RESEARCH ARTICLE

Threshold levels of extracellular L-arginine that trigger NOS-mediated ROS/RNS production in cardiac ventricular myocytes

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L-Arginine (L-Arg) is the substrate for nitric oxide synthase (NOS) to produce nitric oxide (NO), a signaling molecule that is key in cardiovascular physiology and pathology. In cardiac myocytes, L-Arg is incorporated from the circulation through the functioning of system-γ cationic amino acid transporters. Depletion of L-Arg leads to NO uncoupling, with O₂⁻ rather than L-Arg as the terminal electron acceptor, resulting in superoxide formation. The reactive oxygen species (ROS) superoxide (O₂⁻), combined with NO, may lead to the production of the reactive nitrogen species (RNS) peroxynitrite (ONOO⁻), which is recognized as a major contributor to myocardial depression. In this study we aimed to determine the levels of external L-Arg that trigger ROS/RNS production in cardiac myocytes. To this goal, we used a two-step experimental design in which acutely isolated cardiomyocytes were loaded with the dye coelenterazine that greatly increases its fluorescence quantum yield in the presence of ONOO⁻ and O₂⁻. Cells were then exposed to different concentrations of extracellular L-Arg and changes in fluorescence were followed spectrofluorometrically. It was found that below a threshold value of ~100 μM, decreasing concentrations of L-Arg progressively increased ONOO⁻/O₂⁻-induced fluorescence, an effect that was not mimicked by N-arginine or L-lysine and was fully blocked by the NOS inhibitor L-NAME. These results can be explained by NOS aberrant enzymatic activity and provide an estimate for the levels of circulating L-Arg below which ROS/RNS-mediated harmful effects arise in cardiac muscle.

cationic amino acid transporters; nitric oxide; superoxide; peroxynitrite

L-ARGININE (L-Arg) is a multifunctional semiessential amino acid that is synthesized in the small intestine and kidneys and that becomes essential during the physiologic growth of infants or in individuals experiencing catabolic states such as cancer, trauma, stress, sepsis, starvation, and severe burns or with intestine or kidney dysfunction (1). During these metabolic states, L-Arg should be incorporated from exogenous sources to ensure proper extra- and intracellular levels for this amino acid. Arginine is metabolized through multiple pathways (37) and thus its homeostasis and steady-state plasma levels are the result of fine-tuned interactions among endogenous production, dietary protein supply, body protein turnover and the metabolic state of the organism. In addition, some conditions increase L-Arg catabolism (for example, augmented plasma arginase activity; Ref. 37 and references therein) resulting in low circulating levels of this amino acid, a situation that may lead to or worsen disease. Surprisingly, the threshold L-Arg concentration below which pathologic processes are triggered has not been determined.

Among its many roles, L-Arg is the substrate for the enzymatic production of nitric oxide (NO), a signaling molecule that plays a central role in cardiovascular pathophysiology. In heart, NO has negative chronotropic, negative or positive inotropic, and positive lusitropic effects (31), and alterations of the L-Arg-NO pathway have been reported in chronic heart failure (33). NO synthesis requires the presence of L-Arg inside the cells of responsive tissues. While some cell types can synthesize L-Arg from ornithine or citrulline (20, 50), L-Arg is not produced within cardiac myocytes and thus cardiac muscle must import this amino acid from the circulation. Therefore, the carriers responsible for L-Arg transport are expected to play a key role in the metabolism of this amino acid and NO in heart. We have previously solved the kinetic features of the cationic amino acid transporters (CATs) found in cardiac myocytes (28, 40). Using cardiac sarcomemmal vesicles, we discovered high- and low-affinity L-Arg uptake components that function simultaneously. Although with a Km in the millimolar range, the low-affinity CAT, because of its high capacity, was found to be physiologically relevant as it is responsible for ~50% of total L-Arg transport at normal plasma levels of this amino acid (28).

NO biosynthesis is mediated by the enzyme nitric oxide synthase (NOS; EC 1.14.13.39), a dioxygenase composed by reductase and oxidase domains that uses NADPH and O₂ in the oxidation of a guanidino nitrogen from L-Arg to produce NO and l-citrulline (19). This reaction requires flavin mononucleotide, FAD, tetrahydrobiopterin, and, in the constitutively expressed endothelial (eNOS) and neuronal (nNOS) isoforms of the enzyme, a Ca-CaM complex (9, 38). The Ca-CaM complex binds between the oxygenase and reductase domains, after which electrons flow from reduced NADPH through the reduced flavins into the oxidase domain. At the heme site, O₂ is reduced and incorporated into L-Arg to yield the reaction products. Km values of 1.5–3 μM have been reported for L-Arg activation of constitutive NOS isoforms from assays in vitro (Ref. 16 and references therein). Nonetheless, studies by us and others found a low-affinity L-Arg stimulation of NO production at concentrations as much as three orders of magnitude
larger than the $K_m$ for this substrate, an effect known as “the arginine paradox” (6, 11, 55).

