Signal Transduction in Smooth Muscle
Selected Contribution: Synergism between TNF-α and IL-1β in airway smooth muscle cells: implications for β-adrenergic responsiveness

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Moore, Paul E., Thomas Lahiri, Johanne D. Laporte, Trudi Church, Reynold A. Panettieri, Jr., and Stephanie A. Shore. Selected Contribution: Synergism between TNF-α and IL-1β in airway smooth muscle cells: implications for β-adrenergic responsiveness. J Appl Physiol 91: 1467–1474, 2001.—In human cultured airway smooth muscle cells, interleukin (IL)-1β increases cyclooxygenase (COX)-2 expression and PGE2 release, ultimately resulting in decreased β-adrenergic responsiveness. In this study, we aimed to determine whether tumor necrosis factor-α (TNF-α) synergizes with IL-1β in the induction of these events. TNF-α alone, at concentrations up to 10 ng/ml, had no effect on COX-2 protein expression; at concentrations as low as 0.1 ng/ml, it significantly enhanced the ability of IL-1β (0.2 ng/ml) to induce COX-2 and to increase PGE2 release. IL-1β and TNF-α in combination also significantly enhanced COX-2 promoter activity, indicating that synergism between the cytokines is mediated at the level of gene transcription. Although IL-1β and TNF-α each increased nuclear factor-κB activation and induced extracellular regulated kinase and p38 phosphorylation, combined administration of the cytokines did not enhance either nuclear factor-κB or mitogen-activated protein kinase activation. Combined administration of IL-1β (0.2 ng/ml) and TNF-α (0.1 or 1.0 ng/ml) reduced the ability of isoproterenol to decrease human airway smooth muscle cell stiffness, as measured by magnetic twisting cytometry, even though individually these cytokines, at these concentrations, had no effect on isoproterenol responses. Treatment with the selective COX-2 inhibitor NS-398 abolished the synergistic effects of TNF-α and IL-1β on β-adrenergic responsiveness. Our results indicate that low concentrations of IL-1β and TNF-α synergize to promote β-adrenergic hyporesponsiveness and that effects on COX-2 expression and PGE2 are responsible for these events. The data suggest that the simultaneous release in the airway, of even very small amounts of cytokines, can have important functional consequences.

The cyclooxygenase (COX) enzyme catalyzes the conversion of arachidonic acid to prostaglandins and thromboxane. Two isoforms of the enzyme have been described. COX-1 is constitutively expressed in most mammalian cells, whereas COX-2 is induced under conditions of inflammation, for example, by certain cytokines, lipopolysaccharide, and mitogens (6, 15, 21, 28). Interleukin (IL)-1β is among the cytokines with the most potent effects on COX-2 expression. In human airway smooth muscle (HASM) cells, IL-1β induces COX-2 expression and PGE2 release. In these cells, the induction of COX-2 by IL-1β has important functional consequences, including β2-adrenergic hyporesponsiveness (18, 27), a characteristic feature of asthma (4, 5, 12). The precise role of COX-2 in these events has not been firmly established but is thought to involve the release of PGE2, consequent increase in cAMP formation, protein kinase A activation, and phosphorylation of the β2-adrenergic receptor by protein kinase A, uncoupling it from Gs (18).

Although tumor necrosis factor-α (TNF-α) has also been shown to induce COX-2 expression in some cell types (13, 16, 34), HASM cells do not increase their
expression of COX-2 even after treatment with very high (100 ng/ml) concentrations of TNF-α (28). TNF-α alone also has no effect on β-adrenergic responsiveness in HASM cells (27). However, when TNF-α is administered in conjunction with IL-1β, it increases the ability of IL-1β to induce COX-2 (6, 28). The ability of TNF-α to enhance the effects of IL-1β may be particularly important, since increased levels of both of these cytokines have been observed in bronchoalveolar lavage (BAL) fluid of patients with symptomatic asthma (7, 8, 33). Whether TNF-α also enhances the ability of IL-1β to induce β-adrenergic responsiveness has not been established.

