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Salicylate Regulates Cyclooxygenase-2 Expression through ERK and Subsequent NF-κB Activation in Osteoblasts

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The expression of cyclooxygenase-2 (COX-2) is a characteristic response to inflammation and can be inhibited with sodium salicylate. TNF-α plus IFN-γ can induce extracellular signal-regulated kinase (ERK), IKK, IκB degradation and NF-κB activation. The inhibition of the ERK pathway with selective inhibitor, PD098059, blocked cytokine-induced COX-2 expression and PGE_2 release. Salicylate treatment inhibited COX-2 expression induced by TNF-α/IFN-γ and regulated the activation of ERK, IKK and IκB degradation and subsequent NF-κB activation in MC3T3E1 osteoblasts. Furthermore, antioxidants such as catalase, N-acetyl-cysteine or reduced glutathione attenuated COX-2 expression in combined cytokines-treated cells, and also inhibited the activation of ERK, IKK and NF-κB in MC3T3E1 osteoblasts. In addition, TNF-α/IFN-γ stimulated ROS release in the osteoblasts. However, salicylate had no obvious effect on ROS release in DCFDA assay. The results showed that salicylate inhibited the activation of ERK and IKK, IκB degradation and NF-κB activation independent of ROS release and suggested that salicylate exerts its anti-inflammatory action in part through inhibition of ERK, IKK, IκB, NF-κB and resultant COX-2 expression pathway.

Key Words: Salicylate, COX-2, Osteoblast

INTRODUCTION

Prostanoids (prostaglandins and thromboxane A_2) are the metabolic products of the membrane phospholipid arachidonic acid via the COX pathway. Presently, 2 forms of COX enzyme are recognized: the constitutive enzyme COX-1, which is normally expressed in most tissues, and COX-2, which is induced in many cell types in response to various stimuli, including cytokines (Smith et al, 1996).

A number of cytokines are expressed in osteoblasts. These cytokines exert their actions directly on the osteoblast or modulate the expression and release of other mediators, such as prostanoids. Many inflammatory mediators use NF-κB as one of their mechanisms of induction and perpetuation (Yamamoto et al, 1995; Barnes & Karin, 1997). COX-2 is induced by inflammatory cytokines such as tumor necrosis factor-α, which are produced in bone inflammation, via the transcription factor NF-κB (Yamamoto et al,1995).

The anti-inflammatory actions of aspirin and its metabolite, sodium salicylate, have been attributed in part to inhibition of prostaglandin synthesis via inhibition of COX activity (Flower, 1974). Sodium salicylate and aspirin have been shown to inhibit activation of nuclear factor-κB (NF-κB), which is elicited in response to inflammatory agents such as lipopolysaccharide, tumor necrosis factor-α and interleukin-1β (Kopp & Ghosh, 1994). The inhibition, which is ascribed to the ability of salicylate to prevent phosphorylation and subsequent degradation of IκBα (Schwenger et al, 1996; Yin et al, 1998), demonstrates that salicylate specifically inhibits IκB kinase-β activity in vivo and in vitro. IκB kinase-β catalyzes the transfer of phosphate moieties from ATP to IκB, thereby allowing activation of NF-κB and triggering expression of genes involved in the pathogenesis of inflammatory responses.

Mitogen-activated protein kinases (MAPK) play a role in mediating intracellular signal transduction and regulating cytokine production by mononuclear cells in response to a variety of extracellular stimuli (Shapiro & Dinarello, 1995; Shaoiro & Dinarello, 1997). In response to appropriate stimuli, MAPKs are activated by phosphorylation on threonine and tyrosine residues (Kozawa et al, 1999). ERK has been classically associated with growth- and differentiation-inducing signals.

Studies have suggested that the COX gene is regulated at multiple levels: transcriptional, post-transcriptional, and post-translational. However, the role of MAPKs cascades in the control of COX expression has not yet been completely defined.

In this study, we investigated whether the MAPK signaling pathway is involved in the regulation of COX and PGE_2 expression, and whether salicylate influences MAPK activity in osteoblasts. We show that sodium salicylate in-

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ABBREVIATIONS: COX, cyclooxygenase; ERK, extracellularly regulated kinase; PGE_2, Prostaglandin E_2.

hibits MEK1-dependent induction of NF-κB DNA-binding activity and NF-κB-mediated COX gene expression. The findings provide further insights into the mechanisms involved in the action of anti-inflammatory drugs.

METHODS

Materials

Murine recombinant TNF-α and interferon-γ were purchased from Genzyme Corp and R&D Systems. Alpha-MEM, FBS, and tissue culture reagents were obtained from Life Technologies, Inc. [α-^{32}P]-dCTP (10 mCi/ml) and [γ-^{32}P]-ATP (10 mCi/ml) were purchased from DuPont NEN. Phospho-p42/44 MAPK (Thr^{202}/Tyr^{204}) E10 monoclonal antibody was obtained from New England Biolabs, and COX-2 antibody from Calbiochem-Novabiochem Corp. Sodium salicylate (Sigma Chemical Co) was dissolved and diluted with culture medium. The inhibitor, PD098059 (Calbiochem-Novabiochem Corp), which specifically blocks the ERK pathway, was used from a stock solution (25 mM) prepared in DMSO.

