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Cellular Control of Nitric Oxide Synthase Expression and Activity in Rat Cardiomyocytes

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ABSTRACT

Potential ortho- and pathophysiological roles for nitric oxide synthases (NOS) in cardiac functions have been and are continuing to be described. However, cellular signaling mechanisms controlling nitric oxide (NO) production in the heart remain obscure. The aim of this study was to investigate signaling mechanisms involved in regulation of NOS expression and NO generation in cardiomyocytes. Using immunocytochemical methods in conjunction with western blotting, we have found that cultured neonatal rat cardiomyocytes express constitutively all three NOS isoforms targeted predominantly to the particulate component of cardiomyocytes—mitochondria and along contractile fibers, as well as along plasma membrane including T-tubules. Biochemical assay of NO generation has shown that exposure of cultured neonatal rat cardiac cells to isoproterenol (β-adrenergic stimulation), iloprost [stable prostaglandin I_2 (PGI_2) analogue], as well as inflammatory cytokines and dibutyryl adenosine-3′,5′-monophosphate (db-cAMP), resulted in a marked up-regulation of NOS expression by cardiomyocytes. In db-cAMP-stimulated cells, inhibition of protein kinase A (PKA) and protein kinase C (PKC) reduced immunolabeling of NOS and concomitantly lowered NO production. Taken together, these data point to an involvement of β-adrenergic mechanisms, cytokine and PGI_2 receptors, adenylyl cyclase, PKA, and PKC in the control of NO generation and expression of NOS in rat cardiomyocytes. *Antioxid. Redox Signal.* 6, 345–352.

INTRODUCTION

NITRIC OXIDE (NO) acts as an inter- and intracellular messenger in various cell types from all branches of the histogenetic tree (27, 28). NO originates via the oxidative L-arginine pathway catalyzed by a family of enzymes termed NO synthases (NOS). Three distinct NOS isoforms of the enzyme (EC 1.14.13.39) have been described and been shown to represent the products of three distinct genes (11, 22). A basal NO production proceeds presumably via constitutive CA^{2+}/calmodulin (CAM)-dependent forms of NOS, including a neuronal NOS (nNOS, also designated NOS1) and an endothelial NOS (eNOS, also designated NOS3). Produced in trace quantities under normal conditions, NO acts in para- and autocrine fashions to transduce cellular signals. An excessive NO production in response to various pathogenic stimuli is mediated mainly by an inducible NOS isoform (iNOS, also designated NOS2). Thereby, the amount of NO produced by cells can vary over several orders of magnitude, up to micromolar concentrations, and consequences are diverse. Biochemical,

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immunocytochemical, and molecular biological techniques revealed that all cell types present in the heart contribute to NO production and consequently to maintaining cardiac homeostasis (15).

Recognition of the pathophysiological significance of NO as an important biological mediator has led to an intensive research and development of therapies based on the interception of the L-arginine–NO–cyclic GMP signaling cascade. Optimization of corresponding therapeutic protocols requires a better knowledge of the cellular signaling pathways controlling NO production and NOS expression in cardiac cells. To address this issue, we undertook the present study of NOS localization and NO generation in neonatal rat cardiomyocytes cultured under conditions that influence the expression and activity of NOS isoforms and affect the NO signaling in the myocard.

MATERIALS AND METHODS

Animals

Neonatal (1–3 days old), Wistar albino rats, were used in this study. Experiments were performed in accordance with the Helsinki Declaration and The Guiding Principles in the Care and Use of Animals.

Primary culture of neonatal rat cardiocytes

Single cells were dissociated from the minced ventricles of 1–3-day-old Wistar albino rats with 0.2% solution of trypsin. The cells were seeded into 24-well plates (240×10^3 cells per well, for nitrite measurement) or onto coverslips (30×10^3 cells per coverslip) at the bottom of 24-well plates for cytochemical investigation and incubated at 37°C in Halle SM 20-I medium (47).

