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Research Article

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Nitric Oxide Synthase (NOS3) and Contractile Responsiveness to Adrenergic and Cholinergic Agonists in the Heart

Regulation of NOS3 Transcription In Vitro and In Vivo by Cyclic Adenosine Monophosphate in Rat Cardiac Myocytes

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Abstract

Cardiac myocytes express the nitric oxide synthase isoform originally identified in constitutive nitric oxide synthase cells (NOS3), which mediates the attenuation by muscarinic cholinergic agonists of β-adrenergic stimulation of L-type calcium current and contractility in these cells. However, the reciprocal regulation of NOS3 activity in myocytes by agents that elevate cAMP has not been reported. In this study, we show that NOS3 and mRNA and protein levels in cardiac myocytes are reduced both in vitro after treatment with cAMP elevating drugs, and in vivo after 3 d of treatment with milrinone, a type III cAMP phosphodiesterase inhibitor. This effect on NOS3 activity by cAMP is cell type specific because treatment of cardiac microvascular endothelial cells in vitro or in vivo did not decrease NOS3 mRNA or protein in these cells. NOS3 downregulation in myocytes appeared to be at the level of transcription since there was no modification of NOS3 mRNA half-life by agents that increase intracellular cAMP. To determine the functional effects of NOS3 downregulation, we examined the contractile responsiveness of isolated electrically paced ventricular myocytes, isolated from animals that had been treated in vivo with milrinone, to the β-adrenergic agonist isoproterenol and the muscarinic cholinergic agonist carbamylcholine. There was no difference in baseline contractile function in cells that had been pretreated with cAMP elevating agents compared to controls, but cells exposed to milrinone in vivo exhibited an accentuation in their contractile responsiveness to isoproterenol compared to controls and a loss of responsiveness to carbamylcholine. Downregulation of myocyte NOS3 by sustained elevation of cAMP may have important implications for the regulation of myocardial contractile state by the autonomic nervous system. (*J. Clin. Invest.* 1996. 97:1908–1915.) **Key words: cell culture • 3′-5′-cyclic nucleotide phosphodiesterase • L-arginine • calcium • microvascular endothelial cells**

Introduction

The three NO synthase isoforms, originally identified in brain, constitutive neuronal nitric oxide synthase (NOS1)[1] (1), macrophages, inducible nitric oxide synthase (NOS2) (2), and endothelial cells, constitutive endothelial nitric oxide synthase (NOS3), (3) are each encoded by different genes and subsequently have been found expressed in a variety of tissues and cell types. For example, ventricular myocytes, as well as microvascular endothelial cells from adult rat hearts, express NOS2 in response to specific cytokines (4). However, both cardiac myocytes and microvascular endothelial cells also constitutively express the isoform originally identified in large vessel endothelial cells (i.e., NOS3). Among other roles, NOS3 in cardiac myocytes mediates nitric oxide–dependent parasympathetic signaling in these cells (5).

Nitrogen oxides (NO_X), produced by the myocytes themselves or by neighboring microvascular endothelial cells in cardiac muscle, attenuate myocyte inotropic responsiveness to β-adrenergic stimulation (6–8). One of the key second-messenger molecules mediating the positive inotropic effect of β-adrenergic agonists in cardiac myocytes is cAMP. In the amphibian heart, exogenous nitric oxide suppresses the activation of the L-type calcium channel by cAMP by activating guanylate cyclase and cGMP-regulated cyclic nucleotide phosphodiesterases (9). However, the reciprocal regulation of nitric oxide synthase isoform(s) by cAMP in any cell type has been less well characterized. In vascular smooth muscle cells, cAMP was shown to potentiate the induction of NOS2 expression by cytokines (10). Purified NOS1 protein was also shown to be phosphorylated by cAMP-activated protein kinase A in vitro, although the functional relevance of this phosphorylation to nitric oxide synthase (NOS) activity in intact cells remains controversial (11). NOS3 mRNA levels and activity in large vessel endothelial cells are known to be increased by shear stress (12) and decreased by inflammatory cytokines (3, 13). NOS3 expression in cardiac myocytes is also decreased by cytokines. These effects may be mediated by one or more *cis*-regulatory sequences within the promoter region of the NOS3 gene, including shear-stress responsive elements and several AP-1 sites (14, 16). NOS3 promoter analyses have also identified a putative cAMP responsive element in the 5′-flanking region of NOS3, which does not appear to be essential for basal transcriptional activity in bovine aortic endothelial cells (16). However, the regulation of NOS3 expression or activity after in-

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1. *Abbreviations used in this paper:* BAPTA, 1, 2-bis(2-amino phenoxy)ethane-N,N,N′,N′-tetra acetic acid; CMEC, cardiac microvascular endothelial cells; NOS, nitric oxide synthase; NOS1, constitutive neuronal NOS; NOS2, inducible NOS; NOS3, constitutive endothelial NOS.

creases in intracellular cAMP in cardiac myocytes or other cell types has not been characterized.

In this report, we demonstrate that NOS3 mRNA levels, protein abundance, and intracellular activity are decreased after a sustained increase in cAMP in vitro and in vivo in ventricular myocytes, but not in microvascular endothelial cells from adult rat hearts. Finally, the decrease in NOS3 abundance with cAMP in cardiac myocytes was associated with a potentiation of contractile responsiveness to β-adrenergic agonists and a loss of responsiveness to muscarinic cholinergic stimulation.

