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Sodium Salicylate Inhibits Expression of COX-2 Through Suppression of ERK and Subsequent NF-κB Activation in Rat Ventricular Cardiomyocytes

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The expression of cyclooxygenase-2 (COX-2) is a characteristic response to inflammation, which can be inhibited with sodium salicylate. IL-1β and TNF-α can induce extracellular signal-regulated kinase (ERK), IKK, IκB degradation and NF-κB activation. Salicylate inhibited the IL-1β and TNF-α-induced COX-2 expressions, regulated the activation of ERK, IKK and IκB degradation, and the subsequent activation of NF-κB, in neonatal rat ventricular cardiomyocytes. The inhibition of the ERK pathway, with a selective inhibitor, PD098059, blocked the expressions of IL-1β and TNF-α-induced COX-2 and PGE_2 release. The antioxidant, *N*-acetyl-cysteine, also reduced the glutathione or catalase- attenuated COX-2 expressions in IL-1β and TNF-α-treated cells. This antioxidant also inhibited the activation of ERK and NF-κB in neonatal rat cardiomyocytes. In addition, IL-1β and TNF-α stimulated the release of reactive oxygen species (ROS) in the cardiomyocytes. However, salicylate had no inhibitory effect on the release of ROS in the DCFDA assay. The results showed that salicylate inhibited the activation of ERK and IKK, IκB degradation and NF-κB activation, independently of the release of ROS, which suggested that salicylate exerts its anti-inflammatory action through the inhibition of ERK, IKK, IκB and NF-κB, and the resultant COX-2 expression pathway in neonatal rat ventricular cardiomyocytes.

Key words: Cyclooxygenase-2, Nuclear factor-kappaB, *N*-acetyl-L-cysteine, Reactive oxygen species, Interleukin-1, Tumor necrosis factor, Nas sodium salicylate

INTRODUCTION

Aspirin (acetylsalicylic acid) is one of the most widely used drugs worldwide. It acetylates cyclooxygenase, thereby irreversibly blocking the conversion of arachidonic acid to prostanoids. Biotransformation of aspirin yields salicylate, a compound that possesses similar anti-inflammatory potency to aspirin, but lacks aspirin's inhibitory effect on the activity of isolated cyclooxygenase. At present, there is no common agreement about the extent to which salicylate contributes to aspirin's anti-inflammatory properties, or a final conclusion on the mechanisms of action of sodium salicylate. Several possible sites of action of salicylate have been suggested: It has been shown in intact cells, but not in purified enzyme preparations, that sodium salicylate inhibits prostanoid biosynthesis (Amann *et al.*, 2002). This effect seems to be prevented in the presence of high concentrations of arachidonic acid, which has been shown to interfere with the inhibition of salicylate on the cyclooxygenase-2-mediated prostanoid formation *in vitro* (Tegeder *et al.*, 2001). Other possible sites of action, not directly related to cyclooxygenase inhibition, have been suggested based on observations made *in vitro* using high concentrations of aspirin and sodium salicylate. These effects target intracellular signaling mechanisms, such as kinases, including the mitogen activated protein-kinases (MAPK) cascade (Paccani *et al.*, 2002). This may be one reason that, downstream to kinases, the inhibitory effects of salicylates have been observed on several nuclear transcription factors, such as nuclear transcription factor kappa B (NF-κB) and activator protein-1 (AP-1) (Grilli *et al.*, 1996; Frantz and ONeill 1995). The antioxidant properties of salicylates, such as the release of adenosine

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induced by sodium salicylate and aspirin-triggered lipoxin formation, are additional mechanisms that may contribute to the anti-inflammatory properties of aspirin and/or sodium salicylate (Cronstein *et al.*, 1999). The cytokine-induced COX-2 induction in neonatal cardiomyocytes was used as a model system, where the hypothesis that "the effects on mitogen-activated protein kinase (MAPKs) may explain the mechanism of how salicylate exerts its anti-inflammatory effects in the circulatory system" could be tested. These cytokines exert their actions directly on the cardiomyocytes, or modulate the expression and release of other mediators, such as prostanoids. Two kinetically different pathways for many inflammatory mediators use NF-κB in their induction and perpetuation mechanisms (Barnes and Karin 1997). In this study, it has been shown that the combined form of IL-1β and TNF-α expressed COX-2 protein and the induced PGE_2 are released in primary cultures of neonatal rat ventricular cardiomyocytes. IL-1β and TNF-α are key mediators in inflammatory responses and have been shown to activate the transcription factor, nuclear factor-κB (NF-κB), which is critical for the inducible expressions of multiple genes involved in inflammation. In this study; [1] whether the MAPK signaling pathway was involved in the regulation of COX-2 and PGE_2 expression, [2] whether salicylate influenced MAPK activity, [3] whether sodium salicylate inhibits the NF-κB binding affinity and NF-κB mediated COX gene expression via MEK1 dependent mechanism and [4] whether ROS is involved in the mechanism of salicylate-induced regulatory effect on COX-2 have been investigated, which further explain the mechanism involved in the action of anti-inflammatory drugs.

