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Aspirin inhibits expression of the interleukin-1β-inducible group II phospholipase A_2

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Abstract Nonsteroidal anti-inflammatory drugs (NSAIDs) clearly inhibit the synthesis and release of prostaglandins. However, these actions are not sufficient to explain all the anti-inflammatory effects of these drugs. Recently, it has been shown that aspirin and sodium salicylate inhibit the activation of the transcription factor NF-κB. Group II phospholipase A_2 ($sPLA_2$) is expressed in rat glomerular mesangial cells upon exposure to the inflammatory cytokine interleukin-1β (IL-1β) and this induction is attenuated by the NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC). We now report that aspirin inhibits the IL-1β-induced $sPLA_2$ activity in rat mesangial cells in a dose-dependent manner. The IC_{50} value of aspirin for $sPLA_2$ inhibition was 6.5 mM. This decrease in $sPLA_2$ activity was not due to direct inhibition of enzymatic activity but rather to the fact that aspirin inhibits the expression of IL-1β-induced $sPLA_2$ protein and mRNA. Furthermore, by electrophoretic mobility shift analysis we demonstrate reduced DNA binding of the nuclear factor κB, an essential component of the IL-1β-dependent upregulation of $sPLA_2$ gene transcription, after treatment of the cells with aspirin. The study described in this report indicates that the inhibition of $sPLA_2$ expression as induced by pro-inflammatory cytokines potentially represents an additional mechanism of action for aspirin.

Key words: Phospholipase A_2; Interleukin-1β; Aspirin; NF-κB; Rat mesangial cell

1. Introduction

Phospholipase A_2 (PLA_2) is thought to play an important role in the process of inflammation. Mammalian 14 kDA group II PLA_2 is found in soluble form at inflammatory sites such as peritoneal exudates [1] and synovial fluid in rheumatoid arthritis [2,3] and is believed to play an important role in the initiation and propagation of inflammatory processes [4].

High doses of aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs) are particularly effective in the treatment of inflammatory diseases [5]. The mechanisms of action of NSAIDs are explained mainly by their ability to inhibit the cyclooxygenase (COX) reaction [6–8]. COX exists in at least two isoforms, of which COX-1 is expressed constitutively and COX-2 is expressed only following cell activation. COX-2 is induced in a number of cells by pro-inflammatory stimuli and cytokines [9] and it is most likely that the anti-inflammatory actions of aspirin are due to the inhibition of COX-2. However, aspirin is over 100-fold more active towards COX-1 than towards COX-2 [6]. Furthermore, low doses of aspirin and other NSAIDs inhibit the synthesis of prostaglandin in vitro [6,10] and in vivo [11] but much higher doses are required for an anti-inflammatory effect in vivo [12,13]. Another member of the NSAIDs, sodium salicylate, is an ineffective COX inhibitor but is still able to reduce inflammation at doses comparable to those of aspirin [10,14,15]. So, the inhibition of COX does not appear to be sufficient to explain all the anti-inflammatory effects of aspirin.

Recently, it has been reported that aspirin and sodium salicylate inhibit NF-κB activation presumably by inhibiting the phosphorylation and degradation of its inhibitor, IκB, in cultured human Jurkat cells and mouse PD31 pre-B cells [16]. Numerous agents which can cause activation of NF-κB by release from IκB have been identified; these include the inflammatory cytokines interleukin-1β (IL-1β) and tumor necrosis factor α (TNF) [17].

IL-1β and TNF have been shown to induce the synthesis and secretion of non-pancreatic group II PLA_2, also referred to as $sPLA_2$, in many cell types including rat mesangial cells [18,19]. The increase in $sPLA_2$ activity and synthesis is accompanied by an increase in $sPLA_2$ mRNA levels [20,21]. Recently, it has been shown that pyrrolidine dithiocarbamate (PDTC), an antioxidant acting as a potent inhibitor of nuclear factor NF-κB activation, inhibits the increase in $sPLA_2$ mRNA steady state levels in response to IL-1β [22]. These data suggest that NF-κB activation is an essential component in the cytokine signaling pathway responsible for $sPLA_2$ gene regulation.