Methods

Cardiac myocyte and microvascular endothelial cell isolation. Purified ventricular myocytes were prepared as previously described (17) from adult male Sprague-Dawley rats (250 g; Harlan Sprague Dawley Corp., Indianapolis, IN). Cardiac myocytes were plated on laminin and cultured in a defined medium termed ACCITT (17) (i.e., DMEM supplemented with 2 mg/ml albumin, 2 mM L-carnitine, 5 mM creatine, 0.1 μM insulin, 5 mM taurine, 0.1 nM triiodothyronine, 1.3 mM L-glutamine, and 100 IU/ml penicillin/streptomycin) for at least 18 h before initiation of any experimental protocols. In the experiments involving actinomycin D (Sigma Chemical Co., St. Louis, MO), a final concentration of 10 μg/ml was used, with or without addition of forskolin (10 μM), and the cells subsequently were lysed at successive time intervals.

In some experiments, 300-g Sprague-Dawley rats were treated with milrinone (M4856; Sigma Chemical Co.) as described by Sweet et al. (18) with minor modifications. Rats received 4 mg/kg milrinone in the drinking water for the first 24 h, and 2 mg/kg/d for an additional 48 h, and then were killed at 72 h. Fresh ventricular myocyte primary isolates were prepared as described above. Purification of the myocyte fraction included two density-gradient sedimentation steps through a 6% BSA cushion. The myocyte-enriched pellet was used immediately for RNA or protein isolation. The supernatant was collected and centrifuged for 2 min at 100 *g* to sediment any myocytes still in suspension. this step was repeated three times. A subsequent higher speed centrifugation (5 min, 1,000 *g*) was used to pellet the cells remaining in suspension and is referred to as the nonmyocyte fraction. This fraction contained < 5% myocytes and was also used immediately for RNA or protein isolation.

Cardiac microvascular endothelial cells (CMEC) were prepared and cultured as previously described (19), with following modifications: primary isolates of CMEC were cultured for 4 d in DMEM supplemented with 20% FBS (GIBCO BRL, Gaithersburg, MD), and then serum starved for 24 h before the initiation of experimental protocols. CMEC freshly isolated from milrinone-pretreated rats (as described above) were isolated immediately for preparation of protein and total RNA.

Northern blots. Northern blots were performed using standard methods (20, 21). 15 μg of total RNA was electrophoresed through a 1% formaldehyde-agarose gel and transferred to a nylon membrane (Dupont-NEN, Boston, MA). The blots were hybridized with a ^{32}P-radiolabeled 324-bp rat NOS3 cDNA probe (5) for 16 h. They were then washed with 2 × SSC, 0.1% SDS at room temperature, followed by 1 × SSC, 0.1% SDS at 50°C and 0.2 × SSC, 0.1% SDS at 65°C, each for 15 min. Autoradiography was performed at −70°C for 16–24 h.

Western blots. Cells were lysed in a lysis buffer containing 1% Triton X-100, 0.5% NP-40, 150 nM NaCl, 10 mM Tris–HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, and protease inhibitors (1 mM leupeptin, 1 mM PMSF). Protein quantification was performed using a modified Lowry assay (*DC* protein assay, Bio-Rad Laboratories, Richmond, CA). 50–100 μg of denatured protein per lane were loaded and separated on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane. Equal staining and transfer were verified by reversible staining with Ponceau red. After blocking in Tris-buffered saline with 0.01% Tween and 2% BSA, membranes were exposed for 1 h to either NOS3-, NOS2-, or NOS1-specific monoclonal antibodies (Transduction Laboratories, Lexington, KY), followed by six washes and incubation with a goat anti–mouse secondary antibody coupled to peroxidase activity. After six more washes, the membranes were incubated with chemiluminescence detection reagents (Renaissance kit, Dupont-NEN) and exposed to Kodak XAR film (Eastman Kodak Co., Rochester, NY) for 15 s to 2 min.

Measurement of total heart cAMP content. cAMP content was measured in total heart extracts from milrinone-treated and control rats. Rats were killed and hearts were removed, frozen in liquid nitrogen, and kept at −80°C until assayed. Heart tissue was weighed and homogenized with a mortar and pestle in 5 ml 0.1 N HCl containing isobutyl methyl xanthin (0.3 mM). The protein precipitate was separated by centrifugation (10,000 rpm; 20 min, 4°C) and kept for protein measurement. cAMP in the supernatant was measured using a radioimmunoassay kit from Biomedical Technologies Inc. (Stoughton, MA).

Measurements of NOS activity in intact cardiac myocytes. NOS activity in intact myocytes was quantitated by measuring the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline. Cardiac myocytes, isolated from either control or milrinone-treated rats, were plated on laminin-coated dishes and incubated for 3 h in L-arginine–free ACCITT with or without 3 mM L-*N*-nitroarginine (L-NA), a NO synthase inhibitor. 5 μCi/ml of purified L-[^{3}H]arginine (Amersham Corp., Arlington Heights, IL) was then added to each dish, along with 2 mM unlabeled L-arginine, 1 mM L-citrulline, 10 nM isoproterenol, and 10 μM carbamylcholine. The cells were incubated for 20 min at 37°C, then kept on ice and washed three times with ice-cold PBS (GIBCO BRL), and scraped free in ice-cold sodium acetate containing 2 mM EDTA, 0.2 mM EGTA, and 1 mM L-citrulline. Homogenates were prepared by sonication on ice (sonifier 450; Branson Ultrasonics Corp., Danbury, CT) 30 s, and 50-μl aliquots were removed for protein quantification and determination of total radioactivity. The L-[^{3}H]citrulline content was determined by anion-exchange chromatography (5) after subtraction of background radioactivity, defined as L-[^{3}H]citrulline radioactivity from homogenates of myocytes that had been pretreated with L-NA. NOS activity was calculated after normalization to protein content and the total radioactivity incorporated in the cells. The results are expressed in femtomol of L-[^{3}H]arginine converted per mg of protein.

