Diverse Effects of \(L\)-arginine on Cardiac Function of Rats Subjected to Myocardial Ischemia and Reperfusion in vivo

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Abstract  In vivo administration of \(L\)-arginine at different time points during the course of myocardial ischemia and reperfusion (MI/R) has been shown to differentially regulate postischemic apoptosis. Cardiac function is one of the most important indexes used to judge the degree of myocardial injury. The present study attempted to determine whether in vivo administration of \(L\)-arginine at different stages of MI/R has a diverse influence on cardiac function of ischemic reperfused hearts and, if so, to investigate the mechanisms involved. Male adult rats were subjected to 30 min myocardial ischemia followed by 5 h reperfusion. An intravenous \(L\)-arginine bolus was given either 10 min before and 50 min after reperfusion (early treatment) or 3 h and 4 h after reperfusion (late treatment). Early treatment with \(L\)-arginine markedly increased the left ventricular systolic pressure (LVSP) and \(dP/dt_{\text{max}}\), and decreased myocardial nitrotyrosine content. In strict contrast, late treatment with \(L\)-arginine resulted in a significant decrease in LVSP and \(dP/dt_{\text{max}}\) from 4 h to 5 h after reperfusion, and increase in toxic peroxynitrite formation as measured by nitrotyrosine. These results suggest that the administration of \(L\)-arginine at different time points during the course of MI/R leads to diverse effects on cardiac dysfunction. Early supplementation decreased the nitrative stress and improved left ventricular function. However, late treatment with \(L\)-arginine increased the formation of peroxynitrite and aggravated cardiac functional injury.

Key words  myocardial ischemia; reperfusion; cardiac function; nitric oxide; peroxynitrite

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Persistent myocardial ischemia without reperfusion inevitably results in myocyte cell death. Although the beneficial effects of early restoration of blood flow to ischemic myocardium are now well established, results of an increasing number of studies indicate that reperfusion has deleterious effects on the ischemic myocardium, such as regional inflammatory reaction, necrosis and apoptosis, that can accelerate and extend postischemic injury. Herein lies the concept of reperfusion injury [1,2].

Nitric oxide (NO), an endogenous free radical that can be produced in all cell types, is synthesized from \(L\)-arginine by NO synthase (NOS). A lot of research have indicated that NO plays an important role in myocardial ischemia and reperfusion (MI/R) [3–5]. However, both protective and deleterious effects of NO on ischemia/reperfusion injury have been described and the mechanisms responsible for this diversity remain largely unknown. Our previous studies have shown that treatment with \(L\)-arginine during different stages of reperfusion has opposing effects on myocardial apoptosis after MI/R. Early supplementation of \(L\)-arginine can inhibit cardiomyocyte apoptosis, whereas \(L\)-arginine promotes apoptosis if given during the late phase of reperfusion when inducible NOS (iNOS) is expressed [6]. However, it remains unknown whether these opposing effects of \(L\)-arginine on postischemic apoptosis could translate into clinically meaningful protection/damage on cardiac contractile function.
Therefore, the objectives of the present study were: (i) to determine whether early supplementation of L-arginine might ameliorate postischemic cardiac function and, if so, to clarify the relationship between NO production and L-arginine’s protective effect; and (ii) to elucidate whether the supplementation of L-arginine during a late phase of reperfusion might aggravate postischemic cardiac function and, if so, to identify the mechanisms involved.

Materials and Methods

Animal experiment protocol
Adult male Wistar rats weighing 280−320 g were anesthetized with 20% ethylcarbamate (1 g/kg, i.p.) and ventilated with a small animal respirator. A polyethylene catheter connected with a pressure transducer was inserted into the left ventricular cavity through the right carotid artery, and left ventricular pressure (LVP) was measured. A midline thoracotomy was carried out, and myocardial ischemia was produced by opening the pericardium and placing a 6-0 silk suture slipknot around the left anterior descending coronary artery. After 30 min of ischemia, the slipknot was released and the myocardium was reperfused for 5 h. Rats were randomized to receive vehicle (0.9% NaCl, 1 ml/kg) or L-arginine (100 mg/kg; Sigma-Aldrich, St. Louis, USA) [6], whose half-life after a single bolus is approximately 1 h, in the femoral vein either 10 min before and 50 min after reperfusion (early treatment) or 3 h and 4 h after reperfusion (late treatment). Sham-operated control rats (sham MI/R) underwent the same surgical procedures except that the suture passed under the left anterior descending coronary artery and was not tied. At the end of the 5 h reperfusion period, the ligature around the coronary artery was retied and 1 ml of 2% Evans blue dye (Sigma-Aldrich) was injected into the left ventricular cavity. The dye was circulated and uniformly distributed except in that portion of the heart previously perfused by the occluded coronary artery (area-at-risk, AAR). The heart was quickly excised, and the myocardium was processed according to the procedures described below for infarct size, immunohistological and biochemical assays (n=12 for each group).

