Selective β₂-Adrenoreceptor Stimulation Attenuates Myocardial Cell Death and Preserves Cardiac Function After Ischemia–Reperfusion Injury

Shashi Bhushan, Kazuhisa Kondo, Benjamin L. Predmore, Maxim Zlatopolsky, Adrienne L. King, Claire Pearce, Hui Huang, Ya-Xiong Tao, Marah E. Condit, David J. Lefer

Objective—β₂-adrenoreceptor activation has been shown to protect cardiac myocytes from cell death. We hypothesized that acute β₂-adrenoreceptor stimulation, using arformoterol (ARF), would attenuate myocardial ischemia/reperfusion (R) injury via NO synthase activation and cause a subsequent increase in NO bioavailability.

Methods and Results—Male C57BL/6J and endothelial NO synthase (eNOS) knockout mice were subjected to 45 minutes of myocardial ischemia and 24 hours of R. ARF or vehicle was administered 5 minutes before R. Serum troponin-I was measured, and infarct size per area-at-risk was evaluated at 24 hours of R. Echocardiography was performed at baseline and 2 weeks after R. Myocardial cAMP, protein kinase A, eNOS/Akt phosphorylation status, and NO metabolite levels were assayed. ARF (1 μg/kg) reduced infarct size per area-at-risk by 53.1% (P<0.001 versus vehicle) and significantly reduced troponin-I levels (P<0.001 versus vehicle). Ejection fraction was significantly preserved in ARF-treated hearts compared with vehicle hearts at 2 weeks of R. Serum cAMP and nuclear protein kinase A C-α increased 5 and 15 minutes after ARF injection, respectively (P<0.01). ARF increased Akt phosphorylation at Thr308 (P<0.001) and Ser473 (P<0.01), and eNOS phosphorylation at Ser1177 (P<0.01). ARF treatment increased heart nitrosothiol levels (P<0.001) at 15 min after injection. ARF failed to reduce infarct size in eNOS−/− mice.

Conclusion—Our results indicate that β₂-adrenoreceptor stimulation activates cAMP, protein kinase A, Akt, and eNOS and augments NO bioavailability. Activation of this pro-survival signaling pathway attenuates myocardial cell death and preserves cardiac function after ischemia/reperfusion. (Arterioscler Thromb Vasc Biol. 2012;32:1865-1874.)

Key Words: ischemia–reperfusion injury • arformoterol • β₂-adrenoreceptor • NO synthase • nitrite • nitrosothiol • Akt

Coronary heart disease, including acute myocardial infarction (AMI), is one of the leading causes of death among men and women worldwide, despite advances in clinical science. Early intervention at the time of reperfusion after the onset of AMI has been shown to limit infarct size and restore blood flow in the ischemic tissue. Prolonged ischemia and subsequent reperfusion lead to severe myocardial inflammation and tissue damage by several mechanisms. Therefore, it would be beneficial to develop a cardioprotective intervention for patients experiencing AMI.

β-adrenergic receptors (AR) serve as critical regulators of cardiac function at the level of the myocardium and in circulation. In the normal heart, β₂-ARs play a major role in regulating cardiac chronotropy and inotropy. Dysfunction of adrenergic signaling through β₂-AR contributes to arrhythmia. During myocardial infarction and heart failure, β₁-AR signaling in the myocardium is associated with cardiac hypertrophy, ventricular remodeling, and apoptosis. Previous studies have demonstrated that activation of β₂-AR signaling attenuates cardiac remodeling and preserves cardiac contractile function in ischemic heart failure. Furthermore, β₂-AR activation has been implicated in the preservation of cardiac function in a rat model of ischemic heart failure by inhibiting apoptosis and cardiac remodeling. However, the precise role of β₂-adrenergic activation in the cardiovascular system at baseline or during the evolution of AMI remains unclear. Recently, our laboratory has demonstrated the cardioprotective effects of β₂-AR agonists after myocardial ischemia/reperfusion (MI/R) via activation of endothelial NO synthase (eNOS) phosphorylation and upregulation of neuronal NO synthase.

