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**CHARACTERIZATION OF THE  
PROSTANOID TP RECEPTOR POPULATION IN  
HUMAN NONPREGNANT MYOMETRIUM**

By

**MICHELLE SENCHYNA, B.Sc.**

*A Thesis*

*Submitted to the School of Graduate Studies*

*in Partial Fulfilment of the Requirements*

*for the Degree*

*Doctor of Philosophy*

McMaster University

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**CHARACTERIZATION OF THE PROSTANOID TP RECEPTOR  
POPULATION IN HUMAN NONPREGNANT MYOMETRIUM**

**DOCTOR OF PHILOSOPHY (1998)**  
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**McMaster University**  
**Hamilton, Ontario**

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**Population in Human Nonpregnant Myometrium**

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## Abstract

Since the pioneering work of Pickles *et al.* (1965), prostanoids have been implicated in the pain and discomfort of primary dysmenorrhea. Accordingly, current pharmacotherapy is based on the inhibition of prostanoid synthesis. However, 10% to 25% of women who suffer from primary dysmenorrhea fail to gain relief from such therapy. The development of alternative therapies to treat these women has been hindered by the fact that the effects of prostanoids on human nonpregnant myometrium have not been quantified in a rigorous way. The prostanoid thromboxane A<sub>2</sub> causes vascular smooth muscle contraction by interacting with specific prostanoid receptors known as TP receptors, the nomenclature follows recent International Union of Pharmacology recommendations where each prostanoid receptor is designated by the letter P, preceded by a letter signifying the most potent natural prostanoid agonist at that receptor. At the time my thesis was undertaken, several observations suggested that the TP receptor may be involved in the physiological and pathophysiological control of myometrial contractility. Therefore, the purpose of this study was to thoroughly characterize the TP receptor population in human nonpregnant myometrium.

I evaluated the pharmacological characteristics of the myometrial TP receptor via *in vitro* functional and radioligand binding studies and employed reverse transcription-polymerase chain reaction assays to evaluate TP receptor mRNA expression. Both U-46,619 and I-BOP produced concentration-dependent contraction of human myometrial strips *in vitro* ( $pEC_{50} = 6.9 \pm 0.27$ ; and  $7.8 \pm 0.60$ , respectively). The contractile activity induced by U-



## Abstract

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46,619 was attenuated by seven selective TP receptor antagonists. Lastly, the sensitivity of human nonpregnant myometrium was not regulated by anatomical location, tissue orientation or menstrual cycle status of the donor. The binding of [<sup>125</sup>I]-BOP to human myometrial membranes was saturable, selective and displaceable. Equilibrium binding of [<sup>125</sup>I]-BOP identified one class of sites,  $K_d = 3.4$  nM ( $pK_d = 8.7 \pm 0.4$ ) and a maximum binding of  $323.1 \pm 361.5$  fmol/mg protein. The addition of the non-hydrolyzable GTP analog GTP $\gamma$ S (100  $\mu$ M), to the assay had no effect on [<sup>125</sup>I]-BOP binding. The rank order of potency for the seven TP receptor antagonists in displacing [<sup>125</sup>I]-BOP from its binding site was correlated ( $r = 0.75$ ) with the rank order of potency in inhibiting U-46,619-induced contraction of myometrial strips. Ligands selective for other prostanoid receptors were unable to significantly displace [<sup>125</sup>I]-BOP binding. A novel qualitative RT-PCR methodology was developed and with this technique TP receptor mRNA expression was demonstrated in human nonpregnant myometrium excised from different uterine locations, from donors in both the proliferative and secretory phases of the menstrual cycle. The basis for a semi-quantitative RT-PCR methodology was established and an examination of potential influences on TP receptor mRNA expression, such as tissue excision site and donor menstrual cycle status, was begun. Lastly, the semi-quantitative data describing the amplification of TP receptor mRNA was highly variable, however the factor(s) responsible for such high variability remain to be determined. All taken together, these results suggest that a single homogeneous population of TP receptors, most closely resembling the putative low affinity TP receptor population in human platelets, resides in human nonpregnant myometrium.

# Dedication

This work is dedicated to my best friend and father, Gordon Senchyna.

*"A New Confidence*

*A New Attitude*

*A New Beginning"*

Thank You  
Love Michelle

## Acknowledgements

Hmm, where to start....., what to say.....? I have been told that acknowledgements are not supposed to take weeks to write, but for me, I am going on three. I have been laboring over this page simply because I cannot find the words to adequately capture the efforts made by a number of individuals, without which this thesis would not have been completed. However, as flowery prose or Shakespearean diction are really not my style, I have decided to take the simple and honest route - and say what is in my heart.

I would like to thank Dr. Margaret Fahnestock for always having an open door and a minute to listen. Her advise, encouragement and insight allowed me to wade through the murky waters of molecular biology relatively unharmed. The comments and support made by the other members of my committee, Dr. Derek Lobb and Dr. P. K. (Chari) Rangachari, were also very much appreciated.

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*But should I log it????*

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## List of Symbols and Abbreviations

AH 13,205	(±)-trans-2-[4-(1-hydroxyhexyl)phenyl]-5-oxocyclopentaneheptanoic acid
AMP	adenosine monophosphate
BM 13,505	4-(2-(4-chlorobenzenesulfonamido)-ethyl)-benzeneacetic acid
bp	base pairs
B <sub>max</sub>	maximal amount of specifically bound radioligand
BWA868C	3-benxyl-5-(6-carboxyhexyl)-1-(2-cyclohexyl-2-hydroxyethylamino)hydantoin
cAMP	cyclic AMP
cDNA	complementary DNA
cicaprost	5-{ (E) - (1S,5S,6S,7R)-7-Hydroxy-6-[(3S,4S)-3-hydroxy-4-methylnona-1,6-dinnyl]-bicyclo [3.3.0]octan-3-yliden}-3-oxapentanoic acid
cloprostenol	[1R-[1α(Z),2β(1E,3R'),3α,5α]]-7-[2-[4-(3-chlorophenoxy)-3-hydroxy-1-butanyl]-3,5-dihydroxycyclopentyl]-5-heptenoic acid
cpm	counts per minute
CSPD	disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1 <sup>3,7</sup> ]decan}-4-yl)phenyl phosphate
DIG	digoxigenin
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
EC <sub>50</sub>	molar concentration of an agonist which produces 50% of the maximum possible response for that agonist
EDTA	ethylenediamine tetra acetic acid
G3PDH	glyceraldehyde-3-phosphate dehydrogenase
GR 32,191	[1R-[1α(Z),2β,3β,5α]]-(+)-7-[5-[[1,1'-biphenyl]-4-yl]methoxy]-3-hydroxy-2-(1-piperidinyl)cyclopentyl]-4-heptenoic acid
GTPγS	guanosine-5'-O-(3-thio)triphosphate

## List of Symbols and Abbreviations

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HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HMM	human myometrial membranes
I-BOP	[1S[1 $\alpha$ ,2 $\alpha$ (Z),3 $\beta$ (1E,3S <sup>*</sup> ),4 $\alpha$ )]-7-[3-[3-hydroxy-4-(4-iodophenoxy)-1-butenyl]-7-oxabicyclo-[2.2.1]hept-2-yl]5-heptanoic acid
IC <sub>50</sub>	the concentration of competing ligand which displaces 50% of the specific binding of the radioligand
ICI 192,605	4(Z)-6-[(2,4,5 cis)2-(2-chlorophenyl)-4-(2-hydroxyphenyl)1,3-dioxan-5-yl]hexenoic acid
ICI D1,542	(4(Z)-6-[(2S,4S,5R)-2-[1-methyl-1-(2-nitro-4-tolyl-oxy)ethyl]-4-(3-pyridyl)-1,3-dioxan-5-yl]hex-4-enoic acid
IP <sub>3</sub>	inositol 1,4,5-trisphosphate
I-PTA-OH	9,11,-dimethylmethano-11,12-methano-16-(3-iodo-4-hydroxyphenyl)-13,14-dihydro-13-aza-15 $\alpha\beta$ - $\omega$ -tetranor thromboxaneA <sub>2</sub>
I-SAP	7-[1R,2S,3S,5R)-6,6-dimethyl-3-(4-iodobenzenesulfonylamino)bicyclo[3.1.1]hept-2-yl]-5(Z)-heptenoic acid
IUPHAR	International Union of Pharmacology
kb	kilo bases
K <sub>d</sub>	the dissociation constant for a radiolabelled compound
L670,596	(-)-6,8-di-fluoro-9- <i>p</i> -methylsulfonyl benzyl-1,2,3,4-tetra-hydrocarbazol-1-yl-acetic acid
M	molar
mg	milli gram
mL	milli litre
mm	milli meter
mM	milli molar
mN	milli Newton
MOPS	(3-[N-Morpholino]propane-sulfonic acid)
mRNA	messenger RNA
mV	milli volts
n	number of replicates
nM	nano molar

## List of Symbols and Abbreviations

---

OD	optical density
ONO 3,708	(9,11),(11,12)-didedoxa-9 $\alpha$ ,11 $\alpha$ -dimethylmethano-11,12-methano-13,14-dihydro-13-aza-14-oxo-15-cyclo-pentyl-16,17,18,19,20-pentano-15-epi-thromboxane A <sub>2</sub>
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
rRNA	ribosomal RNA
RT	reverse transcription
RT-PCR	reverse transcription-polymerase chain reaction
$pA_2$	the negative log of the concentration of antagonist which would produce a 2-fold shift in the concentration-response curve for an agonist
PCR	polymerase chain reaction
$pEC_{50}$	$-\log[EC_{50}]$
pg	pico gram
$pIC_{50}$	$-\log[IC_{50}]$
PKA	protein kinase A
PKC	protein kinase C
$pK_d$	$-\log[K_d]$
pM	pico molar
PSS	physiological salt solution
S.D.	standard deviation
SDS	sodium dodecyl sulfate
SQ 29,548	[1S-(1 $\alpha$ ,2 $\beta$ (5Z),3 $\beta$ ,4 $\alpha$ )]-7-[3-[[2-[(phenylamino)carbonyl]hydrazino]methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid
SSC	sodium chloride - sodium citrate solution
STA <sub>2</sub>	9,11-epithio-11,12-methano-TxA <sub>2</sub>
sulprostone	(5Z,13E)-(8R,11R,12R,15R)-11,15-dihydroxy-9-oxo-16phenoxy-17,18,19,20-tetranor-5,13-prostadienoic acid-methylsulfonylamide
TBE	tris-borate/EDTA electrophoresis buffer
TE(8)	tris-EDTA buffer
TP	thromboxane A <sub>2</sub> receptor
TxA <sub>2</sub>	thromboxane A <sub>2</sub>

## List of Symbols and Abbreviations

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U	units
U-46,619	15(S)-hydroxy-11 $\alpha$ ,9 $\alpha$ -(epoxymethano)prosta-5Z,13E-dienoic acid
$\mu$ g	micro gram
$\mu$ L	micro litre
$\mu$ M	micro molar
UV	ultra violet
V	volts

# Chapter 1

## Introduction

A considerable body of evidence implicates prostanoids produced by the endometrium at the time of menstruation in the pain and discomfort of primary dysmenorrhea. As such, current pharmacotherapy of primary dysmenorrhea is based on inhibition of prostanoid synthesis. Although an estimated 75% to 90% of women gain relief from primary dysmenorrhea using such therapy, there is growing contention that new and improved treatment strategies are required. That the pathophysiological events leading to the onset of primary dysmenorrhea may result from inappropriate signal reception (hyper- or hyposensitivity), rather than inappropriate signal transmission (hyper- or hyposecretion) has led to the suggestion that selective blockade of prostanoid receptors may provide an alternative therapeutic strategy for those 10% to 25% of women not helped by current pharmacotherapy. However, little work has been devoted to quantifying the effects of prostanoids on human nonpregnant myometrium in a rigorous way. Thus before any advancements can be made with respect to understanding the pathogenesis of primary dysmenorrhea and the development of selective drugs, a thorough characterization of myometrial prostanoid receptors must be assembled. The characterization of one prostanoid receptor in particular, the TP receptor, is the focus of my thesis.

This chapter briefly introduces prostanoids, prostanoid receptors and primary dysmenorrhea. To put this work into context, evidence suggesting an etiological role for



the TP receptor in primary dysmenorrhea will be presented, followed by a brief summary of the fundamental characteristics associated with the molecular and cellular pharmacology of TP receptors.

### 1.1. AN INTRODUCTION TO PROSTANOIDS AND THEIR RECEPTORS

Prostanoids comprise *prostaglandins* (PGs) and *thromboxanes* (TxAs), which collectively represent a family of naturally occurring C<sub>20</sub> arachidonic acid metabolites. Prostanoids are not stored, but are synthesized *de novo* in almost all mammalian cells and released in response to a wide variety of cell stimuli ranging from activation of hormone receptors to simple mechanical trauma (Smith, 1989, Coleman *et al.*, 1990). The first and rate limiting step in the biosynthesis of prostanoids is the liberation of arachidonic acid from membrane glycerophospholipids. Although the biochemical details of the events involved in arachidonate release have not been completely resolved, the key enzyme appears to be phospholipase A<sub>2</sub> (Needleman *et al.*, 1986; Coleman *et al.*, 1990). Arachidonic acid is rapidly converted to the unstable endoperoxide intermediates PGG<sub>2</sub> and PGH<sub>2</sub> by the enzyme, cyclooxygenase [PGG/H Synthase] (Shimizu and Wolfe, 1990). The biologically active prostanoids, which are considered to be PGD<sub>2</sub>, PGE<sub>2</sub>, PGF<sub>2α</sub>, PGI<sub>2</sub> and TxA<sub>2</sub> are synthesized through specific enzymatic conversion of PGH<sub>2</sub> (Moncada and Vane, 1979; Shimizu and Wolfe, 1990). The synthesis of prostanoids is usually cell-specific, in that any given prostanoid forming cell tends to synthesize only one of the five biologically active compounds as its major product (Smith, 1986).

Prostanoids are considered to be local acting hormones [autacoids], and are associated with an extensive and diverse array of physiological and pathophysiological actions involving platelet aggregation, vascular tone, inflammation, and contraction and relaxation of smooth muscle (Campbell, 1990; Coleman *et al.*, 1994). Prostanoids exert their biological actions by interacting with specific membrane bound receptors. Current pharmacological classification recognizes five types of prostanoid receptors on the basis of sensitivity to PGD<sub>2</sub>, PGE<sub>2</sub>, PGF<sub>2α</sub>, PGI<sub>2</sub> and TxA<sub>2</sub>. These receptors are termed P receptors, with a preceding letter indicating the natural prostanoid to which each receptor is most sensitive. Thus the receptors are termed DP, EP, FP, IP and TP respectively. Furthermore, EP is subdivided into four subtypes; EP<sub>1</sub>, EP<sub>2</sub>, EP<sub>3</sub> and EP<sub>4</sub> on the basis of their responsiveness to various agonists and antagonists (Coleman *et al.*, 1994). TP receptor subtypes have been proposed, although definitive identification has yet to occur. In all cases, naturally occurring prostanoids other than the one for which the receptor is named are capable of activating the receptor, albeit often with a lower potency (Coleman *et al.*, 1990; Coleman *et al.*, 1994). Biochemical and cloning strategies have deduced that all prostanoid receptors so far identified belong to the seven transmembrane domain, G-protein-coupled, rhodopsin-like super family (Negishi *et al.*, 1995).

## 1.2. PROSTANOIDS, PROSTANOID RECEPTORS AND PRIMARY DYSMENORRHEA: Evidence Supporting A Pathophysiological Connection

Primary dysmenorrhea (PD), defined as menstrual pain, not associated with pelvic pathology effects between 50% and 70% of postpubescent women, with 10% to 15% of these women being incapacitated for one to three days each month (Ylikorkala and Dawood, 1978; Rosenwaks and Seegar-Jones, 1980). Despite its prevalence and severity, the etiology and pathophysiology of PD are poorly understood and understudied. It is however, widely accepted that PD is largely a consequence of increased uterine muscular activity resulting in increased uterine tone, excessive spasmodic contractions and ischemia as a result of decreased uterine blood flow (Fraser, 1992; Kostrzewska *et al.*, 1996).

Prostanoids have been associated with PD since Pickles *et al.* (1965), found higher levels of PGF<sub>2α</sub> and PGE<sub>2</sub> in menstrual fluid of women with PD than in women with pain free periods. Elevated levels of PGF<sub>2α</sub> and PGE<sub>2</sub> have since been confirmed in menstrual fluid, endometrium and endometrial jet washes from dysmenorrheic women compared to pain free women (Lundström, 1985; Rees, 1990). Little information has been presented regarding other prostanoids, although it is known that the human uterus can produce TxA<sub>2</sub> and PGI<sub>2</sub> (Ylikorkala and Mäkilä, 1985). TxA<sub>2</sub> metabolites have been measured in menstrual fluid (Powell *et al.*, 1985), and Hofer *et al.* (1993), have reported that of the biologically active prostanoids, only TxA<sub>2</sub> and PGF<sub>2α</sub> are found in significant concentrations in menstrual fluid.

Despite a lack of understanding of detailed biochemical mechanisms, the *implication* of irregular prostanoid production combined with considerable evidence suggesting that

prostanoids were potent mediators of uterine contractility, led to the use of prostanoid synthase inhibitors in the treatment of PD (Rees, 1990). For example, aspirin and related non-steroidal antiinflammatory drugs (NSAIDs), inhibit the cyclooxygenase enzyme and thus inhibit all prostanoid production (Smith, 1989). Currently, NSAIDs are the treatment of choice for PD prescribed among family physicians (Apgar, 1997). The failure rate of NSAID therapy is difficult to estimate from the literature as few properly controlled trials have been conducted. Åkerlund (1990), states that an acceptable effect of NSAIDs is obtained in  $\approx$  75% of cases. Rees (1988), estimates a slightly lower failure rate of 10% - 20%.

The foregoing indicates that a clearer understanding of the actions of prostanoids and how they may be regulated is necessary to both advance our understanding of PD and to design novel, efficacious therapies to treat those women not helped by NSAIDs. At present, pharmacological evidence suggests that human myometrium possess all of the currently identified prostanoid receptors with the possible exception of EP<sub>4</sub> (Senior *et al.*, 1991; 1992; Brown and Crankshaw, 1995). Agonist action at four of these receptors (EP<sub>1</sub>, EP<sub>3</sub>, FP and TP) induce myometrial contractions (Senior *et al.*, 1991; 1992). However, the relative contributions of each of these excitatory prostanoid receptors to the overall picture of dysmenorrhea is unknown as most attention has been paid to PGF<sub>2 $\alpha$</sub> , presumably working at the FP receptor.

At the time my thesis was undertaken, a number of facts suggested that pharmacotherapy based on selective blockade at TP receptors be tested as an alternative treatment for PD:

- 1) Thromboxanes are produced by the endometrium (Powell *et al.*, 1985).
- 2) Thromboxane mimetics and presumably thromboxanes themselves are potent uterotonics and these actions can be blocked by TP receptor antagonists (Crankshaw, 1992).
- 3) The TP receptor was the only prostanoid receptor that had been cloned (Hirata *et al.*, 1991).
- 4) Drug development in the area of TP receptors was more productive than for any other of the prostanoids and several potent orally active TP receptor antagonists were available (Patscheke *et al.*, 1987; Ford-Hutchinson *et al.*, 1989; Nagai *et al.*, 1991).
- 5) The sensitivity of human nonpregnant myometrium to thromboxane mimetics was reported to vary greatly from donor to donor, suggesting that TP receptor-mediated responses are subject to regulation (Crankshaw, 1992).

### 1.3. THE TP RECEPTOR

TxA<sub>2</sub> is formed from PGH<sub>2</sub> via thromboxane synthase (Hamberg *et al.*, 1975; Moncada and Vane, 1979). TxA<sub>2</sub> is rapidly hydrolyzed to the chemically stable and biologically inactive metabolite TxB<sub>2</sub> at physiologic pH ( $t_{1/2} \approx 30$  sec). TxB<sub>2</sub> undergoes two major pathways of metabolism resulting primarily in the formation of 2,3-dinor-TxB<sub>2</sub> and 11-dehydro-TxB<sub>2</sub>, both of which represent reliable indices of TxA<sub>2</sub> biosynthesis (Nicosia and Patrono, 1989). The major cellular sites for TxA<sub>2</sub> synthesis are platelets, macrophages, monocytes and lung parenchyma (Nicosia and Patrono, 1989).

Pharmacological studies conducted throughout the mid-1970's and early 80's concluded that TxA<sub>2</sub> and its immediate precursor PGH<sub>2</sub> induced platelet aggregation and

constriction of vascular smooth muscle through interaction with the same protein, putatively identified as the TxA<sub>2</sub>/PGH<sub>2</sub> receptor (Needleman *et al.*, 1976; Coleman *et al.*, 1981; Halushka *et al.*, 1987). Although the ensuing years saw a heightened interest in TxA<sub>2</sub> due to its potential pathophysiological role in cardiovascular and renal disease and gynecological disorders, the extremely liable nature of TxA<sub>2</sub> precluded further characterization of the receptor (Ylilorkala and Mäkilä, 1985; Davis-Bruno and Halushka, 1994; Urayama *et al.*, 1996). Only with the advent of potent and selective agonists and antagonists, in addition to the development of biochemical and molecular methodologies, have strides been made to characterize the complex pharmacology of what is now known as the TP receptor.

### 1.3.1. Pharmacology of the TP Receptor

Studies aimed at elucidating the biological effects of TxA<sub>2</sub> have largely relied on the availability of stable structural mimetics of TxA<sub>2</sub>, most notably U-46,619, I-BOP and STA<sub>2</sub> (Coleman *et al.*, 1981; Katsura *et al.*, 1983; Morinelli *et al.*, 1989). In addition, the synthesis of a plethora of potent and selective TP receptor antagonists and the radiolabeling of several agonists {[<sup>125</sup>I]-BOP; [<sup>3</sup>H]U-46,619} and antagonists {[<sup>3</sup>H]SQ 29,548; [<sup>125</sup>I]PTA-OH} have facilitated the pharmacological characterization of the TP receptor (Morinelli *et al.*, 1989; 1990b).

TP receptors have been identified in a number of human tissues including platelets, vascular smooth muscle, umbilical vessels, endothelial cells, placenta, glomerular mesangial cells, myometrium and peripheral blood mononuclear cells (Senior *et al.*, 1992; Coleman *et*

*al.*, 1994; Davis-Bruno and Halushka, 1994). Despite this broad tissue distribution, extensive functional and binding studies have been performed predominantly on platelets and vascular smooth muscle.

TP receptors mediate platelet aggregation and vascular smooth muscle contraction in response to stimulation by selective agonists such as U-46,619 and I-BOP. As an example of potency, the EC<sub>50</sub> for U-46,619-stimulated platelet aggregation ranges from 87 nM to 121 nM (Dorn, 1991, Morinelli *et al.*, 1990b), whereas for I-BOP, EC<sub>50</sub> values from 4.4 nM to 11 nM have been reported (Dorn, 1991, Morinelli *et al.*, 1990b). Radioligand binding studies on washed platelets, crude and detergent solubilized membranes of human platelets and cultured vascular smooth muscle cells have localized the TP receptor to the plasma membrane. High affinity specific binding has been reported using a number of structurally diverse radiolabeled TP receptor ligands. Among the most common labeled agonists, [<sup>3</sup>H]U-46,619 yields affinity estimates of 20 nM to 131 nM for the platelet TP receptor whereas K<sub>d</sub> values for [<sup>125</sup>I]-BOP range from 234 pM to 3.9 nM (Dorn, 1989; Dorn and DeJesus, 1991). Affinity values for TP receptor antagonists such as [<sup>3</sup>H]SQ 29,548 and [<sup>125</sup>I]PTA-OH range from 2 nM to 30 nM (Saussy *et al.*, 1986; Dorn, 1989, Takahara *et al.*, 1990, Habib *et al.*, 1997).

Studies of the platelet TP receptor have suggested the existence of significant species differences based on comparisons of (1) rank order potencies of TP receptor agonists to induce platelet aggregation; (2) rank order potencies of TP receptor antagonists to inhibit TP receptor-mediated platelet aggregation; and (3) IC<sub>50</sub> rank orders of various TP receptor ligands in competition radioligand binding assays. Of those species studied, the guinea pig TP receptor appears to most closely resemble the human, whereas the dog and

rat receptors differ considerably from the human and each other (Mais *et al.*, 1985b; Halushka *et al.*, 1989). Whether human and rabbit platelet TP receptors differ is unclear as *in vitro* functional and binding studies have produced conflicting results (Narumiya *et al.*, 1986; Halushka *et al.*, 1989; Dorn, 1991). The vascular TP receptors have not been characterized as extensively between species. Mais *et al.* (1985b), concluded that TP receptors in canine and human saphenous veins were similar based on the comparison of rank order potencies of TP receptor antagonists to inhibit U-46,619-induced contraction. In contrast, Dorn (1991), has suggested that rabbit and human vascular smooth muscle TP receptors are different based on the demonstration that the rank order potencies of TP receptor ligands in competition binding assays with [<sup>125</sup>I]-BOP were significantly different. Ogletree and Allen (1992), have argued that significant interspecies differences exist among smooth muscle TP receptors based on their findings of differential rank order potencies of TP receptor antagonists to inhibit U-46,619 induced contraction of guinea pig and rat smooth muscle preparations.

#### 1.3.1.1. TP Receptor Subtypes: Pharmacological Evidence

Early functional investigations suggested that TP receptors found on the platelet may be different from those found on vascular smooth muscle (Lefer *et al.*, 1980; LeDuc *et al.*, 1981; Halushka *et al.*, 1987). However, many of the results obtained from such studies were ambiguous as in many cases only one or a few compounds were examined and/or the comparisons between platelets and vascular preparations were across species.

The first strong evidence for the existence of TP receptor subtypes came from the work of Mais *et al.* (1985a; 1985b), in which the pharmacology of a series of 13-azapinane-



TxA<sub>2</sub> analogs were studied. They found the functional rank order potency for the compounds as antagonists was significantly different in human platelets compared to both human and canine saphenous veins. More recent functional support for TP receptor heterogeneity has come from a number of labs including two studies using a novel series of TxA<sub>2</sub> mimetics [9,11-cyclic carbonate derivatives of PGF<sub>2α</sub>], that were found to differentially induce excitatory activity in human myometrium and rat vasculature compared to human platelets (Krauss *et al.*, 1996; Krauss *et al.*, 1997).

Additional evidence for the existence of tissue specific subtypes has been derived from radioligand binding studies, most notably studies employing [<sup>125</sup>I]-BOP, which in addition to its high specific activity (≈ 2,000 Ci/mmol), is considered to bind to the TP receptor with a higher affinity and specificity compared to other radiolabeled TP receptor ligands (Morinelli *et al.*, 1990a). Reports from Dorn (1991) and Masuda *et al.* (1991), have shown that the rank orders of the binding inhibition constants (IC<sub>50</sub>) for TxA<sub>2</sub> analogs in competition with [<sup>125</sup>I]-BOP were significantly different in cultured human vascular smooth muscle cells compared to platelet preparations derived from humans, rats and rabbits. As a tentative nomenclature, Mais *et al.* (1985b), designated the platelet subtype: α, for aggregation and the vascular receptor subtype: τ, for tone.

Beyond tissue differences, there is also growing evidence that platelets possess two distinct TP receptor subtypes, one which mediates shape change and the other which mediates aggregation. This proposal was derived from radioligand binding studies using [<sup>125</sup>I]-BOP which identified high (K<sub>d</sub> = 234 pM - 270 pM) and low (2.3 nM - 3.9 nM) affinity

sites which corresponded with the concentration of I-BOP causing half-maximal platelet shape change (173 pM - 263 pM) and aggregation (1.8 nM - 4.4 nM) (Dorn, 1989; Dorn and DeJesus, 1991). Additional support for the existence of two receptor subtypes on the platelet has come from the combined functional and binding studies of Takahara *et al.* (1990), who demonstrated that the TP receptor antagonist GR 32,191 non-competitively inhibited U-46,619-induced platelet aggregation by binding irreversibly to a TP receptor, whereas GR 32,191 competitively inhibited U-46,619-induced platelet shape change. In support of this observation, dissociation of [<sup>3</sup>H]GR 32,191 could best be resolved by a two site model, describing a rapidly dissociating site and a more slowly dissociating site. Multiple comparisons of data derived from vascular smooth muscle and platelets has prompted the proposal that the "high" affinity site on platelets is common to that found on vascular smooth muscle, whereas the "low" affinity site is unique to platelets (Dorn 1989; D'Angelo *et al.*, 1994; Allan *et al.*, 1996).

In summary, evidence has been gathered in support of both tissue specific and platelet TP receptor subtypes. However, for two apparent reasons, TP receptor subtypes are currently not recognized under the IUPHAR classification of prostanoid receptors (Coleman *et al.*, 1994). Under this scheme, the classification of prostanoid receptors is based on functional and binding data obtained by means of comparisons of rank orders of agonist activity and where possible, the effects of antagonists (Coleman *et al.*, 1994). As will be discussed in the following sections, a central question common to both subtype proposals that has yet to be resolved is whether the data in support of TP receptor subtypes

reflect two distinct receptors, or, one receptor that is linked to distinct effector systems. In addition, if structural criteria as described by Kenakin *et al.* (1992), are to be included in the resolution of receptor subtypes, then again, as will be described below, clear evidence in support of TP receptor subtypes is lacking.

### 1.3.2. Molecular Characterization of the TP Receptor Gene

The human TP receptor was first purified from human platelets using a high affinity and selective antagonist, S-145 (Ushikubi *et al.*, 1989). Based on a partial amino acid sequence obtained from the purified protein, a cDNA for the TP receptor was cloned and sequenced from a human placental library (Hirata *et al.*, 1991). The protein, consisting of 343 amino acids, was shown to belong to the rhodopsin-like family of G protein-linked receptors, which are monomeric and possess a characteristic seven transmembrane spanning domain topology (Hirata *et al.*, 1991; Yamamoto *et al.*, 1993). Two additional cDNA sequences for the TP receptor were subsequently cloned from platelet-like K562 human chronic myelogenous leukemia cells (D'Angelo *et al.*, 1994), and HEL cells (Allan *et al.*, 1996). Both were identical to the human placental TP receptor apart from a single amino acid substitution (Gly21Lys) in the K562 cell TP receptor clone.

The human TP receptor gene was subsequently characterized by Nüsing *et al.* (1993). The gene, present as a single copy, spans over 15 kilobases (kb) and contains 3 exons divided by 2 introns. The first intron, intron 1, is 6.3 kb and exists in the 5'-noncoding region, whereas intron 2 with a length of 4.3 kb, is located at the end of the sixth transmembrane region, thereby separating it from the downstream coding sequences.

Based on this cloning information, the question of TP receptor subtypes was opened from a new angle. The most compelling evidence in support of only one TP receptor came from Northern Blot analysis of poly(A<sup>+</sup>)RNA derived from human lung and placental tissues and cultured MEG-01 cells. When probed with a fragment of the TP receptor cDNA, a single hybridization band was reported in each case, thus prompting the conclusion that only one TP receptor exists in man (Hirata *et al.*, 1991). This conclusion was supported by the work of Nüsing *et al.* (1993), who found no evidence for alternative splicing of transcripts, as determined by polymerase chain reaction of reverse transcribed poly(A<sup>+</sup>)RNA across the identified exon-intron boundaries.

Contrasting with the above evidence, Raychowdhury *et al.* (1994; 1995), cloned a second cDNA encoding a TP receptor isoform of 407 amino acids from a human endothelial cell cDNA library. Comparison of the placental and endothelial cell TP receptor cDNAs shows that their predicted amino acid sequences are identical for the first 328 amino acids; however from that point a marked divergence is seen with the final 15 amino acids of the placental TP receptor replaced by 79 new amino acids in the endothelial cell TP receptor. Hydropathic analysis reveals that the divergent area is entirely in the carboxy-terminal portion of the receptor, down-stream of the seventh and last transmembrane domain. The divergence in cytoplasmic domains appears to be based on an alternative splicing of the TP receptor transcript. Specifically, the placental transcript is generated from the failure to utilize a potential splice site in the third exon, thus resulting in the encoding of a cytoplasmic domain, that in the endothelial cell TP receptor is an intron (Raychowdhury *et al.*, 1994; 1995).

