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Effect of in Vivo Administration of Epidermal Growth Factor on Prostaglandin Production and NOS Activity in Term Rat Placentae. Possible Participation of Placental EGF Receptors

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Many authors hypothesize that the epidermal growth factor (EGF) is involved in the onset of labor. Previous reports from our laboratory showed that intrauterine administration of EGF delays the beginning of labor. The aims of this study were: 1) to analyze the effect of intrauterine administration of 500 ng EGF on placental prostaglandins and nitric oxide, and 2) to characterize the expression of EGF receptors (EGF-R) in pregnant rat placentae. Saline solution (sham group) and 500 ng EGF (EGF-treated group) were administered via intrauterine injection on day 21 of gestation, and both groups of animals were sacrificed on day 22 (sham rats delivered on day 22). Results showed that EGF treatment: 1) inhibited the production of prostaglandin E ($p < 0.001$) and $F_{2\alpha}$ ($p < 0.01$), 2) increased the synthesis of nitric oxide ($p < 0.001$), and 3) reduced the expression of cyclooxygenase-II, the enzyme responsible for PG synthesis. Placentae were found to express EGF-R and its activated form, and the expressions of both forms were higher at mid and term pregnancy. Hence, EGF is a very interesting molecule for studying the regulation of placental prostaglandin and nitric oxide production related to the parturition process.
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INTRODUCTION

Epidermal growth factor (EGF) was first discovered by Cohen in submaxillary glands of mice [1]. From then on, most EGF studies have been focused on the stimulation of in vivo and in vitro cell proliferation. However, EGF seems to display other biological activities as well. It is present in maternal and fetal blood and in the amniotic fluid [2,27]. It is also believed to be involved in feto-placental growth and development through its mitogenic action, increasing placental and fetal membrane hormone secretion [3]. It has been reported that, in 4–5 week human placentae, EGF and its receptor (EGF-R) are located in cytotrophoblasts, and that EGF increases cytotrophoblast cell proliferation without affecting their ability to secrete human chorionic gonadotrophin (hCG) and human placental lactogen (hPL) [4]. In contrast, in 6–12 week placentae, EGF and its receptor are located in syncytiotrophoblasts and EGF stimulates hCG and hPL secretion without affecting syncytiotrophoblast cell proliferation. Previous studies carried out in our laboratory [5] showed that intrauterine (i/u) administration of 500 ng EGF to 21-day pregnant rats delayed the onset of parturition 19.0 ± 0.6 h (EGF-treated rats delivered on day 23 of pregnancy instead of day 22 as sham animals). Our results also demonstrated that exogenous EGF elicited its effect modulating nitric oxide (NO) and prostaglandin (PG) production in the uterus.

It is well known that, in mammals, NO plays an important role during pregnancy regulating myometrial relaxation and placental circulation [6,7]. Likewise, the ability of the villous vascular tree of human term placenta to both generate and respond to NO, and the significant role of NO in the maintenance of adequate fetal–placental circulation have also been demonstrated [8]. Moreover, three NOS isoforms have been identified in placental tissue: neuronal (nNOS), endothelial (eNOS) and inducible NOS (iNOS) [9].

On the other hand, PGs are active lipid mediators involved in the parturition process [10]. Their biosynthesis is catalyzed by the cyclooxygenase enzyme (COX), of which two isoforms are known: COX-I and COX-II [11]. Even though the uterus is the main target of PGs during parturition, it is not the

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primary source of these lipid mediators. It has been proposed that PGs derived from feto-placental tissues is the way by which they reach the uterus generating the contractions necessary for the expulsion of the fetus.

Since the placenta is a complex tissue located in close contact with the uterus [12], the first aim of our study was to investigate whether the placenta was also related to EGF-delayed labor onset mechanism. Thus, we analyzed if i/u administration of 500 ng EGF administered on day 21 of gestation regulated NOS and COX systems in this tissue.

Specific receptors for EGF were detected in fully developed placentae from pregnant mares [13], cats [14], mice [15] and women [16]. However, no descriptions can be found on the expression of EGF-R in placentae from pregnant rats. Therefore, our second aim was to characterize the expression of EGF-R in placentae from mid and term pregnant rats.

