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Aspirin and indomethacin reduce lung inflammation of mice exposed to cigarette smoke

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ABSTRACT

Neutrophil accumulation response to cigarette smoke (CS) in humans and animal models is believed to play an important role in pathogenesis of many tobacco-related lung diseases. Here we evaluated the lung anti-inflammatory effect of aspirin and indomethacin in mice exposed to CS.

C57BL/6 mice were exposed to four cigarettes per day during 4 days and were treated i.p. with aspirin or indomethacin, administered each day 1 h before CS exposure. Twenty four hours after the last exposure, cells and inflammatory mediators were assessed in bronchoalveolar lavage (BAL) fluid and the lungs used for evaluation of lipid peroxidation, p38 mitogen-activated protein kinase (MAPK) phosphorylation and nuclear transcription factor κB (NF-κB) activation.

Exposure to CS resulted in a marked lung neutrophilia. Moreover, the levels of oxidative stress-related lipid peroxidation, prostaglandin E_2 (PGE_2), interleukin 1β (IL-1β), monocyte chemotactic protein 1 (MCP-1), and activated NF-κB and p38 MAPK were greatly increased in CS group. Aspirin or indomethacin treatment led to a significant reduction of neutrophil influx, but only aspirin resulted in dramatic decrease of inflammatory mediators. Moreover, both drugs reduced lung p38 MAPK and NF-κB activation induced by CS.

These results demonstrate that short-term CS exposure has profound airway inflammatory effects counteracted by the anti-inflammatory agents aspirin and indomethacin, probably through COX-dependent and -independent mechanisms.

1. Introduction

The massive research about the effect of exposure to cigarette smoke (CS) is fully justified by the morbidity and mortality associated to common tobacco-related diseases, such as lung cancer and chronic obstructive pulmonary disease (COPD) [1,2]. Accumulation of inflammatory cells into the lungs is a hallmark response to CS both in humans and animal models, suggesting a key role in the development of COPD [3–5]. Polymorphonuclear leukocyte (PMN) recruitment results from

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the production and secretion of many signaling molecules such as cytokines, chemokines and lipid-derived inflammatory mediators. Among known cytokines, interleukin 1β (IL-1β) release is associated to neutrophil attraction in different situations, such as acute or chronic inflammation and repair [3,6]. In parallel, there is an increase in monocyte chemotactic protein 1 (MCP-1), a chemokine associated with monocyte recruitment [7], which also leads to PMN recruitment to inflamed lungs of mice exposed to CS [3]. In addition, alveolar macrophages and lung fibroblasts stimulated by CS extract produce prostaglandin E_2 (PGE_2) through cyclooxygenases (COX-1 and -2) leading to an increase in blood vessels permeability and dilatation [8].

Non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin and indomethacin, are worldwide available and most frequently used drugs, safely employed by humans in many inflammatory conditions with well-known side-effects [9]. In the case of pulmonary tissue, several reports have shown that NSAIDs can inhibit acute [10,11] as well as chronic [12,13] experimental lung inflammation induced by different agents. Interestingly, in asthmatic patients that are not aspirin-sensitive, aspirin and indomethacin may have bronchial protective effects [14].

In the last few years, growing evidence demonstrated a broad molecular modulation of NSAIDs by interacting with different intracellular pathways other than COX inhibition, suggesting a new therapeutic potential of these drugs. For instance, indomethacin can activate peroxisome proliferator-activated receptor (PPAR) isoforms [15] and is a weak agonist of PGD_2 receptor (CRTh2) [16]. An analog of aspirin-triggered lipoxin A_4, generated by COX acetylation evoked by aspirin, reduces lung inflammation by lipopolysaccharide (LPS) challenge [17]. In addition, a critical target regulated by aspirin and other NSAIDs is the nuclear transcription factor κB (NF-κB) activation [18,19], a common intracellular pathway triggered by a myriad of stimuli [20]. NF-κB is a homo-heterodimer nuclear transcriptional factor composed by p50, c-Rel, Rel-B, p52 and p65 subunits, which p50-p65 form is involved in selected inflammatory-related gene transcription. Under cell resting, NF-κB is complexed in cytoplasm with the inhibitory protein IκBα. After cell activation, IκBα is phosphorylated and releases NF-κB, which, in turn, leaves cytoplasm and enters the nucleus, constituting a transcriptional complex that binds to DNA in order to initiate inflammatory proteins production [20]. Mechanistically, aspirin was shown to inhibit NF-κB by inhibiting IκB kinase (IKK) or upstream modulators in lung cells [19].

An important upstream step in NF-κB translocation to the nucleus involves mitogen-activated protein kinase (MAPK) activity. p38α MAPK, a member of MAPK superfamily, is implicated in intracellular signaling elicited during inflammatory stimuli, such as LPS or CS [21,22] and may be inhibited by aspirin [23]. Therefore, both p38 MAPK and NF-κB pathways are thought to be anti-inflammatory targets for many inflammatory diseases.

The effect of NSAIDs on pulmonary inflammation caused by CS is unknown. Therefore, we evaluated the effect of non-selective NSAIDs aspirin and indomethacin on a mouse model of CS-induced acute lung inflammation. We report that both drugs block PMN recruitment and NF-κB and p38 MAPK activation, but only aspirin inhibits inflammatory mediator production.

2. Material and methods

2.1. Animals and cigarette smoke exposure

The acute model of CS-induced inflammation in mice was performed as we previously described, with slight modifications [3,24–26]. Briefly, male C57BL/6 mice (20–25 g; Universidade Federal Fluminense, Brazil) were put in a smoke-inhalation system and, during 4 days, they were exposed daily to smoke from one, two, four or eight commercial filtered cigarettes, divided in puffs of 100 mL with duration of 60 s per puff. Control animals were exposed to ambient air (AA) in the same smoke-inhalation chamber (n = 5–8). Animal care was in compliance with the Helsinki Convention for the use and care of animals and was approved by the local institutional animal care and use committee.

