Involvement of NF-κB in the regulation of cyclooxygenase-2 protein expression in LPS-stimulated J774 macrophages

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Abstract We investigated the involvement of NF-κB in the regulation of COX-2 protein expression and prostaglandin production in LPS-stimulated J774 macrophages. Incubation of J774 cells with LPS (1 μg/ml) for 24 h caused an increase of COX-2 protein expression and accumulation of both PGE_2 and 6-keto-$PGF_{1\alpha}$ in the cell culture medium. Ammonium pyrrolidinedithiocarbamate (APDC, 0.1, 1, 10 μM) and *N*-α-*p*-tosyl-L-lysine chloromethylketone (TLCK, 1, 10, 100 μM), two inhibitors of NF-κB activation, suppressed in a concentration-dependent manner both LPS-induced COX-2 protein expression and prostanoid generation. Moreover, APDC and TLCK both inhibited the LPS-induced increase of NF-κB DNA binding activity and prevented IκB-α degradation. Our results show for the first time that NF-κB is involved in COX-2 protein expression in LPS-stimulated J774 macrophages and suggest that inhibitors of NF-κB activation may represent a useful tool for the pharmacological control of inflammation.

Key words: COX-2; Prostaglandin; NF-κB; Tosyl-lysine chloromethylketone; Ammonium pyrrolidinedithiocarbamate

1. Introduction

Cyclooxygenase, the enzyme which catalyzes the oxidation of arachidonic acid, is expressed in two isoforms, cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) [1]. COX-1 is generally considered a constitutive enzyme and accounts for the release of prostaglandins (PGs) involved in the regulation of physiological functions [2]. After stimulation with 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 [3]. The promoter region of COX-2 genes in mice [4], rats [5], and humans [6] has been cloned and sequenced. This promoter region contains a canonical TATA box and various putative transcriptional regulatory elements such as CREB, AP2, SP1, GATA box, NF-IL6 and NF-κB. Amongst these elements, NF-IL6 and NF-κB act as positive regulatory elements for the COX-2 transcription in some cell lines [7]. NF-κB is an inducible transcription factor that mediates signal transduction between cytoplasm and nucleus in many cell types [8]. In resting cells, NF-κB is localized in the cytoplasm as a heterodimer composed of two polypeptides of 50 kDa (p50) and 65 kDa (p65), which are non-covalently associated with cytoplasmic inhibitory proteins, including IκB-α. Upon stimulation by a variety of pathogenic inducers (such as viruses, mitogens, bacteria, double-stranded RNA, agents providing oxidative stress and inflammatory cytokines), the NF-κB complex migrates into the nucleus and binds DNA recognition sites in the regulatory regions of the target genes [9]. Activation of NF-κB by LPS induces a cascade of events leading to the phosphorylation of IκB-α and its further proteolytic degradation [10]. Recent observations suggest that the antioxidant ammonium pyrrolidinedithiocarbamate (APDC) and the serine protease inhibitor *N*-α-*p*-tosyl-L-lysine chloromethylketone (TLCK) are potent inhibitors of NF-κB activation [10,11]. Despite the wealth of information derived from studies on COX-2 promoter gene, the potential regulatory role of NF-κB in COX-2 expression and prostaglandin production in inflammatory cells has not been investigated. Therefore, we have studied the effect of NF-κB inhibitors on COX-2 protein expression and prostaglandin production in LPS-stimulated 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% fetal bovine serum, 2 mM glutamine, 100 IU/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 (1×10^7 cells/10 ml/dish) and allowed to adhere for 2 h. Thereafter the medium was replaced with fresh medium and cells were activated with LPS (1 μg/ml). APDC (0.1, 1, 10 μM) or TLCK (1, 10, 100 μM) was added to the cells 5 min before LPS challenge. In some experiments APDC (10 μM) or TLCK (100 μM) was added 12 h after LPS challenge. Cell viability (>95%) was determined with the MTT assay [12].