MATERIALS AND METHODS

Drugs and reagents

Epidermal Growth Factor (murine submaxillary glands, culture grade, PM = 6100) was purchased from Calbiochem, Norabiochem Corporation (La Jolla, CA). [^{14}C]-L-citrulline, [5,6,8,9,11,12,14,15(*n*)-^{3}H]-prostaglandin $F_{2\alpha}$ (160 Ci/mmol) and [5,6,8,9,11,12,14,15(*n*)-^{3}H]-prostaglandin E (130 Ci/mmol) were from Amersham Corporation (Arlington Heights, IL, USA). Monoclonal nNOS, eNOS and iNOS first antibodies were from Transduction Co. (Devon, United Kingdom), whereas polyclonal COX-I, COX-II, EGF-R and EGF-pR were from Santa Cruz Biotechnology Inc. (Copenhagen, Denmark). PGE_2 and $PGF_{2\alpha}$ antiserum, NADPH, valine, EGTA, actin and second antibodies were purchased from Sigma Chemical Co. (St Louis, MI, USA). Dowex AG500-X column (Na^+-form) was from BioRad (CA, USA). All other chemicals were of analytical grade.

Animals

The experimental procedures herein reported were approved by the Animal Care Committee of the Center for Pharmacological and Botanical Studies of the National Research Council (CEFYBO, CONICET) and carried out in accordance with the Declaration of Helsinki.

Wistar female rats were kept in group cages under controlled conditions of light (14 h light, 10 h dark) and temperature (23–25 °C). Animals received food and water ad libitum. Time-mated pregnant rats (200–300 g body weight) were used. The morning the spermatozoa were observed in the vaginal fluid was defined as day 1 of pregnancy. Spontaneous term delivery usually occurs on day 22 of gestation.

Intrauterine EGF administration

Pregnant rats were administered an intrauterine (i/u) injection of a single EGF dose (500 ng, final volume: 250 µl) on day 21 of gestation. Sham animals received i/u injection of 250 µl saline solution (0.9% NaCl, EGF vehicle) on day 21 of pregnancy. Sham and EGF-treated animals were injected as previously described [5]. Briefly, animals were anaesthetized by ether inhalation, and i/u EGF administration (gauge needle 30G) was carried out under direct visualization of the uterine horns. The uterus was surgically exposed and the injection was given inside the uterus lumen. Once injected, the animals were closed and remained in their cages until day 22 of gestation, when they were sacrificed between 10.00 and 11.00 am. Placentae were extracted, cleaned of fat and fetuses.

After the animals were killed it was impossible to asses exactly which was the site of injection in the uterus, so any difference between the placentae extracted was made. The placentae were randomly treated and when we repeated the corresponding measurements (radioimmunoassay, NOS activity or western blot) there were no differences in the results obtained.

Once the animals were killed on day 22, all the placentae from each control ($n = 4$) and each treated animal ($n = 4$) were extracted, cut into four pieces and placed in different petri dishes (one petri dish for each rat). Four placentae pieces from each petri dish were randomly chosen. A separate measurement on a separate placenta from each of the 4 animals was made. This procedure was repeated three times ($n = 12$).

Prostaglandin radioimmunoassay

PGE and $PGF_{2\alpha}$ were measured in placenta samples [17] obtained from sham and EGF-treated rats. Placentae were incubated for 1 h in Krebs–Ringer bicarbonate solution (145 mM Na^+, 6 mM K^+, 2 mM Ca^{++}, 1.3 mM Mg^{++}, 126.1 mM Cl^-, 25.3 mM HCO_3^-, 1.3 mM SO_4^{2-}, 1.2 mM PO_4^{2-} and 11 mM glucose) in an atmosphere of 95% O_2/5% CO_2 at 37 °C. After incubation, the medium was acidified to pH = 3 with 1 N HCl, and PGs were extracted twice with 2 ml ethyl acetate. PG concentrations were determined by radioimmunoassay. $PGF_{2\alpha}$ antiserum was highly specific for $F_{2\alpha}$ and showed low cross reactivity (<0.1% for PGE_1 and PGE_2). PGE antiserum was highly specific for E_1 and E_2 and showed low cross reactivity (<0.1% for $PGF_{2\alpha}$). Sensitivity was 5–10 pg per tube, and 2–5 µl of sample was assayed routinely. Protein concentration was determined by the method of Bradford [18]. Values were expressed as pg PGs/mg protein/1 h.

