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ORIGINAL ARTICLE

The effects of modulating eNOS activity and coupling in ischemia/reperfusion (I/R)

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Abstract The in vivo role of endothelial nitric oxide synthase (eNOS) uncoupling mediating oxidative stress in ischemia/reperfusion (I/R) injury has not been well established. In vitro, eNOS coupling refers to the reduction of molecular oxygen to L-arginine oxidation and generation of L-citrulline and nitric oxide NO synthesis in the presence of an essential cofactor, tetrahydrobiopterin (BH_4). Whereas uncoupled eNOS refers to that the electron transfer becomes uncoupled to L-arginine oxidation and superoxide is generated when the dihydrobiopterin (BH_2) to BH_4 ratio is increased. Superoxide is subsequently converted to hydrogen peroxide (H_2O_2). We tested the hypothesis that promoting eNOS coupling or attenuating uncoupling after I/R would decrease H_2O_2/increase NO release in blood and restore postreperfused cardiac function. We combined BH_4 or BH_2 with eNOS activity enhancer, protein kinase C epsilon (PKC ε) activator, or eNOS activity reducer, PKC ε inhibitor, in isolated rat hearts (ex vivo) and femoral arteries/veins (in vivo) subjected to I(20 min)/R(45 min). When given during reperfusion, PKC ε activator combined with BH_4, not BH_2, significantly restored postreperfused cardiac function and decreased leukocyte infiltration ($p<0.01$) while increasing NO ($p<0.05$) and reducing H_2O_2 ($p<0.01$) release in femoral I/R veins. These results provide indirect evidence suggesting that PKC ε activator combined with BH_4 enhances coupled eNOS activity, whereas it enhanced uncoupled eNOS activity when combined with BH_2. By contrast, the cardioprotective and anti-oxidative effects of the PKC ε inhibitor were unaffected by BH_4 or BH_2 suggesting that inhibition of eNOS uncoupling during reperfusion following sustained ischemia may be an important mechanism.

Keywords eNOS uncoupling/coupling · Nitric oxide · Hydrogen peroxide · Left ventricular developed pressure · Ischemia/reperfusion injury · PKC epsilon

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Introduction

Research throughout the years has focused on the prevention of cardiac injury due to myocardial ischemia followed by reperfusion (I/R) in settings of myocardial infarctions associated with acute ischemic heart disease, coronary angioplasty, coronary bypass, and organ transplantation. Establishing the underlying mechanisms responsible for myocardial I/R injury may lead to novel therapies for patients undergoing heart transplant or those requiring surgery to reestablish blood flow to ischemic tissues.

During the first 10 min of reperfusion following ischemia, reperfusion injury is initiated by endothelial dysfunction, which is a decrease in endothelial derived nitric oxide (NO) release and increased oxidative stress. Endothelial dysfunction can occur in many organ vascular beds such as the kidney (Noiri et al. 2001), intestine (Muià et al. 2005), heart (Teng et al. 2008), and hind limb (Chen et al. 2010). This event serves as a "trigger" to induce increased leukocyte-endothelial interactions (Weyrich et al. 1995; Lefer and Lefer 1996; Scalia et al. 2000). With respect to heart function, transmigrated polymorphonuclear leukocytes (PMNs) are principally responsible for compromised postreperfused cardiac contractile function by releas-

ing cytotoxic substances such as superoxide and proteases (Tsao and Lefer 1990; Tsao et al. 1992; Buerke et al. 1994; Hansen 1995).

Understanding the pathophysiology of vascular endothelial dysfunction is important in identifying sources of oxidative stress. Under normal conditions, endothelial NO synthase (eNOS) produces NO in the presence of an essential cofactor, tetrahydrobiopterin (BH_4), by facilitating the reduction of molecular oxygen to L-arginine oxidation and generation of L-citrulline. This reaction is referred to as eNOS coupling (Schmidt and Alp 2007). Endothelial-derived NO is critical to maintain normal vascular tone and end diastolic pressure (Schulz et al. 2008). Whereas uncoupled eNOS refers to that the electron transfer becomes uncoupled to L-arginine oxidation and superoxide is generated when the dihydrobiopterin (BH_2) to BH_4 ratio is increased (Schmidt and Alp 2007). It has been found in vitro that the increased ratio of BH_2 to BH_4 facilitates BH_2 to displace BH_4 for binding at the eNOS oxygenase domain and allow eNOS to use molecular oxygen to produce superoxide, which can be quickly converted to hydrogen peroxide (H_2O_2) by superoxide dismutase (Vasquez-Vivar et al. 2002). Furthermore, the increased ratio of BH_2 to BH_4 in endothelial cells may be due to incomplete oxidative phosphorylation in mitochondria (Crabtree et al. 2008). In this regard, the oxidative stress during early reperfusion may shift the eNOS profile from NO to superoxide production due to limited availability of BH_4. Until recently, this concept has not been evaluated in the setting of I/R in vivo using real-time measurement of NO and H_2O_2 in blood.

A recent study from our research group showed that BH_4 application during early reperfusion is partially cardioprotective and reduces H_2O_2 release during reperfusion (Chen et al. 2010). By contrast, supplementation of BH_2 during reperfusion results in compromised cardiac function, decreased NO release, and increased H_2O_2 release in blood following I/R. The reason BH_4 has limited utility in the setting of I/R may be that higher doses are not effective due to binding saturation at the oxygenase domain of eNOS. An attractive alternative to deal with this situation is to enhance eNOS activity (i.e., protein kinase C epsilon [PKC ε] activator) combined with promoting eNOS coupling with BH_4 to increase NO release during reperfusion. PKC ε can increase eNOS activity through phosphorylation (Zhang et al. 2005). Moreover, PKC ε is not expressed in PMNs suggesting that cardioprotection involving regulation of PKC ε may be principally mediated by preserving postreperfused endothelium (Dang et al. 2001; Teng et al. 2008). We previously showed that selective PKC ε activator (N-Myr-HDAPIGYD, MW=1,097, Genemed Synthesis, Inc., San Antonio, TX) increased NO release in normal vascular tissue. However, we and others have shown that PKC ε activator is only effective when given prior to ischemia but not when given only during reperfusion (Inagaki et al. 2003; Teng et al. 2008). By contrast, selective PKC ε inhibitor (N-Myr-EAVSLKPT, MW=1054, Genemed Synthesis, Inc. San Antonio, TX) decreased NO release from non-ischemic vascular tissue. However, PKC ε inhibitor significantly reduced H_2O_2 release in the setting of femoral I/R, suggesting that attenuation of eNOS activity is associated with reduced oxidative stress during reperfusion (Teng et al. 2008). The PKC ε activator or inhibitor is a myristoylated peptide that can respectively facilitate or inhibit binding of PKC ε to its receptor for activated C kinase (RACK-1) (Ron and Mochly-Rosen 1995). PKC ε-RACK-1 promotes the translocation of PKC ε from cytosol to cell membrane to interact with substrates, such as eNOS. Myristoylation of peptides promotes rapid diffusion (i.e., 10 s) into the cell (Omiyi et al. 2005). These data suggest that eNOS may change its product profile during I/R conditions. Therefore, an in vivo study examining the effects of BH_4 or BH_2 combined with PKC ε activator or PKC ε inhibitor should provide a fascinating insight into the effects of eNOS coupling/uncoupling mediating oxidative stress in I/R.

