Attenuation of cAMP accumulation in adult rat cardiac fibroblasts by IL-1β and NO: role of cGMP-stimulated PDE2

ÁSA B. GUSTAFSSON1 AND LAURENCE L. BRUNTON2

1Biomedical Sciences Graduate Program, 2Departments of Pharmacology and Medicine, University of California at San Diego, La Jolla, California 92093

Received 2 July 2001; accepted in final form 22 January 2002

Gustafsson, Åsa B., and Laurence L. Brunton. Attenuation of cAMP accumulation in adult rat cardiac fibroblasts by IL-1β and NO: role of cGMP-stimulated PDE2. Am J Physiol Cell Physiol 283: C463–C471, 2002; 10.1152/ajpcell.00299.2001.—Treatment of cultured adult rat cardiac fibroblasts with interleukin-1β (IL-1β) induces the inducible nitric oxide synthase (iNOS) expression, increases nitric oxide (NO) and cGMP production, and attenuates cAMP accumulation in response to isoproterenol by 50%. Reduced cAMP accumulation is due to NO production: the effect is mimicked by NO donors and prevented by Nω-monomethyl-L-arginine, an NOS inhibitor. Effects of NO are not restricted to the β-adrenergic response; the response to forskolin is similarly diminished. NO donors only slightly (12%) decrease forskolin-stimulated adenylyl cyclase (AC) activity in cardiac fibroblast plasma membranes, suggesting that the main effect of NO is not a direct one on AC. An inhibitor of soluble guanylyl cyclase inhibits the effects of IL-1β and NO donors; inhibition of cGMP-dependent protein kinase is without effect. 3-Isobutyl-1-methylxanthine, a nonspecific phosphodiesterase (PDE) inhibitor, and erythro-9-(2-hydroxy-3-nonyl)adenine, a specific inhibitor of the cGMP-stimulated PDE (PDE2), completely restore cAMP accumulation in sodium nitroprusside-treated fibroblasts and largely reverse the attenuated response in IL-1β-treated fibroblasts. Although NO reportedly acts by reducing AC activity in some cells, in cardiac fibroblasts NO production decreases cAMP accumulation largely by the cGMP-mediated activation of PDE2.

NITRIC OXIDE (NO) regulates diverse physiological processes such as vascular tone, immune defense, and neurotransmission (6, 27). NO is synthesized from L-arginine by nitric oxide synthases (NOS). Of the three isoforms of NOS that have been identified, two are constitutively expressed in cells, whereas a third isoform, the inducible NOS (iNOS), is expressed only after stimulation of cells with inflammatory cytokines or lipopolysaccharides (1). The induction of iNOS by inflammatory cytokines has a protective effect by causing the production of large quantities of NO that can kill microorganisms such as bacteria (1, 11). NO is an important mediator within the cardiovascular system, where it can modulate the function of the heart and vessels (1, 18). In some pathological settings such as myocardial infarction, allograft rejection, or sepsis, the induction of iNOS and the subsequent increase in NO production may be deleterious and may be associated with depressed contractile responses and the death of cardiac myocytes (19, 23).

Most studies of NO modulation in the heart have focused on cardiac myocytes and endothelial cells, whereas very little attention has been given to the cardiac fibroblasts. Cardiac fibroblasts are the major nonmyocyte constituent of cardiac tissue, comprising two-thirds of the cell number and one-fifth of the cell mass in the heart (14). Our laboratory has observed that the signaling systems in cardiac fibroblasts are distinct from those of cardiac myocytes. We have previously reported that cardiac fibroblasts express β2-adrenergic receptors that couple to Gi-adenylyl cyclase (AC) but lack β1 receptors, the subtype that predominates on cardiac myocytes (15, 22). We have also reported that interleukin (IL)-1β induces iNOS expression in cardiac fibroblasts and that the β2-adrenergic response (cAMP elevation, cAMP-dependent protein kinase activation) enhances the expression by stabilizing iNOS mRNA (15). Under such conditions, cardiac fibroblasts can be major producers of NO. In a variety of cell types, NO reportedly alters the activities of AC and phosphodiesterase (PDE), thus functionally linking the cAMP and NO pathways. Such interactions could be important in cardiac fibroblasts, in which elevated cAMP can modulate proliferation and collagen production (3, 4, 10). Accordingly, we have continued our studies of cAMP metabolism in cardiac fibroblasts by considering whether NO derived from iNOS or from pharmacologic donors of NO affects the β-adrenergic response. We report here that NO accumulation in cardiac fibroblasts decreases cAMP accumulation in response to β-adrenergic stimulation, in large part by causing the activation of a cGMP-stimulated phosphodiesterase (PDE2).

