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Biosynthesis and catabolism of prostaglandin F_2(alpha) ($PGF_{2\alpha}$) are controlled by progesterone in the rat uterus during pregnancy

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Abstract

Myometrial quiescence is a key factor in all species to accomplish a successful gestation. PGs play a crucial role in mediating parturition events, and their synthesis and metabolism are regulated by cyclooxygenases (COXs) and NAD^+-dependent 15-hydroxy-PG dehydrogenase (PGDH), respectively.

Progesterone (P_4) is the hormone responsible for maintaining uterine smooth muscle quiescence during pregnancy. In this work, we have studied the effect of P_4 on the activity of COXs and PGDH, the uterine enzymes involved in the biosynthesis and metabolism of prostanoids in the rat.

We found that during pregnancy $PGF_{2\alpha}$ production and also protein levels of COX-1 and COX-2 were decreased.

The exogenous administration of P_4 significantly inhibited the uterine production of $PGF_{2\alpha}$ and also the protein level of COX-2.

$PGF_{2\alpha}$, metabolism was assessed by PGDH activity, which resulted high during pregnancy and increased as a result of P_4 administration.

These results indicate that PGs levels were negatively modulated by P_4, which could be exerting its effect by increasing PGs metabolism through stimulation on PGDH activity and an inhibition on COX and that is a major mechanism for maintain uterine quiescence in pregnancy.

Keywords: $PGF_{2\alpha}$; Progesterone; PGDH; Pregnancy

1. Introduction

As pregnancy advances, prostaglandins (PGs) increase in the uterus, leading to elevated uterine contractility. Therefore, regulating the concentration of PGs in the uterus can be a key factor for controlling the duration of pregnancy. PGs play a crucial role in mediating parturition events, and their synthesis and metabolism are regulated by cyclooxygenases (COXs) and NAD^+-dependent 15-hydroxy-PG dehydrogenase (PGDH), respectively.

The enzyme responsible for the rate-limiting step in PGs synthesis exists as two isoforms, COX-1 and COX-2. They perform a two-step conversion of arachidonic acid to PGH_2 via PGG_2. COX-1 is constitutively expressed in most cell types and has been considered a housekeeping gene [1], although more recent evidence shows regulated expression in both the inflammatory and reproductive systems [2,3]. COX-2 is an isoform expressed in macrophages and endothelial cells after being induced by LPS [4].

For the establishment and maintenance of pregnancy, uterine $PGF_{2\alpha}$ production must be suppressed to maintain uterus quiescence, and in some species to prevent luteal regression [5]. Thus, regulation of the synthesis and metabolism of $PGF_{2\alpha}$ within the intrauterine environment is critical in controlling the levels of bioactive PGs reaching the myometrium and cervix, the proposed sites of PG action.

In vivo, $PGF_{2\alpha}$ is rapidly catabolized into its stable, biologically inactive metabolite 13,14-dihydro-15-keto $PGF_{2\alpha}$ (PGFM). Plasma PGFM is often measured instead of $PGF_{2\alpha}$

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as the former is present at much higher levels allowing easier quantitation. The cytosolic enzyme responsible for initial inactivation of several prostaglandins (PGE_1, PGE_2, PGA_2 and $PGF_{2\alpha}$) is PGDH [6,7], which is present at high levels in the lung, liver, and placenta. In the feto-placental unit, PGDH has been implicated in the maintenance of pregnancy, as high PGDH activity in the chorion protects the myometrium from the high levels of $PGF_{2\alpha}$ produced by the amnion [8–10]. A number of correlations between PGDH enzyme activity and progesterone levels in human placental and uterine tissues have led to the suggestion that the enzyme is under steroidal control [11–13]. For example, the lungs of pregnant rabbits increase expression of PGDH in response to progesterone [14] perhaps to provide protection of the corpora lutea of pregnancy from circulating $PGF_{2\alpha}$ [15]. Despite the physiological importance of PGDH, little is understood about the mechanisms involved in regulating its expression. It can be inferred therefore that the COX isoenzymes and PGDH in concert determine the level of biologically active prostaglandins.

In a broader context, we hypothesize that progesterone in uterus would affect the activity of the uterine enzymes involved in the biosynthesis and metabolism of the prostanoids (COXs and PGDH), and these would change at the time of labor. To examine this possibility, we will consider the effects of progesterone on two aspects of prostanoid biosynthesis and metabolism in gestational tissues: conversion of arachidonic acid (AA) to bioactive prostanoids and prostanoid catabolism.