In some experiments, the cell monolayers were cultivated in Dulbecco's modified Eagle medium (DMEM; GIBCO) supplemented with 10% heat-inactivated neonatal calf serum without antibiotics. Cardiocytes grown in DMEM showed a coarse, granular cytoplasm, as reported by Tsujino *et al.* (44), whereas cultivation in Halle SM 20-I medium allowed a clearer distinction of contractile fibers, Golgi complex, and endoplasmic reticulum in the cytoplasm of cultured cardiocytes (Fig. 1). Therefore, Halle SM 20-I was used as a medium of choice for immunocytochemical investigations in this study. The Halle medium SM 20-I was specially developed for cultivation of neonatal cardiomyocytes and commonly used for functional investigations of cardiac cells (14, 47).

Cultivation medium was renewed every 24 h. On day 4, the cells were activated for 24 h with 1 m*M* dibutyryl adenosine-3′,5′-monophosphate (db-cAMP; Boehringer, Mannheim, Germany), 1 μ*M* iloprost [stable prostaglandin I_2 (PGI_2) analogue; Schering, Berlin, Germany), 10 μ*M* isoproterenol or norepinephrine (β-adrenergic stimulation via $G_{s\alpha}$), or a lipopolysaccharide (LPS)-cytokine mixture consisting of interferon-γ (100 U/ml, Boehringer), tumor necrosis factor-α (100 U/ml, Boehringer), interleukin-1β (100 U/ml, Boehringer), and LPS (50 U/ml, *E. coli* serotype 026:B6; Sigma). To trace a possible involvement of protein kinases in the control of NO production and NOS expression, activation of cells with db-cAMP was also performed in the presence of 0.1 μ*M* phorbol ester [*O*-tetradecanoylphorbol 13-acetate (TPA), activator of protein kinase C (PKC); Calbiochem, Bad Soden, Germany], 10 μ*M* *Rp*-adenosine 3′,5′-cyclic monophosphothioate (Rp-cAMPS) [inhibitor of protein kinase A (PKA); Calbiochem], 0.1 μ*M* calphostin C and 0.05 μ*M* staurosporine (inhibitors of PKC; Calbiochem), or 0.5 μ*M* KN 62 (inhibitor of CaM kinase; BIOMOL, Hamburg, Germany).

Immunocytochemical procedure

Cultured cells were rinsed in prewarmed phosphate-buffered saline (PBS), fixed in absolute methanol for 5 min at room temperature, treated for 10 min with methanol containing 0.6% hydrogen peroxide and washed in PBS. PBS was also used for all washes and dilutions of antibodies (AB). After blocking of nonspecific binding sites (F_c-receptors) in PBS containing 10% preimmune goat serum (Sigma) for 30 min, the cell monolayers were immunoreacted for 2 h at room temperature or overnight at 4°C with rabbit primary polyclonal AB recognizing NOS1, NOS2, and NOS3. Anti-NOS1 AB were raised against polypeptides corresponding to NOS1 epitope at the carboxy terminus of either rat origin [amino acid (a.a.) residues 1,400–1,419; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.] or human origin (a.a. residues 1,095–1,289; Transduction Laboratories, Lexington, KY, U.S.A.). Anti-NOS2 AB were raised against polypeptides corresponding to NOS2 epitope of mouse origin either at the amino terminus (a.a. residues 3–22; Santa Cruz Biotechnology) or at the carboxy terminus (a.a. residues 1,126–1,144; Santa Cruz Biotechnology; and a.a. residues 961–1,144, Transduction Laboratories). Anti-NOS3 AB were raised against polypeptides corresponding to NOS3 epitope at the carboxy terminus (a.a. residues 1,030–1,209; Transduction Laboratories).

As in our previous study of myocardial muscle and in blood vessels (6, 7), immunolabeling of NOS with AB purchased from these manufacturers provided similar results irrespective of the mode of visualization: bright-field or fluorescent microscopy. Primary AB were diluted to a final concentration of 1–2.5 μg/ml. Detection of bound primary AB was performed with AffiniPure goat anti-rabbit IgG (Fab) conjugated either with dichlorotriazinylaminofluorescein or with peroxidase (Dianova, Hamburg, Germany) at a dilution of 1:100. For conventional bright-field microscopy, the samples were incubated with a diaminobenzidine–hydrogen peroxide mixture to visualize the peroxidase label, counterstained with Ehrlich hematoxylin for 30 s, and mounted with aqueous mounting medium GelTol (Immunotech, Marseille, France). For fluorescent microscopy, fluorescein-labeled samples were counterstained with 0.0001% propidium iodide for 5 s, mounted with Citifluor (Plano GmbH, Wetzlar, Germany), examined under a Zeiss Axioplan microscope equipped with appropriate filters, and photographed using either a color reversal film Kodak 400 or SensiCam Imaging System (Zeiss, Germany) with A6 digital printer UPD 2500 (Sony). Data shown are representative of at least 10 independent experiments that gave similar results. The controls were (a) omission of incubation with primary AB, (b) substitution of primary AB by rabbit

