The Effects of Modulating Endothelial Nitric Oxide Synthase (eNOS) Activity and Coupling in Coronary, Hindlimb, Renal, and Mesenteric Vascular Inflammation Models

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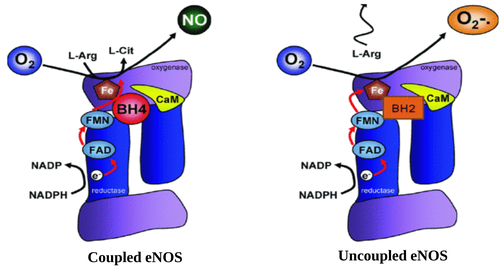
Introduction

Vascular injury is a key component in the pathogenesis of many diseases such as ischemia/reperfusion (I/R), hypertension, and hyperglycemia (1,2). It also a significant risk factor after some medical procedures, such as mechanical vascular trauma induced by extracorporeal shock wave lithotripsy (ESWL) that is used to ablate kidney stones (3,4). The vascular injury is highlighted with increased oxidative stress (i.e., superoxide [O_2^-], hydrogen peroxide [H_2O_2]) and serves as an initiating event that reduces nitric oxide (NO) bioavailability, and enhances leukocyte-endothelial interactions leading to additional cell and organ injury (1,5,6). Endothelial NO synthase (eNOS) uncoupling (i.e., increased dihydrobiopterin [BH_2, 239 g/mol] to tetrahydrobiopterin [BH_4, 314 g/mol] ratio) is a source of O_2^- in both in vitro and in vivo models (figure 1) (2,5). We have previously shown that promoting eNOS coupling by giving BH_4 at the beginning of reperfusion significantly improves post-reperfused cardiac function and reduces of polymorphonuclear leukocyte (PMN) infiltratrion in isolated perfused I/ R hearts. Moreover, BH_4 significantly enhanced NO and reduced H_2O_2 blood levels during hind limb I/R in vivo (5). *These findings led us to explore the combination of modulating eNOS activity and coupling in myocardial and hind limb I/R, ESWL, and mesenteric inflammation models.* Cell-permeable protein kinase C epsilon peptide activator (PKCε+, N-Myr-HDAPIGYD, 1097 g/mol) increases eNOS activity while PKCε inhibitor (PKCε-, N-Myr-EAVSLKPT, 1054 g/mol) reduces eNOS activity. Using a combination of eNOS cofactors BH_4 or BH_2 with PKCε+ we explored the role of promoting eNOS coupling/uncoupling on oxidative stress in I/R, ESWL, and BH_2-induced leukocyte-endothelial interactions in the rat mesenteric circulation. We also explored the role of inhibiting uncoupled eNOS using PKCε- in these four animal models.



Coupled eNOS Uncoupled eNOS

Figure 1. Coupled eNOS and Uncoupled eNOS. BH_4 is the cofactor of coupled eNOS which produces NO. Oxidative stress results in an increased BH_2:BH_4 ratio. BH_2 is the cofactor for uncoupled eNOS which produces superoxide (O_2^-) which is then converted to H_2O_2 by superoxide dismutase {adapted from 2}

Hypothesis

We hypothesized that promoting eNOS coupling and activity with PKCε+/BH_4 or inhibiting uncoupled eNOS activity with PKCε- would attenuate vascular injury in all vascular inflammatory pathologies. We tested this hypothesis in four vascular inflammation models: coronary and hindlimb I/R, ESWL, and BH_2-induced mesenteric inflammation. We predict that there will be: 1) reduction in PMN adherence and tissue infiltration in the coronary vasculature, accompanied by an improvement in post-reperfused cardiac function, 2) enhanced endothelial-derived NO and reduced H_2O_2 blood levels in both hindlimb I/R and ESWL and 3) attenuated BH_2-induced leukocyte-endothelial interactions in the mesentery.

