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SHORT REVIEW

Eicosanoids as Diagnostics for Preterm Labor

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Abstract: Despite continuing significant research efforts and an increasing prevalence of preterm labor (PTL) worldwide, a comprehensive understanding of the mechanisms involved remains to be established; such is the complexity of the interactions involved. Closing the knowledge gaps in this field would afford an opportunity to improve mortality and morbidity rates on a global scale. Early identification of pregnancies at risk of PTL would increase the possibility of delaying birth (where appropriate) prior to initiation of labor. This requires identification of appropriate biomarkers that are readily detectable in easily obtained clinical samples. A family of arachidonic acid metabolites, called eicosanoids. Eicosanoids have shown promise as possible diagnostic biomarkers for PTL. The inherent problems of antibody cross-reactivity in immunoassay methods has, however, resulted in possible misinterpretation of past results. The following mini-review outlines the necessity of such diagnostic targets and the problem with previous research methods and also summarizes recent findings in the field.

Keywords: preterm labor, endocannabinoid, eicosanoid, prostaglandin, prostamide

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Introduction

Although advances in research have been translated into improvements in management of pregnancy and neonatal care, preterm labor (PTL) remains a major obstetric health problem, as rates of preterm delivery (births occurring <37 weeks gestation) have not improved.[1] Even if existing interventions were fully scaled, fewer than 20% of PTL would be prevented,[2] so it is clear that there is much further work to be done. Premature birth is the single most important complication contributing to poor pregnancy and neonatal outcome, as it is strongly associated with low birth weight, increased incidence of perinatal mortality, and greater susceptibility to adult onset diseases.[3] Worldwide, approximately 15 million babies are born prematurely each year,[3] while recent statistics from the Australian Institute of Health and Welfare show that 8.2% of the babies born in Australia in 2009[4] and 8.3% of the babies born in Australia in 2010[5] were preterm. Hence, despite implementing current advised management plans, the occurrence of PTL is not decreasing. The rising morbidity rate has implications not only for quality of life of the affected child, but also for increased demand on health care systems and the associated fiscal burden of ongoing management and treatment of PTL infants[6] through to adult onset of PTL-related complications.[7] Fortunately, study of eicosanoids and the other factors involved in parturition is identifying putative biomarkers of PTL, with the aim of earlier intervention leading to increased deliveries at term and decreased PTL-related mortality and morbidity.

Biosynthesis of Eicosanoids

Eicosanoids are a family of signalling molecules, including prostaglandins and leukotrienes, that are oxygenated derivatives of 20-carbon essential fatty acids. One of the best characterized eicosanoid pathways is the oxygenation of arachidonic acid (an ω-6 fatty acid) to form prostaglandins (Fig. 1).

Arachidonic acid is a polyunsaturdated fatty acid that is present in cell membranes predominantly in an esterified form. It is usually esterified in the sn-2 position of glycophospholipids. The liberation of arachidonic acid is a key event in the biosynthesis of eicosanoids and considered the rate-limiting step in the pathway. The liberation of arachidonic acid from glycerophospholipids occurs by the direct action

