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Roxane Hervé [1] , **Thomas Schmitz** [2 1] , **Danièle Evain-Brion** [1] , **Dominique Cabrol** [2 1] , **Marie-Josèphe Leroy** [1] , **Céline Méhats** [1 *]

[1] *La grossesse normale et pathologique: développement et fonctions du placenta et de l'utérus INSERM : U767 , IFR71 , Université Paris Descartes , Fac Sc Pharmaceutiques et Biologiques 4, Avenue de L'Observatoire 75270 Paris Cedex 06,FR*

[2] *Maternité Port-Royal Assistance publique - Hôpitaux de Paris (AP-HP) , Hôpital Cochin , Université Paris Descartes , 27 rue du Faubourg Saint-Jacques 75659 Paris Cedex 14,FR*

[*]
Correspondence should be adressed to: Céline Méhats <celine.mehats@inserm.fr >

Abstract

Spontaneous preterm delivery is linked to intrauterine inflammation. Fetal membranes are involved in the inflammatory process as an important source of mediators, and the chorion leave produces high levels of the pro-inflammatory cytokine TNFα when stimulated by LPS. The transcription factor NF-κB is the main regulator of this inflammatory process and controls the production of cytokines by the chorion leave. PDE4 inhibitors are recognized for their anti-inflammatory and myorelaxant effects. The purpose of this study was to investigate whether PDE4 inhibition affects the LPS signaling in human cultured chorionic cells. We showed that these cells express TLR4, the main LPS receptor, and exhibit a predominant PDE4 activity. Upon LPS challenge, PDE4 activity increases concomitantly to the induction of the specific isoform PDE4B2 and chorionic cells secrete TNFα. LPS induces the nuclear translocation of the NF-κB p65 subunit and the activation of three different NF-κB complexes in chorionic cells. The presence of the PDE4 inhibitor rolipram reduces the TNFα production and the activation of the three NF-KκB complexes. These data indicate that the PDE4 family interacts with the LPS signaling pathway during the inflammatory response of chorionic cells. PDE4 selective inhibitors may thus represent a new therapeutic approach in the management of inflammation-induced preterm delivery.

MESH Keywords Cells, Cultured ; Chorion ; drug effects ; metabolism ; secretion ; Cyclic Nucleotide Phosphodiesterases, Type 4 ; metabolism ; Female ; Humans ; Lipopolysaccharides ; pharmacology ; NF-kappa B ; metabolism ; Phosphodiesterase 4 Inhibitors ; Protein Binding ; Protein Transport ; Rolipram ; pharmacology ; Toll-Like Receptor 4 ; metabolism ; Tumor Necrosis Factor-alpha ; secretion

Author Keywords human ; cytokines ; lipopolysaccharide ; inflammation ; trophoblast

Introduction

Despite great strides in improving the survival of infants born prematurely (before 37 weeks of postmenstrual age), preterm birth still accounts for 70% of neonatal deaths and up to 75% of neonatal morbidities. A causal link has been established between preterm delivery and infection (1). Microorganisms are often isolated from the amniotic cavity of women with preterm labor (2) and the intraamniotic or intrauterine injection of LPS or heat-killed bacteria is sufficient to promote preterm delivery in mouse, rabbit, and sheep (3). Infection triggers a local inflammation in the gestational tissues, initiating production of cytokines, prostaglandins, and metalloproteases (4 –6). Among the gestational tissues, the amniochorionic membranes are an important source of cytokines, chemokines and prostaglandins. Of particular interest is the chorionic membrane; it is the largest area of contact with the maternal tissues and it is the first tissue colonized by microbial pathogens during an ascending intrauterine infection. In vitro , human choriodecidua produces 10 fold higher level of the pro-inflammatory cytokines TNFα and MCP-1 than human amnion (7 , 8).

LPS signals through ligation to TLR4 (9) and activates the transcription factor NF-κB, a dimer of two subunits, typically p65 and p50 (10). NF-κB has been shown to control the LPS-induced production of pro-inflammatory cytokines by human amnion and choriodecidua (11), as well as activation of the phospholipase A2, an enzyme implicated in the metabolism of prostaglandins (12). Moreover, genetic ablation of TLR4 or pharmacologic inhibition of NF-κB prevents LPS-induced preterm delivery in mice (9 , 13).

The onset and progression of the inflammatory response are sensitive to changes in the steady-state level of the cyclic nucleotide, cAMP (14). Pharmacological manipulation of cyclic nucleotide phosphodiesterases (PDE) [3] , which degrade the cyclic nucleotides cAMP and cGMP, provides a powerful mean of regulation of the biological processes relayed by these intracellular second messengers (15). Among the eleven families of mammalian PDE described to date, the PDE4 family specifically hydrolyzes cAMP and is predominant in all immunocompetent cells (16). Selective PDE4 inhibitors reduce the production of chemokines, pro-inflammatory cytokines, and adhesion molecules in leukocytes, endothelial, and airway epithelial cells (17). The PDE4s are encoded by four genes (A, B, C and D) which generate at least 20 different isoforms. It has been pointed out a role for the PDE4B isoforms in inflammation, as pde4b KO mice are hyporesponsiveness to LPS and resistant to septic shock (18), and a particular function has been given to the short form PDE4B2, predominant in leukocytes and induced by LPS in macrophages (19).

Recently, we have shown that PDE4 inhibition by rolipram, the archetypal PDE4 inhibitor, blocked in vivo a LPS-induced inflammation at the feto-maternal interface and prevented subsequent preterm delivery and fetal demise in mice (20). Persistent intrauterine inflammation was concomitant with nuclear localisation of NF-KκB in a specific set of murine cells at the vicinity of maternal cells and PDE4 inhibition prevented the NF-κB nuclear translocation. Furthermore, we evidenced in a previous study that PDE4 was the main PDE family expressed in human fetal membranes and PDE4 activity was increased by LPS (21). PDE4 inhibitors blocked the release of pro-inflammatory cytokines, prostaglandins, and metalloproteases induced by LPS in these membranes.

In the light of these data, the chorion leave of the fetal membranes is likely to have an integrative part in determining the severity and the extent of the intrauterine inflammation. Therefore, we investigated in this study the ability of cultured human chorionic cells to develop an inflammatory response when challenge with LPS and whether PDE4 are implicated in the control of this response.

