated receptor gamma (PPARγ)(Puigserver et al., 1998) and peroxisome proliferator activated receptor alpha (PPARα) (Vega, Huss & Kelly, 2000) and regulates the expression of uncoupling protein 1 in the brown fat of mice (Puigserver et al., 1998) and genes encoding mitochondrial fatty acid β-oxidation enzymes (Vega et al., 2000). PGC-1α is a master regulator for genes involved in mitochondrial biogenesis (Wu et al., 1999). Most studies have reported that a single bout of exercise increased PGC-1α mRNA level (Baar et al., 2002; Jorgensen et al., 2005; Norrbom et al., 2004; Pilegaard et al., 2005; Pilegaard, Saltin & Neufer, 2003; Terada et al., 2002; Watt et al., 2004) and transcription of PGC-1α in skeletal muscle (Pilegaard et al., 2005), with only rare exceptions (Tunstall et al., 2002). Endurance training is associated with a higher abundance of PGC-1α mRNA in skeletal muscle (Goto et al., 2000; Russell et al., 2003; Short et al., 2003; Terada & Tabata, 2004).

The PPAR isoforms alpha, beta/delta, and gamma control the transcription of genes involved in fatty acid and carbohydrate metabolism (Luquet et al., 2004; Mandard, Muller & Kersten, 2004; Rosen et al., 2000). Endurance exercise training increases the content of PPARα protein (Horowitz et al., 2000; Russell et al., 2003) and mRNA (Russell et al., 2003). Except for one study (Tunstall et al., 2002), most studies found that acute exercise increased transcription (Pilegaard et al., 2005), and mRNA content (Pilegaard et al., 2005; Watt et al., 2004), of PPARα in human skeletal muscle. The difference between Tunstall *et al.'s* (2002) and other studies is likely to be due to the shorter duration of exercise in

Table2. Effect of exercise on the expression of the genes involved in transcriptional regulation of substrate metabolism in human skeletal muscle

Genes	Exercise	Sex	mRNA content	Transcription	Protein	Authors
PGC-1 α	Acute exercise	M	↑	n/a	n/a	Norrbom et al., 2004
		M	↑	↑	n/a	Pilegaard et al., 2005
		M	↑	↑	n/a	Pilegaard, Saltin & Neufer, 2003
		M	↑	n/a	n/a	Watt et al., 2004
		M/F	↔	↔	n/a	Tunstall et al., 2002
	Exercise training	M	↑	n/a	n/a	Russell et al., 2003
		M/F	↑	n/a	n/a	Short et al., 2003
PPARα	Acute exercise	M	↑	↑	n/a	Pilegaard et al., 2005
		M	↑	n/a	n/a	Watt et al., 2004
		M/F	↔	↔	n/a	Tunstall et al., 2002
	Exercise training	F	n/a	n/a	↑	Horowitz et al., 2000
		M	↑	↔	↑	Russell et al., 2003
PPARδ	Acute exercise	M	↑	n/a	n/a	Watt et al., 2004
	Exercise training	F/M	↑	n/a	↑	Fritz et al., 2006
PPARγ	Exercise training	M	↔	n/a	n/a	Russell et al., 2003
SREBP -1c	Acute exercise	M/F	↔	n/a	n/a	Tunstall et al., 2002

Note: ↑, increase; ↔, no change; M, men; F, women; n/a, not available.

Tunstall *et al.'s* study (Watt et al., 2004). Acute exercise (Watt et al., 2004) and exercise training (Fritz et al., 2006) increased the content of PPARδ mRNA in human skeletal muscle. Endurance training also promoted a 2.6-fold upregulation of PPARδ protein in mice (Luquet et al., 2003). Endurance training did not alter the mRNA content of PPARγ in human skeletal muscle (Russell et al., 2003).

Sterol regulatory element-binding protein 1c (SREBP-1c) is a transcription factor which activates the transcription of the genes involved in lipogenesis (Dentin, Girard & Postic, 2005). Acute exercise did not alter the mRNA content of SREBP-1c in human skeletal muscle (Tunstall et al., 2002). Exercise training increased SREBP-1c mRNA in skeletal muscles of mice, with increases of lipogenic genes, such as acetyl-CoA carboxylase-1, stearoyl-CoA desaturase-1, and acyl CoA:diacylglycerol acyltransferase-1 mRNAs (Ikeda et al., 2002).

Exercise also influences the expression the genes involved in fat metabolism pathways (Table 3). Due to difference in exercise protocol, some studies found that exercise did not change the mRNA expression of FA translocase (FAT/CD36), carntine palmitoyltransferase (CPT) I, β-HAD (Tunstall et al., 2002; Watt et al., 2004), plasma membrane-associated FA-binding protein (Tunstall et al., 2002) and HSL mRNA (Watt et al., 2004), while others found that a single exercise bout induced an increase in the mRNA content of FAT/CD36, plasm membrane fatty acid binding protein (FABPpm) (Kiens et al., 2004), CPTI and LPL (Pilegaard et al., 2000; Pilegaard et al., 2005) and the transcription of CPTI and LPL (Pilegaard et al., 2000; Pilegaard et al., 2005) in human skeletal muscle. Acute treadmill running (90 min) did not change CPTI, LPL or UCP3

mRNA in mice (Jorgensen et al., 2005). The training induced higher capacity for lipid oxidation was accompanied by an increased expression of FAT/CD36 and CPTI mRNA. Similarly, FAT/CD36 protein abundance was also upregulated by exercise training (Tunstall et al., 2002).

Table3. Effect of exercise on the mRNA expression of the genes involved in fat metabolism in human skeletal muscle

Genes	Exercise	Sex	mRNA content	Transcription	Authors
FAT/ CD34	Acute exercise	M/F	↔	n/a	Tunstall et al., 2002
		M	↔	n/a	Watt et al., 2004
		M/F	↑	n/a	Kiens et al., 2004
	Exercise training	M/F	↑	n/a	Tunstall et al., 2002
CPTI	Acute exercise	M/F	↔	n/a	Tunstall et al., 2002
		M	↔	n/a	Watt et al., 2004
	Exercise training	M	↑	n/a	Pilegaard et al., 2000; Pilegaard et al., 2005
β-HAD	Acute exercise	M/F	↔	n/a	Tunstall et al., 2002
		M	↔	n/a	Watt et al., 2004
FABPpm	Acute exercise	M/F	↔	n/a	Tunstall et al., 2002
		M/F	↑	n/a	Kiens et al., 2004
HSL	Acute exercise	M	↔	n/a	Watt et al., 2004
LPL	Acute exercise	M	↑	↑	Pilegaard et al., 2000
		M	↑	↑	Pilegaard et al., 2005

Note: ↑, increase; ↔, no change; M, men; F, women; n/a, not available.

Exercise also alters the mRNA expression of the genes involved in carbohydrate and amino acid metabolism pathways (Table 4). Glucose transporter 4 (GLUT4) protein is

Table4. Effect of exercise on the mRNA expression of the genes involved in carbohydrate metabolism in human skeletal muscle

Genes	Exercise	Sex	mRNA content	Transcription	Authors
GLUT4	Acute exercise	M/F	↑	n/a	Kraniou et al., 2000
HKII	Acute exercise	M/F	↑	n/a	Cusi et al., 2001;
		M/F	↑	n/a	Koval et al., 1998
	3-6 h after exercise	M/F	↑	n/a	Kraniou et al., 2000; Nordsborg, Bangsbo & Pilegaard, 2003
		M	↑	n/a	Pilegaard et al., 2003
PFK	Exercise training	M/F	↔	n/a	Vestergaard, 1999
PDK4	Acute exercise	M	↑	n/a	Watt et al., 2004
Glycogenin	1.5 -2h after exercise	M	↑	n/a	Shearer et al., 2005a, 2005b
	3 h after exercise	M/F	↑	n/a	Kraniou et al., 2000
GS	Acute exercise	M/F	n/a	n/a	Cusi et al., 2001; Koval et al., 1998
	Exercise training	M/F	↑	n/a	Vestergaard, 1999

Note: ↑, increase; ↔, no change; M, men; F, women; n/a, not available.

the predominant isoform of glucose transporter protein expressed in the skeletal muscle, which translocates to plasma membrane upon insulin stimulation to facilitate glucose transporting across the plasma membrane (Goodyear et al., 1991; Hirshman et al., 1990). A single bout of exercise is sufficient to increase both GLUT4 transcription (Neufer & Dohm, 1993) and mRNA abundance in human skeletal muscle (Kraniou et al., 2000), although the effect of exercise on the mRNA content of GLUT4 in rodent skeletal muscle is not consistent (Jorgensen et al., 2005; O'Doherty et al., 1994; Ren et al., 1994). Hexokinase II (HK II) catalyzes the phosphorylation of intracellular glucose, and, as a result, it prevents the countertransport of glucose out of the cell membrane (Rose & Richter, 2005). A single bout of exercise increases HKII mRNA and activity in human muscle (Cusi et al., 2001; Koval et al., 1998), rat (Koval et al., 1998; O'Doherty et al., 1994; Weber & Pette, 1990) and the mRNA content of HKII in mice (Jorgensen et al., 2005). An increased rate of gene transcription after exercise accounts for the increased HK II mRNA in rats (O'Doherty et al., 1996). After 3-6 h of recovery from exercise HK II mRNA level was still elevated in human skeletal muscle (Kraniou et al., 2000; Nordsborg, Bangsbo & Pilegaard, 2003; Pilegaard et al., 2003). Endurance training also increased the activity of HK II activity (Phillips et al., 1996). Depending on the intensity and frequency, physical exercise may induce an increased, a decreased or an unaltered level of muscle phorsphofructokinase (PFK) activity (Vestergaard, 1999). In athletes, muscle PFK mRNA is similar to that of sedentary subjects; whereas, the immunoreactive PFK protein concentration was decreased (Vestergaard, 1999). A single bout of exercise increased pyruvate dehydrogenase kinase 4 (PDK4) mRNA content in human (Watt et al., 2004) and mice (Jorgensen et al., 2005). A single bout of exercise is sufficient to cause upregulation of glycogenin mRNA expression

(Kraniou et al., 2000; Shearer et al., 2005a; Shearer et al., 2005b), protein content and activity (Shearer et al., 2005a) in human skeletal muscle. Endurance training in young healthy subjects results an increased total glycogen synthase (GS) activity, increased GS mRNA levels and enhanced insulin-stimulated activation of GS (Vestergaard, 1999). Exercise increased glycogen synthase activity in obese nondiabetic and type 2 diabetic patients (Cusi et al., 2001) and healthy subjects (Koval et al., 1998).

Acute exercise increased the oxidation of leucine in both human and rat associated with an increased activity of branched-chain 2-oxo acid dehydrogenase (BCOAD) (Fujii et al., 1998; Kasperek, Dohm & Snider, 1985; Kasperek & Snider, 1987; McKenzie et al., 2000; Shimomura et al., 1993; Wagenmakers, Schepens & Veerkamp, 1984) and aminotransferase, and decreased activity of branched-chain 2-oxo acid dehydrogenase kinase (BCOADK) (Fujii et al., 1998; Kasperek et al., 1985; Shimomura et al., 1993). Acute exercise did not change the total activity of BCOAD (Wagenmakers et al., 1989). BCOAD was dephosphorylated (activated) during exercise (Rush et al., 1995) due to decreased activity of BCOADK (Fujii et al., 1998; Kasperek et al., 1985; Shimomura et al., 1993). No studies about the mRNA expression of these genes during exercise have been reported.

However, the effect of exercise on mRNA content of FABPc, HSL, SREBP-2, mtGPAT, TFP-α, VLCAD, LCAD, CPTII, GSK3α, PFK, glycogen phosphorylase, AST, BCOAD and BCOADK has not been reported to be evaluated.

(1.5.2) Sex-based difference in skeletal muscle fiber type composition

The molecular mechanisms of the sex differences in skeletal muscle fiber type composition have not been well elucidated. Muscle fiber type specific expression of myosin heavy chain genes (Staron, 1997) makes them good molecular markers for studying potential sex differences in muscle fiber type composition. Fibers coexpressing slow and fast myosin heavy chain isoforms are hybrid fibers under slow-to-fast transformations (Stevens et al., 2004). The difference in the expression of MHC mRNAs between sexes will be useful to further understand potential sex differences in fiber type at the molecular level.

The determination of muscle fiber type is a multi-factorial process; however, several genes have been implicated including, PGC-1α (Lin et al., 2002) and PPARδ (Grimaldi, 2003; Luquet et al., 2003; Wang et al., 2004) and myostatin (Girgenrath, Song & Whittemore, 2005).

PGC-1α encodes a transcriptional coactivator that regulates muscle fiber type determination (Lin et al., 2002). When PGC-1α is expressed at physiological levels in transgenic mice driven by a muscle creatine kinase (MCK) promoter, type II muscles also express proteins characteristic of type I fibres, such as troponin I (slow) and myoglobin, and show a much greater resistance to electrically stimulated fatigue. In cultured muscle cells, PGC-1 alpha activates transcription in cooperation with Mef2 proteins and serves as a target for calcineurin signalling, which has been implicated in slow fiber gene expression (Lin et al., 2002). In agreement with the function of PGC-1α in inducing switch of muscle fiber type II to type I in mice, it was found that exercise and exercise training could

increase the content of PGC-1α mRNA in human skeletal muscle (Norrbom et al., 2004; Pilegaard et al., 2003; Russell et al., 2003) and though no fiber type transition from muscle fiber type II to type I is observed following short-term endurance training (Carter et al., 2001c), long-term exercise training led to transition of type II fibers to typer I fibers in human muscle (Hambrecht et al., 1997).

PPARδ encodes a transcriptional factor of the peroxisome proliferator-activated receptor (PPAR) family (Kersten, Desvergne & Wahli, 2000). It functions as an integrator of transcription repression and nuclear receptor signaling (Shi, Hon & Evans, 2002) and is associated with muscle fiber type determination (Grimaldi, 2003; Luquet et al., 2003; Wang et al., 2004). Overexpression of native or dominant-negative mutant forms of PPARδ in C2C12 myotubes and muscle-specific alteration of PPARδ activity in transgenic mice using a CRE-Lox recombination approach indicate that PPARδ plays a crucial role in myofiber typing determination and regulation of muscle oxidative capability (Grimaldi, 2003). Overexpression in an animal model generated by using a CRE-Lox recombination approach results in a profound change in fiber composition due to hyperplasia and/or shift to more oxidative fiber and, as a consequence, leads to the increase of both enzymatic activities and genes implicated in oxidative metabolism (Luquet et al., 2003). In mice the targeted expression of an activated form of PPARδ in skeletal muscle or treatment of wild-type mice with PPARδ agonist induced a switch from type II fibers to type I fibers and increased the numbers of type I muscle fibers (Wang et al., 2004). Endurance exercise promotes an accumulation of PPARδ protein in muscle of wild-type animals (Luquet et al., 2003).

These results suggest that PPARδ plays an important role in muscle development and adaptive response to environmental changes, such as exercise training.

Myostatin, a member of the transforming growth factor-beta superfamily, is a secreted protein that as a negative regulator of skeletal muscle mass acts to limit the final number of muscle fibers that are formed (Gonzalez-Cadavid et al., 1998; Grobet et al., 2003; Lee, 2004). Myostatin knockout mice displays a larger proportion of fast type II fibers and a reduced proportion of slow type I fibers compared with wild-type animals (Girgenrath et al., 2005), suggesting that sex differential expression of myostatin may lead to sex difference in the number of muscle fibers.

To date, it is unclear whether or not any of the aforementioned genes are differentially expressed between the sexes and whether or not they directionally support the reported sex differences with women showing a higher abundance of type I fibers (Brooke & Engel, 1969b; Roepstorff et al., 2006c; Simoneau & Bouchard, 1989; Staron et al., 2000).

(1.5.3) E2 mediated selective substrate utilization

The potential mechanism(s) of E2 mediated substrate selection has been evaluated in rats (Ellis et al., 1994) and mice (D'Eon et al., 2005; Kamei et al., 2005). E2 increased the activity of LPL (Ellis et al., 1994; Hamosh & Hamosh, 1975; Ramirez, 1981; Wilson et al., 1976), citrate synthase (Beckett, Tchernof & Toth, 2002), and GS (Beckett et al., 2002) in the skeletal muscle of rats, and decreased the activity of hepatic phosphoenol pyruvate carboxy kinase in oophorectomized rodents (Mandour, Kissebah & Wynn, 1977), and

peripheral adipocyte LPL activity in male rats (Ellis et al., 1994). Ovariectomization (Kamei et al., 2005), and E2 replacement in ovariectomized mice (D'Eon et al., 2005), demonstrated that E2 enhanced the expression of the genes involved in transcriptional regulation of fat metabolism and β-oxidation in the skeletal muscle. E2 replacement also decreased the expression of lipogenic genes, such as, SREBP-1c, in the skeletal muscle of ovariectomized mice (Kamei et al., 2005). Replacement of E2 in ovariectomized rats increased mRNA content of PPARα, PPARγ, CPT I and PDK4, and protein content of PPARα, in skeletal muscle (Campbell et al., 2003). In conclusion, E2 increases activity of the enzymes and the mRNA expression of the genes involved in transcriptional regulation and fat oxidation in rodents, which may lead to higher fat oxidation in female than in males. However, the potential mechanism(s) of the E2 mediated alterations in substrate utilization has not yet been evaluated at the mRNA level in human skeletal muscle.

(1.5.4) Menstrual cycle phase differences in substrate utilization

Although many studies have been performed regarding the effect of menstrual cycle phase on substrate utilization both at rest and during exercise (Calloway & Kurzer, 1982; Devries et al., 2006b; Garrel et al., 1985; Hackney et al., 1994; Horton et al., 2002; Hrboticky et al., 1989; Kanaley et al., 1992; Kriengsinyos et al., 2004; Lariviere et al., 1994; Wenz et al., 1997; Zderic et al., 2001), little is known about the molecular mechanism(s) for menstrual phase differences in substrate utilization in human skeletal muscle. It was one of the goals of our study to determine the influence of menstrual cycle on mRNA abundance for genes involved in intermediary metabolism in himan skeletal muscle.

(1.5.5) Sex differential mRNA expression

The effect of sex has been evaluated for several mRNA species involved in fat metabolism in human skeletal muscle (Binnert et al., 2000; Kiens et al., 2004; Roepstorff et al., 2005a) (Table 5). Women have a higher mRNA content of FATm (Binnert et al., 2000), LPL (Kiens et al., 2004), FAT/CD36, FABPpm, and a higher FAT/CD36 protein content (FAT/CD36) (Kiens et al., 2004). Women also have a higher mRNA, but not protein, content of β-HAD (Roepstorff et al., 2005a). CPT I activity was not different

Table 5. Sex differential mRNA expression of the genes involved in substrate metabolism in human skeletal muscle

Genes	Function	Exercise	mRNA content (women vs. men)	Author
LPL	Hydrolysis of lipid	90 min 60%VO2peak	higher	Kiens et al., 2004
FATm	Transport of long chain free fatty acids across plasmic membrane	Rest	higher	Binnert et al., 2000
FAT/CD36	Transport of free fatty acids	90 min 60%VO2peak	higher	Kiens et al., 2004
FABPpm	Transport of long chain free acid across plasmic membrane	90 min 60%VO2peak	higher	Kiens et al., 2004
β-HAD	β –oxidation	90 min 60% VO_2peak	higher	Roepstorff et al., 2005a

between men and women (Berthon et al., 1998). A higher mRNA expression of FATm (Binnert et al., 2000), LPL (Kiens et al., 2004), FAT/CD36 and FABPpm (Kiens et al., 2004) in skeletal muscle of women vs. men may lead to an increased fatty acid transport capacity and an increased fat oxidation in women. However, there are still many key regulatory genes involved in carbohydrate, fat and protein metabolism pathways, which have not been studied at mRNA level with regard to sex differences in substrate utilization. The transcriptomic mechanisms of sex differences in selective utilization of substrate are not well understood.

(1.6) Purpose of thesis

The purpose of the series of studies outlined in this thesis was to elucidate the molecular mechanisms of sex-based differences in substrate utilization during exercise at the transcriptome level. Primarily, we aimed to evaluate the effect of sex, menstrual cycle phase, estrogen and exercise on the mRNA expression of many of the genes involved in fat, CHO and protein metabolism in human skeletal muscle during exercise and provide the potential mechanism(s) for the sex differential utilization of substrate during exercise, menstrual cycle phase difference in substrate utilization, and E2 mediated selective utilization of substrate in men. We hypothesized that the differential mRNA expression for many of the genes involved in lipid, CHO and protein metabolism would reflect these differences in substrate utilization. As such, our primary goal was to discover the genes involved in lipid, CHO and protein metabolism with sex and menstrual cycle phase differential mRNA expression, and E2 mediated alteration in mRNA expression. Because exercise is an inductive stimulus for gene expression and the selective substrate utilization

processes are often observed during exercise, we also intended to characterize the genes involved in lipid, CHO and protein metabolism with exercise stimulated mRNA expression.

At the onset of exercise, signals from inside and outside the muscle cell increase the availability of CHO, fat and amino acids and also 'turn on' the metabolic pathways to provide the fuel required for ATP production. CHO, fat and amino acid oxidation are three sources of aerobic ATP production and the pathways involved in CHO, fat and amino acid metabolism (Figure1) must be heavily regulated during exercise to meet the increased energy demand. Within this paradigm, there is room for shifts between the proportion of energy that is provided from CHO, protein and fat (Spriet & Watt, 2003). It would be expected, for sex differences in substrate utilization, menstrual cycle phase differences in substrate utilization and E2 mediated selective utilization of substrate, that any shift toward increased reliance on fat metabolism might reflect the differential mRNA expression of the genes involved in fat oxidation and lipid synthesis. Therefore, we specifically quantified the abundance of mRNA in skeletal muscle for genes involved in mitochondrial biogenesis (PGC-1α), fat metabolism transcriptional regulation (PPARα, PPARγ, PPARδ), IMCL synthesis (SREBP-1c, SREBP-2, mtGPAT), sarcolemma membrane (FATm), cytosol (FABPc) and mitochondrial (CPTI, CPTII) fatty acid transport, hydrolysis of IMCL (HSL), and β-oxidation (VLCAD, LCAD, TFP-α) (Figure1). The same would be expected for sex, menstrual cycle phase and E2 related selective utilization of CHO and protein, which would reflect the differential mRNA expression of the genes that play key roles in regulating CHO and protein metabolism (Figure1). Inside the muscle these could include glucose uptake (GLUT4) and phosphorylation (hexokinase II), glycogen synthesis

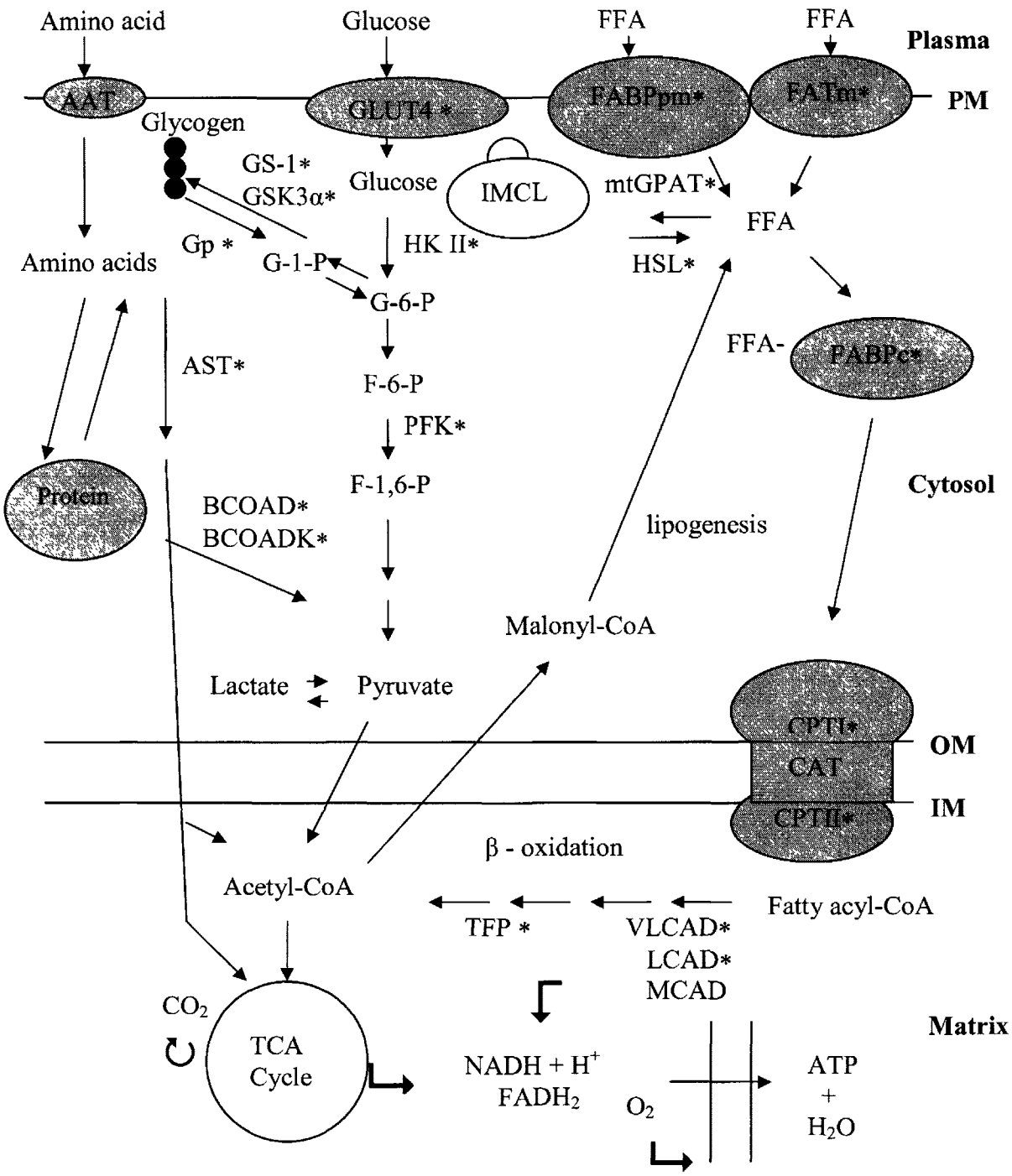

Figure1. Substrate metabolism pathways in human skeletal muscle. AAT, amino acid transporter; PM, plasmic membrane; OM, out mitochondrial membrane; IM, inner mitochondrial membrane; Matrix, mitochondrial matrix. * Targets of this study.

(glycogen synthase kinase3, glycogen synthase, glycogenin), glycogenolysis (glycogen phosphorylase), glycolysis (phosphofructokinase-PFK), deamination (aspartate aminotransferase - AST), dehydrogenation of amino acid backbone (BCOAD) and phosphorylation of BCOAD (BCOADK). As such, we specifically investigated the skeletal muscle mRNA content of GLUT4, HK II, glycogenin, glycogen synthase 1, glycogen synthase kinase 3α, PFK, aspartate aminotransferase-AST, BCOAD and BCOADK.

A second aim of the studies was to investigate the effect of sex and menstrual cycle phase on the mRNA content of the genes involved in determination of sex differences in muscle fiber type composition and provide insights into the molecular mechanism(s) of sex differences in muscle fiber type composition. We hypothesized that the sex differences in muscle fiber type composition would be reflected by the sex differences in mRNA expression of the genes involved in muscle fiber type terminal differentiation (MHCI, MHCIIa, MHCIIx), regulating muscle development (myostatin) and muscle fiber type determination (PGC-1α, PPARδ). We aimed to identify the genes with sex differential mRNA expression in human skeletal muscle.

BAAR, K., WENDE, A. R., JONES, T. E., MARISON, M., NOLTE, L. A., CHEN, M., KELLY, D. P. & HOLLOSZY, J. O. (2002). Adaptations of skeletal muscle to exercise: rapid increase in the transcriptional coactivator PGC-1. *Faseb J* **16,** 1879-86.

BAILEY, S. P., ZACHER, C. M. & MITTLEMAN, K. D. (2000). Effect of menstrual cycle phase on carbohydrate supplementation during prolonged exercise to fatigue. *J Appl Physiol* **88,** 690-7.

BECKETT, T., TCHERNOF, A. & TOTH, M. J. (2002). Effect of ovariectomy and estradiol replacement on skeletal muscle enzyme activity in female rats. *Metabolism* **51,** 1397-401.

BERTHON, P. M., HOWLETT, R. A., HEIGENHAUSER, G. J. & SPRIET, L. L. (1998). Human skeletal muscle carnitine palmitoyltransferase I activity determined in isolated intact mitochondria. *J Appl Physiol* **85,** 148-53.

BINNERT, C., KOISTINEN, H. A., MARTIN, G., ANDREELLI, F., EBELING, P., KOIVISTO, V. A., LAVILLE, M., AUWERX, J. & VIDAL, H. (2000). Fatty acid transport protein-1 mRNA expression in skeletal muscle and in adipose tissue in humans. *Am J Physiol Endocrinol Metab* **279,** E1072-9.

BLAAK, E. (2001). Gender differences in fat metabolism. *Curr Opin Clin Nutr Metab Care* **4,** 499-502.

BOIRIE, Y., GACHON, P., CORDAT, N., RITZ, P. & BEAUFRERE, B. (2001). Differential insulin sensitivities of glucose, amino acid, and albumin metabolism in elderly men and women. *J Clin Endocrinol Metab* **86,** 638-44.

BRAUN, B. & HORTON, T. (2001). Endocrine regulation of exercise substrate utilization in women compared to men. *Exerc Sport Sci Rev* **29,** 149-54.

BROOKE, M. H. & ENGEL, W. K. (1969a). The histographic analysis of human muscle biopsies with regard to fiber types. 1. Adult male and female. *Neurology* **19,** 221-33.

BROOKE, M. H. & ENGEL, W. K. (1969b). The histographic analysis of human muscle biopsies with regard to fiber types. 4. Children's biopsies. *Neurology* **19,** 591-605.

BROOKE, M. H. & KAISER, K. K. (1970). Three "myosin adenosine triphosphatase" systems: the nature of their pH lability and sulfhydryl dependence. *J Histochem Cytochem* **18,** 670-2.

CALLOWAY, D. H. & KURZER, M. S. (1982). Menstrual cycle and protein requirements of women. *J Nutr* **112,** 356-66.

CAMERON-SMITH, D. (2002). Exercise and skeletal muscle gene expression. *Clin Exp Pharmacol Physiol* **29,** 209-13.

CAMPBELL, S. E. & FEBBRAIO, M. A. (2001). Effect of ovarian hormones on mitochondrial enzyme activity in the fat oxidation pathway of skeletal muscle. *Am J Physiol Endocrinol Metab* **281,** E803-8.

CAMPBELL, S. E. & FEBBRAIO, M. A. (2002). Effect of the ovarian hormones on GLUT4 expression and contraction-stimulated glucose uptake. *Am J Physiol Endocrinol Metab* **282,** E1139-46.

CAMPBELL, S. E., MEHAN, K. A., TUNSTALL, R. J., FEBBRAIO, M. A. & CAMERON-SMITH, D. (2003). 17beta-estradiol upregulates the expression of peroxisome proliferator-activated receptor alpha and lipid oxidative genes in skeletal muscle. *J Mol Endocrinol* **31,** 37-45.

CARTER, S., MCKENZIE, S., MOURTZAKIS, M., MAHONEY, D. J. & TARNOPOLSKY, M. A. (2001a). Short-term 17beta-estradiol decreases glucose R(a) but not whole body metabolism during endurance exercise. *J Appl Physiol* **90,** 139-46.

CARTER, S. L., RENNIE, C. & TARNOPOLSKY, M. A. (2001b). Substrate utilization during endurance exercise in men and women after endurance training. *Am J Physiol Endocrinol Metab* **280,** E898-907.

CARTER, S. L., RENNIE, C. D., HAMILTON, S. J. & TARNOPOLSKY. (2001c). Changes in skeletal muscle in males and females following endurance training. *Can J Physiol Pharmacol* **79,** 386-92.

CASAZZA, G. A., JACOBS, K. A., SUH, S. H., MILLER, B. F., HORNING, M. A. & BROOKS, G. A. (2004). Menstrual cycle phase and oral contraceptive effects on triglyceride mobilization during exercise. *J Appl Physiol* **97,** 302-9.

CAVAILLES, V. (2002). Estrogens and receptors: an evolving concept. *Climacteric* **5 Suppl 2,** 20-6.

CHEVALIER, S., MARLISS, E. B., MORAIS, J. A., LAMARCHE, M. & GOUGEON, R. (2005). The influence of sex on the protein anabolic response to insulin. *Metabolism* **54,** 1529-35.

COSTILL, D. L., DANIELS, J., EVANS, W., FINK, W., KRAHENBUHL, G. & SALTIN, B. (1976). Skeletal muscle enzymes and fiber composition in male and female track athletes. *J Appl Physiol* **40,** 149-54.

CUSI, K. J., PRATIPANAWATR, T., KOVAL, J., PRINTZ, R., ARDEHALI, H., GRANNER, D. K., DEFRONZO, R. A. & MANDARINO, L. J. (2001). Exercise increases hexokinase II mRNA, but not activity in obesity and type 2 diabetes. *Metabolism* **50,** 602-6.

D'EON, T. M., SHAROFF, C., CHIPKIN, S. R., GROW, D., RUBY, B. C. & BRAUN, B. (2002). Regulation of exercise carbohydrate metabolism by estrogen and progesterone in women. *Am J Physiol Endocrinol Metab* **283,** E1046-55.

D'EON, T. M., SOUZA, S. C., ARONOVITZ, M., OBIN, M. S., FRIED, S. K. & GREENBERG, A. S. (2005). Estrogen regulation of adiposity and fuel partitioning. Evidence of genomic and non-genomic regulation of lipogenic and oxidative pathways. *J Biol Chem* **280,** 35983-91.

DAVIS, S. N., GALASSETTI, P., WASSERMAN, D. H. & TATE, D. (2000). Effects of gender on neuroendocrine and metabolic counterregulatory responses to exercise in normal man. *J Clin Endocrinol Metab* **85,** 224-30.

DENTIN, R., GIRARD, J. & POSTIC, C. (2005). Carbohydrate responsive element binding protein (ChREBP) and sterol regulatory element binding protein-1c (SREBP-1c): two key regulators of glucose metabolism and lipid synthesis in liver. *Biochimie* **87,** 81-6.

DEVRIES, M. C., HAMADEH, M. J., GRAHAM, T. E. & TARNOPOLSKY, M. A. (2005). 17beta-estradiol supplementation decreases glucose rate of appearance and disappearance with no effect on glycogen utilization during moderate intensity exercise in men. *J Clin Endocrinol Metab* **90,** 6218-25.

DEVRIES, M. C., HAMADEH, M. J., PHILLIPS, S. M. & TARNOPOLSKY, M. A. (2006a). Menstrual cycle phase and sex influence muscle glycogen utilization and glucose turnover during moderate-intensity endurance exercise. *Am J Physiol Regul Integr Comp Physiol* **291,** R1120-8.

DEVRIES, M. C., HAMADEH, M. J., PHILLIPS, S. M. & TARNOPOLSKY, M. A. (2006b). Menstrual Cycle Phase and Sex Influence Muscle Glycogen Utilization and Glucose Turnover During Moderate Intensity Endurance Exercise. *Am J Physiol Regul Integr Comp Physiol.*

ELKIND-HIRSCH, K. E., SHERMAN, L. D. & MALINAK, R. (1993). Hormone replacement therapy alters insulin sensitivity in young women with premature ovarian failure. *J Clin Endocrinol Metab* **76,** 472-5.

ELLIS, G. S., LANZA-JACOBY, S., GOW, A. & KENDRICK, Z. V. (1994). Effects of estradiol on lipoprotein lipase activity and lipid availability in exercised male rats. *J Appl Physiol* **77,** 209-15.

ESBJORNSSON-LILJEDAHL, M., SUNDBERG, C. J., NORMAN, B. & JANSSON, E. (1999). Metabolic response in type I and type II muscle fibers during a 30-s cycle sprint in men and women. *J Appl Physiol* **87,** 1326-32.

ESBJORNSSON, M., SYLVEN, C., HOLM, I. & JANSSON, E. (1993). Fast twitch fibres may predict anaerobic performance in both females and males. *Int J Sports Med* **14,** 257-63.

ESSEN, B., JANSSON, E., HENRIKSSON, J., TAYLOR, A. W. & SALTIN, B. (1975). Metabolic characteristics of fibre types in human skeletal muscle. *Acta Physiol Scand* **95,** 153-65.

FLECHTNER-MORS, M., DITSCHUNEIT, H. H., YIP, I. & ADLER, G. (1999). Sympathetic modulation of lipolysis in subcutaneous adipose tissue: effects of gender and energy restriction. *J Lab Clin Med* **134,** 33-41.

FOX, J., GARBER, P., HOFFMAN, M., JOHNSON, D., SCHAEFER, P., VIEN, J., ZEATON, C. & THOMPSON, L. V. (2003). Morphological characteristics of skeletal muscles in relation to gender. *Aging Clin Exp Res* **15,** 264-9.

FRIEDLANDER, A. L., CASAZZA, G. A., HORNING, M. A., HUIE, M. J., PIACENTINI, M. F., TRIMMER, J. K. & BROOKS, G. A. (1998). Training-induced alterations of carbohydrate metabolism in women: women respond differently from men. *J Appl Physiol* **85,** 1175-86.

FRITZ, T., KRAMER, D. K., KARLSSON, H. K., GALUSKA, D., ENGFELDT, P., ZIERATH, J. R. & KROOK, A. (2006). Low-intensity exercise increases skeletal muscle protein expression of PPARdelta and UCP3 in type 2 diabetic patients. *Diabetes Metab Res Rev.*

FUJII, H., SHIMOMURA, Y., MURAKAMI, T., NAKAI, N., SATO, T., SUZUKI, M. & HARRIS, R. A. (1998). Branched-chain alpha-keto acid dehydrogenase kinase content in rat skeletal muscle is decreased by endurance training. *Biochem Mol Biol Int* **44,** 1211-6.

GARREL, D. R., WELSCH, C., ARNAUD, M. J. & TOURNIAIRE, J. (1985). Relationship of the menstrual cycle and thyroid hormones to whole-body protein turnover in women. *Hum Nutr Clin Nutr* **39,** 29-37.

GIRGENRATH, S., SONG, K. & WHITTEMORE, L. A. (2005). Loss of myostatin expression alters fiber-type distribution and expression of myosin heavy chain isoforms in slow- and fast-type skeletal muscle. *Muscle Nerve* **31,** 34-40.

GOEDECKE, J. H., ST CLAIR GIBSON, A., GROBLER, L., COLLINS, M., NOAKES, T. D. & LAMBERT, E. V. (2000). Determinants of the variability in respiratory exchange ratio

at rest and during exercise in trained athletes. *Am J Physiol Endocrinol Metab* **279,** E1325-34.

GONZALEZ-CADAVID, N. F., TAYLOR, W. E., YARASHESKI, K., SINHA-HIKIM, I., MA, K., EZZAT, S., SHEN, R., LALANI, R., ASA, S., MAMITA, M., NAIR, G., ARVER, S. & BHASIN, S. (1998). Organization of the human myostatin gene and expression in healthy men and HIV-infected men with muscle wasting. *Proc Natl Acad Sci U S A* **95,** 14938-43.

GOODYEAR, L. J., HIRSHMAN, M. F., SMITH, R. J. & HORTON, E. S. (1991). Glucose transporter number, activity, and isoform content in plasma membranes of red and white skeletal muscle. *Am J Physiol* **261,** E556-61.

GORSKI, J., STANKIEWICZ, B., BRYCKA, R. & KICZKA, K. (1976). The effect of estradiol on carbohydrate utilization during prolonged exercise in rats. *Acta Physiol Pol* **27,** 361-7.

GOTO, M., TERADA, S., KATO, M., KATOH, M., YOKOZEKI, T., TABATA, I. & SHIMOKAWA, T. (2000). cDNA Cloning and mRNA analysis of PGC-1 in epitrochlearis muscle in swimming-exercised rats. *Biochem Biophys Res Commun* **274,** 350-4.

GRIMALDI, P. A. (2003). Roles of PPARdelta in the control of muscle development and metabolism. *Biochem Soc Trans* **31,** 1130-2.

GROBET, L., PIROTTIN, D., FARNIR, F., PONCELET, D., ROYO, L. J., BROUWERS, B., CHRISTIANS, E., DESMECHT, D., COIGNOUL, F., KAHN, R. & GEORGES, M. (2003). Modulating skeletal muscle mass by postnatal, muscle-specific inactivation of the myostatin gene. *Genesis* **35,** 227-38.

HACKNEY, A. C., MCCRACKEN-COMPTON, M. A. & AINSWORTH, B. (1994). Substrate responses to submaximal exercise in the midfollicular and midluteal phases of the menstrual cycle. *Int J Sport Nutr* **4,** 299-308.

HAMADEH, M. J., DEVRIES, M. C. & TARNOPOLSKY, M. A. (2005). Estrogen supplementation reduces whole body leucine and carbohydrate oxidation and increases lipid oxidation in men during endurance exercise. *J Clin Endocrinol Metab* **90,** 3592-9.

HAMBRECHT, R., FIEHN, E., YU, J., NIEBAUER, J., WEIGL, C., HILBRICH, L., ADAMS, V., RIEDE, U. & SCHULER, G. (1997). Effects of endurance training on mitochondrial ultrastructure and fiber type distribution in skeletal muscle of patients with stable chronic heart failure. *J Am Coll Cardiol* **29,** 1067-73.

HAMOSH, M. & HAMOSH, P. (1975). The effect of estrogen on the lipoprotein lipase activity of rat adipose tissue. *J Clin Invest* **55,** 1132-5.

HANSEN, F. M., FAHMY, N. & NIELSEN, J. H. (1980). The influence of sexual hormones on lipogenesis and lipolysis in rat fat cells. *Acta Endocrinol (Copenh)* **95,** 566-70.

HARDS, J. M., REID, W. D., PARDY, R. L. & PARE, P. D. (1990). Respiratory muscle fiber morphometry. Correlation with pulmonary function and nutrition. *Chest* **97,** 1037-44.

HATTA, H., ATOMI, Y., SHINOHARA, S., YAMAMOTO, Y. & YAMADA, S. (1988). The effects of ovarian hormones on glucose and fatty acid oxidation during exercise in female ovariectomized rats. *Horm Metab Res* **20,** 609-11.

HELLSTROM, L., BLAAK, E. & HAGSTROM-TOFT, E. (1996). Gender differences in adrenergic regulation of lipid mobilization during exercise. *Int J Sports Med* **17,** 439-47.

HERRERO, P., SOTO, P. F., DENCE, C. S., KISRIEVA-WARE, Z., DELANO, D. A., PETERSON, L. R. & GROPLER, R. J. (2005). Impact of hormone replacement on myocardial fatty acid metabolism: potential role of estrogen. *J Nucl Cardiol* **12,** 574-81.

HIRSHMAN, M. F., GOODYEAR, L. J., WARDZALA, L. J., HORTON, E. D. & HORTON, E. S. (1990). Identification of an intracellular pool of glucose transporters from basal and insulin-stimulated rat skeletal muscle. *J Biol Chem* **265,** 987-91.

HOROWITZ, J. F., LEONE, T. C., FENG, W., KELLY, D. P. & KLEIN, S. (2000). Effect of endurance training on lipid metabolism in women: a potential role for PPARalpha in the metabolic response to training. *Am J Physiol Endocrinol Metab* **279,** E348-55.

HORTON, T. J., MILLER, E. K., GLUECK, D. & TENCH, K. (2002). No effect of menstrual cycle phase on glucose kinetics and fuel oxidation during moderate-intensity exercise. *Am J Physiol Endocrinol Metab* **282,** E752-62.

HORTON, T. J., PAGLIASSOTTI, M. J., HOBBS, K. & HILL, J. O. (1998). Fuel metabolism in men and women during and after long-duration exercise. *J Appl Physiol* **85,** 1823-32.

HOWEL, D. (1994). Statistical analysis of compositional data in anatomy. *Anat Rec* **240,** 625-31.

HRBOTICKY, N., LEITER, L. A. & ANDERSON, G. H. (1989). Menstrual cycle effects on the metabolism of tryptophan loads. *Am J Clin Nutr* **50,** 46-52.

IKEDA, S., MIYAZAKI, H., NAKATANI, T., KAI, Y., KAMEI, Y., MIURA, S., TSUBOYAMA-KASAOKA, N. & EZAKI, O. (2002). Up-regulation of SREBP-1c and lipogenic genes

in skeletal muscles after exercise training. *Biochem Biophys Res Commun* **296,** 395-400.

JACOBS, K. A., CASAZZA, G. A., SUH, S. H., HORNING, M. A. & BROOKS, G. A. (2005). Fatty acid reesterification but not oxidation is increased by oral contraceptive use in women. *J Appl Physiol* **98,** 1720-31.

JORGENSEN, S. B., WOJTASZEWSKI, J. F., VIOLLET, B., ANDREELLI, F., BIRK, J. B., HELLSTEN, Y., SCHJERLING, P., VAULONT, S., NEUFER, P. D., RICHTER, E. A. & PILEGAARD, H. (2005). Effects of alpha-AMPK knockout on exercise-induced gene activation in mouse skeletal muscle. *Faseb J* **19,** 1146-8.

KAMEI, Y., SUZUKI, M., MIYAZAKI, H., TSUBOYAMA-KASAOKA, N., WU, J., ISHIMI, Y. & EZAKI, O. (2005). Ovariectomy in mice decreases lipid metabolism-related gene expression in adipose tissue and skeletal muscle with increased body fat. *J Nutr Sci Vitaminol (Tokyo)* **51,** 110-7.

KANALEY, J. A., BOILEAU, R. A., BAHR, J. A., MISNER, J. E. & NELSON, R. A. (1992). Substrate oxidation and GH responses to exercise are independent of menstrual phase and status. *Med Sci Sports Exerc* **24,** 873-80.

KASPEREK, G. J., DOHM, G. L. & SNIDER, R. D. (1985). Activation of branched-chain keto acid dehydrogenase by exercise. *Am J Physiol* **248,** R166-71.

KASPEREK, G. J. & SNIDER, R. D. (1987). Effect of exercise intensity and starvation on activation of branched-chain keto acid dehydrogenase by exercise. *Am J Physiol* **252,** E33-7.

