The effects of modulating eNOS activity and coupling on leukocyte-endothelial interactions in rat mesenteric postcapillary venules

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Introduction

Endothelial-derived nitric oxide (NO) is essential in the regulation of diastolic blood pressure and promotes an antithrombotic surface which attenuates leukocyte-endothelial interactions associated with vascular injury. Endothelial nitric oxide synthase (eNOS) produces NO from L-arginine in the presence of the essential cofactor tetrahydrobiopterin (BH_4) (Fig. 1). When BH_4 is oxidized to dihydrobiopterin (BH_2) during vascular injury the ratio of BH_2/BH_4 is increased and this promotes eNOS to produce superoxide (SO) instead of NO and is termed eNOS uncoupling (1). Our previous studies have shown administration of BH_2 promotes leukocyte-endothelial interactions (Fig. 2) in the mesenteric circulation in vivo. The pro-inflammatory effects of BH_2 may be due to the increased BH_2/BH_4 ratio causing eNOS uncoupling and reduced endothelial-derived NO bioavailability (2). Protein kinase C epsilon (PKC ε) activates eNOS via phosphorylation on eNOS serine 1177 using PKC ε peptide activator (PKC ε+). Whereas, PKC ε peptide inhibitor (PKC ε-) reduces eNOS activity (3) (Fig. 3). However, the effect of PKC ε+ peptide or PKC ε- to exacerbate or attenuate BH_2-induced leukocyte-endothelial interactions has not yet been determined.
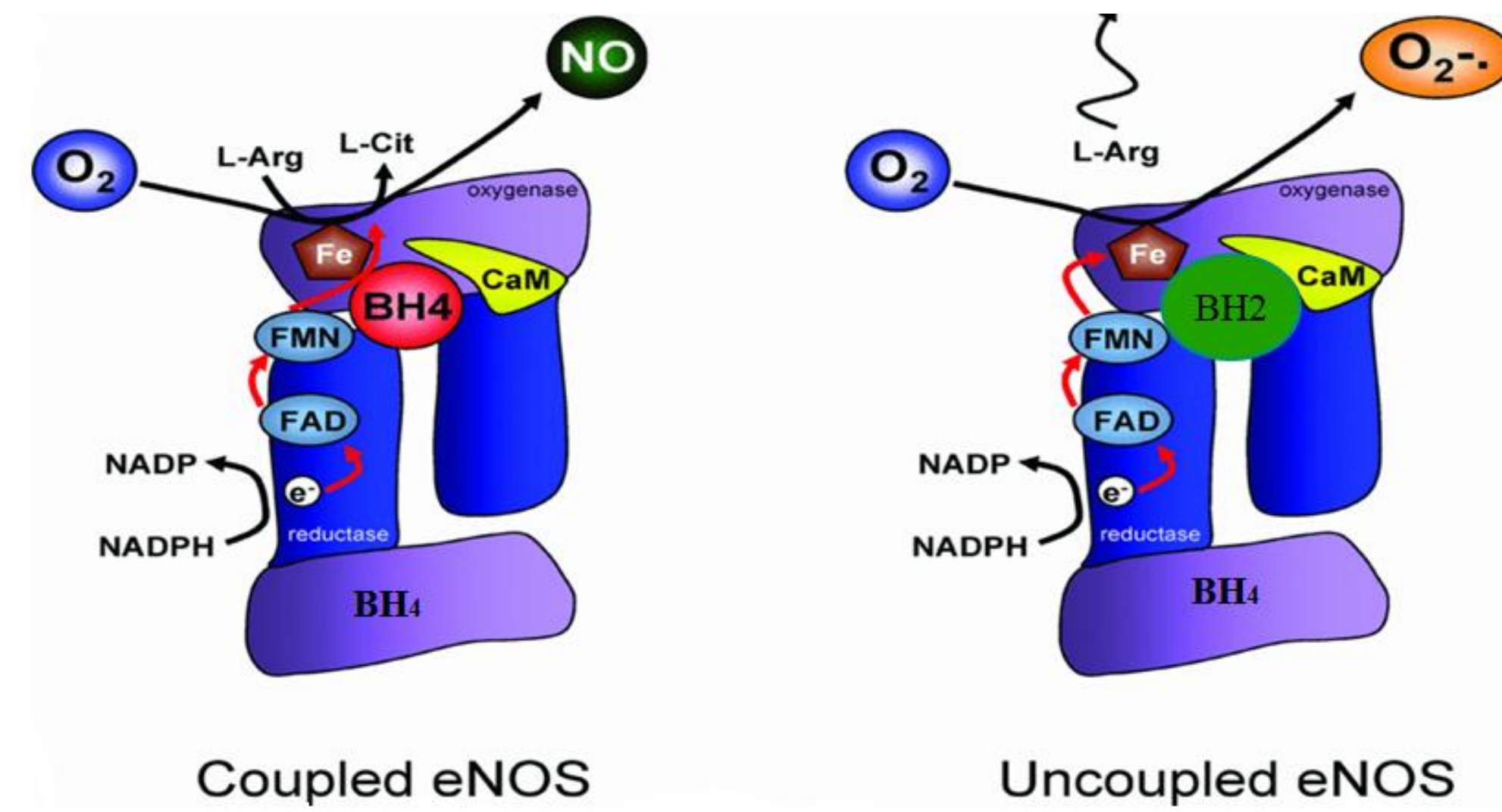