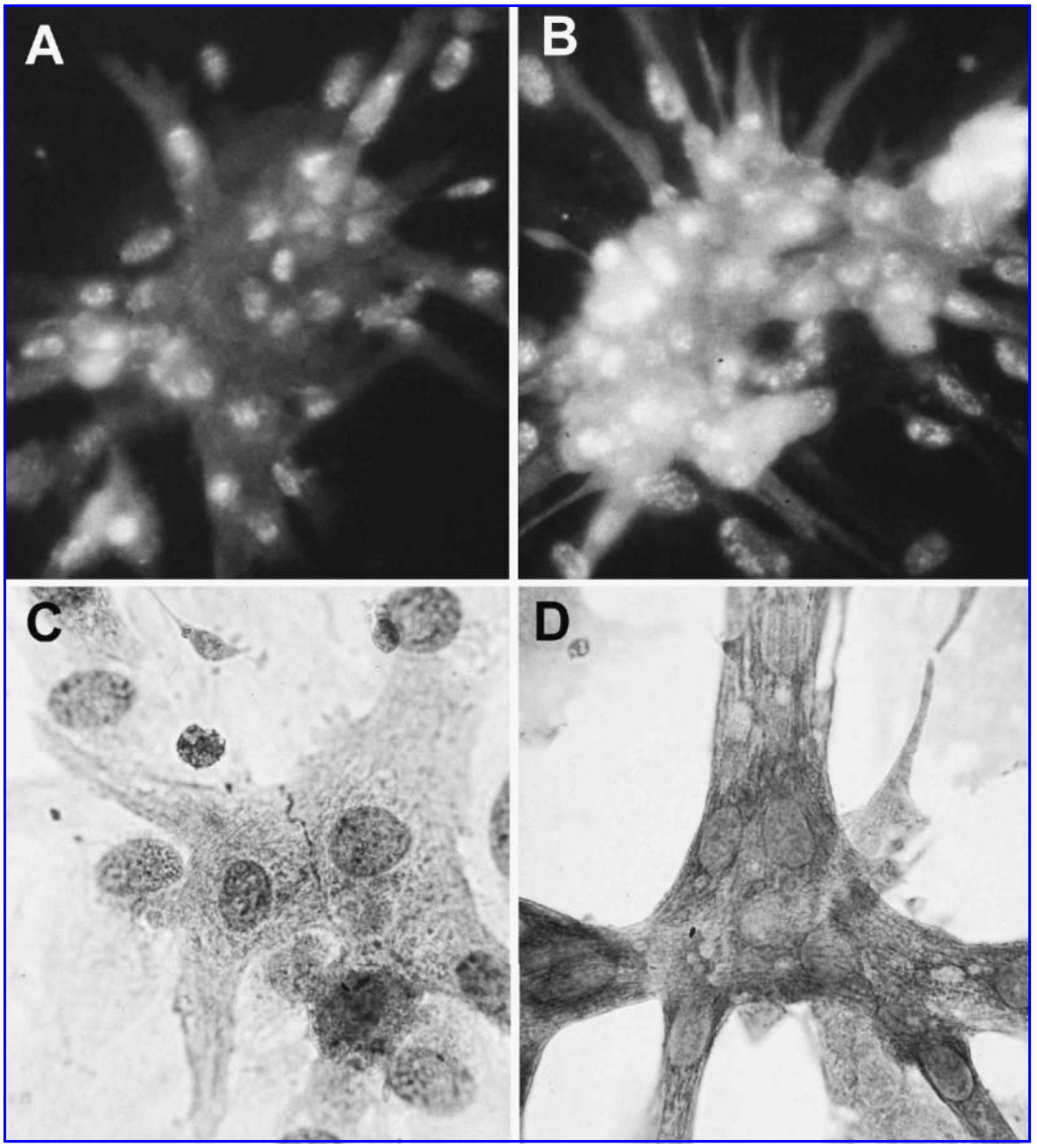

FIG. 1. (**A**) Basal immunofluorescent labeling of NOS1 was observed in unstimulated cardiomyocytes, as well as in the noncardiomyocytes. ×200. (**B**) Stimulation with db-cAMP increased NOS1 immunolabeling in cardiomyocytes. ×200. Higher magnification (×1,000) shows peroxidase-diaminobenzidine immunolabeling of NOS1 in unstimulated cardiomyocytes at the plasma membrane between adjacent cardiomyocytes and in the cytoplasm (**C**) and in cardiomyocytes stimulated with db-cAMP (**D**).

IgG (Dianova) at the same final concentration, and (c) incubation in media containing primary AB that had been preincubated at room temperature for 2 h with a 10-fold molar excess of corresponding control peptides (Santa Cruz).

Measurement of nitrite

Nitrite, a stable metabolic product of NO, in the culture medium was assayed as the sum of NO_2^- and NO_3^- by using a nitrite/nitrate colorimetric test kit with nitrate reductase (Boehringer Mannheim) according to the manufacturer's instruction. Cell number was determined using a Fuchs–Rosenthal chamber. Data are expressed as means ± SD. Student's *t* test was used. A value of $p < 0.05$ was considered statistically significant.

Immunoblotting

Total protein of cells harvested in PBS and sedimented by centrifugation was homogenized in 10 m*M* HEPES, pH 7.5, 0.2 m*M* phenylmethylsulfonyl fluoride and 0.1 m*M* dithiothreitol with Ultra Turrax at 30,000 rpm for 3 × 5 s. The particulate fraction was sedimented by centrifugation at 150,000 *g* for 20 min. The pellet was washed with the homogenization buffer and centrifuged as above. The final pellet was suspended in 0.75 ml of 10 m*M* HEPES, pH 7.5, 250 m*M* sucrose, 0.2

m*M* phenylmethylsulfonyl fluoride, and 0.1 m*M* dithiothreitol and stored at −80°C until use.

