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Aspirin Prevents Apoptosis and NF-κB Activation Induced by H_2O_2 in HeLa Cells

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The classical pathway of nuclear factor-kappa B (NF-κB) activation by several inducers mainly involves the phosphorylation of IκBα by a signalsome complex composed of IκBα kinases (IKKα and IKKβ). However, in some cell types hydrogen peroxide (H_2O_2) has been shown to activate an alternative pathway that does not involve the classical signalsome activation process. In this study, we demonstrate that H_2O_2 induced NF-κB activation in HeLa cells through phosphorylation and degradation of IκB proteins as shown by immunblot analysis. Our studies reveal that a commonly used non-steroid anti-inflammatory drug, acetylsalicylic acid (aspirin) prevents H_2O_2-induced NF-κB activation in a dose-dependent manner through inhibition of phosphorylation and degradation of IκBα and IκBβ. Differential staining and DNA fragmentation analysis also show that aspirin preloading of HeLa cells also prevents H_2O_2-induced apoptosis in a dose-dependent manner with maximum efficiency at 10 mM concentration. Additionally, aspirin effectively prevents caspase-3 and caspase-9 (cysteinyl aspartate-specific proteases) activation by H_2O_2. These results suggest that NF-κB activation is involved in H_2O_2-induced apoptosis and aspirin may inhibit both processes simultaneously.

Keywords: Hydrogen peroxide; Aspirin; Caspases; Apoptosis; NF-κB

Abbreviations: H_2O_2, hydrogen peroxide; NF-κB, nuclear factor-kappa B; ROS, reactive oxygen species; AO, acridine orange; PI, propidium iodide; HO, Hoechst dye

INTRODUCTION

The transcription factor nuclear factor-kappa B (NF-κB) is involved in regulation of cell growth, inflammatory responses and apoptosis. NF-κB is sequestered in the cytoplasm in unstimulated cells and translocates into nucleus when activated by many distinct stimuli including proinflammatory cytokines, bacterial lipopolysaccharide (LPS), oxidative stress, UV radiation, viruses and viral proteins, double-stranded RNA and mitogens.[1–4] NF-κB is mainly composed of p65 and p50 dimers retained in the cytoplasm complexed with inhibitory proteins belonging to IκB family, such as IκBα or IκBβ, which inhibit the translocation of NF-κB into nucleus.[5–7] The classical activation pathway of NF-κB by proinflammatory cytokines and phorbol myristate acetate (PMA) involves the phosphorylation of two serine residues on the N-terminal domain of IκB proteins (Ser-32 and Ser-36 IκBα and Ser-23 and Ser-26 for IκBβ) which is mediated by an upstream macromolecular signalsome complex composed of the IκB kinases (IKKα and IKKβ) and NEMO/IKKγ (NF-κB essential modulator).[8–10] Phosphorylation of IκBα by the signalsome complex promotes the ubiquitination and degradation of IκBα which results in the translocation of NF-κB into the nucleus and target gene expression.[9,10]

Reactive oxygen species (ROS) is involved in the regulation of many cellular functions involving cell proliferation and apoptosis.[11] ROS such as hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$) has been proposed to act as second messenger in signal transduction pathways and the first transcription factor reported to respond directly to oxidative stress is NF-κB.[12–14] H_2O_2 has been demonstrated to directly activate NF-κB in some cell types[15–17] and

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proposed to act as a mediator of NF-κB activation by other agents.[18] In contrast to these studies, H_2O_2 has also been shown to inhibit cytokine induced NF-κB activation and inhibit signalsome complex activity through oxidative modification of a critical cysteine residue in the activation loop of IκB kinases.[19,20] Schoonbroodt *et al.* demonstrated that H_2O_2-induced NF-κB activation involves an alternative pathway that does not involve the serine phosphorylation and degradation of IκB proteins.[21] Interestingly a recent work proposed a discrepant result that H_2O_2 activates IκB kinases.[22] It seems clear that redox regulation of NF-κB activation pathways is complicated and cell-type specific.[23]

NF-κB modulates pro- and anti-apoptotic response of cells, as several cells that have constitutive and inducible NF-κB activity are resistant to apoptosis induced by chemotherapy, radiotherapy and TNFα treatment.[24–27] NF-κB confers resistance to apoptosis via increased expression of inhibitors of apoptotic proteins (cIAP 1 and cIAP 2) and via regulation of the proteins involved in cell cycle control.[28–30] However, NF-κB has also been demonstrated to exert a pro-apoptotic role in prostate cancer cell lines.[31,32]

Caspase (cysteinyl aspartate-specific protease) activation and enzymatic cleavage of their specific target proteins also constitute an important part of oxidative stress-induced apoptotic machinery. The two main executioner caspases; caspase-9 and caspase-3 have been reported to be involved in H_2O_2-induced apoptosis.[44]

