L-Arginine protects human heart cells from low-volume anoxia and reoxygenation

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Received 16 July 2001; accepted in final form 8 November 2001

Shiono, Noritsugu, Vivek Rao, Richard D. Weisel, Muneyasu Kawasaki, Ren-Ke Li, Donald A. G. Mickle, Paul W. M. Fedak, Laura C. Tumiati, Lawrence Ko, and Subodh Verma. L-Arginine protects human heart cells from low-volume anoxia and reoxygenation. Am J Physiol Heart Circ Physiol 282: H805–H815, 2002; 10.1152/ajpheart.00594.2001.—Protective effects of L-arginine were evaluated in a human ventricular heart cell model of low-volume anoxia and reoxygenation independent of alternate cell types. Cell cultures were subjected to 90 min of low-volume anoxia and 30 min of reoxygenation. L-Arginine (0–5.0 mM) was administered during the preanoxic period or the reoxygenation phase. Nitric oxide (NO) production, NO synthase (NOS) activity, cGMP levels, and cellular injury were assessed. To evaluate the effects of the L-arginine on cell signaling, the effects of the NOS antagonist NG-nitro-L-arginine methyl ester, NO donor S-nitroso-N-acetyl-penicillamine, guanylate cyclase inhibitor methylene blue, cGMP analog 8-bromo-cGMP, and ATP-sensitive K+ channel antagonist glibenclamide were examined. Our data indicate that low-volume anoxia and reoxygenation increased NOS activity and facilitated the conversion of L-arginine to NO, which provided protection against cellular injury in a dose-dependent fashion. In addition, L-arginine cardioprotection was achieved by the activation of guanylate cyclase, leading to increased cGMP levels in human heart cells. This action involves a glibenclamide-sensitive, NO-cGMP-dependent pathway.

ventricular myocytes; cardiac surgery; nitric oxide

DURING CARDIAC SURGERY, the heart is arrested with cardioplegia to facilitate surgical intervention and is subsequently reoxygenated after removal of the aortic cross-clamp. Cardioplegic arrest provides a unique clinical situation in which myocardial low-volume anoxia and reoxygenation can be anticipated and allows for interventions to prevent biochemical and functional derangements.

Previous studies have demonstrated that nitric oxide (NO) exerts beneficial effects after low-volume anoxia and reoxygenation (1, 7, 11, 13, 26, 33, 45). The cardioprotective effects of L-arginine and NO were ascribed to endothelial cell preservation, decreased neutrophil activation, improved coronary blood flow, and a reduction in free-radical-mediated injury. The majority of these studies employed isolated whole heart and/or open-chest models (26, 33, 45), and the relative contribution of individual cell types (i.e., endothelial cells, cardiomyocytes, neutrophils, and platelets) toward the beneficial effects of L-arginine could not be determined. The direct, cardioprotective effects of L-arginine on ventricular heart cells have not been previously examined.

We have developed a unique model of low-volume anoxia and reoxygenation in human ventricular heart cells. The quiescent nature of these myocytes exposed to low-volume anoxia simulates the low-flow and non-contractile conditions encountered during cardioplegic arrest. This model has been employed extensively to assess the effects of cardioplegic additives and myocardial preconditioning (8, 18, 19, 22, 30, 37, 41). Importantly, this model facilitates examination of pharmacological interventions independent of other cell types such as endothelial cells, neutrophils, and platelets.

In the present series of experiments, we hypothesized that L-arginine exerts beneficial effects in our human heart cell model of low-volume anoxia and reoxygenation. To this aim, we examined the effects of L-arginine on cell survival and NO production. In addition, we examined the potential mechanisms of L-arginine protective effects.

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MATERIALS AND METHODS

Experimental model of low-volume anoxia and reoxygenation. Our method of culturing human ventricular heart cells has been previously described (19, 22, 41). Briefly, 5–20 mg biopsies were obtained from the right ventricular outflow tract of patients (0.5–4 yr old) undergoing corrective surgery for tetralogy of Fallot, and ventricular myocytes were isolated by collagen digestion.

