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Reduced L-Arginine Transport Contributes to the Pathogenesis of Myocardial Ischemia-Reperfusion Injury

Kylie M. Venardos,* Amanda J. Zatta, Tanneale Marshall, Rebecca Ritchie, and David M. Kaye

Heart Failure Research Group, Baker IDI Heart & Diabetes Institute, Melbourne, Australia

ABSTRACT

Myocardial injury due to ischemia-reperfusion (I-R) damage remains a major clinical challenge. Its pathogenesis is complex including endothelial dysfunction and heightened oxidative stress although the key driving mechanism remains uncertain. In this study we tested the hypothesis that the I-R process induces a state of insufficient L-arginine availability for NO biosynthesis, and that this is pivotal in the development of myocardial I-R damage. In neonatal rat ventricular cardiomyocytes (NVCM), hypoxia-reoxygenation significantly decreased L-arginine uptake and NO production ($42 \pm 2\%$ and $71 \pm 4\%$, respectively, both $P < 0.01$), maximal after 2 h reoxygenation. In parallel, mitochondrial membrane potential significantly decreased and ROS production increased (both $P < 0.01$). NVCMs infected with adenovirus expressing the L-arginine transporter, CAT1, and NVCMs supplemented with L-arginine both exhibited significant (all $P < 0.05$) improvements in NO generation and mitochondrial membrane potentials, with a concomitant significant fall in ROS production and lactate dehydrogenase release during hypoxia-reoxygenation. In contrast, L-arginine deprived NVCM had significantly worsened responses to hypoxia-reoxygenation. In isolated perfused mouse hearts, L-arginine infusion during reperfusion significantly improved left ventricular function after I-R. These improved contractile responses were not dependent on coronary flow but were associated with a significant decrease in nitrotyrosine formation and increases in phosphorylation of both Akt and troponin I. Collectively, these data strongly implicate reduced L-arginine availability as a key factor in the pathogenesis of I-R injury. Increasing L-arginine availability via increased CAT1 expression or by supplementation improves myocardial responses to I-R. Restoration of L-arginine availability may therefore be a valuable strategy to ameliorate I-R injury. J. Cell. Biochem. 108: 156–168, 2009.

KEY WORDS: L-ARGININE; MYOCARDIAL ISCHEMIA-REPERFUSION; OXIDATIVE STRESS; NITRIC OXIDE

Early myocardial reperfusion is without doubt the most effective approach in the management of myocardial ischemia. However, this process is not without risk given the widely recognized phenomenon of reperfusion injury during the restoration of blood flow. This form of cellular damage and dysfunction may contribute to major clinical sequelae including arrhythmia and myocardial damage [Forman et al., 1990; Ambrosio and Chiariello, 1991; Ferrari et al., 1998b]. As such, the existence of reperfusion injury has substantial clinical implications in the recovery of cardiac function to pre-ischemic levels, particularly in the setting of acute coronary syndromes, coronary bypass surgery, and heart transplantation.

The pathogenesis of the injury of ischemic-reperfused myocardium is complex, involving injury of endothelial cells and vascular smooth muscle as well as cardiomyocytes. While the pathogenesis of ischemia-reperfusion (I-R) is not completely understood, there is considerable evidence to implicate reactive oxygen species (ROS) and endothelial dysfunction as initial causes of the injury. Within the myocardium and associated vasculature ROS, can be produced via a number of pathways particularly by NADPH oxidases, which are expressed in various cell types and mitochondria (primarily via the electron transport chain) [Ferrari et al., 1998a; Cadenas and Davies, 2000; Sawyer et al., 2002; Venardos and Kaye, 2007]. By their nature, ROS can attack virtually all cellular targets non-specifically and can initiate lipid peroxidation, oxidize proteins/enzymes to inactive states and cause DNA strand breaks, all potentially damaging to normal cellular function. ROS generation during the early stages of reperfusion are also believed to be involved in contractile dysfunction, arrhythmias, ventricular fibrillation, tachycardia, and premature beating, all of which are

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*Correspondence to: Dr. Kylie M. Venardos, Heart Failure Research Group, Baker IDI Heart & Diabetes Institute, Melbourne, VIC 3004, Australia. E-mail: kylie.venardos@bakeridi.edu.au

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associated with reperfusion and cardiomyocyte death [Hearse and Tosaki, 1987; Kloner et al., 1989; Venardos and Kaye, 2007].

Oxidative stress also exerts important adverse influences over the nitric oxide (NO) system, potentially inactivating NO directly, reducing nitric oxide synthase (NOS) activity, and attenuating agonist-stimulated NO release [Gryglewski et al., 1986; Giraldez et al., 1997; Rezkalla and Kloner, 2002]. A reduction in myocardial NO bioavailability is important by virtue of the important roles it plays in regulating vascular tone and myocardial function, particularly in regard to diastolic function and energetic control [Shah and Lewis, 1992; Grocott-Mason et al., 1994; Shah et al., 1996; Loke et al., 2000]. Whilst NOS is classically considered as an enzyme principally concerned with the generation of NO, it is well recognized that under conditions of substrate (L-arginine) limitation production of superoxide may result. NO and superoxide can also react to form peroxynitrite, a potent reactive nitrogen species which has been shown to attack tyrosine residues in proteins (leading to the formation of 3-nitrotyrosine), thiol groups, and membrane phospholipids resulting in nitrosative stress [Xia et al., 1996; Vasquez-Vivar et al., 1998; Venardos and Kaye, 2007]. In this setting, work from our laboratory has identified the presence of depressed vascular and myocardial L-arginine transport in a range of cardiovascular disease states and in the context of oxidant stress [Kaye et al., 2000; Schlaich et al., 2004; Zhang et al., 2006; Yang et al., 2007], providing a mechanistic explanation for depressed NO bioavailability in conjunction with an increase in oxidative stress in these cardiovascular paradigms. Several groups have previously reported improved post-ischemic myocardial function and smaller infarct sizes in conjunction with enhanced endothelial function following the infusion of L-arginine during reperfusion [Nakanishi et al., 1992; Weyrich et al., 1992], however, the direct effect on cardiomyocytes and the mechanisms involved are yet to be elucidated. In the current study we aimed to test the hypothesis that impaired L-arginine transport plays a pivotal role in the development of myocardial I-R injury, and that its manipulation may provide beneficial effects on myocardial recovery. In addition, we investigated mechanisms involved with L-arginine-induced cardioprotection and the role of the CAT1 transporter in I-R injury.

MATERIALS AND METHODS

The research project was approved by the Alfred Medical Research and Education Precinct Animal Ethics Committee. The study also conforms with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

CARDIOMYOCYTE ISOLATION AND CULTURE

Neonatal ventricular cardiomyocytes (NVCMs) were isolated from 1- to 2-day-old Sprague–Dawley rat pups by enzymatic digestion as previously described [Laskowski et al., 2006]. Cells were then cultured in DMEM containing 10% FBS with antibiotic and antimycotic, then serum deprived for 24 h prior to experiments.

HYPOXIA-REOXYGENATION OF CARDIOMYOCYTES

NVCMs plated in DMEM were subjected to 3 h hypoxia in a humidified 95% N_2-5% CO_2 flushed hypoxic chamber incubated at 37°C, then reoxygenated for up to 2 h at 37°C in 5% CO_2. Normoxic control cells were incubated at 37°C in 5% CO_2 only. Cells were either untreated; supplemented with 1 mmol/L L-arginine (L-arginine–HCl, Sigma, St Louis, MO); transfected with AdCAT1-EGFP or AdEGFP as described below; or L-arginine deprived using L-arginine free media. Additional groups were also pre-treated with the NOS inhibitor L-NAME (1 mmol/L, nitro-L-arginine methyl ester, Sigma) for 2 h prior to and during hypoxia-reoxygenation. DMEM cell culture media used in these studies contains 400 μM L-arginine. NO and ROS production, mitochondrial membrane potential, and lactate dehydrogenase (LDH) release were all measured in these cells, as described below. A minimum of three replicates from four individual experiments were used for each parameter.