2.2. Drug administration

Aspirin (Bayer AG, Leverkusen, Germany) or indomethacin (Sigma–Aldrich, St. Louis, MO, USA) was dissolved in saline with 1% (v/v) dimethyl sulfoxide (DMSO, Merck, Darmstadt, Germany) and administered i.p. at indicated doses, 1 h before each CS exposure. Control group received vehicle only.

2.3. Bronchoalveolar lavage (BAL)

Twenty four hours after the last smoke exposure, animals were sacrificed and BAL was performed. Tracheas were cannulated and BAL fluid was obtained by injecting phosphate-buffered saline, three consecutive times, to a final volume of 1.5 mL and stored on ice. Total cell number was determined in a Z1 Coulter counter (Beckman Coulter, Miami, FL, USA). Differential cell counts were performed on cytospin preparations (Shandon Cytospin, Thermo Fisher Scientific, Waltham, MA, USA) stained with hematoxylin and eosin (Sigma–Aldrich, St. Louis, MO, USA). Over 200 cells were counted and identified according to morphological criteria. Results were expressed as number of cells mL^{-1}. The remaining BAL fluid was centrifuged (400 × g for 10 min), the supernatant collected and stored at −20 °C for determination of mediators.

2.4. Histological analysis of inflammatory lung cells

Histological analysis was performed as described elsewhere [3]. Briefly, animals were sacrificed at indicated time with sodium pentobarbital (60 mg kg^{-1}, i.p.) and exsanguinated. Lungs were distended with 10% phosphate-buffered formalin (pH 7.4), at constant pressure of 2.45 kPa. Lungs were embedded in paraffin and cut into 5 μm sections from each sample. Total neutrophil lung cells were counted in Giemsa-stained sections in 30 random fields of 26,000 μm^2, under 400× magnification, using an Olympus BH-2 microscope equipped with a graticule in eyepiece.

2.5. Determination of total protein, IL-1β, MCP-1 and PGE_2 levels in BAL fluid

IL-1β and MCP-1 levels were detected by enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Abingdon, UK), as well as PGE_2 levels (Cayman Chemical, Ann Arbor, MI, USA) according to manufacturer's instructions, with detection limit of 10 pg mL^{-1}. Total protein was measured by Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA) using BSA (Sigma–Aldrich, St. Louis, MO, USA) as standard.

2.6. Whole lung cytoplasmic and nuclear extracts

Whole lung nuclear extracts were prepared as described by Valença et al. [27], with slight modifications. After BAL fluid withdrawal, perfused lungs were excised, homogenized and incubated for 15 min in 500 μL buffer A [10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.25% (v/v) NP-40, 0.5 mM PMSF, 100 μM orthovanadate, and 1 mM NaF (all purchased from Sigma–Aldrich, St. Louis, MO, USA)] at 4 °C. After centrifugation (16,000 × *g* for 30 s), supernatant were collected and stored at −70 °C for p38 MAPK detection. Pellets were resuspended and incubated in buffer C [20 mM HEPES (pH 7.9), 0.4 M NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 10% (v/v) glycerol, 1 mM dithiothreitol, and 1 mM PMSF] at 4 °C for 15 min. The extracts were centrifuged and supernatants were frozen at −70 °C. Cytoplasmic and nuclear protein levels were measured by at least three independent experiments with Bradford method.

2.7. Electrophoretic mobility shift assay (EMSA)

EMSA was performed as described by Castro et al. [3]. The binding reaction between nuclear factor NF-κB consensus sequence 5′-AGT TTG ATG AGT CAG CCG-3′ and 3′-CGG CTG ACT CAT CAA ACT-5′ with nuclear protein (10 μg) was performed in a final volume of 30 μL in 8 mM HEPES, 10% (v/v) glycerol, 20 mM KCl, 4 mM $MgCl_2$, 1.0 μg poly-dldC (pH 7.0). The oligonucleotides (DNAgency, Malvern, PA, USA) were 5′-end labeled with T4 polynucleotide kinase kit (New England Biolabs, Herts, UK) and [γ-^{32}P] ATP (>5000 Ci $mmol^{-1}$; Amersham Biosciences, Piscataway, USA) and 50,000 cpm of relevant double-stranded oligonucleotides were used per reaction. Binding was allowed to proceed for 30 min at room temperature. Samples were electrophoresed in 6% polyacrylamide non-denaturating gel with 0.5× Tris-borate-EDTA, at 180 V for 2 h. The gels were dried and quantification was achieved on PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA) and expressed as relative optical density arbitrary units. For each gel, coomassie blue staining was performed to serve as a loading control, as done by others [28]. Supershift was performed by adding 200 ng of NF-κB antibodies against the isoform p65 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), 15 min before the binding reaction mixture incubation with labeled oligonucleotide probe and nuclear extracts, at room temperature. Then, samples were resolved by electrophoresis, as described above.

2.8. p38 MAPK immunoblotting

Whole lung cytoplasmic extracts were electrophoresed using 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Amersham Biosciences, Piscataway, USA). Next, membranes were blocked for 1 h with 5% skim dry milk in Tris-buffered saline containing 0.1% Tween 20 followed by incubation for 1 h with polyclonal rabbit antibody against phosphorylated or total p38 MAPK (Invitrogen, Carlsbad, CA, USA) and 1 h with polyclonal anti-rabbit antibody conjugated with peroxidase (Promega Corporation, Madison, WI, USA). Western blots were visualized using ECL system (Amersham Biosciences, Piscataway, USA). Bands were analyzed using QuantityOne software (Bio-Rad Laboratories, Hercules, CA, USA) and expressed as relative optical density arbitrary units. Immunoblots were first probed with anti-phospho-p38 MAPK, then stripped and reprobed with anti-total p38 MAPK to control for equal protein loading in the gels. In order to ensure the linearity of the band intensities, several exposure times were analyzed.