Depletion of L-Arg (and/or tetrahydrobiopterin) leads to NOS uncoupling, i.e., uncoupling of NADPH oxidation and NO synthesis, with $O_2$ rather than L-Arg as the terminal electron acceptor, resulting in the formation of superoxide ($O_2^{−−}$) by all three NOS isoforms (Ref. 52 and references therein). Combination of $O_2^{−−}$ with NO from enzymatic or nonenzymatic sources will result in the production of peroxynitrite (ONO$^{-}$) (5), an oxidizing agent associated with cell damage, decreased myocardial contractility, and congestive heart failure (15). For example, ONOO$^{-}$ infusion into isolated working rat hearts impairs cardiac contractile function by reducing cardiac efficiency, and endogenous formation of ONOO$^{-}$ contributes to myocardial stunning in ischemia/reperfusion injury as well as spontaneous loss of cardiac function (reviewed in Ref. 15).

Peroxynitrite production when NOS is exposed to low L-Arg concentrations has been detected in a kidney cell line (51) and macrophages (53). At the molecular level, ONOO$^{-}$ is responsible for oxidative protein modifications (2, 23), in particular the reversible nitration of Tyr residues in target proteins (12, 26), macrophages (53). At the molecular level, ONOO$^{-}$ is responsible for oxidative protein modifications (2, 23), in particular the reversible nitration of Tyr residues in target proteins (12, 26), and aconitase (10) are particularly sensitive targets for oxidative damage arise within cardiomyocytes.

**MATERIALS AND METHODS**

Adult male Sprague-Dawley rats weighing between 200 and 300 g were injected with pentobarbital sodium (Nembutal; 100 mg/kg ip), and hearts were removed under complete anesthesia. Animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the New Jersey Medical School. Fresh cardiac myocytes were isolated from the number of pixels in the object.

Intensity analysis was performed by NIS element measurements (after magnification) were obtained using a Nikon Eclipse 80i microscope. Confocal images of 3-nitrotyrosine and DAPI distribution (Fig. 1) were analyzed by NIS element software. Background fluorescence was obtained by incubating this reagent ($O_2^{−−}$) to obtain optimal conditions.

**Western blotting.** Rat cardiac myocytes, mouse hepatocytes, HUVEC P7 cells, and mouse whole brain tissue were washed three times with cold PBS and homogenized with the following lysis buffer: RIPA buffer (Sigma-Aldrich, St. Louis, MO), 10 µg/µl leupeptin, 5 µg/µl aprotinin, and 250 µM PMSF. The supernatant was conserved after spinning down this homogenate at 15,000 rpm for 15 min at 4°C. Protein concentration was determined using the BCA assay. For electrophoresis assays, 8% Bis-Tris SDS gels were loaded with 15 µg of control samples or 150 µg of cardiac myocyte or hepatocyte lysates. Gels were run at 140 V until the molecular weight markers were fully separated and banding patterns were transferred to PVDF membranes. Membranes were blocked with 5% nonfat dry milk in TBS-T for 1 h, washed for 10 min using TBS-T and incubated with the primary antibody (anti-mouse eNOS/nNOS) at a 1:2500 dilution, calculated using its molar extinction coefficient (1.670 cm$^{-1}$ M$^{-1}$) and the dilution factor. The calculated TMA-OONO$^{-}$ stock concentration varied between 12 and 15 mM. Solutions with ONOO$^{-}$ concentrations below 12 mM were not used in the experiments. Decomposed TMA-OONO$^{-}$ was obtained by incubating this reagent at pH 7.2 (Langendorff solution, see below) for 60 min at room temperature.

**Fluorescence experiments.** Freshly isolated myocytes were suspended in 1.5 ml of Langendorff solution containing the following (in mM): 135 NaCl, 5.4 KCl, 0.2 CaCl$_2$, 0.33 Na$_2$HPO$_4$, and 10 HEPES-Na pH 7.2 at 23°C, and incubated for 30 min at 23°C with 20 µM of the dye coelenterazine (Fig. 1) in aluminum foil-wrapped glass tubes with occasional mixing. Cells were washed twice to remove the excess coelenterazine and resuspended in 2 ml of Langendorff solution, and 200-µl aliquots were distributed in 96-well plates.

After background fluorescence was recorded, desired concentrations of TMA-OONO$^{-}$/KOH (15 µl) and L-Arg (25 µl) were added and the time course of fluorescence changes followed with a Cary Eclipse spectrofluorometer using a $\lambda_{ex}$ of 400 nm and a $\lambda_{em}$ of 514 nm. The total time elapsed from cardiomycyte isolation to fluorescence measurements was 60–90 min and cells were kept at room temperature.

**Immunocytochemistry assays.** Isolated cardiac myocytes were plated on six-well plates (~1000 cells/sample) and incubated for 1 h at 37°C on laminin-coated (10 µg/ml) coverslips. Attached cells were treated for 30 min with L-Arg (various concentrations), N-nitro-L-arginine methyl ester (L-NAME), L-Arg + nitrotyrosine, or Langendorff’s buffer. Myocytes were then fixed by treatment with 3% paraformaldehyde in PBS for 15 min at room temperature. After being rinsed, plates were incubated with protein G purified mouse monoclonal anti-nitrotyrosine IgG (1:500 dilution; EMD Millipore, Billerica, MA) and Alexa Fluor 555 goat anti-mouse IgG (1:250 dilution; Life Technologies, Grand Island, NY). Nuclei were identified with ProLong gold antifade reagent with DAPI (Invitrogen, Carlsbad, CA). Confocal images of 3-nitrotyrosine and DAPI distribution ($\times40$ magnification) were obtained using a Nikon Eclipse 80i microscope. Intensity analysis was performed by NIS element measurements (after subtracting DAPI staining) and normalized to myocyte area, as estimated from the number of pixels in the object.