Coupled eNOS   Uncoupled eNOS

A.   B.

Figure 1. A. In healthy vascular endothelium, there is an increased BH_4 to BH_2 ratio. BH_4 binds to the ferrous-dioxygen domain complex in the oxygenase domain which results in a coupling reduction of molecular O_2 to L-arginine oxidation and synthesizes NO. B. In diseased conditions, BH_4 is limited and becomes oxidized to BH_2 increasing the BH_2/BH_4 ratio to promote uncoupled eNOS. The electron transfer is uncoupled from L-arginine, the ferrous-dioxygen complex dissociates, and SO is produced from the oxygenase domain from molecular oxygen (1).
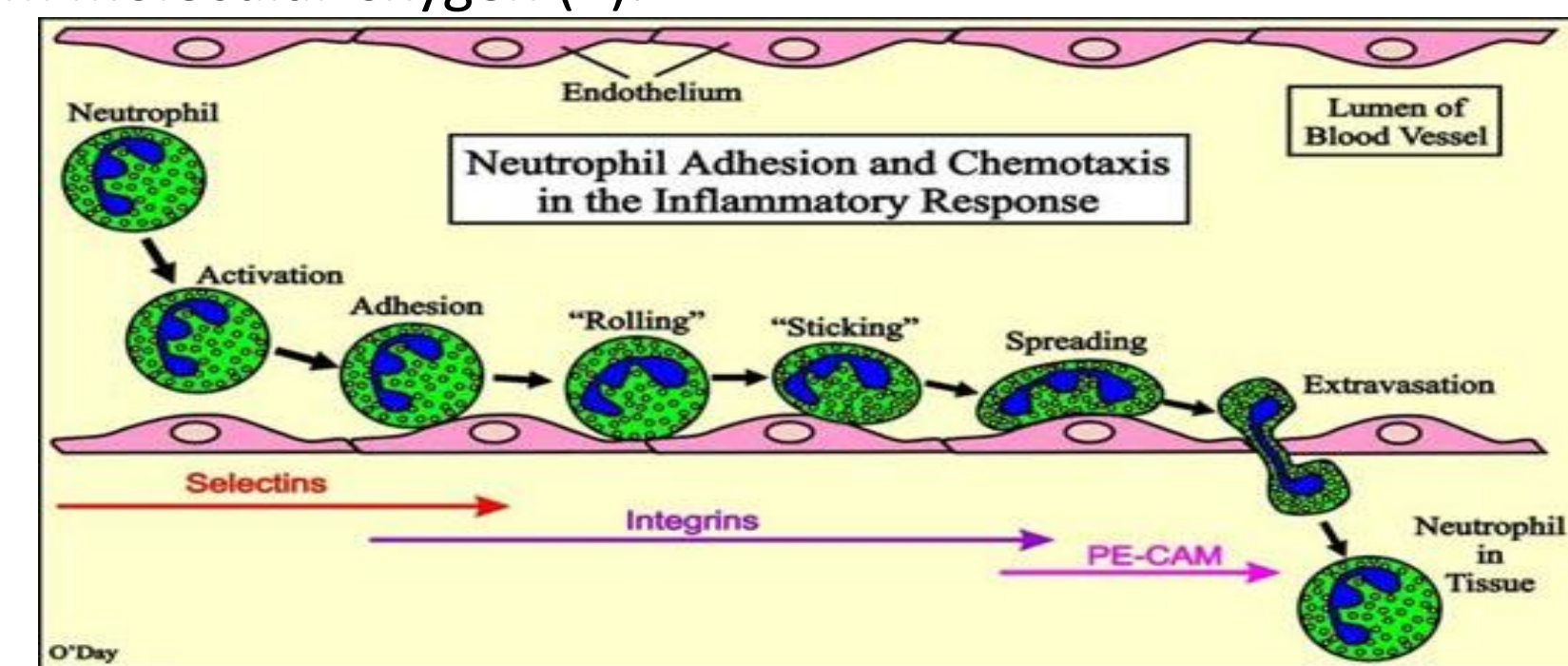

