Asymmetric Dimethyl L-Arginine (ADMA) is a critical regulator of myocardial reperfusion injury

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Abstract

Objective: Endothelial dysfunction by the loss of nitric oxide (NO) is a critical event during reperfusion of ischemic myocardium. Reduced NO availability signals important pathophysiological changes leading to myocardial reperfusion injury. We have recently shown that NO biosynthesis can be disturbed by the endogenous NO synthase (NOS) inhibitor ADMA and that these changes are mediated by an impairment of its metabolism by dimethylarginine dimethylaminohydrolase (DDAH). We therefore analyzed the role of ADMA and its metabolism in the setting of myocardial ischemia and reperfusion.

Methods: C57-bl6 mice underwent myocardial ischemia for exactly 30 min followed by 2, 4, 8, 12, 24, and 72 h of reperfusion achieved by occlusion and re-opening of the left coronary artery. The reperfused left ventricle was subsequently homogenized for measurements of determinants of the NO synthase pathway. Furthermore, the effects and its mechanisms of ADMA on reperfusion injury were analyzed in a genetic mouse model.

Results: A significant accumulation of ADMA was found in myocardial tissue when mice were subjected to 30 min of ischemia followed by reperfusion in our in vivo model. The maximum increase of tissue ADMA at 4 h of reperfusion coincided with reductions of NO tissue concentrations and DDAH activity; protein expression of NOS isoforms, however, was not changed. Furthermore, DDAH overexpression in a genetic mouse model as well as treatment with oral L-arginine markedly reduced reperfusion injury by 40–50% at 4 h of reperfusion. The effects of ADMA on reperfusion injury were shown to be mediated by reduced eNOS activity and phosphorylation, expression of adhesion molecules, and leukocyte activity.

Conclusion: Accumulation of tissue ADMA by impairment of DDAH was found to be a significant determinant of reperfusion injury. Our results indicate that ADMA could be a potential new target for the treatment of myocardial ischemia/reperfusion injury.

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Keywords: Ischemia; Infarction; Nitric oxide synthase; Endothelium; Cell adhesion molecules

1. Introduction

Irreversible injury and subsequent necrosis proceed in a wave front fashion in the myocardium at risk after occlusion and reperfusion of coronary arteries. One of the earliest and most critical events occurring after reperfusion of an ischemic bed is marked endothelial dysfunction characterized by the loss of endothelium derived nitric oxide (NO) [1]. The profound decrease in NO bioavailability triggers a cascade of early pathophysiological events in reperfusion injury, such as up-regulation of adhesion molecules, leukocyte adherence to endothelial cells of reperfused coronary arteries, transmigration of polymorphonuclear cells and subsequent tissue damage due to apoptosis of reperfused myocardium [2].

It is well known that reperfusion injury is exaggerated in metabolic disorders associated with endothelial dysfunction, such as hypercholesterolemia or diabetes [3]. This effect might be due to an impairment of the NO synthase (NOS)
pathway, since endothelial dysfunction in these circumstances is mediated by reduced NO bioavailability. In general, reduced bioavailability of NO is due to reduced NO production (through reduced NOS expression or reduced NOS activity), increased degradation (by superoxide anion) or reduced NO sensitivity by inactivation of soluble guanylate cyclase [4]. Endothelial dysfunction as a consequence of impaired NO biosynthesis may be partly due to increased levels of asymmetrical dimethylarginine (ADMA), an endogenous competitive inhibitor of NOS [5].

ADMA was found to be elevated and closely correlated with impaired vasodilator function in conditions associated with endothelial dysfunction, such as hypercholesterolemia, hypertension, insulin resistance and type 2 diabetes, hyperhomocysteinemia and renal insufficiency [5]. But ADMA also seems to be involved in myocardial ischemia, since its plasma levels predict future coronary events in patients with elevated cardiovascular risk [6]. Indeed, we recently observed elevations of plasma ADMA concentrations in acute coronary events independent of cardiovascular risk factors [7].

ADMA appears to be dynamically regulated, it is formed by methylation of arginine residues on specific proteins, and the majority of ADMA is metabolized by the enzyme dimethylarginine–dimethylaminohydrolase (DDAH) [5]. So far two isoforms of DDAH have been discovered (DDAH-1 and -2), both isoforms have been shown to influence vascular biology [8] and seem to be critical regulators of the NOS pathway and subsequently of endothelial function.