Cell culture

The clonal mouse osteoblastic cell line, MC3T3E1 cell, was maintained in α-MEM supplemented with 10% FBS, penicillin G (100 U/ml), and streptomycin (100 μg/ml).

PGE_2 assay

For determination of PGE_2 synthesis, aliquots were removed from culture medium (1 ml) of 3×10^5 MC3T3E1 cells, and subjected to radioimmunoassay for PGE_2, as described elsewhere (Yosipovitch et al., 1995).

Immunoblot analysis

Immunoblot analysis was performed essentially as described previously (Schwenger et al, 1996). Briefly, whole-cell lysates were prepared in a buffer containing 1% Nonidet P-40, 50 mM HEPES (pH 7.5), 100 mM NaCl, 2 mM EDTA, 1 mM pyrophosphate, 10 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 100 mM sodium fluoride. Equal amounts of lysates were subjected to sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis and then transferred to Immobilon-P membranes (Millipore) in transfer buffer [25 mM Tris, 192 mM glycine, 20% (vol/vol) methanol]. Membranes were first rinsed in Tris-buffered saline [TBS: 10 mM Tris (pH 7.4), 150 mM NaCl] and then blocked for overnight at room temperature in TBS-5% bovine serum albumin (BSA). The anti-IκBα antibody at a dilution of 1 : 200 in TBS-5% BSA was used. The anti-phospho-IκBα, anti-phospho-p38 MAPK, and anti-IκBβ antibodies were each used at a dilution of 1 : 1000 in TBS-5% BSA. Antibody-antigen complexes were detected with the aid of horseradish peroxidase-conjugated protein A or horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Bio-Rad) and a chemiluminescent substrate development kit (Kirkegaard & Perry Laboratories). For IκBα blots, equal loading was ascertained by the presence of an ~70-kDa nonspecific band recognized by the anti-IκBα antibody (not shown).

Electrophoretic mobility shift assay (EMSA)

The nucleus from various agents-treated MC3T3E1 cells was extracted according to modification of the procedure described by Dignam et al (Dignam et al, 1983). The cells were washed twice with ice-cold PBS and lysed with hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM PMSF, 0.5 mM dithiothreitol, 10 μg/ml aprotinin, 20 μM pepstatin A, 100 μM leupeptin). After centrifugation at 1000×g, the nuclear pellets were resuspended in extraction buffer [20 mM HEPES, pH 7.9, 25 %(v/v) glycerol, 0.4 M KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.2 mM PMSF, and 0.5 mM dithiothreitol] and incubated on ice for 10 min. The nuclear proteins in the supernatant were recovered after centrifugation at 15,000 ×g, quantified by a BCA protein assay kit (Sigma Co., Saint Louis, MO) and used to carry out EMSA. To measure the activation of transcription factors including NF-κB, the oligonucleotide probes of NF-κB containing the IgG chain binding site (NF-κB: 5'-CCG GTT AAC AGA GGG GGC TTT CCG AG-3') were used. Two complementary strands of the oligonucleotides were annealed and labeled with [α-^{32}P]-dCTP using random primer labeling kit (rediprime, Amersham Life Science, England). Nuclear extracts (5 μg) with 2~5 ng of the radiolabelled NF-κB or AP-1 probes (50,000~100,000 cpm/ng) were reacted. The reaction was performed in the presence of 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM dithiothreitol, 4% glycerol (final volume: 25 μl) at room temperature for 30 min. The reaction products were then subjected to 4% polyacrylamide gel electrophoresis in 0.5×TBE buffer (50 mM Tris-HCl, pH 8.5, 50 mM borate, and 1 mM EDTA). Gels were dried under vacuum for 1 hr, and DNA binding activity for NF-κB was measured by using PhosphoImager analyzer (BAS, Fuji Co, Japan).

Immunoprecipitation and in vitro kinase assay

Treated cell cultures were washed twice with ice-cold PBS and lysed with lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 12 mM β-glycerophosphate, 5 mM EGTA, 0.5% deoxycholate, 3 mM DTT, 10 mM NaF, 1 mM Na_3VO_4, 2 μM leupeptin, 20 M aprotinin, and 1 mM PMSF). After 30 min on ice, cell lysates were cleared by centrifugation at 12,000 ×g for 20 min. The protein concentration in each sample was quantified by the Bradford method, and immunoprecipitation was performed by incubating 200 μl of the lysates with 2 μg of anti-IKKβ antibody (Santa Cruz Biotechnology) for 1 hr, and then adding 20 μl of protein A-agarose. After incubation for 1 hr at 4°C with end-over-end mixing, the immunocomplex was recovered by centrifugation and washed twice with washing buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EGTA, 2 mM DTT, and 1 mM PMSF). Kinase activity in 55 μl assay buffer (20 mM Tris-HCl, pH 7.5, and 20 mM $MgCl_2$) was assayed by incubating for 20 min at 37°C in the presence of 2 μg substrate (GST-IκBα), 30 μM ATP, and 20 μCi [γ-^{32}P]-ATP. And then, proteins were resolved by SDS-PAGE, and gels were dried and subjected to autoradiography. The relative activity of IKK was quantified by measuring the radioactivity of 32p incorporated into GST-IκBα. A PhosphorImager (Molecular Dynamics, Sunnyvale, CA, U.S.A.) was used to quantify band intensity.