ADCAT1-EGFP VIRAL TRANSFECTION OF CARDIOMYOCYTES

EGFP and mouse CAT1 genes were fused and subcloned into the shuttle vector pShuttle-CMV using *Hin*dIII and *Xba*I to yield pShuttle-CAT1-EGFP. Recombinant adenovirus was generated by bacterial homologous recombination between pShuttle-CAT1-EGFP and pAdEasy1. Large scale amplification of AdCAT1-EGFP (serotype 5) was performed in the cell line HEK293, and subsequent adenovirus used to infect NVCMs. NVCMs were transfected with AdCAT1-EGFP in media for 48 h at 37°C in 5% CO_2 to ensure maximal CAT1-EGFP expression. The adenovirus was then removed, cells washed three times with PBS and fresh media added to dishes. Control cells were also transfected with Ad-EGFP (serotype 5) to control for any viral effects.

L-ARGININE UPTAKE IN CARDIOMYOCYTES

L-Arginine uptake was measured during hypoxia-reoxygenation, post-AdCAT1-EGFP infection and in L-NAME treated cells by measuring ^{3}H-L-arginine accumulation in cells. Cells were washed twice with warm PBS, and L-arginine uptake was measured using radiolabeled ^{3}H-L-arginine (Perkin Elmer Life Sciences, Boston, MA). Total uptake solution containing 100 nmol/L ^{3}H-L-arginine and 100 μmol/L unlabeled L-arginine was prepared in Lockes Buffer consisting of 154 mmol/L NaCl, 5.6 mmol/L KCl, 2 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, 10 mmol/L HEPES, 3.6 mmol/L $NaHCO_3$, and 5.6 mmol/L glucose, pH 7.4. To measure non-specific uptake, a second uptake solution containing an additional 10 mmol/L unlabeled L-arginine was made from the total uptake solution. Total and non-specific uptakes were measured simultaneously by adding 300 μl of the relevant uptake solution to each well and incubating cells for 20 min at 37°C. Uptake was terminated by washing cells 3× with ice-cold PBS. Cells were then lysed with 500 μl 0.2% SDS in 0.2 mol/L NaOH for 60 min at room temperature. Measurement of radioactivity of cell lysates was made using liquid scintillation spectrometry. The remaining cell lysate was used to determine protein content using the Bio-Rad D_C Protein Assay Kit as per the manufacturers instructions. Specific L-arginine uptake was calculated as the difference between total uptake and non-specific uptake, following protein standardization. Data are presented as DPM/mg protein.

MEASUREMENT OF NO AND ROS PRODUCTION, AND MITOCHONDRIAL MEMBRANE POTENTIAL IN ISOLATED CARDIOMYOCYTES DURING HYPOXIA–REOXYGENATION

NO production was measured using 5 μmol/L 4-amino-5-methylamino-2′,7′-dichlorofluorescein diacetate (DAF-FM, Molecular Probes, Eugene, OR), and ROS production was measured with 10 μmol/L 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA, Molecular Probes) both as previously described [Zhang et al., 2006; Yang et al., 2007]. Mitochondrial membrane potential was measured using the fluorescent probe 5,5′,6,6′,-tetrachloro-1,1,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1, Molecular Probes) according to the manufacturers recommendations. Inhibition of the electron transport chain promotes membrane depolarization for which JC-1 specifically detects via a decrease in red/green fluorescence intensity ratio of the probe. Data are presented in raw fluorescent units (RFU). A minimum of five replicates from four individual experiments were measured for each of these parameters.

LANGENDORFF PERFUSION OF ISOLATED MOUSE HEARTS

To establish the role of L-arginine supply directly on the whole heart during I-R, we employed the Langendorff isolated mouse heart model and subjected hearts to I-R ± L-arginine infusion, as described below.

Ten- to 12-week-old wild-type C57B/L mice were anaesthetized with 325 mg/ml sodium pentobarbitone (0.5 ml/kg) (Lethabarb, Virbac, Peakhurst, Australia) via an intraperitoneal injection. A thoracotomy was performed, hearts rapidly removed and immersed in ice-cold perfusion fluid. The aorta was cannulated and hearts perfused on a Langendorff perfusion apparatus as previously described [Headrick et al., 2001]. A fluid-filled balloon constructed of latex was inserted into the left ventricle via the mitral valve for measurement of isovolumic function. Functional parameters measured include peak systolic left ventricular pressure (PSVP), left ventricular developed pressure (LVDP) and its derivatives ±dP/dt, end diastolic pressure (EDP) and heart rate (HR). Coronary flow rate was monitored via a Doppler flow probe (2N probe; Transonic Systems, Inc., Ithaca, NY) in the aortic perfusion line, connected to a T206 Transonic flowmeter. Hearts were perfused for an initial 30 min normoxic period to allow them to stabilize. Coronary flow was then stopped to generate global ischemia. Ischemia was maintained for 22.5 min (a period shown in pilot studies to result in ~50% recovery of contractile function) and hearts were maintained at 37°C in the temperature controlled organ chamber. Flow was then returned to the heart and reperfusion continued for 45 min. L-Arginine (1 mmol/L) was also infused into hearts upon reperfusion and maintained for the duration of reperfusion (n = 8–10/group). At the end of perfusion studies, hearts were removed from the cannula, snap frozen in liquid nitrogen, and stored at −80°C until subsequent biochemical analysis.

MEASUREMENT OF 3-NITROTYROSINE AS A MARKER OF OXIDATIVE DAMAGE AND PEROXYNITRITE FORMATION

The nitration of tyrosine residues in proteins by peroxynitrite or other nitrating agents is a widely used marker of oxidative damage and is frequently reported in many human and animal diseases that involve oxidative stress. 3-Nitrotyrosine was measured as a marker of oxidative/nitrosative injury and as an indicator of peroxynitrite formation in post-ischemic hearts homogenates using a OxiSelect Nitrotyrosine competitive ELISA kit (Cell Biolabs, Inc., San Diego, CA) as per the manufacturers instructions. Equal amounts of protein were assayed for each sample (120 μg), and nitrated BSA standards (0–8,000 nM nitrotyrosine) were assayed alongside the unknown samples allowing for the construction of a standard curve. Data are presented as nM 3-nitrotyrosine.

MEASUREMENT OF LACTATE DEHYDROGENASE AS A MARKER OF CELLULAR NECROSIS

LDH was measured in media collected from NVCMs during hypoxia-reoxygenation and in effluent from Langendorff perfused hearts during equilibration and reperfusion as a marker of necrosis or cell death. Activity was assessed spectrophotometrically by measuring the rate of decrease in absorbance at 340 nm over 2 min. One hundred microliters of 1 mmol/L NADH was added to 800 μl of assay mix containing 125 mmol/L NaH_2PO_4, pH 7.5, and 1.25 mmol/L sodium pyruvate. Two hundred microliters of sample was then added to this mix to start the reaction. The LDH present in the samples reduces pyruvate to lactate using NADH as the electron donor. In the process NADH is oxidized to NAD^+ and no longer absorbs light. LDH standards, ranging from 0 to 1 U/ml, were assayed alongside the unknown samples allowing for the construction of a standard curve. LDH activity is expressed as U/ml.

