Inducible nitric oxide synthase activation after ischemia/reperfusion contributes to myocardial dysfunction and extent of infarct size in rabbits: evidence for a late phase of nitric oxide-mediated reperfusion injury

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Abstract

Background: Ischemia/reperfusion (I/R) leads to the induction of inducible nitric oxide synthase. The present study investigated the effects of selective and continuous inhibition of iNOS on myocardial performance, infarct size and histomorphological changes after I/R in rabbits. Methods and Results: Ischemia/reperfusion (I/R) was induced by occlusion of the circumflex coronary artery for 30 min followed by 48 h of reperfusion. Sham animals (group A) served as control. Three groups were subjected to I/R: (B) placebo; (C) aminoguanidine (AMG; 10 mg/kg bolus) given prior to and 48 h after I/R to test its acute effects; (D) AMG (300 mg/kg/day sc) to test effects of continuous treatment. Hemodynamics, myocardial blood flow, infarct size, iNOS activity, cGMP levels, immunohistochemical analysis of iNOS expression and AMG tissue levels were determined. Continuous AMG treatment improved myocardial performance (hemodynamics and blood flow) compared to placebo group. iNOS was highest in placebo-treated animals. AMG tissue levels were highest in tissues affected by I/R. Infarct size (% of the circumflex region) was significantly smaller in group D when compared to group B. Conclusions: This is the first study showing that activation of myocardial iNOS isozyme during 48 h of reperfusion contributes to a late phase of I/R-induced injury in rabbits. Selective and continuous modulation of iNOS by AMG over this time period exerts protective effects with respect to myocardial performance, coronary blood flow, cellular infiltration and reduction of infarct size; this may be a novel therapeutic approach in the clinical situation to limit irreversible myocardial injury associated with ischemia and reperfusion. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Nitric oxide; Hemodynamics; Regional blood flow; Reperfusion; Infarction

1. Introduction

Several distinct mechanisms are involved in the detrimental process of myocardial reperfusion following ischemic periods; this include neutrophil adhesion to endothelial cells within the microcirculation (leading to the no reflow phenomenon), excessive increase of intracellular Ca ++ and generation of oxygen-derived free radicals [1-6]. Several studies have shown, that some of these detrimental events which take place within seconds or minutes after initiation of reperfusion were abolished by modification of the reperfusate [7-9]. However, injury caused by reperfusion may also occur in later stages (hours or days) after initiation of reperfusion. In this regard, induction and activation of proinflammatory mediators such as cytokines and inducible nitric oxide synthase (iNOS) have been shown to contribute to myocardial injury [10,11]. Upregulation of iNOS leads to excess nitric oxide production for prolonged periods of time and may account for oxynadical-mediated myocardial damage or act as a negative inotrope due to myocardial cGMP production [12]. Previous studies have shown that the inducible nitric oxide synthase is activated 24 to 72 h after onset of myocardial ischemia and is associated with increased NO

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production. In addition, we have previously shown that nonisoform selective inhibition of NOS aggravates ischemia-induced myocardial dysfunction. In contrast, the acute administration of isoform selective inhibitors of iNOS exerts beneficial effects on myocardial performance in this model [13].

Selective iNOS inhibitors have also been tested in several other experimental settings in various disease states. The principle idea is the reduction of toxic amounts of iNOS-derived NO in infiltrating inflammatory cells and cardiomyocytes to avoid nonspecific tissue injury and contractile dysfunction while preserving low concentrations of NO released by the endothelial constitutive NOS (eNOS) which accounts for the physiological vasomotor response and antithrombogenic properties.

Aminoguanidine (AMG), a nucleophilic hydrazone derivative, is currently under investigation as a selective inhibitor of the inducible nitric oxide synthase. It has been shown that AMG exerts beneficial effects in several disease states including acute heart and lung allograft rejection and autoimmune myocarditis [14–18].

Based on our previous findings, the present study investigated the hypothesis that a significant increase of iNOS activity is present 48 h after myocardial ischemia and reperfusion (I/R). In order to confirm, that NO derived from iNOS plays an important role after I/R we investigated the effects of acute and continuous administration of AMG, a selective iNOS inhibitor, on left ventricular hemodynamics, regional myocardial blood flow, infarct size and histomorphological changes in this model.

2. Methods

2.1. Experimental protocol and measurement of left ventricular hemodynamics

The experimental protocol was approved by the institutional Animal Care and Use Committee. New Zealand rabbits were randomly assigned to one of the four groups: A (n=6): sham; B (n=7): ischemia/reperfusion and treatment with placebo (saline; 3.0 ml/sc three times a day); C (n=6): treatment with aminoguanidine (AMG, 10.0 mg/kg/iv bolus) once given upon initiation of reperfusion (absence of iNOS) and again 48 h after I/R (presence of iNOS); D (n=9): continuous treatment with AMG (300.0 mg/kg/day/sc) over 48 h starting upon reperfusion. The daily dose was divided in three dosages and dissolved in 3.0 ml saline. The time points chosen for determination of iNOS-mediated effects on I/R was based on the finding that this isoenzyme was maximally increased 48 h following I/R suggesting that the inhibitor would exert maximal effects on hemodynamics and blood flow. The time course of iNOS activation in the I/R region in this model is depicted in Fig. 1. For this reason additional animals were subjected to the same protocol of I/R. iNOS activity was measured at 0.5 h (n=4), 24 h (n=6), 48 h (n=7), 72 h (n=5), 96 h (n=4) and 120 h (n=4).