2. Materials and methods

2.1. Drugs and chemicals

Progesterone, $PGF_{2\alpha}$, $PGF_{2\alpha}$ antiserum were purchased from Sigma Chemical (St. Louis, MO, USA). Progesterone antiserum was purchased from G.D. Niswender (Colorado State University, Fort Collins, CO) and 13,14-dihydro-15-keto $PGF_{2\alpha}$ antiserum was obtained from Assay Designs Inc. (USA). Mifepristone (RU-486) was obtained from Biomol, Plymouth Meeting, PA.

13,14-Dihydro-15-keto-(5,6,8,9,11,12,14(*n*)-^{3}H)-$PGF_{2\alpha}$ (125 Ci/mmol, 1 mCi/ml), (5,6,8,9,11,12,14,15(*n*)-^{3}H)-$PGF_{2\alpha}$ (160 Ci/mmol, 200 uCi/ml) and 17α-hydroxy-(1,2,6,7-^{3}H-progesterone (60 Ci/mmol, 1 mCi/ml) were obtained from Amersham Corporation (Arlington Heights, IL, USA).

Western blot reagents were obtained from Sigma and Bio-Rad Laboratories. The antibodies for Western blot analysis were obtained from Cayman Chemical (Ellsworth Road, MI, USA). All other chemicals were of analytical grade.

2.2. Animals

Time-mated pregnant rats of the Wistar strain (200–230 g body weight) were used. They were maintained on a 12:12 h light–dark schedule. Animals received an ad libitum supply of animal chow and water. Pregnant animals (n = 6 for each state) were killed on different days of gestation (5, 13, 21, 22) as well as one day postpartum. Spontaneous term labor occurs on the night of the 22nd day (day1: day sperm plug observed). We have also obtained uterine tissue from cycling non-pregnant rats (n = 6) in dioestrus. The experimental procedures reported here were approved by the Animal Care Committee of the Centre for Experimental Pharmacology and Botany of the National Research Council (CEFYBO–CONICET) and were performed in accordance with the Declaration of Helsinki.

(1) Time-mated rats were divided into four groups (6–18 rats in each group).
(2) Rats on day 12 of gestation were injected i.p. with 0.3 ml antagonist of progesterone, RU-486 (10 mg/rat) and killed at 24 h after injection.
(3) Rats on day 12 of gestation were injected i.p. with ethanol (30%, 0.3 ml) and killed at 24 h after injection.
(4) Progesterone (Sigma, 4 mg/rat per day in 0.2 ml of sesame oil) was injected s.c. every 12 h from day 20 to 21 of gestation, and rats were killed on day 22, foregoing labor.
(5) Sesame oil (0.2 ml) was injected s.c. each 12 h from day 20 to 21 of gestation, and rats were killed on day 22 (controls).

All animals were anesthetized with ether and the blood was drawn from the heart. The serum was stored for steroid analysis.

2.3. Progesterone radioimmunoassay

Progesterone was measured in serum samples [16]. Briefly, the blood was allowed to clot and centrifuged at 3500 rpm for 10 min. Serum was removed and frozen until use. Serum was extracted twice with 2 ml diethyl ether, and progesterone concentrations were determined by radioimmunoassay. The progesterone antiserum was highly specific for progesterone and showed low cross reactivity (<2% for 20α-dihydro–progesterone and deoxycorticosterone and 1% for other steroids normally found in serum). The sensitivity was 5–10 pg per tube and 2–5 μl serum assayed routinely. Results were expressed as serum progesterone (ng/ml).

2.4. $PGF_{2\alpha}$ and PGFM determination

$PGF_{2\alpha}$ and PGFM were quantified by specific radioimmunoassay using rabbit antiserum (Sigma Chemical Co., St. Louis, MO, USA). The sensitivity of the assay was 5–10 pg/ml and the cross-reactivity was less than 0.1% with other prostaglandins. Intra- and inter-assay variations were each <8.0%. Results were expressed as ng of prostanglandins synthesized during 1 h/mg protein. The protocol was first described by Jaffe [17]. Briefly, uterus containing both myometrium and endometrium, was removed immediately,

cleaned of fat, placenta, fetuses, fetal membranes and blood vessels, and was then rinsed thoroughly in cold Krebs Ringer bicarbonate buffer (KRB; 145 mM Na^+, 6 mM K^+, 2 mM Ca^{2+}, 1.3 mM Mg^{2+}, 126.1 mM Cl^-, 25.3 mM HCO_3^-, 1.3 mM SO_4^{2-}, 1.2 mM PO_4^{2-}, 11 mM glucose) for $PGF_{2\alpha}$ and PGFM determination. Then, the tissues were incubated in a KRB buffer for 60 min at 37 °C. Uteri were used for measuring the total protein content by the Bradford method [18]. The supernatants were acidified to pH = 3 with HCl 1N and PGs were extracted twice with 2 ml ethyl acetate. PGs concentrations were determined by radioimmunoassay.