Little is known about the possible role of ADMA and DDAH in the setting of myocardial ischemia and reperfusion (mI/R). Systemic administration of other NOS inhibitors either before ischemia or just prior to reperfusion was shown to increase infarct size in previous studies, an effect that could be reversed by administration of L-arginine [9]. Furthermore there is indirect evidence that ADMA could play an important role in mI/R, since L-arginine supplementation itself has been shown to afford protection in some models, although the available evidence suggests that its availability should not be rate-limiting in the generation of NO in vivo.

In conclusion, the reduction in NOS activity during early reperfusion and the beneficial effect of exogenous L-arginine in mI/R injury indicate a possibly important role of the endogenous NOS inhibitor ADMA in the pathophysiology of mI/R. Thus the current study was initiated to test the hypotheses that tissue ADMA levels are elevated in myocardial reperfusion followed by ischemia, that ADMA contributes to the severity of reperfusion injury and that alterations in DDAH activity or expression modulate tissue ADMA levels and the damage after ischemia and reperfusion.

2. Methods

2.1. Animals

C57BL/6N mice were purchased at Charles River Laboratories (Wiga, Germany). Heterozygous hDDAH-1 transgenic mice (TR) were generated as published previously [10]. The colony received from the laboratory of John P. Cooke at Stanford University (U.S.A) was expanded by cross-breeding TR with C57BL/6N mice and offspring were screened for the hDDAH-1 transgene expression by PCR as described earlier [10]. All procedures were performed according to protocols approved by the Institutional Committee for Use and Care of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). The experiments were approved by the Administrative Panel on Laboratory Animal Care of the Medical University of Innsbruck.

2.2. Experimental protocol

First time course studies were performed to evaluate changes of determinants of the NOS/DDAH pathway in our model of mI/R. For these experiments male C57BL/6N mice were either sham operated or subjected to 30 min of ischemia followed by 0, 2, 4, 8, 12, 24 or 72 h of reperfusion (n=6 for each group). Myocardial tissue of the area of infarction was subsequently harvested for determination of L-arginine, ADMA, nitrogen-oxides (NOx), for Western blots and measurements of DDAH enzyme activity.

For comparisons between heterozygous hDDAH-1 transgenic littermates (TR) and age (20–25 weeks) and weight (20–25 g) matched offspring tested negative for the hDDAH-1 transgene (wildtype, WT) 50 male animals of each group were subjected to 30 min of ischemia followed by 4 h of reperfusion. To determine the effects of L-arginine and NOS inhibitors on reperfusion injury, a further set of WT mice (n=20) was fed with L-arginine (6 g/100 ml) in the drinking water starting 1 week prior to surgery, or osmotic mini-pumps (Alzet®, Charles River, Germany) systemically delivering L-NAME (125 and 250 μmol/kg/d) were implanted subcutaneously in the back of a set of TR mice (n=20) 7 d prior to surgery. After completion of surgery whole hearts were collected for sectioning and immuno-histochemistry or myocardial tissue of the LV was harvested for Western blots or analysis of enzyme activity, amino acids and nitrogen-oxides.

2.3. Surgical procedure

Sedation, tracheotomy, intubation, ventilation, and surgery aided by a microscope (Olympus SZH-10) were carried out as previously described [11]. Briefly a 1-mm section of a PE-10 tube was placed around the left anterior descending artery (LAD) to secure ligation of the vessel without damaging the artery. The LAD was ligated with an 8–0 silk and complete occlusion became evident by discoloration of the left ventricle (LV). After 30 min of LAD occlusion, the ligature was removed by cutting the knot on top of the tube and reperfusion was visually confirmed. After a designated time of reperfusion animals were sacrificed and the LVs of the hearts were harvested and cut into portions 1 mm below
the ligation suture for sectioning or tissue of the LV was homogenized for biochemical measurements. The mortality during or right after surgery was approximately 10%, but increased after treatment with high doses of l-NAME (30%).

2.4. Tissue preparation, damage score and area of infarction

Tissues from 26 WT and 23 DDAH-1 transgenic animals were fixed in 4% paraformaldehyde at 4 °C and embedded in paraffin. Sections of 2 μm were cut from the cross area and stained with hematoxylin and eosin (HE) for histological evaluation of tissue damage. In order to have a quantitative score for cardiac damage, sections (n=8 for each animal) were scored by 2 independent observers blinded to the experimental protocol as published previously [11]. The measurement of the infarcted area was performed by reviewing the sections with a BX60 microscope (Zeiss, Jena, Germany) equipped with a Sony 3CCD camera and television monitor. A transmission scanning microscope (Bio-Rad), equipped with a 488 nm argon ion laser and Plan Neofluar 10x/0.3 oculars connected with the program START LSM 510 was used to scan the images. The area of infarction was defined as the region between the living myocytes and the cardiac membrane [11]. Areas were measured and recorded in square micrometers. Differences between WT and hDDAH-1 TR mice were confirmed in 5 WT and 5 DDAH-1 transgenic animals by measurements of the area at risk by injection of Evans Blue into the left ventricle and measurement of the area of infarction by TTC staining as reported earlier [12]. For all experiments the investigator for the analysis was blinded to group assignment.

