Role of Nitric Oxide in Antagonistic Effects of Transforming Growth Factor-β and Interleukin-1β on the Beating Rate of Cultured Cardiac Myocytes

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We have recently shown that transforming growth factor-β (TGFβ) acts in an autocrine manner to maintain the beating rate of neonatal rat cardiac myocytes cultured in serum-free medium on cardiac fibroblast matrix. Interleukin-1β (IL-1β) suppresses the myocyte-beating rate, and TGFβ antagonizes this effect. We now show that TGFβ and IL-1β also have antagonistic effects on the secretion of nitric oxide (NO) by these myocytes, and that NO secretion, the activity of NO synthase (NOS), and expression of the inducible form of NOS correlate inversely with the effects of these two agents on the beating rate. Western blot analysis shows that treatment of myocytes with TGFβ antagonizes the induction of NOS after treatment with IL-1β. Release of NO, induced by IL-1β, is dependent upon the availability of the substrate, L-arginine, and is suppressed by a competitive inhibitor, N^G-monomethyl-L-arginine. L-Arginine (>0.25 mM) also suppresses, and N^G-monomethyl-L-arginine (>0.5 mM) enhances the myocyte-beating rate. Treatment with IL-1β, but not TGFβ, increases cellular cGMP, presumably by activation of guanylate cyclase by NO. Methylene blue, an inhibitor of guanylate cyclase, reverses the suppression of beating caused by IL-1β. Bacterial lipopolysaccharide, present in the serum-free medium, is a coinducer of NO secretion. The suppressive effects of NO on the beating rate can be overcome by altering either the set of cytokines employed to induce NO or the matrix on which the myocytes are cultured, demonstrating that additional parameters are also involved in regulation of the beating rate. (Molecular Endocrinology 6: 1921–1930, 1992)

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INTRODUCTION

It is increasingly appreciated that reactive nitrogen intermediates, including nitric oxide (NO), play critical roles in a wide spectrum of physiological processes, including those of the central and peripheral nervous systems, kidney, immune system, and cardiovascular system (1). Two distinct isoforms of the enzyme NO synthase (NOS) catalyze production of NO by oxidation of a guanidino nitrogen of L-arginine (2). One isoform (cNOS) is constitutive and calcium/calmodulin dependent, as described in the cerebellum (3), penile corpus cavernosum (4), endothelium (5), myocardium (6), and isolated adult myocytes (6). This enzyme can activate NO synthesis within a very short time frame. The other isoform (iNOS) is independent of elevated calcium and exogenous calmodulin (7) and can be induced over a period of several hours in a variety of cell types, including macrophages (8–11), endothelial cells (12), smooth muscle cells (13), renal mesangial cells (14), and adult cardiac myocytes (6). Bacterial endotoxin [lipopolysaccharide (LPS)] and a variety of inflammatory mediators, such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNFα), and interferon-γ (IFNγ), all induce this enzyme activity and NO production. In macrophages and mesangial cells, the transforming growth factors-β (TGFβ) suppress synthesis of NO induced by exposure to proinflammatory cytokines (14, 15); a newly described macrophage-deactivating factor has a similar suppressive activity in macrophages (15).

We have been studying the function of TGFβ in the physiology and pathophysiology of the heart. TGFβ plays a critical role in this tissue, as shown by its acute regulation after experimental myocardial infarction (16), its localization in the mitochondria of cardiac myocytes (17), and its ability to protect the heart from damage resulting from ischemia and reperfusion (18). Moreover, we have shown that TGFβ maintains the function of cultured neonatal cardiac myocytes, both acting in an autocrine fashion to regulate the beating rate of the cells and antagonizing the suppressive effects of IL-1β on the beating rate (19).

Recently, synthesis of NO has been implicated in the regulation of cardiac contractility in several different model systems, including isolated papillary muscles (20), myocardium after reoxygenation injury (21), myocardium of rats treated with endotoxin, and cultured adult rat cardiac myocytes (6). Based on these observations, we have investigated whether the opposing effects of IL-1β and TGFβ on the spontaneous beating rate of cultured neonatal rat cardiac myocytes might be mediated in part by NO. Our results demonstrate that IL-1β induces neonatal myocytes to express iNOS and release NO, and that TGFβ, added alone or together with IL-1β, suppresses the release of NO by reducing the level of iNOS. The effects of each of these cytokines on the activity of iNOS correlate inversely with their effects on the beating rate. However, alteration of the culture conditions demonstrates that the production of NO by neonatal myocytes can occur independently of effects on the beating rate, suggesting that NO is only one of several factors important in the regulation of this aspect of myocyte function.

RESULTS

Neonatal rat cardiac myocytes purified on a Percoll gradient and cultured in serum-free Dulbecco's Modified Eagle's Medium-Ham's F-12 (DMEM/F12) medium supplemented with ITS+ (insulin, transferrin, selenium, linoleic acid, and BSA) were grown on matrix secreted by cultured cardiac fibroblasts, as previously described (19). Under these conditions, the rate of spontaneous beating of the myocytes slowly declines over a 24-h culture period; the rate of decline is accelerated by the addition of IL-1β and reversed by TGFβ, added either alone or together with IL-1β. To ascertain whether neonatal myocytes might secrete NO and whether its production could be correlated with effects on their beating rate, myocytes were treated with IL-1β, TGFβ, or the combination of the two cytokines, each at 5 ng/ml.

