SEX DIFFERENCES IN LIPID METABOLISM

MOLECULAR MECHANISM(S) OF SEX DIFFERENCES IN LIPID METABOLISM IN HUMAN SKELETAL MUSCLE

By

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ABSTRACT

It is well understood that compared with men, women are better able to withstand starvation, have better ultra-endurance capacity, oxidize more fat during endurance exercise, and are more resistant to fat oxidation defects i.e. diet-induced insulin resistance. However, the mechanism(s) for the observed sex differences are unknown. It was my **hypothesis** that women have greater fat oxidation capacity in skeletal muscle than men.

The **objectives** of my thesis were to determine the mechanism(s) by which women oxidize more lipids; including the role of estrogen as a possible regulator. The most **significant findings** were that: 1) mRNA for fatty acid oxidation genes are higher in women compared with men, which was confirmed by Stringent Affymetrix GeneChip array analysis, combined with RT-PCR (chapter 2); 2) long-chain acyl-CoA dehydrogenase in human skeletal muscle is not quantifiable despite the majority (90%) of fatty acids oxidized during exercise are long-chain fatty acids (chapter 3); 3) β-oxidation enzymes: tri-functional protein alpha, very long chain acyl-CoA dehydrogenase, and medium chain acyl-CoA dehydrogenase are significantly higher in women compared with men (chapter 4); 4) Acute (8 days) 17β-estradiol supplementation in men significantly increased protein content of β-oxidation enzymes in skeletal muscle, possibly through the regulation of PGC-1α and microRNA (chapter 5).

In conclusion, my data provided novel insights into the enhanced ability of women to oxidize fat under periods of metabolic stress by showing that: 1) women are transcriptionally (mRNA) "primed" for known physiological differences in metabolism; 2) women have more protein content of the major enzymes involved in long and medium chain fatty acid oxidation; 3) E2 partially regulates lipid metabolism in skeletal muscle by pre-translational modifications of factors involved in β-oxidation. These findings contribute to the molecular understanding of sex differences in substrate utilization.

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ABBREVIATIONS

ACAA2- acetyl-Coenzyme A acyltransferase 2
ACBP- Acyl-CoA binding protein
ACS- Acyl-CoA synthetase
ADP - adenosine diphosphate
AMDR- acceptable macronutrient distribution range
AMP - adenosine monophosphate
AMPK - AMP-activated protein kinase
AST - asparatate aminotransferase
ATGL- adipose triglyceride lipase
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
BMI - body mass index
CAT- carnitine acyltransferase
cDNA - complementary deoxyribonucleic acid
CHO - carbohydrate
CPTI - carntine palmitoyltransferase I
CPTII - carnitine palmityltransferase II
DNA - deoxyribonucleic acid
E2 - 17β- estradiol
ERα - estrogen receptor alpha
ERβ - estrogen receptor beta
ERγ - estrogen receptor gamma
ETC - electron transport chain
FABPc - cytosol fatty acid binding protein
FABPpm - plasm membrane fatty acid binding protein
FAT/CD36 - Fatty acid translocase
FATm - membrane bound fatty acid transport protein 1
FATP- fatty acid transport protein
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
HADHA- hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase/enoyl-Coenzyme A hydratase (trifunctional protein), alpha subunit
HADHB- hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase/enoyl-Coenzyme A hydratase (trifunctional protein), beta subunit

HKII - hexokinase II
HSL - hormone sensitive lipase
IMCL - intramyocellular lipid
IMM- inner mitochondrial membrane
IP3- inositol triphosphate
LCAD - long chain acyl-CoA dehydrogenase
LCFA- long-chain fatty acid
LP- lipoprotein
LPL - lipoprotein lipase
LUT - mid-luteal phase
MCAD- medium chain acyl-CoA dehydrogenase
MHC - myosin heavy chain
MHCI - myosin heavy chain I
MHCIIa - myosin heavy chain IIa
MHCIIx - myosin heavy chain IIx
miRNA- micro ribonucleic acid
mtGPAT - mitochondrial glycerol phosphate acyltransferase
OCT- optimal cutting temperature compound
OMM- outer mitochondrial membrane
OVX – ovarectomized
PANK- pantothenate kinase
PDK1 - pyruvate dehydrogenase kinase 1
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
RDA- recommended dietary allowance
RER - respiratory exchange ratio
RNA - ribonucleic acid
RNase - ribonucleic acid hydrolyase
rRNA - ribosomal ribonucleic acid
RT-PCR - Reverse transcriptase-polymerase chain reaction
SCAD- short-chain acyl-CoA dehydrogenase
SCHAD- short-chain 3-hydroxyacyl-CoA dehydrogenase
SEM- standard error of the mean
SD – standard deviation

SREBP-1c - sterol regulatory element-binding protein 1c
SREBP-2 - sterol regulatory element-binding protein 2
TCA cycle - tricarboxylic acid cycle
TFPα - trifunctional protein alpha subunit
TFPβ- trifunctional protein beta subunit
TG - triglyceride
UCP3 - uncoupling protein 3
VLCAD - very long chain acyl - CoA dehydrogenase
LCAD - long chain acyl - CoA dehydrogenase
VO_2max - maxium volume of oxygen consumption

STATEMENT OF CONTRIBUTIONS

Manuscript 1:
The principal investigator of this study was Mark Tarnopolsky.
Study conception and design was done by Mark Tarnopolsky, Minghua Fu, and Amy Maher. The original subjects were recruited and coordinated by Mazen Hamadeh, Nobuo Yasuda and Elisa Glover, and muscle biopsies were done by Mark Tarnopolsky. RNA extractions, RT-PCR primer design and real-time RT-PCR were performed by Minghua Fu. Microarray experiement was performed, and results were collected, by Proctor and Gamble. Western blot analyses were performed by Amy Maher. Fiber typing was performed by Nobo Yasuda. Interpretation and organization of microarray data, statistical analysis and interpretation RT-PCR, Western blot, and fiber type data were performed by Amy Maher. The entire manuscript was written and prepared by Amy Maher, and edited by Mark Tarnopolsky.

Manuscript 2:
The principal investigator of this study was Mark A Tarnopolsky. Study conception and design was done by Amy Maher and Mark A Tarnopolsky, and the ethics proposal was written by Amy Maher, and edited by Mark Tarnopolsky. The subjects were recruited and coordinated by Amy Maher. The data collection was coordinated by Amy Maher.
The planning/design of the specific genes (RNA and protein) to be analyzed was determined by Amy Maher and Mark Tarnopolsky. Muscle homogenization was coordinated by Amy Maher. Western blotting, and RNA extraction was done by Amy Maher. RT-PCR primer design was done by Amy Maher and Mahmood Akhtar and real-time RT-PCR were performed by Mahmood Akhtar. RT-PCR data collection, statistical analysis and interpretation were performed by Amy Maher. The entire manuscript was written and prepared by Amy Maher, and edited by Mark Tarnopolsky and Mahmood Akhtar.

Manuscript 3: Entirely as above.
Human mitochondrial preparations were done by Amy Maher.
Mouse muscle and mitochondrial preparations were done by Adeel Safdar.

Manuscript 4: Entirely as above.
Plus, all microRNA assays were run and analized by Amy Maher.

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

Chapter 1

Literature Review

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1.0 Introduction

It had been assumed for many years that men and women responded similarly to metabolic stress; however, accumulating evidence supports that sex (gender) plays a role in metabolism. Metabolically, women demonstrate higher relative lipid oxidation and lower amino acid and carbohydrate oxidation at submaximal exercise intensities as compared to men (23, 28, 29, 38, 59, 78, 106, 125, 137, 140).

Changes in metabolism are likely genetically regulated either by predetermined expression of genes or by the regulation of gene expression through cell signaling mechanisms, for example sex hormones (35, 158). Metabolically, the sex hormones appear to have a significant effect on sex and aging related differences in substrate utilization. Estrogen appears to favor lipid oxidation as observed when men and male rats are supplemented with 17β-estradiol (E2)(29, 34). In addition, ovariectomized female rodents demonstrated higher carbohydrate oxidation and lower lipid oxidation that is reversed with estrogen supplementation (68).

Recent advances in modern techniques such as proteomics and gene expression analysis will likely be useful in helping us understand the molecular basis for sex differences in metabolism, and the influence of sex hormones. The implications of sex differences upon substrate metabolism and the impact of this upon recommendations for; nutrition, health care and treatment, drug development, optimal performance and training, etc., are only just beginning to be elucidated and are an interesting practical area for research.

1.2 Muscle Related Differences between Sexes

Skeletal muscle is the most abundant tissue in the human body (63) and one of the main sites for energy substrate utilization of carbohydrates, lipids, and amino acids, especially during exercise. Before discussing differences in muscle metabolism between sexes it is important to review the physiological differences in muscle fiber type between men and women which might ultimately play a role in metabolism, keeping in mind that muscle size and function can be altered by exercise, nutrition, hormones, and aging; and all these factors are correlated with muscle metabolism.

Muscle is composed of three types of muscle fibers; Type I, IIA, and IIX. In terms of sex differences in fiber type, men have a significantly larger type I fiber area in the biceps brachii, and larger type II fiber area in the vastus lateralis then women (24, 98). Women have a greater type I fiber percent area then men in the vastus lateralis (24). Similar results were found in rats where the cross sectional area of type II fibers of the soleus and tibialis were greater in males, and the cross-sectional area of type I fibers was greater in females; however there were no significant differences in the percentage of each individual fiber (37). Metabolically, a higher proportion of type I fibers has been correlated with higher fat oxidation rates. Type I fibers have a greater expression of fatty acid transport

protein, FAT/CD36 (149), and women express almost 50% more FAT/CD36 than men (70).

These findings suggest that sex-related differences exist in muscle fiber type and it is important to consider that this may influence metabolism and adaptive responses to exercise. The role of estrogen in muscle remains an intriguing potential mechanism underlying the observed sex differences. More research is necessary to decipher these potential sex differences and how they affect muscle metabolism.

1.3 Sex Differences in Whole Body Substrate Metabolism during Endurance Exercise

The primary energy source for substrate during exercise is fat and carbohydrate; however, amino acids do contribute a small amount of energy (75). There are sex differences in energy substrate selection with women relying primarily upon lipid oxidation (62%), with men only utilizing approximately 43% lipid during sub-maximal endurance exercise (75, 78, 106, 138). A large scale comparison of 30 published studies were RER for men and women were reported shows that women have a significantly lower (0.87± 0.04) RER compared with men (0.90 ± 0.04) $P \leq 0.01$ (Table 1). As expected, the greater reliance upon lipid oxidation in women during endurance exercise results in a lower proportionate carbohydrate oxidation.

In order to compare between two groups of individuals (i.e., sex), there are several factors that must be controlled for that are known to alter substrate oxidation rate. For example, training status, habitual and pre-exercise dietary intake status are important factors when examining exercise and metabolism. One of the best ways to compare between the sexes is to match men and women for training history, and given the higher percent body fat content for females, they should be compared using VO_{2peak} expressed relative to fat-free mass (144). In addition, with sex comparison studies, the menstrual cycle is known to alter substrate oxidation and must be specified in any study (16, 104). Sex comparison studies should also test men and women at the same time (i.e., during the same experimental period and not with historical data) to control for variation in metabolic carts, different calibration gas supply, and different staff involved in the subject testing.

Irrespective of sex comparisons, key factors that are important in order to accurately measure whole body carbohydrate (and lipid) oxidation are that subjects must be in a steady state, and exercising below the anaerobic threshold. For example, the anaerobic threshold in untrained men and women may be as low as 66 % of $VO_{2peak,}$ (111), and in the moderate to well trained athlete this may be as high as 80 % of VO_{2peak} (106), with no sex difference. Sex comparisons of substrate oxidation rates exceeding the lactate treshold would not yield accurate or valid results.

Table 1. Summary of Studies Where Whole Body Substrate Metabolism was Reported in Men and Women

Reference (mean)	Subjects	Exercise	RER
Costill et al., 1979	12 F, T	60 min run @	F = 0.83
(26)	12 M, T	70% VO_{2max}	M = 0.84
Froberg & Pederson, 1984	7 F, T	to exhaustion @	F = 0.93*
(40)	7 M, T	80+90% VO_{2max}	M = 0.97*
Blatchford, et al., 1985	6 F, T	90 min walk	F = 0.81
(10)	6 M, T	@ 35% VO_{2max}	M = 0.85
Tarnopolsky, et al., 1990	6 F, T	15.5 km run @	F = 0.876
(137)	6 M, T	~ 65% VO_{2max}	M = 0.940
Phillips, et al., 1993	6 F, T	90 min cycle @	F = 0.820
(106)	6 M, T	65% VO_{2max}	M = 0.853
Tarnopolsky, et al., 1995	8 F, T	60 min cycle @	F = 0.892
(140)	7 M, T	75% VO_{2max}	M = 0.923
Tarnopolsky, et al., 1997	8 F, T	90 min cycle @	F = 0.893
(141)	8 M, T	65% VO_{2max}	M = 0.918
Horton, et al., 1998	13 F, T +UT	120 min cycle @	F = 0.84
(59)	14 M, T +UT	45% VO_{2max}	M = 0.86
Friedlander, et al., 1998	17 F, UT→T	60 min cycle @	F = 0.885*
(38)	19 M, UT→T	45 & 65% VO_{2max}	M = 0.932*
Romijn, et al., 2000	8 F, T	20-30 min cycle @	F = 0.81
(121)	5 M, T	65 % VO_{2max}	M = 0.81
McKenzie, et al., 2000	6 F, UT→T	90 min cycle @	F = 0.889
(94)	6 M, UT→T	65% VO_{2max}	M = 0.914
Davis, et al, 2000	8 F, UT	90 min cycle @	F = 0.92
(28)	8 M, UT	50 % VO_{2max}	M = 0.92
Goedecke, et al, 2000	16 F, T	10 min @ 25,50,	F = 0.90*
(46)	45 M, T	and 75 % VO_{2max}	M = 0.92*
Rennie, et al., 2000§	6 F, UT→T	90 min cycle @	F = 0.893
	5, M UT→T	60 % VO_{2max}	M = 0.945
Carter, et al., 2001	8 F, UT→T	90 min cycle @	F = 0.847
(23)	8 M, UT→T	60 % VO_{2max}	M = 0.900
Lamont, et al., 2001	7 F, T + UT	60 min cycle @	F = 0.808
(78)	7 M, T + UT	50 % VO_{2max}	M = 0.868
Roepstorff, et al., 2002	7 F, T	90 min cycle @	F = 0.886
(119)	7 M, T	58 % VO_{2max}	M = 0.905
Melanson, et al., 2002	8 F, T	400 kcal @ 40 +	F = 0.87*
(95)	8 M,T	70 % VO_{2max}	M = 0.91*
Mittendorfer, et al., 2002	5 F, UT	90 min cycle@	F=0.87
(99)	5 M, UT	50 % VO_{2max}	M=0.87
Steffensen, et al., 2002	21 F, T+UT	90 min cycle @	F=0.877

(136)	21 M, T+UT	60 % VO_{2max}	M=0.893
Riddell, et al., 2003	7 F, T	90 min cycle @	F=0.93
(115)	7M, T	60 % VO_{2max}	M=0.93
Lamont, et al., 2003	4 F, UT	60 min cycle @	F=0.82
(79)	4 M, UT	50 % VO_{2max}	M=0.83
M'Kaouar, et al., 2004	6 F, MT	120 min cycle @	F=0.93
(90)	6 M, MT	65% VO_{2max}	M=0.92
Devries, et al., 2005	13 F, MT	90 min cycle @	F=0.91
(30)	10 M, MT	65 % VO_{2max}	M=0.94
Zehnder, et al., 2005	9 F, T	180 min cycle@	F=0.86
(163)	9 M, T	50 % VO_{2max}	M=0.88
Horton, et al., 2006	10 F, MT	90 min cycle@	F=0.865
(58)	11 M, MT	57% VO_{2max}	M=0.880
Roepstorff, et al., 2006	9 F, MT	90 min cycle @	F=0.85
(120)	8 M, MT	60% VO_{2max}	M=0.89
Wallis, et al., 2006	8 F, MT	120 min cycle @	F=0.82
(151)	8 M, MT	67% VO_{2max}	M=0.85
Pillard et al., 2007	9 F, UT	30 min cycle@ 30,50,70%	F=0.85*
(108)	9M, UT	VO_{2max}	M=0.88*
Kang et al., 2009	21 F, MT	20 min cycle @	F=0.89
(66)	11 M, MT	50% VO_{2max}	M=0.91
Mean	n=279 F		F = 0.869 (0.04)
	n=293 M		M = 0.895 (0.04)†

Values are mean (SD). F = females; M = males; T = trained; U = untrained; U→T = longitudinal training study: for longitudinal training studies, the pre/post rides are all collapsed across time for each sex. T+U = trained and untrained in same study. §= master's thesis. * - The RER was a combination of those at both exercise intensities. † - Significant sex difference ($P \leq 0.01$, 2 tailed independent t-test).

1.3.1 Sex Differences in Carbohydrate Metabolism

Carbohydrates (CHO) are a rapidly available source of energy. The Acceptable Macronutrient Distribution Range (AMDR) suggests that 45-65% of the diet should be carbohydrates (164). Carbohydrates circulate in the blood as plasma glucose and are stored in the muscle and liver in the form of glycogen. Glycogen and to a much smaller extent, plasma glucose, provide a rapid source of energy to the cells during exercise.

For years it was assumed that men and women respond similarly to exercise (26); however, the majority of cross-sectional studies summarized in a large scale meta-analysis have found that whole body carbohydrate oxidation rates are lower for women compared to men during endurance exercise at sub-maximal exercise intensities (Table 1)(144, 148). Furthermore, before and after

two or three months of prescribed and controlled exercise training, both men and women show the same sex differences in substrate oxidation (23, 38, 59, 94).

Recent studies examining glucose kinetics in men and women during exercise and post-exercise have demonstrated that the rate of glucose appearance (Ra) and disappearance (Rd) was lower in women than men (30, 58, 119). In order to elucidate the possible biological factors that may be regulating these differences, upstream substrates have been examined including; epinephrine, glucagon, and sex hormones. Interestingly, during exercise the change in epinephrine concentrations are significantly attenuated in women as compared with men (14, 58, 126, 137). Studies have found that the adipocytes from women show higher sensitivity to epinephrine as compared with men (64, 100, 112). The latter results imply that there are sex differences in adrenergic receptor density or post-receptor regulation, at least within the adipocytes.

The data regarding a potential role for glucagon in the regulation of exercise metabolism have not been conclusive. Glucagon plays a role in the release of glucose from the liver to maintain blood glucose levels. Horton et al. (2006) observed that the absolute resting value of glucagon was higher in women than men (58). In contrast, Tarnopolsky et al. (1990) observed that there was no difference in resting glucagon levels between men and women (137). Both studies reported that men had a greater reduction in glucagon during exercise, suggesting that glucagon does not affect glucose levels equally based on sex. Further investigation is needed to establish the role of hormonal factors on substrate metabolism in men and women.

Sex hormones such as estrogen and progesterone show a strong correlation with the observed sex based differences in substrate metabolism. Women in the luteal phase of their menstrual cycle have a significantly lower glucose Ra and glucose Rd at 90 min of exercise (30, 161). Women in the luteal phase also show a reduction in glucose metabolic clearance rates as compared with women in the follicular phase and women in both follicular and luteal states still had a lower glucose Ra, glucose Rd, and metabolic clearance rates as compared with men (30). Furthermore, women in the luteal phase of their menstrual cycle had lower proglycogen, macroglycogen, and total glycogen utilization during exercise compared to women in the follicular phase (30). Although there are changes in Ra and Rd during different phases of the menstrual cycle, the significance is unknown as there seems to be no significant change in whole body substrate metabolism. These data supports the possibility that sex hormones regulate some aspects of carbohydrate metabolism between men and women. The specific effects of estrogen supplementation on substrate metabolism will be discussed later.

Further studies in our lab examined mRNA differences in key genes involved in CHO metabolism (GLUT4, HK II, PFK, glycogenin, GS-1, GSK3α, glycogen phosphorylase) (Table 2)(139). No coordinate or directional differences between sexes in the mRNA abundance of most of the measured genes involved in CHO were supportive of lower CHO oxidation. Suggesting that CHO

utilization in skeletal muscle is not directly regulating the observed differences in whole body substrate oxidation.

Table 2. Summary of the fold difference in mRNA abundance for metabolism related genes.

Gene	Sex Women vs Men	Menstrual Phase FOL vs LUT	Estradiol E2 vs PL	Exercise Post vs Pre
Transcriptional Regulators				
PGC1α	NS	NS	NS	7.9±1.8 PL 3.6±0.8 L
PPARα	1.4±0.1 F	NS	1.4±0.1	NS
PPARδ	2.3±0.1 F	2.2±0.1	2.1±0.2	NS
PPARγ	NS	NS	NS	NS
Fat Metabolism				
VLCAD	NS	NS	NS	NS
TFPα	2.4±0.3 L	NS	1.5±0.1	NS
FATm	4.6±0.5 F 2.9±0.2 L	NS	NS	NS
FABPc	1.7±0.1 F 1.7±0.1 L	NS	NS	1.3±0.1 ALL
CPT1	1.7±0.1 F	NS	1.5±0.1	1.2±0.05 PL 1.5±0.1 L
CPT2	NS	NS	NS	NS
SREBP1c	3.1±0.4 F 4.1±0.7 L	NS	3.0±0.3	NS
SREBP2	NS	NS	NS	NS
mtGPAT	2.0±0.1 F 1.7±0.1 L	NS	1.4±0.1	NS
HSL	NS	NS	NS	1.4±0.2 PL 1.2±0.2 E2
Carbohydrate Metabolism				
GLUT4	2.4±0.3 F	NS	3.9±0.5	2.1±0.2 PL 2.0±0.2 L
HKII	2.9±0.3 F	NS	NS	1.9±0.2 ALL
GP	NS	2.2±0.2	NS	NS
PFK	-3.7±0.0 L	NS	NS	NS
Glycogenin	NS	-1.2±0.1	NS	NS
GS1	NS	NS	1.3±0.1	NS
GSK3α	NS	NS	NS	NS
Amino Acid Metabolism				
AST	NS	NS	NS	1.2±0.1 PL 1.3±0.1 F
BCOAD	NS	NS	NS	NS
BCOADK	1.3±0.1 F 1.2±0.1 L	NS	NS	NS

Data presented as fold difference ± SE. $P<0.05$. NS, not significant; F and FOL, follicular; L and LUT, luteal; PL, placebo; E2, estradiol; All, all groups and conditions showed change. (Fu et. al., Phys Gen. Oct 2009)

1.3.2 Sex Differences in Protein Metabolism

There are 20 different types of amino acids that compose proteins and nine cannot be synthesized by the body and must be supplied through dietary sources, these are considered essential/indispensible. Amino acids can serve as an energy source; however, fats and carbohydrates are the preferred energy sources. In order for proteins to be used as a source of energy they must be broken down into constituent amino acids which can be used as fuel in muscle in several ways (91, 110, 133). The minimal recommended dietary allowance (RDA) for protein intake in men and women over the age of 19 y is 0.80 g/kg/day (reviewed in (105, 164)). Protein requirements are determined based on the minimum necessary supply of indispensable amino acids and the body's ability to maintain nitrogen balance (164). Current dietary recommendations do not make allowances for a possible influence of exercise on amino acid and protein requirements.

The evidence for whole body carbohydrate oxidation being higher for men as compared with women, during endurance exercise, also predicts that amino acid oxidation would be higher in men. Whole body experiments using urea excretion demonstrated that overall amino acid oxidation was less for women versus men (137). Initial studies by our group found that men had higher urinary nitrogen excretion consequent to endurance exercise as compared with women (137), suggesting that amino acid oxidation was higher during endurance exercise for men as compared to women. Further research using stable ^{13}C-leucine isotope methodology in training matched men and women, demonstrated that leucine oxidation was lower for women as compared to men during endurance exercise (72, 78, 79, 94, 106). Interestingly, one study found that women had a lower rate of leucine, but not lysine, oxidation during endurance exercise, as compared with men (78), showing that the observed sex differences in oxidation may be amino acid specific.

Studies have also examined the effect of menstrual cycle phase on amino acid kinetics. Using ^{13}C-leucine tracers Lariviere et al. (1994) demonstrated that women had a higher leucine oxidation during the luteal compared with follicular phase (80), and Lamont et al. (1987) found that urinary urea nitrogen excretion was higher during the luteal compared to follicular phase of the menstrual cycle (76). These findings suggest that sex differences in protein regulation could be related to factors such as estrogen and progesterone levels.

There are many possible metabolic regulators including; substrate availability, regulatory enzyme activity, catecholamine responsiveness, and sex steroid hormones. Substrate availability definitely plays a role in metabolic regulation of protein oxidation (41); however, most well designed sex comparison studies have ensured that men and women were on controlled diets and received comparable protein based on g/kg. In order to examine the potential mechanism(s) behind this apparent sex difference in leucine oxidation, McKenzie et al. (2000) (94) measured the active form of branched chain 2-oxo-acid dehydrogenase (BCOAD) in skeletal muscle of six men and six women before and after a 31 day endurance exercise training program. BCOAD is the rate-

limiting enzyme for muscle branched-chain amino acid oxidation. They found identical BCOAD total activity levels and the acute exercise induced percent activation before and after endurance training (although the basal activation was lower in women) (94). The lack of a sex difference in the acute exercise induced BCOAD activation suggested that some of the attenuation of amino acid oxidation in women may be occurring at the hepatic level. This latter finding was also in keeping with an attenuation of hepatic glycogen utilization during exercise for the women.

Sex differences could also be a result of variation in catecholamine responsiveness (77, 79). When the catecholamine receptors (β1- and β2-adrenergic receptors) are blocked pharmacologically with propranalol, men had a further up-regulation of leucine oxidation with no observed changes in women (77, 79). In light of these initial studies, further research is needed to determine the metabolic regulation of sex-related differences in amino acid utilization.

1.3.3 Sex Differences in Lipid Metabolism

Fats become proportionately more important as a fuel source with prolonged endurance exercise. One gram of fat yields over twice the energy of carbohydrate (110, 133). The AMDR recommends that 20-35% of an adults dietary intake should be fat (164). Fats are predominantly stored as triglycerides in adipocytes; however, fatty acids can also be stored in skeletal muscle as intramyocellular lipids (IMCL's)(101). IMCL's are situated in the sarcoplasma in direct contact with mitochondria, the location of fat oxidation (57), serving as a direct energy source during exercise. IMCL's are highest in oxidative type I muscle fibers (61). Trained athletes have a higher IMCL content compared with sedentary people and IMCL content is lower following prolonged sub-maximal exercise (135). Interestingly, one study found that IMCL utilization was apparent in skeletal muscle of women but not men (136). High dietary fat also increases IMCL content and plays a role in insulin sensitivity in non-athletes (48).

Similar to the sex differences in carbohydrate metabolism, there are sex differences in whole body fat oxidation observed by the lower RER in women (Table 1). The higher oxidation of lipids in women is observed during endurance based exercise. Interestingly, women have higher IMCL's as compared to men (31, 136, 142). The source of the higher lipid use for the women during endurance exercise is likely to be a greater use of IMCL (136), and to a lesser extent, blood born free fatty acids (24, 38). High fat diets in men have been shown to increase fat oxidation and whole body lipolysis by increasing the IMCL, irrespective of changes in plasma free fatty acids (162). Using glycerol tracers, several studies have found that women had a higher lipolytic rate as compared with men during endurance exercise (23, 38, 99). The higher muscle LPL activity for women may drive free fatty acids from blood derived chylomicrons and very low density lipoproteins towards a higher IMCL storage in women with a consequently higher oxidation rate. Trained women runners on a moderate-fat diet restored baseline IMCL content in 22 hours (81), yet it took trained male

cyclists 48 hours to replace IMCL content (147). Interestingly a moderate to higher fat diet (35-68% of energy as fat) can restore IMCL content in athletes within 48 hours, but most athletes only consume ~24% of energy as fat which is not enough to restore IMCL levels within 48 hours (81, 134, 147). It would be of interest to determine if there is a sex difference in the responsiveness to high fat diets between men and women. One would predict that perhaps women may benefit from a high fat recovery diet, whereas men may not. Further studies need to be done to elucidate the effect of high fat diets on exercise performance since some studies demonstrate a positive outcome (74, 124), and others no change (45, 107).

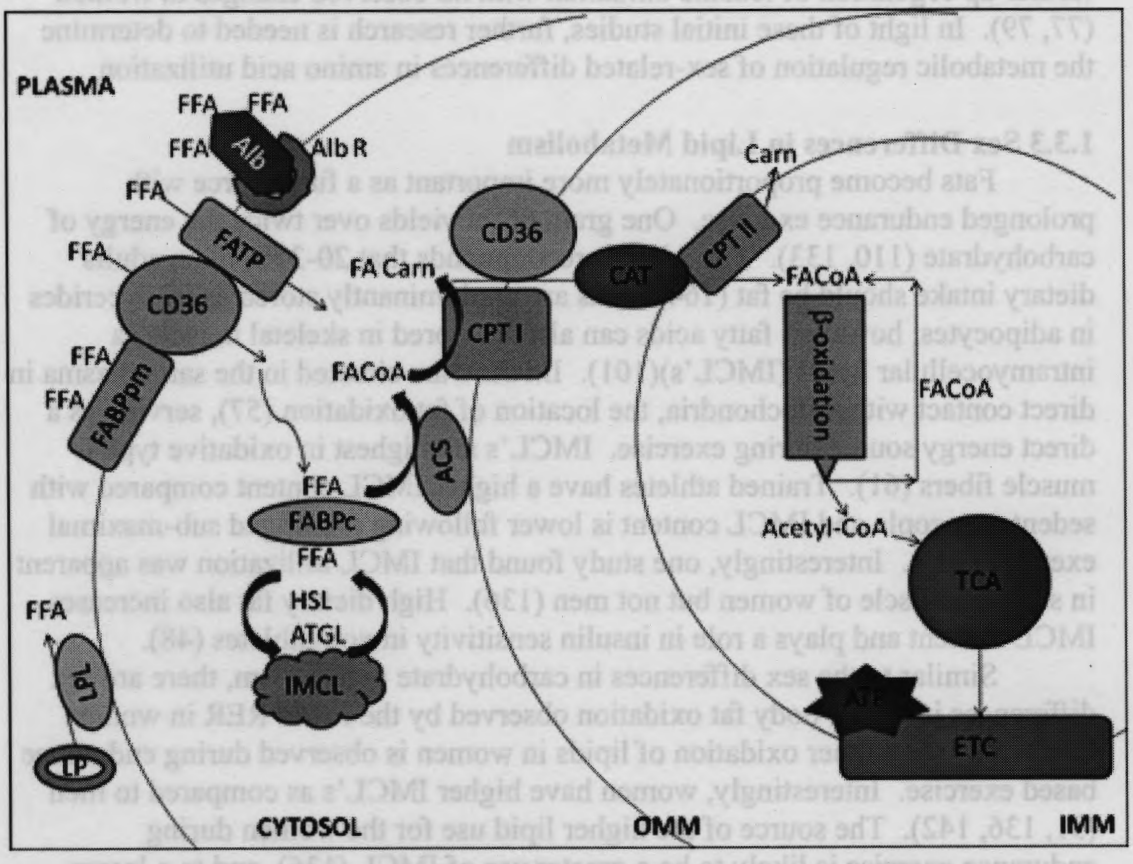

Figure 1. Schematic representation of fatty acid metabolism in skeletal muscle. Alb, albumin; FATP, fatty acid transport protein; FABP, fatty acid binding protein; LP, lipoprotein; LPL, lipoprotein lipase; FFA, free fatty acid; IMCL, intramyocellular lipid; ATGL, adipose triglyceride lipase; HSL, hormone sensitive lipase; ACS, Acyl-CoA synthetase; CPT, carnitine palmitoyltransferase; CAT, carnitine acyltransferase; TCA, tricarboxylic acid cycle; ETC, electron transport chain; OMM, outer mitochondrial membrane; IMM, inner mitochondrial membrane.