Myocyte contractility measurements. Cell-shortening measurements were performed as previously described (6). Freshly isolated cells from control or milrinone-treated rats were plated onto laminin-coated glass coverslips and incubated in ACCITT for 3 h. During this time, the medium was changed every hour to assure removal of any residual milrinone. Coverslips were placed in a superfusion chamber on the thermostated stage of an inverted microscope. One cell per coverslip was selected following previously described criteria (rod-shaped microscopical appearance, absence of spontaneous contraction, symmetrical shortening during electrical stimulation) and edge detection was performed using digital optical video motion analysis.

Statistics. Group comparisons were made using the mean parametric Kruskal-Wallis test. Means±SD for each group are presented for convenience, but no assumptions have been made regarding a normal distribution of the data. P values < 0.05 are considered significant.

Results

Regulation of cardiac myocyte NOS3 mRNA levels by cAMP in vitro. NO, produced by NOS3 in cardiac myocytes, regulates the L-type calcium current and contractile responsiveness to β-adrenergic stimulation in these cells (5, 22). To determine whether sustained alterations of intracellular concentrations of cAMP could regulate the NO synthase pathway in cardiac myocytes, we used several agents known to increase intracellular cAMP content and characterized their effects on NOS3 transcript and protein levels. Figs. 1 and 2 illustrate the effect

of forskolin on NOS3 mRNA abundance in extracts of cardiac myocytes cultures in vitro and analyzed by Northern blot using a rat NOS3 cDNA probe (5).

After 24 h of treatment in vitro, forskolin (1–10 μM) induced a concentration-dependent decrease in NOS3 mRNA levels with an approximate EC_{50} at 3 μM (Fig 1, *A* and *B*) and a maximal inhibition of 70% with 10 μM forskolin (Fig. 1, *A* and *B*). The time dependence for this effect was studied by treating cardiac myocytes with a maximally active (10 μM) concentration of forskolin for 4 to 24 h in vitro (Fig. 1, *C* and *D*). A significant decline in NOS3 transcript levels could be detected within 4 h of exposure to forskolin. These effects were also observed after a 24-h exposure to other agents that elevate cAMP, including 8-Br–cAMP (1 mM; 82±24% inhibition, $n = 4$), isobutyl methyl xanthin (200 μM; 59±8% inhibition, $n = 3$), milrinone (20 μM; 55±5% inhibition, $n = 3$) and cholera toxin (1 nM; 71±12% inhibition, $n = 3$).

Activation of adenylyl cyclase is known to increase L-type calcium channel current by phosphorylation of L-type voltage-sensitive calcium channels (23), thereby increasing intracellular calcium concentration in rat myocytes via calcium-induced release from the sarcoplasmic reticulum. To determine the role of calcium in forskolin's effect on NOS3 transcript levels, we used BAPTA (1, 2-*bis*-[2[amino phenoxy]ethane-*N,N,N′, N′*-tetra acetic acid), an agent known to chelate intracellular calcium, and verapamil, an L-type calcium channel antagonist. Neither BAPTA (10 μM) nor verapamil (10 μM) at concentrations sufficient to prevent excitation–contraction coupling in these cells had any statistically significant effect on NOS3 mRNA abundance at 24 h (BAPTA: 3±15% compared to control, $n = 3$; verapamil: −2±17% compared to control, $n = 3$). Also, neither agent reversed forskolin's downregulation of NOS3 transcript levels (Fig. 2), indicating that increased levels of cAMP, but not calcium, are responsible for reduction of NOS3 expression. These data indicate that cAMP elevation is sufficient by itself for the regulation of NOS3. However, they do not exclude a role for calcium in the regulation of NOS3 mRNA levels.

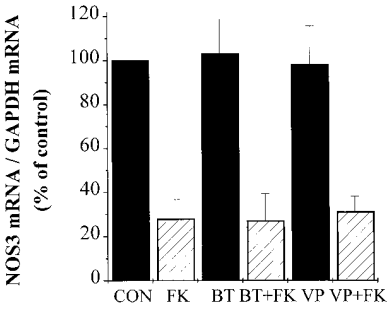

Figure 2. Involvement of calcium in the regulation of NOS3 expression by forskolin. Adult rat ventricular primary isolates were cultured in defined medium for 18 h and then exposed to 10 μM forskolin (*FK*) alone or with either 10 μM BAPTA (*BT*) or 10 μM verapamil (*VP*). Northern analysis was performed as described in the legend to Fig. 1. NOS3 mRNA levels were normalized to GAPDH mRNA levels. After this normalization, data from three separate experiments were expressed as means±SD with the mean NOS3 hybridization intensity in control, untreated cells set to 100%. This experiment was performed three times with similar results.

