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Prostaglandins prevent inducible nitric oxide synthase protein expression by inhibiting nuclear factor-κB activation in J774 macrophages

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Abstract We investigated the effect of PGE_2 and iloprost (a prostacyclin analogue) on inducible nitric oxide synthase (iNOS) protein expression and nuclear factor-κB (NF-κB) activation in lipopolysaccharide (LPS)-stimulated J774 macrophages. Incubation of J774 cells with LPS (10 μg/ml) caused an increase of iNOS protein expression which was prevented in a concentration-dependent fashion by PGE_2 (0.1, 1, 10 μM) and iloprost (0.01, 0.1, 1 μM). Electrophoretic mobility shift assay indicated that both prostanoids blocked the activation of NF-κB, a transcription factor necessary for NO synthase induction. PGE_2 and iloprost also blocked disappearance of IκB-α from cytosolic fraction and nuclear translocation of NF-κB subunits p50 and p65. These results show for the first time that PGE_2 and iloprost down-regulate iNOS protein expression by inhibiting NF-κB activation and suggest a negative feed-back mechanism that may be important for limiting excessive or prolonged NO production in pathological events.

Key words: Prostaglandin; Inducible nitric oxide synthase; Nuclear factor-κB; Lipopolysaccharide

1. Introduction

Prostaglandins (PGs) play an important role as mediators of the inflammatory response. Cyclooxygenase (COX), the key enzyme for PGs generation from arachidonic acid, has been found in two forms [1]. After stimulation with bacterial lipopolysaccharide (LPS) or some cytokines, many cell types, including endothelial cells and macrophages, express the inducible isoform (COX-2) which is responsible for the production of large amounts of proinflammatory PGs at the inflammation site [2,3]. Several lines of evidence suggest that PGs may modulate macrophage activation process by blocking the release of some cytotoxic/cytostatic molecules such as nitric oxide (NO) [4,5]. NO, a short lived mediator, is synthesised by a family of enzymes termed NO-synthase (NOS), which utilise L-arginine as the substrate for the generation of NO and equal amounts of citrulline [6]. Two types of NOS are recognised: constitutive isoforms (endothelial NOS or eNOS and neuronal NOS or nNOS) which are active for relative short time in response to intracellular Ca^{2+} fluctuations, and a Ca^{2+}-independent inducible isoform for which mRNA translation and protein synthesis are required [6,7]. Inducible NOS (iNOS) is regulated by inflammatory mediators, and the excessive production of NO by iNOS has been implicated in the pathogenesis of the inflammatory response [8–11]. The transcription factor NF-κB plays a critical role in the transcriptional regulation of iNOS gene induced by LPS and cytokines [12]. NF-κB is a ubiquitous transcription factor composed of a group of nuclear proteins: c-Rel, p50/p105 (NF-κB1), p65 (Rel A), p52/p100 (NF-κB2) and Rel B. NF-κB complexes are sequestered in the cytoplasm of most resting cells by inhibitory proteins belonging to the IκB family. In response to various stimuli IκB-α is first phosphorylated and then rapidly degraded by the proteasome, allowing NF-κB nuclear translocation and gene activation [13,14]. We have previously shown that PGE_2 and iloprost (a prostacyclin analogue) inhibit NO generation in LPS-stimulated J774 macrophages [4]. Recently, it has been reported that the prostaglandin-cyclic AMP system can affect the LPS-induced iNOS and COX-2 protein expression in J774 macrophages [15]. In the light of these findings, since the activation of NF-κB has been shown to be an essential step in the induction of iNOS expression, we have investigated whether PGE_2 and iloprost were able to down-regulate the iNOS protein expression by inhibiting NF-κB activation in LPS-treated J774 macrophages.

2. Materials and methods

2.1. Cell culture

The murine monocyte/macrophage cell line J774 was cultured at 37°C in humidified 5% CO_2/95% air in DMEM containing 10% foetal bovine serum, 2 mM glutamine, 100 UI/ml penicillin and 100 μg/ml streptomycin. The cells were plated in 24-culture wells at a density of 2.5×10^5 cells/ml/well or 10-cm diameter culture dishes at a density of 1×10^7 cells/ml/dish and allowed to adhere for 2 h. Thereafter the medium was replaced with fresh medium and cells were activated with LPS (10 μg/ml). PGE_2 (1, 10, 100 μM) or iloprost (0.1, 1, 10 μM) were added to the cells 20 min before LPS challenge. In some experiments PGE_2 (10 μM) or iloprost (1 μM) were added 12 h after LPS challenge. NO generation was measured as nitrite (NO_2^-, nmol/10^6 cells) accumulated in the incubation media (6, 12, 24 h after LPS challenge) using a spectrophotometric assay based on Griess reaction [16] and cell viability (>95%) was determined by using MTT assay [17].