An in vitro technique to simulate low-volume anoxia and reoxygenation has been previously described in detail (41). Briefly, after a 30-min stabilization period in 10 ml of normoxic PBS including (in mmol/l) 0.49 MgCl₂, 0.68 CaCl₂, and 3.0 glucose and 150 mmHg of PO₂ at 37°C, the cells were exposed to a low volume (1.6 ml) of anoxic (0 mmHg of PO₂) PBS at 37°C for 90 min. During this period, the cells were placed in an airtight Plexiglas chamber and continuously flushed with 100% nitrogen to maintain anoxic conditions. Cells were then reoxygenated with 10 ml of normoxic PBS at 37°C for 30 min.

The minimum volume of anoxic perfusate was utilized (1.6 ml) to coat the cellular monolayer for preventing cellular dehydration during the anoxic period (19, 41). To verify anoxia, 2 ml of anoxic PBS was placed in a center dish within the sealed chamber and tested at the termination of each anoxic period to ensure a PO₂ of 0 mmHg. Previous studies have demonstrated that this model of low-volume anoxia produced biochemical effects similar to the effects of clinical low-volume anoxia and reoxygenation (8, 18, 19, 22, 30, 37, 41). Heart cells reverted to anaerobic metabolism, producing lactic acidosis, a fall in ATP, and cell injury associated with the release of creatine kinase and troponin I. Importantly, the extent of cellular injury was related to the duration of low-volume anoxia, with a 90-min period producing a profound acidosis and a 50% fall in ATP and a 50% incidence of cell death.

NO synthase enzyme activity. NO synthase (NOS) activities were assessed in the following groups: 1) normoxic: cultures were exposed to 10 ml/plate of normoxic PBS for 150 min; 2) anoxia: low-volume anoxia was carried out as described above, but the cells were harvested before the reoxygenation step; 3) anoxia-reoxygenation as described above, with the cells harvested at the end of the reoxygenation period.

NOS activity was measured by monitoring the conversion of L-[14C]arginine to L-[14C]citrulline (Stratagene). Cells were harvested in PBS + 1 mM EDTA and centrifuged at full speed for 2 min. The supernatant was removed by vacuum aspiration and the pellet was resuspended in homogenization buffer (250 mM Tris-HCl, 10 mM EDTA, and 10 mM EGTA). After centrifugation at full speed for 5 min, the supernatant (soluble fraction) was recovered and the pellet was suspended in homogenization buffer (membrane fraction). The extracts were adjusted to protein concentrations of 5–10 µg/ml.

Western blot analysis. Fifty milligrams of each cell extract were fractionated through a 4% stacking and 10% running SDS-PAGE gel and the fractionated proteins were transferred to a polyvinylidene difluoride membrane. Blots were blocked for 1 h at room temperature with blocking buffer [5% nonfat milk in 10 mM Tris (pH 7.5), 100 mM NaCl, and 0.1% Tween 20]. Two NOS primary antibodies were used: mouse antiendothelial NOS monoclonal IgG (Transduction Laboratories; Lexington, KY), diluted 1:2,500; and mouse anti-inducible NOS monoclonal IgG (Transduction Laboratories), dilution 1:10,000. Primary antibodies were reacted with the blots overnight at 4°C. After being washed (2× for 15 min in 1× Tween 20-Tris-base sodium), the blots were incubated with the secondary antibody (horseradish peroxidase-conjugated goat anti-mouse immunoglobulin antibody; Bio-Rad, Hercules, CA) at 1:3,000 dilution for 1 h at room temperature. Visualization was performed using enhanced chemiluminescence. Inducible NOS (NOS2) positive control was induced from mouse macrophage, whereas endothelial NOS (NOS3) positive control was extracted from human endothelial cells.

Immunohistochemistry. After stabilization, anoxia, or anoxia and reoxygenation, each plate of cells was fixed in absolute ethanol for 5 min followed by 4% paraformaldehyde for 10 min at room temperature. Endogenous peroxidase was inhibited by treatment with 0.3% H₂O₂ in 70% methanol and Triton X-100. Cells were then incubated with BSA. Reactions with NOS primary antibodies using mouse antiendothelial NOS monoclonal IgG (Transduction Laboratories) were performed at 1:1,500 dilution and mouse anti-inducible NOS monoclonal IgG (Transduction Laboratories) at 1:5,000 dilution with overnight incubation at 4°C. After incubation, the cells were treated with peroxidase-conjugated Fab’ fragments of goat anti-mouse IgG at 1:500 dilution, employing an avidin-biotin-peroxidase staining kit. The cells were incubated with diaminobenzidine, followed by counterstaining with hematoxylin.