**Coelenterazine and tetramethylammonium peroxynitrite chemical structure representations.**

![Coelenterazine](image1)

![Tetramethylammonium peroxynitrite](image2)
from BD Transduction Laboratory, San Jose, CA) in 5% milk in TBS-T, overnight at 4°C with gentle shaking. Membranes were then washed three times with TBS-T (10 min each) and the secondary antibody was applied (mouse IgG at a 1:2,500 dilution, from Pierce, Rockford, IL) with gentle shaking for 1 h, after which the washing steps were repeated. Enhanced chemiluminescence (ECL; Pierce, Rockford, IL) was used to visualize the bands.

**Statistics.** Data are presented as means ± SE for the indicated number of experiments. Statistical significance was assessed using Student’s t-test (P < 0.05). Curve fitting was performed with nonlinear least-squares routines included in SigmaPlot (version 10.0, Systat Software, Richmond, CA) using statistical weights proportional to (SE)^−1 or (SE)^−2.

**Reagents.** Tetramethylammonium peroxynitrite was purchased from Axxora (San Diego, CA). Coelenterazine was purchased from Sigma in batches of 50 µg/50 µl 70% ethanol, aliquoted and stored at −80°C. SNAP was from Calbiochem (Billerica, MA; stored at −20°C protected from light), and pyrogallol was purchased from Sigma (stored at room temperature, protected from light). The hydrochloride salts of L-lysine (L-Lys), D-arginine (D-Arg), and L-Arg were purchased from Sigma-Aldrich. t-NAME and uric acid were from Sigma and 7-nitro indazole sodium (7-NINA) was from Alexis Biochemicals (San Diego, CA). Nitrotyrosine was purchased from EMD Millipore. Collagenase type II was obtained from Worthington Biochemical (Lakewood, NJ). Salts and reagents were of analytical reagent grade.

**RESULTS**

Experiments were designed to determine how low must extracellular L-Arg levels fall to trigger NOS-mediated superoxide/peroxynitrite production in cardiac myocytes. Toward this goal, we used coelenterazine, which has been described to produce chemiluminescence upon reaction with superoxide (O_2^−) or peroxynitrite (ONOO^−) but not with NO (17, 29, 46). However, we noticed that coelenterazine chemiluminescence and fluorescence responses were similar and thus in the present work we measured coelenterazine fluorescence to follow O_2^−/ONOO^− production.

The ONOO^− detection system. Acutely isolated cardiac muscle cells from adult rats were suspended in Langendorff solution, incubated with 20 µM coelenterazine, washed, and distributed in 96-well plates. After background fluorescence was recorded, myocytes were exposed to 2.7 µM of the reactive nitrogen species (RNS) tetramethylammonium peroxynitrite (TMA-ONOO^−). Results in Fig. 2 show that ONOO^− produced a sudden increase in fluorescence that was not resolved in the 30-s time interval that followed its addition. This increase in fluorescence, typically from a background level of 150–200 arbitrary units (AU) to 300–350 AU, was not apparent when fresh TMA-ONOO^− (dissolved in 10 mM KOH) was replaced with decomposed TMA-ONOO^− (Fig. 2, open circles). Confocal images of the same cardiomyocyte before and after ONOO^− exposure are included to illustrate the changes in fluorescence (Fig. 2, insets).

Unexpectedly, instead of increasing steadily, fluorescence levels reached a plateau that lasted at least 3 min in the continuous presence of ONOO^−. Considering that the reaction with ONOO^− irreversibly modifies the coelenterazine molecule (see DISCUSSION), such a behavior admits two alternative explanations: either coelenterazine within cardiomyocytes was saturated with ONOO^− or ONOO^− is a short-lived species. We assessed saturation of the fluorescent dye by performing sequential ONOO^− additions to the same batch of coelenterazine-loaded myocytes. Consecutive ONOO^− additions produced corresponding increases in fluorescence that progressively decreased in magnitude approaching saturation (Fig. 3A). Therefore, coelenterazine was not the limiting reagent when ONOO^− was first added.

In terms of the second explanation, the time course of ONOO^− decay was followed by measuring absorbance at 302 nm in either 10 mM KOH (pH 12) or Langendorff buffer. Results in Fig. 3B show that ONOO^− decays much faster in Langendorff buffer (t_{1/2} = 9 s) than in KOH (t_{1/2} = 2910 s). This finding is consistent with the knowledge that ONOO^− is stable in alkaline media (4, 18, 24) and indicates that, for all practical purposes, the added ONOO^− disappears within the first minute, i.e., we were basically exposing myocytes to a pulse of ONOO^−. To further support this conclusion, we exposed coelenterazine-loaded cardiomyocytes to a mixture of 10 µM SNAP and 10 µM pyrogallol. SNAP spontaneously releases NO in solution and pyrogallol is a O_2^− supplier (47, 54). Equimolar combination of these compounds will result in the steady production of peroxynitrite according to NO + O_2^− ↔ ONOO^−. Under these conditions, fluorescence shows an initial increase not resolved in time (similar to that observed with TMA-ONOO^− treatment) but followed by a slower, linearly increasing phase (Fig. 3C). This second phase represents additional coelenterazine molecules reacting with ONOO^− (and likely some O_2^−). Parallel application of SNAP alone confirmed that coelenterazine is not a reporter for NO production (Fig. 3C).

