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Regulation of Cyclo-oxygenase Gene Expression in Rat Smooth Muscle Cells by Catalase

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ABSTRACT. We have studied, in detail, the effect of catalase, one of the naturally occurring antioxidant enzymes, on the expression of cyclo-oxygenase (COX) mRNA and protein in rat aortic smooth muscle cells (RASMC). The activity of COX enzyme within the cells was also determined. Catalase either alone or in combination with interleukin-1β (IL-1β) enhanced mRNA and protein expression for cyclo-oxygenase 2 (COX-2) in a concentration-dependent manner. However, it did not affect the expression of mRNA or protein for cyclo-oxygenase 1 (COX-1). The expression of mRNA for COX-2 induced by catalase was blocked completely by actinomycin D (ACT) or cycloheximide (CHX). In comparison, expression of mRNA for COX-2 stimulated by IL-1β was inhibited by actinomycin D, but not by cycloheximide. This suggests that induction of the synthesis of mRNA for COX-2 by catalase and IL-1β involves different mechanisms. In particular, the induction of mRNA for COX-2 by catalase requires on-going protein and RNA synthesis, but the induction following exposure to IL-1β does not. The increase in expression of mRNA for COX-2 induced by catalase may be related to the ability of catalase to stimulate cyclic AMP response element (CRE) and NF-IL6 transcription factors, but not nuclear factor kappa B (NF-κB), for electrophoretic mobility shift assays (EMSA) showed that catalase enhanced nuclear factor binding to cyclic AMP response element and NF-IL6 but not to NF-κB. Catalase exerted a biphasic effect on prostaglandin synthesis. At low concentrations it enhanced prostaglandin production, but at high concentrations it tended to inhibit it. These findings suggest that catalase has differential and multiple effects on COX expression and activity in rat aortic smooth muscle cells. BIOCHEM PHARMACOL **55**;10:1621–1631, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. catalase; cyclo-oxygenase 2; gene and protein expression; transcription factors

The primary enzyme responsible for initiating the biosynthesis of prostanoids from arachidonic acid is prostaglandin endoperoxide synthase, commonly called COX†. Prostanoids such as PGI_2 and PGE_2 are powerful vasoactive agents, and reductions in their production have been implicated in the pathogenesis of various vasospastic disorders affecting cardiac or cerebral blood flow. COX enzyme exists in two forms, COX-1 and COX-2, encoded by two related genes. The *COX-1* gene is constitutively and ubiquitously expressed, while the *COX-2* gene is only expressed at high levels upon induction by growth factors, cytokines, and extracellular stimuli [1, 2].

Proliferation, migration, and abnormal subintimal accumulation of vascular smooth muscle cells are pathologic hallmarks of primary pulmonary hypertension, coronary atherosclerosis, and restenosis after coronary balloon angioplasty [3, 4]. Interestingly, the proliferation of vascular smooth muscle cells is influenced by prostanoids [5–7]. For instance, PGI_2 inhibits the proliferation of vascular smooth muscle cells while thromboxane A_2 increases it. However, although the synthetic pathways for prostanoids in such smooth muscle cells are well documented [8, 9], the molecular mechanisms regulating these systems are still under intensive investigation. It is known that increases in antioxidant enzyme levels are correlated with decreases in cell proliferation [10], and a deficiency of antioxidant buffering levels is associated with atherosclerosis and hypertension [11, 12]. One of the most important natural antioxidant enzymes, catalase, which converts hydrogen peroxide to water and oxygen, may either increase or decrease prostanoid production within the vasculature, depending upon its concentration [13, 14]. Because of such interactions, there is an increasing interest in studying the relationship between eicosanoid gene expression and redox status [15, 16]. In this connection, we have studied the regulation of COX expression by catalase in rat smooth muscle cells.

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† *Abbreviations:* 6-keto-$PGF_{1\alpha}$, 6-keto-prostaglandin $F_{1\alpha}$; ACT, actinomycin D; AUBF, adenosine-uridine binding factor; CHX, cycloheximide; COX, cyclo-oxygenase; COX-1, cyclo-oxygenase 1; COX-2, cyclo-oxygenase 2; CRE, cyclic AMP response element; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; HRP, horseradish peroxidase; IL-1β, interleukin-1β; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF-κB, nuclear factor kappa B; PGE_2, prostaglandin E_2; PGI_2, prostacyclin; RASMC, rat aortic smooth muscle cells; and RT-PCR, reverse transcriptase-polymerase chain reaction.

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MATERIALS AND METHODS

Materials

IL-1β was purchased from R&D Systems; catalase (from human erythrocytes), 6-keto-$PGF_{1\alpha}$, PGE_2, anti-6-keto-$PGF_{1\alpha}$ antibody, anti-PGE_2 antibody, PBS, antibiotics (penicillin/streptomycin), DTT, ACT, CHX, and arachidonic acid were from the Sigma Chemical Co.; COX-1 antibody was a gift from Merck Frosst; COX-2 antibody was from Cayman Chemical Co.; [^{3}H]PGE_2, 6-keto-[^{3}H]$PGF_{1\alpha}$, Kodak BioMax MS-1 film, Hyperfilm ECL, goat anti-rabbit IgG-HRP horseradish peroxidase and chemiluminescence reagents were from Amersham International plc; Biotin-21-dUTP was from Clontech; Lumi-Phos solution was from Luminogen; RNasin, Acryl 40, Bis-2 and Acryl/Bis (29:1) solution were from Biometra Ltd.; FBS (fetal bovine serum), RPMI 1640 medium, [γ-^{32}P]ATP, and L-glutamine were from ICN Biomedicals Ltd.; TRI REAGENT (RNA isolation reagent) was from the Molecular Research Center; EMSA reagents were from the Promega Co.; poly (dI-dC) and dNTPs were from Pharmacia Biotech Ltd.; random hexanucleotide primers and M-MLV reverse transcriptase were from Life Technologies Ltd.; DNA polymerase and other PCR reagents were from Bioline Ltd.; and Molecular Imager and Imaging Densitometer was from Bio-Rad Laboratories.

Cell Culture

RASMC were prepared by collagenase digestion of rat aortic smooth muscle strips [17] and grown in 6-well plates in RPMI 1640 containing 10% FBS, 2 mM of glutamine, penicillin (100 units/mL), and streptomycin (100 μg/mL). Cell growth was arrested by placement in serum-free RPMI 1640 medium for 48 hr. Quiescent RASMC cultures were stimulated with various agents for different time periods, as indicated. Catalase was dissolved in RPMI 1640 and determined without endotoxin contamination.