TGFβ and IL-1β Have Antagonistic Effects on NO Secretion

As shown in Fig. 1A, treatment of myocytes for 22 h with IL-1β resulted in an increase in the specific activity of NOS in cellular extracts, whereas treatment with TGFβ, either alone or in combination with IL-1β, suppressed that activity. NOS activity reflects the NO synthetic capacity of the myocytes at the time of measurement. In contrast, accumulation of nitrite in the medium represents the summation of enzyme activity throughout the time period studied, since NO secreted by cells rapidly decomposes to more stable products, *i.e.* nitrite and nitrate (10). As shown in Fig. 1B, analysis of the time course of nitrite secretion shows effects of IL-1β and TGFβ similar to those observed on NOS activity. There was a lag phase of nearly 15 h before detectable levels of nitrite accumulated in the medium of myocytes treated with IL-1β; thereafter, there was a steady increase in nitrite secretion, persisting for at least 48 h

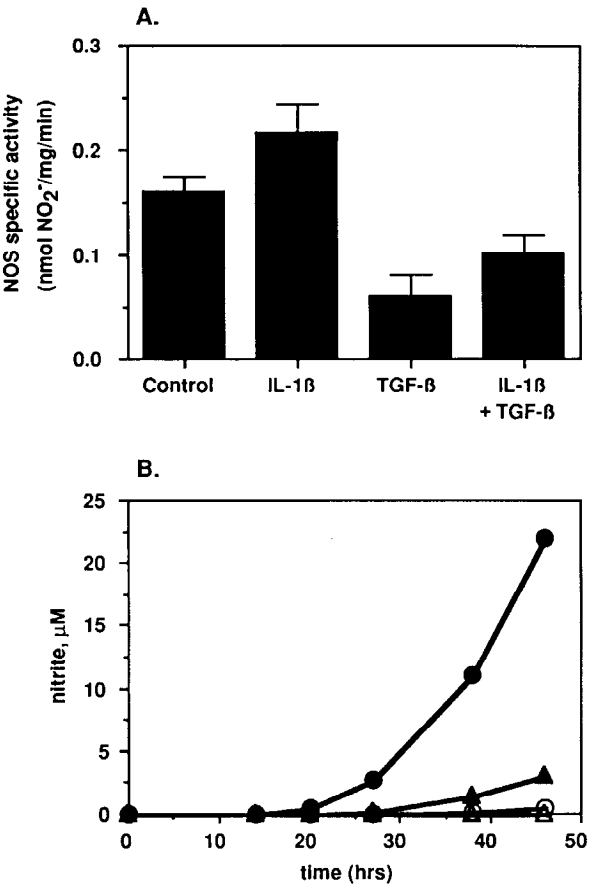

Fig. 1. Effects of IL-1β and TGFβ on the Specific Activity of NOS and Nitrite Secretion by Neonatal Myocytes

Myocytes were cultured, as described, on cardiac fibroblast matrix in serum-free medium containing ITS+ (high LPS) and IL-1β, TGFβ, or the combination of the two, each at 5 ng/ml. A, Specific activity of NOS in extracts of cells cultured for 22 h under experimental conditions. Results represent the mean ± SEM of two experiments, each containing cells pooled from five individual wells. B, Nitrite levels measured in aliquots of medium pooled from four wells. Cells were cultured in 1 ml medium containing vehicle (○), IL-1β (●), TGFβ (△), or IL-1β plus TGFβ (▲). At each time point beginning at 14 h, 100-µl aliquots were removed from each well, pooled, and assayed. Data are from a representative experiment.

(Fig. 1B). The suppressive effects of TGFβ also persisted throughout the 48-h observation period; myocytes treated with both TGFβ and IL-1β secreted less than 15% the amount of nitrite secreted by myocytes treated with IL-1β alone.

Accumulation of Nitrite Is Dependent on Oxidation of L-Arginine

When myocytes were cultured in DMEM lacking L-arginine, the secretion of nitrite induced by treatment of myocytes with IL-1β was markedly stimulated by the addition of L-arginine, the substrate for NOS. In contrast, the addition of N^G-monomethyl-L-arginine (NMA), a competitive inhibitor of NOS, suppressed nitrite secretion in the presence of L-arginine (Fig. 2).

NO activates guanylate cyclase by formation of a nitrosyl-heme complex at the active site of the enzyme (22). Its product, cGMP, has been shown to mediate many of the biological effects of NO (23). While nitrite was measured after accumulating for 24 h in culture, cGMP was assayed for only a 10-min period at the conclusion of the experiment. Nonetheless, as shown in Fig. 2, there was a correlation between cellular cGMP and nitrite accumulation by myocytes cultured in L-arginine-free medium. The reduced levels of both nitrite and cGMP in cells treated with NMA in the presence of added arginine as well as the L-arginine-dependent increase in each of these parameters demonstrate the role of L-arginine oxidation in regulation of levels of both nitrite and cGMP.