Acetylsalicylic acid (aspirin) is a widely used analgesic and anti-pyretic drug (Fig. 1). Aspirin exerts its anti-inflammatory effects, at least in part by irreversible inhibition of cyclooxygenases and prostaglandin synthesis. Aspirin and its metabolite, sodium salicylate can inhibit activation of the NF-κB pathway as a result of their specific binding to IKKβ to reduce ATP binding *in vitro* and *in vivo*.[33,34] Furthermore, aspirin and sodium salicylate were shown to possess neuroprotection via inhibition of NF-κB activation that indicates a pro-apoptotic role of NF-κB in neuron cells.[35] Although aspirin has been demonstrated to inhibit NF-κB activation by various agents in many cell types, aspirin-induced NF-κB activation has also been proposed as a mechanism of apoptosis in colon cancer cells.[36] These results imply that effects of aspirin including cancer chemoprevention and neuroprotection are potentially through modulation of NF-κB activation in a cell-type specific manner.

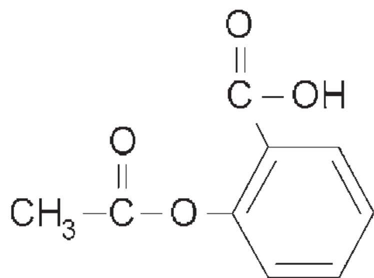

FIGURE 1 Molecular structure of aspirin.

In the present study, we investigated the effect of H_2O_2 on activation of NF-κB in HeLa cells and potential involvement of IκB proteins and modulation of H_2O_2-induced NF-κB activation by pretreatment with aspirin. H_2O_2-induced apoptotic response of HeLa cells and effect of aspirin on this process is also evaluated.

We present here that H_2O_2-induced NF-κB activation in HeLa cells involves phosphorylation and degradation of IκBα and to a lesser extent degradation of IκBβ. Our results show that aspirin prevents phosphorylation and degradation of IκBα and IκBβ and thereby H_2O_2-induced NF-κB activation. Aspirin also prevented H_2O_2-induced apoptosis indicating a crucial role for NF-κB activation in H_2O_2-induced apoptotic signaling. Furthermore, aspirin also prevents H_2O_2-induced caspase-3 and caspase-9 activation, which points out the potential crosstalk between NF-κB and caspase activation mechanisms. To our knowledge, this is the first study implying NF-κB as a mediator of cytoprotective function of aspirin against H_2O_2-induced apoptosis.

MATERIALS AND METHODS

Cell Line and Reagents

Human cervix carcinoma HeLa cells were obtained from HUKUK (Ankara, Turkey). Eagle's minimal essential medium (EMEM), Fetal bovine serum (FBS) and all materials such as H_2O_2, poly (dI–dC)–poly (dI–dC), albumin, acridine orange (AO), propidium iodide (PI), Hoechst dye (HO) and aspirin were obtained from Sigma (Darmstadt, Germany) unless otherwise indicated. Anti-p50, anti-p65, anti-IκBα, p-IκBα, anti-IκBβ, caspase-3 and caspase-9 antibodies were purchased from Santa Cruz Biotechnologies Inc. (Santa Cruz, CA, USA) NF-κB-specific oligonucleotides were from Promega (Madison, WI, USA).

Cell Culture and Treatments

HeLa cells were cultured in EMEM containing 2 mM glutamine with 10% FBS and penicillin/streptomycin (100 U/ml, respectively) in a humidified incubator at 37°C and 5% CO_2. At 90% confluency, cells were starved with EMEM plus 0.2% FBS for 12 h, preloaded with or without specified concentrations of aspirin in Tris–HCl, pH 7.4, medium was changed with fresh EMEM

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0.2% FBS and subsequently treated with or without H_2O_2 at indicated times.

Nuclear and Cytoplasmic Protein Extraction

Nuclear and cytoplasmic proteins were isolated as described before with minor modifications.[21] Briefly, cells were treated as indicated and washed with cold PBS, then scraped and harvested by centrifugation (300*g*, 5 min). They were resuspended in 1 ml of cold PBS and transferred to 1.5-ml microfuge tubes. After centrifugation at 13,000*g* for 30 s cells were resuspended in 1 ml of cold wash buffer [10 mM Hepes–KOH (pH 7.9), 20 mM KCl, 2 mM $MgCl_2$ and 0.1 mM EDTA]. Cells were then lysed by incubation for 30 s in 1 ml of cold buffer B [10 mM Hepes–KOH, (pH 7.9), 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), 0.5 mM PMSF, protease inhibitors and Nonidet P-40 (0.2%)]. After centrifugation at 13,000*g* for 30 s, supernatants containing cytoplasmic proteins were removed and stored at −70°C. The nuclear pellet was washed and nuclear protein isolation was carried out by incubation for 20 min on ice in a cold saline buffer [20 mM Hepes–KOH (pH 7.9), 1.5 mM $MgCl_2$, 0,2 mM EDTA, 650 mM NaCl, glycerol (25%, v/v), 1 mM DTT, 0.5 mM PMSF and protease inhibitors]. After centrifugation at 13,000*g* for 30 min at 4°C, supernatants containing nuclear proteins were removed and stored at −70°C. For caspase-3 and caspase-9 immunblots, cells were harvested and washed with phosphate-buffered saline and lysed on ice in a solution containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EGTA, 1 mM PMSF, 0.1% SDS, protease inhibitors and Nonidet P-40 (0.2%) and after cell lysis cell debris was removed by centrifugation 10 min at 13,000*g*. Protein concentrations were determined by Bradford reagent (Bio-Rad, Munich, Germany).