**Effect of low L-Arg concentrations.** Since intracellular coelenterazine appears to be in excess, our design is well suited to detect further O_2^−/ONOO^− production. Thus, after background fluorescence was recorded, myocytes were treated with 2.7 µM TMA-ONOO^− and the system was allowed to reach a plateau in fluorescence (Fig. 4A). After 3 min, enough L-Arg to yield a final concentration of 50 µM was added to the well.
plates. L-Arg enters cardiac muscle cells through high- and low-affinity CATs (28). Once inside, among other roles, L-Arg becomes the substrate for NO synthesis via NOS activity. However, 50 μM L-Arg further increased fluorescence on top of the TMA-ONOO⁻ plateau (Fig. 4A), suggesting that this concentration of L-Arg mediated the endogenous synthesis of O₂⁻⁻¹ rather than NO. Another aliquot of coelenterazine-loaded myocytes from the same batch was treated with 200 μM L-Arg. In this case, no further increase in fluorescence was observed on top of the plateau (Fig. 4A), suggesting that this L-Arg concentration was enough to keep NOS activity in a NO-producing mode. L-Arg transport and NOS activity were involved in the effect observed with 50 μM L-Arg, since 50 μM D-Arg, neither a transported species nor a NOS substrate, and 50 μM L-Lys, a transported species but not a NOS substrate, both failed to produce an increase in fluorescence (Fig. 4B). Furthermore, the increase in fluorescence produced with 50 μM L-Arg was completely blocked by the general NOS inhibitor 1-NAME (Fig. 4C). The L-Arg concentration dependence of fluorescence increase indicates that the lower the L-Arg concentration, the larger the increase in fluorescence, i.e., the more O₂⁻⁻¹/ONOO⁻ produced (Fig. 4D). In fact, the change in fluorescence was found to be a decreasing function of L-Arg concentration with abscissa equal to 130 μM (Fig. 4E). This abscissa value represents the minimal L-Arg concentration that produces NO rather than O₂⁻⁻¹ under these experimental conditions (2.7 μM TMA-ONOO⁻⁻¹).

Dependence on added ONOO⁻⁻¹. The concentration of added TMA-ONOO⁻⁻¹ must necessarily have an effect on the amount of L-Arg needed to produce an increase in fluorescence. Thus experiments such as those presented in Fig. 4, D and E, were carried out with myocytes initially exposed to different concentrations of ONOO⁻⁻¹. It was found that the lower the ONOO⁻⁻¹ concentration, the lower the L-Arg concentration required to produce increases in fluorescence (Fig. 5A). Linear regression analysis at each ONOO⁻⁻¹ concentration yielded abscissa values that were a hyperbolic increasing function of ONOO⁻⁻¹ concentration (Fig. 5B). The ordinate of this hyperbola at [ONOO⁻⁻¹] = 0 was found to have a value of 64 ± 16 μM. This value represents the extracellular L-Arg concentration below which NOS begins synthesizing O₂⁻⁻¹, eventually leading to ONOO⁻⁻¹ production.