Statistical evaluation

Values are expressed as mean±S.E.M of at least four experiments. Student's *t*-test was used to assess the statistical significance of the differences, a *P* value of less than 0.05 being considered statistically significant.

RESULTS

TNF-α and IFN-γ stimulate COX-2 expression and PGE_2 release in osteoblasts

Murine MC3T3E1 cells were chosen to investigate the effect of inflammatory cytokines on cyclooxygenase (COX), especially COX-2 expression, since they are established from newborn mouse calvaria and differentiate into osteoblasts, showing calcification in vitro similar to primary osteoblasts. When MC3T3E1 cells were incubated with TNF-α (10 ng/ml) in the presence or absence of IFN-γ (100 U/ml) for 24 hr, the combined cytokines (TNF-α/IFN-γ) led to significant increase in COX-2 protein (Fig. 1A). IFN-γ by itself had no apparent effects, whereas TNF-α stimulated the expression of COX-2. However, TNF-α (10 ng/ml) in the presence of IFN-γ (100 U/ml) had a synergistic effect on the induction of COX-2 expression in MC3T3E1 cells. COX-1 levels were not affected by treatment with either TNF-α (10 ng/ml) or IFN-γ (100 U/ml) alone or together. To elucidate the molecular basis for sodium salicylate-mediated COX-2 inhibition, the cells were first treated with TNF-α (10 ng/ml) and/or IFN-γ (100 U/ml). When these cells were subsequently treated with salicylate, a dose-dependent decrease of TNF-α and IFN-γ-induced COX-2 protein expression was observed (Fig. 1B). The decrease in salicylate-mediated COX-2 protein levels resulted in reduced PGE_2 release (Fig. 1C).

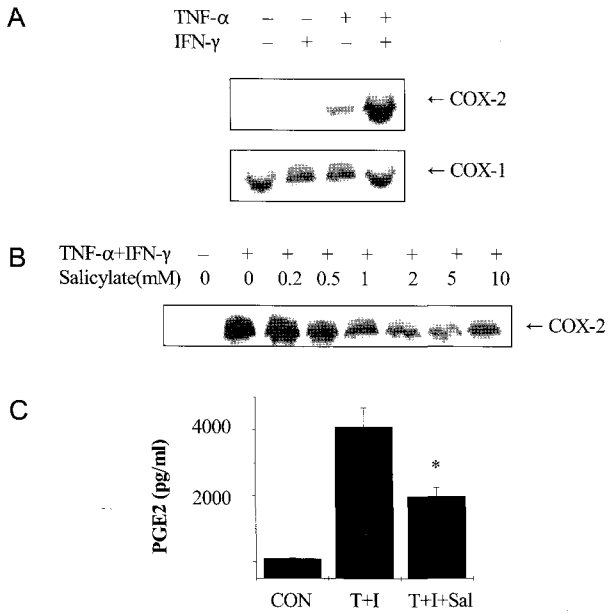

Fig. 1. Salicylate regulates TNF-α/IFN-γ-stimulated COX-2 expression and PGE_2 release in MC3T3E1 osteoblasts. (A) Cells were treated with IFN-γ (50 U/ml) or TNF-α (10 ng/ml) or TNF-α+IFN-γ for 8 h. The cell extracts were subjected to Western blot analysis using antibodies specific toward COX-2 (1; control, 2; IFN-γ, 3; TNF-α, 4; TNF-α+IFN-γ). (B) Cells were incubated with TNF-α (10 ng/ml) and IFN-γ (50 U/ml) in the presence (0, 0.2, 0.5, 1, 2, 5 and 10 mM) or absence of salicylate for 8 h. Then, the cell extracts were subjected to western blot analysis using antibodies specific against COX-2. (C) Cells were treated with TNF-α/IFN-γ for 12 h in the presence (10 mM) or absence of salicylate. And the culture media were collected and the amount of PGE_2 was measured by PGE_2 assay kit, as described in Materials and Methods. Data show means±S.E.M of four experiments. *, $P<0.05$ vs. TNF-α+IFN-γ-treated cells.

