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β-Adrenergic Stimulation of Rat Cardiac Fibroblasts Enhances Induction of Nitric-Oxide Synthase by Interleukin-1β via Message Stabilization

ÅSA B. GUSTAFSSON and LAURENCE L. BRUNTON

Biomedical Sciences Graduate Program (Å.B.G.), Departments of Pharmacology and Medicine (L.L.B.), University of California at San Diego, La Jolla, California

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ABSTRACT

We have investigated factors modulating expression of inducible NO synthase (iNOS) in isolated adult rat cardiac fibroblasts. Treatment of cardiac fibroblasts with interleukin-1β (IL-1β) promotes induction of iNOS mRNA and protein and production of NO. Simultaneous incubation of cells with isoproterenol enhances the response to IL-1β, even though isoproterenol alone is without effect. N^G-nitro-L-arginine methyl ester inhibits the effect of isoproterenol + IL-1β on NO production. β_2-Adrenergic receptors appear to mediate this effect of isoproterenol. Reverse transcriptase-polymerase chain reaction analyses show that β_2-receptor mRNA is the predominant β-receptor message; in pharmacologic studies, ICI-118,551 significantly antagonizes isoproterenol-stimulated cyclic AMP production whereas CGP20712A does not. Dibutyryl-cyclic AMP and forskolin mimic the synergistic effect of isoproterenol on IL-1β-induced NO production; H-89, a cyclic AMP-dependent protein kinase (PKA) inhibitor, antagonizes the enhancing effect of isoproterenol. Nuclear run-off experiments indicate that enhancement of iNOS by isoproterenol does not occur at the level of transcription. Message stability studies demonstrate that isoproterenol increases the half-life of iNOS mRNA from 1.0 to 1.9 h; this change is sufficient to account for the observed augmentation of iNOS mRNA and protein. Thus, cardiac fibroblasts produce significant amounts of NO in response to IL-1β via induction of iNOS; β-adrenergic stimulation enhances the IL-1β effect by stabilizing the iNOS message. These data suggest that cardiac fibroblasts could participate in a paracrine mechanism whereby the direct positive inotropic effect of β_1-adrenergic stimulation of myocytes is opposed by β_2-adrenergic enhancement of NO production, a negative inotropic event, in neighboring fibroblasts.

Nitric oxide (NO) is involved in myriad physiological functions including vasodilation, cytotoxicity, and neurotransmission (Wright et al., 1992; Bredt and Snyder, 1994). Within the heart, NO causes a negative inotropic effect on myocytes and relaxation of vascular smooth muscle (Balligand and Cannon, 1997). NO production is normally catalyzed by the two Ca^{2+}-calmodulin dependent isoforms of NO synthase. However, a third isoform, inducible NO synthase (iNOS), may be induced in cells stimulated by cytokines and lipopolysaccharides (Balligand and Cannon, 1997). In the course of studying the capacity of different cardiac cell types to produce NO (Villegas and Brunton, 1996), we have recently focused our attention on cardiac ventricular fibroblasts as a source of NO in the heart. In this study, we report on conditions that promote the expression of iNOS in ventricular fibroblasts isolated from adult rat heart.

The up-regulation of iNOS reportedly occurs in a variety of cardiac diseases, such as allograft rejection (Yang et al., 1994), myocardial infarction (Wildhirt et al., 1997), and septic shock (Wright et al., 1992). The relatively large quantities of NO released as a consequence of this induction may serve helpful roles, such as causing local vasodilation and fighting bacterial infections (Gazzinelli et al., 1992). However, a sustained NO production can also contribute to myocardial dysfunction, since NO acts as a negative modulator of contractile function (Brady et al., 1992; Balligand and Cannon, 1997), and cause damage to cells via the formation of reactive oxygen intermediates (Stamler et al., 1992).

Inducible NO synthase activity has been detected in a variety of cardiovascular cell types, including endothelial (Estrada et al., 1992) and smooth muscle cells (Schini et al.,

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ABBREVIATIONS: NO, nitric oxide; iNOS, inducible nitric-oxide synthase; IL, interleukin; TNF, tumor necrosis factor; IFN, interferon; L-NAME, N^G-nitro-L-arginine methyl ester; PKA, cyclic AMP-dependent protein kinase; DMEM, Dulbecco's modified Eagle's medium; BSA, bovine serum albumin; Iso, isoproterenol; DRB, 5,6-dichloro-1-β-ribofuranosyl benzimidazole; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-PCR; IBMX, isobutylmethylxanthine; db-cAMP, dibutyryl-cAMP; EPI, epinephrine; NE, norepinephrine; bp, base pair.

1992) and cardiac myocytes (Balligand et al., 1993). However, the expression of iNOS in cardiac fibroblasts is less well characterized and has not been studied in fibroblasts isolated from mature myocardium. Since fibroblasts represent approximately two-thirds of the cardiac cell population by cell number (Grove et al., 1969), the induction of iNOS in these cells could provide a sizable source of diffusable NO within the heart.

Recent studies have demonstrated that cyclic AMP can modulate NO production and iNOS expression in several cell types. For instance, elevated intracellular cyclic AMP induces expression of iNOS in rat renal mesangial (Kunz et al., 1994) and vascular smooth muscle cells (Koide et al., 1993). Furthermore, cyclic AMP has been reported to enhance cytokine-induced iNOS expression in cardiac myocytes (Ikeda et al., 1996) but to inhibit cytokine-mediated expression of iNOS in rat primary astrocytes and Kupffer cells (Pahan et al., 1997; Mustafa and Olson, 1998). Thus, there is not a single pattern that describes the effects of cytokines and cyclic AMP on iNOS expression; rather, the effects appear to vary among different cell types.

Using isolated adult rat ventricular fibroblasts, we have measured the capacity of a variety of cytokines to induce iNOS; we have also defined the effect of increased intracellular cyclic AMP on iNOS induction. We report for the first time that adult cardiac fibroblasts can be major producers of cardiac NO, that only one of the major cytokines, IL-1β, can induce iNOS, and that elevated cellular cyclic AMP enhances the effects of IL-1β by increasing the stability of the iNOS message. These findings suggest that β-adrenergic stimulation of cardiac tissue results in direct effects and can also contribute to an anti-adrenergic paracrine response.

Experimental Procedures

Materials. Rat recombinant IL-1β was purchased from Sigma-Aldrich (St. Louis, MO). Collagenase I and trypsin were obtained from Worthington (Freehold, NJ). The cDNA mouse iNOS probe, generated by reverse transcriptase-polymerase chain reaction (RT-PCR), was kindly provided by Dr. Carol L. Macleod (UCSD). [α-^{32}P]dCTP and sheep anti-mouse IgG-horseradish peroxidase were from Amersham Pharmacia Biotech (Piscataway, NJ). [α-^{32}P]UTP was from ICN (Los Angeles, CA). GAPDH was obtained from Ambion (Austin, TX). A monoclonal anti-iNOS antibody was obtained from Transduction Laboratories (Lexington, KY). All other reagents and chemicals were of reagent grade from Sigma-Aldrich or Calbiochem-Novabiochem (La Jolla, CA).