2.2. Preparation of cytosolic and nuclear extracts

Extracts of macrophages stimulated for 2 h with LPS (10 μg/ml) in the presence or absence of PGE_2 (1, 10, 100 μM) or iloprost (0.1, 1, 10 μM) were prepared as described [18]. Briefly, harvested cells (2×10^7) were washed two times with ice-cold PBS and centrifuged at $180\times g$ for 10 min at 4°C. The cell pellet was resuspended in 100 μl of ice-cold hypotonic lysis buffer (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM phenylmethylsulfonylfluoride, 1.5 μg/ml soybean trypsin inhibitor, 7 μg/ml pepstatin A, 5 μg/ml leupeptin, 0.1 mM benzamidine, 0.5 mM DTT) and incubated on ice for 15 min. The cells were lysed by rapid passage through a syringe needle for 5–6 times and the cytoplasmic fraction was then obtained by centrifugation for 1 min at $13\,000\times g$ for 1 min. The nuclear pellet was resuspended in 60 μl of high salt extraction buffer (20 mM HEPES, pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% v/v glycerol, 0.5 mM phenylmethylsulfonylfluoride, 1.5 μg/ml soybean trypsin inhibitor, 7 μg/ml

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Abbreviations: iNOS, inducible nitric oxide synthase; NF-κB, nuclear factor-κB; LPS, lipopolysaccharide

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pepstatin A, 5 μg/ml leupeptin, 0.1 mM benzamidine, 0.5 mM DTT) and incubated with shaking at 4°C for 30 min. The nuclear extract was then centrifuged for 15 min at 13 000×*g* and supernatant was aliquoted and stored at −80°C. Protein concentration was determined by the Bio-Rad protein assay kit.

2.3. Electrophoretic mobility shift assay (EMSA)

Double stranded oligonucleotides containing the NF-κB recognition sequence (5′-AGT TGA GGG GAC TTT CCC AGG-3′) were end-labeled with [γ-^{32}P]ATP. Nuclear extracts containing 5 μg proteins were incubated for 30 min with radiolabeled oligonucleotides (2.5–5.0×10^4 cpm) in 20 μl reaction buffer containing 2 μg poly(dI-dC), 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 μg/ml bovine serum albumin, 10% (v/v) glycerol. Nuclear protein-oligonucleotide complexes were resolved by electrophoresis on a 6% non-denaturing polyacrylamide gel in 0.5× TBE buffer at 150 V for 2 h at 4°C. The gel was dried and autoradiographed with intensifying screen at −80°C for 20 h. Subsequently, the relative bands in nuclear fractions were quantified by densitometric scanning of the X-ray films with a GS 700 Imaging Densitometer (Bio-Rad) and a computer programme (Molecular Analyst, IBM).

2.4. Western blot analysis

Immunoblotting analysis of iNOS, IκB-α, p50 and p65 proteins was performed on J774 cells incubated with LPS (10 μg/ml) in the presence or absence of PGE_2 or iloprost at different time points. Cytosolic fraction proteins were mixed with gel loading buffer (50 mM Tris/10% SDS/10% glycerol/10% 2-mercaptoethanol/2 mg of bromophenol per ml) in a ratio of 1:1, boiled for 3 min and centrifuged at 10 000×*g* for 10 min. Protein concentration was determined and equivalent amounts (75 μg) of each sample were electrophoresed in a 12% discontinuous polyacrylamide minigel. The proteins were transferred onto nitrocellulose membranes, according to the manufacturer's instructions (Bio-Rad). The membranes were saturated by incubation at 4°C overnight with 10% non-fat dry milk in PBS and then incubated with (1:1000) anti-iNOS, anti-IκB-α, anti-p50 and anti-p65 antibodies for 1 h at room temperature. The membranes were washed three times with 1% Triton 100-X in PBS and then incubated with anti-rabbit immunoglobulins coupled to peroxidase (1:1000). The immunocomplexes were visualised by the ECL chemiluminescence method (Amersham). Subsequently, the relative expression of iNOS, IκB-α, p50 and p65 proteins in cytosolic and nuclear fractions was quantified by densitometric scanning of the X-ray films with a GS 700 Imaging Densitometer (Bio-Rad) and a computer programme (Molecular Analyst, IBM).

