Expression and Regulation of Prostaglandin Receptors in Human Fetal Membranes and Placenta

By

Elif Unlugedik

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Abstract of Thesis

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Elif Unlugedik
Ph.D., 2006, Department of Physiology, University of Toronto

Preterm birth is a leading cause of neonatal mortality and morbidity. Despite the advances in medical care, we still cannot prevent the long or short term consequences of preterm delivery such as cerebral palsy, respiratory problems, deafness, blindness and complications of neonatal intensive care. PGs are considered as the key mediators of parturition in most mammalian species including human. The actions of PGs are mediated through distinct G protein coupled receptors (GPCRs). Prostaglandin receptors are divided according to functional data as DP, EP, FP, IP and TP for the natural occurring prostanoids, PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 and TXA2, respectively.

The short-term objectives of this thesis were **i)** to describe the distribution of PG receptor subtypes (EP1-4 and FP) in intrauterine tissues at term and preterm birth, and to understand the mechanisms that control the expression of PG receptors in vivo; **ii)** to define the effect of cytokines on PG receptor expression; **iii)** to identify the effect of different oxygen tensions on PG receptor expression in placenta *in vivo* and *in vitro.*

I have shown the presence of PG receptors (EP1-4 and FP) in placenta and fetal membranes using immunohistochemistry and Western Blot analysis. Labor associated changes were observed in EP1, EP3 and FP receptors both in fetal membranes and placenta at term. Betamethasone treatment increased EP1, EP3 and FP receptor protein

expressions at early gestational ages. Chorioamnionitis was associated with a decrease in all the receptor subtypes that were expressed in the fetal membranes. Western Blot analysis revealed that proinflammatory cytokines can upregulate EP3 and FP expression in the absence or presence of prostaglandins in both JEG-3 cells and primary chorion trophoblast cells, respectively. Finally, EP4 was down-regulated and EP3 was upregulated in preeclamptic placentas. Exposure to decreased oxygen tensions mimicked the same results.

We suggest that the differential expression and regulation of PG receptors in the fetal membranes and placenta during term and preterm parturition may contribute to breakdown of the membranes, and alter the generation of bioactive glucocorticoids, with later effects on PG synthesis and metabolism.

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List of Abbreviations in Alphabetical Order

ACTH	adrenocorticotropin hormone
ANOVA	analysis of variance
BSA	bovine serum albumin
cAMP	cyclic 3', 5'-adenosine monophosphate
CAP	contraction associated protein
cDNA	complementary deoxyribonucleic acid
cGMP	cyclic guanosine monophosphate
COX	cyclooxygenase
CRH	corticotrophin releasing hormone
DAB	diaminobenzidine
DNA	deoxyribonucleic acid
DP	prostaglandin D receptors
EP	prostaglandin E receptors
FBS	fetal bovine serum
FP	prostaglandin F receptors
G-protein	guanine nucleotide binding protein
h	hour
11β-HSD	11beta-hydroxysteroid dehydrogenase
HPA	hypothalamic–pituitary–adrenal
IHC	immunohistochemistry
IL	interleukin
IP	prostacyclin receptors
IP3	inositol-3-phosphate
JEG-3	human choriocarcinoma cell line
kb	kilobase
kDa	kilo Dalton
LP	lipoxin
LT	leukotriene
MEL	meloxicam
MMP	matrix metalloproteinase

mRNA	messenger ribonucleic acid
NAD+	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NF-IL6	nuclear factor-interleukin 6
NFκB	nuclear factor kappa B
NSAIDs	non-steroid anti-inflammatory drugs
OT	oxytocin
P450c17	cytochrome P450 17α hydroxylase/17, 20 lyase
PDTC	pyrrolidine dithiocarbamate (PDTC
PG	prostaglandin
PGD_2	prostaglandin D2
PGDH	prostaglandin dehydrogenase
PGE_2	prostaglandin E2
$PGF_{2\alpha}$	Prostaglandin F2α
PGG_2	prostaglandin G endoperoxide
PGH_2	prostaglandin H endoperoxide
PGHS	prostaglandin synthase
PGI_2	prostacyclin
PKA	protein kinase A
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PCR	polymerase chain reaction
PPE	6-amino-4-(4-phenoxyphenylethylamino) quinazoline
PTHrP	parathyroid hormone related peptide (PTHrP)
SDS	sodium dodecyl sulfate
SEM	standard error of mean
TIMP	tissue inhibitors of matrix metalloproteinases
TP	thromboxane receptors
WISH	amnion derived cell line

CHAPTER I

General Introduction

General Introduction

I-1 Human Parturition

Parturition is an integrated series of complex biochemical and physiological processes that results in the expulsion of the fetus from the uterus to the outside world. It is one of the most vital events in animals for it ensures the continuation of the genetic heritage of the species. During human parturition uterine contractions, cervical dilatation and rupture of the fetal membranes are achieved in a synchronized manner via endocrine, paracrine and autocrine biochemical pathways (Bryant-Greenwood and Millar, 2000). Normal term labor in human occurs between 37-42 weeks of gestational ages when progressive cervical dilatation accompanies regular uterine contractility. Although the mechanisms and pathways responsible for the initiation of parturition have been thoroughly investigated over the years, the trigger for parturition still remains obscure. At the time of parturition, synchronous maturation of the fetus and stimulus to increased uterine activity are desired (Challis et al., 2000). Moreover, much evidence suggests that it is the fetus itself that controls the timing of labor.

I-1.1 Preterm Birth

Labor that occurs after 20 weeks and before 37 completed weeks of gestation is defined as preterm birth. Even though preterm birth occurs in approximately 5-10% of the pregnancies, it is the leading cause of neonatal mortality and morbidity (Challis et al., 2002). Despite its importance, the etiology of preterm birth and the keys to its prevention remain poorly understood.

Spontaneous preterm labor (PTL) affects both developed and underdeveloped countries (Creasy and Merkatz, 1990). According to Health Canada, the preterm birth rate in Canada has been increasing gradually, from 6.4% of live births in 1981 to 7.1% of live births in 1996. Among singleton births, it increased by 5 percent in the same time period while the rates resulting from multiple gestations increased dramatically by 25 percent (Joseph et al., 1998). It is associated with an increased incidence of cerebral palsy, respiratory problems, deafness, blindness and complications of neonatal intensive care (Challis et al., 2002; Copper et al., 1993; Lopez Bernal et al., 1995; Lumley, 1993;

Morrison, 1990). In North America, the estimated cost of maintaining premature neonates in the intensive care nursery for the first months of life is $5-6 billion annually (Motquin JM et al., 1996). These figures do not include the cost of chronic health care and special education needs of those infants left with major motor and/or mental disabilities, nor do they account for the extraordinary financial and emotional burdens to the family of a premature infant.

Most preterm birth prevention programs are based upon the early recognition of preterm labor followed by prompt tocolysis (Joseph et al., 1998). Thus, prevention of PTL presents three clinical challenges: (1) early, accurate diagnosis; (2) defining and understanding the risk factors; and (3) subsequent appropriate management. As Eastman pointed out almost 60 years ago, 'only when the factors causing prematurity are clearly understood can any intelligent attempt at prevention be made' (Eastman, 1947). Numerous risk factors for PTL have been established including previous low birth weight or preterm delivery, multiple second trimester abortions, multiple gestations, placental anomalies, cervical and/or uterine anomalies, work during pregnancy, gestational bleeding, *in vitro* fertilization, hydramnios, infection, associated conditions such as chronic hypertension, cigarette smoking, alcohol consumption, single marital status, low socio-economic status and being an African-American (Creasy and Herron, 1981; Honest et al., 2004; Mercer et al., 1996). Many scoring systems considering these risk factors have been developed for screening in early pregnancy in an attempt to distinguish those at high risk. However, currently none of the approaches have proven to be adequate enough for accurate assessment of risk to warrant intervention (Honest et al., 2004). Thus, each clinical situation of PTL must be critically evaluated to determine the probability of delivery. The causes of PTL and subsequent delivery have been classified in three main categories: (1) iatrogenic- where there is a certain maternal and/or fetal complication that demands delivery, 20% of cases; (2) idiopathic- where there is no identifiable cause of PTL, 40-50% of cases; and (3) intrauterine infection, occurring in 20-30% of cases.

Even though PTL can be accurately diagnosed, there is no therapeutic intervention that prolongs pregnancy that shows clear benefits in neonatal survival and reduces the disability (Klam and Leduc, 2004). At present, the management of preterm

delivery includes tocolytic agents, antibiotics and hormones. Tocolytic agents were shown to prolong the gestation long enough for administration of corticosteroids, without any improved perinatal outcomes (Berkman et al., 2003; Gyetvai et al., 1999). Antimicrobials have been used to arrest preterm labor especially with premature rupture of the membranes. However, results have been disappointing as different groups failed to demonstrate a clear overall benefit of antimicrobial treatment for PTL (Goldenberg, 2002; Kenyon et al., 2001; King and Flenady, 2002). In 1995, a National Institutes of Health Consensus Development Panel recommended corticosteroids for fetal lung maturation in threatened preterm birth. Currently, this is the only therapeutic intervention shown to improve neonatal survival and outcome by inducing the fetal lung surfactant production and preventing neonatal respiratory disease (Katz and Farmer, 1999; Liggins and Howie, 1972). Immediate and long-term adverse effects, however, have been reported with multiple courses of corticosteroid treatment such as early-onset neonatal sepsis, chorioamnionitis, and neonatal death (Lee et al., 2004; Vermillion et al., 2000).

While preterm delivery has an enormous medical and economical impact, until now no effective diagnostic indicators or effective treatment has been found. Current medical interventions tend to increase the rate of preterm birth without corresponding improvement in outcomes. An important reason for the inability to prevent preterm birth and its consequences is attributed to the lack of understanding of the basic molecular mechanisms underlying human parturition. Thus, it is of a critical importance to define the biochemical cascades involved in the spontaneous onset and progression of normal term labor.

I-1.2 Mechanisms of Human Parturition

Parturition is comprised of interdependent physiological events such as myometrial contractility, cervical dilatation, membrane rupture, placental separation and uterine involution. Throughout pregnancy the uterus is maintained in a relative state of quiescence in order to accommodate and protect the growing fetus. Separate or combined activities of inhibitors such as progesterone, prostacyclin (PGI_2), relaxin, parathyroid hormone related peptide (PTHrP), calcitonin gene-related peptide, adrenomedullin, vasoactive intestinal peptide, nitric oxide, and corticotrophin-releasing hormone (CRH),

which is considered also as a uterotonin, are involved in the maintenance of the quiescent state of uterus (Challis et al., 2002; Challis et al., 2000). There is a switch in the pattern of myometrial activity as the contractions change from irregular (long-lasting, low-frequency) to regular manner (high-intensity, high-frequency) (Nathanielsz et al., 1997). Meanwhile, effacement and dilation of the uterine cervix occurs and this is usually followed by the rupture of the fetal membranes (Duff et al., 1984).

Increased uterine contractility results from two distinct yet integrated phases : *activation* and *stimulation* of myometrium both at term and preterm (Challis et al., 2000) (Figure I-1, page 42). "Activation" is defined as the transition of myometrium from a quiescent to an active state (Challis et al., 2000) and is essential to enable the myometrial muscle to function as a single, coordinated contractile unit in response to stimulators. It is regulated by a group of proteins collectively referred to as "contraction associated proteins" (CAPs) (Challis et al., 2000; Lye et al., 1988; Lye, 1994). CAPs include ion channel components (Boyle et al., 1987), GAP junction components such as connexin 43 (Cx43) that permits cell to cell coupling, the oxytocin receptors and prostaglandin receptors (EP1-4 and FP) (Challis et al., 2002; Cunningham et al., 1993; Lye, 1994). The expression of CAPs is organized in such a way that would allow a contraction to be initiated at the fundus of the uterus and rapidly spread through the uterine body while there is a relaxation of the lower segment of uterus and dilatation of the cervix (Challis et al., 2002; Cunningham et al., 1993; Lye, 1994). After this activation occurrs the myometrium can then undergo "*stimulation*" in response to endogenous and/or exogenous agonists referred as uterotonins. Two important uterotonins produced within the intrauterine tissues are *oxytocin* (OT) and *prostaglandins* (PG) (Lye, 1994).

Activation can be provoked by: (1) mechanical stretch of uterus; and (2) by an endocrine pathway resulting from increased activity of fetal hypothalamic–pituitary–adrenal (HPA) axis. During the first trimester, the uterus undergoes a rapid growth mainly due to hyperplasia. A second phase dominated by hypertrophy follows and continues until the final stages of gestation (Lye et al., 1998). For the duration of this second phase, growth of the uterus is closely matched to increased fetal size. The growing fetus, the placenta and increased amniotic fluid volume causes stretch that in turn induces the hypertrophy. According to the 'Law of Laplace' this stretch should

generate increased tension in the uterine wall. However, focal adhesion kinase (FAK) mediates the remodeling of the myocyte attachments to the extracellular matrix (Macphee and Lye, 2000). Thus, the myocytes detach from the matrix, stretch to accommodate the increasing uterine contents and reform focal extracellular matrix adhesions. In rodents, FAK activity increases through gestation and is maintained by placental progesterone (Macphee and Lye, 2000). In the final stages of gestation there is a decline in uterine growth compared to fetal growth which increases the uterine wall stretch and tension (Challis et al., 2000; Lye et al., 1998). Near term, the fall in progesterone, as observed in most animal species, or the functional withdrawal of it, as is believed to occur in human (Dong et al., 2005), results in decreased FAK activity, thus the remodeling process becomes inhibited (Macphee and Lye, 2000).

As mentioned earlier, activation can also be provoked by an endocrine pathway relying on the activation of the fetal HPA axis. The critical role of the HPA axis was first defined by Liggins and colleagues when they observed that in pregnant ewes a markedly hypoplastic pituitary and adrenal glands prolonged the gestation length (Liggins et al., 1973). Subsequently, more *in vivo* studies demonstrated the critical role of the fetal HPA axis in the determination of gestation length and the timing of labor. Fetal hypophysectomy or adrenalectomy, disruption of the fetal hypothalamic-pituitary stalk, hypothalamic paraventricular nuclear lesions caused a prolongation of gestation length (Challis et al., 2002; Challis et al., 2000; Gluckman et al., 1991; McDonald et al., 1992; McDonald and Nathanielsz, 1991) whereas in utero infusion of adrenocorticotropin hormone (ACTH) or of glucocorticoids to the fetal lamb caused premature parturition (McLaren et al., 1996; Thorburn et al., 1991). In sheep fetuses, studies demonstrated an increased expression of CRH mRNA in parvocellular neurones of the hypothalamus and of pro-opiomelanocortin (POMC) mRNA in the pars distalis of the fetal pituitary in late pregnancy. In particular the increase in CRH correlates with increased concentrations of adrenocorticotrophic hormone (ACTH 1–39) in the fetal circulation. ACTH acts on the fetal adrenal gland to increase expression of key enzymes required for cortisol production (especially P450 C17). In both the sheep and primate fetus the fetal adrenal produces increased amounts of cortisol in late gestation (Challis et al., 2000). Levels of cortisol can also be modified within the fetal membranes through the activity of 11ß-hydroxysteroid

dehydrogenase (11ß-HSD) enzymes (Challis et al., 2000; Yang et al., 1995). Recent studies have demonstrated that the rise in glucocorticoid levels directs the increase in prostaglandin E_2 (PGE_2) followed by an increase in prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (Challis et al., 2002; Challis et al., 2000; Gyomorey et al., 2000). Consequently, the fetus provides both mechanical and endocrine signals that can trigger a cascade of events causing elevated levels of PGs and leads to parturition (Challis et al., 2002).

Over the years PGs have become the focus of attention as the key mediator in the event of parturition. In this thesis the expression and regulation of PG receptors within the fetal membranes and placenta will be discussed in depth. However, prior to focusing on PG receptors a brief overview of PGs and their participation in the local feed-forward cascades within the intrauterine tissues will be given.

I-1.2.1 Oxytocin

Oxytocin is a nanopeptide hormone synthesized by maternal and fetal hypothalamus (Chard, 1989; Dawood, 1983; Zingg and Lefebvre, 1988), and intrauterine tissues (Chibbar et al., 1993; Miller et al., 1993; Mitchell and Chibbar, 1995). Oxytocin promotes myometrial contractility during late pregnancy and parturition, and stimulates the milk release from the mammary gland in lactation (Chard, 1989; Fuchs et al., 1987; Fuchs et al., 1984). Thus it has been widely used alone or in combination with prostaglandins for the induction of labor. However, the role of oxytocin in the process of labor is still unclear. Studies have shown that the maternal plasma oxytocin levels do not change with the onset of labor (Casey and MacDonald, 1988; Chard, 1989; Fuchs et al., 1982) whereas mRNA and protein levels of oxytocin was found to be elevated in human chorio-decidua at the onset of labor (Chibbar et al., 1993; Mauri et al., 1995). Oxytocin receptor antagonists significantly decrease uterine activity in women with threatening preterm labor (Goodwin et al., 1994), but cannot prevent the onset of labor (Chan and Chen, 1992; Honnebier et al., 1989). Moreover, the use of oxytocin antibodies did not change the timing for the onset of labor (Kumaresan et al., 1971). Finally, mice bearing a null mutation in the OT gene have normal pregnancies and labors (Gross et al., 1998; Muglia, 2000; Nishimori et al., 1996; Young et al., 1996). Taken together, these studies

imply that while oxytocin contributes to labor, it may not be an essential element (Challis et al., 2000; Honnebier et al., 1989).

I- 1.3 CRH and Parturition

During human pregnancy, the concentrations of CRH, a 41 amino acid peptide hormone, in maternal blood, amniotic fluid, cord plasma and maternal urine increase exponentially, reaching maximum levels at the most advanced stages of cervical dilatation (Campbell et al., 1987; Chan et al., 1990; Economides et al., 1987; Goland et al., 1993; Goland et al., 1988; Goland et al., 1986; Laatikainen et al., 1988; Okamoto et al., 1989; Petraglia et al., 1996; Petraglia et al., 1990; Sasaki et al., 1990; Sorem et al., 1996; Stalla et al., 1989; Wolfe et al., 1988). In addition to the production by hypothalamus in response to stress CRH is also produced by human intrauterine tissues such as syncytiotrophoblast, intermediate trophoblast and fetal membranes (Petraglia et al., 1987; Petraglia et al., 1992; Riley and Challis, 1991; Riley et al., 1991; Saijonmaa et al., 1988; Shibasaki et al., 1982; Vale et al., 1983). Increased levels of CRH in the maternal blood correlate with increased CRH mRNA and CRH peptide in placental tissues and decidua (Frim et al., 1988; Grino et al., 1987; Petraglia et al., 1992; Schulte and Healy, 1987). Placental CRH has been postulated as the major source of CRH in maternal and fetal compartments (Goland et al., 1988; Maser-Gluth et al., 1987). It has been suggested that maternal free CRH value might be a component of the trigger to the labor process and predict the patient at risk of preterm labor (Korebrits et al., 1998; McLean et al., 1995). However, it has been concluded that the assessment of the maternal CRH alone does not have sufficient sensitivity or specificity as a predictive value for preterm birth (Challis et al., 2005). Regulation of CRH production in placenta and fetal membranes is mutifactorial (Challis et al., 2002; Petraglia et al., 1996).

Recent studies suggest a role for CRH, produced locally within the human intrauterine tissues, in tissue breakdown of fetal membranes (Li and Challis, 2005). CRH was able to stimulate the matrix metalloproteinase-9 (MMP-9), but not MMP-2 output from cultured human fetal membrane cells revealing yet another feed-forward cascade in the parturition mechanisms.

I-2 Prostaglandins

Prostaglandins (PGs) and their related compounds, PGI_2, thromboxane (TX), leukotrienes (LT), and lipoxins (LP) constitute a unique class of polyunsaturated, hydroxylated, 20 carbon fatty acids that are categorized as eicosanoids (Corey et al., 1980). The discovery of eicosanoids (from the Greek *eicosa* = twenty; for twenty carbon fatty acid derivatives) was made in the 1930s when human seminal fluid was shown to contain a substance(s) that caused a rapid decrease in blood pressure and contraction of uterine and intestinal smooth muscle (Burr and Burr, 1930; Kurzrok and Lieb, 1930; von Euler, 1934). However, it was not until 30 years later when Bergstrom and Samulesson linked those observations with the classical prostaglandins (1981). Thereafter began an era of eicosanoid research. Over the years it has been demonstrated that these substances are involved in the regulation of inflammation, pain, fever, allergy and immunity, mitogenesis, differentiation, cancer, homeostasis and reproductive processes such as ovulation, luteolysis, menstruation, implantation and parturition (Gilroy et al., 1999; Kelly, 1996; Lupulescu, 1996; Matsuoka et al., 2000; Murata et al., 1997; Narumiya and FitzGerald, 2001; Olofsson and Leung, 1996; Patrono et al., 2001; Rocca et al., 1999; Smith, 1989; Tilley et al., 2001; Ushikubi et al., 1998).

PGs are derived from phospholipase-released arachidonic acid. They are produced in close proximity to their site of action and act in an autocrine/paracrine manner over a short lifetime. They are not stored in the cells (Piper and Vane, 1971). In most cases the serum concentrations of PGs are too low ($<10^{11}$ M) to elicit an endocrine effect (Ferreira and Vane, 1967; Smith, 1986). However, PGE_2 secreted from placenta by cortisol induction can act as a positive mediator of fetal HPA axis (Louis et al., 1976; Young and Thorburn, 1994).

I-2.1 Prostaglandin Biosynthesis

PG synthesis starts with the mobilization of the precursor arachidonic acid (AA) from membrane phospholipids by the action of phospholipases, including phospholipase A_2 (PLA_2) and phospholipase C enzymes, forming unesterified arachidonic acid (Clark et al., 1991; Dennis, 1987; FitzGerald et al., 2000) (Figure I-2, page 43). Under basal conditions AA exists primarily in an esterified form within the plasma membrane.

Immunohistochemical localization of phospholipase A_2 isoforms was demonstrated in human fetal membranes and myometrium during pregnancy and parturition (Skannal et al., 1997). PLA_2 might exist as cytosolic form ($cPLA_2$) (85-110 kDa) or the extracellular secretory types I, II, and III, 14-kDa forms. Their enzyme activities are distinguished biochemically by virtue of their preference for hydrolyzing specific fatty acids in the sn-2 position, with $sPLA_2$ having little preference, whereas $cPLA_2$ preferentially mobilizes AA (Clark et al., 1990). The activity of PLA_2 within the fetal membranes increases with gestational age (Farrugia et al., 1993; Lopez Bernal et al., 1992; Okazaki et al., 1981), whereas phospholipase C does not change in association with labor (Bala et al., 1990; Di Renzo et al., 1981), suggesting that it is PLA_2 that has a role in mediating the uterine AA release. After the gradual increase in PLA_2 levels in human fetal membranes throughout gestation, there is no exceptional increase of PLA_2 at the time of labor (Challis et al., 2000; Olson et al., 1995).

Conversion of AA to prostaglandin H_2 (PGH_2) by PGHS enzymes, which have both cyclooxygenase and peroxidase activities, is the second stage in PG biosynthesis pathway (Smith and Dewitt, 1996). Two isoforms of PGHS, coded by two distinct genes, have been well described: (1) the constitutive form (PGHS-1); and (2) the inducible form (PGHS-2). Even though they are products of distinct genes, they have 60-70% cDNA homology (Xu et al., 1995). Both isoforms are located in the lumen of the nuclear envelope and endoplasmic reticulum, a fact that is further supported by numerous studies using fluorescence and immuno-electron microscopy (DeWitt et al., 1981; Liou et al., 2000; Morita et al., 1995; Ren et al., 1995; Rollins and Smith, 1980; Smith et al., 1981). PGHS-2 staining appears to be predominantly localized in the nuclear envelope (Morita et al., 1995). Both PGHS-1 and -2 undergo self-inactivation in the presence of excess substrate (Egan et al., 1976; Ogino et al., 1978; Ohki et al., 1977; Smith and Lands, 1972). While PGHS-1 is constitutively expressed, PGHS-2 expression can be regulated by glucocorticoids, growth factors and inflammation (DeWitt and Meade, 1993; Jones et al., 1993; Kujubu and Herschman, 1992; Masferrer et al., 1995). PGHS is of pharmacologic importance because it has been used as a target for nonsteroidal anti-inflammatory drugs since 1970s (Vane, 1971). Recently a third isoform PGHS-3 has been described (Gilroy et al., 1999; Willoughby et al., 2000). PGHS-3 does not produce

proinflammatory prostanoids like PGHS-1 and PGHS-2, but produces anti-inflammatory members of that family.

The final stage of PG synthesis pathway involves the conversion of the PG intermediate, PGH_2, to prostaglandins of the E, F, thromboxane, and prostacyclin series by specific synthase enzymes (DeWitt and Smith, 1983; Haurand and Ullrich, 1985; Moonen et al., 1982; Smith, 1992; Smith et al., 1983; Smith et al., 1990; Smith et al., 1981; Suzuki-Yamamoto et al., 1999; Urade et al., 1985; Watanabe et al., 1985). In general this process is cell specific with a dominating prostanoid in various differentiated cells (Lands, 1979; Smith, 1992; Sun et al., 1977). Among the prostaglandins PGE_2 and $PGF_{2\alpha}$ are involved in muscle contraction (Lands, 1979). PGI_2 and TXA_2 modulate platelet aggregation, smooth muscle contraction of blood vessels, and vessel wall repair in an antagonistic manner (Hornstra, 1982; Moncada and Vane, 1984; Ylikorkala and Makila, 1985).

Arachidonic acid may also be metabolized through different lipoxygenase pathways including 5-lipoxygenase, platelet-type-12-lipoxygenase, leukocyte-type-12-lipoxygenase, and 15-lipoxygenase (Needleman et al., 1986). Conversion through 5-lipoxygenase forms 5 H(P)ETE, and 5 H(P)ETE can be converted to leukotriene A4 (LTA4). Subsequently, LTA4 is hydrolyzed to LTB4 or LTC4. 12-Lipoxygenase or 15-lipoxygenase activity results in the formation of 12-H(P)ETE and 15H(P)ETE (Samuelsson et al., 1979; Samuelsson et al., 1980). Parturition can be initiated in the presence of high concentrations of leukotrienes even in the absence of PGs in rhesus monkey (Walsh, 1991). Although the presence and production of lipoxygenase compounds during pregnancy and labor were demonstrated, functional data suggest that PGs are the main AA products directing the event of parturition. However, the relative importance of lipoxygenase in pregnancy and parturition remains largely unexplored.

I-2.2 PG Catabolism

The major pathway in the metabolism of PGE_2 and $PGF_{2\alpha}$ involves the action of a type 1 NAD^+- dependent hydroxyprostaglandin dehydrogenase (Type I PGDH) (Nakano et al., 1969). The rate limiting step in the catabolism of PGs is the initial oxidation of the 15-hydroxyl group of PGs to 15-keto metabolites with reduced biological activity

(Anggard et al., 1971; Nakano et al., 1969). PGDH is expressed in most adult tissues, including lung and placenta, where it has a high specific activity (Challis et al., 1999; Jarabak, 1972; Schlegel et al., 1974).

Another enzyme capable of oxidizing the 15 hydroxyl group of PGs is NADP-dependent PGDH (Type II PGDH) which is also referred to as carbonyl reductase (Okita and Okita, 1996; Wermuth, 1981). Carbonyl reductase requires higher concentrations of PGs for optimal activity and NADPH is a less available compound in mammalian cells compared to NADH (Hansen, 1976). Thus, NAD-dependent PGDH is regarded as the primary enzyme responsible for inactivation of PGs *in vivo* (Challis et al., 1999; Challis et al., 2000). In vitro studies have shown that PGE_2, $PGF_{2\alpha}$, PGA_2, PGI_2 and TXA_2 are all substrates for PGDH (McGuire and Sun, 1980). PGDH has been purified from many tisues including placenta, kidney and lung (Hansen, 1976; Jarabak, 1972; Schlegel et al., 1974). Moreover, PGDH cDNA and genomic DNA were cloned from the human placenta (Ensor et al., 1990). No tissue or species specificity of PGDH activity and substrate affinity was found (Hansen, 1976; Zhang et al., 1997).

PGDH expression and activity were found to be decreased in choriodecidual tissue of women at spontaneous and preterm labor (Challis et al., 1999). Thus, it has been speculated that PGDH may have a central role to play in determining biologically active prostaglandin concentrations within human fetal membranes and placenta at the time of labor, at term or preterm.

I-2.3 Prostaglandin transporters

PGs cannot cross the cell membrane for they are charged organic anions at physiologic pH levels (Uekama et al., 1979). They diffuse poorly through the lipid bilayer of plasma membrane (Baroody and Bito, 1981; Bito and Baroody, 1975). However, they need to be transported out of the cell in order to exert their biological effects via specific membrane bound PG receptors. Moreover, cellular uptake is essential to facilitate the inactivation of PGs by the intracellular catabolic enzymes. It has been demonstrated that they can move through the cell membrane via highly specific PG transporters (Bito et al., 1977; Bito et al., 1976). Facilitated diffusion was shown to be effective in a number of species and tissues including rabbit and rat lung (Anderson and

Eling, 1976; Eling et al., 1975), rabbit liver (Bito, 1972), kidney (Irish, 1979), uterus and vagina (Bito and Spellane, 1974; Jones and Harper, 1983), sheep blood-brain barriers (Krunic et al., 1997; Krunic et al., 2000).

The PG transporter has been cloned in rat (Kanai et al., 1995), mouse (Pucci et al., 1999), and human (Lu et al., 1996). In human, it has been localized in a number of organs including placenta and ovary, heart, brain, lung, liver, skeletal muscle, pancreas, kidney, spleen, prostate, small intestine and colon (Lu et al., 1996). It has been mapped to chromosome 3 of the human genome, and exists as a single copy comprised of 14 exons with a length of 95 kilobases (kb) (Lu and Schuster, 1998). PG transporter has a high affinity for primary PGs, in particular biologically active ones (Itoh et al., 1996) suggesting a potential role for the removal of PGs from the extracellular fluids for catabolism (Itoh et al., 1996; Schuster et al., 2000). Indeed, in the lungs PGI_2 and TXA_2 could escape pulmonary metabolism because they are not the substrates for the PG transporter in lungs (Anderson and Eling, 1976; Dusting et al., 1978; Horton and Jones, 1969; Pitt et al., 1983).

The promoter region of the human PG transporter contains a TATA box, 2 Spl sequences and a CRE (Lu and Schuster, 1998). Biomechanical stimuli generated by the blood flow induced the gene expression of prostaglandin transporter in human vascular endothelium (Topper et al., 1998). However, many other potential stimuli including endotoxin and pro-inflammatory cytokines such as interleukin-1beta (IL-1β) and tumor necrosis factor-alpha (TNFα) did not alter the expression of PG transporter (Topper et al., 1998). Recently, the expression of PG transporter has been characterized in bovine uterus (Banu et al., 2003) and fetal membranes (Banu et al., 2005). It is yet to be explored whether this transporter is expressed in human fetal membranes and whether they have a critical role throughout the pregnancy and parturition.

I-3 Prostaglandin receptors

The actions of PGs are mediated through distinct G protein coupled receptors (GPCRs). The first studies that suggested the existence of distinct receptors for prostanoids were performed by Pickles in 1967, when he demonstrated different patterns of activity with a range of different prostanoids (Pickles, 1967). Subsequent studies

supported the existence of multiple receptor types (Andersen and Ramwell, 1974; Gardiner and Collier, 1980). However, there was not a working classification of these receptors until 1982 when Kennedy divided them according to functional data as DP, EP, FP, IP and TP for the natural occurring prostanoids, PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 and TXA_2, respectively (Kennedy et al., 1982). Studies with PGE_2 demonstrated different activities to specific agonists and antagonists, and this multiplicity of PGE_2 actions prompted a further division of EP receptors into four subtypes, EP1, EP2, EP3, and EP4 (Coleman et al., 1987; Coleman et al., 1994). Recently, another receptor that binds PGD_2 has been identified in T helper type 2 cells, eosinophils and basophils (Hirai et al., 2001). However, this seven-transmembrane PGD_2 receptor contains characteristic molecular signatures of chemokine receptors rather than PG receptors (Nagata et al., 1999).

I-3.1 Molecular Biology of Prostaglandin Receptors

Molecular cloning, coupling with G-protein and sensitivity to agonist induced sensitization revealed that PG receptors are Rhodopsin type receptors (Avdonin et al., 1985; Murata et al., 1997). They contain 7 hydrophobic segments of transmembrane domains, an extracellular amino terminus, an intracellular carboxy terminus, three intracellular and three extracellular loops (Coleman et al., 1994; Narumiya et al., 1999; Wright et al., 2001) (Figure I-3, page 44). The most conserved regions of the prostanoid receptors are the seventh transmembrane domain and the second extracellular loop that play fundamental roles in ligand binding and recognition (Coleman et al., 1994; Narumiya et al., 1999; Savarese and Fraser, 1992; Wright et al., 2001). Two conserved Cys residues, one in the 1st loop and the other in the 2nd extracellular loop, contribute to the stabilization of GPCRs in the membrane by forming a disulfide bond (Dohlman et al., 1991). In the rabbit EP_3 receptor, when an Ala residue was substituted for the Cys residue in the second extracellular loop, no effect was observed on ligand binding (Audoly and Breyer, 1997). However, in human TP receptor, after the substitution of Ser for the analogous Cys, ligand binding was completely abolished (Chiang et al., 1996; D'Angelo et al., 1996). Similar results were obtained when the Cys of the first extracellular loop was mutated, supporting evidence for a loss of agonist binding to TP after chemical perturbation of these Cys residues (Dorn, 1990).

The eight known types of prostaglandin receptors are each encoded by an individual gene. The EP1, TP, and IP genes were mapped to chromosome 19 at positions 19p13.1, 19p13.3, and 19q13.3, respectively (Duncan et al., 1995). The FP and EP3 genes mapped to chromosome 1 at positions 1p31.1 and 1p31.2, respectively, and EP2 gene mapped to chromosome band 5p13.1 (Duncan et al., 1995). However, Smock et al reported that EP2 gene was located to chromosome 14q22 (Smock et al., 1999), and finally it was EP4 gene that was located to 5p13.1 (Foord et al., 1996).

Phylogenetic analyses indicate that receptors sharing a common signaling pathway have higher sequence homology (Boie et al., 1995; Regan et al., 1994; Toh et al., 1995). For example the receptors that induce smooth muscle relaxation, EP_2, EP_4, DP, and IP are more closely related to each other. Similarly, EP_1, FP, and TP receptors cause smooth muscle contraction and have more sequence homology with each other than the other PG receptors. Overall there is 24-30% homology among the contractile and relaxatory receptor groups (Myatt and Lye, 2004). On the basis of their phylogenetic analyses, it has been suggested that the COX pathway may have evolved from PGE_2 and an ancestral EP receptor (Narumiya et al., 1999). Subsequent evolution of the EP receptor types from this ancestral prostanoid receptor would have linked PGE_2 to different signal transduction pathways. It has been speculated that other PG receptors would have then evolved by gene duplication of these different EP receptor subtypes.

The cloning of the prostanoid receptors showed alternative mRNA splicing, thus revealing a further heterogeneity for EP1, EP3, TP and FP. Alternative mRNA splicing involves the formation of multiple mRNA and protein products from a single gene. Nine splice variants for human EP3 has been identified: $EP_{3\text{-}1a}$, $EP_{3\text{-}1b}$, $EP_{3\text{-}II}$, $EP_{3\text{-}III}$, $EP_{3\text{-}IV}$, $EP_{3\text{-}V}$, $EP_{3\text{-}VI}$, $EP_{3\text{-}e}$, and $EP_{3\text{-}f}$ (Adam et al., 1994; Kotani et al., 2000; Kotani et al., 1997; Regan et al., 1994). Splice variants for EP3 in mouse (Irie et al., 1993; Negishi et al., 1993; Sugimoto et al., 1993), rat (Takeuchi et al., 1994), bovine (Namba et al., 1993) and rabbit (Breyer et al., 1994) have also been reported. Human TP receptor also has two splice variants, TPα and TPβ, according to variation by the tail of their carboxyl-terminal end (Raychowdhury et al., 1994). Two subtypes for each of the rat EP1 and ovine FP were identified (Okuda-Ashitaka et al., 1996; Pierce et al., 1997). All the splice variants

of PG receptors differ between each other by their carboxyl-terminal portions. The regulation of the alternative splicing of PG receptors has yet to be studied.

I-3.2 Signaling Pathways of Prostaglandin Receptors

Early studies of the second messengers downstream of the prostanoids focused on cyclic nucleotides (Coleman et al., 1994). PGE_2 and $PGF_{2\alpha}$ were reported to stimulate cAMP (Butcher and Sutherland, 1967) and cGMP (Dunham et al., 1974), respectively. Since then, other signal transduction pathways have been suggested such as mobilization of free Ca^{2+} and increases in inositol phosphate (Narumiya et al., 1999). The molecular cloning of prostaglandin receptors revealed information about their coupling to heterotrimeric G proteins. G proteins are composed of three structural subunits: α, β and γ (Hamm, 1998); and two functional subunits: after the receptor activation, $G\alpha$ subunit can dissociate from $G\beta\gamma$ subunits both of which can act as effectors in signal transduction (Clapham and Neer, 1997; Rens-Domiano and Hamm, 1995). The signal transduction pathways underlying the mechanisms of prostaglandin action are shared within the group. According to shared secondary pathway mechanisms, three clusters of related receptors have been defined: (1) DP, IP, EP_2, and EP_4; (2) EP_1, FP, and TP; and (3) EP_3 (Wright et al., 2001) (Figure I-4, page 45). The PGs within the same group were also the ones that have higher sequence homology than receptors sharing a common prostanoid as their preferential ligand.

The first group of receptors, DP, IP, EP2 and EP4 are linked to $G\alpha$ subunits. Recombinant human DP (Boie et al., 1995), IP (Boie et al., 1994; Katsuyama et al., 1994; Nakagawa et al., 1994), EP2 (Bastien et al., 1994; Regan et al., 1994), and EP4 (An et al., 1993) all increase the cAMP concentration via stimulation of adenylate cyclase, consistent with their ability to relax smooth muscle in vivo (An et al., 1993; Bastien et al., 1994; Regan et al., 1994; Wright et al., 2001). Recombinant human IP receptors can mediate inositol phosphate production and increase free Ca^{2+} levels via $G\alpha_q$ (Namba et al., 1994). Similarly, in choroid EP_2, EP_4, and DP receptors couple to eNOS rather than adenylate cyclase (Abran et al., 1997; Abran et al., 1997). This may be induced by $G\beta\gamma$ action on phosphatidylinositol 3-kinase (Clapham and Neer, 1997) that activates protein kinase B (PKB) (Vanhaesebroeck et al., 1997) and eNOS (Dimmeler et al., 1999).

Fujino and colleagues have shown that the stimulation of EP2 and EP4 receptors can activate T-cell factor signaling (Fujino et al., 2002). While EP2 did this via cAMP-dependent protein kinase-dependent pathway, EP4 receptors primarily utilized a phosphatidylinositol 3-kinase. The same group also demonstrated that activation of EP4 receptors can lead to phosphorylation of the extracellular signal regulated kinases (ERKs) (Fujino et al., 2003) and inhibition of protein kinase A (PKA) (Fujino et al., 2005) again through a phosphatidylinositol 3-kinase dependent pathway.

Stimulation of human EP1, FP and TP receptors leads to IP3 generation by activation of phospholipase C via Gq and increased intracellular Ca concentrations (Narumiya et al., 1999). This pathway has been clearly demonstrated for EP1 when its stimulation led to an increase in inositol phosphate in brain and ocular vasculature (Abran et al., 1995). In rat luteal cells, inositol phosphate turnover was demonstrated following the exposure of the cells to $PGF_{2\alpha}$ (Raymond et al., 1983). The Gq pathway is the main effector pathway also for TP as it has been demonstrated in human platelet cells following the exposure to TXA2 (Shenker et al., 1991).

The variants of EP3, considered as the third group of PG receptor family, mainly decrease cAMP generation by the inhibition of adenylate cyclase through the $G_{i\alpha}$-family (Negishi et al., 1988). However, there are additional signaling mechanisms including Gs and Ca release (An et al., 1994; Audoly et al., 1999; Namba et al., 1993), the small G protein Rho (Aoki et al., 1999; Katoh et al., 1996) and protein kinase C (Asboth et al., 1996; Zacharowski et al., 1999).

I-3.3 Nuclear Prostaglandin Receptors

Several lines of evidence suggest a novel intracrine signaling mechanism for PG receptors. The existence of human PG transporter (Kanai et al., 1995; Lu et al., 1996; Lu and Schuster, 1998) provides one of the potential mechanisms by which PGs can be delivered to an intracellular or nuclear site of action. Accumulating evidence on the perinuclear localization of PG synthesizing enzymes has further supported the existence of intracrine signaling pathways that PGs can activate. Cytosolic phospholipase A2 has been localized in the nucleus (Neitcheva and Peeva, 1995; Tamiya-Koizumi et al., 1989), and the nuclear membrane has been defined as an important site of arachidonate

metabolism (Capriotti et al., 1988; Neufeld et al., 1985). COX-1 and COX-2 were also identified in the nuclear perinuclear region (Coffey et al., 1997; Marvin et al., 2000; Morita et al., 1995; Parfenova et al., 1997; Parfenova et al., 2001; Regier et al., 1995; Spencer et al., 1998). Specific binding of PGE_2 and PGD_2 to nuclear fractions were clearly demonstrated in pig brain and myometrium (Bhattacharya et al., 1998). Functional EP3 and EP4 receptors were also shown in the nuclear envelope of a variety of cells (Bhattacharya et al., 1999; Gobeil et al., 2002). Moreover, a distinct functional role for the nuclear and plasma membrane receptors has been reported in porcine brain demonstrating PGE_2-induced eNOS expression via perinuclear EP3 receptors (Gobeil et al., 2002). The existence of functional nuclear PG receptors adds yet another level of complexity of PG signaling pathways in gestational tissues. Currently, there is no information whether the nuclear G-protein coupled PG receptors are present in these tissues.

I-3.4 Evidence Provided by Disruption of Prostaglandin Receptors

Until recently the physiological role of the prostaglandins was for the most part determined by using COX inhibitor non-steroid anti-inflammatory drugs. However, the cloning of prostaglandin receptors and development of animals with disrupted genes of these receptors has tremendously advanced our understanding of the functions of these receptors. In this section the phenotypes as a result of PG receptor disruption will be discussed in detail. Some studies demonstrated a direct involvement of PG receptors in reproductive biology. However, there are some other functions shown in different cells that might be important during the process of pregnancy and parturition (Table I-1, page 41).

Among the PGE_2 receptors, the most widely distributed subtypes are EP3 and EP4 which are expressed in almost every tissue (Coleman et al., 1994; Narumiya et al., 1999). EP1 and EP2 are present in vascular/nonvascular smooth muscle (Chemtob et al., 1996; Coleman et al., 1994), and in lung, placenta, endometrium, renal tubules, brain synaptosomes, heart (Li et al., 1993; Regan et al., 1994), respectively. Finally, FP is expressed especially in corpus luteum, iris sphincter muscle, trabecular meshwork and vascular smooth muscle (Coleman et al., 1994; Narumiya et al., 1999).

I-3.4.1 FP disruption

$PGF_{2\alpha}$ has been known as a potent constrictor of the uterus since the 1970s (Horton and Poyser, 1976). $PGF_{2\alpha}$ and its analogues can cause premature regression of the corpus luteum (Olofsson and Leung, 1994) normally caused by $PGF_{2\alpha}$ produced by uterus (Horton and Poyser, 1976). In the rat, FP receptor mRNA is expressed in corpus luteum and can be detected at lower levels in the remainder of ovary (Olofsson and Leung, 1996). Moreover, modulation of FP receptor expression could regulate the ovarian responsiveness to $PGF_{2\alpha}$ (Ristimaki et al., 1997). Sugimoto and colleagues produced mice lacking FP gene (Sugimoto et al., 1997). FP-/- mice developed with normal estrous cycles, ovulation, fertilization and implantation. However, they were unable to deliver normal fetuses at term and did not respond to exogenous oxytocin. The progesterone levels in their serum remained high. Induction of the oxytocin receptor could be restored by ovariectomy in late gestation which is translated as the removal of progesterone. The authors concluded that parturition in the mouse is initiated when $PGF_{2\alpha}$ interacts with FP in ovarian luteal cells to induce luteolysis. Subsequently, the same group demonstrated that FP-/- mouse does not induce the myometrial PGHS-2 at parturition although this could be restored by ovariectomy (Tsuboi et al., 2000). In human parturition, however, the corpus luteum is not required to support pregnancy. Instead uterine FP receptors have been implicated as the principal site of action for $PGF_{2\alpha}$. Even though other prostaglandin receptor null mice have been generated none of them had phenotypes that affect parturition.

I-3.4.2 EP1 disruption

There are individual reports of the generic disruption of the EP1 gene (EP1-/-) (Audoly et al., 1999; Takeuchi et al., 1999; Watanabe et al., 1999). EP1 and EP3 are both required for LPS-induced HPA axis activation (Matsuoka et al., 2003). Again both receptors were shown to be involved in the activation of neurons in paraventricular nucleus of the hypothalamus. However, the most striking observation was the contribution of PGE_2 to colon cancer acting through EP1. In a mouse model, preneoplastic lesions of colon cancer were decreased in EP1-/- mice relative to wild type mice (Watanabe et al., 1999). Subsequent studies also suggest that EP1 antagonists may

be useful as chemopreventive agents for colon cancer (Kawamori et al., 2005; Kawamori and Wakabayashi, 2002; Watanabe et al., 1999). On the other hand, EP1 revealed cytoprotective actions in the gastric mucosa following the exposure to HCl and/or ethanol (Araki et al., 2000; Komoike et al., 2003; Takeuchi et al., 2001) or indomethacin induced gastric damage (Kunikata et al., 2001).

A gender specific vasodepressor effect was observed in EP1-/- mice (Audoly et al., 1999). Male EP1-/-mice exhibited less PGE_2-induced hypotension relative to wild-type mice, but the mechanism was not understood. It has been demonstrated that EP1 contributes to enhanced PGE_2 mediated salt and water excretion along with EP3 and EP4 (Nusing et al., 2005), and it is involved in an increase of renal vascular tone (Schweda et al., 2004).

Systemic inflammation induced fever was also mediated via the action of both EP1 and EP3 in central nervous system (Oka et al., 2003). Recently Ahmad and colleagues demonstrated that EP1 aggravates neurotoxicity and modulation of this receptor can determine the focal ischemic neuronal damage (Ahmad et al., 2006).

