Atrial natriuretic peptide increases inflammation, infarct size, and mortality after experimental coronary occlusion

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Submitted 2 July 2008; accepted in final form 28 December 2008

CARDIOVASCULAR DISEASE is the major cause of death in the United States and worldwide. Occlusion of a coronary artery causes tissue ischemia that leads to myocardial infarction (MI) with death of cardiomyocytes (27). Ischemia and reperfusion trigger neutrophil migration into the myocardium via endothelial adhesion molecules (e.g., P-selectin) (11, 12, 19, 25). These infiltrating neutrophils are activated after the onset of ischemia, increase cytokine levels in the blood (e.g., tumor necrosis factor-α and IL-6), and have potent cytotoxic effects on the myocardium (9, 11, 21). A depletion of neutrophils or an interference with their function has been shown to significantly reduce infarct size (15, 37).

Atrial natriuretic peptide (ANP) is rapidly secreted from cardiomyocytes during ischemia brought on by coronary artery occlusion (29, 44). ANP plays an important role in regulating blood pressure and volume through its natriuretic and vasodilatory effects (31, 38). Recently, it also has been recognized that ANP and its primary receptor guanylyl cyclase-A (GC-A) are expressed in immune cells and that ANP has proinflammatory effects (43). ANP primes neutrophils for activation, which leads to superoxide anion production and neutrophil degranulation processes that contribute to the cytotoxic effects of these cells (4, 44).

The effects of the physiological levels of ANP on inflammation and MI are unknown. However, ANP has been recently administered in pharmacological or supraphysiological doses to humans with myocardial ischemia/infarction undergoing coronary artery reperfusion. In these studies, ANP reduced infarct size, diminished dilation of the left ventricle (LV), and slightly improved heart function 30 days after treatment, though there was no difference in mortality (14, 23). In experimental models, pharmacological doses of ANP administered during coronary ischemia and reperfusion also reduced infarct size; this was attributed to the fact that ANP increased coronary collateral blood flow and lowered end-diastolic pressure, etc (36, 41). However, in these studies, the effects of ANP on neutrophil infiltration, ventricular remodeling, and survival were not examined. The proinflammatory effects of ANP on neutrophil activation and superoxide production raise concerns that ANP may have a deleterious effect on infarct size or survival when coronary artery reperfusion is delayed or unsuccessful. Consequently, we used ANP knockout mice to examine the effect of ANP on myocardial infarct size, neutrophil infiltration, and survival following coronary artery occlusion without reperfusion.

METHODS

Mice. Congenic ANP-deficient (ANP−/−) (18) and ANP+/+ mice on a C57BL/6J background were obtained from Jackson Laboratory (Bar Harbor, ME), and a breeding colony was maintained. Mice were housed in microisolation cages on a constant 12-h:12-h light-dark cycle with controlled temperature and humidity and given access to food and water ad libitum. Experiments adhered to the guidelines set forth in the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1996) and were performed under protocols approved by the Medical College of Georgia’s Institutional Animal Care and Use Committee.
Coronary occlusion. MI was induced as described by Tarnavski et al. (42) with minimal modifications by an investigator blinded to genotype. Male 6–24-wk-old mice were anesthetized with pentobarbital sodium (60 mg/kg ip) and ventilated with 0.5% isoflurane (TOPO Dual Mode Ventilator, Kent Scientific, Torrington, CT). Body temperature was maintained at 37°C with a warming pad. A left thoracotomy was performed between the fourth and fifth ribs. Under a microscope, the left anterior descending coronary artery (LAD) was permanently ligated 1 to 2 mm below the tip of the left auricle with 8-0 nylon sutures (Syneture, Covidien, Mansfield, MA). Successful LAD ligation was verified by the observed blanching of the anterior wall of the LV distal to the ligation. The chest cavity was closed by bringing together the fourth and fifth ribs with nylon sutures (6-0, S&T, Neuhausen, Switzerland), followed by suturing the muscle and skin layers. The mice were allowed to recover. A sham operation procedure was performed in additional animals by placing a nonrestrictive suture around the artery. At 24 h and 30 days after MI, the hearts were excised under isoflurane on the day of the implant and 5–7 days after pump placement. Retroorbital blood was collected using Natelson blood collection tubes (Fisher Scientific, Pittsburgh, PA) pretreated with 1% AquaSil Siliconizing Fluid (Pierce, Rockford, IL) for 10 min, rinsed with 100% methanol, and air dried overnight. Collected blood was deposited into iced microcentrifuge tubes containing 2 μl of 500 mM EDTA (pH 8.0) and 10 μl of 0.6 trypsin inhibitory units of aprotinin (Sigma-Aldrich). Blood was spun at 1,460 × g for 20 min, and plasma was collected and stored at −80°C. ANP levels were later analyzed with an enzyme-linked immunosorbent assay as described by the manufacturer (Cat. No. EK-005-24, Phoenix Pharmaceuticals, Burlingame, CA). Eleven days after minipump insertion, the mice underwent ligation of the LAD as described above. Twenty-four hours later, the hearts were excised under deep anesthesia and subjected to analysis.

