Enhanced early after-myocardial infarction concentration of TNF-α subsequently increased circulating and myocardial adrenomedullin in spontaneously hypertensive rats

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Abstract. Both inflammatory cytokine tumor necrosis factor-α (TNF-α) and the cardiac protective peptide adrenomedullin (AM) are increased in cardiac tissues and plasma in patients with myocardial infarction (MI) and chronic heart failure. Recently they have been increasingly recognized as important factors in the pathophysiology of MI and resultant congestive heart failure. Compared with sham-operated spontaneously hypertensive rats (SHR), we investigated myocardial immunoreactivity of TNF-α and AM and also their mutual relations *in vivo* in SHR+MI. Residual myocardial depression after MI was studied also in isolated perfused hearts.

In chronic experiments, 24 and 48 h after permanent ligation of the descending anterior branch of the left coronary artery, we examined hemodynamics, plasma and myocardial peptide levels. Left ventricular function was assessed in isolated perfused hearts subjected to "global ischemia and reperfusion" and after induction of "calcium paradox". Circulating and myocardial TNF-α concentrations increased early after MI in SHR. Studies with global ischemia and calcium paradox in isolated heart showed early myocardial depression and calcium-dependent gradual increase of left-ventricular end-diastolic pressure. In the SHR+MI myocardial AM concentrations were increased 9- and 49-fold after respective 24 h and culminated 48 h following MI.

Circulating and myocardial AM was increased in SHR+MI in association with TNFα-induced myocardial depression. The both studied cardiac parameters displayed the beneficial effect of the enhanced myocardial AM concentration.

Key words: Adrenomedullin — Tumor necrosis factor-α — Radioimmunoassays — Spontaneously Hypertensive rat — Myocardial infarction — Isolated perfused heart — Left ventricular end-diastolic pressure

Introduction

Despite advances in the management of myocardial infarction (MI), risks of excessive mortality and recurrent episodes persist in patients who recover from an acute attack. Findings of elevated blood levels of cytotoxic tumor necrosis factor-α (TNF-α) and occurrence of chronic heart failure in these patients were arguments for formulating the hypothesis that TNF-α is the main contributing factor in cardiac pathology. We recently showed that cytotoxic TNF-α and proliferative adrenomedullin (AM) were elevated in hypoxic human cells (Drimal et al. 2005, 2006). Previous studies reported elevated circulating TNF-α levels in patients with acute MI (Maury and Teppo 1989; Lissini et al. 1992; Latori et al. 1994). Increased plasma AM accumulation was found in patients with acute MI and congestive heart failure (Kobayashi et al. 1996; Nishikimi et al. 1997; Nagaya et al. 1999a,b; Yu et al. 2001). AM generation in cardiovascular tissues is regulated by inflammatory cytokines, thus TNF-α strongly stimulates secretion of AM (Sugo et al. 1995; Isumi et al. 1998). The elevated plasma AM has been identified as having a putative beneficial role in re-

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duced cytokine expression in the infarcted myocardium, early after acute MI (Hamid and Baxter 2005; Deten et al. 2003; Ono et al. 1998). This may accentuate the possible regulatory role of AM in cardiac function, especially following acute MI. However, little is known about the mechanisms through which the proliferative hormone AM and myocardial depressant TNF-α exert their effects in the infarcted heart. Similarly little understood is the role played by AM in TNF-α expression and cardiac pathology. In a rat model of experimental MI, we therefore investigated the pathophysiological relevance of AM and TNF-α early after occlusion of the coronary artery.