To date, synergism between TNF-α and IL-1β in HASM cells has been demonstrated only at fairly high concentrations of these cytokines (10 ng/ml each) and the signaling cascade leading to this interaction is not known. The first purpose of this study was to determine whether there is also synergism between TNF-α and IL-1β at lower concentrations (in the 0.1–1.0 ng/ml range) and to examine the mechanistic basis for this synergism. Such effects may be particularly important to establish because concentrations of TNF-α and IL-1β measured in BAL fluid of asthmatic subjects fall within this range (7, 8). To determine whether the interaction between the cytokines occurs at the transcriptional level, we used a COX-2 promoter/luciferase reporter construct. Because the extracellular regulated kinase (ERK) and p38 mitogen-activated protein (MAP) kinases and the transcription factor nuclear factor (NF)-κB have been implicated in the induction of COX-2 by IL-1β and/or TNF-α either in HASM cells or in other cell types (16, 17, 19, 24, 34), we also determined whether there was synergism between IL-1β and TNF-α at the level of MAP kinase or NF-κB activation.

The second purpose of the study was to determine whether TNF-α and IL-1β can also synergize in the induction of β-adrenergic hyporesponsiveness. β-Adrenergic responsiveness was determined by measuring isoproterenol-induced changes in cell stiffness using magnetic twisting cytometry. We and others have established that changes in stiffness can be used as a surrogate for force generation in these cells (9, 14, 20, 31). Because our results indicated synergism at the level of COX-2 and prostanooid synthesis as well as β-adrenergic responsiveness and because we have previously reported that COX-2 expression is required for the β-adrenergic hyporesponsiveness induced by IL-1β alone (18), we also assessed the effects of the selective COX-2 inhibitor NS-398 on the decreased responsiveness to β-agonists that was observed in cells treated with low concentrations of TNF-α and IL-1β.

METHODS

Cell culture. Human tracheas were obtained from lung transplant donors, in accordance with procedures approved by the University of Pennsylvania Committee on Studies Involving Human Beings. Tracheal smooth muscle cells were harvested from the tracheas as previously described (18, 26, 29). Cells were plated in plastic flasks at 10^4 cells/cm^2 in Ham’s F-12 medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (1 mg/ml), amphotericin-B (2 mg/ml), NaOH (12 mM), CaCl_2 (1.7 μM), L-glutamine (2 mM), and HEPES (25 mM). Culture medium was replaced every 3–4 days. Cells were passaged with 0.25% trypsin and 1 mM EDTA every 10–14 days. Confluent serum-deprived HASM cells in passages 5–7 were used in the studies described below. When cells were serum deprived, they were supplemented with 5.7 μg/ml insulin and 5.0 μg/ml transferrin 24–48 h before use.

Western blotting for measurement of COX-2 expression and ERK and p38 phosphorylation. Confluent HASM cells were serum deprived and treated with IL-1β (0.2 ng/ml) alone or in combination with TNF-α (0.1 and/or 1.0 ng/ml). Cytokine treatment was for 15 min in the case of ERK and p38 activation and for 20 h in the case of COX-2 expression. Medium was removed, and cells were washed with PBS and then lysed in 400 μl of extraction buffer (10 mM Tris-HCl buffer with 50 mM NaCl, 50 mM NaF, 10 mM d-merine, 1 mM EDTA, 1 mM ECTA, 1% SDS, 1% Triton X-100, 0.2 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 1 μg/ml pepstatin, 10−2 U/ml aprotinin). Cells were scraped off flasks, passed through a 25 and 58-gauge needle, and solubilized by sonication. Western blot analysis using antibodies to phosphorylated p38, phosphorylated p42/p44 ERK (New England Biolabs, Beverly, MA), or COX-2 (Oxford Biomedical Research, Oxford, MI) was performed as previously described (17–19). The standards for phosphorylated p42 ERK and phosphorylated p38 and for COX were also obtained from New England Biolabs and Oxford Biomedical Research. The bands visualized at 38 kDa (p38), 42 and 44 kDa (ERK), or 70 kDa (COX-2) were quantified by laser densitometry.

PGE_2 release. Cells were plated in 24-well plates, grown to confluence, and serum deprived for 24 h. Cells were then either left untreated or treated with IL-1β (0.02–2.0 ng/ml), TNF-α (0.01–1.0 ng/ml), or combinations of these cytokines. Approximately 22 h later, cell medium was removed, cells were washed with PBS, and 0.5 ml of fresh medium was added to each well. After a 15-min incubation at 37°C, the supernatants were harvested and stored at −20°C until assayed with a PGE_2 enzyme immunoassay kit (Caymen Chemical, Ann Arbor, MI). The antibody to PGE_2 had <1% cross-reactivity to 6-keto-GF_2, and <0.01% to thromboxane B2 and other prostaglandins, according to the manufacturer’s specifications.