Although it is tempting to conclude that the existence of multiple TP receptor cDNAs resolves the issue of TP receptor subtypes, there is in fact very little known regarding the functioning of the alternatively-spliced endothelial cell TP receptor, or how the pharmacology of the two isoforms compare. It is possible, but unproven that the unique carboxy-terminal tails of the two splice variants confer unique signalling and regulatory properties on the two TP receptor isoforms, that in turn explain the differential functional and ligand binding characteristics of TP receptors observed in pharmacological experiments.

### 1.3.3. TP Receptor Signalling

Studies of the relationship between TP receptor occupancy and second messenger generation have been conducted largely on platelets and vascular smooth muscle preparations.

The pathway linked to contraction of vascular smooth muscle appears to involve activation of phospholipase C (PLC) via a pertussis toxin-insensitive G protein and subsequent inositol 1,4,5-trisphosphate (IP<sub>3</sub>)-dependent Ca<sup>2+</sup> mobilization and activation of protein kinase C (PKC) through diacylglycerol (DG) formation (Coleman *et al.*, 1994). The identity of the G protein has yet to be resolved but is suggested to reside in the G<sub>q</sub> family (Coleman *et al.*, 1994).

TxA<sub>2</sub>-induced platelet activation appears more complex and is suggested to consist of at least two receptor-effector systems; one linked to PLC activation, resulting in platelet aggregation and the other one mediating Ca<sup>2+</sup> mobilization and platelet shape change through a PLC-independent process (Coleman *et al.*, 1994; Negishi *et al.*, 1995) A number

of reports suggest that platelet TP receptor(s) couple to one or more pertussis toxin-insensitive members of both the  $G_q$  and  $G_{12/13}$  families of G proteins (Shenker *et al.*, 1991; Knezevic *et al.*, 1993; D'Angelo *et al.*, 1994; Offermanns *et al.*, 1994). The pertussis toxin sensitive G protein  $G_{i2}$ , has also recently been implicated in platelet TP receptor signalling (Ushikubi *et al.*, 1994; Hirata *et al.*, 1996).

How this information relates to the putative existence of TP receptor subtypes is debatable. In support of heterogeneity, one could argue that multiple TP receptor isoforms may be expressed that couple to distinct G proteins. Precedent for such a phenomenon has been set within the prostanoid receptor family by the  $EP_3$  receptor. Alternatively-spliced mRNA transcribed from a single  $EP_3$  receptor gene results in a number of  $EP_3$  receptor isoforms, which differ only in their carboxy-terminal domains, and which exhibit distinct functional properties, including different efficiencies in activating G proteins and different specificities in coupling to G proteins (An *et al.*, 1994; Schmid *et al.*, 1994; Hirata *et al.*, 1996). Hirata *et al.* (1996), reported the identification of two TP receptor isoforms in human platelets, which differed only in their carboxy terminal tails. The two receptors, when expressed in cultured cells, utilized multiple and distinct signalling pathways. This study however, could not conclude that the two cloned isoforms represented the functional TP receptors previously identified in the platelet by pharmacological methods. Proponents of TP receptor homogeneity argue that differential binding and cell signalling may come as a result of a single TP receptor displaying differential coupling to multiple G proteins. D'Angelo *et al.* (1994) and Allan *et al.* (1996), have provided evidence to suggest that the affinity state of a single TP receptor can be altered via the interaction with different G proteins. Furthermore, Ushikubi *et al.* (1994) and Hirata *et al.* (1996), have demonstrated

that a single platelet TP receptor could functionally couple to G<sub>α</sub> and G<sub>β</sub> in reconstituted lipid vesicles or G<sub>α</sub> and G<sub>s</sub>/G<sub>i</sub> in transfected cells.

### 1.3.4. TP Receptor Regulation

Studies have suggested that the response of platelets to TP receptor stimulation is regulated by homologous desensitization of the TP receptor (Murray and Fitzgerald, 1989). The mechanism(s) is currently unknown, but the sequence of events appear to involve initial uncoupling of the receptor from a G protein followed by a more gradual loss of surface TP receptors due to internalization and degradation (Murray and Fitzgerald, 1989, Dorn and Davis, 1992; Habib *et al.*, 1997). The carboxy-terminal tails of the placental and endothelial cell TP receptors contain six and seventeen serine and threonine residues respectively and show some homology to the adrenoceptors, especially the β<sub>1</sub>-adrenoceptor (Hirata *et al.*, 1991; Raychowdhury *et al.*, 1994; 1995). These amino acids, along with two conserved potential PKC sites in the second cytoplasmic loop of both isoforms (Okwu *et al.*, 1992), are potential targets for phosphorylation (Hausdorff *et al.*, 1990; Premont *et al.*, 1995), and a number of reports suggest that phosphorylation of the TP receptor may be involved in the process of homologous desensitization (Kinsella *et al.*, 1994; Habib *et al.*, 1997). Furthermore, as the two TP receptor isoforms differ in the number of serine and threonine residues, it has been proposed that one aspect of TP receptor function that may differ between the isoforms is agonist-induced desensitization. In support of this notion, Yukawa *et al.* (1997), demonstrated that the mechanisms of desensitization of the two isoforms, stably transfected in CHO cells differ in two ways; (1) only the placental TP receptor is

downregulated following a 24 hour exposure to agonist and (2) activation of PKC effectively uncouples  $\text{Ca}^{2+}$  mobilization from the interaction of I-BOP with the endothelial cell TP receptor, but not the placental TP receptor. In contrast, Habib *et al.* (1997), have demonstrated that the rate and extent of agonist-induced phosphorylation of the two cloned TP receptor isoforms expressed in HEK 293 cells is similar, and that neither PKC nor PKA play a major role in homologous desensitization.

Whether additional mechanisms exist to regulate TP receptor function or expression have yet to be fully elucidated. Two putative N-glycosylation sites have been identified from the deduced amino acid sequence of the placental TP receptor (Hirata *et al.*, 1991), suggesting that post-translational glycosylation of the TP receptor may modify its interaction with ligands. TP receptor gene regulation has been suggested by Raychowdhury *et al.* (1994), who demonstrated that the expression of alternatively spliced TP receptor mRNA was decreased six-fold in endothelial cells preincubated with 20  $\mu\text{M}$  U-46,619 compared to control, untreated endothelial cells.

### 1.3.5. The Myometrial TP Receptor

Knowledge of the TP receptor status in human myometrium is incomplete. TP receptor agonists cause concentration-dependent contractions of human myometrium *in vitro* from both pregnant (Dyal and Crankshaw, 1988; Senior *et al.*, 1993) and nonpregnant (Crankshaw, 1992; Senior *et al.*, 1992), donors with a potency similar to that observed for both contraction of human vascular smooth muscle and platelet aggregation (Dorn, 1991). The TP receptor antagonists GR 32,191 (Lumley *et al.*, 1989), L670,596 (Ford-Hutchinson *et al.*, 1989) and BM



13,505 (Bush and Smith, 1986), block U-46,619-induced contractions of human myometrium (Clayton *et al.*, 1990; Senior *et al.*, 1992; Crankshaw, 1995). Swanson *et al.* (1992), have demonstrated by autoradiography that the TP receptor antagonist [<sup>125</sup>I]PTA-OH binds to human myometrial smooth muscle cells. However, this study was inconclusive as binding was not displaced by the selective TP receptor agonist U-46,619.

That the TP receptor may be involved in the pathogenesis of PD is suggested in part by the work of Crankshaw (1992; 1995), who reported that the sensitivity of nonpregnant myometrium to TxA<sub>2</sub> mimetics varies greatly from donor to donor, while the sensitivity at EP and FP receptors remains relatively constant. Such observations suggest that TP receptor-mediated responses may be subject to regulation. Although the nature of this regulation and its potential physiological significance are currently unknown, two potential explanations are as follows:

- 1) It has been reported that both the uterine response to prostanoids (Gannon, 1985), and the expression of prostanoid receptors (Hoffman *et al.*, 1993), may be dependent upon levels of ovarian hormones. As the pain of PD usually begins at around the onset of menstrual bleeding (Rosenwaks and Seegar Jones, 1980), myometrial sensitivity to TP receptor stimulation may be regulated by fluctuating hormone levels in accordance with the menstrual cycle.

- 2) Heterogeneity of tissue sensitivity to agonist stimulation can often be attributed to gradients in tissue responsiveness which coincide with anatomical location (Kenakin, 1984). The human uterus has been suggested to possess such regional differences with

respect to prostanoid sensitivity (Wikland *et al.*, 1984). As Crankshaw (1992; 1995), did not report the retrieval of myometrial tissue from a standardized excision site, it is possible that such inconsistency is responsible for the observed variability in the sensitivity of TP receptor-mediated myometrial contraction.

Thus, other than functional evidence, little else is known regarding the myometrial TP receptor. This deficiency is due in part to the lack of established techniques with which to study the TP receptor. Immunohistochemical and Western blot techniques are currently not feasible as a specific TP receptor antibody has yet to be produced. Analysis of TP receptor mRNA via *in situ* hybridization, Northern blotting or reverse transcriptase-polymerase chain reaction (RT-PCR), are all possible based on the availability of human TP receptor cDNA and gene sequence information. However, to date only four studies have been published regarding human TP receptor mRNA expression in the *uterus* using any of the aforementioned techniques and the only definitive conclusion that came from such investigation was that "*TP receptor mRNA is expressed in the uterus*" (Hirata *et al.*, 1991; Swanson *et al.*, 1992; Nüsing *et al.*, 1993; D'Angelo *et al.*, 1994).

In light of the previously mentioned evidence supporting a role for the TP receptor in PD, it seems prudent to suggest that a thorough characterization of the TP receptor be undertaken so as to further evaluate this claim. I have chosen this task as the topic of my thesis.

## 1.4. THESIS OBJECTIVES

The goal of my thesis was to thoroughly characterize the TP receptor population in human nonpregnant myometrium. To achieve this goal I set two primary objectives.

First, I set out to expand our pharmacological knowledge of the myometrial TP receptor via *in vitro* contractility and binding studies. I sought to:

- 1) compare and contrast the functional and binding properties of the myometrial TP receptor population with the published functional and binding properties of the TP receptor populations in human vascular smooth muscle and human platelets.
- 2) evaluate the potency of available TP receptor antagonists in human nonpregnant myometrium
- 3) examine potential regulatory mechanisms responsible for the reported variability in TP receptor-mediated responses by searching for a correlation between the sensitivity and/or maximum response of TP receptor-mediated myometrial contraction with anatomical location, tissue orientation and menstrual cycle status of the donor.

My second objective was to further explore potential mechanisms of TP receptor-mediated response variability. As a TP receptor antibody is not available and binding studies are not sufficiently sensitive, direct quantification of TP receptor protein is currently not feasible. I therefore chose to investigate whether TP receptor mRNA expression is subject to regulation. Specifically, I sought to determine whether regional differences exist with respect to TP receptor mRNA expression and whether TP receptor mRNA expression changes throughout the course of the menstrual cycle. In order to achieve this objective, I first had to develop a method by which TP receptor mRNA could be quantified. As I

desired a high throughput, sensitive and rapid methodology, I chose to develop a semi-quantitative reverse transcription-polymerase chain reaction assay.

These experiments were made possible due in part to the co-operation of the operating room staff and Pathology Department at both McMaster University and the Henderson General Hospital. With such assistance I was able to retrieve human nonpregnant myometrium from standardized excision sites as well as obtain information concerning the phase of the menstrual cycle the patient was in at the time of hysterectomy.

## Chapter 2

### Materials and Methods

#### 2.1. MATERIALS

U-46,619, I-BOP and [<sup>125</sup>I]-BOP were purchased from Cayman Chemical (Ann Arbor, MI). GR 32,191 and AH 13,205 were from Glaxo Group Research (Ware, Herts, UK). L670,596 was from Merck Frosst (Point Claire-Dorval, PQ, Canada). ONO 3,708 was from ONO Pharmaceuticals (Osaka, Japan). SQ 29,548 was from the Squibb Institute for Medical Research (Princeton, NJ, USA). ICI 192,605 and ICI D1,542 were from Zeneca Pharmaceuticals (Alderley Park, Cheshire, UK). BM 13,505 was from Boehringer Mannheim (Mannheim, Germany). BW A868C was from the Wellcome Research Laboratories (Beckenham, Kent, UK). Cicaprost and sulprostone were from Schering (Berlin, Germany). Cloprostenol was purchased from Coopers Agropharm (Ajax, ON, Canada). On the day of the experiment appropriate serial dilutions were made from stored stock concentrations in double distilled water. SuperScriptII Reverse Transcriptase and 100 bp DNA molecular weight standard were purchased from Gibco/BRL (Grand Island, NY, USA). *Thermus aquaticus* (*Taq*) DNA polymerase was purchased from Perkin-Elmer/Cetus (Norwalk, CT, USA). Restriction enzymes were purchased from Boehringer Mannheim (Mannheim, Germany) or Pharmacia LKB Biotechnology (PQ, Canada). RQ1 DNase

was purchased from Promega (Madison, WI, USA). TP receptor cDNA in Bluescript2 plasmid was a gift from Dr. M. Abramovitz (Merck Frosst, Point Claire-Dorval, PQ, Canada). Additional kits and equipment used in the analysis of TP receptor mRNA expression are detailed in Section 2.5. All other reagents were of the finest quality available and obtained from either Sigma (St. Louis, MO, USA) or BDH (Toronto, ON, Canada).

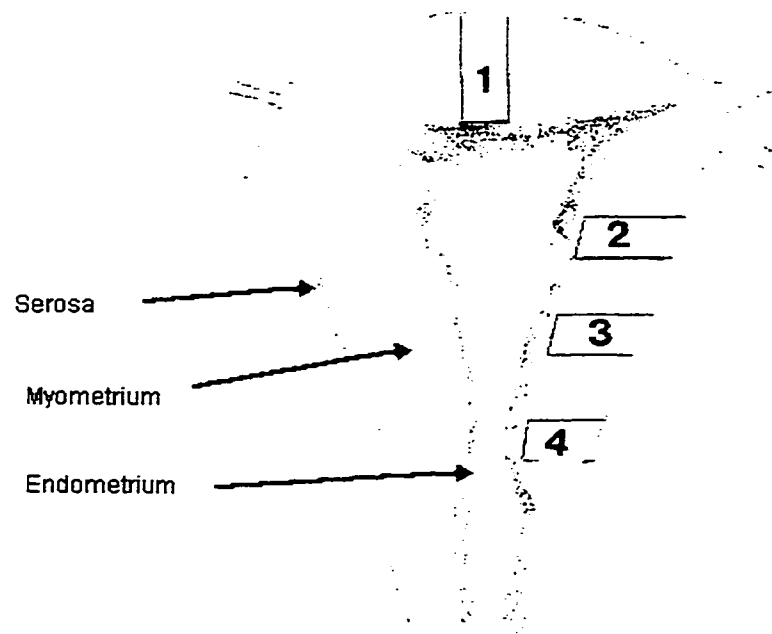
## **2.2. MYOMETRIAL TISSUE COLLECTION**

Human myometrial samples were obtained from premenopausal, nonpregnant women (aged 22 - 49), undergoing hysterectomy for benign disorders such as fibroids, menorrhagia and uterine prolapse. The tissue collection protocol was approved by the Research Advisory Group of the Faculty of Health Sciences, McMaster University and at the Henderson General Hospital, Hamilton, Ontario, Canada. At the time of surgery none of the women had used oral contraceptives or received any hormone therapy, however use of prostaglandin synthase inhibitors was not assessed. Only specimens from women with regular 28 day cycles were used, and phase of the cycle at the time of collection was determined by patient recollection of their last menstrual period. Specimens were generally taken from the anterior aspect of the corpus uteri. For purposes of data analysis, all tissues were grouped as proliferative phase or secretory phase tissues, where proliferative phase represents the time between the onset of menses to the 14<sup>th</sup> day of the cycle, and secretory phase represents the second half of the menstrual cycle, starting fifteen days after the onset of menses. Specimens intended for *in vitro*

contractility studies were excised from three possible locations (top, lateral wall, lower uterine segment), as diagrammed in Figure 1. Except where specified, experiments were performed on tissue excised from the lateral wall (Position 2 in Figure 1). Tissues that could not be used immediately, were maintained for up to 24 hours post-operatively in oxygenated (95% O<sub>2</sub>, 5% CO<sub>2</sub>) PSS of the following composition (mM): KCl 4.6; MgSO<sub>4</sub> 1.16; NaH<sub>2</sub>PO<sub>4</sub> 1.16; CaCl<sub>2</sub> 2.5; NaCl 115.5; NaHCO<sub>3</sub> 21.9 and *D*-glucose 11.1 with indomethacin at 10 μM.

Specimens intended for radioligand binding studies were excised from the lateral wall (Position 2 in Figure 1), and were maintained at 4°C for up to 2 hours in Sucrose-MOPS buffer (250 mM sucrose; 20 mM MOPS; 25 mM EDTA; adjusted with NaOH to pH 6.5).

Specimens collected for the isolation of total RNA were excised from four possible locations (Figure 1). Following removal of serosal, endometrial and fibrous tissues (Figure 1), myometrial samples were blotted dry, weighed and stored at -20°C for a maximum of two years.



**Figure 1: Myometrial Tissue Excision Sites.** Myometrial tissue was excised routinely from the anterior aspect of the corpus uteri from one or more of the following sites: (1) Top of the uterine fundus; (2) Lateral wall; (3) Lower uterine segment; (4) Cervix. Arrows denote the location of the *Serosal*, *Myometrial* and *Endometrial* tissue layers of the uterus. Diagram adapted from Netter (1977).



## 2.3. *IN VITRO* CONTRACTILITY STUDIES

### 2.3.1. Concentration-Effect Experiments

Myometrial tissue strips were prepared as previously described (Crankshaw, 1990; Crankshaw and Dyal, 1994). Whole tissue specimens were trimmed of endometrial, serosal, fat and fibrous tissue. Up to sixteen strips of myometrium (15 x 2 x 3 mm) from the longitudinal muscle layer directly adjacent to the serosa were cut, tied at each end with silk thread and mounted longitudinally in individual 10 or 15 mL jacketed muscle baths containing oxygenated PSS at 37°C. In a limited number of experiments, tissue strips were also prepared from the sub-endometrial muscle layer and/or mounted in a horizontal orientation as described in Section 3.1.3. One end of each strip was anchored in the bath, the other was attached to an FT-03 force displacement transducer writing to either a 7D polygraph (Grass Instruments, Quincy, MA, USA) or a custom-made amplifier writing to the data collection software, In Vitro Collection System Ver 4.0 (J. Milton, Dundas, ON, Canada), running on a personal computer. An optimum resting force of 25 mN was applied to each strip (Crankshaw and Dyal, 1994). This usually declined quite rapidly and was readjusted throughout the next hour to obtain a steady baseline force of 25 mN. Tissues were then challenged with potassium chloride (90 mM). The criterion for inclusion in the study was that tissue strips should develop a minimum force of 10 mN within 30 seconds of the KCl challenge. Failure to develop this minimum response would have resulted in removal from the experiment, however, in this study no strip failed to meet this criterion. Thorough wash-out of potassium chloride was followed by a three hour equilibration period.

Following the equilibration period the mean force developed by the individual muscle strips during a 10 minute control period was determined and was used as a measure of their contractility. This technique has been used routinely in this laboratory (Dyal and Crankshaw, 1988) and others (Cheuk *et al.*, 1993) since it can be used to successfully quantify drug effects in tissues that develop significant spontaneous activity and that respond to stimulation by changes in both tonic and phasic activity (Crankshaw, 1990). Mean force was determined as described by Wainman *et al.* (1988), using the In Vitro Collection Software. Agonists (U-46,619 or I-BOP) were then added to the baths, in a cumulative fashion every 11 minutes. A total of 8 to 10 additions were made to ensure maximum response had been achieved. The mean force recorded in the 10 minute period immediately following agonist addition, minus the mean control force was considered to be the force developed in response to that concentration of agonist. Agonist concentration-effect curves (percentage of maximum response versus log molar agonist concentration) were then constructed from these data by fitting the equation:

$$E = E_{\min} + (E_{\max} - E_{\min}) / (1 + e^{-k \cdot (\log C - \log EC_{50})})$$

where E is the effect of the agonist, C is the molar concentration of the agonist, k is a power coefficient and  $EC_{50}$  is the molar concentration of the agonist that produces a half-maximal response. The value of  $-\log EC_{50}$  is equivalent to the  $pEC_{50}$ .

Concentration-effect curves for U-46,619 and I-BOP were also determined in the absence and in the presence of various TP receptor antagonists. Only one concentration-effect

experiment could be performed on each tissue strip. Therefore, antagonists' effects were investigated by incubating separate tissue strips from the same donor in the absence or in the presence of one of three concentrations of antagonist for 1 hour prior to and throughout the duration of an agonist concentration-effect experiment. Normally, two strips were used at each concentration of antagonist and two strips for controls. EC<sub>50</sub> values were calculated as described above. Antagonist pA<sub>2</sub> values were then determined according to the equation:

$$pA_2 = \log((EC_{50A}/EC_{50C})-1)-\log[B]$$

where EC<sub>50A</sub> is the agonist EC<sub>50</sub> in the presence of antagonist, EC<sub>50C</sub> is the control agonist EC<sub>50</sub> and [B] is the molar concentration of antagonist. The final pA<sub>2</sub> reported represents a mean of the individual pA<sub>2</sub> values calculated at each antagonist concentration, as individual pA<sub>2</sub> values were not concentration-dependent.

### 2.3.2. Statistics

All data are expressed as mean ± S.D. Statistical comparisons of U-46,619 potency were performed using a Students' t-Test and One-way Anova. Analysis of antagonists' rank order potencies was performed using Spearman's Rank correlation coefficient. In each case, data were considered to be significant when P < 0.05.

## 2.4. RADIOLIGAND BINDING STUDIES

### 2.4.1. Preparation of Human Myometrial Membranes (HMM)

Enriched membrane fractions were prepared from myometrial tissues within 2 hours of tissue retrieval as previously described (Crankshaw *et al.*, 1979). Myometrial tissue was cleared of any endometrial, serosal and fibrous tissue, lightly blotted dry and weighed. Tissue was minced with a razor blade and suspended in 6 volumes of Sucrose-MOPS buffer and homogenized with a Polytron PT20 (Brinkmann; Westbury, NY, USA), giving three bursts of 10 second duration each at a rheostat setting of 10. The Polytron probe was washed with 6 volumes of Sucrose-MOPS buffer giving one 10 second burst at a rheostat setting of 10. The homogenates and washings were combined and centrifuged at 3,000 rpm (1,400 g) for 15 minutes at 4°C in a Sorvall RC5 centrifuge with a SS-34 rotor (Fisher Scientific, Nepean, ON, Canada). Supernatants were filtered through three layers of cheese cloth, so as to remove cellular debris and the collected filtrate was centrifuged at 35,000 rpm (87,000 g) for 30 minutes at 4°C in a Beckman L7 ultracentrifuge using a Beckman 60Ti rotor (Beckman; Rexdale, ON, Canada). Supernatants were discarded and the final pellet, which represented the enriched membrane fraction was resuspended in Sucrose-MOPS buffer to an approximate protein concentration of 1 mg/mL. Membrane preparations were aliquoted and stored at -20°C for no more than 2 months. Final protein concentrations were measured according to the method of Bradford (1976), using bovine serum albumin as standard.

### 2.4.2. Saturation Radioligand Binding Studies

Saturation experiments using HMM were performed with the iodinated TP receptor agonist [<sup>125</sup>I]-BOP. Saturation experiments were carried out in silanized glass tubes (13x100 mm) in a final volume of 250  $\mu$ L. Incubations contained 100  $\mu$ g of membrane protein with [<sup>125</sup>I]-BOP (~15,000 cpm, 30 pM) and increasing concentrations of [<sup>127</sup>I]-BOP (Morinelli *et al.*, 1990b). In addition, each reaction contained: 4.6 mM MgCl<sub>2</sub>; 0.5 mM MOPS; 6 mM indomethacin; and 2.3 mM phenylmethylsulfonylfluoride. The incubation period was 60 minutes at 30°C. All reactions were performed in triplicate, were initiated by the addition of the HMM fraction and were terminated by the addition of 4 mL of ice cold Sucrose-MOPS buffer (250 mM sucrose; 20 mM MOPS; pH 7.4) followed by filtration under vacuum through Whatman GF/C glass fiber filters (Whatman INC; Clifton, NJ, USA). The filters were washed with three additional 4 mL volumes of ice cold buffer and then counted for bound radioactivity in a LKB 1261 Gamma Counter (Pharmacia; Baie D'Urfe, PQ, Canada). The filtration procedure was complete within ten seconds. Non-specific binding was defined as the amount of radioactivity bound in the presence of the TP receptor antagonist L670,596 (10 mM) (Morinelli *et al.*, 1990b), specific binding was determined by subtracting non-specific binding from total binding. Binding data were fitted to one- and two-site models according to:

$$RL = (R_t * L)/(K_d + L)$$

and

$$RL = ((R_{t1} * L)/(K_{d1} + L)) + ((R_{t2} * L)/(K_{d2} + L))$$

respectively, where  $RL$  is the molar concentration of specifically bound ligand,  $R_t$ ,  $R_{t1}$ , and  $R_{t2}$ , represent the total concentration of each site,  $K_d$ ,  $K_{d1}$ ,  $K_{d2}$ , the concentration of ligand that produces half-maximal saturation of each site, and  $L$  is the molar concentration of free ligand. The significance of the fit between the two- and one-site models was determined using the F test as described by Munson and Rodbard (1980).

### 2.4.3. Saturation Radioligand Binding Studies with GTP $\gamma$ S

The effect of GTP $\gamma$ S on [ $^{125}$ I]-BOP binding was studied using two different buffer systems, Sucrose-MOPS and HEPES (25 mM HEPES; 125 mM NaCl; 10 mM MgCl $_2$ ; pH 6.5) with and without the addition of 100 $\mu$ M GTP $\gamma$ S. Binding was performed as described above. In the case of membranes suspended in HEPES buffer, termination of reactions and filter washing was performed with a buffer consisting of 25 mM HEPES and 125 mM NaCl, pH 6.5.

### 2.4.4. Binding Kinetics

Kinetic analysis of [ $^{125}$ I]-BOP binding to HMM was performed at 30°C. Association and dissociation rates were determined from single binding reactions of 4.125 mL which contained the same molar concentrations of reagents as described above and 1 nM [ $^{125}$ I]-BOP. The time course of association was determined by removing individual 250  $\mu$ L aliquots of the reaction at specified time points from 1 to 60 minutes after initiation of the reaction. The binding reaction

was stopped by placing the aliquot in a test tube containing 4 mL of ice cold buffer. This sample was then filtered and the test tube was washed three additional times with ice cold buffer as described above. Dissociation was monitored by the addition of ICI 192,605 (10  $\mu$ M) to parallel samples immediately after the 50 minute aliquot was drawn. The time course of dissociation was determined from 52 to 120 minutes exactly as described above. Non-specific binding was determined in the presence of 10  $\mu$ M L670,596. The effective association rate constant ( $k_{obs}$ ) and the dissociation rate constant ( $k_{21}$ ) were calculated by non-linear analysis of ligand association and dissociation curves as described by Hulme and Birdsall (1992). Ligand association was given by:

$$RL = RL_{eq}(1 - e^{-(k_{12} \cdot L + k_{21}) \cdot t})$$

and ligand dissociation was given by:

$$RL = RL_0 \cdot e^{-k_{21} \cdot t}$$

where  $RL_0$  is the molar concentration of bound ligand at  $t = 0$  and  $RL_{eq}$  is the molar concentration of bound ligand at equilibrium.

The association rate constant ( $k_{12}$ ) was calculated from the equation:

$$k_{obs} = (k_{12} \cdot L) + k_{21}$$

The kinetically determined dissociation constant ( $K_d$ ) was given by  $K_d = k_{21}/k_{12}$

#### 2.4.5. Competition Binding

Competition for specific binding of [ $^{125}$ I]-BOP (1 nM) was studied using non-labelled compounds within a concentration range of  $10^{-11}$  to  $10^{-4}$  M. Incubations and termination of binding reactions were performed exactly as described above. Inhibition constants ( $IC_{50}$ ) characterizing competing drugs were determined according to Steinberg *et al.* (1985) using the equation:

$$B_{obs} = 100 * (IC_{50}^h)/(IC_{50}^h + L^h)$$

where  $B_{obs}$  is the percent maximum specific binding,  $IC_{50}$  is the molar concentration of ligand that produces half-maximal inhibition,  $h$  is the Hill coefficient and  $L$  is the molar concentration of competing ligand.

#### 2.4.6. Statistics

All data are expressed as mean  $\pm$  S.D. Statistical comparisons of saturation binding data were performed using a One-way Anova. For competition binding experiments, 95% confidence intervals were calculated for each competitor to determine if the mean Hill coefficients differed significantly from unity. Analysis of rank order potencies was performed using



Spearman's Rank correlation coefficient. In each case, data were considered to be significant when  $P < 0.05$ .

## **2.5. ANALYSIS OF TP RECEPTOR mRNA EXPRESSION**

### **2.5.1. Isolation of Total RNA**

Human myometrial samples were prepared from frozen myometrial tissue by a modified guanidium thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi, 1987). Frozen myometrial tissue samples (0.5 g - 1.0 g), were homogenized in 10 mL of Solution D (4M guanidine thiocyanate, 25 mM sodium-citrate, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol) with a Polytron PT20 (Brinkmann; Westbury, NY, USA), for 10 seconds at a rheostat setting of 12. Sequentially, 1 mL of 2 M sodium acetate pH 4.0; 10 mL of water-equilibrated phenol and 2 mL of chloroform-iso-amyl alcohol mixture (49:1) were added to the homogenate, with thorough mixing by vortexing of the homogenate after the addition of each reagent. The final suspension was vortexed for 10 seconds and placed on ice for 15 minutes. Samples were centrifuged at 10,000 rpm (4,700 x g) for 20 minutes at 4°C in a Sorvall RC5 centrifuge with a SS-34 rotor (Fisher Scientific, Nepean, ON, Canada). The aqueous phase was transferred to a fresh tube, mixed with 1 volume of isopropanol and placed at -20°C for a minimum of 2 hours and a maximum of 24 hours. The solutions were centrifuged at 10,000 rpm (4,700 x g) for 20 minutes at 4°C, the supernatants were discarded, the RNA pellets were dissolved in 1.5 mL of Solution D and precipitated with 1

volume of isopropanol at  $-20^{\circ}\text{C}$  for 2 hours. After centrifugation for 20 minutes at 10,000 rpm ( $4,700 \times g$ ) at  $4^{\circ}\text{C}$ , the RNA pellets were washed with 75% ethanol and centrifuged at 5,000 rpm ( $2335 \times g$ ) for 10 minutes at  $4^{\circ}\text{C}$ . The ethanol was removed by aspiration and the pellets were dried overnight in a desiccator. The RNA pellets were resuspended in 3 ml of RNA solubilization buffer (1 mM EDTA, 0.1 % SDS, 10 mM Tris-HCl pH 7.5); 1.0 mL 2 M sodium acetate pH 5.2 and 8.0 mL absolute ethanol. The concentration and purity of total RNA was determined by UV light absorption using a LKB 4050 Biochrom spectrophotometer (LKB Biochrom Ltd. Cambridge, England). Preparations were discarded if they had a ratio of optical densities at 260nm / 280nm that was lower than 1.6 (Sambrook et al., 1989). To assess the presence of intact RNA molecules 5  $\mu\text{g}$  of total RNA from each sample was loaded onto a 1% agrose-formaldehyde gel and subjected to electrophoresis. Following ethidium bromide staining of the gel, RNA isolates were considered intact if the UV fluorescence of the 28S rRNA band was two times as intense as the 18S rRNA band and when no UV fluorescence was detected below the 18S rRNA band. RNA isolates were stored in RNA solubilization buffer at  $-20^{\circ}\text{C}$  for a maximum of three years.