We tested the combination of PKC ε activator with BH_4 or BH_2 in isolated I/R hearts reperfused with PMNs. We also measured NO and H_2O_2 release from femoral I/R veins. This model is intended to address the role of eNOS coupling/uncoupling in vascular function/dysfunction associated with reperfused cardiac function. The femoral I/R model is more appropriate to measure H_2O_2 and NO in blood in real-time due to accessibility and size of the rat femoral blood vessels compared to the coronary vessels. We hypothesize that the combination of PKC ε activator with BH_4 given during reperfusion will significantly restore cardiac function, attenuate leukocyte transmigration, increase NO release, and decrease H_2O_2 release compared to non-drug control groups. By contrast, we hypothesize that the PKC ε activator combined with BH_2 will be the opposite of the effects of the combination with BH_4 in these parameters.

We also tested the combination of PKC ε inhibitor with BH_4 or BH_2 given during reperfusion in isolated I/R hearts and in femoral I/R. We hypothesize that PKC ε inhibitor will significantly restore postreperfused cardiac function, decrease H_2O_2, and increase NO release in blood when combined with either BH_4 or BH_2, since eNOS is being inhibited.

Methods

Isolated rat heart preparation

The Institutional Animal Care and Use Committee of the Philadelphia College of Osteopathic Medicine approved all

animal protocols performed in this study. Male Sprague–Dawley rats (275–325 g; Ace Animals, Boyertown, PA) were injected with pentobarbital sodium (60 mg/kg) and sodium heparin (1,000 U) intraperitoneally (i.p.) after which the hearts were rapidly excised. The hearts were subjected to retrograde perfusion with a modified Krebs' buffer (in mmol/l: 17.0 dextrose, 120.0 NaCl, 25.0 $NaHCO_3$, 2.5 $CaCl_2$, 0.5 EDTA, 5.9 KCl, and 1.2 $MgCl_2$) maintained at 37°C. The perfusate was aerated with 95% O_2–5% CO_2 and equilibrated at a pH of 7.3–7.4. Three side arms in the perfusion line proximal to the heart inflow cannula allowed infusion of PMNs, plasma (control hearts), plasma containing BH_4 (5 μM) or BH_2 (100 μM), PKC ε activator (10 μM) or PKC ε inhibitor (5 μM), and N^G-nitro-L-arginine methyl ester (L-NAME; 50 μM; NOS inhibitor) independently or in various combinations.

The isolated rat heart was cannulated via the aorta onto a perfusion needle and immersed in a water-jacketed reservoir that contained 160 ml of Krebs' buffer. Coronary flow was monitored by a flowmeter (T106; Transonic Systems, Inc., Ithaca, NY). Left ventricular developed pressure (LVDP), which is the left ventricular end-systolic pressure (LVESP) minus left ventricular end-diastolic pressure (LVEDP), and the maximal rate of LVDP ($+dP/dt_{max}$) were monitored using a pressure transducer (SPR-524; Millar Instruments, Inc., Houston, TX) positioned in the left ventricular cavity and recorded using a Powerlab Station acquisition system (ADInstruments, Grand Junction, CO). Coronary flow, LVDP, and $+dP/dt_{max}$ were measured every 5 min for 15 min to obtain baseline measurements. Data continued to be recorded every 5 min for 45 min postreperfusion.

After baseline, ischemia was induced for 20 min by reducing the flow of Krebs' buffer to zero. Hearts were infused at the beginning of reperfusion with 200×10^6 PMNs resuspended in 5 ml of Krebs' buffer and 5 ml of plasma at a rate of 1 ml/min for 5 min. PKC ε activator, PKC ε inhibitor, BH_4 or BH_2 combined with PKC ε activator or PKC ε inhibitor were added to the plasma corresponding to the specific drug groups. Moreover, we also had another group in which the combination of PKC ε activator, BH_4, and L-NAME was used to test if any cardioprotective effects were mediated by a NO mechanism. Isolated rat hearts were placed in 4% paraformaldehyde and stored at 4°C for later histological analysis at the end of the experimental protocol.

Groups of isolated perfused hearts

Table 1 indicates the 14 groups (control and treatment conditions) of isolated perfused rat hearts used in the study. Three types of control groups were used in the study based on the well-established PMN-induced myocardial I/R model (Lefer et al. 1997). (1) Sham hearts were not subjected to ischemia and were not perfused with PMNs, as described by Teng et al. 2008. (2) I/R hearts were subjected to 20 min of ischemia followed by 45 min of reperfusion, and were perfused with 5 ml of plasma (1 ml/min) during the first 5 min of reperfusion. This group was employed to show that these hearts would recover to near baseline values by the end of the 45 min of reperfusion without PMNs. (3) I/R + PMN hearts were subjected to 20 min of ischemia and were reperfused with PMNs (200×10^6, resuspended in 5 ml Krebs' buffer and 5 ml of plasma (1 ml/min)) during the first 5 min of reperfusion. This control group was employed to show that 20 min of ischemia followed by 45 min reperfusion in the presence of PMNs resulted in sustained cardiac contractile dysfunction throughout the entire reperfusion period. PKC ε activator (10 μM) or PKC ε inhibitor (5 μM) was perfused separately or combined with BH_4 (5 μM) or BH_2 (100 μM) dissolved in plasma and perfused into hearts in order to show that these combinations did not exert cardiotonic or cardiodepressant effects in sham or I/R settings. Previous data showed that each drug individually did not exert cardiotonic or cardiodepressant effects in this model (Teng et al. 2008; Chen et al. 2010). L-NAME (50 μM) was used throughout the 45-min reperfusion in PKC ε activator combined with BH_4 treated I/R + PMN hearts to determine if an NO mechanism was involved in the cardioprotective effect. The dose of L-NAME used in this study did not significantly affect LVDP in sham hearts (Young et al. 2000).

