Autonomic regulation of pacemaker activity: role of heart nitric oxide synthases

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Autonomic regulation of pacemaker activity: role of heart nitric oxide synthases. Am J Physiol Heart Circ Physiol 291: H1246–H1254, 2006. —In autonomic-blocked rats treated with L-NAME (7.5 mg/kg), heart rate increased 18% and mean arterial pressure increased 48%. Thyroidectomy, along with autonomic blockade, hampered the chronotropic response but did not modify the effect on blood pressure. After 150 min of autonomic blockade, the experimental end point, total nitric oxide (NO) production by heart NO synthases (NOS) decreased 61%: from 54 to 21 mmol NO·min⁻¹·g heart⁻¹. Mitochondrial NOS (mitNOS) and sarcoplasmic reticulum endothelial NOS activities decreased 74% and 52%, respectively. Mitochondria isolated from whole heart showed a well-coupled oxidative phosphorylation with high respiratory control and ADP-to-O ratios, decreased mitNOS activity (55–60%), and decreased mitNOS protein expression (70%). Immunohistochemistry with anti-inducible NOS antibody linked to gold particles localized mitNOS at the inner mitochondrial membranes. Histochemical right atrial NOS (NADPH-diaphorase) decreased 55% after heart denervation. The effects of autonomic denervation on the NO system were partially prevented by thyroidectomy performed simultaneously with autonomic blockade. Western blot analysis indicated a very rapid mitNOS protein turnover (half time = 120 min) with a process of protein expression that was upregulated by thyroidectomy and a degradation process that was downregulated by the autonomic nervous system. The observations suggest that NO-mediated pathways contribute to pacemaker heart activity, likely through the NO steady-state levels in the right atrium and the whole heart.

heart rate; autonomic nervous system; thyroid gland; mitochondrial nitric oxide synthase; endothelial nitric oxide synthase

REGULATION OF CARDIAC function by the autonomic nervous system is a complex process in which nitric oxide (NO) is a mediator and thyroid status has a role (12, 29, 30, 32, 38). Dysfunction of the cardiac autonomic nervous system is considered a determinant of a negative prognosis in cardiovascular diseases (11). Classic concepts concerning heart function and NO are as follows: 1) NO is a regulator of cardiac function through indirect vascular-dependent mechanisms (33) and by direct actions on the myocardium (26) and 2) the three known genomic isoforms of NO synthase (NOS) (2, 3) are present and functionally active in the heart. Recently, Gonzales et al. (22) and Zaobornyj et al. (44) reported that NO is produced in the myocardium in physiologically relevant quantities by two isoforms of NOS: 1) an isoenzyme located in the mitochondria [mitochondrial NOS (mtNOS)], which accounts for ~55–62% of total heart NO production, and 2) an isoenzyme located in the cytosolic fraction, the endothelial NOS (eNOS) of the caveolae of the sarcoplasmic reticulum and plasma membranes, which accounts for the remaining 38–45% of total heart NO production. The physiological roles of NO are interpreted to be mediated through activation of cytosolic guanylate cyclase (26) and inhibition of mitochondrial cytochrome oxidase (1, 9, 13). Although NO was recognized primarily as a mediator of endothelial control of vascular smooth muscle, it is conceived as a paracrine autacoid involved in modulation of cardiac autonomic control and contractility (23). The mechanisms by which NO regulates heart contractility and contraction rate and the relations of the heart cycle to NO diffusion between mitochondria and cytosol are physiological processes that are starting to be understood.

We have observed that L-NAME administration to autonomic-blocked rats induces tachycardia, along with an increase in blood pressure, in a physiological process that is partially prevented by thyroidectomy and is not affected by adrenalectomy (18, 19). It is well known that the heart is a target organ for thyroid hormones and that alterations in thyroid status influence cardiac contractile and electrical activities by a direct action of triiodothyronine (T3) in cardiomyocyte receptors (16, 38). Moreover, a functional relation involving the thyroid gland, endothelial cells, and NO, which is able to modulate cardiovascular function, has been described (41), and it has been claimed that thyroid hormones are able to regulate heart rate by an NO-mediated mechanism in the absence of autonomic regulation (18).