Figure 2. Leukocyte-endothelial interaction cascade in the basement membrane of blood vessel that begins with initial leukocytes rolling and concluding with extravasation through the vascular endothelial cells. Attenuation of this process reduces inflammation-mediated tissue damage.
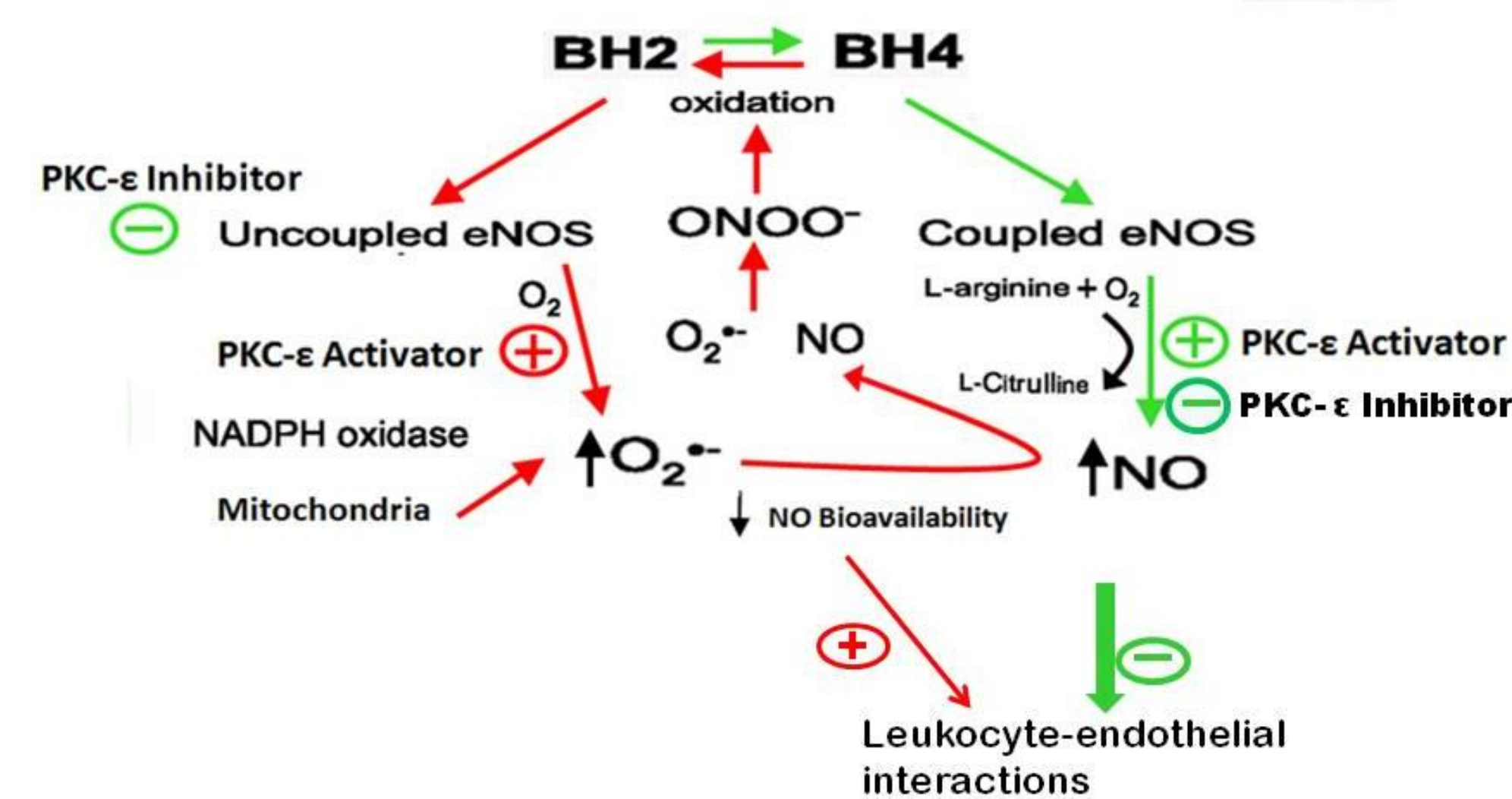