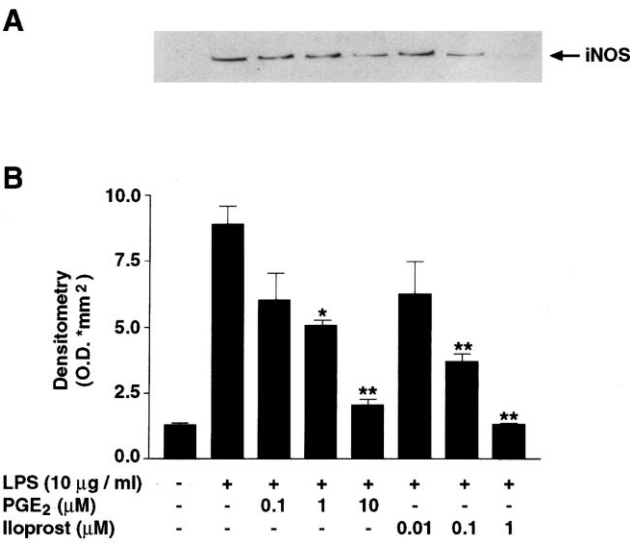

Fig. 1. Effect of different concentrations of PGE_2 and iloprost on iNOS protein expression in J774 macrophages stimulated with LPS (10 μg/ml) for 24 h. Representative Western blot of iNOS protein (A) as well as the densitometric analysis (B) shows the effect of PGE_2 (0.1, 1, 10 μM) and iloprost (0.01, 0.1, 1 μM). Data are expressed as mean ± S.E.M. of three separate experiments. *$P < 0.05$; **$P < 0.01$ vs. control (LPS alone).

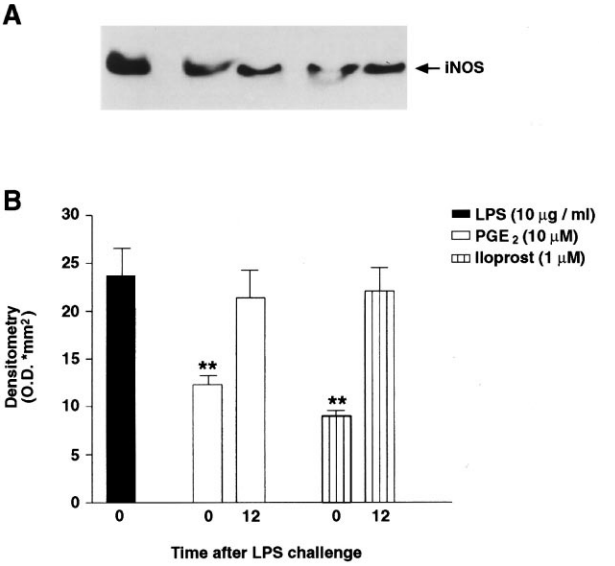

Fig. 2. Effect of delayed addition of PGE_2 and iloprost on iNOS protein expression in J774 macrophages stimulated with LPS (10 μg/ml) for 24 h. Representative Western blot of iNOS protein (A) as well as the densitometric analysis (B) shows the effect of PGE_2 (10 μM) and iloprost (1 μM) added to the cells at the same time as the LPS (time 0) or 12 h after LPS challenge on iNOS protein expression. Data are expressed as mean ± S.E.M. of three separate experiments. **$P < 0.01$ vs. control (LPS alone at time 0).

2.5. Statistics

Results were expressed as the mean ± S.E.M. of *n* experiments. Statistical analysis was determined by Student's unpaired *t*-test with $P < 0.05$ considered significant.

2.6. Reagents

LPS (from *S. typhosa*) was from Difco. Phosphate buffer saline was from Celbio. DL-Dithiothreitol, phenylmethylsulfonylfluoride, soybean trypsin inhibitor, pepstatin A, leupeptin and benzamidine were from Calbiochem. [γ-^{32}P]ATP was from Amersham. Poly(dI-dC) was from Boehringer. Anti-iNOS, anti-IκB-α, anti-p50, anti-p65 and anti-c-Rel antibodies were from Santa Cruz. Non-fat dry milk was from Bio-Rad. All the other reagents were from Sigma.