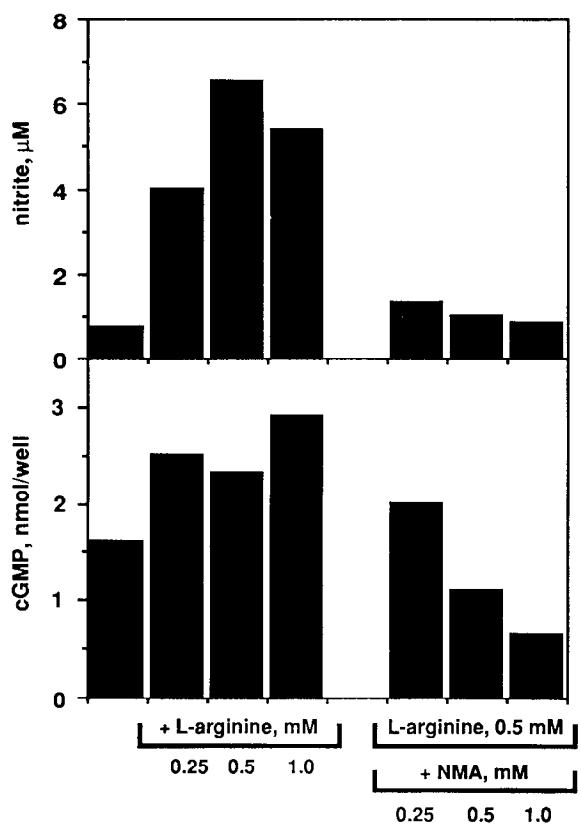

Fig. 2. Effects of L-Arginine and NMA on Nitrite Release by Myocytes Treated with IL-1β

Cells were cultured in L-arginine-free DMEM for 22 h in the presence of 5 ng/ml IL-1β and the indicated concentrations of L-arginine and NMA. Nitrite and cGMP were determined, as described, in samples pooled from five replicate wells. Data are from one of five similar experiments.

LPS Is a Coinducer of iNOS

Since LPS can act as a coinducer of nitrite secretion by macrophages (8, 9) as well as by short term cultures of adult cardiac myocytes (6), we determined the LPS levels in solutions used to prepare the myocytes and in the culture medium. Although myocytes were exposed to LPS during separation on the Percoll gradients, medium collected from myocytes 48 and 72 h after the initial plating contained undetectable levels of LPS (<5 pg/ml). However, an unexpected source of significant concentrations of LPS was the ITS+ supplement (Collaborative Research, Waltham, MA), which contributed approximately 50–70 pg/ml LPS to the final serum-free culture medium. For this reason, we examined the effects of replacing this supplement with another ITS preparation (Biofluids, Rockville, MD) together with a lipid-rich BSA preparation, Albumax (Gibco, Grand Island, NY). DMEM/F12 supplemented with these reagents (ITS/Albumax) had approximately 1% the LPS present in medium supplemented with ITS+.

As shown in Fig. 3, culture of myocytes on cardiac fibroblast matrix using serum-free medium supplemented with ITS/Albumax (low LPS) markedly reduced both basal and IL-1β-induced levels of nitrite secretion and NOS activity compared to those of myocytes cultured in medium supplemented with ITS+ (high LPS). Nitrite levels of myocytes cultured in ITS/Albumax medium and treated with IL-1β averaged 37% those of corresponding cultures supplemented with ITS+ (mean of seven different experiments comprising 41 individual wells). Both the release of nitrite and the NOS activity of myocytes cultured in low LPS medium were increased by the inclusion of LPS in addition to IL-1β. The highest levels of both nitrite and NOS activity were seen in cultures treated with TNFα and IFNγ in addition to IL-1β. These data demonstrate that the inflammatory mediators TNFα and IFNγ, like LPS, can each synergize with IL-1β to induce NO, and that LPS present in the ITS+ (high LPS) supplement probably serves as a coinducer of NO secretion together with IL-1β.

TGFβ Suppresses Accumulation of iNOS

The above data suggest that TGFβ acts by either suppressing the activity of iNOS or preventing its accumulation. As shown in Fig. 4, iNOS was not detectable in an immunoblot of lysates of myocytes made at the time of their initial isolation, 72 h later at the time of switching the cells to serum-free culture conditions, or after 24 h of culture in serum-free low LPS medium supplemented with ITS/Albumax. However, the inten-

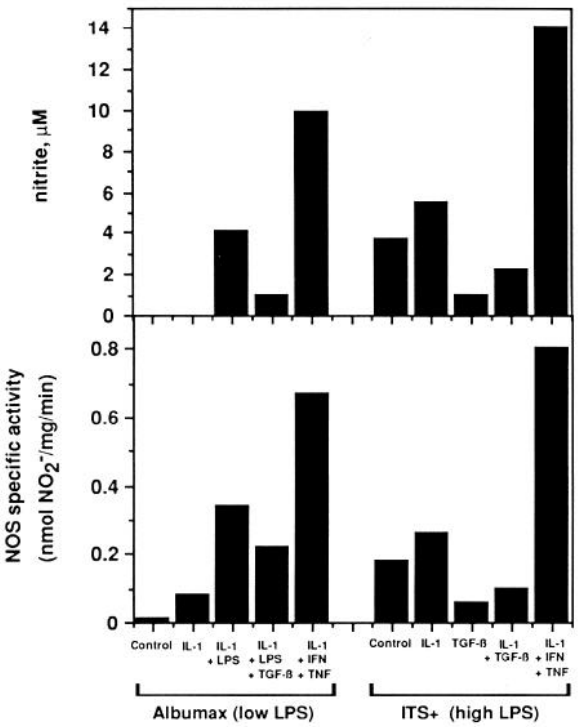

Fig. 3. Comparison of Effects of Medium Supplements Containing Different Levels of LPS on the Ability of Cytokines to Induce NOS Activity and Nitrite Release in Neonatal Myocytes

Cells were cultured for 22 h in medium containing either a low LPS supplement [ITS (Biofluids) plus Albumax] or a higher LPS supplement (ITS+; Collaborative Research). Cytokines were added at the following concentrations: IL-1β and TGFβ, 5 ng/ml; IFNγ, 9 ng/ml; TNFα, 25 ng/ml, and LPS, 20 μg/ml. Nitrite release and NOS specific activity were measured in pools of medium or cells of 12 and 10 replicate wells, respectively. Results are from one of more than four experiments of similar design.