Assessment of cellular injury. Nonconfluent cell cultures (~121,000 cells/plate) were used to assess cellular injury. At the end of the reoxygenation period (after 150-min incubation for the normoxic groups), plates were incubated with 0.4% Trypan blue dye and assessed for injury under an inverted light microscope at ×200 magnification. Injured cells were unable to exclude the large molecular weight dye and were stained blue. These blue-stained cells were counted and expressed as a percentage of the total cell number. A single blinded observer performed all counts.

Measurement of NO production by total nitrite and nitrate. NO production can be detected spectrophotometrically by measuring its final stable equimolar degradation products, nitrite and nitrate (3, 35). Total nitrite was quantified after the reduction of all nitrates with nitrate reductase (Boehringer Mannheim). After the conversion of nitrate to nitrite, total nitrite was determined spectrophotometrically at 540 µm by employing the Griess reaction (35). Cell plates were grown to near confluence (~415,000 cells/plate). Measurement of nitrite was performed in a total of 5.0 ml of Tris-buffered saline composed of (in mmol/l) 25 Tris, 138 NaCl, 0.49 MgCl₂, 0.68 CaCl₂, and 3.0 glucose (pH 7.4) to avoid phosphate interference with the assay. Extracellular fluid was collected after 30 min of stabilization and again after 30 min of reoxygenation. In the normoxic groups, extracellular fluid was collected after both 30 and 150 min of incubation. The extracellular fluid was then concentrated by freeze-drying and reconstituted in glass-distilled water.

Measurement of intracellular cGMP. Confluent cell cultures (~475,000 cells/plate) were used for the measurement of intracellular cGMP by enzyme immunoassay kit (model RPN 226, Amersham; Mississauga, Ontario, Canada). Immediately posttreatment, ice-cold ethanol (65%) was added to the plates. Cells were scraped and then centrifuged at 2,000 rpm for 15 min at 4°C. Supernatant was transferred to fresh tubes and freeze-dried. The extracts were dissolved in 100 µl of the manufacturer’s assay buffer before analysis. Extracted intracellular cGMP was assayed by the enzyme immunoassay kit. The cross-reactivity of this assay kit was <0.00008 for cAMP and <0.000004 for GMP, where the reactivity was 100 for cGMP.
L-Arginine treatment. Experimental protocols are shown in Fig. 1. The following groups were studied: 1) normoxic control, 2) normoxic treatment (cells were exposed to 5.0 mmol/l L-arginine in 10 ml of normoxic PBS (50 mmol/l Tris, pH 7.6/0.2) for 150 min; 3) low-volume anoxia and reoxygenation control where cells were stabilized in normoxic PBS for 30 min, followed by 90 min of low-volume anoxia in anoxic PBS, and then 30 min of reoxygenation in normoxic PBS. D: reoxygenation treatment groups were subjected to stabilization and low-volume anoxia, followed by reoxygenation in PBS with either L-arginine, L-NAME, combination of L-arginine and L-NAME, or the nitric oxide (NO) synthase (NOS)-independent NO donor S-nitroso-N-acetyl-penicillamine (SNAP). E: preanoxic treatment groups were stabilized in PBS with L-Arg, followed by simulated low-volume anoxia and reoxygenation. n = 8 plates/group.

NOS antagonist treatment. Nω-nitro-L-arginine methyl ester (L-NAME), a competitive inhibitor of NOS, was added during reoxygenation to assess its effect on heart cells. The effect of exogenous NO on heart cells after low-volume anoxia and reoxygenation was studied by employing S-nitroso-N-acetyl-penicillamine (SNAP), which produces NO independently of NOS activity. Treatment protocols are shown in Fig. 1. The following groups were studied: 1) normoxic control; 2) normoxic with SNAP (50 μmol/l) or L-NAME (100 μmol/l) treatment; 3) low-volume anoxia and reoxygenation control; 4) low-volume anoxia and reoxygenation with L-arginine (3.0 mmol/l), SNAP (50 μmol/l), L-NAME (100 μmol/l), or the combination of L-NAME and L-arginine treatment during reoxygenation. Nonconfluent plates were used, and Trypan blue staining (0.4%) was employed to assess cellular injury. Confluent plates were used, and NO production was measured in extracellular fluid collected at the end of each of the stabilization and reoxygenation steps (at 30 and 150 min for the normoxic controls). NO production was not measured for SNAP treatment groups; it is well established that SNAP produces NO independently of cellular metabolism (6, 12, 14, 29, 36).