I-3.4.3 EP2 disruption

Of all of the prostanoid receptors, the ablation of the EP_2 receptor gene (EP_2-/-) has been one of the most intensely studied. Even though EP2 has been implicated in reproductive system and blood pressure homeostasis (Ushikubi et al., 2000) it is essential for the actions of PGE_2 in a variety of other systems such as tumor biology, neurological system, immune response, pulmonary system and bones.

Female EP2-/- mice exhibit a reduced litter size at term delivery, a decrease in ovulation number, and a severely impaired fertilization rate (Hizaki et al., 1999; Kennedy et al., 1999; Tilley et al., 1999). Studies demonstrated that expansion of cumulus cells, which is an indispensable step for a successful fertilization, is mediated by PGE_2 via EP2 receptors (Hizaki et al., 1999). Apart from these direct evidences of the involvement of EP2 in reproductive functions, other studies demonstrated the actions of PGs via EP2 that might contribute to physiology and physiopathology of pregnancy and parturition. EP2 has been implicated in regulation of apoptosis/proliferation, smooth muscle function, and regulation of the immune system.

In pulmonary endothelial cells, EP2-/- cells showed reduced cell migration compared to control groups (Kamiyama et al., 2006). On the other hand, EP2 receptor plays a significant role in the protumorigenic action of PGE_2 in skin tumor development (Sung et al., 2005) and overexpression of EP2 results in enhanced skin tumor development (Sung et al., 2006). Genetic deletion of EP2 reduced radiation-induced apoptosis (Tessner et al., 2004). Moreover, the regulatory effect of PGE_2 on the proliferation of fibroblasts and lymphocytes are also mediated via EP2 (Moore et al., 2005; Nataraj et al., 2001).

Contradictory effects of the $EP_2^{-/-}$ phenotype are reported for blood pressure homeostasis. Both hypotension (Tilley et al., 1999) and hypertension (Kennedy et al., 1999) were observed in EP2-/- mice. EP2 stimulation can either cause hypotensive response via vasorelaxant effects or hypertensive response by activation of renin-angiotensin system (Tilley et al., 1999). In female mice EP2 has been defined as one of the main mediators of the vasodepressor response to PGE_2 (Audoly et al., 1999). Reduced systolic blood pressure following the disruption of EP2 receptor has been demonstrated (Tilley et al., 1999). When these animals were placed on a high-salt diet, blood pressure increased (Kennedy et al., 1999) which suggests that the EP2 receptor may be involved in sodium handling by the kidney (Tilley et al., 1999). Moreover, renin release from the kidney is stimulated by PGE_2 via EP2 and EP4 (Schweda et al., 2004). Both receptor subtypes also contribute to the control of renal vascular tone by decreasing it. The relaxatory effects of EP2 receptors were also demonstrated in airway. Gene disruption of EP2 results in loss of bronchodilation by PGE_2 (Fortner et al., 2001; Sheller et al., 2000; Tilley et al., 2003).

Deleting EP2 had a protective effect on oxidative damage from activation of innate immunity, particularly induction of nitric oxide synthase (NOS) activity, in the cerebrum (Montine et al., 2002). EP2 contributes to acute inflammation as well. In mouse peritoneal neutrophils, the effect of PGE_2 on granulocyte colony-stimulating factor production, which is a hemopoietic growth factor that mediates the differentiation of progenitor cells, is mediated via EP2 receptor subtype (Sugimoto et al., 2005). PGE_2 has an inhibitory effect on the proliferation of lymphocytes and this is mediated via EP2 receptors which also regulate the function of antigen presenting cells (Nataraj et al.,

2001). While PGE_2 is a potent inhibitor of fibroblast function, in EP2-/- mice PGE_2 was unable to suppress the proliferation or collagen synthesis in fibroblasts (Moore et al., 2005). Hypercalcemia was stimulated by PGE_2 via EP2 receptors in vivo (Li et al., 2002).

I-3.4.4 EP3 disruption

EP3 receptor subtype is involved in tumor biology via its mediating effect on the proliferation of the cells. Tumor associated angiogenesis and expression of vascular endothelial growth factor (VEGF) were markedly suppressed in tumor implanted EP3-/- mice (Amano et al., 2003) demonstrating the significance of PGE_2-EP3 receptor signaling in tumor development. In the skin, EP3 receptor has been implicated in the development of squamous cell carcinomas (Shoji et al., 2005). However, it is also apparent that EP3 plays an important role in suppression of colon cancer cell proliferation and its downregulation enhances colon carcinogenesis at a later stage (Shoji et al., 2004).

The action of PGs on vasoconstriction and vasodilation can ben mediated via EP3 as well. In the kidney, EP3 mediates vasoconstriction and is capable of buffering renal vasodilation mediated via EP2/EP4 receptor subtypes (Audoly et al., 2001; Schweda et al., 2004). EP3 was also implicated in PGE_2-induced hypotension in male mice due to gender specific vasopressor responses (Audoly et al., 1999). Studies have shown that EP3 may function in duodenum and intestine towards the maintenance of mucosal integrity. In the duodenum EP3 was required for bicarbonate secretion (Takeuchi et al., 1999; Takeuchi et al., 1999). Intestinal cytoprotective actions of PGE_2 following the exposure to indomethacin are also mediated via EP3 receptors (Kunikata et al., 2001; Kunikata et al., 2002). Genetic deletion of EP3 protected against the formation of intravascular clots in a venous inflammation model (Fabre et al., 2001) suggesting that the proaggregatory actions of low PGE_2 are mediated via EP3 receptor.

EP3 has been shown to be crucial in some of the neurologic system functions, such as fever generation. Fever is elicited by endogenous pyrogens such as inflammatory cytokines acting on brain (Kluger, 1991). The production of these cytokines can be stimulated by exogenous pyrogens such as lipopolysaccharide (LPS) or by non-infectious inflammatory insults. PGE_2 has been proposed as the main mediator of fever (Milton and Wendlandt, 1970) and aspirin-like drugs could suppress fever (Vane, 1971). Studies have

clearly shown that EP_3 is involved in PGE_2-induced pyrexia because $EP_3^{-/-}$ mice fail to mount a febrile response to exogenous (i.e., lipopolysaccharide) and endogenous (i.e., IL-1β) pyrogens (Ushikubi et al., 1998). More recent studies have also concluded that EP3 is involved in fever induced by systemic inflammation but not in stress-induced hyperthermia (Oka et al., 2003).

Mice lacking EP3 developed allergic inflammation that was more pronounced that in wild-type mice (Kunikata et al., 2005). Finally, spinal EP3 receptors were shown to be involved in PGE_2 induced hyperalgesia (Minami et al., 2001).

I-3.4.5 EP4 disruption

The principal observation in EP4-/- mice is their inability to close the ductus arteriosus, which connects the pulmonary artery with the descending aorta during the fetal period, immediately after birth. Loss of the EP4 receptor was not lethal in utero, but 95% of EP4-/- neonates became pale and lethargic in about 24 hours and died within 72 hours (Segi et al., 1998). Histological examination revealed that the difference between the dead and alive mice was the closure of the ductus arteriosus. The ductus arteriosus remained open in dead neonates and in situ hybridization study showed that there is strong EP4 mRNA expression in the ductus. Interestingly, this patency did not coincide with the relaxant actions of PGE_2 on ductus arteriosus that are mediated via EP4 (Smith et al., 1994).

PGE_2-EP4 signaling facilitates the migration and maturation in dendritic cells, and potently suppresses the activation and proliferation of T cells (Narumiya, 2003). PGE_2 modulates macrophage function via EP4 resulting in the inhibition of cytokine release (Nataraj et al., 2001). PGE_2 is known to inhibit the lipopolysaccharide (LPS)-induced tumor necrosis factor alpha formation in Kupffer cells via an increase in cAMP (Fennekohl et al., 2002) and it has been demonstrated that EP4 is the more physiologically relevant receptor subtype in this regulation. In skin, PGE_2-EP4 signaling facilitates the initiation of immune responses by promoting the migration and maturation of Langerhans cells (Kabashima et al., 2003).

In intestinal tissue PGE_2 has cytoprotective properties against indomethacin that are mediated by both EP3 and EP4 receptor subtypes (Kunikata et al., 2001; Kunikata et

al., 2002). EP4 has been implicated in the maintenance of intestinal homeostasis by maintaining mucosal integrity and downregulating immune response (Kabashima et al., 2002), as elevated expression of genes associated with immune response and reduced expression of genes with mucosal repair and remodeling in the colon of EP4-deficient mice were shown.

Finally, bone resorption is primarily mediated by EP4 (Miyaura et al., 2000; Ono et al., 2003; Sakuma et al., 2000; Suzawa et al., 2000). EP4 receptors are essential for osteoclast formation (Ono et al., 2003) and impaired induction of osteoclast formation was observed in EP4 knockouts (Li et al., 2000; Li et al., 2002). Absence of the EP4 receptor decreases bone mass and impairs fracture healing in aged mice (Li et al., 2005) indicating the essential role of PGE_2-EP4 signaling in the maintenance of bone mass and fracture healing.

I-4 The Role of Prostaglandins in Parturition

Prostaglandins (PGs) are the key mediators of parturition in most mammalian species including human (Challis et al., 2002; Novy and Liggins, 1980; Okazaki et al., 1981). They have been shown to induce myometrial contractility (Bennett et al., 1987; Haluska et al., 1987; Ritchie et al., 1984; Wiqvist et al., 1983) and regulate changes in extracellular matrix metabolism associated with cervical ripening (Ellwood et al., 1980; Keirse, 1993; Wiqvist et al., 1983). In addition, PGs have also been implicated in the up-regulation of the fetal HPA axis (Challis et al., 2000); rupture of membranes (So, 1993; Vadillo Ortega et al., 1994); fetal adaptation to the labor process including inhibition of fetal movements and breathing to conserve energy (Kitterman, 1987; Thorburn, 1992), and maintenance of uterine and placental blood flow (Carter, 1998; Challis et al., 2000; Rankin, 1976; Sastry et al., 1997).

Over the years, four main lines of evidence have been proposed as support of the importance of PGs during parturition: (1) with the progression of labor the amount of PGs are increased in maternal plasma, maternal urine and amniotic fluid; (2) blocking PG synthesis delays the onset of labor, reduces the contractions and prolongs the process of labor; (3) administration of PGs induces labor in the third trimester; (4) PGs can stimulate uterine contractility *in vitro* (Olson, 2005).

Several studies have shown the increase in PGs associated with parturition. Increased concentration of PGs in the amniotic fluid and of PG metabolites in maternal plasma were found with the progression of cervical dilatation (Karim and Devlin, 1967; Novy and Liggins, 1980). When the amniotic fluid was assessed for the amount of PGs that it contains, there was an increase during labor (Dray and Frydman, 1976; MacDonald and Casey, 1993; Salmon JA and J-J, 1973). Although the fetus itself can be the source of this increased level of PGs (Gleason, 1987), it has a modest contribution to the amount of overall amniotic PG levels (Casey et al., 1983; Mitchell et al., 1985). Interestingly, the distribution of PGs within the amniotic fluid is not homogenous. Amniotic fluid obtained after amniocentesis revealed that the concentration of PGs are higher at the cervical compared to upper compartment (MacDonald and Casey, 1993; Romero et al., 1994).

PGs can induce contraction in myometrium in vivo or in vitro. Thus, blocking PG synthesis to prevent preterm labor was proposed as early as the 1970s (Harper and Skarnes, 1972; Lewis and Schulman, 1973; Okazaki et al., 1981; Reiss et al., 1976; Skarnes and Harper, 1972). When women took asprin (ASA) for the last 6 months of their pregnancy, the average length of gestation, frequency of postmaturity and mean duration of spontaneous labor was significantly prolonged (Lewis and Schulman, 1973). Conversely, exogenously administered PGs were able to stimulate myometrial contraction and cervical ripening at any gestational age thereby inducing abortion in early pregnancy (MacDonald et al., 1974) or labor (Embrey, 1969; Embrey, 1970; Husslein, 1991; Karim and Filshie, 1970; Karim and Filshie, 1970; Karim et al., 1968; Macer et al., 1984).

Cervical ripening is a prerequisite for the normal onset and progression of labor. Premature cervical ripening can lead to premature labor (Anderson and Turnbull, 1969; Bouyer et al., 1986; Leveno et al., 1986; Papiernik et al., 1986). It can be controlled by hormones such as estrogen (Rajabi et al., 1991; Stjernholm et al., 1996) and progesterone (Radestad et al., 1990; Sato et al., 1991) enhancing or inhibiting the ripening, respectively. A variety of studies were able to demonstrate the effect of PGs (especially PGE_2) on cervical softening, effacement and dilation (Calder and Embrey, 1973; Keirse, 1993; Ulmsten et al., 1982). These PGs can be produced by the cervical tissue at term from the fibroblasts and infiltrating neutrophils and eosinophils (Junqueira et al., 1980;

Kelly, 1994; Romero et al., 1988). Consistent with this hypothesis it has been shown that in human cervical tissue, PGDH mRNA expression was reduced during term and preterm labor (Tornblom et al., 2004). Fetal membranes are another possible source for the increased PG levels. In chorion, immunoreactivity and expression of PGDH was significantly lower in the region closest to cervical os (Van Meir et al., 1997). Thus, the reduced activity and expression of PGDH in this specific region would provide elevated levels of PGs for the remodeling of cervical connective tissue.

I-4.1 Prostaglandins and Membrane Rupture

The fetal membranes (FM) surrounding the amniotic cavity are composed of amnion and chorion which are closely adherent layers of several specialized cells embedded in extracellular collagenous matrix. The main cell types consist of epithelial, mesenchymal and trophoblast cells (Bourne, 1960). FMs are genetically identical to the fetus. With the placenta they form the highly specialized points of maternal-fetal interactions that have been recognized as needed for the survival of the pregnancy as well as for a normal parturition (Liggins, 1985). They retain amniotic fluid, secrete substances into amniotic fluid and towards the uterus, and protect the fetus from ascending infection (Parry and Strauss, 1998).

Throughout pregnancy FM should be able to accommodate the growing fetus by replication or hypertrophy of the cells and/or extracellular remodeling (Bryant-Greenwood, 1998). Although chorion is thicker, it is amnion that maintains the tensile strength of FM throughout the late stages of normal pregnancy (Helmig et al., 1993; Oxlund et al., 1990; Parry and Strauss, 1998).

Rupture of the fetal membranes usually occurs after the onset of regular uterine contractions. In 8-10% of the pregnancies, rupture of the FM takes place before the onset of these contractions and this is defined as 'premature rupture of the fetal membranes' (PROM) (Parry and Strauss, 1998). However, in around 1% of pregnancies PROM occurs before the 37 weeks of gestational age, which is defined as 'preterm premature rupture of the fetal membranes' (PPROM), and this is associated with 30-40% of preterm deliveries (Parry and Strauss, 1998). Thus, PPROM is the leading identifiable cause of preterm deliveries.

Rupture of the membranes is a consequence of coordinated progression of events preceding and during parturition that result in controlled degradation of collagen within the fetal membranes. Infection/inflammation, hormones, programmed cell death and membrane stretch have been defined as clinical factors associated with the rupture of the membranes. However, the mechanism underlying PROM are relatively unknown despite the many hypotheses that have been proposed (Bryant-Greenwood and Millar, 2000; Naeye, 1982; Parry and Strauss, 1998). The epidemiologic and clinical factors associated with an increased risk for PPROM are the same with those for PTL such as infection, smoking, alcohol abuse, substance abuse, malnutrition, coitus during pregnancy, multiple gestation, gestational bleeding (Alger and Pupkin, 1986; French and McGregor, 1996; Krohn et al., 1995; Polzin and Brady, 1991; Polzin and Brady, 1998; Shubert et al., 1992).

Intra-amniotic infection and histologic chorioamnionitis have been associated with more than 50% of PPROM (Gibbs and Blanco, 1982). Bacterial toxins and extracellular matrix (ECM) degrading enzymes produced by bacteria have been considered among the risk factors for membrane rupture (McGregor et al., 1986). The inflammatory response to bacterial infection is mediated by polymorphonuclear neutrophils and macrophages that are recruited to the site of infection and produce cytokines, matrix metalloproteinases (MMPs) and PGs (Parry and Strauss, 1998). A programmed collagenolytic remodeling process which is primarily mediated by matrix metalloproteinases (MMPs) exists in amniochorion throughout the pregnancy (Bryant-Greenwood and Yamamoto, 1995; Skinner and Liggins, 1981). At the tissue level the action of MMPs is mediated by the tissue inhibitors of metalloproteinases (TIMPs). There are 28 members of the MMP family of proteins along with four member of TIMP family identified so far (Menon and Fortunato, 2004). The expression of TIMP was first identified in amnion by Murphy and colleagues (Murphy et al., 1981). Human FMs express all four types of TIMP (Menon and Fortunato, 2004). The integrity of FM remains stable during most part of gestation in part due to a combination of MMPs activity and a relatively higher concentration of TIMP-1 (Vadillo-Ortega et al., 1996). Closer to the time of delivery the balance shifts towards the proteolytic degradation of the ECM of the FM (Draper et al., 1995; Hampson et al., 1997; So, 1993; Vadillo Ortega et

al., 1995). In human, amnion and chorion MMP-9 activity increases and TIMP-1 concentrations decrease dramatically during labor (Vadillo-Ortega et al., 1995; Vadillo-Ortega et al., 1996). Moreover, MMP-1 and MMP-3 activities were found elevated before labor or during labor, respectively (Bryant-Greenwood and Yamamoto, 1995). Inappropriate MMP activity comprises part of the pathogenic mechanism associated with PPROM (Athayde et al., 1998; Fortunato et al., 2000). Inflammatory cytokines increase the activity of MMP-1 and MMP-3, and decrease TIMP production in the FMs (Katsura et al., 1989; So et al., 1992). These cytokines can also increase the production of PGs by acting on PG biosynthesis pathways as will be discussed in detail. PGs in turn diminish the collagen synthesis in FM and increase the activity of MMP1 and MMP-3 (DiBattista et al., 1994; Tjugum and Norstrom, 1985).

Hormones are involved in the mechanism of membrane rupture as well. Both progesterone and estradiol suppress ECM remodeling in reproductive tissues. In the cervical fibroblasts of rabbits, both hormones decreased the concentrations of MMP-1 and MMP-3 and increased the concentrations of TIMP (Sato et al., 1991). In guinea pigs, progesterone had paradoxical effects on the production of collagenase. At high concentrations it decreased the production of collagenase while at low concentrations stimulated it (Rajabi et al., 1991). Relaxin is a locally expressed protein hormone that regulates the remodeling of connective tissue. Relaxin gene is increased before labor in human fetal membranes at term (Bryant-Greenwood and Yamamoto, 1995). It opposes the inhibitory effects of progesterone and estradiol by increasing MMP-3 and MMP-9 activities in fetal membranes (Qin et al., 1997). However, the involvement of the hormones in the process of FM rupture remains to be defined.

Programmed cell death, or apoptosis, is associated with the remodeling of various reproductive tissues, including those of the uterus and cervix. Lei et al has reported that in rats amnion epithelial cells undergo apoptosis as labor approaches following the start of ECM degradation (Lei et al., 1996). In human amnion increased apoptosis is associated with labor at term (Hsu et al., 2000). Regionally increased incidence of apoptosis was observed in the cytotrophoblastic layer of the membrane overlying the cervix (McLaren et al., 1999). Increased apoptosis of human fetal membranes were implicated in the pathophysiology of rupture of the membranes (Kataoka et al., 2002). Locally produced

glucocorticoids are involved in apoptotic cell death of chorion leave in human fetal membrane (Ohyama et al., 1998). Cyclooxygenase and/or prostaglandins play a role in the apoptosis of amnion-derived WISH cells in vitro (Moore et al., 1999). Recently, it has been shown that prostaglandin release occurs coincident with apoptosis in both amnion epithelial and mesenchymal cells (Moore et al., 2003).

Uterine overdistention induces stretch of the membranes and increases the risk of PROM. Mechanical stretch up-regulates the production of a number of amniotic factors including PGs and IL-8 (Maradny et al., 1996). MMP-activity is also elevated due to stretch (Maradny et al., 1996). In turn increased PGs increase the uterine irritability and works towards the rupture of the membranes as stated before. Taken together, PGE_2 is involved in the mechanism of the structural changes of fetal membranes and, hence, in parturition associated with membrane rupture. Figure I-5 summarizes the events leading towards rupture of the membranes (page 46).

I-4.2 Prostaglandin Synthesis and Metabolism During Human Pregnancy

During pregnancy there is a discreet compartmentalization of PG production and metabolism within the intrauterine tissues (Challis et al., 2002; Challis et al., 2000). Human amnion is the major site of PG synthesis (Challis and Olson, 1988; Duchesne et al., 1978; Gibb and Sun, 1996; Lundin-Schiller and Mitchell, 1990; Olson et al., 1995; Olson et al., 1991). PGHS-1 and PGHS-2 were localized in both amnion epithelium and amnion fibroblast cells at term, but amnion fibroblasts express higher levels of PGHS-2, and thus produce more PGE_2 than the amnion epithelial cells (Sun et al., 1996). Both PGHS activity and mRNA levels were increased in amnion layer at term (Bennett et al., 1992; Gibb and Sun, 1996; Keirse and Turnbull, 1976; Mitchell et al., 1978; Okazaki et al., 1981) and preterm (Hirst et al., 1995; Skinner and Challis, 1985; Teixeira et al., 1993). There is very low or below the detectable levels of PGDH enzyme present in human amnion (Cheung et al., 1990; Keirse and Turnbull, 1975; Okazaki et al., 1981). Chorion, on the other hand, expresses a very high concentration of PGDH localized to the trophoblast cells (Keirse et al., 1985; Keirse et al., 1978; Keirse et al., 1976; Keirse and Turnbull, 1975; Van Meir et al., 1997). These high levels of chorionic PGDH prevent intact PGs from the fetal or maternal compartments from crossing over to the other side

(McCoshen et al., 1990; Mitchell et al., 1993). However, in infection induced preterm birth or in about 15% of idiopathic preterm births the specific activity of PGDH in chorion decreases, potentially allowing greater amount of PGs crossing across the chorion to facilitate myometrial contraction (Van Meir et al., 1996). PGHS is also abundant within chorion (Gibb and Sun, 1996). PGHS-2 mRNA is found increased in chorion with the onset of labor (Slater et al., 1998; Slater et al., 1995). PG synthetic capacity of membranes was found considerably lower at preterm birth than at term birth (Sadovsky et al., 2000; Teixeira et al., 1994).

Human decidua, a well vascularized maternal tissue that consists of decidualized stromal cells, bone-marrow derived macrophages and other cells, contains low concentration of both PGHS-1 and PGHS-2, and minimal PGDH (Casey and MacDonald, 1988; Cheung et al., 1990; Liggins, 1976; Okazaki et al., 1981; Teixeira et al., 1994). Most groups did not detect a significant change in PGHS-2 production in decidua correlated with labor (Casey and MacDonald, 1988; Fuentes et al., 1996; Gibb and Sun, 1996; Harper et al., 1983). In brief, amnion and choriodecidua produce increasing amounts of PGs throughout gestation, but a further increase in the PGHS-2 mRNA is only observed in amnion and chorion layers (Freed et al., 1995; Hirst et al., 1995; Mijovic et al., 1997; Olson et al., 1983; Reddi et al., 1990; Skinner and Challis, 1985; Slater et al., 1995; Teixeira et al., 1994). Finally, one group has reported an increase in PGHS-1 mRNA in preterm labor group versus preterm non-labor group (Mijovic et al., 1998).

Placenta is composed of several different trophoblast phenotypes that have specialized functions such as transport/exchange or hormone production (Challis and Lye, 1994). Even though both PGHS-1 and PGHS-2 have been localized to human placental syncytiotrophoblast and to intermediate trophoblast cells (Johansen et al., 2000; Pomini et al., 1999; Wetzka et al., 1997; Woodworth et al., 1994), PGHS-2 was the predominant isoform localized in placenta at term (Anteby et al., 1997; Macchia et al., 1997). The locally produced PGs can mediate endocrine function or uteroplacental blood flow (Challis et al., 2000; Rankin, 1976; Rankin and Phernetton, 1976; Sastry et al., 1997). Placental villi produce more PGE_2 before labor compared to during labor (Harper et al., 1983). Since the PG is in close proximity to the myometrium, the contribution of

placental PGs to the uterine contraction has been evaluated. Different reports demonstrated that the PGs produced by placenta passes into the fetal or maternal circulation without being completely metabolized (Glance et al., 1986; Greystoke et al., 2000). Human placenta also has high PG catabolic activity. PGDH could be localized in placenta as early as 7 to 8 weeks of gestation (Greystoke et al., 2000; Hansen, 1976; Jarabak, 1972; Keirse et al., 1976; Kinoshita et al., 1980; Tai et al., 1985). The rate of PG metabolism in the human placenta exceeds the rate of PG synthesis (Keirse et al., 1985). PGDH action in placenta has been proposed as a barrier that prevents the PG transfer between the maternal and fetal circulation (Greystoke et al., 2000). No significant difference in PGDH levels in relation to labor was reported (Harper et al., 1983; Van Meir et al., 1997).

I-4.3 Prostaglandins and Preeclampsia

Preeclampsia is a pregnancy specific syndrome that occurs only in the presence of placenta. Even though it affects 5-10% of all pregnancies, preeclampsia is still considered among the leading causes of maternal morbidity and mortality in developed countries. It is diagnosed by hypertension, proteinuria and edema during the third trimester of pregnancy (Redman and Roberts, 1993). The pathogenesis of preeclampsia is not clearly understood yet, but histological evidence from intrauterine tissues and prompt resolution following the removal of placenta designate the central role of placenta in this syndrome.

Placentation occuring from weeks 6-18 of pregnancy involves the critical process of cytotrophoblast invasion of the placental bed (Fisher et al, 2004). In normal placentation, the cytotrophoblasts penetrate deep into the myometrium to infiltrate the spiral arteries which are transformed into conduits to supply blood flow to the placenta. Later, the endothelium is replaced by trophoblast cells. In preeclampsia, cytotrophoblast invasion is abnormally shallow and the spiral arteries keep their endothelial lining and remain narrow (Van Wijk et al, 2000). Smaller spiral arteries cause poor blood supply to the placenta.

The contribution of prostaglandins to the pathogenesis of preeclampsia has been proposed as early as 1970s when Demers and Gabbe measured reduced levels of PGE_2 and elevated levels of $PGF_{2\alpha}$ in the placental tissue of preeclampsia compared to normal

placentas (Demers and Gabbe, 1976). Inhibition of prostaglandin synthesis caused a rise in diastolic blood pressure hence imitated the increased vascular responsiveness of preeclampsia (Everett et al., 1978). Even though subsequent studies failed to report consistent results regarding the amount of prostaglandins (Hillier and Smith, 1981; Pedersen et al., 1983; Robinson et al., 1979; Valenzuela et al., 1983) inhibition of prostaglandin synthesis with low-dose aspirin has been proposed as an effective preventive treatment for preeclampsia (Heyborne, 2000; Walsh, 1990). Currently there is no information regarding the expression or regulation of PG receptors during preeclampsia.

I-5 Local Feed-forward Cascades in Human Intrauterine Tissues

Many studies show that birth in the human is an integrated series of autocrine-paracrine loops which results from processes leading to increased PG output. Cortisol has a central role in those processes. Human placental CRH output rises progressively throughout pregnancy and it is elevated paradoxically by cortisol. Cortisol is locally produced in amnion epithelium and chorion trophoblast cells by 11β-HSD type 1 activity that increases with labor (Alfaidy et al., 2003; Alfaidy et al., 2001; Sun et al., 1997; Sun et al., 1997). While cortisol and CRH seem to be involved in a feed-forward endocrine cascade, they are also involved in the local pathways that affect the levels of PGs. Increased CRH levels upregulate PGHS-2 and downregulate PGDH (Challis et al., 2002). Cortisol also promotes increased PG levels. In amnion mesenchyme, PGE_2 production is increased by cortisol, mainly through induction of cPLA(2) and PGHS-2 expression (Sun et al., 2003). In trophoblast cells, cortisol decreases the PGDH activity (Patel et al., 1999). In turn, PGE_2 and $PGF_{2\alpha}$ increase the activity of 11β-HSD1 which potentially increases the bioactive cortisol levels. Moreover, FP receptor subtype was involved in this local regulation (Alfaidy et al., 2001).

Over the years, animals, especially sheep, have been successfully used as a model in order to enhance our understanding of parturition. However, there is a crucial difference that limits the application of those models to human. In sheep placenta, activated HPA axis results in elevated levels of PGE_2 via cortisol (Challis et al., 2002; Challis et al., 2000). Towards term, this PGE_2 activates P450-C17 hydroxylase enzyme

that converts pregnenolone to estrogen, thus causing a progesterone withdrawal. P450-C17 hydroxylase enzyme was not found in human placenta and human parturition is not associated with a progesterone withdrawal (Challis et al., 2000). However, recent studies suggest a functional withdrawal of progesterone which involves PG-progesterone interactions (Condon et al., 2003; Dong et al., 2004). Progesterone maintains the PGDH activity, while PGHS-2 may be involved in inhibiting progesterone production (Narayansingh et al., 2004; Narayansingh et al., 2004). PGs can also differentially regulate the expression of progesterone receptors, and thus contribute to the activity or inactivity of progesterone.

Term parturition may represent a modest inflammatory response. Several studies have shown that increased intrauterine cytokine production is associated with both term and preterm labor (Dudley, 1997; Gomez et al., 1997). Gestational tissues are important sources of cytokines (Bowen et al., 2002). The levels of intrauterine cytokines, in particular IL-1β and TNFα, increased with preterm labor, term labor and preterm deliveries that are complicated with infection (Dudley et al., 1996; Dudley et al., 1996; Gunn et al., 1996; Opsjon et al., 1995; Romero et al., 1989; Romero and Mazor, 1988; Romero et al., 1988; Romero et al., 1990; Romero et al., 1992; Romero et al., 1993). IL-1β and TNFα act at multiple points of the prostanoid biosynthetic pathway. Production of PGs in amnion (Bry and Hallman, 1992; Romero et al., 1989), chorion (Lundin-Schiller and Mitchell, 1991), decidua (Mitchell et al., 1990), and myometrium (Hertelendy et al., 1993; Pollard and Mitchell, 1996) is enhanced by IL-1β and TNFα stimulation. Conversely, PGDH expression and activity is decreased by IL-1β and TNFα in fetal membranes and cultured chorionic trophoblast cells (Brown et al., 1998; Mitchell et al., 2000; Pomini et al., 1999).

In addition to proinflammatory cytokines, gestational tissues produce anti-inflammatory cytokines as well such as IL-10 (Cassatella et al., 1993; Denison et al., 1998; Trautman et al., 1997). In the term placenta IL-10 levels decline prior to labor and remain low through labor (Hanna et al., 2000). IL-10 inhibits both cytokine and PG production within the human chorion, decidual and placental cells in vitro (Barsig et al., 1995; Fortunato et al., 1997; Trautman et al., 1996). The effect of TNFα on PGDH mRNA levels could be reversed by IL-10 (Pomini et al., 1999).

All these hormones, PGs and cytokines work in a harmony for the maintenance of uterine quiescence throughout the pregnancy and mediate normal parturition at term. All these factors have a variety of different effects within the intrauterine tissues. These effects include stimulation of ECM degradation, programmed cell death, upregulation of steroidogenic enzymes. In conclusion, human birth involves a series of positive feed-forward cascades involving cytokines, prostaglandins and steroid hormones (Challis et al, 2005).

I-6 The Role of Prostaglandin Receptors During Pregnancy and Parturition

PG receptors are expressed both in pregnant and non-pregnant human myometrium (Senior et al., 1991; Senior et al., 1993; Senior et al., 1992). However, the expression of PG receptors varies considerably. The FP, EP2 and EP3 receptor mRNA levels were found to be low in pregnant uterus compared to non-pregnant uterus (Matsumoto et al., 1997). Uterine FP mRNA expression decreased significantly with gestational age in patients not in labor and at term increased significantly with labor (Brodt-Eppley and Myatt, 1999). These data also explain the in vitro human myometrial contractile data. The administration of $PGF_{2\alpha}$ or PGE_2 generated a much stronger response in non-pregnant myometrium compared to pregnant myometrium suggesting that the number of contractile receptors is reduced during pregnancy for the maintenance of uterine quiescence (Senior et al., 1991; Senior et al., 1993; Senior et al., 1992).

Conversely, labor in the pregnant baboon myometrium was associated with a reduction in relaxatory EP_2 receptor mRNA (Smith et al., 1998; Smith et al., 2001). In the myometrium of pregnant rats, expression of FP mRNA increased significantly from late gestation until delivery, while the levels of the relaxatory EP_2 receptor were the inverse to those of FP (Brodt-Eppley and Myatt, 1998). In one exception, rat myometrial FP mRNA levels were higher on the 18^{th} gestational day than in non-pregnant tissues, but similar to high term levels raising the possibility of initiated labour-associated changes (Dong and Yallampalli, 2000). The myometrium of the sheep displays a significant increase in the mRNA expression of PG receptors including EP2 with the onset of labour at term. The elevated levels of EP2 may be important for passage of the fetus (Ma et al., 1999).

The maintenance of pregnancy is probably related to an increase in relaxatory EP receptor (Senior et al., 1993) and increased coupling of $G_{\alpha s}$ to adenylate cyclase (Europe-Finner et al., 1994). There is an increase in EP2 mRNA levels in the myometrium of women late in gestation and not in labor compared to women at term not in labor (Brodt-Eppley and Myatt, 1999). Data from pregnant baboons (Smith et al., 2001; Smith et al., 2001) and mice (Docherty, Sathananthan et al, 1999) support these findings. Thus, myometrial quiescence may change to an active contractile state in concert with an up-regulation of contractile receptors and a loss of relaxatory receptors.

The regional expression of receptors within the uterus may also be important. Contraction-promoting EP1 and EP3 receptors are more abundant in the fundus, whereas relaxation-promoting EP2 and EP4 receptors are found in the lower uterine segment (Smith et al., 2001). All the receptors work towards allowing the passage of the fetal head by the relaxation of lower part and concurrent contraction of the upper part resulting in the expulsion of fetus. However, the lower segment responds by contraction to $PGF_{2\alpha}$ challenge and lower doses of PGE_2 in in vitro studies (Senior et al., 1993). The concept of functional regionalization has recently gained credence but should be definitively proven.

The relative levels of PG receptors in human myometrium at preterm have been studied as well (Europe-Finner et al., 1994). FP mRNA levels from lower uterine segment myometrium were higher at preterm birth with or without labor than at term without labour. The EP_2 mRNA levels were highest at preterm not in labour.

I-6.1 Prostaglandin Receptors in Fetal Membranes and Placenta

The existence of PG receptors within the intrauterine tissues was first reported some 20 years ago by Fukai et al (Fukai et al., 1984). In this study the existence of FP in human amnion and decidua was shown and it has been reported that the binding of FP did not change during pregnancy. Spaziani and colleagues have shown the expression of EP1 receptor subtype in the amnion (Spaziani et al., 1999). PGE_2 downregulated the EP1 subtype in the amnion, whereas IL-1β stimulated the receptor levels. The histochemical localization of EP1 within the amnion cells revealed a cytoplasmic identity and a possibility of plasma membrane localization (Gould et al., 1999). The same group studied

the expression of PG receptors within an immortalized human cell line, amnion derived WISH cells (Spaziani et al., 1998; Spaziani et al., 2000; Spaziani et al., 1997). In the WISH cells IL-1β and IL-4 increased the EP1 but not EP3 receptor levels (Spaziani et al., 1997). EP1 receptor protein and mRNA expression was also elevated with increasing concentrations of TNFα and CRH (Spaziani et al., 1998; Spaziani et al., 2000).

The expression of different PG receptors subtypes in gestational tissues was proposed when exogenous PGE_2 had different functional effects on placental and uterine blood vessels (Sastry et al., 1997). When exogenous PGE_2 was administered, a vasoconstriction in placental vessels and vasodilation in uterine blood vessels was observed suggesting the activation of two different subtypes of PG receptors. Human placental EP2 receptor subtype was characterized (Smock et al., 1999). Human FP receptor mRNA was also shown in placenta with Northern blot analysis (Vielhauer et al., 2004). In sheep placenta EPs and FP receptor expression were shown with a marked increase in EP2 and EP3 receptor subtypes with the onset of labor (Palliser et al., 2005).

The expression of PG receptors in the fetal membranes was demonstrated also in pregnant baboon (Smith et al., 1998; Smith et al., 2001). EP2, EP3 and EP4 mRNA were all detected in the decidua and chorion of baboon (Smith et al., 1998). Labor associated changes were observed in the receptor levels. EP1 and FP mRNA levels in decidua and EP4 mRNA in chorion was lower with advancing gestational age. Expression of EP2 receptor gene in decidua was lower in labor. It has been concluded that the complex pattern of change in the expression of PG receptors in chorion and decidua with advancing gestational age and in association with labor might have an important role in primates during parturition.

There are a limited number of studies about the function of PG receptors in the intrauterine tissues. Alfaidy et al (2003) showed that PGs can act locally in the placenta and fetal membranes and regulate cortisol production at term (Alfaidy et al., 2001). In placenta, PGE_2 inhibited 11-β hydroxysteroid dehydrogenase type 2 (11β-HSD2) which is responsible for cortisol metabolism. The inhibitor effect of prostaglandins, PGE_2 and $PGF_{2\alpha}$, was observed in human choriocarcinoma cell line, JEG-3 cells as well (Hardy et al., 1999). Interestingly, in the chorion trophoblast cells, both PGE_2 and $PGF_{2\alpha}$ activated the 11β-HSD1, the enzyme responsible for cortisol production from cortisone (Alfaidy et

al., 2001). At least FP receptor subtype was involved in this regulation, although the exact mechanism is still unknown.

The roles of PGE_2 and PGE_2 mediated signaling was tested in the migration of extravillous trophoblast cells (Nicola et al., 2005). Using different EP receptor agonist and antagonists, migration assays and measurements of intracellular calcium and calpain activity were performed. A functional predominance for EP1 and EP4 receptors were demonstrated in migration assays. PGE_2 was shown to stimulate the EVT migration by signaling through EP1 receptors and activating calpain, a group of Ca^{2+}- dependent proteases.

Placental tissues were shown to express both forms of DP receptor, DP1 and DP2, identified to date (Helliwell et al., 2006). Placental PGD_2 production was stimulated following inflammatory activation and this PGD_2 has a potent inhibitory effect on placental cytokine production. The locally produced PGD_2 and its receptors have been implicated in immunoregulation, feto-placental communication and possibly in the regulation of parturition (Helliwell et al., 2006).

I-6.2 Mechanisms Regulating the PG Receptors

The chromosomal locations of prostaglandin receptor genes have been mapped in human, but there is currently little data available on isolation and characterization of the promoter regions (Duncan et al., 1995). 5'-flanking region and the first intron of prostanoid receptor genes contain several consensus sequences in the *cis*-acting regulatory elements (Narumiya et al., 1999). Basal promoter motifs for transcription have been identified in the 5'-flanking region of the transcription initiation site of some of the prostanoid receptor genes. The promotor regions of the human EP3, and the human EP_4 receptor genes have a TATA-like box, and two CCAAT boxes, respectively. There is an AP-1 site and an AP-2 site in the mouse EP1 receptor gene (Batshake et al., 1995). Human EP3 receptor gene contains a *sis*-inducible factor (SIF) binding element, E boxes, AP-2 sites, an interferon (IFN)-γ responsive element (γ-IRE), a c-Myb, and a GC box (Kotani et al., 1997). Several responsive motifs were identified for proinflammatory agents such as NF-IL6, NFκB, and H-apf-1 in addition to a Y box, AP-1 sites, and AP-2 sites in the human EP_4 receptor gene (Foord et al., 1996).

The FP gene has been sequenced from human (Abramovitz et al., 1994), bovine (Sakamoto et al., 2002), mouse (Sugimoto et al., 1994), and sheep (Graves et al., 1995). The FP gene is approximately 10 kb in length and is composed of three exons separated by two introns. Exon 1 is noncoding, the translation initiation start site is located in exon 2, and exon 3 contains large regions of noncoding sequences. Two isoforms of ovine FP were reported, FP_A and FP_B, arise from alternative splicing within exon 3 (Pierce et al., 1997). Although the gene structure is known the FP promoter has not been well characterized. The DNA sequence from the 5′ flanking region of the human (Betz et al., 1999), mouse (Hasumoto et al., 1997), bovine (Ezashi et al., 1997), and rat (Neuschafer-Rube et al., 2000) FP gene has been described. In bovine FP gene, two promoter regions were defined, promoter region A and B (Ezashi et al., 1997) with several potential binding sites for transcription factors in particular within promoter B. Recently, several AP-1 sites, a STAT-1 site, a potential estrogen response element (ERE), a progesterone response element (PRE), an NFκB site were identified in human FP gene (Zaragoza et al., 2004). Since cytokines and steroids directly and indirectly interact with these sites they can be considered as potential regulators of FP expression at term and preterm. When the human FP full-length promoter was coupled to a green fluorescent protein reporter and transfected into HeLa cells a repressor region and an enhancer region were revealed (Zaragoza et al., 2004) providing a molecular basis for the regulation of FP receptor during pregnancy and parturition.

The mouse EP_2 receptor gene is composed of two exons and one intron, and approximately 16 kb in length (Katsuyama et al., 1998). Exon 1 contains a 5′ untranslated region and the coding region for the transmembrane domain. Exon 2 encodes the residual part of the coding region and the 3′ untranslated region. Interestingly, uterine EP2 mRNA was found to have longer 5'-untranslated region than the macrophage EP2 transcript suggesting a specificity of expression or regulation of translation in the uterus. A potential progesterone response element (PRE) was found upstream of the transcription start site in the 5′-flanking sequence (Tsuchiya et al., 2003). The 2 kb segment containing the immediate 5′ flanking and 5′ non-coding regions contain consensus sequences for the NF-kappaB binding site, NF-IL6 binding site, AP-2, AP-4, and a cAMP response element and the progesterone response element (Katsuyama et al., 1998).

These reported motifs are well correlated with the results that the PG receptor genes are regulated by both hormonal and proinflammatory stimuli. It has been reported that increased myometrial expression of FP receptor mRNA levels during term and preterm labor was associated with progesterone withdrawal (Ou et al., 2000). Other studies with rats have also shown that steroids regulate the expression of PG receptors (Dong and Yallampalli, 2000). Administration of the progesterone receptor antagonist, RU486, increased FP and decreased EP_2 mRNA. While antagonism of estrogen action decreased FP mRNA, administration of estradiol enhanced FP expression. Progesterone administration alone promoted EP_2 expression, in accordance with the defined PRE within the promoter region. Estradiol potentiated the effect of progesterone.

In different models, LPS and pro-inflammatory cytokines induced the expression of EP2, EP4 and EP3, respectively (Harizi et al., 2003; Spaziani et al., 1999; Spaziani et al., 1997). IL-1β (10 ng/mL for 24 hours) stimulated FP mRNA expression also in human granulosa-luteal cells (Narko et al., 1997). Further studies demonstrating that PGs in turn can regulate the cytokine levels via cAMP linked receptors suggest a local feed-forward cascade. The production of TNFα, IL-6, IL-12 was decreased via EP2, while PGE_2 increased the release of IL-10 and IL-6 through EP2 and EP1, EP3, respectively (Gomi et al., 2000; Jozefowski et al., 2003).

Another potential regulator of prostaglandin receptors' expression is their own ligands. Both exogenous and endogenous $PGF_{2\alpha}$ decreased the number of $PGF_{2\alpha}$ binding sites in rat myometrium, suggesting ligand-induced receptor down regulation (Molnar and Hertelendy, 1990). $PGF_{2\alpha}$ also induced the internalization of FP_A in HEK-293 cells (Srinivasan et al., 2002).

I-6.3 FP receptor antagonists as a new therapeutic agent

Recently, a new G-protein–coupled receptor antagonist was developed that inhibits $PGF_{2\alpha}$-stimulated contractile activity (Peri et al., 2002). THG113.31 is a specific, noncompetitive, reversible octapeptide inhibitor of the FP receptor that blocks the interaction of the receptor with $G_{\alpha q}$, thereby inhibiting further signal transduction and ultimately preventing the increase in intracellular $[Ca^{2+}]$. Only 1% or less of iodinated

antagonist crosses the mouse placenta (Olson, 2005). THG113 was effective in delaying LPS induced (50 μg, intraperitoneally, twice at 3-hour intervals) preterm birth on gestational day 16 in pregnant mice (Peri et al., 2002). THG113 (10 μmol/kg bolus followed by 0.8 μmol/kg/h) given 4 to 6 hours after administration of LPS delayed preterm birth by more than 40 hours in LPS-treated dams. Fetuses from THG113-treated dams were born alive with higher birth weights than control-treated dams and appeared healthy. THG113.31 was also effective on delaying RU486-induced preterm labor and delivery in pregnant sheep (Hirst et al., 2005). The time to delivery after RU486 was extended in the THG113.31-treated ewes, an average delay of 7.1 hours (Hirst et al., 2005). However, results with human tissues were not as consistent. One group have reported that THG113.31 exerted a significant relaxant effect on human spontaneous and oxytocin-induced contractility but did not alter $PGF_{2\alpha}$-elicited contractility raising questions about the exact mechanism of THG113.31 (Friel et al., 2005).