Left ventricular end systolic pressure (LVESP) response to various intravenous concentrations of AMG (1.0, 3.0, 10.0 30.0 and 50.0 mg/kg) were determined in additional animals at various time points after I/R (prior to I/R and 30 min after I/R: n=10; 24 h after I/R: n=5; 48 h after I/R: n=5) and is shown in Fig. 2. To account for acute effects of AMG (10.0 mg/kg/iv bolus) between the 30 min and 48 h time intervals, we used additional animals at 24 h (n=6) after initiation of reperfusion. The AMG dosages selected for acute and continuous inhibition of iNOS were chosen from preliminary experiments showing potent iNOS inhibition in vivo without any signs of adverse events.

In the placebo group (B) and continuous AMG treatment group (D) the myocardial response to the β-adrenoceptor agonist isoproterenol (30 ng/kg) given via a left atrial catheter was tested 48 h after I/R in the presence of increased iNOS activation (placebo group) and absence of iNOS activation (continuous AMG treatment). The dose assured significant cardiac specific response without significant systemic effects, which in turn would have resulted in alterations of the hemodynamic response.

The time interval chosen (48 h after I/R) was based on the time course of iNOS induction in this model. Inducible NOS activity significantly increased 24 to 72 h (max. at 48 h) after I/R and subsequently decreased over time comparable to findings described previously [13]. We hypothesized that administration of the inhibitor during the time period of enzyme activation exerted maximal effects on hemodynamics and changes in myocardial blood flow.

Heart rate, left ventricular end systolic (LVESP) and end diastolic pressures (LVEDP), mean arterial pressure (MAP), maximum positive and negative dP/dt;
The effects of various concentrations of aminoguanidine (AMG) on left ventricular end-systolic pressure (LVESP) response over time is shown. A dose-dependent increase of LVESP is observed only 48 h after initiation of I/R. In addition, intravenous administration of AMG at dosages of 30.0 mg/kg and 100.0 mg/kg does not further increase the pressure response observed after 3.0 and 10.0 mg/kg/iv (A). Prior to I/R and 30 min after I/R: n=5; 24 h after I/R: n=5; 48 h after I/R: n=5. Data are given as mean±SD. * P<0.05 vs. response to similar concentrations given prior to I/R.

Fig. 2. The effects of various concentrations of aminoguanidine (AMG) on left ventricular end-systolic pressure (LVESP) response over time is shown. A dose-dependent increase of LVESP is observed only 48 h after initiation of I/R. In addition, intravenous administration of AMG at dosages of 30.0 mg/kg and 100.0 mg/kg does not further increase the pressure response observed after 3.0 and 10.0 mg/kg/iv (A). Prior to I/R and 30 min after I/R: n=5; 24 h after I/R: n=5; 48 h after I/R: n=5. Data are given as mean±SD. * P<0.05 vs. response to similar concentrations given prior to I/R.

2.2. Surgical preparation

Anesthesia was maintained with pentobarbital as described [19]. An 18-g catheter was inserted into the left common carotid artery and advanced into the aortic root for measurement of hemodynamics and withdrawal of reference blood samples. An 18-g catheter was inserted into the left ventricle via the left ventricular apex for continuous measurement of hemodynamics. A 20-g catheter was inserted into the left atrium for injection of microspheres and for administration of isoproterenol in groups B and D. After completion animals were allowed to stabilize for 30 min prior to the of start the experimental protocol.

To produce myocardial ischemia the circumflex coronary artery was occluded midway between left atrial appendage and apex, using a 4-0 prolene suture. In sham animals the suture was kept in place without ligation for later determination of the circumflex region. Appearance of cyanosis and bulging of the anterolateral aspect of the left ventricle documented successful coronary occlusion. After 30 min of complete ischemia the reperfusion phase was initiated by re-opening the occluded artery. The sutures were kept in place without ligation. At the end of the experiment the chest was closed in layers and animals were inspected daily until their scheduled re-operation.

2.3. Measurement of myocardial blood flow

Myocardial blood flow was measured by injecting four different colors of fluorescent microspheres in random order at definite time points: (1) when animals had returned to stable hemodynamic conditions (baseline); (2) after initiation of ischemia, (3) after initiation of reperfusion and (4) 48 h after I/R.

Approximately 250 000 fluorescent microspheres (15 μm in diameter; blue, blue–green, yellow–green, orange or red; Triton Technology, San Diego, CA) were vortexed, sonicated and injected into the left atrial catheter followed by a flush of heparinized, prewarmed saline (3.0 ml; 37°C) [13]. Reference blood samples were withdrawn from the carotid artery catheter at a rate of 1.36 ml/min (Harvard Apparatus, Dover, MA) starting 20 s before injection of microspheres for a total of 120 s. During microsphere filtration, lipids, triphenyl tetrazolium chloride and Evans blue dye were removed by rinsing filters with a solution consisting of 2% TWEEN 80 in distilled water (60%) and
ethyl alcohol (40%). Measurement of emission peaks was performed using a fluorescence spectrophotometer.