Inhibition of guanylate cyclase. Effects of guanylate cyclase inhibition with methylene blue on L-arginine-induced cardioprotection were then examined using the experimental protocol shown in Fig. 2. The following groups were studied:

Fig. 2. Experimental design. Sham groups were incubated in normoxic PBS. Normoxic treatment groups were incubated with methylene blue. Low-volume anoxia-reoxygenation control groups were stabilized for 30 min in PBS, exposed to 90 min of low-volume anoxia in anoxic PBS, and then 30 min of reoxygenation in normoxic PBS. D: reoxygenation treatment groups were subjected to stabilization and low-volume anoxia, followed by reoxygenation in PBS with either L-arginine, L-NAME, combination of L-arginine and L-NAME, or the nitric oxide (NO) synthase (NOS)-independent NO donor S-nitroso-N-acetyl-penicillamine (SNAP). E: preanoxic treatment groups were stabilized in PBS with L-Arg, followed by simulated low-volume anoxia and reoxygenation.

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Fig. 3. Specific activities of NOS2 and NOS3 in cultured human heart cells in response to low-volume anoxia and reoxygenation. Low levels of Ca\(^{2+}\)-insensitive (NOS2) and Ca\(^{2+}\)-sensitive (NOS3) activity were detected in both soluble and membrane fractions in the normoxic controls. Soluble NOS2 activity increased dramatically during low-volume anoxia, decreased during reoxygenation, but remained significantly elevated compared with the normoxic control. NOS3 activity remained low during low-volume anoxia but increased during reoxygenation in both soluble and membrane fractions. \(n = 6\) plates/group. *\(P < 0.01\) vs. control; +\(P < 0.01\) vs. low-volume anoxia.

1) heart cells were incubated in normoxic PBS for 150 min (sham); 2) heart cells were stabilized for 30 min in PBS and exposed to 90 min of anoxia and 30 min of reoxygenation (low-volume anoxia and reoxygenation control); 3) heart cells were incubated in normoxic PBS with methylene blue (50 mmol/l) for 150 min; and 4) heart cells were exposed to preanoxic treatment with L-arginine (3.0 mmol/l), the combination of L-arginine and methylene blue, SNAP (50 mmol/l), the combination of SNAP and methylene blue, or methylene blue alone in PBS for 30 min followed by 90 min of anoxia and 30 min of reoxygenation. Cellular injury was assessed immediately after each experiment.

Stimulation of cGMP. To determine whether the effects of L-arginine could be mimicked by stimulation of cGMP, we examined the effects of 8-bromo-cGMP (8-BrcGMP), a membrane-permeable analog of cGMP. The effect of cGMP on heart cells after anoxia and reoxygenation was studied employing 1, 10, and 100 \(\mu\)mol/l 8-BrcGMP. The following groups were studied: 1) sham (see Fig. 2); 2) normoxic 8-BrcGMP treatment (100 \(\mu\)mol/l); 3) 8-BrcGMP (1, 10, and 100 \(\mu\)mol/l) preanoxic treatment; and 4) low-volume anoxia and reoxygenation control. Cellular injury was assessed by Trypan blue staining as described above.

Inhibition of ATP-sensitive K\(^+\) channels. We examined the hypothesis that one effector of L-arginine cardioprotection involves opening of ATP-sensitive K\(^+\) (K\(_{ATP}\)) channels. We examined the effects of L-arginine with and without glibenclamide, an inhibitor of K\(_{ATP}\) channels. The following groups were studied: 1) sham (Fig. 2); 2) normoxic glibenclamide treatment (20 \(\mu\)mol/l); 3) preanoxic treatment with L-arginine (3.0 mmol/l), the combination of L-arginine and glibenclamide, 8-BrcGMP (10 \(\mu\)mol/l), the combination of 8-BrcGMP and glibenclamide, and glibenclamide alone; and 4) low-volume anoxia and reoxygenation control. Cellular injury was assessed by Trypan blue staining as described above.