KENDRICK, Z. V. & ELLIS, G. S. (1991). Effect of estradiol on tissue glycogen metabolism and lipid availability in exercised male rats. *J Appl Physiol* **71,** 1694-9.

KENDRICK, Z. V., STEFFEN, C. A., RUMSEY, W. L. & GOLDBERG, D. I. (1987). Effect of estradiol on tissue glycogen metabolism in exercised oophorectomized rats. *J Appl Physiol* **63,** 492-6.

KERSTEN, S., DESVERGNE, B. & WAHLI, W. (2000). Roles of PPARs in health and disease. *Nature* **405,** 421-4.

KIENS, B., ROEPSTORFF, C., GLATZ, J. F., BONEN, A., SCHJERLING, P., KNUDSEN, J. & NIELSEN, J. N. (2004). Lipid-binding proteins and lipoprotein lipase activity in human skeletal muscle: influence of physical activity and gender. *J Appl Physiol* **97,** 1209-18.

KNECHTLE, B., MULLER, G., WILLMANN, F., KOTTECK, K., ESER, P. & KNECHT, H. (2004). Fat oxidation in men and women endurance athletes in running and cycling. *Int J Sports Med* **25,** 38-44.

KOVAL, J. A., DEFRONZO, R. A., O'DOHERTY, R. M., PRINTZ, R., ARDEHALI, H., GRANNER, D. K. & MANDARINO, L. J. (1998). Regulation of hexokinase II activity and expression in human muscle by moderate exercise. *Am J Physiol* **274,** E304-8.

KRANIOU, Y., CAMERON-SMITH, D., MISSO, M., COLLIER, G. & HARGREAVES, M. (2000). Effects of exercise on GLUT-4 and glycogenin gene expression in human skeletal muscle. *J Appl Physiol* **88,** 794-6.

KRIENGSINYOS, W., WYKES, L. J., GOONEWARDENE, L. A., BALL, R. O. & PENCHARZ, P. B. (2004). Phase of menstrual cycle affects lysine requirement in healthy women. *Am J Physiol Endocrinol Metab* **287,** E489-96.

LAMONT, L. S., MCCULLOUGH, A. J. & KALHAN, S. C. (2001). Gender differences in leucine, but not lysine, kinetics. *J Appl Physiol* **91,** 357-62.

LAMONT, L. S., MCCULLOUGH, A. J. & KALHAN, S. C. (2003). Gender differences in the regulation of amino acid metabolism. *J Appl Physiol* **95,** 1259-65.

LARIVIERE, F., MOUSSALLI, R. & GARREL, D. R. (1994). Increased leucine flux and leucine oxidation during the luteal phase of the menstrual cycle in women. *Am J Physiol* **267,** E422-8.

LEE, S. J. (2004). Regulation of muscle mass by myostatin. *Annu Rev Cell Dev Biol* **20,** 61-86.

LIN, J., WU, H., TARR, P. T., ZHANG, C. Y., WU, Z., BOSS, O., MICHAEL, L. F., PUIGSERVER, P., ISOTANI, E., OLSON, E. N., LOWELL, B. B., BASSEL-DUBY, R. & SPIEGELMAN, B. M. (2002). Transcriptional co-activator PGC-1 alpha drives the formation of slow-twitch muscle fibres. *Nature* **418,** 797-801.

LUQUET, S., LOPEZ-SORIANO, J., HOLST, D., FREDENRICH, A., MELKI, J., RASSOULZADEGAN, M. & GRIMALDI, P. A. (2003). Peroxisome proliferator-activated receptor delta controls muscle development and oxidative capability. *Faseb J* **17,** 2299-301.

LUQUET, S., LOPEZ-SORIANO, J., HOLST, D., GAUDEL, C., JEHL-PIETRI, C., FREDENRICH, A. & GRIMALDI, P. A. (2004). Roles of peroxisome proliferator-activated receptor delta (PPARdelta) in the control of fatty acid catabolism. A new target for the treatment of metabolic syndrome. *Biochimie* **86,** 833-7.

MANDARD, S., MULLER, M. & KERSTEN, S. (2004). Peroxisome proliferator-activated receptor alpha target genes. *Cell Mol Life Sci* **61,** 393-416.

MANDOUR, T., KISSEBAH, A. H. & WYNN, V. (1977). Mechanism of oestrogen and progesterone effects on lipid and carbohydrate metabolism: alteration in the insulin: glucagon molar ratio and hepatic enzyme activity. *Eur J Clin Invest* **7,** 181-7.

MANNION, A. F., DUMAS, G. A., COOPER, R. G., ESPINOSA, F. J., FARIS, M. W. & STEVENSON, J. M. (1997). Muscle fibre size and type distribution in thoracic and lumbar regions of erector spinae in healthy subjects without low back pain: normal values and sex differences. *J Anat* **190 (Pt 4),** 505-13.

MARLISS, E. B., KREISMAN, S. H., MANZON, A., HALTER, J. B., VRANIC, M. & NESSIM, S. J. (2000). Gender differences in glucoregulatory responses to intense exercise. *J Appl Physiol* **88,** 457-66.

MATUTE, M. L. & KALKHOFF, R. K. (1973). Sex steroid influence on hepatic gluconeogenesis and glucogen formation. *Endocrinology* **92,** 762-8.

MCKENZIE, S., PHILLIPS, S. M., CARTER, S. L., LOWTHER, S., GIBALA, M. J. & TARNOPOLSKY, M. A. (2000). Endurance exercise training attenuates leucine oxidation and BCOAD activation during exercise in humans. *Am J Physiol Endocrinol Metab* **278,** E580-7.

MILLER, A. E., MACDOUGALL, J. D., TARNOPOLSKY, M. A. & SALE, D. G. (1993). Gender differences in strength and muscle fiber characteristics. *Eur J Appl Physiol Occup Physiol* **66,** 254-62.

MITTENDORFER, B., HOROWITZ, J. F. & KLEIN, S. (2002). Effect of gender on lipid kinetics during endurance exercise of moderate intensity in untrained subjects. *Am J Physiol Endocrinol Metab* **283,** E58-65.

MOLLER, S. E., MOLLER, B. M., OLESEN, M. & FJALLAND, B. (1996). Effects of oral contraceptives on plasma neutral amino acids and cholesterol during a menstrual cycle. *Eur J Clin Pharmacol* **50,** 179-84.

NEUFER, P. D. & DOHM, G. L. (1993). Exercise induces a transient increase in transcription of the GLUT-4 gene in skeletal muscle. *Am J Physiol* **265,** C1597-603.

NORDSBORG, N., BANGSBO, J. & PILEGAARD, H. (2003). Effect of high-intensity training on exercise-induced gene expression specific to ion homeostasis and metabolism. *J Appl Physiol* **95,** 1201-6.

NORRBOM, J., SUNDBERG, C. J., AMELN, H., KRAUS, W. E., JANSSON, E. & GUSTAFSSON, T. (2004). PGC-1alpha mRNA expression is influenced by metabolic perturbation in exercising human skeletal muscle. *J Appl Physiol* **96,** 189-94.

O'DOHERTY, R. M., BRACY, D. P., GRANNER, D. K. & WASSERMAN, D. H. (1996). Transcription of the rat skeletal muscle hexokinase II gene is increased by acute exercise. *J Appl Physiol* **81,** 789-93.

O'DOHERTY, R. M., BRACY, D. P., OSAWA, H., WASSERMAN, D. H. & GRANNER, D. K. (1994). Rat skeletal muscle hexokinase II mRNA and activity are increased by a single bout of acute exercise. *Am J Physiol* **266,** E171-8.

OTIS, C. L., DRINKWATER, B., JOHNSON, M., LOUCKS, A. & WILMORE, J. (1997). American College of Sports Medicine position stand. The Female Athlete Triad. *Med Sci Sports Exerc* **29,** i-ix.

PERSEGHIN, G., SCIFO, P., PAGLIATO, E., BATTEZZATI, A., BENEDINI, S., SOLDINI, L., TESTOLIN, G., DEL MASCHIO, A. & LUZI, L. (2001). Gender factors affect fatty acids-induced insulin resistance in nonobese humans: effects of oral steroidal contraception. *J Clin Endocrinol Metab* **86,** 3188-96.

PHILLIPS, S. M., ATKINSON, S. A., TARNOPOLSKY, M. A. & MACDOUGALL, J. D. (1993). Gender differences in leucine kinetics and nitrogen balance in endurance athletes. *J Appl Physiol* **75,** 2134-41.

PHILLIPS, S. M., HAN, X. X., GREEN, H. J. & BONEN, A. (1996). Increments in skeletal muscle GLUT-1 and GLUT-4 after endurance training in humans. *Am J Physiol* **270,** E456-62.

PILEGAARD, H., ORDWAY, G. A., SALTIN, B. & NEUFER, P. D. (2000). Transcriptional regulation of gene expression in human skeletal muscle during recovery from exercise. *Am J Physiol Endocrinol Metab* **279,** E806-14.

PILEGAARD, H., OSADA, T., ANDERSEN, L. T., HELGE, J. W., SALTIN, B. & NEUFER, P. D. (2005). Substrate availability and transcriptional regulation of metabolic genes in human skeletal muscle during recovery from exercise. *Metabolism* **54,** 1048-55.

PILEGAARD, H., SALTIN, B. & NEUFER, P. D. (2003). Exercise induces transient transcriptional activation of the PGC-1alpha gene in human skeletal muscle. *J Physiol* **546,** 851-8.

PRINCE, F. P., HIKIDA, R. S., HAGERMAN, F. C., STARON, R. S. & ALLEN, W. H. (1981). A morphometric analysis of human muscle fibers with relation to fiber types and adaptations to exercise. *J Neurol Sci* **49,** 165-79.

PUIGSERVER, P., WU, Z., PARK, C. W., GRAVES, R., WRIGHT, M. & SPIEGELMAN, B. M. (1998). A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis. *Cell* **92,** 829-39.

RAMIREZ, I. (1981). Estradiol-induced changes in lipoprotein lipase, eating, and body weight in rats. *Am J Physiol* **240,** E533-8.

REN, J. M., SEMENKOVICH, C. F., GULVE, E. A., GAO, J. & HOLLOSZY, J. O. (1994). Exercise induces rapid increases in GLUT4 expression, glucose transport capacity, and insulin-stimulated glycogen storage in muscle. *J Biol Chem* **269,** 14396-401.

RIDDELL, M. C., PARTINGTON, S. L., STUPKA, N., ARMSTRONG, D., RENNIE, C. & TARNOPOLSKY, M. A. (2003). Substrate utilization during exercise performed with and without glucose ingestion in female and male endurance trained athletes. *Int J Sport Nutr Exerc Metab* **13,** 407-21.

ROEPSTORFF, C., DONSMARK, M., THIELE, M., VISTISEN, B., STEWART, G., VISSING, K., SCHJERLING, P., HARDIE, D. G., GALBO, H. & KIENS, B. (2006a). Gender differences in hormone-sensitive lipase expression, activity and phosphorylation in skeletal muscle at rest and during exercise. *Am J Physiol Endocrinol Metab.*

ROEPSTORFF, C., SCHJERLING, P., VISTISEN, B., MADSEN, M., STEFFENSEN, C. H., RIDER, M. H. & KIENS, B. (2005a). Regulation of oxidative enzyme activity and eukaryotic elongation factor 2 in human skeletal muscle: influence of gender and exercise. *Acta Physiol Scand* **184,** 215-24.

ROEPSTORFF, C., STEFFENSEN, C. H., MADSEN, M., STALLKNECHT, B., KANSTRUP, I. L., RICHTER, E. A. & KIENS, B. (2002). Gender differences in substrate utilization during submaximal exercise in endurance-trained subjects. *Am J Physiol Endocrinol Metab* **282,** E435-47.

ROEPSTORFF, C., THIELE, M., HILLIG, T., PILEGAARD, H., RICHTER, E. A., WOJTASZEWSKI, J. F. & KIENS, B. (2006b). Higher skeletal muscle {alpha}2AMPK activation and lower energy charge and fat oxidation in men than in women during submaximal exercise. *J Physiol* **574,** 125-38.

ROEPSTORFF, C., THIELE, M., HILLIG, T., PILEGAARD, H., RICHTER, E. A., WOJTASZEWSKI, J. F. & KIENS, B. (2006c). Higher skeletal muscle alpha2AMPK activation and lower energy charge and fat oxidation in men than in women during submaximal exercise. *J Physiol* **574,** 125-38.

ROEPSTORFF, C., VISTISEN, B. & KIENS, B. (2005b). Intramuscular triacylglycerol in energy metabolism during exercise in humans. *Exerc Sport Sci Rev* **33,** 182-8.

ROMIJN, J. A., COYLE, E. F., SIDOSSIS, L. S., ROSENBLATT, J. & WOLFE, R. R. (2000). Substrate metabolism during different exercise intensities in endurance-trained women. *J Appl Physiol* **88,** 1707-14.

ROONEY, T. P., KENDRICK, Z. V., CARLSON, J., ELLIS, G. S., MATAKEVICH, B., LORUSSO, S. M. & MCCALL, J. A. (1993). Effect of estradiol on the temporal pattern of exercise-induced tissue glycogen depletion in male rats. *J Appl Physiol* **75,** 1502-6.

ROSE, A. J. & RICHTER, E. A. (2005). Skeletal muscle glucose uptake during exercise: how is it regulated? *Physiology (Bethesda)* **20,** 260-70.

ROSEN, E. D., WALKEY, C. J., PUIGSERVER, P. & SPIEGELMAN, B. M. (2000). Transcriptional regulation of adipogenesis. *Genes Dev* **14,** 1293-307.

ROSEN, G. J., O'BRYANT, E. L., SWENDER, D. & WADE, J. (2004). Fiber type composition of the muscle responsible for throat fan extension in green anole lizards. *Brain Behav Evol* **64,** 34-41.

RUBY, B. C., COGGAN, A. R. & ZDERIC, T. W. (2002). Gender differences in glucose kinetics and substrate oxidation during exercise near the lactate threshold. *J Appl Physiol* **92,** 1125-32.

RUBY, B. C. & ROBERGS, R. A. (1994). Gender differences in substrate utilisation during exercise. *Sports Med* **17,** 393-410.

RUBY, B. C., ROBERGS, R. A., WATERS, D. L., BURGE, M., MERMIER, C. & STOLARCZYK, L. (1997). Effects of estradiol on substrate turnover during exercise in amenorrheic females. *Med Sci Sports Exerc* **29,** 1160-9.

RUSH, J. W., MACLEAN, D. A., HULTMAN, E. & GRAHAM, T. E. (1995). Exercise causes branched-chain oxoacid dehydrogenase dephosphorylation but not AMP deaminase binding. *J Appl Physiol* **78,** 2193-200.

RUSSELL, A. P., FEILCHENFELDT, J., SCHREIBER, S., PRAZ, M., CRETTENAND, A., GOBELET, C., MEIER, C. A., BELL, D. R., KRALLI, A., GIACOBINO, J. P. & DERIAZ, O. (2003). Endurance training in humans leads to fiber type-specific increases in levels of peroxisome proliferator-activated receptor-gamma coactivator-1 and peroxisome proliferator-activated receptor-alpha in skeletal muscle. *Diabetes* **52,** 2874-81.

RUSSELL, A. P., HESSELINK, M. K., LO, S. K. & SCHRAUWEN, P. (2005). Regulation of metabolic transcriptional co-activators and transcription factors with acute exercise. *Faseb J* **19,** 986-8.

SALTIN, B. & PILEGAARD, H. (2002). [Metabolic fitness: physical activity and health]. *Ugeskr Laeger* **164,** 2156-62.

SCHANTZ, P. G. & HENRIKSSON, J. (1987). Enzyme levels of the NADH shuttle systems: measurements in isolated muscle fibres from humans of differing physical activity. *Acta Physiol Scand* **129,** 505-15.

SCHIAFFINO, S. & REGGIANI, C. (1996). Molecular diversity of myofibrillar proteins: gene regulation and functional significance. *Physiol Rev* **76,** 371-423.

SHEARER, J., GRAHAM, T. E., BATTRAM, D. S., ROBINSON, D. L., RICHTER, E. A., WILSON, R. J. & BAKOVIC, M. (2005a). Glycogenin activity and mRNA expression in response to volitional exhaustion in human skeletal muscle. *J Appl Physiol* **99,** 957-62.

SHEARER, J., WILSON, R. J., BATTRAM, D. S., RICHTER, E. A., ROBINSON, D. L., BAKOVIC, M. & GRAHAM, T. E. (2005b). Increases in glycogenin and glycogenin mRNA accompany glycogen resynthesis in human skeletal muscle. *Am J Physiol Endocrinol Metab* **289,** E508-14.

SHI, Y., HON, M. & EVANS, R. M. (2002). The peroxisome proliferator-activated receptor delta, an integrator of transcriptional repression and nuclear receptor signaling. *Proc Natl Acad Sci U S A* **99,** 2613-8.

SHIMOMURA, Y., FUJII, H., SUZUKI, M., FUJITSUKA, N., NAOI, M., SUGIYAMA, S. & HARRIS, R. A. (1993). Branched-chain 2-oxo acid dehydrogenase complex activation by tetanic contractions in rat skeletal muscle. *Biochim Biophys Acta* **1157,** 290-6.

SHORT, K. R., VITTONE, J. L., BIGELOW, M. L., PROCTOR, D. N., RIZZA, R. A., COENEN-SCHIMKE, J. M. & NAIR, K. S. (2003). Impact of aerobic exercise training on age-related changes in insulin sensitivity and muscle oxidative capacity. *Diabetes* **52,** 1888-96.

SIMONEAU, J. A. & BOUCHARD, C. (1989). Human variation in skeletal muscle fiber-type proportion and enzyme activities. *Am J Physiol* **257,** E567-72.

SIMONEAU, J. A., LORTIE, G., BOULAY, M. R., THIBAULT, M. C., THERIAULT, G. & BOUCHARD, C. (1985). Skeletal muscle histochemical and biochemical characteristics in sedentary male and female subjects. *Can J Physiol Pharmacol* **63,** 30-5.

SPRIET, L. L. & WATT, M. J. (2003). Regulatory mechanisms in the interaction between carbohydrate and lipid oxidation during exercise. *Acta Physiol Scand* **178,** 443-52.

STARON, R. S. (1997). Human skeletal muscle fiber types: delineation, development, and distribution. *Can J Appl Physiol* **22,** 307-27.

STARON, R. S., HAGERMAN, F. C., HIKIDA, R. S., MURRAY, T. F., HOSTLER, D. P., CRILL, M. T., RAGG, K. E. & TOMA, K. (2000). Fiber type composition of the vastus lateralis muscle of young men and women. *J Histochem Cytochem* **48,** 623-9.

STEFFENSEN, C. H., ROEPSTORFF, C., MADSEN, M. & KIENS, B. (2002). Myocellular triacylglycerol breakdown in females but not in males during exercise. *Am J Physiol Endocrinol Metab* **282,** E634-42.

STEVENS, L., BASTIDE, B., BOZZO, C. & MOUNIER, Y. (2004). Hybrid fibres under slow-to-fast transformations: expression is of myosin heavy and light chains in rat soleus muscle. *Pflugers Arch* **448,** 507-14.

TARNOPOLSKY, L. J., MACDOUGALL, J. D., ATKINSON, S. A., TARNOPOLSKY, M. A. & SUTTON, J. R. (1990). Gender differences in substrate for endurance exercise. *J Appl Physiol* **68,** 302-8.

TARNOPOLSKY, M. A. (2000). Gender differences in substrate metabolism during endurance exercise. *Can J Appl Physiol* **25,** 312-27.

TARNOPOLSKY, M. A., RENNIE, C. D., ROBERTSHAW, H. A., FEDAK-TARNOPOLSKY, S. N., DEVRIES, M. C. & HAMADEH, M. J. (2006). The Influence of Endurance Exercise Training and Sex on Intramyocellular Lipid and Mitochondrial Ultrastructure, Substrate Use, and Mitochondrial Enzyme Activity. *Am J Physiol Regul Integr Comp Physiol.*

TARNOPOLSKY, M. A., ROY, B. D., MACDONALD, J. R., MCKENZIE, S., MARTIN, J. & ETTINGER, S. (2001). Short-term 17-beta-estradiol administration does not affect metabolism in young males. *Int J Sports Med* **22,** 175-80.

TARNOPOLSKY, M. A. & RUBY, B. C. (2001). Sex differences in carbohydrate metabolism. *Curr Opin Clin Nutr Metab Care* **4,** 521-6.

TARNOPOLSKY, M. A. & SARIS, W. H. (2001). Evaluation of gender differences in physiology: an introduction. *Curr Opin Clin Nutr Metab Care* **4,** 489-92.

TERADA, S., GOTO, M., KATO, M., KAWANAKA, K., SHIMOKAWA, T. & TABATA, I. (2002). Effects of low-intensity prolonged exercise on PGC-1 mRNA expression in rat epitrochlearis muscle. *Biochem Biophys Res Commun* **296,** 350-4.

TERADA, S. & TABATA, I. (2004). Effects of acute bouts of running and swimming exercise on PGC-1alpha protein expression in rat epitrochlearis and soleus muscle. *Am J Physiol Endocrinol Metab* **286,** E208-16.

TUNSTALL, R. J., MEHAN, K. A., WADLEY, G. D., COLLIER, G. R., BONEN, A., HARGREAVES, M. & CAMERON-SMITH, D. (2002). Exercise training increases lipid

metabolism gene expression in human skeletal muscle. *Am J Physiol Endocrinol Metab* **283,** E66-72.

VEGA, R. B., HUSS, J. M. & KELLY, D. P. (2000). The coactivator PGC-1 cooperates with peroxisome proliferator-activated receptor alpha in transcriptional control of nuclear genes encoding mitochondrial fatty acid oxidation enzymes. *Mol Cell Biol* **20,** 1868-76.

VENABLES, M. C., ACHTEN, J. & JEUKENDRUP, A. E. (2005). Determinants of fat oxidation during exercise in healthy men and women: a cross-sectional study. *J Appl Physiol* **98,** 160-7.

VESTERGAARD, H. (1999). Studies of gene expression and activity of hexokinase, phosphofructokinase and glycogen synthase in human skeletal muscle in states of altered insulin-stimulated glucose metabolism. *Dan Med Bull* **46,** 13-34.

VOLPI, E., LUCIDI, P., BOLLI, G. B., SANTEUSANIO, F. & DE FEO, P. (1998). Gender differences in basal protein kinetics in young adults. *J Clin Endocrinol Metab* **83,** 4363-7.

WAGENMAKERS, A. J., BROOKES, J. H., COAKLEY, J. H., REILLY, T. & EDWARDS, R. H. (1989). Exercise-induced activation of the branched-chain 2-oxo acid dehydrogenase in human muscle. *Eur J Appl Physiol Occup Physiol* **59,** 159-67.

WAGENMAKERS, A. J., SCHEPENS, J. T. & VEERKAMP, J. H. (1984). Effect of starvation and exercise on actual and total activity of the branched-chain 2-oxo acid dehydrogenase complex in rat tissues. *Biochem J* **223,** 815-21.

WANG, Y. X., ZHANG, C. L., YU, R. T., CHO, H. K., NELSON, M. C., BAYUGA-OCAMPO, C. R., HAM, J., KANG, H. & EVANS, R. M. (2004). Regulation of muscle fiber type and running endurance by PPARdelta. *PLoS Biol* **2,** e294.

WATT, M. J., SOUTHGATE, R. J., HOLMES, A. G. & FEBBRAIO, M. A. (2004). Suppression of plasma free fatty acids upregulates peroxisome proliferator-activated receptor (PPAR) alpha and delta and PPAR coactivator 1alpha in human skeletal muscle, but not lipid regulatory genes. *J Mol Endocrinol* **33,** 533-44.

WEBER, F. E. & PETTE, D. (1990). Rapid up- and down-regulation of hexokinase II in rat skeletal muscle in response to altered contractile activity. *FEBS Lett* **261,** 291-3.

WENZ, M., BEREND, J. Z., LYNCH, N. A., CHAPPELL, S. & HACKNEY, A. C. (1997). Substrate oxidation at rest and during exercise: effects of menstrual cycle phase and diet composition. *J Physiol Pharmacol* **48,** 851-60.

WILSON, D. E., FLOWERS, C. M., CARLILE, S. I. & UDALL, K. S. (1976). Estrogen treatment and gonadal function in the regulation of lipoprotein lipase. *Atherosclerosis* **24,** 491-9.

WU, Z., PUIGSERVER, P., ANDERSSON, U., ZHANG, C., ADELMANT, G., MOOTHA, V., TROY, A., CINTI, S., LOWELL, B., SCARPULLA, R. C. & SPIEGELMAN, B. M. (1999). Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. *Cell* **98,** 115-24.

YASUDA, N., GLOVER, E. I., PHILLIPS, S. M., ISFORT, R. J. & TARNOPOLSKY, M. A. (2005). Sex-based differences in skeletal muscle function and morphology with short-term limb immobilization. *J Appl Physiol* **99,** 1085-92.

ZDERIC, T. W., COGGAN, A. R. & RUBY, B. C. (2001). Glucose kinetics and substrate oxidation during exercise in the follicular and luteal phases. *J Appl Physiol* **90,** 447-53.

CHAPTER 2: Manuscript 1

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Acute endurance exercise, sex and menstrual cycle influence the mRNA content for genes involved in lipid metabolism in human skeletal muscle

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ABSTRACT

An increase in fat oxidation is frequently observed during endurance exercise in females vs. males. Consequently, we hypothesized that sex and menstrual cycle phase would influence basal and exercise induced mRNA content for genes involved in lipid metabolism in human skeletal muscle. Twelve men and 12 women had muscle biopsies taken from their vastus lateralis before and after a 90 min bout of cycling at 65 % VO_{2peak}. Women were studied in the mid-follicular (FOL) and mid-luteal (LUT) phases (~ 2 weeks apart). mRNA content was determined using TaqMan® RT-PCR with β2-microglobulin as an internal standard for genes involved in intramyocellular lipid synthesis (SREBP-1c, SREBP-2, mtGPAT) and hydrolysis (HSL), sarcolemmal and mitochondrial FFA transport (FATm, FABPc, CPTI, CPTII), β-oxidation (VLCAD, LCAD, TFP-α), and transcriptional regulation (PGC-1α, PPARα, PPARγ, PPARδ). Exercise ($P < 0.05$) increased mRNA content of PGC-1α (men, LUT), FABPc (men, FOL, LUT), CPTI (men, LUT), and LCAD (LUT) (1.1-7.9 fold). Women had a higher ($P \leq 0.05$) mRNA content of FATm, FABPc, SREBP-1c and mtGPAT (FOL, LUT), PPARα (FOL), CPTI (FOL), LCAD (FOL) and TFPα (LUT) pre- and post- exercise, PPARδ (FOL) at rest, and PPARα (LUT) and LCAD(LUT) after exercise (1.4- 3.3 fold), than men. The mRNA content of PPARδ and LCAD were higher ($P < 0.05$) for FOL vs. LUT phase (1.7-2.6 fold), while post-exercise FABPc mRNA content increased to a greater extent in LUT vs. FOL phase ($P = 0.0001$). In conclusion, sex, acute endurance exercise and menstrual cycle influence the content of mRNA species involved in lipid turnover in human skeletal muscle and these changes would support a higher capacity for fat oxidation in women vs. men.

INTRODUCTION

A number of studies found that women oxidize more fat during endurance exercise as compared with men (6, 13, 17, 25, 34, 39, 42, 51, 53). Expectedly, this has often translated into lesser carbohydrate oxidation during endurance exercise for women as compared with men (6, 13, 33, 42, 47). Animal studies have also implicated sex differences in exercise metabolism with female rats showing higher intramyocellular lipid (IMCL) content (36), lipid oxidation and muscle LPL activity, as compared with male rats (11, 22). Furthermore, oophorectomy of female rats reduced the oxidation of fat during endurance exercise (23). The potential mechanism(s) of such sex differences have only recently been evaluated in human based research (4, 24, 44).

Several studies have found that women have higher IMCL content (38, 53) and lipoprotein lipase (LPL) mRNA content (24) as compared to men matched for fitness level. Women have higher whole body lipolysis than men (35). Women also show a greater skeletal muscle uptake of plasma free fatty acids (35), potentially due to higher sarcolemmal free fatty acid (FFA) transport protein content (FAT/CD36) (24). It has also been reported that compared to men, women have higher mRNA levels of membrane fatty acid transport protein 1 (FATm) (4), FAT/CD36 and FABPpm (24). Women also have a higher mRNA, but not protein, content

of β-hydroxyacyl-CoA dehydrogenase (β-HAD) (44); however, CPT I activity was not different between men and women (2). Given that there are many proteins involved in lipid metabolism in human skeletal muscle, there are many more potential mRNA species that require evaluation in order to comprehensively evaluate potential molecular mechanisms for the observed differences in substrate oxidation between men and women (6, 13, 17, 25,

34, 39, 42, 51, 53). Furthermore, most studies have either not considered menstrual phase in the evaluation of sex differences in metabolism or have studied women in only the follicular (FOL) phase of the menstrual cycle (2, 4, 42).

Studies about the influence of menstrual cycle phase on fat oxidation during endurance exercise show controversial results (14, 16, 21, 58, 59). Although some studies did not find menstrual cycle phase influences on substrate metabolism during endurance exercise (16, 21), several have found that lipid oxidation was greater during the luteal (LUT) as compared to the FOL phase during low- to moderate-intensity endurance exercise (14, 58, 59). In agreement with these observations, plasma glucose turnover is lower during the LUT phase (59), yet somewhat paradoxically, amino acid oxidation was found to be higher in the LUT phase (26, 27).

Given that sex differences in lipid metabolism are predominantly apparent only during exercise (6, 13, 17, 25, 34, 39, 42, 51, 53), and that acute exercise is a potent stimulus for mRNA induction (5), it is important to consider sex and menstrual cycle differences in mRNA expression in response to acute exercise. A single bout of endurance exercise in men alters the mRNA content of a diverse set of genes involved in substrate oxidation in skeletal muscle (5). One of the most robust and well characterized mRNA species to show an up-regulation is the "master regulator" of mitochondrial biogenesis, peroxisome proliferator activated receptor-γ coactivator-1α (PGC-1α) (31, 37, 41, 55), due to an increase in mRNA transcription (41). Acute exercise also increased the transcription (41) and mRNA content (41, 57) of peroxisome proliferator activated receptor - alpha (PPARα), and peroxisome proliferator activated receptor - delta (PPARδ) mRNA (57) in

human skeletal muscle. Exercise also acutely increased the mRNA content of genes involved in FFA sarcolemmal transport (FAT/CD36 and FABPpm) (24), and both content and transcription of CPTI and LPL in human skeletal muscle (40, 41).

The purpose of this study was to comprehensively evaluate whether sex, acute exercise, or menstrual cycle influenced the mRNA content of genes involved in many aspects of lipid metabolism in human skeletal muscle. Specifically, we evaluated mRNA content for genes involved in the transcriptional regulation of mitochondrial biogenesis (PCG-1α) and lipid metabolism (peroxisome proliferator activated receptor-γ (PPARγ), PPARα and PPARδ), IMCL (sterol regulatory element binding protein –1c (SREBP-1c), mitochondrial glycerol phosphate acyltransferase (mtGPAT)) and membrane lipid (sterol regulatory element binding protein-2 (SREBP-2)) synthesis, IMCL hydrolysis (hormone sensitive lipase(HSL), sarcolemmal (membrane fatty acid transport protein 1 (FATm), cytosol fatty acid binding protein(FABPc)) and mitochondrial free fatty acid (FFA) transport (carnitine palmityltransferase I (CPTI) and carnitine palmityltransferase II (CPTII)) free fatty acid (FFA) transport, and β-oxidation (very long chain acyl-CoA dehydrogenase (VLCAD), long chain acyl-CoA dehydrogenase (LCAD) and trifunctional protein alpha subunit (TFP-α). We specifically hypothesized that the mRNA content of many of the genes involved in lipid metabolism would be higher in women as compared to men before and after acute exercise and that menstrual cycle would influence the mRNA content of fewer genes as compared with the sex difference, given the less consistent evidence for a phase related influence on exercise mediated substrate selection. Finally, given that men and women appear to respond similarly to endurance exercise training with

respect to increases in mitochondrial capacity (7), we hypothesized that PCG-1α would increase similarly after exercise for men and women, independent of menstrual phase. We also planned to evaluate sex-based and exercise-induced differences in a number of mRNA species involved in lipid metabolism that had not previously been evaluated in human skeletal muscle (FABPc, HSL, SREBP-2, GPAT, TFP-α, VLCAD, LCAD, CPTII).

MATERIAL AND METHODS

Subjects. Twenty-four, young (21.9 ± 2.2 y) healthy, non-smoking, non-obese, recreationally active men (n = 12) and women (n = 12) participated in the current study. They were involved in recreational physical activity but were not trained athletes. In addition, their diet and activity patterns were constant in the 3 - 4 weeks prior to commencing the study as determined by a questionnaire. The female subject characteristics were: age, 22 ± 2 y (mean ± SD); weight, 61 ± 9 kg; BMI, 23 ± 3 kg/m^2; fat-free mass, 73 ± 5 % body weight; VO_{2peak}, 55 ± 9 mL O_2/kg FFM/min. The male subject characteristics were: age, 22 ± 6 y; weight, 78 ± 11 kg; BMI, 25 ± 3 kg/m^2; fat-free mass, 81 ± 5 % body weight; VO_{2peak}, 55 ± 9 mL O_2/kg FFM/min. Before inclusion into the study, potential subjects were required to complete a health questionnaire to ensure that they were healthy and fit to participate. Each subject was given an information sheet describing all testing procedures, was informed of the purposes and associated risks, and gave written consent prior to participation. The projects conformed to the standards set by the Declaration of Helsinki, and were approved by the McMaster University Hamilton Health Sciences Human Research Ethics Board.

Study design. All subjects maintained their normal activity level throughout the course of the study. Food intake was also recorded for 4-days prior to commencing the study and was used to control diet during the second arm of the study. Women were tested in the mid-follicular (FOL, days 7-9) and mid-luteal (LUT, days 19-21) phases of the menstrual cycle. Ovulation was determined by usage of an ovulation test kit (Clear Plan Easy, Novartis). The remaining six females were taking a triphasic pill and thus determination of

ovulation was not required. Women taking oral contraceptives were tested on days 7-9 and 19-21 following their previous menstrual cycle. VO_2max was determined at least 7 d before the first testing day (15). On the morning of the test day the subjects reported to the lab. Muscle biopsies were taken from the vastus lateralis muscle both before and immediately after the acute exercise bout of 90 min cycling at 65% VO_2 peak. Samples were taken from the vastus lateralis of the nondominant leg ~20 cm proximal to the lateral knee joint of each subject under local anesthesia using manual suction and the women had the second set of biopsies (in the other menstrual cycle phase) taken from the contralateral leg. All biopsies were taken from separate incisions ~3cm apart, to minimize the potential effect of the biopsy itself on gene expression (37). Approximately 150 mg of muscle was taken from each biopsy, and this was immediately examined for and if needed, dissected from adherent fat and connective tissue. The first piece (~ 25 mg) dissected from this muscle sample was rapidly placed in an RNase-free polyethylene tube, flash-frozen in liquid nitrogen, and stored at –86°C until being processed for analysis.

Preparation of RNA. Total RNA was extracted from frozen human muscle according to Tarnopolsky et al. (52) and Mahoney at el. (31). Briefly, 25 -50 mg of human skeletal muscle biopsy was homogenized in 1 ml of TRIzol reagent (Invitrogen, Carlsbad, CA) at 4^0C, left at room temperature for 5 – 10 minutes, followed by addition of 0.2 ml of chloroform, vortexing for 15 s, and then centrifuged at 12,000 rpm at 4^0C for 15 min. The supernatant aqueous layer was transferred to a fresh tube and mixed with 0.5 ml of isopropanol ethanol, stood at ~ 22^0C for 10 min, and centrifuged at 12,000 rpm at 4^0C for 10 min. The RNA pellet was washed twice with 0.5 ml of 75% ethanol and air dried and

dissolved in 14 µl of Depc-treated ddH_2O. Aliquots of 2 µl each was made and stored at –80 ^{0}C. The concentration and purity of the RNA was determined using a UV spectrophotometer (Shimadzu UV-1201; Mandel Scientific, Guelph, Ontario) by measuring the absorbance at 260 (OD_{260}) and 280 (OD_{280}) nm. Measurements were done in duplicate and had an average coefficient of variation (CV) of <10%. The average purity (OD_{260}/OD_{280}) of the samples was ~1.5. RNA integrity was assessed in a randomly chosen subset of samples using agarose gel electrophoresis, and the OD ratio of 28S to 18S rRNA was consistently greater than 1 for each sample checked, indicating high quality RNA.

TaqMan® real-time RT-PCR. Prior to TaqMan® real-time RT-PCR, RNA samples were treated with DNase I for 25 min at 37°C to remove any contaminating DNA using DNA-free™ (Ambion Inc, Austin, TX) according to the manufacturer's instructions. Specific primers and probe to each target gene (Table 1, Table 2) were designed based on the cDNA sequence in GenBank (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi) with primer 3 designer (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Their specificity was checked using Blast (http://www.ncbi.nlm.nih.gov/BLAST/). Their thermal dynamics was manipulated through calculating delta G with Analyzer of Oligo (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/Default.aspx). All target gene probes were labeled with FAM at their 5' ends and BHQ-1 at their 3' ends. Human β2- microglobulin (β2-M) was used as an internal standard. Human β2- M primers and probe were same as used by Mahoney *et al.* (31). To validate human β2- M as an internal standard for quantification of mRNA content of the target genes with TaqMan® real-time RT-PCR (31), we tested the effect of acute exercise, sex and menstrual cycle phase on the

mRNA content of β2- M in the same samples used for this study under the same RT- PCR reaction conditions as used by Mahoney *et al.* (31) and found no effect of any of these parameters on mRNA content (Figure1A, B). Duplex RT-PCR was performed on iCycler real-time PCR system (Bio-Rad Laboratories, Hercules, CA) in the One-step TaqMan® RT-PCR Master Mix Reagents (Roch, Branchburg, New Jersey, part no. 4309169) according to the manufacture's instruction with target gene primers and probe and internal standard gene primers and probe in the same reaction. We optimized each PCR reaction condition so that the amplification efficiency for both target gene and internal reference was close to 1. The difference between the slopes of the regression curves of each target gene and the corresponding internal standard gene (Ct V.S. amount of RNA) was less than 10%. Agarose gel electrophoresis was used to confirm the specificity of the priming. The optimized RT-PCR conditions for each target gene are showed in Table 3. RT-PCR profiling was: 1cycle, 48^0C, 30 min; 1cycle, 95^0C, 10 min; 50 cylces, 95^0C, 15s; 60^0C, 1min; and 1cycle, 4^0C, ∞. All samples were run in duplicate simultaneously with RNA- and RT-negative controls. Fluorescence emission was detected through a filter corresponding to the reporter dye at the 5'end of each probe, and C_T was automatically calculated and displayed.

Statistical Analysis. All statistical analyses about mRNA expression of the genes tested were performed on linear data 2^{-CT} for evaluation of internal standards, $2^{-\Delta CT}$ for target gene normalized with internal reference (29). Data from evaluation of internal standards about sex effect and exercise effect were analyzed using a two-way ANOVA of sex X exercise, with sex as a between group factor. Data from evaluation of internal standards,

about menstrual phase and exercise effect were analyzed using a two way repeated measurement ANOVA of menstrual cycle phase X exercise with both menstrual cycle phase and exercise as within group factors. All results from evaluation of internal standards are expressed as mean fold change between sexes, menstrual phases and pre- vs. post- exercise (±SE), using $2^{-\Delta CT}$. Data on sex differences about target gene mRNA expression were analyzed using a two-way ANOVA of sex X exercise with sex as a between group factor. Data on menstrual cycle phase effect on the target gene mRNA expression were analyzed using a two-way repeated measurement ANOVA of menstrual cycle phase X exercise. Post-hoc tests were performed only when an interaction between sex and exercise or menstrual phase and exercise was significant ($P < 0.05$). All data displayed in the figures are expressed as mean (±SE), using $2^{-\Delta CT}$ normalized with β2- M. The differences in mRNA expression between between sexes, menstrual cycle phases or pre- and post- exercise, are expressed as mean fold change (±SE), using $2^{-\Delta\Delta CT}$. We also checked the six women who were taking oral contraceptives, as compared with the six who were taking an oral triphasic contraceptive pill and did not find any differential influence on mRNA abundance; consequently, all data for women were analyed and presented using N = 12. All analyses were done using computerized statistics software (Statistica; Statsoft, Tulsa, OK). Statistical significance was set at $\alpha \leq 0.05$, and statistical trends were considered at $0.05 < \alpha \leq 0.08$.

RESULTS

It has been reported with the same subjects as investigated in this study that both FOL and LUT women had a lower RER, glucose Ra, glucose Rd and MCR during exercise, as compared with men (9). Men used more proglycogen than LUT women (9). The sex differential mRNA expression of PPARα, PPARδ, LCAD and TFP-α observed in this study directly supports the substrate utilization study that women oxidize more fat than men during exercise in the same subjects (9).

Mitochondrial biogenesis

PGC-1α

Exercise increased PGC-1α mRNA content in men (7.9 ± 3.7 fold, P= 0.047) and LUT women (3.6 ± 1.7 fold, P = 0.021), but not FOL women (Figure 2A). There was no significant difference between men and women (FOL and LUT), pre- or post-exercise. There was no significant difference between FOL and LUT, pre- or post-exercise (Figure 2A).

Lipid metabolism

PPARα

Exercise did not significantly alter PPARα mRNA content in men or women (FOL and LUT) (Figure 2B). FOL women had higher PPARα mRNA content as compared with men (1.6 ± 0.3 fold, P = 0.04). Post-exercise PPARα mRNA content was higher in LUT

women as compared with men (1.4 ± 0.2 fold, P = 0.04). There was no significant difference between FOL and LUT, pre- or post-exercise (Figure 2B).

PPARγ

Exercise did not significantly alter PPARγ mRNA content in men or women (FOL and LUT) (Figure 2C). PPARγ mRNA content tended to be higher in FOL women as compared with men (1.6 ± 0.3 fold, P = 0.07). There was no significant difference between men and LUT women or between FOL and LUT, pre- or post exercise (Figures 2C).

PPARδ

Exercise tended to increase PPARδ mRNA content in men (1.6 ± 0.3 fold, P = 0.08), but did not significantly alter its content in women (FOL and LUT) (Figure 2D). PPARδ mRNA content was higher in FOL women as compared with men at rest (2.3 ± 0.4 fold, P = 0.014), but not after exercise. There was no significant difference between LUT women and men, pre- or post-exercise (Figure 2D). PPARδ mRNA content was higher in FOL women as compared with LUT women, pre- and post-exercise (1.7 ± 0.3 fold, P = 0.0007) (Figure2D).

Sarcolemmal fatty acid transport

FATm

Exercise did not significantly alter FATm mRNA content in men or women (FOL and LUT) (Figure 3A). Women had higher FATm mRNA content as compared with men (FOL, 3.3 ± 1.1 fold, P = 0.039; LUT, 2.2 ± 0.6 fold, P = 0.05). There was no significant difference between FOL and LUT, pre- or post-exercise (Figure 3A).

FABPc

Exercise increased FABPc mRNA content in men (1.2 ± 0.1 fold, P= 0.004) and women (FOL, 1.1 ± 0.2 Fold, P = 0.005; LUT, 1.6 ± 0.2 fold, P = 0.005), with LUT increasing to a greater extent than men (P = 0.001) (Figure 3B). FOL (1.7 ± 0.3 fold, P = 0.05) and LUT (2.0 ± 0.4 fold, P = 0.008) women had higher FABPc mRNA content as compared with men. There was no significant difference between FOL and LUT, pre- or post-exercise (Figure 3B).

Mitochondrial fatty acid transport

CPTI and CPTII

Exercise increased CPTI mRNA content in men (1.2 ± 0.1 fold, P = 0.014) and LUT women (1.5 ± 0.3 fold, P = 0.014) (Figure 3C). FOL women had higher CPTI mRNA content as compared with men (1.5 ± 0.2 fold, P = 0.023). There was no significant difference between FOL and LUT, pre- or post-exercise (Figure 3C).

CPTII mRNA content was not significantly altered by exercise, sex or menstrual phase (Figure 3D).

IMCL synthesis

SREBP-1c

Exercise did not significantly alter the SREBP-1c mRNA content in men and women (FOL and LUT) (Figure 4A). FOL (3.1 ± 1.0 fold, P = 0.014) and LUT (4.0 ±1.5 fold, P = 0.011) women had higher SREBP-1c mRNA content as compared with men. There was no significant difference between FOL and LUT, pre- or post-exercise (Figure 4A).

mtGPAT

Exercise did not significantly alter the mtGPAT mRNA content in men and women (FOL and LUT) (Figure 4B). FOL (2.0 ± 0.3 fold, P = 0.002) and LUT (1.6 ± 0.2 fold, P = 0.004) women had higher mtGPAT mRNA content as compared with men. There was no significant difference between FOL and LUT, pre- or post-exercise (Figure 4B).

Membrane lipid synthesis

SREBP-2

SREBP-2 mRNA content was not significantly altered by exercise, sex or menstrual phase (Figure 4C).

IMCL hydrolysis

HSL

Exercise tended to increase HSL mRNA content in men (1.4 ± 0.4 fold, P = 0.08) and FOL women (1.1 ± 0.3 fold, P = 0.08), with no significant change in LUT women

(Figure 4D). There were no significant difference between men and women (FOL and LUT). There was no significant difference between FOL and LUT, pre- or post-exercise (Figure 4D).

β-oxidation

LCAD

Exercise tended to decrease LCAD mRNA content in men (0.8 ± 0.1 fold, P = 0.074) and FOL women (0.5 ± 0.2 fold; P = 0.074) (Figure 5A). Exercise increased LCAD mRNA content in LUT (2.1 ± 0.6 fold, P = 0.038). FOL women had higher LCAD mRNA content as compared with men, pre- and post-exercise (2.7 ± 0.7 fold, P = 0.019), whereas LUT women had higher LCAD mRNA content as compared with men only post-exercise (2.1 ± 0.6 fold, P = 0.025). FOL women had higher LCAD mRNA content as compared with LUT women (2.6 ± 0.7 fold, P = 0.027), pre- and post-exercise (Figure 5A).

VLCAD

VLCAD mRNA content was not significantly altered by exercise, sex or menstrual phase (Figure 5B).