2.9. Lipid peroxidation

Lung tissue oxidative damage was evaluated by thiobarbituric acid reactive species (TBARS), as described by Bezerra et al. [25], with modifications. Lungs were minced in ice-cold buffer A [10 mM HEPES-KOH (pH 7.9), 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA (pH 8.0)] and centrifuged for 30 s at 14,000 × *g*. Supernatants were incubated with 10 mM butylated hydroxytoluene (Sigma–Aldrich, St. Louis, MO, USA), 8.1% (w/v) SDS (Sigma–Aldrich, St. Louis, MO, USA) and 0.8% (w/v) thiobarbituric acid (Sigma–Aldrich, St. Louis, MO, USA) in dry bath at 95 °C for 90 min. Afterwards, samples were kept at 4 °C for 5 min and centrifuged at 14,000 × *g* for 5 min. Supernatants were collected and analyzed at 532 nm by spectrophotometer. TBARS level (malondialdehyde equivalents per milligram) is presented as percentage of control (AA) mice value.

2.10. Statistical analysis

Comparisons were made by analysis of variance (ANOVA). Post-hoc tests (Dunnett's) were also used to identify differences between values. Results are expressed as mean ± SEM and values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. CS exposure induces lung neutrophil recruitment and oxidative damage

A special feature of acute inflammation process is neutrophil infiltration. Accordingly, we have already seen that CS is able to stimulate pulmonary neutrophilia in acute rather than in chronic exposure [3,24–26]. Here, the effect of increasing cigarette doses in BAL number of neutrophils is shown in Fig. 1A. Neutrophil recruitment was only detected in BAL fluid after exposure to four or eight cigarettes per day. Yet considered a high amount of CS, no neutrophils were

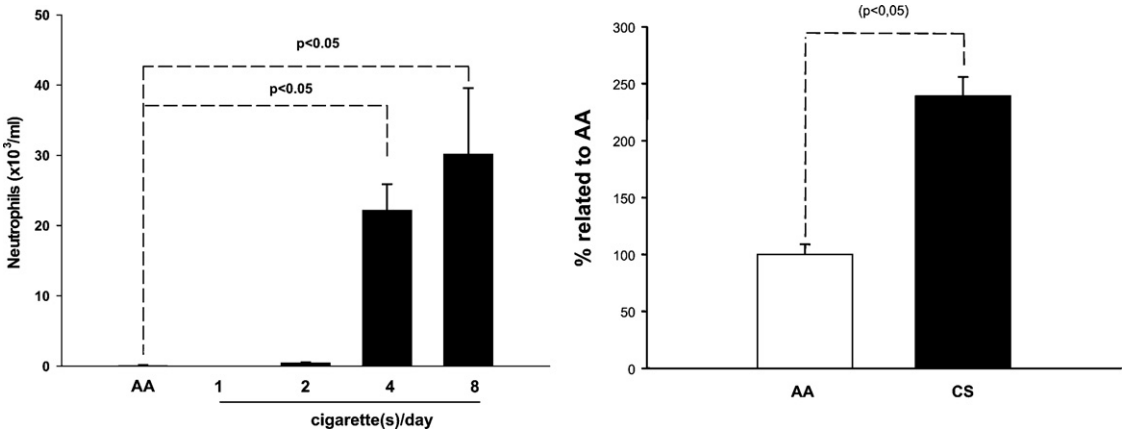

Fig. 1 – (A) Dose-response effect of cigarette smoke (CS) exposure on mice bronchoalveolar lavage (BAL) fluid cellularity. Neutrophils were collected from BAL fluid and counted after 4 days of exposure to ambient air (AA) or different amounts of CS. (B) Peroxidation of lung tissue lipids after 4 days of exposure to ambient air (AA) or CS from four cigarettes per day. Results are expressed as mean ± SEM (n = 5–8).

observed after 1 day of exposure to eight cigarettes per day, but just alveolar macrophages as in AA group (data not shown). Alveolar oxidants are more abundant after CS inhalation because CS has high levels of reactive oxidant species and it also promotes the migration of inflammatory cells in alveoli, which spontaneously release oxidants [25]. In our experiments, at the lung tissue level, 4 days of exposure to CS from four cigarettes per day led to tissue damage when compared with AA group, shown by a significant (almost 2.5-fold) increase of lipid peroxidation using TBARS protocol (Fig. 1B). Together, these results demonstrate that CS triggers an active lung inflammation associated with marked oxidative stress.

3.2. *CS-induced lung neutrophil recruitment is inhibited by aspirin or indomethacin*

NSAIDs are used to treat a large variety of inflammatory diseases and have been shown to ameliorate acute lung inflammation in animal models [10,11]. Thus, we evaluated the effect of aspirin and indomethacin on pulmonary inflammation caused by CS. Groups of mice exposed to four cigarettes per day for 4 days were simultaneously treated daily with 40 or 80 mg kg^{-1} aspirin i.p. As shown in Fig. 2B, this led to a significant decrease in neutrophil number collected from BAL fluid of CS-exposed mice treated with vehicle (almost 95% inhibition with 80 mg kg^{-1}). Indomethacin also inhibited