Genotypes	Phenotypes
FP	Loss of parturition due to disturbed induction of luteolysis
EP1	Loss of LPS induced HPA axis function
EP2	Impaired ovulation and fertilization Vasopressor or impaired vasodepressor response to intravenous PGE_2 Loss of bronchodilation with PGE_2
EP3	Development of tumor via impaired apoptosis/ proliferation Vasopressor response to intravenous PGE_2 Impaired febrile response to pyrogens
EP4	Patent ductus arteriosus Impaired vasodepressor response to intravenous infusion of PGE_2 Loss of bronchodilation with PGE_2

Table I-1. Roles of prostaglandin receptors revealed from studies using mice lacking specific prostaglandin receptors. FP receptor subtype mediates the action of $PGF_{2\alpha}$, while the action of PGE_2 is mediated via four distinc receptor subtypes (EP1-4). [Modified from Ushikubi et al, 2000]

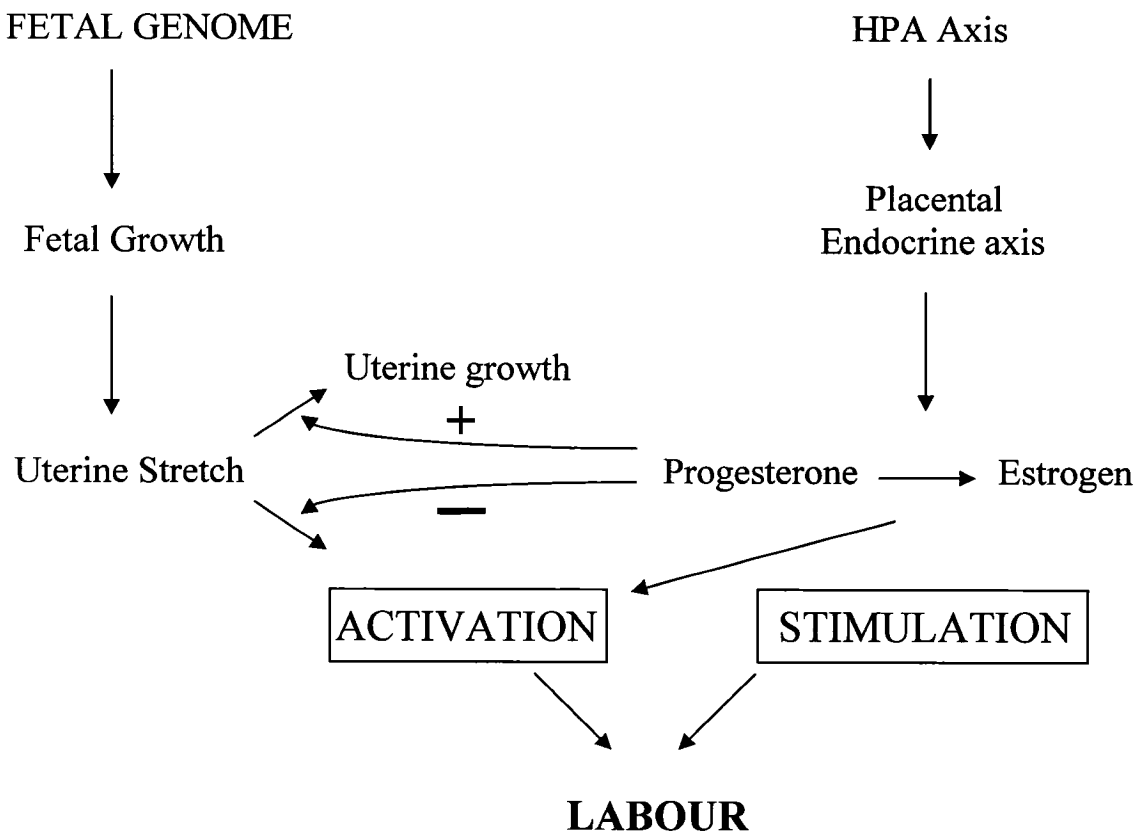


Figure I-1. Activation and stimulation of labor via two interdependent pathways. [Modified from Challis et al, 2000]

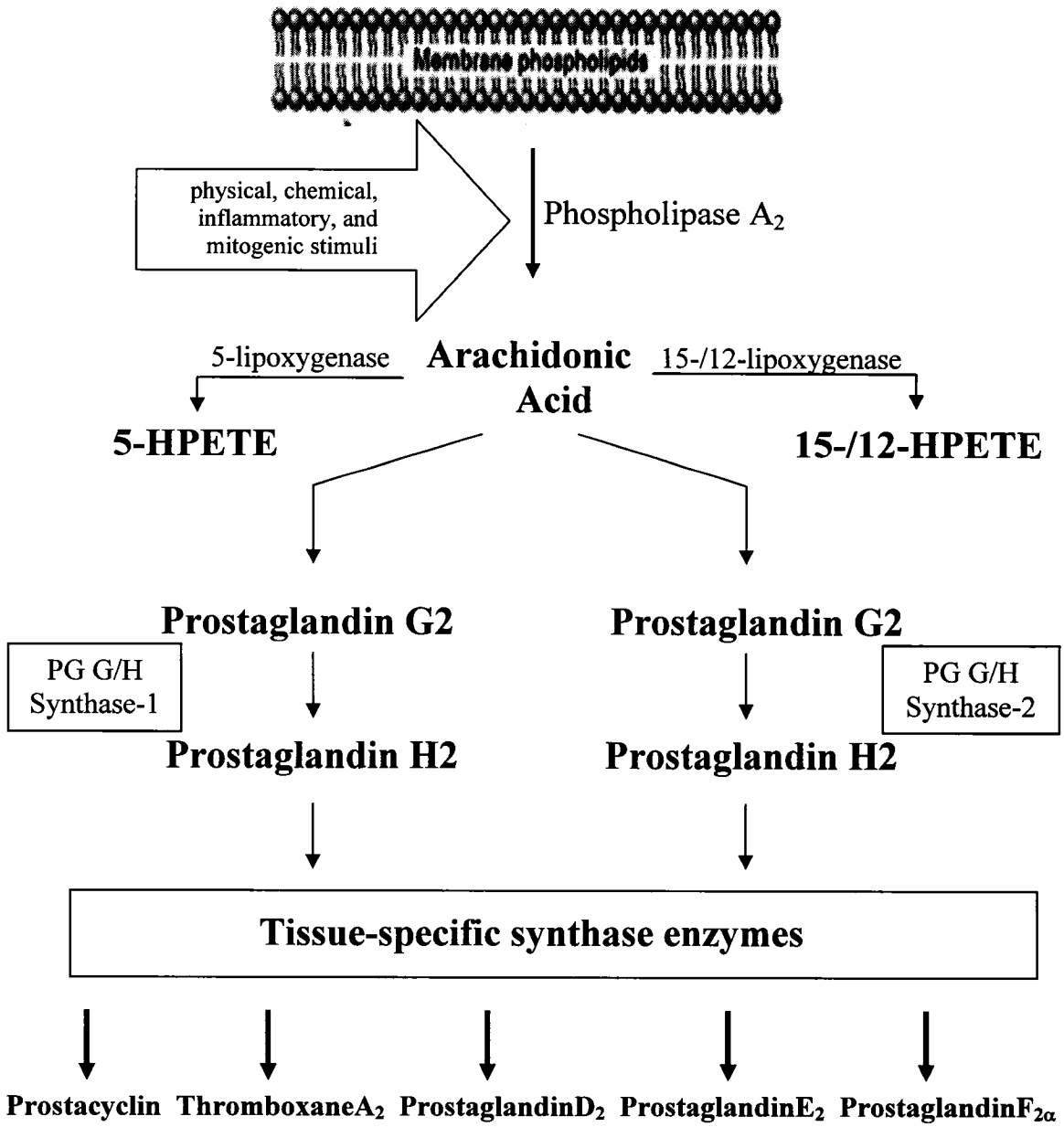


Figure I-2. Diagrammatic representation of enzymatic synthesis of primary prostaglandins and their major relatives 5-HPETE and 15-/12-HPETE. [Modified from Fitzgerald et al, 2000]

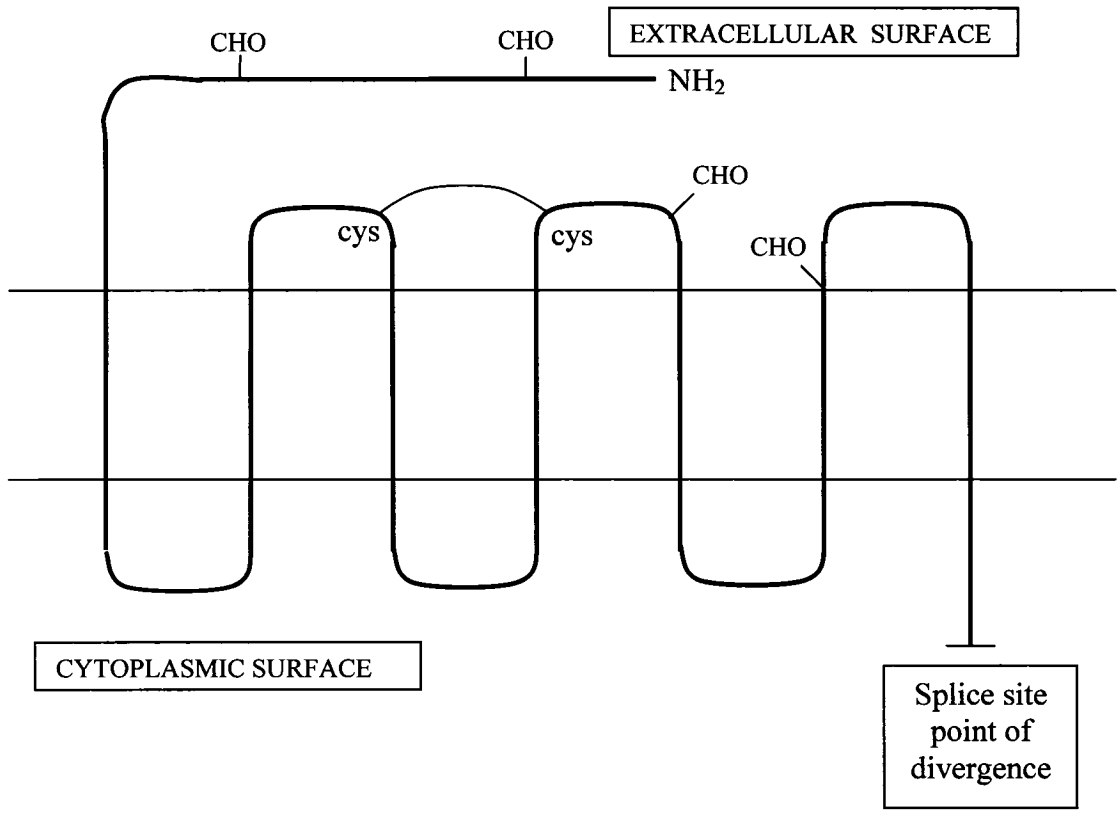


Figure I-3. The structure of G-protein coupled prostaglandin receptors. PG receptors consist of an extracellular amino terminus, an intracellular carboxy terminus and seven transmembrane and seven segments of transmembrane domains. [Modified from Narumiya et al, 1999]

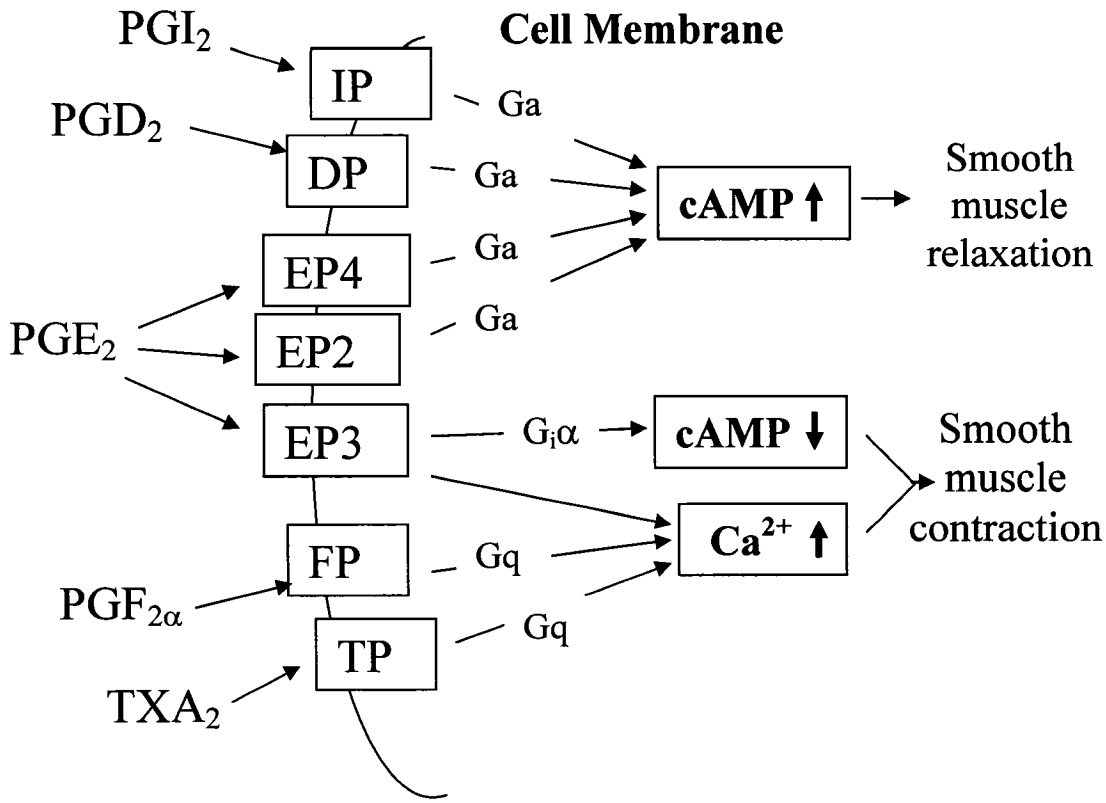


Figure I-4. Diagrammatic representation of membrane prostaglandin receptors and the principal secondary messenger pathways they activate. [Modified from Myatt and Lye, 2004]

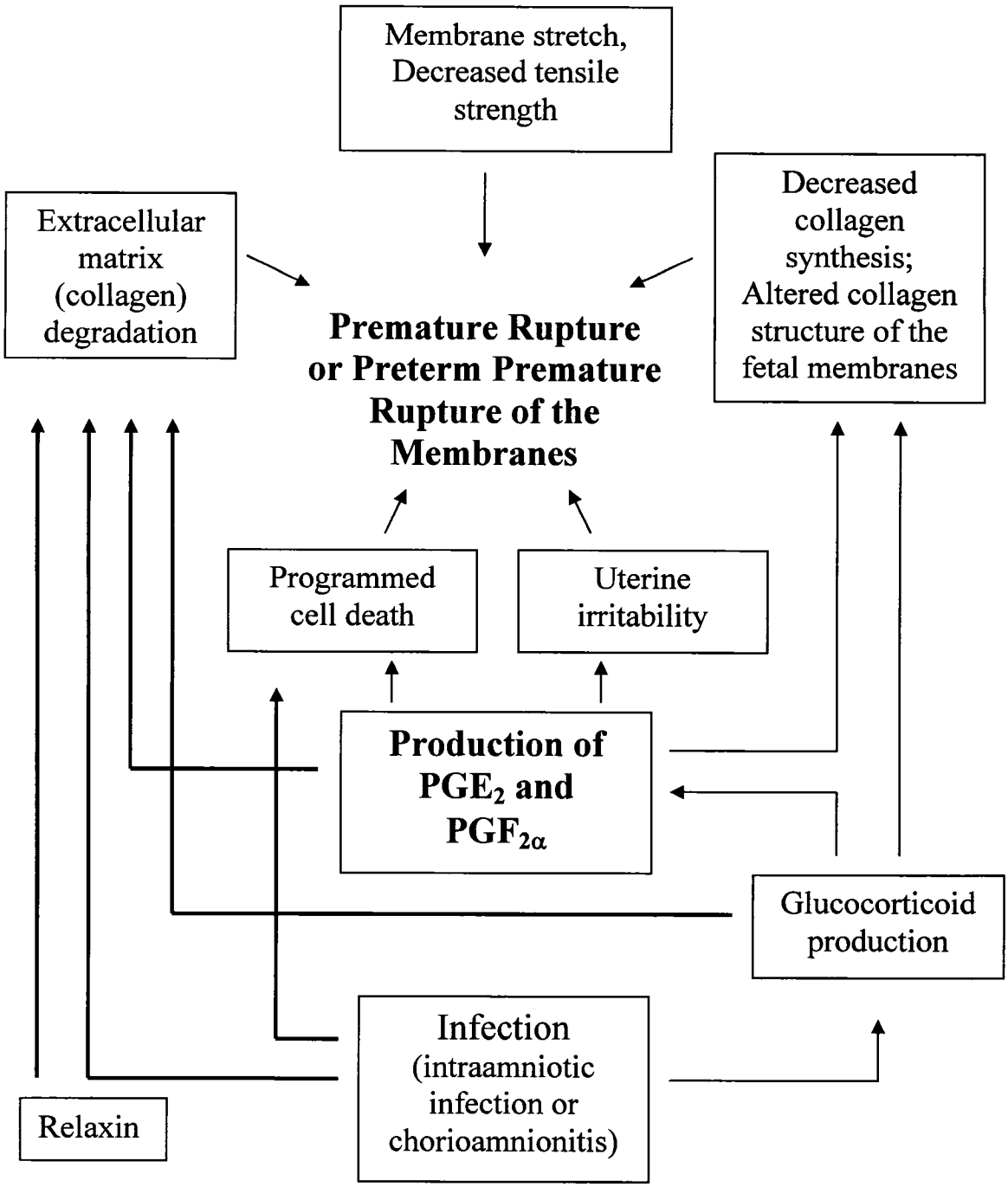
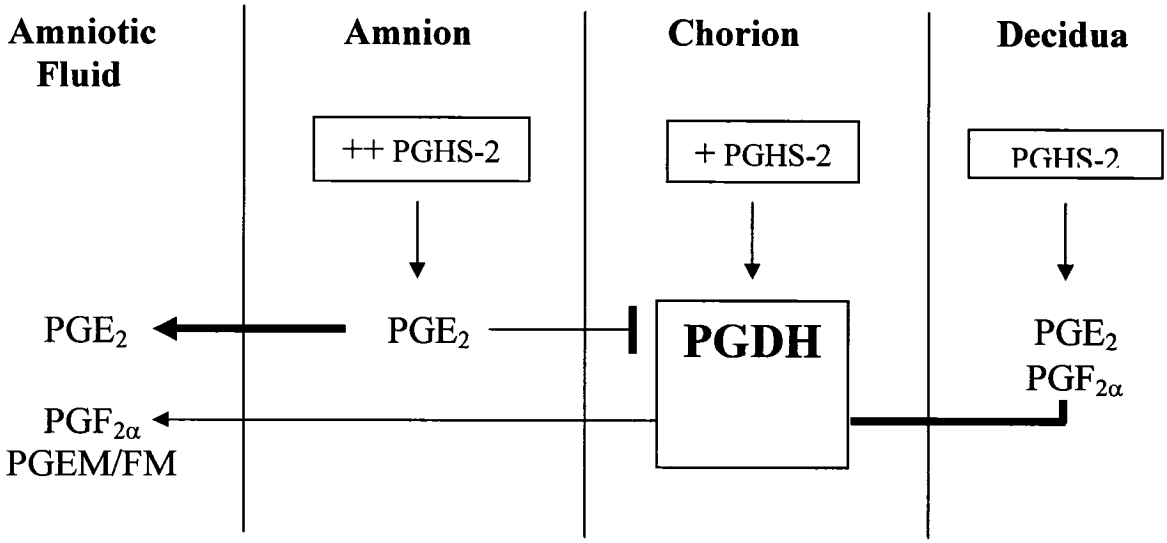


Figure I-5. Diagrammatic representation of the factors leading towards the premature rupture or preterm premature rupture of the membranes. [modified from Parry et al, 1998]

A. Term Labor



B. Preterm Labor

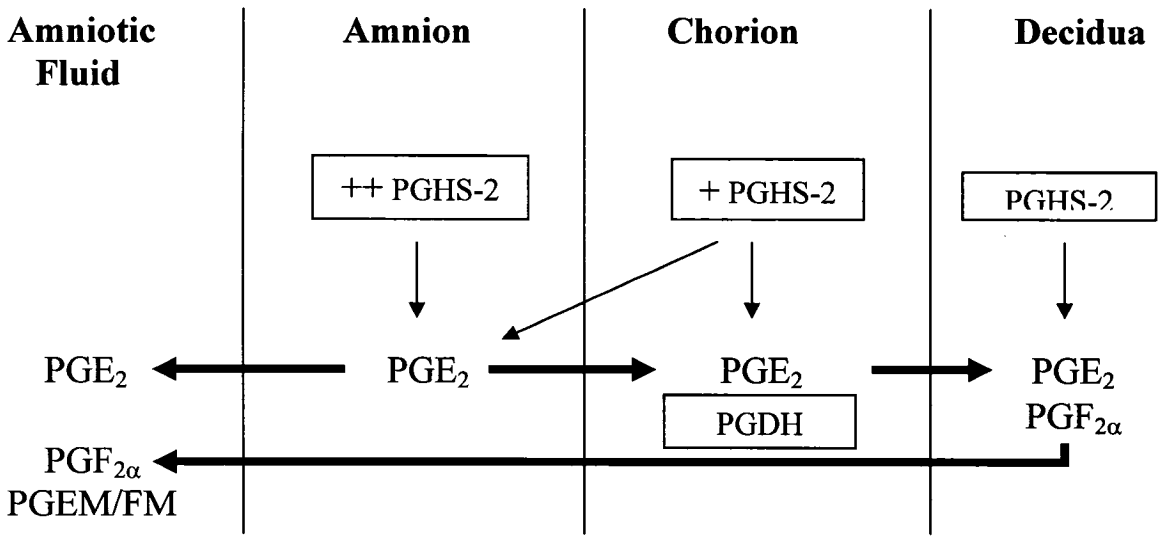


Figure I-6. Compartmentalization of prostaglandin synthesis and metabolism within the human fetal membranes and decidua et term and preterm labor. PGDH (prostaglandin dehydrogenase); PGHS-2 (prostaglandin H synthase type 2). [Modified from Challis et al, 2000]

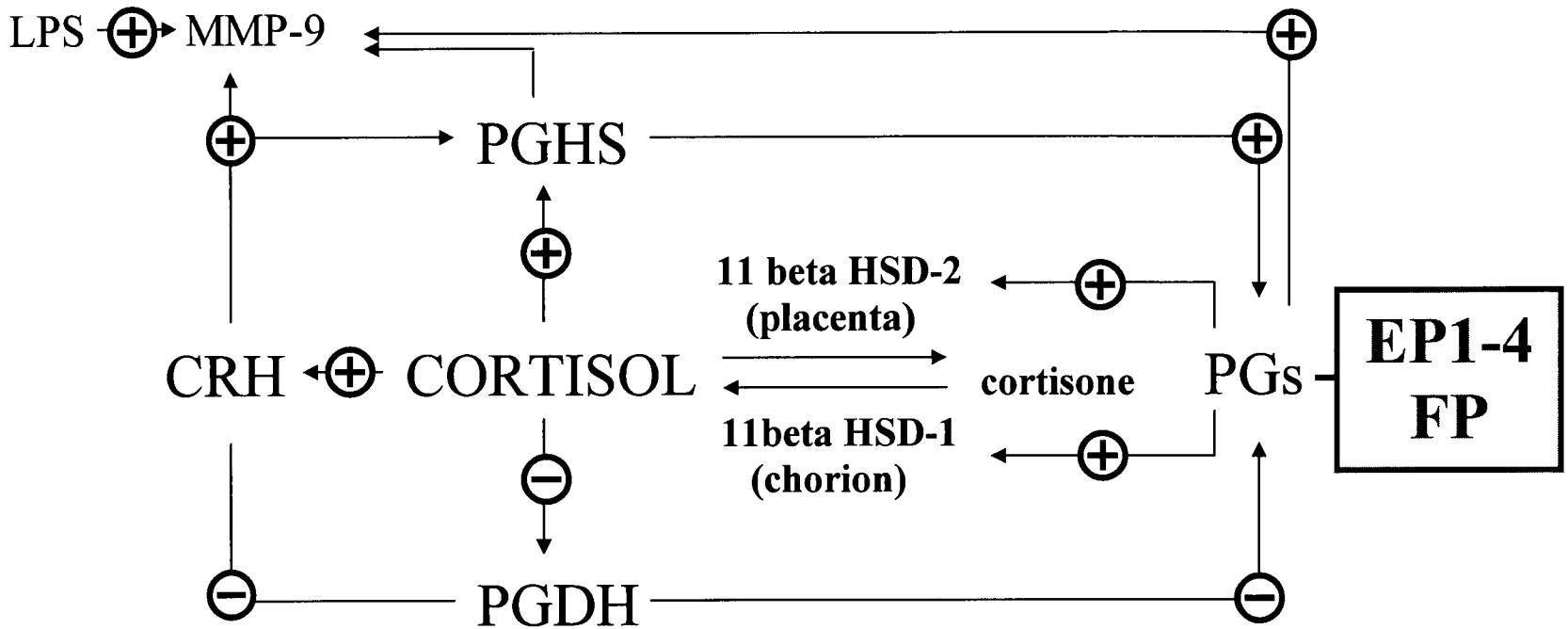


Figure I-7. Local paracrine interactions in human intraueterine tissues. [Modified from Challis et al, 2005]

CHAPTER II

Rationale, Hypothesis, and Specific Aims

Rationale, Hypothesis, and Specific Aims

II-1 Rationale and Hypothesis

Preterm birth remains the major problem of obstetrics. Therefore, understanding the mechanisms involved in parturition is of a great importance. Thus, the long-term objectives of this research program are to define the mechanisms that control local hormonal regulation during pregnancy and to use this information to develop effective treatments for preterm birth to improve the outcome of neonates. The short-term objective of this study is to gain a better understanding of the local expression and regulation of prostaglandin receptors within human fetal membranes and placenta.

Regulation of birth at term and preterm involves complex endocrine, paracrine and autocrine interactions between fetus, placenta and mother. The activated fetal hypothalamic-pituitary-adrenal (HPA) axis causes increased expression of steroidogenic enzymes in placenta which in turn elevates the output of prostaglandins (PGs). At present, the regulation of locally produced PG actions in the intra-uterine tissues is not clear. These prostaglandins act on their specific receptors (EP1-4 and FP) identified in myometrium causing either relaxation or contraction. In chorion trophoblast cells, FP receptors are instrumental in the regulation of 11β-HSD type 1 activity which increases bioactive cortisol levels in the presence of cortisone. Both PGE_2 and $PGF_{2\alpha}$ increased the activity of 11β-HSD1. Cortisol, on the other hand is known to stimulate PGHS-2 in intrauterine tissues, thus increasing PG output. These results suggest the existence of a locally controlled feed-forward cascade in the fetal membranes and placenta that would result in parturition.

Based on previous studies in the literature we hypothesized that i) PG receptors are expressed in human placenta and fetal membranes and their expression differs among layers of placenta and changes during labor at term and preterm; ii) in fetal membranes proinflammatory cytokines up-regulate the PG receptor levels; iii) in placenta, different oxygen tensions can regulate the receptor levels in a fashion that might contribute to the pathophysiology of preeclampsia.

II-2 Specific Aims

II-2.1 Chapter III: Expression and Regulation of Prostaglandin Receptors in the Human Placenta and Fetal Membranes at Term and Preterm.

In this chapter we hypothesized that PG receptors are expressed in all layers of placenta and in the specialized cells (amnion epithelium, amnion mesenchyme, chorion trophoblast, placental trophoblast) and that their expression would increase at the time of labor, with betamethasone treatment and chorioamnionitis. The specific aims were as follows:

1. To establish the presence of PG receptors (EP1-4 and FP) in human fetal membranes, and placenta at term.
2. To establish the presence of PG receptors in cultured amnion epithelium, amnion mesenchyme, chorion and placental trophoblast cells.
3. To determine the effect of labor on the receptor levels at term.
4. To determine the effect of betamethasone treatment and chorioamnionitis on the receptor protein expression at preterm deliveries.

II-2.2 Chapter IV: Pro-Inflammatory Cytokine Regulation of Prostaglandin Receptors in JEG-3 Cell Line and Human Chorion Torphoblast Cells

In this chapter we hypothesized that the protein levels of PG receptors expressed in human choriocarcinoma cell line, JEG-3, and human chorion trophoblast cells can be up-regulated by pro-inflammatory cytokines. We propose to examine the following:

1. To establish the presence of PG receptors (EP1-4 and FP) in human choriocarcinoma cell line.
2. To determine whether pro-inflammatory cytokines, IL-1β and TNFα affect the protein expression of PG receptors.
3. To determine whether the effect of pro-inflammatory cytokines can be regulated by anti-inflammatory cytokines, meloxicam, and/or NFκB inhibitors.

II-2.3 Chapter V: Protein Levels of Prostaglandin Receptors during Preeclampsia and at Reduced Oxygen Tensions

In this chapter we hypothesized that in the placental trophoblast cells the levels of PG receptors protein can be altered during preeclampsia and regulated by different oxygen tensions. We propose to examine the following:

1. To examine the protein levels of EP1-4 and FP receptor subtypes in the preeclamptic placentas.
2. To determine the effect of different oxygen tensions on the receptor expression.

CHAPTER III

Expression and Regulation of Prostaglandin Receptors in the Human Placenta and Fetal Membranes at Term and Preterm

III-1 Introduction

Prostaglandins (PGs) play a central role in the initiation of the onset of labor at term and preterm by regulating cervical ripening and myometrial contractility in several species, including humans (Challis et al., 1997). Local control of PG synthesis and metabolism occurs in the fetal membranes, decidua, myometrium and placenta (Challis et al., 2002). Increased output of PG, especially PGE_2 and $PGF_{2\alpha}$ at the time of labor results from elevated expression and activity of PGHS-2 in amnion and chorion (Bennett et al., 1992; Economopoulos et al., 1996; Gibb and Sun, 1996; Keirse and Turnbull, 1976; Mitchell et al., 1978; Okazaki et al., 1981; Slater et al., 1998; Slater et al., 1995) and reduced expression and activity of the PG metabolizing enzyme, 15-hydroxprostaglandin dehydrogenase (PGDH) in chorion trophoblasts (Sangha et al., 1994; Van Meir et al., 1997; Van Meir et al., 1996).

PGs act through a family of G protein-coupled receptors (prostaglandin receptors receptors) with 7 transmembrane domains (Coleman et al., 1994). Prostaglandin receptors are classified on the basis of agonist binding; thromboxane to TP, prostacyclin to IP, $PGF_{2\alpha}$ to FP, and PGE_2 to EP. EP receptors are classified further into subtypes, EP1, EP2, EP3 and EP4 depending on the second messenger produced. At least two FP receptor subtypes have been described so far (Coleman et al., 1994). Stimulation of EP1 and FP leads to an increase in intracellular calcium, whereas EP3 stimulation generally leads to an inhibition of adenylate cyclase. Thus signalling through these three receptors can result in smooth muscle contraction. Stimulation of EP2 and EP4 receptors activates adenylate cyclase thereby increasing cAMP, leading to relaxation. It has been suggested that changes in the relative expression of relaxant or contractile PG receptors may participate in both maintenance of uterine quiescence for the majority of gestation and the switch to contractions at the time of delivery (Sugimoto et al., 1997). Prostaglandin E2 therefore has a wide spectrum of physiologic actions depending on the tissue distribution of receptor subtypes. Differential expression of the PG receptors may play an essential role in parturition by allowing PGs to exert their actions in a tissue specific manner.

The myometrium is a major site of PG action during pregnancy and labor leading to modulation of uterine contractility (Novy and Liggins, 1980). PGs can also act locally at

the sites of their synthesis in the placenta and fetal membranes to regulate the activity of key enzymes of glucocorticoid metabolism and the expression of matrix metalloproteinase (MMP) (Alfaidy et al., 2001). The local action of PGs suggests that specific receptors for PGE_2 and $PGF_{2\alpha}$ might be expressed in the placenta and FM.

In this study, we examined the distribution and relative abundance of prostaglandin receptor protein in human placenta, choriodecidua and amnion at term. We further localized prostaglandin receptor expression in amnion epithelium, amnion mesenchyme, chorion trophoblast and placental trophoblast cells. We determined changes in their relative expression at the time of labor in term tissue and the effect of betamethasone treatment and chorioamnionitis in preterm tissue. We hypothesized that EP 1-4 and FP receptors would be expressed in the human placenta and fetal membranes at term and preterm labor. Their expression would increase during labor at term, and with betamethasone treatment and chorioamnionitis at preterm in a fashion that would enhance prostaglandin activity.

III-2 Materials and Methods

III-2.1 Tissue Collection

Patient consent and ethical approval was obtained prior to the onset of the study and tissue collection, according to the guidelines of Mount Sinai Hospital, Toronto, Canada and the University of Toronto. For the first part of this study, placenta (PL) with attached fetal membranes (FM) and decidua (n=12) were collected at term (38 – 41 weeks gestation) from uncomplicated pregnancies after elective cesarean section (ie. term not in labor) (NL) or spontaneous labor (ie. term vaginal delivery) (L). None of the patients had received any prostaglandin synthesis inhibitors or corticosteroids. Placentas collected from normal term (>37 weeks of gestation) pregnancies after elective cesarean delivery (nonlabor, n = 5) were used in *in vitro* studies.

Intrauterine tissues were collected also from preterm deliveries. Whole thickness FMs and PL samples were collected from women giving birth between 28-32 weeks of gestational age at St Joseph's Health Centre, London, Ontario. Three groups were identified; i) Idiopathic preterm labor (PTL) without chorioamnionitis or betamethasone (BM) treatment, (n=9), ii) Idiopathic PTL that received BM with no chorioamnionitis, (n=9), and iii) Pregnancies that were complicated with chorioamnionitis and had no BM. (n=6). Patients who had multiple gestation, preeclampsia, induction of labor, or any other clinical pathology were excluded.

Pieces of intrauterine tissue were fast frozen directly in dry-ice cooled isopentane and stored at –80°C for Western blot analysis. For immunohistochemistry rolls of fetal membranes and placental tissue were fixed in 4% paraformaldehyde and kept in 70% ethanol until embedded in paraffin. All the tissues were sectioned at 5μm on a microtome (Histocut, Reichert-Jung, Cambridge Instruments, and W. Germany), placed on Superfrost Plus slides (Fisher Scientific, USA) and processed as described below.

III-2.2 Cell Purification and Culture

III-2.2.1 Placental and chorionic trophoblast cell cultures.

Placental and chorionic trophoblast cells were prepared using a modification of the method of Kliman *et al.* (Kliman et al., 1986) as described previously (Li et al., 2004). The placental tissue was pooled and digested with 0.125% trypsin (Sigma, St. Louis, MO) and 0.02% DNase I (Sigma) in DMEM (Life Technologies, Inc., Grand Island, NY) three times for 30 min each. The chorion with adherent decidua was peeled away from the amnion and digested three times for 60 min each time with DMEM as above, containing 0.2% collagenase (Sigma). Subsequently, the placental or chorio-decidual cells were loaded onto a 5–75% Percoll (Sigma) gradient at step increments of 5% Percoll and then centrifuged at 37 C at 2500 x *g* for 20 min to separate different cell types. Cytotrophoblasts between the density markers of 1.049 and 1.062 g/ml were collected, and 0.5 ml of 10^6 cells/ml per well (for immunostaining) in eight-well chamber slides (Labtek; Nunc, Naperville, IL) or 8×10^6 cells (for western blot analysis) were plated in 60-mm diameter dishes (Labtek; Nunc, Naperville, IL) in DMEM culture medium containing 10% fetal calf serum (Life Technologies, Inc.). The cells were cultured for 3 days at 37 C in 5% CO_2-95% O_2. Under these conditions, the placental cells aggregate to form a syncytium, whereas the chorionic trophoblast cells form clumps or remain as single cells. The purity of the cell preparation was assessed at the end of each experiment by histochemical staining for cytokeratin, an epithelial cell lineage marker (DAKO Corp., Glostrup, Denmark), or vimentin, a mesenchymal cell lineage marker (DAKO Corp.); cells were counterstained with Carrazzi's hematoxylin.

III-2.2.2 Isolation and culture of amniotic epithelial and mesenchymal cell.

Term human placenta with attached fetal membranes were collected immediately after elective caesarean section (n = 5). The amnion was peeled from the chorion, cut approximately 2 cm from the placenta disk, and washed in PBS (Dulbecco's PBS, pH 7.5; Life Technologies, Inc./BRL, Burlington, Ontario, Canada). The amnion epithelial cells

were isolated as described previously (Li et al., 2004). The whole amnion was cut into five pieces, and the tissues were treated with 0.2% trypsin and incubated at 37 C with shaking. The supernatant of the first time period (15 min) was discarded, and epithelial cells from the second digestion period (20–30 min) were used for cell culture. Subsequently the cells were digested two more times for 30 and 15 minutes, respectively. The remaining tissue was washed and digested further in DMEM containing 1% collagenase (Sigma) for one and a half hour, and mesencyhmal cells were collected. After isolation, both cell types were filtered through 100-μm nylon mesh and were pelleted by centrifugation at 2500 x *g* for 10 min. The pellets were suspended and washed in DMEM medium. Cell suspensions (0.4 ml/well of 10^6 cells/ml for immunostaining and 8 x 10^6 cells for western blot analysis) were plated in eight-well chamber slides and in 60-mm diameter dishes in DMEM medium supplemented with 10% fetal calf serum (Life Technologies, Inc.) and antibiotics (1000 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.23 μg/ml Amphotericin; Sigma). After 2 hours in these conditions the culture medium of amnion mesenchymal cells were changed in order to eliminate the contamination of epithelial cells and then the cells were maintained in culture at 37 C in 5% CO_2-95% O_2. Cultures were immunostained to determine the proportion of cytokeratin- (an epithelial cell marker) or vimentin- (a fibroblast cell marker) positive cells and were counterstained with Carazzi's hematoxylin.

III-2.3 Immunohistochemical (IHC) analysis

Slides were incubated in xylene substitute (ED Diagnostic Systems, NJ, USA) to remove the paraffin and then re-hydrated in a graded series of ethanol dilutions and a final 0.01 M PBS [pH 7.4; 150 mM NaCl, 19 mM Na_2HPO_4; 1.5 mM NaH_2PO_4] wash. Slides were blocked with 3% normal goat serum (NGS) for one hour. Subsequently, they were incubated with rabbit anti-human PG receptor antibodies (EP1, 2, 3, 4 and FP). EP1-4, FP antibodies and their respective blocking peptides were purchased from Cayman Chemical, USA. EP1-4 blocking peptides were derived from human and the specific receptor sequences were as follows: C-terminal amino acids 380-402 (GLTPSAWEASSLRSSRHSGLSHF) for EP1, C-terminal amino acids 335-358 (SLRTQDATQTSCSTQSDASKQADL) for EP2, amino acids 308-327

(NQTSVEHCKTHTEKQKECNF) for EP3 and amino acids 459-488 (GSGRAGPAPKGSSLQVTFPSETLNLSEKCI) for EP4. FP receptor blocking peptide was derived from murine (2-16; SMNSSKQPVSPAAGL). All antibodies were used at 1/100 dilution (10μg/ml) for tissue sections or 1/200 dilution for chamber slides in antibody dilution buffer. The avidin-biotin-peroxidase technique (Vectastain ABC Kit; Vector Laboratories, CA, USA) for immunostaining was utilized with diaminobenzidine (DAB; Sigma Chemical Co., Miss., USA) as the substrate. Slides were counterstained with Carazzi's haemotoxylin followed by dehydration in a graded series of ethanols, cleared in xylene substitute, and mounted with Permount (Fisher Scientific, Fair Lawn, NJ, USA). Negative control sections were treated with PG receptor (EP 1-4, FP) antibodies that had been preabsorbed with the appropriate antigenic peptide (1:5) overnight at 4^0C before application on the tissue sections.

III-2.4 Western Blot Analysis

Frozen amnion, choriodecidua and placental tissues samples were homogenized on ice for 1 min in RIPA lysis buffer [50 nM TrisHCl (pH 7.5), 150 mM NaCl, 1% (wt/vol) sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 100 mM sodium orthovanadate (Sigma), 1% (vol/vol) Triton X-100 (Fisher Chemicals, Fiarlawn, NJ), and Complete MiniEDTA-free protease inhibitors (Roche Molecular Biochemicals; Dorval, Canada). The cells were also collected in the same RIPA lysis buffer containing protease inhibitors. Homogenates and cell suspensions were then centrifuged at 4°C at 15,000 X g for 25 min, and supernatants were collected. Protein concentrations were determined by the Bradford assay as previously described (Bradford, 1976). Proteins (70 μg/well for tissue and 20 μg/well for purified cells) were separated by polyacrylamide gel electrophoresis (12% acrylamide gel), and then transferred electrophoretically to a 0.45 μm-pore nitrocellulose membrane (Bio-Rad Laboratories, Inc.). Transfer was confirmed by protein visualization with Ponceau S (Sigma). Blots were washed with PBS-T [150 mM NaCl, 10 mM Na_2HPO_4, 1.5 mM NaH_2PO_4, and 0.1% Tween-20 (Sigma); pH 7.5] and incubated overnight with blocking solution (3% Goat serum in PBS-T). Subsequently, blots were incubated with primary antibody EP1-4 (1:600 dilution, 5μl/3ml, in blocking solution) or FP (1:200 dilution, 15μl/3ml) for 1 hr. Blots were then rinsed six times for 5 min each with

PBS-T and incubated with secondary rabbit antiserum conjugated with horseradish peroxidase (1:3000 dilution in blocking solution; Amersham Pharmacia Biotech) for 1 hr. Blots were washed six times, 5 min each, and the antibody-antigen complex was detected using the Amersham Pharmacia Biotech ECL detection system (Amersham Pharmacia Biotech). The membranes were then exposed to X-OMAT blue film (Kodak Scientific Imaging Products, Rochester, NY). The intensities of immunoreactive bands were measured by scanning (6200C scanner, Hewlett Packard (Canada) Ltd., Mississauga, Ontario) and analyzing the image on a desktop computer using Scion Image software (v.4.0.2; Scion Corporation, Frederick, MD). Protein bands were digitized and the mean pixel density for each band was analyzed to obtain relative optical density units for each protein. Control blots were treated with PG receptor (EP 1-4 and FP) antibodies that had been preabsorbed with the appropriate antigenic peptide overnight at 4^0C .

III-2.5 Statistical Analysis

Results are expressed as the mean +/- standard error of the mean (SEM). Statistical comparisons were made using one-way ANOVA test. Subsequently, Student t-test (for the term tissue) and Tukey test (for preterm tissue) were used (SigmaStat; Jandel Scientific Software, San Rafael, CA). For all the tests, a value of $P < .05$ was considered statistically significant.

III-3 Results

III-3.1 Prostaglandin receptors in human placenta, choriodecidua and amnion at term

Using western blotting, immunoreactive proteins with molecular weights corresponding to the different prostaglandin receptors were detected in all tissues examined. Placenta, amnion and chorion expressed EP1-4 and FP receptor subtypes in twelve non-labouring patients at term. The apparent molecular weight for the five receptors was as follows: EP1 (42 kDa), EP2 (45 kDa), EP3 (52 kDa), EP4 (52 kDa) and FP (64 kDa). Figures: III-1A, 1B, 1C, and 2A, 2B show representative western blots for each of the four receptors in the three different tissues examined. Immunoreactivity was abolished or reduced when the primary antibodies for EP1, EP2, EP3, EP4 and FP were substituted with antibodies that had been preabsorbed with the antigenic peptide. Densitometric analysis of the signals showed that EP3 proteins were significantly more abundant in the chorion and placenta compared to amnion (Figure III-1B) ($p<0.05$). EP1 levels in amnion and FP levels in chorion were lower than the other tissues compared (Figure III-1A, 1C). There was no significant difference in the level of EP2 expression between the three tissue types (Figure III-2A). The EP4 receptor was more abundant in the placenta compared to the chorion and amnion tissues (Figure III-2B).

Because of the existence of different isoforms for EP3 and FP receptors, due to the alternative mRNA splicing, multiple bands were present on those blots; whereas discrete bands were observed for EP1 and EP2 receptors. The EP4 blot showed a second band around 64 kDa in intrauterine tissues, possibly a splice variant, specific for this tissue. In the placenta and fetal membranes there were distinctive larger molecular size bands for EP3 that were abolished completely by preabsorption, but have not been identified. Finally, two bands in close proximity were detected for FP both of which were considered during the statistical analysis.

III-3.2 Expression of prostaglandin receptors in purified placental trophoblast (PT), chorion trophoblast (CT), amnion mesenchymal (AM) and amnion epithelial (AE) cells.

In order the characterize the cultured cells, chamber slides were immunostained with cytokeratin- (an epithelial cell marker) or vimentin- (a fibroblast cell marker) and were counterstained with Carazzi's hemotoxylin (Figure III-3). Immunohistochemical analysis showed that the separated amniotic epithelial cells were more than 95% cytokeratine positive and vimentin negative. Approximately 90-95% of placental and chorionic cells were cytokeratin positive as expected with a minority of vimentin stained fibroblast contamination. Amnion mesenchymal cells, on the other hand, were vimentin positive and cytokeratin negative.

All EP1-4 and FP receptor subtypes could be localized in the four different types of cells examined. Interestingly the amounts of the proteins used as internal control (β-actin and α-tubulin) were different among the different cell types. Results are represented as raw data, normalized with β-actin, and normalized with α-tubulin. According to raw data EP1 was highest in the AE cells, and unlike whole thickness of choriodecidual layer there was a weak signal in CT cells alone (Figure III-4A). EP3 levels were highest in the AE, and lowest in AM (Figure III-4B). When the cells were purified the highest amount of FP was observed in the Pl and AM layer, and lower levels were found in CT and AE cells (Figure III-4C). EP2 and EP4 levels did not differ among the different cell types (Figure III-5A and 5B).

III-3.3 The effect of labor on the expression of prostaglandin receptors in human intra-uterine tissues.

III-3.3.1 Immunohistochemistry.

Sections from human placenta and fetal membranes were labeled with antibodies to human EP1, EP2, EP3, EP4 and FP receptor subtypes, or with the same antibodies preabsorbed with the peptides against which the antibodies were raised. In the placenta, EP1, EP3 and FP were expressed mainly in fetal blood vessels, both in endothelial and

smooth muscle cells, with a fainter staining in the syncytiotrophoblast (Figure III-6A, 6B, 6G, 6H, 6M, 6N). EP2 was expressed mainly in syncytiotrophoblast with a weak or no detectable staining in the blood vessels (Figure III-6D, 6E) whereas EP4 was essentially absent from trophoblast and present in villous blood vessels (Figure III-6J, 6K).

All receptors were detected in amnion epithelial layer and chorion trophoblast layer of the fetal membranes, but each had different pattern. EP1 (Figure III-7A, 7B) and EP3 (Figure III-7G, 7H) receptors were localized abundantly in the chorion trophoblast layer. No change in EP2 (Figure III-7D, 7E) and EP4 (Figure III-7J, 7K) expression was observed with labor.

III-3.3.2 Western blot analysis of tissue.