Isolation of Adult Ventricular Fibroblasts. Cardiac fibroblasts were isolated from adult male Sprague-Dawley rats weighing between 250 and 275 g, by a modification of a previously described protocol (Villarreal et al., 1995). Briefly, two to three hearts were excised and the atria were removed. The ventricles were minced and placed in a solution containing 100 U/ml collagenase I and 0.1% trypsin. The ventricles were subjected to periods of digestion at 37°C for 10 min; cells from the second to ninth digestion were pooled, centrifuged, and suspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and penicillin/streptomycin (100 U/ml). The cell suspension was divided between two uncoated plastic culture dishes (100 mm) for 30 min to allow for the preferential attachment of fibroblasts, after which unattached cells were rinsed off. The fibroblasts became confluent within 72 h and were subsequently passaged with trypsin. All cells used in experiments were from passages 2 through 4. The purity of these cultures were determined by immunofluorescent staining with anti-vimentin, anti-von Willebrand factor, and anti-α-smooth muscle cell actin for identification of fibroblasts, endothelial cells, and smooth muscle cells, respectively. The purity of these cultures was assessed to be >95% fibroblasts since the fibroblasts in culture at passage 1 or greater stained positive for vimentin and negative for von Willebrand factor and α-smooth muscle cell actin. Only minimal contamination was observed at passage 1 and was subsequently eliminated by passaging of the cells.

Measurement of Nitrite Levels. Fibroblasts were plated on 60-mm culture dishes and grown to 80 to 90% confluency. For experiments, cells were incubated in 1.5 ml of DMEM (phenol red-free, serum-free) supplemented with 1.5 mM L-arginine, 0.1 mg/ml BSA, 10 μg/ml leupeptin, and 100 U/ml penicillin/streptomycin with vehicle or drug added for 24 h. Nitrite in the medium was measured by mixing 150 μl of the medium with 900 μl of Griess reagent (one part 0.075% *N*-1-naphthylethylene-diamine dihydrochloride and one part 0.75% sulfanilamide in 0.5 N HCl). The absorbance at 543 nm was measured, and the nitrite concentration was determined using a standard curve of 150-μl aliquots of sodium nitrite (in concentrations ranging from 0.1 to 100 μM). Data are expressed as micromolar concentration of nitrite in the 150 μl of medium.

Assay of Cyclic AMP Accumulation. Cardiac fibroblasts were incubated with DMEM without serum for 2 h and then treated as described in the text at 37°C, after which the medium was aspirated and ice-cold 5% trichloroacetic acid was added. The trichloroacetic acid extracts were purified over Dowex-50, and the cyclic AMP content was determined according to the method of Gilman (1970).

RT-PCR. Total RNA was isolated using RNeasy kit (QIAGEN, Valencia, CA) and subjected to RT-PCR using SUPERSCRIPT Preamplification System (Life Technologies, Inc., Grand Island, NY). Sense (5′-CGCTCACCAAACCTCTTCATCATGTCC-3′) and antisense (5′-CAGCACTTGGGGTCGTTGTAGGAGC-3′) primers for the β_1-adrenergic receptor, sense (5′-CACCAACTACTTCATAACCTC-3′) antisense (5′-GGCAATCCTGAAATCTGGGCTCCGGCAG-3′) primers for the β_2-adrenergic receptor, and sense (5′-TGCGCCCAT-CATGAGCCAGTGGTG-3′) and antisense (5′-GCGAAAGTC-CGGGCTGCGGCAGTA-3′) primers for the β_3-adrenergic receptor were synthesized and used to amplify transcripts for the receptors (Troispoux et al., 1998, Wangemann et al., 1999). The samples were subjected to 40 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The PCR products were separated by electrophoresis through a 1% agarose gel and visualized by exposure to UV light.

For the nuclear run-off transcription assay, sense (5′-ATGGCTT-GCCCCTGGAAGTTTCTC-3′) and antisense (5′-CCTCTGATGGT-GCCATCGGGCATCTG-3′) primers for iNOS (Nunokawa et al., 1993), and sense (5′-AGCGGGAAATCGTGCGTG-3′) and antisense (5′-CAGGGTACATGGTGGTGCC-3′) for β-actin were synthesized (Reiling et al., 1998). The RT-PCR products of the expected sizes (~830 bp and ~350 bp, respectively) were ligated into pCR2.1 plasmids using a TA cloning kit (Invitrogen, Carlsbad, CA). The plasmids were linearized and denatured, and 5 μg of each was slot-blotted onto 0.45-μm nitrocellulose membranes.

Northern Analysis. Northern blots were performed as previously described (Farivar et al., 1996). Fibroblasts were grown on 100-mm culture dishes until they were 80 to 90% confluent. Cells were then incubated in serum-free media with vehicle or drug for 24 h. Total RNA was isolated using RNeasy kit (QIAGEN) and 10-μg aliquots of RNA were electrophoresed on a 1% formaldehyde gel, transferred to a nylon membrane, and cross-linked using a UV Stratalinker 2400 (Stratagene, La Jolla, CA). The membrane was hybridized with cDNA probes for mouse iNOS and GAPDH mRNA that were labeled with [α-^{32}P]dCTP by random priming (Stratagene), followed by washing under increasingly stringent conditions. Blots were exposed to X-ray film at −70° overnight.

Western Analysis. Fibroblasts (on 60-mm culture dishes) were lysed at 4°C in a buffer containing 50 mM β-glycerolphosphate (pH 7.5 at 4°C), 1 mM EGTA, 10 mM $MgCl_2$, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and, 10 μg/ml leupeptin. Equal amounts of total protein per lane were loaded and separated on a

7.5% SDS-polyacrylamide gel, then transferred to an Immobilon-P membrane (Millipore, Bedford, MA). After blocking in 5% nonfat milk, the membrane was incubated with an iNOS-specific monoclonal antibody overnight at 4°C, followed by a series of washes and incubation with a secondary antibody coupled to horseradish peroxidase for 1 h at 20°C. iNOS was detected using enhanced chemiluminescence (Amersham Pharmacia Biotech).

Nuclear Run-Off Transcription. Cells were scraped in ice-cold buffer A (20 mM Hepes, pH 7.4, at 4°C, 3 mM $MgCl_2$, 0.1 mM EGTA, 0.1 mM EDTA, 12.5 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 1% NP-40) and homogenized in a Dounce homogenizer on ice. The nuclei were isolated by centrifuging at 500*g* for 5 min, washed once in ice-cold buffer A, resuspended in storage buffer (50 mM Tris-HCl, pH 8.3, 40% glycerol, 5 mM $MgCl_2$, and 0.1 mM EDTA) and stored at −70°C. The nuclear run-off reaction was performed as described previously (Greenberg and Bender, 1997). For the run-off assay, about 1×10^7 nuclei were thawed and incubated in reaction buffer (10 mM Tris-HCl, 5 mM $MgCl_2$, 0.3 mM KCl, and 1 mM each of ATP, CTP, and GTP, and 100 μCi [^{32}P]UTP) at 30°C for 30 min. The ^{32}P-labeled RNA transcripts were isolated and equal amounts of labeled RNA were added to scintillation vials containing slot blot strips and allowed to hybridized at 42°C for 72 h. After hybridization, the membranes were washed, dried, and subjected to autoradiography for 7 to 9 days at −70°C.

Protein determination. Protein content was estimated by the method of Bradford (1976) using bovine serum albumin as a standard.