3. Results

3.1. Effect of PGs on LPS-induced nitrite production and iNOS protein expression

The production of nitrite by unstimulated J774 macrophages was undetectable (< 1 nmol/10^6 cells; $n = 3$). The stimulation of cells with LPS (10 μg/ml) resulted in a time-dependent accumulation of nitrite in the medium which reached 5.2 ± 0.5 nmol/10^6 cells at 6 h ($n = 4$), 18.1 ± 1.2 nmol/10^6 cells at 12 h ($n = 6$), 45 ± 1.3 nmol/10^6 cells at 24 h ($n = 8$). Moreover, upon stimulation with LPS (10 μg/ml) for 24 h, cells expressed a significant high level of iNOS protein expression when compared to control, untreated cells (Fig. 1). Pre-treatment of cells with PGE_2 (1, 10, 100 μM) and iloprost (0.1, 1, 10 μM) reduced in a concentration-dependent manner nitrite accumulation at 24 h (by 15.1 ± 0.6%, 25.3 ± 1.1%, 43.2 ± 1.2% and 13.0 ± 0.5%, 27.2 ± 1.3%, 46.7 ± 0.9%, respectively, $n = 6$). The reduction of nitrite accumulation by prostanoids was comparable to the inhibition of iNOS protein expression (Fig. 1). In contrast, $PGF_{2\alpha}$ (10 μM) and U46619 (a stable analogue of thromboxane A_2, 10 μM) did not affect iNOS protein expression and nitrite production (data not shown).

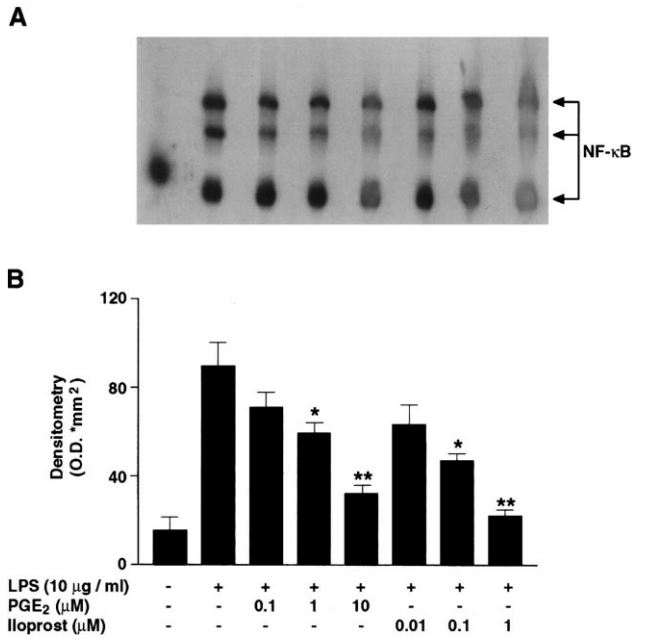

Fig. 3. Effect of different concentrations of PGE_2 and iloprost on NF-κB/DNA binding in J774 macrophages stimulated with LPS (10 μg/ml) for 2 h. Representative EMSA of NF-κB (A) as well as the densitometric analysis (B) shows the effect of PGE_2 (0.1, 1, 10 μM) and iloprost (0.01, 0.1, 1 μM) on LPS-induced NF-κB/DNA binding activity. Data are expressed as mean ± S.E.M. of three separate experiments. $*P < 0.05$; $**P < 0.01$ vs. control (LPS alone).

The addition of PGE_2 (10 μM) and iloprost (1 μM) to the cells 12 h after LPS challenge did not affect either iNOS protein expression (Fig. 2) or nitrite accumulation at 24 h (44.9 ± 1.6 nmol/10^6 cells, $n = 4$ and 41.2 ± 1.8 nmol/10^6 cells, $n = 4$, respectively) indicating that both prostanoids did not cause direct inhibition of iNOS activity.