Electrophoretic Mobility Shift Assay

The EMSA method was performed as described before.[21] Briefly, the oligonucleotide probe was labelled with γ-^{32}P-dATP (3000 Ci/mmol) using T_4 polynucleotide kinase and then labelled oligonucleotide was purified on a Sephadex G-25 column. Five μg of nuclear proteins were incubated for 30 min at room temperature with 0.2 ng of ^{32}P-labelled oligonucleotide probe in gel shift binding buffer 1 μg of BSA and 1.25 μg of poly (dI–dC)–poly (dI–dC) in 20 mM Hepes–KOH, 75 mM NaCl, 1 mM EDTA, 5% (v/v) glycerol, 0.5 mM $MgCl_2$, 1 mM DTT (pH 7.9) (final volume 10 μl). DNA-protein complexes then resolved on a non-denaturating 6% polyacrylamide gel run for 3 h at 180 V. The gel was then dried and autoradiographed on Kodak X-ray film. The consensus sequences of the oligonucleotides used in this work were 5′-AGTTGAGGGGACTTTCCCAGGC-3′ and 3′-TCAACTCCCCTGAAAGGGTCCG-5′. For competition experiments unlabelled probe was added in excess (50 ×) in the binding buffer and for supershift experiment incubation with anti p65/p50 was performed before addition of ^{32}P-labelled oligonucleotide probe into the binding buffer.

Western Blot Analysis

IκBα, pIκBα, IκBβ, caspase-3 and caspase-9 were detected by Western blot analysis using specific antibodies (Santa Cruz, CA, USA) 30 μg cytoplasmic protein was resolved on 10% Tris–glycine SDS-PAGE gels and electro-transferred to PVDF membrane. The membranes were then incubated with specific primary and HRP-conjugated secondary antibodies. Proteins were finally analyzed using an enhanced chemiluminescence (ECL-Plus, Amersham Pharmacia Biotech, Freiburg, Germany). For detection of active caspase-3 and caspase-9 fragments, longer exposure times were used. For densitometric analysis, images were digitalized and relative band densities were determined by using Image-Pro Plus software (MediaCybernetics).

Determination of DNA Fragmentation and Apoptosis

DNA fragmentation was detected as described before with minor modifications.[37] Briefly HeLa cells (1×10^6 cells/well) were grown on 6-well plates, starved with EMEM plus 0.2% FBS for 12 h. After indicated treatments, cells were harvested by scraping, washed twice with ice cold PBS and lysed in lysis buffer [10 mM Tris–HCl (pH 8.0), 10 mM EDTA, and 0.2% Triton X-100] on ice for 40 min. Cells were subsequently centrifuged at 13,000*g* at 4°C for 10 min; supernatant was collected and transferred to a new tube. Supernatant was incubated with RNase A (200 μg/ml) at 37°C for 1 h and then incubated with Proteinase K (5 mg/ml) with 1% SDS solution at 50°C for 2 h. Soluble DNA was isolated by phenol–chloroform–isoamylalcohol extraction and ethanol precipitation. Vacuum dried DNA pellets were dissolved in TE buffer and resolved on 1.5% agarose gel for 2 h. DNA fragments were visualized by staining with ethidium bromide.

Quantification of Apoptosis

For the determination of apoptosis HeLa cells (1×10^5 cells/well) were grown on glass bottom dishes in DMEM with 10% FBS. After confluency, cells were incubated with DMEM plus 0.2% FBS for 12 h. After indicated treatments, cells were washed 3 times with PBS and then fixed with 2% paraformaldehyde and stained with AO, PI and HO and

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cell death/apoptosis were assessed as described before.[38] At least 400 cells were counted in 5 high-power fields using Olympus BX51 microscope and data were expressed as expressed as mean ± SEM of percent apoptosis.

Statistical Analysis

The data are given as the mean ± SEM. Significance testing was performed using one-way analysis of variance (ANOVA) followed by Bonferoni's *post hoc* test. Values of $p < 0.1$ and $p < 0.01$ were considered significant.