Materials and Methods

All procedures were approved by the Animal Care and Use Committee at the Istitute of Experimental Pharmacology and are conform to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, publication No. 85-23, revised 1996 and State Veterinary and Food Product Inspection (Slovakia)). MI was induced surgically in spontaneously hypertensive rats (SHR) (Dobrá Voda, Slovakia). Male SHR weighing 230 to 250 g were anesthetized by intraperitoneal injection of sodium pentobarbital (30 mg/kg), intubated and ventilated with a small-animal respirator with setting of the respiratory pump according to nomograms for small laboratory animals (Biology Data Book, Altman and Dittmer 1964). The heart was exposed *via* left anterior thoracotomy and the left anterior descending coronary artery was ligated by permanent suture (Ethicon). The thorax was closed by layers and pneumothorax was evacuated. The control SHR underwent sham operation consisting of thoracotomy, pericardiotomy but without coronary ligature. In MI animals, spontaneous respiration was reconstituted within 3 min of intermittently activated pump. The rats were injected with morphine sulphate 60 min after MI. The surviving rats were maintained on standard rat chow, each in a separate box, water ad libitum, in the animal house.

Langendorff heart perfusion

Male SHR were anesthetized, heparinized (1000 i.u./kg), the thoracic cavity was opened, the aorta cannulated in situ, the heart was then excised, mounted in the perfusion apparatus and perfused according to the Langendorff method with a modified Krebs bicarbonate buffer (in mmol/l: 118.5 NaCl; 4.2 KCl; 1.75 $CaCl_2$; 1.2 Mg SO_4; 1.2 KH_2PO_4; 12.5 glucose; 25.0 $NaHCO_3$) saturated with O_2 and 5% CO_2, under constant flow rate (12 ml/min). For *in vitro* functional left ventricular measurements, a water-filled elastic balloon was inserted into the left ventricle and the left ventricular diastolic pressure was adjusted to approximately 10 mm Hg at the beginning of the experiment. The coronary flow, left ventricular pressure (with the balloon catheter), ECG and heart rate (Statham cardiovascular analyzer) were constantly recorded on a six-channel NEK-6T physiograph. After 20 min of equilibration and 10 min of recording, saline (control) or pharmacologically active peptides were shortly infused with an infusion pump (0.1 ml/min, *via* the side arm in the perfusion line). The infusion was followed by 30-min global normothermic ischemia and subsequent by 30-min reperfusion.

Measurements of AM and TNF-α

Plasmatic levels of inflammatory cytokines (TNF-α and AM) were measured by RIA and ELISA on using commercially available kits (Amersham Biosciences, UK) and microplate multiscan reader (Finnland). Ventricular tissue was homogenized in phosphate-buffered saline which contained 1% Triton along with a protease inhibition cocktail. The homogenate was centrifuged at 4.500 × *g* for 25 min at 4°C. The supernatant was colleted and the levels of peptides were measured using sandwich ELISA kits (Amersham Biosciences) for rat AM and TNF-α. The assays were performed according to the manufacturer´s instructions. Absorbance of standards and samples was determined with the aid of multiscan reader. Protein concentration was determined by the method of Bradford (1976) using bovine serum as standard.

Chemicals

Human $AM_{(1–52)}$ (Calbiochem), human angiotensin-II (Calbiochem), aprotinin, BSA, leupeptin, pepstatin A, pentoxifylline (Calbiochem), PMSF, p-nitroblue tetrazolium (Sigma) sep-pack $C_{(18)}$ (Amprep), human TNF-α (Bachem).

Statistical analysis

All data are expressed as mean ± SEM, where *n* is number of animals, or experiments. Data were compared statistically by ANOVA. Values of $p < 0.05$ were considered significant.

Results

SHR were continuously monitored during the whole surgical procedure (Table l). The SHR model of chronic MI was characterized electrocardiographically immediately, 24 and 48 h after induction of coronary occlusion. ECG showed the signs of acute MI with a significant elevation of the ST-segment, manifested mostly in the standard lead II and III with rather unchanged duration of PQ and QRS intervals in the MI group. The arrhythmias occurred 24 and 48 h after MI, the response of the heart to permanent occlusion was manifested by ventricular extrasystoles (single or in volleys), mostly polytopic (or monotopic) in character. Comparable with the occurrence of ventricular arrhythmias, there was de-