Isolation of plasma and PMNs

As previously described, 5 ml of plasma was collected from the abdominal aorta of the same rat from which the heart was isolated for each cardiac perfusion experiment in order to simulate the conditions in vivo (Peterman et al. 2004). PMNs were prepared after peritoneal lavage from male Sprague–Dawley rats (350–400 g; Ace Animals, Boyertown, PA) after receiving 0.5% glycogen, as previously described (Omiyi et al. 2005).

Measurement of NO and H_2O_2 release from rat femoral veins during reperfusion

A novel technique was developed to measure NO and H_2O_2 release from femoral veins in rats (Teng et al. 2008; Chen et al. 2010). One leg was subjected to I/R by clamping the femoral artery/vein for 20 min followed by 45 min reperfusion (similar to the I/R time course in our isolated myocardial I/R model) and the other is a non-ischemic sham control (Kuntscher et al. 2002; Teng et al. 2008; Chen et al. 2010). Rats were anesthetized with sodium pentobarbital (i.p. 60 mg/kg for induction and 28 mg/kg for maintenance). The NO or H_2O_2 microsensors (100 μm;

Table 1 Groups of rat hearts and treatment received at the beginning of reperfusion

Group	Sham	I/R	I/R + PMN	I/R + PMN + L-NAME
Control (no drug)	n=6	n=7	n=11	
PKC ε activator (10 μM) + BH_4 (5 μM)		n=6	n=7	n=6
PKC ε activator (10 μM)			n=7	
PKC ε activator (10 μM) + BH_2 (100 μM)		n=7	n=6	
PKC ε inhibitor (5 μM)			n=7	
PKC ε inhibitor (5 μM) + BH_4 (5 μM)		n=6	n=6	
PKC ε inhibitor (5 μM) + BH_2 (100 μM)		n=7	n=6	

World Precision Instruments (WPI) Inc., Sarasota, FL) were inserted into 24-gauge catheters and placed inside each femoral vein and connected to a free radical analyzer (Apollo 4000; WPI Inc.) as previously described (Teng et al. 2008; Chen et al. 2010). PKC ε activator (0.9 mg/kg, about 10 μM in blood) or PKC ε inhibitor (0.4 mg/kg, about 5 μM in blood) alone, or combined with BH_4 (0.8 mg/kg diluted in saline, about 30 μM in blood) or BH_2 (2 mg/kg, about 100 μM in blood), or saline (non-drug control group) were applied through tail vein injection at the beginning of reperfusion. The changes in NO and H_2O_2 release during reperfusion (in pA) were expressed as relative changes to baseline (initial). Thereafter, the values were converted to the concentration of NO (nM) or H_2O_2 (μM) after correction to the calibration curve of NO and H_2O_2 microsensors. Five to six experiments were conducted by using PKC ε activator or PKC ε inhibitor alone, or PKC ε activator/inhibitor combined with BH_4/BH_2 as indicated in the figure legends.

Determination of total intravascular and infiltrated PMNs

Three or four rat hearts that were closest to the group mean of the cardiac function studies from the 14 groups of perfused rat hearts were used for histological analysis. First, heart tissues were embedded in plastic and cut into 2.5-μm serial sections. Next, sections were stained with hematoxylin and eosin by previously established methods (Omiyi et al. 2005). Under light microscopy, ten areas of each rat heart from the left ventricle were counted for PMN vascular adherence and infiltration into the heart tissue and expressed as total intravascular and infiltrated PMNs/mm^2.

Statistical analysis

All data in the text and figures were presented as means± SEM. The data were analyzed by ANOVA using post-hoc analysis with the Bonferroni/Dunn test for heart function data and Student–Newman–Keuls test for femoral vein data. Probability values of <0.05 were considered statistically significant.

Results

Combination: PKC ε activator with BH_2 or BH_4

Cardiac function

PKC ε activator was combined with BH_2 or BH_4 during reperfusion in isolated perfused hearts to determine the effects on postreperfused cardiac function. Figure 1 illustrates the time course of cardiac contractile function for LVDP (left panel) and LVEDP (right panel) for the I/R + PMN, I/R + PMN + BH_2 (100 μM) + PKC ε activator (10 μM), and I/R + PMN + BH_4 (5 μM) + PKC ε activator (10 μM) groups. The hearts in the I/R + PMN control group exhibited sustained cardiac contractile and diastolic dysfunction, 45±6% of initial LVDP and 55±4 mmHg in LVEDP, respectively, at 45 min reperfusion. This result was consistent with our previous published results and suggested that PMNs were principally responsible for the sustained cardiac contractile dysfunction in this model of I/R (Teng et al. 2008). Compared to I/R + PMN hearts, I/R + PMN + BH_2 (100 μM) + PKC ε activator (10 μM) hearts showed similar sustained contractile and diastolic dysfunction, recovering to only 38±9% in LVDP and 55±11 mmHg in LVEDP. By contrast, I/R + PMN + BH_4 (5 μM) + PKC ε activator (10 μM) hearts significantly increased final LVDP to 92±11% (p<0.01), and significantly decreased final LVEDP to 29±5 mmHg (p<0.05) compared to I/R + PMN control. Additionally, the individual effects of BH_4 (5 μM) or BH_2 (100 μM) did not restore postreperfused cardiac function and were not significantly different from I/R + PMN control in our previous publications (Teng et al. 2008; Chen et al. 2010). These data suggest that PKC ε activator combined with BH_4 exerted a significant restoration in postreperfused cardiac contractile and diastolic function.