1.3.3.1 Long-chain Fatty Acid Up-take and Oxidation in Skeletal Muscle

Fatty acids (FAs), specifically long-chain fatty acids (LCFAs), are an important contributor to ATP production in skeletal muscle both at rest and during exercise. For example, during exercise Fas contribute anywhere from 30-70% of substrate utilized, depending on exercise intensity and duration (39). Approximately 90% of Fas that contribute to energy production during exercise are derived from LCFAs (53). The percentage of the predominant LCFA to the composition of plasma FFAs are ~43% oleate, ~30% palmitate and ~14% stearate (52). This ratio is consistent at rest and does not change significantly during exercise (52) despite the large change in flux. During steady state exercise adipose triglycerides and plasma FFA accounts for approximately 50%, the plasma triglycerides 10%, and intramyocellular lipid (IMCL) 40% of total FA oxidation (121).

Until recently, pathways regarding LCFA oxidation have remained largely unexplored. It is becoming more apparent that the LCFA oxidation pathway has many levels of regulation that are getting increasingly important in understanding lipid metabolism.

Levels of regulation for LCFA oxidation in skeletal muscle (figure 1);

1) Plasma delivery and transport of LCFAs across the plasma membrane for skeletal muscle use.

Skeletal muscle utilizes FFAs released from adipose tissue stores, chylomicrons, and lipoproteins. Lipoproteins and chylomicrons in the plasma can be hydrolyzed by lipoprotein lipase (LPL) into FFA for transport across the membrane (reviewed in (47)). Short-chain and medium-chain FAs can diffuse across the membrane; however LCFAs require protein-mediated transport across the membrane (reviewed in (11).

There are three types of FA transport proteins; fatty acid binding protein located on the interstitial side of the plasma membrane (FABPpm), fatty acid translocase (FAT/CD36), and a family of six fatty acid transport proteins (FATP1-6). FAT/CD36 is highly glycosylated and has at least two transmembrane domains, and the FATP family of transporters has at least six transmembrane domains. The FATP family remains largely unexplored, except FATP1 and FATP4 have been shown to play a role in LCFA transport and oxidation rates in skeletal muscle (32, 71, 103). FABPpm, FAT/CD36 and FAT4 expression is correlated with the rate of LCFA transport and oxidation (103). Expression of these transporters on the plasma membrane is to some degree transient. For example, FAT/CD36, FATP1 and FABPpm are translocated to the plasma membrane in the presence of insulin or contraction stimulation (86, 87, 159) and content is reduced following muscle denervation (73). Lastly, chronic muscle stimulation can elevate the protein expression of FAT/CD36 and FABPpm (73). Taken together, skeletal muscle can tightly regulate LCFA oxidation at the level of cellular uptake in response to physiological demand.

2) Transport of LCFAs through the cytosol and addition of a CoA thioester.

Once LCFAs cross the plasma membrane they are either bound by fatty acid binding protein in the cytosol (FABPc) or the acyl-CoA moieties are bound by acyl-CoA binding protein (ACBP). FABPc is highly expressed in skeletal muscle and only a small portion of the pool is thought to be bound to LCFA at a time, and necessary for optimal oxidation (12, 88). The exact mechanism(s) by which bound LCFA are chaperoned is unknown; however, LCFA are chaperoned to the site of activation by acyl-CoA synthetase (ACS) (42). ACS uses energy to couple LCFA to CoASH yielding acyl-CoA. Once, LCFAs are activated by ACS they can be esterified into IMCL or delivered to the mitochondria for oxidation (reviewed in (60)).

3) Utilization of IMCL stores.

In healthy skeletal muscle, intramyocellular lipids (IMCL) are a low cellular constituent (4-10 mmol kg^{-1} wet weight); however, IMCL can contain more than 50% of the energy stored in glycogen, and the close proximity of IMCL to the mitochondria suggests that IMCLs are important in metabolism((142) and reviewed in (56)). The lipolysis of IMCL occurs through the enzymatic activation of hormone sensitive lipase (HSL), diacylglycerol (DAG) and monoacylglycerol (MAG) lipase, and adipose triacylglyceride lipase (ATGL). Once LCFA are released from IMCL stores they are bound by cytsolic proteins FABPc or ACBP, same as LCFA transported from the plasma, and processed/metabolized in a similar fashion (56).

4) Transport into the mitochondria.

Due to the polarity of fatty acyl-CoAs they cannot passively cross the mitochondrial membrane; consequently, carnitine palmitoyltransferase-I (CPT I) trans-esterifies fatty acyl-CoA to fatty acyl-carnitine, a neutral intermediate prior to translocation (as reviewed in (93)). Fatty acyl-carnitine can then be transported into the mitochondrial by carnitine acyltransferase (CAT). Fatty acyl-carnitine gets converted back into fatty acyl-CoA by CPT II on the inner mitochondrial membrane and can enter the β-oxidation pathway.

Until recently, the CPT pathway was thought to be the sole mediator of FA uptake into the mitochondria. It is now known that FAT/CD36 (6) and FABPpm (4) are also located on the mitochondrial membrane in human skeletal muscle. Although the mitochondrial population of FABPpm does not seem to directly regulate LCFA oxidation (55), pharmacological manipulation of the mitochondrial population of FAT/CD36 *in vitro* does (3). Furthermore, FAT/CD36 co-immunoprecipitates with CPT1 (18, 128), and multiple regression analysis of FAT/CD36 and CPT1 as predictors, significantly correlated with palmitate oxidation, where independently they did not (6). The exact mechanism(s) and consequence of multiple LCFA transports on the mitochondrial membrane remains an intriguing area for future research.

5) β-oxidation and the reduction of various co-factors to produce ATP.

The primary site of lipid oxidation in skeletal muscle is the mitochondria through a process called β-oxidation (figure 2). There are four major classes of enzymes in the β-oxidation pathway; acyl-CoA dehydrogenases, enoyl-CoA hydrateses, 3-hydroxyacyl-CoA dehydrogenases, and 3-ketoacyl-CoA thiolases (152). Very long-chain acyl-CoA dehydrogenases (VLCAD), long-chain acyl-CoA dehydrogenases (LCAD), medium-chain acyl-CoA dehydrogenases (MCAD), and short-chain acyl-CoA dehydrogenases (SCAD) are four of the nine most commonly identified acyl-CoA dehydrogenases. Trifunctional protein is a heterooctamer composed of four α- and four β-subunits. The alpha subunit, from the HADHA gene (160), also commonly referred to as long-chain hydroxyacyl-CoA dehydrogenase (LCHAD), has enzyme activity to hydrolyze long-chain enoyl-CoA and oxidize long-chain 3-hydroxyacyl-CoAs of 10 carbon chain-length and greater (146). The beta subunit, from the HADHB gene, catalyzes the 3-ketoacyl-CoA thiolase activity. Another member of the 3-hydroxyacyl-CoA dehydrogenase gene family is SCHAD, which catalyzes the oxidation of medium-chain-length fatty acids, specifically short-chain 3-hydroxyacyl-CoAs. Lastly, acetyl-Coenzyme A acyltransferase 2 (ACAA2) (also called ketothiolase) catalyzes the last step of the mitochondrial fatty acid beta-oxidation spiral.

Each passage through the β-oxidation pathway removes two carbons and yields an acetyl-CoA. LCFA can continually cycle through the β–oxidation pathway until they are completely reduced. Acetyl-CoA enters the tricarboxylic acid cycle (TCA) where reducing equivalents ($NADH + H^+$ and $FADH_2$) are formed and transferred to the electron transport chain (ETC) to produce ATP.

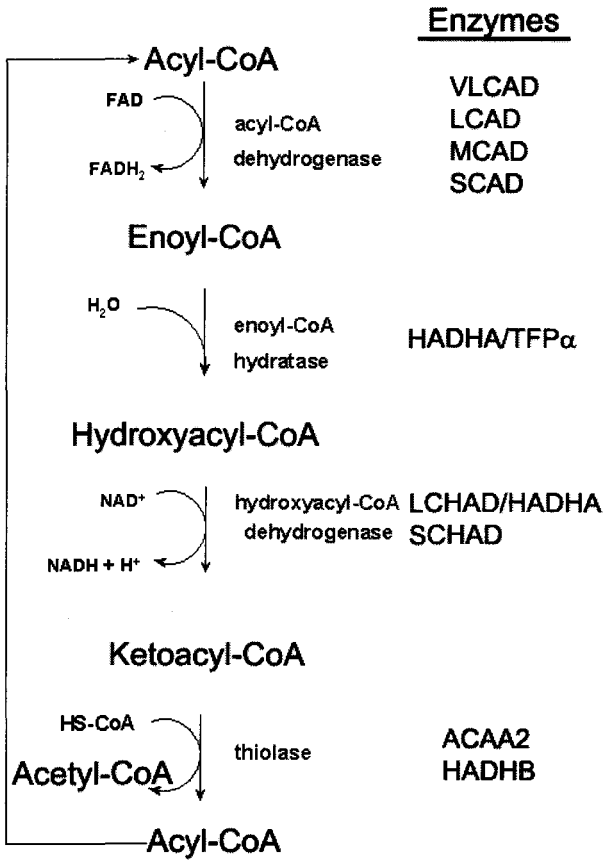

Figure 2. Schematic representation of the β-oxidaiton pathway and the enzymes responsible for the removal a 2-carbon Acetyl-CoA. VLCAD, very long-chain acyl-CoA dehydrogenases; LCAD, long-chain acyl-CoA dehydrogenases; MCAD, medium-chain acyl-CoA dehydrogenases; SCAD, short-chain acyl-CoA dehydrogenases; HADHA, trifunctional protein alpha; LCHAD; long-chain hydroxyacyl-CoA dehydrogenase; HADHB, trifunctional protein beta; SCHAD, straight-chain 3-hydroxyacyl-CoA; ACAA2, acetyl-Coenzyme A acyltransferase 2.

1.3.2.2 Sex Differences in the Regulation of LCFA Oxidation

Recent research has shown that women compared to age and fitness matched men have higher mRNA content for LPL (69), membrane fatty acid transport protein 1 (FATm) (9), FAT/CD36 transporter and plasma membrane fatty acid binding protein (FABPpm) (69), citrate synthase (118), β-hydroxyacyl-CoA dehydrogenase (β-HAD) (118), and hormone sensitive lipase (117). Furthermore, recent studies in our lab comparing men and women have shown that women have a higher mRNA expression of genes involved in intramyocellular lipid synthesis (SREBP-1c and mtGPAT) and hydrolysis (HSL), sarcolemmal and mitochondrial FFA transport (FATm, FABPc, CPTI), β-oxidation (TFP-α), and transcriptional regulation (PPARα and PPARδ) (Table 2)(139). Sex differences in mRNA content, and limited protein expression data, appear to be directionally consistent with the observed metabolic differences present during exercise, implying that fat oxidation is regulated and CHO and protein oxidation follows based on metabolic demand.

Although the above data support increased transport of FFA into skeletal muscle and higher capacity for lipid synthesis and hydrolysis, there are no sex differences in lipid oxidation via CPT1 activity (5). Similarly, there is no sex differences in muscle enzyme activity of 3-beta-hydroxyacyl CoA dehydrogenase, citrate synthase, succinate-cytochrome c oxidoreductase, and cytochrome c oxidase (24). It is unknown if there are sex differences in mitochondrial FAT/CD36 that might contribute to greater mitochondrial FA uptake and thus greater lipid oxidation. Furthermore, sex differences in mitochondrial β-oxidation enzymes (example; VLCAD, LCAD, MCAD, TFP) have not been explored.

1.4 Sex Chromosomes and their Role in Regulation of Metabolic Genes

During human embryological development the deciding factor for genetic sex determination is the presence or absence of a Y-chromosome; XX female or XY male. With any sex related comparison, the genetic basis for sex needs to be considered. Both men and women express a compliment of genes off one X-chromosome. The second X-chromosome in women is inactive as a result of X-chromosome inactivation (43, 89). The male-specific region of the Y chromosome, the MSY, differentiates the sexes and comprises 95% of the chromosome's length (~58 Mb). There are 156 known transcription units on the Y-chromosome, which include 78 protein-coding genes that collectively encode 27 distinct proteins (131). None of the protein coding genes on the Y-chromosome encode for genes involved in metabolism.

The human X-chromosome was sequenced by Ross et al, 2005. It was determined that there are a total of 1098 genes on the X-chromosome; 699 known genes, 132 novel coding sequences, 166 novel transcripts and 101 putative transcripts (123). There are also 700 pseudogenes of which 644 are processed and 56 are not. While both men and women express this compliment of genes, some genes on the second X-chromosome in women escape inactivation and get expressed from both X-chromosomes (15, 19, 20), potentially leading to sexual

dimorphic traits. Research by Carrel and Willard (2005), in human fibroblast cells, indicated that ~15% of X-linked genes escape inactivation, and an additional 10-20% show variable patterns of inactivation. Carrel and Willard (2005) were able to test 634 genes found on the X inactivated chromosome (21), which resulted in 190 genes of known homology that escape inactivation.

As an initial screen, I have reviewed the gene list and results published by Carrel and Willard (2005) to determine if X-inactivation in women may account for the observed sex differences in substrate selection. This screen revealed that there are approximately 21 metabolic and mitochondrial related genes (Appendix Table 1); however, none of the genes were considered to be likely candidates to explain the sex differences in CHO and lipid utilization. Furthermore, it is unknown if differences in X-inactivation are tissue specific. Comparison of tissue specific X-inactivated gene lists with gene array results and other known metabolic pathways may shed more light on the potential influence of X-inactivation.

1.5 The Effects of Sex Hormones on Substrate Metabolism during Endurance Exercise

1.5.1 Estrogen and Sex Differences

Estrogen is the primary sex hormone in women and is well characterized in the regulation of reproduction. Estrogen is also present in lower levels in men and post-menopausal women (130). Estrogens belong to a group of steroid compounds produced by the enzymatic alteration of androgens, specifically testosterone produces estradiol (130). Estrogen regulates many physiological functions of the musculoskeletal (132), gastrointestinal, immune (155), neural (92), and cardiovascular systems (96). In ovulating women, estradiol is highest in the late follicular phase of the menstrual cycle, specifically the week prior ovulation (130). Studies using ovariectomized rodents or oral administration of 17β-estradiol to rodents and humans have shown that estrogen has a major influence upon carbohydrate metabolism at the skeletal and hepatic level (22, 67, 122, 126). Although several of the earlier studies that examined the effects of 17β-estradiol on substrate metabolism used the hormone at a supra-physiological dose (34, 51, 67), the same metabolic effects were seen with physiologically relevant doses (29, 68).

Initial studies examining the effects of an ovariectomy on muscle metabolism in female rats revealed that female rats showed male-like metabolic pattern with an increase in glycogen utilization and lower lipid utilization in both skeletal muscle and heart (51, 68). These effects could be reverted back to the normal female-like metabolic pattern of higher lipid and lesser glycogen utilization by supplementing the rats with 17β-estradiol (51, 68). In rodents, the administration of 17β-estradiol attenuated glycogen degradation in the liver during exercise (67, 122). Results have shown when male or ovariectomized female rats are supplemented with 17β-estrdiol, and exercised, there is a sparing

of muscle and liver glycogen, and an increase in free fatty acid use (67), leading to an overall improvement in exercise performance. 17β-estradiol also influences lipid storage in mice by increasing intra-muscular triglyceride content in both heart and skeletal muscle (34).

In humans, women demonstrate a lower glycogen utilization rate during endurance exercise (23, 30, 125). Some studies have found that there does not appear to be a sex difference in basal muscle glycogen content, and women do not show differences in glycogen content at either phase of the menstrual cycle (62, 94, 141, 143). However, during endurance exercise women use less muscle glycogen compared to men (137), and have a significantly lower proglycogen, macroglycogen, and total glycogen utilization in the luteal versus follicular phase (30). Interestingly, administration of 17β-estradiol in men reduced the basal level of total muscle glycogen at rest and after exercise (29). Administration of 17β-estradiol in both men and women attenuated hepatic glucose production during endurance exercise (22, 126). Tarnopolsky et al. (2001) (143) found that administration of 17β-estradiol to men increased their plasma 17β-estradiol concentration to mid-follicular levels without effecting muscle glycogen breakdown during exercise; however, men given 17β–estradiol had a lower RER which reflected a reduced reliance upon carbohydrate (CHO) substrate utilization and an increase in lipid metabolism, similar to what was observed in women (29). Studies have found that glucose rate of appearance and disappearance (38), and glucose metabolic clearance rate (22), were lower for exercising women as compared with men. Interestingly, men given 17β-estradiol had lower proglycogen, total glycogen, hepatic glucose production and glucose uptake suggesting whole body glycogen sparing (29). These findings suggested that 17β-estradiol reduced hepatic glucose production during exercise.

Estrogen also seems to be acting on lipid metabolism as men supplemented with 17β–estradiol had a lower RER which reflected an increase in lipid metabolism (29, 50). Mechanistically, Ellis et al. (1994)(34) demonstrated that administration of 17β-estradiol increased LPL activity in skeletal muscle, and decreased it in adipocytes, suggesting that estrogen might play a role in the preferred storage of lipids in the skeletal muscle for immediate availability in oxidation. Recent studies have shown that women have 160 % higher mRNA for LPL then men, but there was no observed sex differences in LPL activity (70). There are also sex differences in the expression of lipid binding proteins. Fatty acid translocase (FAT/CD36) protein is approximately 50 % higher in women compared with men (70). One study has demonstrated that women have twice the amount of plasma membrane fatty acid transporter (FATP-1) mRNA compared to men (9); however more research is needed to determine whether or not this finding translates into functional significance. There appears to be no significant sex differences in carnitine palmitoyl transferase-1 (CPT-1) or β-3-OH-acyl-CoA-dehydrogenase activity in humans; however, 17-β-estradiol supplementation in ovariectomized rats lead to higher maximal enzyme activities for carnitine

palmitoyl transferase-1 (CPT-1) and β-3-OH-acyl-CoA-dehydrogenase (17). Lastly, recent studies showed 17β-estradiol administration significantly increased the mRNA content of PGC-1α, PPARδ, TFPα, CPT1, SREBP-1c, mtGPAT, GLUT4, GS1 and AST (table 2)(139). Menstrual cycle had a small affect on PPARδ, GP and glycogenin mRNA content (table 2)(139). Taken together, women have greater mRNA content for several genes involved in lipid metabolism as opposed to CHO, which is partially due to an effect of 17β-estradiol.

Estrogen elicits its effects by binding estrogen receptors (ER) -α and -β, which are known transcription factors for the regulation of genes. ERα and ERβ mRNA and protein have been documented in skeletal muscle of humans (85, 153), rats (83) and mice.(2) A recent study by Wiik et al. (2005) (154) found that ERα and ERβ expression was higher in endurance trained men compared to moderately active men. However, Lemoine et al. (2002) (83) found that exercising female rats for seven weeks increased ERα mRNA expression with no significant change in males. The change in muscle ER expression due to training seems to be muscle type specific (84). In rats, ER expression is higher in slow twitch oxidative muscle than in fast twitch oxidative-glycolytic and glycolytic muscle (84). Differences in ER expression in muscle fiber type, and increased expression during exercise, suggests that ER's are involved in muscle adaptation to exercise, most likely act at the level of gene regulation.

Murine studies are helping to lead the way in understanding the mechanism and other physiological related outcomes of the effects of estrogen based sex differences. Estrogen has been shown to modulate insulin sensitivity in women (44), possibly by altering insulin related gene expression (102, 150). The overexpression of GLUT4 in a transgenic murine model resulted in an increase in the percent of glucose disposal through glycolysis in male animals and an increase in that directed towards glycogen storage in female animals (145). Interestingly, estrogen receptor-α knockout mice exhibit insulin resistance (54). In a study by Barros et al. (2006) (2) estrogen receptor-α was shown to be a positive regulator, and estrogen receptor-β a negative regulator, of GLUT-4 expression. More studies are needed to determine the exact signaling pathway for estrogen related GLUT-4 expression but estrogen has been shown to regulate inositol triphosphate (IP3) signaling (129), and IP3 is downstream of PI3K, a signaling molecule that has been shown to play a role in GLUT-4 translocation to the sarcolemma (65).

A transgenic peripheral peroxisome activating receptor knockout (PPARα$^{-/-}$) murine model demonstrated that most of the male PPARα$^{-/-}$ mice died with severe hypoglycemia when an inhibitor of CPT activity (etomoxir) was given, yet the majority of female mice survived (33). Furthermore, males administered 17β-estradiol avoided the fatal effects of CPT inhibition (33). This study demonstrates the inter-relatedness of glucose and lipid oxidation and the relationships to 17β-estradiol. Ovariectomized rodent models typically become obese which can be prevented by administering 17β-estradiol (114). Further investigation into the molecular mechanism of 17β-estradiol in ovariectomized mice showed that

lipogenic genes were down-regulated in adipocytes, liver and skeletal muscle (27). 17β-estradiol also up-regulated the expression of PPARδ, and activated AMP-activated protein kinase in mice, suggesting that estrogen promotes the partitioning of free fatty acids toward oxidation (27).

In summary, 17β-estradiol appears to be involved in sex related differences in the use of glucose/glycogen and lipid oxidation during endurance exercise. The exact mechanism by which 17β-estradiol is eliciting these differences has yet to be determined; however, there may be differences in the regulation of genes and/or protein involved in fatty acid transport and triglyceride hydrolysis (muscle LPL) mediated at the molecular level. Although 17β-estradiol offers protection from fatal hypoglycemia in a CPT inhibitor transgenic model of human fatty acid oxidation defects, a significant sex difference in the CPT system and enzymes involved in β-oxidation is apparent only at the mRNA level in human studies. Together, the aforementioned data may imply that the sex differences in metabolism occur at a locus distal to the CPT pathway and implicate some component of β-oxidation. Current gene array analysis techniques will hopefully help elucidate the role of estrogen in metabolic fuel selection.

1.5.2 Sex Differences due to Testosterone

The primary sex hormone in men is testosterone. Men have approximately 10 times higher testosterone concentration as compared with women. Testosterone is a steroid based hormone that promotes secondary sex characteristics in men, including increased muscle mass. Testosterone unequivocally has a stimulatory effect on protein synthesis, resulting in an increase in fat-free mass (7, 49). Similarly, gains in strength and muscle size have been observed with the exogenous administration of testosterone (8). Research has shown that testosterone administration increased the fractional rate of mixed muscle protein synthesis with no change in fractional protein breakdown rate (36, 49). Furthermore, the protein synthetic stimulation effect was not mediated by an increase in amino acid transport, but rather was due to an increase in the reutilization of intracellular amino acids (36). Despite these changes in strength and muscle size, reduction and/or elevation of testosterone in men does not alter substrate metabolism (13). Braun et al. (2005) (13) evaluated three levels of testosterone (low (~0.8 ng/ml), normal (~5.5 ng/ml), and high (~11 ng/ml)) in men and found that there were no significant differences in carbohydrate oxidation, glucose Rd, plasma glucose, or plasma free fatty acids at any dose vs saline. In conclusion, testosterone does not appear to be a candidate in the regulation of sex observed differences in substrate utilization during endurance exercise.

1. 6 MicroRNA as a Possible Regulating Factor in Post-Transcriptional Gene Expression

MiRNA are small 19-20 nt single-stranded RNA's that regulate gene expression at the post transcriptional level, usually by repression (113). There are

thought to be over 500 micro-RNA (miRNA or miR) species in the human genome (157). MiRNA genes can exist singly or can be co-transcribed poly-cistronicly with the target mRNA(s) (25). MiRNA are transcribed from both introns and more commonly exons of protein coding genes by RNA polymerase II (82). Large precursor RNA (pre-miRNA) gets cleaved in the nucleus and exported to the cytoplasm where it joins the RNA-induced silencing complex (RISC)-like complex (Argonaute proteins) and one strand is eliminated and the remaining strand can base pair with specific sequence of target mRNA, inhibiting mRNA translation (157). MiRNA are predominantly located in the cytoplasm but have been found within nucleoli of skeletal muscle (109).
MiRNA have been shown to be involved in the regulation of proliferation and differentiation of skeletal muscle. Specifically, MiR-1 promotes myogenesis by regulating HDAC4, and MiR-133a, represses SRF to regulate proliferation and myogenesis in cluture (25). There has been multiple miRNA hypothesized to be involved in the regulation of metabolic genes. MiR-23b is predicted to target mRNA for PGC1α, and in one study in mice lower expression of miR-23 was associated with higher expression of PGC-1α mRNA and protein along with several downstream targets of PGC-1α including ALAS, CS, and cytochrome *c* mRNA (127). MiR-24 is predicted to target many mRNA in the lipid metabolism pathway including PGC-1α (156). MiR-29b is one of a few published miRNA's that have been found to regulate a metabolic pathways (97). Specifically, in HEK 293 cells, miR-29b regulates branch chain α-ketoacid dehydrogenase (BCKD) complex which is involved in the metabolism of amino acids. MiR-29b is also predicted to regulate PGC-1α, PPARδ, and pyruvate dehydrogenase kinase 1 (PDK1) (156). Bioinformatics and literature reviews suggest that miR -103, and -107 paralogs may regulate multiple mRNA in metabolic pathways (156). Both miR-103/7 sequences are found within introns of the gene that encodes for pantothenate kinase (PANK) (156). Specifically, miR-103 is associated with PANK2/3, and miR-107 with PANK1 (1). PANK enzymes are the rate limiting step in generating Co-enzyme A (CoA) (116). The role of miRNA in metabolism and sex based differences remains unexplored but could be an interesting and important area of genetic and proteomic regulation.

1.7 Purpose of the Thesis

The purpose of the study was to determine the cellular differences, in human skeletal muscle, of men and women, which might contribute to the observed differences in whole body substrate metabolism. Primarily, we aimed to evaluate the effect of sex on the mRNA and protein expression of many of the genes involved in substrate metabolism to provide a potential mechanism(s) for the sex difference in utilization of substrate during exercise. Furthermore, given the E2 mediated shift in whole body utilization of substrate in men after supplementation, we wanted to determine if 17β-estradiol is involved in regulating protein expression of lipid metabolism genes in skeletal muscle.

1.8 Hypothesis

We hypothesized that sex differences in substrate utilization are due to gene and protein expression differences in skeletal muscle. We hypothesized that the differences would be in lipid metabolism related genes. We hypothesized that the molecular differences are due in part to estrogen related signaling of metabolic genes, protein expression and function.

1.9 Thesis Objectives

1) To identify sex differences in gene expression patterns related to metabolic function and signaling using unbiased microarray techniques.
2) To determine if sex related differences in gene expression patterns translate into functional differences in the expression of mRNA and protein.
3) To investigate the effect of sex on the mRNA and protein content of the genes involved in the determination of muscle fiber type composition. Specifically the genes involved in muscle fiber type terminal differentiation (MHCI, MHCIIa, MHCIIx), regulation of muscle development (myostatin), and muscle fiber type determination (PGC-1α, PPARδ).
4) To determine if sex differences in lipid utilization are due to differences in mitochondrial oxidative capacity, specifically focusing on key enzymes involved in β-oxidation and the genes that play a role in mitochondrial biogenesis and transcriptional regulation of lipid genes.
5) To determine if E2 is a mechanism governing the expression of metabolic genes in skeletal muscle by giving exogenous E2 to men, altering whole body substrate metabolism to physiologically mimic that observed in women, and ascertaining the differences in mRNA and protein expression of lipid related genes.

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Chapter 2

Manuscript 1

Published

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Sex differences in global mRNA content of human skeletal muscle

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Abstract

Women oxidize more fat as compared to men during endurance exercise and several groups have shown that the mRNA content of selected genes related to fat oxidation are higher in women (e.g. hormone sensitive lipase, β-hydroxyacyl-CoA dehydrogenase, CD36). One of the possible mechanisms is that women tend to 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 and protein content for genes involved in metabolism and the determination of muscle fiber type. Muscle biopsies from the *vastus lateralis* were collected from healthy men and women. We examined mRNA content globally using Affymetrix GeneChips, and selected genes were examined and/or confirmed by RT-PCR. Furthermore, we examined protein content by Western blot analysis. Stringent gene array analysis revealed 66 differentially expressed genes representing metabolism, mitochondrial function, transport, protein biosynthesis, cell proliferation, signal transduction pathways, transcription and translation. Stringent gene array analysis and RT-PCR confirmed that mRNA for; acyl-coenzyme A acyltransferase 2 (ACAA2), trifunctional protein β (HADHB), catalase, lipoprotein lipase (LPL), and uncoupling protein-2 (UCP-2) were higher in women. Targeted gene analysis revealed that myosin heavy chain I (MHCI), peroxisome proliferator-activated receptor (PPAR)δ were higher in women compared with men. Surprisingly, there were no significant sex based differences in protein content for HADHB, ACAA2, catalase, PPARδ, and MHC1. In conclusion, the differences in the basal mRNA content in resting skeletal muscle suggest that men and women are transcriptionally "primed" for known physiological differences in metabolism however the mechanism behind sex differences in fiber type remains to be determined.

Key Words: metabolism, skeletal muscle, adults, muscle biopsy

Introduction

Skeletal muscle is the most abundant tissue in the human body [1], and there are major differences between women and men in energy metabolism, fiber type composition, and contractile speed [2,3,4]; however, the mechanisms behind these differences are unknown. A number of groups have reported differences in exercise related substrate metabolism between men and women, specifically that women oxidize more lipid and less carbohydrate than men [5,6,7,8,9,10,11]. Similar studies in rats have also found sex differences in lipid metabolism, specifically that female rats have greater lipid oxidation and muscle lipoprotein lipase (LPL) activity, as compared with male rats [12,13]. Furthermore, when female rats are oophorectomized, lipid oxidation is lower during endurance exercise [14]. The exact mechanisms behind such observations are unclear; however, sex differences in mRNA content and protein expression appear to be directionally consistent with the observed metabolic differences present during exercise. Specifically, women compared to age and fitness matched men have higher mRNA content for LPL [15], membrane fatty acid transport protein 1 (FATm) [16], FAT/CD36 transporter and plasma membrane fatty acid binding protein (FABPpm) [15], citrate synthase [17], β-hydroxyacyl-CoA dehydrogenase (β-HAD) [17], and hormone sensitive lipase [11]. On the whole body level, women show greater lipolysis [5,18], and greater uptake of plasma free fatty acids [18], and use more intramuscular triacylglycerol [11,19,20] than men.

Futhermore, a sex difference in muscle fiber composition has been found in different skeletal muscles, such as, *erector spinae* [21], *internal and external intercostal, latissimus dorsi* [22], *biceps brachii* [23], *vastus medialis* [24], *and vastus lateralis* [5,25,26,27,28,29,30]. Although studies concerning sex differences in skeletal muscle fiber type have shown inconsistent results [4,25,26,28,29,31,32], women generally have a greater relative type I fiber area [4,5,26,27,30], a smaller relative area of type II fibers and a greater percentage area of type I fibers [4,5,26,27,28,32]. The potential molecular mechanisms behind these sex differences have not been evaluated for the mRNA species of the genes involved in muscle development, or fiber-type determination and differentiation.

The molecular mechanisms for the observed differences in substrate oxidation and fiber type between men and women are unknown. In this study we used a targeted approach (RT-PCR) to evaluate mRNA species of the genes involved in muscle development, fiber-type determination and differentiation, and a microarray comparison to evaluate the many more potential mRNA species that are required for lipid metabolism and fiber type in human skeletal muscle, which may yield important data for understanding potential novel mechanisms. Gene array technology has provided a rapid and efficient way to screen a large number of mRNAs in order to identify potential targets and pathways for further research. Recently, several groups have used gene arrays to identify novel transcriptional programs related to human muscle repair, inflammation, protein synthesis and cellular control in skeletal muscle after various interventions such as exercise

[33,34,35], immobilization [36], and drug treatments [37]. A few groups have done targeted gene expression profiles to examine sex differences in humans [38,39] and mice [40]. In this study, skeletal muscle biopsies from healthy, young men and women were analyzed for mRNA abundance of over 23,000 genes by Affymetrix gene array analysis with an *a priori* hypothesis that mRNA species involved in lipid oxidation, muscle development, and fiber-type determination and differentiation would be different in women compared with men. Furthermore, we hypothesized that this global analysis would identify novel mRNA targets that are relevant to biological pathways that differ in skeletal muscle between men and women.