To evaluate whether the ERK pathway is involved in the regulation of COX induction, we examined the ability of salicylate to regulate TNF-α and IFN-γ-induced ERK activation in MC3T3E1 osteoblasts. As shown in Fig. 2A, the combination of these two cytokines induced a rapid and transient phosphorylation of ERK1 and 2, which increased within 10 min and then returned to baseline after 30 min. To demonstrate that the observed ERK phosphorylation correlated with changes in kinase activity, the lysates were immunoprecipitated with ERK1 antibody and in vitro kinase assays were performed using MBP as a substrate. A significant increase in ERK1 kinase activity was noted at 10 min of treatment in parallel with ERK phosphorylation. Treatment of MC3T3E1 cells with salicylate (10 mM) in the presence of TNF-α and IFN-γ attenuated the ERK 1 and 2 phosphorylation and reduced ERK1 kinase activity (Fig. 2A and B). Next, to address the role of MEK1

Salicylate regulates TNF-α and IFN-γ-activated ERK activation in MC3T3E1 osteoblasts

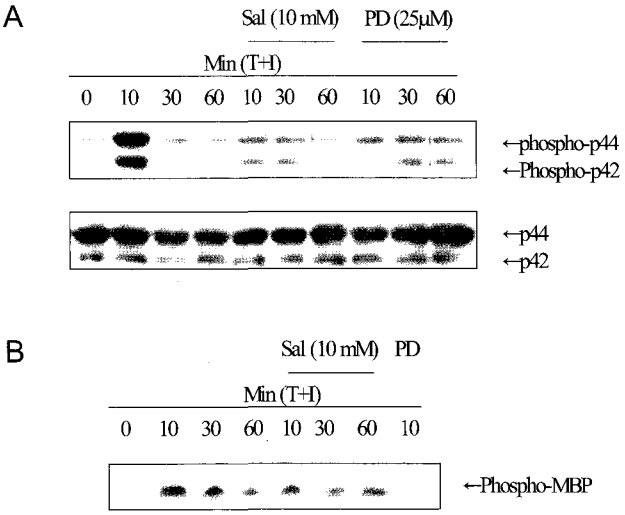

Fig. 2. Salicylate inhibits TNF-α/IFN-γ-induced ERK activation in MC3T3E1 osteoblasts. Cells were exposed to TNF-α (10 ng/ml)/IFN-γ (50 U/ml) for various periods (0, 10, 30 and 60 min) in the presence (10 mM) or absence of salicylate or PD098059 (25 μM). (A). The proteins were then subjected to immunoblot analysis, using antibodies specific for the active (phosphorylated) form of ERK. Parallel blots were run with anti-total ERK antibody, which served as controls. (B) Endogenous ERK MAP kinase activity was examined by immunocomplex assays, as described in Materials and Methods. These are representative results from four independent experiments.

in the cytokine-mediated induction of COX-2, MC3T3E1 cells were treated with the specific MEK1 inhibitor PD090859, and the levels of ERK and ERK kinase activity were found to be reduced in TNF-α and IFN-γ-treated cells.

PD098059 also has a regulatory effect on the TNF-α and IFN-γ-induced COX-2 expression in MC3T3E1 osteoblasts

To examine the effect of PD098059, a specific MEK 1 inhibitor, on TNF-α and IFN-γ-induced COX-2 expression, MC3T3E1 cells were treated with TNF-α (10 ng/ml) and IFN-γ (100 U/ml) in the presence (0, 6.25, 12.5 or 25 μM) or absence of PD098059. As seen in Fig. 3A, a dose-dependent decrease in cytokine-mediated COX-2 protein expression was induced by the MEK 1 inhibitor. Next, to verify whether the decrease of cytokines-induced COX-2 protein levels by PD098059 resulted in decreased PGE_2 release, PGE_2 levels were measured. As expected, PD098059 dramatically inhibited the PGE_2 release in the TNF-α and IFN-γ-treated MC3T3E1 cells (Fig. 3B). This result suggests a role for ERK signaling pathway in the regulation of COX-2 expression by IFN-γ and TNF-α in MC3T3E1 osteoblasts, in agreement with observations made in other cell types (Chen et al, 2001).

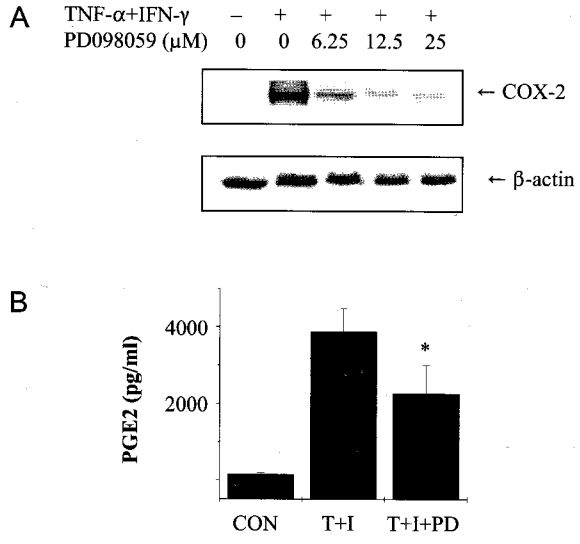

Fig. 3. PD098059, a specific MEK1 inhibitor, inhibits TNF-α and IFN-γ-induced COX-2 expression in MC3T3E1 osteoblasts. (A) Cells were incubated with TNF-α (10 ng/ml) and IFN-γ (50 U/ml) in the presence (0, 6.25, 12.5 and 25 μM) or absence of PD098059 for 8 h. Then, the cell extracts were subjected to Western blot analysis using antibodies specific towards COX-2. (B) Cells were treated with TNF-α/IFN-γ for 12 h in the presence (25 μM) or absence of PD098059. And the culture media were collected and the amount of PGE_2 was measured by PGE_2 assay kit. Data show means ± S.E.M. of four experiments. *, $P < 0.05$ vs. TNF-α + IFN-γ -treated cells.