Magnetic twisting cytometry. Confluent cells were serum deprived for 24 h and treated with TNF-α (0.1 or 1.0 ng/ml) alone or in combination with IL-1β (0.2 ng/ml). Eighteen hours later, cells were harvested by brief exposure to trypsin and EDTA, resuspended in serum-free hormone supplemented medium with or without cytokines, and plated at 20,000 cells/well on collagen I (500 ng/cm^2)-coated bacteriological plastic dishes (6.4 mm, 96-well Removawells, Immunon II). Two to six hours later, measurements of cell stiffness were made using magnetic twisting cytometry as previously described (17–19, 22, 23, 29). Cumulative concentration-response curves to isoproterenol were performed as follows. First, three to five measurements of cell stiffness were made under baseline conditions. After these measurements, 2 μl of a solution containing the isoproterenol were added to the well that contained 200 μl of medium. After a 1-min incubation with agent, two to four measurements of cell stiffness were again obtained. This procedure was repeated with concentrations of isoproterenol increased from 10−8 to 10−5 M.

To examine the effect of NS-398 on IL-1β- and TNF-α-induced changes in cell stiffness responses to isoproterenol, four flasks of cells from the same passage of the same donor
were used. Two flasks were treated with NS-398 (10^{-5} M). Two hours later, IL-1β (0.2 ng/ml) and TNF-α (0.2 ng/ml) were added to one of the flasks treated with NS-398 and was also added to an untreated flask. Eighteen hours later, the cells were harvested and used for cell stiffness measurements as described above.

**HASM cell transfection.** After passage, HASM cells were grown for 72 h (60–80% confluence) in complete medium in six-well tissue culture plates. Before transfection with the COX-2 promoter/luciferase reporter construct (COX2-S), the medium was changed to contain only serum-free media, to avoid growth factor induction of COX-2. HASM cells were cotransfected with 0.5 μg of COX2-S and 0.5 μg of a β-galactosidase control vector (Promega, Madison, WI), using Fu gene 6 (Roche, Indianapolis, IN) according the manufacturer’s protocol. The β-galactosidase control vector was used to normalize for differences in transfection efficiency. The ~500-bp fragment of the promoter region of human COX-2 was constructed by creating SacI (~471 bp) and Nhel (~4 bp upstream of ATG) sites from a larger 2.0-kb fragment of the COX-2 promoter (gift of Stephen M. Prescott), and this fragment was cloned into the respective sites of the pGL3-basic vector (Promega). Cells were then incubated for 15 h with either IL-1β (0.2 ng/ml) or TNF-α (1.0 ng/ml) alone or with the two cytokines in combination. Cells were lysed with reporter lysis buffer (Promega), harvested, and assayed for luciferase activity by spectrophotometry using the β-galactosidase enzyme assay system (Promega).

The results of the experiments are reported as mean luciferase activity normalized for β-galactosidase activity. With the use of this system, transfection efficiency typically ranges between 10 and 20%, as assessed by flow cytometry of cells transfected with a green fluorescence protein-expressing vector.

To examine the ability of IL-1β and TNF-α to synergize in the activation of NF-kB, HASM cells were cotransfected with 0.5 μg of pNF-xB-Luc, designed for monitoring the NF-xB signal transduction pathway (Clontech, Palo Alto, CA) and with 0.5 μg of a β-galactosidase control vector as described above, except that, for these experiments, the medium was changed from 10% to 1% fetal bovine serum just before transfection. Cells were treated with cytokines and luciferase and β-galactosidase activities were measured as described above.

**Reagents.** Tissue culture reagents and drugs used in this study were obtained from Sigma Chemical (St. Louis, MO), with the exception of amphotericin and trypsin-EDTA, which were obtained from Gibco (Grand Island, NY), and TNF-α and IL-1β, which were obtained from R&D Systems (Minneapolis, MN). Isoproterenol (10^{-1} M in distilled water) was made fresh each day. Because isoproterenol is rapidly oxidized, dilutions of isoproterenol in medium were made immediately before addition to the cells. NS-398 was dissolved in DMSO at 10^{-2} M and diluted in culture medium.