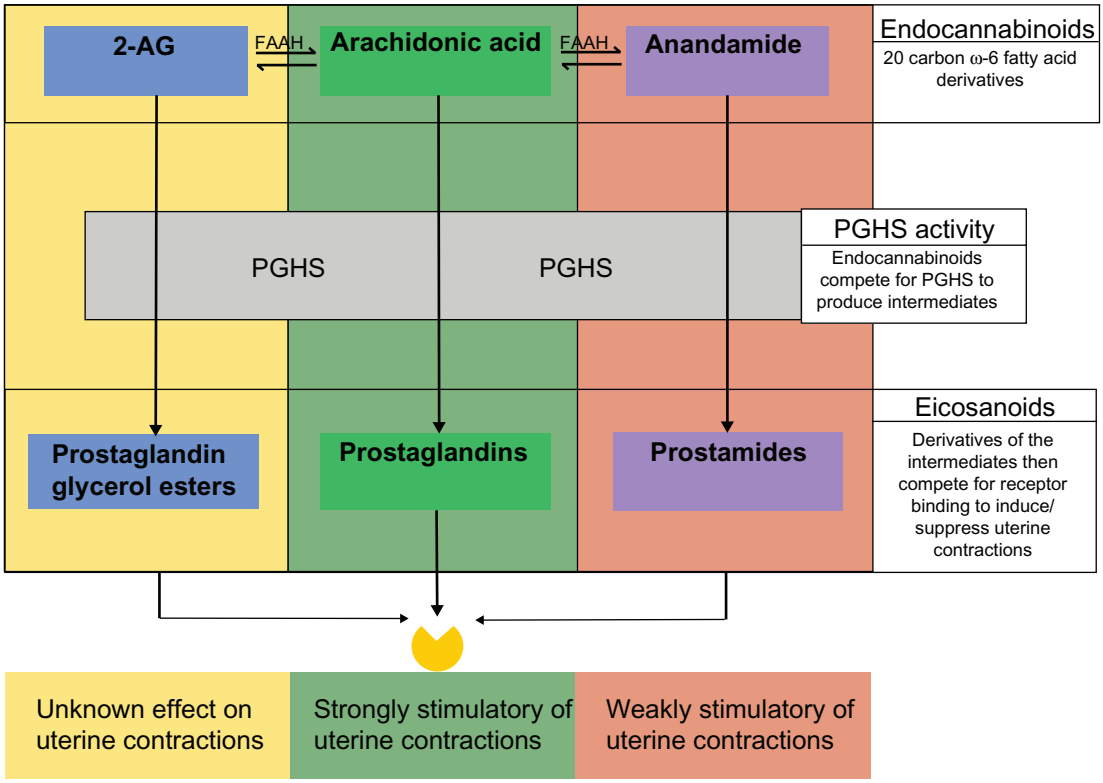
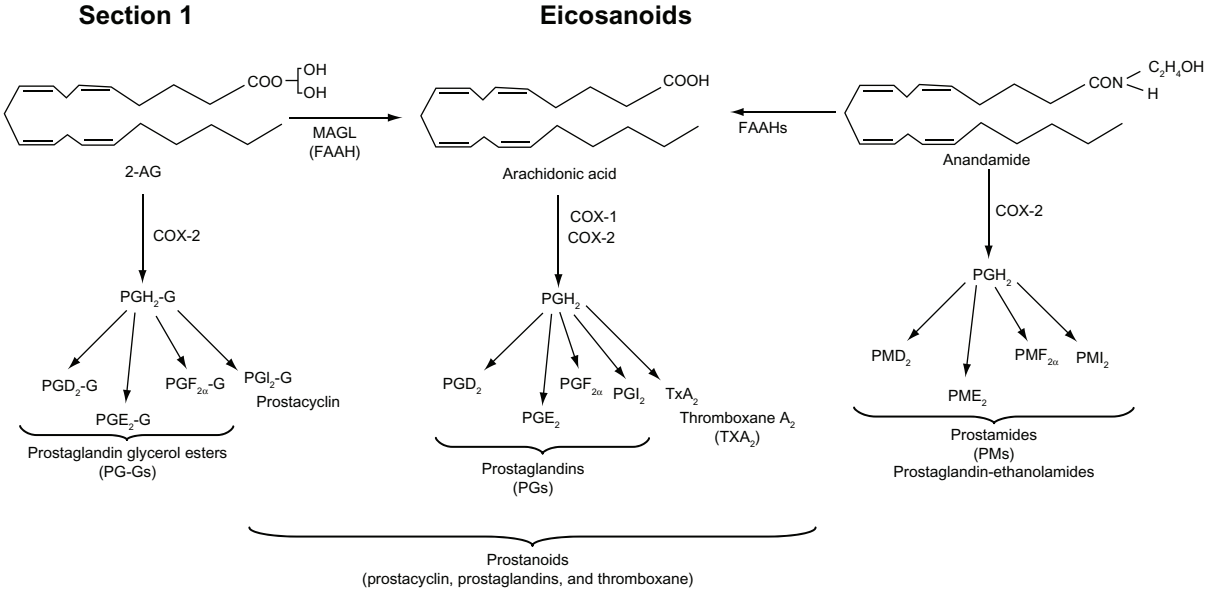


Figure 1. Endocannabinoid interrelationships and the effect of their metabolites on uterine contractions. 2-AG, 2-arachidonoylglycerol. FAAH, fatty acid amide hydrolase. PGHS, prostaglandin H synthase.

of phospholipase A_2 or indirectly by the action of phospholipase C and diacyl- and mono-acylglycerol lipase.