Salicylate has a regulatory effect on TNF-α and IFN-γ-induced NF-κB DNA-binding activity and Ikb protein levels in osteoblasts

It has been known that NF-κB activation is an essential process for the induction of COX-2 expression (Fiebich et al, 2000). Thus, we examined the effects of salicylate and PD098059 on cytokine-induced NF-κB DNA-binding activity by EMSA. As shown in Fig. 4A, incubation of MC3T3E1 cells with TNF-α plus IFN-γ generated prominent NF-κB complex binding. In the cells treated with salicylate, however, the nuclear level of NF-κB DNA-binding activity was significantly reduced. Immunoblot analysis of IκB proteins in the cytosol of MC3T3E1 cells indicated that the salicylate-induced inhibition of NF-κB activation was associated with suppression of IκB degradation (Fig. 4B).

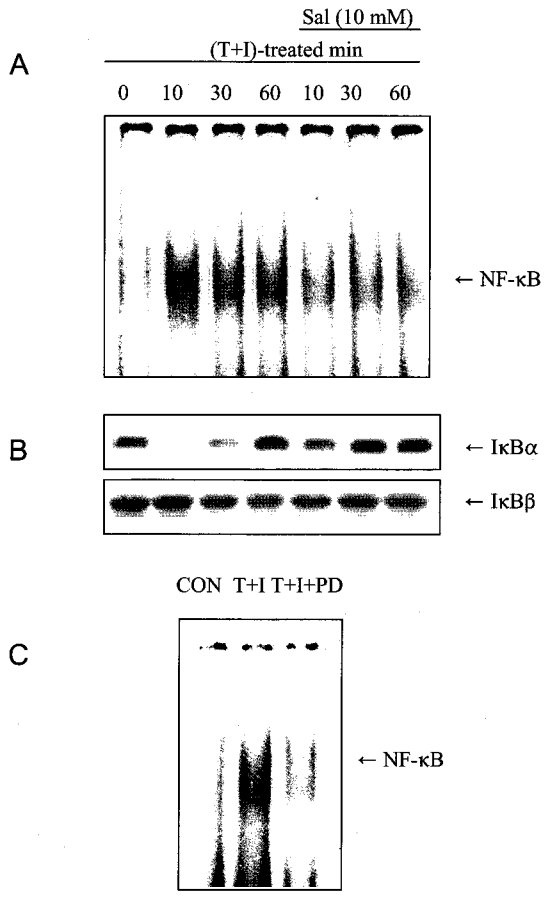

Fig. 4. Salicylate inhibits TNF-α/IFN-γ-induced NF-κB activation in MC3T3E1 osteoblasts. (A) Cells were exposed to TNF-α (10 ng/ml) and IFN-γ (50 U/ml) in the presence (10 mM) or absence of salicylate for various time intervals (0, 10, 30 or 60 min). Nuclear proteins were analyzed in an EMSA with α-^{32}P-labelled oligonucleotide encompassing the NF-κB binding site. And then, the cytosolic extracts were examined by protein immunoblotting for IκB-α and IκB-β degradation. (B) Cells were exposed to TNF-α (10 ng/ml) and IFN-γ (50 U/ml) in the presence (25 μM) or absence of PD098059 for 30 min. Nuclear proteins were analyzed in an EMSA with α-^{32}P-labelled oligonucleotide encompassing the NF-κB binding site. These data shown are a representative of two experiments (A to B).

Stimulation of the cells with TNF-α plus IFN-γ induced significant degradation of IκBα and IκBβ within 30 min. However, the addition of salicylate blocked the degradation of both IκB proteins, suggesting that salicylate inhibited an intermediate step in the signal pathway toward NF-κB activation. In addition, PD098059 inhibited NF-κB activation and IκB degradation in TNF-α/IFN-γ-treated MC 3T3E1 osteoblasts (Fig. 4C).

Salicylate has a regulatory effect on TNF-α and IFN-γ-induced IKK activation in osteoblasts

Degradation of IκB protein is shown to occur after signal- induced phosphorylation of IκB protein at specific serine residues by IKK. To determine whether salicylate and PD 098059 inhibited signaling pathways leading to IκB phosphorylation, we measured IKK activity in TNF-α /IFN-γ-treated cells in the presence(10 mM) or absence of salicylate or PD098059 (20 μM). Thus, the cell lysates were immunoprecipitated with an anti-IKKα Ab and incubated with GST-IκBα and [γ-^{32}P] ATP. As shown in Fig. 5A, IKK activity was barely detectable in non-stimulated cells, whereas incubation with the pro-inflammatory cytokines induced a remarkable increase in IKK activity, which was detectable within 10 min, peaked at 30 min, then gradually declined over a 2h period. When was salicylate applied in the presence of PD98059, the induction of IKK was blocked (Fig. 5B), thus showing that salicylate inhibits COX-2 expression via ERK, IKK and subsequent by NF-κB activation in MC3T3E1 osteoblasts.