TFP- α

Exercise did not significantly alter the TFPα mRNA content in men and women (FOL and LUT) (Figure 5C). LUT women had higher TFPα mRNA content as compared with men (2.4 ± 0.5 fold, P = 0.021). There was no significant difference between FOL and LUT, pre- or post-exercise (Figure 5C).

DISSCUSSION

This study evaluated the independent and interactive influences of sex, acute endurance exercise and menstrual cycle phase on the mRNA content of genes involved in fat metabolism; specifically mitochondrial biogenesis, transcriptional regulation, fatty acid transport, IMCL hydrolysis, IMCL synthesis and β-oxidation. We found sex differences in the mRNA content of genes involved in fat oxidation that support previous findings that women have more IMCL than men and oxidize more fat during endurance exercise. Moderate intensity endurance exercise increased the mRNA content of genes involved in mitochondrial biogenesis, cytosolic and mitochondrial fatty acid transport and β-oxidation in men and women, particularly LUT women. The menstrual cycle phase differences in mRNA content of PPARδ, LCAD and FABPc did not coordinately support that women use more fat during the LUT phase than the FOL phase (14, 58, 59). We also found that some genes involved in fat oxidation showed different patterns of mRNA expression in response to exercise during the different phases of the menstrual cycle.

Sex differences in the capacity for fat oxidation

We found that the mRNA content of PPARα, PPARγ, FATm, FABPc, CPTI, SREBP-1c, mtGPAT and LCAD was significantly higher in women during FOL phase than men at rest and immediately after exercise, and that of PPARδ was significantly higher in FOL women than men at rest only; the mRNA content of FATm, FABPc, SREBP-1c, mtGPAT and TFPα was significantly higher in women during LUT phase than men at rest and immediately after exercise, and that of PPARα and LCAD was significantly higher in

LUT women than men after exercise only. PPARα transcriptionally activates MCAD, CPTI, CPTII, FABP and FATP (32, 56), whereas PPARδ transcriptionally activates FABPc, FAT/CD36, LPL, CPTI, and genes involved in β-oxidation (10, 12, 30). Taken together with the findings of a sex difference in FATm mRNA (4), FABPm protein and mRNA, FAT/CD36 protein and mLPL (24) and beta-hydroxyacyl-CoA dehydrogenase (HAD) (44), these results indicate that as compared with men, FOL women at rest and during exercise, and LUT women at rest, have a higher capacity for transcriptional activation of the genes responsible for fat oxidation downstream of PPARα or PPARδ. Both FOL and LUT women have a higher capacity for sarcolemmal, cytosolic and mitochondrial fatty acid transport, synthesis of IMCL, and oxidation of fatty acid within mitochondria at rest and during exercise. These results are in agreement with our hypothesis and directly support previous observations that women have higher IMCL content (38, 43, 46, 54) than men and oxidize more fat during endurance exercise (6, 9, 13, 17, 25, 34, 39, 42, 45, 51, 53, 54).

The co-existence of the sex differences in the mRNA content of genes involved in fat metabolism suggests that there may be some regulatory relationship among them. PPARα (32, 49, 56), PPARδ (10, 12, 30), PPARγ (48) and SREBP-1c (50) are transcription factors that regulate the expression of the genes involved in lipid oxidation and IMCL synthesis, respectively. Higher mRNA content of PPARα in FOL women at rest and during exercise and PPARδ in FOL women only at rest may lead to higher mRNA content of FATm, FABPc, CPTI, and LCAD in FOL women than men. Higher mRNA content of PPARα in LUT women versus men during exercise may ultimately result in higher mRNA content of FATm, FABPc and TFPα. Higher mRNA content of PPARγ in FOL women

and SREBP-1c in both FOL and LUT women may lead to higher mRNA content of mtGPAT both at rest and during exercise and this observation would directionally support higher IMCL content in women (38, 43, 46, 54).

Menstrual cycle phase differences in the capacity to oxidize fat

We found that the mRNA content of PPARδ and LCAD were significantly higher in the skeletal muscle of FOL women as compared with LUT women both before and after exercise. Post-exercise FABPc mRNA content was more abundant in LUT women versus FOL women. There were no significant differences in the mRNA content of PPARγ, FATm, CPTI, CPTII, SREBP-1c, mtGPAT, SREBP-2 or HSL between the different phases. These rather minimal, at least relative to the robust sex differences, findings support our hypothesis, and the suggestions by others (8), that menstrual phase has a small influence on exercise substrate selection, at least compared with sex differences and exercise intensity. PPARδ selectively activates the transcription of genes involved in fatty acid transport and fatty acid oxidation (30). FABPc (3) and LCAD (19) are involved in cytosolic fatty acid transport and β-oxidation, respectively. Women in the FOL phase may have a higher capacity for the transcriptional activation of genes involved in fat oxidation by PPARδ and a higher transcriptional capacity for β-oxidation due to a higher level of LCAD mRNA. It is unlikely that the higher abundance of FABPc seen post-exercise in the LUT phase could be implicated in the finding that women use more fat during the LUT phase than FOL phase (14, 58, 59). Our results also imply that there is no difference in the transcriptional capacity for synthesis of IMCL and membrane lipid, IMCL hydrolysis, or

fatty acid transport across plasma and mitochondrial membranes between the different phases of the menstrual cycle.

Acute exercise increases the transcriptional capacity for fatty acid metabolism and mitochondrial biogenesis.

Acute and chronic endurance exercise training increase the proportionate utilization of fat (1). We found that moderate intensity endurance exercise increased the mRNA content of PGC-1α, PPARδ, FABPc, and CPTI, and decreased the mRNA content of LCAD in men; increased the mRNA content of PGC-1α and FABPc in FOL women; and increased the mRNA content of FABPc, CPTI and LCAD in LUT women. Our observations are consistent with previous findings that acute exercise increases the mRNA content of PGC-1α (31, 37), PPARδ (57) and CPTI (40, 41) in human skeletal muscle. Our finding of a non-significant trend for an increase in HSL mRNA content in men after exercise is similar to another study using a nearly identical protocol (90 min at 60 % VO_{2peak}) where a small, non-significant, directional increase in HSL mRNA was seen only in men after acute exercise (Roepstorff *et al.*, 2006a). The minimal and non-significant acute increase in HSL mRNA abundance also in agreement with the observation by Watt et al. (57). These findings are likely due to the fact that HSL appears to be regulated acutely by post-translational modifications such as Ser^{659} phosphorylation leading to higher HSL activity in both men and women (Roepstorff *et al.*, 2006a). The coordinate induction of the mRNA content for genes involved in mitochondrial biogenesis, hydrolysis of IMCL, cytosolic and mitochondrial fatty acid transport and β-oxidation are directionally supportive

of the fact that acute endurance exercise increases the transcriptional capacity for fat oxidation in men, and FOL and LUT women.

Endurance exercise training increases IMCL content in skeletal muscle (Tarnopolsky *et al.*, 2006), and acute exercise appears to reduce IMCL content, primarily in women (Watt *et al.*, 2004; Roepstorff *et al.*, 2005b; Roepstorff *et al.*, 2006a). In contrast to the immediate post-exercise increase in mRNA abundance for many genes involved in fat oxidation, we did not find higher abundance of mRNA species for genes involved in IMCL synthesis (SREBP-1c, mtGPAT). These results are consistent with studies investigating the mRNA abundance of SREBP-1c (55) in response to acute exercise in human skeletal muscle. Our finding of no change in mtGPAT is also consistent with a lack of an acute exercise induced increase in GPAT activity in exercising men (Watt *et al.*, 2004). In addition to the mtGPAT mRNA data, we are also the first to measure and report the lack of and acute exercise effect on SREBP-2 abundance (involved in membrane biosynthesis). Together, the above data imply that acute endurance exercise does not change the transcriptional capacity for synthesis of IMCL or membrane lipids in men or women.

The co-alteration in the mRNA expression of PGC-1α, PPARδ, FABPc, CPTI, HSL, LCAD in men; the mRNA expression of PGC-1α, FABPc in FOL women; and the mRNA expression of FABPc, CPTI and LCAD in LUT women during exercise suggests that there may be some regulatory relationship among these genes in the corresponding group of subjects. PGC-1α is a transcriptional coactivator of PPARα, which in turn increases gene expression of enzymes involved in mitochondrial fatty acid beta-oxidation including; medium chain acyl-CoA dehydrogenase (MCAD), CPTI (32, 56), CPTII (32),

FATm and FABP (49). Even in the absence of PPARα mRNA induction, increased PGC-1α expression may still increase the expression of PPARα target genes through co-activation (56). PPARδ is a transcription factor (12) that specifically induces the genes involved in lipid metabolism, such as heart FABPc, FAT/CD36, LPL and CPTI (10, 30). Exercise may induce the expression of these transcription factors, triggering an increase in the mRNA expression of the genes downstream of these regulators. The exact regulatory relationship needs to be further elucidated.

Given that men and women have similar mitochondrial volume densities (7) and enzyme activities (2, 7), it was surprising that consistently found acute induction of PGC-1α following endurance exercise was robustly found in men (↑ 7.9 fold), lower in LUT women (↑ 3.6 fold) and not seen women during the FOL phase of the menstrual cycle. These results suggest that the response of PGC-1α mRNA expression to exercise may depend on the ratio of progesterone to estrogen. In LUT women, the level of progesterone is greater than estrogen, while the level of progesterone is less than estrogen in FOL women (20). Because estrogen stimulates the transcription of estrogen related receptor-α (28), and estrogen related receptor-α represses the transcription of PGC-1α (18), estrogen may repress the increase of PGC-1α mRNA expression with exercise. In support of the estrogen mediated repression of PGC-1α mRNA expression, we recently found that 17-β-estradiol attenuated the exercise-induced increase in PGC-1α mRNA in men (Fu et al, unpublished observations, 2006). The mechanisms regulating PGC-1α mRNA expression in response to exercise and sex hormones requires further elucidation.

We conclude that both FOL and LUT women have a higher mRNA capacity for transcriptional activation of genes responsible for fat oxidation, and a higher mRNA capacity for sarcolemmal, cytosolic and mitochondrial fatty acid transport, synthesis of IMCL, and oxidation of fatty acids at rest and during exercise, as compared with men. These observations directly support previous research demonstrating that women have more IMCL than men and oxidize more fat during endurance exercise. Exercise increases the mRNA capacity of fatty acid metabolism in men and women, specifically in the LUT phase. The menstrual cycle influences the profile of mRNA involved in lipid metabolism at rest and its response to endurance exercise.

References

1. **Bennard P, Imbeault P, and Doucet E.** Maximizing acute fat utilization: effects of exercise, food, and individual characteristics. *Can J Appl Physiol* 30: 475-499, 2005.

2. **Berthon PM, Howlett RA, Heigenhauser GJ, and Spriet LL.** Human skeletal muscle carnitine palmitoyltransferase I activity determined in isolated intact mitochondria. *J Appl Physiol* 85: 148-153, 1998.

3. **Binas B, Han XX, Erol E, Luiken JJ, Glatz JF, Dyck DJ, Motazavi R, Adihetty PJ, Hood DA, and Bonen A.** A null mutation in H-FABP only partially inhibits skeletal muscle fatty acid metabolism. *Am J Physiol Endocrinol Metab* 285: E481-489, 2003.

4. **Binnert C, Koistinen HA, Martin G, Andreelli F, Ebeling P, Koivisto VA, Laville M, Auwerx J, and Vidal H.** Fatty acid transport protein-1 mRNA expression in skeletal muscle and in adipose tissue in humans. *Am J Physiol Endocrinol Metab* 279: E1072-1079, 2000.

5. **Cameron-Smith D.** Exercise and skeletal muscle gene expression. *Clin Exp Pharmacol Physiol* 29: 209-213, 2002.

6. **Carter SL, Rennie C, and Tarnopolsky MA.** Substrate utilization during endurance exercise in men and women after endurance training. *Am J Physiol Endocrinol Metab* 280: E898-907, 2001.

7. **Carter SL, Rennie CD, Hamilton SJ, and Tarnopolsky.** Changes in skeletal muscle in males and females following endurance training. *Can J Physiol Pharmacol* 79: 386-392, 2001.

8. **Casazza GA, Jacobs KA, Suh SH, Miller BF, Horning MA, and Brooks GA.** Menstrual cycle phase and oral contraceptive effects on triglyceride mobilization during exercise. *J Appl Physiol* 97: 302-309, 2004.

9. **Devries MC, Hamadeh MJ, Phillips SM, and Tarnopolsky MA.** Menstrual cycle phase and sex influence muscle glycogen utilization and glucose turnover during moderate-intensity endurance exercise. *Am J Physiol Regul Integr Comp Physiol* 291: R1120-1128, 2006.

10. **Dressel U, Allen TL, Pippal JB, Rohde PR, Lau P, and Muscat GE.** The peroxisome proliferator-activated receptor beta/delta agonist, GW501516, regulates the expression of genes involved in lipid catabolism and energy uncoupling in skeletal muscle cells. *Mol Endocrinol* 17: 2477-2493, 2003.

11. **Ellis GS, Lanza-Jacoby S, Gow A, and Kendrick ZV.** Effects of estradiol on lipoprotein lipase activity and lipid availability in exercised male rats. *J Appl Physiol* 77: 209-215, 1994.

12. **Fredenrich A and Grimaldi PA.** PPAR delta: an uncompletely known nuclear receptor. *Diabetes Metab* 31: 23-27, 2005.

13. **Friedlander AL, Casazza GA, Horning MA, Huie MJ, Piacentini MF, Trimmer JK, and Brooks GA.** Training-induced alterations of carbohydrate metabolism in women: women respond differently from men. *J Appl Physiol* 85: 1175-1186, 1998.

14. **Hackney AC, McCracken-Compton MA, and Ainsworth B.** Substrate responses to submaximal exercise in the midfollicular and midluteal phases of the menstrual cycle. *Int J Sport Nutr* 4: 299-308, 1994.

15. **Hamadeh MJ, Devries MC, and Tarnopolsky MA.** Estrogen supplementation reduces whole body leucine and carbohydrate oxidation and increases lipid oxidation in men during endurance exercise. *J Clin Endocrinol Metab* 90: 3592-3599, 2005.

16. **Horton TJ, Miller EK, Glueck D, and Tench K.** No effect of menstrual cycle phase on glucose kinetics and fuel oxidation during moderate-intensity exercise. *Am J Physiol Endocrinol Metab* 282: E752-762, 2002.

17. **Horton TJ, Pagliassotti MJ, Hobbs K, and Hill JO.** Fuel metabolism in men and women during and after long-duration exercise. *J Appl Physiol* 85: 1823-1832, 1998.

18. **Ichida M, Nemoto S, and Finkel T.** Identification of a specific molecular repressor of the peroxisome proliferator-activated receptor gamma Coactivator-1 alpha (PGC-1alpha). *J Biol Chem* 277: 50991-50995, 2002.

19. **Ikeda Y, Okamura-Ikeda K, and Tanaka K.** Purification and characterization of short-chain, medium-chain, and long-chain acyl-CoA dehydrogenases from rat liver mitochondria. Isolation of the holo- and apoenzymes and conversion of the apoenzyme to the holoenzyme. *J Biol Chem* 260: 1311-1325, 1985.

20. **Jabbour HN, Kelly RW, Fraser HM, and Critchley HO.** Endocrine regulation of menstruation. *Endocr Rev* 27: 17-46, 2006.

21. **Kanaley JA, Boileau RA, Bahr JA, Misner JE, and Nelson RA.** Substrate oxidation and GH responses to exercise are independent of menstrual phase and status. *Med Sci Sports Exerc* 24: 873-880, 1992.

22. **Kendrick ZV and Ellis GS.** Effect of estradiol on tissue glycogen metabolism and lipid availability in exercised male rats. *J Appl Physiol* 71: 1694-1699, 1991.

23. **Kendrick ZV, Steffen CA, Rumsey WL, and Goldberg DI.** Effect of estradiol on tissue glycogen metabolism in exercised oophorectomized rats. *J Appl Physiol* 63: 492-496, 1987.

24. **Kiens B, Roepstorff C, Glatz JF, Bonen A, Schjerling P, Knudsen J, and Nielsen JN.** Lipid-binding proteins and lipoprotein lipase activity in human skeletal muscle: influence of physical activity and gender. *J Appl Physiol* 97: 1209-1218, 2004.

25. **Knechtle B, Muller G, Willmann F, Kotteck K, Eser P, and Knecht H.** Fat oxidation in men and women endurance athletes in running and cycling. *Int J Sports Med* 25: 38-44, 2004.

26. **Kriengsinyos W, Wykes LJ, Goonewardene LA, Ball RO, and Pencharz PB.** Phase of menstrual cycle affects lysine requirement in healthy women. *Am J Physiol Endocrinol Metab* 287: E489-496, 2004.

27. **Lariviere F, Moussalli R, and Garrel DR.** Increased leucine flux and leucine oxidation during the luteal phase of the menstrual cycle in women. *Am J Physiol* 267: E422-428, 1994.

28. **Liu D, Zhang Z, and Teng CT.** Estrogen-related receptor-gamma and peroxisome proliferator-activated receptor-gamma coactivator-1alpha regulate estrogen-related receptor-alpha gene expression via a conserved multi-hormone response element. *J Mol Endocrinol* 34: 473-487, 2005.

29. **Livak KJ and Schmittgen TD.** Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25: 402-408, 2001.

30. **Luquet S, Lopez-Soriano J, Holst D, Gaudel C, Jehl-Pietri C, Fredenrich A, and Grimaldi PA.** Roles of peroxisome proliferator-activated receptor delta (PPARdelta) in the control of fatty acid catabolism. A new target for the treatment of metabolic syndrome. *Biochimie* 86: 833-837, 2004.

31. **Mahoney DJ, Carey K, Fu MH, Snow R, Cameron-Smith D, Parise G, and Tarnopolsky MA.** Real-time RT-PCR analysis of housekeeping genes in human skeletal muscle following acute exercise. *Physiol Genomics* 18: 226-231, 2004.

32. **Mandard S, Muller M, and Kersten S.** Peroxisome proliferator-activated receptor alpha target genes. *Cell Mol Life Sci* 61: 393-416, 2004.

33. **Marliss EB, Kreisman SH, Manzon A, Halter JB, Vranic M, and Nessim SJ.** Gender differences in glucoregulatory responses to intense exercise. *J Appl Physiol* 88: 457-466, 2000.

34. **McKenzie S, Phillips SM, Carter SL, Lowther S, Gibala MJ, and Tarnopolsky MA.** Endurance exercise training attenuates leucine oxidation and BCOAD activation during exercise in humans. *Am J Physiol Endocrinol Metab* 278: E580-587, 2000.

35. **Mittendorfer B, Horowitz JF, and Klein S.** Effect of gender on lipid kinetics during endurance exercise of moderate intensity in untrained subjects. *Am J Physiol Endocrinol Metab* 283: E58-65, 2002.

36. **Neumann-Haefelin C, Kuhlmann J, Belz U, Kalisch J, Quint M, Gerl M, Juretschke HP, and Herling AW.** Determinants of intramyocellular lipid concentrations in rat hindleg muscle. *Magn Reson Med* 50: 242-248, 2003.

37. **Norrbom J, Sundberg CJ, Ameln H, Kraus WE, Jansson E, and Gustafsson T.** PGC-1alpha mRNA expression is influenced by metabolic perturbation in exercising human skeletal muscle. *J Appl Physiol* 96: 189-194, 2004.

38. **Perseghin G, Scifo P, Pagliato E, Battezzati A, Benedini S, Soldini L, Testolin G, Del Maschio A, and Luzi L.** Gender factors affect fatty acids-induced insulin resistance in nonobese humans: effects of oral steroidal contraception. *J Clin Endocrinol Metab* 86: 3188-3196, 2001.

39. **Phillips SM, Atkinson SA, Tarnopolsky MA, and MacDougall JD.** Gender differences in leucine kinetics and nitrogen balance in endurance athletes. *J Appl Physiol* 75: 2134-2141, 1993.

40. **Pilegaard H, Ordway GA, Saltin B, and Neufer PD.** Transcriptional regulation of gene expression in human skeletal muscle during recovery from exercise. *Am J Physiol Endocrinol Metab* 279: E806-814, 2000.

41. **Pilegaard H, Osada T, Andersen LT, Helge JW, Saltin B, and Neufer PD.** Substrate availability and transcriptional regulation of metabolic genes in human skeletal muscle during recovery from exercise. *Metabolism* 54: 1048-1055, 2005.

42. **Riddell MC, Partington SL, Stupka N, Armstrong D, Rennie C, and Tarnopolsky MA.** Substrate utilization during exercise performed with and without glucose ingestion in female and male endurance trained athletes. *Int J Sport Nutr Exerc Metab* 13: 407-421, 2003.

43. **Roepstorff C, Donsmark M, Thiele M, Vistisen B, Stewart G, Vissing K, Schjerling P, Hardie DG, Galbo H, and Kiens B.** Gender differences in hormone-sensitive lipase expression, activity and phosphorylation in skeletal muscle at rest and during exercise. *Am J Physiol Endocrinol Metab*, 2006.

44. **Roepstorff C, Schjerling P, Vistisen B, Madsen M, Steffensen CH, Rider MH, and Kiens B.** Regulation of oxidative enzyme activity and eukaryotic elongation factor 2 in

human skeletal muscle: influence of gender and exercise. *Acta Physiol Scand* 184: 215-224, 2005.

45. **Roepstorff C, Thiele M, Hillig T, Pilegaard H, Richter EA, Wojtaszewski JF, and Kiens B.** Higher skeletal muscle alpha2AMPK activation and lower energy charge and fat oxidation in men than in women during submaximal exercise. *J Physiol* 574: 125-138, 2006.

46. **Roepstorff C, Vistisen B, and Kiens B.** Intramuscular triacylglycerol in energy metabolism during exercise in humans. *Exerc Sport Sci Rev* 33: 182-188, 2005.

47. **Romijn JA, Coyle EF, Sidossis LS, Rosenblatt J, and Wolfe RR.** Substrate metabolism during different exercise intensities in endurance-trained women. *J Appl Physiol* 88: 1707-1714, 2000.

48. **Rosen ED, Walkey CJ, Puigserver P, and Spiegelman BM.** Transcriptional regulation of adipogenesis. *Genes Dev* 14: 1293-1307, 2000.

49. **Schoonjans K, Staels B, and Auwerx J.** Role of the peroxisome proliferator-activated receptor (PPAR) in mediating the effects of fibrates and fatty acids on gene expression. *J Lipid Res* 37: 907-925, 1996.

50. **Tarling E, Salter A, and Bennett A.** Transcriptional regulation of human SREBP-1c (sterol-regulatory-element-binding protein-1c): a key regulator of lipogenesis. *Biochem Soc Trans* 32: 107-109, 2004.

51. **Tarnopolsky LJ, MacDougall JD, Atkinson SA, Tarnopolsky MA, and Sutton JR.** Gender differences in substrate for endurance exercise. *J Appl Physiol* 68: 302-308, 1990.

52. **Tarnopolsky M, Parise G, Fu MH, Brose A, Parshad A, Speer O, and Wallimann T.** Acute and moderate-term creatine monohydrate supplementation does not affect creatine transporter mRNA or protein content in either young or elderly humans. *Mol Cell Biochem* 244: 159-166, 2003.

53. **Tarnopolsky MA.** Gender differences in substrate metabolism during endurance exercise. *Can J Appl Physiol* 25: 312-327, 2000.

54. **Tarnopolsky MA, Rennie CD, Robertshaw HA, Fedak-Tarnopolsky SN, Devries MC, and Hamadeh MJ.** The Influence of Endurance Exercise Training and Sex on Intramyocellular Lipid and Mitochondrial Ultrastructure, Substrate Use, and Mitochondrial Enzyme Activity. *Am J Physiol Regul Integr Comp Physiol*, 2006.

55. **Tunstall RJ, Mehan KA, Wadley GD, Collier GR, Bonen A, Hargreaves M, and Cameron-Smith D.** Exercise training increases lipid metabolism gene expression in human skeletal muscle. *Am J Physiol Endocrinol Metab* 283: E66-72, 2002.

56. **Vega RB, Huss JM, and Kelly DP.** The coactivator PGC-1 cooperates with peroxisome proliferator-activated receptor alpha in transcriptional control of nuclear genes encoding mitochondrial fatty acid oxidation enzymes. *Mol Cell Biol* 20: 1868-1876, 2000.

57. **Watt MJ, Southgate RJ, Holmes AG, and Febbraio MA.** Suppression of plasma free fatty acids upregulates peroxisome proliferator-activated receptor (PPAR) alpha and delta and PPAR coactivator 1alpha in human skeletal muscle, but not lipid regulatory genes. *J Mol Endocrinol* 33: 533-544, 2004.

58. **Wenz M, Berend JZ, Lynch NA, Chappell S, and Hackney AC.** Substrate oxidation at rest and during exercise: effects of menstrual cycle phase and diet composition. *J Physiol Pharmacol* 48: 851-860, 1997.

59. **Zderic TW, Coggan AR, and Ruby BC.** Glucose kinetics and substrate oxidation during exercise in the follicular and luteal phases. *J Appl Physiol* 90: 447-453, 2001.

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Figure legends

Figure 1. Skeletal muscle β2-microglobulin mRNA content in men and women (FOL and LUT). FOL, follicular phase; LUT, luteal phase. There was no significant difference between pre- and post-exercise. Values are means ± SEM.

Figure 2. Skeletal muscle mRNA content of the genes involved in the transcriptional regulation of mitochondrial biogenesis (PGC-1α) and lipid metabolism (PPARα, PPARγ, PPARδ) in men and women (FOL and LUT). Panel A: PGC-1α; * Post-exercise higher than pre-exercise (men, P = 0.05; LUT, P = 0.02). Panel B: PPARα; † FOL higher than men (P = 0.04); ‡ Post-exercise, LUT higher than men (P = 0.04). Panel C: PPARγ; ¶ Trend for FOL to be higher than men (P = 0.07). Panel D: PPARδ; † At rest, FOL higher than men (P = 0.01); & Trend for post-exercise to be higher than pre-exercise (P = 0.08); ¶ FOL higher than LUT (P = 0.007). FOL, follicular phase; LUT, luteal phase. β2-microglobulin was used as an internal standard. Values are means ± SEM.

Figure 3. Skeletal muscle mRNA content of the genes involved in the sarcolemmal (FATm, FABPc) and mitochondrial FFA transport (CPTI, CPTII) in men and women (FOL and LUT). Panel A: FATm; † FOL (P=0.039) and LUT (P = 0.05) higher than men. Panel B: FABPc; * Post-exercise higher than pre-exercise (men, P = 0.004; FOL, P = 0.005; LUT, P = 0.005); & Increase to a greater extent during exercise in LUT than men (P = 0.001); † FOL (P = 0.05) and LUT (P = 0.008) higher than men. Panel C: CPTI; * Post-exercise higher than pre-exercise (P = 0.014); ‡ FOL higher than men (P = 0.023). Panel D: CPTII. β2 - M was used as an internal standard. Values are means ± SE.

Figure 4. Skeletal muscle mRNA content of the genes involved in the IMCL (SREBP-1c, mtGPAT) and membrane lipid (SREBP-2) synthesis in men and women (FOL and LUT). Panel A: SREBP-1c; † FOL (P = 0.014) and LUT (P = 0.011) higher than men. Panel B: mtGPAT; † FOL (P = 0.002) and LUT (P = 0.004) higher than men. Panel C, SREBP-2.

Panel D: HSL; ¶ Trend for post-exercise to be higher than pre-exercise (P = 0.08); $ Trend for post-exercise in FOL women to be higher than men at rest (P = 0.08). β2 - M was used as an internal standard. Values are means ± SE.

Figure 5. Skeletal muscle mRNA content of the genes involved in the (-oxidation (LCAD, VLCAD, TFP-alpha) in men and women (FOL and LUT). Panel A: LCAD; (Post-exercise higher than pre-exercise (P = 0.038); & Trend for post-exercise to be lower than pre-exercise in men (P = 0.074) and FOL (P = 0.074); (FOL higher than men (P = 0.019); ‡ Post-exercise, LUT higher than men (P = 0.025); ¶ FOL higher than LUT (P = 0.027). Panel B: VLCAD. Panel C: TFP-α; ‡ LUT higher than men (P = 0.021). β2 - M was used as an internal standard. Values are means ± SE.

Table 1. Targeted genes involved in fat metabolism in human skeletal muscle

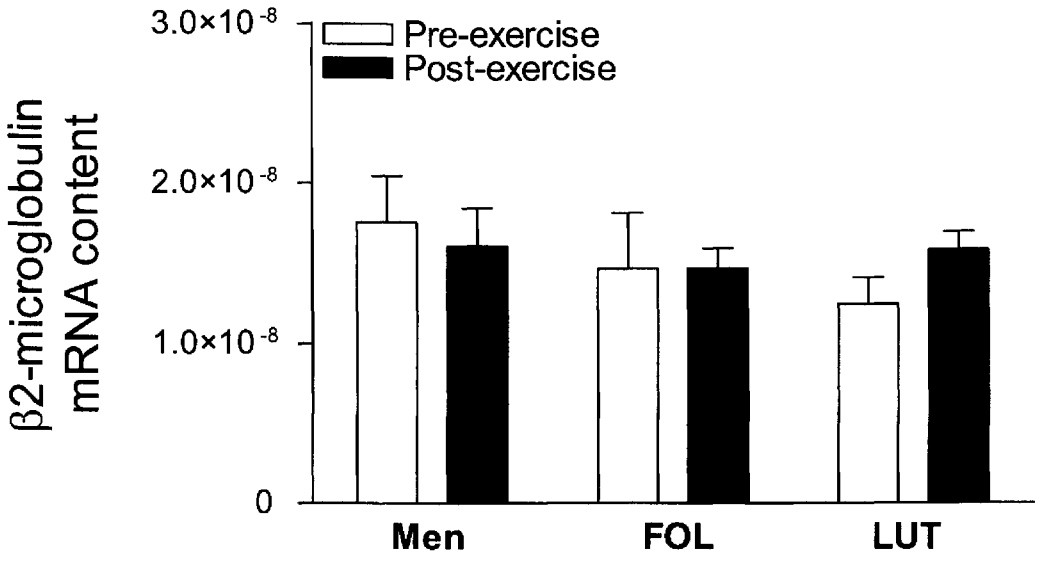
Fat metabolism pathways	Gene Symbol	Function	GenBank access No.
Transcriptional regulation of mitochondiral biogenesis and lipid metabolism	PGC-1α	Oxidative capacity	NM_ 013261
	PPARα	Oxidative capacity	NM_005036
	PPARγ	Oxidative capacity	NM_138712
	PPARδ	Oxidative capacity	NM_006238
Fatty acid transport	FATm	Sarcolema membrane fatty acid transport	NM_198580
	FABP	Cytosol fatty acid transport	X56549
	CPTIB	Mitochondrial membrane fatty acid transport	NM-004377
	CPTII	Mitochondrial membrane fatty acid transport	NM_000098
IMCL and membrane lipid synthesis	SREBP-1c	IMCL synthesis	NM_004176
	mtGPAT	IMCL synthesis	NM_020918
	SREBP-2	Membrane lipid synthesis	BC051799
IMCL hydrolysis	HSL	IMCL hydrolysis	NM_005357
β-oxidation	LCAD	oxidation	NM_001608
	VLCAD	Oxidation	BC000399
	TFP-α	Oxidation	BC009235

Table 2. Primers and probe sets

Variables / Genes	GenBank acess No	Primers and probe set
PGC-1α	NM_013261	Left primer: 5'-ttgctaaacgactccgagaa-3'; Right primer 5'-tgcaaagttccctctctgct-3' Probe: 5'- aacagttgggctgtcaacattcaaagc
PPARα	NM_005036	Left primer 5'-gacacgctttcaccagctt-3', Right primer 5'- gcagattctacattcgatgttc-3' Probe 5'-tcctcggtgacttatcctgtggtcc-3'
PPARγ	NM_138712	Left primer 5'-cctccttgatgaataaagatg-3', Right primer 5'-gggctccataaagtcaccaa-3' Probe 5'-caaggcttcatgacaagggagtttctaaa-3'
PPARδ	NM_006238	Left primer: 5'-actgagttcgccaagagcat-3', Right primer 5'-gtgcacgccatacttgagaa-3' Probe: 5'-cagcctcttcctcaacgaccaggttac-3'
FATm	NM-198580	Left Primer 5'- cgctgctcaacgtgaacct -3'; Right Primer 5'-cagatgcccgctcacttc-3' Probe 5'-ccgccaccatttctcctccaaagatca-3'
FABPc	X56549	Left primer 5'- ggagttcgatgagacaacag-3'; Right primer 5'-aacaagtttccctccatcca-3' Probe 5'-tgtcacaatggacttgaccttcctgtcat-3'
CPTIB	NM_004377	Left primer 5'- ctctcatggtgaacagcaacta-3'; Right primer 5'-gtccagtttacggcgatacat-3' Probe 5'- atgtcatggaccttgtgctcatcaaga-3'
CPTII	NM_000098	Left primer 5'- ctacctggaccctgcatacg -3'; Right primer 5'-agcatacccaacaccaaagc-3' Probe 5'- cagataaaccacaatgtcctgtccacg -3'
SREBP-1c	NM_004176	Left primer 5'-cagactcgctgcttctgaca-3'; Right primer 5'-ggactgttgccaagatggtt-3' Probe 5'-ccttcacagtggctccgtctgtct t -3'
mtGPAT	NM_020918	Left primer 5'-gtgttgctgggaaaccaagt-3'; Right primer 5'-ggatgccaagcccttactg-3' Probe 5'-cacctggaaacttacaatgcactttagcg-3'
SREBP-2	BC000399	Left primer 5'- ctctgaccagcacccacact-3'; Right primer 5'-cacaccatttaccagccataag-3' Probe 5'- agagtaggcatcatccagtcaaaccagc-3'
HSL	NM_005357	Left primer 5'-gagcggatcacacagaacct-3'; Right primer 5'-ccagagacgatagcacttcca-3' Probe 5'- aaagccttctggaacatcaccgaga-3'
LCAD	NM_001608	Left primer 5'-ttatcaagggacgaaagctaca-3'; Right primer 5'-cactagctggcaaccgtatatc-3' Probe 5'-aagcccaggataccgcagaactattctttg-3'
VLCAD	BC000399	Left primer 5'-tccgagatcttcgcatcttc-3'; Right primer 5'-agcattcccaaagggattctt-3' Probe 5'-acattcttcggctgtttgtggctct-3'
TFP-α	BC009235	Left primer 5'-gtcagcatctttgcccttct-3'; Right primer 5'-ccaggtcccactttctctca-3' Probe 5'-tccctccctaaggtgctgcctagaat-3'

Table 3. TaqMan™ Real-time RT-PCR reaction conditions

Variables / Target Genes	Target gene left primer (μM)	Target gene right primer (μM)	Target gene probe (μM)	β-2M left primer (μM)	β-2M right primer (μM)	β-2M Probe (μM)	RNA Template (ng)	Reaction Volume (μl)
PGC-1α	0.9	0.9	0.1	0.3	0.3	0.05	5.0	25
PPARα	0.3	0.3	0.1	0.6	0.6	0.05	2.5	25
PPARγ	1.2	1.2	0.1	0.1	0.1	0.05	15.0	50
PPARδ	0.9	0.9	0.2	0.6	0.6	0.05	7.5	25
FATm	0.3	0.05	0.1	0.4	0.4	0.05	5.0	25
FABPc	0.9	0.9	0.05	0.1	0.1	0.05	10.0	50
CPTIB	0.6	0.6	0.15	0.4	0.4	0.05	5.0	25
CPTII	0.6	0.2	0.15	0.6	0.6	0.05	5.0	25
SREBP-1c	0.6	0.2	0.15	0.4	0.4	0.05	7.5	25
mtGPAT	0.9	0.3	0.2	0.6	0.6	0.05	7.5	25
SREBP-2	0.3	0.9	0.05	0.3	0.3	0.05	4.0	25
HSL	0.3	0.3	0.1	0.4	0.4	0.05	15.0	50
LCAD	0.9	0.9	0.1	0.2	0.2	0.05	5.0	25
VLCAD	0.6	0.6	0.15	0.1	0.1	0.05	10.0	50
TFP-α	0.6	0.6	0.05	0.4	0.4	0.15	7.5	25


β2-microglobulin mRNA content
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2.0×10-8
1.0×10-8
0
Pre-exercise
Post-exercise
Men
FOL
LUT

Figure 1

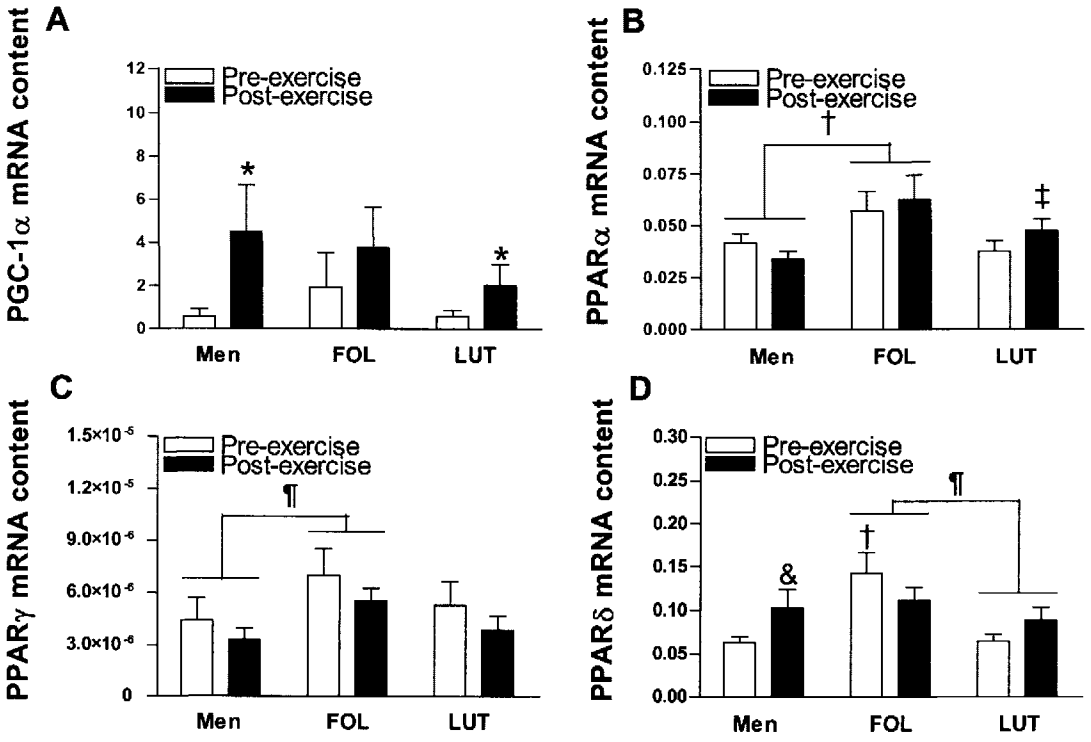
A
PGC-1α mRNA content
Pre-exercise
Post-exercise
12
10
8
6
4
2
0
*
*
Men
FOL
LUT
B
PPARα mRNA content
Pre-exercise
Post-exercise
0.125
0.100
0.075
0.050
0.025
0.000
†
‡
Men
FOL
LUT
C
PPARγ mRNA content
Pre-exercise
Post-exercise
1.5×10⁻⁵
1.2×10⁻⁵
9.0×10⁻⁶
6.0×10⁻⁶
3.0×10⁻⁶
0
¶
Men
FOL
LUT
D
PPARδ mRNA content
Pre-exercise
Post-exercise
0.30
0.25
0.20
0.15
0.10
0.05
0.00
&
†
¶
Men
FOL
LUT

Figure 2

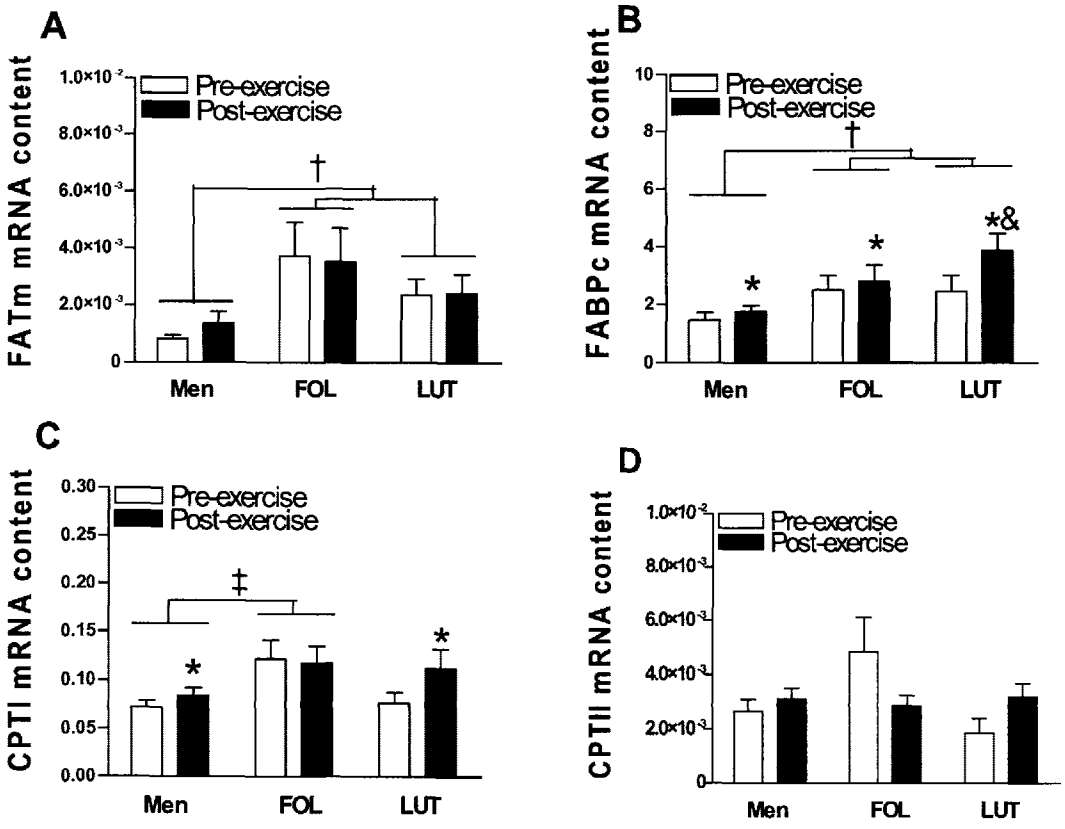
A
FATm mRNA content
1.0×10⁻²
8.0×10⁻³
6.0×10⁻³
4.0×10⁻³
2.0×10⁻³
0
Pre-exercise
Post-exercise
†
Men
FOL
LUT
B
FABPc mRNA content
10
8
6
4
2
0
Pre-exercise
Post-exercise
†
*
*
*&
Men
FOL
LUT
C
CPTI mRNA content
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Pre-exercise
Post-exercise
‡
*
*
Men
FOL
LUT
D
CPTII mRNA content
1.0×10⁻²
8.0×10⁻³
6.0×10⁻³
4.0×10⁻³
2.0×10⁻³
0
Pre-exercise
Post-exercise
Men
FOL
LUT

Figure 3

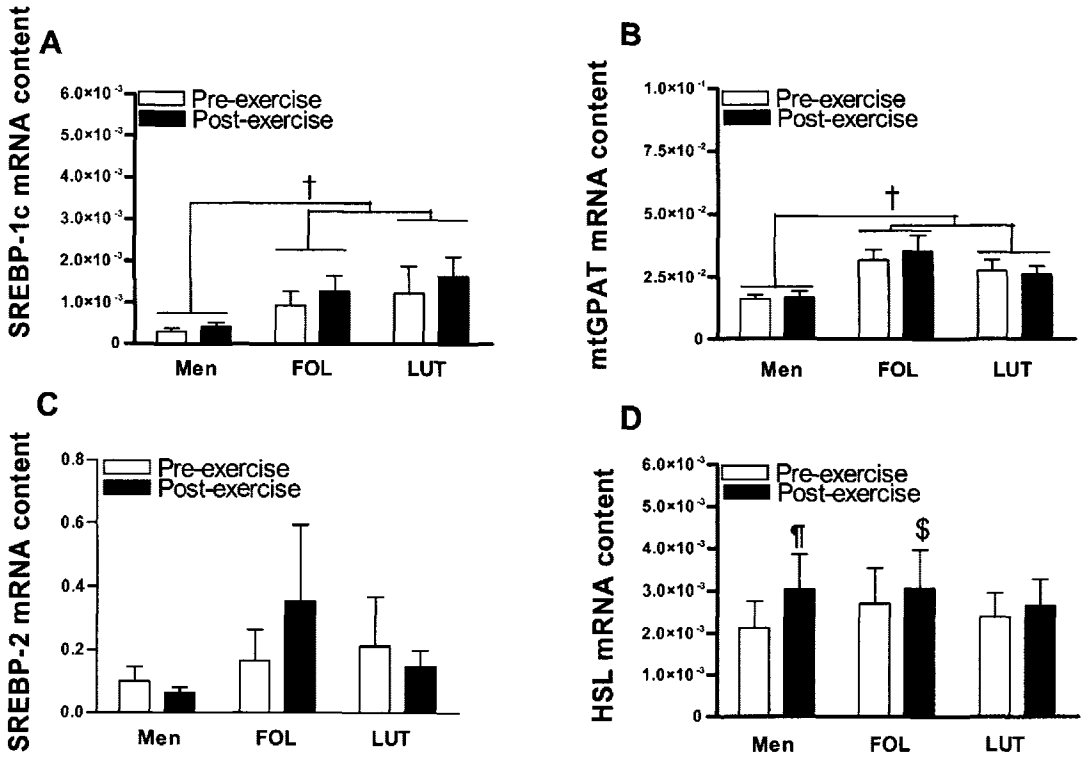
A
SREBP-1c mRNA content
Pre-exercise
Post-exercise
†
Men
FOL
LUT
B
mtGPAT mRNA content
Pre-exercise
Post-exercise
†
Men
FOL
LUT
C
SREBP-2 mRNA content
Pre-exercise
Post-exercise
Men
FOL
LUT
D
HSL mRNA content
Pre-exercise
Post-exercise
¶
$
Men
FOL
LUT

Figure 4

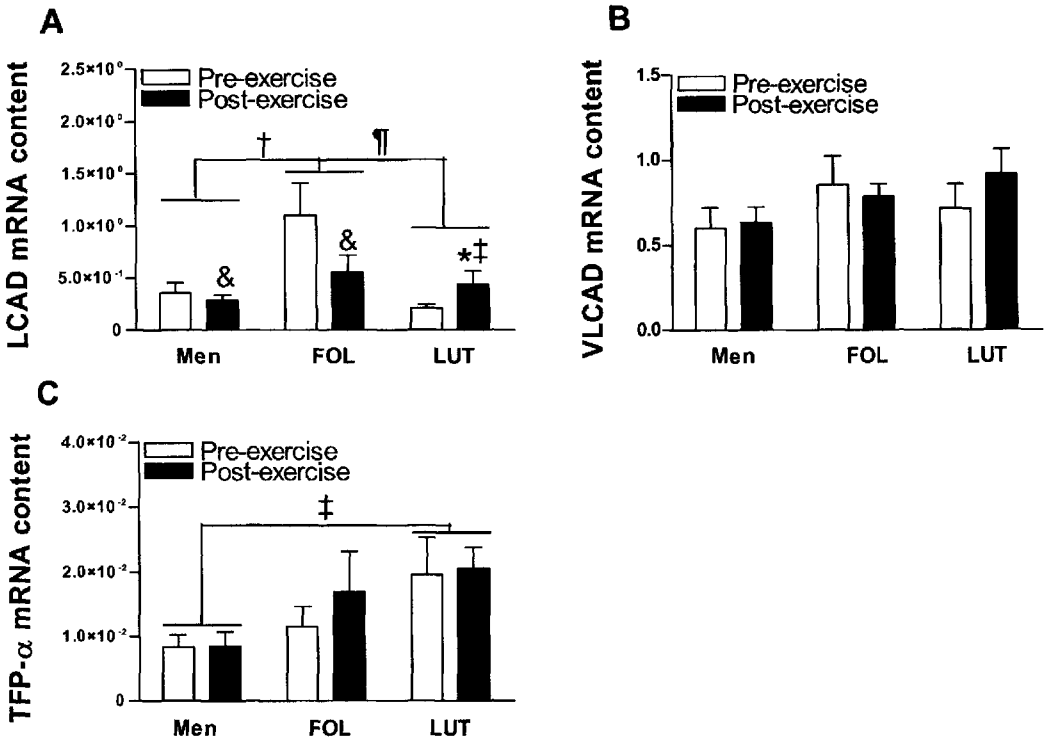
A
LCAD mRNA content
2.5×10⁰
2.0×10⁰
1.5×10⁰
1.0×10⁰
5.0×10⁻¹
0
Pre-exercise
Post-exercise
†
¶
&
&
*‡
Men
FOL
LUT
B
VLCAD mRNA content
1.5
1.0
0.5
0.0
Pre-exercise
Post-exercise
Men
FOL
LUT
C
TFP-α mRNA content
4.0×10⁻²
3.0×10⁻²
2.0×10⁻²
1.0×10⁻²
0
Pre-exercise
Post-exercise
‡
Men
FOL
LUT

Figure 5

CHAPTER 3: Manuscript 2

Formated for American Journal of Physiology

Acute exercise, sex and menstrual cycle influence the mRNA content for genes involved in carbohydrate and protein metabolism in human skeletal muscle

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Running head: Exercise and sex influences on carbohydrate and protein metabolism gene expression in muscle

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ABSTRACT

Lower carbohydrate and protein oxidation is often observed during endurance exercise in females as compared with males. Consequently, we hypothesized that sex and menstrual phase would influence mRNA content for genes involved in carbohydrate and protein metabolism in skeletal muscle. Twelve men and 12 women had muscle biopsies taken before and after a 90 min bout of cycling at 65 % VO_{2peak}. Women were studied in the mid-follicular (FOL) and mid-luteal (LUT) phases. mRNA content was determined using TaqMan® RT-PCR for genes involved in glucose transport (GLUT4), glucose phosphorylation (HKII), glycogen synthesis (glycogenin, GS-1, GSK3α), glycogenolysis (glycogen phosphorylase), glycolysis (PFK), transamination (AST), dehydrogenation (BCOAD) and BCOAD phosphorylation (BCOADK). Exercise significantly ($P < 0.05$) increased the mRNA content of HKII, AST and GLUT4 in men and women. Women had significantly ($P < 0.05$) higher mRNA content for HKII (FOL) and BCOADK (FOL, LUT) pre- and post- exercise, and GLUT4 (FOL) at rest, and lower mRNA content for PFK (LUT) pre- and post- exercise vs. men. FOL women had significantly ($P < 0.05$) higher glycogen phosphorylase and lower glycogenin mRNA content pre- and post- exercise, and tended to have higher GLUT4 mRNA content ($P = 0.056$) at rest, vs. LUT women. In conclusion, exercise induced mRNA changes that support a higher capacity for glucose phosphorylation, amino acid transamination, and post-exercise glucose uptake; whereas only the higher BCOADK mRNA content was directionally supportive of previously observed sex differences in exercise metabolism. The menstrual cycle had a minor impact on the mRNA content for several genes involved in CHO and protein metabolism.

