The Journal of Immunology

RESEARCH ARTICLE | AUGUST 01 2008

The PDE4 Inhibitor Rolipram Prevents NF-κB Binding Activity and Proinflammatory Cytokine Release in Human Chorionic Cells[1] FREE

Roxane Hervé; ... et. al

J Immunol (2008) 181 (3): 2196–2202.
https://doi.org/10.4049/jimmunol.181.3.2196

The PDE4 Inhibitor Rolipram Prevents NF-κB Binding Activity and Proinflammatory Cytokine Release in Human Chorionic Cells[1]

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

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 proinflammatory cytokine TNF-α 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. Phosphodiesterase 4 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 TNF-α. 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 TNF-α production and the activation of the three NF-κB 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. ***The Journal of Immunology,*** **2008, 181: 2196–2202.**

Despite great strides in improving the survival of infants born prematurely (before 37 wk 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 intra-amniotic 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 a 10-fold higher level of the proinflammatory cytokines TNF-α 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 proinflammatory 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, proinflammatory 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 hyporesponsive 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 localization of NF-κB in a specific set of murine cells at the vicinity of maternal

---

*Institut National de la Santé et de la Recherche Médicale, Unité 767, Paris, France; †Université Paris Descartes, Paris, France; and ‡Assistance Publique-Hôpitaux de Paris, Hôpital Cochin, Maternité Port-Royal, Paris, France

Received for publication October 26, 2007. Accepted for publication June 2, 2008.

[1] This work was supported by March of Dimes Birth Defects Foundation Grant 6-FY03-6 and is integrated in the European Preterm Labour Group.

[2] Address correspondence and reprint requests to Dr Céline Méhats, Institut National de la Santé et de la Recherche Médicale, Unité 767, Faculté de Pharmacie 4 Avenue de l'Observatoire, 75270 Paris cedex 06, France. E-mail address: celine.mehats@inserm.fr

[3] Abbreviations used in this paper: PDE, phosphodiesterase; CK7, cytokeratin 7.

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 proinflammatory 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 challenged 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 nonlaboring women after a normal term (>37 wk 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 and informed consent was obtained from all donors. In brief, fetal membranes were dissected from the 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-Aldrich) and 0.2% collagenase B (Roche Diagnostics) in DMEM-F12 (Invitrogen) at 37°C for 3 h. 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), 1× 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-Aldrich) 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 mAbs 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 3 days at 37°C in 5% CO_2 and 95% air before experimentation. For some experiments, macrophages or mesenchymal cells were immunodepleted using CD45 mAbs or vimentin monoclonal using the Dynabeads system according to the manufacturer's instructions. Chorionic cells were transferred to serum-deprived medium 2 h 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 1640 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 48 h and then replaced in fresh medium without PMA for 24 h.

Assessment of the chorionic cell 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 cytokeratin 7 (CK7) and vimentin Abs, cells were permeabilized by incubation in 0.1% Triton X-100 in 10% FCS-PBS for 15 min and for binding of CD45 Abs, cells were incubated in 10% FCS-PBS without Triton for 15 min. Incubation with primary mAbs directed against CK7 (dilution 1/500, DakoCytomation), vimentin (dilution 1/500, DakoCytomation), and CD45 (dilution 1/500, DakoCytomation) was performed overnight at 4°C. Incubation with secondary Abs was performed with the anti-mouse IgG1 TRITC-labeled solution (dilution 1/1200, Southern Biotechnology Associates) for 45 min at room temperature. 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 conducted 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 Abs directed against NF-κB p65 (dilution 1/500, sc-109, Santa Cruz Biotechnology) was performed overnight at 4°C. Incubation with secondary Abs was performed with the AlexaFluor R-488 donkey anti-rabbit IgG FITC-labeled solution (dilution 1/500, Interchim) for 45 min at room temperature. 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 conducted by using a nonimmune 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 labeled 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, 25 mM 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-Aldrich)) 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) with BSA as a standard.