Three recent studies have identified a cAMP response element and several AP-1 sites in the 5′-flanking region of NOS3 (14, 16), all of which could potentially mediate the transcriptional regulation of this gene. To exclude the possibility that a decrease in NOS3 mRNA abundance by cAMP elevating agents might be due to a posttranscriptional regulation of mRNA half-life, we measured the effect of forskolin in cardiac myocytes cotreated with actinomycin D. As shown in Fig. 3, the half-life of NOS3 mRNA was not significantly affected by forskolin (7.4±2.5 h vs 6.8±2.7 h, with and without forskolin, respectively), suggesting that elevation of intracellular cAMP

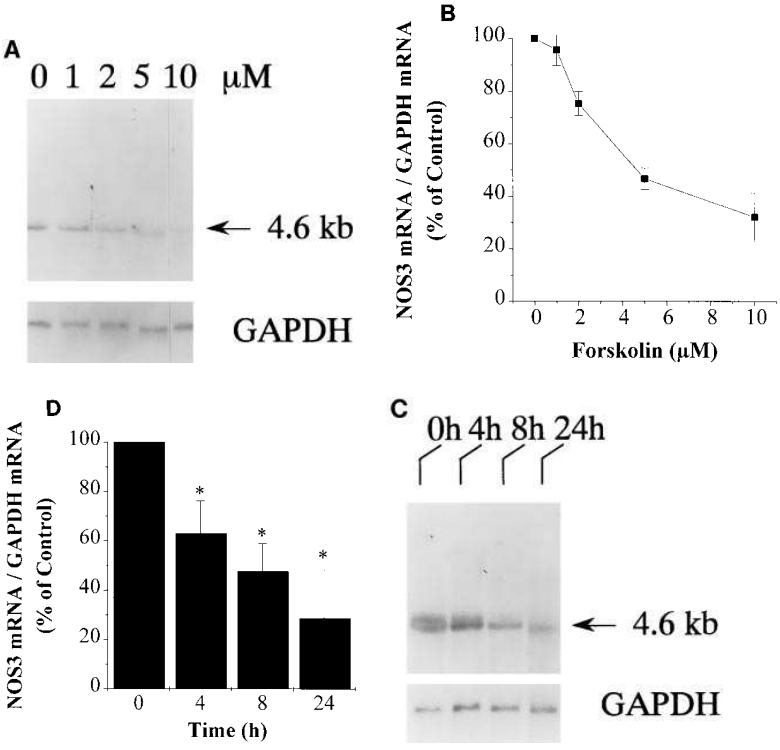

Figure 1. Concentration dependence and time course of the decrease in cardiac myocyte NOS transcript levels after forskolin exposure. Primary isolates of adult rat ventricular myocytes were cultured in a defined medium (ACCITT; see Methods) for 18 h and then exposed to forskolin as indicated. Total RNA was extracted from homogenates and analyzed by Northern blot using a radiolabeled 324-bp cDNA probe for NOS3 as described in Methods. Hybridization of the same blot with a radiolabeled probe for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (Clontech, Palo Alto, CA) was performed to indicate the amount of RNA loaded per lane (15 μg). (*A*) Northern blot of NOS3 mRNA from cardiac myocytes treated with 1–10 μM forskolin for 24 h. (*B*) Concentration-response curve of corrected NOS3 mRNA levels normalized to the hybridization signal for GAPDH (mean±SEM, $n = 3$ experiments). (*C*) Northern blot of mRNA from cardiac myocytes exposed to 10 μM forskolin for intervals from 4 to 24 h. (*D*) Time course of corrected NOS3 mRNA levels normalized to the hybridization signal for GAPDH (mean±SD, $n = 4$ experiments; $^*P < 0.01$).

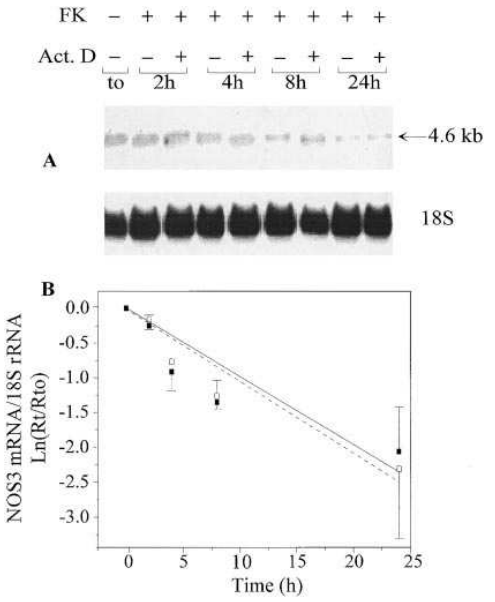

Figure 3. Forskolin does not modify NOS3 mRNA stability in cardiac myocytes. (*A*) Northern blot of total RNA from myocytes treated with actinomycin D (10 μM), with or without forskolin (10 μM), hybridized with a NOS3-specific probe or 18S rRNA probe, according to the techniques described in the legend to Fig. 1 and Methods. (*B*) The time course of the decrease in NOS3 mRNA levels is illustrated after treatment with 10 μg/ml actinomycin D, either with (*open squares, dotted line*) or without (*filled squares, solid line*) 10 μM forskolin. The calculated $t_{1/2}$ of NOS3 mRNA was ~ 7 h in the absence or presence of forskolin. Each curve was generated from NOS3 mRNA signals, which had been corrected for total RNA loading using 18S hybridization signals, from three separate experiments. After normalization of the result at each time point to the control value at time 0, the Naperian logarithms of the ratios were plotted as a function of time.

does not alter the stability of NOS3 mRNA in cardiac myocytes under these conditions.

Regulation of cardiac myocyte NOS3 mRNA levels by milrinone in vivo. To determine whether sustained elevations of intracellular cAMP content could also regulate NOS3 mRNA in vivo, adult rats were treated for 3 d with the type III phosphodiesterase inhibitor milrinone added to their drinking water, as previously described by Sweet et al. (18). As expected, milrinone increased the total heart cAMP content in treated rats over levels in control, untreated animals over a 3-d period (0.9±0.26 vs 7.2±1.55 pmol/mg protein, $n = 3$ for each condition, $P < 0.01$). Over the same time interval, NOS3 mRNA abundance in extracts of freshly isolated cardiac myocytes from milrinone-treated rats decreased by 85±24% ($n = 3$) compared with extracts from control animals (Fig. 4).

