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Sepiapterin reduces postischemic injury in the rat heart

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Abstract A reduced availability of tetrahydrobiopterin (BH4), an essential cofactor for NO-synthesis, is causally involved in the development of endothelial dysfunction associated with ischemia/reperfusion. We, therefore, investigated the effect of sepiapterin, a substrate for BH4 synthesis, on *postischemic* injury in myocardial infarction and myocardial stunning. In rats, myocardial stunning was induced by repetitive ischemia (5×10-min ligation of the left coronary artery, 5×20-min reperfusion) and myocardial infarction by 50-min ligation and 60-min reperfusion. Myocardial blood flow was determined by H₂-clearance, regional myocardial function by pulsed Doppler and infarct size by tetrazolium staining. Myeloperoxidase (MPO) activity was measured as a marker of neutrophil extravasation. cGMP was determined in rat serum as an indicator of increased NO synthesis. In animals treated with sepiapterin, regional myocardial function was significantly improved in both myocardial stunning and infarction and infarct size was significantly reduced. MPO activity decreased with sepiapterin treatment in both models. The systemic level of cGMP was reduced both following myocardial stunning and myocardial infarction in the control group. Pretreatment with sepiapterin induced a significant increase of cGMP level at the end of the protocol in both models. Substitution of sepiapterin reduces postischemic injury both in myocardial stunning and infarction apparently by ameliorating the availability of NO, thereby attenuating the activation of neutrophils in ischemia/reperfusion.

Keywords Endothelial function · Leukocytes · Myocardial infarction · Myocardial stunning · Nitric oxide

Introduction

A reduced bioavailability of nitric oxide (NO) has been shown to play an important role in the development of ischemia/reperfusion injury by the following mechanisms: (1) an increased expression of several adhesion molecules that facilitate the binding of leukocytes to the endothelium [11]; (2) a decrease in coronary reserve [27]; (3) increased oxygen consumption and decreased calcium sensitivity and contractile function during ischemia [6]; and (4) thrombus formation by increased platelet aggregation [18]. The underlying reasons for the reduction of NO during ischemia have not yet been clarified. The synthesis of NO from L-arginine is catalyzed by NO-synthase (NOS) and several cofactors. An essential cofactor of all NOS isoforms is tetrahydrobiopterin (BH4), which is intracellularly produced from GTP via GTP-cyclohydrolase I or, alternatively, from sepiapterin [26]. BH4 regulates the activity of NOS in several ways: it stabilizes the enzyme, acts as a redox factor and facilitates the binding to L-arginine [14, 26]. Recently it has become evident that under conditions when BH4 availability is reduced, NOS catalyzes the production of superoxide anions rather than NO [24, 29]. Superoxide anions rapidly react with NO to form toxic peroxynitrite which increases reperfusion injury. In isolated perfused coronary arterioles from pigs, following ischemia/reperfusion, exogenous application of different tetrahydrobiopterin metabolites attenuates endothelial dysfunction [27]. Studies of gastric, renal and pulmonary ischemia/reperfusion injury propose a protective effect of sepiapterin and BH4 [8, 10, 20]. To date, there have been no *in vivo* studies of the heart. The aim of the present study was therefore to investigate if substitution of BH4 attenuates cardiac injury following ischemia/reperfusion. We used two different experimental approaches in *in situ* perfused rat hearts: first, a model of myocardial stunning and, second, a model of myocardial infarction. As a measure of the effect of sepiapterin on NO production, we determined the cGMP level in serum from the experimental animals. Since the extravasation of neutrophils as

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a marker of reperfusion injury is prevented by NO, we determined MPO activity in myocardial tissue.

Materials and methods

Animal studies

Female Wistar-Furth rats of 215 ± 30 g body weight were anesthetized with intraperitoneal barbiturate (Inactin Byk-Gulden, 0.1 mg/100 g intraperitoneally) and ventilated with a volume of 2–3 ml at a rate of 60–75 per minute (small animal respirator, Harvard). Body temperature was maintained at 37°C using a heated operating table. The carotid artery was cannulated for continuous measurement of mean arterial blood pressure with a Statham pressure transducer and the jugular vein was cannulated to allow administration of fluid and drugs. The heart was exposed by removing an oblong portion of chest wall and sternum, and a tie was placed around the left coronary artery. The tie was then passed through a plastic cannula to allow ligation and reopening of the vessel.