RESULTS

H_2O_2 Induces NF-κB Activation via Phosphorylation and Degradation of IκBα and Degradation of IκB-β

To investigate the effect of H_2O_2 on NF-κB activation, HeLa cells were grown to confluency on 6-well plates, starved for 12 h and treated with 600 μM H_2O_2 for indicated times. Five μg of protein from nuclear extracts was used for performing electrophoretic mobility shift assay (EMSA) for the assessment of NF-κB activation. H_2O_2 induces NF-κB activation with a maximum activation in 2 h (Fig. 2A). The specificity of NF-κB band was confirmed by competition experiments with an excess of unlabelled oligonucleotide and mutated oligonucleotide and supershift assays with antibodies directed against various members of Rel family indicating that composition of the bands are p50 homodimer and p50/p65 heterodimer (Fig. 2A).

To examine the effect of H_2O_2 on cytoplasmic IκB proteins, we followed the cytoplasmic levels of total and phosphorylated IκBα and total IκBβ. HeLa cells were grown to confluency on 6-well plates, starved for 12 h and treated with 600 μM H_2O_2. Cytoplasmic proteins were isolated as described in "Materials and methods" section, and analyzed by means of immunblot. H_2O_2 induces nearly total degradation of IκBα in 2 h similar to NF-κB activation.

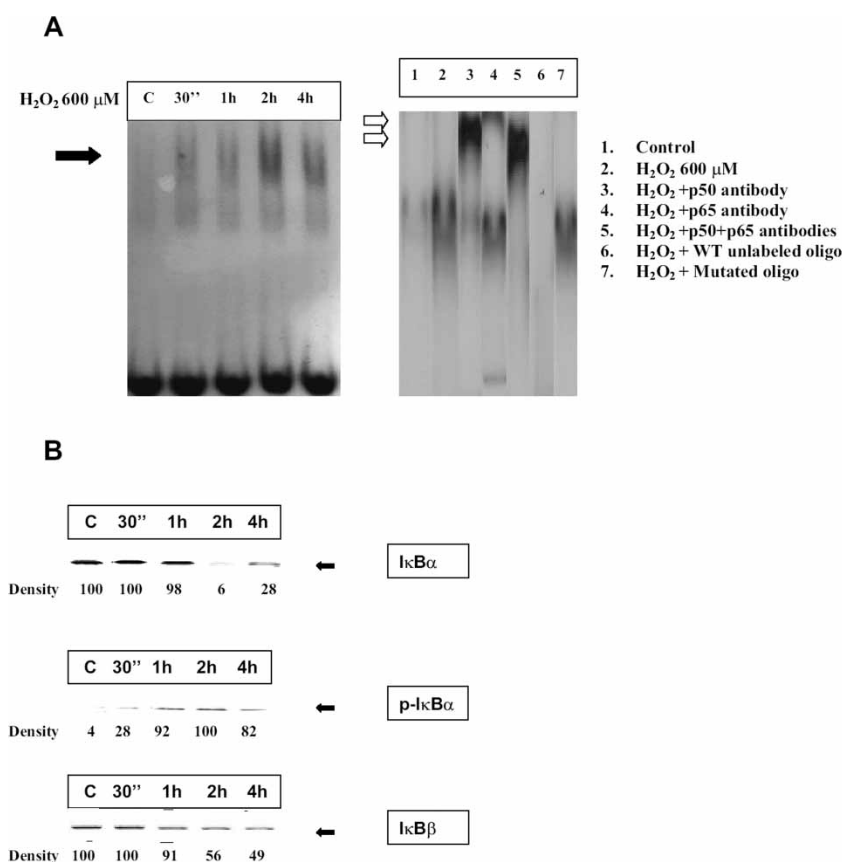

FIGURE 2 Effect of H_2O_2 on NF-κB induction and IκB proteins. (A) HeLa cells were grown on 6-well plates (1×10^6 cells/well) and treated with 600 μM H_2O_2 for indicated times. EMSAs performed using ^{32}P-labelled probe. Black arrow indicates the NF-κB complex. NF-κB bands are composed of p50/p50 and p65/p50 dimers as identified by supershift experiments. The specificity of binding was examined by competition with the unlabeled probe and mutated probe. Black arrow indicates the specific NF-κB complexes and open arrows indicate antibody supershifts. (B) HeLa cells 5×10^5 cells/well were stimulated with 600 μM H_2O_2 for indicated times and nuclear/cytoplasmic proteins were isolated as described before. Cytoplasmic proteins were loaded at equal amounts (30 μg) to SDS-PAGE gel and run at 100 V for 1.5 h. Proteins were blotted to PVDF membrane at 25 V o/n and then detected by using a polyclonal antibody to IκBα, IκBβ, and p-IκBα. (arrow indicates IκBα, p- IκBα and IκBβ bands, respectively).

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An increased level of serine 32-phosphorylated IκBα (p-IκBα) is observed in 1 h preceding degradation of IκBα. IκBβ degradation by H_2O_2 was less significant in contrast to IκBα (Fig. 2B), as determined by densitometric analysis. These results indicate that signalsome complex is involved in H_2O_2-induced NF-κB activation.