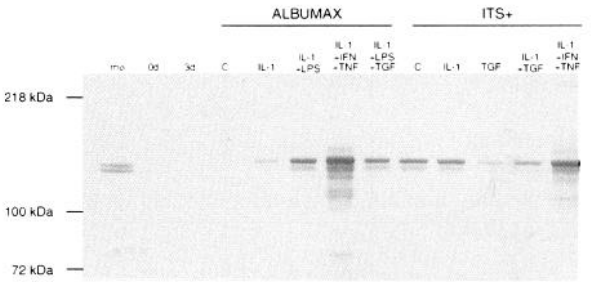

Fig. 4. Western Blot of iNOS Induced in Cardiac Myocytes

Cultures of rat neonatal myocytes were prepared, as described (0 day), and cultured in 5% calf serum for 72 h (3 day). Cells were then washed with serum-free medium and placed in fresh serum-free medium containing ITS (Biofluids) and Albumax (Gibco) or ITS+ (Collaborative Research). Cells were left untreated [control (C)] or treated with the indicated combinations of IL-1β (5 ng/ml), LPS (20 μg/ml), TGFβ (5 ng/ml), TNFα (25 ng/ml), and IFNγ (9 ng/ml) for 22 h before being harvested, lysed, and subjected to SDS-polyacrylamide gel electrophoresis (15 μg protein/lane), followed by immunoblot with anti-iNOS IgG, as described. *Numbers on the left side* indicate the apparent mol wt of prestained markers. An extract of macrophages treated with IFNγ was used as a control (MØ).

sity of a 130-kilodalton band corresponding to iNOS increased progressively in cultures treated with IL-1β alone or together with LPS or with the combination of TNFα and IFNγ; these results correlate closely with the findings shown in Fig. 3. Levels of iNOS were somewhat higher in cells cultured in the low LPS medium in the presence of IL-1β and LPS than in the high LPS medium with the addition of IL-1β alone, possibly reflecting the higher level of LPS added. Most importantly, the addition of TGFβ inhibited the accumulation of iNOS, regardless of the medium supplements.

NO Is a Regulator of the Beating Rate of Neonatal Myocytes

We showed previously that the beating rate of the cultured myocytes begins to decrease only after 10–14 h (19). This is the time period when levels of nitrite begin to accumulate to detectable levels in the medium (Fig. 1B), suggesting that NO might affect the beating rate. As shown in Fig. 5, the beating rate of myocytes treated with TGFβ, IL-1β, or the combination of the two correlated inversely with the levels of nitrite found in the medium. Figure 6A shows that the effects of IL-1β on suppression of the beating rate or enhancement of nitrite secretion were dose dependent, as was the ability of TGFβ to reverse the effects of IL-1β (Fig. 6B). ED_{50} values for IL-1β alone or TGFβ in the presence of 5 ng/ml IL-1β were approximately 0.3 and 0.02 ng/ml, respectively, for effects on nitrite secretion and 0.08 and 0.1 ng/ml, respectively, for effects on the beating rate.

Significantly, the beating rate of neonatal myocytes was also a function of L-arginine oxidation, as shown in Fig. 7. The addition of L-arginine to myocytes cultured in L-arginine-free medium suppressed the beating rate of both control (not shown) and IL-1β-treated myocytes in a dose-dependent fashion, whereas the competitive inhibitor NMA, added in the presence of 0.25 mM L-arginine, increased the beating rate. Comparison of Figs. 3 and 7 again shows a strong inverse correlation between the effects of either L-arginine or NMA on the beating rate compared to their effects on levels of either secreted nitrite or intracellular cGMP. Moreover, the approximately 53% decrease in cGMP and 320% increase in the beating rate (average of three experiments) of myocytes treated with IL-1β and methylene blue, an inhibitor of guanylate cyclase, support a role for cGMP as a more proximal mediator of the effects of NO on this aspect of myocyte function.

Culture of myocytes using serum-free medium supplemented with ITS/Albumax (low LPS) markedly reduced the suppressive effects of IL-1β on the beating rate compared with its effects in medium supplemented with the original ITS+ preparation (high LPS), again showing an inverse relationship of beating rate with nitrite release and iNOS activity (see Figs. 3 and 4). The

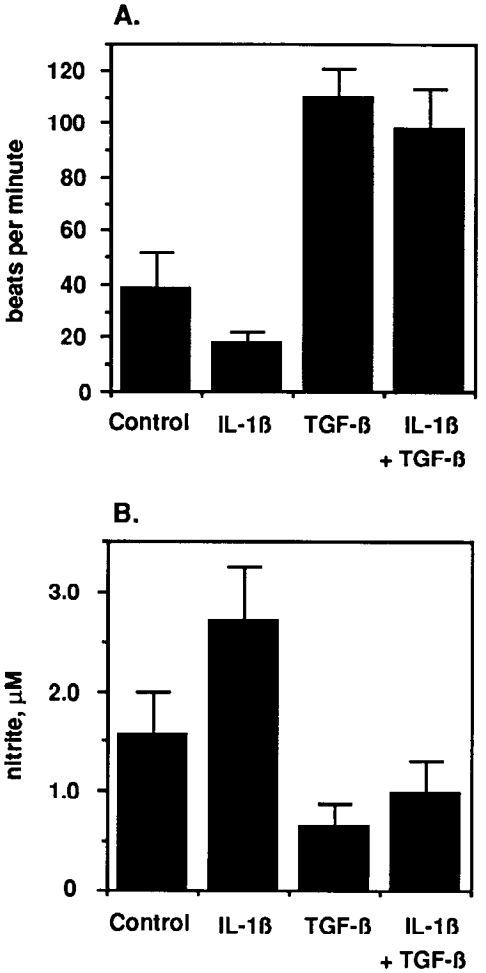

Fig. 5. Inverse Correlation of the Beating Rate and Nitrite Release of Neonatal Myocytes Cultured on Cardiac Matrix in Serum-Free Medium Supplemented with ITS+ (High LPS)