Phospholipids from cell membranes are cleaved by phospholipase A_2 resulting in the release of arachidonic acid and lysophospholipid. The action of fatty acid cyclooxygenase (COX), also known as prostaglandin H synthase (PGHS), on arachidonic acid is a key step in the production of prostanoids; arachidonic acid is converted by isoforms of COX (COX-1 constitutive [PGHS-1], or COX-2 inducible [PGHS-2]) to an intermediate, PGH_2, which is further modified, by a different enzyme in each case, to create a family of prostaglandins (PGs) (PGD_2, PGE_2, $PGF_{2\alpha}$, and PGI_2),



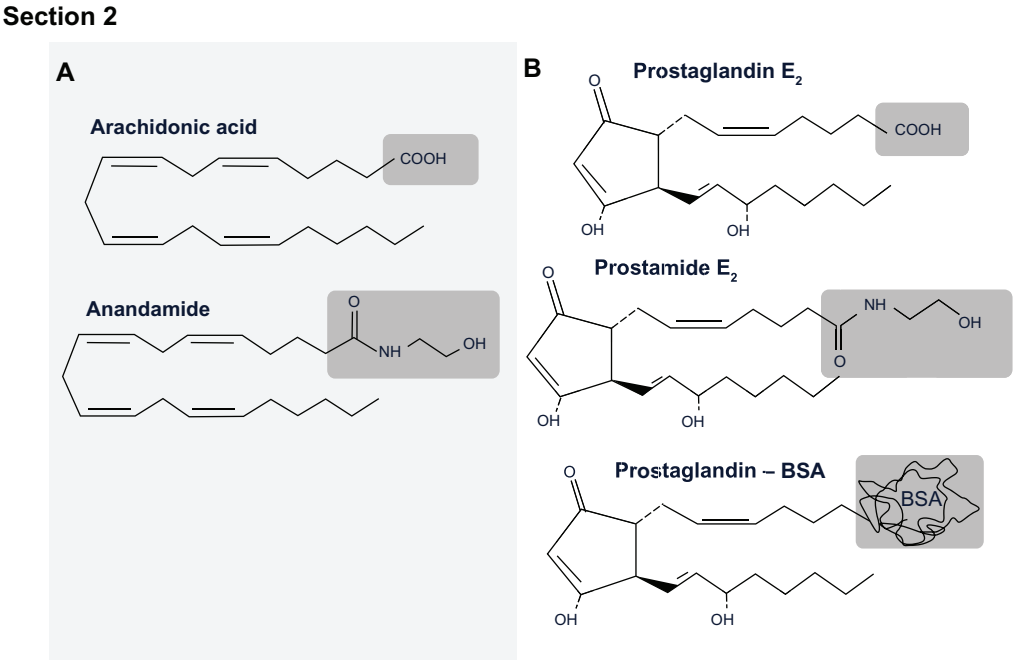


Figure 2. Section 1 The eicosanoid biosynthesis pathway demonstrating the relationship between subclasses of eicosanoids and the enzymes involved. 2-AG, 2-arachidonoylglycerol. FAAH, fatty acid amide hydrolase. MAGL, monoacylglycerol lipase. COX, cyclooxygenase (isoforms 1 and 2). **Section 2** Structural similarities between arachidonic acid and anandamide in panel A, and their metabolites prostaglandin E_2 and prostamide E_2, respectively, in panel B. Prostaglandin E_2 (PGE_2) conjugated to bovine serum albumin (BSA) is also shown in panel B; a conjugate commonly used as an antigen to raise antibodies to PGE_2. Note that prostamide E_2 has greater structural similarity to the antigen (PGE_2-BSA) than PGE_2 itself, due to the larger moiety at the carboxyl end (Modified from Woodward et al, 2008).[8]

as well as prostacyclin (PGI_2-G) and thromboxane A_2 (TXA_2)[8] (see Fig. 2, Section 1). Condensation of arachidonic acid with ethanolamine produces anandamide, an endocannabinoid (eCB) and the precursor of prostamides (PMs). As the term eicosanoid applies to all metabolites of arachidonic acid, the abovementioned factors have similar chemical structures with subtle differences (see Fig. 2, Section 2).

Factors Affecting Parturition

Parturition is effected by a combination of paracrine, endocrine, and mechanical stimuli[9,10] from both the mother and baby[11] that results in 4 distinct physiological events: cervical remodeling, uterine contraction, cervical dilatation, and fetal membrane rupture. These events are coordinated by multiple effector pathways, such as nuclear factor-κB (NF-κB) for the activation of inflammation,[10] and eCB and free corticotrophin-releasing hormone (CRH) for the onset of uterine contractions.[12,13] It follows, therefore, that PTL may occur when these signaling pathways are blocked, mimicked, or subverted such that the effector pathways are induced irrespective of fetal development.