Aspirin Prevents H_2O_2-induced NF-κB Activation through Inhibition of Phosphorylation and Degradation of IκBα and IκBβ in a Dose-dependent Manner

In order to investigate, the effect of aspirin on H_2O_2-induced NF-κB activation, cells were preloaded with various concentrations of (0, 0.1, 1 and 10 mM) aspirin for 2 h and subsequently treated with 600 μM H_2O_2 for 2 h. Aspirin inhibited H_2O_2-induced NF-κB activation in a dose dependent manner with a maximum inhibition at 10 mM concentration (Fig. 3A).

We then followed the phosphorylation and degradation profiles of cytoplasmic IκB proteins (IκBα, p-IκBα, IκBβ) to evaluate the mechanism for the inhibitory effect of aspirin on H_2O_2-induced NF-κB activation. HeLa cells were pretreated with various concentrations of (0, 0.1, 1 and 10 mM) aspirin for 2 h and subsequently treated with 600 μM H_2O_2 for 2 h. Immunblot analysis of cytoplasmic proteins indicates that aspirin pretreatment resulted in a significant dose-dependent increase in the levels of total IκBα and total IκBβ and decrease in p-IκBα with the most significant effect at 10 mM concentration (Fig. 3B), as confirmed by densitometric analysis. These findings support that the inhibitory effect of aspirin on H_2O_2-induced NF-κB activation is mediated by blocking the phosphorylation and degradation of IκB proteins.

Aspirin Prevents H_2O_2-induced Apoptosis in a Dose-dependent Manner

NF-κB activation may have both pro- and anti-apoptotic effects. We evaluated the possible modulation of H_2O_2-induced apoptosis by aspirin via its inhibitory effect on NF-κB activation. HeLa cells grown on glass bottom dishes were treated with 600 μM H_2O_2 for 24 h alone or preloaded with 0, 0.1, 1, 10 mM aspirin for 2 h and then 600 μM H_2O_2 24 h. Cells were fixed and stained with AO, PI and HO for evaluation of apoptosis and data were expressed as mean ± SEM of percent apoptosis. Aspirin significantly blocks H_2O_2-induced apoptosis at 1 mM

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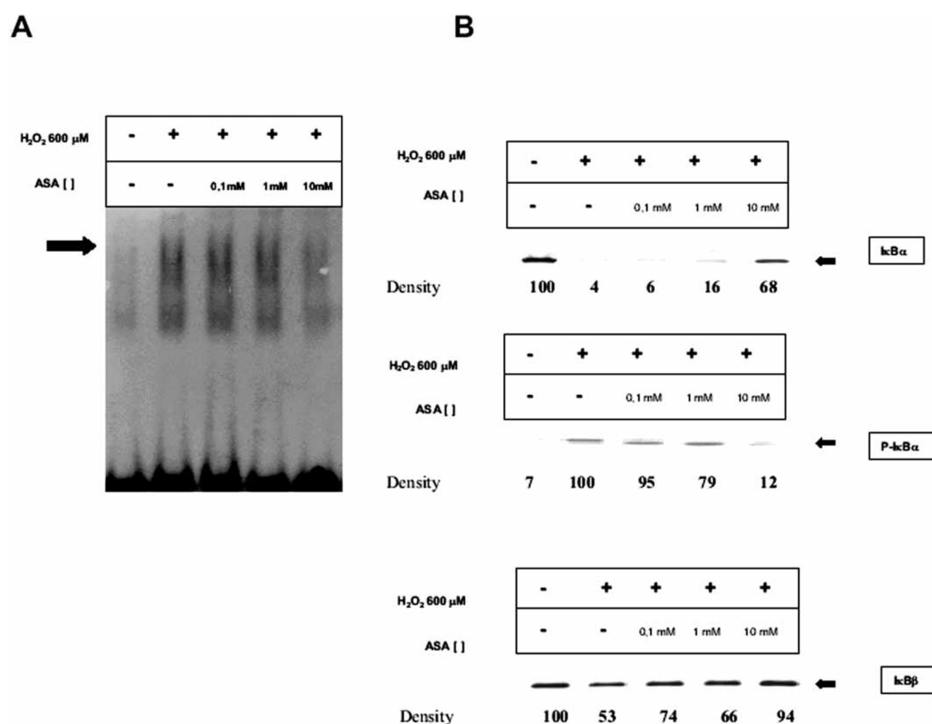

FIGURE 3 Effect of acetylsalicylic acid (aspirin) on H_2O_2 induced NF-κB activation and IκB proteins. (A) HeLa cells were grown on 6-well plates (1×10^6 cells/well) and treated with 600 μM H_2O_2 for 2 h after preloading with aspirin for 2 h at indicated concentrations. EMSAs performed using ^{32}P-labelled probe. Arrow indicates the specific NF-κB complexes. (B) HeLa cells (1×10^6 cells/well) were treated with 600 μM H_2O_2 for 2 h after preloading with aspirin for 2 h at indicated concentrations nuclear/cytoplasmic proteins were isolated as described before. Cytoplasmic proteins were loaded at equal amounts(30 μg) to SDS-PAGE gel and run at 100 V for 1.5 h. Proteins were blotted to PVDF membrane at 25 V o/n and then detected by using a polyclonal antibody to IκBα, IκBβ and p-IκBα. (arrow indicates IκBα, p-IκBα and IκBβ bands, respectively).