The effect of labor on EP1, EP2, EP3, EP4 and FP levels in placenta, chorion and amnion tissue was examined using western blotting analysis on tissues from 12 non-labouring patients and 12 patients in labor (Figure III-8, 9, 10, 11, 12). The results substantiated the information obtained with immunohistochemistry. There was a significant increase in the levels of expression of EP1, EP3 and FP (Figure III- 8, 10, 12) in amnion, chorion and placenta ($p<0.05$) with the onset of labor. There was no significant change in EP2 (Figure III-9) or EP4 (Figure III-11) expression in the chorion, amnion or placenta.

III-3.4 Expression and regulation of prostaglandin receptors during early (28-32 weeks) gestational ages in human intra-uterine tissues.

III-3.4.1 Immunohistochemistry.

Placentas and fetal membranes from idiopathic preterm labor (PTL), PTL that received betamethasone treatment (BM), and preterm labor that were complicated with chorioamnionitis (CHA), but did not receive betamethasone were studied for the localization of prostaglandin receptor expression.

In the placenta, EP1 staining was localized both in syncytial layer and blood vessels of placenta (Figure III-13A, 13C, 13E), but staining was quite weak in all groups. In the fetal membranes all receptors were immunolocalized in PTL (Figure III-13B) and BM (Figure III-13D) groups in the amnion epithelial layer. Fetal membranes from the CHA

group were not intact (Figure III-13F). There was a decreased or no staining in the fetal membranes of placentas complicated with chorioamniontis. EP2 was immunolocalized mainly in the syncytial layer in the BM group (Figure III-14C), but blood vessels also expressed EP2 (Figure III-14A, C). EP3 could be immunolocalized both in syncytial layer and blood vessels of placenta (Figure III-15A, C, and E). There was a distinct staining in the mesenchymal layer of EP3 receptors in BM group (Figure III-15D). In the fetal membranes EP4 was immunolocalized in PTL and BM groups in a relatively diffuse manner (Figure III-16 B, D). FP was immunolocalized both around the blood vessels and in syncytial layer of all groups (Figure III-17A, C, E). In fetal membranes, FP staining could be also localized in amnion and chorion layers (Figure III-17D).

III-3.4.2 Western Blot Analysis.

Whole thickness of fetal membranes was compared in order to determine the protein levels of prostaglandin receptors. In fetal membranes, EP1, EP3 and FP receptor levels were significantly elevated in the BM group compared to PTL group, consistent with the changes observed in tissue due to labor (Figure III-18). EP2 and EP4 levels were not affected by betamethasone treatment (Figure III-19). For EP1, EP3, EP4 and FP receptors there was a reduction in the protein levels in CHA group compared to BM group. In the placenta, however, the receptor levels were consistent among the different patient groups (Figure III-20, 21).

III-4 Discussion

In the present study, I have demonstrated the presence of EP (1-4) and FP in human placenta and fetal membranes at term and preterm. I also showed the expression of EP1-4 and FP proteins in the purified cells of AE, AM, CT and PT. Our results demonstrate that in the same tissues EP1, EP3 and FP are differentially expressed among the different layers of intrauterine tissues. Furthermore these receptor subtypes are localized to blood vessels and syncytiotrophoblast in placenta and to trophoblast and amnion epithelium in the fetal membranes. With labor, the expression of EP1, EP3 and FP increased in placenta and fetal membranes. However EP2 and EP4, while in general localized to similar cell types, exhibited no change with labor, suggesting some specificity to regulation of PG receptors in these tissues at the time of labor.

When the tissue samples from preterm deliveries were examined, betamethasone treatment was found to have effects similar to labor at term on the fetal membranes. EP1, EP3 and FP receptor levels were increased in the fetal membranes from patients that received betamethasone treatment. There was no significant difference in these receptor levels in the placental tissue. EP2 and EP4 receptor levels were not altered after the betamethasone treatment in whole thickness of fetal membranes or placenta. It is interesting to note that the immunostaining for all receptors were generally localized to the amnion epithelial layer rather than the mesenchymal layer. Only a strong EP3 staining was observed in amnion mesenchyme of BM treated group. Since PGHS-2 is mainly localized in the amnion mesenchyme layer, this difference we found in receptor localization suggests that PGs produced in amnion mesenchymal cells would act on their specific receptors that are expressed in amnion epithelial cells. However, the physiological actions of PG receptors expressed in amnion epithelium remains to be elucidated.

Interestingly, all the receptor subtypes were found significantly reduced in fetal membranes that were affected by chorioamnionitis. Not only the receptor levels but also β-actin was consistently lower in CHA group. The reduction in receptor protein levels might in part be explained by the loss of trophoblast cells due to chorioamnionitis. However, further studies are needed to clarify the effect of chorioamnionitis on prostaglandin receptor regulation.

Whereas we found one or two major immunoreactive bands for EP1, EP2 and EP4 by immunoblotting, we detected several immunoreactive bands for EP3 and FP. For the most part these were abolished by preabsorbing the antibody with the specific peptide against which the antibodies have been raised. In our analysis we assessed changes only in expression of the major bands, for insistence 52 kDa for EP3, reported in previous studies. We suspect that the additional bands may represent alternatively processed forms of immunoreactive proteins, but have not yet been characterized.

Recent studies have reported the expression of PG receptors within human placenta and fetal membranes. Based on immunohistochemistry and mRNA evidence Grisby et al have shown the presence of EP receptors in human fetal membranes and placenta in late pregnancy (Grigsby et al., 2005; Grigsby et al., 2006; Grigsby et al., 2006; Grigsby et al., 2005). However, Grisby et al reported that only EP3 is expressed in the syncytiotrophoblast layer (Grigsby et al., 2006). Moreover, they reported no significant difference in the receptor levels associated with labor. These results are somewhat different from what I have observed. I have demonstrated the existence of PG receptors including EP1, EP2 and EP4 in placental tissue and in particular in the purified placental trophoblast cells by Immunohistochemistry and Western Blot Analysis. Immunostaining was diminished with the preabsorption of the primary antibody with the specific antigen indicating the specificity of the antibody used.

Studies with knockout mice have provided strong evidence for an obligatory role of PG receptors during parturition. Mice carrying a null mutation for FP receptors failed to deliver (Sugimoto et al., 1997). More recently, it has been shown that LPS induced preterm labor in mice was delayed when a selective FP antagonist, THG113 was administered concurrently in late pregnancy (Peri et al., 2002).

Although the expression of PG receptors has been studied extensively in the myometrium in relation to the control of contractility (Myatt and Lye, 2004), there is limited information available regarding the expression of these receptors in human PL and FM. In subhuman primates, it is known that PG receptors exhibit temporal and regional variations in the uterus during pregnancy. In the baboon uterus, EP2 is highly expressed in the lower uterine segment compared to the fundus, while EP3 exhibits the opposite pattern with no change in EP4 and FP expression (Smith et al., 2001). Furthermore, in the baboon,

Smith and colleagues have reported EP1, EP2, EP4 and FP expression at the mRNA level in both chorion and decidua, with changes in EP2 expression in the decidua with the onset of labor (Smith et al., 2001). Results from studies with human tissues are generally limited to those obtained from cell lines. The expression of the EP1 and EP3 receptors was demonstrated in amnion WISH cells (Spaziani et al., 2000; Spaziani et al., 1999), but a better understanding of the distribution of EPs in PL and FM is important for an appreciation of the action of PGs in different target tissues. In the present study, specific immunostaining for EP1/EP3 was localized to blood vessels in the placenta, perhaps supporting a role of these receptors in the regulation of placental blood flow. Factors responsible for the regulation of PG receptor expression in these cells remain unknown. LPS and pro-inflammatory cytokines induced the expression of EP2, EP4 and EP3, respectively, in different models (Harizi et al., 2003; Spaziani et al., 1999; Spaziani et al., 1997). If these findings can be extrapolated to primary trophoblast cells, they would suggest that PG receptor expression may be altered in the presence of increased proinflammatory cytokines, for example in the presence of infection, or with non-symptomatic chorioamnionitis, in the circumstance of preterm labor, or labor at term.

In rat myometrium, increased expression of FP receptor mRNA levels during term and preterm labor was associated with progesterone withdrawal (Ou et al., 2000). Furthermore, a novel progesterone-binding sequence was identified in the promoter region of mouse EP2 gene (Tsuchiya et al., 2003) but effects of locally generated progesterone on PG receptor expression have not been reported. In amnion WISH cells, expression of the EP1 receptor was stimulated by corticotrophin-releasing hormone (CRH) (Spaziani et al., 2000) and in choriocarcinoma-derived cells, EP3 and FP expression is increased by TNFα, as indicated above (Unlugedik et al., 2004).

The primary prostaglandins PGE_2 and $PGF_{2\alpha}$ can act locally in the chorion trophoblast or amnion, at or adjacent to their sites of synthesis, to regulate glucocorticoid metabolism at term (Alfaidy et al., 2001). We showed that $PGF_{2\alpha}$ and PGE_2 both increased the activity of 11β–HSD1 in chorion trophoblasts, favoring cortisol synthesis locally within these cells. Moreover, Hardy et al. (1999) have shown that PGE_2 reduces the activity of 11β-HSD2 in JEG-3 cells thereby reducing cortisol metabolism in these cells (Hardy et al., 1999). These findings suggest that the effects of PGs on cortisol synthesis and metabolism

may depend on the specific expression of PG receptors in discrete tissue types at term. Because cortisol increases prostaglandin output in these tissues by increasing PGHS-2 and decreasing PGDH expression and activities, it would appear that EP receptors play a central role in regulating this positive cascade that potentially contributes to the labor process.

During parturition, there is also an ongoing degradation and remodeling of extracellular matrix (ECM), which may be orchestrated by the proteolytic enzymes, matrix metalloproteinases (MMPs), interacting with tissue inhibitors of MMP (TIMPs). MMPs are well known to facilitate membrane degradation at both preterm and term (Li et al., 2004). $PGF_{2\alpha}$ increased activity of MMP-2 and -9 (Ulug et al., 2001), in the rhesus monkey (Vadillo-Ortega et al., 2002). We have reported recently that LPS-induced release of MMP-9 in placental and chorion trophoblast cells is inhibited by meloxicam, a relatively specific inhibitor of prostaglandin synthase -2 (PGHS-2) (Li et al., 2004; Li et al., 2004). This effect of meloxicam can be overcome in a dose–dependant manner by PGE_2 and by $PGF_{2\alpha}$, consistent with a local action of PGs. We have suggested that this interaction may contribute to rupture of the membranes at term, but also may contribute to preterm premature rupture of the membranes, a major predisposing factor to preterm labor. These data suggest that a local network involving cytokine-PG-MMP and steroid interconversion must exist in the placenta and chorion at the time of labor (Mitchell et al., 2000). The function of this network will depend on the subtype of PG receptors expressed by these tissues.

In this present study, I have demonstrated the intrauterine expression of key receptors for PG action (EP1-4 and FP) in human amnion, chorion and placenta. We suggest that the differential expression and regulation of PG receptors in the fetal membranes during term and preterm parturition may contribute to breakdown of the membranes, and alter the generation of bioactive glucocorticoids, with later effects on PG synthesis and metabolism. Thus PG receptors are likely to play a key role in the feed-forward cascades that lead to birth. Moreover, the central role of PG receptors indicates their potential as therapeutic targets for the prevention and treatment of preterm labor and delivery.

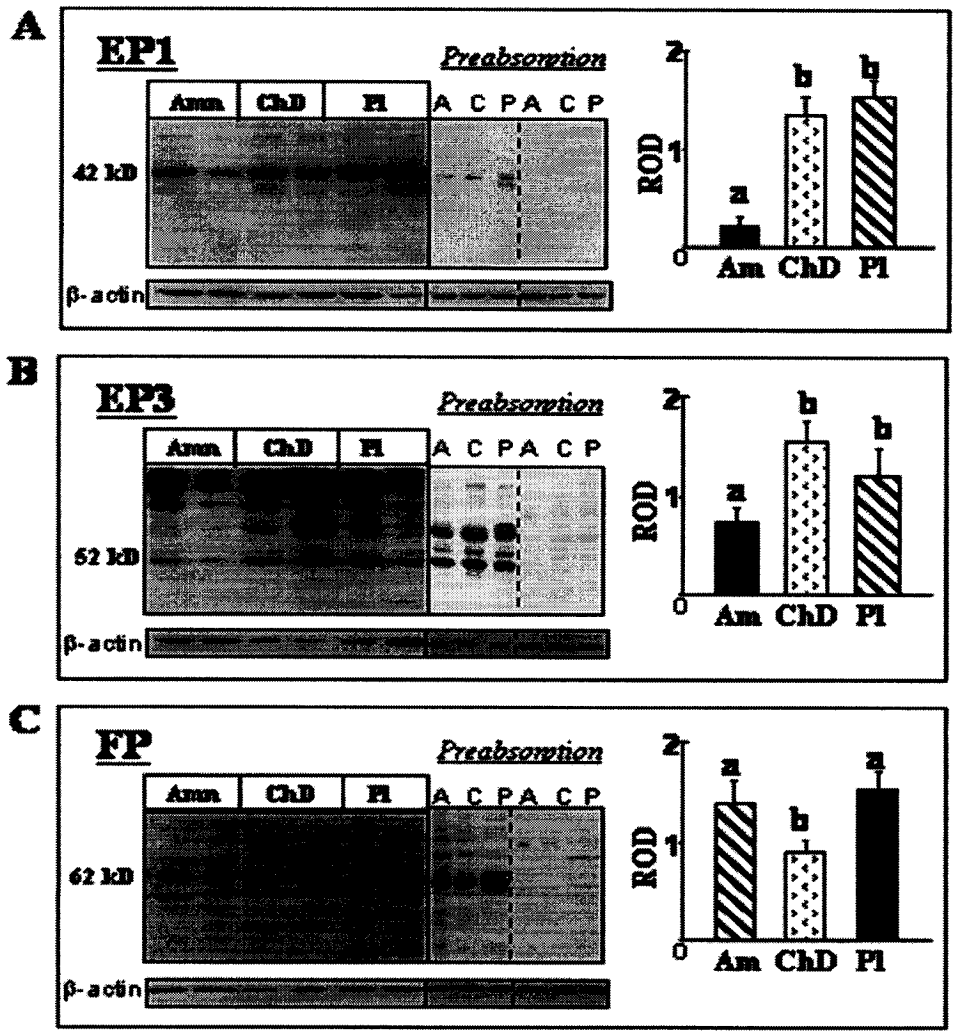


Figure III-1 Representative Western Blots for EP1, EP3 and FP receptors at term human placenta. Corresponding amnion and choriodecidua and placental tissues were obtained from 12 non-labouring patients. In this figure two representative samples from each group are shown. Amnion, choriodecidua and placenta samples (one sample from each group is shown) from the same placenta were then run on a separate gel. One part of the membrane was incubated with primary antibody and the second part with the preabsorbed primary antibody. Preabsorption with the specific antigen eliminated the specific immunoreactive band. Am/A= amnion; ChD/C= chorio decidua; Pl/P= placenta. Data are mean ± SEM. Histograms with different superscripts (a,b) are significantly different from each other ($p<0.05$).

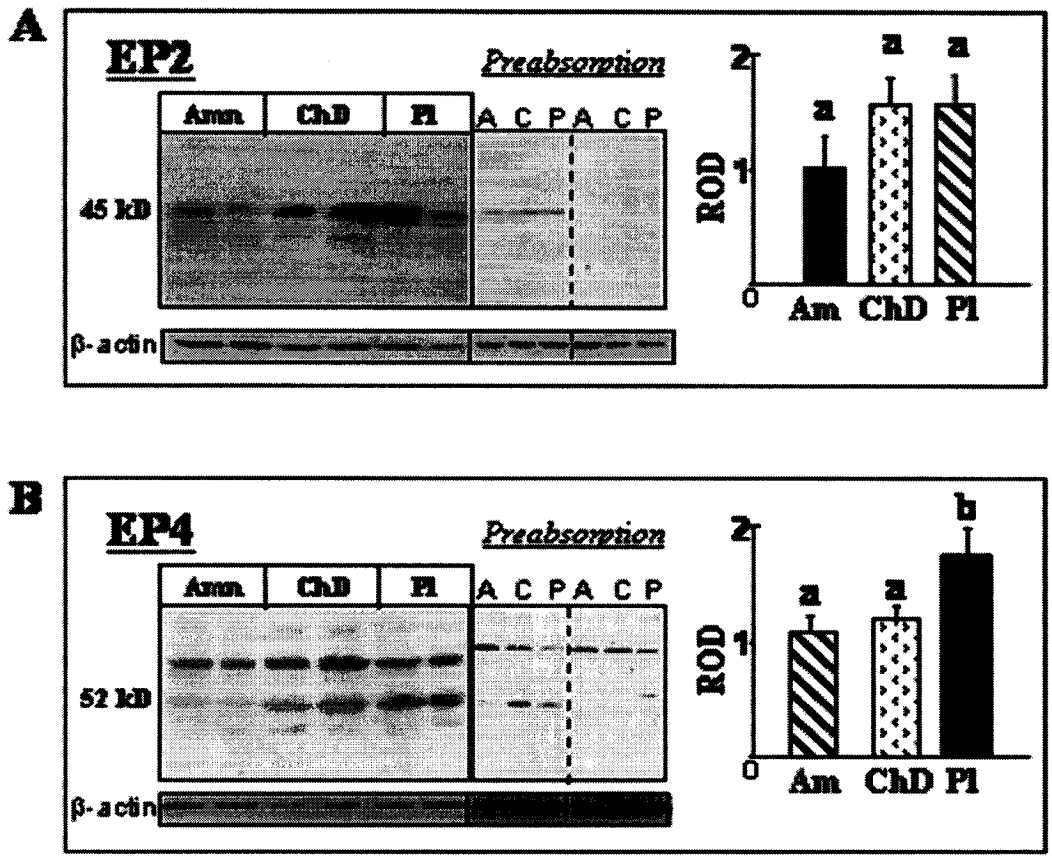


Figure III-2. Representative Western Blots of signals for EP2 and EP4 receptors. Human placenta amnion and chorion tissues were obtained from 12 non-labouring patients. In this figure 2 representative samples from each group are shown. Preabsorption with the specific antigen eliminated the specific immunoreactive band. Am/A= amnion; ChD/C= chorio decidua; Pl/P= placenta. Data are mean ± SEM. Histograms with different subscripts (a,b) are significantly different from each other ($p<0.05$).

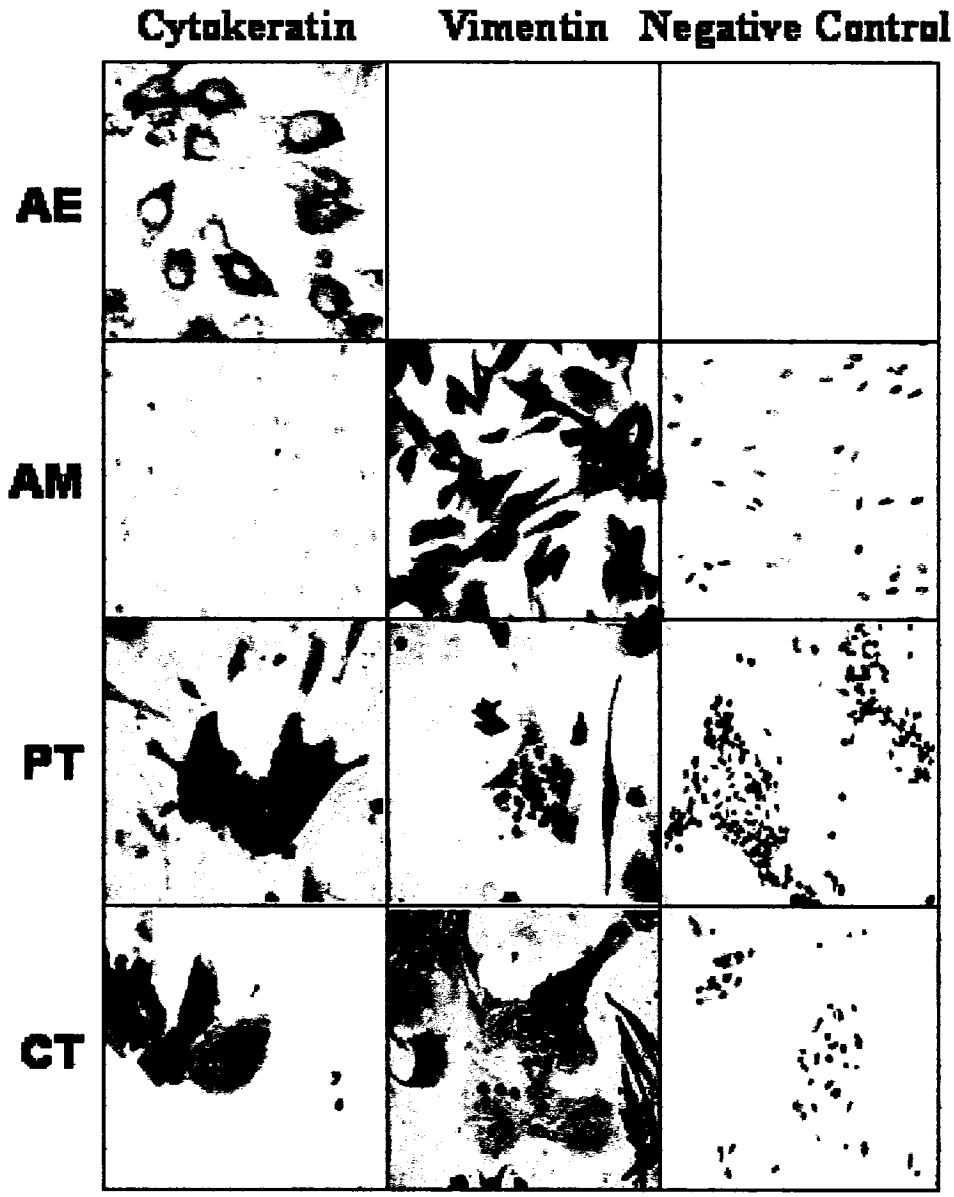


Figure III-3 Characterization of cultured cells: amnion epthelium (AE), amnion mesenchyme (AM), placental trophoblast (PT) and chorion trophoblast (CT) cells. Cytokeratin (an epthelial cell marker) and vimentin (a fibroblast cell marker) were used. AE, CT and PT were cytokeratin positive. There were a few fibroblast cells that were stained vimentin positive. AM cultures were vimentin positive and cytokeratin negative. Cells were stained with cytokeratin and vimantin after every culture.

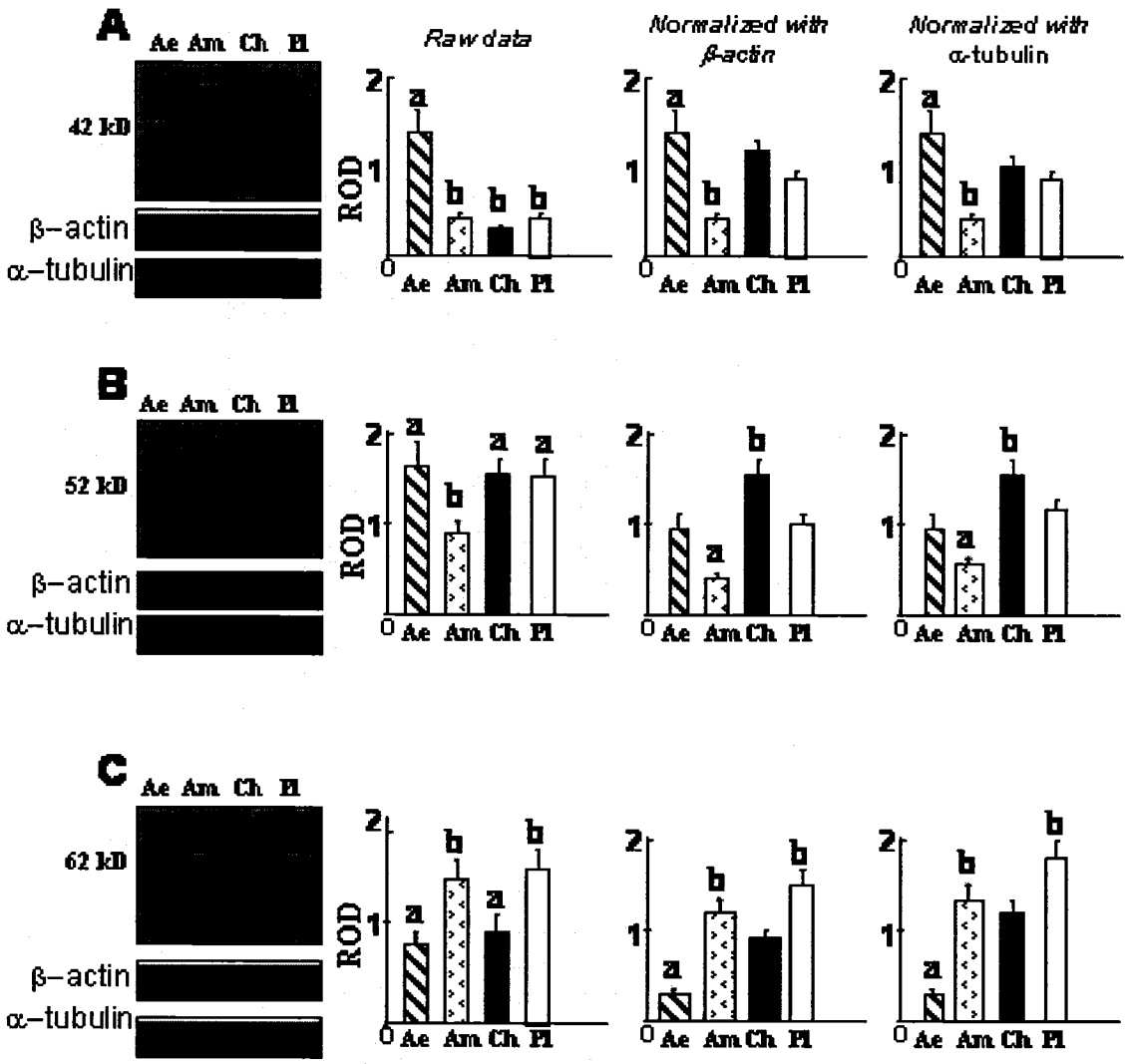


Figure III-4. Western blot analysis of EP1 (A), EP3 (B) and FP (C) in cultured cells from intrauterine tissues. Ae, Am, Ch and Pl cells obtained from the same placenta were compared. One representative sample of each cell type, from the same placenta is shown in this figure. Results are presented as raw data or normalized with b-actin or a-tubulin. [amnion epthelium (Ae) (n=5), amnion mesenchyme (Am) (n=5), placental trophoblast (Pl) (n=5) and chorion trophoblast (Ch) (n=5)]. Data are mean ± SEM, Histograms with different superscripts (a,b) are significantly different from each other ($p<0.05$).

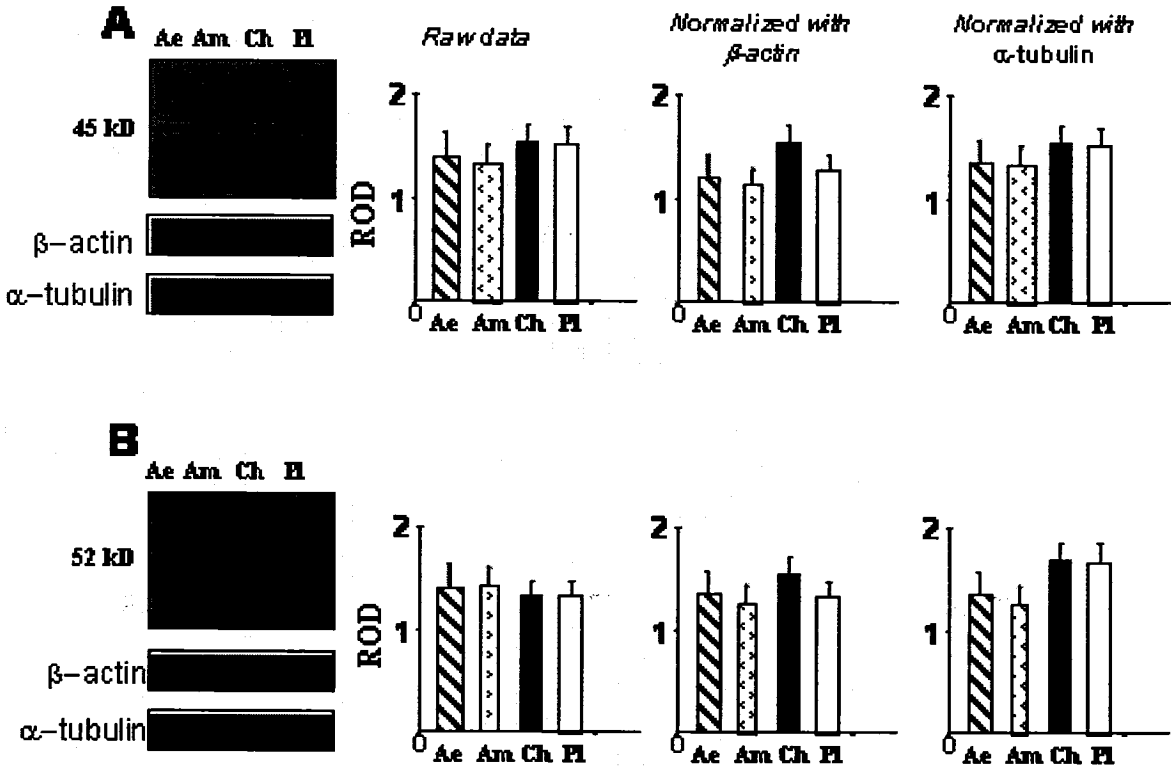


Figure III-5. Western blot analysis of EP2 (A) and EP4 (B) in cultured cells from intrauterine tissues. Ae, Am, Ch and Pl cells obtained from the same placenta were compared. One representative sample of each cell type, from the same placenta is shown in this figure. [amnion epthelium (AE) (n=5), amnion mesenchyme (AM) (n=5), placental trophoblast (PT) (n=5) and chorion trophoblast (CT) (n=5)]. Results are presented as raw data or normalized with b-actin or a-tubulin. Data are mean ± SEM.

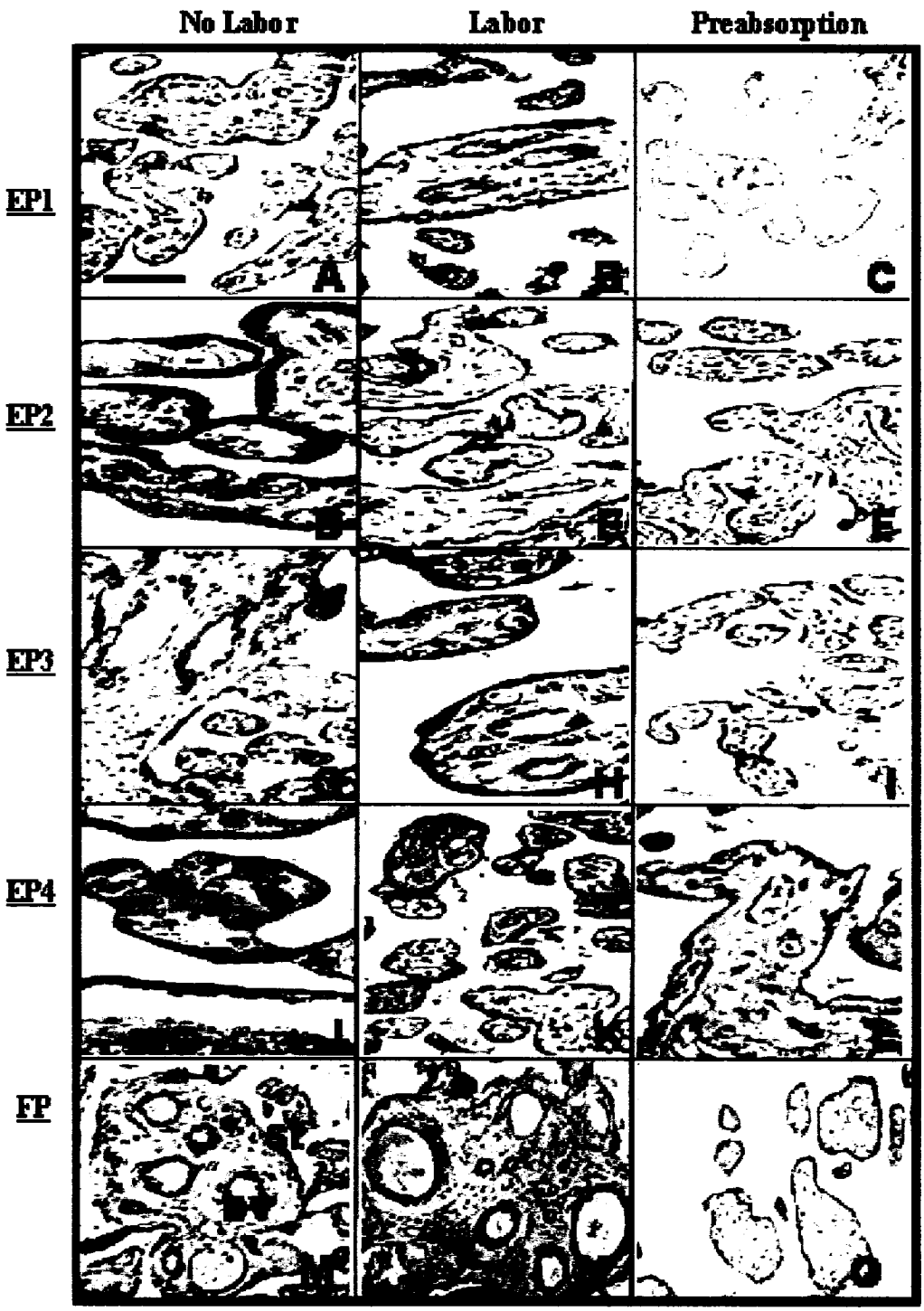


Figure III-6. Representative staining for EP1, EP2, EP3, EP4 and FP in placenta in the absence (A, D, G, J, M) or presence (B, E, H, K, N) of labor. No staining was observed when primary antibodies were preabsorbed with the blocking peptides (C, F, I, L, O). n=4, Scale bar 100 μm. (st: Syncytiotrophoblast layer; bv: Blood vessels.)

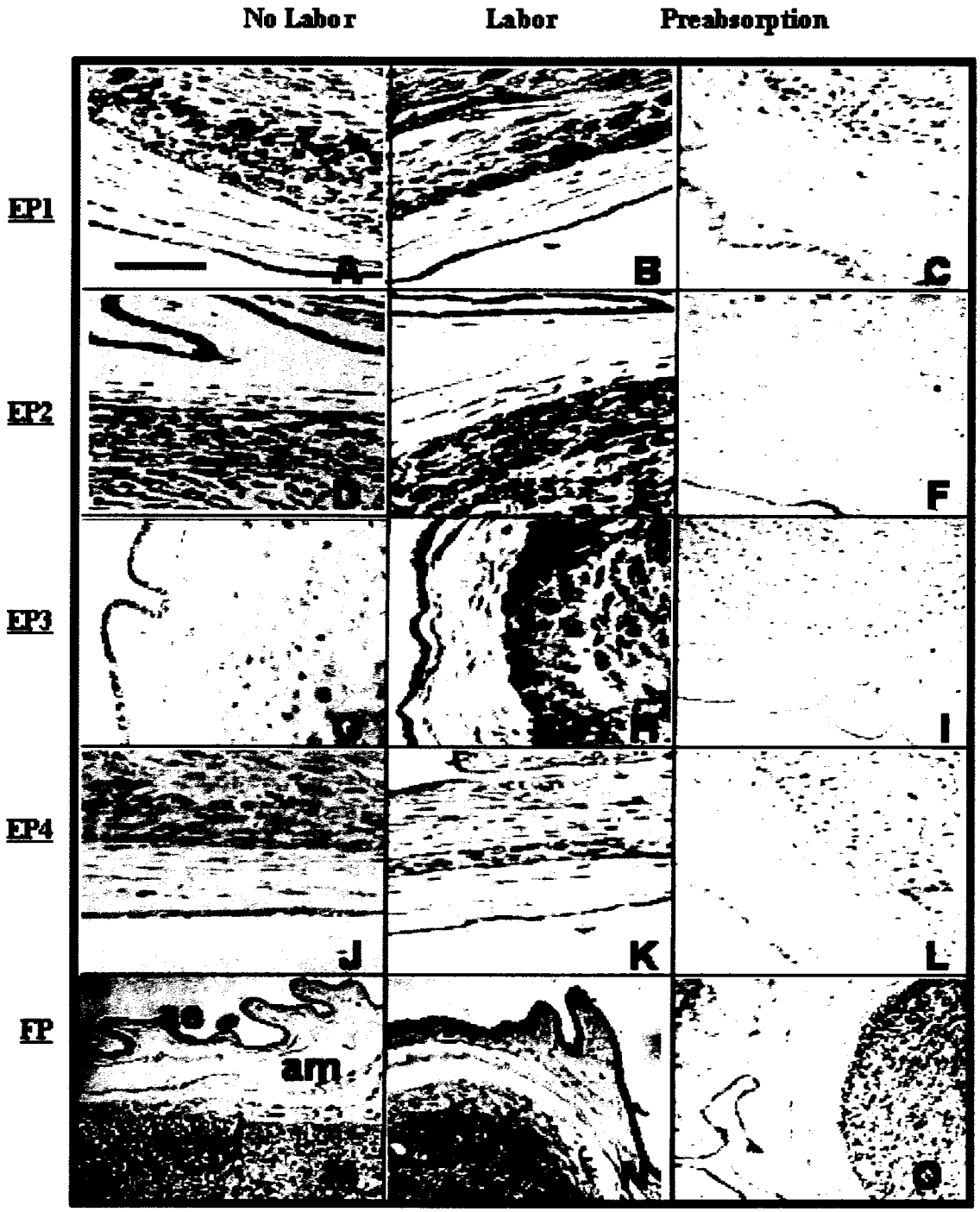


Figure III-7. Representative staining for EP1, EP2, EP3, EP4 and FP in human fetal membranes in the absence (A, D, G, J, M) or presence (B, E, H, K, N) of labor. No staining was observed when primary antibodies were preabsorbed with the blocking peptides (C, F, I, L, O). n=4, Scale bar 100 μm. (ae: Amnion epithelium; am: Amnion Mesenchyme; chd: Choriodecidua)

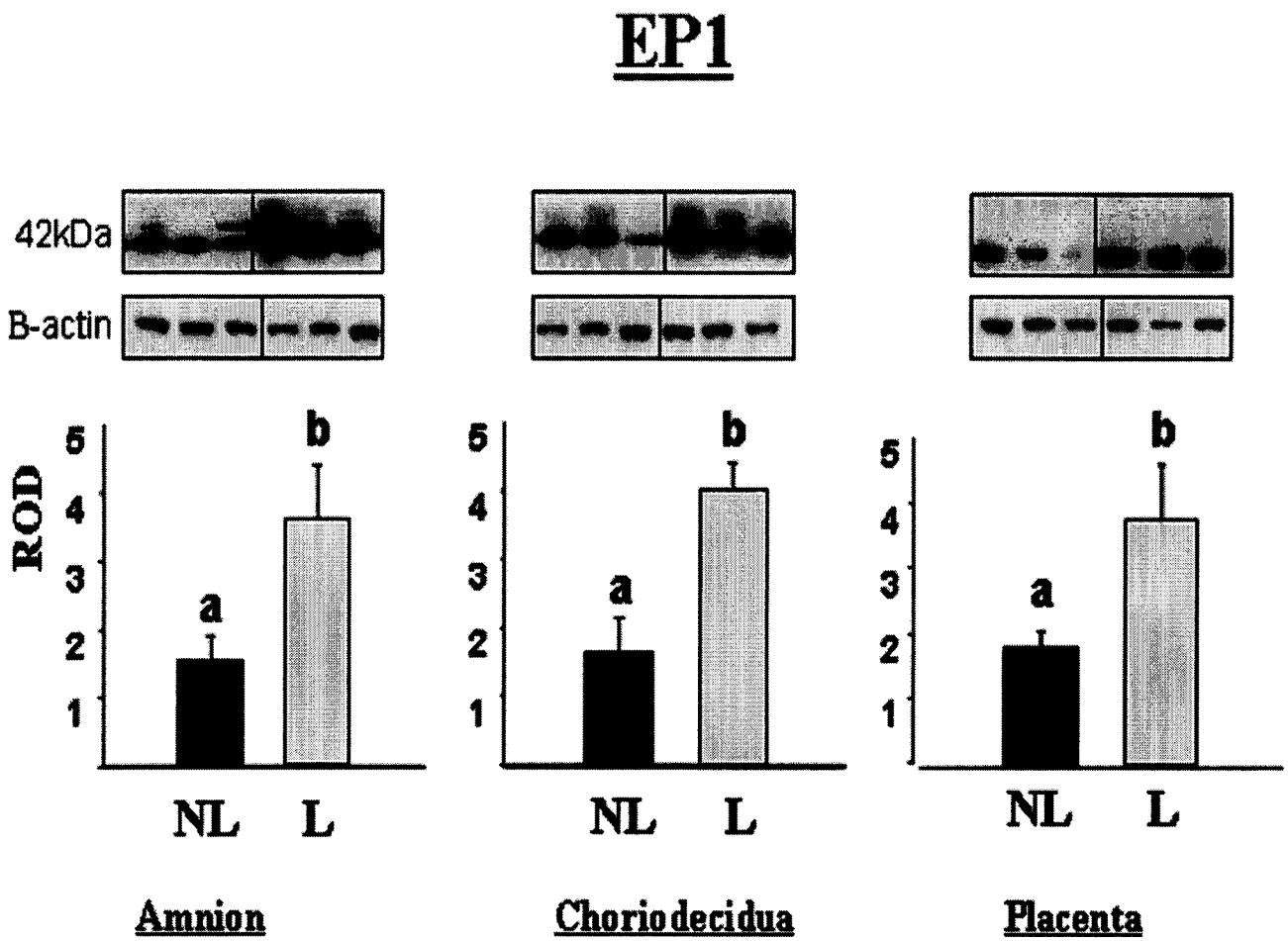


Figure III-8. Effect of labor on the expression of EP1 receptor subtype in human amnion, choriodecidua and placental tissue samples. Tissue samples from patients in labor (L) (n=12) were compared to placentas from non-laboring patients (NL) (n=12). 3 representative samples for each group were shown in the Western Blots. All values are means +/-SEM. Histograms with different subscripts (a,b) are significantly different from each other ($p<0.05$).

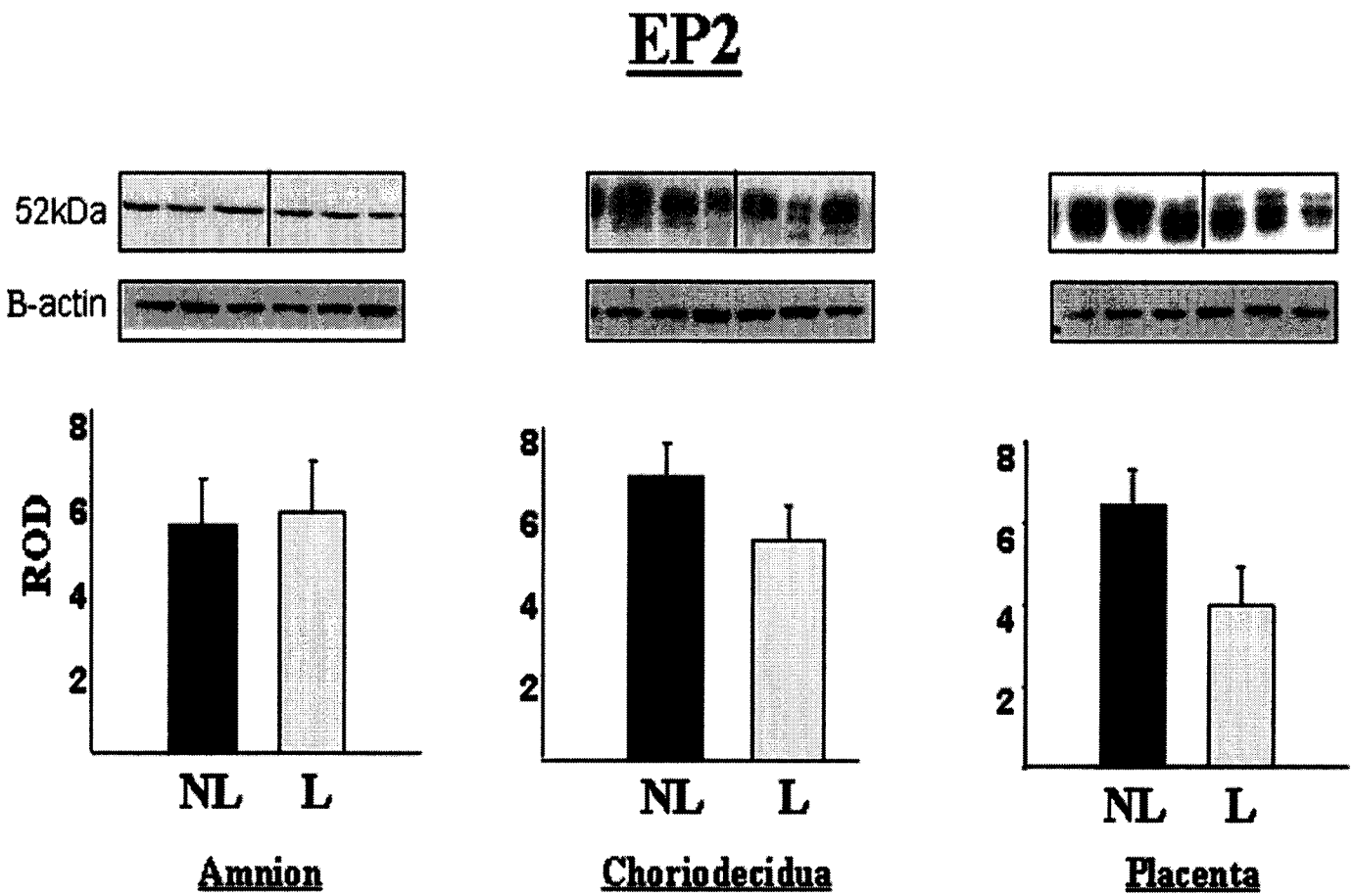


Figure III-9. Effect of labor on the expression of EP2 receptor subtype in human amnion, choriodecidua and placental tissue samples (n=24). Tissue samples from patients in labor (L) (n=12) were compared to placentas from non-laboring patients (NL) (n=12). 3 representative samples for each group were shown in the figure. All values are means +/-SEM. There was no significant difference among the groups compared.