Protein samples (40 µg) solubilized in sodium dodecyl sulfate sample buffer were separated by 7.5% Laemmli polyacrylamide gel electrophoresis (18). Separated proteins were electrotransferred onto polyvinylidene difluoride membranes. Processing for immunoblotting was performed as described by Towbin *et al.* (43). Dilution of primary anti-NOS AB was performed according to the manufacturer's instructions (Santa Cruz and Transduction Laboratories). The secondary AB was peroxidase-labeled anti-rabbit IgG (Sigma; 1:15,000). The immunoreaction was visualized using an ECL kit (Amersham).

RESULTS

NOS expression in cardiomyocytes in vitro

Morphological examination of cultured neonatal cardiac cells revealed that the seeding technique resulted in 50–70% confluent monolayers of adherent cells consisting of ~80% typical cardiomyocytes. They could be morphologically distinguished by lengthened cytoplasm with well expressed contractile fibers and small, round, dense nuclei. Nonmyocyte contamination in this primary culture of cardiac cells was represented by round or polyhedral cells with more distended phase-lucent cytoplasm and larger nuclei with diffuse chromatin. The majority of nonmyocytes seem to be fibroblast-like cells (36, 37) or mast cell precursors (24). After the first 24 h of cultivation, neonatal rat cardiomyocytes revealed their inclination to merge into asterisk-like cell congregates (Fig. 1), and after 48 h they beat synchronously.

AB purchased from two different manufacturers (Santa Cruz and Transduction Laboratories) revealed a moderate expression of NOS1, NOS2, and NOS3 in cultured neonatal cardiac cells, but cultivation for 2 and more days induced a notable increase in the immunolabeling of the cytoplasmic structures as reported by us earlier (6).