Key words: sex, menstrual cycle phase, carbohydrate and protein metabolism, mRNA

INTRODUCTION

Several studies have found that women oxidize less carbohydrate (CHO) and more fat during endurance exercise as compared with men (8, 12, 15, 24, 35, 44, 51, 54, 63). In agreement with the latter observation, men had higher glucose turnover and metabolic clearance rate during exercise as compared with women (10, 12, 15). Animal studies have also implicated sex differences in CHO metabolism with female rats showing less glycogen depletion and CHO oxidation, as compared with male rats (31, 57). Furthermore, oophorectomy of female rats reduced CHO oxidation during endurance exercise (32).

Sex differences in protein metabolism have also been found in humans with females oxidizing less leucine than males both at rest (3, 44, 68) and during exercise (40, 44, 51), probably due to a lower skeletal muscle BCOAD activation (44). To date, we are not aware of any studies that have examined the influence of sex and menstrual cycle on the basal and acute exercise induced alterations in mRNA content for genes involved in carbohydrate or protein metabolism.

The potential molecular mechanisms of such sex differences in exercise substrate selection have mainly been evaluated for genes involved in fat metabolism (2, 16, 34, 55). For example, women have higher skeletal muscle mRNA levels of membrane bound fatty acid transporter (2, 33), membrane fatty acid binding protein, lipoprotein lipase (33) and beta-hydroxyacyl-CoA dehydrogenase (55), and higher mRNA and protein content of FAT/CD36 (33), as compared with men. Women also have a higher mRNA (16), but not activity (1), of carnitine palmitoyltransferase I, suggesting that there may be regulations at translation level, post-translation level. These findings are directionally supportive of a higher proportionate fat oxidization in women as compared with men during endurance

exercise (8, 12, 15, 24, 35, 44, 51, 54, 63). Because carbohydrate, protein and fat metabolism are interdependent processes, it is unclear whether or not the sex difference in substrate selection during endurance exercise is/are due to a primary sex or sex hormone regulated effect on lipid metabolism with a secondary effect on carbohydrate and protein metabolism and/or whether carbohydrate and protein metabolism are processes regulated by sex and/or sex hormones.

Studies concerning the influence of menstrual cycle phase on substrate utilization during endurance exercise show more variable results (5, 12, 18, 20, 23, 25, 28, 38, 41, 72, 73). Some have found no effect of menstrual cycle on substrate metabolism during endurance exercise (18, 23, 28), while others reported that CHO oxidation was lower during low- to moderate-intensity endurance exercise (20, 72, 73), and protein turnover was higher (5, 18, 38, 41, 55) in women in the LUT vs. the FOL phase of the menstrual cycle. In agreement with these observations, LUT women had a lower plasma glucose turnover (12, 58) , and lower pro-glycogen, macro-glycogen and total glycogen utilization during exercise (12), and a lower plasma amino acid concentration (38, 46), a higher requirement of lysine (38), a higher turnover of leucine (41), tryptophan (25) and phenylalanine (38), and a higher nitrogen excretion (5), as compared with FOL women.

Since sex differences in CHO (8, 12, 15, 24, 35, 44, 51, 54, 63) and protein (4, 66), metabolism are predominantly apparent during exercise, and acute exercise is a potent stimulus for mRNA induction (6), it is important to evaluate sex and menstrual cycle differences in mRNA expression in response to exercise. A single bout of endurance exercise changes the mRNA content of a diverse set of genes involved in CHO oxidation in human skeletal muscle (6). Acute exercise increased transcription of GLUT4 (47), and

mRNA content of GLUT4 (34), HKII (10, 27, 36, 37, 49, 52, 71), pyruvate dehydrogenase kinase 4 (PDK4) (70) and glycogenin (37, 60, 61). With respect to protein metabolism, we are not aware of any studies examining the effect of acute exercise in mRNA content; however, the activity of aspartate aminotransferase (AST) (34) increased in response to an exercise stimulus. In addition, the branched-chain 2-oxo acid dehydrogenase (BCOAD) enzyme is activated by exercise via de-phosphorylation (17, 29, 30, 44, 51, 59, 62, 69) due to reduced kinase activity (BCOADK) (17, 29, 62) in skeletal muscle.

The purpose of this study was to evaluate whether sex, acute exercise, or menstrual cycle influenced the mRNA content for genes involved in several aspects of CHO and protein metabolism in human skeletal muscle. Specifically, we evaluated the mRNA content for genes involved in the sarcolemmal glucose transport (GLUT4), glucose phosphorylation (hexokinase II – HKII), glycogen hydrolysis (glycogen phosphorylase - GP), glycolysis (phosphofructokinase – PFK), glycogen synthesis (glycogenin, glycogen synthase 1 – GS-1, glycogen synthase kinase-3α - GSK3α), transamination (AST), dehydrogenation (branched-chain 2-oxo acid dehydrogenase - BCOAD) and phosphorylation/inactivation of BCOAD (BCOADK). Given that the female sex and estradiol appear to attenuate glucose uptake (Rd and metabolic clearance rate) and attenuate glycogen use in both human and animal based research (8, 12, 15, 31, 57), we specifically hypothesized that the mRNA content for genes involved in glucose uptake (GLUT4, HKII) and glycogen breakdown (phosphorylase) would be lower in abundance in women as compared with men. Furthermore, with lower glucose turnover and glycogenolysis seen in LUT vs. FOL women (12, 58), we hypothesized that GLUT4, HKII, and phosphorylase

would be lower in LUT as compared with FOL women. We also hypothesized that the mRNA content for AST and BCOAD would be lower, while BCOAD would be higher, in women as compared to men before and after acute exercise. To our knowledge, this is also the first report of the influence of acute endurance exercise, sex and menstrual cycle on the mRNA content for GS-1, GSK3α, glycogen phosphorylase, AST, BCOAD, and BCOADK in humans.

MATERIAL AND METHODS

Subjects. Subjects for this study included twenty-four, young (22 ± 6 y) healthy, non-smoking, non-obese, recreationally active men (n=12) and women (n=12). They participated in recreational physical activity but were not trained athletes. Questionnaires indicated that their diet and activity patterns were constant in the 3-4 weeks prior to commencing the study. The female subject characteristics were: age, 22 ± 2 y (mean ± SD; weight, 61 ± 9 kg; body mass index, 23 ± 3 kg/m^2; fat-free mass, 73 ± 5 % body weight; VO_{2peak}, 55 ± 9 mL O_2/kg FFM/min. The male subject characteristics were: age, 22 ± 6 y; weight, 78 ± 11 kg; body mass index, 25 ± 3 kg/m^2; fat-free mass, 81 ± 5 % body weight; VO_{2peak}, 55 ± 9 mL O_2/kg FFM/min. Data from these subjects regarding CHO, protein and fat oxidation has been previously reported (12), and demonstrated that women had higher fat oxidation and lower protein and carbohydrate oxidation during endurance exercise as compared with males. Subjects were included in the study after they completed a health questionnaire to ensure that they were healthy and fit to participate. Each subject provided informed written consent and the study was approved by the McMaster University Hamilton Health Sciences Human Research Ethics Board.

Study design. Subjects did not change their habitual activity level throughout the study. Food intake was recorded for 4-days prior to commencing the study and was used to control diet (check-list provided to subjects) during the testing phase of the study. Women were tested in the mid-follicular (FOL, days 7-9) and mid-luteal (LUT, days 19-21) phases of the menstrual cycle with ovulation determined using a test kit (Clear Plan Easy,

Novartis) for the six females who were not taking a triphasic oral contraceptive pill. VO_2peak was measured at least 7 d before the first testing day (21).

On the morning of the test day, the subjects reported to the laboratory after an overnight fast. Muscle biopsies were taken from the *vastus lateralis* before and immediately (< 5 min) after the acute exercise bout of 90 min cycling at 65% VO_2peak. Muscle biopsies were randomly taken from the dominant or non-dominant leg ~15 cm proximal to the lateral knee joint of each subject under local anesthesia using manual suction and the women had the second set of biopsies (in the other menstrual cycle phase) taken from the contralateral leg. All biopsies were taken from different incisions ~4 cm apart, to minimize the potential influence of the biopsy itself on gene expression (50). Each biopsy weighed ~150 mg, and was immediately examined for and if needed, dissected from adherent fat and connective tissue. The first piece of muscle (~ 25 - 50 mg) was rapidly put into an RNase-free polyethylene tube, flash-frozen in liquid nitrogen, and stored at –86°C until being processed for analysis (see below).

Preparation of RNA. Total RNA was isolated from frozen human muscle according to Tarnopolsky et al. (64) and Mahoney et al. (43). Briefly, 25 -50 mg of muscle was homogenized in 1 ml of TRIzol reagent (Invitrogen, Carlsbad, CA) at 4^0C. After being left at room temperature for 5 – 10 min, followed by addition of 0.2 ml of chloroform, vortexing for 15 s, and standing at room temperature for 10 min, the mixture was centrifuged at 12,000 rpm at 4^0C for 15 min. The upper aqueous layer was transferred to a fresh tube and mixed with 0.5 ml of isopropanol ethanol. After standing at ~ 22^0 C for 10 min, the precipitated RNA was centrifuged at 12,000 rpm at 4^0 C for 10 min. The RNA pellet was washed twice with 0.5 ml of 75% ethanol. When the RNA pellet was air dried, it

was dissolved in 14 µl of DEPC-treated ddH_2O. Aliquots of 2 µl were stored at –80 ^{0}C. The concentration and purity of the RNA was tested using a UV spectrophotometer (Shimadzu UV-1201; Mandel Scientific, Guelph, Ontario) by measuring the absorbance at 260 (OD_{260}) and 280 (OD_{280}) nm. Measurements were done in duplicate and had an average coefficient of variation (CV) of <10%. The average purity (OD_{260}/OD_{280}) of the samples was ≥ 1.5 (note - before DNAse treatment). RNA integrity was examined through using agarose gel electrophoresis. When the density ratio of 28S to 18S rRNA was greater than 1, the RNA was considered of high quality.

TaqMan® real-time RT-PCR. Prior to TaqMan® real-time RT-PCR, RNA samples were treated with DNase I at 37°C for 25 min to remove any contaminating DNA using DNA-free™ (Ambion Inc, Austin, TX) according to the manufacturer's instructions. Specific primers and probe to each target gene (Table 1, Table 2) were designed based on the cDNA sequence in GenBank (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi) with primer 3 designer (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Their specificity was monitored using Blast (http://www.ncbi.nlm.nih.gov/BLAST/). After being calculated with Analyser of Oligo (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/Default.aspx), only primer and probe sets with delta G > - 9 kcal.mole^{-1} for hairpin structures and dimers were selected. All target gene probes were labeled with FAM at their 5' ends and BHQ-1 at their 3' ends (Sigma Aldrich Canada, Sigma-Genosys, Oakville, Ontario, Canada). Human β2-microglobulin (β2-M) was used as an internal standard since acute exercise, sex and menstrual cycle phase did not influence β2-M mRNA content in skeletal muscle in the

current study (data not shown). Human β2-M primers and probes were same as used by Mahoney *et al.* (43). Duplex RT-PCR was performed on an iCycler real-time PCR system (Bio-Rad Laboratories, Hercules, CA) in the One-step TaqMan® RT-PCR Master Mix Reagents (Roch, Branchburg, New Jersey, part no. 4309169) according to the manufacture's instruction with target gene primers and probe and β2-M primers and probe in the same reaction. We optimized each PCR reaction condition so that the amplification efficiency for both target gene and β2-M was close to 1 and the difference between the slopes of the regression curves of each target gene and β2-M (Ct vs. amount of RNA) was less than 10% (Table 3). The specificity of the priming was confirmed by using agarose gel electrophoresis. All samples were run in duplicate simultaneously with RNA- and RT-negative controls. RT-PCR profiling was: 1cycle, 48^0C, 30 min; 1cycle, 95^0C, 10 min; 50 cycles, 95^0C, 15s; 60^0C, 1min; and 1cycle, 4^0C, ∞. Fluorescence emission was detected through filters corresponding to the reporter dye at the 5'ends of the probes, and C_T was automatically displayed.

Statistical Analysis. All statistical analyses about mRNA expression of the genes tested were performed on linear data $2^{-\Delta CT}$ normalized with β2-M as an internal reference (42). Data on sex differences in target gene mRNA expression were analyzed using a two-way ANOVA of sex X exercise with sex as a between group factor. Data on menstrual cycle phase effect on the target gene mRNA expression were analyzed using a two-way repeated measures ANOVA of menstrual cycle phase X exercise with both menstrual cycle phase and exercise as within group factors. Post-hoc test was performed only when interaction between sex and exercise or menstrual phase and exercise was significant ($P < 0.05$). All

data displayed in the figures are expressed as mean (±SE), using $2^{-\Delta CT}$ normalized with β2-M. The differences in mRNA expression between sexes, menstrual cycle phases or pre- and post- exercise are expressed as mean fold change (±SE), using $2^{-\Delta\Delta CT}$ (42). We also checked the six women who were taking oral contraceptives, as compared with the six who were taking an oral triphasic contraceptive pill and did not find any differential influence on mRNA abundance; consequently, all data for women were analyed and presented using N = 12. All statistical analyses were done using computerized statistics software (Statistica; Statsoft, Tulsa, OK) with statistical significance set at $\alpha \leq 0.05$ and statistical trends at $0.05 < \alpha \leq 0.08$.

RESULTS

It has been reported with the same subjects as investigated in this study that both FOL and LUT women had a lower RER, glucose Ra, glucose Rd and MCR during exercise, as compared with men (11). Men used more proglycogen than LUT women (11). The sex differential mRNA expression of GLUT4, hexokinase II, PFK and BCOADK observed in this study does not directly support the substrate utilization study that women oxidize less CHO than men during exercise in the same subjects (11).

Genes involved in glucose transport and glucose phosphorylation

GLUT 4

Acute exercise significantly increased the mRNA content of GLUT4 in men (2.1 ± 0.5 fold, P = 0.002) and LUT women (2.0 ± 0.3 fold, P = 0.002) (Figure 1A). No significant exercise effect was detected for the mRNA content of GLUT4 in FOL women (Figure 1A). In contrast, FOL women had a significantly higher GLUT4 mRNA content than men at rest (2.4 ± 0.7 fold, P = 0.032) (Figure 1A). There was no significant difference in GLUT4 mRNA content between LUT women and men pre- and post-exercise (Figure 1A). FOL women tended to have a higher mRNA content of GLUT4 than LUT women at rest (2.2 ± 0.6, P = 0.056) (Figure 1A).

HKII

Exercise significantly increased the mRNA content of HK II in men (2.0 ± 0.5 fold, P = 0.03), FOL women (1.5 ± 0.3 fold, P = 0.04) and LUT women (2.4 ± 0.4 fold, P = 0.04) (Figure 1B). FOL women had higher HKII mRNA content than men (2.4 ± 0.6, P = 0.016)

pre- and post-exercise (Figure 1B). There was no significant difference in mRNA content of HKII between LUT women and men (Figure 1B), and menstrual cycle phase had no influence on HK II mRNA content (Figure 1B).

Genes involved in glycogenolysis and glycolysis

glycogen phosphorylase

No significant exercise or sex effect was seen for the mRNA content of glycogen phosphorylase (Figure 1C). Glycogen phosphorylase mRNA content was significantly higher in FOL vs LUT women (1.6 ± 0.4, P = 0.041) (Figure 1C).

PFK

No significant exercise effect was seen for PFK mRNA content in men, FOL or LUT women (Figure 1D). FOL women and men had similar levels of PFK abundance (Figure 1D). Men had a significantly higher PFK mRNA content than LUT women (3.4 ± 1.5 fold, P = 0.031) (Figure 1D). Finally, the menstrual cycle phase did not affect PFK mRNA content (Figure 1D).

Genes involved in glycogen synthesis

glycogenin

No significant exercise effect or sex effect on mRNA content of glycogenin were detected (Figure 2A). The mRNA content of glycogenin in LUT women was significantly higher than that in FOL women (1.2 ± 0.2 fold, P = 0.02) (Figure 2A).

GS-1 and GSK3α

No exercise, sex or menstrual phase effects were seen on the mRNA content of GS (Figure 2B). No exercise or menstrual phase effects were seen on the mRNA content of GSK3α (Figure 2C). The mRNA content of GSK3α in FOL women tended to be higher than that in men (1.8 ± 0.4 fold, P = 0.065).

Genes involved in amino acid metabolism

AST

There were no differences in mRNA content of AST between men and women during FOL and LUT phases pre- and post- exercise (Figure 3A). Exercise increased AST mRNA content in men (1.2 ± 0.2 fold, P = 0.038) and women in FOL (1.3 ± 0.2 fold, P = 0.032) and LUT (1.3 ± 0.3 fold, P = 0.063) phases (Figure 3A). No menstrual phase effect was detected on AST mRNA content (Figure 3A) pre- or post- exercise.

BCOAD

No significant exercise effect, sex differences or menstrual phase effect were detected for BCOAD mRNA content (Figure 3B).

BCOADK

The mRNA content of BCOADK was significantly higher in FOL and LUT women than men pre- and post- exercise (FOL, 1.4 ± 0.2 fold, P = 0.026; LUT, 1.3 ± 0.2 fold, P = 0.05) (Figure 3C). Exercise did not significantly influence the mRNA content of BCOADK in men, FOL women, or LUT women pre- and post exercise (Figure 3C). No

significant menstrual phase effect on the mRNA content of BCOADK was detected pre- and post- exercise (Figure 3C).

DISSCUSSION

In contrast to the observation that the mRNA content for many genes involved in skeletal muscle fat metabolism are directionally supportive of the observed higher proportional fat oxidation in women (16), we did not find a coordinate or directional influence of sex on the mRNA abundance of most of the measured genes involved in carbohydrate and protein metabolism that were supportive of a lower carbohydrate (8, 12, 15, 24, 35, 44, 51, 54, 63) and protein (4, 40, 44, 51, 66) oxidation in women during exercise. The only mRNA species that was supportive of lower protein oxidation during exercise was BCOADK (the kinase inhibits BCOAD activity). Consistent with the observation that the phase of the menstrual has a comparatively minor influence on substrate selection during endurance exercise (20, 23, 25, 28, 72, 73), we did not find any consistent effects on mRNA abundance for genes involved in carbohydrate or protein metabolism. Acute exercise increased the mRNA abundance for HKII, GLUT4 and AST but had no immediate effect on the mRNA abundance for glycogenin, GSK-3α, GS-1, PFK, phosphorylase, BCOAD or BCOADK. The mRNA content of glycogenin increases at 1.5 – 3 hours after exercise (37, 60, 61). Whether or not increases are seen at a later time point post-exercise for the rest of the genes cannot be determined from the current data. These data are the first to report on the influence of acute exercise on mRNA abundance for GSK-3α, PFK, phosphorylase, AST, BCOAD and BCOADK in human skeletal muscle.

Sex differences in the capacity for carbohydrate utilization

In apparent contradiction to the observation of lower muscle glucose uptake (10), metabolic clearance rate (8, 15) and attenuation of muscle glycogen use during endurance exercise in women (8, 12, 15, 24, 35, 44, 51, 54, 63), we found novel data showing that women in the FOL phase had significantly higher GLUT4 and HKII mRNA content at rest and post-exercise, and similar mRNA content of glycogenin, GS-1, GSK3α and glycogen phosphorylase as compared with men. The higher mRNA abundance for HKII and GLUT4 in women may be expected to result in higher rates of muscle glucose uptake and glycogen synthesis during and/or after exercise (9, 36). However, it is likely that the metabolic clearance rate/skeletal muscle uptake (Rd) for glucose are regulated by the availability of substrate and intracellular signals, with exercise substrate selection being primarily mediated by a higher rate of fat oxidation in women (8, 12, 15, 24, 35, 44, 51, 54, 63). The metabolic reciprocal interrelatedness of lipid and carbohydrate metabolism during exercise has been conceptualized for decades through the classical Randle cycle (13) and more recently, as the cross-over concept (13). For example, a greater elevation of plasma FFA in women during exercise (45) may spare muscle glycogen utilization by inactivation of phosphorylase during exercise (14). Alternative explainations for the apparent lack of directional consistency between GLUT4 and HKII mRNA abundance and metabolism could be that the mRNA levels do not correlate with protein levels (57), or, in the case of GLUT 4, that intracellular location is more important than total content (67). It has also been suggested that GLUT4 content is more important in post-exercise recovery as compared with during exercise (39); however, the higher GLUT4 mRNA abundance would

predict (assuming a similar increase in protein) that women would have higher rates of post-exercise glycogen re-synthesis, yet this is not the case (65).

The finding of a lower mRNA abundance in women as compared with men is consisent with reports that PFK activity is lower in women as compared with men (19, 26). In theory a lower PFK activity in women could lead to lower net carbon flux through glycolysis and this could relate to lower glycogenolysis and/or glucose uptake; however, during endurance exercise it is more likely that higher lipid oxidation is a more important mediator of sex differences in glucose uptake/clearance and glycogen utilization (8, 12, 15, 24, 35, 44, 51, 54, 63). Therefore, the sex differential mRNA expression of the genes involved in CHO metabolism was not coordinately or directionally supportive of the sex difference in CHO oxidation in human skeletal muscle during aerobic activity. Little is known about the transcriptional regulation of GLUT4, HKII and PFK in regard to their sex differential mRNA expression in human skeletal muscle and it is not possible to determine whether or not the protein for the respective mRNA species was directionally consistent, although the PFK mRNA was consistent with previous reports of sex differences in PFK activity (19, 26).

Sex differences in the capacity for amino acid utilization

In apparent contradiction to the observation of less amino acid oxidation during exercise in women vs. men (40, 44, 51), we did not find sex differences in mRNA content of AST or BCOAD between men and women during either the FOL or LUT phases. Importantly, our finding of higher mRNA abundance for BCOADK in FOL and LUT women is consistent with higher phosphorylation potential for BCOAD, and consequently

lower activity (44). In addition to potentially higher BCOADK protein abundance, a lower oxidation of amino acids in women vs. men could also be a response to higher fat oxidation leading to lower carbohydrate oxidation in women as compared with men (8, 12, 15, 24, 35, 44, 51, 54, 63). The transcriptional regulatory mechanism underlying the sex differential BCOADK mRNA expression is not clear but does not appear to be due to energy charge or AMPK signaling, given that these are not different between sexes in the resting state (56) and exercise did not alter the mRNA content of BCOADK in men (current study) even though AMPK activation increased during exercise in men (56).

Exercise increases the capacity for utilization of carbohydrate and protein

Exercise increased the mRNA content of GLUT 4 in men and LUT (but not FOL) women, and HKII and AST in men and women. We are the first to measure and report that exercise did not influence the mRNA content of GS-1, GSK3α, or glycogen phosphorylase in human skeletal muscle. Our observations are consistent with the studies measuring the mRNA content of GLUT4 (51), HK II (10, 36, 37, 49, 52), but inconsistent with studies showing an acute effect of exercise increasing GS-1 activity (10, 36) due to dephosphorylation by activation of phosphatase and inactivation of kinases (48). The menstrual cycle phase differential response of GLUT4 to exercise suggests that there may be sex hormonal influences involved in the regulation of GLUT4 mRNA expression in women at different phases of the menstrual cycle phases during exercise (7, 22). We confirm that acute endurance exercise influences the mRNA content of the genes involved in glucose transport (GLUT4), phosphorylation and trapping (HKII) in human skeletal

muscle, which may increase the capacity for glucose uptake, disposal and oxidation. Our observations are also in agreement with that training increased AST activity in human muscle (53), and show that this is a very early response induced immediately after a single bout of exercise. The exercise-mediated increase in percentage of activated BCOAD (30, 44) is due to lower phosphorylation (59) associated with a reduction in BCOADK protein and activity (29). Because we did not find any significant exercise effect on the mRNA content of BCOADK in men, FOL women, or LUT women (Figure 1C, 2C), the exercise mediated decrease in BCOADK protein and activity (29) appears to be primarily regulated at the translation and post-translation levels in human skeletal muscle.

Menstrual cycle phase differences in the capacity for carbohydrate and protein utilization

We found that FOL women had a significantly higher mRNA content of GLUT4 at rest and glycogen phosphorylase pre- and post- exercise than LUT women, and LUT women had a significantly higher mRNA content of glycogenin than FOL women pre- and post- exercise. Menstrual cycle phases had no influence on mRNA content of HK II, PFK, GS-1, GSK3α, AST, BCOAD or BCOADK. These observations suggest that FOL women may have a higher capacity for glucose uptake at rest, a higher capacity for hydrolysis of glycogen and a lower capacity for glycogen synthesis pre- and post-exercise vs. LUT women, which are consistent with that LUT women have a lower plasma glucose turnover (12, 73) and a lower total glycogen utilization (12), and spare more glycogen than FOL women during exercise (12). However, we previously reported that menstrual cycle phase

did not influence the mRNA content of PPARα and PGC-1α (16), implicating that the capacity for transcriptional activation of the genes involved in carbohydrate utilization was similar between FOL and LUT women. Furthermore, the current study indicates that FOL and LUT women have similar glycolysis capacity and the capacity for oxidation of amino acids. Therefore, the influences of menstrual cycle phases on mRNA content do not consistently support that LUT women oxidized less CHO (20, 72, 73) and more protein (5, 18, 38, 41, 46) than FOL women. The menstrual phase differences in CHO and amino acid oxidation may be mediated at translation, post-translation or substrate levels. The regulatory relationship among the mRNA expression of GLUT4, glycogen phosphorylase and glycogenin in regard to menstrual cycle phase differences needs to be elucidated in the future.

We conclude that women in the FOL phase had higher GLUT4 and hexokinase II mRNA content at rest and post-exercise, and similar mRNA content of glycogenin, GS-1, GSK3α and glycogen phosphorylase, and LUT women a higher PFK mRNA content, as compared with men. These observations were not directionally supportive of a lower carbohydrate and protein oxidation in women during exercise. Both FOL and LUT women had a higher mRNA abundance for BCOADK, which is the only mRNA species that was supportive of lower protein oxidation during exercise. Exercise increases the mRNA capacity of CHO and protein metabolism. The menstrual cycle influences the profile of mRNA involved in CHO metabolism at rest and its response to endurance exercise.

REFERENCES

1. **Berthon PM, Howlett RA, Heigenhauser GJ, and Spriet LL.** Human skeletal muscle carnitine palmitoyltransferase I activity determined in isolated intact mitochondria. *J Appl Physiol* 85: 148-153, 1998.

2. **Binnert C, Koistinen HA, Martin G, Andreelli F, Ebeling P, Koivisto VA, Laville M, Auwerx J, and Vidal H.** Fatty acid transport protein-1 mRNA expression in skeletal muscle and in adipose tissue in humans. *Am J Physiol Endocrinol Metab* 279: E1072-1079, 2000.

3. **Boirie Y, Gachon P, Cordat N, Ritz P, and Beaufrere B.** Differential insulin sensitivities of glucose, amino acid, and albumin metabolism in elderly men and women. *J Clin Endocrinol Metab* 86: 638-644, 2001.

4. **Braun B and Horton T.** Endocrine regulation of exercise substrate utilization in women compared to men. *Exerc Sport Sci Rev* 29: 149-154, 2001.

5. **Calloway DH and Kurzer MS.** Menstrual cycle and protein requirements of women. *J Nutr* 112: 356-366, 1982.

6. **Cameron-Smith D.** Exercise and skeletal muscle gene expression. *Clin Exp Pharmacol Physiol* 29: 209-213, 2002.

7. **Campbell SE and Febbraio MA.** Effect of the ovarian hormones on GLUT4 expression and contraction-stimulated glucose uptake. *Am J Physiol Endocrinol Metab* 282: E1139-1146, 2002.

8. **Carter SL, Rennie C, and Tarnopolsky MA.** Substrate utilization during endurance exercise in men and women after endurance training. *Am J Physiol Endocrinol Metab* 280: E898-907, 2001.

9. **Christ-Roberts CY, Pratipanawatr T, Pratipanawatr W, Berria R, Belfort R, Kashyap S, and Mandarino LJ.** Exercise training increases glycogen synthase activity and GLUT4 expression but not insulin signaling in overweight nondiabetic and type 2 diabetic subjects. *Metabolism* 53: 1233-1242, 2004.

10. **Cusi KJ, Pratipanawatr T, Koval J, Printz R, Ardehali H, Granner DK, Defronzo RA, and Mandarino LJ.** Exercise increases hexokinase II mRNA, but not activity in obesity and type 2 diabetes. *Metabolism* 50: 602-606, 2001.

11. **Devries MC, Hamadeh MJ, Phillips SM, and Tarnopolsky MA.** Menstrual cycle phase and sex influence muscle glycogen utilization and glucose turnover during moderate-intensity endurance exercise. *Am J Physiol Regul Integr Comp Physiol* 291: R1120-1128, 2006.

12. **Devries MC, Hamadeh MJ, Phillips SM, and Tarnopolsky MA.** Menstrual Cycle Phase and Sex Influence Muscle Glycogen Utilization and Glucose Turnover During Moderate Intensity Endurance Exercise. *Am J Physiol Regul Integr Comp Physiol*, 2006.

13. **Dulloo AG, Gubler M, Montani JP, Seydoux J, and Solinas G.** Substrate cycling between de novo lipogenesis and lipid oxidation: a thermogenic mechanism against skeletal muscle lipotoxicity and glucolipotoxicity. *Int J Obes Relat Metab Disord* 28 Suppl 4: S29-37, 2004.

14. **Dyck DJ, Peters SJ, Wendling PS, Chesley A, Hultman E, and Spriet LL.** Regulation of muscle glycogen phosphorylase activity during intense aerobic cycling with elevated FFA. *Am J Physiol* 270: E116-125, 1996.

15. **Friedlander AL, Casazza GA, Horning MA, Huie MJ, Piacentini MF, Trimmer JK, and Brooks GA.** Training-induced alterations of carbohydrate metabolism in women: women respond differently from men. *J Appl Physiol* 85: 1175-1186, 1998.

16. **Fu MH HM, Ye CH and Tarnopolsky MA.** Acute exercise, sex and menstrual cycle influence the mRNA content for genes involved in fat metabolism in human skeletal muscle, 2006. In press.

17. **Fujii H, Shimomura Y, Murakami T, Nakai N, Sato T, Suzuki M, and Harris RA.** Branched-chain alpha-keto acid dehydrogenase kinase content in rat skeletal muscle is decreased by endurance training. *Biochem Mol Biol Int* 44: 1211-1216, 1998.

18. **Garrel DR, Welsch C, Arnaud MJ, and Tourniaire J.** Relationship of the menstrual cycle and thyroid hormones to whole-body protein turnover in women. *Hum Nutr Clin Nutr* 39: 29-37, 1985.

19. **Green HJ, Fraser IG, and Ranney DA.** Male and female differences in enzyme activities of energy metabolism in vastus lateralis muscle. *J Neurol Sci* 65: 323-331, 1984.

20. **Hackney AC, McCracken-Compton MA, and Ainsworth B.** Substrate responses to submaximal exercise in the midfollicular and midluteal phases of the menstrual cycle. *Int J Sport Nutr* 4: 299-308, 1994.

21. **Hamadeh MJ, Devries MC, and Tarnopolsky MA.** Estrogen supplementation reduces whole body leucine and carbohydrate oxidation and increases lipid oxidation in men during endurance exercise. *J Clin Endocrinol Metab* 90: 3592-3599, 2005.

22. **Hansen PA, McCarthy TJ, Pasia EN, Spina RJ, and Gulve EA.** Effects of ovariectomy and exercise training on muscle GLUT-4 content and glucose metabolism in rats. *J Appl Physiol* 80: 1605-1611, 1996.

23. **Horton TJ, Miller EK, Glueck D, and Tench K.** No effect of menstrual cycle phase on glucose kinetics and fuel oxidation during moderate-intensity exercise. *Am J Physiol Endocrinol Metab* 282: E752-762, 2002.

24. **Horton TJ, Pagliassotti MJ, Hobbs K, and Hill JO.** Fuel metabolism in men and women during and after long-duration exercise. *J Appl Physiol* 85: 1823-1832, 1998.

25. **Hrboticky N, Leiter LA, and Anderson GH.** Menstrual cycle effects on the metabolism of tryptophan loads. *Am J Clin Nutr* 50: 46-52, 1989.

26. **Jaworowski A, Porter MM, Holmback AM, Downham D, and Lexell J.** Enzyme activities in the tibialis anterior muscle of young moderately active men and women: relationship with body composition, muscle cross-sectional area and fibre type composition. *Acta Physiol Scand* 176: 215-225, 2002.

27. **Jorgensen SB, Wojtaszewski JF, Viollet B, Andreelli F, Birk JB, Hellsten Y, Schjerling P, Vaulont S, Neufer PD, Richter EA, and Pilegaard H.** Effects of alpha-AMPK knockout on exercise-induced gene activation in mouse skeletal muscle. *Faseb J* 19: 1146-1148, 2005.

28. **Kanaley JA, Boileau RA, Bahr JA, Misner JE, and Nelson RA.** Substrate oxidation and GH responses to exercise are independent of menstrual phase and status. *Med Sci Sports Exerc* 24: 873-880, 1992.

29. **Kasperek GJ, Dohm GL, and Snider RD.** Activation of branched-chain keto acid dehydrogenase by exercise. *Am J Physiol* 248: R166-171, 1985.

30. **Kasperek GJ and Snider RD.** Effect of exercise intensity and starvation on activation of branched-chain keto acid dehydrogenase by exercise. *Am J Physiol* 252: E33-37, 1987.

31. **Kendrick ZV and Ellis GS.** Effect of estradiol on tissue glycogen metabolism and lipid availability in exercised male rats. *J Appl Physiol* 71: 1694-1699, 1991.

32. **Kendrick ZV, Steffen CA, Rumsey WL, and Goldberg DI.** Effect of estradiol on tissue glycogen metabolism in exercised oophorectomized rats. *J Appl Physiol* 63: 492-496, 1987.

33. **Kiens B, Roepstorff C, Glatz JF, Bonen A, Schjerling P, Knudsen J, and Nielsen JN.** Lipid-binding proteins and lipoprotein lipase activity in human skeletal muscle: influence of physical activity and gender. *J Appl Physiol* 97: 1209-1218, 2004.

34. **King SW, Statland BE, and Savory J.** The effect of a short burst of exercise on activity values of enzymes in sera of healthy young men. *Clin Chim Acta* 72: 211-218, 1976.

35. **Knechtle B, Muller G, Willmann F, Kotteck K, Eser P, and Knecht H.** Fat oxidation in men and women endurance athletes in running and cycling. *Int J Sports Med* 25: 38-44, 2004.

36. **Koval JA, DeFronzo RA, O'Doherty RM, Printz R, Ardehali H, Granner DK, and Mandarino LJ.** Regulation of hexokinase II activity and expression in human muscle by moderate exercise. *Am J Physiol* 274: E304-308, 1998.

37. **Kraniou Y, Cameron-Smith D, Misso M, Collier G, and Hargreaves M.** Effects of exercise on GLUT-4 and glycogenin gene expression in human skeletal muscle. *J Appl Physiol* 88: 794-796, 2000.

38. **Kriengsinyos W, Wykes LJ, Goonewardene LA, Ball RO, and Pencharz PB.** Phase of menstrual cycle affects lysine requirement in healthy women. *Am J Physiol Endocrinol Metab* 287: E489-496, 2004.

39. **Kuo CH, Browning KS, and Ivy JL.** Regulation of GLUT4 protein expression and glycogen storage after prolonged exercise. *Acta Physiol Scand* 165: 193-201, 1999.

40. **Lamont LS, McCullough AJ, and Kalhan SC.** Gender differences in leucine, but not lysine, kinetics. *J Appl Physiol* 91: 357-362, 2001.

41. **Lariviere F, Moussalli R, and Garrel DR.** Increased leucine flux and leucine oxidation during the luteal phase of the menstrual cycle in women. *Am J Physiol* 267: E422-428, 1994.

42. **Livak KJ and Schmittgen TD.** Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25: 402-408, 2001.

43. **Mahoney DJ, Carey K, Fu MH, Snow R, Cameron-Smith D, Parise G, and Tarnopolsky MA.** Real-time RT-PCR analysis of housekeeping genes in human skeletal muscle following acute exercise. *Physiol Genomics* 18: 226-231, 2004.

44. **McKenzie S, Phillips SM, Carter SL, Lowther S, Gibala MJ, and Tarnopolsky MA.** Endurance exercise training attenuates leucine oxidation and BCOAD activation during exercise in humans. *Am J Physiol Endocrinol Metab* 278: E580-587, 2000.

45. **Mittendorfer B, Horowitz JF, and Klein S.** Effect of gender on lipid kinetics during endurance exercise of moderate intensity in untrained subjects. *Am J Physiol Endocrinol Metab* 283: E58-65, 2002.

46. **Moller SE, Moller BM, Olesen M, and Fjalland B.** Effects of oral contraceptives on plasma neutral amino acids and cholesterol during a menstrual cycle. *Eur J Clin Pharmacol* 50: 179-184, 1996.

47. **Neufer PD and Dohm GL.** Exercise induces a transient increase in transcription of the GLUT-4 gene in skeletal muscle. *Am J Physiol* 265: C1597-1603, 1993.

48. **Nielsen JN and Wojtaszewski JF.** Regulation of glycogen synthase activity and phosphorylation by exercise. *Proc Nutr Soc* 63: 233-237, 2004.

49. **Nordsborg N, Bangsbo J, and Pilegaard H.** Effect of high-intensity training on exercise-induced gene expression specific to ion homeostasis and metabolism. *J Appl Physiol* 95: 1201-1206, 2003.

50. **Norrbom J, Sundberg CJ, Ameln H, Kraus WE, Jansson E, and Gustafsson T.** PGC-1alpha mRNA expression is influenced by metabolic perturbation in exercising human skeletal muscle. *J Appl Physiol* 96: 189-194, 2004.

51. **Phillips SM, Atkinson SA, Tarnopolsky MA, and MacDougall JD.** Gender differences in leucine kinetics and nitrogen balance in endurance athletes. *J Appl Physiol* 75: 2134-2141, 1993.

52. **Pilegaard H, Saltin B, and Neufer PD.** Exercise induces transient transcriptional activation of the PGC-1alpha gene in human skeletal muscle. *J Physiol* 546: 851-858, 2003.

53. **Raimondi GA, Puy RJ, Raimondi AC, Schwarz ER, and Rosenberg M.** Effects of physical training on enzymatic activity of human skeletal muscle. *Biomedicine* 22: 496-501, 1975.

54. **Riddell MC, Partington SL, Stupka N, Armstrong D, Rennie C, and Tarnopolsky MA.** Substrate utilization during exercise performed with and without glucose ingestion in female and male endurance trained athletes. *Int J Sport Nutr Exerc Metab* 13: 407-421, 2003.

55. **Roepstorff C, Schjerling P, Vistisen B, Madsen M, Steffensen CH, Rider MH, and Kiens B.** Regulation of oxidative enzyme activity and eukaryotic elongation factor 2 in human skeletal muscle: influence of gender and exercise. *Acta Physiol Scand* 184: 215-224, 2005.

56. **Roepstorff C, Thiele M, Hillig T, Pilegaard H, Richter EA, Wojtaszewski JF, and Kiens B.** Higher skeletal muscle {alpha}2AMPK activation and lower energy charge and fat oxidation in men than in women during submaximal exercise. *J Physiol* 574: 125-138, 2006.

57. **Rooney TP, Kendrick ZV, Carlson J, Ellis GS, Matakevich B, Lorusso SM, and McCall JA.** Effect of estradiol on the temporal pattern of exercise-induced tissue glycogen depletion in male rats. *J Appl Physiol* 75: 1502-1506, 1993.

58. **Rose AJ and Richter EA.** Skeletal muscle glucose uptake during exercise: how is it regulated? *Physiology (Bethesda)* 20: 260-270, 2005.

59. **Rush JW, MacLean DA, Hultman E, and Graham TE.** Exercise causes branched-chain oxoacid dehydrogenase dephosphorylation but not AMP deaminase binding. *J Appl Physiol* 78: 2193-2200, 1995.

60. **Shearer J, Graham TE, Battram DS, Robinson DL, Richter EA, Wilson RJ, and Bakovic M.** Glycogenin activity and mRNA expression in response to volitional exhaustion in human skeletal muscle. *J Appl Physiol* 99: 957-962, 2005.

61. **Shearer J, Wilson RJ, Battram DS, Richter EA, Robinson DL, Bakovic M, and Graham TE.** Increases in glycogenin and glycogenin mRNA accompany glycogen resynthesis in human skeletal muscle. *Am J Physiol Endocrinol Metab* 289: E508-514, 2005.

62. **Shimomura Y, Fujii H, Suzuki M, Fujitsuka N, Naoi M, Sugiyama S, and Harris RA.** Branched-chain 2-oxo acid dehydrogenase complex activation by tetanic contractions in rat skeletal muscle. *Biochim Biophys Acta* 1157: 290-296, 1993.

63. **Tarnopolsky LJ, MacDougall JD, Atkinson SA, Tarnopolsky MA, and Sutton JR.** Gender differences in substrate for endurance exercise. *J Appl Physiol* 68: 302-308, 1990.

64. **Tarnopolsky M, Parise G, Fu MH, Brose A, Parshad A, Speer O, and Wallimann T.** Acute and moderate-term creatine monohydrate supplementation does not affect creatine transporter mRNA or protein content in either young or elderly humans. *Mol Cell Biochem* 244: 159-166, 2003.