Direct L-Arg concentration dependence. Once the detection system and the L-Arg and ONOO⁻⁻¹ concentration dependence of fluorescence were validated, it was anticipated that limiting concentrations of L-Arg would themselves produce fluorescence increases. Therefore, after background fluorescence was recorded, coelenterazine-loaded cardiomycocytes were exposed to 20, 50, or 100 μM L-Arg. The increase in fluorescence was larger at lower L-Arg concentrations, displaying an initial fast phase not resolved in time followed by a much slower, mildly increasing second component (Fig. 6). Notice that, according to the rate constants of ONOO⁻⁻¹ decay as a function of pH. The decay of 4 μM TMA-ONOO⁻⁻¹ dissolved either in 10 mM KOH (gray squares, n = 3) or added to a 0.2-mM calcium-containing Langendorff buffer (filled circles, n = 3) was followed spectrophotometrically at a wavelength of 302 nm. Open circles represent the absorbance of 0.1 mM tetramethylammonium chloride (TMACl; n = 4) that was then subtracted. Single exponential functions were fitted to the data, from which rate constants k of 4.62 min⁻¹ (Langendorff) and 0.014 min⁻¹ (KOH) were obtained. Symbols represent the means ± SE for the indicated number of experiments. C: time course of fluorescence by ONOO⁻⁻¹ produced from a S-nitroso-N-acetyl-L-cysteine-penicillamine (SNAP) + pyrogallol mixture. At the time indicated by the arrow, a mixture of 10 μM SNAP plus 10 μM pyrogallol already releasing O₂⁻⁻¹ (●) or only 10 μM SNAP (○) were added to coelenterazine-loaded cardiomycocytes suspended in calcium-containing Langendorff solution. Results are representative of 3 independent experiments.
Fig. 4. Effect of L-arginine (L-Arg) on peroxynitrite-induced fluorescence. **A**: time courses of fluorescence changes at 50 (○) and 200 µM L-Arg (●). The arrow shows the time at which L-Arg was added. Symbols represent the means ± SE of 5 experiments performed in triplicate for each L-Arg concentration. **B**: time courses of fluorescence changes in the presence of 50 µM D-Arg (●) or L-Lys (○). Symbols represent the means ± SE of 5 experiments for D-Arg and 3 for L-Lys, each performed in triplicate. **C**: effect of 50 µM L-Arg in the absence (○, n = 5) and presence of 1 mM L-NAME (●, n = 3). Cells were preincubated with L-NAME for 5 min before adding peroxynitrite. **D**: L-Arg concentration dependence of average fluorescence increases for the range 20–200 µM. **E**: increase in fluorescence above the baseline as a function of L-Arg concentration. Symbols (as well as bars in D) represent the means ± SE of 24 time points, each of which summarizes the values from 5 experiments at those same times. The line through the data points is the best-fit linear regression, with an x-axis intercept of 130.3 µM.
concentrations and ONOO$^-$ response further confirms the relationship between low L-Arg concentration. Lines through the data points represent linear regressions, with the $y$-intercept (Abs$_{0}$ = $y_0 + (\text{Absmax} \times [\text{ONOO}^-]/K_{\text{on}} + [\text{ONOO}^-]))$, yielding a value of $y_0 = 64 \mu$M. This value represents the minimal L-Arg concentration required for NOS to produce NO in the absence of exogenous ONOO$^-$. A more realistic estimate. A frequently neglected fact is that the blood that irrigates cardiac myocytes contains L-Arg but also L-Lys and L-ornithine (L-Orn), which are equally efficient transported by the system-$\gamma^+$ cationic amino acid carriers present on the cell membrane (13, 40). Considering a plasma concentration of 250 $\mu$M for L-Arg and L-Lys, and 100 $\mu$M for L-Orn (39, 48), and assuming that every L-Arg molecule that enters the cell is converted into NO, a maximum of two out of five transport events will likely result in NO production. This scenario suggests that a larger extracellular L-Arg concentration would be required to avoid NOS-mediated ONOO$^-$/O$_2$•− production. To determine this new, more realistic L-Arg concentration, experiments similar to those described in Figs. 4 and 5 were performed in the presence of 250 $\mu$M L-Lys and 100 $\mu$M L-Orn. A 533-nM TMA-ONOO$^-$ concentration was selected because its L-Arg abscissa value represents a good approximation to that extrapolated at zero ONOO$^-$. 

The L-Arg concentration dependence of fluorescence increase is plotted in Fig. 7A together with the corresponding curve in the absence of L-Lys and L-Orn from Fig. 5A. Clearly, the presence of these two amino acids shifted the curve to the right, indicating that a larger L-Arg concentration will still deviate NOS activity toward the production of aberrant by-products. Linear regression yielded an abscissa value of 106 ± 7 $\mu$M L-Arg in the presence of L-Lys and L-Orn. This value is, in average, ~70% larger than that obtained in the absence of these amino acids (Fig. 7B). Therefore, at extracellular L-Arg concentrations of ~100 $\mu$M or lower, some NOS units will switch from NO to O$_2$•− production in the presence of physiological concentrations of other cationic amino acids.

to the error bars, changes in fluorescence brought about by 100 $\mu$M L-Arg were not significantly different from zero, so that the L-Arg concentration that first triggers O$_2$•−/ONOO$^-$ production must fall within the range 50 $\mu$M < [L-Arg] < 100 $\mu$M, a result consistent with the value found in the previous section. Two controls are also presented in Fig. 6. First, a complete block of fluorescence increase was observed when myocytes were incubated with 1 mM L-NAME before being exposed to 20 $\mu$M L-Arg (open diamonds). This result clearly indicates the involvement of NOS activity in the fluorescence increases observed without the inhibitor. Second, myocyte incubation with 200 $\mu$M uric acid, a well-known ONOO$^-$ scavenger (Ref. 49 and references therein), also prevented fluorescence increases in the presence of 20 $\mu$M L-Arg (gray squares). Except for an initial mild increase, likely showing that O$_2$•− production precedes that of ONOO$^-$, this lack of response further confirms the relationship between low L-Arg concentrations and ONOO$^-$ production.

Fig. 5. Peroxynitrite concentration dependence of L-Arg-induced increases in fluorescence. A: Fluorescence, expressed as a percent of increase over the level obtained at each ONOO$^-$ concentration, as a function of L-Arg concentration. Symbols, which are time averages, correspond to 0.53 (●), 2.7 (○), 13 (■), and 67 (▲) $\mu$M ONOO$^-$ and represent the means ± SE of 5 experiments for each concentration. Lines through the data points represent linear regressions, with $0.955 < R^2 < 0.997$. B: L-Arg concentration that produces nitric oxide synthase (NOS)-mediated endogenous ONOO$^-$ as a function of ONOO$^-$ concentration. The following hyperbolic function was fitted to the data: Abs = $y_0 + (\text{Absmax} \times [\text{ONOO}^-]/K_{\text{on}} + [\text{ONOO}^-])$, yielding a value of $y_0 = 64 \mu$M. This value represents the minimal L-Arg concentration required for NOS to produce NO in the absence of exogenous ONOO$^-$. 