PAKT, PTROPONIN I, PPHOSPHOLAMBAN AND SERCA PROTEIN EXPRESSION IN POST-ISCHEMIC MOUSE HEARTS

Left ventricles from post-ischemic hearts were ground to a fine powder using a mortar and pestle under liquid N_2 then placed in phospholysis buffer (10 mmol/L Tris–HCl, pH 7.5, 50 mmol/L NaCl, 1% Triton-X, 30 mmol/L sodium pyrophosphate, 0.1 mmol/L sodium orthovanodate, 1 mmol/L DTT) containing protease inhibitors and lysed on ice for 30 min. Samples were centrifuged for 10 min at 10,000 rpm at 4°C then soluble extracts were collected and stored at −80°C until analysis. Samples (20–30 μg) were subjected to SDS–PAGE analysis using either 7.5% or 10% acrylamide gels. After transfer to polyvinylidene di-fluoride (PVDF) membranes, immunoblotting was performed for pAkt (1:1,000 in 5%, w/v BSA, 1× TBS, 0.1% Tween-20, Cell Signalling Technology, Beverly), pTroponin I (1:1,000 in 5%, w/v BSA, 1× TBS, 0.1% Tween-20, Cell Signalling Technology), pPhosopholamban (1:1,000 in 1× TBS, 0.1% Tween-20, Abcam, Cambridge, UK), and SERCA (1:1,000 in 1× TBS, 0.1% Tween-20, Santa Cruz Biotechnology, Santa Cruz) overnight on a roller at 4°C after blocking for 1 h at room temperature in 5%, w/v skim milk in 1× TBS with 0.1% Tween-20. Appropriate secondary antibodies were then used (all at 1:1,000), and signal detection was performed by chemiluminescence. Quantitative densitometric analysis was performed (Bio-Rad laboratories, Hercules) and specific protein expression levels were indexed according to appropriate total protein expression as indicated.

STATISTICAL ANALYSIS

Data were analyzed with one-way analysis of variance (ANOVA) followed by Bonferonni post hoc tests for multiple comparisons. All

statistical tests were performed using GraphPad Prism program, version 4.01 (GraphPad Software, San Diego), and $P < 0.05$ was considered indicative of statistical significance. All data are presented as mean ± SEM.

RESULTS

HYPOXIA–REOXYGENATION OF CARDIOMYOCYTES

Exposure to the conditions of hypoxia with reoxygenation significantly reduced NVCM L-arginine uptake and NO production maximal after 2 h reoxygenation (both $P < 0.01$, Table I). The depression of L-arginine transport occurred in the absence of a change in CAT1 protein expression (data not shown). Both L-arginine uptake and NO production started to increase again after 4 h reoxygenation, although levels were still significantly lower than normoxic controls. In parallel, ROS production significantly increased during reoxygenation, peaking 2 h after reoxygenation ($P < 0.01$, Table I). In conjunction, NVCM mitochondrial membrane potential significantly decreased during both hypoxia and reoxygenation with the largest decrease observed after 2 h reoxygenation ($P < 0.01$, Table I). Also of interest to this finding was our observation that H_2O_2 exposure elicited significant concentration-dependent decrease in NVCM L-arginine uptake (Table I). Given that the largest and most significant changes were observed after 3 h hypoxia and 2 h reoxygenation, the remainder of the hypoxia-reoxygenation studies were done with this time course.

EFFECT OF L-ARGININE AVAILABILITY ON CARDIOMYOCYTES DURING HYPOXIA–REOXYGENATION

To investigate whether the adverse effect of hypoxia-reoxygenation on L-arginine/NO metabolism contributed to cellular injury, we evaluated whether enhancing the availability of intracellular arginine would protect the myocardium. NVCMs were infected with AdCAT1-EGFP as shown in Figure 1A,B, and Ad-EGFP as a control vector as shown in Figure 1C,D. Vector expression remained consistent and stable between experiments. ^{3}H-L-arginine uptake was significantly increased by 43% in AdCAT1-EGFP NVCM compared to AdEGFP NVCM ($P < 0.01$, n = 3 individual experiments with four replicates for each, Fig. 2E). There was no change in ^{3}H-L-arginine uptake in NVCMs pre-treated with the NOS inhibitor L-NAME (n = 3 independent experiments with four replicates for each, data not shown). There was a modest but significant increase in NO production in AdCAT1 and L-arginine supplemented normoxic cells compared to untreated controls. L-NAME and L-arginine deprivation significantly reduced NO production in normoxic cells as shown in Table II.

No significant changes in ROS production were observed with L-arginine supplementation, in AdCAT1 or AdEGFP cells, nor with L-arginine deprivation or L-NAME in normoxic time control cells (Table II). Similar results were also observed for mitochondrial membrane potential (Table II). There were also no changes in LDH release (data not shown). Given that these treatments did not alter these parameters in normoxic time control cells, and seeing as one of our aims was to investigate the cardioprotective effects of L-arginine manipulation in myocardial I-R injury, we chose to compare and analyze our data against the untreated reoxygenated controls.

TABLE I. Effect of Hypoxia-Reoxygenation and Oxidative Stress on Isolated Cardiomyocytes

	Normoxic time control	3 h hypoxia	30 min reoxygenation	1 h reoxygenation	2 h reoxygenation	4 h reoxygenation	25 μM H_2O_2	50 μM H_2O_2	100 μM H_2O_2	150 μM H_2O_2
L-Arginine uptake	100	78 ± 1	81 ± 3#	67 ± 1#	41 ± 2#	47 ± 2#	92 ± 3	63 ± 9#	38 ± 3#	33 ± 3#
NO production	100	79 ± 4#	81 ± 2#	75 ± 2#	71 ± 4#	80 ± 1#				
ROS production	100	55 ± 3*	108 ± 2	117 ± 1#	129 ± 4#	114 ± 2*				
Mitochondrial membrane potential	100	78 ± 1*	87 ± 3	71 ± 5#	66 ± 5#	73 ± 2#				

L-Arginine uptake, NO production, ROS production, and mitochondrial membrane potential were measured as described in Materials and Methods Section during 3 h hypoxia and various lengths of reoxygenation. L-Arginine uptake in NVCMs following treatment with various concentrations of hydrogen peroxide was also measured (n = 3 individual experiments with five replicates for each experiment). Data are presented as mean ± SEM (*$P < 0.05$ and #$P < 0.01$ vs. normoxic or untreated controls, respectively).