Nuclear extracts were prepared using the Nuclear Extraction Kit (Imgenex), 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 1 h in 5% nonfat dried milk in 10 mM Tris, 150 mM NaCl, and 0.1% Tween 20 (pH 7.6; TBST) and incubated overnight at 4°C with Abs 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, donated by Dr. M. Conti, Stanford University, Palo Alto, CA, (24)), or β-actin (dilution 1/2,000, A-2006, Sigma-Aldrich). After washing, membranes were incubated with HRP-conjugated rabbit secondary Abs (dilution 1/7,000, sc-2313, Santa Cruz Biotechnology) and immunoreactive bands were visualized by chemiluminescence (Amersham Biosciences ECL reagents, GE Healthcare).

Measurement of TNF-α and MCP1

Detection of TNF-α in the supernatant of cultured chorionic cells was performed with an ELISA kit (Pierce) 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.

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.

EMSA

Binding reactions were performed with 5 μg of nuclear extracts from chorionic cells, using the LightShift Chemiluminescent EMSA kit (Pierce) with 1× binding buffer, 50 ng/μl poly (dI:dC), 5% glycerol, 0.05% Nonidet P-40, 0.5 mM EDTA, and 20 fmol biotin end-labeled NF-κB p65 oligonucleotide probe (5′-AGTTGAGGGGAACTTCCCAGGCAGGGGAATTTCCCAGGC-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% nondenaturing polyacrylamide gel in 0.5× Tris-borate-EDTA 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 preincubated for

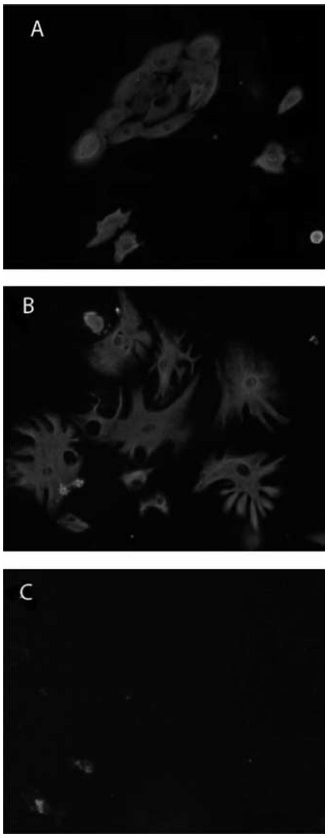


FIGURE 1. Characterization of cultured chorionic cells. Immunofluorescence analysis of CK7 (*A*), vimentin (*B*), and CD45 (*C*) in cultured chorionic cells showed the clustered pattern of trophoblasts, the expansion of mesenchymal cells and a few macrophages.

1 h at 4°C with 1 μg Abs directed against NF-κB p65 (sc-372, Santa Cruz Biotechnology) or NF-κB p50 (sc-7178, Santa Cruz Biotechnology) before addition of the labeled probe.

Statistical analysis

The significance of the difference was assessed by the 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 24 h of culture. After 72 h 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 (Fig. 1). Depletion of the macrophages during the isolation of the cells did not affect the microscopic aspects of the chorionic cells after 72 h of culture, whereas depletion of mesenchymal cells induced major death of CK7-positive cells (data not shown).