Materials and Methods

Primary culture of human chorionic cells

Chorionic cells were prepared using a modification of the technique described by Kliman et al (22). Placentas were obtained from non laboring women after a normal term (> 37 weeks of gestation) singleton-pregnancy delivered by elective caesarean section. This study was approved by the « Comité Consultatif de Protection des Personnes pour la Recherche Biomédicale » (Paris-Cochin, France) and informed consent was obtained from all donors. Briefly, fetal membranes were dissected from placenta under sterile conditions and chorion with adherent decidua was peeled off amnion and placed in PBS. After the removal of blood clots, chorion was cut in small pieces and digested with 0.5% trypsin (Sigma, St Louis, MO) and 0.2% collagenase B (Roche Diagnostics, Mannheim, Germany) in DMEM-F12 (Invitrogen, Cergy-Pontoise, France) at 37°C for 3h. After the addition of DMEM-F12 containing 5% FCS and 100 μM EDTA, the cell suspension was filtered through a 100 μm nylon gauze and centrifuged at 400 g for 10 min. The cell pellet, resuspended in complete medium (DMEM-F12 containing 5% FCS, 2.5 μg/ml amphotericin, 100 IU/ml penicillin (Invitrogen), 100 μg/ml streptomycin (Invitrogen), 1x Insulin Transferrin Selenium and 0.02 μg/ml Epidermal Growth Factor), was loaded onto a continuous Percoll gradient (40% Percoll, 10% HBSS, 10 mM Hepes, Sigma) and centrifuged at 1,200 g for 20 min. Cells that accumulate around 1/3 superior of the tube were collected and incubated on a Petri dish coated with monoclonal antibodies against human HLA class I for 20 min, to remove the maternal cells (23). Unattached cells were pelleted by centrifugation, resuspended in complete medium and plated at a density of 10^5 cells/cm^2 for immunocytochemistry and immunofluorescence analysis, and at a density of 2.10^5 cells/cm^2 for other experiments on dishes coated with collagen type I 5 μg/cm^2 , and cultured in complete medium for three days at 37°C in 5% CO$_2$ and 95% air before experimentation. For some experiments, macrophages or mesenchymal cells were immuno-depleted using CD45 monoclonal antibodies or vimentin monoclonal using the Dynabeads system according to the manufacturer's instructions. Chorionic cells were transferred to serum-deprived medium 2h before treatment and then incubated with 100 ng/ml LPS at 37°C, with or without 10^{-5} M rolipram for the indicated times.

U937 cells

U937 cells, obtained from ATCC, were cultured in RPMI supplemented with 10% FCS and 2 mM glutamine, at 37°C in 5% CO$_2$ and 95% air. U937 cells were differentiated in macrophages with 100 nM PMA for 48h and then replaced in fresh medium without PMA for 24h.

Assessment of the chorionic cells preparation purity by immunofluorescence

Chorionic cells were cultured in 24-well dishes on collagen-coated glass coverslips. After fixation for 15 min with 4% para-formaldehyde in PBS, for binding of CK7 and vimentin antibodies, cells were permeabilized by incubation in 0.1% Triton X-100 in 10% FCS–PBS for 15 min and for binding of CD45 antibodies, cells were incubated in 10% FCS–PBS without Triton for 15 min. Incubation with primary monoclonal antibodies directed against CK7 (dilution 1:500, DakoCytomation, Carpinteria, CA), vimentin (dilution 1:500, DakoCytomation) and CD45 (dilution 1:500, DakoCytomation) was performed overnight at 4°C. Incubation with secondary antibodies was performed with the anti-mouse IgG1 TRITC-labeled solution (dilution 1:1200, SouthernBiotech, Birmingham, AL) for 45 min at RT. Nuclei were labeled with Hoechst 33342, diluted 1:500 in water for 2 min. Coverslips were mounted on slides using fluorescent mounting medium (DakoCytomation). Negative controls were carried out by using mouse IgG1.

Quantitative analysis of the NF-κB p65 nuclear translocation by immunofluorescence

Chorionic cells were cultured in 24-well dishes on collagen-coated glass coverslips. After fixation for 15 min with 4% para-formaldehyde in PBS, cells were permeabilized by incubation in 0.1% Triton X-100 in 10% FCS–PBS for 15 min. Incubation with primary antibodies directed against NF-κB p65 (dilution 1:500, sc-109, Santa Cruz Biotechnology) was performed overnight at 4°C. Incubation with secondary antibodies was performed with the Alexa Fluor R-488 donkey anti-rabbit IgG FITC-labeled solution (dilution 1:500, Interchim, Montluçon, France) for 45 min at RT. Nuclei were labeled with Hoechst 33342, diluted 1:500 in water for 2 min. Coverslips were mounted on slides using fluorescent mounting medium (DakoCytomation). Negative controls were carried out by using a non immune rabbit serum.

For quantitative analysis, total and unlabelled nuclei were counted in six distinct random fields per coverslip. The difference corresponds to nuclei containing NF-κB p65. The result was expressed in percentage of labelled nuclei. All coverslips were counted by a single investigator for internal consistency. Random fields were counted by a blinded independent investigator for external verification of the results.

Cell lysates and nuclear extracts

For whole lysates of chorionic cells, cells were homogenized in ice-cold buffer (50 mM NaCl, 25mM Hepes, 2.5 mM EDTA, 50 mM NaF, 3 mM $Na_4P_2O_7$, 0.5 mM Na_3VO_4, 10% glycerol, 1% NP 40 and a protease inhibitor cocktail (P2714, Sigma)) for 15 min on ice. Homogenates were centrifuged for 5 min at 14,000 g and supernatants were collected. Protein concentrations were determined using the BioRad protein assay (Bio-Rad Laboratories, Hercules, CA, USA) with BSA as a standard.

Nuclear extracts were prepared using the Nuclear Extraction Kit (Imgenex, San Diego, CA), according to the manufacturer′s instructions. Nuclear protein concentrations were determined using the DC BioRad protein assay (Bio-Rad Laboratories) with BSA as a standard.