The investigation conforms with the "Guide for the care and use of laboratory animals" published by the US National Institutes of Health (NIH publication No 85–23, revised 1996).

Determination of myocardial blood flow

Myocardial blood flow (MBF) was measured by the hydrogen clearance technique using platinum electrodes inserted into the myocardial tissue for measuring tissue H_2 pressure (PH_2). A discussion of the basic theory of determining tissue blood flow by inert gas clearance and demonstration of the applicability of H_2 as an indicator is given elsewhere [1]. In brief, the target tissue is saturated with hydrogen at a constant PH_2 by adding H_2 (30%) in inspired air. Thereafter, H_2 is removed from the breathing air and the tissue H_2 is washed out by H_2 -free blood draining the tissue yielding an exponential concentration decline. The tissue blood flow ($\text{ml} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$) is derived from the half-time of PH_2 decay as tissue blood flow = $\ln 2 / t_{1/2}$. The exponential fit of the measured PH_2 curve for determination of flow was done with a computer-aided device. The logarithmic regression coefficient of all curves was >0.98 . The electrodes used in this study were constructed from 50 μm epoxide insulated platinum wires with a 300–500 μm non-insulated PH_2 -sensing tip. The electrodes were inserted into the myocardium for approximately 2 mm and polarized against an intrarectally positioned silver/silver chloride reference electrode by -300 mV.

The H_2 -clearance method has been validated for use in isolated perfused rat hearts. At different perfusion rates, reliable values for MBF could be obtained. In the in situ perfused rat heart, measurements of MBF could be reliably reproduced with 5–10% changes.

Determination of regional left ventricular function

Regional left ventricular function was assessed by determination of myocardial systolic thickening fraction (FT) by use of the pulsed Doppler technique with a single epicardial transducer (Crystal Biotech) as described previously [5]. The Doppler crystal (probe size validated for rats) was placed on the ischemic area of the left ventricular wall and sutured to the epicardium with a fine stitch (prolene, 7.0). Myocardial wall thickening was evaluated by integrating the velocity of myocardial layers passing through a sample volume at a selected depth which was determined by characteristic acoustic and echo-signals on an oscilloscope. Myocardial thickening fraction was estimated by dividing systolic excursion by sample volume depth. Measurements could be reliably reproduced with 5–10%.

Determination of myeloperoxidase activity

Tissue-associated myeloperoxidase (MPO) activity was determined by the method of Suzuki as modified by Sirsjo [22]. At the end of the experiments, tissue obtained from the ischemic area of the left ventricle and from a control area of the right ventricle were centrifuged at 12,000 g for 5 min at 5°C to pellet the insoluble cellular debris. The pellet was then rehomogenized in an equivalent volume of 0.05 M potassium phosphate buffer (PBS, pH 6) containing 0.05% hexadecyltrimethylammonium bromide (HTAB) and 0.01 M ethylenediaminetetraacetic acid (EDTA). MPO activity was assessed by measuring H_2O_2 -dependent oxidation of 3,3',5,5'-tetramethylbenzidine (TMB). Extinction was measured in a spectrophotometer (model 24, Beckmann) at 655 nm. Horse radish peroxidase (Boehringer Mannheim, Germany) was used as a standard.

Determination of myocardial infarct size and area at risk

Myocardial infarct size was determined by tetrazolium staining. At the end of the MI protocol, the LCA was briefly reoccluded and Evans blue dye solution (3 ml, 2%) was injected *into the left ventricle* to allow perfused (stained blue) and non-perfused (unstained) areas of the heart to be distinguished. After removal of the hearts, the left ventricle was divided into six slices perpendicular to the apex–base axis. To distinguish between area at risk and infarcted myocardium, slices were incubated in *p*-nitroblue tetrazolium (NBT, 0.5 $\text{mg} \cdot \text{ml}^{-1}$ for 20 min at 37°C). In the presence of intact dehydrogenase enzyme systems, NBT forms blue-colored precipitates while areas of necrosis lack dehydrogenase activity and therefore do not stain. Tissue slices were photographed and area at risk, infarcted and non-infarcted areas were then determined using a computer-based system (Adobe Photoshop and NIH Image). Area at risk and infarcted area were expressed as a percentage of the left ventricle.