Analysis of myocardial infarct size. Twenty-four hours after actual or sham-operated LAD ligation, the animals were deeply anesthetized with pentobarbital sodium, and 1 ml of 2% Evans blue dye (Sigma-Aldrich) was perfused into the right jugular vein. The dye distributed throughout the patent vasculature, allowing the identification of the nonperfused or ischemic area in the heart. The heart was then excised, the right ventricle was removed, and the LV was sliced into four 2-mm cross sections below the ligature. Each section was weighed and placed on a Plexiglas holder and secured with spring clamps, and both sides were digitally photographed for quantification. Myocardial tissue was then stained in 1% 2,3,5-triphenyl-2H-tetrazolium chloride (Sigma-Aldrich) wt/vol in sodium phosphate buffer (pH 7.4) at 37°C for 15 min. Viable myocardium stained brick red, and the infarcted area appeared pale white. Both sides of the tissue were digitally photographed for quantification, and the heart slices were fixed in 10% neutral-buffered formalin (Sigma-Aldrich). Digital images of the infarct area (IN), the ischemic area at risk for infarction (AAR), and the total LV area were assessed by two observers blinded to the genotypes using analysis software (NIH ImageJ). For each photograph, the planimetric ratios of AAR to LV area and IN to LV area were multiplied by the weight of the heart slice. The weighted ratios were averaged and expressed as percentages to estimate the amount of injured tissue within each heart.

Immunohistological analysis. Twenty-four hours after actual or sham-operated coronary ligation, 2-mm-thick heart sections were fixed in 10% neutral-buffered formalin. Each section was paraffin embedded and sliced into 4-μm samples that were mounted on slides (Superfrost plus, VWR, Swumee, GA). After air drying and heating at 60°C for 30 min, the slides were deparaffinized and pretreated with Target Retrieval Solution (pH 6.0) (Dako, Carpintera, CA). Endogenous peroxidase activity was quenched with 0.3% H2O2. As previously described, infiltrating neutrophils were identified by their myeloperoxidase staining (17). Myocardial sections were incubated with a rabbit anti-myeloperoxidase antibody (1:1,000, 30 min, Dako). After being rinsed, the sections were incubated with a secondary peroxidase-conjugated AffiniPure [F(ab')2] fragment donkey anti-goat antibody (1:100 dilution, Jackson ImmunoResearch, West Grove, PA) for 1 h. After the sections were rinsed twice with phosphate-buffered saline at room temperature, the bound antibody was detected with diaminobenzidine substrate (Dako). Negative control slides were processed in a similar manner but lacked the primary antibody. The slides were counterstained with hematoxylin (Richard-Allan Scientific, Kalamazoo, MI). The slides were analyzed under a light microscope at ×1,000 magnification. Myeloperoxidase-stained cells were counted in 20 randomly selected fields in noninfarct zone and averaged.

Additional sections were probed for P-selectin by immunohistochemistry using a goat anti-P-selectin antibody (1:100 dilution, Santa Cruz Biotechnology, Santa Cruz, CA) for 30 min at room temperature. The P-selectin antibody was detected as described above. The average P-selectin stained area in each heart was assessed in seven randomly selected fields containing vessel sections at ×200 magnification using the computerized planimetry analysis software program (NIH ImageJ).

Hearts excised from mice surviving 30 days post-MI were similarly pretreated in 10% neutral-buffered formalin, embedded, cut in 4-μm sections, and stained with Masson’s trichrome to assess the extent of fibrosis. The total amount of collagen staining was assessed in whole heart slices by computerized planimetry (NIH ImageJ). The amount of fibrosis in the noninfarcted area was calculated from six randomly selected fields in the LV at ×100 magnification.