The aim of this study was to evaluate the changes produced by autonomic blockade and thyroidectomy in heart rate and in whole heart NO production, which is considered the sum of heart NOS activities.

MATERIALS AND METHODS

Experimental Animals

Male Sprague-Dawley rats (230–260 g body wt) were housed in a humidity- and temperature-controlled environment with an automatic 12:12-h light-dark cycle and fed standard rat chow and tap water ad libitum. Animal handling and use was in accordance with the American Physiological Society “Guiding Principles in the Care and Use of Animals,” and with the 63/44/96 regulation of Argentinian National Drug Food and Medical Technology Administration (ANMAT).

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Anesthesia was initiated with urethane (1.0 g/kg ip) and maintained with additional small doses of urethane throughout the experiment (27). A tracheotomy was performed, and a 3.5-mm tube was inserted into the trachea. Polyethylene cannulas were inserted into the right and left femoral veins for drug administration. Arterial pressure was measured through a cannula inserted into the right femoral artery connected to a pressure transducer (Statham P23 ID, Gould Instruments, Cleveland, OH) and recorded with a polygraph (Physiograph E & M, Houston, TX). Heart rate was determined from the pulsatile pressure signal by beat-to-beat conversion with a tachograph amplifier (model S77-26 tachometer, Coulbourn Instruments, Allentown, PA). Body temperature was monitored with a rectal probe and maintained at 37.0 ± 0.2°C with heating lamps. The Labtech Notebook program (Laboratory Technology, Wilmington, MD) was used for data acquisition.

Surgical Procedures for Autonomic Blockade and Thyroidectomy

**Drug treatments.** Basal heart rate and arterial pressure were recorded for 10 min before initiation of the surgical protocols for autonomic blockade and thyroidectomy. Control rats (n = 72) were tracheotomized, and autonomic blockade (n = 63) was produced by bilateral supracvacular vagotomy and hexamethonium bromide injection (10 mg/kg iv) every 20 min to maintain blockade of ganglionic transmission. An infusion of phenylephrine (4 – 6 μg·kg⁻¹·min⁻¹ iv) was initiated immediately after autonomic blockade to maintain arterial pressure in the basal range (18, 19, 36, 37). Thyroidectomy (n = 18) and bilateral adrenalectomy (n = 18) were performed immediately before autonomic blockade. The rats received L-NAME (7.5 mg/kg iv) 40 min after surgery. The pressor and chronotropic responses were continuously monitored. The end point was set at 150 min after autonomic blockade. Control experiments (n = 9) in autonomic-blocked rats were performed using N⁵-nitro-δ-arginine methyl ester, at the same dose, to verify the specificity of L-NAME-induced pressor and chronotropic responses.

**Plasma levels of thyroid hormones.** T₃ and thyroxine (T₄) were determined by radioimmunoassay (8) in serum samples obtained at the experimental end point.

**Isolation of rat heart mitochondria.** Control, autonomic-blocked, and autonomic-blocked + thyroidectomy animals were killed by decapitation, and the hearts were immediately removed and placed in 0.23 M mannitol, 0.07 M sucrose, 1 mM EDTA, and 10 mM Tris·HCl (pH 7.4) (MSET). The hearts were finely minced and homogenized in a Teflon Potter-Elvejhem homogenizer in MSET (5 ml/g of heart). All procedures were carried out at 0–2°C. Protein contents were determined with the Folin reagent, with BSA as standard. The content of mitochondria in the heart, determined from the ratios of cytochrome oxidase activity in the mitochondrial and postmitochondrial (cytosolic) fractions to that in the organ homogenate, was 49 ± 2 and 91 ± 4 mg cytosolic protein/g heart.