Functional response of chorionic cells to LPS

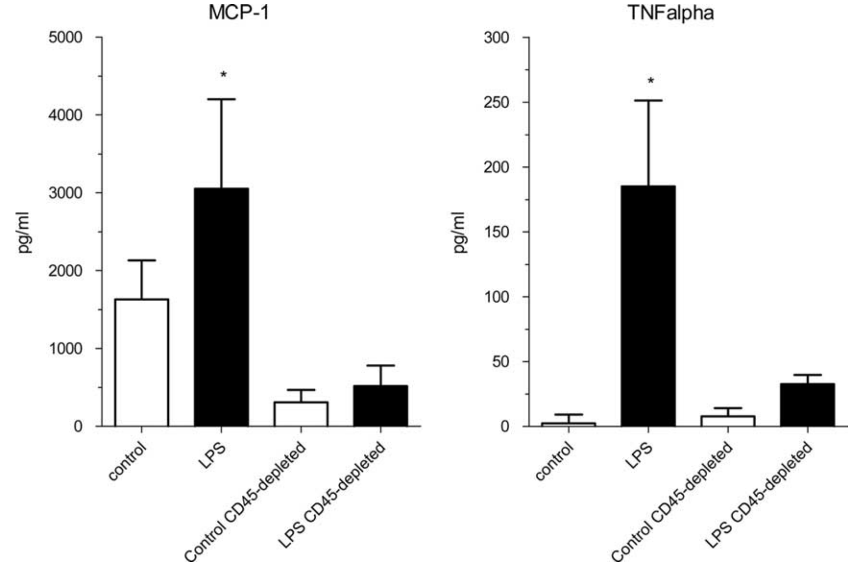
Chorionic cell preparations were performed without or with depletion of the macrophages and challenged with 100 ng/ml of LPS. The proinflammatory cytokines MCP-1 and TNF-α concentrations were measured in the cell medium (Fig. 2). In the presence of macrophages, MCP-1 was present in the culture medium in the control condition, whereas TNF-α was merely detectable. LPS induced a significant increase of MCP-1 and TNF-α concentrations in the culture medium. After depletion of the macrophages, MCP-1 and TNF-α 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 cannot 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.

FIGURE 2. Response of chorionic cells to LPS in the presence and absence of macrophages. Concentration of TNF-α and MCP-1 were measured in cell media collected after 4 h of treatment with LPS 100 ng/ml or its diluent. The cell preparations were or were not depleted of CD45 positive cells. Data are expressed as the mean ± SEM of five different preparations. $*$, $p < 0.05$, significantly different from control, not CD45-depleted cell preparation.



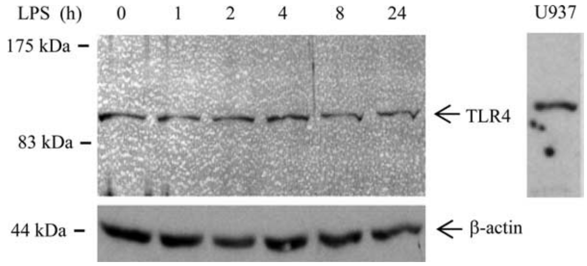


FIGURE 3. Analysis of TLR4 expression by chorionic cells. Lysates of cells treated with LPS 100 ng/ml for the indicated times were subjected to Western blot (15 μg of proteins per lane) and probed with the TLR4 Abs. Lysate of U937 cells served as a positive control. The detection of β-actin in each sample served as a loading control. A representative experiment is shown, reproduced three times.

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. 4*A*, PDE4 activity was predominant in chorionic cells, as it represents >58% of the total cAMP-PDE activity in basal condition, and increased significantly in the presence of LPS. PDE4 activity reached a maximum at 2 h of LPS treatment and then declined up to 8 h. 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.

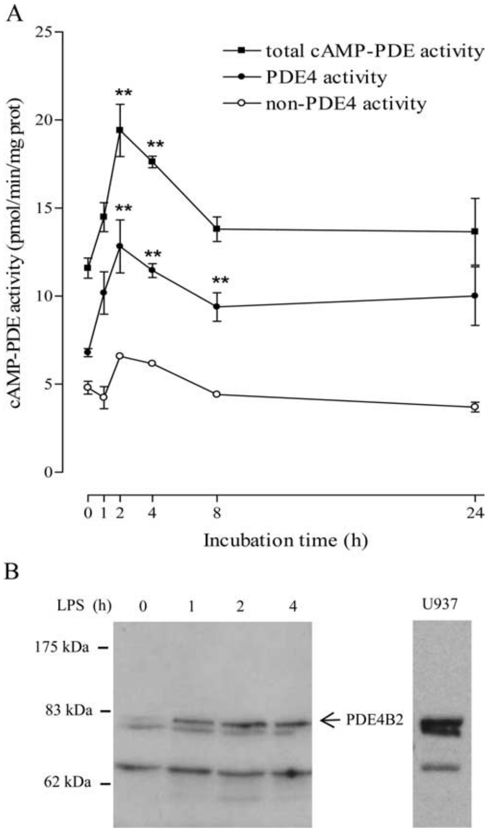


FIGURE 4. Induction of PDE4 by LPS in chorionic cells. *A*, Time-dependent effect of LPS on PDE4 activity. Data reported the mean ± SEM of four separate experiments. **, $p < 0.01$. *B*, Effect of LPS on the PDE4B expression. Lysates of cells treated with LPS 100 ng/ml for the indicated times were subjected to Western blot (15 μg of proteins per lane) and probed with the PDE4B Abs. Lysate of U937 cells served as a positive control. A representative experiment is shown, reproduced three times.

