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THE ROLE OF PROSTAGLANDIN ENDOPEROXIDE H SYNTHASE IN THE INITIATION AND MAINTENANCE OF TERM AND PRETERM LABOR IN HUMANS.

BY

Jane Elizabeth Kondejewski

A thesis submitted to the Faculty of Graduate Studies and Research as a requirement for the degree of Doctor of Philosophy.

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ABSTRACT

Prostaglandins (PG) play an important role in the initiation and maintenance of labor in women. The fetal membranes and decidua are a major source of PG at term and during labor. Since PG endoperoxide H synthase (PGHS) catalyzes the rate-limiting step of PG synthesis, we studied the changes in PGHS specific activity, and determined the levels and localization of PGHS-1 and PGHS-2 mRNA during gestation and with term and preterm labor in the amnion, chorion and decidua. PGHS specific activity and PGHS-2 mRNA abundance showed a significant increase ($P<0.05$) in association with term labor in the fetal membranes, but not decidua. In addition, in the amnion and chorion, but not decidua, there was a significant rise in PGHS enzyme activity at term before the onset of labor; correlation analyses indicated this was likely due to an increase in the functional expression of the PGHS-2 isoenzyme. Furthermore, at this time there was an association between PGHS-2 mRNA levels in the amnion and chorion and fetal fibronectin (fFn) concentrations in the cervico-vaginal fluids; fFn is a biochemical marker predictive of labor onset. These data are indicative that PGHS-2 induction in the fetal membranes has a role in labor initiation and in the maintenance of the labor process at term. At preterm labor there was an increase in PGHS enzyme activity in amnion and chorion and a rise in the abundance of both PGHS-1 and PGHS-2 mRNAs. The induction of PGHS in the fetal membranes at term labor occurred in both the epithelial and the mesenchymal cells, whereas at preterm labor only the mesenchymal tissue components expressed PGHS. Our data suggest inhibition of PGHS may be an effective therapeutic measure for the management of preterm labor, however, as we also documented the presence of both PGHS-1 and PGHS-2 in a variety of fetal tissues in the third trimester and with labor such treatment may have potentially detrimental effects on fetal well-being. This research provides new information concerning the mechanisms responsible for the initiation and maintenance of parturition in humans.

For my family

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LIST OF ABBREVIATIONS

ACTH	adrenocorticotropin
ANOVA	analysis of variance
AVP	arginine vasopressin
b.p.	boiling point
cDNA	complementary deoxyribonucleic acid
cRNA	complementary ribonucleic acid
CRH	corticotropin-releasing hormone
CS	cesarean section
DEPC	diethylpyrocarbonate
DEX	dexamethasone
DDC:DTC	dithiocarbamic acid
DHEA/DHEAS	dihydroepiandrosterone/sulfate
DIG	digoxigenin
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EP	prostaglandin E receptor
ER	estrogen receptor
FP	prostaglandin F receptor
GAPDH	glyceraldehyde-phosphate dehydrogenase
GSH	reduced glutathione
HETE	hydroxyeicosatetraenoic acid
11βHSD	11β-hydroxysteroid dehydrogenase
IC_{50}	half maximal inhibition
IR	immunoreactive
kb	kilobase
kDa	kilodalton
K_m	Michaelis-Menten constant
LPS	lipopolysaccharide
LT	leukotriene
mRNA	messenger ribonucleic acid
NSAIDS	nonsteroidal anti-inflammatory drugs
OA	okadaic acid
OT	oxytocin

PBS	phosphate buffered saline
PG	prostaglandin
PGDH	15-hydroxyprostaglandin dehydrogenase
PGEM	13,14-dihydro,15-keto PGE
PGFM	13,14-dihydro,15-keto $PGF_{2\alpha}$
PGHS	prostaglandin endoperoxide H synthase
PGI	prostacyclin
pH	negative log of hydrogen ion concentration
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PP	protein phosphatase
PR	progesterone receptor
PRE	progesterone response element
PVN	paraventricular nucleus
r	coefficient of correlation
RIA	radioimmunoassay
RU486	17β-hydroxy-11β-(4-methylaminophenol)-17α-(prop-1-ynyl) estra-4,9-diene-3-one
SD	standard deviation
SE;SEM	standard error of the mean
SL	spontaneous labor
tRNA	transfer RNA
TPA	12-*O*-tetradecanoylphorbol-13-acetate
Trp	tryptophan
TXA_2	thromboxane
V_{max}	maximal velocity
v/v; vol/vol	volume per volume

1. BACKGROUND

1.1. Introduction

Parturition is the process of giving birth. It is a highly orchestrated sequence of events resulting in the birth of a baby and the regeneration of normal (non-pregnant) cyclic uterine physiology. Parturition has four distinct but overlapping phases (Casey and MacDonald, 1993). Phase 0 comprises about the first 95% of pregnancy when the uterus is maintained in a state of relative quiescence. In phase 1 there are multiple changes in uterine physiology which collectively bring about specific alterations in preparation for labor. This is the period of uterine "activation" or "awakening". There is altered function and activity of ion channels, and increases in the expression of contraction-associated proteins (CAPs) including connexin-43 (Cx-43), the major protein comprising gap junctions between myocytes, and receptors for oxytocin (OT) and prostaglandins (PGs). The cervix undergoes the process of softening and effacement. Effective labor is only possible after the uterine modifications of phase 1 are complete. Phase 2 is the time of myometrial stimulation. The uterus can now be stimulated by the action of uterotonins, amongst which OT and PGs are believed to have a predominant role. Phase 3 follows delivery of the fetus, placenta and fetal membranes and results in uterine involution.

The transition of the uterine myometrium from the relative quiescent state of pregnancy to the contractile state of labor is the essence of the control of parturition. The purpose of this thesis is to examine the role of a group of locally produced factors called prostaglandins in relation to the events associated with the onset of human labor. Specifically, we will investigate the involvement of PGs in the initiation of human parturition.

1.2. Prostaglandin Synthesis and Metabolism

1.2.1. Prostaglandin Synthesis: An Overview

Primary PGs and related eicosanoids are formed from the 20 carbon polyunsaturated fatty acid, 5,8,11,14-cis eicosatetraenoic acid (arachidonic acid) in three sequential steps:

1) The mobilization of arachidonic acid from cellular phospholipids. Free arachidonic acid is liberated through the activities of one or more isoenzymes of phospholipase C or of forms of phospholipase A_2.

2) The sequential conversion of liberated arachidonic acid to the PG endoperoxides, PGG_2 and PGH_2, respectively. Formation of PGH_2 is catalyzed by the enzyme prostaglandin endoperoxide synthase (PGHS) which is rate-limiting in the regulation of PG formation in a number of systems. Two forms of PGHS have now been identified, cloned and characterized.

3) Isomerization or reduction of PGH_2 to the biologically active eicosanoids. Most PG-forming cells produce only one type of eicosanoid because of the predominance of a single PGH_2 metabolizing enzyme. PG formation occurs through the action of a set of PG isomerases that produce PGs of the D, E and I series, or via a two electron reduction of PGH_2 to produce $PGF_{2\alpha}$ (for a review see Funk, 1993).

The levels of biologically active PGs are regulated by the rate of metabolism. The initial step in PG metabolism is catalyzed by a 42 kD cytosolic enzyme 15-hydroxyprostaglandin dehydrogenase (PGDH). Two isoforms of this enzyme have been identified, type I which is NAD+ requiring and type II which is NADP+ requiring. PGDH catalyzes oxidation of the 15-OH of PGs of the E and F series. This results in the formation of 15-keto metabolites. Once formed, the 15-keto PGs are acted on by a γ-reductase enzyme to yield the 13,14-dihydro-15 keto PG

metabolites which are decreased significantly in their ability to elicit physiological responses.

Metabolism of arachidonic acid may also occur through one of at least four lipoxygenase pathways. These include 5-lipoxygenase, leukocyte-type 12-lipoxygenase, platelet-type 12-lipoxygenase and 15-lipoxygenase. Five-lipoxygenase activity results in the formation of 5-H(P)ETE; this can then be converted to leukotriene A_4 (LTA_4), which in turn may be hydrolyzed to LTB_4 or LTC_4. Arachidonic acid is converted through 12- and 15-lipoxygenase activities to 12-H(P)ETE and 15-H(P)ETE, respectively.

1.2.2. Phospholipases

The initiation of PG synthesis begins with the liberation of arachidonic acid from cellular sources by the action of specific acyl hydrolases or phospholipases. The two major species of phospholipases involved in PG generation are phospholipase A_2 (PLA_2) and phospholipase C (PLC) (for reviews see Bleasdale and Johnston, 1985; Olson and Zakar, 1993).

PLA_2 catalyses the hydrolysis of the *sn*-2- ester bonds of glycerophospholipids resulting in the formation of free fatty acids and lysophospholipids. It has preferential substrate specificity for phosphatidylethanolamine, phosphatidylcholine, and plasmalogens. Arachidonic acid is esterified preferentially in the *sn*-2 position of phospholipids, therefore PLA_2 action often results in the release of free arachidonate. There are two categories of PLA_2: extracellular low molecular weight PLA_2 (secreted PLA_2) classified into four types (I-IV) on the basis of their structural characteristics and intracellular high molecular weight cytosolic PLA_2 ($cPLA_2$) (Rice, 1995). Although $cPLA_2$ is isolated from the cytosol, it can translocate to membrane substrate in the presence submicromolar

calcium (Ca^{2+}). There is substantial evidence that hormonally induced eicosanoid production is mediated through a $cPLA_2$ with selectivity for arachidonyl phospholipids (Clark *et al.*, 1991; Dennis, 1994).

PLC is involved in the indirect release of arachidonic acid and catalyses the hydrolysis of the phosphodiester bond between diacyglycerol and the phosphate moiety of the head group of phospholipids. At the present time, at least 10 distinct PLC isoforms have been cloned and sequenced in mammalian tissues, including four PLC-β isoforms, two PLC-γ isoforms and four PLC-δ isoforms (Rhee and Choi, 1992). PLC preferentially hydrolyzes phosphatidylinositol resulting in the release of diacylglycerols and inositol phosphates. Diacylglycerol is then further metabolized through the sequential action of diacyl- and monoacylglycerol lipases to yield glycerol and free fatty acids, including arachidonic acid. Diacylglycerol may also function to activate PLA_2 via the phosphorylation of lipocortins (inhibitors of PLA_2 activity) or mobilization of Ca^{2+} in response to 1,4,5-inositol trisphosphate. The net result of both pathways is an increase in the availability of arachidonic acid for further processing.

1.2.3. Metabolism of Arachidonic Acid

Once arachidonic acid is released, it can be acted on by prostaglandin endoperoxide H synthase (PGHS) to produce the intermediate prostaglandins PGG_2 and PGH_2.

To date, two isoforms of PGHS have been identified, PGHS-1 and PGHS-2. These are homodimeric, heme-containing, glycosylated proteins which catalyze both a cyclooxygenase reaction in which arachidonate is converted to PGG_2, and a peroxidase reaction in which PGG_2 undergoes a two-electron reduction to PGH_2 (for a review see Smith and DeWitt, 1996).

1.2.3.1. PGHS isoenzymes

Primary structures of PGHS isoenzymes

PGHS-1 was originally purified from ovine and bovine vesicular glands in the mid-1970s. Complementary DNAs encoding murine, human, rat and ovine PGHS-1 have now all been cloned. In 1989 Simmons *et al.* identified a second inducible form of PGHS, known as PGHS-2. This isoform was independently identified by differential screening of a phorbol ester-stimulated Swiss-3T3 fibroblast cDNA library and by many other groups (reviewed by Smith *et al.*, 1996). Human PGHS-2 has subsequently been cloned and expressed.

The overall length of the 2 isoenzymes is almost identical, varying from 599 (human) to 602 (mice) amino acids for PGHS-1, and 603 (mice) to 604 (human) amino acids for PGHS-2 (reviewed by Xie *et al.*, 1992). For a given PGHS isoform there is approximately 90% (81 to 98%) identity between species, but there is only 59 to 62% homology between the PGHSs at the nucleic acid and amino acid levels. The major differences are that PGHS-1 contains a sequence of 17 amino acids in its amino terminus that is not present in PGHS-2, whereas PGHS-2 has an additional insert of 18 amino acids in its carboxy terminus. However, the amino acid residues thought to be important for catalysis are conserved (DeWitt *et al.*, 1993), and the two isoforms have about the same affinity (K_m) and capacity (V_{max}) to convert arachidonic acid to PGH_2 (Percival *et al.*, 1994). It is possible the subtle differences in primary amino acid sequence may cause differences in the secondary and tertiary structure of the protein that could affect the active site of the two enzymes. For example, after aspirin treatment PGHS-1 is completely inhibited, whereas PGHS-2 converts arachidonic acid to 15-*R*-HETE functioning as a 15-lipoxygenase (Capdevila *et al.*, 1995; Lecomte *et al.*, 1994); PGHS-2 is also able to metabolize C18 and C20:3 fatty acids as substrates while PGHS-1 is mainly restricted to arachidonic acid (C20:4) (Meade *et*

al., 1993): and, PGHS-1 and PGHS-2 have different pharmacological profiles in response to various nonsteroidal anti-inflammatory drugs.

Inhibition of PGHS-1 and PGHS-2 by nonsteroidal anti-inflammatory drugs:

The cyclooxygenase activities of both PGHS-1 and PGHS-2 can be inhibited by most NSAIDs, which compete with arachidonate for binding to the cyclooxygenase active site; in general, NSAIDs do not affect peroxidase activity. There are significant functional differences among these drugs in the manner with which they interact with the cyclooxygenase site after binding to this site. Accordingly, it is convenient to group NSAIDs that inhibit cyclooxygenase activity into classes I-III. Class I inhibitors (e.g., ibuprofen, mefenamic acid) are typical reversible competitive cyclooxygenase inhibitors. Class II inhibitors (e.g., indomethacin, flurbiprofen and meclofenamate) exhibit time-dependent reversible inhibition. Binding of these drugs to PGHSs yields an initial EI complex typical of a reversible competitive inhibitor, but this EI complex slowly (seconds to minutes) rearranges to an EI* complex from which the drug dissociates very slowly (minutes to hours). Class III inhibitors (aspirin, valerylsalicylate) convert EI to an EI* complex by covalent modification (acylation) of the protein; once an EI* complex is formed, it is not possible for the protein to revert to EI. Thus, a cell treated with a class III inhibitor must synthesize new PGHS to regain cyclooxygenase activity (for a review see Smith and DeWitt, 1995).

All currently available NSAIDs inhibit both PGHS-1 and PGHS-2, these compounds are effective anti-inflammatory agents, but they are also ulcerogenic. Many pharmaceutical companies have developed new cyclooxygenase inhibitors that selectively inhibit PGHS-2 driven by the notions that:

1) PGHS-2 is the relevant enzyme in inflammation

2) PGHS-1, but not PGHS-2, is responsible for the production of PGs that mediate "housekeeping" functions such as regulation of renal H_2O and Na^+ metabolism, stomach acid secretion and hemostasis.

PGHS-2 inhibitors have been reported to be anti-inflammatory and analgesic and to lack gastrointestinal toxicity (Seibert *et al.*, 1994). These include DuP697, SC52125, L-745-337, NS398, and meloxicam. These agents are all relatively poor inhibitors of PGHS-1 but are time-dependent, reversible inhibitors of PGHS-2 (for a review see Smith and DeWitt, 1995). The selectivity of these agents for PGHS-2 is probably due to the one amino-acid difference between PGHS-1 and PGHS-2 within the hydrophobic cyclooxygenase channel. PGHS-2 has a valine at position 509, while the corresponding amino acid in PGHS-1 is an isoleucine (Gierse *et al.*, 1996; Guo *et al.*, 1996).

Regulation

PGHS-1 and PGHS-2 are encoded by separate genes located on different chromosomes. PGHS-1 is composed of 11 exons and 10 introns spanning 22.5 kb. The human PGHS-1 locus maps to chromosome 9 (Kraemer *et al.*, 1992). By comparison, the human PGHS-2 gene is only 8.3 kb in size. This reduction in size is primarily due to smaller introns. Most of the exons are conserved, with the exception of the absence of exon 2 in PGHS-2. Fluorescence *in situ* hybridization localized human PGHS-2 to chromosome 1 (Kosaka *et al.*, 1994). It is of interest to note that one of the phospholipase genes (PLA2 G4) is in extremely close proximity to the PGHS-2 gene on chromosome 1 (MacPhee *et al.*, 1995), the close proximity of PGHS-2 and PLA2 may have some biological relevance. The small size of the PGHS-2 gene is consistent with its characterization as an immediate-early gene (Herschman, 1991). The transcripts of PGHS-1 and PGHS-2 differ significantly in

size and are of 2.8-3.6 kb and 4.0-4.1 kb. respectively (Xie *et al.*, 1992; Smith and DeWitt, 1996; Herschman, 1996).

The single best characterized distinction between PGHS-1 and PGHS-2 is their differential regulation of expression. PGHS-1 can be detected in most tissues although not within all cells of a tissue (Smith and DeWitt, 1996). In cultured cells PGHS-1 is typically expressed at constant levels throughout the cell cycle (DeWitt and Meade, 1993). PGHS-2 is expressed only in response to cytokines, growth factors or tumor promoters (Herschman, 1996). Hence, PGHS-1 has become known as the *constitutive* isoenzyme, and PGHS-2 is known as the *inducible* isoform. Constitutive PGHS-1 expression suggests that cells use PGHS-1 to produce PGs involved in the rapid response to stimulation by circulating hormones. Because of the time lag required for PGHS-2 induction in a cell or tissues, this enzyme is available to produce PGs after initiation of specific physiological events such as inflammation, mitogenesis and ovulation. However, this classification is actually an oversimplification; PGHS-2 is expressed constitutively in brain (Yamagata *et al.*, 1993), testes (Simmons *et al.*, 1991), tracheal epithelia (Walenga *et al.*, 1996) and the macula densa in kidney (Harris *et al.*, 1994); while PGHS-1 levels change during development as in ovine vasculature (Brannon *et al.*, 1994), and its expression can be down-regulated in endothelial cells in response to acidic fibroblast growth factor (Hla and Maciag, 1991) and up-regulated in mast-cells treated with stem cell factor plus dexamethasone (Samet *et al.*, 1995).

The structures of the promoters of the two PGHS genes are predictive of their mode of regulation. PGHS-1 has no TATA box (Kraemer *et al.*, 1992), a promoter element commonly lacking in constitutively expressed housekeeping genes. Virtually nothing is known about the details of regulation of PGHS-1 gene expression, although, recently Hansen *et al.* (1998 b) characterized the 5'-upstream region of the PGHS-1 promoter in human amnion WISH cells. They identified a putative

transcription start site, Sp-1 sites and a peroxisome proliferator activated receptor half site, as well as a GC box. The physiological significance of these findings is presently unknown.

The PGHS-2 promoter, contains a TATA box and PGHS-2 transcription is linked to multiple signaling pathways involving protein kinases A and C, tyrosine kinases, phosphatases, and *src* : and those pleiotropic pathways activated by growth factors (PDGF, EGF), bacterial endotoxin (lipopolysaccharide) and inflammatory cytokines. Experiments with reporter plasmids containing the PGHS-2 upstream 5'-flanking sequences have identified several specific regulatory DNA sequences that can activate PGHS-2 gene transcription. For example, the control elements necessary for activation of the mouse PGHS-2 gene by PDGF and serum are located within the first 371 nucleotides upstream of the mouse PGHS-2 transcription start site (Fletcher *et al.*, 1992). An NF-IL-6/C/ECβ regulatory element in the rat promoter can mediate, at least partially, the increased PGHS-2 gene transcription in rat follicles following exposure to follicle-stimulating hormone, luteinizing hormone, or forskolin (Sirois *et al.*, 1992; Pall *et al.*, 1997). And, functional cAMP response elements (CRE) have been identified in the mouse and human promoters. The location and identity of a number of *cis*-acting DNA elements that are conserved between the mouse, rat and human include NF-KB, SP1 and ETS elements, but these have yet to be tested for functionality (for reviews see Williams and DuBois, 1996; Herschman, 1996, Smith and DeWitt, 1996).

There have been reports of splice variants being produced from the PGHS-1 primary transcript as a result of TGF-β1, IL-1β, TNF-α, phorbol esters and serum stimulation (Diaz *et al.*, 1992). There is also evidence for alternative splicing of the PGHS-2 transcript. Xie *et al.* (1991) observed that in nonproliferating chick fibroblasts expressing the potent PGHS-2 inducer temperature-sensitive v-*src*, the predominant PGHS-2 mRNA possessed an unspliced intron that prohibited

translation. Upon transformation and mitogenic stimulation, the majority of the PGHS-2 mRNA produced was of the completely spliced derivative and was translated into PGHS-2 protein. Additional work characterizing the tissue prevalence of this alternative transcript or the machinery involved in the differential splicing has not been reported.

Although the primary level of regulation of PGHS-2 synthesis appears to be transcriptional, post-transcriptional regulation of PGHS-2 may also occur. PGHS-2 mRNA is unstable compared to PGHS-1 mRNA, a feature that probably results from the multiple Shaw Kamens sequences (ATTA) present in the 3'-untranslated region (UTR). This motif is present in many immediate early genes and is thought to be involved in modulating the rate of mRNA degradation (Kosaka *et al.*, 1994). Interestingly, these sequences are conserved between murine and human PGHS-2 genes suggesting an important role for their presence.

Factors that increase or decrease the half-life of PGHS-2 mRNA can also presumably increase or decrease the efficiency of its turnover during translation. IL-1 appears to regulate PGHS-2 by this mechanism: in the human endothelial cell line ECV204, IL-1 not only increases PGHS-2 gene transcription, but also PGHS-2 mRNA stability (Ristimaki *et al.*, 1994). In many cells dexamethasone inhibits transcription of PGHS-2 (DeWitt and Meade, 1993) and reduces PGHS-2 mRNA stability (Evett *et al.*, 1993) although the biochemical mechanism is not known.

PGHS-2 expression may also be directly regulated at the level of translation. Dexamethasone only partially inhibits transcription and PGHS-2 mRNA accumulation in serum-stimulated fibroblasts (DeWitt and Meade, 1993) and in IL-1 stimulated mesangial cells (Rzymkiewicz *et al.*, 1994), but dexamethasone completely inhibits PGHS-2 protein expression. The presence of PGHS-2 mRNA in these cells with no protein synthesis suggests that dexamethasone can act directly to inhibit translation of PGHS-2 mRNA.

Localization

The tissue distribution of PGHS-1 and PGHS-2 differs between species and can be heterogeneous within the same tissue. For example, PGHS-2 is the major isoform in rat and mouse brain (Seibert *et al.*, 1994; Breder and Saper, 1996; Yamagata *et al.*, 1993), whereas similar levels of both isoforms have been detected in human brain (O'Neill and Ford-Hutchinson, 1993). Both isoforms have been detected in the stomach, with PGHS-1 being the most abundant in mouse and rat (Seibert *et al.*, 1994, 1995), whereas PGHS-2 is expressed to a similar extent as PGHS-1 in human tissue (O'Neill and Ford-Hutchinson, 1993). In the rat stomach, PGHS-2 was found in the surface mucous cells, while PGHS-1 was found in mucous neck cells (Iseki, 1995). Similarly, heterogeneous distribution was observed in the kidney, with PGHS-2 localized in the macula densa and possibly contributing to the regulation of salt, volume and blood pressure homeostasis (Seibert *et al.*, 1994; Smith and Wilkin, 1977; Harris *et al.*, 1994; Jensen and Kurtz, 1997).

In addition, PGHS isoenzymes also partition at the cellular level since PGHS-1 functions primarily in the endoplasmic reticulum, whereas PGHS-2 activity is located both in the endoplasmic reticulum and in the nuclear envelope (Morita *et al.*, 1995). Furthermore, PGHS-2 immunoreactivity was twice as concentrated in the nuclear envelope as in the endoplasmic reticulum. This compartmentalization suggests that PGHS isoenzymes may represent two temporally and spatially separated prostanoid biosynthetic systems (Smith and DeWitt, 1996). PGHS-1 generates products that end up outside the cell and function via G protein linked receptors; this is also true for the subset of PGHS-2 molecules found in the ER. However, the nuclear activity of PGHS-2 leads to the prediction that there is a unique nuclear prostanoid biosynthetic system that forms products that act through nucleoplasmic or nuclear membrane targets in association with cell differentiative or replicative events.

For example, peroxisomal proliferator-activated receptor γ has been shown to be activated by PGJ_2 (Forman *et al.*, 1995). PGJ_2 metabolites also activate the unfolded protein response element (Odani *et al.*, 1996). Identification of other nuclear targets for other prostanoids is expected.

Finally, the hypothesis of a functional compartmentalization of PGHS isoenzymes is further supported by their ability to metabolize arachidonic acid from different pools (reviewed by Herschman, 1996). For example, mast cells stimulated with IgE show a biphasic production of PGD_2, possibly related to different eicosanoid synthetic pathways (Murakami *et al.*, 1994; Kawata *et al.*, 1995):

1) An early PGHS-1-mediated pathway using arachidonate released by soluble PLA_2;

2) A late PGHS-2 mediated pathway, using arachidonate released by cytosolic PLA_2.

Although this hypothesis has not been confirmed in other cell types it suggests the possibility that there is a transcellular pathway of PG biosynthesis involving circulating soluble PLA_2 as an arachidonate-generating system for cellular PGHS-1.

PGHS knockout mice

The compartmentalization of PGHS isoenzymes is an attempt to explain their respective roles at a cellular level, while gene disruption techniques may allow an understanding of their biological relevance.

Mice homozygous for PGHS-1 deficiency survived well, had no gastric pathology, and showed either less indomethacin-induced gastric ulceration and/or a smaller ulcerated area of the stomach surface than mice with the wild type PGHS-1. In the ear odema model, there was reduced platelet aggregation and decreased inflammatory response to arachidonic acid, but not to phorbol ester stimulation (Langenbach *et al.*, 1995). Studies with PGHS-1 null mice demonstrated that neither

male nor female fertility was affected: but that a complete lack of PGHS-1 as occurs in homozygous by homozygous matings exhibited a markedly prolonged gestation when compared to wild type, and decreased pup survival perinatally by 100%. These data indicate labor was impaired by this mating. Pregnancies of PGHS-1 null females and PGHS-1 wild type or heterozygous males demonstrated an equally prolonged gestation indicating a necessity for maternal production of PGHS-1 in labor initiation. Transfer of PGHS-1 knockout or wild type blastocysts to pseudopregnant wild type foster mothers resulted in equivalent gestational length suggesting fetal PGHS-1 activity is not essential. Induction of PGHS-1 mRNA in endometrium and a rise in uterine $PGF_{2\alpha}$ concentration was demonstrated at day 16.5 of gestation, with a fall to non-gravid levels just prior to delivery (term=19.6 days) (Gross *et al.*, 1998). The regulatory cascade culminating in parturition in the mouse is initiated by regression of the corpus luteum and these data indicate that the essential role of PGHS-1 generated PGs in labor initiation in this species is the induction of luteolysis.

PGHS-2 null mice had a reduced lifespan and no innate gastrointestinal pathology, but had kidney abnormalities that caused a progressive deterioration as the animals aged. They had a normal inflammatory response to arachidonic acid and phorbol ester stimulation (Morham *et al.*, 1995; Dinchuk *et al.*, 1995). PGHS-2 has been implicated in the ovulation process (Sirois *et al.*, 1992); and PGHS-2 knockouts had multiple female reproductive failures including a virtual absence of corpora lutea, and impaired fertilization, implantation and decidualization (Lim *et al.*, 1997). They have not been studied in relation to gestational length.

1.3. Parturition

1.3.1. Role of the Fetus in the Initiation of Labor

For centuries a critical question with regard to the mechanisms that regulate the onset of parturition is "Does the signal originate within the mother or the fetus?" The concept that the fetus is aware of the proper time to initiate its own delivery dates from 460 BC. The Greek philosopher, Hippocrates, suggested that the fetus could sense when the placenta was failing to keep up with its nutritional needs and when the time was opportune to exit the uterus. Two and a half millennia later, as a result of experiments with sheep, Sir Joseph Barcroft concluded that the fetus monitors its own oxygen supply. When oxygen becomes deficient, mechanisms are activated to initiate parturition. Both ideas are similar in suggesting that fetal awareness of a deficiency of essential substances produces the need to be born. These views introduced the theory that the signal for birth originates at the level of the fetal brain (Swaab *et al.*, 1976; Thorburn, 1994).

In the 16th century the Italian anatomist, Fabricius ab Aquapendente, proposed that the chief agent of parturition was the muscular action of the uterus. The importance of the link between the maturation of the fetal hypothalamic-pituitary-adrenal axis and the initiation of labor can now be appreciated, at least in some species.

1.3.2. Initiation of Labor in the Sheep

In 1963 a congenital cyclopian-type malformation associated with prolonged pregnancy was identified in fetuses of range sheep in Idaho. This teratogenic effect was a consequence of maternal ingestion of skunk cabbage (*Veratrum californicum*) on the 14th day of gestation. In these animals gestation lasted 200-250 days, longer than normal term of 150 days. The affected lambs had pituitary glands, but the neural connections with the hypothalamus were either missing or abnormal.

In 1965 Liggins developed a technique for ablating the pituitary gland of fetal sheep. When more than 70% of the pituitary gland was destroyed, pregnancy was prolonged. These fetuses had hypoplasia of the adrenal gland and retarded somatic development. Similar results were seen when the fetal hypothalamus was destroyed or the fetal pituitary stalk sectioned. Conversely, stimulating the fetal adrenal by infusion of adrenocorticotropin (ACTH) or direct administration of a glucocorticoid hormone into the fetal lamb *in utero* led to preterm delivery within a predictable number of days. The timing of parturition was unaltered by maternal hypophysectomy or by the administration of ACTH or synthetic glucocorticoids to the mother at similar doses and times in pregnancy (Liggins *et al.*, 1973).

In sheep, therefore, it is likely that the initial signal for parturition originates in the fetal hypothalamus. In the adult of most species, the hypothalamic paraventricular nucleus (PVN) secretes corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) which regulates anterior pituitary corticotrope biosynthesis and ACTH release, which itself regulates adrenocortical glucocorticoid production. *In situ* hybridization methods demonstrated messenger RNA (mRNA) for both CRH and AVP in the fetal PVN increased significantly between days 105 and 128 of gestation (Brieu *et al.*, 1989; Brooks *et al.*, 1989). Bilateral stereotaxically-placed lesions of the fetal ovine hypothalamic PVN prolonged gestation significantly and abolished the preparturient fetal plasma ACTH and cortisol rise. This method also showed that at day 130 the PVN signaled the onset of the expression (via ACTH) of fetal adrenal P_{450} steroid hydroxylase enzymes necessary for *de novo* cortisol synthesis (McDonald and Nathanielsz, 1991; Gluckman *et al.*, 1991). It is these signals for the activation of the fetal adrenal gland that can be considered the first fundamental step in the initiation of the endocrine cascade of ovine parturition.

Cortisol from the fetal adrenal then brings about changes in maternal plasma hormone levels which lead to uterine activation and stimulation. In the sheep,

parturition is preceded by a large decrease in progesterone and a sharp rise in estrogens in the maternal blood. These steroids are synthesized by the placenta throughout the majority of gestation. Mean fetal plasma cortisol levels are highest during the final 2-3 days of intrauterine life. At these levels cortisol induces placental 17α-hydroxylase ($P450_{C17}$) synthesis which converts pregnenalone to 17α-hydroxypregnenalone, an estrogen substrate. Therefore, maternal plasma progesterone levels decrease 1-2 days before labor onset and estrogen levels sharply increase in the 36 hours leading up to labor. This promotes uterine activation, increased synthesis of uterotonic contractile agents such as $PGF_{2\alpha}$, and results in myometrial contractions. These changes are induced prematurely in ewes where *in vivo* administration of ACTH or synthetic glucocorticoid to the fetus results in premature parturition (for a review see Challis and Lye, 1994).

Interestingly, cortisol not only effects placental enzyme synthesis but also acts to induce systems in the fetal lung that bring about maturation of this organ. In this way, cortisol in the sheep coordinates fetal lung maturation with the timing of birth.

1.3.3. Initiation of Labor in the Human

Evidence for the role of the fetus in the initiation of parturition in the human is more circumspect. In fact, for obvious ethical reasons, much of our knowledge concerning the physiology of human parturition is often from purely anecdotal sources. Because of this, it is often pertinent to refer to studies using animal models. While this is recognized as a necessity, its physiological significance is not always clear, therefore, in these discussions it will be kept to a minimum.

1.3.3.1. Observations on the fetal control of birth in the human

In 1933 an English obstetrician, Malpas, reported a series of cases of prolonged pregnancy in humans associated with fetal anencephaly. He observed that

the front portions of the fetal brain failed to develop and concluded the timing of the onset of labor is determined by the fetus. He suggested that the fetal adrenal, pituitary and nervous system, in combination, trigger the neuro-muscular mechanisms effecting labor.

Further observations on the effects of anencephaly on gestational length in humans have been carried out. Where a large number of patients have been monitored, the mean length of gestation of the anencephalic group was not found to be significantly different from that of the control population. However, in the anencephalic group there was a much wider variation around the mean; the second and third stages of labor were significantly longer, and manual extraction of the placenta was more frequent (Swaab *et al.*, 1976). These latter two observations may be due to an influence of fetal neurohypophyseal peptides on myometrial contractility and the duration of labor, that is absent in the anencephalic group. These observations suggest the human fetus plays a role different from its ovine counterpart in determining the timing of labor onset and its maintenance thereafter.

In sharp contrast to the fetal sheep, exogenous glucocorticoids fail to induce parturition in both monkeys and humans except in some patients already classified as postterm. The amounts of glucocorticoids given intra-amniotically to such patients are large and their effect is inconsistent. In addition, dexamethasone administration results in a decrease of maternal estrogen levels (Liggins *et al.*, 1976). In humans as in the sheep, increasing maternal estrogen concentrations are implicated in uterine activation and uterotonin synthesis. These data suggest fetal cortisol is unlikely to serve as the physiological trigger to parturition in humans. However, closer analysis of more recent research may yet reveal an essential role for fetal glucocorticoids in these processes.

1.3.3.2. The human fetal adrenal gland

It is established that the human fetal adrenal is involved in parturition through the synthesis and secretion of steroids such as the C_{19} estrogen precursors, dehydroepiandrosterone (DHEA), and its sulfated form, DHEAS. The placenta is the major site of steroid production after the sixth to ninth week of human pregnancy. The primate placenta cannot synthesize estrogens, *de novo*, from acetate as it lacks the 17α-hydroxylase/C17-20 lyase enzymatic system responsible for this. However, it is capable of converting C_{19} steroids into estrogen. In human and nonhuman primates maternal plasma concentrations of unconjugated estrogens rise progressively with time of gestation reaching peak levels at term. Evidence suggests this estrogen production is closely related to the secretion of DHEAS from the fetal adrenal gland (Pepe and Albrecht, 1990a, b).

In the rhesus monkey spontaneous delivery is preceded by rising concentrations of DHEAS in fetal blood, but not maternal blood or amniotic fluid. Furthermore, *in vivo* data support the concept of rapid fetal adrenal maturation before birth; and fetectomy in rhesus monkeys and baboons (Valenzuela *et al.*, 1993), fetal decapitation, fetal death or suppression of the fetal adrenocortical axis with glucocorticoids in rhesus monkeys (Novy and Haluska, 1988) causes peripheral maternal estrogen levels to fall and a significant increase in gestational length. Conversely, an increase in the production of fetal adrenal androgens and consequently maternal estrogens similar to that seen at spontaneous term labor (184 days) has also been found in pregnant baboons at 155-165 days of gestation treated to cause fetal hypoxic stress which would normally result in preterm birth at this time (Shepherd *et al.*, 1992). Furthermore, circulating maternal estrogens rise after administration of ACTH or DHEAS to the fetus, and administration of androstenedione to the rhesus monkey fetus causes a switch in myometrial activity from contractures to contractions and an increase in circulating maternal estrogen levels (Figueroa *et al.*, 1989),

inducing premature labor (Mecenas *et al.*, 1996). In humans it is also likely that steroidal precursors for estrogen production arise in the fetus as pregnancy with an anencephalic fetus is associated with subnormal levels of urinary estrogen, estriol secretion and fetal adrenal hypoplasia; and low estriol secretion is also observed after fetal death and after suppression of the fetal adrenocortical axis with synthetic glucocorticoid (Challis and Lye, 1994). While DHEAS and androstenedione administered to the fetus cause increases in uterine contractile activity, these results cannot be replicated with estrogen administration in the non-human primate. This may be due to serum binding or metabolism of the estrogen, or it may reflect a local, placental effect of estrogen formed from the androgen which cannot be replicated by exogenous estrogen administration. Alternatively, androgen may have its own intrinsic effect upon uterine contraction. More investigation must be performed to resolve this discrepancy.

The fetal adrenal gland of the human shows functional specialization. There is an outer adult-type definitive zone or neocortex, a transitional zone, and an inner fetal cortical zone which comprises between 80 and 90% of the gland during the majority of gestation. The fetal zone exhibits a period of rapid growth from day 150 of gestation. It secretes primarily the C_{19} steroid precursors of estrogen in increasing quantities throughout gestation in accordance with its increasing mass. The adult cortex secretes primarily aldosterone, and the transitional zone produces cortisol and is responsible for the increase in fetal plasma cortisol levels which occurs in late gestation (Mesiano and Jaffe, 1997). At mid to late gestation fetal adrenal size and weight in anencephalic human fetuses are considerably reduced when compared with normal. This suggests factors of fetal pituitary origin are important to fetal adrenal growth at this time. These include ACTH and other proopiomelanocortin-derived peptides (POMC) as well as fetal and placental growth factors. Anterior pituitary

POMC expression is regulated by the synthesis of neuropeptide releasing factors such as CRH and AVP (for a review see Pepe and Albrecht, 1990b).

In human fetuses CRH- and AVP-like immunoreactivities were found to be present in hypothalamic tissue by 12 weeks, and the concentration of bioactive CRH rose between 12 and 27 weeks (Ackland *et al.*, 1986). There is also evidence for the production of placental CRH and POMC-derived peptides (Riley and Challis, 1991). Studies show amniotic fluid and maternal and fetal plasma immunoreactive CRH (IR-CRH) rise steadily from mid second trimester to 35 weeks, after which time they increase rapidly to term (ca. 40 weeks) (Sasaki *et al.*, 1990; Wolfe *et al.*, 1988). These increasing concentrations correlate with increasing IR-CRH levels in placental extracts. Furthermore, in the last few weeks of gestation the levels of CRH-binding protein decrease thereby causing a further increase in free CRH (Linton *et al.*, 1992). Although a topic of controversy, some studies indicate glucocorticoids increase placental CRH gene expression and mRNA levels (Jones *et al.*, 1989; Robinson *et al.*, 1988); and that CRH enhances PG production by fetal membranes, the myometrium and the placenta and exerts a priming and potentiating effect on the myometrial contractile response to OT (Jones and Challis, 1990a, b; Quartero and Fry, 1989). Based on these observations it is possible that late in gestation when the human fetal adrenal adult cortex has matured and begins to secrete cortisol, the increased glucocorticoid concentrations could then act to stimulate placental CRH expression. This would initiate an intraplacental positive cascade that stimulates PG production by paracrine/autocrine interactions and modulates the sensitivity of the myometrium to contractile effectors. Once concentrations of CRH in amniotic fluid, maternal and fetal plasma increase, they may further drive the pituitary-adrenal axis in the fetus and possibly the mother. When this process has been initiated, feedback control must be diminished to allow propagation of this set of signals to stimulate the onset and maintenance of labor. The enzymes, 11β-hydroxysteroid dehydrogenase (11β-HSD)

type 1 and 2, catalyze the oxidation and reduction of cortisol and cortisone, respectively, and are present in the placenta and fetal membranes of humans throughout gestation. *In vitro* data using cultured placental syncytiotrophoblast suggests placental 11β-HSD type 2 is involved in a local positive feedback of glucocorticoids on CRH and PGE_2 release by inactivating cortisol to cortisone (Sun *et al.*, 1998). Alternatively, OT and PGs maintain the intraplacental cascade; OT stimulates human placental CRH release, and PGs stimulate ACTH and cortisol release from the pituitary-adrenal axis of fetal sheep (Jones and Challis, 1990a, b; Quartero and Fry, 1989; Petraglia *et al.*, 1989). In this case the timing of the maturation of the fetal adrenal adult definitive zone plays a critical role in signaling the onset of parturition in primates. Moreover, placental CRH output is increased with preterm labor (Warren *et al.*, 1992) and worsening metabolic acidosis (Nodwell *et al.*, 1998) further implying fetal adaptive processes are playing a role in the endocrine events of labor. The failure of exogenous glucocorticoid administration to induce premature labor in primates might be due to the fact that treatments were given too early in gestation when negative feedback control was still in operation. This together with the resultant decrease in estrogen precursors would simply serve to prolong gestation.

While this is an attractive theory explaining the control of the onset of parturition in primates it should be noted that several studies have failed to find a stimulatory effect of glucocorticoids on CRH and PG production form the human placenta and myometrium (Siler-Khodr *et al.*, 1997; Kang and Siler-Khodr, 1998); and, preliminary data indicate there is a decrease in the expression of the CRH receptor type 1 in myometrium during pregnancy and in association with term labor (Rodriguez-Linares *et al.*, 1998).

1.3.3.3. Primate fetal adrenal-pituitary maturation

Evidence suggests the primate fetus has a role in the timing of the maturation of its own hypothalamic-pituitary-adrenal axis. During the majority of intrauterine life the primate fetus has limited capacity to produce cortisol. The fetus obtains this hormone by transplacental transfer from the mother. At midgestation 100% of fetal serum cortisol is derived from the mother while at term less than 50% originates from hormone produced by the maternal adrenal gland. Placental cortisol metabolism controlled by 11β-HSD changes with advancing gestation from reduction at midgestation to oxidation near term. The increase in placental cortisol oxidation is regulated by estrogen, the production of which increases to term.

Pepe and Albrecht (1990a, b) have proposed the following model for primate fetal pituitary-adrenal maturation. At midgestation cortisol is the principal corticosteroid arriving within the fetal circulation; fetal pituitary activity and thus ACTH release is suppressed; this limits the growth of the adult definitive zone of the fetal adrenal including development of the enzyme systems for producing glucocorticoids. The growth of the fetal cortex and consequent DHEA and DHEAS production continues under the influence of fetal and placental growth factors. With advancing gestation, under the influence of increasing estrogen dictated by increasing fetal adrenal C_{19} precursors, transplacental metabolism is changed, and the oxidation of cortisol to cortisone is increased. Concentrations of maternal cortisol in the fetal circulation drop, resulting in increased production and release of ACTH of fetal pituitary origin, maturation of the fetal adrenal adult definitive zone and de novo cortisol production.

In this way the primate fetus, initially through secretion of estrogen precursors by the adrenal gland and then ACTH and cortisol from the pituitary-adrenal axis, has the potential to exert a greater control over the timing of its own birth than was previously thought. It is noteworthy that in the human, as in the sheep, cortisol also

acts to induce systems to bring about maturation of many fetal organ systems, especially the lung. Furthermore, in primates fetal lung surfactant in the amniotic fluid can stimulate PG output from the amnion and is therefore another way of linking fetal lung maturity to a trigger for labor onset (Ban *et al.*, 1986). While much of this scheme is still speculative and active investigation is continuing in this field, these data, nevertheless, support the contention that activation of the fetal hypothalamic-pituitary-adrenal axis is responsible for the initiation of parturition in primates as well as in sheep.

1.3.4. The Roles of Maternal Sex Steroids in the Maintenance and Termination of Pregnancy and the Initiation of Labor

1.2.4.1. The progesterone block theory

Ever since Csapo (1956, 1976) published his progesterone "block" theory, the role of progesterone in human parturition has been a topic of debate. Under progesterone dominance, the myometrium is characterized by its refractoriness to contract when stimulated by OT or $PGF_{2\alpha}$. This is the state of uterine quiescence that is maintained throughout pregnancy, but is lost at parturition and labor. A large number of studies have defined a reduction in the concentration of circulating progesterone as the point at which the initiation of parturition occurs in many species (Challis and Lye, 1994). In the rabbit, for instance, parturition is preceded by a decrease in the concentration of progesterone in maternal peripheral plasma and in the myometrium. Uterine quiescence can be removed by ovariectomy (progesterone withdrawal), a response that may be blocked by administration of exogenous progesterone (Csapo, 1956).

The nonpregnant sheep uterus conforms to the classic progesterone block theory of Csapo. Progesterone inhibits the sheep myometrium and blocks the action of OT and PGs. Furthermore, in the pregnant sheep there is a decrease in peripheral

progesterone 1-2 days before labor onset and a switch from progesterone to estrogen domination (for reviews see Challis *et al.*, 1985; Challis and Lye, 1994). However, it can be concluded from the above discussion that this is in response to fetal signals and thus is a part of the endocrine cascade leading to labor as Csapo originally proposed, not the trigger.

1.3.4.2. The estrogen: progesterone ratio in late pregnancy

Progesterone

There is essentially no evidence that systemic maternal progesterone withdrawal is a prerequisite for the initiation of primate parturition. The rhesus monkey plasma progesterone levels remain at midluteal phase concentrations during the second half of gestation, with either no change or a small increase in plasma progesterone at the onset of parturition (Walsh *et al.*, 1974). Ovariectomy can be performed during early pregnancy without inducing abortion, and, after fetectomy, the placenta is retained and the pregnancy continued without the development of normal uterine activity even though basal systemic progesterone concentrations drop significantly below control (intact) levels. In humans, maternal peripheral progesterone levels increase progressively towards term (Tulchinsky *et al.*, 1972).

Progesterone withdrawal may take place at a paracrine rather than an endocrine level involving the intrauterine tissues. (Mitchell *et al.*, 1987). Mitchell and Wong (1993) have shown that human amnion and chorion contain 17β,20α-hydroxysteroid dehydrogenase activity that is reversible, having the ability to oxidate and reduce C_{18} and C_{21} steroids. Its oxidate activity usually predominates, favoring the production of estrone and progesterone. They demonstrated, using explant experiments, an increase in its reductive activity with labor onset, which leads to a doubling of the relative formation of the five-times more biologically active estradiol and inactive 20α-dihydroprogesterone. This may result in a significant increase in the

estrogen/progesterone ratio of both the amnion and chorion. They suggested this produces a local withdrawal time of labor onset, which is not reflected in the plasma, but may be important in controlling uterine activity. However, determinations of amnion, chorion and decidua tissue levels of estrone, estradiol, and progesterone showed that no change in the absolute concentrations of steroids or in the estrogen/progesterone ratio occurred with labor onset. Hence, no evidence exists for actual systemic or local withdrawal of progesterone at term or with labor.

To test whether systemic or local progesterone withdrawal could result in the physiological changes associated with the onset of parturition, experiments using synthetic progesterone receptor antagonists and inhibitors of progesterone synthesis have been performed. RU486 (mifepristone) is a progesterone receptor antagonist effective in nearly all species so far studied. Haluska and Novy (1993) found that RU486 administered orally to rhesus monkeys in the mid-third trimester of pregnancy stimulated intense uterine activity, but did not produce the orderly sequence of contractile events observed during normal parturition. The contraction pattern was quantitatively different from the contractions observed in control animals either at night or during spontaneous labor. Although there was a dramatic increase in gap junctions in the myometrium and estrogen receptor in the myometrium and decidua, which was greater than that observed after spontaneous labor and parturition, amniotic fluid $PGF_{2\alpha}$, PGFM and PGEM levels increased 40 hours after the onset of increased uterine activity. In control animals that delivered at term, amniotic fluid PGs increased 24 to 48 hours before the prelabor increase in uterine activity. Cesarean section was performed 72 hours after the start of RU486 treatment because the cervix did not dilate, and there was evidence of fetal asphyxia.

In a set of equivalent studies, when RU486 was replaced with epostane, a 3β-hydroxysteroid-dehydrogenase blocker, there was an 80% to 90% decrease in maternal, fetal and amniotic fluid progesterone levels; cervical ripening occurred; a

pattern of uterine contractility identical to that seen at spontaneous term labor was observed; and vaginal delivery occurred within 48 hours. Substitution with exogenous progesterone prevented the epostane-induced delivery. However, epostane also caused a decrease in circulating estrogen and cortisol levels, and, as there is no such decrease in the latter or in systemic progesterone at spontaneous labor and delivery at term, these events cannot be interpreted to suggest the initiation of parturition is purely the result of a decrease in progesterone synthesis. Furthermore, it has not been possible to block normal parturition and extend pregnancy by the exogenous administration of progesterone in humans (Hendricks, 1970) or nonhuman primates (Haluska and Novy, 1993). In humans, RU486 and epostane are clinically used for termination of early and second-trimester pregnancies. However, when either of these agents is used alone, the frequency of complete abortion seldom exceeds 70% irrespective of the dose of drug used and the duration of treatment. Additionally, in early pregnancy there is an increased risk of heavy bleeding associated with expulsion. The outcome of treatment is quite different when RU486 is combined with PG therapy, which results in a 94% success rate (Bygdeman *et al.*, 1991)

Last, lower progesterone levels do not necessarily imply improved contractile activity. Human serum concentrations of progesterone in both the fetal cord and maternal vein have been found to be significantly lower in women with OT-resistant dystocia than in women in spontaneous normal labor and those with OT-induced labor and normal progression (Lofgren and Bachstrom, 1992).

In summary, chemical withdrawal of progesterone in primates increases uterine contractility. However, neither the contractile patterns nor other physiological events mimic those of normal labor at term, preterm labor, or labor induced with PGs. Therefore, the data do not indicate that a withdrawal of progesterone is the cause of the normal physiological initiation of term and preterm labor in women and

nonhuman primates. To suggest otherwise would require more experimentation. From these data, we must conclude that progesterone's role is the maintenance of pregnancy, and initiation of labor-like contractile activity occurs in its presence and action.

Estrogen

The concept of uterine activation, includes but is not limited to, changes in the resting membrane potential of the myometrial cell, an increase in myometrial cell actin and myosin concentrations, the formation of gap junctions between myometrial cells, the expression of receptors for OT and PGs on myometrial cells, and the enhancement of post-receptor coupling mechanisms (for a review see Challis and Lye, 1994). Most if not all the events of activation are considered to be a result of the action of estrogen, and there is evidence that maternal estrogens have a role in triggering parturition in primates.

In human and nonhuman primates, maternal plasma concentrations of unconjugated estrogens rise progressively during gestation, reaching peak levels at term whereas plasma progesterone level also increase linearly with increasing gestational age (Albrecht and Pepe, 1990). Therefore, an increase in the plasma estrogen to progesterone ratio does not occur during late gestation or at term, which is unlike the situation in several nonprimate species (Challis and Lye, 1994). However, saliva steroid levels reflect unbound unconjugated steroids, and there is a rise in the saliva estriol/progesterone ratio beginning approximately 3 weeks before the spontaneous onset of labor at term and an increased estriol/progesterone ratio in women with idiopathic preterm labor (Moran *et al.*, 1992). The mean saliva estriol/progesterone ratio has also been shown to increase more than 2 weeks before spontaneous labor at 42 weeks, with no increase occurring in those women whose labor was induced at 42 weeks.