2.2. Determination of prostaglandin production

The accumulation of PGE_2 and 6-keto-$PGF_{1\alpha}$ in the culture medium was measured, without prior extraction or purification, by radioimmunoassay (RIA). The anti-PGE_2 rabbit antibody showed the following cross-reactivity (%): PGE_1 15, $PGF_{2\alpha}$ 0.41, 6-keto-$PGF_{1\alpha}$ 0.015, TXB_2 <0.0006. The relative cross-reaction (%) of the anti-6-keto-$PGF_{1\alpha}$ rabbit antibody was: PGE_2 0.4, $PGF_{2\alpha}$ 1.12, PGD_2 0.03, TXB_2 <0.009.

2.3. Preparation of cytosolic fractions and nuclear extracts

Extracts of macrophages stimulated for 2 h with LPS (1 μg/ml) in the presence or absence of APDC (0.1, 1, 10 μM) or TLCK (1, 10, 100 μM) were prepared as described [13]. Briefly, harvested cells (2×10^7) were washed twice 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 phenylmethylsulfonyl fluoride, 1.5 μg/ml soybean trypsin inhibitor, pepstatin A 7 μg/ml, leupeptin 5 μg/ml, 0.1 mM benzamidine, 0.5 mM DTT) and incubated in ice for 15 min. The cells were lysed by rapid passage through a syringe needle five or six 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,

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1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% v/v glycerol, 0.5 mM phenylmethylsulfonyl fluoride, 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 with shaking at 4°C for 30 min. The nuclear extract was then centrifuged for 15 min at 13000×*g* and supernatant was aliquoted and stored at −80°C. Protein concentration was determined with the Bio-Rad protein assay kit.

2.4. Electrophoretic mobility shift assay (EMSA)

Double-stranded oligonucleotides containing the NF-κB recognition sequence was end-labeled with [γ-^{32}P]ATP. Nuclear extracts (5 μg) 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 5% non-denaturing polyacrylamide gel in 0.25×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.

2.5. Western blot analysis

Immunoblotting analysis of COX-2 and IκB-α proteins was performed on J774 cells incubated with LPS (1 μg/ml) in the presence or absence of APDC or TLCK 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 bromophenol per ml) in a ratio of 1:1, boiled for 3 min and centrifuged at 10000×*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 anti-IκB-α or anti-COX-2 antibodies (1:1000) for 1 h at room temperature. The membranes were washed three times with 1% Triton X-100 in PBS and then incubated with anti-rabbit immunoglobulins coupled to peroxidase (1:1000). The immune complexes were visualized by the ECL chemiluminescence method (Amersham).

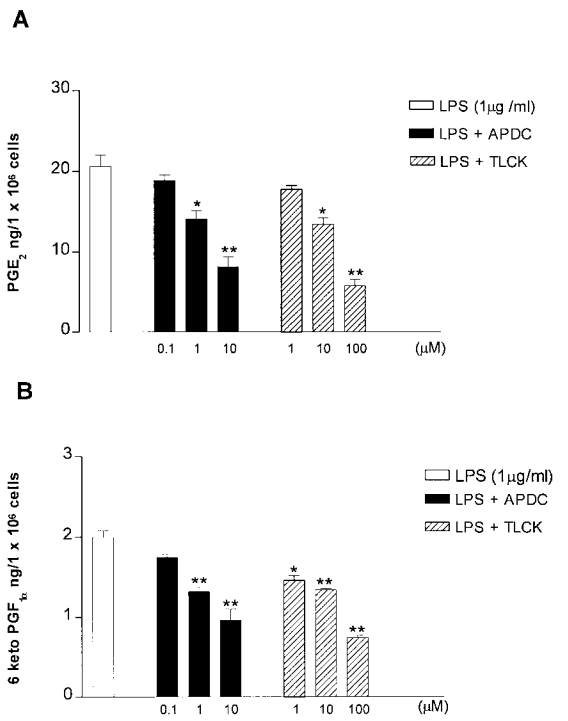

Fig. 1. Effect of different concentrations of APDC and TLCK on PGE_2 (A) and 6-keto-$PGF_{1\alpha}$ (B) production by J774 macrophages stimulated with LPS (1 μg/ml) for 24 h. APDC and TLCK were added to the cells at the same time as LPS. The values are expressed as means ± S.E.M. of four experiments performed in triplicate. *$P < 0.05$, **$P < 0.01$ vs. control (LPS alone).