RNA Isolation and RT-PCR

Cell monolayers were washed with PBS, and then RNA was isolated as previously described [18]. Total RNA (0.5 μg) was converted to cDNA by the standard method [19]. PCR conditions were optimized such that only the desired product was produced. PCR was performed using an OmniGene™ thermocycler. Initial denaturation was done at 94° for 2 min followed by 30–36 cycles of amplification. Each cycle consisted of 35 sec of denaturation at 94°, 35 sec of annealing at 58°, and 45 sec for enzymatic primer extension at 72°. After the final cycle, the temperature was held at 72° for 10 min to allow reannealing of the amplified products. PCR products were then size-fractionated through a 2% agarose gel, and the bands were visualized using ethidium bromide. Rat COX-1 and COX-2 primers were designed according to the published sequences of rat COX-1 [20] and COX-2 [21] mRNA, respectively. The sequences of the rat COX-1 and COX-2 primers were: 5′-TAA GTA CCA GGT GCT GGA TGG-3′ (sense, bases 772–792) and 5′-GGT TTC CCC TAT AAG GAT GAG G-3′ (antisense, bases 1036–1015) and 5′-TAC AAG CAG TGG CAA AGG C-3′ (sense, bases 1299–1317) and 5′-CAG TAT TGA GGA GAA CAG ATG GG-3′ (antisense, 1602–1580), respectively. The PCR products were 265 and 304 bp, respectively. Rat *G3PDH*, a constitutively expressed gene, was chosen as a control gene [22]. The primer sequences were: 5′-ACC ACA GTC CAT GCC ATC AC-3′ (sense, bases 562–581) and R:5′-TCC ACC ACC CTG TTG CTG TA-3′ (antisense, bases 1013–1032). The PCR product was 452 bp in size. To quantitate better the observed differences in mRNA expression, G3PDH and COX cDNAs were co-amplified in the same reaction tube.

Nuclear Extracts and EMSA

Nuclear extracts were prepared basically as described by Schreiber *et al.* [23] with slight modifications. Briefly, after washing with PBS, cells were resuspended in 500 μL of Buffer A (10 mM of HEPES, pH 7.9, 4.5 mM of KCl, 7 mM of Na_2HPO_4, 0.5 mM of DTT, 0.5 mM of phenylmethylsulphonyl fluoride) and incubated in ice for 15 min. Afterwards, 30 μL of 10% Nonidet P-40 was added to the mixture, and the samples were mixed on a Vortex machine for 10 sec. Nuclei were collected following a 30-sec spin and resuspended in 50 μL of cold Buffer C (5 mM of HEPES, pH 7.9, 26% glycerol, 1.5 mM of $MgCl_2$, 0.2 mM of EDTA, 0.5 mM of DTT, 0.5 mM of phenylmethylsulphonyl fluoride). The samples were rocked vigorously for 15 min at 4°, and the supernatants were frozen at −70°.

EMSA was performed essentially as described earlier with some modification [24, 25]. Binding reaction mixtures (10 μL) containing 2 μg protein of nuclear extract, 1 μg of poly (dI-dC), ^{32}P-labelled probe, 4% glycerol, 1 mM of $MgCl_2$, 0.5 mM of EDTA, 0.5 mM of DTT, 50 mM of NaCl, and 10 mM of Tris–HCl (pH 7.5) were incubated for 30 min at 25°. For specificity controls, a 100-fold excess of unlabelled probe was applied, and in some experiments 100-fold noncompetitor probe was also used as an additional control. Proteins were separated by electrophoresis through a native 4% polyacrylamide gel at 4° in a running buffer of 12.5 mM of Tris borate, 0.25 mM of EDTA (pH 8.0), followed by autoradiography. The sequences of the NF-κB-, CRE- and NF-IL6-specific probes (binding sites are underlined) were 5′-AGT TGA GGG GAC TTT CCC AGG C-3′, 5′-AGA GAT TGC CTG ACG TCA GAG AGC TAG-3′, and 5′-CAC CGG GCT TAT GCA ATT TTT TTA A-3′, respectively. The oligonucleotides were labelled with [^{32}P]ATP by T4 polynucleotide kinase, and the labelled probe was purified by passing it through a G-25 column.

Northern Blot Analysis

A non-isotopic method was used to perform Northern analysis [26]. Briefly, 5 μg of total RNA was fractionated in a 1.2% agarose-formaldehyde gel. Samples from the gel were transferred to nylon membrane. The membranes were prehybridized for 1 hr and then hybridized to a biotinylated rat *COX-2* probe or *G3PDH* probe. The signals on the membranes were detected by adding chemiluminescent substrate (Lumi-Phos 530), and the image was captured on X-ray film.

Western Blot Analysis

The samples for the immunoblot assay were prepared as described previously [27]. Briefly, samples were boiled in sample buffer and separated on 10% SDS–polyacrylamide gels. Proteins were then transferred electrophoretically from the gel onto nitrocellulose membranes, and the membranes were blocked for 1 hr in PBS–Tween buffer containing 5% dry milk powder (fat free) at room temperature. Then the membranes were incubated with antisera for 1 hr at room temperature. After washing, the membranes were incubated with goat anti-rabbit IgG-HRP. Finally, the membrane was treated with the reagents in the chemiluminescent detection kit according to the manufacturer's instructions.

COX Activity

COX activity was estimated by the conversion of exogenous arachidonic acid to 6-keto-$PGF_{1\alpha}$ (stable hydrolysis product of PGI_2) and PGE_2. Cells were exposed to medium containing 30 μM of arachidonic acid for 10 min at 37°. The resulting amounts of 6-keto-$PGF_{1\alpha}$ and PGE_2 in the supernatants were measured by radioimmunoassay [28].

Cell Viability

Cell respiration, an indicator of cell viability, was assessed by the mitochondrial dependent reduction of MTT to formazan [29]. Following the removal of conditioned medium, cells in 96-well plates were incubated with MTT (0.2 mg/mL) for 1 hr. The medium was aspirated, and cells were solubilized in 200 μL of DMSO. The optical density of each sample at 550 nm was measured using a Molecular Devices microplate reader. The optical density of the medium is proportional to the number of viable cells.

Image Analysis

The densities of the bands corresponding to amplification products were determined with the Molecular Imager and Imaging Densitometer from Bio-Rad Laboratories. The data were then analyzed by Molecular analysis, Microsoft Excel and InStat computer softwares.