Haemodynamic measurement. Mice that survived 30 days after LAD ligation were assessed for heart function as we have previously described (16). Anesthesia was induced with 3% followed by 1.5% isoflurane once the mice were intubated. Body temperature was maintained at 37°C by a warming pad. A midline incision in the neck was made, and the right common carotid artery was cannulated using a Millar catheter (model SPR-671, Millar Instruments, Houston, TX). The catheter was advanced into the aorta, passed through the aortic valve, and stabilized in the LV cavity. Ventricular catheterization was successful in all but two mice. The catheter was connected to a pressure transducer (model TCB-600, Millar Instruments), and real-time measurements were recorded using PowerLab software (ADInstruments, Colorado Springs, CO). The aortic blood pressure, heart rate (HR), and systolic and diastolic blood pressures were measured for 10 min in each mouse, and the values for each measurement category were averaged by PowerLab Chart 5 software from the real-time data collected during the observation period. The HR (in beats/min) and systolic and diastolic LV pressures (in mmHg) were recorded and averaged. The peak maximum and minimum first derivative of pressure (dP/dt max and dP/dt min, respectively, in mmHg/s) were obtained from the LV.

Statistics. Data are expressed as the means ± SD unless otherwise indicated. Differences in survival were assessed by a Kaplan-Meier analysis using GraphPad Prism 4.0 (La Jolla, CA). An unpaired Student’s t-test was used to assess the significance of variance between group means. A P value of <0.05 was considered to be significant.
RESULTS

We examined whether ANP affected mortality after coronary occlusion by analyzing the survival of ANP−/− and congenic ANP+/+ (wild-type) male mice. After coronary occlusion, the survival in wild-type mice was at 20% at 30 days, with most of the mortality occurring within the first week. In contrast, ANP−/− mice had markedly better survival with 56% surviving 30 days (P < 0.01, Fig. 1). Nearly all ANP−/− mice also died in the first week following MI.

Cardiac catheterization was performed to determine whether there were significant differences in the hemodynamics of ANP−/− and ANP+/+ mice. There were no differences between the groups in HR or mean, diastolic, and systolic blood pressures (Table 1). LV end-diastolic pressures were normal, indicating that there was no heart failure. However, the ANP−/− mice had lower indexes of contractility than ANP+/+ mice as assessed by dP/dmax and dP/dmin (P < 0.05). When hearts were harvested at 30 days, the ratio of heart weight to body weight was greater in the ANP−/− mice than in wild-type mice (5.5 ± 0.7 vs. 3.5 ± 0.7 mg/g, P ≤ 0.001) as previously reported (33). Masson’s trichrome staining showed that the collagen scar occupied 20.9% of the total myocardium of ANP+/+ mice versus 16.9% of ANP−/− mice, but this did not achieve statistical significance. Outside of the infarct area, there was increased collagen deposition in the hearts of the ANP−/− versus ANP+/+ mice (P < 0.01).

The high mortality rate of mice within the first few days after MI suggested that survival may be due to differences in infarct size. Consequently, we assessed the myocardial AAR and compared it with the infarct area 24 h after actual or sham-operated coronary ligation. In mice undergoing sham-operated coronary ligation, there were no detectable areas of myocardium at risk for ischemia, and there were no detectable infarctions in either ANP−/− or ANP+/+ mice (Fig. 2, E–H and I). In contrast to the 30-day postinfarct data, heart sizes normalized to body weight were the same in both ANP+/+ and ANP−/− mice [3.1 ± 0.2 vs. 3.3 ± 0.2 mg heart/g body wt, P = not significant (NS)]. In mice subjected to coronary ligation, the AAR was not significantly different between ANP−/− mice versus ANP+/+ mice (Fig. 2, A, C, and D). However, the total size of the infarct was significantly smaller (~38%) in ANP−/− mice (Fig. 2, B, D, and I) when expressed as a proportion of the AAR (P < 0.01) or ~48% smaller when expressed as a fraction of LV mass (infarct mass:LV mass, P < 0.01).

To be certain that these differences were due to ANP and not to secondary changes that may be related to congenital deficiency of ANP, we examined infarct size in ANP−/− mice supplemented with ANP via an osmotic minipump for 11 days before LAD ligation. In ANP−/− mice supplemented with ANP, circulating levels of ANP rose significantly (from 0.3 ± 0.3 to 9.9 ± 8.5 ng/ml, P < 0.01). After LAD ligation, the infarct size (IN/AAR) in ANP−/− mice supplemented with ANP (83.7 ± 10.2%) was significantly larger than in ANP−/− mice (62.6 ± 12.1%, P < 0.05) but not statistically different from wild-type mice alone (100.8 ± 3.8%, Fig. 2I). This indicated that ANP itself played a mechanistic role in infarct size after coronary occlusion.