Regulation of NO generation in activated neonatal cardiomyocytes

To investigate putative involvement of protein kinases in intracellular signaling pathways regulating expression and activity of NOS in cardiomyocytes, we exposed neonatal rat cardiac cells to db-cAMP in the presence of agents modulating protein kinase activity. In this part of the present study, db-cAMP activation of neonatal rat cardiac cells was performed in the presence of 10 µ*M* Rp-cAMPS (inhibitor of PKA), 0.1 µ*M* calphostin C and 0.05 µ*M* staurosporine (inhibitors of PKC), 0.1 µ*M* TPA (activator of PKC), or 0.5 µ*M* KN 62 (inhibitor of CaM kinase). Along with enhancing NOS1, NOS2, and NOS3 protein expression, db-cAMP dramatically increased the production of nitrite, thereby indicating a stimulation of NOS activity (Fig. 2). Rp-cAMPS, calphostin C, and TPA markedly attenuated a db-cAMP-induced increase in NO content in the culture supernatants with a concomitant decrease in immunolabeling of NOS2 and, to a lesser extent, of NOS1 and NOS3. KN 62 and staurosporine exerted no effect on the immunostaining intensity and NO production in db-cAMP-activated neonatal rat cardiac cells.

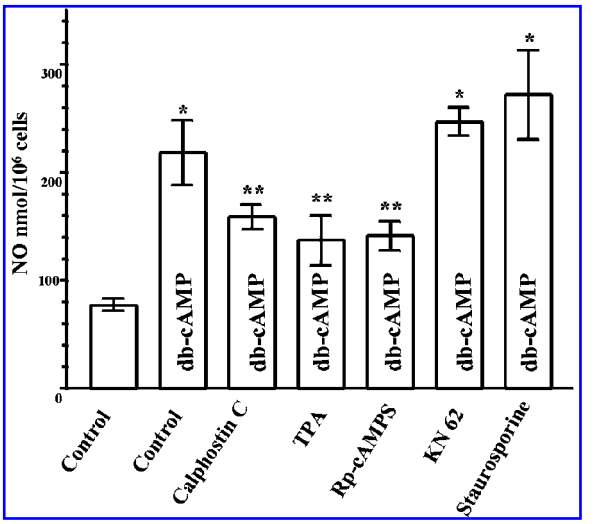

FIG. 2. NO production in cultured neonatal rat cardiomyocytes measured by photometry (Griess reagent). Cardiomyocytes in Halle SM 20-I medium were exposed to 1 m*M* db-cAMP for 24 h in the presence of 0.1 µ*M* calphostin C, 0.1 µ*M* TPA, 10 µ*M* Rp-cAMPs, 0.5 µ*M* KN 62, or 0.05 µ*M* staurosporine. NO production was assayed as the sum of NO_3^- and NO_2^- accumulated in culture supernatants. Data are means ± SD of five experiments. *$p < 0.01$ compared with control samples; **$p < 0.01$ compared with db-cAMP.

Regulation of NOS expression in activated neonatal cardiomyocytes

As the subcellular events occurring in cardiomyocytes during induction of NOS2 are not well studied, we have investigated the influence of some agents affecting the expression of NOS2 in this study. Exposure of cultured neonatal rat cardiac cells for 24 h to 10^{-3} *M* db-cAMP markedly enhanced immunolabeling of not only NOS2, as has been already reported by us (6), but also of NOS1 (Fig. 1) and NOS3 (data not shown). NOS immunolabeling was also increased in noncardiomyocytes, but to a lesser extent. As with db-cAMP, similar up-regulation of NOS expression was observed after exposure to LPS-cytokine mixture and, to a lesser extent, after exposure to 1 µ*M* iloprost, 10 µ*M* isoproterenol, and 10 µ*M* norepinephrine (data not shown).

Western blotting

Western blotting characterization of rabbit primary polyclonal anti-NOS AB used in this study is described elsewhere (6, 7). Stimulation of cultured neonatal rat cardiomyocytes with db-cAMP resulted in NOS2 up-regulation, which was confirmed by western blotting of the particulate fraction of cardiomyocytes that permitted the demonstration of characteristic immunoreacting protein bands at 130 kDa (Fig. 3). In stimulated cardiomyocytes, an appearance of a characteristic double band might be accounted for by an existence of two NOS isoforms or by differences in the molecular weights of phosphorylated and dephosphorylated forms.