MATERIALS AND METHODS

Materials. Rat recombinant IL-1β was purchased from Sigma-Aldrich (St. Louis, MO). Collagenase I and trypsin

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
were from Worthington (Freehold, NJ). S-nitroso-N-acetyl-
penicillamine (SNAP) was purchased from Alexis Biochemi-
cals (San Diego, CA). A monoclonal antibody to iNOS was
obtained from Transduction Laboratories (Lexington, KY).
[α-32P]ATP and [3H]cAMP were from Perkin Elmer Life-
sciences (Boston, MA). All other reagents and chemicals were
of reagent grade from Sigma-Aldrich or Calbiochem-Novabio-
chem (La Jolla, CA).

Isolation of adult ventricular fibroblasts. Rat cardiac fibro-
blasts were isolated from adult male Sprague-Dawley rats
(250–275 g) as recently described (15). Briefly, the hearts
were minced and placed in a trypsin/collagenase digestion
solution. The digestions were pooled, centrifuged, and resus-
pended in Dulbecco’s modified Eagle’s medium (DMEM) sup-
plemented with 10% fetal bovine serum and penicillin-strep-
tomycin (100 μU/ml). The cells were plated for 30 min, after
which unattached cells were rinsed off. All cells used in
experiments were from passages 2 through 4. The purity of
these cultures was >95% as determined by immunostaining
(15).

Western blot analysis. Fibroblasts (on 60-mm culture dis-
hes) were lysed at 4°C in a buffer containing 50 mM
β-glycerophosphate (pH 7.5 at 4°C), 1 mM EGTA, 10 mM
MgCl2, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluo-
ride (PMSF), and 10 μg/ml leupeptin. Equal amounts of total
protein per lane were loaded and separated on a 7.5% SDS-
polyacrylamide gel and then transferred to an Immobilon-P
membrane (Millipore, Bedford, MA). After being blocked in
5% nonfat milk, the membrane was incubated with a mono-
clonal iNOS 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 by
using enhanced chemiluminescence (Amersham Life Sci-
ence).

Measurement of nitrite levels. NO production was esti-
ated by measuring nitrite accumulation in the culture medium.
Fibroblasts were plated on 60-mm culture dishes and grown
up to 90–95% confluency. For experiments, cells were incu-
bated in phenol-red-free DMEM 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 the method of
Griess et al (13).

Assay of cGMP accumulation. Cells were treated with
IL-1β for 24 h; 1 mM 3-isobutyl-1-methylxanthine (IBMX) was
added to each plate 45 min before the assay was termi-
nated. In experiments with a NO donor, the cells were pre-
treated for 15 min with 1 mM IBMX and then stimulated
with 1 mM sodium nitroprusside (SNP) for 45 min. All
experiments were terminated by aspiration of the medium
and addition of ice-cold 5% trichloroacetic acid (TCA). The
TCA extracts were extracted four times with water-saturated
ether, and the cGMP content was determined by RIA (16).
Data are expressed as picomoles of cGMP per milligram of
protein.

Assay of cAMP accumulation. Cardiac fibroblasts were
incubated with DMEM without serum for 2 h and then
treated as described in RESULTS, after which the medium
was aspirated and ice-cold 5% TCA was added. The extracts were
purified over Dowex-50, and the cAMP content was deter-
mined by the method of Gilman (12). Data in the figures are
expressed as percentages of maximal responses in the par-
cular experimental protocol. Actual mean cyclic contents
were as follows: basal, 4 ± 1; isoproterenol (1 μM, 5 min),
48 ± 6; and forskolin (30 μM, 5 min), 193 ± 8 pmol/mg
protein. IBMX (1 mM), a nonspecific PDE inhibitor, in-
creased the effect of isoproterenol 26-fold; the PDE2-specific
inhibitor erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA; 0.2
mM), doubled the β-adrenergic response.