Table 1. Spontaneously hypertensive rats (SHR), heart rate and rate-dependent functions, perioperative record, immediately after coronary ligature

Parameter	Procedure (n = 48)	
	Sham SHR	SHR+MI
Body weight (g)	293 ± 11	305 ± 24
Heart rate (b/min)	360 ± 27	389 ± 8
PQ interval (ms)	82 ± 13	78 ± 6
QRS interval (ms)	28 ± 4	27 ± 3
QTC interval (ms)	133 ± 12	120 ± 11
ST-segment (mm)	0 ± 0	3.00 ± 0.15*
MI infarct (size in %)	–	39 ± 5*

Mortality and final number of animals: the total mortality among rats subjected to coronary ligation was 38.2%, of these 21% died either immediately, or up ro 30 min after procedure (exitus in tabula) and remaining 7% died up to 12 h after ligature. Heart rate of these animals after ligature was significantly higher (448 ± 14 b/min, $p < 0.05$) and also ST-segment was elevated (+4.1 ± 0.2 mm). MI, myocardial infarction; * statistically significant.

pletion of creatine-phosphokinase activity 24 and 48 h after the intervention. At postmortem examination one week after MI, macroscopically the area of infarction was slightly elevated, with the pericardium healed up to the myocardial surface, with marked signs of neovascularization. Histologically, there was evidence of focal perivascular lymphocyte infiltrations, extravasation, activation of fibroblasts, signs of focal inflammation in the area of occlusion.

Hemodynamic variables

As expected SHR showed higher systemic mean arterial blood pressure (188 ± 9, $p < 0.05$). A further increase in mean arterial blood pressure was observed in SHRs after short intravenous infusion (25 μg/kg) of norepinephrine (+27 ± 10%), 10 μg/kg of endothelin-1 (+26 ± 5%) and 50 μg/ kg of angiotensin-II (+60 ± 8%). The maximal response of arterial blood pressure 24 and 48 h after induction of infarction was slightly reduced to +19 ± 6% after norepinephrine, to +17 ± 8% after endothelin-1 and +30 ± 6% after angiotensin-II. Similarly, the response of left ventricular dP/dt (as an index of myocardial contractility) to 25 μg/kg of isoprenaline was significantly reduced in SHR with MI (Fig. 1). Furthermore, the response of diastolic arterial blood pressure to slow intravenous infusion of 25 μg/kg of acetylcholine was reduced from –49 ± 8% in SHR to respective –16 ± 7 and –22 ± 11% after MI 24 and 48 h in the group of SHR.

Effects of AM on global ischemia in SHR hearts

Functional studies with isolated heart showed pronounced decrease in left ventricular systolic pressure (LVSP) immediately after the beginning of global ischemia. Fig. 2 demonstrates the contractile behavior of the isolated perfused

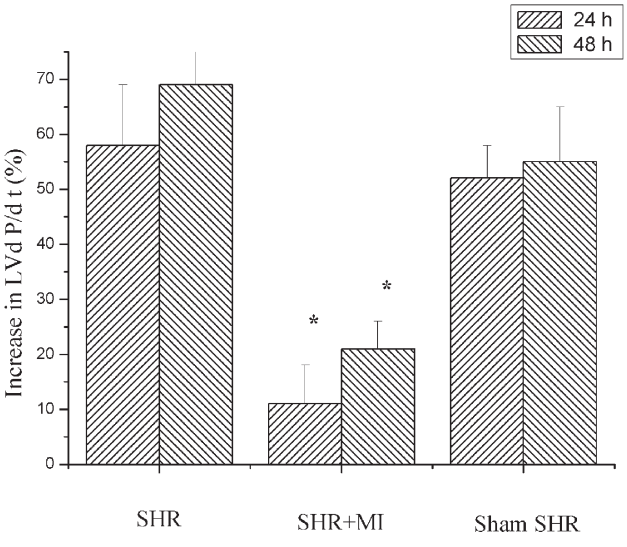

Figure 1. Left ventricular contractile force (LVdP/dt) response to intravenous test-dose (25 μg/kg) of isoprenaline in SHR, SHR+MI and sham SHR (n = 6). Values are mean ± SEM (in percent of control). MI, myocardial infarction; SHR, spontaneously hypertensive rats; sham SHR, sham-operated SHR; * statistically significant response.