**Mitochondrial Respiration and mtNOS Functional Activity**

O₂ uptake was determined polarographically with a Clark-type electrode in a 1.5-ml chamber at 30°C in a reaction medium consisting of 0.23 M mannitol, 0.07 M sucrose, 20 mM Tris·HCl, 5 mM phosphate, 1 mM EDTA, and 2 mg/ml BSA (pH 7.4), saturated with air (225 μM O₂), and 0.5–1.0 mg mitochondrial protein/ml. Mitochondria were supplemented with 7 mM succinate or 6 mM malate and 6 mM glutamate as substrates in the presence (state 3) or absence (state 4) of 0.2–0.5 mM ADP (6). O₂ uptake is expressed as nanograms-atoms O₂ per minute per milligram protein. Respiratory control (state 3/state 4) and ADP-to-O ratio were calculated as described elsewhere (6). The mtNOS-regulated state 3 respiration, also termed mtNOS functional activity, was calculated as the difference in state 3 mitochondrial respiration between a condition of maximal intramitochondrial NO levels, with mitochondria added with 1 mM arginine and 0.5 μM CuZn-superoxide dismutase (SOD), and a condition of minimal intramitochondrial NO levels, with mitochondria added with 2 mM L-NAME and 10 μM oxyhemoglobin (HBO₂) (42).

**NO Production**

NO production was measured by spectrophotometric (model DU 7400 diode array spectrophotometer, Beckman) monitoring (at 577–591 nm) of the oxidation of HBO₂ to methemoglobin at 37°C (5). The reaction medium was 50 mM phosphate buffer (pH 7.4), 1 mM L-arginine, 1 mM CaCl₂, 0.1 mM NADPH, 10 μM dithiothreitol, 2 μM SOD, 0.1 μM catalase, and 30 μM HBO₂ heme. NO production was determined in heart mitochondrial membranes and in the postmitochondrial supernatant at 0.70–1.30 mg protein/ml. Mitochondrial membranes were obtained from mitochondria that were disrupted by two freeze-thaw cycles and by homogenization through a hypodermic needle. Control experiments, in which 1 mM N⁵-methyl-L-arginine (L-NMMA) was added, were performed to consider only L-NMMA-sensitive hemoglobin oxidation as due to NO formation and expressed as nanomoles NO per minute per milligram protein. Addition of L-NMMA resulted in an 88–94% inhibition of the rate of hemoglobin oxidation.

**Histochemical NOS Activity**

The NADPH-diaphorase assay was used for total NOS activity in right atria that were removed at the end point and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The tissues were cryoprotected in 15% sucrose and frozen. The sections (15 μm) were cut on a cryostat, mounted on gelatin-coated glass slides, and incubated with a reaction mixture containing 1.2 mM NADPH and 0.24 mM nitro blue tetrazolium in phosphate buffer added with 0.3% Triton X-100 for 60 min at 37°C. This technique assesses NOS activities that are inhibited by preincubation with diphenyleneiodonium and other NOS inhibitors (7, 39, 43). A Zeiss Axiohot microscope was used for observation, absorption determination, and photography. Computerized image analysis of stained sections was carried out using a Kontron-Zeiss Vidas analyzer, and mean absorption values were calculated from 5 areas of each section and from 10 different sections. The determinations were performed blindly and under similar light, gain, offset, and magnification conditions.

No reaction product was found when NADPH was omitted. In control experiments, 5 mM L-NAME was added to consider the specific NADPH-diaphorase staining due to NOS activity.

**Western Blot Analysis**

The proteins of mitochondrial membranes and of the postmitochondrial supernatant (0.10 mg protein/lane) were separated by electrophoresis in 7.5% SDS-polyacrylamide gels and transferred to a nitrocellulose membrane (Bio-Rad, Munich, Germany) and then incubated with rabbit polyclonal anti-NOS antibodies [1:500 dilution: anti-inducible NOS (iNOS), epitope at the COOH terminus; anti-eNOS, epitope at the NH₂ terminus; and anti-neuronal NOS (nNOS), epitope at the NH₂ terminus] and a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:5,000 dilution). Samples were revealed by chemiluminescence using the enhanced chemiluminescence reagent (Amersham Pharmacia Biotechnology, Uppsala, Sweden) for 2–4 min. Quantification of the bands was performed by digital image analysis using a Hewlett-Packard scanner and Tottallab analyzer software (Biodynamics, Seattle, WA). All experiments were performed in triplicate.