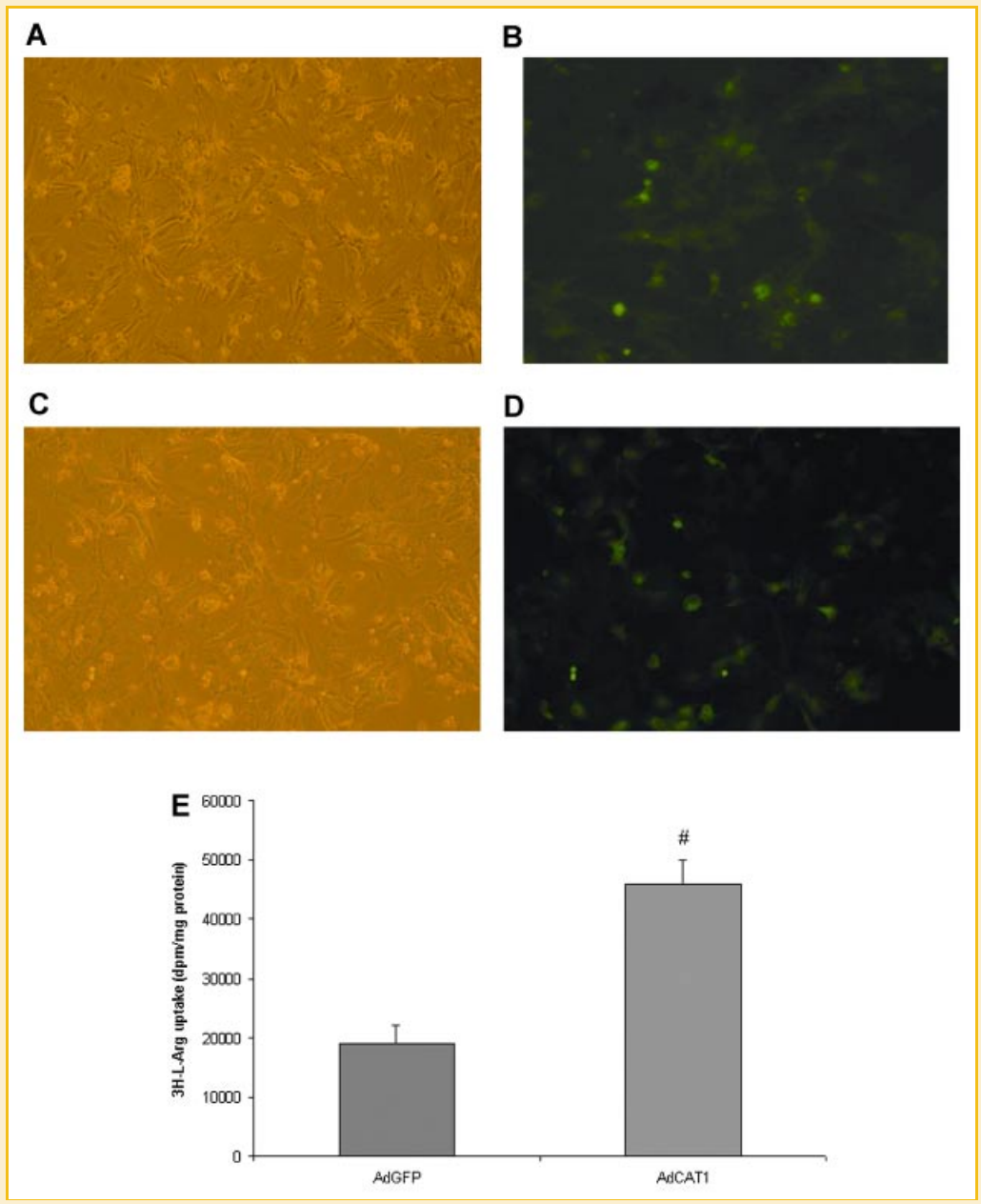

Fig. 1. Neonatal ventricular cardiomyocytes were infected with adenovirus expressing either CAT1-EGFP or EGFP for 48 h before being subjected to hypoxia and reoxygenation. A,C: represents AdCAT1-EGFP and Ad-EGFP infected NVCMs, respectively under bright light and (B,D) under UV fluorescence. L-Arginine uptake in NVCMs 48 h post-infection is shown in (E). Data are shown as mean ± SEM, n = 3 independent trials with four replicates per trial ($^{\#}P < 0.01$ vs. AdEGFP infected controls).

NVCMs infected with adenovirus expressing the CAT1 L-arginine transporter and NVCMs supplemented with 1 mmol/L L-arginine during hypoxia-reoxygenation both exhibit significantly reduced ROS generation, significantly improved mitochondrial membrane potentials and significantly increased NO production (all $P < 0.001$), whilst L-arginine deprived NVCM had significantly worsened responses to hypoxia-reoxygenation (Table III, all n = 4 independent experiments with five replicates per experiment). Cells pre-treated with L-NAME displayed significantly lower NO production ($P < 0.001$) and mitochondrial membrane potentials ($P < 0.01$) following 2 h reoxygenation, however, there was no significant change in ROS generation (Table III). L-Arginine supplemented and AdCAT1 NVCMs pre-treated with L-NAME also displayed significantly reduced ROS production and cell injury compared to untreated controls, however, NO production was significantly ($P < 0.001$) reduced following 2 h reoxygenation in these treatment groups. Whilst NO production was significantly reduced in all L-NAME treated groups after reoxygenation, NO production was significantly ($P < 0.001$) higher in both the L-arginine + L-NAME and AdCAT1 + L-NAME treated groups than L-NAME treatment

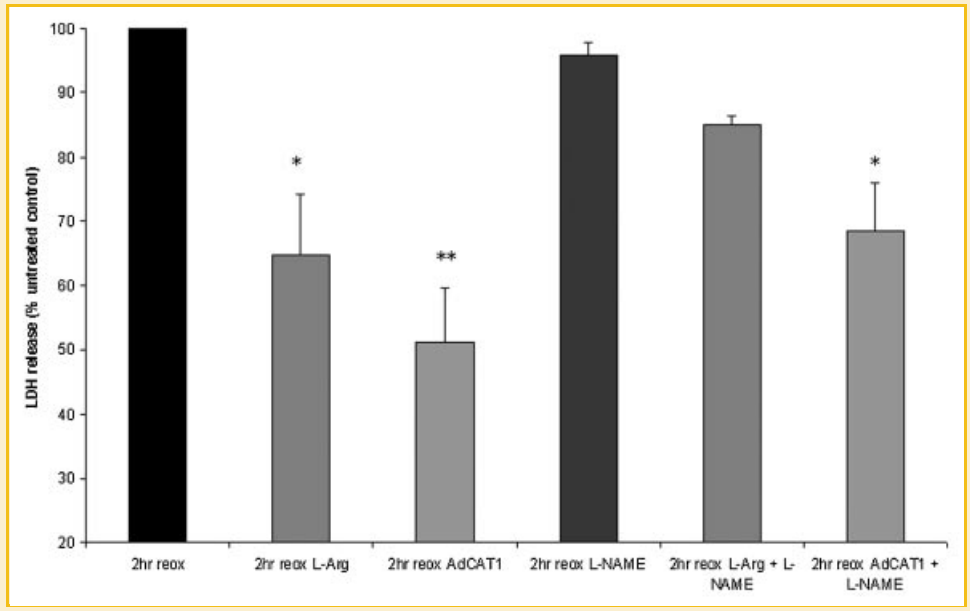

Fig. 2. LDH was measured in media collected from cardiomyocytes after 3 h hypoxia–2 h reoxygenation as a marker of cell death (n = 3 independent experiments). NVCMs were either untreated; supplemented with 1 mmol/L L-arginine or infected with AdCAT1-EGFP, all ±L-NAME co-treatment. Data represents mean ± SEM ($^{*}P < 0.05$ and $^{**}P < 0.01$ vs. untreated controls after 2 h reoxygenation).

alone. There were no significant differences in ROS generation, mitochondrial membrane potential or NO production after 2 h reoxygenation in AdEGFP NVCMs compared to untreated controls (Table III).

LDH release was significantly ($P < 0.001$) increased after 2 h reoxygenation in untreated NVCMs compared to normoxic controls, and there was no significant change in LDH release during reoxygenation in AdEGFP NVCMs compared with untreated controls (data not shown). L-Arginine deprived NVCMs had considerably higher (~500%) LDH release after 2 h reoxygenation compared to untreated controls ($P < 0.001$, data not shown). L-Arginine supplemented and AdCAT1 NVCMs had significantly lower LDH release than untreated controls (65 ± 9%, $P < 0.05$ and 51 ± 8% of controls, $P < 0.01$, respectively, Fig. 2 n = 3). L-NAME pre-treatment alone did not significantly alter LDH release during reoxygenation (96 ± 2% of controls), however, AdCAT1 cells treated with L-NAME had significantly lower LDH release than untreated controls (69 ± 7% of controls, $P < 0.05$). LDH release was limited to 85 ± 1% in L-arginine + L-NAME treated NVCMs.