Statistical Analysis

All values are expressed as means ± SEM. Statistical comparisons were performed using one-way ANOVA and the Dunnett's test. A *P*-value of less than 0.05 was taken as statistically significant.

RESULTS

Effect of Catalase on the Expression of mRNA for COX

Incubation of RASMC with various concentrations of catalase (0–6750 U/mL) or catalase plus IL-1β (10 U/mL) for 4 hr induced concentration-dependent increases in the expression of mRNA for COX-2 (Figs. 1 and 2). The topmost concentration of catalase (6750 U/mL) increased the expression of mRNA for COX-2 by approximately 58% over that in RASMC treated with a low concentration of catalase (250 U/mL). Furthermore, exposure of cells to catalase (6750 U/mL) plus IL-1β (10 U/mL) led to an increase in mRNA for COX-2 that was 106% greater than that in cells treated with IL-1β alone, and 95% greater than that in cells treated with catalase (250 U/mL) plus IL-1β (10 U/mL), indicating a synergistic induction. Concentrations of catalase higher than 6750 U/mL caused significant cell death in our experimental model and so were not employed in any of the subsequent studies. Heat-denatured catalase had no effect on the induction of mRNA for COX-2 (data not shown). Levels of mRNA for COX-1 were unaffected by any of the above conditions (data not shown). Northern blot analysis confirmed the RT-PCR evidence that the expression of mRNA for rat COX-2 was increased by catalase (Fig. 3). Heat-inactivated catalase (100°, 10 min) abolished its effect on COX-2 expression (Fig. 1C), suggesting that the COX-2 mRNA expression was due to the activity of catalase itself.

To determine the extent to which the accumulation of mRNA for COX-2 in response to catalase was dependent upon increased mRNA synthesis, we investigated the effects of ACT, an inhibitor of DNA-dependent RNA transcription. Pretreatment of cells with ACT completely abolished the increase in mRNA for COX-2 induced by catalase or catalase plus IL-1β (Fig. 4A, compare lanes 5 and 6 with lanes 2 and 3).

Requirement of On-going Protein and RNA Syntheses for the Induction of mRNA for COX-2 by Catalase

The accumulation of mRNA for COX-2 in response to catalase was blocked almost completely by CHX (Fig. 4, compare lane 8 with lane 4). However, the increase in mRNA for COX-2 induced by IL-1β was unaffected by CHX treatment (compare lane 7 with lane 3), suggesting that IL-1β might target the *COX-2* gene directly. Interestingly, treatment of the cells with CHX alone resulted in the accumulation of mRNA for COX-2 (compare lane 10 with lane 1), indicating that CHX alone affects the expression of mRNA for COX-2 in RASMC.

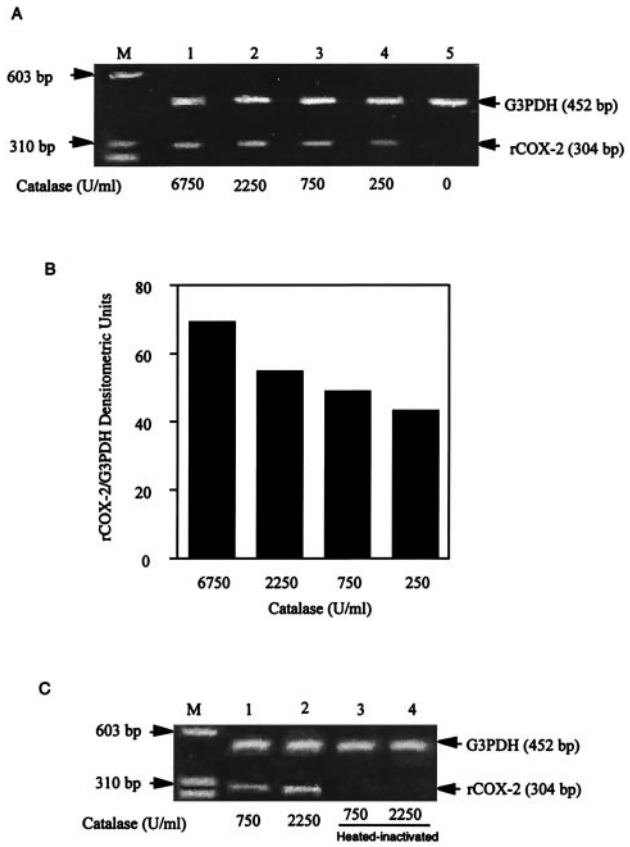

FIG. 1. Concentration-dependent induction of mRNA for COX-2 by catalase. RASMC were incubated for 4 hr either in medium alone (lane 5) or in medium supplemented with catalase (250–6750 U/mL) (lanes 1–4). Following cell lysis and RNA isolation, the levels of mRNA for COX-2 and G3PDH were assessed by RT-PCR. Data are reported as a photograph of a typical agarose gel stained with ethidium bromide (A) or as a graph of the results obtained by quantitative densitometric analysis of photographic signals (B). Results of heat-inactivated catalase on COX-2 expression (C).

Time Course of Catalase-Induced Expression of mRNA for COX-2

Exposure of RASMC to either IL-1β, catalase, or IL-1β plus catalase enhanced the expressions of mRNA for COX-2, with a peak effect at about 4 hr (Fig. 5). Normalization of the densitometrically scanned COX-2 signals to the internal G3PDH controls showed that catalase, and catalase plus IL-1β, caused 1.17- and 1.26-fold greater inductions, respectively, after a 4-hr incubation than the lowest detectable level within a 36-hr time period (Fig. 5, B and C). Levels of mRNA for COX-2 declined after a 4-hr incubation. No expression of mRNA for COX-2 was detected after incubation of cells with IL-1β for 24 hr or with catalase for 36 hr.

Effects of Catalase on DNA Binding of Transcription Factors

Previous studies have suggested that three transcription factors, NF-κB, CRE, and NF-IL6, are important regulators of the expression of *COX-2* gene [15, 30–33]. In our RASMC, catalase significantly increased the binding of CRE and NF-IL6 but not that of NF-κB (Fig. 6). Catalase increased binding of NF-IL6 in a concentration-dependent fashion (Fig. 6B), but produced a maximum effect on CRE binding at a concentration of 2250 U/mL (Fig. 6A). Conversely, catalase at high concentrations tended to inhibit NF-κB binding (Fig. 6C). Binding of the transcription factors to specific DNA sites in each case were blocked completely or almost completely by 100-fold excess of the related unlabelled probes, indicating that binding was specific.