65. **Tarnopolsky MA, Bosman M, Macdonald JR, Vandeputte D, Martin J, and Roy BD.** Postexercise protein-carbohydrate and carbohydrate supplements increase muscle glycogen in men and women. *J Appl Physiol* 83: 1877-1883, 1997.

66. **Tarnopolsky MA and Ruby BC.** Sex differences in carbohydrate metabolism. *Curr Opin Clin Nutr Metab Care* 4: 521-526, 2001.

67. **Tremblay F, Dubois MJ, and Marette A.** Regulation of GLUT4 traffic and function by insulin and contraction in skeletal muscle. *Front Biosci* 8: d1072-1084, 2003.

68. **Volpi E, Lucidi P, Bolli GB, Santeusanio F, and De Feo P.** Gender differences in basal protein kinetics in young adults. *J Clin Endocrinol Metab* 83: 4363-4367, 1998.

69. **Wagenmakers AJ, Brookes JH, Coakley JH, Reilly T, and Edwards RH.** Exercise-induced activation of the branched-chain 2-oxo acid dehydrogenase in human muscle. *Eur J Appl Physiol Occup Physiol* 59: 159-167, 1989.

70. **Watt MJ, Southgate RJ, Holmes AG, and Febbraio MA.** Suppression of plasma free fatty acids upregulates peroxisome proliferator-activated receptor (PPAR) alpha and delta and PPAR coactivator 1alpha in human skeletal muscle, but not lipid regulatory genes. *J Mol Endocrinol* 33: 533-544, 2004.

71. **Weber FE and Pette D.** Rapid up- and down-regulation of hexokinase II in rat skeletal muscle in response to altered contractile activity. *FEBS Lett* 261: 291-293, 1990.

72. **Wenz M, Berend JZ, Lynch NA, Chappell S, and Hackney AC.** Substrate oxidation at rest and during exercise: effects of menstrual cycle phase and diet composition. *J Physiol Pharmacol* 48: 851-860, 1997.

73. **Zderic TW, Coggan AR, and Ruby BC.** Glucose kinetics and substrate oxidation during exercise in the follicular and luteal phases. *J Appl Physiol* 90: 447-453, 2001.

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Figure legends

Fig.1. Skeletal muscle mRNA content of the genes involved in the glucose transport, glucose phosphorylation, glycogenolysis and glycolysis in men and women (FOL and LUT). Panel A: GLUT4; ‡ At rest FOL higher than men (P = 0.032); * Post- exercise higher than pre-exercise (P = 0.002); & trend for FOL to be higher than LUT at rest (P = 0.056). Panel B: HK II; † FOL higher than men (P = 0.016); * Post-exercise higher than pre-exercise (men, P = 0.03; FOL, P = 0.04; LUT, P = 0.04). Panel C: GP; ¶ FOL higher than LUT (P = 0.041). Panel D: PFK; † LUT lower than men (P = 0.031). FOL, follicular phase; LUT, luteal phase. β2-M was used as an internal standard. Values are means ± SE.

Fig.2. Skeletal muscle mRNA content of the genes involved in the glycogen synthesis in men and women (FOL and LUT). Panel A: Glycogenin; † LUT higher than FOL (P = 0.02). Panel B: GS-1. Panel C: GSK3α; ¶ trend for FOL to be higher than men (P = 0.065). FOL, follicular phase; LUT, luteal phase. β2-M was used as an internal standard. Values are means ± SE.

Fig.3. Skeletal muscle mRNA content of the genes involved in the oxidation of amino acids in men and women (FOL and LUT). Panel A: AST; * significant increase during exercise (P = 0.038); ¶ trend for post-exercise to be higher than pre-exercise (P = 0.063). Panel B: BCOAD. Panel C: BCOADK; † Both FOL (P = 0.026) and LUT (P = 0.05) higher than men. FOL, follicular phase; LUT, luteal phase. β2-M was used as an internal standard. Values are means ± SE.

Table1. Targeted genes involved in carbohydrate and protein metabolism

Metabolism pathways	Gene Symbol	Function	GenBank access No.
Glucose transport and glucose phosphorylation	GLUT4	Membrane glucose transport	NM_001042
	HK II	Glucose phosphorylation	BC021116
Glycogenolysis and glycolysis	GP	Glycogen hydrolysis	NM_005609
	PFK	Glycolysis	BC021203
Glycogen synthesis	Glycogenin	Glycogen synthesis	U44131
	GS-1	Glycogen synthesis	NM_002103
	GSK3α	Glycogen synthesis	BC051865
Amino acid metabolism	AST	Transamination	M37400
	BCOAD	Dehydrogenation	NM_183050
	BCOADK	Phosphorylation of BCOAD and inactivation of BCOAD	NM_005881

Table2. Primers and probe sets

Genes	GenBank acess No	Primers and probe set	
GLUT4	NM_001042	Left primer	5'-gtg gct ggt ttc tcc aac tg-3'
		Right primer	5'-cgcaaatagaaggaagacgta -3'
		Probe	5'-agcaacttcatcattggcatgggtttc-3'
HKII	BC021116	Left primer	5'-gaccaacttccgtgtgcttt-3';
		Right primer	5'-gtcctcagggatggcataga -3'
		Probe	5'-agcccattgtccgttactttcaccc-3'
GP	NM_005609	Left primer	5'-gcggagaacatctctcgtgt-3';
		Right primer	5'-gccgaacttggaagacttga-3'
		Probe	5'-aagcaggagtatttcgtggtggctg-3'
PFK	BC021203	Left primer	5'-tgaccattggcactgactct-3';
		Right primer	5'-tctgggcagtggtagtgatg-3'
		Probe	5'-atctacaatttccatgatccgatgcag-3'
Glycogenin	U44131	Left primer	5'-ttgtcaaagacacctgctcata-3';
		Right primer	5'-ccatcgttccttccgttct-3'
		Probe	5'-tcaggagccatatcacatctgtcccttt-3'
GS-1	NM_002103	Left primer	5'-tgcccactgtgaaaccacta-3';
		Right primer	5'-catgggaaagggatcagtatg-3'
		Probe	5'-tccagatctagaagccaggacctagaacc-3'
GSK3α	BC051865	Left primer	5'-tctttgatgaactgcgatgtct-3';
		Right primer	5'-agacggttggatggagagtt-3'
		Probe	5'-tctcttcaacttcagtgctggtgaactc-3'
AST	M37400	Left primer	5'-tccaagaacttcgggctcta-3';
		Right primer	5'-gaccaagtaatccgcacgat-3'
		Probe	5'-aacctgagagcatcctgcaagtccttt-3'
BCOAD E1 B-subunit	NM_183050	Left primer	5'-tatacccagaagccctttcca-3';
		Right primer	5'-tgggacagtgggatgttgta-3'
		Probe	5'-cttcttccgctgctgccctgtaaagta-3'
BCOADK	NM_005881	Left primer	5'- ccacaatggagagtcacctaga-3';
		Right primer	5'-ttcctccaccacggtctga-3'
		Probe	5'-ccaacaatgatgtcgatctgatcatc-3'

Table3. TaqMan ™ Real-time RT-PCR reaction conditions

Target genes	TL (μM)	TR (μM)	TP (μM)	β2-ML (μM)	β2-MR (μM)	β2-MP (μM)	RNA (ng)	Reaction volume(μl)
GLUT4	0.3	0.3	0.2	0.4	0.4	0.05	5.0	25
HKII	0.3	0.9	0.1	0.6	0.6	0.05	12.5	25
GP	0.2	0.2	0.25	0.6	0.6	0.05	5.0	25
PFK	0.3	0.3	0.25	0.6	0.6	0.05	12.5	25
Glycogenin	0.6	0.2	0.1	0.2	0.2	0.05	5.0	25
GS-1	0.6	0.6	0.2	0.6	0.6	0.05	5.0	25
GSK3α	0.6	0.6	0.2	0.6	0.6	0.05	5.0	25
AST	0.3	0.3	0.1	0.4	0.4	0.05	5.0	25
BCOAD	0.6	0.2	0.25	0.1	0.1	0.05	5.0	25
BCOADK	0.6	0.6	0.1	0.6	0.6	0.05	7.5	25

Note: TL, target gene left primer; TR, target gene right primer; TP, target gene probe; β2-ML, β2-M left primer; β2-MR, β2-M right primer; β2-MP, β2-M probe.

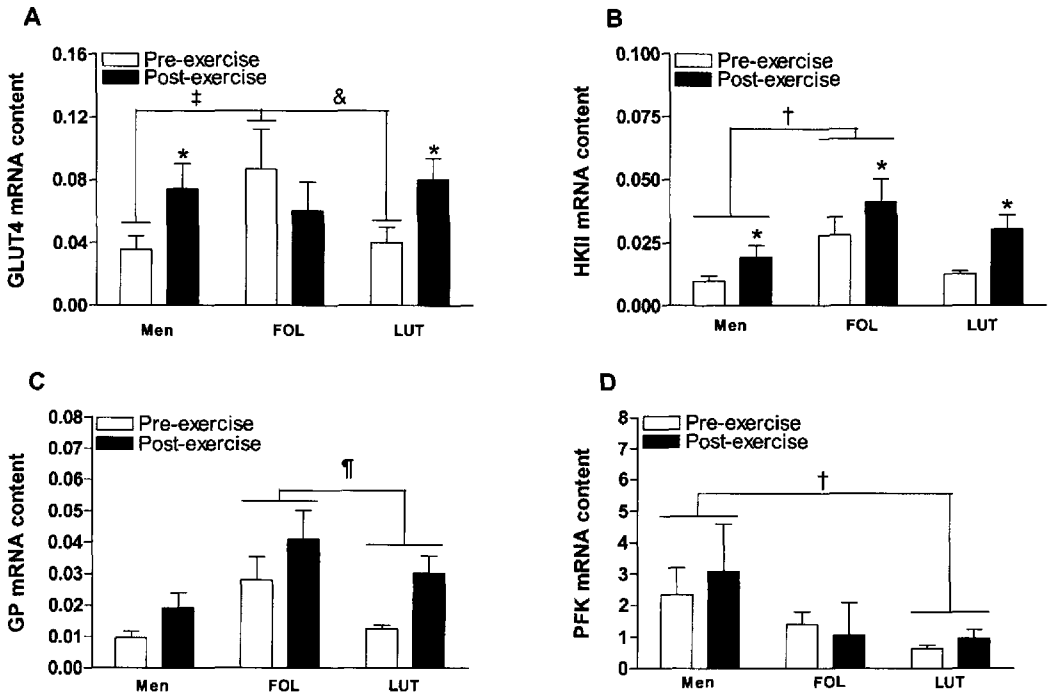
A
GLUT4 mRNA content
Pre-exercise
Post-exercise
0.16
0.12
0.08
0.04
0.00
‡
&
*
*
Men
FOL
LUT
B
HKII mRNA content
0.100
0.075
0.050
0.025
0.000
†
*
*
*
C
GP mRNA content
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
¶
D
PFK mRNA content
8
7
6
5
4
3
2
1
0
†

Figure1

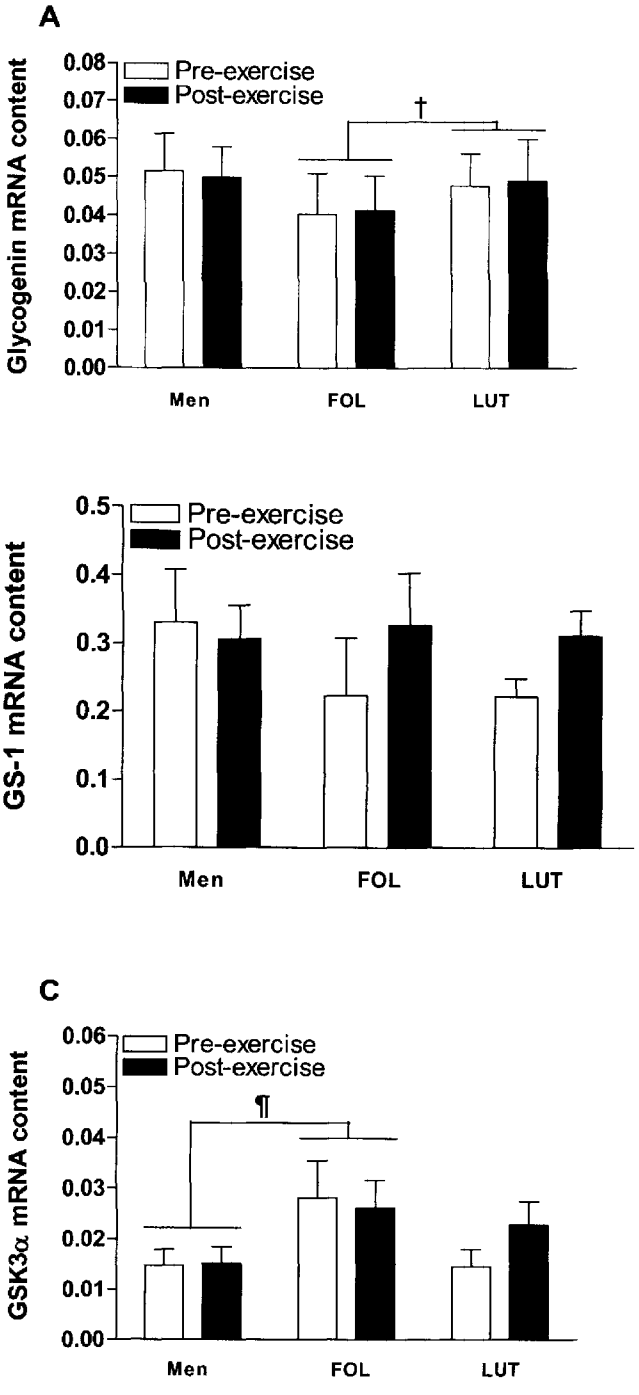
A
Glycogenin mRNA content
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
Pre-exercise
Post-exercise
†
Men
FOL
LUT
GS-1 mRNA content
0.5
0.4
0.3
0.2
0.1
0.0
Pre-exercise
Post-exercise
Men
FOL
LUT
C
GSK3α mRNA content
0.06
0.05
0.04
0.03
0.02
0.01
0.00
Pre-exercise
Post-exercise
¶
Men
FOL
LUT

Figure2

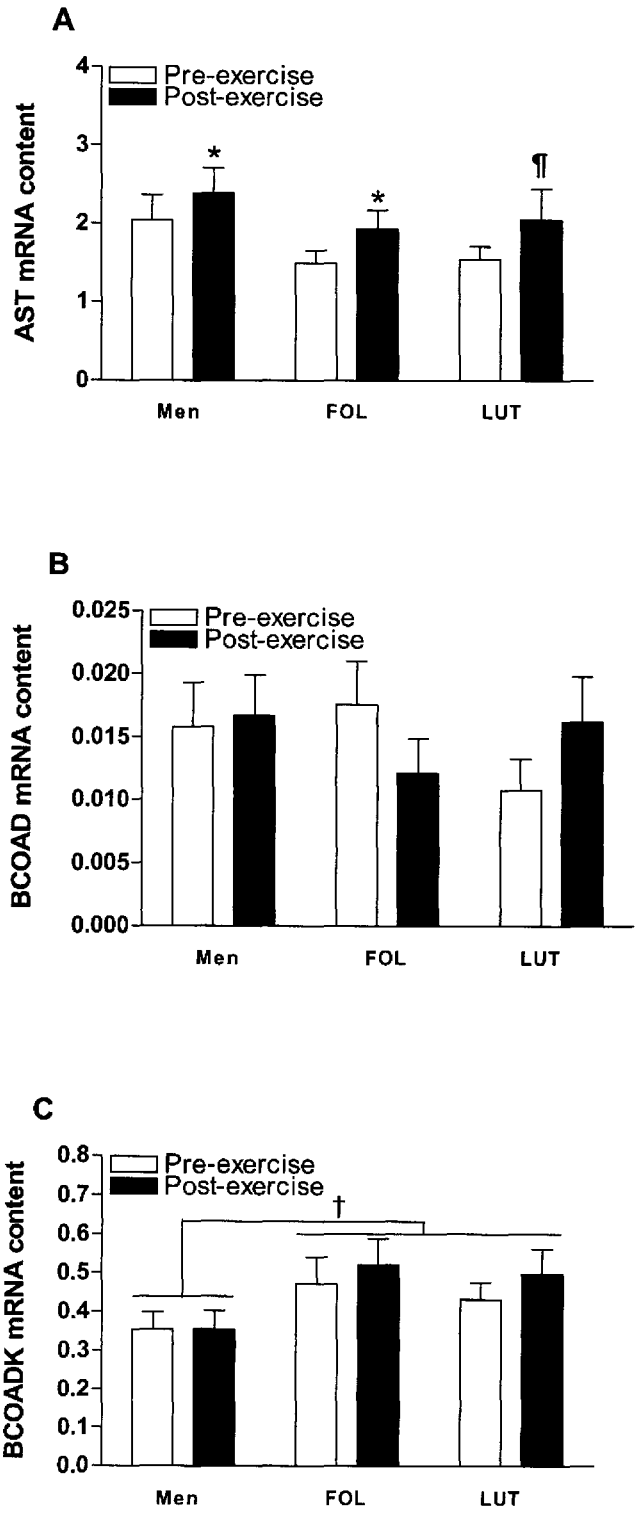
A
Pre-exercise
Post-exercise
AST mRNA content
4
3
2
1
0
*
*
¶
Men
FOL
LUT
B
Pre-exercise
Post-exercise
BCOAD mRNA content
0.025
0.020
0.015
0.010
0.005
0.000
Men
FOL
LUT
C
Pre-exercise
Post-exercise
BCOADK mRNA content
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
†
Men
FOL
LUT

Figure 3

CHAPTER 4: Manuscript 3

Journal of Clinic Endocrinology and Metabolism

17β-estradiol supplementation influences the mRNA content of the genes involved in lipid, carbohydrate and protein metabolism in the skeletal muscle of men.

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ABSTRACT

Context and Objective: 17β-estradiol (E2) supplementation increases the oxidation of lipid, reduces the oxidation of carbohydrate and amino acids in men. Consequently, we hypothesized that E2 would alter basal and exercise-induced mRNA content for genes involved in many aspects of lipid, carbohydrate and protein metabolism in skeletal muscle. **Subjects and Methods:** Twelve men were randomly allocated to both E2 (2mg/day) and placebo (400mg/day) for 8 days and had muscle biopsies taken from their vastus lateralis before and after a 90 min bout of cycling at 65 % $VO_{2peak.}$ mRNA content was determined using TaqMan® RT-PCR with β2-microglobulin as an internal standard for genes involved in mitochondrial biogenesis (PGC-1α), transcription regulation (PPARα, PPARγ, PPARδ, SREBP-1c, SREBP-2), and metabolism of lipid (FATm, FABPc, HSL, CPTI, CPTII, mtGPAT, VLCAD, LCAD, TFP-α), carbohydrate (GLUT4, HK II, PFK, glycogenin, GS-1, GSK3α, glycogen phosphorylase) and amino acids (AST, BCOAD, BCOADK). **Results:** E2 significantly increased mRNA content of PPARα, CPTI, SREBP-1c, mtGPAT, LCAD, TFP-α, GLUT4 and GS-1 ($P < 0.05$) in the skeletal muscle of men at rest and during exercise, and PPARδ at rest ($P < 0.05$). E2 significantly attenuated the exercise- induced increase in PGC-1α mRNA content ($P < 0.05$). Exercise significantly increased the mRNA content of PGC-1α, FABPc, HSL, GLUT4, HK II and AST ($P < 0.05$). **Conclusions:** Acute exercise altered the mRNA content of genes involved in substrate metabolism that directionally supported mitochondrial biogenesis and glycogen replenishment following exercise. The influence of E2 on mRNA content was supportive of a higher lipid oxidation in women vs. men.

Key words: estrogen, exercise metabolism, substrate utilization, mRNA

INTRODUCTION

As compared with men, women oxidize more fat, less carbohydrate and protein during exercise (1, 2). To elucidate the role of 17β-estradiol (E2) in the sex differences in substrate utilization, a wide range of studies have been conducted with animal models (3-5) and human subjects (6-9) through alteration of E2 plasma concentration by ovariectomization in animals and E2 supplementation in animals or human subjects. Studies in animals have generally concluded that E2 increases lipid oxidation and decreases carbohydrate oxidation (3-5, 10), resulting in improved exercise performance (4, 5), sparing of muscle and liver glycogen during exercise (4, 5), increased free fatty acid (FFA) availability for oxidation (3, 5), elevated intramyocellular lipid (IMCL) concentration (10), and decreased lactate concentration (5). In general, the consistency of the results from studies looking at the metabolic effects of E2 administration upon substrate selection during exercise showing a higher fat and lower carbohydrate oxidation suggests that some of the sex differences in substrate selection are hormonally mediated and not solely due to a higher proportion of type I muscle fibers (11) or other factors such as training differences (12) or body fat content between men and women favoring higher fat oxidation (13).

The administration of E2 to amenorrheic females (9) and to males (6-8) was reported to decrease the respiratory exchange ratio during exercise (6-9). E2 reduced whole body carbohydrate oxidation, lucine oxidation, and increased lipid oxidation at rest and during exercise in men (8). E2 supplementation lowered resting pro-glycogen and total glycogen concentration (7), but did not attenuate skeletal muscle glycogen utilization during exercise in males (6, 7). E2 supplementation altered fuel selection in human by

increasing plasma FFA concentration (9), reducing glucose Ra (primarily liver glucose production) (6, 7, 9), Rd (primarily muscle glucose uptake) and metabolic clearance rate (6, 7). Supplementation of an E2 and progesterone combination to young women with premature ovarian failure (14) or to eumenorrheic women (15) indicated that E2 may work synergistically (16) or antagonistically (17) with progesterone in the regulation of substrate metabolism.

The potential mechanism(s) of E2 mediated substrate selection has been evaluated in rats (10, 18-22) and mice (23, 24). E2 increased the activity of lipoprotein lipase (LPL) (10), citrate synthase (18), and glycogen synthase (18) in the skeletal muscle of rats, and decreased the activity of hepatic phosphoenol pyruvate carboxy kinase in oophorectomized rodents (25), and peripheral adipocyte LPL activity in male rats (10). Ovariectomization (24), and E2 replacement in ovariectomized mice (23), demonstrated that E2 enhanced the expression of the genes involved in transcriptional regulation of fat metabolism and β-oxidation, and decreased the expression of lipogenic genes, such as, SREBP-1c, in the skeletal muscle. Replacement of E2 in ovariectomized rats increased mRNA content of PPARα, PPARγ, CPT I and PDK4, and protein content of PPARα (19), but not mRNA (21, 22) or protein (20) content of GLUT4, in skeletal muscle. In humans, the potential mechanism(s) of the E2 mediated alterations in substrate utilization has not yet been evaluated at the mRNA level.

In contrast, there has been some evaluation of the potential molecular mechanism(s) of the sex differences in substrate utilization during exercise (1, 2), through analysis of the steady-state mRNA content for the genes involved in fat, carbohydrate and protein

metabolism (26-30). Women have a higher mRNA content of membrane fatty acid transport protein (FATm)(26), FAT/CD36, FABPpm, LPL (29), beta-hydroxyacyl-CoA dehydrogenase (30), and a higher level of FAT/CD36 protein (29), as compared with men. We also found that women have a higher mRNA content peroxisome proliferator activated receptor-α(PPARα), peroxisome proliferator activated receptor-delta (PPARδ), peroxisome proliferator activated receptor-γ (PPARγ), sterol regulatory element binding protein-1c (SREBP-1c), mitochondrial glycerol phosphate acyltransferase (mtGPAT), cytosol fatty acid binding protein (FABPc), carnitine palmitoyltransferase I (CPTI), long chain acyl-CoA dehydrogenase (LCAD), trifunctional protein alpha subunit (TFP-α), hexokinase II (HKII) and glucose transporter 4 (GLUT4), a lower mRNA content of phosphofructokinase (PFK) and branch chain keto-acid dehydrogenase kinase (BCOADK) as compared with men (27, 28). However, there were no sex differences in protein content of beta-hydroxyacyl-CoA dehydrogenase (30) or activity of CPTI (31).

A single bout of exercise increased the transcription of peroxisome proliferator activated receptor-γ coactivator-1α (PGC-1α) (32), PPARα (32), CPT I, LPL (32, 33) and GLUT4 (34), and the mRNA content of PGC-1α (27, 32, 35-37), PPARα (32, 37), PPARδ (27, 37), FAT/CD36 and FABPpm (29), FABPc, CPTI, LCAD, hormone sensitive lipase (HSL) (27), GLUT4 (28, 38), HKII (27, 36, 38), pyruvate dehydrogenase kinase 4 (37), glycogenin (38) and aspartate aminotransferase (AST) (28) in skeletal muscle. Given that sex differences in substrate utilization (1, 2), and E2 mediated substrate utilization (6-9), are predominantly apparent during exercise (1, 2, 6-9), we have evaluated the effect of E2 on mRNA expression during exercise.

The purpose of this study was to evaluate whether E2 influenced the mRNA content of genes involved in several aspects of lipid, carbohydrate and protein metabolism at rest and after acute exercise. Specifically, we evaluated the mRNA content for genes involved in mitochondrial biogenesis (PGC-1α), transcriptional regulation of CHO and fat metabolism (PPARα, PPARγ, PPARδ), sarcolemmal (FATm, FABPc) and mitochondrial (carnitine palmityltransferase I and II (CPTI, CPTII) FFA transport, intramyocellular (IMCL) synthesis (SREBP-1c, sterol regulatory element binding protein-2 (SREBP-2), mtGPAT) and hydrolysis (HSL), β- oxidation (very long chain acyl-CoA dehydrogenase (VLCAD), LCAD, TFP-α), glycogen synthesis (glycogenin, glycogen synthase1 (GS-1), glycogen synthase kinase 3α (GSK3α), glycogen hydrolysis (glycogen phosphorylase), glucose transport (GLUT4), phosphorylation of glucose (HK II), glycolysis (PFK), and amino acid metabolism (AST, branch chain keto-acid dehydrogenase (BCOAD) and BCOADK). We specifically hypothesized that the mRNA content for many genes involved in lipid, carbohydrate and protein metabolism would be altered by E2 supplementation in men before and after acute exercise and that these genes would directionally represent those found to be differentially expressed between sexes, given that E2 plays a role in selective substrate utilization (6-9).

MATERIAL AND METHODS

Subjects. Twelve healthy, recreationally active men (n=12) participated in this study. Breifly, they were healthy, non-smokers, non-obese and participated in recreational physical activity but were not trained athletes. In addition, their diet and activity patterns were constant in the 3 - 4 weeks prior to commencing the study as determined by a questionnaire. The subject characteristics were: age, 22 ± 6 y (Mean ± SD); weight, 78 ± 11 kg; height, 177 ± 5 cm; BMI, 25 ± 3 kg/m^2; fat-free mass, 63 ± 3 kg; fat mass, 16 ± 6kg; VO_{2peak}, 55 ± 9 mL O_2/kg FFM/min. Before inclusion into the study, potential subjects were required to complete a health questionnaire to ensure that they were healthy and fit to participate. Each subject was given an information sheet describing all testing procedures, was informed of the purposes and associated risks, and gave written consent prior to participation. The projects were approved by the McMaster University Hamilton Health Sciences Human Research Ethics Board and conformed to the Declaration of Helsinki guidelines.

Study design. All subjects were randomly assigned to receive oral doses of either 17-β-estradiol (E2, 2mg Estrace/day, Shire Biochem Inc., St. Laurent, Quebec, Canada) or placebo (PL) (400 mg/d Polycose; Abbott Laboratories, Ross Division, St. Laurent, Quebec, Canada) for 8 days in a randomized, double-blind manner. The 17ß-estradiol tablets were placed in gelatin capsules filled with Polycose. Subjects were instructed to take the capsules at the same time every day and to return unused capsules. All subjects reported 100% compliance. During the 8-day dose period subjects maintained their normal activity level. Food intake was recorded for the first 4 days of the dose period and was

used to control for food intake during the second arm of the study. On the 9th day subjects reported to the lab for testing (see below). A 14-day washout period was used and then subjects were crossed over and repeated the 9-day protocol on the opposite treatment. VO_2max was determined at least 7 d before the first testing day (8). On the morning of the test day the subject reported to the lab. Muscle biopsies were taken from the *vastus lateralis* of the nondominant leg ~20 cm proximal to the lateral knee joint of each subject under local anesthesia using manual suction both before and immediately after the acute exercise bout of 90 min cycling at 65% VO_2max from the same legs for both arms of the study. All biopsies were taken from separate incisions ~ 4 cm apart, to minimize the potential effect of the biopsy itself on gene expression (35). Approximately 150 mg of muscle was taken from each biopsy, and this was immediately examined for and if needed, dissected from adherent fat and connective tissue. The first piece (~ 25 mg) dissected from the muscle sample was rapidly placed in an RNase-free polyethylene tube, flash-frozen in liquid nitrogen, and stored at –86°C until being processed for analysis.

Preparation of RNA. Total RNA was extracted from frozen human muscle with TRIzol reagent (Invitrogen, Carlsbad, CA) (27, 28). Briefly, 25-50 mg of human skeletal muscle biopsy was homogenized in 1 ml of TRIzol reagent at 4^0C, left at room temperature for 5 – 10 min, followed by addition of 0.2 ml of chloroform, vortexing for 15 s, and then centrifuged at 12,000 rpm at 4^0C for 15 min. The supernatant aqueous layer was transferred to a fresh tube and mixed with 0.5 ml of isopropanol ethanol, stood at ~ 22^0C for 10 min, and centrifuged at 12,000 rpm at 4^0C for 10 min. The RNA pellet was washed twice with 0.5 ml of 75% ethanol and air dried and dissolved in 14 μl of DEPC-treated

ddH2O. Aliquots of 2 μl each was made and stored at -80^0C. The concentration and purity of the RNA was determined using a UV spectrophotometer (Shimadzu UV-1201; Mandel Scientific, Guelph, Ontario) by measuring the absorbance at 260 (OD260) and 280 (OD280) nm. Measurements were done in duplicate and had an average coefficient of variation (CV) of <10%. The average purity (OD260/OD280) of the samples was ~1.5. RNA integrity was assessed in a randomly chosen subset of samples using agarose gel electrophoresis, and the OD ratio of 28S to 18S rRNA was consistently greater than 1 for each sample checked, indicating high quality RNA.

TaqMan® real-time RT-PCR. TaqMan® real-time RT-PCR analysis of mRNA content for each target gene was exactly the same as Fu et al. (27, 28). Prior to TaqMan® real-time RT-PCR, RNA samples were treated with DNase I for 25 min at 37°C to remove any contaminating DNA using DNA-free™ (Ambion Inc, Austin, TX) according to the manufacturer's instructions. Specific primers and probe to each target gene (27, 28) were designed based on the cDNA sequence in GenBank (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi) with primer 3 designer (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Their specificity was checked using Blast (http://www.ncbi.nlm.nih.gov/BLAST/). Delta G of the hairpin structures and dimmers of the candidate primer and probe sets was calculated using Analyzer of Oligo (http://www.idtdna.com/analyzer/). Only the primer and probe sets with delta G >-9 kcal/mole for every hairpin and dimmer were selected. All target gene probes were labeled with FAM at their 5' ends and BHQ-1 at their 3' ends. β-2 microglobulin (β2-M) primers and probe and the RT-PCR reaction conditions for evaluation of β2-M as internal standard

were same as used by Fu et al. (27, 28). Since acute exercise and E2 did not influence the mRNA content of β2-M (Figure1A) human β2- M was used as internal standard. Duplex RT-PCR was performed on iCycler real-time PCR system (Bio-Rad Laboratories, Hercules, CA) in the One-step TaqMan® RT-PCR Master Mix Reagents (Roch, Branchburg, New Jersey, part no. 4309169) according to the manufacture's instruction with target gene primers and probe and internal standard gene primers and probe in the same reaction. The sequences of the primers and probes and the RT-PCR conditions, for the genes involved in fat metabolism and regulation of metabolism were same as Fu et al.(27), for the genes involved in carbohydrate and protein metabolism were same as Fu et al. (28). RT-PCR profiling was: 1cycle, 48^0C, 30 min; 1cycle, 95^0C, 10 min; 50 cylces, 95^0C, 15s; 60^0C, 1min; and 1cycle, 4^0C, hold. All samples were run in duplicate simultaneously with RNA- and RT-negative controls. Fluorescence emission was detected through a filter corresponding to the reporter dye at the 5'end of each probe, and CT was automatically calculated and displayed.

Statistical Analysis. All statistical analyses about mRNA expression of the genes tested were performed on linear data 2^{-CT} for evaluation of internal standard, $2^{-\Delta CT}$ for target gene normalized with internal reference (39). Data from evaluation of internal standard about E2 effect and exercise effect were analyzed using a two-way repeated measurement ANOVA of E2 supplementation X exercise, with both E2 supplementation and exercise as within group factors. All results from evaluation of internal standard are expressed in the figure as mean (±SE), using 2^{-CT}. Data about the effect of E2 on target gene mRNA expression were analyzed using a two-way repeated measurement ANOVA of E2 supplementation X

exercise with E2 supplementation and exercise as within group factors. Post-hoc test was performed only when interaction between E2 supplementation and exercise was significant ($P < 0.05$). Given our *a priori* data showing a higher mRNA content in skeletal muscle from women as compared with men (27), we used a directional one-tailed test for the E2 vs. PL comparisons for PPARα, PPARδ, mtGPAT and LCAD. The data are expressed in the figures as mean (±SE) normalized with β2-M, using $2^{-\Delta CT}$. The effects of E2 and exercise on target gene mRNA expression are expressed as mean fold change between placebo and estrogen (±SE) or pre- and post-exercise (±SE), using $2^{-\Delta\Delta CT}$. All analyses were done using computerized statistics software (Statistica; Statsoft, Tulsa, OK) with statistical significance set at $\alpha \leq 0.05$.

RESULTS

Genes involved in mitochondrial biogenesis and transcriptional regulation

E2 supplementation increased the mRNA content of PPARα at rest and following exercise (1.4 ± 0.3 fold, P = 0.038) (Figure 1C) and PPARδ only at rest (2.1 ± 0.5 fold, P = 0.034) (Figure 1E), while not altering the mRNA content of PPARγ (Figure 1D) in the skeletal muscle of men. E2 significantly attenuated the increase in PGC-1α mRNA content during exercise (P = 0.041), but not the resting/basal PGC-1α mRNA content (Figure 1B). Exercise increased PGC-1α (7.9 ± 3.8 fold, P = 0.047) and PPARδ (2.1 ± 0.4 fold, P = 0.08) RNA content (Figure 1B, E), but did not significantly alter PPARα (Figure 1C) or PPARγ (Figure 1D) mRNA content.

Genes involved in fatty acid transport

E2 significantly increased CPTI mRNA content (1.3 ± 0.2 fold, P = 0.041) (Figure 2C) while not altering the mRNA content of FATm (Figure 2A), FABPc (Figure 2B) or CPTII (Figure 2D) in the skeletal muscle of men both pre- and during exercise. Exercise significantly increased FABPc mRNA content (1.4 ± 0.2 fold, P = 0.004) (Figure 2B) while not influencing the mRNA content of FATm (Figure 2A), CPTI (Figure 2C) or CPTII (Figure 2D).

Genes involved in lipogenesis and IMCL hydrolysis

E2 significantly increased SREBP-1c (2.9 ± 1.3 fold, P = 0.05) and mtGPAT (1.3 ± 0.2 fold, P = 0.043) (Figure 3A, C), while not altering the mRNA content of SREBP-2 (Figure 3B) or HSL (Figure 3D) in skeletal muscle at rest or during exercise. Exercise significantly increased HSL (1.4 ± 0.4 fold, P = 0.037) (Figure 3D) mRNA content, while

not altering SREBP-1c (Figure 3A), SREBP-2 (Figure 3B) or mtGPAT (Figure 3C) mRNA content in skeletal muscle.

Genes involved in β-oxidation

E2 significantly increased mRNA content of LCAD mRNA (1.6 ± 0.4 fold, P = 0.033) (Figure 4B) and TFP- α (1.5 ± 0.3 fold, P = 0.033) (Figure 4C), with no effect upon VLCAD (Figure 4A) mRNA content in the skeletal muscle of men at rest or during exercise. Exercise did not alter the mRNA content of VLCAD (Figure 4A), LCAD (Figure 4B) or TFP-α (Figure 4C) in skeletal muscle.

Genes involved in glycogen synthesis and hydrolysis

E2 supplementation significantly increased the mRNA content of GS-1 (1.4 ± 0.2 fold, P = 0.02) (Figure 5B), but not for glycogenin (Figure 5A), GSK3α (Figure 5C) or glycogen phosphorylase (Figure 5D). Acute exercise did not alter glycogenin (Figure 5A), GS-1 (Figure 5B), GSK3α (Figure 5C) or glycogen phosphorylase (Figure 5D) mRNA content.

Genes involved in glucose transport, phosphorylation and glycolysis

E2 supplementation significantly increased the mRNA content of GLUT4 (2.0 ± 0.5 fold, P = 0.025) (Figure 6A), but not HK II (Figure 6B) or PFK (Figure 6C) mRNA content either pre- or post-exercise. Exercise significantly increased the mRNA content of GLUT4 (2.1 ± 0.5 fold, P = 0.039) (Figure 6A) and HK II (2.1 ± 0.6 fold, P = 0.05) (Figure 6B), but not PFK (Figure 6C).

Genes involved in oxidation of amino acids

E2 supplementation did not alter the mRNA content of BCOAD or BCOADK (Figure 6E, F). E2 may attenuate the increase in the mRNA content of AST by exercise in men supplemented with E2 (Figure 6D), because exercise significantly increased the mRNA content of AST in men on PL (1.1 ± 0.1 fold, $P = 0.03$) (Figure 6D), but not in men on E2. Exercise did not influence the mRNA content of BCOAD or BCOADK (Figure 6E, F).

DISCUSSION

This is the first study to examine the effect of short-term E2 supplementation on basal and exercise-induced changes in mRNA content for genes involved in substrate metabolism in human skeletal muscle. In general, E2 altered both basal and exercise induced mRNA content for several genes involved in intermediary substrate metabolism. The changes induced by E2 directionally supported the observation of a higher lipid and lower carbohydrate and amino acid oxidation in women as compared to men. An unexpected finding was that E2 completely abolished the exercise induced induction of mRNA content for PGC-1α, which could lead to future insights into PCG-1α regulation in human skeletal muscle.

Influence of E2 and exercise on the mRNA for genes involved in mitochondrial biogenesis and lipid oxidation capacity

One of the most interesting, but unexpected, findings of the current study was an attenuation of the exercise induced increase in PGC-1α mRNA content. PGC-1α is a transcriptional co-activator involved in mitochondrial biogenesis (40) and substrate metabolism (41). During exercise, contraction stimulated elevation of $[Ca^{2+}]$, [AMP]/[ATP] ratio and free radicals contribute to a robust increase in PGC-1α transcription (32) and mRNA content (27, 32, 35-37). PGC-1α has been considered a "master regulator" of mitochondrial biogenesis (40). Our results demonstrate that E2 alters the signaling pathways involved in transcriptional and/or post-transcriptional factors involved in determining steady-state mRNA content. We do feel that this attenuation is a

true finding due to the robustness of the effect and the fact that we have previously demonstrated an attenuated exercise induced increase in PGC-1α mRNA content in women during the follicular phase of their menstrual cycle (when E2/progesterone ratio is elevated) as compared to the luteal phase. Furthermore, the magnitude of the exercise induced PGC-1α mRNA response was lower in luteal phase women (3.6 fold) as compared with men (7.9 fold) in our previously reported data (27). Although our study did not elucidate a mechanism for the E2 mediated attenuation of exercise induced PGC1-α mRNA induction, E2 stimulates the transcription of estrogen related receptor-α (42), which, in turn, represses the transcription of PGC-1α (43). E2 is also an antioxidant which may attenuate exercise induced free radical production (44) and repress free radical induced PGC-1α transcription (45). Future studies will be required to determine whether the E2 mediated attenuation of exercise induced PGC-1α induction is solely due to a receptor mediated pathway and/or an indirect effect of E2 as an anti-oxidant.

PGC-1α also interacts with the various PPARs and these are involved in mitochondrial biogenesis and fat oxidation. An E2 mediated increase in the mRNA content of PPARα and PPARδ suggests that E2 increases the capacity for transcriptional activation of the genes involved in fat oxidation. PPARδ is involved in muscle fiber specification, mitochondrial biogenesis and elevation of mitochondrial fatty acid oxidative capacity (46, 47). PPARδ specifically activates the transcription of the genes involved in fat oxidation (46). In the current study, E2 increased the mRNA content of PPARδ at rest, but attenuated the exercise induced induction of PPARδ with acute exercise. The similarity of the PPARδ response to that of PGC-1α, and the know interaction between PGC-1α and PPARs,

suggests that E2 may influence the mRNA content for these species via similar mechanisms. The basal elevation of PPARδ with E2 supplementation is directionally consistent with higher fat oxidation in women (1, 2). Given that overall mitochondrial capacity (as assessed by citrate synthase activity) is not different between men and women (48), it is possible that higher basal levels of PPARδ induced by E2 (via PPARδ's effect on mitochondrial biogenesis (47) could partially compensate for an attenuation of the exercise induced PGC-1α response to E2. These results are in agreement with our previous data showing that exercise tended to increase the PPARδ mRNA content in the skeletal muscle of men, but not women during both follicular and luteal phases of the menstrual cycle (27). PPARα is a transcription factor that increases the transcription of the genes involved in fat oxidation and carbohydrate metabolism (41, 49). The higher basal levels of PPARα that we observed with E2 administration were directionally similar to that we found with PPARδ, suggesting a coordinate process involved in the steady state level for these transcription factors. Furthermore, these findings are also consistent with the fact that many of the downstream targets of these transcription factors change in a coordinate fashion in response to E2 (see below). We did not find an effect of E2 or acute exercise on PPARγ mRNA content in skeletal muscle; however, given its relatively greater importance in adipocyte substrate regulation (50), it will be of interest to determine the effects of E2 in this tissue in the future.

Influence of E2 and exercise on the mRNA for genes involved in fat metabolism

One of the most consistent effects of E2 appeared to be on the mRNA content for genes involved in skeletal muscle β-oxidation (LCAD and TFP-α) and mitochondrial transport (CPTI) of long chain free fatty acids. CPT I catalyzes the conversion of long-chain fatty acyl-CoAs to acyl carnitines in the presence of L-carnitine, a rate-limiting step in the transport of long-chain fatty acids from the cytoplasm to the mitochondrial matrix (51). LCAD is responsible for reducing acyl-CoAs of chain lengths C14-C16 in the first reaction of β-oxidation (52). TFP-α is a subunit of mitochondrial trifunctional protein which is a heterooctamer of four α- and four β-subunits and catalyses the last three steps in the β-oxidation of long-chain fatty acids (53). The α-subunit contains the long-chain enoyl-CoA hydratase and long-chain 3-hydroxyacyl-CoA dehydrogenase activities (53). These results are consistent with murine data showing that E2 up-regulated genes involved in transcription regulation and oxidation of fat and increased the capacity for fat oxidation in skeletal muscle (23, 24).

Consistent with the observation that IMCL content is higher in women as compared with men (54), we found an increase in both SREBP-1c and mtGPAT mRNA content with E2 administration. These latter results implicate E2 as a key factor to explain the sex difference in IMCL content (54), given that SREBP-1c and mtGPAT are involved in the transcriptional regulation of and biochemical synthetic pathways for IMCL synthesis, respectively (55, 56). Further support for an E2 mediated effect was the observation that ovariectomization decreased the mRNA expression of the genes involved in lipogenesis in mice (24).

Influence of E2 and exercise on the mRNA for genes involved in carbohydrate and protein metabolism

E2 increased the mRNA content of GLUT4 and GS-1 in the skeletal muscle of men. GLUT4 is a contraction-stimulated glucose transporter (57), and GS-1 catalyses the incorporation of uridine diphosphateglucose into glycogen in skeletal muscle (58). The estrogen receptor, ERα is a positive regulator of GLUT4 expression, whereas ERβ has a suppressive role in mice muscle (59). These latter findings indirectly suggest that although estrogen receptors are present in human male skeletal muscle (Tarnopolsky and Parshad, unpublished findings, 2003), the ERα form may be predominant and this postulation is worthy of further study. Given that ovariectmization removing both estrogen and progesterone did not influence GLUT4 protein and glycogen content in rat skeletal muscle (20), our observations suggest that progesterone may antagonistically reduced the expression of GLUT4 and GS-1 in normal rats. The increase in both GS-1 and GLUT4 would predict that muscle glycogen would be higher in women after E2 administration; however, this is in contrast to our findings that E2 lowers basal muscle glycogen content (7), reduces glucose rate of disappearance and metabolic clearance rate (6, 7), and several studies that have not found sex differences in basal glycogen content (60). In contrast, E2 did not alter the mRNA content of glycogen phosphorylase, indicating no change in the potential for glycogen breakdown, which is consistent with the observation that E2 did not attenuate the use of glycogen during exercise (6, 7). The lack of consistency between the directional change in the mRNA content for genes involved in carbohydrate metabolism and physiological observations in men vs. women and with E2 administration indirectly suggests that fat metabolism appears to be the main regulated process and carbohydrate

metabolism follows by physiological regulation (1).

We did find a small but significant exercise induced increase in AST mRNA content in men on placebo, which was attenuated by E2. These findings are consistent with our finding of an exercise induced increase in amino acid metabolism that was greater for men as compared with women (1, 2). In contrast to the AST finding, the mRNA content for BCOAD and BCOAD kinase were not significantly altered by exercise or E2. Given the known regulation of BCOAD by phosphorylation (61), the fact that BCOAD activity is acutely increased with exercise (62), the fact that women have a lower BCOAD enzyme activity (63), and the lack of effect on mRNA content for the enzyme and its inactivating kinase (current data) suggests that BCOAD regulation is not at the mRNA level.