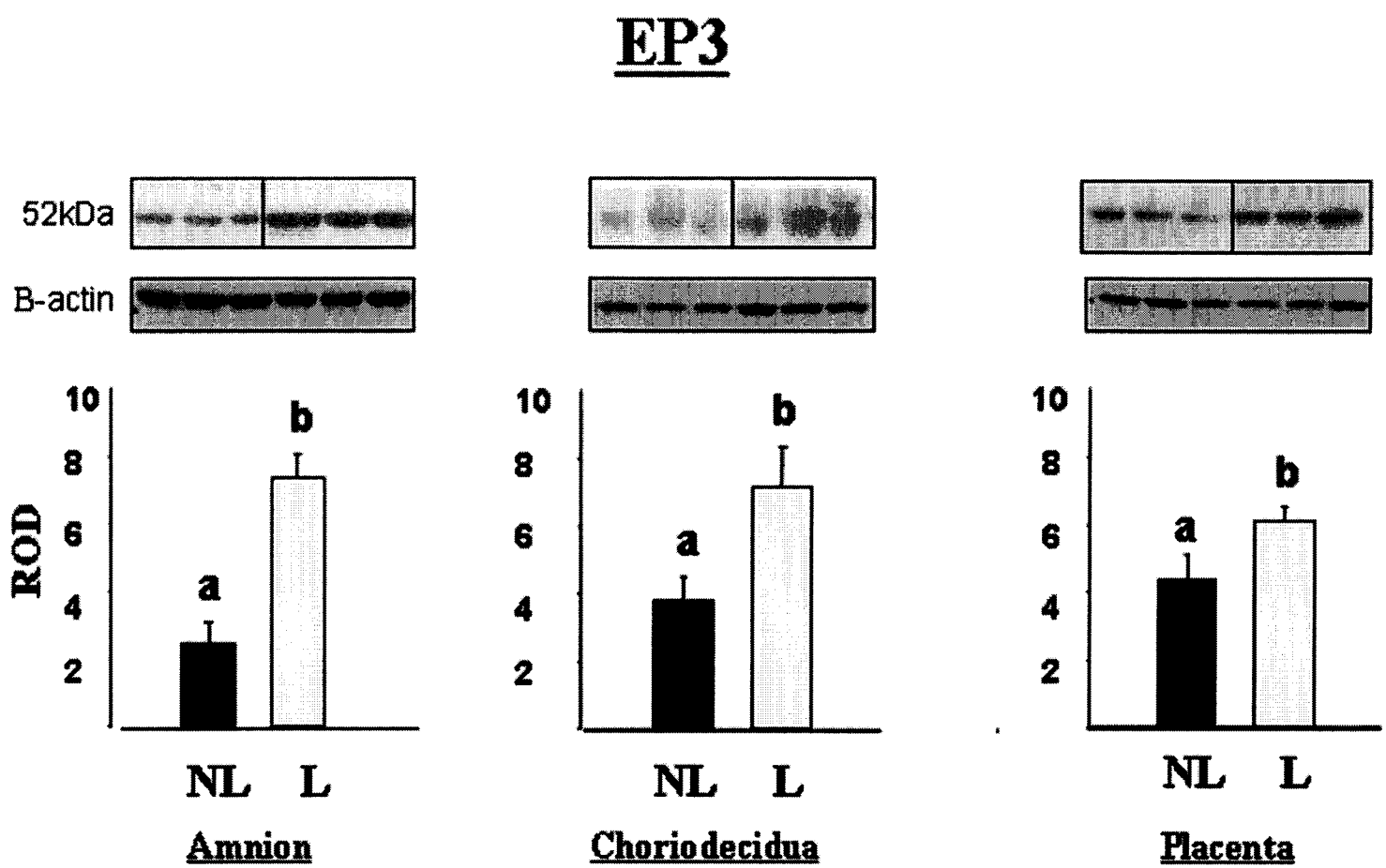


Figure III-10. Effect of labor on the expression of EP3 receptor subtype in human amnion, choriodecidua and placental tissue samples (n=24). Tissue samples from patients in labor (L) (n=12) were compared to placentas from non-laboring patients (NL) (n=12). 3 representative samples for each group were shown in the figure. All values are means +/-SEM. Histograms with different subscripts (a,b) are significantly different from each other ($p<0.05$).

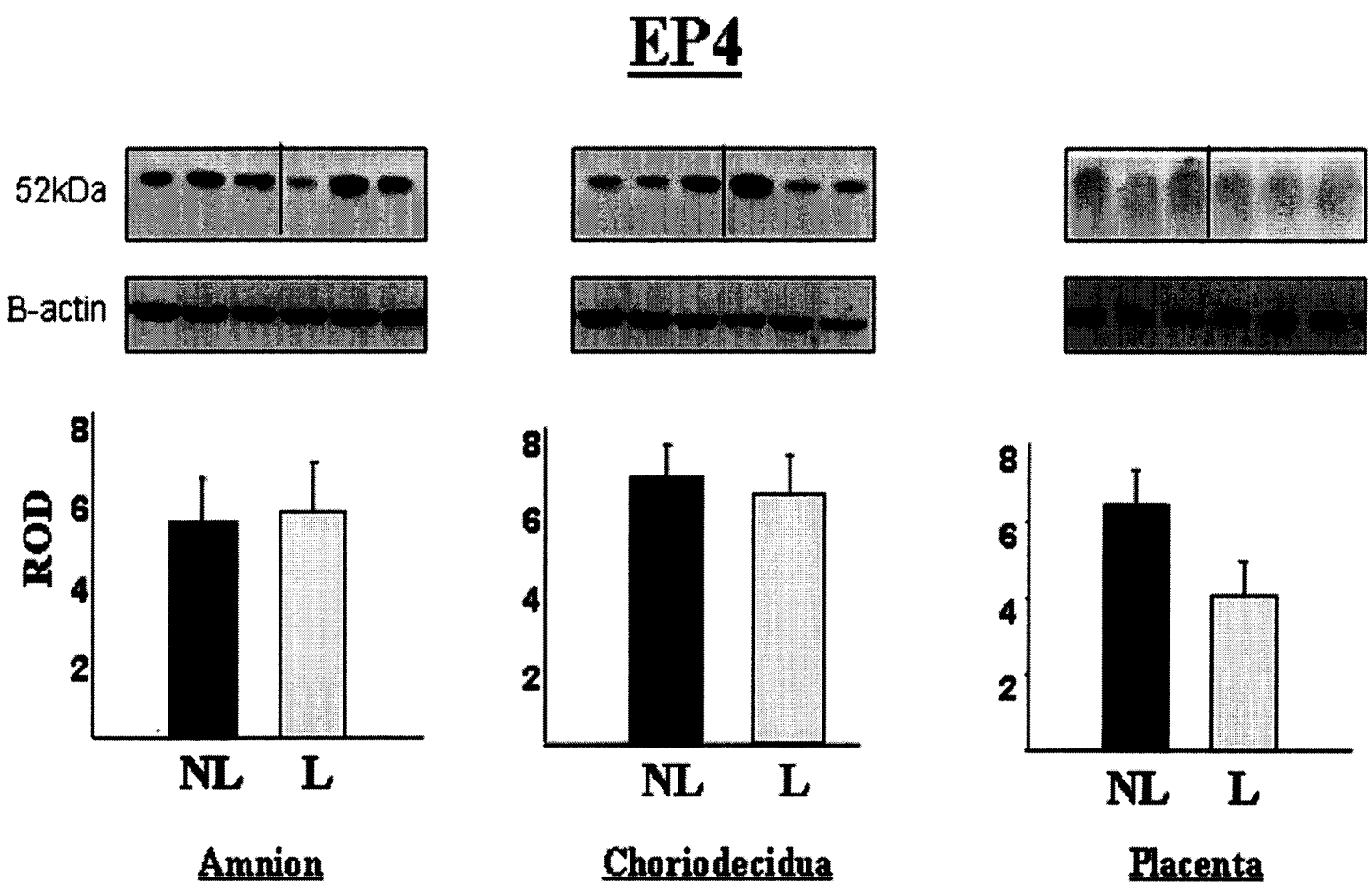


Figure III-11. Effect of labor on the expression of EP4 receptor subtype in human amnion, choriodecidua and placental tissue samples (n=24). Tissue samples from patients in labor (L) (n=12) were compared to placentas from non-laboring patients (NL) (n=12). 3 representative samples for each group were shown in the figure. All values are means +/-SEM. There was no significant difference among the groups compared.

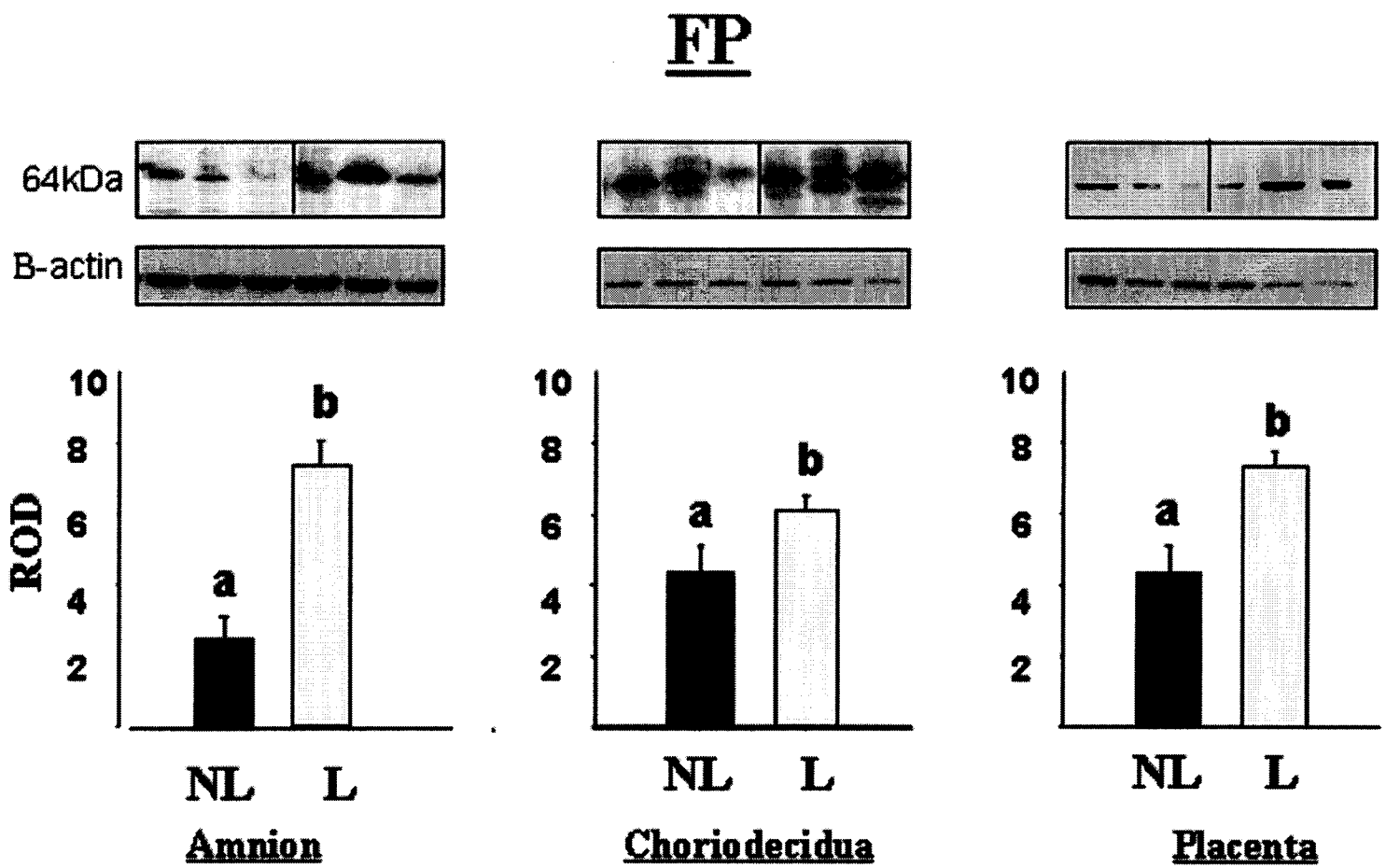


Figure III-12. Effect of labor on the expression of FP receptor subtype in human amnion, choriodecidua and placental tissue samples (n=24). Tissue samples from patients in labor (L) (n=12) were compared to placentas from non-laboring patients (NL) (n=12). 3 representative samples for each group were shown in the figure. All values are means +/-SEM. Histograms with different subscripts (a,b) are significantly different from each other ($p<0.05$).

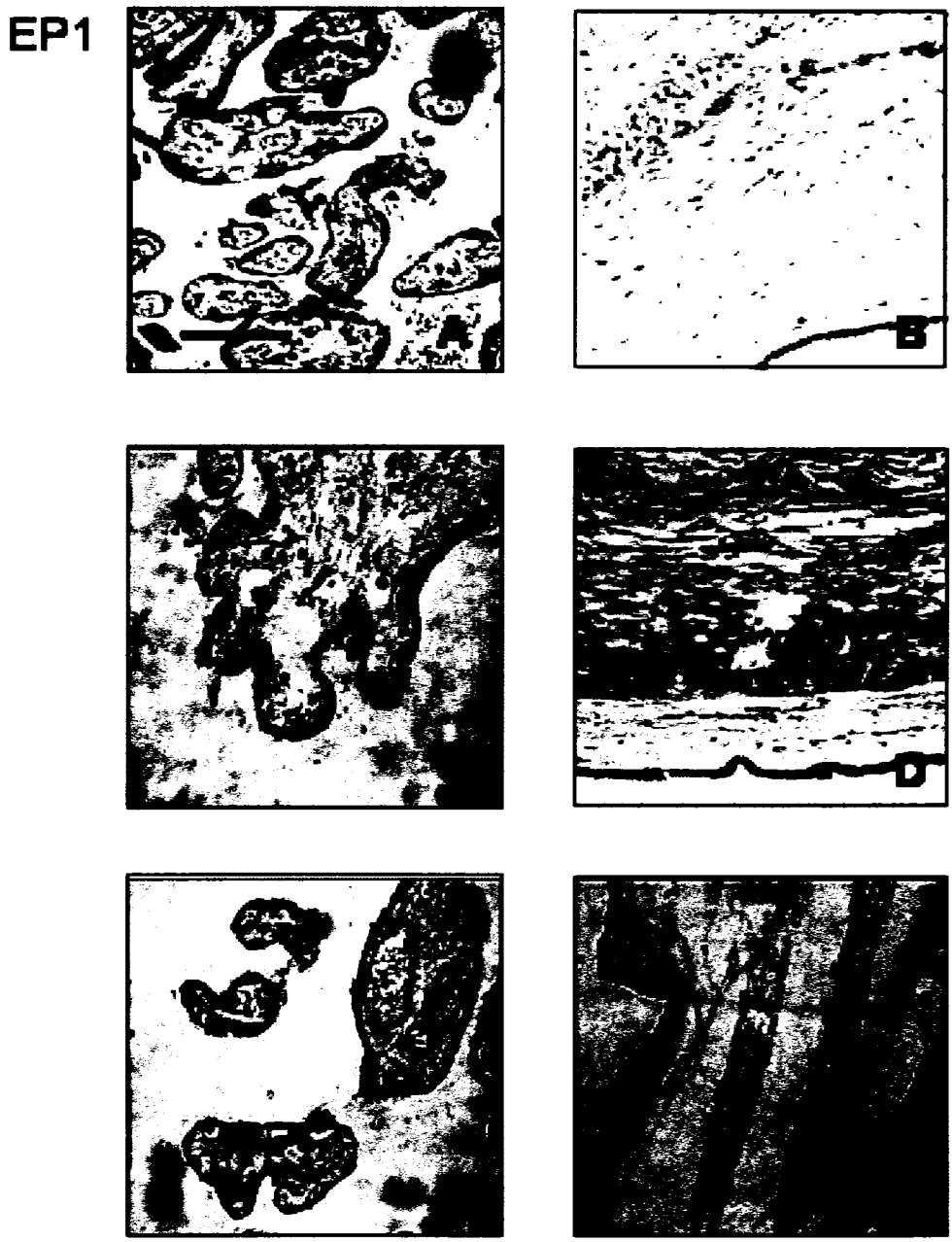


Figure III-13. Immunohistochemical analysis of EP1 receptor subtype in placenta and fetal membranes at preterm. A and B represents EP1 immunostaining in placenta and fetal membranes, respectively from idiopathic preterm labor (PTL) deliveries. C and D represents EP1 immunostaining in tissues from patients that received betamethasone (BM) treatment. E and F are samples from placentas complicated with chorioamnionitis (CHA), stained for EP1. n=3, Scale bar 100 μm.

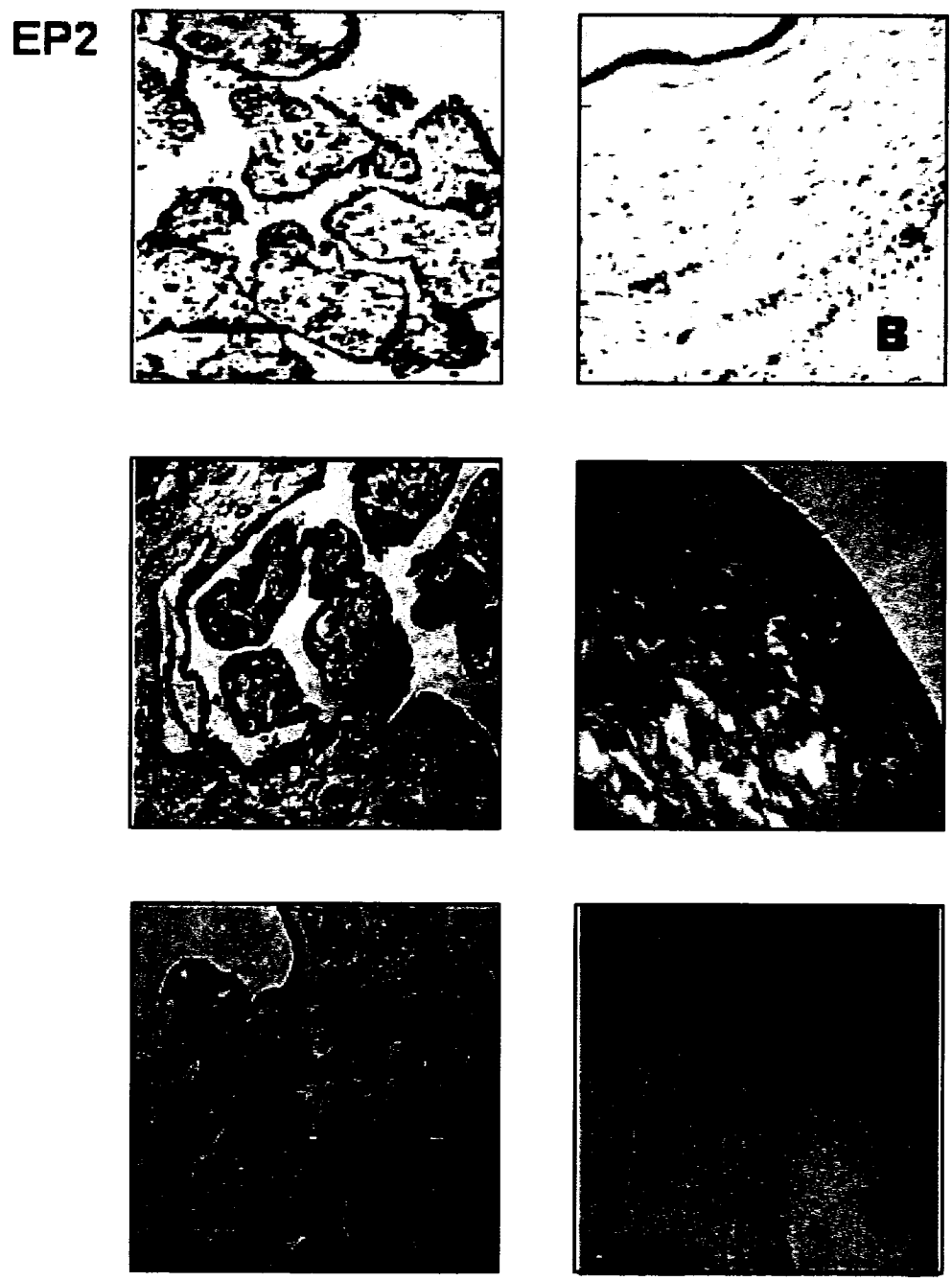


Figure III-14. Immunohistochemical analysis of EP2 receptor subtype in placenta and fetal membranes at preterm. A and B represents EP2 immunostaining in placenta and fetal membranes, respectively from idiopathic preterm labor (PTL) deliveries. C and D represents EP2 immunostaining in tissue from patients that received betamethasone (BM) treatment. E and F are samples from placentas complicated with chorioamnionitis (CHA), stained for EP2. n=3, Scale bar 100 μm.

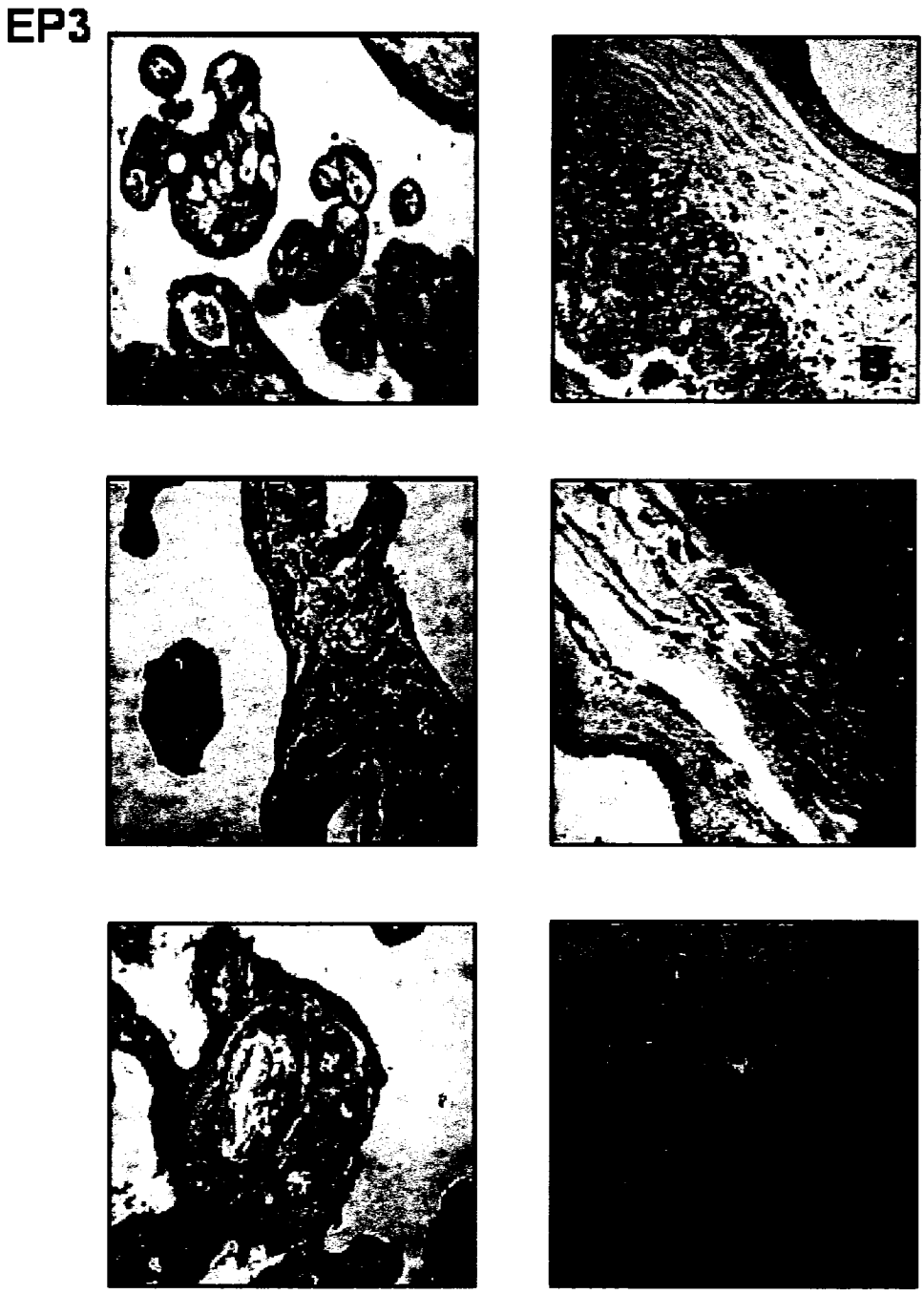


Figure III-15. Immunohistochemical analysis of EP3 receptor subtype in placenta and fetal membranes at preterm. A and B represents EP3 immunostaining in placenta and fetal membranes, respectively from idiopathic preterm labor (PTL) deliveries. C and D represents EP3 immunostaining in tissue from patients that received betamethasone (BM) treatment. E and F are samples from placentas complicated with chorioamnionitis (CHA), stained for EP3. n=3, Scale bar 100 μm.

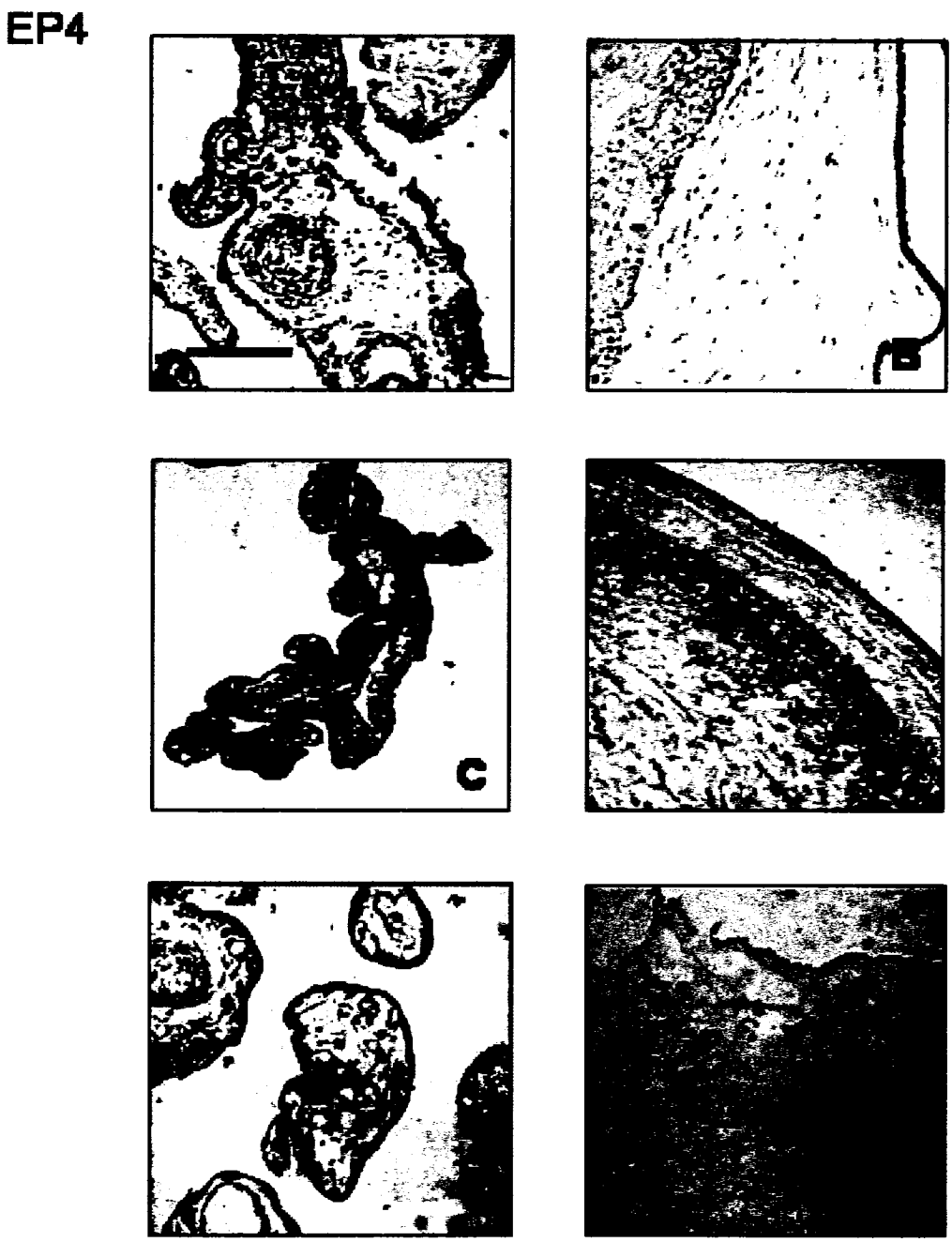


Figure III-16. Immunohistochemical analysis of EP4 receptor subtype in placenta and fetal membranes at preterm. A and B represents EP4 immunostaining in placenta and fetal membranes, respectively from idiopathic preterm labor (PTL) deliveries. C and D represents EP4 immunostaining in tissue from patients that received betamethasone (BM) treatment. E and F are samples from placentas complicated with chorioamnionitis (CHA), stained for EP4. n=3, Scale bar 100 μm.

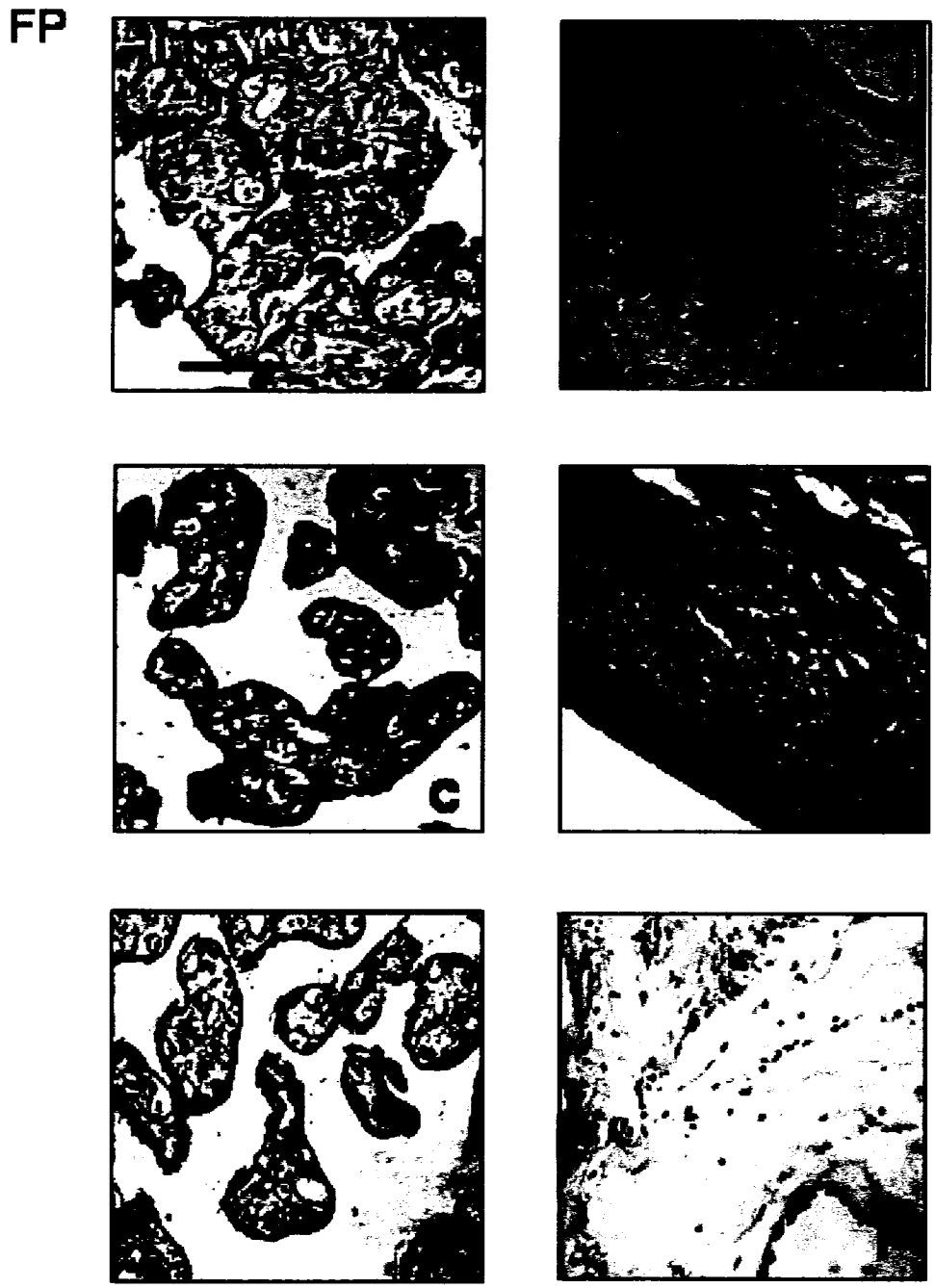


Figure III-17. Immunohistochemical analysis of FP receptor subtype in placenta and fetal membranes at preterm. A, C and E represents FP immunostaining in PTL (idiopathic preterm labor), betamethasone (BM) treated and chorioamnionitis (CHA) groups of placentas, respectively. B, D, F are immunostaining of FP in the fetal membranes of IPL, BM and CHA groups. n=3, Scale bar 100 μm.

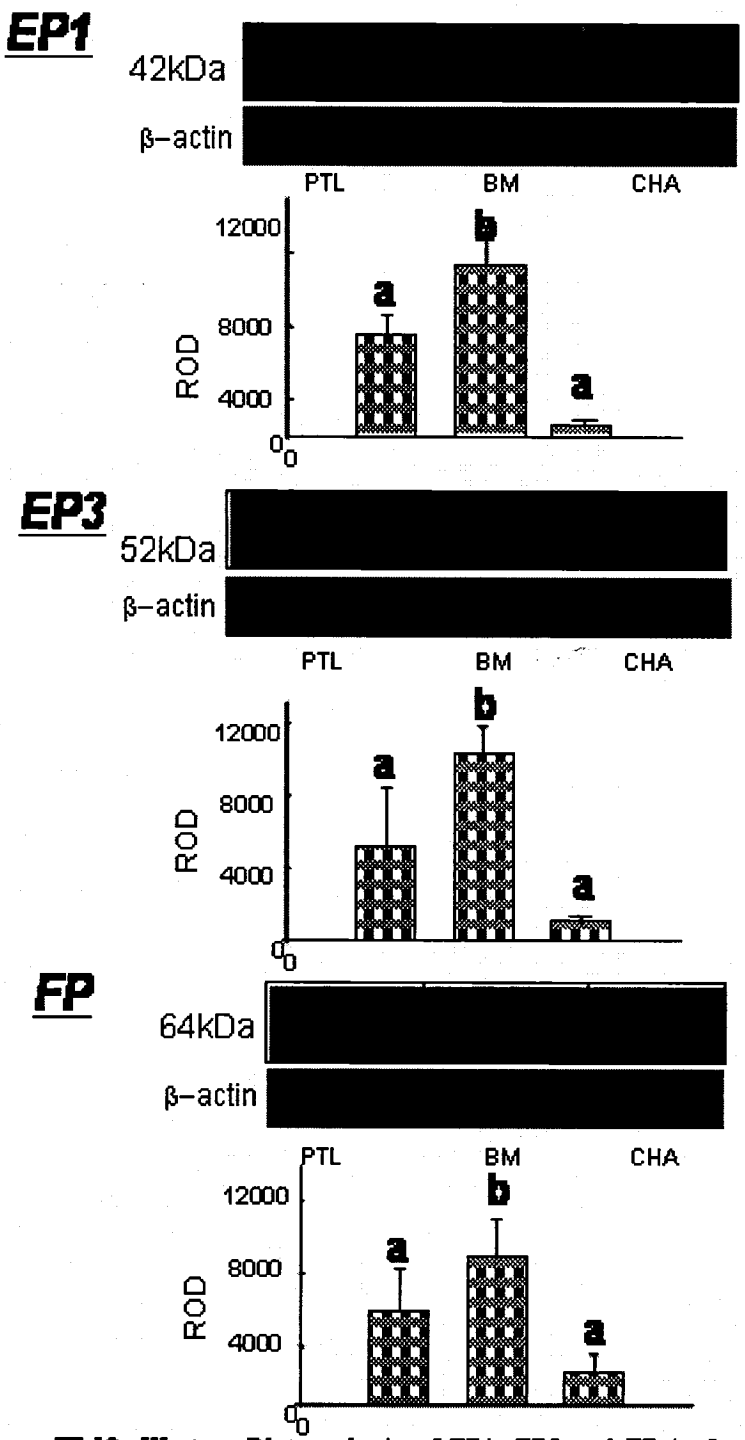


Figure III-18. Western Blot analysis of EP1, EP3 and FP in fetal membranes from preterm deliveries. The receptor levels were elevated after the betamethasone (BM) treatment compared to idiopathic preterm deliveries (PTL) chorioamnionitis group (CHA) for EP1, EP3 and FP. Histograms with different superscripts (a,b) are significantly different from each other ($p<0.05$). n=9 for PTL and BM, n=6 for CHA.

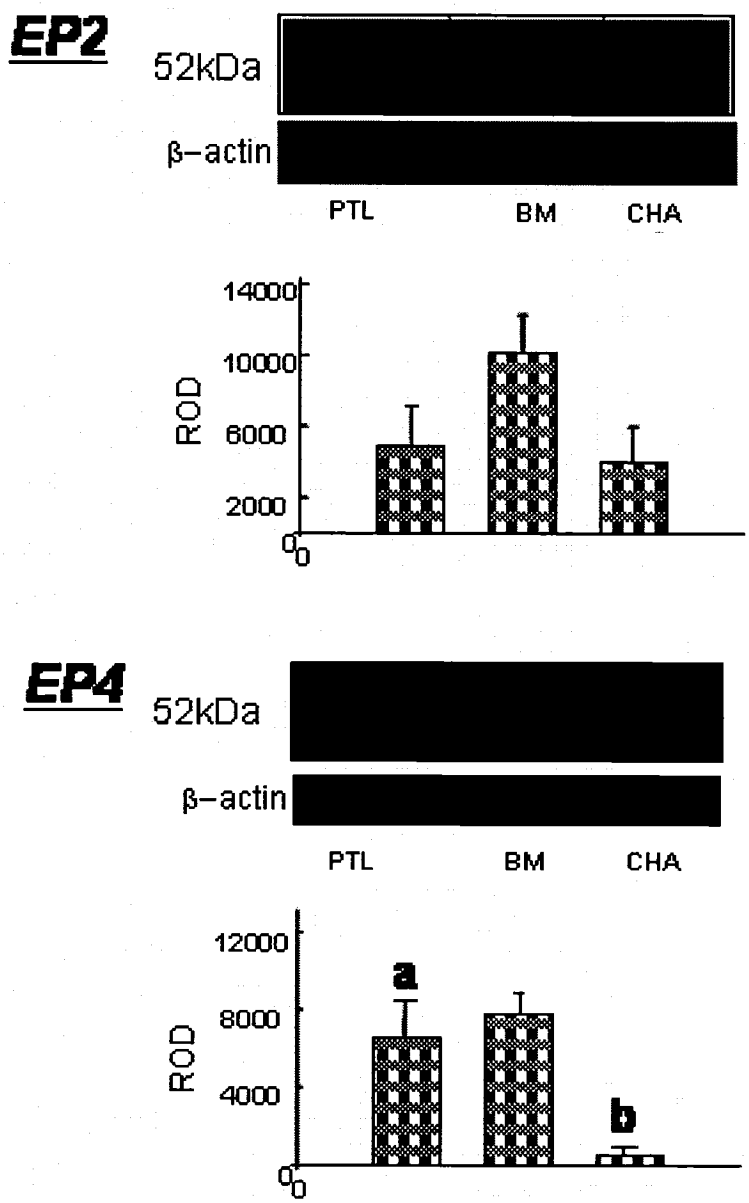


Figure III-19. Western Blot analysis of EP2 and EP4 in fetal membranes from preterm deliveries. There was no significant difference in the receptor levels in the betamethasone (BM) treatment compared to idiopathic preterm deliveries (PTL) for EP2 or EP4 protein levels. EP4 receptor subtype was significantly reduced in the chorioamnionitis group (CHA) compared to PTL group. Histograms with different superscripts (a,b) are significantly different from each other ($p<0.05$). n=9 for PTL and BM, n=6 for CHA.

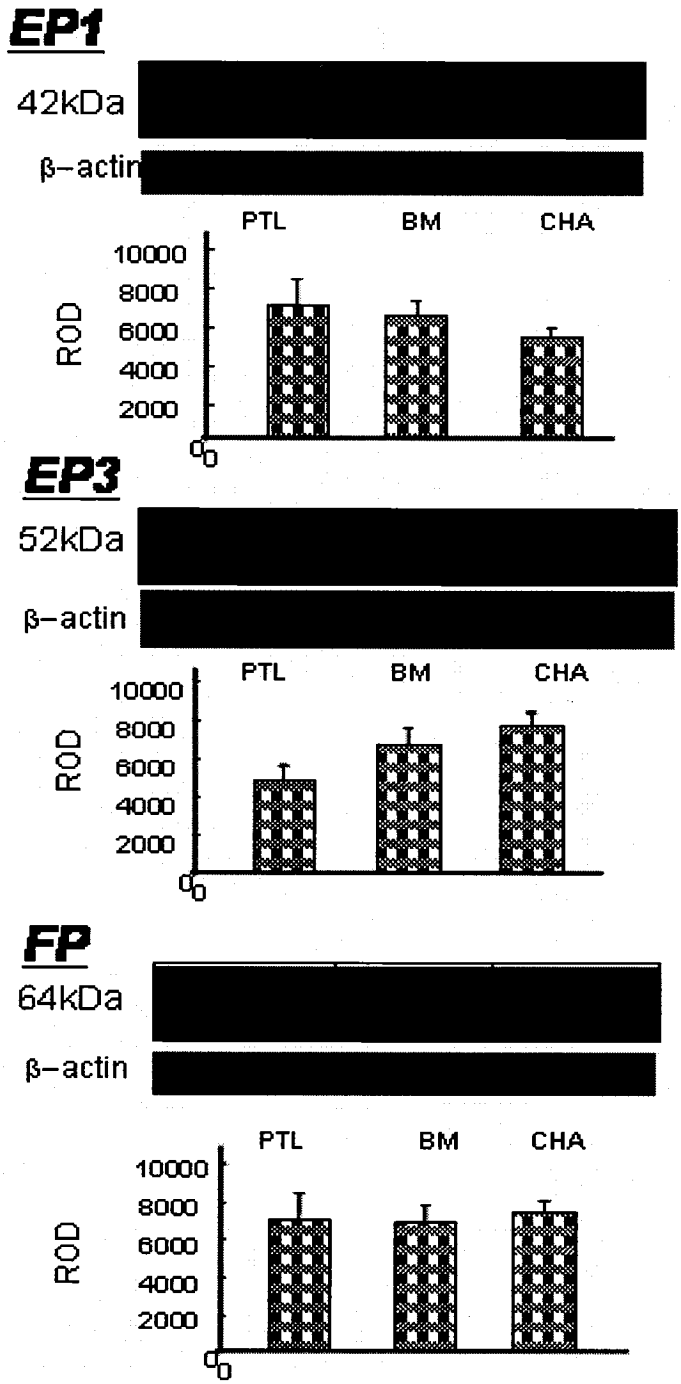


Figure III-20. Western Blot analysis of EP1, EP3 and FP receptor subtypes in the placental tissue of idiopathic preterm labor (PTL), betamethasone treated group (BM), and chorioamnionitis (CHA) group. n=9 for PTL and BM, n=6 for CHA.

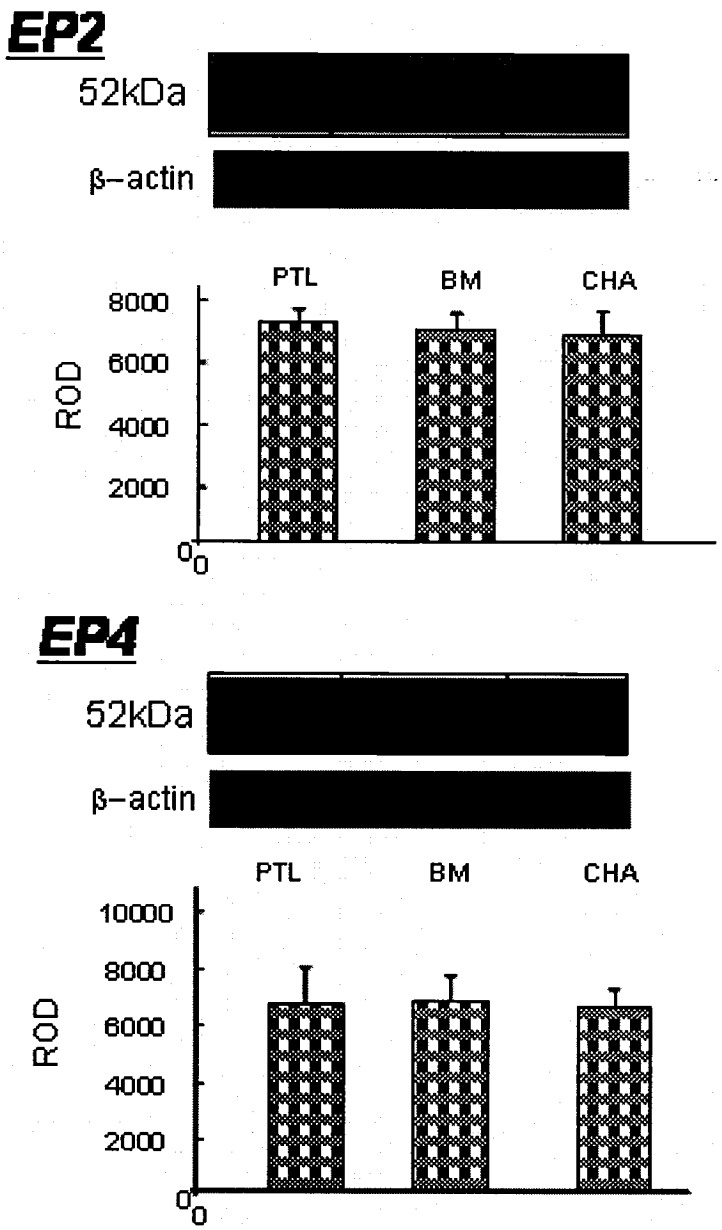


Figure III-21. Western Blot analysis of EP2 and EP4 receptor subtypes in the placental tissue of idiopathic preterm labor (PTL), betamethasone treated group (BM), and chorioamnionitis (CHA) group. n=9 for PTL and BM, n=6 for CHA.