### 2.5.2. cDNA Synthesis

RNA was reversed transcribed into cDNA following a protocol outlined in the GeneAmp RNA PCR Kit (Perkin-Elmer/Cetus, Norwalk, CT, USA), with some modifications. A 10  $\mu\text{L}$  reverse transcription reaction containing 0.5 $\mu\text{g}$  of total RNA; 1X first strand buffer

(75 mM KCl, 50 mM Tris-HCl pH 8.3, 3.0 mM MgCl<sub>2</sub>); 1.7 mM MgCl<sub>2</sub>; 1 mM each dNTP; 10 mM dTT; 2.5 μM oligo (dT)<sub>18</sub> and 5 U/μL of SuperScriptII Reverse Transcriptase was incubated at 42°C for 60 minutes, heated at 95°C for 5 minutes then cooled at 4°C for a minimum of 5 minutes and a maximum of 30 minutes.

### 2.5.3. Primers Used for Amplification

Oligonucleotide primers used to amplify TP receptor and G3PDH mRNA were synthesized by the Central Facility of the Institute of Molecular Biology and Biotechnology at McMaster University and are detailed in Table 1. Primers for both messages were designed to span intron - exon boundaries to distinguish between amplification of mRNA and genomic DNA. TP receptor primers (TP-A and TP-S), were designed based on the cloned full length human cDNA (Hirata *et al.*, 1991) (Gene Bank accession numbers D38081 and U27325). A second set of primers was synthesized for the amplification of a variant form of TP receptor mRNA that was reported to arise from alternative splicing of the human TP receptor gene (Raychowdhury *et al.*, 1994; 1995). This primer pair, labeled ALT-TP-A and ALT-TP-S, was used *only* in a limited number of experiments designed to probe for this splice variant. Primers designed to amplify G3PDH mRNA were originally designed by Clontech (Clontech Laboratories Inc, Palo Alto, California, USA), based on the published human cDNA sequence (Arcari *et al.*, 1984).

**Table 1. Oligonucleotide Primer Sequence Information**

Primer Name	Sequence	Reference Location	Mp <sup>a</sup> (°C)	Product Size	Digestion Products
TP-S	5' GGT GAC CGG TAC CAT CGT GGT GTC 3'	237-260 <sup>b</sup>	78		300 / 173
TP-A	5' CTT CCT ACT GCA GCC CGG AGC GCT G 3'	1012-1036 <sup>b</sup>	84	799	/ 326 <sup>b</sup>
G3P-S	5' TGA AGG TCA GAG TCA ACG GAT TTG GT 3'	71-96 <sup>c</sup>	76		---
G3P-A	5' CAT GTG GGC CAT GAG GTC CAC CAC 3'	1030-1053 <sup>c</sup>	78	983	
ALT-TP-S	5' GAG ATG ATG GCT CAG CTC CT 3'	724-743 <sup>d</sup>	62		---
ALT-TP-A	5' TGG GCC ACA GAG TGA GAC TC 3'	986-1005 <sup>d</sup> / 1645-1664 <sup>e</sup>	64	282 <sup>f</sup> / 941 <sup>g</sup>	

<sup>a</sup> Estimated melting point of primer

<sup>b</sup> Based on published human cDNA sequence (Hirata., *et al*, 1991)

<sup>c</sup> Based on published human cDNA sequence (Arcari., *et al*, 1984)

<sup>d,e</sup> Based on published human cDNA sequence (Raychowdhury., *et al*, 1994), where two different annealing locations are expected referring to TP receptor cDNA<sup>d</sup> and alternatively spliced TP receptor cDNA<sup>e</sup>

<sup>f,g</sup> Expected size of amplification product, where two products are possible due to co-amplification of alternatively spliced TP receptor mRNA<sup>f</sup> and TP receptor mRNA<sup>g</sup>

<sup>h</sup> Expected product lengths following endonuclease digestion of TP Receptor mRNA PCR product with SmaI

#### 2.5.4. Polymerase Chain Reaction

PCR was performed on 5  $\mu$ L of cDNA preparation, to which was added 44  $\mu$ L of a PCR master mix containing 1X PCR buffer (55 mM KCl, 13 mM Tris-HCl pH 8.3); 1 mM MgCl<sub>2</sub>; 10% dimethylsulphoxide (DMSO); 1.25 U/50  $\mu$ L *Taq* DNA polymerase and 0.2  $\mu$ M each sense and antisense primer in a total volume of 50  $\mu$ L. The amplification time profile involved denaturing at 92°C for 45 seconds, annealing at 66°C for 45 seconds and extending at 72°C for 1 minute. Thirty-five cycles of PCR was performed during preliminary experiments designed to amplify TP receptor or alternatively spliced TP receptor mRNA. To validate semi-quantitative assessment of TP receptor gene expression, the kinetics of both TP receptor and G3PDH mRNA amplification were studied empirically to determine the

points at which amplification was in the exponential phase (see Section 3.3.4 and 3.3.5). Precautions were taken to avoid PCR product contamination. PCR set-up, amplification and product processing were performed using dedicated equipment in separate areas. In addition, several control reactions were routinely run in parallel during RT-PCR analysis including RT reactions run in the absence of the reverse transcriptase enzyme to confirm the absence of genomic DNA and/or cDNA contamination and RT reactions without RNA to check for reagent contamination. PCR amplification of 10 pg of cloned human TP receptor cDNA purified from Bluescript2 plasmid (Abramovitz, 1992) or 1.5 ng of human genomic DNA served respectively as positive and negative controls. The cloned TP receptor cDNA used in these experiments was determined to have a 60 bp internal deletion in the coding sequence (Abramovitz, 1992; personal communication), and as the TP receptor primers spanned this deletion site the anticipated PCR amplification product was 739 bp.

### **2.5.5. Analysis of PCR Products**

#### **2.5.5.1. Electrophoresis**

PCR products containing 0.4 vol of loading dye (30% glycerol, 0.4% bromophenol blue, 0.4% xylene cyanol) were size fractionated on 1.5% agarose gels containing 0.5 µg/mL ethidium bromide. Unless otherwise stated, electrophoresis was performed in TBE buffer (45 mM Tris-HCl pH 8.0, 45 mM boric acid, 2 mM EDTA (disodium)) for 2 hours at 95 V. 500 ng of a 100 bp DNA molecular weight ladder was included as a size marker. PCR

product bands within the gel were visualized by UV transillumination. A photograph of each gel was obtained using a Polaroid DS34 direct screen instant camera and Polaroid 667 Negative Instant Pack film (Polaroid Co, USA). Product identification was made by comparison to the molecular weight ladder and/or the positive control.

### **2.5.5.2. Endonuclease Digestion**

Digestion of TP receptor amplification product was performed using 20 U of SmaI endonuclease enzyme in a reaction volume of 25  $\mu$ L. Following digestion, products were resolved by 2.5 hours of electrophoresis at 90 V on a 2.0% agarose-TBE gel stained with 0.5  $\mu$ g/mL ethidium bromide. Gels were illuminated by UV light and photographed. Confirmation of appropriate splice products was made by comparison to 500 ng of a 100 bp DNA molecular weight ladder included on each gel.

### **2.5.5.3. Hybridization**

In experiments designed to validate the TP receptor amplification product, TP receptor RNA was synthesized and labeled with digoxigenin-11-ddUTP (DIG) and used as a probe for Southern blot analysis. 2  $\mu$ g of Bluescript2 plasmid containing cloned human TP receptor cDNA was linearized with 15 U of HINDIII and purified on an Elutip-R minicolumn (Schleicher and Schuell, USA). RNA was transcribed and labeled with a DIG-RNA Labeling Kit using T7 Polymerase (Boehringer Mannheim, Germany). The resulting DIG-labeled RNA was treated with 20 U of RQ1 RNase-free DNase and purified over a glycogen gradient. The

final ethanol precipitated pellet was suspended in 50  $\mu$ L of TE(8) (10 mM Tris-HCl pH 8.0, 1 mM EDTA (disodium) and stored at  $-20^{\circ}\text{C}$ . Southern blotting (Southern, 1975), was performed following a procedure outlined in *The DIG System User's Guide for Filter Hybridization* (Boehringer Mannheim, Germany). TP receptor PCR amplification products were subjected to electrophoresis on a 1.5% agarose-TBE gel for 2.5 hours at 90 V. Following denaturing and neutralization treatments, the PCR products were blotted from the gel by capillary transfer in 20X SSC (3 M NaCl; 0.3 M sodium citrate) onto a positively charged nylon membrane (Dupont, USA). Fixation of the DNA was accomplished by baking the membrane at  $100^{\circ}\text{C}$  for 40 minutes. Dried membranes were sealed in hybridization bags and stored at  $4^{\circ}\text{C}$ . Hybridization and development of Southern blots was performed following procedures outlined in *The DIG System User's Guide for Filter Hybridization* (Boehringer Mannheim, Germany). Membranes were prehybridized in prehybridization buffer (5X SSC, 50% deionized formamide, 0.1% sodium-lauroylsarcosine, 0.02% SDS, 2% blocking reagent), for 2 hours at  $50^{\circ}\text{C}$ . Hybridization was conducted in the presence of 100 ng/mL TP receptor DIG-labeled RNA probe in the same solution as described for prehybridization. Membranes were hybridized overnight at  $50^{\circ}\text{C}$ . Both prehybridization and hybridization were conducted in sealed plastic bags and the volumes of prehybridization and hybridization buffer added were calculated based on 10 mL / 100  $\text{cm}^2$  membrane surface and 4.0 mL / 100  $\text{cm}^2$  membrane surface respectively. Before development, blots were washed twice in 2X washing solution (2X SSC, 0.1% SDS), once in 0.5X washing solution and once in 0.1X washing solution. Each wash was for 15 minutes at room temperature. Chemiluminescent detection was performed with an anti-DIG antibody tagged with alkaline

phosphatase, diluted 1:5,000 and CSPD substrate. Before exposure to film, blots were incubated at 37°C for 10 minutes. Blots were typically exposed to film for 5 minutes.

### **2.5.6. Detailed Procedures for Semi-Quantitative RT-PCR**

#### **2.5.6.1. Semi-Quantitative Analysis of PCR Products**

A photographic image was acquired using a Paper Port 6000 flat-bed scanner (Visioneer Inc, USA) and Photoshop software (Adobe System). The quantity of PCR product was determined by calculating the integrated density of each PCR product band from the positive photographic image using Scion Image for Windows software (freeware, Scion Corporation, USA), where integrated density is defined as the sum of the optical density of each pixel defining the product band. Data are reported as uncalibrated optical density (OD) units as image analysis was performed using an uncalibrated reference scale. Semi-quantitative assessment of TP mRNA expression was made by comparing TP receptor mRNA expression relative to that of an external standard. Specifically, data are reported as the ratio of the integrated density of TP receptor mRNA amplification product / the integrated density of G3PDH mRNA amplification product.



### **2.5.6.2. Investigation of TP Receptor mRNA Expression in Myometrial Tissue Derived from a Single Excision Site**

For each of the four excision sites diagrammed in Figure 1, eight RNA preparations were studied, where four preparations represented proliferative phase tissues and four represented secretory phase tissues. Each preparation had previously been assigned a numerical code during the RNA isolation procedure, thus the order of PCR tube preparation followed this numerical code in ascending order to avoid any potential experimental bias. G3PDH and TP receptor mRNA were amplified in parallel in separate tubes for 29 and 35 cycles respectively, as determined from kinetic studies. Each set of eight RNA preparations was subjected to three separate RT-PCR analyses. Failure to detect a product due to lack of product intensity or RT-PCR failure was noted.

### **2.5.6.3. Investigation of TP Receptor mRNA Expression in Myometrial Tissue Derived from Different Excision Sites**

Eight RNA preparations were studied simultaneously, in which each of the four sites under investigation were represented by two samples. All eight RNA preparations were derived from tissues excised from donors in the proliferative phase of the menstrual cycle. G3PDH and TP receptor mRNA were amplified in separate tubes for 29 and 35 cycles respectively. Three separate RT-PCR analyses were performed. Failure to detect a product due to lack of product intensity or RT-PCR failure was noted.

#### **2.5.6.4. Investigation of TP Receptor mRNA Expression in Different Human Tissues**

Seven different human total RNA preparations [brain, heart, kidney, lung, placenta, skeletal muscle and small intestine], were purchased from Clontech Laboratories (Palo Alto, Ca, USA), so as to examine the relative tissue distribution of TP receptor mRNA. Total RNA (0.5 µg), from each preparation as well as human myometrial total RNA derived from tissue excised from the uterine lateral wall was reverse transcribed and amplified over 29 or 35 cycles to identify G3PDH and TP receptor mRNA respectively. Three separate RT-PCR analyses were performed. Failure to detect a product due to lack of product intensity or RT-PCR failure was noted.

#### **2.5.7. Statistical Analysis**

All data are expressed as mean  $\pm$  S.D. TP receptor and G3PDH mRNA amplification kinetics were analysed by linear regression. Statistical comparisons of TP receptor/G3PDH ratios derived from RNA preparations from the same site and RNA preparations from four different sites were performed using the Kruskal-Wallis test. The Mann-Whitney test was used to test for significance between TP receptor/G3PDH ratios in proliferative phase and secretory phase groups. Correlation coefficient, linear regression and One-way Anova were used to assess the reproducibility of RT-PCR data. In each case, data were considered to be significant when  $P < 0.05$ .

## Chapter 3

### Results

#### 3.1. *IN VITRO* CONTRACTILITY STUDIES

##### 3.1.1. Agonist Induced Myometrial Contraction

Figure 2 is a representative tracing of the effect of U-46,619 on a strip of human myometrium obtained from the uterine lateral wall of a nonpregnant donor *in vitro*. As reported by others (Senior *et al.*, 1992; Senior *et al.*, 1993), the spontaneous force developed by individual strips of myometrium from nonpregnant donors was variable. However, the peak tension induced by both U-46,619 and I-BOP was consistent when compared to the corresponding KCl response, being  $1.02 \pm 0.18$  ( $n = 10$ ) and  $1.08 \pm 0.16$  ( $n = 10$ ) times greater respectively. The concentration-effect curves resulting from such experiments were often bell-shaped in appearance. For purposes of analysis, data points falling below and to the right of the concentration-effect asymptote were omitted. The  $pEC_{50}$  for U-46,619-stimulated myometrial contraction was  $6.9 \pm 0.27$  ( $n = 34$ ). The maximum response induced by U-46,619 was  $2.3 \pm 1.2$  N/cm<sup>2</sup>. The  $pEC_{50}$  and maximum response values for I-BOP-stimulated contraction were  $7.8 \pm 0.60$  and  $2.1 \pm 0.4$  N/cm<sup>2</sup> respectively ( $n = 6$ ).

### 3.1.2. Effect of TP Receptor Antagonists on U-46,619-Induced Activity

Figures 3 and 4 show the effect of the TP receptor antagonist ICI 192,605 on the U-46,619-induced contractile response of myometrial smooth muscle strips. Increasing the concentration of ICI 192,605 caused a parallel rightward displacement in the concentration-effect curve. Table 2 summarises the  $pA_2$  values for each of the TP receptor antagonists examined in this study. As can be seen in Table 2, the potency of TP receptor antagonists on human myometrium was similar to what has been reported for human platelets.

**Table 2: Potencies of TP Receptor Antagonists on Human Myometrium and on Human Platelets *in vitro***

ANTAGONIST	HUMAN MYOMETRIUM <sup>a</sup>		HUMAN PLATELETS <sup>b</sup>		
	$pA_2 \pm S.D.$	n	$pA_2$	$pIC_{50}$	REFERENCE
ICI 192,605	$9.2 \pm 0.3$	7	8.2		Brownrigg et al., 1992
ICI D1,542	$9.1 \pm 0.3$	6	8.4		Jessup et al., 1988
L670,596	$8.6 \pm 0.3$	6		6.96	Ford-Hutchinson et al., 1989
GR 32,191	$8.6 \pm 0.2$	6	8.79		Lumley et al., 1989
SQ 29,548	$8.2 \pm 0.5$	6		7.92	Ogletree et al., 1985
ONO 3,708	$8.1 \pm 0.3$	7		6.21	Kondo et al., 1989
BM 13,505	$7.4 \pm 0.2$	5		6.44	Bush and Smith et al., 1986

<sup>a</sup> Antagonist potency determined in this study as described in Section 2.3.1.

<sup>b</sup> Antagonist potency determined as described in the reference cited.

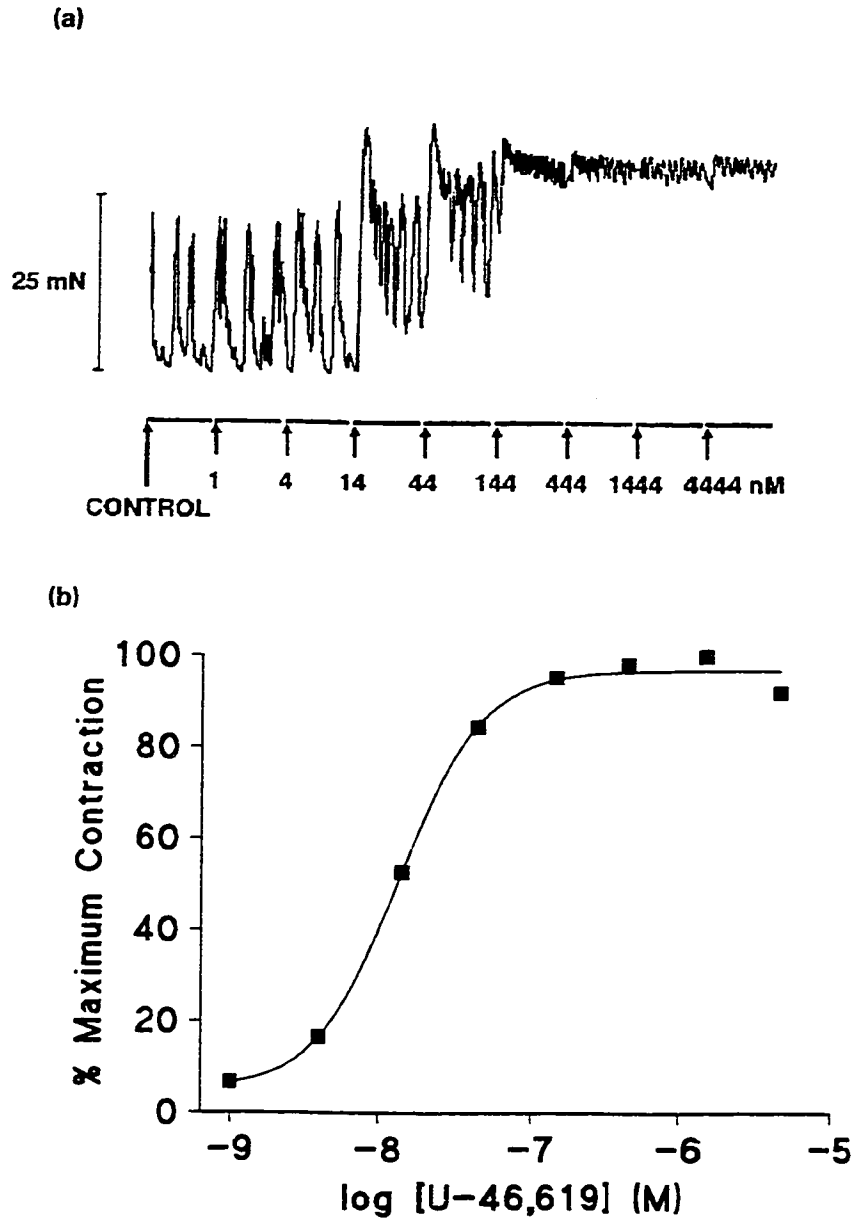
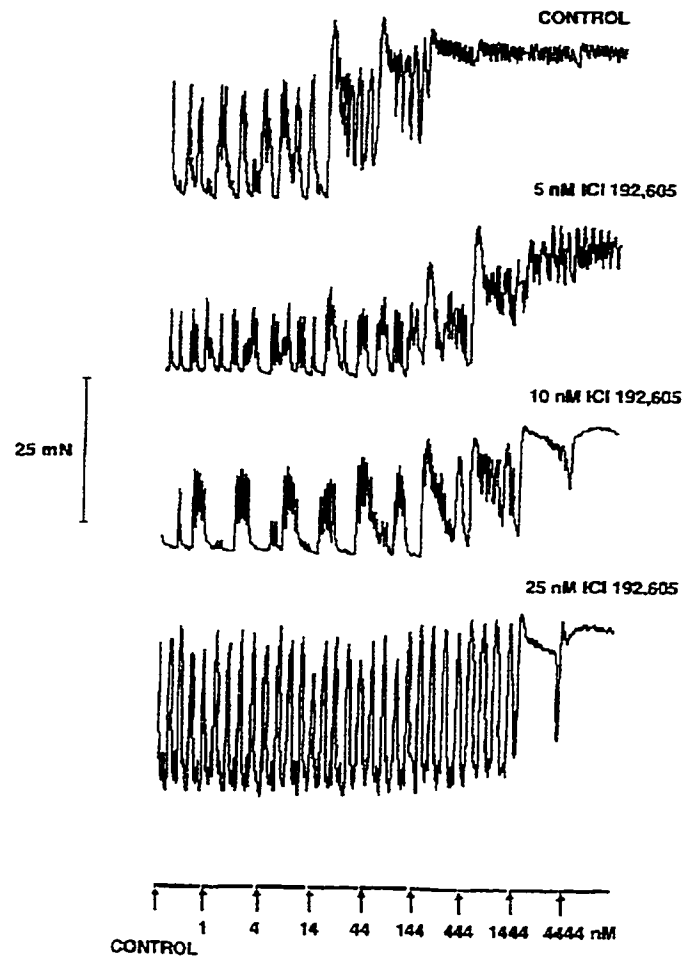


Figure 2: The Effect of U-46,619 on Force Development by a Strip of Human Myometrium from the Uterine Lateral Wall of a Nonpregnant Donor. (a) Analog recording of contractile activity. Horizontal bars indicate 10 minute collection periods, the first of which is the control. Thereafter, U-46,619 was added cumulatively at the arrows to give bath concentrations ranging from 1 nM to 4.4  $\mu$ M. (b) The concentration-effect curve from the data in (a). The last collection in this experiment produced activity that was lower than the maximum, and therefore was not included in the determination of the  $pEC_{50}$ .



**Figure 3: Analog Representation of the Effect of the TP receptor Antagonist ICI 192,605 on U-46,619-Induced Contraction of Strips of Human Myometrium from the Same Nonpregnant Donor.** Analog recordings of contractile activity in the presence of different concentrations of ICI 192,605. Horizontal bars indicate 10 minute collection periods, the first of which is the control. Thereafter, U-46,619 was added cumulatively at the arrows to give bath concentrations ranging from 1 nM to 4.4  $\mu$ M.

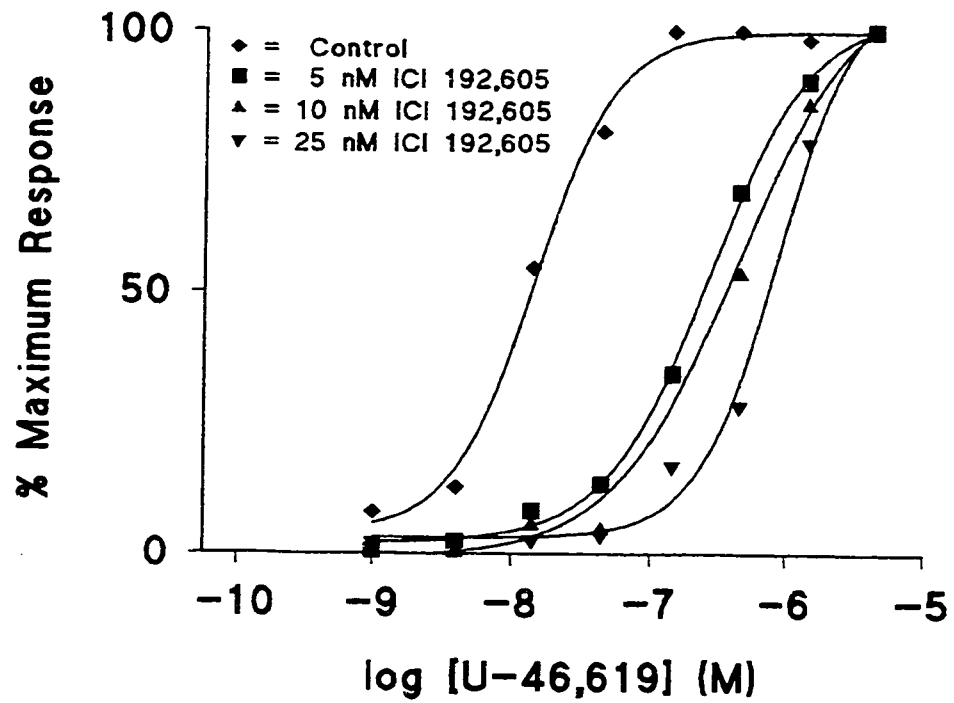


Figure 4: The Effect of the TP Receptor Antagonist ICI 192,605 on U-46,619-Induced Contraction of Strips of Human Myometrium from the Same Nonpregnant Donor. Concentration-effect curves from data in Figure 3.

### 3.1.3. Effect of Anatomical Location, Orientation and Menstrual Cycle Status on U-46,619-Induced Contractile Activity

To examine the effect of excision site, tissue orientation and menstrual status of the donor on the response of nonpregnant myometrium to U-46,619, tissues were obtained from the sub-serosal layer of the top, lateral wall and lower uterine segment and from the sub-endometrial layer of the lateral wall of the uterine fundus. Experiments were conducted as detailed in Section 2.3.1. Graded responses to U-46,619 were obtained in all strips tested, and resulting concentration effect curves were often bell-shaped in appearance. No significant difference ( $P < 0.05$ ), was found in either  $pEC_{50}$  or maximum response values between groups (Table 3).

**Table 3: Effect of Excision Site, Orientation and Menstrual Cycle Status of the Donor on U-46,619-Induced Contractile Activity of Human Nonpregnant Myometrium**

EXCISION SITE	PROLIFERATIVE PHASE		SECRETORY PHASE	
	$pEC_{50}$	MAXIMUM RESPONSE (N/cm <sup>2</sup> )	$pEC_{50}$	MAXIMUM RESPONSE (N/cm <sup>2</sup> )
Top	6.9 ± 0.2	2.6 ± 1.0	6.9 ± 0.3	1.8 ± 0.9
Lateral Wall	6.9 ± 0.3	1.4 ± 0.9	7.1 ± 0.3	3.1 ± 1.0
Lateral Wall sub-endometrial	6.8 ± 0.1	0.9 ± 0.8	7.0 ± 0.4	1.1 ± 0.4
Lateral Wall horizontal orientation	6.8 ± 0.2	1.3 ± 0.7	6.7 ± 0.2	1.1 ± 0.6
Lower Uterine Segment	6.8 ± 0.3	2.4 ± 2.4	7.0 ± 0.2	2.2 ± 0.9

(values are means ± S.D. means, n = 5 in all cases)



## 3.2. RADIOLIGAND BINDING STUDIES

### 3.2.1. Saturation Binding of [<sup>125</sup>I]-BOP

Initial studies indicated that displaceable binding of [<sup>125</sup>I]-BOP was linear over a protein range of 40 to 120 µg protein/tube, with a pH optimum of 6.5 (data not shown). All subsequent experiments were carried out at pH 6.5 with 100 µg protein/tube. Binding of [<sup>125</sup>I]-BOP to HMM was saturable over a concentration range of 300 to 4000 pM (Figure 5). Analysis of the data by non-linear regression yielded a best fit of the data to a one-site model with a mean  $K_d$  of 3.4 nM ( $pK_d = 8.7 \pm 0.4$ ) and a  $B_{max}$  of  $323.1 \pm 361.5$  fmol/mg protein ( $n = 20$ ).

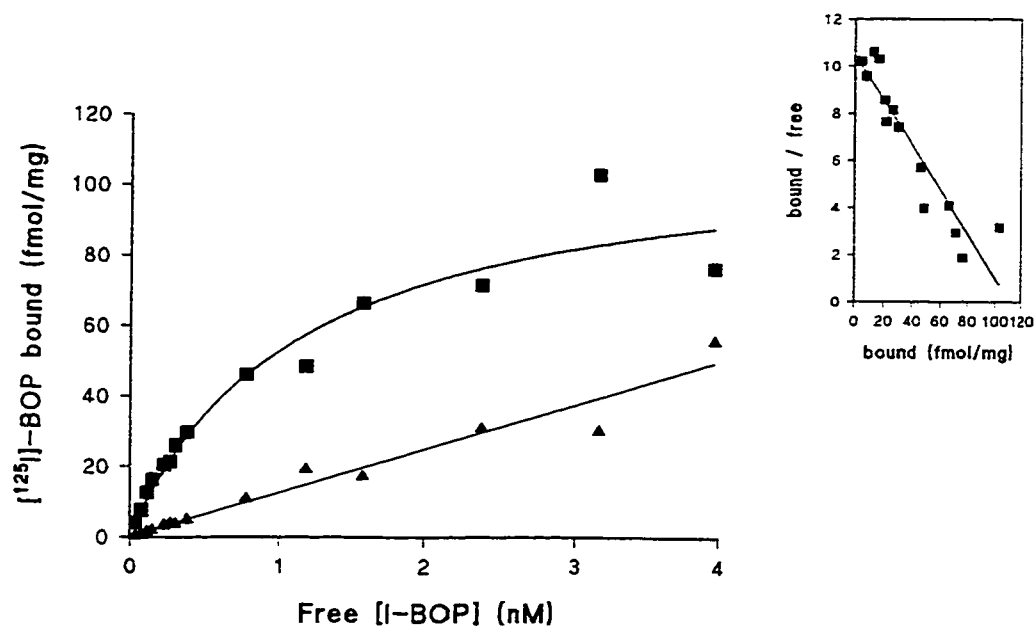
### 3.2.2. Saturation [<sup>125</sup>I]-BOP Binding Studies with GTP $\gamma$ S

The addition of 100 µM GTP $\gamma$ S in Sucrose-MOPS or HEPES buffered experiments did not have any effect on [<sup>125</sup>I]-BOP binding as demonstrated in Table 4 ( $P < 0.05$ ).

Table 4: Effect of GTP $\gamma$ S and Buffer on [<sup>125</sup>I]-BOP Binding to Human Myometrial Membranes

BUFFER	$K_d$ (nM)		$pK_d$		$B_{max}$ (fmol/mg protein)	
	+GTP $\gamma$ S	CONTROL	+GTP $\gamma$ S	CONTROL	+GTP $\gamma$ S	CONTROL
Sucrose-MOPS	4.4	4.7	$8.4 \pm 0.3$	$8.4 \pm 0.3$	$578.1 \pm 87.8$	$583.4 \pm 151.8$
HEPES	1.3	1.1	$9.0 \pm 0.3$	$9.3 \pm 0.5$	$1266.5 \pm 530.4$	$821.3 \pm 410.3$

Data are expressed as means  $\pm$  S.D. from 3 experiments.



**Figure 5. Effect of the Free Concentration of  $[^{125}\text{I}]\text{-BOP}$  on the Amount of  $[^{125}\text{I}]\text{-BOP}$  Bound by Membranes Prepared from Human Myometrium from a Nonpregnant Donor.** The conditions of the binding assay were as described in Section 2.4.2. **Upper Curve:** specific binding; **Lower Curve:** non-specific binding; **Inset:** specific binding in the form of a Scatchard plot. The saturation isotherm of specific binding fit a one-site model with a  $K_d$  of 1.1 nM and a  $B_{\text{max}}$  of 109 fmol/mg protein. Representative of 20 experiments.