Total NOS enzyme assay

NOS enzyme activity was quantified by the modified method of Bredt and Snyder [19] that measured the conversion of [^{14}C]-L-arginine into [^{14}C]-L-citrulline. NO and L-citrulline were produced in equimolar amounts.

Sham and EGF-treated placentae were homogenized (Ultra Turrax, T25 basic, IKA Labortechnik) and incubated at 37 °C in HEPES buffer (20 mM HEPES, 25 mM L-valine, 0.45 mM $CaCl_2$, 100 mM DTT) containing 0.6 µCi/ml [^{14}C]-L-arginine and 0.5 mM NADPH. After 15 min of incubation, samples were centrifuged for 10 min at 3000*g*. Then they were applied into a 1 ml DOWEX AG500-X column (Na^+-form), and

[^{14}C]-L-citrulline was eluted in 3 ml distilled water. [^{14}C]-L-citrulline radioactivity was measured by liquid scintillation counting. NOS activity was determined as the difference between [^{14}C]-L-citrulline produced in samples with or without 1 mM EGTA and 2 mM L-NAME. Protein concentration was measured by the Bradford assay [18]. Enzyme activity was expressed as pmoles [^{14}C]-L-citrulline/mg protein/15 min.

Western blot analysis

Isolated placentae from sham and EGF-treated animals were homogenized in 20 mM Tris buffer, pH = 7.4, containing 1 mM EDTA, 2 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml DTT, 100 μg/ml soybean trypsin inhibitor, 1 mg/ml caproic acid, 1 mg/ml benzamidine and 1 mM sodium orthovanadate. For NOS and COX determinations, homogenates were sonicated (Ultrasonic Cell Disrupter, Microson, Heat systems Inc.) for 30 s and centrifuged at 1500*g* for 5 min to remove cellular debris. For EGF-R and EGF-pR determinations, samples were centrifuged at 2000*g* for 10 min after homogenization. Protein concentration was determined by the Bradford assay [18]. Each point represented pooled material from 4 different animals. The experiment was repeated three times ($n = 3$). Homogenates were boiled for 5 min in sample buffer (0.3% bromophenol blue, 0.5 M Tris pH = 6.8, 1% SDS, 5% β-mercaptoethanol, 10% glycerol). One hundred micrograms of total protein was loaded in each lane. Positive control aliquots were also loaded. Membrane fractions of human endothelial cells were used for eNOS, mouse macrophage lysate for iNOS and COX-II, rat pituitary lysate for nNOS, goat seminal glands for COX-I and rat salivary glands for EGF-R and EGF-pR. Samples were run on 7.5% (for COX and NOS) and 6% (for EGF-R and EGF-pR) SDS polyacrylamide gel electrophoresis (0.03 A). Then they were transferred to a nitrocellulose membrane (40 V overnight at 4 °C). Membranes were first blocked for 1 h at room temperature in Tris/saline (50 mM Tris/HCl pH = 7.5, 500 mM NaCl) containing 5% milk powder, and then incubated overnight at 4 °C with primary antibodies diluted in Tris/saline buffer (NOS: 1/500, COX-I: 1/600, COX-II: 1/1000, EGF-R: 1/600 and EGF-pR: 1/300). Then they were washed three times with Tris/saline buffer containing 0.2% Tween-20, incubated for 1 h at room temperature with actin (1/1000) and washed out three times again. Then, membranes were incubated for 1 h at room temperature with the second antibody (1/5000, goat anti-mouse IgG alkaline phosphatase and goat anti-rabbit IgG alkaline phosphatase) and washed out as described above. The developing solution was nitroblue tetrazolium with 5-bromo-4-chloro-3-indol phosphate. Molecular weight standards were run under the same conditions to identify protein bands. Blots were scanned with a scanning densitometer UMAX Astra 1220S, and band intensities were determined using the Sigma Plot program. Results were expressed as the relative optic density compared with actin.

Statistics

Statistical analyses were performed using the Graph Pad Prism 3.0 Program (Graph Pad Software, San Diego, CA, USA). Comparisons between group values were performed using one way ANOVA. Significance was determined using Tukey's multiple comparison test for unequal replicates. All values represent means ± SEM. Mean differences were considered significant when $p \leq 0.05$.

RESULTS

Effect of i/u EGF administration on PG synthesis

We decided to investigate if the delay in the onset of labor caused by the administration of EGF was due to a modulation on PG synthesis in the placenta. Therefore, we compared PG production in sham and EGF-treated rats.