Analysis of Data. Analysis and graphing of data were performed with Prism 2.0 (GraphPad Software, San Diego, CA). All experiments were replicated at least three times using cells obtained from different fibroblast preparations; thus, in figure legends, n = the number of independent experiments. Data are expressed as mean ± S.E.M. Statistical analysis was performed by ANOVA or Student's t test. P values less than .05 were considered to indicate significance.

Results

Effects of Cytokines on NO Production. We examined the ability of several common inflammatory cytokines, known to induce iNOS in other cell types (see Balligand and Cannon, 1997 and *Discussion*), to induce expression of iNOS in cardiac fibroblasts. Twenty-four hour treatment of cardiac fibroblasts with IL-1β (10 ng/ml) resulted in a 10-fold increase in NO production compared with untreated cells, whereas treatment of cells with IL-2 (10 ng/ml), IL-6 (10 ng/ml), TNF-α (100 ng/ml), or IFN-γ (500 U/ml) for 24 h resulted in no detectable increase in NO production (Fig. 1A). We also examined the induction of iNOS in response to cytokine treatment by Western analysis (Fig. 1B). Consistent with the levels of NO production, cardiac fibroblasts treated with IL-2, IL-6, TNF-α, or IFN-γ expressed no detectable levels of iNOS protein, whereas cells treated with IL-1β for 24 h showed expression of iNOS (Fig. 1B). Thus, IL-1β was the only common cytokine that noticeably stimulated NO production and induced expression of iNOS in rat cardiac fibroblasts. Addition of 1 mM N^G-nitro-L-arginine methyl ester (L-NAME), a competitive inhibitor of NO synthase, inhibited the effect of IL-1β, indicating that the NO assayed is likely derived from the activity of NO synthase (Fig. 1C).

Effects of β-Adrenergic Agonists on NO Production and iNOS mRNA Accumulation. We have previously determined that rat cardiac fibroblasts have β-adrenergic receptors that couple to G_s-adenylyl cyclase (Meszaros et al., 2000). To determine whether β-adrenergic stimulation could affect IL-1β-stimulated NO production, we incubated the cells simultaneously with 10 μM isoproterenol (Iso) and 10 ng/ml IL-1β for 24 h. Isoproterenol alone had no effect on NO production, whereas a simultaneous treatment of cells with Iso and IL-1β doubled NO production compared with the effect of IL-1β treatment alone (Fig. 2A). Inclusion of L-NAME (1 mM) abolished the effect of isoproterenol and IL-1β on NO production, confirming that the assessed NO derived from NO synthase activity.

The naturally occurring catecholamines, epinephrine and norepinephrine, also caused this same effect. Simultaneous

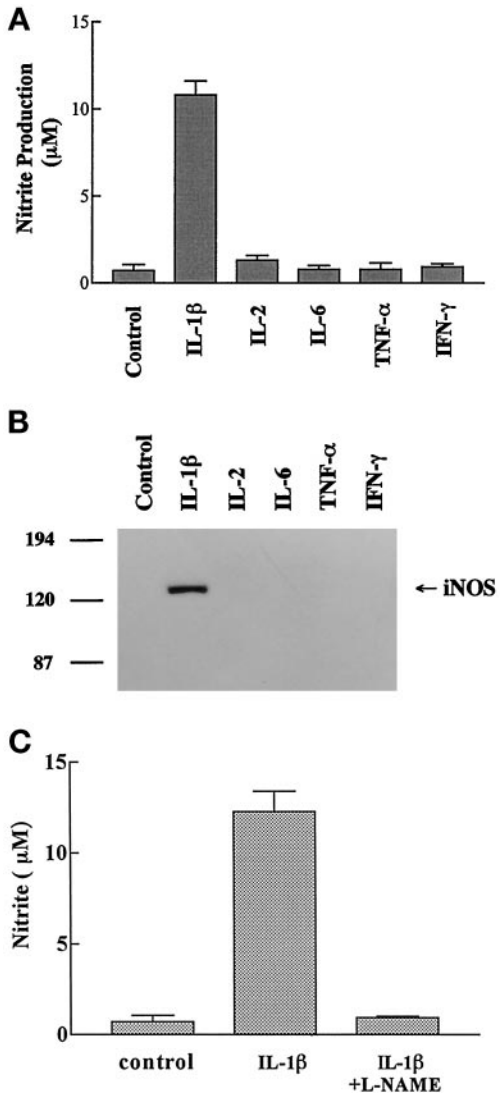

Fig. 1. IL-1β induces iNOS and stimulates NO production in adult rat cardiac fibroblasts. A, cardiac fibroblasts were incubated with diluent, 10 ng/ml IL-1β, 10 ng/ml IL-2, 10 ng/ml IL-6, 100 ng/ml TNF-α, or 500 U/ml IFN-γ for 24 h. Nitrite accumulating in the medium was measured as described under *Experimental Procedures*. IL-1β caused a significant increase in NO production over basal ($P < .001$, $n = 4$), whereas the other cytokines had no significant effect. B, cells were treated as described above for 24 h. Samples were subjected to SDS-polyacrylamide gel electrophoresis, followed by immunoblot analysis using a monoclonal antibody against iNOS. A 130-kDa band corresponding to iNOS is detected in lysates from cells stimulated with IL-1β. Blot is representative of three replicate experiments. C, the fibroblasts were treated with diluent, 10 ng/ml IL-1β or 10 ng/ml IL-1β + 1 mM L-NAME for 24 h. L-NAME completely inhibited the IL-1β-induced nitrite accumulation. Data are mean ± S. E., $n = 4$.

incubation of cells with 10 ng/ml IL-1β and 10 μM norepinephrine or 10 μM epinephrine for 24 h resulted in approximately a doubling of NO production compared with the effect of IL-1β treatment alone (Fig. 2B). Norepinephrine and epinephrine alone had no effect on NO production.

The enhancing effects of β-adrenergic stimulation could be due to an increase in the cellular content of iNOS or to a direct effect on the enzymatic activity of iNOS. We tested the idea of covalent activation of iNOS by adding isoproterenol (10 μM, 10 min) to cells induced by IL-1β (10 ng/ml for 24 h); in such a protocol, isoproterenol was without effect on NO production (data not shown); thus, we concluded that the β-adrenergic effect was not explained simply as the activation of existing enzyme by phosphorylation.

Next, we examined whether Iso induced an increase in iNOS mRNA levels in cardiac fibroblasts. Consistent with the levels of NO production, control cardiac fibroblasts expressed no detectable iNOS mRNA, whereas cells treated with IL-1β for 24 h expressed a large amount of iNOS mRNA and simultaneous incubation with Iso and IL-1β for 24 h enhanced iNOS mRNA expression about 2-fold compared with IL-1β-stimulated cells (Fig. 3A). In additional replications of this experiment, the mean enhancement by Iso of the IL-1β effect was 2.1- $\pm$ 0.2-fold (Fig. 3B). These data suggest that the basis of the isoproterenol-enhanced NO production is at the level of iNOS mRNA expression.