AC assay. Membranes were prepared by Dounce homoge-
nization of the cells in buffer A (50 mM HEPES, pH 7.6, 5 mM
MgCl2, 1 mM EGTA, 0.3 mM PMSF, and 10 μg/ml leupeptin).
The membranes were collected by centrifugation at 3000 g
for 5 min and resuspended in buffer A. Membrane protein
(40 μg) was incubated with 1 mM SNP or vehicle on ice for 30
min, and AC activity was assayed by monitoring the conver-


RESULTS

Induction of functional iNOS by IL-1β: effect on the
β-adrenergic and forskolin responses. We investigated
whether IL-1β treatment results in increased expres-
sion of iNOS by immunoblotting with an antibody
against iNOS. Expression of iNOS was not detected in
unstimulated cells; however, treatment with IL-1β (10
ng/ml) caused a increase in the iNOS protein expres-
sion in a time-dependent manner (Fig. 1, A and B). The
iNOS protein band, molecular mass ~130 kDa, was
first detected after exposure to IL-1β for 6 h, reaching
a maximum after 24 h of treatment. Moreover, treat-
ment for 24 h with IL-1β caused a concentration-de-
pendent increase in NO production with an EC50 of 3
ng/ml (~0.17 nM) (Fig. 1C).

The induction of iNOS (24-h treatment with IL-1β,
10 ng/ml) caused a 10-fold increase in NO production
compared with untreated cells (Fig. 2). Addition of 1
mM N6-monomethyl-L-arginine (L-NMMA), a com-
tpetitive inhibitor of NO synthase, inhibited NO production
by IL-1β-treated cells. These data indicate that the NO
is enzymatically produced and that its production can
be readily inhibited pharmacologically.

Having defined the time course and magnitude of
iNOS induction and NO production in IL-1β-treated
cells, we proceeded to determine whether IL-1β treat-
ment would affect the β-adrenergic response. Treat-
ment of cardiac fibroblasts with 10 ng/ml IL-1β for 24 h
resulted in a 55% decrease in isoproterenol-stimulated
cAMP accumulation (Fig. 3A). Forskolin-stimulated
cAMP accumulation was similarly attenuated after
IL-1β treatment (Fig. 3B).

Rationale for subsequent studies. Having established
that exposure to IL-1β causes iNOS induction and
presses responses to β-adrenergic agonists and fors-
kolin, we designed experiments to elucidate the mech-
anism of this effect by answering the following ques-
tions. 1) Is the effect on cAMP accumulation due
to iNOS induction and NO production, or is it due to other
effects of IL-1β? 2) Is the effect on an identifiable
component of hormone-sensitive AC? 3) Are the ex-
pected cellular consequences of NO production re-
quired for the depression of cAMP accumulation? If
cGMP production is involved, does it mediate the effect via cGMP-dependent protein kinase (PKG), by modulating cAMP hydrolysis or by other means?

NO dependence of the IL-1β effect, mimicry by NO donors, and reversibility. Exposure to IL-1β (10 ng/ml, 24 h) reduced cAMP accumulation in response to isoproterenol and forskolin (Fig. 3, A and B). Inclusion of L-NMMA fully prevented the effect of IL-1β to reduce these responses (Fig. 3, A and B). These data indicate that these effects of IL-1β are dependent on enzymatic NO production and, thus, on the induction of iNOS.

By way of substantiating this conclusion, we found that the effect of IL-1β could be reproduced by exposing fibroblasts to NO donors. We examined the effect of two structurally different NO donors, SNP and SNAP, on isoproterenol-stimulated cAMP accumulation. Pretreatment of cells with SNP or SNAP for 45 min caused decreases in the β-adrenergic response (Fig. 4A) comparable to that due to the iNOS induction occurring with a 24-h treatment with IL-1β (Fig. 3A). Thus we could readily simulate the effect of iNOS induction by providing NO pharmacologically.