2.4. Determination of infarct size

After euthanasia hearts were removed, mounted on a Langendorff apparatus (Hugo Sachs Electronics) and perfused with saline for 1 min. To account for the circumflex region, the suture was re-occluded prior to perfusion. Infarcted regions were determined using triphenyltetrazolium–chloride and Evans Blue Dye [20]. Left ventricular rings were placed between clear overlays and regions were traced on paper. Areas were planimetered using appropriate software. Infarct size was expressed as a percentage of the risk (circumflex) region.

2.5. Nitric oxide synthase assay

Approximately 40.0 mg of tissue from the infarcted and non-infarcted regions were homogenized in 1.0 ml of Tris buffer (0.05 M, pH 7.4). Supernatants were adjusted to a protein content of 2.0 mg/ml using protein assay kit PS656 (Sigma) with bovine serum albumin as standard, and were used for the enzyme activity assay immediately. L-[14C]arginine was purified by column cation-exchange chromatography. Constitutive NOS (cNOS) activity was measured in the presence of NADPH (1 mM), calmodulin (30 nM), Ca++ (2 mM) and tetrahydrobiopterin (5 μM) (all from Sigma). Inducible NOS (iNOS) was determined in the presence of the above factors and EGTA (5 mM) and without Ca++. NOS activity (fmol/μg/min) was linear with respect to time for at least 30 min.

2.6. Determination of myocardial cGMP levels

Left ventricular myocardium was analyzed in duplicates using a radio immunoassay kit (TRK 500 Amersham, Arlington Heights, IL). Frozen myocardial samples were homogenized with 0.5 ml of 6% trichloroacetic acid (TCA) at 4°C in a glass tissue grinder and centrifuged at 4°C (10 000 g; 10 min). The supernatant was removed and extracted four times with 5 ml of water-saturated diethyl ether. The aqueous phase was lyophilized and the residue was dissolved in 0.3 ml of assay buffer. The protein pellet was solubilized by addition of 1.5 ml of 1 M NaOH for 24 h. Cyclic GMP concentration was expressed as pmol/mg of protein [21].

2.7. Determination of myocardial aminoguanidine tissue levels

Approximately 50 mg of left ventricular tissue from normal and I/R myocardial regions were homogenized. Homogenates were reacted with p-nitrobenzaldehyde in the presence of HCL at 100°C. This converted AMG to a derivative which was yellow in alkaline solution. After alkanisation of the sample the yellow product was extracted into ethyl acetate and back into an aqueous phase of dilute HCL in the presence of heptane. After alkanisation by HCL extraction the yellow product was assayed by spectrophotometry. The concentration of AMG (μM) was derived from standards using known amounts of AMG added to tissue homogenates and carried through the entire procedure [22].

2.8. Immunohistochemical analysis

Consecutive sections were washed in PBS (pH 7.4) and incubated with a primary monoclonal antibody against macrophage iNOS (dilution 1:25; Transduction Laboratories, Lexington, KY). In addition, infiltrating mononuclear cells were identified with a monoclonal rabbit anti-macrophage (RAM-11; dilution 1:50; DAKO) antibody. The incubation medium consisted of PBS (pH 7.4) containing 0.4% Triton X-100 and incubation was performed at room temperature for 1–2 h. Control sections were incubated with mouse-serum (dilution 1:100) (Dako Corp.). Visualization was performed with a fluorescein conjugated antimouse IgG and the Vectastain Elite ABC Kit (Vector Laboratories) [23].

2.9. Statistical analysis

Mean values are shown with their standard deviations (SD). Paired t-test was used to assess differences in hemodynamics and blood flow within each individual animal. Analysis of variance (ANOVA) was used to assess differences between groups. Correlations were determined by single regression analysis. P-values of 0.05 or less were considered statistically significant.

3. Results

3.1. Effects of AMG on left ventricular hemodynamics

In Sham animals, no significant changes in left ventricular and systemic hemodynamics were noted, indicating that the surgical procedure per se had no significant effects on cardiac function (Table 1).

All animals subjected to I/R in groups B, C, D showed a significant reduction in +dP/dr and increase in LVEDP indicating left ventricular dysfunction 30 min after I/R (dP/dr: I/R-placebo: baseline: 3287±299 vs. 30 min I/R: 2032±237, P=0.02; I/R+AMG bolus: baseline: 3166±265 vs. 30 min after I/R: 2143±277, P=0.03; I/R+AMG cont.: baseline: 3266±365 vs. 30 min after I/R: 2098±212, P=0.008; LVEDP (mmHg): I/R-placebo: baseline: 11.1±0.8 vs. 30 min I/R: 6.1±0.6, P=0.002; I/R+AMG bolus: baseline: 1.2±0.7 vs. 30 min after I/R: 5.3±1.1, P=0.007; I/R+AMG cont.: baseline: 1.5±0.8 vs. 30 min after I/R: 5.1±0.9, P=0.01).
The hemodynamic effects of acute and chronic AMG treatment are summarized in Table 1. Fig. 2 shows the effects of various concentrations of AMG on LVESP response at various time points. A dose dependent increase in LVESP and effects of various concentrations of AMG on LVESP during sufficient iNOS inhibition (continuous AMG treatment) 48 h after I/R. In both groups a significant increase in LVESP 48 h after I/R was not due to increased reperfusion was significantly lower as compared to sham animals. In addition, no significant changes in coronary vascular resistance (CVR) were noted in AMG-treated animals as shown in Table 1.