Characterization of β-Adrenergic Receptor Subtypes. We have previously suggested that the β_2-adrenergic receptor predominates in the cardiac fibroblasts based on the order of potency data (EPI > NE) (Meszaros et al., 2000) and on the fact that ventricular myocytes express few β_2-receptors but there are many β_2-receptors in the intact rat ventricle (Buxton and Brunton, 1985), that is, there are β_2-receptors on the non-myocyte components. To determine more definitively which β-adrenergic receptor subtype is responsible for the enhancement of NO production in response to isoproterenol, we performed RT-PCR analysis using primers specific for the β_1-, β_2- and β_3-adrenergic receptors to identify the receptor subtype messages expressed in cardiac fibroblasts. Reactions with primers for the β_1- and β_2-adrenergic receptors revealed RT-PCR products of the expected sizes, 376 and 805 bp, respectively (Fig. 4A). The predominant β-receptor subtype expressed in cardiac fibroblasts appeared to be the β_2-subtype. A faint, but observable and reproducible signal for the β_1-adrenergic receptor suggests that the cardiac fibroblasts express a few β_1-receptors as well. In contrast, no product was detected using the primers specific for

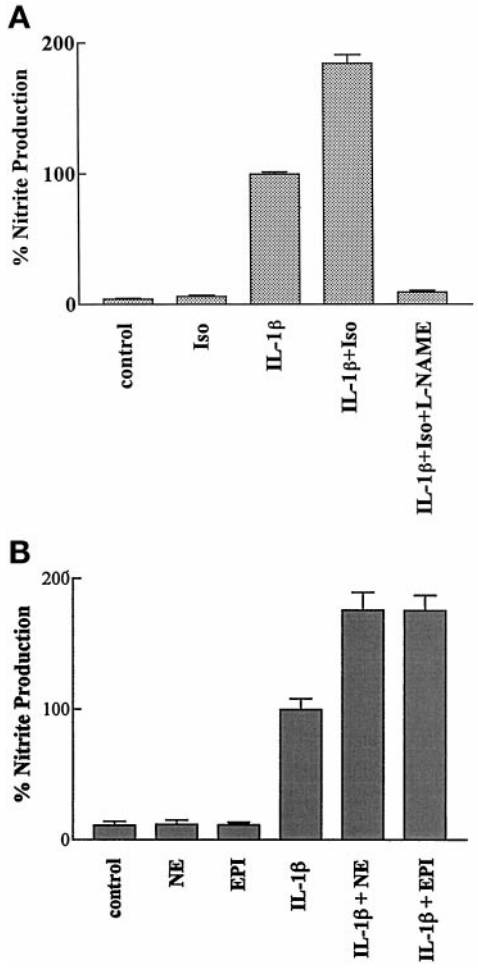

Fig. 2. Catecholamines enhance NO production in IL-1β-stimulated fibroblasts. A, cells were incubated with diluent, Iso (10 μM), IL-1β (10 ng/ml), IL-1β + Iso, and IL-1β + Iso + L-NAME (1 mM) for 24 h. Nitrite accumulation was assayed as described for Fig. 1. IL-1β + Iso caused a significant increase in NO production compared with IL-1β alone ($P <$ .0001). Data are mean $\pm$ S.E.M., $n = 4$. B, cells were incubated with diluent, norepinephrine (NE, 10 μM), epinephrine (EPI, 10 μM), IL-1β (10 ng/ml), IL-1β + NE, or IL-1β + EPI for 24 h. IL-1β + NE and IL-1β + EPI caused a significant increase in NO production over IL-1β-stimulated cells ($P <$ .005). Data are mean $\pm$ S.E., $n = 3$.

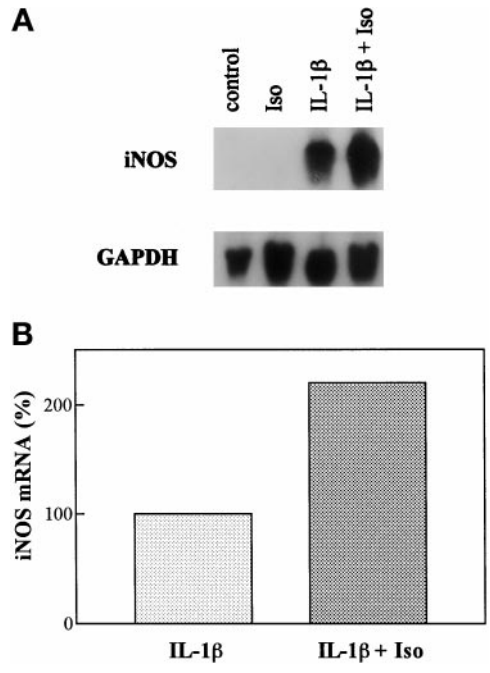

Fig. 3. Isoproterenol enhances iNOS mRNA accumulation in IL-1β-stimulated cells. A, Total RNA was extracted from fibroblasts that had been incubated with diluent (control), Iso (10 μM), IL-1β (10 ng/ml), and IL-1β + Iso for 24 h. Ten micrograms of RNA/lane were electrophoresed and analyzed by Northern blotting for the mRNA for iNOS (upper panel) and GAPDH (lower panel), as described under *Experimental Procedures*; each lane was run in duplicate. Data are from a representative experiment replicated four times. B, quantitation. To correct for loading differences, the densitometric signal of each iNOS mRNA sample was divided by the corresponding GAPDH mRNA density; the effect of IL-1β alone was set to 100% and the values for IL-1β + Iso were plotted as a percentage of IL-1β signal. IL-1β treatment induced iNOS mRNA accumulation; Iso enhanced the effect of IL-1β about 2-fold compared with IL-1β alone ($P <$ .05). Data are mean $\pm$ S.E., $n = 4$.

the β_3-adrenergic receptor, suggesting that the fibroblasts do not contain β_3-adrenergic receptors. The specificities of the primers were verified by PCR analysis using genomic DNA from the fibroblasts; the PCR products were distinct and of the predicted molecular sizes.

We further investigated the functional presence of β_1- and β_2-adrenergic receptors in the cardiac fibroblasts pharmacologically using the subtype-specific antagonists ICI-118,551 and CGP20712A (Fig. 4B). CGP20712A (10 nM), a β_1 selective antagonist, did not significantly reduce the capacity of 10 nM isoproterenol to cause cyclic AMP accumulation. Using literature values for the binding constants (Kaumann, 1997), we calculate that this concentration of CGP20712A should occupy 90% of β_1- and 0.1% of β_2-receptors in the presence of 10 nM Iso. The β_2-selective antagonist ICI-118,551, at 10 nM, which should occupy 4% of β_1- and 90% of β_2-receptors in the presence of 10 nM Iso, reduced the response to isoproterenol by about 90%. These data suggest that the β_2-adrenergic receptor is the predominant subtype expressed and functionally coupled to cyclic AMP production in the cardiac fibroblasts.