2.5. Western blot analysis

Uteri were homogenized immediately after collection in an Ultra-Turrax homogenizer (IKA, Staufen, Germany) in 20 mM Tris buffer (pH = 7,4), containing 1 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mg/kg benzamidine, 1mM DTT and 10 μg ml-1 soybean trypsin inhibitor. Then, the samples were sonicated for 10 s. The homogenate was spun at 7800 g for 10 min at 4 °C to remove debris and the supernatants were collected and kept at −70 °C until Western blot analysis. Data presented are pooled results from four animals and experiments were repeated three times. Protein concentrations were determined by the Bradford method, using bovine serum albumin as a standard. Equal amounts of total protein for each sample (70 μg) were loaded in 10% SDS-polyacrylamide gel and separated by electrophoresis. In the present study, we used mouse macrophage lysate as a positive control for COX-2 and sheep seminal vesicles as a positive control for COX-1.

Membranes were then incubated with either polyclonal rabbit anti-rat COX-1 antibody (1:600) or primary COX-2 polyclonal antibody (1:1000) (Cayman Chemical, Ellsworth Road, MI, USA) for 2 h and then, incubated with alkaline phosphatase-conjugated anti-rabbit IgG (Sigma, 1:5000) as the secondary antibody. After three washes, the membranes were developed with 5-bromo-4-chloro-3-indolyl-phosphate toluidine salt (BCIP) and nitroblue tetrazolium (NBT). Molecular weight markers (Bio-Rad) identified protein bands. Each blot was repeated three times, with different samples from different animals. Pictures of the membranes were taken, scanned densitometrically and analyzed using a Dekmate III scanner (UMAX, Dallas, TX, USA) and SigmaGel software (Sigma Chemical).

2.6. Statistics

Statistical analysis was performed using the Instat Program (Graph Pad Software, San Diego, CA). Comparisons between values of groups were performed using one- and two-way ANOVA. Significance was determined using Tukey's multiple comparison test for unequal replicates.

All values presented in this study are mean ± S.E.M. Differences between means were considered significant at $P < 0.05$.

3. Results

3.1. Temporal relationship between $PGF_{2\alpha}$ and progesterone

To determine the temporal relationship between uterine $PGF_{2\alpha}$ synthesis and the fall in circulating progesterone, we measured by radioimmunoassay the serum progesterone concentration at different days during pregnancy (5, 13, 21 and 22), one day after parturition and in cycling dioestrus rat. In the same groups of animals, we evaluated uterine $PGF_{2\alpha}$ synthesis.

These studies showed that uterine $PGF_{2\alpha}$ levels remain low through early and mid-gestation, being minimum on day 13 of pregnancy and then increase sharply at the end of gestation. The peak in uterine $PGF_{2\alpha}$ levels occurs at day 22 of gestation, just before the onset of labor. On the other hand, the serum progesterone concentration rises in the rat during early and mid-gestation. The levels of hormone in serum is maintained at a high concentration until a sharp decline in