MATERIALS AND METHODS

Materials

The rat recombinant TNF-α and IL-1β were purchased from R&D Systems (Minneapolis, MN, U.S.A.). The phospho-p42/44 MAPK (Thr^{202}/Tyr^{204}) E10 monoclonal antibody, PD98059 (2-(2′-amino-methoxyphenyl)-oxanaphalen-4-one) and SB203580 were obtained from New England Biolabs (Beverly, MA, U.S.A.). The anti-ERK1, anti-actin and myelin basic protein (MBP) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The COX-2 antibody and pansorbin were purchased from Calbiochem-Novabiochem Corp (La Jolla, CA). All cell culture media and reagents were obtained from Life Technologies, Inc. (Gaithersburg, MD, USA).

Isolation and cultures of neonatal myocytes

Primary cultures of cardiac myocytes were prepared from the ventricles of 1-2 day-old Wistar rats. The ventricles were separated from arterial tissue and washed briefly in digestion solution (116 mM NaCl, 20 mM HEPES, 1 mM NaH_2PO_4, 5.5 mM glucose, 5.4 mM KCl, 0.8 mM $MgSO_4$ [pH 7.35], collagenase [95 U/mL] and pancreatin [0.6 mg/mL]). The myocytes were dissociated in fresh digestion buffer and collected by centrifugation. The isolated cells, which were a mixture of myocytes and nonmyocyte fibroblasts, were suspended in plating media (Dulbeccos modified Eagles medium), and plated onto 150-mm-diameter noncoated culture dishes for 1 h to reduce contamination by cardiac fibroblasts. The myocytes were purified using a Percoll gradient, replated at a density of 1 $\times 10^5$ in 25-mm-diameter etched coverslips, or in 12 or 6 well plates precoated with 1% collagen, and grown in plating media. After 24 h, more than 98% of the cells were found to be myocytes, as determined by the cell morphology and myosin staining.

PGE_2 assay

To determine the PGE_2 synthesis, aliquots were removed from culture medium (1 mL) containing 3×10^5 neonatal rat ventricular cardiomyocytes, and subjected to radioimmunoassay for PGE_2, as described previously (Walch and Morris, 2000).

Western blot analysis

Western blot analysis was performed, essentially as described previously (Chae *et al.*, 2001). Briefly, whole-cell lysates were generated using a buffer consisting of 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, which were then transferred to Immobilon-P membranes (Millipore) in transfer buffer (25 mM Tris, 192 mM glycine and 20% [vol/vol] methanol). The membranes were blocked in TBS-5% skimmed milk, rinsed, and then incubated at 4°C overnight, with primary antibodies in TBS. The membranes were washed four times in TBS-T, and 0.1 μg/mL peroxidase-labeled secondary antibody was then added for 1 h. Following four washes in TBS, the bands were visualized by ECL, and exposed to X-ray film.

Electrophoretic mobility shift assay (EMSA)

The nucleus from neonatal rat ventricular cardiomyocytes was extracted according to a modification to the procedure described previously (Newton *et al.*, 1997). 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 and 100 μM leupeptin). After centrifugation at 1000×g, the nuclear pellet was 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 were used for the 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 a random primer labeling kit (rediprime, Amersham Life Science, England). Nuclear extracts (5 μg) were reacted with 2-5 ng of the radiolabeled NF-κB probes (50,000-100,000 cpm/ng). The reaction was performed in the presence of 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM dithiothreitol and 4% glycerol (final volume: 25 μL) for 30 min at room temperature. The reaction products were subjected onto 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 h. The DNA binding activity for the NF-κB was measured using a PhosphoImager analyzer (BAS, Fuji Co, Japan).

Immunoprecipitation and *in vitro* kinase assay

Treated cell cultures were washed twice 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 μM aprotinin and 1 mM PMSF). Thirty minutes later, the cell lysates were cleared by centrifugation at 12,000×g for 20 min. Immunoprecipitation was performed by incubating 200 μL of lysates with 2 μg of anti-IKKβ antibody (Santa Cruz Biotechnology) for 1 h, and then adding 20 μL of protein A-agarose. After incubation for 1 h 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 was assayed for 20 min at 37°C in the presence of 2 μg of substrate (GST-IκBα), 30 μM ATP, and 20 μCi [γ-^{32}P]-ATP in 55 μL assay buffer (20 mM Tris-HCl, pH 7.5 and 20 mM $MgCl_2$). The proteins were then resolved by SDS-PAGE, and the gels dried and subjected to autoradiography. The relative activity of IKK was quantified by measuring the radioactivity of phosphorus-32 incorporate into the GST- IκBα.