Statistical analysis. The StatView program (Abacus Concept; Berkeley, CA) was employed for statistical analysis. Results are expressed as means ± SE. ANOVA was employed to identify significant differences between control and treatment groups. Statistical differences were specified using Scheffe’s test. Two-way ANOVA was used to determine the presence of any interactions among preanoxic, anoxic, and reoxygenation groups, with respect to both cellular injury and nitrite concentrations. A \(P\) value of <0.05 was considered to be statistically significant.

RESULTS

NOS enzyme activity. Ca\(^{2+}\)-insensitive (NOS2) and Ca\(^{2+}\)-sensitive (NOS3) activities are displayed in Fig. 3 as the specific activity of the conversion of L-[\(^{14}\)C]arginine to L-[\(^{14}\)C]citrulline. NOS2 activity during stabilization was primarily in the soluble fraction. Activity of NOS2 increased dramatically in the soluble fraction after anoxia. Whereas soluble NOS2 activity then declined during reoxygenation, it remained significantly elevated compared with stabilization. In the membrane fraction, NOS2 activity was low and did not show a significant change after low-volume anoxia or reoxygenation.

NOS3 activity in the stabilization group was observed mainly in the membrane fraction. During anoxia, NOS3 activity in the membrane fraction decreased and remained low in the soluble fraction. After reoxygenation, NOS3 activity increased significantly in both soluble and membrane fractions.

Western blot analysis. Levels of both NOS2 and NOS3 proteins in soluble and membrane fractions derived from cultured human heart cells are shown in Fig. 4. NOS2 and NOS3 were detected as bands of 130 and 140 kDa, respectively. NOS2 was not detected in the membrane fraction at any stage. After anoxia and reoxygenation, NOS2 was detected strongly in the soluble fraction. During stabilization, NOS3 protein was detected at a moderate level in the membrane fraction but not in the soluble fraction. After reoxygenation, NOS3 was detected strongly in both soluble and membrane fractions.

Non-Anoxic Anoxia Reoxygenation Controls

Fig. 4. Western blot analysis of NOS2 and NOS3 protein in cultured human heart cells in response to low-volume anoxia and reoxygenation. NOS1 isoform-specific Western blots were performed on soluble (S) and membrane (M) fractions. NOS2 protein (130 kDa) was observed only in the soluble fraction, increasing dramatically during low-volume anoxia and dropping during reoxygenation but remained elevated compared with the normoxic control. NOS3 protein (140 kDa) was observed largely in the membrane fraction, except during reoxygenation when levels increased in both membrane and soluble fractions.
Immunohistochemistry. Figure 5 shows the results of immunohistochemical staining for NOS2 and NOS3 proteins. No immunoreactive products of NOS2 were observed during stabilization, but the cytoplasm, perinuclear, and nuclear regions of the heart cells stained strongly during both low-volume anoxia and reoxygenation. NOS3 immunoreactivity was seen at the plasma membrane during stabilization, low-volume anoxia and reoxygenation. After reoxygenation, the cytoplasm also stained strongly for NOS3.

**L-Arginine treatment.** Figure 6 demonstrates a comparison between cellular injury in preanoxic and reoxygenation L-arginine treatment groups. L-Arginine had no effect on the normoxic group compared with normoxic controls. Preanoxic treatment with L-arginine at doses ranging from 0.1 to 5.0 mmol/l afforded protection in a dose-dependent manner \((P < 0.01)\). L-arginine treatment during reoxygenation also protected the heart cells from low-volume anoxia-reoxygenation injury \((P < 0.01)\). The 1.0 and 3.0 mmol/l doses of L-arginine applied during reoxygenation afforded greater protection than the same doses applied in the preanoxic stabilization period \((P < 0.05)\). Preanoxic treatment provided increasing protective effect with increasing doses in the range studied (up to 5.0 mmol/l).

Figure 7 depicts the percent change in NO production as measured by supernatant nitrite concentrations after L-arginine (3.0 mmol/l) treatment. Changes in NO production in the normoxic control, normoxic L-arginine, and preanoxic L-arginine, and preanoxic L-arginine treatment groups were not significant. Low-volume anoxia-reoxygenation had revealed a decrease in NO production compared with controls \((P < 0.05)\), whereas reoxygenation treatment with L-arginine had an increase in NO production compared with low-volume anoxia-reoxygenation controls \((P < 0.01)\).