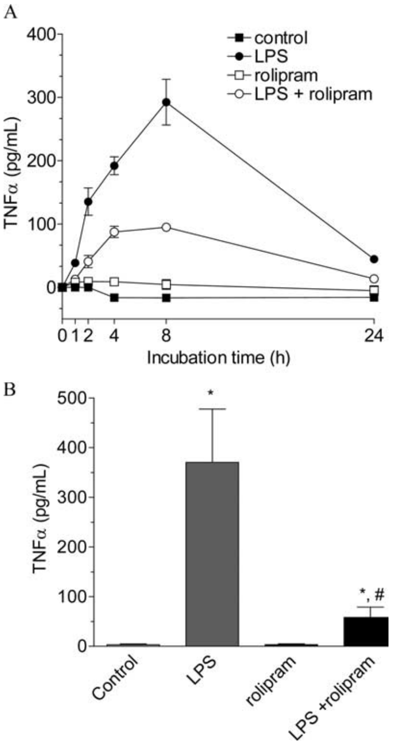


FIGURE 5. Effect of LPS and rolipram on TNF-α secretion by chorionic cells. *A*, Time-course of TNF-α secretion by chorionic cells. Cells were incubated in the absence or the presence of LPS 100 ng/ml with or without rolipram 10^{-5} M for the indicated times. A representative experiment is shown, data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

Western blot analysis (Fig. 4*B*) performed with the PDE4B specific Abs K118 revealed three bands, two that migrated at ~80 kDa and one that migrated at ~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 nontreated cells, appeared at 1 h 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 Ab (24). Positive control done in differentiated U937 (29) shows 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 TNF-α secretion in chorionic cells