CHAPTER IV

Pro-Inflammatory Cytokine Regulation of Prostaglandin Receptors in JEG-3 Cell Line and Human Chorion Trophoblast Cells

IV-1 Introduction

In the previous chapter, I have identified four subtypes of PGE_2 receptor (EP1-4) and $PGF_{2\alpha}$ receptor (FP) within the placenta and fetal membranes. When I examined the receptor levels, I found an increase in EP1, EP3 and FP protein levels with the onset of labor in human choriodecidual layer. Chorioamnionitis was associated with a reduction in all the receptor levels in fetal membranes compared to gestational age matched controls. However, the mechanism of this regulation of prostaglandin receptors is poorly understood.

During pregnancy and parturition the fetal membranes act as a mechanical barrier as well as an endocrine organ. Rupture of the membranes usually follows the onset of regular uterine contractions. However, occasionally, this membrane ruptures precedes uterine activity and is defined as premature rupture of fetal membranes (PROM). If PROM occurs before the 37^{th} week of gestational age, then it is identified as preterm premature rupture of the fetal membranes (PPROM). Even though PPROM occurs in only 1% of the pregnancies this is associated with 30-40% of preterm deliveries (Parry and Strauss, 1998). Thus, PPROM is the leading identifiable cause of preterm deliveries (Parry and Strauss, 1998).

Although the exact mechanism of the initiation and progress of parturition is not clear, prostaglandins have been implicated as the main mediators of these events including rupture of the membranes (Challis et al., 2000; Vadillo Ortega et al., 1994). Alfaidy et al showed that PGs are involved in a local feed forward cascade within the fetal membranes by potentiating the activity of 11β-HSD1 (Alfaidy et al., 2001). This enzyme converts biologically inactive cortisone into cortisol, thereby potentially increasing the production of cortisol in the chorion trophoblast cells. Evidence was presented to suggest that this action of $PGF_{2\alpha}$ was mediated via functional G-protein coupled FP receptor localized in trophoblast cells (Alfaidy et al., 2001).

Previous studies have shown an increase in intrauterine cytokine production both at term and preterm labor. This coincides with and may be casual to the upregulation of PGs. As reviewed by Keelan et al. (2003), mRNA expression and amniotic fluid concentrations of the pro-inflammatory cytokines, in particular interleukin-1beta (IL-1β) and tumor necrosis factor-alpha (TNFα) are elevated in the amniotic fluid at the onset of labor

(Keelan et al., 2003). Gestational tissues also produce anti-inflammatory cytokines such as interleukin-10 (IL-10) that reduce the release and/or inhibit the action of proinflammatory cytokines, including TNFα and IL-1β (Cassatella et al., 1993; Niiro et al., 1997). IL-1β rapidly induces PGHS-2 mRNA expression and PGE_2 production in primary human amnion cells, chorion and decidua (Mitchell et al., 1993; Mitchell et al., 1994; Mitchell et al., 1993; Tahara et al., 1995; Trautman et al., 1996) and in an amnion-derived cell line (WISH cells) (Xue et al., 1995). In cultured placental trophoblast cells, IL-1β and TNFα stimulate PGE_2 production (Goodwin et al., 1998). These effects of IL-1β were reversed by co-incubation with the anti-inflammatory cytokine, IL-10, in placenta and chorion. p50 and p65, key members of the NF-kB Rel family of proteins, are present in trophoblast cells (Kniss, 1999). They are thought to be mediators of cytokine-induced upregulation of PGHS-2 expression (Kniss, 1999). However, the stimulation of PG synthesis caused by cytokines cannot be explained solely due to increase in PGHS activity (Edwin et al., 1996). These results imply multiple sites of action for cytokines on the prostaglandin biosynthesis pathway all of which would contribute to the net stimulation of PG output. Indeed, multiple sites of action for cytokines have been demonstrated in different studies. IL-1β induced $cPLA_2$ mRNA expression in WISH cells (Xue et al., 1996) while IL-1β, IL-10 and TNFα downregulated PGDH activity and expression. Taken together, the local interactions between eicosanoids and cytokines are critical for the regulation of the final response of the tissue and the amount of PG produced (Challis et al., 2002; Keelan et al., 1997).

Some interactions between pro-inflammatory cytokines and prostaglandin receptors have been described previously in different in vitro cell models. In amnion WISH cells, IL-1β, IL-4 and TNFα increased the expression of EP1 (Spaziani et al., 1999; Spaziani et al., 1997). In turn, PGs can regulate the cytokine levels via cAMP linked receptors. In murine Kupfer cells, TNFα expression was inhibited by exogenous PGE_2 via EP4 (Fennekohl et al., 2002), and in bone morrow-dendritic cells PGE_2 decreased the production of TNFα, IL-6, IL-12 and increased the release of IL-10 via EP2 in a dose dependent manner (Jozefowski et al., 2003). In murine bone marrow-derived mast cells (BMMC), PGE_2 increased the production of IL-6 via EP1and EP3 (Gomi et al., 2000). There is little information regarding regulation of PG receptors in response to cytokines during parturition.

While the pro-inflammatory cytokines regulate the production of PGs, they might also be effective at the prostaglandin receptor levels leading to a potentiation in the action of prostaglandins pregnancy and parturition. In this study we investigated the effect of proinflammatory cytokines on the expression of FP and EP1-4 receptor subtypes. We choose JEG-3 cells, a human choriocarcinoma cell line, as a stable and consistent model for human trophoblast cells. Since JEG-3 cells cannot produce PGs unless arachidonic acid is given, PGs would not interfere with the effect of cytokines on the trophoblast cells, thus, providing a simpler system for identifying the effect of the pro-inflammatory cytokines. We further examined the expression of PG receptors in primary cultures of chorion trophoblast cells in response to cytokine treatment.

IV-2. Materials and Methods

IV-2.1. JEG-3 Cell Cultures

Human choriocarcinoma cell line (ATCC, Manassas, VA, USA) were seeded into sterile vented 75 cm^2 polyethylene tissue culture flasks. The culture medium was supplemented with 10% fetal bovine serum (Sigma, St Louis, MO, USA) without antibiotics. Cultures were grown to 80% confluence in a humidified incubator at 37^0 C and 5% CO_2. The cells were passaged with 2 ml 0.25 % trypsin, 0.003% EDTA solution for four times. Each time 2 culture plates (60 mm) were prepared for the treatments.

For immunohistochemistry JEG-3 cells were seeded into sterile, 8 wells chamber slides (Labtek, Nunc, Naperville, Il, USA) at a concentration of approximately $1x10^5$ per well. After incubating overnight, the cells were fixed with acetone:ethanol (1:1) solution, at 4^0 C for 10 minutes following 1 hour incubation at 37^0 C (non-humidified). The slides were kept at -80^0 C until they were used.

IV-2.2 Collection of Fetal Membranes

Placentas were collected from normal term (>37 weeks of gestation) pregnancies after elective cesarean delivery (nonlabor, n = 5) from Mount Sinai Hospital, Toronto. Multiple gestation, preeclampsia, or induction of labor were exclusion criteria. None of the patients had received any prostaglandin synthesis inhibitors or corticosteroids. Patient consent and ethical approval were obtained before tissue collection in accordance with the Canadian Tri-Council guidelines and the regulations of Mount Sinai Hospital, Toronto, and the University of Toronto.

IV-2.3. Purification of Chorion Trophoblast Cells

Chorion trophoblast cells were prepared using a modification of the method of Kliman *et al.* (Kliman et al., 1986) as described in chapter III and in previous publications

(Li et al., 2004). Briefly, the placental tissue was pooled and digested with 0.125% trypsin (Sigma, St. Louis, MO) and 0.02% DNase I (Sigma) in DMEM (Life Technologies, Inc., Grand Island, NY) three times for 1 h each. Subsequently, the trophoblast cells were loaded onto a 5–75% Percoll (Sigma) gradient at step increments of 5% Percoll and then centrifuged at 37 C at 2500 x *g* for 20 min to separate different cell types. Trophoblasts were collected, and 8×10^6 cells (for western blot analysis) were plated in 60-mm diameter dishes (Labtek; Nunc, Naperville, IL) in DMEM culture medium containing 10% fetal calf serum (Life Technologies, Inc.).

IV-2.4. In vitro treatment

Once JEG-3 cultures reached 80% confluence cells were serum depleted overnight. Primary chorion trophoblast cells were serum starved after 72 hours incubation. Subsequently, cells were treated with increasing concentrations of pro-inflammatory cytokines [IL-1β (0.01-10 ng/ml) and TNFα (0.1-20 ng/ml)] for 24 h. Primary trophoblast cells were exposed to cytokines for various times up to 24 hours (3-6-12 and 24 hours). In further experiments, JEG-3 cells were treated both with IL-1β (1 ng/ml) and TNFα (10ng/ml) combined individually with IL-10 (10 ng/ml), an anti-inflammatory cytokine, meloxicam (10^{-5} M), a COX-2 inhibitor, and pyrrolidine dithiocarbamate (PDTC; 10^{-5} M), NFκB inhibitor. Primary trophoblast cells were treated with TNFα (10ng/ml) in the presence of IL-10 (10ng/ml), meloxicam (10^{-5}M), PDTC (10^{-5} M) or 6-amino-4-(4-phenoxyphenylethylamino) quinazoline (PPE) an inhibitor of NFκB activation (10^{-5}M) (Calbiochem) Each treatment was performed in duplicate for each preparation of cells.

IV-2.5. Immunohistochemistry (IHC) Analysis

Slides were immunostained with the specific prostaglandin receptors as described in Chapter III. All antibodies were used at 1/100 dilution in antibody dilution buffer and the slides were kept at 4^0C for overnight. The avidin-biotin-peroxidase technique (Vectastain

ABC Kit; Vector Laboratories, CA, USA) was utilized for immunostaining with diaminobenzidine (DAB; Sigma Chemical Co., Miss., USA) as the substrate. Slides were counterstained with Carazzi's haemotoxylin.

IV-2.6. Western Blot Analysis

At the end of each experiment, the cells were collected with 1 ml of 0.01 M PBS while the plates were on ice. After centrifuging at 4^0 C for 10 min at 5,000 x g, the PBS was removed and 200 µL of RIPA lysis buffer [50 nM TrisHCl (pH 7.5), 150 mM NaCl, 1 per cent (wt/vol) sodium deoxycholate, 0.1 per cent sodium dodecyl sulfate (SDS), 100 mM sodium orthovanadate (Sigma), 1 per cent (vol/vol) Triton X-100 (Fisher Chemicals, Fiarlawn, NJ), and Complete MiniEDTA-free protease inhibitors (Roche Molecular Biochemicals; Dorval, Canada)] was added. After 1 h, the lysates were centrifuged at 4°C at 15,000 x g for 15 min, and supernatants were collected. Protein concentrations were determined by the Bradford assay as previously described (Bradford, 1976). 20 µg of protein was used for each sample for the Western Blot Anlaysis as described in Chapter III. ß-actin protein was detected as an internal control. Exposure of the membranes to X-ray film (X-Omat LS; Eastman Kodak Co, Rochester, NY) was used for protein visualization. Computerized image analysis (MCID Imaging Research, St Catharines, Canada) was used to determine the relative optical densities of the relevant protein bands.

IV-2.7. Statistical analysis

Results are expressed as the mean +/- standard error of the mean (SEM). Statistical comparisons were made using one-way Anova test followed by Dunnett's Test. The criterion for significance was $P < 0.05$. Calculations were carried out using SigmaStat (Jandel Scientific Software, San Rafael, CA).

IV-3. Results

IV-3.1 Expression of EPs and FP in JEG-3 cells

EP1-4 and FP receptors were each immunolocalized to the JEG-3 cells (Figure IV-1, 2). Perinuclear localization of the receptors could not be detected with immunostaining for any of the receptor subtypes. Staining was eliminated when the same antibodies were preabsorbed with the peptides against which the antibodies were raised. Nuclear expression was not observed except EP4 receptor subtype (Figure IV-2).

Using western blotting, immunoreactive proteins with molecular weights corresponding to the reported values for the different PG receptors were also detected in untreated JEG-3 cells (Figure IV-3). Major immunoreactive bands were 42 kDa for EP1, 52 kDa for EP2, EP3 and EP4, 64 kDa for FP. Immunoreactivity was abolished or reduced when the primary antibodies for EP1-4 and FP were substituted with antibodies that had been preabsorbed with the specific antigenic peptides.

IV-3.2. Regulation of EPs and FP in JEG-3 cells

EP1 protein levels increased in a dose dependent manner following exposure to increasing concentrations of TNFα for 24 h (Figure IV-4A). In contrast IL-1β at the concentrations that were used did not have a significant effect on the protein levels of EP1. In further experiments, IL-10 was able to reverse the effect of TNFα on EP1 protein expression (Figure IV-4B). When NFκB was blocked with PDTC (10^{-5} M), the EP1 levels of TNFα treated cells were reduced back to the control levels (Figure IV-4B). Meloxicam modestly reduced the levels of EP1 that were up-regulated with TNFα, but this effect was not significant. There was no significant effect on EP1 protein in cells treated with either IL-10 or meloxicam alone (Figure IV-4B).

Similar results were observed with EP3 and FP protein levels (Figure IV-5, 6). TNFα but not IL-1β increased protein levels of both receptors in a dose dependent manner. The effect of TNFα on EP3 and FP could be blocked with IL-10 and with PDTC. When

TNFα treatment was combined with meloxicam, receptor levels were lower than the group that was treated with TNFα alone, but this was not statistically significant.

The protein levels of EP2 and EP4 were also evaluated in cells treated with increasing concentrations of TNFα and IL-1β. Neither of the pro-inflammatory cytokines had any significant effect on EP2 or EP4 levels under the conditions of these experiments (Figure IV-7).

IV-3.3 The Effect of Pro-inflammatory Cytokines in Chorion Trophoblast Cells

Figure IV-8 and IV-9 represents the dose dependent effect of two pro-inflammatory cytokines, IL-1β and TNFα. IL-1β had no significant effect on the protein expression of EP3 or FP receptors, consistent with our observations using JEG-3 cell line. There was a significant dose dependent increase in EP3 and FP (Figure IV-8), but no change was observed in EP1, EP2 or EP4 when the cells were treated with TNFα at 10ng/ml and 20ng/ml (Figure IV-9). The stimulatory effect of TNFα (10ng/ml) was demonstrable after 24 h culture, but not in earlier (3h-12h) time points (Figure IV-10).

IV-3.5. The Regulation of the Effect of TNF-alpha in Chorion Trophoblast Cells

Stimulation of both EP3 and FP receptor protein by TNFα at 24 h was significantly altered by co-addition of IL-10, an anti-inflammatory cytokine (Figure IV-10, 11). IL-10 was able to reverse the stimulating effect of TNFα. There was no effect of meloxicam, added at a concentration we have shown to reduce endogenous PG output by 70% by chorion trophoblast cells (Li et al, 2006), on TNFα-stimulated EP3 or FP proteins, indicating independence of endogenous PG effect. However, the action of TNFα was blocked by 6-Amino-4-(4-phenoxyphenylethylamino) quinazoline, an inhibitor of NFκB activation, although they were unaltered by addition of PDTC, an inhibitor of NFκB (Figure IV-11). This amount of PDTC was effective in reversing the stimulation of EP3 and FP receptor protein by TNFα in JEG-3 cells.

IV-4 Discussion

In this chapter, I have demonstrated the effect of proinflammatory cytokines on the protein expression of EP1-4 and FP receptors both in JEG-3 cells and human chorion trophoblast cells. I found that TNFα increased EP1, EP3 and FP receptors in a dose dependent manner in JEG-3 cells. The stimulating effect of TNFα could be blocked by IL-10 and NFκB inhibition. Both IL-10 and PDTC reduced the levels of EP1, EP3 and FP to control levels in cells that were stimulated with TNFα. A second pro-inflammatory cytokine, IL1-β, did not have a significant effect on the levels of the same receptors in JEG-3 cells under the conditions of our experiments. This indicates some specificity of cytokine actions in JEG-3 cells, a difference that was emphasized further by the lack of effect of either TNFα or IL-1β on EP2 and EP4 receptor levels.

In the second part of the study I have shown that TNFα, but not IL-1β, increased EP3 and FP protein levels also in cultured human chorion trophoblast cells in a time and dose dependent manner. This effect of TNFα could be reversed by IL-10 and inhibition of NFκB, consistent with our observations in JEG-3 cells. When TNFα treatment was combined with meloxicam, the receptor levels were found always lower than TNFα alone, but there was no significant difference with the concentrations used.

The results in this chapter support the idea that the effect of chorioamnionitis observed in Chapter III is probably caused by the loss of trophoblast cells in the fetal membranes. Thus, the regulation of PG receptor expression in the presence of intrauterine infection still remains to be clarified.

This study was performed *in vitro* with an immortalized human choriocarcinoma cell line, JEG-3 cells and trophoblast cells purified from human chorion. Previously, positive immunostaining for PGHS-2 was shown in JEG-3 cells, but they did not produce PGs with the exposure to various pro-inflammatory cytokines unless arachidonic acid was added to the cell cultures (Premyslova et al., 2006). Since the consequences of increased PGs on PG receptor expression are unknown, the lack of PG production in JEG-3 cells provided a relatively simple system for observing the effect of pro-inflammatory cytokines on PG receptor levels in the absence of stimulated PG output. Clearly, however, our studies

have been conducted using cells in culture, and we recognize that the in vitro culture conditions do not reproduce faithfully the cellular milieu in vivo.

Parturition is commonly regarded as an inflammatory event with increasing levels of cytokines and prostaglandins in amniotic fluid. Among the cytokines that are associated with the inflammatory process, IL-1β and TNFα has been shown to increase PG levels during labor (Hansen et al., 1998; Keelan et al., 2003; Mitchell et al., 2000; Xue et al., 1996). The concentrations of IL-1β (0.01-10 ng/ml) and TNFα (0.1-20 ng/ml) used in this study were comparable to concentrations released in vitro by cultured human trophoblast cells at term and preterm labor (Steinborn et al., 1996) and similar to amounts utilized with effect in other studies from our laboratory.

Interleukin-10 is an important anti-inflammatory cytokine with a central role in maintaining normal pregnancy. It is locally produced in a gestational age-dependent manner and down-regulated at term (Hanna et al., 2000). It has been shown that the effect of pro-inflammatory cytokines, IL-1β and TNFα, on PG output through synthesis and metabolism can be opposed by IL-10 (Pomini et al., 1999). Others have shown that IL-10 itself can inhibit PGHS-2 expression in cultured placental explants from preterm labor deliveries (Hanna et al., 2006). In our experiments IL-10 displayed a potent bioactivity in down-regulating the activities of TNFα. We further co-treated the cells with meloxicam, a PGHS-2 inhibitor, to ensure the absence of PG production in JEG-3 cells. There was no effect of meloxicam on PG receptor response to the cytokines, substantiating that the effects of cytokines on PG receptors are independent of increased PGs.

The molecular mechanisms responsible for the multiple biological activities of TNFα are due to its ability to activate multiple signal transduction pathways (Baud and Karin, 2001). Binding of TNFα to its receptors causes activation of two major transcription factors: AP-1 and NFκB (Barnes and Karin, 1997; Karin et al., 1997). Even though there is not enough information about the promoter regions of prostaglandin receptors, several AP-1 sites and an NFκB site were identified in human FP gene (Zaragoza et al., 2004) which might explain our results. The TNFα promoter itself also contains AP-1 and NFκB binding sites that makes TNFα susceptible to a positive autoregulation (Baud and Karin, 2001). Among the signals generated at TNFα receptors, NFκB plays a critical role in cell

proliferation and survival; functions that have been implicated for TNFα within the fetal membranes. TNFα causes phosphorylation of inhibitory-κBa (IkBa) that holds NFκB in its inactive form (McKay and Cidlowski, 1999). Subsequently, NFκB is released from the complex and migrates into the nucleus, where it regulates gene expression (McKay and Cidlowski, 1999). Interestingly, when we blocked the NFκB activation in the presence of TNFα in primary cell cultures, receptor expression was reduced below the control levels. This result suggest the existence of other pathways, linked to NFκB signaling, that also contribute to the regulation of prostaglandin receptor expression, thus potentially regulating the local activity of prostaglandins.

The regulation of IL-1β and its mechanism of actions within the human fetal membranes appear to be more complicated. Human fetal membranes can produce IL-1β (Menon et al., 1995) and high concentrations of IL-1 receptors were found in chorion leave (Alnaif et al., 1994). On the other hand, amnion, chorion and mainly decidua express IL-1 receptor antagonist (Fidel et al., 1994; Kelly et al., 1995), and IL-1β was able to stimulate the production of IL-1 receptor antagonist (Fidel et al., 1994). In our experimental design it is speculated that the enhanced stimulation of IL-1ra by IL-1β, thus explaining the lack of IL-1β effect on the receptor levels. However, in parallel studies we have reported that IL-1β but not TNFα stimulates a natural antimicrobial, HBD-2, mRNA expression again in cultured primary chorion trophoblast cells (King et al., 2006). Taken together, these results indicate different functions and mechanisms of actions for IL-1β and TNFα within the fetal membranes during pregnancy and parturition.

Additional studies examining the role of PG receptors have shown that they are involved in regulation of apoptosis, proliferation, and matrix metalloproteinase expression. Moreover, current knowledge indicates that rupture of the fetal membranes is likely to be an integrated process that involves impaired balance between apoptosis and proliferation and/or the digestion of the fetal membranes by locally produced matrix metalloproteinase. The change in the balance of proliferation and apoptosis was first recognized by Parry-Jones and Priya (1976), when they found that fetal membranes cease to expand during the latter half of pregnancy (Parry-Jones and Priya, 1976). Recent studies also indicated that apoptosis may have important implications for fetal membrane rupture (McLaren et al.,

1999; Runic et al., 1998). Observations from other tissues revealed that PGE_2 acting through EP2 and EP4 inhibits growth in the human gastric carcinoma cell lines (Okuyama et al., 2002) whereas EP1 increases the mitogenic activity (Kimura et al., 2001). In contrast, TNFα induces cell death in human villous trophoblast cells (Yui et al., 1994). Considering the increasing effects of TNFα on PG receptors we found in this study, it can be argued whether the apoptotic effect of this cytokine is regulated solely by its own receptor activation or involves the activation of PG receptors as well.

Matrix metalloproteinase (MMPs) the main mediators of extracellular matrix degradation are thought to have a major contribution to preterm membrane rupture (Vadillo-Ortega and Estrada-Gutierrez, 2005). Recent studies showed that extracellular matrix metalloproteinase inducer (EMMPRIN) is expressed in the placenta and fetal membranes and that there is a significant increase in EMMPRIN expression in term labor chorio-decidua and amnion, compared with non-labor (Li et al., 2004). $PGF_{2\alpha}$ induced MMP-2 and -9 (Ulug et al., 2001), while IL-1β caused a progressive increase in the MMP-9 levels, both in amnion and chorion, in the rhesus monkey (Vadillo-Ortega et al., 2002). The stimulatory effect of LPS on MMP-2 and -9 levels was inhibited by the anti-inflammatory cytokine IL-10 (Fortunato and Menon, 2001). All these observations are consistent with the proposition that interdependent pathways involving cytokines, PGs, MMPs and PG receptors work towards rupture of the fetal membranes.

In summary, I have shown that, in trophoblast cells, pro-inflammatory cytokines such as TNFα can affect prostaglandin receptor levels in addition to increasing prostaglandin production, hence mediating the amplitude of biological actions of prostaglandins. Within the fetal membranes there are clearly a number of local feed forward cascades that potentiate each other contributing to the rupture of membranes and labor. The PG receptors occupy a central position in those cascades, suggesting possibilities for therapeutic intervention to accelerate a delay in membrane rupture and labor.

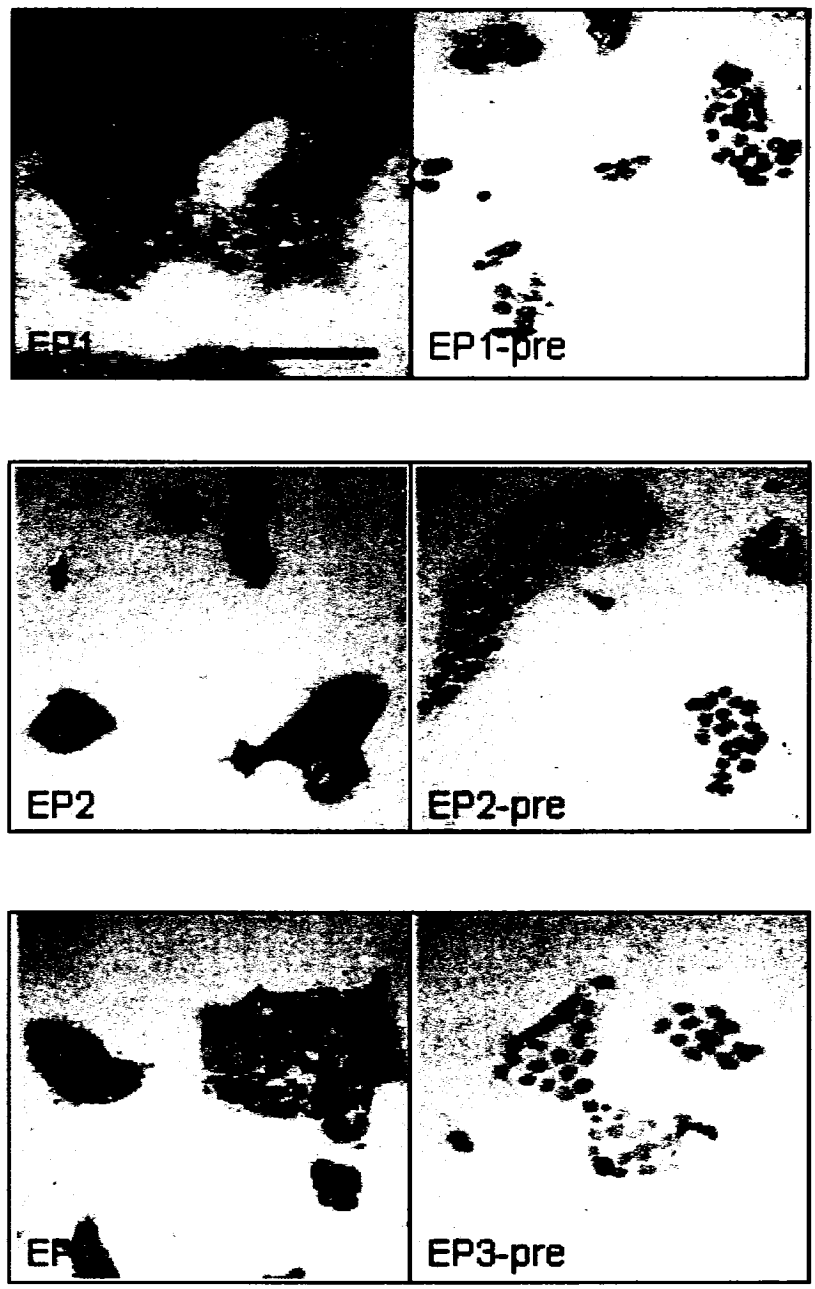


Figure IV-.1. Immunohistochemical localization of EP1, EP2 and EP3 receptors in JEG-3 choriocarcinoma cell line. Preabsorption of the primary antibody eliminated the positive staining with IHC. Scale bar 100 μm.

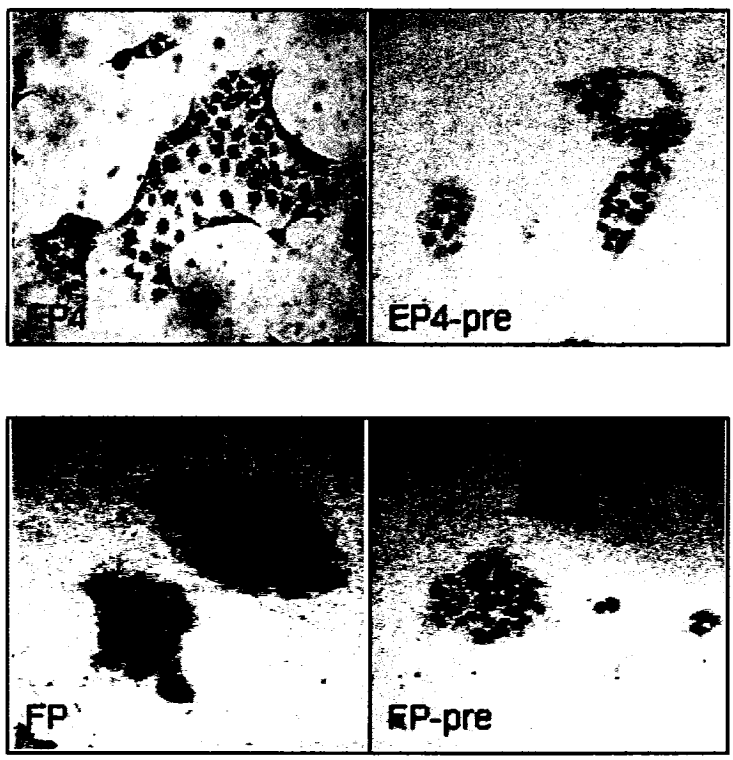


Figure IV-2 Immunohistochemical localization of EP4 and FP receptors in JEG-3 choriocarcinoma cell line. Preabsorption of the primary antibody eliminated the positive staining with IHC. Original magnification x40.

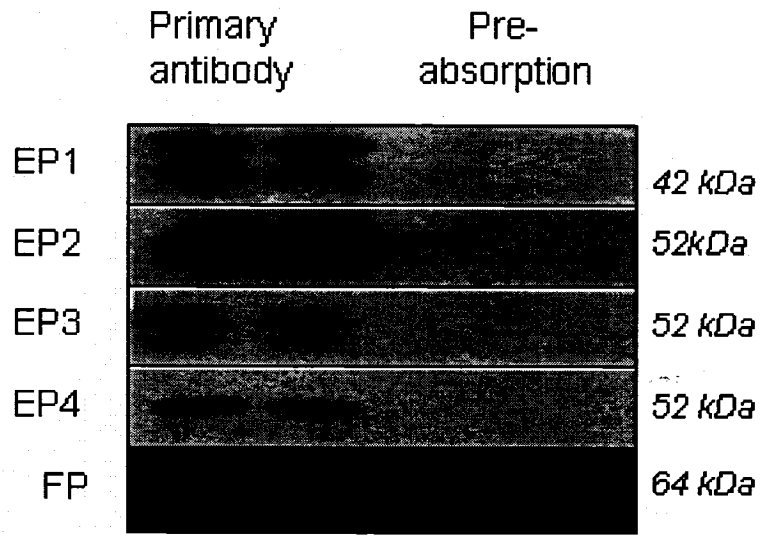


Figure IV-3 Western Blot analysis of prostaglandin receptors in JEG-3 cell line. Preabsorption of the primary antibody eliminated the specific band with Western Blot analysis.

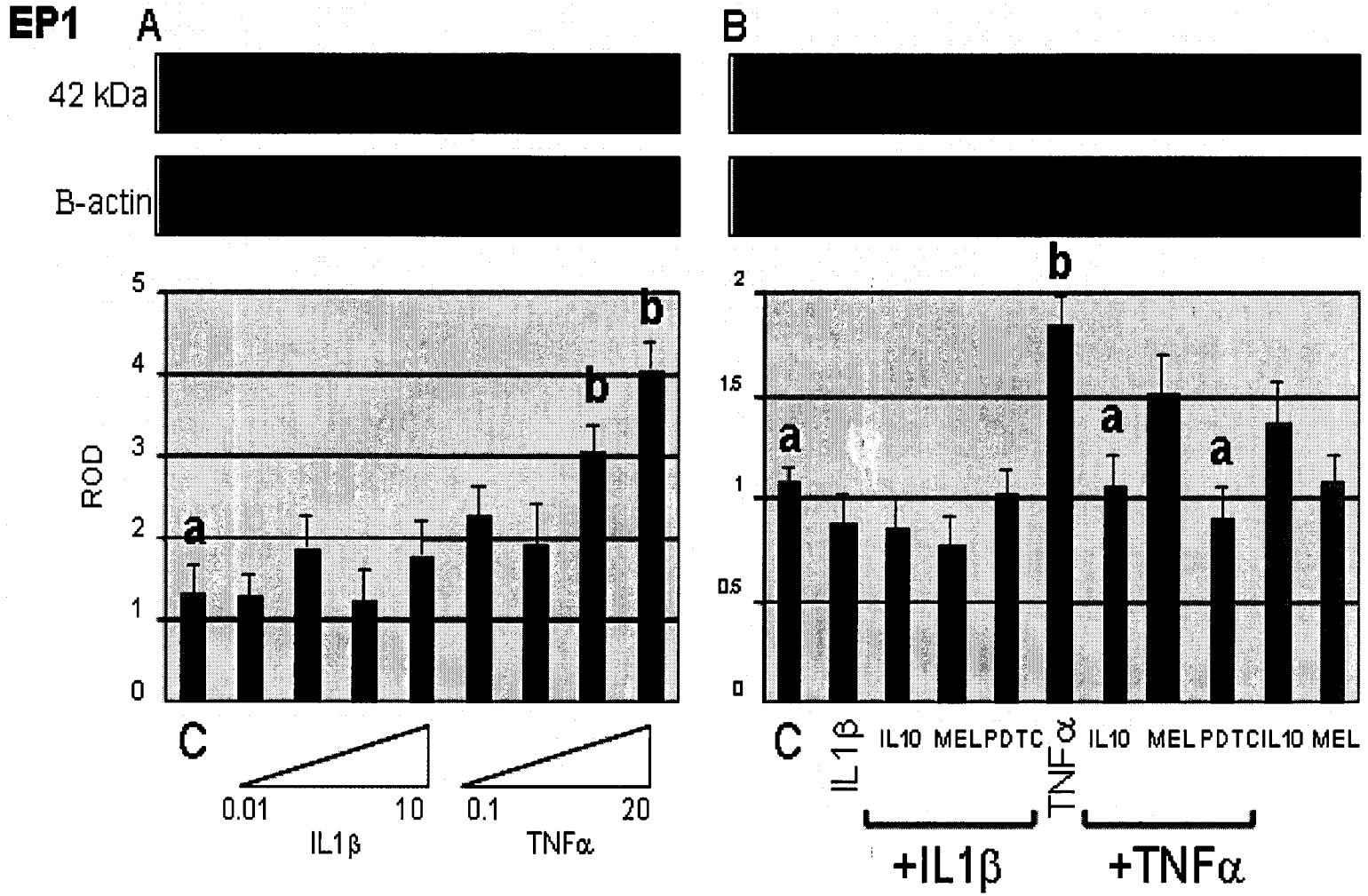


Figure IV-4. The effect of pro-inflammatory cytokines, interleukin-1beta (IL1β) and tumor necrosis factor alpha (TNFα) on EP1 protein expression in JEG-3 cell line. TNFα (10 and 20 ng/ml) stimulated the protein expression of EP1 and this effect of TNFα could be reversed by IL-10 (10 ng/ml) and NFκB inhibition (PDTC, 10-5 M), but not with meloxicam (MEL). Histograms with different subscripts (a,b) are significantly different from each other. n=5, $p<0.05$.

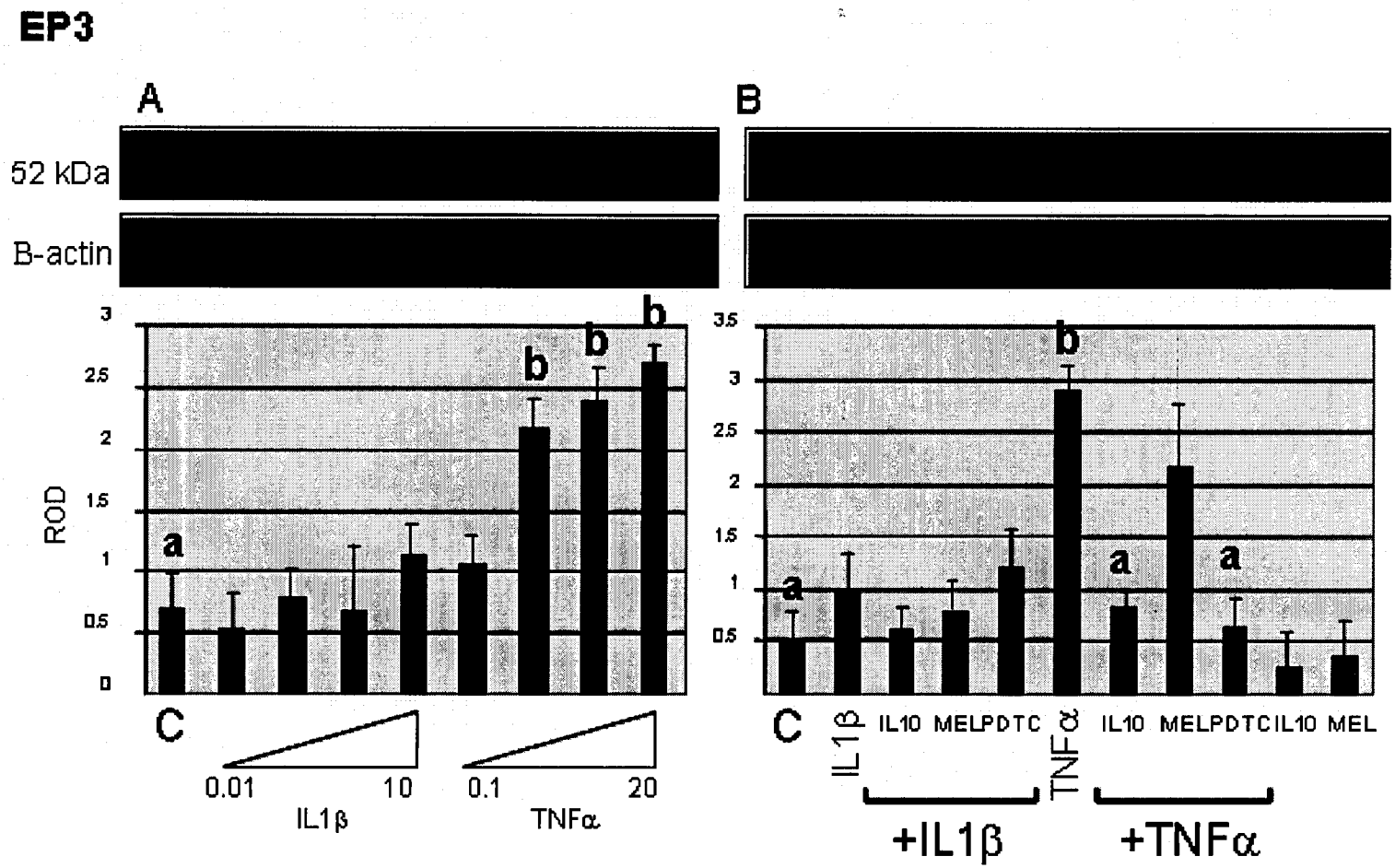


Figure IV-5. The effect of pro-inflammatory cytokines, interleukin-1beta (IL1β) and tumor necrosis factor alpha (TNFα) on EP3 protein expression in JEG-3 cell line. The stimulating effect of TNFα could be reversed by IL-10 and NFκB inhibition, but not with meloxicam (MEL). Histograms with different subscripts (a,b) are significantly different from each other. n=5, $p<0.05$

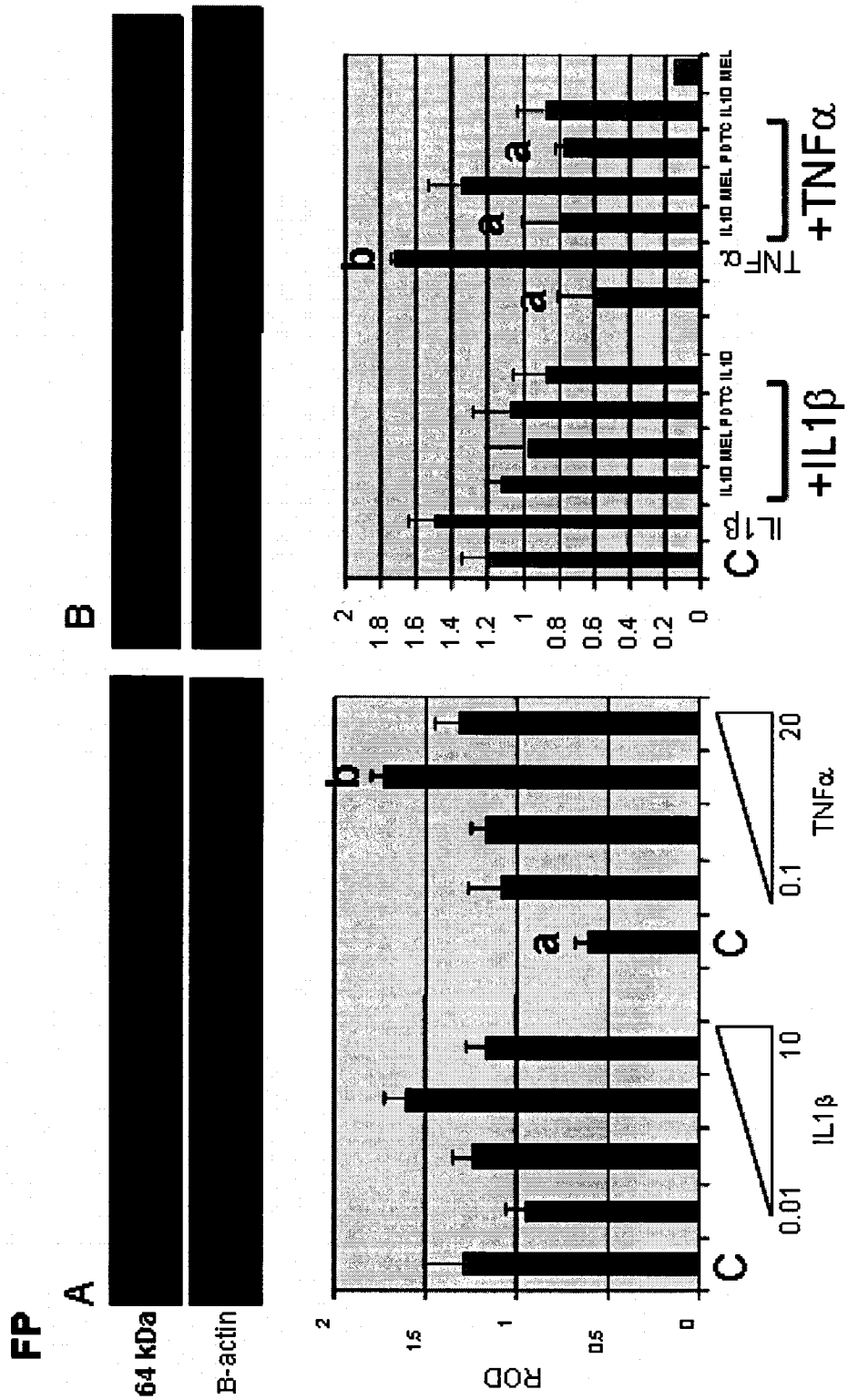


Figure IV-6. The effect of pro-inflammatory cytokines, interleukin-1beta (IL1β) and tumor necrosis factor alpha (TNFα) on FP protein expression in JEG-3 cell line. The stimulating effect of TNFα could be reversed by IL-10 and NFκB inhibition, but not with meloxicam (MEL). Histograms with different subscripts (a,b) are significantly different from each other. n=5, $p<0.05$.

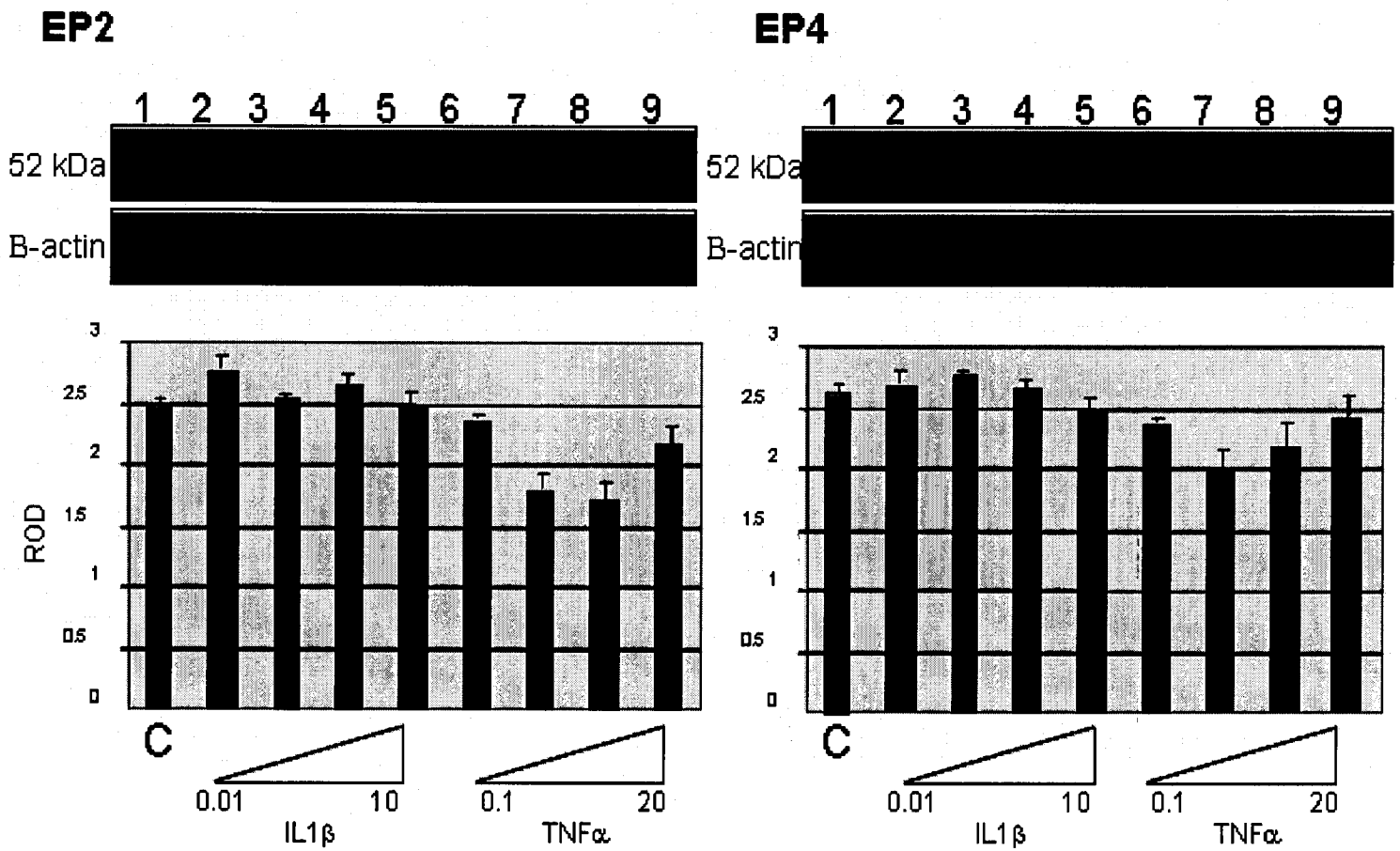


Figure IV-7. The effect of pro-inflammatory cytokines, interleukin-1beta (IL1β) and tumor necrosis factor alpha (TNFα) on EP2 and EP4 protein expression in JEG-3 cell line. There were no significant difference of cytokine treatments on EP2 or EP4 receptor subtypes. n=5.