Myocytes were cultured for 22 h under experimental conditions in the presence of IL-1β, TGFβ, or the combination of the two, each at 5 ng/ml. Beating rate (A) and levels of nitrite in the medium (B) were determined as described. Results represent the mean ± SEM of 10 individual experiments containing a total of 50 wells.

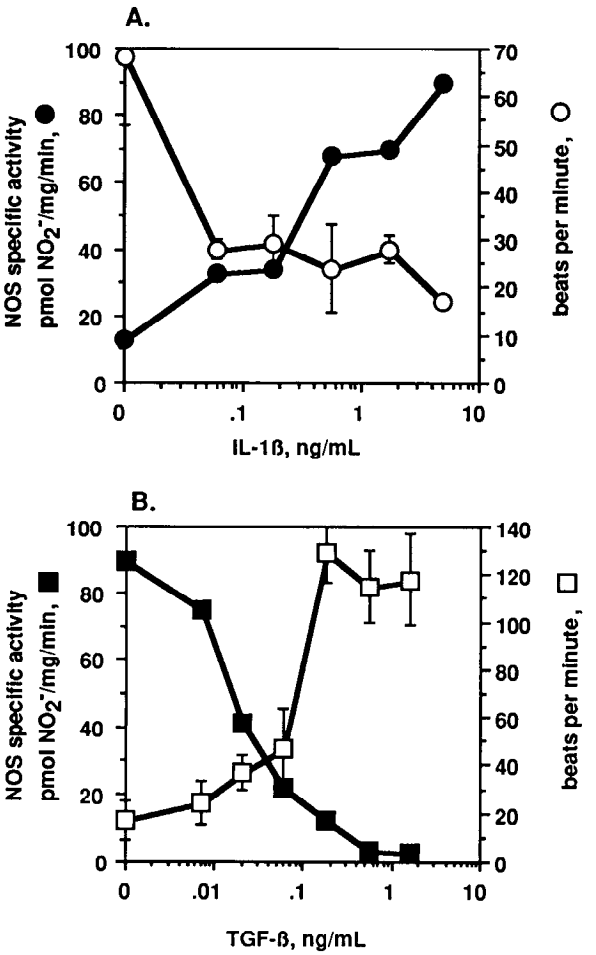

Fig. 6. Inverse Dose-Response Relationship of NOS Specific Activity and Myocyte-Beating Rate to Increasing Concentrations of IL-1β or TGFβ

Myocytes were cultured for 24 h, as described, in the presence of varying concentrations of IL-1β (A) or varying concentrations of TGFβ in the presence of 5 ng/ml IL-1β (B). ○ and □, Beating rate (mean ± SEM) measured at 17 h; ● and ■, specific activity of NOS in cell lysates (22 h). Data are from a representative experiment, with five or six replicate wells per point.

beating rates in beats per minute (bpm) of myocytes treated with IL-1β and cultured in medium supplemented with ITS+ or ITS/Albumax were 28 ± 5 *vs.* 120 ± 9 bpm (±SEM), respectively (average of 12 wells in 3 separate experiments). In contrast, the change in supplements had little effect on the beating rate in the presence of either TGFβ (140 ± 5 *vs.* 140 ± 4 bpm, respectively) or the combination of TGFβ and IL-1β (130 ± 11 *vs.* 140 ± 8 bpm, respectively).

Effects of Nitric Oxide on Beating Rate Can Be Attenuated by Culture Conditions

Correlations among the beating rate, cGMP levels, and nitrite secretion of myocytes appear to be limited to a narrowly defined set of culture conditions, namely those confined to myocytes cultured on fibroblast matrix in medium supplemented with ITS+ (high LPS) and treated with either TGFβ or IL-1β. Similar correlations cannot be found for myocytes treated with TNFα and/or IFNγ or for myocytes cultured on gelatin (denatured collagen), rather than native fibroblast matrix. As shown in Table 1, treatment of myocytes with TNFα and IFNγ in combination with IL-1β either failed to increase or even attenuated the suppressive effects of IL-1β on the beating rate, even though levels of nitrite and cGMP increased substantially. Moreover, although nitrite and cGMP levels of myocytes treated with IL-1β and cultured on gelatin are similar to those of myocytes cultured on fibroblast matrix, the beating rate of the cells on gelatin is not suppressed, in agreement with previous observations (19). These data demonstrate that the induction of NO and cGMP represents only one of

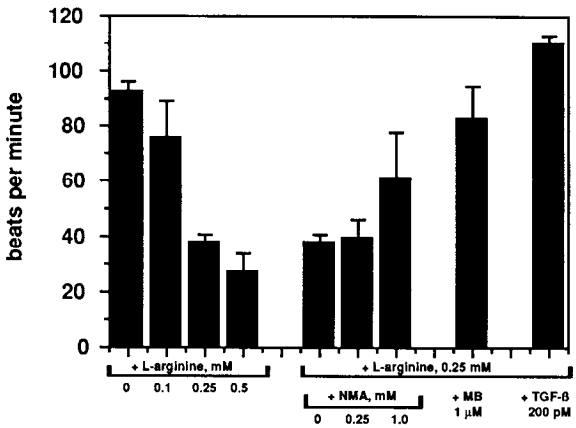

Fig. 7. Effects of L-Arginine and NMA on the Beating Rate of Myocytes Treated with IL-1β

Myocytes were cultured as described for 22 h in L-arginine-free DMEM supplemented with ITS+ (high LPS) and containing 5 ng/ml IL-1β. Results represent the mean ± SEM of five replicate wells in one experiment of four of similar design. MB, Methylene blue. TGFβ, 200 pM = 5 ng/ml.

multiple parameters involved in the regulation of the myocyte-beating rate.