Measurement of ROS

Cells were treated with various agents and incubated with 100 μM DCF-DA (Dichloro fluorescein-diacetate), or the equivalent amount of DMSO, for 20 min at 37°C. After incubation, the cells were washed with PBS, an aliquot was taken for cell counting, and the rest used to determine the relative amounts of ROS by measuring the fluorescence with a spectrophotofluorimeter (excitation, 495 nm; emission, 520 nm). The cell numbers were determined in parallel, and the fluorescence values normalized to the number of cells per sample.

Statistical evaluation

Values are expressed as the mean±standard error of the mean of at least three experiments. Students *t*-test was used to assess statistically significance differences, with a *P* value of less than 0.05 being considered statistically significant.

RESULTS

IL-1β and TNF-α stimulate COX-2 expression and PGE_2 release in cardiomyocytes

Neonatal rat ventricular cardiomyocytes were chosen to investigate the effect of inflammatory cytokines on cyclooxygenase (COX), especially the COX-2 expression in the heart system. When the cardiomyocytes were incubated with IL-1β (2 ng/mL) in the presence, or absence, of TNF-α (2 ng/mL) for 24 h, the combined cytokines (IL-1β/TNF-α) led to a significant increase of the COX-2 protein in the cardiomyocytes (Fig. 1A). The TNF-α had no apparent effects, but the IL-1β had a rather stimulatory effect, on the expression of COX-2. However, the IL-1β (2 ng/mL), in the presence of TNF-α (2 ng/mL), had a synergistic effect on the induction of COX-2 in the cardiomyocytes. The COX-1 levels were not affected by the treatment with IL-1β (2 ng/mL) and TNF-α (2 ng/mL). To elucidate the molecular basis for sodium salicylate-induced COX-2 inhibition, the cells were first treated with IL-1β (2 ng/mL) and TNF-α (2 ng/mL). There was a dose-dependent decrease in the expression of the COX-2 protein induced by the IL-1β and TNF-α by treatment with salicylate (Fig. 1B). The decrease in the COX-2 protein level was mirrored by a decrease in the COX activity, as assessed by PGE_2 production (Fig. 1C).

Salicylate regulates IL-1β and TNF-α-induced ERK activation in rat ventricular cardiomyocytes

To evaluate whether the ERK pathway is involved in the regulation of COX-2 induction, the ability of salicylate to regulate the IL-1β and TNF-α-induced ERK activation was examined in ventricular cardiomyocytes. 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 minutes, and then decreased back to the baseline after 30 minutes. The treatment with salicylate (10 mM) in the presence of IL-1β and TNF-α attenuated the ERK 1 and 2 phosphorylations. Next, to address the role of ERK in the cytokine-mediated induction of COX-2,

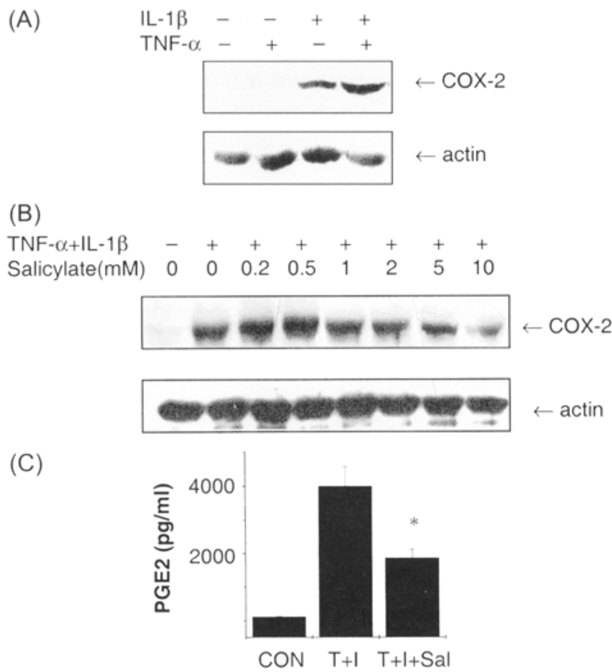

Fig. 1. Salicylate regulates IL-1β/TNF-α-stimulated COX-2 expression and PGE_2 release in rat neonatal ventricular cardiomyocytes. (A) Cells were treated with IL-1β (2 ng/mL) or TNF-α (2 ng/mL) or in combination; IL-1β+TNF-α for 24 h. The cell extracts were subjected to western blot analysis using antibodies specific to COX-2 (1; control, 2; IL-1β, 3; TNF-α, 4; IL-1β+TNF-α). (B) Cells were incubated with IL-1β (2 ng/mL) and TNF-α (2 ng/mL) in the presence, or absence, of salicylate (0, 0.2, 0.5, 1, 2, 5 and 10 mM) for 24 h. The cell extracts were then subjected to western blot analysis using antibodies specific to COX-2. (C) Cells were treated with IL-1β/TNF-α for 24 h in the presence, or absence, of salicylate (10 mM). The culture media were collected and the PGE_2 measured by a PGE_2 assay kit, as described in Materials and Methods. The data shown is the means ± S.D. of four experiments. *P< 0.05 vs. control.