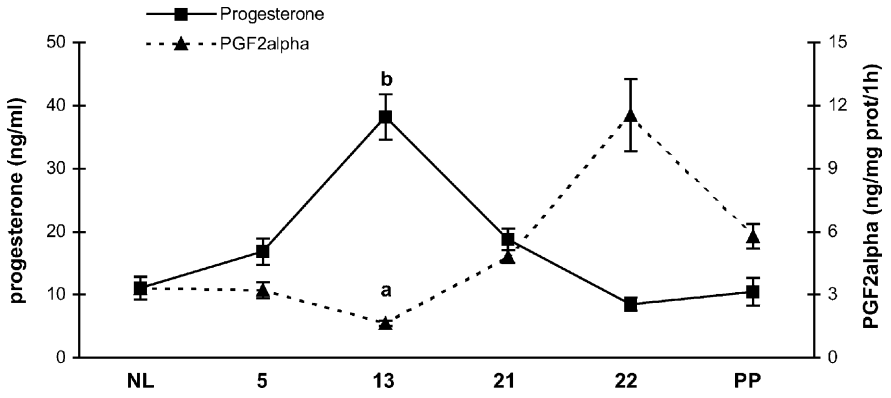

Fig. 1. Uterine $PGF_{2\alpha}$ production (dotted line) and serum progesterone (full line) of non-labor (NL, dioestrus), pregnant animals in different days of gestation (5, 13, 21 and 22) and 24 h postpartum. Each point represents the mean ± S.E.M. from three different experiments with $n = 6$. (a) $P < 0.001$ vs. day 22. (b) $P < 0.001$ when compared with the other groups.

concentration occurs between day 21 and 22 of pregnancy, just before labor (Fig. 1).

3.2. Effect of progesterone on uterine $PGF_{2\alpha}$ production

The temporal association of the increase in uterine $PGF_{2\alpha}$ and the decline in serum progesterone suggest that uterine $PGF_{2\alpha}$ production could be inhibited by progesterone. This indicates that steroids may play an important role in the control of $PGF_{2\alpha}$.

To examine the effects of progesterone on $PGF_{2\alpha}$ synthesis in the rat uterus, we measured the synthesis of this PG in progesterone-treated rats. As shown in Fig. 2A, treatment of rats with progesterone from day 20 to 21 of gestation prevented the increase of $PGF_{2\alpha}$ production that occurred during pregnancy on day 22. In addition, we observed that the administration of progesterone at the end of gestation delayed labor 24 h (Fig. 2B). These data provide further evidence that progesterone is necessary for maintaining the $PGF_{2\alpha}$ levels low during pregnancy.

To further ascertain the role of progesterone in PG regulation during pregnancy, we measured the synthesis of $PGF_{2\alpha}$ in the uterus from rats treated with the antigestagen mifepristone (RU-486) in mid-gestation. As shown in Fig. 3A, RU-486-induced preterm labor was associated with a significant ($P < 0.05$) increase in $PGF_{2\alpha}$ synthesis, when compared with the synthesis of prostaglandin in the rat uterus from day 13 (control). All rats given RU-486 delivered pups within 72 h after injections (Fig. 3B). This confirms that progesterone is required for inhibiting PG production during pregnancy.

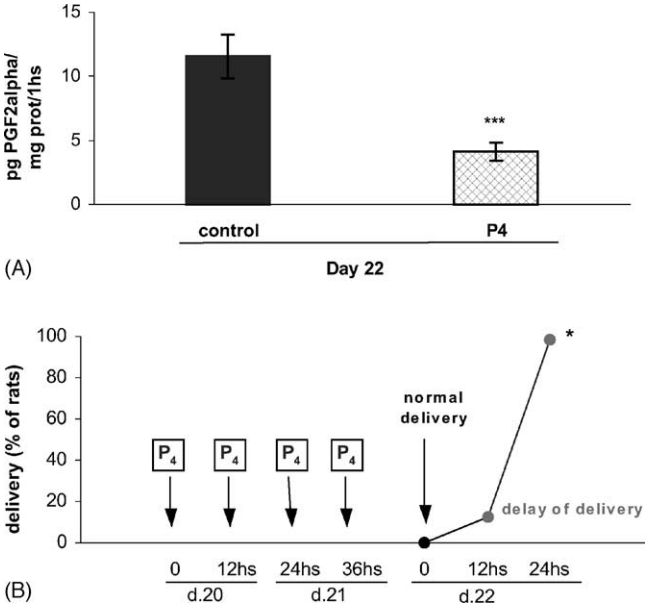

Fig. 2. Effect of progesterone (8 mg/kg, on day 20 and 21 of gestation) on uterine $PGF_{2\alpha}$ production (A). Each bar represents the mean ± S.E.M. from three experiments with $n = 6$. $^{***}P < 0.001$ vs. control. (B) Onset of labor.