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

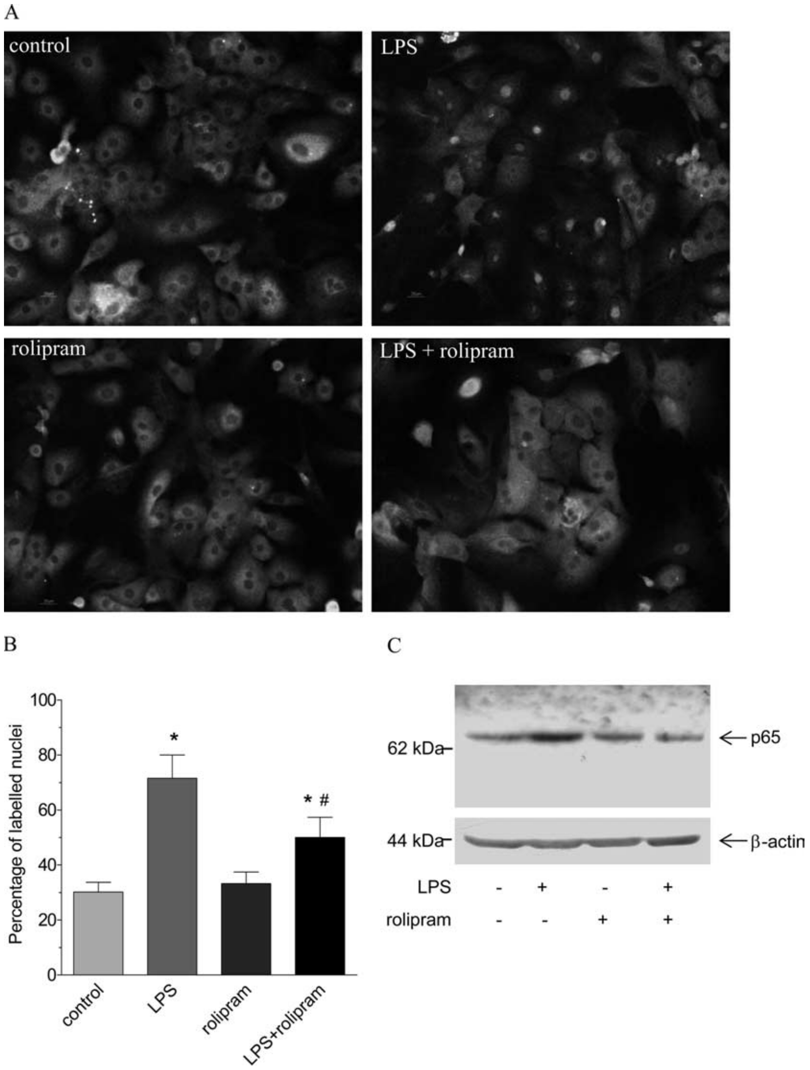


FIGURE 6. Effect of LPS and rolipram on the NF-κB p65 nuclear translocation in chorionic cells. *A*, p65 nuclear translocation assessed by immunofluorescence under LPS 100 ng/ml with or without rolipram 10^{-5} M for 2 h. A representative experiment is shown, reproduced seven times. *B*, Chorionic cells were incubated in the absence or in the presence of LPS and rolipram for 2 h. p65 nuclear translocation was assessed by immunofluorescence and quantified as described in *Materials and Methods*. Data are expressed as the mean ± SEM of the seven 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 2 h were subjected to Western blot (25 μg of proteins per lane) and probed with the p65 Abs. The detection of β-actin in each sample served as a loading control. A representative experiment is shown, reproduced three times.

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. 6*A*). In the control cells, p65 was mainly localized in the cytoplasm of clustered cells and single cells. Addition of LPS for 2 h 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. 6*B*). 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. 6*C*). A band that migrated at 65 kDa was detected in nontreated 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

To assess whether rolipram affects the increase in NF-κB binding activity induced by LPS, we performed EMSA on nuclear extracts of chorionic cells. As shown in Fig. 7*A*, two NF-κB complexes were present under control condition. Intensity of these complexes was increased as early as 1 h after LPS treatment and peaked at 2 h. A third weak complex that migrated slower appeared at 2 h 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 2 h, the peak time point of activation (Fig. 7*B*). Addition of Abs against the p50 protein supershifted the fast and middle complexes. Abs 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.

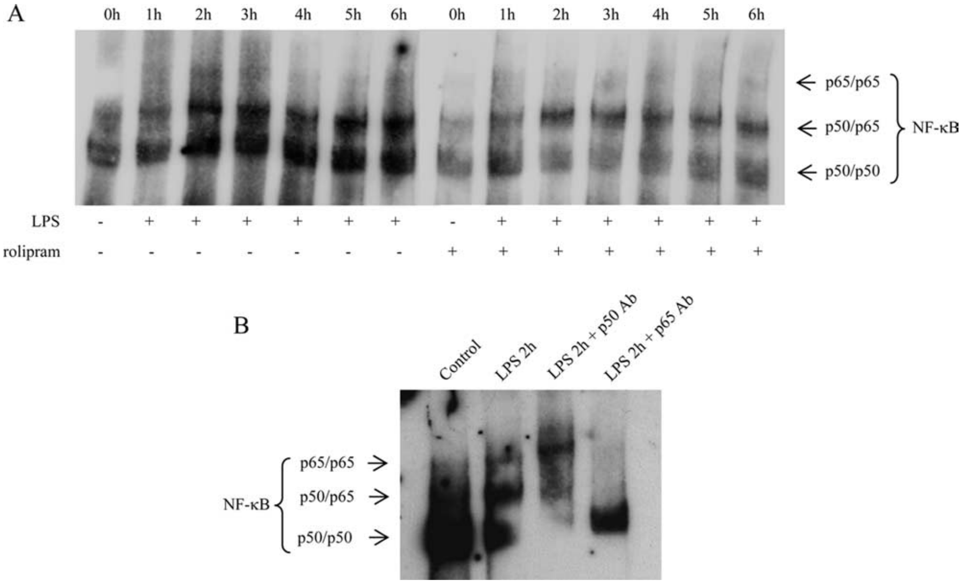


FIGURE 7. Effect of LPS and rolipram on the NF-κB binding activity in chorionic cells. Representative experiments are shown, reproduced three times. *A*, Time-course of NF-κB binding activity in chorionic cells under LPS 100 ng/ml with or without rolipram 10^{-5} M. Nuclear extracts from chorionic cells 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 2 h of LPS treatment. Nuclear extracts were preincubated with Abs against p50 or p65.

