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Dual effects of acetylsalicylic acid on mast cell degranulation, expression of cyclooxygenase-2 and release of pro-inflammatory cytokines

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Abstract

Several studies have demonstrated that nonsteroidal anti-inflammatory drugs, such as acetylsalicylic acid (ASA), can have inhibitory or enhancing effects on inflammatory cell function. These effects seem independent of cyclooxygenase activity and prostaglandin synthesis inhibition. Here, we examined the effect of ASA on bone marrow-derived mast cells in more detail. ASA blocked the expression of cyclooxygenase-2, the production of tumor necrosis factor-α and interleukin-6, and the release of granule mediators from mast cells in a concentration-dependent fashion. Concomitantly, ASA inhibited nuclear factor (NF)-κB activity, as well as the phosphorylation and breakdown of the inhibitory protein IκB-α. We thus propose that the anti-inflammatory effects of ASA in mast cells are due to suppression of IκB kinase activity, thereby inhibiting subsequent phosphorylation and degradation of IκB-α, activation of NF-κB, and transcription of proinflammatory cytokines. The inhibition of BMMC degranulation was independent of NF-κB activation, however. Interestingly, the expression of cyclooxygenase-2 was not inhibited at 1 mM ASA, but was even enhanced significantly. The latter might contribute to the adverse effects of ASA in ASA-sensitive asthmatics.

Keywords: Mast cells; NF-κB; Allergy; Inflammation; IgE and antigen; ASA

1. Introduction

Acetylsalicylic acid (ASA) and its analogs are among the most widely used drugs on a worldwide basis. For more than three decades, the anti-inflammatory actions of ASA and salicylate have been attributed primarily to inhibition of prostaglandin synthesis [1]. However, a growing number of studies have provided evidence for immunomodulatory effects unrelated to inhibition of prostaglandin synthesis. For example, Casolaro et al. have reported that various NSAIDs such as ASA have dual effects on degranulation of human basophils and mast cells [2]. Moreover, a major finding was the discovery that ASA and salicylate specifically inhibit the activation of the transcription factor nuclear factor κB (NF-κB) by inflammatory stimuli [3]. NF-κB is an inducible transcription factor comprised of subunits that can include cRel, RelA, RelB, p50 and p52. In most cells the NF-κB prototype is a heterodimer composed of the RelA (p65) and NF-κB1 (p50) subunits. Cytoplasmic NF-κB is inactive when bound to an inhibitory protein IκB-α. NF-κB is activated by a wide variety of agents, including phorbol esters, IL-1β, TNF-α, lipopolysaccharide (LPS), double-stranded RNA, cAMP, bacteria, and viral transactivators [4,5]. Upon cell stimulation, the signal-responsive kinases IKKα and β are activated which directly phosphorylate Ser^{32} and Ser^{36} in IκB-α, triggering ubiquitination at Lys^{21} and Lys^{22}, and rapid degradation of IκB-α, in 26S proteasome [6]. This process liberates NF-κB, allowing it to translocate to the nucleus. In the nucleus, NF-κB binds to its cognate site, κB element, which regulates transcription of downstream genes. Transcription of most genes for inflammatory proteins, such as TNF-α, IL-2, IL-9, GM-CSF, and COX-2 is dependent on regulation by the transcription factor NF-κB in inflammatory or immune cells [4,7,8].

Abbreviations: ASA, acetylsalicylic acid; Anti-DNP IgE, anti-dinitrophenol IgE; COX, cyclooxygenase; DNP-HSA, dinitrophenol human serum albumin; HPRT, hypoxanthine-guanine phosphoribosyltransferase; MTT, 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide; NaSal, sodium salicylate; RT-PCR, reverse transcription polymerase chain reaction

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Mast cells and basophils are the main effector cells in IgE-mediated pathological symptoms seen in allergic disorders, such as urticaria, hay fever, and asthma [9,10]. Mast cells are also implicated in non-allergic acute and chronic inflammatory disease [11,12]. The signaling pathway leading to degranulation of mast cells after engagement of the FcεRI receptor by antigen and antibody has been extensively characterized [13,14]. Rapid activation of phosphorylation of intracellular target proteins, generation of IP3 and a subsequent increase in the cytosolic calcium level ($[Ca^{2+}]_i$) is considered to be essential in the mechanism of degranulation of mediators by mast cells [14]. The various cytokines and several bioactive substances, e.g. neuropeptides, complement, and kinins which are supplied from tissue microenvironments, are believed to act as positive or negative regulators of mast cell function [15]. Recently it has been shown that mast cells, through release of their pro-inflammatory mediators, are involved in aspirin attacks in aspirin-sensitive patients [16,17]. This prompted us to investigate the effects of ASA on the various functions of mast cells with respect to modulation of the NF-κB pathway.