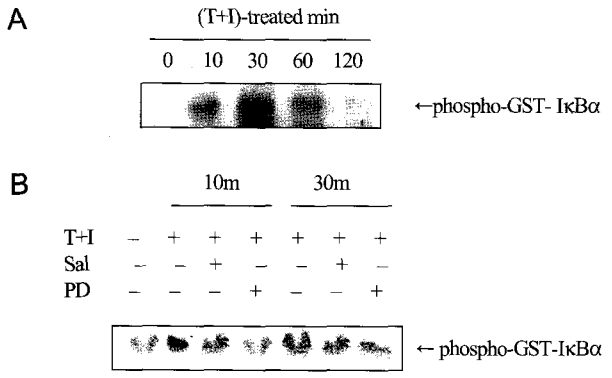

Fig. 5. Salicylate inhibits TNF-α/IFN-γ-induced IKK activation in MC3T3E1 osteoblasts. (A) Cells were exposed to TNF-α (10 ng/ml) and IFN-γ (50 U/ml) for the times indicated (0, 10, 30, 60 or 120 min), and were lysed. IKK was immunoprecipitated with anti-IKKα Ab, and the precipitate was used in *in vitro* kinase reactions with GST-IκBα (aa 1~54) and ^{32}γ-ATP. Phosphorylated GST-IκBα was visualized by SDS-PAGE and autoradiography. (B) IKK immune complex was obtained from MC3T3E1 osteoblasts stimulated with TNF-α (10 ng/ml) and IFN-γ (50 U/ml) in the presence (10 mM) or absence of salicylate or PD098059 (25 μM) for 10 or 30 min. And, the IKK immunoprecipitates were used in *in vitro* kinase assays with GST-IκBα and [γ-^{32}P]. These data represent four independent experiments (A to B).

Various antioxidants, including catalase, N-acetyl cysteine or reduced glutathione, have a regulatory effect on the TNF-α and IFN-γ-induced COX-2 expression in osteoblasts

To examine the effect of antioxidants, especially H_2O_2 scavenger, on TNF-α and IFN-γ-induced COX-2 expression, MC3T3E1 cells were treated with TNF-α (10 ng/ml) plus IFN-γ (50 U/ml) in the presence or absence of antioxidants; catalase (300 U/ml), N-acetyl cysteine (NAC; 10 μM), or reduced glutathione (10 μM). Fig. 6A shows that cytokines-induced COX-2 expression was regulated by all of these antioxidants. NAC, however, was less effective in

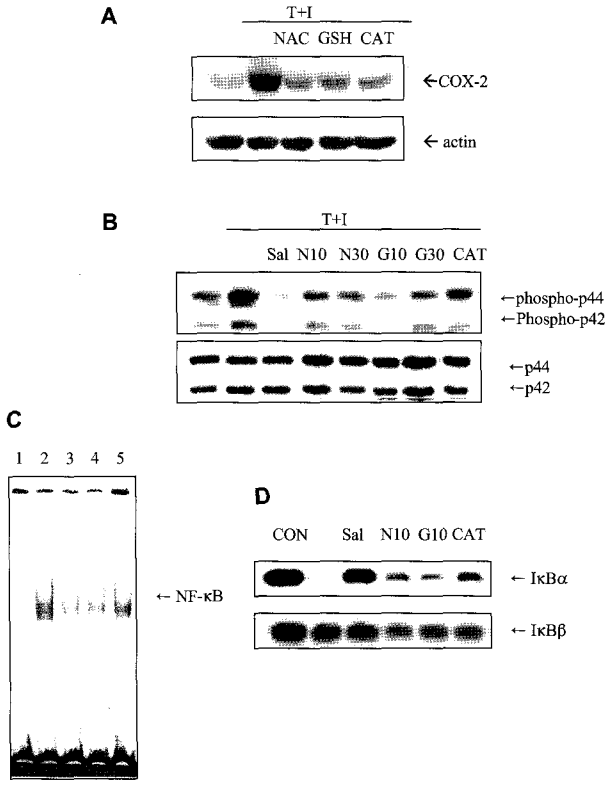

Fig. 6. Catalase, NAC, or GSH inhibits TNF-α and IFN-γ-induced COX-2 expression through the inhibition of ERK, IKK and subsequent NF-κB activation. (A) Cells were incubated with TNF-α (10 ng/ml) and IFN-γ (50 U/ml) in the presence or absence of catalase (300 U/ml), NAC (10 mM) or GSH (10 mM) for 8 h. Then, the cell extracts were subjected to Western blot analysis using antibodies specific toward COX-2. (B) Cells were incubated with TNF-α (10 ng/ml) and IFN-γ (50 U/ml) in the presence or absence of salicylate (10 mM), NAC (10 mM), (30 mM), GSH (10 mM), (30 mM) or catalase (300 U/ml) for 10 min. Then, the cell extracts were subjected to Western blot analysis using antibodies specific towards phosphorylated ERK 1 and 2. (C) Cells were incubated with TNF-α (10 ng/ml) and IFN-γ (50 U/ml) in the presence or absence of NAC (10 mM), GSH (10 mM) or catalase (300 U/ml) for 30 min. Nuclear proteins were analyzed in an EMSA with α-^{32}P-labelled oligonucleotide encompassing the NF-κB binding site. (D) Cells were incubated with TNF-α (10 ng/ml) and IFN-γ (50 U/ml) in the presence or absence of salicylate (10 mM), NAC (10 mM), GSH (10 mM) or catalase (300 U/ml) for 30 min. Cytosolic extracts were examined by immunoblotting for IκB-α and IκB-β degradation. Results shown are a representative of four experiments (A to D).

result suggests a role for ROS signaling pathway in the regulation of COX-2 expression.