We found that i/u administration of 500 ng EGF on day 21 of gestation significantly decreased both PGE (Figure 1A) and $PGF_{2\alpha}$ (Figure 1B) production in placentae. Placentae from EGF-treated rats produced less PGs during day 22 than those from sham animals (50% PGE_2 and 33% $PGF_{2\alpha}$).

Effect of i/u EGF administration on NO production

Following the same approach adopted for PGs, we analyzed whether NOS activity was affected by the administration of 500 ng EGF on day 21 of gestation.

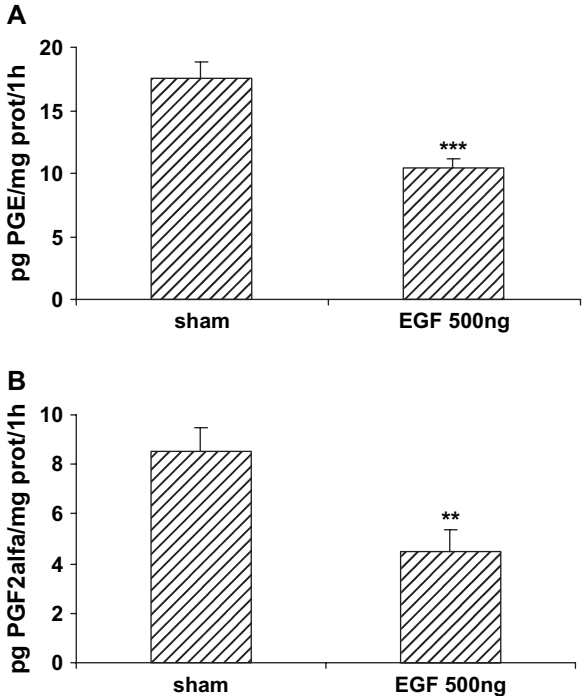

Figure 1. Effect of i/u administration of 500 ng EGF on day 21 of gestation on PGE (A) and $PGF_{2\alpha}$ (B) syntheses in rat placenta. Values are expressed as mean ± SEM. Mean differences were collectively analyzed by one way ANOVA followed by Tukey's multiple comparison. $^{**}p < 0.01$ vs sham, $^{***}p < 0.001$ vs sham, $n = 12$.

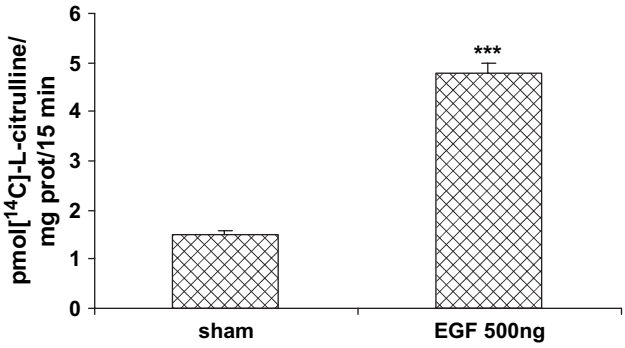

Figure 2. Effect of i/u administration of 500 ng EGF on day 21 of gestation on NOS activity in rat placenta. Values are expressed as mean ± SEM. Mean differences were collectively analyzed by one way ANOVA followed by Tukey's multiple comparison. ***$p < 0.001$ vs sham, $n = 12$.

Placentae from animals treated with EGF showed higher NOS activity compared to placentae from sham animals ($p < 0.001$, Figure 2).

Effect of EGF administration on the expression of COX and NOS isoforms

Since the production of PG and NO in the placentae were affected by the treatment with EGF, we decided to analyze if i/u administration of 500 ng EGF on day 21 of gestation affected NOS and COX activities or also their expression.

A positive band for COX-I with an approximate molecular mass of 72 kDa was detected in placentae from sham and EGF-treated rats (Figure 3A). COX-I expression was not altered by EGF treatment when compared to sham protein level. As it was expected, COX-II was identified as a single band of an approximate molecular mass of 70 kDa in placentae from sham and EGF-treated rats (Figure 3B). The administration of 500 ng EGF on day 21 of gestation resulted in a significant decrease of COX-II expression ($p < 0.01$).