Classic studies conducted by Mont Liggins, a New Zealand specialist in obstetrics, provided what is still today our basic outline of the mechanisms of parturition.[14] Unfortunately, those studies in sheep have not been transferable to humans without significant anomalies. The traditional theory of activation of the fetal hypothalamic-pituitary-adrenal axis and the coordinated stimulation of intrauterine PG production does not fit easily into the mechanisms of the onset of labor in humans although some parts can be translated successfully.[9] It is now unequivocal, however, that in all mammalian species studied, there is an absolute requirement for increased intrauterine PG production during the parturient process; treatment with PGs will induce labor, and inhibition of PG biosynthesis will prevent labor and delivery.[15] Increased intraunterine PG production during term labor is a cause rather than a result of labor,[16] and amniotic fluid PG concentrations increase prior to the initiation of labor.[17] Research has also shown that intrauterine-associated infection is the major defined, and only known, cause of PTL.[18] Indeed, it appears that PTL is associated with an exaggerated, more vigorous production of cytokines[19] that then stimulate intrauterine tissues to produce PGs.[18] Hence, the mechanisms that cytokines use to stimulate PG biosynthesis need to be thoroughly defined, with the aim of detecting biomarkers and devising intervention procedures in pregnancies at risk of PTL.

Etiology of Pregnancy Complications

A theory in contemporary obstetrics is that events during the first trimester of pregnancy that adversely affect placentation and the conversion of maternal spiral arteries may increase the risk of complications of pregnancy and contribute to poor pregnancy outcome. In particular, conditions that affect the function and responsiveness of placental cells may dramatically compromise placental perfusion and function[20–23] and the subsequent growth and development of the fetus.[24–35] The severity of dysfunction may determine outcome and result in a spectrum of clinical presentations that share a common etiology, with severe dysfunction being associated with miscarriage and lesser dysfunction leading to preeclampsia, intrauterine growth restriction, and a predisposition to PTL. It follows that the spectrum of complications may stem from the same or related triggering factors or conditions, with the extent of complication being proportional to the variation from normal physiological levels of a biological factor (or combination of factors). Evidence has been accumulating that points to PGs being a possible factor in complications of pregnancy, as discussed further in the following section.

Eicosanoids in Parturition

PGs play a critical role in the mechanisms of miscarriage and parturition both at term and preterm;[9,15] PGE_2, $PGF_{2\alpha}$, and TXA_2 initiate uterine contractions, ripening of the cervix, and even membrane rupture.[36–38] We observed a 2- to 6-fold increase in PG production in human amnion tissue explants and choriodecidua treated with eCB and synthetic cannabinoids (CP55,940–CB1/2 agonist).[39] This could be one mechanism by which eCB promotes labor onset. A similarly strong stimulation was observed when the experiment was repeated on isolated chorion tissue explants but not when tested on decidual tissue explants,[39] a finding that is in synchrony with those of Reese et al,[11] who used a COX knockout mouse model with embryo transfer. As the pregnant mice lacked the necessary enzyme (COX-1) to synthesize maternal PG,[11] the authors suggested that fetally derived factors alone may be enough

to successfully induce labor. From the above results, it appears that fetal tissues have a more pronounced response to cannabinoid triggers than maternal tissues and that fetal tissues may be a sufficient source of PG synthesis to coordinate parturition.

Prostamides are ethanolamide esters of PGs (See Fig. 2, Section 1). $PMF_{2\alpha}$, E_2, and D_2 were measured by liquid chromatography tandem mass spectrometry in liver, kidney, lung, and small intestine tissue in anandamide-treated wild type and fatty acid amide hydrolase (FAAH) knockout mice. It was found that concentrations of these PMs were at least 3-fold higher in the tissues of anandamide-treated FAAH knockout mice compared with their wild-type controls.[40] In most tissues, PM concentrations in wild type mice were below the limit of quantitation, indicating that reduced FAAH activity drives PM production. Paradoxically, anandamide has a relaxant effect on oxytocin-induced contractions in human myometrial tissue obtained at elective caesarean delivery performed at term.[12] This effect is suggested to be due to increased anandamide concentrations being linked to decreased uterine PG production.[41] One explanation for this relates to the actions of PMs. Bimatoprost, a synthetic PM which activates PM-sensitive receptors, weakly elicits contractions in both pregnant and nonpregnant human isolated myometrial tissue, whereas the tissues exhibit a stronger response to 17-phenyl-prostaglandin $F_{2\alpha}$.[42] The weak contractions elicited by PMs could result from their low affinity for PG receptors because the affinity of PME_2 for PGE_2 receptors is at least 500-fold lower than that of PGE_2.[43] If anandamide is being converted to PMs (as opposed to arachidonic acid and subsequently to PGs), we propose that increased PM concentrations may have a less stimulatory effect on myometrial contractions and hence promote labor quiescence rather than activation (see Fig. 3). However, alternative routes to contraction induction exist and are discussed in the section below titled A New Model of Preterm Spontaneous Onset of Labor.