**Postembedding Immunogold Electron Microscopy**

Mitochondrial pellets from control rats were fixed in 4% paraformaldehyde and 0.75% glutaraldehyde in 0.1 M phosphate buffer (pH
7.4) for 2 h, washed overnight with 0.32 M sucrose in the same buffer, dehydrated in 70% ethanol, and preembedded overnight in LR White acrylic resin with benzoyl peroxide as initiator. All these operations were performed at 4°C. Final embedding and polymerization were performed at 50°C for 48 h. Ultrathin (90-nm) sections were obtained in a Porter-Blum ultramicrotome and mounted on 300-mesh nickel grids. Postembedding immunostaining was carried out in sections that were preincubated in 3% BSA diluted in phosphate buffer + 0.01% Triton X-100 at 4°C for 30 min and then incubated overnight at 4°C with iNOS (1:25 dilution) or eNOS (1:20 dilution) monoclonal antibody. Sections were washed in phosphate buffer (6 times for 10 min each) and incubated in a 10-nm gold-labeled goat anti-mouse IgG antibody (1:50 dilution) in Tris-buffered saline-BSA (pH 8) at room temperature for 60 min. The grids were washed with Tris-buffered saline and double-distilled water and immersed in 2% glutaraldehyde solution for 5 min. Finally, sections were stained with 1% uranyl acetate and lead citrate (Reynolds solution). Microphotographs were obtained using an electron microscope (model C10, Zeiss). For controls, primary antibodies were omitted, and an antibody against complex I (1:500 dilution) of the respiratory chain was used (35).

**Data Analysis**

Values are means ± SE. ANOVA followed by Bonferroni’s ad hoc test was used for multiple comparisons. The 5% probability level was used as a criterion for biological significance. GraphPad Prism software (San Diego, CA) was used for statistical analysis.

**Materials**

The antibodies against the three isoforms of NOS [iNOS, eNOS, and neuronal NOS (nNOS)], anti-complex I of the respiratory chain, and anti-β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); anti-cytochrome c oxidase antibody from BD Bioscience (San Diego, CA); Western blot detection system Hybond-ECL membranes from Amersham Pharmacia Biotech; and biochemicals from Sigma Chemical (St. Louis, MO).

**RESULTS**

**Hemodynamic Basal Parameters**

The baseline hemodynamic parameters, heart rate and mean arterial pressure, were similar for the five groups of experimental animals at the initial time point (0 min): 353 ± 2 beats/min and 80 ± 1 mmHg for control, 354 ± 2 beats/min and 84 ± 3 mmHg for autonomic-blocked rats, 358 ± 5 beats/min and 82 ± 1 mmHg for autonomic-blocked + thyroidectomized rats, 350 ± 3 beats/min and 81 ± 3 mmHg for autonomic-blocked + adenalecctomized rats, and 339 ± 5 beats/min and 85 ± 4 mmHg for autonomic-blocked + d-NAME-treated rats.

**Effect of Inhibition of NO Synthesis on Heart Rate and Arterial Pressure**

The administration of l-NAME had a positive chronotropic effect in autonomic-blocked rats: heart rate was increased by a statistically significant 18% to 417 ± 2 beats/min (P < 0.05 vs. basal). This chronotropic effect was ~50% prevented by thyroidectomy, with an end-point heart rate of 387 ± 3 beats/min (P < 0.05 vs. basal), but not by adenalectomy (Fig. 1A). However, the marked pressor-induced increase (48%) in mean arterial pressure by l-NAME in autonomic-blocked rats was not affected by adenalectomy or thyroidectomy (Fig. 1B). In contrast to l-NAME, d-NAME had no effect on mean arterial pressure and heart rate in autonomic-blocked animals (88 ± 4

mmHg and 341 ± 3 beats/min, respectively) compared with basal values.

Plasma levels of T3 and T4 were determined after 150 min of autonomic blockade + thyroidecetomy. Thyroidectomy decreased T3 concentration by 28% (1.25 ± 0.03 and 0.90 ± 0.02 ng/ml in control and thyroidectomized rats, respectively, P < 0.01) but had no effect on T4 (3.66 ± 0.19 and 3.44 ± 0.22 μg/dl in control and thyroidectomized rats, respectively).

**Mitochondrial Respiratory Function**

Heart mitochondria isolated from control, autonomic-blocked, and autonomic-blocked + thyroidectomized rats showed high rates of respiration and high respiratory control and ADP-to-O ratios, indicating that the organelles were well coupled and able to effectively carry out oxidative phosphorylation (Table 1). No differences in respiratory control and ADP-to-O ratios were observed between the experimental groups. The succinate-supported state 3 respiration was increased after autonomic blockade, and this change was prevented by thyroidectomy (Table 1). No differences in malate-glutamate-supported state 3 respiration were observed between the experimental groups.