**Statistics.** The effect of TNF-α and IL-1β on changes in stiffness induced by isoproterenol was examined by repeated-measures ANOVA using drug and experimental day as main effects. Follow-up tests were performed to determine at which concentration of isoproterenol the drug treatment effect was observed. The Bonferroni rule was used to correct for multiple comparisons. The effect of TNF-α and IL-1β on PGE_2 release was assessed by ANOVA using experimental day, TNF-α dose, and IL-1β dose as main effects. The effect of TNF-α and IL-1β on COX-2 expression, COX-2 promoter activity, and NF-xB luciferase activity was determined by ANOVA using experimental day and drug treatment as main effects. Follow-up paired t-tests were performed to compare among drug treatment groups. A P value 0.05 was considered significant.

**RESULTS**

TNF-α at concentrations up to 10 ng/ml had no effect on COX-2 expression in HASM cells from four different donors (data not shown). However, TNF-α did synergize with IL-1β in the induction of COX-2 expression. For these experiments, cells were treated with a concentration of IL-1β (0.2 ng/ml) that induced a small but less than maximal increase in COX-2 expression (18) and with concentrations of TNF-α in the same range (0.1–1.0 ng/ml). A representative COX-2 Western blot is shown in Fig. 1A. As previously described, COX-2 was not expressed in control, untreated cells. IL-1β induced COX-2 expression, whereas TNF-α did not. However, when the two cytokines were administered simultaneously, there was a marked increase in COX-2 expression compared with cells treated with IL-1β alone. Similar results were obtained in cells from six HASM cell donors (Fig. 1B). There was also synergism between IL-1β and TNF-α at the level of PGE_2 release. IL-1β caused a concentration-related increase in PGE_2 release (P < 0.001), as previously described (18), whereas TNF-α failed to increase PGE_2 release. PGE_2

![Fig. 1. A: Western blot showing cyclooxygenase-2 (COX-2) expression in human airway smooth muscle (HASM) cells pretreated for 20 h with interleukin (IL)-1β (0.2 ng/ml); tumor necrosis factor-α (TNF-α, 0.1 or 1.0 ng/ml), or IL-1β and TNF-α in combination. The lane on the far left is a COX-2 standard (ST). B: densitometric analysis of COX-2 Western blots. Results are means ± SE of data obtained on 13 experimental days in cells from 6 different donors. *P < 0.05 compared with untreated cells; #P < 0.05 compared with cells treated with IL-1β alone.](http://jap.physiology.org/ by 10.220.33.4 on June 9, 2017)
release into HASM cell supernatants averaged 0.40 ± 0.15 ng/ml in control cells and 0.36 ± 0.10, 0.37 ± 0.15, 0.62 ± 0.25, and 0.47 ± 0.16 ng/ml in cells treated with 0.01, 0.1, 1.0, or 10.0 ng/ml TNF-α for 20 h (P > 0.05). Although TNF-α alone had no effect on PGE2 release, it significantly augmented the effects of IL-1β (P < 0.05 by ANOVA) (Fig. 2). This was particularly apparent at 1.0 ng/ml TNF-α but was also observed at 0.1 ng/ml TNF-α.

To determine whether the effects of IL-1β and TNF-α we observed are mediated at the level of COX-2 transcription, HASM cells were transiently transfected with a COX-2 promoter/luciferase construct and treated with IL-1β (0.2 ng/ml) and TNF-α (1.0 ng/ml) alone or in combination. As shown in Fig. 3, neither cytokine on its own increased COX-2 promoter activity; however, in combination, there was a significant increase (P < 0.05) in COX-2 promoter activity that was only slightly smaller in magnitude than that induced by IL-1β at a 100-fold higher concentration (20 ng/ml). We reasoned that this effect on COX-2 promoter activity might be the result of IL-1β and TNF-α synergizing in their ability to activate NF-κB, since consensus binding sites for NF-κB are present in the COX-2 gene in the region of the promoter that we used in our construct (2). To address this possibility, HASM cells were transiently transfected with an NF-κB/luciferase reporter construct (Fig. 4). Although IL-1β (0.2 ng/ml) and TNF-α (1.0 ng/ml) each increased NF-κB promoter activity, the combinations of these cytokines did not significantly augment the increase observed with IL-1β on its own.