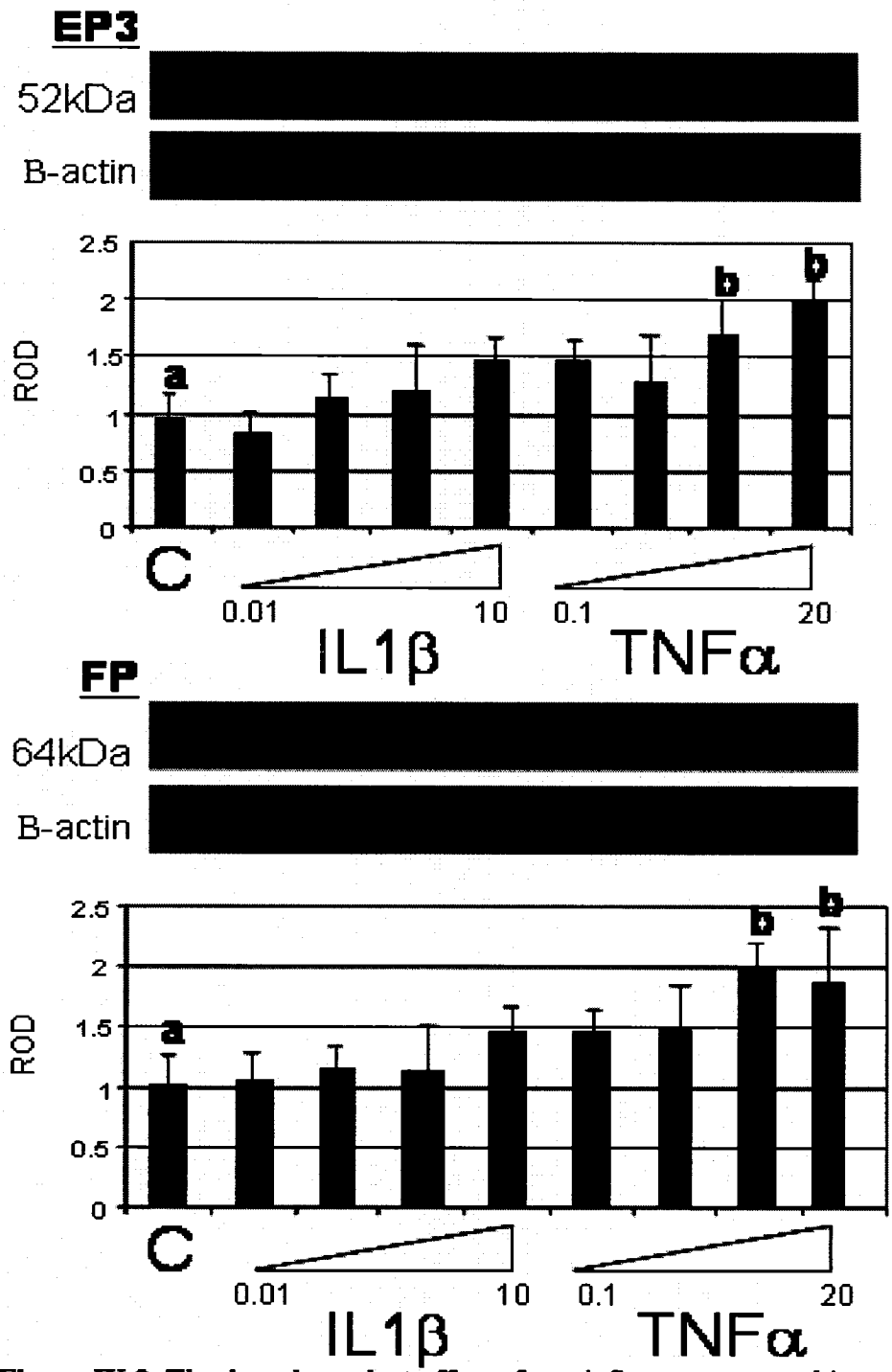


Figure IV-8. The dose dependent effect of pro-inflammatory cytokines on EP3 and FP receptor subtype in primary human chorion trophoblast cells. TNFα, but not IL-1β increased the receptor levels in a dose dependent manner. Histograms with different superscripts (a,b) are significantly different from each other ($p<0.05$). (n=5)

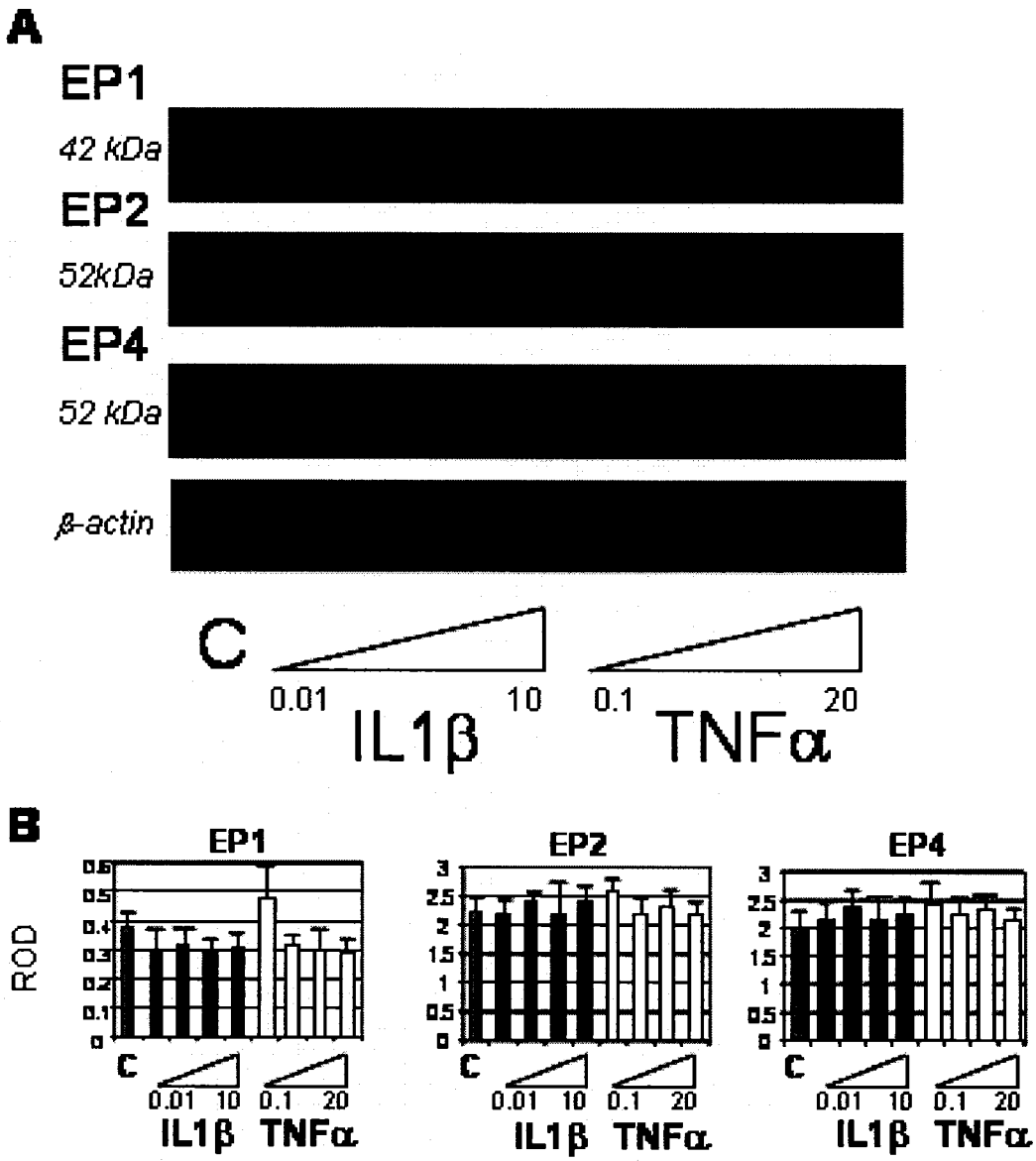


Figure IV-9. The protein levels of EP1, EP2 and EP4 after cytokine treatments in human chorion trophoblast cells. There was no significant effect of IL1β or TNFα on the protein expression of EP1, EP2 or EP4 at the doses used. (n=5)

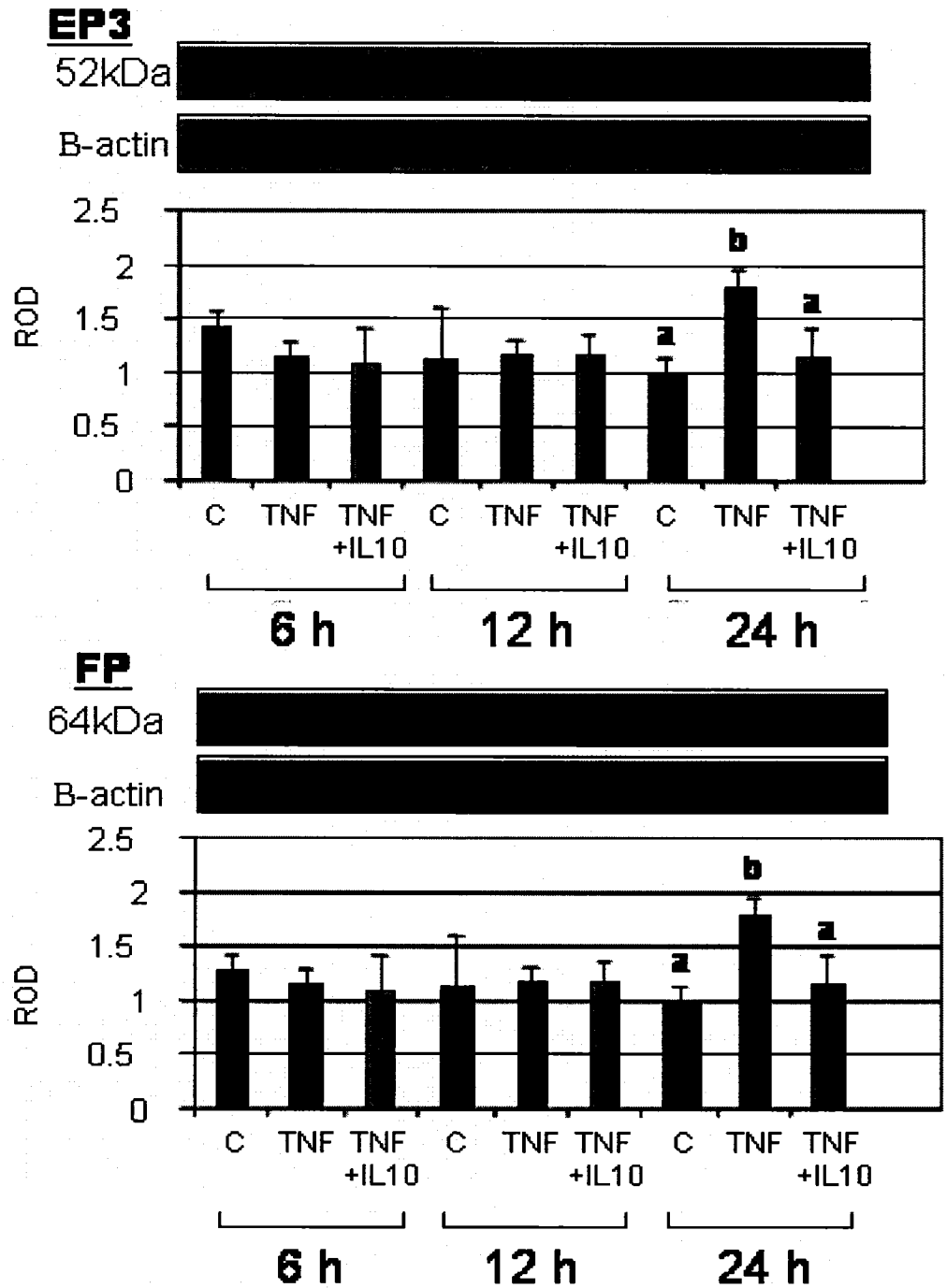


Figure IV-10. The time dependent effect of TNF-α on EP3 and FP receptor subtype in primary human chorion trophoblast cells. Cells were treated with 10 ng/ml of TNF-α alone or combined with IL-10 up to 24 hours. At the end of 24 hours the stimulating effect of TNF-α was significant on the EP3 and FP receptor levels. IL-10 could reverse the effect of TNF-α at the 24 hour time point. Histograms with different superscripts (a,b) are significantly different from each other ($p<0.05$). ($n=5$)

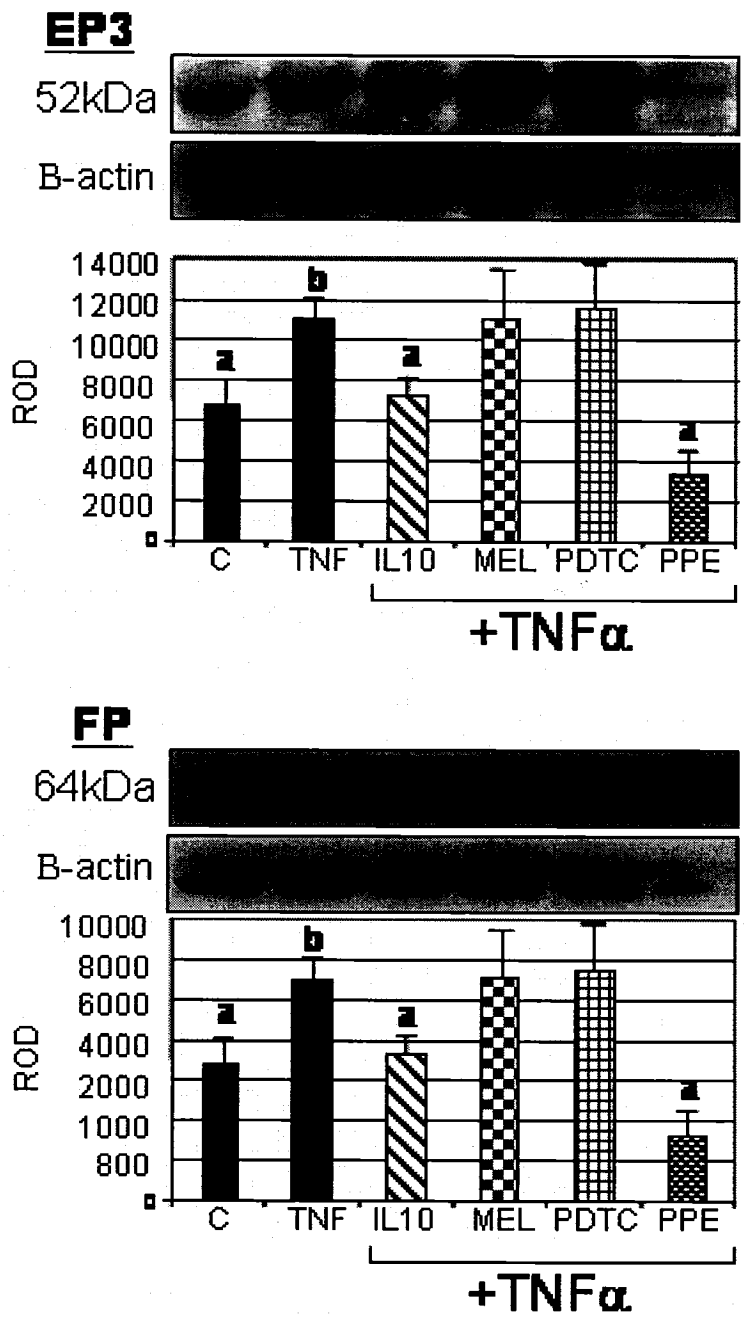


Figure IV-11. The regulation of stimulating effect of TNFa on chorion trophoblast cells. Increasing effect of TNFα on EP3 could be reversed by IL-10, an anti-inflammatory cytokine, and PPE, but not with meloxicam (MEL) or PDTC. Histograms with different superscripts (a,b) are significantly different from each other ($p<0.05$). (n=5)

CHAPTER V

Protein Levels of Prostaglandin Receptors during Preeclampsia and at Reduced Oxygen Tensions

V-1 Introduction

Prostaglandins (PGs) play a pivotal role in the initiation and maintenance of labor at term and preterm. These PGs elicit diverse biological responses via distinct G protein coupled receptors (GPCRs). In chapter III, I have clearly shown that prostaglandin receptors, EP1-4 and FP are expressed in fetal membranes and placenta, hence intrauterine tissues should be considered as an important site for PG actions beside myometrium. In the placenta, they were mainly localized in syncytiotrophoblast cells and around the blood vessels. In the fetal membranes, EP1, EP3 and FP receptor subtypes were upregulated with betamethasone treatment. However, the receptor levels were not altered by betamethasone or chorioamnionitis in the placentas from preterm deliveries, suggesting a differential regulation of the prostaglandin receptors in different layers of the intrauterine tissues.

Normal placental development is required for the birth of a healthy infant at term. The trophoblast layer of placenta is a highly specialized part of the placenta that provides an extensive surface area for nutrient and gas exchange, and triggers a series of physiological reactions in order to promote maternal blood flow to the implantation site with the hormones it secretes (Cross, 2006). Histological examinations revealed an increased proliferation of villous cytotrophoblast cells in preeclamptic placentas (Cross, 2006; Fox, 1964; Jones and Fox, 1980; Redline and Patterson, 1995). Moreover, the striking modifications of uterine spiral arteries, which provide the blood supply to placenta, following the trophoblast invasion do not take place in preeclampsia (Brosens et al., 1972; Gerretsen et al., 1981; Khong et al., 1986). A strong body of evidence supports the central role for the local oxygen availability in human trophoblast cell differentiation leading to appropriate placental development and function (Kingdom and Kaufmann, 1999) and it has been well established that preeclampsia is associated with reduced oxygenation of the placenta (Soleymanlou et al., 2005).

Even though there are contradicting results about the prostaglandin levels during preeclampsia (Hillier and Smith, 1981; Pedersen et al., 1983; Robinson et al., 1979; Valenzuela et al., 1983), inhibition of prostaglandin synthesis with low-dose aspirin is still

proposed as an effective preventive treatment for preeclampsia (Heyborne, 2000; Walsh, 1990).

While prostaglandins were accepted to be involved in the pathogenesis of preeclampsia, there is little information about the expression or function of prostaglandin receptors during preeclampsia. Thus, this study aims to investigate the protein levels of prostaglandin receptors at preeclampsia *in vivo* and the effect of different oxygen tensions on the receptor levels *in vitro*.

V-2 Materials and Methods

V-2.1 Tissue collection

Patient consent and ethical approval were obtained before tissue collection in accordance with the Canadian Tri-Council guidelines and the regulations of Mount Sinai Hospital, Toronto, and the University of Toronto. Preeclamptic (PE) (n=12) and age-matched control group placentas (n=12) were obtained from patients between 25–36 wk gestation. PE was defined as an increase in blood pressure to at least 140/90 mm Hg after the 20th wk of gestation, an increase in diastolic blood pressure of at least 15 mm Hg from the level measured before the 20th wk, or an increase in systolic blood pressure of at least 30 mm Hg from the level measured before the 20th wk, combined with proteinuria (protein excretion, at least 0.3 g/24 h) or edema (Chesley, 1985). The age-matched control group was primiparous with no clinical or pathological signs of preeclampsia or any other placental disease. Term human placentas were obtained from uncomplicated pregnancies after elective cesarean section delivery between 38–40 wk gestation.

V-2.2 Placental Trophoblast Cell Culture

Placental trophoblast cells were isolated from term placental cotyledons and cultured using a modification of the technique described in chapter III. Briefly, cotyledonary tissue (~60 g) was removed randomly from the maternal side and digested three times for 30 min each time with 0.125% trypsin (Sigma, St. Louis, MO) and 0.02% deoxyribonuclease I (Sigma) in DMEM (Life Technologies, Inc., Grand Island, NY) containing 10% FCS. The dispersed placental cells were filtered through a 200-μm pore size nylon gauze and loaded onto a 5–75% Percoll (Sigma) gradient at step increments of 5% Percoll and then centrifuged at 37 C at 1200 x *g* for 20 min to separate different cell types. Cytotrophoblasts between the density markers of 1.049 and 1.062 g/ml were collected. Cells were plated in six well culture plates (Falcon, Becton Dickinson, Franklin Lakes, NJ) at a density of 3×10^6 cells/well (for western blot analysis) in DMEM culture medium containing 10% fetal calf serum (Sigma) and 1% antibiotic–antimycotic solution (Sigma; penicillin, streptomycin, amphotericin B).

The dispersed trophoblast cells were cultured (n=4) for 12 hours at 37^0C in 5% CO_2-95% O_2 air to allow attachment. The cells were then divided into three culture conditions: (1) in standard tissue culture condition (20% O_2/95% air/5% CO_2), (2) in an atmosphere of 8% O_2/93% air/5% CO_2, and (3) in an atmosphere of 3% O_2/93% air/5% CO_2. For each condition cells were cultured in triplicates and for each of three different time periods. The cells were collected at the end of 24, 48 and 72 hours.

V-2.3 Immunohistochemistry

Prostaglandin receptors were localized in placenta using standard immunohistochemical procedures as described in Chapter III. All primary antibodies were used at 1/100 dilution in antibody dilution buffer and the slides were kept at 4^0C for overnight. Cells were then incubated with the biotinylated secondary antibody diluted in non-immune blocking serum, followed by an avidin–biotin peroxidase detection system (both for 2 hours at room temperature; Vectastain ABC Kit; Vector Laboratories, CA, USA). Diaminobenzidine (Sigma) was used to identify positive staining. Cells were counterstained in Mayer's haemotoxylin (Sigma), dehydrated in ascending grades of ethanol and mounted from xylene with Permount (Fisher Scientific, Fair Lawn, NJ, USA).

V-2.4 Western blotting for prostaglandin receptors

Western analysis of proteins was performed by SDS-PAGE on homogenates from the two different sources of placental tissue described earlier. Protein concentrations were determined by the Bradford assay as previously described (Bradford, 1976). The protein samples (40 μg for placental tissue and 20 μg for cultured cells) were separated by polyacrylamide gel electrophoresis using 11% separating gels and a 4% stacking gel as described in Chapter III. Computerized image analysis (MCID Imaging Research, St Catharines, Canada) was used to determine the relative optical densities of the protein bands in question.

V-2.5 Statistical Analysis

The results are presented as the mean ± SEM. Data were analyzed using 1-way or 2-way analysis of variance and subsequent pairwise multiple comparison procedures (Tukey method) to determine differences between two patient groups as well as within each group (for the different gestational ages). For all the tests, a value of $P < .05$ was considered statistically significant.

V-3 Results

V-3.1 Immunolocalization of Prostaglandin Receptors in Preeclampsia

Prostaglandin receptors were immunolocalized in the preeclamptic placentas (n=4) and age-matched control groups (n=4). A faint staining for EP1 was observed in the syncytiotrophoblast layer in both groups (Figure V-1A and B). EP2 staining was strong in both groups. EP2 expression was positive around the blood vessels and trophoblast layer. The localization did not seem to be changed in two groups (Figure V-2). In control groups EP3 was immunolocalized in the syncytiotrophoblast layer and around the blood vessels (Figure V-3C and D). EP4 staining in the trophoblast layer was almost diffuse in the control groups (Figure V-4C and D). EP4 receptors could be immunolocalized also around the blood vessels. FP receptors were mainly localized in the synctial layer (Figure V-5).

V-3.2 Protein Levels of Prostaglandin Receptors in Preeclampsia

Placentas that were affected with preeclampsia and age-matched control placentas with no identifiable pathology were compared. EP4 protein levels were significantly reduced in the preeclamptic placentas (Figure V-6). There was no significant difference in receptor levels related to gestational age. Conversely, EP3 was found elevated in the PE group (Figure V-7). Difference in the gestational age did not affect the results. EP1, EP2 and FP protein levels were consistent in the two groups studied. Preeclampsia did not seem to have a significant effect on the protein expression of these receptors (Figure V-8).

V-3.3 The Effect of Different Oxygen Tensions on Prostaglandin Receptors

In order to observe the effect of different oxygen tensions, purified placental trophoblast cells obtained from normal term placentas were cultured in decreasing oxygen concentrations (20%, 8%, and 3%). EP3 and EP4 levels were determined with western blot analysis. In our preliminary data, EP4 receptor levels were decreased when exposed to lower oxygen and this difference was significant at the end of 72 hours with 3% oxygen (Figure V-9). EP3, on the other hand was found at elevated levels in the same comditions compared to control groups (Figure V-10). .The results were not normalized with β-actin.

V-4 Discussion

In the current study I have investigated the prostaglandin receptor levels in placentas from preeclamptic patients and compared these receptor levels with the placentas from idiopathic preterm deliveries at the same gestational age. I showed a reduction in EP4 receptor subtypes in preeclamptic placentas whereas EP3 protein levels were found elevated in the same samples. Immunohistochemistry results indicate that the changes in the receptor levels are mainly due to syncytiotrophoblast layer. In further experiments, I have shown that in vitro decreased oxygen tension mimicked the changes that are observed in preeclampsia in vivo. When the syncytiotrophoblast cells were exposed to 3% oxygen tension, EP4 receptor subtype was decreased while EP3 protein levels were found elevated which are consistent with the observations from in vivo environment.

The preeclamptic placentas in this study were compared to the placentas from the same gestational ages with no identifiable pathology. Therefore, the two groups can be simply classified as (1) preterm placentas with preeclampsia; and (2) preterm placentas without preeclampsia. In further experiments in vivo environment was mimicked by an in vitro experimental model. This transition often results in loss of physiological parameters and in particular in experiments with different oxygen tensions (Lyall, 2006). Thus, for the in vitro part of our study we have applied exactly the same culture conditions as it was used before in our laboratory (Alfaidy et al., 2002; Manduch et al., 2006)

Studies have shown that, normally, cytotrophoblast invasion is accompanied by a reduction in the cells' proliferative capacity (Fisher et al., 1989). However in preeclampsia the invasion of cytotrophoblasts is shallow (Lunell, Nylund et al, 1982). Moreover the balance between proliferation and apoptosis has been disrupted. There seems to be an increased ability to proliferate (Redline and Patterson, 1995) with an increase in trophoblast cell death and shedding (Allaire et al., 2000; Crocker et al., 2001; Crocker et al., 2003; DiFederico et al., 1999; Ishihara et al., 2002; Levy et al., 2002; Sargent et al., 2003). Prostaglandins might potentially contribute to the pathology of preeclampsia by both mediating the invasion and cell cycle of cytotrophoblast cells. They have been shown to stimulate the migration of the cytotrophoblast cells in a well-characterized extravillous trophoblasts line HTR-8/Svneo and EP1 was shown to be involved in this process (Nicola

et al., 2005). On the other hand, they are also involved in the regulation of apoptosis and proliferation in various tissues, and, interestingly, this effect of prostaglandins seems to be cell-specific. While in some tissues prostaglandins induced apoptosis such as T- and B-lymphocytes (Brown et al., 1992; Goetzl et al., 1995; Mastino et al., 1992; Shimozato and Kincade, 1999); in rat spinal horn they displayed anti-apoptotic properties (Kawamura et al., 1997). Moreover, PGE_2 enhanced colon carcinogenesis through induction of cell proliferation and reduction of apoptosis (Kawamori et al., 2003).

In this current study the expression of two receptors were found altered in the preeclamptic tissues: EP4 receptor levels were reduced and EP3 was increased. EP4 is linked to $G\alpha$ subunits, thus activation of EP4 causes an increase in the cAMP concentration via stimulation of adenylate cyclase (An et al., 1993). EP3, on the other hand, mainly decrease cAMP generation by the inhibition of adenylate cyclase through the $G_i\alpha$-family (Negishi et al., 1988). Taken together, our results suggest reduced levels of cAMP via activation of different subtypes of prostaglandin receptors in preeclamptic placentas.

Cyclic AMP has been implicated in PGE_2 mediated apoptosis and proliferation. In hippocampal neurons PGE_2 induced caspase dependent apoptosis via elevated levels of cAMP (Takadera et al., 2004). Conversely, activation of cAMP inhibited apoptosis in gastric mucosal cells (Hoshino et al., 2003), and in the intestinal epithelial cells (Joseph et al., 2005). Furthermore, EP3 and EP4 have been implicated for the regulatory action of prostaglandins on apoptosis or proliferation in different systems. In neutrophils, EP3 activation promoted a novel form of cell death which could not be quite defined as apoptosis or necrosis (Liu et al., 2000). Alternatively, prostaglandin E2 promotes cell survival of glomerular epithelial cells via EP4 receptors by decreased apoptosis, and increased cell proliferation (Aoudjit et al., 2006). The anti-apoptotic effect of EP4 could be demonstrated on the mice hepatocytes (Kataoka et al., 2005) and TNFα induced apoptosis in neuron cells (Lee et al., 2003). Apart from apoptosis, in mice, EP4 was also involved in the proliferation and hypertrophy of vascular smooth muscle cells (Fujino et al., 2002). Taken together these results clearly demonstrate that PGE_2 can mediate apoptosis or proliferation of different cells at least in part via a cAMP dependent pathway.

A number of studies demonstrated that the abnormalities in cytotrophoblast differentiation that occur in preeclampsia can be duplicated by culturing normal cells in a

hypoxic environment suggesting that hypoxia can be an important proximal event leading to preeclampsia. In mammalian cells, oxygen tension could regulate both ability of the cytotrophoblasts to differentiate and expression of proteins that are critical to the invasion process (Anderson et al., 1989; Heacock and Sutherland, 1986; Sutherland et al., 1986). In vitro studies also demonstrated that the reduced oxygenation leads to increased levels of trophoblast proliferation (Caniggia et al., 2000; Genbacev et al., 1996; Genbacev et al., 1997; Soleymanlou et al., 2005). Under hypoxia, primary cytotrophoblast cells exhibit increased levels of apoptosis (Crocker et al., 2003; Levy et al., 2000). The main cellular pathway by which oxygen regulates gene expression is the formation of hypoxia inducible factor (HIF) (Caniggia et al., 2000; Wang et al., 1995; Wang and Semenza, 1993). HIF is a heteromeric protein complex comprised of two distinct subunits α and β. When oxygen tension is low, the labile α subunit forms a heterodimer with the constitutively expressed β subunit. Subsequently the heterodimer activates transcription by binding to Hypoxia Responsive Elements (HRE) in the promoter regions of a variety of genes (Caniggia et al., 2000; Forsythe et al., 1996; Iyer et al., 1998; Semenza et al., 1994; Semenza and Wang, 1992). Thus hypoxia can explicitly alter a cell's biology parallel to that seen in preeclampsia. In this study, I have also demonstrated that in cultured primary trophoblast cells EP4 and EP3 can be down-regulated or up-regulated, respectively, in hypoxic conditions consistent with our results from preeclamptic tissue in vivo. However, there is currently no information whether EP3 and EP4 genes contain HRE in their promoter regions.

In summary, prostaglandins are involved in the pathology of preeclampsia by mediating the migration and invasion of the cytotrophoblast cells or the balance of proliferation and apoptosis. Previous studies have demonstrated that these effects of prostaglandins in different tissues are associated with cAMP levels and prostaglandin receptors. Our study has clearly demonstrated the altered protein expression of prostaglandin receptors in preeclamptic placentas versus gestational age matched controls in vivo, or under the hypoxic conditions in vitro, which might be translated as a reduction in cAMP levels. Even though the contribution of prostaglandin receptors to the pathology of preeclampsia is currently unknown, this study suggests new targets for the therapeutic treatment of preeclampsia.

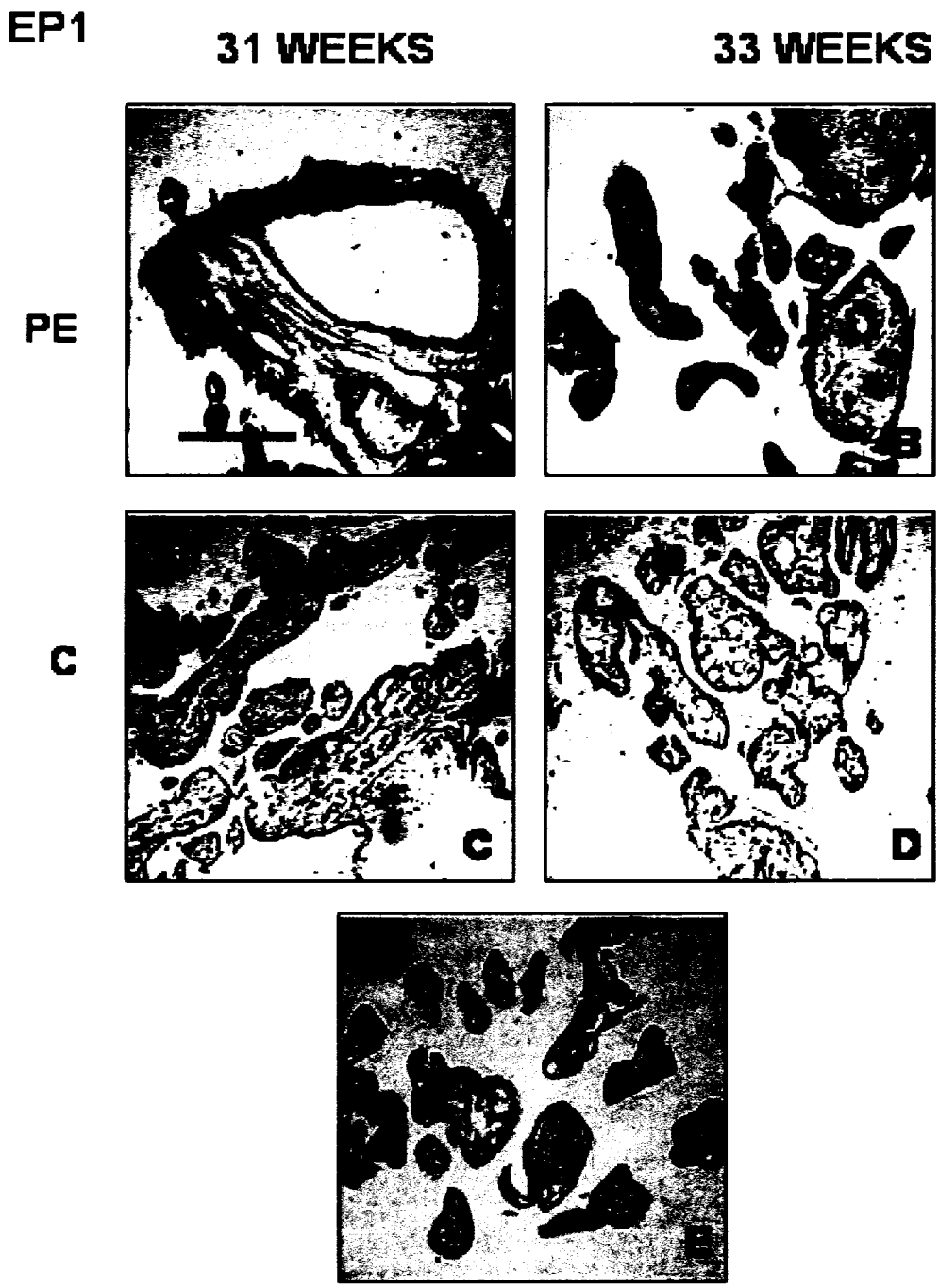


Figure V-1 Immunohistochemical analysis of EP1 receptor in preeclamptic tissue (PE) and idiopathic preterm deliveries (C). Placentas were collected from preeclamptic tissues at 31 weeks (A) or 33 weeks (B) of gestational ages, and age matched control groups (C, D). Preabsorption of the primary antibody eliminated the positive staining. Scale bar 100 μm.

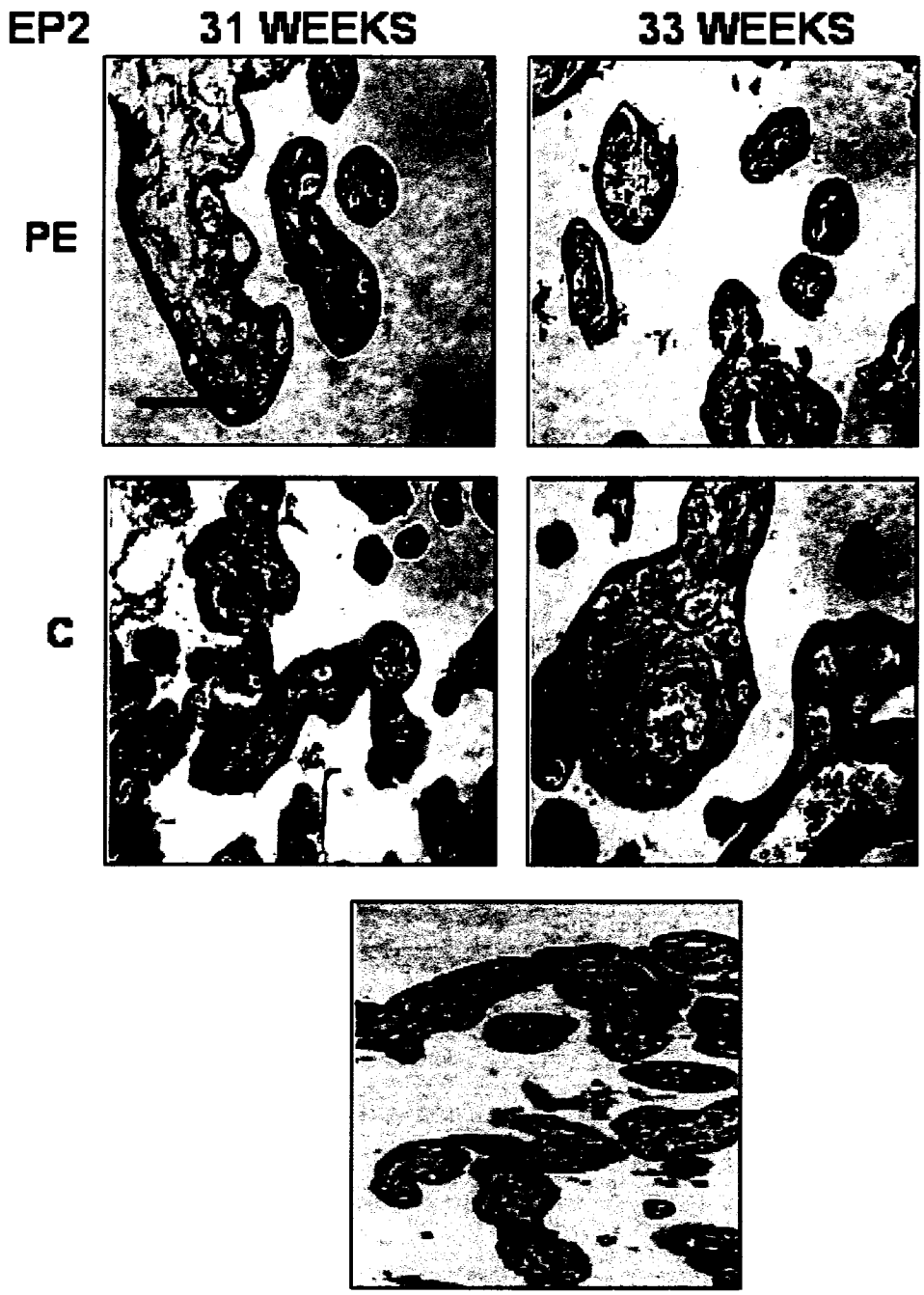


Figure V-2 Immunohistochemical analysis of EP2 receptor in preeclamptic tissue (PE) and idiopathic preterm deliveries (C). Placentas were collected from preeclamptic tissues at 31 weeks (A) or 33 weeks (B) of gestational ages, or age matched control groups (C, D). Preabsorption of the primary antibody eliminated the positive staining. Scale bar 100 μm.

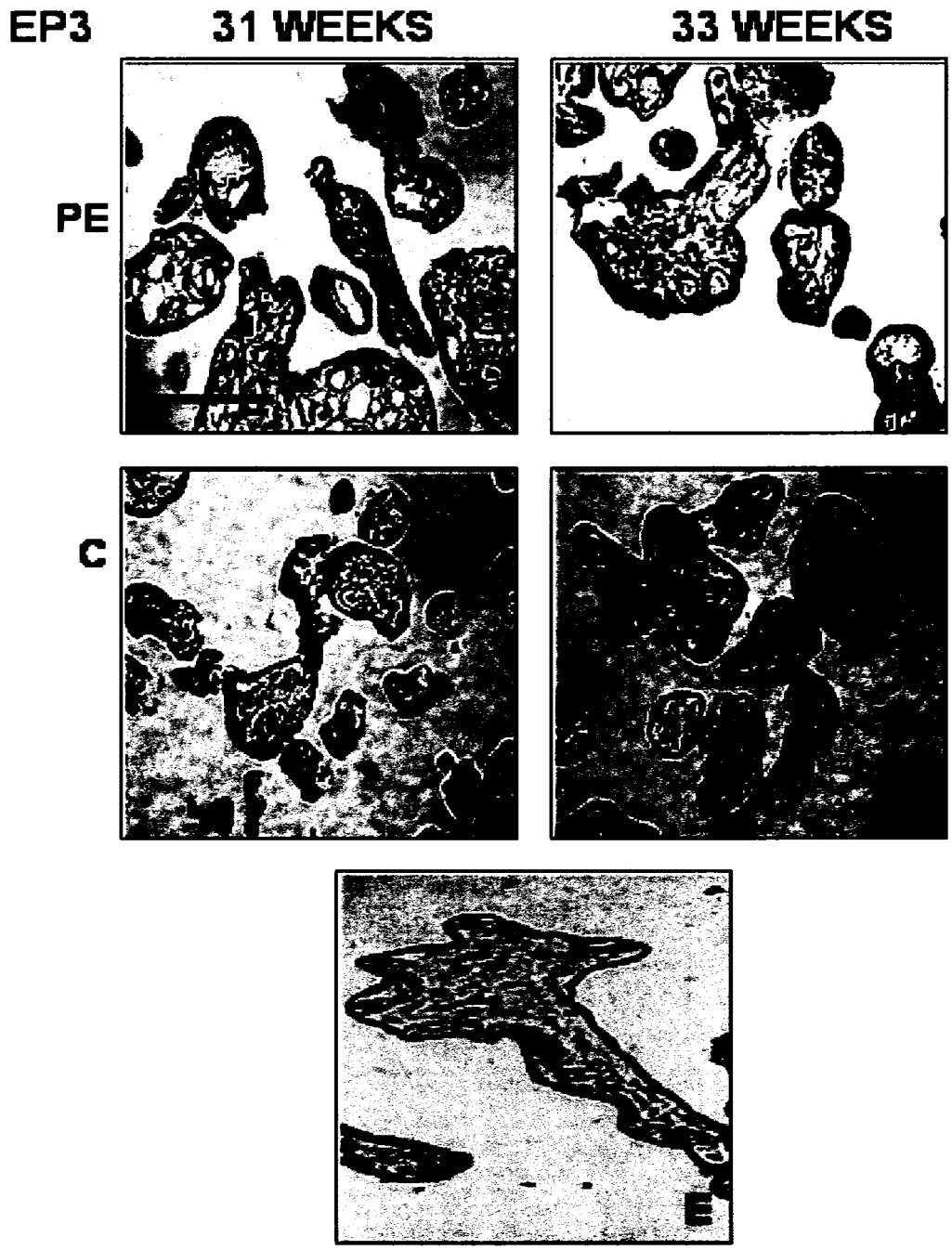


Figure V-3 Immunohistochemical analysis of EP3 receptor in preeclamptic tissue (PE) and idiopathic preterm deliveries (C). Placentas were collected from preeclamptic tissues at 31 weeks (A) or 33 weeks (B) of gestational ages, or age mathced control groups (C, D). Preabsorption of the primary antibody eleiminated the positive staining. Scale bar 100 μm.