Role of E2 in sex differences in substrate utilization

Men supplemented with E2 have been used as a model to elucidate the role of estrogen in mediating the sex differences in substrate utilization (6-8). E2 supplementation altered the mRNA content of PGC-1α, PPARα, PPARδ, CPTI, LCAD, TFP-α, SREBP-1c and mtGPAT in the skeletal muscle of men, and increased capacity for fat oxidation and synthesis of IMCL, which is in agreement with that women oxidize more fat (1, 2) and synthesize more IMCL (64) than men, and support our hypothesis that estrogen would influence the mRNA content of some genes whose mRNA expression was influenced by sex (27) and play a role in mediating the sex differences in substrate utilization (1, 2). However, among the genes with sex differential mRNA expression (26, 27, 29, 30), we found that the mRNA content of FATm, FABPc, PPARr was not influenced by E2 in men,

suggesting that other factors than E2 may involve in mediating the sex differential mRNA expression of these genes. Furthermore, exercise increased CPTI mRNA content in LUT women (27) but not in men or FOL women (27), and exercise increased LCAD mRNA content in LUT women but not in men, and tended to decrease in FOL women (27). Though CPTI and LCAD were differentially expressed between men and women (27) and E2 increased the mRNA content of these genes in men, the response patterns of these genes to exercise suggest that the mRNA expression of CPTI and LCAD was regulated synergistically by E2 and other factors. E2 only partially mediates the sex differences in fat utilization during exercise at the mRNA level. These results are in agreement with that both E2 and progesterone are involved in sex differences in fat utilization during exercise (17).

E2 increased the mRNA content of GLUT4 and GS -1 and caused a higher capacity for glucose uptake and glycogen synthesis in the skeletal muscle of men, which is in agreement with that women spare more glycogen during exercise than men (2, 7, 10). The mRNA expression of HKII, GLUT4 and PFK was sex differential and involved in causing sex differences in utilization of carbohydrate (28). Only the mRNA content of GLUT4, but not HKII or PFK, was upregulated by E2, implicating that E2 only influence the capacity for glucose uptake but not the capacity for glucose phosphorylation and glycolysis. That exercise increased the content of GLUT4 mRNA in men and LUT women but not FOL women (28), and that E2 increased the content of GLUT4 mRNA both pre- and post-exercise in men, further indicate that even the mRNA expression of GLUT4 in women is not regulated by E2 only. Though the mRNA content of GS-1 was upregulated by E2 in men, there was not sex differences in GS-1 mRNA content between sexes (28), indicating

that the mRNA expression of GS-1 is antagonistically regulated by other factors than E2 in women. Therefore, estrogen plays a role in mediating the sex differences in glycogen spare, but not in glucose phosphorylation or glycolysis, at the mRNA level. There should be other factors involved in mediating sex differences in carbohydrate oxidation (16).

E2 did not influence the mRNA content of BCOAD or BCOADK. Though estrogen might lead to a lower capacity for oxidation of amino acids during exercise through ceasing the increase in mRNA content of AST by exercise in men, the mRNA content of AST was not influenced by sex (28), probably other factors encountered the effect of estrogen on the mRNA expression of AST in women. While the mRNA expression of BCOADK was sex differential and involved in the sex differences in amino acid oxidation (28), its mRNA content was not influenced by E2. Estrogen did not mediate the sex differences in oxidation of amino acids at the mRNA level. There should be other factors mediating the sex differential expression of BCOADK in human skeletal muscle.

In summary, the current data support a major role for E2 to mediate steady state mRNA levels for genes primarily involved in skeletal muscle fat metabolism. There were several mRNA species however, that we previously reported to be different in men and women and not influenced by E2. This latter finding suggests that other factors such as fiber type (48), progesterone concentration/menstrual phase (65), and/or duration of exposure or pulsatility of sex hormones could influence the previously observed sex differences in mRNA content (27, 28).

REFERENCES

1. **Braun B, Horton T** 2001 Endocrine regulation of exercise substrate utilization in women compared to men. Exerc Sport Sci Rev 29:149-154

2. **Tarnopolsky MA, Ruby BC** 2001 Sex differences in carbohydrate metabolism. Curr Opin Clin Nutr Metab Care 4:521-526

3. **Hatta H, Atomi Y, Shinohara S, Yamamoto Y, Yamada S** 1988 The effects of ovarian hormones on glucose and fatty acid oxidation during exercise in female ovariectomized rats. Horm Metab Res 20:609-611

4. **Kendrick ZV, Ellis GS** 1991 Effect of estradiol on tissue glycogen metabolism and lipid availability in exercised male rats. J Appl Physiol 71:1694-1699

5. **Kendrick ZV, Steffen CA, Rumsey WL, Goldberg DI** 1987 Effect of estradiol on tissue glycogen metabolism in exercised oophorectomized rats. J Appl Physiol 63:492-496

6. **Carter S, McKenzie S, Mourtzakis M, Mahoney DJ, Tarnopolsky MA** 2001 Short-term 17beta-estradiol decreases glucose R(a) but not whole body metabolism during endurance exercise. J Appl Physiol 90:139-146

7. **Devries MC, Hamadeh MJ, Graham TE, Tarnopolsky MA** 2005 17beta-estradiol supplementation decreases glucose rate of appearance and disappearance with no effect on glycogen utilization during moderate intensity exercise in men. J Clin Endocrinol Metab 90:6218-6225

8. **Hamadeh MJ, Devries MC, Tarnopolsky MA** 2005 Estrogen supplementation reduces whole body leucine and carbohydrate oxidation and increases lipid oxidation in men during endurance exercise. J Clin Endocrinol Metab 90:3592-3599

9. **Ruby BC, Robergs RA, Waters DL, Burge M, Mermier C, Stolarczyk L** 1997 Effects of estradiol on substrate turnover during exercise in amenorrheic females. Med Sci Sports Exerc 29:1160-1169

10. **Ellis GS, Lanza-Jacoby S, Gow A, Kendrick ZV** 1994 Effects of estradiol on lipoprotein lipase activity and lipid availability in exercised male rats. J Appl Physiol 77:209-215

11. **Roepstorff C, Thiele M, Hillig T, Pilegaard H, Richter EA, Wojtaszewski JF, Kiens B** 2006 Higher skeletal muscle {alpha}2AMPK activation and lower energy charge and fat oxidation in men than in women during submaximal exercise. J Physiol 574:125-138

12. **Ruby BC, Robergs RA** 1994 Gender differences in substrate utilisation during exercise. Sports Med 17:393-410

13. **Blaak E** 2001 Gender differences in fat metabolism. Curr Opin Clin Nutr Metab Care 4:499-502

14. **Elkind-Hirsch KE, Sherman LD, Malinak R** 1993 Hormone replacement therapy alters insulin sensitivity in young women with premature ovarian failure. J Clin Endocrinol Metab 76:472-475

15. **D'Eon TM, Sharoff C, Chipkin SR, Grow D, Ruby BC, Braun B** 2002 Regulation of exercise carbohydrate metabolism by estrogen and progesterone in women. Am J Physiol Endocrinol Metab 283:E1046-1055

16. **Matute ML, Kalkhoff RK** 1973 Sex steroid influence on hepatic gluconeogenesis and glucogen formation. Endocrinology 92:762-768

17. **Campbell SE, Febbraio MA** 2001 Effect of ovarian hormones on mitochondrial enzyme activity in the fat oxidation pathway of skeletal muscle. Am J Physiol Endocrinol Metab 281:E803-808

18. **Beckett T, Tchernof A, Toth MJ** 2002 Effect of ovariectomy and estradiol replacement on skeletal muscle enzyme activity in female rats. Metabolism 51:1397-1401

19. **Campbell SE, Mehan KA, Tunstall RJ, Febbraio MA, Cameron-Smith D** 2003 17beta-estradiol upregulates the expression of peroxisome proliferator-activated receptor alpha and lipid oxidative genes in skeletal muscle. J Mol Endocrinol 31:37-45

20. **Hansen PA, McCarthy TJ, Pasia EN, Spina RJ, Gulve EA** 1996 Effects of ovariectomy and exercise training on muscle GLUT-4 content and glucose metabolism in rats. J Appl Physiol 80:1605-1611

21. **Sugaya A, Sugiyama T, Yanase S, Shen XX, Minoura H, Toyoda N** 2000 Expression of glucose transporter 4 mRNA in adipose tissue and skeletal muscle of ovariectomized rats treated with sex steroid hormones. Life Sci 66:641-648

22. **Sugaya A, Sugiyama T, Yanase S, Terada Y, Toyoda N** 1999 Glucose transporter 4 (GLUT4) mRNA abundance in the adipose tissue and skeletal-muscle tissue of ovariectomized rats treated with 17 beta-estradiol or progesterone. J Obstet Gynaecol Res 25:9-14

23. **D'Eon TM, Souza SC, Aronovitz M, Obin MS, Fried SK, Greenberg AS** 2005 Estrogen regulation of adiposity and fuel partitioning. Evidence of genomic and non-genomic regulation of lipogenic and oxidative pathways. J Biol Chem 280:35983-35991

24. **Kamei Y, Suzuki M, Miyazaki H, Tsuboyama-Kasaoka N, Wu J, Ishimi Y, Ezaki O** 2005 Ovariectomy in mice decreases lipid metabolism-related gene expression in adipose tissue and skeletal muscle with increased body fat. J Nutr Sci Vitaminol (Tokyo) 51:110-117

25. **Mandour T, Kissebah AH, Wynn V** 1977 Mechanism of oestrogen and progesterone effects on lipid and carbohydrate metabolism: alteration in the insulin: glucagon molar ratio and hepatic enzyme activity. Eur J Clin Invest 7:181-187

26. **Binnert C, Koistinen HA, Martin G, Andreelli F, Ebeling P, Koivisto VA, Laville M, Auwerx J, Vidal H** 2000 Fatty acid transport protein-1 mRNA expression in skeletal muscle and in adipose tissue in humans. Am J Physiol Endocrinol Metab 279:E1072-1079

27. **Fu MH HM, Ye CH and Tarnopolsky MA** 2006a. In press Acute endurance exercise, sex and menstrual cycle influence the mRNA content for genes involved in lipid metabolism in human skeletal muscle. . In:

28. **Fu MH HMaTM** 2006b. In press. Acute endurance exercise, sex and menstrual cycle influence the mRNA content for genes involved in carbohydrate and protein metabolism in human skeletal muscle. . In:

29. **Kiens B, Roepstorff C, Glatz JF, Bonen A, Schjerling P, Knudsen J, Nielsen JN** 2004 Lipid-binding proteins and lipoprotein lipase activity in human skeletal muscle: influence of physical activity and gender. J Appl Physiol 97:1209-1218

30. **Roepstorff C, Schjerling P, Vistisen B, Madsen M, Steffensen CH, Rider MH, Kiens B** 2005 Regulation of oxidative enzyme activity and eukaryotic elongation factor 2 in human skeletal muscle: influence of gender and exercise. Acta Physiol Scand 184:215-224

31. **Berthon PM, Howlett RA, Heigenhauser GJ, Spriet LL** 1998 Human skeletal muscle carnitine palmitoyltransferase I activity determined in isolated intact mitochondria. J Appl Physiol 85:148-153

32. **Pilegaard H, Osada T, Andersen LT, Helge JW, Saltin B, Neufer PD** 2005 Substrate availability and transcriptional regulation of metabolic genes in human skeletal muscle during recovery from exercise. Metabolism 54:1048-1055

33. **Pilegaard H, Ordway GA, Saltin B, Neufer PD** 2000 Transcriptional regulation of gene expression in human skeletal muscle during recovery from exercise. Am J Physiol Endocrinol Metab 279:E806-814

34. **Neufer PD, Dohm GL** 1993 Exercise induces a transient increase in transcription of the GLUT-4 gene in skeletal muscle. Am J Physiol 265:C1597-1603

35. **Norrbom J, Sundberg CJ, Ameln H, Kraus WE, Jansson E, Gustafsson T** 2004 PGC-1alpha mRNA expression is influenced by metabolic perturbation in exercising human skeletal muscle. J Appl Physiol 96:189-194

36. **Pilegaard H, Saltin B, Neufer PD** 2003 Exercise induces transient transcriptional activation of the PGC-1alpha gene in human skeletal muscle. J Physiol 546:851-858

37. **Watt MJ, Southgate RJ, Holmes AG, Febbraio MA** 2004 Suppression of plasma free fatty acids upregulates peroxisome proliferator-activated receptor (PPAR) alpha and delta and PPAR coactivator 1alpha in human skeletal muscle, but not lipid regulatory genes. J Mol Endocrinol 33:533-544

38. **Kraniou Y, Cameron-Smith D, Misso M, Collier G, Hargreaves M** 2000 Effects of exercise on GLUT-4 and glycogenin gene expression in human skeletal muscle. J Appl Physiol 88:794-796

39. **Livak KJ, Schmittgen TD** 2001 Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402-408

40. **Wu Z, Puigserver P, Andersson U, Zhang C, Adelmant G, Mootha V, Troy A, Cinti S, Lowell B, Scarpulla RC, Spiegelman BM** 1999 Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. Cell 98:115-124

41. **Vega RB, Huss JM, Kelly DP** 2000 The coactivator PGC-1 cooperates with peroxisome proliferator-activated receptor alpha in transcriptional control of nuclear genes encoding mitochondrial fatty acid oxidation enzymes. Mol Cell Biol 20:1868-1876

42. **Liu D, Zhang Z, Gladwell W, Teng CT** 2003 Estrogen stimulates estrogen-related receptor alpha gene expression through conserved hormone response elements. Endocrinology 144:4894-4904

43. **Ichida M, Nemoto S, Finkel T** 2002 Identification of a specific molecular repressor of the peroxisome proliferator-activated receptor gamma Coactivator-1 alpha (PGC-1alpha). J Biol Chem 277:50991-50995

44. **Duckles SP, Krause DN, Stirone C, Procaccio V** 2006 Estrogen and mitochondria: a new paradigm for vascular protection? Mol Interv 6:26-35

45. **Lee HC, Wei YH** 2005 Mitochondrial biogenesis and mitochondrial DNA maintenance of mammalian cells under oxidative stress. Int J Biochem Cell Biol 37:822-834

46. **Luquet S, Lopez-Soriano J, Holst D, Gaudel C, Jehl-Pietri C, Fredenrich A, Grimaldi PA** 2004 Roles of peroxisome proliferator-activated receptor delta (PPARdelta) in the control of fatty acid catabolism. A new target for the treatment of metabolic syndrome. Biochimie 86:833-837

47. **Wang YX, Zhang CL, Yu RT, Cho HK, Nelson MC, Bayuga-Ocampo CR, Ham J, Kang H, Evans RM** 2004 Regulation of muscle fiber type and running endurance by PPARdelta. PLoS Biol 2:e294

48. **Carter SL, Rennie CD, Hamilton SJ, Tarnopolsky** 2001 Changes in skeletal muscle in males and females following endurance training. Can J Physiol Pharmacol 79:386-392

49. **Mandard S, Muller M, Kersten S** 2004 Peroxisome proliferator-activated receptor alpha target genes. Cell Mol Life Sci 61:393-416

50. **Rosen ED, Walkey CJ, Puigserver P, Spiegelman BM** 2000 Transcriptional regulation of adipogenesis. Genes Dev 14:1293-1307

51. **McGarry JD, Brown NF** 1997 The mitochondrial carnitine palmitoyltransferase system. From concept to molecular analysis. Eur J Biochem 244:1-14

52. **Ikeda Y, Okamura-Ikeda K, Tanaka K** 1985 Purification and characterization of short-chain, medium-chain, and long-chain acyl-CoA dehydrogenases from rat liver mitochondria. Isolation of the holo- and apoenzymes and conversion of the apoenzyme to the holoenzyme. J Biol Chem 260:1311-1325

53. **Olpin SE, Clark S, Andresen BS, Bischoff C, Olsen RK, Gregersen N, Chakrapani A, Downing M, Manning NJ, Sharrard M, Bonham JR, Muntoni F, Turnbull DN, Pourfarzam M** 2005 Biochemical, clinical and molecular findings in LCHAD and general mitochondrial trifunctional protein deficiency. J Inherit Metab Dis 28:533-544

54. **Steffensen CH, Roepstorff C, Madsen M, Kiens B** 2002 Myocellular triacylglycerol breakdown in females but not in males during exercise. Am J Physiol Endocrinol Metab 282:E634-642

55. **Tarling E, Salter A, Bennett A** 2004 Transcriptional regulation of human SREBP-1c (sterol-regulatory-element-binding protein-1c): a key regulator of lipogenesis. Biochem Soc Trans 32:107-109

56. **Thuresson ER** 2004 Inhibition of glycerol-3-phosphate acyltransferase as a potential treatment for insulin resistance and type 2 diabetes. Curr Opin Investig Drugs 5:411-418

57. **Rose AJ, Richter EA** 2005 Skeletal muscle glucose uptake during exercise: how is it regulated? Physiology (Bethesda) 20:260-270

58. **Nielsen JN, Richter EA** 2003 Regulation of glycogen synthase in skeletal muscle during exercise. Acta Physiol Scand 178:309-319

59. **Barros RP, Machado UF, Warner M, Gustafsson JA** 2006 Muscle GLUT4 regulation by estrogen receptors ERbeta and ERalpha. Proc Natl Acad Sci U S A 103:1605-1608

60. **Roepstorff C, Steffensen CH, Madsen M, Stallknecht B, Kanstrup IL, Richter EA, Kiens B** 2002 Gender differences in substrate utilization during submaximal exercise in endurance-trained subjects. Am J Physiol Endocrinol Metab 282:E435-447

61. **Popov KM, Hawes JW, Harris RA** 1997 Mitochondrial alpha-ketoacid dehydrogenase kinases: a new family of protein kinases. Adv Second Messenger Phosphoprotein Res 31:105-111

62. **van Hall G, MacLean DA, Saltin B, Wagenmakers AJ** 1996 Mechanisms of activation of muscle branched-chain alpha-keto acid dehydrogenase during exercise in man. J Physiol 494 (Pt 3):899-905

63. **McKenzie S, Phillips SM, Carter SL, Lowther S, Gibala MJ, Tarnopolsky MA** 2000 Endurance exercise training attenuates leucine oxidation and BCOAD activation during exercise in humans. Am J Physiol Endocrinol Metab 278:E580-587

64. **Perseghin G, Scifo P, Pagliato E, Battezzati A, Benedini S, Soldini L, Testolin G, Del Maschio A, Luzi L** 2001 Gender factors affect fatty acids-induced insulin resistance in nonobese humans: effects of oral steroidal contraception. J Clin Endocrinol Metab 86:3188-3196

65. **D'Eon T, Braun B** 2002 The roles of estrogen and progesterone in regulating carbohydrate and fat utilization at rest and during exercise. J Womens Health Gend Based Med 11:225-237

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Figure legends

Fig.1. Skeletal muscle mRNA content of β2-M and the genes involved in the transcriptional regulation of mitochondrial biogenesis (PGC-1α) and substrate metabolism (PPARα, PPARγ, PPARδ) in men on PL or E2. Panel A: β2-M; No significant effect of exercise or estrogen on the mRNA content of β2-M was detected. Panel B: PGC-1α; † Post-exercise, men on PL higher than men on E2 (P = 0.041); * Post-exercise higher than pre-exercise (P = 0.047). Panel C: PPARα; † Men on E2 higher than men on PL (P = 0.038). Panel D: PPARγ. Panel E: PPARδ; † At rest, men on E2 higher men on PL (P = 0.034); ¶ Trend for post-exercise to be higher than pre-exercise (P = 0.08). PL, placebo; E2, 17β-estradiol. For PPARα, PPARδ and PGC-1α, β2-M was used as an internal standard. Values are means ± SE.

Fig.2. Skeletal muscle mRNA content of the genes involved in fatty acid transport in men on PL or E2. Panel A: FATm; Panel B: FABPc; * Post-exercise higher than pre-exercise (P = 0.004). Panel C: CPTI; † Men on E2 higher than men on PL (P = 0.041). Panel D: CPTII. PL, placebo; E2, 17β-estradiol. β2- M was used as an internal standard. Values are means ± SE.

Fig.3. Skeletal muscle mRNA content of the genes involved in lipid synthesis and IMCL hydrolysis in men on PL or E2. Panel A: SREBP-1c; † Men on E2 higher than men on PL (P = 0.05). Panel B: SREBP-2. Panel C: mtGPAT; † Men on E2 higher than men on PL (P = 0.043). Panel D: HSL; * Post-exercise higher than pre-exercise (P = 0.037). PL, placebo; E2, 17β-estradiol. β2-M was used as an internal standard. Values are means ± SE.

Fig.4. Skeletal muscle mRNA content of the genes involved in β - oxidation of fatty acids in men on PL or E2. Panel A: VLCAD. Panel B: LCAD; † Men on E2 higher than men on PL (P = 0.033). Panel C: TFP-α; † Men on E2 higher than men on PL (P = 0.033). PL,

placebo; E2, 17β-estradiol. β2-M was used as an internal standard. Values are means ± SE.

Fig.5. Skeletal muscle mRNA content of the genes involved in glycogen synthesis and hydrolysis in men on PL or E2. Panel A: Glycogenin. Panel B: GS -1; † Men on E2 higher than men on PL (P = 0.02). Panel C: GSK3α. Panel D: Glycogen phosphorylase. PL, placebo; E2, 17β-estradiol. β2-M was used as an internal standard. Values are means ± SE.

Fig.6. Skeletal muscle mRNA content of the genes involved in glucose transport and phosphorylation, glycolysis and oxidation of amino acids in men on PL or E2. Panel A: GLUT4; † Men on E2 higher than men on PL (P = 0.025); * Post-exercise higher than pre-exercise (P = 0.039). Panel B: HKII; * Post-exercise higher than pre-exercise (P = 0.05). Panel C: PFK; Panel D: AST; * Post-exercise higher than pre-exercise (P = 0.03). Panel E: BCOAD. Panel F: BCOADK. PL, placebo; E2, 17β-estradiol. β2-M was used as an internal standard. Values are means ± SE.

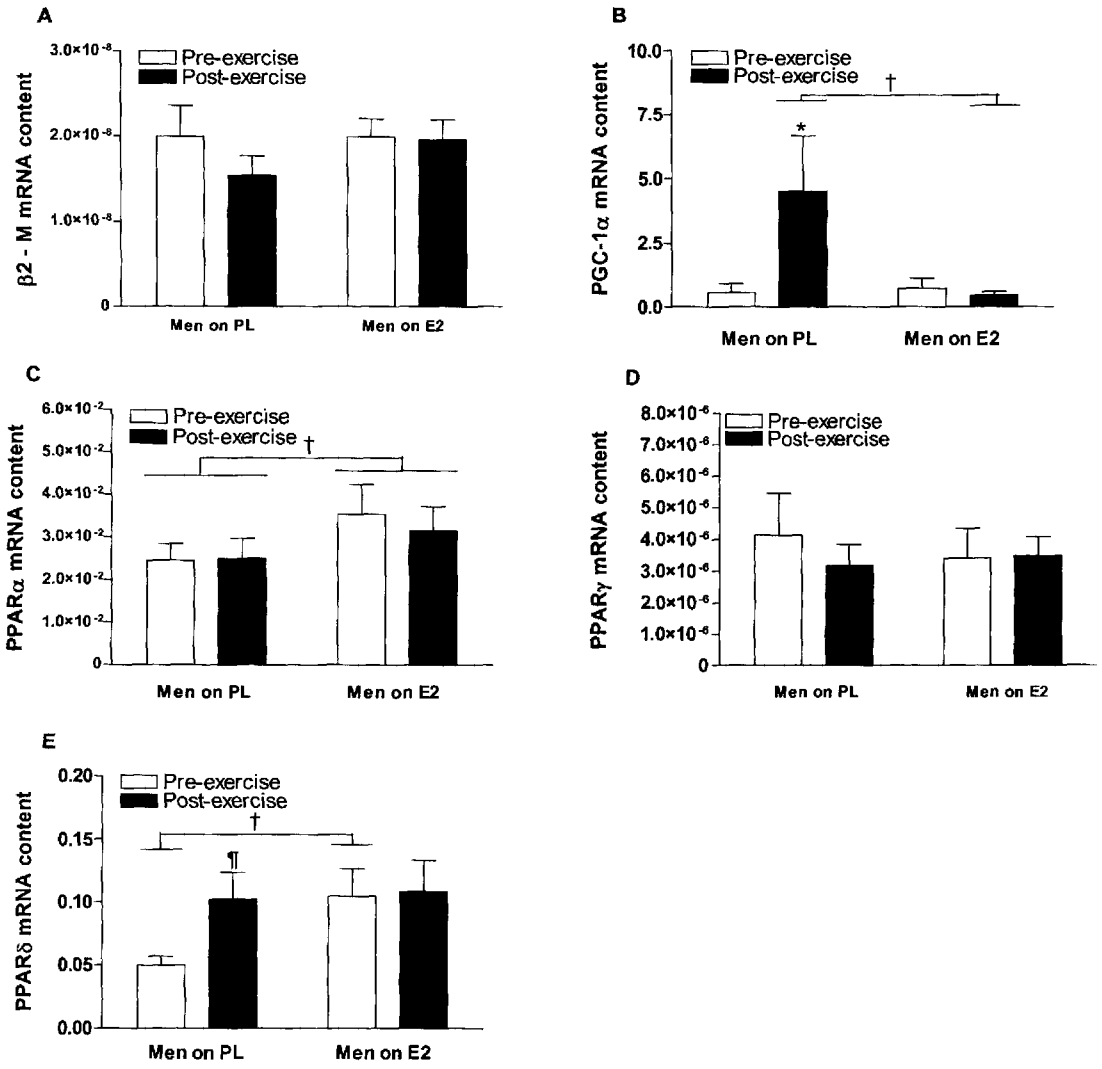
A
β2 - M mRNA content
Pre-exercise
Post-exercise
Men on PL
Men on E2
B
PGC-1α mRNA content
*
†
C
PPARα mRNA content
D
PPARγ mRNA content
E
PPARδ mRNA content
¶

Figure 1

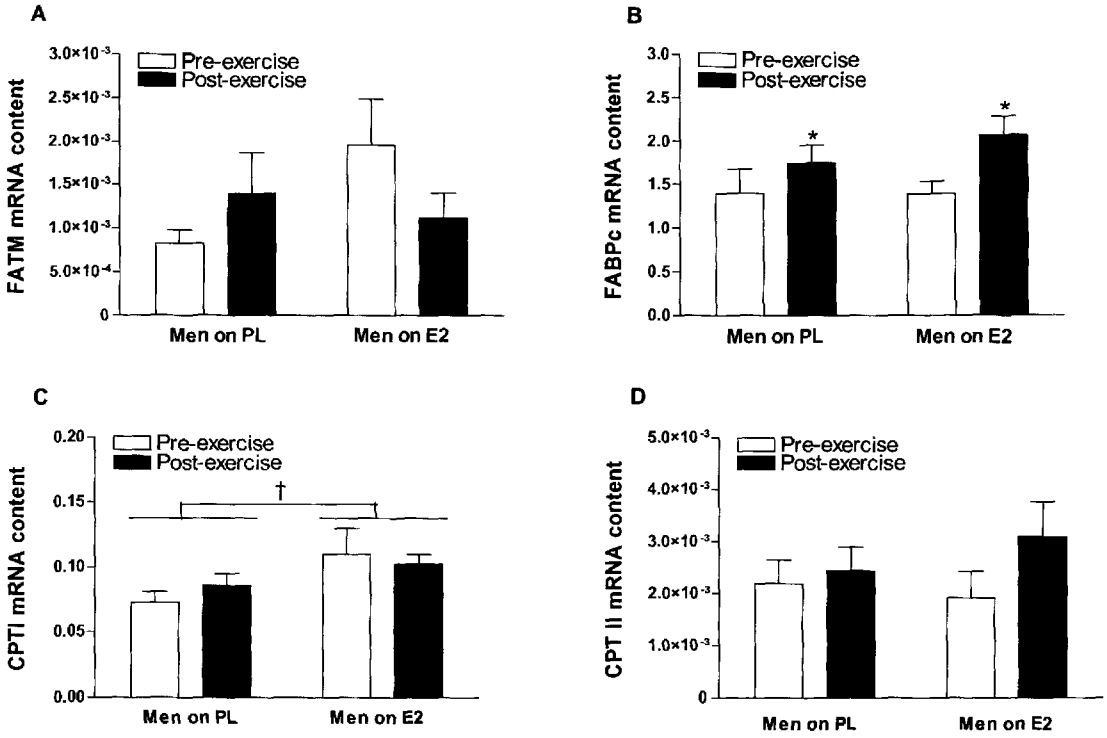
A
FATM mRNA content
3.0×10⁻³
2.5×10⁻³
2.0×10⁻³
1.5×10⁻³
1.0×10⁻³
5.0×10⁻⁴
0
Pre-exercise
Post-exercise
Men on PL
Men on E2
B
FABPc mRNA content
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
Pre-exercise
Post-exercise
Men on PL
Men on E2
C
CPTI mRNA content
0.20
0.15
0.10
0.05
0.00
†
Pre-exercise
Post-exercise
Men on PL
Men on E2
D
CPT II mRNA content
5.0×10⁻³
4.0×10⁻³
3.0×10⁻³
2.0×10⁻³
1.0×10⁻³
0
Pre-exercise
Post-exercise
Men on PL
Men on E2

Figure 2

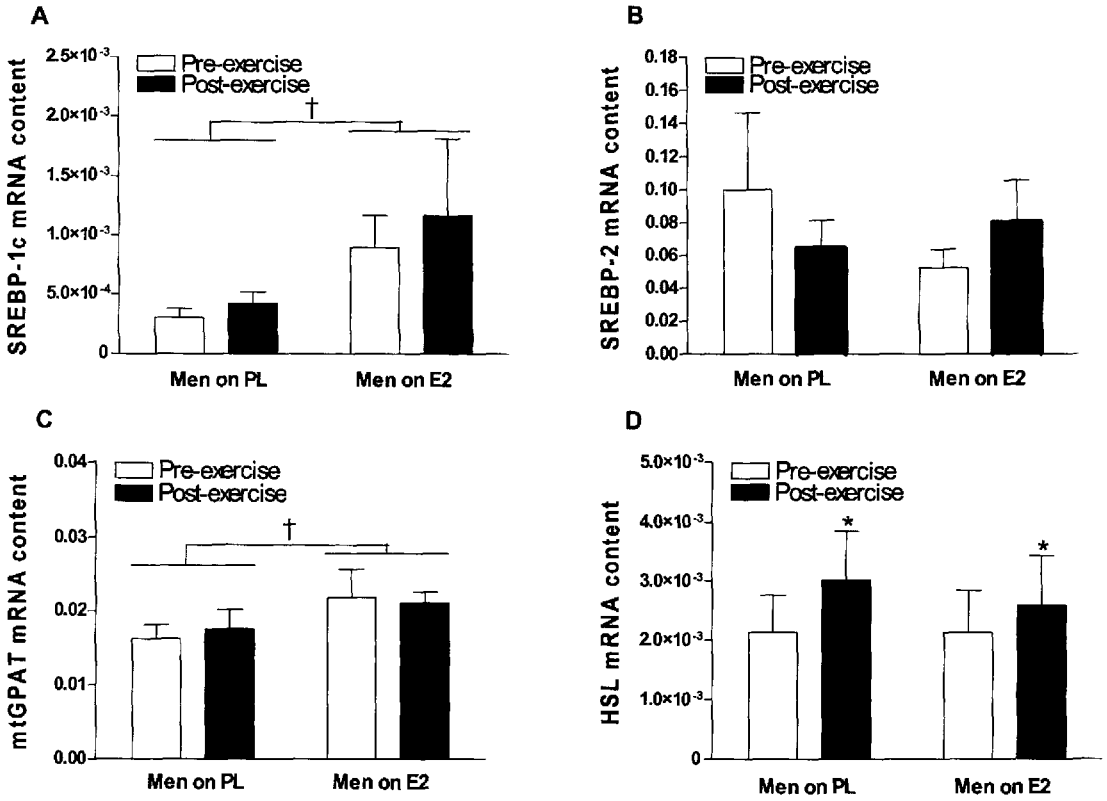
A
SREBP-1c mRNA content
2.5×10⁻³
2.0×10⁻³
1.5×10⁻³
1.0×10⁻³
5.0×10⁻⁴
0
Pre-exercise
Post-exercise
†
Men on PL
Men on E2
B
SREBP-2 mRNA content
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
Pre-exercise
Post-exercise
Men on PL
Men on E2
C
mtGPAT mRNA content
0.04
0.03
0.02
0.01
0.00
Pre-exercise
Post-exercise
†
Men on PL
Men on E2
D
HSL mRNA content
5.0×10⁻³
4.0×10⁻³
3.0×10⁻³
2.0×10⁻³
1.0×10⁻³
0
Pre-exercise
Post-exercise
*
*
Men on PL
Men on E2

Figure 3

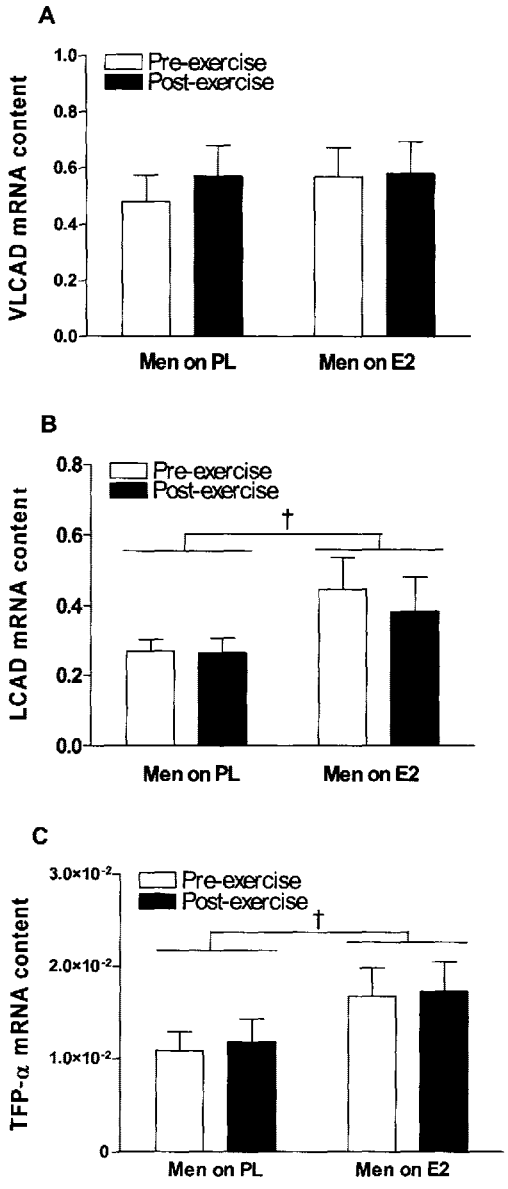
A
VLCAD mRNA content
1.0
0.8
0.6
0.4
0.2
0.0
Pre-exercise
Post-exercise
Men on PL
Men on E2
B
LCAD mRNA content
0.8
0.6
0.4
0.2
0.0
Pre-exercise
Post-exercise
†
Men on PL
Men on E2
C
TFP-α mRNA content
3.0×10⁻²
2.0×10⁻²
1.0×10⁻²
0
Pre-exercise
Post-exercise
†
Men on PL
Men on E2

Figure 4

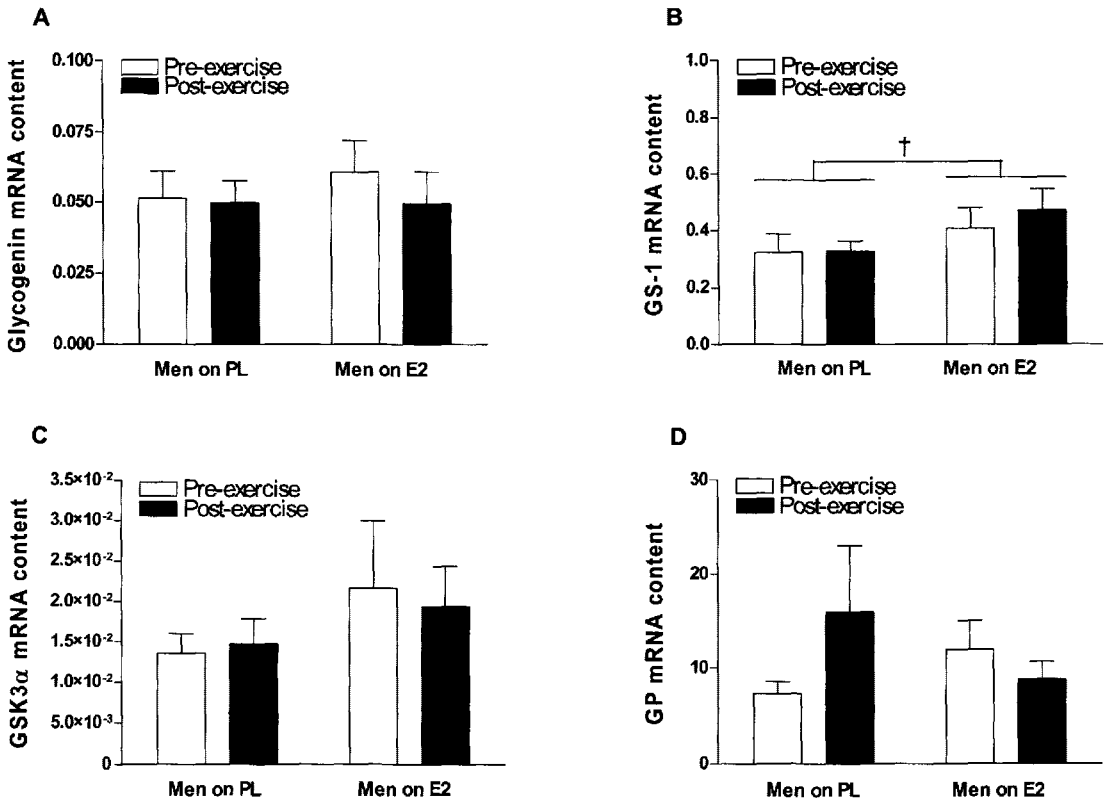
A
Glycogenin mRNA content
0.100
0.075
0.050
0.025
0.000
Pre-exercise
Post-exercise
Men on PL
Men on E2
B
GS-1 mRNA content
1.0
0.8
0.6
0.4
0.2
0.0
†
Pre-exercise
Post-exercise
Men on PL
Men on E2
C
GSK3α mRNA content
3.5×10-2
3.0×10-2
2.5×10-2
2.0×10-2
1.5×10-2
1.0×10-2
5.0×10-3
0
Pre-exercise
Post-exercise
Men on PL
Men on E2
D
GP mRNA content
30
20
10
0
Pre-exercise
Post-exercise
Men on PL
Men on E2

Figure 5

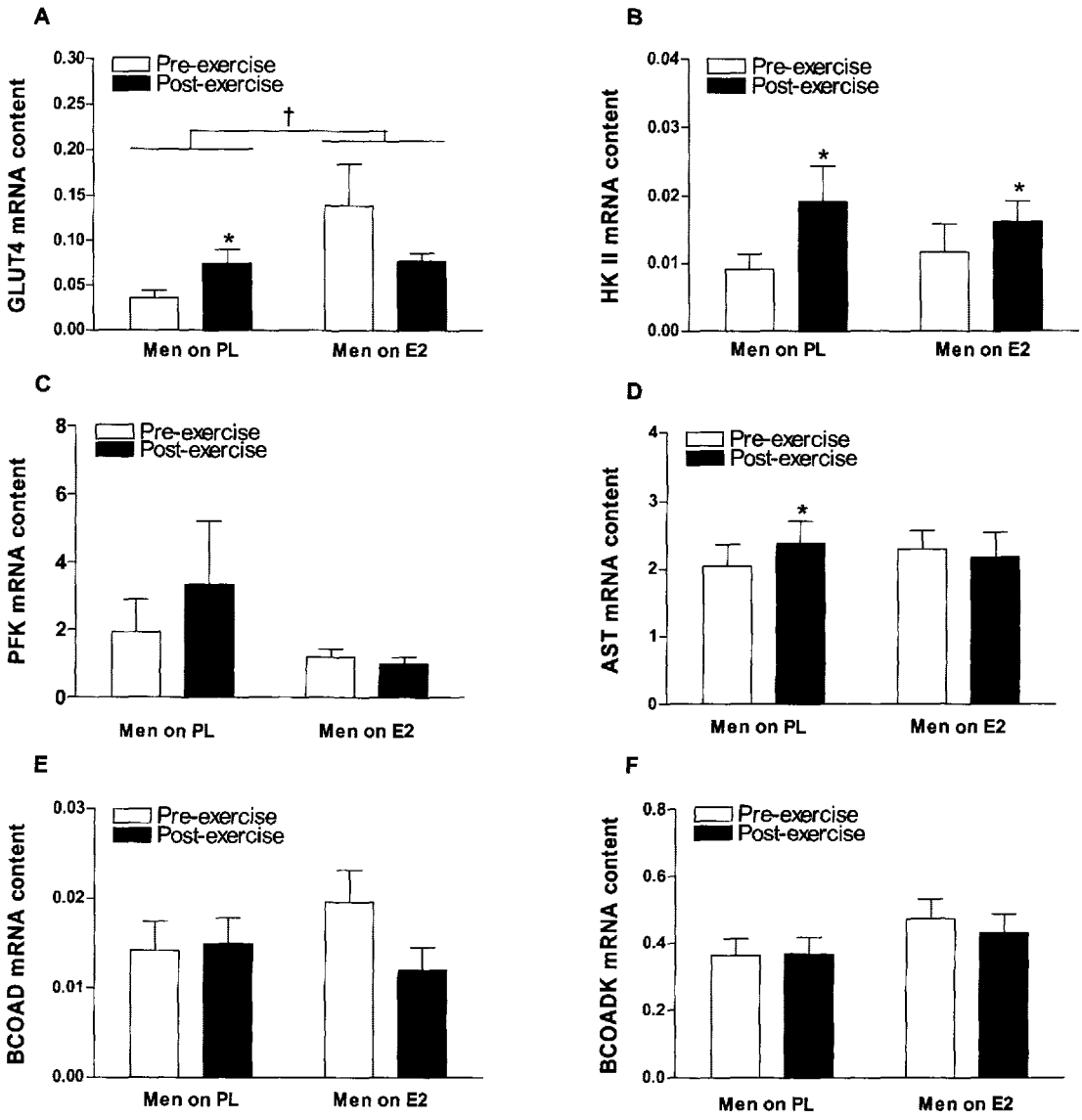
A
GLUT4 mRNA content
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Pre-exercise
Post-exercise
†
*
Men on PL
Men on E2
B
HK II mRNA content
0.04
0.03
0.02
0.01
0.00
Pre-exercise
Post-exercise
*
*
Men on PL
Men on E2
C
PFK mRNA content
8
6
4
2
0
Pre-exercise
Post-exercise
Men on PL
Men on E2
D
AST mRNA content
4
3
2
1
0
Pre-exercise
Post-exercise
*
Men on PL
Men on E2
E
BCOAD mRNA content
0.03
0.02
0.01
0.00
Pre-exercise
Post-exercise
Men on PL
Men on E2
F
BCOADK mRNA content
0.8
0.6
0.4
0.2
0.0
Pre-exercise
Post-exercise
Men on PL
Men on E2

Figure 6

CHAPTER 5: Manuscript 4

Formated for American Journal of Physiology

Sex influences the mRNA content for genes involved in muscle fiber type determination in human skeletal muscle

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Running head: Sex influences mRNA content in muscle fiber determination

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ABSTRACT

Women have a higher area percentage (%) of type I skeletal muscle fibers as compared with men. Consequently, we hypothesized that sex would influence the basal mRNA content for genes involved in the determination of muscle fiber type. A total of 24 women and 22 men had muscle biopsies taken from their vastus lateralis. Twelve women were studied in both follicular (FOL) and mid-luteal (LUT) phases. Twelve other women were studied in LUT phase. Muscle fiber composition was determined from ATPase staining in 12 men and 12 women studied in both FOL and LUT phases. mRNA content was determined in all samples using TaqMan® RT-PCR for genes involved in determination of muscle fiber (PGC-1α, PPARδ), determination of the proportion of type II fibers to type I fibers (myostatin), and terminal differentiation (MHCI, MCHIIa, MCHIIx). Women had lower mean area of individual fibers for type IIa and type IIx, and a higher area % of type I fibers and lower area % of type II fibers vs. men ($P < 0.05$). Women had higher mRNA content of MHCI (FOL, 2.6 ± 0.7 fold; LUT, 2.0 ± 0.5 fold; $P \leq 0.05$) and PPARδ (FOL, 2.3 ± 0.4 fold, $P < 0.05$), and a similar mRNA content of MHCIIa, MHCIIx, PGC-1α, myostatin and PPARδ (LUT), vs. men. FOL women had a significantly higher mRNA content of PPARδ than LUT women (2.2 ± 0.4 fold, $P = 0.0007$). Menstrual cycle phase did not significantly alter the mRNA content of MHCI, PGC-1α or myostatin. In conclusion, sex influences the mRNA content of the genes involved in muscle fiber determination and the change in PPARδ mRNA content supports a greater area % of type I fibers in women than men.

Key words: gender difference, muscle fiber area, PPARδ, MHC, myostatin, PGC-1α

INTRODUCTION

The potential for sex difference in muscle fiber composition has been studied in animals (8) and humans (1, 6, 7, 13, 14, 23, 24, 33, 34, 36, 40). In rats (8), sex differences were not found in the fiber type composition of the soleus and tibialis anterior muscles; however, the area of type I fibers was larger in females as compared with males (consequently, the area % occupied by type I fibers was higher). In humans, a sex difference in muscle fiber composition has been found in different skeletal muscles, such as, erector spinae (23), internal intercostal, external intercostal, and latissimus dorsi muscles (13), biceps brachii (24), vastus medialis (14), and vastus lateralis (1, 3, 6, 7, 33, 36, 40). In human vastus lateralis, there are generally three muscle fibers, type I, type IIa and type IIx, that can be identified with myosin-ATPase based histochemical analysis (2, 3, 40), distinguished by their myosin heavy chain (MHC) isoforms (30). Although studies concerning sex differences in skeletal muscle fiber type have shown inconsistent results (1, 4, 6, 28, 33, 34, 36), women generally have a greater relative type I fiber area (3, 6, 7, 34, 40), a smaller relative area of type II fibers and a greater percentage area of type I fibers (3, 6, 7, 28, 33, 34). The potential molecular mechanisms behind these sex differences have not been evaluated for the mRNA of the genes involved in muscle development, or fiber-type determination and differentaition.