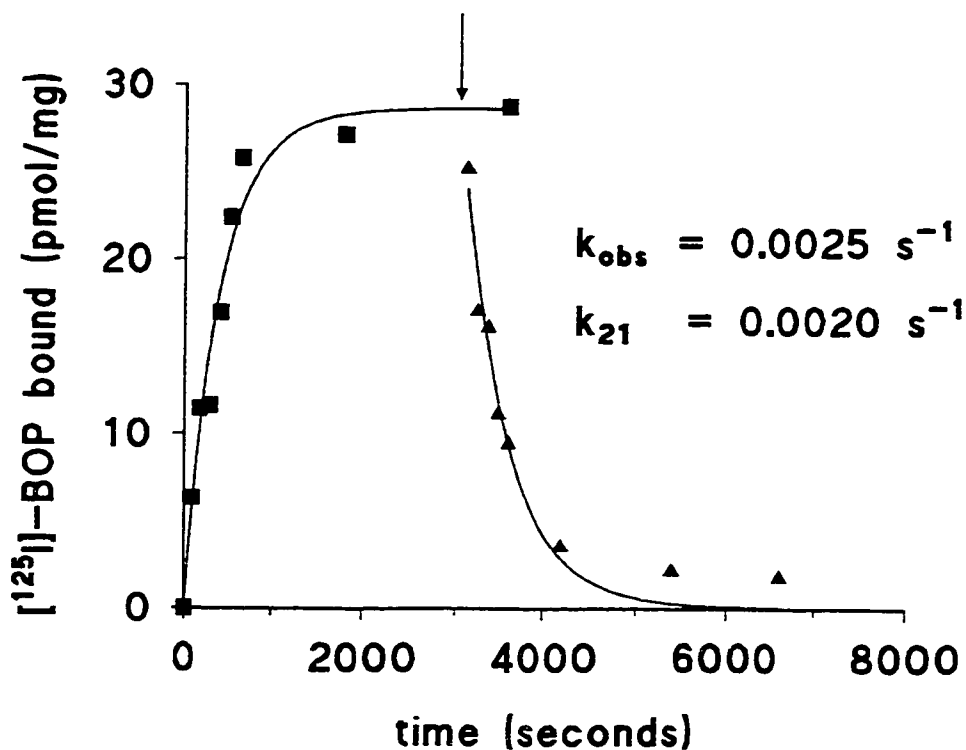
### 3.2.3. Kinetics of [<sup>125</sup>I]-BOP Binding

The  $K_d$  of [<sup>125</sup>I]-BOP binding was also determined kinetically. The association rate constant ( $k_{12}$ ) for the ligand-binding site complex was determined from the time course of [<sup>125</sup>I]-BOP binding. At a concentration of 1 nM, equilibrium binding occurred with a  $t_{1/2}$  of 4.8 minutes (Figure 6). The effective association rate constant ( $k_{obs}$ ) was  $0.0024 \text{ sec}^{-1}$ . The dissociation rate constant ( $k_{21}$ ) was determined from the time course of dissociation of [<sup>125</sup>I]-BOP from its binding site in the presence of  $10 \mu\text{M}$  ICI 192,605 to prevent reassociation of the ligand, and was  $0.002 \text{ sec}^{-1}$ . As can be seen in Figure 6, dissociation was an exponential process although the data do not perfectly fit the mathematical model as residual specific binding remained after 120 minutes. The observed dissociation  $t_{1/2}$  was 5.8 minutes. The association rate constant was  $4.8 \times 10^5 \text{ M sec}^{-1}$  and the kinetically determined dissociation constant ( $K_d$ ) given by  $K_d = k_{21}/k_{12}$  was 4.1 nM.

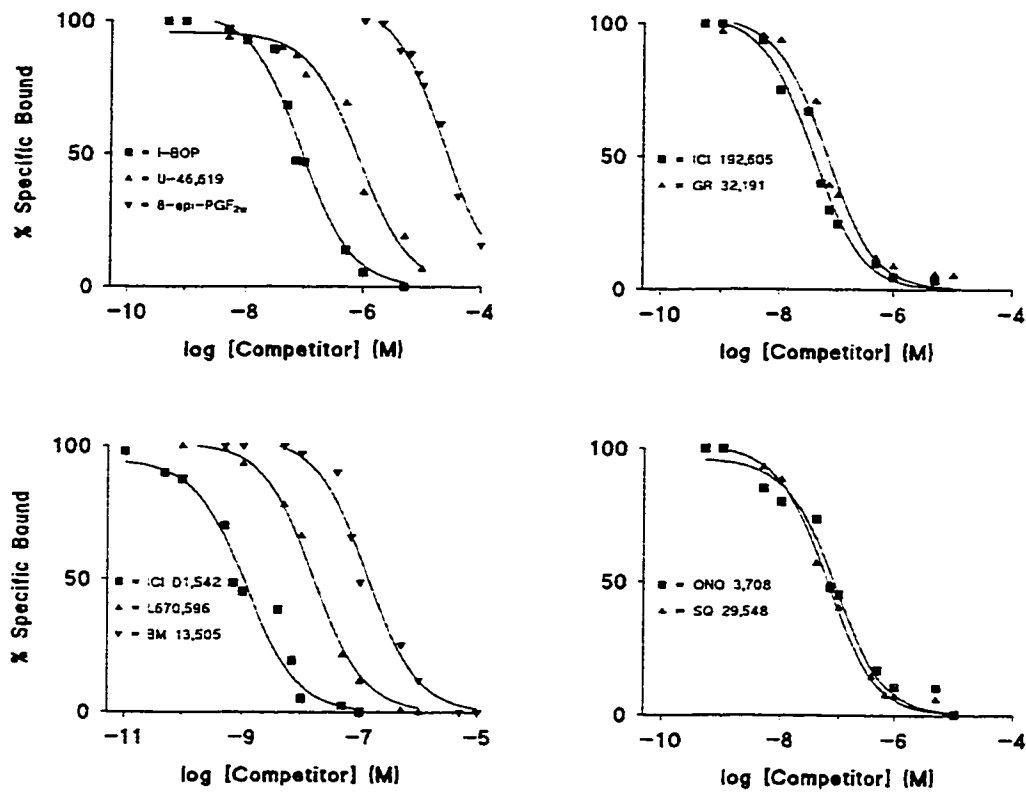
### 3.2.4. Competitive Inhibition of [<sup>125</sup>I]-BOP Binding

To ascertain that [<sup>125</sup>I]-BOP was interacting with TP receptors on HMM, competition binding studies were performed using the TP receptor agonists U-46,619, I-BOP and 8-epi-PGF<sub>2 $\alpha$</sub>  (Takahashi *et al.*, 1992) and the TP receptor antagonists ICI D1,542 (Jessup *et al.*, 1988), ICI 192,605 (Brownrigg *et al.*, 1992), GR 32,191 (Lumley *et al.*, 1989), BM 13,505 (Bush and Smith, 1986), ONO 3,708 (Kondo *et al.*, 1989), SQ 29,548 (Ogletree *et al.*, 1985) and L670,596 (Ford-Hutchinson *et al.*, 1989). Figure 7 shows the cumulative displacement curves for each of the compounds tested. In all cases, displaceable binding was found to be  $\geq 90\%$  and Hill coefficients were not significantly different from unity ( $P < 0.05$ ). The rank order for the

agonists to displace [<sup>125</sup>I]-BOP was I-BOP > U-46,619 > 8-epi-PGF<sub>2α</sub>, whereas for the antagonists it was ICI D1,542, L670,596 > ICI 192,605 > ONO 3,708 > SQ 29,548 > GR 32,191 > BM 13,505 (Table 5). For both agonists and antagonists, the rank orders for displacement correlated with the pharmacological rank orders ( $r = 0.75$ ). In addition, the ability of a variety of prostanoids to compete with [<sup>125</sup>I]-BOP binding was examined to further evaluate its selectivity in this system. BW A868C (DP receptor); sulprostone (EP<sub>1</sub> and EP<sub>3</sub> receptors); AH 13,205 (EP<sub>2</sub> receptor); cloprostenol (FP receptor); and cicaprost (IP receptor), (Coleman *et al.*, 1994) each at 10 μM, displaced 11%, 3%, 0%, 15% and 22% of specifically bound [<sup>125</sup>I]-BOP respectively. Since the  $pIC_{50}$  value for I-BOP did not agree with the equilibrium or kinetic  $K_d$  of I-BOP, I questioned whether I-BOP might be binding to a very low affinity site that was missed by my original saturation experiments. Therefore, I conducted six saturation experiments using an expanded I-BOP concentration range and fit the resulting data to both one- and two-site models. In four cases data were collected suggesting that a lower affinity site may be present, but I was unable to obtain statistical significance in support of this because of high non-specific binding at high ligand concentrations.



**Figure 6: The Kinetics of [<sup>125</sup>I]-BOP Binding to Membranes Prepared from Human Myometrium from a Nonpregnant Donor.** The conditions of the binding assay were as described in Methods. At the point indicated by the arrow, ICI 192,605 was added to give a final concentration of 10  $\mu\text{M}$  in order to prevent re-binding of [<sup>125</sup>I]-BOP. Association and dissociation data were fitted as described in Section 2.4.4. Representative of 3 experiments.



**Figure 7: The Effect of the Concentration of TP Receptor Ligands on the Binding of [<sup>125</sup>I]-BOP to Membranes Prepared from Human Myometrium from a Nonpregnant Donor.** The conditions of the binding assay were as described in Section 2.4.5. The points represent the means of triplicate determinations and are representative of three to five independent experiments. Data were fitted as indicated in Section 2.4.5. Error bars have been omitted for clarity.

**Table 5: Inhibition of [<sup>125</sup>I]-BOP Binding to Human Myometrial Membranes by TP Receptor Ligands**

COMPETITOR	pIC <sub>50</sub>	IC <sub>50</sub> (nM)	n	h	Rank	
					pIC <sub>50</sub>	pEC <sub>50</sub>
<b>AGONISTS</b>						
I-BOP	7.2 ± 0.2	48.5	4	1.2 ± 0.2	1	1
U-46,619	6.2 ± 0.4	660.7	5	1.0 ± 0.1	2	2
8-epi-PGF <sub>2α</sub>	4.5 ± 0.06	30,902	3	1.0 ± 0.3	3	3
<b>ANTAGONISTS</b>						
					Rank	
					pIC <sub>50</sub>	pK <sub>a</sub>
ICI D1,542	8.3 ± 0.4	5.01	4	1.0 ± 0.3	1	2
L670,506	7.9 ± 0.1	12.30	3	1.0 ± 0.1	2	3
ICI 192,605	7.5 ± 0.06	30.90	3	1.2 ± 0.2	3	1
ONO 3,708	7.2 ± 0.04	63.10	3	1.0 ± 0.2	4	5
SQ 29,548	7.2 ± 0.1	64.56	3	1.1 ± 0.2	5	4
GR 32,191	7.0 ± 0.2	107.15	4	1.1 ± 0.1	6	3
BM 13,505	6.8 ± 0.07	177.83	3	1.0 ± 0.3	7	7

r = 0.75

Data are expressed as means ± S.D. from n experiments. Rank orders of pIC<sub>50</sub> values for both agonists and antagonists are compared to the rank orders of functional potencies (pEC<sub>50</sub> and pA<sub>2</sub> values) determined in this study.

### **3.3. ANALYSIS OF TP RECEPTOR mRNA EXPRESSION**

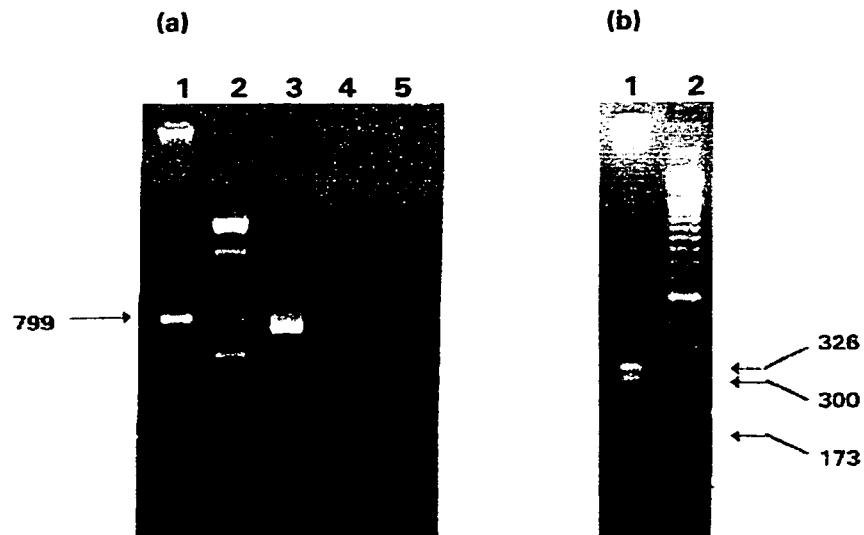
#### **3.3.1. Verification of PCR Amplification Product as TP Receptor mRNA**

The amplification product derived from 0.5  $\mu$ g of myometrial total RNA was identical to the predicted size of 799 bp based on the published human TP receptor cDNA sequence (Figure 8a, lane 1). There was no PCR product visible when either the RT reaction was carried out in the absence of reverse transcriptase enzyme (Figure 8a, lane 4), or when 1.5 ng of human genomic DNA was used as the PCR template (Figure 8a, lane 5). Amplification of 10  $\mu$ g of cloned TP receptor cDNA yielded a single PCR band of 739 bp, which was predicted based on the 60 bp internal deletion in this cDNA (Figure 8a, lane 3).

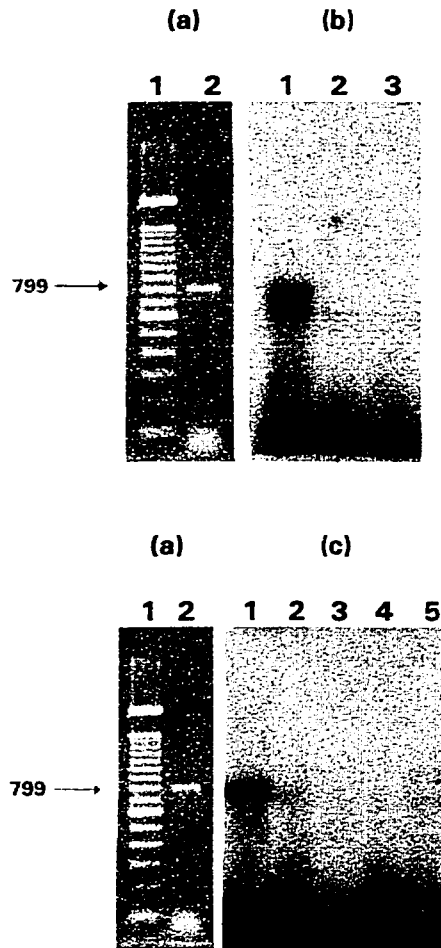
Further verification of the identity of the TP receptor PCR product was obtained through specific endonuclease digestion with the enzyme *Sma*I, where the anticipated splice fragments of 326 bp, 300 bp and 173 bp were obtained (Figure 8b, lane 1).

TP receptor amplification products subjected to Southern blotting and hybridization with a DIG-labeled TP receptor RNA probe produced two hybridization signals differing by approximately 60 bp (Figure 9b, lane 1). The higher molecular weight signal was identified as TP receptor PCR amplification product (799 bp). Hybridization bands were not detected in lanes containing negative control amplification products (Figure 9b, lanes 2 and 3), suggesting that the appearance of the lower molecular weight hybridization signal was not due to contamination of reagents or RNA with cloned TP receptor cDNA. Hybridization bands were also not seen when DIG-labeled TP receptor RNA was used to probe Southern blots containing PCR amplification products representing prostanoid FP, EP<sub>2</sub>, EP<sub>3</sub> and EP<sub>4</sub> receptor mRNA (Figure 9c, lanes 2 - 5) (Senchyna and Crankshaw, 1995).





**Figure 8. Verification of TP Receptor mRNA Amplification Product Identity.** Ethidium bromide staining pattern of TP receptor mRNA amplificates resolved by electrophoresis, where PCR was carried out for 35 cycles. (a) lane 1: RT-PCR amplification of 0.5  $\mu$ g of total RNA derived from nonpregnant human myometrium excised from the uterine lateral wall; lane 2: 100 bp DNA molecular weight ladder; lane 3: PCR amplification of 10 pg of cloned TP receptor cDNA; lane 4: negative control, where RT of 0.5  $\mu$ g of myometrial total RNA was carried out in the absence of reverse transcriptase enzyme; lane 5: PCR amplification of 1.5 ng of human genomic DNA. (b) lane 1: endonuclease digestion of TP receptor mRNA amplification product with 20 U of *Sma*I; lane 2: 100 bp DNA molecular weight ladder. Arrows mark bp approximation of resolved bands as assessed by comparison to the molecular weight ladder.



**Figure 9: Southern Blot of TP Receptor mRNA Amplification Products.** Following electrophoresis of TP receptor mRNA amplification products, a portion of the gel was excised and stained with ethidium bromide. The remaining gel underwent Southern blotting by capillary transfer onto a nylon support. The blot was hybridized overnight with a DIG-labeled TP receptor RNA probe. **(a)** Ethidium bromide stained portion of agarose gel. lane 1: 100 bp DNA molecular weight ladder; lane 2: TP receptor mRNA amplification product. **(b)** Southern blot. lane 1: TP receptor mRNA amplification product; lane 2: negative control, where RT reaction with 0.5  $\mu$ g of myometrial total RNA was carried out in the absence of reverse transcriptase enzyme; lane 3: negative control, where the RT reaction was carried out in the absence of RNA. PCR was performed for 35 cycles. **(c)** Southern blot. lane 1: TP receptor mRNA amplification product; lane 2: prostanoid FP receptor mRNA amplification product; lane 3: prostanoid EP<sub>2</sub> receptor mRNA amplification product; lane 4: prostanoid EP<sub>3</sub> receptor mRNA amplification product; lane 5: prostanoid EP<sub>4</sub> receptor mRNA amplification product. Arrow marks bp approximation of resolved bands as assessed by comparison to the molecular weight ladder.

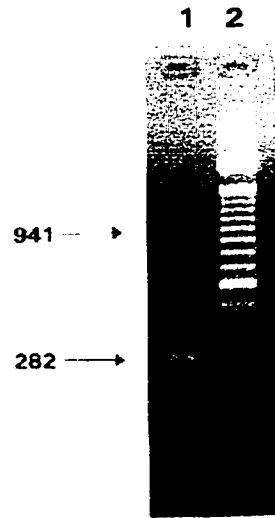
Over the course of these studies, this lower molecular weight signal was seen in approximately 50% of TP receptor amplification products that were subjected to Southern blotting (Figure 9b, lane 1 and Figure 9c, lane 1), and as detailed below, was also seen on approximately 30% of ethidium bromide stained agrose gels.

### **3.3.2. Identification of Alternatively Spliced TP Receptor mRNA**

Two products of the anticipated size were obtained when 0.5  $\mu$ g of myometrial RNA was reverse transcribed and subjected to 35 cycles of PCR using ALT-TP-A and ALT-TP-S primers, indicating the amplification of both TP receptor (941 bp) and alternatively spliced TP receptor mRNA (282 bp) (Figure 10, lane 1).

### **3.3.3. Standardization of Integrated Density Measurements from Scanned Polaroid Film Images**

To examine the accuracy and sensitivity of using scanned images for densitometric analysis of 1-D gels, various volumes of G3PDH mRNA PCR amplification product were resolved by electrophoresis and photographed on Polaroid 667 Negative film. As PCR product was routinely added to wells located at the top and middle of a gel, the limits of product detection were studied in both these locations. Furthermore, to ensure that experimental PCR amplification products would be maximally visible, yet fall within the accurate limits of detection, TP receptor and G3PDH mRNA PCR amplification products were

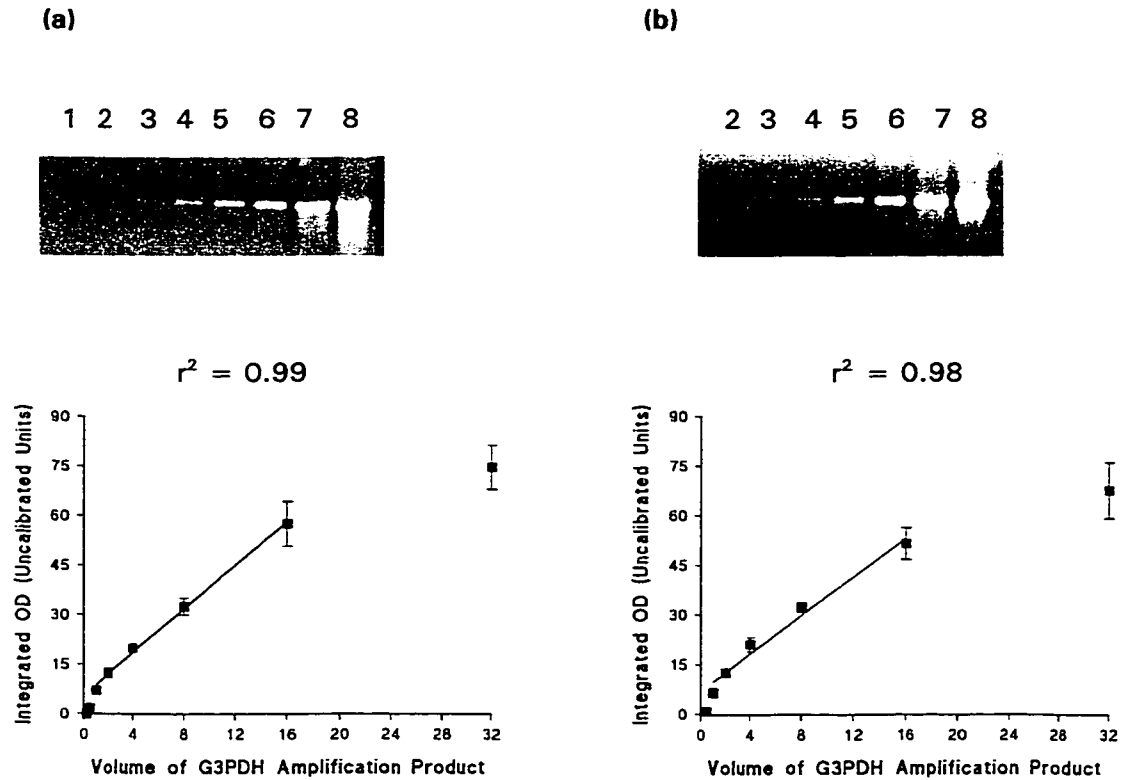


**Figure 10. Identification of an Alternately Spliced TP Receptor mRNA Transcript.** lane 1: ethidium bromide staining pattern of amplification products representing TP receptor mRNA (941 bp) and alternatively spliced TP receptor mRNA (282 bp), obtained from reverse transcription of 0.5  $\mu$ g of myometrial total RNA derived from the uterine lateral wall and 35 cycles of PCR using ALT-TP-S and ALT-TP-A primers; lane 2: 100 bp DNA molecular weight ladder.

run in parallel. As the degree of film exposure, scanner settings and volume of PCR product loaded all effect the intensity of the resolved DNA, these variables were optimized by evaluating the sensitivity of densitometric measurement after changing one parameter at a time. Optimized variables are detailed in Table 6, and were employed for all semi-quantitative analyses. As shown in Figure 11, the density of the bands measured from this standardized measurement procedure correlated well with the volume of DNA loaded on the gel in the range between 1  $\mu\text{L}$  to 16  $\mu\text{L}$  for both the top and middle of the gel. Density measurements for representative TP receptor and G3PDH mRNA amplification products fell on this linear range (data not shown).

**Table 6: Settings for Densitometric Measurements**

VARIABLE	SETTING
Camera: lens aperature control : shutter speed control	2 f/6.2
Scanner: brightness : contrast : resolution	53 50 300dpi
Volume of TP Receptor Amplification Product Loaded on Gel	20 $\mu\text{L}$
Volume of G3PDH Amplification Product Loaded on Gel	3 $\mu\text{L}$



**Figure 11. Relationship Between Volume of Applied Amplification Product and Measured Band Integrated Density.** Ethidium bromide staining pattern of G3PDH mRNA amplicates (29 cycles) resolved by electrophoresis, where product was added to the top (a) or middle (b) of the gel. lane 1 = 0.25 $\mu$ L, lane 2 = 0.5  $\mu$ L; lane 3 = 1  $\mu$ L; lane 4 = 2  $\mu$ L; lane 5 = 4  $\mu$ L; lane 6 = 8  $\mu$ L; lane 7 = 16  $\mu$ L; lane 8 = 32  $\mu$ L. Each band in lanes 1 - 8 was quantified from the scanned Polaroid image and a regression curve plotted from the data obtained through densitometric analysis. Values are expressed in uncalibrated units of optical density and represent the mean of 8 experiments.

During the course of experimentation however, approximately 10% of the TP receptor mRNA amplification products that were generated fell beyond both the high and low ends of the accurate range of detection. Only those integrated density values that fell within the linear range of detection were included in kinetic studies as an accurate indication of product accumulation was required in order to estimate the point at which the PCR entered the plateau phase. Outside of kinetic studies, all integrated density values were included in the assessment of TP receptor mRNA as it was accepted that these values would represent very high or very low levels of TP receptor mRNA expression.

#### **3.3.4. TP Receptor mRNA Amplification Kinetics**

As an initial broad assessment of the point at which the plateau phase was reached, eight identical PCR reactions, using the same cDNA preparation, were subjected to a variable number of amplification cycles (20, 25, 30, 35, 40, 45, 50, 55). Resulting amplification products were resolved by electrophoresis and the extent of product accumulation was assessed by densitometric analysis of the scanned Polaroid image. As shown in Figure 12a and 12c, no product was visible between 20 and 30 cycles (Figure 12a, lanes 1 - 3). At 35 cycles, a faint product band was detected (Figure 12a, lane 4), followed by the onset of plateau at 40 cycles (Figure 12a, lane 5). Due to the questionable sensitivity<sup>1</sup> of ethidium bromide staining in allowing for the visualization of low abundance amplification products, it was thought possible that the failure to detect product prior to the

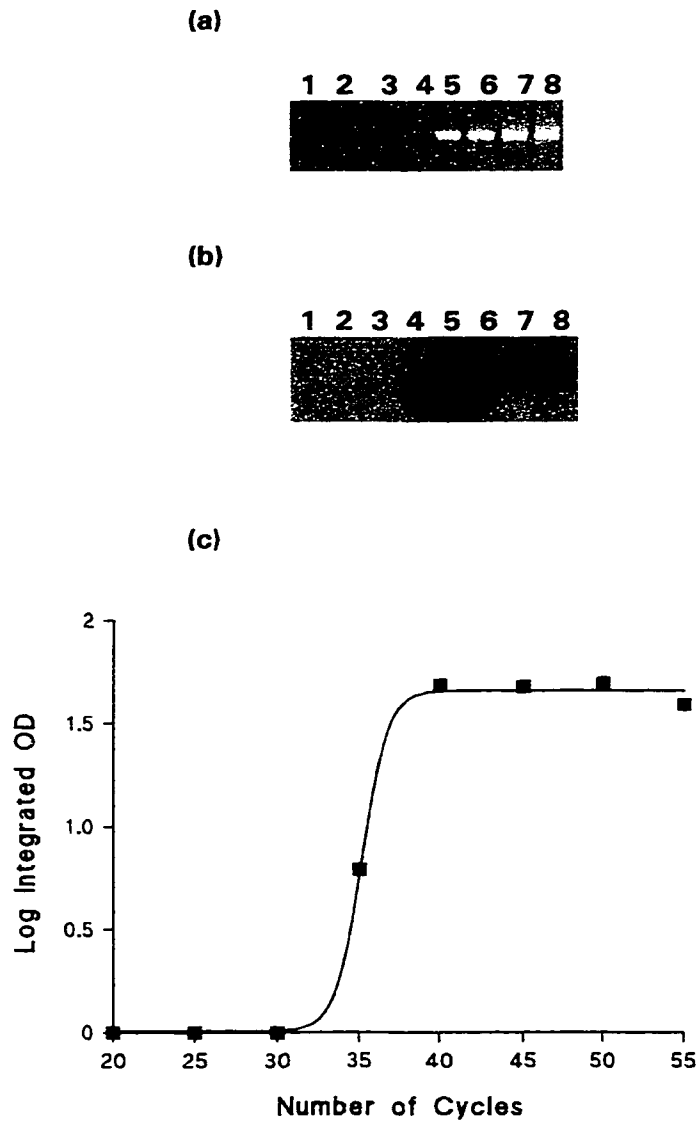
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<sup>1</sup> Discussed in Section 4.2

35 cycle time point was due to limited detection ability. Southern blotting of amplification products however, confirmed the absence of product prior to the 35 cycle time point and the onset of plateau at the 40 cycle time point (Figure 12b). In addition, Southern blotting revealed a second PCR product, differing by approximately 60 bp from the TP receptor mRNA band which also appeared at 35 cycles (Figure 12b, lane 4). The near "all or none" nature of these results was repeated with different RNA preparations, derived from myometrial tissue excised from different uterine locations.

To establish a more accurate range of exponential amplification, truncated time course studies were designed. As the semi-quantitative survey of TP receptor mRNA expression planned in these studies involved a survey of many different RNA preparations derived from both proliferative and secretory phase myometrium excised from four different locations, the kinetics of TP receptor mRNA amplification was assessed in each of these cases. To account for potential tube-to-tube variability, a single 2X PCR was set up and 15  $\mu$ L aliquots were removed at three cycle intervals, starting on or after the beginning of the 30<sup>th</sup> cycle and thereafter for four additional samplings at three cycle intervals (Table 7). It should be noted that in preliminary experiments, all timecourses were initially started at the beginning of the 30<sup>th</sup> cycle. However, it was often found that amplification product was not visible at any of the cycle time points when sampling began at this time point, where as visible bands were attainable by delaying the start of the experiment by one or more cycles. Following PCR, amplification products were resolved by electrophoresis and the integrated density of each band was determined. Under these conditions, amplification product was





**Figure 12. Accumulation of TP Receptor mRNA Amplification Product Following Different Numbers of Amplification Cycles.** A single 4X RT master mixture was prepared containing 2.0  $\mu\text{g}$  of myometrial total RNA. Following cDNA synthesis, the mixture was divided into 8 - 5  $\mu\text{L}$  aliquots and subjected to PCR amplification using TP-A and TP-S primers. Individual tubes were removed from the thermocycler at 5 cycle intervals. (a) Ethidium bromide staining pattern of TP receptor mRNA amplification products resolved by electrophoresis. (b) Southern blot of TP receptor mRNA amplification products following hybridization with a DIG-labeled TP receptor RNA probe. (c) Each band in lanes 1 - 8 of the ethidium bromide stained gel was quantified from the scanned Polaroid image and the data obtained through densitometric analysis were plotted. Values are expressed as the logarithm of optical density (uncalibrated units) and are representative of six experiments.

visible at at least three of the time points sampled and there was a clear increase in product yield (Figure 13). Regression analysis of the densitometric data was used to define the region of linearity representative of exponential amplification for each sample tested. Table 7 summarizes the findings from these kinetic studies. Although there were differences in terms of the point at which a visible band appeared and in the absolute region of linearity, 35 cycles was a common point where all samples were within a range of exponential amplification. Thus all subsequent PCR reactions in which TP receptor mRNA was being amplified were performed for 35 cycles. It has previously been shown that the slope of the straight line portion of the graph of log (product accumulation) [as reported as integrated OD in this study] versus cycle number can be used to calculate the efficiency of product amplification from the formula:

$$[1 + e = 10^x]$$

where  $x$  = slope of the line and  $e$  = efficiency of amplification ranging from 0 (no amplification) to 1 (100% efficiency) (Saiki *et al.*, 1985; Chelly *et al.*, 1988; Bishop *et al.*, 1997). Using this equation, the efficiency of TP receptor mRNA amplification was calculated to be  $25\% \pm 7\%$  ( $n = 9$ ). No significant difference ( $P < 0.05$ ), was found in the amplification efficiency between RNA derived from different excision sites or from donors in the proliferative phase versus donors in the secretory phase of the menstrual cycle.