Immunoblot analysis

Proteins were resolved by 8% or 10% SDS/PAGE and transferred onto polyvinylidene fluoride membranes. Membranes were then blocked for 1h in 5% non fat dried milk in 10mM Tris, 150 mM NaCl and 0.1% Tween 20, pH 7.6 (TBST) and incubated overnight at 4°C with antibodies directed against TLR4 (dilution 1:250, sc-10741, Santa Cruz Biotechnology), NF-κB p65 (dilution 1:3,000, sc-372, Santa Cruz Biotechnology), NF-κB p50 (dilution 1:1,000, sc-7178 Santa Cruz Biotechnology), PDE4B (K118, dilution 1:2,000, kindly donated by Dr M. Conti, Stanford University, CA, (24)) or β-actin (dilution 1:2,000, A-2006, Sigma). After washing, membranes were incubated with HRP-conjugated rabbit secondary antibodies (dilution 1:7,000, sc-2313, Santa Cruz Biotechnology) and immunoreactive bands were visualised by chemiluminescence (Amersham Biosciences ECL reagents, GE Healthcare, Buckinghamshire, UK).

Measurement of TNFα and MCP1

Detection of TNFα in the supernatant of cultured chorionic cells was performed with an ELISA kit (Pierce, Rockford, IL) according to the manufacturer′s instructions. Sensitivity of the kit was < 2 pg/ml and the standard curve range was 16–1,000 pg/ml. Detection of MCP1 was performed using the Searchlight™ multiplex sample testing by Endogen, PerbioScience (Berbieres, France).

cAMP-phosphodiesterase assay

Aliquots of the cell lysates were assayed for cAMP-PDE activity according to the method of Thompson and Appleman as detailed previously (25). PDE activities were measured with 1 μM [^{3}H]-cAMP as a substrate. PDE4 activity was defined as the fraction of cAMP-PDE activity inhibited by 10^{-5} M rolipram and expressed in pmoles/min/mg protein.

Electrophoretic Mobility Shift Assay

Binding reactions were performed with 5 μg of nuclear extracts from chorionic cells, using the LightShift Chemiluminescent EMSA kit (Pierce) with 1x binding buffer, 50 ng/μl poly (dI.dC), 5% glycerol, 0.05% NP-40, 0.5 mM EDTA and 20 fmol biotin end-labeled NF-κB p65 oligonucleotide probe (5′-AGTTGAG<u>GGGAACTTCC</u> CAGGCAG<u>GGGAATTTCC</u> CAGGC-3′) for 20 min. NF-κB sites (underlined) correspond to the sequences described in the MCP-1 promoter (26). After addition of the loading buffer, complexes were separated on a 6% non denaturing polyacrylamide gel in 0.5X TBE and then transferred onto a nylon membrane. Complexes were fixed by UV exposure and visualized by chemiluminescence. For supershift analysis, 5 μg nuclear extracts were pre-incubated for 1h at 4°C with 1 μg antibodies directed against NF-κB p65 (sc-372, Santa Cruz Biotechnology) or NF-κB p50 (sc- 7178, Santa Cruz Biotechnology) before addition of the labelled probe.

Statistical analysis

The significance of the difference was assessed by Kruskall-Wallis test followed by a Wilcoxon matched pair test using the Prism-Graph Pad software (GraphPad Software). The difference was considered significant when p was < 0.05.

Results

Characterization of dispersed chorionic cells

As observed by immunofluorescence analysis, routine preparation of chorionic cells yield to a mixed combination of CK7-positive cells (trophoblasts, >90% of the total cells), CD45-positive cells (macrophages, 1–5% of the total cells) and vimentin-positive cells (mesenchymal cells, 1–5%) after 24h of culture. After 72h of culture, trophoblasts clustered and were present as clumps or scarce single cells, macrophages remained as single cells and mesenchymal cells covered the remaining space of the wells (Figure 1). Depletion of the

macrophages during the isolation of the cells did not affect the microscopic aspects of the chorionic cells after 72h of culture, whereas depletion of mesenchymal cells induced major death of CK7-positive cells (data not shown).

Functional response of chorionic cells to LPS

Chorionic cell preparations were performed without or with depletion of the macrophages and challenged with 100 ng/mL of LPS. The pro-inflammatory cytokines MCP-1 and TNFα concentrations were measured in the cell medium (Figure 2). In the presence of macrophages, MCP-1 was present in the culture medium in the control condition, whereas TNFα was merely detectable. LPS induced a significant increase of MCP-1 and TNFα concentrations in the culture medium. After depletion of the macrophages, MCP-1 and TNFα were detected in a small amount in the culture medium in the control condition and were not induced by LPS. Because resident macrophages in the chorionic leave of fetal membranes are described throughout pregnancy and because we can not rule out a cooperation of chorionic trophoblasts and macrophages in the response to LPS in term of cytokine production, we performed the next experiments with cell preparations not depleted in macrophages.

TLR4 expression by chorionic cells

The expression of TLR4, the main LPS receptor, was investigated in human chorionic cells by western blot, revealing a unique band of 96 kDa corresponding to TLR4 (27). The intensity of the band remained unchanged upon LPS challenge (Fig 3), indicating that chorionic cells constitutively express TLR4 and this receptor is not regulated by LPS in these cells.

Induction of PDE4 by LPS in chorionic cells

We measured the cAMP-PDE activity in chorionic cells challenged by the endotoxin. As shown in Fig 4A , PDE4 activity was predominant in chorionic cells, as it represents more than 58% of the total cAMP-PDE activity in basal condition, and increased significantly in the presence of LPS. PDE4 activity reached a maximum at 2h of LPS treatment and then declined up to 8h. The non-PDE4 activity increased slightly but the difference failed to reach significance, suggesting that the effect of LPS on cAMP-PDE activity was limited to PDE4 activity.

Western blot analysis (Fig. 4B) performed with the PDE4B specific antibodies K118 revealed three bands, two that migrated at about 80 kDa and one that migrated at about 70 kDa. Among the two close bands, the upper signal was first described as a protein corresponding to the short form product of the PDE4B gene, the PDE4B2 isoform (28). This band, almost undetectable in non-treated cells, appeared at 1h upon LPS treatment. The lower signal was described as nonspecific in human samples and the 70 kDa-band was previously reported as non-specific with the K118 antibody (24). Positive control done in differentiated U937 (29), show the same three bands pattern with K118. These data suggest that the specific isoform PDE4B2 is involved in the LPS-induced inflammatory pathway in chorionic cells.