In view of the above findings we investigated whether aspirin affected the IL-1β-induced $sPLA_2$ activity in rat renal mesangial cells. Aspirin was tested for its capacity to inhibit both $sPLA_2$ activity and gene expression. We report that aspirin inhibits $sPLA_2$ expression and activity in rat mesangial cells stimulated with IL-1β, although at therapeutic concentrations the inhibition is only partial. In addition, we show with electrophoretic mobility shift analysis that these results can be explained by the observed inhibition by aspirin of the IL-1β-induced activation of NF-κB. We therefore conclude that inhibition of $sPLA_2$ expression and activity may represent another mechanism of action of aspirin and that at least part of the anti-inflammatory action of this drug in chronic inflammatory states may be due to inhibition of $sPLA_2$ synthesis.

2. Materials and methods

2.1. Materials

Recombinant IL-1β was prepared by the Biotechnology Depart-

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Abbreviations: IL-1β, interleukin-1β; $sPLA_2$, secretory phospholipase A_2; NF-κB, nuclear factor κB; NSAIDs, nonsteroidal anti-inflammatory drugs

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ment of Ciba Geigy Ltd., Basel, Switzerland. Forskolin and fatty acid-free bovine serum albumin (BSA) were obtained from Sigma, St. Louis, MO, USA. Aspirin was purchased from Janssen Chimica, Beerse, Belgium. Nitrocellulose and Nytran nylon membranes were obtained from Schleicher and Schuell, Dassel, Germany. Double-stranded poly(dI-dC) was a product from Pharmacia Biotech, Roosendaal, The Netherlands. NF-κB and SP-1 consensus oligonucleotides (1.75 pmol/μl), T4 polynucleotide kinase (PNK) (5–10 U/μl) and T4 PNK 10× buffer were part of a Gel Shift Assay System from Promega Corporation BNL, Leiden, The Netherlands.

2.2. Cell culture and incubation

Cultivation and characterization of rat mesangial cells were performed as described previously [23]. For the experiments passages 8–20 were used. Confluent mesangial cells cultured in 75 cm^2 flasks were rinsed once with PBS and incubated with 10 ml serum-free DMEM containing 0.1 mg/ml fatty acid-free bovine serum albumin (Sigma) and treated with aspirin and immediately stimulated with IL-1β for 20 h. Solutions containing aspirin were brought to pH 7.4 with concentrated NaOH prior to use [24].

2.3. PLA_2 assay

PLA_2 activity was assayed using 0.2 mM *sn-2*-labeled [1-^{14}C]linoleoylphosphatidylethanolamine (specific activity 3000 dpm/nmol) in 0.2 ml of 0.1 M Tris/HCl, pH 8.5, in the presence of 10 mM $CaCl_2$. Reactions were stopped after 30 min and the liberated ^{14}C-labeled fatty acids were extracted by a modified Dole extraction procedure [25]. The substrate 1-acyl-2[1-^{14}C]linoleoylphosphatidylethanolamine was prepared as described earlier [25].

2.4. Immunoblotting

Cells were washed once with PBS, scraped in homogenization buffer (50 mM HEPES pH 7.4, 0.25 M sucrose, 1 mM EDTA, 1 mM EGTA, 10 mM leupeptin, 0.1 mM pepstatin and 0.15 mM PMSF) and homogenized through a 27 G needle. Western blot analysis of protein extracts from mesangial cells and culture media using a monoclonal antibody specific for $sPLA_2$ was performed as previously described [19,21].

2.5. Northern blot analysis

Total mesangial cell RNA was extracted using the guanidinium thiocyanate/phenol/chloroform method and blotted on Nytran nylon membrane as described before [21]. The RNA was fixed to the membrane by 3 min treatment in a microwave oven at full setting (650 W) [26]. Prehybridization, hybridization and autoradiography were performed as previously described [21]. The ribosomal RNA was stained on the filters with methylene blue [27] in order to asses equal RNA loading and transfer.