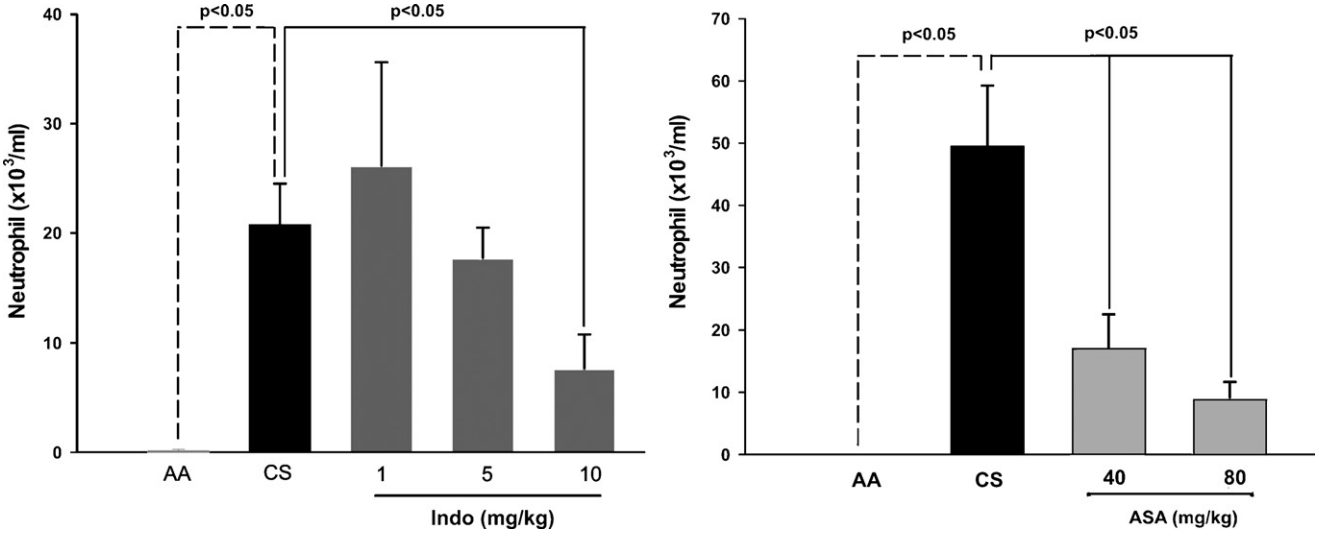

Fig. 2 – Effect of aspirin or indomethacin administration on bronchoalveolar lavage (BAL) fluid cellularity from mice exposed to cigarette smoke (CS). Dose-response effect on the number of neutrophils collected in BAL fluid from mice exposed to four cigarettes per day during 4 days and treated with indicated doses of indomethacin (A) or aspirin (B). Results are expressed as mean ± SEM (n = 5–8). AA: mice exposed to ambient air; CS: CS group treated with vehicle; Indo: CS group treated with indomethacin (i.p.); ASA: CS group treated with aspirin (i.p.).

neutrophil accumulation at 10 mg kg^{-1} but not at 1 or 5 mg kg^{-1} (Fig. 2A). Similarly, the histological analysis of lung tissue revealed an increased neutrophil number after CS exposure that was completely inhibited after treatment with aspirin and indomethacin (Fig. 3). The reduction of neutrophil recruitment by both aspirin and indomethacin might be due to a non-specific inflammation caused by the irritation of the peritoneal cavity. To eliminate this possibility, we performed a peritoneal lavage and the cellularity pattern obtained showed no difference among the groups AA, treated with vehicle, aspirin or indomethacin (data not shown). Moreover, no significant differences in BAL fluid cells pattern as well as lung tissue cells and titres of inflammatory mediators were found among AA group and animals that received only vehicle,