PDE4 inhibition blocks LPS-induced TNFα secretion in chorionic cells

We investigated the effect of the PDE4 inhibitor rolipram on the release of TNFα, induced by LPS in chorionic cells. As shown in Fig 5A , TNFα concentration increased to reach a maximum at 8h of LPS treatment and declined to basal level after 24h. In the presence of rolipram, TNFα release is reduced as soon as 1h of treatment and plateaus at 4h. At this time point, LPS induced a TNFα release significantly different from control and rolipram alone conditions (Fig 5B). Addition of rolipram to LPS significantly reduced the TNFα release. These data indicate that the PDE4 inhibitor has an anti-inflammatory effect on LPS-stimulated chorionic cells.

PDE4 inhibition reduces LPS-induced NF-κB p65 translocation in chorionic cells

As the stimulation of TLR4 triggers the activation of NF-κB, starting with its nuclear translocation, we investigated by immunofluorescence whether rolipram affects the effect of LPS on the cellular localization of the p65 subunit of NF-κB in chorionic cells (Fig 6A). In the control cells, p65 was mainly localized in the cytoplasm of clustered cells and single cells. Addition of LPS for 2h triggered a translocation of p65 from the cytoplasm into the nucleus which was partially blocked in the presence of rolipram. Rolipram alone had no effect on the nuclear translocation of p65.

To evaluate the magnitude of the rolipram effect on LPS-induced translocation of p65, the percentage of cells showing a nuclear immunostaining was determined (Fig 6B). LPS treated cells showed a significant difference from control and rolipram conditions. Addition of rolipram to LPS significantly reduced the number of p65-stained nuclei.

The presence of the p65 subunit of NF-κB in the chorionic cell nuclei was also confirmed by Western Blot analysis (Fig 6C). A band that migrated at 65 kDa was detected in non-treated cells and its intensity was increased in cells treated by LPS. In cells treated by rolipram alone and rolipram in addition to LPS, the intensity of the band was comparable to the one observed in basal condition.

These data indicate that LPS activates the NF-κB pathway in chorionic cells and that PDE4 inhibition reduces the LPS-induced nuclear translocation of NF-κB p65.

PDE4 inhibition decreases LPS-induced NF-κB binding activity in chorionic cells

In order to assess whether rolipram affects the increase in NF-κB binding activity induced by LPS, we performed electrophoretic mobility shift assay on nuclear extracts of chorionic cells. As shown in Fig 7A , two NF-κB complexes were present under control condition. Intensity of these complexes was increased as early as 1h after LPS treatment and peaked at 2h. A third weak complex that migrated slower appeared at 2h of LPS treatment. The addition of rolipram strongly reduced the activation of the two fast complexes and suppressed the third complex.

Supershift analysis was performed to further identify the subunits of the NF-κB complexes present at 2h, the peak time point of activation (Fig 7B). Addition of antibodies against the p50 protein supershifted the fast and middle complexes. Antibodies against p65 decreased the binding activity of the slow weak complex as well as the middle complex. This indicated that the fast activated complex in chorionic cells consisted of a p50 homodimer, the middle complex consisted of a p65 and p50 heterodimer and the weak slow complex consisted of a p65 homodimer.

These results suggest that three different NF-κB complexes having a DNA binding activity in LPS-stimulated chorionic cells are sensitive to PDE4 inhibition.

Discussion

Knowing the causal link between intrauterine inflammation and preterm birth, it is essential to understand the mechanisms that take place at the feto-maternal interface to develop strategies preventing this event. In this study, we showed that the PDE4 inhibitor rolipram has an anti-inflammatory effect on LPS-stimulated chorionic cells. Upon LPS stimulation, the transcription factor NF-κB is activated in these cells which release the pro-inflammatory cytokines TNFα and MCP-1. PDE4 inhibition reduces this NF-κB activation. Because NF-κB is thought to have a central role in human preterm labor, selective targeting of its activation at the feto-maternal interface may offer an alternative way to prevent preterm delivery.

Several teams have developed protocols to isolate disperse chorionic cells; the most widely used is adapted from the Kilman's protocol to isolate villous trophoblast of the placenta, as in the current study. These protocols yield to an enriched preparation of CK7-positive cells, i.e. trophoblasts, and a workshop about primary culture of human trophoblasts recommended using preparations with 50% or more CK7 positive cells (30). We routinely obtained more than 90% of CK7 positive cells with little contamination by macrophages and mesenchymal cells. In order to improve purity of the preparation, methods based on negative selection can be used; however we found major changes in the survival of isolated trophoblasts in the absence of mesenchymal cells and in the global response to the endotoxin LPS of the chorionic cells in the absence of macrophages. Because chorionic leave of fetal membranes is composed mainly of chorionic trophoblasts, but also of mesenchyme and extracellular matrix as well as of resident macrophages, our chorionic cells model reflects this specific combination of cells.

Chorion leave is in the direct contact with the maternal decidua and the cervix and in the vicinity of the myometrium throughout pregnancy. Because chorionic cells metabolize prostaglandins throughout pregnancy and this function is modified towards parturition, it has been postulated that these cells regulate myometrial contractility at term and preterm (31). Herein, we provide evidence that TLR4, the main LPS receptor, is expressed by human chorionic cells and is not regulated by LPS. Moreover, chorionic cells respond functionally to LPS in that they produce the proinflammatory cytokines TNFα and MCP-1. As described in human choriodecidua (21), PDE4 activity is also predominant in cultured human chorionic cells. PDE4 activity was increased in these cells upon LPS challenge and the expression of the specific isoform PDE4B2 was induced. PDE4 was previously reported to be predominant in monocytes and macrophages, wherein LPS strongly stimulates the mRNA expression of the PDE4B subtype, and in neutrophils, wherein PDE4B is constitutively expressed at high levels. The specific isoform implicated in the PDE4 activity increase under LPS in monocytes was identified as PDE4B2 (19 , 32 , 33). It is well-documented that PDE4-selective inhibitors produce profound inhibitory effect on LPS-stimulated TNFα production in circulating monocytes (34). The use of mice deficient in PDE4B gene allowed to determine that the PDE4B induction by LPS was responsible for the two-thirds of the TNFα production in monocytes and macrophages (32). In our model of chorionic cells, we have shown that the inhibition of PDE4 by rolipram decreased the TNFα release in the same proportion than in leukocytes. As we were able to identify an upregulation of the specific isoform PDE4B2 in chorionic cells, we suggest that PDE4B2 is involved in the LPS-induced TNFα production in these cells. Such data, together with our previous studies, confirm PDE4B2 as a promising pharmaceutical target in the management of preterm labor related to inflammation. PDE4 inhibitors have been developed for use in a variety of human health conditions such as depression, asthma, or inflammatory disorders. Heretofore their most frequent adverse effects, namely nausea and emesis, and high dosage dependent cases of arteritis in rodents and dog have hindered their therapeutic application (35). Although not yet available, we can expect that specific PDE4 subtype inhibitors such as PDE4B2 inhibitors may result in a broader safety window compatible with a clinical use during pregnancy.