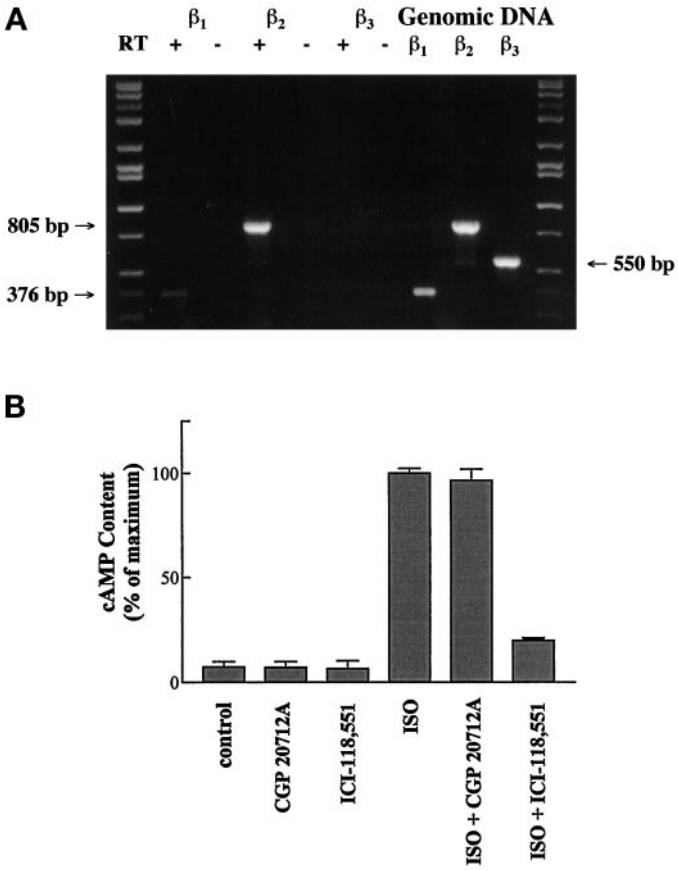

Fig. 4. A, RT-PCR analysis of β-adrenergic receptor mRNA levels in cardiac fibroblasts. Total RNA was isolated and subjected to RT-PCR as described under *Experimental Procedures*. Products were separated on a 1% agarose gel. All reactions were carried out in the presence (+) or absence (−) of reverse transcriptase. The specificities of the primers were verified by PCR analysis using genomic DNA from the fibroblasts. The PCR products were distinct and of the predicted molecular sizes. B, effects of β-adrenergic antagonists on isoproterenol-stimulated cyclic AMP accumulation. CGP20712A (10 nM) had no significant effect on isoproterenol-stimulated cAMP accumulation in cells treated with 10 nM isoproterenol ($P > .5$), whereas 10 nM ICI-118,551 significantly inhibited the response to isoproterenol ($P < .0001$). Data are mean ± S.E., $n = 3$.

Effects of Dibutyryl-Cyclic AMP, Forskolin, and a PKA Inhibitor. The enhancement of the IL-1β effect by β-adrenergic stimulation presumably results from $G_{s\alpha}$ stimulation of adenylyl cyclase and the consequent activation of PKA by cyclic AMP. However, it is possible that $G_{\beta\gamma}$ heterodimers, mobilized by β-adrenergic stimulation, mediate a non-cyclic AMP-dependent effect. We tested this possibility by using treatments that by-pass the receptor-$G_{s\alpha}$ mechanism and by employing an inhibitor of cyclic AMP-dependent protein kinase (PKA).

Dibutyryl-cyclic AMP (db-cAMP), a membrane permeable analog of cyclic AMP, and forskolin, a direct activator of adenylyl cyclase, reproduced the effect of Iso on iNOS induction. Simultaneous incubation of 100 μM db-cAMP or 20 μM forskolin with 10 ng/ml IL-1β for 24 h resulted in a significant increase in NO production compared with the effect of IL-1β alone (Fig. 5), indicating that the effect is mediated by cyclic AMP and does not necessarily involve alternative explanations.

Most of our experiments have used a relatively high concentration of the β-agonist, isoproterenol. We have also assessed the capacity of a full range of concentrations of Iso to stimulate cyclic AMP accumulation (a 5-min exposure) and to enhance IL-1β-induced NOS induction (assessed as nitrite accumulation measured after 24 h), both assessed in the absence of any phosphodiesterase inhibitor. These data (Fig. 6) show that low concentrations of isoproterenol (e.g., in the range of 10^{-10} M) that produce no more than a doubling of cellular cyclic AMP are sufficient to enhance the IL-1β effect. Furthermore, the data demonstrate that the capacity of the cells to accumulate cyclic AMP in response to isoproterenol far exceeds what is needed to enhance iNOS induction/NO production. Maximal NO accumulation occurred at a concentration of isoproterenol, ~5 nM, that caused half-maximal cyclic AMP accumulation.

This sensitivity of iNOS induction to modest elevations of cyclic AMP is also demonstrated by the effects of phosphodiesterase inhibition. Isobutylmethylxanthine, at 0.2 mM, doubled the basal level of cyclic AMP accumulation in the cells (Fig. 7A). This result suggests that cultured cardiac fibro-

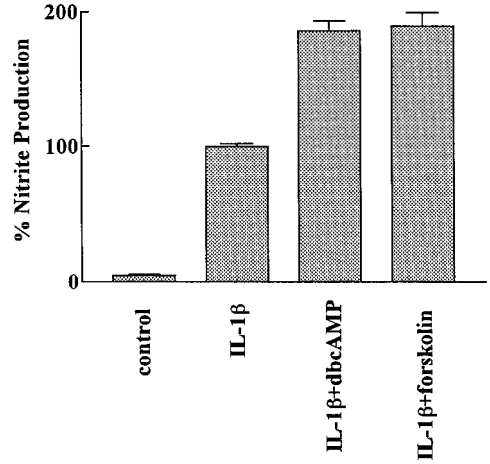

Fig. 5. Dibutyryl-cyclic AMP and forskolin enhance NO production in IL-1β-stimulated fibroblasts. Cells were incubated with diluent (control), IL-1β (10 ng/ml), IL-1β + db-cAMP (100 μM), or IL-1β + forskolin (20 μM) for 24 h. Nitrite accumulation was assessed as described for Fig. 1. Both db-cAMP and forskolin increased IL-1β-induced NO production ($P < .05$). Data are mean ± S.E., $n = 3$.

blasts express a relatively high basal activity of adenylyl cyclase and active phosphodiesterases. As consequence, 0.2 mM IBMX, by itself, accentuated the effect of IL-1β (Fig. 7B), again indicating that modest elevations in cellular cyclic AMP are able to enhance the induction of iNOS by IL-1β and that the elevation of cyclic AMP suffices to cause the enhancement; there is no need to invoke other receptor-mediated membrane events.

As confirmation of this conclusion and to determine whether cyclic AMP is acting via the cyclic AMP-dependent protein kinase, we tested the effect of H-89, a protein kinase A inhibitor, on NO production and iNOS protein expression.

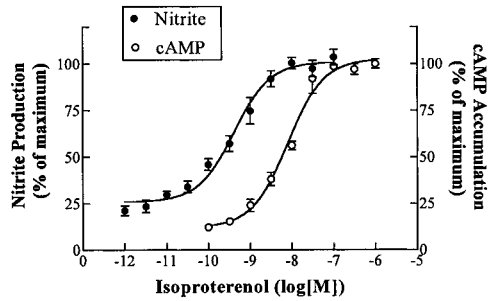

Fig. 6. Concentration-dependent stimulation of NO production and cyclic AMP accumulation by isoproterenol. For nitrite production, cardiac fibroblasts were incubated with 10 ng/ml IL-1β and Iso for 24 h, at which time nitrite accumulation (●) in the medium was measured as described for Fig. 1. For cyclic AMP accumulation, cells were stimulated with Iso for 5 min and the cyclic AMP content (○) was determined as described under *Experimental Procedures*. Data are mean ± S.E. of three experiments and are expressed as percentage of maximal response.