2. Materials and methods

2.1. Materials

Recombinant mouse IL-1β, stem cell factor (SCF) and IL-10 were purchased from PeproTech. LPS (*Escherichia coli* 055.B5), Tyrode's salts and MTT were purchased from Sigma. RPMI 1640, fetal calf serum (FCS), and nonessential amino acids were purchased from GibCo BRL. Penicillin, streptomycin, 1-glutamine, sodium pyruvate, 2-mercaptoethanol, dinitrophenol-human serum albumin (DNP-HSA), ASA and sodium salicylate (NaSal) were obtained from Sigma. ASA was dissolved in deionized water at a concentration of 20 mM to be used as stock solution.

The ELISA kits for mouse TNF-α and IL-6 were purchased from Biosource. Rabbit polyclonal anti-IκBα and anti-p65 Abs were obtained from Santa Cruz Biotechnology. Rabbit polyclonal anti-phospho-IκBα Ab (Ser 32) was supplied by New England Biolabs. Goat anti-rabbit and rabbit anti-goat secondary Abs conjugated with horseradish peroxidase (HRP), were purchased from Dako. Proteasome inhibitor MG-132 (Z-Leu-Leu-Leu-H) and NF-κB SN50 were purchased from Calbiochem. Nuclear and cytoplasmic extraction reagents and Light Shift Chemiluminescent EMSA kits were purchased from Pierce. The NF-κB primer corresponding to NF-κB consensus sequence in the κ light chain enhancer in B cells (5′-AGT TGA GGG GAC TTT CCC AGG C-3′), end labeled with biotin and purified with HPLC, was purchased from Isogen Life Science.

2.2. Mouse bone marrow cultures

BMMC were generated from bone marrow of male BALB/cBy mice. Briefly, mice were sacrificed, and intact femurs were removed. Sterile endotoxin-free medium was repeatedly flushed through the bone shaft using a needle and syringe. The suspension of bone marrow cells was centrifuged at $320 \times g$ for 10 min, and cultured at a concentration of 0.5×10^6 nucleated cells/ml in RPMI 1640 with 10% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin (Life technology), 10 μg/ml gentamycine, 2 mM L-glutamine and 0.1 mM nonessential amino acids (referred to as enriched medium). Pokeweed mitogen-stimulated spleen cell conditioned medium (PWM-SCM, see below) 20% (v/v) was added to the enriched medium. Flasks were then incubated at 37 °C in a 5% CO_2 humidified atmosphere. Nonadherent cells were transferred to fresh medium at least once a week. After 3–4 weeks, mast cell purity of >95% was achieved as assessed by toluidine blue staining.

2.3. Pokeweed mitogen-stimulated spleen cell conditioned medium (PWM-SCM)

Spleen cells from BALB/c mice (Charles River Breeding Laboratories) were cultured at a density of 2×10^6 cells/ml in RPMI 1640 medium containing 4 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.1 mM nonessential amino acids (complete RPMI 1640) containing lectin (8 μg/ml) and placed in 75-cm^2 tissue culture flasks. The cells were incubated at 37 °C in a 5% CO_2 humidified atmosphere. After 5–7 days, medium was collected, centrifuged for 15 min at $3200 \times g$, filtered through a 0.22-μm Millipore filter, and used as PWM-SCM.

2.4. Survival assay

Viability of cells treated with ASA was determined by assaying the ability of mitochondrial dehydrogenases to convert a soluble tetrazolium, MTT, into an insoluble purple formazan by cleavage of the tetrazolium ring [18]. Briefly, cells were treated with 10 mM of ASA for 6 h. Then, the cells were washed, and their viability was measured by MTT assay as described previously [18].

2.5. Activation of BMMC

BMMC were washed once and suspended at a cell density of 10^7/ml in PWM-SCM 20% (v/v), and were sensitized with 10 μg/ml monoclonal anti-dinitrophenyl IgE for 45 min at 37 °C. After washing twice with enriched medium, cells were suspended at 2×10^6/ml in enriched medium containing SCF (100 ng/ml), IL-1β (5 ng/ml) and LPS (100 ng/ml). Then ASA or NaSal was added at

concentrations varying between 0.01 and 10 mM, after which the cells were stimulated with DNP-HSA (10 ng/ml). Subsequent incubation times were chosen for optimal effects, i.e. 30 min for NF-κB activity and IκB-α and phospho-IκB-α expressions, 5 h for COX-1 and COX-2 protein and 2 h for mRNA determinations, and 5 or 6 h for TNF-α and IL-6, respectively. Incubations were stopped by centrifugation at 1200 × *g* for 5 min at 4 °C.