2.6. Assessment of aspirin cytotoxicity

The cytotoxic effect of aspirin on rat mesangial cells was assessed by lactate dehydrogenase (LDH) release according to the method described by Moldeus et al. [28]. LDH was measured in cell culture supernatants after 20 h incubation in the presence or absence of IL-1β and different concentrations of aspirin. A separate sample was treated with 1% Triton X-100 for 30 min at 23°C, for the measurement of total LDH activity.

2.7. Nuclear extract preparation

Nuclear extracts from cultured mesangial cells were isolated by a rapid method modified from Dignam et al. [29,30]. For the preparation of nuclei, cells from confluent 75 cm^2 flasks were gently washed twice with ice-cold PBS and scraped off in 1 ml ice-cold hypotonic buffer (buffer A: 10 mM HEPES pH 7.9; 10 mM KCl; 0.1 mM EDTA; 0.1 mM EGTA; and freshly added 1 mM DTT and 0.5 mM PMSF) [31]. The cells were allowed to swell on ice for 15 min after which a 10% solution of Nonidet P-40 was added (0.1% final concentration). Thereafter, the cells were vigorously vortexed for 10 s and the nuclei were pelleted by centrifugation at 12000×*g* for 15 s at 4°C. The nuclei were gently resuspended in 100 μl ice-cold high salt extraction buffer (buffer C: 20 mM HEPES pH 7.9; 0.4 M NaCl; 1 mM EDTA; 1 mM EGTA; and freshly added 1 mM DTT and 0.5 mM PMSF) and incubated for 20 min on ice while shaking. The lysates were centrifuged at 12000×*g* for 5 min at 4°C and the supernatants were transferred to new Eppendorf tubes and then further supplemented with 0.5 mM PMSF, aliquoted and stored at −80°C. Between steps the samples were always kept on ice. Protein concentration was determined by the method of Bradford [32] with bovine serum albumin as standard.

2.8. Electrophoretic mobility shift assay (EMSA)

Double stranded oligonucleotides containing the NF-κB consensus sequence (5′-AGTTGA*GGGGACTTTCCC*AGGC-3′; Promega) were end-labelled using T4 polynucleotide kinase and [γ-^{32}P]ATP (Amersham, 3000 Ci/mmol, 10 mCi/ml). 10 μg of nuclear protein was preincubated for 10 min at RT in a 19 μl binding mixture containing binding buffer (4% Ficoll; 20 mM HEPES pH 7.9; 50 mM KCl; 1 mM EDTA; 1 mM DTT; 0.25 mg/ml BSA), 2 μg poly(dI-dC) and 0.3 μg herring sperm DNA. Thereafter, binding reactions were performed for 25 min on ice with 200 nCi of radiolabelled oligonucleotide in a final volume of 21 μl. Nucleoprotein-oligonucleotide complexes were resolved by electrophoresis on a 4% acrylamide (acrylamide/bisacrylamide, 30:1) gel at 100 V for 3 h. Prior to sample loading, the gel was run for 1 h at 100 V [33]. After electrophoresis, the gel was exposed to an X-ray film for 12–24 h at −80°C. Specificity of binding was determined by prior addition of a 10-fold excess of non-labelled competitor (NF-κB) or non-competitor (SP-1; 5′-ATTCGATCGGGGCGGGGCGAGC-3′; Promega) consensus oligonucleotide.

3. Results

3.1. Effect of aspirin on IL-1β-induced $sPLA_2$ activity

In mesangial cells the pro-inflammatory cytokine IL-1β can induce the synthesis and secretion of $sPLA_2$ [19]. Previous work has suggested that NF-κB activation is an essential component in the cytokine signaling pathway responsible for group II PLA_2 gene regulation in rat mesangial cells [22]. In addition it has been shown that the anti-inflammatory drug aspirin can inhibit the activation of NF-κB [16]. In view of these facts we examined the effect of aspirin on IL-1β-induced $sPLA_2$ activity. Rat mesangial cells were activated with IL-1β to induce $sPLA_2$ and simultaneously incubated with vehicle or different concentrations of aspirin (2–25 mM). In Fig. 1 a concentration-dependent inhibition of $sPLA_2$ activity by aspirin is shown in both cells and culture media. Suprapharmacological concentrations of aspirin (5 and 10 mM) decreased $sPLA_2$ activity to 54 ± 10% and 34 ± 15% in the media, and 61 ± 17% and 38 ± 13% in the cells, respectively, compared to total $sPLA_2$ activity in IL-1β-stimulated cells. At a concentration of 15 mM of aspirin the activity completely decreased to control level. At pharmacological concentrations of aspirin (2 mM) a decrease in $sPLA_2$ activity of approximately 14% was found in both cells and culture medium.