2.5. Determination of serum troponin T

From each mouse heparinized blood was collected from the V. cava inferior when it was sacrificed. Serum levels of troponin T (TnT) were determined in plasma by using the quantitative TnT rapid assay from Roche Diagnostics (Mannheim, Germany).

2.6. Determination of l-arginine and ADMA

l-arginine and ADMA concentrations in homogenized myocardial tissue were measured by reversed phase high-performance liquid chromatography (HPLC) with pre-column derivatization with o-phthalaldehyde (OPA) as previously described [13]. The recovery rate for ADMA was 95% and the intra-sample variation was less than 5%. The detection limit of the assay was 0.05 μM.

2.7. Determination of nitrogen oxides

Concentration of nitrogen-oxides (NOx) in the homogenized tissue of the LV was determined with a chemiluminescence apparatus (Dasibi 2108) after reduction of the samples in boiling acidic vanadium (III) chloride at 98 °C [13]. Standard curves for NOx were linear over a range between 100 nM and 100 μmol/L (R²=0.99). The detection limit of this assay was 10 nM. Intra- and inter-assay variability was <6%.

2.8. Western blotting

Myocardial tissues were homogenized, cell lysates were centrifuged at 2000 g for 45 min and supernatants were collected for DC protein assay (Biorad, Germany) and for Western blotting. Hybond ECL nitrocellulose membranes (Amersham) with proteins transferred from SDS-PAGE gels were incubated with monoclonal antibodies raised against purified rat DDAH-1 (cross-reacting with mouse DDAH-1), purified human DDAH-1, mouse DDAH-2 (Abcam, U.K.), mouse eNOS (BD Biosciences, U.S.A.), mouse nNOS and iNOS (Transduction Lab., U.S.A.), goat serine 1177 phosphorylated-eNOS (Santa Cruz Biotechnology, U.S.A.), mouse ICAM-1 (Abcam, U.K.), as well as human P-selectin (BD Biosciences, U.S.A.) and were probed with anti-mouse Ig G horseradish peroxidase (HRP)-linked antibody (Amer-sham, U.S.A.) followed by detection by ECL Western blotting reagents (Amersham).

2.9. Immunohistochemical analysis

Serial 5 μm frozen sections were cut from cryo-preserved tissue blocks, fixed in ice cold 1:1 acetone for 10 min followed by acetone–methanol (1:1) for 5 min, and washed with TRIS buffer for 3×3 min. Sections were then blocked with MeOH and H2O2, washed with TRIS buffer, stained with goat monoclonal antibodies against serine 1177

<table>
<thead>
<tr>
<th>Table 1</th>
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<tbody>
<tr>
<td>The NOS/DDAH pathway is dynamically regulated in myocardial ischemia/reperfusion</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>NOx (μmol/g)</th>
<th>Sham</th>
<th>0 h</th>
<th>2 h</th>
<th>4 h</th>
<th>8 h</th>
<th>12 h</th>
<th>24 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>l-arginine (mmol/g)</td>
<td>6.9±0.5</td>
<td>9.1±0.7</td>
<td>7.3±1.1</td>
<td>5.5±1.8</td>
<td>4.1±0.5</td>
<td>4.0±0.2</td>
<td>4.2±0.7</td>
<td>2.3±1.0</td>
</tr>
<tr>
<td>ADMA (mmol/g)</td>
<td>86±17</td>
<td>29±6</td>
<td>238±48</td>
<td>276±55</td>
<td>199±40</td>
<td>94±19</td>
<td>83±17</td>
<td>112±22</td>
</tr>
<tr>
<td>l-arginine activity (U/g protein)</td>
<td>2489±498</td>
<td>1055±211</td>
<td>1267±253</td>
<td>2011±402</td>
<td>2111±422</td>
<td>2540±508</td>
<td>3019±604</td>
<td>4321±864</td>
</tr>
<tr>
<td>DDAH-1 activity (U/g protein)</td>
<td>2522±487</td>
<td>881±128</td>
<td>654±292</td>
<td>358±177</td>
<td>692±139</td>
<td>864±262</td>
<td>1655±487</td>
<td>1686±563</td>
</tr>
<tr>
<td>DDAH-2 activity (U/g protein)</td>
<td>611±334</td>
<td>670±418</td>
<td>549±196</td>
<td>440±146</td>
<td>520±212</td>
<td>287±224</td>
<td>593±262</td>
<td>586±232</td>
</tr>
</tbody>
</table>