2.6. Degranulation assays

As parameter for in vitro mast cell degranulation, the release of granular β-hexosaminidase was measured. Bone marrow cells were cultured for 3 weeks under conditions as described above. Approximately 2–3 × 10^6 cells from each culture were resuspended in culture medium (enriched medium) and incubated with 1 μg/ml anti-DNP IgE from hybridoma clone 26.82 for 1 h in 5% CO_2 at 37 °C. After sensitization with IgE, cells were washed twice with modified Tyrode's buffer (containing 137 mM NaCl, 2.7 mM KCI, 0.4 mM NaH_2PO_4; 1 mM $CaCI_2$; 5.6 mM glucose) supplemented with 0.1% BSA and 10 mM Hepes, pH 7.2 and resuspended at a density of 0.6 × 10^6 cells/ml. Cells were aliquoted in 96 well plates (3 × 10^4 cells per well) and activated with indicated concentrations of DNP-HSA or combination of cytokines and LPS for 30 min in the presence or absence of various concentrations of ASA. After incubation supernatants were collected. Cells were subsequently lyzed using 0.1% NP-40 (Pierce) in order to quantify the total β-hexosaminidase activity present in these cells. Samples were incubated with 4-methylumbelliferyl glucosaminide (4-MUG) (Sigma) in 0.1 M citrate buffer (pH 4.5) for 1 h at 37 °C. 4-MUG hydrolysis was determined by fluorimetric measurement (λ_{ex}: 360 nm/λ_{em}: 452 nm) using a Millipore Cytofluor 2350 microplate reader. The percentage of β-hexosaminidase released, was calculated as follows: 100% × fluorescence supernatant/fluorescence cell lysate, corrected for the β-hexosaminidase activity present in the supernatant of non-challenged cells.

2.7. Western blot analysis

After activation and incubation time, BMMC were washed once with PBS (10 mM phosphate buffer, pH 7.4, containing 150 mM NaCl) and lysed in lysis buffer containing 1% Triton X-100 or NP-40, NaCI, Tris and Mini™ protease inhibitors (Roche). The protein concentration was determined by BCA protein assay kit (Pierce). The lysate (25 or 50 μg) was subjected to SDS/PAGE [10% (w/v) gel]. The separated proteins were electroblotted on PVDF membranes (Bio-Rad). Membranes were then washed once with Tris/HCI, pH 7.4, containing 159 mM NaCI and 1% Tween 20 (TBS-T), and then blocked in super-blocking buffer (Pierce) for 1 h. After the membranes had been washed with TBS-T, antibodies against COX-1 or COX-2, IκB-α, P65, or antibody specific for phosphorylated IκB-α were added at a dilution of 1:3000 in TBS-T and incubated for 1 h. After three washes with TBS-T, membranes were treated for 1 h with HRP-conjugated goat anti-rabbit IgG for COX-1 and COX-2, rabbit-anti goat IgG for IκB-α and phosphorylated IκB-α, diluted to 1:20,000 in TBS-T. After three washes with TBS-T, immunoreactive protein bands were revealed with an enhanced chemiluminescence Western blot analysis system (Amersham Pharmacia Biotech). Films were scanned and analyzed on a GS7-10 Calibrated Imaging Densitometer equipped with Quantity One v. 4.0.3 software (Bio-Rad).

2.8. Measurement of cytokines

TNF-α and IL-6 concentrations were quantitated using ELISA kits (sensitivity 10 pg/ml) according to the manufacturer's instructions.

2.9. Preparation of cytoplasmic and nuclear extracts

Cells were washed twice with PBS and allowed to equilibrate for 5 min in ice-cold cytoplasmic extraction reagent (Pierce) containing protease inhibitors (Mini™ protease inhibitors, cocktail). Cells were lysed on ice for 5 min in mentioned reagent. Following centrifugation at 3500 × *g* for 5 min, the supernatants (cytoplasmic extracts) were collected and frozen at −70 °C. The pellets were suspended in nuclear extraction buffer containing protease inhibitors. After vigorous mixing and incubating for 10 min on ice, the solution was clarified by centrifugation at 14,000 × *g* for 5 min, and the supernatant (nuclear extract) was collected and stored at −70 °C. Protein concentrations were determined by using a BCA protein assay kit (Pierce). The lysates (25 μg) from cytoplasmic or nuclear fractions were subjected to SDS/PAGE [10% (w/v) gel] for detection of P65 expression.

2.10. EMSA

The NF-κB double-stranded oligonucleotide corresponding to NF-κB consensus in the κ light chain enhancer in B cells (5′-AGT TGA GGG GAC TTT CCC AGG C-3′) was end-labeled with biotin and purified with HPLC.

Nuclear extracts (in a volume of 3 μl, according to the protocols of the nuclear extraction kits) were added to biotin labeled NF-κB oligonucleotide in a binding buffer containing 50% glycine, 200 mM EDTA, and 1 μg/μl poly (dI − dC).

Reaction mixtures were incubated for 20 min at room temperature. Supershift assays were performed by incubation of oligonucleotide for 20 min with the nuclear extract, and then 0.4 μg of anti-p65 or p50 Ab were added and allowed to react for 45 min at room temperature. DNA-protein complexes were resolved on 4% nondenaturing polyacrylamide gel (29:1) and subsequently blotted onto

nylon for 30 min and then detected by using Light Shift TM Chemiluminescent EMSA Kit.

2.11. RT-PCR

Expression of COX-2 mRNA was assessed using RT-PCR standardized by coamplifying COX-2 with the housekeeping gene HPRT.