EFFECT OF L-ARGININE AVAILABILITY ON THE ISOLATED MOUSE HEART DURING ISCHEMIA-REPERFUSION

In an attempt to establish the role of L-arginine supply directly on the whole heart during I-R, we employed the Langendorff isolated heart model and subjected mouse hearts to zero-flow global ischemia and reperfusion.

In isolated mouse hearts subjected to I-R, we found that infusion of 1 mmol/L L-arginine in hearts during reperfusion significantly improved recovery when compared to that of untreated hearts for: LVDP (74.6 ± 6.9% vs. 53.1 ± 5.3% pre-ischemic LVDP, $P < 0.05$); rate pressure product (83 ± 8.4% vs. 54.7 ± 7.3% pre-ischemic RPP, $P < 0.05$); and +dP/dt (98.7 ± 6.2% vs. 78.5 ± 3.6% pre-ischemic +dP/dt, $P < 0.05$) (Table IV). These hearts also displayed significantly improved −dP/dt (108.5 ± 7% vs. 74.8 ± 10.6% pre-ischemic −dP/dt, $P < 0.01$) and significantly lower EDP (8.35 ± 4.2 mm Hg vs. 32.16 ± 3.4 mm Hg, $P < 0.01$) after 45 min reperfusion indicating improved ventricular relaxation and reduced diastolic stiffness in these hearts. There were no significant differences in pre-ischemic or post-ischemic coronary flow in these hearts, and there were no

TABLE II. Effect of L-Arginine Availability on Cardiomyocytes Under Normoxic Conditions

	Control	L-Arginine	AdCAT1	AdEGFP	Arg deprive	L-NAME	L-Arginine + L-NAME	AdCAT1 + L-NAME
ROS production	100	100 ± 1	99 ± 1	101 ± 1	103 ± 1	99 ± 1	101 ± 1	100 ± 1
Mitochondrial membrane potential	100	100 ± 1	102 ± 1	97 ± 1	94 ± 1*	98 ± 1	99 ± 1	99 ± 2
NO production	100	112 ± 1#	115 ± 2#	98 ± 1	94 ± 2	70 ± 1***	84 ± 1†	93 ± 3†

NVCMs were either untreated; supplemented with 1 mmol/L L-arginine; infected with AdCAT1-EGFP or AdEGFP; or L-arginine deprived. Additional cells were also pre-treated with L-NAME. ROS generation, mitochondrial membrane potential and NO production were measured in these cells using the fluorescent probes H_2DCF-DA, JC1, and DAF-FM, respectively (n = 4 individual experiments with five replicates per experiment). Data represents mean ± SEM ($^{*}P < 0.05$, $^{\#}P < 0.01$, and $^{***}P < 0.001$ vs. untreated normoxic time controls; $^{\dagger}P < 0.01$ vs. L-NAME).

TABLE III. Effect of L-Arginine Availability on Cardiomyocytes During Hypoxia-Reoxygenation

	2 h reoxygenation	2 h reoxygenation L-arginine	2 h reoxygenation AdCAT1	2 h reoxygenation AdEGFP	2 h reoxygenation Arg deprive	2 h reoxygenation L-NAME	2 h reoxygenation L-arginine + L-NAME	2 h reoxygenation AdCAT1 + L-NAME
ROS production	$131 \pm 1^{\#}$	$120 \pm 1^{***}$	$118 \pm 1^{***}$	133 ± 2	$139 \pm 4^{**}$	126 ± 2	$123 \pm 1^{**}$	$121 \pm 1^{**}$
Mitochondrial membrane potential	$73 \pm 1^{\#}$	$82 \pm 1^{***}$	$86 \pm 1^{***}$	69 ± 1	$61 \pm 1^{***}$	$68 \pm 1^{**}$	$77 \pm 1^{**,\dagger,\S}$	$79 \pm 1^{***,\dagger\dagger,\S}$
NO production	$74 \pm 1^{\#}$	$86 \pm 1^{***}$	$90 \pm 1^{***}$	71 ± 1	$66 \pm 3^{***}$	$51 \pm 1^{***}$	$62 \pm 1^{***,\dagger,\S}$	$66 \pm 1^{***,\dagger\dagger,\S}$

NVCMs were either untreated; supplemented with 1 mmol/L L-arginine; infected with AdCAT1-EGFP or AdEGFP; or L-arginine deprived; then subjected to 3 h hypoxia-2 h reoxygenation. Additional cells were also pre-treated with L-NAME prior to and during hypoxia-reoxygenation. ROS generation, mitochondrial membrane potential and NO production were measured in these cells using the fluorescent probes H_2DCF-DA, JC1, and DAF-FM, respectively (n = 4 individual experiments with five replicates per experiment). Data represents mean ± SEM ($^{\#}P < 0.01$ vs. normoxic controls; $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. untreated controls after 2 h reoxygenation; $^{\dagger}P < 0.01$ vs. L-arginine supplemented and $^{\dagger\dagger}P < 0.001$ vs. AdCAT1 after 2 h reoxygenation; $^{\S}P < 0.001$ vs. L-NAME after 2 h reoxygenation).

TABLE IV. Myocardial Function in Langendorff Perfused Isolated Mouse Hearts Both Pre- and Post-Ischemia-Reperfusion

	Controls	1 mM L-arginine
Pre-ischemia		
LVDP (mm Hg)	169.8 ± 11.2	157 ± 7.4
HR (bpm)	301 ± 15.3	309 ± 16.1
+dP/dt (mm Hg/s)	$4{,}898 \pm 260$	$4{,}878 \pm 230$
−dP/dt (mm Hg/s)	$3{,}407 \pm 143$	$3{,}230 \pm 154$
Coronary flow (ml/min)	2.47 ± 0.25	2.55 ± 0.33
45 min reperfusion		
EDP (mm Hg)	32.16 ± 3.4	$8.35 \pm 4.2^{**}$
% recovery LVDP[a]	53.1 ± 5.3	$74.6 \pm 6.9^{*}$
HR (bpm)	303 ± 19.5	343 ± 14.7
% recovery RPP[a]	54.7 ± 7.3	$83.0 \pm 8.4^{*}$
% recovery +dP/dt[a]	78.5 ± 3.6	$98.7 \pm 6.2^{*}$
% recovery −dP/dt[a]	74.8 ± 10.6	$108.5 \pm 7.0^{**}$
Coronary re-flow (ml/min)	2.64 ± 0.17	2.77 ± 0.24

Data are presented as mean ± SEM ($^{*}P < 0.05$ and $^{**}P < 0.01$ vs. untreated controls).
LVDP, left ventricular developed pressure; HR, heart rate; +dP/dt, rate of LV positive pressure development (contraction) over time; −dP/dt, rate of negative pressure development (relaxation) over time; EDP, LV end diastolic pressure; RPP, rate pressure product.
[a]Percentage recovery versus baseline pre-ischemic function.

significant differences in baseline (pre-ischemic) contractile function observed in any of these animals (Table IV).

Protein oxidation by peroxynitrite, measured as 3-nitrotyrosine, was significantly ($P < 0.001$) lower in L-arginine infused mouse hearts (97.8 ± 2.0 nM) versus untreated controls (378.9 ± 5.6 nM, Fig. 3A). LDH release was also significantly lower in L-arginine infused hearts compared to controls after 45 min reperfusion (0.048 ± 0.004 U/ml vs. 0.078 ± 0.006 U/ml, $P < 0.01$, Fig. 3B).

