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ARTYKUŁ ORYGINALNY / *ORIGINAL ARTICLE*

The endothelial nitric oxide synthase cofactor tetrahydrobiopterin shields the remote myocardium from apoptosis after experimental myocardial infarction *in vivo*

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Abstract

Background: Following myocardial infarction (MI), apoptosis occurs early in the remote myocardium and contributes to the processes of myocardial remodelling. Increased nitrosative stress is a well-known and potent inductor of myocardial apoptosis. Excess activation of endothelial nitric oxide synthase (eNOS) increases its uncoupling potential and results in nitrosative stress via formation of peroxynitrite. However, the pathophysiological role of eNOS signalling in the remote myocardium after MI is as yet undefined.

Aim: The impact of eNOS activation on pro- and anti-apoptotic signalling in the remote myocardium and the influence of pretreatment with the eNOS cofactor tetrahydrobiopterin (BH4) on eNOS activation, nitrosative stress level, and apoptosis induction and execution were studied in a rat MI model *in vivo*.

Results: Twenty-four hours after anterior MI, eNOS activity in animals treated with left anterior descending coronary artery ligation (LIG) significantly increased in the posterior left ventricular (LV) myocardium as did protein nitrosylation when compared to sham treatment. This was paralleled by induction of apoptosis via the extrinsic and intrinsic pathways. Moreover, anti-apoptotic signalling via protein kinase B/Akt and glycogen synthase-kinase 3 beta was suppressed. Notably, pretreatment with the eNOS cofactor BH4 reduced eNOS activation, prevented excess protein nitrosylation, blunted apoptosis induction, facilitated anti-apoptotic signalling, and eventually prevented apoptosis execution.

Conclusions: Here we showed that 24 h after experimental MI in rats *in vivo*, apoptosis was induced in the posterior non-infarcted LV wall. Evidence is presented that pretreatment with the eNOS cofactor BH4 resulted in less nitrosative stress and weakened apoptotic processes, although the stabilisers contained did participate in this phenomenon. Because apoptosis is a crucial component of myocardial remodelling, influencing eNOS signalling might be an interesting pharmacological target for the development of novel anti-remodelling therapies.

Key words: myocardial infarction, myocardial remodelling, apoptosis, endothelial nitric oxide synthase, nitric oxide, nitrosative stress

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INTRODUCTION

Despite advances in treatment, cardiovascular disease and especially acute coronary syndromes are still leading causes of morbidity and mortality worldwide [1]. In the early phase after myocardial infarction (MI), acute ischaemic symptoms are prevailing whereas in the late phase loss of viable myocardium may cause progressive left ventricular (LV) dysfunction and development of symptomatic LV heart failure (HF). From a pathophysiological perspective, the central underlying process contributing to the development of HF is ventricular myocardial remodelling, involving the key processes of apoptosis, necrosis, and hypertrophy [2]. While development

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of HF due to progressive LV dysfunction after acute MI phenomenologically is acknowledged, the molecular mechanisms promoting hypertrophy, apoptosis, necrosis, and fibrosis are still not completely understood.

Although the loss of viable, contractile myocardium by apoptosis and necrosis can in part be compensated by hypertrophy, interstitial fibrosis resulting in scar healing usually cannot be totally prevented. Progressive HF with reduced LV ejection fraction (LVEF) is the consequence, and it results in systemic compensatory mechanisms, namely activation of the renin–angiotensin–aldosterone system [3]. These initially beneficial and compensatory effects on haemodynamics nourish, however, in the mid- and long-term negative adaptive remodelling processes of the heart itself, further promoting the progression of LV dysfunction (vicious circle).

Apoptosis is a crucial part in post-MI ventricular remodelling [4]. More recently, besides apoptosis, the phenomena of autophagy and necroptosis have also been implicated in the ventricular remodelling process and are subjected to extensive ongoing research [5]. While being extensively studied in the infarct zone and border zone after MI, the emphasis of apoptosis in the non-infarcted remote myocardium is less pronounced and is still discussed controversially. We previously demonstrated that apoptotic markers are activated in the non-infarcted remote myocardium as early as one day after MI, with persistence for up to four weeks [6]. Intriguingly, when considering total myocardial apoptosis after MI, apoptosis in the remote myocardium affects up to 70% of all myocardial apoptotic cells after 12 weeks [7]. Induction of apoptosis is multifactorial with neuro-humoral, mechanical, and, notably, oxidative and nitrosative stress being among the triggering factors [4].

Activation of apoptosis occurs in two phases with the initiation phase preceding the effector phase [8]. Initiation of apoptosis can be achieved via the extrinsic and intrinsic pathways [8] and is tightly controlled by physiological inhibitors such as protein kinase B/Akt (PKB/Akt) [9].

A well-known and potent inductor of apoptosis is increased nitrosative stress [10]. Nitric oxide (NO) synthases (NOSs) are commonly believed to exhibit cardioprotective effects after MI due to NO production, given the availability of the essential NOS cofactors NADPH, FAD, FMN, calcium/calmodulin, and tetrahydrobiopterin (BH4) [10]. The endothelial NOS (eNOS) is the predominant cardiac isoform and is detectable in endothelial cells and cardiomyocytes [11]. Phosphorylation increases eNOS activity, with Ser1177 being one of the most important phosphorylation sites for increasing NO production [12]. Of note, improper electron transfer may result in eNOS uncoupling, a condition when a superoxide anion is produced instead of NO [13]. Various causes of eNOS uncoupling are described, including non-stable eNOS dimerisation and eNOS phosphorylation at position Thr495 [13]. In general, eNOS uncoupling is promoted by facilitating electron transfer to oxygen with subsequent superoxide anion formation [13]. Superoxide anions do not possess pro-apoptotic potential. However, they easily react with NO to form peroxynitrite [14]. Notably, peroxynitrite not only increases nitrosative stress but also inactivates the eNOS cofactor BH4 that acts as a stabiliser of non-stable eNOS dimers, further propagating the uncoupling process [10]. A vicious circle is established. However, the role of BH4 on eNOS activation, nitrosative stress and induction and execution of apoptosis in the remote myocardium after MI remains undefined and constitutes the subject of this study.