Determination of myocardial infarct size
After staining, as described above, the heart was excised, frozen in −20 °C, and sliced into 1 mm thick sections perpendicular to the long axis of the heart. Slices were incubated individually using a 24-well culture plate in 1% triphenyltetrazolium chloride (TTC) in phosphate buffer at pH 7.4 at 37 °C for 10 min, and photographed with a digital camera. Evan’s blue stained area (area-not-at-risk, ANAR), TTC stained area (red staining, ischemic but viable tissue), and TTC staining negative area (infarct myocardium) were digitally measured using SigmaScan (SPSS, Chicago, USA). The myocardial infarct size was expressed as a percentage of infarct area over AAR.

Determination of cardiac function injury
MI/R-induced cardiac dysfunction was continuously monitored during the entire MI/R period. The LVP, including left ventricular systolic and diastolic pressure (LVSP and LVDP), was digitally processed using a hemodynamic analyzing system (Powerlab Hardware; AD Instruments, Charlotte, USA). Heart rate, and maximal positive and negative values of the instantaneous first derivative of LVP (+dP/dt max and −dP/dt max) were measured by computer algorithms. The postischemic recovery of cardiac function was expressed as a percentage of the pre-ischemic value.

Determination of total NO x content in cardiac tissue
Cardiac tissue samples from AAR were rinsed, homogenized in deionized water (1:10, W/V), and centrifuged at 14,000 g for 10 min. The tissue NO and its in vivo metabolic products (NO2 and NO3) in the supernatant, collectively known as NOx, were determined using a chemiluminescence NO detector (SIEVER 280i NO Analyzer; Ionics Instruments, Boulder, USA), as described in our previous study [7].

Immunohistochemical detection of nitrotyrosine formation
Myocardial tissue was removed and fixed with 4% formalin for less than 48 h. Fixed myocardial tissues were dehydrated and embedded in paraffin, and sections were cut at 5 μm and mounted onto glass slides. Immunohistochemical detection of nitrotyrosine was carried out as described previously [8].

Quantitation of tissue nitrotyrosine content
Nitrotyrosine content, a footprint of in vivo ONOO− formation, was determined using an enzyme-linked immunosorbent assay (ELISA) method described in our previous publication [8]. The results were presented as nmol nitrotyrosine per g protein.

Statistical analysis
Data are reported as the mean±SE. Differences among
the experimental groups were analyzed by two-way repeated-measures ANOVA (time and group) for hemodynamic variables or by a one-way ANOVA for other indices when appropriate, followed by Bonferroni post hoc tests. Probabilities (\(P\)) of 0.05 or less were considered to be statistically significant.

Results

Supplementation of \(L\)-arginine at early and late stages led to opposing effects on postischemic cardiac dysfunction

Consistent with previous studies, MI/R resulted in significant decrease in cardiac function (Figs. 1–3). \(L\)-arginine had no effects on heart rate or LVDP before, during or after ischemia (data not shown). However, early treatment with \(L\)-arginine markedly improved LVSP and \(+dP/dt_{\text{max}}\). Specifically, LVSP (from 1 h to 5 h reperfusion), \(+dP/dt_{\text{max}}\) (from 1 h to 3.5 h reperfusion) and \(-dP/dt_{\text{max}}\) (from 1 h to 3 h reperfusion) in rats treated with \(L\)-arginine 10 min before reperfusion were significantly higher than those in rats treated with vehicle. On the contrary, supplementation of \(L\)-arginine 3 h after reperfusion not only failed to ameliorate the postischemia injury but also further worsened the values of LVSP and \(dP/dt_{\text{max}}\). Most noticeably, all of the three parameters of cardiac function reduced markedly compared with those of the vehicle group from 4 h to 5 h after reperfusion (Figs. 4–6).