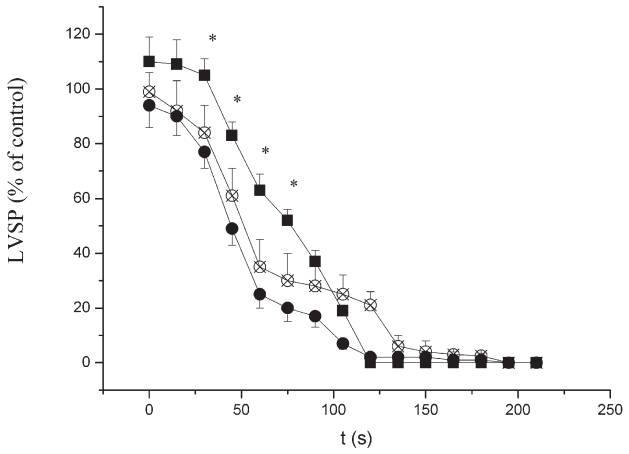

Figure 2. Effects of adrenomedullin (AM) infusion (0.5 μg/ml/30 s; ■) on left ventricular systolic pressure (LVSP) after global ischemia in isolated spontaneously hypertensive rat hearts. In control group (sham SHR; ○) rats were thoracotomized, and the thoracic cavity was closed without induction of MI, in SHR+MI group (●) MI was induced surgically and the thoracic cavity closed. SHR hearts were isolated 48 h after induction of MI and retrogradely perfused with Krebs solution. Values are mean ± SEM, (n = 36). * significant response when compared to control.

SHR heart in three groups of experiments: i) with infusion of saline (control), ii) AM (0.5 μg/ml/30 s) infused in slow infusion directly into inflow cannula, and iii) sham animals. AM significantly attenuated the decrease in LVSP in SHR+MI and in sham SHR groups. The functional studies in SHR hearts subjected to global ischemia (stop-flow for 20 min) showed additional depression after reperfusion (Fig. 3). In the second minute of reperfusion, the left ventricular end-diastolic pressure (LVEDP) gradually increased up to +55 ± 11 mm Hg ($p < 0.05$) and remained there for 3 to 4 min, to decrease then gradually. Increase in the LVEDP was followed by its gradual decrease in 7–8 min and the procedure terminated with paroxysmal ventricular tachycardia and ventricular fibrillation. Infusion of AM significantly reduced the secondary increase in the LVEDP after reperfusion of global ischemia in AM SHR+MI hearts. To investigate the mechanism(s) responsible for the beneficial effect of AM, three groups of retrogradely perfused SHR hearts were subjected to global ischemia and at appropriate time intervals LVSP was continuously measured up to the occurrence of asystole: first in sham SHR, second in the group of SHR+MI, and third after short AM infusion. Infusion of AM significantly delayed the decline in the LVSP early after stop-flow ischemia in the SHR+MI group. On reperfusion of the isolated rat heart, infusion of AM, administered shortly before cardioversion, significantly reduced dyskinesis of isolated heart and increased the possibility to defibrillate (ellectrically) the heart to permanent sinus rhythm (in 5 of 6 cases, Fig. 3).