3.2. Effect of PGs on NF-κB activation

The effects of different concentrations of prostanoids on NF-κB/DNA binding activity in J774 macrophages stimulated for 2 h with LPS (10 μg/ml) were tested by EMSA. A low basal level of NF-κB/DNA binding activity was detected in nuclear proteins from unstimulated macrophages. Conversely, a retarded band was clearly detected following stimulation with LPS (10 μg/ml). Treatment of cells with PGE_2 (1, 10, 100 μM) or iloprost (0.1, 1, 10 μM) caused a concentration-dependent inhibition of NF-κB/DNA binding activity (Fig. 3). The specificity of NF-κB/DNA binding complex was evident by the complete displacement of NF-κB/DNA binding in the presence of a 50-fold molar excess of unlabeled NF-κB probe (W.T. 50×) in the competition reaction. In contrast a 50-fold molar excess of unlabeled mutated NF-κB probe (Mut. 50×) or SP-1 oligonucleotide (SP-1 50×) had no effect on this DNA-binding activity. The composition of the NF-κB complex activated by LPS was determined using specific antibodies against p50 (p50), p65 (p65) and c-Rel (c-Rel) subunits of NF-κB proteins. Addition of either anti-p50 or anti-p65 but not c-Rel to the binding reaction resulted in a marked reduction of NF-κB band intensity (Fig. 4).

3.3. Effects of PGs on degradation of IκB-α and nuclear translocation of NF-κB subunits

The effects of prostanoid treatment on the presence of IκB-α in the cytosolic fraction or p50 and p65 subunits in nuclear fraction were examined by immunoblotting analysis. Unstimulated cells expressed a basal level of either IκB-α in the cytosolic fraction or p50 and p65 in the nuclear fraction. When the cells were activated with LPS (10 μg/ml), IκB-α disappeared from the cytosolic fraction whereas p50 and p65 level was increased in the nuclear fraction. Treatment of cells with PGE_2 (10 μM) and iloprost (1 μM) prevented either

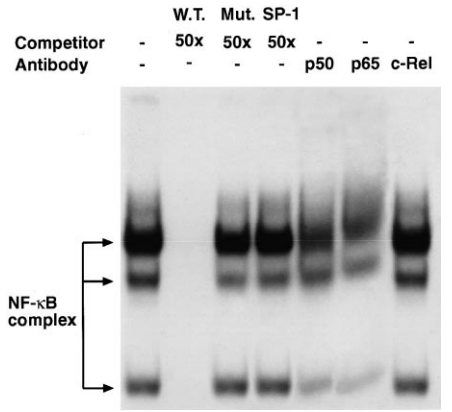

Fig. 4. Identification of LPS-induced NF-κB/DNA binding proteins in J774 macrophages. Nuclear extracts from LPS treated J774 macrophages were prepared as described in Section 2 and incubated with ^{32}P-labelled NF-κB probe. In competition reaction nuclear extracts were incubated with radiolabeled NF-κB probe in the absence or presence of identical but unlabeled oligonucleotides (W.T. 50×), mutated non-functional κB probe (Mut. 50×) or unlabeled oligonucleotide containing the consensus sequence for SP-1 (SP-1 50×). In supershift experiments nuclear extracts were incubated with antibodies against p50 (p50), p65 (p65) or c-Rel (c-Rel) 30 min before incubation with radiolabeled NF-κB probe. Data illustrated are from a single experiment and are representative of three separate experiments.