Administration of exogenous NO or eNOS activation has been shown to be highly protective in the setting of MI/R injury. Systemic overexpression of eNOS, as well as cardiac-specific overexpression of eNOS, attenuates cardiac dysfunction,
improves survival in congestive heart failure, and protects against MI/R injury in mice.16–18 Genetic deletion of eNOS results in increased injury from MI/R injury.19–21 Agents that augment NO bioavailability and signaling, including NO donors, ACE inhibitors, statins, sildenafil, and so on, confer profound beneficial effects on the pathophysiological states in the cardiovascular system.22 Furthermore, supplementation of endogenous NO by exogenous administration of sodium nitrite, which can be converted back to NO in vivo during ischemic conditions, protects the heart against MI/R injury.23–25

Previous studies have shown that β2-AR signaling promotes eNOS activation, at least in part, through the β2-AR in vitro.26,27 In the present study, we investigated whether a highly specific β2-AR agonist protects against MI/R injury in an in vivo murine model of AMI. For this study, we used the specific, β2-adrenergic agonist arformoterol (ARF), administered at the time of reperfusion. We hypothesized that β2-AR stimulation would attenuate MI/R injury via NOS activation and increased NO bioavailability.

**Methods**

**Animals**

Male C57BL/6J mice, 10–12 weeks of age (Jackson Labs, Bar Harbor, ME), and eNOS-deficient (eNOS−/−) mice19 (10–12 weeks) were used in the present study. All animals were housed in a temperature-controlled animal facility with a 12-hour light/dark cycle, with water and rodent chow provided ad libitum. All animals received humane care in compliance with the Principles of Laboratory Animal Care formulated by the National Society of Medical Research and the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (Publication 85-23, Revised 1996). All animal procedures were approved by the Emory University Institutional Animal Care and Use Committee.

**MI/R Protocol and Assessment of Myocardial Infarct Size**

Mice were fully anesthetized via intraperitoneal injection of ketamine (50 mg/kg) and pentobarbital sodium (60 mg/kg), intubated, and connected to a rodent ventilator. A median sternotomy was performed to gain access to and identify the left coronary artery (LCA). The LCA was surgically ligated with a 7-0 silk suture mated to a rodent ventricle against trauma. Mice were subjected to 45 minutes of LCA clamping to neutralize the perchloric acid. Samples were spun at 6000 g for 10 minutes, and the supernatants containing cAMP were collected. The LCA was surgically ligated with a 7-0 silk suture mated to a rodent ventricle to inject 7.0% Evans blue (1.2 mL) to rapidly excise and cross-sectioned into 1-mm-thick sections, which were then incubated in 1.0% 2,3,5 triphenyl tetrazolium chloride for 10 minutes, and the myocardial area-at-risk and infarct per left ventricle were determined by a blinded observer as previously described.15

**Echocardiography**

Baseline echocardiography was performed 1 week before the MI/R surgical protocol. Transthoracic echocardiography was performed to obtain B-mode and M-mode images using a 30-MHz probe connected to a Vevo 2100 (VisualSonics, Toronto, Ontario, Canada) imaging system. During the procedure, mice were under light anesthesia with isoflurane supplemented with 100% O2. Echocardiography was performed in the same manner at 2 weeks after the MI/R protocol. To determine cardiac structure and function, intraventricular septal end diastolic dimension, left ventricular end diastolic dimension, left ventricular end systolic dimension, left ventricular ejection fraction, and % left ventricular fractional shortening were analyzed from M-mode images.

**Serum Troponin-I Level Measurement**

Blood samples were collected via a carotid artery catheter at 24 hours of reperfusion. Cardiac troponin-I level was measured in serum using the Life Diagnostic high-sensitivity mouse cardiac troponin-I ELISA kit (Mouse Cardiac Tn-I ELISA Kit; Life Diagnostics, Inc; West Chester, PA) as previously described.15