Methods

Experiments were performed on male Sprague-Dawley rats (275-325g, Charles River, Springfield, MA) which were anesthetized with sodium pentobarbital (60 mg/kg) intraperitoneally. A maintenance dose (30 mg/kg) was administered as needed. All data was presented as means ±SEM. The data for each time-point were compared by ANOVA using post-hoc analysis with the Bonferroni/Dunn test (coronary data), Student Newman Keuls test (hindlimb and ESWL data), and Fisher's PLSD test (mesenteric data). Probability values of less than 0.05 were considered to be statistically significant.

Coronary I/R Model**:** Hearts were rapidly excised, and retrograde perfusion of the hearts was initiated with modified Kreb's Buffer and maintained at 80 mmHg pressure at 37°C. After a stable baseline was established, hearts were subjected to I (20 min)/R (45 min) and given PMNs ($200x10^6$) and plasma in the presence or absence of drug given at reperfusion (5). Experimental groups included I/R + PMNs (control), I/R + PMNs + PKCε- (Genemed Synthesis), I/R + PMNs + PKCε+ (Genemed Synthesis) /BH_4, and I/R + PMNs + PKCε+/BH_2.

Hindlimb & Renal ESWL Models: NO or H_2O_2 microsensors were inserted into angiocatheters in the rat femoral veins or left renal vein. These microsensors receive an electrical signal proportional to the free radical concentration through an oxidation/reduction reaction on the sensor membrane. The TBR 4100 Free Radical Analyzer (WPI, Inc., Sarasota, FL) produces a trace of real-time measurements of NO or H_2O_2 release in picoamps (pA) throughout the experiment. NO readings were converted to nM, and H_2O_2 readings were converted to µM based on their own calibration curves. All measurements post-I/R or post-ESWL were expressed as relative change from baseline. In the hindlimb I/R model, one femoral artery/vein was clamped for 20 min ischemia followed by releasing the clamp for 45 min reperfusion. The other femoral artery/vein was a non-ischemic sham control (5). In the renal ESWL model, ESWL treatment was administered for 13 min with a Dornier Epos Ultra Lithotripter (16kV, 1.3mHz), and measurements were taken through 30 min post-ESWL. At reperfusion or immediately following ESWL, 0.5 mL of saline or drug bolus was administered via the left jugular vein (4). Experimental groups included a post-I/R or post-ESWL infusion of the following: 0.8 mg/kg PKCε-, 0.9 mg/kg PKCε+ with 0.8 mg/kg BH_4 (Cayman Chemicals), and 0.9 mg/kg PKCε+ with 2 mg/kg BH_2 (Cayman Chemicals).

Vascular Inflammation Model: Intravital microscopy was performed on anesthetized rats. One loop of mesentery was placed on a viewing pedestal to observe microcirculation in post-capillary venules in real-time. All groups received a superfusion of BH_2 over the mesentery to initiate inflammation. Leukocyte-endothelial interactions were recorded for 2 min at 30 min intervals after baseline. Superfused mesenteric tissue was harvested at the end of experiment for later hematoxylin & eosin (H&E) staining to confirm intravital microscopy observations (6). Experimental groups included Krebs' control, 100µM BH_2 control, 100µM BH_2 with 10µM PKCε-, 100µM BH_2 with 10µM PKCε+ and 100µM BH_4, and 100µM BH_2 with 10µM PKCε+.

Results

Table 1. Initial and Final LVDP and +dP/dt$_{max}$ among Different Experimental Groups

Group	Initial LVDP	Final LVDP	Initial +dP/dt$_{max}$	Final +dP/dt$_{max}$
I/R + PMNs control (n=11)	94.4 ± 4.1	42.0 ± 5.8$^{\#}$	2469.2 ± 116.9	807.9 ± 97.8$^{\#}$
I/R + PMNs + 5µM PKCε- (n=7)	91.1 ± 2.6	90.0 ± 4.9**	2505.9 ± 97.7	2240.5 ± 103.4**
I/R + PMNs + 10µM PKCε+/5µM BH_4 (n=7)	85.3 ± 1.2	78.8 ± 9.4*	2174.6 ± 56.5	1806.6 ± 157.8**
I/R + PMNs + 10µM PKCε+/100µM BH_2 (n=6)	92.6 ± 2.2	35.2 ± 8.4	2438.4 ± 105.3	751.4 ± 204.9