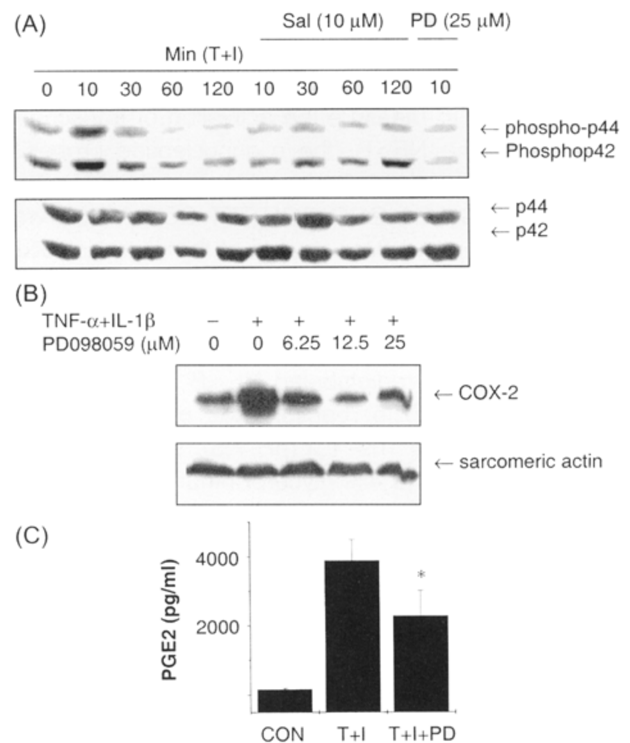

Fig. 2. Salicylate inhibits IL-1β/TNF-α-induced ERK activation in rat ventricular cardiomyocytes. (A) Cells were exposed to IL-1β (2 ng/mL)/ TNF-α (2 ng/mL) for various periods (0, 10, 30 and 60 min) in the presence, or absence, of salicylate (10 mM) or PD090850 (20 μM). The proteins were then subjected to immunoblot analysis, using antibodies specific for the active (phosphorylated) forms of ERK 1 and 2. Parallel blots, run with anti-total ERK antibody, which served as controls. (B) Cells were incubated with IL-1β (2 ng/mL) and TNF-α (2 ng/mL) in the presence, or absence, of PD098059 (0, 6.25, 12.5 and 25 μM) for 24 h. The cell extracts were then subjected to western blot analysis using antibodies specific to COX-2. (C) Cells were treated with IL-1β and TNF-α for 24 h in the presence, or absence, of PD098059 (20 μM). The culture media were collected, and the amount of PGE_2 measured as descried in Materials and Methods. These are typical results from three independent experiments.

cardiomyocytes were treated with the MEK1-specific inhibitor, PD098059. The PD compound reduced the levels of ERK phosphorylation in the IL-1β and TNF-α-treated cells.

To examine the effect of PD098059, a specific MEK 1 inhibitor, on the IL-1β and TNF-α-induced COX-2 expressions, the cardiomyocytes were treated with IL-1β (2 ng/ mL) and TNF-α (2 ng/mL) in the presence, or absence, of PD098059 (0, 6.25, 12.5 and 25 μM). There was a dose-dependent decrease in the expression of the COX-2 protein induced by the IL-1β and TNF-α due to the treatment of the MEK 1 inhibitor (Fig. 2B). Next, to verify whether the decrease in the combined cytokines-induced COX-2 protein levels caused by the PD098059 resulted in the inhibition of the release of PGE_2, a PGE_2 assay was performed. As expected, the PD098059 (20 mM) significantly inhibited the release of PGE_2 in the IL-1β and TNF-α-treated cardiomyocytes (Fig. 2C). This result suggests a role for the ERK signaling pathway in the regulation of the expression of COX-2 by IL-1β and TNF-α in neonatal rat ventricular cardiomyocytes.