Total RNA was obtained from 5×10^6 cells using TRIZOL (Invitrogen). Total RNA (1 μg) was used as a template for reverse transcription, using oligo d(T) and M-MLV (Invitrogen) reverse transcriptase. Different dilutions of cDNA were amplified using specific primers for COX-2 and HPRT. Primers sequences for mouse COX-2 (GenBank: J00944967) were 5′-CAA GCA GTG GCA AAG GCC TCC A-3′ (forward primer) and 5′-GGC ACT TGC ATT GAT GGT GGC T-3′ (reverse primer) (Isogen Bioscience) and for HPRT (GenBank J00423) primers sequences were for the forward primer 5′-AGT CCC AGC GTC GTG ATT AGC GAT GA-3′ and the reverse primer 5′-TGG CCT GTA TCC AAC ACT TCG AGA GGT-3′ (Amersham Pharmacia). PCR products were separated on 1.5% agarose gels containing 0.5 μg/ml of ethidium bromide and visualized using a Gel Doc 1000 system (Bio-Rad).

2.12. Statistical analysis

Experimental results are expressed as mean ± S.E.M. Results were tested statistically by an unpaired two-tailed Student's *t*-test or one-way ANOVA, followed by Newman–Keuls test for comparing all pairs of groups. Analyses were performed by using GraphPad Prism (version 2.01). Results were considered statistically significant when $p < 0.05$.

3. Results

3.1. Inhibition of mast cell degranulation by ASA

We determined whether ASA can influence degranulation of BMMC in response to activation with IgE and antigen (Ag) plus a combination of cytokines (IL-1β, SCF) and LPS. IgE-mediated degranulation was monitored by β-hexosaminidase release. ASA at concentrations between 0.01 and 10 mM did not affect degranulation of non-activated cells (Fig. 1). Treatment of activated cells with ASA caused dose-dependent inhibition of β-hexosaminidase release (Fig. 1). Similar inhibitory effects of ASA were found when BMMC were activated with IgE/Ag alone, in the absence of cytokines and LPS (data not shown).

3.2. Attenuation of TNF-α and IL-6 production by ASA

To test the effect of ASA on the production of TNF-α and IL-6, BMMC were treated with different concentrations of ASA and simultaneously stimulated with IgE/Ag alone or with cytokines and LPS. Significant inhibition of TNF-α production was seen at 1 mM and 10 mM of ASA (Fig. 2A), while IL-6 production was reduced to a somewhat lesser extent only at 10 mM (Fig. 2B). Cell viability was not affected by 10 mM ASA (92 ± 1% versus

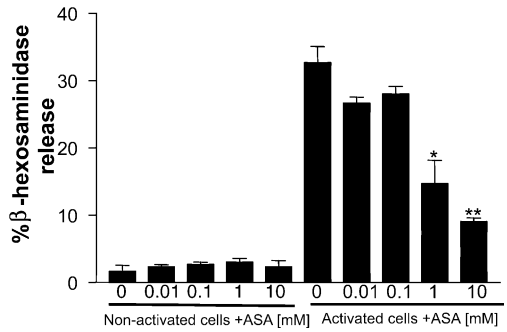

Fig. 1. ASA inhibits degranulation of activated mast cells. BMMC were sensitized with DNP-specific IgE, followed by activation with DNP-HSA (10 ng/ml), cytokines and LPS. At the same time of activation ASA at 0.01–10 mM was added. Degranulation was assessed by release of β-hexosaminidase. The percentage of degranulation was calculated as: $(a - b)/(t - b) \times 100$, where a is the amount of β-hexosaminidase released from stimulated cells, b that of β-hexosaminidase released from unstimulated cells and t is the total cellular content of β-hexosaminidase. Data are mean ± S.E.M. of quadruplicate samples. The asterisks represent significant differences compared with activated cells alone ($^{*}p < 0.05$; $^{**}p < 0.01$).

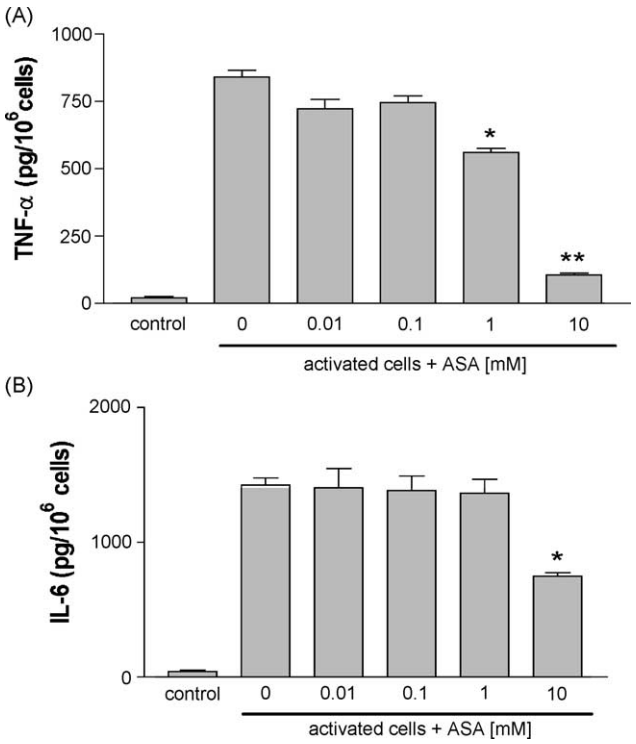

Fig. 2. ASA attenuates cytokine production in activated cells. BMMC were incubated with the indicated concentrations of ASA and simultaneously stimulated, as described in Section 2, for production of TNF-α (A) and IL-6 (B). The levels of cytokines in the supernatants were estimated by ELISA methods. Data shown are mean ± S.E.M. of three independent experiments. The asterisks represent significant differences compared with activated cells alone ($^{*}p < 0.05$; $^{**}p < 0.01$).