Effect of \(L\)-arginine treatment on myocardial infarct size

Consistent with our previous reports, MI/R resulted in marked myocardial infarction. When given 10 min before reperfusion, \(L\)-arginine markedly reduced myocardial infarct size. This result was consistent with previous results in other groups [9,10]. In contrast, \(L\)-arginine given 3 h after reperfusion not only failed to reduce postischemic myocardial injury, but also further worsened myocardial

![Fig. 1](image1)

**Fig. 1** Time course of left ventricular systolic pressure (LVSP) in sham myocardial ischemia and reperfusion (MI/R) group of rats or MI/R group treated with vehicle

I, ischemia; R, reperfusion. *\(P<0.05\) versus sham MI/R (\(n=12\)).

![Fig. 2](image2)

**Fig. 2** Time course of \(+dP/dt_{\text{max}}\) in sham myocardial ischemia and reperfusion (MI/R) group of rats or MI/R group treated with vehicle

I, ischemia; R, reperfusion. *\(P<0.05\) versus sham MI/R (\(n=12\)).

![Fig. 3](image3)

**Fig. 3** Time course of \(-dP/dt_{\text{max}}\) in sham myocardial ischemia and reperfusion (MI/R) group of rats or MI/R group treated with vehicle

I, ischemia; R, reperfusion. *\(P<0.05\) versus sham MI/R (\(n=12\)).
reperfusion injury as evidenced by a significantly enlarged infarct size (Fig. 7).

Fig. 4  Effect of L-arginine treatment at different time points (arrow) on left ventricular systolic pressure (LVSP) in rats subjected to myocardial ischemia and reperfusion (MI/R)
I, ischemia; R, reperfusion. *P<0.05 versus MI/R+vehicle (n=12).

Fig. 5  Effect of L-arginine treatment at different times (arrow) on +dP/dt max in rats subjected to myocardial ischemia and reperfusion (MI/R)
I, ischemia; R, reperfusion. *P<0.05 versus MI/R+vehicle (n=12).

Fig. 6  Effect of L-arginine treatment at different times (arrow) on –dP/dt max in rats subjected to myocardial ischemia and reperfusion (MI/R)
I, ischemia; R, reperfusion. *P<0.05 versus MI/R+vehicle (n=12).

Fig. 7  Myocardial infarct size in the different treatment groups
(A) Representative photomicrographs of heart sections obtained from rats subjected to sham myocardial ischemia and reperfusion (sham MI/R), MI/R treated with vehicle (MI/R+vehicle), and MI/R treated with L-arginine in the early phase [MI/R+L-arginine (E)] and late phase [MI/R+L-arginine (L)]. Blue portion, nonischemic, normal region; red portion, ischemic reperfused, but not infarcted region; negatively stained portion, ischemic reperfused, infarcted region. (B) Column graph of myocardial infarct size expressed as a percentage of total ischemic reperfused area [area-at-risk (AAR); mean±SE of 10 rats per group]. Inf, infarct area. #P<0.01 versus sham MI/R; *P<0.05 versus MI/R+vehicle; **P<0.01 versus MI/R+vehicle.

Effect of L-arginine treatment on myocardial total NO content
To establish a link between L-arginine’s improving and deteriorating effects on cardiac function and its NO stimulatory effect, we directly measured myocardial total NO content in hearts with different treatments. Compared with sham MI/R hearts, myocardial total NO content was significantly increased in hearts subjected to MI/R. Interestingly, although the supplementation of L-arginine before reperfusion significantly increased myocardial to-
Effect of L-arginine treatment on myocardial nitrotyrosine formation and its content

NO itself has low reactivity with most biological molecules and is cytoprotective. In contrast, the secondary reaction products between NO and other reaction species are highly reactive and often cytotoxic. Specifically, peroxynitrite (ONOO\(^{-}\)), the reaction product between NO and superoxide, reacts with a variety of biological molecules [11–13], and has been reported to cause cell death in vitro and in vivo [14]. To determine whether the properties of improving and deteriorating cardiac function by L-arginine supplementation at different time points were related to their effects on ONOO\(^{-}\) generation, myocardial nitrotyrosine content, an in vivo footprint marker of ONOO\(^{-}\) formation, was determined. As illustrated in Fig. 9, 30 min of myocardial ischemia and 5 h of reperfusion resulted in a 3.6-fold increase in nitrotyrosine formation, indicating a significant increase in ONOO\(^{-}\) formation in ischemic/reperfused cardiac tissue. Of particular interest, early and late treatment with L-arginine exerted opposite effects on nitrotyrosine content (Fig. 9). Specifically, supplementation of L-arginine before reperfusion reduced ONOO\(^{-}\) formation (2.2-fold increase over sham), whereas late treatment increased ONOO\(^{-}\) formation (6.3-fold increase over sham). This result suggests that the opposing effects of L-arginine treatment at different times during the course of MI/R might be related to inhibitory and promoting effects on peroxynitrite formation and resultant nitrative stress.