Salicylate has a regulatory effect on IL-1β and TNF-α-induced NF-κB binding activity in rat ventricular cardiomyocytes

The activation of NF-κB is known to be an essential process for the induction of COX-2 expression (Newton *et al.*, 1997). The electrophoretic mobility shift assay (EMSA) showed that the IL-1β and TNF-α markedly increased the NF-κB in the nuclear fraction at 10 and 30 min (Fig. 3A). The addition of 10 mM sodium salicylate, which significantly inhibited the expression of COX-2 induced by IL-1β (2 ng/mL) and TNF-α (2 ng/mL), blocked the activation

and translocation of the NF-κB induced by this combination of cytokines. Immunoblot analysis for IκB proteins in the cytosol of rat ventricular cardiomyocytes indicated that the salicylate-induced inhibition of the NF-κB activation was associated with the suppression of IκB degradation (Fig. 3B). Stimulation of cells with IL-1β/TNF-α induced significant IκBα and IκBβ degradation within 10 min. However, the addition of salicylate blocked both IκB protein degradations, suggesting that salicylate inhibits an intermediate step in the signal pathway toward the activation of NF-κB. In addition, PD098059, a MEK1 inhibitor, inhibited the NF-κB activation in the IL-1β/TNF-α-treated cardiomyocytes (Fig. 3C). These data show that salicylate, an anti-inflammatory agent, regulates the expression of COX-2 and the resultant release of PGE_2 through ERK and the subsequent activation of NF-κB in rat ventricular cardiomyocytes.

To determine whether salicylate and PD 098059 inhibit the signal pathway that leads to NF-κB, the IKK activity was measured in IL-1β/TNF-α-treated cells in the presence, or absence, of salicylate (10 mM) or PD098059 (20 μM). The cell lysate was immunoprecipitated with an anti-IKKβ Ab and incubated with GST-IκBα and [γ-^{32}P] ATP. IKK activity was barely detectable in the non-stimulated cells, whereas the incubation with the pro-inflammatory cytokines induced a remarkable increase in the IKK activity within 10 min. When the induction of the IKK activity was measured in the cells treated with salicylate and PD098059, the kinase activation was blocked by both the agents (Fig. 3D) Our results suggest that salicylate inhibits the expression of COX-2 via ERK, IKK and subsequent activation of NF-κB in rat ventricular cardimyocytes.

Various antioxidants-catalase, *N*-acetyl cysteine and reduced glutathione- have regulatory effects on the IL-1β/TNF-α-induced COX-2 expression in rat ventricular cardiomyocytes

To examine the effect of antioxidants, especially H_2O_2 scavengers, on the IL-1β and TNF-α-induced COX-2 expression, rat ventricular cardiomyocytes were treated with IL-1β (2 ng/mL)/TNF-α (2 ng/mL) in the presence, or absence, of antioxidants-catalase (300 U/mL), *N*-acetyl cysteine (10