Material and Methods

Participants

These studies were approved by the Human Research Ethics Board of McMaster University. The present study used muscle samples from two different studies that recruited subjects using identical criteria and subject characteristics were not significantly different (Table 1). All subjects were between the ages of 18 and 35, healthy, recreationally active, and non-smokers. Highly trained athletes were excluded from the studies. All female participants were eumenorrheic. The present study only compared muscle samples collected at baseline (before manipulation) thus termed "resting muscle" from the two studies. In *study 1*, 14 women and 13 men volunteered to participate but we only used samples from 12 women and 12 men. Subject characteristics have been previously published [30] but can be viewed in Table 1. In *study 2*, 24 healthy, recreationally active men (n=11) and women (n=13) participated [41]. Data from these subjects regarding diet, CHO, protein and fat oxidation has been previously reported [41], and demonstrated that women had higher fat oxidation and lower protein and carbohydrate oxidation during endurance exercise as compared with men. The muscle samples collected pre-exercise were used for the present study and only samples from women in the follicular phase of their menstrual cycle were used as there was little difference in mRNA content of metabolic related genes between follicular and luteal phase women (Figure S2) compared to differences due to sex (Figure S1) (unpublished data). *Study 1* samples were used to acquire the gene array data, histochemical data is a combination of a representative subset of subjects from both studies, and RNA and protein data was acquired from *study 2* samples. A comparison of subject characteristics is reported in Table 1. All study participants gave written consent to participate in the study.

Acquisition of muscle samples

Muscle samples were obtained from the *vastus lateralis* muscle, ~ 15 cm proximal to the lateral knee joint line, using a custom suction-modified Bergstrom needle. Biopsies were taken at rest and all subjects refrained from any exercise

for at least 3 days before the muscle biopsy. All biopsies were completed in the morning. Approximately 60 mg of muscle tissue was divided and snap-frozen in liquid nitrogen and stored in a -80°C freezer for RNA and protein analysis.

Histochemical analysis

Histochemical analyses were conducted on samples from *study 1and 2*, as described by Yasuda *et al.* [30]. 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% $CaCl_2$ and 2% $CoCl_2$ for 3 min, and then incubated in 1% ammonium sulphide for 30 s 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 (μm^2) were determined using an image analysis program (Image Pro, V6.0, Media Cybernetics Inc., Silver Spring, MD).

Microarray Preparation

Muscle samples from *study 1* were used to extract RNA, and the RNA was prepared according to Affymetrix recommendations (Santa Clara, CA). In brief, muscle samples were homogenized in TRIzol Reagent (Life Technologies, Rockville, MD) by shaking in a mixer mill with tungsten carbide beads as recommended by the manufacturer (Qiagen, Life Technologies, Rockville, MD). RNA was purified using an RNeasy Mini Kit (Qiagen, Chatsworth, CA). Ten micrograms of purified RNA was reverse transcribed using SuperScript II Reverse Transcriptase and T7-$(dT)_{24}$ primer followed by second strand DNA synthesis as per the manufacturer's instructions (Life Technologies, Rockville, MD). Contaminants were removed from the samples by phenol-chloroform-isoamyl alcohol extraction, and the clean cDNA was recovered by ethanol precipitation, using a RNA Transcript Labeling Kit (Enzo Diagnostics, Farmingdale, NY), it was further converted into biotin-labeled cRNA, as per manufacturer's instructions. Moreover, cRNA was purified using a RNeasy Mini Kit (Quiagen, Life Technologies, Rockville, MD) and fragmented in pieces <200 bases by incubation in fragmentation buffer. Samples were stored at -20°C until hybridization.

Samples were hybridized on human HG-U133 Plus 2.0 Arrays using protocols as recommended by Affymetrix (Affymetrix, Santa Clara, CA). In brief, 24 gene chips were used to compare 12 women to 12 men. Biotinylated cRNA was hybridized for 16 h at 45°C in a GeneChip Hybridization Oven 640.

Arrays were placed in a GeneChip Fluidic Station 400 for a series of washes, followed by incubation with streptavidin-conjugated phycoerythrin. Finally, arrays were scanned with a GeneArray Scanner (Agilent, Palo Alta, CA) and analyzed using GeneChip Analysis software (Affymetrix, Santa Clara, CA).

Statistical Analysis of MicroArray

The statistical analysis was based on the Affymetrix signal (MAS 5.0 algorithm). Exploratory statistical tools were used to check data quality. There were no quality control problems with the data. The data was then filtered based on the given algorithms for gene content levels, which filtered out genes that have low content levels compared to background (Affymetrix Absent Calls). When a minimum of 19 out of 24 gene replicates read as Affymetrix Absent Calls, the gene is filtered out. An ANOVA model with log (base 2) of the Affymetrix signal as a response is fitted for each one of the genes that are not filtered based on Affymetrix Absent Calls. Significant differential content is calculated by the NLOGP ($-\log_{10}$(P-value)). A gene was considered to be differentially expressed if the NLOGP measure was greater than the 4, and the fold change was at least 1.2. Table S1. Proctor and Gamble use these criteria for their Affymetrix gene array analyses [42]. The negative log of the *p* value (NLOGP) was calculated from ($-\log_{10}$ (NLOGP 4 = *p* value threshold of 0.0001), where the *p* value is a summary measure of the statistical significance for the corresponding comparison.

Gene annotation and functional analysis

Gene function and characterization information was obtained from the Affymetrix website: http://www.affymetrix.com/analysis/index.affx. Further description was obtained from various databases at the National Center for Biotechnology Information. iPath™ (Invitrogen) was used to compared differentially regulated genes against 225 signaling and metabolic human biological pathway maps created for Invitrogen™ by GeneGo. (http://escience.invitrogen.com/ipath). Differentially regulated gene were also run against a Connectivity Map, which is a gene expression database with statistical scoring algorithm to link drug treatment, genes, and diseases.

All gene array data can be accessed via Gene Expression Omnibus (GEO) http://www.ncbi.nlm.nih.gov/geo/. Accession #GSE14901. The data used for this study were the PRECAST values (Table S1) only from a larger study "Limb immobilization induces a coordinate down-regulation of mitochondrial and other metabolic pathways in men and women".

RNA isolation for RT-PCR

Muscle biopsy samples from *study 2* were used to isolated RNA as described previously[43]. Briefly, frozen muscle was thawed in a Tenbroeck homogenizer with 1 ml of TRIzol reagent (Invitrogen, Carlsbad, CA) and homogenized on ice. The homogenate was extracted with 200 µl of chloroform. The aqueous phase was removed and the RNA was precipitated at room

temperature using 500 µl of iso-propanol and washed twice with 75% ethanol. The final RNA pellet was air dried at room temperature and resuspended in 14 µl ddH_2O and treated with DNaseI. The RNA samples were quantified by spectrophotometer ($A_{260}/A_{280} \geq 1.5$) and the quality was assessed by agarose gel electrophoresis.

TaqMan® Real-time RT-PCR

TaqMan® real-time RT-PCR was conducted on total RNA. Duplex RT-PCR was performed using an iCycler real-time PCR system (Bio-Rad Laboratories, Hercules, CA). One-step TaqMan® RT-PCR Master Mix Reagent (Roch, Branchburg, New Jersey) was combined with RNA, target gene primers and probes, and internal standard gene primers and probes in the same reaction. 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/primer3www.cgi.). Their specificity was monitored using Blast (http://www.ncbi.nlm.nih.gov/BLAST/). Primer sequences are listed in Table 2.

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). Human β2- microglobulin (β2-M) was used as an internal standard as there was no significant sex difference in expression (data not shown). 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.

Western blot analysis

Thirty mg of tissue was used for protein content analysis. Muscle tissue was homogenized in a phosphate lysis buffer; 50mM K_2HPO_4, 1mM EDTA, pH7.4, 0.1mM DDT, PhosSTOP (Roach Diagnostics, Mannheim, Germany), Protease inhibitor cocktail tablets (Roach). Protein concentrations were calculated by Bradford assay (Biorad) and equal amounts of protein were boiled in Laemmli buffer, resolved by SDS-PAGE, transferred to nitrocellulose paper and immunoblotted with desired antibodies. Primary antibodies; HADHB, ACAA2, catalase, PPARδ, MHC I, MHC II and β-actin were all purchased from (Abcam, Cambridge, MA). Secondary antibodies conjugated to horseradish peroxidase (Amersham Bioscience, UK) and specific antibody binding was detected using the chemiluminescence detection reagent ECL+ (Amersham BioScience, UK). Scanned films were analyzed using ImageJ 1.40 software (Wayne Rasband National Institute of Health, USA).

Statistical analysis

All statistical analyses, for mRNA content of the genes, was performed on linear data 2^{-CT} for evaluation of internal standards, $2^{-}\Delta^{CT}$ for target gene

normalized with internal reference [44]. Data on sex differences about target gene mRNA content were analyzed using a Student's *t* test. All results from evaluation of target gene are expressed as mean ±SEM, using $2^{-}\Delta^{CT}$. Western blot data was normalized by the loading control (β-actin) and a Student's *t* test was preformed to test for a difference between men and women. Data are presented as means ± SEM. The data regarding muscle fiber composition were analyzed using a single factor ANOVA and expressed as mean ± SEM. All analyses were done using statistics software (Statistica version 5.0; Statsoft, Tulsa, OK). Statistical significance was set at $\alpha \leq 0.05$.

Results

Sex alters mRNA content in skeletal muscle; microarray

The mRNA abundance in skeletal muscle between men and women was significantly different for 66 genes (using a stringent NLOGP≥4, fold-change >1.2), after Y-linked genes were removed (Table 3; GEO accession #GSE14901). Of these 66 genes, 49 genes have known functions in metabolism, mitochondrial function, transport, protein biosynthesis, cell proliferation, signal transduction pathways, transcription and translation (Table 3). Conversely, 17 of the genes identified in the current analysis do not have known functions. Of the 49 genes with known function, women had higher content of 25 genes (mean 1.7 ± 0.4), compared to men. Subsequently, women have lower content of 24 genes (mean -2.1 ± 2.0), compared to men.

iPath™ (Invitrogen) was used to compared differentially regulated genes against 225 signaling and metabolic human biological pathway maps, and these gene were found to be involved in 39 different signal transduction pathways. Differentially regulated genes were also run against a Connectivity Map to compare the expression profile to that of known drug treatments, genes, and diseases. Interestingly, differentially expressed genes between genders were found to share a similar expression pattern with the transcript profiling of estradiol (which is estrogenic), genistein (which could be estrogenic or anti-estrogenic) and tretinoin (a vitamin A derivative) drug treatments.

Sex alters mRNA content of genes involved metabolism and mitochondrial biogenesis

Sex altered content of six genes involved in metabolism and three genes involved in the function of mitochondria and energy production (Table 3). HADHB (trifunctional protein β), ACAA2, LPL, UCP-2, catalase, aldehyde dehydrogenase 2 family (ALDH2), and argininosuccinate synthase 1 (ASS1) all had higher mRNA content in women compared to men by 1.3- to 2.2-fold. ALDH1A1 and ATP synthase mitochondrial F1 complex assembly factor 1 were significantly lower in women compared to men by 1.9 fold and 1.5-fold respectively (Table 3).

The mRNA content of genes involved in lipid metabolism, HADHB, ACAA2, and catalase, were confirmed by real time RT-PCR (Figure 1, 2a, 3a, 4a) using *study 2* samples. The mRNA content of UCP-2, LPL, and ALDH1A1 were also confirmed by real time RT-PCR (Figure 1, 5), and fold-change of the RT-PCR results correlated to the fold-change reported by the microarray (Figure 1).

To determine if sex differences in mRNA content were consistent with protein content we resolved HADHB, ACAA2, and catalase on a western blot and found no significant difference in protein content between men and women (Figure 2c, 3c, 4c;*study 2 samples*).

Sex difference in muscle fiber composition

Fiber composition data for a subset of *study 1* subjects has been published previously [30]. Fiber composition data for a subset of study 2 subjects was compared to study 1 and when no differences were observed the data was combined to increase the n-value and the data is presented in table 4. Fiber data was consistent with other published data [4,5,26,27] showing women had a significantly higher area % of Type I fibers (women, 32.9 ± 1.3; men, 27.3 ± 1.0; P = 0.001) and a significantly lower area % of Type II fibers (type IIa + type IIx) (women, 67.1 ± 1.3 %; men, 72.7 ± 1.0 %; P = 0.001) than men (Table 4). Women had significantly smaller mean individual fiber area for type IIa (women, 4777.2 ± 347.8 μm^2; men, 6066.9 ± 408.3.8 μm^2; P = 0.013), while there was no significant difference in the type I fiber area or type IIx between women and men (Table 4).

Sex differences in mRNA content for genes involved in muscle type determination

The stringent microarray analysis revealed no significant genes related to muscle type determination. A more biased targeted RT-PCR approach resulted in the content of MHCI mRNA being significantly higher in the skeletal muscle of women (2.6 ± 0.7 fold, P = 0.035) than men (Figure 6a). No significant difference in the mRNA content of MHCIIa or MHCIIx was found between women and men (Figure 6c,d). Women had a significantly higher mRNA content of PPARδ than men (2.3 ± 0.4 fold, P = 0.004) (Figure 7a). There were no significant differences in the mRNA content of PGC-1α or myostatin in the skeletal muscle of men vs. women (Figure 8a, 9a). Western blot analysis showed no significant sex difference in the protein content of MHC I, MHC II, PPARδ, PGC-1α or myostatin (Figure 6b,e, 7c, 8c, 9c). *All data was acquired from study 2.

Discussion

The purpose of this study was to identify novel differences in genes related to metabolism, muscle development, and fiber-type determination and differentiation in mRNA and protein content in skeletal muscle between men and

women at rest using microarray, Real Time-PCR analysis, and protein analysis. Microarrays are a useful tool for the identification of novel mRNA expression patterns and can help to understand potential pathways involved in regulating cellular activity in skeletal muscle [33,38,45,46]. The results from these studies indicate that there are significant differences in mRNA content between men and women. Roth et al. (2002) and Welle et al. (2008) have previously shown that there are significant differences in skeletal muscle mRNA content between men and women, as well as showing sex differences are significantly greater than age and/or strength training effects on mRNA content [38,39]. Approximately 30% of the genes we found to have changed due to sex correspond to the results of Welle and colleagues [39]. Results likely vary between studies due to differences in age of the subject populations, fitness variations, low sample numbers, pooled samples on one gene chip versus individual gene chips per subject, and different gene array technology [38,39]. The methodology used in this study greatly strengthens the data regarding sex based differences in skeletal muscle mRNA for we examined over 23,000 genes with updated annotation, with 12 subjects per group (N = 24 total) hybridized to individual gene chips for analysis and used stringent statistical analysis with an NLOGP >4. The criteria for differential expression is stringent compared to other array studies that generally use an NLOGP>2, however, necessary for the most accurate unbiased account of gene content differences. For the microarray experiment we used samples from study one which included 7 women in the follicular phase and 5 women in the luteal phase, 6 on oral contraceptives and 6 not on oral contraceptives which gives a good representation of the female population. Given the high n-value of the analysis and stringent array analysis criteria we wanted to reduce any variability in gene expression due to these factors in order to only identify specific and significant gene differences that can be applied to a larger population. The goal of the microarray was not to identify genetic differences due menstrual cycle phase but identify sex related differences between an average population of men and women. In another manuscript "in preparation" from study 2 we used a targeted PCR approach to examine differences in metabolic related genes in men compare to women in both the follicular and luteal phases of the menstrual cycle and found that menstrual cycle had little effect on metabolic related mRNA species, compared to the robust difference that sex has (Figures S1 and S2). Due to these findings, the little amount of precious human muscle sample, and the relatively consistent use of follicular phase women in other gender related studies [5,11,17,18,19,20,47] we compared the mRNA and protein of women in the follicular phase only. It is also important to note that Devries et al (2006) report no physiological difference in exercise performance due to menstrual cycle phase including average RER, glycogen utilization, glucose rate of appearance, rate of disappearance, and metabolic clearance rate averaged over the exercise period [41].

The first focus was on the mRNA content of genes involved in intermediary substrate metabolism due to the known fact that women oxidize

more fat during endurance exercise as compared with men [5,6,7,8,10,11,48]. Array results identified six genes related to metabolism that were differentially regulated between men and women. We chose to confirm the mRNA content of five of these genes by RT-PCR. Results identified a novel sex-based difference in the mRNA content of ACAA2 and HADHB (TFP-β). Also, the mRNA content for catalase, lipoprotein lipase, and uncoupling protein-2 were higher in women compared with men. Lastly, ALDH1A1 mRNA content was lower in women compared with men. There was also sex based differences in the mRNA content of genes involved in protein biosynthesis, cell proliferation, signal transduction, transcription, and translation with a particular interest in those genes which are directly involved in muscle function and/or structure.

HADHB (TFPβ) is a multi-enzyme complex found in the mitochondria that is involved in the β-oxidation of fatty acids [49]. Specifically, the TFP enzyme catalyzes the last three steps of long chain fatty acid β-oxidation for long-chain specific acyl-CoA moieties. We are the first to measure and report a sex difference in the mRNA content for this gene. An enhanced β-oxidation capacity distal to transport of FFA into the mitochondria may allow for maintenance of β-oxidation when the cell is under metabolic stress. For example, male transgenic peripheral peroxisome activating receptor knockout (PPARα$^{-/-}$) animal develop severe hypoglycemia when an inhibitor of CPT activity (etomoxir) was given, yet the majority of female mice survived [50]. Furthermore, CPTII deficiency is an autosomal recessive condition and yet many more cases have been documented in men as compared with women, possible due to enhanced β-oxidation capacity in women. Others have reported that the short-chain specific isoform of HAD (SCHAD) has higher mRNA and protein abundance in women compared with men [2,51].

These results also indicate a significant sex based difference in the mRNA content of ACAA2 (acetyl-Coenzyme A acyltransferase 2). ACAA2 is one of two isoforms of ACAA, which is an intracellular enzyme that biosynthesizes cholesteryl esters. Specifically, ACAA is involved in storing cholesteryl esters as lipid droplets, in absorbing dietary cholesterol, and in providing cholesteryl esters as part of the core lipid for lipoprotein synthesis and assembly [52,53,54]. Although ACAA appears to be present in many cell types such as hepatocytes, adrenal cells, skin cells, intestinal enterocytes, neurons, and macrophages it has not been well characterized in skeletal muscle [55,56]. In the aforementioned cell types it has been shown that the ACAA1 isoform is the predominant enzyme compared to ACAA2 [55]. Our gene array results suggest that men and women have no significant difference in the expression of ACAA1 but women have an increased expression of ACAA2 compared to men. Therefore, the total ACAA expression should be significantly higher in women than men, which may explain why lipids are more readily available for substrate utilization during endurance exercise in women. Future studies are needed to determine the expression profile of ACAA1 and ACAA2 in human skeletal muscle as well as the importance of ACAA2 over-expression in women compared to men.

Catalase is one of the three primary antioxidant enzymes. RT-PCR confirmed that mRNA content for catalase is significantly higher in women compared to men. Interestingly, Fano et al (2001) have shown that the enzyme activity of catalase, in the *vastus lateralis*, is significantly higher in women than men [57]. Sex differences have also been observed in other antioxidant enzyme, including manganese-superoxide dismutase [58], suggesting that women are better protected against reactive oxygen species (ROS) as compared with men. We also demonstrated that mRNA content for UCP2 was higher in women compared to men. UCP's can affect energy metabolism efficiency by uncoupling ATP production from mitochondrial respiration. UCP2 is involved in the regulation of energy metabolism and might play a role in obesity [59,60,61]. UCP2 has also been suggested to affect the production of reactive oxygen species (ROS) [62,63,64], and regulate the [ATP]/[ADP] ratio [64,65,66]. Although we are the first to show a sex specific difference in mRNA content in the *vastus lateralis*, this may help to explains why young women are protected against ROS (reviewed in [67]). Furthermore, if UCP2 does play a role in obesity the higher expression in women might help to regulate lipid oxidation.

ALDH1A1 is an isoform of the aldehyde dehydrogenase superfamily primarily responsible for the oxidation of endogenous and exogenous aliphatic and aromatic aldehydes including acetaldehyde, benzaldehyde, 4-hydroxynonenal, malondialdehyde, and retinaldehyde [68,69,70]. Recently ALDH1A1 was also shown to convert 3-deoxyglucosone into 2-keto-3-deoxygluconate [71]. In this study we found that the mRNA content was lower in women compared with men, suggesting that men might be able to metabolize aldehydes (i.e. alcohol) more efficiently than women. ALDH1A1 has not been well characterized in humans, let alone skeletal muscle, but in the mouse liver and human colon there appears to be no sex differences in activity [72,73]. Further studies into the implications of ALDH1A1 mRNA sex differences in skeletal muscle need to be conducted.

These results also showed sex specific differences in genes involved in cell transport, protein biosynthesis, cell proliferation, signal transduction pathways, transcription and translation. Of interest, women had a 2.2-fold reduction in the solute carrier family 1, member 4 that is involved in glutamate/neutral amino acid transport which could be important in metabolism. Women had a 1.7-fold increase in mRNA content of angiopoietin 1, a factor involved in increasing vascularization to a specific tissue, in this case, muscle; however, there does not seem to be a significant difference in capillarization of the *tibialis anterior* or *vastus lateralis* muscle of women compared to men [74,75]. Women also had a 1.7- and 1.5-fold reduction in Dishevelled associated activator of morphogenesis 2, and spectrin- beta- non-erythrocytic 1 which are involved in actin cytoskeleton organization and biogenesis, and barbed-end actin filament capping, respectively. Both are important in muscle cell shape and function. There was an interesting trend in the expression of signal transduction genes and transcription factor genes. Women had a significant up-regulation of the majority of signal transduction related genes and a significant down-regulation

of the majority of transcription factor genes. Despite these trends, there was no evidence that one signal transduction pathway was favored over another as the identified genes spanned multiple pathways including the MAPK pathway, frizzled signaling pathway, G-protein coupled receptor protein signaling pathway, and insulin receptor signaling pathway.

It is important to note that gene array analysis works on the assumption that basal mRNA equates into changes at the protein level and thus activity level. However, recent studies have been demonstrating that is not always the case. For example Kiens et al. (2004) demonstrated that women have a significantly higher LPL mRNA content; however there was no observed differences in LPL activity between men and women [47]. Similarly, Roepstorff et al. demonstrated that although mRNA and protein expression of hormone sensitive lipase (HSL) was higher in women, phosphorylation activation was significantly higher in men [11]. Another study in skeletal muscle biology also found discrepancies in the correlation between mRNA and protein content of a number of genes related to fatty acid oxidation [76]. Part of the discrepancy between mRNA abundance and protein and enzyme assays may relate to higher variance in Western blots and activity assay techniques, and/or that the transcriptome abundance regulates multiple interacting and synergistic pathways that combine to influence flux through metabolic pathways at the protein level that is below the detectable threshold for statistical changes in a single given protein to be manifested. In order to fully understand cellular differences between men and women it is important to understand pre-translational (mRNA abundance), translational (protein) and post-translational (phosphorylation) levels of control.

It has recently been hypothesized that some of the sex differences in exercise substrate selection may be due to fiber-type compositional differences [32]. Subject fiber type characteristics were the same in this study as previously reported [4,5,26,27,30,32]; specifically, the proportionate area (area %) of type I fibers was higher, while that of type II fibers was lower, in women compared with men. Previous findings of a sex difference in type I fiber proportion [25,28,29,32] and a larger type I fiber area [4,5,27,30], in women as compared with men were not confirmed. Nevertheless, it is the proportion of the total muscle area represented by a given fiber type (area X proportion = area %) that should determine the overall abundance of a given transcript or protein in a homogenate of skeletal muscle. Examination of mRNA expression of myosin heavy chain genes, which are specifically expressed in their corresponding muscle fiber types [77], are good markers of the 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 compared with men. The difference in MHCI mRNA did not translate into differences in MHCI protein expression, consistent with previous findings [30].

Similarly, we found sex differences in the mRNA content, but not the protein content, of PPARδ; which plays a role in the conversion of muscle fiber type II into type I and maintenance of the number of type I fibers [78], and

increases the capacity for oxidative metabolism of muscle fibers through hyperplasia of type I fibers [79,80] in transgenic mice.

Strong evidence in transgenic mice [81], and controversial evidence in humans [82,83,84], suggests that PGC-1α is important in the determination of muscle fiber type and induces a fiber type transformation from type II into type I muscle fibers. We did not find an influence of sex on the mRNA or protein content of PGC-1α in skeletal muscle in spite of the fact that women had a higher % area of type I fibers. We also found that there was no sex difference in the mRNA content of myostatin, a negative regulator of skeletal muscle growth [85,86,87].

At rest, there are no significant differences in protein content of the select genes examined, which are involved in metabolism or fiber type. Consistently, there are no observed sex differences in substrate utilization at rest [7,8,88,89,90]. However, mRNA content suggest that men and women are "primed" differently for specific cellular events, and future studies are need to determine if exercise induces changes at the translational and post-translational levels.

Overall, these results identified sex-based differences in mRNA content of metabolic related genes that might lead the way towards an understanding of the sex-based differences in metabolic fuel selection during endurance exercise. Furthermore, this study emphasizes the importance of the influence of sex based differences in gene expression. At the mRNA level there are no inconsistencies in our data or in the literature, which supports that women have higher mRNA abundance for genes involved in fat metabolism as compared with men. Furthermore, men and women demonstrate varied regulation of genes involved in mitochondrial function, transport, protein biosynthesis, cell proliferation, signal transduction pathways, transcription and translation, even at rest.

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Figure Legends

Figure 1. Comparison of microarray data to RT-PCR results for 6 genes found to be significantly different between men and women. Shown as fold change in women versus men. N (microarray) = 12 men, 12 women; N (RT-PCR) = 12 men, 12 women.

Figure 2. Sex differences in HADHB. Differences in HADHB muscle mRNA content between men and women shown by Real time RT-PCR, adjusted to β2-M mRNA (A). Protein content of HADHB in skeletal muscle of men and women, adjusted to β-actin (B,C). Lanes 1-4 are men, lanes 5-8 women, representative of all blots. N=12 men and 12 women. *P<0.05.

Figure 3. Sex differences in ACAA2. Differences in ACAA2 muscle mRNA content between men and women shown by Real time RT-PCR, adjusted to β2-M mRNA (A). Protein content of ACAA2 in skeletal muscle of men and women, adjusted to β-actin (B,C). Lanes 1-4 are men, lanes 5-8 women, representative of all blots. N=12 men and 12 women. *P<0.05.

Figure 4. Sex differences in catalase. Differences in Catalase mRNA content between men and women shown by Real time RT-PCR, adjusted to β2-M mRNA (A). Protein content of catalase in skeletal muscle of men and women, adjusted to β-actin (B,C). Lanes 1-4 are men, lanes 5-8 women, representative of all blots. N=12 men and 12 women. *P<0.05.

Figure 5. Skeletal muscle mRNA content of LPL, UCP-2 and ALDH1A1 in men and women. LPL is higher in women than men (P=0.009) (A). UCP-2 is higher in women than men (P=0.05) (B). ALDH1A1 is lower in women than men (P=0.01) (C). β2-M mRNA was used as an internal standard. N=12 men and 12 women.

Figure 6. Sex differences in MHC isoforms. Differences in MHC I muscle mRNA content between men and women shown by Real time RT-PCR, adjusted to 28 S rRNA (A). Protein content of MHCI in skeletal muscle of men and women, adjusted to β-actin (B). MHC IIa (C) and MHC IIx (D) muscle mRNA content in men and women shown by Real time RT-PCR, adjusted to 28 S rRNA. Protein content of MHC II in skeletal muscle of men and women (E). Lanes 1-4 are men, lanes 5-8 women, representative of all blots. N=12 men and 12 women. *P<0.05.

Figure 7. Sex differences in PPARδ. Differences in PPARδ muscle mRNA content between men and women shown by Real time RT-PCR, adjusted to β2-M mRNA (A). Protein content of PPARδ in skeletal muscle of men and women,

adjusted to β-actin (B,C). Lanes 1-4 are men, lanes 5-8 women, representative of all blots. N=12 men and 12 women. *P<0.05.

Figure 8. Sex differences in PGC1α. Differences in PGC1α muscle mRNA content between men and women shown by Real time RT-PCR, adjusted to β2-M mRNA (A). Protein content of PGC1α in skeletal muscle of men and women, adjusted to β-actin (B,C). Lanes 1-4 are men, lanes 5-8 women, representative of all blots. N=12 men and 12 women. *P<0.05.

Figure 9. Sex differences in myostatin. Differences in myostatin muscle mRNA content between men and women shown by Real time RT-PCR, adjusted to β2-M mRNA (A). Protein content of myostatin in skeletal muscle of men and women, adjusted to β-actin (B,C). Lanes 1-4 are men, lanes 5-8 women, representative of all blots. N=12 men and 12 women.

Figure S1. Sex differences in resting mRNA content of genes related to substrate metabolism. Genes are expressed as mean fold difference women/men ± SEM. β2-M mRNA was used as an internal standard. N=12 men and 12 women. *P<0.05.

Figure S2. Menstrual cycle differences in resting mRNA content of genes related to substrate metabolism. Genes are expressed as mean fold difference follicular/luteal ± SEM. β2-M mRNA was used as an internal standard. N=12 men and 12 women. *P<0.05.

Tables

Table 1. Subject characteristics from study #1 and study #2.

	Study #1		Study #2	
	Men (n=12)	Women (n=12)	Men (n=11)	Women (n=13)
Age (yr)	21±1	22±1	21±1	22±2
Weight (kg)	79±4	61±2*	80±3	63±2*
Height (cm)	179±2	164±1*	178±1	165±1*
BF (%)	18±1	25±1*	19±5	29±5*
FFM (kg)	64±3	44±1*	59±1	52±1*
BMI	25±1	23±1	25±1	23±1
VO2peak (ml*kg body wt-1*min-1)	NA	NA	45±1	39±2*
(ml*kg FFM-1*min-1)	NA	NA	56±1	54±3
Menstral cycle		7=Fol, 5=Lut		Fol
Oral Contraceptive use		6=OC, 6=NOC		6=OC, 7=NOC
Feeding state	Boost® 2hrs before biopsy		Fasted (10-12 hrs)	

*significant difference between men and women for each study ($P<0.05$). There is no significant difference between the men or the women in study 1 compared with study 2. BF; body fat, BMI; body mass index, Fol; follicular phase, Lut; luteal phase, OC; oral contraceptives.

Table 2. Primer Sequences

Gene Name	Forward Primer	Reverse Primer
beta2-microglobulin	ggctatccagcgtactccaa	gatgaaacccagacacatagca
Catalase	actgaggtccaccctgactac	tcgcattcttaggcttctca
Lipoprotein Lipase (LPL)	gaaaggcacctgcggtatt	catgccgttctttgttctgta
Uncoupling protein-2 (UCP-2)	tcatcacctttcctctggatac	agaatggtgcccatcacac
Acyl-coenzyme A acyltransferase-2 (ACAT2)	ggcaggagaagcaagatga	gcaccattgaaggaacctatg
Aldehyde dehydrogenase 1 family member A1 (ALDH1A1)	cgccagacttacctgtcctact	ctcctcagttgcaggattaaag
Trifunctional protein β subunit (HADHB)	aaacaagcaatgtggctagaga	ggcttggttggcagagatac
Myosin Heavy Chain I (MHCI)	cctggaacatctggagacct	agctgctttcggaccttct
Myosin Heavy Chain IIa (MHCIIa)	caatctagctaaattccgcaagc	tcacttatgacttttgtgtgtgaacct
Myosin Heavy Chain IIx (MHCIIx)	aaatggtggaaagaagagagtcc	aatacagcttcatccagggc
PGC-1alpha	ttgctaaacgactccgagaa	tgcaaagttccctctctgct
PPARdelta	actgagttcgccaagagcat	gtgcacgccatacttgagaa
Myostatin	gacccgtcgagactcctaca	aataccagtgcctgggttca

*All primer sequences are shown 5' to 3', left to right.