Histological assessment of myocardial tissue

To rule out the presence of necrosis following the repetitive ischemia protocol, at the end of the protocol, hearts were perfused with glutaraldehyde and imbedded into Epon araldite. Without knowledge of the applied experimental protocol, representative semi-thin dibasic stained transmural sections of the left ventricular free wall and of the interventricular septum were evaluated in a blinded fashion with respect to tissue edema, hemorrhage and necrosis by light microscopy at magnification $\times 400$.

Experimental protocol

After the preparation and a stabilization period of 30 min, four control measurements of MBF and FT were obtained before any intervention. The mean value of these four baseline measurements was taken as 100% in all groups. All subsequent measurements were related to this baseline value. Mean arterial blood pressure (MAP) and heart rate (HR) were continuously determined throughout the experiments.

Following determination of baseline values, a bolus of sepiapterin (2 $\text{mg} \cdot \text{kg}^{-1}$) was applied through the jugular vein catheter followed by a continuous intravenous infusion of 10 $\text{mg} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ in the intervention groups or saline (0.9%, 2 $\text{ml} \cdot \text{h}^{-1}$) in the control groups, respectively. Administration of sepiapterin was started 20 min prior to the first ischemia and continued throughout the experiment. An additional four control measurements of MBF and FT were taken to rule out possible effects of sepiapterin on baseline values. The doses of sepiapterin used in this study are equivalent to those used in similar investigations [9, 10, 11].

Following baseline measurements, in the *myocardial stunning* (MS) protocol, the LCA was five times ligated for 10 min, followed by 20 min of reperfusion. Values of MBF and FT were obtained

during ligation and 1, 5, and 15 min following reperfusion. At the end of the protocol, hearts were removed, tissue samples for MPO determination obtained and the hearts fixed in glutaraldehyde for histological investigation.

In the *myocardial infarction* (MI) protocol, the LCA was occluded for 50 min and reperused for 60 min. MBF and FT were measured during ischemia and at 1, 10, 20, 30 and 60 min of reperfusion. At the end, hearts were infused with Evans blue dye to mark the area at risk and fixed in glutaraldehyde after removal of tissue samples for MPO determination.

Determination of cGMP in rat serum

Systemic cGMP levels were determined at baseline and at the end of the experimental protocol. cGMP was determined in rat serum with a commercially available Elisa-kit (Amersham).

Statistical analysis

The data are expressed as mean \pm one standard error of the mean. To test for significance between means, as appropriate, the data were analyzed by Student's *t*-test for paired data or (repeated) analysis of variance followed by the Bonferroni *t*-test. For all tests, a probability value $p < 0.05$ was considered indicative of a statistically significant difference.

Results

In the control groups as well as in the intervention groups of both ischemic protocols, MBF was reduced by approximately 80% in the ischemic area in all experiments during ligation, whereas in the non-ischemic control area MBF was not statistically different from baseline values, indicating effective ischemia by coronary ligation. In all groups, HR remained fairly constant throughout the experiments. Baseline MAP was similar in all groups and at the end of the experimental protocol, there was an average reduction by 15% to 95 mmHg (n.s.). There was no significant change in HR, MAP, MBF, and FT due to intravenous infusion of sepiapterin.

Myocardial stunning

Under baseline conditions, FT was $22.9 \pm 0.4\%$. During ischemia, it was greatly reduced and following each ischemic episode there was a gradual decrease. At the end of the protocol, FT was $12.8 \pm 0.6\%$ ($p < 0.01$), a significant decline as compared to baseline (Fig. 1). In sepiapterin-treated animals, FT was initially $22.6 \pm 0.4\%$. In the course of repetitive ischemia/reperfusion, there was gradual decrease of FT to finally $17.2 \pm 0.6\%$. This reduction is significantly less as in the control group ($p < 0.001$; Fig. 1).