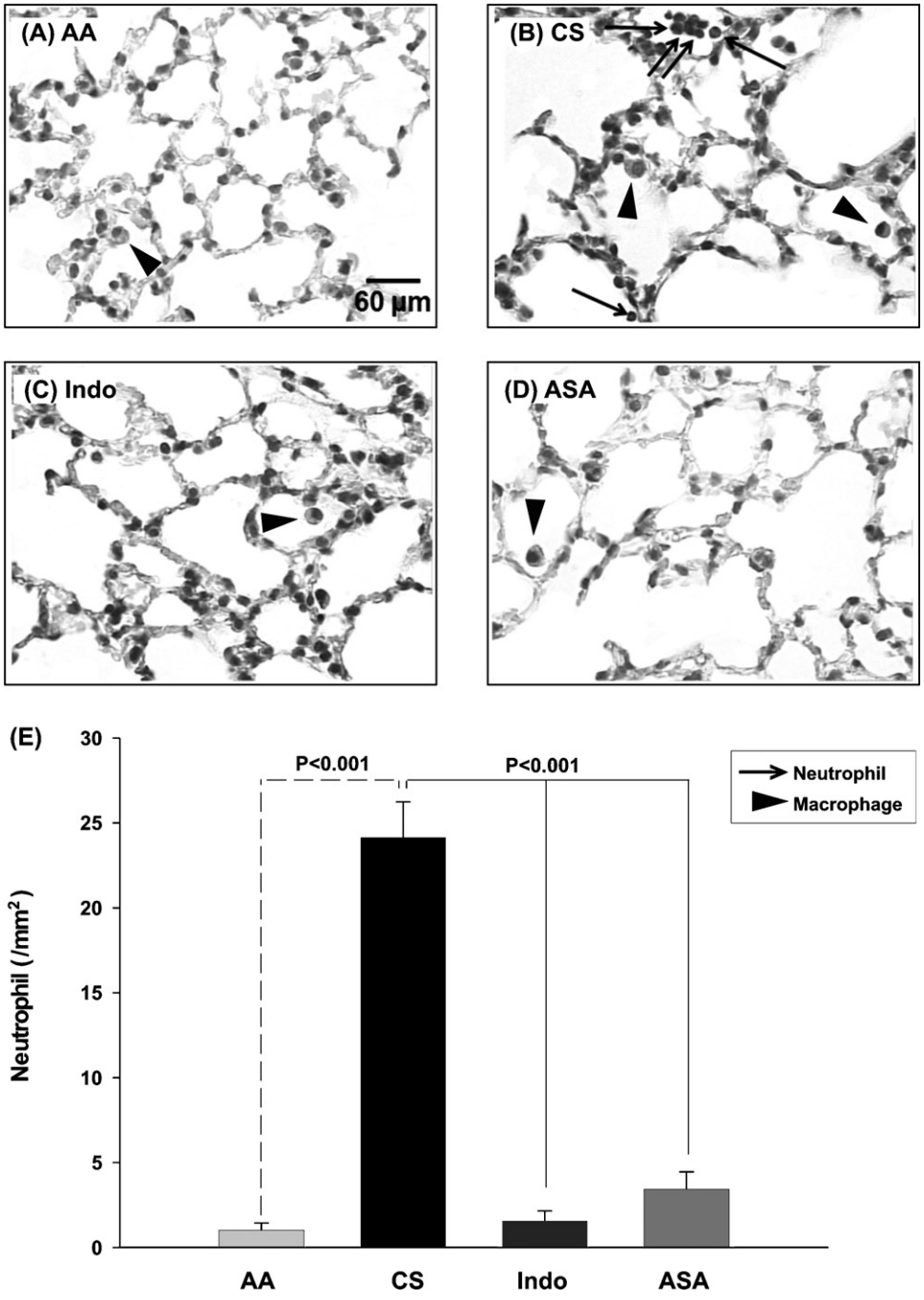

Fig. 3 – Effect of aspirin or indomethacin administration on lung inflammatory cell accumulation from mice exposed to cigarette smoke (CS). Total lung neutrophil counted after four cigarettes per day during 4 days from mice treated with aspirin or indomethacin. Mice exposed to ambient air (AA) and CS treated with 10 mg kg^{-1} i.p. indomethacin (Indo) or 80 mg kg^{-1} i.p. aspirin (ASA) present only alveolar machophages (arrowhead) but no neutrophils (A, C and D, respectively). CS mice treated with vehicle show neutrophil infiltration (arrows, B). Quantitative analysis of neutrophil number in lung histological sections (E). Results are expressed as mean ± SEM (n = 5–8).

10 mg kg^{-1} indomethacin or 80 mg kg^{-1} aspirin, during 4 days (data not shown). These data evidence that lung neutrophil infiltration induced by CS exposure, an essential step in inflammation, is blocked by NSAIDs.

3.3. CS-induced lung inflammatory mediators are inhibited by aspirin but not indomethacin

Different cytokines, chemokines and lipid signaling molecules are generated and released during inflammation. CS is a known stimulating factor of several of these substances in lungs [3]. As NSAIDs was shown to reduce neutrophil migration to lungs, we investigated their effect on the tissue inflammatory mediators. Groups of mice exposed to four cigarettes per day for 4 days were simultaneously treated daily with 80 mg kg^{-1} aspirin or 10 mg kg^{-1} indomethacin. Fig. 4 reveals that treatment with aspirin inhibited the augmentation of total protein, IL-1β, MCP-1 and PGE_2 levels evaluated in BAL fluid. On the other hand, indomethacin did not significantly inhibit any of these factors, although there was a trend toward lower levels of PGE_2 (Fig. 4D). These results show that, as expected, CS exposure resulted in the augment of different pro-inflammatory products and this is blocked by aspirin. Surprisingly, indomethacin was unable to produce the same effect.

3.4. CS-induced lung NF-κB activation is inhibited by aspirin or indomethacin

Our data indicated that, at least for indomethacin, a COX-independent action might be involved in its anti-inflammatory effect. NF-κB is a key player in inflammation and distinct NSAIDs have been shown to affect NF-κB activation [18]. To further investigate the effect of NSAIDs on cellular signaling, we evaluated NF-κB activation in lungs of mice exposed to AA or CS and the latter were treated with NSAIDs as described above. Fig. 5 reveals that treatment with indomethacin or aspirin completely inhibited NF-κB activation, as demonstrated in two representative gels (Fig. 5A and B, respectively) and averaged densitometries (Fig. 5D and E). Similar total protein loading was observed among gel lanes (data not shown). Specificity was determined by addition of 50-fold