Atkinson *et al.* (1992) reported unusually high estradiol concentrations in the maternal plasma of a rhesus monkey after day 100 of pregnancy, which delivered a nonviable premature infant on day 139 of pregnancy (term=168 days). Novy (1977) has used estradiol injections to provoke delivery of a monkey fetus after intrauterine death, and in human patients with placental sulfatase deficiency, estradiol is low, the pregnancy is prolonged, and there may be failure to respond to induction (Liggins *et al.*, 1977). In the baboon during the third trimester of pregnancy, plasma estradiol and progesterone surge nocturnally with the increase in estradiol after that of progesterone (Wilson *et al.*, 1991). During the last 10 to 12 days of pregnancy, there is a forward shift in the initiation of the nocturnal estradiol surge so that it precedes the progesterone surge. This forward shift results in a period of 3 to 5 hours every day when the ratio of estradiol to progesterone is elevated, which coincides with the beginning of nocturnal uterine activity and is postulated to be the signal for the initiation of parturition. Conversely, primate parturition is not delayed indefinitely in situations where maternal plasma estrogens are low such as fetectomy. The administration of estradiol benzoate to two pregnant monkeys at midgestation in a manner that replicated the normal prepartum increase in serum estradiol concentrations failed to induce premature delivery of the fetus (Weiss *et al.*, 1976).

It is apparent that estrogen formed within the placenta is involved in the regulation of progesterone formed by this tissue during primate pregnancy. Administration of the antiestrogen, MER-25, a receptor antagonist, to baboons in the last third of gestation was associated with a 50% decline in serum concentration and production rate of progesterone. These data suggest estrogen regulates the biosynthesis of placental progesterone (Pepe and Albrecht, 1990a, b).

Pepe and Albrecht (1990a, b) have shown that a regulatory system exists *in utero* at midgestation in which there is negative feedback control of placental estrogen on the secretion of the fetal adrenal DHEAS, possibly by attenuating responsivity to

trophic peptides (ACTH). During late gestation this effect is lost resulting in the increased fetal DHEAS and maternal estrogens seen at term. Presumably this does not result in a parallel increase in placental progesterone, as it is proposed that normally the placenta is maximally stimulated by estrogen. Therefore, these processes would lead to an increase in the estrogen:progesterone ratio at term. As placental estrogen formation is dependent on fetal androgen precursors from the adrenal gland, this process is ultimately directed by the fetus.

The role of steroid hormone receptors

A change in the estrogen:progesterone ratio may also occur at the receptor level. Classically, estrogen and progesterone exert their effects by binding first to a cytoplasmic macromolecule to form a receptor steroid complex. This complex undergoes translocation to specific nuclear sites where it stimulates RNA synthesis which is responsible for modifying cell growth and function. Thus it is the nuclear receptors that are biologically active. The cytoplasmic receptors are replenished by recycling of the receptor macromolecule and by de novo synthesis.

In rat intrauterine tissue, cytosol and nuclear estrogen receptor (ER) concentrations increase abruptly and are maximal at labor. Cytosol progesterone receptor (PR) concentrations increase throughout pregnancy until term, whereas nuclear PR concentrations fall prior to delivery, paralleling the changes in steroid concentrations at this time (Mahesh *et al.*, 1996). In human myometrium, low or absent levels of PR and ER have been shown throughout pregnancy. These low concentrations may be due to antagonism of receptor synthesis by high progesterone concentrations, however, the estrogen:progesterone ratio may also be important in this regard. In the lower myometrial segment of women at spontaneous labor or undergoing elective cesarean section without labor, ER and PR appeared equally distributed between the cytosol and the nucleus. No differences in ER were evident

between the two groups, but a decreased PR concentration was seen in the labored tissues (Giannopoulos and Tulchinsky, 1979). Estrogen and PR also exist in the fetal membranes and decidua. Estrogen receptor mRNA concentrations increase three- to four-fold around the time of labor onset, whereas PR mRNA remains unchanged (Mitchell *et al*., 1992). These data suggest that an altered action of estrogen relative to progesterone may be observed with the onset of labor in humans as a consequence of increased intrauterine tissue ER relative to PR. Furthermore, Casey and MacDonald (1993) have suggested a mechanism by which an endogenously produced, naturally occurring growth factor, TGF-β, may serve to regulate progesterone-responsive genes in an opposing manner to progesterone. Effectively, this could be equivalent to progesterone withdrawal. However, studies in rhesus monkeys using a type I-antiprogestin, ZK98229 (which interferes with the specific binding of PR to progesterone response elements [PRE]), showed that its effects on uterine contractility, amniotic fluid PG levels, and cervical ripening were indistinguishable from RU486 and so cannot be described as mimicking spontaneous term labor and delivery (Haluska and Novy, 1993).

The mRNAs for ER and PR are encoded by 8 exons. Several mRNA transcripts for PR varying from 1-11kb in size have been identified . Three isoforms of PR have been characterized at the mRNA and protein level, and the two most commonly studied are PRA and PRB (Kraus *et al*., 1993). PRA and PRB isoforms differ in their interactions with other proteins and coactivators of steroid receptors. The ratio of PRA and PRB is of interest as the two isoforms display distinct biological functions. For example, *in vitro* studies with HeLa cells show higher transcription from promoters containing a PRE by PRB compared to PRA. And, when the two isoforms are coexpressed together, PRA acts as a dominant negative inhibitor of PRB (Tung *et al*., 1993; Vegeto *et al*., 1993). The physiological significance of these two isoforms is not yet clear but, in the chick oviduct, relative

expression of the A and B isoforms is under seasonal and developmental control (Boyd and Spelsberg, 1979); rabbit uterine tissues express only PRB (Loosfelt *et al.*, 1984). In humans, a switch in PR isoforms may function to diminish the effects of progesterone. In support of this Haluska *et al.* (1996) have demonstrated a switch from the predominant PRB to PRA isoform in the rhesus monkey myometrium at the time of parturition .

Several ER variants have also been characterized. These ER variants are products of differential splicing events resulting in isoforms that are constitutively active dominant positive isoforms designated ER-Δ5 and ER-Δ7; or dominant negative isoforms which interfere with binding of the wild type ER (ER_{WT}) to target DNA (ER-Δ2 and ER-Δ3) (Lemieux and Fuquo, 1996; Pfeffer, 1996). Both ER isoforms have been identified in ovine myometrium (Wu *et al.*, 1998a), and in the rhesus monkey amnion, chorion, and myometrium (Wu *et al.*, 1998b). In the sheep, the expression of both variants was significantly increased in tissues collected following spontaneous term labor, and differentially regulated by estrogen and progesterone. However, as with the PR isoforms, the physiological significance of the ER variants is unknown. There may be regulation of cellular levels of dominant negative or positive isoforms of ER in the intrauterine tissues. During pregnancy, the dominant negative form of ER may predominate while a switch to the dominant positive form may occur to activate the uterus at parturition.

Finally, it is now becoming increasingly apparent that sex steroids may alter cell function in other ways than through classic receptor mechanisms. These include binding to the cell membrane thereby affecting its structure and fluidity and altering the interactions of many agonists-membrane receptor-second messenger systems. In this context a relaxation action of progestins on rat myometrium via a membrane effect which inhibits extracellular calcium influx by calcium channels is known to exist. Progesterone may act by modulating calcium channel opening (Mahesh *et al.*,

1996). Therefore, extrapolating data about steroid activity from receptor studies must be viewed with care considering the emergence of evidence suggesting non-receptor mediated effects.

1.3.4.3. Myometrial uterotonins

The increased sensitivity of the myometrium to contractile agents is determined by increased receptor numbers and enhanced receptor coupling to second-messenger systems (Challis and Lye, 1994). In sheep, the change in the steroid environment to estrogen dominance promotes the formation of receptors for OT and PGs. In women, estrogen treatment in late pregnancy increases oxytocin sensitivity by stimulating an increase in OT receptor concentrations. PGs can be identified as positive feedback agents in this cascade as they further enhance estrogen-induced expression of OT receptors (Fuchs, 1995).

In addition, estrogens stimulate endocrine and paracrine production of the OT peptide. The release and synthesis of OT in the hypothalamus is increased due to stimulation of OT gene expression by estrogen (Robinson *et al.*, 1976). Furthermore, in human amnion, chorion and decidua, estrogen stimulated a four-fold increase in OT mRNA *in vitro* which is an increase similar to that seen in tissues obtained around the time of spontaneous parturition relative to those obtained earlier in gestation. Progesterone had little effect on this response alone and inhibited the estrogen response in some experiments (Chibbar *et al.*, 1993; Lefebvre *et al.*, 1994; Mitchell *et al.*, 1992). Oxytocin stimulates phosphatidylinositol hydrolysis in myometrial cells resulting in increased inositol triphosphate generation which is a second messenger step leading to myometrial contraction. Diacylglycerol is also a product of this hydrolysis; the action of cellular lipases on diacylglycerol releases arachidonic acid, the substrate of PGs. Also, the release of intracellular Ca^{2+} initiated by phosphatidylinositol hydrolysis can cause further mobilization of arachidonic acid

from phospholipid stores as the phospholipases responsible for this reaction are Ca^{2+} dependent. Furthermore, OT has been shown to induce the production of the uterotonin, endothelin-I, in decidua. Comparable to all these stimulatory agents, endothelin-I induces Ca^{2+} influx into myometrial cells and is equipotent to OT in stimulating uterine contractions (Casey and MacDonald, 1993).

The production of PGs by human intrauterine tissues is also regulated hormonally. In general, estrogen stimulates PG production while progesterone can either antagonize or augment estrogen action (Abel and Baird, 1980). In culture, estrogen has been shown to stimulate the output of PGE_2 from decidual cell preparations obtained from patients at elective Cesarean section (Olson *et al.*, 1983). In the myometrium, dexamethasone decreased prostacyclin synthesis by 90%. Prostacyclin (PGI_2) causes myometrial relaxation, and the increase in fetal glucocorticoid production at term described previously may have a further role in decreasing PGI_2 synthesis and causing a switch to the production of stimulatory $PGF_{2\alpha}$ and PGE_2 (Russo-Marie, 1990).

Estradiol and progesterone have no effect on the expression of the two isoforms of PGHS, the enzyme catalyzing the rate limiting step of the conversion of arachidonic acid to PGE_2 in cultured amnion cells (Zakar *et al.*, 1995). The PG metabolizing enzyme PGDH is found in most intrauterine tissues, especially chorion. The activity of this enzyme is stimulated by progesterone (Van Meir, 1996; Patel *et al.*, 1998a). Therefore, under the progesterone domination of pregnancy, PGDH enzyme levels will be high, reducing the bioactivity of any PGs formed. Local withdrawal of progesterone at term may reduce PDGH activity thus increasing PG bioactivity. In support of this are the observations of women treated early in pregnancy with the progesterone receptor antagonist, RU486. Such treatment is associated with a dramatic decrease in decidual PGDH activity and increased spontaneous uterine contractility (Cheng *et al.*, 1993).

Progesterone withdrawal is also thought to be responsible for increased placental CRH release at parturition (Van Meir, 1996). *In vitro* studies of human cell cultures have shown that progesterone inhibits CRH secretion while progesterone antagonists stimulate its release. *In vivo* studies measure plasma CRH as an index of increased placental output. However, preliminary studies in patients at term who received oral administration of the progesterone antagonist mifepristone showed no significant change in systemic CRH (Byrne *et al.*, 1998). These data indicate local withdrawal of progesterone action may alter CRH release on the cellular level.

1.3.5. The Role of Prostaglandins in the Initiation of Labor

The role of the uterotonins, OT, platelet-activating factor, endothelin, and the lipoxygenase products, which have important actions *in situ* are beyond the scope of this chapter. Rather, attention will be devoted to those recent advances in PGs that provide information about their roles as uterine contractile stimulants.

1.3.5.1. Prostaglandins and ovine parturition

As discussed previously, parturition is initiated in animals such as the sheep by the fetus through activation of the fetal hypothalamic-pituitary adrenal axis. In this species birth is preceded by a decrease in progesterone output and an increase in estrogen production by the placenta. In late gestation, fetal plasma cortisol concentrations rise and are responsible for inducing placental synthesis of the enzyme $P450_{C17}$, thereby allowing placental metabolism of C_{21} steroids to estrogen. The changes in steroid output are accompanied by an increase in the concentrations of $PGF_{2\alpha}$ in the maternal utero-ovarian venous blood during the last 12-24 h before delivery occurs. In the fetal circulation, the principle PG is PGE_2, and its concentration increases progressively over the last 15-20 days of gestation (Olson *et al.*, 1985). The difference in the profiles of PGE_2 and $PGF_{2\alpha}$ in the fetal and

maternal circulation raises the possibility that these may be derived from different tissues. PGE_2 in the fetal circulation may originate from placental trophoblast (fetal tissue), whereas $PGF_{2\alpha}$ in the maternal circulation may be derived from the endometrium and myometrium (Challis, 1997).

Metabolism of arachidonic acid in the ovine amnion, chorion and placenta occurs through both the prostaglandin synthase and lipoxygenase pathways from day 50 of gestation. During much of pregnancy the metabolism of arachidonic acid by the amnion is greater than by either the chorion or the placenta. However, at term the placenta is the major site of arachidonic acid metabolism, and there is a preferential increase in the formation of PGs over lipoxygenase products (Langlois *et al.*, 1993). An increase in the PG:lipoxygenase product ratio reflecting predominantly an increase in PGHS activity has also been reported in human amnion obtained at the time of labor (Bennett *et al.*, 1993). The increase in PGHS activity and PGHS protein in sheep placenta with advancing gestation is due to increased expression of PGHS-2 mRNA in the trophoblast. There was no change in PGHS-1 mRNA in placenta over this period of time (Gibb *et al.*, 1996). Furthermore, glucocorticoid infusion to the fetal lamb *in utero* results in an increase in PGHS activity in the placenta, and in PGHS-2, but not PGHS-1 mRNA in placental trophoblasts (Challis, 1997). It is doubtful that estrogen mediates these changes in PGHS activity and expression in the ovine placenta at term, as although estrogen can increase levels of PGHS-2 mRNA and alter the $PGF_{2\alpha}$ content in myometrium and endometrium from non-pregnant sheep, it has no effect in the fetal part of the placenta of the pregnant sheep (Wu *et al.*, 1997; Liggins, 1973).

It is known that glucocorticoids have a direct stimulatory effect on PG production in human fetal tissues (Zakar *et al.*, 1995), therefore, in sheep the rising levels of fetal cortisol in late gestation may directly upregulate PGHS-2 expression in placental trophoblast. This leads to increased PGE_2 synthesis and output from the

placenta. PGE_2 is a potent ACTH secretagogue whose activity does not diminish as adrenal maturation proceeds (Young *et al.*, 1996 a,b), and PGE_2 has also been reported to directly stimulate cortisol secretion by the fetal adrenal glands (Thorburn and Liggins, 1994). Thus, a positive feedback loop between the fetal pituitary, adrenal gland and placenta may be proposed. Therefore, PGE_2 acting in a paracrine/autocrine fashion alters placental $P450_{C17}$ expression resulting in a fall in progesterone and increased estrogen output. Placental estrogen is then responsible for upregulating the contraction-associated proteins (Cx-43, OT-receptor, PG receptors) of the endometrium and myometrium. Estrogen may also be responsible for increased PGHS activity in maternal tissues as one of the final maternal events leading to increased uterotonin production and birth (Challis, 1997).

1.3.5.2. Prostaglandins and human parturition

The sites of PG synthesis in the human and higher primates are the intrauterine tissues. The lines of evidence supporting PGs as important in the initiation and maintenance of labor include PGs and their metabolites rise in amniotic fluid and maternal plasma and urine in late gestation prior to term, at term around the expected time of labor onset, and again with the progression of labor; blocking PG synthesis delays labor onset reduces contractions and prolongs the process of labor; the administration of PG from midpregnancy to term induces labor; and PGs stimulate uterine contractility, *in vitro*. However, the involvement of PGs in the initiation of primate parturition should not be viewed as an established biological fact, and controversy continues to exist as to whether PGs are a cause or a consequence of labor in humans.

Prostaglandins in biological fluids

The major circulating metabolite of $PGF_{2\alpha}$ is 13,14-dihydro-15-keto-$PGF_{2\alpha}$ (PGFM). The plasma concentrations of PGFM change little during pregnancy with the possibility of a small rise during the final month; a significant increase in PGFM does, however, occur during labor (Mitchell *et al.*, 1978). It appears therefore, that during labor there is an increasing rate of production of $PGF_{2\alpha}$, presumably by tissues within the uterus. This $PGF_{2\alpha}$ is subsequently metabolized by the lungs so that only the levels of a metabolite can be detected in the circulation. Surprisingly, the circulating concentration of the major metabolite of PGE_2 is not elevated during labor (Mitchell *et al.*, 1982). This is probably because PGE_2 is converted to $PGF_{2\alpha}$ by enzymes in the decidua vera before it can reach the maternal circulation (Schlegel *et al.*, 1984).

In numerous studies (for a review see Keirse, 1979) it has been shown that concentrations of PGE_2 and $PGF_{2\alpha}$ in amniotic fluid increase slowly from mid-pregnancy until term, rise sharply with the onset of labor and continue to increase in parallel with cervical dilatation. Since, during labor, amniotic fluid concentrations of PGFM exhibit changes similar to those of $PGF_{2\alpha}$, it is assumed that the increased concentrations of $PGF_{2\alpha}$ (and PGE_2) reflect enhanced rates of PG biosynthesis rather than reduced rates of catabolism. This assumption is being proven correct since it has been demonstrated that the activities of PG-catabolizing enzymes within intrauterine tissues change little or decrease during labor (Sangha *et al.*, 1994; Van Meir, 1996). Changes in the PG synthetic capacity of these tissues throughout gestation and with labor is an active area of research and the main topic of this thesis. Both 6-keto $PGF_{1\alpha}$ and TXB_2, the major hydrolysis products of prostacyclin and thromboxane A_2, respectively, have been detected in the amniotic fluid. Although the concentrations of these products are elevated in labor, concentrations do not tend to rise further as labor progresses (Mitchell, 1981). Hence it has been suggested that in

human labor, as discussed earlier with reference to pregnant sheep, there is a redistribution of the flow through different prostanoid pathways favoring PGE_2 and $PGF_{2\alpha}$. As a result, labor is characterized by an increasing ratio in the rates of formation of PGs that contract the uterus versus those that are inhibitory or have no action on uterine contractility.

While this evidence appears strongly supportive of a role for PGs in labor initiation it is by no means conclusive. Principal among the reasons is the fact that the major increase in amniotic fluid and maternal plasma PGs occurs after labor has started rather than before. Furthermore, many of these observations have been considered invalid because amniotic fluid for these studies was obtained transvaginally. Prostaglandin determinations in fluid collected transvaginally may yield spurious results because of contamination of the sample with vaginal secretions which have a high PG content. Moreover, PG concentrations in amniotic fluid obtained transvaginally may not be representative of physiological events affecting the entire amniotic cavity and membranes. Prostaglandin concentrations in fluid retrieved transvaginally are significantly higher than those found in fluid obtained transabdominally from the same patient. This difference has been attributed to the fetal membranes and attached decidua coming into contact with vaginal fluids and the bacteria contained therein. This, combined with decidual necrosis, leads to enhanced local production of PGs which is reflected by a gradient of PG concentrations within the amniotic fluid that is highest in the cervical end and lowest in the fundal region (MacDonald and Casey, 1993). Consequently, studies using amniotic fluid obtained transabdominally in early labor were required to determine if a change in PG concentration occurs during the initial stages of labor.

Studies in which amniotic fluid was obtained transabdominally from women in spontaneous term labor demonstrated that concentrations of $PGF_{2\alpha}$, PGFM and PGE_2 were significantly higher in patients in early labor (cervical dilatation $\leq$3cm)

than in patients not in labor. Significant increases in amniotic fluid concentrations of $PGF_{2\alpha}$ and PGFM were also found in patients with advanced cervical dilatation (8-10cm) in comparison with those in early labor (<3cm) (Romero *et al.*, 1994). Additionally, amniotic fluid concentrations of PGE_2 and $PGF_{2\alpha}$ were found to increase prior to spontaneous labor onset (Romero *et al.*, 1996). Furthermore, even though transcervical amniocentesis can be criticized because amniotic fluid can come into contact with vaginal secretions, the procedure was performed identically in each patient, therefore, the changes observed in late gestation amniotic fluid PG concentrations in these studies must be considered valid. Taken together these findings strongly support a critical role for PGs in the onset and progression of labor.

Prostaglandin synthesis inhibitors and uterine contractility

A further criterion for PG involvement with labor is that inhibitors of PG synthesis block uterine contractility. Novy *et al.* (1974) administered indomethacin to three pregnant rhesus monkeys starting on day 150. This treatment prolonged gestation to at least day 185 (term=161 days) when the pregnancies were terminated by Cesarean section. Hsu *et al.* (1989) found indomethacin was able to revert spontaneous contractions to contractures in a rhesus monkey at day 132 of gestation. Uterine activity converted to contractions on removal of indomethacin. A retrospective controlled study of pregnant women who had taken aspirin during the last 6 months of pregnancy showed they had longer gestations (286±13.3 days versus 278.6±6.91 days), and the length of labor was nearly double the control group (Lewis and Schulman, 1973). Also, when indomethacin was administered to sixteen women at term, and in labor, seven had complete cessation of labor, and another seven had the duration of labor prolonged (Reiss *et al.*, 1976). These studies are very suggestive of a role for PGs in labor initiation, uterine activation and labor maintenance. However, the proposition that pharmacological inhibitors of PG synthesis will prevent

or arrest labor in human pregnancy has not been rigorously tested because of fear that these agents will adversely affect the fetus (Keirse, 1990). More data may be forthcoming with the development of inhibitors specific for PGHS-1 or PGHS-2 activity.

Prostaglandin administration and labor induction

Prostaglandin administration induces abortion and labor throughout most of gestation (for a review see Keirse, 1990). Both PGE_2 and $PGF_{2\alpha}$ are efficacious, and the most accepted routes of administration are endocervical and vaginal. However, this in itself is not grounds for the conclusion that PGs are the physiological uterotonins that initiate parturition. Many agents, almost certainly not involved in the initiation of spontaneous parturition will cause myometrial smooth muscle to contract. The success of PG administration to induce labor or abortion may be completely independent of the physiological processes that initiate parturition. In addition, the amount of PGE_2 or $PGF_{2\alpha}$ instilled into the amniotic fluid necessary to cause abortion is orders of magnitude greater than the maximal amount of these PGs that accumulate in amniotic fluid during labor. However, although these levels may appear pharmacological, as little as 0.25 to 0.50 mg PGE_2 is effective in inducing cervical ripening and myometrial contractions. This is in the nanomolar range when distributed throughout the entire body and not considering the rapid metabolism. Moreover, the likelihood of not being delivered vaginally within a specified period of time is markedly reduced when PG is used for labor induction as compared to the use of OT.

Prostaglandins and uterine contractility in vitro

Prostaglandins have the ability to stimulate uterine contractility *in vitro*. However, frequently these studies have been performed on rat or rabbit muscle.

Furthermore, PGE_2 and $PGF_{2\alpha}$ when applied to human myometrial tissue strips obtained before or after the spontaneous onset of labor, were relatively ineffective in causing contraction (Word *et al.*, 1992). This discrepency may be explained by investigations into changes in the expression and abundance of receptors for PGs in the myometrium.

Molnar and Hertelendy (1990) showed that endogenous PGs regulate their own receptors numbers in myometrium *in vivo*. In pregnant rats, when the endogenous source of PGs was removed by indomethacin treatment or fetectomy, the number of both PGE_2 and $PGF_{2\alpha}$ receptors increased in myometrium, and a late gestation increase in receptors occurred coinciding with labor onset. Without fetectomy, no late gestation change in receptor number was evident, and when exogenous PGs were administered, the number of receptors decreased. Senior *et al.* (1991) studied the types of PGE receptors present in nonpregnant human myometrium and determined three types of receptor exist: EP_1 and EP_3, which lead to contraction, and EP_2, which leads to relaxation. Biphasic responses of contraction followed by relaxation were evident. These data indicate that the lack of contractile response elicited by exogenous PGs when applied to myometrium in vitro is to be expected.

Prostaglandin synthesis

In women PG production is discreetly compartmentalized within the fetal membranes. The two steps in the biosynthetic pathway leading to PGs that are the most amenable to control are the deacylation, or release, of arachidonic acid and its conversion to endoperoxide.

The first area in this field that has received recent attention is at the level of the phospholipases (for reviews see Olson and Zakar, 1993; Rice, 1995). It is doubtful that phospholipases are rate-limiting in the pathway of PG biosynthesis at

parturition in human gestational tissues as there is evidence of a large amount of unesterified arachidonic acid in the intrauterine compartment both before and after term and pretrem labor (Bleasdale and Johnston, 1985). However, some recent findings in this area are particularly notable. PLA_2 enzymatic activities have been identified in the amnion, chorion, decidua and placenta. Original observations indicate that PLA_2 enzymatic activity in the amnion increases significantly during gestation (Okazaki *et al.*, 1981) with no further rise in relation to term labor (Lopez-Bernal *et al.*, 1992) despite a 6-10 fold increase in the concentration of free arachidonic acid in the amniotic fluid (MacDonald *et al.*, 1974) and a decrease in the content of arachidonic acid in the amnion (Okita *et al.*, 1982). However, the activity assays utilized in these studies did not distinguish intracellularly and extracellularly active enzymes; and these data have been based on the quantification of *in vitro* net PLA_2 enzymatic activity and the contribution made by individual PLA_2 enzymes was not accounted for.

More recently, three PLA_2 isoenzymes have been identified in human gestational tissues: $sPLA_2$ II, $sPLA_2$ IV, and $cPLA_2$; only the expression of $sPLA_2$ II has been studied in detail. Aitken *et al.* (1990, 1993) have established the presence of $sPLA_2$ II mRNA and immunoreactive protein in amnion, choriodecidua and placenta. The immunoreactive and catalytic activity of this $sPLA_2$ II increases in amnion and placenta taken from patients in labor. There is no corresponding change in mRNA suggesting an acute regulation at a post-transcriptional site (Rice *et al.*, 1994). In addition, preliminary data indicate an increase in the mRNA levels of $cPLA_2$ in placental tissue taken from patients in late gestation. Furthermore, some insight on the role of $cPLA_2$ in labor initiation can be obtained from $cPLA_2$ deficient pregnant female mice. These animals fail to undergo labor at term (18.5-20.5 days gestation) and deliver only a few live offspring at day 21.5-22.5 gestation which do not survive. Labor could be induced and viable neonates obtained by administration of RU486. In

the mouse at least, these data indicate that $cPLA_2$ is involved in the induction of labor by the production of $PGF_{2\alpha}$ which in turn causes luteolysis (Uozumi *et al.*, 1997). Further study will be needed to identify the mechanism by which $cPLA_2$ and $sPLA_2$ affect pregnancy and labor in the human.

The second point of control of PG synthesis is the PGHS step. This is most likely the rate-limiting enzyme of PG biosynthesis in human gestational tissues. PGHS-1 and PGHS-2 mRNA have been identified in human gestational tissues by Northern blot analysis (Bennett *et al.*, 1993), reverse transcriptase polymerase chain reaction (Mitchell *et al.*, 1993 and solution hybridization (Hirst *et al.*, 1995). In amnion, PGE_2 is the principal PG formed and there is an increase in PGHS activity and PGHS-2 mRNA in tissues collected from patients at term spontaneous labor compared to term Cesarean section (Hirst *et al.*, 1995). Furthermore, there is an increase in human amnion PGHS enzyme activity in late gestation, before the onset of labor, which parallels the prelabor changes in amniotic fluid PGs (Teixeira *et al.*, 1994). Decidua also has the potential for PG production, and PGHS-1 and PGHS-2 mRNA and immunoreactive proteins have been identified in this tissue (Slater *et al.*, 1995; Gibb and Sun, 1996). In addition, preliminary data suggest that labor is associated with increases in PGHS-2 but not PGHS-1 expression in the human myometrium (Challis, 1997).

The chorion, interposed between the amnion and decidua, has both PGHS and PGDH activities, but the metabolizing enzyme predominates (Cheung *et al.*, 1990). It has been suggested that for much of pregnancy the chorion forms a metabolic barrier preventing passage of PGs generated within the amnion and chorion from reaching underlying decidua and myometrium (Nakala *et al.*, 1986). This suggests that at term unless the synthetic capacity of the amnion/chorion exceeds the metabolic potential of the chorion, PGs driving the myometrium would have to be generated within decidua tissue, or the myometrium itself. The ability of the intrauterine tissues to synthesize

PGs is measured by the expression and activity of the PGHS isoenzymes. Changes in the PG biosynthetic pathway at the level of the PGHS enzyme in the amnion, chorion and decidua throughout gestation and with term and preterm labor is an active area of research and the focus of this thesis.

The factors at term which control the expression of PGHS-2 remain to be identified, but notably glucocorticoids have been shown to increase PGHS-2 mRNA in cultured amnion cells (Zakar *et al.*, 1995). In human fetal membranes in late gestation, expression of PGHS-2 mRNA occurs in amnion epithelium, in sub-epithelial fibroblasts, and in chorion trophoblasts. PGHS-2 mRNA localizes to blood vessels in decidual tissue, and is expressed at low levels in decidual stromal cells of patients at term, in the absence of active labor (Gibb and Sun, 1996; Slater *et al.*, 1995). The distribution of PGHS-2 mRNA in the human fetal membranes is similar to the pattern of localization of glucocorticoid receptors (GR), detected by immunohistochemistry. Interestingly, the number of cells that were immunopositive for Type-2 GR was significantly higher in tissues collected from patients at preterm labor (Sun *et al.*, 1996).

In vitro glucocorticoids directly stimulate PGHS-2 mRNA and activity in human amnion (Gibb and Lavoie, 1990; Potestio *et al.*, 1988, Zakar *et al.*, 1995). Evidence suggests the effects of glucocorticoids on amnion PGHS-2 expression could also be indirect via the production of locally acting peptides, such as CRH (Jones and Challis, 1990). Recently, it has been shown that CRH treatment increased levels of PGHS-2 mRNA several fold in amnion cells maintained in culture compared to control, and this correlated with an increase in PGE_2 output by the cells (Challis, 1997). These data suggest glucocorticoids may stimulate amnion cells to produce CRH, which in turn stimulate increased PGHS-2 expression and PG production in the same or adjacent cell layer.

These data are of particular significance when considering the evidence presented earlier discussing the role of fetal cortisol in the initiation of labor in humans. It is possible that elevated levels of glucocorticoids produced in late gestation by the fetal hypothalamic-pituitary-adrenal axis stimulate PG production by increasing expression of PGHS-2 at least in amnion cells, and thereby increasing output of PGE_2. Glucocorticoids also decrease the activity of PGDH (see later), and stimulate output of CRH. CRH further promotes PGHS expression and PG output in the amnion. Collectively, these pathways contribute to increased PG production by intrauterine tissues. Increased production of these uterotonins acting through the appropriate receptors participate in the drive to myometrial contractility, cervical remodeling, membrane rupture and expulsion of the fetus.

Obviously, these pathways are not exclusive and do not exist in isolation, indeed, *in vitro* studies have identified many other factors that increase PG output by human fetal membranes (Romero *et al.*, 1991). Growth factors including EGF and TGF promote PG biosynthesis. Cytokines can upregulate expression of PLA_2 (Hansen *et al.*, 1998a) and PGHS-2, and increase PG output. At present however, although these latter observations may be especially relevant to the pathology of preterm birth in the setting of infection, the significance of many of these *in vitro* measurements to the physiological regulation of PG production *in vivo* remains speculative, and should be treated with caution.

Prostaglandin metabolism

PGDH is found in the placenta and the fetal membranes. In the fetal membranes chorion trophoblast cells express abundant PGDH while this enzyme is virtually undetectable in the amniotic tissue and the underlying decidua (Cheung *et al.*, 1990). Evidence suggests there is a decrease in the activity and expression of PGDH in the chorion trophoblast of pateints at term and preterm labor. The mean

level of PGDH activity ($PGF_{2\alpha}$ to PGFM conversion) and PGDH mRNA in chorion was lower in patients at spontaneous labor compared to that at Cesarean section at term (Van Meir *et al.*, 1997). Studies by Sangha *et al.* (1994) showed that 10-15% of patients in idiopathic preterm labor in the absence of infection had very low levels or absent PGDH activity and mRNA in the chorion. The activity and levels of IR-PGDH in membranes from patients in preterm labor with an underlying infective process were also found to be extremely low (Van Meir *et al.*, 1996); this was associated with the destruction and loss of the chorionic trophoblast cells. In all cases there were no changes in PGDH activity in placental tissue from these same groups of patients indicating the loss of PGDH expression was specific for chorion.

Recent data suggests *in vivo* regulation of PGDH activity and expression may reflect a balance between opposing effects of cortisol and progesterone exerted in a paracrine or autocrine manner (Patel *et al.*, 1998a). Cortisol had a significant dose-dependent inhibitory effect on PGDH activity and levels of mRNA in both syncytiotrophoblast cells and chorion trophoblast cells maintained in culture. PGDH activity was increased in the presence of the stable progestagen analogue R5020 and inhibited by RU486 and trilostane. Exogenous progesterone reversed the inhibition after trilostane, causing a dose-dependent increase in PGDH activity. It appears, therefore, that cortisol inhibits PGDH activity exerting its effect, in part, at the level of PGDH mRNA. Furthermore, the effects of cortisol on PGDH are modified by the tissue specific expression of 11β-HSD. In placenta, the effects of cortisol are reduced by 11β-HSD2 oxidation, whereas in chorion the presence of 11β-HSD1 allows activation of cortisone by converting it to cortisol (Patel *et al*, 1998 b).

In normal pregnancy, PGDH expression and activity in chorion is maintained at a high level due to the predominance of progesterone in the environment. Prostaglandins generated within amnion or chorion are rapidly metabolized and pass to the decidua and myometrium in only very small amounts. At term and in some

patients in idiopathic preterm labor, expression and activity of chorionic PGDH are reduced under the influence of local progesterone withdrawal and increasing concentrations of cortisol from the fetal adrenal, and changes in the balance of cortisone-cortisol metabolism within the chorion itself. In these patients PGs generated within the amnion or chorion in response to a wide variety of potential stimuli would be metabolized only poorly, and could therefore pass to underlying decidua tissue and myometrium. In patients with infection where trophoblasts are destroyed, PGDH activity is lost. In these patients the PG synthetic capacity of the intrauterine tissues predominates (Romero *et al.*, 1991), and in the absence of metabolism these PGs drive myometrial contractions.

Prostaglandin receptors

Prostaglandins act through specific receptors including the four main subtypes EP_1, EP_2, EP_3, and EP_4 for PGE_2, and FP receptors for $PGF_{2\alpha}$. EP_1 and EP_3 receptors mediate contractions of smooth muscle in a number of tissues through mechanisms that include calcium mobilization and inhibition of intracellular cyclic AMP (cAMP). EP_3 receptors exist as a number of isoforms produced following alternative RNA splicing of a single gene product. EP_2 and EP_4 receptors relax smooth muscle through increased cAMP formation (Ushikubi *et al.*, 1995).

Expression of the four main receptor subtypes in primate myometrium and fetal membranes during pregnancy and parturition has now been established (Senior *et al.*, 1991; Brodt-Eppley and Myatt, 1998; Smith *et al.*, 1998a, b). Prior to term there is an increase in the EP_2 receptor mRNA in human myometrium which presumably plays a role in maintaining uterine quiescence during pregnancy (Brodt-Eppley and Myatt, 1998). At term, PGs acting through EP_1, EP_3 and FP receptors in the uterus promote contractility. In addition, Smith *et al.* (1998a) have found significant increases in the expression of the EP_2 receptor in the myometrium of the

lower uterine segment (LUS) of the rhesus monkey with the onset of labor. These data suggest the effects of PGE_2 on EP_2 receptors may be important to relax smooth muscle during labor, particularly in the LUS, and allow passage of the fetus out of the uterus.

Loss of inhibition is another mechanism for initiation of contraction

Other physiological regulators of the PG biosynthetic pathway have been identified and postulated to play a role in the suppression of labor. It is hypothesized that withdrawal of these factors at parturition results in increased PG synthesis and uterine contractility.

Lipocortins are phospholipase A_2 inhibitors, but their physiological activity is questionable; they are found in large quantities which argues against a regulatory role; they appear to interact with the substrate rather than the enzyme which means they may not be specific for the PG biosynthetic pathway; furthermore, inducible levels have not been found to correlate with phospholipase inhibition or PG synthesis (Davidson and Dennis, 1989). Uteroglobulin is a progesterone-induced protein inhibitor of phospholipase A_2, and, although no physiological role for uteroglobulin has been established yet, it may be involved in the mediation of progesterone-controlled uterine quiescence (for a review see Olson *et al.*, 1993).

Gravidin is a purported protein inhibitor of phospholipase A_2 which is present in human amniotic fluid where it is identical to the secretory component of IgA. The greatest production of the gravidin protein has been found in the chorion. Assays in chorion taken before and after labor onset showed gravidin activity before labor onset was much greater than that after labor onset, thus it is possible gravidin becomes inactive or its synthesis stops at parturition. Measurements of gravidin have also been made in amniotic fluid and serum in a group of patients admitted in preterm labor. Mothers going into preterm labor had lower levels of gravidin than those who went

on to term. In addition, serum gravidin-IgA falls to non-pregnant levels on administration of RU486, therefore it appears gravidin action may be mediated by progesterone. These data provide evidence of a role for gravidin in the maintenance of pregnancy (Wilson, 1993).

Human pregnancy plasma has been found to have the ability to inhibit PG synthesis. This has been ascribed to the presence of a circulating endogenous inhibitor of PG synthesis (EIPS) which inhibits cyclooxygenase (Saeed *et al.*, 1982). The physiological significance of this in the initiation of parturition is doubtful as EIPS levels did not change in studies of maternal plasma during pregnancy, parturition and labor. However, it may act at a local level. Further to this, a pregnancy-associated prostaglandin synthase inhibitor (PAPSI) has been located specifically in human amniotic epithelial cells in women before the onset of labor and found to be absent in the amniotic epithelium of women in labor (for a review see Olson *et al.*, 1993). The results from such studies may become more meaningful when re-evaluated with reference to the recent findings concerning the differential expression of the PGHS -1 and PGHS-2 isoforms with parturition.

1.3.6. Other Physiological Events of Parturition

The essential aspect of successful delivery of the fetus is not only the development of rhythmical, sustained and coordinated contractions of uterine muscle, but rupture of the fetal membranes and ripening and dilatation of the cervix must occur also so that the fetus can be released from the uterine compartment.

1.3.6.1. Fetal membrane rupture

The process of labor and delivery results initially from a change in the relationship of the external fetal membranes and uterine wall. This involves

separation of the chorion from the uterine decidual layer. There is enhanced glycosylation of a fetal-fibronectin glycoprotein localized in an area where the placenta and its membranes meet the uterine wall. The enhanced glycosylation of this placental fibronectin substantially reduces its binding affinity for other components of the extracellular matrix and therefore facilitates the separation of the chorion from the decidual layer. When this occurs fetal fibronectin is released into the cervicovaginal secretions. As fetal fibronectin is different to the fibronectin extracted from adult tissues, its appearance in these secretions is frequently used as a predictor of the onset of preterm and term labor and delivery (Lockwood *et al.*, 1991).

Rupture of the fetal membranes is the result of mechanical and enzymatic processes. The biomechanical properties of fetal membranes collected after spontaneous labor or after cesarean section in the absence of labor are different. Following vaginal delivery the strength of the amnion decreases, and the extensibility of the chorion increases. Thus, during labor there are mechanical changes in the fetal membranes that facilitate rupture. Other evidence suggests collagenases and fibrinolytic factors are involved in this process. Term amniotic fluids are capable of inducing the synthesis of collagenases and other proteases in fibroblasts while nonterm amniotic fluids fail to do the same. A number of fibrinolytic activators and inhibitors are present in the fetal membranes during gestation. It is postulated that a balance favoring the production of activators over inhibitors triggers membrane rupture at term (Watanabe *et al.*, 1993)

There is evidence that membrane rupture at term may involve apoptosis. In the rat, the cells of the amnion membrane undergo apoptosis in late gestation (Lei *et al.*, 1996). The factors controlling this process are unknown, but evidence from the rabbit suggests progesterone withdrawal and the presence of transforming growth factor β-1 causes uterine epithelial cells to undergo apoptosis (Gerschenson and Rotello, 1992). *In vitro* data are suggestive of a role for PGHS and/or PGs; in

amnion-derived WISH cells maintained in culture indomethacin blocks agent-induced apoptosis in a concentration and time-dependent manner (Moore *et al.*, 1998).

1.3.6.2. Cervical ripening

Further to membrane rupture, cervical ripening towards term and dilatation at delivery are essential processes. In the first part of gestation the cervix is hard and firmly holds the uterine contents. The biochemical process of "cervical maturation" commences at about the 34th week of pregnancy until the cervical os is fully dilated at delivery. The close cooperation between the myometrium and cervix is essential for normal uterine function, and defects in this relationship cause maternal and fetal morbidity.

There are three main structural components in the cervix of women: smooth muscle, collagen and the connective tissue "ground-substance". The last contains the cervical glucosaminoglycans: dermatan sulfate, chondroitin sulfates and hyaluronic acid. In humans, smooth muscle has not been shown to have a role in cervical dilatation. The enzymatic breakdown of collagen is a key factor in cervical softening. The collagen fragments become soluble and leave the ripened cervical tissue. The degradation of collagen occurs as an action of the enzymes collagenase and leukocyte elastase. The latter is located in the azurophil granules of polymorphonuclear leukocytes. Leukocyte infiltration and degranulation occurs in the term cervix in a similar manner to that seen in inflammatory reactions (Uldjerg and Malmstrom, 1991; Jeffery, 1991). Indeed, it was Liggins who first proposed that cervical ripening was similar to an inflammatory reaction. Cervical dermatan sulfate concentrations diminish along with those of collagen and the cervix becomes swollen and soft due to increased hyaluronic acid and water content. The increased hyaluronic acid and water content accounts for the soft, fragile texture of the ripened cervix, whereas the

breakdown and loss of collagen and dermatan/chondroitin sulfates facilitate flexibility and distensibility.

The biochemical events underlying cervical maturation indicate it is an active cellular process and is thus subject to regulatory control. The activity of collagenase and other proteolytic enzymes rises with the increasing intrauterine estrogen dominance in late gestation. Conversely, in nonpregnant human cervix explants, collagen breakdown is diminished by progesterone administration. Prostaglandins, especially PGE_2, are clearly involved in cervical ripening at term in women. Prostaglandins have been used clinically for years to induce first and second trimester abortions and cervical ripening. Further, in humans ripening of the cervix is associated with increased PGI_2 and HETE production (for reviews see Huzar, 1991; Challis and Lye, 1994). HETEs and their metabolites are potent chemoattractants; PGI_2 is involved in increasing vascular permeability during inflammatory reactions. Based on these observations it is possible production of these mediators summons the polymorphonuclear leukocytes known to infiltrate the cervix at term and leads to enzyme secretion and collagen degradation

In addition to evidence of their local production by the cervix, PGs have been shown to have effects on cervical ripening *in vivo*. In late pregnant sheep treated with epostane, a 3βHSD inhibitor which decreases progesterone synthesis, there was an increase in utero-ovarian plasma PGE_2 and $PGF_{2\alpha}$. This was accompanied by increases in uterine activity and cervical softening. Addition of the PG synthesis inhibitor, mefenamic acid, caused PG levels to fall and uterine activity and cervical softening to cease. Both PGHS-1 and PGHS-2 protein have been localized to the ovine cervix. Treatment of pregnant sheep with the specific PGHS-2 inhibitor, nimesulide, indicated that this isoform was responsible for a local increase in PGE_2 output from the cervix during spontaneous term labor (Westerhausen-Larsen *et al.*, 1998).

The hormone relaxin is also postulated to be involved in cervical ripening. In non-pregnant, estrogen-primed rhesus monkeys, relaxin administration induced histologic, biochemical and biomechanical changes that were similar to normal cervical ripening. Relaxin receptors are present in the human cervix and local administration of relaxin to women is beneficial in cervical dilatation (for reviews see Huzar, 1991; Challis and Lye, 1994).

Last, a relationship between cervical maturation and the initiation of labor is well demonstrated. In one study women received labor preinduction treatment of oxytocin infusion alone or oxytocin infusion and intracervical PGE_2 gel. The contractile activity was no different in the two groups. However, in the PGE_2 treated group, the women proceeded to spontaneous labor and delivered fast; the length of the active phase and the second stage of labor were shorter, and the incidence of cesarean sections was lower. Thus, pretreatment of the cervix with PG suppositories causing maturation facilitates a more efficient labor process without an increase in myometrial activity (Huzar and Walsh, 1991).

1.4. Summary

It is evident that parturition results from a complex interplay of maternal and fetal factors. It is the result of the sequential maturation of an endocrine organ communication system. In sheep and potentially primates, the sequence can be seen to begin at the level of the fetal brain with increased cortisol production from the fetal adrenal providing the trigger to the subsequent evolution of maternal endocrine changes. In sheep, the regulatory mechanisms responsible for the changes in fetal adrenal glucocorticoid secretion can be considered to be the initial signals for the onset of parturition. In primates, these mechanisms are estrogen dependent, the synthesis of which is directed by the arrival of C_{19} steroid precursors at the placenta.

These estrogen precursors are also synthesized by the fetal adrenal. Throughout gestation, progesterone acts to maintain pregnancy. Interestingly, in primates placental progesterone synthesis is for the most part dependent on estrogen synthesis by the same tissue and therefore, ultimately, the fetus. Towards term, the influence of progesterone is either withdrawn locally and/or overcome by rising estrogen levels. The role of the fetal adrenal in providing increased estrogen precursors for the latter process in primate parturition is at least, firmly established. Under the increasing estrogen dominance of the intrauterine tissues, activation of the myometrium and an increased capacity for uterotonin synthesis occurs. This leads to the rhythmic contractions of labor that lead to the expulsion of the uterine contents. Rupture of fetal membranes and dilatation of a ripened cervix are essential to complete a successful delivery. A glucocorticoid signal from the fetus, or, another as yet undefined signal, initiates a feedforward cascade of events which results in secretion of myometrial stimulants, uterine contraction, membrane rupture, cervical dilatation and labor. The ultimate signals for birth may yet be found to reside at the level of the primate fetal brain as in the sheep. These signals initiate an array of fetal and maternal endocrine and paracrine processes that result in the timely delivery of a viable neonate.

1.5. Rationale

Increased PG levels within the intrauterine compartment play a pivotal role in the processes associated with labor in women. The biochemical pathways of PG production involve both the release and metabolism of arachidonic acid. The enzymes PLA_2 and PLC are involved in arachidonate mobilization in the gestational tissues. While the activities of these enzymes increase over gestation in the amnion and placenta, neither is apparently responsible for the increased plasma and amniotic

fluid PG levels observed at the time of labor. Furthermore, the activities of PG-catabolizing enzymes within intrauterine tissues change little or decrease during labor. Therefore, altered activity of the arachidonic acid metabolizing enzyme, PGHS, is considered the rate-limiting step of PG biosynthesis at labor. Indeed, the capacity of the amnion to produce PGs increases significantly in association with labor due to the enhanced expression of PGHS-2, the inducible isoform of this enzyme. The chorion laeve is positioned between the amnion and the decidua, and knowledge of its contribution to intrauterine tissue PG synthesis throughout gestation and with labor is limited. The close proximity of the decidua to the myometrium suggests that decidual activation involving the elevated production of $PGF_{2\alpha}$ may be important for labor onset. The contribution of the two PGHS isoenzymes to PG synthesis in decidua is unknown. Changes in the PG synthetic capacity of the amnion, chorion laeve and decidua throughout gestation, and of the chorion laeve and decidua with labor will be investigated in this thesis.

The physiological function of intrauterine PGs synthesized during labor is generally believed to be the maintenance and/or augmentation of myometrial contractions, cervical dilatation, and membrane rupture. However, the involvement of PGs in the initiation of labor is disputed. Therefore, the temporal relationship of labor onset and any change in PGHS expression in the amnion, chorion laeve and decidua will be examined.

Preterm birth accounts for 5-10% of all births, and 70% of all perinatal deaths are associated with prematurity. The majority of this thesis will attempt to gain insight into the fundamental physiological processes of normal term birth. These will then be applied to the pathophysiology of preterm birth. The expression, activity and localization of PGHS will be examined in intrauterine tissues collected from patients before and after preterm labor.

In the amnion, PGHS-2 is believed to be responsible for PG production at term labor. If preterm labor occurs due to the premature activation of events that normally occur at term, preterm birth may be prevented and gestation prolonged by inhibition of the expression or activity of the PGHS-2 isoenzyme. If PGHS-2 is induced only in the intrauterine tissues with increasing gestational age, and not elsewhere in the fetus, blocking its expression and/or activity may be a safe, effective therapeutic approach to dealing with preterm birth. However, no data are available describing the gestational-age dependent profile and prevalence of PGHS-1 and PGHS-2 in fetal tissues; and, little information exists on the source of the PGs essential for fetal development and the regulation of their production. Therefore, in a preliminary attempt to define the role of PGHS and its isoforms in regulating fetal organ maturation and fetal/neonatal physiology, and to test the feasibility of using a specific PGHS-2 inhibitor as an efficacious tocolytic, we determined the changes in PGHS-1 and PGHS-2 mRNA abundance with increasing gestational age in a variety of fetal tissues.

Finally, the *in vitro* mechanisms by which PGHS may act to regulate PG production throughout gestation and at labor will be investigated.

The **objective** of this research is to understand the regulation of PGHS-2 expression in intrauterine tissues and its role in the initiation and maintenance of term and preterm labor, fetal development and organogenesis. The **hypothesis** is that induction of the functional expression of one or more isoforms of PGHS at term or preterm labor occurs in fetal membranes, and also most tissues of fetal origin to produce PGs involved in the initiation of parturition and other aspects of fetal development.