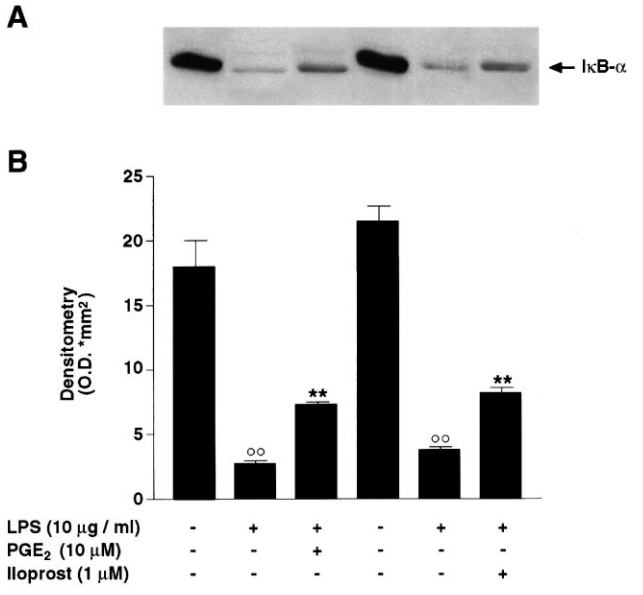

Fig. 5. Effect of PGE_2 and iloprost on LPS-induced degradation of IκB-α. Representative Western blot of IκB-α (A) as well as the densitometric analysis (B) shows the effect of PGE_2 (10 μM) and iloprost (1 μM) on degradation of IκB-α in J774 macrophages collected 30 min after LPS (10 μg/ml) challenge. Data are expressed as mean ± S.E.M. of three separate experiments. °°$P < 0.01$ vs. control (unstimulated cells); $**P < 0.01$ vs. LPS-stimulated cells.

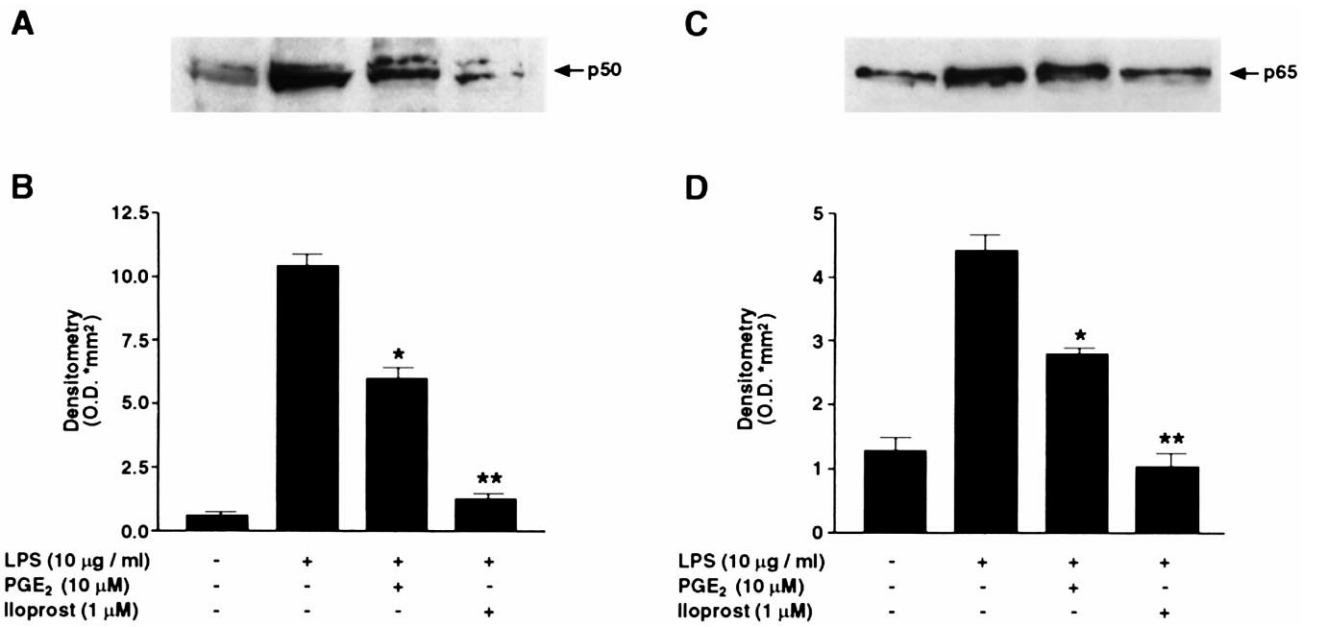

Fig. 6. Effect of PGE_2 and iloprost on p50 and p65 nuclear translocation in J774 macrophages stimulated with LPS (10 μg/ml) for 2 h. Representative Western blot of p50 (A) and p65 (C) as well as the densitometric analysis (B and D, respectively) show the effect of PGE_2 (10 μM) and iloprost (1 μM) on LPS-induced p50 and p65 nuclear translocation. Data are expressed as mean ± S.E.M. of three separate experiments. $*P < 0.05$; $**P < 0.01$ vs. control (LPS alone).

IκB-α degradation or p50 and p65 nuclear translocation (Figs. 5 and 6, respectively).