Fig. 6. Effect of low concentrations of L-Arg on fluorescence produced by coelenterazine-loaded cardiac myocytes. Fluorescence increases at each L-Arg concentration were converted into percentages after subtracting the corresponding baselines. Symbols represent the means ± SE of 3 time courses for each of the L-Arg concentrations shown above the respective curves. Also shown are time courses obtained in the presence of 20 $\mu$M L-Arg for myocytes that were incubated with 1 mM L-NAME (open diamonds) or 200 $\mu$M uric acid (gray squares). Symbols represent the means of 3 experiments for each condition but error bars were omitted for the sake of clarity.

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A pertinent question relates to the identity of the NOS isoform that is responsible for ROS/RNS generation in adult rat cardiomyocytes. Western blots from freshly isolated myocyte lysates treated with specific antibodies (see MATERIALS AND METHODS) showed a prominent band for the endothelial isoform (eNOS) whereas the neuronal isoform (nNOS) was hardly detected (Fig. 8A). Consistent with this finding, 20\(\mu\)M of the NOS inhibitor 7-NINA, reported to block nNOS in vitro with an IC50 of 0.47\(\mu\)M (36) without affecting eNOS (43, 44), failed to reduce the increase in fluorescence produced by direct treatment of myocytes with 20\(\mu\)M L-Arg, an increase that was completely prevented by preincubation with 1 mM of the NOS general inhibitor L-NAME (Fig. 8B). These results suggest that eNOS is responsible for NO and \(\text{O}_2^-/\text{ONOO}^-\) production in adult rat cardiomyocytes.

The NOS isoform. A pertinent question relates to the identity of the NOS isoform that is responsible for ROS/RNS generation in adult rat cardiomyocytes. Western blots from freshly isolated myocyte (> 95% purity) lysates treated with specific antibodies (see MATERIALS AND METHODS) showed a prominent band for the endothelial isoform (eNOS) whereas the neuronal isoform (nNOS) was hardly detected (Fig. 8A). Consistent with this finding, 20\(\mu\)M of the NOS inhibitor 7-NINA, reported to block nNOS in vitro with an IC50 of 0.47\(\mu\)M (36) without affecting eNOS (43, 44), failed to reduce the increase in fluorescence produced by direct treatment of myocytes with 20\(\mu\)M L-Arg, an increase that was completely prevented by preincubation with 1 mM of the NOS general inhibitor L-NAME (Fig. 8B). These results suggest that eNOS is responsible for NO and \(\text{O}_2^-/\text{ONOO}^-\) production in adult rat cardiomyocytes.

Effect of endogenous \(\text{ONOO}^-\). To test the underlying hypothesis that \(\text{ONOO}^-\) is the ultimate by-product of NOS aberrant activity at limiting L-Arg concentrations, we investigated the presence of \(\text{ONOO}^-\) fingerprints, i.e., the nitration in position 3 of aromatic rings in protein tyrosine residues. Nitrotyrosine formation was monitored by immunocytochemistry. Incubation of cardiomyocytes for 30 min with 20 \(\mu\)M L-Arg resulted in a prominent 3-NO2-Tyr staining (Fig. 9, top left), an effect that was ameliorated in a dose-dependent manner by increasing the concentration of external L-Arg (Fig. 9, top middle and right). The staining observed in the presence of 20 \(\mu\)M L-Arg was completely blocked by preincubation of the primary antibody with 1 mM nitrotyrosine, confirming the specificity of the immunological reaction (Fig. 9, bottom left). This staining was also prevented by preincubating the cardiomyocytes with 1 mM L-NAME (not shown, but see Table 1), confirming the involvement of NOS activity in nitrotyrosine formation. Interestingly, incubation of myocytes with L-Arg-

Fig. 7. Effect of physiological levels of L-Lys and L-Orn on L-Arg-mediated increases in fluorescence. A: fluorescence, expressed as a percentage of increase over the level obtained with 0.53 \(\mu\)M \(\text{ONOO}^-\), as a function of L-Arg concentration. Symbols correspond to the absence (●) and the presence of 250 \(\mu\)M L-Lys plus 100 \(\mu\)M L-Orn (○) and represent time averages of the mean ± SE of 5 experiments for each concentration. Lines through the data points represent linear regressions, with \(R^2\) values of 0.957 and 0.999 for the absence and the presence of L-Lys and L-Orn, respectively. B: abscissa values from the linear regression analysis. Closed bars represent the absence and open bars represent the presence of 250 \(\mu\)M L-Lys plus 100 \(\mu\)M L-Orn.