Discussion

In the present study, we showed that supplementation with L-arginine at different time points can either improve or reduce cardiac function during the course of MI/R. In the early stage of reperfusion, treatment with L-arginine reduced the myocardial nitrotyrosine content, partially reversed the impaired cardiac function induced by reperfusion injury and resulted in a decrease in infarct size.
In strict contrast, when L-arginine was supplemented at a later stage, the toxic peroxynitrite formation was further increased and the cardiac dysfunction was further aggravated with an increased infarction.

NO, a molecule that plays a very important role in physiological and pathophysiological conditions, is a free radical that is synthesized by three NOS from L-arginine [15, 16]. Two isoforms (endothelial NOS and neuronal NOS, eNOS and nNOS) are constitutively expressed and are acutely regulated by calcium/calmodulin and phosphorylation, whereas the third (iNOS) is induced during inflammation and produces higher levels of NO for a longer period. It has been reported that significant iNOS expression was markedly upregulated 3 h after reperfusion [6]. Therefore, the myocardial reperfusion period was divided into early and late stages in the present experiment. In addition, because the half-life of L-arginine given as a single bolus is approximately 1 h, in order to maintain its content in blood, L-arginine was supplemented 1 h after its first injection into the femoral vein at the same dose.

Our previous experiment [6] provided direct evidence that the early supplementation of L-arginine inhibited iNOS expression and its NO production and attenuated toxic ONOO⁻ formation. At high concentrations (0.1–100 nM) and/or highly reactive nitrogen species (RNS) have been shown to cause apoptotic cell death, the negative feedback of early, physiological amounts of NO production on subsequent toxic levels of NO production might contribute to the anti-apoptotic effect of early L-arginine supplementation, as observed in the present study. Apoptosis might contribute to cardiac dysfunction by several possible mechanisms. Loss of cardiomyocytes leads to loss of cardiac mass and hence diminished pumping power. Loss of cardiomyocytes might also result in electrical conduction inhomogeneity that might lead to arrhythmias. Finally, apoptosis might lead to cardiac remodeling due to realignment of neighboring cardiomyocytes. This latter mechanism is unique to the heart, as its function is extremely dependent on optimal geometrical and structural alignment. Thus apoptosis, even if limited in scope, could result in widespread mechanical and electrical disturbances [17–19]. Our present experimental results provide further support that the inhibition of apoptosis during MI/R by early supplementation of L-arginine is beneficial to the myocardial injury and can indeed be translated into a clinically meaningful cardiac functional improvement.

Another finding in our experiment was that cardiac function was worsened with supplementation of L-arginine 3 h after reperfusion. To explain the mechanisms involved, total NO content and ONOO⁻ content were determined and the results showed that both NO and ONOO⁻ were increased significantly when L-arginine was given 3 h after reperfusion. Biochemical and in vitro experiments have shown that NO and O₂⁻ react at the diffusion-limited rate (three times faster than its detoxification reaction with superoxide dismutase) [20] to produce ONOO⁻, a highly reactive species that might oxidize (e.g., cysteine oxidation) or nitrate (e.g., tyrosine nitration) a variety of molecules, thus resulting in cell death and tissue injury through multiple signaling pathways [21–24]. Moreover, ONOO⁻ has been reported to increase apoptotic cell death in a variety of cell types. Increased ONOO⁻ formation might increase apoptosis and, therefore, further aggravate cardiac function. Additionally, ONOO⁻-induced cardiac dysfunction during the course of MI/R might be related to the abnormality of myocardial energy metabolism. Lee et al. [25] reported that treatment with an ONOO⁻ biosynthesis inhibitor or an ONOO⁻ scavenger produced a substantial recovery of left ventricular developed pressure, cardiac work, efficiency of O₂ utilization and a partial recovery of ATP in isolated perfused heart subjected to ischemia/reperfusion.

In summary, L-arginine given at different time points during the course of MI/R results in diverse effects on cardiac dysfunction. Early supplementation can improve left ventricular function through L-arginine’s antinitrative effect. In contrast, late treatment might aggravate left ventricular function by increasing nitrate stress and resultant tissue injury.

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