Original Article

Adrenomedullin Infusion during Ischemia/Reperfusion Attenuates Left Ventricular Remodeling and Myocardial Fibrosis in Rats

Hiroyuki OKUMURA, Noritoshi NAGAYA*, and Kenji KANGAWA

Recent studies have demonstrated that the activation of protein kinase Akt attenuates myocardial ischemia/reperfusion injury. However, it remains unknown whether adrenomedullin (AM), which is also a potent Akt activator, has cardioprotective effects after ischemia/reperfusion. In the present study, Sprague-Dawley rats were exposed to a 30-min period of ischemia induced by ligation of the left coronary artery followed by 24-h reperfusion. They were randomized to receive intravenous administration of AM (0.05 μg/kg/min) or saline for 60 min after coronary ligation. We examined the hemodynamics and myocardial apoptosis 24 h after ischemia/reperfusion. Echocardiographic measurements were performed 4 weeks after ischemia/reperfusion. Myocardial infarct size was also measured histologically. AM significantly reduced left ventricular (LV) end-diastolic pressure (17 ± 2 to 8 ± 2 mmHg, \( p < 0.05 \)) and the number of apoptotic nuclei in myocytes (387 ± 39 to 147 ± 72 per field, \( p < 0.05 \)). AM significantly increased LV dP/dtmax (4,803 ± 228 to 5,672 ± 199 mmHg/s, \( p < 0.05 \)). AM significantly increased LV fractional shortening (23 ± 2 vs. 28 ± 2%, \( p < 0.05 \)), and significantly reduced LV diastolic dimension (7.4 ± 0.1 to 6.9 ± 0.1 mm, \( p < 0.05 \)) and myocardial infarct size (33 ± 2 to 20 ± 2%, \( p < 0.01 \)) 4 weeks after ischemia/reperfusion. In conclusion, AM infusion during ischemia/reperfusion attenuated the development of LV remodeling and myocardial fibrosis in rats. Based on these results, the cardioprotective effects of AM may be attributed at least partly to its anti-apoptotic effect.


Key Words: reperfusion injury, apoptosis, myocardial infarction, hemodynamics, left ventricular remodeling

Introduction

The development of revascularization therapy, including catheter intervention, has significantly improved the prognosis in patients with ischemic heart disease. However, there are still serious problems with such therapy, including ischemia/reperfusion injury, restenosis, and ischemic cardiomyopathy. Therefore, to resolve the problem associated with ischemia/reperfusion injury is an urgent business in cardiologists.

Adrenomedullin (AM) is a potent vasodilatory peptide that was first purified from human pheochromocytoma (1). AM has been shown to exert not only a vasodilatory effect but also a variety of cardioprotective effects (2, 3). Interestingly, a recent genetic approach has revealed that AM is necessary for vasculature development during the embryonal stage and regulation of postnatal blood pressure via nitric oxide (NO) production (4). More recently, AM has been shown to induce endothelium-dependent vasodilation through Akt activation followed by NO production (5). Protein kinase Akt, which serves as an oncogene, plays a key role in cancer

From the Department of Biochemistry, National Cardiovascular Center Research Institute, Suita, Japan and * Department of Internal Medicine, National Cardiovascular Center, Osaka, Japan.

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Address for Reprints: Noritoshi Nagaya, M.D., National Cardiovascular Center, 5–7–1 Fujishirodai, Suita 565–8565, Japan. E-mail: nagayann@hsp.ncvc.go.jp

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progression by stimulating cell proliferation and inhibiting apoptosis (6). Earlier studies have demonstrated that Akt activation attenuates myocardial ischemia/reperfusion injury, and that this attenuation occurs via the anti-apoptotic effect of Akt (7, 8). Thus, we hypothesized that AM administration in the acute phase of ischemia/reperfusion attenuates myocardial injury partly through an Akt-dependent anti-apoptotic effect.

The aims of the present study were 1) to determine whether AM infusion during ischemia/reperfusion attenuates myocardial injury through an anti-apoptotic effect and 2) to investigate whether AM improves left ventricular (LV) function and remodeling in the late phase of ischemia/reperfusion.

**Methods**

**Reperfusion Model**

We used male Sprague-Dawley rats weighing 180–220 g. Ligation of the left coronary artery was performed as described previously (9). In brief, under anesthesia with pentobarbital sodium (30 mg/kg) and artificial ventilation, the heart was exposed via a left thoracotomy, and the left coronary artery was ligated 2–3 mm from its origin between the pulmonary artery conus and the left atrium using a 6-0 Prolene suture. The heart was subjected to regional ischemia for 30 min, followed by coronary reperfusion through release of the tie. After ligation of the left coronary artery, AM (0.05 µg/kg/min) or placebo (0.9% saline) was administered for 60 min through a catheter inserted into the left jugular vein. Sham-operated rats only underwent left thoracotomy. The chest wall was then closed, and the animal was allowed to recover. This protocol resulted in the creation of three groups: sham-operated rats (Sham group: n = 5), placebo-treated rats with ischemia/reperfusion (I/R-Placebo group: n = 20), and AM-treated rats with ischemia/reperfusion (I/R-AM group: n = 20). For the measurement of hemodynamics and apoptosis at 24 h after ischemia/reperfusion, we used all 5 rats from the Sham group, 8 rats from the I/R-Placebo group, and 8 rats from the I/R-AM group.