METHODS

Experimental MI, sampling, and determination of infarct size

Left anterior descending coronary artery (LAD) ligation (LIG) and sham operation (sham) in adult male Wistar rats were performed as described previously [6, 15, 16]. Pretreatment was administered 1 h prior to operation, if applicable. After the operation, anaesthesia was stopped and animals were kept alive and conscious. 24 h after the operation, animals were re-anesthetised and transmural drill biopsies from the posterior non-infarcted wall of the LV were taken from eight animals per treatment group. Hearts from another three animals per group were kept for immunofluorescence experiments. Preliminary determination of infarct sizes was performed as previously described [6]. Briefly, following 30 min of reperfusion, 10 mg/kg body weight propidium iodide (Fluka Biochemica, Switzerland) at a concentration of 1 mg/mL was administered as bolus intravenously to demarcate the infarcted area. After another 15 min of reperfusion, the hearts were excised and mounted on a Langendorff perfusion setup at the aortic root. Hearts and coronaries were perfused retrogradely at constant pressure (100 cmH_2O) with 0.9% saline to wash out residual blood from the coronaries. Next, the LAD ligation was re-occluded and 4–9 μm fluorescent microspheres (Duke Scientific, Palo Alto, USA) were infused to demarcate the viable myocardium from the area at risk. Hearts were weighed, snap frozen in liquid nitrogen, and stored at –20°C until further processing. Within two weeks, transverse cryo-sections of 2 mm were prepared and ultraviolet light microscopy (wave length 340 nm) was performed. Planimetry was used to determine the infarct zone (propidium iodide, bright red fluorescence), the area at risk (infarct zone + non-fluorescent area), and the viable myocardium (fluorescent microspheres, bright green fluorescence). All animal procedures were in agreement with the "Guide for the Care and Use of Laboratory Animals" and were approved by the Local Ethics Committee (# 24-9168.11-1/2012-53).

Treatment groups

Adult male Wistar rats were allocated to one of six treatment groups, each group consisting of a total of 11–15 animals (Table 1). Tetrahydrobiopterin (BH4) is marketed under the brand name Kuvan®, not as a pure substance but in combination with the stabilisers ascorbic acid and riboflavin. Hence, the

Table 1. Treatment groups. Pretreatment was administered 1 h prior to operation orally by gastric feeding tube, if applicable

Group	Abbreviation	N	Pretreatment	Operation
1	LIG	15	None	LAD ligation
2	Sham	11	None	Sham
3	LIG + Kuvan®	15	tetrahydrobiopterin (BH4) (20 mg/kg bw) ascorbic acid (0.1 mg/kg bw) riboflavin (0.006 mg/kg bw)	LAD ligation
4	Sham + Kuvan®	11	tetrahydrobiopterin (BH4) (20 mg/kg bw) ascorbic acid (0.1 mg/kg bw) riboflavin (0.006 mg/kg bw)	Sham
5	LIG + stabiliser	15	ascorbic acid (0.1 mg/kg bw) riboflavin (0.006 mg/kg bw)	LAD ligation
6	Sham + stabiliser	11	ascorbic acid (0.1 mg/kg bw) riboflavin (0.006 mg/kg bw)	Sham

bw — body weight; LAD — left anterior descending coronary artery; LIG — treatment group experiencing true LAD ligation; sham — control group that experienced a sham operation without LAD ligation; Kuvan® — Kuvan® pretreatment consisting of tetrahydrobiopterin (active compound) and the stabilisers ascorbic acid and riboflavin; n — number of animals

experimental design was adapted accordingly to address for potential confounding immanent anti-oxidative and potential anti-apoptotic effects of the stabiliser formulation (treatment groups 5 and 6).

Protein determination

Protein determination was performed according to the method of Bradford et al. as described previously [6, 15, 16].

Immunoblotting analysis

For immunoblotting analysis, aliquots of 30 μg total protein were separated on SDS-polyacrylamide gels and transferred to PVDF membranes as reported previously [6, 16]. Pre-stained molecular weight standards were electrophoresed and transferred in parallel. Primary antibodies targeting bcl-2, bax, Akt, GSK-3b, phospho-GSK-3b, eNOS, phospho-eNOS(Ser1177), caspase 3 and 8, cleaved caspase 3 and 8, and nitrotyrosin (all Cell Signaling, Danvers, USA; except anti-nitrotyrosin [Upstate, Lake Placid, USA], anti-bax [Calbiochem, Schwalbach, GER] and anti-bcl-2 [Santa Cruz, Heidelberg, GER]) were polyclonal and rabbit raised and detected by a goat-anti-rabbit-IgG HRP-linked secondary antibody (Santa Cruz, Heidelberg, GER). The primary monoclonal mouse anti-phospho-Akt(Ser473) antibody (Cell Signalling, Danvers, USA) was detected by a sheep-anti-mouse-IgG HRP-linked secondary antibody (Amersham, Buckinghamshire, GB). A comprehensive synopsis of the primary and secondary antibodies, their respective hosts, and dilutions is given in Table 2. Detection of the specific protein bands was performed using Western Lightning ECL chemiluminescence kit (Perkin Elmer). For semiquantitative analysis, intensities of protein bands were quantified by densitometry using QuantityOne software (BioRad, Hercules, USA) and normalised to the respective inactive or non-phosphorylated protein (caspase 3 and 8, eNOS, Akt, GSK-3b) or Ponceau-S protein staining (0.05%; Sigma, Steinheim, GER), as reported previously [6]. The protein ratios of p-GSK-3b/GSK-3b, p-eNOS(Ser1177)/eNOS and p-Akt(Ser473)/Akt were determined sequentially from one single membrane each after protein stripping. Each sample was analysed in three independent repetitions. Sample normalisation to compare target protein expression levels between different samples on the same blot or across several blots was performed with a loading control to normalise the data. In preliminary experiments, target protein aliquots were analysed in the presence of excess antigenic peptide. The peptides fully blocked staining of the specific bands (not shown). Increased amounts of proteins were analysed on the gels to ensure linearity of the analysis in the protein range used (not shown).

Immunofluorescence, detection of apoptosis (M30, TUNEL)

Cryo-sections were prepared as described previously [15, 16]. Apoptotic cells were detected by the means of M30 antibody, recognising caspase 3 cleaved ceratin-18 neo-epitope (M30 CytoDeath™, Enzo Life Sciences, Lörrach, GER; 1:100). The secondary antibody used was a Cy-3 conjugated anti-mouse antibody (Cy™, Jackson, UK; 1:200). Counterstaining was done with 4',6-diamidin-2-phenylindol (DAPI) for 3 min (0.5 μg/mL in PBS; Applichem, Darmstadt, GER). Final fixation was done using Mount Flour (BroCyl, Luckenwalde, GER). Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labelling (TUNEL) was performed as described previously [15] using the "in situ cell death detection kit" (Roche, Mannheim, GER), then counterstaining with DAPI and final fixation were done as described above. Samples were viewed with an inverted fluorescence microscope (Axio Vert S100, Zeiss, Göttingen, GER) at a 10- to 40-fold magnification. Im-

