NO modulates myocardial $O_2$ consumption in the nonhuman primate: an additional mechanism of action of amlodipine

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Forfia, Paul R., Xiaoping Zhang, Delvin R. Knight, Andrew H. Smith, Christopher P. A. Doe, Eric A. Wolfgang, David M. Flynn, Michael S. Wolin, and Thomas H. Hintze. NO modulates myocardial $O_2$ consumption in the nonhuman primate: an additional mechanism of action of amlodipine. Am. J. Physiol. 276 (Heart Circ. Physiol. 45): H2069–H2075, 1999.—Recent evidence from our laboratory and others suggests that nitric oxide (NO) is a modulator of in vivo and in vitro oxygen consumption in the murine and canine heart. Therefore, the goal of our study was twofold: to determine whether NO modulates myocardial oxygen consumption in the nonhuman primate heart in vitro and to evaluate whether the seemingly cardioprotective actions of amlodipine may involve an NO-mediated mechanism. Using a Clark-type $O_2$ electrode, we measured oxygen consumption in ex vivo and endogenous sources of NO and that isolated coronary microvessels were a significant source of NO (45, 47). Therefore, the first goal of our study was to determine whether NO is involved in the modulation of myocardial oxygen consumption in the nonhuman primate and whether the coronary microvasculature represents a likely source of NO production. A recent clinical study revealed that the calcium-channel blocker amlodipine may reduce the risk of morbidity and mortality in patients with nonischemic dilated cardiomyopathy (29). In marked contrast, numerous clinical trials have concluded that calcium-channel blockers have negligible or even detrimental effects on the outcome of patients with severe heart failure (27, 11). Historically, only two drugs have consistent beneficial effects in the treatment of patients with heart failure: organic nitrates (11) and angiotensin-converting enzyme (ACE) inhibitors (34). Both drugs are known to work, at least in part, through the release of NO (44). Whether the beneficial effects of the calcium-channel blocker amlodipine can be explained by a similar NO-dependent mechanism is unknown. Therefore, the second goal of our study was to determine whether the actions of amlodipine may involve an NO-mediated mechanism.

METHODS

Male ($n = 18$) and female ($n = 13$) monkeys (Macaca fascicularis), weighing between 2.8 and 7.2 kg, were used in this study. All hemodynamic recordings as well as euthanasia procedures were performed in the Department of Cardiovascular Diseases at Pfizer Central Research (Groton, CT).

Measurement of hemodynamics. On the day of study, monkeys (2.8–6.8 kg) were anesthetized with ketamine (15 mg/kg im; Ketaject, Phoenix Scientific, St. Joseph, MO) and a limb-lead electrocardiogram (ECG) was established (ECG/Biotach amplifiers, Gould, Cleveland, OH). The left ventricle was imaged from a left parasternal approach using two-dimensional M-mode echocardiography (7.5 MHz, Hewlett-Packard Sonos 100 CF, Hewlett-Packard, Glastonbury, CT). The echocardiogram and ECG were recorded for 2–5 min to calculate left ventricular end-diastolic and end-systolic dimensions (48). To measure left ventricular pressure, a solid-state pressure catheter (SPR-524, Millar Instruments, Houston, TX) was used.
TX) was advanced into the left ventricle via the left common carotid artery. The catheter was subsequently withdrawn into the root of the aorta to measure aortic blood pressure. Pressure and ECG signals were continuously recorded on a strip-chart recorder (MT95000, Astromed, West Warwick, RI). After completion of the hemodynamic recording, the primates were euthanized with pentobarbital sodium (≥ 100 mg/kg iv; Sigma Chemical, St. Louis, MO), and the hearts were rapidly excised, rinsed in ice-cold saline, and transported to the Department of Physiology, New York Medical College (Valhalla, NY) in ice-cold saline for in vitro studies. The hearts were delivered within 3 h from the start of the study.

Preparation of cardiac muscle tissue. Atria and great vessels were removed from the base of the heart, and total cardiac weights were obtained. The left and right ventricular free walls and septum were dissected free and their respective weights recorded. The septum of each heart was used for in vitro measurement of oxygen consumption, whereas left ventricular free walls were used for the coronary microvessel preparation. The right ventricles were discarded.