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2. MATERIALS AND METHODS

2.1. Materials

Leupeptin, phenylmethylsulfonylfluoride, diethyldithiocarbamic acid, tryptophan (Trp), 1, 4- piperazine-diethanesulfonic acid (PIPES), Ficoll-400, diethylpyrocarbonate (DEPC), Denhardt's solution and Cytokeratin immunohistochemical detection kits were purchased from Sigma Chemical Co. (St. Louis, MO). [5,6,8,11,12,14,15-N-^{3}H]Prostaglandin E_2 (PGE_2) (SA, 140 Ci/mmol) was obtained from Amersham Canada (Oakville, Canada), and [α-^{32}P]CTP was purchased from DuPont Canada (Mississauga, Canada). Arachidonic acid was obtained from NuChek Preparations (Elysian, MN). Reduced glutathione, proteinase-K, ribonuclease-A, ribonuclease-T1, ribonuclease free deoxyribonuclease-1, and DIG nucleic acid labeling and detection kits were purchased from Boehringer Mannheim Canada (Laval, Canada). VIP Substrate kit for peroxidase was bought from Vector Laboratories (Burlingame, CA). T7 and T3 RNA polymerase was obtained from BRL (Gaithersburg, MD). Sep-Pak C_{18} cartridges were products of Waters-Millipore (Milford, MA). Fetal fibronectin enzyme linked immunosorbant assay kits were a gift from Adeza Biomedical (Sunnyvale, California USA). All other chemicals were of analytical (ACS) purity.

2.2. Methods

2.2.1. PGHS Enzyme Activity Assay

To study PGHS enzyme activity in intrauterine tissues we adapted a previously described PGHS enzyme assay (Smieja *et al.*, 1993; Teixeira *et al.*, 1993, 1994).

Sample preparation

Placentas were collected from singleton pregnancies immediately upon delivery and placed in ice cold physiological saline. The placenta was separated from the fetal membranes by cutting around the placental margins. Membranes were washed in physiological saline to remove excess blood. Reflected amnion was separated from the choriodecidua by blunt dissection; decidual tissue was then scraped from the chorion with a blade. Histological examination of the membranes after scraping indicated that although most of the decidua was removed, the chorion laeve epithelium remained intact and covered by a thin layer of decidua. Amnion, chorion and decidua samples were cut into strips (10 x 20 mm), washed repeatedly with physiological saline to remove clotted blood, and snap-frozen in liquid nitrogen. All frozen tissues were stored at -70°C and processed within three weeks of collection. The collected tissue samples were examined histologically and only those free of infection, as determined by neutrophil invasion, were used in the studies. The tissue collection protocol was approved by the University of Alberta Ethics Review Committee.

Microsomal preparation

Each frozen tissue sample was crushed in mortars precooled with liquid nitrogen and placed on ice in seven volumes of homogenization buffer containing 50 mM TrisCl, 2 mM EDTA, 0.25 M sucrose and supplemented with 1 mM dithiodicarbamic acid (DTC), 4 mM Trp and 10 μg/mL leupeptin, (pH 8). The tissue sample was homogenized (3 x 30 s setting 5, 3 x 30 s setting 6 Brinkman Polytron), and centrifuged at 1000 x *g* for 15 min at 4°C, and the supernatant collected. A crude particulate fraction containing microsomes was then prepared by centrifugation of the low speed homogenate at 105000 x *g* for 60 min at 4°C. The resulting microsomal pellet was placed on ice, resuspended in buffer containing 50 mM TrisCl, 2 mM

EDTA, supplemented with 1 mM DTC and 4 mM Trp, (pH 8), and hand homogenized to form a suspension.

Microsomal incubation

All microsomal incubations were carried out in a 37°C water-bath for 4 mins in acid-washed 12 x 75 mm test tubes. The specific activity of the enzyme at 20 μM arachidonic acid produces a value statistically equal to the V_{max} of the enzyme (Fig. 2-1). Therefore, an incubation system using 20 μM arachidonate was used to quantitate PGHS enzyme activity in these tissues. Each microsomal preparation was assayed in triplicate, at three different protein concentrations, to establish a linear relationship between enzyme activity and the protein content of the reaction mixture. The reactions were started by the addition of 25 μL of the prepared microsomes to an incubation mixture containing 1 mM GSH, 1 mM Trp, and 20 μM arachidonate. Following incubation, the reactions were stopped by the addition of two volumes of 50 mM citrate buffer (pH 3) containing 15% (v/v) ethanol. Samples were either extracted immediately or stored at -20°C for no more than 72 h.

Extraction and radioimmunoassay

Since the cofactor conditions used during the incubations favored PGE_2 production, this was used to evaluate PGHS enzyme activity. PGE_2 produced during this incubation period was extracted using reverse phase C_{18} cartridges (Powell, 1982). Samples were loaded onto activated C_{18} Sep-pak cartridges and rinsed with 2 mL citrate buffer (50 mM, pH 3) plus 15% (v/v) ethanol and 2 mL distilled water. Unconverted arachidonate was eluted by the addition of 3 mL petroleum ether (b.p. 30-60°C) while PGE_2 was eluted and collected by the addition of 3 mL methyl formate. The extract was dried under vacuum and redissolved in phosphate buffered saline (PBS). Under these conditions, [^{3}H]PGE_2 recovery values were consistently 90% or greater; corrections were made for extraction losses. Quantification of PGE_2

was performed using a specific and well characterized radioimmunoassay (Olson *et al.*, 1984).

Protein assay

Microsomal protein was quantitated using the method described by Bradford (1976). The protein (50 μL aliquots of microsomal suspension) was dissolved in 0.8 mL of 0.2 N NaOH, covered, and warmed at 37°C overnight. The next day the pH of the samples was returned to neutrality with 0.2 mL of 0.8 M HCl before assay. Bovine serum albumin was used as the standard.

Data analysis

Results were expressed as picograms of PGE_2 produced per μg microsomal protein per minute.

2.2.2. Ribonuclease Protection Assay

Total tissue PGHS activity is likely the sum of the activities of the PGHS-1 and PGHS-2 enzymes (Barnett *et al.*, 1994). The relative contributions of the isoforms to total PGHS expression in the amnion, chorion laeve and decidua were assessed by measuring the levels of PGHS-1 and PGHS-2 mRNA and establishing the correlation of PGHS activity with the levels of isoform-specific mRNAs.

We have found the measurement of mRNA abundance to be a more reliable indicator of enzyme expression in the intrauterine tissues than the assessment of total tissue enzyme activity or the detection of steady-state immunoreactive PGHS protein for a number of reasons. First, the intracellular location of the two PGHS isoenzymes has been reported to differ slightly in several cell types. PGHS-2 has been localized in both the endoplasmic recticulum and the nuclear envelop while PGHS-1 has been found predominantly bound to the endoplasmic recticulum (Morita *et al.*, 1995). Protocols measuring PGHS enzyme specific activity and immunoreactive protein require removal of the nuclear fraction for the homogenates by low speed

centrifugation. It is possible that the subtle difference in location of the PGHS isoenzymes may result in the preferential extraction and measurement of PGHS-1 protein from the tissues.

Furthermore, previous studies show PGHS enzymes have a relatively short half-life of 5 min because they undergo inactivation during catalysis (Smith and Marnett, 1991). Therefore, levels of immunoreactive PGHS protein in tissues may not be an accurate estimate of enzyme abundance, as at any point in time a portion of these protein pools might be inactive (Teixeira *et al.*, 1994). To maintain steady-state levels of the active enzyme, these proteins must be synthesized continuously, and their rate of synthesis must increase to raise enzyme activity levels. In a situation such as this, elevated levels of gene expression, in terms of increased levels of mRNA, result in increased protein.

Therefore, an isoform-specific analysis of PGHS expression was performed by determining the abundance of PGHS-1 and PGHS-2 mRNAs using ribonuclease protection assays.

Sample preparation

To measure PGHS-1 and PGHS-2 mRNA levels in intrauterine tissues, we adapted a ribonuclease protection assay that has previously been described (Hirst *et al.*, 1995). Samples were collected as for the PGHS enzyme activity assay. Where possible data relating to PGHS enzyme activity and mRNA abundance were obtained using the same tissue.

RNA preparation

Total RNA was isolated from tissue samples by the acid-guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). One gram of frozen tissue was homogenized in denaturing solution consisting of 4 M guanidnium thiocyanate, 25 mM sodium citrate (pH 7); 0.5% sarcosyl, 0.1 M 2-mercaptoethanol. Sequentially, 0.1 mL of 2 M sodium acetate (pH 4), 1 mL phenol

(water saturated), and 0.2 mL of choloroform-isoamyl alcohol (49:1) were added to the homogenate with thorough mixing by inversion after the addition of each solution. The final suspension was shaken vigorously for 10 s and cooled on ice for 15 min. Samples were centrifuged at 10,000 x *g* for 20 min at 4°C. After centrifugation, RNA was present in the aqueous phase whereas DNA and proteins were present in the interphase and phenol phase. The aqueous phase was transferred to a fresh tube, mixed with 1 mL of isopropanol, and placed at -20°C for at least 1 h to precipitate the RNA. Sedimentation at 10,000 x *g* for 20 min resulted in an RNA pellet, which was then redissolved in 0.3 mL of denaturing solution. The solubilized RNA was transferred into a 1.5 mL eppendorf tube, and reprecipitated with 1 vol isopropanol at -20°C for 1 h. After centrifugation for 10 min at 4°C the RNA pellet was washed with 0.75% ethanol, sedimented, vacuum dried (10 min), and dissolved in 100 µL 1mM EDTA (pH 8). The concentration and purity of the RNA was assessed by the absorption of the preparations at 260 and 280 nm, and by electrophoresis in a 1% agarose minigel stained with ethidium bromide.

Complementary DNAs

Human PGHS-1 (Funk *et al.*, 1991), PGHS-2 (Hla and Neilson, 1992), γ-actin (Gunning *et al.*, 1983), and GAPDH (Tso *et al.*, 1985) complementary DNAs (cDNA) were used for the production of the complementary RNA (cRNA) probes for use in the ribonuclease protection assay (Hirst *et al.*, 1995).

A 309-nucleotide sequence from the center of the amino-acid coding region of human PGHS-1 (ORF 1066-1374) was subcloned into the PstI site of Bluescript-2 (Stratagene, La Jolla, CA) for generation of the PGHS-1 cRNA probe.

For PGHS-2, a 400-nucleotide sequence from the 3'-end of the amino-acid coding region of the PGHS-2 cDNA (ORF 1516-1915) was directionally subcloned into the EcoRI-PstI sites of Bluescript-2. The PGHS-2 nucleotide sequence contained the region that encodes the 18-amino acid C-terminal of the enzyme that is unique to

PGHS-2 (Xie *et al.*, 1992). These sequences were selected to render the respective probes highly specific for either PGHS-1 or PGHS-2 mRNA.

Human GAPDH cDNA was obtained from American Type Tissue Culture as a 1.2 kb insert in the Pst1 site of pBR322 (Tso *et al.*, 1985). A 387-base sequence (ORF 539-926) was isolated from the cDNA by digestion with Acc1 and Xba1 (5' and 3' respectively) and was directionally subcloned into the corresponding sites of Bluescript 2 (Zakar *et al.*, 1998). This sequence was selected since Xba1 cuts uniquely into the coding region of the functional gene in the genome.

The human γ-actin probe was transcribed from a cDNA derived from the pHFgA cDNA clone (Gunning *et al.*, 1984). A 270 nucleotide sequence of the C-terminal amino acid coding region subcloned into pGEM-3 for the production of template for the generation of the γ-actin probe.

The identity of all the subcloned sequences was verified by restriction analysis (Fig. 2-2).

In vitro transcription

Antisense riboprobes were transcribed from the linearized plasmids in the presence of 0.15 mCi ^{32}P-CTP using 0.25 μg template with 75 units T3 or T7 RNA polymerase, 0.9 μL of 200 μM dithiothreitol (DTT), 1 μL each of 4 mM unlabelled ATP, UTP and GTP, and 30 units placental ribonuclease inhibitor in transcription buffer (200 mM TrisCL [pH 8], 40 mM $MgCl_2$, 10 mM spermidine, 250 mM NaCl). At the completion of the transcription reaction, the template DNA was removed from the reaction mixture by the addition of 150 units ribonuclease-free deoxyribonuclease-1. After phenol-chloroform extraction and ethanol precipitation, radiolabeled probes were purified by electrophoresis in 6% polyacrylaminde gels. The specific activities of the PGHS-1, PGHS-2, γ-actin and GAPDH probes were 3-5 x 10^8 cpm/μg, 5-8 x 10^8 cpm/μg, 1-3 x 10^8 cpm/μg and 2-6 x 10^8 cpm/μg respectively.

Sense cRNA probes for use as negative controls were also prepared using the conditions described above.

Hybridization

Total tissue RNA (40 μg) was hybridized in solution with either the PGHS-1 or PGHS-2 probe. Each hybridization was incubated for 16h at 55°C in a 40 μL reaction volume with 1-1.5 x 10^6 cpm/probe in the presence of 80% formamide and 20% 1,4-PIPES (pH 6.4) in buffer containing 1 mM EDTA, 0.2 mM NaCl.

To verify the uniform abundance of the mRNA in the tissue RNA samples, the level of the constitutively expressed γ-actin or GAPDH was also measured. Ten μg total RNA of the same samples as used for PGHS-1 and PGHS-2 mRNA determination was hybridized with the γ-actin or GAPDH cRNA probe under identical conditions.

Hybridizations of sample RNA with sense cRNA probes, along with yeast tRNA and antisense cRNA probes, served as negative controls

Digestion

Following hybridization, the reaction mixture was subjected to digestion with 2.5 mg/mL ribonuclease A (20 min; 30°C) and 3000 units ribonuclease T1 (20 min; 37°C) in buffer containing 10 mM TrisCl (pH 7.5), 300 mM NaCl and 5mM EDTA. At the conclusion of these digestions, the ribonucleases were removed by treatment with 20 μg/mL proteinase K in the presence of 0.1% sodium dodecyl sulfate (30 min; 37°C), followed by phenol-chloroform extraction and ethanol precipitation.

Detection

The protected RNA fragments were separated by electrophoresis in 8% polyacrylamide denaturing gels. The gels were dried and exposed for an appropriate time to XAR X-ray film. The sizes of the protected radioactive bands were estimated using ^{32}P end-labeled *Hinf*I-digested OX174 DNA size markers.

Data analysis

PGHS-1 and PGHS-2 mRNA levels in tissue samples were evaluated by quantification of the protected bands on autoradiographs by densitometry with a GS-670 Bio-Rad imaging densitometer. Peaks corresponding to the protected bands were integrated with a software package supplied by the manufacturer. In all sets of hybridization reactions, a pooled tissue RNA preparation were included as PGHS-1, PGHS-2, γ-actin or GAPDH standards. The standards were assigned a densitometric value of one on the autoradiograms, and PGHS-1, PGHS-2, γ-actin and GAPDH densitometric intensities were normalized to that value. The PGHS densitometric values were divided by the γ-actin or GAPDH mRNA densitometric values measured in the same RNA preparations. The resultant ratio is referred to as PGHS mRNA band intensity and is interpreted as representing PGHS-1 and PGHS-2 mRNA levels corrected for variations between individual RNase protection assays and RNA sample quality. Backgrounds due to nonspecific probe protection in each assay were determined by hybridizing labeled antisense probes to yeast tRNA (40 μg), and labeled sense probes to sample RNA. Undigested probe was also electrophoresed in each assay to further verify specific protection.

2.2.3. Fetal Fibronectin Assay

Sample preparation

Cervico-vaginal swabs were collected from term patients immediately before the time of sectioning and assayed for fetal fibronectin. Patients were excluded on the basis of membrane rupture as defined by the presence of two or more of the following: gross pooling of the amniotic fluid, an alkaline vaginal pH on Nitrazine paper, ferning of dried vaginal secretions on microscopy, and ultrasoniographic diagnosis of a decreased volume of amniotic fluid. Cervico-vaginal fluids were sampled using Dracon swabs. Each swab was left in place for 10 seconds and then

withdrawn and placed in 750 µL of sample buffer. Since the Dracon swab held 150 µL of fluid when saturated, the approximate dilution of cervico-vaginal secretions in the buffer was 1 to 5.

Fetal fibronectin enzyme immunoassay

Concentrations of fetal fibronectin antigen were measured with a sensitive immunoassay. This assay used the fetal fibronectin specific monoclonal antibody FDC-6 raised against the epitope unique to the fetal form of fibronectin. Cervico-vaginal samples were incubated in microtitre wells coated with an affinity purified mouse monoclonal antibody to human fetal fibronectin. The resulting antibody-antigen complex was washed to remove non-specifically bound material, then reacted with a goat antihuman plasma fibronectin IgG conjugated to alkaline phosphatase. Following formation of the antigen-antibody sandwich, the microtitre wells were washed to remove unbound labeled antibody, then incubated with a phenolphthalein monophosphate enzyme substrate. The amount of fetal fibronectin contained in each standard and sample was determine in duplicate at a wavelength of 550 nm with an automated microtiter-plate reader.

Data analysis

Fetal fibronectin concentrations were derived from the SoftMax software program. The interassay and intraassay coefficients of variation were less than 10%, and fetal fibronectin was detected in the range of 0.02 to 4 µg per mL.

2.2.4. *In Situ* Hybridization (Mijovic *et al.*, 1997, 1998).

Sample preparation

Small pieces of full thickness membranes were rolled and fixed in 10% formalin in PBS for 24-48 h at room temperature. These fixed tissues were then dehydrated and embedded in paraffin blocks. Five µm thick sections were cut and

collected on ribonuclease free, 3-aminopropylethoxysilane coated slides and stored at room temperature until assay.

Pretreatments

Sections were dewaxed and rehydrated by passing the slides through a graded series of xylenes and ethanols and then DEPC treated water. Slides were placed in 0.2 M hydrochloric acid for 30 min at room temperature, followed by 3% Triton-X 100 for 15 min at room temperature. The sections were then treated with 0.1 M TrisCl (pH 7.5), and 0.5 mM EDTA containing 100 μg/mL proteinase K for 30 min at 37°C. Following this, they were rinsed in 0.1 M TrisCl (pH 7.5), 0.1 M NaCl containing 0.2 % glycine; postfixed for 5 min with 10% formalin in PBS; acetylated for 10 min with 0.25% acetic anhydride containing 0.1 M triethanolamine at room temperature; and prehybridized for 60 min at 37°C in solution containing 50% formamide, 2xSSC (3 M NaCl, 0.34 M sodium citrate, [pH 7.0]), 1 x Denhardt's solution (0.02% (w/v) each of BSA, Ficoll 400 and polyvinylpyrrolidone), 1 μg/mL tRNA, 50 mM PBS, 1 mM EDTA and 5% dextran sulfate.

In vitro transcription

Digoxigenin-labeled sense and antisense probes were synthesized from the human PGHS-1 and PGHS-2 cDNA used in the ribonuclease protection assay. 1 μg template cDNA was incubated with 40 units T3 or T7 RNA polymerase, 1mM each of ATP, CTP and GTP, 0.65 mM UTP, and 0.35 mM digoxigenin-conjugated UTP, and 20 units of placental ribonuclease inhibitor, in transcription buffer (200 mM TrisCL [pH 8], 40 mM $MgCl_2$, 10 mM spermidine, 250 mM NaCl). Following transcription, template DNA was removed by digestion with 20 units ribonuclease free deoxyribonuclease-1 at 37°C for 20 minutes. The labeled RNA was isolated by ethanol precipitation in the presence of 4 M lithium chloride. The transcripts were analyzed by agarose-gel electrophoresis and yields were estimated by absorbance at 260 nm.

Hybridization

The tissue sections were overlaid with probe solution (500 ng/mL, in hybridization buffer), covered with microscope coverslips and incubated overnight in a humid chamber at 37°C. The hybridization solution consisted of 50% formamide, 2 x SSC, 1 x Denhardt's solution, 1 μg/mL tRNA, 50 mM phosphate buffered saline, 1 mM EDTA, and 5% dextran sulfate. The unhybridized probe was washed away with 2 x 15 min washes in 2 x SSC, 1 x 10 min wash in 1 x SSC at room temperature, and 1 x 10 min wash in 0.1 x SSC at 37°C.

Detection

The hybridized probe was then visualized by enzyme linked immunoassay using the DIG Nucleic Acid Detection kit (Boehringer Mannheim). The DIG labeled RNA probes were detected using an antibody-conjugate (anti-digoxigenin alkaline phosphatase conjugate, Anti-DIG-AP). A subsequent enzyme catalyzed color reaction with 5-bromo-4-chloro-3-indolyl phosphate (X-phosphate) and nitroblue tetrazolium salt (NBT) produced an insoluble purple precipitate that visualized the hybrid molecules.

Counterstaining

The tissue sections were counterstained using a cytokeratin immunohistochemistry detection kit according to the manufacturer's instructions (Sigma). Initially, endogenous peroxide was quenched with 3% hydrogen peroxide. A mouse monoclonal anti-α-cytokeratin primary antibody was then applied to the tissue sections. Following a brief wash, the section was incubated with a biotinylated goat anti-mouse IgG secondary antibody. The ExtrAvidin-conjugated peroxidase reagent was added and a stable avidin-biotin complex was formed with the bound biotinylated antibody. The sites of antibody deposition were visualized by the addition of freshly prepared substrate which contained hydrogen peroxide and the chromagen 3-amino-9-ethyl-carbazole (AEC; an electron donor). The bound

peroxidase catalyzed the oxidation of the AEC to form a reddish-brown insoluble precipitate at the antigen sites

Data analysis

Slides were examined by light field microscopy and photographed. Six sections from membranes from each patient chosen for analysis by *in situ* hybridization were examined; this yielded approximaltely 5-10 fields of view per section, and 30-60 fields of view per patient.

Figure 2-1 Saturation of PGHS in chorion microsomes by increasing concentrations of arachidonic acid. Increasing concentrations of arachidonate were incubated in the presence of enzyme cofactors and microsomes for 4 minutes. Prostaglandin E_2 produced was extracted and measured by radioimmunoassay. The data was normalized for microsomal protein content and expressed as picograms of PGE_2 produced per μg microsomal protein per minute. Results are expressed as mean ± SEM of five individual data points obtained from five individual chorions.

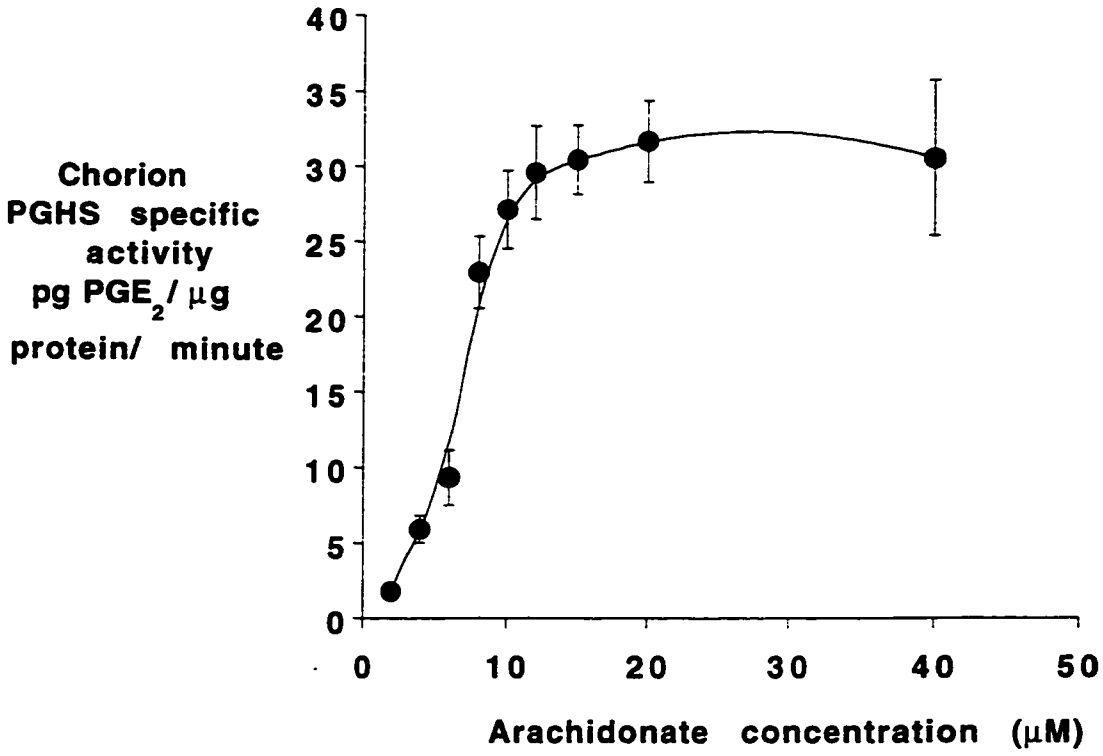

Chorion
PGHS specific
activity
pg PGE2 / µg
protein/ minute
40
35
30
25
20
15
10
5
0
0
10
20
30
40
50
Arachidonate concentration (µM)

Figure 2-2 A Restriction analysis identifying the 387-base sequence (ORF 539-926) isolated from the GAPDH cDNA by digestion with Acc1 and Xba1 (5' and 3' respectively) and directionally subcloned into the corresponding sites of Bluescript-2 (Zakar *et al.*, 1998). This sequence was selected since Xba1 cuts uniquely into the coding region of the functional gene in the genome. This restriction site is not present in the nonfunctional GAPDH gene-related sequences known to exist, some of which are processed pseudogenes.

Lane 1 Intact Bluescript-2 plasmid containing GAPDH cDNA insert in the subcloning site.

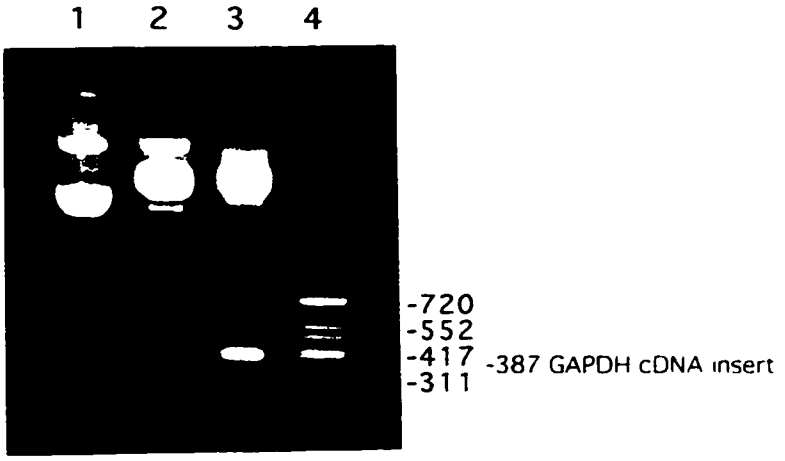
Lane 2 Restriction analysis of Bluescript-2 containing the subcloned GAPDH cDNA fragment digested with Xba1.

Lane 3 Restriction analysis of Bluescript-2 containing the subcloned GAPDH cDNA fragment in Bluescript 2 digested with Xba1 and Acc1.

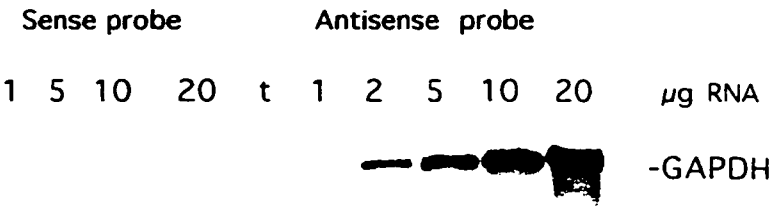
Lane 4 OX174 DNA/*Hinf* 1 molecular weight size markers.

B Autoradiograph showing GAPDH mRNA in human decidua measured by ribonuclease protection assay. The results of this experiment show a parallel rise in band intensity when increasing amounts of decidua mRNA (1-20 μg) were hybridized with equal amounts of the labelled GAPDH probe. No bands were detected when the probe was hybridized to 10 μg transfer RNA (tRNA, lane t) serving as a negative control or when decidua RNA was hybridized with labeled sense GAPDH probe.

A


1
2
3
4
-720
-552
-417
-311
-387 GAPDH cDNA insert

B


Sense probe
Antisense probe
1 5 10 20 t 1 2 5 10 20
µg RNA
-GAPDH

2.3. References

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3. CHANGES IN PGHS ACTIVITY AND mRNA LEVELS IN HUMAN INTRAUTERINE TISSUES WITH LABOR AT TERM

3.1. Introduction

The preceding chapter (Chapter 1) has described the evidence indicating that PGs are important in labor initiation in humans and other species; and documented the observed increases in PG levels in the biological fluids of women at parturition. The origins of these elevated PG levels must now be considered. The fetal membranes (amnion and chorion) and maternal decidua have a unique anatomical location and enzymatic capability and are therefore proposed to play a crucial role in producing the elevated PG concentrations seen at labor. An active area of research involves assessing the relative contributions of each of these tissues to the elevated PG levels at parturition by the quantification and localization of the PG synthesizing and metabolizing enzymes.

The amnion is an avascular fetal membranes that is bathed by amniotic fluid on one side and is contiguous with the chorion on the other. To date, the amnion has been proposed to be the major source of intrauterine PGs at labor in humans (Challis and Olson, 1988). Indeed, in the amnion there is an increase in PGHS specific activity at this time which has been attributed to a selective rise in the abundance of PGHS-2 mRNA (Teixeira *et al.*, 1994; Hirst *et al.*, 1995).

The chorion laeve is positioned between the amnion and the decidua and contains high concentrations of PG metabolizing enzymes (McCoshen *et al.*, 1987; Mitchell *et al.*, 1993; Sangha *et al.*, 1994). Most previous studies indicate there is no change or a decrease in the PG metabolic capacity of this tissue at term and with labor onset in humans (Sangha *et al.*, 1994; Van Meir, 1996). The chorion laeve also contains PGHS activity (Okazaki *et al.*, 1981), but knowledge of its contribution to PG synthesis at parturition is limited. Based on these observations, we developed the

hypothesis that an increase in PG synthesis in the chorion may result in a rise in the net concentration of biologically active PGs in the intrauterine compartment in association with labor in women.

The maternally derived decidua is found adjacent to the fetal chorion on one side and the myometrium on the other. Fuchs *et al.* (1982) suggest that the decidua is the major source of intrauterine $PGF_{2\alpha}$ during late gestation and labor; and $PGF_{2\alpha}$ production by decidual tissue has been reported to increase in association with labor onset by several groups of investigators (Skinner and Challis, 1985; Norwitz *et al.*, 1992; Khan *et al.*, 1992). The close proximity of the decidua to the myometrium suggests that decidual activation involving the elevated production of $PGF_{2\alpha}$ may be important at parturition (Casey and MacDonald, 1988). *In situ* hybridization showed both PGHS-1 and PGHS-2 mRNAs are present in term decidua (Slater *et al.*, 1995), however, there is no information regarding the level of their expression before and after labor. The contribution of the two PGHS isoenzymes to PG synthesis in term decidua is unknown.

In order to define the potential contribution of the chorion and decidua to the PGs involved in the processes causing birth in humans further studies on the PG synthetic capacity of these tissues at labor are needed. In the present study we have compared the level of PGHS specific activity in chorion and decidua obtained before and after labor at term. Further, we have determined the abundance of PGHS-1 and PGHS-2 mRNA in these tissues as a reliable isoform-specific measure of PGHS expression. We used these values to assess the contribution of the two PGHS isoenzymes to the PG synthetic capacity of the chorion and decidua at term labor. Finally, the localization of PGHS-1 and PGHS-2 mRNAs was studied to detect labor associated changes in the tissue distribution of these enzymes.

3.2. Methods

3.2.1. Tissue Collection

Patient data

Placentas with attached fetal membranes were obtained from a total of 50 uncomplicated singleton pregnancies. Twenty-seven were collected after term elective cesarean section (CS) (37-41 weeks; mean ± SEM = 38.5 ± 0.8 weeks [gestational age calculated from the first day of the last menstrual period]) in the absence of labor (defined as ≤ 1 uterine contraction/10 min, ≤ 2 cm cervical dilatation as determined by pelvic examination, and intact membranes); and 23 were collected following noninduced, nonaugmented term spontaneous vaginal delivery (SL) (mean ± SEM = 38.7 ± 0.3 weeks). Women with clinical signs of inflammation or genital infection (fever, foul vaginal discharge) or bacterial vaginosis were excluded from the study. Additionally, all patients were routinely tested for the presence of group B streptococcus in the vaginal flora, and those found positive were excluded from the study.

In order that accurate comparisons could be made from tissues collected from a group of women at term CS compared to those following SL the placentas and membranes collected from the SL group of patients were used only if they were delivered within twenty to thirty minutes following birth of the infant. This ensured prolonged contact of tissues with cytokines, toxins and bacterial flora in the vaginal environment was avoided. These agents are known to induce PGHS-2 expression and PG output from many tissues and therefore may have caused us to draw false conclusions. Evidence from the literature suggests that these regulators take 2-4 h to induce increased PGHS expression in gestational tissues and that an even longer period of exposure is needed for an increase in PG output to occur (Alnaif *et al.*, 1993). Therefore, we are confident our data reflect real physiological events rather than artifacts resulting from the tissue collection protocol.

The mean gestational ages of SL and CS patients were not significantly different. The use of these tissues was approved by the University of Alberta Ethical Review Committee.

Tissue processing

The placenta was separated from the fetal membranes immediately after delivery by cutting around the placental margins. Membranes were washed in physiological saline to remove excess blood. Small pieces of full-thickness membrane were cut, rolled, and fixed in 10% formalin in phosphate buffered saline (PBS) for 24-48 h at room temperature (RT). The remaining amnion was separated from the chorio-decidua by blunt dissection; decidual tissue was then scraped from the chorion using a blade. Histological examination revealed that almost all the decidua had been separated from the chorion. Chorion and decidua samples were cut into strips (10 x 20 mm), washed repeatedly with physiological saline to remove clotted blood and snap frozen in liquid nitrogen. The effects of the trauma of isolation on tissue PG production was minimized by limiting preparation time to < 20 min. Each sample of frozen tissue was pulverized using a dry-ice cooled pestle and mortar and separated into batches for RNA extraction and enzyme activity assay.

The formalin fixed tissue samples were processed for *in situ* hybridization and histological analysis by dehydration and embedding in paraffin block.

Full thickness membrane samples from all patients were examined histologically for evidence of neutrophil invasion as a sign of inflammation; tissues that showed positive for inflammation were excluded from the study.

3.2.2. PGHS Enzyme Activity Assay

The method to assay PGHS activity in amnion tissue has been characterized in our laboratory (Smieja *et al.*, 1993; Teixeira *et al.*, 1993, 1994). This methodology

was validated for use in the chorion and decidua and is described in detail in Chapter 2.

3.2.3. Ribonuclease Protection Assay

Total RNA was isolated from chorion and decidua tissues by the guanidine thiocyanate-phenol-choloroform method (Chomczynzki and Sacchi, 1987).

The ribonuclease protection assay for the measurement of PGHS-1 and PGHS-2 mRNA levels in amnion has previously been reported (Hirst *et al.*, 1995). The protocol (Chapter 2) was found to be optimal for use with chorion and decidua, as well as amnion.

3.2.4. *In Situ* Hybridization (Mijovic *et al.*, 1997).

This methodology was designed and validated as part of these studies and is described in detail in Chapter 2.

3.2.5. Data Assessment and Statistical Analysis

PGHS enzyme specific activity values are expressed as picograms of PGE_2 produced /μg microsomal protein /minute.

PGHS-1 and PGHS-2 mRNA levels in tissue samples were evaluated as previously described (Chapter 2).

Differences between PGHS enzyme activity levels and PGHS-1 and PGHS-2 mRNA levels in samples from CS and SL patients were analyzed using the nonparametric Wilcoxon signed rank test (Significance $P<0.05$). This method of analysis was considered to be most appropriate as the variance of the values in the two groups was found to be heterogeneous (Bartlett's test).

PGHS mRNA levels measured as densitometric band intensities were compared with enzyme activity by linear regression. Significance was achieved at $P<0.05$.

In situ hybridization data were analyzed qualitatively as described in Chapter 2.

3.3. Results

3.3.1. PGHS Enzyme Activity in Intrauterine Tissues during Term Labor

PGHS specific activity was determined in the microsomal fraction of chorion and decidua homogenates. These values reflect total enzyme activity of all isoforms, and results are presented in Figure 3-1.

Chorion

PGHS specific enzyme activity in the chorion in the term postlabor SL group (mean ± SEM, 32.1 ± 1.6; median, 31.45; range, 19.5-42.8; pg of PGE_2 /μg protein /min; n=14) was significantly higher than in tissues collected from patients at term CS (mean ± SEM, 17.4 ± 2.7; median, 14.72; range, 4.28-38.09; pg of PGE_2 /μg protein /min; n=17) (Wilcoxon signed rank test; $P=0.001$) (Fig. 3-1A).

Decidua

There was no significant difference in the PGHS specific activity levels in decidua collected from term CS (mean ± SEM, 111.5 ± 31.2; median, 74.5; range, 34-360; pg of PGE_2 /μg protein /min; n=10) and SL patients (mean ± SEM, 110 ± 27.5; median, 85; range, 23-275; pg of PGE_2 /μg protein /min; n=10). (Wilcoxon signed rank test; $P>0.05$) (Fig. 3-1B).

3.3.2. PGHS-1 and PGHS-2 mRNA Abundance in Intrauterine Tissues during Term Labor

Chorion

A composite autoradiograph detecting PGHS-1 mRNA in five CS and five SL chorion samples is shown in Figure 3-2Aa. The corresponding γ-actin mRNA bands are also shown. Altogether, seventeen chorion samples from CS patients and fourteen samples from SL patients were analyzed for PGHS-1 mRNA abundance. PGHS-1 mRNA was detected in all but two chorion samples collected from patients following SL. When quantified these data showed there was no significant difference in the pattern of abundance between CS (mean ± SEM, 0.24 ± 0.05; median, 0.17; range, 0.06-0.91; relative densitometric units; n=17) and SL tissues (mean ± SEM, 0.25 ± 0.05; median, 0.25; range, 0-0.65; relative densitometric units; n=14) (Wilcoxon signed rank test; $P>0.05$) (Fig. 3-2Ab).

PGHS-2 mRNA levels from the same patients were also measured. The results obtained are shown in Figure 3-2B. PGHS-2 was detected in all but two patients from the term CS group. PGHS-2 band intensities in the chorion of SL patients (mean ± SEM, 1.3 ± 0.14; median, 1.27; range, 0.36-2.52; relative densitometric units; n=14) were significantly higher than those collected at CS (mean ± SEM, 0.43 ± 0.11; median, 0.3; range, 0-1.91; relative densitometric units; n=17) (Fig. 3-2Bb) (Wilcoxon signed rank test; $P=0.0001$).

PGHS mRNA and PGHS activity levels were correlated in patients where both parameters were determined. There was no statistically significant correlation between PGHS-1 mRNA levels and PGHS specific enzyme activity ($P>0.05$) (Fig. 3-3A). However, there was a significant positive correlation between PGHS-2 mRNA levels and total PGHS enzyme activity in these samples ($r=0.87$; $P<0.05$) (Fig. 3-3B).

Decidua

The expression of PGHS isoenzymes in the decidua was also examined by ribonuclease protection assays. Figure 3-4Aa shows a composite autoradiograph of PGHS-1 mRNA levels in six CS and eight SL patients. Altogether, twenty-six decidua samples from CS patients and twenty samples from SL patients were analyzed for PGHS-1 mRNA abundance. As demonstrated in Figure 3-4Ab, there was no significant difference in the levels of PGHS-1 mRNA between the two groups (CS: mean ± SEM, 0.28 ± 0.05; median, 0.24; range, 0.04-1.2; relative densitometric units; n=26; SL: mean ± SEM, 0.4 ± 0.07, median, 0.26; range, 0.09-1.56; relative densitometric units; n=20; $P>0.05$).

A similar study of decidual PGHS-2 mRNA levels is shown in Figure 3-4B. Decidua from twenty-seven CS and twenty-three SL patients were analyzed in this series of ribonuclease protection assays and results of samples from eight CS and ten SL patients are presented in Figure 3-4Ba (composite autoradiograph). No significant change in PGHS-2 mRNA abundance was detected with labor (CS: mean ± SEM, 0.9 ± 0.26; median, 0.32; range, 0.01-4.6; relative densitometric units; n=27; SL: mean ± SEM, 0.71 ± 0.18; median, 0.47; range, 0.05-3.7; relative densitometric units; n=23; $P>0.05$; Fig. 3-4Bb).

When PGHS-1 and PGHS-2 mRNA abundance was correlated with PGHS enzyme activity in these tissues, a significant positive correlation between mRNA abundance and enzyme activity was found in the case of both isoforms (Fig. 3-5). These data indicate that both PGHS-1 and PGHS-2 contribute to the total PGHS activity present in the decidua.

3.3.3. *In Situ* Hybridization

PGHS-1 and PGHS-2 mRNA localization in term CS and SL membranes visualized by *in situ* hybridization is shown in Figures 3-6 and 3-7. Tissues were counterstained with the epithelial cell marker cytokeratin. Cytokeratin positive amnion and decidual epithelial cells, and chorionic trophoblast cells stained brown, while hybridization of the PGHS-mRNA probes resulted in purple immunostaining superimposed on the brown color.

Panels A and C, Figure 3-6, shows staining for PGHS-1 mRNA throughout term full thickness membranes collected at CS and SL respectively. B and D indicate the respective negative controls stained for cytokeratin and with only slight nonspecific hybridization of the sense PGHS-1 probe. In a representative tissue collected from a patient at term CS, PGHS-1 mRNA was located in the amnion epithelium, the cytokeratin negative cells of the amnion mesoderm, the chorion trophoblast, the cytokeratin negative cells of the chorion mesoderm, and the decidua The pattern of staining appeared markedly heterogeneous since some cells exhibited strong hybridization while others, often adjacent, showed low levels of hybridizing material; this is best visualized under higher magnification (Fig. 3-6; Panel E and F). In membranes collected from a representative patient following term labor, the localization of PGHS-1 mRNA did not differ from that described in membranes collected from patients before labor at term CS (Fig. 3-6; Panel A and C).

Membranes from the same patients show the localization of PGHS-2 mRNA at term CS and SL (Fig. 3-7, Panel A and C respectively). Panel B and D are the negative controls indicating little nonspecific hybridization. The localization of PGHS-2 mRNA at term CS (Panel A) appeared similar to that of PGHS-1 (Fig. 3-6; Panel A). Heterogeneous staining was seen in the amnion epithelial cells, the cytokeratin-negative component of the amnion mesoderm, the chorion trophoblast, and the decidua, with stronger staining in the fibroblastic component of the chorion.

In tissues collected from patients at term SL (Panel C), a greater number of cells throughout the fetal membranes appeared to express PGHS-2 mRNA. In decidua, labor had no apparent effect on the distribution of this mRNA among the cells. Although the pattern of staining was more intense in amnion and chorion following term SL, it remained heterogeneous especially in the epithelial component of the membranes as shown by higher magnification (Panels E and F).

3.4. Discussion

3.4.1. PGHS Specific Activity and PGHS-1 and PGHS-2 mRNA Abundance in Chorion during Term Labor

In this study we report differences in the specific activity of PGHS in human chorion before and after the onset of spontaneous labor at term. The data indicate that PGHS specific activity is approximately two-fold greater in chorion tissues collected following term labor compared to those at CS of the same gestational age. This is consistent with the findings of Olson *et al.* (1983) who reported PG output from dispersed chorion cells maintained in vitro. They demonstrated a 1.5-2 fold greater PG output of the E and F series in cells from chorion tissues collected at term SL compared to CS.

However, others have demonstrated that a similar increase in the output of biologically active PGs from such tissues did not occur, rather showing up to 4-fold increases in the production of PG metabolites (Mitchell, 1988; Okazaki *et al.*, 1981; Skinner and Challis, 1985). The chorion is known to have a considerable capacity to metabolize PGs predominantly by the enzyme PG dehydrogenase (PGDH) (Kierse and Turnbull, 1976). Mitchell *et al.* reported a 2-fold increase in the activity of PGDH in chorion at SL compared to CS (Mitchell *et al.*, 1993). Other groups found no such change in activity in choriodecidua and reported a concomitant decrease in

the PGDH protein and mRNA content of these tissues with labor (Sangha *et al.*, 1994; Van Meir, 1996). These results are not consistent, probably due to the use of different tissue preparations (chorion vs choriodecidua), incubation protocols, or patients selected by varying clinical criteria. Nonetheless, even the greatest change in metabolic capacity of the chorion reported (Mitchell *et al.*, 1993) cannot solely account for the increase in the output of PG metabolites from this tissue seen with labor. Rather, for a large elevation in the product of the metabolic enzymes to occur with little or no change in the activity or expression of these enzymes, there must be an increase in the amount of their substrate; therefore, there must be a rise in PG synthesis.

Previously, it has been shown that the amnion is an important intrauterine source of biologically active PGs at term (Mitchell, 1988; Okazaki *et al.*, 1981; Olson *et al.*, 1983; Skinner and Challis, 1985, Teixeira *et al.*, 1994). In the present study we show equivalent levels of PGHS specific activity are present in term chorion as were detected in amnion of the same gestational age; we have also observed a 2-fold increase in PGHS enzyme specific activity in chorion with labor onset, similar to that seen in amnion (Teixeira *et al.*, 1994). Taken together, these data suggest there is a significant increase in the PG synthetic capacity of the chorion at term SL. Khan *et al.* (1991) calculated that the sum of PG production by the choriodecidua at this time outweighed that of amnion by >100-fold. This in itself is not surprising due to the greater tissue mass of the choriodecidua compared to the amnion. However, as the chorion metabolizes >98% of this production it can be estimated that a 1% decrease in this metabolism by the chorion would increase PG levels 2-3 fold, while a 5% decrease would elevate PG levels to 10-50 ng/ml. These are levels similar to those detected in amniotic fluid during labor. Alternatively, a 1-5% increase in the PG synthetic capacity of the chorion would have a similar effect. Thus the increase in PGHS specific activity seen here in tissue collected following term labor, and

reported by others (Okazaki *et al.*, 1981), raises the possibility of an important role for chorion PG production in the process of term labor. While this role has previously been assigned to the amnion, the latter comment requires important consideration as the mass of chorion tissue is much greater than that of the amnion in term membranes.

In this study, we found a significant 2-fold rise in PGHS-2 mRNA levels with term labor in chorion tissue. No change was seen in the expression of PGHS-1 mRNA. Bennett *et al.* (1992) reported a 2.5-fold rise in PGHS mRNA expression in human chorion trophoblast using Northern hybridization, however they could not specify if this was due to PGHS-1 or PGHS-2. In another study Freed and coworkers (1995) found no change in PGHS-1 mRNA levels in human choriodecidua using a specific sheep PGHS-1 cRNA probe and Northern analysis. They did not estimate PGHS-2 mRNA abundance. Our data indicate that increased PGHS-2 expression is responsible for the increase in total PGHS specific enzyme activity seen in chorion tissue after spontaneous labor at term. This is further supported by our observation of a positive correlation between PGHS-2 mRNA abundance and total tissue PGHS enzyme activity in all the tissues studied. There was no similar correlation with PGHS-1 mRNA.

In summary, we report an increase in the levels of PGHS activity and PGHS-2 mRNA after spontaneous labor in term human chorion. Our results suggest that induction of PGHS-2 is of principal importance in determining PGHS activity in late gestation and with labor onset in this tissue. We propose that subtle changes in the balance between PG synthesis and metabolism in the chorion may result in a substantial increase in the levels of active PG reaching the maternal side of the membranes. This is likely one source of the PGs that are involved in the stimulation of normal term labor in humans.

3.4.2. PGHS Specific Activity and PGHS-1 and PGHS-2 mRNA Abundance in Decidua during Term Labor

There is discrepancy in the literature regarding PG output from the human decidua at term parturition. Willman and Collins (1976) determined the content of PGs in decidua and found a significant increase in both PGE_2 and $PGF_{2\alpha}$ content with the onset of labor. Satoh *et al.* (1981) reported the output of PGF, but not of PGE, by minced decidua was significantly higher from tissue collected during labor, than from tissue collected before labor, or at delivery. Furthermore, Skinner and Challis (1985) as well as Norwitz *et al.* (1992) measured increased output of $PGF_{2\alpha}$ from dispersed decidua cells with labor onset. However, no labor related change in PGE_2 or PGD_2 output was found in the latter study. Also, decidual stromal cells prepared from tissues collected following labor when separated by density gradient centrifugation were reported to release more PGE_2 and $PGF_{2\alpha}$ compared to cell preparations from tissues collected at term prior to labor (Khan *et al.*, 1992). Finally, Ishihara *et al.* (1996) reported recently that Percoll gradient-separated decidual cells produced more PGE_2 with labor onset. In contrast, Olson *et al.* (1993) showed only a small increase in PGE and PGF output by dispersed decidual cells with labor which did not reach statistical significance, and Lopez-Bernal *et al.* (1987) reported no change in PGE_2 output from decidual cell suspensions with term labor onset.

We have shown the regulated expression of PGHS in the amnion (Teixeira *et al.*, 1994; Hirst *et al.*, 1995) and chorion plays a major role in the control of PG production at term. To determine whether a similar type of regulation exists in the decidua, we have measured the specific activity of PGHS and the abundance of PGHS-1 and PGHS-2 mRNA in this tissue before and after labor at term.

We found no change in PGHS specific activity in decidua with labor. This is in agreement with previous studies where the arachidonic acid-promoted PG output of dispersed decidua cells or decidua homogenates was not affected by labor onset

(Lopez-Bernal *et al.*, 1987; Okazaki *et al.*, 1981). No differences in the mean levels of PGHS-1 or PGHS-2 mRNAs were detected in association with labor in decidua, and correlation analysis suggested that both isoenzymes contributed to the steady-state activity of PGHS. This is in contrast to the observations described in the human amnion (Hirst *et al.*, 1995) and chorion which showed enzyme activity was correlated exclusively with PGHS-2 mRNA abundance, and that an increase in enzyme activity and PGHS-2 mRNA occurred with labor onset at term. The lack of such increases in the decidua clearly indicates that PG production is controlled by a different mechanism in this tissue.

In the absence of changing PGHS expression, alternative mechanisms may play a role in the regulation of decidual PG biosynthesis. For example, both PGHS isoenzymes require glycosylation for full activity (O'Neill *et al.*, 1994), and each isoenzyme may require different concentrations of peroxide for the same level of activity (Kulmacz and Wang, 1995). Additionally, PGHS activity is affected by arachidonate availability. The gestational tissues are rich in esterified arachidonic acid, and contain substantial phospholipase activity (Olson *et al.*, 1993). Phospholipases may be regulated acutely and transiently, explaining some of the discordant findings regarding the PG output of isolated decidua tissue and cells before and after labor. Furthermore, evidence suggests endogenous and exogenous arachidonic acid are utilized differently by the two isoenzymes of PGHS (Reddy and Herschmann, 1994, 1996) leading to differential access of substrate to the two PGHS isoenzymes. Finally, the presence of a 55-60 kDa protein in human decidua which inhibits PGHS activity and may act in a gestational age or labor-related manner on PGHS-1 and/or PGHS-2 has been reported (Kinoshita *et al.*, 1977; Ishihara *et al.*, 1990).

In summary, we report that neither PGHS-2 nor PGHS-1 expression increases with term labor onset in human decidua. Although there is no change in the total

PGHS specific activity with labor onset, both PGHS mRNAs are positively correlated with enzyme activity. These data suggest that further studies exploring phospholipase activation or post-translational regulation of PGHS enzyme activity may be fruitful in gaining greater insight into PG synthesis regulation in human decidua.