**Western Blot Analysis**

Myocardial tissue samples (75 mg) were taken and weighed, and the myocardial area-at-risk and infarct per left ventricle were determined by a blinded observer as previously described to obtain either whole cell or fractionated lysates to be used for Western blot analysis. Protein concentrations were measured with the DC protein assay (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein were loaded into lanes of SDS-PAGE gels. The gels were electrophoresed, followed by transfer of the protein to a polyvinylidene fluoride membrane. The membrane was then blocked and probed with primary antibodies overnight at 4°C. The following primary antibodies were used: eNOS (1:5000; BD Biosciences, San Jose, CA); Phospho-eNOS Ser1177 (eNOS-Pser1177) (1:2000; Cell Signaling Technology, Danvers, MA); Phospho-eNOS Thr495 (eNOS-Pth495) (1:2000; Cell Signaling Technology); GAPDH (1:40 000; Cell Signaling Technology, Danvers, MA); inducible NO synthase (1:5000, BD Biosciences, San Jose, CA); neuronal NO synthase (1:5000; BD Biosciences); Phospho-Akt Thr308 (1:5000; Cell Signaling Technology); Akt (1:5000, Cell Signaling Technology); protein kinase A (PKA) (1:5000; Abcam, Cambridge, MA). Immunoblots were then probed with the appropriate secondary antibodies (Cell Signaling) for 1 hour at room temperature. Immunoblots were visualized with the SuperSignal West Dura Extended Duration Substrate (Thermo Scientific, Rockford, IL), followed by exposure to x-ray film.

**Analysis of Nitrite, Nitrate, and RXNO Levels in Cardiac Tissue and Plasma**

Nitrite and nitrate concentrations were quantified by ion chromatography (ENO20 Analyzer; Eicom, San Diego, CA), and total nitrosothiols (RXNO) were quantified using a chemiluminescent detector (Eco Physics, Ann Arbor, MI) as previously described.28

**PKA Activity Assay**

PKA activity was measured in heart lysates and serum using PKA Kinase Activity Kit (Enzo Life Sciences, Farmingdale, NY) according to the manufacturer’s instructions.

**Measurement of cAMP in Plasma and Heart Tissue**

Frozen heart samples, 100–300 mg, were homogenized by KCl and transferred to prechilled glass tubes with 800 mL of 0.5N perchloric acid containing 180 mg/mL theophylline. The tissues were vortexed vigorously until all tissue particles had been broken down. Then, 400 mL of 0.72 mol/L KOH/0.6 mol/L KHCO3 was added to neutralize the perchloric acid. Samples were spun at 600g for 10 minutes, and the supernatants containing cAMP were collected. Plasma samples were assayed directly by diluting with water. The
concentrations of cAMP in tissue extracts and plasma were assayed by radioimmunoassay.

Hemodynamics
Mice were first lightly anesthetized with isoflurane (1.0–2.0 L/min) in 100% oxygen. The right carotid artery was exposed for a length of 5 vessels. A 1.2F Scisense pressure catheter (Scisense Inc, Ontario, Canada) connected to the computerized data-acquisition system (iWorx 404; iWorx Systems, Inc, Dover, NH) was advanced through the carotid artery and into the aorta to record heart rate and systolic, diastolic, and mean arterial pressures. These values were recorded with LabScribe2 (iWorx Systems, Inc).

Statistics
All data are expressed as mean±SEM. Student t test or 1-way ANOVA with Tukey or Dunnett post hoc analysis was performed using Prism 5 (GraphPad Software, La Jolla, CA). Values >2 SDs outside the mean were considered as outliers. A P value <0.05 was considered statistically significant.

Results

β2-Adrenergic Activation Limits MI/R Injury and Preserves Cardiac Function
Male, wild-type mice were subjected to 45 minutes of LCA ischemia and 24 hours of reperfusion. ARF (500 ng/kg to 2 µg/kg) or vehicle (VEH) was administered into the cardiac lumen at the time of reperfusion. Myocardial infarct size in each group was evaluated at 24 hours of reperfusion. Representative midventricular cross-sectional images of mice treated with ARF and VEH are shown in Figure 1A. Among all doses of ARF that mice received (Figure 1B) at the time of reperfusion, the 1 µg/kg dose of ARF significantly reduced the myocardial infarct size per area-at-risk by 53% (22.0±3.0%) compared with the VEH-treated mice (47±4.0%). In addition, we measured circulating plasma levels of cardiac troponin-I as a marker of myocardial injury, and these data are presented in Figure 1C. We observed that 1 µg/kg of ARF significantly reduced circulating troponin levels in the treatment group to 9.87±2.5 µg/mL compared with the VEH level of 37.1±4.1 µg/mL. A single dose of ARF (1 µg/kg) proved to be most effective for infarct size reduction and was used as the standard dose subsequently in this study. Given that ARF is a proven vasodilator, we evaluated the effects of acute ARF treatment on systemic hemodynamics (Figure 1 in the online-only Data Supplement). We observed a significant, transient reduction in systolic, diastolic, and mean arterial pressures at 30 seconds after administration of ARF. Blood pressure returned to baseline levels within 5 minutes, and we did not observe any changes in heart rate after ARF injection.