An examination of the molecular mechanisms of LPS-induced inflammation in chorionic cells has shown the nuclear translocation of the p65 subunit of NF-κB and the global activation of three distinct NF-κB complexes. A fraction of p50/p50 was already active in control conditions while NF-κB complexes containing p65 were activated by LPS. Because p50 lacks of a transactivation domain, p50/p50 is thought to be a repressor of transcription that would explained its presence in control conditions (36). Conversely, p65 subunit is a potent transcriptional activator (37). Schreiber et al (38) demonstrated that the different NF-κB family members act coordinately to regulate gene expression in LPS-stimulated human monocytes. They have shown that p50 is bound to the promoters of many NF-κB target genes in resting cells while p65 binds to its target genes, including some prebound by p50, only in LPS stimulated cells. In the chorionic cells, a part of complexity in the NF-κB signaling is supplied by the presence of three complexes; each one could be responsible for the activation and/or repression of specific genes. The PDE4 inhibition partially blocked the p65 nuclear translocation and reduced the NF-κB global binding activity. Prevention of NF-κB nuclear translocation by rolipram was previously shown in a LPS-induced uveitis model in rat (39) and in a LPS-induced preterm delivery model in mouse (20). Our data highlight an involvement of PDE4 in the NF-κB signaling of human chorionic cells and such interaction may be a key event in the fetal response to inflammation. Moreover because nuclear translocation of NF-κB-p65 subunit was observed in most of the cells, cooperation in a paracrine manner between trophoblasts and the other cell types at the feto-maternal interface may occur in response to LPS.

In conclusion, human chorionic cells are able to mount an inflammatory response against bacterial products that is readily reduced by PDE4 inhibition. Our previous works showed that PDE4 inhibition is not only able to abolish metalloprotease activation and prostaglandin synthesis in fetal membranes but is also able to block spontaneous contractions of myometrial strips (21 , 40). Given its dual myorelaxant and anti-inflammatory properties (41), selective PDE4 inhibition may represent a novel approach in the management of inflammation-induced preterm delivery.

Footnotes:

Disclosure statement The authors have no financial conflict of interest.

Abbreviations

PDE : phosphodiesterase

CK7 : cytokeratin 7

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Fig 1

Characterization of cultured chorionic cells

Immunofluorescence analysis of CK7 (A), vimentin (B) and CD45 (C) in cultured chorionic cells showed the clustered pattern of trophoblasts, the expansion of mesencymal cells and a few macrophages.
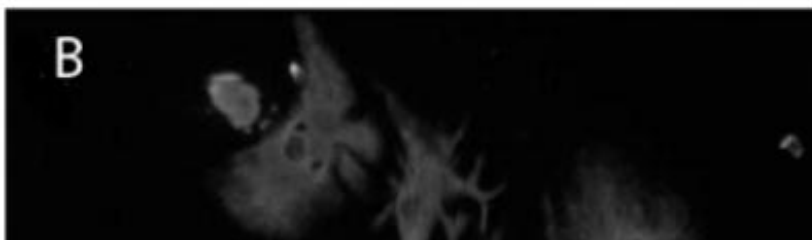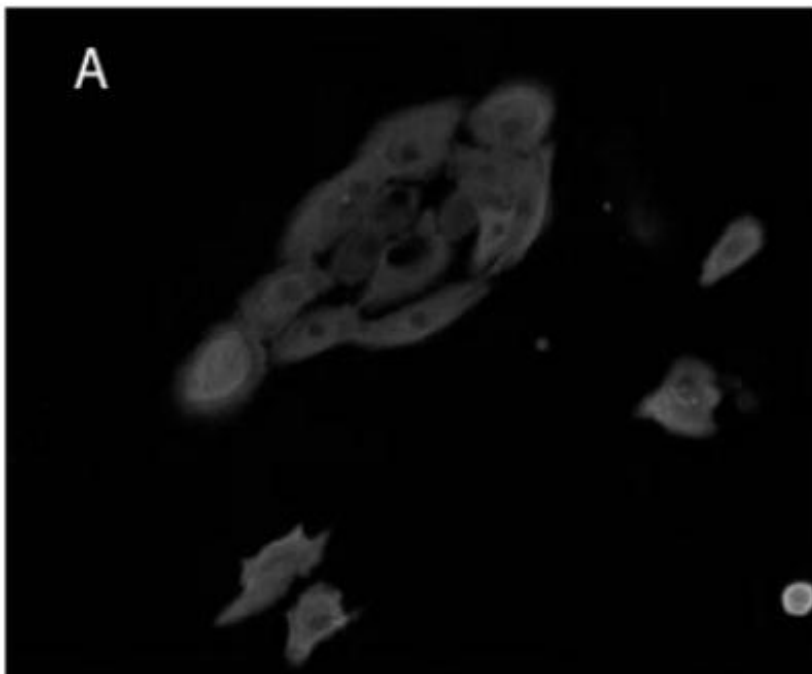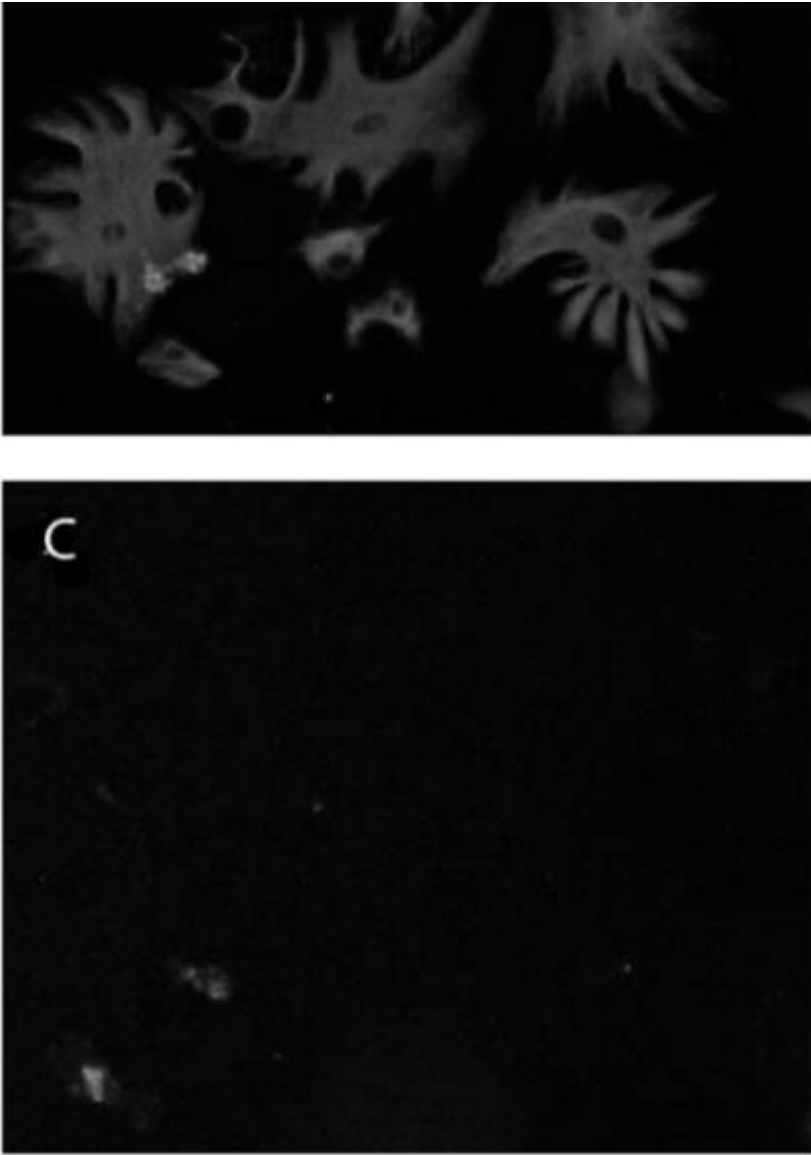
Fig 2