In response to ischemia, there is a rapid infiltration of neutrophils into all regions of the heart (6, 15). Neutrophils are believed to be a major cause of death for marginally viable myocytes, mediated in part through the effects of myeloperoxidase (2, 15). Consequently, we examined neutrophil infiltration into the noninfarced region of the myocardium 24 h after actual or sham-operated coronary occlusion (Fig. 3, A and B). In mice undergoing sham-operated occlusion, neutrophil counts in the myocardium were indistinguishable between ANP−/− and ANP+/+ mice (Fig. 3B). There was a 2.7-fold increase in the number of neutrophils in ANP+/+ mice after coronary occlusion compared with the ANP+/+ sham-operated ligated mice (P < 0.0005). Interestingly, there was no increase in neutrophils in the myocardium of ANP−/− mice following the actual ligation versus sham operation (P = NS). After coronary occlusion, ANP−/− mice had significantly fewer myocardial neutrophils than both the ANP+/+ (P < 0.0005) and ANP−/− mice supplemented with ANP (P < 0.0005, Fig. 3, A and B). Wild-type and ANP−/− mice supplemented with ANP showed similar neutrophil levels in the myocardium after coronary occlusion (P = NS).

Neutrophil migration into the tissue is facilitated by the increased P-selectin expression that occurs throughout the ischemic myocardium (34). We examined whether the decreased neutrophil infiltration seen in ANP−/− mice was associated with diminished P-selectin expression in the vasculature of the myocardium. In the ANP−/− mice, there was significantly less P-selectin staining than in ANP+/+ mice or in the ANP−/− mice supplemented with ANP (P < 0.002, Fig. 4).

DISCUSSION

The cardiovascular effects of ANP on vasodilation and natriuresis have provided the rationale for using ANP in the treatment of heart failure and myocardial ischemia. Only more recently has ANP been recognized to have proinflammatory effects on the immune system (43). To determine the integrative biological effects of ANP during MI, we compared ANP−/− with ANP+/+ mice undergoing experimental coronary occlusion. The infarct size in ANP−/− mice was comparable with that reported for wild-type mice undergoing permanent coronary occlusion (28, 30). A deficiency of ANP markedly diminished the 30-day mortality after coronary occlusion with the greatest apparent effect within the first week. Previous studies have shown that early mortality is associated with...
larger myocardial infarcts (3). Consistent with this observation, ANP−/− mice had much smaller infarcts than ANP+/+ mice as determined by the mass of infarcted LV or by the percentage of the AAR. Pharmacological supplementation of ANP−/− mice with ANP for days before coronary occlusion significantly increased the size of the infarcts, so that they were comparable in magnitude with those seen in ANP+/+ mice. This provides strong evidence that infarct size is directly related to the effects of ANP and not to other potential confounding factors.

Neutrophils migrate into the tissue after the onset of ischemia and increase infarct size (15, 37). P-selectin is an adhesion molecule that facilitates neutrophil migration from the vasculature into the underlying tissue, and it has been implicated in myocardial cell death following ischemia (34). After coronary occlusion, there was significantly less vascular P-selectin expression in the ANP−/− mice versus ANP+/+ mice or ANP−/− mice supplemented with ANP. Similarly, Izumi et al. (17) also found that there was substantially less expression of P-selectin following MI in mice that lacked the GC-A receptor for ANP (17). P-selectin is expressed by both activated platelets and endothelial cells; unfortunately, neither of our studies pinpointed whether the differences in P-selectin expression were due to a particular cell type. However, ANP does not affect platelet function (e.g., aggregation) in
vitro (7, 26) or when infused in human volunteers (24). In contrast, ANP markedly augments NF-κB activation and associated P-selectin expression induced by H$_2$O$_2$. This suggests that under conditions of oxidative stress such as a MI, ANP increases endothelial cell expression of P-selectin (17, 22, 39). This may be due to the fact that ANP deficiency decreases NF-κB activation, which plays a role in P-selectin expression (17). Consistent with the recently described proinflammatory effects of ANP on neutrophils, there were substantially more infiltrating neutrophils in the myocardium of ANP$^{+/+}$ mice and of ANP$^{-/-}$ mice supplemented with ANP than in the myocardium of ANP$^{-/-}$ mice alone. Taken together, these data provide strong evidence that ANP increases myocardial infarct size, vascular P-selectin expression, neutrophil infiltration, and mortality following experimental coronary occlusion.