Regulation of cardiac myocyte NOS3 protein levels by cAMP in vivo and in vitro. To determine whether the decrease in NOS3 mRNA levels associated with increased cAMP was accompanied by decreases in NOS3 protein abundance, extracts of cardiac myocytes were analyzed by Western blot using an mAb against human NOS3 as previously described (5). As shown in Fig. 5 *A*, exposure of cardiac myocytes in vitro to forskolin for 24 h decreased protein levels by 52±15%. A qualitatively similar effect was observed with 8-Br–cAMP (Fig. 5 *A*). In extracts of cardiac myocytes isolated from rats treated with

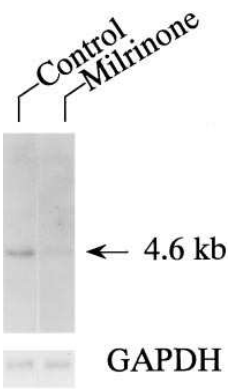

Figure 4. Regulation of NOS3 mRNA from cardiac myocytes isolated from rats pretreated with milrinone in vivo. As described in Methods, male Sprague-Dawley rats were given milrinone in their drinking water, or water alone for 3 d. Total RNA from freshly isolated ventricular myocytes was analyzed by Northern blot using a 324-bp radiolabeled cDNA for NOS3. The same blot was then rehybridized with a radiolabeled probe for GPDH to estimate the amount of total RNA loaded in each lane. Similar results were obtained in three different experiments.

milrinone as described above, NOS3 protein abundance was decreased by 80±33% after a 3-d treatment, compared with NOS3 protein levels from control rats (Fig. 5 *B*).

Aliquots of the same protein extracts from control cardiac myocytes and from myocytes with elevated cAMP were analyzed by Western blotting using monoclonal antibodies against either murine NOS2 or human NOS1. These reagents did detect their respective NOS protein isoforms in control samples from rat brain (for NOS1) or rat macrophages (for NOS2) (data not shown). However, no specific band was detected in myocyte extracts, either under control conditions or after interventions elevating cAMP, indicating the absence of constitutive expression of these NOS isoforms in cardiac myocytes or their upregulation with agents that elevate cAMP in myocytes.

Decreased NOS3 activity and cardiac myocyte contractile function after milrinone treatment in vivo. The decrease in NOS3 protein abundance after milrinone treatment in vivo, as described above, was accompanied by a decline in NOS3 activity, measured by the rate of conversion of L-[^{3}H]arginine into L-[^{3}H]citrulline in freshly isolated intact cardiac myocytes from milrinone-treated rats, as shown in Fig. 6. Although intact cardiac myocytes from control animals exhibited a detectable rate of L-[^{3}H]arginine conversion, NOS activity could not be detected above background in cardiac myocytes from the milrinone-treated rats. It is important that the intracellular tritium activity, which corresponds to the total amount of incorporated L-[^{3}H]arginine, was not statistically different between cells from control and milrinone-treated rats, suggesting that milrinone treatment had no effect on L-arginine transport into cardiac myocytes.

We have demonstrated previously that muscarinic cholinergic attenuation of increased L-type calcium current and contractile amplitude after β-adrenergic stimulation in cardiac myocytes is mediated by the activation of NOS3 (5). Therefore, the contractile responsiveness to adrenergic and cholinergic agonists was studied in electrically paced cardiac myocytes isolated from control and milrinone pretreated rats. As described in Methods, freshly isolated cells were plated for 3 h with hourly changes of media before contractile function was measured, thereby allowing cell recovery and milrinone washout after the cell isolation procedure. Indeed, cAMP levels, measured in plated myocytes after 3 h plating, were identical in control and treated rats (13±4 vs 11±6 pmol/mg protein, respectively, $n = 4$, $P > 0.05$). As shown in Fig. 7, basal contractile function (i.e., in the absence of adrenergic or cholinergic

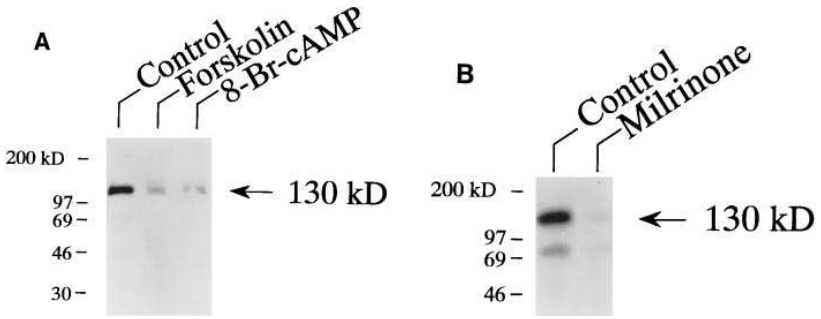

Figure 5. Effect of cAMP-elevating drugs on NOS3 protein abundance in cardiac myocytes in vitro and in vivo. (*A*) NOS3 protein abundance in cardiac myocytes in vitro: cardiac myocytes were exposed for 24 h to 10 μM forskolin or 1 mM 8-Br–cAMP. 60 μg protein was loaded in each lane and analyzed by Western blot using an mAb against human NOS3 (see Methods). For all Western blot analyses, equal loading among lanes was verified by staining with Ponceau red. The presence of immunoblotted NOS3 was revealed with a goat anti–mouse secondary antibody coupled to peroxidase activity using a chemiluminescence kit. Similar results were obtained in five different experiments. (*B*) NOS3 protein abundance in cardiac myocytes in vivo: proteins from purified cardiac myocytes from control or milrinone-pretreated rats were analyzed using the same technique. Similar results were obtained from five different experiments.