MBF was $4.2 \pm 0.5 \text{ ml} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ under baseline conditions and was reduced to $2.3 \pm 0.3 \text{ ml} \cdot \text{g}^{-1} \cdot \text{min}^{-1}$ at the end of the fifth ligation ($p < 0.01$). Immediately following reperfusion, there was a reactive hyperemia which lost intensity with the number of ischemia/reperfusion episodes (Fig. 2). In sepiapterin-treated animals, MBF, initially $4.1 \pm 0.3 \text{ ml} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$, was reduced to $2.4 \pm 0.2 \text{ ml} \cdot \text{g}^{-1} \cdot \text{min}^{-1}$ at the end of the protocol. The

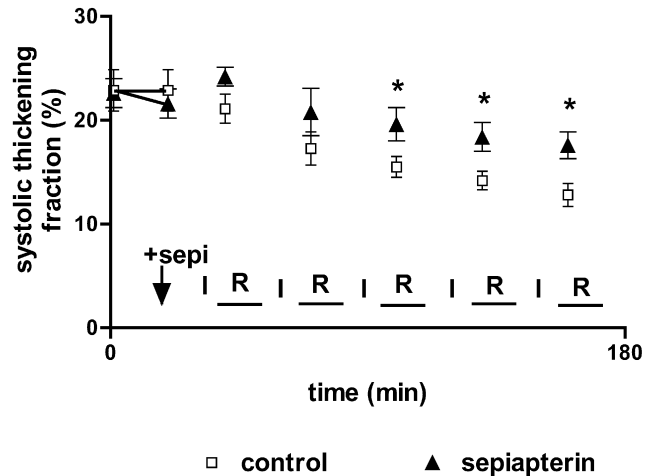


Fig. 1 Myocardial function in control ($n=7$) and sepiapterin-treated ($n=8$) animals from the stunning protocol during ischemia (I) and reperfusion (R). Data are presented as mean \pm SEM. * $p < 0.05$ vs. control

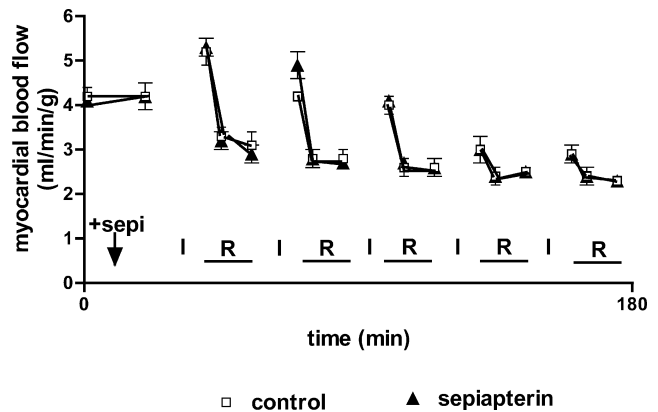


Fig. 2 Myocardial blood flow in control ($n=7$) and sepiapterin-treated ($n=8$) animals from the stunning protocol during ischemia (I) and reperfusion (R). Data are presented as mean \pm SEM

reduction of MBF was not significantly improved by sepiapterin as compared to the control group (Fig. 2).

By histological examination, only slight tissue edema without necrosis or hemorrhage was seen in the MS experiments both in the control and in the intervention groups indicating that there were no signs of irreversible ischemic myocardial damage as expected in myocardial stunning.

Myocardial infarction

FT, initially $21.3 \pm 1.9\%$ was reduced to $9.6 \pm 1.2\%$ during ischemia. During reperfusion, there was no significant increase of FT and after 60 min of reperfusion, FT was $12.3 \pm 1.3\%$ in the control group (Fig. 3). In sepiapterin-treated animals, FT, initially $21.9 \pm 0.9\%$, was reduced during ischemia to $10.3 \pm 1\%$ which is not different from

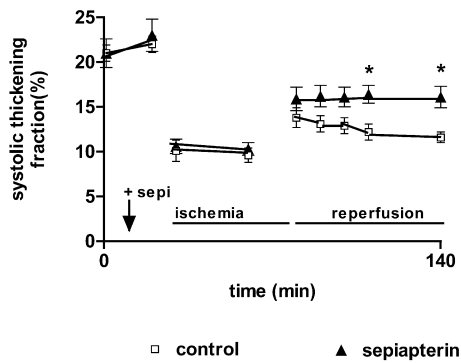


Fig. 3 Myocardial function in control ($n=12$) and sepiapterin-treated ($n=13$) animals from the infarct protocol. Data are presented as mean \pm SEM. * $p<0.05$ vs. control