Tissue concentrations of NO-metabolites, ADMA, l-arginine, as well as DDAH enzyme activity (all per g total protein) and DDAH-1 and -2 protein expression (pixels in densitometry) were measured in the homogenized left ventricle of sham operated C57BL/6N mice (sham), animals that underwent 30 min of ischemia only (0 h) or 30 min of ischemia followed by different times of reperfusion (2–72 h); data are expressed as mean±SEM of 6 different specimens; * p<0.05 vs. sham, † p<0.05 vs. 0 h.
phosphorylated-eNOS, CD-31 or MAC-1 (Santa Cruz Biotechnology, U.S.A.) and subsequently counterstained with hematoxylin. Immunofluorescence staining was carried out using the primary antibodies mentioned above and Alexa fluor 568 donkey anti goat (red) and 488 donkey anti-rat (green) secondary antibodies.

2.10. DDAH and MPO enzyme activity assay

DDAH enzyme activity in homogenized myocardium was assayed by determining L-citrulline formation as previously described [13]. The activity of myeloperoxidase (MPO), an enzyme specific for neutrophil activity, was determined in the homogenized myocardial tissue as published previously [14]. Tissue was stored in liquid nitrogen until the start of the experiment.

2.11. Calculations and statistical analyses

Data are reported as mean ± SEM. Group differences were examined by Statview software (SAS Institute Inc.) using analysis of variance (ANOVA) and post hoc analysis was performed using Fisher’s protected least significant difference test. Statistical significance was set at p < 0.05.

3. Results

3.1. Derangements of the NOS pathway during myocardial ischemia and reperfusion

Generation of nitric oxide measured by NOx tissue concentration increased significantly after 30 min of ischemia compared to the hearts of sham operated animals. However, when the LV was reperfused after 30 min of ischemia, a marked decrement of NOx concentrations was observed in our in vivo model already at 2 h reperfusion, and NOx significantly declined further with prolonged reperfusion (Table 1). The observed changes of NO metabolites were not mediated by altered eNOS or nNOS expression, as determined by Western blots (not shown). Furthermore no protein expression of iNOS was found during ischemia or 2 to 12 h of reperfusion, only a minimal induction of iNOS expression was observed at 24 and 72 h of reperfusion (not shown). The observed stable NOS expression suggests that alterations of NO generation during ischemia and reperfusion are due to changed NOS enzyme activity. Interestingly, tissue concentrations of the endogenous NOS inhibitor ADMA were markedly reduced during ischemia; however they dramatically increased already 2 h after reperfusion of the hearts to a maximum at 4 h (Table 1). After prolonged reperfusion tissue ADMA subsequently declined to initial levels at 12 h. By contrast ADMA levels in the non-ischemic right ventricle were not found to be changed during ischemia or reperfusion (data not shown). Tissue concentrations of the NOS precursor L-arginine decreased significantly after ischemia as well as 2 h of mI/R, and then significantly accumulated during longer reperfusion times (Table 1).

3.2. Mechanism of dynamic changes of ADMA during ischemia/reperfusion: role of DDAH

No significant differences were found in DDAH-1 protein expression during ischemia or reperfusion, when expression and enzyme activity of DDAH were analyzed (Table 1). Moreover Western blotting for the DDAH-2 isoform did not reveal any significant changes during ischemia alone or a short duration of reperfusion. At longer reperfusion times, however, dramatic increases of DDAH-2 expression were found up to a 6-fold increase at 72 h of reperfusion (Table 1). Total DDAH activity (measuring the activity of both isoforms) in homogenized heart tissue was significantly reduced after myocardial ischemia. In the early phase of reperfusion DDAH enzyme activity further decreased to a minimum at 4 h of reperfusion, however later DDAH activity was again increased and was restored up to initial levels in the late phase of reperfusion (Table 1). Dynamic changes in DDAH enzyme activity were therefore independent of the reduction of ADMA in ischemia, but mirrored ADMA elevations in the early phase of reperfusion. Whereas reductions of DDAH enzyme activity during ischemia and early reperfusion did not seem to be due to reductions of protein expression of either isoform, the restoration of