Induction of COX Protein Expression

The expression of COX-2 protein, which was not detected at time 0, increased at 4 hr and remained elevated for at least 36 hr, following exposure of the cells to catalase (Fig. 7). This increase was also somewhat catalase concentration-related (Figs. 8 and 9). Catalase at 6750 U/mL induced a 40.1% greater increase in protein than it did at 250 U/mL. Similarly, the expression of COX-2 protein was increased by 51.8 and 48.4% by catalase (6750 U/mL) plus IL-1β (10 U/mL), compared with the expression induced by IL-1β alone, and by catalase (250 U/mL) plus IL-1β (Fig. 9). Obviously, these increases were lower than the accompanying changes in gene expression (58 and 94%, respectively; Figs. 1 and 2). COX-1 protein expression was not affected by either catalase or catalase plus IL-1β (Fig. 10).

Effects of Catalase on COX Activity

Exposure of RASMC to catalase (250–6750 U/mL) plus IL-1β (10 U/mL) for 24 hr increased the production of 6-keto-$PGF_{1\alpha}$ and PGE_2 induced by arachidonic acid by 47.1 and 35.9%, respectively, compared with cells treated with IL-1β alone. However, high concentrations of catalase (> 2250 U/mL) reduced the production of both prostaglandins to levels below those caused by IL-1β alone. Catalase alone did not affect prostaglandin production (Fig. 11, A and B).

Cell Viability

None of the treatments used, except catalase at concentrations > 6750 U/mL, significantly affected cell viability, as assessed by the MTT assay.

DISCUSSION

Here we present clear evidence that catalase stimulated the expression of mRNA and protein for COX-2 in RASMC, and that these effects were enhanced by IL-1β. There was an accompanying increase in COX activity at low concentrations of catalase, although high concentrations inhibited prostaglandin synthesis. Catalase did not affect the expression of either mRNA or protein for COX-1.

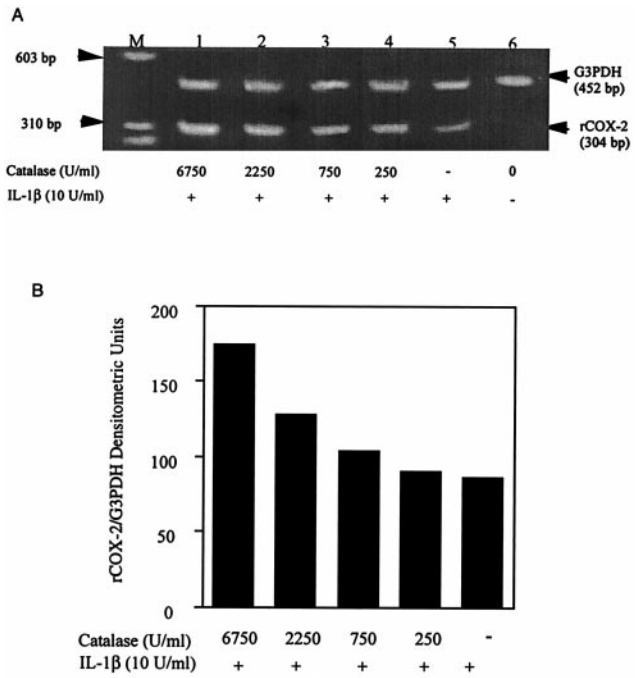

FIG. 2. Concentration-dependent induction of mRNA for COX-2 by catalase plus IL-1β. RASMC were incubated for 4 hr in medium alone (lane 6), medium supplemented with IL-1β (lane 5), or medium supplemented with IL-1β plus catalase (250–6750 U/mL) (lanes 1–4). For other conditions, refer to the legend of Fig. 1.

Increased levels of mRNA are often interpreted as being indicative of increased levels of gene expression, resulting in an enhanced rate of translation of the corresponding protein. This interpretation may be particularly true for COX. The mRNAs for COX-1 and COX-2 are unstable, as they have relatively short half-lives and undergo degradation during catalysis [2, 34, 35]. Therefore, to maintain constant enzyme activity, mRNA for COX must be transcribed continuously. In our study, we found the expression of mRNA for COX-2 to increase from 1 hr after the addition of IL-1β, to reach a maximum at 4 hr, and then to persist at steady-state levels for up to 12 hr. However, in the presence of IL-1β plus catalase, the steady-state level of the mRNA for COX-2 was maintained for 24 hr. This difference in mRNA survival may be conferred by binding factors. For instance, it is known that the AUUUA sequence repeats, and this motif located in the 3′ untranslated region of mRNA is related to decreased stabilities of a number of mRNAs, including that for COX-2 [36, 37]. Malter and Hong [38] have identified an AUBF, which complexes to a variety of labile RNAs containing multiple reiterations of the AUUUA sequence and stabilizes the mRNA. Importantly, AUBF activity can be blocked or inhibited by oxidizing agents, such as diamide and *N*-ethylmaleimide, suggesting that antioxidant agents may have a protective role. However, it is not known whether AUBF plays a role in stabilizing the mRNA for COX-2 produced in response to catalase and IL-1β. Interestingly, the ability of catalase to induce the expression of mRNA and protein for COX-2 in RASMC correlates with the observation that Zellweger syndrome, an autosomal recessive disease characterized by the absence of peroxisomes in which endogenous catalase is compartmentalized, is associated with a reduced production of prostaglandins due to a reduced expression of mRNA and protein for COX [39].

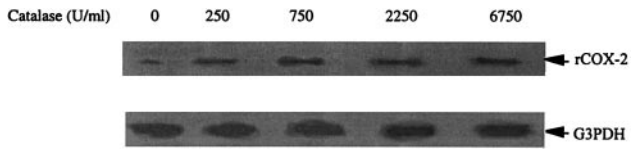

FIG. 3. Northern blot analysis of rat COX-2 RNA expression. RASMC were incubated for 4 hr either in medium alone or in medium supplemented with catalase, as indicated in the figure. Total RNA was fractionated by agarose/formaldehyde gel electrophrosis, and the RNA was transferred onto a nylon membrane. The rat COX-2 RNA message was detected by chemiluminescent assay.