Response of chorionic cells to LPS in the presence and in the absence of macrophages

Concentration of TNFα and MCP1 were measured in cell media collected after 4h of treatment with LPS 100 ng/ml or its diluent. The cell preparations were either or not depleted of CD45 positive cells. Data are expressed as the mean ± sem of 5 different preparations. *, $p < 0.05$, significantly different from control, not CD45-depleted cell preparation
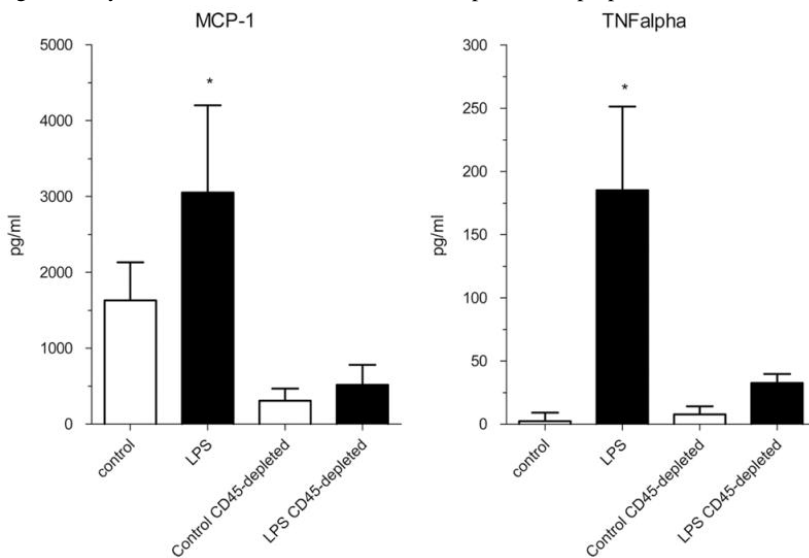
Fig 3

Analysis of TLR4 expression by chorionic cells

Lysates of cells treated with LPS 100 ng/mL for the indicated times were subjected to Western Blot (15 μg of proteins per lane) and probed with the TLR4 antibodies. Lysate of U937 cells served as a positive control. The detection of β-actin in each sample served as a loading control. A representative experiment is shown, reproduced 3 times.
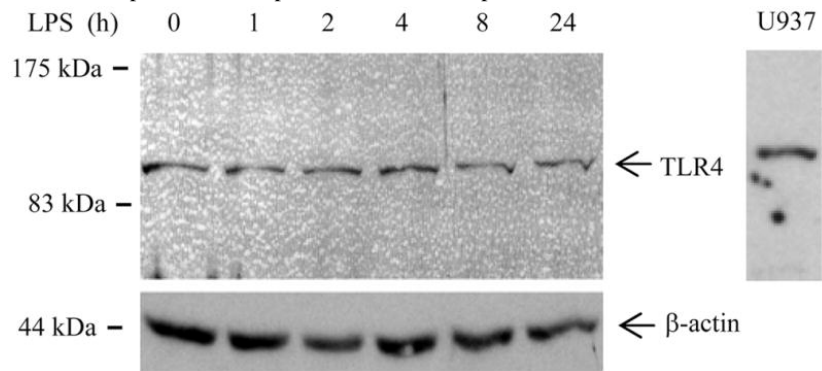
Fig 4