The septum was freed of both endocardial and epicardial surfaces under a dissecting microscope. The tissue was then cut into pieces (≈ 2 × 2 × 0.5–1 mm (length × width × thickness)) using a tissue chopper (Mickle Laboratory Engineering, Guildford, UK). Tissue slices from the septum of each animal were then incubated separately in Krebs bicarbonate buffer containing (in mmol/l) 118 NaCl, 4.7 KCl, 2.5 CaCl2, 25 NaHCO3, 1.1 MgSO4, 1.2 KH2PO4, and 5.6 glucose at 37°C. The buffer solution was bubbled with a mixture of 21% O2–5% CO2–74% N2. Incubation was performed for 2 h before tissue respiration studies were conducted.

Measurement of in vitro oxygen consumption. Tissue oxygen uptake was measured polarographically with a model 5300 Biological Oxygen Monitor and Clark-type oxygen electrode (YSI, Yellow Springs, OH) with the electrode response recorded on a strip-chart recorder. Cardiac tissue slices from each septum were placed in separate 1- to 10-ml chambers containing 3 ml of air-saturated, 37°C Krebs solution buffered with 10 mmol/l HEPES at a pH of 7.4 and stirred continuously. Tissue oxygen consumption was calculated as the rate of decrease of oxygen concentration from the bath, assuming an initial concentration of 224 nmol/ml (43). Tissue oxygen consumption was calculated by analyzing the slope of the line on the chart recorder and was expressed as nanomoles of oxygen consumed per minute per gram of tissue. The rate during the administration of a drug was divided by the initial rate to calculate the percent change in oxygen consumption. Although the baselines can be found in the text, all data will be expressed as percent change. Each septum from a single animal was considered n = 1.

Effects of agonists on cardiac tissue respiration. To assess the effects of exogenous and endogenous NO on tissue respiration, S-nitroso-N-acetylpenicillamine (SNAP), carbachol, or bradykinin was added to the tissue bath in a cumulative and concentration-dependent (10−7–10−4 M) manner. The effects on cardiac oxygen consumption of the ACE inhibitor ramiprilat (10−7–10−4 M) and the calcium-channel blocker amlodipine (10−7–10−5 M) were also recorded. Dose responses to all drugs were performed in the presence and absence of nitro-L-arginine methyl ester (L-NAME; 10−4 M).

Preparation of coronary microvessels. The left ventricles from five to six primates received on a single day were pooled for the coronary microvessel preparation. Coronary microvessels were obtained from the left ventricular free wall using the methods of Gerritsen and Printz (12). Briefly, the myocardium was cut into small pieces, minced, and suspended in ice-cold phosphate-buffered saline (PBS). The suspension was then subjected to a series of steps involving homogenization and glass bead purification to obtain coronary microvessels devoid of large arteries and veins as well as myocytes. We have used these methods previously (47). In these studies, N = 1 represents the results from pooled hearts on a single day.

Effects of agonists on nitrite production from coronary microvessels. Coronary microvessels were collected and transferred into a tissue bath containing PBS that was oxygenated with 95% O2–5% CO2 for 30 min. Approximately 20 mg (wet weight) of coronary microvessels were placed in 5-ml plastic tubes and incubated with either 500 µl of PBS (control) or 450 µl of PBS and 50 µl of drugs (treatment). The drugs were used to either stimulate (bradykinin, carbachol, ramiprilat, and amlodipine) or inhibit L-NAME, HOE-140, 3,4-dichloroisocoumarin (DCIC), atropine) the release of nitrite from the coronary microvessels. To determine the contribution of NO to the release of nitrite from the coronary microvessels, the specific NO synthase inhibitor L-NAME was used (10−4 M). The specific bradykinin B2-receptor antagonist HOE-140 (10−5 M) was used to assess the role of kinins on nitrite release, whereas the serine protease inhibitor DCIC (10−6 M) was used to elucidate a role for endogenous kinin-forming enzymes. Atropine (10−4 M) was used to block muscarinic receptors.

At the end of the incubation period, microvessels were removed from the tubes and sulfinilamide (1%, 450 µl) and N-(1-naphthyl)ethylenediamine were added to the incubate for diazotization of sulfinilic acid by NO. NO release was measured as nitrite, the major metabolite of NO in aqueous solution. A standard curve was generated using known concentrations of sodium nitrite. Absorbance of standards was plotted, and nitrite production (pmol/mg tissue) was calculated from the standard curve.