The inhibition of $sPLA_2$ activity occurred at drug concentrations that were not toxic to the cells as assessed by LDH release in the culture medium. The percent of total cellular LDH released in control cultures activated with IL-1β alone was less than 7% as measured in three independent experiments. No appreciable cytotoxicity (LDH release equivalent to control) was observed with aspirin at all concentrations tested (results not shown). Thus, it can be concluded that cytotoxicity did not account for the inhibitory effects on $sPLA_2$ activity.

3.2. Effect of aspirin on $sPLA_2$ protein levels

It is known that aspirin inhibits COX activity by acetylating the enzyme in an irreversible way. Inhibition of IL-1-induced COX expression by aspirin in cultured endothelial cells has also been reported [34]. This raised the question how aspirin inhibits IL-1β-induced $sPLA_2$ activity. Aspirin may decrease the expression of $sPLA_2$ protein and, therefore, decrease the

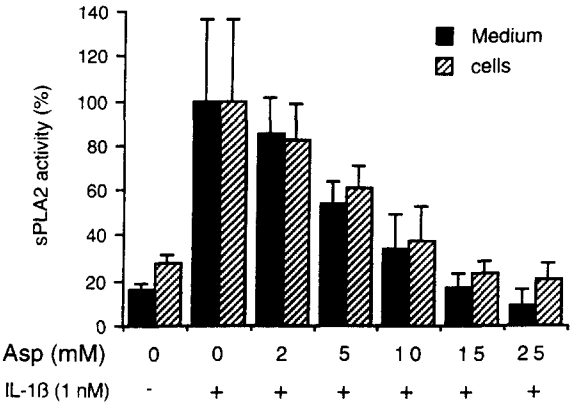

Fig. 1. Effect of aspirin on IL-1β-induced $sPLA_2$ activity. Rat mesangial cells were treated with the indicated concentrations of aspirin (ASP) and immediately activated by addition of IL-1β. The $sPLA_2$ activity was determined 20 h later in the culture media and cell homogenates. Results are means ± S.E. ($n = 5$). The 100% values for IL-1β-stimulated cells amounted to 1.63 ± 0.59 nmol/min/ml and 1.52 ± 0.59 nmol/min/mg protein for media and cells, respectively.

specific activity of the enzyme or it may decrease only the specific activity of the enzyme without influencing the expression of $sPLA_2$ protein levels. To differentiate between these possibilities we analyzed by immunoblotting $sPLA_2$ protein expression in IL-1β- and aspirin-treated cells. Fig. 2 shows a dose-dependent decrease in $sPLA_2$ expression in cells and medium treated with different concentrations of aspirin which is already clearly visible at 2 mM aspirin. Aspirin, at concentrations up to 20 mM, did not affect $sPLA_2$ activity measurements (data not shown). From these results it can be concluded that inhibition of $sPLA_2$ protein expression accounts for the decrease in $sPLA_2$ activity in cells treated with aspirin.

3.3. Effect of aspirin on expression of IL-1β-induced $sPLA_2$ mRNA levels

Additional studies were performed to evaluate whether the effect on $sPLA_2$ activity and protein levels was due to altered $sPLA_2$ gene expression. We addressed this question because down-regulation of $sPLA_2$ mRNA levels is not always found in experiments where IL-1β-induced $sPLA_2$ protein levels are decreased, as we showed previously for the inhibition of $sPLA_2$ expression by dexamethasone in rat mesangial cells [21]. In order to determine whether the inhibition of IL-1β-dependent stimulation of $sPLA_2$ expression by aspirin was due to down-regulation of $sPLA_2$ steady state mRNA levels we performed Northern blot analyses. Fig. 3 shows a clear decrease in $sPLA_2$ mRNA levels by aspirin. This decrease is in parallel with the reduction in IL-1β-induced $sPLA_2$ activity (Fig. 1) and protein levels (Fig. 2) by aspirin.