Table 3. Differential expression of mRNA in women vs men.

Gene Name	Symbol	Fold Change	NLOG P	Biological Process
Metabolism				
hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase/enoyl-Coenzyme A hydratase (trifunctional protein), beta subunit	HADHB	1.32	4.00	lipid metabolism, fatty acid metabolism, fatty acid beta-oxidation
acetyl-Coenzyme A acyltransferase 2 (mitochondrial 3-oxoacyl-Coenzyme A thiolase)	ACAA2	1.61	5.20	lipid metabolism, fatty acid metabolism, cholesterol biosynthesis
lipoprotein lipase	LPL	1.85	4.30	fatty acid metabolism, circulation, lipid catabolism
aldehyde dehydrogenase 2 family (mitochondrial)	ALDH2	1.46	4.70	carbohydrate metabolism, alcohol metabolism
argininosuccinate synthetase 1	ASS1	2.19	6.60	urea cycle, arginine biosynthesis, amino acid biosynthesis
aldehyde dehydrogenase 1 family, member A1	ALDH1A1	-1.88	6.00	aldehyde metabolism
Mitochondrial function/oxidative stress				
catalase	CAT	1.7	6.60	electron transport, response to oxidative stress, hydrogen peroxide catabolism
uncoupling protein 2 (mitochondrial, proton carrier)	UCP2	1.5	4.20	proton transport in the mitochondria
ATP synthase mitochondrial F1 complex assembly factor 1	ATPAF1	-1.49	4.00	protein complex assembly
Transport				
solute carrier family 25, member 34	SLC25A34	2.37	4.20	transport (mitochondrial carrier)
solute carrier family 1 (glutamate/neutral amino acid transporter), member 4	SLC1A4	-2.2	4.80	dicarboxylic acid transport, neutral amino acid transport
Signal transduction				
Rap guanine nucleotide exchange factor (GEF) 2	RAPGEF2	1.21	4.00	intracellular signal transduction, MAPK cascade, cAMP-mediated signaling ,
transducin-like enhancer of split 1 (E(sp1) homolog, Drosophila)	TLE1	1.7	4.00	regulation of transcription, signal transduction, frizzled signaling pathway
kalirin, RhoGEF kinase	KALRN	1.31	4.00	protein amino acid phosphorylation, signal transduction, vesicle-mediated transport
amyloid beta (A4) precursor-like protein 2	APLP2	1.32	5.60	G-protein coupled receptor protein signaling pathway
growth factor receptor-bound protein 10	GRB10	2.41	7.80	intracellular signaling cascade , cell-cell signaling , insulin receptor signaling pathway

Cyclin-dependent kinase inhibitor 1C (p57, Kip2)	CDKN1C	1.71	5.20	regulation of cyclin-dependent protein kinase activity , G1 phase of mitotic cell cycle
family with sequence similarity 13, member A1	FAM13A 1	1.32	4.20	signal transduction
mitogen-activated protein kinase 6	MAPK6	-1.34	4.00	protein amino acid phosphorylation , cell cycle , signal transduction
WW domain containing E3 ubiquitin protein ligase 1	WWP1	-1.52	7.40	signal transduction, negative regulation of transcription , protein ubiquitination, protein modification , ubiquitin cycle
Rho guanine nucleotide exchange factor (GEF) 10-like	ARHGEF 10L	-1.31	4.10	regulation of Rho protein signal transduction
Transcription				
Sine oculis homeobox homolog 1 (Drosophila)	SIX1	1.34	4.00	regulation of transcription, muscle development
small nuclear ribonucleoprotein polypeptide N, SNRPN upstream reading frame	SNRPN, SNURF	-1.6	5.90	mRNA metabolism
nuclear receptor interacting protein 1	NRIP1	-1.59	4.60	negative and positive regulation of transcription from RNA polymerase II promoter , androgen receptor signaling pathway
TBC1 (tre-2/USP6, BUB2, cdc16) domain family, member 1	TBC1D1	-1.45	4.40	DNA metabolism , chromosome organization and biogenesis
cyclin H	CCNH	-1.21	4.80	regulation of cyclin-dependent protein kinase activity , DNA repair , regulation of transcription
LAG1 longevity assurance homolog 6 (S. cerevisiae)	LASS6	-1.82	4.10	regulation of transcription, lipid biosynthesis
iroquois homeobox protein 3	IRX3	-10.92	11.40	regulation of transcription
zinc finger protein 33A	ZNF33A	-1.57	4.00	regulation of transcription
Protein Biosynthesis and Translation				
eukaryotic translation initiation factor 1A, X-linked	EIF1AX	1.38	7.30	protein biosynthesis, translational initiation
eukaryotic translation initiation factor 2, subunit 3 gamma, 52kDa	EIF2S3	1.45	7.20	protein biosynthesis
FK506 binding protein 9, 63 kDa	FKBP9	1.32	4.00	protein folding
similar to Caspase-4 precursor (CASP-4) (ICH-2 protease) (TX protease) (ICE(rel)-II)	LOC648 470	1.9	4.50	proteolysis
JTV1 gene	JTV1	-1.42	5.80	protein biosynthesis
ubiquitin specific peptidase 31	USP31	-1.42	4.10	ubiquitin-dependent protein catabolism
ring finger and CHY zinc finger domain containing 1	RCHY1	-1.31	5.10	ubiquitin cycle
ADP-ribosylhydrolase like 1	ADPRHL 1	-1.61	4.70	protein amino acid ADP-ribosylation
Cell Proliferation				
zinc finger protein 36, C3H type-like 2	ZFP36L2	1.48	4.80	cell proliferation

monocyte to macrophage differentiation-associated	MMD	1.67	4.10	cytolysis
angiopoietin 1	ANGPT1	1.74	4.30	angiogenesis, signal transduction, cell differentiation, development
plexin C1	PLXNC1	1.94	4.70	cell adhesion, development
cell division cycle associated 7-like	CDCA7L, RAM2	2.45	4.40	positive regulation of cell proliferation
tumor protein D52	TPD52	-1.72	5.70	morphogenesis, B cell differentiation, secretion
Down syndrome critical region gene 1-like 1	DSCR1L 1	-1.67	5.90	central nervous system development, calcium-mediated signaling
cytokine induced apoptosis inhibitor 1	CIAPIN1	-1.31	4.90	apoptosis, anti-apoptosis
CD24 molecule	CD24	-4.19	7.00	humoral immune response
dishevelled associated activator of morphogenesis 2	DAAM2	-1.71	4.20	actin cytoskeleton organization and biogenesis
attractin-like 1	ATRNL1	-1.83	4.80	development
spectrin, beta, non-erythrocytic 1	SPTBN1	-1.52	5.30	barbed-end actin filament capping
UNKNOWN				
ubiquitously transcribed tetratricopeptide repeat, X chromosome	UTX	2.13	9.35	---
zinc finger, BED-type containing 5	ZBED5	-1.31	4.30	---
Nedd4 family interacting protein 2	NDFIP2	-1.4	4.60	---
chromosome 2 open reading frame 25	C2orf25	-1.23	4.00	---
tryptophan rich basic protein	WRB	-1.47	6.00	---
PQ loop repeat containing 3	PQLC3	1.77	4.30	---
CDNA FLJ25488 fis, clone CBR00232	---	-1.34	5.20	---
family with sequence similarity 79, member B	FAM79B	5.94	8.80	---
X (inactive)-specific transcript	XIST	192.07	32.77	---
CDNA FLJ33569 fis, clone BRAMY2010317	---	-1.47	4.00	---
hypothetical protein	LOC387 882	3.04	5.30	---
chromosome 8 open reading frame 22	C8orf22	2	4.20	---
chromosome Y open reading frame 15A	CYorf15 A	-2.01	5.10	---
KIAA1155 protein	KIAA115 5	1.71	5.10	---
Prader-Willi syndrome chromosome region 1	PWCR1	-1.61	5.00	---
Heparan-alpha-glucosaminide N-acetyltransferase, similar to transmembrane protein 76	HGSNAT , LOC643 642	1.53	5.30	---
hypothetical gene CG018	CG018	1.61	6.60	---

*Microarray significance >1.2 fold increase or decrease. NLOGP, negative log of the *p* value, >4.0. N=12 men and 12 women.

Table 4. Fiber type composition in the *vastus lateralis* muscle of women and men.

	Men (N=10)	Women (N=16)
Fiber type composition (area%)		
Type I	27.3 ± 1.0	32.9 ± 1.3 *
Type IIa + IIx	72.7 ± 1.0	67.1 ± 1.3 *
Mean area per fiber (μm^2)		
Type I	4218.6 ± 225.0	4691.7 ± 477.9
Type IIa	6066.9 ± 408.3	4777.2 ± 347.8 *
Type IIx	5208.7 ± 220.3	4677.7 ± 389.8

Sex differences in fiber type composition between men and women. Due to experimental difficulties data is a combined subset of samples from both study #1 and study#2. Means ± SEM * $P < 0.05$.

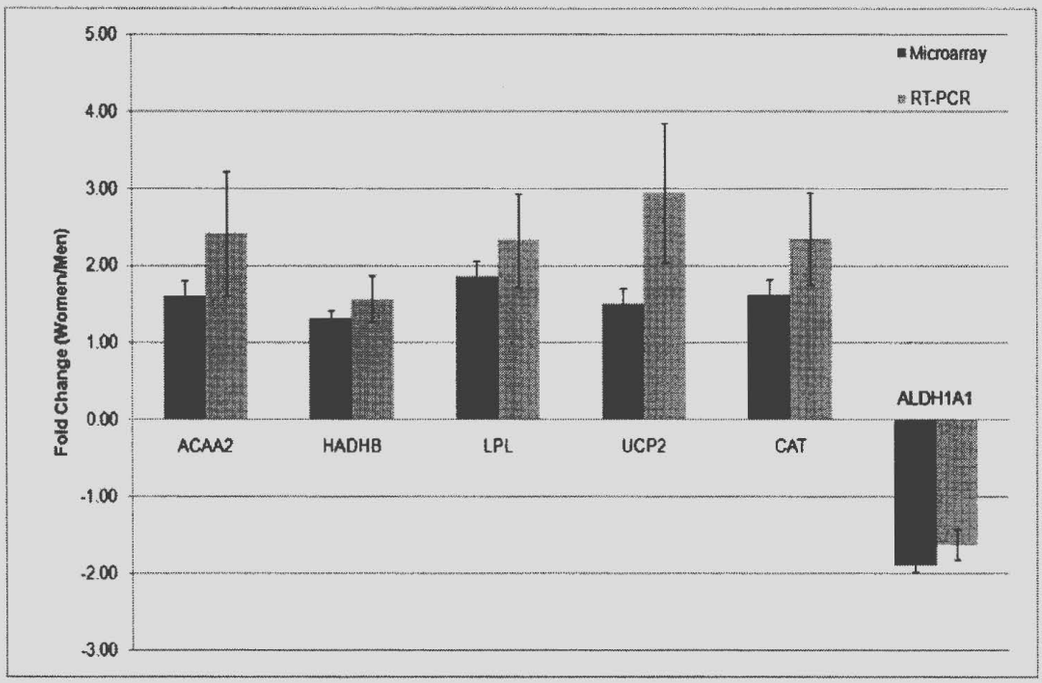
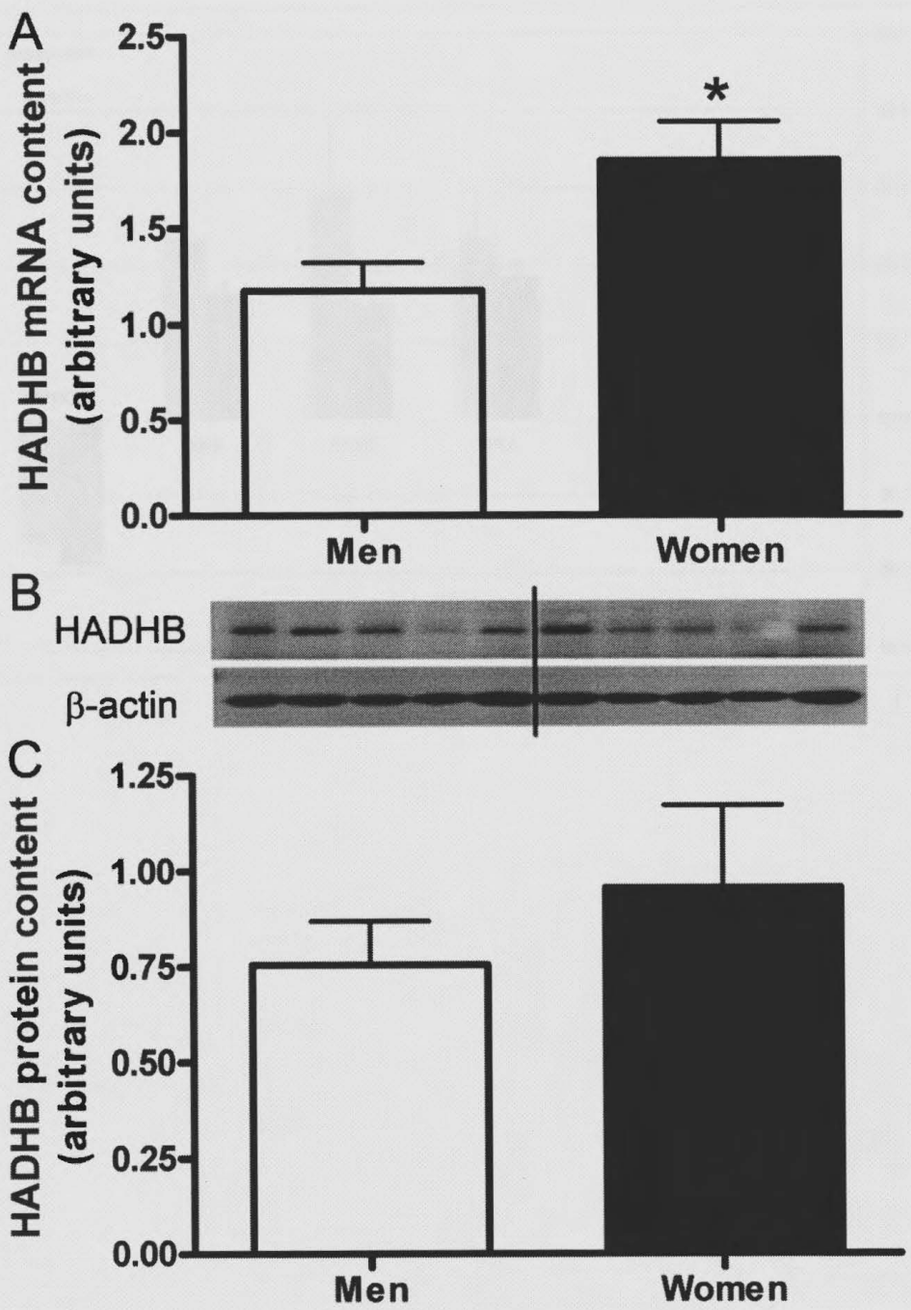

5.00
4.00
3.00
2.00
1.00
0.00
-1.00
-2.00
-3.00
Fold Change (Women/Men)
Microarray
RT-PCR
ACAA2
HADHB
LPL
UCP2
CAT
ALDH1A1

Figure 1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HADHB mRNA content (arbitrary units)
2.5
2.0
1.5
1.0
0.5
0.0
*
Men
Women
B
HADHB
β-actin
C
HADHB protein content (arbitrary units)
1.25
1.00
0.75
0.50
0.25
0.00
Men
Women

Figure 2

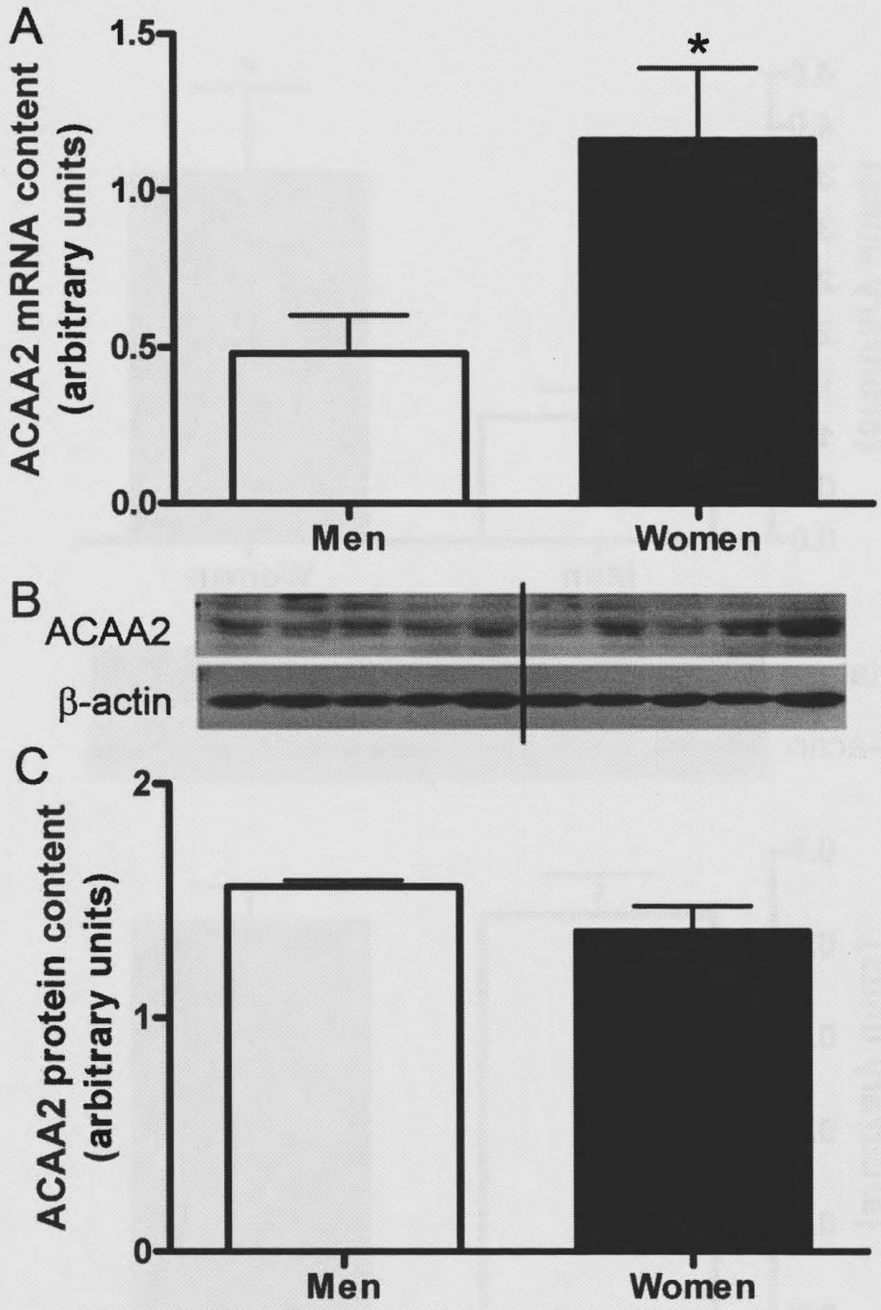

A
ACAA2 mRNA content (arbitrary units)
1.5
1.0
0.5
0.0
*
Men
Women
B
ACAA2
β-actin
C
ACAA2 protein content (arbitrary units)
2
1
0
Men
Women

Figure 3

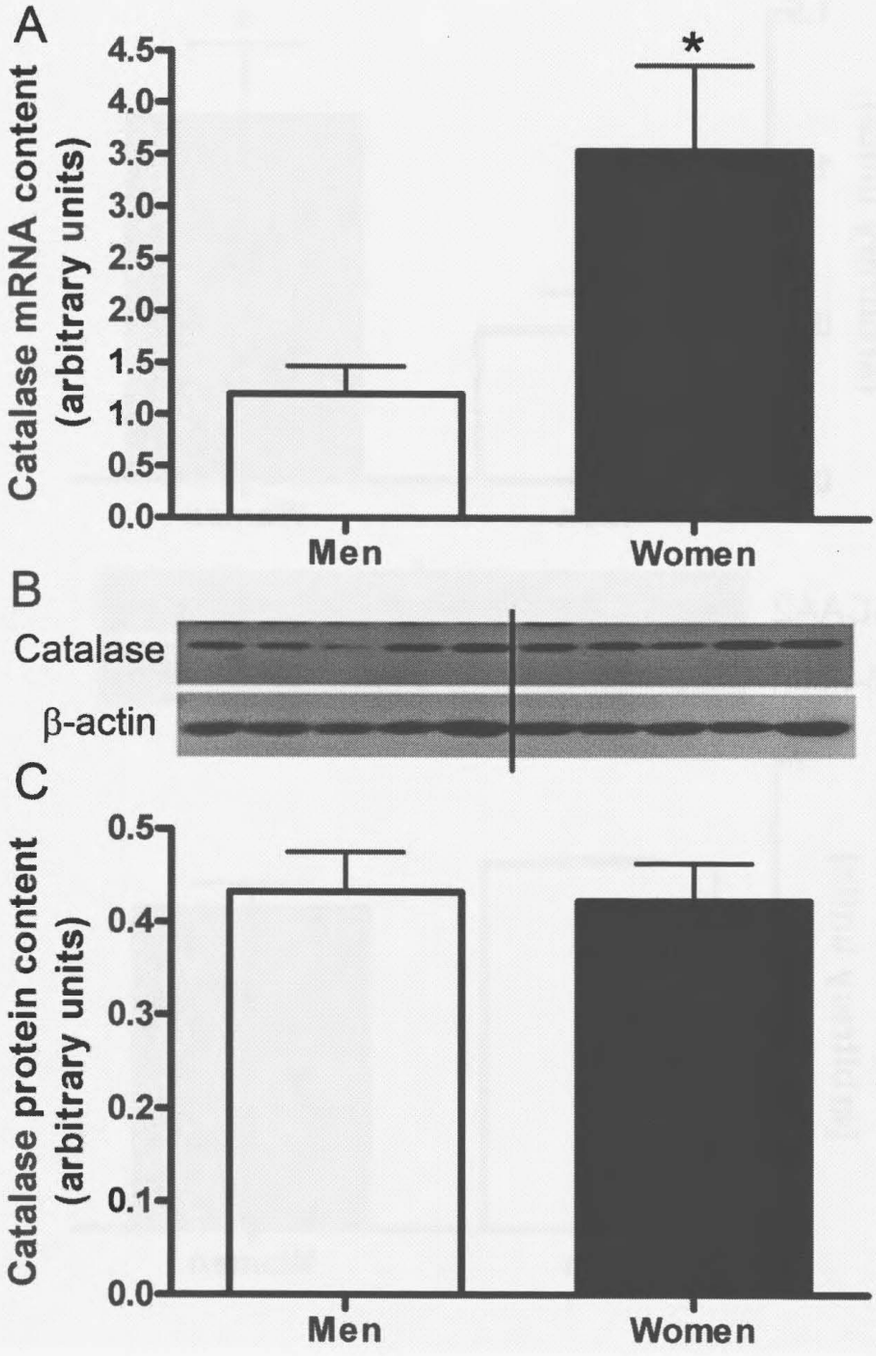

A
Catalase mRNA content (arbitrary units)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
Men
Women
B
Catalase
β-actin
C
Catalase protein content (arbitrary units)
0.5
0.4
0.3
0.2
0.1
0.0
Men
Women

Figure 4

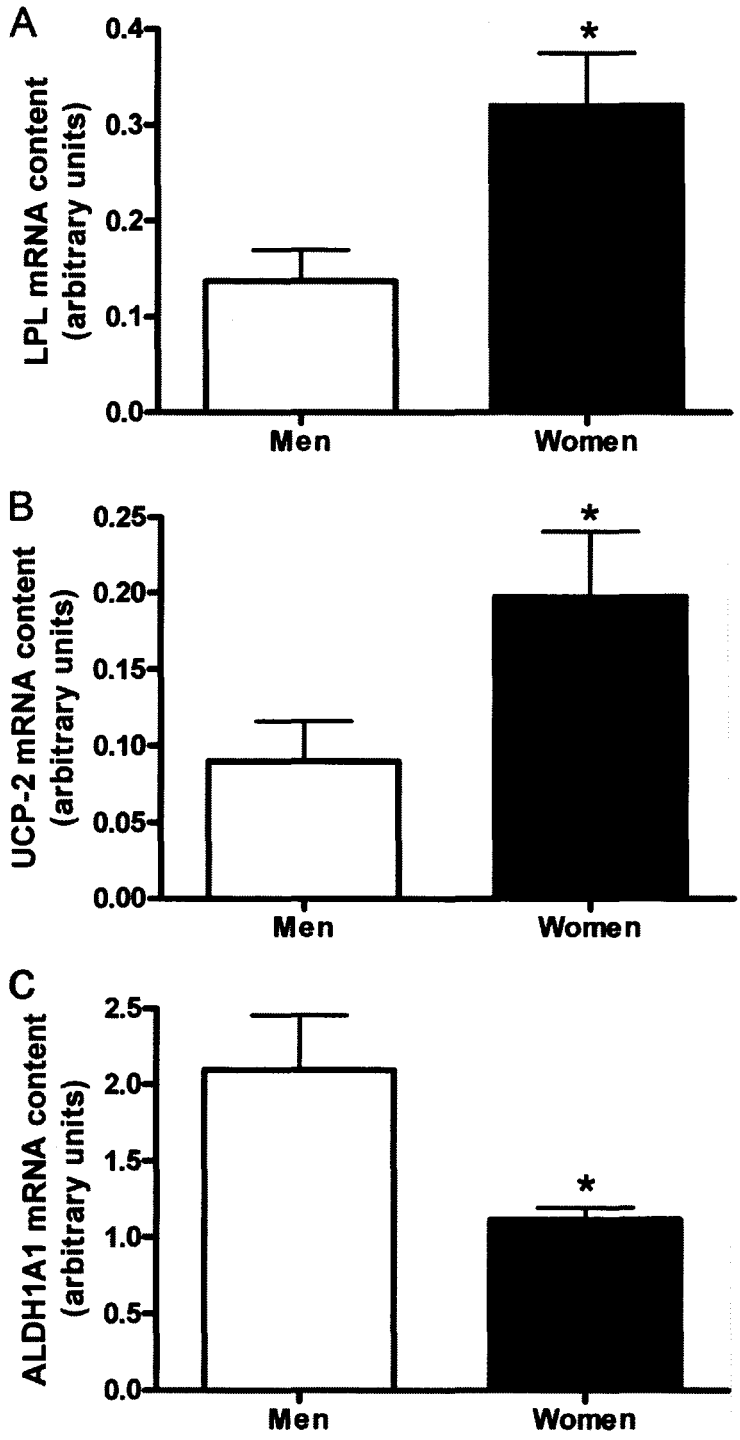

A
LPL mRNA content (arbitrary units)
0.4
0.3
0.2
0.1
0.0
*
Men
Women
B
UCP-2 mRNA content (arbitrary units)
0.25
0.20
0.15
0.10
0.05
0.00
*
Men
Women
C
ALDH1A1 mRNA content (arbitrary units)
2.5
2.0
1.5
1.0
0.5
0.0
*
Men
Women

Figure 5

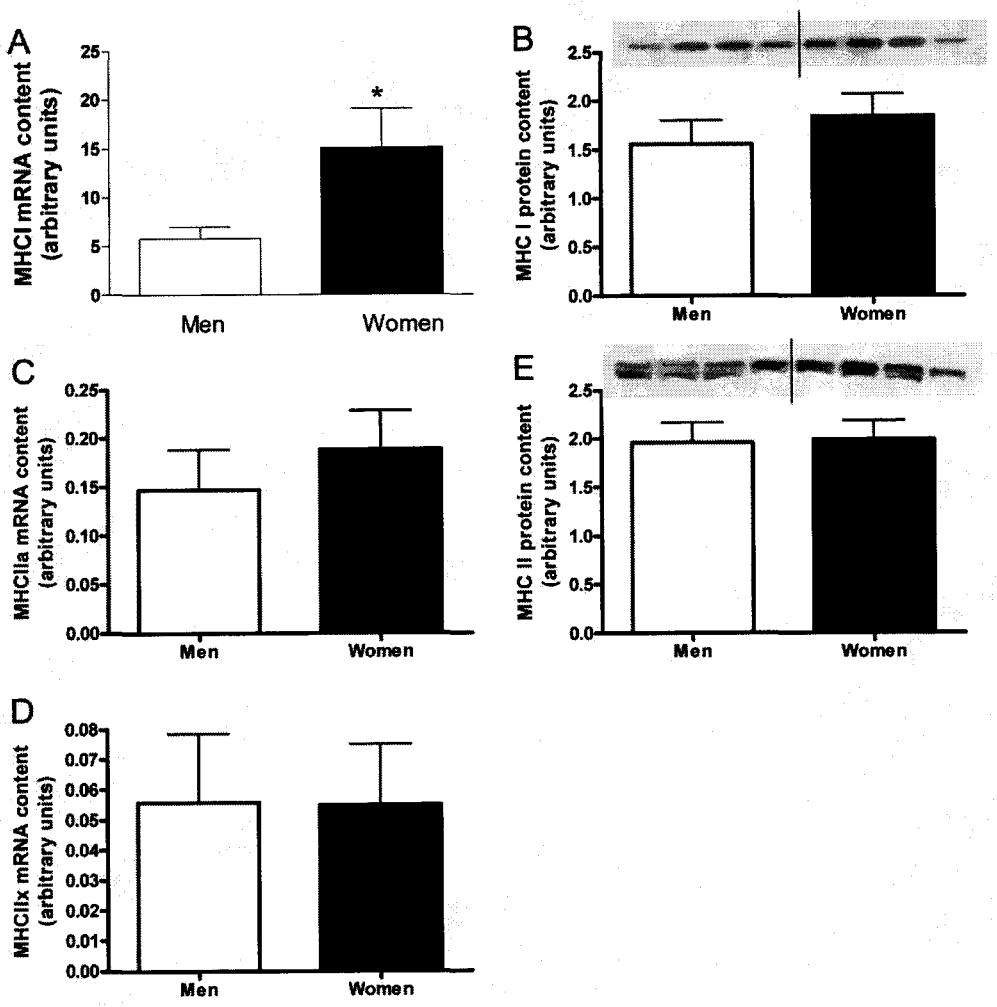

A
MHCI mRNA content (arbitrary units)
25
20
15
10
5
0
*
Men
Women
B
MHC I protein content (arbitrary units)
2.5
2.0
1.5
1.0
0.5
0.0
Men
Women
C
MHCIIa mRNA content (arbitrary units)
0.25
0.20
0.15
0.10
0.05
0.00
Men
Women
E
MHC II protein content (arbitrary units)
2.5
2.0
1.5
1.0
0.5
0.0
Men
Women
D
MHCIIx mRNA content (arbitrary units)
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
Men
Women