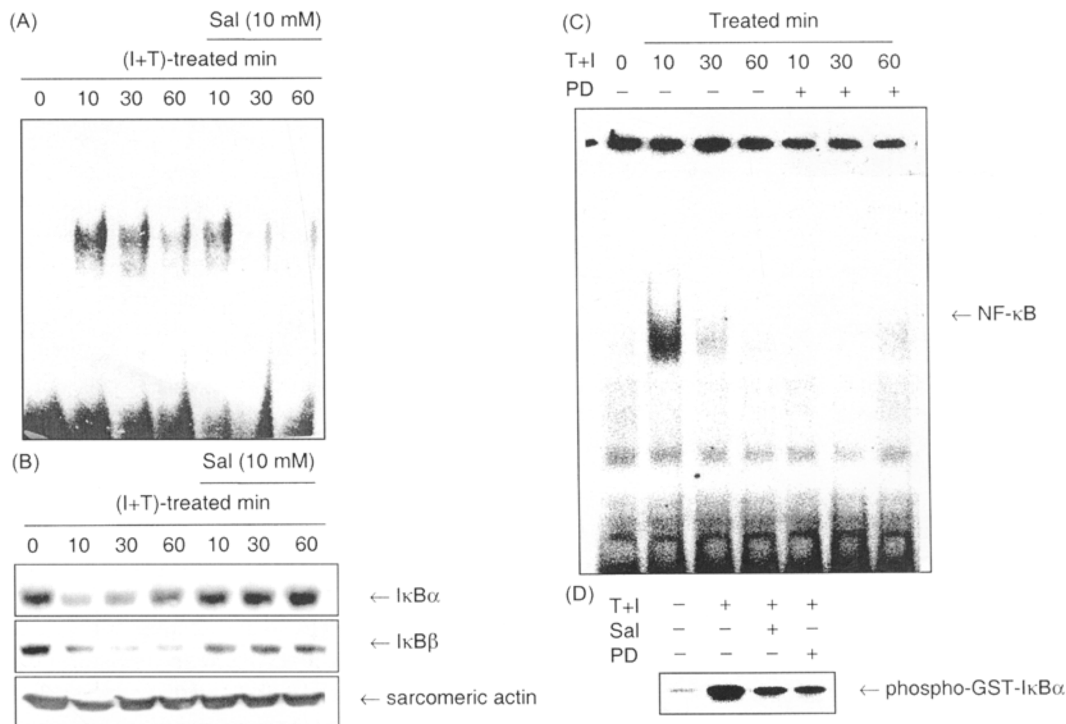

Fig. 3. Salicylate inhibits IL-1β/TNF-α-induced NF-κB activation in rat ventricular cardiomyocytes. (A) Cells were exposed to IL-1β (2 ng/mL) and TNF-α (2 ng/mL) in the presence, or absence, of salicylate (10 mM) for various time intervals (0, 10, 30 or 60 min). Nuclear proteins were analyzed in an EMSA, with an α-^{32}P-labelled oligonucleotide encompassing the NF-κB binding site. (B) Cells were also exposed to IL-1β (2 ng/mL) and TNF-α (2 ng/mL) in the presence, or absence, of salicylate (10 mM) for various time intervals (0, 10, 30 or 60 min). The cytosolic extracts were examined by protein immunoblotting for IκB-α and IκB-β degradation. (C) Cells were exposed to IL-1β (2 ng/mL) and TNF-α (2 ng/mL) in the presence, or absence, of PD098059 (20μM) for various time intervals (0, 10, 30 or 60 min). Nuclear proteins were analyzed in an EMSA, with an α-^{32}P-labelled oligonucleotide encompassing the NF-κB binding site. (D) Cells were exposed to IL-1β (2 ng/mL) and TNF-α (2 ng/mL) in the presence, or absence, of salicylate (10 mM) or PD098059 (20 μM) for 10 min. The IKK kinase activity was measured as descried in Materials and Methods. The results shown are representative of two experiments.