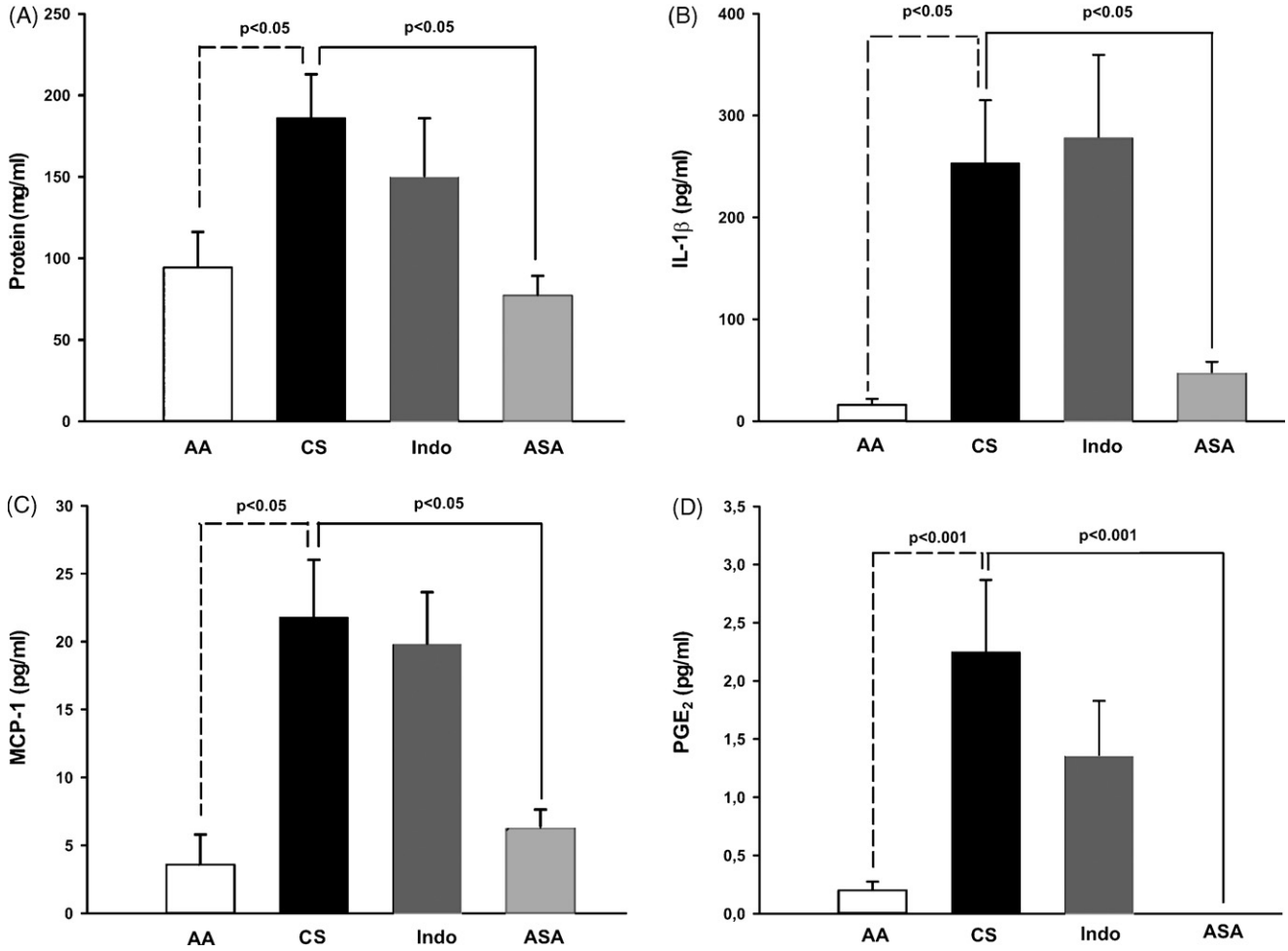

Fig. 4 – Effect of indomethacin or aspirin administration on bronchoalveolar lavage (BAL) fluid total protein, IL-1β, MCP-1 and PGE_2 levels from mice exposed to cigarette smoke (CS). Total protein (A), IL-1β (B), MCP-1 (C) and PGE_2 (D) levels in BAL fluid from mice exposed to four cigarettes per day during 4 days and treated with indomethacin or aspirin. Results are expressed as mean ± SEM (n = 5–8). AA: mice exposed to ambient air; CS: CS group treated with vehicle; Indo: CS group treated with indomethacin (10 mg kg^{-1} i.p.); ASA: CS group treated with aspirin (80 mg kg^{-1} i.p.).