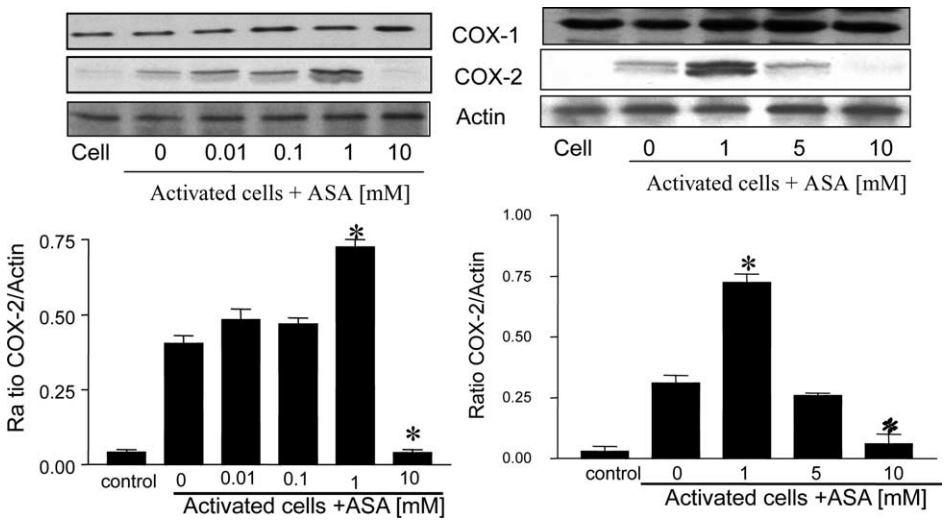

Fig. 3. Effect of various concentrations of ASA on COX-1 and COX-2 protein expression. BMMC were incubated with the indicated concentrations of ASA and simultaneously stimulated, as described in Section 2. The expression of COX-1, COX-2, and actin as a housekeeping protein, on the same samples were assessed by Western blot analysis. The ratios of COX-2 to actin expression from three separate gels are shown in the lower panels. Data are mean ± S.E.M. of triplicate samples. The asterisks represent significant differences compared with activated cells alone ($^{*}p < 0.05$).

97 ± 1% in control). For comparison, NaSal at a concentration of 10 mM was also effective in suppressing TNF-α production (83% ± 3% inhibition) and IL-6 production (46% ± 7% inhibition), but was less effective at the concentration of 1 mM (TNF-α, 20% ± 5% and IL-6, 6% ± 2% inhibition).

3.3. Regulation of COX-2 protein expression by ASA

In previous experiments it has been demonstrated that co-incubation of BMMC with IgE/Ag plus IL-1β, SCF and LPS induces enhanced expression of COX-2 [19,20]. Indeed, activation of BMMC with this combination resulted in significant expression of COX-2 (Fig. 3). ASA had a differential effect on COX-2 expression. At low concentrations (up to 1 mM) ASA increased expression of COX-2 (Fig. 3, left panel), which was not observed at higher concentrations (Fig. 3, right panel). At 10 mM ASA the COX-2 expression was even completely inhibited (Fig. 3, both panels). No effect of any concentration of ASA on the expression of COX-1 was observed (Fig. 3), which enzyme is constitutively expressed in BMMC. We can exclude that the observed effects of ASA can be attributed to the acidity of the drug, since we observed similar inhibitory effects of ASA after pH adjustment of the incubation media. NaSal at a concentration of 10 mM likewise downregulated COX-2 protein expression (data not shown).

3.4. Suppression of COX-2 mRNA expression by ASA

In the next experiments, we determined whether the effects of ASA on COX-2 protein expression were due to increased or decreased expression of mRNA. To assess the expression of COX-2 mRNA, RT-PCR was carried out using specific primers for COX-2. It has been reported that COX-2 mRNA can be detected in cells as early as 2 h after stimulation [20]. We found that COX-2 mRNA was undetectable in non-activated BMMC, and its expression was up-regulated in activated cells after 2 h (Fig. 4). ASA at 10 mM suppressed the expression of COX-2 mRNA, while at 1 mM the expression was not significantly inhibited (Fig. 4). No effect on the expression of mRNA for the household enzyme HPRT was observed. These results