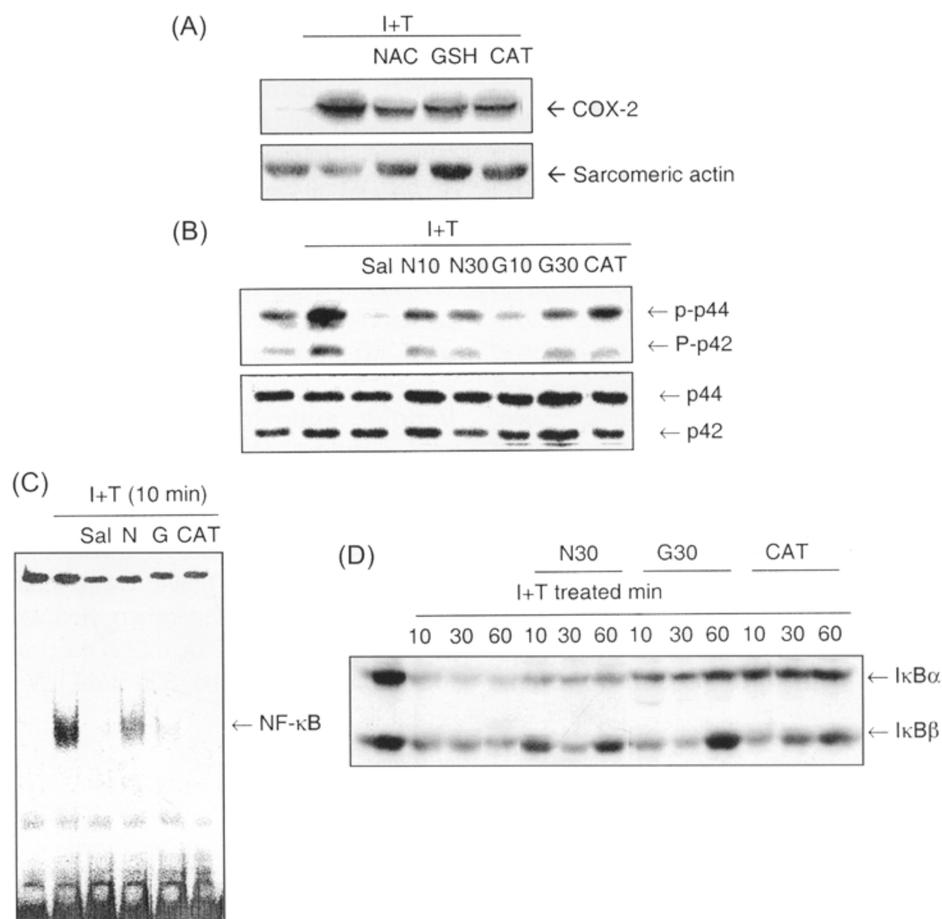

Fig. 4. Various antioxidants, catalase, NAC or GSH inhibit IL-1β and TNF-α-induced COX-2 expression in rat ventricular cardiomyocytes. (A) Cells were incubated with IL-1β (2 ng/mL) and TNF-α (2 ng/mL) in the presence, or absence, of catalase (300 U/mL), NAC (30 mM) or GSH (30 mM) for 24 h. The cell extracts were then subjected to western blot analysis using antibodies specific to COX-2. (B) Cells were incubated with IL-1β and TNF-α (2 ng/mL) in the presence, or absence, of catalase (300 U/ml), NAC (30 mM) or GSH (30 mM) for 10 min. The cell extracts were subjected to western blot analysis using antibodies specific to phospho-specific ERK 1 and 2 (p-p44 and p-p42, and p42 and p44). (C) Cells were also exposed to IL-1β (2 ng/mL) and TNF-α (2 ng/mL) in the presence, or absence, of salicylate (10 mM) for various time intervals (0, 10, 30 or 60 min). The cytosolic extracts were examined by protein immunoblotting for IκB-α and IκB-β degradation. (D) Cells were incubated with IL-1β and TNF-α (2 ng/mL) in the presence, or absence, of salicylate (10 mM), catalase (300 U/mL), NAC (30 mM) or GSH (30 mM) for 10 min. EMSA was performed as described in Materials and Methods. The results shown are representative of three experiments.

or 30 mM) or reduced glutathione (10 or 30 mM). Fig 4A revealed the combined cytokines-induced COX-2 expression was regulated by these antioxidants. The role of reactive oxygen species (ROS) in the control of COX-2 expression has recently been implicated in the pathogenesis of inflammation (Nakamura *et al.*, 2001).

The role of ROS in the signaling pathways, including those of ERK and NF-κB, has recently been investigated (Haddad *et al.*, 2002). The ROS, including H_2O_2, can alter gene expressions through the phosphorylation of transcription factors via the activation of MAP kinase signaling pathways (Sano *et al.*, 2001). Since the induction of the COX gene in IL-1β/TNF-α-exposed cardiomyocytes was mediated by H_2O_2 scavengers, the effect of antioxidants were tested on the combined cytokines-induced ERK, IκBα, IκBβ and NF-κB activation. Fig. 4B shows that the treatment with NAC (10 or 30 mM), GSH (10 or 30 mM) or catalase (300 U/mL), in the presence of IL-1β and TNF-α, attenuated the ERK 1 and 2 phosphorylations. Next, the nuclear κB-binding activity was measured by EMSA (Fig. 4C). In cells treated with the antioxidants (30 mM NAC, 30 mM GSH or 300 U/mL catalase), the nuclear level of the NF-κB was significantly reduced. In addition, immunoblot analysis of IκB proteins in the cytosol of cardiomyocytes indicated that the antioxidants-induced inhibition of the NF-κB activation was associated with the suppression of IκB degradation (Fig. 4D). These data suggest that ROS play an important role in ERK and NF-κB activation as a mediator of the salicylate-associated COX inhibition in rat neonatal cardiomyocytes.

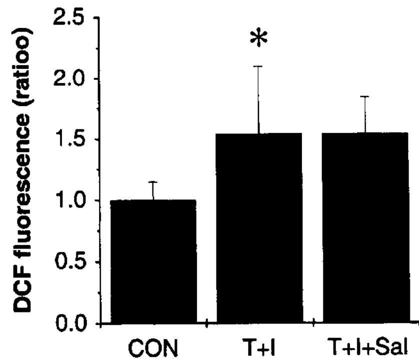

Fig. 5. Salicylate does not have any effect on oxygen radical production in IL-1β/TNF-α-treated rat ventricular cardiomyocytes. Cells were exposed to IL-1β (2 ng/mL)/TNF-α (2 ng/mL) in the presence, or absence, of salicylate (10 mM) for 10 min. The cells were then labeled with 100 μM DCF-DA for 20 min. The fluorescence was measured as described in Material and Methods. The data represent the mean ± S.D. of three cultures. Significant difference from non-treated control; *P<0.05.

Salicylate dose not have any inhibitory effect on the release of ROS in IL-1β and TNF-α-exposed neonatal rat ventricular cardiomyocytes

The reactive oxygen species (ROS) produced in cardiomyocytes were determined by measuring the fluorescence after loading with DCFDA, a dye that is oxidized to a highly fluorescent form in the presence of peroxides. DCFDA can be oxidized by any peroxide or hydroperoxide, including H_2O_2. To specify the active oxygen species responsible for the oxidation of DCF, whether these antioxidants could prevent the oxidation of DCF was examined. Cells were exposed to IL-1β and TNF-α in the absence, or presence, of salicylate (10 mM). The pretreatment with salicylate had no effect on the DCF oxidation (Fig. 5). Also, the antioxidants-catalase and NAC inhibited the increase in the DCF oxidation measured 10 minutes after exposure to the IL-1β and TNF-α in neonatal rat ventricular cardiomyocytes (data not shown).