Fig. 8. Immunohistochemical and functional characterization of NOS isoforms. A: Western blots for NOS isoforms in rat cardiac ventricular myocytes (CM). Positive controls: endothelial (e)NOS, human umbilical vein endothelial cells (HUVEC P7); neuronal (n)NOS, mouse whole brain tissue homogenate. B: pharmacological manipulations. Acutely isolated myocytes were loaded with coelenterazine and incubated for 5–6 min with either Langendorff buffer (●), 20 \(\mu\)M 7-NINA (○), or 1 mM L-NAME (△). Treatment groups were then exposed to 20 \(\mu\)M L-Arg. Background fluorescence was subtracted from all 3 groups. The displayed behavior is representative of 3 experiments each performed in duplicate.
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Fig. 9. Immunocytochemical detection of nitrotyrosine formation. Isolated myocytes plated on 6-well plates were exposed for 30 min to the treatments described and then incubated with protein G purified mouse monoclonal anti-3-nitrotyrosine IgG followed with Alexa Fluor 555 goat anti-mouse IgG. Nuclei were identified with DAPI although this staining was not included in the image analysis. Confocal images (×40 magnification) were obtained using a Nikon Eclipse 80i microscope. All solutions were prepared in Langendorff buffer; for the ONOO− treatment (bottom right), myocytes were exposed to a mixture of 10 μM of SNAP + 10 μM pyrogallol. Calibration bar, 20 μm.

Table 1. Immunocytochemical analysis of 3-NO2-tyrosination in cardiac myocytes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean Intensity ± SE* (Average Pixel Value)</th>
<th>Percentage of ONOO− Fluorescence After Subtracting Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>ONOO−</td>
<td>2,316 ± 156</td>
<td>100</td>
</tr>
<tr>
<td>20 μM L-Arg</td>
<td>1,756 ± 110</td>
<td>72 ± 8</td>
</tr>
<tr>
<td>100 μM L-Arg</td>
<td>870 ± 58</td>
<td>27 ± 4</td>
</tr>
<tr>
<td>2 mM L-Arg</td>
<td>524 ± 58</td>
<td>10 ± 3</td>
</tr>
<tr>
<td>20 μM L-Arg + 1 mM NO2-tyrosine</td>
<td>386 ± 25</td>
<td>3 ± 2†</td>
</tr>
<tr>
<td>20 μM L-Arg + 1 mM 1-NAME</td>
<td>397 ± 30</td>
<td>3 ± 2†</td>
</tr>
<tr>
<td>Langendorff buffer</td>
<td>330 ± 28</td>
<td>0</td>
</tr>
</tbody>
</table>

*n = 10 myocytes for each treatment condition. †,‡Not significantly different from zero.

DISCUSSION

A novel experimental design was set up for the detection of O2−/ONOO− production in cardiac myocytes to determine the levels of extracellular L-Arg that trigger NOS biosynthesis of these by-products. The fluorescent dye coelenterazine, when loaded into myocytes, proved to be sensitive to both externally added ONOO− and its endogenous production mediated by limiting levels of L-Arg. Although addition of TMA-ONOO−, SNAP + pyrogallol, or limiting L-Arg concentrations all increased fluorescence in coelenterazine-loaded myocytes, the initial response and secondary fluorescence increases appear to be of different nature. Exposure to TMA-ONOO− in Langendorff buffer (pH 7.2) resulted in a sudden increase in fluorescence followed by a plateau. This behavior likely represents only modification of the most accessible pool of coelenterazine molecules given the short half-life of ONOO− at this pH value. In fact, mass spectrometry assays showed nitration in position 3 (meta) of aromatic rings in the coelenterazine molecule (see MATERIALS AND METHODS) upon brief exposure to TMA-ONOO− in Langendorff buffer (Li H, Peluffo RD, unpublished results). The SNAP + pyrogallol ONOO− producing system showed fast and slow fluorescence components. The latter component may represent ROS/RNS reaction with coelenterazine molecules located in more distant and/or less accessible compartments. A similar explanation applies to the endogenous production of O2−/ONOO− mediated by limiting levels of extracellular L-Arg. However, intracellular diffusion of L-Arg molecules to reach more distant/less accessible pools of NOS enzyme must also be considered in this case as the reason for the second, slower component.

The effect of L-Arg showed an inversely proportional concentration dependence on fluorescence. D-Arg, which is not transported by system-y+ members, and L-Lys, which although transported is not a substrate for NO synthesis, did not increase fluorescence at similar concentrations. The notion that the increase in fluorescence requires NOS activity was con-
firmed by the lack of effect when 50 μM L-Arg was applied to myocytes preincubated with the NOS inhibitor L-NAME. Altogether, these findings strongly suggest that limiting L-Arg concentrations are promoting the synthesis of O₂⁻, which by itself or through the reaction NO + O₂⁻ → ONOO⁻ enhances coelenterazine fluorescence.

Our quantification of “limiting substrate conditions” indicates that, in the absence of externally added ONOO⁻, a plasma [L-Arg] ≤ 64 μM will result in ROS/RNS production in cardiac myocytes. However, given the presence in the extracellular fluid of physiological concentrations of other cationic amino acids that efficiently compete with L-Arg transport (13, 28, 40), a more realistic limiting value of 106 ± 7 μM L-Arg was obtained in the simultaneous presence of L-Lys and L-Orn. Therefore, at extracellular [L-Arg] ≤ 100 μM, while some NOS units will still produce NO, a subset of this enzyme will begin producing O₂⁻. It is anticipated that the lower the L-Arg concentration the larger the recruited number of O₂⁻-synthesizing NOS units. These observations also fit well with the detection of H₂O₂ formation at concentrations of L-Arg below 100 μM using purified brain NOS preparations (21). Of particular note, even if a limiting value of 64 μM L-Arg is considered, we can see the “arginine paradox” at work (6, 27, 32), provided that this [L-Arg] is ~20-fold larger than the K₀.₅ for L-Arg activation of constitutive NOS activity in vitro under saturating conditions for cosubstrates and cofactors (Ref. 16 and references therein).