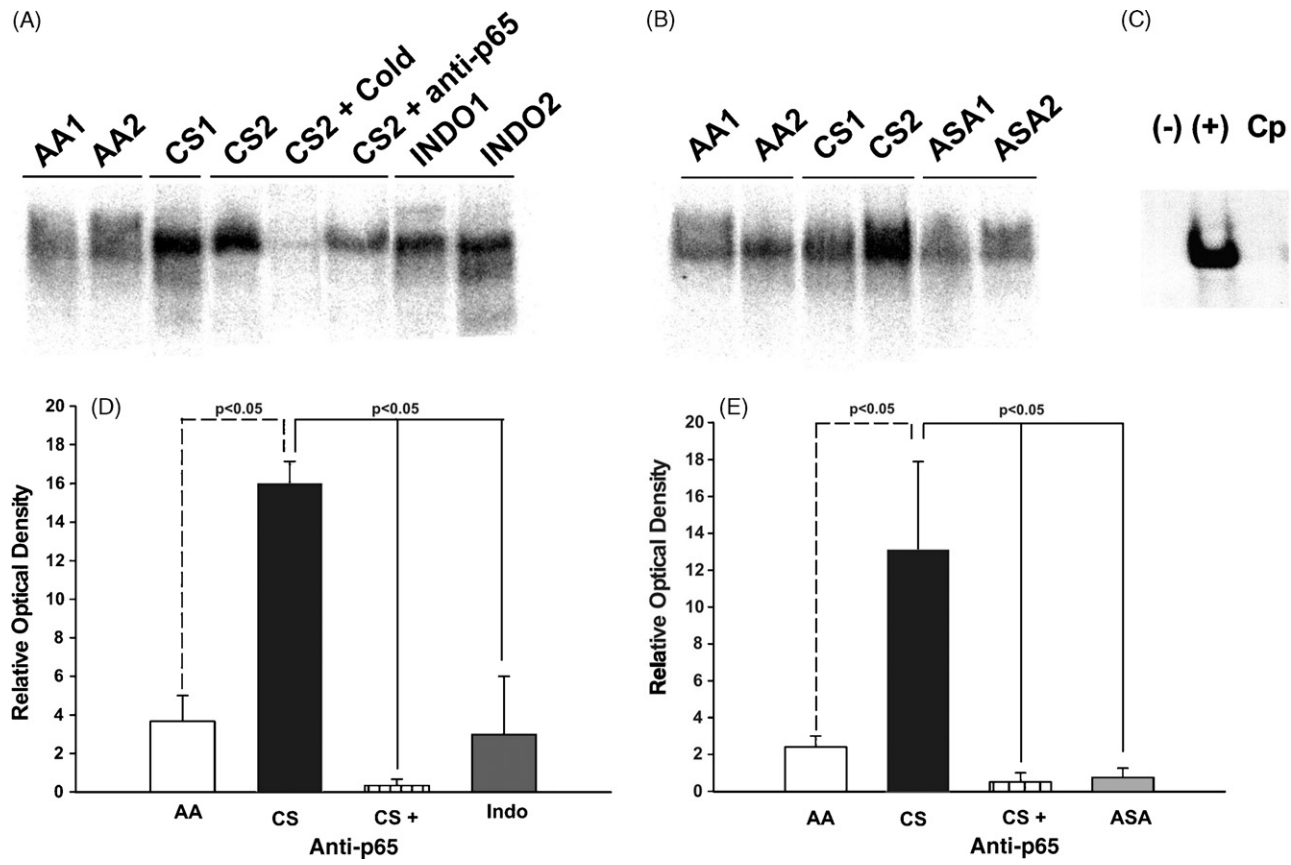

Fig. 5 – Effect of indomethacin or aspirin administration on NF-κB activation in whole lung of mice exposed to cigarette smoke (CS). Mice were exposed to smoke of four cigarettes per day during 4 days and treated with indomethacin or aspirin (A and B, respectively, as representative blots). Whole lung nuclear extract was incubated with DNA probe and electrophoretic mobility shift assay was performed. Panels (D) and (E) show gel relative optical density in arbitrary units of blots from three independent experiments. Panel (C) exhibits the negative control without nuclear extract (−), nuclear extracts from HeLa cells incubated with 1 mg mL^{-1} of LPS for 1 h (+) and specific competition with unlabeled NF-κB oligonucleotide (Cp). Results are expressed as mean ± SEM. AA: mice exposed to ambient air; CS: CS group treated with vehicle; Indo: CS group treated with indomethacin (10 mg kg^{-1} i.p.); ASA: CS group treated with aspirin (80 mg kg^{-1} i.p.); CS + anti-p65: CS group plus antibody against NF-κB p65 subunit for supershift assay; CS + cold: CS group plus 50-fold excess unlabeled NF-κB oligonucleotide.

excess unlabeled oligonucleotide (Fig. 5A and C). Fig. 5 also shows the supershift assay to demonstrate the presence of NF-κB p65 subunit. Here we demonstrate that both drugs are able to impair NF-κB activation in vivo.

3.5. *CS-induced lung p38 MAPK activation is inhibited by aspirin or indomethacin*

As a stress signaling kinase, p38 MAPK has been uncovered as an important component in inflammation and has been implicated in the activation of NF-κB in this condition [46]. The activational status of p38 MAPK as well as the effect of NSAIDs were examined in our model. Activation of p38 MAPK was evaluated through its phosphorylated form in lung tissue. Groups of mice were exposed to AA or CS and the latter were treated with NSAIDs as described above. Exposure to CS promoted the phosphorylation (activation) of p38 MAPK that was significantly prevented in mice treated with NSAIDs (Fig. 6A and B). Total p38 MAPK expression did not exhibit any significant difference among the experimental groups studied (Fig. 6A, densitometric data not shown). Therefore, two sequential signaling partners stimulated in inflammation, p38 MAPK and NF-κB, are blocked by both NSAIDs in vivo.

4. Discussion

Here we demonstrated for the first time that (1) NSAIDs could significantly reduce acute lung inflammation induced by CS; (2) p38 MAPK and NF-κB activation were inhibited by both NSAIDs in vivo; and (3) COX-dependent and -independent effects were discriminated for different NSAIDs in vivo. The present findings are in line with our previous works, in which acute exposure to CS for a few days with an average of four to six cigarettes day^{-1} resulted in a considerable inflammatory response with increased neutrophil number collected from

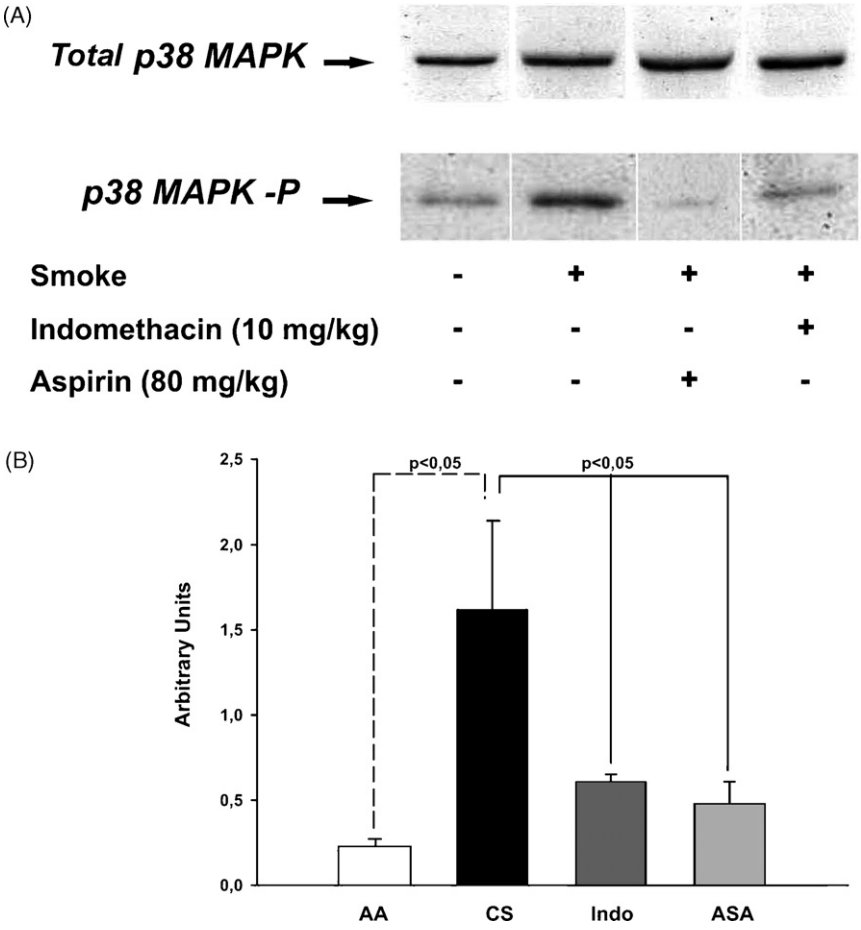

Fig. 6 – Effect of indomethacin or aspirin administration on p38 mitogen-activated protein kinase (MAPK) activation in whole lung of mice exposed to cigarette smoke (CS). Mice were exposed to four cigarettes per day during 4 days and treated with indomethacin or aspirin (A as representative blots for total and phosphorylated p38 MAPK). Panel (B) shows gel relative optical densitometry in arbitrary units of phosphorylated p38 MAPK blots from three independent experiments. Results are expressed as mean ± SEM. AA: mice exposed to ambient air; CS: CS group treated with vehicle; Indo: CS group treated with indomethacin (10 mg kg^{-1} i.p.); ASA: CS group treated with aspirin (80 mg kg^{-1} i.p.).