4. Discussion

Prostaglandins modulate the immune and inflammatory response by controlling the production of some mediators such as NO [19]. Previous studies demonstrated that PGE_2 and iloprost were able to inhibit the LPS-stimulated NO production in J774 macrophages [4]. A similar role for PGs on the NO pathway has been shown in murine peritoneal macrophages primed in vivo and activated in vitro with LPS [5]. The molecular mechanisms by which these prostanoids modulate iNOS expression are not yet known. The results of the present study show that PGE_2 or iloprost concentration-dependently suppresses the LPS-induced iNOS protein expression in J774 macrophages. Inhibition by iloprost was more effective than that exhibited by PGE_2. In fact, the LPS-induced iNOS protein expression was abolished by 1 μM iloprost compared to 10 μM PGE_2. The inhibition of iNOS protein expression by prostanoids was not dependent on direct inhibition of iNOS activity since both compounds, when added to the cells 12 h after LPS challenge, did not affect the enzyme catalytic activity suggesting the possibility that they may act at transcriptional level. NF-κB activation has a key role in the regulation of iNOS expression [12]. We investigated the effect of PGE_2 and iloprost on LPS-induced NF-κB activation. Our data show that both prostanoids inhibited in a concentration-related manner NF-κB/DNA binding activity. In this respect, it is also interesting to note that iloprost was more effective than PGE_2 in inhibiting NF-κB/DNA binding activity. These results obtained with EMSA analysis were virtually comparable to those observed with Western blotting. PGE_2 and iloprost also inhibited disappearance of IκB-α from the cytosolic fraction and translocation of NF-κB subunits into the nuclear fraction. The mechanisms by which PGE_2 and iloprost block NF-κB activation may be related to an inhibitory effect on IκB-α degradation. Furthermore, the nuclear translocation of p50 and p65 subunits in LPS-stimulated J774 macrophages was prevented by PGE_2 and iloprost at the same concentrations that blocked proteolytic degradation of IκB-α, reinforcing the hypothesis that these compounds inhibit NF-κB activation by stabilising IκB-α. These results indicate that PGE_2 and iloprost inhibit NO synthase induction by preventing NF-κB activation. Several in vitro studies have defined LPS and cytokine responsive elements on the iNOS gene promoter and have identified NF-κB as the primary transcription factor regulating iNOS expression after LPS or cytokine challenge. The presence of NF-κB binding sequences in the NO synthase promoter [7] and requirement of nuclear translocation of NF-κB in NO synthase induction [12] were supportive for the necessity of NF-κB activation in NO synthase gene expression. Our results are consistent with this hypothesis and suggest that the inhibition of NO synthase induction by prostanoids occurs through the prevention of NF-κB activation. The mechanism of action of prostaglandins on the NO pathway has been attributed to increased intracellular levels of the second messenger cAMP. A role of cAMP as an intermediate in the down-regulation of iNOS by PGE_2 has been suggested in murine peritoneal macrophages primed in vivo [5] and in vitro in J774 macrophages activated with LPS [20]. In addition, NO production by LPS-stimulated J774 murine macrophages was inhibited by prolonged elevation of intracellular cAMP, suggesting that in these cells cAMP may act as a mediator of the PGE_2 inhibition of iNOS induction [21]. A recent study showed that cAMP elevating agents inhibited the functional activity of endogenous NF-κB/Rel complexes in human monocytic and endothelial cells [22]. The molecular mechanism by which PGE_2 and iloprost possibly inhibit iNOS expression could

be related to their ability to increase intracellular cAMP levels which in turn inhibit NF-κB/DNA binding activity. This view is supported by the inability of $PGF_{2\alpha}$ and U46619 (a stable analogue of thromboxane A_2) both of which do not affect cAMP to modulate iNOS expression. Five different classes of prostanoid receptors have been described [23]. Amongst these, EP_2 and EP_4 receptor subtypes (selective agonist PGE_2) and IP receptor (selective agonist iloprost) have been shown to elevate the cAMP content in different cell lines [24,25]. Although our work does not establish which of these receptors are operative in J774 cells, our results showing that iloprost is more effective than PGE_2 suggest a prevalent role of IP receptors in mediating the above effects. Nevertheless, delineation of the mechanism(s) of negative transcriptional regulation of iNOS promoter by cAMP remains to be shown. In any case, the down-regulation of iNOS gene expression by endogenous prostaglandins may represent a relevant feedback mechanism in modulating the sustained NO production which occurs in many pathological events.

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