### Table 2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>NOx (mmol/g)</th>
<th>ADMA (mmol/g)</th>
<th>ADMA (mmol/g)</th>
<th>DDAH-1 protein n.d.</th>
<th>I/R</th>
<th>I/R + L-arg</th>
<th>I/R</th>
<th>I/R + L-NAME</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>6.9 ± 0.5</td>
<td>8.3 ± 0.3†</td>
<td>9.1 ± 0.7</td>
<td>12.1 ± 0.7*</td>
<td>6.2 ± 0.6</td>
<td>9.2 ± 0.5†</td>
<td>8.7 ± 1.1*</td>
<td>5.9 ± 0.4†</td>
</tr>
<tr>
<td>DDAH-1</td>
<td>86 ± 17</td>
<td>70 ± 5</td>
<td>29 ± 6</td>
<td>49 ± 11*</td>
<td>282 ± 53</td>
<td>256 ± 58</td>
<td>65 ± 5*</td>
<td>n.d.</td>
</tr>
</tbody>
</table>
| 1-arginine (mmol/g)
| 2489 ± 498 | 2869 ± 215 | 1055 ± 211 | 2098 ± 116* | 3605 ± 1331 | 7706 ± 4680† | 5534 ± 974* | 5702 ± 520* |
| hDDAH-1 protein n.d. | 4632 ± 733* | n.d. | 4372 ± 659* | n.d. | 4465 ± 878* | 5011 ± 463* |

Tissue concentrations of NO-metabolites (NOx), ADMA, L-arginine (per g total protein), as well as hDDAH-1 protein expression (pixels in Western blot) were measured in the homogenized left ventricle after sham operation (sham), 30 min of ischemia (ischemia) and 30 min of ischemia and 4 h of reperfusion (I/R). Mice tested negative for hDDAH-1 (WT) were compared with WT animals treated with oral L-arginine (l-arg; 6 g/100 ml) prior to surgery, heterozygous hDDAH-1 TR littermates (DDAH-1), and hDDAH-1 TR animals systemically treated with l-NAME (125 μmol/kg/d). Data are expressed as mean ± SEM of 6 different specimens; * p < 0.05 vs. control, † p < 0.05 vs. I/R.
3.3. Effects of DDAH-overexpression on the ADMA/NOS pathway

Tissue concentrations of NO metabolites, ADMA, L-arginine and hDDAH-1 protein expression are given in Table 2: Myocardium of DDAH-1 transgenic mice contained significantly elevated NO\textsubscript{x} in sham operated animals, after ischemia and after mI/R. Similarly, ADMA was significantly reduced in the left ventricle after mI/R, and there was a trend towards reductions of ADMA in sham operated animals and elevations of ADMA during myocardial ischemia (Table 2). Finally L-arginine concentration was found to be elevated by DDAH-1 overexpression in ischemia and mI/R, but was not affected by sham operation.

Table 3
The DDAH/ADMA pathway determines myocardial injury after 30 min of ischemia and 4 h of reperfusion

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>WT+ L-arginine</th>
<th>DDAH-1</th>
<th>DDAH-1+ L-NAME</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum troponin (ng/ml)</td>
<td>0.39±0.05</td>
<td>0.21±0.02*</td>
<td>0.22±0.02*</td>
<td>0.35±0.05†</td>
</tr>
<tr>
<td>Area of infarction (10\textsuperscript{6} μm\textsuperscript{2})</td>
<td>1.34±0.15</td>
<td>0.85±0.09*</td>
<td>0.91±0.07*</td>
<td>1.15±0.08</td>
</tr>
<tr>
<td>Damage score</td>
<td>2.47±0.19</td>
<td>1.62±0.18*</td>
<td>1.51±0.21*</td>
<td>2.58±0.36†</td>
</tr>
</tbody>
</table>

Myocardial injury (determined by serum concentration of troponin T, area of infarction and histological grading using a damage score) was measured in mice tested negative for hDDAH-1 (WT, n=14), WT animals treated with L-arginine (6 g/100 ml) in the drinking water (WT+L-arginine, n=12), hDDAH-1 TR mice (DDAH-1, n=15) and hDDAH-1 TR mice systemically treated with 125 μmol/kg/d L-NAME (DDAH-1+L-NAME, n=8). All data are expressed as mean±SEM; * p<0.01 vs. control, † p<0.05 vs. DDAH.