3.4.3. Localization of PGHS-1 and PGHS-2 mRNA in Fetal Membranes and Decidua during Term Labor

Localization of PGHS-1 and PGHS-2 mRNA was determined by *in situ* hybridization of digoxigenin-labeled PGHS-1 and PGHS-2 cRNA probes to full thickness human membranes (Fig. 3-6 and 3-7). The degree of staining for PGHS-1 mRNA in amnion, chorion and decidua did not seem to change with labor onset. A greater number of cells throughout the fetal membranes appeared to express the PGHS-2 isoform in tissues collected following term SL compared to CS. In decidua there was no detectable variation in numbers and types of cells stained or intensity of staining for PGHS-2 mRNA with labor.

The literature concerning the distribution of PGHS-1 and PGHS-2 mRNA in fetal membranes and decidua determined by *in situ* hybridization is different. Slater *et al.* (1995) detected PGHS-1 and PGHS-2 mRNA in amnion epithelium and fibroblasts and decidua; but found only PGHS-1 exists in chorion. Khan *et al.* (1992) detected increased staining for PGHS protein in decidual stromal cells following labor although the isoform specificity of the antibodies used in this study was not clear. The variations in these findings are probably due to different assay conditions and treatment protocols. Our cRNA probes have previously been verified as being specific from their use in ribonuclease protection assays (Hirst *et al.*, 1995), and our *in situ* hybridization methodologies have been optimized for maximum sensitivity.

Notably, there was extensive variability in the abundance of both PGHS-1 and PGHS-2 mRNAs among individual cells of the same type in all tissues. Such

heterogeneity has been reported for PGHS protein and mRNA in previous studies (Slater *et al.*, 1995), but also for the PG metabolizing enzyme PGDH in the chorion (Sangha *et al.*, 1994). It is possible that cells that do not contain PGDH represent areas through which PGs may pass, unmetabolized, from the fetal to the maternal side of the membranes. While no overall changes with PGDH are seen during term labor, the increased overall synthetic capacity of the chorion (and the amnion [Hirst *et al.*, 1995; Teixeira *et al.*, 1994]) at this time may mean elevated levels of active PG reach the myometrium.

3.5. Conclusion

The purpose of these studies was to assess the contribution of the chorion and decidua to the elevated PG levels in the intrauterine compartment at labor in humans. We investigated the PG synthetic capacity of these tissues in an attempt to identify some of the fundamental physiological processes associated with term labor in humans. Previous work from our laboratory demonstrated that the specific activity of PGHS increases in human amnion with labor, and that this correlates significantly and exclusively with a rise in the abundance of the PGHS-2 isoenzyme (Teixeira *et al.*, 1994, Hirst *et al.*, 1995). Our present data show an equivalent increase in PGHS activity and PGHS-2 levels in chorion tissues collected following term labor compared to nonlabored tissues of the same gestational age, but that similar events do not occur in the decidua.

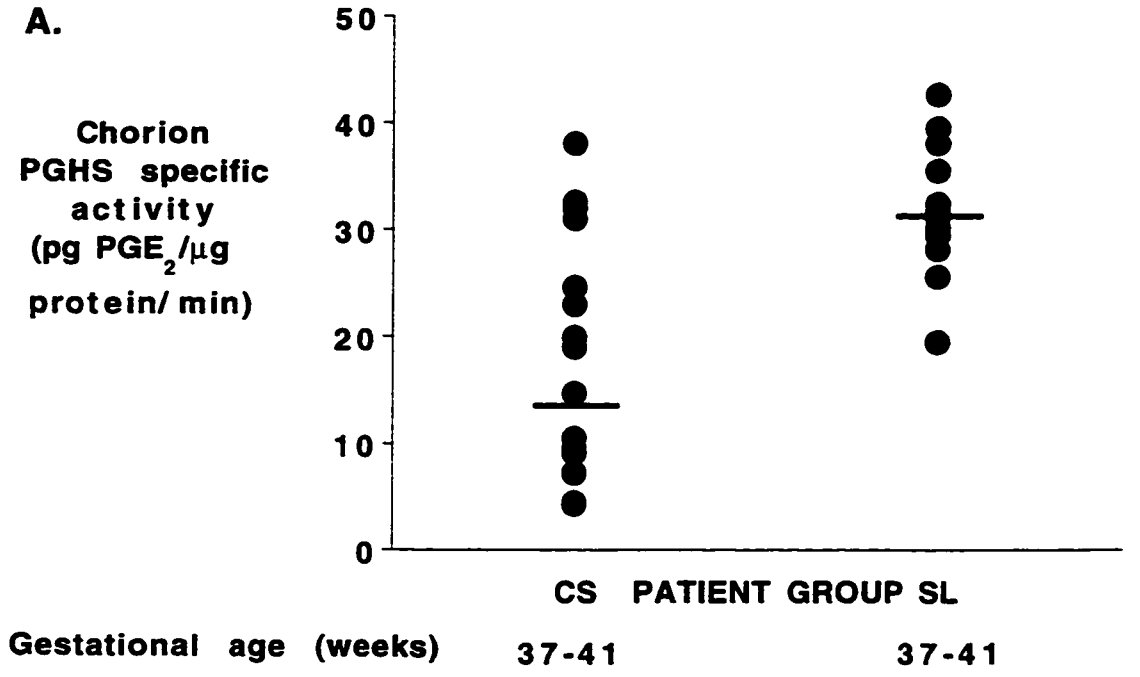
The amnion and chorion are membranes of fetal origin, the decidua is a maternal tissue. Therefore, we propose selective induction of PGHS-2 expression and activity occurs exclusively in the intrauterine tissues of fetal origin at term labor. We suggest that this is one process contributing to changes in the balance between PG

synthesis and metabolism resulting in an increase in the production of biologically active PGs in the intrauterine compartment during parturition.

Figure 3-1 PGHS specific activity in human chorion and decidua after term CS and term SL. PGHS specific activity is expressed as pg PGE_2 /μg protein/min; each point represents an individual patient. Medians are indicated by a solid horizontal bar.

A PGHS specific activity in chorion was significantly higher following term labor (median. 31.45 pg of PGE_2 /μg protein/min; n=14) than in samples obtained at elective SL (median. 14.72 pg of PGE_2 /μg protein/min; n=17) (Wilcoxon signed rank test; P=0.001).

B There was no significant difference in the specific activity of PGHS in decidua obtained at term before the spontaneous onset of labor (CS: median. 74.5 pg of PGE_2 /μg protein/min; n=10), and after spontaneous delivery (SL: median. 85 pg of PGE_2/μg protein/min; n=10).


A.
Chorion PGHS specific activity (pg PGE_2/μg protein/ min)
50
40
30
20
10
0
CS PATIENT GROUP SL
Gestational age (weeks)
37-41
37-41

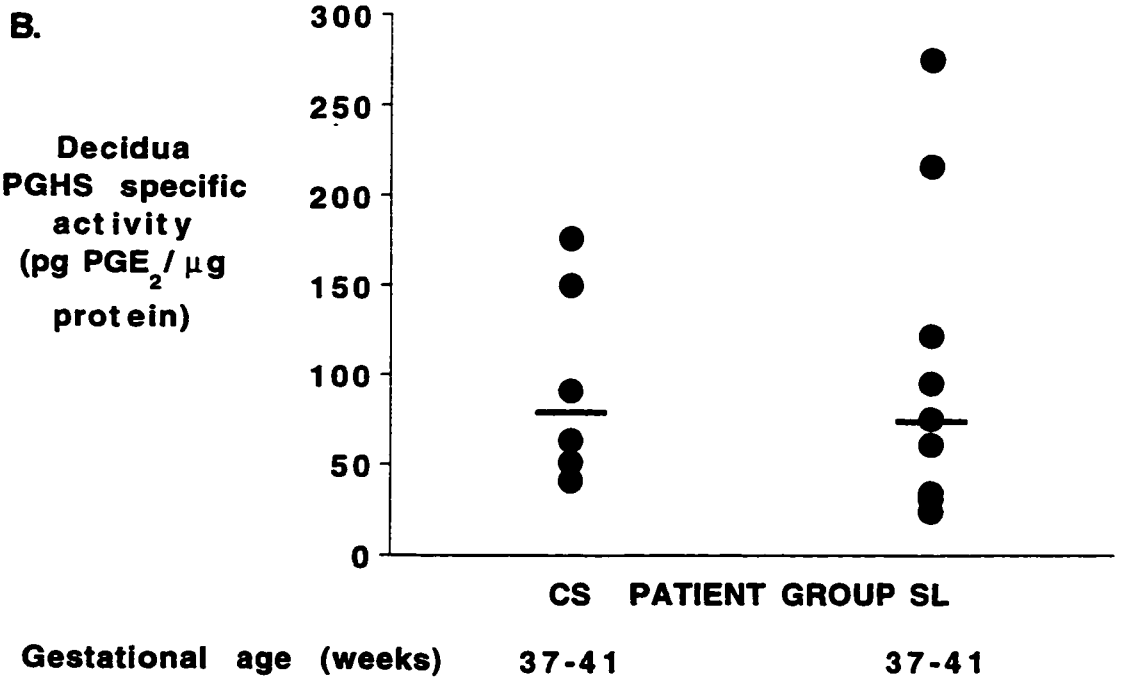

B.
Decidua PGHS specific activity (pg PGE_2/ μg protein)
300
250
200
150
100
50
0
CS PATIENT GROUP SL
Gestational age (weeks)
37-41
37-41

Figure 3-2 A a: A composite autoradiograph detecting PGHS-1 mRNA in CS and SL chorion samples. γ-actin mRNA levels are shown for reference. The positions of the 300 and 400 nucleotide long oligonucleotides are shown at the left side of the upper panel. The size of the protected PGHS-1 probe fragment representing PGHS-1 mRNA abundance is 309 nucleotides. Lane t shows the assay background. b: Quantification of these data showed there was no significant difference in the pattern of abundance between CS (median, 0.17 relative densitometric units; n=17) and SL tissues (median, 0.25 relative densitometric units; n=14) (Wilcoxon signed rank test; $P>0.05$). Each point on the graph represents an individual patient; medians are indicated by a solid horizontal bar.

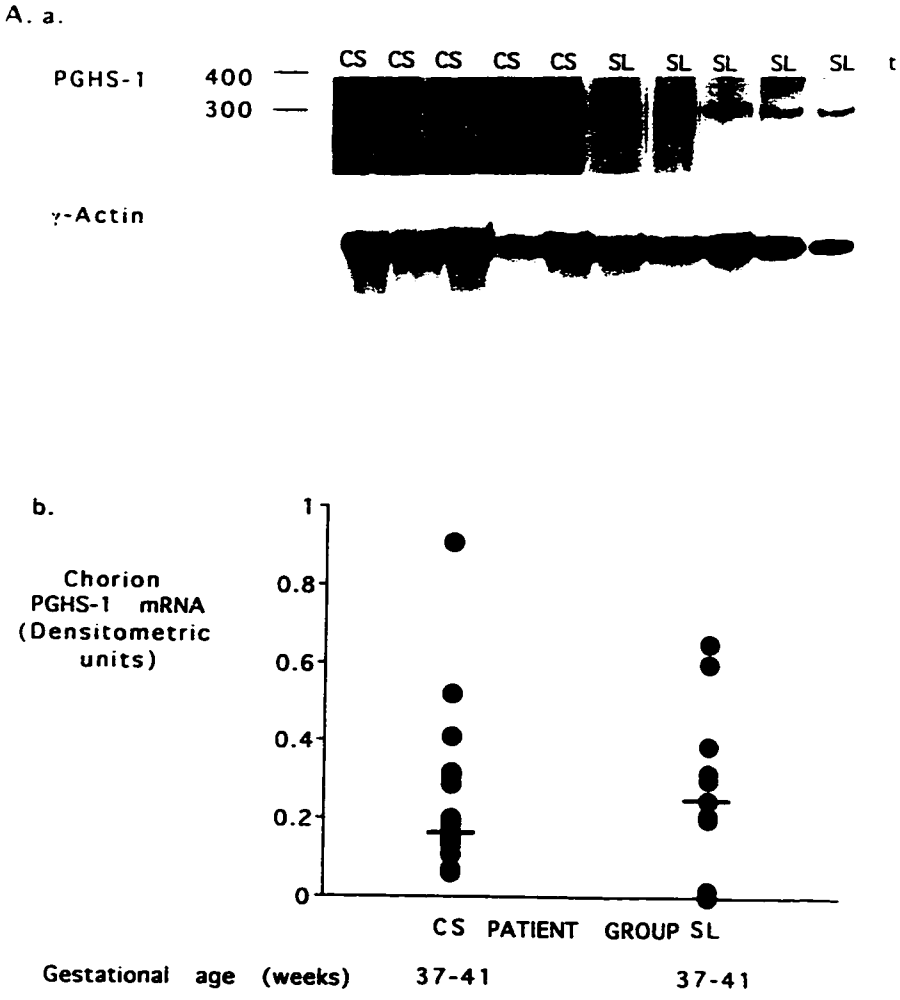

A. a.
PGHS-1
400
300
CS CS CS CS CS SL SL SL SL SL t
γ-Actin
b.
Chorion PGHS-1 mRNA (Densitometric units)
1
0.8
0.6
0.4
0.2
0
CS PATIENT GROUP SL
Gestational age (weeks)
37-41
37-41

Figure 3-2 B a: A composite autoradiograph detecting PGHS-2 mRNA in CS and SL chorion samples. γ-actin mRNA levels are shown for reference. The size of the protected PGHS-2 probe fragment representing PGHS-2 mRNA abundance is 400 nucleotides. b: PGHS-2 mRNA levels were significantly higher in samples obtained after SL (median. 1.27 relative densitometric units: n=14) when compared to those obtained at elective CS (median. 0.3 relative densitometric units: n=17): (Wilcoxon signed rank test: P=0.0001).

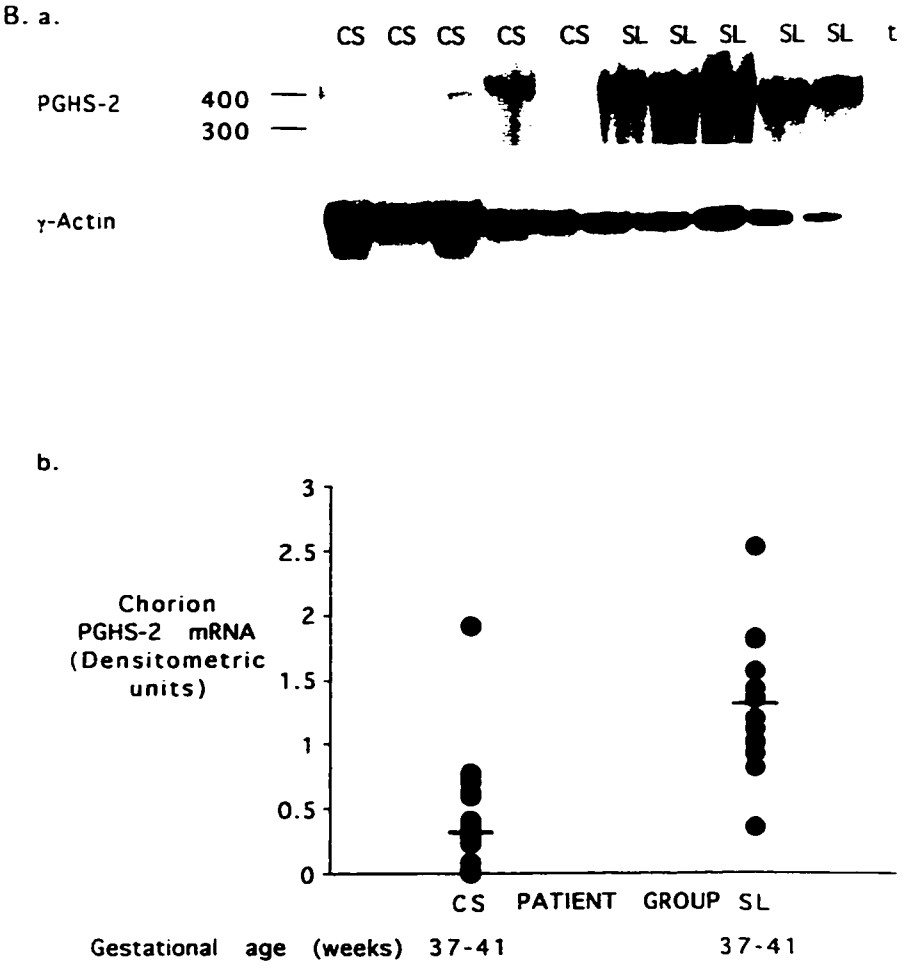
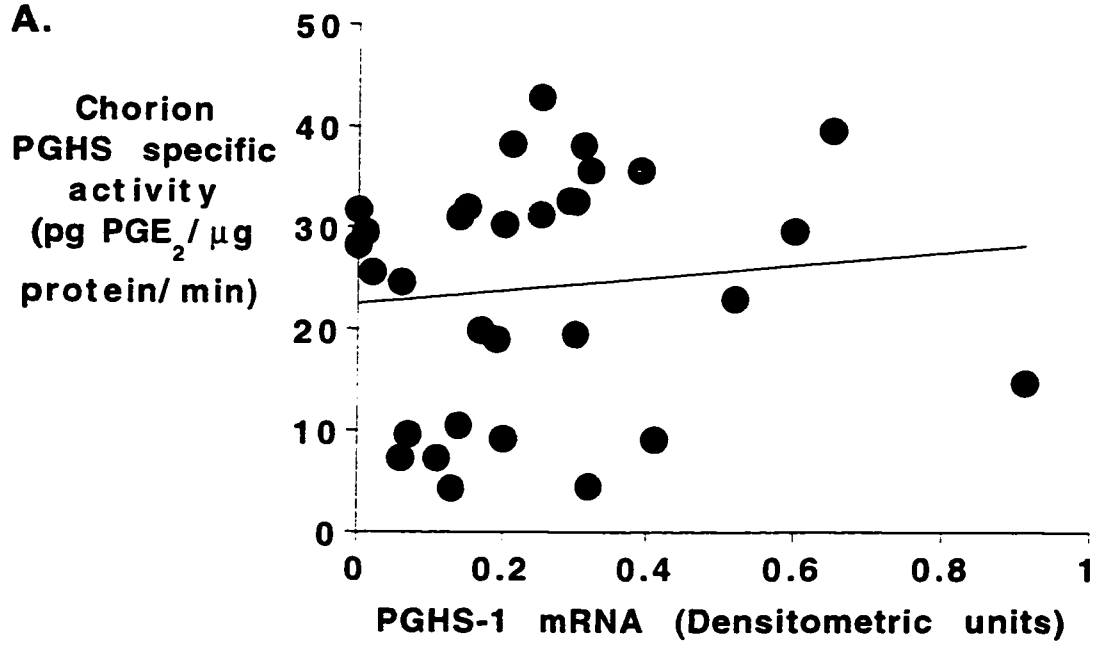

B. a.
CS CS CS CS CS SL SL SL SL SL t
PGHS-2
400
300
γ-Actin
b.
Chorion PGHS-2 mRNA (Densitometric units)
3
2.5
2
1.5
1
0.5
0
CS PATIENT GROUP SL
Gestational age (weeks) 37-41 37-41

Figure 3-3 Correlation of total PGHS enzyme activity (pg PGE_2 /μg protein/min) with PGHS-1 (A) and PGHS-2 (B) mRNA levels in term chorion CS and SL tissues. Linear regression analysis indicated a significant positive correlation between total enzyme activity and PGHS-2 mRNA levels (r=0.87; n=31; $P<0.05$). There was no correlation between PGHS enzyme activity and PGHS-1 mRNA levels (r=0.11; n=31; $P>0.05$).

A.


Chorion PGHS specific activity (pg PGE2 / µg protein/ min)
50
40
30
20
10
0
0
0.2
0.4
0.6
0.8
1
PGHS-1 mRNA (Densitometric units)

B.

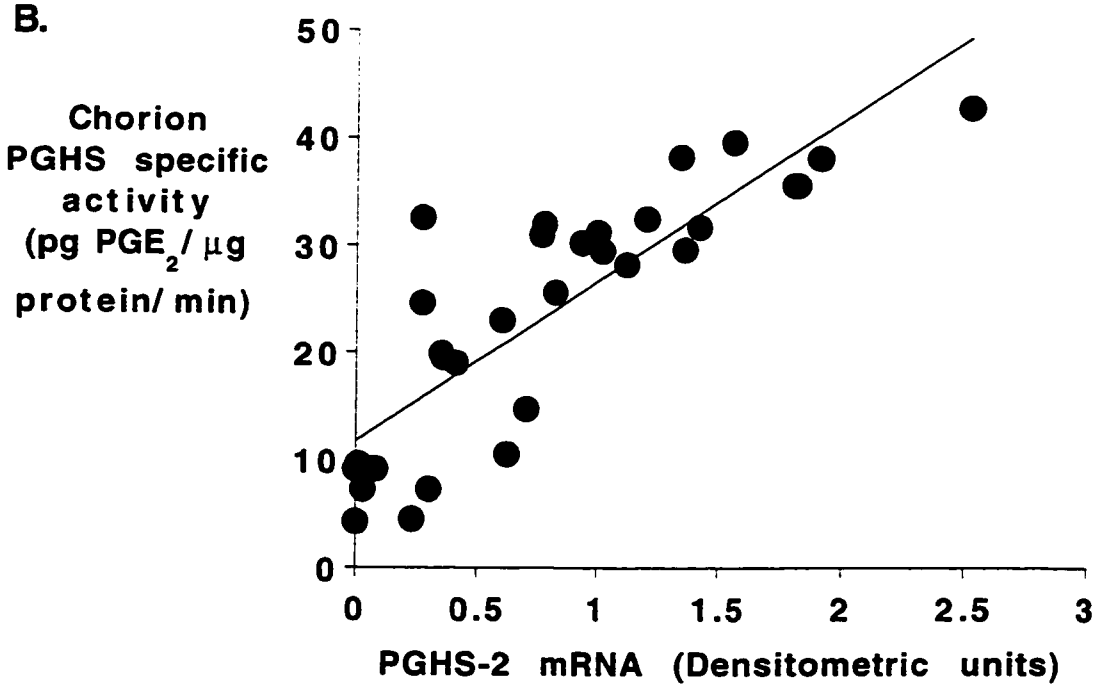
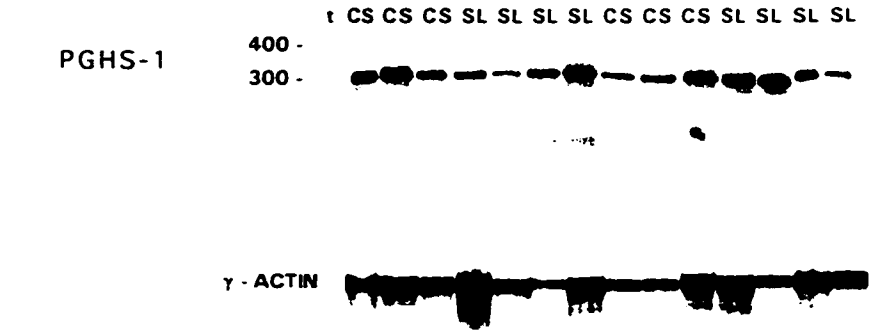

Chorion PGHS specific activity (pg PGE2 / µg protein/ min)
50
40
30
20
10
0
0
0.5
1
1.5
2
2.5
3
PGHS-2 mRNA (Densitometric units)

Figure 3-4 A a: Ribonuclease protection assays demonstrating the abundance of PGHS-1 and γ-actin mRNAs in the decidua of several patients before (CS) and after (SL) spontaneous labor. b: Quantification of these data indicate there was no significant difference in the abundance of PGHS-1 mRNA between CS (median, 0.24 relative densitometric units: n=26) and SL (median, 0.26 relative densitometric units: n=20) patients (Wilcoxon signed rank test: $P>0.05$). Each point on the graph represents an individual patient: medians are indicated by a solid horizontal bar.

A. a.


t CS CS CS SL SL SL SL CS CS CS SL SL SL SL
PGHS-1
400 -
300 -
γ - ACTIN

b.

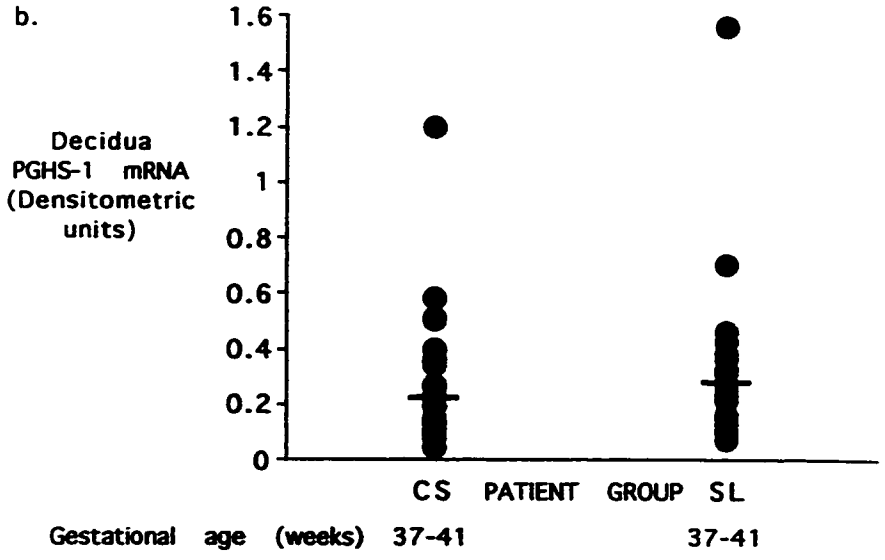
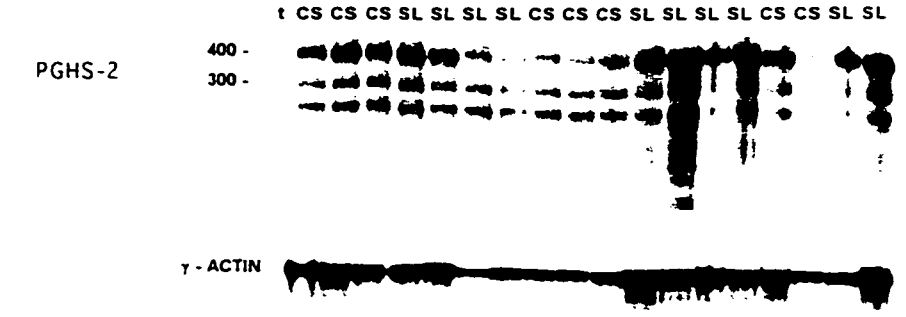

Decidua
PGHS-1 mRNA
(Densitometric
units)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
CS PATIENT GROUP SL
Gestational age (weeks) 37-41 37-41

Figure 3-4 B a: Ribonuclease protection assays demonstrating the abundance of PGHS-2 and γ-actin mRNAs in the decidua of several patients before (CS) and after (SL) spontaneous labor. b: There was no significant difference in the abundance of PGHS-2 mRNA between CS (median, 0.32 relative densitometric units; n=27) and SL (median, 0.5 relative densitometric units; n=23) patients. (Wilcoxon signed rank test; $P>0.05$).

B. a.


t CS CS CS SL SL SL SL CS CS CS SL SL SL SL CS CS SL SL
PGHS-2
400 -
300 -
γ - ACTIN

b.

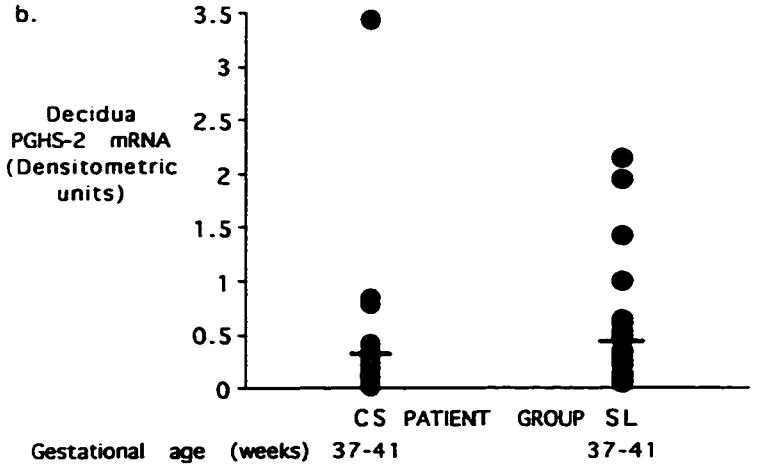
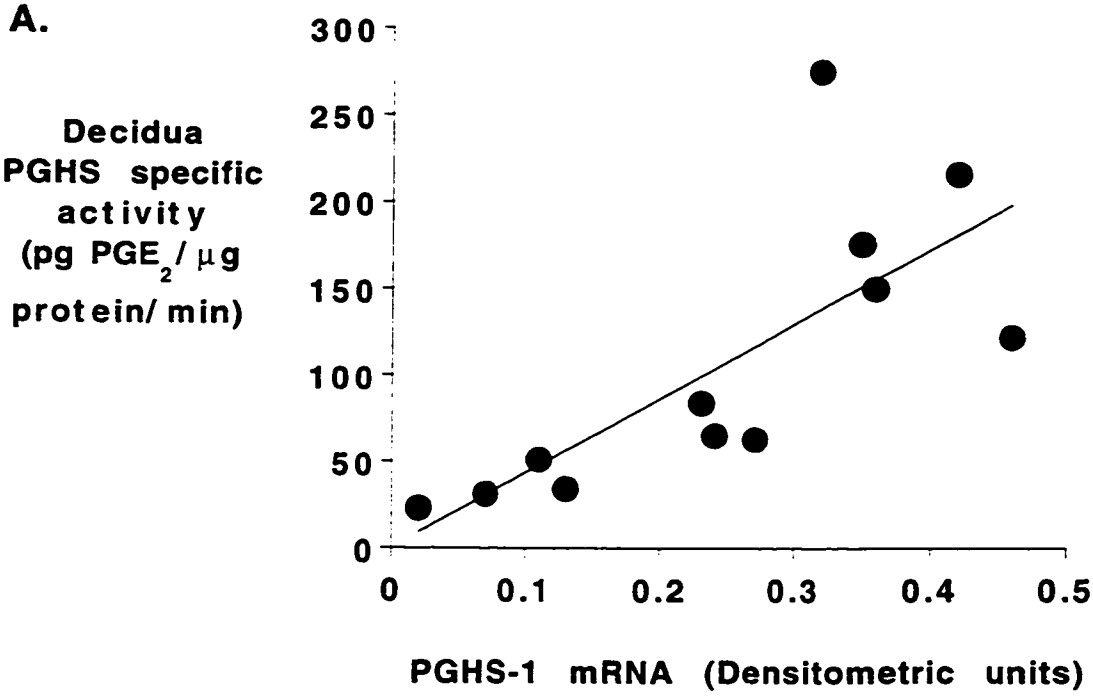

Decidua PGHS-2 mRNA (Densitometric units)
3.5
3
2.5
2
1.5
1
0.5
0
CS PATIENT GROUP SL
Gestational age (weeks) 37-41 37-41

Figure 3-5 Correlation of total PGHS enzyme activity (pg PGE_2 /μg protein/min) with PGHS-1 (A) and PGHS-2 (B) mRNA levels in term decidua CS and SL tissues Significant correlation was found in both cases (A: r=0.77; n=12; *P*=0.005; B: r=0.74; n=15; *P*=0.002).

A.


Decidua PGHS specific activity (pg PGE2/ µg protein/ min)
300
250
200
150
100
50
0
0
0.1
0.2
0.3
0.4
0.5
PGHS-1 mRNA (Densitometric units)

B.

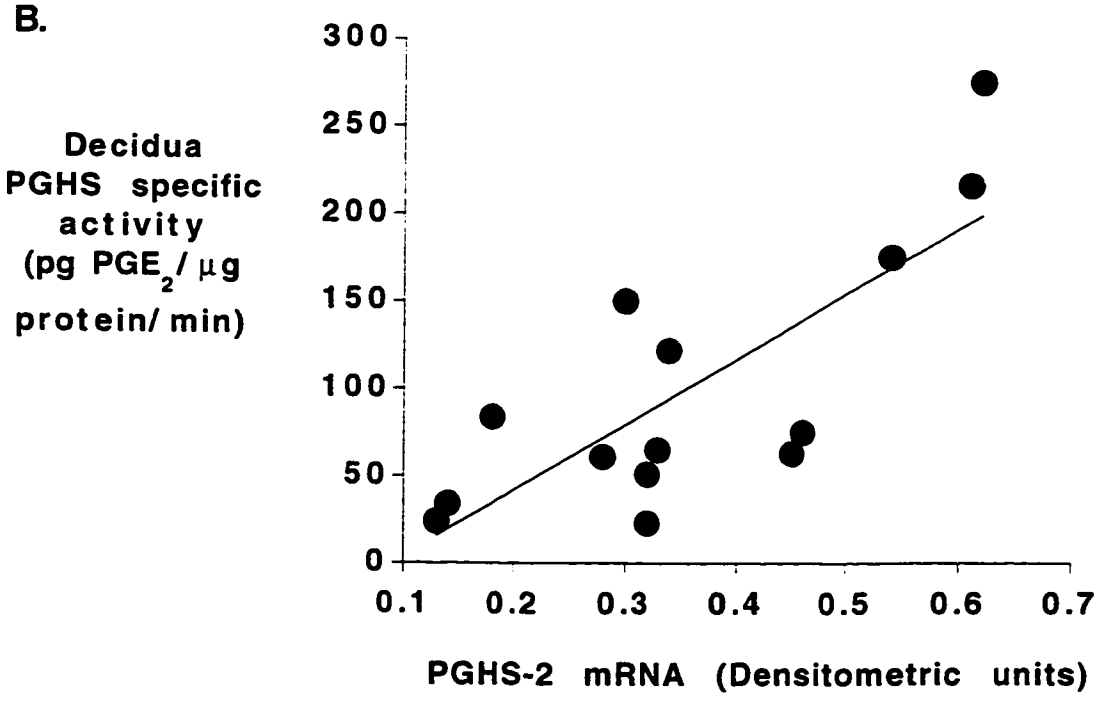

Decidua PGHS specific activity (pg PGE2/ µg protein/ min)
300
250
200
150
100
50
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
PGHS-2 mRNA (Densitometric units)

Figure 3-6 PGHS-1 mRNA distribution in term fetal membranes visualized by *in situ* hybridization. PGHS-1 mRNA was visualized by the dark purple stain. cytokeratin counterstaining was brown.

A PGHS-1 mRNA expression in full thickness membranes collected at term CS: X10. ae: amnion epithelium; am: amnion mesoderm; cm: chorion mesoderm; ct: chorion trophoblast; f: fibroblast cells; d: decidua.

B CS negative control stained for cytokeratin and hybridized with sense probe: X10.

C PGHS-1 mRNA expression in full thickness membranes collected following term SL: X10.

D SL negative control stained for cytokeratin and hybridized with sense probe: X10.

E Expression of PGHS-1 mRNA in term SL amnion. Staining for PGHS-1 mRNA was faint and heterogeneous in epithelial cells with stronger staining in the fibroblastic component: X20.

F Chorion of term SL membranes demonstrating faint heterogeneous staining in trophoblast for PGHS-1 mRNA with stronger staining in the fibroblastic component: X20.

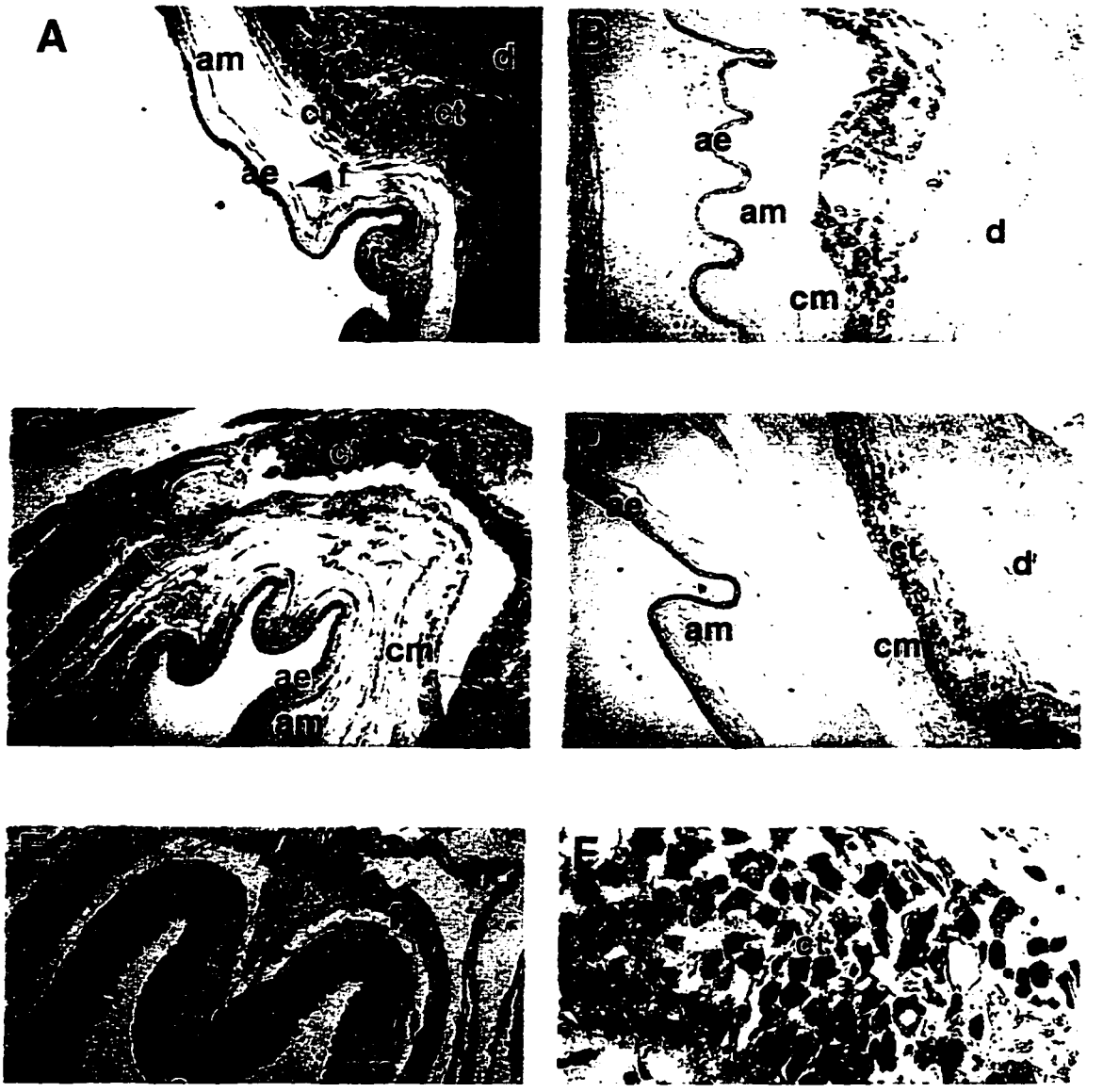

A
am
d
ae
f
ae
am
d
cm
cm
ae
am
am
cm
d
cm

Figure 3-7 PGHS-2 mRNA distribution in term fetal membranes visualized by *in situ* hybridization. PGHS-2 mRNA was visualized by the dark purple stain. cytokeratin counterstaining was brown.

A PGHS-2 mRNA expression in full thickness membranes collected at term CS: X10. ae: amnion epithelium; am: amnion mesoderm; cm: chorion mesoderm; ct: chorion trophoblast; f: fibroblast cells; d: decidua; X10

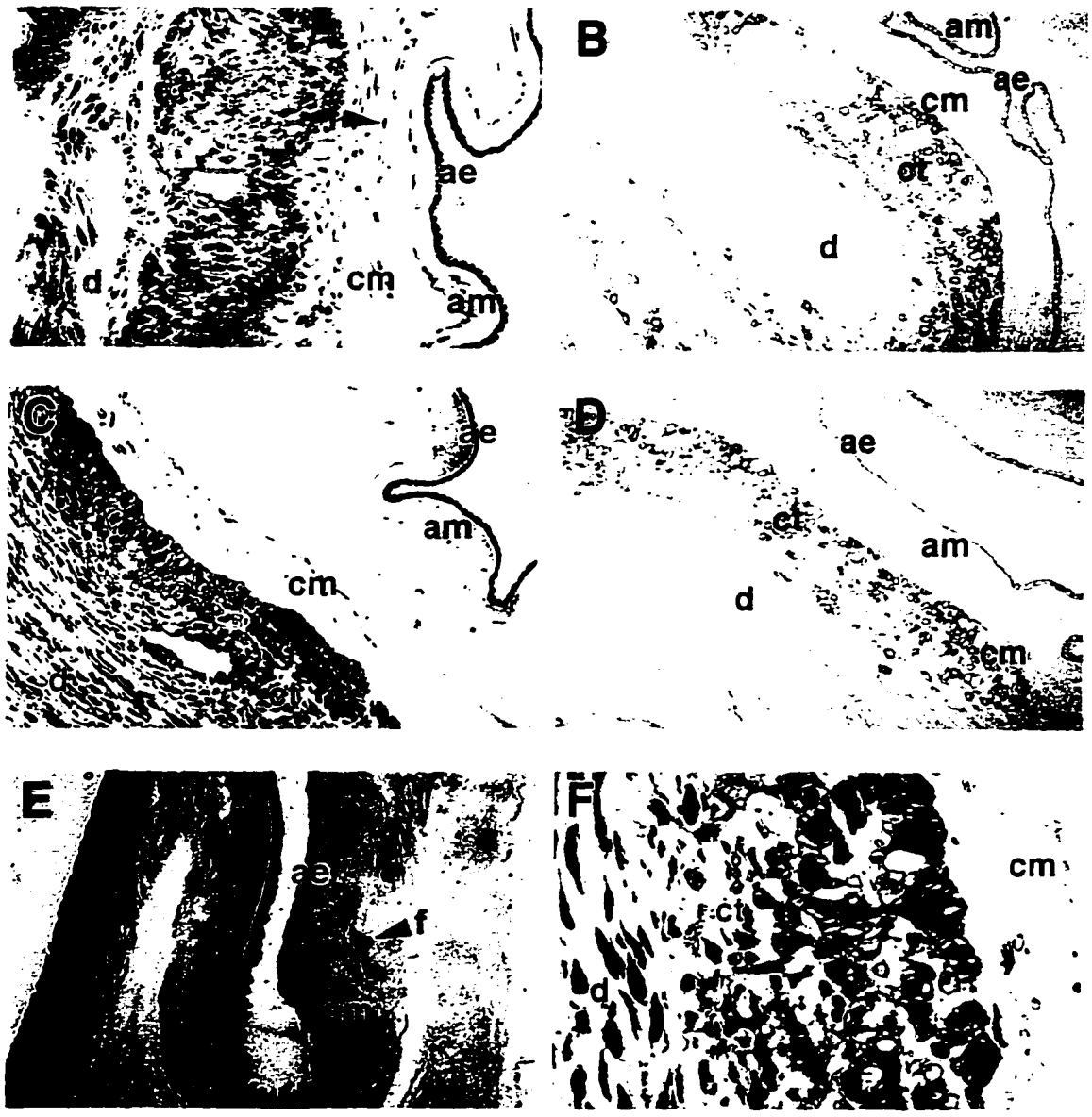
B CS negative control stained for cytokeratin and hybridized with sense probe: X10.

C PGHS-2 mRNA expression in full thickness membranes collected following term SL: X10.

D SL negative control stained for cytokeratin and hybridized with sense probe; X10.

E PGHS-2 mRNA expression in amnion collected at term SL. Staining was strong and heterogeneous in epithelial and fibroblastic cells: X20.

F PGHS-2 mRNA expression in term SL membranes demonstrating strong heterogeneous staining in chorion trophoblast and the cytokeratin negative cells of the decidua; X20.


B
am
ae
cm
ct
d
ae
d
cm
am
C
ae
am
cm
d
D
ae
ct
am
d
cm
E
ae
f
F
cm
ct
d

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4. PGHS ACTIVITY AND mRNA LEVELS IN HUMAN AMNION, CHORION, AND DECIDUA THROUGHOUT GESTATION

4.1. Introduction

Previously, a selective increase in the activity and abundance of the PGHS-2 isoenzyme in amnion and chorion tissues collected following term labor in humans has been described (Chapter 3, Teixeira *et al.*, 1993, 1994; Hirst *et al.*, 1995; Fuentes *et al.*, 1996; Mijovic *et al.*, 1997, 1998). These observations indicate that the expression of PGHS-2 in the fetal tissues of the uterus is elevated during term labor in humans, and implicate a role for PGs in the process of human parturition.

It has also been demonstrated that PGHS specific activity rises in the amnion in late gestation before labor (Teixeira *et al.*, 1993, 1994). This suggests that PGHS induction in the fetal membranes may contribute to the increased intrauterine PG production observed before the onset of labor (Chapter 1). In the present study we have explored this possibility further by examining the activity and abundance of PGHS in the amnion, chorion laeve and the decidua during the course of gestation before term labor. In addition, we have determined the localization of PGHS-1 and PGHS-2 mRNAs among the various cell types of the fetal membranes and the decidua vera during gestation by *in situ* hybridization. The results from these studies have given us important information concerning some of the mechanisms responsible for the control of PGs at term birth in humans.

4.2. Methods

4.2.1. Tissue Collection

Patient data

Placentas with attached fetal membranes were obtained from a total of 30 uncomplicated singleton pregnancies. Of these, 8 were collected before 20 weeks of pregnancy (gestational age calculated from the first day of the last menstrual period) after elective abortion by the Central Laboratory for Human Embryology at the University of Washington; 5 were collected following emergency preterm cesarean section (CS) (<37 weeks; mean ± SEM = 31.9 ± 1.4 weeks) due to pregnancy induced hypertension or fetal distress; and 17 were collected after term (37-41 weeks ; mean ± SEM = 38.3 ± 0.6 weeks) elective CS. All samples were collected from patients in the absence of labor defined as ≤ 1 uterine contraction/10 min, ≤ 2 cm cervical dilatation as determined by pelvic examination, and intact membranes. Women with clinical signs of inflammation or genital infection (fever, foul vaginal discharge) or bacterial vaginosis were excluded from the study. Additionally, all patients were routinely tested for the presence of group B streptococcus in the vaginal flora, and those found positive were not included in the study.

The use of these tissues was approved by the University of Alberta Ethical Review Committee.

Tissue processing

The placenta was separated from the fetal membranes immediately after delivery by cutting around the placental margins. Membranes were washed in physiological saline to remove excess blood. Small pieces of full-thickness membrane were cut, rolled, and fixed in 10% formalin in phosphate buffered saline (PBS) for 24-48 h at room temperature (RT). The remaining amnion was separated from the chorio-decidua by blunt dissection; decidual tissue was then scraped from the chorion using a blade. Histological examination revealed that almost all the

decidua had been separated from the chorion. Amnion, chorion and decidua samples were cut into strips (10 x 20 mm), washed repeatedly with physiological saline to remove clotted blood and snap frozen in liquid nitrogen. The effects of the trauma of isolation on tissue PG production was minimized by limiting preparation time to < 20 min. Each sample of frozen tissue was pulverized using a dry-ice cooled pestle and mortar and separated into batches for RNA extraction and enzyme activity assay.

The formalin fixed tissue samples were processed for *in situ* hybridization and histological analysis by dehydration and embedding in paraffin blocks.

Full thickness membrane samples from all patients were examined histologically for evidence of neutrophil invasion as a sign of inflammation: tissues that showed positive for inflammation were excluded from the study.

4.2.2. PGHS Enzyme Activity Assay

The method to assay PGHS activity in amnion tissue has been characterized in our laboratory (Smieja *et al.*, 1993; Teixeira *et al.*, 1993, 1994). This methodology was validated for use in the chorion (Chapter 2) and decidua (Hirst *et al.*, 1998).

4.2.3. Ribonuclease Protection Assay

Total RNA was isolated from amnion, chorion and decidua tissues by the guanidine thiocyanate-phenol-choloroform method (Chomczynzki and Sacchi, 1987).

The ribonuclease protection assay for the measurement of PGHS-1 and PGHS-2 mRNA levels in amnion has previously been reported (Hirst *et al.*, 1995). The protocol (Chapter 2) was found to be optimal for use with chorion and decidua, as well as amnion.

4.2.4. *In Situ* Hybridization (Mijovic *et al.*, 1997).

This methodology was designed and validated as part of these studies and is described in detail in Chapter 2, Materials and Methods.

4.2.5. Data Assessment and Statistical Analysis

PGHS enzyme specific activity values are expressed as picograms of PGE_2 produced /μg microsomal protein/minute.

PGHS-1 and PGHS-2 mRNA levels in tissue samples were evaluated as previously described (Chapter 2).

Patients were grouped according to those who delivered in the absence of labor before term, and at term (World Health Organization, 1977; Table 4.1). Enzyme activity and mRNA levels were compared between these two groups using the Wilcoxon signed rank test. This nonparametric test was used as the variance of the values in each patient group were heterogeneous (Bartlett's test).

Polynomial regression was used for longitudinal analysis of changes in enzyme activity and expression (Mini-Tab Statistical Software Co., State College, PA; Significance $P<0.05$).

Simple linear regression analysis was used to compare PGHS mRNA abundance in chorion with enzyme activity in the same tissues, and PGHS-1 and PGHS-2 mRNA levels in amnion, chorion, and decidua. (Significance $P<0.05$).

In situ hybridization data was analyzed qualitatively as described in Chapter 2.

4.3. Results

4.3.1. PGHS Enzyme Activity in Intrauterine Tissues during Gestation

A comprehensive study describing the gestational age dependent changes in PGHS specific enzyme activity in amnion has already been published (Teixeira *et al.*, 1993, 1994). We carried out similar analyses in chorion and decidua.

In Table 4-1 the patients in our study were grouped into those who delivered before term, and at term respectively. The mean and range of the gestational ages of the patients included in the two groups is shown. PGHS specific activity in the amnion and chorion from patients who delivered at term was significantly higher than in patients whose pregnancies were terminated before term. There was no significant difference in PGHS specific activity between the two groups in decidua.

Longitudinal regression analysis of PGHS enzyme activity as a function of gestational age in amnion (after Teixeira *et al.*, 1993, 1994), chorion and decidua is presented in Figure 4-1A, B, and C, respectively. In amnion (Figure 4-1A) and chorion (Figure 4-1B), polynomial regression indicated that enzyme activity was low in the first and second trimester of gestation (<10 pg $PGE_2/\mu g$ protein/min), and predicted a three fold increase following 35-37 weeks of pregnancy. Maximal levels of PGHS activity in these tissues was observed immediately before labor onset. The increase in PGHS specific activity throughout gestation in amnion was best described by the equation $y = 1.64\ x - (6.69 \times 10^{-2}\ x^2) + (2.4 \times 10^{-5}\ x^3$; $P<0.05$; Teixeira *et al.*, 1993, 1994); and in chorion by the equation $y = 4.6\ x - (2.3 \times 10^{-1}\ x^2) + (3.7 \times 10^{-3}\ x^3)$ ($P<0.05$), where y = PGHS specific activity and x = gestational age. Figure 4-1C shows there was no significant change in enzyme activity with increasing gestational age in the decidua.