Figure 6

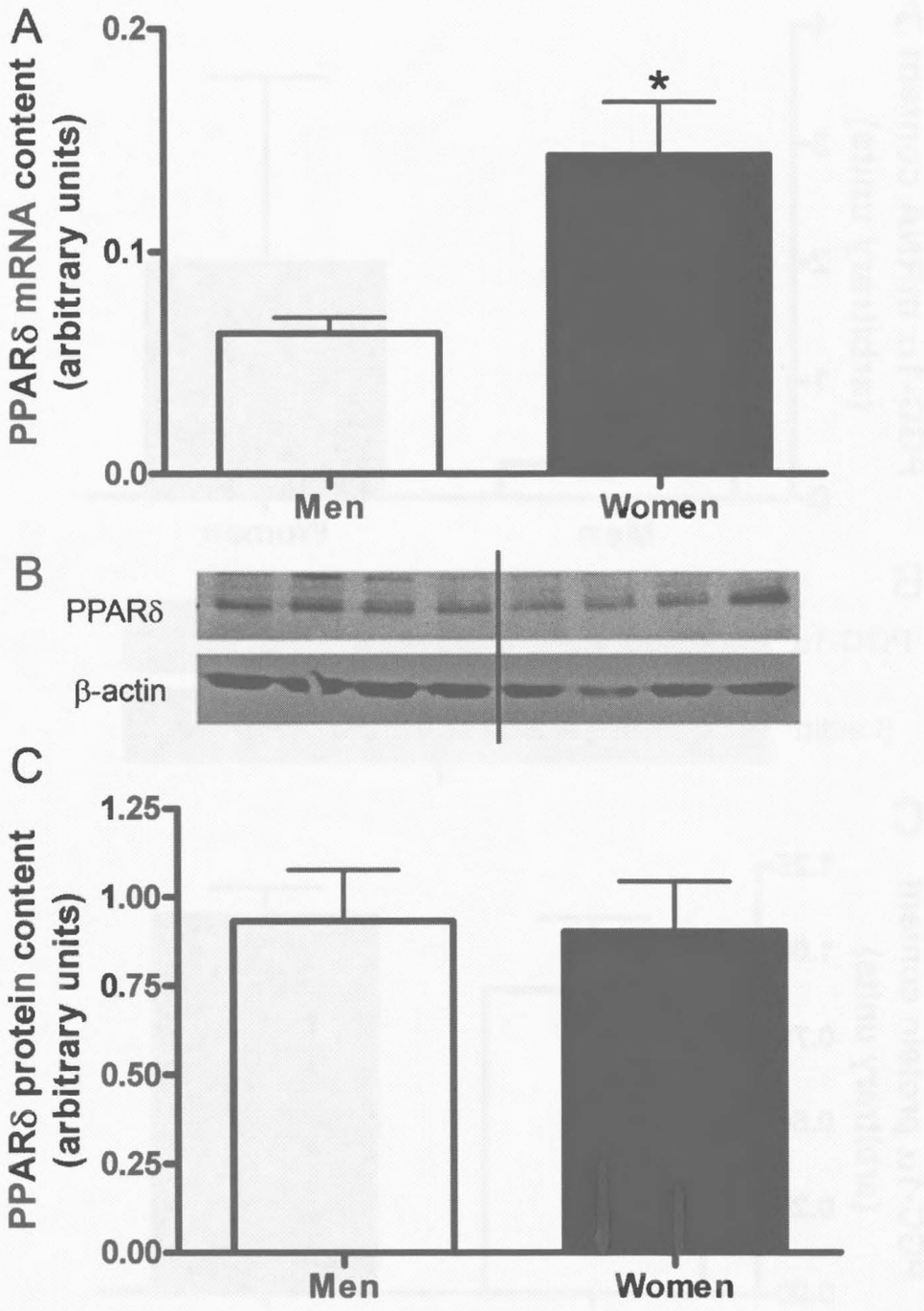

A
PPARδ mRNA content (arbitrary units)
0.2
0.1
0.0
*
Men
Women
B
PPARδ
β-actin
C
PPARδ protein content (arbitrary units)
1.25
1.00
0.75
0.50
0.25
0.00
Men
Women

Figure 7

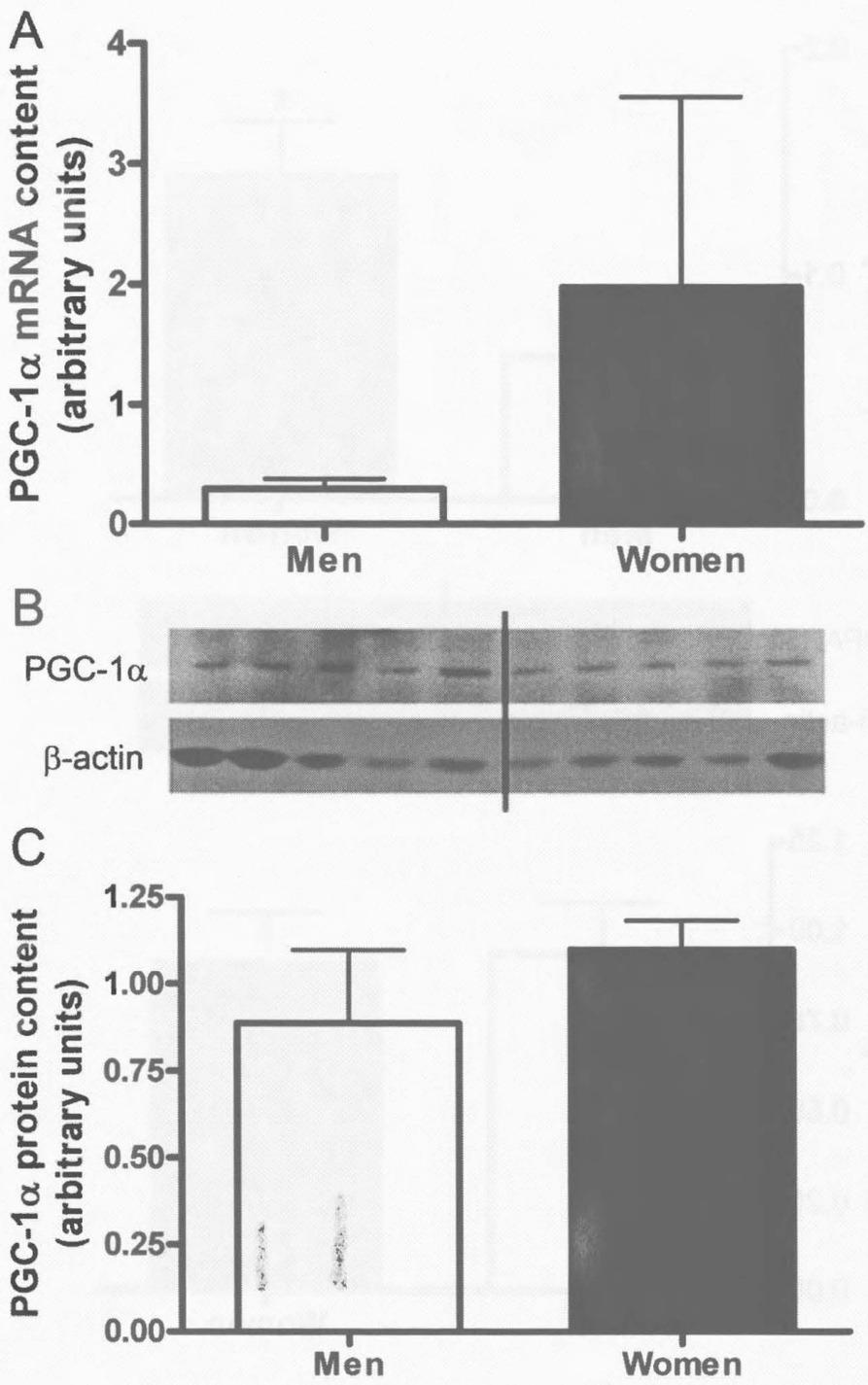

A
PGC-1α mRNA content (arbitrary units)
4
3
2
1
0
Men
Women
B
PGC-1α
β-actin
C
PGC-1α protein content (arbitrary units)
1.25
1.00
0.75
0.50
0.25
0.00
Men
Women

Figure 8

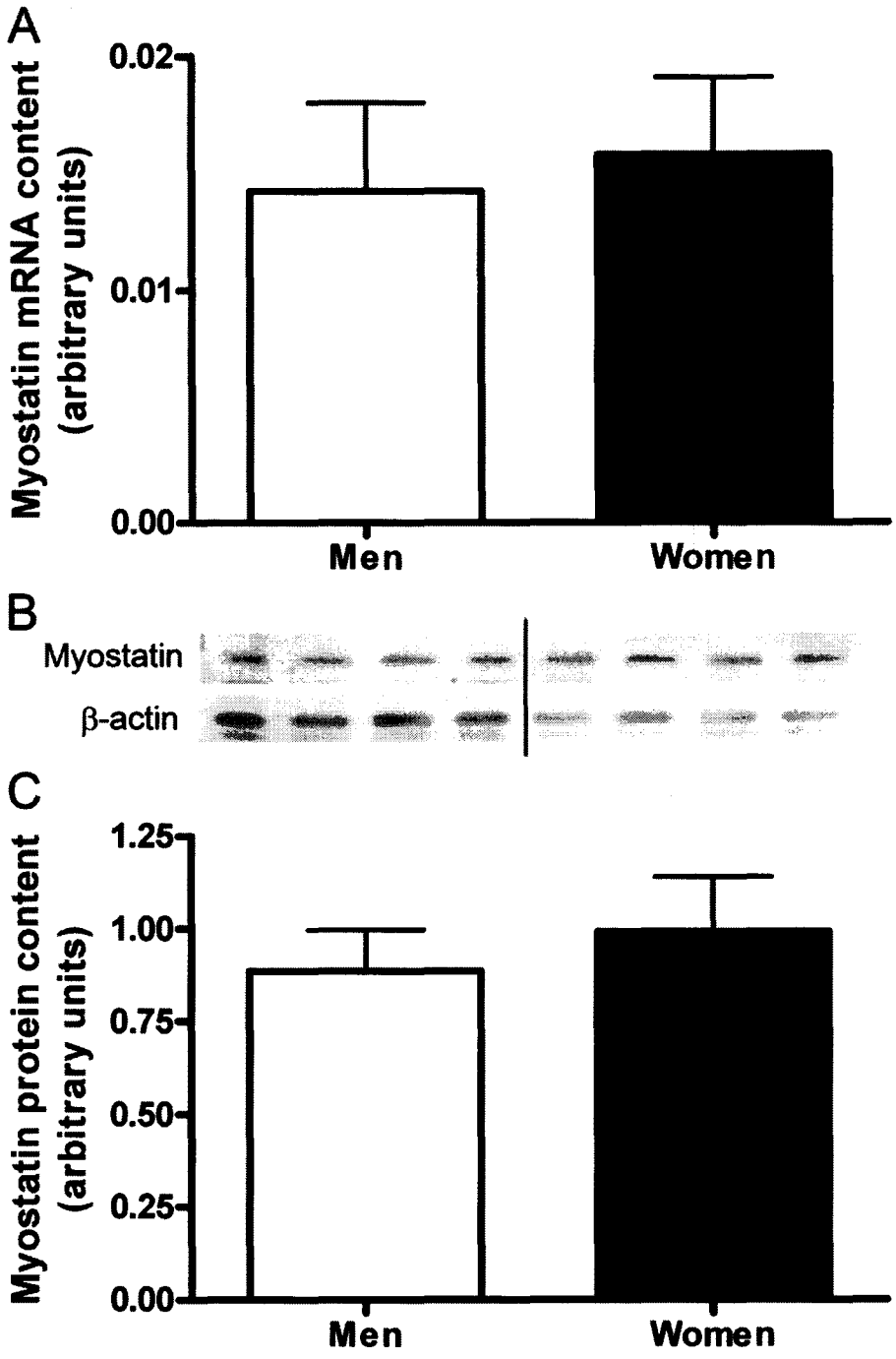
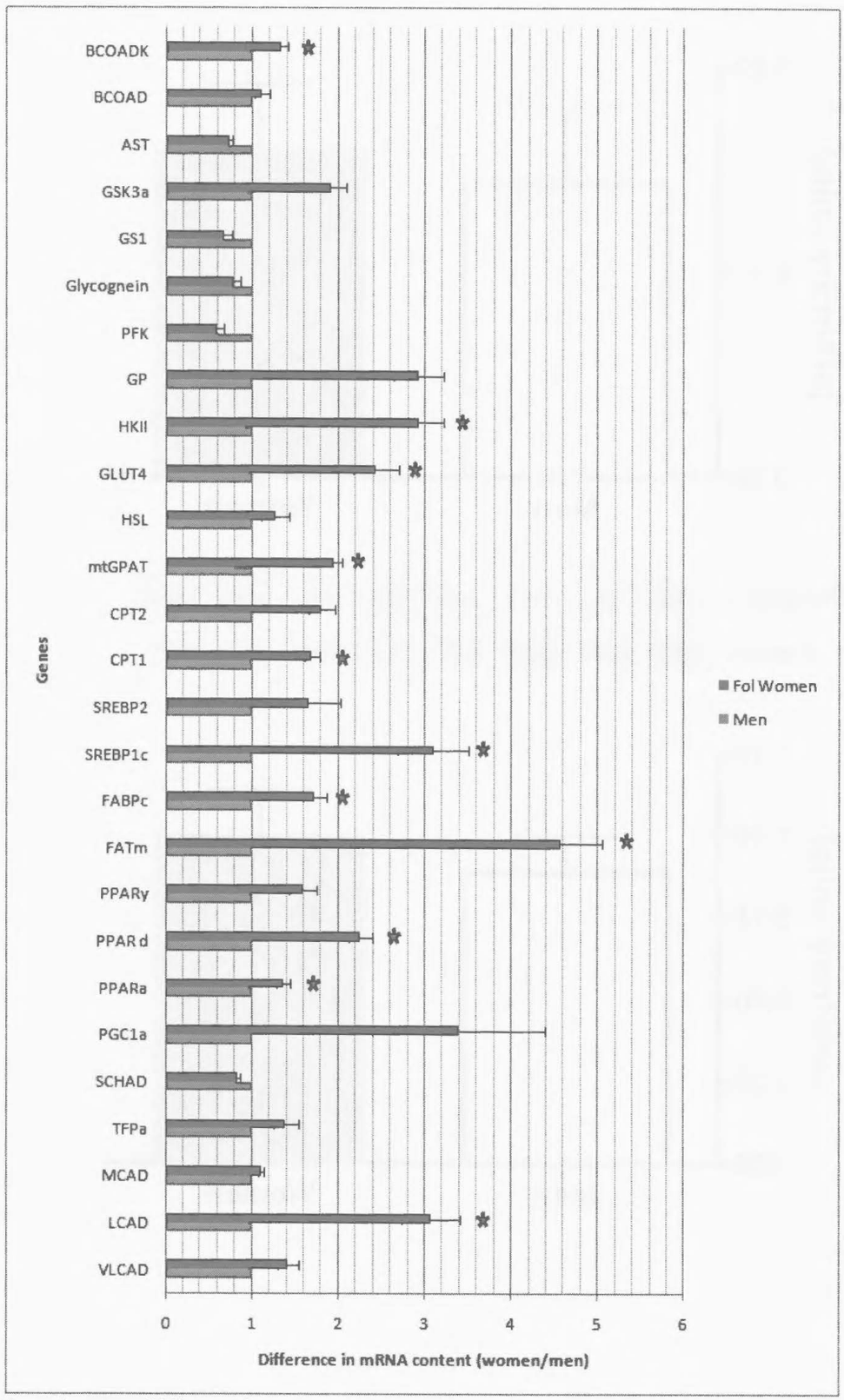
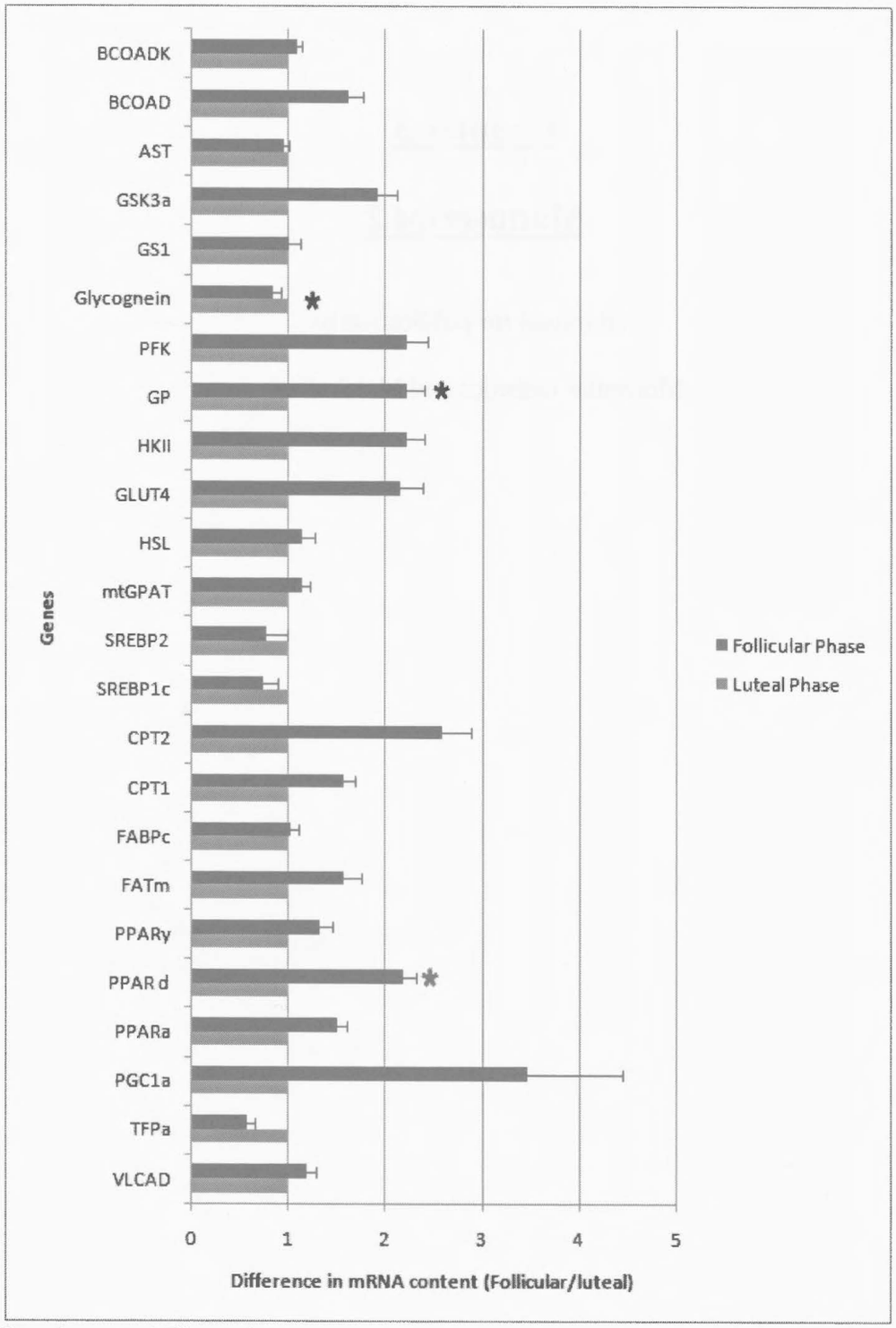

A
Myostatin mRNA content (arbitrary units)
0.02
0.01
0.00
Men
Women
B
Myostatin
β-actin
C
Myostatin protein content (arbitrary units)
1.25
1.00
0.75
0.50
0.25
0.00
Men
Women

Figure 9



Supplemental Figure 1



Supplemental Figure 2

Chapter 3

Manuscript 2

Submitted for publication to

Molecular Genetics and Metabolism

Low expression of LCAD in human skeletal muscle

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Running head: LCAD in human skeletal muscle

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Abstract

Long-chain acyl-CoA dehydrogenase (LCAD) belongs to a family of mitochondrial flavoenzyme involved in fatty acid metabolism. LCAD was identified in humans in the 1950's and was one of the first identified acyl-CoA dehydrogenases. It is thought to be one of the major enzymes responsible for long-chain (LC) free fatty acid (FFA) oxidation. During exercise, skeletal muscle accounts for approximately 97% of total LC-FFA oxidation. Surprisingly, recent studies have shown LCAD is not very highly expressed in human tissue. In this study we investigated the expression levels of LCAD in human skeletal muscle. We obtained muscle biopsies from the *vastus lateralis* of moderately active men and women, and examined mRNA and protein content of LCAD, including isolated mitochondrial protein from an elite endurance athlete. We compared LCAD abundance of skeletal muscle to human heart, liver. We show that LCAD is so lowly expressed in human skeletal muscle, that its role is likely minor or redundant in skeletal muscle fatty acid metabolism.

Key words; LCAD, skeletal muscle, human, protein, RNA

Introduction

Although all tissues are capable of oxidizing substrate, skeletal muscle is the most abundant tissue in the human body and the primary tissue responsible for the clearance of dietary lipids, proteins and carbohydrates in order to maintain metabolic homeostasis (13). The process of lipid utilization, which occurs via mitochondrial fatty acid (FA) β-oxidation (FAO), is essential for energy production, especially during exercise, starvation, and other metabolic stresses. During exercise FA's contribute anywhere from 30-70% of substrate utilized (depending on exercise intensity) (7), of which ~90% are LC-FA's (8). FAO is a process that through a series of enzymatic reactions removes carbon bonds to generate energy. There are thought to be three major enzymes that can contribute to LC-FAO based on their overlapping enzymatic affinity for varying carbon lengths; very long chain acyl-CoA dehydrogenase (VLCAD), long chain acyl-CoA dehydrogenase (LCAD) and medium chain acyl-CoA dehydrogenase (MCAD). VLCAD and MCAD are essential to maintain metabolic homeostasis and muscle function as mutations in them result in disruption of muscle function with myopathy and/or cardiomyopathy (18). However, the role of LCAD in normal muscle physiology remains unclear. Research in our lab, and by others, have shown that MCAD and VLCAD are highly expressed in human skeletal muscle and exercise training increases the expression of these enzymes (10). In contrast, LCAD has been reported to be expressed to a much lower level than VLCAD in human muscle, calling into question its role in the generation of energy under physiologic stress (9). Here we compare the expression of LCAD protein in human skeletal muscle, liver and heart and look specifically and the mitochondrial isolated population of LCAD in these tissues.

Material and Methods

Preparation of RNA

Fifty milligrams (mg) of human skeletal muscle from the *vastus lateralis* of men and women was used to isolate mRNA using an Ambion mirVana™ miRNA isolation kit (Ambion Inc., Austin, TX #AM1561). In brief, muscle tissue was homogenized in Lysis/Binding buffer in a glass homogenizer. The miRNA/RNA was extracted organically using Acid-Phenol:Chloroform and ethanol precipitation. Final isolation was done using provided filter cartridges. RNA was eluted in nuclease-free water and quantity and quality of RNA was assessed using a NanoDrop Spectrophotometer. Measurements were done in duplicate and had an average coefficient of variation (CV) of <10%. The average purity (OD_{260}/OD_{230}) of the samples was > 1.5.

RNA was synthesized into cDNA using Applied Biosystems High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Inc., Foster City, CA Cat#4368814) and a BioRad iCycler iQ® real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA) as per manufactures instructions.

TaqMan® real-time RT-PCR

Gene content was quantified using 7300 Real-time PCR System (Applied Biosystems Inc., Foster City, CA) and SYBR® Green chemistry (PerfeCTa SYBR® Green Supermix, ROX, Quanta BioSciences, Gaithersburg, MD) as previously described (16), with the exception that cDNA was not diluted in order to be able to amplify LCAD. Specific primers to each target gene were designed based on the cDNA sequence in GenBank with MIT primer 3 designer software. Specificity was checked using Blast. MCAD; forward 5'-tgccagagaggaaatcatcc-3', reverse 5'- tctcggacccttgaaccaaa-3', LCAD; forward 5'-cccaggataccgcagaacta-3', reverse 5'- gaaggtgtcctttccgacaa-3', VLCAD; forward 5'-gtggccgctttctgtctaac-3', reverse 5'-ccttcgttcgaaacctagtc-3'.

Muscle homogenate preparation

Thirty mg of skeletal muscle was isolated from the *vastus lateralis* of healthy moderately active men (N=12) and women (N=11), and used for protein content analysis. All subject data has been published (Maher et al, 2009 submitted). All subjects gave informed 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.

Muscle tissue was homogenized in a phosphate lysis buffer; 50mM K_2HPO_4, 1mM EDTA, pH7.4, 0.1mM DDT, PhosSTOP (Roach Diagnostics, Mannheim, Germany), Protease inhibitor cocktail tablets (Roach). Protein concentrations were calculated by Bradford assay (Bio-Rad). Equal concentrations of muscle homogenate from each subject were pooled to give an accurate account of total human skeletal muscle LCAD content. Human liver homogenate (ab29889) and human heart homogenate (ab29431) were purchased from Abcam Inc. (Cambridge, MA).

Muscle mitochondrial preparation

Human mitochondria were isolated from the *vastus lateralis* muscle of a 30 year old female who was an elite endurance runner. Mouse mitochondria were isolated from the *quadriceps* of a male wild-type C57BL/6J (breed in-house McMaster University Medical Centre Animal Facilities). Animal care followed strict guidelines put forth by Canadian Council of Animal Care and McMaster University Animal Research Ethics Board. Human isolated liver mitochondria and human isolated heart mitochondria were acquired from MitoSciences (MitoSciences Inc., Eugene, OR).

Mitochondrial isolation protocol was as follows: Fresh muscle was rinsed in ice cold wash buffer (PBS plus 10mM EDTA pH7.4), and 100 mg of wet weight muscle was weighed out and added to 2mls of homogenization buffer A (67mM Sucrose, 50mM Tris, 50mM KCl, 10mM EDTA, 0.2% BSA, pH7.4). Muscle was minced using a Polytron, then transferred to a Dounce homogenizer and homogenized. Homogenate was centrifuged at 700x g for 15 min at 4 degrees. Supernatant was transferred to a fresh tube and centrifuged at 12000x g

for 20 min. at 4 degrees. The pellet was washed once in 1.5 mls buffer B (250mM Sucrose, 3mM EGTA, 10mM Tris, pH7.4) and re-suspended in 75 µl of buffer B, flash frozen and stored at -80 degrees.

Immunoblotting analysis

Protein homogenate were boiled in Laemmli buffer, resolved by SDS-PAGE, transferred to PVDF. Protein was immunoblotted with purified recombinant human LCAD antibody (3) and secondary anti-rabbit antibody conjugated to horseradish peroxidase (Amersham Bioscience, UK). Specific antibody binding was detected using Millipore Immobilon™ Western chemiluminescent HRP substrate (Millipore Corp., Billerica, MA). Scanned films were analyzed using ImageJ 1.40 software (Wayne Rasband National Institute of Health, USA) for quantification of protein.

Results

One hundred micrograms of purified RNA was used to synthesis cDNA. When cDNA was diluted 1:10 for RT-PCR amplification LCAD was virtually undetectable. RT-PCR amplification of undiluted cDNA yielded a small but detectable amount of LCAD in human skeletal muscle, which was significantly less abundant than MCAD and VLCAD (Figure 1).

Comparison of LCAD protein content in human skeletal muscle using a human recombinant LCAD antibody was challenging. Initial examination of 10, 20, 40 and 80 µg of human muscle homogenate protein yielded no observable bands. Comparison of mouse muscle homogenate and isolated mitochondrial protein from mouse muscle yielded an abundant band at the predicted 43 KDa. To rule out antibody species specificity western blot comparison of 10 µg of isolated human heart and liver mitochondria to 30 µg isolated mitochondria from skeletal muscle of human and mouse showed that the antibody was not species specific and LCAD was expressed in human liver mitochondria, to a lesser extent in human heart mitochondria, and almost undetectable in human skeletal muscle mitochondria (Figure 2). When we compared 20ug of whole liver, heart and skeletal muscle homogenate, LCAD was detectable in human liver and was virtually undetectable in human heart and skeletal muscle (Figure 3).

Discussion

In order to fully understand the process of FAO in skeletal muscle we set out to examine LCAD expression in human skeletal muscle. Previous studies have shown that MCAD and VLCAD are highly expressed in human skeletal muscle and exercise training increases the expression of these enzymes (10). Our data showed that LCAD is so lowly expressed in human skeletal muscle that it cannot readily be detected in whole muscle homogenate. We were able to detect the protein in isolated skeletal muscle mitochondria from an elite endurance athlete, who we would expect to have the highest abundance, confirming that LCAD is expressed in skeletal muscle but in low amounts. The protein

expression is consistent with the mRNA expression of LCAD in skeletal muscle being low but detectable in high enough concentrations. Previous studies show that of all the tissues skeletal muscle has the highest number of expressed sequence tags (EST) counts albeit hardly detectable (5). Taken together, our results indicate that LCAD has very low expression in human skeletal muscle.

The functional importance of LCAD in human FAO has been developing. Until 1992 it was thought that all LC-FA's were oxidized via LCAD, and mutations in the LCAD gene were the cause of FAO disorders primarily resulting in muscle dysfunction with myopathy and/or cardiomyopathy (18). In 1992 Izai and coworkers identified VLCAD (12), which lead to the recognition that VLCAD is the main if not exclusive enzyme involved in palmitate β-oxidation (17) and LCAD may only oxidize branched chain acyl-CoA substrates (3, 17). It was shortly after the discovery of VLCAD that fibroblast derived from patients thought to have LCAD deficiency were found to have normal LCAD protein and mRNA yet palmitate oxidation was severely deficient (11, 19), and thus these patients were ultimately diagnosed with VLCAD deficiency (4). Currently there are no reported cases of LCAD deficiency (2, 19). To try and elucidate the role of LCAD in metabolism knockout mouse models have been developed; however, studies using LCAD and VLCAD knockout mice are not strictly representative of human FAO disorders as both enzymes share a role in the oxidation of long chain fats in this species. The phenotype of $LCAD^{-/-}$ mice have a more sever phenotype than $VLCAD^{-/-}$ mice, and the $LCAD^{-/-}$ mice display more of the metabolic symptoms similar to human VLCAD deficiency (6, 14). Although enzyme assays reveal that in knockout mice VLCAD or LCAD can functionally compensate for loss of the other, leading to a less sever phenotype, compared with human VLCAD deficiency where oleic acid oxidations is ablated causing sever disease (5). Recently, Chegary *et. al.*, compared EST counts in mice compared to humans and found that mice have an equal abundance of EST transcripts for LCAD and VLCAD in all tissues; whereas, VLCAD EST transcripts in humans were approximately double that of the mouse in most tissues, and LCAD transcripts were virtually undetectable (5). Taken together, this data supports that LCAD is not functionally necessary for substrate utilization in humans.

To validate the effectiveness of our reagents and corroborate our findings, we were able to detect LCAD in mice as previously reported (14). Although protein coding regions of LCAD are well conserved (15), database searches reveal that there are significant differences in the LCAD promoter region between mice and humans (15, 20), and the number of microRNA's estimated to regulate LCAD expression, most likely through inhibition, is significantly higher in human (18 microRNA) compared with mouse (2 microRNA) (http://microrna.sanger.ac.uk). The differences in promoter region and microRNA expression directionally support the difference gene expression, regulation, and apparent function of LCAD in mice and humans and suggest that genetic alterations occurred after evolutionary divergence of rodent and primate.

To further corroborate our findings, we were able to detect LCAD in human whole liver tissue homogenate as previously reported (1, 5). The higher expression of LCAD in human liver could be due to the unique intrinsic function of the organ to metabolize FFA's to produce ketone bodies, an important fuel for extra-hepatic organs, specifically brain, heart, skeletal muscle and kidney, during periods of starvation. Although, LCAD has been shown to have specific activity towards branched chain acyl-CoA substrates of varying chain length (3), further studies into the liver's ability to utilize straight-chain versus branch-chain fatty acids specially during periods of metabolic stress are warranted. Lastly, we could not detect LCAD in human heart tissue as previously reported (1, 5), although we were able to detect LCAD in isolated mitochondria from human heart tissue.

In conclusion, LCAD is in such low abundance in skeletal and cardiac muscle that the functionality of LCAD is likely minor or redundant in human fatty acid metabolism, and likely has little contribution to substrate metabolism during exercise.

Acknowledgements

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Figure legends

Figure 1. mRNA expression of LCAD is barely detectable in human skeletal muscle. Real-time RT-PCR expression of VLCAD, LCAD and MCAD genes in young moderately active men and women (N=6).