Table 2. Synopsis of the primary and secondary antibodies

Antibody	Host	Dilution	Company
Primary antibodies			
Anti-caspase 8 (D35G2)	Rabbit	1:1000	Cell Signalling, Danvers, USA
Anti-cleaved caspase 8 (Asp387)	Rabbit	1:250	Cell Signalling, Danvers, USA
Anti-caspase 3	Rabbit	1:1000	Cell Signalling, Danvers, USA
Anti-cleaved caspase 3 (Asp175/5A1)	Rabbit	1:100	Cell Signalling, Danvers, USA
Anti-GSK-3β	Rabbit	1.500	Cell Signalling, Danvers, USA
Anti-phospho-GSK-3β	Rabbit	1:250	Cell Signalling, Danvers, USA
Anti-Akt	Rabbit	1:500	Cell Signalling, Danvers, USA
Anti-phospho-Akt (Ser473)	Mouse	1:250	Cell Signalling, Danvers, USA
Anti-eNOS	Rabbit	1:500	Cell Signalling, Danvers, USA
Anti-phospho-eNOS (Ser1177)	Rabbit	1:200	Cell Signalling, Danvers, USA
Anti-bax (PC66)	Rabbit	1:100	Calbiochem, Schwalbach, GER
Anti-bcl-2 (N-19)	Rabbit	1:100	Santa Cruz, Heidelberg, GER
Anti-nitrotyrosin	Rabbit	1:500	Upstate, Lake Placid, USA
Secondary antibodies			
IgG HRP-linked anti-mouse	Sheep	1:10,000	Amersham, Buckinghamshire, GB
IgG HRP-linked anti-rabbit	Goat	1:5000	Santa Cruz, Heidelberg, GER

age acquisition was done using the Spot RT KE Slider camera (SPOT Imaging, Sterling Heights, USA). For quantification, three randomly chosen representative visual fields from the infarcted anterior zone and the non-infarcted posterior zone of the LV wall from each of the four section levels were analysed using MetaMorph software (Molecular Devices, Sunnyvale, USA). Data was recorded as the number of apoptotic cells in relation to total cell count per visual field.

Statistical analysis

Data analysis was done using the unilateral variance test without repetition with *post hoc* testing by means of the Tukey Kramer test. Additionally, Student's t-test was used for comparison of two groups. All values are given as means ± standard error of the mean (SEM) or standard deviation (SD), as indicated. $P < 0.05$ was considered statistically significant, and $p < 0.2$ was defined as a trend towards statistical significance.

RESULTS

Tetrahydrobiopterin prevents eNOS activation and reduces protein nitrosylation

Activation of eNOS increases the likelihood of its uncoupling and may lead to increased nitrosative stress by peroxynitrite formation [13]. Therefore, we first studied eNOS activity in the non-infarcted posterior wall of the LV. Induction of MI in the LIG group resulted in significantly increased eNOS activity, as expressed by increased p-S^{1177}-eNOS/eNOS ratio (Fig. 1A). Of note, BH4 pretreatment prevented the ischaemia-induced increase in eNOS activation by phosphorylation at Ser1177 in LIG animals, whereas pretreatment with the stabilisers only had no effect (Fig. 1A). Uncoupling of eNOS can be transient and the short-lived products cannot be neutralised during *in vivo* experiments by the administration of toxic trapping substances for ethical reasons. Therefore, we assessed protein modification by peroxynitrite using an anti-nitrotyrosin antibody as a surrogate parameter of nitrosative stress [17]. Paralleling increased eNOS activity in non-pretreated and stabiliser-only pretreated LIG animals, we found a trend towards increased protein nitrosylation in these groups (Fig. 1B). Of note, between-group comparison showed a significant reduction of protein nitrosylation in BH4 pretreated LIG animals compared to non-pre-treated LIG animals (Fig. 1B). Because there is a correlation between the extent of protein nitrosylation and peroxynitrite formation and the latter permitting conclusions on nitrosative stress level [17], we conclude that BH4 shields the remote myocardium from excess nitrosative stress.

Tetrahydrobiopterin ameliorates apoptosis induction

To study pro-apoptotic signalling in the non-infarcted posterior LV wall, marker proteins indicative for activation of the extrinsic and intrinsic pathways and the effect of pretreatment with BH4 and stabiliser only were assessed. In terms of the extrinsic pathway, protein expressions of the initiator caspase 8 and effector caspase 3 were assessed, both in their inactive (pro-caspase) and catalytically active, cleaved forms (Fig. 2). In terms of the intrinsic pathway, the bcl-2/bax ratio was stud-

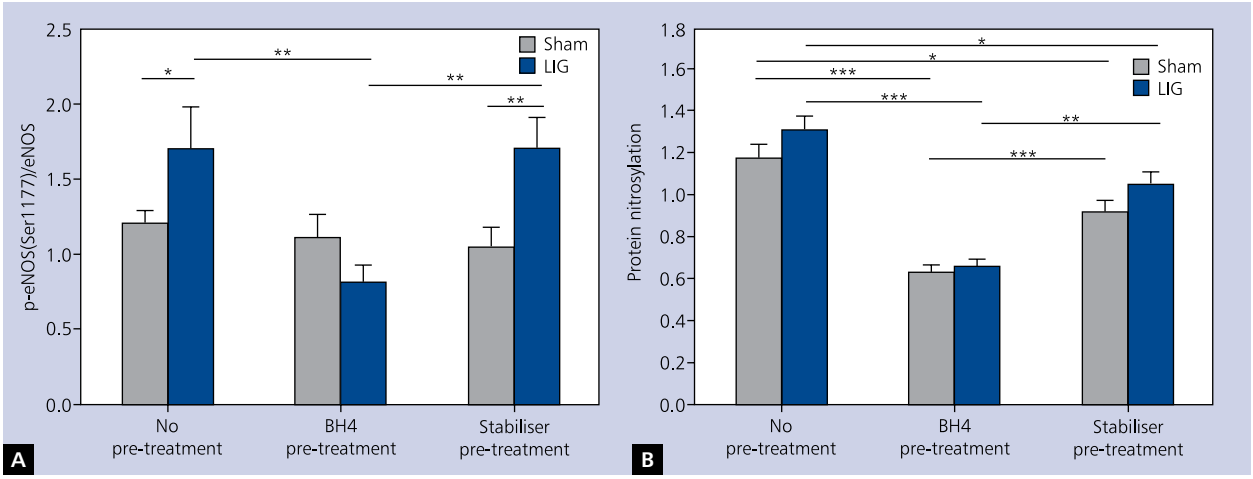

Figure 1. Tetrahydrobiopterin (BH4) prevents endothelial nitric oxide synthase (eNOS) activation and reduces protein nitrosylation in the posterior left ventricular (LV) myocardium 24 h after anterior myocardial infarction. **A.** LIG treatment causes a significant increase in eNOS phosphorylation at Ser1177. BH4 pretreatment significantly reduced p-eNOS(Ser1177)/eNOS protein ratio in LIG animals. Stabiliser-only pretreatment did not have significant implications on the p-eNOS(Ser1177)/eNOS protein ratio. **B.** BH4 pretreatment reduced posterior LV myocardium protein nitrosylation, indicative of less nitrosative stress. A similar effect, although to a lesser extent, was observed in stabiliser-only pretreated animals; LIG — treatment group experiencing true LAD ligation; sham — control group that experienced a sham operation without LAD ligation; LAD — left anterior descending coronary artery (n = 8–10 per group, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001; values are means ± SEM)