4.3.2 PGHS-1 and PGHS-2 mRNA Abundance in Intrauterine Tissues during Gestation

PGHS-1 and PGHS-2 mRNA levels were determined in amnion, chorion and decidua tissues by ribonuclease protection assay. Forty μg of total tissue RNA was hybridized with the PGHS-1 or PGHS-2 cRNA probe and produced protected bands in the expected 309 and 400 nucleotide positions, respectively. PGHS-1 and PGHS-2 mRNA abundance was quantified as described in Chapter 2, Materials and Methods. Figure 4-2 shows an example of some results collected by ribonuclease protection assay; the expression of PGHS-1 and PGHS-2 mRNAs in the chorion of a group of patients at different gestational ages is demonstrated. Data collected for the amnion, chorion and decidua were quantified as described in Chapter 2 and are presented in Table 4-1 and Figure 4-3.

Table 4-1 shows the statistical comparison of PGHS-1 and PGHS-2 mRNA levels in the amnion, chorion and decidua of the patients when grouped into those who delivered at term in the absence of labor and those who delivered earlier, also in the absence of labor. PGHS-1 as well as PGHS-2 mRNA levels in amnion and chorion from patients who delivered at term was significantly higher than in patients whose pregnancies were terminated before term; in the decidua there was no significant difference in the levels of PGHS-1 or PGHS-2 mRNA between these two groups of patients.

Figure 4-3A shows the gestational age dependent pattern of PGHS-1 and PGHS-2 mRNA abundance in individual amnion tissues. In early gestation, PGHS-1 mRNA was detectable at low levels, and there was a small, significant increase in the abundance of this isoenzyme after 35-37 weeks of gestation. This change is best described by the equation $y = 0.01\ x - (2.9 \times 10^{-3}\ x^2) + (2.3 \times 10^{-4}\ x^3)$ (polynomial regression; $P<0.05$). PGHS-2 mRNA was undetectable in amnion collected in the first and second trimester; levels were low in the early third trimester, and there was a

sharp rise in abundance in tissues collected from patients after 35-37 weeks of gestation [$y = 0.56\ x - (8.0 \times 10^{-2}\ x^2) + (2.4 \times 10^{-3}\ x^3)$; polynomial regression; $P<0.05$]. Figure 4-3B shows similar changes in the abundance of PGHS-1 mRNA [$y = 0.04\ x - (2.3 \times 10^{-3}\ x^2) + (4.04 \times 10^{-5}\ x^3)$; polynomial regression: $P<0.05$]; and PGHS-2 mRNA [$y = 0.25\ x - (1.3 \times 10^{-2}\ x^2) + (2.0 \times 10^{-5}\ x^3)$; polynomial regression: $P<0.05$] were seen in chorion tissues collected throughout gestation. However, in contrast to our data for amnion and chorion, Figure 4-3C shows there was no significant change in the abundance of PGHS-1 or PGHS-2 mRNA in decidua with increasing gestational age of the tissue.

The aim of this part of the project was to assess the importance of PGHS expression and PG biosynthesis by the intrauterine tissues in the processes involved in the initiation of term labor in humans. During active labor we found an increase in PGHS specific activity in the fetal membranes and attributed this to an exclusive induction of the PGHS-2 isoenzyme (Teixeira *et al.*, 1994; Hirst *et al.*, 1995; Mijovic *et al.*, 1997, 1998; Chapter 3). However, our gestational age dependent data suggest that both PGHS-1 and PGHS-2 may contribute to the increase in PGHS enzyme activity in the third trimester, and that induction of both these isoenzymes may be important in the mechanisms involved in the initiation of parturition. To investigate this possibility we carried out further data analysis.

First, we compared PGHS mRNA abundance in chorion collected from patients at term with enzyme activity in the same tissues. Since we had paired enzyme activity and message data from these chorion samples it was possible to investigate their correlation in individual patients. Linear regression analysis revealed a significant positive correlation between PGHS-2 mRNA levels and PGHS enzyme activity ($r=0.78$ $P=0.0002$; Fig. 4-4A), while similar analysis resulted in no correlation between PGHS-1 mRNA abundance and enzyme activity ($r=0.15$; $P=0.56$; Fig. 4-4B). This suggests that the increase in PGHS activity in chorion at term was

predominantly the consequence of PGHS-2 induction. We could not carry out similar analyses in the amnion as we did not have paired activity and PGHS mRNA data, however, inferring from our knowledge that the processes occurring during term labor are the same in the amnion as in the chorion. we proposed that induction of the PGHS-2 isoenzyme is also responsible for the changes in PGHS specific activity seen in third trimester amnion tissues (Teixeira *et al*., 1993, 1994).

To further substantiate these statements we correlated the abundance of PGHS-1 and PGHS-2 mRNA in the amnion, chorion and decidua of the individual patients sampled at term. Results are presented in Figure 4-5. Figure 4-5A shows there was no correlation when a comparison of PGHS-1 mRNA abundance in the amnion and chorion of individual patients was carried out; however, Figure 4-5B shows there was a significant positive correlation of PGHS-2 mRNA levels in these tissues. Results from correlations in amnion and decidua, and chorion and decidua, showed there was no relationship between PGHS-1 or PGHS-2 mRNA abundance in these tissues.

4.3.3. *In Situ* Hybridization

PGHS-1 and PGHS-2 mRNA localization was studied by *in situ* hybridization in sections of full thickness membranes (amnion-chorion with attached decidua) collected at different times of gestation from patients not in labor. Epithelial and mesenchymal cell types were identified by immunohistochemical staining for the epithelial cell marker, cytokeratin. The cytokeratin-positive amnion epithelial and chorionic trophoblast cells stained brown, while hybridization of the PGHS mRNA probes resulted in purple immunostaining superimposed on the brown color. Negative controls were stained for cytokeratin and hybridized with the PGHS-1 mRNA or PGHS-2 mRNA sense probe. We assessed this to be the most useful negative control since it included the non-specific hybridization as well as the

aspecific immunostaining components of the background. Very weak non-specific staining was detected with both sense probes.

In situ hybridization results are presented in Figure 4-6. As shown in Panels A and B (upper and lower parts of the figure), cytokeratin positive amnion epithelial and chorion trophoblast cells were essentially devoid of PGHS-1 and PGHS-2 mRNA before term. Low intensity, sporadic staining for PGHS-1 was observed in the cytokeratin negative (mesenchymal) cells of the fetal membranes during this period, and for PGHS-2 mRNA close to term. In tissues collected at term (Panels C), PGHS-1 and PGHS-2 expression was pervasive throughout the epithelial and mesenchymal components of the amnion and chorion. However, the pattern of staining appeared markedly heterogeneous, since some cells exhibited strong hybridization while others, often adjacent, showed lower levels of hybridizing material. Decidua cells exhibited variable intensity of staining for both mRNAs throughout gestation.

4.4. Discussion

In the present investigation we explored the changes of PGHS activity and abundance in human amnion, chorion and decidua during gestation prior to the onset of labor. The tissues were collected from patients who underwent elective termination of pregnancy in the absence of labor at various gestational ages. The results show that PGHS activity and the abundance of both isoenzymes is increased in amnion and chorion at term as compared with earlier during gestation. Additionally, the distribution of PGHS enzyme activity and PGHS-1 and PGHS-2 mRNA abundance vs. gestational age in the fetal membranes was consistent with a sharp increase in enzyme expression between the 36th and 41st weeks of pregnancy. Furthermore, PGHS-2 mRNA abundance, but not PGHS-1 mRNA abundance, showed significant positive correlation with PGHS enzyme activity levels, at least in

the chorion, suggesting that the inducible PGHS-2 isoenzyme was expressed in a functionally predominant and increasing manner in the fetal membranes during the last weeks of gestation. In decidua we found no gestational age dependent change in the activity of PGHS or in the level of expression of its isoenzymes. In agreement with these changing patterns of enzyme expression in the amnion and chorion, PGE_2 and $PGF_{2\alpha}$ concentrations have been found to rise severalfold in the amniotic fluid at late gestation prior to labor onset (Dray and Frydman, 1976; Mitchell *et al.*, 1978; Salmon and Amy, 1974; Neider and Augustin, 1983; Romero *et al.*, 1996). Taken together, these observations suggest that an induction of PGHS (with PGHS-2 as the functionally dominant isoform) occurs in the fetal membranes shortly before labor and likely contributes to the increased intrauterine PG production at this time. The resulting rise in PG concentrations may have a pivotal influence on the timing of labor.

It is well documented that PGs accumulate in the amniotic fluid during labor (Dray and Frydman, 1976; MacDonald and Casey, 1993; Romero *et al.*, 1994). Elevated PG production by explants or cells from fetal membranes obtained after spontaneous labor, as compared to tissues collected before labor, has also been reported (Mitchell, 1988; Olson *et al.*, 1983; Skinner and Challis, 1985, McCoshen *et al.*, 1987; Brennand *et al.*, 1995). Furthermore, PGHS activity and PGHS-2 mRNA levels are higher in the amnion and chorion laeve following spontaneous delivery than prior to labor at term (Teixeira *et al.*, 1994; Hirst *et al.*, 1995; Mijovic *et al.*, 1997; Slater *et al.*, 1995, 1996). The increase of intrauterine PG production associated with term labor has been considered a consequence of labor, caused mainly by tissue trauma and the exposure of the fetal membranes and the decidua to stimulating factors in the vaginal fluids via the opening cervix (MacDonald and Casey, 1993). However, the increased expression of PG biosynthetic enzymes in intrauterine tissues before labor is evidently not attributable to factors and conditions

brought about by the process of labor itself. It is therefore reasonable to suggest that the induction of PGHS in the amnion and chorion at term as demonstrated in these investigations, is part of the process that leads to labor, possibly in a causative fashion.

The chorion is known to have a considerable capacity to metabolize PGs predominantly by the enzyme 15-hydroxyprostaglandin dehydrogenase (PGDH). The mean level of PGDH activity and mRNA in chorion is lower in patients at SL compared to that at CS at term (Sangha *et al.*, 1994; Van Meir, 1996). These data suggest that for much of pregnancy the chorion forms a metabolic barrier diminishing passage of PGs to the myometrium. At term, an elevated expression of PGHS-2 in the amnion and chorion may result in the production of more PGs, and they may not be degraded as rapidly. In vitro studies have identified many factors that increase PGHS-2 mRNA and protein abundance, and PG output by human fetal membranes (Smith and DeWitt, 1996). For example, cytokines and growth factors can upregulate expression of PGHS-2 and increase PG output from these tissues. Importantly, the distribution of PGHS-2 mRNA in the human fetal membranes is similar to the pattern of localization of glucocorticoid receptors, detected by immunohistochemistry (Gibb and Sun, 1996). In vitro, glucocorticoids stimulate PGHS-2 mRNA and activity in amnion (Zakar *et al.*, 1995; Economopoulos *et al.*, 1996). Furthermore, the activity of the PGDH enzyme is known to be decreased by glucocorticoids (Patel *et al.*, 1997). These observations indicate one possible physiological regulation of enhanced PG output from amnion and chorion *in vivo* in relation to term and labor.

Localization of PGHS-1 and PGHS-2 mRNAs by in situ hybridization showed that both were present in the cytokeratin positive and negative cells of the chorion laeve and amnion and revealed that levels increased with advancing gestation. This indicates that both the amnion and the chorion undergo maturation at term before the onset of labor resulting in the enhanced expression of both the PGHS-1 and the

PGHS-2 gene. The maturation process appears to involve both the cytokeratin positive epithelial and cytokeratin negative mesenchymal cell types in the fetal membranes. During term labor, only the inducible PGHS-2 gene is expressed in an increasing manner predominantly in the epithelial cells of the amnion and chorion leave (Chapter 3: Mijovic *et al*.. 1997).

The factors and control mechanisms that influence the structural integrity and functional properties of the gestational tissues in preparation for labor are largely undefined. The existence of a "placental clock" has been proposed recently with the purported function of determining the pace of maturation of the placenta and the membranes as gestation proceeds (McLean *et al*.. 1995). The evidence presented here strongly suggests that the fetal membranes mature at term in preparation for labor and the process includes the enhanced expression of both PGHS isoenzymes. Although our data indicate PGHS-2 is the functionally prevalent isoenzyme at this time, a requirement for PGHS-1 during parturition cannot be ruled out.. Studies using PGHS-1 knockout mice showed this isoenzyme is implicated in the parturition process in these animals (Chapter 1; Langenbach *et al*.. 1995: Morham *et al*., 1995; Gross *et al*., 1998). Further investigations are warranted to explore the significance of PGHS-1 in the context of human labor.

4.5. Conclusion

We have documented the presence of PGHS-1 and PGHS-2 in human amnion, chorion and decidua throughout gestation. We have shown a gestational age-dependent increase in PGHS specific enzyme activity and the relative abundance of PGHS-1 and PGHS-2 mRNA in human amnion and chorion, but not decidua. We propose that induction of PGHS in late pregnancy in amnion and chorion could contribute to changes in the balance between PG synthesis and metabolism resulting

in a net increase in the production of biologically active PGs ideally situated to stimulate the myometrium. These regulatory events may be of principal importance in bringing about the increase in PG concentrations seen in the amniotic fluid and intrauterine tissues that are believed to be responsible for the onset of spontaneous labor. Furthermore, as the amnion and chorion are membranes of fetal origin, but the decidua is a maternal tissue, we suggest that the gestational tissues from the fetus are the dominant sites of PG synthesis during this process. Therefore, these data provide evidence in support of a role for the fetus in controlling the timing of its own birth.

Table 4-1 PGHS activity and abundance in amnion, chorion laeve and decidua before the onset of labor. Samples were collected from patients who delivered by elective termination of pregnancy in the absence of labor at different times during gestation. The tissues were analysed for specific activity and PGHS-1 and PGHS-2 mRNA abundance as described in Materials and Methods.

		PGHS Activity (pg PGE_2/μg protein/minute)		
		Patient data (Gestational age weeks)	Mean ±SEM	Median (range)
Amnion				
	Before term	N/A[b]	N/A	N/A
	At term[a]			
Chorion				
	Before term	19.1 (7.5-35) n=13	7.23±0.94	7.4 (1.5-15.25)
	At term	38.3 (37.1-40.1) n=17	18.47±2.8**	16.87** (4.28-38.1)
Decidua				
	Before term	15.6 (7.5-35) n=9	26.38±6.57	20 (2.5-55)
	At term	38.3 (37.1-40.1) n=9	41.3±9.5	32 (3-82)

		PGHS-1 mRNA (Densitometric units)			PGHS-2 mRNA (Densitometric units)		
		Patient data (Gestational age weeks)	Mean ±SEM	Median (range)	Patient data (Gestational age weeks)	Mean ±SEM	Median (range)
Amnion							
	Before term	17.8 (7.5-35) n=12	0.12±0.03	0.11 (ND[c]-0.33)	19.1 (7.5-35) n=13	0.18±0.08	0.00 (ND-0.97)
	At term[a]	38.6 (37.1-40.1) n=18	0.31±0.05**[d]	0.3** (0.01-0.83)	38.5 (37.1-40.1) n=21	1.04±0.15**	0.96** (0.06-2.8)
Chorion							
	Before term	19.1 (7.5-35) n=13	0.038±0.01	0.02 (ND-0.13)	19.1 (7.5-35) n=13	0.014±0.01	0.00 (ND-0.12)
	At term	38.3 (37.1-40.1) n=17	0.27±0.05**	0.19** (0.06-0.91)	38.3 (37.1-40.1) n=17	0.52±0.13**	0.35** (ND-1.91)
Decidua							
	Before term	17.9 (7.5-35) n=12	0.63±0.16	0.55 (ND-1.78)	17.6 (7.5-35) n=15	1.25±0.45	0.00 (ND-4.7)
	At term	38.6 (37-1-40.1) n=19	0.30±0.06	0.23 (0.04-1.2)	38.5 (37.1-40.1) n=19	0.46±0.18	0.21 (0.01-3.44)

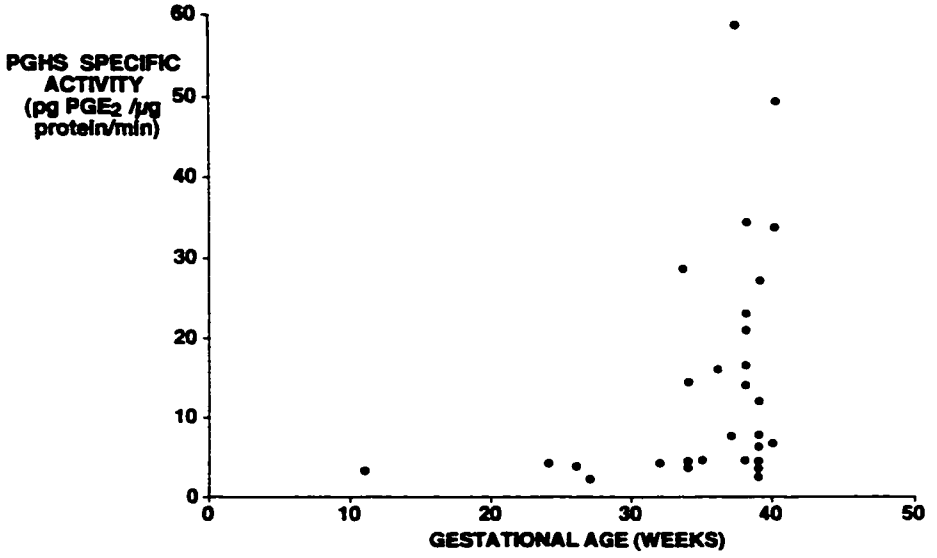
[a] Defined as delivery between 37-41 completed weeks of gestation (World Health Organization, 1977)

[b] Data not available

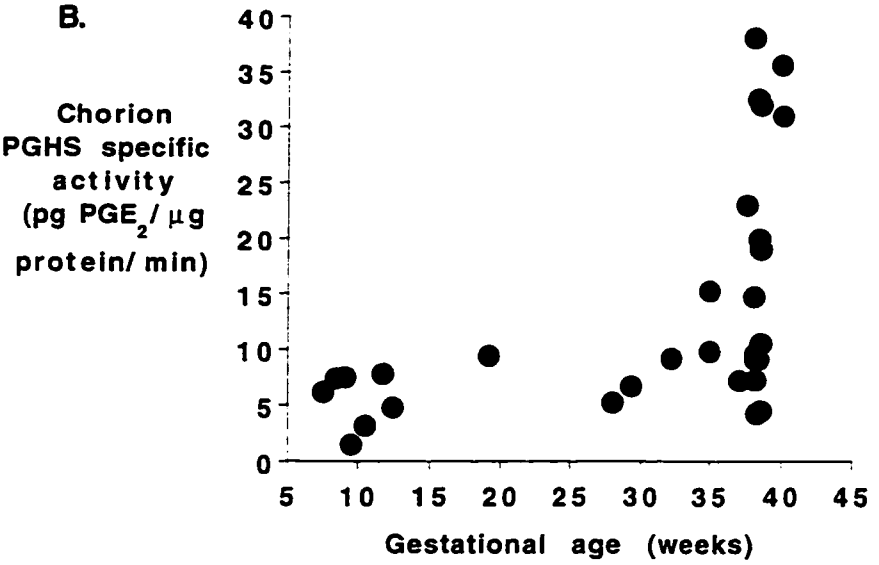
[c] ND, not detected, entered as zero in statistical calculations.

**[d] Denotes statistical significance $P<0.01$ as tested by Wilcoxon's signed rank test

Figure 4-1 PGHS specific activity in human amnion (A: after Teixeira *et al.*, 1994), chorion (B), and decidua (C) during gestation. PGHS specific activity is expressed as pg PGE_2/μg protein/min: Each point represents a tissue sample from one patient. In amnion and chorion, PGHS enzyme activity (pg PGE_2/μg protein/min) remained low in the first and second trimesters of pregnancy. Significantly higher enzyme activity levels were found in tissues from patients at term, before the expected time of labor onset: >37 weeks (polynomial regression analysis: $P<0.05$). In decidua there was no change in PGHS specific activity with gestational age.


PGHS SPECIFIC ACTIVITY (pg PGE2 /µg protein/min)
60
50
40
30
20
10
0
0
10
20
30
40
50
GESTATIONAL AGE (WEEKS)

B.


Chorion PGHS specific activity (pg PGE2 / µg protein/ min)
40
35
30
25
20
15
10
5
0
5 10 15 20 25 30 35 40 45
Gestational age (weeks)

C.

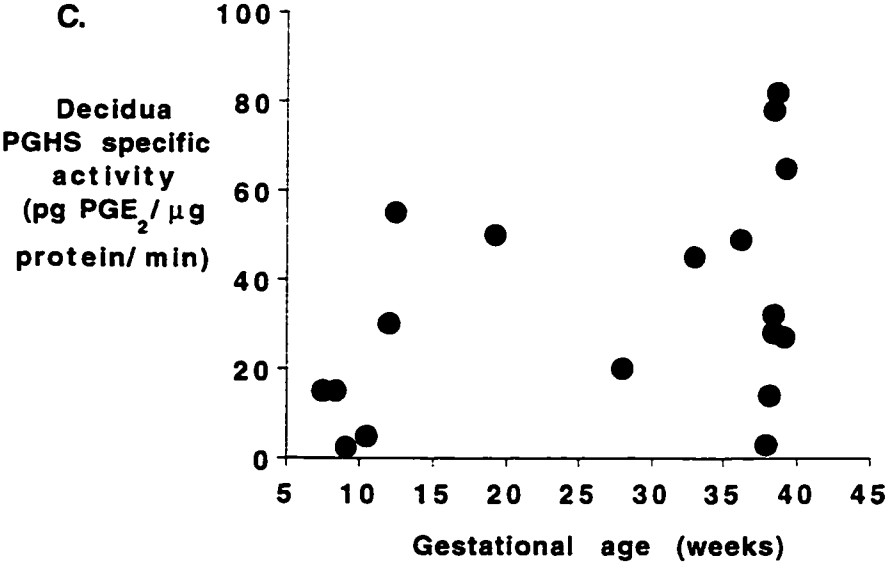

Decidua PGHS specific activity (pg PGE2 / µg protein/ min)
100
80
60
40
20
0
5 10 15 20 25 30 35 40 45
Gestational age (weeks)

Figure 4-2 A A representative autoradiograph detecting PGHS-1 mRNA in chorion samples collected between 8 and 21 weeks (1st and 2nd trimesters) of gestation. between 28 and 36 weeks (third trimester) of gestation. and at term CS (37-41 weeks of pregnancy). respectively. Patients were not in labor. The positions of the 300 and 400 nucleotide long oligonucleotides are shown at the left side of the upper panel. The size of the protected PGHS-1 probe fragment representing PGHS-1 mRNA abundance is 309 nucleotides. Lane t shows the assay background. Each lane represents a single patient.

B A representative autoradiograph detecting PGHS-2 mRNA in the chorion of the same patients. The size of the protected PGHS-2 probe fragment indicating PGHS-2 mRNA abundance is 400 nucleotides.

C γ-actin mRNA levels are shown for reference.

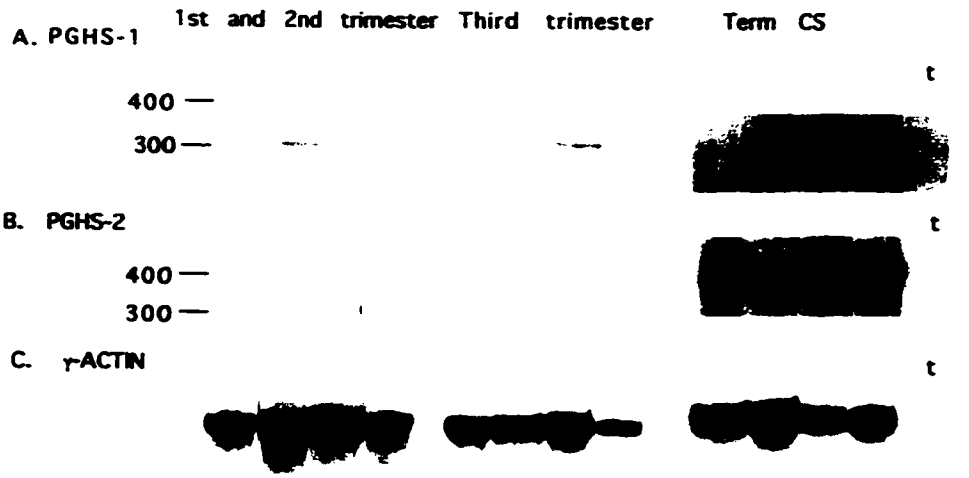

A. PGHS-1
1st and 2nd trimester
Third trimester
Term CS
t
400
300
B. PGHS-2
t
400
300
C. γ-ACTIN
t

Figure 4-3 PGHS-1 and PGHS-2 mRNA levels in amnion (A) chorion (B) and decidua (C) at various times during gestation in the absence of labor. Each point represents tissue from a single patient. PGHS mRNA levels were determined by ribonuclease protection assays and quantified by densitometry as described in Chapter 2, materials and Methods. In amnion and chorion PGHS-1 mRNA was detectable throughout gestation with significantly higher abundance in the third trimester immediately before the expected time of labor onset (polynomial regression analysis: $P<0.05$). PGHS-2 mRNA expression was undetectable or very low in the first and second trimesters of pregnancy however it reached high levels at term immediately before the expected time of labor onset (polynomial regression analysis: $P<0.05$). In decidua there was no change in PGHS-1 or PGHS-2 mRNA abundance with increasing gestational age.

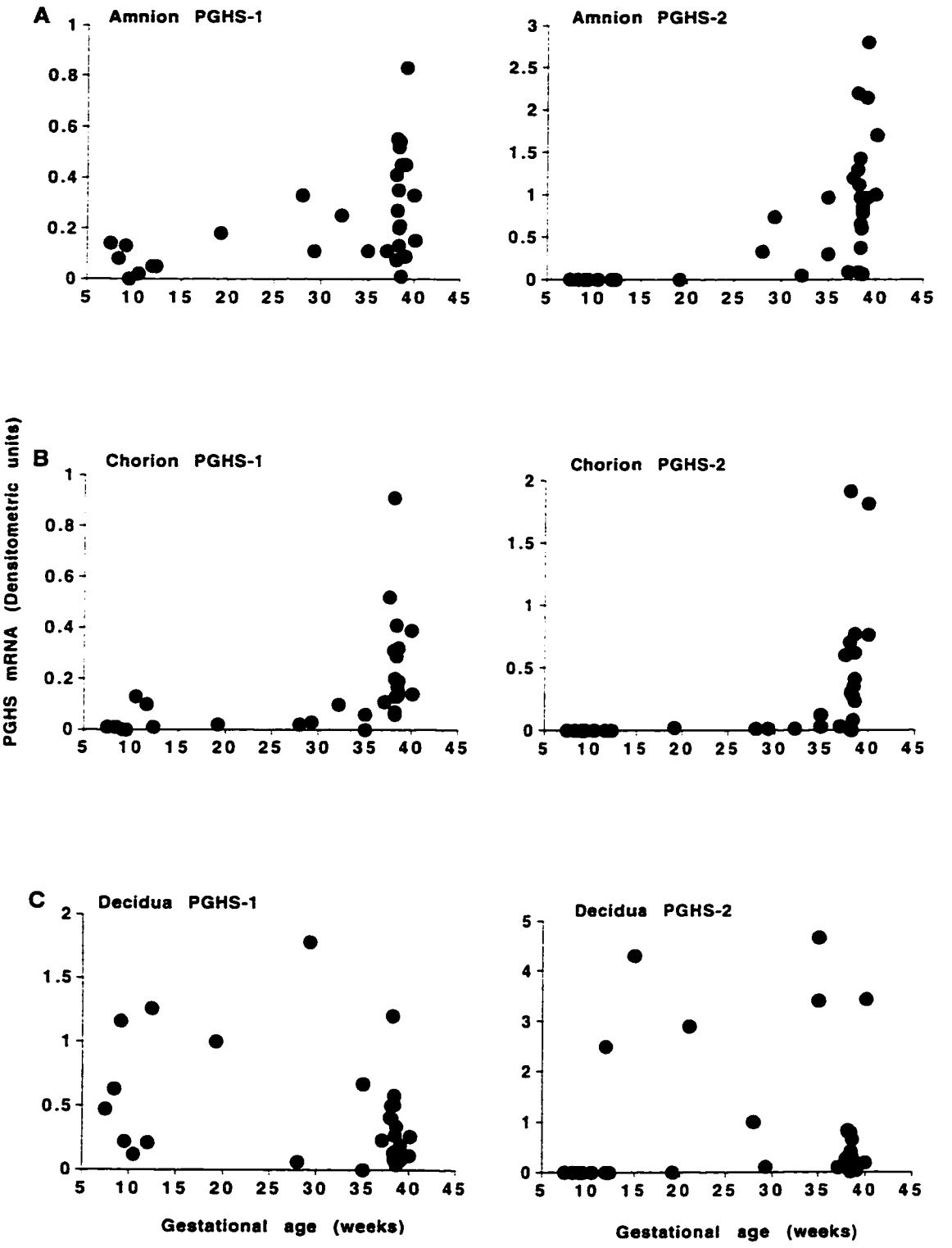
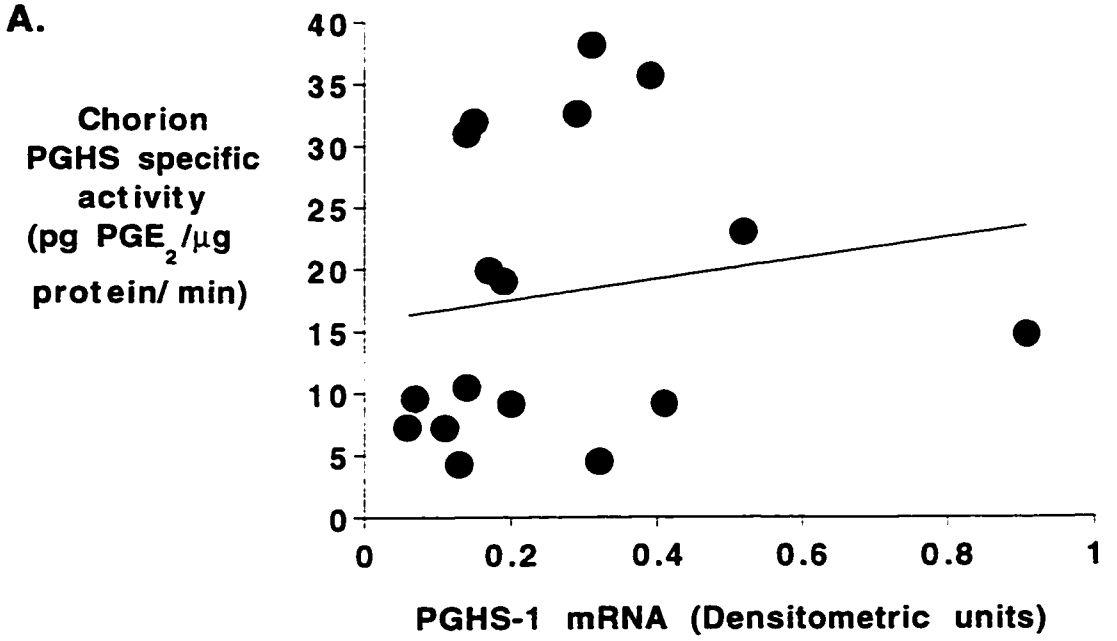

A
Amnion PGHS-1
Amnion PGHS-2
B
Chorion PGHS-1
Chorion PGHS-2
C
Decidua PGHS-1
Decidua PGHS-2
PGHS mRNA (Densitometric units)
Gestational age (weeks)
Gestational age (weeks)

Figure 4-4 Correlation of PGHS-1 (A) and PGHS-2 (B) mRNA levels with total PGHS enzyme activity (pg PGE_2/μg protein/min) in chorion collected at term. Each point is a single chorion sample collected from individual patients between 37 and 41 weeks of gestation in the absence of labor. Linear regression analysis indicated a significant positive correlation between PGHS-2 mRNA levels and total enzyme activity (r=0.78: *P*=0.0002). There was no correlation between PGHS-1 mRNA levels and PGHS enzyme activity (r=0.15: *P*=0.56).

A.


Chorion PGHS specific activity (pg PGE2/µg protein/ min)
40
35
30
25
20
15
10
5
0
0
0.2
0.4
0.6
0.8
1
PGHS-1 mRNA (Densitometric units)

B.

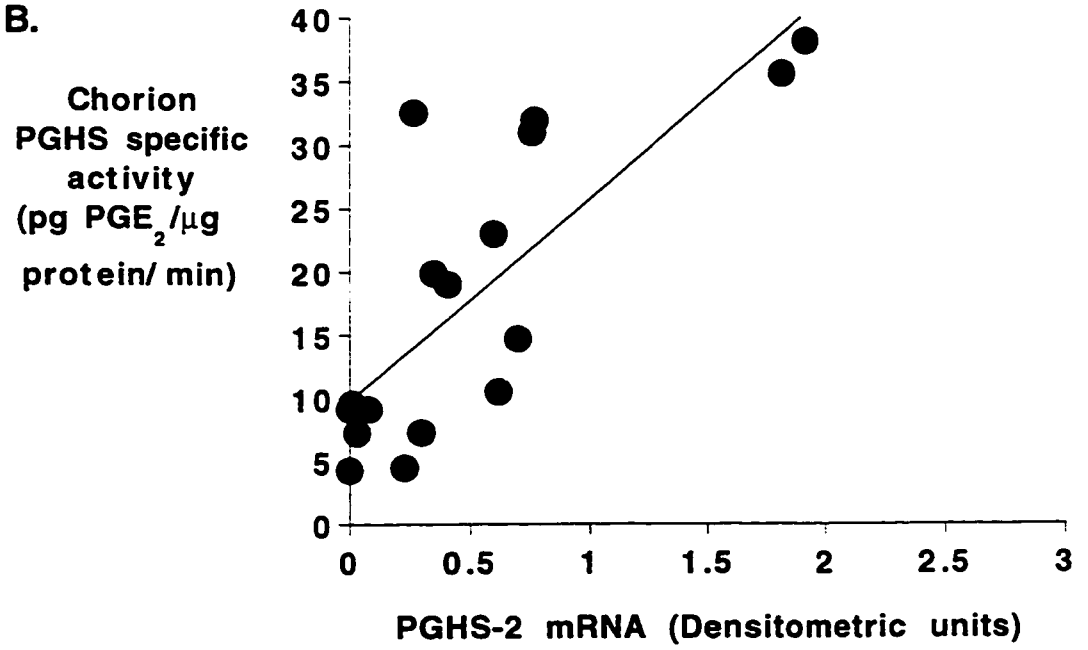
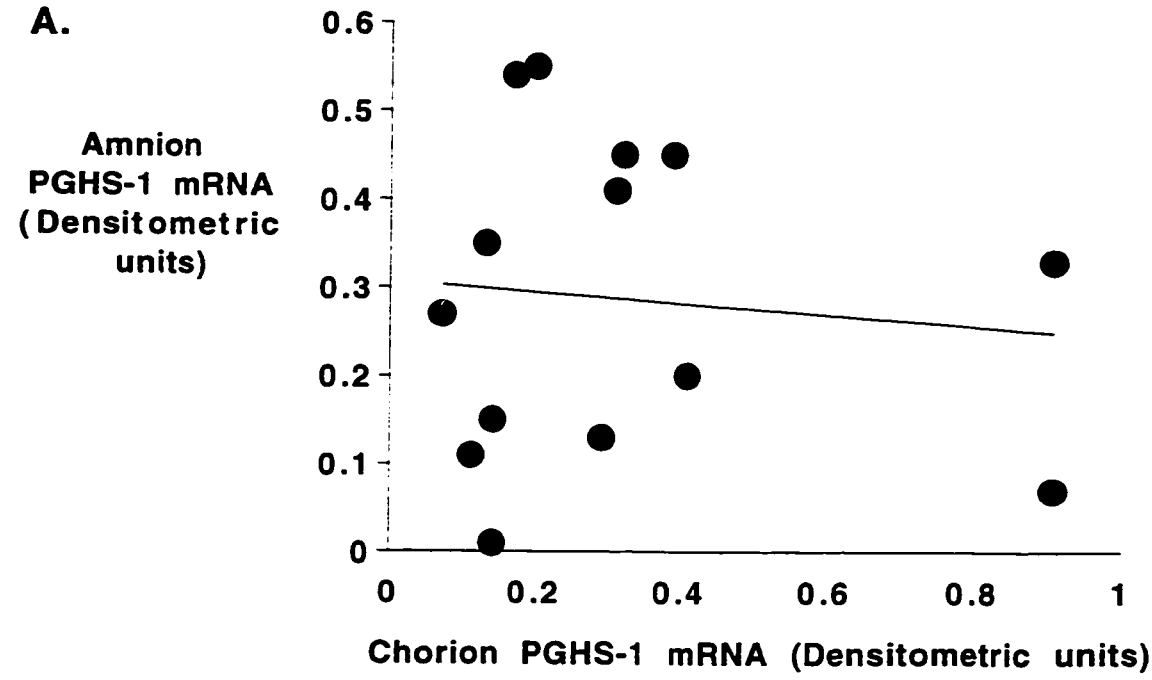

Chorion PGHS specific activity (pg PGE2/µg protein/ min)
40
35
30
25
20
15
10
5
0
0
0.5
1
1.5
2
2.5
3
PGHS-2 mRNA (Densitometric units)

Figure 4-5 Correlation of PGHS-1 mRNA (A) and PGHS-2 (B) mRNA levels in amnion and chorion collected from patients at term in the absence of labor. Each point is a single sample from a different patient. Linear regression analysis indicated a significant positive correlation of PGHS-2 mRNA levels in amnion and chorion ($r=0.60$; $P=0.002$). There was no correlation of PGHS-1 mRNA levels ($r=0.15$; $P=0.45$) in these tissues.


A.
Amnion PGHS-1 mRNA (Densitometric units)
0.6
0.5
0.4
0.3
0.2
0.1
0
0
0.2
0.4
0.6
0.8
1
Chorion PGHS-1 mRNA (Densitometric units)

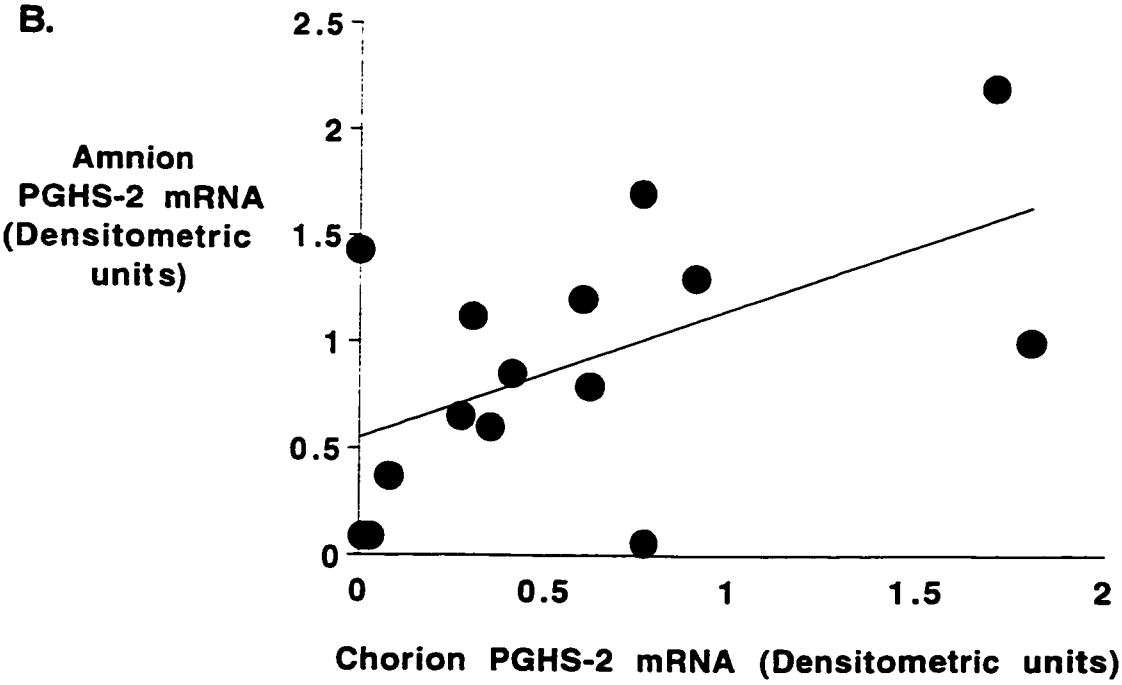

B.
Amnion PGHS-2 mRNA (Densitometric units)
2.5
2
1.5
1
0.5
0
0
0.5
1
1.5
2
Chorion PGHS-2 mRNA (Densitometric units)

Figure 4-6 PGHS-1 (upper panel) and PGHS-2 (lower panel) mRNA localization in full thickness membrane samples collected at various gestational ages before labor onset as visualized by *in situ* hybridization. PGHS-1 and PGHS-2 mRNA was visualized by the dark purple stain. cytokeratin counterstaining was brown.

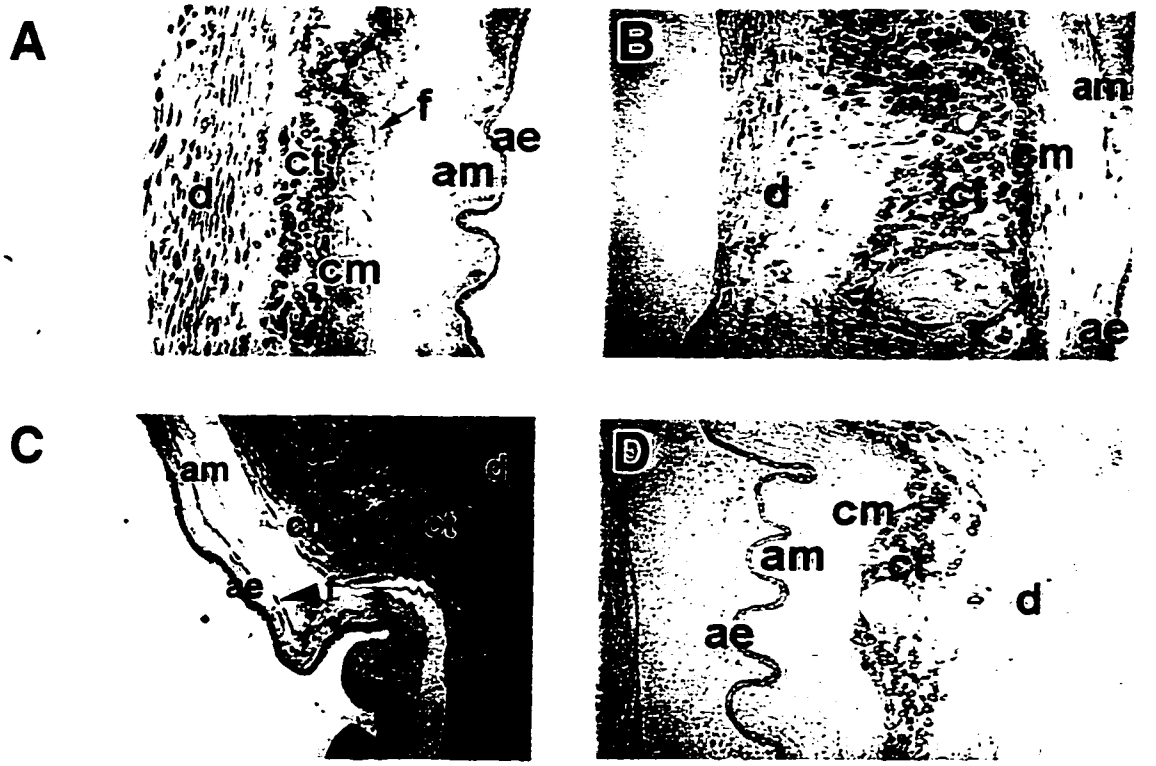
A PGHS-1 and PGHS-2 mRNA expression in full-thickness membranes obtained at 12 weeks of gestation: X10. ae: amnion epithelium: am: amnion mesoderm: cm: chorion mesoderm: ct: chorion trophoblast: f: fibroblast cells: d: decidua.

B PGHS-1 and PGHS-2 mRNA expression in full-thickness membranes obtained at 28 weeks of gestation: X10.

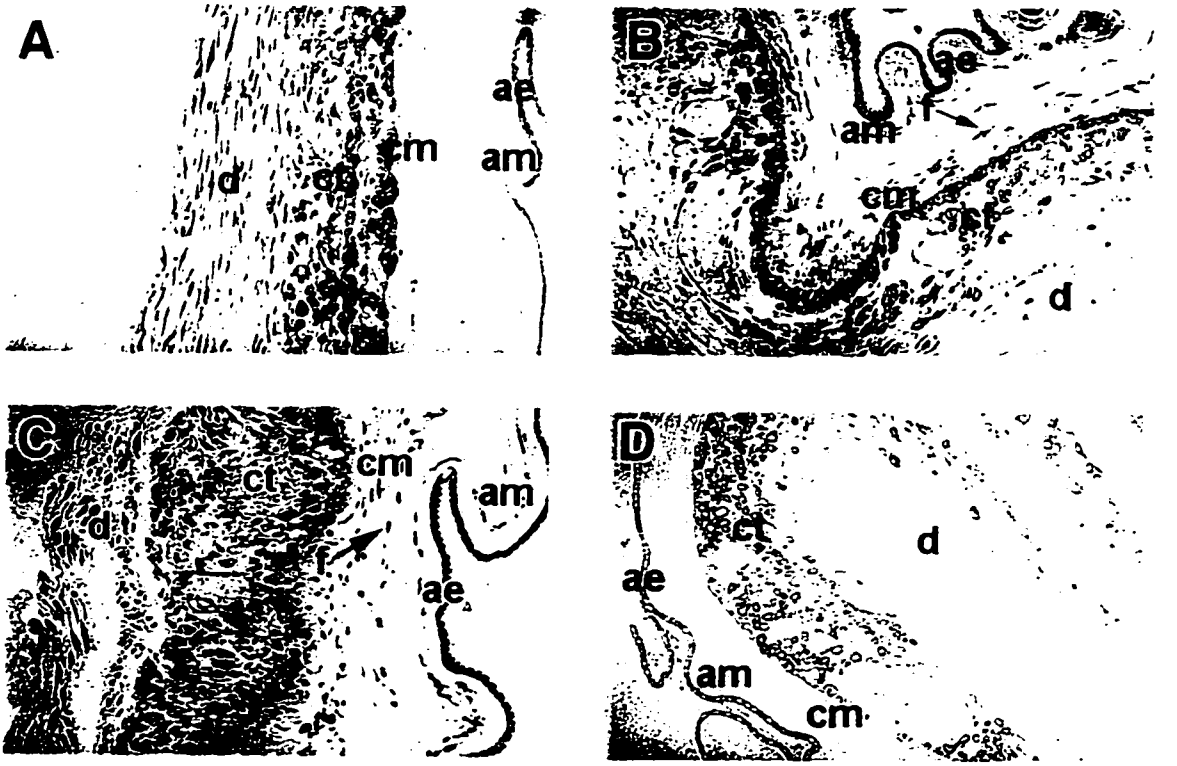
C PGHS-1 and PGHS-2 mRNA expression in full-thickness membranes obtained at term. 38 weeks of gestation: X10.

D Negative controls from a patient at term. stained for cytokeratin and hybridized with sense probes: X10.

PGHS - 1


A
f
ae
ct
am
d
cm
B
am
cm
d
ct
ae
C
am
ae
D
cm
am
ct
d
ae

PGHS - 2


A
ae
cm
am
d
ct
B
ae
f
am
cm
d
C
cm
ct
am
d
f
ae
D
ct
d
ae
am
cm

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5. PGHS ABUNDANCE IN THE FETAL MEMBRANES CORRELATES WITH FETAL FIBRONECTIN CONCENTRATION IN THE CERVICO-VAGINAL FLUIDS AT TERM: EVIDENCE OF ENZYME INDUCTION BEFORE THE ONSET OF LABOR.

5.1. Introduction

Considerable evidence supports the importance of prostaglandins (PGs) in the initiation of human parturition. PGE_2 and $F_{2\alpha}$ concentrations rise in the amniotic fluid at term before the onset of labor (Romero *et al.*, 1996); the administration of PGs promotes cervical ripening and induces labor (Jacobs, 1986), and drugs that inhibit the biosynthesis of PGs prolong pregnancy and protract labor (Gamissans *et al.*, 1993). Furthermore, the specific activity of the key enzyme of PG biosynthesis, prostaglandin endoperoxide H synthase (PGHS), and the relative abundance of its isoforms PGHS-1 and PGHS-2 rise in the fetal membranes prior to the onset of term labor (Chapter 4; Teixeira *et al.*, 1993, 1994; Mijovic *et al.*, 1998a, b).

These observations indicate that elevated synthesis of PGs through a rise in the expression of PGHS-1 and PGHS-2 in the gestational tissues is one event associated with the initiation of parturition in humans. The aim of the present investigation was to study in more detail the relationship between changes in the PG synthetic capacity of the intrauterine tissues and the timing of labor onset.

Data describing the increased expression and activity of PGHS in gestational tissues at term around the expected time of labor onset has been obtained from patients who delivered by elective cesarean section (CS) following 37 weeks of pregnancy. This ensured that clinically recognizable labor (no uterine contractions, intact membranes, cervical dilatation $\leq$ 2.0 cm) did not interfere with the postulated pre-labor changes in enzyme expression (Chapter 4; Teixeira *et al.*, 1993, 1994;

Mijovic *et al.*, 1998a, b). However, we know the process of parturition begins with subclinical changes occurring in the intrauterine tissues that are undetectable by normal obstetric monitoring and can only be identified by the measurement of biochemical markers. These changes include the early lesion of the chorio-decidual interface, which results in a rise in (onco)fetal fibronectin (fFn) levels in the cervico-vaginal secretions (Lockwood *et al.*, 1991). Fibronectins are a ubiquitous group of glycoproteins found in the plasma and extracellular fluid which have many different functions. A unique fibronectin present in the placenta, amniotic fluid, and certain malignancies contains an epitope called the "oncofetal domain" and is recognized by the antibody FDC-6 (Lockwood *et al.*, 1991). Fetal fibronectin has been identified in the extracellular matrix surrounding the extravillous trophoblast at the uteroplacental junction and in the chorion (Lockwood *et al.*, 1991). This immunolocalization of fFn suggests that it may be "leaked" into the vagina before the onset of labor. Available data indicate that the concentration of fFn in cervico-vaginal swabs obtained at the time of CS is reliably predictive of the remaining length of gestation, even in patients with clinically intact membranes (Lockwood *et al.*, 1991; Ahner *et al.*, 1995a, b; Iams *et al.*, 1995; Goldenberg *et al.*, 1996; Garite *et al.*, 1996; Peaceman *et al.*, 1997). The predictive value of cervico-vaginal fFn offered an opportunity to examine the temporal relationship of PGHS induction in the gestational tissues and labor onset.