### 3.3. Effects of AMG on regional myocardial blood flow and coronary vascular resistance

Myocardial blood flow in left ventricular regions not affected by I/R did not differ significantly among groups. Myocardial blood flow measured 30 min after initiation of reperfusion was significantly lower as compared to sham animals and baseline blood flow determined prior to I/R (Fig. 4). A further reduction of myocardial blood flow in the same region was observed in the placebo-treated group determined 48 h after I/R. In contrast, administration of AMG bolus (10.0 mg/kg) as well as continuous AMG treatment (300.0 mg/kg/day) restored myocardial blood flow to levels comparable to sham animals. In addition, no significant changes in coronary vascular resistance (CVR) were noted in AMG-treated animals as shown in Table 1.

### 3.4. Effects of AMG on myocardial NOS activity

Endothelial constitutive NOS (ecNOS) activity tended to be decreased in placebo and AMG-treated animals. However, AMG treatment did not significantly affect ecNOS activity.
Fig. 3. Cardiac performance of animals receiving AMG continuously over a time period of 48 h improves significantly with respect to max. $\pm dP/dt$ (A) and LVEDP (B). This is less pronounced after acute intravenous administration of AMG (10.0 mg/kg). In contrast animals subjected to I/R placebo develop left ventricular dysfunction. No significant changes are observed in sham operated animals. Sham ($n=6$); ischemia/reperfusion (I/R) placebo (saline; 3.0 ml/sc three times a day ($n=7$); I/R treatment with aminoguanidine (AMG, 10.0 mg/kg/iv bolus; $n=6$); I/R + continuous treatment with AMG (300.0 mg/kg/day/sc; $n=9$). Data are given as mean±SD; * $P<0.05$ vs. sham; †$P<0.05$ vs. I/R + placebo.

Activity in the different animal groups indicating that AMG is selective for iNOS activity in this model (Fig. 5A).

The time course of iNOS activation is shown in Fig. 1. Inducible NOS activity was significantly increased in placebo-treated animals 48 h after I/R. Bolus of AMG given 48 h after I/R significantly inhibited iNOS activity. In addition, AMG continuously administered significantly inhibited iNOS activity (Fig. 5A).

3.5. Effects of AMG on myocardial cGMP levels

Myocardial cGMP levels significantly increased 48 h
after I/R in placebo-treated animals and were completely blocked by continuous AMG treatment (Fig. 5B). Elevated cGMP levels were also observed in animals which received AMG as bolus (Fig. 5B).

### 3.6. Myocardial aminoguanidine levels

In order to confirm that the inhibitor administered reaches the site of increased iNOS activation within myocardial tissue, we measured the AMG concentration in the affected myocardium 48 h after I/R. No AMG levels were detected in sham and placebo-treated animals. In contrast, a significant amount of AMG within the myocardium was measured in animals treated with AMG bolus.

Highest AMG concentration was determined in animals which received continuous AMG treatment over 48 h. In this group, AMG concentration was significantly higher in the affected myocardium when compared to regions not subjected to I/R, indicating a higher uptake of AMG in regions of increased iNOS expression and activation (Fig. 5C). One explanation of increased AMG tissue content at the site of inflammation may be the selectivity of the compound to iNOS in this model.

In addition, there was a strong correlation between iNOS activation in the placebo group and AMG tissue levels in the continuously treated group over time further supporting the specificity of AMG on iNOS inhibition (Fig. 5D).

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**Table 2**

<table>
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<th>Group</th>
<th>HR (bpm)</th>
<th>LVESP (mmHg)</th>
<th>LVEDP (mmHg)</th>
<th>MAP (mmHg)</th>
<th>+dP/dt (mmHg)</th>
<th>−dP/dt (mmHg)</th>
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<tr>
<td>Placebo</td>
<td>203±25</td>
<td>56.2±6.3</td>
<td>4.7±0.6</td>
<td>53.6±4.8</td>
<td>1855±319</td>
<td>1439±249</td>
</tr>
<tr>
<td>Placebo+Iso</td>
<td>232±18</td>
<td>73.4±5.2</td>
<td>3.6±0.5</td>
<td>69.4±6.3</td>
<td>2466±258</td>
<td>1866±231</td>
</tr>
<tr>
<td>AMG cont.</td>
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<td>76.6±6.7</td>
<td>3.7±0.6</td>
<td>73.8±4.6</td>
<td>2763±231</td>
<td>2138±221</td>
</tr>
<tr>
<td>AMG cont.+Iso</td>
<td>231±16</td>
<td>89.4±4.8</td>
<td>2.1±0.7</td>
<td>85.9±5.5</td>
<td>4828±258</td>
<td>2794±207</td>
</tr>
</tbody>
</table>

*a Values are mean±SD.

b P<0.05 vs. baseline.

c P<0.05 vs. placebo.

d P<0.05 vs. isoproterenol.