ied (Fig. 3). No significant intra-group differences between sham and LIG treated animals were observed in terms of total caspase 8 protein expression (Fig. 2A). However, in terms of catalytically active, cleaved caspase 8, a trend towards increased protein expression and therefore activity was evident in non-pretreated LIG animals compared to sham treatment (Fig. 2B). Of note, between-group comparison showed caspase 8 activation effectively prevented in BH4 pretreated LIG animals, yet a similar preventive effect was observed in stabiliser-only pretreated LIG animals (Fig. 2B). Downstream of caspase 8 we studied effector caspase 3 protein expression and activity. Analogous to total caspase 8 protein expression, no significant changes within treatment groups were observed for total caspase 3 protein expression in the non-infarcted posterior LV wall (Fig. 2C). In terms of caspase 3 activation, however, there was a trend towards increased cleaved caspase 3 protein expression in non-pretreated LIG animals, resembling the results for caspase 8 (Fig. 2D). Remarkably, active caspase 3 in terms of cleaved caspase 3 protein expression was not detectable by western blotting in the BH4 or in the stabiliser-only pretreated LIG and sham animals (Fig. 2D). We conclude that BH4 pretreatment ameliorates apoptosis induction in the remote myocardium by the extrinsic pathway, but this effect is partially mediated by the stabiliser compounds. The ratio of anti-apoptotic bcl-2 to pro-apoptotic Bax is one of the key regulatory mechanisms controlling apoptosis induction via the intrinsic pathway. We showed that LIG treatment caused a significant shift of the bcl-2/Bax ratio towards pro-apoptotic Bax in the non-infarcted posterior LV wall (Fig. 3). Of note, BH4 pretreatment ameliorated apoptosis activation via the intrinsic pathway (Fig. 3), but this effect seems partially mediated by the stabiliser compounds (Fig. 3).

Tetrahydrobiopterin promotes anti-apoptotic signalling via PKB/Akt and GSK-3β

Phosphorylated protein kinase B/Akt (phospho-Akt) is a well-known apoptosis regulator, preventing apoptosis propagation by the phosphorylation and inactivation of key pro-apoptotic mediators, including caspases and glycogen synthase-kinase 3 beta (GSK-3β) [9]. LIG treatment resulted in a trend towards decreased phospho-Akt(Ser473)/Akt protein ratio, indicating a pro-apoptotic environment (Fig. 4A). Of note, BH4 pretreatment reversed this ratio and comparison between non-pretreated and BH4 pretreated animals showed the phospho-Akt(Ser473)/Akt protein ratio significantly increased in BH4 pretreated LIG animals, reflecting a shift towards apoptosis amelioration (Fig. 4A). Of note, although stabiliser-only pretreatment similarly inversed the phospho-Akt(Ser473)/Akt ratio, it failed to elicit significant effects when compared to animals that received no pretreatment (Fig. 4A). GSK-3β in its active, non-phosphorylated form inhibits anti-apoptotic transcription factors such as CREB or heat shock factor-1 and promotes apoptosis through p53 [9]. GSK-3β is inhibited by phosphorylation at Serin 9 via activated,