Endocannabinoids and Early Prediction of Preterm Labor Risk

Endocannabinoid signaling is tightly regulated throughout normal pregnancy. Plasma anandamide

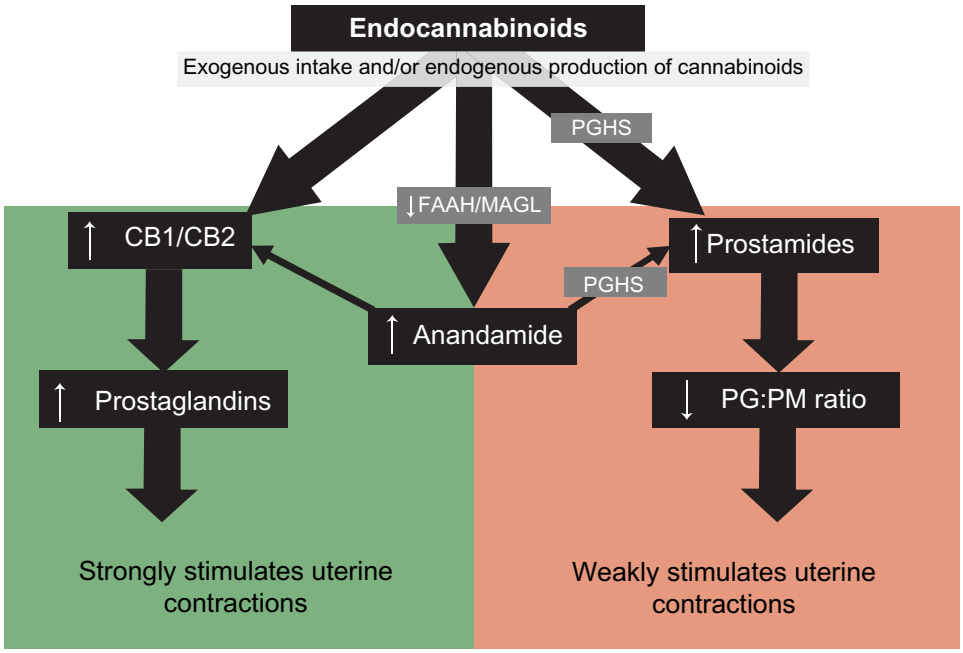


Figure 3. A new model of preterm spontaneous onset labor, implicating fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) activity, cannabinoid receptor (CB) expression, prostaglandins (PG) and prostamides (PM) as strong and weak stimulators of uterine contractions. Preterm labor may occur as a result of premature or abnormal fluctuations of concentrations of said factors. When FAAH and/or MAGL concentrations are low, conversion of 2-arachidonoylglycerol (2-AG) and anandamide to arachidonic acid is limited, which reduces availability of PG precursor (arachidonic acid) directly inhibiting prostaglandin production; if prostaglandin H synthase (PGHS) is present, the anandamide can be converted to PM. Together, the decreased PG and increased PM production lower the PG:PM ratio, causing weak stimulation of uterine contractions. Conversely, increased anandamide concentration increases placental CB receptor expression which has been associated with increased prostaglandin production, strongly stimulating uterine contractions.

concentrations (measured by mass spectrometry) are remarkably elevated in association with term labor.[44–46] Interestingly, plasma anandamide concentrations measured in the first trimester of women presenting with signs of potential miscarriage were 3-fold higher in those who subsequently miscarried compared with those who progressed to term,[47] and FAAH concentrations measured after less than 8 weeks' gestation were significantly lower in women who subsequently miscarried than in those women whose pregnancies continued to term.[48] Also, FAAH protein and mRNA expression, as well as FAAH activity in peripheral lymphocytes, were found to be markedly reduced in women who conceived unassisted but subsequently miscarried spontaneously, or failed to maintain pregnancy in the first trimester after undergoing *in vitro* fertilisation, compared to gestational age-matched women undergoing voluntary pregnancy termination.[49–52] This suggests that high plasma anandamide concentrations may be associated with uterine activation and labor onset and, therefore, may be surrogate biomarkers for placental dysfunction and/or poor pregnancy outcome.