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Fig. 4. Myocardial blood flow to the I/R region as measured by injection of fluorescent microspheres is shown. Ischemia results in complete reduction of blood flow in the affected region. Following 30 min of reperfusion blood flow is still significantly lower in the I/R groups when compared to sham animals and baseline flows. A further reduction of myocardial blood flow in the same region is noted in the placebo-treated group determined 48 h after I/R. In contrast, administration of AMG bolus (10.0 mg/kg) as well as continuous AMG treatment (300.0 mg/kg/day) restores myocardial blood flow to levels observed in sham animals. Sham (n=6); ischemia/reperfusion (I/R)+placebo (saline; 3.0 ml/sc three times a day (n=7); I/R+treatment with aminoguanidine (AMG, 10.0 mg/kg/iv bolus, (n=6); I/R+continuous treatment with AMG (300.0 mg/kg/day/sc; n=9). Data are given as mean±SD; * P<0.05 vs. sham; † P<0.05 vs. values 30 min after I/R in both AMG groups.
Fig. 5. Enzyme activity of inducible and endothelial constitutive nitric oxide synthase (iNOS, ecNOS) as determined by the conversion of L-[14C]-arginine to L-[14C]-citrulline is shown (A). Myocardial iNOS activity is significantly increased within 48 h after initiation of I/R in the placebo group. Both, bolus and continuous AMG treatment significantly reduces iNOS activity (A). In contrast, endothelial constitutive NOS (ecNOS) activity remains unchanged after I/R, placebo as compared to sham; in addition, both bolus and continuous AMG administration do not exert significant inhibitory effects on ecNOS activity when compared to placebo-treated animals (A). Cyclic guanosine monophosphate (cGMP) levels as measured by RIA increases significantly after 48 h of I/R; cGMP is significantly inhibited by both, acute and continuous AMG treatment (B). AMG tissue distribution is shown in C. No specific signal is detected in sham animals. In addition, measurement of AMG concentration after 48 h of bolus (group C) and continuous AMG treatment (group D) reveals low levels in nonaffected myocardium. In contrast, the myocardium affected by I/R with subsequent upregulation of iNOS shows significantly higher AMG tissue levels compared to nonaffected myocardium in the same animals. In particular animals treated continuously show the highest AMG levels in the affected cardiac tissue (C). Moreover, a significant correlation between myocardial iNOS activity and AMG tissue levels over time is found (D); Sham (n=6); ischemia/reperfusion (I/R)+placebo (saline; 3.0 ml/sc three times a day (n=7); I/R+pretreatment with aminoguanidine (AMG, 10.0 mg/kg/iv bolus, (n=6); I/R+continuous treatment with AMG (300.0 mg/kg/day/sc; n=9). Data are given as mean±SD.; * P<0.05 vs. sham; † P<0.05 vs. placebo.

3.7. Effects of AMG on myocardial infarct size

No infarcts were present in sham operated animals. Total risk regions (RCX regions as % of the left ventricle) determined 48 h after I/R did not differ significantly between groups (B: 31.7±4.3; C: 29.8±4.8; D: 32.5±3.6). Infarct size, expressed as percentage of risk region (RCX region) in placebo group was 62.3±7%. No significant differences were noted in animals treated with AMG bolus (56.7±8.2%). In contrast, animals which received AMG continuously (300.0 mg/kg/day/sc) had significantly smaller infarcts (percentage of the risk region) when compared to placebo-treated animals (44.2±6%, P = 0.003) (Fig. 6).

3.8. Histopathology

In placebo-treated animals, no infarcts were present in the sham group. Animals which received placebo showed typical signs of reperfused myocardial infarcts including hemorrhage, myocyte coagulation necrosis, contraction bands, abundant infiltration of inflammatory cells and calcification (Fig. 7A). Animals treated with AMG showed similar signs of reperfused infarcts (Fig. 7E). There was
Fig. 6. Infarct sizes as percentage of the circumflex regions is shown. Injection of Evans Blue Dye was used to determine the total circumflex region as a percentage of the whole left ventricle. As shown for groups B–D no significant differences were noted between the three groups (I/R + placebo: 31.7±4.3%; I/R + AMG bolus: 29.8±4.8%; I/R + AMG continuous: 32.5±3.6%). The infarcted region as a percentage of the circumflex region was determined by triphenyltetrazolium chloride (TTC) staining. As shown, a significant reduction of the necrotic region was observed by continuous treatment with AMG over the time period of 48 h following I/R (I/R + placebo: 62.3±7%; I/R + AMG bolus: 56.7±8.2%, P=ns vs. I/R + placebo; I/R + AMG continuous: 44.2±6%, * P=0.003 vs. I/R + placebo). Ischemia/reperfusion (I/R) + placebo (saline: 3.0 ml/sc) three times a day (n=7); I/R + treatment with aminoguanidine (AMG, 10.0 mg/kg/iv bolus, n=6); I/R + continuous treatment with AMG (300.0 mg/kg/day/sc; n=9). Data are given as mean ± SD.

3.9. Immunohistochemical analysis

Specific immunoreactivity for macrophages and iNOS were absent in hearts of sham operated animals (not shown).