Treatment of the cells with ACT, an RNA polymerase inhibitor, abolished the increase in mRNA for COX-2 induced by IL-1β, or catalase plus IL-1β, suggesting that the stimulatory effects of IL-1β and catalase on the expression of the *COX-2* gene are mainly transcriptional events. Furthermore, treatment of the cells with CHX, an inhibitor of protein synthesis, revealed two potentially important features of the regulatory mechanisms involved. First, accumulation of mRNA for COX-2 in response to catalase was abolished completely by pretreatment of cells with CHX, whereas CHX did not affect the expression of mRNA for COX-2 induced by IL-1β. This indicates strongly that the mechanisms leading to induction of mRNA for COX-2 following stimulation by catalase or IL-1β are different. In the case of catalase, the synthesis of intermediate proteins appears to be a prerequisite for efficient enhancement of transcription of the *COX-2* gene. Conversely, and particularly with regard to CRE, NF-IL6, and NF-κB, this may not be the case for IL-1β. However, whether other transcription factors are involved in the induction of *COX-2* gene expression by IL-1β is not known.

Enhanced gene expression is believed to involve an increase in the phosphorylation of one or more of the transcription factors necessary for gene transcription. In particular, studies have suggested that the transcription factor NF-κB is involved in the regulation of *COX-2* gene expression [15, 30]. However, we found that catalase did not enhance the activity of NF-κB in RASMC. Conversely, catalase significantly increased the activities of NF-IL6 and CRE transcription factors, suggesting that these two transcription elements, rather than NF-κB, are responsible for the increase in *COX-2* gene expression seen following exposure of RASMC to catalase. These findings are not surprising if we consider the following facts. First, there is no exact NF-κB binding sequence within the promoter region of the *COX-2* gene in the rat. Moreover, the sequences homologous to the consensus NF-IL6 and CRE sites are conserved in the *COX-2* genes from a number of species, including human, mouse, and rat [31–33].

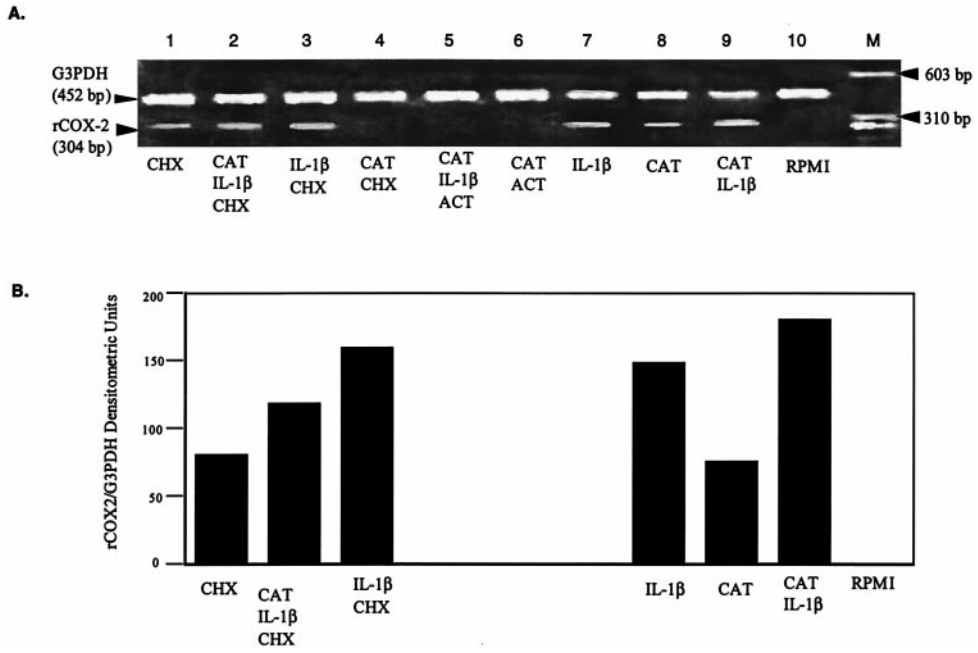

FIG. 4. Effect of RNA synthesis and protein synthesis inhibitors on the induction of mRNA for COX-2 by catalase, and catalase plus IL-1β. Cells were treated with ACT (2 μg/mL) or CHX (20 μg/mL) for 30 min before the addition of catalase (2250 U/mL) and/or IL-1β (10 U/mL). Then cells were incubated for a further 4 hr with different combinations of ACT (2 μg/mL), CHX (20 μg/mL), catalase (2250 U/mL), and IL-1β (10 U/mL), as indicated in Fig. 4A. For other conditions, refer to the legend of Fig. 1.

Second, transient transfection experiments have indicated that NF-IL6 and CRE play key roles in regulating the induction of the *COX-2* gene [40, 41]. Third, stable transfection of catalase inhibits NF-κB [42], although overexpression of catalase does not inhibit NF-κB activation in some circumstances [43]. Fourth, calpain inhibitor I, an apparent NF-κB inhibitor [44], does not suppress the expression of mRNA for COX-2 in our model (data not shown). It should be noted that results from our study do not exclude the possibility that other transcription factors such as AP-1 are involved in the increased expression of mRNA for COX-2 induced by catalase.

Catalase increased not only the expression of mRNA for COX-2 but also the amount of COX-2 protein. Interestingly, the expression of mRNA for COX-2 increased by more than 10-fold, from a low basal level, whereas the amount of immunogenic protein increased only 51.8%. This indicates that not all the mRNA for COX-2 induced was translated, suggesting that there is translational control of expression of mRNA for COX-2. It is unlikely that the imbalance between the expression of mRNA and protein for COX-2 is due to an increased turnover of the latter, because the half-life for the mRNA for COX-2 is only a few minutes, which is much shorter than that of COX-2 protein (3 hr or longer) [2, 34, 35]. It is also evident in the present study that the increase in the COX-2 protein was sustained for at least 36 hr, whereas the significant elevation of mRNA for COX-2 was only maintained until 24 hr. The detailed pathways by which the signal of catalase goes into cells and stimulates the transcription factors in RASMC are unclear at present. In addition to its well-known antioxidant property, it has been reported that catalase is able to increase the production of IL-1 in culture cells [45].