Table 2 also showed the initial and final values for LVDP and + dP/dt_{max}, respectively, from drug treated experimental groups. There was no significant difference between the initial baseline values of LVDP and + dP/dt_{max} in all the groups studied. There was no significant difference between the initial and final values of LVDP and + dP/dt_{max} in control or drug treated I/R or sham hearts

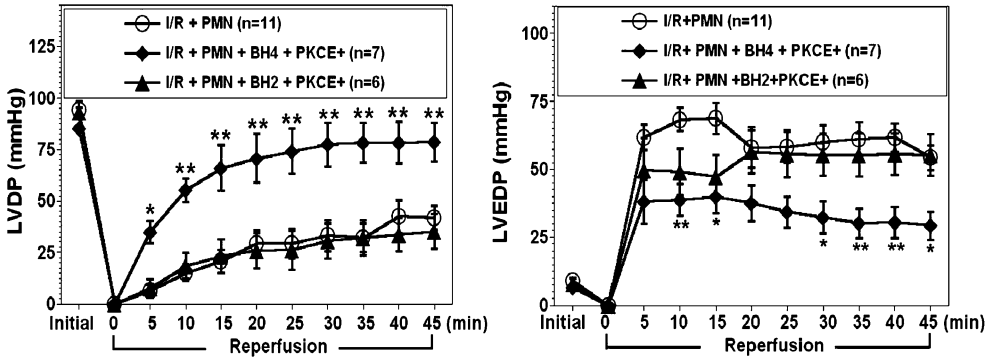

Fig. 1 Time course of LVDP (*left panel*) and LVEDP (*right panel*). LVDP or LVEDP is shown at initial (*baseline*) and reperfusion from 0 to 45 min after 20 min ischemia. The I/R + PMN + BH_2 + PKC ε activator (E+) group (n=6) exhibited a significant and sustained reduction in LVDP and increased LVEDP, which is similar as the I/R + PMN group (n=11). The I/R + PMN + BH_4 + PKC ε activator group (n=7) showed significant recovery on cardiac contractile and diastolic function throughout reperfusion. (*p<0.05, **p<0.01 compared to I/R + PMN values)

(data not shown). PKC ε activator (10 μM)-treated postreperfused IR + PMN hearts recovered to only 60±8% LVDP and 54±8%+dP/dt_{max} and was similar to control I/R + PMN hearts. However, IR + PMN hearts given PKC ε activator combined with BH_4 significantly restored postreperfused cardiac function recovering to 92±11% and 83±7% of initial LVDP (p<0.05) and + dP/dt_{max} (p<0.01), respectively, compared to control IR + PMN hearts. These results suggest a synergistic effect on LVDP and + dP/dt_{max} since neither drug restored postreperfused cardiac function individually. Moreover, these cardioprotective effects were blocked by L-NAME since LVDP and + dP/dt_{max} recovered to 54±8% and 41±8% of initial values, respectively. This result suggests that the restoration of postreperfused cardiac function was mediated by a mechanism involving NO. By contrast, the combination of I/R + PMN + BH_2 + PKC ε activator resulted in the most severe cardiac contractile dysfunction throughout reperfusion among all study groups (Table 2). These results suggest an additive effect of BH_2 and PKC ε activator on compromising postreperfused cardiac function.

H_2O_2 release in rat femoral I/R

Figure 2 represents the effects of PKC ε activator (10 μM) independently and in combination with BH_4 (30 μM) or BH_2 (100 μM) on H_2O_2 release compared to saline control. In the saline infused group, blood H_2O_2 level started at 2.6 μM increase at 5 min of reperfusion and continued to 3.2 μM by 45 min reperfusion. This result indicates femoral I/R caused an increase in oxidative stress due to the increase of H_2O_2. The independent effects of PKC ε activator, BH_4 or BH_2 (data not shown) on H_2O_2 release were not significantly different from saline control. However, the PKC ε activator combined with BH_4 group significantly decreased H_2O_2 release compared to control (p<0.01). These data suggest that the combination of PKC ε activator and BH_4 exerted a

Table 2 Initial and final LVDP and + dP/dt_{max} among different experimental groups

	Initial LVDP	Final LVDP	Initial + dP/dt_{max}	Final + dP/dt_{max}
I/R (n=7)	88.91±6.08	82.90±4.19	2,385.31±134.47	2,232.23±229.55
I/R + PMN (n=11)	94.37±4.09	42.04±5.83#	2,469.18±116.89	807.90±97.75#
I/R + PMN + E + (n=7)	90.92±3.08	54.39±7.66	2,462.45±84.68	1,319.20±209.05
I/R + PMN + E + BH_4 (n=7)	85.30±1.21	78.76±9.44*	2,174.55±56.5	1,806.56±157.79**
I/R + PMN + E + BH_4 + L-NAME (n=6)	88.97±2.09	48.25±7.41	2,422.35±92.06	993.54±182.76
I/R + PMN + E + BH_2 (n=6)	92.56±2.22	35.16±8.43	2,438.44±105.31	751.41±204.94
I/R + PMN + E−(n=7)	91.12±2.58	90.03±4.87**	2,505.85±97.67	2,240.54±103.38**
I/R + PMN + E− + BH_4 (n=6)	89.15±1.64	72.81±3.98*	2,497.66±47.16	1,720.63±77.25*
I/R + PMN + E− + BH_2 (n=6)	90.05±2.61	80.14±2.28**	2,412.03±63.31	1,865.94±75.27**

#p<0.01 compared to initial LVDP or + dP/dt_{max} of I/R + PMN group

*p<0.05, **p<0.01 compared to I/R + PMN final LVDP or + dP/dt_{max}

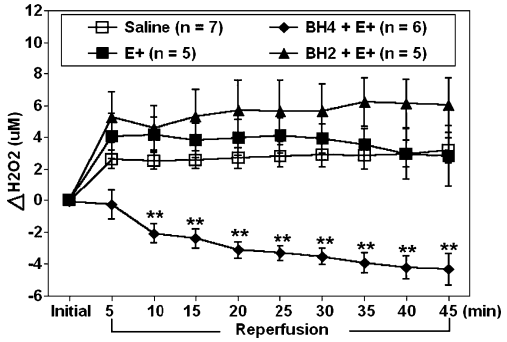

Fig. 2 Relative difference in H_2O_2 release between I/R and sham femoral veins during reperfusion in saline control (n=7), PKC ε activator (E+) (n=5), PKC ε activator with BH_4 (n=6), and PKC ε activator with BH_2 (n=5) groups. The data is expressed as the change (Δ) in H_2O_2 release from initial baseline. PKC ε activator showed a similar increase in H_2O_2 release as control group. By contrast, BH_4 + PKC ε activator showed a significant decrease in H_2O_2 release from 10 to 45 min reperfusion. Meanwhile, PKC ε activator + BH_2 showed a trend to increase H_2O_2 compared to control. (**p<0.01)

synergistic effect on reducing H_2O_2 release, whereas the combination of PKC ε activator and BH_2 showed a trend to augment H_2O_2 release.