To evaluate the chronic cardioprotective effect of ARF, we performed 2-dimensional echocardiography at 1 week before MI/R and 2 weeks after MI/R. During the MI/R procedure, ARF (1 µg/kg) or saline VEH was administered into the cardiac lumen at the time of reperfusion. At 2 weeks after reperfusion, echocardiography was performed to assess postischemic left ventricular structure and function. Intraventricular septal end diastolic dimension significantly decreased in the VEH group 2 weeks after MI/R (P<0.01), but not in the ARF-treated group (Figure 1D). Left ventricular end diastolic dimension significantly increased in the VEH group 2 weeks after MI/R (P<0.001), but not in the ARF-treated group (Figure 1E). Left ventricular end diastolic dimension 2 weeks after MI/R was also significantly greater in the VEH group compared with ARF-treated group (P<0.05) (Figure 1E). Left ventricular end systolic dimension significantly increased in the VEH group 2 weeks after MI/R (P<0.001), but not in the ARF-treated group (Figure 1F). Left ventricular end systolic dimension 2 weeks after MI/R was also significantly greater in the VEH group compared with the ARF-treated group (P<0.01) (Figure 1F). Ejection fraction was significantly lower in both the VEH-treated (P<0.001) and ARF-treated (P<0.01) groups 2 weeks after MI/R; however, the ARF-treated group had a significantly greater ejection fraction (P<0.001) than the VEH-treated group (Figure 1G).

Preconditioning With β2-Adrenergic Agonist Limits MI/R Injury
Next, we also examined whether ARF had preconditioning effects against MI/R. Male C57BL6/J mice were pretreated with ARF (1 µg/kg) through tail vein injection at 30 minutes or 24 hours before 45 minutes of ischemia followed by 24 hours of reperfusion (Figure II in the online-only Data Supplement). Interestingly, we observed significant reduction in infarct size by 40.5% in the mice receiving ARF 30 minutes before ischemia (27.1±5.0%) compared with the VEH-treated mice (45.6±3.5%), but mice failed to show any cardioprotection when preconditioned 24 hours before inducing ischemia.

Treatment With Fenoterol Limits MI/R Injury
We also evaluated the effects of a chemically distinct β2-AR agonist, fenoterol, on myocardial injury after MI/R injury (Figure III in the online-only Data Supplement). Fenoterol (1µg/kg) significantly (P<0.01) attenuated myocardial infarct size per area-at-risk (37% reduction) (Figure IIIA in the online-only Data Supplement) and also significantly (P<0.03) reduced serum levels of troponin-I (Figure IIIB in the online-only Data Supplement). In separate groups of animals, we inhibited β2-AR receptors with ICI 118 551, which is a highly selective β2-AR antagonist. As expected, we failed to observe any significant reduction in myocardial infarct size after administration of ARF (1 µg/kg) in the presence of β2-AR receptor antagonism compared with the VEH group, demonstrating that specific agonism of β2-AR is necessary for cardioprotection (Figure IIIC in the online-only Data Supplement).