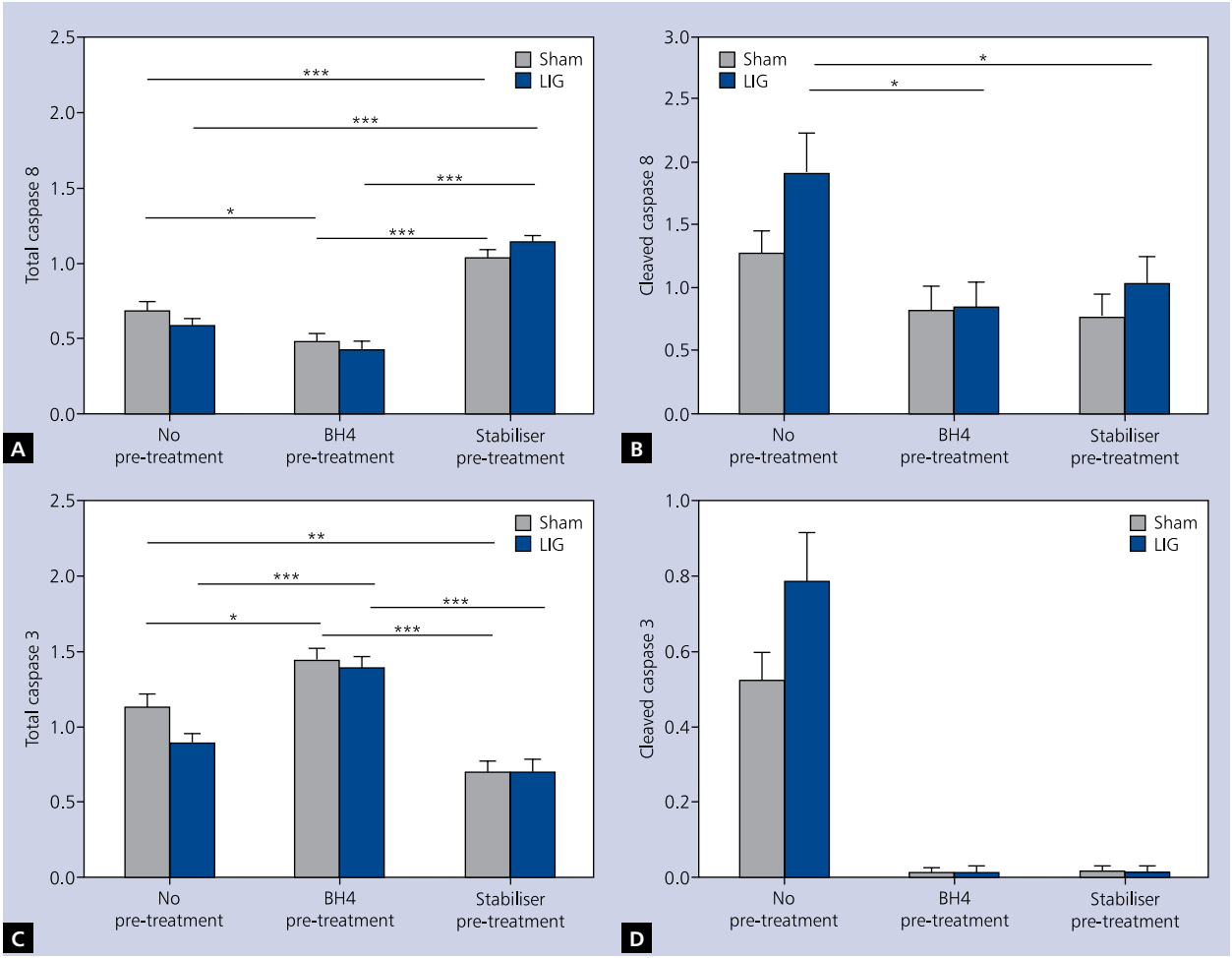

Figure 2. Tetrahydrobiopterin (BH4) ameliorates caspase activation in the posterior left ventricular myocardium 24 h after anterior myocardial infarction. **A.** Total caspase 8 protein expression was highest after stabiliser pretreatment. No significant intra-group differences between sham and LIG-treated animals were observed. **B.** LIG treatment resulted in a trend towards increased caspase 8 activation, as expressed by increased cleaved caspase 8 protein expression. Caspase 8 activation was prevented by BH4 and stabiliser-only pretreatment. **C.** Total caspase 3 protein expression was highest following BH4 pretreatment. No significant intra-group differences between sham and LIG treated animals were seen. **D.** LIG treatment resulted in increased caspase 3 activation, as expressed in increased cleaved caspase 3 protein expression levels. Remarkably, caspase 3 activation assessed by cleaved caspase 3 protein expression was not detectable in BH4 or in stabiliser-only pretreated animals; LIG — treatment group experiencing true LAD ligation; sham — control group that experienced a sham operation without LAD ligation; LAD — left anterior descending coronary artery (n = 8–10 per group, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001; values are means ± SEM)

i.e. phosphorylated, Akt [9]. Here we show that LIG treatment resulted in a significantly decreased phospho-GSK-3β/GSK-3β protein ratio, indicating a pro-apoptotic environment (Fig. 4B). Of note, BH4 pretreatment preserved the phospho-GSK-3β/GSK-3β protein ratio with a significantly higher phospho-GSK-3β/GSK-3β ratio compared to not pre-treated LIG animals. Similar effects were observed in stabiliser-only pretreated LIG animals (Fig. 4B).

Tetrahydrobiopterin prevents myocardial apoptosis execution

Early execution of apoptosis can be detected by immunohistochemistry using M30 antibody. Because cleavage of cytokeratin-18 occurs early during apoptosis, M30 immunohistochemistry has emerged as established method for the detection of apoptosis onset [18]. Here we show that 24 h after anterior MI, apoptosis execution in LIG animals occurs in the infarcted anterior wall of the LV as well as in the non-infarcted posterior LV wall, albeit to a lesser extent (Fig. 5). Of note, BH4 pretreatment prevented apoptosis execution in the anterior and posterior LV walls, with apoptotic cell count almost reaching sham level. Remarkably, no cardioprotective effects were seen after stabiliser-only pretreatment (Fig. 5). Furthermore, late-phase apoptosis execution was studied using TUNEL technique. LIG animals showed a significant increase in TUNEL-positive cells at 24 h after MI both in the

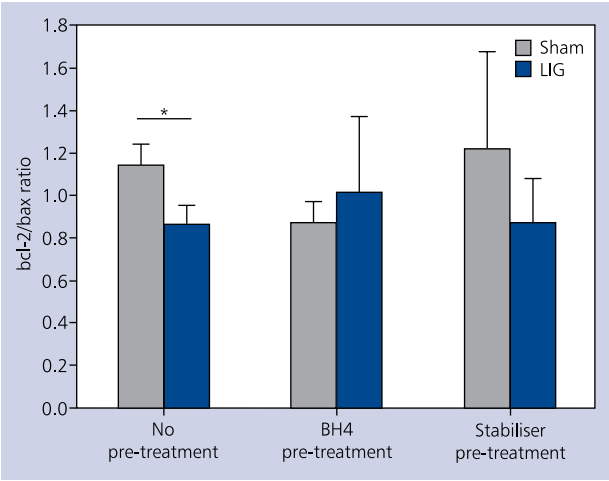

Figure 3. Tetrahydrobiopterin (BH4) ameliorates activation of the intrinsic apoptotic pathway in the posterior left ventricular myocardium 24 h after anterior myocardial infarction. LIG animals showed a significant shift of the bcl-2/bax ratio towards pro-apoptotic bax. Pretreatment with BH4 prevented activation of the intrinsic pathway. Similar effects were seen after stabiliser pre-treatment; LIG — treatment group experiencing true LAD ligation; sham — control group that experienced a sham operation without LAD ligation; LAD — left anterior descending coronary artery (n = 8–10 per group, *p ≤ 0.05; values are means ± SEM)

anterior and posterior LV walls (Fig. 6). Of note, BH4 pretreatment again effectively prevented apoptosis execution, closely resembling the results for the early apoptotic phase (M30). Remarkably, again no cardioprotective effects were observed in stabiliser-only pretreated LIG animals (Fig. 6).

DISCUSSION

Apoptosis execution as a consequence of induced pro- and simultaneously suppressed anti-apoptotic signalling was detected in the non-infarcted posterior LV wall 24 h after experimental anterior MI in rats *in vivo*. Induction of apoptosis was paralleled by eNOS activation and an increase in nitrosative stress. Pretreatment with the eNOS cofactor BH4 prevented eNOS activation, reduced the nitrosative stress level, and ameliorated induction and execution of apoptosis. Taken together, our results suggest that pretreatment with the eNOS cofactor BH4 shields the remote myocardium from apoptosis after MI, attenuating myocardial remodelling processes and progressive HF development.

Following acute MI, the majority of cell loss occurs within the first 24 h due to necrosis and apoptosis in the infarcted area as well as in the remote non-ischaemic remodelling zone. Execution of apoptosis in the remodelling zone occurs as early as 3–6 h following MI [6, 7, 15]. It is believed that the total amount of myocardial cell loss due to apoptosis exceeds necrotic cell loss, especially in the remote myocardium distant from the infarcted area where myocardial remodelling processes prevail [6, 7]. Therefore, we studied apoptosis induction and execution in the posterior non-infarcted LV wall 24 h after experimental anterior MI. However, focusing on one single time point, the results presented in this study may not be applicable to different time points of the remodelling process.