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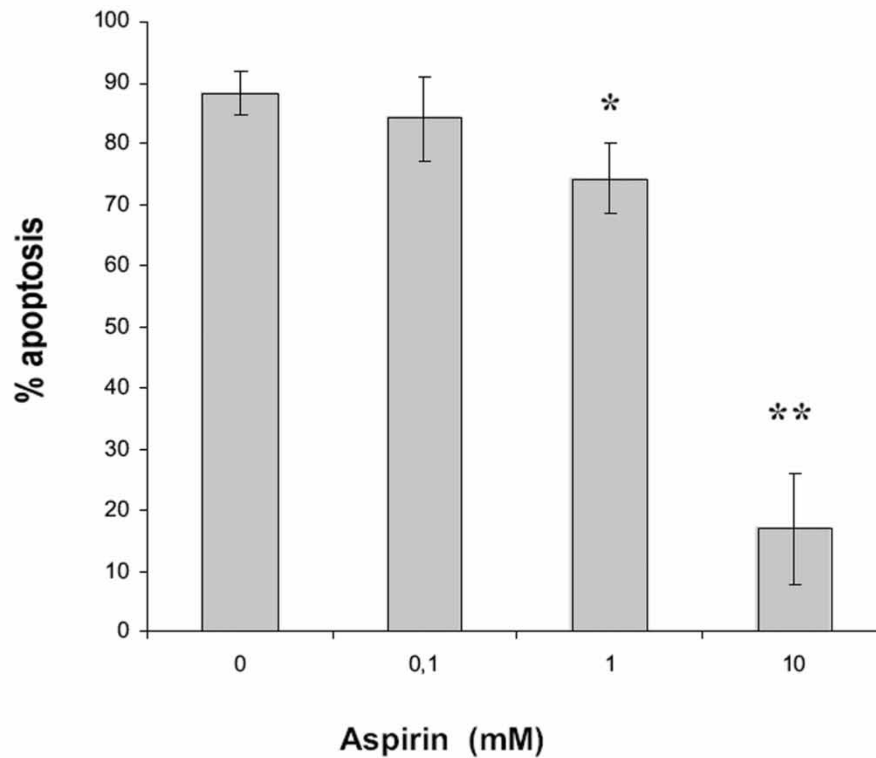

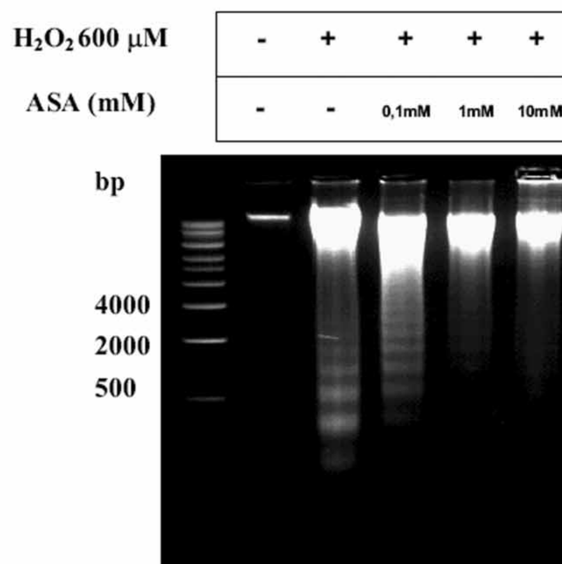

FIGURE 4 Aspirin prevents H_2O_2-induced apoptosis in HeLa cells. (A) HeLa cells were treated with 600 μM H_2O_2 with or without pretreatment with aspirin at indicated concentrations, fixed and stained with AO, HO and PI. Early/late apoptotic cells were scored as described in "Materials and methods" section. In each case, at least 400 cells were counted in five different microscopic fields. Data are expressed as mean ± SEM of percent apoptosis and representative of the from six experiments. *$p < 0.1$, **$p < 0.01$.

(74.2 ± 5.78, $p < 0.1$) and 10 mM (16.84 ± 9.2, $p < 0.01$) concentrations (Fig. 4A).