3.4. Effect of aspirin on NF-κB activation

Previously it has been reported that NF-κB is an essential transcription factor mediating $sPLA_2$ gene activation in response to IL-1β [22]. In addition, it has been reported that aspirin inhibits the activation of NF-κB [16] by preventing the degradation of the NF-κB inhibitor, IκB. This led us to investigate the effect of aspirin on the activation of NF-κB by IL-1β in mesangial cells. To detect NF-κB binding activity we performed EMSAs. In control cells hardly any complex was observed (Fig. 4, left, lane 2), whereas IL-1β elicited a substantial rise in NF-κB DNA binding activity (Fig. 4, left, lane 3). Following IL-1β treatment two NF-κB DNA-protein complexes were identified in the nuclear extract of cultured mesangial cells. The slower migrating complex comprises the p65/p50 heterodimer of NF-κB and the faster migrating complex is likely to represent the p50/p50 homodimer which has been previously demonstrated to be present in the extracts of mesangial cells [35]. After simultaneous addition of IL-1β and aspirin neither the p65-containing DNA-protein complex nor the p50/p50-like complex was detectable, indicating that aspirin abolished IL-1β-mediated activation of NF-κB (Fig. 4, left, lane 4). Competition analysis further determined the specificity of the DNA-protein complexes observed. As shown in Fig. 4, right, lane 2, inclusion of a 10-fold excess of unlabelled double stranded NF-κB oligonucleotides in the DNA binding reaction effectively abolished the IL-1β-induced formation of specific DNA-protein complexes. When a 10-fold excess of SP-1 consensus oligonucleotides was added to the binding reaction the DNA-protein complex formed was unaffected thereby assessing the specificity of the complexes seen (Fig. 4, right, lane 3).

4. Discussion

NSAIDs are widely used in the treatment of pain and inflammation associated with acute injury or chronic diseases. The action of aspirin and other NSAIDs on the COX is well known. However, this action is not likely to be solely responsible for the total anti-inflammatory effects of these drugs because compared to doses that inhibit prostaglandin syn-

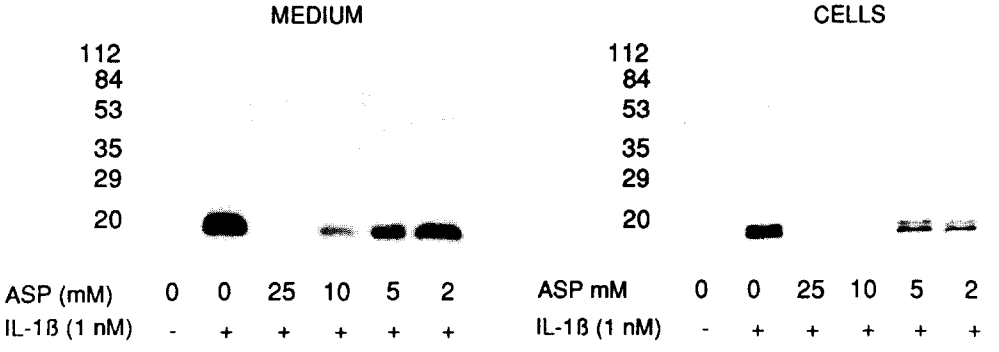

Fig. 2. Effect of aspirin on IL-1β-induced $sPLA_2$ protein levels in cells and culture medium. Rat mesangial cells were treated with the indicated concentrations of aspirin (ASP) and immediately activated by addition of IL-1β (1 nM). Thereafter, aliquots of 800 μl of the culture supernatants or 20 μg of cellular protein were used for Western blotting and $sPLA_2$ immunostaining.

thesis in vivo [11], much higher doses are needed in vivo for an anti-inflammatory effect [12,13].