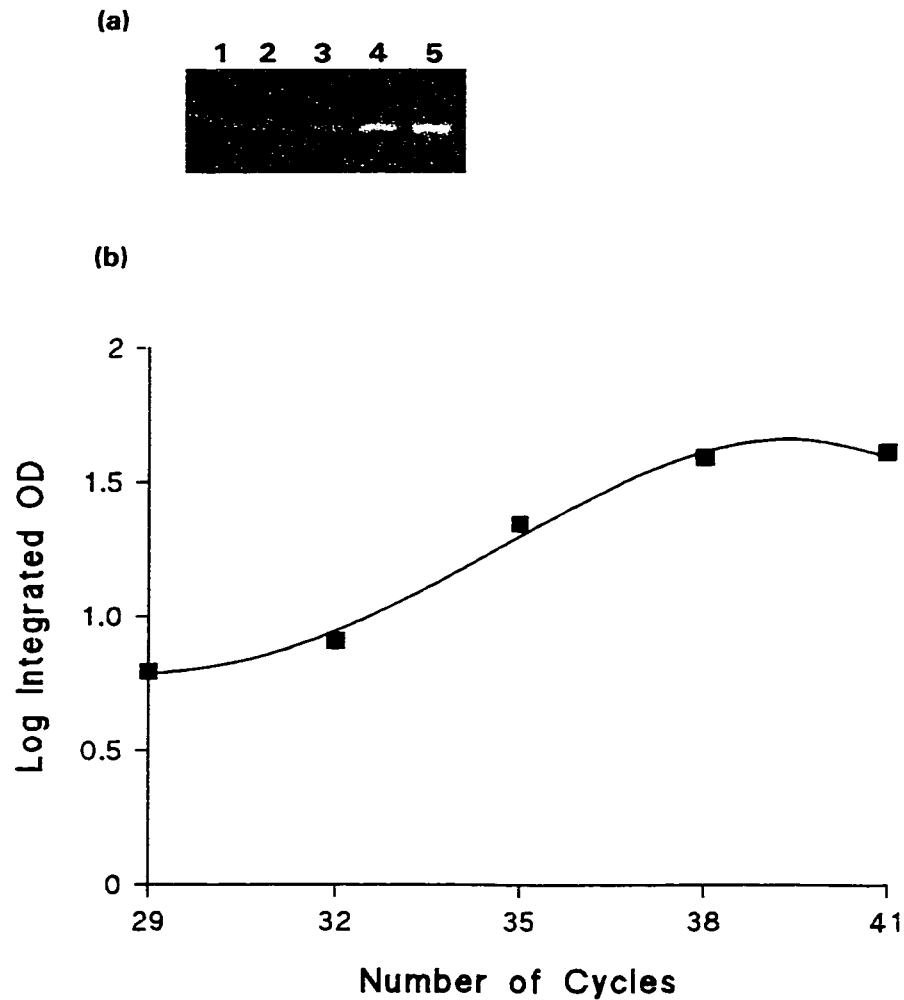
Table 7: Summary of TP Receptor mRNA Amplification Kinetics

EXCISION SITE OF MYOMETRIAL TISSUE AND MENSTRUAL STATUS OF THE DONOR	CYCLE RANGE FOR WHICH KINETICS WERE ASSESSED	CYCLE RANGE FOR WHICH LINEAR AMPLIFICATION WAS RECORDED	r <sup>2</sup> VALUE*	SLOPE OF REGRESSION LINE
<b>PROLIFERATIVE PHASE</b>				
Top	29 - 41	29 - 35	0.99	0.15
Lateral Wall	31 - 43	34 - 40	0.96	0.08
Lower Uterine Segment	31 - 43	34 - 40	0.99	0.10
Cervix	32 - 44	35 - 38	1.00	0.07
<b>SECRETORY PHASE</b>				
Top	32 - 44	35 - 44	0.98	0.10
Lateral Wall	29 - 41	32 - 38	0.99	0.10
Lower Uterine Segment	29 - 41	32 - 35	0.96	0.08
Cervix	32 - 44	35 - 44	0.99	0.06

\* r<sup>2</sup> value determined from regression analysis

### 3.3.5. G3PDH mRNA Amplification Kinetics

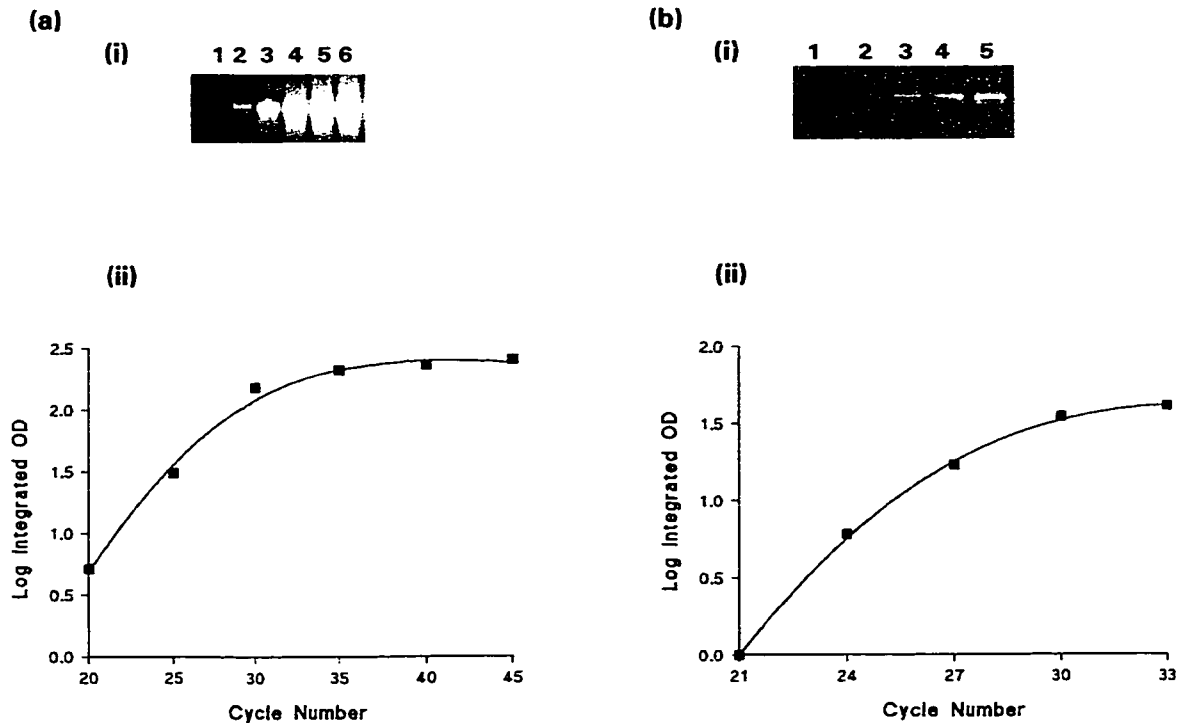
The assessment of G3PDH mRNA amplification kinetics was approached in a similar manner to that described above for TP receptor mRNA. Preliminary studies employed six identical PCR reactions which were subjected to a variable number of amplification cycles (20, 25, 30, 35, 40, 45). Resulting amplification products were resolved by electrophoresis and the extent of product accumulation assessed by densitometric analysis of the scanned Polaroid image. Under these conditions, amplification products were visible at each time point sampled and a clear increase in product yield occurred between 20 and 35 cycles (Figure 14a, lanes 1 - 3), followed by the onset of plateau (Figure 14a, lane 4). To accurately establish the upper boundary of exponential product accumulation, truncated timecourse studies were performed using a single 2X PCR reaction in which 15  $\mu$ L aliquots



**Figure 13. Kinetics of Amplification of TP Receptor mRNA PCR Product.** A single 2X PCR reaction was prepared and a 15  $\mu$ L aliquot of the PCR product was removed from the tube every 3 cycles, starting after the 29<sup>th</sup> cycle and ending after the 41<sup>st</sup> cycle. (a) Ethidium bromide staining pattern of TP receptor mRNA amplification products resolved by electrophoresis. (b) Semi-logarithmic representation of the relative extent of TP receptor mRNA PCR product accumulation measured by densitometric analysis of the scanned Polaroid image. Regression analysis defined the region of 32 - 38 cycles to be linear and thus representative of exponential amplification ( $r^2 = 0.98$ ). Data representative of 9 experiments.

were removed at three cycle intervals, starting after the 21<sup>st</sup> cycle and ending after the 33<sup>rd</sup> cycle. The determination of the upper boundary of exponential amplification was necessary as all semi-quantitative studies of TP receptor mRNA expression required that TP receptor and G3PDH mRNA be co-amplified in the same PCR reaction. Based on the information described above regarding the *sensitivity* of TP receptor mRNA amplification, it was felt that performing as many PCR cycles as possible before pausing the thermocycler to remove tubes would be advantageous.

G3PDH mRNA amplification products derived from truncated kinetic studies were resolved by electrophoresis and the integrated density of each band was determined. Again, a clear consistent increase in product yield was observed in such studies over progressive sampling points. In contrast to TP receptor mRNA kinetic studies, the G3PDH amplification profile for each of eight RNA preparations derived from myometrial tissue excised from different anatomical locations from donors in either phase of the menstrual cycle was invariant. Regression analysis of the densitometric data defined the region of 21 - 27 cycles as linear in two cases ( $r^2 = 0.98$ ), and the region of 24 - 30 cycles as linear in six cases ( $r^2 = 0.97 - 0.99$ ). The efficiency of G3PDH mRNA amplification was estimated to be  $31\% \pm 4\%$  ( $n = 8$ ).

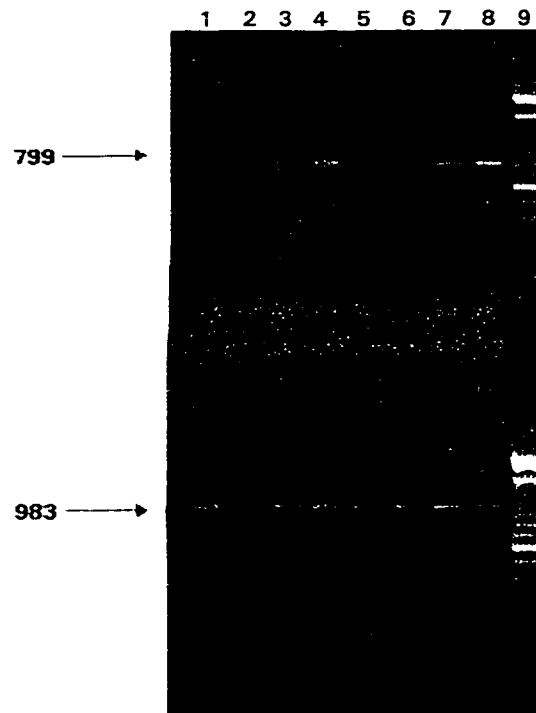


**Figure 14. Kinetics of Amplification of G3PDH mRNA PCR Product.** (a) A single 3X RT master mixture was prepared containing 1.5  $\mu\text{g}$  of total myometrial RNA. Following cDNA synthesis, the mixture was divided into 6 - 5  $\mu\text{L}$  aliquots and subjected to PCR amplification using G3P-A and G3P-S primers. Individual tubes were removed from the thermocycler at 5 cycle intervals (i) Ethidium bromide staining pattern of 15  $\mu\text{L}$  of G3PDH mRNA amplification products resolved by electrophoresis; (ii) Semi-logarithmic representation of the relative extent of G3PDH mRNA PCR product accumulation measured by densitometric analysis of the scanned Polaroid image. (b) A single 2X PCR reaction was prepared and a 15  $\mu\text{L}$  aliquot of the PCR product was removed from the tube every 3 cycles, starting after the 21<sup>st</sup> cycle and ending after the 33<sup>rd</sup> cycle. (i) Ethidium bromide staining pattern of 3  $\mu\text{L}$  of G3PDH mRNA amplification products resolved by electrophoresis. (ii) Semi-logarithmic representation of the relative extent of G3PDH mRNA PCR product accumulation measured by densitometric analysis of the scanned Polaroid image. Data in (b) are representative of eight experiments.

### 3.3.6. Semi-Quantification of TP Receptor mRNA Expression in Human Nonpregnant Myometrium

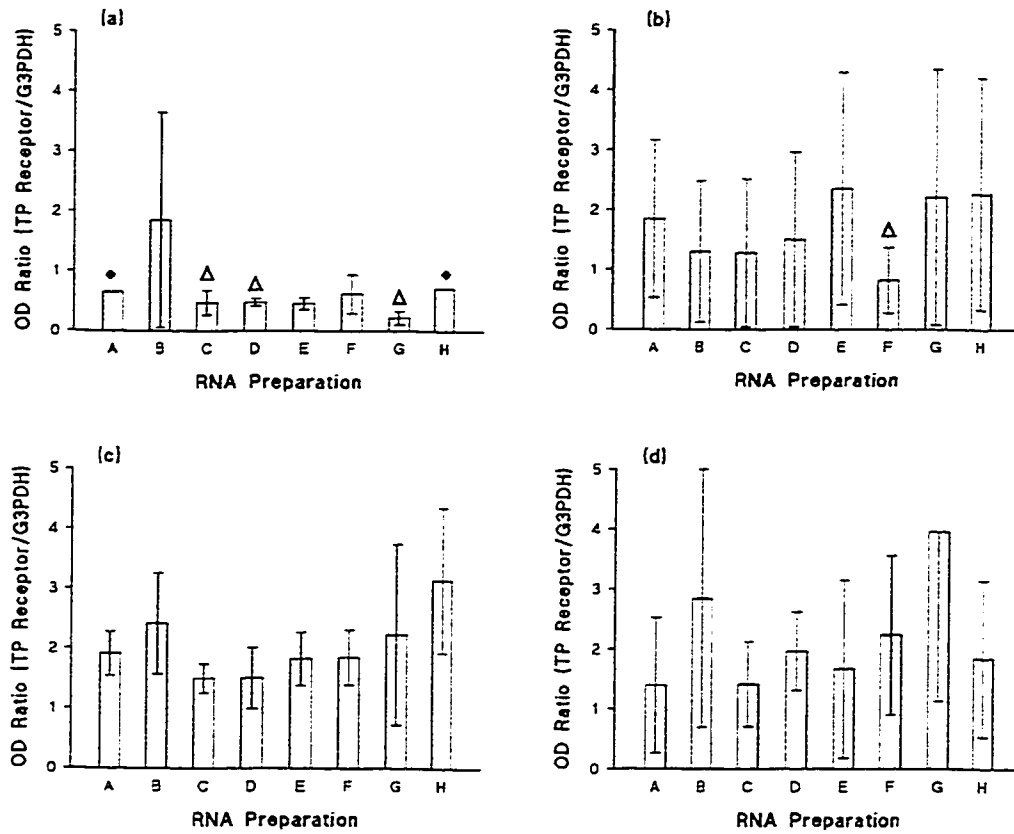
#### 3.3.6.1. Comparisons Made Within a Single Excision Site

Figure 15 represents a typical result, where TP receptor mRNA amplification products were always resolved on the upper portion of a gel and G3PDH mRNA amplification products were always loaded on the middle portion of the gel. Data derived from each of the four sites investigated are displayed in Figure 16. Although TP receptor mRNA expression appeared to vary among samples within a given site, a significant difference was not found in any of the four sites studied ( $P < 0.05$ ). Nor was a significant difference found between proliferative and secretory phase tissues in any of the four sites ( $P < 0.05$ ). The mean  $\pm$  S.D. of TP receptor / G3PDH ratios were:  $0.74 \pm 0.94$ ;  $1.75 \pm 1.65$ ;  $2.05 \pm 0.97$  and  $2.16 \pm 1.80$  for the top, lateral wall, lower uterine segment and cervix respectively. Due to the uncalibrated OD measurement scale, I could not determine from these data whether TP receptor mRNA expression differed between excision site groups. However, such a comparison was directly investigated as detailed in section 3.3.6.2. Complicating the data collection was the appearance of a second TP receptor PCR product, which was approximately 60 bp smaller than the band of interest. The second product was seen *only* in experiments using RNA prepared from tissues excised from the lower uterine segment and cervix. In the lower uterine segment group, the second product was seen in each sample in each of the three RT-PCR experiments performed, although the intensity of the band varied from sample to sample. In the cervix group, bands of varying intensity



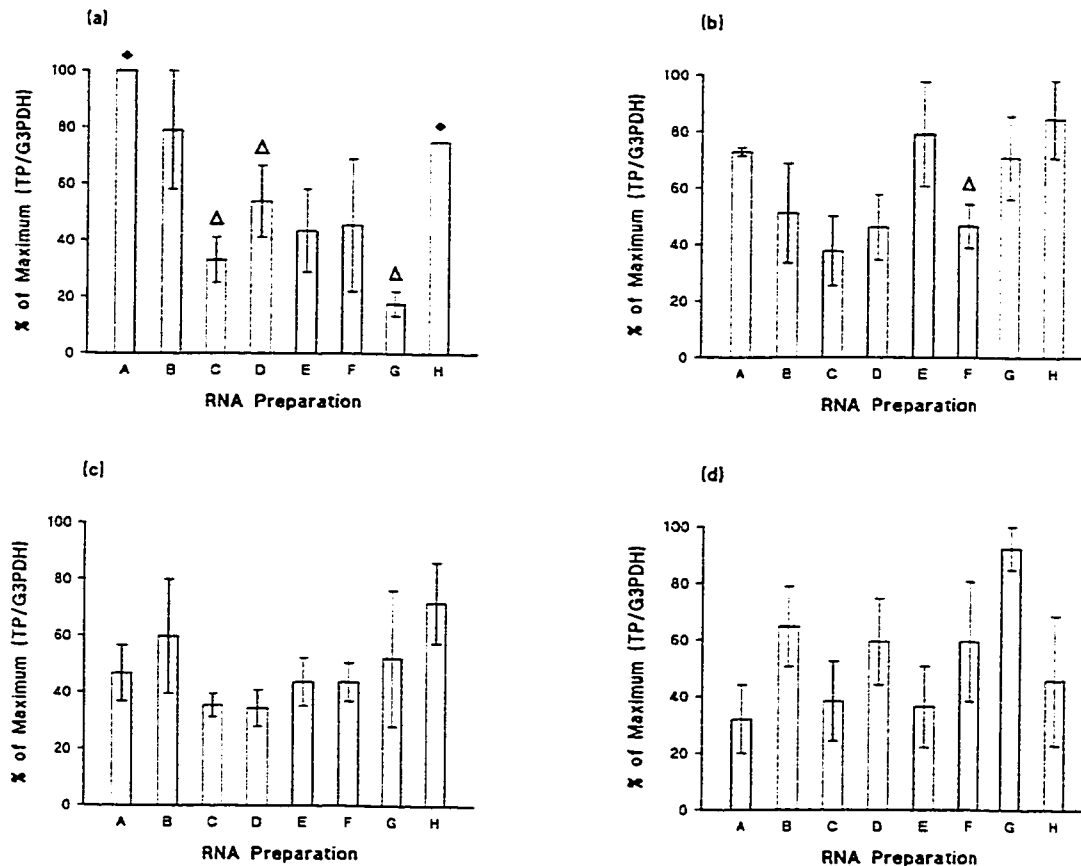
**Figure 15. Semi-Quantitative Measurement of TP Receptor mRNA.** Representative ethidium bromide staining of TP receptor mRNA and G3PDH mRNA amplification products obtained from 35 and 29 cycles of PCR respectively and resolved by electrophoresis, as described in Sections 2.5. and 3.3.6.1. 8 different RNA preparations derived from myometrial tissue excised from the uterine lateral wall were run in parallel. 4 donors were in the proliferative phase (lanes 3 - 6) and 4 donors were in the secretory phase (lanes 1 & 2, lanes 7 & 8) of the menstrual cycle. Lane 9: 100 bp DNA molecular weight ladder. Arrows mark the positions of TP receptor mRNA amplification products (799 bp) and G3PDH mRNA amplification products (983 bp).





**Figure 16: Graphical Representation of TP Receptor mRNA Expression Reported as the Ratio of TP Receptor mRNA Amplification Product Integrated Density / G3PDH mRNA Amplification Product Integrated Density.** Data were collected as described in Sections 2.5. and 3.3.6.1. Capital letters indicate individual RNA preparations, (A - D): myometrial tissue derived from donors in the proliferative phase of the menstrual cycle; (E - H): myometrial tissue derived from donors in the secretory phase of the menstrual cycle. Results are presented as means  $\pm$  S.D. (n=3) except where indicated: ( $\Delta$ ) = mean of two RT-PCR experiments, where third experiment yielded a band that was too faint to quantify; ( $\diamond$ ) data represents one RT-PCR experiment, where the other two RT-PCR experiments either failed or yielded product that was too faint to analyze. (a - d) Tissue excision site, (a) Top; (b) Lateral Wall; (c) Lower Uterine Segment; (d) Cervix.

representing this second unidentified amplification product were seen in five of the eight samples tested, although not consistently. It was not possible to separate the lower molecular weight band from the band of interest during the calculation of integrated density, and thus reported values may have been artificially high. To determine if "removal" of the contribution of the lower molecular weight band would alter the conclusions made with respect to TP receptor mRNA expression, single point measurements were made, in which the density of a 3x3 pixel window placed directly over the middle of an individual band were calculated. Although this method of data collection is not considered as accurate a reflection of overall product yield (Vivino, 1998), the data collected followed a very similar trend to the integrated density values for each band. Statistical analysis of these single point measurements yielded the same conclusions as described above, where no significant difference was found in any comparison made ( $P < 0.05$ ). A review of the raw data indicated that one possible reason for the extraordinarily large standard deviations associated with the mean ratio data was fluctuation in product yield from one experiment to the next. This variation was especially obvious for TP receptor mRNA amplification product, where integrated density measurements differed by 1- to 15- fold from one experiment to the next. In an attempt to minimize the influence of variable product yield, data from each experiment were converted from raw ratio to *% of maximum ratio* numbers. Specifically, the raw data was normalized to the largest ratio in each experiment, which was designated as 100%. The mean data for the *% of maximum ratio* values were calculated and the resulting numbers were graphed (Figure 17) and analyzed for significance. Again, despite the appearance of varying amounts of TP receptor mRNA among different samples within a single site, a significant difference was not found ( $P < 0.05$ ). Nor was there a significant



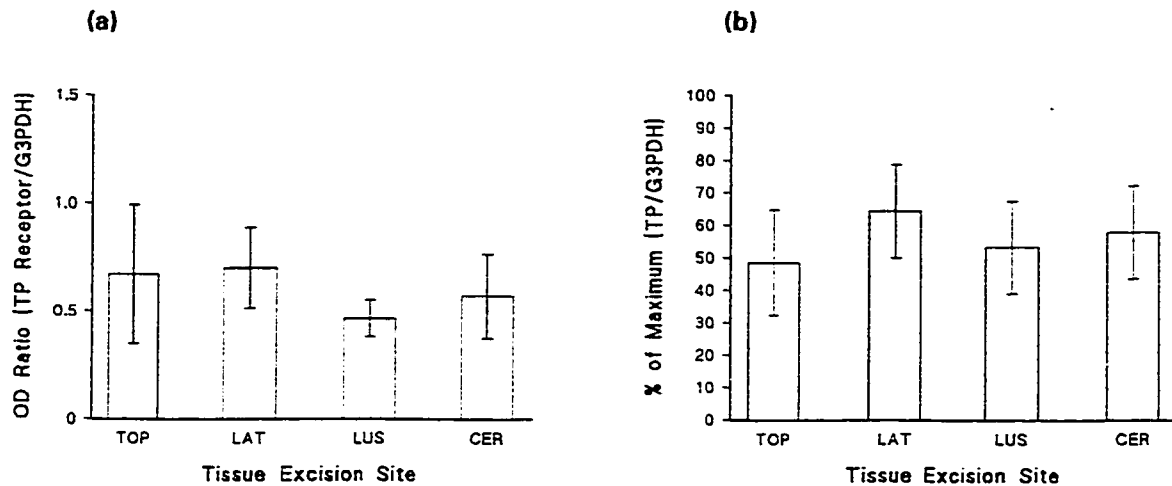
**Figure 17: Graphical Representation of TP Receptor mRNA Expression Reported as % of Maximum (TP Receptor/G3PDH) Ratio.** Data were collected as described in Sections 2.5. and 3.3.6.1. Capital letters indicate individual RNA preparations, (A - D): myometrial tissue derived from donors in the proliferative phase of the menstrual cycle; (E - H): myometrial tissue derived from donors in the secretory phase of the menstrual cycle. Results are presented as means  $\pm$  S.D. (n=3) except where indicated: ( $\Delta$ ) = mean of two RT-PCR experiments, where third experiment yielded a band that was too faint to quantify; ( $\diamond$ ) data represents one RT-PCR experiment, where the other two RT-PCR experiments either failed or yielded product that was too faint to analyze. (a - d) Tissue excision site, (a) Top; (b) Lateral Wall; (c) Lower Uterine Segment; (d) Cervix.

difference in TP receptor mRNA expression between proliferative and secretory phase tissues for any of the four excision sites tested ( $P < 0.05$ ). As is described above, the single point measurements of band density made for the lower uterine segment and cervix groups also failed to produce significant differences among any of the comparisons made.

Although the means of transformed (% of maximum ratio), data were associated with smaller standard deviations compared to mean values calculated from raw data, a large amount of variability remained. In other words, from one experiment to the next, the sample that represented the highest ratio of TP receptor/G3PDH amplification product fluctuated. The source of this additional variability was further investigated and summarized in section 3.3.7.

### **3.3.6.2. Comparisons Made Between Different Excision Sites**

Results from the direct comparison of TP receptor mRNA expression in different uterine locations are diagrammed in Figure 18. Statistical analysis of both raw ratio and transformed % of maximum ratio data failed to find a significant difference in TP receptor mRNA expression between individual samples or between the four sites investigated ( $P < 0.05$ ). In contrast to the single site studies detailed in section 3.3.6.1., the lower molecular weight PCR product associated with TP receptor mRNA amplification was not seen in the lower uterine segment or cervix samples studied in these experiments.

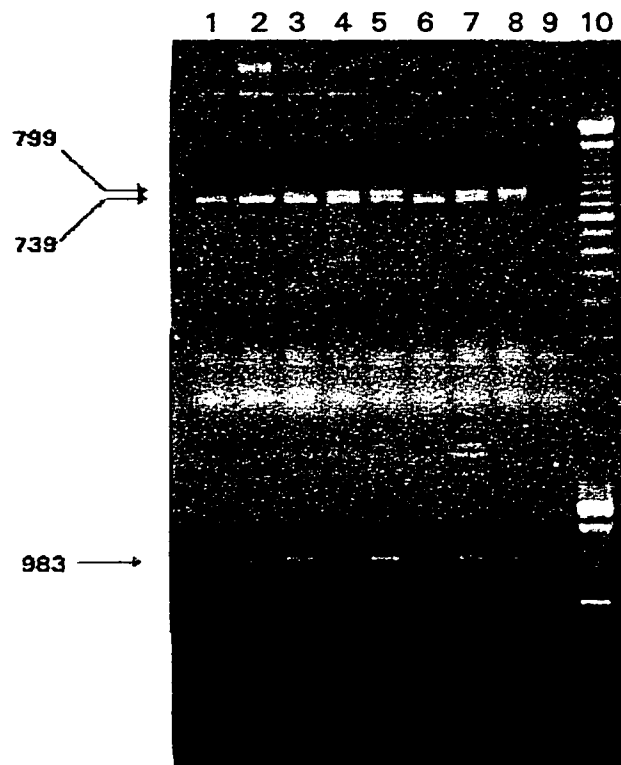


**Figure 18: Comparison of TP Receptor mRNA Expression in Four Different Uterine Locations.** Data were collected as described in Sections 2.5. and 3.3.6.2. and presented graphically in both raw ratio (a) and transformed % of maximum ratio (b) forms. x axis: Tissue excision site: TOP = top; LAT = lateral wall; LUS = lower uterine segment; CER = cervix. Results are presented as means  $\pm$  S.D. (n = 3).

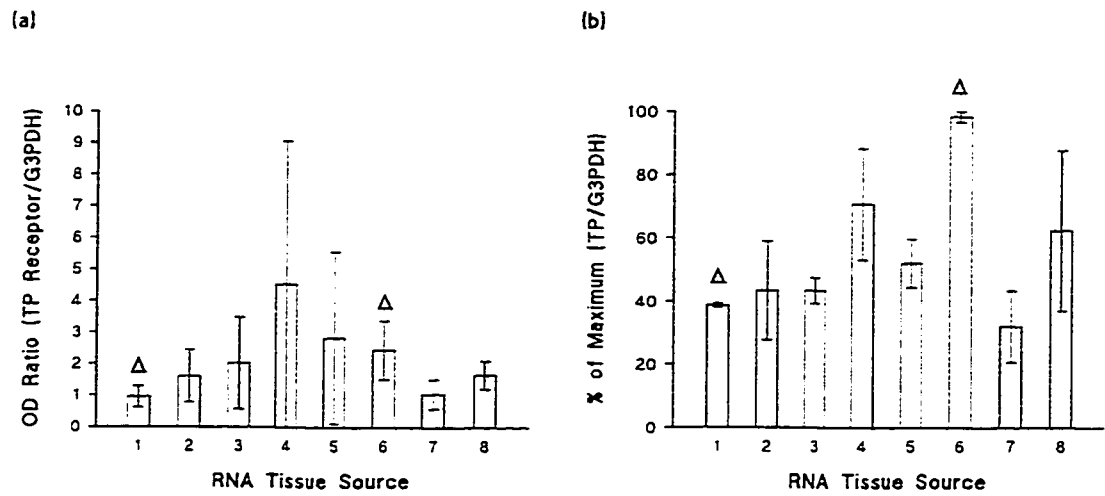
### 3.3.6.3. Comparisons Made Between RNA Preparations Derived from Different Human Tissues

Figure 19 is representative of results obtained from the survey of TP receptor mRNA expression in different human tissues. PCR amplification product indicative of TP receptor mRNA was seen in all tissues studied. Results however, were not completely consistent, as amplification of human brain and skeletal muscle total RNA failed to yield a TP receptor amplification product in 1 of 3 RT-PCR experiments. The failure to see product in each of these cases was not attributed to failed RT as amplification of G3PDH mRNA was successful.

Data were expressed as the mean of triplicate experiments in which the ratio of TP receptor/G3PDH mRNA amplification product was determined. Statistical analysis failed to find a significant difference in TP receptor mRNA expression among any of the eight tissues studied regardless of whether raw ratio or transformed % of maximum ratio data was compared ( $P < 0.05$ ) (Figure 20). Complicating the density analysis however, was the presence of a second amplification product approximately 60 bp lower than the TP receptor mRNA product (Figure 19). Single point density analysis was performed as described in Section 3.3.6.1. and statistical analysis of the resulting data found no significant difference in TP receptor mRNA expression in any of the eight tissues examined.



**Figure 19: Survey of TP Receptor mRNA Expression in Different Human Tissues.** Representative ethidium bromide staining of TP receptor mRNA and G3PDH mRNA amplification products obtained from 35 and 29 cycles of PCR respectively and resolved by electrophoresis, as described in Sections 2.5. and 3.3.6.3. Each lane represents a different total RNA preparation derived from human, lane 1: brain; lane 2: heart; lane 3: kidney; lane 4: lung; lane 5: placenta; lane 6: skeletal muscle; lane 7: small intestine; lane 8: nonpregnant myometrium. Lane 9: negative control where the RT reaction with 0.5  $\mu$ g of myometrial total RNA was performed in the absence of reverse transcriptase enzyme; lane 10: 100 bp molecular weight ladder. Arrows mark the positions of TP receptor mRNA amplification products (799 bp), unidentified amplification products (739 bp) and G3PDH mRNA amplification products (983 bp).