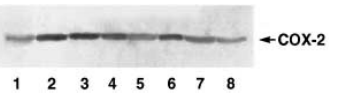

Fig. 2. Effect of APDC and TLCK on LPS-induced COX-2 protein expression. Western blot analysis shows the effect of APDC (10 μM) and TLCK (100 μM) on COX-2 protein expression in J774 macrophages stimulated with LPS (1 μg/ml) for 24 h. Basal level of COX-2 protein expression by unstimulated cells (lane 1). Induction of COX-2 protein expression by LPS-stimulated cells (lane 2). Inhibition of LPS-induced COX-2 protein expression by APDC (0.1, 1, 10 μM; lanes 3, 4 and 5) and TLCK (1, 10, 100 μM; lanes 6, 7 and 8). The data illustrated are from a single experiment and are representative of a total of three separate experiments.

2.6. Immunofluorescence of COX-2

J774 macrophages were fixed in 3% formaldehyde for 5 min and subsequently permeabilized with 0.2% Triton X-100 for 10 min. Non-specific binding sites were blocked by 10% FCS for 30 min. Samples were incubated with anti-COX-2 (1:200) antibody, followed by visualization of IgG with a fluorescein-conjugated second antibody. The coverslips were mounted in 0.1% *p*-phenylenediamine and 90% glycerol solution in PBS and analyzed by fluorescence microscopy with a Zeiss Axiovert 100 microscope.

2.7. 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.

2.8. Reagents

Phosphate buffer saline was from Celbio. DL-Dithiothreitol, phenylmethylsulfonyl fluoride, soybean trypsin inhibitor, pepstatin A, leupeptin and benzamidine were from Calbiochem. [γ-^{32}P]ATP and fluorescein-conjugated second antibody were from Amersham. Poly dI-dC was from Boehringer-H. Anti-IκB-α was from Santa Cruz. Anti-COX-2 antibody was a gift from G. Folco. Non-fat dry milk was from Bio-Rad. All other reagents were from Sigma.

3. Results

3.1. Effect of APDC and TLCK on LPS-induced prostaglandin production and COX-2 expression

Treatment of J774 macrophages with LPS (1 μg/ml) caused an accumulation of either PGE_2 (20.62 ± 1.4 ng/10^6 cells; $n = 4$) or 6-keto-$PGF_{1\alpha}$ (2.0 ± 0.08 ng/10^6 cells; $n = 4$) as compared to the release by unstimulated cells (1.09 ± 0.1 ng/10^6 cells and 0.22 ± 0.09 ng/10^6 cells respectively; $n = 4$). The addition of APDC (0.1, 1, 10 μM) to the cells 5 min before LPS challenge inhibited in a concentration-dependent manner the generation of both prostanoids. Thus, PGE_2 production was reduced by 8.0 ± 0.27%, 32 ± 2.24%, 60 ± 9.0% and 6-keto-$PGF_{1\alpha}$ by 13 ± 0.3%, 34 ± 1.3%, 52 ± 7.3%, respectively. Similarly, TLCK (1, 10, 100 μM) was able to suppress the production of PGE_2 by 14 ± 3.78%, 35 ± 1.9% and 72 ± 10% and 6-keto-$PGF_{1\alpha}$ by 27 ± 1.08%, 33 ± 0.5% and 63 ± 2.5%, respectively (Fig. 1A,B). Moreover, the stimulation of the cells with LPS (1 μg/ml) resulted in an increase of COX-2 protein expression which was prevented by co-incubation with APDC or TLCK, as demonstrated by immunoblotting experiments (Fig. 2). Addition of APDC (10 μM) or TLCK (100 μM) to the cells after 12 h LPS challenge did not significantly affect COX-2 protein expression, PGE_2 (18.4 ± 1.3% and 17 ± 1.3% respectively; $n = 3$) and 6-keto-$PGF_{1\alpha}$ production (11 ± 0.2% and 22 ± 0.12% respectively; $n = 3$), indicating that both agents did not cause direct inhibition of COX-2 activity (Fig. 3A,B,C respectively). The MTT assay showed that none of