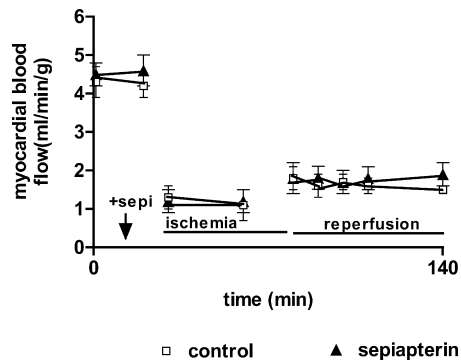


Fig. 4 Myocardial blood flow in control ($n=12$) and sepiapterin-treated ($n=13$) animals from the infarct protocol. Data are presented as mean \pm SEM

the control group. However, at the end of the reperfusion period, the reduction of FT was significantly lower in the sepiapterin-treated group as compared to the control group ($16.1 \pm 1.2\%$; $p=0.002$; Fig. 3).

MBF, initially $4.3 \pm 0.2 \text{ ml}\cdot\text{g}^{-1}\cdot\text{min}^{-1}$, was reduced to $1.1 \pm 0.2 \text{ ml}\cdot\text{g}^{-1}\cdot\text{min}^{-1}$ during ischemia. After reopening of the ligature, there was only a slight increase of MBF. Following 60 min of reperfusion, MBF was $1.5 \pm 0.1 \text{ ml}\cdot\text{g}^{-1}\cdot\text{min}^{-1}$, which is a reduction to 39.5% of baseline (Fig. 4). In sepiapterin-treated animals, MBF was initially $4.5 \pm 0.2 \text{ ml}\cdot\text{g}^{-1}\cdot\text{min}^{-1}$. There was a slight, non-significant increase following application of sepiapterin to $4.7 \pm 0.1 \text{ ml}\cdot\text{g}^{-1}\cdot\text{min}^{-1}$. During ischemia, MBF was reduced to $1.2 \pm 0.2 \text{ ml}\cdot\text{g}^{-1}\cdot\text{min}^{-1}$. At the end of the reperfusion episode, MBF was $1.9 \pm 0.3 \text{ ml}\cdot\text{g}^{-1}\cdot\text{min}^{-1}$ which is not significantly different from the control group ($p=0.09$; Fig. 4).

Mean area at risk of the left ventricle was $49.6 \pm 2.1\%$ and the infarcted area was $31.3 \pm 1.1\%$. Thus, the infarcted area was $63.1 \pm 1.6\%$ of the area at risk (Fig. 5). In sepiapterin-treated animals, the area at risk of the left ventricle was $52.3 \pm 2\%$ and the infarcted area $23.3 \pm 1\%$. Thus, the infarcted area was $44.5 \pm 2.3\%$ from the area at risk which is significantly less as compared to the control group (Fig. 5).

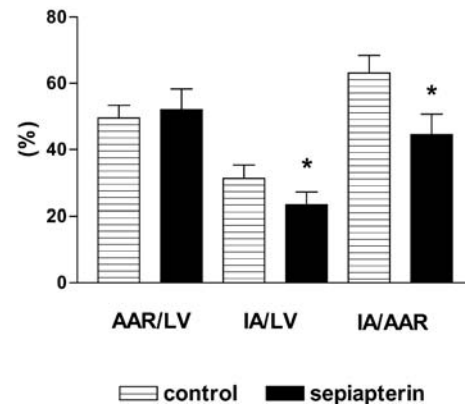


Fig. 5 Myocardial infarct size in the control ($n=12$) and sepiapterin-treated ($n=13$) group. Shown are the area at risk from the left ventricle (AAR/LV), the infarcted area from the left ventricle (IA/LV) and the infarcted area from the area at risk (IA/AAR) in % \pm SEM. * $p<0.05$ vs. control