BAL fluid plus a slight (present work, not shown) but significant macrophage influx [3,24–26]. These occurred in parallel with lung lipid peroxidation [25,26] and increased levels of IL-1β, MCP-1 [3], total protein and PGE_2. It has been demonstrated that CS extract induces COX-2 and PGE_2 synthase transcription, justifying the increased PGE_2 levels in BAL fluid after CS exposure [29,30].

Treatment with NSAIDs inhibited neutrophil influx in mouse lungs exposed to CS. According to Tegeder et al. [31], aspirin negatively modulates PGE_2 production and NF-κB activation and decreases IL-1β, MCP-1 and PGE_2 levels in BAL fluid. There was no CS-induced NF-κB activation after treatment with aspirin. This could be explained by interactions of the drug on different intracellular pathways. NF-κB is an important intracellular signaling molecule with strong association to inflammatory response. The mechanism underlying aspirin inhibition of NF-κB translocation to the nucleus, demonstrated in lung epithelial cells [19], may be explained by the ability to decrease IKKβ activity, which is thought to be mediated by competition with ATP in its ATP-binding site or even by affecting an upstream factor [19,32]. Consequently, the NF-κB inhibitor protein, known as IκBα, is prevented from being phosphorylated and keeps NF-κB in cytoplasm.

This effect of aspirin on IKKβ activity may be extended to other kinases, such as those involved in p38 MAPK activation. Here we found that treatment with aspirin decreased p38 MAPK phosphorylation triggered after exposure to CS. In T lymphocytes, neutrophils and endothelial cells, aspirin and aspirin-triggered lipoxin A_4 stable analog ATL-1 were shown to block p38 activation, which seems to be stimulus- and cell-specific [23,33,34]. Interestingly, acrolein, an aldehyde present in tobacco smoke, induced COX-2 expression and PGE_2 formation in endothelial cells by means of p38 MAPK activation [35], which further links CS, p38 MAPK activation and inflammation.

To further investigate for a specific effect of aspirin, mice were also treated with indomethacin. Indomethacin is another NSAID first assigned only as reversible COX inhibitor. However, it has been described to have an effect on cellular apoptosis and inflammatory cell migration via a COX-independent mechanism [31,36]. Here we showed that treatment with indomethacin also decreased neutrophil influx, inhibited NF-

κB and p38 MAPK activation, but unexpectedly failed to reduce inflammatory mediators, including PGE_2. Indomethacin is described to inhibit COX-2 activity at lower concentrations than aspirin in pulmonary and non-pulmonary cells in vitro [37,38], yet it seems slightly more selective to COX-1 [37,39]. In our experiments only aspirin reduced BAL fluid PGE_2 levels after CS exposure. The dose range and route of administration we utilized are in accordance to previous studies on pulmonary inflammation using aspirin [40,41] or indomethacin [11,41–43]. Therefore, our data suggest that the main mechanisms underlying neutrophil inhibition by indomethacin are probably COX-independent. In agreement with our findings, indomethacin is able to block NF-κB in different cells [18,44,45] but, to our knowledge, it was the first time that indomethacin was shown to inhibit p38 MAPK stimulation. Recently, Liu et al. [46] clearly evidenced that activated p38 MAPK is critical to LPS-induced lung injury, and p38 MAPK inhibition attenuated NF-κB activation and PMN infiltration. Thus, we suggest that indomethacin (and aspirin) might have an anti-inflammatory effect in our model by a similar mechanism.

Other mechanisms may be involved in NSAIDs effects. For instance, indomethacin has a well-known PPAR-γ binding property and this receptor, which is expressed in both alveolar macrophages and neutrophils, has been shown to broadly regulate inflammatory and reparative responses, with direct suppressive effects on migration of neutrophils, monocytes and eosinophils [36,47,48]. Moreover, PPAR-γ activation in alveolar epithelial cells resulted in suppression of NF-κB transcriptional activity in vitro [49]. On the other hand, aspirin has also an anti-inflammatory effect in IL-1β-stimulated mouse mesentery through 15-epi-lipoxin A_4 production [50]. Likewise, ATL-1 effectively inhibited LPS-induced acute pulmonary inflammation [17]. Oxidative damage evoked by CS may also be a target for the antioxidative effects of NSAIDs [51]. These putative mechanisms, however, were not considered in our study.

The molecular mechanisms involved in pathogenesis of lung diseases associated to CS are not fully understood. The use of aspirin or indomethacin modulates CS-induced lung inflammatory reaction in the current murine model. Lung inflammation underlies several tobacco-related diseases, as seen in COPD, and these findings may help to achieve new therapeutic strategies based on well-known clinical drugs available. In addition, available therapeutic options have a mild effect over the decreasing pulmonary function. Macrophages and neutrophils are increased in BAL fluid and lung tissue from animals exposed to CS [3,25,26], and neutrophils are believed to play a key role in pathogenesis of COPD. As lung neutrophil accumulation due to CS is refractory to corticosteroid therapy, alternative pharmacological approaches are needed [52,53].

In conclusion, we showed that acute exposure to CS led to cellular activation of NF-κB and p38 MAPK resulting in neutrophil recruitment into the lungs and oxidative damage, and these effects can be blocked by treatment with NSAIDs aspirin and indomethacin. Nevertheless, aspirin, but not indomethacin, remarkably prevented the increase of inflammatory mediators, including the COX-derived prostanoid PGE_2. This suggests that, in such experimental model, COX-independent mechanisms are at least as important as COX-dependent ones to the anti-inflammatory effect of these NSAIDs. The evaluation of the effect of COX-selective inhibitors, corticosteroids, and on chronic inflammation induced by CS exposure with aspirin and indomethacin is under investigation.

Conflict of interest statement

The authors declare no conflicting financial interests.

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