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 proinflammatory cytokines TNF-α 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. 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 toward 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 TNF-α 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 TNF-α production in circulating monocytes (34). The use of mice deficient in *pde4b* gene allowed us to determine that the PDE4B induction by LPS was responsible for the two-thirds of the TNF-α production in monocytes and macrophages (32). In our model of chorionic cells, we have shown that the inhibition of PDE4 by rolipram decreased the TNF-α release in the same proportion than in leukocytes. As we were able to identify an up-regulation of the specific isoform PDE4B2 in chorionic cells, we suggest that PDE4B2 is involved in the LPS-induced TNF-α 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 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 an 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.

Disclosures

The authors have no financial conflict of interest.

References

1. Goldenberg, R. L., J. C. Hauth, and W. W. Andrews. 2000. Intrauterine infection and preterm delivery. *N. Engl. J. Med.* 342: 1500–1507.
2. Romero, R., M. Sirtori, E. Oyarzun, C. Avila, M. Mazor, R. Callahan, V. Sabo, A. P. Athanassiadis, and J. C. Hobbins. 1989. Infection and labor. V. Prevalence, microbiology, and clinical significance of intraamniotic infection in women with preterm labor and intact membranes. *Am. J. Obstet. Gynecol.* 161: 817–824.
3. Elovitz, M. A., and C. Mrinalini. 2004. Animal models of preterm birth. *Trends Endocrinol. Metab.* 15: 479–487.
4. Hulboy, D. L., L. A. Rudolph, and L. M. Matrisian. 1997. Matrix metalloproteinases as mediators of reproductive function. *Mol. Hum. Reprod.* 3: 27–45.
5. Keelan, J. A., M. Blumenstein, R. J. Helliwell, T. A. Sato, K. W. Marvin, and M. D. Mitchell. 2003. Cytokines, prostaglandins, and parturition: a review. *Placenta* 24 (*Suppl.* A): S33–S46.
6. Romero, R., J. Espinoza, T. Chaiworapongsa, and K. Kalache. 2002. Infection and prematurity and the role of preventive strategies. *Semin. Neonatol.* 7: 259–274.
7. Denison, F. C., R. W. Kelly, A. A. Calder, and S. C. Riley. 1998. Cytokine secretion by human fetal membranes, decidua and placenta at term. *Hum. Reprod.* 13: 3560–3565.
8. Leroy, M. J., E. Dallot, I. Czerkiewicz, T. Schmitz, and M. Breuiller-Fouche. 2007. Inflammation of choriodecidua induces tumor necrosis factor α-mediated apoptosis of human myometrial cells. *Biol. Reprod.* 76: 769–776.
9. Elovitz, M. A., Z. Wang, E. K. Chien, D. F. Rychlik, and M. Phillippe. 2003. A new model for inflammation-induced preterm birth: the role of platelet-activating factor and Toll-like receptor-4. *Am. J. Pathol.* 163: 2103–2111.
10. Hayden, M. S., and S. Ghosh. 2004. Signaling to NF-kappaB. *Genes Dev.* 18: 2195–2224.
11. Lappas, M., M. Permezel, H. M. Georgiou, and G. E. Rice. 2002. Nuclear factor kappa B regulation of proinflammatory cytokines in human gestational tissues in vitro. *Biol. Reprod.* 67: 668–673.
12. Lappas, M., M. Permezel, and G. E. Rice. 2003. N-Acetyl-cysteine inhibits phospholipid metabolism, proinflammatory cytokine release, protease activity, and nuclear factor-kappaB deoxyribonucleic acid-binding activity in human fetal membranes in vitro. *J. Clin. Endocrinol. Metab.* 88: 1723–1729.