**NOS antagonist treatment.** Figure 8 demonstrates cellular injury after treatment with SNAP (50 μmol/l) or L-NAME (100 μmol/l) during reoxygenation with or without simultaneous L-arginine (3.0 mmol/l) reoxy-
Normoxic treatment with SNAP or L-NAME had no effect on cellular injury. The addition of SNAP during reoxygenation protected heart cells from anoxia and reoxygenation injury ($P < 0.05$), but the protection was less than that afforded by the optimum doses of L-arginine administered during reoxygenation. L-NAME treatment during reoxygenation led to a significant decrease in NO production compared with the low-volume anoxia-reoxygenation control ($P < 0.05$). Reoxygenation L-NAME treatment had no significant effect on NO production compared with the low-volume anoxia-reoxygenation controls. L-arginine reoxygenation treatment significantly increased NO production ($P < 0.05$). Reoxygenation treatment with the combination of L-NAME and L-arginine blocked the increase in NO production seen with L-arginine reoxygenation treatment ($P < 0.05$), and the combination treatment group was not significantly different from the low-volume anoxia-reoxygenation control.

**Methylene blue treatment with L-arginine and SNAP.** Figure 10 shows the cellular injury after preanoxic treatment of cultured heart cells with methylene blue and L-arginine or SNAP. Normoxic treatment with methylene blue had no effect on cellular injury compared with sham. Both L-arginine and SNAP treatment groups demonstrated a significant decrease in cellular injury compared with the low-volume anoxia-reoxygenation control ($P < 0.05$). The methylene blue treatment group displayed greater cellular injury than the low-volume anoxia-reoxygenation control ($P < 0.05$). Also, methylene blue abolished the protective effect of both L-arginine and SNAP ($P < 0.05$). The combination of methylene blue and SNAP treatment resulted in the same level of cellular injury as the untreated low-volume anoxia-reoxygenation control, but the combination of methylene blue and L-arginine treatment caused greater cellular injury than the control ($P < 0.05$).

**Intracellular cGMP with L-arginine, SNAP, methylene blue and L-NAME.** Figure 11 illustrates intracellular cGMP levels after diverse pharmacological interventions. The PBS control groups showed no change compared with baseline groups. Both L-arginine and
SNAP treatments led to increases in cGMP levels relative to the PBS control group ($P < 0.01$). Treatment with either methylene blue or L-NAME alone decreased cGMP levels compared with PBS control ($P < 0.05$). The combination of methylene blue with either L-arginine or SNAP abolished increases in cGMP ($P < 0.01$). Both combination treatments resulted in cGMP levels similar to the PBS control. It is important to note that methylene blue is not as specific as 1H-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one for guanylate cyclase inhibition.

8-BrcGMP treatment. Figure 12 demonstrates cellular injury after preanoxic treatment of cultured heart cells with various doses of 8-BrcGMP. Normoxic treatment with 8-BrcGMP had no effect on cellular injury compared with sham. In the preanoxic treatment groups, 8-BrcGMP at all doses tested led to a decrease in cellular injury compared with the low-volume anoxia-reoxygenation control ($P < 0.01$). 8-BrcGMP-mediated protection was less than that exhibited by L-arginine or SNAP treatment (see Fig. 10).

Glibenclamide treatment with L-arginine and cGMP. Figure 13 demonstrates cellular injury after preanoxic treatment of cells with glibenclamide, both alone and combined with L-arginine or 8-BrcGMP. Normoxic treatment with glibenclamide had no effect on cellular injury compared with sham. Preanoxic glibenclamide treatment alone led to greater cellular injury than the low-volume anoxia-reoxygenation control ($P < 0.05$). Glibenclamide also abolished the protective effects of both L-arginine and 8-BrcGMP ($P < 0.01$).