To assess the regulation by catalase of the activity of COX, RASMC were treated with arachidonic acid, which is converted to prostaglandin H_2 by COX. This latter conversion was quantified by measuring the formation of two prostaglandin H_2 products, 6-keto-$PGF_{1\alpha}$ (the stable hydrolysis product of PGI_2) and PGE_2. In the presence of IL-1β, catalase had a biphasic effect on the production of both prostaglandins. Low concentrations of catalase increased their syntheses, whereas high concentrations inhibited them. This is in agreement with the earlier observation that antioxidants at low concentrations stimulate COX, but at high concentrations inhibit it [46]. Similarly, others have also demonstrated that antioxidants increase prostaglandin production [47], while hydrogen peroxide decreases their syntheses [13, 14]. These observations might be explained as follows. The COX enzyme, a combined endoperoxide synthase and hydroperoxidase, is prone to self-inactivation. This is due to the production of reactive oxygen species in the course of the metabolism of arachidonic acid to prostaglandin H_2. In addition, the speed of formation of prostaglandin H_2 from the intermediate hydroperoxide compound, prostaglandin G_2, is enhanced by oxidizable cosubstrates. At the same time, a trace of hydroperoxide is required to react with iron(III) haem at the active site of COX, forming a peroxy radical that can stereospecifically abstract a hydrogen atom from arachidonic acid and so start the process of prostaglandin G_2 formation. This model can account, therefore, both for the stimulation of the activity of COX by antioxidants and also for the inhibition afforded by hydroperoxide scavengers. One might also envisage a mechanism whereby catalase increases prostaglandin production by providing an excess of molecular oxygen (Reaction 1), which is one of the substances required for COX activity (Reaction 2).

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2 \qquad \text{(Reaction 1)}$$

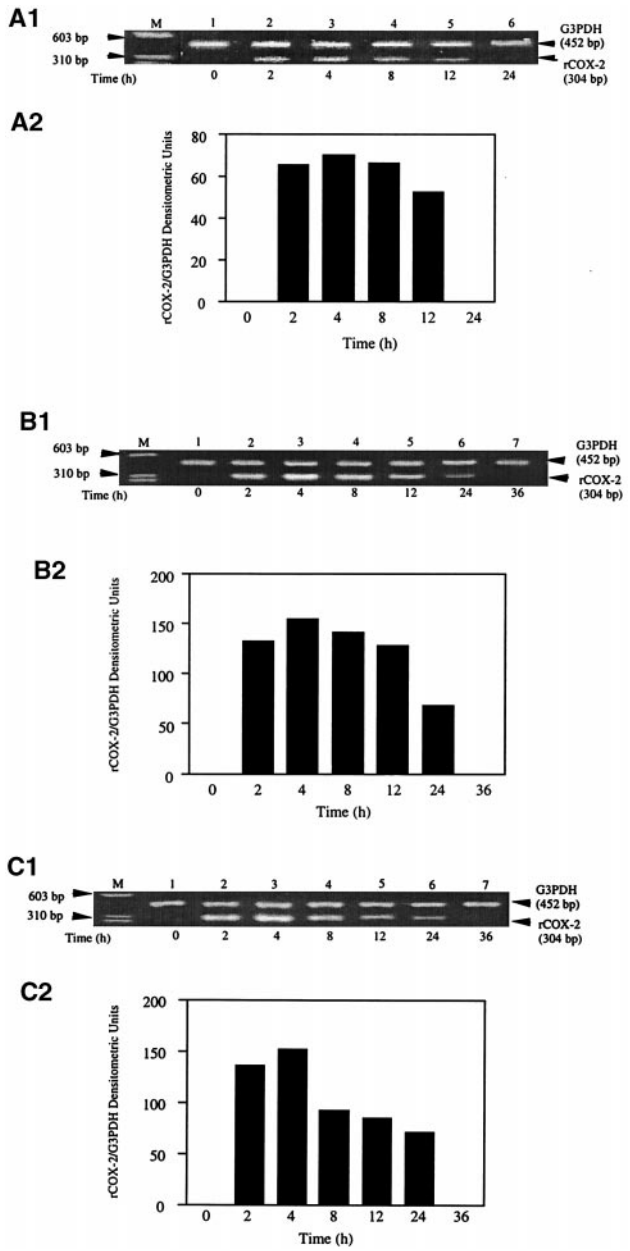

FIG. 5. Kinetics of the induction of mRNA for COX-2 by IL-1β (A), catalase (B), and catalase plus IL-1β (C). RASMC were incubated for the indicated times in medium supplemented with IL-1β (10 U/mL), catalase (2250 U/mL), and catalase (2250 U/mL) in combination with IL-1β (10 U/mL), respectively. Following cell lysis and RNA isolation, the levels of mRNA for COX-2 and G3PDH were assessed at each time point by RT-PCR. Data are reported as photographs of a typical agarose gel stained with ethidium bromide (A1, B1, and C1) or as graphs of the results obtained by quantitative densitometric analysis of photographic signals (A2, B2, and C2).

$$\text{Arachidonic acid} + 2O_2 \xrightarrow{\text{endoperoxide synthase}} \text{prostaglandin } G_2 \quad \text{(Reaction 2)}$$

Finally, the increase in the activity of COX caused by low concentrations of catalase may also result from increases in