13. Condon, J. C., P. Jeyasuria, J. M. Faust, and C. R. Mendelson. 2004. Surfactant protein secreted by the maturing mouse fetal lung acts as a hormone that signals the initiation of parturition. *Proc. Natl. Acad. Sci. USA* 101: 4978–4983.
14. Moore, A. R., and D. A. Willoughby. 1995. The role of cAMP regulation in controlling inflammation. *Clin. Exp. Immunol.* 101: 387–389.
15. Mehats, C., C. B. Andersen, M. Filopanti, S. L. Jin, and M. Conti. 2002. Cyclic nucleotide phosphodiesterases and their role in endocrine cell signaling. *Trends Endocrinol. Metab.* 13: 29–35.
16. Torphy, T. J. 1998. Phosphodiesterase isozymes: molecular targets for novel antiasthma agents. *Am. J. Respir. Crit. Care Med.* 157: 351–370.
17. Souness, J. E., D. Aldous, and C. Sargent. 2000. Immunosuppressive and anti-inflammatory effects of cyclic AMP phosphodiesterase (PDE) type 4 inhibitors. *Immunopharmacology* 47: 127–162.
18. Jin, S. L., L. Lan, M. Zoudilova, and M. Conti. 2005. Specific role of phosphodiesterase 4B in lipopolysaccharide-induced signaling in mouse macrophages. *J. Immunol.* 175: 1523–1531.
19. Wang, P., P. Wu, K. M. Ohleth, R. W. Egan, and M. M. Billah. 1999. Phosphodiesterase 4B2 is the predominant phosphodiesterase species and undergoes differential regulation of gene expression in human monocytes and neutrophils. *Mol. Pharmacol.* 56: 170–174.
20. Schmitz, T., E. Souil, R. Herve, C. Nicco, F. Batteux, G. Germain, D. Cabrol, D. Evain-Brion, M. J. Leroy, and C. Mehats. 2007. PDE4 inhibition prevents preterm delivery induced by an intrauterine inflammation. *J. Immunol.* 178: 1115–1121.
21. Oger, S., C. Mehats, E. Dallot, D. Cabrol, and M. J. Leroy. 2005. Evidence for a role of phosphodiesterase 4 in lipopolysaccharide-stimulated prostaglandin E2 production and matrix metalloproteinase-9 activity in human amniochorionic membranes. *J. Immunol.* 174: 8082–8089.
22. Kliman, H. J., J. E. Nestler, E. Sermasi, J. M. Sanger, and J. F. Strauss, 3rd. 1986. Purification, characterization, and in vitro differentiation of cytotrophoblasts from human term placentae. *Endocrinology* 118: 1567–1582.
23. Hsi, B. L., C. J. Yeh, and W. P. Faulk. 1984. Class I antigens of the major histocompatibility complex on cytotrophoblast of human chorion laeve. *Immunology* 52: 621–629.
24. Iona, S., M. Cuomo, T. Bushnik, F. Naro, C. Sette, M. Hess, E. R. Shelton, and M. Conti. 1998. Characterization of the rolipram-sensitive, cyclic AMP-specific phosphodiesterases: identification and differential expression of immunologically distinct forms in the rat brain. *Mol. Pharmacol.* 53: 23–32.
25. Thompson, W. J., G. Brooker, and M. M. Appleman. 1974. Assay of cyclic nucleotide phosphodiesterases with radioactive substrates. *Methods Enzymol.* 38: 205–212.
26. Leung, T. H., A. Hoffmann, and D. Baltimore. 2004. One nucleotide in a kappaB site can determine cofactor specificity for NF-kappaB dimers. *Cell* 118: 453–464.
27. Ma, Y., G. Krikun, V. M. Abrahams, G. Mor, and S. Guller. 2007. Cell Type-specific expression and function of Toll-like receptors 2 and 4 in human placenta: implications in fetal infection. *Placenta* 28: 1024–1031.