Then we studied the regulation of NOS isoforms expression after the treatment with EGF. Neuronal NOS was not detected in placentae from sham rats, or in placentae from EGF-treated animals (Figure 4A). On the contrary, eNOS was easily detected in placental tissue at a molecular mass of 142 kDa (Figure 4B). Intrauterine EGF administration did not modify eNOS expression in comparison with protein level observed in sham placental samples. Similar results were obtained for iNOS isoform. A positive band for iNOS of approximately 130 kDa was detected in sham placentae, and EGF i/u administration did not alter its expression (Figure 4C).

Expression of EGF-R in placental tissue during pregnancy

Since there are no reports on EGF-R expression in rat placenta, and we have observed that i/u EGF administration modified both PG and NO synthesis in this tissue, we decided to investigate the placental expression of EGF-R during gestation.

Our results showed that placentae expressed EGF-R. A double band of approximately 170 kDa and 150 kDa was found in placentae at days 13, 18, 19, 20, 21 and 22 of pregnancy. We observed that both bands were regulated during gestation in the placentae. The expression of the 170 kDa band was high at mid (day 13) and term (day 22) gestation ($p < 0.01$, Figure 5A), whereas the 150 kDa band remained constant until day 22, when its expression increased significantly ($p < 0.001$, Figure 5A).

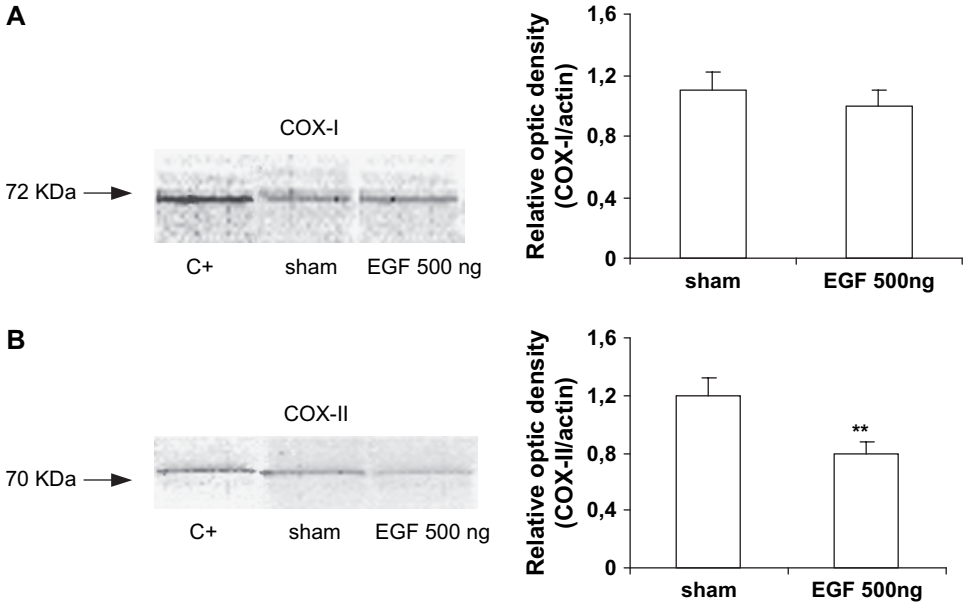

Figure 3. Effect of i/u administration of 500 ng EGF on day 21 of gestation on COX-I (A) and COX-II (B) expression in rat placenta. A representative experiment is shown. The experiment was performed in triplicate. Values are expressed as mean ± SEM. Mean differences were collectively analyzed by one way ANOVA followed by Tukey's multiple comparison. Data were normalized with actin values. C+: positive control, **$p < 0.01$ vs sham, $n = 3$.