Drugs. Bradykinin, L-NAME, carbachol, atropine, and DCIC were all purchased from Sigma Chemical. Ramiprilat and HOE-140 were generously supplied by Hoechst-Roussel (Somerville, NJ). Amlodipine was supplied by Pfizer (Groton, CT), whereas SNAP was synthesized as described previously (17). Ramiprilat was dissolved in 0.1 N sodium hydroxide, whereas all other chemicals were dissolved in sterile water.

Statistical analysis. All data are expressed as means ± SE. Differences in mean values were analyzed using a Student’s t-test for group comparisons. Statistical significance was assumed at P < 0.05. All statistics were performed using a commercially available statistics program (Jandel SigmaStat, version 2.0).

RESULTS

Hemodynamics. With the exception of larger diastolic and systolic ventricular diameters reported in the male hearts compared with the female hearts, there were no statistically significant differences with respect to hemodynamic data (Table 1). The total heart weight in male primates was significantly larger than in female primates. Combined data from male and female primates showed that total heart weight was 14.1 ± 0.9 g, left ventricular weight was 7.2 ± 0.5 g, septum weight was 3.5 ± 0.2 g, and right ventricular weight was 3.3 ± 0.2 g (n = 31).

Effects of agonists on cardiac tissue respiration. The effects of bradykinin on tissue respiration in male and female primate hearts were not significantly different
Results for SNAP, ramiprilat, and amlodipine on cardiac tissue respiration represent the combined data for both male and female primates (Figs. 2 and 3) because there were no differences between tissues from male and female monkeys.

Cardiac tissue slices treated with SNAP showed significant reductions in oxygen consumption at each dose from $10^{-7}$–$10^{-4}$ M (Fig. 2). At its highest dose, SNAP reduced cardiac tissue respiration by 38±5.8%. Bradykinin also caused dose-dependent reductions in tissue respiration, with the effect of each dose reaching statistical significance (Fig. 2). Similarly, ramiprilat suppressed tissue respiration (Fig. 3) with a maximum reduction at $10^{-2}$ M (28±2.3%). In addition, amlodipine exerted significant effects on tissue respiration with a 23±4.5% reduction from control at $10^{-2}$ M (Fig. 3).

In tissue preincubated with L-NAME, the effects of bradykinin and ramiprilat on cardiac tissue respiration were essentially abolished (Figs. 2 and 3). Interestingly, the effects of the calcium-channel blocker amlodipine also were markedly attenuated in tissue preincubated with L-NAME (Fig. 3). In contrast, L-NAME had no effect on the inhibition of tissue oxygen consumption by SNAP (Fig. 1).

Effects of agonists on nitrite release from coronary microvessels. Primate coronary microvessels released nitrite in a dose-dependent manner in response to bradykinin (Fig. 4) and carbachol (Fig. 4), with significant increases from control reported from $10^{-8}$–$10^{-5}$ M. Bradykinin and carbachol increased nitrite release by 104±19 and 93±21%, respectively, at their highest dose (Fig. 5). Ramiprilat increased nitrite production by a maximum of 94±21%, whereas amlodipine accounted for an 89±15% increase in nitrite at its highest dose (Fig. 5). In contrast, the increase in nitrite production to the highest concentrations of either ramiprilat or amlodipine was markedly attenuated in microvessels preincubated with N^G-nitro-L-arginine methyl ester (L-NAME) (Figs. 4 and 5). In addition, the effects of bradykinin on nitrite release were blunted by HOE-140, whereas those to carbachol were significantly reduced by atropine ($10^{-5}$ M). Nitrite production during stimulation with the highest concentrations of either ramiprilat or amlodipine was significantly attenuated in the presence of HOE-140. Nitrite production to ramiprilat or amlodipine was inhibited by DCIC; however, only the effects of ramiprilat reached statistical significance (Fig. 5).