In this study we assessed the PG synthetic capacity of the amnion, chorion and decidua of patients admitted for elective CS at term by measuring the abundance of PGHS mRNAs using a ribonuclease protection assay. We correlated these levels with fFn concentrations in the cervical secretions of the same patients. The hypothesis was that, among term pregnant women not in labor, high PGHS expression in the gestational tissues was associated with high fFn concentrations in the cervico-vaginal fluids since in these cases labor onset was close. The purpose of the study was to test

this hypothesis. The results provide a better assessment of the temporal sequence of changing PGHS mRNA expression and labor onset.

5.2. Methods

5.2.1. Study Patients

This study was carried out at the Royal Alexandra Hospital in Edmonton, Alberta, Canada. A written consent was signed after detailed information was given to every patient selected for the study. Permission for this study was obtained from the University of Alberta Ethical Review Committee and the Royal Alexandra Hospital Investigational Review Committee. Twenty four pregnant women at term (between 37 and 41 weeks gestation) admitted to the hospital at the time of elective CS with singleton pregnancies in the absence of clinical signs of labor were recruited to the study. Gestational age was established according to last menstrual period and by ultrasonographic fetometry at < 20 weeks of gestation. Patients were included on the basis of absence of uterine contractions, cervical dilatation ≤ 2.0 cm, and clinically intact membranes. Membrane rupture was defined by the presence of two or more of the following: gross pooling of the amniotic fluid, an alkaline vaginal pH on Nitrazine paper, ferning of dried vaginal secretions on microscopy, and ultrasonographic diagnosis of a decreased volume of amniotic fluid. Furthermore, women with clinical or histological signs of inflammation, or genital infection (fever, foul vaginal discharge), or with bacterial vaginosis, or with the presence of Group B streptococcus in the vaginal flora, were not included in the study.

5.2.2. Fetal Fibronectin Assay

Specimens for quantitative analysis of fFn were obtained no later than 30 minutes before the time of elective CS by taking a sample of the cervical and

posterior vaginal forniceal mucus with a sterile Dracon swab. The fFn assay was performed as described in Chapter 2.

5.2.3. Ribonuclease Protection Assay

Placentas with attached fetal membranes were obtained at the time of the elective CS. The placenta was separated from the fetal membranes immediately after delivery by cutting around the placental margins. Membranes were washed in physiological saline to remove excess blood. The amnion was separated from the chorio-decidua by blunt dissection; decidual tissue was then scraped from the chorion using a blade. The removal of decidual tissue from the chorion was monitored by histological examination indicating there were places in which very small areas of decidua remained. The tissue samples were cut into strips (10 x 20 mm), washed repeatedly with physiological saline, and snap frozen in liquid nitrogen. The effects of trauma of isolation on tissue PG production were minimized by limiting preparation time to < 20 mins. Samples from all membranes were examined histologically for evidence of neutrophil invasion as a sign of inflammation; only tissues that were shown to be negative for inflammation were used in the studies.

The frozen tissue was pulverized using a dry ice-cooled pestle and mortar. Total RNA was extracted from tissues using the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). The RNA concentration of each sample was determined by absorbance at 260 nm. Samples were then assayed for PGHS-1 and PGHS-2 mRNA abundance by ribonuclease protection as described in Chapter 2.

5.2.4. Data assessment and Statistical Analysis

Fetal fibronectin values are presented as averaged values of duplicate results.

PGHS-1 and PGHS-2 mRNA abundance in tissue samples were evaluated as previously described (Chapter 2).

Following application of all the exclusion criteria defined for this study, paired cervico-vaginal swabs and intrauterine tissue samples from a total of twenty-four patients were obtained for our analyses. Patients were assigned to groups according to the frequency distribution of fFn values. PGHS-1 and PGHS-2 mRNA abundance between patient groups was compared by the Wilcoxon signed rank test. This nonparametric test was used as the variance of the values in each patient group were heterogeneous (Bartlett's test).

PGHS mRNA levels in amnion, chorion and decidua were correlated with fFn in the cervico-vaginal swabs of the individual patients by simple linear regression analysis.

In all statistical analyses a P value < 0.05 was considered significant.

5.3. Results

Figure 5-1 shows the frequency distribution of fFn concentrations in the cervico-vaginal fluids of the patients involved in the study. As demonstrated in the histogram and the corresponding numerical data presented in Table 5-1, the patients fell into two groups according to the values of the fFn concentrations. In Group A, fFn concentrations ranged between 20-250 ng/ml; in Group B, concentrations of fFn were between 600 and 810 ng/ml of cervico-vaginal fluid and were significantly higher than in Group A ($P<0.05$). No patients exhibited concentrations between 250 and 600 ng fFn/ml. Statistical analysis showed that the mean gestational ages of the two groups were not different. This indicates that the difference in fFn levels

between the groups was not due to differing gestational ages, but because of the different lengths of time that was to elapse before the spontaneous onset of labor. Therefore, the relationship of PGHS abundance in the gestational tissues with the time of labor onset was examined.

First, the level of PGHS-1 and PGHS-2 mRNAs in the amnion, chorion laeve, and decidua of patients in Groups A and B were compared. The tissues were isolated following CS performed shortly (within 30 min) after the fibronectin swabs were obtained. The results are presented in Table 5-2. PGHS-2 mRNA abundance in the amnion and the chorion laeve, and PGHS-1 mRNA abundance in the chorion laeve of patients in Group B, were significantly ($P<0.05$, Wilcoxon signed rank test) higher than in the respective tissues of patients in Group A. No significant difference was found in decidua PGHS-1 or PGHS-2 mRNA levels and amnion PGHS-1 mRNA levels between the two groups. Thus, higher fFn concentrations in the cervico-vaginal fluids were associated with increased PGHS-1 and PGHS-2 mRNA levels in the chorion laeve and with enhanced PGHS-2 mRNA abundance in the amnion.

The relationship of cervico-vaginal fFn with PGHS mRNA levels was evaluated further by correlating PGHS mRNA abundance values with fFn concentrations in individual patients using linear regression analysis. A significant positive correlation was found between cervico-vaginal fluid fFn concentrations and PGHS-1 mRNA abundance in the chorion laeve (Fig. 5-3A) and between fFn concentrations and PGHS-2 mRNA abundance in the amnion (Fig. 5-2B) and the chorion laeve (Fig. 5-3B). No significant correlation was detected with amnion PGHS-1 mRNA (Fig. 5-2A) and decidua PGHS-1 or PGHS-2 mRNA abundance values (Fig. 5-4A, B).

5.4. Discussion

In several published studies, fFn levels of 50-60 ng/ml, or higher, in the cervico-vaginal fluid were found to be indicative of impending labor and birth in patient populations with threatened preterm labor, and in term and post-term pregnancies (Lockwood *et al.*, 1991, 1994; Ahner *et al.*, 1995a, b; Iams *et al.*, 1995; Goldman *et al.*, 1996; Garite *et al.*, 1996; Peaceman *et al.*, 1997). Lockwood *et al.* (1991) were the first to report that fibronectin in cervicovaginal secretions may predict preterm delivery. The presence of fFn (defined as > 50ng/ml) in cervical and vaginal swabs identified women who delivered before term with a sensitivity of 81.7% and a specificity of 82.5%. Ahner *et al.* (1995a) assessed that fFn is also a useful predictor of term delivery: in women admitted in the absence of clinical signs of labor 86% who tested positive for fFn (>50 ng/ml) delivered within 48 hours after sampling (negative predictive value, 88%). Additionally, low fibronectin values at 39 weeks (<60 ng/ml) are predictive of postdate pregnancies continuing beyond 41 weeks of gestation (22/23 negative predictive value, 95.8%). While vaginal fFn values of 60 ng/ml at 39 weeks of gestation predict delivery within 10 days (Lockwood *et al.*, 1994). Finally, the presence of fibronectin independently identifies patients who will have a shorter and easier induction of labor and lower CS rates. Multivariate analysis indicates that fFn (>50 ng/ml) is an independent, statistically significant predictor of delivery within 24 h of induction (Ahner *et al.*, 1995b; Garite *et al.*, 1996).

In the present investigation, all but two of the twenty-four patients involved had levels of fFn in their cervico-vaginal secretions higher than this 50-60 ng/ml "cutoff" range. Therefore, it is reasonable to assume that most of the patients in our study (mean gestational age ± SEM: 38.5 ± 0.17 weeks) would have delivered within 10 days (Lockwood *et al.*, 1994) had CS not been performed shortly after sampling. Conspicuously, none of the patients exhibited fFn levels between 250 and 600 ng/ml

cervico-vaginal fluid. Although the reason for this particular distribution of values is unclear, it has allowed us to separate the patients into two groups, one with fFn levels of ≤ 250 ng/ml (Group A), and one with fFn levels of ≥ 600ng/ml (Group B). Using a cutoff value of 500 ng/ml, Mouw *et al.*, (1995) found that delivery within 3 days could be predicted with a sensitivity of 94% and a positive predictive value of 100% in a group of patients with pregnancies of 41 weeks of longer. Furthermore, the mean gestational lengths in the two groups of the present study were equal indicating that the different fFn levels could not be attributed to different gestational ages. Collectively, these data suggest that Group B patients were closer to spontaneous labor onset at the time of fFn sampling and the subsequent CS than patients in group A.

Since Groups A and B were identified as comprising patients who were at different times before spontaneous labor onset, differences in PGHS expression in the gestational tissues could also be interpreted as a consequence of being more or less close to labor. The comparison of PGHS-1 and PGHS-2 mRNA levels in Groups A and B (Table 5-2) thus clearly indicated that the abundance of PGHS-1 and PGHS-2 mRNAs in the chorion laeve and of PGHS-2 mRNA in the amnion increased with approaching labor onset. In addition, the results imply that the induction of these enzymes at term, as reported previously (Chapter 4; Mijovic *et al.*, 1998a, b), is associated with the processes leading to labor initiation.

The rise in PGHS-1 mRNA levels in the amnion at term could not be linked to the time of labor onset on the basis of fFn values. Similarly, no labor-related change of PGHS abundance was found in the decidua, which is in agreement with the lack of gestational age dependent changes of enzyme levels in this tissue (Mijovic *et al.*, 1998b). These conclusions were confirmed by the correlational analysis of cervico-vaginal fFn concentrations vs. PGHS mRNA levels in individual patients. Fetal

fibronectin levels correlated positively and significantly with chorion laeve PGHS-1 and PGHS-2 mRNA and amnion PGHS-2 mRNA abundance values only.

The chorion laeve undergoes maturation at term, as indicated by the increased expression of PGHS-1, a developmentally regulated enzyme, and of PGHS-2, which is inducible by agonists (Mijovic *et al.*, 1998a, b). The tight correlation of fFn concentrations in the cervico-vaginal fluids with PGHS-1 and PGHS-2 mRNA abundance in the chorion laeve therefore links the release of fFn with the maturation of the chorion in preparation for labor. By the same token, the lack of correlation of amnion PGHS-1 expression with cervico-vaginal fFn makes it unlikely that fFn present in the amnion basement membrane appears in the cervical secretion before labor, despite the fact that PGHS-1 and PGHS-2 expression is enhanced at term in the amnion too (Chapter 4). It is tempting to speculate that the agonist(s) responsible for the induction of PGHS-2 in both tissues might originate, or emanate from, the chorion laeve. It may be relevant in this context, that culture medium conditioned by isolated chorion laeve cells was reported to contain factor(s) promoting PGHS activity and PG output in amnion cells (Lundin-Schiller *et al.*, 1990).

The mechanisms responsible for the release of fetal fibronectin into the cervico-vaginal secretions before term or preterm labor are unknown. Immunohistochemical studies have indicated that possible sources of the fFn may be the area of the uteroplacental junction and/or the chorio-decidual interface in the membranes (Lockwood *et al.*, 1991). Experimental evidence of increased PG, protease and cytokine metabolism using *in vitro* models of amniotic, chorionic and decidual cells indicate pathways exist supporting a relationship between increased PG synthesis, fFn release and impending labor (Lundin-Schiller *et al.*, 1990; Jackson *et al.*, 1996). The first proposed pathway is mechanical. Arachidonic acid is liberated from membrane phospholipids and then used as a substrate for the production of PGE_2 and $PGF_{2\alpha}$ by the PGHS pathway. PGE_2 and $PGF_{2\alpha}$ stimulate cervical change

and uterine activity which in turn mechanically stimulate choriodecidual extracellular membrane degradation and the release of fFn in cervico-vaginal secretions (Chapter 1). A second proposed pathway is related to localized inflammation at the choriodecidual interface. Activation of the maternal inflammatory response initiates cytokine and protease synthesis and secretion by constituent white cells. The cytokine/protease pathway leads to amniotic membrane degradation and the release of fetal fibronectin (Inglis *et al.*, 1994). Induction of cytokine synthesis also promotes decidual activation and synthesis and secretion of PGs which initiate cervical remodeling and increased uterine activity as previously described (Romero *et al.*, 1989). Additionally, experimental evidence suggests that cytokines and PGs indirectly facilitate degradation of the extracellular matrix by independently decreasing synthesis of matrix components (Huzar, 1991). Coordinated and continuous inductions of fetal membrane PG synthesis and maternal inflammatory response mechanisms are predicted to cause rapid cervical change, increased uterine contractions, rupture of amniotic membranes and delivery.

In conclusion, by correlating fFn concentrations in cervico-vaginal secretions with PGHS-1 and PGHS-2 expression in the gestational tissues, we have obtained evidence supporting the possibility that PGHS induction in the fetal membranes is associated with term labor onset. Further studies are required to define the exact role of these enzymes in the regulatory cascade that determines the timing of birth.

Table 5-1 Gestational age and cervical fluid fetal fibronectin concentration.

	Group A	Group B	
Number of patients	16	8	
Gestational age (weeks) mean ± SEM	38.3 ± 0.17	38.8 ± 0.36	NS
Fibronectin (ng/ml) mean ± SEM [range]	154 ± 19 [20-250]	732 ± 28 [600-810]	$P<0.0001$ [1]

NS not significant
[1] t-test

Table 5-2 PGHS-1 and PGHS-2 mRNA abundance in the gestational tissues of women with different levels of fetal fibronectin in their cervical fluid.

Tissue, and mRNA species	Group A		Group B		
	Number of patients	mRNA band intensity mean ± SEM median [range]	Number of patients	mRNA band intensity mean ± SEM median [range]	
Amnion PGHS-1	11	0.3 ± 0.05 0.27 [0.11-0.55]	7	0.45 ± 0.12 0.45 [0.075-0.9]	NS
Amnion PGHS-2	15	0.82 ± 0.12 0.83 [0.09-1.7]	7	2.67 ± 0.15 2.75 [2.14-3.2]	P=0.0002 [1]
Chorion PGHS-1	16	0.21 ± 0.03 0.16 [0.06-0.52]	6	0.57 ± 0.11 0.57 [0.3-0.91]	P=0.0057
Chorion PGHS-2	16	0.39± 0.13 0.25 [ND-1.91]	6	1.3 ± 0.29 1.2 [0.6-2.3]	P=0.0045
Decidua PGHS-1	15	0.36 ± 0.08 0.27 [ND-1.2]	6	0.58 ± 0.21 0.45 [0.08-1.3]	NS
Decidua PGHS-2	15	1.05 ± 0.39 0.3 [0.01-4.67]	6	0.97 ± 0.54 0.2 [0.15-3.2]	NS

NS, Not significant
ND, Not detectable
[1] Wilcoxon signed rank test

Figure 5-1 Frequency distribution of fetal fibronectin concentrations in the cervico-vaginal fluid of patients involved in the study.

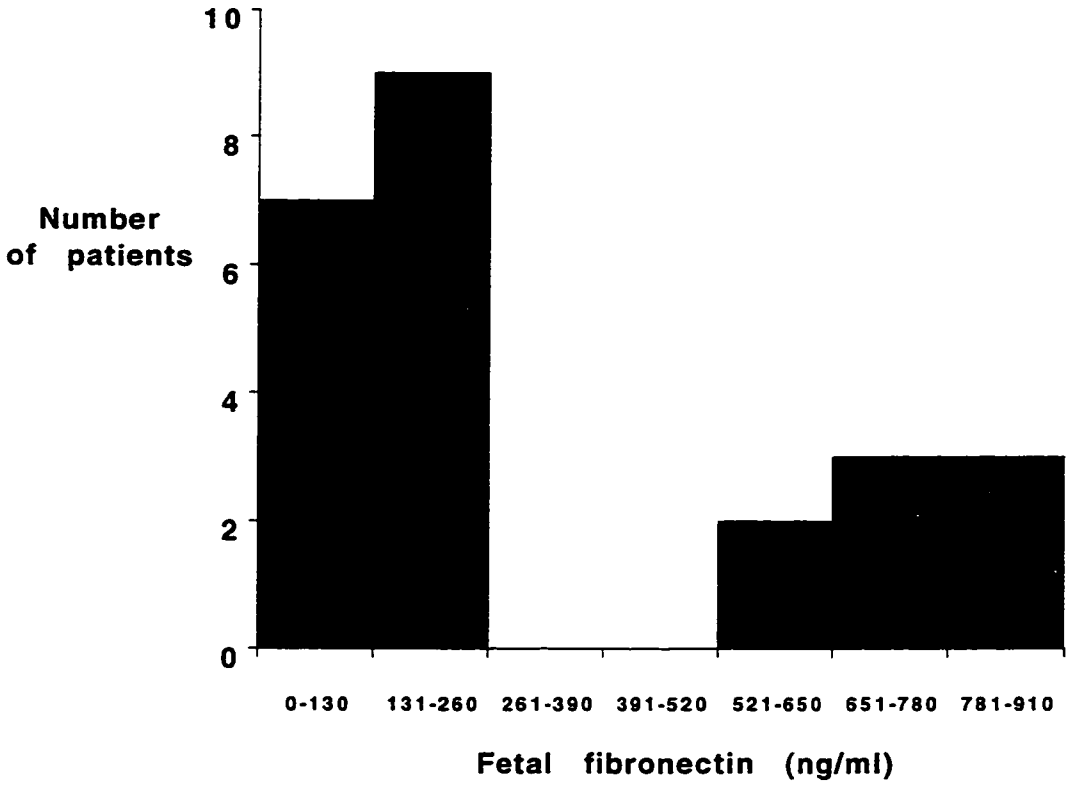
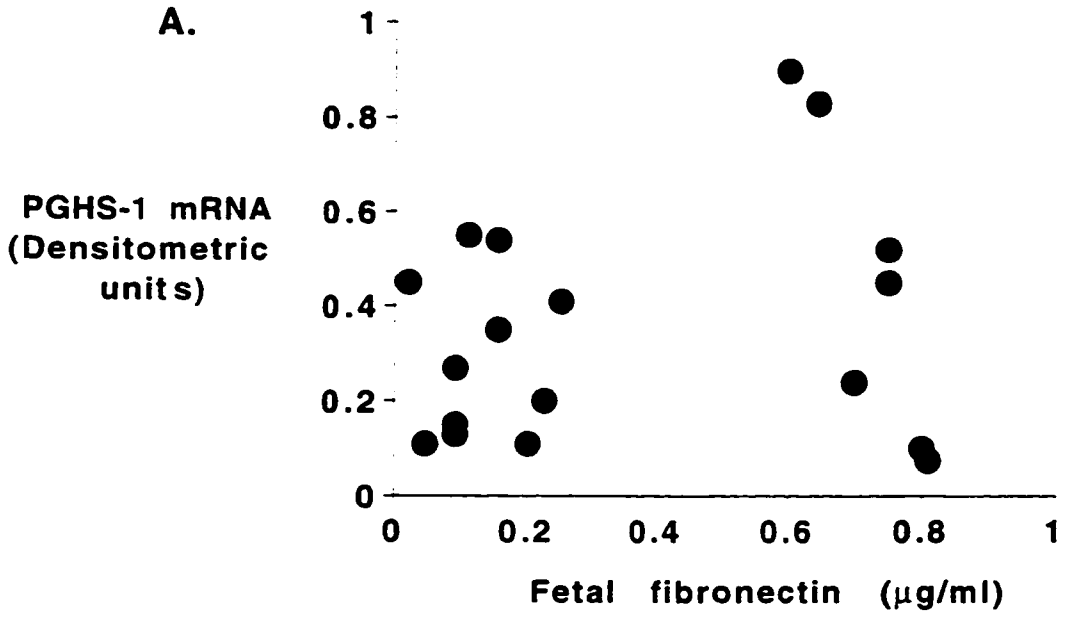

10
8
6
4
2
0
Number of patients
0-130
131-260
261-390
391-520
521-650
651-780
781-910
Fetal fibronectin (ng/ml)

Figure 5-2 Correlation of cervico-vaginal fluid fetal fibronectin concentration with PGHS-1 mRNA (A: r=0.19: P=0.46: n=18) and PGHS-2 mRNA (B: r=0.88: P<0.0001: n=22: linear regression analysis) abundance in the amnion of patients.


A.
PGHS-1 mRNA (Densitometric units)
1
0.8
0.6
0.4
0.2
0
0
0.2
0.4
0.6
0.8
1
Fetal fibronectin (µg/ml)

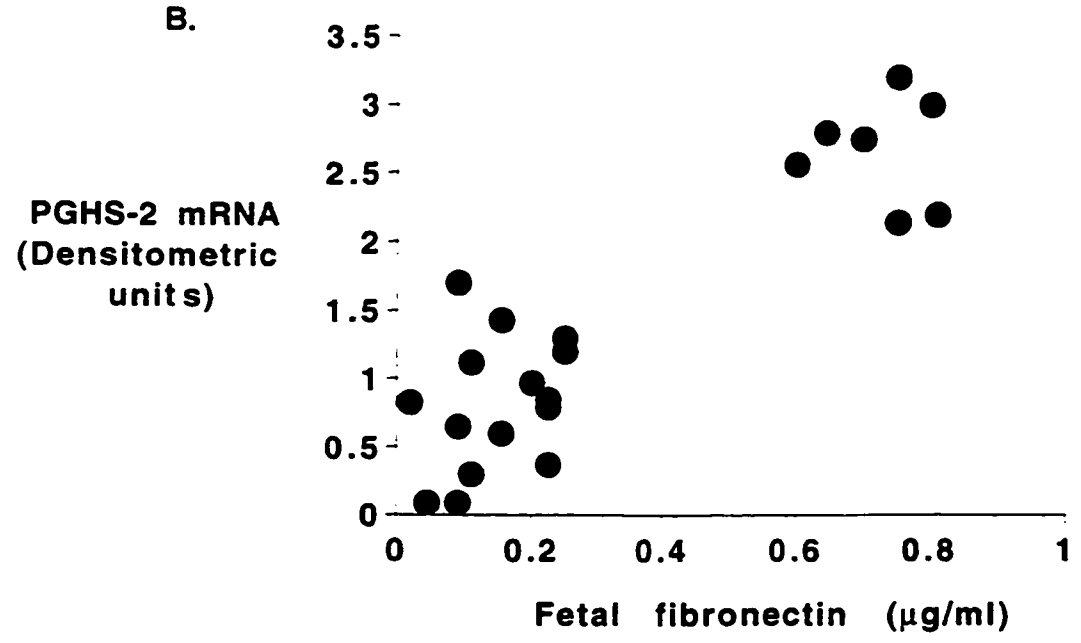

B.
PGHS-2 mRNA (Densitometric units)
3.5
3
2.5
2
1.5
1
0.5
0
0
0.2
0.4
0.6
0.8
1
Fetal fibronectin (µg/ml)

Figure 5-3 Correlation of cervico-vaginal fluid fetal fibronectin concentration with PGHS-1 mRNA (A: r=0.72; *P*=0.0002; n=22) and PGHS-2 mRNA (B: r=0.69; *P*=0.004; n=22; linear regression analysis) abundance in the chorion laeve of patients.

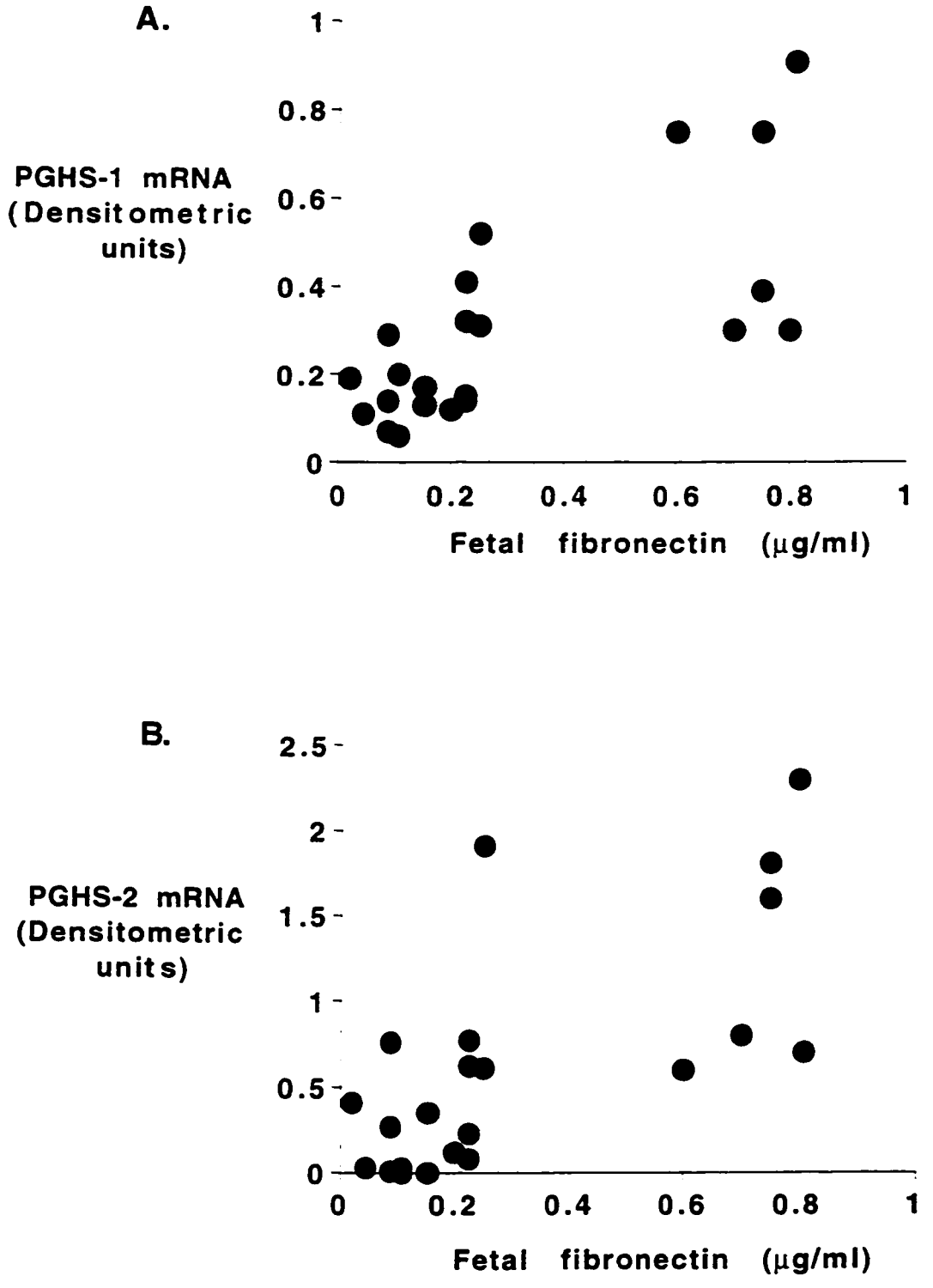
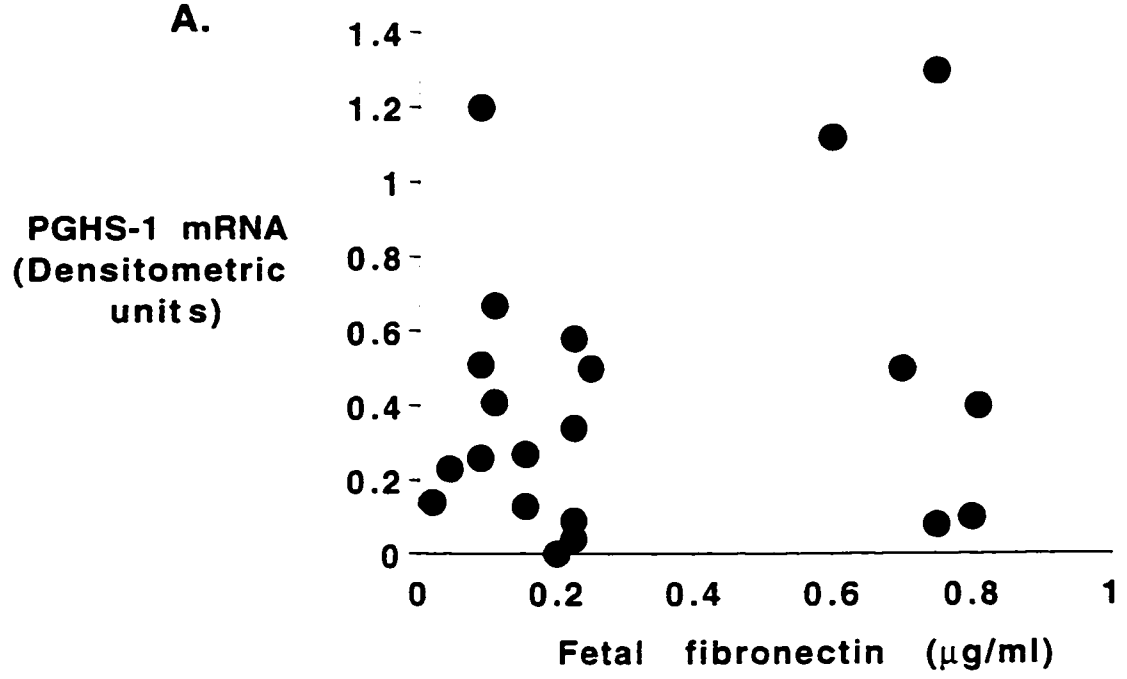
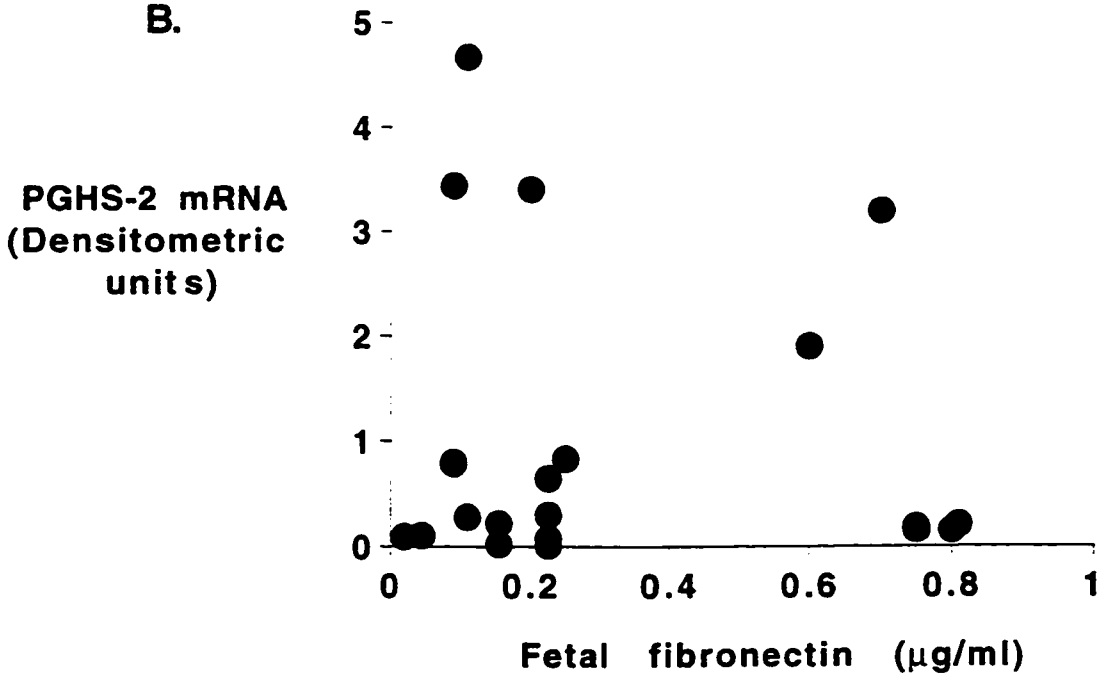

A.
PGHS-1 mRNA (Densitometric units)
1
0.8
0.6
0.4
0.2
0
0
0.2
0.4
0.6
0.8
1
Fetal fibronectin (µg/ml)
B.
PGHS-2 mRNA (Densitometric units)
2.5
2
1.5
1
0.5
0
0
0.2
0.4
0.6
0.8
1
Fetal fibronectin (µg/ml)

Figure 5-4 Correlation of cervico-vaginal fluid fetal fibronectin concentration with PGHS-1 mRNA (A: r=0.18 P=0.43; n=21) and PGHS-2 mRNA (B: r=0.089; P=0.70; n=21; linear regression analysis) abundance in the decidua of patients.

A.


PGHS-1 mRNA
(Densitometric
units)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
0.2
0.4
0.6
0.8
1
Fetal fibronectin (μg/ml)

B.


PGHS-2 mRNA
(Densitometric
units)
5
4
3
2
1
0
0
0.2
0.4
0.6
0.8
1
Fetal fibronectin (μg/ml)

5.5. References

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6. PROSTAGLANDIN ENDOPEROXIDE H SYNTHASE AND PRETERM BIRTH: CONSIDERATIONS AND CONSEQUENCES

6.1. Introduction

Substantial evidence has been presented suggesting that PGs are of critical importance in the initiation and maintenance of human labor at term. In this study we have attempted to apply our knowledge of the fundamental physiological processes occurring at term birth to the pathophysiology, and methods of prevention of preterm labor and delivery.

In the past it has often been assumed that preterm labor differs little from labor at term, except that it occurs too early. In the absence of major complications that override physiological processes, the mechanisms that initiate preterm labor have been thought to be precocious activation of the same events that cause labor at term. For these reasons suppression of endogenous PG synthesis was suggested as a logical approach to the inhibition of preterm labor.

The first reports on the application of the principle of blocking endogenous PG synthesis as an approach to the inhibition of preterm labor were based on two studies that purported to compare indomethacin with placebo (Niebyl *et al.*, 1980; Zuckerman *et al.*, 1984). However, neither of these studies was placebo-controlled since a number of women in each study received other tocolytic drugs; but, delivery was delayed for 48 h or more, pregnancy was prolonged for more than 1 week, and the incidences of preterm birth and of low birthweight were lower in the indomethacin treated group (Zuckerman *et al.*, 1984).

These data suggest that the use of indomethacin in preterm labor may prolong gestation and improve fetal and neonatal mortality and morbidity. More recently, studies specifically designed to evaluate the outcome of infants exposed to short antenatal courses of indomethacin for tocolysis have been described (Norton *et al.*,

1993; Major *et al.*, 1994; Eronen *et al.*, 1994). Despite significantly prolonging gestation, infants born < 120 h after indomethacin exposure suffered from an increased incidence of bronchopulmonary dysplasia (BPD), respiratory distress syndrome (RDS), necrotizing enterocolitis (NES), patent ductus arteriosus (PDA) and an increased need for surgical ligation of the PDA. In addition, platelet dysfunction and fluctuations in intracranial pressure in the fetus resulting in a higher risk of intracranial hemorrhage (ICH) in infants were documented. In another study, Van den Veyver and Moise (1993) found maternal indomethacin use caused prolonged prenatal constriction of the DA resulting in persistent pulmonary hypertension and tricuspid insufficiency in the newborn. Furthermore, indomethacin has been found to affect fetal renal blood flow and tubular function causing a decrease in fetal urine output and oligohydramnios. And, transient postnatal renal dysfunction has been described resulting in significant metabolic and volume derangements in premature newborn infants (Vanhaesebrouck *et al.*, 1988).

Maternal side-effects of indomethacin use include peptic ulceration, gastrointestinal bleeding, thrombocytopenia, allergic reactions, masking of signs of infection, and prolonged bleeding times (Gamissans and Balasch, 1984).

The undesirable side-effects of PG synthesis inhibitors on fetal and maternal well being when used in an attempt to prolong gestation is not surprising due to the ubiquitous nature of the PGs. For example, the increased incidence of RDS and BPD in infants exposed to indomethacin can be attributed to the inhibitory effects of this drug on surfactant production which is stimulated by PGE_1 and PGE_2. Furthermore, prevention of PG production increases the amounts of unmetabolized arachidonic acid; this can then be converted to other proinflammatory mediators resulting in lung damage (Eronen *et al.*, 1994). In addition, the central role of PGE_2 in maintaining patency of the DA during pregnancy determines that constriction of this vessel will be

an outcome of tocolytic treatments utilizing inhibition of PG synthesis as their mode of action (Brezinka *et al.*, 1993).

The rate-limiting, and committing step, of PG biosynthesis is catalyzed by the enzyme prostaglandin endoperoxide H synthase (PGHS). Tocolytics such as indomethacin that act by blocking PG synthesis inhibit PGHS. The effects of these drugs on pregnancy and maternal and fetal physiology must be re-interpreted since the identification of two PGHS isoenzymes (Sirois *et al.*, 1992; Herschman, 1994), especially as studies in mice (Langenbach *et al.*, 1995; Morham *et al.*, 1995; Gross *et al.*, 1998) and our observations in human tissues (Hirst *et al.*, 1995, 1998; Mijovic *et al.*, 1997, 1998a, b; Chapters 3,4,5) suggest that PGHS-1 and PGHS-2 may have unique roles in the initiation of normal term labor; and, as indomethacin has been found to be 40-fold more potent against PGHS-1 compared to PGHS-2 (Smith and DeWitt, 1995). Inferring from these data we predict that the use of indomethacin as a tocolytic results in detrimental side-effects to fetal (and maternal) well-being due to the inhibition of PG production by the PGHS-1 enzyme, but prolongs gestation due to inhibition of the PGHS-2 isoform. And, by making the assumptions:

1) that PGHS-1 is responsible for production of the PGs involved in fetal growth and development during the third trimester; and,

2) that PGHS-2 is induced only in the intrauterine tissues with increasing gestational age, and not elsewhere in the fetus;

we suggest a safer more effective therapeutic approach to dealing with preterm birth may be the administration of a specific PGHS-2 inhibitor to women at risk.

However, the scientific evidence in support of these assumptions is limited: the extent of our knowledge of the physiological processes occurring at preterm labor is minimal, and, the source of the PGs essential for fetal development and the regulation of their production is unknown. Therefore, in a preliminary set of studies we attempted to substantiate our speculations. We determined the activity, abundance

and distribution of PGHS-1 and PGHS-2 mRNA in the amnion, chorion laeve and decidua vera of patients before and after preterm labor; and, we studied the gestational age-dependent profile of the expression of these isoenzymes in a variety of fetal tissues.

6.2. Methods

6.2.1. Tissue Collection

Fetal membranes and decidua

Placentas with attached fetal membranes were obtained from a total of ten singleton pregnancies at 28-36 weeks of gestation. Five samples were collected at preterm CS (mean ± SEM, 31 ± 1.4 weeks [gestational age calculated from the first day of the last menstrual period]) in the absence of labor (defined as ≤ 1 uterine contraction/10 min, ≤ 2 cm cervical dilatation as determined by pelvic examination, and intact membranes) and five samples were collected from patients following spontaneous preterm labor (mean ± SEM, 30.8 ± 0.8 weeks). Preterm CS was performed due to pregnancy induced hypertension, placenta previa, or fetal distress. Patients in preterm labor presented with intact membranes and delivered within 72 h. The mean gestational ages of CS and SL patients were not significantly different.

The placenta was separated from the fetal membranes immediately after delivery by cutting around the placental margins. Membranes were washed in physiological saline to remove excess blood. Small pieces of full-thickness membrane were cut, rolled, and fixed in 10% formalin in phosphate buffered saline (PBS) for 24-48 h at room temperature (RT). The remaining amnion was separated from the chorio-decidua by blunt dissection; decidual tissue was then scraped from the chorion using a blade. Histological examination revealed that almost all the decidua had been separated from the chorion. Chorion and decidua samples were cut

into strips (10 x 20 mm), washed repeatedly with physiological saline to remove clotted blood and snap frozen in liquid nitrogen. The effects of the trauma of isolation on tissue PG production was minimized by limiting preparation time to < 20 min. Each sample of frozen tissue was pulverized using a dry-ice cooled pestle and mortar and separated into batches for RNA extraction and enzyme activity assay.

The formalin fixed tissue samples were processed for *in situ* hybridization and histological analysis by dehydration and embedding in paraffin block.

Fetal tissues

Human embryonic and fetal tissues were obtained at elective abortion by the Central Laboratory for Human Embryology at the University of Washington. This laboratory is supported by the National Institute of Health and supplied tissues from normal embryos and fetuses ≤ 20 weeks of gestation. Specimens were obtained within minutes of passage and tissues were aseptically identified and immediately snap frozen in liquid nitrogen. The effects of the trauma of isolation on tissue PG production was minimized by limiting preparation time to < 20 mins. Samples were shipped in dry ice and stored at -80°C until assayed.

Tissues from third trimester and term fetuses were obtained from patients in the absence of labor (defined as ≤ 1 uterine contraction/10 min, ≤ 2 cm cervical dilatation as determined by pelvic examination, and intact membranes) by the Department of Laboratory Medicine and Pathology, University of Alberta Hospital, and from the National Disease Research Institute in Philadelphia (NDRI). Tissues were snap frozen in liquid nitrogen at the time of autopsy. These fetuses and neonates had been stored at 4°C for no longer than two days prior to autopsy. Additionally, tissues from postdelivery fetuses were supplied by the NDRI.

Gestational age was calculated from the first day of the last menstrual period.

In all cases women with clinical signs of inflammation or genital infection (fever, foul vaginal discharge) or bacterial vaginosis were excluded from the study. Additionally, patients were routinely tested for the presence of group B streptococcus in the vaginal flora, and those found positive were not included in the study. Furthermore, small pieces of full thickness fetal membranes and decidua were rolled and fixed in 10% formalin in PBS for 24-48 hours at room temperature. These formalin-fixed tissue samples were processed for histological analysis and were examined for neutrophil invasion as a sign of inflammation; only tissue samples from patients with no histological signs of infection were used.

6.2.2. PGHS Enzyme Activity Assay

The method to assay PGHS activity in amnion tissue has been characterized in our laboratory (Smieja *et al.*, 1993; Teixeira *et al.*, 1993, 1994) and is described in detail in Chapter 2. PGHS specific activity was measured in chorion obtained from patients before and after preterm labor.

6.2.3. Ribonuclease Protection Assay

PGHS-1 and PGHS-2 mRNA abundance was measured in amnion, chorion and decidua collected from patients at preterm CS and SL. In addition, the established methodology for the ribonuclease protection assay was validated for use in fetal tissues; the probes and protocol used were as described previously (Hirst *et al.*, 1995; Chapter 2). However, the possibility that the mRNA in the fetal and neonatal tissues collected at autopsy and stored at 4°C had undergone degradation by the action of ribonucleases during the time between sampling and freezing had to be investigated. A sample of amnion tissue was collected and pieces were either snap frozen in liquid nitrogen immediately and stored at -80°C, or stored for varying lengths of time in physiological saline at 4°C before the freezing process. The

objective of this preliminary investigation was an attempt to mimic the treatment of the sample tissues being used in this study. Ribonuclease protection assays were then carried out to measure the levels of PGHS-1, PGHS-2 and γ-actin mRNA in these amnion samples and to compare the effect of snap freezing of tissues with storage at 4°C on mRNA levels. As we found no difference in the abundance of any of these mRNAs in the amnions subjected to the various treatments we concluded that no significant degradation of RNA would occur in our term fetal and neonatal samples and that meaningful results could be obtained from this project. We therefore assayed all samples by the standard protocol.

6.2.4. *In Situ* Hybridization (Mijovic *et al.*, 1997).

The distribution of PGHS-1 and PGHS-2 mRNA in full thickness membranes obtained before and after preterm labor was determined by *in situ* hybridization. This methodology is described in detail in Chapter 2.

6.2.5. Data Assessment and Statistical Analysis

PGHS enzyme specific activity values are expressed as picograms of PGE_2 produced /μg microsomal protein /minute.

PGHS-1 and PGHS-2 mRNA levels in tissue samples were evaluated as previously described (Chapter 2).

Differences between PGHS enzyme activity levels in chorion and PGHS-1 and PGHS-2 mRNA levels in amnion, chorion and decidua samples from the preterm CS and SL patients were analyzed using the nonparametric Wilcoxon signed rank test. This method of analysis was considered to be most appropriate as the variance of the values in the two groups was found to be heterogeneous (Bartlett's test).

Longitudinal changes in PGHS mRNA abundance in fetal tissues as a function of gestational age were assessed by polynomial regression.

In situ hybridization data was analyzed qualitatively as described in Chapter 2. In all statistical analyses, significance was achieved at $P<0.05$.

6.3. Results

6.3.1. PGHS Enzyme Activity and PGHS-1 and PGHS-2 mRNA Abundance in Intrauterine Tissues during Preterm Labor

PGHS enzyme activity in intrauterine tissues during preterm labor

Figure 6-1 shows PGHS specific activity in chorion tissues collected from patients at preterm CS compared to those following preterm SL. PGHS specific activity was significantly higher in the preterm SL group (mean ± SEM, 21.61 ± 2.8; median, 19.9; range, 15.2-32.2 pg of PGE_2 /μg protein /min) compared to the preterm, not in labor CS group (mean ± SEM, 9.2 ± 1.7; median, 9.2; range, 5.3-15.3; Wilcoxon signed rank test; $P<0.05$). These data are similar to those in amnion reported by Teixeira *et al.* (1994). They found the specific activity of PGHS increased from 5.9 ± 1.8 pg of PGE_2 /μg protein /min in tissues obtained at preterm CS to 28.3 ± 6.8 pg of PGE_2 /μg protein /min after spontaneous labor. To date, similar studies have not been carried out in decidua.

PGHS -1 and PGHS-2 mRNA abundance in intrauterine tissues during preterm labor

PGHS-1 and PGHS-2 mRNA levels were determined in amnion, chorion and decidua tissues by ribonuclease protection assay. Forty μg of total tissue RNA was hybridized with the PGHS-1 or PGHS-2 cRNA probe and produced protected bands in the expected 309 and 400 nucleotide positions, respectively. Figure 6-2 shows an example of some results collected by ribonuclease protection assay; the expression of PGHS-1 and PGHS-2 mRNAs in the chorion of patients at preterm CS and SL is demonstrated. Data collected for the amnion, chorion and decidua were quantified as

described in Chapter 2 and are presented in Figure 6-3. Statistical analysis indicated there was a significant increase in the abundance of both PGHS-1 and PGHS-2 mRNA in amnion and chorion tissue (Amnion PGHS-1 preterm CS: mean ± SEM. 0.2 ± 0.05; median. 0.18; range 0.1-0.33; n=4; preterm SL: mean ± SEM. 0.81 ± 0.16; median. 0.66; range. 0.43-1.34; n=5; Amnion PGHS-2 preterm CS: mean ± SEM. 0.47 ± 0.16; median. 0.33; range 0.05-0.97; n=5; preterm SL: mean ± SEM. 1.34 ± 0.25; median. 1.64; range. 0.74-1.94; n=4; Fig. 6-4A; Chorion PGHS-1 preterm CS: mean ± SEM. 0.04 ± 0.01; median. 0.03; range 0-0.1; preterm SL: mean ± SEM. 0.2 ± 0.03; median. 0.31; range. 0.19-0.35; n=5; Chorion PGHS-2 preterm CS: mean ± SEM. 0.04 ± 0.02; median. 0.01; range 0.01-0.12; preterm SL: mean ± SEM. 0.31 ± 0.06; median. 0.33; range. 0.1-0.41; n=5; densitometric units; Fig. 6-3B; Wilcoxon signed rank test; $P<0.05$). In decidual tissues collected following preterm labor there was a decrease in the abundance of both PGHS-1 and PGHS-2 mRNA compared to those at CS from patients of the same gestational age.

In situ hybridization

In Figure 6-4, the localization of PGHS-1 and PGHS-2 mRNAs in membranes collected at preterm CS in the absence of labor and in membranes obtained after spontaneous preterm delivery are presented. Figure 6-4C and D are the negative controls hybridized with the corresponding sense probes. *In situ* hybridization patterns in Figure 6-4A and B (upper section) clearly indicate a marked increase in the abundance of PGHS-1 mRNA in the cytokeratin-negative cells of the chorion laeve and in the amnion after spontaneous preterm labor. Hybridization patterns in the lower section of Figure 6-4A and B, indicate an increase in abundance of PGHS-2 mRNA with preterm labor, which was also localized to the cytokeratin-negative cells of the chorion laeve and amnion mesoderm. The cytokeratin positive chorion laeve trophoblasts and amnion epithelium exhibited little or no increase of either PGHS-1

or PGHS-2 mRNA hybridization with preterm labor. however. cells in the decidua stained positive for PGHS-2 mRNA at this time.

6.3.2. Gestational Age-Dependent Changes in PGHS-1 and PGHS-2 mRNA Abundance in Fetal Tissues

Gestational age dependent changes in PGHS-1 and PGHS-2 mRNA abundance in human fetal intestine. kidney. lung and heart collected at various times during gestation were investigated by ribonuclease protection assay. Twelve sets of tissues were collected following elective early termination of pregnancy, one set was obtained from a preterm patient. and five sets were collected at term. All the fetuses were delivered by Cesarean section. In addition three sets of tissues were obtained from neonates following term labor.

Longitudinal regression analysis of PGHS-1 and PGHS-2 mRNA abundance as a function of gestational age in human fetal intestine (A). kidney (B). lung (C) and heart (D) is shown in Figure 6-5. These results indicate that in fetal lung and heart there is a small but significant decrease in the abundance of the PGHS-1 isoenzyme with increasing gestational age. In the intestine and kidney and other fetal and neonatal tissues such as the brain and the liver. there was no change in PGHS-1 mRNA levels from the first to the third trimester.

However. in intestine. kidney. and lung. PGHS-2 mRNA levels were low or undetectable in tissues collected form the 59-135 day age group but showed a significant increase in tissues collected from third trimester fetuses and postdelivery neonates. The apparent induction of PGHS-2 in these tissues was not an event common to all tissues sampled. For example in the heart. liver and brain there was no change in PGHS-2 mRNA levels throughout gestation or with term labor.

At present, due to difficulty in obtaining appropriate tissues we have no data describing the effect of preterm labor on fetal tissue PGHS-1 and PGHS-2 mRNA abundance, therefore these studies are still incomplete, but ongoing.