NO release in rat femoral I/R

Figure 3 represents the results of the PKC ε activator (10 μM) treatments in terms of NO release compared to saline control. In the saline infused group, there was not a significant change of blood NO release throughout the reperfusion period. By contrast, the PKC ε activator group showed an initial increase in NO release during the first 5 min of reperfusion, however, thereafter a sustained decrease in NO release throughout the remainder of

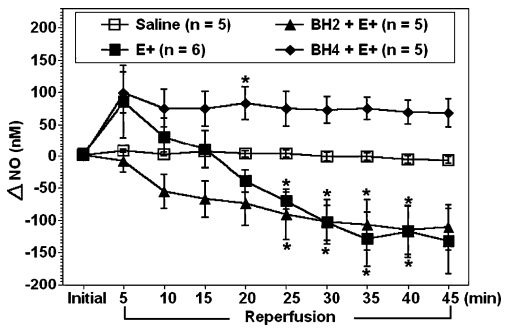

Fig. 3 Relative difference in NO release between I/R and sham femoral veins during reperfusion in saline control (n=5), PKC ε activator (E+) (n=6), PKC ε activator with BH_4 (n=5), and PKC ε activator with BH_2 (n=5) groups. The data is expressed as the change (Δ) in NO release from initial baseline. The PKC ε activator significantly decreased NO release from 25 to 40 min. BH_4 + PKC ε activator showed a significant increase in NO release at 20 min of reperfusion, whereas BH_2 + PKC ε activator groups significantly decreased NO release compared to control. (*p<0.05)

reperfusion that was significantly different compared to the control from 25 to 40 min reperfusion (p<0.05). PKC ε activator and BH_2 also showed a significant decrease in NO release from 25 to 40 min reperfusion (p<0.05), but did not show an initial increase. This suggests that an additive effect on reducing NO release resulted from this combination. By contrast, the PKC ε activator and BH_4 treatment showed as much as 100 nM increase in NO release compared to the control group suggesting that these two drugs had a synergistic effect on increasing NO release (p<0.05).

Combination: PKC ε inhibitor with BH_2 or BH_4

Cardiac function

PKC ε inhibitor was combined with BH_2 or BH_4 individually and was applied to the isolated perfused hearts to determine the effects on postreperfused cardiac function. Figure 4 illustrates the time course of cardiac contractile function (LVDP, left panel) and diastolic function (LVEDP, right panel), respectively, for the I/R + PMN, I/R + PMN + BH_2 (100 μM) + PKC ε inhibitor (5 μM), and I/R + PMN + BH_4 (5 μM) + PKC ε inhibitor (5 μM) groups. Compared to I/R + PMN hearts, I/R + PMN + BH_4 (5 μM) + PKC ε inhibitor (5 μM) sustained increased contractile and diastolic function, recovering to 82±5% in LVDP and 25±6 mmHg in LVEDP. Similarly, I/R + PMN + BH_2 (100 μM) + PKC ε inhibitor (5 μM) provided significantly increased final LVDP of 89±3% of initial baseline, and decreased final LVEDP to 18±5 mmHg (both p<0.01). These data suggest that neither BH_4 nor BH_2 significantly influenced PKC ε inhibitor's effects on postreperfused cardiac contractile and diastolic function.

As illustrated in Table 2, IR + PMN + PKC ε inhibitor significantly restored postreperfused cardiac function recovering to 99±3% of initial LVDP and 89±4% of initial + dP/dt_{max}, respectively, compared to IR + PMN hearts (both p<0.01). Similarly, the combination of PKC ε inhibitor and BH_4 (p<0.05) or BH_2 (p<0.01) significantly restored postreperfused cardiac function compared to IR + PMN hearts. These results suggest that the effects of PKC ε inhibitor are not appreciably influenced by either BH_4 or BH_2.

H_2O_2 release in rat femoral I/R

Figure 5 shows the effects of PKC ε inhibitor (5 μM) in combination with BH_4 (30 μM) or BH_2 (100 μM) on H_2O_2 release. All PKC ε inhibitor groups significantly decreased H_2O_2 release in femoral I/R veins compared to saline control (p<0.05). These data suggest that neither BH_2 nor

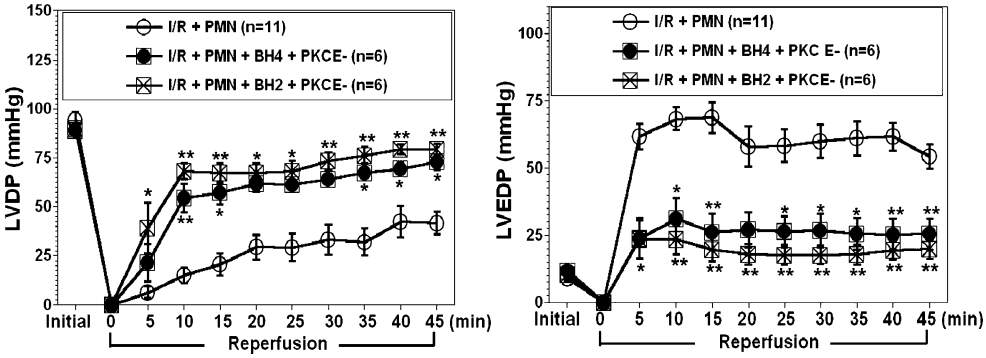

Fig. 4 Time course of LVDP (*left panel*) and LVEDP (*right panel*). LVDP or LVEDP is shown at initial (*baseline*) and reperfusion from 0 to 45 min after 20 min ischemia. All combinations with PKC ε inhibitor (E−) showed significant recovery on cardiac contractile and diastolic function throughout reperfusion compared to I/R + PMN hearts. (*$p<0.05$, ** $p<0.01$ compared to I/R + PMN values)

BH_4 exerted a significant influence on the PKC ε inhibitor's effect on H_2O_2 release in femoral I/R veins.

NO release in rat femoral I/R

Figure 6 shows the effects of PKC ε inhibitor (5 μM) in combination with BH_4 (30 μM) or BH_2 (100 μM) on NO release. All PKC ε inhibitor groups significantly increased NO release in femoral I/R veins compared to saline control. The PKC ε inhibitor group significantly increased NO release at 10 min of reperfusion and maintained increased NO release between 100 and 120 nM throughout reperfusion compared to controls ($p<0.05$). Similarly, the PKC ε inhibitor with BH_4 group also significantly increased NO release, which was maintained between 80 and 100 nM throughout the 45-min reperfusion period ($p<0.05$). The PKC ε inhibitor with BH_2 group also significantly increased NO release up to 50–60 nM from 20 to 45 min compared to controls ($p<0.05$). This suggests that neither BH_2 nor BH_4 significantly influenced the effects of PKC ε inhibitor on NO release in femoral I/R veins.