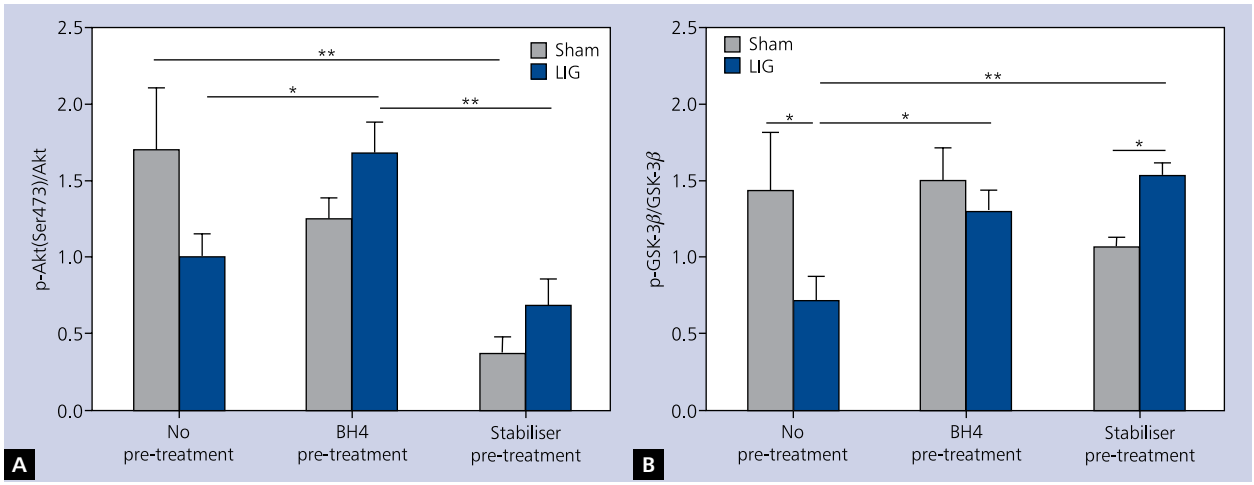

Figure 4. Tetrahydrobiopterin (BH4) promotes anti-apoptotic signalling while facilitating Akt and GSK-3β/phosphorylation in the posterior left ventricular myocardium 24 h after anterior myocardial infarction. **A.** LIG treatment resulted in a decreased phospho-Akt(Ser473)/Akt protein ratio, indicative of a pro-apoptotic environment. BH4 pretreatment inversed the phospho--Akt(Ser473)/Akt protein ratio and promoted a significant increase of the phospho-Akt(Ser473)/Akt protein ratio compared to the none pre-treatment group. **B.** LIG treatment caused a significant decrease in the phospho-GSK-3β/GSK-3β protein ratio, indicative of a pro-apoptotic environment. BH4 pre-treatment preserved the phospho-GSK-3β/GSK-3β protein ratio that was significantly higher compared to non-pretreated LIG animals. A similar effect was observed in stabiliser-only pretreated LIG animals; LIG — treatment group experiencing true LAD ligation; sham — control group that experienced a sham operation without LAD ligation; LAD — left anterior descending coronary artery (n = 8–10 per group, *p ≤ 0.05, **p ≤ 0.01; values are means ± SEM)

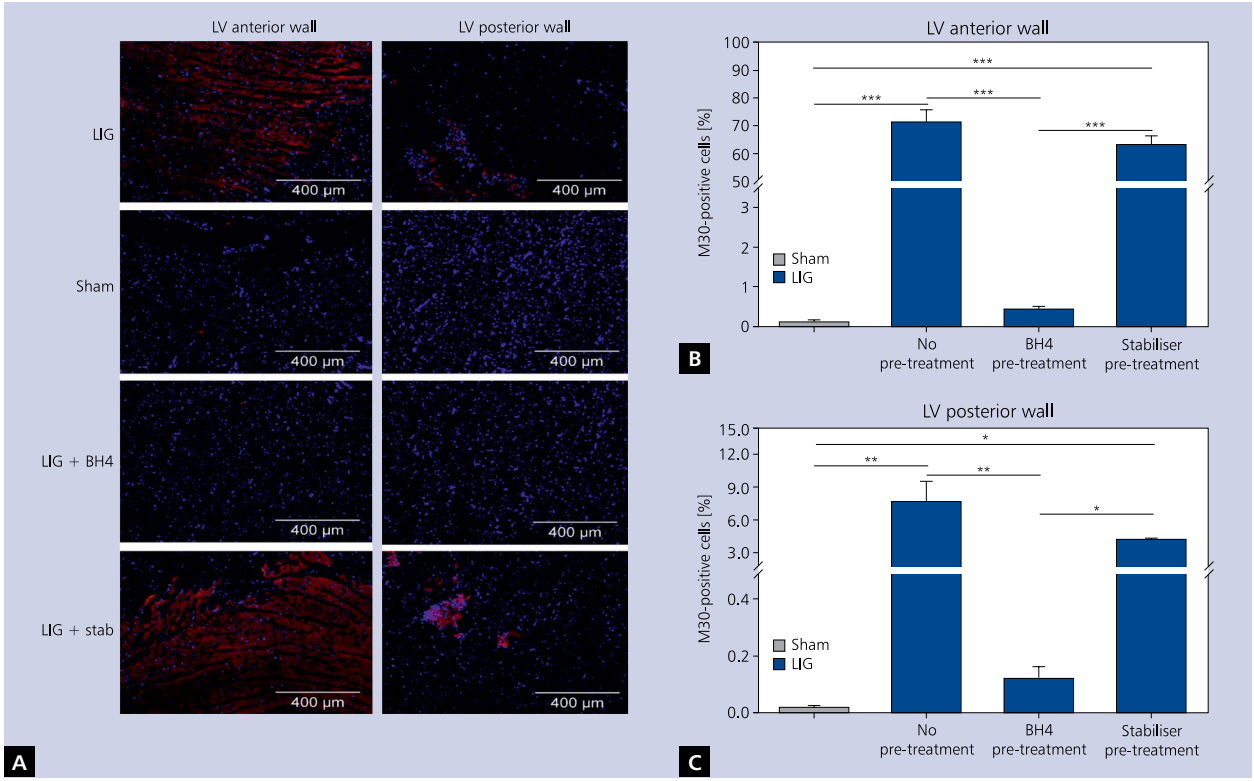

Figure 5. Tetrahydrobiopterin (BH4) prevents early myocardial apoptosis execution. **A.** Immunohistochemistry, representative images are shown. In LIG animals, apoptosis execution was present in the anterior and, to a lesser extent, posterior walls of the left ventricle (first row). As expected, sham treatment did not cause apoptosis (second row). BH4 pretreatment prevented apoptosis execution (third row). No protective effect was observed in stabiliser-only pretreated LIG animals (fourth row). Semi-quantification of results for the anterior and posterior left ventricular (LV) walls is depicted in panel **B** and panel **C**, respectively; Red colour — M30 positive cells; Blue colour — DAPI nuclei counterstaining; LIG — treatment group experiencing true LAD ligation; sham — control group that experienced a sham operation without LAD ligation; stab — stabiliser pretreatment; LAD — left anterior descending coronary artery (n = 3 per group, *p ≤ 0.05, **p ≤ 0.01, ***p < 0.001; values are means ± SEM)