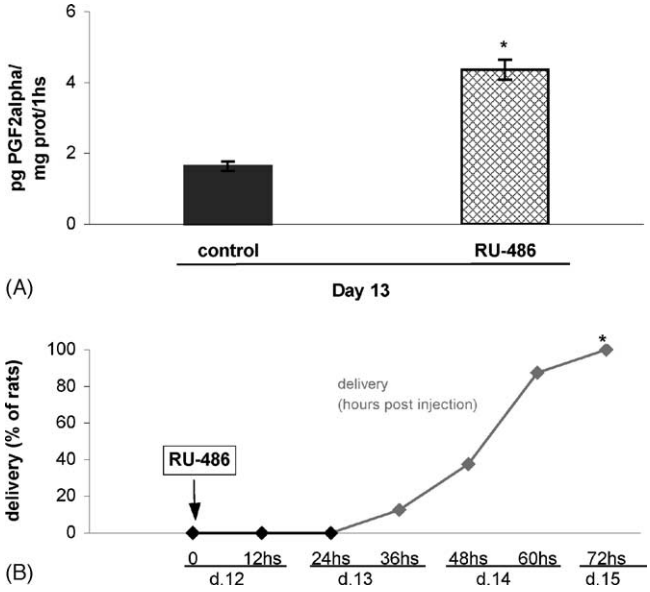

Fig. 3. Effect of RU-486 (10 mg/kg, on day 12 of gestation) on uterine $PGF_{2\alpha}$ production (A). Each bar represents the mean ± S.E.M. from three experiments with $n = 6$. $^{*}P < 0.05$ vs. control (day 13). (B) Onset of labor.

3.3. Expression of COX-1 and COX-2 during gestation

Cyclooxygenase (COX) catalyzes the committing step in prostaglandin biosynthesis and exists as two related but unique isoforms, COX-1 and COX-2. Western blot experiments were used to determine the presence of each isoform in rat uterus. Fig. 4 shown the expression of both immunoreactive proteins at different stages of pregnancy, in dioestrus and after parturition.

The levels of protein COX-1 (70 kDa) was low early in gestation followed by a significant induction ($P < 0.05$) at day 21 and remained elevated on day 22. That expression was markedly reduced in uteri collected after parturition (Fig. 4A).

The polyclonal antibody to COX-2 reacted with the appropriate band corresponding to the 72 kDa protein from mouse macrophage lysate. A band at 72 kDa was expressed at detectable levels in uteri in all the states studied. Densitometric analysis revealed that expression of COX-2 was low during gestation, but it abruptly increased ($P < 0.01$) on day 21 and 22 of pregnancy, whereas the protein COX-2 was not detectable after parturition (Fig. 4B).

The increases in COX-1 and COX-2 were coincident with the increased $PGF_{2\alpha}$ levels observed at end of pregnancy (day 21 and 22).

3.4. Effect of Progesterone on COX-1 and COX-2 expression

We investigated whether progesterone was able to modulate protein levels of COXs in uterus of pregnant rats. The treatment of rats with progesterone at day 20 and 21 of gestation prevented the increase of COX-1 (Fig. 5A) and COX-2