The purpose of this study was to evaluate whether sex and/or menstrual cycle phase would influence the mRNA content of several genes involved in aspects of muscle fiber-type determination in human skeletal muscle. Specifically, we evaluated the mRNA content for genes involved in the transcriptional regulation of muscle fiber switch and mitochondrial biogenesis (peroxisome proliferator activated receptor-γ coactivator-1α,

PGC-1α; peroxisome proliferator activated receptor – delta, PPARδ), determination of the proportion of type II fibers to type I fibers during development (myostatin), and terminal differentiation (myosin heavy chain type I, MHCI; myosin heavy chain type IIa, MHCIIa; myosin heavy chain type IIx, MHCIIx). Given that women generally have a greater relative type I fiber area (3, 6, 7, 34, 40), a smaller relative area of type II fibers and a greater percentage area of type I fibers (3, 6, 7, 28, 33, 34), we hypothesized that the mRNA content of MHCI would be higher, and the mRNA content of MHCIIa and MHCIIx would be lower, in women as compared to men. Because PPARδ plays a role in the conversion of muscle fiber type II into type I and maintenance of the number of type I fibers (11, 21, 38), PGC-1α plays roles in determination of muscle fiber type and induces a fiber type transformation from type II into type I muscle fibers (19), and myostatin negatively regulates skeletal muscle growth (10, 12, 18) and plays roles in determination of the proportion of type II fibers to type I fibers during muscle development (9), we also hypothesized that there would be sex differential mRNA expression for PGC-1α, PPARδ and myostatin. Given that oestradiol affects muscle fiber characteristics in mice (31) and rats (17, 26), and plays some role in the determination of muscle fiber size and myosin heavy chain expression in rats (17, 26) and mice (31), we also planed to evaluate the effect of menstrual cycle phase on the mRNA content of the aforementioned genes.

MATERIALS AND METHODS

Subjects. The present study used the muscle samples from two studies where some aspects of the study outcomes from one of the studies, *study 2*, have been reported (5). In *study 1*, 12 females and 10 males volunteered to participate. The characteristics of the female subjects were: age, 25 ± 6 y (mean ± SD); weight, 64 ± 12 kg; height, 163 ± 8 cm; body mass index, 24 ± 3 kg/m^2; fat-free mass, 42 ± 5 kg; maximum O_2 consumption (VO_2max), 37 ± 7 ml O_2/kg body weight/min; and RER at 56% VO_2max, 0.88 ± 0.03. The characteristics of the male subjects were: age, 26 ± 4 y; weight, 83 ± 13 kg; height, 176 ± 6 cm; body mass index, 27 ± 4 kg/m^2; fat-free mass, 67 ± 8 kg; VO_2max, 42 ± 9 ml O_2/kg body weight/min; and RER at 56% VO_2max, 0.91 ± 0.03. Women were tested in the mid-luteal (days 19-21) phase of the menstrual cycle. In *study 2*, 24 healthy, recreationally active men (n=12) and women (n=12) (age: 22 ± 2 y) participated (5). Data from these subjects regarding CHO, protein and fat oxidation has been previously reported (5), and demonstrated that women had higher fat oxidation and lower protein and carbohydrate oxidation during endurance exercise as compared with males. The female subject characteristics were: age, 22 ± 2 y (mean ± SD); weight, 63 ± 9 kg; body mass index, 23 ± 3 kg/m^2; fat-free mass, 44 ± 5 kg; VO_2max, 40 ± 7 mL O_2/kg body weight/min. The male subject characteristics were: age, 22 ± 6 y; weight, 78 ± 11 kg; body mass index, 25 ± 3 kg/m^2; fat-free mass, 63 ± 7 kg; VO_2max, 45 ± 5 mL O_2/kg body weight/min. Women were tested in the mid-follicular (days 7-9) and mid-luteal (days 19-21) phases of the menstrual cycle. Ovulation was determined by usage of an ovulation test kit (Clear Plan Easy, Novartis). The remaining six females were taking a triphasic pill and thus

determination of ovulation was not required. All subjects from both studies were healthy, non-smokers and non-obese who participated in normal physical activity but were not trained athletes. In addition, their diet and training regime did not change within the 3-4 weeks prior to commencing the study. Before inclusion into the study, potential subjects were required to complete a health questionnaire to ensure that they were healthy and fit to participate. Each subject was given an information sheet describing all testing procedures, was informed of the purposes and associated risks, and gave written consent prior to participation. The study was approved by the McMaster University Hamilton Health Sciences Human Research Ethics Board and conformed to the Declaration of Helsinki guidelines.

Study design. Subjects reported to the laboratory on the morning of test day. For both *study1* and *study 2*, muscle biopsies were taken at rest from the vastus lateralis of the nondominant leg ~20 cm proximal to the lateral knee joint of each subject under local anesthesia using manual suction. If required, the sample was immediately dissected from excess fat and connective tissue, sectioned into a 25 – 50 mg piece, placed in an RNase-free polyethylene tube, flash-frozen in liquid nitrogen, and stored at –86°C until being processed for analysis. Muscle for histochemical analysis was placed into optimum cutting temperature embedding medium (OCT Tissue-Tek, Sakura Finetek, Torrance, CA), with the orientation of the fibers perpendicular to the horizontal plane, and was quickly frozen in isopentane cooled by liquid nitrogen and stored at –80°C until subsequent histochemical analysis (40).

Histochemical analysis. Histochemical analyses were conducted with the muscle biopsies only from the subjects of study 2 as described by Yasuda et al. (40). Briefly, the OCT mounted muscle samples were serially sectioned to 10μm thickness, and slides were preincubated at a pH value of 4.60 (in 50 mM potassium acetate, 17.5 mM calcium chloride) for 7 min. Slides were rinsed with distilled, deionized water (ddH20) between each of the following steps. Slides were incubated in 3 mM ATP using an alkaline solution (75 mM glycine, 40.5 mM calcium chloride, 75 mM NaCl, 67.5 mM NaOH, pH 9.4) for 45 min at 37°C with agitation at regular intervals. They were incubated consecutively in 1% CaCl2 and 2% CoCl2 for 3 min, and then incubated in 1% ammonium sulphide for 30 seconds at room temperature. Sections were photographed at 200 X magnifications with a microscope (Olympus America Inc., Melville, NY) in conjunction with a SPOT digital camera (Model: SP401-115, SPOT Diagnostic Instruments Inc., MI). Based on the staining intensity at pH 4.60, the three fiber types were classified as type I (dark), IIa (light) and IIx (intermediate). Cross-sectional areas of the muscle fibers (μm2) were determined using an image analysis program (Image Pro, V6.0, Media Cybernetics Inc., Silver Spring, MD). Total fiber numbers counted to distinguish the three fiber types and determine CSA were 294 ± 73 for men and 317 ± 77 for women (type I = 126 ± 29 men, 146 ± 12 women; type IIa = 122 ± 50 men, 132 ± 52 women; and type IIx = 46 ± 10 men, 41 ± 14 women).

Preparation of RNA. Total RNA was prepared from frozen human muscle and treated with DNaseI according to Tarnopolsky et al. (37) and Mahoney at el. (22). Briefly, 25-50 mg of human skeletal muscle biopisy was homogenized in 1 ml of TRIzol reagent (Invitrogen, Carlsbad, CA) at 4^0C. After the homogenate stood at room temperature for 5 –

10 min, 0.2 ml of chloroform was added and vortexed for 15 s. After standing at room temperature for 5 min, it was centrifuged at 12,000 rpm at 4^0C for 15 min. The aqueous layer was transferred to a fresh tube and mixed with 0.5 ml of isoproponal ethanol and left at room temperature for 10 min, followed by centrifugation at 12,000 rpm at 4^0C for 10 min. The RNA pellet was washed with 0.5 ml of 75% ethanol twice at room temperature and air-dried and dissolved in 14 μl of DEPC-treated ddH_2O. Aliquots of 2 μl each was made and stored at –80 ^{0}C. The concentration and purity of the RNA was determined in duplicate using a UV spectrophotometer (Shimadzu UV-1201; Mandel Scientific, Guelph, Ontario) by measuring the absorbance at 260 (OD_{260}) and 280 (OD_{280}) nm, with an average coefficient of variation (CV) of <10%. The average purity (OD_{260}/OD_{280}) of the samples was ≥ 1.5 (before DNAse treatment). RNA integrity was assessed using agarose gel electrophoresis with the OD ratio of 28S to 18S rRNA greater than 1 considered to be high-quality RNA.

TaqMan® real-time RT-PCR. Prior to TaqMan® real-time RT-PCR, RNA samples were treated with DNase I for 25 min at 37°C to remove any contaminating DNA by using DNA-free™ (Ambion Inc, Austin, TX) according to the Manufacturer's instructions. Specific primers and probes to each target gene were designed based on the cDNA sequence in GenBank (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi) with primer 3 designer (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) (Table1). Their specificity was monitored using Blast (http://www.ncbi.nlm.nih.gov/BLAST/). We manipulated their thermal dynamics through calculating delta G with Analyzer of Oligo (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/Default.aspx). All probes

were dual-labeled with fluorophores, with a fluorescent reporter dye at the 5' end (FAM, TET, HEX or TAMRA) and a corresponding quencher dye at its 3' end (3BHQ-1 or 3BHQ-2) (Table1). Human 28s rRNA and β2- microglobulin (β2-M) were evaluated (22) and used as internal standards because there was no sex differential mRNA expression of these genes in human skeletal muscle (Figure 1A, C) or menstrual phase cycle differential mRNA expression of β2-M (Figure 1B). For testing the target gene mRNA expression, duplex RT-PCR was performed on a real-time PCR system, iCycleriQ® Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) in the One-step TaqMan® RT-PCR Master Mix Reagents (Roch, Branchburg, New Jersey, part no. 4309169) according to the Manufacture's instruction with target gene primers and probe and internal standard gene primers and probe in the same reaction. RT- PCR reaction conditions were optimized so that the amplification efficiency for both target gene and internal reference was close to 1 and the difference between the slopes of the regression curves of each target gene and the corresponding internal standard gene (Ct V.S. amount of RNA) was less than 10 % (Table 2). Agarose gel electrophoresis was used to confirm the specificity of the priming. All samples were run in duplicate simultaneously with RNA- and RT-negative controls. Fluorescence emission was detected through a filter corresponding to the reporter dye at the 5' end of each probe, and C_T was automatically calculated and displayed.

Statistical Analysis. All statistical analyses about mRNA expression of the genes tested were performed on linear data 2^{-CT} for evaluation of internal standards, $2^{-\Delta CT}$ for target gene normalized with internal reference (20). Data from evaluation of internal standards

about sex effect were analyzed using a single factor ANOVA. Data from evaluation of internal standards regarding menstrual phase effects were analyzed using a paired *t*-test. All evaluation results are displayed in the figures as mean (±SE), using 2^{-CT}. Data from *study 1* and *study 2* regarding sex differences in target gene mRNA expression were analyzed using a single factor ANOVA. Data from *study 2* regarding the menstrual cycle phase effect on target gene mRNA expression were analyzed using a paired *t*-test between women at different menstrual cycle phases. All data are displayed in the figures as mean (± SE). The differences in target gene mRNA expression are expressed as mean fold change (±SE) between sexes or menstrual cycle phases, using $2^{-\Delta\Delta CT}$. The data regarding muscle fiber composition were analyzed using a single factor ANOVA and expressed as mean (± SE). Pearson's product-moment correlations were computed to examine the relationships between the mRNA content of MHCI and PPARδ and muscle fiber type I area, muscle fiber type I area % and muscle fiber type II (type IIa + type IIx) area %. All analyses were done using computerized statistics software (Statistica; Statsoft, Tulsa, OK) with statistical significance set at $\alpha \leq 0.05$.

RESULTS

Sex difference in muscle fiber composition: Women had significantly smaller mean individual fiber area for type IIa and type IIx (P = 0.046), while there was no significant difference in the type I fiber area between women and men (Table 3). Women had a significantly higher area % of type I fibers (P = 0.046) and a significantly lower area % of type II fibers (type IIa + type IIx) (P = 0.046) than men (Table 3). There was no significant difference in muscle fiber number composition (number %) between women and men (Table 3).

MHCI mRNA expression: Because neither 28S rRNA nor β2–M mRNA content was different between sexes (Figure1A, B), MHCI mRNA content was first tested in the skeletal muscle of the subjects from *study1* with 28S rRNA and β2–M as internal standards. The mRNA content of MHCI was significantly higher in women than men (3.6 ±1.8 fold, normalized to 28S rRNA; 3.4 ± 1.2 fold, normalized to β2 –M; P = 0.05) (Figure 2A, B). MHCI mRNA content was further tested in the skeletal muscle of the subjects from *study 2* with β2-M as an internal standard. The content of MHCI mRNA was significantly higher in the skeletal muscle of women (FOL, 2.6 ± 0.7 fold, P = 0.035; LUT, 2.0 ± 0.5 fold, P = 0.04) than men (Figure 2C, D). No significant difference in MHCI mRNA content between LUT and FOL women was detected (Figure 2E).

MHCIIa and MHCIIx mRNA expression: No significant difference in the mRNA content of MHCIIa or MHCIIx was found between women and men (Figure 3A, B).

PGC-1α mRNA expression: There were no significant differences in the mRNA content of PGC-1α in the skeletal muscle of men vs. women at FOL and LUT phases (Figure 4A,

B). The mRNA content of PGC-1α was not significantly different between women at FOL and LUT phases (Figure 4C).

PPARδ mRNA expression: FOL women had a significantly higher mRNA content of PPARδ than men (2.3 ± 0.4 fold, P = 0.004) (Figure 5A). No significant difference was detected in PPARδ mRNA content in the skeletal muscle between men and LUT women (Figure 5B). The PPARδ mRNA content was significantly higher in FOL women than LUT women (2.2 ± 0.4 fold, P = 0.005) (Figure 5C).

Myostatin mRNA expression: There were no significant differences in myostatin mRNA content in the skeletal muscles between men and women at FOL and LUT phases (Figure 6A, B). No significant difference in mRNA content of myostatin was detected in the skeletal muscles between LUT women and FOL women (Figure 6C).

Correlation coefficients: Significant correlations were found between the mRNA content of MHCI and area % of type I fibers ($r = 0.65$, $P < 0.042$, $n = 10$) and area % of type II (type IIa + type IIx) fibers ($r = -0.65$, $P < 0.04$, $n = 10$) in men and women (Figure 7A, B). Significant correlations were also found between the mRNA content of PPARδ and area % of type I fibers ($r = 0.62$, $P \leq 0.05$, $n = 10$) and area % of type II (type IIa + type IIx) fibers ($r = -0.84$, $P < 0.002$, $n = 10$) in men and women (Figure 7C, D). No significant correlation was found between type I fiber area and the mRNA content of MHCI or PPARδ.

DISCUSSION

This study is the first to evaluate the influence of sex and menstrual cycle phase on the mRNA content for genes involved in muscle fiber type determination in human skeletal muscle. We found that only MHCI and PPARδ mRNA were differentially expressed in men and women. The sex influenced changes in PPARδ and MHCI mRNA are consitent with the observation of a greater area % of type I fibers in women than men.

Myosin heavy chain genes are specifically expressed in the corresponding type of muscle fibers (35) and are good markers of terminal differentiation of muscle fibers. In this study, we found a significantly higher mRNA content of MHCI and a similar mRNA content of MHCIIa and MHCIIx in the skeletal muscles of women vs. men. These mRNA results are directionally consistent with previous reports of proportionally more type I fibers (1, 28, 33, 36), larger type I fiber area (3, 7, 34, 40), and higher area % of type I fibers in women as compared with men (3, 6, 7, 28, 34, 40). Although we did not confirm previous findings of a sex difference in type I fiber area or number % of type I or type II fibers, we did find that the proportionate area (area %) of type I fibers was higher, while that of type II fibers was lower, in women vs. men (3, 6, 7, 28, 34, 40). While a smaller fiber area % of type IIa and type IIx fibers in women vs. men was not directionally consistent with the finding of a similar content of mRNA (current study) and protein (40) for MHCIIa and MHCIIx in women vs. men, the positive correlation between the mRNA content of MHCI and area % of type I fibers, the negative correlation between the mRNA content of MHCI and area % of type II fiber and a higher mRNA content of MHCI in women vs. men directly support that a higher area % of type I fibers and a smaller fiber area % of type IIa

and type IIx fibers in women vs. men. Our observation of a higher MHCI mRNA expression is consistent with a previous report from our group showing that the MHCI protein content was higher for women (47 %) as compared to men (41 %), though, this latter finding was not statistically significant (40). Using SDS-PAGE, in combination with silver staining (39) and immunohistochemistry with specific anti-MHC monoclonal antibodies (32) muscle fibers coexpressing different MHC isoforms have been identified in human vastus lateralis muscle according to the MHC isoform they express at the protein level, types I, I+IIA, IIA, IIAX and IIX. mATPase histochemistry in this study may underestimate hybrid fibres coexpressing two isoforms (32). Further evaluation of sex differences in muscle fiber composition with immunohistochemistry is warranted.

In transgenic mice, it has been well established that PGC-1α is important in determination of muscle fiber type and induces a fiber type transformation from type II into type I muscle fibers (19). In contrast, studies regarding the role of PGC-1α in fiber type determination has shown conflicting results in humans (3, 25, 27, 29). Some studies reported no muscle fiber type conversion in men or women after endurance training (3), in spite of other studies showing increases in PGC-1α expression after exercise training (29). In contrast, some studies have shown that exercise training increased the expression level of PGC-1α mRNA and protein and the number of type I muscle fibers in human skeletal muscle (25, 27, 29). In the future, studies should examine whether or not the acute or chronic (training) induced changes in PGC-1α mRNA and protein change similarly in men and women. We did not find an influence of sex or menstrual cycle on the mRNA content of PGC-1α in the skeletal muscle in spite of the fact that women had a higher area % of

type I fibers. To make sure if PGC-1α plays a role in mediating the sex-based difference in muscle fiber area%, effect of sex on PGC-1α protein content should be evaluated.

In contrast, we did find that the mRNA content of PPARδ was higher in FOL women but not LUT women than men; and that mRNA content of PPARδ was higher in FOL women than LUT women. The mRNA expression of PPARδ may depend on the ratio of progesterone to estrogen. In LUT women, the level of progesterone is greater than estrogen while level of progesterone is less than estrogen in FOL women(15). Ovectomization of mice to remove both estrogen and progesterone (16) and 17β-estradiol supplementation of men (data not shown) demonstrated that estrogen increased the mRNA content of PPARδ, suggesting that progesterone may inhibit the mRNA expression of PPARδ. PPARδ plays a role in the conversion of muscle fiber type II into type I and maintenance of the number of type I fibers (38), and increases the capacity for oxidative metabolism of muscle fibers through hyperplasia of type I fibers (11, 21) in transgenic mice. The positive correlation between the mRNA content of PPARδ and area % of type I fibers, the negative correlation between the mRNA content of PPARδ and area % of type II fiber and a higher mRNA content of PPARδ in FOL women vs. men suggest that PPARδ may play a role in determination and maintenance of the sex difference in fiber area % in human.

Myostatin is a negative regulator of skeletal muscle growth (10, 12, 18) and plays roles in determination of the proportion of type II fibers to type I fibers during muscle development (9). We found that there was no sex difference in the mRNA content of myostatin between men and women at FOL and LUT phases. Women at FOL phase had

the same amount of myostatin mRNA expression in their skeletal muscle as women at LUT phase. All these results suggest that myostatin does not seem to play a role in determination of the sex-based differences in muscle fiber composition in human skeletal muscle. However, mRNA level is not always correlated to prtein level of a gene (), further evaluation aout the effect of sex and menstrual phase on the content of myostatin protein is required.

We conclude that MHCI mRNA abundance was higher in the skeletal muscle of women during both the FOL and LUT phases of the menstrual cycle as compared with men. There was no sex difference in the mRNA expression of MHCIIa and MHCIIx in spite of the higher area % of type IIx and IIa fibers in men. Sex and menstrual cycle phase influence the mRNA content of PPARδ in human skeletal muscle. PPARδ may play a role in the determination and/or maintenance of the sex difference in muscle fiber area % in human skeletal muscle. The role of PGC-1α, myostatin and PPARδ in mediating the sex-based difference in muscle fiber area % should be further evaluated for their protein content and tested with transgenic mice.

REFERENCES

1. **Brooke MH and Engel WK.** The histographic analysis of human muscle biopsies with regard to fiber types. 1. Adult male and female. *Neurology* 19: 221-233, 1969.

2. **Brooke MH and Kaiser KK.** Three "myosin adenosine triphosphatase" systems: the nature of their pH lability and sulfhydryl dependence. *J Histochem Cytochem* 18: 670-672, 1970.

3. **Carter SL, Rennie C, and Tarnopolsky MA.** Substrate utilization during endurance exercise in men and women after endurance training. *Am J Physiol Endocrinol Metab* 280: E898-907, 2001.

4. **Costill DL, Daniels J, Evans W, Fink W, Krahenbuhl G, and Saltin B.** Skeletal muscle enzymes and fiber composition in male and female track athletes. *J Appl Physiol* 40: 149-154, 1976.

5. **Devries MC, Hamadeh MJ, Phillips SM, and Tarnopolsky MA.** Menstrual cycle phase and sex influence muscle glycogen utilization and glucose turnover during moderate-intensity endurance exercise. *Am J Physiol Regul Integr Comp Physiol* 291: R1120-1128, 2006.

6. **Esbjornsson-Liljedahl M, Sundberg CJ, Norman B, and Jansson E.** Metabolic response in type I and type II muscle fibers during a 30-s cycle sprint in men and women. *J Appl Physiol* 87: 1326-1332, 1999.

7. **Esbjornsson M, Sylven C, Holm I, and Jansson E.** Fast twitch fibres may predict anaerobic performance in both females and males. *Int J Sports Med* 14: 257-263, 1993.

8. **Fox J, Garber P, Hoffman M, Johnson D, Schaefer P, Vien J, Zeaton C, and Thompson LV.** Morphological characteristics of skeletal muscles in relation to gender. *Aging Clin Exp Res* 15: 264-269, 2003.

9. **Girgenrath S, Song K, and Whittemore LA.** Loss of myostatin expression alters fiber-type distribution and expression of myosin heavy chain isoforms in slow- and fast-type skeletal muscle. *Muscle Nerve* 31: 34-40, 2005.

10. **Gonzalez-Cadavid NF, Taylor WE, Yarasheski K, Sinha-Hikim I, Ma K, Ezzat S, Shen R, Lalani R, Asa S, Mamita M, Nair G, Arver S, and Bhasin S.** Organization of the human myostatin gene and expression in healthy men and HIV-infected men with muscle wasting. *Proc Natl Acad Sci U S A* 95: 14938-14943, 1998.

11. **Grimaldi PA.** Roles of PPARdelta in the control of muscle development and metabolism. *Biochem Soc Trans* 31: 1130-1132, 2003.

12. **Grobet L, Pirottin D, Farnir F, Poncelet D, Royo LJ, Brouwers B, Christians E, Desmecht D, Coignoul F, Kahn R, and Georges M.** Modulating skeletal muscle mass by postnatal, muscle-specific inactivation of the myostatin gene. *Genesis* 35: 227-238, 2003.

13. **Hards JM, Reid WD, Pardy RL, and Pare PD.** Respiratory muscle fiber morphometry. Correlation with pulmonary function and nutrition. *Chest* 97: 1037-1044, 1990.

14. **Howel D.** Statistical analysis of compositional data in anatomy. *Anat Rec* 240: 625-631, 1994.

15. **Jabbour HN, Kelly RW, Fraser HM, and Critchley HO.** Endocrine regulation of menstruation. *Endocr Rev* 27: 17-46, 2006.

16. **Kamei Y, Suzuki M, Miyazaki H, Tsuboyama-Kasaoka N, Wu J, Ishimi Y, and Ezaki O.** Ovariectomy in mice decreases lipid metabolism-related gene expression in adipose tissue and skeletal muscle with increased body fat. *J Nutr Sci Vitaminol (Tokyo)* 51: 110-117, 2005.

17. **Kobori M and Yamamuro T.** Effects of gonadectomy and estrogen administration on rat skeletal muscle. *Clin Orthop Relat Res*: 306-311, 1989.

18. **Lee SJ.** Regulation of muscle mass by myostatin. *Annu Rev Cell Dev Biol* 20: 61-86, 2004.

19. **Lin J, Wu H, Tarr PT, Zhang CY, Wu Z, Boss O, Michael LF, Puigserver P, Isotani E, Olson EN, Lowell BB, Bassel-Duby R, and Spiegelman BM.** Transcriptional co-activator PGC-1 alpha drives the formation of slow-twitch muscle fibres. *Nature* 418: 797-801, 2002.

20. **Livak KJ and Schmittgen TD.** Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25: 402-408, 2001.

21. **Luquet S, Lopez-Soriano J, Holst D, Fredenrich A, Melki J, Rassoulzadegan M, and Grimaldi PA.** Peroxisome proliferator-activated receptor delta controls muscle development and oxidative capability. *Faseb J* 17: 2299-2301, 2003.

22. **Mahoney DJ, Carey K, Fu MH, Snow R, Cameron-Smith D, Parise G, and Tarnopolsky MA.** Real-time RT-PCR analysis of housekeeping genes in human skeletal muscle following acute exercise. *Physiol Genomics* 18: 226-231, 2004.

23. **Mannion AF, Dumas GA, Cooper RG, Espinosa FJ, Faris MW, and Stevenson JM.** Muscle fibre size and type distribution in thoracic and lumbar regions of erector spinae

in healthy subjects without low back pain: normal values and sex differences. *J Anat* 190 (Pt 4): 505-513, 1997.

24. **Miller AE, MacDougall JD, Tarnopolsky MA, and Sale DG.** Gender differences in strength and muscle fiber characteristics. *Eur J Appl Physiol Occup Physiol* 66: 254-262, 1993.

25. **Norrbom J, Sundberg CJ, Ameln H, Kraus WE, Jansson E, and Gustafsson T.** PGC-1alpha mRNA expression is influenced by metabolic perturbation in exercising human skeletal muscle. *J Appl Physiol* 96: 189-194, 2004.

26. **Piccone CM, Brazeau GA, and McCormick KM.** Effect of oestrogen on myofibre size and myosin expression in growing rats. *Exp Physiol* 90: 87-93, 2005.

27. **Pilegaard H, Saltin B, and Neufer PD.** Exercise induces transient transcriptional activation of the PGC-1alpha gene in human skeletal muscle. *J Physiol* 546: 851-858, 2003.

28. **Roepstorff C, Thiele M, Hillig T, Pilegaard H, Richter EA, Wojtaszewski JF, and Kiens B.** Higher skeletal muscle {alpha}2AMPK activation and lower energy charge and fat oxidation in men than in women during submaximal exercise. *J Physiol* 574: 125-138, 2006.

29. **Russell AP, Feilchenfeldt J, Schreiber S, Praz M, Crettenand A, Gobelet C, Meier CA, Bell DR, Kralli A, Giacobino JP, and Deriaz O.** Endurance training in humans leads to fiber type-specific increases in levels of peroxisome proliferator-activated receptor-gamma coactivator-1 and peroxisome proliferator-activated receptor-alpha in skeletal muscle. *Diabetes* 52: 2874-2881, 2003.

30. **Schiaffino S and Reggiani C.** Molecular diversity of myofibrillar proteins: gene regulation and functional significance. *Physiol Rev* 76: 371-423, 1996.

31. **Sciote JJ, Horton MJ, Zyman Y, and Pascoe G.** Differential effects of diminished oestrogen and androgen levels on development of skeletal muscle fibres in hypogonadal mice. *Acta Physiol Scand* 172: 179-187, 2001.

32. **Serrano AL, Perez M, Lucia A, Chicharro JL, Quiroz-Rothe E, and Rivero JL.** Immunolabelling, histochemistry and in situ hybridisation in human skeletal muscle fibres to detect myosin heavy chain expression at the protein and mRNA level. *J Anat* 199: 329-337, 2001.

33. **Simoneau JA and Bouchard C.** Human variation in skeletal muscle fiber-type proportion and enzyme activities. *Am J Physiol* 257: E567-572, 1989.

34. **Simoneau JA, Lortie G, Boulay MR, Thibault MC, Theriault G, and Bouchard C.** Skeletal muscle histochemical and biochemical characteristics in sedentary male and female subjects. *Can J Physiol Pharmacol* 63: 30-35, 1985.

35. **Staron RS.** Human skeletal muscle fiber types: delineation, development, and distribution. *Can J Appl Physiol* 22: 307-327, 1997.

36. **Staron RS, Hagerman FC, Hikida RS, Murray TF, Hostler DP, Crill MT, Ragg KE, and Toma K.** Fiber type composition of the vastus lateralis muscle of young men and women. *J Histochem Cytochem* 48: 623-629, 2000.

37. **Tarnopolsky M, Parise G, Fu MH, Brose A, Parshad A, Speer O, and Wallimann T.** Acute and moderate-term creatine monohydrate supplementation does not affect creatine transporter mRNA or protein content in either young or elderly humans. *Mol Cell Biochem* 244: 159-166, 2003.

38. **Wang YX, Zhang CL, Yu RT, Cho HK, Nelson MC, Bayuga-Ocampo CR, Ham J, Kang H, and Evans RM.** Regulation of muscle fiber type and running endurance by PPARdelta. *PLoS Biol* 2: e294, 2004.

39. **Williamson DL, Godard MP, Porter DA, Costill DL, and Trappe SW.** Progressive resistance training reduces myosin heavy chain coexpression in single muscle fibers from older men. *J Appl Physiol* 88: 627-633, 2000.

40. **Yasuda N, Glover EI, Phillips SM, Isfort RJ, and Tarnopolsky MA.** Sex-based differences in skeletal muscle function and morphology with short-term limb immobilization. *J Appl Physiol* 99: 1085-1092, 2005.

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Figure Legends

Fig.1. Skeletal muscle mRNA content of housekeeping genes in men and women. Panel A: β2-M; no significant difference between men and women of *study 1*. Panel B: 28S rRNA; no significant difference between men and women of *study 1*. Panel C: β2-M; no significant differences between men and FOL women, men and LUT women or FOL and LUT women of *study 2*. FOL, follicular phase; LUT, luteal phase. Values are means ± SE.

Fig.2. Skeletal muscle MHCI mRNA content in men and women (FOL and LUT). Panel A: subjects of *study 1* normalized to 28S rRNA; * Women higher than men (P = 0.05). Panel B: subjects of *study 1* normalized to β2-M mRNA; * Women higher than men (P = 0.05). Panel C: men vs. FOL of *study 2* normalized to β2-M; * FOL higher than men (P = 0.035). Panel D: men vs. LUT of *study 2* normalizeded to β2-M; * LUT higher than men (P = 0.04). Panel E: FOL vs. LUT of *study 2* normalized to β2-M. FOL, follicular phase; LUT, luteal phase. Values are means ± SE.

Fig.3. Skeletal muscle mRNA content of MCHIIa and MHCIIx in men and women. Panel A: MHCIIa. Panel B: MHCIIx. 28S rRNA was used as an internal standard. Values are means ± SE.

Fig.4. Skeletal muscle mRNA content of PGC-1α mRNA content in men and women (FOL and LUT) of *study 2*. Panel A: FOL vs. men. Panel B: LUT vs. men. Panel C: FOL vs. LUT. FOL, follicular phase; LUT, luteal phase. β2-M was used as an internal standard. Values are means ± SE.

Fig.5. Skeletal muscle mRNA content of PPARδ in men and women (FOL and LUT) of *study 2*. Panel A: FOL vs. men; * FOL higher than men (P = 0.004). Panel B: LUT vs. men. Panel C: FOL vs. LUT; † FOL higher than LUT (P = 0.005). FOL, follicular phase; LUT, luteal phase. β2-M was used as an internal standard. Values are means ± SE

Fig.6. Skeletal muscle mRNA content of myostatin in men and women (FOL and LUT) of *study 2*. Panel A: FOL vs. men. Panel B: LUT vs. men. Panel C: FOL vs. LUT. FOL, follicular phase; LUT, luteal phase. β2-M was used as an internal standard. Values are means ± SE.

Fig.7. Correlations of skeletal muscle mRNA content of MHCI and PPARδ to area % of type I fibers or type II (type IIa + type IIx) fibers to in men and women of *study 2*. Panel A: MHCI mRNA content to area % of type I fibers ($r = 0.65$, $P < 0.05$, $n = 10$). Panel B: MHCI mRNA content to area % of type II (type IIa + type IIx) fibers ($r = -0.65$, $P < 0.05$, $n = 10$). Panel C: PPARδ mRNA content to area % of type I fibers ($r = 0.62$, $P = 0.002$, $n = 10$). Panel D: PPAR δ mRNA content to area % of type II (type IIa + type IIx) fibers ($r = -0.84$, $P = 0.002$, $n = 10$).

Table1: Primer and probe sets

Variables / Genes	GenBank access No.	Primer and probe set	Probe labeling
28S rRNA	M11167	Left primer: 5'-cccagtgctctgaatgtcaa-3' Right primer: 5'-agtgggaatctcgttcatcc-3' Probe: 5'-ccttaagagagtcatagttactcccgccgt-3'	5' end -TAMRA 3' end-3BHQ-1
β2 – M	NM_004048	Left primer: 5'- ggctatccagcgtactccaa-3' Right primer: 5'- gatgaaacccagacacatagca-3' Probe: 5'-tcaggtttactcacgtcatccagcagag-3'	5' end -TAMRA 3' end -3BHQ-2
MHCI	M21665	Left primer: 5'-cctggaacatctggagacct-3' Right primer:5'-agctgctttcggaccttct-3' Probe: 5'-atagtctttccgctggaacccaactgct-3'	5' end – FAM 3' end - 3BHQ-1
MHCIIa	AF111784	Left primer: 5'-caatctagctaaattccgcaagc-3' Right primer:5'-tcacttatgacttttgtgtgtgaacct-3' Probe: 5'-ttcacccgcagtttgttcacctggga-3'	5' end -TET 3' end -3BHQ-1
MHCIIx	AF111785	Left primer: 5'-aaatggtggaaagaagagagtcc-3' Right primer:5'-aatacagcttcatccagggc-3' Probe:5'-catccagtacaatgtccgtgccttca-3'	5' end- HEX 3' end - 3BHQ-1
PGC-1α	NM_013261	Left primer:5'-ttgctaaacgactccgagaa-3' Right primer: 5'-tgcaaagttcccctctgct-3' Probe: 5'- aacagttgggctgtcaacattcaaagc-3'	5' end - FAM 3' end - 3BHQ-1
PPARδ	NM_006238	Left primer: 5'-actgagttcgccaagagcat-3' Right primer: 5'-gtgcacgccatacttgagaa-3' Probe: 5'-cagcctcttcctcaacgaccaggttac-3'	5' end -FAM 3' end -3BHQ-1
Myostatin	AF104922	Left primer:5' –gacccgtcgagactcctaca-3' Right primer: 5' –aataccagtgcctgggttca-3' Probe: 5'-tgtgcaaatcctgagactcatcaaacc-3'	5' end - FAM 3' end -3BHQ-1

Table 2: TaqMan ™ real-time RT-PCR reaction conditions

Target genes / Parameters	28S rRNA	β-2 M	MHCI	MHCIIa	MHCIIx	PGC-1α	PPARδ	Myostatin
arget gene left rimer	0.3μM	0.9 μM	0.6 μM	0.3μM	0.3μM	0.9 μM	0.9 μM	0.6 μM
arget gene ight primer	0.3μM	0.9 μM	0.6 μM	0.3μM	0.3μM	0.9 μM	0.9 μM	0.2 μM
arget gene probe	0.15μM	0.05μM	0.1μM	0.1μM	0.1μM	0.1μM	0.2 μM	0.15μM
3-2 M eft primer	n/a	n/a	0.6 μM	n/a	n/a	0.3μM	0.6 μM	0.3μM
3-2 M ight primer	n/a	n/a	0.6 μM	n/a	n/a	0.3μM	0.6 μM	0.3μM
3-2 M probe	n/a	n/a	0.05μM	n/a	n/a	0.05μM	0.05μM	0.05μM
28SrRNA eft primer	n/a	n/a	n/a	0.3μM	0.3μM	n/a	n/a	n/a
28SrRNA ight primer	n/a	n/a	n/a	0.3μM	0.3μM	n/a	n/a	n/a
28SrRNA robe	n/a	n/a	n/a	0.1μM	0.1μM	n/a	n/a	n/a
RNA template	15 ng	15 ng	2.5 ng	25 ng	25 ng	5.0 ng	7.5 ng	5.0 ng
eaction volume	25 μl	25 μl	25 μl	50μl	50μl	25 μl	25 μl	25 μl

Table3. Fiber type composition in the *vastus lateralis* muscle of women and men.

	Women		Men
Fiber type composition (number %)			
type I	47.8 ± 3.4		45.2 ± 3.5
type IIa	37.0 ± 2.2		34.9 ± 1.2
type IIx	17.8 ± 2.8		17.2 ± 3.6
Fiber type composition (area %)			
type I	31.4 ± 1.6	*	27.2 ± 1.1
type IIa + type IIx	68.6 ± 1.6	*	72.8 ± 1.1
Mean area per fiber (μm^2)			
type I	3891.0 ± 379.8		4304.1 ± 257.6
type IIa	4753.5 ± 461.2	*	6439.4 ± 398.8
type IIx	3943.0 ± 500.9	*	5132.8 ± 263.0

Data are mean ± SE. Sex difference, * $P < 0.05$. n = 24.

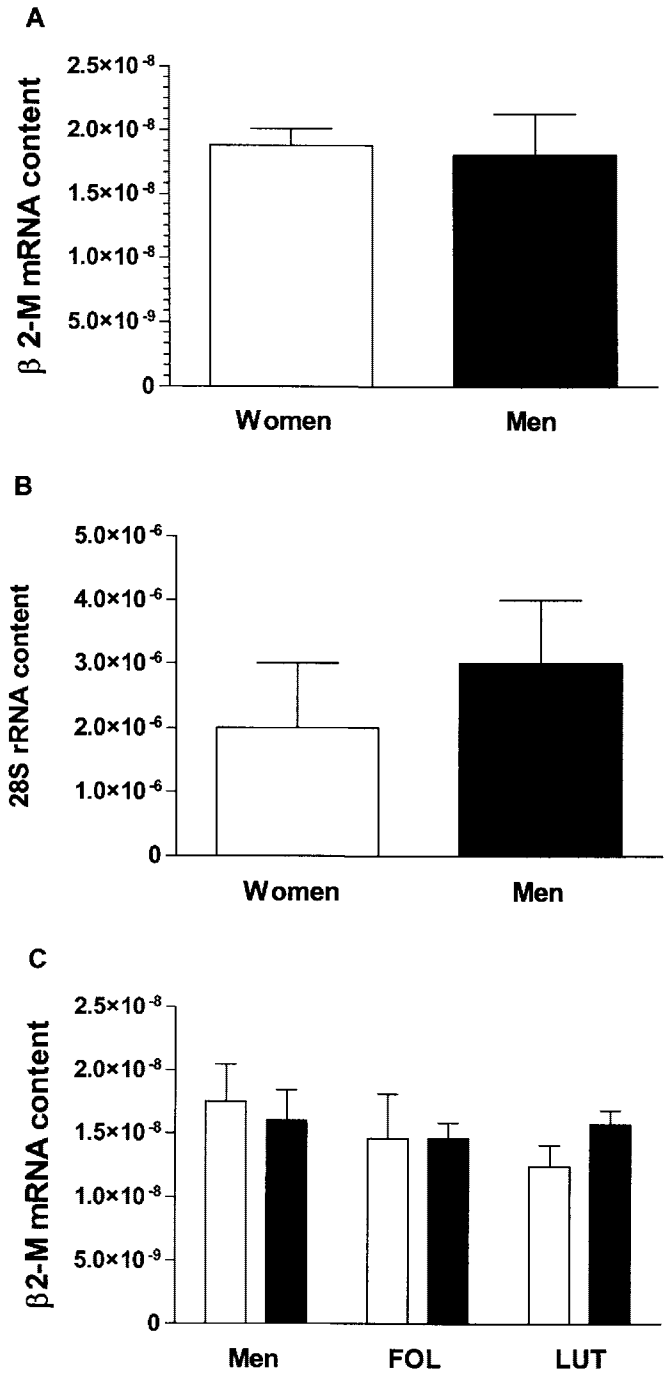
A
β 2-M mRNA content
2.5×10-8
2.0×10-8
1.5×10-8
1.0×10-8
5.0×10-9
0
Women
Men
B
28S rRNA content
5.0×10-6
4.0×10-6
3.0×10-6
2.0×10-6
1.0×10-6
0
Women
Men
C
β2-M mRNA content
2.5×10-8
2.0×10-8
1.5×10-8
1.0×10-8
5.0×10-9
0
Men
FOL
LUT

Figure 1

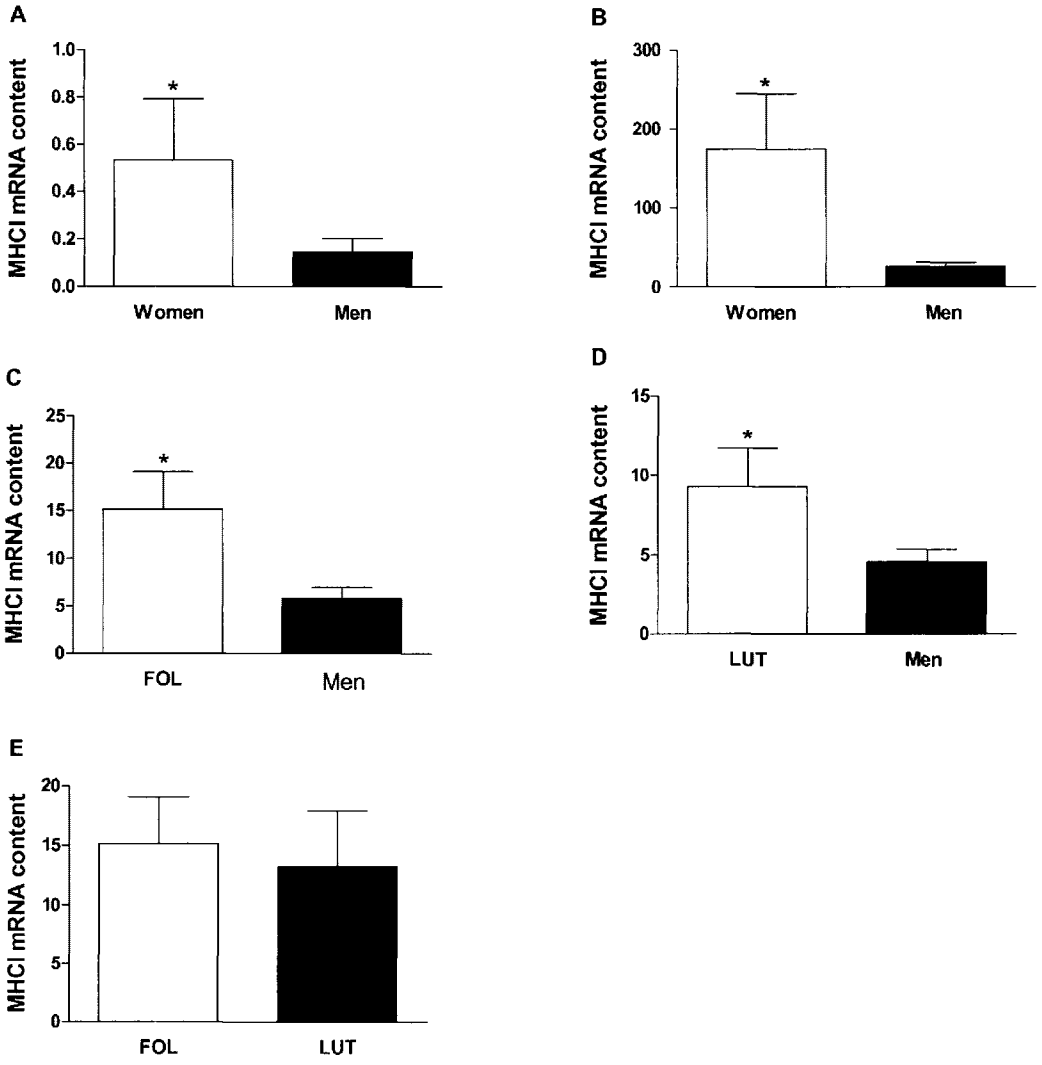
A
MHCI mRNA content
1.0
0.8
0.6
0.4
0.2
0.0
*
Women
Men
B
MHCI mRNA content
300
200
100
0
*
Women
Men
C
MHCI mRNA content
25
20
15
10
5
0
*
FOL
Men
D
MHCI mRNA content
15
10
5
0
*
LUT
Men
E
MHCI mRNA content
20
15
10
5
0
FOL
LUT

Figure 2

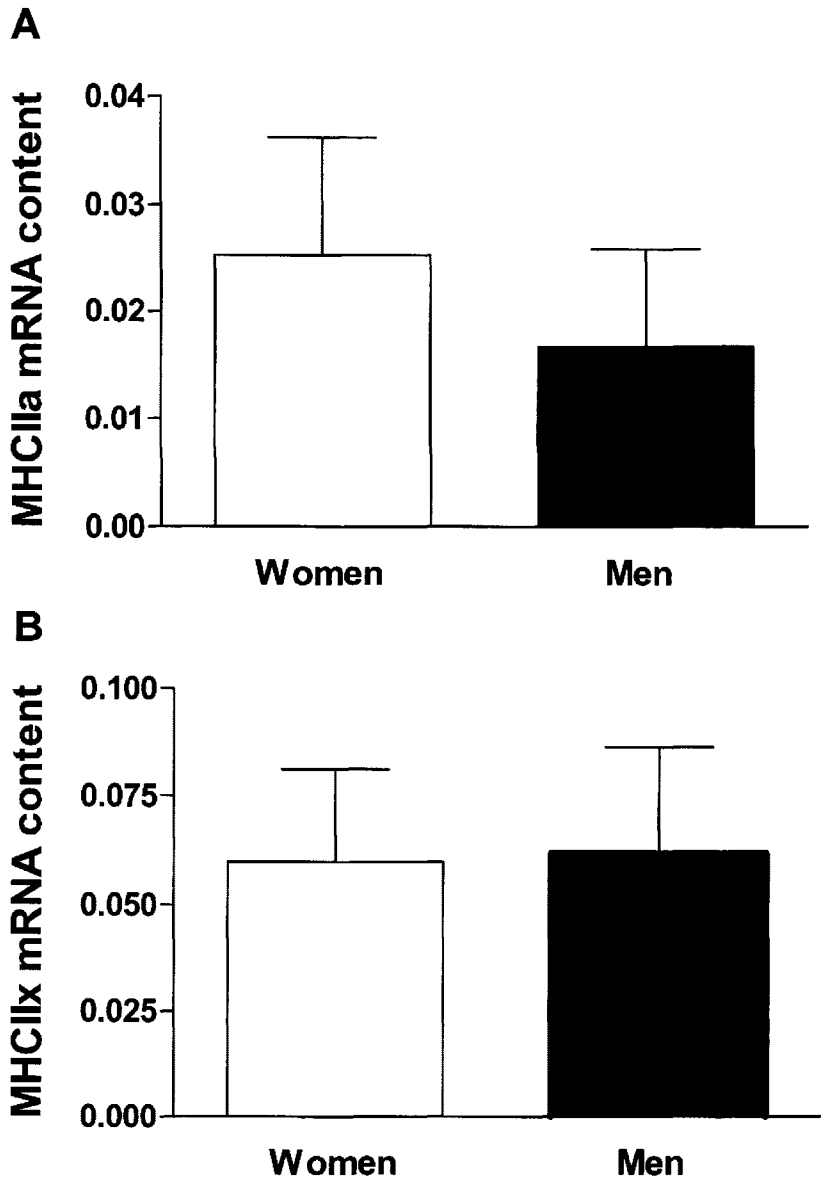
A
MHCIIa mRNA content
0.04
0.03
0.02
0.01
0.00
Women
Men
B
MHCIIx mRNA content
0.100
0.075
0.050
0.025
0.000
Women
Men