Figure 2. LCAD protein expression in isolated mitochondria. LCAD protein is detectable in 10 μg of isolated mitochondria from human heart and liver homogenate (lanes 1&2). Thirty μg of isolated mitochondria from human skeletal muscle was required to quantify LCAD expression (lane 3). Thirty μg of isolated mitochondria from mouse quadriceps muscle was loaded as a comparison (lane 4). The quantified bands were normalized for 10 μg of protein to give an estimated abundance of LCAD in the tissues compared (see graph). LCAD expression in mouse skeletal muscle is approximately 16 times that of human skeletal muscle.

Figure 3. LCAD protein expression in tissue homogenate. LCAD protein content in 20 μg of tissue homogenate from human heart, liver, and skeletal muscle (lanes 1-3). Human and mouse skeletal muscle mitochondria (20μg) are shown (lanes 4&5) to give an appreciation of LCAD abundance.

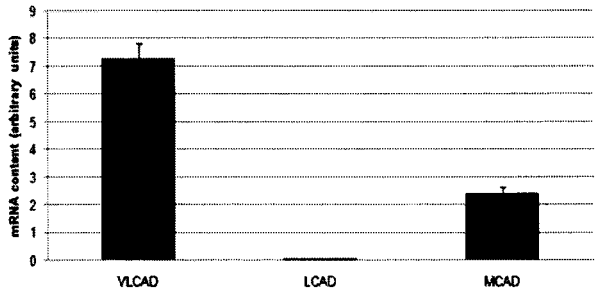


Figure 1.

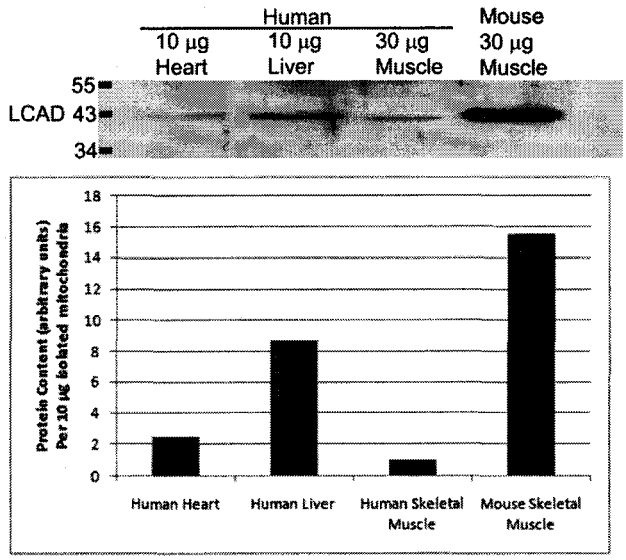
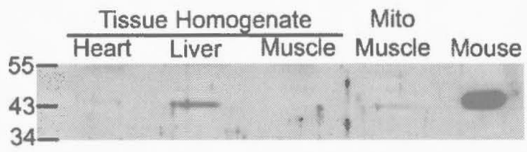

Human
Mouse
10 µg Heart
10 µg Liver
30 µg Muscle
30 µg Muscle
55
LCAD 43
34
Protein Content (arbitrary units) Per 10 µg isolated mitochondria
18
16
14
12
10
8
6
4
2
0
Human Heart
Human Liver
Human Skeletal Muscle
Mouse Skeletal Muscle

Figure 2.


Tissue Homogenate
Mito
Heart
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Muscle
Muscle
Mouse
55
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34

Figure 3.

Chapter 4

Manuscript 3

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Women have higher protein content of β-oxidation enzymes in skeletal muscle than men

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Running header: Sex influences β-oxidation

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Abstract

Women oxidize more fat during endurance exercise as compared with men. Several groups have shown that the mRNA and protein content of genes related to transport of fatty acids into the muscle is convincingly greater in women than men; however, the mechanism(s) for the observed sex differences in fat oxidation remains to be determined. Muscle biopsies from the *vastus lateralis* were obtained from moderately active men (N=12) and women (N=11) at rest to examined mRNA and protein content of genes involved in lipid oxidation. Our results demonstrate that women have significantly higher protein content for tri-functional protein alpha (TFPα), very long chain acyl-CoA dehydrogenase (VLCAD), and medium chain acyl-CoA dehydrogenase (MCAD). There was no significant sex difference in the expression of short-chain hydroxyacyl-CoA dehydrogenase (SCHAD), or peroxisome proliferator activated receptor (PPAR)-α, or PPARγ, genes involved in the transcriptional regulation of lipid metabolism. In conclusion, women have more protein content of the major enzymes involved in long and medium chain fatty acid oxidation which could account for the observed differences in fat oxidation during exercise. Furthermore, these differences appear to be post-transcriptionally regulated.

Key words: sex differences, lipid metabolism, tri-functional protein, acyl-CoA dehydrogenase

Introduction

A number of studies have shown that women have a lower respiratory exchange ratio (RER) compared with men indicating higher lipid oxidation and lower carbohydrate (CHO) oxidation during moderate intensity endurance exercise (1-11). Furthermore, women have higher whole body lipolysis and greater skeletal muscle uptake of plasma free fatty acids (FFA) (12), higher intramyocelluar lipid (IMCL) content (13-16) and higher net IMCL utilization (10, 17), as compared to men during endurance exercise. The potential mechanism(s) of such sex differences have only recently been evaluated in human based research (11, 18, 19), but include sex based differences in gene expression at the transcriptional and post-transcriptional levels, sex differences in response to an acute bout of endurance exercise, and/or hormonal regulation of pathways involved in metabolism. Several studies have shown that sex differences alone are greater predictors of substrate selection than are age, menstrual cycle phase, 17β-estradiol supplementation, endurance and strength training effects on mRNA content (20-22).

Skeletal muscle from women shows higher mRNA and protein content of fatty acid transporter (FAT/CD36) (19), and hormone sensitive lipase (HSL) (10) than in men. Women also have higher mRNA expression of lipoprotein lipase (LPL) (19), membrane fatty acid transport protein 1 (FATm) (18), plasma membrane fatty acid binding protein (FABPpm) (19), CPT I (23), trifunctional protein-β (TFPβ) (Maher et al 2009), peroxisome proliferator activated receptor-α and δ (PPARα, PPARδ), cytosolic fatty acid binding protein (FABPc), sterol regulatory element binding protein –1c (SREBP-1c), and mitochondrial glycerol phosphate acyltransferase (mtGPAT) (22) than men. Taken together, these results suggest that women are transcriptionally programmed for greater fatty acid transport into the skeletal muscle, β-oxidation, and IMCL synthesis than men.

Lipid oxidation occurs in the mitochondria through β-oxidation. Fatty acids (FAs) enter the mitochondria by transporters CPTI, CPTII and FAT/CD36 (24) or diffusion, depending on chain length. Women have significantly higher mRNA for CPTI than men; however, there is no significant sex difference in CPT1 protein or activity (23). Recent evidence suggests that there are FAT/CD36 transporters on the mitochondrial membrane (25) and although sex differences in the mitochondria specific population have not been examined women have significantly greater FAT/CD36 protein in whole muscle homogenate compared with men (26), which may allow for greater mitochondrial uptake of long chain fatty acids (LCFAs) in women (24).

There are four major classes/steps of enzymes in the β-oxidation pathway; acyl-CoA dehydrogenases, enoyl-CoA hydratases, 3-hydroxyacyl-CoA dehydrogenases, and 3-ketoacyl-CoA thiolases (27). Sex based differences of β-oxidation enzymes have only recently been studied. The mRNA content of β-hydroxyacyl-CoA dehydrogenase (β-HAD, also known as short-chain β-OH acyl-CoA dehydrogenase (SCHAD)) is greater in women compared with men; however, there is no sex difference in β-HAD activity (11). Similarly, women

have higher levels of acyl-CoA acyltransferase 2 (ACAA2) mRNA with no sex specific difference in protein content(28). Both β-HAD (SCHAD) and ACAA2 are responsible for short chain FA oxidation, but long chain- and medium chain-acyl-CoA oxidation has not been compared between the sexes. Due to the convincing evidence that women have greater whole body FA oxidation and uptake of FAs during exercise than men, we hypothesized that there would be sex differences in the protein capacity for LCFA oxidation in human skeletal muscle.

The purpose of this study was to comprehensively evaluate the effect of sex on the mRNA expression and protein content of genes involved in β-oxidation (VLCAD, MCAD, TFP-α, SCHAD) and regulators of these proteins (PPARα, and PPARγ) in human skeletal muscle. We specifically hypothesized that the mRNA and protein content for the genes involved in lipid metabolism would be higher in women than men.

Material and Methods

Subjects. Twenty-three, young (22 ± 2 y) healthy, non-smoking, non-obese, recreationally active men (n = 12) and women (n = 11) participated in the current study. The subject characteristics are described in Table 1. Men and women were matched based on peak O_2 consumption (VO_2peak) expressed as milliliters per kilogram fat-free mass per minute. Women were in the follicular phase of their menstrual cycle (day 3-13). All subjects gave informed 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. At least one week prior to testing, subjects completed a whole body DEXA scan and progressive VO_{2peak} test on a stationary electronically braked cycle ergometer and a computerized open-circuit gas collection system (Moxus Modulator VO_2 system with O_2 analyzer S-3A/I and CO_2 analyzer CD-3A, AEI Technologies Inc., Pittsburgh, PA) as previously described (1, 5, 7, 8). Subjects completed diet records for three days leading up to testing, and did not exercise 48 h before testing. Subjects were asked to refrain from eating after 2100 h the day before testing and were given a defined formula drink (Ensure Plus®, Abbott Laboratory Inc., Saint-Laurent, Quebec, Canada) 2 h before baseline blood and muscle biopsies were acquired. All samples were taken in the morning. Blood was collected and resting glucose and lactate levels were determined using a blood-gas analyzer (Radiometer ABL800 FLEX, Copenhagen, Denmark). Serum estradiol levels were evaluated using a human estradiol ELISA kit; Fertigenix-E2-EASIA (Biosource Europe S.A, Nivelles, Belgium). Muscle biopsies (~150 mg) were taken from the *vastus lateralis* muscle before, immediately after, and 3 h after, 90 min of stationary cycling at 65% of their VO_{2peak}. The muscle was rapidly placed in an RNase-free polyethylene tube, flash-frozen in liquid nitrogen, and stored at –80°C until being processed for

analysis. Respiratory measures (oxygen uptake, expired CO2, and RER) were taken at 30 and 60 min using the computerized open-circuit gas collection system and averaged together to represent RER over 30 min in the middle of the exercise bout. Fat and glucose oxidation rates were determined using the non-protein respiratory quotient (29), and percentage fat and glucose utilized was determined as previously described (2).

Preparation of RNA. Fifty mg of human muscle was used to isolate mRNA using an Ambion mirVana™ miRNA isolation kit (Ambion Inc., Austin, TX #AM1561). In brief, muscle tissue was homogenized in Lysis/Binding buffer in a glass homogenizer. The RNA was extracted organically using acid-phenol:chloroform and ethanol precipitation. Final isolation was done using provided filter cartridges. RNA was eluted in nuclease-free water and quantity and quality of RNA was assessed using a NanoDrop Spectrophotometer (Thermo Scientific, Wilmington, DE). Measurements were done in duplicate and had an average coefficient of variation (CV) of <10%. The average purity (OD_{260}/OD_{230}) of the samples was > 1.5.

TaqMan® real-time RT-PCR. First-strand cDNA synthesis from 100 μg of total RNA was performed with random primers using Applied Biosystems High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Inc., Foster City, CA Cat#4368814) according to manufacturer's directions. Gene expression was quantified using 7300 Real-time PCR System (Applied Biosystems Inc., Foster City, CA) and SYBR® Green chemistry (PerfeCTa SYBR® Green Supermix, ROX, Quanta BioSciences, Gaithersburg, MD) as previously described (30). Specific primers to each target mRNA (Table 2) were designed based on the cDNA sequence in GenBank (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi) with MIT primer 3 designer software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Thermal dynamics was optimized through calculating delta G with Analyzer of Oligo (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/Default.aspx). Primer specificity was checked using Blast (http://www.ncbi.nlm.nih.gov/BLAST/), and RT-PCR dissociation curves. All samples were run in duplicate on a 96-well plate. Each target gene was run in parallel with human β2- microglobulin (β2-M) as an internal standard with RNA- and RT-negative controls.

Immunoblotting analysis. Thirty mg of tissue was used for protein content analysis. Muscle tissue was homogenized in a phosphate lysis buffer; 50mM K_2HPO_4, 1mM EDTA, pH7.4, 0.1mM DDT, PhosSTOP (Roach Diagnostics, Mannheim, Germany), Protease inhibitor cocktail tablets (Roach). Protein concentrations were calculated by Bradford assay (Biorad) and equal amounts of protein were boiled in Laemmli buffer, resolved by SDS-PAGE, transferred to nitrocellulose paper or PVDF and immunoblotted with desired antibodies.

Primary antibodies; VLCAD (Dr. J. Vockley), MCAD (Dr. J. Vockley), HADHA (Protein Tech Group, Chicago, IL), SCHAD (Abcam), PPARα (Santa Cruz), PPARγ (Cell Signaling), actin (Abcam) and citrate synthase(CS) (Dr. B. Robinson, Sick Kids Hospital, Toronto). There were no sex differences in protein content of citrate synthase (mitochondrial content) or actin (total myofibrillar and cytosolic protein loading). CS was used as a loading control for VLCAD, MCAD, HADHA, and SCHAD. Actin was used as a loading control for PPARα and PPARγ. Secondary antibodies conjugated to horseradish peroxidase (Amersham Bioscience, UK) and specific antibody binding was detected using the chemiluminescence detection reagent ECL Plus (Amersham BioScience, UK). Scanned films were analyzed using ImageJ 1.40 software (Wayne Rasband National Institute of Health, USA).

Statistical Analysis. Statistical analyses on 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 to β2-M (31). Statistical analysis of gender differences in mRNA and protein content (±SE) were calculated using a Student's t-test. Statistical significance was set at $\alpha \leq 0.05$.

Results

Subject Characteristics

Women had significantly lower body weight, height, fat free mass (FFM), and VO_{2peak} per kilogram body weight ($P<0.05$) compared with men (Table 1). When VO_{2peak} was expressed relative to FFM there was no significant difference between men and women. There was no significant difference in resting serum estradiol, glucose or lactate between men and women (Table 3). During exercise women had a significantly lower RER (0.87 ± 0.02) than men (0.91 ± 0.01)($P<0.05$) (Table 4).

Sex differences in key enzymes of the β-oxidation pathway

Women had a 2.5 ± 0.4 fold ($P=0.001$) higher protein content of VLCAD compared with men (Figure 1B,C). There was no sex difference in the mRNA abundance of VLCAD (Figure 1 A). Women had a 2.6 ± 0.6 fold ($P=0.007$) higher protein content of MCAD than men (Figure 2 B,C). There was no sex difference in the mRNA abundance of MCAD (Figure 2 A). Women had a 1.5 ± 0.1 fold ($P<0.001$) higher protein content of TFP-α than men (Figure 3B,C). Women had a significantly lower abundance (-1.4 ± 0.1 fold $p=0.004$) of TFP-α mRNA compared with men (Figure 3A). There was no sex difference in the mRNA abundance or protein content of SCHAD (Figure 4).

Regulation of lipid metabolism

There was no sex difference in the protein content of PPARα and PPARγ (Figure 5); two transcription factors involved in the regulation of mitochondrial FA oxidation proteins.

Disscussion

This study shows that women have a higher protein content of VLCAD, MCAD and HADHA (also known as trifunctional protein α) than men, suggesting that women have a higher capacity for β-oxidation of LCFAs. Previous research has also shown that there is a sex difference in FATm mRNA (18), FABPpm protein and mRNA, FAT/CD36 protein and mLPL (19) these results indicate that as compared with men, women at rest and during exercise, have a higher capacity for fatty acid transport. Taken together, these results suggest women have a higher capacity than men for LCFA utilization; including skeletal muscle up-take and β-oxidation within mitochondria at rest and during exercise. These results are in agreement with our hypothesis and directly support previous observations that women oxidize more fat than men during endurance exercise (1-9, 15, 32, 33).

We conducted a comparative analysis by matching men and women based on their aerobic capacity expressed per unit fat-free mass (Vo_{2peak}·kg FFM^{-1}·min^{-1}). We (1, 6-8, 34-38) and others (2, 3, 39) consistently match men and women using Vo_{2peak} per kg FFM per min because endurance training is known to alter substrate utilization, increasing reliance on lipid sources and reducing the reliance on carbohydrates (5, 40, 41). Furthermore, we controlled for mitochondrial content by comparing CS protein content and observed no sex difference in total amount. Taken together with previous findings that matched men and women have similar mitochondrial volume densities (34) and enzyme activities of CPT1 (23) and 3-beta-hydroxyacyl CoA dehydrogenase, citrate synthase, succinate-cytochrome c oxidoreductase, and cytochrome c oxidase (34) we believe our paired subjects to be well matched.

Women, having a higher abundance of VLCAD and MCAD than men, also showed a higher utilization of straight-chain FAs. VLCAD and MCAD are two of the nine identified acyl-CoA dehydrogenases, and have broad chain-length enzyme activity raging from 4 -16 carbon FA (MCAD) and 12 - 24 carbon (VLCAD) (42). Trifunctional protein is a heterooctamer composed of 4α- and 4β-subunits. The alpha subunit, from the HADHA gene (43), has enzyme activity to hydrolyze long-chain enoyl-CoA and oxidize long-chain 3-hydroxyacyl-CoAs of 10 carbon chain-length and greater (44). During exercise, FAs contribute anywhere from 30-70% of substrate utilized (depending on exercise intensity) (45), of which ~90% are LCFAs (46). The percentage of the predominant LCFA to the composition of plasma FFA are ~43% oleate, ~30% palmitate and ~14% stearate (47). This ratio is consistent at rest and does not change significantly during exercise (47) despite the large change in flux. However, LCFA kinetics in skeletal muscle are different than plasma with women showing an increase in IMCL utilization during exercise compared with men (17). Given the higher IMCL content, the close proximity of IMCL's to mitochondria (48), and the significantly greater protein abundance of VLCAD, MCAD and HADHA in women compared to men mechanistically explains the observed sex difference lipid oxidation seen during endurance exercise.

In order to elucidate the mechanism regulating the increase protein content of VLCAD, MCAD and HADHA we looked at protein content of PPARα and PPARγ. Our data shows no significant difference in the total amount of these transcription factors in whole muscle homogenate. Recent work from our lab showed that there is a greater transcriptional activation of the genes responsible for fat oxidation PPARα or PPARδ in women compared with men (28). PPARα is a transcriptional activator of MCAD, CPTI, CPTII, FABP and FATP (49, 50), whereas PPARδ transcriptionally activates FABPc, FAT/CD36, LPL, CPTI, and genes involved in β-oxidation (51-53). Here we show that there is no significant difference between men and women in PPARα or PPARγ protein content. The lack of difference in protein content of PPAR α despite differences in mRNA is consistent with similar findings with PPARδ (28). Although changes in total protein content are not different between men and women, transcription factors rely on nuclear abundance and translocation into the nucleus to elicit changes in RNA expression. Future studies should include sex differences in nuclear abundance of these transcription factors. Also, we cannot negate that recent studies in skeletal muscle biology have also found discrepancies in the correlation between mRNA and protein content of a number of genes related to fatty acid oxidation (10, 26, 54). Part of the discrepancy between mRNA abundance, protein content, and enzyme assays may be that the transcriptome abundance regulates multiple interacting and synergistic pathways that combine to influence flux through metabolic pathways at the protein level that is below the detectable threshold for statistical changes in a single given protein to be manifested. It could also be the technical sensitivity of RT-PCR compared with the higher variance in Western blots and activity assay techniques. Regardless, in order to fully understand cellular differences between men and women it is important to understand pre-translational (mRNA abundance), translational (protein) and post-translational (phosphorylation) levels of control.

One of the possible mechanisms for the observed sex difference could be estrogen effects. Research using rodents (55-57) and humans (35, 37, 58, 59) has suggested that estrogen may play a role in regulating substrate utilization. The administration of E2 to amenorrheic women (59) and to men (35, 37, 58) lowers the respiratory exchange ratio during exercise (35, 37, 58, 59), reduces whole body carbohydrate and leucine oxidation and increases lipid oxidation in men (37), increases plasma FFA concentration (59) and reduces glucose rate of appearance (35, 58, 59), rate of disappearance and metabolic clearance rate (35, 58). In favor of a direct role for estrogen in sex differences in substrate utilization, database searches reveal that MCAD, SCHAD, PPARα, PPARδ, and PPARγ have up-stream estrogen response elements (ERE) (60) suggesting these genes may be regulated by estrogen.

In conclusion, our results show that women have higher VLCAD, MCAD, and HADHA protein than age and fitness matched men. Our findings offer an explanation for the observed sex differences in lipid oxidation. Taken together with the evidence that women have greater FA transport capabilities in skeletal

muscle, we can conclude that women are more efficient at using lipid as a substrate during endurance exercise than men.

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Figure legends

Figure 1. VLCAD protein content is higher in women than men. Differences in VLCAD mRNA content between men and women shown by Real time RT-PCR, adjusted to β2-M mRNA (A). Protein content of VLCAD in skeletal muscle of men and women, adjusted to CS (B,C). Representative western blot (B) Lanes 1-4 are men, lanes 5-8 women. N=12 men and 11 women. *P<0.05.

Figure 2. MCAD protein content is higher in women than men. Differences in MCAD mRNA content between men and women shown by Real time RT-PCR, adjusted to β2-M mRNA (A). Protein content of MCAD in skeletal muscle of men and women, adjusted to CS (B,C). Representative western blot (B) Lanes 1-4 are men, lanes 5-8 women. N=12 men and 11 women. *P<0.05.

Figure 3. HADHA protein content is higher in women than men. Differences in HADHA mRNA content between men and women shown by Real time RT-PCR, adjusted to β2-M mRNA (A). Protein content of HADHA in skeletal muscle of men and women, adjusted to CS (B,C). Representative western blot (B) Lanes 1-5 are men, lanes 6-10 are women. N=12 men and 11 women. *P<0.05.

Figure 4. SCHAD protein content is equal in women and men. SCHAD mRNA content between men and women shown by Real time RT-PCR, adjusted to β2-M mRNA (A). Protein content of SCHAD in skeletal muscle of men and women, adjusted to CS (B,C). Representative western blot (B) Lanes 1-4 are men, lanes 5-8 women. N=12 men and 11 women.

Figure 5. No sex differences in PPARα or PPARγ protein content. Protein content of PPARα (A) and PPARγ (B) in skeletal muscle of men and women, adjusted to actin. Representative western blots; lanes 1-4 are men, lanes 5-8 women. N=12 men and 11 women.

Table 1. Subject characteristics

	Men (n=12)	Women (n=11)
Age (yr)	21 ± 1	20 ± 1
Weight (kg)	83 ± 5	66 ± 1 a
Height (cm)	181 ± 3	167 ± 1 a
Body Fat (%)	17 ± 2	32 ± 1 b
Fat Mass(kg)	14 ± 2	20 ± 6 b
Fat Free Mass (kg)	66 ± 3	42 ± 1 a
VO_2peak (ml*kg body wt-1*min-1)	44 ± 2	39 ± 2 a
VO_2peak(ml*kg FFM-1*min-1)	56 ± 2	60 ± 3

Data are means ±SE. a; significantly lower in women $P<0.05$, b; significantly higher in women $P<0.05$.

Table 2. Real-time PCR primer sequences

Gene	Accession	Forward primer (5'-3')	Reverse primer (5'-3')
VLCAD	BC012912	gtggccgctttctgtctaac	ccttcgttcgaaacctagtc
MCAD	AF251043	tgccagagaggaaatcatcc	tctcggacccttgaaccaaa
HADHA	BC009235	gagttgacccgaagaagctg	aaccacacctacatcgcttt
SCHAD	BC000306	cgttgtccacagcacagact	gacctgttcaaacgacgact
β2M	NM_004048	ggctatccagcgtactccaa	gatgaaacccagacacatagca

Table 3. Resting blood glucose, lactate, and estradiol levels in men and women

	Men	Women
Glucose (mmol/L)	4.95 ± 0.39	4.63 ± 0.16
Lactate (mmol/L)	1.16 ± 0.18	0.96 ± 0.11
Estradiol (pg/ml)	50.1 ± 4.5	61.2 ± 5.8

Data are means ±SE. Estradiol intra-assay CV<7%.

Table 4. Substrate oxidation in men and women cycling at 65% VO_{2peak}

	Men	Women
RER	0.91 ± 0.01	0.87 ± 0.02 *
Fat oxidation (g/min)	0.36 ± 0.06	0.38 ± 0.05
Glucose oxidation (g/min)	2.36 ± 0.14	1.31 ± 0.12 *
Fat oxidation (%)	29.6 ± 3.5	45.0 ± 4.9 *
Glucose oxidation (%)	70.4 ± 3.5	55.0 ± 4.9 *

Data are means ±SE, RER; respiratory exchange ratio, *P<0.05.

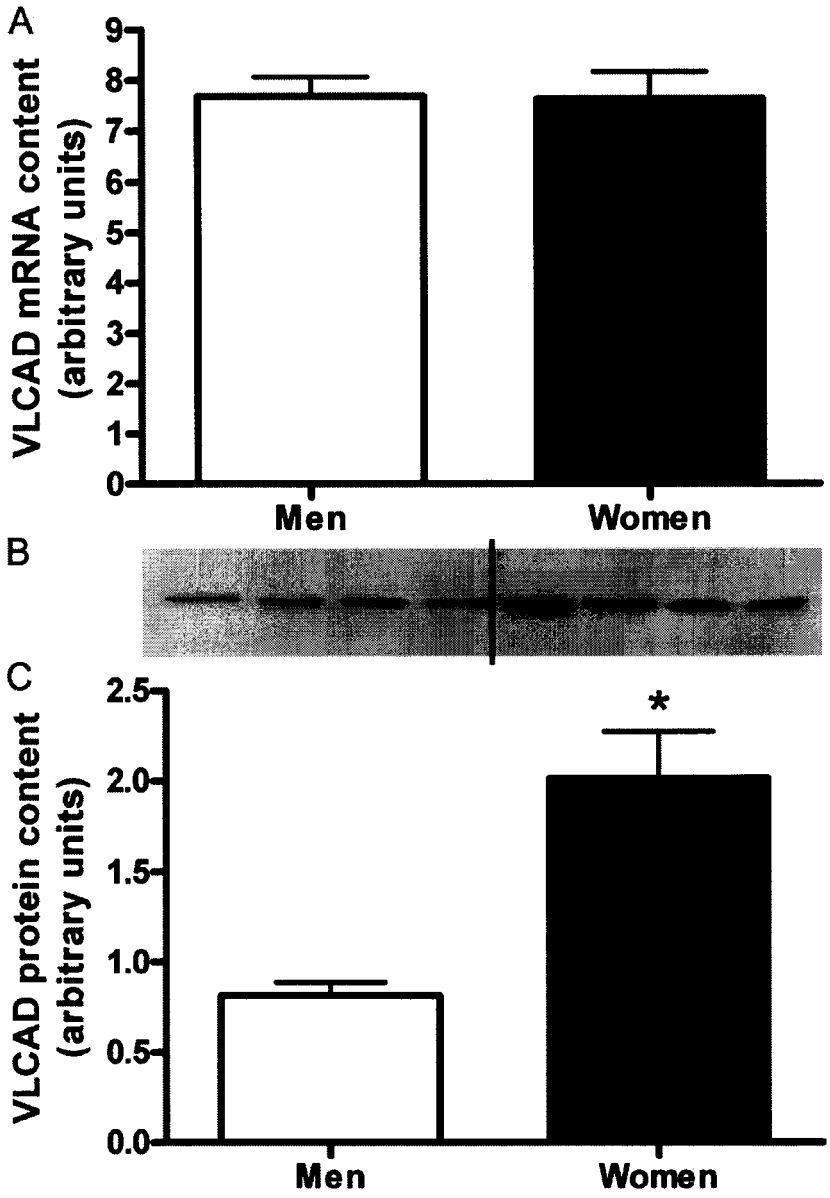
A
VLCAD mRNA content (arbitrary units)
9
8
7
6
5
4
3
2
1
0
Men
Women
B
C
VLCAD protein content (arbitrary units)
2.5
2.0
1.5
1.0
0.5
0.0
*
Men
Women

Figure 1

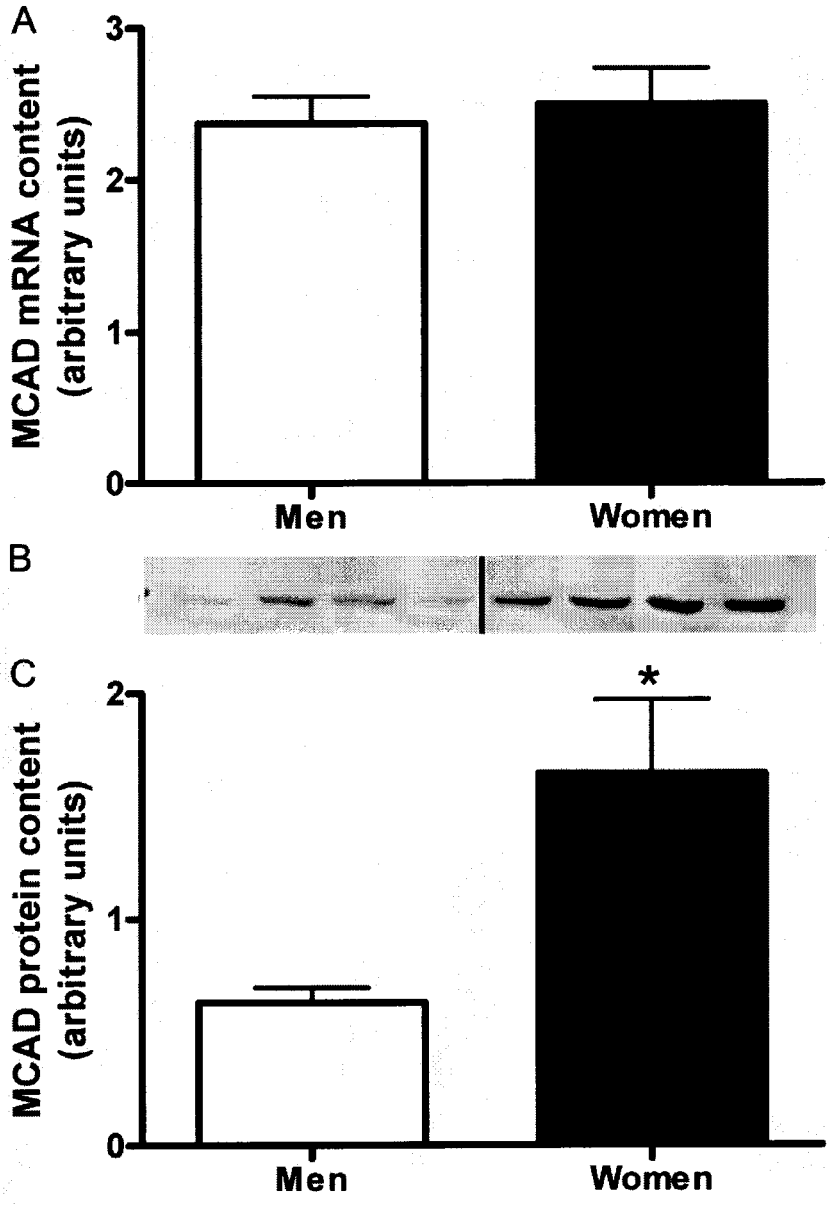
A
MCAD mRNA content
(arbitrary units)
3
2
1
0
Men
Women
B
C
MCAD protein content
(arbitrary units)
2
1
0
*
Men
Women