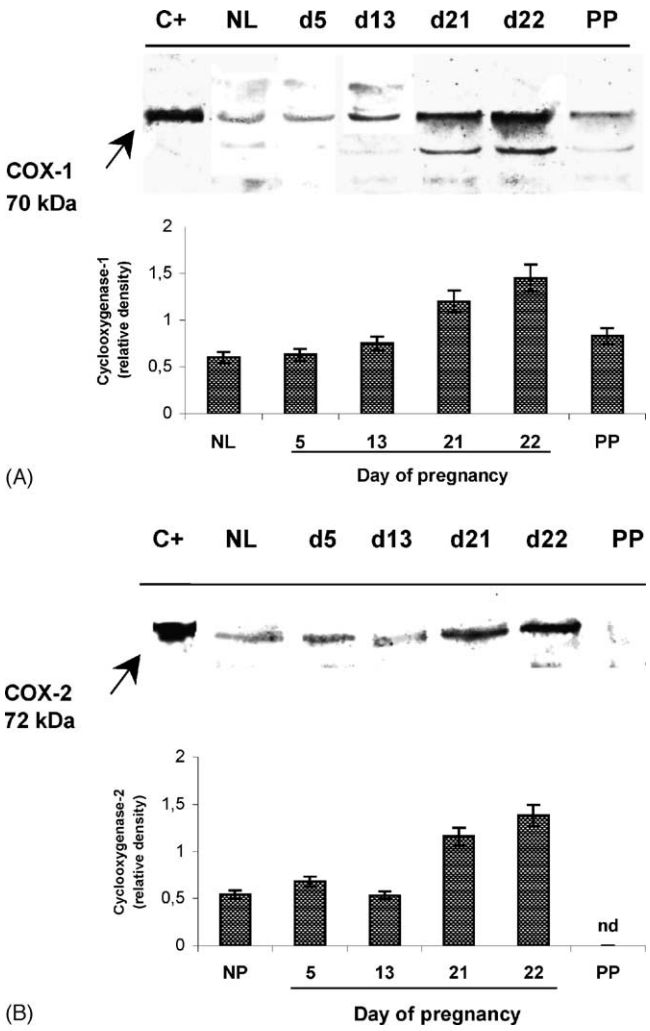

Fig. 4. Western blotting using polyclonal antibodies against (A) COX-1 and (B) COX-2. Rat uterine homogenates from non-pregnant (NL), pregnant (day 5, 13, 21 and 22) and one day postpartum (PP) were loaded. A representative experiment is shown. The lower panels show quantified and normalized COX-1 and COX-2 protein levels. The bars represent the mean ± S.E.M. of triplicate determinations from five separate animals.

expression (Fig. 5B) that occurred during pregnancy on day 22.

We measured the COX-1 and COX-2 expression in uterus of rats treated with RU-486. The administration of RU-486 on day 13 of gestation induced an increase of COX-2 expression (Fig. 5B), while COX-1 expression was not affected by this treatment (Fig. 5A).

3.5. $PGF_{2\alpha}$ and PGFM ratio during gestation

In addition to enzymes that regulate the production of PGs, certain enzymes modulate PG levels by catalyzing the degradation of PGs into inactive metabolites. We evaluated alterations in the efficiency of PG degradation by examining the ratio between 13,14-dihydro-15-keto $PGF_{2\alpha}$ (PGFM) and $PGF_{2\alpha}$ in non-pregnant (dioestrus) rats, pregnant animals in different days of gestation (5, 13, 21 and 22) and post-labor. In Fig. 6, each value represents the ratio between PGFM and $PGF_{2\alpha}$ synthesized in the uterus in the different days studied.

The ratio between PGFM and $PGF_{2\alpha}$ is high in day 13 of gestation, but begins to decrease during late gestation and it reaches its lowest level at day 22 of pregnancy. Following labor, the relationship between PGFM and $PGF_{2\alpha}$ again begins to increase. This profile is consistent with a high PGDH activity at mid-gestation compared to that at the end of pregnancy.

3.6. Effect of progesterone on $PGF_{2\alpha}$ and PGFM ratio

We observed that there was a temporal relationship between the rise in uterine $PGF_{2\alpha}$ and the fall in its metabolite synthesis, which suggested a possible modulation of PGFM synthesis by progesterone.

As show in Fig. 7, treatment of rats with progesterone from days 20 to 21 of gestation increased the PGFM/ $PGF_{2\alpha}$ ratio (22%, $P < 0.05$).

RU-486, a progestin antagonist, significantly inhibited PGFM/ $PGF_{2\alpha}$ ratio output (82%, $P < 0.05$), which suggests that PGDH enzymatic activity is lower when animals were administrated with RU-486; progesterone had an inhibitory activity. On the other hand, the administration of progesterone at the end of gestation increased the PGFM/$PGF_{2\alpha}$ ratio. This suggests an increased in PGDH activity at this period.

4. Discussion

Endogenous prostaglandins, particularly $PGF_{2\alpha}$ and PGE_2, play a role in preparing the uterus and the cervix for delivery. The changes in uterine PGF production appear to be involved in the regulation of uterine activity in the rat during pregnancy and labor. In the present study we found that the levels of uterine $PGF_{2\alpha}$ was negatively regulated by progesterone, which would act increasing degradation $PGF_{2\alpha}$ through a PG dehydrogenase (PGDH) stimulation and by inhibiting COX-1 and COX-2, both isoforms responsible of uterine $PGF_{2\alpha}$ synthesis.

In our experiments, $PGF_{2\alpha}$ production was low during pregnancy, and the synthesis was increased abruptly on day 22, the moment just before labor. Additionally, the levels of proteins of both Cyclooxygenase (COX-1 and COX-2) presented the same profile, increasing significantly on days 21 and 22. Additionally, our assay shows that progesterone administration at the end of the pregnancy significantly inhibited the uterine production of $PGF_{2\alpha}$. In contrast, the treatment with RU-486 increased the production of $PGF_{2\alpha}$. Previous studies showed a direct correlation of COX-2 mRNA levels to $PGF_{2\alpha}$ secretion by uterine cells in response to steroids [19].