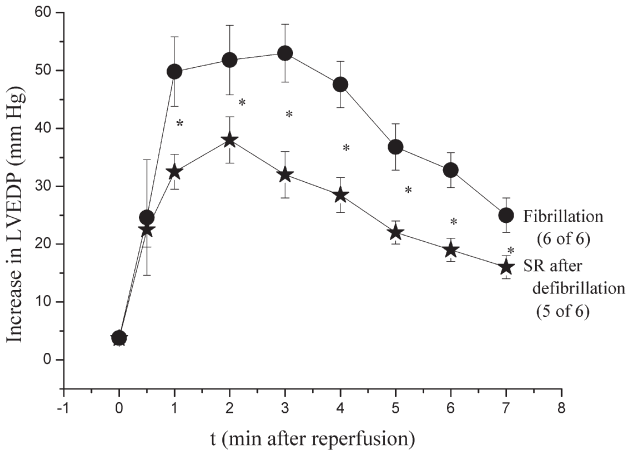

Figure 3. Increase in left ventricular end-diasolic pressure (LVEDP) in isolated rat hearts ($n = 12$), perfused with Krebs bicarbonate solution, after reperfusion of global ischemia (20 min). Explanation and symbols: ● SHR hearts after experimental MI; ★ SHR hearts, rats were pretreated with AM (0.5 μg/ml/30 s), then SHR hearts were isolated, mounted to perfusion stand, perfused 20 min with Krebs solution and then exposed to global ischemia and reperfused. SHR+MI hearts fibrillated permanently after reperfusion. In contrast, AM-pretreated SHR+MI hearts (in 5 of 6 cases) were successfully defibrillated and permanent sinus rhythm (SR) supervened. Values are mean ± SEM ($n = 12$). * significant response.

Left ventricular Ca^{2+} -dependent early depressive response ("calcium paradox")

The possible participation of TNF-α in the regulation of the LVEDP was studied in two groups of SHR, one pretreated with 60 mg/kg of pentoxifylline (vasodilator, TNF-α synthesis and T-cell adhesion inhibitors (Langstein et al. 1991)), and the other with saline. SHR were subjected to surgically induced MI, 48 h after procedure the heart was isolated and for 20 min perfused with normal Krebs solution, then the perfusion fluid was changed to low Ca^{2+} solution (Krebs without bivalent cations + 0.5 mg/ml Na_2-EDTA for 5 min). On reperfusion with normal Krebs solution, "calcium paradox" (CP) developed (i.e. secondary increase in LVEDP). The response to CP was almost identical in both groups, so was the increased response to the test-dose of norepinephrine (25 μmol/ml) in the second minute of reperfusion, only the restitution phase of the response (recovery) was much faster in the pretreated group.

Circulating arterial and myocardial concentrations of TNF-α and AM in SHR with experimental MI

As shown in Fig. 4, in comparison with sham SHR, more than 3.4-fold increase in circulating TNF-α (mixed venous blood sampled from the right ventricle) concentration was detected in the group of SHR+MI. Increased circulating

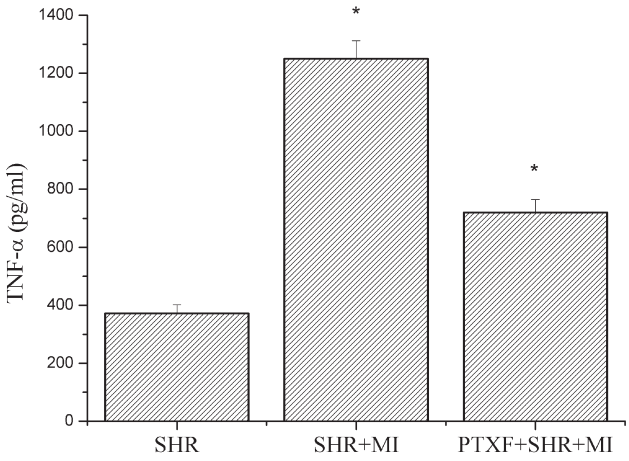

Figure 4. Circulating tumor necrosis factor-α (TNF-α) concentrations in spontaneously hypertensive rats (SHR) subjected to experimental myocardial infarction (MI) and SHR pretreated with pentoxifylline (PTXF, 60 mg/kg intraperitoneally, prior to MI). * statistical significance compared to response in SHR.