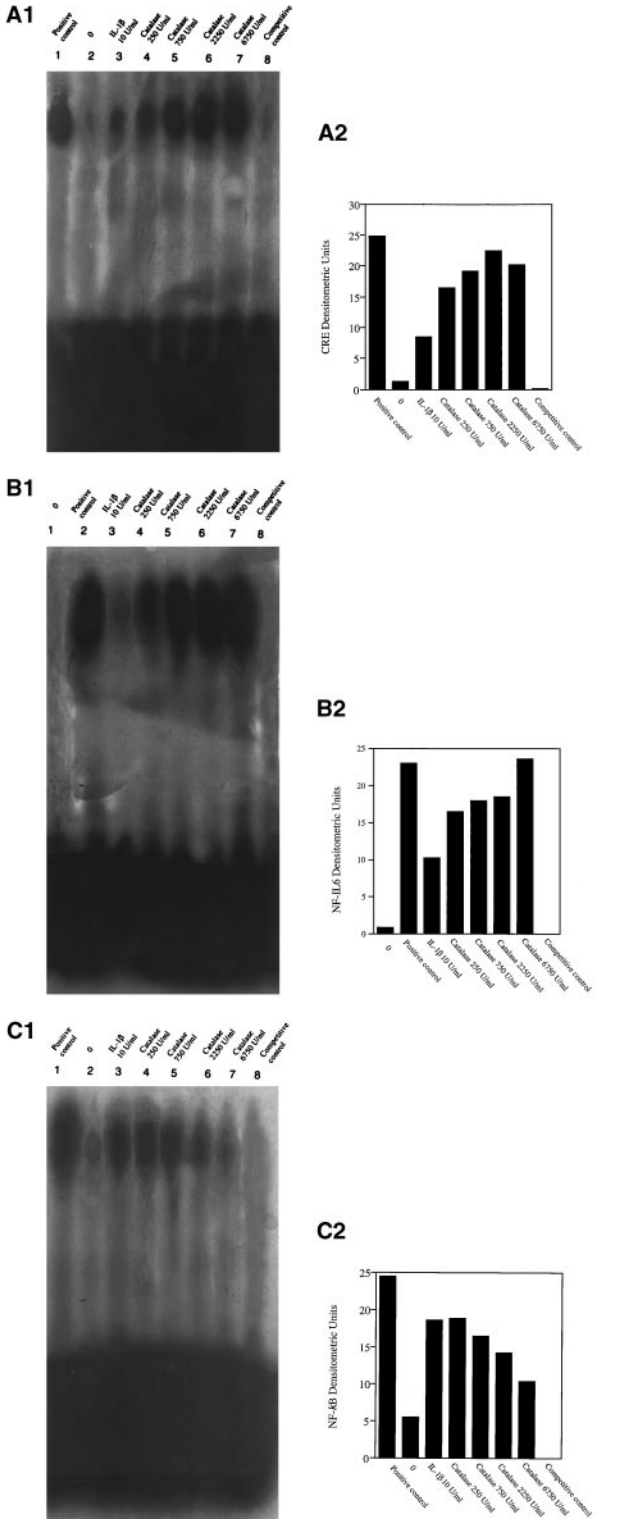

FIG. 6. Induction by catalase of nuclear factor binding to the CRE site (A), the NF-IL6 site (B) and the NF-κB site (C). RASMC were incubated with catalase (250–6750 U/mL) for 4 hr before preparation of nuclear extracts. Two micrograms of each extract was incubated with ^{32}P-labelled oligonucleotides, and the DNA–protein complexes formed were visualized on a 4% nondenaturing polyacrylamide gel (A1, B1, and C1). A Bio-Rad Image analysis system was used to quantitate the signals on the radiophotograph (A2, B2, and C2). A 100-fold molar excess of unlabelled oligonucleotides served as a competitive control (lane 8).

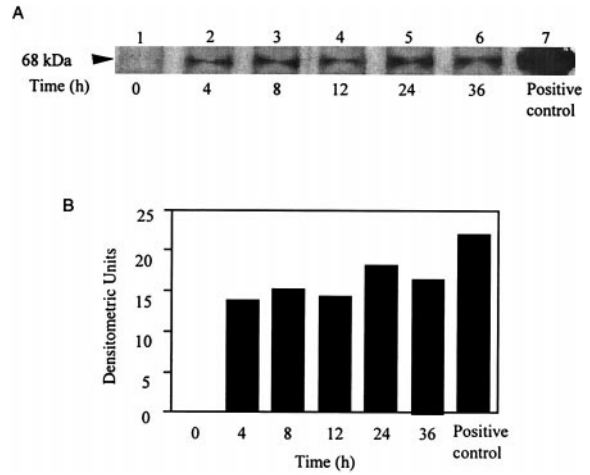

FIG. 7. Time–course study of the induction of COX-2 protein by catalase. RASMC were incubated for the indicated times in medium supplemented with catalase (2250 U/mL). Following cell lysis and protein isolation, equal amounts of cell protein (50 μg) were subjected to western blot analysis using COX-2 (1:1000) specific antibodies. Protein bands were visualized using a chemiluminescent detection kit. The top panel (A) shows actual protein bands detected by western blot. The bottom panel (B) is a graph of the results obtained by quantitative densitometric analysis of photographic signals.

the expressions of mRNA and protein for COX-2. However, it is clear that these increases in mRNA and protein for COX-2 are not the sole mechanisms underlying the effects of catalase on the activity of COX seen here. Currently, we cannot explain why catalase by itself has no significant effect on prostaglandin production, although others have found similarly that catalase cannot act alone

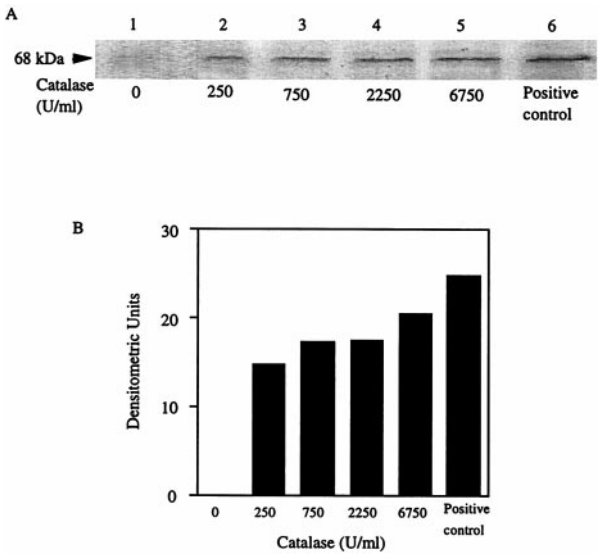

FIG. 8. Concentration–response analysis of the induction of COX-2 protein by catalase. RASMC were incubated for 4 hr either in medium (lane 1) or in medium supplemented with catalase (250–6750 U/mL) (lanes 2–5). The positive control is shown in lane 6. Following cell lysis and protein isolation, COX-2 protein levels were assessed in all samples by western blot analysis. For other conditions, refer to the legend of Fig. 7.

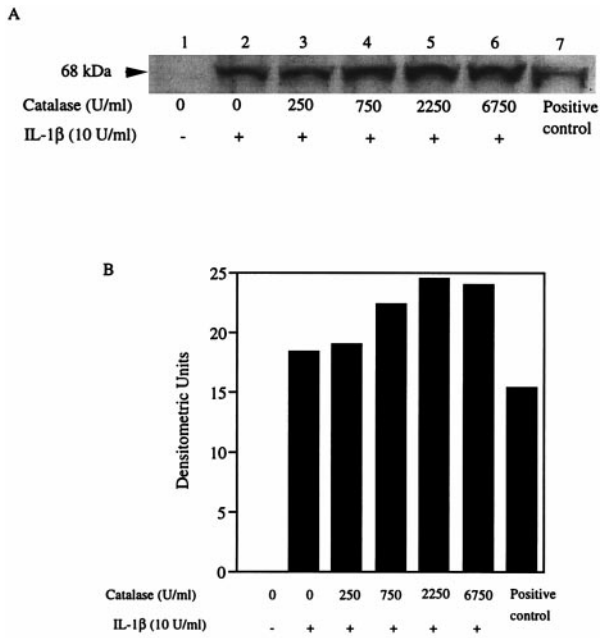

FIG. 9. Concentration–response analysis of the induction of COX-2 protein by catalase plus IL-1β. RASMC were incubated for 4 hr either in medium alone (lane 1), medium supplemented with IL-1β (lane 2), or medium supplemented with catalase (250–6750 U/mL) plus IL-1β (10 U/mL) (lanes 3–6). The positive control is shown in lane 7. For other conditions, refer to the legend of Fig. 7.