To identify the isoforms responsible for the production of $PGF_{2\alpha}$ during gestation, we examined COX-1 and COX-2 levels in the rat uterus by Western blot. This study also shows that the levels of COX-1 and COX-2 in the uterus were low

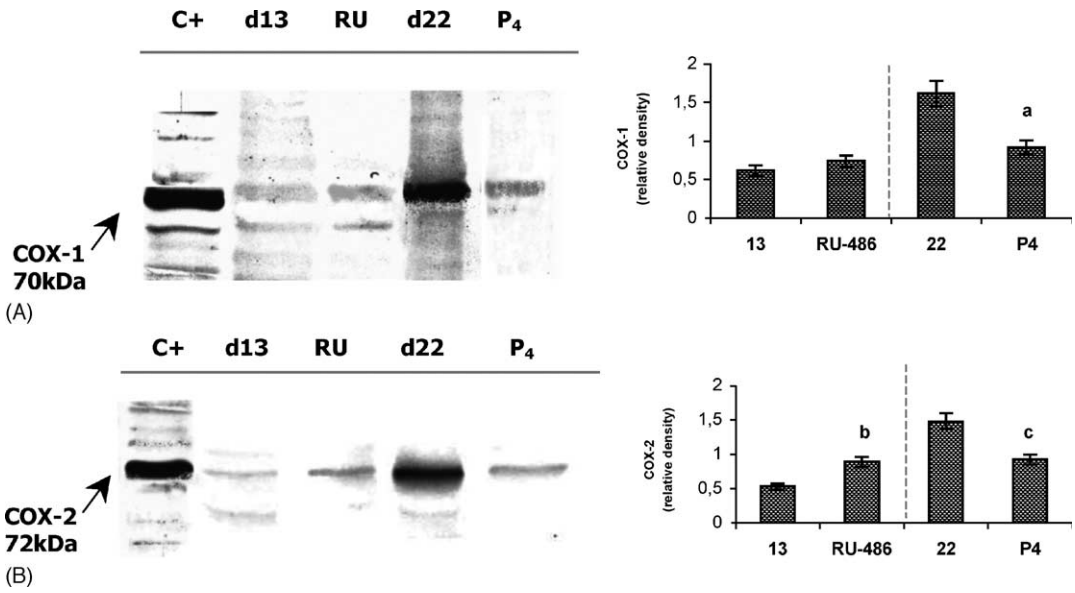

Fig. 5. Effect of RU-486 (10 mg/kg, on day 12 of gestation) and progesterone (8 mg/kg, on day 20 and 21 of gestation) on (A) COX-1 and (B) COX-2 protein levels in rat uterine tissue. In all cases, the experiments were repeated three times using uterine homogenates from five different animals. The differences between means were collectively analyzed by a one-way ANOVA followed by a Turkey's multiple comparison. (a) $P < 0.05$ vs. day 22; (b) $P < 0.05$ vs. day 13; and (c) $P < 0.05$ vs. day 22.

during early and mid-pregnancy and increased significantly at the term of gestation.

These data indicate that COX-1 as well as COX-2 are regulated during gestation and that the levels of both isoforms increase towards the time of labor.

We also found that in the rat uterus, COX-2 levels were reduced in animals treated with progesterone and increased in animals treated with the antiprogestin RU-486, indicating that the steroid hormone may modulate COX-2 protein levels.

These results are coincident with the reports of Doualla Bell [20] who observed that progesterone suppressed the expression of COX-2 mRNA in bovine myometrium. Loudon et. al. [21] also reported an inhibiting effect of progesterone on amnion COX-2, while studies carried out in pregnant mouse uterus [22] showed a clear inhibition of myometrial COX-2 expression by this hormone.

The modulation of the levels of COX-1 by progesterone seems to be more complex. While the administration of this