A large number of infiltrating mononuclear cells (RAM-11) were identified in placebo-treated animals subjected to I/R (Fig. 7B, C). Consecutive sections revealed distinct iNOS expression in these cells (Fig. 7D). There was no significant immunostaining for iNOS detected in cardiomyocytes.

Animals treated with AMG showed significantly less infiltration of inflammatory cells when compared to placebo-treated animals (Fig. 7F, G). Immunohistochemical analysis revealed that these cells were positive for iNOS expression 48 h after I/R (Fig. 7H).

4. Discussion

The novel and clinically important finding of the present study is that late after ischemia and reperfusion, the high output inducible nitric oxide synthase (iNOS)/NO pathway is activated contributing to left ventricular dysfunction and extent of infarct size. Moreover, the deleterious effects mediated by iNOS activation are reversed by continuous isoform selective inhibition of this isoenzyme leading to a significant reduction of infarct size, an important determinant of survival after myocardial injury. The data presented here indicate that the delayed augmentation of nitric oxide production by activation of iNOS late after initiation of reperfusion leads to a NO-dependent myocardial injury which contrasts an early myocardial and endothelial damage caused at least in part by an impaired endothelial production/release of NO.

The investigational drug aminoguanidine used in this study is a nucelophilic hydrazine derivative and a selective iNOS inhibitor in vitro and in vivo [18,24]. A recent study by Foote and colleagues described in detail the pharmacokinetics of the drug in humans; they showed that the compound’s half-life in humans with normal renal function is approx. 4.4 h and is directly proportional to glomerular filtration [25]. The tissue distribution of AMG in various animal models has been shown by Beaven et al [22]. As shown in Fig. 5C, AMG tissue levels were significantly higher in cardiac tissue subjected to ischemia followed by 48 h of reperfusion. In addition, iNOS activity and AMG tissue levels were directly associated indicating a high selectivity of the drug for iNOS isoenzyme (Fig. 5D).

AMG treatment improves left ventricular hemodynamics (Figs. 2, 3), enhances myocardial blood flow (Fig. 4), most likely due to the improvement of the hemodynamic state of the myocardium and without significant effects on CVR; it reduces infarct size when given continuously over a time period of 48 h after initiation of reperfusion (Fig. 6) and leads to a reduction of the cellular infiltrate within the affected regions (Fig. 7).

Acute, intravenous administration of AMG early after reperfusion (absence of iNOS activity) does not exert specific effects on myocardial function indicating that the drug does not inhibit endothelial cNOS activity; this would have resulted in an increase in blood pressure and systemic vascular resistance and reduction of myocardial blood flow due to vasoconstrictive effects. However, a significant pressure response, improvement of cardiac performance and enhanced myocardial blood flow is observed when aminoguanidine is given in the presence of increased iNOS activation 48 h after I/R (Fig. 1). The time course of iNOS activation (Fig. 1) and the fact that AMG bolus administration did not exert significant beneficial effects on hemodynamics and blood flow 24 h after I/R suggests that there is a certain time window in which the selective inhibition of iNOS is protective on the myocardium. However, this cannot be clearly identified from the present data; the fact that the isoenzyme is regulated by protein de novo synthesis within hours upon I/R suggests that initiation of therapy at certain time points after I/R results in protective effects with regard to hemodynamics, histological changes and infarct size.
Fig. 7. Representative photomicrographs from placebo-treated animals are shown on the left hand side (A–D). Histology and immunohistochemical results from AMG-treated animals are shown on the right panel (E–H). No histomorphological differences are noted with respect to I/R regions between placebo and AMG-treated groups and consist of myocyte coagulation necrosis, interstitial and intracellular edema and intramyocardial bleeding. (A vs. E). However, immunohistochemical staining with a specific anti-macrophage antibody (RAM-11, DAKO, Carpenteria, CA) reveals a significant reduction of the number of infiltrating mononuclear cells in the AMG-treated animals (B and C vs. F and G). Staining of adjacent sections with antibodies against the inducible nitric oxide synthase shows specific iNOS immunostaining in both groups. However, a significant reduction of the number of cells positive for specific iNOS expression is noted in AMG-treated animals (D vs. H).
4.1. Potential mechanisms of iNOS-mediated myocardial dysfunction

Previous studies from our and other laboratories have shown that increased iNOS activation parallels an enhanced production of myocardial cyclic guanosine monophosphate (cGMP), a second messenger which acts as a negative inotrope on cardiomyocytes [13]. In this regard, Joe et al. reported that iNOS-mediated cardiac myocyte contractile dysfunction is mediated by a decrease in intracellular cAMP levels, which is at least in part mediated by a cGMP-dependent mechanism [21]. Moreover, in patients with heart failure but not in healthy controls, inhibition of nitric oxide synthase potentiated the response of the myocardium to β-adrenergic stimulation [26].

Aminoguanidine is known to selectively inhibit cGMP levels produced by iNOS as shown in endotoxemic rats [24,27]. As shown here, animals treated with AMG over a time period of 48 h showed a significant reduction of myocardial cGMP production and a significant improvement of cardiac contractile function.