**Hemodynamic Studies**

We measured hemodynamic parameters 24 h after ischemia/reperfusion. A 1.5 F micromanometer-tipped catheter was advanced into the left ventricle through the right carotid artery, and a polyethylene catheter (PE-50) was advanced into the right ventricle through the right jugular vein to measure right ventricular (RV) pressure. Heart rate was also monitored with an electrocardiogram. All animal experiments were conducted in accordance with the principles and procedures outlined in the National Cardiovascular Center Guide for the Care and Use of Laboratory Animals—which adheres strictly to the animal experimental guidelines of the National Institutes of Health—with the approval of the National Cardiovascular Center Animal Experimental Committee.

**TUNEL Staining**

Fixed, paraffin-embedded, 5-µm thick myocardial sections were used for the terminal dUTP nick-end labeling (TUNEL) assay as described previously (10). In brief, after deparaffinization and enzyme-mediated antigen retrieval, TUNEL staining was performed using a commercially available kit (Apop Tag Plus, Intergen Co., New York, USA). Samples were incubated with monoclonal anti-desmin antibody (Sigma, St. Louis, USA) followed by tetramethylrhodamine isothiocyanate-conjugated rabbit anti-mouse antibody (DAKO, Glostrup, Denmark). Finally, these slides were mounted with Vector Shield (Vector Laboratories, Burlingame, USA) containing an anti-fade reagent. We measured the number of TUNEL-positive nuclei in myocytes and non-myocytes by means of confocal microscopy (Fluoview 500; Olympus, Tokyo, Japan). Quantitative analysis was performed on 60 high power fields (magnification × 600), using at least ten randomly selected fields per section.

**Echocardiographic Measurements**

Echocardiography was performed using a Sonos 5500 (Philips Medical Systems, Best, the Netherlands) with a 6–15 MHz ultraband intraoperative linear array ultrasound transducer (15-6L; Philips Medical Systems) in 24 rats (12 rats in the I/R-Placebo group and 12 rats in the I/R-AM group) 4 weeks after the ischemia/reperfusion procedure. 2D targeted M-mode tracings were obtained at the level of the papillary muscles. Anterior and posterior end-diastolic wall thickness, LV end-diastolic and end-systolic dimensions, and LV fractional shortening were measured by the American Society for Echocardiology leading-edge method.

**Assessment of Infarct Size**

Four weeks after the ischemia/reperfusion procedure, the heart was removed and perfused with a Langendorff’s apparatus to wash out the blood, and then fixed with 10% neutral buffered formalin. The heart was sliced transversely from the apex to the atrioventricular groove in sections of 2.5-mm thickness. Within 24 h after fixation, each section was embedded in paraffin. Serial 5-µm myocardial sections were cut using microtome and mounted on siliconized slides. After Masson trichrome staining, the infarct size of each slice was analyzed by microscopy. The infarct area was outlined and measured by planimetry. Finally, the % infarct area was determined as the total infarct area divided by the total LV area.
Statistical Analysis

Numerical data were expressed as the mean ± SEM. Comparisons of parameters between the I/R-Placebo and I/R-AM groups were made by one-way ANOVA. Comparisons of parameters among three groups were made by one-way ANOVA for repeated measures, followed by Scheffe’s test. Values of \( p < 0.05 \) were considered to indicate statistical significance.

Results

Surgical procedures were successfully performed in about 90% of rats. All deaths due to the ischemia/reperfusion procedures occurred during the 30-min ischemic period. During the 4-week follow-up period, all rats survived without any clinical events.

AM-Induced Hemodynamic Improvement

Mean aortic pressure was significantly lower in the I/R-Placebo group than in the I/R-AM and Sham groups, although there was no significant difference in heart rate among the three groups (Table 1). LV end-diastolic pressure showed a marked elevation in the I/R-Placebo group; the elevation was significantly attenuated in the I/R-AM group (\( p < 0.05 \)). LV \( \frac{dP}{dt_{\text{max}}} \) was higher in the I/R-AM group than in the I/R-Placebo group (\( p < 0.05 \)). LV \( \frac{dP}{dt_{\text{min}}} \) was signifi-
cantly decreased in the I/R-AM group compared with that in the I/R-Placebo group (\(p < 0.05\)). RV systolic pressure was significantly higher in the I/R-placebo group than in Sham group (\(p < 0.01\)). The elevation of RV systolic pressure was significantly attenuated in the I/R-AM group.