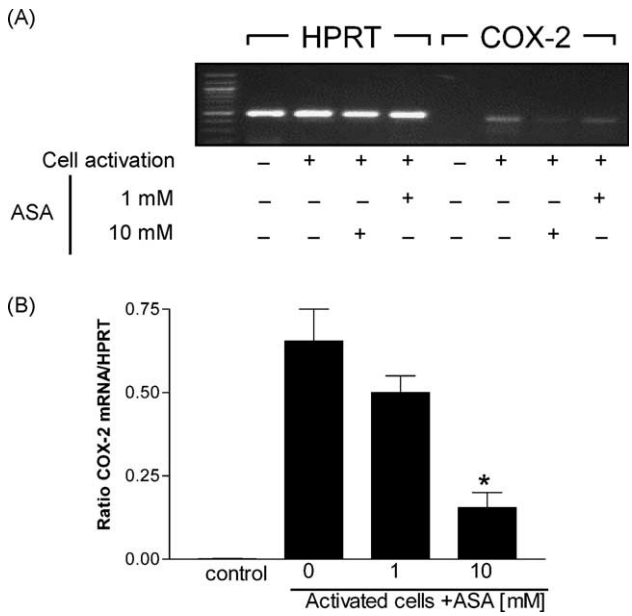

Fig. 4. Inhibition of COX-2 mRNA by ASA in mast cells. Total RNA was isolated from BMMC that had been activated and treated with or without ASA at 1 and 10 mM for 2 h. COX-2 mRNA was assayed by a semi-quantitative RT-PCR procedure using serial dilutions for comparing relative amounts of RNA. (A) RT-PCR analysis for COX-2 and HPRT mRNA. (B) Quantitative expression of COX-2 mRNA, as a ratio to HPRT mRNA. Representative results of three independent experiments are shown.

suggest that decreased expression of COX-2 protein at 10 mM of ASA correlated with a decrease of mRNA levels. However, the increased expression of COX-2 at 1 mM could not be explained by increased mRNA levels.

3.5. Suppression of NF-κB activation by ASA

To test the possibility that ASA-induced suppression of TNF-α, IL-6 and COX-2 expression was due to blocking of NF-κB activation, we measured NF-κB activity after stimulation in the presence or absence of ASA. Thus, cells were treated with SCF, IL-1β, LPS and IgE/Ag with or without ASA for 30 min. The nuclear extracts were subjected to EMSA with κB site DNA probe. ASA caused a clear inhibition of NF-κB activity at 10 mM, whilst at 1 mM a less pronounced inhibition was observed (Fig. 5A).

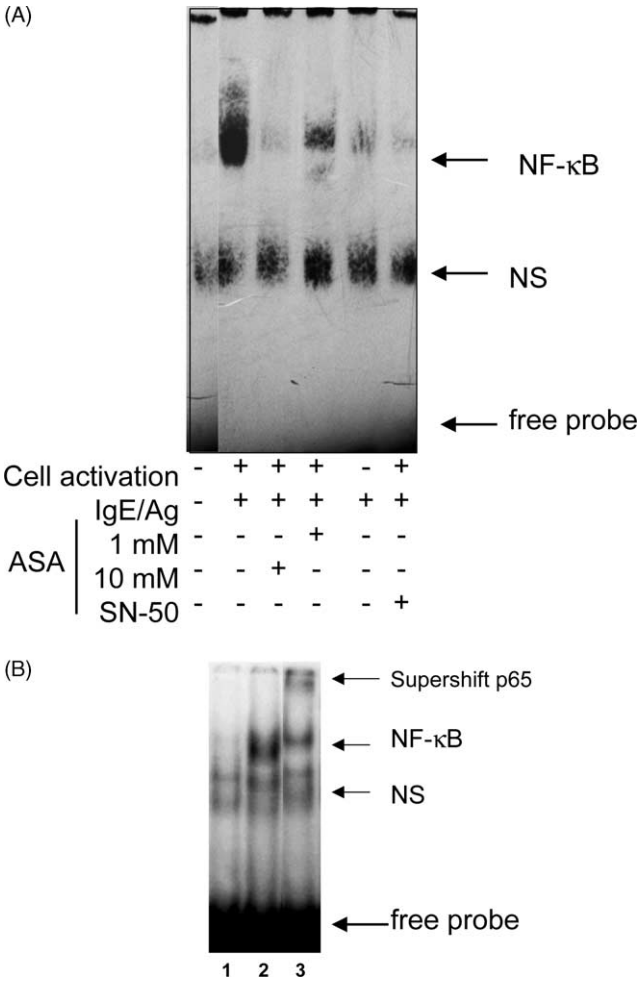

Fig. 5. NF-κB binding activity suppressed by ASA in activated mast cells. BMMC were incubated with the indicated concentrations of ASA and simultaneously stimulated, as described in Section 2. (A) Nuclear extracts were prepared and subjected to EMSA with κB site DNA probe. As a positive control for inhibition of NF-κB activity, cells were pretreated with a specific inhibitor of NF-κB (SN-50) for 30 min. (B) Supershift assay with an anti-p65 IgG antibody demonstrating the specificity of DNA-nuclear protein binding; lane 1: unactivated cells, lane 2: activated cells + IgE/Ag, lane 3: activated cells + IgE/Ag with P65 antibody. NS denotes nonspecific binding. Representative results of three independent experiments are shown.