The issue of the reversibility of the effect of NO offers a clue as to mechanism because the interaction between heme and NO is rapidly reversible, whereas other NO-mediated modifications, such as tyrosine nitrosation, are not readily reversible in cells (25). To determine whether the inhibition of cAMP accumulation by NO was reversible, we investigated whether cardiac fibroblasts that had been treated with an NO donor could recover the ability to produce cAMP in response to isoproterenol. Cells were preincubated with 1 mM SNP for 45 min, washed once with medium, and incubated in fresh medium without SNP. At the indicated times after the removal of SNP, the cells were stimulated with isoproterenol for 5 min and the cAMP levels were determined. With no washout (i.e., at 0 min in Fig. 4B), isoproterenol-stimulated cAMP accumulation was inhibited by 55% by the SNP treatment, as expected. However, isoproterenol-stimulated cAMP accumulation was progressively less inhibited as time after removal of SNP increased. At 5, 10, and 20 min after SNP removal, the cardiac fibroblasts displayed 20, 12, and 8% inhibition of isoproterenol-stimulated cAMP accumulation, respectively, thus recover-
ing with a half-time of ~5 min. At 30 min, the fibroblasts appeared to have completely recovered from the effects of SNP; the β-adrenergic response was comparable to that of untreated cells. Thus the effect of exogenous NO seems fully reversible by the simple expedient of washing the cells, suggesting that long-lived covalent changes are not involved.

Effect of IL-1β/NO does not localize to hormone-sensitive AC. IL-1β reduced responses to both isoproterenol and forskolin (Fig. 3, A and B). Because forskolin directly activates AC and also activates the αs-AC complex, we conclude that this NO-dependent inhibition of cAMP accumulation is not occurring at the level of the β-adrenergic receptor, on a mechanism regulating receptor refractoriness, or at the receptor-Gs protein interface.

It seemed possible that the reduced response to isoproterenol and forskolin could result from an activation of the Gs pathway by NO. We have demonstrated the existence of an active Gs pathway in these cells by measuring pertussis toxin (PTx)-sensitive inhibition of extracellular signal-regulated kinase (ERK) by lyso-phosphatidic acid (unpublished observation). We found that treatment of cells overnight with PTx, sufficient to inhibit this activation of Gs, did not alter the effect of SNP to decrease isoproterenol-stimulated cAMP accumulation (Fig. 5A). In fact, SNP treatment decreased cAMP accumulation to the same extent in cells that had been treated with PTx as in untreated cells, indicating that the inhibitory action of NO is not mediated through activation of the Gs pathway.

Recent studies suggest that NO can inhibit AC activity directly in N18TG2 neuroblastoma cells and that the inhibition is specific to AC isoforms 5 and 6 (17, 21). Because types 5 and 6 are known to be expressed in the
Fig. 5. Effects of NO are not mediated by G_i activation or adenylyl cyclase (AC) inhibition. A: pertussis toxin (PTx) treatment does not alter the effect of SNP. Cells were incubated with or without 0.1 μM PTx for 18 h, after which cells were treated with 1 mM SNP for 45 min, and cAMP accumulation was assessed after a 5-min exposure to 1 μM Iso. PTx did not significantly alter the effect of SNP to decrease Iso-stimulated cAMP accumulation (* vs. **, P > 0.5; mean ± SE; n = 3). B: SNP has a small effect on AC activity. Membranes were incubated on ice for 30 min with or without 1 mM SNP. Forskolin-stimulated AC activity was assessed by using 20 μg of membrane protein per tube. The effect of SNP was slight but significant (** vs. ***, P < 0.05). Data are means ± SE, n = 3; 100% = 568 pmol·mg⁻¹·min⁻¹.

heart (8), we investigated whether NO would have an effect on AC activity in the membranes of rat cardiac fibroblasts. Using the same protocol employed by McVey et al. (21), we found that preincubation of membranes with 1 mM SNP for 30 min had a small but significant effect on AC activity in the fibroblasts: SNP treatment reduced activity to about 88% of control enzyme activity (Fig. 5B). This 12% diminution of AC activity by NO in cardiac fibroblasts is much smaller than the 50–75% reduction reported in membranes from N18TG2 cells (21). This small effect seems unlikely to account for the 50% decrease in isoproterenol-stimulated cAMP accumulation in response to NO that we observed in the intact fibroblast. To the extent that the NO effect described by McVey et al. (21) is diagnostic of the presence of AC5 and AC6, we also conclude that these isoforms are not major contributors to cAMP production by rat ventricular fibroblasts.