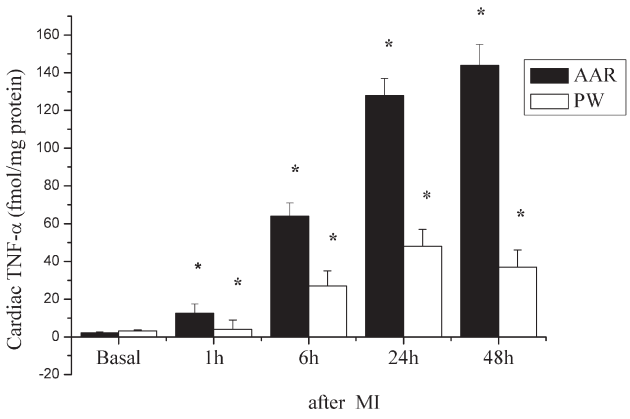

Figure 5. Cardiac tumor necrosis factor-α (TNF-α) concentrations were significantly higher in the infarcted area (area at risk, AAR) in the noninfarcted heart (posterior wall (PW) of the left ventricle; $n = 12$). * statistically significant increase. MI, myocardial infarction.

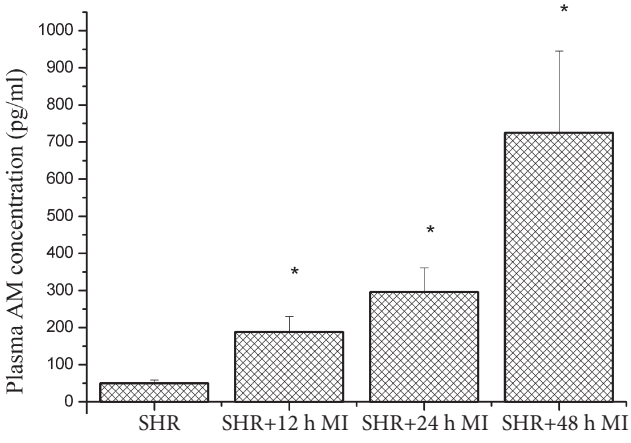

Figure 6. Circulating adrenomedullin (AM) concentration in spontaneously hypertensive rats (SHR) and SHR 12, 24 and 48 h after myocardial infarction (MI). The value at each time interval represents the normalized mean ± SEM for 6 SHR. Basal plasma AM values in SHR were 9.28 ± 1.16 pg/ml ($n = 24$), the control Wistar rats showed low plasma AM levels (3.21 ± 0.26 pg/ml). * statistical significance ($p < 0.05$) compared with SHR group.

TNF-α concentrations were markedly reduced (–42 ± 8%, $p < 0.05$) in pentoxifylline-pretreated SHR+MI animals. Impairment in cardiac performance and significantly increased levels of myocardial TNF-α concentrations were observed early after induction of MI (Fig. 5). The myocardial concentrations of TNF-α increased simultaneously and significantly in both myocardial locations, with a major increase in the myocardium near the area of occlusion (always the anterior descending branch of the left coronary artery (area