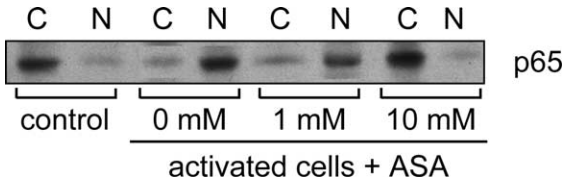

Fig. 6. Suppression of NF-κB translocation by ASA. BMMC were activated in the presence or absence of the indicated concentrations of ASA, as described in Section 2. Cytoplasmic (C) or nuclear (N) fractions of cell lysates (25 μg) were subjected to Western blot by using p65 antibody of NF-κB subunits. Representative results of three independent experiments are shown.

A supershift assay demonstrated the presence of the p65 subunits of NF-κB (Fig. 5B). As a positive control for inhibition of NF-κB activity, cells were pretreated with a specific inhibitor of NF-κB (SN-50) for 30 min. Treatment with NS-50 inhibited NF-κB DNA binding in mast cells (Fig. 5A).

To confirm that nuclear p65 levels were reduced in the presence of ASA, as well as to verify the identity of the nuclear NF-κB subunits, cytoplasmic and nuclear extracts from control and activated cells were subjected to Western blot analysis. The majority of p65 was located in the cytoplasmic fraction of control cells at basal condition, and 30 min of activation of cells caused an increase in the nuclear expression of p65. Nuclear p65 levels were greatly reduced by treatment with 10 mM of ASA (Fig. 6), whilst cytoplasmic p65 was increased concomitantly.

3.6. Inhibition of IκB-α degradation by ASA

NF-κB exists as an inactive form bound to the inhibitory protein IκB-α in the cytoplasm, and degradation of IκB-α must occur in order for NF-κB to translocate to the nucleus. We explored the possibility that ASA affects degradation of IκB-α in activated cells.

Protein levels of IκB-α were analyzed by immunoblotting of whole cell extracts with IκB-α specific Ab. Activation of cells resulted in a reduction of IκB-α levels (Fig. 7), which was prevented by 1 and 10 mM ASA. ASA had no effect on IκB-α in non-activated cells (data not shown). IκB-α degradation by a proteasome dependent pathway is

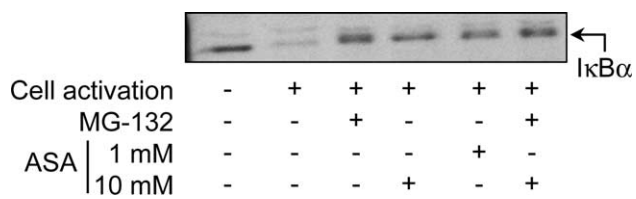

Fig. 7. ASA stabilizes IκB-α in mast cells. Cells were pretreated with or without the proteasome inhibitor MG-132 (50 μM) for 1 h and then treated with ASA and activated, as described in Section 2. Western blot analysis for IκB-α was carried out with whole cell extracts (50 μg) with rabbit polyclonal anti-IκB-α antibody. Representative results of three independent experiments are shown.

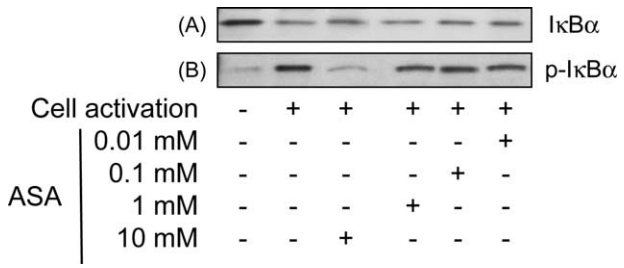

Fig. 8. Phosphorylation of IκB-α is inhibited by ASA. Cells were pretreated with the proteasome inhibitor MG-132 (50 μM) for 1 h and then treated with ASA and activated, as described in Section 2. (A) IκB-α measured by Western blot analysis of whole cell lysates, using an anti-IκB-α antibody. (B) Phosphorylated kB-a measured by Western blot analysis of whole cell lysates, using a specific anti-P-IκB-α antibody. Representative results of three independent experiments are shown.

preceded by phosphorylation of two serine residues (Ser^{32} and Ser^{36}) and subsequent ubiquitination [4,21]. To address the mechanism involved in the stabilization of IκB-α by ASA, we examined phosphorylation of IκB-α by Western blot analysis. The cells were pretreated with the proteasome inhibitor MG-132 for 1 h at 50 μM, which allows the phosphorylated IκB-α to accumulate in the cell [22]. Indeed, MG-132 prevented the disappearance of IκB-α in BMMC after cell activation (Fig. 7 and Fig. 8A). Immunoblotting with an antibody specific for phosphorylated IκB-α on Ser^{32} showed that 10 mM ASA prevented the increase of IκB-α phosphorylation after BMMC activation (Fig. 8B), whilst lower ASA concentrations were uneffective in this respect.