6.4. Discussion

6.4.1. PGHS Enzyme Activity and PGHS-1 and PGHS-2 mRNA Abundance in Intrauterine Tissues during Preterm Labor

Premature delivery continues to be a major cause of infant morbidity and death. Because PGs play an important role in the initiation and maintenance of term labor, inhibitors of PG synthesis have been used for the prevention of preterm labor since the early 1970s. However, it is meaningful to question how comparable the physiological process occurring at term and preterm labor really are; and how appropriate it is to develop tocolytic treatment based on the assumption that the two events are synonymous.

To date the evidence supporting a role for PGs in the mechanisms responsible for preterm labor is less firm than for term labor. Levels of PG have been reported to be either normal (Neider and Augustin, 1984) or increased (Novy and Liggins, 1980) in both plasma and amniotic fluid of women with preterm labor. The discrepancies between these studies may be attributed to the heterogeneous nature of the disease causing preterm labor. Teixeira *et al.* (1994) suggested a common mechanism associated with both term- and idiopathic preterm labor is an increase in the PGHS specific activity of the amnion. Our data indicate preterm labor is also associated with an increase in the specific activity of PGHS in the chorion.

However, despite these observations indicating that both term and preterm labor are characterized by an increase in PGHS specific activity in the fetal membranes, PGHS expression patterns at preterm labor appear substantially different

from those seen with labor at term. There was an increase in the levels of both PGHS isoforms at preterm labor and the increases were localized predominantly to the mesenchymal cells of the amnion and the chorion laeve. There was apparently no significant induction of either isoenzyme in the epithelial cells in association with preterm labor in our group of patients. This is in contrast to the events associated with term labor which involve an exclusive increase in PGHS-2 expression throughout amnion and chorion epithelium and mesenchymal cells, and decidua (Chapter 3; Mijovic *et al.*, 1997, 1998a). Furthermore, the virtual absence of epithelial cell involvement in PGHS-1 and PGHS-2 induction clearly distinguishes preterm labor from tissue maturation at term before labor. Although, this process also involves increased expression levels of both PGHS-1 and PGHS-2 it is localized to the epithelial and mesenchymal components of the fetal membranes (Chapter 4; Mijovic *et al.*, 1998b). The available data did not allow the determination of whether the changes in PGHS expression occur shortly before or during preterm labor but they do suggest that there is a preterm maturation of the mesenchymal components of the fetal membranes (as indicated by enhanced PGHS-1 mRNA expression), accompanied by a preterm induction of PGHS-2, also in the mesenchymal cells.

The factors responsible for regulating the increases in PGHS expression at preterm labor have not been identified. Considerable controversy exists concerning the identity of the initiators responsible for premature labor onset in humans, especially in the so-called 'idiopathic' cases of preterm labor. Clinical and subclinical infection, hemorrhage, utero-placental ischemia, stress and physiological factors (multiple fetuses) have all been listed as possible causes of the pathogenesis of preterm labor (Lockwood, 1995). These initiators result in the potentiated expression of PG, cytokines and ECM-degrading proteases in the intrauterine compartment leading to cervical change, separation of the chorion from the decidua and uterine contractions. However, it is possible that preterm labor in any setting is initially a

premature activation of the fetal hypothalamic-pituitary-adrenal axis in response to maternal and fetal stress caused by the pathogenic processes listed above. This would result in an increase in fetal cortisol output causing enhanced placental, decidual and amnio-chorion CRH expression and the premature onset of an endocrine cascade involving PGHS induction specifically in the fibroblastic component of the fetal membranes. Unless intervention was successful these processes would lead to labor and premature delivery of an infant. In this context it should be noted that the stimulation by glucocorticoids of PGHS-2 expression in cultured amniotic fibroblasts has been reported (Economopoulos *et al.*, 1996) warranting further studies to explore the significance of this phenomenon in the processes occurring at preterm labor.

In summary, these data indicate that although an increase in PGHS activity in the fetal membranes is a common event associated with term and preterm labor. PGHS expression in preterm labor appears to be limited to the mesenchymal cells of the membranes indicating that tissue regulation in this condition is different from that in normal labor at term.

While this study is preliminary, the results do suggest the extrapolation of our knowledge of the processes responsible for the intimation of labor at term to that occurring preterm may be misleading and imply the use of caution when developing potential therapeutic treatments for the prevention of premature labor based on such information.

6.4.2. PGHS-1 and PGHS-2 mRNA Abundance in Fetal Tissues during Gestation

The preceding discussion emphasizes the importance of the implications of studies into the physiological mechanisms controlling preterm labor. Our data confirm that inhibitors of PGHS should prolong gestation in patients presenting with symptoms of preterm labor, however, the effectiveness of such treatment relative to

the adverse effects on maternal and fetal well-being must also be considered. Traditionally, tocolytics such as indomethacin are believed to significantly prolong gestation by inhibition of PGHS-2, while prevention of PG synthesis via PGHS-1 is thought to cause detrimental effects on the fetus. This implies the development of a specific PGHS-2 inhibitor may efficiently prevent preterm labor, and result in minimal fetal and maternal side-effects. However, no data exists to substantiate these statements. Indeed, our observations imply both PGHS-1 and PGHS-2 are involved in the events causing the onset of preterm labor. Furthermore, the increased expression of both these isoenzymes appears to occur mainly in the fetal membranes raising the possibility that an elevation in the expression of PGHS-1 and PGHS-2 is a general phenomena occurring in many fetal tissues in late gestation. In this case, both isoforms of PGHS may contribute to the PGs responsible for fetal development and organogenesis indicating development of a tocolytic devoid of potential side-effects may be more complex than originally thought. In order to investigate this possibility further we determined the profile and prevalence of the expression of PGHS-1 and PGHS-2 in a variety of fetal tissues collected throughout pregnancy.

We found a significant increase in PGHS-2 mRNA abundance and no change or a concomitant decrease in PGHS-1 mRNA levels in a variety of fetal tissues during the last trimester of gestation and with term labor. It is possible that PGHS-1 may be responsible for PG production in the early fetus, with PGHS-2 becoming more important towards term. Initially PGs produced by the constitutive PGHS-1 may be important for general cell proliferation and growth. Later in gestation the inducible isoenzyme may be responsible for controlling tissue specific differentiation and maturation resulting in the development of the term fetus. It is of interest that the tissues that showed significant increases in PGHS-2 expression to term are those detrimentally effected by indomethacin treatment.

The significance of the gestational age-dependent changes in PGHS-1 and PGHS-2 mRNA abundance in the human fetus can be interpreted from the available knowledge of fetal physiology, and from studies using various animal models.

It is now known that PGs have a profound effect on fetal lung development and the cardiopulmonary transition that occurs at birth. Prostaglandin E_2 tonically suppresses fetal breathing movements and postnatal breathing movements at the level of the respiratory neurones in the brain stem. At birth, occlusion of the umbilical cord leading to a decrease in the concentration of inhibitory substances such as PGs in the fetal circulation is one factor contributing to the establishment of rhythmic continuous breathing in the infant. Furthermore, there is a decrease in pulmonary vascular resistance in the newborn which is mediated in part by PGHS products such as PGI_2 and PGD_2. Indomethacin administration increases fetal breathing movements, *in utero*, and reduces the fall in pulmonary vascular resistance in the newborn. In sheep, the synthesis of PGI_2 and other vasodilatory PGs in the whole lung and pulmonary artery rises during late gestation and in the newborn due to a significant increase in PGHS-1 mRNA and protein . This increase was shown to attenuate hypoxic pulmonary vasoconstriction in the neonate, to modulate vascular cell growth, and to be involved in maturational changes in pulmonary vessel structure before and after birth (Brannon *et al.*, 1994). In humans, an increase in the functional expression of PGHS-2 in the lung and pulmonary vasculature may be responsible for parallel processes.

The significance of a gestational age-dependent elevation of PGHS-2 expression in the intestine can be explained by observations on the role of PGs in colon cancer. Case control and prospective studies have found a reduced occurrence of colon cancer and lower mortality from the disease among frequent aspirin users (Giovannucci *et al.*, 1995). Three research groups have examined PGHS-1 and PGHS-2 expression in normal and malignant colon tissues (Eberhart *et al.*, 1994;

Kargman *et al.*, 1995; Sano *et al.*, 1995). Each of these studies concluded that PGHS-1 is expressed constitutively, and its levels are similar in normal and transformed colon tissue; PGHS-2 is not expressed in nontumor tissue but is elevated in 90% of colonic carcinomas. Furthermore, Tsujii and DuBois (1995) have examined the effects that elevated PGHS-2 has on rat intestinal epithelia (RIE) *in vitro*. They demonstrated that when normal RIE were plated onto extracellular matrices that mimic the basement membrane, they undergo apoptosis. However, RIE expressing high levels of PGHS-2 are refractive to apoptosis when plated onto the same matricies. Importantly, the resistance to apoptosis in PGHS-2 transfected cells could be reversed by the PGHS inhibitor sulindac sulfide. These experiments indicate that PGHS-2 is involved with the normal differentiative processes of cultured intestinal cells, an event that could be mimicked during development of the fetal intestine *in vivo*. Moreover, RIE in culture show a mitogenic response and a 16-fold increase in the $PGF_{2\alpha}$ metabolite 6-keto-$PGF_{1\alpha}$ when stimulated with the cytokine TGFα, as well as significant increases in immunoreactive PGHS-2 protein. PGHS inhibitors prevent the induction of 6-keto-$PGF_{1\alpha}$, and the mitogenic response to TGFα (DuBois *et al.*, 1994). Taken together, these observations suggest PGHS-2 may produce certain eicosanoids that play a role in the growth regulation of intestinal epithelial cells; and that a developmental process may account for the increased PGHS-2 expression seen in the human fetal intestine during late gestation.

Experiments by Langenbach *et al.* (1995) and Morham *et al.* (1995) describing the construction and phenotypic characterization of transgenic mice deficient in PGHS-1 and PGHS-2 are indicative of the importance of the PGHS-2 isoenzyme in fetal and neonatal kidney development. PGHS-1 deficient mice (Ptgs1 -/-) had few phenotypic abnormalities, however homozygous mice lacking PGHS-2 (Ptgs2-/-) began to die around 8 weeks of age with few surviving as long as 16 weeks. All tissues examined in these mice were normal except the kidney. Nephropathy was

observable by 6 weeks and increased in severity until death. Kidney maturation ceased prematurely in these mice after only a small percentage of nephrons developed and the vast majority of glomeruli and tubules remained small and immature. As the animals grew older, the functional nephrons became overworked and began to atrophy, with increased glomerular sclerosis, interstitial inflammation, and fibrosis, ultimately leading to kidney failure. Further insight into the roles of PGHS-1 and PGHS-2 in the development of the human kidney have been gained by immunohistochemistry and *in situ* hybridization studies (Komhoff *et al.*, 1997). In fetal kidney PGHS-1 is primarily expressed in collecting duct cells and podocytes, while PGHS-2 was detected in endothelial and smooth muscle cells of the veins and arteries. Glomerular staining for PGHS-2 was present in podocytes only at the end of renal development. Taken together, the data documenting renal pathologies in transgenic mice and the studies describing PGHS localization in human fetal kidney suggest that PGHS-2 is required for renal perfusion and glomerular haemodynamics during kidney development, and in its absence only a few nephrons develop. Additionally, PGHS-2 may produce PGs involved in the production of growth factors required for development of the fetal and neonatal renal system.

In summary, in this study we found a significant increase in PGHS-2 mRNA abundance and no change or a concomitant decrease in PGHS-1 mRNA levels in a variety of fetal tissues in the third trimester of gestation and with term labor. We have presented evidence that indicates the induction of PGHS-2 may be important in some aspects of fetal physiology and organogenesis with increasing gestational age at least in the intestine, kidney and lung.

6.5. Conclusion

Our observations describing the changes in activity and expression of PGHS in intrauterine tissues at preterm labor suggest there is a preterm increase in the abundance of both PGHS-1 and PGHS-2 mRNA exclusively in the mesenchymal components of the fetal membranes. At present the function and contribution of these isoenzymes to the processes occurring at preterm labor are unknown. In addition, in this study we have documented a significant rise in PGHS-2 expression towards term in a variety of other fetal tissues. These data indicate the selection of a therapeutic method for the prevention of preterm birth based on isoform selectivity of PGHS may be more complex that previously thought. A specific PGHS-2 inhibitor may prolong gestation in patients presenting with preterm labor, but they may also prove detrimental to the well-being of the fetus. Assuming PGHS-1 makes a significant contribution to the events responsible for the initiation and maintenance of preterm labor this study raises the possibility that a selective PGHS-1 inhibitor may be more appropriate.

The obvious deficit in the data described here concerns the situation in third trimester and preterm fetuses. It is important to extend our preliminary studies to determine the timing of the induction of PGHS-2 expression in fetal tissues and its significance in terms of contribution to total PGHS enzyme activity. We can speculate that induction of PGHS-2 involved in the maturation of organ systems and such processes may not have been initiated or completed in a premature fetus. Thus PGHS-2 mRNA levels and activities may be significantly lower in preterm tissues while PGHS-1 is still the prevalent form. If this is the case a selective inhibitor of this isoenzyme may not prove to be an option.

On the other hand, the use of PGHS-2 inhibitors can be considered as safe and effective tocolytics only if fetal PGHS-2 increases very close to term (39-40 weeks) or the enzyme is inactive for some reason. If induction occurs earlier, or there is a

large variation between subjects, such treatments will endanger the welfare of the developing fetus. At present our studies are ongoing and for obvious ethical reasons a full study on the cause and effects of preterm labor and pharmaceutical methods to prevent it would be most thoroughly carried out in an animal model. Therefore, we have developed and characterized a mouse model of preterm birth for use as part of a quick, effective system for initial screening of new drugs for potential management of preterm labor in humans (Appendix).

Figure 6-1 PGHS specific activity in the chorion at preterm labor. Tissues were collected following idiopathic preterm labor (SL: n=5). and after CS performed at a similar gestational age in the absence of labor (CS: n=5). Each point represents an individual patient: medians are indicated by solid horizontal bars. CS: mean ± SEM. 9.2 ± 1.7; median. 9.2: range, 5.3-15.3; SL: mean ± SEM. 21.61 ± 2.8; median. 19.9; range. 15.2-32.2 pg of PGE_2 /μg protein /min. (Wilcoxon signed rank test: $P<0.05$).

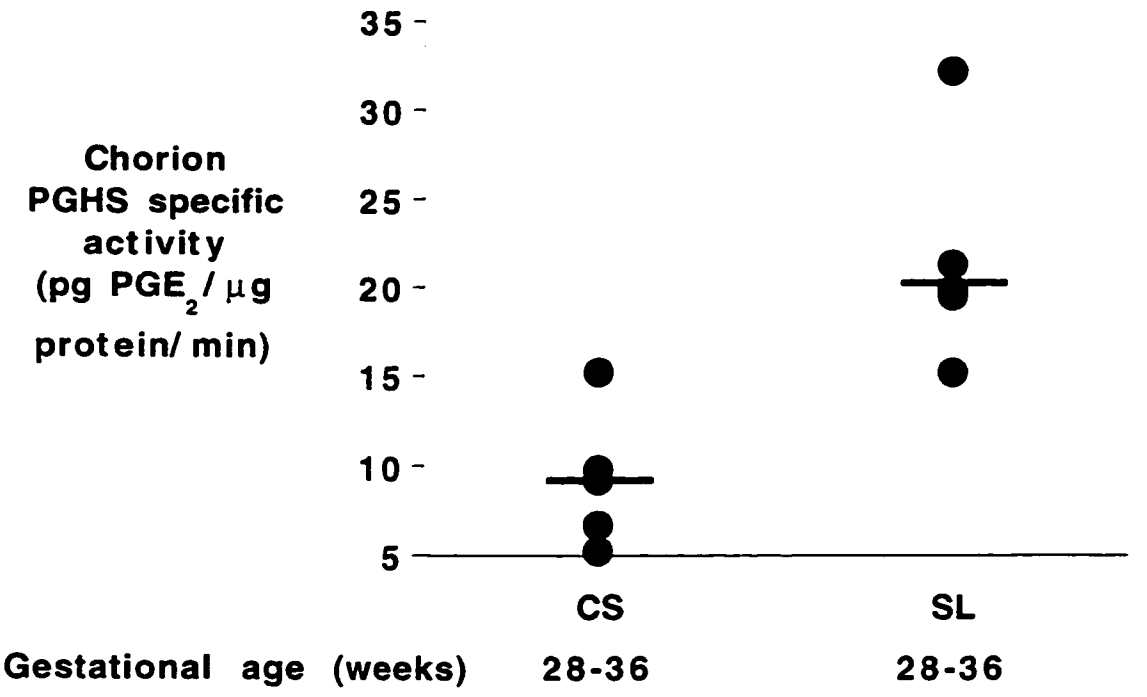

35
30
25
20
15
10
5
Chorion PGHS specific activity (pg PGE2 / µg protein/ min)
CS
SL
Gestational age (weeks)
28-36
28-36

Figure 6-2 PGHS-1 mRNA (A), PGHS-2 mRNA (B), and γ-actin mRNA (C) levels in human chorion obtained after preterm labor (SL) and preterm CS in the absence of labor (CS), measured by ribonuclease protection assays. Each lane represents an individual patient. The three mRNA species were determined in the same tissue samples, and results are presented in corresponding order in panels. Positions of the 300 and 400 base standards are shown on the left. Assay backgrounds measured using only carrier yeast tRNA for hybridization are also shown (t).

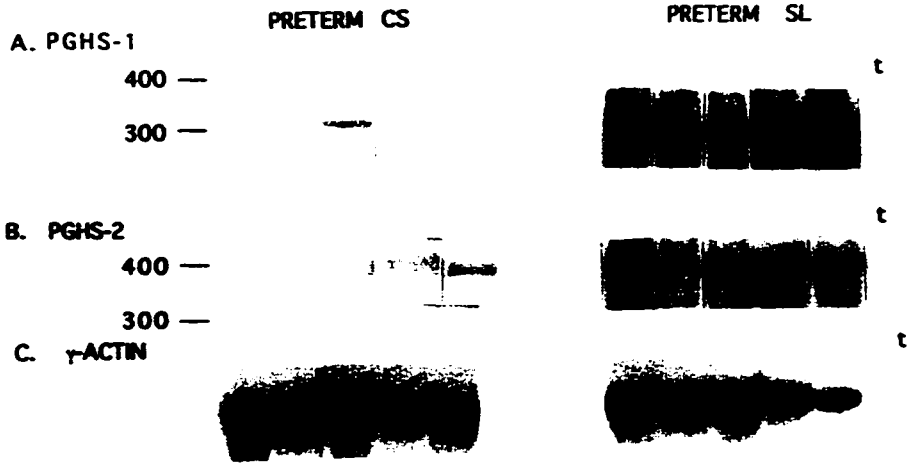

PRETERM CS
PRETERM SL
A. PGHS-1
400
300
t
B. PGHS-2
400
300
t
C. γ-ACTIN
t

Figure 6-3 PGHS-1 and PGHS-2 mRNA abundance in amnion (A), chorion (B) and decidua (C) after preterm labor (SL) and CS at a similar gestational age (CS). PGHS-1 and PGHS-2 mRNA intensities on autoradiographs were quantified by densitometry and normalized to the γ-actin band intensity in the same tissue. Each point represents an individual patient; medians are indicated by solid horizontal bars. Both PGHS-1 and PGHS-2 mRNA levels were significantly higher in the amnion and chorion following preterm labor than in the absence of preterm labor (Wilcoxon signed rank test; $P<0.05$).

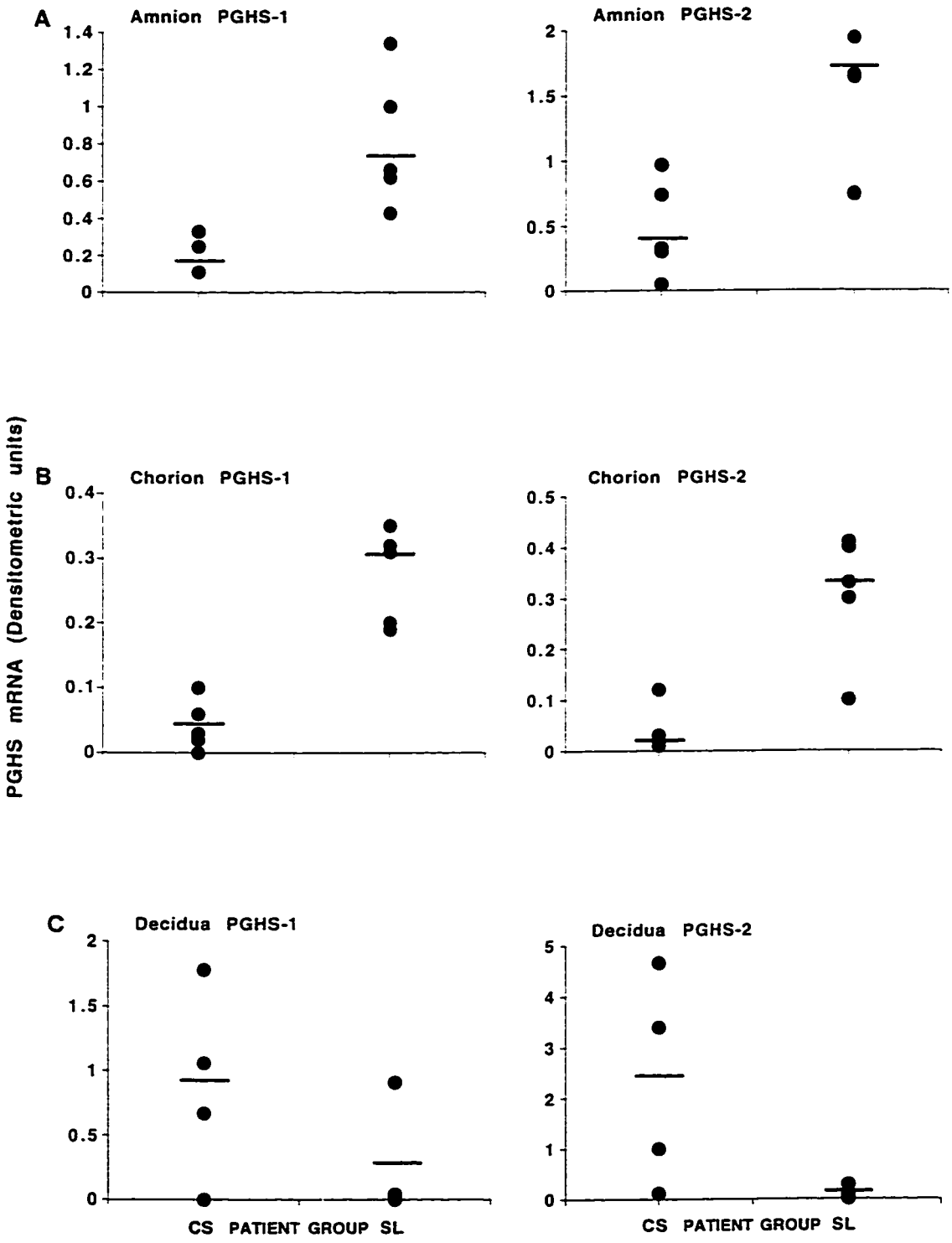
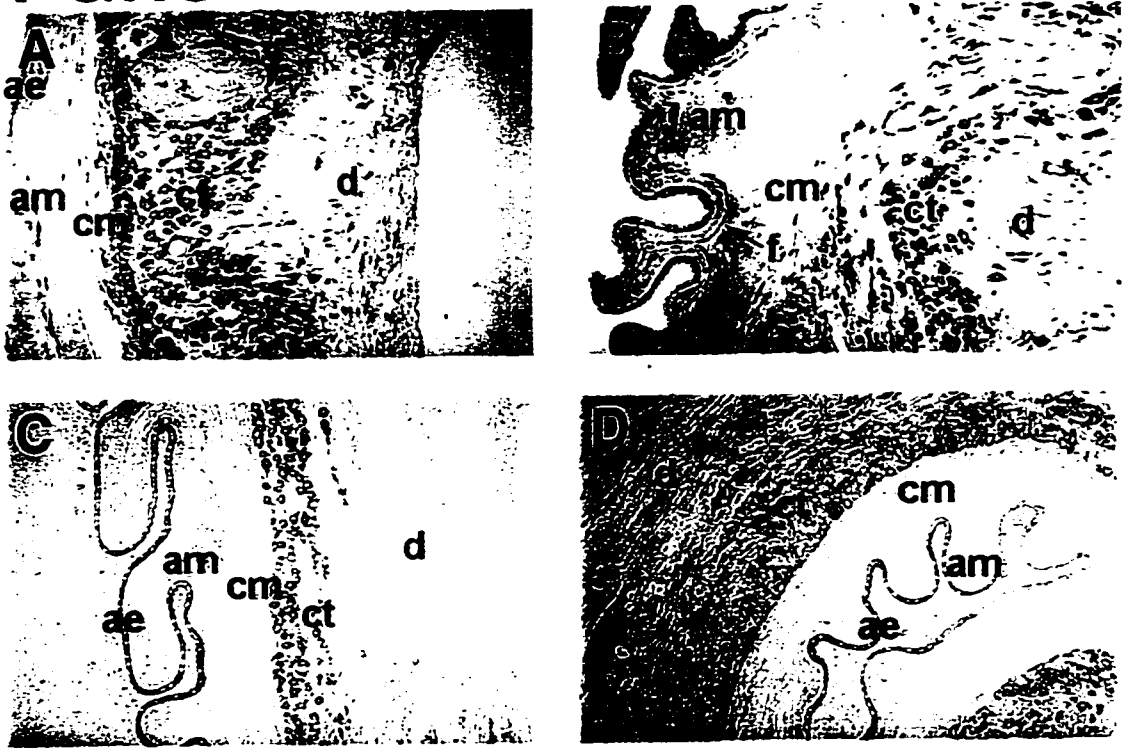

A
Amnion PGHS-1
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Amnion PGHS-2
2
1.5
1
0.5
0
B
Chorion PGHS-1
0.4
0.3
0.2
0.1
0
Chorion PGHS-2
0.5
0.4
0.3
0.2
0.1
0
PGHS mRNA (Densitometric units)
C
Decidua PGHS-1
2
1.5
1
0.5
0
Decidua PGHS-2
5
4
3
2
1
0
CS PATIENT GROUP SL
CS PATIENT GROUP SL

Figure 6-4 PGHS-1 and PGHS-2 mRNA localization in full-thickness membranes collected at idiopathic preterm labor (B: 231 days of gestation) and after preterm CS in the absence of labor (A: 254 days) as visualized by *in situ* hybridization. C and D. respective negative controls from the same patients. Purple color shows hybridization. whereas brown immunohistochemical staining shows cytokeratin-positive amnion epithelium and chorion trophoblast. Magnification: X10. ae: amnion epithelium: am: amnion mesoderm: cm: chorion mesoderm: ct: chorion trophoblast: f: fibroblast cells: d: decidua.

PGHS - 1


A
ae
am
cm
ct
d
am
cm
ct
d
f
C
am
cm
ae
ct
d
D
cm
am
ae

PGHS - 2

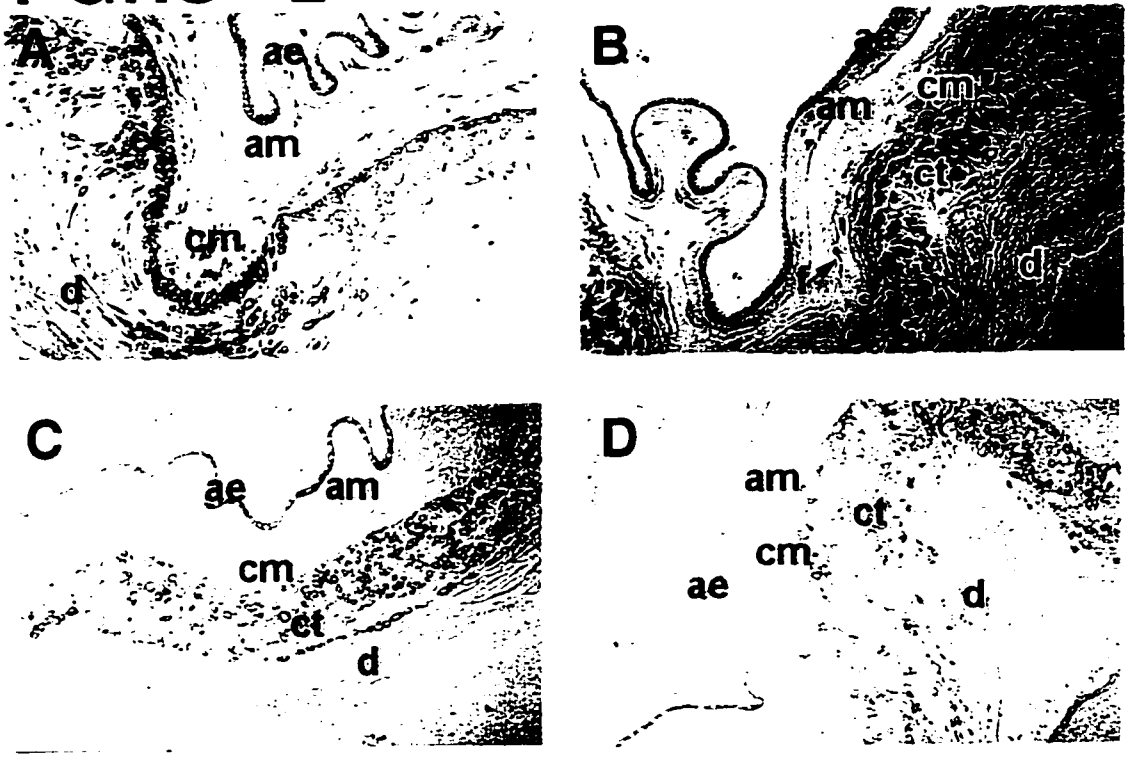
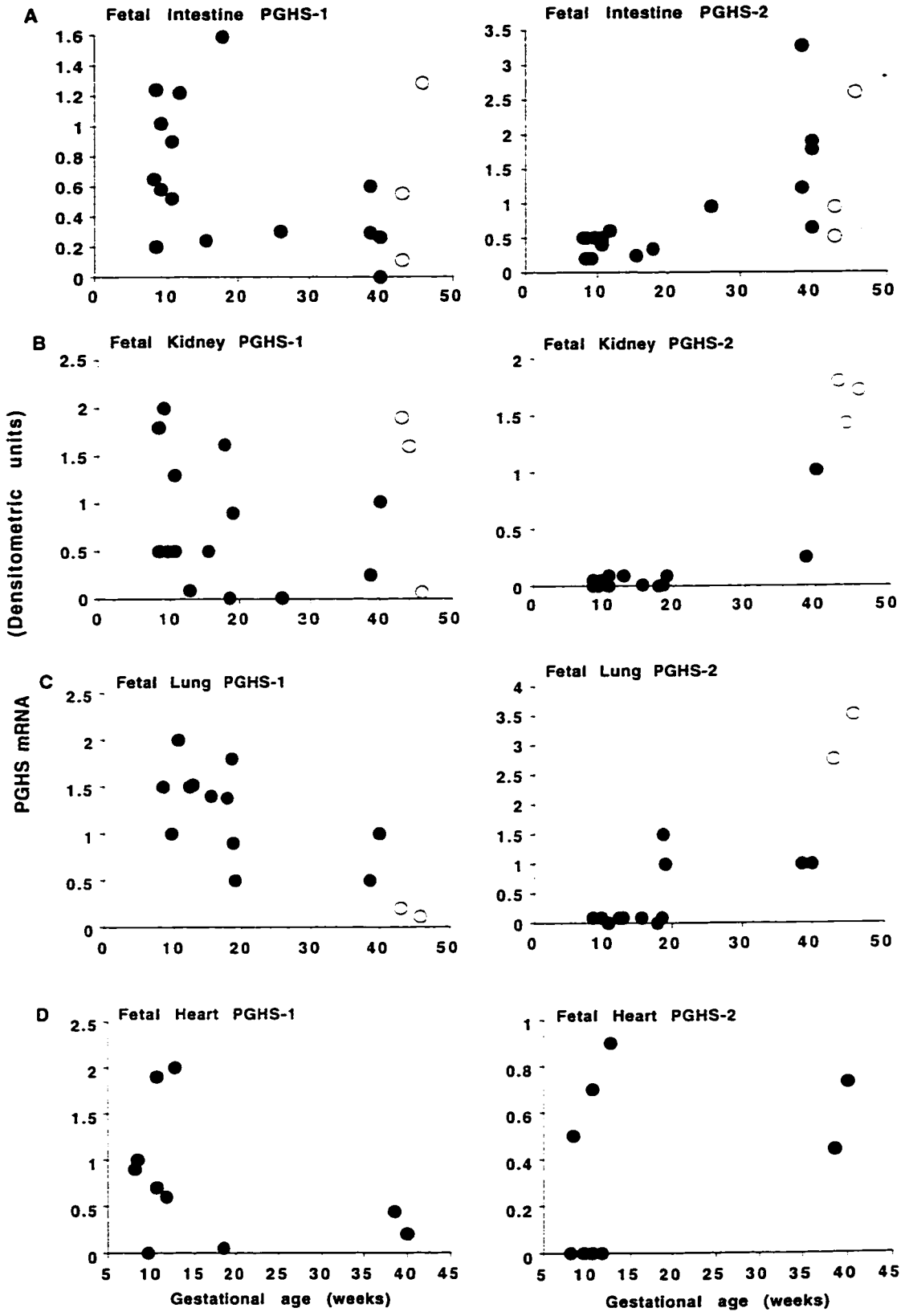

A
ae
am
cm
d
B
cm
am
ct
d
C
ae
am
cm
ct
d
D
am
ct
cm
ae
d

Figure 6-5 Changes in the abundance of PGHS-1 and PGHS-2 mRNA in human fetal intestine (A), kidney (B), lung (C), and heart (D) with increasing gestational age (closed circles) and in neonatal tissues (open circles) collected following term labor. Each point represents a single tissue from an individual fetus or neonate. Preliminary data suggest a gestational age dependent increase in PGHS-2 mRNA in a variety of fetal tissues with a concomitant decrease or no change in the abundance of PGHS-1 mRNA (polynomial regression analysis: $P<0.05$).


A
Fetal Intestine PGHS-1
Fetal Intestine PGHS-2
B
Fetal Kidney PGHS-1
Fetal Kidney PGHS-2
(Densitometric units)
C
Fetal Lung PGHS-1
Fetal Lung PGHS-2
PGHS mRNA
D
Fetal Heart PGHS-1
Fetal Heart PGHS-2
Gestational age (weeks)
Gestational age (weeks)

6.6. References

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7. GENERAL CONCLUSION

Based on the data presented in this thesis, the answers to several previously asked questions (Challis, 1997) can be expanded upon:

1) Are PGs responsible for the initiation of parturition?

We know the answer to this is no. The initiation of parturition probably occurs at the time of uterine activation, and most likely even earlier. It is possible to propose a scheme involving PGs but beginning at the level of the fetal hypothalamic-pituitary-adrenal axis. Progesterone maintains pregnancy. During this time the fetal adrenal obtains the capacity to produce increasing amounts of estrogen precursors. The placenta converts these precursors to estrogen. Towards term there are increasing concentrations of estrogen in the intrauterine compartment mirroring the increasing DHEAS output from the fetal adrenal. During late gestation maturation of the adult zone of the fetal adrenal occurs resulting in elevated levels of cortisol in the fetal circulation and the gestational tissues. Alternatively, at term the fetus may produce increased cortisol as an adaptive response to an environmental stress such as a decreased capacity of the placenta to deliver oxygen and nutrients; this proposal revisits the early theories of Hippocrates and Barcroft. Preterm birth may be a premature activation of this response, and/or a reaction of the fetus to a pathological condition such as infection or hemorrhage. The processes responsible for the changing glucocorticoid and estrogen levels in the fetal circulation and intrauterine tissues must be considered the initiators of parturition. In fact, it is doubtful there is one event or one time point at which it is possible to say "parturition has begun". More likely, it is a gradual process, but still, for the most part, exquisitely controlled by physiological changes in the fetus and the mother. The initial triggers to labor onset may be a threshold level of cortisol production together with an elevated estrogen output in the face of a high progesterone environment and/or a local

progesterone withdrawal at the tissue/cellular level. Estrogens cause uterine activation. Glucocorticoids act to increase placental CRH and initiate the feedforward cascade controlling the production of intrauterine uterotonins. A crucial part of this process is the induction of PGHS-2 in the gestational tissues directly by cortisol, and indirectly via placental CRH. The feedforward cascade is maintained by 11β-HSD type 2 in the syncytiotrophoblast which inactivates cortisol to cortisone and prevents a negative feedback of glucocorticoids on CRH and PGE_2 release; chorion 11-βHSD1 acts locally to prevent conversion of cortisol to cortisone and so maintains high glucocorticoid levels in this tissue. This decreases PGDH expression and activity and so prevents catabolism of newly synthesized PGs produced by PGHS-2 induced in the amnion and chorion. Additionally, progesterone withdrawal inhibits PGDH activity. Collectively, these pathways contribute to increased PG production by intrauterine tissues. Increased production of these uterotonins acting through the appropriate receptors participate in the drive to myometrial contractility, cervical remodeling, membrane rupture and expulsion of the fetus.

2) Do PGs play an obligatory role in the process of parturition?

Based on experiments with gene null mutations and transgensis in mice (Langenbach *et al*., 1995; Morham *et al*., 1995) we know the answer to this is also no. In light of these observations and the discussions pertaining to question one, therefore, it is meaningful to ask if it is relevant to develop blockers of PGHS expression and activity for use in the prevention of preterm birth? Preliminary observations in a mouse model suggest that it may be (Appendix). But, pregnancy in the mouse is corpus luteum dependent and PGs cause luteolysis resulting in parturition. Any luteotropic substance, physiological or pharmacological will prolong gestation in this species. In women, PGHS-2 is implicated as being the functionally important isoenzyme at labor. Most research is concentrating on developing a

specific PGHS-2 inhibitor as a tocolytic with minimal maternal and fetal side-effects. However, PGHS-2 is present in the fetus during the third trimester although its function is unknown, therefore, inhibition of this isoenzyme may effectively prolong gestation but may also be detrimental to fetal well-being. The prelabor increase in PGHS-1 expression in human amnion and chorion should not be overlooked, especially as evidence in mice suggest PGHS-1 is necessary for parturition (Gross *et al.*, 1998). In humans, the fetal side-effects caused by indomethacin treatment are attributed to an inhibition of PGHS-1. However, it is possible a specific PGHS-1 inhibitor would be a more efficacious tocolytic if administered within a "window of opportunity" identified to maximize the length of gestation but minimize detrimental effects on fetal development. Obviously, more research into the role of the PGHS isoenzymes and PGs in fetal physiology and development, and at parturition is required before pharmaceutical intervention can safely and effectively be used for the treatment of preterm birth.

Therefore,

3) What are the possible functions of PGs produced by the intrauterine tissues at parturition and labor, and in the fetus?

Prostaglandins are usually thought of as stimulators of uterine contractility. And, the attention paid to the smooth muscle-stimulating activity of PGs has probably obscured their possible alternative functions. Support for a role of PG that is different from myometrial contractility comes from data presented in this thesis documenting rises in the PG synthetic capacity of the amnion and chorion before subclinical and clinical signs of labor. This suggests PGs may have a role in uterine activation. There is evidence that PGs stimulate the formation of gap junctions in rat myometrium. Indomethacin administration is associated with decreased numbers of gap junctions (Garfield *et al.*, 1980), and naproxen delayed the formation of gap

junctions (Chan *et al.*, 1988). Such a mechanism may account for the preterm increase in human myometrial connexin-43 mRNA expression (Chow and Lye, 1994) which occurs without any known change in maternal steroid levels. Furthermore, naproxen also prevented the term increase in OT receptors in rats, (Chow and Lye, 1994), and administration of PG gel induces labor at any stage of gestation. These data imply PGs have a role prior to uterine stimulation to achieve birth.

In addition, the prelabor rise in PGHS expression appears to be exclusive to tissues of fetal origin and includes the amnion and chorion, but also the lung, kidney and gut. In the amnion and chorion this prelabor increase in PG synthetic capacity appears to be due to an increase in the abundance of both PGHS isoforms, while in other fetal tissues, an exclusive induction of PGHS-2 is occurring. Amnion and fetal kidney PGs may play a role in fluid or ion balance. PGs are potent mediators of transmembrane ion flow (Ramwell and Shaw, 1970), and PGE_2 is active in the control of ion transport in many cells, including those of the kidney (Frazier and Yorio, 1992), colon (Kreusel *et al.*, 1991), duodenum (Yao *et al.*, 1993) and gall bladder (Saunders-Kirkwood *et al.*, 1993). The turnover of amniotic fluid is due partly to fetal swallowing and partly to absorption by the amnion (Lotgering and Wallenburg, 1986), and this might be affected by PGs. Such an explanation would account for the oligohydramnios associated with indomethacin treatment in humans (Darwood, 1993) and monkeys (Manaugh and Novy, 1976), although this fluid loss could be attributed to an effect on the fetal kidney. An epidermal growth factor like-factor has been reported in amniotic fluid which increases PGHS in amnion cells (Casey *et al.*, 1988). This factor originates in the fetal kidney; could this factor be coordinating fetal kidney and amnion to ensure correct fluid and/or ion balance with PG as the mediator?

Localization studies describing the distribution of PGHS in fetal tissues at term were not completed as part of this thesis. However, evidence suggests PG

production is associated with many epithelial cell layers, the most obvious being gastrointestinal (Mezey and Palkovits, 1992), lung and trachea (Hume *et al.*, 1993). Therefore, it is possible that the PGs are involved in modulating mucosal immunity in the fetus. Since the amnion might form an integral part of the emerging fetal immune system (Cleveland *et al.*, 1991), is PGE_2 a critical immune regulator?

What is the function of PG production in the decidua? Our data suggests the specific activity of PGHS in the decidua is much higher than in any other intrauterine tissue studied, and is present at these levels throughout gestation. It is likely the PGs produced by the decidua during gestation are acting locally due to the high concentrations of PGDH adjacent to their site of production. And, it is probable that they do not stimulate intense myometrial contractility during this time due to a decreased sensitivity of the uterus. Rather, decidual PGs may be involved in the maintenance of pregnancy having an immunosuppressive role. By regulating leukocyte function and cytokine secretion (Kelly, 1994) these PGs could allow accommodation of the maternal immune system and the conceptus for the length of gestation. Evidence in support of this comes from studies in mice which suggest that many of the observed changes in leukocyte behavior within the decidua responsible for the maintenance of pregnancy are due to PGE_2 (Linnemeyer and Pollack, 1993). Furthermore, observations using cultured decidual cells indicate that they produce an immunosuppressive agent which has been identified as PGE_2 since its activity is substantially reduced by both indomethacin and anti-PGE antiserum, and correlates with the PGE_2 content of the culture medium (Parhar *et al.*, 1988).

In apparent contradiction to the above, PGs are known to be involved in increasing vascular permeability during inflammatory reactions and so allowing leukocyte infiltration. However, it is likely that for the majority of pregnancy the high concentrations of systemic progesterone maintain PGDH activity in the vasculature and so avoid a large influx of leukocytes. At term, as the PG metabolic

capacity of the intrauterine tissues decreases, decidual PGs may participate in leukocyte infiltration to initiate what has been termed "decidual activation". The resultant neutrophils allow the cervix to soften, initiate membrane rupture, and contribute to uterotonin production necessary for birth. Ultimately, in the presence of contractile receptors and correct post-receptor mechanisms, PGs and other uterotonins produced by the decidua and invading cells are available to stimulate the myometrium and effect expulsion of the fetus.

So, what is the significance of the increased PG biosynthetic potential of the amnion and chorion at term and with labor? For a number of reasons it is doubtful these tissues produce the PGs that are necessary to initiate uterine contractility, although they may contribute to the process. Our data indicate the specific activity of PGHS in the amnion and chorion is so much lower than in the decidua that it is difficult to imagine that PGs produced by the fetal membranes could make the difference between the presence or absence of contractile activity, unless of course the threshold of uterotonin concentration required to initiate this event is maintained within very close limits. Furthermore, many studies have addressed the dynamics of PG metabolism in the fetal membranes and decidua at term (Mitchell *et al.*, 1993; Bennett *et al.*, 1990; Nakla *et al.*, 1986). Transfer studies indicate that PGE_2 passes from the amnion to the decidua at a rate of 1-4%/hr, that only a small proportion of the transferred hormone remains in the active form, and, that this changes little at the time of parturition. However, the significance of these data is unclear as they were based on *in vitro* observations and so may not represent physiological conditions. Nonetheless, it is likely that increased PG output from the amnion and chorion at term and with labor has a function other than the initiation of uterine contractility. More probable, these PGs contribute to the extensive tissue remodeling that must occur at parturition. Cervical ripening and membrane rupture are both the result of collagenase activity (matrix metalloproteinases; MMPs) secreted by infiltrating

leukocytes. It is likely the PGs produced in the fetal membranes facilitate the entry of these invading cells into the appropriate tissues. Fetal membrane rupture in the rat is accompanied by a decrease in type I collagen content of the amnion and an increase in interstitial collagenase activity (Lei *et al.*, 1996). In the human amnion and chorion, parturition is associated with elevated levels of type IV collagenase (MMP-9) (Vadillo-Oterga *et al.*, 1995). These enzymes participate in degradation of the basement membrane and connective tissue collagens transferring the tissues from resilient fetal membranes to an amorphous gel. Although the role of PGs produced by the amnion and chorion in these events is speculative, their possible involvement highlights an important point. That is, that too often parturition and labor are considered mainly at the level of uterine contractility and because PGs are potent uterotonins they are frequently not considered in any other capacity. In fact, before the function of the PGs produced by the intrauterine tissues can be fully appreciated the identity of these PGs and their receptors must be characterized. The data presented in this thesis requires that the role of PGs at parturition be revisited and expanded to include the multiple physiological events that must be occurring in addition to the muscular contraction of the myometrium. The exact nature of these events, however, remains to be elucidated.

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APPENDIX

A.1. *In Vitro* Studies

A.1.1 Prostaglandin Endoperoxide H Synthase-2 Expression in Human Amnion Cells: Involvement of Tyrosine Kinases

PGHS-2 induction in amnion cell cultures

Cell cultures derived from gestational tissues are suitable models to investigate the regulation of intrauterine PG production. In our laboratory Dr. Zakar has established a primary amnion cell culture system in order to study the control of PGHS expression in term human amnion. He has found PGHS activity is low in confluent, serum-starved amnion cells, but can be increased by agonists such as dexamethasone (DEX), epidermal growth factor (EGF), the protein kinase C (PKC) activating phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA) (for a review see Olson and Zakar, 1993), and okadaic acid (OA), which is a marine toxin that modulates cellular regulation by inhibiting the protein phosphatases PP1 and PP2A (Cohen *et al.*, 1990). Furthermore, down-regulation of PKC by prolonged phorbol ester treatment, or co-incubation with the specific PKC inhibitor chelerythine, did not block the stimulation of PGHS activity by OA. This suggests OA acts by a mechanism that is independent of PKC.

Using specific ribonuclease protection assays we found that DEX increases the abundance of PGHS-2 mRNA in the cell cultures within 4 hours; EGF, TPA, and OA induce PGHS-2 expression within 2 hours; while PGHS-1 mRNA is undetectable (Fig. A-1). Inhibition of protein synthesis by cycloheximide potentiates the effect of the agonists suggesting the stimulation is not mediated by an intervening protein or peptide. In addition, since the non-passaged primary cultures essentially reflect the cellular composition and heterogeneity of amnion tissue, we employed *in situ* hybridization to examine the distribution of DEX,- EGF-, TPA-, and OA-responsive

cells in the cultures. Nearly all cells exhibited higher levels of PGHS-2 mRNA after treatment with each of the three agonists, indicating that DEX,- EGF-, TPA-, and OA-responsive pathways co-exist in the amnion cells. The results for EGF, TPA, and OA are shown in Figure A-2.

Involvement of tyrosine kinases

The involvement of tyrosine kinases in the induction of PGHS-2 expression was examined using herbimycin A and the tyrphostins A1, AG126, and A1288. Herbimycin A is a cell permeable, potent inhibitor of a variety of receptor and non-receptor tyrosine kinases, but has no effect on the major serine- and threonine specific protein kinase types (Uehara and Fukazawa, 1991). Table A-1 shows herbimycin A pretreatment completely prevented (>90%) the induction of PGHS activity by EGF and TPA, and partially (≈60%) inhibited the induction of the enzyme by OA. The increase of PGHS-2 mRNA was also blocked indicating that the tyrosine kinase dependent step(s) was involved in the accumulation of the mRNA encoding the enzyme (Fig. A-3 and A-4). Immunoblot analysis demonstrated early signaling through tyrosine phosphorylation in response to EGF and TPA. Within 5 minutes of EGF treatment tyrosine-phosphorylation of the EGF receptor and the 120, 110, and 77 kDa protein fractions were detected. TPA treatment resulted in increased tyrosine phosphorylation of the same three cellular fractions within 5 minutes. Dexamethasone and OA affected the phosphotyrosine content of these proteins after 1 to 2 hours. In cells pretreated with herbimycin A (0.5 μM for 16 h) followed by EGF, the phosphorylation of the EGF receptor decreased. The phosphorylation of the 110 kDa protein was also reduced by herbimycin A in unstimulated cells, and following DEX- TPA- and OA-stimulation (Fig. A-5). Taken together, these data indicate functionally upstream targets of herbimycin A include the EGF receptor and a tyrosine kinase with preference for a 110 kDa cellular substrate. Increased tyrosine phosphorylation was not detected early after OA treatment although herbimycin A

reduced the induction of PGHS-2 suggesting a downstream step(s) in the signaling pathway of OA was affected.

Tyrphostins inhibit protein tyrosine kinases by competing with ATP and/or the protein substrate (Levitzki, 1992). The various tyrphostins exhibit distinct specificity toward the members of the tyrosine kinase family. In an *in vitro* protein tyrosine kinase assay, tyrphostin A1 was shown to be a less potent inhibitor than either AG126 or AG1288, whereas, AG126 was demonstrated to be as efficient as AG1288 in suppressing the tyrosine kinase dependent actions of endotoxin in peritoneal macrophages (Novogrodsky *et al.*, 1994). Figure A-6 shows the effect of the tyrphostins A1, AG126, and AG1288 on PGHS enzyme activity in DEX treated amnion cells. The tyrosine kinase inhibitors suppressed the induction of PGHS activity in a concentration dependent manner. The relative efficacies of the compounds were AG126>AG1288>A1 as shown by the IC_{50} values in Table A-2 and was in general agreement with the findings of Novogrodsky *et al.* (1994) suggesting that their actions in amnion cells were mediated by tyrosine kinase inhibition. The drugs did not affect PGHS activity in control experiments where they were added to the cultures together with arachidonic acid after DEX treatment. This indicates that the induction and not the activity of the enzyme was blocked. In all experiments cell viability was unimpaired as no LDH release in the culture medium was detected.