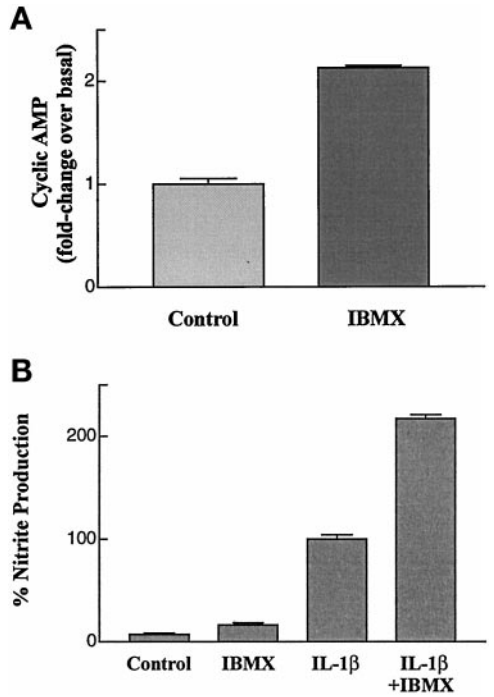

Fig. 7. Inhibition of phosphodiesterase activity by IBMX enhances NO production in IL-1β-stimulated fibroblasts. A, effect of IBMX on cellular cyclic AMP content. Confluent cultures of cardiac fibroblasts were treated with 0.2 mM IBMX for 15 min, after which cyclic AMP content was determined. IBMX significantly elevates cellular cyclic AMP content ($P < .0001$, $n = 3$). B, cardiac fibroblasts were treated with 0.2 mM IBMX, 10 ng/ml IL-1β, and IBMX + IL-1β for 24 h. IBMX significantly enhances NO production in IL-1β-stimulated cardiac fibroblasts ($P < .0001$; data are mean ± S.E., $n = 3$).

Inclusion of 10 μM H-89 virtually obliterated the enhancing effect of β-adrenergic stimulation but did not reduce the induction in response to IL-1β (Fig. 8). The changes in NO

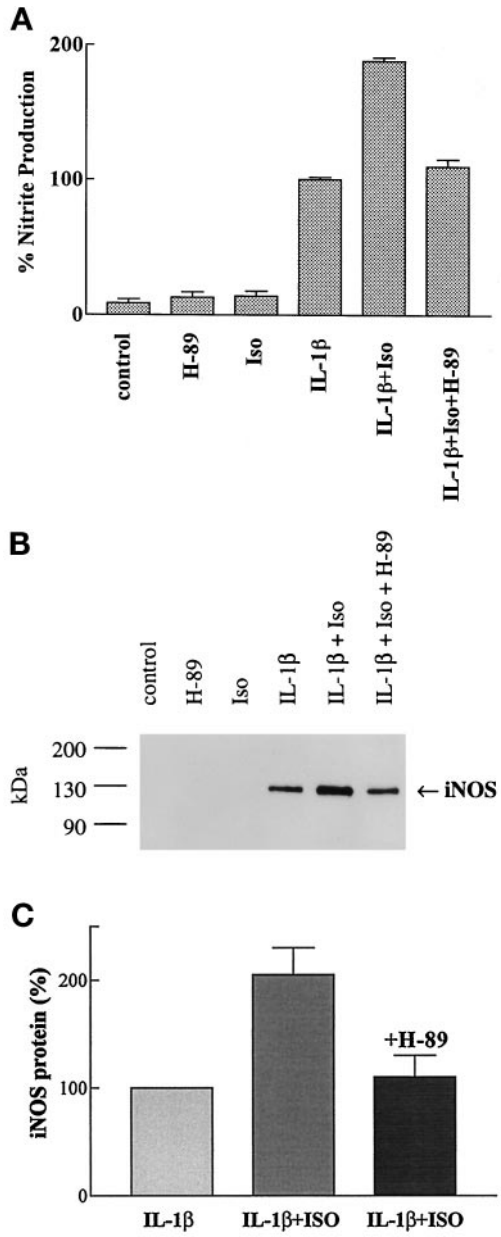

Fig. 8. PKA mediates effects of cyclic AMP on iNOS expression. A, H-89 inhibits isoproterenol-enhanced NO production. Cells were exposed to IL-1β (10 ng/ml) + Iso (10 μM) in the presence or absence of H-89 (10 μM, added 30 min before IL-1β and Iso). Nitrite in the culture medium was measured as described under *Experimental Procedures*. H-89 significantly inhibited the effect of Iso on NO production in IL-1β-stimulated cells ($P < .05$; data are mean ± S.E., $n = 3$). B, H-89 inhibits isoproterenol-enhanced iNOS expression. Cells were pretreated with H-89 (10 μM) as described above. Cell lysates were subjected to SDS-polyacrylamide gel electrophoresis followed by immunoblot analysis using a monoclonal anti-iNOS antibody. IL-1β treatment induced iNOS protein expression (130 kDa); cotreatment with Iso (10 μM) markedly increased the expression of iNOS protein; the addition of H-89 (10 μM) reduced the expression of iNOS to the level due to IL-1β alone. C, quantitation. The iNOS signals were quantified using NIH Image software and then plotted as a percentage of IL-1β stimulation. Iso + IL-1β treatment significantly increased the expression of iNOS protein compared with IL-1β treatment alone ($P < .05$); inclusion of H-89 reduced the expression of iNOS to the level due to IL-1β alone ($P > .5$ compared with IL-1β). Data are mean ± S.E., $n = 3$.

production (Fig. 8A) correlated with changes in iNOS protein content (Fig. 8, B and C); that is, simultaneous incubation of cells with IL-1β and Iso resulted in a significant increase not only in NO production but also of the iNOS protein compared with the effect of IL-1β alone. Furthermore, the inhibitory effect of H-89 was manifest in both NO production and protein content.

Effects of Isoproterenol on the Transcriptional Rate of iNOS. The data above suggest that the effect of elevated cyclic AMP is to increase the level of iNOS mRNA. Either an increase in transcription of the iNOS gene or an increase in the stability of the iNOS message following transcription could account for the changes in the iNOS mRNA and protein levels that we observe. To investigate the mechanism by which Iso enhances iNOS expression in IL-1β stimulated cells, we measured the effect of Iso treatment on the rate of transcription of the iNOS gene. Data from nuclear run-off experiments (Fig. 9A) show that iNOS transcripts are not detected in control cells or cells that were treated with Iso (10 μM) alone for 20 h, whereas cells that were treated with IL-1β (10 ng/ml) or IL-1β + Iso for 20 h show about the same level of iNOS transcripts. By densitometry, the ratio of nascent iNOS transcript to β-actin was 1.77 $\pm$ 0.1 in IL-1β-stimulated cells and 1.66 $\pm$ 0.2 in IL-1β + Iso-stimulated cells (Fig. 9B); these ratios are not significantly different ($P >$ .5; data are mean $\pm$ S.E., n = 3). These results indicate that the observed increase in iNOS mRNA and protein is not due to an increase in transcription of the iNOS gene.