Induction of apoptosis depends on activation of either the extrinsic or intrinsic pathway, or both [4]. Crucial for apoptosis induction via the extrinsic pathway are the initiator caspase 8 and the effector caspase 3 [8]. Here we show that 24 h after acute MI, increased caspase 8 and caspase 3 activities were detected in the remote myocardium. Although statistically not significant in the present set of experiments, these data are in agreement with previous studies that showed increased pro-apoptotic signalling via the extrinsic pathway in the non-infarcted remote myocardium as early as 3 h after MI [6, 15]. Crucial for apoptosis induction via the intrinsic pathway on the other hand are bcl-2 protein family members in the mitochondria [4, 8]. A shift towards pro-apoptotic bcl-2 like protein 4 (bax) of the well balanced bcl-2/bax protein ratio indicates apoptosis activation. Here we show activation of the intrinsic apoptotic pathway 24 h after acute MI in the remote myocardium. Activation of the intrinsic pathway in LIG animals paralleled pro-apoptotic signalling via the extrinsic pathway in this study and is in agreement with previous studies [6, 15, 16]. Apoptosis induction is the result of prevailing pro-apoptotic conditions. One of the most important anti-apoptotic signalling pathways depends on protein kinase B/Akt (PKB/Akt) signalling [9]. In this pathway, PKB/Akt in its active (phosphorylated) form inhibits induction of apoptosis by phosphorylation and inactivation of key pro-apoptotic proteins, including GSK-3β. GSK-3β is ubiquitously expressed and constitutively active with abundant physiologic roles, controlling apoptosis induction being one of them. However, its role in cardiomyocyte signalling still is controversial. Transgene and pharmacological stimulation of PKB/Akt signalling in a rat ischaemia–reperfusion model resulted in less apoptotic cell loss and infarct size reduction [19]. To date there is only limited evidence for the role of PKB/Akt signalling in the non-infarcted remote myocardium [15]. Here we show impaired anti-apoptotic signalling by a reduction of phosphorylated PKB/Akt 24 h after experimental acute MI in the remote myocardium, which translates into less anti-apoptotic phosphorylated GSK-3β. Notably, activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway has been shown to exert anti-apoptotic and cardioprotective

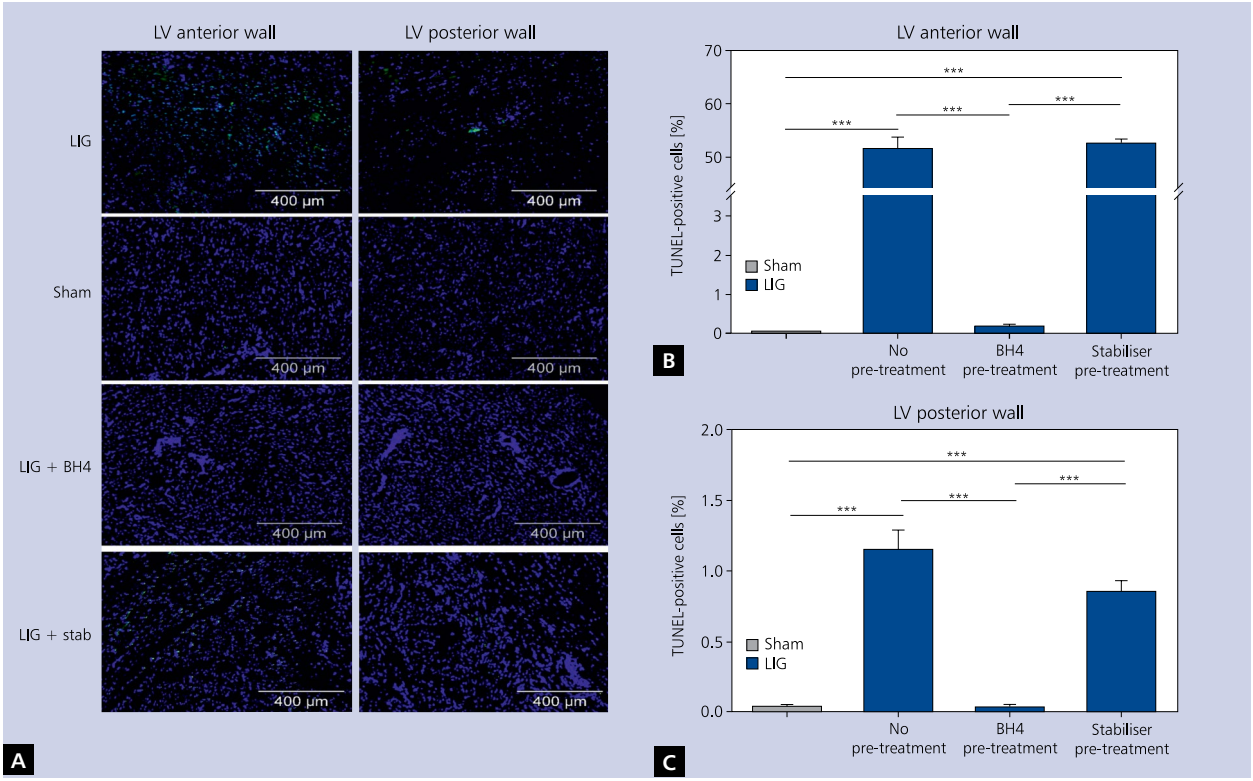

Figure 6. Tetrahydrobiopterin (BH4) prevents late myocardial apoptosis execution. **A.** Immunohistochemistry, representative images are shown. In LIG animals, apoptosis execution was present in the anterior and, to a lesser extent, posterior walls of the left ventricle (first row). As expected, sham treatment did not cause apoptosis (second row). BH4 pretreatment prevented apoptosis execution (third row). No protective effect was observed in stabiliser-only pretreated LIG animals (fourth row). Semi-quantification of results for the anterior and posterior left ventricular walls is depicted in panel **B** and panel **C**, respectively; Green colour — TUNEL positive cells; Blue colour — DAPI nuclei counterstaining; LIG — treatment group experiencing true LAD ligation; sham — control group that experienced a sham operation without LAD ligation; stab — stabiliser pretreatment; LAD — left anterior descending coronary artery (n = 3 per group, ***p ≤ 0.001; values are means ± SEM)

properties in animal models of ischaemia-reperfusion, leading to eNOS phosphorylation and subsequently increased NO production [9]. However, pathophysiology and signalling pathways in the infarcted area and in the non-ischaemic remote zone after MI are distinct and different to the situation in ischaemia-reperfusion injury. Intriguingly, the beneficial and harmful effects of eNOS activation are not mutually exclusive. In fact, the transformation of eNOS from cardioprotection to a contributor of oxidative stress has been scientifically acknowledged [10]. With respect to PI3K/Akt signalling in the remote non-ischaemic myocardium after MI, we have previously shown that apoptosis inhibition facilitated anti-apoptotic signalling via PI3K/Akt and, vice versa, inhibition of PI3K/Akt signalling promoted apoptosis execution [16].