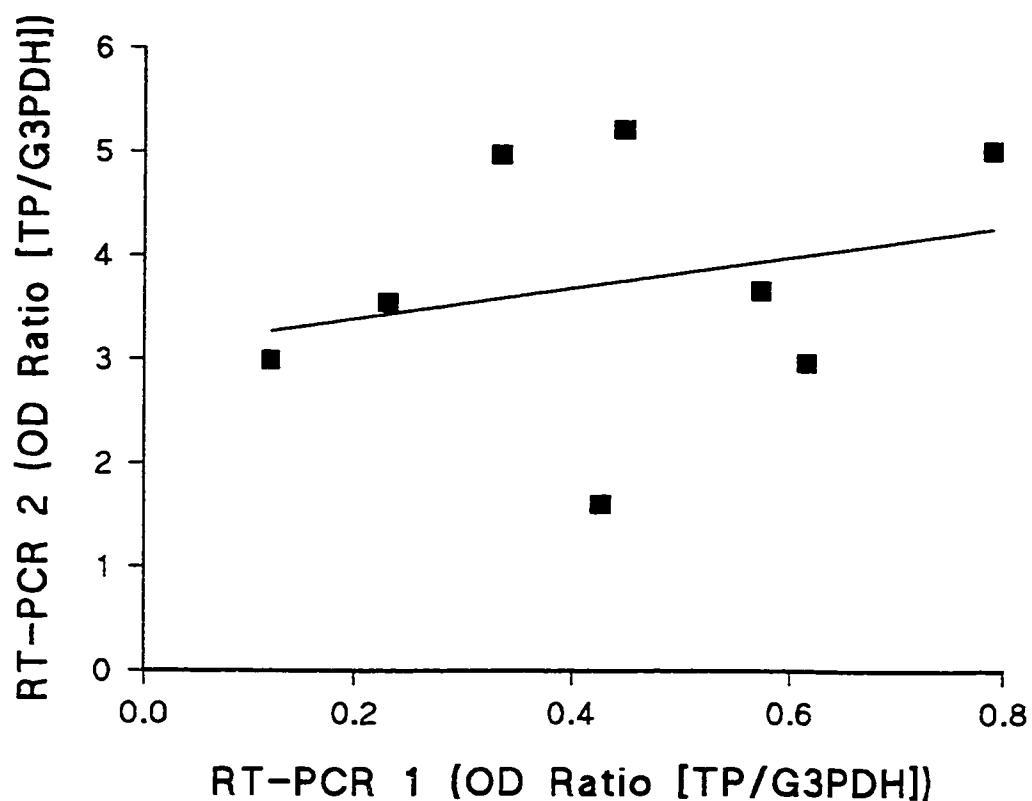


**Figure 20: Graphical Representation of TP Receptor mRNA Expression in Different Human Tissues.** Data were collected as described in Sections 2.5. and 3.3.6.3. and are presented in both raw ratio (a) and transformed % of maximum ratio (b) forms. x axis: Human Tissue: 1 brain; 2 heart; 3 kidney; 4 lung; 5 placenta; 6 skeletal muscle; 7 small intestine; 8 nonpregnant myometrium. Results are presented as means  $\pm$  S.D. ( $n = 3$ ), except where indicated: ( $\Delta$ ): results are presented as the mean of two RT-PCR experiments, where third experiment failed to yield product.



### 3.3.7. Estimation of Data Reproducibility

To partially account for the high standard deviations associated with the semi-quantitative data presented above, it was hypothesized that data were not consistently replicated. Shown graphically, Figure 21 represents a comparison of the TP receptor/G3PDH ratios obtained from two separate RT-PCR experiments performed on the same eight RNA samples. Two points are noteworthy from such a comparison. One, a global increase in product yield occurred in RT-PCR 2 compared to RT-PCR 1. Although the absolute increase in product yield cannot be assessed due to the uncalibrated nature of the data, the comparison was considered valid as preliminary experiments indicated that identical concentrations of PCR product loaded on separate gels and individually analyzed resulted in integrated density measurements that varied by 1% - 30% (Figure 22). A comparison of the two data sets used to construct the curve in Figure 21 indicated that integrated density values for TP receptor mRNA amplification product increased by 122% to 1,031% in RT-PCR 2 compared to RT-PCR 1. In contrast, G3PDH mRNA amplification product integrated densities decreased by 40% to 69% in RT-PCR 2 compared to RT-PCR 1. Clearly the differences in yield seen between the two RT-PCR assays represented by Figure 21 are not due to the method of data collection. In light of this observation, I reviewed all of the semi-quantitative data presented in the previous sections. 5% to 1,000% fluctuations in product yield were noted from one experiment to the next and larger fluctuations were noted for TP receptor mRNA compared to G3PDH mRNA. However, normally both TP receptor and G3PDH mRNA integrated densities would fluctuate in the same direction, ie: either both

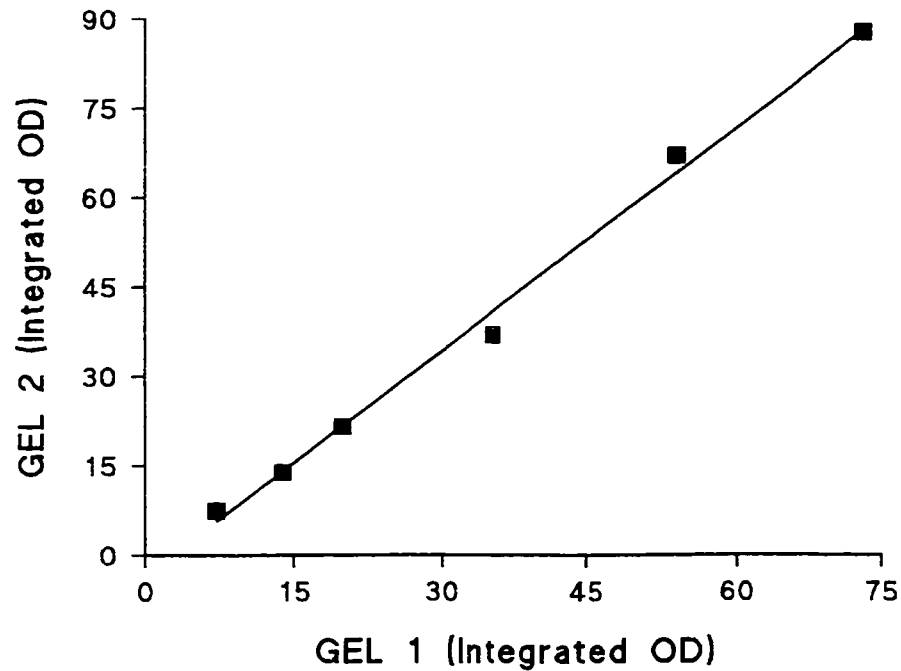


**Figure 21: Correlation of Results from RT-PCR 1 and RT-PCR 2.** Integrated density ratios of TP receptor/G3PDH mRNA amplification product were calculated for eight RNA samples in two identical RT-PCR experiments (RT-PCR 1 and RT-PCR 2) and the data compared as outlined in Section 3.3.7. There was no correlation between the two experiments as assessed by linear regression ( $r^2 = 0.06$ ) and correlation coefficient ( $r = 0.36$ )

(a)



(b)



**Figure 22: Correlation Between Integrated Density Measurements Derived from Two Separate Gels.** (a) Ethidium bromide staining pattern of varying volumes of G3PDH mRNA amplificates (29 cycles) resolved by electrophoresis on two separate 1.5% agarose-TBE gels. lane 1 = 1  $\mu$ L; lane 2 = 2  $\mu$ L; lane 3 = 4  $\mu$ L; lane 4 = 8  $\mu$ L; lane 5 = 16  $\mu$ L; lane 6 = 32  $\mu$ L. (b) Each band in lanes 1 - 6 was quantified from the scanned Polaroid image and a regression curve was plotted from the obtained data through densitometric analysis. Values are expressed in uncalibrated units of optical density. The data was highly correlated [ $r^2 = 0.99$ ,  $p < 0.001$ ,  $r = 0.98$ ]

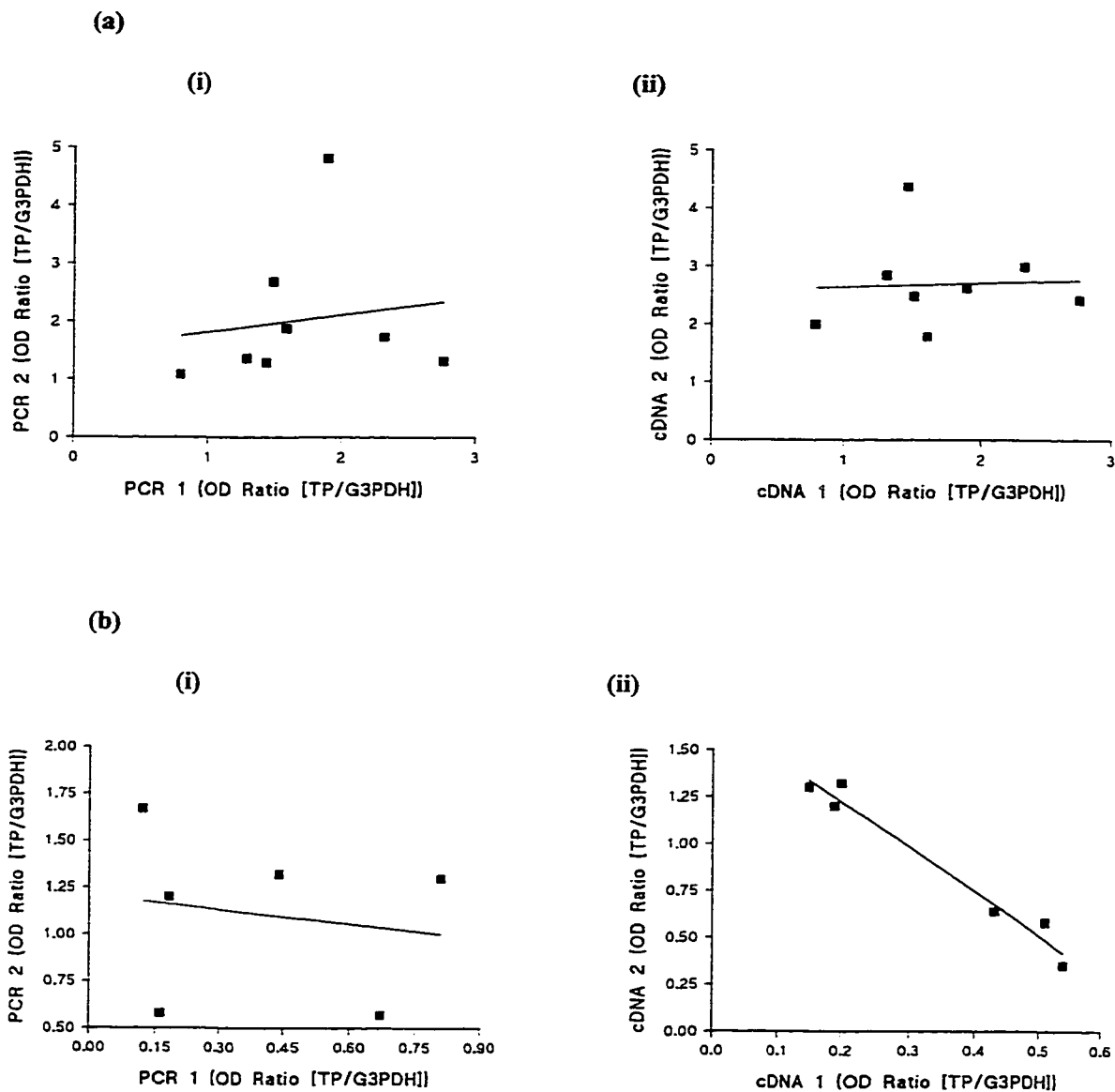
would increase or both would decrease. Second, there was no correlation between data obtained from RT-PCR 1 compared to RT-PCR 2 as determined by linear regression ( $r^2 = 0.06$ ) and correlation coefficient analysis ( $r = 0.36$ ).

In order to isolate potential causes for this inconsistency, experiments were set up to directly assess the variability of PCR and to indirectly assess the variability associated with cDNA synthesis.

To assess the reproducibility of the PCR, a single 2X RT reaction was performed and two separate PCR reactions (PCR 1 and PCR 2) were conducted. The reproducibility of reverse transcription cannot readily be measured directly, thus an indirect estimate was obtained by comparing the results from a single PCR on two separate cDNA syntheses of identical RNA samples (cDNA 1 and cDNA 2). Both these comparisons were performed twice, using in the first case eight RNA preparations derived from myometrial tissue excised from the uterine lateral wall and in the second case, eight RNA preparations representing the four myometrial excision sites under investigation in this study.

As can be seen in Figure 23, the PCR reaction was not reproducible as no correlation was found between results obtained in PCR 1 compared to results obtained in PCR 2. Correlation coefficients for the two trials were:  $r = 0.43$  and  $-0.31$ . Analysis of the data by linear regression produced  $r^2$  values of 0.02 and 0.03. The comparison of cDNA synthesis variability also indicated a lack of reproducibility as in one case no correlation was found between cDNA 1 and cDNA 2 ( $r = 0.1$ ,  $r^2 = 0.003$ ), and in the second case a significant negative correlation was found ( $r = -0.829$ ,  $r^2 = 0.97$ ,  $P < 0.0001$ ).

Following these findings, all semi-quantitative data described in Section 3.3.6 were analyzed using linear regression and correlation coefficient. In no case was a significant



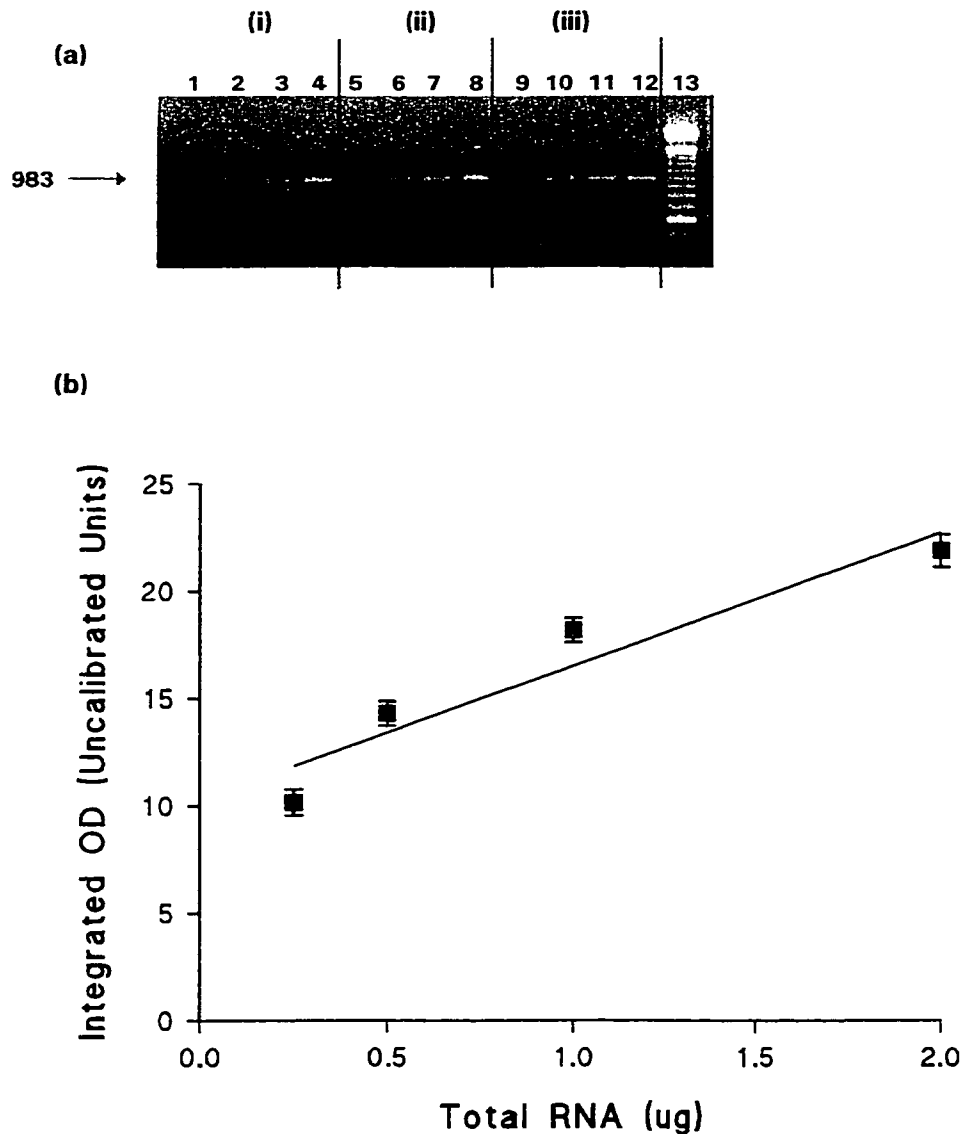
**Figure 23: Assessment of RT-PCR Data Reproducibility.** Integrated density ratios of TP receptor/G3PDH mRNA amplification product were calculated from (i) two identical PCR assays (PCR 1 and PCR 2) or from (ii) 1 PCR using two cDNA preparations (cDNA 1 and cDNA 2) and the data compared as outlined in Section 3.3.7. (a) Case 1, eight RNA preparations derived from myometrial tissue excised from the uterine lateral wall; (b) Case 2, eight RNA preparations derived from different uterine excision sites.

( $P < 0.05$ ), correlation found (negative or positive), with the range of  $r$  values falling between  $= -0.351$  to  $0.476$ .

### 3.3.8. Evaluation of the Semi-Quantitative RT-PCR Assay

Based on the lack of reproducibility of RT-PCR data detailed in section 3.3.7., I questioned whether the technique employed in these studies *could* detect differences in RNA expression. Thus experiments were performed where varying amounts of total RNA were added to the RT reaction and the resulting cDNA was amplified as previously described. As depicted in Figure 24, increasing initial amounts of total RNA from  $0.5 \mu\text{g}$  to  $2.0 \mu\text{g}$ , resulted in a linear increase in G3PDH mRNA amplification product ( $r^2 = 0.97$ ,  $n = 3$ ). One-way analysis of variance indicated a significant difference in RT-PCR product in each of the four concentration groups tested ( $P < 0.05$ ). The reproducibility of this significant result was tested by re-running the entire experiment. In this second trial, a significant difference was found between all concentration groups tested except between  $0.25 \mu\text{g}$  and  $0.5 \mu\text{g}$  (data not shown).

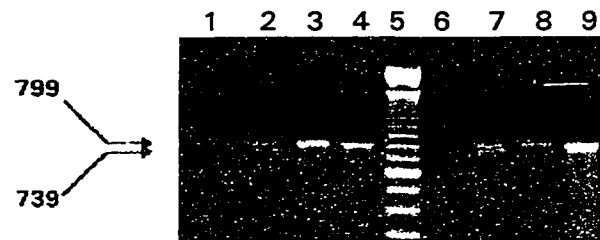
The same ability to detect differences in RNA abundance was not found when TP receptor mRNA was amplified. Figure 25 depicts a representative result, where increasing initial amounts of total RNA ( $0.5 \mu\text{g}$  to  $2.0 \mu\text{g}$ ), failed to produce a linear increase in TP receptor amplification product across the concentration range tested. An additional finding from these studies was the *differential* appearance of a second PCR product, estimated as



**Figure 24: Correlation Between the Initial Concentration of Starting RNA and Resulting Integrated Density of G3PDH mRNA Amplification Product.** (a) Ethidium bromide staining pattern of G3PDH mRNA amplification products derived from RT of varying amounts of myometrial total RNA (lane 1 = 0.25  $\mu\text{g}$ ; lane 2 = 0.5  $\mu\text{g}$ ; lane 3 = 1.0  $\mu\text{g}$ ; lane 4 = 2  $\mu\text{g}$ ) and 29 cycles of PCR, resolved by electrophoresis on a 1.5% agarose-TBE gel. Lane 13 = 100 bp DNA molecular weight ladder. Arrow marks the position of the G3PDH mRNA amplification products. (i - iii) represent three separate RNA preparations. (b) Each band in lanes 1 - 4 was quantified from the scanned Polaroid image and a regression curve plotted from the mean data. Values are expressed in uncalibrated units of optical density.

approximately 60 bp smaller than the TP receptor mRNA product band. This second band appeared as a faint signal when 0.5  $\mu\text{g}$  of RNA was reverse transcribed (Figure 25, lanes 2 & 7), was not visible when initial input RNA was doubled to 1.0  $\mu\text{g}$  (Figure 25, lanes 3 & 8), and was the only product seen at the final RNA concentration of 2.0  $\mu\text{g}$  (Figure 25, lanes 4 & 9). The expected TP receptor mRNA amplification product was also seen as a faint signal at 0.5  $\mu\text{g}$  of starting RNA, and was the sole product visible when 1.0  $\mu\text{g}$  of RNA was used for reverse transcription. This pattern of PCR product appearance was consistently seen in three separate experiments.





**Figure 25: TP Receptor mRNA Amplification Products Derived from PCR Following Reverse Transcription of Different Concentrations of RNA.** Ethidium bromide staining pattern of TP receptor mRNA amplicates derived from RT of varying amounts of myometrial total RNA (lane 1 & 6 = 0.25  $\mu\text{g}$ ; lane 2 & 7 = 0.5  $\mu\text{g}$ ; lane 3 & 8 = 1.0  $\mu\text{g}$ ; lane 4 & 9 = 2  $\mu\text{g}$ ) and 35 cycles of PCR, resolved by electrophoresis on a 1.5% agarose-TBE gel. Lane 5 = 100 bp DNA molecular weight ladder. Arrows mark the positions of TP receptor mRNA amplification products (799 bp) and unidentified amplification products (739 bp).

## **Chapter 4**

### **Discussion**

#### **4.1. PHARMACOLOGICAL CHARACTERIZATION OF THE TP RECEPTOR POPULATION IN HUMAN NONPREGNANT MYOMETRIUM**

The first objective of my thesis was to pharmacologically characterize the TP receptor in human nonpregnant myometrium. I broke this objective down into three tasks. Firstly, using the selective TP receptor agonists U-46,619 and I-BOP, I examined the functional and binding characteristics of the myometrial TP receptor and compared these data with the functional and binding properties of TP receptors found in human vascular smooth muscle and human platelets. Secondly, I evaluated the potency of seven TP receptor antagonists and compared these data to that gathered from studies on human platelets. Lastly, I examined potential regulatory mechanisms responsible for the reported variability of TP-receptor mediated responses. Specifically, I searched for a correlation between the sensitivity and maximum response of human nonpregnant myometrium with anatomical location, tissue orientation and menstrual cycle status of the donor.

As will be discussed below, the data gathered in these studies suggests that human nonpregnant myometrium possesses a single, homogeneous population of TP receptors, pharmacologically similar to that currently identified as the putative low affinity TP receptor

in human platelets. In addition, it appears that the contractile response of human nonpregnant myometrium induced by U-46,619 is not regulated by anatomical location, tissue orientation or menstrual cycle status of the donor.

#### 4.1.1. The Human Factor

Beyond methodological considerations, the study of human tissue introduces a great deal of complexity with regard to data interpretation compared to studies performed in animal models. The cause for such complexity is that in contrast to laboratory bred animals, human beings are *inherently variable*. Relating directly to the studies conducted in this thesis, numerous factors including age, weight, parity, caffeine, nicotine, and medication consumption, underlying disease states and indication for hysterectomy all could potentially influence the contractile properties of myometrial tissue (Sobczyk, 1980; Kenakin, 1984; Young, 1994). While some of these variables are more difficult to control than others, a confounding factor in the collection of myometrial tissue is availability. Thus the inclusion criteria outlined in Section 2.2. were established in rather broad terms to accommodate low supply. Such leniency must be considered when interpreting the data.

While human variability may pose a disadvantage, certainly a widely recognized advantage of utilizing human tissue is that such studies are directly relevant to human physiology and thus may significantly enhance the efficiency of the "discovery process" (Pharmagene Laboratories Ltd., 1997). This point is of particular importance to the delineation of potential TP receptor-mediated effects in human myometrium as an acceptable animal model has yet to be defined.

#### 4.1.2. *In Vitro* Contractility Studies

One property that distinguishes isolated myometrium from other smooth muscles studied *in vitro* is the development of high levels of spontaneous contractile activity (Crankshaw, 1990). The nature of this spontaneous activity has previously been reported to vary both between different donors and between tissue strips from the same donor (Senior *et al.*, 1991; 1992; 1993; Crankshaw, 1995). The results obtained in this study agree with such observations. Without manipulation of the ionic environment, such variability in spontaneous activity make reliable concentration-effect data difficult to obtain. Two quite different *in vitro* techniques have been developed to address such difficulty (Wainman *et al.*, 1988; Senior *et al.*, 1991). In this study, I employed the equilibrium method used by Wainman *et al.*, (1988), as it, unlike the superfusion technique used by Senior *et al.* (1991), allows for the determination of  $pEC_{50}$  values. It should be noted that although both techniques *account* for spontaneous activity, how the underlying mechanisms responsible for spontaneous activity may affect agonist-induced myometrial contractility cannot be assessed. Once more, as there is little evidence to suggest that such spontaneous activity occurs *in vivo*, the effect of agonists *in vitro* might be quite different from their effect *in vivo*.

Minimal requirements for the functional definition of a receptor include the determination of antagonist affinities, establishment of a potency ratio for agonists and estimation of the relative intrinsic efficacies of agonists (Kenakin *et al.*, 1992). In order to fulfill these requirements, I first examined the ability of two potent TP receptor agonists,

U-46,619 and I-BOP, to induce contractile activity in myometrial tissue obtained from the lateral wall of the corpus uteri and then examined the potency of seven selective TP receptor antagonists to inhibit U-46,619-induced myometrial contractions.

#### **4.1.2.1. Evaluation of the Potency of U-46,619 and I-BOP in Human Nonpregnant Myometrium Obtained from the Uterine Lateral Wall**

U-46,619 has previously been shown to be a potent stimulant of human myometrium from nonpregnant donors *in vitro* (Crankshaw, 1992; Senior *et al.*, 1992), but this is the first full report of an equilibrium study from which a  $pEC_{50}$  value was determined (Senchyna and Crankshaw, 1996). In addition, to my knowledge, this is the first full report of the effect of I-BOP in this tissue (Senchyna and Crankshaw, 1996). The mean  $EC_{50}$  values for U-46,619 and I-BOP are 125 nM and 16 nM respectively. The lack of variation in tissue sensitivity to U-46,619 (mean  $pEC_{50} = 6.9 \pm 0.27$ ), was not expected as previous studies have demonstrated large inter-donor differences in the sensitivity to this agonist (Crankshaw, 1992; 1995). That methodological differences could explain the lack of variation in my experiments will be addressed below. The maximum responses induced by U-46,619 and I-BOP were highly variable;  $2.3 \pm 1.2$  N/cm<sup>2</sup> and  $2.1 \pm 0.4$  N/cm<sup>2</sup> respectively. Such variability was not attributable simply to inter-donor differences, as significantly different maximum responses were recorded in strips prepared from the same donor. Variability of this nature has been recognized previously and a number of attempts to normalize maximum response data have been suggested (Crankshaw and Popat, 1997). However, experiments performed in our laboratory have indicated that for reasons we are

unable to explain, a uniform maximum tissue response cannot be achieved (unpublished observation). Thus the maximum response variability observed in my work could not be eliminated and as a result, important information may have been lost.

Although both U-46,619 and I-BOP evoked excitatory activity in all tissues, a down-turn in the concentration-effect curves was often observed at high agonist concentrations. The point at which the down-turn occurred and the extent of down-turn were variable, although a down-turn was never observed before the fifth cumulative addition of agonist. Bell-shaped curves for a number of prostanoid mimetics, including U-46,619 have been previously described in uterine tissue (Crankshaw and Gaspar, 1995; Crankshaw, 1995; Popat, 1998) and also for U-46,619-induced platelet aggregation (Takahara *et al.*, 1990). Although Senior *et al.* (1992), did not address the issue, U-46,619 concentration-effect curves reported in their study of human nonpregnant myometrium were also bell-shaped. Such an observation is significant because unlike the superfusion technique used by Senior *et al.* (1992), where agonists are added as a bolus dose and concentration-effect curves constructed sequentially, the technique I used involved a cumulative design. It has been argued that cumulative additions may not always produce the same results as when the bath is washed between additions (Kenakin, 1984). Possible explanations to support this argument include the formation of biologically active metabolites, alterations in the pH of the bathing solution and receptor desensitization due to prolonged exposure to drug (Kenakin, 1984). However, based on the fact that the concentration-effect curves reported by myself and by Senior *et al.* (1992), are similar, methodology can be ruled out as a factor responsible for the bell-shaped appearance. Another possibility that can be discounted as having a role in the down-turn of the concentration-effect curve is tissue relaxation induced

by PGI<sub>2</sub>. It has been demonstrated that both TxA<sub>2</sub> and U-46,619 can stimulate PGI<sub>2</sub> release from cultured human endothelial cells and human umbilical arteries and that PGI<sub>2</sub> release may be important in the feedback control of responses to TxA<sub>2</sub>. (Bj ero, 1986; Hunt *et al.*, 1992). That such a regulatory loop exists in myometrial tissue is possible. However in my experiments, all studies were performed in the presence of a concentration of indomethacin that has been shown to inhibit prostanoid production in a similar tissue (Dubin *et al.*, 1982).

Thus, having ruled out methodology and PGI<sub>2</sub>-induced relaxation, four additional explanations;

- 1) TP receptor desensitization
- 2) TP receptor coupling to physically and functionally different and pharmacologically antagonistic signalling pathways
- 3) TP receptor isomerization
- 4) TP receptor agonists, in high concentrations, have activity at an inhibitory prostanoid receptor

remain, that as explained below, may account for the bell-shaped curves reported in this study.

Homologous desensitization of the TP receptor has been demonstrated in platelets and transfected cell lines (Murray and Fitzgerald, 1989; Okwu *et al.*, 1992; Yukawa, *et al.*, 1997). Although the mechanisms have yet to be completely elucidated, phosphorylation of the TP receptor appears to mediate the attenuation in downstream signalling events (Okwu *et al.*, 1994; Yukawa *et al.*, 1997; Habib *et al.*, 1997). From a limited number of kinetic experiments Habib *et al.* (1997), demonstrated that U-46,619-induced IP<sub>3</sub> formation in TP receptor-expressing HEK 293 cells plateaus when concentrations of U-46,619 exceed 100 nM - 300 nM and that pre-

treatment with 300 nM U-46,619 for ten minutes results in 70% - 90% inhibition of IP<sub>3</sub> formation in response to a subsequent addition of U-46,619. Although direct comparisons are not possible, such data are consistent with the notion that homologous desensitization may account for the bell-shaped concentration-effect curves reported in this study.

That TP receptors have the potential to couple to multiple signalling pathways is supported by Ushikubi *et al.* (1994), who demonstrated that a single TP receptor partially purified from human platelet membranes could functionally couple to both purified G<sub>q</sub> and G<sub>12</sub> in reconstituted lipid vesicles. Similarly, Hirata *et al.* (1996), expressed two different isoforms of TP receptors in cultured cells and demonstrated that the two isoforms were able to activate phospholipase C activity, but oppositely regulated adenylyl cyclase activity. Lastly, Allan *et al.* (1997), transiently expressed a TP receptor cloned from HEL cells and demonstrated coupling to both  $\alpha_{13}$  and  $\alpha_q$ . Whether such differential coupling occurs in myometrial smooth muscle cells is not known and to date no signalling studies have been performed with the TP receptor in human myometrium. Members of the G<sub>s</sub>, G<sub>i</sub> and G<sub>q</sub> families are known to be present in nonpregnant myometrium (Europe-Finner *et al.*, 1993; Zumbihl *et al.*, 1994; Popat, 1998). Evidence for G<sub>12/13</sub> in human myometrium is lacking. If one can assume that G<sub>q</sub> plays a role, then such a pathway would most likely mediate, at least in part, the excitatory effect of TP receptor stimulation on myometrial smooth muscle contraction. At high agonist concentrations, a switch in G protein coupling in favour of G<sub>s</sub> may account for the bell-shaped appearance of the concentration effect curves as stimulation of adenylyl cyclase results in the inhibition of smooth muscle contraction. In support of this notion, Hirata *et al.* (1996), have reported that agonist induced-activation of adenylyl cyclase activity could only be seen in TP



receptor transfected cells when high ( $> 10$  nM) I-BOP concentrations were used, whereas phospholipase C activation appeared much more sensitive to agonist exposure.