DISCUSSION

Aspirin, as an anti-inflammatory agent, is routinely used for the primary and secondary prevention of coronary artery disease, which is a common cause of heart failure (Matalka and Deedwania 1999).

As COX-2 has emerged as a major culprit responsible for the synthesis of proinflammatory prostaglandins, the pharmacological action of most NSAIDS has been explained by the inhibition of COX-2 activity. The action of salicylate, however, remains paradoxical. It has comparable anti-inflammatory properties to aspirin (Vane *et al.*, 1994; Xiao *et al.*, 1999), but unlike aspirin does not inhibit COX-2 activity when added to a purified enzyme (Cromlish *et al.*, 1996). By demonstrating COX-2 gene suppression and its mechanism, our findings add a new dimension to Vane's report (1994) regarding the actions of aspirin and salicylate.

In this study, it has been shown that IL-1β/TNF-α-activated and increased the levels of COX-2 protein, which was followed by the release of PGE_2 (Fig. 1). Since the addition of IL-1β and TNF-α brought about such a typical and prominent induction of COX-2 in rat ventricular cardiomyocytes, attempts to elucidate the signal transduction of the COX-2 gene in this system were made.

Treatment of cardiomyocytes with PD098059, a specific ERK inhibitor, decreased the IL-1β/TNF-α-mediated phosphorylation of ERK1/2 MAPK and COX-2 expressions, which has been linked to the activation of MAPK pathways (Fig. 2). ERK1/2 activation can regulate the expression of numerous genes by activating NF-κB (Tuyt *et al.*, 1999). Our data suggest that NF-κB is important in mediating the induction of COX-2, through ERK1/2, in neonatal rat cardiomyocytes.

Recent studies have shown that salicylate inhibits NF-κB- and AP-1-mediated gene transcriptions (Kopp and Ghosh, 1994; Dong *et al.*, 1997). A selective inhibition of IκB kinase by salicylate has also been reported. IκB kinase α and β phosphorylate IkB cause its degradation, resulting in NF-κB translocation and activation of the genes involved in inflammation (Yin *et al.*, 1998). Hence, salicylate may exert its anti-inflammatory action by suppressing the expression of NF-κB-activated inflammatory genes. It is important to re-examine whether the suppressive effect of salicylate on COX-2 induction is mediated by IκB kinase inhibition. Another possible mechanism for the action of salicylate may be mediated by peroxisome proliferator-activated receptors. It has been suggested that NSAIDs, including salicylate, may exert their anti-inflammatory activities through peroxisome proliferator-activated receptor activation (Jürgen *et al.*, 1997; Jiang *et al.*, 1998). However, salicylate does not bind and activate either form of the peroxisome proliferator-activated receptors (Jürgen *et al.*, 1997). It is unlikely that the action of salicylate on COX-2 is mediated by the peroxisome proliferator-activated receptor pathway. As salicylate is capable of suppressing the COX-2 expressions induced by diverse stimuli, where the activation of the COX-2 transcription involves distinct regulatory elements and transactivators, besides NF-κB (Hiroyasu *et al.*, 1995; Kei *et al.*, 1995), it is possible that salicylate suppresses the COX-2 induction by altering a common signaling pathway, reducing the transcriptional activation of COX-2. Further work is needed to define the mechanism and to determine if it is distinct from its action on IκB kinase.

Reactive oxygen species (ROS) have been considered an important regulator of inflammation and an activator of

the transcription factor, NF-κB, which regulates inflammatory cytokine gene expression (Kim *et al*., 2000). The COX-2 expression was regulated by antioxidants, such as NAC, GSH and catalase, and the antioxidants inhibited the activation of ERK and NF-κB in neonatal rat ventricular cardiomyocytes. This study has shown that ROS are one of the mediators in the expression of COX-2, but that salicylate has no regulatory effect on the IL-1β and TNF-α-induced ROS release, as determined by the dichloro fluorescein diacetate dye (Fig. 5). The salicylate-induced COX-2 regulation appears to be independent of the ROS in IL-1β and TNF-α-treated cardiomyocytes. Tomohiro and Kazuichi (2001) showed that COX-2 mRNA was highly accumulated in H_2O_2-treated cells. In Bradykinin receptor-induced dilation of cerebral arterioles, ROS can also modulate gene expressions, including the expression of the inducible isoform of COX-2 (Brian *et al*., 2001). In addition, the involvement of the MAPK pathway in regulating the LPS-mediated TNF-α secretion is redox-dependent and NF-κB-sensitive, and is attenuated by *N*-acetyl-L-cysteine (NAC) and other antioxidants (Haddad and Land, 2001).

This study has shown how salicylate has a regulatory effect on the expression of COX-2. Furthermore, it suggests that another therapy, with potent antioxidants modulating ERK and NF-κB activation, may be of therapeutic benefit.

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