MOLECULAR MECHANISMS FOR THE PROTECTIVE EFFECTS OF L-ARGININE

On the basis of the evidence showing the augmentation of L-arginine availability improves the function of, and protects the reperfused myocardium, we next investigated the potential molecular basis. By Western analysis, levels of phosphorylated troponin I were significantly increased in L-arginine-infused post-ischemic mouse hearts when compared to post-ischemic control hearts (1.19 ± 0.04 vs. 0.81 ± 0.04, $P < 0.001$, Fig. 4A,B). Similarly, levels of phosphorylated Akt were significantly increased in these hearts (1.30 ± 0.08 vs. 1.09 ± 0.06 in control post-ischemic hearts ($P < 0.05$, Fig. 4C,D). No significant changes in phospholamban, phosphorylated phospholamban, or SERCA protein expression were apparent (data not shown).

DISCUSSION

This study tested the hypothesis that depressed L-arginine availability due to reduced cellular uptake plays a critical role in the pathogenesis of myocardial I-R injury. Our principle findings are that cardiomyocyte L-arginine transport and NO production are significantly reduced during hypoxia-reoxygenation whilst ROS production is significantly increased. In support of this hypothesis, augmenting L-arginine availability significantly improved both cardiomyocyte and whole heart responses to I-R. We show that in

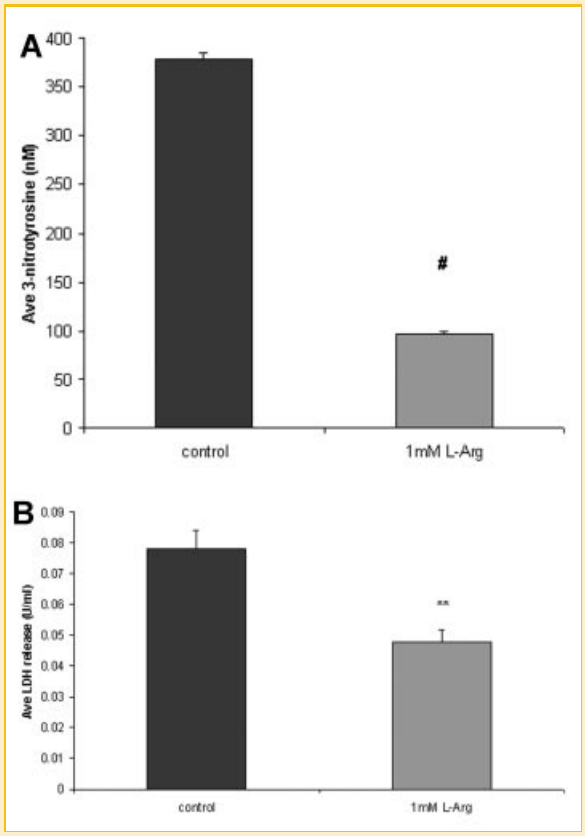

Fig. 3. Protein oxidation and cell death was measured in our hearts following 22.5 min ischemia–45 min reperfusion. Protein oxidation was assessed as a maker of oxidative damage and indicator of peroxynitrite formation by measuring 3-nitrotyrosine levels in tissue extracts (A), and necrosis measured by LDH release into coronary effluent (B). Both 3-nitrotyrosine levels and LDH release are significantly reduced in L-arginine infused hearts after 45 min reperfusion. Data are presented as mean ± SEM ($^{\#}P < 0.001$ and $^{**}P < 0.01$ vs. untreated controls).

cultured cardiomyoctyes ROS production was reduced, significantly higher NO production was achieved, and significantly improved mitochondrial membrane potentials in conjunction with a significant reduction in cell injury or death (LDH release).

Further confirmation of the importance of L-arginine in cardioprotection from I-R injury is that L-arginine deprived cardiomyocytes showed significantly worsened responses to hypoxia-reoxygenation with increased ROS production, reduced NO production, and significantly higher cell injury as indicated by higher LDH release and lower mitochondrial membrane potentials. In addition, significant improvements were also seen following hypoxia-reoxygenation in L-arginine supplemented and AdCAT1 cardiomyocytes pre-treated with the NOS inhibitor L-NAME, perhaps suggesting L-arginine itself may also be cardioprotective via mechanisms independent of NO. Given that L-arginine supplementation and CAT1 overexpression did not significantly alter ROS production, mitochondrial membrane potential, or LDH release in normoxic time control cells, we do not believe the beneficial effects we have observed following hypoxia-reoxygenation are due to an offset phenomenon.

In the isolated whole heart, there were significant improvements in left ventricular contractile function (LVDP and RPP), left ventricular relaxation (EDP and maximum $-dP/dt$) as well as significantly reduced 3-nitrotyrosine levels and LDH release into coronary effluent indicating reduced cellular injury and/or necrosis. These improved responses were independent of changes in coronary flow and were associated with increases in phosphorylation of both Akt and troponin I.

Our group has recently demonstrated that depressed vascular and myocardial L-arginine transport plays a key role in the pathophysiology of a range of cardiovascular disorders such as hypertension and heart failure [Kaye et al., 2000; Schlaich et al., 2004; Chin-Dusting et al., 2007; Yang et al., 2007]. In addition we have also previously found reduced CAT1 expression in the failing heart [Kaye et al., 2000]. Hypoxic inhibition of L-arginine transport has previously been reported in PAEC following long term (24 h) hypoxia; however, the effects of acute hypoxia have not been reported [Zharikov and Block, 2000]. We have extended these findings by showing decreased L-arginine transport in cardiomyocytes during I-R without any changes in CAT1 protein expression. Although the exact mechanism behind this remains to be elucidated, it is possible CAT1 protein is susceptible to oxidative or nitrosative attack, with a resultant diminution in transport capacity. This notion is supported by our finding that H_2O_2 exposure elicits a significant concentration-dependent decrease in NVCM L-arginine uptake, and by our previous studies suggesting ROS may reduce L-arginine transport in endothelial cells [Zhang et al., 2006]. By their nature ROS can attack virtually all cellular targets non-specifically, including proteins, causing a loss in normal protein and enzyme activity. Changes in cellular oxidative status can also alter signal transduction, DNA and RNA synthesis, protein synthesis, and enzyme activation [Kloner et al., 1989; Lucchesi, 1990; Venardos and Kaye, 2007], thus, it is possible ROS contribute to the diminished transport capacity of CAT1 via one of these mechanisms.

There is considerable evidence implicating increased oxidative stress, endothelial dysfunction, and reduced NO production in the pathogenesis of I-R, and our findings further support this [Kloner et al., 1989; Lucchesi, 1990; Giraldez et al., 1997; Flood et al., 2002; Venardos and Kaye, 2007]. In addition, our finding of reduced L-arginine transport during both hypoxia and reoxygenation may provide a mechanistic explanation for the reduced NO production and endothelial dysfunction observed post-I-R, particularly when considering the conflicting data as to whether NOS activity increases or decreases during ischemia [Depre et al., 1997; Wang et al., 1997; Prasan et al., 2007]. Although Gao et al. [2007] have reported a twofold increase in arginase I protein expression in coronary arteries following 30 min ischemia and 90 min reperfusion, we have previously found no evidence of an alteration in the myocardial mRNA expression of arginase I or II in the setting of I-R (unpublished data). This observation excludes the possibility that increased metabolism of intracellular arginine by arginase is a key contributing mechanism in the reduction of NO generation during I-R. Whilst there are reports of ROS specifically inactivating NO and reducing NOS activity in endothelial cells [Gryglewski et al., 1986; Giraldez et al., 1997], reduced L-arginine transport may also