It has been shown that placental cannabinoid receptor (CB1/CB2) expression is elevated 2.5-fold in spontaneous miscarriage during the first trimester compared to voluntary termination.[51] Conversely, CB2 expression is relatively unchanged throughout the first trimester,[53] and its expression in the second and third trimester remains to be determined. Recently, we were the first to publish evidence that eCBs and synthetic cannabinoids stimulate fetal membrane production of PGE_2 in a CB1-dependent manner.[39] There is an association between anandamide signaling and early labor onset; the intermediate processes remain unknown (ie, from CB receptor activation to intracellular signaling to biological outcome).

A New Model of Preterm Spontaneous Onset of Labor

Based on the studies described above and recent data from our laboratory, we propose a new model for spontaneous onset labor where the eCB pathway couples to PG synthesis in the placenta (Fig. 3).

Increased plasma anandamide concentration with reduced PG:PM ratio

Firstly, reduced FAAH activity is associated with premature labor. Low FAAH activity reduces anandamide conversion to arachidonic acid, therefore limiting arachidonic acid availability for conversion to PGs (which have a strong stimulatory effect on myometrial contraction). In the presence of PGHS, the anandamide is converted to PMs driving the PG:PM ratio down, and therefore has only a weak stimulatory effect on uterine contractions.

Increased CB receptor expression with PG production

If plasma anandamide concentrations are elevated, the maternal circulation will expose placental tissues to the eCB, inducing increased CB receptor expression. As CB receptors are activated by eCB, CB1 expression has been shown to be elevated in spontaneous miscarriage, and data from our laboratory that provides a link between CB1 expression and uterine PGE_2 concentration, it is possible that the enhanced CB1 expression in placenta may induce uterine contractions and labor onset via PGE_2 stimulation.

Issues with Previous Measurement Methods

Due to their common arachidonic acid precursor, PGs and PMs share the same lipid backbone with differing polar head groups at the position through which a large molecule is attached to provide antigenicity and thus raise antisera. The nature of antigenicity specificity is such that the antibody will recognize the lipid structure less well near the site of conjugation and will tend to favor similar molecules to the antigen—in this case a PG structure with a large functional group at the carboxyl end (In Fig. 2, Section 2, panel B demonstrates prostamide E_2 [PME_2] having a larger functional group at the carboxyl end than prostaglandin E_2 [PGE_2]). Hence, not only will an antibody raised to PGE_2 recognize PGE_2 as well as PME_2 but it will actually bind more avidly to the PM species.[54] The same will hold true for $PGF_{2\alpha}$, and so on.[54] Hence, we had hypothesized that antisera raised against PGs linked to a large molecule such as bovine serum albumin (BSA) at the carboxyl functional group (see Fig. 2, Section 2, panel B) would