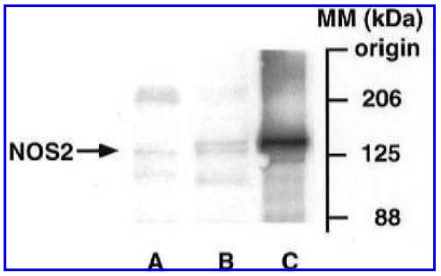

FIG. 3. Western blotting of NOS2 in the particulate fraction from unstimulated cultured neonatal rat cardiomyocytes (lane A) and after activation with db-cAMP (lane B). Positive control (lane C) was performed with activated mouse macrophage lysate.

DISCUSSION

Using a biochemical assay of NO generation by cultured neonatal rat cardiomyocytes in conjunction with immunocytochemical methods and western blotting, we have shown in this study that neonatal rat cardiomyocytes express constitutively all three NOS isoforms and specified definite factors involved in the control of NO generation by these cells.

Cardiomyocytes have been reported to express constitutively two NOS isoforms, *i.e.,* NOS1 and NOS3, and NOS2 in disease states (38, 39, 45). Tsujino *et al.* (44) and later Oddis and Finkel (30) reported on NOS2 expression by cultured neonatal rat cardiomyocytes after stimulation with cytokines, whereas nonstimulated cells failed to immunoreact with anti-NOS2 AB. However, we have reported earlier that cardiomyocytes (4–6, 41, 42) express NOS2 not only under pathophysiological conditions, but also constitutively.

Data accumulated over the past few years show that constitutive expression of NOS2 in cardiomyocytes does not seem to be a rare phenomenon and indicate that NOS2 can be expressed constitutively *in vivo* also in other cell types. Thus, NOS2 has been reported to be expressed constitutively in human colorectal epithelia (23), in human peripheral blood mononuclear cells (1), in human megakaryocytes and eosinophils (9, 46), in rat ovaries (48), in the pregnant rat uterus (smooth muscle and epithelia) and placenta (sinusoids and macrophages) osteoblast-like cells (8, 17, 20), in the mouse ileal mucosa (16), in smooth muscle cells (7), in renal cells (3), and in skeletal muscles (13, 31–33).

In this study, we found all three NOS isoforms constitutively expressed in neonatal rat cardiomyocytes. The pattern of NOS isoform expression in cardiomyocytes and noncardiomyocytes was notably different and was found to be specific for each isoform, which permits exclusion of a possible cross-reactivity of AB used and indirectly attests to their specificity. Thus, premature intercalated discs in cultured cardiomyocytes immunoreacted only with anti-NOS1 AB, whereas perinuclear space, endoplasmic reticulum, and Golgi complex were immunolabeled only with anti-NOS2 AB. Immunolabeling with anti-NOS3 AB was more diffuse. However, mitochondria and contractile fibers in cardiomyocytes positively immunoreacted with anti-NOS1, anti-NOS2, and anti-NOS3 AB, which could be seen also with immunogold labeling of both cultured cardiomyocytes and myocard of adult rats (3).

In cardiomyocytes, we observed an up-regulation of NOS2, and to a lesser extent also of the two other NOS isoforms, after stimulation with cyclic AMP. However, in some other cell types, cyclic AMP could exert opposite effects. Regulation of the NOS2 gene transcription appears to be the primary mechanism of action of cyclic AMP, and whether it is stimulatory or inhibitory hinges on the cell-specific regulation of transcription factors including CREB, nuclear factor-κB, and C/EBP (12). Cyclic AMP must therefore be considered a modulator rather than a suppressor of NOS2 expression.

In the present study, a notable increase in immunolabeling of NOS2 was observed, not only after activation with an exogenous cyclic AMP derivative, but also with iloprost (stable PGI_2 analogue), isoproterenol (β-adrenergic stimulation via $G_{s\alpha}$), and with an LPS-cytokine mixture. This suggests that cellular signaling pathways involved in the control of NOS2 expression include adenylyl cyclase, β-adrenergic mechanisms, PGI_2, and cytokine receptors, and $G_{s\alpha}$. The biochemical assay of NO generation by db-cAMP-activated cultured cardiac cells in the presence of phorbol ester (TPA, activator of PKC), Rp-cAMPS (inhibitor of PKA), calphostin C and staurosporine (inhibitors of PKC), or KN 62 (inhibitor of CaM kinase), taken together, suggests an involvement of PKC and PKA, but not of CaM kinase in the control of NOS2 expression and activity.