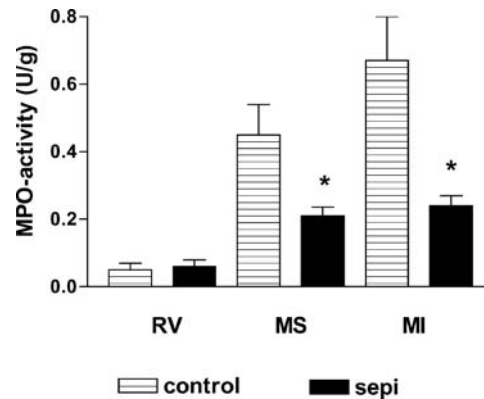


Fig. 6 Myeloperoxidase activity (MPO) in samples from the ischemic area of the left ventricle and from non-ischemic right ventricle (RV). In non-ischemic tissue, MPO was $0.05 \pm 0.02 \text{ U/g}$ tissue in the control group ($n=8$) and $0.06 \pm 0.02 \text{ U/g}$ tissue in the sepiapterin-treated group ($n=8$). MPO was greatly increased following myocardial stunning (MS) and myocardial infarction (MI) (MS: $0.45 \pm 0.05 \text{ U/g}$ tissue, $n=7$; MI: $0.67 \pm 0.04 \text{ U/g}$ tissue, $n=8$). Sepiapterin treatment significantly reduced the increase in MPO (MS: $0.21 \pm 0.02 \text{ U/g}$ tissue, $n=8$; MI: $0.24 \pm 0.03 \text{ U/g}$ tissue, $n=9$). * $p<0.05$ vs. control

MPO activity

Stunning group

MPO activity in tissue from the ischemic area was significantly increased as compared to that in non-ischemic tissue from the right ventricle. In sepiapterin-treated animals, MPO activity in tissue from the ischemic area was significantly lower as compared to that in tissue from the control group indicating reduced neutrophil extravasation in treated animals (Fig. 6).

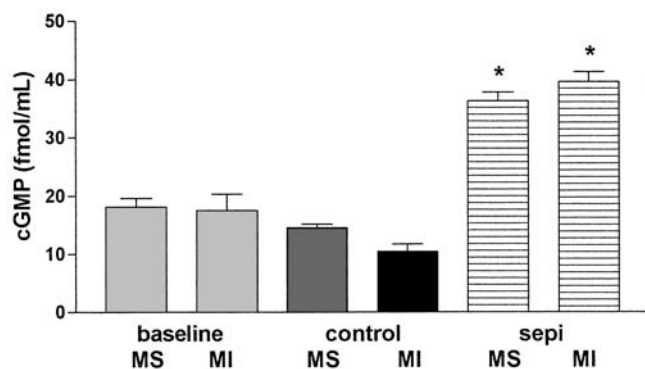


Fig. 7 Systemic level of cGMP in rats at baseline and following MS and MI in the control groups ($n=6$) and the sepiapterin-treated groups ($n=6$) in fmol/ml \pm SEM. * $p<0.001$ vs. control

Infarct group

MPO activity in tissue from the ischemic area was significantly increased as compared to that in non-ischemic tissue from the right ventricle. In sepiapterin-treated animals, MPO activity in tissue from the ischemic area was, again, significantly lower as compared to that in the control group (Fig. 6).

Influence of sepiapterin on cGMP levels

In the control groups, following both MS and MI, systemic cGMP levels were reduced at the end of the experiments. In the sepiapterin-treated groups, there was a significant increase in serum cGMP level, about twofold at the end of both the MS and the MI protocol. (Fig. 7).

Discussion

Summary

Intravenous application of sepiapterin, a substrate for endogenous synthesis of tetrahydrobiopterin which is an essential cofactor of NOS, improved the reduction of regional myocardial function in myocardial stunning and myocardial infarction and reduced the extent of necrosis in myocardial infarction. Furthermore, MPO activity as a marker of leukocyte extravasation into the myocardial tissue was significantly decreased by sepiapterin in both models. The effect of sepiapterin is at least partially mediated via increased availability of NO since it increased the production of cGMP. The results of this study provide evidence that sepiapterin is a protective agent in the prevention of ischemia/reperfusion injury in the heart.

Physiological and pathophysiological implications

Is NO good or bad regarding the pathophysiology of postischemic injury? NO has been shown to reduce infarct size in eNOS knockout mice [9] and to contribute to the development of cardiac hibernation [6]. L-Arginine, the substrate of NO synthesis, attenuates reperfusion injury [25, 32]. Several protective mechanisms of NO regarding reperfusion injury have been suggested (see Introduction) [6, 11, 17].