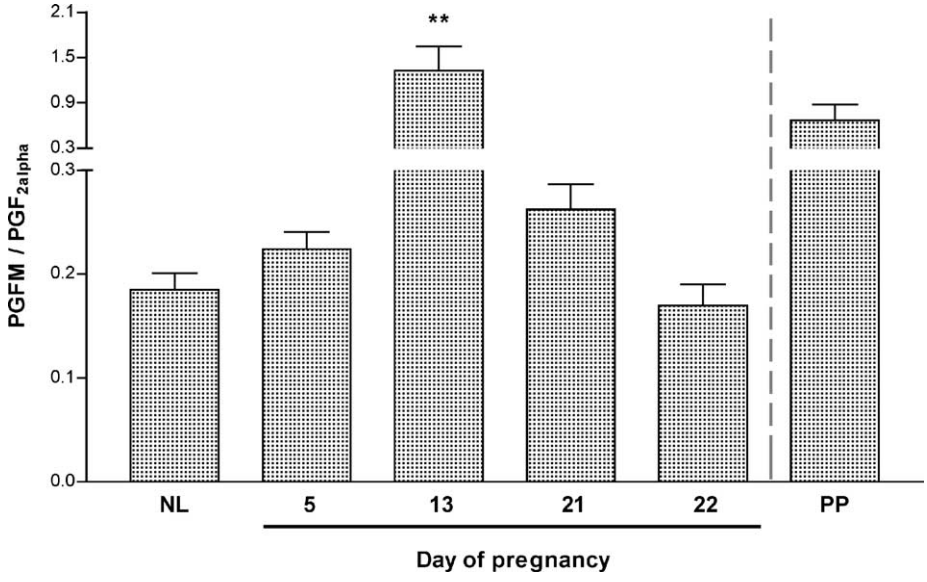

Fig. 6. Relationship between PGFM/$PGF_{2\alpha}$ in rat uterus during pregnancy. In each analyzed sample, the levels of $PGF_{2\alpha}$ and its metabolite, 13,14-dihydro-15-keto $PGF_{2\alpha}$ (PGFM), were determined. The results represent the mean ± S.E.M. of two carried out experiments, with $n = 8$. $^{**}P < 0.01$vs. dioestrus and days 5, 21 and 22 of gestation.

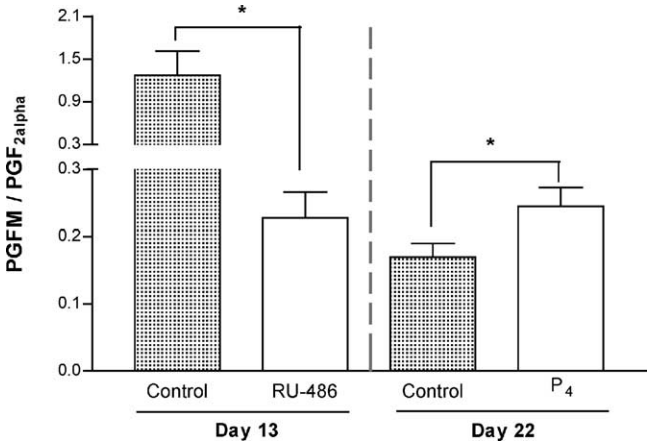

Fig. 7. Effect of RU-486 and progesterone on the relationship between PGFM/$PGF_{2\alpha}$ in rat uterus during pregnancy. The results indicate the media of the relationship PGFM/$PGF_{2\alpha}$ obtained for each analyzed sample ($n = 8$).

hormone at the end of pregnancy resulted in a decrease in COX-1 protein levels; the progesterone antagonist, RU-486, was not able to modify this isoform levels in a doses that was effective for COX-2.

However, previous studies carried out in uterine fibroblasts of rabbit reported that progesterone did not affect neither the activity nor the expression of COX-1, but it diminished COX-2 level and activity [23]. These data suggest that the regulation of COX-1 should be mediated for more than one factor during pregnancy.

We evaluated PG degradation by examining the ratio between PGFM and $PGF_{2\alpha}$. Our results show that $PGF_{2\alpha}$ metabolism was high during mid pregnancy and post labor, a result that coincides with the minimum levels of PGs previously obtained.

Finally, we evaluated whether progesterone was able to modulate the activity of PGDH in rat uterus during mid-gestation, when progesterone levels were maximum. Our results showed that RU-486 was able to diminish significantly the activity of PGDH in mid-gestation. On the other hand, the administration of progesterone in days 20 and 21 of pregnancy increased the activity of this enzyme. In agreement with these results it has been reported that PGDH mRNA increased significantly in stromal cells by the addition of progesterone [19], and that this hormone induces the promoter of the PGDH gene [24].

The results obtained in this study provide evidence that progesterone regulates the levels of uterine PGs through two mechanisms: (1) diminishing the protein concentrations of COX-1 and COX-2, and therefore resulting in a decreased formation of prostanoids and (2) stimulating the activity of PGDH, the enzyme responsible for their metabolization to inactive the intermediates.

Taken together, these results suggest that the regulation of $PGF_{2\alpha}$ levels is a complex process involving the coordinate induction of synthesizing enzymes and an inhibition of enzymes involved in prostaglandin metabolism.

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