A possibility that has been presented previously to explain bell-shaped concentration-effect curves describing carbachol binding to the muscarinic receptor is that a receptor can possess two distinct binding sites, which are selective for agonist and antagonist binding respectively. In the case of carbachol, it is proposed that its inhibitory effect on adenylyl cyclase activity is antagonized by its own binding to the antagonistic site (Järv, *et al.*, 1993). Such binding characteristics have never been proposed for TP receptor agonists, although it has been suggested through studies investigating the pH dependency of ligand binding, that TP receptor agonist and antagonist binding characteristics are different (Mayeux *et al.*, 1991).

Lastly, it is possible that U-46,619 in high concentrations, has activity at inhibitory prostanoid receptors. The DP receptor-selective antagonist BW A868C has no effect on U-46,619-induced myometrial activity (Crankshaw, 1995), however the contribution of IP and EP<sub>2</sub> receptor activity is unknown. The issue of TP receptor subtypes in human nonpregnant myometrium has yet to be formally proposed. As will be discussed in following sections, data gathered in this thesis *suggest* that two populations of TP receptors may exist in this tissue. The possibility that one such population regulates inhibitory activity in myometrial tissue cannot be substantiated by any work performed in this thesis. However, Elmhurst *et al.* (1997), have described inhibitory TP receptors on canine colonic epithelium, the presence of which could only be defined after significant stimulatory activity had been induced by excitatory agonists. Despite the lack of supporting evidence, I attempted to fit my data to a two-receptor model according to the methods of Szabadi (1977) and Pliška (1994). I was, however, unable to make reliable

estimates of  $pEC_{50}$  values for the two components of the curves from the data. Therefore I opted to omit data points falling below and to the right of the concentration-effect asymptote. Although this was the only practical method, it may lead to some inaccuracies in the estimation of location parameters on concentration-effect curves.

The mean  $EC_{50}$  values I report are similar to those found to induce aggregation of human platelets, where the  $EC_{50}$  for U-46,619 ranges from 87 nM to 121 nM (Dorn, 1991; Morinelli *et al.*, 1990b) and for I-BOP from 1.8 nM to 11 nM (Dorn, 1991; Dorn and DeJesus, 1991; Morinelli *et al.*, 1990b), but they are substantially lower than that reported for contraction of human vascular smooth muscle where the  $EC_{50}$  of U-46,619 is 6.4 nM and of I-BOP is 0.33 nM (Dorn, 1991). In the context of putative TP receptor subtypes, the mean  $EC_{50}$  I report for I-BOP is similar to the I-BOP  $EC_{50}$  range of 1.8 nM to 4.4 nM found to induce human platelet aggregation, but is much higher than the concentration of I-BOP found to induce human platelet shape change, where the mean  $EC_{50}$  ranges from 0.173 nM to 0.263 nM (Dorn, 1989; Dorn and DeJesus, 1991). Due to the variability in maximal response, it was difficult to ascertain whether U-46,619 and I-BOP represented full or partial agonists. No statistical difference was found between the maximal response data gathered for both agonists ( $P < 0.05$ ), and thus I assumed both U-46,619 and I-BOP were full agonists in this system. Based on this assumption, the potency ratio ( $EC_{50}$  I-BOP /  $EC_{50}$  U-46,619) determined in this study was 1:8. The potency ratio ( $EC_{50}$  I-BOP /  $EC_{50}$  U-46,619), when the drugs were tested for their ability to stimulate platelet aggregation was reported as 1:11 (Morinelli *et al.*, 1990a) and 1:16 (Dorn 1991), while a ratio of 1:19 was reported when the two agonists were used to stimulate human vascular smooth muscle contraction (Dorn, 1991). Thus although I-BOP is consistently more potent than

U-46,619, the potency ratio I reported is lower compared to other systems. These results should not however be interpreted as evidence in support of TP receptor subtypes as I could not conclusively demonstrate the equal efficacy of I-BOP and U-46,619. Recently, Krauss *et al.* (1996), provided evidence to suggest that a novel TxAz mimetic, AGN 192093, could be used to distinguish between TP receptor subtypes. They demonstrated that AGN 192093 was a highly potent contractile agonist at rat vascular smooth muscle and human myometrium, whereas it had a very weak proaggregatory effect on human platelets. This difference in potency was considered evidence for the existence of TP receptor subtypes. However, the non-equilibrium technique of Senior *et al.* (1991; 1992), was used to determine AGN 192093 potency in human myometrium whereas equilibrium methods were employed on rat vasculature. The non-equilibrium superfusion technique will tend to record differences in the kinetic variables of an agonist as differences in potency, whereas the equilibrium method will not (Brown *et al.*, 1997). Thus it is difficult to ascertain from the work of Krauss *et al.* (1996), if TP receptor subtypes exist in the human. The difference in AGN 192093 potency between rat vasculature and human platelets may be explained by species differences. As AGN 192093 is not commercially available, I attempted to obtain this agonist from the authors for my own experiments, but my requests were refused.

The potencies of the two agonists reported in this work further support the claim that TP receptors are present in human myometrium from nonpregnant donors (Senior *et al.*, 1992). Initial comparisons suggest that the myometrial TP receptor is similar to that found in human platelets, and more specifically, is similar to that putatively recognised as the low affinity site in human platelets.

#### 4.1.2.2. Evaluation of the Potency of TP Receptor Antagonists to Inhibit U-46,619-Induced Myometrial Contractions

Prior to this study,  $pA_2$  values for only two TP receptor antagonists (BM 13,505 and GR 32,191), in human myometrium had been published. To further advance our understanding of the potency of available TP receptor antagonists, seven different compounds were evaluated in my work. As a minimum of three concentration-effect experiments were to be performed for each antagonist, only U-46,619 was used to induce myometrial contractions. The potency order that I determined is consistent with that obtained with human platelets, although there are some notable exceptions (Table 1). Thus the effect of these TP receptor antagonists on U-46,619-induced contractions add further support to the presence of a platelet-like TP receptor in human nonpregnant myometrium. Similar data were not available to facilitate a comparison with human vascular smooth muscle TP receptors. Clayton *et al.* (1990) reported a  $pA_2$  of 7.1 for BM 13,505 in nonpregnant human myometrium which agrees well with my  $pA_2$  of 7.4. The  $pA_2$  of 7.1 for GR 32,191 in human nonpregnant human myometrium reported by Senior *et al.* (1992) is significantly lower than my  $pA_2$  value of 8.6 which is close to the  $pA_2$  of 8.79 reported by Lumley *et al.* (1989) for human platelets. GR 32,191 has, in several cases, been reported to act as an irreversible antagonist (Takahara *et al.*, 1990; Hirata *et al.*, 1996). Furthermore, GR 32,191 has been shown to produce an apparent down-regulation of TP receptors in human platelets after a 30 minute exposure *in vitro*, the mechanism of which is not understood (Armstrong *et al.*, 1993). Such inconsistencies in the reported mechanism(s) of GR 32,191 action make it difficult to resolve the discrepancy between the two  $pA_2$  values reported in human nonpregnant myometrium by Senior *et al.* (1992), and myself. It is worth noting,

however, that Senior *et al.* (1992), used a shorter preincubation period with GR 32,191 compared to the protocol used in my studies, suggesting that desensitization of TP receptors may account for at least some of the difference in the apparent potency of this antagonist.

The effects of TP receptor antagonists on I-BOP-induced myometrial contraction was also assessed, but on a much smaller scale. L670,596 had a mean  $pA_2$  value of 8.6 ( $n = 2$ ), which is consistent with the value of 8.6 reported when U-46,619 was used as the agonist. Two additional TP receptor antagonists, BM 13,505 and ONO 3,708 yielded single experiment  $pA_2$  values of 7.5 and 7.9 respectively, which also agree with data collected with U-46,619, and thus support activity at TP receptors.

Determination of an antagonist's dissociation constant ( $K_b$ ) and estimates of receptor homogeneity from functional experiments are facilitated by Schild analysis, which is a mathematical model based on the relationship between the antagonist concentration and the shift in location of the agonist's concentration-effect curve (Aruniakshana and Schild, 1959, Kenakin, 1984). Although I employed the equation described by Schild in the determination of  $pA_2$  values, I could not assume that my reported  $pA_2$  values were equal to the  $K_b$  values of the respective antagonists as two fundamental requirements of Schild analysis were not satisfied in my experiments (Lew and Angus, 1995). Specifically, Schild analysis requires a minimum of two concentration-effect curves be completed in a single tissue. Only one concentration-effect experiment could be performed per tissue in my experiments as changes in myometrial tissue sensitivity to agonist stimulation occur over prolonged wash out procedures (Cheuk *et al.*, 1993; Crankshaw and Dyal, 1994). Secondly, Schild analysis requires accurate quantification of three aspects of the concentration-effect curve: maximum, slope and  $pEC_{50}$ . As discussed above, the

variability in maximal response observed in my experiments and the omission of data points falling below and to the right of the concentration-effect asymptote may have led to slight inaccuracies in the calculation of these location parameters. Thus so as to avoid inappropriate conclusions regarding the affinity of the antagonists employed in this study,  $K_b$  values were not reported. My *inability* to accurately estimate  $K_b$  values is not unique, and consequently new methods have been proposed (Lew and Angus, 1995). Following the completion of my experiments, a novel method for calculating  $K_b$  values was presented by Lew and Angus (1995), that seems to account for the deficiencies in my experiments. The fact that the  $pA_2$  values I report were not concentration-dependent and appear not to be agonist-dependent, suggests that their actions are directed at a single TP receptor. This is consistent with data from Senior *et al.* (1992), who reported a Schild plot slope for GR 32,191 of 0.95.

#### **4.1.2.3. Effect of Excision Site, Tissue Orientation and Menstrual Cycle Status of the Donor on the Mean Sensitivity of Human Nonpregnant Myometrium to U-46,619 Stimulation**

Two hypotheses were explored in an attempt to explain the nature of the differential sensitivity of human myometrium to TP receptor stimulation observed by Crankshaw (1992; 1995). First, previous reports have suggested that the uterine response to prostanoids (Gannon, 1985), and the expression of prostanoid receptors (Hoffman *et al.*, 1993), may be dependent upon levels of ovarian hormones. As the pain of PD usually begins at around the onset of menses (Rosenwaks and Seegar Jones, 1980), I investigated whether myometrial sensitivity to U-46,619 stimulation may be regulated by fluctuating hormone levels in accordance with the menstrual cycle. I anticipated secretory phase tissue to yield a higher

mean sensitivity compared to proliferative phase tissue, indicating a “priming” of the tissue prior to the onset of menstruation. Secondly, as detailed by Kenakin (1984), heterogeneity of tissue sensitivity to agonist stimulation can often be attributed to regional differences in tissue responsiveness. The human uterus has been suggested to possess such regional differences with respect to prostanoid sensitivity (Wikland *et al.*, 1984). Wikland *et al.* (1984), demonstrated using human pregnant myometrium that tissue excised from ‘upper’ and ‘lower’ segments of the uterus displayed marked differences in contractile activity induced by PGE<sub>2</sub> and PGF<sub>2 $\alpha$</sub> . Further variability was imposed on the response to either prostanoid depending on whether the tissue was excised before or during labor. Gradients of tissue responsiveness may also occur in a “width wise” direction. Anderson *et al.* (1981) and Tuross *et al.* (1987), have demonstrated that circular (sub-endometrial) and longitudinal (sub-serosal) muscle in rat myometrium responds differently upon exposure to the same agonist. Kenakin (1984), has also suggested that geometrical orientation can influence tissue responsiveness in *in vitro* experiments when measuring responses isometrically. As the two hypotheses I presented appeared not to be mutually exclusive, I investigated the effect of menstrual cycle status of the donor and tissue excision site simultaneously.

As is detailed in Table 3, I found no significant difference in the mean sensitivity or mean maximal response between any of the groups I examined (Senchyna and Crankshaw, 1997). The mean  $pEC_{50}$  values obtained for tissue excised from the lateral wall from donors in the proliferative ( $pEC_{50} = 6.9 \pm 0.3$ ) or secretory phase ( $pEC_{50} = 7.1 \pm 0.3$ ) of the menstrual cycle are consistent with the  $pEC_{50}$  value of  $6.9 \pm 0.3$  reported in the previous section of this thesis. Again, bell-shaped concentration-effect curves were often recorded in

each group I examined and the point at which the downturn began was consistent with my earlier observations. Wikland *et al.* (1984), reported biphasic effects from pregnant, non-laboring human myometrial tissue excised from the upper segment in response to PGE<sub>2</sub>, whereas tissue obtained during active spontaneous labor yielded purely contractile responses to PGE<sub>2</sub>. These and other observations lead Wikland to coin the theory of Fundal Dominance, where the endocrine events responsible for partuition alter myometrial responses to prostanoids such that they facilitate contraction of the upper segment but relax the lower segment. The fact that similar responses to U-46,619 were observed in my experiments suggest that the mechanism(s) responsible for the bell-shaped concentration-effect curves are not regulated by either ovarian hormones or anatomical location and that TP receptor-mediated fundal dominance does not occur in preparation for menstruation. Melin *et al.* (1988), have reported that spontaneous contractile activity and graded contractile responses elicited by arginine-vasopressin (AVP), differ between serosal and endometrial sections of human nonpregnant myometrium. Specifically, these authors noted that the spontaneous activity of muscle strips from the endometrial region was of a much higher frequency and the response to AVP was more differentiated compared to the response of the serosal part. Although these are purely qualitative observations, I reviewed my raw data for a similar pattern. Although I did observe differences in the pattern of both spontaneous and agonist-induced phasic and tonic activity between sub-endometrial and sub-serosal tissues, it was not a consistent finding.



My data are in contrast to previously reported large inter-donor differences in sensitivity to U-46,619. The studies by Crankshaw (1992; 1995), utilized smaller sample numbers and did not employ the exclusion criteria I specified with respect to hormone replacement therapy or irregular menstrual cycle length. I suggest that the combined differences between the studies by Crankshaw and myself may be responsible for the inconsistency of our data.

The data that I have presented represent the first study to systematically evaluate the effects of anatomical location, tissue orientation and donor menstrual cycle status on the response of human nonpregnant myometrium to TP receptor stimulation. That the response appears invariant bears significance to future investigations on myometrial TP receptor-mediated responses as the rigorous and time consuming steps required to obtain tissue from one standardized location may not be necessary. However, I will stress caution on the interpretation of my data and as I explain below, that due to methodological reasons, differences that may be present in terms of TP receptor-mediated responses, both *in vitro* and/or *in vivo* were not captured.

The mean maximal responses were highly variable within all groups studied. My inability to accurately evaluate maximal response may have affected my ability to observe differences between the groups IF the parameter that is variable is tissue maximal response as opposed to tissue sensitivity. An increased maximal tissue response to TP receptor stimulation could occur via an increase in TP receptor expression. That maximal response would increase in the absence of a change in the sensitivity of the tissue to TP receptor stimulation would imply the absence of spare TP receptors. It is also possible that an

increase in maximal tissue response could occur through the *facilitation*, rather than the initiation of contractile activity. As discussed below, three possible events;

- 1) Alkalinization of the myometrium
- 2) Upregulation of TP receptor signal transduction machinery
- 3) Formation of gap junctions

may facilitate increased myometrial contractile activity.

The myometrial TP receptor may couple to G<sub>12</sub> or G<sub>13</sub> in addition to G<sub>q</sub>.  $\alpha_{12}$  and  $\alpha_{13}$  subunits have been reported to modulate the activity of Na<sup>+</sup>/H<sup>+</sup> exchangers (Dhanasekaran *et al.*, 1994; Voyno-Yasenetskaya *et al.*, 1994). It has been demonstrated that acute alkalinization of nonpregnant myometrium results in a significant increase in the frequency of spontaneous contractile activity presumably as a consequence of increased membrane calcium currents (Phoenix and Wray, 1993; Wray, 1993; Parratt *et al.*, 1995). Whether such an increase in pHi occurs *in vivo* immediately prior to menses to (a) aid in endometrial sloughing, or (b) result in the strong and sustained contractions observed *in vivo* in women with PD (Ulmsten, 1985), remains to be determined. However, one may assume if alkalinization does occur, it is regulated to coincide with the onset of menses. Three potential regulatory mechanisms may be (a) regulated coupling of TP receptors to G<sub>12/13</sub>; (b) regulated expression of an alternatively spliced TP receptor that couples to G<sub>12/13</sub>; (c) regulated expression of G<sub>12/13</sub>.

An increased maximal response to TP receptor stimulation may be facilitated by regulation of the signal transduction machinery involved in propagating the TP receptor

signal. Information gathered from pregnant myometrial tissue suggests that G protein levels are regulated throughout term so as to control myometrial contractility (Europe-Finner *et al.*, 1993; Zumbihl *et al.*, 1994). To my knowledge, comparisons of G protein levels or enzyme concentrations such as PLC, during different phases of the menstrual cycle have not been conducted.

Lastly, it has been reported that an upregulation of gap junctions between cells facilitates more efficient coupling of electrical, metabolic and mechanical activity (Huizinga *et al.*, 1992). Furthermore, it has been suggested that steroid hormones and prostanoids provoke the formation of myometrial gap junctions and that prostanoids may control the permeability of myometrial gap junctions (Garfield, 1994). That TxA<sub>2</sub> specifically is involved in regulating either the presence or function of myometrial gap junctions has yet to be determined. However, regardless of mechanism, increased coupling between adjacent myometrial smooth muscle cells may increase the maximal response induced by a stimulatory agonist as has been proposed by a number of investigators (Garfield and Hayashi, 1980; Pulkkinen, 1983; Chow and Lye, 1994; Garfield, 1994).

Whether my data are truly representative of the *in vivo* response to TP receptor stimulation may be questioned due to the "disadvantages" of *in vitro* experimentation. By isolating myometrial tissue from its natural environment one in the process removes potential endogenous mediators and tissue interactions both of which may directly or indirectly influence myometrial contractility. Specifically, the contractile activity of human myometrium has been shown to be dependent upon the steroid hormone milieu (Thornburn and Challis, 1979). While it has been demonstrated with rabbit myometrium that the

control these hormones exert on prostanoid function does decrease over time *in vitro*, the rate of decrease is relatively slow and perhaps should not be significant if experiments are performed within a limited time frame (Currie and Jeremy, 1979). As such, my experiments were always performed within 24 hours of tissue retrieval. However, as Crankshaw (1990), has reviewed, we know very little about quickly reversible forms of control of myometrial activity. Pulkkinen (1983), has suggested that high prostanoid concentrations in the uterus may not be painful *unless* they are associated with a suitable hormonal environment. It is known that women who are anovulatory do not have menstrual pain (Rosenwaks and Seegar Jones, 1980), thus implying that hormonal stimuli that coincide with follicle maturation may mediate the underlying mechanisms of PD. Whether such stimuli are lost *in vitro* is not known. Interactions between longitudinal and circular muscle layers and between smooth muscle and both the endometrium and the vasculature have been removed in my experiments through selective dissection. The *in vivo* interplay between these tissues could all potentially effect the contractile activity of the uterus. Thus the *in vitro* technique in itself is incapable of modeling precisely the forces that result from these complex interactions.

In summary, while the technique I have employed to study the functional response of human nonpregnant myometrium to TP receptor stimulation may not provide complete information regarding the contractile response of the *uterus*, it does provide an understanding of underlying mechanisms and variables regulating a discrete section. Without question, such information has been indispensable in the elucidation of receptor

classification and in the discovery of potential therapeutic targets. Based on the methodological limitations I described, I can only suggest that the sensitivity of human nonpregnant myometrium to TP receptor stimulation is not regulated by anatomical location, tissue orientation or hormonal events associated with the menstrual cycle. Until the *in vitro* methodology is refined to enable the accurate estimation of maximal response, future investigations of myometrial TP receptor-mediated responses and potential mechanisms of regulation may focus in part, on events other than contractile activity, such as myometrial alkalization and expression of proposed components of the TP receptor signal transduction pathway.

#### 4.1.3. Radioligand Binding Studies

Having functionally defined the myometrial TP receptor population, radioligand binding experiments were performed to gain further insight into TP receptor pharmacology and to estimate receptor density, which is not possible in isolated tissue studies (Kenakin, 1984). As with functional experiments, minimum requirements, including the demonstration of high affinity binding which is saturatable, reversible and displaceable must be fulfilled in order to define a receptor site with radioligand binding data (Laduron, 1984). In an attempt to meet these criteria, I employed the radioligand [<sup>125</sup>I]-BOP, which is considered the most potent radiolabeled TP receptor agonist yet described (Dorn, 1989), and performed saturation, kinetic and competition analyses. Prior to the start of my thesis, only a single study suggested that the TP receptor antagonist [<sup>125</sup>I]PTA-OH binds to human myometrial smooth muscle cells.

This report then represents the first comprehensive investigation of myometrial TP receptor binding characteristics (Senchyna and Crankshaw, 1996).

#### 4.1.3.1. Estimation of $K_d$ and $B_{max}$ Values for [ $^{125}$ I]-BOP Binding to HMM

Saturation analysis suggests that [ $^{125}$ I]-BOP interacts with a single class of sites in human myometrial membranes (HMM). The  $K_d$  values determined by saturation and kinetic analyses are in good agreement, although [ $^{125}$ I]-BOP dissociation did not fit a one-site model perfectly. A lower affinity site may be present, and as such I attempted to fit my data to a two-site model. A low affinity site ( $K_d = 1.4 \mu\text{M}$ ), detected by [ $^3\text{H}$ ]U-46,619 has been reported to exist on the human platelet, although the authors did concede that it was difficult to characterize and may have represented displaceable but non-specific binding (Morinelli *et al.*, 1987). I rejected the two-site data that I calculated as a significant F statistic could not be obtained because of high non-specific binding at high ligand concentrations. Thus, I conclude that the affinity of the myometrial TP receptor for [ $^{125}$ I]-BOP is similar to that reported for the putative low affinity site in human platelets ( $K_d = 2.2 - 3.9 \text{ nM}$ ) (Dorn, 1989; Morinelli *et al.*, 1989; Dorn and DeJesus, 1991; Masuda *et al.*, 1991) and for the stably expressed HEL cell TP receptor ( $K_d \approx 3.8 \text{ nM}$ ), which possesses the pharmacological characteristics of the low affinity site in human platelets (Allan *et al.*, 1996).

Previous studies have suggested that the  $B_{max}$  for platelet TP receptors can increase *in vivo* in patients with acute myocardial infarction compared to control patients (Dorn *et al.*, 1990). As I suggested in Section 4.1.2.3., that an increase in tissue maximum response may be regulated by an increase in TP receptors, binding of [ $^{125}$ I]-BOP to HMM was examined as a function of donor menstrual status. Saturation analysis revealed that  $pK_d$  and  $B_{max}$  values were

invariant over the course of the menstrual cycle (proliferative phase  $pK_d = 8.3 \pm 0.3$ ,  $B_{max} = 412 \pm 319$  fmol/ mg protein,  $n = 5$ ; secretory phase  $pK_d = 8.5 \pm 0.4$ ,  $B_{max} = 369 \pm 192$  fmol/mg protein,  $n = 6$ ) (Senchyna and Crankshaw, 1994). As such, these values were pooled and incorporated into the mean  $K_d$  and  $B_{max}$  values reported in my results (Section 3.2.1.). Swanson *et al.* (1992), demonstrated through an autoradiographic technique that the expression of TP receptors in human myometrial smooth muscle cells was invariant over the course of the menstrual cycle. These data were inconclusive however as the ligand employed in their study ( $[^{125}I]$ -PTA-OH), could not be displaced by U-46,619. Although my data are consistent with the observations of Swanson *et al.*, the high variability associated with my  $B_{max}$  values may have hindered my ability to detect significant changes in TP receptor expression.

#### 4.1.3.2. The Effect of GTP $\gamma$ S and Buffer on $[^{125}I]$ -BOP Saturation Binding Characteristics

That the affinity of  $[^{125}I]$ -BOP binding was not decreased by the presence of GTP $\gamma$ S has been reported previously (Saussy *et al.*, 1991), although it is inconsistent with both functional and structural data suggesting that TP receptors are coupled to a member of the  $G_q$  family of G proteins (Hirata *et al.*, 1991; Coleman *et al.*, 1994). Insight has been gained into this apparent paradox from studies by D'Angelo *et al.* (1994), and Allan *et al.* (1996). Both groups cloned TP receptor cDNAs; D'Angelo *et al.*, from platelet-like K562 cells and Allan *et al.*, from HEL cells. Amino acid analysis demonstrated that both clones are encoded by the same sequence as the placental cDNA. D'Angelo *et al.*, demonstrated that binding of  $[^{125}I]$ -BOP to placental membranes was sensitive to GTP $\gamma$ S whereas binding to membranes derived from HEK293 cells transfected with the K562 clone was not.  $[^{125}I]$ -BOP binding to K562 cells, which was best characterized by

a single  $K_d$  of  $\approx 5$  nM, similarly was not affected by  $GTP\gamma S$ . Taken together, these results suggested that either a G protein that can alter the affinity state of a single TP receptor on placental membranes is lacking in HEK293 and K562 cells, or, multiple TP receptor isoforms that couple to distinct G proteins exist in different cell types. The work by Allan *et al.*, lent support to the former suggestion. This group first demonstrated that the affinity of the HEL cell TP receptor for [ $^{125}$ I]-BOP was decreased when coexpressed in COS-7 cells with  $G\alpha_{13}$  or  $G\alpha_q$ , but not by  $G\alpha_{12}$  or  $G\alpha_2$ . Secondly, they demonstrated that  $GTP\gamma S$  had no effect on the binding of [ $^{125}$ I]-BOP to COS-7 cell membranes transfected with either the TP receptor clone alone or with  $G\alpha_{12}$ . In contrast, in COS-7 cell membranes cotransfected with the HEL TP receptor clone and  $G\alpha_{13}$ , there was a significant decrease in the affinity for [ $^{125}$ I]-BOP in the presence of  $GTP\gamma S$ . Thus, in light of these studies, the fact that I did not see an effect of  $GTP\gamma S$  may be attributed to the fact that the effect of  $GTP\gamma S$  on TP receptors is dependent on the tissue source of the receptors; or more specifically, on the G protein repertoire expressed by that tissue. A difference in experimental conditions was ruled out as I was unable to detect  $GTP\gamma S$  sensitivity using the HEPES buffer of D'Angelo *et al.* (1994). Interestingly, it has been reported that the affinity of  $G_q$  for  $GTP\gamma S$  is very low ( $\approx 960$  nM) (Ushikubi *et al.*, 1994). Allan *et al.* (1996), noted that  $G\alpha_{12}$  and  $G\alpha_{13}$  also display a low affinity for GTP analogs and as such performed their incubations with  $GTP\gamma S$  for 2 hours. I performed my incubations with  $GTP\gamma S$  for 1 hour whereas the protocol by D'Angelo *et al.* (1994), utilized a 30 minute incubation period. Thus it is possible that interactions with  $G_q$  may affect the affinity of the TP receptor and the experimental procedures employed by myself and D'Angelo *et al.* were not appropriate to detect such action. Finally, although all the data discussed in this section are significant in that they provide a possible explanation for the variations in TP receptor pharmacology observed between different tissues



(and in the platelet itself), the possibility that multiple TP receptors exist cannot be excluded. Clearly further efforts are needed to determine the role of G proteins in the coupling of TP receptors and the regulation of their affinity states.

In light of the fact that G protein interactions may alter the affinity of the myometrial TP receptor, I attempted to investigate whether a low affinity site would still be suggested by my data if I employed a radiolabeled antagonist. I performed six saturation experiments with the iodinated TP receptor antagonist [<sup>125</sup>I]-SAP (Naka *et al.*, 1992), but was unable to obtain more than  $\approx 35\%$  specific binding. As a similar lack of specific binding was found to occur with human vascular smooth muscle cells (Morinelli, 1994, personal communication), I abandoned the use of [<sup>125</sup>I]-SAP.

That I reported similar affinity values for [<sup>125</sup>I]-BOP using either HEPES buffer or sucrose-MOPS buffer, but significantly higher maximal binding with HEPES buffer is interesting as there appears to be evidence in the literature to support a role for buffer and / or pH in the determination of TP receptor affinity and density values for [<sup>125</sup>I]-BOP. Morinelli *et al.* (1990a; 1990b), have demonstrated that [<sup>125</sup>I]-BOP binds to a single class of TP receptors on human platelets when saturation and / or kinetic studies are performed with Tris-NaCl buffer, pH 7.4. However, by: (a) decreasing the pH of the Tris-NaCl buffer to pH 6.0 and extending the incubation time from one to two hours, or (b) using HEPES buffer, pH 7.6, these authors reported that the affinity of [<sup>125</sup>I]-BOP is increased and the resulting data are best resolved by a two-site model. A similar effect of buffer has been seen in studies of human vascular smooth muscle cells, where in Hanks Balanced Salt Solution, a single equilibrium  $K_d$  of 2.6 nM was reported for [<sup>125</sup>I]-BOP (Morinelli *et al.*, 1990c), whereas use of HEPES buffer at pH 7.6

significantly decreased the  $K_d$  to 390 pM (Dorn, 1991). The effect of pH has been further probed by Mayeux *et al.* (1991), who demonstrated that in washed human platelets a change in the pH of Tris-NaCl buffer from 7.4 to 6.0 increased both receptor affinity and density for [ $^{125}$ I]-BOP. An increase in  $K_d$  but not  $B_{max}$  was also observed in solubilized membranes at pH 6.5 compared to pH 7.4. I cannot explain the increased maximal binding I report associated with the use of HEPES buffer. One methodological difference other than buffer between my studies and that of D'Angelo *et al.* (1994), is that my sucrose-MOPS wash buffer was at pH 7.4, whereas the HEPES wash buffer was at pH 6.5. Although Mayeux *et al.* (1991), did not observe an effect of pH on the  $B_{max}$  of solubilized membranes with Tris-NaCl buffer, the pH difference between the two wash buffers in my studies may be significant. Whether further manipulations of buffer or increasing the incubation time of saturation experiments will uncover a second class of binding sites and /or resolve the increase in  $B_{max}$  remains to be determined.

#### 4.1.3.3. Competitive Inhibition of [ $^{125}$ I]-BOP Binding

The notion that [ $^{125}$ I]-BOP was binding to TP receptors was supported by competition studies in which the radioligand was completely displaced by all TP receptor ligands tested (Table 2). The fact that ligands with a high degree of selectivity for other prostanoid receptors were relatively ineffective in competing for [ $^{125}$ I]-BOP binding suggests that the compound binds to sites with the expected pharmacological profile of TP receptors. I-BOP was 8 times more potent than U-46,619 in provoking contractions of human myometrium (Table 1) and 14 times more potent at displacing [ $^{125}$ I]-BOP from HMM (Table 3). 8-epi-PGF $_{2\alpha}$  was 100 times less potent than U-46,619 in causing contraction (Crankshaw, 1995) and 50 times less potent in the binding assay (Table 3). There was good correlation between antagonist functional ( $pA_2$  values, Table 1)

and binding ( $pIC_{50}$  values, Table 3) potencies. One clear exception to this was GR 32,191 which showed much lower potency in the binding assay than would be predicted from its  $pA_2$  against U-46,619. As discussed above, GR 32,191 has been shown to produce an apparent down-regulation of TP receptors in human platelets after a 30 minute exposure *in vitro* (Armstrong *et al.*, 1993). If a similar process occurs in human myometrial strips *in vitro* I may have over-estimated the potency of the compound. The rank order of I-BOP > SQ 29,548 > U-46,619 is consistent with that previously reported for human platelets (Morinelli *et al.*, 1989), human vascular smooth muscle cells (Morinelli *et al.*, 1990c), K562 cells, placental membranes and HEK 293 cells transfected with cloned K562 cell TP receptor cDNA (D'Angelo *et al.*, 1994), and for COS-7 cells transfected with cloned HEL cell TP receptor cDNA (Allan *et al.*, 1996). Although in agreement with the literature, no insight could be gained from this comparison as to whether the myometrial TP receptor more closely represents the putative low affinity TP receptor on platelets or the TP receptor found on vascular smooth muscle. This similarity in rank order was not unexpected as it is now generally accepted in the literature that structurally dissimilar compounds such as I-BOP and SQ 29,548 are incapable of differentiating between platelet and vascular TP receptors (Masuda *et al.*, 1991; Krauss *et al.*, 1996). In contrast, numerous reports attest to the ability of a series of 13-azapinane  $TxA_2$  analogs to discriminate between platelet and vascular TP receptors (Mais *et al.*, 1985a; 1985b; Masuda *et al.*, 1991; Allan *et al.*, 1996). Unfortunately, I realized the utility of the 13-azapinane class of TP receptor antagonists following the completion of my experiments. Future use of these compounds may yield valuable information into the pharmacology of the myometrial TP receptor.