One of the hallmarks of apoptosis is the endonuclease mediated degradation of chromatin giving rise to DNA laddering. For the assessment of DNA integrity, were treated with 600 μM H_2O_2 for 24 h alone or preloaded with 0, 0.1, 1, 10 mM aspirin for 2 h and then 600 μM H_2O_2 24 h and

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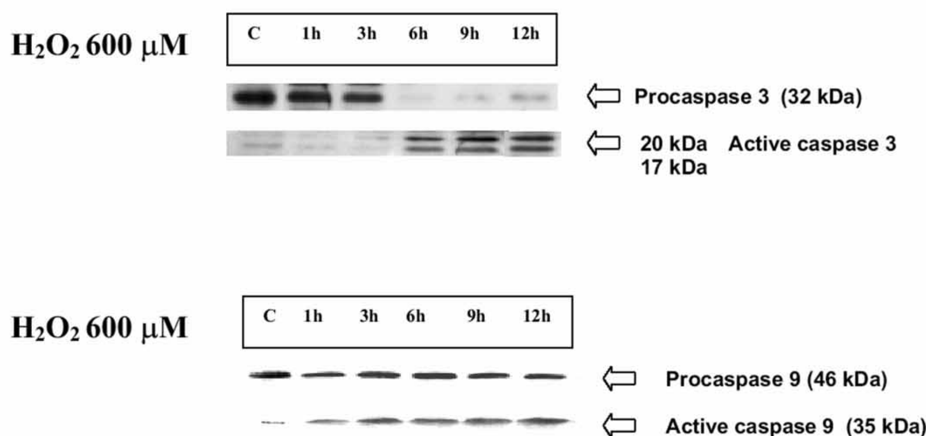

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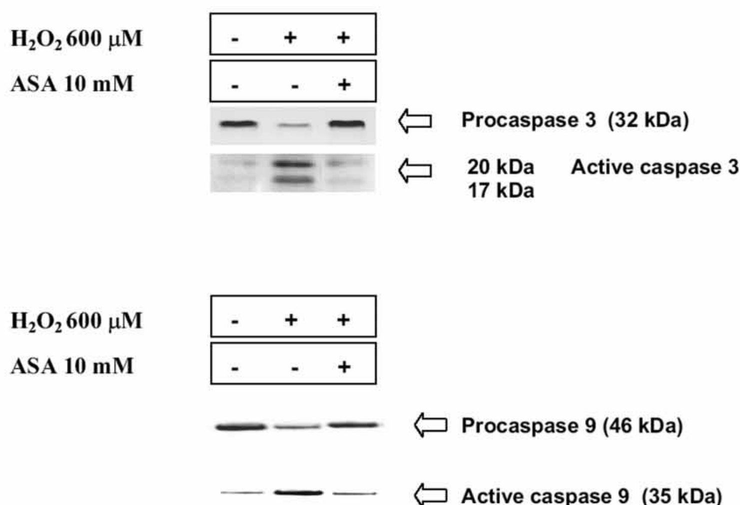

FIGURE 5 Aspirin prevents H_2O_2-induced caspase activation in HeLa cells. (A) HeLa cells were grown on 6-well plates (1×10^6 cells/well) and treated with 600 μM H_2O_2 for indicated times. Total proteins were loaded at equal amounts (40 μg) to SDS-PAGE gel and run at 100 V for 1.5 h. Proteins were blotted to PVDF membrane at 25 V o/n and then detected by using polyclonal antibodies to caspase-3 and caspase-9. (arrows indicate procaspase-3/active caspase-3 fragments and caspase 9/active caspase 9 fragments, respectively). (B) HeLa cells (1×10^6 cells/well) were treated with 600 μM H_2O_2 for 6 h after preloading with or without 10 mM aspirin for 2 h and total proteins were loaded at equal amounts(30 μg) to SDS-PAGE gel and run at 100 V for 1.5 h. Proteins were blotted to PVDF membrane at 25 V o/n and then detected by using a polyclonal antibodies to caspase-3 and caspase-9 (arrows indicate procaspase-3/active caspase-3 fragments and caspase 9/active caspase 9 fragments, respectively).

DNA was isolated as described in "Materials and methods" section. Treatment of cells with 600 μM H_2O_2 for 24 h results in DNA laddering indicating the fragmentation of genomic DNA in the process of apoptosis. Pretreatment with aspirin prevents H_2O_2-induced DNA laddering in a dose dependent manner with the most impressive effect at 10 mM concentration (Fig. 4B). Aspirin alone does not have any effect for apoptosis (data not shown). All these results support the role of NF-κB activation in H_2O_2-induced apoptosis and potential inhibitory effect of aspirin in both processes.