Anti-Apoptotic Effect of AM

TUNEL-positive myocytes were less frequently observed in the I/R-AM group than in the I/R-Placebo group. The number of TUNEL-positive nuclei in cardiomyocytes was significantly lower in the I/R-AM group than in the I/R-Placebo group (147 ± 72 vs. 387 ± 39 per field, \(p < 0.05\)) (Fig. 1, left panel). Furthermore, the number of TUNEL-positive nuclei in non-cardiomyocytes was significantly lower in the I/R-AM group than in the I/R-Placebo group (307 ± 57 vs. 865 ± 69 per field, \(p < 0.01\)) (Fig. 1, right panel). Immunofluorescence analysis revealed that TUNEL-positive nuclei were more frequently observed in non-cardiomyocytes than in cardiomyocytes, and that TUNEL-positive nuclei in endothelial cells were fewer than in interstitial cells (data not shown).

Inhibitory Effect of AM on LV Remodeling

LV diastolic dimension was significantly smaller in the I/R-AM group than in the I/R-Placebo group (7.4 ± 0.1 vs. 6.9 ± 0.1 mm, \(p < 0.05\); Fig. 2, left panel). LV systolic dimension tended to be smaller in the I/R-AM group than in the I/R-Placebo group (4.9 ± 0.3 vs. 5.5 ± 0.2 mm, \(p = 0.1\); Fig. 2, right panel); however, the difference did not reach the statistical significance. LV fractional shortening was higher in the I/R-AM group than in the I/R-Placebo group (23 ± 2 vs. 28 ± 2\%, \(p < 0.05\); Fig. 3). There was no difference in LV wall thickness either in the anterior wall or in the posterior wall (Fig. 4).

Reducing Effect of AM on Myocardial Infarct Size after Ischemia/Reperfusion

Histological analysis demonstrated moderate infarcts defined as a blue-stained fibrotic area at 4 weeks after ischemia/reperfusion. AM infusion during the acute phase of ischemia/reperfusion significantly reduced myocardial infarct area compared with placebo infusion (20 ± 2 vs. 33 ± 2\%, \(p < 0.01\); Fig. 6).

Discussion

In the present study, we demonstrated that 1) AM administration reduced the number of apoptotic myocytes and improved hemodynamics in the acute phase of ischemia/reperfusion, and 2) AM reduced myocardial infarct size and attenuated the development of LV remodeling. These results suggest that AM infusion during the acute phase of ischemia/reperfusion has cardioprotective effects.

Earlier studies have reported that AM inhibits vascular endothelial cell apoptosis (11, 12). However, the mechanism of its anti-apoptotic effect remains unknown. Recently, AM has been shown to elicit endothelium-dependent vasorelaxation through Akt-dependent NO production (5). On the other
hand, the activation of Akt, which serves as an oncogene, has been reported not only to inhibit apoptosis in several tumor cells but also to attenuate myocardial ischemia/reperfusion injury partly through its anti-apoptotic effect (7, 8). In addition, more recent studies have shown that insulin and corticosteroid (13, 14) exerts cardioprotective effects against ischemia/reperfusion injury through Akt activation, which is thought to play a pivotal role in these effects. These studies have also demonstrated that endothelial NO synthase induced by Akt activation mediates the cardioprotective effect. These findings raise the possibility that AM attenuates ischemia/reperfusion injury partly through its Akt/NO-dependent anti-apoptotic effect. We have already ascertained that AM activates Akt in cardiac tissues, and that wortmannin, a phosphatidylinositol 3-kinase inhibitor, reverses the cardioprotective effect induced by AM (data not shown). In the present study, AM infusion markedly reduced myocardial infarct size after ischemia/reperfusion. In addition, AM infusion during ischemia/reperfusion reduced TUNEL-positive nuclei both in cardiomyocytes and in non-cardiomyocytes. Therefore, it is possible that AM-induced Akt activation contributes to its cardioprotective effect.

In the present study, AM infusion during ischemia/reperfusion produced hemodynamic improvement in the acute phase. In light of the potent anti-apoptotic effect of AM, the attenuation of infarct size as well as the vasodilatory effect induced by AM may cause beneficial hemodynamic effects.

There is a growing body of evidence that apoptosis of cardiomyocytes is one of the major contributors to the development of myocardial infarcts (15, 16), which is related to the pathogenesis of heart failure. The present study revealed that earlier administration of intravenous AM during ischemia/reperfusion inhibited myocyte apoptosis and attenuated the development of LV remodeling. Therefore, it is possible to speculate that myocardial protection in the acute phase of ischemia/reperfusion inhibits LV remodeling and prevents the development of heart failure.

In conclusion, AM infusion during ischemia/reperfusion improved LV function, associated with an attenuation of the development of LV remodeling and myocardial fibrosis in rats. The cardioprotective effects of AM may be attributed at
least in part to its anti-apoptotic effect.

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