Western blot experiments detected eNOS (NOS3) as the constitutive isoenzyme to be almost exclusively present in isolated rat cardiac ventricular myocytes. This finding, although indirect, points to eNOS as responsible for ROS/RNS production in cardiac muscle cells. The lack of effect of the fairly selective nNOS inhibitor 7-NiNA on L-Arg-induced fluorescence further supports this conclusion.

In an independent approach, immunocytochemistry experiments clearly show that the lower the extracellular L-Arg concentration the stronger the 3-NO₂-Tyr staining in rat cardiac myocytes, a fingerprint for ONOO⁻ production (12). These results demonstrate that limiting concentrations of L-Arg are able to produce substantial amounts of a Tyr-nitrating agent and seem to be at variance with previous reports indicating the lack of Tyr nitration by NOO⁻ generated from NO and O₂⁻ at pH 7.4 in vitro (41). Furthermore, Tyr nitration of proteins upon exposure to low concentrations of extracellular L-Arg took place despite the anticipated presence of physiological levels of intracellular GSH and SOD that might diminish ONOO⁻ formation in acutely isolated cardiac myocytes.

Interestingly, incubation with 100 μM L-Arg did not completely abolish the nitration of Tyr residues in cardiac muscle proteins although it reduced immunostaining to roughly one-third of that obtained with 20 μM L-Arg. A possible explanation to reconcile this finding with our threshold L-Arg value of 106 μM is that the 30 min-long myocyte incubation in immunocytochemistry assays could have uncovered additional sources for the production of O₂⁻/ONOO⁻ such as mitochondria (42). This mitochondrial ROS/RNS production that has been reported to be responsible for the 3-NO₂-tyroosination of several critical proteins in the mitochondrial matrix, inner and outer membranes, as well as the intermembrane space (42), might have a more complex relationship with extracellular L-Arg levels (54).

Two additional features of our immunocytochemistry assays are noticeable. First, millimolar L-Arg concentrations prevented immunostaining of 3-NO₂-tyrosinated proteins in cardiac myocytes, a result that can be interpreted as a full inhibition of O₂⁻/ONOO⁻ production by these concentrations of L-Arg. This observation is consistent with our previous reports showing that millimolar concentrations of extracellular L-Arg promote full production of NO by NOS (40, 55) and might explain the finding that improvement of both clinical symptoms of cardiovascular disease and the outcome of patients with congestive heart failure are observed upon L-Arg supplementation but only when its plasma levels reach concentrations of 3–6 mM (7, 25). In agreement, skeletal muscle from rabbits treated with ~2 mM L-Arg showed both inhibition of NOS-mediated O₂⁻ production and reduction of ischemia/reperfusion injury (22).

The second feature is the lack of staining when myocytes were incubated with L-Arg-free Langendorff buffer, a condition that could be visualized as the lowest possible L-Arg concentration and thus should result in full production of ROS/RNS and nitrotyrosine fingerprints. Previous studies using a similar immunocytochemical approach on nNOS-overexpressing HEK 293 cells found prominent nitrotyrosine immunostaining upon prolonged incubation in L-Arg-free medium (51). This treatment resulted in an estimated intracellular L-Arg of ~16 μM after 24 h. We have previously reported the presence of high-affinity/low-capacity and low-affinity/high-capacity passive CATs functioning in parallel in rat cardiac ventricular myocytes (28). In particular, due to its high capacity, the low-affinity transporter was shown to be responsible of at least 50% of total cationic amino acid transport in this cell type (28). To the best of our knowledge, a low-affinity/high-capacity component of L-Arg transport has not been reported in HEK cells. Therefore, passive L-Arg efflux could be much more efficient in cardiomyocytes incubated with L-Arg-free medium thus completely depleting intracellular pools in the ~1.5 h of total myocyte manipulations. With L-Arg concentrations approaching zero, NOS isoforms will only produce O₂⁻ from rabbits treated with ~2 mM L-Arg showed both inhibition of NOS-mediated O₂⁻ production and reduction of ischemia/reperfusion injury (22).

Constitutive NOS isoforms appear to be highly conserved between rats and humans (>90% amino acid identity) (16), and circulating L-Arg plasma levels have been reported to be similar in these two species (39, 48). Accordingly, the present results suggest routine evaluation of L-Arg plasmatic levels in patients and provide a lower concentration limit for corrective therapeutic interventions in the clinical practice.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

J.R. performed experiments; J.R. and R.D.P. analyzed data; J.R. and R.D.P. edited and revised manuscript; J.R. and R.D.P. approved final version of manuscript; R.D.P. conceived and designed the research; R.D.P. interpreted results of experiments; R.D.P. prepared figures; R.D.P. drafted manuscript.

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