Figure 2

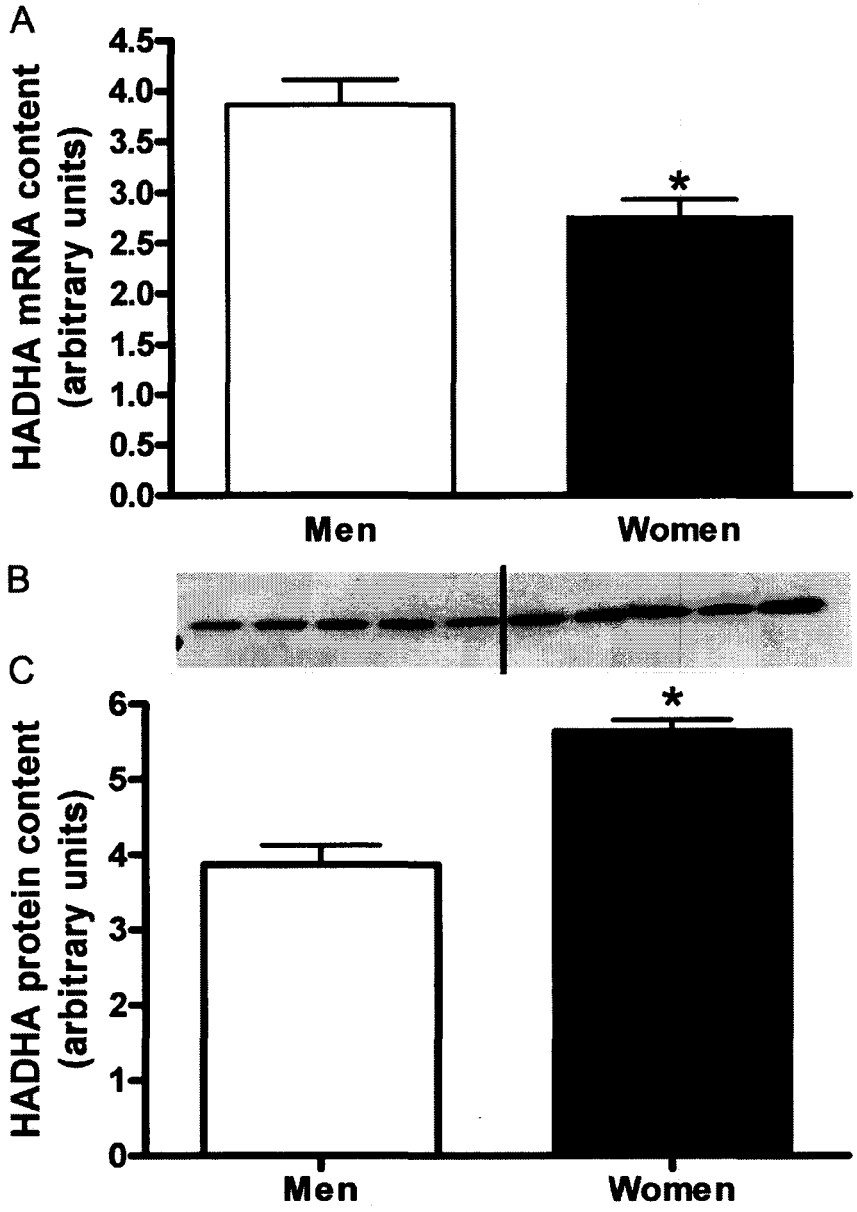
A
HADHA mRNA content
(arbitrary units)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
Men
Women
B
C
HADHA protein content
(arbitrary units)
6
5
4
3
2
1
0
*
Men
Women

Figure 3

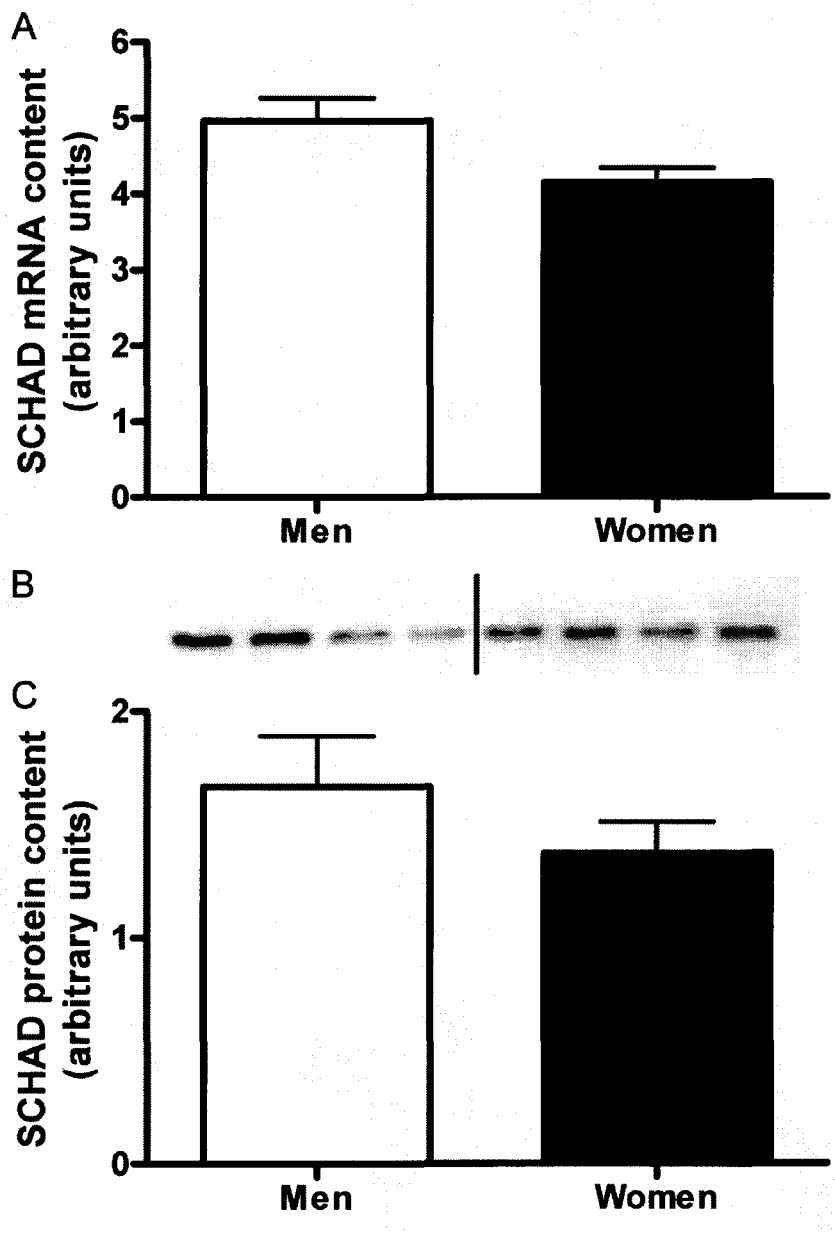
A
SCHAD mRNA content
(arbitrary units)
6
5
4
3
2
1
0
Men
Women
B
C
SCHAD protein content
(arbitrary units)
2
1
0
Men
Women

Figure 4

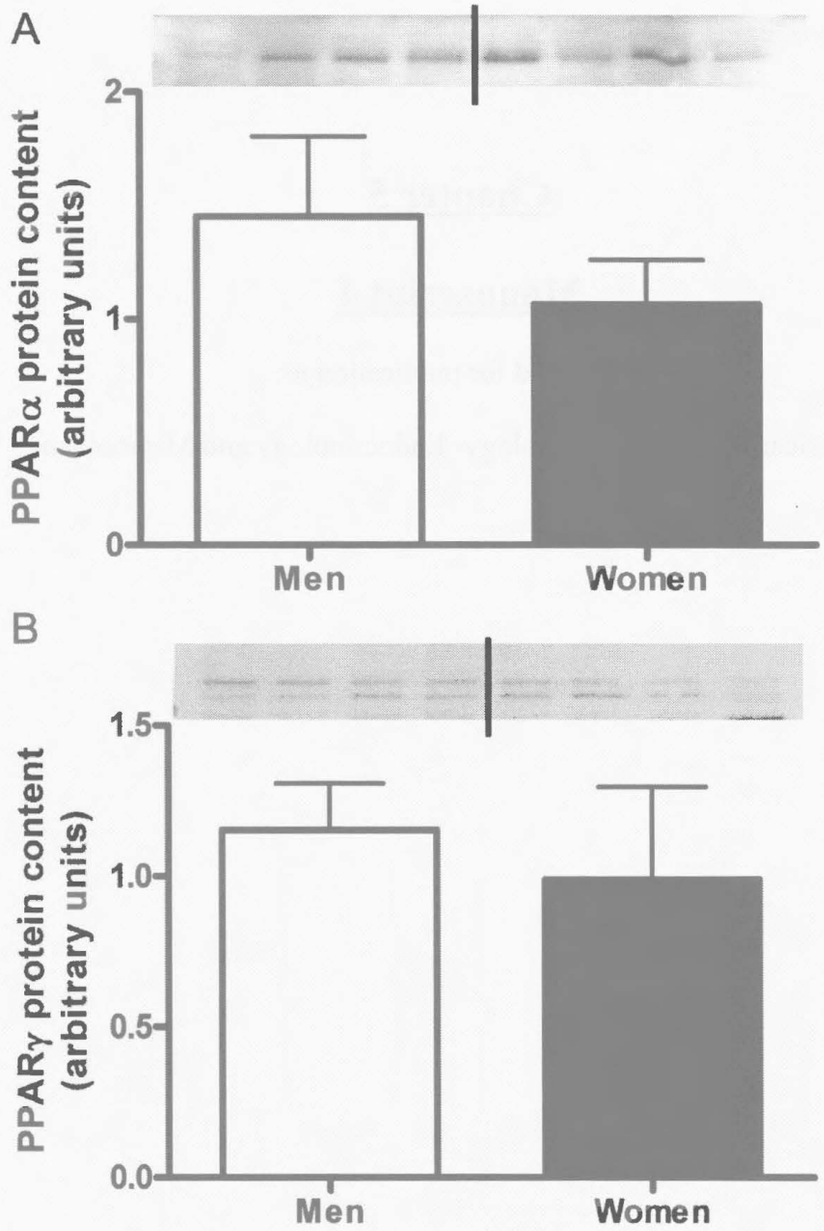
A
PPARα protein content (arbitrary units)
2
1
0
Men
Women
B
PPARγ protein content (arbitrary units)
1.5
1.0
0.5
0.0
Men
Women

Figure 5

Chapter 5

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Men supplemented with 17β-estardiol have increased β-oxidation capacity

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Running header: Estrogen and lipid oxidation

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ABSTRACT

During endurance exercise women have lower carbohydrate and higher lipid oxidation compared with men. Supplementation of humans and rodents with 17β-estradiol (E2) lowers the respiratory exchange ratio, glucose rate of appearance and disappearance, and metabolic clearance rate. The mechanism(s) for the observed estrogen effects in substrate utilization remains to be determined; however, we hypothesized that estrogen would increase the mRNA content for genes involved in β-oxidation. Ten moderately active men were supplemented with placebo or E2 for 8 days in a randomized double-blind cross-over design. Following supplementation, men cycled for 90 min at 65% VO_{2peak} and muscle biopsies were obtained from the *vastus lateralsis* and examined for differences in mRNA, microRNA and protein content of genes involved in lipid oxidation. E2 increased the protein abundance of medium chain acyl-CoA dehydrogenase, and tri-functional protein alpha ($P<0.05$). PGC-1α mRNA was significantly higher after E2 supplementation ($P<0.05$), and microRNA 103 and 29b (predicted to regulate PGC1α) were significantly lower ($P<0.05$). In conclusion, E2 regulates lipid metabolism in skeletal muscle by pre-translational modifications of factors involved in β-oxidation.

Key words: human, skeletal muscle, lipid metabolism, mRNA, protein, microRNA

INTRODUCTION

A number of studies have shown that women have a lower respiratory exchange ratio (RER) compared with men (7, 13, 17, 24, 29, 36, 37, 40, 41, 46, 47). Furthermore, women have higher whole body lipolysis and greater skeletal muscle uptake of plasma free fatty acids (FFA) (31), higher intramyocelluar lipid (IMCL) content (35, 39, 43, 49) and greater net IMCL utilization (40, 42). At the molecular level, there is growing evidence that women are genetically "primed" for greater fatty acid transport into the skeletal muscle, IMCL synthesis and β-oxidation efficiency. For example, resting skeletal muscle of women has; higher mRNA and protein content of fatty acid transporter (FAT/CD36) (22), and hormone sensitive lipase (HSL) (40); higher mRNA expression of lipoprotein lipase (LPL) (22), membrane fatty acid transport protein 1 (FATm) (3), plasma membrane fatty acid binding protein (FABPpm) (22), CPT I (2), trifunctional protein-β (TFPβ) (Maher et al 2009), peroxisome proliferator activated receptor-α and δ (PPARα, PPARδ), cytosolic fatty acid binding protein (FABPc), sterol regulatory element binding protein –1c (SREBP-1c), and mitochondrial glycerol phosphate acyltransferase (mtGPAT) (48), as compared with men. A clear understanding of the mechanism(s) for these observed sex differences remains unknown.

One potential mechanism is the hormonal regulation of metabolic pathways. Research using rodents (15, 20, 21) and humans (6, 9, 14, 44), have both suggested that estrogen may play a role in regulating substrate utilization. The administration of E2 to amenorrheic women (44) and to men (6, 9, 14) lowers RER during exercise (6, 9, 14, 44), reduces whole body carbohydrate and leucine oxidation and increases lipid oxidation in men (14), increases plasma FFA concentration (44), and reduces glucose rate of appearance (6, 9, 44), rate of disappearance and metabolic clearance rate (6, 9). At the molecular level, animals supplemented with 17β-estradiol have higher skeletal muscle enzyme activity of lipoprotein protein lipase (11), carnitine palmitoyl transferase-1, and short-chain β-3-OH-acyl-CoA-dehydrogenase (βHAD) (5). These results have yet to be confirmed in humans supplemented with E2, although there has been no observed sex differences in βHAD (29), CPT1 (2) or LPL (23) activity between men and women. In humans, E2 has also been shown to increase the mRNA abundance of PPARα, PPARδ, CPT1, SREBP-1c, TFP-α, GLUT4, and glycogen synthase (48). Taken together, these data suggest that E2 might be involved primarily in regulating genes and enzymes involved in lipid utilization.

The purpose of this study was to comprehensively evaluate the potential mechanism by which E2 alters substrate utilization in human skeletal muscle. We did a targeted approach specifically comparing mRNA expression and protein content of genes involved in β-oxidation (VLCAD, MCAD, TFP-α, SCHAD) and mitochondrial biogenesis (PGC-1α, PPARα, and PPARδ) in human skeletal muscle. We also examined changes in microRNA (miR-103, -107, -29b, -23b, -24), which has been hypothesized to regulate some of the genes involved in lipid metabolism (53). We specifically hypothesized that the mRNA and protein

content for the genes involved in lipid metabolism would be higher in men after E2 supplementation as compared with their placebo state, with a decrease in microRNA species involved in their regulation.

MATERIAL AND METHODS

Subjects. Ten, young healthy, non-smoking, non-obese, recreationally active men participated in the current study. The subject characteristics are described in Table 1. All subjects gave informed 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. At least 10 days prior to testing subjects completed a whole body DEXA scan and progressive VO_{2peak} test on a stationary electronically braked cycle ergometer and a computerized open-circuit gas collection system (Moxus Modulator VO_2 system with O_2 analyzer S-3A/I and CO_2 analyzer CD-3A, AEI Technologies Inc., Pittsburgh, PA) as previously described (7, 29, 37, 46). Subjects were randomly assigned in a double-blind crossover fashion to receive either placebo (400mg/day Polycose, Abbott Laboratories, St. Laurent, Quebec) or E2 (1mg/day for 2 days, 2mg/day for 6 days)(Estrance; Shire BioChem, Inc., St. Laurent, Quebec). Subjects supplemented for 8 days prior to testing in which they were asked to maintain normal activity and nutrition. Forty-eight hours before testing subjects completed diet records, which were returned to them for the second arm of testing so nutrition could be the same. Subjects were asked not to exercise 48hr before testing and refrain from eating after 2100 h the day before testing. In the morning subjects were given a controlled beverage (Ensure Plus®, Abbott Laboratory Inc., Saint-Laurent, Quebec, Canada) 2 h before baseline blood and muscle biopsies were acquired. Water was given adlib throughout the testing. All testing was done in the morning. Blood was collected and resting glucose and lactate levels were determined using a blood-gas analyzer (Radiometer ABL800 FLEX, Copenhagen, Denmark). Serum estradiol levels were evaluated using a human estradiol ELISA kit; Fertigenix-E2-EASIA (Biosource Europe S.A, Nivelles, Belgium). Muscle biopsies (~150 mg) were taken from the *vastus lateralis* muscle before, immediately after, and 3 h after, 90 min of stationary cycling at 65% of their VO_{2peak}. The muscle was rapidly placed in an RNase-free polyethylene tube, flash-frozen in liquid nitrogen, and stored at –80°C until being processed for analysis. Respiratory measures (oxygen uptake, expired CO2, and RER) were taken at 30 and 60 min using the computerized open-circuit gas collection system and averaged together to represent RER over 30 min in the middle of the exercise bout. Fat and glucose oxidation rates were determined using the non-protein respiratory quotient (34), and percentage fat and glucose utilized was determined as previously described (13).

Preparation of RNA/microRNA. Fifty mg of human muscle was used to isolate mRNA and microRNA using an Ambion mirVana™ miRNA isolation kit (Ambion Inc., Austin, TX #AM1561). In brief, muscle tissue was homogenized in lysis/binding buffer in a glass homogenizer. The RNA was extracted organically using acid-phenol:chloroform and ethanol precipitation. Final isolation was done using provided filter cartridges. RNA was eluted in nuclease-free water and quantity and quality of RNA was assessed using a NanoDrop Spectrophotometer. Measurements were done in duplicate and had an average coefficient of variation (CV) of <10%. The average purity (OD_{260}/OD_{230}) of the samples was > 1.5.

TaqMan® real-time RT-PCR. First-strand cDNA synthesis from 100 ng of total RNA was performed with random primers using Applied Biosystems High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Inc., Foster City, CA Cat#4368814) according to manufacturer's directions. Gene expression was quantified using 7300 Real-time PCR System (Applied Biosystems Inc., Foster City, CA) and SYBR® Green chemistry (PerfeCTa SYBR® Green Supermix, ROX, Quanta BioSciences, Gaithersburg, MD) as previously described (27). Specific primers to each target mRNA (Table 2) were designed based on the cDNA sequence in GenBank (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi) with MIT primer 3 designer software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Thermal dynamics was optimized through calculating delta G with Analyzer of Oligo (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/Default.aspx). Primer specificity was checked using Blast (http://www.ncbi.nlm.nih.gov/BLAST/), and RT-PCR dissociation curves. All samples were run in duplicate on a 96-well plate. Each target gene was run in parallel with human β2- microglobulin (β2-M) as an internal standard with RNA- and RT-negative controls.

MicroRNA expression analyses. The miRNA expression was quantified in real-time using TaqMan® miRNA assays for human microRNA (hmiR) -103, -107, -23b, -24, and -29b according to the manufacturer's instructions (Applied Biosystems Inc., Foster City, CA). Briefly, reverse transcriptase (RT) reactions were performed with microRNA-specific RT primers and 5 ng of total microRNA for 30 min at 37°C followed by 10 min incubation at 95°C to inactivate the RT enzyme. End-point PCR was then performed using the RT product and microRNA-specific PCR primers for 40 cycles (two steps: 95°C for 15 s followed by 60°C for 30 s). Human *Rnu6b* (U6 small nuclear RNA TaqMan® miRNA assay) was used as endogenous control for miRNA expression analyses.

Immunoblotting analysis. Thirty mg of tissue was used for protein content analysis. Muscle tissue was homogenized in a phosphate lysis buffer; 50mM K_2HPO_4, 1mM EDTA, pH7.4, 0.1mM DDT, PhosSTOP (Roach Diagnostics,

Mannheim, Germany), Protease inhibitor cocktail tablets (Roach). Protein concentrations were calculated by Bradford assay (Biorad) and equal amounts of protein were boiled in Laemmli buffer, resolved by SDS-PAGE, transferred to PVDF membrane and immunoblotted with desired antibodies. Primary antibodies; VLCAD (Dr. J. Vockley), MCAD (Dr. J. Vockley), HADHA (Protein Tech Group, Chicago, IL), SCHAD (Abcam), actin (Abcam) and citrate synthase(CS) (Dr. B. Robinson, Sick Kids Hospital, Toronto). There was no effect of E2 on the protein content of CS or actin (data not shown). CS was used as a control for mitochondrial content. Actin and ponseau staining were used to confirm equal protein loading. Secondary antibodies conjugated to horseradish peroxidase (Amersham Bioscience, UK) and specific antibody binding was detected using the chemiluminescence detection reagent ECL Plus (Amersham BioScience, UK). Scanned films were analyzed using ImageJ 1.40 software (Wayne Rasband National Institute of Health, USA).

Statistical Analysis. Statistical analyses on mRNA and microRNA expression of the genes tested were performed on linearized $2^{-\Delta Ct}$ normalized with internal reference to human β2-microglobulin (β2M) and human Rnu6b respectively. Statistical analysis of RER and indirect calorimetry, and protein content differences were calculated using a paired Student's t-test. Linear regression was carried out to define correlations. Statistical significance was set at $P \leq 0.05$. Data are presented as the means ± standard error (SE).

RESULTS

Hormone concentrations, RER and metabolism

Eight days of supplementation with E2 significantly increased (90 ± 8 pg/ml) circulating plasma levels of E2 in men from baseline (50 ± 5 pg/ml) (Figure 1a). During exercise men supplemented with E2 had a significantly lower RER (0.89 ± 0.01) compared with placebo (0.91 ± 0.01)($P<0.05$) (Figure1b). Total fat oxidation significantly increased with E2 supplementation (Figure 1b), which significantly increased the percent energy from lipid (31 ± 4% vs 39 ± 4%, p=0.05), and reduced the percent energy from CHO (69 ± 4% vs 60 ± 4%, p=0.05).

E2 regulation of β-oxidation pathway enzymes

Eight days of supplementation with E2 significantly increased the protein content of MCAD (2 fold, p=0.05) (Figure 3B), which significantly correlated with [E2] (R=0.44, P=0.03). There was a strong trend for E2 supplementation to increase HADHA (1.7 fold. P=0.08) (Figure 4B), which also trended to correlate with [E2] (R=0.30, p=0.09). Eight days of E2 supplementation in men had no effect on the protein content of VLCAD or SCHAD (Figure 2B, 5B). E2 supplementation also had no effect on mRNA content for MCAD, HADHA, VLCAD and SCHAD (Figure 2A, 3A, 4A, 5A).

Pre-translational changes in metabolic regulation

Eight days of E2 supplementation significantly increased PGC-1α mRNA content (Figure 6A), but did not alter the mRNA content of PPARα (Figure 6B) or PPARδ (Figure 6C). miR-29b expression, a putative regulator of PGC-1α mRNA translation (53), was significantly decreased (3 fold, p=0.007), and there was a very strong trend for a reduction in miR-103 (1.5 fold, p=0.06), another putative regulator of PGC-1α mRNA translation (53)(Table 3).

DISCUSSION

Results of this study show that E2 supplementation in men for eight days alters the protein content of β-oxidation enzymes MCAD and HADHA, increases PGC-1α mRNA and decreases microRNA-103, -29b content. These findings suggest that E2 mechanistically influences pre-translational regulation of factors involved in lipid metabolism. In addition these findings, in combination with other observed difference in whole body substrate utilization due to E2 (9, 14), support the hypothesis that E2 directly or indirectly alters lipid metabolism in human skeletal muscle.

Eight days of E2 supplementation slightly but significantly lowered RER during cycling at 65% VO_{2peak}. These findings are consistent with other studies where the administration of E2 to amenorrheic women (44) and to men (6, 9, 14) lowered the respiratory exchange ratio during exercise (6, 9, 14, 44). We found, using respiratory measures, that lipid utilization was significantly increased and CHO utilization was decreased after supplementation with E2. These indices have been also been studied using tracer methods, whereby E2 supplementation reduced whole body carbohydrate and leucine oxidation and increased lipid oxidation in men (14), increased plasma FFA concentration (44) and reduced glucose rate of appearance (6, 9, 44), rate of disappearance and metabolic clearance rate (6, 9). Taken together, these results show E2 supplementation in humans influences whole body substrate utilization.

We show that MCAD and HADHA protein content was higher after 8 days of E2 supplementation. MCAD and HADHA are two of the main enzymes involved in the oxidation of long-chain fatty acids (LC-FA). MCAD has a broad chain-length enzyme activity raging from 4 -16 carbon FA (19). The alpha subunit, from the HADHA gene (TFPα) (54), has enzyme activity to hydrolyze long-chain enoyl-CoA and dehydrate long-chain 3-hydroxyacyl-CoA of 10 carbon chain-length and greater (50). Of the total contribution of FFA to oxidation, ~90% are LC-FA (16), which would mechanistically explain the observed increase in lipid oxidation in men after E2 supplementation. MCAD is predicted to have up-stream estrogen response elements (ERE) (4), suggesting this gene may be directly regulated by estrogen, especially given the significant correlation between [E2] and protein abundance. Albeit, there was no difference in mRNA abundance, indirectly implying that the effects of E2 on MCAD and HADHA content are post-transcriptionally regulated; however, it is plausible that mRNA

expression increased earlier on during supplementation, increased protein content, and returned to baseline by day eight. These results are consistent with previous findings showing that there are significant sex difference in the protein abundance, but not mRNA, of MCAD and HADHA in women compared with men (Maher *et., al.*, 2009, submitted to FASEB). Taken together, these data suggest that the observed sex difference in lipid metabolism maybe in part be hormonally regulated.

In order to elucidate the mechanism regulating the increase protein content of MCAD and HADHA, we looked at the mRNA expression of PGC-1α, PPARα and PPARδ. Our data showed a significant increase in PGC-1α mRNA with no significant difference in the total amount of mRNA for PPARα and PPARδ. PGC-1α is a transcriptional co-activator of PPARα, which in turn increases gene expression of enzymes involved in mitochondrial fatty acid beta-oxidation including MCAD (28, 51). PPARδ has also been shown to transcriptionally activate genes involved in β-oxidation (10, 12, 26). Although we did not observe difference in PPARα mRNA expression it has been shown that increased PGC-1α expression along, even in the absence of PPARα mRNA induction, can still increase the expression of PPARα target genes through co-activation (51). It is also likely PGC-1α is up-regulating MCAD by co-activation of estrogen related receptor α (ERRα), given that ERRα is up-regulated by E2 (25), and is a promoter of MCAD (45). Of further note, although no differences in PPARα and PPARδ were observed, these transcription factors rely on nuclear abundance and translocation into the nucleus to elicit changes in their corresponding genes. Future studies should include the effect of E2 on differences in nuclear abundance of these transcription factors.

MicroRNA's are a class of small regulatory molecules that modulate protein production by binding to their associated mRNA and degrading (32) or inhibiting (18) mRNA translation. Bioinformatics and literature reviews suggest that miR-23b, -24, -29b, -103, -107 paralogs may regulate multiple mRNA in the metabolic pathways (53), as listed in part in Table 3. All five miR have been predicted to regulate PGC-1α. Given the observed increase in PGC-1α and the reduction in miR-103 and -29b after E2 supplementation, it is directionally consistent that these two miR's could be regulating metabolism in this manner. Furthermore, miR-103 sequences are found within introns of the gene that encodes for pantothenate kinase (PANK) 2 and 3 (1, 53); enzymes are the rate limiting step in generating Co-enzyme A (CoA) (38). The role of miR-103 is only predicted at this point and more research is needed to determine specific gene targets. MiR-29b has a broader role in skeletal muscle and is also involved in the regulation of branch chain α-ketoacid dehydrogenase (BCKD) complex which is involved in the metabolism of amino acids (30), muscle cell differentiation (52), and carcinogenesis (33, 52). MiR-29b has been shown to be regulated by NF-κB and Yin Yang 1(YY1)(52). Furthermore, PGC-1α and YY1 have been shown to regulate mitochondrial gene expression (8). Future research is require to determine if the effects of E2 on PGC-1α and microRNA-103 and -29b expression

is direct or indirect, and what other signalling pathways and molecules may be involved.

In summary, our data show that an acute increase in estradiol in men increases the protein content of β-oxidation enzymes, possibly through PGC-1α mediated transcription and microRNA mediated events. These results partially explain the metabolic effects of E2 increasing lipid oxidation in human skeletal muscle. Although future studies are required to fully understand the signalling mechanism of E2, taken together E2 supplementation alters the PGC-1α, miR-29b/-103 pathway which leads to increased mitochondrial gene expression (MCAD and HADHA), and thus lipid utilization.

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Figure legends

Figure 1. Serum estradiol levels, RER, and substrate oxidation in men after 8days of placebo or E2 supplementation. Eight days of E2 supplementation significantly increased serum E2 levels (A). Eight days of E2 supplementation significantly lowered RER (B) during 90 min of cycling at an intensity of 65% VO_{2max}, increasing lipid oxidation (C). Data are means ±SE. N=10, *P<0.05. Estradiol intra-assay CV<7%. PL, placebo; E2, 17β-estradiol; CHO, carbohydrate.

Figure 2. MCAD protein content increases in men after E2 supplementation. Eight days of E2 supplementation had no effect on MCAD mRNA content (A) but significantly increased the abundance of MCAD protein (B). N=10, *P<0.05.

Figure 3. HADHA expression in men after E2 supplementation. HADHA mRNA content in men after eight days of placebo or E2 supplementation shown by Real time RT-PCR, adjusted to β2-M mRNA (A). Protein content of HADHA in skeletal muscle of men, adjusted to CS (B), and representative western blot. T, trend, N=10.

Figure 4. E2 does not alter VLCAD mRNA or protein. Eight days of E2 supplementation had no effect on VLCAD mRNA content shown by Real time RT-PCR, adjusted to β2-M mRNA (A). Protein content of VLCAD in skeletal muscle of men after eight days of E2 or placebo supplementation adjusted to citrate synthase (CS) (B) and depicted with a representative western blot (B). N=10.

Figure 5. SCHAD protein content is equal in women and men. SCHAD mRNA content after eight days of placebo or E2 supplementation shown by Real time RT-PCR, adjusted to β2-M mRNA (A). Protein content of SCHAD in skeletal muscle of men, adjusted to CS (B) and representative western blot (B). N=10 men.

Figure 6. Effects of E2 supplementation on PGC-1α, PPARα, and PPARδ. Eight days of E2 supplementation significantly increases PGC-1α content in skeletal muscle (A). E2 supplementation does not alter PPARα (B) or PPARδ (C) mRNA, adjusted to β2-M. N=10, *P<0.05.

Table 1. Subject characteristics

	Men (N=10)
Age (yr)	21 ± 1
Weight (kg)	87 ± 5
Height (cm)	183 ± 2
Body Fat (%)	18 ± 2
Fat Mass(kg)	14 ± 2
Fat Free Mass (kg)	68 ± 3
VO_2peak (ml*kg body wt-1*min-1)	43 ± 2
VO_2peak(ml*kg FFM-1*min-1)	55 ± 2

Data are means ±SE.

Table 2. Real-time PCR primer sequences

Gene	Accession	Forward primer (5'-3')	Reverse primer (5'-3')
VLCAD	BC012912	gtggccgctttctgtctaac	ccttcgttcgaaacctagtc
MCAD	AF251043	tgccagagaggaaatcatcc	tctcggacccttgaaccaaa
HADHA	BC009235	gagttgacccgaagaagctg	aaccacacctacatcgcttt
SCHAD	BC000306	cgttgtccacagcacagact	gacctgttcaaacgacgact
PGC-1α	AF106698	catcaaagaagcccaggtaca	catacgtgttgagtcgttcagg
PPARα	NM_001001928	ttcgactcaagctggtgtatg	agtgttcacggaaagacagc
PPARδ	NM_006238	atccgcatgaagctggagta	atgacggcgaaggtcttcac
β2-M	NM_004048	ggctatccagcgtactccaa	gatgaaacccagacacatagca

VLCAD, very-long chain acyl-CoA dehydrogenase; MCAD, medium chain acyl-CoA dehydrogenase; HADHA, trifunctional protein alpha subunit; SCHAD, short chain hydroxyacyl-CoA dehydrogenase; PGC-1α, peroxisome proliferator activated receptor-γ coactivator-1α; PPAR, peroxisome proliferator activated receptor; β2-M, β2-microglobulin.