Roles of soluble guanylyl cyclase, cGMP, and PKG. A major target of NO is the soluble guanylyl cyclase (sGC); NO activates sGC and thereby enhances production of cGMP (9). Treatment of cardiac fibroblasts with IL-1β stimulated NO production (Fig. 2); treatment with either a NO donor or IL-1β enhanced accumulation of cGMP (Fig. 6, A and B). The competitive inhibitor of NO synthase, l-NMMA (1 mM) inhibited cGMP accumulation in response to IL-1β (10 ng/ml, 24 h): control, 39 ± 32; l-NMMA, 32 ± 25; IL-1β, 641 ± 81; and IL-1β plus l-NMMA, 46 ± 24 pmol cGMP/mg (means ± SE, n = 3; Fig. 6, A and B). These data support the idea that cGMP produced by IL-1β-treated cells results from the effect of NO to activate sGC.

Indeed, 1H[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), a specific inhibitor of sGC, inhibited cGMP production in response to SNP and IL-1β (see Fig. 6, A and B). ODQ also fully restored isoproterenol-stimulated cAMP accumulation in SNP-stimulated cardiac fibroblasts (Fig. 6C), suggesting that sGC-cGMP mediates the effects of NO donors on cAMP production. Similarly, ODQ substantially restored isoproterenol-stimulated cAMP accumulation in IL-1β-treated cells (Fig. 6D). We interpret this data to mean that NO-stimulated cGMP production mediates most of the effects of IL-1β on cAMP accumulation.

Many effects of cGMP are mediated via activation of PKG. The effect of NO/cGMP to reduce cAMP is not one of those effects: treatment of cells with 1 μM KT-5823, an inhibitor of PKG, did not alter the effect of SNP to decrease isoproterenol-stimulated cAMP accumulation (Fig. 7). These data indicate that the inhibitory action of NO on cAMP accumulation is not mediated through activation of PKG.

Role of cyclic nucleotide PDEs. Because the NO/cGMP effect does not appear to require PKG activation, we turned our attention to the other major mediator of cGMP’s effects, the cyclic nucleotide PDE activities that are modulated by cGMP. To investigate whether enhanced PDE activity mediates the inhibition of isoproterenol-stimulated cAMP production, we determined the effects of PDE inhibitors in fibroblasts treated with either SNP or IL-1β. Treatment of cells with 1 mM SNP for 45 min, in the absence of a PDE inhibitor, resulted in the expected 50% decrease in cAMP accumulation in response to isoproterenol (Fig. 8A). Pretreatment of cells with 1 mM IBMX, a nonspecific PDE inhibitor (Fig. 8B), or 0.2 mM EHNA, a PDE2 specific inhibitor (Fig. 8C), completely restored cAMP accumulation. We also investigated whether specific inhibitors of other PDE isoforms (types 1, 3, and 4) could restore the attenuated cAMP accumulation in response to isoproterenol. However, neither 25 μM vinpocetine (PDE1) nor 10 μM milrinone (PDE3) or 10 μM rolipram (PDE4) restored cAMP accumulation in SNP-treated cells, verifying that the NO/cGMP response reflects the altered activity of an ENHA-sensitive isoform, PDE2, specifically.
The findings were similar, but not identical, in IL-1β-treated cells. Neither IBMX nor EHNA completely reversed the attenuated cAMP response in IL-1β-treated cells. Both inhibitors largely (to 85% of maximum) reversed the attenuation in cAMP accumulation in IL-1β-treated fibroblasts (Fig. 9); thus most of the IL-1β effect is attributable to activation of PDE2. There is, however, a small component of the IL-1β effect on cAMP accumulation that is not restored by inhibitors of PDE2, which is in accord with the results obtained with the sGC inhibitor ODQ (Fig. 6D). As previously demonstrated (Fig. 3), an NO synthase inhibitor will fully restore cAMP accumulation, so the effect of IL-1β is NO dependent. We hypothesize that this EHNA/ODQ-resistant component reflects an effect of long-term IL-1β exposure on expression of other proteins involved in cAMP production and metabolism.