3.4. Pathophysiological role of ADMA in determining myocardial reperfusion injury

We then wanted to find out, if reperfusion injury could be influenced by alterations of the DDAH/ADMA pathway. Since the maximum increase in tissue ADMA was observed after 30 min ischemia and 4 h reperfusion, this time point was chosen. Interestingly, all three measures of reperfusion injury could be reduced by oral L-arginine: The area of infarction could be reduced by almost 40%, the histological damage score was significantly reduced and serum troponin T fell by 50% in mice fed with L-arginine compared to mice fed with regular tap water (Table 3). Similarly, constitutive overexpression of human DDAH-1 resulted in significant amelioration of reperfusion injury after 30 min of ischemia followed by 4 h of reperfusion: The area of infarction could be reduced by approximately 40%, the histological damage score fell and serum troponin T was reduced in TR mice (Table 3). To prove that the beneficial effects on reperfusion

Fig. 1. hDDAH-1 over-expression is associated with increased eNOS phosphorylation and reduced inflammation in murine myocardium after ischemia and reperfusion: Protein expression of phosphorylated eNOS (a, upper scan), eNOS (a, lower scan), of ICAM-1 (b, upper scan) and human DDAH-1 (b, lower scan) in the left ventricle of hDDAH-1 negative and transgenic mice that underwent either sham operation (sham) or 30 min of ischemia and 4 h of reperfusion (I/R). 40 μg of protein was loaded in each lane. All experiments were performed in triplicates.

Fig. 2. Distribution of leucocytes and their activity is reduced by DDAH-1 overexpression after myocardial ischemia/reperfusion: Distribution of neutrophil granulocytes (stained for MAC-1 in sections of the left ventricle) and enzyme activity of myeloperoxidase (MPO) in homogenated myocardium of DDAH-1 negative mice (WT, open bars) and in transgenic animals over-expressing DDAH-1 (black bars) in sham-operated animals and after 30 min of ischemia and 4 h of reperfusion; data are expressed as percent of control±SEM in 4 different specimens; * p<0.05 vs. WT.
injury in the TR animals were specific to DDAH-1 overexpression, another set of mice was treated with L-NAME, an NOS inhibitor that is not metabolized by DDAH. As expected reperfusion injury could be augmented to levels seen in WT animals, when DDAH-1 transgenic mice were systemically treated with L-NAME: Serum troponin increased, the damage score was elevated and area of infarction increased (Table 3). Since the increase of area of infarction did not reach statistical significance, a higher dose of L-NAME was applied to the animals (250 μg/kg/d), which augmented area of infarction to $1.44 \times 10^6 \mu m^2 \ (p=0.03$ vs. DDAH-1 without treatment), but also increased peri-procedural mortality of the animals (30% of mice died during surgery). With our current method to determine reperfusion injury we are not able to synchronically measure the area at risk (AAR). However, AAR measured by Evans blue staining was highly homogenous and comparable between DDAH-1 transgenic mice and WT animals (data not shown). Furthermore the effect of DDAH-1 overexpression on reperfusion injury was confirmed by TTC staining (area of infarction/AAR 18.4 vs. 12.2%; area of infarction/area of left ventricle 6.4 vs. 3.3%; both $p<0.05$).

3.5. Mechanism of ADMA induced myocardial injury after ischemia and reperfusion

It has been speculated that decreased NO bioavailability triggers reperfusion injury by up-regulation of cellular adhesion molecules, leading to exaggerated leukocyte adherence, transmigration of polymorphonuclear cells and

![Fig. 3.](image-url)
subsequent tissue damage. We therefore hypothesized that the observed inhibition of reperfusion injury in DDAH-1 transgenic mice could be a consequence of increased NOS activity, reduction of adhesion molecules in the myocardial tissue and subsequently decreased inflammation. Indeed, we observed a significant reduction of protein expression of ICAM-1 (Fig. 1b, upper scan) and P-selectin (not shown) in DDAH-1 transgenic after 4 h of reperfusion, whereas these adhesion molecules were not detectable in sham operated animals. Furthermore leukocyte activity (enzyme activity of myeloperoxidase; Fig. 2a) and distribution (MAC-1 staining in immuno-histochemistry; Fig. 2b) were markedly increased by mL/R, but both parameters were significantly reduced in the hearts of mice overexpressing DDAH-1 compared with wildtype animals. The effect of mL/R on inflammatory cells in WT mice can be potentially explained by reduced eNOS-phosphorylation after mL/R (365±97 pixels; p<0.05), which was preserved in transgenic animals (1524±374 pixels). P-eNOS expression, however, was not altered by DDAH-1 overexpression in sham operated animals (1448±357 and 1297±395 pixels; Fig. 1a, upper scan) indicating an effect specific for reperfusion. eNOS phosphorylation was reported to be mediated by VEGF and phosphorylation of the serine/threonine protein kinase akt/PKB [15]. This mechanism, however, or frank angiogenesis were not operative in our model, since VEGF-protein and phosphorylated akt isoforms were not detectable in the myocardium after mL/R (data not shown) and the number of endothelial cells (CD-31 in immuno-histochemistry) did not change by DDAH-1 overexpression before and after mL/R.