In this experiment, TPA attenuated the activating effect of db-cAMP on NO production and NOS expression after 24 h, but enhanced the NO generation by these cells after a shorter treatment (30 min; data not shown). This suggests that on the one hand TPA up-regulates NOS activity, but on the other hand down-regulates its expression. Likewise, prolonged incubation of cells with phorbol esters down-regulated PKC activity and reduced NO production in murine macrophage cell lines J774 and RAW 264.7 activated with interferon-γ (35). The mechanism by which PKC induces NOS2 expression apparently involves PKC-dependent pathways via activation of phospholipase or phosphatidylcholine- and phosphatidylinositol-specific phospholipase (34) and the activation of nuclear factor-κB (10).

In contrast to calphostin C, a nonselective inhibitor of PKC, staurosporine, failed to attenuate the activating effect of db-cAMP on NO production and NOS2 expression after 24 h. This apparent contradiction can be explained in view of the fact that, although inhibiting PKC, staurosporine on the other hand can elicit an extreme up-regulation of NOS2 gene expression. Apparently, similar mechanisms of staurosporine action are applicable to NOS1 and NOS3. The up-regulation of NOS3 expression by staurosporine and analogues appears to be a transcriptional event because staurosporine enhanced the activity of a 1.6-kb human NOS3 promoter fragment transiently transfected into EA.hy 926 endothelial cells (19). Data obtained with other kinase inhibitors (and stimulators) indicated, however, that the effect of staurosporine on NOS expression was unrelated to the activities of PKC, PKA, PKG, or tyrosine kinase(s). These findings demonstrate that staurosporine belongs to compounds positively interacting with the transcription of the NOS genes. Such compounds may prove

useful in the prophylaxis and therapy of cardiovascular diseases (19).

The molecular mechanisms underlying these regulations are currently being studied in several laboratories (2, 12, 19, 25, 26, 40). Potential physiological and pathophysiological roles for NOS in cardiac functions have been and are continuing to be described (21, 22, 29). Data obtained in this study on neonatal cardiomyocytes contribute to a better knowledge of NO signaling mechanisms. With definite limitations, this information may be applied to the adult myocard, with the idea that in ontogenesis the organ protein profile may undergo a more quantitative rather than qualitative modification.

To summarize, we described here the subcellular localization of NOS1, NOS2, and NOS3 in rat cardiomyocytes and found that these NOS isozymes are constitutively expressed both *in vitro* and *in vivo*. Physiological and pathophysiological roles of NOS isoforms seem to be determined by their preferential association with mitochondria and along contractile fibers.

This study leads to the following conclusions: (a) Rat cardiomyocytes express constitutively all three NOS isoforms. (b) NOS1 and NOS2 are associated predominately with the particulate component of cardiomyocytes—mitochondria and along contractile fibers, as well as along plasma membrane including T-tubules; NOS1 is bound also to intercalated discs; NOS3 immunolabeling was more diffuse. (c) Targeting of NOS to mitochondria and contractile fibers in cardiomyocytes may indicate a potential structure–functional relationship of NO with contractile function and energy production in the cardiac muscle. (d) Cellular signals controlling NOS in cardiomyocytes involve β-adrenergic mechanisms, PGI_2, and cytokine receptors, adenylyl cyclase, $G_{s\alpha}$, PKA, and PKC.

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ABBREVIATIONS

a.a., amino acid; AB, antibodies; CaM, Ca^{2+}/calmodulin; db-cAMP, dibutyryl adenosine-3′,5′-monophosphate; DMEM, Dulbecco's modified Eagle medium; LPS, lipopolysaccharide; NO, nitric oxide; NOS, nitric oxide synthase; NOS1, neuronal NOS (nNOS); NOS2, inducible NOS (iNOS); NOS3, endothelial NOS (eNOS); PBS, phosphate-buffered saline; PGI_2, prostaglandin I_2; PKA, protein kinase A; PKC, protein kinase C; Rp-cAMPS, *Rp*-adenosine 3′,5′-cyclic monophosphothioate; TPA, *O*-tetradecanoylphorbol 13-acetate.

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