β2-AR Agonist Increases cAMP Levels and Activates PKA
We investigated the effect of β2-AR stimulation on cAMP generation and PKA activation at different time points (0 minutes, 5 minutes, 15 minutes, 30 minutes, 1 hour, and 2 hours) after administration of ARF (Figures 2 and 3). We observed a significant rise in serum cAMP levels at 5, 15, and 30 minutes compared with baseline in control animals (Figure 2A). There were no significant changes in cardiac
Figure 1. A, Representative midventricular photomicrographs of mouse hearts after 45 minutes of myocardial ischemia and 24 hours of reperfusion treated with vehicle (VEH) or arformoterol (ARF) (1 μg/kg) at the time of reperfusion. B, Bar graph of myocardial area-at-risk (AAR) per left ventricle (LV) and infarct (INF) per AAR in mice treated with ARF. C, Troponin levels in VEH- and ARF-treated mice at 24 hours after reperfusion. D, Intraventricular septal end diastolic dimension (IVSd); (E) left ventricular end diastolic diameter (LVEDD); (F) left ventricular end systolic diameter (LVESD); and (G) left ventricular ejection fraction (EF). Values are means±SEM. Numbers inside bars indicate the number of animals investigated in each group.
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cAMP levels at any measured time points (Figure 2B). We then investigated the effect of β₂-AR stimulation on cAMP generation after MI/R. Serum cAMP levels significantly increased in ARF-treated animals after MI/R compared with both sham- and VEH-treated animals (Figure 2C). However, there was no significant change in cAMP levels in the heart after ARF treatment (Figure 2D). We observed a 4-fold increase at 5 minutes and a 3.1-fold increase at 30 minutes in PKA C-α in nucleus fractions after intracardiac injection of ARF (Figure 3A and 3B). Furthermore, although sham-operated mice treated with ARF did not show increased activity of PKA (Figure 3C and 3D), PKA activity was significantly higher in the plasma (P < 0.001) and heart (P < 0.01) of mice treated with ARF after MI/R (Figure 3E and 3F).

**β₂-AR Agonism Mediates Akt Activation**

We next evaluated the expression and phosphorylation status of Akt in cardiac tissue after intracardiac administration of ARF (Figure 4). Representative total Akt and phosphorylated Akt are shown in Figure 4A. Total Akt remained unchanged at 15 minutes after intracardiac injection of ARF (1 µg/kg) in control animals (Figure 4B). However, we observed significant increases in Akt phosphorylation at Thr308 (Akt-P Thr308 ) and Ser473 (Akt-P Ser473 ) (Figure 4C and 4D).

**β₂-AR Agonist Activates eNOS and Increases Nitrosothiol Levels**

We examined the expression and phosphorylation status of eNOS in cardiac tissue after 15 minutes of intracardiac injection of ARF (1 µg/kg) in control animals at eNOS-P Ser1177 to check for activation of eNOS. Representative graph blots are shown in Figure 5A. There were no significant changes in total eNOS expression (Figure 5B) between VEH- and ARF-treated animals. Interestingly, ARF significantly increased eNOS-P Ser1177 at 15 minutes after administration of drug (Figure 5C), but eNOS-P Thr495 remained unchanged (Figure 5D). We further evaluated the plasma and cardiac nitrite and RXNO levels in plasma and heart after administration of ARF (1 µg/kg) in vivo at 15 minutes and 2 hours and in mice undergoing MI/R with administration of ARF (1 µg/kg) at reperfusion at 15 minutes and 2 hours of reperfusion. Plasma nitrite levels exhibited no significant change at 15 minutes and 2 hours after ARF injection (Figure VA in the online-only Data Supplement). Heart nitrite levels significantly increased 2 hours after ARF injection (P < 0.05) (Figure VB in the online-only Data Supplement). Heart nitrite levels significantly increased 2 hours after ARF injection (P < 0.05) (Figure VB in the online-only Data Supplement). Interestingly, RXNO significantly increased in the plasma 15 minutes after ARF injection (P < 0.001; Figure 5E) but did not significantly increase in the heart after ARF injection (Figure 5F). Neither plasma nor heart nitrite levels significantly increased when animals were subjected to MI/R, and ARF was injected at reperfusion (Figure VC andVD in the online-only Data Supplement). However, plasma RXNO did significantly increase 15 minutes after ARF injection at reperfusion (P < 0.01; Figure 5G), whereas heart RXNO significantly decreased 2 hours after ARF injection at reperfusion (P < 0.001; Figure 5H). We also investigated the expression of other NOS isoforms (inducible NO synthase and neuronal NO synthase) after ARF therapy. There were no significant changes in either inducible NO synthase or neuronal NO synthase expression (Figure IV in the online-only Data Supplement).