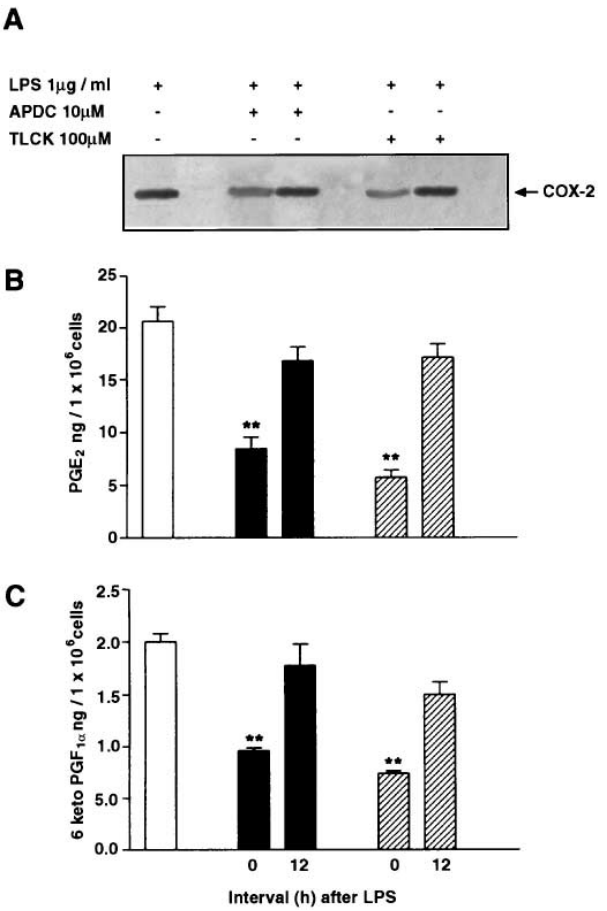

Fig. 3. Effect of delayed addition of APDC and TLCK on COX-2 protein expression (A), PGE_2 (B) and 6-keto-$PGF_{1\alpha}$ (C) production by J774 macrophages stimulated with LPS (1 μg/ml) for 24 h. APDC (10 μM) and TLCK (100 μM) were added to the cells at the same time as LPS (time 0) or 12 h after LPS challenge. A: Western blot analysis shows the effect of APDC and TLCK on COX-2 protein expression. The data illustrated are from a single experiment and are representative of a total of three separate experiments. B: Effect of APDC and TLCK on PGE_2 production. The values are expressed as means ± S.E.M. of three experiments performed in triplicate. $^{**}P < 0.01$ vs. control (LPS alone). C: Effect of APDC and TLCK on 6-keto-$PGF_{1\alpha}$ production. The values are expressed as means ± S.E.M. of three experiments performed in triplicate. $^{**}P < 0.01$ vs. control (LPS alone).

the tested compounds was cytotoxic at the concentrations used.

3.2. COX-2 immunofluorescence

Fig. 4 shows photographs of COX-2 immunofluorescence studies in J774 macrophages. Control cells showed only background staining, whereas cells stimulated with LPS (1 μg/ml, for 24 h) revealed a diffuse accumulation of COX-2 protein in the cytoplasm. Treatment of cells with APDC (10 μM) or TLCK (100 μM) suppressed the appearance of COX-2 immunostaining in the cytoplasm, confirming the results obtained with immunoblotting analysis.