Aspirin Prevents H_2O_2-induced Caspase Activation

Caspase-3 and caspase-9 are two main executioners of apoptosis induced by oxidative stress; therefore we investigated the possible modulation of H_2O_2-induced caspase activation by aspirin. Treatment of cells with 600 μM H_2O_2 leads to activation of caspase

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9 and caspase-3 in a sequential manner since active caspase-9 fragments were detected even at 1 h of H_2O_2-treatment and active caspase 3 fragments were detected at 6 h of H_2O_2-treatment (Fig. 5A). Immunblot analysis demonstrated that 10 mM aspirin preloading of HeLa cells down-regulates protein levels of active forms of caspase -9 and caspase-3 (Fig. 5B).

DISCUSSION

The inducible eukaryotic transcription factor NF-κB regulates the expression of a number of genes involved in inflammation, proliferation and apoptosis. NF-κB can be activated by many diverse stimuli via phosphorylation of the two major forms of IκBs, IκBα and IκBβ at their N-terminal serines, followed by their degradation and NF-κB activation.[8–10] ROS both may either act as second messengers for NF-κB activation[12–14] or directly induce its activation in a cell-specific manner.[15–17] Recently, H_2O_2 has been demonstrated to stimulate serine/threonine kinases IKKα and IKKβ, which are components of the NF-κB activation signalsome.[22] Redox regulation of NF-κB activation may not always follow the classical cytokine/mitogen pathway described above. Oxidative stress has been shown to activate NF-κB through phosphorylation of amino-terminal Tyr42 of IκBα and its subsequent degradation in a T lymphocytic cell line.[21] Involvement of tyrosine kinases has been shown to activate an atypical mechanism of NF-κB activation during reoxygenation stress in microvascular endothelial cells.[39] Therefore, ROS-induced NF-κB activation is mediated through multiple signalling pathways and different tyrosine and serine/threonine kinases. This type of complex regulation network may be accepted as a naive way of differential gene expression via activation of different NF-κB dimers by each signalling cascade. In contrast, redox active molecules and H_2O_2 has been shown to inhibit IKK activation via oxidative modification of critical cysteine residues in the activation loop of these enzymes.[19,20]

In our experimental system, 600 μM H_2O_2 induces NF-κB activation, phosphorylation and degradation of IκBα and in a lesser extent degradation of IκBβ in 2 h. Proinflammatory cytokines and mitogens activate NF-κB in a shorter period of time, usually not more than 30 min.[1–4] One of the most important findings by Kamata *et al.* is that H_2O_2 activates IKKs interdependently in contrast to their sequential activation pattern by cytokines.[22] This differential degradation profiles of IκBα and IκBβ may be due this interdependent activation manner of IKKα and IKKβ.

Aspirin is a widely used non-steroid anti-inflammatory drug used mainly for its analgesic, anti-pyretic and anti-thrombosis properties. Aspirin acts partly through inhibition of cyclooxygenases (COX-1 and COX-2). Additionally, aspirin has been demonstrated to interfere with signalsome complex activation[33,34] and silica induced NF-κB activation.[40] In contrast, aspirin has been shown to induce apoptosis activate via activation of NF-κB and in a colon cancer cell line.[36] Our results indicate that aspirin prevents NF-κB activation via inhibiton of phosphorylation and degradation of IκBα and degradation of IκBβ, which confirms IKK signalsome complex as a target for aspirin.

Recent studies focuses on the pro- or anti-apoptotic role of NF-κB that mediates the survival of cells. Regulation of apoptotic behavior by NF-κB either in pro-apoptotic or anti-apoptotic manner is determined by the nature of the apoptotic stimuli and overexpression of a transdominant negative form of IκBα protects against H_2O_2-induced cytotoxicity.[41] Inhibition of inducible or constitutive NF-κB activation confers sensitivity to apoptosis-inducing therapies such as TNFα in some cancer cell types,[24–27] but controversial results has also been observed.[31,32] Indeed, aspirin prevents neural cell death via inhibition of NF-κB activation that indicates a pro-apoptotic role for NF-κB in neural cells.[35] Aspirin has been demonstrated to protect against oxidative stress in endothelial cells,[42] our results clearly show that aspirin prevents H_2O_2-induced apoptosis in HeLa cells at similar concentrations and incubation times that it confers its inhibitory effect on NF-κB activation.

H_2O_2-induced apoptosis has been shown to require the mitochondrial ROS production and NF-κB activation in Jurkat cells,[43] in consistency with our observations. Thus, the apoptosis-inducing ability of H_2O_2 depends on NF-κB activation and aspirin may be used a potential modulator of all these processes. We have further investigated the involvement of caspase-3 and caspase-9 activation in H_2O_2-induced apoptosis in HeLa cells. In our experimental system, 600 μM H_2O_2 induces caspase-9 activation in 1h and caspase-3 activation in 6 h. Aspirin pretreatment prevents both H_2O_2-induced caspase-3 and caspase-9 activation. As a conclusion, it is still an unavoidable issue to identify exact molecular mechanisms involved in H_2O_2-induced NF-κB activation at upstream of signalsome activation and specific target gene expression as well as potential crosstalk between NF-κB and caspase activation pathways.

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