Effects of Isoproterenol on iNOS mRNA Half-Life. Since the enhancement of iNOS induction did not appear to be at the level of transcription, we investigated the effect of Iso treatment on the stability of iNOS mRNA in IL-1β-stimulated cardiac fibroblasts. To assay mRNA half-life, cardiac fibroblasts were stimulated with IL-1β alone or IL-1β/Iso for 24 h, then treated with 65 μM 5,6-dichloro-1-β-ribofuranosyl benzimidazole (DRB), an inhibitor of transcription (Harrod et al., 1991). Control experiments (data not shown) demonstrated that DRB fully inhibited transcription of iNOS message in cells treated with either IL-1β or IL-1β + Iso. To determine the half-life of iNOS message, total RNA was extracted for Northern analysis from induced cells at 0, 2, 4, and 8 h after the addition of DRB (Fig. 10A). The half-life of iNOS mRNA, when normalized against GAPDH mRNA, was 1.0 $\pm$ 0.2 h in IL-1β-treated cells, whereas the mRNA half-life increased significantly, to 1.9 $\pm$ 0.2 h, in cells treated with IL-1β + Iso (mean $\pm$ S.E., n = 3; Fig. 10B). This doubling of mRNA stability is sufficient to account for the observed increases in iNOS protein and NO production, as will be argued under *Discussion*.

Discussion

Our data demonstrate that rat cardiac fibroblasts can be a significant source of nitric oxide. In particular, IL-1β induces iNOS in these cells, and hormones that elevate cellular cAMP enhance the induction. The effects of IL-1β and elevated cAMP are manifest as an increased level of mRNA for iNOS, as more of the enzyme and as enhanced NO production. The

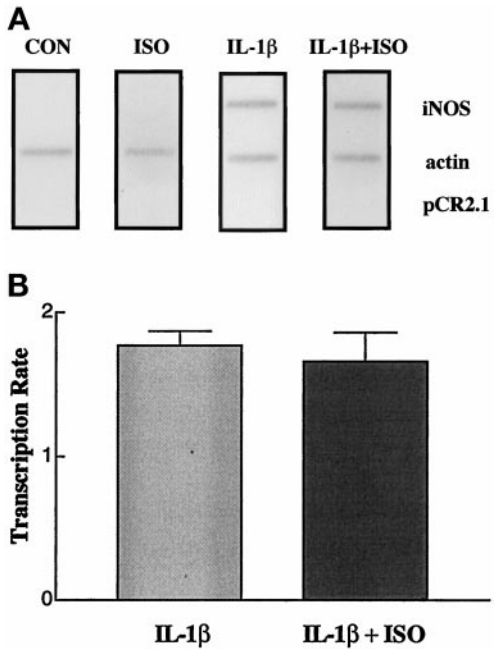

Fig. 9. Effects of isoproterenol on iNOS gene transcription. A, nuclear run-off. Cardiac fibroblasts were treated with diluent, 10 mM Iso, 10 ng/ml IL-1β, or Iso + IL-1β. After 20 h, nuclei were prepared and subjected to nuclear run-off assay as described under *Experimental Procedures*, assessing iNOS and actin messages, with the pCR2.1 plasmid serving to monitor nonspecific binding. B, the data from the nuclear run-off experiments were quantified with NIH Image software and iNOS data were normalized to β-actin signals, this unitless ratio being termed the "transcription rate" (ordinate). IL-1β treatment stimulated transcription of the iNOS gene; Iso did not significantly enhance the rate of transcription in IL-1β-stimulated fibroblasts (n = 3, $P >$ .5).

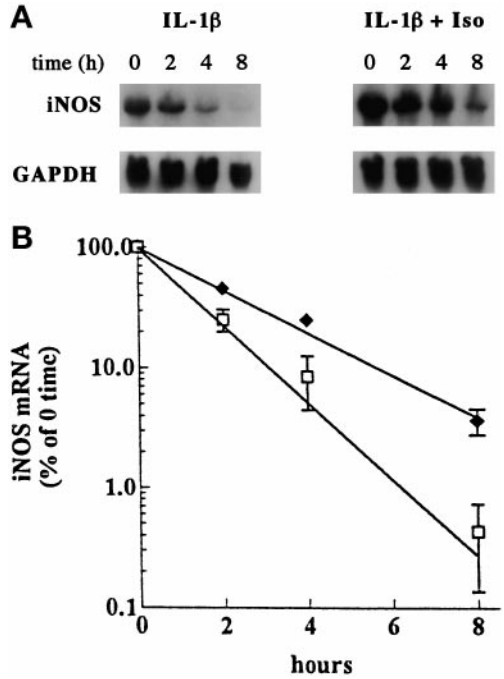

Fig. 10. Isoproterenol stabilizes iNOS mRNA in IL-1β-stimulated cardiac fibroblasts. Cells were stimulated for 24 h with IL-1β (10 ng/ml) or IL-1β (10 ng/ml) and Iso (10 μM). After 24 h of treatment, DRB was added and total RNA was extracted at the indicated times after the administration of DRB. Northern analysis was performed with cDNAs for iNOS and GAPDH as probes, as described under *Experimental Procedures*. A, representative Northern blots; B, time course of iNOS mRNA decay. Densities of iNOS mRNA were normalized to GAPDH mRNA values and plotted as a percentage of zero time values. iNOS mRNA half-lives are 1.0 h for IL-1β (□) and 1.9 h for IL-1β + Iso (◆); the effect of isoproterenol is significant, $P <$ .05. Each data point represents the mean $\pm$ S.E. of three independent experiments.

activation of PKA mediates the effect of cyclic AMP, resulting in a stabilization of iNOS mRNA.

Several aspects of cyclic nucleotide metabolism in cardiac fibroblasts bear discussion. First, from the effect of IBMX on basal cAMP accumulation, we infer that cultured cardiac fibroblasts have an active basal adenylyl cyclase and a basal cAMP content that is limited by active phosphodiesterases. Second, slight elevations of cAMP are sufficient to activate PKA and stabilize iNOS message. To the extent that primary cultures of ventricular fibroblasts mimic the cells in situ, physiologically relevant concentrations of catecholamines can enhance iNOS induction by IL-1β. Third, if the rat cardiac fibroblast mimics the human equivalent, then from the therapeutic point of view it may be significant that modest inhibition of phosphodiesterase activity suffices to cause stabilization of iNOS message (the 0.2 mM IBMX used in our experiments achieves about 25% of maximal inhibition). PDE inhibitors used to enhance contractility by elevating cAMP and enhancing the sequelae of PKA activation in myocytes may also promote an indirect negative inotropic effect by enhancing the production of NO by cardiac fibroblasts in the presence of IL-1β. Fourth, the β-receptor subtype mediating cAMP accumulation in the rat cardiac fibroblast is, by several criteria [order of agonist potency (Meszaros et al., 2000), efficacy of subtype-specific receptor antagonists (Fig. 4A), analysis of β-receptor transcripts (Fig. 4B)], largely, if not exclusively, the β_2-receptor. In contradistinction, the predominant β-receptor subtype on the rat ventricular myocyte is the β_1; thus, it may be possible to selectively modulate β-adrenergic effects in these two adjacent cell types.