Various antioxidants-catalase, NAC or reduced glutathione- have a regulatory effect on TNF-α and IFN-γ -mediated signal transduction in osteoblasts

Role of reactive oxygen species (ROS) in the signaling pathways including ERK and NF-κB has recently been investigated (Smith et al, 1987). ROS can alter gene expression through phosphorylation of transcription factors via the activation of MAP kinase signaling pathways (Smith, 1992). Since induction of COX-2 gene in TNF-α/IFN-γ -exposed osteoblasts was suppresed by H_2O_2 scavengers, we tested the effect of antioxidants on the cytokines-induced ERK, IKK, IκBα, IκBβ and NF-κB activation. First, Nuclear Factor-κB-DNA-binding activity was measured by EMSA (Fig. 6C). In the cells treated with antioxidants, the nuclear level of NF-κB was significantly reduced. In addition, immunoblot analysis of IκB proteins in the cytosol of MC3T3E1 cells indicated that antioxidants-mediated inhibition of NF-κB activation was associated with suppression of IκB degradation (Fig. 6D). Degradation of IκB proteins is shown to occur after signal-induced phosphorylation of IκB proteins at specific serine residues by IKK. Therefore, to determine whether the antioxidants inhibited signal pathways leading to IκB phosphorylation, we measured IKK activity in TNF-α/IFN-γ-stimulated MC3T3E1 cells. When we measured induction of IKK activity in cells treated with catalase, NAC or GSH, but not by superoxide dismutase (SOD) or TEMPO, superoxide scavenger (data not shown), the inhibitory effect of the antioxidants appeared at the same concentrations which inhibited NF-κB activation. These data suggest that ROS play an important role in ERK, IKK, IκB degradation and subsequent by NF-κB activation in MC3T3E1 osteoblasts.

ROS is released in TNF-α/IFN-γ-exposed MC3T3E1 osteoblasts

ROS produced in osteoblasts were determined by measuring fluorescence after loading with dichlorofluorescein-diacetate (DCF-DA), a dye that is oxidized into a highly fluorescent form in the presence of peroxides. DCF-DA can be oxidized by any peroxidase and hydroperoxide, including H_2O_2. We examined whether salicylate prevented the oxidation of DCF. Therefore, MC3T3E1 cells were exposed to TNF-α plus IFN-γ in the presence of salicylate (10 mM) or absence. Pretreatment of the cells with salicylate did not have any effect on DCF oxidation. However, ROS generation was markedly reduced when the cytokines-stimulated cells were exposed to salicylate. These data indicate that, when the MC3T3E1 osteoblasts were exposed to TNF-α plus IFN-γ, free oxygen radicals were generated, leading to ERK and IKK activation, IκB degradation, and NF-κB activation and resulting in COX-2 expression in the osteoblasts. However, our data suggested that the signaling transduction of salicylate did not include ROS inhibition in MC3T3E1 osteoblasts.

DISCUSSION

The factors that may regulate bone metabolism during inflammation are of considerable importance in understanding the pathogenesis of a number of common inflammatory diseases, including rheumatoid arthritis, osteoarthritis and the periodontal diseases. The effects of cytokines such as interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ) on bone cells have extensively been studied in the past and are known to have complex effects on bone metabolism (Takahasi et al, 1994). TNF-α stimulates bone resorption and can inhibit markers of osteoblast activity such as alkaline phosphatase and collagen synthesis (Bertolini et al, 1986), and IFN-γ can directly inhibit cell proliferation and alkaline phosphatase activity in osteoblast cultures (Smith et al, 1987).

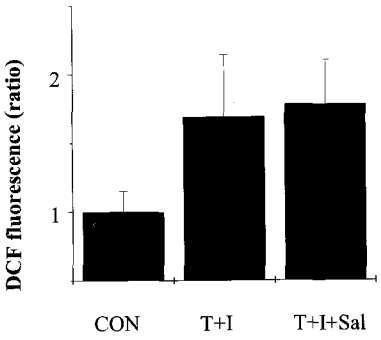

Fig. 7. Salicylate does not have any effect on hydrogen peroxide production in TNF-α/IFN-γ-treated MC3T3E1 osteoblasts. Cells were exposed to TNF-α (10 ng/ml)/IFN-γ (50 U/ml) in the presence (10 mM) or absence of salicylate. Cells were labeled with 100 μM DCF-DA and treated with DMSO alone (control) or TNF-α (10 ng/ml)/IFN-γ (50 U/ml) in the presence or absence of salicylate. After incubation for 10 min, fluorescence was measured with a spectrophotofluorometer. Data represent mean±S.E.M. of four cultures.