Other iNOS/NO-mediated factors may also contribute to myocardial dysfunction after I/R i.e. formation of oxyradicals such as peroxynitrite by binding of NO to superoxide, singlet oxygen or to hydrogen peroxide; it may lead to cytotoxic effects on adjacent cardiomyocytes due to initiation of lipid peroxidation, inhibition of mitochondrial respiratory chain enzymes, inhibition of membrane Na⁺/K⁺-ATPase activity, or inactivation of sodium membrane channels [28–32]. Nitric oxide may also enhance oxyradical production via release of iron from ferritin [33]. Moreover, recent evidence suggests that cytokine stimulated iNOS activity may also contribute to the decline in velocity of myocyte shortening due to a decrease in sensitivity of the myofilaments to \([Ca^{2+}]\) [34]. This is supported by the observation that components of the NO pathway downstream to NO appear to desensitize cardiac myofilaments to \([Ca^{2+}]\), an effect, which is, at least in part mediated by phosphorylation of troponin I [35].

4.2. Effects of iNOS on myocardial blood flow late after ischemia/reperfusion

Several studies have investigated the effects of endothelium derived nitric oxide release on coronary vasomotor function in the early phase after ischemia and reperfusion. In this regard, Quillen et al. showed that 1 h of ischemia followed by 1 h of reperfusion resulted in a marked reduction of endothelium-dependent response in the coronary microcirculation in dogs [36]. In addition, Bolli and colleagues found a prolonged impairment of the vasodilator responsiveness and a prolonged increase in vascular resistance after a reversible (15 min) ischemic injury. This was unrelated to the contractile depression and the authors suggested that a microvascular stunning in addition to myocardial stunning might have developed which lasted for at least 4 h [37]. In addition, Amrani and co-workers demonstrated that impaired nitric oxide release is associated with reduction of coronary flow following ischemia reperfusion [38]. There is evidence that in the early phase after reperfusion the receptor-dependent production of endothelium derived nitric oxide is impaired, or that nitric oxide scavenging is enhanced due to increased production of oxyradicals including superoxide [39,40]. Further support for the involvement of an altered trans-arginine/nitric oxide pathway in endothelial dysfunction is provided by Pernow et al. and Hiramatsu et al.; they showed, that trans-arginine treatment resulted in enhanced myocardial protection and improvement of endothelial function in pigs [41,42]. Other methods of preservation of cardiac endothelial NO production/release have been described, including exposure of hearts to heat stress and modulation of the temperature during cardioplegic arrest [43,44]. These findings indicate that early after I/R the availability of NO is impaired resulting in alterations of coronary flow and flow reserve under these conditions.

However, increased NO production due to activation of iNOS during the late phase of I/R may also be deleterious for various reasons. As shown in our study, AMG bolus 48 h after I/R (in the presence of iNOS activation) as well as continuous treatment improved cardiac performance and led to an increase in blood flow. In addition, as shown in Table 1, AMG treatment did not exert significant changes in coronary vascular resistance, which confirms that the eNOS activity and eNOS derived NO production/release was not inhibited. The increase in myocardial blood flow as observed in our study is therefore not due to direct effects of AMG on the endothelial NO, but rather due to improvement of left ventricular hemodynamics leading to a reduction in wall stress. Moreover, the inhibitory effect of AMG on cGMP production may also contribute to improved regulation of regional blood flow. Similar findings have been shown by Avontuur et al. They reported that high amounts of myocardial NO production during endotoxemia resulted in autoregulatory dysfunction and altered reactive hyperemia which were reversed by NOS inhibition [45].

In addition, Worral et al. reported that AMG treatment prevented vascular barrier dysfunction in a rat model of acute allograft rejection by inhibition of enhanced iNOS activity; this resulted in a significant reduction of perivascular edema formation and improved organ function [46]; it suggests that iNOS-derived NO alters vascular homeostasis and blood flow regulation. In this regard, Wu and colleagues demonstrated that the vascular hyporeactivity of aortic rings from LPS-treated rats was reversed by AMG administration without affecting the response of these vessel rings to endothelium dependent (eNOS-dependent) vasodilations, suggesting that iNOS activation is directly involved in circulatory failure in this model [17]. In contrast to AMG, L-NAME did have significant inhibitory effects on acetylcholine-induced vasodilations as
shown by Joly et al. [47]. These findings suggest that selective inhibition of iNOS may lead to improved blood flow and flow reserve under conditions of acute inflammation.

Interestingly, myocardial blood flow after acute administration of AMG bolus was comparable to values obtained in the continuously treated group. The fact that the last subcutaneous dose of AMG (100 mg/kg) was given 4 h prior to flow measurement indicates that the drug may have a lasting effect. This is in line with the pharmacologic profile of the drug as described by Beaven et al. and Foote and co-workers [22,25].

4.3. iNOS-mediated effects on cardiac function: myocardial dysfunction versus myocardial protection

Several recent findings suggest that iNOS activation in myocardial tissues may exert different effects on organ function.