This is not the first report of cytokine-induced NO production in cardiac fibroblasts. We have previously reported that a combination of TNF-α and IL-1β induces NO production in ventricular fibroblasts and myocytes isolated from adult rat heart and that induction couples functionally to increased cGMP content of the cells (Villegas and Brunton, 1996). Shindo et al. (1995) reported that lipopolysaccharides or IL-1β can induce iNOS in myocytes but *not* in fibroblasts isolated from neonatal rat hearts. IFN-γ and IL-1β, by themselves, are reportedly ineffective at inducing iNOS expression in neonatal cardiac fibroblasts; the combination of the two cytokines is required (Farivar et al., 1996). Ours is the first report demonstrating that IL-1β, *alone*, is able to induce iNOS expression in cardiac fibroblasts and that IL-2, IL-6, TNF-α, and IFN-γ are without effect. With the addition of our data, it is now clear that IL-1β, by itself, induces iNOS in all adult rat cardiac cell types tested, whereas IFN-γ induces iNOS in myocytes but not in cardiac microvascular endothelial cells and fibroblasts (our data; Balligand et al., 1994; Imai et al., 1994; Singh et al., 1996; Kinugawa et al., 1997). Thus, different responses to specific cytokines may serve to target the induction of iNOS to specific cells or regions within the heart.

Reported effects of cAMP on iNOS induction are variable and may reflect differentiated properties of the cells under study. In adult rat vascular smooth muscle and renal mesangial cells, cAMP, alone, promotes iNOS induction and enhances the effect of cytokines (Koide et al., 1993; Imai et al., 1994; Kunz et al., 1994). In rat myocytes, Oddis et al. (1995) and Ikeda et al. (1996) found little effect of cAMP alone but synergy between cAMP and IL-1β. By contrast, elevated cAMP reduced iNOS induction by lipopolysaccharide in rat astrocytes and Kupffer cells, in part by preventing activation of NF-κB; cAMP has just the opposite effect on NF-κB in macrophages (Pahan et al., 1997; Mustafa and Olson, 1998). The available data suggest that pathways modulating iNOS expression vary significantly among cell types. Our data are clear. Elevation of cAMP and activation of PKA have no noticeable effect on basal iNOS levels in cardiac fibroblasts but dramatically enhance the effect of IL-1β. This enhancement requires activation of PKA (H-89 inhibits the effect) and reflects accumulation of iNOS mRNA to a higher level, an effect that we believe is due to altered stability of the mRNA for iNOS.

The regulation of mRNA degradation is an increasingly studied mechanism by which the level of gene expression is controlled in mammalian cells (Sachs, 1993). With respect to the mRNA for iNOS, the effect of cAMP on message stability is reported to be positive or none, depending on the system studied. For instance, Oddis et al. (1995) reported that cAMP enhances NO production in IL-1β-stimulated neonatal cardiac myocytes. Employing semiquantitative RT-PCR, these workers found that cAMP increased the abundance of mRNA for iNOS, primarily through the induction of a lag preceding mRNA degradation, rather than through a change in the rate of degradation, itself. By contrast, Koide et al. (1993) found that forskolin enhanced the inductive effect of interferon on iNOS but did not affect the half-life of iNOS mRNA in IFN-γ-stimulated vascular smooth muscle cells; rather, the proposed mechanism was an increase in the rate of transcription of the iNOS gene by cAMP. In rat kidney mesangial cells, cAMP regulated iNOS mRNA at the levels of transcription and mRNA degradation (Kunz et al., 1994). In rat cardiac fibroblasts, we find no evidence for an effect of cAMP on transcription of the iNOS gene; rather, we find that an elevation of cAMP nearly doubles the half-life of the iNOS message from 1 h (after stimulation with IL-1β), comparable to that in IL-1β-induced rat mesangial cells (Kunz et al., 1994), to 1.9 h (after stimulation with IL-1β + Iso).

To determine whether the observed increase in mRNA half-life could account for the enhancement of iNOS message, we estimated how a change in mRNA degradation rate might affect the steady-state level of message. Assuming that IL-1β induces a constant rate of mRNA production (k_{+1}) and that the rate of the message degradation is first order (where k_{-1} is the rate constant for degradation and Q is the iNOS mRNA content), at steady state, production will equal degradation: $k_{+1} = k_{-1}Q$. If β-adrenergic treatment alters the degradation rate constant to k'_{-1}, then after isoproterenol, a new steady-state level, Q', will be reached, such that $k'^{+1} = k'_{-1}Q'$. The ratio Q' to Q will be $Q'/Q = k_{-1}/k'_{-1}$ or $Q'/Q = t'_{1/2}/t_{1/2}$ where $t_{1/2}$ = mRNA half-life after IL-1β, $t'_{1/2}$ = mRNA half-life after IL-1β + Iso, and where the degradation constant and the half-life are related by the expression, $k = \ln 2/t_{1/2}$. The measured effect of Iso is a doubling of half-life (from 1 to 2 h), which should lead to a doubling of mRNA content ($Q'/Q = 2$). This is exactly what we observe. Although we could hypothesize other effects of β-adrenergic stimulation, we do not detect changes in transcription or in activity of iNOS; this doubling of mRNA half-life is sufficient to account for the observed effects on mRNA content, and the increased mRNA translates proportionately to enzyme content and activity. Applying the law of parsimony, we conclude that the effect of β-adrenergic stimulation is via stabilization of iNOS mRNA.

The mechanism by which activation of PKA mediates changes in the stability of the iNOS message transcripts has yet to be determined. Recent molecular cloning and sequencing of the rat iNOS gene have revealed the presence of four "AUUUA" motifs in the 3′-untranslated region of the transcript (Keinanen et al., 1999). These AU-rich sequences in the 3′-untranslated region of many genes may function as destabilizing elements that target the mRNA for rapid degradation (Shaw and Kamen, 1986). Perhaps the activation of PKA affects these AUUUA destabilizing sequences and leads to a more stable iNOS message.

The most important features of our observations are: 1) demonstration that a single cytokine, IL-1β, can induce iNOS and increase NO production to high levels in adult cardiac fibroblasts; 2) that cyclic AMP elevation and activation of PKA enhance iNOS induction and NO production; and 3) that the enhancement of iNOS occurs via stabilization of the iNOS message, with no effect on the rate of transcription. The data clearly establish fibroblasts as a major source of NO in adult rat heart. Thus, NO produced by fibroblasts may have paracrine effects on neighboring cells and may impair the contractile function of cardiac myocytes. The data also pose an interesting paradox. In response to local stress or immune activation, cardiac fibroblasts will synthesize iNOS and begin to make large quantities of NO that will have negative contractile effects on the myocytes. A typical compensatory response to this reduced contractility could be increased adrenergic tone, with release of norepinephrine and activation of cardiac β-receptors, including the β_2-adrenergic receptors on fibroblasts. This activation will lead to an increase in cAMP within the fibroblasts and thence to an enhancement of iNOS induction, beginning the cycle again. Whether this is a deleterious cycle, a futile cycle, or one that serves a homeostatic role of maintaining cardiac function within certain useful limits remains to be seen. In any event, the inducibility of iNOS in adult cardiac fibroblasts may account for substantial quantities of NO within the heart and very likely constitutes an important variable in the adult heart's response to hormones, stress, and disease.

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Send reprint requests to: Åsa B. Gustafsson, Department of Pharmacology 0636, UCSD School of Medicine, 9500 Gilman Dr., La Jolla, CA 92093-0636. E-mail: agustafsson@ucsd.edu.