DISCUSSION

We have examined the effect of NO production on the β-adrenergic response in cardiac fibroblasts. Our data show that induction of iNOS by IL-1β treatment attenuates the increase in intracellular cAMP in response to isoproterenol or forskolin. This is similar to some previous reports. For instance, Chung et al. (7) reported that isoproterenol-stimulated cAMP accumulation in rat ventricular myocytes was inhibited by conditioned medium containing cytokines from activated immune cells. However, these workers also reported that the effect was not apparent when the cells were stimulated with forskolin, suggesting that the effect of NO occurred at the level of the β-adrenergic receptor or G protein coupling in the myocytes (7). Balligand et al. (2) reported that incubation of adult rat cardiac myocytes with macrophage-conditioned medium was accompanied by induction of iNOS, an increase in NO production, and a depression of the contractile response to β-adrenergic stimulation. Joe et al. (19) reported a decrease in the β response but not in the forskolin response (cAMP accumulation) in rat cardiac myocytes.
expressing iNOS, suggesting that some of the effect of NO was occurring proximal to AC.

Our data in cardiac fibroblasts are similar to the extent that we observed that IL-1β/H9252 induces iNOS and causes NO production, resulting in a decrease in isoproterenol-stimulated cAMP accumulation. In contrast to the case in myocytes (7, 19), we found that forskolin-stimulated cAMP accumulation is also attenuated in IL-1β/H9252-treated fibroblasts, suggesting that the β-adrenergic receptor and Gs are not the targets of NO in these cells. If data on myocytes prove to be correct, then this is another way in which cardiac myocytes and fibroblasts differ (22). Our data also indicate that activation of the Gi pathway (defined ± PTx) is not the mechanism by which NO exerts its effect on cAMP accumulation.

There is no mechanism proposed that accounts for the effect of NO on β-adrenergic-Gs coupling reported in myocytes (7, 19). There is, however, a developing literature on effects of NO on AC. Tao et al. (26) demonstrated that NO can inhibit both hormone- and forskolin-stimulated AC activity in N18TG2-cultured cells.

Fig. 9. PDE inhibitors largely restore Iso-stimulated cAMP accumulation in IL-1β/H9252-treated cardiac fibroblasts. Cells were treated with IL-1β (10 ng/ml, 24 h) and then incubated with diluent (A), 1 mM IBMX (B), or 0.2 mM EHNA (C) for 15 min before Iso treatment (1 μM, 5 min), after which cells were stimulated with 1 μM Iso for 5 min and the cAMP content was determined. A: SNP significantly reduced Iso-stimulated cAMP accumulation (* vs. **, P < 0.001). In the presence of IBMX (B) or EHNA (C), Iso-stimulated cAMP accumulation was completely restored (* vs. **, P > 0.05). Data are means ± SE, n = 3.

Fig. 8. Phosphodiesterase (PDE) inhibitors restore Iso-stimulated cAMP accumulation in SNP-treated cardiac fibroblasts. Cardiac fibroblasts were incubated with diluent (A), 1 mM 3-isobutyl-1-methylxanthine (IBMX), a nonspecific PDE inhibitor (B), or 0.2 mM erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA), a PDE2-specific inhibitor (C), for 15 min before SNP treatment (1 mM, 45 min), after which cells were stimulated with 1 μM Iso for 5 min and the cAMP content was determined. A: SNP significantly reduced Iso-stimulated cAMP accumulation (* vs. **, P < 0.001). In the presence of IBMX (B) or EHNA (C), Iso-stimulated cAMP accumulation was completely restored (* vs. **, P > 0.05). Data are means ± SE, n = 3.
neuroblastoma cells. Specifically, NO seems to selectively inhibit AC5 and AC6 with no effect on isoforms 1 or 2 (17). We found that pharmacologically produced NO caused a small (12%) but significant reduction in forskolin-stimulated AC activity in membranes isolated from cardiac fibroblasts. This inhibition is insufficient to fully account for the 50% decrease in responses to forskolin or isoproterenol that we observe in intact fibroblasts. In addition, we did not detect this inhibition of AC activity by NO in intact cardiac fibroblasts in the presence of a PDE inhibitor. This difference can be explained by several factors. The release of NO from NO donors can be influenced by a number of variables, including the incubation medium, light, temperature, and pH. For example, the differences in medium or the temperature at which experiments were performed could be factors in the different effects of NO on AC activity in whole cells vs. membranes. Preliminary RT-PCR and Western analysis indicate expression of AC2, 3, 4, 5, 6, and 7 in the fibroblasts (unpublished observation). Even though the NO-sensitive isoforms (5 and 6) are present in the fibroblasts, our data (little effect of NO on hormone-sensitive AC activity) suggest that these two forms are a small component of the total AC activity. Thus the major functional isoforms of AC present in cardiac fibroblasts are likely to be NO insensitive.