Because we have previously demonstrated that both the ERK and p38 MAP kinases are required for induction of COX-2 by higher concentrations of IL-1β (2.0 ng/ml) (17, 19), we reasoned that the synergism between IL-1β and TNF-α might be mediated at the level of MAP kinase activation. Hence, we treated HASM cells with IL-1β (0.2 ng/ml) and TNF-α (0.1 ng/ml) alone or in combination and examined their effects on ERK and p38 phosphorylation. As shown in Fig. 5, IL-1β and TNF-α at these concentrations each caused phosphorylation of ERK and p38. However, when the two cytokines were combined, there was no further augmentation of the effects of IL-1β by TNF-α. Similar results were obtained in cells from three donors (data not shown).

We have previously reported that COX-2 expression and PGE2 release are involved in the decreased β-adrenergic responsiveness induced by IL-1β in HASM cells (18). Hence, we sought to determine whether the ability of TNF-α to synergize with IL-1β in the induction of COX-2 expression and PGE2 release (Figs. 1 and 2) might lead to increased β-adrenergic desensitization. Isoproterenol-induced changes in HASM cell stiffness, as measured by magnetic twisting cytometry, were used as the index of β-adrenergic responsiveness. Because the greatest synergism in terms of PGE2 release was observed when IL-1β at 0.2 ng/ml was administered with TNF-α at 0.1 or 1.0 ng/ml (Fig. 2), we
examined the effects of these combinations of cytokines on responses to isoproterenol. We have previously reported that IL-1β (0.2 ng/ml) alone has no effect on cell stiffness responses to isoproterenol (29), although higher concentrations do influence these responses. Similarly, TNF-α alone at 0.1 ng/ml did not alter responses to isoproterenol: isoproterenol caused a concentration-dependent decrease in cell stiffness in control cells, and the magnitude of this decrease in stiffness was not significantly different in cells treated with TNF-α (Fig. 6). TNF-α at 1.0 ng/ml did have a statistically significant effect on cell stiffness responses to isoproterenol, but the magnitude of the effect was small and was apparent only at 10^{-7} and 10^{-6} M isoproterenol. Note that baseline stiffness was not affected by TNF-α, averaging 117 ± 7.0, 137 ± 11.6, and 125 ± 8.4 dyn/cm^2 in control cells and cells treated with 0.1 or 1.0 ng/ml TNF-α, respectively (P > 0.05).

Whereas no effect of IL-1β and either no or a very small effect of TNF-α depending on the concentration was observed when the cytokines were administered separately, the cytokines had profound effects on HASM cell responses to isoproterenol when they were administered together, virtually abolishing the response to isoproterenol (Fig. 7). None of the cytokine combinations had any significant effect on baseline cell stiffness.

To determine whether the effects of combined IL-1β and TNF-α treatment on β-adrenergic responsiveness were the result of COX-2 expression and PGE2 release, we examined the effect of the selective COX-2 inhibitor NS-398 (10^{-5} M) on IL-1β and TNF-α-induced changes in the ability of isoproterenol to evoke cell stiffness changes (Fig. 8). Neither NS-398 nor the combination of IL-1β and TNF-α alone or with NS-398 had any significant effect on baseline stiffness. Repeated-measures ANOVA indicated a significant effect of drug treatment on cell stiffness responses to isoproterenol (P < 0.01). Follow-up analysis indicated that the treatment effect lay in the combined IL-1β and TNF-α treatment group in which ISO responses were significantly different from control at all isoproterenol concentrations (P < 0.05). In contrast, neither cells treated with NS-398 alone nor cells treated with NS-398 in combination with IL-1β and TNF-α were significantly different from control cells. Note that the data in Fig. 8 were derived from donors different from those in Fig. 7, which likely accounts for the difference in the efficacy of isoproterenol in the control cells.