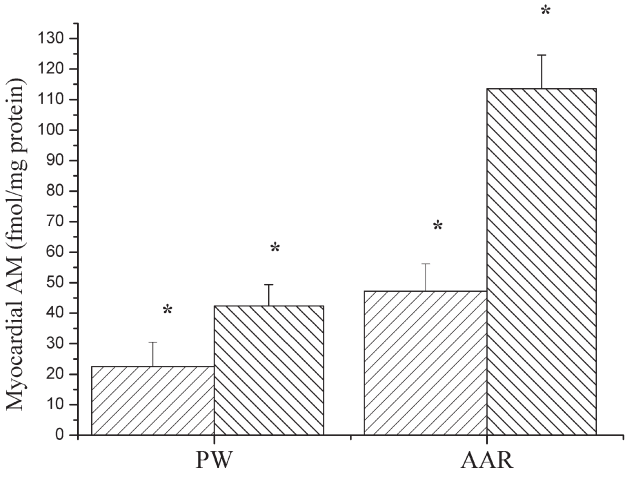

Figure 7. Myocardial concentration of adrenomedullin (AM) in SHR 24 h (first column) and 48 h (second column) after induction of MI. Basal concentration of AM in control Wistar rats: 5.23 ± 1.9 fmol/mg of protein. PW, posterior wall of the left ventricule; AAR, area at risk (from the drainage of the anterior branch of the left coronary artery, where the suture was always situated); * significantly increased concentration of myocardial AM. Note: maximal increase in myocardial concentration of AM in SHR was seen in the compromized area (AAR) of the left ventricle.

at risk, AAR), and with a minor increase in TNF-α was seen somewhat later also in the noninfarcted myocardium (posterior wall of the left ventricle). In SHR+MI, the venous plasma AM concentrations (again the mixed venous blood, sampled from the right heart) were significantly higher then the corresponding arterial AM concentrations. Venous AM concentration 24 and 48 h after MI was significantly higher (172 ± 6 and 244 ± 32 pg/ml; $p < 0.05$) than were the corresponding concentrations of AM in the arterial blood (149 ± 25 and 241 ± 8 pg/ml). Circulating arterial AM concentrations (Fig. 6) increased significantly 24 h after MI. The arterial plasma AM levels in the infarcted SHR reached their peak in approximately 48 h after MI and decreased gradually thereafter to the values seen after 12 h of ischemia, while arterial blood concentrations of AM remained elevated in SHR+MI for more than 7 days (Fig. 7).

Ventricular AM concentrations in infarcted and noninfarcted myocardium

Ventricular AM concentrations 24 and 48 h after induction of MI increased 4.3 and 8.0-fold in the noninfarced posterior wall of the left ventricle, and much more elevated concentrations of AM (9.0-fold and 49-fold increase) were measured out in the infarcted area of the left ventricular myocardium. Ventricular AM concentrations in SHR+MI tended to be increased compared with those of sham SHR for more than 7 days after MI.

Discussion

In the present study, we clearly demonstrated that i) both circulating and myocardial TNF-α concentrations were increased in SHR with MI as an early signal, ii) circulating and myocardial concentrations of TNF-α were increased in SHR after MI and that increase was much more pronounced in the infarcted myocardium, iii) plasma and myocardial AM concentrations were increased with a latency and that the maximal increase in myocardial and circulating AM was seen 48 h after induction of experimental MI with the maximally increased myocardial AM concentration in the infarcted area of the left ventricle, and iv) furthermore, by using RIA of TNF-α and AM concentrations in circulating arterial and venous blood, we demonstrated the adverse effects of TNF-α on the heart. The SHR+MI rats pretreated with TNF-α synthesis inhibitor pentoxifylline, expectedly, showed lower circulating concentrations of TNF-α. Previous studies in rat infarction reported 1.7-fold increase in myocardial AM peptide levels in the noninfarcted region and 1.5- fold increase in AM in the infarcted area (Oie et al. 2000; Nagaya et al. 2000). AM infused after induction of MI in rats improved survival in cardiac heart failure (Nakamura et al. 2004). In this study, we clearly showed a marked elevation of myocardial AM concentration in the area of infarction (9- and 49-fold increase 24 and 48 h after MI). Significantly lower were the myocardial AM concentrations in the noninfarcted left ventricular wall (showing 4.3- and 8-fold increase). The findings of short latency of the increased circulating and myocardial AM concentration after MI in SHR in the present study and the protective effects of short intravenous infusion of AM administered before induction of infarction in the spontaneously hypertensive rodent model may be indicative of the beneficial effects of AM on cardiac failure in SHR with MI. Based on the data presented here, the following hypothesis is forwarded: myocardial and circulating AM may play a role in protecting the heart from damage during MI and TNF-α production in the cardiovascular system. TNF-α depresses myocardial function and AM production early after MI in SHR was found to be beneficial.

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