Histology assessment

The infiltration of PMNs into the myocardium within the 45-min reperfusion period was closely correlated with the cardiac contractile dysfunction associated with I/R in this model. Histological data (Fig. 7) shows a considerable amount of PMN tissue infiltration in post-reperfused heart tissue in control I/R + PMN (Fig. 7a) and PKC ε activator with BH_2 (Fig. 7c). By contrast, PKC ε activator with BH_4 (Fig. 7b) showed reduced PMN tissue infiltration. Combination of PKC ε inhibitor with either BH_4 (Fig. 7d) or BH_2 (Fig. 7e) resulted in substantial reduction in PMN tissue infiltration. This data suggests that the cofactor status of eNOS has a negligible effect in the presence of PKC ε inhibitor and has a pronounced

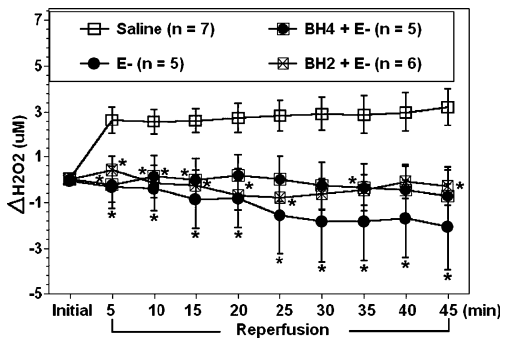

Fig. 5 Relative difference in H_2O_2 release between I/R and sham femoral veins during reperfusion in saline control ($n=7$), PKC ε inhibitor (E−) ($n=5$), PKC ε inhibitor with BH_4 ($n=5$) and PKC ε inhibitor with BH_2 ($n=6$) groups. The data is expressed as the change (Δ) in H_2O_2 release from initial baseline. All PKC ε- groups significantly decreased H_2O_2 release in femoral I/R veins during reperfusion compared to control. (*$p<0.05$)

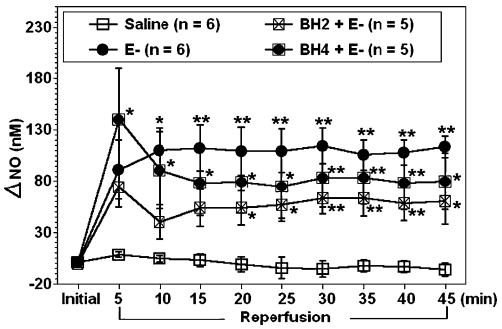

Fig. 6 Relative difference in NO release between I/R and sham femoral veins during reperfusion in saline control ($n=5$), PKC ε inhibitor (E−) ($n=6$), PKC ε inhibitor with BH_2 ($n=5$) and PKC ε inhibitor with BH_4 ($n=5$) groups. The data is expressed as the change (Δ) in NO release from initial baseline. All PKC ε inhibitor groups showed a significant increase in NO release when compared to control. (*$p<0.05$, **$p<0.01$)

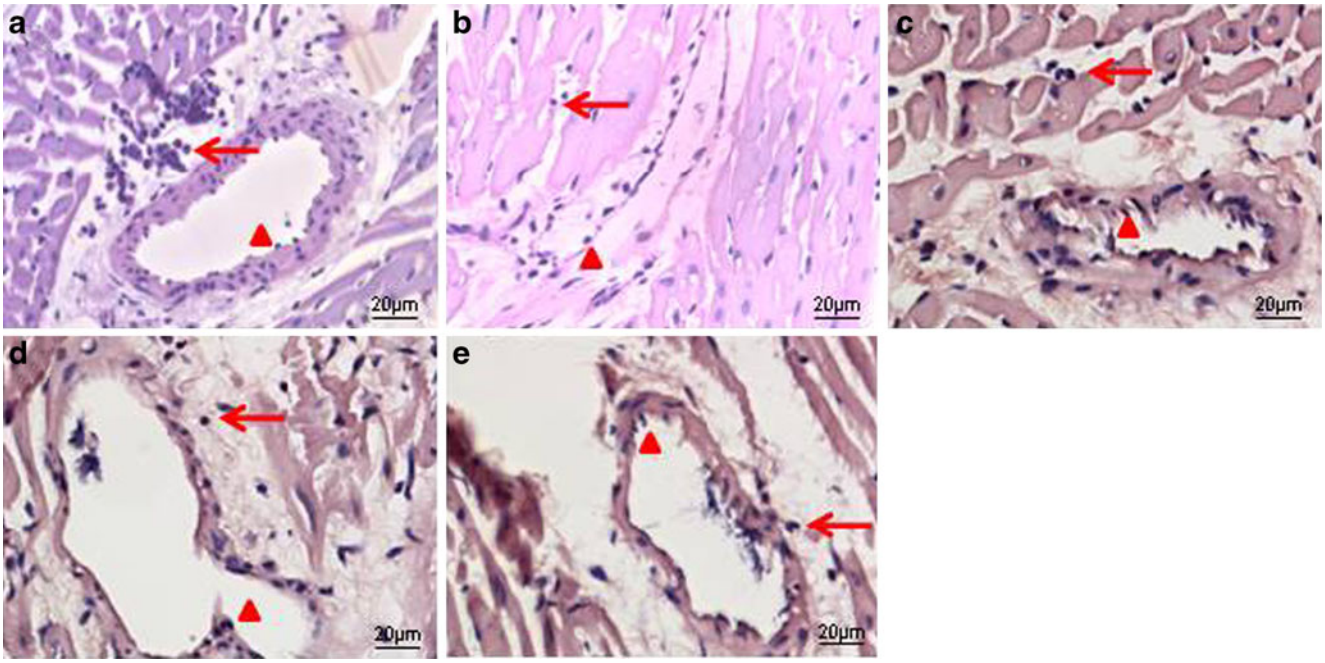

Fig. 7 Light microscopy photographs of hematoxylin and eosin-stained rat hearts. **a** ×40 magnification of I/R + PMN heart. **b** ×40 magnification of I/R + PMN + BH_4 + PKC ε activator heart. **c** ×40 magnification of I/R + PMN + BH_2 + PKC ε activator heart. **d** ×40 magnification of I/R + PMN + BH_4 + PKC ε inhibitor heart. **e** ×40 magnification of I/R + PMN + BH_2 + PKC ε inhibitor heart. *Arrowhead* indicates PMNs rolling/adherence, *full arrow* indicates PMNs transmigration (scale bar: 20 μm)

effect when eNOS is activated by PKC ε activator. The total intravascular and infiltrated PMNs from different experimental groups are listed in Table 3. Compared to total intravascular and infiltrated I/R + PMN values, the PKC ε activator with BH_4 and all PKC ε inhibitor groups significantly reduced total neutrophils in the postreperfused heart tissue (all $p<0.01$). The adhered PMNs from all groups were not found to be significantly different from those in the I/R + PMNs group; a possible explanation for this result is that the majority of PMNs had infiltrated by the end of the 45-min period.