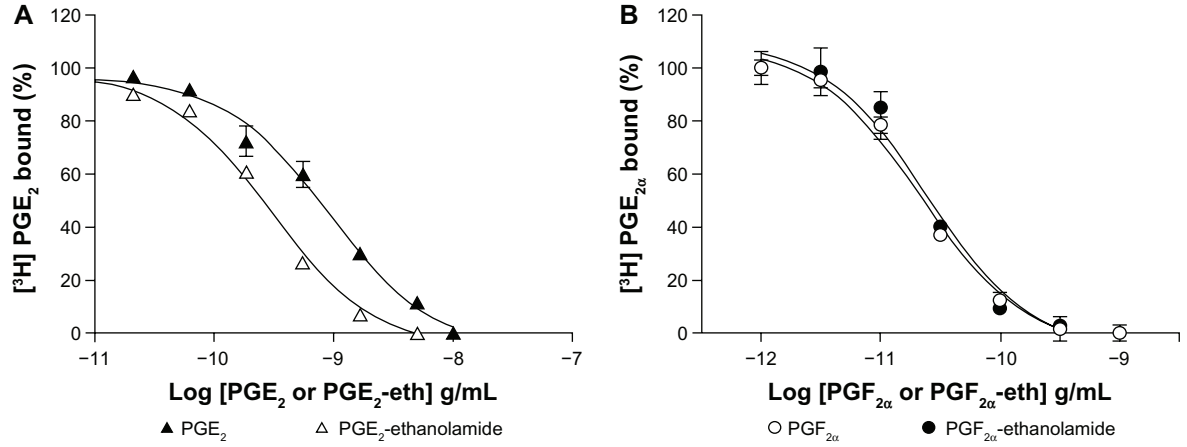


Figure 4. Cross-reactivity of prostamides in standardized prostaglandin radioimmunoassays was determined by the ability of prostamides to displace the titrated prostaglandin from the antibody compared with the parent compound. (**A**) Displacement of [3H]prostaglandin E_2 (PGE_2) from in-house-generated antibody by PGE_2 (closed triangles) or PGE_2-ethanolamide (PGE_2-eth; open triangles). (**B**) Displacement of [3H]prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) from anti-$PGF_{2\alpha}$ antibody by $PGF_{2\alpha}$ (open circles) or $PGF_{2\alpha}$-ethanolamide ($PGF_{2\alpha}$-eth; closed circles). Error bars represent standard error of the mean (Modified from Glass et al., 2005).[54]

also recognize PMs (eCB metabolites), leading to highly misleading interpretations of data. In a finding that is as exciting as it is concerning, we found this to be true, with major cross-reactivity of commercial antisera raised to PGs with eCB metabolites (Fig. 4) providing evidence that anandamide acts as substrate for PM production and that stimulation with cytokines induces mainly PM output rather than PG output.[54] This has important implications for the methods that rely on antibody-antigen interactions to detect and quantitate PGs, such as immunoassays, as all presently available antisera to PGE_2 recognize the related PM species. Hence, during inflammatory reactions when substrate is targeted to the site, it may be a PM that is secreted rather than a PG. Furthermore, in a well-characterized cell line (WISH) or primary amnion tissue explants, eCB treatment led to increased production of eCB metabolites as opposed to primary PGs. This was apparent only after separation of products by thin-layer chromatography (TLC)