The involvement of $sPLA_2$ in the pathophysiology of various diseases has been well documented. $sPLA_2$ can release a fatty acid, often arachidonic acid, from the *sn-2* position of membrane phospholipids with concomitant production of a lysophospholipid. The free arachidonic acid serves as precursor for the COX pathway producing thromboxanes, prostacyclins and other prostaglandins, but it also serves as a substrate for the 5-lipoxygenase pathway producing leukotrienes. Alkyl lysophosphatidylcholine is converted to platelet activating factor (PAF), another important lipid mediator. Thus, if $sPLA_2$ is inhibited, in principle all three inflammatory pathways discussed above can be blocked. In addition, it has recently been shown that aspirin can inhibit NF-κB [16] and this transcription factor is involved in the activation of $sPLA_2$ by pro-inflammatory cytokines in rat mesangial cells [22]. Thus, $sPLA_2$ expression is a likely candidate for the additional action of NSAIDs.

In the present study we showed that aspirin can inhibit the activity of IL-1β-induced $sPLA_2$ in a dose-dependent manner (Fig. 1). Western blot analysis revealed that the decrease in $sPLA_2$ activity occurs in parallel with the decrease in $sPLA_2$ protein levels (Fig. 2). Thus, the decrease in $sPLA_2$ protein level by aspirin accounts for inhibition of $sPLA_2$ activity. This finding is in agreement with a recent report in which the authors showed that aspirin had no direct effect on the enzymatic activity of group II PLA_2 as measured in an in vitro assay system [36]. Northern blot analysis of rat mesangial cells treated with aspirin showed suppression of $sPLA_2$ mRNA induction by IL-1β (Fig. 3). The fact that the transcription of IL-1β-induced $sPLA_2$ mRNA is inhibited by aspirin is explained most easily by the hypothesis that also in rat mesangial cells activation of NF-κB is inhibited by aspirin. By EMSAs we established that IL-1β-induced NF-κB-activation is indeed inhibited in rat mesangial cells. The results obtained show that the IL-1β-activated DNA binding activity of NF-κB is drastically reduced under the action of aspirin (Fig. 4, left, lanes 3 and 4). Competition analysis further determined the specificity of the DNA-protein complexes observed (Fig. 4, right). The inhibition of DNA binding activity of NF-κB may be the key step in the suppression of the cytokine induced $sPLA_2$ expression by aspirin in rat mesangial cells.

The action of aspirin on the induction of $sPLA_2$ by IL-1β is consistent with results from a recent study from Kopp and Ghosh [16]. They showed that aspirin (3 mM) and sodium salicylate (5 mM) inhibit NF-κB-dependent transcription,

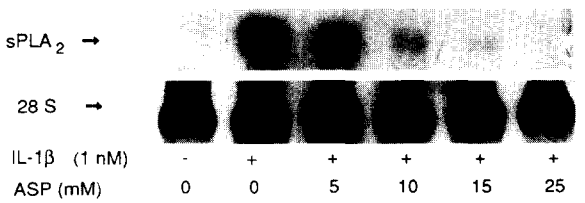

Fig. 3. Effect of aspirin on expression of IL-β-induced $sPLA_2$ mRNA in rat mesangial cells. Indicated concentrations of aspirin were added to the culture medium of rat mesangial cells followed by the induction of $sPLA_2$ by adding IL-1β (1 nM) to each flask for 20 h. After the end of this period, total RNA was isolated and 20 μg was used for Northern blot hybridizations for $sPLA_2$ mRNA. RNA loading and tranfer was assessed by staining of 28S RNA with methylene blue.