agents) was identical in both groups. However, cardiac myocytes isolated from milrinone-pretreated rats showed a greater response to the β-adrenergic agonist isoproterenol (10 nM) than cells from the control group (75±24% above baseline in control group, $n = 7$; 162±66% above baseline in the milrinone group, $n = 1$, $P < 0.01$). Carbamylcholine (10 μM) reduced the isoproterenol-stimulated contractile shortening in myocytes from control animals by 62±16%, but no attenuation was observed with carbamylcholine in cardiac myocytes from milrinone-treated rats.

We have shown previously that inhibition of endogenous NO production with both methylene blue and selective NOS inhibitors potentiates the amplitude of shortening, in response to isoproterenol, of freshly isolated ventricular myocytes from normal rats (6). We have also demonstrated that methylene blue or L-arginine analogue NOS inhibitors attenuate muscarinic cholinergic inhibition of β-adrenergic signaling (5). In the present study, methylene blue (10 μM), a free radical scavenger and inhibitor of NOS and guanylyl cyclase activities, also increased the isoproterenol-stimulated contractile shortening in control cardiac myocytes paced at 1 Hz as expected (55± 24% increase above the response to isoproterenol found in the absence of methylene blue, $n = 4$, $P < 0.01$). However, no significant effect of methylene blue was observed on the contractile responsiveness to isoproterenol of cardiac myocytes in milrinone-pretreated animals (+6±4% compared to the control response to isoproterenol, $n = 4$, $P > 0.05$). The data from these methylene blue experiments confirm the absence of the normal inhibitory feedback effect of NO generated by NOS3 on isoproterenol-stimulated contractility in cells from treated animals. However, downstream signaling by NO-dependent pathways appeared to be intact in myocytes from milrinone-treated animals since *S*-nitroso-acetylcysteine (10 μM), a nitrosothiol NO donor, depressed the contractile responsiveness of myocytes from these animals in a manner indistinguishable

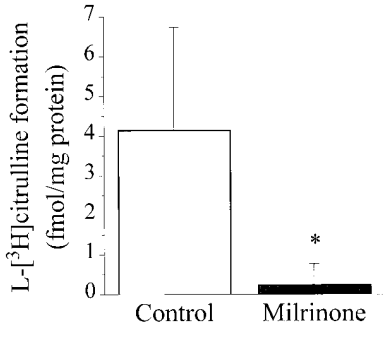

Figure 6. NO synthase activity measured in intact cardiac myocytes from animals pretreated with milrinone. NO synthase activity was measured as the enzymatic conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline in intact adult rat ventricular myocyte primary isolates as described in Methods. The NOS-specific radioactivity (i.e., after subtraction of radioactivity from homogenates of myocytes pretreated with 3 mM L-nitroarginine, a NOS inhibitor) was normalized to total radioactivity incorporated by the cells and to protein content. The results are expressed in femtomoles per milligram per protein per 20-min incubation (mean±SD, five control rats, seven milrinone-treated rats, each measurement in duplicate; $^*P < 0.01$).

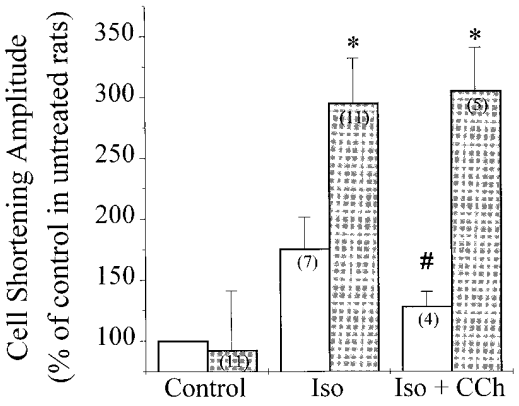

Figure 7. Loss of NOS3 activity in cardiac myocytes from milrinone-pretreated animals and isolated myocyte contractile responsiveness to b-adrenergic and muscarinic cholinergic agonists. Ventricular myocytes were isolated from animals that had been pretreated with milrinone for 3 d and from control animals, a time period sufficient to lower NOS3 protein and activity levels (see Figs. 5 and 6). Myocytes were isolated, plated, and washed with milrinone-free buffer to deplete the cells of the drug before initiation of cell-shortening measurements. Myocyte contractile amplitude was determined at a 2-Hz stimulation frequency at 37°C. Baseline contractile amplitude was determined, and then cells were exposed to 10 nM isoproterenol (*Iso*) alone, followed by Iso washout and recovery of baseline contractile amplitude, followed by Iso with 10 μM carbamycholine (*CCh*) protocol, allowing paired data analysis. Results are expressed as a percentage of the mean control values in cells from control animals. Solid bars represent data from control rats, shaded bars from milrinone-treated rats (means±SD; $n = 4$–11 in each condition for both experimental groups; $^{\#}P < 0.01$, significantly different from previous condition in the same cell; $^*P < 0.01$, significantly different from the same condition between control and treated rats).

from untreated control animals (46±13% vs 55±20%, respectively, $n = 3$ in each group, $P > 0.05$).