**DISCUSSION**

**Primary observations.** The present study tested the hypothesis that L-arginine exerts cardioprotective effects in heart cells independent of other cell types such as endothelial cells, platelets, or leukocytes. Using a well-established human ventricular heart cell model of low-volume anoxia-reoxygenation and a variety of pharmacological interventions, we demonstrated 1) L-
arginine exerts dose-dependent cardioprotective effects after low-volume anoxia-reoxygenation and treatment during reoxygenation is more effective than pretreatment at the same dose; these effects are mediated by an increase in NO production; and the final effector of L-arginine cardioprotection involves a glibenclamide-sensitive, NO-cGMP-dependent pathway. In addition, we characterize NOS2 and NOS3 and their subcellular distribution in human heart cells in low-volume anoxia and reoxygenation. These data are novel as they describe direct cardioprotective effects of L-arginine in isolated human heart cells in the absence of alternate cell types.

Characterization of NOS isoforms in human heart cells after low-volume anoxia and reoxygenation. With respect to NOS1 isoform characterization, we found a rise in soluble Ca\(^{2+}\)-insensitive NOS activity (NOS2) and NOS2 protein levels during low-volume anoxia. During reoxygenation, NOS2 activity and protein levels fell, but remained significantly elevated compared with preanoxia. It has been demonstrated that inflammatory cytokines can induce NOS2 expression in ventricular myocytes (42). Whereas some evidence that NOS2 can be induced by low-volume anoxia has been presented (5), those studies were performed in vivo, and it is unclear whether the NOS2 induction in the cardiomyocytes was secondary to cytokine release by other cell types such as leukocytes or endothelial cells.

Our results show for the first time that simulated low-volume anoxia-induced NOS2 production in isolated heart cells.

Ca\(^{2+}\)-sensitive NOS activity (NOS3) also increased in our experiments, but only during the reoxygenation period. Whereas both NOS1 (neuronal NOS) and previously defined as endothelial NOS3 (constitutive NOS) are Ca\(^{2+}\)-sensitive, NOS1 has been detected only in the neurons of human myocardium. In contrast, NOS3 has long been known to be present in cardiomyocytes. Thus NOS1 was unlikely to contribute significantly to Ca\(^{2+}\)-sensitive NOS activity in our isolated heart cell model, which lacks both neurons and endothelial cells; the activity was attributed to NOS3. Increase in activity was concurrent with an apparent translocation of NOS3 protein from membrane to the cytosol (31, 32). In endothelial cells, NOS3 is known to be phosphorylated in response to agonists and is also subject to depalmitoylation with concurrent translocation to cytosol. Phosphorylation of NOS3 may contribute to its regulation by controlling the translocation of the enzyme (25). Rise in Ca\(^{2+}\)-sensitive NOS activity during reoxygenation may be due to phosphorylation of NOS3, facilitating its activation and transfer to the cytosol. Intracellular translocation of NOS3 in heart cells may be an important mediator of the biological effects of NO during reoxygenation. The presence of active NOS enzymes in our isolated heart cell low-volume anoxia-reoxygenation model suggested that it...
was possible for NO production from L-arginine to occur, despite the absence of other cell types.

**Effects of L-arginine on cellular injury and NO production after low-volume anoxia and reoxygenation.** The data from our L-arginine treatment experiments demonstrate that L-arginine conferred a direct and dose-dependent protective effect in isolated heart cells. L-arginine treatment also reversed the decrease in NO production noted in the low-volume anoxia-reoxygenation control group. The administration of L-arginine during reoxygenation provided greater protection and produced more NO compared with the preanoxic treatment of L-arginine. Reoxygenation treatment may be more effective because of heightened activities of both NOS2 and NOS3 during reoxygenation. Beneficial effects of L-arginine were found over a narrow range of doses and higher doses were not beneficial. The narrow dose-response relation may limit the clinical usefulness of L-arginine and increase the need to understand the mechanism of benefit afforded to human ventricular heart cells.

To determine whether the beneficial effects of L-arginine were mediated by an increase in NO production, we examined the effects of L-arginine in the presence and absence of L-NAME (a specific NOS1 inhibitor) and SNAP (an exogenous NOS-independent NO donor) on cellular injury. Notably, L-NAME blocked L-arginine-mediated NO production and abolished the protective effect of L-arginine, whereas the NO donor SNAP mimicked the protective effect of L-arginine. These data support our hypothesis that L-arginine exerts its protective effects by NOS-mediated NO production in human heart cells subjected to low-volume anoxia and reoxygenation.