3.3. Effect of APDC and TLCK on NF-κB activation

To detect NF-κB DNA binding activity, EMSA was performed using nuclear extracts from LPS-stimulated J774 cells and a labeled oligonucleotide containing the NF-κB binding site. As shown in Fig. 5, while DNA-protein complexes were

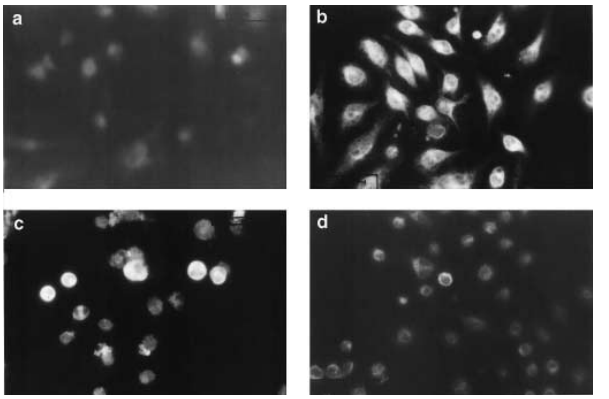

Fig. 4. Indirect immunofluorescence staining of COX-2 in J774 macrophages. Background staining of unstimulated cells (a). Positive staining and diffuse cytoplasmic localization of COX-2 in cells stimulated with LPS (1 μg/ml) (b). Disappearance of COX-2 immunostaining in the cytoplasm of cells incubated with LPS (1 μg/ml) in the presence of APDC (10 μM; c) and TLCK (100 μM; d).

slightly detectable in nuclear extracts from control cells, a retarded band was clearly detected following stimulation of cells with LPS (1 μg/ml) evaluated at 2 h after LPS challenge. Incubation of cells with TLCK (1, 10, 100 μM) caused a concentration-dependent inhibition of LPS-stimulated NF-κB binding. APDC (0.1, 1, 10 μM) also inhibited in a concentration-dependent manner LPS-stimulated NF-κB activation. The specificity of the retarded complex was demonstrated by the fact that it was abrogated in the presence of a 100-fold molar excess of the same unlabelled oligonucleotide included in the reaction mixture.

3.4. Effect of APDC and TLCK on IκB-α degradation

The appearance of IκB-α in the cytosolic fractions was investigated by immunoblotting analysis. A basal level of IκB-α was detectable in the cytosolic fraction of unstimulated cells whereas, after challenge with LPS, IκB-α was still present at 5 and 15 min while it had disappeared at 30 and 45 min. APDC (10 μM) as well as TLCK (100 μM) prevented IκB-α degradation, in fact the IκB-α band remained unchanged at both time points considered (Fig. 6).

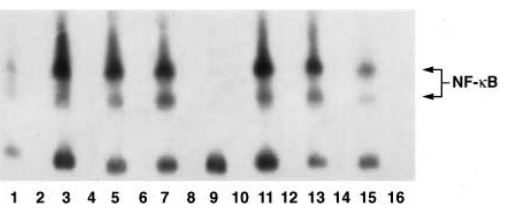

Fig. 5. Inhibition of NF-κB DNA binding by APDC and TLCK. The EMSA shows the effect of APDC or TLCK on LPS-induced NF-κB DNA binding in nuclear extracts from J774 macrophages. NF-κB DNA binding from unstimulated cells (lane 1). Increase of NF-κB DNA binding from cells stimulated with LPS (1 μg/ml) (lane 3). Inhibition of NF-κB DNA binding from cells stimulated with LPS (1 μg/ml) in the presence of TLCK (1, 10, 100 μM; lanes 5, 7 and 9 respectively) or APDC (0.1, 1, 10 μM; lanes 11, 13 and 15 respectively). Specificity of retarded complexes is demonstrated by the addition of an excess of the same unlabeled oligonucleotide used as probes (lanes 2, 4, 6, 8, 10 and 12). The data illustrated are from a single experiment and are representative of a total of three separate experiments.