Induction of PDE4 by LPS in chorionic cells

A. Time-dependent effect of LPS on PDE4 activity. Data reported the mean ± SEM of 4 separate experiments. **, p < 0.01. B. Effect of LPS on the PDE4B expression. Lysates of cells treated with LPS 100 ng/mL for the indicated times were subjected to Western Blot (15 μg of proteins per lane) and probed with the PDE4B antibodies. Lysate of U937 cells served as a positive control. A representative experiment is shown, reproduced 3 times.
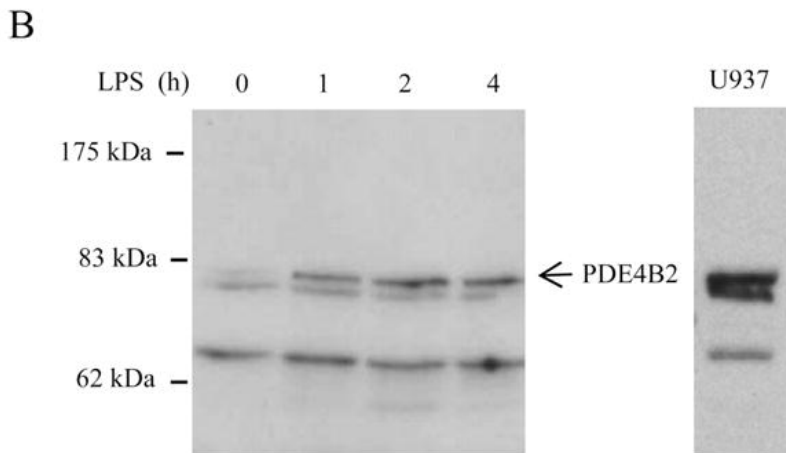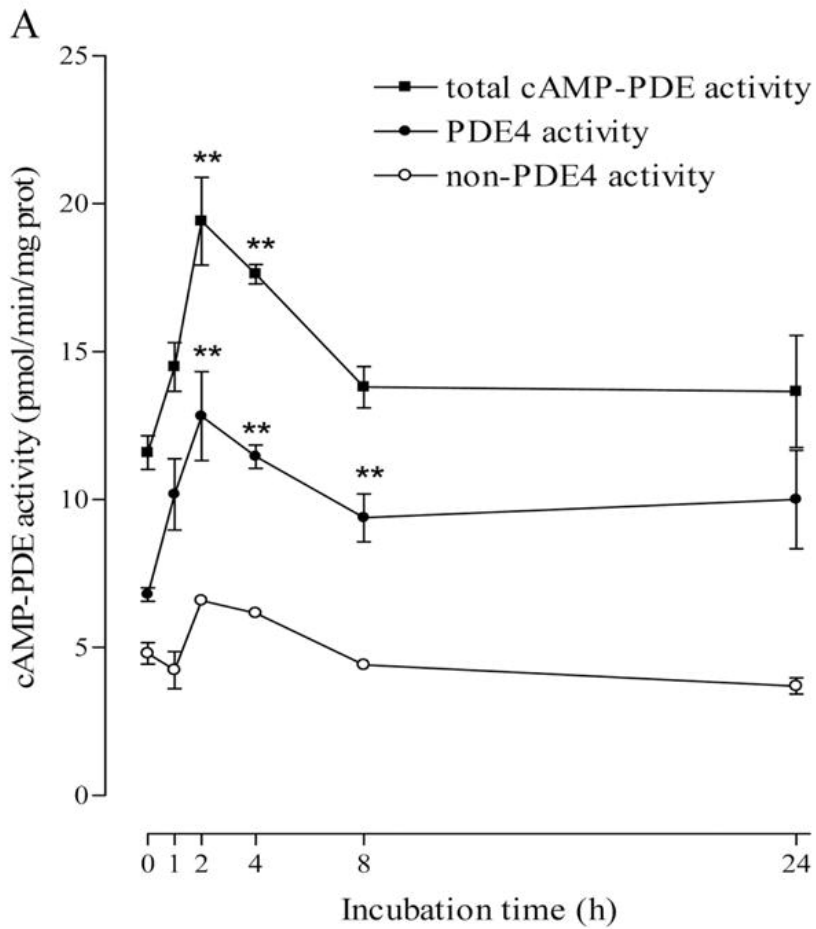
Fig 5

Effect of LPS and rolipram on TNFα secretion by chorionic cells

A. Time-course of TNFα secretion by chorionic cells. Cells were incubated in the absence or the presence of LPS 100 ng/mL with or without rolipram 10^{-5} M for the indicated times. A representative experiment is shown, data reported the mean ± SD of triplicates in the assay, reproduced 3 times. B. Chorionic cells of 10 different placentas were incubated in the absence or presence of LPS with or without rolipram for 4h. Data are expressed as the mean ± SEM of the 10 separate experiments. *, $p < 0.01$, significantly different from control and rolipram. [#], $p < 0.01$, significantly different from LPS treatment.
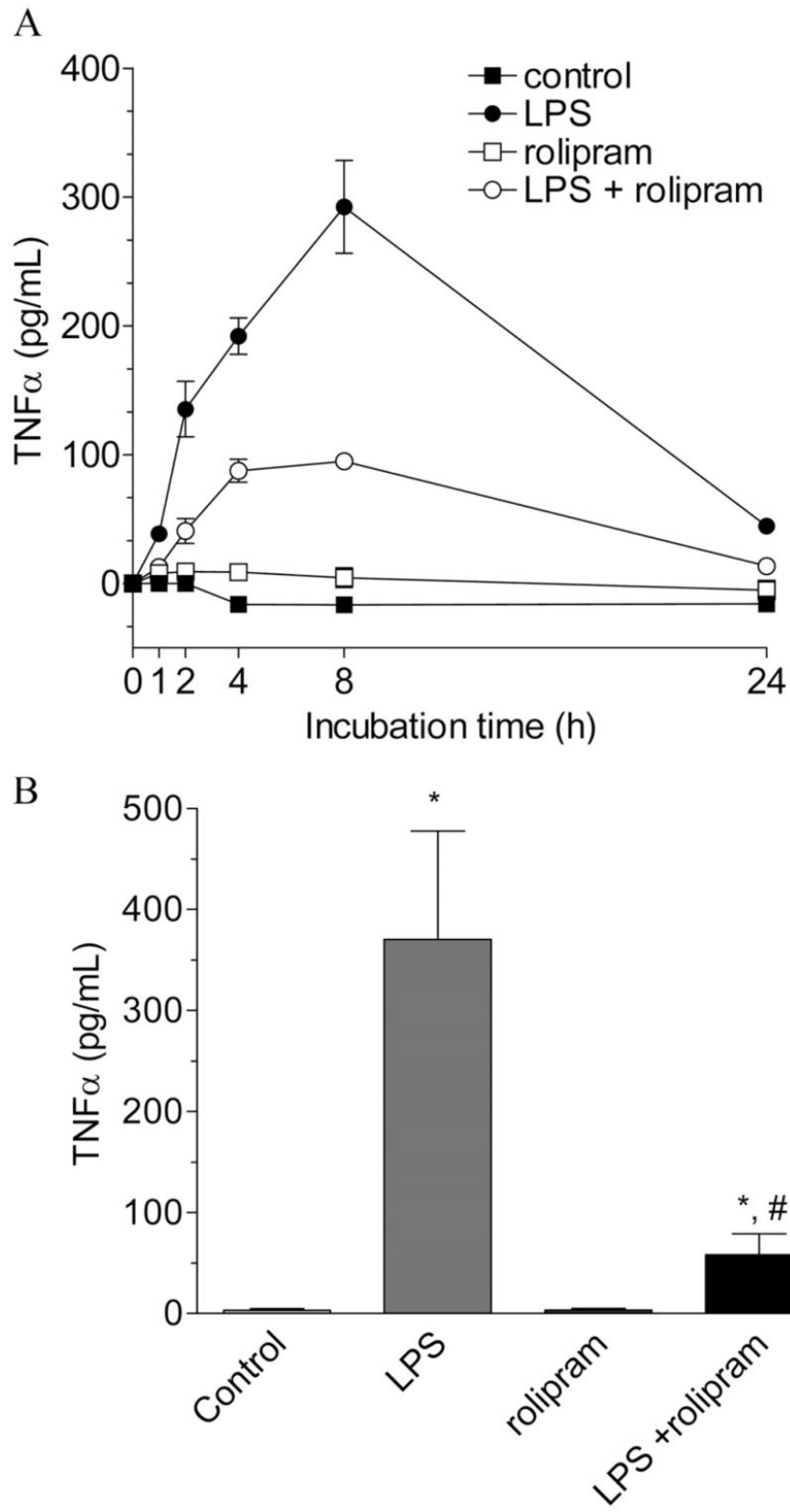
Fig 6