Figure 3

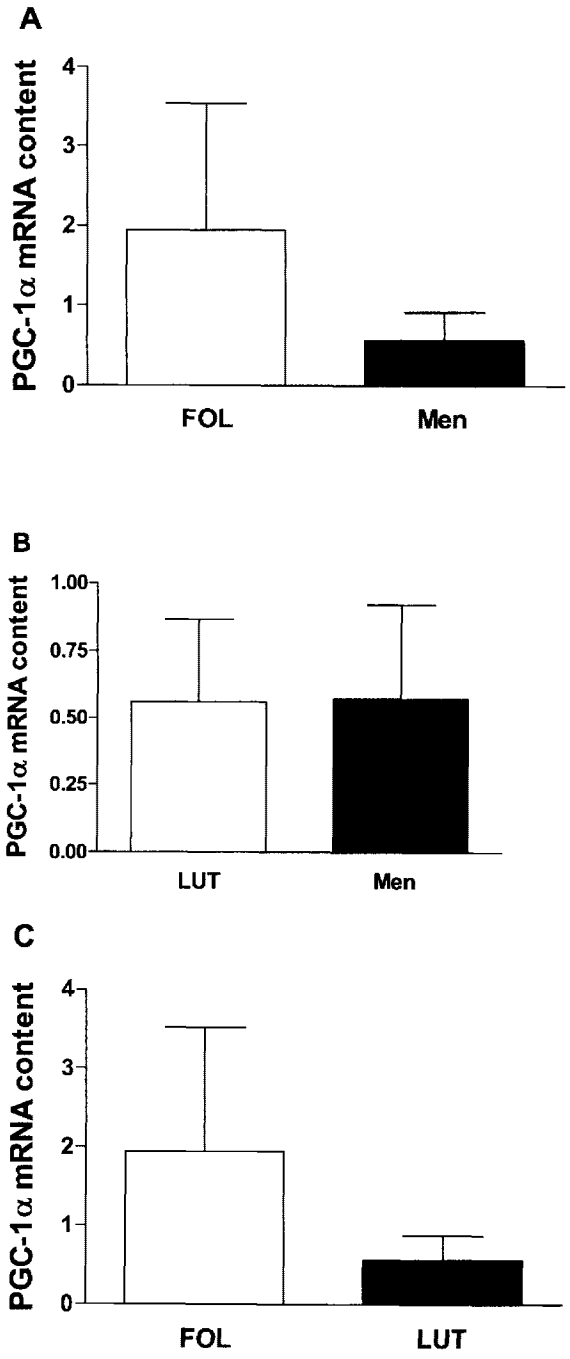
A
PGC-1α mRNA content
4
3
2
1
0
FOL
Men
B
PGC-1α mRNA content
1.00
0.75
0.50
0.25
0.00
LUT
Men
C
PGC-1α mRNA content
4
3
2
1
0
FOL
LUT

Figure 4

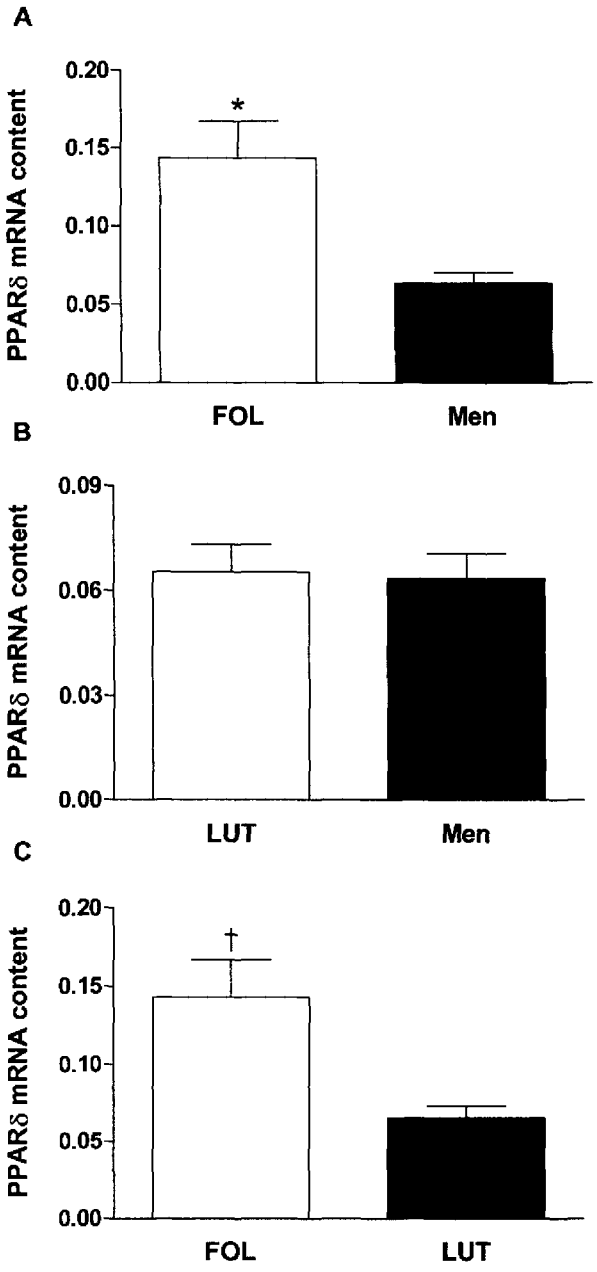
A
PPARδ mRNA content
0.20
0.15
0.10
0.05
0.00
*
FOL
Men
B
PPARδ mRNA content
0.09
0.06
0.03
0.00
LUT
Men
C
PPARδ mRNA content
0.20
0.15
0.10
0.05
0.00
†
FOL
LUT

Figure 5

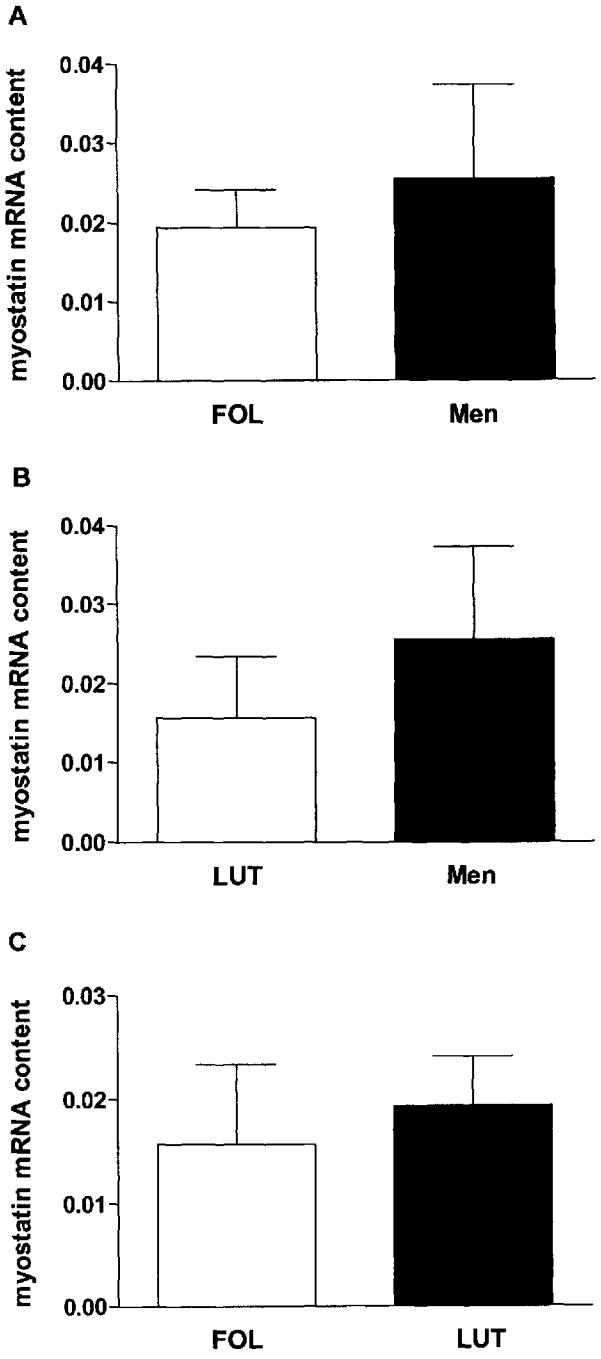
A
myostatin mRNA content
0.04
0.03
0.02
0.01
0.00
FOL
Men
B
myostatin mRNA content
0.04
0.03
0.02
0.01
0.00
LUT
Men
C
myostatin mRNA content
0.03
0.02
0.01
0.00
FOL
LUT

Figure 6

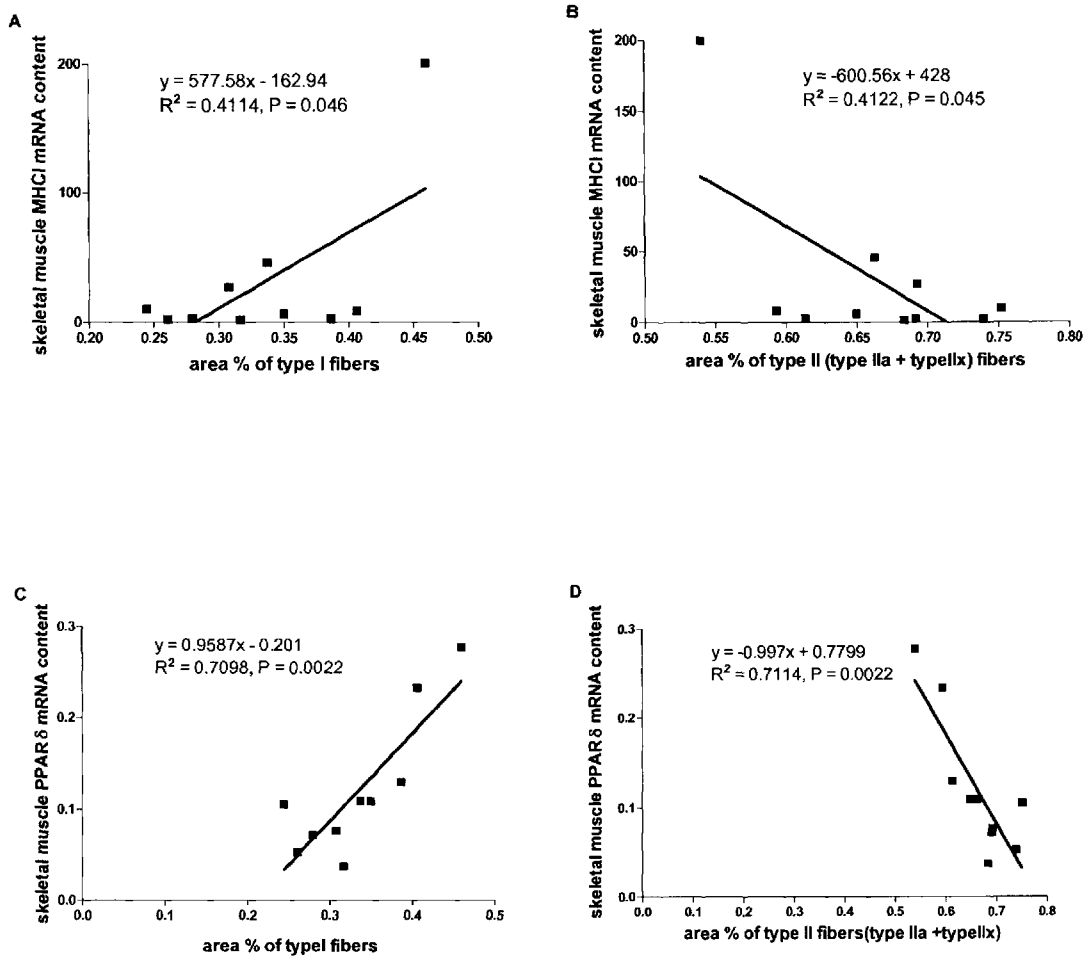
A
skeletal muscle MHCI mRNA content
y = 577.58x - 162.94
R² = 0.4114, P = 0.046
area % of type I fibers
B
skeletal muscle MHCI mRNA content
y = -600.56x + 428
R² = 0.4122, P = 0.045
area % of type II (type IIa + typeIIx) fibers
C
skeletal muscle PPARδ mRNA content
y = 0.9587x - 0.201
R² = 0.7098, P = 0.0022
area % of typeI fibers
D
skeletal muscle PPARδ mRNA content
y = -0.997x + 0.7799
R² = 0.7114, P = 0.0022
area % of type II fibers(type IIa +typeIIx)

Figure 7

CHAPTER 6: OVERALL DISSCUSSION

6.0 Introduction

We have provided novel data regarding sex-based differences in the abundance of mRNA for genes involved in fat, CHO and protein metabolism. Our findings are directionally supportive of substrate-derived data on the well characterized sex-based differences in substrate utilization during exercise, menstrual cycle phase differential utilization of substrate, and the role of estrogen in mediating the sex differences in substrate utilization during exercise. We have also evaluated several of the mRNA species involved in determination of muscle fiber (PGC-1α, PPARδ), determination of the proportion of type II fibers to type I fibers (myostatin), and terminal differentiation (MHCI, MCHIIa, MCHIIx) and showed that sex-based difference in PPARδ mRNA content may be a potential basis for sex differences in muscle fiber composition in human skeletal muscle. Collectively, our study demonstrated that acute endurance exercise, menstrual cycle phase, sex and E2 influence the mRNA content of the genes involved in fat, CHO and protein metabolism. These changes in mRNA content of the genes support that sex selective utilization of substrate may be mainly defined through regulation of the mRNA content for genes involved in fat metabolism, while CHO and protein metabolism appears to respond passively. E2 also mediates the selective substrate utilization in men predominantly through targeting the genes involved in fat metabolism at mRNA level. Sex differential mRNA expression of PPARδ may play a role in the determination of the sex differences in muscle fiber composition in human skeletal muscle. However, it must be acknowledged that there are multiple levels of regulation of gene expression beyond mRNA content that may influence the physiological processes studied in this thesis. Translation efficiency may also influence the protein content of a gene. Post-translation

modification (phosphorylation), compartment and metabolites may influence the activity of a protein. Further evaluation for the molecular mechanisms of the interested physiological processes need to be performed at these levels. Functional confirmation of the roles for the genes with changes in mRNA content is necessary.

6.1 Exercise stimulated mRNA expression for genes involved in substrate utilization: implication for exercise stimulated fat, glucose and protein oxidation

It has been well documented that acute exercise stimulates the expression of many genes involved in fat and glucose metabolism (Cameron-Smith, 2002), which potentially increase the mRNA capacity for lipoprotein hydrolysis (Pilegaard et al., 2000; Pilegaard et al., 2005), FFA transport (Kiens et al., 2004; Pilegaard et al., 2000; Pilegaard et al., 2005), fat oxidation (Watt et al., 2004) and glycolysis (Pilegaard et al., 2000; Watt et al., 2004). This thesis was the first study that comprehensively evaluated the effect of exercise on the mRNA content of genes involved in fat, CHO and protein metabolism as an integrated theme, and investigated the mRNA content of FABPc, HSL, SREBP-2, mtGPAT, TFP-α, VLCAD, LCAD, CPTII, GSK3α, PFK, glycogen phosphorylase, AST, BCOAD and BCOADK that have not been studied previously. We found that exercise increased the mRNA content of PGC-1α (men, LUT), FABPc (men, FOL, LUT), CPTI (men, LUT), LCAD (LUT), HKII, AST and GLUT4 (men and women). Taken together with the findings that exercise increases the mRNA expression of PPARα (Pilegaard et al., 2005; Watt et al., 2004), PPARδ (Watt et al., 2004), FAT/CD36, and FABPpm (Kiens et al., 2004), carnitine palmitoyltransferase I and lipoprotein lipase (Pilegaard et al., 2000; Pilegaard et al., 2005), pyruvate dehydrogenase kinase 4 (PDK4) (Watt et al.,

2004) and glycogenin (Kraniou et al., 2000; Shearer et al., 2005a; Shearer et al., 2005b) in human skeletal muscle, our observations suggest that exercise increase the mRNA capacity for fat, glucose and protein oxidation.

6.2 Menstrual cycle phase differential mRNA expression: implications for menstrual cycle phase differences in substrate utilization

Although studies regarding menstrual cycle phase differences in substrate utilization in human are controversial, most studies found women oxidized more fat, more amino acid and less CHO during LUT phase than FOL phase (Calloway & Kurzer, 1982; Garrel et al., 1985; Hackney, McCracken-Compton & Ainsworth, 1994; Kriengsinyos et al., 2004; Lariviere, Moussalli & Garrel, 1994; Moller et al., 1996; Wenz et al., 1997; Zderic, Coggan & Ruby, 2001). Menstrual cycle phases have been considered as factors influencing the sex differences in substrate utilization during exercise. In this study, we are the first to comprehensively evaluate the effect of menstrual cycle phases on the mRNA content for genes involved in fat, carbohydrate and protein metabolism. We found that FOL women had a significantly higher PPARδ, LCAD and glycogen phosphorylase and lower glycogenin mRNA content pre- and post-exercise, and tended to have higher GLUT4 mRNA content at rest, vs. LUT women. Post-exercise FABPc mRNA content increased to a greater extent in LUT vs. FOL phase. Though a higher mRNA content of FABPc, GLUT4 and glycogen phosphorylase and a lower mRNA content of glycogenin may lead to a higher capacity for cytosol fatty acid transport, membrane glucose transport, glycogen hydrolysis, and a lower capacity for glycogen synthesis, in women during LUT phase vs. FOL phase, a higher mRNA content

of PPARδ and LCAD may lead to a higher capacity for fat oxidation in women during FOL phase vs. LUT phase. Our observations did not coordinately or directionally support a higher oxidation of fat and amino acids and a lower oxidation of CHO in women during LUT phase vs. FOL phase of the menstrual cycle at rest or during exercise.

6.3 Sex differential mRNA expression: implications for sex differences in substrate utilization

Previous research had established that women have a higher mRNA content of membrane bound fatty acid transport protein 1 (FATm) (Binnert et al., 2000), lipoprotein lipase (LPL) (Kiens et al., 2004), FAT/CD36, FABPpm (Kiens et al., 2004), and a higher protein content for FAT/CD36 (Kiens et al., 2004). Women also have a higher mRNA, but not protein, content of β-hydroxyacyl-CoA dehydrogenase (β-HAD) (Roepstorff et al., 2005), and CPT I activity was not different between men and women (Berthon et al., 1998). We found that women had a significantly higher mRNA content of FATm, FABPc, SREBP-1c and mtGPAT (FOL, LUT), PPARα (FOL), CPTI (FOL), LCAD (FOL), TFPα (LUT), HKII (FOL) and BCOADK (FOL, LUT) pre- and post- exercise, PPARδ (FOL) and GLUT4 (FOL) at rest, and PPARα (LUT) and LCAD (LUT) after exercise, and lower mRNA content for PFK (LUT) pre- and post- exercise, than men. The male-female difference in mRNA abundance of the genes involved in fat metabolism supports the data based on substrate oxidation that women use more fat than men. Our data show that mRNA differences may well bring about the higher capacity of women versus men for fat oxidation, IMCL synthesis, sarcolemma and mitochondrial fatty acid

transport and fat oxidation, and coordinately supports a higher oxidation of fat during exercise in women than men. The sex differential mRNA expression for genes involved in CHO and protein metabolism implicate that women have a higher capacity for membrane glucose transport and muscle glucose phosphorylation, and a lower capacity for glycolysis and amino acid backbone dehydrogenation. These observations did not directionally support a lower CHO and protein oxidation in women than men during exercise. However, it must be acknowledged that there are multiple levels of regulation of metabolic flux beyond gene transcription that may influence the observation of a lower carbohydrate and protein oxidation in women. In addition to mRNA level, translation efficiency may also influence the protein content of a gene. Post-translation modification (phosphorylation), compartment and metabolites may influence the activity of a protein. Our observation, combined with the previous findings, suggest that the observed sex-based differences in substrate utilization in human during exercise may be predominantly and coordinately defined by the genes involved in fat metabolism, while the metabolism of CHO and protein follows.

6.4 E2 mediated alteration in mRNA expression: implications for E2 mediated selective substrate utilization in men and role of E2 in sex selective substrate utilization during exercise

E2 supplementation in men increased fat oxidation and decreased CHO and amino acid oxidation (Devries et al., 2005; Hamadeh, Devries & Tarnopolsky, 2005). We first comprehensively evaluated the effect of E2 on mRNA content for genes involved in fat, CHO and protein metabolism in the skeletal muscle of men. We found that E2

significantly increased the mRNA content of PPARα, CPTI, SREBP-1c, mtGPAT, LCAD, TFP-α, GLUT4 and GS-1 in men at rest and during exercise, and PPARδ at rest. E2 significantly attenuated the exercise induced increase in PGC-1α mRNA content. Our observations implicate that E2 increased the capacity for IMCL synthesis and fat oxidation and support an E2 mediated increase in fat oxidation while not directionally support an E2 mediated decrease in CHO and protein oxidation in men. E2 mediates selective substrate utilization in men predominantly through influencing the expression of the genes involved in fat metabolism while the decrease in CHO and protein oxidation is a secondary consequence following increased fat utilization. E2 mediated increase in PPARδ mRNA expression may compensate for the attenuation of the acute exercise stimulated increase of PGC-1α mRNA.

6.5 Sex differential mRNA expression of MHCI and PPARδ: implications for sex differences in muscle fiber composition

Although the results of studies regarding sex differences in muscle fiber type characteristics are controversial (Brooke & Engel, 1969; Costill et al., 1976; Esbjornsson-Liljedahl et al., 1999; Simoneau & Bouchard, 1989; Simoneau et al., 1985; Staron et al., 2000), women in general have a greater relative type I fiber area (Carter et al., 2001; Esbjornsson-Liljedahl et al., 1999; Esbjornsson et al., 1993; Simoneau et al., 1985; Yasuda et al., 2005), a smaller relative area of type II fibers and a greater type I % area (Carter et al., 2001; Yasuda et al., 2005). Our ATPase staining study further confirmed that women have a higher area % of type I fibers and lower area % of type II fibers vs. men. In this study we also found that women had higher mRNA content of MHCI (FOL,

LUT) and PPARδ (FOL) vs. men at rest, which supports the observed sex differences in fiber type composition.

6.6 Future directions

To fully understand the molecular mechanisms of the processes related to sex-based differences in substrate utilization during exercise in human skeletal muscle, we should do further studies with regard to (1), potential mechanisms for sex differences in substrate utilization during exercise at transcription activity, mRNA stability, and proteomic levels; (2), role of progesterone in sex, menstrual cycle phase and exercise influenced substrate utilization; (3), molecular mechanisms of the attenuation of the increase in PGC-1α mRNA expression during exercise by E2; (4), the role of E2 in sex differences in muscle fiber composition in humans and confirmation of the role of PPARδ in determination of sex difference in muscle fiber composition; (5), functional confirmation and regulatory relationship among the genes with exercise, sex, menstrual cycle phase and E2 influenced mRNA expression.

6.6.1 Mechanisms for sex-based differences in substrate utilization during exercise at transcription, mRNA stability and proteomic levels

Gene expression is regulated at transcriptional and post-transcription level. In turn, proteins are regulated at the translation level and post-translation level. The steady state level of mRNA of a gene is the result of transcription and mRNA degradation (Hargreaves & Cameron-Smith, 2002). In the present study, we have evaluated the effects of sex, menstrual cycle phase, estrogen and exercise on the steady level of mRNA

for the genes involved in substrate utilization and muscle fiber type determination, and found that the mRNA content of many genes were influenced by sex, menstrual cycle phase, E2 and exercise. There may be differences in the transcription activity or mRNA stability with these mRNA species between sexes, menstrual cycle phases. Estrogen may influence the steady level of mRNA of the genes in men through altering the transcription activity of these genes or the stability of these mRNA species. Exercise may alter the steady level of these mRNA species through influencing the transcription activity of these genes or the stability of these mRNA species. Transcription activity assays (Marinovic, Mitch & Price, 2000; Southern, Brown & Hall, 2006) and mRNA stability assay (Freyssenet et al., 1999) may be employed to answer these questions. Gel shift assay may be used to identify the putative transcription factors that are involved in mediating the differences in the transcription activity of the genes (Tschritter et al., 2006). Through mRNA and protein binding assay (Marquis et al., 2006), the putative mRNA stabilizing factors and mRNA binding proteins which play roles in translation regulation (Wells, 2006) may also be identified.

Although the steady state content of mRNA generally reflect the content of protein in the tissues, some exceptions have been documented that the steady state of mRNA is not correlated with the content of the corresponding proteins (Cox, Kislinger & Emili, 2005). For enzymes in particular, the physiological outcome is more influenced by the activity of proteins and not merely their protein content. To identify novel proteins that may be influences by sex or E2, two dimensional gel electrophoresis may be used (Kubota, Kosaka & Ichikawa, 2005), followed by mass spectroscopy to further

characterize the proteins. Functional proteomics may be used to measure the activity of these proteins (Zhu et al., 2001; Zhu, Bilgin & Snyder, 2003).

6.6.2 Role of progesterone in sex, menstrual cycle phase and exercise influenced substrate utilization

Progesterone is also a sex hormone and has been found to play some roles in regulating substrate utilization and involved in sex, menstrual cycle phase and exercise influenced substrate utilization (Braun & Horton, 2001). We found some genes that showed different response patterns to exercise between menstrual cycle phase, indicating that estrogen and progesterone may interact to influence the mRNA expression of these genes. Furthermore, there were mRNA species that were altered between the sexes, but not influenced by E2 in men, indirectly indicating that progesterone may influence the mRNA content of these genes. To definitively elucidate the independent and interactive effects of E2 and progesterone on mRNA abundance of these genes, studies using amenorrheic women with different combinations of E2 and progesterone could be performed. Pharmacological suppression through injection of Ganirelix to suppress endogenous production of gonadotropin-releasing hormone and replacement of different combinations of E2 and progesterone in women may also be employed (D'Eon et al., 2002). We could also use ovariectomized mice or rats and supplement them with different combinations of E2, progesterone and E2 plus progesterone. After exercise, the effect of different combinations of E2 and progesterone on the mRNA expression, and protein content and activity of these genes could be evaluated.

6.6.3 Molecular mechanisms of attenuation of increase in PGC-1α mRNA expression during exercise by E2.

In this study, we found that E2 supplementation significantly attenuated the increase of PGC-1α mRNA during exercise in men. During exercise, contraction stimulated elevation of $[Ca^{2+}]$, [AMP]/[ATP] ratio and free radicals contribute to a robust increase in PGC-1α transcription (Pilegaard et al., 2005) and mRNA content (Norrbom et al., 2004; Pilegaard et al., 2005; Pilegaard, Saltin & Neufer, 2003; Watt et al., 2004). PGC-1α has been considered the "master regulator" of mitochondrial biogenesis (Wu et al., 1999). It has also been found that E2 stimulates the transcription of estrogen related receptor-α (Liu, Zhang & Teng, 2005), which, in turn, represses the transcription of PGC-1α (Ichida, Nemoto & Finkel, 2002). E2 is also an antioxidant which may attenuate exercise induced free radical production (Duckles et al., 2006) and this in turn could repress free radical induced PGC-1α transcription (Lee & Wei, 2005). To further explore the aforementioned phenomena, one could look at the effect of E2 on the affinity of transcription factors to the PGC-1α promoter. Transcription activity assays and mRNA stability assays may be used to look at if E2 influenced the transcription activity or stability of PGC-1α mRNA during contraction of electrically stimulated muscle cells. One could also use E2 blockers to block some E2 pathways and identify the putative pathway which is involved in the attenuation. For example, we can block E2 receptor pathways with Tamoxifen or Raloxifene and look at the role of E2 as antioxidant in the attenuation of the increase of PGC-1α mRNA expression during exercise.

6.6.4 Role of E2 in sex differences in muscle fiber composition in human and confirmation of the role of PPARδ in determination of sex difference in muscle fiber composition

We found that there are sex differences in muscle fiber area % and muscle fiber area. MHCI sex differential expression and PPARδ sex differential expression appear to play roles in mediating the differences. E2 increased the mRNA expression of PPARδ in men and may play a role in mediating the sex difference in muscle fiber composition in human. Estrogen has been found to influence the muscle fiber composition in animals (Kobori & Yamamuro, 1989; Piccone, Brazeau & McCormick, 2005; Sciote et al., 2001). But it has not been investigated if E2 influences the muscle composition in humans. To tackle this problem, we could supplement men with E2 for a longer-term and look at if E2 alters muscle fiber composition in men. One could also look at whether E2 influences the expression of the genes involved in muscle fiber type determination and muscle fiber terminal differentiation in addition to those measured in the current study.

Our finding of sex-based differences on the mRNA abundance of PPARδ implies that PPARδ may play a role in determination of sex differences in muscle fiber composition. This should be further functionally confirmed in transgenic mice. We could overexpress PPARδ in male mice, or knock out or knock down PPARδ in female mice. Comparison of the muscle fiber type composition between transgenic mice with control mice will provide some data to confirm the predicted role of PPARδ in determination of sex differences in muscle fiber composition.

6.6.5 Functional confirmation and regulatory relationship among the genes with exercise, sex, menstrual cycle phase and E2 influenced mRNA expression

Our observations that the mRNA content of many genes we studied was different between sexes, altered by exercise, menstrual cycle phase and E2 administration, indicate that there may be some regulatory relationship among them. The transcriptional regulatory relationship among the genes with exercise induced mRNA expression, sex differential mRNA expression, menstrual cycle phase differential expression and E2 altered expression may be experimentally elucidated through using DNA – protein binding assays. The exact transcriptional regulatory relationship among E2 and the genes with E2 influenced mRNA expression may also be elucidated through analysis of the *cis*-elements within the promoters of these genes using promoter and protein interaction assays.

Although the observed changes in the mRNA content of these genes are potent molecular mechanisms for observed sex-based differences in substrate utilization during exercise, menstrual cycle phase differences in substrate utilization and E2 mediated selective substrate utilization in men, it is not certain whether or not the changes in the mRNA content of these genes really cause sex, menstrual cycle phase and E2 mediated differences in substrate *in vivo*. Functional confirmation of the role for these genes with changes in mRNA content is necessary. For sex differences in substrate utilization, we could make transgenic mice with vectors containing cassettes of the coding sequences of the genes with the observed sex differential mRNA expression and all are driven by a sex differential promoter by measureing substrate utilization during exercise in these animals. Through introduction of vectors with different combination of the genes with sex

differential mRNA expression, the role of each gene in substrate utilization may be differentiated. In the same way, for exercise stimulated genes, gene expression cassette driven by exercise activation promoter may be used. For menstrual cycle phase differences, gene expression cassette driven by menstrual cycle phase specific promoter may be used. For E2 mediated differences, gene expression cassette driven by E2 activated promoter may be used.

6.7 Conclusions

We concluded that exercise increased the mRNA content of many genes involved in fat, glucose and protein oxidation. There were differences in mRNA content of PPARδ, LCAD, FABPc, glycogen phosphorylase, glycogenin and GLUT4 in LUT women vs. FOL women. Sex-based differences in mRNA content were observed with many genes involved in fat, CHO and protein metabolism, which support the substrate data that women oxidize more fat, less CHO and protein during exercise as compared with men. E2 influenced the mRNA content of some genes involved in fat and CHO metabolism, which provides some mechanisms for E2 mediated selective utilization of substrate during exercise in men. A higher mRNA content of MHCI and PPARδ was correlated with a higher type I fiber area percentage in women vs men, implicating that PPARδ may play a role in mediating the sex-based difference in muscle fiber area percentage. Further work needs to be performed to elucidate the molecular mechanisms for sex-based differences in substrate utilization during exercise at transcription, mRNA stability, translation, post-translation and protein activity levels. The role of the genes

with the observed sex differential mRNA expression and E2 mediated alteration need to be functionally confirmed.

BERTHON, P. M., HOWLETT, R. A., HEIGENHAUSER, G. J. & SPRIET, L. L. (1998). Human skeletal muscle carnitine palmitoyltransferase I activity determined in isolated intact mitochondria. *J Appl Physiol* **85,** 148-53.

BINNERT, C., KOISTINEN, H. A., MARTIN, G., ANDREELLI, F., EBELING, P., KOIVISTO, V. A., LAVILLE, M., AUWERX, J. & VIDAL, H. (2000). Fatty acid transport protein-1 mRNA expression in skeletal muscle and in adipose tissue in humans. *Am J Physiol Endocrinol Metab* **279,** E1072-9.

BRAUN, B. & HORTON, T. (2001). Endocrine regulation of exercise substrate utilization in women compared to men. *Exerc Sport Sci Rev* **29,** 149-54.

BROOKE, M. H. & ENGEL, W. K. (1969). The histographic analysis of human muscle biopsies with regard to fiber types. 1. Adult male and female. *Neurology* **19,** 221-33.

CALLOWAY, D. H. & KURZER, M. S. (1982). Menstrual cycle and protein requirements of women. *J Nutr* **112,** 356-66.

CAMERON-SMITH, D. (2002). Exercise and skeletal muscle gene expression. *Clin Exp Pharmacol Physiol* **29,** 209-13.

CARTER, S. L., RENNIE, C. D., HAMILTON, S. J. & TARNOPOLSKY. (2001). Changes in skeletal muscle in males and females following endurance training. *Can J Physiol Pharmacol* **79,** 386-92.

COSTILL, D. L., DANIELS, J., EVANS, W., FINK, W., KRAHENBUHL, G. & SALTIN, B. (1976). Skeletal muscle enzymes and fiber composition in male and female track athletes. *J Appl Physiol* **40,** 149-54.

COX, B., KISLINGER, T. & EMILI, A. (2005). Integrating gene and protein expression data: pattern analysis and profile mining. *Methods* **35,** 303-14.

D'EON, T. M., SHAROFF, C., CHIPKIN, S. R., GROW, D., RUBY, B. C. & BRAUN, B. (2002). Regulation of exercise carbohydrate metabolism by estrogen and progesterone in women. *Am J Physiol Endocrinol Metab* **283,** E1046-55.

DEVRIES, M. C., HAMADEH, M. J., GRAHAM, T. E. & TARNOPOLSKY, M. A. (2005). 17beta-estradiol supplementation decreases glucose rate of appearance and disappearance with no effect on glycogen utilization during moderate intensity exercise in men. *J Clin Endocrinol Metab* **90,** 6218-25.

DUCKLES, S. P., KRAUSE, D. N., STIRONE, C. & PROCACCIO, V. (2006). Estrogen and mitochondria: a new paradigm for vascular protection? *Mol Interv* **6,** 26-35.

ESBJORNSSON-LILJEDAHL, M., SUNDBERG, C. J., NORMAN, B. & JANSSON, E. (1999). Metabolic response in type I and type II muscle fibers during a 30-s cycle sprint in men and women. *J Appl Physiol* **87,** 1326-32.

ESBJORNSSON, M., SYLVEN, C., HOLM, I. & JANSSON, E. (1993). Fast twitch fibres may predict anaerobic performance in both females and males. *Int J Sports Med* **14,** 257-63.

FREYSSENET, D., CONNOR, M. K., TAKAHASHI, M. & HOOD, D. A. (1999). Cytochrome c transcriptional activation and mRNA stability during contractile activity in skeletal muscle. *Am J Physiol* **277,** E26-32.

GARREL, D. R., WELSCH, C., ARNAUD, M. J. & TOURNIAIRE, J. (1985). Relationship of the menstrual cycle and thyroid hormones to whole-body protein turnover in women. *Hum Nutr Clin Nutr* **39,** 29-37.

HACKNEY, A. C., MCCRACKEN-COMPTON, M. A. & AINSWORTH, B. (1994). Substrate responses to submaximal exercise in the midfollicular and midluteal phases of the menstrual cycle. *Int J Sport Nutr* **4,** 299-308.

HAMADEH, M. J., DEVRIES, M. C. & TARNOPOLSKY, M. A. (2005). Estrogen supplementation reduces whole body leucine and carbohydrate oxidation and increases lipid oxidation in men during endurance exercise. *J Clin Endocrinol Metab* **90,** 3592-9.

HARGREAVES, M. & CAMERON-SMITH, D. (2002). Exercise, diet, and skeletal muscle gene expression. *Med Sci Sports Exerc* **34,** 1505-8.

ICHIDA, M., NEMOTO, S. & FINKEL, T. (2002). Identification of a specific molecular repressor of the peroxisome proliferator-activated receptor gamma Coactivator-1 alpha (PGC-1alpha). *J Biol Chem* **277,** 50991-5.

KIENS, B., ROEPSTORFF, C., GLATZ, J. F., BONEN, A., SCHJERLING, P., KNUDSEN, J. & NIELSEN, J. N. (2004). Lipid-binding proteins and lipoprotein lipase activity in human skeletal muscle: influence of physical activity and gender. *J Appl Physiol* **97,** 1209-18.

KOBORI, M. & YAMAMURO, T. (1989). Effects of gonadectomy and estrogen administration on rat skeletal muscle. *Clin Orthop Relat Res,* 306-11.

KRANIOU, Y., CAMERON-SMITH, D., MISSO, M., COLLIER, G. & HARGREAVES, M. (2000). Effects of exercise on GLUT-4 and glycogenin gene expression in human skeletal muscle. *J Appl Physiol* **88,** 794-6.

KRIENGSINYOS, W., WYKES, L. J., GOONEWARDENE, L. A., BALL, R. O. & PENCHARZ, P. B. (2004). Phase of menstrual cycle affects lysine requirement in healthy women. *Am J Physiol Endocrinol Metab* **287,** E489-96.

KUBOTA, K., KOSAKA, T. & ICHIKAWA, K. (2005). Combination of two-dimensional electrophoresis and shotgun peptide sequencing in comparative proteomics. *J Chromatogr B Analyt Technol Biomed Life Sci* **815,** 3-9.

LARIVIERE, F., MOUSSALLI, R. & GARREL, D. R. (1994). Increased leucine flux and leucine oxidation during the luteal phase of the menstrual cycle in women. *Am J Physiol* **267,** E422-8.

LEE, H. C. & WEI, Y. H. (2005). Mitochondrial biogenesis and mitochondrial DNA maintenance of mammalian cells under oxidative stress. *Int J Biochem Cell Biol* **37,** 822-34.

LIU, D., ZHANG, Z. & TENG, C. T. (2005). Estrogen-related receptor-gamma and peroxisome proliferator-activated receptor-gamma coactivator-1alpha regulate estrogen-related receptor-alpha gene expression via a conserved multi-hormone response element. *J Mol Endocrinol* **34,** 473-87.

MARINOVIC, A. C., MITCH, W. E. & PRICE, S. R. (2000). Tools for evaluating ubiquitin (UbC) gene expression: characterization of the rat UbC promoter and use of an unique 3' mRNA sequence. *Biochem Biophys Res Commun* **274,** 537-41.

MARQUIS, J., PAILLARD, L., AUDIC, Y., COSSON, B., DANOS, O., LE BEC, C. & OSBORNE, H. B. (2006). CUG-BP1/CELF1 requires UGU-rich sequences for high affinity binding. *Biochem J.* **400,** 291-301.

MOLLER, S. E., MOLLER, B. M., OLESEN, M. & FJALLAND, B. (1996). Effects of oral contraceptives on plasma neutral amino acids and cholesterol during a menstrual cycle. *Eur J Clin Pharmacol* **50,** 179-84.

NORRBOM, J., SUNDBERG, C. J., AMELN, H., KRAUS, W. E., JANSSON, E. & GUSTAFSSON, T. (2004). PGC-1alpha mRNA expression is influenced by metabolic perturbation in exercising human skeletal muscle. *J Appl Physiol* **96,** 189-94.

PICCONE, C. M., BRAZEAU, G. A. & MCCORMICK, K. M. (2005). Effect of oestrogen on myofibre size and myosin expression in growing rats. *Exp Physiol* **90,** 87-93.

PILEGAARD, H., ORDWAY, G. A., SALTIN, B. & NEUFER, P. D. (2000). Transcriptional regulation of gene expression in human skeletal muscle during recovery from exercise. *Am J Physiol Endocrinol Metab* **279,** E806-14.

PILEGAARD, H., OSADA, T., ANDERSEN, L. T., HELGE, J. W., SALTIN, B. & NEUFER, P. D. (2005). Substrate availability and transcriptional regulation of metabolic genes in human skeletal muscle during recovery from exercise. *Metabolism* **54,** 1048-55.

PILEGAARD, H., SALTIN, B. & NEUFER, P. D. (2003). Exercise induces transient transcriptional activation of the PGC-1alpha gene in human skeletal muscle. *J Physiol* **546,** 851-8.

ROEPSTORFF, C., SCHJERLING, P., VISTISEN, B., MADSEN, M., STEFFENSEN, C. H., RIDER, M. H. & KIENS, B. (2005). Regulation of oxidative enzyme activity and eukaryotic elongation factor 2 in human skeletal muscle: influence of gender and exercise. *Acta Physiol Scand* **184,** 215-24.

SCIOTE, J. J., HORTON, M. J., ZYMAN, Y. & PASCOE, G. (2001). Differential effects of diminished oestrogen and androgen levels on development of skeletal muscle fibres in hypogonadal mice. *Acta Physiol Scand* **172,** 179-87.

SHEARER, J., GRAHAM, T. E., BATTRAM, D. S., ROBINSON, D. L., RICHTER, E. A., WILSON, R. J. & BAKOVIC, M. (2005a). Glycogenin activity and mRNA expression in response to volitional exhaustion in human skeletal muscle. *J Appl Physiol* **99,** 957-62.

SHEARER, J., WILSON, R. J., BATTRAM, D. S., RICHTER, E. A., ROBINSON, D. L., BAKOVIC, M. & GRAHAM, T. E. (2005b). Increases in glycogenin and glycogenin mRNA accompany glycogen resynthesis in human skeletal muscle. *Am J Physiol Endocrinol Metab* **289,** E508-14.

SIMONEAU, J. A. & BOUCHARD, C. (1989). Human variation in skeletal muscle fiber-type proportion and enzyme activities. *Am J Physiol* **257,** E567-72.

SIMONEAU, J. A., LORTIE, G., BOULAY, M. R., THIBAULT, M. C., THERIAULT, G. & BOUCHARD, C. (1985). Skeletal muscle histochemical and biochemical characteristics in sedentary male and female subjects. *Can J Physiol Pharmacol* **63,** 30-5.

SOUTHERN, M. M., BROWN, P. E. & HALL, A. (2006). Luciferases as reporter genes. *Methods Mol Biol* **323,** 293-305.

STARON, R. S., HAGERMAN, F. C., HIKIDA, R. S., MURRAY, T. F., HOSTLER, D. P., CRILL, M. T., RAGG, K. E. & TOMA, K. (2000). Fiber type composition of the vastus lateralis muscle of young men and women. *J Histochem Cytochem* **48,** 623-9.

TSCHRITTER, O., MACHICAO, F., STEFAN, N., SCHAFER, S., WEIGERT, C., STAIGER, H., SPIETH, C., HARING, H. U. & FRITSCHE, A. (2006). A new variant in the human Kv1.3 gene is associated with low insulin sensitivity and impaired glucose tolerance. *J Clin Endocrinol Metab* **91,** 654-8.

WATT, M. J., SOUTHGATE, R. J., HOLMES, A. G. & FEBBRAIO, M. A. (2004). Suppression of plasma free fatty acids upregulates peroxisome proliferator-activated receptor

(PPAR) alpha and delta and PPAR coactivator 1alpha in human skeletal muscle, but not lipid regulatory genes. *J Mol Endocrinol* **33,** 533-44.

WELLS, D. G. (2006). RNA-binding proteins: a lesson in repression. *J Neurosci* **26,** 7135-8.

WENZ, M., BEREND, J. Z., LYNCH, N. A., CHAPPELL, S. & HACKNEY, A. C. (1997). Substrate oxidation at rest and during exercise: effects of menstrual cycle phase and diet composition. *J Physiol Pharmacol* **48,** 851-60.

WU, Z., PUIGSERVER, P., ANDERSSON, U., ZHANG, C., ADELMANT, G., MOOTHA, V., TROY, A., CINTI, S., LOWELL, B., SCARPULLA, R. C. & SPIEGELMAN, B. M. (1999). Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. *Cell* **98,** 115-24.

YASUDA, N., GLOVER, E. I., PHILLIPS, S. M., ISFORT, R. J. & TARNOPOLSKY, M. A. (2005). Sex-based differences in skeletal muscle function and morphology with short-term limb immobilization. *J Appl Physiol* **99,** 1085-92.

ZDERIC, T. W., COGGAN, A. R. & RUBY, B. C. (2001). Glucose kinetics and substrate oxidation during exercise in the follicular and luteal phases. *J Appl Physiol* **90,** 447-53.

ZHU, H., BILGIN, M., BANGHAM, R., HALL, D., CASAMAYOR, A., BERTONE, P., LAN, N., JANSEN, R., BIDLINGMAIER, S., HOUFEK, T., MITCHELL, T., MILLER, P., DEAN, R. A., GERSTEIN, M. & SNYDER, M. (2001). Global analysis of protein activities using proteome chips. *Science* **293,** 2101-5.

ZHU, H., BILGIN, M. & SNYDER, M. (2003). Proteomics. *Annu Rev Biochem* **72,** 783-812.

Appendex