4. Discussion

In this paper we show that ASA at high concentrations (10 mM) fully inhibited several functional characteristics of mast cells, i.e. mast cell degranulation, the release of TNF-α and of IL-6. In addition, the expression of the inducible COX-2 enzyme, both at the mRNA and protein level, was also inhibited, whilst the constitutive COX-1 was unaltered. The effects of ASA in mast cells are likely due to interference with the transcription factor NF-κB. ASA decreased the appearance of the NF-κB subunit p65 in the nucleus, whereas it prevented the disappearance of this subunit from the cytoplasm after activation of the cells. Kopp and Ghosh (1994) were the first to describe an inhibitory action of ASA and sodium salicylate on NF-κB activity in the human Jurkat T cell line and the mouse pre-B cell line, PD31 [3]. Similar findings were reported for human primary endothelial cells [23] and murine primary macrophages [24]. Other NSAIDs, including flurbiprofen [25], aminosalicylate [26], and ibuprofen [27] have also been shown to inhibit NF-κB activity. In human monocytes, however, ibuprofen was without effect on NF-κB [28]. Effects on alternative pathways may be responsible for inhibition by NSAIDs. Many studies have shown that the NSAID indomethacin does not directly affect the NF-κB pathway, but may indirectly display an inhibitory effect through activation of p38 MAP kinase [29]. In addition, cell type differences exist in the action of NSAIDs on the NF-κB signalling pathway. Indeed, hepatocytes were shown not to display inhibitory effects on the NF-κB signalling pathway by ASA, sodium salicylate, indomethacin, ibuprofen or a derivative of rofecoxib [30].

In mast cells the mode of action of ASA in inhibiting NF-κB activation turns out to be a stabilizing effect on the inhibitory protein IκB-α by blocking the phosphorylation of IκB-α and its subsequent degradation (present study). These findings are in agreement with results reported for other cells [3,24,31]. Other mechanisms may also apply, since flurbiprofen was shown to inhibit NF-κB activation independent of IκB-α phosphorylation and degradation [25]. The inhibitory effects of ASA and other NSAIDs on the NF-κB signalling pathway, as shown by others and us, may well explain the observed attenuation of TNF-α and IL-6 release from primary mast cells. Both genes contain a NF-κB consensus sequence site in the promoter region [24,32]. With respect to mast cell degranulation, protein synthesis is not strictly necessary for this response, and thus the NF-κB pathway may not have been involved in the inhibitory action of ASA. Alternatively, it has previously been demonstrated that NSAIDs, including ASA, disrupt gel-like regions of the plasma membrane and thereby affect nucleotide-protein and protein-protein interactions within the lipid bilayer [33]. This direct effect on mast cell membrane or on intracellular signalling pathways might account for the inhibition of mast cell degranulation.

The high concentrations of ASA, as used in our in vitro experiments described above, reflect situations that may very well occur in vivo after chronic administration of this drug. Serum concentrations have been reported to be as high as 2 mM in patients treated for chronic inflammatory diseases [34]. Moreover, the acidic nature of ASA results in accumulation in acidic body compartments, such as inflamed tissues, because of the increased lipophilicity of ASA under these conditions [35,36].

At lower concentrations (10^{-4}–10^{-7} M), we did not detect any inhibitory effect of ASA on TNF-α or IL-6 production, mast cell degranulation, COX-2 protein expression, and the NF-κB signaling pathway. It is at these concentrations that NSAIDs have been reported to inhibit COX enzyme activity [37] but not gene expression [38].

Considering these data, and also similar findings in literature [34], it appears that ASA most often has an inhibitory effect towards intracellular kinases and transcription factors alike, explaining its anti-inflammatory efficacy. However, we observed that 1 mM ASA upregulated the expression of the pro-inflammatory COX-2 protein in mast cells. It seems that this effect is not mediated at the transcriptional level since at this ASA concentration NF-κB activity was not dramatically affected. Moreover, mRNA

levels for COX-2 were not altered. This is in agreement with findings of Ferguson et al. [39], who observed an increase in COX-2 protein expression in kidney cortical collecting duct cells by the COX-2 selective drug NS-398, independently of any change in the level of COX-2 mRNA expression. Likewise, NS-398, and also indomethacin, were shown to increase COX-2 protein without any effect on COX-2 mRNA in fetal hepatocytes [40]. Thus, post-transcriptional or post-translational processes might be involved. This increase in expression of COX-2 may have serious consequences when NSAID levels fall below therapeutic (COX-2 inhibitory) concentrations.

We conclude that in mast cells ASA may display anti-inflammatory effects through a dose-dependent inhibition of the NF-κB pathway. This notion is corroborated by our observations that NaSal, from which it is known that it displays anti-inflammatory effects but is not an inhibitor of COX enzyme activity but does inhibit NF-κB [3,34], also inhibited cytokine release and COX-2 expression in our mast cells.

Since dysregulation of NF-κB is associated with many disease states such as AIDS, atherosclerosis, asthma, arthritis, cancer, diabetes, inflammatory bowel disease, muscular dystrophy, stroke, and viral infections [41], our findings may have clinical relevance with respect to treatment with ASA in these disease states.

We find it of particular interest that ASA, at a lower concentration (1 mM), was able to increase the expression of the proinflammatory COX-2 protein. This may aid to the understanding of the role of mast cells in aspirin-induced airway exacerbations in aspirin-sensitive asthmatics.

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