Cyclooxygenase-2 (COX-2), also known as prostaglandin H synthase, catalyzes the rate-limiting steps in formation of prostaglandin endoperoxides (Smith et al, 1996). Two distinct cyclooxygenases have been identified and they are encoded by separate genes. COX-1 is expressed constitutively in many tissues (Smith, 1992). In contrast, the more recently identified COX-2 gene is expressed at a very low basal level in most tissues, but is rapidly and transiently induced by a wide variety of mitogens, hormones and other ligands (Takahasi et al, 1994).

An osteogenic MC3T3E1 cell line was established from newborn mouse calvaria, and the cells differentiate into osteoblasts and show calcification in vitro (Chen et al, 2001). Cyclooxygenase-2 induction has been demonstrated with the MC3T3E1 cells by prostaglandins (Sato et al, 1987; Pilbeam et al, 1993), TNF-α has been shown to increase the PGE_2 production and the cyclooxygenase activity in mouse osteoblastic cell line MC3T3E1 (Rapuano & Bockman, 1991; Yanaga et al, 1992; Saunders & Jetten, 1994). IFN-γ is a proinflammatory cytokine that is principally produced by activated T-lymphocytes and natural killer cells, and affects a vast array of different cellular processes (Rapuano & Bockman, 1991; Yanaga et al, 1992).

Since the addition of TNF-α and IFN-γ resulted in a typical and prominent induction of cyclooxygenase-2 in MC3T3E1 cells, we attempted in the present study to elucidate the transcriptional regulation of the cyclooxy-

genase-2 gene.

A requirement of NF-κB activity in COX-2 induction has been suggested in many cell lines (Saunders & Jetten, 1994; Yamamoto et al, 1995). However, many studies which implicate NF-κB activation in COX-2 induction include the use of chemical inhibitors (Yanaga et al, 1992) and peptide inhibitors (Inoue & Tanabe, 1998), which may affect NF-κB activity at another site on the COX-2 promoter.

To investigate whether ERK1/2 MAPK mediated the induction of COX-2 by TNF-α, PD098059, a specific inhibitor of MAPK kinase, was used to block the activation of ERK1/2 MAPK. Treatment of MC3T3E1 cells with 20 μM PD098059 decreased TNF-α-mediated phosphorylation of ERK1/2 MAPK. COX-2 expression has been shown to have linked with activation of MAPK pathways (Sato et al, 1987; Abate et al, 1998; Suh et al, 1998). In the present study, we demonstrated that salicylate inhibited COX-2 by activating ERK1/2 MAPK. Since ERK1/2 MAPK activation can regulate the expression of numerous genes by activating NF-κB (Subbaramaiah et al, 1998), our data suggest that NF-κB is important for mediated the induction of COX-2 by TNF-α.

It has been shown that sodium salicylate inhibits NF-κB activation by preventing the phosphorylation and subsequent degradation of IκBα (Kopp & Ghosh, 1994; Lapointe & Isenovic, 1999). Salicylate inhibits IκBα phosphorylation and degradation induced by TNF-α, but not by IL-1 (Pierce et al, 1996; Schwenger et al, 1998). The ability of salicylate to inhibit TNF-α-induced COX-2 is dependent upon inhibition of TNF-α-induced NF-κB activation.

The data reported here suggest that salicylate inhibited COX-2 expression induced by cytokines in part through the suppression of the ERK1/2 MAPK signaling pathway. TNF-α and IFN-γ increased the phosphorylation of ERK and COX-2 expression, and inhibitor of ERK1/2 MAPK (PD 098059) suppressed COX-2 protein and NF-κB binding activity induced by cytokines. Salicylate inhibited the phosphorylation of ERK. We further showed that pretreatment of cells with salicylate blocked the TNF-α plus IFN-γ induced phosphorylation of ERK. Taken together, these data suggest that at least some of the anti- inflammatory effects of salicylate may be due to the inhibition of ERK.

It has been proposed that salicylate exerts a global inhibitory effect an TNF-α signaling by acting at a TNF-α receptor proximal site (Schwenger et al, 1996). The results described herein provide a possible mechanism involved in the anti-inflammatory effects of salicylate and suggest that ERK activation by cytokines may be involved in the subsequent induction of COX-2 expression.

Salicylate prevents the TNF-α-induced degradation of the NF-κB receptor, IκBα, in some epithelial cell lines (Madge et al, 1999). Because IκBα degradation is necessary for NF-κB translocation to the nucleus, this correlates with decreased activation of the transcription factor. Inhibition of NF-κB by salicylate has also been demonstrated in lymphocytes (Kopp & Ghosh, 1994).

Vietor et al (1993) reported that TNF-α activated p42/p44 MAPK in normal human fibroblasts, and the overall purpose of this study was to further characterize the activation of p42/p44 MAPK and COX-2 by TNF-α. It is possible that this newly demonstrated inhibition of cytokine signaling contributes to the anti-inflammatory action of salicylate.

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