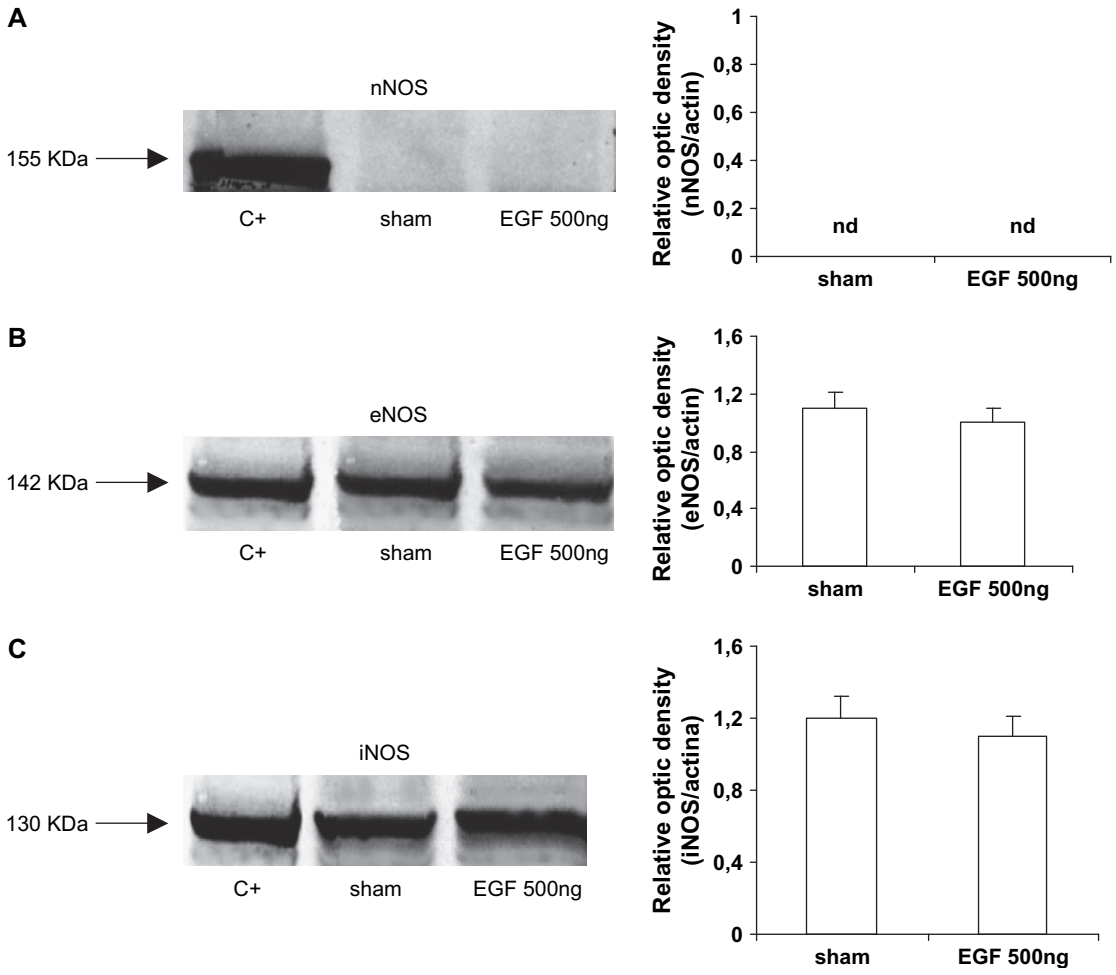

Figure 4. Effect of i/u administration of 500 ng EGF on day 21 of gestation on nNOS (A), eNOS (B) and iNOS (C) expression in rat placenta. A representative experiment is shown. The experiment was repeated three times. The difference between means was collectively analyzed by a one way ANOVA followed by a Tukey's multiple comparison. There were no significant differences between the treatments. Data were normalized with actin values. C+: positive control, $n = 3$.

The fact that EGF-R has endogenous tyrosine kinase activity [20] led us to investigate whether the receptor was phosphorylated during gestation in the placenta, and if the protein level of this receptor form (EGF-pR) was also regulated. Thus, we used a specific primary antibody against tyrosine 1173, the amino acid which is specifically phosphorylated once the receptor is activated. EGF-pR was easily detectable at 170 kDa in placentae from days 13 and 18 to 22 of gestation (Figure 5B), and the regulation of its expression agreed with that of the 170 kDa band corresponding to EGF-R (Figure 5A).

DISCUSSION

The placenta is known to be an exceedingly rich source of receptors for several growth factors including EGF. This factor, which is primarily mitogenic for a variety of cells and also affects differentiated cellular functions in the absence of a mitogenic effect, is presumed to play a role not only in feto-placental development, but also as a signal during labor.

Previous results from our laboratory show that intrauterine administration of EGF to pregnant rats delays the onset of labor [5]. Specifically, i/u administration of 500 ng EGF on day 21 of gestation delays 19.0 $\pm$ 0.6 h the onset of labor. This effect is mediated through modulation over PGs, NO and progesterone, three important uterine regulators that participate in the modulation of myometrial contractions.