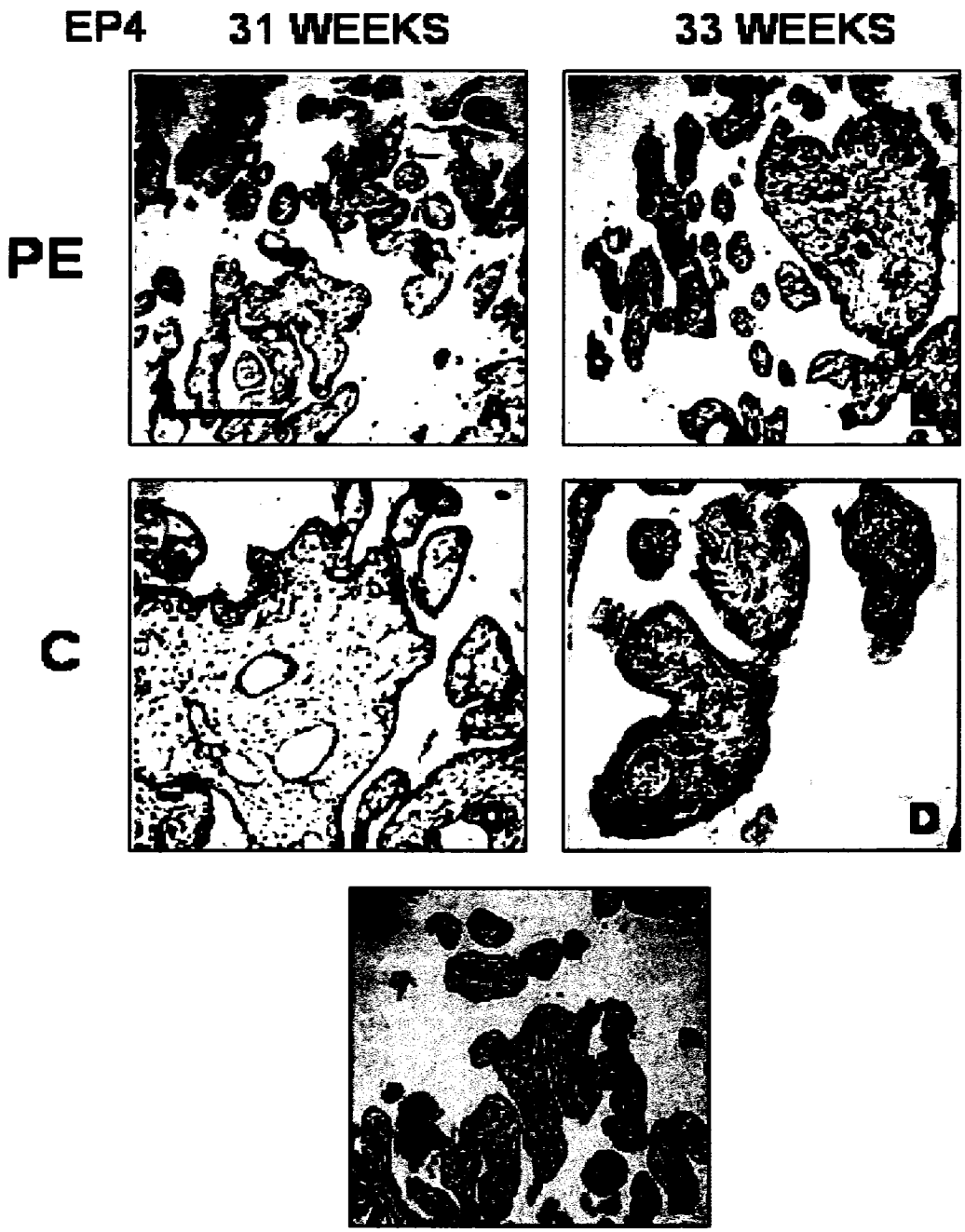


Figure V-4 Immunohistochemical analysis of EP4 receptor in preeclamptic tissue (PE) and idiopathic preterm deliveries (C). Placentas were collected from preeclamptic tissues at 31 weeks (A) or 33 weeks (B) of gestational ages, or age matched control groups (C, D). Preabsorption of the primary antibody eliminated the positive staining. Scale bar 100 μm.

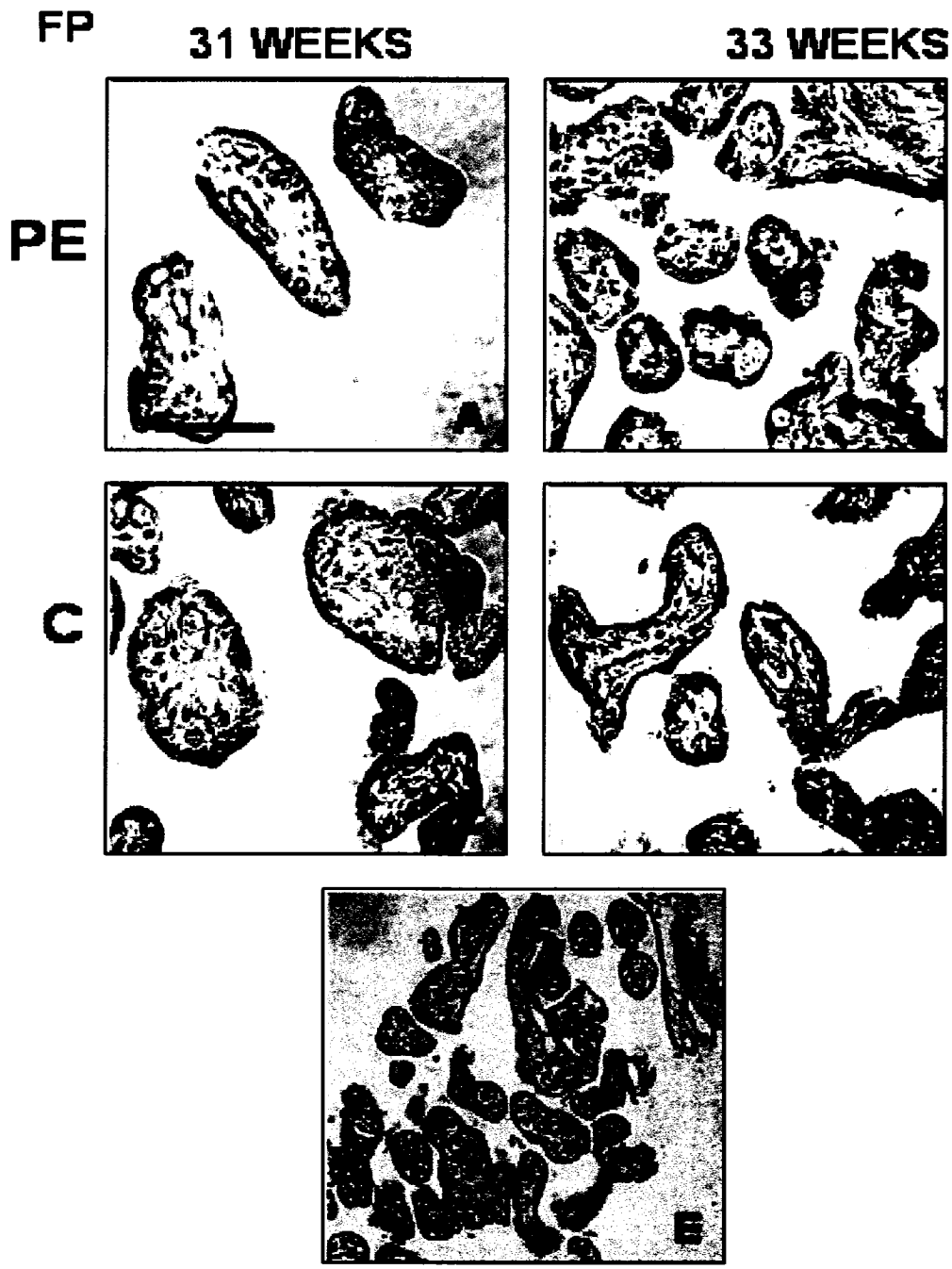


Figure V-5 Immunohistochemical analysis of FP receptor in preeclamptic tissue (PE) and idiopathic preterm deliveries (C). Placentas were collected from preeclamptic tissues at 31 weeks (A) or 33 weeks (B) of gestational ages, or age matched control groups (C, D). Preabsorption of the primary antibody eliminated the positive staining. Scale bar 100 μm.

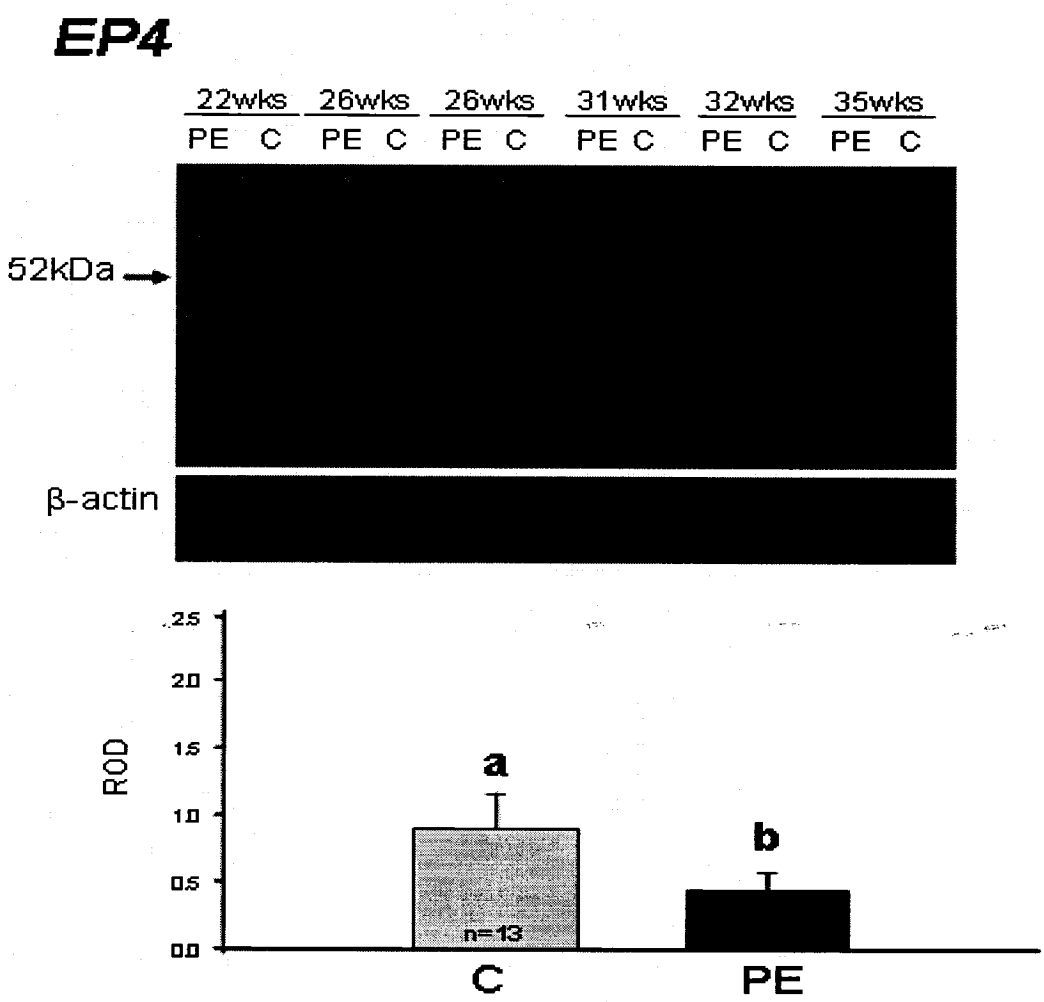


Figure VI-6 Western Blot analysis of EP4 receptor subtype in preeclamptic and age matched control placentas. Preeclamptic placentas (PE) (n=13) from 27th to 35th gestational ages were compared to age matched control groups (C) (n=13). Histograms with different subscripts (a,b) are significantly different from each other ($p<0.05$).

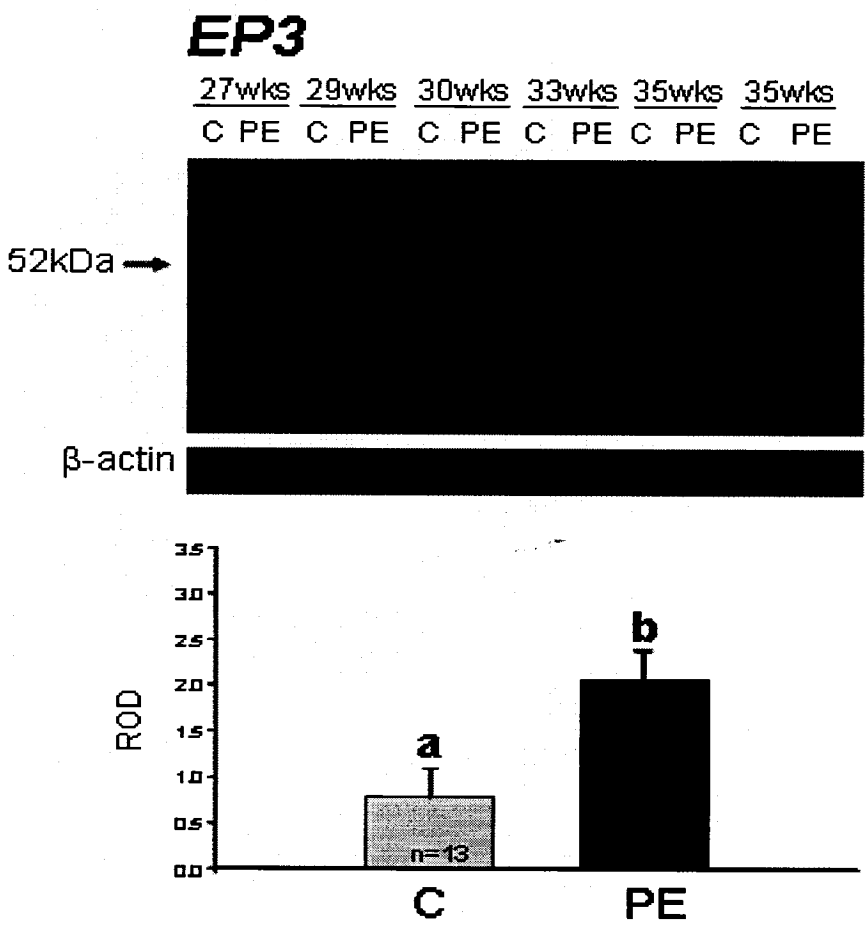


Figure VI-7 Western Blot analysis of EP3 receptor subtype in preeclamptic and age matched control placentas. Preeclamptic placentas (PE) (n=13) from 27th to 35th gestational ages were compared to age matched control groups (C) (n=13). Histograms with different subscripts (a,b) are significantly different from each other ($p<0.05$).

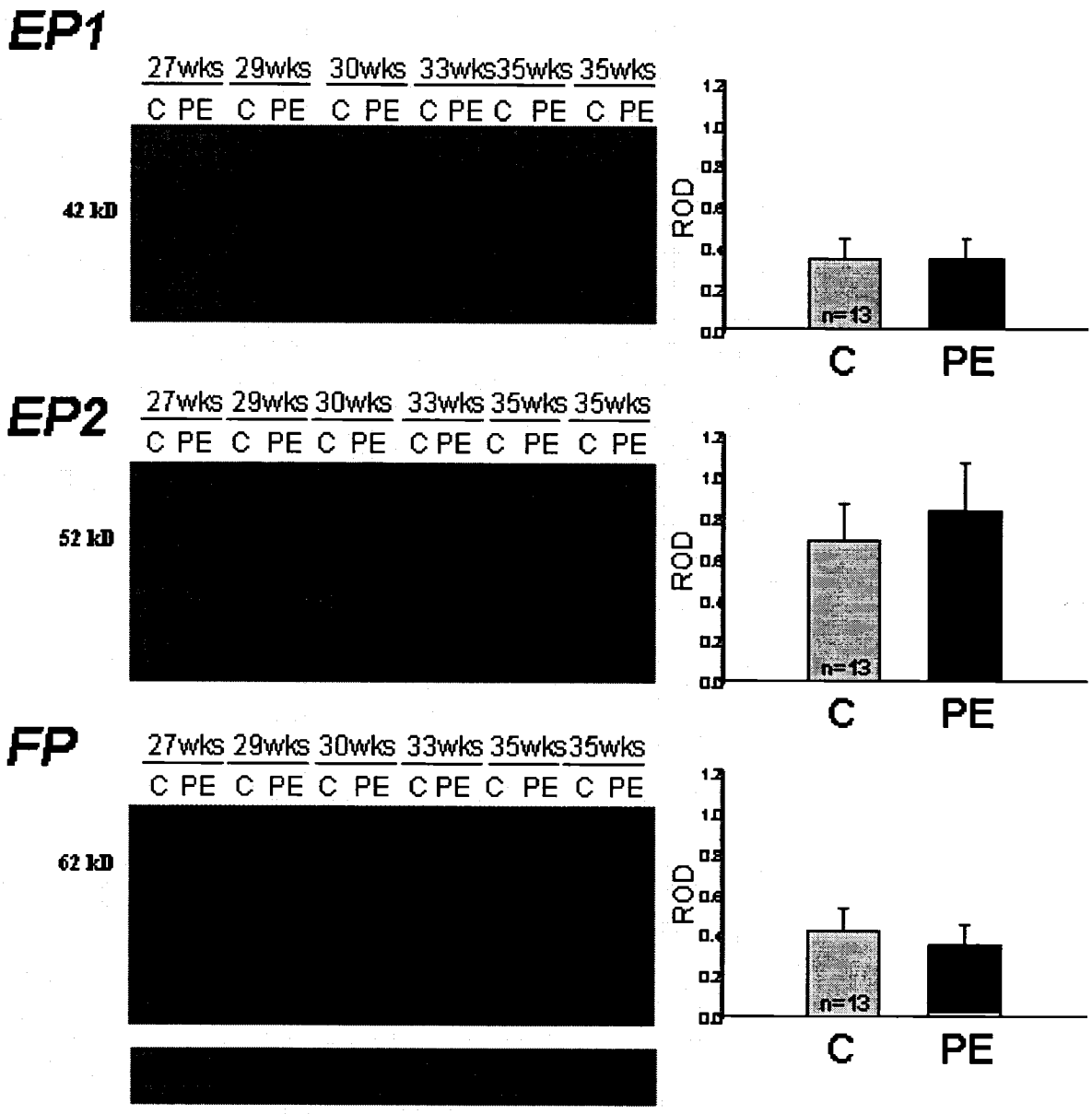


Figure VI-8 Western Blot analysis of EP1, EP2 and FP receptor subtype in preeclamptic and age matched control placentas. Preeclamptic placentas (n=13) from 27th to 35th gestational ages were compared to age matched control groups (C) (n=13). EP1, EP2 and FP protein levels did not show a significant difference between the two groups.

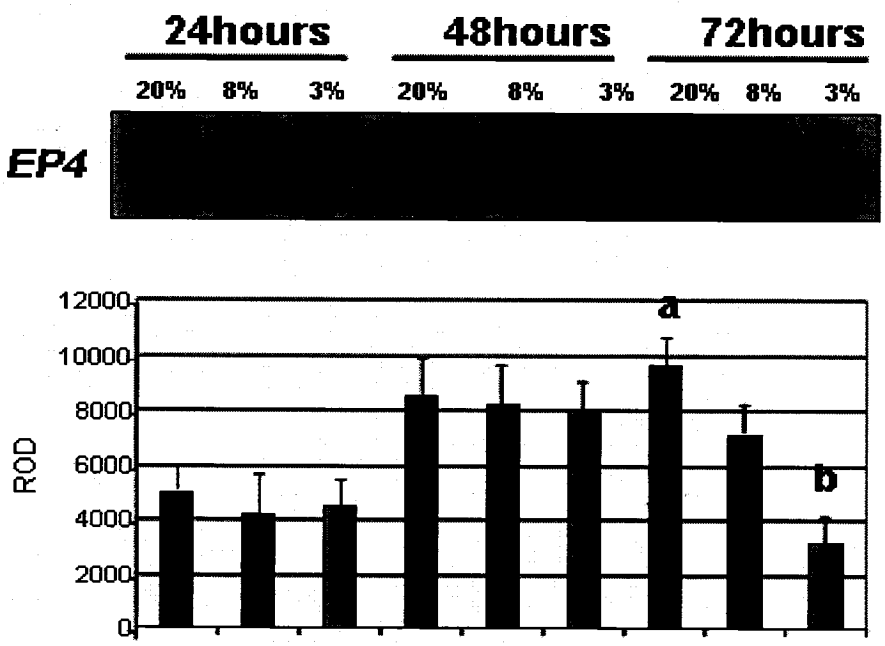


Figure VI-9 The effect of decreased oxygen tension on EP4 protein levels in human placental trophoblast cells. Cultured cells were exposed to 20%, 8% or 3% oxygen tension for up to 72 hours (24h, 48h, 72h), (n=4). Histograms with different subscripts (a,b) are significantly different from each other ($p<0.05$).

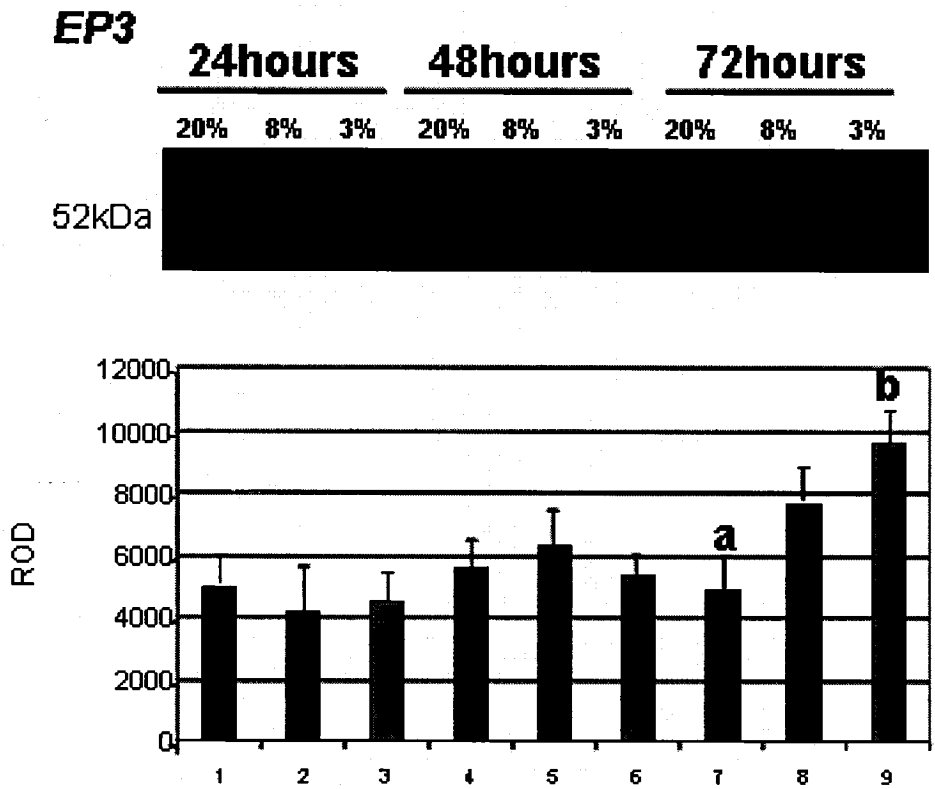


Figure VI-10 The effect of decreased oxygen tension on EP3 protein levels in human placental trophoblast cells. Cultured cells were exposed to 20%, 8% or 3% oxygen tension for up to 72 hours (24h, 48h, 72h), (n=4). Histograms with different subscripts (a,b) are significantly different from each other ($p<0.05$).

CHAPTER VI

Final Discussion

VI-1. Introduction to Final Discussion

Preterm birth remains the major problem of obstetrics. It is the leading cause of neonatal mortality and morbidity. While preterm delivery has an enormous medical and economic impact, to date no effective diagnostic indicators or efficient treatment have been found. Therefore, understanding the mechanisms that lead to parturition is of great importance. Recent studies support the thesis that parturition in women is affected by local paracrine/autocrine interactions within the uterus and fetal membranes. The activated fetal hypothalamic-pituitary-adrenal (HPA) axis causes increased steroidogenic enzymes in placenta which in turn elevates the output of prostaglandins (PGs). These prostaglandins act on their specific receptors (EP1-4 and FP) identified in myometrium causing either relaxation or contraction.

At the start of this thesis, 4 years ago, there was a very limited amount of information concerning the expression and function of prostaglandin receptors in human intrauterine tissues. As I started to write this thesis two other studies reported the expression of PG receptors within human placenta and fetal membranes. Based on immunohistochemistry and mRNA evidence Myatt's group has shown the presence of EP receptors in human fetal membranes and placenta (Grigsby et al., 2005; Grigsby et al., 2006; Grigsby et al., 2006; Grigsby et al., 2005). No coordination in receptor expression between the maternal and fetal tissues was reported indicating different regulatory mechanisms as well as different functions for PG receptors in the human fetal membranes and myometrium.

The long-term objectives of this research program were to understand the mechanisms responsible for the onset of human labor at term and preterm. The short term objectives of this thesis were i) to determine the localization of PG receptors within human fetal membranes and placenta at term and preterm, and to examine the changes in the receptor expression *in vivo*; iii) to determine the role of cytokines and oxygen tension in altering the expression of PG receptors in fetal membranes and placenta, respectively.

My overall hypothesis was that the expression of PG receptors differs among the layers of placenta and fetal membranes, and changes during labor at term and preterm; and that the expression can be affected by pro-inflammatory cytokines and/or oxygen tension. Studies from this thesis have clearly established the existence of prostaglandin receptors,

EP1-4 and FP, in human fetal membranes and placenta. I have shown labor associated changes in the term placenta and fetal membranes by comparing tissues from vaginal delivery and elective cesarean sections. EP1, EP3 and FP protein levels were significantly increased with labor. EP2 and EP4 levels were stable during the parturition process.

In preterm deliveries, in the whole thickness fetal membranes EP1, EP3 and FP receptor levels were found elevated in response to betamethasone treatment. This result indicates that the up-regulation in PG receptor levels observed during labor might, at least in part, be attributed to elevated levels of glucocorticoids. Interestingly, all the receptor levels were found reduced in the chorioamnionitis group. There is a strong possibility that this reduction might be due to loss of trophoblasts rather than the direct effect of infection. However, further studies are needed to define the effect of infection on PG receptor levels. In contrast, there was no significant effect of betamethasone or chorioamnionitis on the placenta indicating the existence of different mechanisms that control the expression of prostaglandin receptors in the different layers of human placenta.

In further experiments I have shown the immunolocalization of PG receptors in the specialized cells of the intrauterine tissues such as amnion epithelium (AE), amnion mesenchyme (AM), chorion trophoblast (CT) and placental trophoblast (PT). There were some differences in the amount of PG receptor protein between the tissue and cell culture samples. These differences might be due to following reasons: i) the samples that I collect for cell culture and tissue were from different locations. For the cell cultures almost the whole membranes were used, whereas the pieces of tissues collected were from the site of rupture; ii) with cell cultures we obtain a certain type of single cells (such as amnion epithelial or mesenchymal cells) without any extracellular matrix components or other cells that are localized in the membranes and placenta which might contribute to the protein levels of the receptors.

After describing the localization and regulation of PG receptors in vivo in Chapter III, I have explored the effects of cytokines on the receptors expressed in the chorion in Chapter IV. Finally, since the prostaglandin receptors are implicated in the vasculopathology, I have examined the effects of preeclampsia and different oxygen tensions on receptor levels in Chapter V.

VI-2 Regulation of PG receptors in Chorion with the Cytokines

There are strong data suggesting a correlation between the concurrent increase in intrauterine cytokine-prostaglandin (PG) production and labor even without any detectable infection. Two of the cytokines associated with the inflammatory process, IL-1β and TNFα, are elevated in the amniotic fluid at the onset of labor (Keelan et al., 2003). Both IL-1β and TNFα increase PG levels by acting at multiple points of PG biosynthesis pathway. They increase prostaglandin levels via elevated phospholipase and PGHS expression in gestational tissues (Hansen et al., 1998; Xue et al., 1996). Both pro-inflammatory cytokines also decrease the ratio of PG metabolites to PGs ($PGFM/PGE_2$) indicating a decrease in the activity of PGDH (Mitchell et al., 2000) (Figure VI-1).

I have clearly demonstrated that TNFα, but not IL-1β, both of which are pro-inflammatory cytokines, increased EP3 and FP protein levels in a time and dose dependent manner (Figure IV-8). These results with pro-inflammatory cytokines further support the hypothesis that the reduction we observed in chorioamnionitis was due to loss of trophoblast cells.

Studies indicate that IL-10 can mediate the activity of TNFα on synthesis and metabolism of prostaglandins (Hansen et al., 1999; Keelan et al., 2003) consistent with what I have shown in this thesis. In human chorion trophoblast cells, IL-10 was able to reverse the effects of TNFα on prostaglandin receptor levels which strengthens the role of IL-10 as a key mediator in the control of local inflammatory events. Inhibition of NFκB, on the other hand, decreased the receptor expression below the control levels implying a regulatory effect for NFκB independent of exogenous pro-inflammatory cytokines.

The effect of prostaglandin themselves on their specific receptors were not studied in this thesis. Prostaglandins are considered as potential negative regulator of prostaglandin receptors' expression. Both exogenous and endogenous $PGF_{2\alpha}$ decreased the number of binding sites in rat myometrium, suggesting ligand-induced receptor down regulation (Molnar and Hertelendy, 1990). Moreover, the interactions between PGs and cytokines have been clearly established as previously mentioned. Therefore, I examined the effect of pro-inflammatory cytokines in a system where I could eliminate the effect of PGs. JEG-3 cells were ideal for this purpose since they are unable to produce PGs unless arachidonic acid is provided (Premyslova et al., 2006). The results in JEG-3 cells suggest that cytokines can mediate the expression of PG receptors, independent of prostaglandins.

In summary, in Chapter IV, I have demonstrated that pro-inflammatory cytokines can mediate the expression of PG receptors in human chorion trophoblast cells while increasing the output of PGs within the intrauterine tissues. Thus, by increasing both the PG levels and expression of PG receptors they can potentially mediate the PG actions and enhance the activity of PGs during labor.

VI-3 Regulation of PG receptors in Placenta

Prostaglandin receptors have been implicated in the response of the vasculature to adjustments in perfusion pressure and oxygen and carbon dioxide tension, as well as mediation of the actions of numerous factors (Wright et al., 2001). Since preeclampsia is associated with vasculopathology, I hypothesized that there might be an abnormality in the prostaglandin receptor expression in the preeclamptic placenta. Moreover, I hypothesized that I can mimic these changes in placental cells that are exposed to decreased oxygen tension as hypoxia has been proposed to play a key role in the pathogenesis of preeclampsia. Examination of preeclamptic placentas has revealed a decrease in EP4 and an increase in EP3 receptor subtypes compared to age-matched control groups. In further experiments when the cells from normal term placentas were exposed to decreased oxygen tension the same observations were observed.

Previous studies in different systems suggest that prostaglandin receptors might be effective on the migration and cell cycle of the cytotrophoblast cells. Prostaglandins were shown to mediate the migration of the cytotrophoblast cells (Nicola et al., 2005). Moreover, they were involved in apoptosis and/or proliferation in a number of tissues in a cell-specific manner, indicating the differentially mediated action via different receptor subtypes (Goetzl et al., 1995; Kawamori et al., 2003; Kawamura et al., 1997; Shimozato and Kincade, 1999). Cyclic AMP has been implicated in PGE_2 mediated apoptosis and proliferation in these actions of prostaglandins (Hoshino et al., 2003; Joseph et al., 2005; Takadera et al., 2004). In accordance, both EP3 and EP4 are known to mediate cAMP levels. EP4 causes an increase (An et al., 1993) whereas EP3 mainly decreases cAMP generation. Thus the changes in these receptor levels might be translated as a potential decrease in cAMP levels during preeclampsia.

The exact mechanism of the regulation of prostaglandin receptors during preeclampsia or the contribution of the differentially regulated receptor expression in the

pathogenesis of preeclampsia is currently unknown. However, the results I have presented in this thesis might provide potential new targets for the therapeutic treatment of preeclampsia.

VI-4 Local feed-forward cascades

Previous studies from our laboratory have demonstrated differential actions of prostaglandins in different layers of the intrauterine tissues. Alfaidy et al (2001) has shown that in chorion trophoblast cells, PGs act on FP receptors and increase the activity of 11β-HSD1 which results in elevated levels of cortisol. On the other hand, in placental trophoblast cells, PGE_2 inhibited 11-b hydroxysteroid dehydrogenase type 2 (11β-HSD2), which is responsible for cortisol metabolism. Taken together these results indicated the presence of different prostaglandin receptor subtypes localized in fetal membranes and placenta, and that they might be differentially regulated in the different layers of intrauterine tissues.

I have shown the existence of EP1-4 and FP receptor subtypes within the fetal membranes and placenta, in each of the specialized cells. Further studies revealed a differential in vivo regulation of prostaglandin receptors in different layers of placenta. In chapter III, I have shown the regulation of these receptors in the fetal membranes in response to labor at term, and to betamethasone treatment and chorioamnionitis at preterm. I have examined the effect of cytokines on the receptor expression in *in vitro* conditions as well, and observed a time and dose dependent increase in the EP3 and FP receptor levels in response to TNFα, but not IL-1β. In chapter V, on the other hand, I have shown that oxygen tension can affect the different receptor subtypes expressed in placental trophoblast cells.

Taken together, the results in this thesis demonstrate the involvement of prostaglandin receptors in the local feed-forward cascades that are previously proposed (Challis et al., 2005; Challis et al., 2002; Challis et al., 2000) (Figure VI-2).

Glucocorticoids seem to play a central role in the regulation of these local feed-forward cascades. They have been shown to stimulate local CRH production in chorion and placenta (Jones et al., 1989; Karalis et al., 1996; Robinson et al., 1988). Both glucocorticoids and CRH can stimulate PGHS-2 in amnion and chorion (Alvi et al., 1999; Gibb and Lavoie, 1990; Mitchell et al., 1988; Potestio et al., 1988; Whittle et al., 2000;

Zakar et al., 1992; Zakar and Olson, 1989). Glucocorticoids can also down-regulate the PGDH in chorion (Patel et al., 1999), thus acts in favor of increased net output of available PGs within the intrauterine tissue with the onset of labor. Prostaglandins that are mainly produced in the amnion mesenchymal cells in turn act on the 11-beta hydroxysteroid dehydrogenase enzyme and can potentially mediate the biologically active cortisol levels.

Prostaglandin receptors are also involved in the modest inflammatory response implicated during labor. Term and preterm labor have been shown to be associated with an intrauterine increase in cytokine levels (Dudley, 1997; Gibbs et al., 1992; Gomez et al., 1997) that are locally produced (Bowen et al., 2002). Pro-inflammatory cytokines, in particular TNFα and IL-1β act at multiple points of the prostanoid biosynthetic pathway resulting in elevated levels of PGs within the intrauterine tissues (Brown et al., 1998; Bry and Hallman, 1992; Lundin-Schiller and Mitchell, 1991; Mitchell et al., 1990; Mitchell et al., 2000; Pomini et al., 1999; Romero et al., 1989). I have clearly demonstrated that TNFα but not IL-1β increases also the receptor levels revealing a quite strong feed-forward cascade within the intrauterine tissues. IL-10 could reverse the effect of TNF-a. Moreover, NFκB is involved in the expression of PG receptors partly through the stimulation with cytokines.

It is clear from these results that once the process of parturition is triggered there are feed-forward cascades that involve cytokines, glucocorticoids and PG receptors. This cascade seems to get stronger at every step and most probably results in the rupture of the membranes and labor (Figure VI-1). As well as potentiating each other's activity, both inflammatory cytokines and prostaglandins were shown to increase the activity of matrix metalloproteinases (DiBattista et al., 1995; Katsura et al., 1989; So et al., 1992; Tjugum and Norstrom, 1985) which are thought to be the main mediators of degradation of extracellular matrix. Recent data from our laboratory indicates that PGs are involved in the regulation of MMP-9 in chorion trophoblast cells (Li and Challis, 2006). Prostaglandin receptors have been implicated in the proliferation and/or apoptosis of the cells, and the changed balance in these processes has been implied to play a role both in rupture of the membranes and pathogenesis of preeclampsia.

Taken together, prostaglandin receptors are important in mediating the local effects of increased prostaglandins at the time of labor. They act in favor of the local feed-forward cascades by interacting with locally produced hormones and cytokines, and most probably

mediate the responses of the cells towards the rupture of the membranes and labor. The trigger for parturition might not be the changes in the expression and regulation of prostaglandin receptors, but it is obvious that existence of prostaglandin receptors within the fetal membranes and placenta revealed new therapeutic sites for the treatment and prevention of preterm birth and possibly preeclampsia.

VI-5 Difficulties and Future Directions

The main advantage of using an in vitro system is working with a simplified system. In this thesis I have purified the specialized cells of the intrauterine tissues and examined the local possible interactions. However, I do recognize that the in vitro environment might not faithfully reproduce the in vivo milieu. The main problem with the in vitro studies I foresee is the lack of interactions between the different cell types that are in close proximity within the tissue. Moreover, several factors such as culture media, oxygen tension, handling of the cells, and loss of structural integrity might alter the results of in vitro studies. Thus, I do recognize that the study presented in this thesis is the first step in the establishment of the role and importance of prostaglandin receptors in the physiology of term and pathology of preterm labor.

I have demonstrated the regulation of prostaglandin receptors with cytokines and oxygen tension. I have shown the effect of betamethasone treatment on the protein levels of prostaglandin receptors, but did not examine the effect of locally produced hormones in vitro. Although the prostaglandin receptors have been cloned, there is a limited amount of information regarding the regulation of prostaglandin receptors. Interestingly, a potential estrogen response element (ERE), a progesterone response element (PRE) for human FP gene (Zaragoza et al., 2004), and a progesterone response element (Katsuyama et al., 1998) have been defined for EP2.

We have speculated that the locally expressed prostaglandins are instrumental in the activity of elevated prostaglandins which might be involved in the rupture of the membranes or preeclampsia. However, further studies are needed in order to determine the role of prostaglandin receptors expressed within the fetal membranes and placenta. Moreover, results are needed to be verified in models where structural integrity is maintained and in animal models.

VI-6 Clinical Implications

Preterm delivery has an enormous medical and economic impact. However, currently there are no effective diagnostic indicators or effective treatment. Lack of understanding of the basic molecular mechanisms underlying human parturition seems to be the main obstacle in the prevention of preterm birth.

Corticosteroids were recommended for fetal lung maturation in threatened preterm birth. Currently, this is the only therapeutic intervention shown to improve neonatal survival and outcome by inducing the fetal lung surfactant production and preventing neonatal respiratory disease (Katz and Farmer, 1999; Liggins and Howie, 1972). However, adverse effects have been reported with multiple courses of corticosteroid treatment such as early-onset neonatal sepsis, chorioamnionitis, and neonatal death (Lee et al., 2004; Vermillion et al., 2000). At present, the management of preterm delivery includes prostaglandin synthesis inhibitors as tocolytic agents. Serious side effects have been reported with PG synthesis inhibitors as well (Glock and Morales, 1993; Lopez Bernal et al., 1993; Papatsonis et al., 2005; Papatsonis et al., 1997; Zuckerman et al., 1984; Zuckerman et al., 1984).

The existence of prostaglandin receptors during pregnancy and parturition might provide the development of more specific drugs with minor side effects.

VI-7 Conclusion

I have presented a focused set of experiments in this thesis that clearly demonstrates the existence of prostaglandin receptors in human fetal membranes and placenta and their involvement in the local feed-forward cascades. I have shown that the locally expressed prostaglandin receptors can be regulated at the time of labor at term and preterm. I have shown that there is a differential regulation of prostaglandin receptors within the different cell types of intrauterine tissues and proposed locally produced hormones and cytokines, and the decreased oxygen tension as the regulatory factors. Although the mechanisms by which the prostaglandin receptors contribute to the rupture of the membranes and uterine activity is currently unknown, results derived from various studies are highly suggestive of effectiveness of prostaglandin receptors antagonism as tocolytic therapy in the treatment of preterm labor.

This study contributes to our understanding of the mechanism of human parturition. It provides insights about the local regulation of the key mediators of parturition, prostaglandins, at the time of labor, thus reveals new target sites for the treatment of preterm labor.

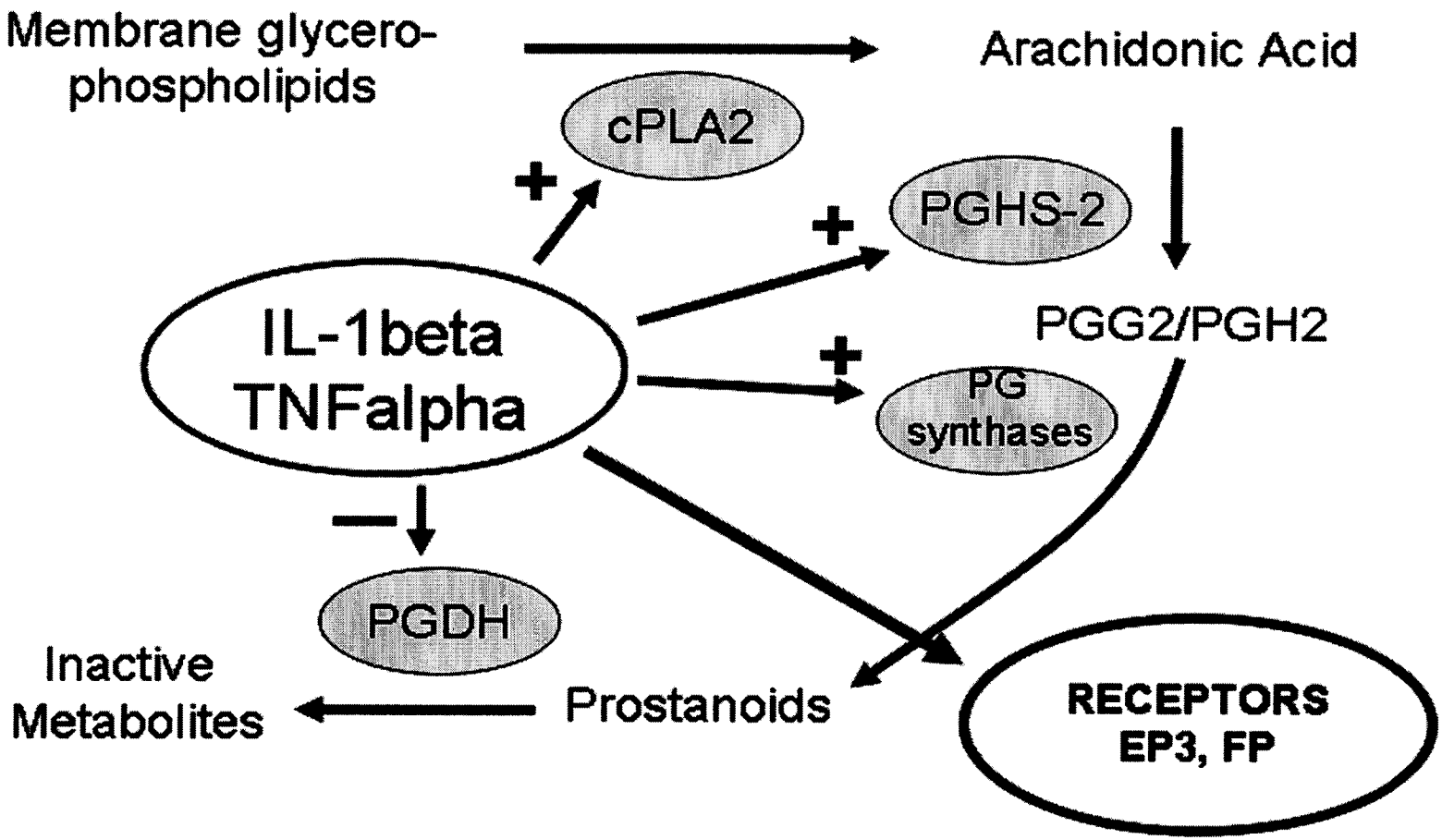


Figure VII-1 The effect of pro-inflammatory cytokines on prostaglandin biosynthesis pathway and prostaglandin receptors. Proinflammatory cytokines can increase the PG output by acting on multiple points of PG biosynthesis pathway. I have shown that they can also increase the receptor levels thus potentially enhance the local PG activity.

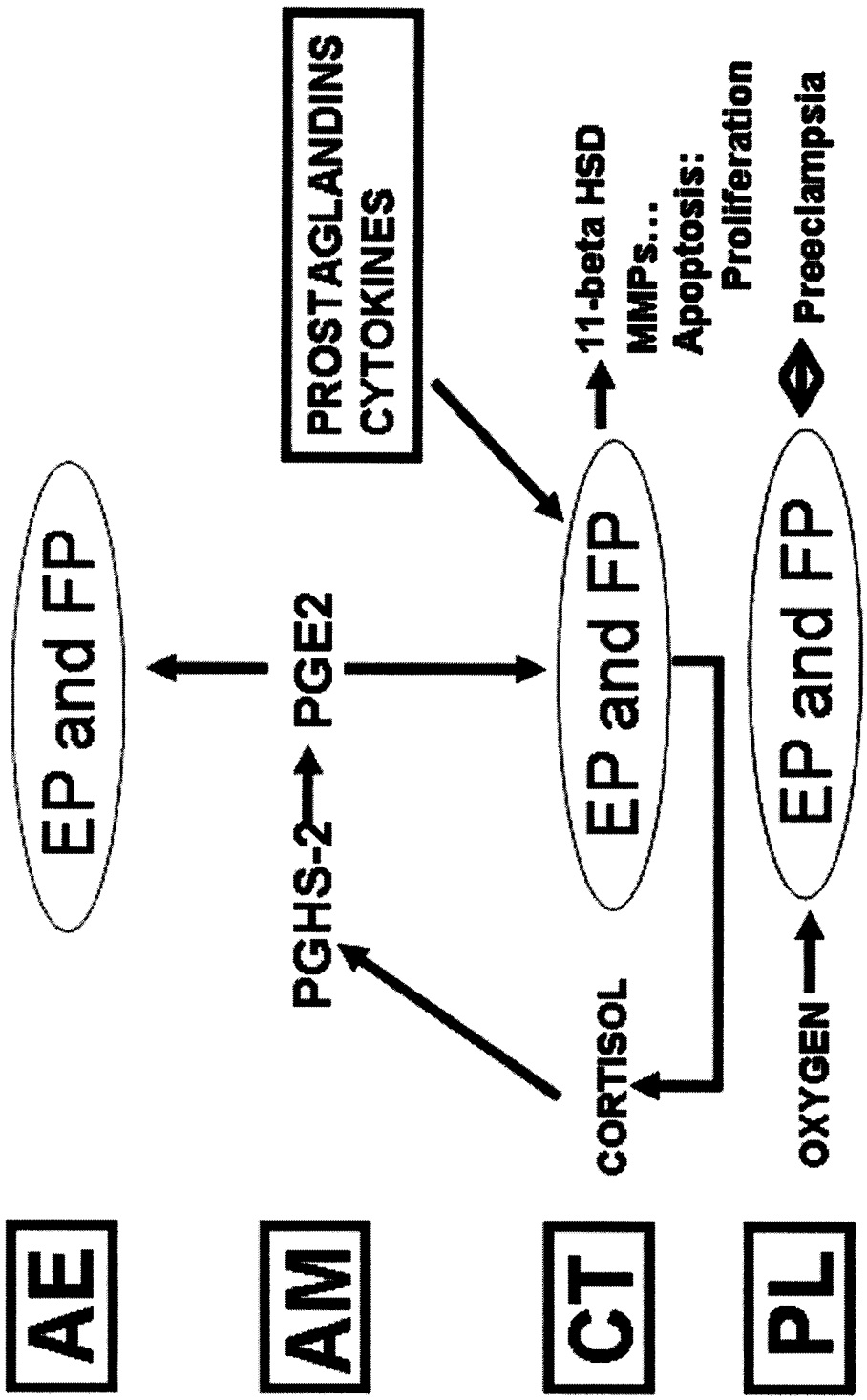


Figure VII-2. Proposed local feed-forward cascades involving prostaglandin receptors.

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