Table 3 Histological assessment of PMNs in isolated perfused rat heart samples

Group PMNs	Total intravascular and infiltrated PMNs
Sham	26.6±7.1*
I/R alone	35.7±3.6*
I/R + PMNs	193.2±7.3
I/R + PMNs + PKC ε inhibitor	111.5±11.0*
I/R + PMNs + PKC ε activator	177.1±11.3
I/R + PMNs + PKC ε inhibitor + BH_2	105.5±8.5*
I/R + PMNs + PKC ε inhibitor + BH_4	111.8±17.1*
I/R + PMNs + PKC ε activator + BH_2	142.3±12.8
I/R + PMNs + PKC ε activator + BH_4	86.1±6.4*
I/R + PMNs + PKC ε activator + BH_4 + L-NAME	206.3±17.1

*$p<0.01$ compared to total intravascular and infiltrated I/R + PMN values

Discussion

Summary of major findings

The major findings of this study are as follows: (1) Treatment of rat hearts with PKC ε activator and BH_4 resulted in restoration of postreperfused cardiac function (i.e., LVDP and + dP/dt_{max}), and was associated with a significant increase in NO release and a decrease in H_2O_2 release in femoral I/R veins in vivo. (2) The cardioprotective effects of PKC ε activator and BH_4 treated rat hearts were blocked by L-NAME. (3) Rat hearts treated with PKC ε activator and BH_2 sustained the most compromised cardiac contractile function, and this was associated with increased H_2O_2 and decreased NO release. (4) All hearts treated with PKC ε inhibitor significantly restored postreperfused cardiac function, and all PKC ε inhibitor treated groups decreased H_2O_2 release and increased NO release in femoral I/R. (5) The histology data correlated with the degree of postreperfused cardiac function (i.e., LDVP and + dP/dt_{max}) with regard to total intravascular and infiltrated PMNs. These results are consistent with the hypotheses that facilitating eNOS activity in its coupled state or attenuating activity in its

uncoupled state reduces oxidative stress and thus attenuates endothelial dysfunction and subsequent cardiac contractile dysfunction following I/R.

The effect of PKC ε activator combined with BH_4 or BH_2 on cardiac function and H_2O_2/NO release following I/R

This study confirmed the effectiveness of the combination of PKC ε activator and BH_4 in significantly restoring postreperfused cardiac contractile function. The increased LVDP was principally attributed to lower LVEDP relative to the control IR + PMN hearts during reperfusion. LVEDP serves as an index for the heart's ability to relax to allow for appropriate filling with blood. Moreover, this data also correlates with a significant decrease in H_2O_2 release during reperfusion compared to control femoral I/R, suggesting that the large decrease in oxidative stress during reperfusion may be the principal mechanism responsible for the improved cardiac function in I/R. This combination also significantly increased NO release that was sustained during reperfusion in rat femoral veins. These findings suggest that a synergistic effect occurred on improving postreperfused cardiac function and attenuating oxidative stress in I/R, since neither drug restored postreperfused cardiac function or reduced oxidative stress individually. Moreover, the improvement in postreperfused cardiac function also correlated with significantly decreased total intravascular and infiltrated PMNs compared to I/R + PMN control hearts. Furthermore, the effects of PKC ε activator and BH_4 combination on cardiac contractile dysfunction and infiltrated PMNs were blocked by L-NAME, suggesting that coupled eNOS was principally responsible for mediating the cardioprotective effects.

By contrast, the combination of BH_2 with PKC ε activator resulted in severe cardiac contractile dysfunction throughout reperfusion. This group demonstrated the most compromised postreperfused cardiac function among all study groups. Additionally, final LVEDP for this combination was similar to IR + PMN controls and was significantly different from the PKC ε activator and BH_4 group. Moreover, the compromised postreperfused cardiac function correlated with sufficient total and infiltrated PMNs that was similar to control I/R + PMN hearts. Likewise, the combination of PKC ε activator and BH_2 significantly decreased NO release and showed a trend to increase H_2O_2 release more than the controls during reperfusion in femoral I/R. This suggests that the large increase in oxidative stress during reperfusion may principally correlate to the severe sustained cardiac contractile dysfunction observed in the rat hearts post reperfusion.

Collectively, the data provides indirect evidence to support the hypothesis that promoting eNOS coupling (i.e., BH_4) combined with eNOS enhancer (i.e., PKC ε activator) during reperfusion may be primary mechanisms to attenuate oxidative stress and reperfusion injury. Previous studies have only documented a cardioprotective role for PKC ε activator when given as pretreatment prior to ischemia to stimulate cardiac preconditioning (Inagaki et al. 2003; Teng et al. 2008). Preconditioning is clinically relevant in that it could protect cardiac function prior to invasive procedures such as organ transplantation, coronary artery bypass, and angioplasty. However, pretreatment/preconditioning is often not an option in these clinical situations; therefore, attenuating reperfusion injury may be best accomplished by giving compounds during the reperfusion phase. Furthermore, activating PKC ε in the absence of BH_4 supplementation during reperfusion has shown to result in compromised postreperfused cardiac function (Inagaki et al. 2003; Teng et al. 2008). This study is noteworthy in that the PKC ε activator can restore postreperfused cardiac function when given during reperfusion combined with a supplemental dose of BH_4.

The combination of PKC ε activator and BH_2 had an additive effect on promoting postreperfused cardiac contractile dysfunction and oxidative stress in I/R since neither drug when given independently produced an equivalent degree of cardiac contractile dysfunction or oxidative stress. Therefore, promoting eNOS uncoupling (i.e., BH_2) combined with eNOS enhancer (i.e., PKC ε activator) during reperfusion may exacerbate oxidative stress and reperfusion injury. This data is consistent with the notion that increased oxidative stress is observed when BH_2 is the binding cofactor to eNOS at the oxygenase domain (Vasquez-Vivar et al. 1998, 2002), especially when eNOS activity is enhanced by PKC ε activator.