Being constitutively expressed, eNOS is the leading NOS-isoform in the heart [11]. While implication of inducible NOS in increased nitrosative stress and apoptosis induction in the infarct and remodelling zones after MI has already been described [20], the impact of eNOS is still a matter of debate and was the subject of this study. Uncoupling of NOS refers to a condition where superoxide anions are generated instead of NO [13]. It should be acknowledged that eNOS uncoupling is not the sole source of cardiovascular superoxide anion production. NADPH oxidases also contribute to cardiovascular superoxide anion and peroxynitrite production [10], and this is a limitation of our study. Superoxide anions per se are not pro-apoptotic but easily react with NO to form peroxynitrite, with the latter bearing a marked pro-apoptotic potential [14]. A lack of the essential eNOS cofactor BH4 is believed to be one of the crucial factors leading to uncoupling of that enzyme [10, 21]. Direct cardioprotective properties of NO causing decreased apoptosis induction via the extrinsic pathway by caspase inhibition are generally known [22]. Because BH4 pretreatment did not cause enhanced eNOS phosphorylation at position Ser1177, which is the most important phosphorylation site for an increase in NO production [12], an increase in NO formation due to eNOS activation is probably not the reason for the cardioprotective effects of BH4 pretreatment on nitrosative stress reduction and the prevention of apoptosis induction and execution in this study.

Of note, stabiliser-only pretreatment (i.e. pretreatment with ascorbic acid and riboflavin) did not prevent eNOS activation in LIG animals. However, we observed a reduction in nitrosative stress as expressed in reduced protein nitrosylation in stabiliser-only pretreated animals, resembling the effects of BH4 pretreatment, although to a lesser extent. In addition, stabiliser-only pretreated animals also showed blunted caspase 8 and 3 activation and reduced pro-apoptotic activity via the intrinsic pathway, again resembling the effects of BH4 pretreatment. Anti-apoptotic properties of ascorbic acid have previously been demonstrated for the prevention of FAS-ligand-induced apoptosis via the extrinsic pathway and reduction of nitrosative stress due to inhibition of NADPH-oxidase [23]. This might explain the observed cardioprotective properties of the stabiliser formation in our study. Moreover, prevention of BH4 oxidation to BH3, mimicking the effects of BH4 supplementation in this study, and inhibition of GSK-3β has been described for ascorbic acid and might contribute to the beneficial effects of the stabiliser formation [24]. Therefore, future studies assessing the cardioprotective properties of BH4 supplementation may use pure BH4 to eliminate potential confounding effects of the stabiliser formulation. Remarkably, however, although stabiliser-only pretreatment closely resembled the beneficial effects of BH4 pretreatment in terms of reduced nitrosative stress, pro-apoptotic activity, and facilitation of anti-apoptotic signalling, final execution of apoptosis detected by TUNEL assay and detection of cleaved ceratin-18 neo-epitope (M30 antibody) exclusively was prevented by BH4 pretreatment, with the apoptotic cell count almost reaching sham level. Our data are consistent with previous publications [6, 15]. The detection of cleaved ceratin-18 neo-epitope using the M30 antibody and TUNEL assay were used in this study to assess apoptosis execution. This is because the specificity of the TUNEL method has been doubted recently [25], and in order to capture early- and late-phase myocardial apoptosis execution.

CONCLUSIONS

Here we showed that 24 h after experimental MI in rats *in vivo*, apoptosis was induced in the posterior LV myocardium remote of the infarcted area. There is evidence that pretreatment with BH4 resulted in less nitrosative stress and ameliorated pro-apoptotic pathways, although the contained stabiliser effect did participate in this phenomenon. Because myocardial cell loss due to apoptosis is important for myocardial remodelling, influencing eNOS signalling might become an important pharmacological target for the development of novel tailored therapies to prevent myocardial remodelling after MI.

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Tetrahydrobiopteryna, kofaktor śródbłonkowej syntazy tlenku azotu, chroni odległe obszary miokardium przed apoptozą po wywołanym doświadczalnie zawale serca w warunkach *in vivo*

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Streszczenie

Wstęp: Apoptoza w odległych rejonach miokardium występuje wcześnie po zawale serca (MI) i przyczynia się do remodelingu mięśnia sercowego. Zwiększenie stresu nitrozacyjnego jest znanym silnym czynnikiem wywołującym apoptozę miokardium. Nadmierna aktywacja śródbłonkowej syntazy tlenku azotu (eNOS) zwiększa jej rozprzęganie i powoduje stres nitrozacyjny w wyniku syntezy peroksynitrytu. Jednak nie poznano dotychczas patofizjologicznej roli eNOS w przesyłaniu sygnałów do odległych rejonów miokardium po MI.

Cel: Celem niniejszej pracy było przeanalizowanie wpływu aktywacji eNOS na przekazywanie sygnałów pro- i anty-apoptycznych w odległych rejonach miokardium oraz wpływu wstępnego leczenia kofaktorem, tetrahydrobiopteryną (BH4) na aktywację NOS, poziom stresu nitrozacyjnego oraz indukowanie i przebieg apoptozy w szczurzym modelu *in vivo* zawału serca.

Wyniki: Dwadzieścia cztery godziny po zawale ściany przedniej stwierdzono istotne zwiększenie w porównaniu z wartościami obserwowanymi w grupie kontrolnej w zakresie aktywności eNOS w miokardium tylnej ściany lewej komory u zwierząt, u których podwiązano gałąź międzykomorową przednią lewej tętnicy wieńcowej, podobnie jak w przypadku nitrozylacji białek. Jednocześnie zaobserwowano indukcję apoptozy zarówno w mechanizmie zewnątrz-, jak i wewnątrzpochodnym. Ponadto zahamowane zostało przekazywanie sygnałów anty-apoptotycznych za pośrednictwem ścieżki sygnałowej kinazy proteinowej B/Akt i kinazy syntazy glikogenu 3 beta. Warto zaznaczyć, że podanie wcześniej kofaktora eNOS, BH4, zmniejszyło aktywację eNOS, zapobiegło nadmiernej nitrozylacji białek, stłumiło indukcję apoptozy, ułatwiło przekazywanie sygnałów anty-apoptotycznych i zatrzymało proces apoptozy.

Wnioski: Wykazano, że 24 godziny po wywołanym eksperymentalnie MI u szczurów *in vivo* nastąpiła indukcja apoptozy w tylnej ścianie lewej komory nieobjętej zawałem. Przedstawiono dowody naukowe potwierdzające, że wcześniejsze podanie kofaktora eNOS, BH4, spowodowało zmniejszenie stresu nitrozacyjnego i osłabienie procesów apoptozy, chociaż zawarte w nim stabilizatory miały w tym swój udział. Ze względu na to, że apoptoza jest ważnym elementem remodelingu miokardium, oddziaływanie na ścieżkę sygnałową eNOS może być interesującym celem leczenia w opracowywaniu nowych terapii przeciwdziałających remodelingowi.

Słowa kluczowe: zawał serca, remodeling miokardium, apoptoza, śródbłonkowa syntaza tlenku azotu, tlenek azotu, stres nitrozacyjny

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