A comparison of binding data obtained from saturation and competition experiments shows that the  $IC_{50}$  value for I-BOP is significantly greater than the  $K_d$ . Although this observation

could be explained by the binding of I-BOP to an additional site with considerably lower affinity, I was unable to confirm this hypothesis with expanded saturation experiments. In addition, the Hill coefficient of 1.2 for I-BOP competition is inconsistent with such a hypothesis. Therefore at this time I am unable to resolve this discrepancy. One methodological difference that may improve the ability to resolve the binding characteristics of [<sup>125</sup>I]-BOP is to use membranes isolated from cultured myometrial smooth muscle cells. Such a procedure would remove the possible influence of non-myometrial smooth muscle tissue, such as stroma, glands and vasculature on the binding of [<sup>125</sup>I]-BOP. As was the case with my functional experiments, I chose not to calculate affinity ( $K_d$ ) values for the ligands employed in the competition binding assays via the Cheng-Prusoff transformation of  $IC_{50}$  values (Craig, 1993), as I could not conclusively demonstrate that the  $K_d$  value I reported for [<sup>125</sup>I]-BOP represented binding at a single site.

The difference in apparent binding affinity of I-BOP necessitates two possible explanations to describe the relationship between I-BOP occupancy and I-BOP-induced contractile response. In the first case, the saturation  $K_d$  for [<sup>125</sup>I]-BOP is  $\approx$  one fourth the calculated  $EC_{50}$  value for stimulating myometrial contractions. This difference may reflect positive cooperativity of [<sup>125</sup>I]-BOP binding, where affinity increases as the proportion of occupied TP receptors increases. However, arguing against this possibility is the fact that the Hill coefficients determined from the competition binding data did not significantly differ from unity. An alternative explanation is variance in experimental conditions; namely a difference in the buffer and the pH in which functional and binding experiments were performed. My binding experiments were performed in Sucrose-MOPS at pH 6.5 whereas contractility experiments were performed in PSS at pH 7.4. As I discussed above, both the type of buffer used and the

pH of the buffer can alter the affinity of the TP receptor for [<sup>125</sup>I]-BOP. That the increase in affinity noted with a decrease in pH is associated with a change in function as opposed to simply a binding phenomenon has been demonstrated by Mayeux *et al.* (1991). This group reported that the EC<sub>50</sub> value for I-BOP to induce shape change in platelets suspended at pH 6.0 was two-fold lower than in platelets suspended at pH 7.4. Thus had my functional experiments been performed at a lower pH, the apparent potency of I-BOP may have increased in accordance with an increased affinity for the TP receptor. However, as a decrease in pH may alter the contractile activity of human myometrium (Wray, 1993), an alternative may be to employ HEPES buffer at pH 7.6 in both functional and binding experiments. Again, HEPES buffer appears to enable the resolution of a high affinity binding site equally as well as a decrease in buffer pH, although the mechanism is not understood (Dorn, 1989; Morinelli *et al.*, 1990a). In the second case, the IC<sub>50</sub> value for [<sup>125</sup>I]-BOP ( $\approx$  49 nM) is over three times greater than the EC<sub>50</sub> value for I-BOP (16 nM). Such a difference in this case may be explained by the presence of a TP receptor reserve, in which case less than 50% of the binding sites need be occupied to elicit 50% of maximum response.

#### 4.1.4. Summary

In summary, I have presented the first in-depth characterization of TP receptors in human myometrium from nonpregnant donors.

In this report, I presented evidence for an excitatory receptor in human nonpregnant myometrium at which U-46,619 and I-BOP are potent agonists. I have shown that contractile activity induced by U-46,619 can be blocked by selective TP receptor

antagonists and I have shown that both agonist and antagonist potencies are consistent with those found in human platelets. I have documented the first evidence to suggest that the sensitivity of human nonpregnant myometrium to U-46,619 stimulation is not influenced by anatomical location, tissue orientation or menstrual cycle status of the donor. Lastly, I have demonstrated for the first time, displaceable binding of the radiolabeled TP receptor agonist [<sup>125</sup>I]-BOP in this tissue and determined that the affinity of [<sup>125</sup>I]-BOP for the myometrial TP receptor is similar to that reported for the putative low affinity site on human platelets.

Taken together, these data support the notion that a single homogeneous TP receptor population resides in human nonpregnant myometrium. Comparing the functional and binding data I gathered to that reported for human platelets and human vascular smooth muscle suggests that the myometrial TP receptor population most closely resembles the putative low affinity TP receptor population found in human platelets.

That my results demonstrate the potent inhibitory action of currently available TP receptor antagonists on TP receptor-induced myometrial activity suggests that selective blockade of the myometrial TP receptor be tested as an alternative pharmacotherapy for PD. However, by no means does this work exclude the possible contribution of one or more additional prostanoid receptors in the etiology of PD. I chose to focus on the TP receptor based on a number of suggestive observations implicating the TP receptor as an etiological factor in PD. Only following a thorough characterization of myometrial DP, EP, FP and IP receptors, will we be able to gain a more complete understanding of the complex pharmacology that may mediate PD.

## 4.2. ANALYSIS OF TP RECEPTOR mRNA EXPRESSION

The second objective of my thesis was to extend my functional investigation of potential mechanisms of TP receptor-mediated response variability. As the direct quantification of TP receptor protein is currently not feasible, I chose to investigate TP receptor mRNA expression. Based on the current lack of scientific tools, other than pharmacological, I developed a method by which TP receptor mRNA could be quantified. I then examined whether regional differences exist with respect to TP receptor mRNA expression and whether TP receptor mRNA expression changes throughout the course of the menstrual cycle.

As will be detailed below, I first developed a qualitative reverse transcription-polymerase chain reaction assay (RT-PCR), with which I conclusively demonstrated the expression of TP receptor mRNA in human nonpregnant myometrial tissue. Secondly, I developed a semi-quantitative RT-PCR assay with which the relative amount of TP receptor mRNA in a given RNA preparation was estimated relative to that of the housekeeping gene glyceraldehyde-3-phosphate-dehydrogenase. However, I was unable to determine whether TP receptor mRNA is differentially expressed in myometrial tissue excised from different uterine locations or from donors in either the proliferative or secretory phase of the menstrual cycle due to the lack of reproducibility associated with the semi-quantitative amplification of TP receptor mRNA.

### 4.2.1. Rationale for Study Design

Kinsella *et al.* (1994), suggested that the cellular responses to TxA<sub>2</sub> are mediated by both phosphorylation of the TP receptor by different protein kinases and by regulated gene expression. Whether these or other mechanisms play a role in pathophysiologies suggested to involve the TP receptor such as PD, renal disease and a wide variety of cardiovascular diseases (Davis-Bruno and Halushka, 1994), have yet to be established. Swanson *et al.* (1992), attempted to investigate whether the expression of the TP receptor changes during different reproductive states in women. As I have previously stated, their autoradiographic assessment of TP receptor expression was inconclusive due to methodological inconsistencies. Via *in situ* hybridization, this group did identify TP receptor mRNA expression in human myometrial smooth muscle cells. However, only three proliferative phase tissues were examined. That steroids may play a role in the expression of the TP receptor is suggested by Matsuda *et al.* (1993), who demonstrated that testosterone, but not estrogen significantly increased the density without any significant change in the affinity of TP receptors expressed on HEL cells. The potential effects of progesterone were not investigated nor was the possibility that the effect of steroids on TP receptor expression may be dependent on tissue type (Matsuda *et al.*, 1993). The mechanism by which the increase in TP receptor density occurred was also not investigated. A direct effect of testosterone on the TP receptor gene is unlikely based on the absence of a steroid response element [other than a glucocorticoid response element], in either of the two promoter regions 5' to the coding sequence (Nüsing *et al.*, 1993). More likely then, if



steroids exert transcriptional control, they function through intermediate transcription factors.

I chose to develop an assay with which TP receptor mRNA expression could be quantified. With this technique, I could determine whether anatomical location or menstrual status of the donor played a role in the regulation of the TP receptor gene. Although I had demonstrated pharmacologically that neither anatomical location nor menstrual status of the donor influenced the sensitivity of human nonpregnant myometrium to TP receptor stimulation or altered TP receptor density, limitations in both *in vitro* contractility and binding assays left the possibility open that changes in TP receptor density may occur. Of course, firm conclusions regarding protein expression based on mRNA levels could not be made from the investigations I planned as there are many regulatory steps in the pathway leading from DNA to protein (Derman *et al.*, 1981; Darnell, 1982). However, based on the lack of available immunological tools with which to study TP receptor protein expression, I felt the development of a *molecular* technique would be a valuable tool with which to study the TP receptor and at the very least, would lend valuable insight into potential regulatory factors responsible for TP receptor expression.

Of the resources available to me, Northern blotting, ribonuclease protection, *in situ* hybridization and reverse transcription-polymerase chain reaction (RT-PCR), were all techniques which I could have optimized to quantify TP receptor mRNA expression. I chose RT-PCR for the following reasons. Northern blotting compared to RT-PCR requires considerably more starting RNA (Malucelli *et al.*, 1996). I conducted a number of preliminary Northern blots and determined that a minimum of 20  $\mu\text{g}$  of total RNA was

required to visualize TP receptor mRNA (data not shown). As I was unable to obtain more than 0.5 g of myometrial tissue from different excision sites, I was unable to isolate sufficient quantities of total RNA to accommodate Northern blotting. In addition, I assumed that the increased sensitivity of RT-PCR compared to both Northern blotting and ribonuclease protection (Flick and Anson, 1995), would enhance my ability to quantify the TP receptor message. *In situ* hybridization can be used with small samples, but at the time I started this work, RT-PCR was perceived by some as a more accurate method of quantification (Murphy *et al.*, 1990) and also RT-PCR was less labor intensive, thus facilitating the investigation of a larger number of samples.

Once decided on RT-PCR, the next decision was how to accommodate quantification. Many strategies have been developed for performing semi-quantitative and quantitative RT-PCR and each has its own advantages and disadvantages (Morrison and Gannon, 1994; Xiao *et al.*, 1996). I specifically set out to devise an inexpensive, rapid, high throughput and non-radioactive methodology. Thus I chose to model the semi-quantitative technique first described by Chelly *et al.* (1988), in which an estimate of the relative amount of a message (TP receptor mRNA in my case), in various RNA preparations can be made using a known housekeeping gene as an internal control. The housekeeping gene I chose was glyceraldehyde-3-phosphate-dehydrogenase (G3PDH), as it is one of the most frequently used internal markers for gene expression (Murphy *et al.*, 1990; Takamiyagi *et al.*, 1996; Santagati *et al.*, 1997). As for the visualization of resulting amplification products, I chose to directly quantify photographic images from ethidium bromide stained agarose gels. This technique has been widely reported to possess sufficient

sensitivity to accurately assess PCR product accumulation (Santagati *et al.*, 1993; Ferré *et al.*, 1994; Malucelli *et al.*, 1996; Takamiyagi *et al.*, 1996)

#### **4.2.2. Development of the Semi-Quantitative RT-PCR Assay: Step 1 - Amplification of TP Receptor mRNA**

As the first step to developing semi-quantitative RT-PCR, I had to demonstrate the amplification of TP receptor mRNA (Senchyna *et al.*, 1995; Senchyna and Crankshaw, 1995). As depicted in Figures 8 and 9, the identity of the TP receptor mRNA amplification product was established by (1) the size of the fragment stained by ethidium bromide; (2) by restriction endonuclease digestion; (3) by its specific hybridization in stringent conditions with a TP receptor RNA probe. By using primers annealing to two different exons, I precluded any artefactual amplification of contaminating DNA. This was also excluded by control experiments including performing the RT reaction in the absence of reverse transcriptase enzyme and the PCR of human genomic DNA. Although I was able to confirm the presence of myometrial TP receptor mRNA, I was unable to conclude that the TP receptor amplification product was derived *entirely* from myometrial smooth muscle cell RNA. Swanson *et al.* (1992), have demonstrated via *in situ* hybridization, the presence of TP receptor mRNA in myometrial stromal cells, smooth muscle cells and blood vessels. The appearance of a second non-specific amplification product, approximately 60 bp smaller than the TP receptor mRNA amplification product was noted on approximately 30% of ethidium bromide stained agarose gels. Neither the identity of this product nor the cause for its inconsistent appearance were determined, although contamination by cloned plasmid cDNA was ruled out again through negative controls. That this product represents a prostanoid

receptor mRNA other than the TP receptor is *possible* as amino acid sequence comparison of all currently recognized prostanoid receptors shows 28% to 45% sequence identity between any two members (Boie *et al.*, 1995). As the transmembrane spanning domains appear to represent the areas of highest sequence homology (Boie *et al.*, 1995), I purposely designed the TP receptor mRNA primers to fall between any two such domains. However, the possibility that uncharacterized prostanoid receptor sequences are being amplified cannot be discounted. That this unidentified amplification product appears to be the same size as the cloned TP receptor cDNA used in this study is interesting. This cDNA was originally made by RT-PCR of human placental poly(A<sup>+</sup>)RNA using the same primers as I employed. Sequence analysis of the PCR amplified cDNA revealed the 60 bp deletion (Abramovitz, personal communication, 1992), however "why" the deletion in the sequence exists was not determined. The size of the unknown fragment ( $\approx$  739 bp), indicates that it does not represent the alternatively spliced TP receptor transcript identified by Raychowdhury *et al.* (1994; 1995). D'Angelo *et al.* (1994), reported the presence of a partial TP receptor cDNA in a human lung cDNA library. The antisense primer I used shares identical sequence compatibility with this partial clone, but it is not known whether the sense primer also shared a complementary sequence. Obviously, further work is required to determine the identity and possible physiological significance of this amplification product. Furthermore, that this sequence may have affected the efficiency of amplification of TP receptor mRNA will be discussed below.

### 4.2.3. Amplification of Alternatively Spliced TP Receptor mRNA

I demonstrated, using a second set of TP receptor primers that the alternatively spliced TP receptor mRNA is also expressed in myometrial tissue (Figure 10) (Senchyna and Crankshaw, 1995). Although it is tempting to speculate based on the relative intensities of the two resolved amplification products, that the alternatively spliced TP receptor mRNA (282 bp) is present in much higher starting concentrations compared to TP receptor mRNA (941 bp), the more likely explanation for the different product yields is different amplification efficiency associated with the two messages. Chelly *et al.* (1990), have demonstrated using PCR, that the yield of amplification is inversely proportional to the length of the amplified product. Furthermore, Chelly *et al.* (1990), concluded that it is impossible to directly compare by PCR amplification, the abundance of different transcripts without calculating the amplification efficiency for each analysed sequence. Thus an accurate statement regarding the relative abundance of the two TP receptor mRNA transcripts in human myometrium cannot be made from the data I collected. Previous to the description by Raychowdhury *et al.* (1994; 1995), of this alternatively spliced TP receptor transcript, seemingly conclusive molecular evidence supported TP receptor homogeneity in man (Hirata *et al.*, 1991; Nüsing *et al.*, 1993). However, the failure to detect an alternatively spliced transcript in early investigations may be attributed to the fact that Northern analysis was insufficiently sensitive to differentiate between the two transcripts and/or primers designed for PCR fell before the point where the two messages diverge (Raychowdhury *et al.*, 1994). Recently, Hirata *et al.* (1996), demonstrated that both isoforms exist in human platelets.

Unfortunately, this study failed to determine whether they represented the two forms of functional TP receptors identified by pharmacology in human platelets. Thus although the capacity of the isoforms to subserve distinct biological functions is illustrated by differential coupling of the expressed isoforms to adenylyl cyclase in COS cells (Hirata *et al.*, 1996), the functional significance of this observation and whether or not the alternatively spliced TP receptor protein is expressed under physiological conditions remain unknown.

**4.2.4. Development of the Semi-Quantitative RT-PCR Assay:  
Step 2 - Evaluation of the Sensitivity and Linearity of the  
Scanning / Densitometric Method by Which PCR  
Amplification Products Would be Analyzed**

The scanning/densitometric analysis of the Polaroid film photographed directly from the ethidium bromide stained product bands correlated well with the amount of product loaded on the gel within a narrow range. Through the optimization of camera and scanner settings, the maximum variation in integrated density values observed for each point was 10% or less, when constrained to this linear range (Figures 11 and 23). Outside the linear range, variability of up to 30% in integrated density values was noted for the same product resolved on different gels. Variation of this magnitude has been reported and is considered *acceptable* for semi-quantitative studies (Siripurkpong *et al.*, 1997). Unfortunately, a broader perspective of "acceptable" variability could not be gained as very few publications demonstrate calibrations or determinations of limits of detection. As the most accurate integrated density results are obtained when products are resolved at a concentration that resides within the linear range (Santagati *et al.*, 1997), I established a standardized loading

volume for both TP receptor and G3PDH mRNA amplification products. Although reproducible, the sensitivity of the measurements was less than 100%, as a doubling in the amount of PCR product loaded on the gel resulted in only a 60% to 75% increase in integrated density. The significance of this lack of sensitivity will be considered in the interpretation of the kinetic data discussed below.

#### **4.2.5. Development of the Semi-Quantitative RT-PCR Assay: Step 3 - Determining the Kinetics of TP Receptor and G3PDH mRNA Amplification**

Relative or absolute comparisons of gene expression via PCR is only valid if the PCR is sampled during the exponential phase of amplification (Morrison and Gannon, 1994). Thus I assessed the kinetics of amplification of both TP receptor and G3PDH mRNA. For reasons that I cannot explain, the assessment of TP receptor mRNA amplification product accumulation was very difficult. That methodology was responsible for my inability to generate time course data for TP receptor mRNA amplification was discounted based on my ability to establish exponential product accumulation for G3PDH mRNA. It has been reported that ethidium bromide staining is not sensitive enough to quantify small amounts of DNA produced early in the exponential phase of the PCR process (Santagati *et al.*, 1993; Siddiqi *et al.*, 1996; Xiao *et al.*, 1996). Therefore, I employed Southern hybridization in an attempt to increase the sensitivity of my detection system. However, as demonstrated in Figure 12, Southern hybridization only confirmed the lack of TP receptor mRNA product accumulation prior to 30 cycles of PCR. I was able to establish an estimate of the kinetics

of TP receptor mRNA amplification by employing a “truncated” time courses methodology. Although there were differences in the range in which individual RNA preparations exhibited exponential kinetics, a common overlap was 35 cycles. The only *explanation* I formed from these experiments was that interrupting the thermocycler prior to 29 cycles of PCR resulted in the inability to generate PCR product. Unfortunately, at this time, I cannot support this explanation with any published literature.

I studied the amplification of G3PDH mRNA in RNA preparations derived from myometrium excised from each of the sites under investigation and also from donors in either the proliferative or secretory phase of the menstrual cycle, as it has been suggested that the expression of G3PDH may vary within organisms depending on the stage of proliferation and differentiation (Barbu and Dautry, 1989; Takamiyagi *et al.*, 1996). My results suggest however, that G3PDH mRNA expression in human nonpregnant myometrium is invariant.

From the kinetic data I gathered, I was able to estimate the efficiency of the PCR reactions. Theoretically, if the repetitive yield of the PCR cycle is 100%, than amplification of the target DNA sequence (A) can be described by  $2^n(A)$ , where n represents the number of PCR cycles (Chelly *et al.*, 1990). However, the efficiency of each PCR under my conditions was  $25\% \pm 7\%$  for TP receptor cDNA and  $31\% \pm 4\%$  for G3PDH cDNA. Such low efficiency may partially be explained based on the results of Chelly *et al.* (1990), who demonstrated using differential primer design that efficiency is roughly inversely proportional to the distance separating two primers on their analyzed template. The largest fragment studied in the work by Chelly was 564 bp, which amplified with an estimated efficiency of



35%. Thus amplification of a much smaller fragment of the TP receptor and G3PDH cDNAs may have improved the efficiency of the reactions and overall product yield. Whether or not this would have aided in the determination of TP receptor mRNA amplification kinetics remains to be tested. The low sensitivity of the detection system employed in my experiments may also have contributed to the calculation of low efficiency. As mentioned in Section 4.2.4., a doubling in product results in only a 60% to 75% increase in the integrated density of the resolved band. Thus, the values I plotted to determine the linear range of amplification and reaction efficiency were most likely underestimates of the true extent of product accumulation. It has been suggested that to obtain a more accurate description of reaction kinetics, a more sensitive method of detection such as phosphorimaging or autoradiography, be employed (Siddiqi *et al.*, 1996; Xiao *et al.*, 1996).

#### **4.2.6. Development of the Semi-Quantitative RT-PCR Assay: Step 4 - Attempt to Co-Amplify TP Receptor and G3PDH mRNA**

The last step in the development of this semi-quantitative RT-PCR assay was to demonstrate co-amplification of TP receptor and G3PDH mRNA under conditions that favored exponential amplification for both messages. In this regard, G3PDH serves as an internal control to which tube-to-tube variation and inaccuracies in RNA measurement and aliquoting can be normalized (Chelly *et al.*, 1988; Morrison and Gannon, 1994). I did not display these data in my results, as shall be outlined below, I was unable to achieve this task. I first attempted to add the G3PDH primers to the reaction after seven cycles of PCR. However, as previously discussed, this "pause" in the thermocycler resulted in the failure to

generate TP receptor mRNA amplification product. Thus, I opted to decrease the concentration of G3PDH primers so that both TP receptor and G3PDH primers could be added simultaneously. Again, I was unable to generate TP receptor mRNA amplification product. It has been demonstrated previously that co-amplification of target and standard within the same reaction tube results in either premature attenuation of the exponential phase of the PCR reaction, or an "all-or-none" pattern of product accumulation similar to what I described in Figure 12 (Murphy *et al.*, 1990; Delp *et al.*, 1997). Thus, based on my failure to co-amplify TP receptor and G3PDH mRNA, I chose to amplify the messages in separate tubes within the same experiment, as has been previously described for semi-quantitative methods employing a variety of housekeeping genes (Murphy *et al.*, 1990; Iriyoshi *et al.*, 1996). It should be noted that although two of the eight time courses I performed to assess the kinetics of G3PDH mRNA product accumulation plateaued before 29 cycles, I amplified G3PDH mRNA for 29 cycles in the semi-quantitative studies. Again, this was necessitated based on the apparent "sensitivity" of TP receptor mRNA PCR product accumulation to interruptions during the amplification process. It is well recognized that following the onset of plateau, similar amounts of amplified product are obtained regardless of the abundance of initial transcript concentration (Chelly *et al.*, 1990; Morrison and Gannon, 1994). Therefore, the possibility that G3PDH was amplified outside the exponential range combined with my inability to co-amplify G3PDH and TP receptor mRNA may have significantly decreased the value of G3PDH as an indicator of tube-to-tube and RNA loading variability.

#### 4.2.7. Evaluation of the Semi-Quantitative RT-PCR Data and the Method Itself

With the semi-quantitative RT-PCR methodology developed, I performed a number of experiments to investigate TP receptor mRNA expression in different uterine locations and during different phases of the menstrual cycle. The results from these experiments represent the first report of this kind and suggest that TP receptor gene expression is invariant in human nonpregnant myometrium. These results were not entirely expected based on the data gathered on TP receptor mRNA amplification kinetics. In performing the TP receptor mRNA amplification time courses, I noted that the cycle in which TP receptor mRNA amplification product was first visible varied among the different RNA preparations. One interpretation of these data is that samples where PCR product appears at earlier cycle numbers contain more TP receptor mRNA than samples where the PCR product appears later (Bishop *et al.*, 1997). Individual semi-quantitative experiments also suggested that TP receptor mRNA may be differentially expressed according to either tissue excision site or donor menstrual status. Precluding the possibility of finding any significant difference however, was the extraordinarily high variability associated with my data over replicate experiments. Based on this finding of high variability, I focused my attention on determining its source(s). Two points became clear. Firstly, global fluctuations in product yield were common from one experiment to the next, with TP receptor mRNA amplification products displaying over ten-fold greater differences compared to G3PDH amplicates. Varying product yields among different RT-PCR experiments have previously been reported and have been attributed to a multitude of possible factors such as differences in cDNA synthesis

and/or PCR efficiency and/or in PCR quantification (Bishop *et al.*, 1997). Secondly, comparisons between individual RT-PCR, RT or PCR experiments all demonstrated the lack of ability to reproduce data. This finding has also been previously reported and in fact was a common criticism of early attempts at quantitative RT-PCR (Gilliland *et al.*, 1990; Clementi *et al.*, 1993; Bishop *et al.*, 1997). However, the literature also contains overwhelming evidence in favor of the reproducibility of quantitative RT-PCR data based on recent improvements in PCR instrumentation and methodology (Chelly *et al.*, 1990; Santagati *et al.*, 1993; Kuehnelt *et al.*, 1994; Bishop *et al.*, 1997). Specifically, "second-generation", block-type thermocyclers are reported to provide excellent well-to-well temperature uniformity and faster ramping times in comparison to open-air, first generation thermocyclers such as I used (Katz *et al.*, 1993; Kuehnelt *et al.*, 1993). In terms of methodology, several lines of evidence have suggested that competitive PCR (and competitive RT-PCR) is the most reliable approach for nucleic acid quantification (Wang *et al.*, 1989; Gilliland *et al.*, 1990; Clementi *et al.*, 1993; Santagati *et al.*, 1993; 1997). The strategy of this method involves co-amplification of a competitive template of known concentration that uses the same primers as the target template so that the yield of the amplification products depends only on the relative initial amounts of the competitor and the cDNA of interest as any variable affecting amplification has the same effect on both sequences. One can only speculate that increased consistency in my data may have occurred had I utilized these innovations. However, I did demonstrate reproducible results when only the G3PDH message was studied. Figure 24 shows that the amount of amplified G3PDH mRNA was proportional to the quantity of input total RNA during the exponential

phase of the amplification process when the initial starting concentration of RNA ranged from 0.5  $\mu\text{g}$  to 2.0  $\mu\text{g}$ . Furthermore, these results demonstrated that my technique is capable of detecting as little as two- to four-fold changes in gene expression. Figure 25 demonstrates the lack of proportionality between TP receptor mRNA amplification product and input RNA. In addition, varying the initial amount of starting RNA resulted in the differential appearance of a second PCR product, estimated as approximately 60 bp smaller than the TP receptor mRNA band. As mentioned above, this second product was visible by ethidium bromide staining in approximately 30% of my experiments. It appears from Figure 25, that the generation of this unknown product is partially dependent on the concentration of starting RNA and on the yield of TP receptor amplification product. Such results *resemble* the appearance of amplification products that would be obtained from the competitive PCR methodology described above, where the amount of each product that is amplified is dependent on the relative initial concentrations of the two templates (Wang *et al.*, 1989). That a similar competitive situation *inherently* exists in my studies is possible. In other words, a sequence similar to TP receptor mRNA may be present in total RNA preparations, but in varying amounts. Based on the relative abundance of TP receptor mRNA and this additional sequence, either one or both sequences appear as amplification products. The inconstant nature of this competition has been described in the literature (Murphy *et al.*, 1990; Morrison and Gannon, 1994), and obviously has a significant impact on the ability to accurately assess the level of gene expression of the target of interest. It is important to note that this additional PCR template was not unique to myometrial total RNA, or as a result of my RNA isolation procedure, as the 739 bp amplification product was also seen in RT-PCR experiments in which the starting RNA was purchased from Clontech and derived

from human tissues other than myometrium (Figure 19). This survey of different human tissues suggested that TP receptor mRNA is equally expressed in human brain, heart, kidney, lung, placenta, skeletal muscle, small intestine and myometrium. However, as I mentioned previously, the contribution of RNA from individual cell types cannot be assessed using total RNA preparations derived from whole tissue sections. It might be expected that every tissue would test positive for TP receptor mRNA due to the presence of blood vessels.

Thus it appears from the discussion above that amplification of TP receptor mRNA largely represents the “unreproducible factor” in my experiments. This suggestion is further supported by additional work I conducted with this semi-quantitative technique in which amplification of EP<sub>3</sub> receptor mRNA varied in yield by no more than 50% from one experiment to the next (unpublished observation). Therefore, I propose that the method I developed is reproducible and can accurately detect changes in RNA expression greater than 2-fold. However, I would also propose that this method is not acceptable for the semi-quantification of all mRNA species. In other words, I do not consider this technique appropriate for what I intended; semi-quantification of TP receptor mRNA expression.

To improve this technique, I would in fact significantly change the methodology. In light of the evidence supporting the reproducibility and accuracy of competitive RT-PCR (cPCR), I would suggest that this method be tried. As a competitive template already exists in the form of the cloned TP receptor cDNA with a 60 bp deletion, much of the initial work required to initiate cPCR has been eliminated. However, I have doubts as to whether cPCR will be an appropriate method for the amplification and quantification of TP receptor

mRNA. Such skepticism is based on the observed differential appearance of TP receptor mRNA amplification product and “unknown” amplification product in my experiments where I varied the initial amount of input RNA (Figure 25). Such concentration-dependent variability may lead to the generation of erroneous results in cPCR experiments. As an alternative procedure, I suggest adopting a non-competitive methodology in which the techniques used by Murphy *et al* (1990) and Bishop *et al* (1997) are employed. Specifically, such a methodology would allow for the reliable determination of differences between samples as opposed to determining absolute concentration. The technique would involve constructing a time course for *both* TP receptor mRNA and G3PDH mRNA for *each* RNA sample to be investigated. One could then directly evaluate both the amount of TP receptor PCR product and the efficiency of the TP receptor amplification reaction from the exponential phase of the reaction. In addition, errors in RNA aliquoting could be normalized to G3PDH. However, contingent on any new methodology yielding reproducible data is the elimination of nonspecific amplification products. New primer design for TP receptor mRNA, producing an amplification product of no more than 300 bp may aid in this endeavor.

Lastly, the failure of this semi-quantitative technique poses an interesting problem in that *I am not sure why such difficulty arose in the amplification of TP receptor mRNA*. Since the PCR was devised, the majority of the work carried out on the polymerase chain reaction has been designed around the useful application of the reaction, largely in terms of quantification and clinical diagnostics (Morrison and Gannon, 1994). It might be fair to say that many researches like myself have trusted with blind faith the ability of the PCR to generate meaningful quantitative data if a few obligatory steps are followed. That we really

don't understand why the PCR reaction plateaus or how the presence of competitor sequences affect overall product yield (Morrison and Gannon, 1994), suggests that quantitative RT-PCR results may not be as accurate or meaningful as we would like to believe. Perhaps until a greater understanding of the features which limit the RT-PCR reaction are gained a number of questions and methodological inconsistencies will remain unanswered.

#### **4.2.8. Summary**

In summary, I have developed a novel qualitative RT-PCR methodology with which to identify TP receptor mRNA. Using this qualitative assay, I have demonstrated that TP receptor mRNA is expressed in human nonpregnant myometrial tissue excised from different uterine locations, from donors in either the proliferative or secretory phase of the menstrual cycle. These results further support the presence of TP receptors in human nonpregnant myometrium. I have also developed a novel qualitative RT-PCR methodology to amplify alternatively spliced TP receptor mRNA. In so doing, I have provided the first documented evidence to support the presence of alternatively spliced TP receptor mRNA in human nonpregnant myometrial tissue. I established the basis for a semi-quantitative RT-PCR assay with which potential influences on TP receptor mRNA expression can be evaluated and began to examine whether tissue excision site or donor menstrual status regulates TP receptor gene expression. Lastly, I have demonstrated that the semi-quantitative RT-PCR data describing the amplification of TP receptor mRNA is significantly more variable than



data describing the amplification of G3PDH mRNA and thus propose that the factor(s) responsible for the variable amplification of TP receptor mRNA be further explored.

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