Figure 2. cAMP levels in serum and heart with and without myocardial ischemia/reperfusion (MI/R) injury. A, Serum levels of cAMP after administration of arformoterol (ARF) (1 µg/kg) without MI/R. B, Myocardial cAMP levels after administration of ARF (1 µg/kg) without MI/R. C, Serum levels of cAMP at 15 minutes after administration of ARF (1 µg/kg) with MI/R. D, Myocardial cAMP levels at 15 minutes after administration of ARF (1 µg/kg) with MI/R. Values are means±SEM. Numbers inside bars indicate the number of animals per group.
Cardioprotective Actions of ARF Are Abrogated by Inhibition of eNOS Pathway

We investigated the protective effects of ARF by blocking all NOS isoforms with the NOS inhibitor, L-NG-Nitroarginine methyl ester (12.5 mg/kg). The cardioprotective effects of β2-AR activation were significantly attenuated in mice treated with L-NG-Nitroarginine methyl ester in vivo (Figure 6A). We then investigated the extent of myocardial infarction in eNOS−/− mice after administration of ARF (1 μg/kg) to activate β2-AR signaling. We observed a loss of cardioprotection in eNOS−/− mice treated with ARF compared with VEH (Figure 6B).

Discussion

The present study investigated the effects of β2-AR activation in the setting of acute MI/R injury. We demonstrate that β2-AR activation results in a significant reduction in myocardial infarct size and preservation of cardiac function after acute administration of ARF. In addition, we have been able to reduce MI/R injury using another chemically distinct β2-AR activator, fenoterol. Previous studies have shown that catecholamines improve diastolic function in the human heart by inducing relaxation of the ventricular myocardium through β1- and β2-AR. Furthermore, chronic stimulation of β2-AR
attenuates the progression of dilated cardiomyopathy in a rat model of heart failure.13,30

The present study investigated the role of β2-AR subtype activation in the setting of MI/R injury in vivo. The primary mechanisms we found by which β2-AR activation protects the ischemic heart are as follows: (1) increased circulating levels of cAMP and PKA activity, (2) Akt activation via phosphorylation at Thr308 and Ser473, (3) phosphorylation of eNOS at the activation site (ie, Ser1177), and (4) an increase in circulating NO levels (ie, increased plasma nitrosothiols).

β2-ARs play a pivotal role in the signal transduction in the cardiovascular system.21 β2-AR stimulation increases cAMP-mediated PKA phosphorylation.32 PKA activation further phosphorylates β2-AR, which plays a significant role in the switching of G-protein coupling from Gs to Gi, a prerequisite for cardioprotection.33,34 We observed a significant rise in the circulating level of cAMP after administration of a highly selective β2-AR agonist, but failed to see a rise of cAMP in the myocardium. However, we observed an increase in PKA C after ARF treatment and an increase in the activity of PKA in both serum and heart tissue with ARF treatment after MI/R.

The exact molecular mechanisms related to the signaling pathway of specific β-AR subtypes still remain to be fully elucidated. However, in cardiomyocytes, β2-ARs are predominantly localized in caveola, which negatively affects cAMP accumulation.35 This may explain our finding of no significant change in cAMP levels in the heart after β2-AR agonist administration in vivo. However, future study is needed to understand the complexity of β2-AR-mediated signaling in cardiomyocytes and their implication in the diseased heart.

The role of endothelial β2-AR–mediated vasorelaxation is well established in the human vasculature system.36 Recent studies have demonstrated that β2-ARs facilitate vasodilation mainly through the endothelium and are endothelium dependent in a variety of organs.36–39 β2-AR gene therapy is also efficacious in the setting of endothelial dysfunction in hypertension.40 This indicates that β2-AR activation may predominantly affect endothelial cells in the vasculature to increase NO and modulate cytoprotection. This may be the case in the present study where we observed significant increases in circulating levels of cAMP after administration of ARF. The subsequent increase in NO and NO metabolites may protect the underlying cardiac myocytes against MI/R injury.

Tong et al34 show an exacerbation of damage in a β2-AR–heart exposed to ischemia–reperfusion injury. Similarly, our results demonstrate a loss of cardioprotection in male C57BL/6J mice when the β2-AR receptors are antagonized with ICI 118 551 during acute MI/R injury.