DISCUSSION

We have shown that neonatal cardiac myocytes express iNOS and secrete NO after treatment with LPS, IL-1β plus LPS, or the combination of IL-1β, IFNγ, and TNFα. TGFβ, added simultaneously, antagonizes the effects of these cytokines and suppresses NO synthesis by blocking accumulation of iNOS. The inverse relationship of effects of these cytokines on nitrite secretion or cGMP production compared to their effects on the beating rate of the myocytes suggests that the L-arginine-NO-cGMP pathway may play a critical role in suppression of myocyte rhythmicity.

The recent demonstration that TGFβ also suppresses expression of iNOS in macrophages (Vodovotz, Y., C. Bogdan, and C. Nathan, manuscript in preparation) suggests that this may be a general mechanism of TGFβ action in inhibiting NO production by iNOS in a variety of tissues. Since overproduction of NO by iNOS induced by either endotoxin or cytokines can result in inappropriate vasodilation and cell injury, new avenues of therapy that specifically target iNOS and not cNOS are being sought (24). Our findings raise the possibility that TGFβ itself or agents that induce local expression of TGFβ (25) could be useful as an adjunct to combination therapy targeted to iNOS. Such therapy holds promise for prevention or treatment of myocardial dysfunction in situations where NO may be generated in response to LPS or proinflammatory cytokines, as in some of the newer antitumor cyokine therapies, inflammatory diseases of the heart, and septic shock (6, 24, 26).

Our observations that increased NO secretion leads to suppression of the myocyte-beating rate are consistent with previously described relaxant effects of NO in vascular smooth muscle cells (4, 27, 28). To our knowledge, this is the first demonstration of a direct link between NO secretion and the function of cultured neonatal cardiac myocytes. Schulz *et al.* (6) recently showed that both intact ventricular myocardium and cultured adult cardiac myocytes secrete NO and elevate cGMP production in response to treatment with endotoxin *in vivo* or IL-1β and TNFα *in vitro*; however, no functional consequences were demonstrated.

We have demonstrated a profound effect of traces of LPS on the physiology of cultured cardiac myocytes. Beating rate, cGMP levels, NO production, iNOS induction, response to cytokines, and perhaps even response to extracellular matrix were all markedly influenced by LPS present in the ITS+ supplement at picogram per ml concentrations. Other investigators working with phagocytes and other cell types have long recognized that they must contend with LPS as a common contaminant of cell separation media, culture components, cytokines, and chemicals (29, 30). Our findings underscore the caution required in interpreting and comparing

Table 1. Effect of Matrix on Responses of Myocytes to Cytokines

Treatment[a]	Fibroblast Matrix			Gelatin		
	bpm[b]	Nitrite (μM)	cGMP (fmol)	bpm[b]	Nitrite (μM)	cGMP (fmol)
IL-1β	0	0.75	0.54	85 ± 12	0.63	0.41
TGFβ	160 ± 8	0	0.25	120 ± 4	0	0.24
IL-1β + TGFβ	150 ± 36	0	0.39	120 ± 5	0	0.41
IL-1β + IFNγ	89 ± 25	9.0	1.2	120 ± 14	9.3	2.9
IL-1β + TNFα	58 ± 26	3.0	0.94	76 ± 3	3.3	1.1
IL-1β + IFNγ + TNFα	120 ± 16	13	1.2	100 ± 14	14	1.8
IL-1β + IFNγ + TNFα + TGFβ	120 ± 9	7.7	1.2	120 ± 11	8.6	0.84

[a] Results are from a representative experiment following 22-h culture in the presence of the following concentrations of cytokines: IL-1β and TGFβ, 5 ng/ml; IFNγ, 9 ng/ml; and TNFα, 25 ng/ml. Cardiac fibroblast matrix or gelatin coatings are as previously described (19).

[b] Mean ± SEM of five replicate wells.

the results of studies in which the LPS content of the reagents has not been reported.

This concern may bear on the recent findings of Finkel *et al.* (20), who reported that NO mediated a negative inotropic effect of cytokines on heart muscle within 2–3 min. The effect was reversed by NMA, and the researchers concluded that the cytokines activated a cNOS in the myocardium, since the effects were too rapid to reflect induction of iNOS. These results contradict those of Schulz *et al.* (6), who demonstrated that IL-1β and TNFα induce expression of iNOS in adult cardiac myocytes, but have no effect on the activity of cNOS. The immunoblot (Fig. 4) and the prolonged time course of the effects of IL-1β in our experimental system (Fig. 1) also clearly implicate iNOS in NO production induced by cytokine treatment of neonatal cardiac myocytes. Indeed, activation of cNOS by cytokines is unprecedented (1). On the other hand, LPS has been reported to activate cNOS within minutes (31), a role distinct from its participation in the transcriptional induction of iNOS over a period of hours (2), as in the present study. Thus, the possibility should be considered that contaminating LPS may have been responsible for the effects observed by Finkel *et al.* (20).