Differential regulation of NOS3 in cardiac myocytes and CMEC by cAMP. CMEC represent a prominent cell type in ventricular muscle, and are known to express NOS3 protein constitutively (5). Despite the absence of detectable NOS3 activity in long-term, confluent cultures of CMEC (24), short-term, subconfluent primary cultures of CMEC, initially established in the presence of 20% FBS and then serum starved, do express detectable quantities of NOS3 by immunoblotting using the monoclonal anti-NOS3 antibody described above (Fig. 8). Using the same culture conditions, NOS3 protein could also be detected by immunohistochemistry using the same antibody (data not shown).

To determine whether NOS3 would be regulated by agents that elevate cAMP in CMEC, as in cardiac myocytes, we first examined the effect of a 24- or 48-h treatment with forskolin (10 μM), known to increase cAMP in endothelial cells (25), on the abundance of NOS3 protein in subconfluent, short-term CMEC primary cultures. In contrast to the effect of forskolin in myocytes, there was no reduction in NOS3 protein abundance in CMEC in vitro (Fig. 8). To verify these findings in vivo, the abundance of NOS3 protein was examined in the total nonmyocyte fraction (mainly composed of CMEC and fibroblasts) from collagenase-digested rat hearts. There was no difference between the abundance of NOS3 protein in these fractions from control and treated animals (data not shown). We repeated these experiments using CMEC primary isolates from milrinone-treated adult rats for 3 d, and determined NOS3 abundance by immunoblotting. As shown in Fig. 8, milrinone treatment did not result in any reduction of NOS3 protein abundance in CMEC, in contrast to the effect observed in the myocyte fraction.

Discussion

The findings presented here can be summarized as follows: (*a*) sustained increases in intracellular cAMP within cardiac myocytes in vivo or in vitro are accompanied by a decrease in NOS3 mRNA abundance through a calcium-independent mechanism, in the absence of any measurable alteration in NOS3 mRNA half-life; (*b*) the decrease in NOS3 mRNA abundance with cAMP is paralleled by a decrease in NOS3 protein abundance and NOS3 enzymatic activity in intact myocytes; (*c*) these changes are accompanied by an increase in myocyte contractile responsiveness to β-adrenergic agonists and by a loss of responsiveness to muscarinic cholinergic agonists; and (*d*) NOS3 protein content is regulated differently by cAMP in cardiac myocytes and in microvascular endothelial cells.

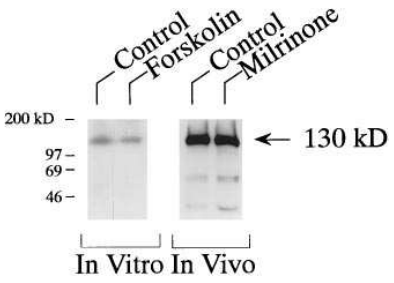

Figure 8. Regulation of NOS3 protein by cAMP-elevating drugs in CMEC in vitro and in vivo. Protein extracts (60 μg) from CMEC were analyzed by Western blotting as in Fig. 5. For in vitro experiments, CMEC were isolated from control rats and cultured for 5 d as described in Methods, and then exposed to defined medium with or without 10 μM forskolin for 24 h. For in vivo experiments, using the same dissociation procedure, CMEC were purified from control animals that had been pretreated with milrinone for 3 d (as in Fig. 4 *B*). Similar results were obtained in three independent experiments.

A decrease in NOS3 mRNA abundance with cAMP could be explained by either a decrease in NOS3 gene transcription, a decrease in NOS3 mRNA stability, or both. Previous characterization of the 5′-flanking region of the human NOS3 gene revealed the presence of a number of potential *cis*-regulatory DNA sequences, including several AP-1 sites and a putative cAMP response element (5′-TGCGTCA-3′) (14, 16), all of which could mediate an increase or decrease in NOS3 transcription after increases in intracellular cAMP (26). However, the role of these specific sequences in the transcriptional control of NOS3 expression in any cell type is not yet clear. In one study, deletion of the putative cAMP responsive element binding sequence in the NOS3 promotor did not alter its basal transcriptional activity in transfected bovine aortic endothelial cells but the response to elevated levels of cAMP was not reported (16). Although the experiments reported here did not measure directly the rate of NOS3 transcription in response to elevated cAMP levels because of the difficulty in obtaining transcriptionally active nuclei from adult cardiac myocytes, NOS3 mRNA half-life was not altered by exposure of these cells to agents that elevated cAMP. These results are most consistent with transcriptional regulation of NOS3 mRNA levels by cAMP in cardiac myocytes under the experimental conditions used.

The downregulation of NOS3 mRNA in vitro was reproduced by several agents that elevate intracellular cAMP, including forskolin, cholera toxin, 8-Br–cAMP, and milrinone, suggesting the involvement of cAMP per se as opposed to other drug-related effects. Since elevation of cAMP is followed by an increase in the intracellular calcium concentration, we studied the involvement of calcium in forskolin-mediated downregulation of NOS3 transcripts. Neither BAPTA nor verapamil were able to reverse forskolin's effect.

We extended these in vitro findings by analyzing the regulation of NOS3 mRNA and protein in cardiac myocytes freshly isolated from rats pretreated with milrinone. Milrinone is an inhibitor of the cyclic GMP-inhibited cyclic AMP-phosphodiesterase (or type III PDE [27]), the predominant type of PDE expressed in the rat heart (28–30). Milrinone is known to increase the intracellular concentration of cAMP and to produce a positive inotropic effect in mammalian cardiac muscle. We verified that oral dosing with milrinone for 3 d elevated total heart cAMP content by sevenfold in our study. Under these conditions, NOS3 mRNA abundance also was reduced in myocytes from milrinone-treated animals. Although these data in vivo with milrinone are consistent with our findings in vitro with forskolin and other agents that elevate cAMP, we cannot exclude other factors in addition to the effect of elevated cAMP in this in vivo model, such as the reduction in ventricular wall stress during contraction due to a decline in systemic vascular resistance with milrinone.