Table 1. Hemodynamics in anesthetized male and female primates

<table>
<thead>
<tr>
<th>Primes</th>
<th>Primate</th>
<th>Male</th>
<th>Female</th>
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<tbody>
<tr>
<td>LV systolic pressure, mmHg</td>
<td>150±6</td>
<td>151±5</td>
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<tr>
<td>LV end-diastolic pressure, mmHg</td>
<td>4.8±0.3</td>
<td>4.9±0.5</td>
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<tr>
<td>LV dP/dt, mmHg/s</td>
<td>5,550±284</td>
<td>6,461±491</td>
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<tr>
<td>LV end-diastolic diameter, mm</td>
<td>7.1±0.06</td>
<td>5.3±0.06</td>
<td></td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>149±6</td>
<td>163±4</td>
<td></td>
</tr>
<tr>
<td>Body weight, kg</td>
<td>4.9±0.3</td>
<td>3.3±0.1</td>
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Values are means ± SE. LV, left ventricular; dP/dt, first derivative of LV pressure over time.
The present study demonstrates that the release of NO from either exogenous or endogenous sources may regulate tissue respiration in the primate heart. These findings were supported by experiments showing that cardiac tissue respiration was suppressed by the NO donor SNAP, the endogenous NO-releasing agent bradykinin, the ACE inhibitor ramiprilat, and the calcium-channel antagonist amlodipine. With the exception of SNAP, these effects were prevented by the NO synthase inhibitor L-NAME. In addition, coronary microvessels isolated from the left ventricle of the primate heart released nitrite in response to the same drugs, affirming the importance of the coronary microvasculature as a potential source of NO. The increase in nitrite production to carbachol was prevented with L-NNA or atropine, whereas the stimulatory effects of bradykinin, ramiprilat, and amlodipine were prevented by either L-NNA or HOE-140. These findings suggest a link between the microvascular production of kinins: NO and the control of mitochondrial respiration in the adjacent cardiac myocytes. In addition, the ability of amlodipine to generate NO, coupled with its NO-dependent action on tissue respiration, may represent an alternative mechanism of action for this calcium-channel antagonist.

The first major finding in the present study is the dose-dependent reduction in oxygen uptake to SNAP, bradykinin, ramiprilat, and amlodipine in primate myocardium. The most potent effects were seen with the NO-releasing agent SNAP, which caused a 38 ± 5.8% decrease in tissue respiration at its maximal dose. Inhibitory effects of bradykinin, ramiprilat, and amlodipine were also noted, with maximal reductions in tissue respiration of 19 ± 3.9, 28 ± 2.3, and 23 ± 4.5%, respectively. These drugs did not have significant inhibitory effects on cardiac tissue respiration in the presence of the NO synthase inhibitor L-NAME. In addition, the lack of significant differences in the ability of these agonists to suppress oxygen consumption in microvessels from male or female monkeys suggests that these actions are not gender specific. These results, in part, are consistent with previous in vitro studies from our laboratory (35, 47) and, for the first time, establish a role for NO in the control of oxygen consumption in the primate myocardium.

Almost two decades ago, Granger et al. (14) found that a macrophage-derived product could inhibit mitochondrial respiration in neoplastic cells in vitro. Numerical...
Amlodipine modulates nitric oxide release

Potential sites of mitochondrial inhibition by NO in our studies include aconitase, complex I, complex II, and cytochrome oxidase. However, with the exception of cytochrome oxidase, inhibition of these enzymes has only been reported with concentrations of NO in the micromolar range (15, 41). Several laboratories (6, 42) have reported significant inhibition of cytochrome oxidase at nanomolar concentrations of NO. Brown et al. (6) estimated the inhibitory constant of NO for cytochrome oxidase to be between 60 and 270 nM. Considering that in vivo levels of NO have been estimated in the nanomolar range (22), it appears that cytochrome oxidase is the most likely target for regulation by physiological concentrations of NO. In this context, biochemical (23) and spectrophotometric evidence (33) suggests that a strong similarity exists between the electron-accepting domain CuA of mitochondrial cytochrome oxidase and that of bacterial N2O reductase. Brudwig et al. (7) and others (4) have shown that the mitochondrial cytochrome oxidase is capable of reducing NO and N2O. Thus some investigators have proposed that the interaction of NO with mitochondrial cytochrome oxidase may reflect some rudimentary N2O reductase activity (7, 32). The highly lipophilic nature of NO would allow access to hydrophobic enzymes such as those embedded in the inner mitochondrial membrane, whereas its paramagnetic properties would lend to its avid binding with catalytic iron centers, leading to disruption of enzyme function (20). Nevertheless, if nanomolar levels of NO are found in tissues, then the Michaelis-Menten constant (Km) for oxygen will be higher in a system in which NO is present. Indeed, this could potentially explain the observations that the apparent Km for oxygen in tissues is consistently higher than expected from values in vitro (5, 18).