Our data demonstrate that elevated NO secretion and cGMP synthesis do not always result in suppression of the myocyte-beating rate. We had previously shown that IL-1β had little effect on the beating rate of myocytes cultured on a coating of denatured type I collagen (gelatin) (19). These myocytes assume a distinctly different, more spread, flattened shape than myocytes cultured on fibroblast matrix. Since levels of cGMP or nitrite secreted by myocytes cultured on either of these matrices are comparable (Table 1), the possibility that the myocyte phenotype itself or growth factors bound to the native matrix (32) may modulate the cellular response to NO, must be considered. Another notable exception to the inverse correlation between nitrite levels and beating rate is the inability of high levels of nitrite resulting from treatment with the combination of IL-1β, IFNγ, and TNFα to suppress the beating rate of myocytes cultured on either fibroblast matrix or gelatin. Interestingly, whereas it has been shown that IL-1β and TNFα suppress myocardial contractility (18) and that TNFα mimics the injury seen after myocardial ischemia-reperfusion (33), pretreatment of animals with IL-1β, TNFα, or LPS provides protection against subsequent damage following myocardial ischemia-reperfusion injury (34–36). Since IL-1β and TNFα pretreatment induce manganous superoxide dismutase (34), and LPS pretreatment induces catalase (36), it has been suggested that the consequent detoxification of superoxide anions might play a role in the protection. Accordingly, the paradoxical effects of treatment with combinations of these cytokines on the myocyte-beating rate might result from a multiplicity of both early and later effects on gene expression, depending on the relative contributions of reactive nitrogen and reactive oxygen intermediates (1).

The mechanism by which NO regulates the beating rate of the myocytes is unknown. However, it has been shown that elevation of cellular cGMP, resulting from activation of soluble guanylate cyclase by NO, can stimulate Na^+/Ca^{2+} exchange in vascular smooth muscle cells (37). This has been proposed to lead to a reduction of intracellular Ca^{2+}, which, in turn, causes relaxation of these cells with consequent vasodilation. Moreover, cGMP is known to exert a negative inotropic effect on cardiac myocytes, thought to result from activation of an endogenous cGMP-dependent protein kinase that regulates the Ca^{2+} channel current (38). Recent data obtained in Sertoli cells demonstrate that treatment with TGFβ for more than 6 h stimulates the uptake of Ca^{2+} (Grasso, P., L. E. Reichert, M. B. Sporn, and T. A. Santa-Coloma, submitted). Preliminary experiments suggest that TGFβ also stimulates and IL-1β inhibits Ca^{2+} uptake by neonatal myocytes (Santa-Coloma, T., personal communication).

Other mechanisms could also contribute to suppression of myocyte contractility. Thus, nitrosylation of key iron-containing enzymes of the respiratory chain has been shown to lead to dysfunction of mitochondrial energy metabolism (39, 40). However, since cellular ATP levels in neonatal myocytes treated with IL-1β are not significantly different from those in myocytes treated with TGFβ (19), it is unlikely that these effects play a significant role in our system. Nevertheless, other, as yet unidentified, key regulatory proteins, including cytokines secreted by the myocytes, may also be targets for nitrosylation, resulting in alteration of their biological activity (41–43).

MATERIALS AND METHODS

Reagents

Porcine TGFβ1 was obtained from R & D Systems (Minneapolis, MN), recombinant human IL-1β and murine TNFα from Michael Palladino, Genentech, Inc. (South San Francisco, CA), recombinant rat IFNγ from Gibco, and LPS (*E. coli* serotype) from Sigma (St. Louis, MO). All factors were stored in concentrated solutions at −70 C and added to the culture medium as 50-fold concentrated solutions in medium supplemented with ITS (insulin, transferrin, and selenium; Biofluids) and Albumax (lipid-rich BSA; 1 mg/ml final concentration; Gibco). NMA, acetate salt, was obtained from Calbiochem (La Jolla, CA), and all other reagents were purchased from Sigma. LPS was determined using a Chromogenic Limulus Amebocyte Lysate assay kit (Whitaker Biochemicals, Walkersville, MD).

RIA grade BSA, nitroblue tetrazolium (NBT), 5-bromo-4-chloro-3-indolylphosphate, NADPH (reduced), flavin adenine dinucleotide, phenylmethylsulfonylfluoride, aprotinin, chymostatin, and pepstatin-A were obtained from Sigma. Tetrahydrobiopterin was obtained from Dr. B. Schirks Laboratories (Jona, Switzerland). Dithiothreitol was obtained from Gibco-Bethesda Research Laboratories (Gaithersburg, MD). Rabbit antimouse iNOS immunoglobulin G (IgG) was produced as previously described (2).