The mtNOS-regulated state 3 respiration, also named mtNOS functional activity, was markedly decreased (55–60%) by autonomic blockade (Table 1). This observation was consistent
with the concept that NO produced by mtNOS inhibits cytochrome oxidase, O₂ consumption in the assay, and physiological conditions. The assay determines the difference in respiration between two mitochondrial situations: one with the highest NO level (supplementation with arginine and SOD) and the other with the lowest NO level (supplementation with l-NAME and HbO₂); in both cases the source of NO in isolated mitochondria is mtNOS, and its product NO is the active molecule that inhibits the rate of respiration. Thyroidectomy hampered the change induced by autonomic denervation and decreased the effect to 16–21% (Table 1).

**Whole Heart NOS Activities**

The capacity of the whole heart to produce NO was evaluated by the determination of the two significant sources of cardiac NO, mtNOS and sarcoplasmic reticulum eNOS (21, 41), which were determined in isolated mitochondria and in the postmitochondrial supernatant with the subcellular fractions fully supplemented with substrates and cofactors. After 150 min of autonomic blockade, there was a 61% decrease in the total NO production by heart NOS: from 54 to 21 nmol NO·min⁻¹·g⁻¹·heart⁻¹. The two partial activities, mtNOS and sarcoplasmic reticulum eNOS, were decreased by 74% and 52%, respectively (Table 2). Thyroidectomy partially prevented the effect on mtNOS and totally prevented the effect on sarcoplasmic reticulum eNOS (Table 2). The individual values of mtNOS biochemical activity significantly correlated (multivariate analysis) with the values of mtNOS functional activity (r = 0.92, P < 0.01).

**Histochemical NOS Activity**

The histochemical NADPH-diaphorase reaction in right atrial sections showed a marked decrease (55%) after autonomic blockade that was reduced to 21% in thyroidectomized animals (Fig. 2). The decreases in histochemical NO activity in the right atrium (to 55% and 21%) are in quantitative agreement and correlated with the decreases of biochemical NOS activities in the whole heart (to 61% and 17%, r = 0.95, P < 0.01). Addition of l-NAME inhibited the histochemical NADPH-diaphorase staining in the tissues by 85–90%.

**Heart mtNOS Protein Expression**

The decrease in mtNOS activity observed after autonomic blockade was further characterized by determination of mtNOS protein expression. Western blot analysis of heart mitochondrial fractions from control rats showed a 130-kDa protein, identified as mtNOS, which reacted with an anti-iNOS antibody (Fig. 3). The same mitochondrial fractions showed a very weak reaction with the anti-eNOS antibody and no reaction with the anti-nNOS antibody (Fig. 3) (22). The purity of the fractions was controlled by assay of a mitochondrial marker (cytochrome oxidase) and a cytosolic marker (β-actin). The heart postmitochondrial supernatant showed a clear reaction with the anti-eNOS antibody, a weak reaction with the anti-iNOS antibody, and no reaction with the anti-nNOS antibody (Fig. 3). The mitochondrial membranes from autonomic-blocked animals exhibited a highly decreased (70%) expression of mtNOS protein, an effect that was partially attenuated (to 53%) by thyroidectomy (Fig. 4). The densitometric quantitation of the Western blots of Fig. 4 is highly consistent with the biochemical data of Table 2, and the individual values were quantitatively correlated (r = 0.89, P < 0.01). A marked proteolytic degradation of heart mtNOS was observed in control, autonomic-blocked, and autonomic-blocked + thyroidectomized rats. Low-molecular-mass (54- and 80-kDa) fragments reacting with anti-iNOS were observed in heart mitochondria.