**The effector of L-arginine cardioprotection involves cGMP-mediated KATP channel opening.** After our initial studies confirming that L-arginine protects human heart cells via production of NO, we hypothesized that the final effector involves NO-mediated stimulation of cGMP and the resultant opening of KATP channels (see Fig. 14). Recent studies from Ockaili et al. (27) have shown the role of KATP channels in cardioprotection. We therefore examined the inhibition of guanylate cyclase (with methylene blue), stimulation of cGMP (8-BrcGMP) and antagonism of KATP channels (with glibenclamide) on cellular injury and the responses of L-arginine. Activation of cGMP may afford cardioprotection through several possible mechanisms including inhibition of Ca²⁺ influx (10, 39, 40), reduction in myocardial O₂ consumption (43), decrease in lactate accumulation within hypoxic cardiomyocytes (23), and/or the activation of K⁺ channels (4). cGMP has...
recently been shown to be an endogenous intracellular cardioprotectant against reoxygenation-induced arrhythmia (28). Also, cGMP may activate K+ channels and increase whole cell potassium currents in smooth muscle cells (4).

We demonstrated that inhibition of guanylate cyclase with methylene blue blunted cGMP accumulation and abolished the protection from low-volume anoxia-reoxygenation injury conferred by either L-arginine or SNAP. Furthermore, glibenclamide inhibited the cardioprotective effects of L-arginine. These results suggest that cGMP-regulated K_ATP channels are possibly important in L-arginine-mediated cardioprotection. Opening K_ATP channels during reoxygenation after low-volume anoxia may facilitate the recovery of aerobic mitochondrial ATP production (8, 18, 30). Mitochondrial K_ATP channel opening may improve electron transport (8). L-arginine may protect heart cells by stimulating many of the protective processes involved in preconditioning.

Study limitations. Cardioprotective effects of 8-BrcGMP, a membrane-permeable cGMP analog, appeared to be less than either L-arginine or SNAP. Therefore, NO may contribute to myocardial protection via both cGMP-dependent and -independent mechanisms. Also notable are the high concentrations of glibenclamide used in the study, which may also inhibit Ca2+-activated channels and render the results regarding K_ATP channels inconclusive.

In summary, many studies have demonstrated the cardioprotective effects of L-arginine in different animal models. (1, 7, 13, 17, 26, 27, 33, 34, 44) Initial studies found that NO generated from L-arginine reduced neutrophil adherence to endothelial cells (26, 33, 45). Subsequent studies revealed that L-arginine improved endothelial cell function, and the resulting improvements in coronary reflow were associated with enhanced cardiac recovery (11). Although cardiomyocytes have been shown to express constitutive Ca2+-sensitive NOS (9, 20), and Ca2+-insensitive NOS2 expression has been induced in vivo after low-volume anoxia-reoxygenation (5), the physiological role of NOS1 in human cardiomyocytes remains unknown. The direct effect of L-arginine-derived NO on cardiomyocytes, in the absence of other cell types, has not been reported. In the present study, we provide evidence that human heart cells express NOS and that heightened levels of active NOS1 isoforms in heart cells during low-volume anoxia and reoxygenation facilitate the conversion of administered L-arginine to NO, which in turn provided dose-dependent protection against cellular injury. In addition, our findings suggested that the cardioprotective mechanisms of L-arginine include the activation of guanylate cyclase that led to increased cGMP levels in human heart cells. The final effector of NO/cGMP-mediated protection may be the opening of K_ATP channels (27). Developing strategies and pharmacological targets to limit perioperative low-volume anoxia-reoxygenation injury may improve the outcomes of cardiac surgery in high-risk populations. An important pharmacological target may be tetrahydrobipterin, an essential cofactor for NOS production. Tetrahydrobipterin is seen as the gatekeeper facilitating NOS-mediated NO production versus superoxide production. Recent data from our laboratory have demonstrated that tetrahydrobipterin restores functional recovery after global low-volume anoxia and reoxygenation (S. Verma and R. D. Weisel, unpublished observations).

This work was supported by Heart and Stroke Foundation of Ontario Grant-In-Aid B4177 (to R. D. Weisel) and R-K. Li and the Career Investigator at the Heart and Stroke Foundation of Ontario. P. W. M. Pedak and S. Verma are Fellows of the Heart and Stroke Foundation of Canada and Medical Research Council of Canada.

REFERENCES