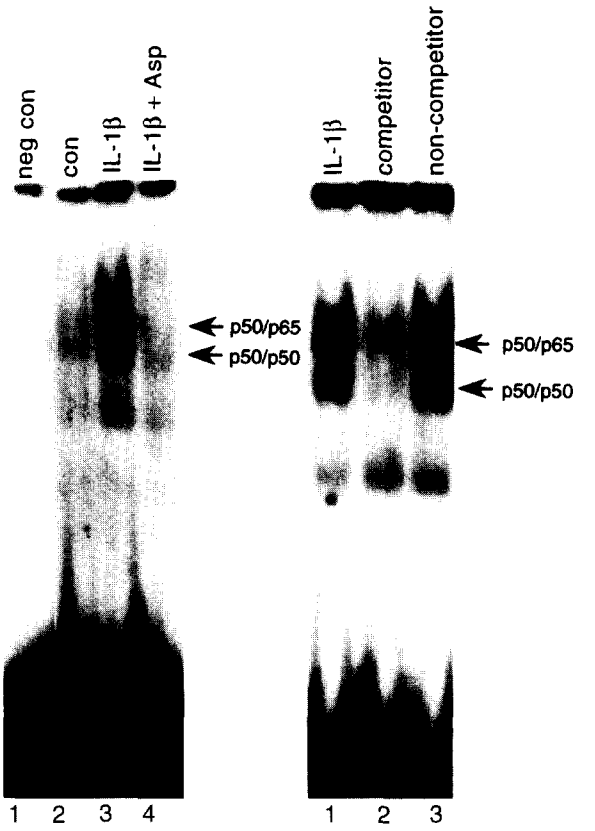

Fig. 4. Effect of aspirin on IL-1β-induced NF-κB activation. Left: Mesangial cells were stimulated with vehicle (con, lane 2), IL-1β (1 nM, lane 3), IL-1β (1 nM) plus aspirin (15 mM, lane 4) for 20 h. Nuclear extracts were prepared as described in Section 2 and equal amounts were incubated with a radiolabelled NF-κB oligonucleotide probe. No nuclear protein was added to the negative control (lane 1). DNA-protein complexes were analyzed on a 4% polyacrylamide gel. The position of p50/65 heterodimer and p50/p50 homodimer is shown. Right: Specificity of the DNA-protein complexes. Nuclear proteins were incubated in the presence of a 10-fold excess of either unlabelled NF-kB (competitor, lane 2) or of SP-1 (non-competitor, lane 3) consensus oligonucleotides.

using sensitive assays based on plasmids containing two IgK-κB sites driving a luciferase reporter gene. However, the same concentrations of these drugs had no significant effect on NF-κB activation as examined by mobility shift analysis in which case effects on NF-κB were only observed at relatively high concentrations of 5–20 mM [16]. According to the authors mM aspirin concentrations may be maintained in the plasma for treatment of chronic inflammatory states like arthritis [37].

Plasma and synovial fluid concentrations of aspirin and sodium salicylate in rheumatics are close to those shown to be inhibitory for NF-κB activation by Kopp and Gosh [16] and for $sPLA_2$ induction in the present study. However, the relevance of comparing the effectiveness of drug levels in tissue culture studies in vitro with those in plasma in vivo is questionable. During inflammation, capillary damage leading to extravasation of protein-bound NSAIDs and an acidic environment of cells can enhance concentration and uptake of acidic NSAIDs [38] in inflamed joint tissues. Thus, the concentration of NSAIDs may be higher in inflamed tissue than in plasma making it difficult to compare these concentrations with those used in cell culture studies.

In summary, the inhibitory effects of aspirin reported herein lend further support to the conclusion that IL-1β-induced $sPLA_2$ expression is mediated by NF-κB activation as previously noticed from the inhibition by pyrrolidine dithiocarbamate [22]. It is clear that aspirin does not inhibit $sPLA_2$ expression completely at pharmacological concentrations. However, partial suppression of cytokine inducible $sPLA_2$ may be sufficient to mitigate an inflammatory response as has been proposed for the inhibition of iNOS by aspirin [39]. The partial inhibition of IL-1β-induced $sPLA_2$ expression may represent an additional mechanism of action for aspirin and perhaps also other aspirin-like drugs. The anti-inflammatory action of aspirin-like drugs may then be due not only to inhibition of prostaglandin synthesis but also to the suppression of inducible $sPLA_2$ gene expression.

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