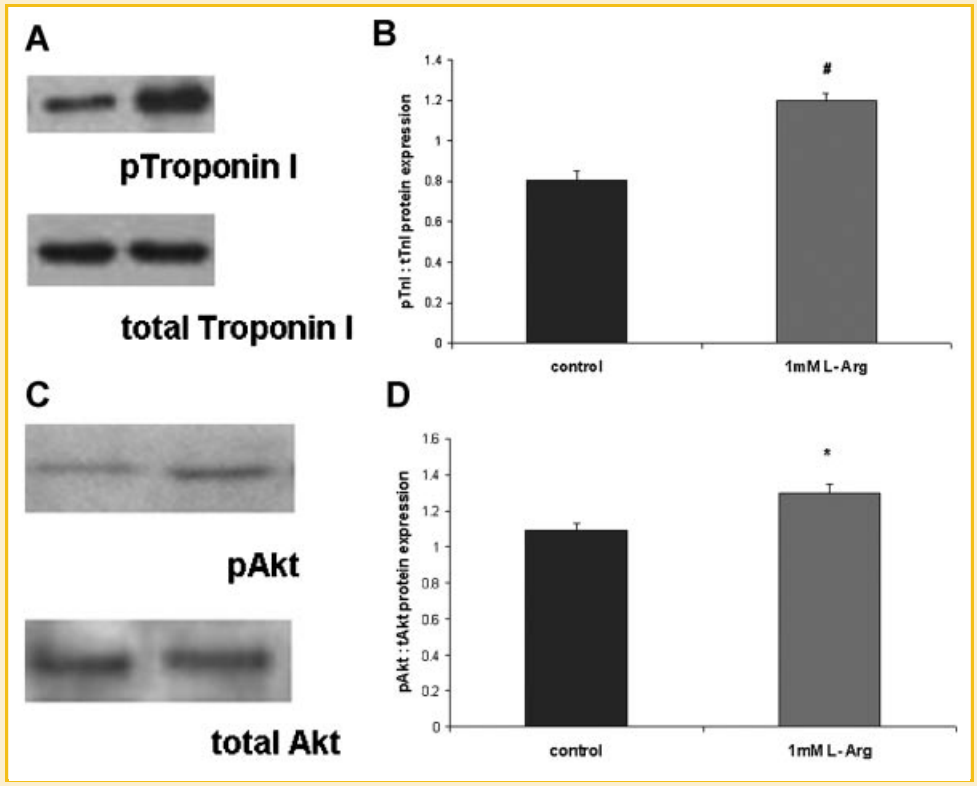

Fig. 4. Western blot analysis of pTroponin I and pAkt protein expression in post-ischemic mouse hearts. Levels of phosphorylated troponin I (A,B) and phosphorylated Akt (C,D) were significantly increased in the L-arginine infused hearts after 22.5 min ischemia–45 min reperfusion. Representative blots for pTroponin I and total troponinI, and for pAkt and total Akt protein expression in untreated control (lane 1) and L-arginine infused (lane 2) hearts are shown in (A) and (C), respectively. Densitometry results of pTroponin I and pAkt expression standardized to total proteins are displayed in (B,D), respectively. Data represents mean ± SEM ($^{*}P < 0.05$ and $^{\#}P < 0.001$ vs. untreated controls).

contribute to reduced NO production and the endothelial dysfunction observed post-ischemia.

We demonstrated that increased L-arginine availability resulted in improved functional recovery after myocardial ischemia, but this is not associated with an increase in coronary flow during reperfusion. Although we did not observe "no-reflow" in our study, this phenomenon is not generally a characteristic of the buffer perfused isolated heart model as "no-reflow" is thought to require blood components and extrinsic factors [Flood et al., 2002; Rezkalla and Kloner, 2002]. Our observation of unaltered coronary flow following changes in L-arginine availability is, however, consistent with a study by Takeuchi et al. [1995] who showed that L-arginine supplementation did not increase or change coronary flow. It is possible that the protective effects of heightened NO production in our isolated hearts might be mediated by paracrine actions on cardiomyocytes although flow did not change. Coronary microvascular endothelial cells are known to modulate left ventricular contractile behavior, particularly ventricular relaxation and diastolic function, by modifying cardiac myofilament properties and/or altering cytosolic Ca^{2+} transients [Shah and Lewis, 1992; Grocott-Mason et al., 1994; Shah et al., 1996]. These effects are mediated by the release of NO, which elevates myocardial cyclic GMP and have also been shown to be independent of coronary flow [Shah and Lewis, 1992; Grocott-Mason et al., 1994].

Several groups have previously reported improved myocardial function after I-R following treatment with L-arginine, however, the mechanism for the protective effect has not been addressed to date. Prasan et al. [2007] found enhanced post-ischemic myocardial function in rabbit hearts with L-arginine independent of changes in NO synthesis whilst Engelman et al. [1995] reported that L-arginine treatment was associated with both improved cardiac function following I-R together with an increase in NO synthesis in the pig heart. Nakanishi et al. [1992] and Weyrich et al. [1992] both demonstrated improved post-ischemic function and smaller infarct sizes in conjunction with enhanced endothelial function and reduced neutrophil accumulation following the infusion of L-arginine during reperfusion. These later studies support our finding of increased NO production in L-arginine supplemented and AdCAT1 cardiomyocytes, and reduced NO in L-arginine deprived cells during hypoxia-reoxygenation. These increases in NO production with increased L-arginine availability were significantly reduced by pre-treating cardiomyocytes with the NOS inhibitor L-NAME.

Although the intracellular concentration of L-arginine is well in excess of the K_m of NOS under normal physiological conditions, it is readily appreciated that extracellular L-arginine is required for NO synthesis [Gold et al., 1989; McDonald et al., 1997]. Further, it has been well shown that the intracellular content of L-arginine in endothelial cells falls rapidly (by 50% within 1 h) in the absence of

an exogenous supply [Gold et al., 1989]. In addition, it has also been shown that the concurrent cellular transport of L-arginine by CAT1 may be more important than the intracellular L-arginine concentration per se for the production of NO by eNOS [McDonald et al., 1997; Baydoun et al., 1999]. These factors help to provide an explanation for increased NO production in our L-arginine supplemented and AdCAT1 transfected cardiomyocytes during hypoxia-reoxygenation. Whilst we have shown the rate of L-arginine transport is reduced following hypoxia-reoxygenation, supplementing cells, or infusing hearts with L-arginine during reperfusion significantly improves responses to I-R, most likely by increasing the intracellular supply of L-arginine. Given that we observed significant improvements in responses to hypoxia-reoxygenation in cardiomyocytes with increased CAT1 expression but without L-arginine supplementation also demonstrates the importance of the CAT1 transporter itself in I-R injury.

NOS itself can also be a notable source of ROS. Specifically, substrate deplete NOS has the capacity to produce substantial quantities of superoxide, via electron transfer to molecular oxygen at the oxygenase domain of NOS [Xia et al., 1996; Vasquez-Vivar et al., 1998]. Thus, depending on the specific biochemical milieu, NOS is an important source of both NO and superoxide, therefore, increasing L-arginine availability may also limit superoxide production by preventing the uncoupling of NOS. This is supported by our finding of significantly reduced 3-nitrotyrosine levels in post-ischemic hearts infused with L-arginine during reperfusion. Peroxynitrite ($ONOO^-$) is formed by the iron-independent reaction of superoxide and NO, which have an overwhelming affinity for each other [Venardos and Kaye, 2007]. Peroxynitrite can attack tyrosine residues in proteins leading to the formation of 3-nitrotyrosine, a widely used marker of oxidative damage in human and animal disease states. Increasing L-arginine availability in post-ischemic hearts appears to prevent the uncoupling of NOS leading to reduced superoxide production and subsequent peroxynitrite formation, thereby protecting the hearts not only by increasing NO production, but reducing the amount of oxidative/nitrosative damage to proteins. Given that ROS have also been implicated in contractile dysfunction and hypercontracture [Venardos and Kaye, 2007], this reduction in superoxide production, peroxynitrite generation and protein oxidation may also contribute to the enhanced contractile responses, particularly the improved ventricular relaxation, observed in L-arginine infused hearts.