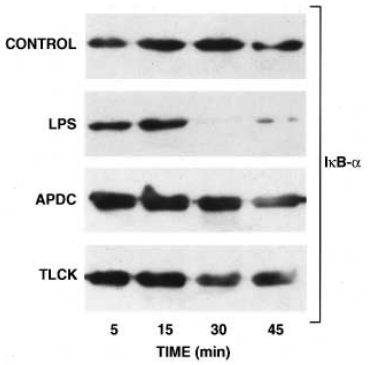

Fig. 6. Effect of APDC and TLCK on LPS-induced degradation of IκB-α. Western blot analysis shows the effect of APDC (10 μM) and TLCK (100 μM) on degradation of IκB-α in J774 macrophages collected at 5, 15, 30 and 45 min after LPS (1 μg/ml) challenge. Control: basal level of IκB-α band was present in the cytosolic fraction of unstimulated cells. LPS: IκB-α band has disappeared from the cytosolic fraction at 30 and 45 min. APDC and TLCK: IκB-α band remained unchanged at all time points considered. The data illustrated are from a single experiment and are representative of a total of three separate experiments.

4. Discussion

A growing body of evidence suggests that high levels of PGs, synthesized by COX-2, are involved in mediating both acute and chronic inflammation [1]. NF-κB was demonstrated to play a relevant role in the expression of many genes involved in the inflammatory process [14]. The molecular signaling mechanisms by which pathogenic stimuli induce COX-2 expression and PG production by macrophages have not been investigated. Here, we report that two inhibitors of NF-κB activation, APDC and TLCK, reduced in a concentration-related manner PGE_2 and 6-keto-$PGF_{1\alpha}$ production by LPS-stimulated J774 macrophages. Inhibition of prostaglandin production by PDTC and TLCK was not dependent on direct inhibition of COX-2 activity since both compounds, when added to the cells 12 h after LPS challenge, did not affect the enzyme catalytic activity. Moreover, the reduction of PG production seems to be related to inhibition of COX-2 gene expression, because both compounds reduced the level of COX-2 protein expression, as demonstrated by immunoblotting and immunofluorescence experiments. Our results are in agreement with recently reported data demonstrating that PDTC inhibits IL-1-induced PGE_2 production and COX-2 protein expression in rat mesangial cells and inhibits COX-2 protein expression at the post-transcriptional level without affecting COX-2 mRNA [15]. Previous observations indicated that NF-κB activation requires the liberation of free NF-κB in the cytoplasm, after which NF-κB migrates into the nucleus and binds to the appropriate DNA region to promote transcription [8]. One of the most important intracellular events for NF-κB activation is the proteolytic cleavage of inhibitor proteins such as IκB-α, which allows the liberation of free NF-κB [10]. Inhibition of this proteolytic pathway results in the inhibition of NF-κB activation. This study demonstrates that the serine protease inhibitor TLCK, which is known to block the degradation of IκB-α and prevent the activation of NF-κB [10], inhibits PG production and COX-2 protein expression by interfering with NF-κB activation. Recent evidence suggests that the activation of NF-κB may also be under the control of oxidant/antioxidant balance [16]. This hypothesis is mainly based on the observation that low doses of peroxides, including H_2O_2 and *tert*-butyl-hydroperoxide, induce NF-κB activation whereas some antioxidants prevent it [17,18]. Our results are in agreement with this hypothesis since it is conceivable that APDC inhibited NF-κB activation through an antioxidant mechanism. Moreover, the transient loss of IκB-α which occurs in LPS-stimulated J774 cells was prevented by either APDC and TLCK suggesting that these compounds inhibit NF-κB activation by stabilizing IκB-α. Taken together, our results show that the antioxidant APDC and the serine proteinase inhibitor TLCK are able to reduce COX-2 protein expression and PG production by activated macrophages and suggest an involvement of NF-κB in the induction of COX-2. However, since we have not investigated the effect of PDTC and TLCK on binding of NF-κB proteins to the COX-2 promoter, further studies on COX-2 gene transcription will better clarify the mechanism by which NF-κB modulates COX-2 protein expression. This study suggests that inhibitors of NF-κB activation may represent a useful tool for investigating the mechanisms which bring about the inflammatory process as well as potential agents for its pharmacological control.

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