**DISCUSSION**

Our results indicate that TNF-α alone has no effect on COX-2 expression and PGE2 release. However, even

Fig. 5. A: representative Western blot showing p38 mitogen-activated protein (MAP) kinase phosphorylation in HASM cells pretreated for 20 h with IL-1β (0.2 ng/ml) and TNF-α (0.1 ng/ml) alone or in combination. The lane on the far left is a phosphorylated p38 standard (ST). B: representative Western blot showing phosphorylation of p42 and p44 extracellular regulated kinase MAP kinase in HASM cells pretreated for 20 h with IL-1β (0.2 ng/ml) and TNF-α (0.1 ng/ml) alone or in combination. The lane on the far left is a phosphorylated p42 standard.
very small concentrations of TNF-β can augment the ability of IL-1β to induce these effects in HASM cells (Figs. 1 and 2). Furthermore, synergism between IL-1β and TNF-α appears to occur at the level of COX-2 gene transcription (Fig. 3), but this transcriptional effect does not appear to involve increased activation of NF-κB (Fig. 4); NF-κB activation in cells treated with IL-1β and TNF-α was similar to that induced by IL-1β alone. Similarly, both IL-1β and TNF-α alone induced p38 and ERK phosphorylation, but we did not observe any synergism between IL-1β and TNF-α in their effects on MAP kinase activation (Fig. 5). The combination of TNF-α and IL-1β also caused marked β-adrenergic hyporesponsiveness in HASM cells (Fig. 7), and this effect was abolished by the selective COX-2 inhibitor, NS-398 (Fig. 8).

TNF-α alone did not increase COX-2 expression or PGE₂ release. These results confirm those of Pang and Knox (28) in HASM cells. TNF-α also has no effect on COX-2 expression in the human macrophage U-937 cell line (3), although it does induce COX-2 expression and/or PGE₂ release in human monocytes, human HT-29 epithelial cells, and the murine MC3T3-E1 osteogenic cell line (13, 16, 34). It is not clear why there are cell type-related differences in the expression of COX-2 by TNF-α.

Synergism between IL-1β and TNF-α in their effects on COX-2 expression and PGE₂ in HASM cells has been reported by other investigators (6, 28). However, these investigators used 10- to 100-fold higher concentrations of the cytokines in their studies and did not examine effects of cytokine combinations on β-adrenergic responsiveness or the mechanism of interaction between IL-1β and TNF-α. To our knowledge, we are the first to report synergistic effects of these cytokines at these lower concentrations on HASM cells. These findings may be of particular importance, since TNF-α and IL-1β are often released together, and the concentrations of TNF-α and IL-1β measured in BAL fluid of symptomatic asthmatics fall within this range (7, 8). We do not know whether the concentrations of IL-1β and TNF-α in the vicinity of the airway smooth muscle are greater or less than the concentrations in BAL fluid, but our results suggest that even very mild airway inflammation, if it results in the release of both IL-1β and TNF-α, may lead to COX-2 expression and β-adrenergic hyporesponsiveness.

Synergism between IL-1β and TNF-α in their effects on COX-2 protein expression and/or PGE₂ release has also been reported in other cell types (10, 32, 35). Because the cytokines also exert synergistic effects on COX-2 mRNA expression (10, 35), their effects are likely to be mediated at the level of COX-2 transcription or message stabilization. Indeed, both IL-1β and TNF-α have been shown to increase COX-2 transcription in some cell types (10, 24, 34). Our results (Fig. 3), using a COX-2 promoter/luciferase reporter construct, suggest that the synergistic effects of IL-1β and TNF-α are due to transcriptional effects, at least in part. IL-1β and TNF-α have also been demonstrated to stabilize COX-2 mRNA in a murine osteogenic cell line (13). There is an AU-rich region in the 3'-untranslated region of the COX-2 gene, and protein binding to this region has been postulated to increase COX-2 mRNA stability (30). We cannot rule out the possibility that such effects also contribute to the synergism between IL-1β and TNF-α.

The promoter region of the COX-2 gene contains consensus sequences for binding of the transcription factor NF-κB (2). In addition, activation of NF-κB occurs in response to both IL-1β and TNF-α in HASM cells (1, 19, 23) and has been reported to play a role in the transcriptional regulation of COX-2 by IL-1β and TNF-α in some cell types (16, 24, 34). Although NF-κB does not appear to contribute to IL-1β-induced COX-2 expression in HASM cells (19), we reasoned that it might still be involved in the synergistic effects exerted by IL-1β and TNF-α. However, our results suggest that this is unlikely. Although both IL-1β and to a lesser extent TNF-α caused activation of NF-κB even at fairly low concentrations, the combination of the two cytokines did not increase the magnitude of NF-κB activation that was effected by IL-1β alone (Fig. 4).