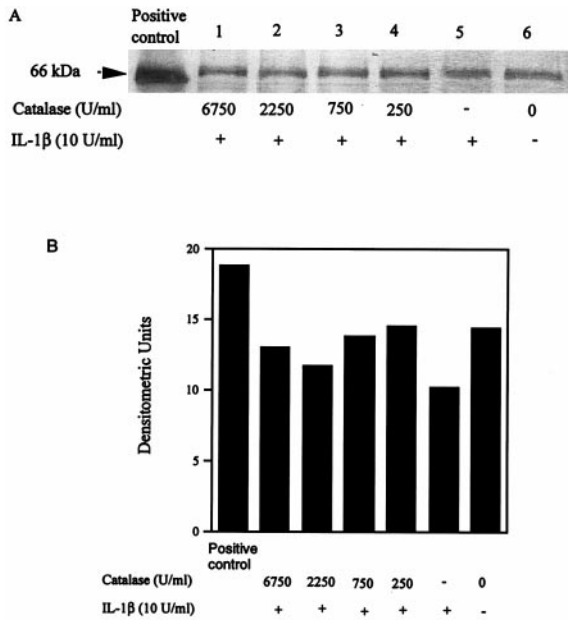

FIG. 10. Concentration–response analysis of the induction of COX-1 protein by catalase plus IL-1β. RASMC were incubated for 4 hr either in medium (lane 6), medium supplemented with IL-1β (lane 5) or medium supplemented with catalase (250–6750 U/mL) plus IL-1β (10 U/mL) (lanes 1–4). Following cell lysis and protein isolation, equal amounts of cell protein (50 μg) were subjected to western blot analysis using COX-2 (1:5000) specific antibodies. For other conditions, refer to the legend of Fig. 7.

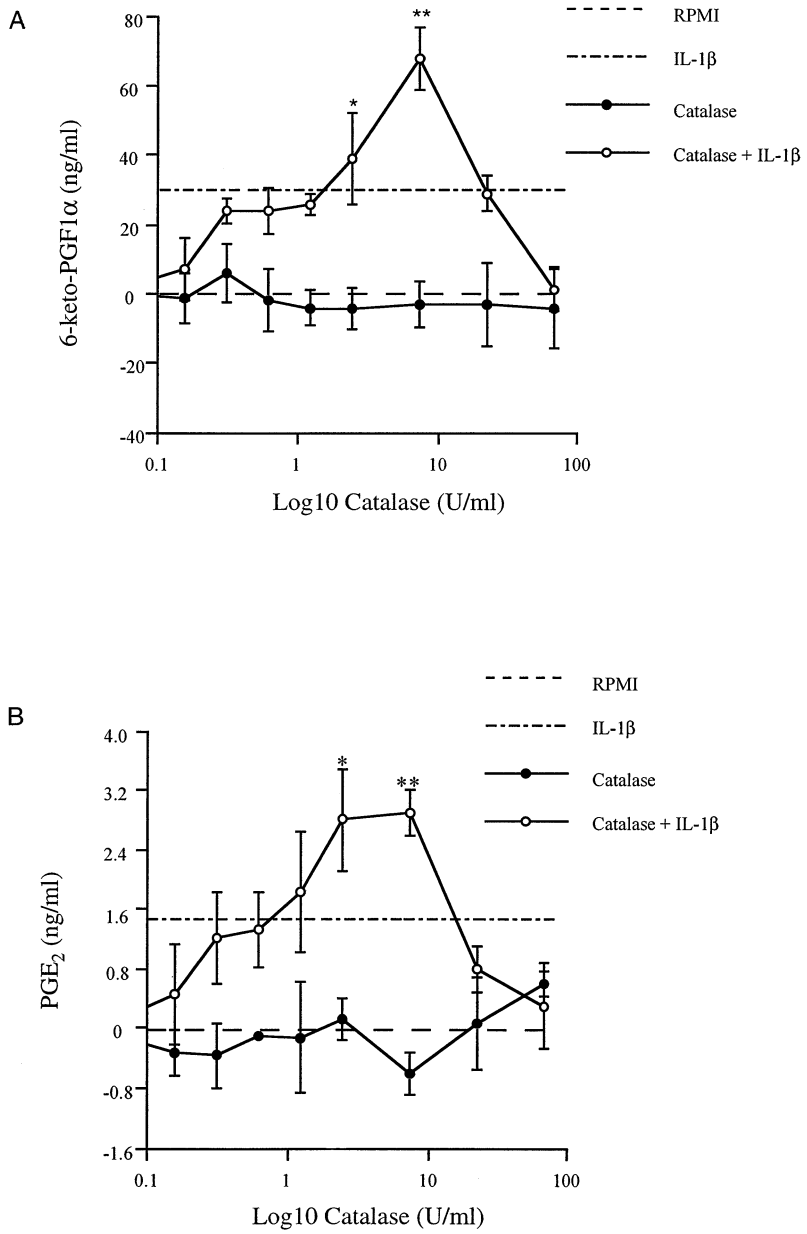

FIG. 11. Effect of IL-1β, catalase, and their combination on COX activity. COX activity was estimated by measuring the formation of 6-keto-$PGF_{1\alpha}$ (stable hydrolysis product of PGI_2) (A) and PGE_2 (B). RASMC were incubated with IL-1β, catalase, or catalase plus IL-1β for 24 hr. After this time the cells were exposed to medium containing arachidonic acid (30 μM) for 10 min at 37°. The amounts of 6-keto-$PGF_{1\alpha}$ and PGE_2 present in the supernatants were then measured by radioimmunoassay. Values are expressed as means ± SD, N = 6. *$P < 0.05$, and **$P < 0.01$, when compared with cells treated with IL-1β alone.

to regulate this production [48]. Only a comprehensive analysis of the interactions of various types of antioxidants and free radical species with COX, and a better understanding of the mechanisms regulating the enzyme function at molecular levels, will help resolve this issue.

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