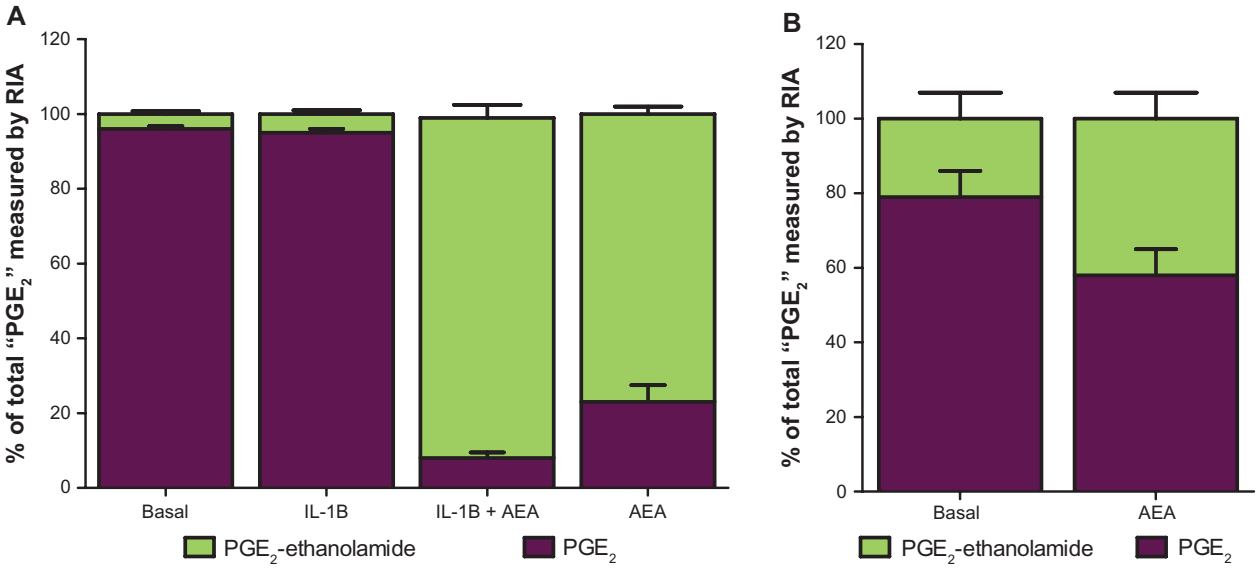


Figure 5. Relative proportions of prostaglandin to prostamide production by stimulated (**A**): WISH cells or (**B**): primary amnion explants. Results of thin layer chromatography (TLC) separation of prostaglandin E_2 (PGE_2) and PGE_2-ethanolamide followed by radioimmunoassay (RIA) with in-house antibody raised to PGE_2 on WISH cells and primary amnion explants, respectively. The purple portion of the bar represents the percentage of the total "PGE_2" signal that eluted with PGE_2 standards; green indicates the percentage that eluted with PGE_2-ethanolamide standards on the TLC plate. AEA, anandamide. Error bars represent standard error of the mean (Modified from Glass et al., 2005).[54]

because they were measured as PGs by radioimmunoassay (RIA) (Fig. 5).

Consequences of Misidentification

A few early studies of explant culture media[55] and plasma[56,57] used gas chromatography mass spectrometry (GC-MS)—a method not subject to the pitfalls of cross-reactivity between eicosanoid species—to demonstrate that an increase in PG concentrations occurs in human labor, but the overwhelming majority of studies (>99% upon review) have used immunoassays, and, hence, the resultant data interpretations may be unsound due to the reasons described in the previous section. Consider the variation that the cross-reactions induce across studies and conclusions and how this would affect reproducibility of results for a diagnostic. Moreover, all studies of cytokine effects on PG biosynthesis—a critical step in the mechanisms of intrauterine infection induced PTL—may have dramatically different results, potentially hiding some key information on a specific cytokine. This may include our own findings that eCB can stimulate PG production by human gestational tissues.[39]

The cross-reactivity of eicosanoids has particular importance in the field of PTL research because the major identifiable cause of PTL is intrauterine infection,[18] which may provoke enhanced secretion of the cross-reacting PMs; anandamide release has been described in response to hemorrhagic shock,[58] lipopolysaccharide treatment of macrophages,[59] and lipopolysaccharide challenge of human peripheral lymphocytes.[60] Likewise, PGHS-2 is induced by a range of inflammatory stimuli, such as IL-1β and lipopolysaccharide.[61] These findings suggest that at the site of inflammatory/infectious challenges, both anandamide and PGHS-2 may be increased to synergistically increase PME_2 production.

Clearly, our view of the role of PGs in human pregnancy and parturition and the utility of measuring these bioactive lipids as a diagnostic for PTL has been based upon what might be highly flawed interpretation of immunoassay results. The variability in measurements and thus lack of clinical utility as a diagnostic or predictor of PTL may be entirely due to a dynamic changing contribution of PMs and structurally-related similar compounds at different times throughout pregnancy.

Conclusions

Since increased intrauterine PG production is considered a critical step in the mechanisms of human parturition, it is possible that a potentially clinically useful role for PGs as diagnostic and predictive factors for PTL has been missed due to erroneous interpretation of immunoassay-related data. Advances in mass spectrometry, however, mean that this issue can now be addressed. Overall, this review presents potential biomarkers for risk of PTL (FAAH, COX-2, PM, PG, anandamide, and CB1) and has demonstrated why measurement by mass spectrometry is optimal for obtaining meaningful data and conclusions. Future studies may endeavor to isolate a tocolytic effective in humans with fewer adverse effects than those currently available, such as that which was investigated by Olson and Ammann.[62] Another avenue of further research would be to define, for each potential biomarker, the magnitude at which fluctuation from the normal gestational range indicates that a prophylactic tocolytic should be administered in order to improve neonatal morbidity and mortality rates.

Author Contributions

Conceived and designed the experiments: MDM. Analyzed the data: GER. Wrote the first draft of the manuscript: NCM. Contributed to the writing of the manuscript: MDM, GER, NCM. Agree with manuscript results and conclusions: MDM, GER, NCM. Jointly developed the structure and arguments for the paper: MDM, GER, NCM. Made critical revisions and approved final version: MDM, GER, NCM. All authors reviewed and approved of the final manuscript.

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Disclosures and Ethics

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under consideration for publication nor published elsewhere, of their compliance with legal and ethical guidelines concerning human and animal research participants (if applicable), and that permission has been obtained for reproduction of any copyrighted material. This article was subject to blind, independent, expert peer review. The reviewers reported no competing interests.

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