We have previously established that both the ERK and p38 MAP kinases are required for the COX-2 expression and β-adrenergic hyporesponsiveness induced by higher concentrations of IL-1β (2 ng/ml) in HASM cells (17, 19). ERK and p38 are activated by IL-1β, and relatively selective inhibitors of each of these pathways cause marked decreases in IL-1β-induced COX-2 expression and PGE₂ release and abolish the effects of IL-1β on β-adrenergic responsiveness in HASM cells. Because TNF-α has also been demonstrated to induce ERK and p38 activation in HASM cells (25), we reasoned that one explanation for the

**Fig. 8.** Effect of NS-398 (10⁻⁶ M, 24 h) in the presence or absence of IL-1β (0.2 ng/ml) and TNF-α (0.1 ng/ml) each for 22 h, on changes in ISOPROTERENOL (log M) STIFFNESS (% baseline) COX-2 protein expression and/or PGE₂ release has also been reported in other cell types (10, 32, 35). Because the cytokines also exert synergistic effects on COX-2 mRNA expression (10, 35), their effects are likely to be mediated at the level of COX-2 transcription or message stabilization. Indeed, both IL-1β and TNF-α have been shown to increase COX-2 transcription in some cell types (10, 24, 34). Our results (Fig. 3), using a COX-2 promoter/luciferase reporter construct, suggest that the synergistic effects of IL-1β and TNF-α are due to transcriptional effects, at least in part. IL-1β and TNF-α have also been demonstrated to stabilize COX-2 mRNA in a murine osteogenic cell line (13). There is an AU-rich region in the 3'-untranslated region of the COX-2 gene, and protein binding to this region has been postulated to increase COX-2 mRNA stability (30). We cannot rule out the possibility that such effects also contribute to the synergism between IL-1β and TNF-α.

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synergistic effects of TNF-α and IL-1β in HASM cells might be effects at the level of ERK and/or p38 activation. Our results confirm that both IL-1β and TNF-α cause p38 and ERK phosphorylation even at fairly low concentrations. However, when administered together, the cytokines did not augment phosphorylation of these MAP kinases above the levels induced by either cytokine alone (Fig. 5). Our results also indicate that, although activation of p38 and ERK are required for induction of COX-2 expression (17, 19), their activation is not sufficient for COX-2 expression because TNF-α alone induced phosphorylation of both proteins but did not induce COX-2.

We have previously reported that IL-1β at concentrations 2.0 ng/ml and greater causes β-adrenergic hyporesponsiveness in HASM cells and that the mechanistic basis for this effect is likely to involve uncoupling of the β-receptor from Gs (29). The observations that COX-2 is induced by IL-1β, that PGE2 mimics the effects of IL-1β, and that COX-2 inhibitors ablate the effects of IL-1β on β-adrenergic responses suggest that COX-2-generated prostanoids are involved in the signal transduction pathway leading from IL-1β to effects on β-adrenergic responsiveness (18) (27). One hypothesis is that COX-2-generated PGE2 leads to increased cAMP formation, activation of protein kinase A, and consequent phosphorylation of the β-receptor (18). We now report that TNF-α at concentrations that on their own have either no effect or very minimal effects on responses to β-agonists (Fig. 6) has marked effects on β-adrenergic responsiveness (Fig. 7) if administered simultaneously with IL-1β at a concentration that on its own has no effect (29). The same concentrations of IL-1β and TNF-α synergize in the induction of COX-2 expression and PGE2 release (Figs. 1 and 2). Taken together with the observation that the relatively selectively COX-2 inhibitor, NS-398, abolishes the effects of combined treatment with low concentrations of IL-1β and TNF-α (Fig. 8), the results suggest that COX-2-generated prostanoids mediate the effects of the combination of IL-1β and TNF-α on β-adrenergic responsiveness.

Decreased β-adrenergic responsiveness is a characteristic feature of human asthma. Decreased bronchodilator responses to β-agonists have been observed in asthmatic airways both in vivo (5) and in vitro (4, 12), as well as in animal models of asthma (11). β-Agonists are currently one of the most important forms of therapy for asthma, and understanding the mechanistic basis for the β-adrenergic receptor dysfunction in asthma may prove to be an important step in improving the efficacy of these agents. Our results suggest that the release of IL-1β and TNF-α in the asthmatic airway may contribute to the β-adrenergic hyporesponsiveness of asthma. Furthermore, the data suggest that the release of even very small amounts of these cytokines can have important functional consequences if they are released simultaneously.

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