Although there were no significant differences in heart weight to body weight within 24 h of infarction, ANP$^{-/-}$ mice had significantly greater hypertrophy than did ANP$^{+/+}$ mice 30 days after infarction. ANP$^{-/-}$ mice also displayed greater collagen deposition in the noninfarcted region of the myocardium. ANP opposes the effects of the renin-angiotensin-aldosterone system on ventricular remodeling and hypertrophy (31, 38). Several lines of data indicate the hypertrophy and increased scarring in ANP$^{-/-}$ hearts 30-days post-MI is in large part due to the effects of angiotensin II and the angiotensin II type 1a receptor. Levels of angiotensin II are higher in ANP knockout mice than in normal mice (1, 32), and there are significant increases in angiotensin II post-MI (40). Similar to our study, Nakanishi et al. (30) showed that mice lacking the receptor for ANP (GC-A) have enhanced collagen fibrosis post-MI. However, that fibrosis was completely blocked in ANP receptor-deficient mice which also lacked the gene for angiotensin II type 1a receptors. The selective blockade of the angiotensin II type 1a receptor is sufficient to block post-MI fibrosis and hypertrophy (35, 40). These data argue that the enhanced fibrosis and hypertrophy we detected in ANP$^{-/-}$ mice were due in part to the unopposed activity of the angiotensin system on ventricular remodeling in ANP$^{-/-}$ mice.

There have been no previous studies that have investigated the effect of ANP on myocardial ischemia without reperfusion. In a model of acute MI similar to ours, an overexpression of brain natriuretic peptide (BNP) increased neutrophil infiltration, which is consistent with the proinflammatory effects of ANP we observed in these experiments (20). In a model of acute MI followed by reperfusion, mice lacking the GC-A receptor (the receptor for both ANP and BNP) had smaller infarcts and decreased neutrophil infiltrates in the myocardium, though a survival advantage was not shown. This was attributed to decreased P-selectin expression due to the diminished activation of NF-κB (17). However, when MI was induced without reperfusion, GC-A receptor knockout mice had diminished survival, which was attributed to the development of heart failure due to a diminished urinary excretion of salt (30).

In contrast to the lack of data on the effects of ANP on MI without reperfusion, there are several studies of the effects of pharmacological doses of ANP on ischemia-reperfusion. A pharmacological administration of ANP has been reported to reduce infarct size in vivo, but it also increased collateral blood flow, reduced end-diastolic pressure, etc. (36, 41). In these experimental studies, the effects of ANP administration on inflammation and survival were not determined. In limited clinical trials in humans with myocardial ischemia/infarction undergoing reperfusion, the pharmacological administration of ANP reduced apparent infarct size, diminished dilation of the LV, and slightly improved heart function 30 days after treatment; however, there was no difference in mortality (14, 23).

When considered in the light of previous data, it appears that ANP may exert different effects after brief versus prolonged ischemia. Brief ischemia with reperfusion and prolonged ischemia elicit different patterns of protein and gene expression, thereby activating different pathways in the heart (8). In addition, ANP like other molecules (e.g., P-selectin; see Ref. 34) may exert different effects on the myocardium depending on the duration of ischemia. Moreover, like the angiotensin II type 1A receptor, which affects late, but not early survival (13), ANP may have unique, but divergent, effects during the ischemic, reperfusion, inflammation, and wound healing/remodeling phase of MI. Given the increasing use of ANP as a treatment for human MI and concerns about the potential safety and efficacy of the natriuretic peptides (10), it will be important to clarify the role of ANP in these processes.

ACKNOWLEDGMENTS

We thank Kimberly Smith and Doris Cawley at the Georgia Research Pathology Core and Drs. Daniel Rudic, Inna Gladysheva, and Michael Brands for assistance. J. Tronolone is currently at the University of Rochester (Rochester, NY). A. Kerner is currently at Washington University (St. Louis, MO).

GRANTS

This work was supported in part by National Heart, Lung, and Blood Institute Grants HL-508496 and HL-78562 (to G. L. Reed) and by research funds provided through the Medical College of Georgia in support of research training (to J. Tronolone, A. Kerner, and A. Mohamad).
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