It is well known that the placenta is in close contact with the uterus tissue, and now is becoming clearer that EGF might act as a fetal signal for the beginning of parturition. Based on the evidence described above, we decided to investigate if i/u administration of 500 ng EGF on day 21 of gestation also regulated the production of placental PGs and NO.

Previous works have demonstrated that PGs play a key role in the onset of parturition [10], and particular attention has been paid to the importance of growth factors and cytokines in this process. Such consideration evolves from data that indicate the co-expression of a variety of growth factors and cytokines with that of eicosanoid enzymes in uterine and feto-placental tissues acting as regulators of PG biosynthesis [21].

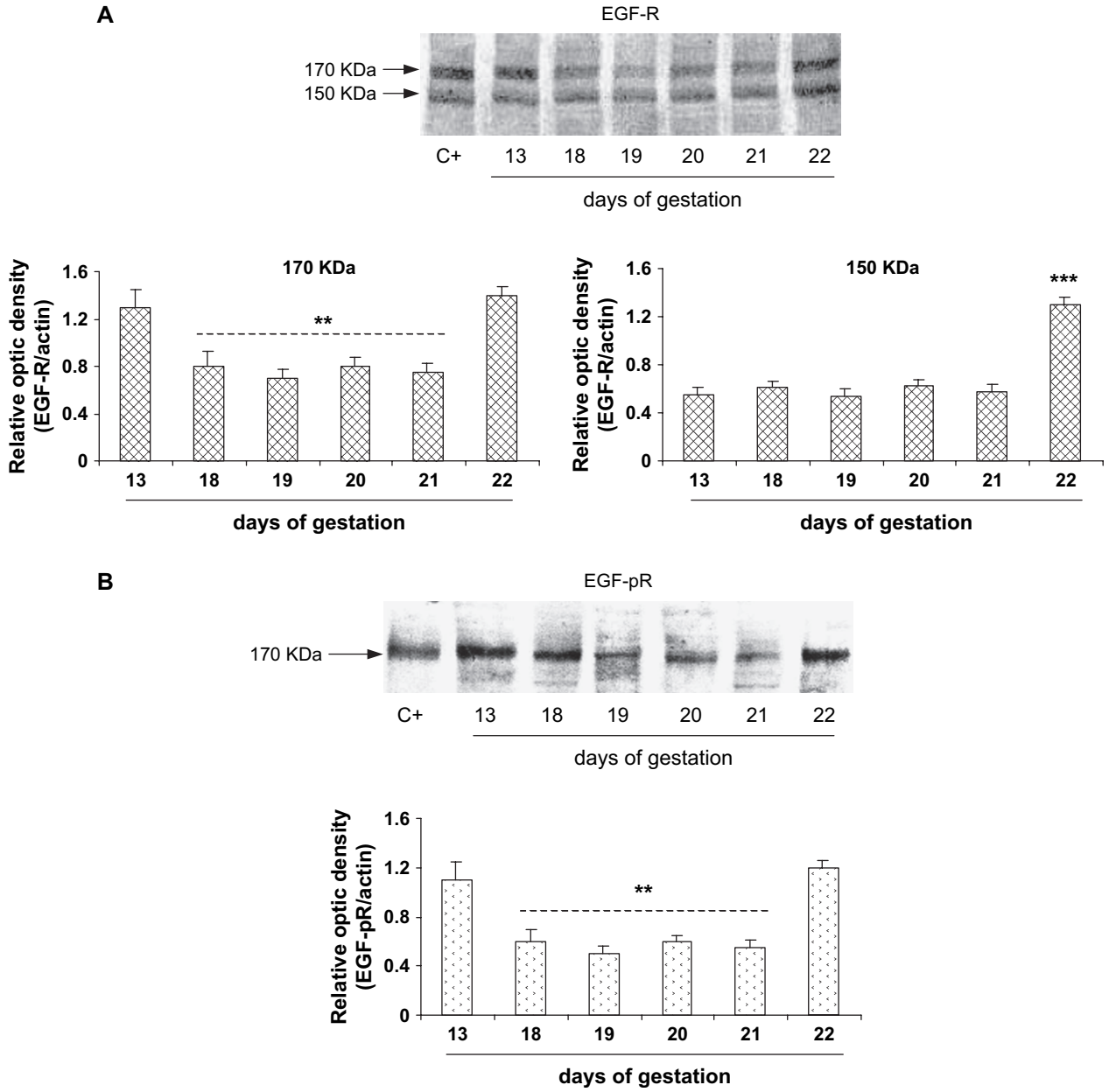

Figure 5. Western blotting using polyclonal antibody against EGF-R (A) and EGF-pR (B) in placentae from mid (day 13) and term (days 18–22) pregnant rats. A representative experiment is shown. The experiment was performed in triplicate. Mean differences were collectively analyzed by one way ANOVA followed by Tukey's multiple comparison. Data were normalized with actin values. C+: positive control, $^{**}p < 0.01$ vs days 13 and 22 of gestation, $^{***}p < 0.001$ vs the other days of gestation, $n = 3$.

Our results showed that i/u EGF administration decreased PGE and $PGF_{2\alpha}$ production in the placenta. This agrees with the effect observed in uterine PG synthesis, in which EGF also inhibited PG production when compared to sham pregnant rats [5]. Other authors found that EGF not only regulates COX activity but also its expression and RNA messenger [21].

The following step was to study whether EGF-inhibited PG biosynthesis was due only to COX activity inhibition, or if EGF was also regulating COX expression. We found that while i/u EGF administration did not modify COX-I expression, it significantly inhibited COX-II expression when compared to sham protein level. These observations supported the notion that EGF affected not only COX activity in rat term placentae but also its expression. Faber and colleagues [22] reported that the level of EGF immunostaining paralleled that of eicosanoids in myometrial tissues from failed and normal labors in humans. These data suggest that, in addition to the contribution of the feto-placental production of eicosanoids and growth factors, essential for the normal progression of labor, there seems to be a correlation between eicosanoid levels and EGF expression in myometrial tissue during this period. The relation between growth factors and prostaglandins during the labor process may be relevant not only for the uterus, but also for the placenta.