Finally, we analyzed the location of increased p-eNOS expression after 30 min of ischemia and 4 h of reperfusion by immuno-histochemistry. As quantified in the Western-blot we observed a markedly increased staining for p-eNOS in the hearts of hDDAH-1 transgenic mice (Fig. 3a and b). Although there was apparent unspecific red staining in vascular smooth muscle cells, an increased eNOS phosphorylation was found in the endothelium of arterioles, which was very intense at some locations (arrows in Fig. 3), but less visible in capillaries branching between myocytes. However, a slight change of p-eNOS distribution in DDAH-overexpressing mice cannot be excluded at the present stage.

4. Discussion

The salient findings of our study are: (1) The NO-synthase pathway is dynamically regulated during myocardial ischemia and reperfusion, specifically elevated tissue concentrations of the endogenous NOS inhibitor ADMA are associated with reductions of bioavailable NO in our in vivo mouse model. (2) In the early phase of reperfusion reductions in DDAH-enzyme activity cause the observed increased ADMA levels in myocardial tissue, whereas the gradual reduction of ADMA in the late phase of reperfusion is associated with increases of DDAH-2 protein expression. (3) ADMA is a dominant player in reperfusion injury, since expression of adhesion molecules, leukocyte distribution and activity and subsequent reperfusion injury are blunted, if ADMA is reduced by DDAH overexpression or if animals were treated with exogenous L-arginine.

One of the earliest and most critical events occurring after reperfusion of an ischemic bed is marked endothelial dysfunction characterized by the loss of endothelium derived nitric oxide: In the Langendorff-perfused model impaired endothelium-dependent vasodilatation develops in the first 2.5 min of myocardial reperfusion, it is exacerbated and sustained over 270 min of the post-reperfusion observation period [14], and is characterized by reduced NO release [16]. As a potential mechanism total eNOS activity has been shown to decrease already after 2 h of reperfusion [17]. Several days after reperfusion NO then accumulates by up-regulation iNOS and increased NO generation in this phase can prove toxic and exacerbate tissue injury [18]. By contrast, the significant reduction in NO release in the early phase of reperfusion is associated with increases in leukocyte adherence to endothelial cells of reperfused coronary arteries starting 20 min post-reperfusion and further increasing after 270 min of reperfusion [16]. Considerable evidence has accumulated showing that nitric oxide is not just an innocent bystander in this process, but seems to be a critical regulator of leukocyte adherence to endothelial cells after mL/R: Incubation of coronary segments with L-arginine following mL/R significantly attenuates the increase in leukocyte adherence [16]. Likewise donor agents for exogenous NO (e.g. sydnonimine or nitrate) inhibit adherence of leukocytes to the endothelium as well as transmigration of polymorphonuclear cells into the area of necrosis [19]. Along the same lines NOS inhibition was shown to exacerbate certain aspects of the injury response, including neutrophil adherence in some models of reperfusion injury. These data strongly point toward an important anti-neutrophil effect of NO in mL/R.

In our model NO tissue concentration was reduced already in early reperfusion (4 and 8 h), the constitutive NOS isoforms however were not altered and iNOS was not induced, suggesting diminished NO generation due to altered NOS activity as the operative mechanism in this phase of reperfusion. This hypothesis is supported by our finding that accumulation of the endogenous NOS inhibitor ADMA in myocardial tissue coincided with the decrement of NO formation. This observation provides a new mechanism for the profound endothelial dysfunction and decreased NO availability in early myocardial ischemia/reperfusion.

A growing body of data indicates that ADMA may be responsible for endothelial vasodilator dysfunction in individuals with coronary and peripheral arterial disease, and those with risk factors for atherosclerosis, particularly hypercholesterolemia, hypertension, type 2 diabetes and insulin resistance as well as hyperhomocyst(e)inemia, since...
plasma ADMA has been found to be elevated and closely correlated with impaired vasodilator function in these conditions [5]. However, ADMA also seems to play a role in the physiological regulation of regional blood flow: When different regions of the normal heart of dogs were separated into high-flow and low-flow sections, ADMA concentrations in the homogenized myocardial tissue were significantly reduced (to 25%) in low perfused – but non-ischemic – myocardium compared to high-flow tissue [20]. Furthermore there is at least indirect evidence that ADMA might also regulate myocardial blood flow in the diseased state, since plasma ADMA concentrations are statistically related to hyperemic myocardial blood flow induced by dipyridamole in subjects with borderline hypertension and familial hypercholesterolemia [21]. So far little was known about the possible role of ADMA in the setting of myocardial ischemia and reperfusion. Transgenic mice with low ADMA concentrations have been found to develop less transplant vasculopathy after heterotopic heart transplantation [22] (a procedure also involving myocardial ischemia and reperfusion). Furthermore we have shown in a recent study that plasma ADMA is significantly elevated in patients with acute coronary syndromes [7]. The current study supports these findings and also provides a potential mechanism for the accumulation of tissue ADMA in mI/R.