The effect of PKC ε inhibitor combined with BH_4 or BH_2 on cardiac function and H_2O_2/NO release following I/R

This study confirmed the role of PKC ε in I/R injury and the data was consistent with our previous study suggesting that PKC ε inhibitor was involved in the cardioprotection and subsequent reduction of oxidative stress during the reperfusion phase after prolonged acute ischemia (Teng et al. 2008). PKC ε inhibitor alone, or combined with BH_2 or BH_4 significantly restored postreperfused cardiac function, resulting in significantly increased final LVDP and decreased final LVEDP compared to controls. Moreover, the improvement in postreperfused cardiac function also correlated with significantly decreased total intravascular and infiltrated PMNs compared to I/R + PMN control hearts. These results correlate with a significant decrease in oxidative stress (i.e., H_2O_2 release) in rat femoral I/R. PKC ε inhibitor and BH_2 or BH_4 significantly decreased H_2O_2 release compared to controls during reperfusion, suggesting that decreased oxidative stress was the principal

mechanism responsible for the cardiac protection in the rat hearts. Additionally, both combinations significantly increased NO bioavailability compared to controls during reperfusion. These findings suggest that BH_2 or BH_4 did not significantly influence the effects of PKC ε inhibitor on H_2O_2 or NO release and postreperfused cardiac function, contrary to the effects of BH_4 or BH_2 observed with the PKC ε activator.

Collectively, the data provides indirect evidence to support the hypothesis that PKC ε inhibitor attenuates eNOS activity in its uncoupled state, reducing the quenching of NO by superoxide/H_2O_2 and subsequently increasing NO bioavailability during reperfusion regardless of the eNOS cofactor. The data further supports the hypothesis that postreperfused cardiac function is restored and oxidative stress is reduced when eNOS uncoupling is attenuated and that PKC ε inhibitor may be a vital factor in attenuating reperfusion injury. Further support of this concept comes from a recent study that showed improved cardiac function when PKC ε inhibitor was given systemically after transplantation (Koyanagi et al. 2007).

In summary, these data demonstrate the dual role of PKC ε in that activation is beneficial prior to ischemia to induce preconditioning, or needs to be combined with a priming dose of BH_4 (e.g., 5 μM) to be effective when given during reperfusion. Whereas, inhibition is helpful when given during reperfusion to attenuate additional injury and is independent of the eNOS cofactor status (Lasley et al. 1997; Young et al. 2005). This would have clinical benefits to patients suffering from heart attacks due to ischemic heart disease requiring coronary artery bypass, angioplasty, and/or organ transplantation.

The role of eNOS activity in endothelial dysfunction in the setting of I/R

PKC is a key enzyme in the regulation of eNOS activity (Young et al. 2005) that can increase or decrease endothelial NO release when the respective PKC isoforms are activated by second messengers such as diacylglycerol (DAG) upon receptor stimulation or directly by selective PKC isoform activators/inhibitors (Li et al. 1998; Young et al. 2005). PKC ε can form a three-member PKC ε-Akt-eNOS signaling module to increase NO release via phosphorylation at serine 1177 (Zhang et al. 2005; Newton and Messing 2010).

Moreover, PKC ε activation or inhibition had no effect on PMN superoxide release which is consistent with the absence of PKC ε expression in PMNs (Dang et al. 2001; Teng et al. 2008). These observations lead us to further utilize PKC ε activator or inhibitor to determine the role of eNOS uncoupling during reperfusion as the principal mechanism mediating endothelial and subsequent cardiac contractile dysfunction during reperfusion. Therefore, it would be expected that PKC ε activation would restore postreperfusion cardiac function only when given prior to ischemia when eNOS function is coupled. In support of this concept, other compounds (e.g., C-peptide) that have increased eNOS expression and/or activity as a primary mechanism to attenuate reperfusion injury required animal and/or organ pretreatment (Scalia et al. 2000; Young et al. 2000). However, the novelty of the current study suggests that activating eNOS exclusively during reperfusion can be beneficial when the BH_4/BH_2 ratio is increased or can be deleterious when the BH_2/BH_4 ratio is increased.

By contrast, PKC ε inhibition when given during reperfusion by itself exhibits remarkable postreperfusion cardiac function suggesting that uncoupled eNOS was effectively inhibited during reperfusion and was principally responsible for attenuating H_2O_2 release by this mechanism since neither BH_4 nor BH_2 significantly altered the effects compared to PKC ε inhibitor by itself. Moreover, vascular endothelium is the principal cell type that is influenced in this model of myocardial I/R since the concentrations of PKC ε activator, PKC ε inhibitor, BH_4, or BH_2 independently (Teng et al. 2008; Chen et al. 2010) or in combination did not elicit cardiotonic or cardiodepressant effects in I/R alone conditions. These data suggest that potential diffusion into cardiac myocytes did not exert any significant effect. Additionally, no observable blood pressure or respiratory abnormalities were observed in the in vivo femoral I/R model.

The effects of PKC ε on other tissues can be derived from PKC ε knockout or transgenic studies in mice (Takeishi et al. 2000; Saurin et al. 2002; Gray et al. 2004). These studies suggest that the absence or overexpression of PKC ε does not result in abnormal developmental or physiological effects. However, these animal models are limited in that the absence or overexpression of PKC ε has occurred prior to any ischemic event. Therefore, these models do not adequately address the role of PKC ε under I/R conditions compared to a normal PKC ε expression level.

Future studies

To further elucidate the role of eNOS uncoupling during reperfusion, other regulators of eNOS activity could be used in conjunction with BH_4 or BH_2 in myocardial and femoral I/R studies. For example, palmitic acid is known to inhibit the association of eNOS with L-arginine and therefore may possibly promote eNOS uncoupling. Inhibiting the mobilization of palmitic acid may attenuate the potential uncoupling effect of fatty acids on eNOS activity and potentially identify another pharmacological tool that may be useful to attenuate reperfusion injury.

Significance of findings

Collectively, this study provides indirect in vivo evidence that eNOS uncoupling may be a significant mechanism mediating oxidative stress under I/R conditions. By contrast, promotion of eNOS coupling can also reduce oxidative stress and I/R injury. Therefore, PKC ε activator supplemented with BH_4 during reperfusion elicits cardioprotection and reduces oxidative stress. Whereas, PKC ε activator given with BH_2 during reperfusion promoted eNOS uncoupling, exacerbated postreperfused cardiac dysfunction, and was associated with increased oxidative stress. By contrast, all PKC ε inhibitor groups significantly restored postreperfused cardiac function suggesting that attenuation of uncoupled eNOS can be an effective means of reducing oxidative stress.

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