Many authors emphasized the importance of the regulation of NO generation in the placenta [8]. In our study, NOS activity was detectable in sham term placentae, and while nNOS isoform expression was not detectable by western blot, eNOS and iNOS protein levels were readily detected. Intrauterine EGF administration increased NOS activity but did not alter the expression of any of its isoforms. These results agree with those observed in the uterus [5]. As we already mentioned, NO seems to be responsible for uterine quiescence and placental perfusion. Our observations suggest that placental NO may be an important modulator of fetal homeostasis, regulating uterine contractility by inhibiting PG synthesis in the uterus and maintaining adequate fetal–maternal perfusion.

Since we observed that EGF treatment affected PG and NO production in the placenta, we decided to study if this tissue expressed EGF receptors during pregnancy. Results show that the placenta expressed both the receptor (EGF-R) and its activated form (EGF-pR) all along pregnancy, and that these forms were regulated during gestation. We think that EGF might be exerting its effect on placental PG and NO production by a specific binding and activation to these receptors. Other authors reported similar results. Chegini and Rao [23] found that all cellular types, but not non-cellular elements, found in human amnion, chorion, decidua and placenta of mid and term pregnancy contained several silver grains after incubation with [^{125}I]-EGF. Moreover, placental EGF binding sites increased throughout pregnancy, in parallel to the development of the syncytial layer and human hPL secretion [24]. Even though EGF may be directly exerting its effect binding to EGF-R in the placenta, we could not discard an indirect effect based on a previous report [5]. We showed that i/u EGF administration increases serum progesterone levels, thus having an effect not only in the uterus but also in the ovary. The regulation of EGF receptors in the placenta may be modulating the effect of endogenous EGF during the onset of labor. However, this approach needs to be studied in depth.

It is not clear which signal molecules control placental PG and NO production, but EGF has been proposed to be one of these ones. Since EGF levels in human amniotic fluid increase steadily from 30 to 40 weeks of gestation and EGF modulates PG biosynthesis in several reproductive tissues [21], some authors proposed that EGF plays a significant role in perinatal development and parturition [25]. Moreover, EGF has been suggested to derive, at least in part, from the fetal kidney, thus supporting the hypothesis that the signal triggering labor emanates from the fetus [26].

EGF is, therefore, a very attractive molecule to study the regulation of placental PG and NO production related to the parturition process.

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