Another important finding in this study is that coronary microvessels isolated from the primate left ventricle released nitrite in a dose-dependent manner to carbachol, bradykinin, ramiprilat, and amlodipine. The effects of carbachol were mediated through a muscarinic receptor to produce NO, because nitrite release after stimulation with carbachol was blunted in the presence of L-NNa, HOE-140, and 3,4-dichloroisocoumarin (DCIC). The source of NO is presumed to be via the constitutive isoform of NO synthase in endothelial cells, because baseline nitrite release was low in this preparation and could be stimulated by agonists such as carbachol and bradykinin. The possibility exists that NO could have also come from type III NOS in myocytes (2), but because microvessel preparation contains <10% myocytes (43) and because most of these are broken fragments, we feel that this source would be minor. The myocardium is thought to possess ~3,000 capillaries per square millimeter (30), a tremendous surface area for endothelial cells and thus, NO production. Because of this tremendous capillary density in the myocardium, the average distance from capillary to cardiac myocyte has been estimated at no greater than 16 µm (39), which is well within the range of diffusion of NO (19).
Ramiprilat caused a dose-dependent increase in nitrite release in microvessels isolated from the primate myocardium. This can be explained based on the fact that ACE and kininase II are the same enzyme (21), located on the luminal surface of the endothelial cell, and represent one of two major enzymes responsible for the breakdown of kinins. Therefore, inhibition of kininase II with an ACE inhibitor leads to a local accumulation of kinins (21, 44), which are known to act on the bradykinin B1 receptor (24, 25) to produce NO. Interestingly, DCIC also prevented an increase in nitrite release after the maximal dose of ramiprilat, suggesting that an endogenous kinin-generating system is present in the isolated coronary microvessels from the primate.

The second major finding in this study is that the L-type calcium-channel antagonist amlodipine caused reductions in oxygen consumption in cardiac tissue slices and significantly increased nitrite production in isolated coronary microvessels from the primate heart. The suppression of tissue oxygen consumption was prevented in tissue preincubated in the presence of L-NAME. Meanwhile, increases in nitrite production in isolated coronary microvessels were blunted by HOE-140 and by L-NNA. Inhibitory effects of DCIC were also reported; however, these did not reach statistical significance.

Amlodipine is a dihydropyridine calcium-channel blocker that, like the other drugs in its class, is known to selectively inhibit L-type calcium channels (31). However, the use of calcium-channel blockers as vasodilator therapy in heart failure remains controversial. In fact, clinical trials have reported that the dihydropyridine calcium-channel blocker nifedipine (15), as well as others (26, 27, 28), are ineffective or even harmful in the treatment of severe heart failure. These adverse effects may be attributable to a direct cardiodepressant action of these drugs on an already dysfunctional myocardium (27, 31). In contrast to these reports, a recent clinical study by Packer et al. (29) showed that amlodipine was beneficial in the treatment of patients with dilated cardiomyopathy. This study reported a 31% decrease in morbidity and mortality and a 46% reduction in risk of death in patients receiving amlodipine treatment compared with the placebo group. Thus it appears that amlodipine acts somehow differently from other calcium-channel blockers during the treatment of heart failure. In the present study, amlodipine releases NO from isolated primate coronary microvessels through the action of local kinins and reduces tissue oxygen consumption via an NO-dependent mechanism. This effect is most likely unique to amlodipine, because a recent study from our laboratory demonstrated that amlodipine, but not nifedipine or diltiazem, released NO from large and small coronary arteries and the aorta from the dog (46). Interestingly, organic nitrates and ACE inhibitors are the only vasodilator agents shown to produce consistent long-term benefits in patients with severe heart failure (1, 9, 11, 34, 37). Both of these drugs release NO.

In summary, SNAP, bradykinin, ramiprilat, and amlodipine all caused dose-dependent reductions in oxygen uptake in cardiac tissue slices from the primate heart. These results suggest that tissue respiration can be modulated by the exogenous and endogenous release of NO in the primate heart. In addition, isolated coronary microvessels released NO in response to bradykinin and carbachol as well as to ramiprilat and amlodipine. These results confirm the ability of the primate coronary microvasculature to produce NO, and they also support the conclusion that microvasculature-derived NO caused the reductions in tissue respiration seen in the present study. Indeed, amlodipine and the ACE inhibitor ramiprilat appear to share a common mechanism of action that could potentially account for the benefit of these drugs in patients with severe heart failure.

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