**Table 1. Respiration of heart mitochondria isolated from autonomic-blocked and thyroidectomized rats**

<table>
<thead>
<tr>
<th>Condition</th>
<th>O₂ Uptake, ng atoms O₂·min⁻¹·mg protein⁻¹</th>
<th>Respiratory Control Ratio</th>
<th>ADP-to-O Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>State 4</td>
<td>State 3</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>61 ± 7</td>
<td>278 ± 25</td>
<td>4.4 ± 0.3</td>
</tr>
<tr>
<td>Autonomic blockade</td>
<td>84 ± 8</td>
<td>362 ± 34*</td>
<td>3.2 ± 0.4</td>
</tr>
<tr>
<td>Autonomic blockade + thyroidectomy</td>
<td>63 ± 7</td>
<td>272 ± 24†</td>
<td>4.3 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>Succinate</td>
<td>Maltate-glutamate</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>22 ± 4</td>
<td>125 ± 12</td>
<td>31 ± 6</td>
</tr>
<tr>
<td>Autonomic blockade</td>
<td>20 ± 4</td>
<td>140 ± 13</td>
<td>14 ± 4*</td>
</tr>
<tr>
<td>Autonomic blockade + thyroidectomy</td>
<td>21 ± 5</td>
<td>133 ± 14</td>
<td>26 ± 5</td>
</tr>
</tbody>
</table>

Values are means ± SE (n = 9). mtNOS, mitochondrial nitric oxide synthase. *P < 0.05 vs. control. †P < 0.05 vs. autonomic blockade.

**Table 2. Heart NO production rates in autonomic-blocked and thyroidectomized rats**

<table>
<thead>
<tr>
<th>Condition</th>
<th>NO Production</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mtNOS, nmol/min⁻¹·mg protein⁻¹</td>
</tr>
<tr>
<td>Control</td>
<td>0.53 ± 0.02</td>
</tr>
<tr>
<td>Autonomic blockade</td>
<td>0.14 ± 0.03*</td>
</tr>
<tr>
<td>Autonomic blockade + thyroidectomy</td>
<td>0.32 ± 0.05†</td>
</tr>
</tbody>
</table>

Values are means ± SE (n = 9). Whole heart nitric oxide (NO) production rates were calculated as follows: heart NO production (nmol/min⁻¹·g heart⁻¹) = [mtNOS production (nmol NO·min⁻¹·mg protein⁻¹ × mitochondrial content (mg protein/g heart)) + [cytosolic production (nmol NO·min⁻¹·mg protein⁻¹) × cytosolic protein content (mg protein/g heart)] and as described in Ref. 22. *P < 0.05 vs. control. †P < 0.05 vs. autonomic blockade.
isolated from control rats [total 491 densitometric units (du)]. The amount of these fragments was slightly increased (to 539 du) by autonomic blockade. Thyroidectomy not only did not prevent the presence of these low-molecular-mass fragments but markedly increased their amount (to 1,183 du; Fig. 4).

**Electron Microscopy**

Immunogold electron microscopy using iNOS antibody showed gold particles in the inner mitochondrial membranes of isolated rat heart (Fig. 6, C and D). The number of gold particles was similar in isolated mitochondria and mitochondria from myocardial tissue (not shown). Immunostaining was specific to the anti-iNOS antibody, because control mitochondria (Fig. 6A) or mitochondria treated with the anti-eNOS antibody did not show labeling (Fig. 6B). Controls using an anti-complex I antibody were positive.

**DISCUSSION**

The autonomic blockade by administration of supraclavicular denervation + hexamethonium bromide to rats constitutes an animal model for investigation of autonomic nervous system regulation of heart function (18, 19, 36). Also this animal model mimics autonomic dysfunctions, such as those associated with heart transplant and some cardiovascular diseases, in humans (4). The present study shows that the in vivo pressor effect produced by L-NAME, an NOS competitive inhibitor, in autonomic-blocked rats is independent of adrenal and thyroid glands (18) and is mediated through the decrease of NO synthesis in the microvascular endothelium. Although the effect induced by L-NAME administration on heart rate is controversial (25, 34), our findings clearly show a tachycardia following L-NAME administration in autonomic-blocked rats (17). This phenomenon is counteracted by thyroidectomy, whereas adrenalectomy has no effect, indicating that adrenal catecholamines are not involved.