28. Mehats, C., G. Tanguy, E. Dallot, D. Cabrol, F. Ferre, and M. J. Leroy. 2001. Is up-regulation of phosphodiesterase 4 activity by PGE2 involved in the desensitization of β-mimetics in late pregnancy human myometrium? *J. Clin. Endocrinol. Metab.* 86: 5358–5365.
29. Shepherd, M. C., G. S. Baillie, D. I. Stirling, and M. D. Houslay. 2004. Remodelling of the PDE4 cAMP phosphodiesterase isoform profile upon monocyte-macrophage differentiation of human U937 cells. *Br. J. Pharmacol.* 142: 339–351.
30. Frank, H. G., D. W. Morrish, A. Potgens, O. Genbacev, B. Kumpel, and I. Caniggia. 2001. Cell culture models of human trophoblast: primary culture of trophoblast: a workshop report. *Placenta* 22 (*Suppl.* A): S107–S109.
31. Sangha, R. K., J. C. Walton, C. M. Ensor, H. H. Tai, and J. R. Challis. 1994. Immunohistochemical localization, messenger ribonucleic acid abundance, and activity of 15-hydroxyprostaglandin dehydrogenase in placenta and fetal membranes during term and preterm labor. *J. Clin. Endocrinol. Metab.* 78: 982–989.
32. Jin, S. L., and M. Conti. 2002. Induction of the cyclic nucleotide phosphodiesterase PDE4B is essential for LPS-activated TNF-α responses. *Proc. Natl. Acad. Sci. USA* 99: 7628–7633.
33. Ma, D., P. Wu, R. W. Egan, M. M. Billah, and P. Wang. 1999. Phosphodiesterase 4B gene transcription is activated by lipopolysaccharide and inhibited by interleukin-10 in human monocytes. *Mol. Pharmacol.* 55: 50–57.
34. Souness, J. E., M. Griffin, C. Maslen, K. Ebsworth, L. C. Scott, K. Pollock, M. N. Palfreyman, and J. A. Karlsson. 1996. Evidence that cyclic AMP phosphodiesterase inhibitors suppress TNF α generation from human monocytes by interacting with a "low-affinity" phosphodiesterase 4 conformer. *Br. J. Pharmacol.* 118: 649–658.
35. Bender, A. T., and J. A. Beavo. 2006. Cyclic nucleotide phosphodiesterases: molecular regulation to clinical use. *Pharmacol. Rev.* 58: 488–520.
36. Tong, X., L. Yin, R. Washington, D. W. Rosenberg, and C. Giardina. 2004. The p50–p50 NF-kappaB complex as a stimulus-specific repressor of gene activation. *Mol. Cell. Biochem.* 265: 171–183.
37. Ballard, D. W., E. P. Dixon, N. J. Peffer, H. Bogerd, S. Doerre, B. Stein, and W. C. Greene. 1992. The 65-kDa subunit of human NF-kappa B functions as a potent transcriptional activator and a target for v-Rel-mediated repression. *Proc. Natl. Acad. Sci. USA* 89: 1875–1879.
38. Schreiber, J., R. G. Jenner, H. L. Murray, G. K. Gerber, D. K. Gifford, and R. A. Young. 2006. Coordinated binding of NF-kappaB family members in the response of human cells to lipopolysaccharide. *Proc. Natl. Acad. Sci. USA* 103: 5899–5904.
39. Chi, Z. L., S. Hayasaka, X. Y. Zhang, Y. Hayasaka, and H. S. Cui. 2004. Effects of rolipram, a selective inhibitor of type 4 phosphodiesterase, on lipopolysaccharide-induced uveitis in rats. *Invest. Ophthalmol. Visual Sci.* 45: 2497–2502.
40. Leroy, M. J., I. Cedrin, M. Breuiller, Y. Giovagrandi, and F. Ferre. 1989. Correlation between selective inhibition of the cyclic nucleotide phosphodiesterases and the contractile activity in human pregnant myometrium near term. *Biochem. Pharmacol.* 38: 9–15.
41. Mehats, C., T. Schmitz, S. Oger, R. Herve, D. Cabrol, and M. J. Leroy. 2007. PDE4 as a target in preterm labour. *BMC Pregnancy Childbirth* 7 (*Suppl.* 1): S12.