Table 3. The influence of 17β-estradiol on microRNA expression in human skeletal muscle.

MicroRNA species	Predicted regulated genes	E2 effects (Fold)
miR-103	PGC-1α, SCHAD, PDK4, PANK2	-1.5 ± 0.1 P=0.06
miR-107	PGC-1α, SCHAD, CPT1β, PANK1	NS
miR-29b	PGC-1α, PPARδ, PDK1, BCKD	-3.0 ± 0.3 P=0.007
miR-23b	PGC-1α, ACAA2	NS
miR-24	PGC-1α	NS

All data expressed as fold ± SE. P, p-value; miR, microRNA; PGC-1α, peroxisome proliferator activated receptor-γ coactivator-1α; SCHAD, short chain hydroxyacyl-CoA dehydrogenase; PDK, pyruvate dehydrogenase kinase; PANK, pantothenate kinase; CPT1β, carnitine palmitoyl transferase Iβ; PPARδ, peroxisome proliferator activated receptor delta; BCKD, branched-chain keto-dehydrogenase; acyl-CoA Acyltranferase.

Figure 1

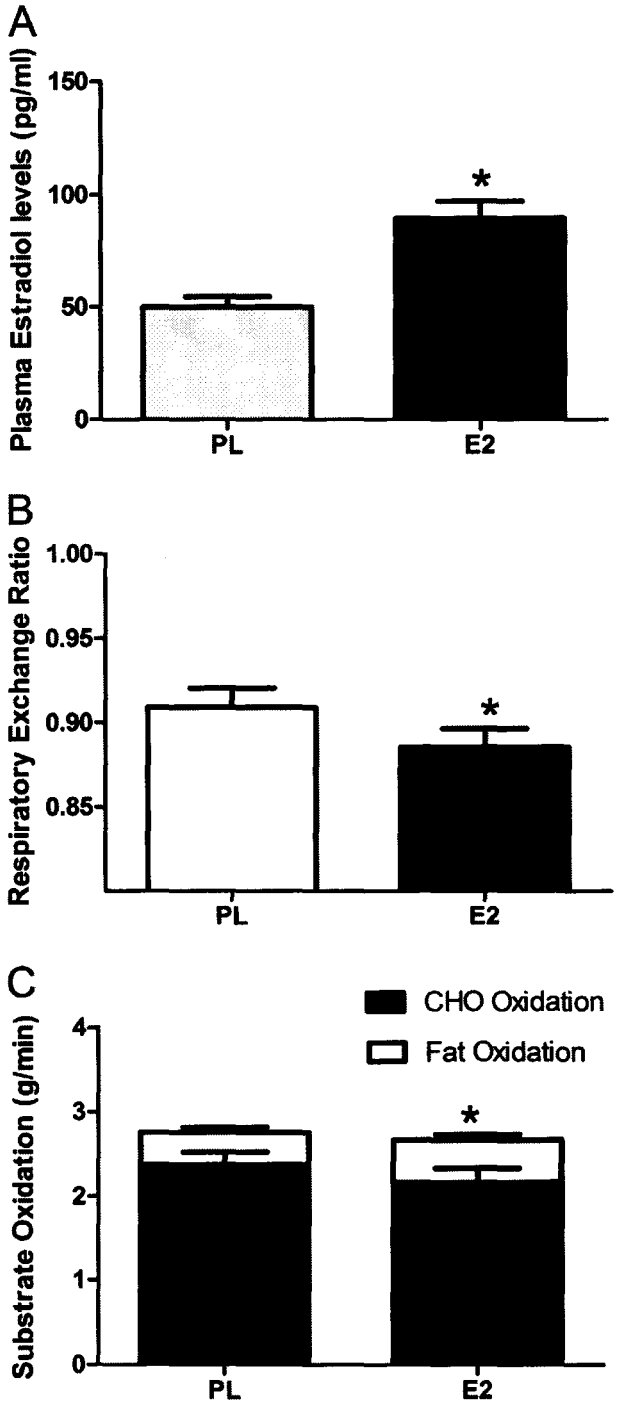
A
Plasma Estradiol levels (pg/ml)
150
100
50
0
*
PL
E2
B
Respiratory Exchange Ratio
1.00
0.95
0.90
0.85
*
PL
E2
C
Substrate Oxidation (g/min)
CHO Oxidation
Fat Oxidation
4
3
2
1
0
*
PL
E2

Figure 1

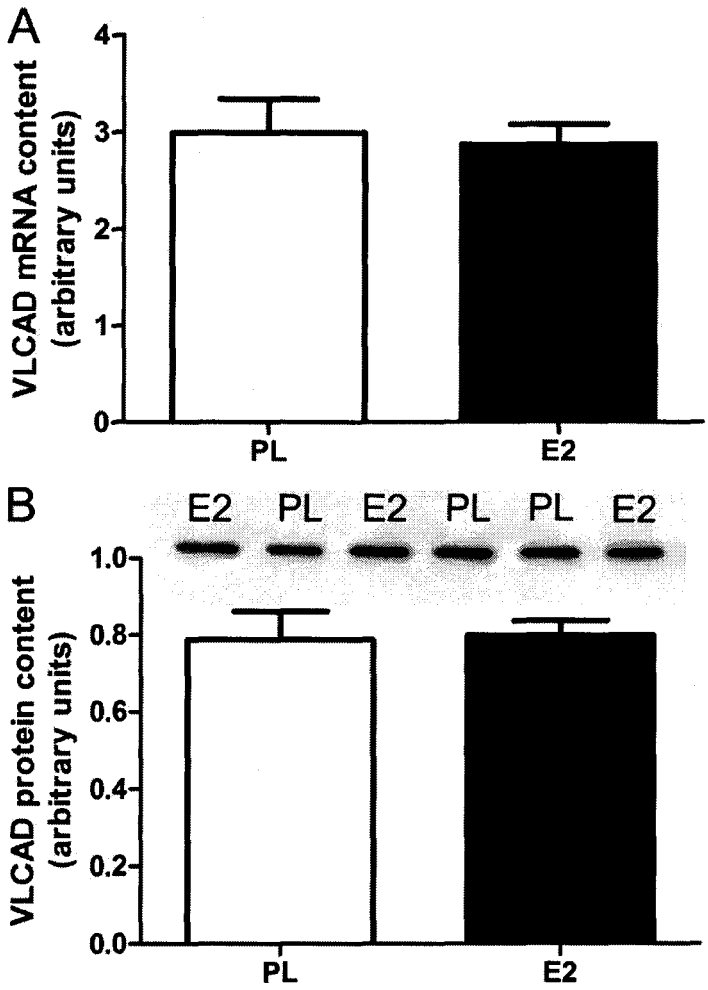

Figure 2

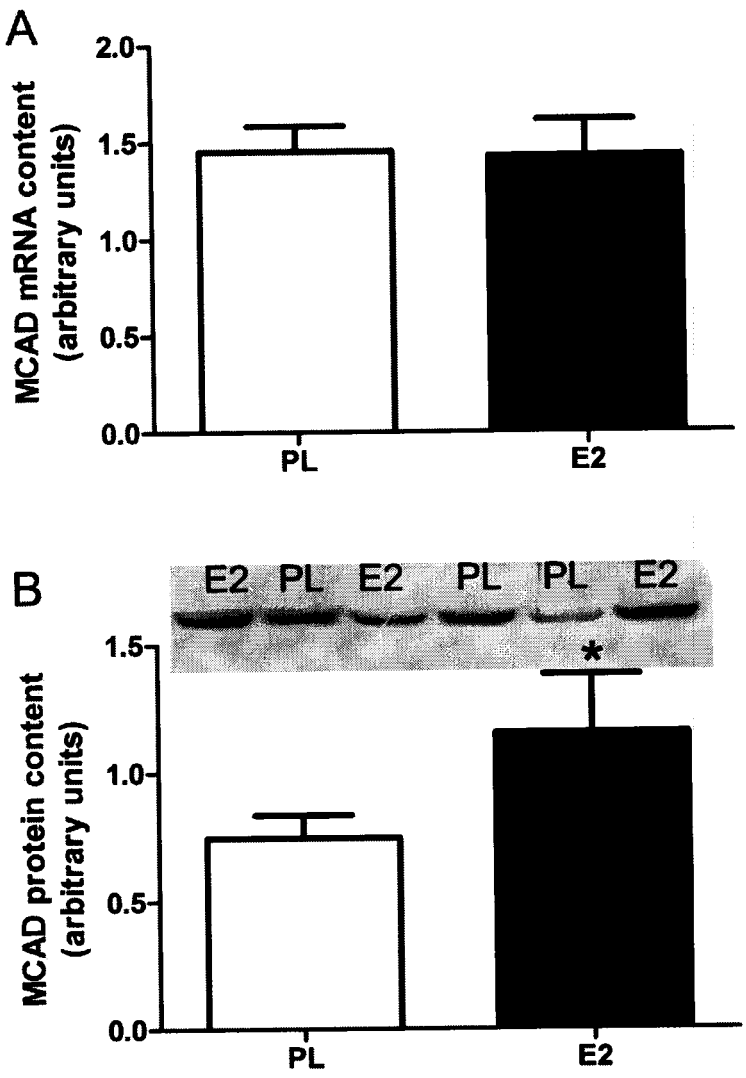
A
MCAD mRNA content (arbitrary units)
2.0
1.5
1.0
0.5
0.0
PL
E2
B
E2 PL E2 PL PL E2
MCAD protein content (arbitrary units)
1.5
1.0
0.5
0.0
*
PL
E2

Figure 3

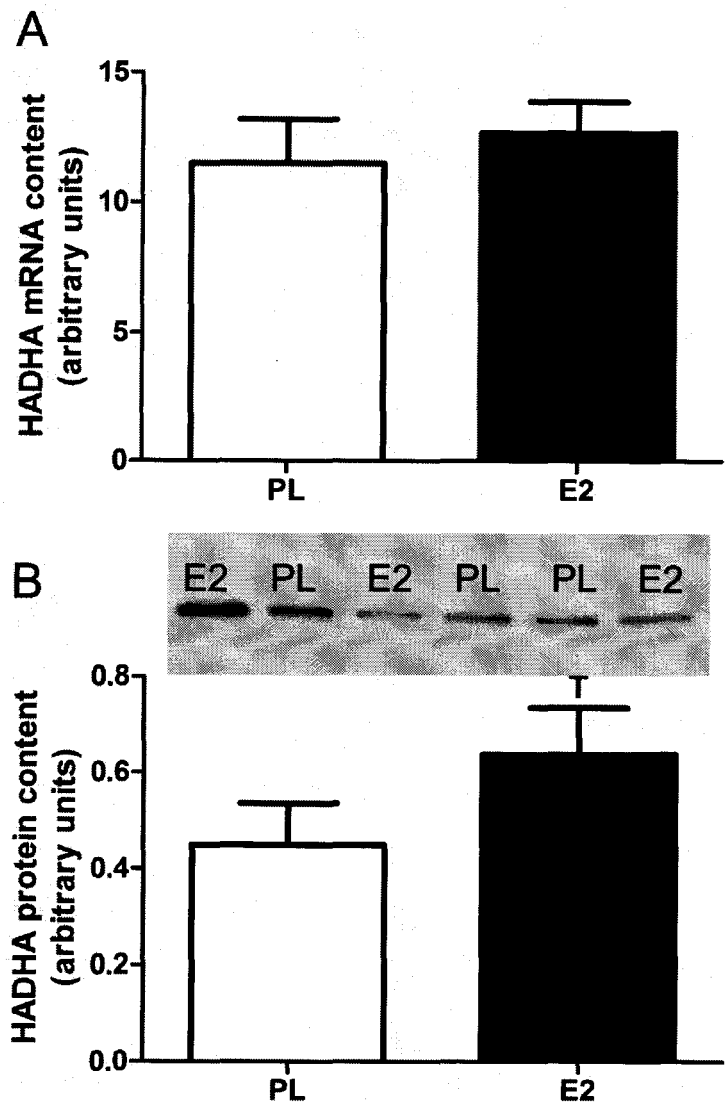
A
HADHA mRNA content (arbitrary units)
15
10
5
0
PL
E2
B
E2 PL E2 PL PL E2
HADHA protein content (arbitrary units)
0.8
0.6
0.4
0.2
0.0
PL
E2

Figure 4

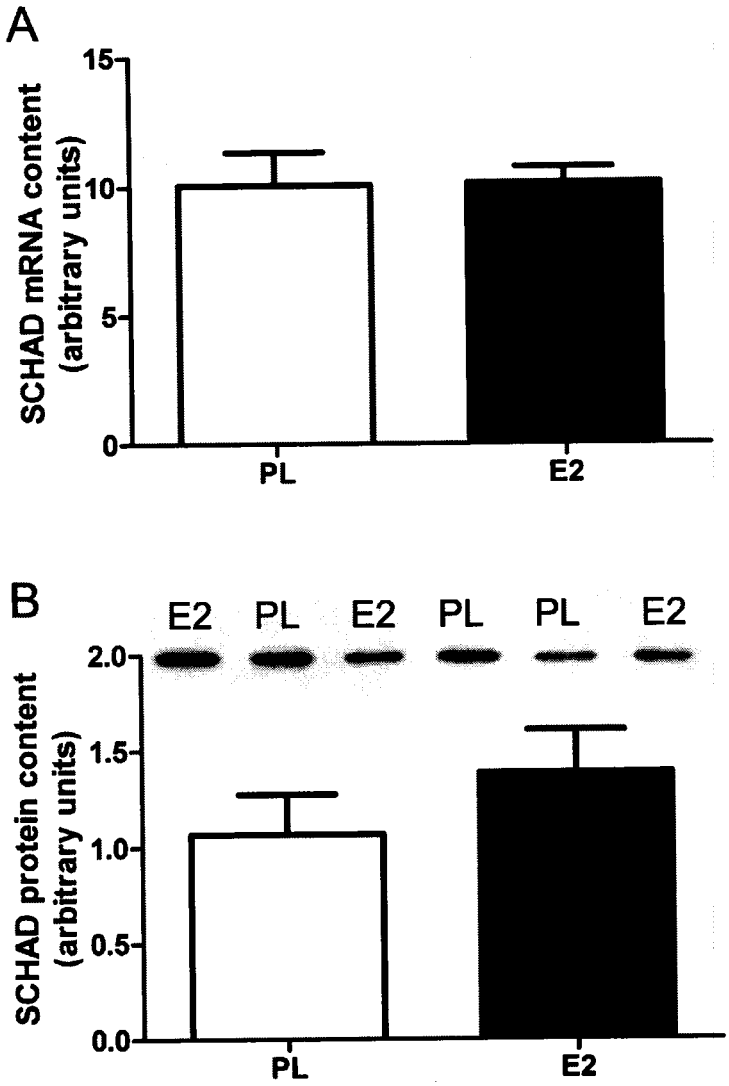
A
SCHAD mRNA content (arbitrary units)
15
10
5
0
PL
E2
B
E2 PL E2 PL PL E2
SCHAD protein content (arbitrary units)
2.0
1.5
1.0
0.5
0.0
PL
E2

Figure 5

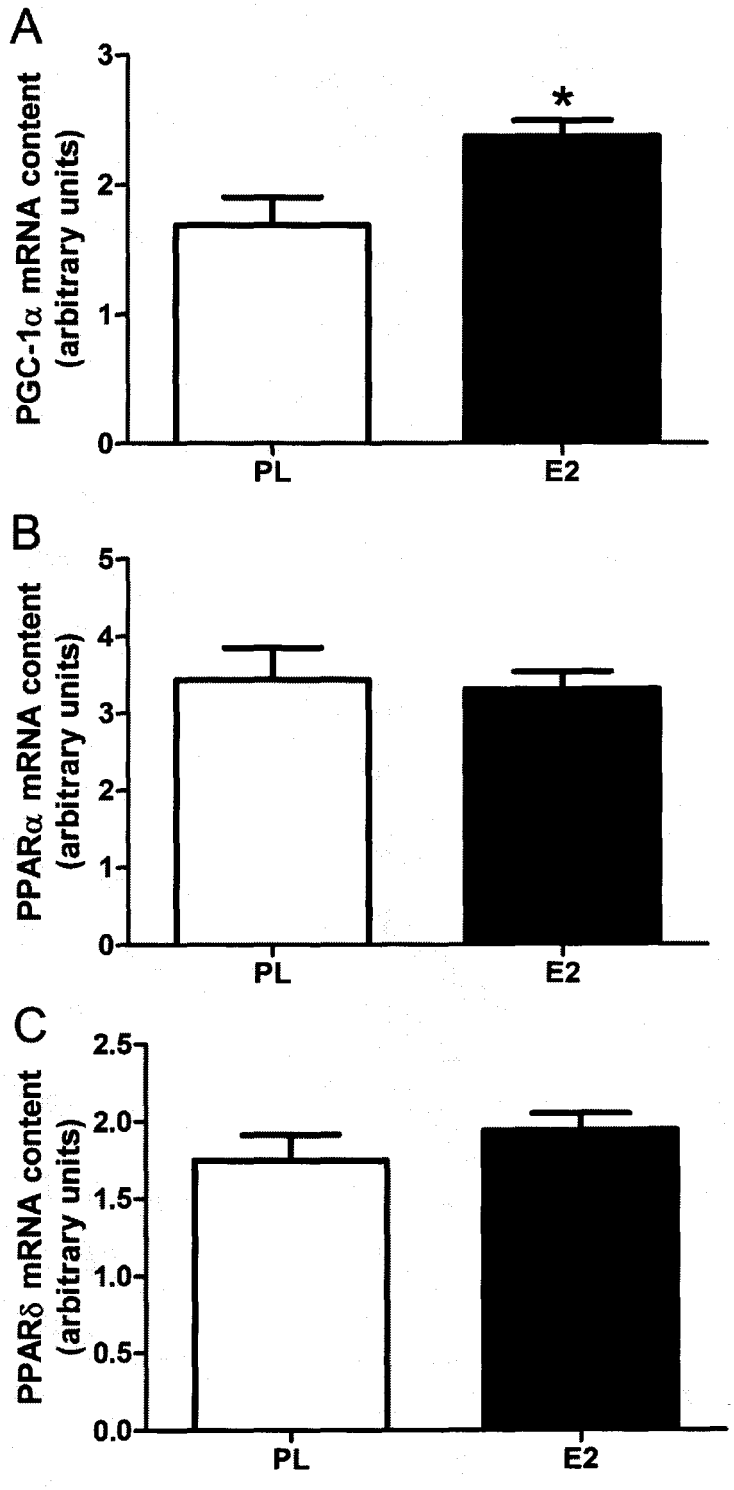
A
PGC-1α mRNA content (arbitrary units)
3
2
1
0
*
PL
E2
B
PPARα mRNA content (arbitrary units)
5
4
3
2
1
0
PL
E2
C
PPARδ mRNA content (arbitrary units)
2.5
2.0
1.5
1.0
0.5
0.0
PL
E2

Figure 6

Chapter 6

General Discussion and Future Directions

6.0 General Discussion

Substrate metabolism, the utilization of lipid and CHO as a source of energy, can be influenced by many factors including; diet, obesity, fitness level, and age. However, even when these factors are controlled for there are still significant differences in whole body substrate utilization between men and women during exercise, characterized by a higher lipid and lower carbohydrate and amino acid oxidation (5, 9, 10, 15, 20, 23, 26, 30, 36, 38). Recent studies have implicated E2 as a possible mechanism by which these sex differences are mediated, due to observed differences in whole body metabolism during different phases of the menstrual cycle (11), menopause and overectomized rodents (8, 18), and in men and rodents supplemented with E2 (4, 16, 21, 29, 31). The exact cellular mechanism by which these changes are occurring has remained a mystery despite isolated findings in one gene or another. Furthermore, there are tissue specific differences in gene expression and pathways involved in metabolism and the organ primarily responsible for regulating metabolic flux has not been ascertained. It has been assumed that skeletal muscle plays a major role in the regulation of substrate metabolism due to its relative size and specific contribution to exercise performance. In this thesis we have provided novel data regarding sex-based differences in lipid metabolism in skeletal muscle. Our findings are supportive of the data demonstrating the well characterized sex-based differences in substrate utilization during exercise, and the role of estrogen in mechanistically mediating the sex differences. Collectively, this thesis has demonstrated that sex and E2 influence the mRNA and protein content of genes involved in fat metabolism, specifically at the level of β-oxidation, in skeletal muscle. These differences support that sex selective utilization of substrate may be mainly defined through regulation of the genes involved in fat metabolism, while CHO and protein metabolism appears to respond to the primary change in fat metabolism. These findings have implications for exercise performance, and nutritional recommendations, and exemplify the importance of continued research in the field of sex differences to optimize pharmacological and health recommendations.

6.1 Implications for Sex Differences in Lipid Utilization

In chapter 2 we show that by unbiased, stringent (NLOGP4) microarray analysis the mRNA abundance for ACAA2, trifunctional protein β, and LPL where significantly higher in women compared with men. Previous research had established that women have a higher mRNA content of FATm, (2), LPL (22), FAT/CD36, FABPpm (22), FABPc, SREBP-1c, mtGPAT, PPARα, PPARδ, CPTI, TFPα, (37), and β-HAD (28), than men. The sex difference in mRNA abundance of the genes involved predominantly in fat metabolism, including a higher capacity for IMCL synthesis, sarcolemma and mitochondrial fatty acid transport and fat oxidation, coordinately supports a higher ability to flux FA towards oxidation during exercise in women as compared to men. There had been observed sex differences in the mRNA content for genes involved in CHO

(GLUT4, HKII (37)) and protein (BCOADK (37)) metabolism: however, our unbiased microarray analysis did not confirm these findings or generate any other mRNA differences involved in CHO and protein oxidation. Our observations, combined with the previous findings, suggest that the observed sex-based differences in substrate utilization in humans during exercise may be predominantly and coordinately defined by the genes involved in fat metabolism, while the metabolism of CHO and protein follows by metabolic demand. We recognize 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, which warrants further investigation.

Gene array results were the first indicator that increased lipid oxidation was occurring at the level of the mitochondria with genes involved in β-oxidation; specifically ACAA2 and TFPβ. Although we were unable to find statistical differences in the protein content of these genes we did find significant differences in the up-stream LCFA enzyme proteins, VLCAD, MCAD, and TFPα (chapter 3). Investigation into the other presumed LCFA enzyme LCAD, lead to the discovery that it is not present in human skeletal muscle and the majority of LCFAs are oxidized through the over-lapping chain-length specificity of VLCAD, MCAD, and TFPα (chapter 4), amplifying the important consequence of greater VLCAD, MCAD, and TFPα in women than men. Inevitably, confirming sex differences in enzyme activity of these proteins would strengthen these findings. Taken together with the finding by Kiens et al (2004) that women have higher protein content for FAT/CD36 and FABPpm (22), in combination with FAT/CD36 and FABPpm expression being directly correlated with the rate of LCFA transport and oxidation in vitro (25), the implication is that women should be able to transport and oxidize a larger amount of FFA in skeletal muscle as compared with men. These findings mechanistically explain the greater lipid oxidation in women compared with men seen during exercise.

To our surprise, we found that steady state mRNA and protein abundance did not correlate. We are not the first to find discrepancies in mRNA vs protein expression of genes involved in lipid metabolism (1, 19, 22, 27). Gene expression is regulated at multiple levels including; transcriptional, pre-translational, and post-translational level. The steady state level of mRNA of a gene is the result of transcription and mRNA degradation/stability (17), which can be studied by employing techniques such as transcription activity assays (24, 34) and mRNA stability assay (14). The role of microRNA in regulating pre-translational gene expression is also in its infancy and microRNA arrays and targeted approaches could be used to investigate these sex differences. Post-translational activity of proteins can also be determined by phosphorylation status and enzyme activity assays.

6.2 E2 as a Mechanism for Selective Substrate Utilization

We observed a significant decrease in RER in men supplemented with E2 (chapter 5). These findings were consist with previous finding showing E2

supplementation in men increases fat oxidation and decreased CHO and amino acid oxidation (10, 16). Again, previous findings looking at broad range of genes found that E2 supplementation predominantly increased genes involved in IMCL synthesis and fat oxidation (37), which supported an E2 mediated increase in fat oxidation while not directionally supporting an E2 mediated decrease in CHO and protein oxidation in men.

In support of the hypothesis that E2 directly or indirectly alters lipid metabolism in human skeletal muscle, we showed that PGC-1α (a regulator of mitochondrial biogenesis) mRNA increased and microRNA-103, -29b (putative repressors of PGC-1α mRNA expression) content decreased, and the protein content of β-oxidation enzyme MCAD and HADHA was higher in women compared to men The significant E2 mediated change in the abundance of β-oxidation proteins suggests a greater capacity to oxidize more lipids. Future studies should look at the protein content of FAT/CD36 and FABPpm at the plasma membrane and mitochondrial membrane to determine if E2 regulates FA uptake into the skeletal muscle.

To further explore the role of E2 in lipid oxidation, one could look at the effect of E2/ER on the affinity of metabolic gene promoters using DNA – protein binding assays or through analysis of the *cis*-elements within the promoters of these genes using promoter and protein interaction assays. Transcription activity assays and mRNA stability assays may be used to determine if E2 influenced the transcription activity or stability of mRNA. One could also use E2 blockers Tamoxifen or Raloxifene or ER knockout mice to identify the putative pathway which are involved in E2 mediated cellular changes.

6.3 Implications for Sex Differences in Muscle Fiber Composition

Although the results of studies regarding sex differences in muscle fiber type characteristics are controversial (3, 7, 12, 32, 33, 35), it is generally reported that women have a greater relative type I fiber area (6, 12, 13, 33, 39), consistent with our findings (chapter 2). This data partly explains the higher fat oxidation observed in women during endurance exercise (6); however, acute E2 supplementation also altered substrate metabolism (10), implying that fiber type is not the only factor contributing to the observed sex difference. Furthermore, we were unable to account for the cellular mechanism of the % area difference as there were no sex differences in total protein content of MHCI, MHCII, myostatin, or PPARδ. Further research into this area is needed.

6.4 Conclusions

In conclusion, the studies comprising this thesis demonstrate that substrate metabolism is different for men and women, specifically at the level of oxidative capacity (chapters 2,3,4), and E2 is a partial mechanism regulating these differences (chapter 5). It is essential to move forward in our understanding of these sex differences in health and disease. For years results generated from physiological research studies, based on male responses, were assumed to equally

apply to women. It is becoming increasingly clear that the information we have gathered on men cannot be expected to elicit the same response in women, which is of utmost importance when making recommendation on training, nutrition, health, disease prevention and treatment.

Although the work in this thesis greatly advances the field in sex-related differences in lipid metabolism, to fully understand the molecular mechanisms of the processes related to sex-based differences in substrate utilization, we should do further studies with regard to (1), differences in gene expression due to pre-determined genetic makeup, and/or differences in cellular signaling that may regulate gene expression at the level of transcription activity, mRNA stability, phosphorylation, and enzyme activity; (2) regulatory relationship among the genes with exercise, nutrition and disease.

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Appendix

Appendix Table 1. Genes that escape X-chromosome inactivation. Data reviewed and generated from screens by Carrel and Willard (2005).

Accession	Gene	Gene Name	Percent Xi expression
Metabolism			
Hs.378766	PLCXD1	Phosphatidylinositol phospholipase C, X domain 1	100%
Hs.11779	DHRSX	Dehydrogenase/reductase (SDR) family	100%
Hs.380757	GYG2	Glycogenin 2	100%
Hs.350927	SLC25A6	Solute carrier family 25, member 6	100%
Hs.226469	HDHD1A	Haloacid dehalogenase-like hydrolase domain 1	100%
Hs.264	PNPLA4	Patatin-like phospholipase domain containing 4	100%
Hs.424966	PIR	Pirin	100%
Hs.31535	CA5BL	Carbonic anhydrase VB-like	100%
Hs.162613	CA5B	Carbonic anhydrase VB, mitochondrial	100%
Hs.414156	CTPS2	CTP synthase II	100%
Hs.183109	MAOA	Monoamine oxidase A	100%
Hs.433291	ARD1	N-acetyltransferase	56%
Hs.432170	COX7B	Cytochrome c oxidase subunit VIIb	33%
Hs.69089	GLA	Galactosidase, alpha	22%
Hs.298885	ACATE2	Mitochondrial Acyl-CoA Thioesterase	22%
Hs.412707	HPRT1	Hypoxanthine phosphoribosyltransferase 1	20%
Hs.447877	TIMM8A	Translocase of inner mitochondrial membrane 8A	20%
Hs.171280	HADH2	Hydroxyacyl-Coenzyme A dehydrogenase, type II	11%
Hs.606	ATP7A	ATPase, Cu++ transporting, alpha polypeptide	44%
Hs.88252	ATP11C	ATPase, Class VI, type 11C	11%
Hs.183434	ATP6AP2	ATPase, H+ transport, lysosomal accessory protein 2	11%
Signal Transduction			
Hs.522840	PGPL	Pseudoautosomal GTP-binding protein-like	100%
Hs.43505	IKBKG	inhibitor of kappaB kinase gamma	100%
Hs.444327	RAB9A	Ras-related GTP-binding protein	100%
Hs.40368	AP1S2	Adaptor-related complex 1, sigma 2 subunit	100%
Hs.406078	RBBP7	Retinoblastoma binding protein 7	100%
Hs.171834	PCTK1	PCTAIRE kinase 1	100%

Hs.118494	GAB3	GRB2-associated binding protein 3	89%
Hs.76536	TBL1X	Transducin (beta) like 1	78%
Hs.74124	GPR143	G protein-coupled receptor 143	78%
Hs.3109	ARHGAP4	Rho GTPase activating protein 4	78%
Hs.73883	GRPR	Gastrin releasing peptide receptor	67%
Hs.652	TNFSF5	Tumor necrosis factor superfamily member 5	44%
Hs.27453	RAB40A	RAS oncogene family member	33%
Hs.446641	TIMP1	Tissue inhibitor of metalloproteinase 1	33%
Hs.25010	NXT2	NTF2-related export protein 2	22%
Hs.390616	PAK3	p21-activated kinase 3	22%
Hs.54941	PHKA2	Phosphorylase kinase alpha 2	22%

Cell Membrane			
Hs.21595	DXYS155E	Lymphocyte surface protein	100%
Hs.283477	CD99	CD99 antigen	100%
Hs.129614	TMEM27	Transmembrane protein 27	100%
Hs.639	CALB3	Calbindin 3	100%
Hs.2524	AVPR2	Arginine vasopressin receptor 2	100%
Hs.380742	PLXNB3	Plexin B3	100%
Hs.406228	IL9R	Interleukin 9 receptor	100%
Hs.445201	L1CAM	L1 cell adhesion molecule	89%
Hs.72157	DKFZP564I1922	Adlican	78%
Hs.417091	CLCN4	Chloride channel 4	56%
Hs.17109	ITM2A	Integral membrane protein 2A	56%
Hs.54570	CLIC2	Chloride intracellular channel 2	33%
Hs.408	COL4A6	Collagen, type IV, alpha 6	33%
Hs.58367	GPC4	Glypican 4	22%
Hs.512825	CLDN2	Claudin 2	11%
Hs.169470	DMD	Dystrophin	11%
Hs.144700	EFNB1	Ephrin-B1	11%
Hs.2700	GLRA2	Glycine receptor, alpha 2	11%
Hs.27812	GPR23	G protein-coupled receptor 23	11%

Modifiers			
Hs.124942	PR48	Protein phosphatase 2A 48 kDa subunit	100%
Hs.458420	ASMTL	Acetylserotonin O-methyltransferase-like	100%
Hs.201790	DUSP21	Dual specificity phosphatase 21	100%
Hs.147996	PRKX	Protein kinase, X-linked	78%
Hs.4552	UBQLN2	Ubiquilin 2	22%