Recently, it was reported that only two NOS isoenzymes, mtNOS and sarcoplasmic reticulum eNOS (22, 44), contribute significantly to total heart NO production. The main finding of this study is that autonomic blockade causes a fast and significant (61%) decrease in whole heart NO production, with decreases of 74% and 52% in mtNOS and eNOS activity, respectively. The speed of this response is remarkable, if we consider that protein half times of mtNOS and eNOS are 120 and 150 min, respectively. However, this is not the first time that a similar phenomenon was observed: the mtNOS activity of thymocytes was increased six times, with a half time of 15 min after cell Ca2+ overload (10). In the present work, the dowregulation of eNOS expression could be a consequence of the absence of vagal regulation on the enzyme expression.

Moreover, the decreased activity of mtNOS in autonomic-blocked (74%) and autonomic-blocked + thyroidectomized (40%) rats is consistent with the reduction of 1) mtNOS functional activity (55–60% and 16–21%, respectively), 2) Western blot protein expression (70% and 53%, respectively), and 3) right atrial NADPH-diaphorase histochemical activity (55% and 21%, respectively).
The biochemical and biological status of mtNOS is a subject of open and active research: the NOS isoform located in heart mitochondria (mtNOS) shows a classical biochemical NOS activity (13, 20, 29, 41, 44). This NOS isoform reacts in Western blots with antibodies against iNOS, and similar observations were made in mitochondria isolated from other organs, such as liver (11), kidney (5), and thymus (10), where iNOS-like immunoreactivity was

Fig. 3. Representative Western blot analysis of control rat heart mitochondrial and postmitochondrial subcellular fractions to an anti-NOS antibody. iNOS, eNOS, and nNOS, inducible, endothelial, and neuronal NOS. Values [means ± SE (n = 5)] below blots represent mean of densitometric units (du) after background subtraction. All experiments were performed in triplicate. Each blot was normalized to expression of the respective marker (i.e., cytochrome oxidase or β-Actin) from the same gel.

Fig. 4. Representative Western blot analysis of rat heart mitochondrial and postmitochondrial subcellular fractions to an anti-iNOS antibody in control, autonomic-blocked, and autonomic-blocked plus thyroidectomized rats. Values [means ± SE (n = 5)] below blots represent mean of densitometric units after background subtraction. All experiments were performed in triplicate. *P < 0.001 vs. control rats. Each blot was normalized as described in Fig. 3 legend.
also found. Liver mtNOS, which reacted with anti-iNOS antibodies in the first purification steps, has been fully purified, sequenced, and identified as a transcript of genomic nNOS \textit{H9251} -isoform, phosphorylated in a serine reside at the reductase domain in the COOH terminus, and myristoylated in the oxidase domain at the NH2 terminus (17). The apparent discrepancies referred to mtNOS reactivities with the available anti-NOS antibodies can be considered as arising from cross-reactions and homology in NOS sequences (21, 31). Immunohistochemistry using anti-iNOS antibodies, antibody with conjugated gold particles, and electron microscopy confirmed the localization and the isoenzyme specificity of the mtNOS in the inner mitochondrial membranes. Thus our results support the belief that iNOS would be the main heart mitochondrial NO source in our experimental condition. However, we cannot rule out that there would be more than one mtNOS variant, depending on the type of tissue and animal species.

The molecular basis and the physiological significance of the rapid mtNOS turnover in cardiac tissue represent an interesting and a challenging aspect of a biological phenomenon. Rapid enzymatic turnover is usually associated with highly regulated enzymes and with high physiological significance. The fragmentation of the protein that reacts with the anti-iNOS antibody in the mitochondrial fractions was observed in the three experimental groups. The degree of mtNOS expression is associated with the levels of mtNOS fragmentation: the more expression, the more fragmentation. This finding suggests that mtNOS is compartmentalized in the mitochondrial space. The proteolytic fragments and whole NOS enzymes were recognized by the same anti-iNOS antibody. In addition, downregulation of the mtNOS in autonomic-blocked animals was consistent with a lower NO production and proteolytic fragmentation. These observations raise questions related to NOS protein turnover of the intact mtNOS enzyme. However, the rate at which this steady state is obtained is a function of the relative rates of synthesis and degradation and requires additional kinetic data.