Effect of LPS and rolipram on the NF-κB p65 nuclear translocation in chorionic cells

A. p65 nuclear translocation assessed by immunofluorescence under LPS 100 ng/mL with or without rolipram 10^{-5} M for 2h. A representative experiment is shown, reproduced 7 times. B. Chorionic cells were incubated in the absence or in the presence of LPS and rolipram for 2h. p65 nuclear translocation was assessed by immunofluorescence and quantified as described in Materiel and Methods. Data are expressed as the mean ± SEM of the 7 separate experiments.*, $p < 0.05$, significantly different from control and rolipram. [#], $p < 0.01$, significantly different from LPS treatment. C. Nuclear extracts from chorionic cells treated with LPS with or without rolipram for 2h were subjected to Western Blot (25 μg of proteins per lane) and probed with the p65 antibodies. The detection of β-actin in each sample served as a loading control. A representative experiment is shown, reproduced 3 times.
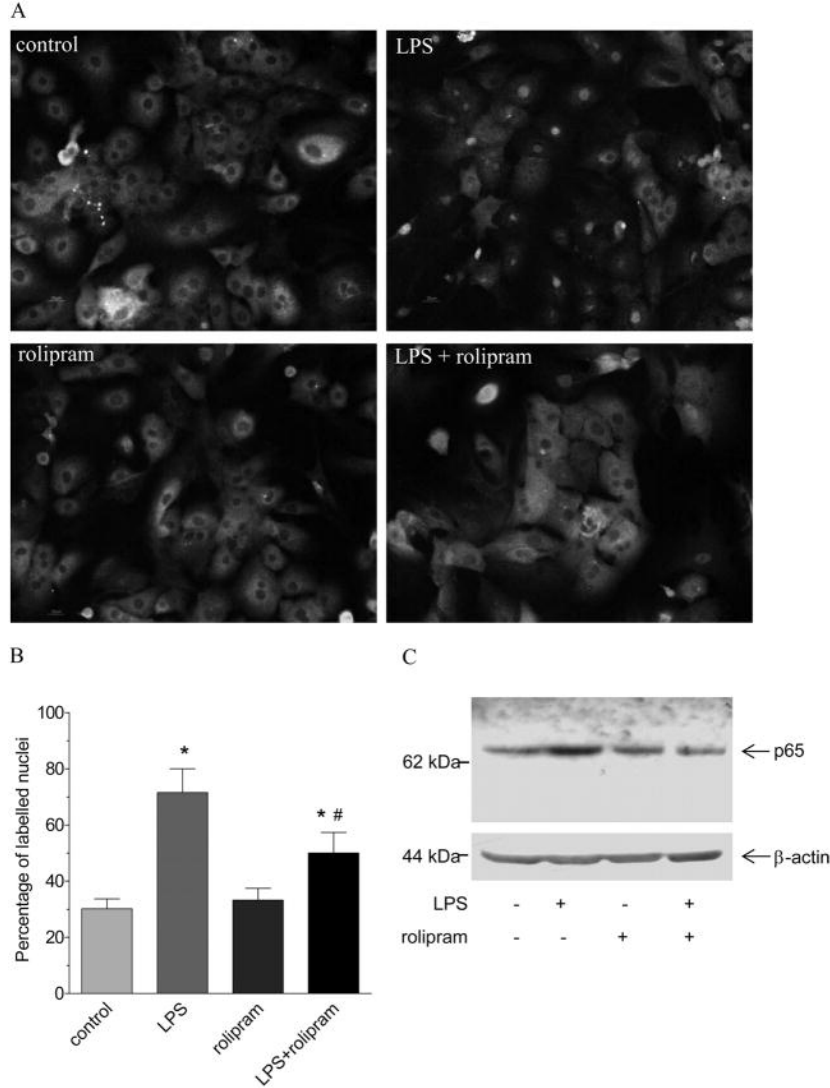
Fig 7

Effect of LPS and rolipram on the NF-κB binding activity in chorionic cells. Representative experiments are shown, reproduced 3 times. A. Time-course of NF-κB binding activity in chorionic cells under LPS 100 ng/mL with or without rolipram 10^{-5} M. Nuclear extracts from chorionic cells were incubated with biotin end-labeled oligonucleotides containing the NF-κB consensus sequence and subjected to EMSA. B. Supershift analysis of the NF-κB complexes present at 2h of LPS treatment. Nuclear extracts were pre-incubated with antibodies against p50 or p65.