In contrast, however, there are reports indicating that endogenously formed NO significantly contributes to the development of reperfusion injury [4], that inhibition of NOS with L-NAME reduces myocardial reperfusion injury in rabbit hearts [17] and that infusion of L-arginine into isolated rabbit hearts potentiates ischemia/reperfusion injury [28]. As another source of ambiguity, it has been suggested that whereas basal release of endogenous NO may not be involved in the pathophysiology of reperfusion injury, under conditions of increased kinin or prostaglandin metabolism, NO may be cardioprotective [12] and that NO produced by eNOS is cardioprotective whereas the excessive amount of NO released by iNOS may be detrimental to the heart [30, 33].

The solution for the conflicting data reported from the literature may be the bimodal function of NOS. Under physiological conditions, there is a balance between endothelial production of NO and oxygen-derived free radicals by NOS. Under pathological conditions there is a shift of this balance towards the production of vasotoxic oxygen-derived free radicals [3]. An increased production of toxic radicals is involved in the pathophysiology of myocardial stunning and myocardial infarction [2, 13]. An important regulator for the control of the generation of NO is the essential NOS cofactor tetrahydrobiopterin: when tetrahydrobiopterin is depleted, NOS generates superoxide anions instead of NO [3, 29]. A reduced bioactivity of tetrahydrobiopterin under conditions such as ischemia/reperfusion would lead to increased production of vasotoxic superoxide anions and peroxynitrite not necessarily at the cost of reduced generation, but a reduced availability of NO due to an increased conversion to peroxynitrite. Indeed, there are convincing data demonstrating an increase of NO, superoxide and peroxynitrite production following ischemia/reperfusion [13, 31, 35]. Peroxynitrite finally results in amino acid nitritation and cellular injury [31, 34, 35]. The theoretical background would therefore strongly support the findings of our study, namely that sepiapterin improves the sequelae of ischemia/reperfusion. This is furthermore in line with the results of other investigations, showing that exogenous substitution of tetrahydrobiopterin or sepiapterin improves endothelial dysfunction by increasing the synthesis and/or stability of NO in vessels from patients with hypercholesterolemia and atherosclerosis [23] and in animals following ischemia/reperfusion [27]. In addition, endothelial cell damage can be prevented by pretreatment with sepiapterin via increased intracellular levels of tetrahydrobiopterin and finally NO [7]. We applied

sepiapterin prior to the onset of ischemia. Thus, we cannot rule out a primary effect on ischemic damage rather than on reperfusion injury.

In the present study, we found that extravasation of neutrophils during ischemia is prevented by pretreatment with sepiapterin. A variety of investigations demonstrated that neutrophil activation is involved in the development of myocardial stunning as well as reperfusion injury [15]. Inhibition of neutrophil extravasation reduced myocardial infarct size in different animal models [19, 21]. We, therefore, propose that at least part of the protective effect of sepiapterin is mediated by the prevention of leukocyte extravasation.

Another possible protective mechanism of sepiapterin is an improvement of endothelium-dependent vasodilation in coronary resistance vessels as it has been demonstrated in canine arterioles from hearts exposed to ischemia/reperfusion [24] and in resistance vessels from patients with diabetes and hypercholesterolemia [23]. However, in the present study, sepiapterin did not significantly improve MBF in contrast to an improvement of myocardial function and a reduction of myocardial infarct size. This may be explained by the fact that a decrease of myocardial perfusion is likely not of major importance regarding the pathophysiology of MS [16].

We do not know if the effect of sepiapterin is exclusively the result of increased availability of NO, or is mediated by a protective effect of sepiapterin or tetrahydrobiopterin itself as well, for example on neutrophil activation or as a radical scavenging substance. Furthermore, since we applied sepiapterin prior to left coronary artery occlusion, the protective effect could theoretically occur during ischemia. These questions need to be addressed in further investigations.

Conclusions

This investigation demonstrates that application of sepiapterin reduces ischemia/reperfusion injury by decreasing the extravasation of activated neutrophils most likely via improving the synthesis of NO. We conclude that an altered bioavailability of tetrahydrobiopterin is involved in the pathophysiology of postischemic injury in the heart. Substitution of this NOS cofactor might therefore be a promising therapeutic approach in the prevention and treatment of cardiac ischemia/reperfusion injury.

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