There was no significant difference between initial baseline values of left ventricular developed pressure (LVDP) and +dP/dT$_{max}$ in all study groups. I/R + PMNs hearts recovered to only 45% and 31% of LVDP and +dP/dt$_{max}$, respectively. Addition of PKCε- resulted in almost complete recovery for both LVDP and +dP/dt$_{max}$. Similarly, I/R + PMNs + PKCε+/BH_4 recovered to 92% and 83% for LVDP and +dP/dt$_{max}$, respectively. In contrast, I/R + PMNs + PKCε+/BH_2 hearts recovered to 38% for LVDP and 31% for +dP/dt$_{max}$ similar to controls. (#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$_{max}$)

Table 2. Histological Assessments of PMNs in Isolated Perfused Rat Hearts

The infiltration of PMNs into the myocardium closely correlated with cardiac contractile dysfunction associated with I/R. PKCε- or PKCε+/BH_4 treated hearts significantly attenuated PMN infiltration and was associated with improved post-reperfused cardiac function. (*p<0.01 from total intravascular and infiltrated I/R + PMN values)

Group	Total Intravascular and Infiltrated PMNs
I/R + PMNs control	193.2 ± 7.3
I/R + PMNs + PKCε-	111.5 ± 11.0*
I/R + PMNs + PKCε+/BH_4	86.1 ± 6.4*
I/R + PMNs + PKCε+/BH_2	142.3 ± 12.8

Effects of PKCε-, PKCε+/BH_4, and PKCε+/BH_2 on NO/H_2O_2 blood levels in Hindlimb I/R:

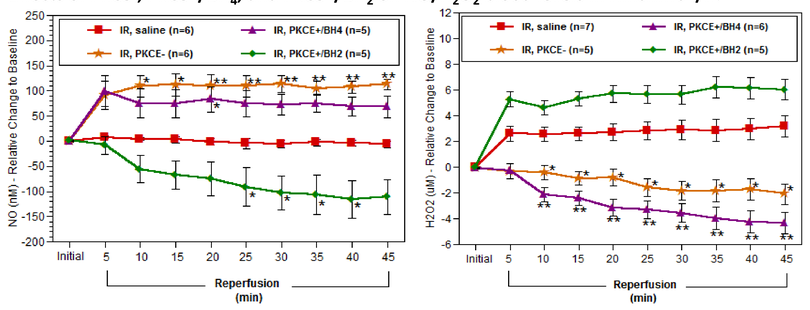


Figure 2. Relative difference in blood NO and H_2O_2 release between I/R and sham femoral veins during reperfusion in saline controls, PKCε-, PKCε+/BH_4, or PKCε+/BH_2 treated groups. By contrast to saline group, PKCε- significantly increased NO and decreased H_2O_2 and PKCε+/BH_4 significantly decreased H_2O_2 from 10-45 min and increased NO at 20 min reperfusion; whereas PKCε+/BH_2 significantly decreased NO from 25-40 min and increased H_2O_2, (not significant). (*p≤0.05, **p≤0.01, compared to saline controls)

Effects of PKCε-, PKCε+/BH_4, and PKCε+/BH_2 on NO/H_2O_2 blood levels post-ESWL:

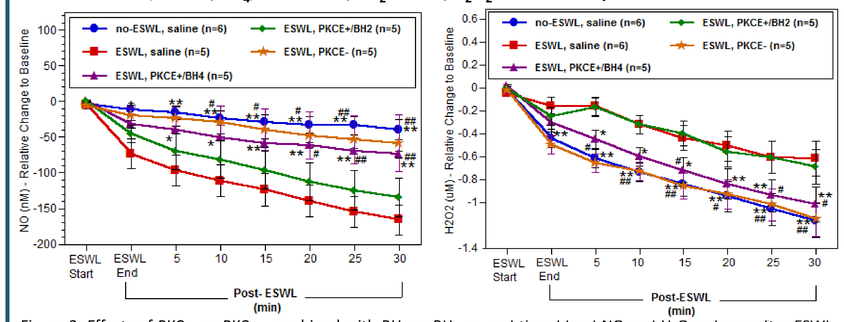


Figure 3. Effects of PKCε- or PKCε+ combined with BH_4 or BH_2 on real-time blood NO and H_2O_2 release after ESWL. ESWL significantly decreased NO release and increased H_2O_2 release (5-30 min post-ESWL) compared to no-ESWL controls. PKCε- administration after ESWL attenuated the ESWL-induced effects resulting in increased NO and decreased H_2O_2 release, similar to no-ESWL controls. Administration of PKCε+/BH_4 after ESWL was also similar to no-ESWL controls, significantly increasing NO and decreasing H_2O_2release (5-30 min post-ESWL) compared to ESWL controls. Post-ESWL infusion of PKCε+/BH_2 was similar to the ESWL control group in both NO and H_2O_2 release. (*p≤0.05, **p≤0.01, compared to ESWL controls; #p≤0.05, ##p≤0.01, compared to ESWL with PKCε+/BH_2)

Leukocyte vascular adherence/transmigration in superfused mesenteric tissues:

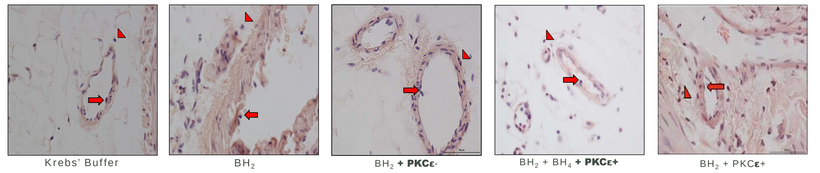
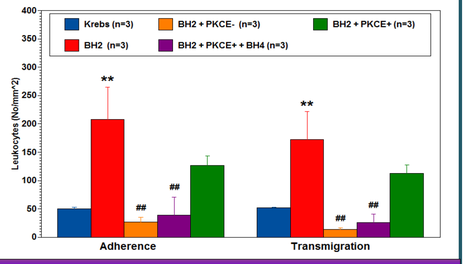


Figure 4. Representative pictures of leukocyte adherence (arrow) and transmigration (arrow head) by H&E staining of mesenteric tissues from experimental groups (top). Compared to Krebs', the BH_2 group exhibited a significant increase in adhered and transmigrated leukocytes. By contrast, PKCε+/BH_4 or PKCε- significantly decreased the BH_2 induced effects on these parameters. (*P<0.05,**P<0.01 from Krebs; #P<0.05, ##P<0.01 from 100 µM BH_2)



Conclusions

Collectively, the data suggest that eNOS uncoupling is a significant and common mechanism mediating inflammation in vascular injuries such as coronary and hindlimb I/R, ESWL, and mesenteric vascular inflammation. The results from the four vascular inflammation studies support our hypothesis that stimulating eNOS activity and promoting coupling using PKCε+/BH_4 or attenuating eNOS uncoupled activity using PKCε-: 1) significantly attenuated PMN coronary vascular adherence and tissue infiltration and improved post-reperfused cardiac function; 2) increased NO and decreased H_2O_2 blood levels in hindlimb I/R and ESWL compared to I/R or ESWL controls; 3) decreased leukocyte vascular adherence and tissue infiltration in BH_2-induced mesenteric vascular inflammation. By contrast, the stimulation of uncoupled eNOS activity using PKCε+/BH_2 reversed these effects in these four vascular models. These results suggest that PKCε+/BH_4 or PKCε- based therapies may mitigate vascular pathologies in the coronary, hindlimb, renal, and mesenteric vasculature.

References

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