On the other hand, the results show that thyroidectomy partially prevented the marked decreased mtNOS expression and biochemical mtNOS activity, inasmuch as it partially prevented the increase of heart rate produced by L-NAME in autonomic-blocked rats. The concept of hormonal regulation of mtNOS activity was mentioned by Carreras et al. (11) and Costa et al. (14). Carreras et al. observed that mtNOS activity and protein expression in liver and skeletal muscle are regulated by the thyroid status. Thyroidectomy-induced upregulation of mtNOS protein expression and activity in the autonomic-blocked animals is in agreement with previous reports in which hypothyroidism was associated with upregulation of mtNOS activity and T3 administration was associated with downregulation of the enzyme (11).

Mitochondria isolated from the animals of the three experimental groups showed high rates of respiration and high respiratory control and ADP-to-O ratios, which indicate that autonomic blockade and/or thyroidectomy had no effect on the basic mitochondrial processes for the provision of ATP and energy for cellular needs. In addition, mitochondria exhibited an increase in succinate-supported respiration after autonomic blockade, which indicates a close and fast regulation by the autonomic nervous system of the level of

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**Fig. 5.** Representative Western blot analysis of rat heart mitochondrial and postmitochondrial subcellular fractions to an anti-eNOS antibody in control, autonomic-blocked, and autonomic-blocked + thyroidectomized rats. Values [means \( \pm \) SE \((n = 5)\)] below blots represent mean of densitometric units after background subtraction. All experiments were performed in triplicate. *\( P < 0.001 \) vs. control rats. Each blot was normalized as described in Fig. 3 legend.
mitochondrial proteins in the heart. Disorders in myocardial energy balance were reported in sympathectomized rats (37, 40), and the loss of muscarinic stimulation was claimed to contribute to the development of heart failure (24). The downregulation of NOS activities after autonomic blockade should lead to decreased levels of cGMP and increased rates of O₂ uptake and ATP synthesis in the cardiomyocytes, if we consider the known effects of NO on guanylate cyclase and cytochrome oxidase (1, 9, 13, 25). Either a lower level of cGMP or a higher ATP availability should afford the molecular mechanism a higher rate of impulse generation by the sinusal node. Our data are consistent with the fact that modulation of the L-type Ca²⁺ channel in the heart involves two different biochemical pathways: 1) cGMP-dependent activation of a protein kinase (PKG), which is necessary for the reduction in the L-type Ca²⁺ channel, and 2) the cGMP-mediated activation of phosphodiesterases, which selectively breaks down cAMP and attenuates the L-type Ca²⁺ channel (15, 23). A hypothesis centered in the mitochondrial NO steady-state levels in the sinusal node would focus on the NO-mediated regulation of O₂ uptake and ATP levels and consider that decreased and suboptimal rate-limiting levels of ATP lead to decreased rates of sinusal activity and heart contraction. Such concepts are supported by the results reported here and by the observation by Kanai et al. (28) of an inverse relation between contraction and mtNOS activity in isolated cardiomyocytes. Contractility, as determined via video monitoring of cardiomyocyte shortening, was decreased by ~50% in cardiomyocytes from mdx mice, a strain that overexpresses mtNOS. The addition of the general NOS competitive inhibitor l-NMMA recovered about half of the lost contractility, which was to be mediated by NO and was mitochondrial in origin (28).

The systems centered in NO, inasmuch as signaling molecules largely modulate cardiac function and the cardiovascular system. Similarly, changes in heart NO production are associated with the autonomic regulation of heart rate. Autonomic blockade produces a marked decrease in mtNOS and eNOS expression and activity. The experimental data also provided evidence that thyroidectomy counteracts the effect of autonomic blockade. Thyroidectomy was associated with increased cardiac mtNOS expression and activity and attenuates the l-NAME-induced chronotropic response. NO steady-state level may act as a messenger to modulate the mitochondrial bioenergetic function, resulting in an NO-mediated regulation of the heart pacemaker activity.

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Fig. 6. Representative immunoelectron micrographs of isolated mitochondria from control animals. A: negative control of isolated mitochondria in which primary antibody was omitted. B: isolated mitochondria immunolabeled using an eNOS antibody. C: isolated mitochondria immunolabeled using an iNOS antibody. D: higher magnification of isolated mitochondria immunolabeled using an iNOS antibody. Observe round and dense 10-nm gold particles (arrows) on inner mitochondria membranes. Scale bars, 200 nm (A–C) and 317 nm (D).
CARDIAC NO PRODUCTION IN DENERVATED RAT HEART

REFERENCES


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