Worral and co-workers showed that continuous inhibition of iNOS by AMG prolonged graft survival [15]. Hirono and co-workers showed that under conditions of acute inflammation in a rat model of myocarditis administration of AMG significantly reduced iNOS activity, the amount of inflammatory cells and improved cardiac hemodynamics when compared to non-treated rats [14]. Ishiyama and colleagues reported a significant inhibition of superoxide release and nitrotyrosine formation in AMG-treated rats. In line with these findings they also showed a significant reduction of the CK-MB levels and improvement of hemodynamics indicating a reduction of myocardial cell damage by inhibition of iNOS-mediated formation of oxyradicals such as peroxynitrite. Their histomorphological analysis revealed a significant reduction in myocardial inflammation, reduced cellular infiltrate and absence of nitrotyrosine staining in animals treated with AMG, indicating expression of iNOS but only minimal production of iNOS derived NO. They concluded from their findings that AMG selectively inhibits iNOS-mediated production of the potent radical peroxynitrite [48].

In line with these findings, immunohistochemical analysis revealed distinct iNOS expression in infiltrating macrophages within the affected myocardium. No specific immunoreactivity was noted in cardiomyocytes. As shown in Fig. 7 there were significantly less cellular infiltrate in animals treated with AMG when compared to placebo group. Even though infiltrating mononuclear cells in the infarcted regions of AMG-treated animals expressed iNOS (Fig. 7H), overall iNOS activity was significantly reduced. This was most likely caused by direct effects of AMG on iNOS and also due to the reduction of the cellular infiltrate within the region affected by I/R. Similar findings were reported by others [46,49].

The mechanism, which leads to reduction of infiltrating inflammatory cells in the AMG group, remains unclear. It is not known whether or not AMG exerts direct effects on cell migration or influences induction of pro-inflammatory cytokines as a main stimulus for regional cell migration. However, it seems likely that inhibition of massive cytotoxic NO production in cells infiltrating the infarcted region may limit the amount of non-salvageable tissue damage which in turn results in reduction of further cellular infiltration. Subsequent reduction of enzyme activity, NO and cGMP production as observed in our study (Fig. 3B) may explain, at least in part, the improvement of myocardial function and reduction in infarct size.

In contrast to these observations, several investigators have shown that iNOS expression plays a protective role in ischemic and pharmacological preconditioning. Tosaki and co-workers reported expression of iNOS mRNA by pretreatment with Monophosphoryl Lipid A (MLA) which was associated with improved posts ischemic functional recovery of isolated rat hearts [50]. In this study, the beneficial effect of MLA was inhibited by L-nitro-arginine-methyl-ester (L-NAME) further supporting that the NO pathway might be involved [50]. A major difference in the study design as compared to the present study may explain the intriguing findings. The authors used a non-isoform selective NOS inhibitor to account for iNOS-mediated effects on myocardial performance; some of the detrimental effects mediated by L-NAME may have been caused by significant inhibition of endothelial constitutive NOS, thereby leading to reduction of organ blood flow and flow reserve, reduction of LV $dP/dt$ as well as the increased incidence of ventricular arrhythmias [50]. In this regard, various other investigators have shown that inhibition of ecNOS leads to a significant reduction in organ blood flow associated with impaired organ function in increased mortality [51].

As reviewed by Elliot et al., MLA-mediated cardioprotection involves various biochemical endpoints such as adenosine, heat shock protein 70, catalase, manganese superoxide dismutase, adenosine kinase and others suggesting that various factors may be involved in the beneficial effects of these drugs on heart function [52].

Further research is therefore necessary to account for the various mechanisms in which NO production under pathological conditions is protective or needs to be inhibited to prevent further tissue damage. The design of new, more selective inhibitors of the NOS isoforms will certainly help to understand the various effects of NO in different disease states which enables us to more specifically direct the endogenous NO pathway into a therapeutic tool.

4.4. Study limitations

Aminoguanidine is a non-amino acid analogue of L-arginine and a selective iNOS inhibitor in vivo. However, it can not be excluded that AMG exerts other iNOS independent effects, which may be of benefit after I/R.

In this regard, in a recent study by Picard and co-
workers it has been shown that AMG inhibits the oxidative modification of low density lipoproteins and the subsequent increase in uptake by macrophage scavenger receptors. Moreover, these authors demonstrated that AMG in high concentrations acts directly as an antioxidant as shown by the decreased formation of conjugated dienes [53]. Others who showed that AMG significantly reduced protein modification by lipid peroxidation derived aldehydes reported similar findings [54].

In addition, AMG inhibits the formation of advanced glycation end-products. In particular, AMG reacts with carbonyl groups of early glycation products, thus preventing the electrophilic attack of these compounds on lysine residues of adjacent proteins [55]. It is noteworthy that in these studies the iNOS pathway was not part of the investigation and some of the effects observed may also have been attributed to modulation of this isoenzyme. However, these potential mechanisms may also contribute to the cardioprotection observed in our study.

5. Conclusions

In conclusion, this is the first study which provides evidence that continuous administration of AMG within 48 h following acute myocardial ischemia and reperfusion exerts protective effects with respect to myocardial performance, coronary blood flow and cellular infiltration, all of which may contribute to reduction of cellular necrosis and infarct size. AMG is a selective inhibitor of iNOS and this may contribute to its mechanism of action in this setting.

Based on these findings, we suggest that continuous administration of selective iNOS inhibitors over this time period may therefore be of clinical advantage under pathophysiologic conditions involved in ischemia/reperfusion including myocardial infarction or coronary angio-plastic procedures.

References

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