One target of cGMP is the cGMP-stimulated PDE (PDE2), to which binding of cGMP causes activation, resulting in a decrease in cAMP content in cells (20). Not all workers find that this pathway is involved in cytokine action. Chung et al. (7) reported that cytokine-induced inhibition of cAMP accumulation in cardiac myocytes was not altered by IBMX. Joe et al. (19) reported that IBMX only partly reversed the attenuation of the β-adrenergic response in cardiac myocytes expressing iNOS. Our data are very clear: the NO-induced decrease in cAMP accumulation in response to isoproterenol or forskolin reflects enhanced degradation of cAMP in cardiac fibroblasts. The mechanism that we propose is shown in Fig. 10: IL-1β induces iNOS; iNOS makes NO, which activates the sGC; and the resultant cGMP activates PDE2, thereby reducing cAMP accumulation.

In nitroprusside-treated cells, the same scheme would apply, with the NO activating sGC with the same sequelae. Again, the evidence is similar: preincubation with IBMX or EHNA completely restored isoproterenol-stimulated cAMP accumulation to control levels, suggesting that the effect of NO is attributable to PDE2 activation by cGMP. However, in cells treated with IL-1β to induce iNOS, IBMX or EHNA only partially restored cAMP accumulation to control levels: PDE inhibitors reduced the NO effect from ~55 to 15% inhibition of maximal isoproterenol-stimulated cAMP accumulation. These results are also in agreement with the data obtained from experiments where guanylyl cyclase was inhibited by ODQ: inhibiting cGMP production completely restored isoproterenol-stimulated cAMP accumulation in SNP-treated cells and largely, but not completely, restored isoproterenol-stimulated cAMP accumulation in IL-1β treated cells. These results suggest that some of the effect of iNOS induction is not dependent on acute cGMP production and PDE2 activation. The main difference be-

---

Fig. 10. Testable mechanisms by which IL-1β, iNOS, and NO could affect cAMP accumulation. Solid arrows indicate the proposed pathway. Dashed lines indicate sites of action of pharmacologic inhibitors used diagnostically in our experiments. Shaded lines indicate pathways found not to play a role in the effect of NO on cAMP accumulation in cardiac fibroblasts.
between the two experimental protocols is duration of NO exposure (acute vs. iNOS induction over 24 h). In experiments with SNP, the cells were exposed to the NO donor for 45 min before isoproterenol stimulation; in IL-1β stimulated cells, the cells were exposed to NO production for an extended period of time. We detected significant iNOS protein after 6 h of IL-1β treatment; thus when we stimulated the cells with isoproterenol after 24 h of IL-1β treatment, the cells had been exposed to increasing levels of NO production by iNOS for at least 18 h. It is clear that the effects on cAMP accumulation by IL-1β treatment are due to an increase in NO production and not to some other effect of IL-1β, because the cAMP accumulation can be completely restored by the inclusion of an iNOS inhibitor. Nevertheless, the prolonged exposure to NO may cause transcriptional changes in the cells, possibly due to the activation of PKG (9). Activation of PKG may induce proteins that influence second messenger generation. It is also possible that chronic NO and cGMP production could result in downregulation of portions of the response pathway, such as the β-adrenergic receptor, Gβ, or AC. Thus the acute effects of NO production on cAMP accumulation are through the activation of PDE2, whereas chronic NO and cGMP production also increase PDE2 activity but may also bring other factors into play that also reduce the β-adrenergic response to a lesser extent.

In conclusion, we have demonstrated that cardiac fibroblasts can produce large quantities of NO in response to immune activation and the consequent induction of iNOS. The resulting NO depresses cAMP accumulation within the cardiac fibroblast. The mechanism of this effect is not via an inhibition of AC by NO or via stimulation of PKG by the resultant cGMP. Rather, the effect is via stimulation of sGC by NO and stimulation of PDE2 by the elevated cGMP.

This work was supported by grants from the University of California San Diego Academic Senate and the California Tobacco-Related Diseases Research Program and by a predoctoral fellowship to A. B. Gustafsson from the American Heart Association, Western States Affiliates.

REFERENCES