Preparation and Culture of Neonatal Rat Cardiac Myocytes

Myocytes were prepared from the left ventricles of 2-day-old rat pups, as previously described (19). Briefly, after differential

plating to remove fibroblasts, myocytes were further purified on a gradient system consisting of Percoll (Sigma) in Minimum Essential Medium (Gibco) adjusted to final densities of 1.082, 1.061, and 1.050 g/ml (44). The purified myocytes banding between the 1.082 and 1.061 g/ml layers were cultured in DMEM/F12 containing 10–15 mM HEPES buffer (Gibco) and supplemented with 5% calf serum, penicillin (50 U/ml), and streptomycin (50 μg/ml). Cells were seeded at a density of 2.2 × 10^5 cells/ml·16-mm well on Falcon (Oxnard, CA) or Costar (Cambridge, MA) 24-well plates coated with either gelatin (ICN Biochemicals, Costa Mesa, CA) or matrix, prepared as previously described (45), from cardiac fibroblasts used at the first passage after the differential plating. Cells were fed fresh medium containing 5% calf serum 48 h after plating. Twenty-four hours later, at which time the myocytes had formed a syncytium and beat synchronously, they were washed with serum-free medium, incubated in the same medium for 30 min, and then placed in fresh serum-free medium containing the added factors (time zero). Medium consisted of either DMEM/F12 or L-arginine-free DMEM supplemented with ITS+ (Collaborative Research), or DMEM/F12 supplemented with ITS (Biofluids) plus Albumax (Gibco). Under these conditions, nonmyocytes (endothelial cells, smooth muscle cells, and fibroblasts) represent only 5–10% of the cell population. The beating rate of the myocytes was measured by counting the time required for 30 beats of the myocytes using an enclosed heated stage maintained at 37 C (Nikon incubator NP-2, Nikon, Inc., Melville, NY). Each treatment consisted of 4–16 replicate wells.

Determination of cGMP

At the end of the observation period, each well was washed with 1 ml of a solution consisting of 5.5 mM dextrose and 10 mM HEPES, pH 7.3, in PBS and incubated for 10 min at 37 C in 200 μl of the same solution containing the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine at a final concentration of 0.5 mM. The reaction was terminated by the addition of $HClO_4$ to a final concentration of 0.7 M, cells were extracted on ice for 30 min, and aliquots of the pooled extracts of four to six wells were stored at −20 C. Aliquots were neutralized with 4 N K_2CO_3, and the supernatant was assayed in duplicate using a cGMP RIA kit (Amersham, Arlington Heights, IL). The range of duplicate determinations was less than 5%, while the SD of values of individual wells comprising the pool averaged 10% of the mean.

Preparation of Cell Lysates

At the end of the experiment, each well was washed with 1 ml PBS, and the cells were released with 1 ml trypsin-EDTA (Gibco). Pooled cells from 8–12 wells were pelleted by low speed centrifugation, resuspended in PBS, and recentrifuged. The final cell pellet was overlaid with 100 μl PBS, immediately frozen on dry ice, and stored at −20 C. The cell pellet was resuspended in 500 μl lysis buffer [100 mM phosphate (pH 7.4), 25 mM NaCl, 5 μg/ml pepstatin-A, 1 μg/ml chymostatin, 5 μg/ml aprotinin, and 100 μM phenylmethylsulfonylfluoride]. After sonication, an aliquot was removed for determination of protein using the Bradford assay (Bio-Rad, Richmond, CA), and RIA grade BSA was added to give a final protein concentration of 1–3 mg/ml. Aliquots were stored at −70 C.

Western Blotting of iNOS

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out according to the method of Laemmli (46) on 7.5% acrylamide, 1.5-mm thick slab gels, using prestained high mol wt markers (Bethesda Research Laboratories). Proteins were transferred to 0.2-μm pore size nitrocellulose membranes using a buffer consisting of 20% methanol, 25 mM Tris, and 192 mM glycine, pH 8.3, at 4 C. The membrane was blocked for either 1 h at room temperature or overnight at 4 C with 1% BSA in TBS [25 mM Tris (pH 7.5), 150 mM NaCl, and 0.02% NaN_3] containing 0.2% Tween-20. The membrane was washed once in TBS and incubated for 2–3 h with rabbit antimouse iNOS IgG (2) used at a 1:2000–3000 dilution. After successive washes in TBS, TBS-0.2% Tween-20, and TBS, the membrane was incubated for 45 min with donkey antirabbit IgG, $F(ab)_2'$, conjugated to alkaline phosphatase (Jackson Laboratories, Bar Harbor, ME; 1:10,000 final dilution). After successive washes as before, the membrane was equilibrated for 5 min in 100 mM Tris (pH 9.5), 100 mM NaCl, and 5 mM $MgCl_2$ and developed in a solution of 167 μg/ml each of NBP and 5-bromo-4-chloro-3-indolylphosphate in the same buffer.

Measurement of Nitrite Accumulation

Nitrite accumulation in the supernatant of cultured cells was used as an indicator of NO production and was measured by the Griess reaction, as described previously (9, 15). Briefly, 100 μl Griess reagent were added to 100 μl of each pooled supernatant in triplicate. The plates were read using a Dynatech MR5000 ELISA plate reader (Los Angeles, CA) at 505 nm. Nanomoles of NO_2^- were determined by comparison to a standard curve of $NaNO_2$. The SD of values of five individual wells comprising the pool was 7–10% of the mean.

NOS Enzyme Assay

The NOS activity of cell lysates was determined essentially as described previously (47). The reaction was carried out in duplicate in 96-well plates (Corning, Corning, NY) in 100 μl 20 mM Tris-HCl, pH 7.9, containing 4 μM each of flavin adenine dinucleotide and tetrahydrobiopterin, and 3 mM dithiothreitol. The reaction was started by adding L-arginine and NADPH, each to a final concentration of 2 mM, and continued for 2–3 h at 37 C. As a control, an additional well for each sample contained all reagents except L-arginine and NADPH. Residual NADPH was oxidized enzymatically with lactate dehydrogenase and pyruvate (48). The plates were read as described above, and the optical density value from the control well was subtracted from the mean of the test wells before comparison to the standard curve of $NaNO_2$ plus lactate dehydrogenase.

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