Conclusion

The results of these studies indicate that glucocorticoid and growth factor stimulation, PKC activation and protein phosphatase inhibition all result in elevated PGHS activity and PGHS-2 mRNA accumulation in amnion cells. These effects are similar to some of the changes occurring in the amnion membrane at term and preterm birth. PGHS-2 expression is regulated by multiple signaling mechanisms in these cells, however, the significance of the individual pathways in the in vivo regulation of enzyme activity is unclear. Our data indicate that tyrosine kinases are

involved at different levels in all the regulatory pathways investigated in these studies. This suggests the possibility that tyrosine kinase inhibitors administered *in vivo* may block the induction of PGHS-2 in the gestational tissues thereby delaying the onset of labor. Such treatment may result in isoform and tissue-specific inhibition of PGHS-2, and, depending on the timing of drug administration may lead to reduced side-effects and more efficacious therapy. Therefore, the purpose of the next study was to test whether administration of tyrosine kinase inhibitors *in vivo* would delay term and/or preterm birth in mice.

A.2. *In Vivo* Studies

A.2.1 The Influence of Tyrosine Kinase Inhibitors on Term and Preterm Parturition in Mice

Maternal infection is a cause of spontaneous abortion and preterm labor in humans (Romero and Mazor, 1988), but the pathophysiology is unclear. The aim of this investigation was to evaluate the physiological parameters responsible for preterm parturition by analyzing the mechanism of action of agents effective against it.

We administered bacterial lipopolysaccharide (LPS; endotoxin) to pregnant Balb/c mice in an attempt to establish an animal model that reproducibly gave preterm delivery in all individuals treated. We expect that PGs play an important role in infection-driven pregnancy loss and that one method of prolonging gestation and preventing preterm birth may be by the inhibition of the action and/or expression of one or both isoforms of PGHS. Evidence suggests the expression of PGHS-2 is blocked by tyrosine kinase inhibitor drugs in many cells and tissues, including the amnion (see above), possibly by interfering with intracellular signaling pathways. Therefore, we tested the possibility that tyrosine kinase inhibitors delay preterm

parturition in mice. We selected AG126 and AG1288 as our therapeutic agents because previous studies have shown tyrphostins of the AG126 family provide the most protection against lethal LPS-induced toxicity *in vivo* (Novogrodsky *et al.*, 1994).

Materials and methods

Animals

This protocol was approved by the Ethics Review Committee at the University of Alberta. Male and female Balb/c mice were used throughout these studies for the production of timed-pregnant females. Mice were housed individually in a viral antigen-free area of the animal facility; ambient temperature was controlled at approximately 72°F and a light dark cycle of 12 h each was maintained. Animals had free access to food and water. Females in estrus, as determined by the appearance of the vaginal epithelium were placed with Balb/c males for a period of 24 h. Visualization of a vaginal plug was used as evidence of a positive pregnancy and was termed day 0 of gestation. Pregnancies were confirmed by weight gain through the first 14 days of gestation; animals were treated on day 17.

Experimental design

Treatment of mice with lipopolysaccharide: Escherichia coli lipopolysaccharide (LPS), serotype 026, isolated by phenol extraction (Sigma, St. Louis) was used for these studies. Lipopolysaccharide was dissolved in phosphate-buffered saline (PBS) and injected into the mice intraperitoneally in a volume of 0.5 ml. The first series of studies were conducted to determine a dose of bacterial LPS that would consistently induce preterm parturition without maternal death. For these studies, LPS was administered on day 17 of gestation according to the following protocols:

1) One dose of 0, 5, 10, 20, 50 100, and 150 μg/mouse;

2) Two doses of 0, 5, 10, and 20 μg/mouse at a 3 h interval;

Animals receiving 0 μg were given PBS. The mice were observed on a continual basis and sacrificed after delivery. Signs of fever and vaginal bleeding were recorded for each animal. The presence or absence of preterm and term deliveries (>20, and 20 or 21 days of pregnancy, respectively) was observed in the early mornings and late afternoons during the experimental period. If the mice were not delivered by day 22 of pregnancy, the animals were killed while they were under ether anesthesia to check for intrauterine fetal death.

Treatment of mice with tyrphostins: Stock solutions of tyrphostin AG126 and AG1288 (Alexis Corp, San Diego, CA) were prepared in dimethylsulfoxide (DMSO). To evaluate the therapeutic efficacy of the drugs, the lowest dose of LPS (two doses at a 3 h interval of 10 μg/mouse) that gave consistent preterm delivery of live pups without maternal death was administered intraperitoneally. The mice were treated with tyrphostins AG126 or AG1288 administered intraperitoneally in a volume of 0.5 ml according to the following protocols

1) 2 h before LPS treatment;

2) at the same time as LPS treatment, and

3) 2 h after LPS treatment at doses of 0, 25, 50, 100, 200, 300, 400, 500, 700 and 1000 μg/mouse.

Animals receiving 0 μg were given vehicle of PBS/DMSO; the concentration of DMSO in the treatment solutions was less than 0.1% (v/v) and had no *in vivo* toxicity. The presence or absence of preterm deliveries was noted as mentioned above. When the offspring were born alive the litter size and their body weights at birth and at weaning were measured. Their postnatal viability was examined according to the methods of Irwin (1968).

Statistical analysis: The results of each experiment were expressed as the incidence of preterm and term deliveries. One-way ANOVA was used to analyze differences between each drug-dosed group and the corresponding control group.

Results

Effects of lipopolysaccharide on preterm parturition in pregnant mice

A dose-response of LPS-mediated preterm parturition was performed to establish a concentration that would consistently induce preterm birth of live pups without maternal death. Preterm parturition was defined as delivery at 2 SDs from the mean gestational age (from fertilization to delivery) of PBS-treated mice (mean ± SD: 20.43 ± 0.1 days, n=5). Mice treated with 10 μg LPS twice at a 3 h interval on day 17 of pregnancy revealed the highest incidence (100%) of preterm delivery of live pups (19.03 ± 0.19 days: n=4: ANOVA: $P<0.05$) (Fig. A-7).

Neither maternal death nor severe depression of spontaneous movement was observed under the experimental conditions used. Offspring delivered preterm before day 18 of pregnancy were all dead. Phosphate-buffered saline-treated mice did not have vaginal bleeding during periods defined as preterm. In LPS-treated mice bleeding was usually observed within 6 h of delivery.

Effect of tyrphostins on lipopolysaccharide-induced preterm parturition in pregnant mice

The effects of tyrphostin AG126 and AG1288 on LPS-induced preterm birth in pregnant mice were evaluated. At two doses of 10 μg/mouse within a 3 h interval on day 17 of gestation LPS induced 100% preterm delivery in pregnant mice compared to PBS-treated controls. The administration of ≥300 μg AG126 or ≥400 μg AG1288 2 h before the first LPS treatment prevented preterm birth (delivery times: 20.3 ± 0.19 days, n=5, vehicle treated controls; 19.03 ± 0.19 days; n=4 LPS treated; 21.13 ± 0.53 days, n=3, AG126/LPS; 20.7 ± 0.33 days, n=3, AG1288/LPS; ANOVA; not different from the control group) (Fig. A-8). Simultaneous administration of AG126 or AG1288 with LPS, or, administration of the tyrphostins 2 h after LPS treatment had essentially no preventative effect against LPS induced preterm delivery. The ability of AG126 and AG1288 to prevent preterm birth in the LPS treated mice

was dose-dependent. A dose of 300 μg AG126 and 400 μg AG1288 was the minimal dose that provided essentially 100% protection against LPS-induced preterm parturition. These tyrphostin treatments had no adverse effects on newborn viability, weight, sucking ability, and postnatal weight gain (Fig. A-9).

Effect of tyrphostins on term parturition in pregnant mice

In addition, the effect of tyrphostin AG126 and AG1288 on term parturition in Balb/c x Balb/c pregnant mice was investigated. Mice were injected with either vehicle (n=5), 300 μg AG126 (n=3), or 400 μg AG1288 (n=3) intraperitoneally on day 17 of gestation. AG126 significantly prolonged gestation compared to controls (delivery times: 20.38 ± 0.09 days, n=5, vehicle treated controls; 21.26 ± 0.12 days, AG126 treated; ANOVA: $P<0.05$), while the effect of AG1288 was not significant (20.77 ± 0.18 days) (Fig. A-10).

Conclusion

By use of this animal model for preterm delivery, we tested the preventative effect of several drugs. The results suggest that tyrosine kinase dependent mechanisms are involved in normal and LPS induced preterm labor in mice. Tyrphostins are a newly recognized group of compounds capable of increasing the length of gestation with little or no maternal and fetal side-effects. While we recognize that many genes are sensitive to tyrosine kinases, we postulate that one mechanism by which tyrphostins prolong pregnancy is by the inhibition of PGHS-2 induction before the onset of labor.

Table A-1 Inhibition by Herbimycin A of PGHS activity stimulated by EGF, TPA and OA (after Zakar et al., 1998a)

Treatment	PGE_2 output (pg/μg protein/h; mean ± SEM; n=6	
Control	2.00 ± 0.14	
TPA (10nM, 4h)	14.82 ± 0.62	
Herbimycin A plus TPA	0.62 ± 0.093	$P<0.05$ vs. TPA
Control	0.39 ± 0.06	
EGF (10ng/ml, 2h)	17.11 ± 0.81	$P<0.05$ vs. EGF
Herbimycin A plus EGF	1.22± 0.13	
OA (100nM, 16h)	76.17 ± 1.4	
Herbimycin A plus OA	32.47 ± 2.04	$P<0.05$ vs. OA

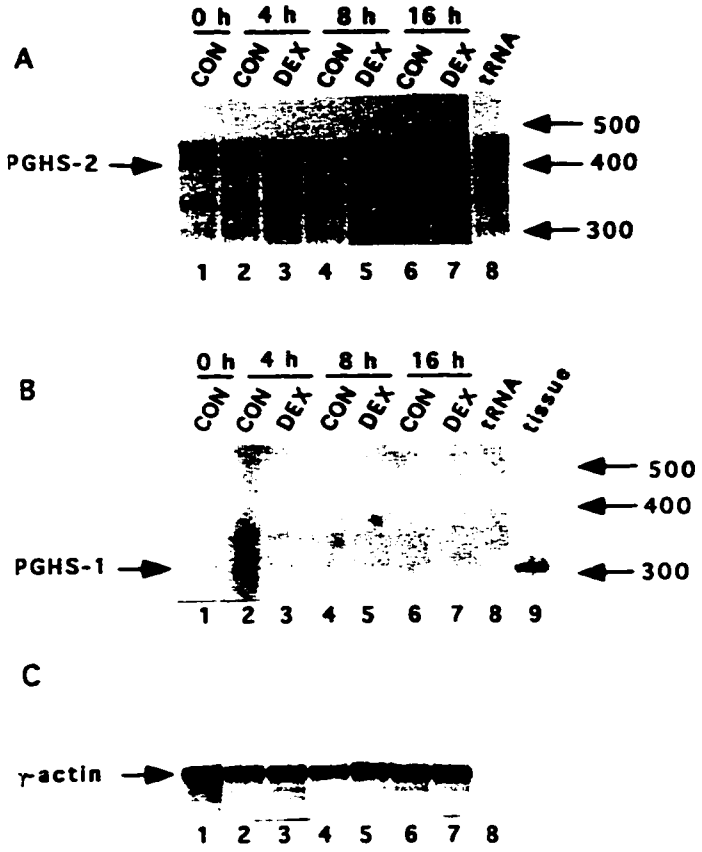
Cells were pretreated with Herbimycin A (1 μM) for 16 h, followed by agonists as indicated. PGHS activity was determined by incubating the cells with 10 μM arachidonic acid for 1 h, and measuring PGE_2 output with radioimmunoassay. Separate representative experiments are presented for TPA and the other two agonists, respectively. ANOVA and the Newman-Keuls test were performed to statistically analyze the data.

Table A-2 The efficacy of tyrosine kinase inhibitors as blockers of PGHS activity induction by dexamethasone in primary amnion cell cultures (after Zakar et al., 1998b)

Inhibitor	IC_{50}[a]
Herbimycin A	54 nM
AG126	21 µM
AG1288	42 µM
AI	77 µM

[a] Values were determined for each compound using pooled data from 3 or 4 independent experiments

Figure A-1 The effect of dexamethasone (DEX), epidermal growth factor (EGF), protein-kinase C activating phorbol ester (TPA), and okadaic acid (OA) on PGHS-2 and PGHS-1 mRNA abundance. Confluent primary amnion cell cultures were treated with 100 nM DEX, 10 ng/ml EGF, 10 nM TPA, or 100 nM OA for 0-16 h as indicated. PGHS-2 mRNA (upper panels), PGHS-1 mRNA (middle panels) and for reference, γ-actin or GAPDH mRNA (lower panels) levels were determined in total cytoplasmic RNA fractions by ribonuclease protection assays. The positions of the undigested probes (P), included as controls in the assays, and probe fragments protected by mRNA are shown by arrows. Assay backgrounds determined by hybridizing the probes with carrier yeast tRNA only, are also presented (t). CON vehicle in DEX assays; C, tissue is the positive control for PGHS-1 mRNA (amnion tissue).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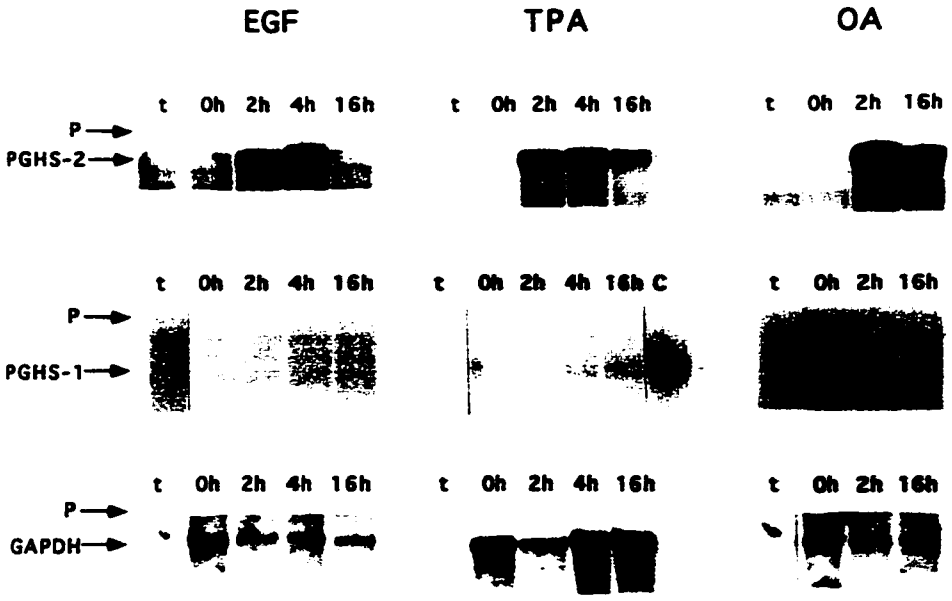
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P
GAPDH

Figure A-2 The localization of PGHS-2 mRNA in cultured amnion cells. Amnion cell cultures were incubated with EGF (10 ng/ml for 2h, panels b,c), TPA (10 nM for 4h, panels d,e) or OA (100 nM for 2 h, panels f,g), and processed for *in situ* hybridization using digoxigenin-labeled antisense (panels a,b,d,f), or sense (panels c,e,g) riboprobes for PGHS-2 mRNA. Control cells were not treated with agonist (panel a). Probes were visualized by immunoreaction with an alkaline phosphatase-conjugated anti-digoxigenin antibody followed by a chromogenic enzyme reaction resulting in purple color. Magnification X10.

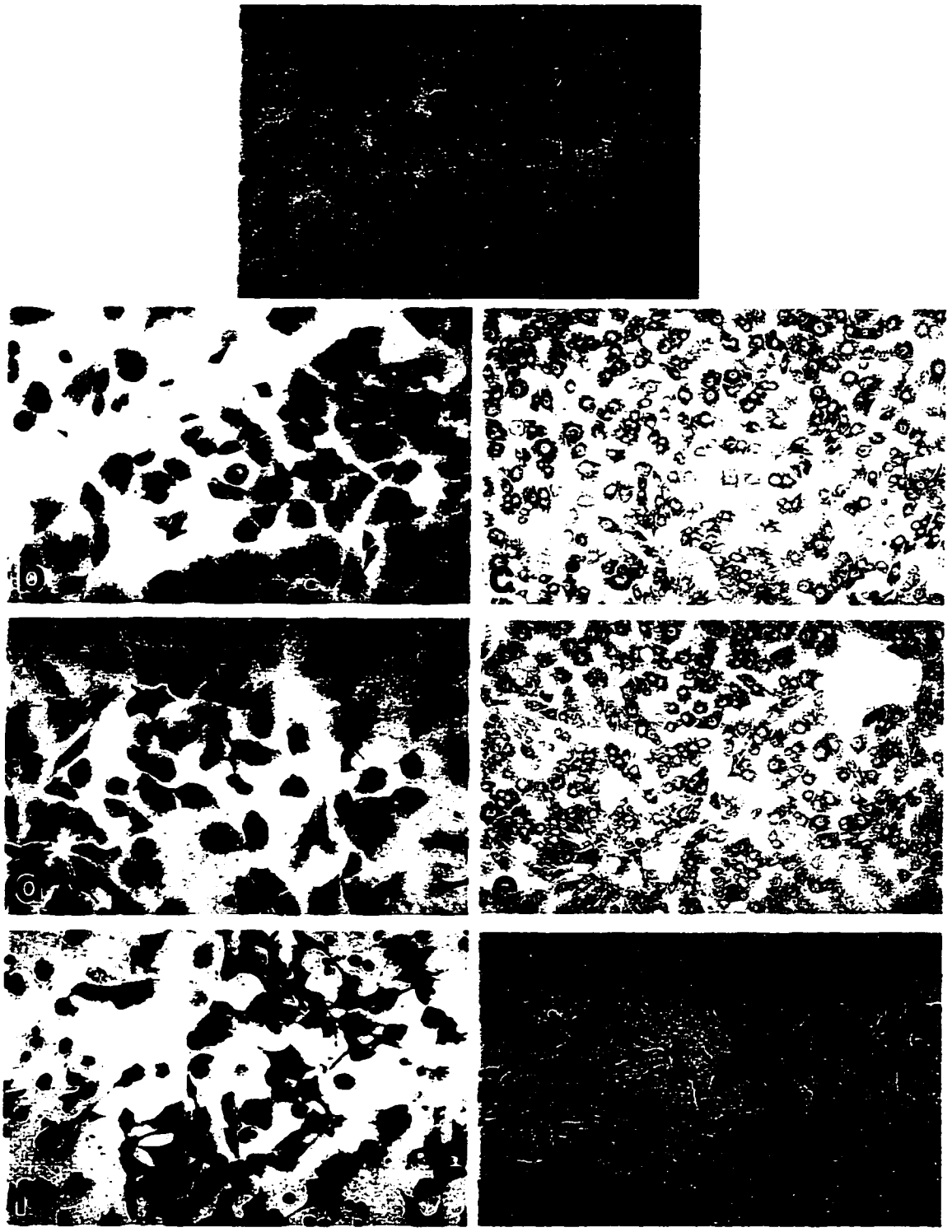
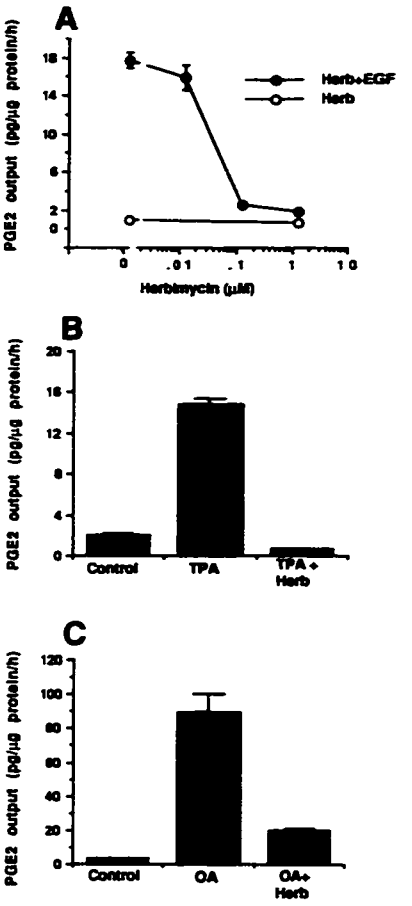


Figure A-3 The effect of Herbimycin A on PGHS activity and PGHS-2 mRNA levels stimulated by EGF, TPA, and OA. A, B, and C: Amnion cells were pretreated with 0-1 μM (panel A), or 0.5 μM (panels B and C) Herbimycin A (Herb) for 16 h. Following pretreatment, the cultures were stimulated with EGF (10 ng/ml for 2 h), TPA (10 nM for 4 h), or OA (100 nM for 16 h) in the continuing presence of Herbimycin A. PGHS activity was measured as arachidonic acid-promoted PGE_2 output. Control cultures were incubated with vehicle only. Means ± S.E.M. of results with 6 parallel treated cultures are presented. Herbimycin A, (at concentrations of 0.1 μM and 1 μM in Panel A) significantly ($P<0.05$) inhibited the enhancement of PGHS activity by the three stimulants. D: Untreated and Herbimycin A-pretreated (0.5 μM for 16 h) amnion cells were stimulated with 10 ng/ml EGF for 2 h, 10 nM TPA for 4 h, or 100 nM OA for 2 h (E and H + E, T and H + T, OA and H + OA, respectively). Control cultures were either untreated (C) or pretreated with Herbimycin A only (H). PGHS-2 (upper panels) and GAPDH (lower panel) mRNA levels were determined by ribonuclease protection assay. The positions of the undigested (P) and the protected probes are indicated by the arrows. Assay backgrounds are also shown (t). Data represent results from 2-3 independent experiments.


A
PGE2 output (pg/µg protein/h)
Herb+EGF
Herb
Herbimycin (µM)
B
PGE2 output (pg/µg protein/h)
Control
TPA
TPA + Herb
C
PGE2 output (pg/µg protein/h)
Control
OA
OA+ Herb

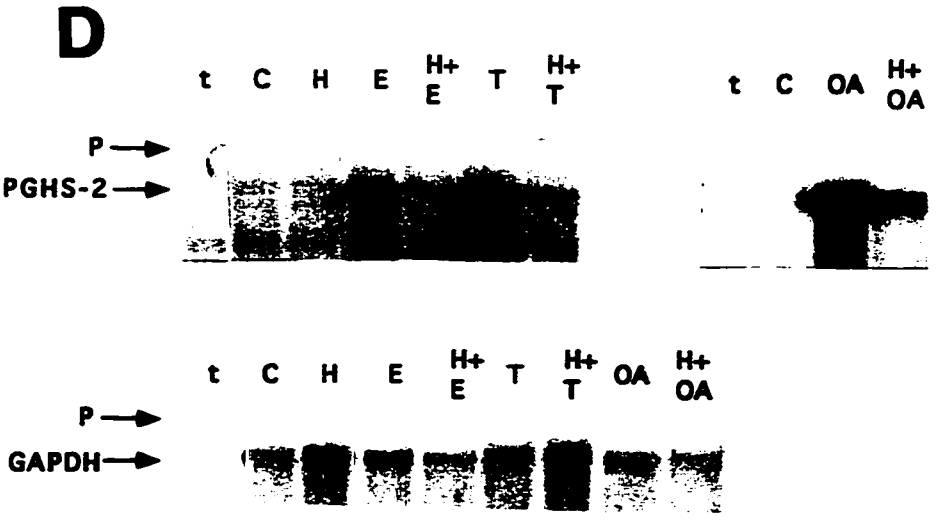
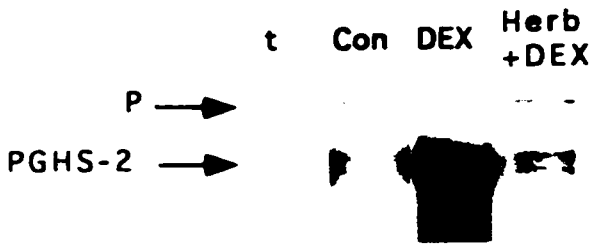
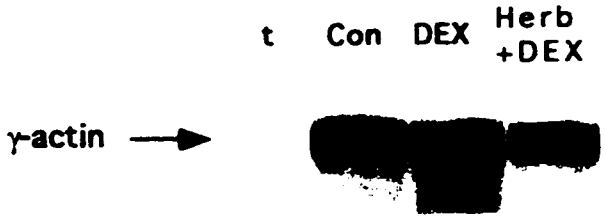

D
t C H E H+ E T H+ T
t C OA H+ OA
P
PGHS-2
t C H E H+ E T H+ T OA H+ OA
P
GAPDH

Figure A-4 The effect of Herbimycin A on PGHS-2 mRNA levels stimulated by dexamethasone. Amnion cell cultures were treated with dexamethasone alone (DEX: 50 nM for 16 h) or with dexamethasone and Herbimycin A (Herb + DEX). Control cultures were incubated without the drugs. PGHS-2 (Panel A) and γ-actin mRNA (for reference; Panel B) abundance in the total cytoplasmic RNA fractions were determined by ribonuclease protection assays. The positions of the protected probes and the unprotected PGHS-2 cRNA probe (P) are indicated by the arrows. Lane t represents the assay background determined in the presence of yeast tRNA carrier only. The ratios of the PGHS-2 and γ-actin mRNA band intensities, measured by densitometry, are shown in Panel C. Representative results of three independent experiments are presented.

A


t
Con
DEX
Herb
+DEX
P
PGHS-2

B


t
Con
DEX
Herb
+DEX
γ-actin

C

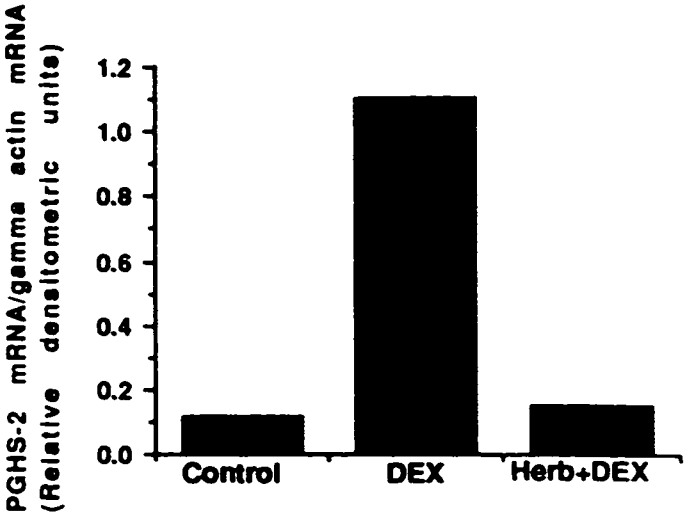

PGHS-2 mRNA/gamma actin mRNA
(Relative densitometric units)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
DEX
Herb+DEX

Figure A-5 The influence of dexamethasone and Herbimycin A on tyrosine-phosphorylated proteins in amnion cell extracts. Amnion cell cultures were preincubated with Herbimycin A (H) for 16 h followed by dexamethasone (D) for 1 or 2 h. Control cultures were not treated with drugs (C) or treated with Herbimycin A alone (H). Proteins extracted with 1% Triton X-100 containing buffer were separated with SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane, and treated with a peroxidase-conjugated monoclonal anti-phosphotyrosine antibody. Immunoreactive bands were detected by enhanced chemiluminescence. The positions of molecular weight standards, separated on a parallel line, are indicated by arrows on the right. Major tyrosine-phosphorylated protein bands, denoted by Roman numerals (I,II,III) are indicated on the left. The experiment was repeated twice with consistent results. (After Zakar *et al*., 1998b).

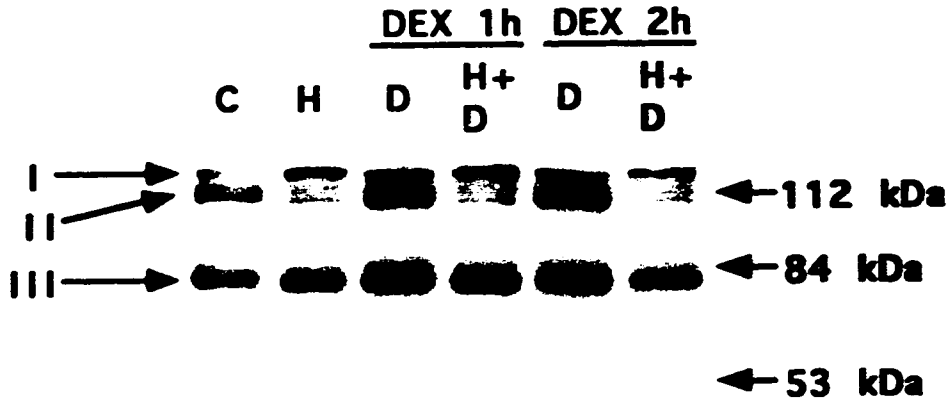

DEX 1h
DEX 2h
C
H
D
H+ D
D
H+ D
I
II
III
112 kDa
84 kDa
53 kDa

Figure A-6 The effect of Herbimycin A (Panel A), tyrphostin AG126 (Panel B), tyrphostin AG1288 (Panel C), and tyrphostin A1 (Panel D) on PGHS activity induced by dexamethasone in primary amnion cell cultures. Cell cultures were preincubated with increasing concentrations of Herbimycin A or tyrphostins for 16 h or 30 min, respectively, and then stimulated by 50 nM dexamethasone for 16 h in the continuous presence of the drugs. PGHS activity was determined after stimulation by adding 10 μM arachidonic acid to the cells in fresh medium for 1 h, and measuring PGE_2 output by radioimmunoassay. Points represent the mean ± S.E.M. of four (A) or three (B,C,D) independent experiments with 6-parallel-treated cultures each. Asterisks denote significant ($P<0.05$) inhibition relative to steroid-only controls. (After Zakar *et al.*, 1998b).

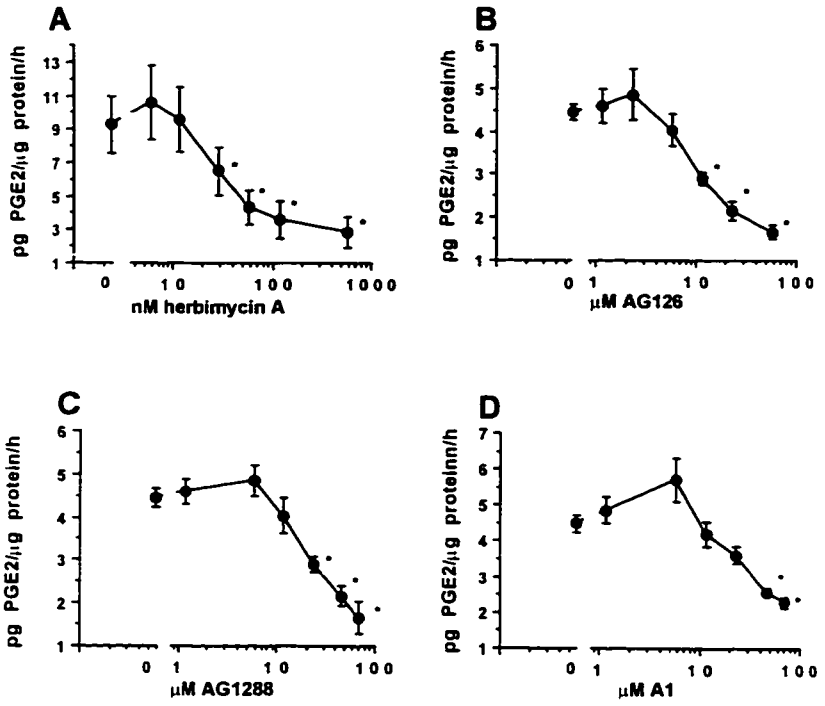

A
pg PGE2/µg protein/h
nM herbimycin A
B
pg PGE2/µg protein/h
µM AG126
C
pg PGE2/µg protein/h
µM AG1288
D
pg PGE2/µg proteinn/h
µM A1

Figure A-7 Effect of two doses at a 3 h interval of 10 μg/animal lipopolysaccharide (LPS) on preterm parturition in pregnant Balb/c mice. On day 17 of gestation mice were randomized to receive either LPS or phosphate buffered saline solution (PBS) injected intraperitoneally (i.p.). Mice were observed for preterm delivery. Asterisks denote a significant (ANOVA: $P<0.05$) decrease in the length of gestation in the LPS treated animals relative to controls.

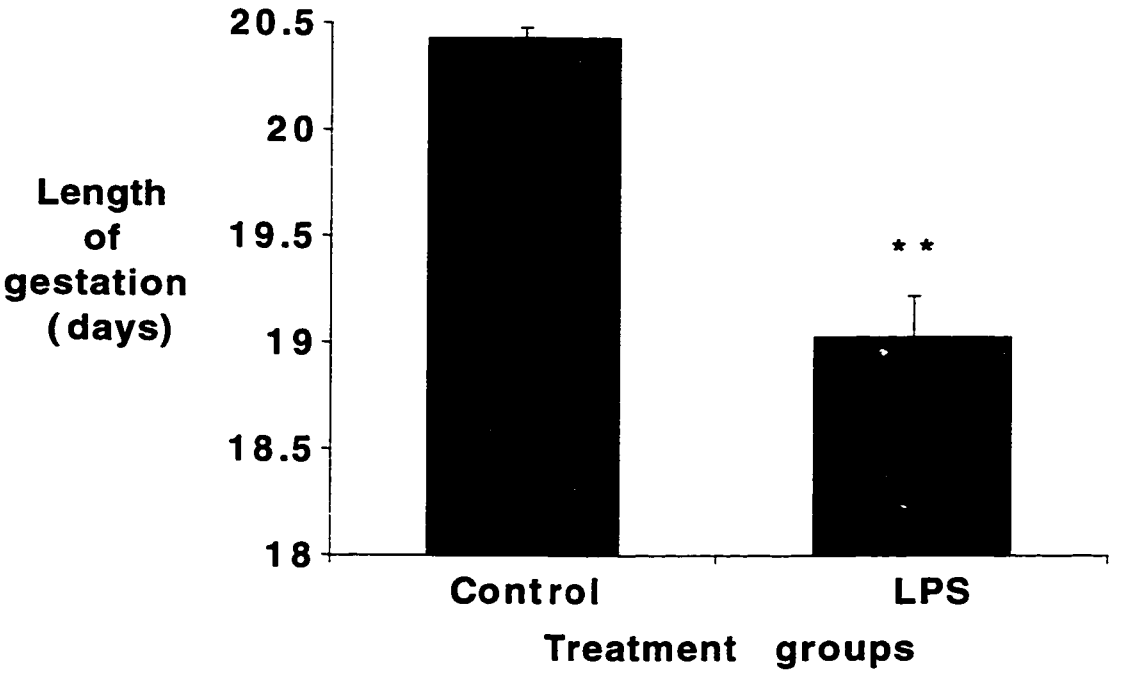

20.5
20
19.5
19
18.5
18
Length of gestation (days)
* *
Control
LPS
Treatment groups

Figure A-8 Effect of ≥300 μg/animal tyrphostin AG126 and ≥400 μg/animal tyrphostin AG1288 on LPS-induced preterm labor in pregnant Balb/c mice. Mice were injected (i.p.) with tyrphostin or vehicle control (PBS/dimethysulfoxide [DMSO]) 2 h before injection (i.p) of LPS (2 doses of 10 μg/animal administered at a 3 h interval). Tyrphostin AG126 (≥300 μg/animal) and tyrphostin AG1288 (≥400 μg/animal) prevented LPS-induced preterm delivery in these mice.

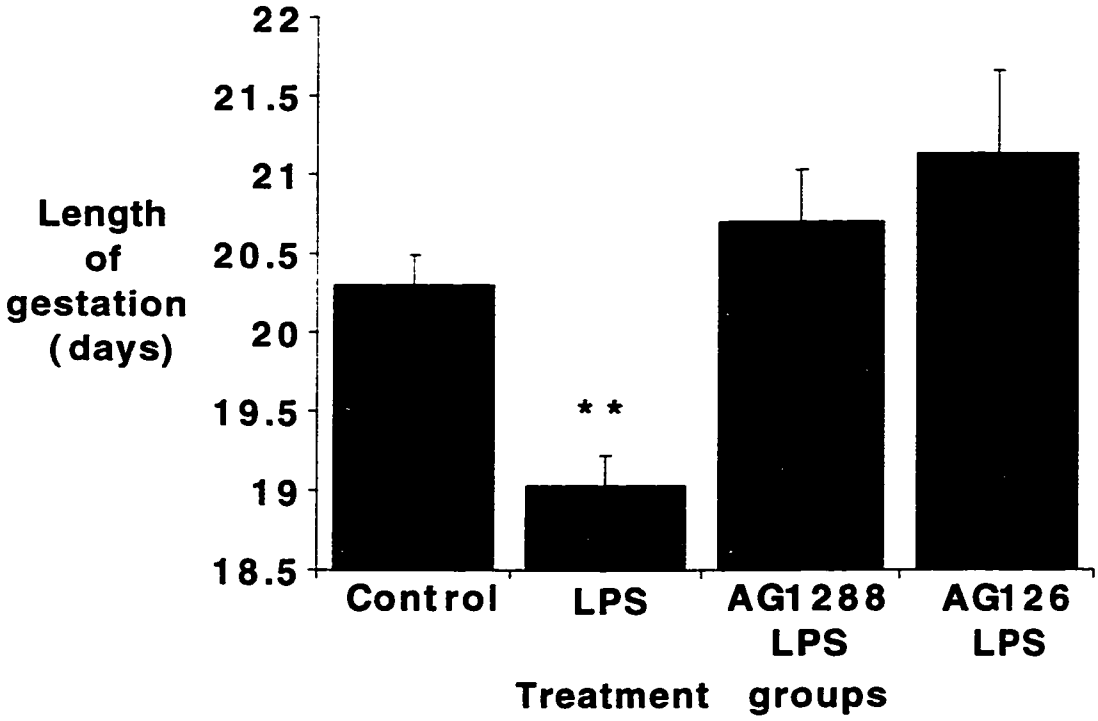
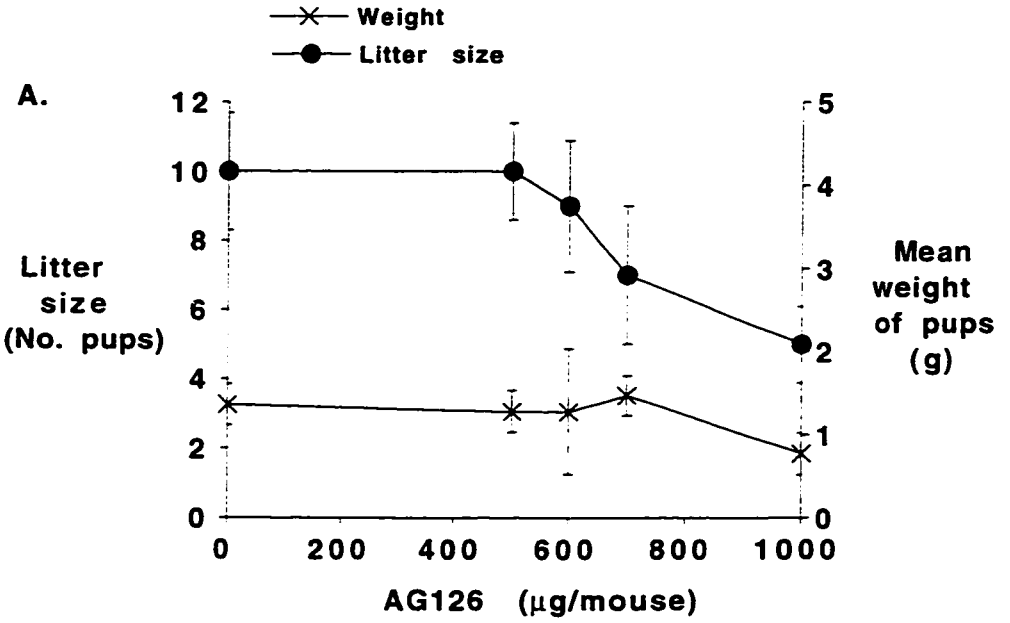

22
21.5
21
20.5
20
19.5
19
18.5
Length of gestation (days)
* *
Control
LPS
AG1288 LPS
AG126 LPS
Treatment groups

Figure A-9 Effect of different concentrations of tyrphostin AG126 (A) and tyrphostin AG1288 (B) on pup viability in pregnant Balb/c mice. Animals receiving 0 μg tyrphostin were given vehicle (PBS/dimethysulfoxide [DMSO]). Pup viability was assessed at delivery by litter size and weight.


Weight
Litter size
A.
Litter size (No. pups)
Mean weight of pups (g)
0
2
4
6
8
10
12
0
1
2
3
4
5
0
200
400
600
800
1000
AG126 (µg/mouse)

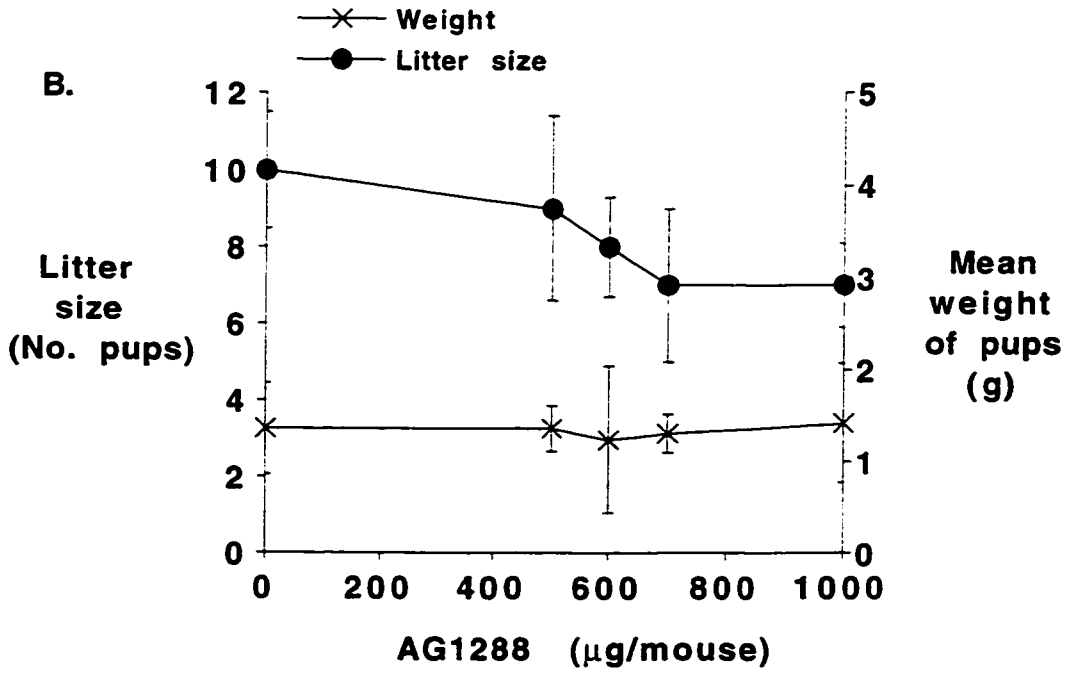
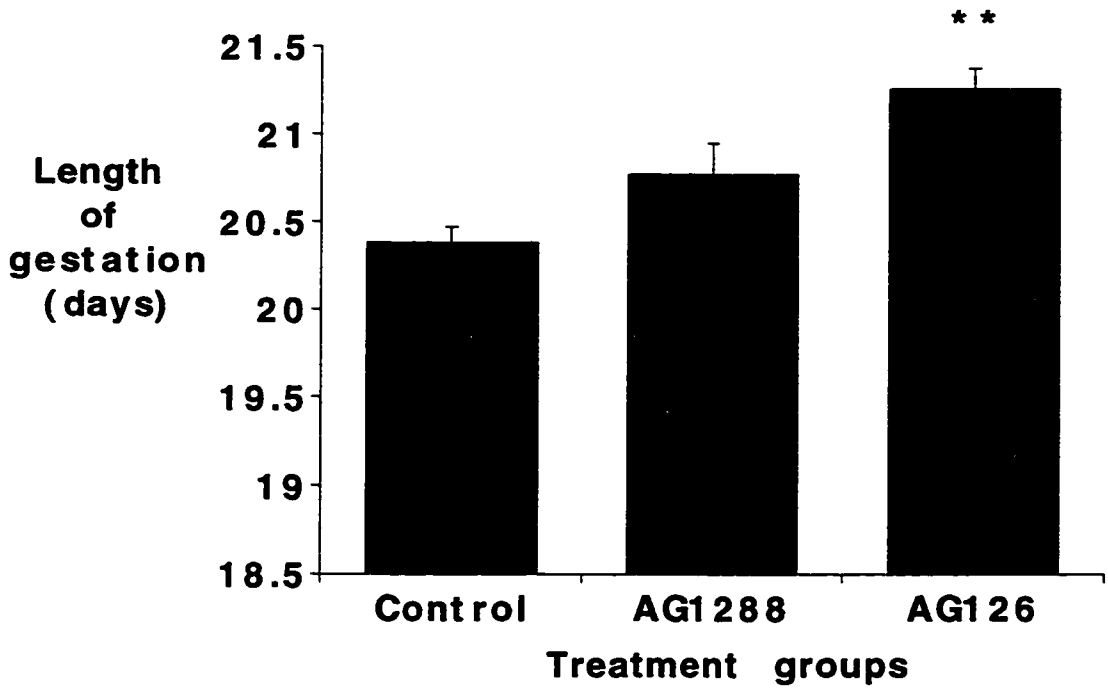

Weight
Litter size
B.
Litter size (No. pups)
Mean weight of pups (g)
0
2
4
6
8
10
12
0
1
2
3
4
5
0
200
400
600
800
1000
AG1288 (µg/mouse)

Figure A-10 Effect of ≥300 μg/animal tyrphostin AG126 and ≥400 μg/animal tyrphostin AG1288 on term parturition in pregnant Balb/c mice. On day 17 of gestation mice were randomized to receive either tyrphostin or vehicle controls (PBS/DMSO) injected intraperitoneally. The time of delivery was observed. Asterisks denote a significant (ANOVA; $P<0.05$) increase in the length of gestation in the AG126 treated animals relative to controls.


* *
21.5
21
20.5
20
19.5
19
18.5
Length of gestation (days)
Control
AG1288
AG126
Treatment groups

A.3. References

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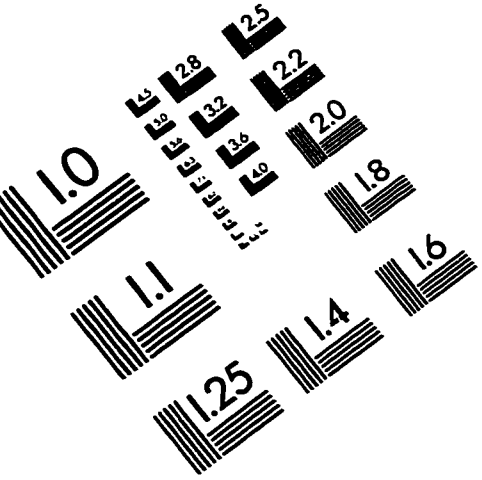
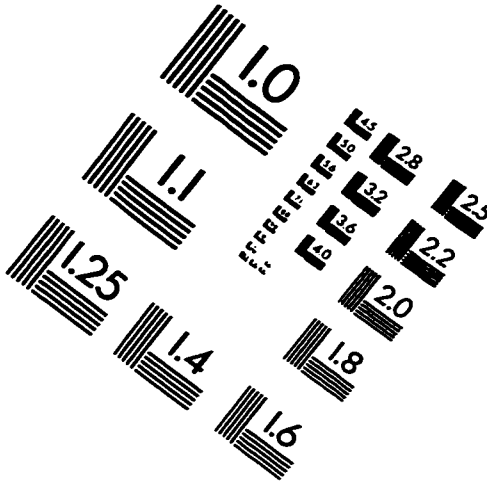
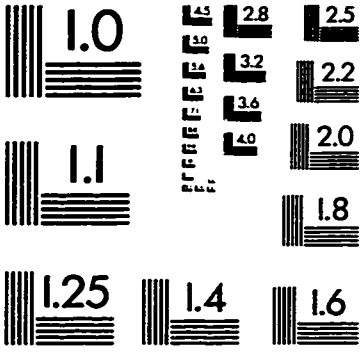
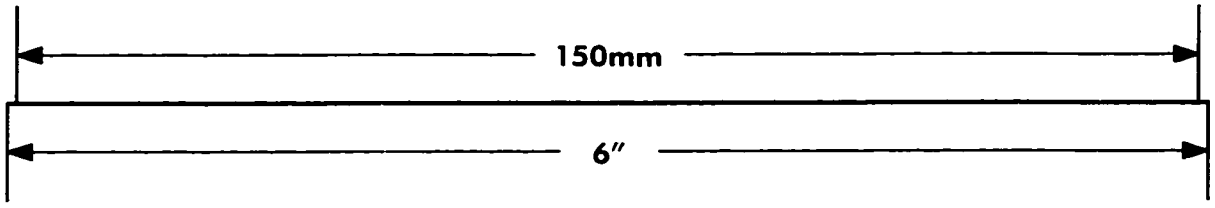
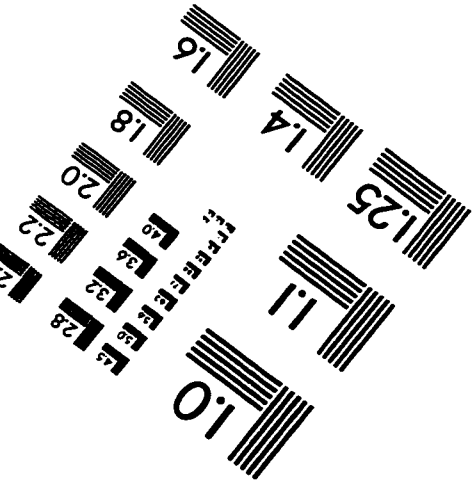
IMAGE EVALUATION
TEST TARGET (QA-3)

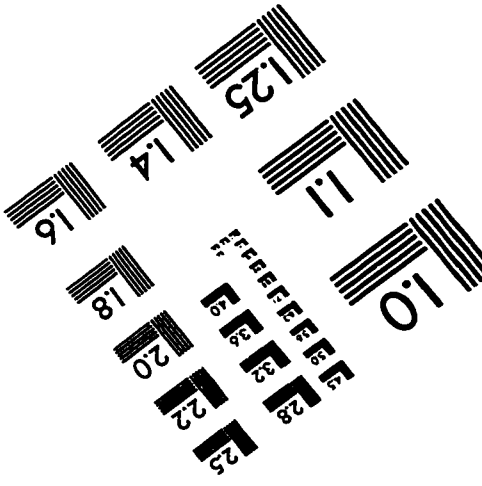












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