In addition to changes in NO synthesis, L-arginine has been shown to have other protective properties. This may explain why L-arginine has protective effects in several conditions such as atherosclerosis and I-R of various organs, where oxygen metabolites are thought to mediate endothelial, myocardial, and cellular injury [Hearse and Tosaki, 1987; Kaneko et al., 1989; Kloner et al., 1989; Suessenbacher et al., 2002; Schneider et al., 2003]. In ischemic acute renal failure, L-arginine supplementation not only improved the recovery phase and the expression of NO signaling proteins in rats, it also significantly reduced superoxide generation suggesting L-arginine has some antioxidant or ROS scavenging properties [Schneider et al., 2003]. In support of this, we observed a significant decrease in ROS production in cardiomyocytes during reoxygenation in L-arginine supplemented and AdCAT1 cells. This effect was not significantly prevented by the NOS inhibitor L-NAME, moreover, cardiomyocytes treated with both L-arginine + L-NAME and AdCAT1 + L-NAME still had significantly lower ROS generation than untreated controls. These results also suggest L-arginine has effects beyond NO which may include the inhibition of ROS production and/or the scavenging of ROS. This is consistent with other data showing that L-arginine can act as a scavenger of free radicals in the vasculature and reduce free radical injury in primary cultured human cardiomyocytes [Wascher et al., 1997; Nonami et al., 1998]. The observation by some that D-arginine as well as L-arginine had similar cardioprotective effects also suggests that the protection might be due to a direct chemical interaction of arginine with oxygen radicals [Lass et al., 2002; Suessenbacher et al., 2002]. Taken together, these results also provide an explanation for the increased ROS production observed in L-arginine deprived cardiomyocytes during hypoxia-reoxygenation. The diminution in ROS production observed in our study may also contribute to the improved mitochondrial membrane potentials seen in cardiomyocytes treated with L-arginine and AdCAT1, especially after taking into consideration that mitochondria are a major source of ROS, particularly during I-R [Kloner et al., 1989; Ferrari et al., 1998a; Venardos and Kaye, 2007].

Although NOS inhibition via L-NAME reduced mitochondrial membrane potential after 2 h reoxygenation in cardiomyocytes treated with L-arginine and AdCAT1, these cells still displayed significantly higher mitochondrial membrane potentials than the untreated controls indicating that L-arginine-induced cardioprotection is not solely mediated via NO. This protection is further supported by the LDH results demonstrating reduced cell death or injury in cells treated with both L-arginine + L-NAME and AdCAT1 + L-NAME compared to untreated controls. L-Arginine deprived cardiomyocytes displayed the worse mitochondrial membrane potentials after 2 h reoxygenation and also had the highest LDH release than any other group.

At the molecular level, we made the novel observation that the extent of Akt phosphorylation (activation) was increased significantly in L-arginine infused hearts after I-R. Akt/PKB and several downstream pathways are activated in various disease states including myocardial I-R, and this forms part of the reperfusion-induced-salvage-kinase (RISK) pathway [Hausenloy and Yellon, 2007]. Akt/PKB controls a variety of regulatory responses in cells including inhibition of apoptosis as well as the regulation of cellular proliferation and metabolism [Oudit et al., 2004]. Accumulation of pAkt in the mitochondria has also been suggested to trigger additional protective mechanisms such as the opening of mitochondrial K_{ATP} channels [Bijur and Jope, 2003; Yellon and Downey, 2003; Oudit et al., 2004]. Ischemic preconditioning also leads to increased pAkt, and although the precise connection is unclear, this has been linked to preserved post-ischemic function and smaller infarct sizes [Yellon and Downey, 2003; Oudit et al., 2004; Hausenloy and Yellon, 2007]. Animal models with increased Akt signaling have enhanced cell survival and smaller infarct size during I-R, and furthermore, mesenchymal stem cells overexpressing Akt/PKB prolonged survival, prevented cardiac remodeling and improved contractile performance in infarcted hearts [Fujio et al., 2000; Matsui et al., 2001; Yamashita et al., 2001; Mangi et al., 2003; Oudit et al., 2004]. Additionally, a study by Cittadini et al. [2006]

demonstrated increased ventricular contractility and enhanced intracellular calcium handling in rat hearts transfected with AdAkt. Thus, activation of Akt/PKB may be a means of L-arginine-induced cardioprotection contributing to both the reduced LDH release and improved contractile function observed in these hearts. Whilst a direct relationship or interaction between L-arginine and pAkt has not been investigated or demonstrated, we have shown that L-arginine-induced cardioprotection does involve increased activity of this protective pathway.

In addition to Akt activation, phosphorylation of troponin I was also significantly increased in L-arginine infused hearts after I-R. Troponin I degradation increases with the severity of ischemic injury, and this has been demonstrated to be in part responsible for the contractile dysfunction observed in I-R injury [Westfall and Solaro, 1992; Gao et al., 1997; Van Eyk et al., 1998; McDonough et al., 1999]. However, phosphorylation of troponin I prevents this ischemia-induced degradation [McDonough et al., 1999]. In addition, Arteaga et al. [2005] reported that increases in troponin I phosphorylation and myofilament Ca^{2+} sensitivity are able to reduce the effect of I-R on cardiac function. Therefore, the increase in phosphorylated troponin I in our post-ischemic hearts with increased L-arginine availability may also provide an explanation for the significant improvements in contractile dysfunction, in particular the improved ventricular relaxation, (as shown by significantly lower EDP and $-dP/dt$) observed in these hearts. Phosphorylation of troponin I decreases Ca^{2+} binding of troponin C causing tropomyosin to block the interaction between actin and myosin resulting in improved relaxation and reduced ventricular stiffness [Gao et al., 1995; Solaro and Van Eyk, 1996; McDonough et al., 1999]. Although the mechanism responsible for L-arginine related increases in phosphorylated troponin I remain unknown, it may be via increased production of NO, and does appear to be independent of changes in SERCA and phosphorylated phospholamban protein expression.

CONCLUSION

This study evaluated the possibility that reduced L-arginine transport plays a major role in the genesis of I-R injury. Collectively, our data indeed implicate reduced L-arginine availability as a key factor in the pathogenesis of myocardial I-R injury. Increasing L-arginine availability either via augmented transport or increased extracellular supply, reduces oxidative stress, restores NO levels, and decreases necrosis, thereby improving cardiomyocyte response to hypoxia-reoxygenation. Left ventricular function in isolated mouse hearts subjected to I-R is also significantly improved in hearts infused with L-arginine and this was associated with changes in the expression of pAkt and pTroponinI. These observations were independent of coronary flow alterations but were associated with a decrease in nitrotyrosine formation. Taken together, it is possible increasing L-arginine availability post-ischemia results in increased NO and decreased ROS (superoxide, peroxynitrite), leading to increases in pTroponinI and pAkt expression and improved mitochondrial function, thereby enhancing cardiomyocyte and whole heart responses to I-R. Our findings also demonstrate that the CAT1 transporter itself plays an important role in I-R injury, and that L-arginine is directly important for cardiomyocytes, both of which are also novel findings. Ultimately, therapeutic manipulation of L-arginine availability may therefore be a valuable strategy to ameliorate I-R injury.

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