ADMA is derived from the catabolism of proteins containing methylated arginine residues, which are hydrolyzed to release free intracellular ADMA. ADMA may then be excreted into the urine, but is predominantly metabolized by the enzyme DDAH. There are 2 isoforms of this enzyme, DDAH-1 and -2. DDAH-2 is the predominant isoform in heart and vascular tissue [8], but both isoforms may play an important role in regulating ADMA levels. We now observed that DDAH enzyme activity is inhibited in the first hours of reperfusion – coinciding with accumulation of ADMA and reduced NO-elaboration – independently of DDAH protein expression. In the late phase of reperfusion (24 h to 72 h) however, DDAH activity and ADMA tissue concentrations return to the baseline level and at the same time expression of DDAH-2 dramatically increases. In our model the exact mechanism of perturbation of DDAH-activity still needs to be clarified. We have recently shown that DDAH-activity is inhibited in vitro by oxidative stress induced by hyperhomocysteinemia [13] and that DDAH-activity can be preserved and subsequently ADMA accumulation can be blunted by antioxidants. Indeed, a massive load of oxygen-based free radicals (superoxide anion and hydroxyl radicals) is generated in the first few minutes of post-ischemic organ reperfusion [1,23]. Therefore it is logical to hypothesize that the observed early inhibition of DDAH is initiated by a burst of oxidative stress during ischemia, leading to ADMA accumulation. Furthermore it is currently not clear whether increased tissue ADMA after mI/R is derived from inflammatory cells, from the endothelium or from myocytes. Thus, further experiments are planned to elucidate the exact mechanism of DDAH inhibition during reperfusion and to clarify the source of ADMA in mI/R.

At this point the exact mechanism, how DDAH overexpression and/or ADMA elimination lead to increased eNOS phosphorylation, also needs further clarification. This phenomenon seems to be specific to mI/R, since it was not found in sham operated animals (Fig. 1a). One DDAH isoform (DDAH-2) has been reported to enhance VEGF mRNA expression in endothelial cells [24], however phosphorylation of eNOS was not mediated by VEGF and akt/PKB in our model, since VEGF expression or phosphorylation of akt/PKB were not observed after 4 h of reperfusion. But DDAH might be able to regulate protein signaling independent of the NOS pathway: DDAH-2 protein directly binds to protein kinase A and subsequently phosphorylates the transcription factor Sp1 without increasing NO production [25]. Therefore DDAH protein seems to be involved in regulation of intracellular pathways by protein–protein interaction additionally to its known function in ADMA metabolism. Alternatively also ADMA could have pathophysiological roles – such as direct or indirect regulation of NOS phosphorylation – additionally to its known inhibitory effect on NOS activity. These new hypotheses are currently tested in in vitro studies in our laboratory.

With our current method to measure myocardial injury we were not able to determine the area at risk as an internal control. However this parameter was measured in a subset of animals, where we could show that the AAR was very homogenous and comparable between DDAH-1 transgenic mice and WT animals. Still we cannot rule out that the concentration of ADMA, DDAH and NOx after ischemia or mI/R were influenced by different areas at risk in the two mouse strains. Furthermore exogenous sources of nitrogen-oxides and a washout phenomenon during reperfusion could have influenced our measurements of NO bioavailability. However, animals were kept on a stable diet and on nitrate free drinking water, and we think that measurements of NOS activity by conversion of labeled l-arginine also have its limitations in this setting. Finally, regulation of ADMA/DDAH pathway seems to be different in ischemia than it is in mI/R. Specifically, reduction of ADMA during the short phase of ischemia could not be explained by changes in DDAH and might not be responsible for the observed increases in NOx due to the fact that l-arginine was equally reduced.

5. Conclusion

In summary, we provide a new mechanism for the impairment of the NO synthase pathway during myocardial ischemia and reperfusion: The endogenous NOS inhibitor ADMA is elevated in the early phase of reperfusion due to inhibition of its metabolism by the enzyme DDAH, leading to a reduction of NO bioavailability. Partial elimination of ADMA in a genetic mouse model and amelioration of the
deleterious effects of ADMA by exogenous l-arginine drastically reduced markers of inflammation and subsequently the amount of m I/R injury. These insights may lead to new diagnostic modalities and therapeutic interventions for myocardial infarction, such as modulators of DDAH activity or expression.

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