Previous studies demonstrate that Akt (protein kinase B) promotes survival of murine cardiomyocytes and protects against ischemia–reperfusion injury in vitro.41 Phosphoinositide kinase-3-Akt signaling cascade activation has demonstrated protection against ischemia–reperfusion injury.42 β2-AR stimulation leads to increased phosphoinositide kinase-3 activity and further activation of its downstream target, Akt. This signaling cascade is essential for myocyte survival under ischemic stress.43 Thus, we investigated whether β2-AR activation could salvage myocardial cell death through the Akt signaling pathway. Our findings suggest that β2-AR stimulation increases phosphorylation of Akt protein at Thr308 and Ser473, in vivo, and facilitates cardiomyocyte survival under ischemic stress. Dimmeler et al44 show that activation of Akt enhances eNOS phosphorylation and cardioprotective signaling through NO. NO therapy is very beneficial in the setting of MI/R injury.45 Elrod et al47 have shown the importance of cardiomyocyte-specific eNOS overexpression and its role in attenuating infarct size by increasing cardiac NO bioavailability. Recent research has demonstrated the cardioprotective effects of protein S-nitrosylation.46–48 Furthermore, circulating RXNO can act as mediators of NO bioavailability and potentiate their release under stress conditions including ischemia.49 In the present study we fail to demonstrate a significant increase in plasma nitrite levels after administration of ARF, but we do observe a significant increase in plasma RXNO levels in both

Figure 4. A, Representative immunoblots of Akt-PThr308, Akt-PSer473, total Akt, and GAPDH in tissue samples taken from the left ventricle of sham-operated mice at 15 minutes after receiving either vehicle (VEH) or arformoterol (ARF) (1 μg/kg) via intracardiac injection. B, Akt protein remained constant compared with VEH. C, ARF increased the phosphorylation of Akt at Thr308 at 15 minutes compared with VEH (P<0.001). D, ARF increased the phosphorylation of Akt at Ser473 at 15 minutes compared with VEH (P<0.01). Values are means±SEM. Numbers inside bars indicate the number of animals studied in each group.
These data suggest that circulating RXNO may prove to be a more crucial NO donor than either nitrite or nitrate and provide a compelling case for its use as a more powerful indicator of endogenous NO levels.

The present study is not without limitations. Because a mouse model was used, these data may not accurately predict human disease. The mice used in this study are without any known cardiovascular risk factors such as hypertension, obesity, hypercholesterolemia, and diabetes mellitus. Future studies need to be conducted in more translational large animal models. Another limitation with the use of β₂-AR agonist agents such as ARF is the severe hypotension that occurs with high doses administered systemically. In fact, we did observe significant hypotension at dosages of ARF >1 µg/kg that resulted in increased mortality during MI/R in the present study (data not shown).

In summary, our results demonstrate that low-dose administration of β₂-AR agonist leads to significant cardioprotection against MI/R injury via NOS activation and increased bioavailability of NO. These data suggest that β₂-AR activation may be beneficial in the treatment of MI.
Figure 6. A. Myocardial infarct size in C57BL/6J mice treated with L-N^3-hydroxy-N^6-nitro-L-arginine methyl ester (L-NAME) (12.5 mg/kg) and mice treated with L-NAME (12.5 mg/kg) and arformoterol (ARF) (1 μg/kg) at reperfusion. B. Myocardial infarct size in endothelial NO synthase-deficient (eNOS−/−) mice receiving either vehicle (VEH) or ARF (1 μg/kg) at reperfusion. Values are means±SEM. Numbers inside bars indicate the number of animals per group.

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Disclosures
None.

References


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Supplemental Figure IV

A

nNOS  iNOS  GAPDH

155 kDa  130 kDa  37 kDa

VEH    ARF

B

C

p = NS

p = NS

iNOS/GAPDH (Relative Intensity)

nNOS/GAPDH (Relative Intensity)

VEH    ARF

VEH    ARF
Supplemental Figure V

(A) Plasma Nitrite (µM)

(B) Heart Nitrite (µM)

(C) Plasma Nitrite (µM)

(D) Heart Nitrite (µM)