The decrease in NOS3 mRNA was paralleled by a decrease in NOS3 protein abundance after cAMP elevation in cardiac myocytes both in vitro and in vivo. It is of interest that we could verify that the reduced NOS3 protein abundance with cAMP was not compensated by an increase in cardiac myocytes of either of the two other known NOS isoforms. In vascu-

lar smooth muscle cells, for example, agents that increase cAMP have been shown to potentiate the induction of NOS2 mRNA with cytokines (10). Since we had previously demonstrated that expression of NOS2 and protein are increased in response to cytokines in cardiac myocytes (4), and that IL-1β and IFN-γ exposure decrease the expression of NOS3 mRNA in these same cells (5), it was important to exclude the possibility that cAMP could have induced NOS2 expression accompanying the downregulation of NOS3 in the present experiments. No transcript or protein for NOS2 was detected in cardiac myocytes from control or milrinone-treated rats using a rat-specific NOS2 cDNA. Also, we could not detect any signal for NOS1 protein by Western blotting in myocyte protein extracts from either control or milrinone-treated animals. This is consistent with previous work excluding the constitutive expression of NOS1 mRNA by reverse transcriptase-PCR in cardiac myocytes (5), and supports the view that NOS3 is the sole isoform constitutively expressed in these cells.

In this report, we have demonstrated also that NOS3 activity, measured by the intracellular conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline, is reduced to background levels in intact cardiac myocytes isolated from milrinone-treated rats, even though the abundance of NOS3 protein measured by Western blotting in the same extracts is only reduced by $\sim$ 80% compared to extracts from control animals. Intracellular measurement of L-[^{3}H]arginine conversion offers several advantages in comparison with the measurement of NOS activity in protein extracts. These include the integration of any changes in transmembrane L-arginine transport, which was unaffected by milrinone treatment in our experiments, while avoiding loss of reversible posttranslational modifications of NOS3 during in vitro processing of myocyte cellular lysates that could have affected enzyme activity in situ. However, several potentially important variables are unknown with this technique, and may confound the interpretation of results, including the intracellular concentrations of L-arginine and of essential cofactors such as tetrahydrobiopterin (BH_4) that are accessible to NOS in situ. Moreover, the sensitivity of this technique in intact cells is lower than that for maximal NOS activity in cell lysates, and therefore may not reveal a functionally significant activity despite the presence of detectable residual protein by immunoblot.

In addition to measuring changes in NOS3 transcript and protein levels, we also examined the functional consequences of NOS3 downregulation by analyzing a NOS-dependent physiological response in cardiac myocytes: the NO-dependent decrease in myocyte contractile responsiveness to β-adrenergic agonists induced by muscarinic cholinergic agonists. Consistent with the data on NOS enzymatic activity, we found that the muscarinic agonist carbamylcholine did not attenuate isoproterenol-stimulated contraction in cardiac myocytes from milrinone-treated rats, compared to a 60% reduction in the inotropic response to β-adrenergic agonists with carbamylcholine in myocytes from control animals.

Even in the absence of muscarinic cholinergic agonists, activation of NOS3 in response to isoproterenol in isolated electrically paced cardiac myocytes decreases their contractile responsiveness to the β-adrenergic agonist. This expected counterregulatory effect of NOS on β-adrenergic stimulation was lost in cardiac myocytes from milrinone-treated rats, resulting in a potentiation of the contractile response to isoproterenol compared to cardiac myocytes from control animals. The NO donor *S*-nitroso-*N*-acetyl cysteine, a source of bioactive NO that is not dependent on enzymatic release mechanisms, was able to reproduce the inhibition of contractility in response to β-adrenergic stimulation, indicating that downstream NO signaling pathways remained functional under the conditions of these experiments. It is unlikely that this potentiation resulted from a residual inhibition of phosphodiesterase-type III by retained intracellular milrinone in the treated group for several reasons: (*a*) the time elapsed between the end of milrinone treatment (i.e., the death of the animal) and the functional experiments on isolated cells was $\sim$ 5 h, during which the cells were repeatedly washed in milrinone-free buffers, both during the isolation procedure and for a minimum of 3 h after plating; (*b*) intracellular cAMP levels and the baseline contractile amplitude of the cells (i.e., in the absence of isoproterenol) were identical between the two groups, which argues against residual phosphodiesterase-type III inhibition in cardiac myocytes from the treated groups; and (*c*) the increase in contraction amplitude after perfusion with milrinone in vitro was identical in cells from the two groups (not shown).

These data provide evidence for the downregulation of NOS3 mRNA, protein, and enzymatic activity in cardiac myocytes in response to sustained elevation of intracellular cAMP. This results in a potentiation of the contractile response to β-adrenergic agonists and a loss of parasympathetic inhibition of β-adrenergic stimulation in cardiac myocytes. Decreased expression of NOS3 in response to elevated cAMP is cell type specific since NOS3 expression was unaffected by cAMP in microvascular endothelial cells from the heart. This could have important implications in diseases or drug regimens characterized by elevated intracellular cAMP levels that could result in a loss of the counterregulatory effect of NO in response to β-adrenergic stimulation and decreased responsiveness to parasympathetic agonists.

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