Figure 3. Hypothesis diagram of PKC ε+/PKC ε- combined with BH_4/BH_2 on vascular endothelial function and leukocyte-endothelial interactions.

Hypothesis

We predict that BH_2 with the addition of PKC ε- (N-Myr-EAVSLKPT, MW=1054, Genemed Synthesis, Inc., San Antonio, TX) will inhibit uncoupled eNOS, increase endothelial-derived NO bioavailability and reduce endothelial-leukocyte interactions. By contrast, when the PKC ε+ (N-Myr-HDAPIGYD, MW=1097, Genemed Synthesis) is administered with BH_2, it will augment uncoupled eNOS activity and/or sustain the BH_2-induced leukocyte-endothelial interactions. However, when the BH_4/BH_2 ratio is increased and the coupled eNOS status is more favorable, we predict that the addition of PKC ε+, will promote NO release from eNOS and attenuate the BH_2 induced inflammation. Whereas, PKC ε- will attenuate BH_2 induced inflammation even in the presence of increased BH_4 .

Methods

Intravital microscopy was performed on one loop of mesentery of male Sprague-Dawley rats (275-325 g, Ace Animals, Boyertown, PA) and the mesentery was placed on a viewing pedestal to observe mesenteric venules under light microscopy (Fig. 4). A right carotid artery cannulation was performed to monitor mean arterial blood pressure. During the experiment, test solutions (listed in the following experimental groups) were superfused over the mesentery and the number of rolling (number that passed a set reference point per minute), adhered (number that remained firmly adhered to the endothelial surface for >30 seconds within 100 μm length), and transmigrated (number that had emigrated through the endothelium within 10 μm on either side of the 100 μm length of venule) leukocytes were recorded (4).

Experimental Groups
1. Control: superfusion of Krebs' buffer (n=4)
2. BH_2: superfusion of 100 μM BH_2 (n=5)
3. BH_2 + PKC ε+: superfusion of 100 μM BH_2 + 10 μM PKC ε+ (n=6)
4. BH_2 + PKC ε-: superfusion of 100 μM BH_2 + 10 μM PKC ε- (n=5)
5. BH_2 + BH_4: superfusion of 100 μM BH_2 / 100 μM BH_4 (n=5)
6. BH_2 + BH_4 + PKC ε+: superfusion of 100 μM BH_2/100 μMBH_4 + 10 μM PKC ε+ (n=5)
7. BH_2 +BH_4 + PKC ε-: superfusion of 100 μM BH_2/100 μMBH_4 + PKC ε- (n=5)

Figure 4. Experimental setup for intravital microscopy. Inserted picture in upper right: exteriorized loop of mesenteric tissue undergoing superfusion of test solution.
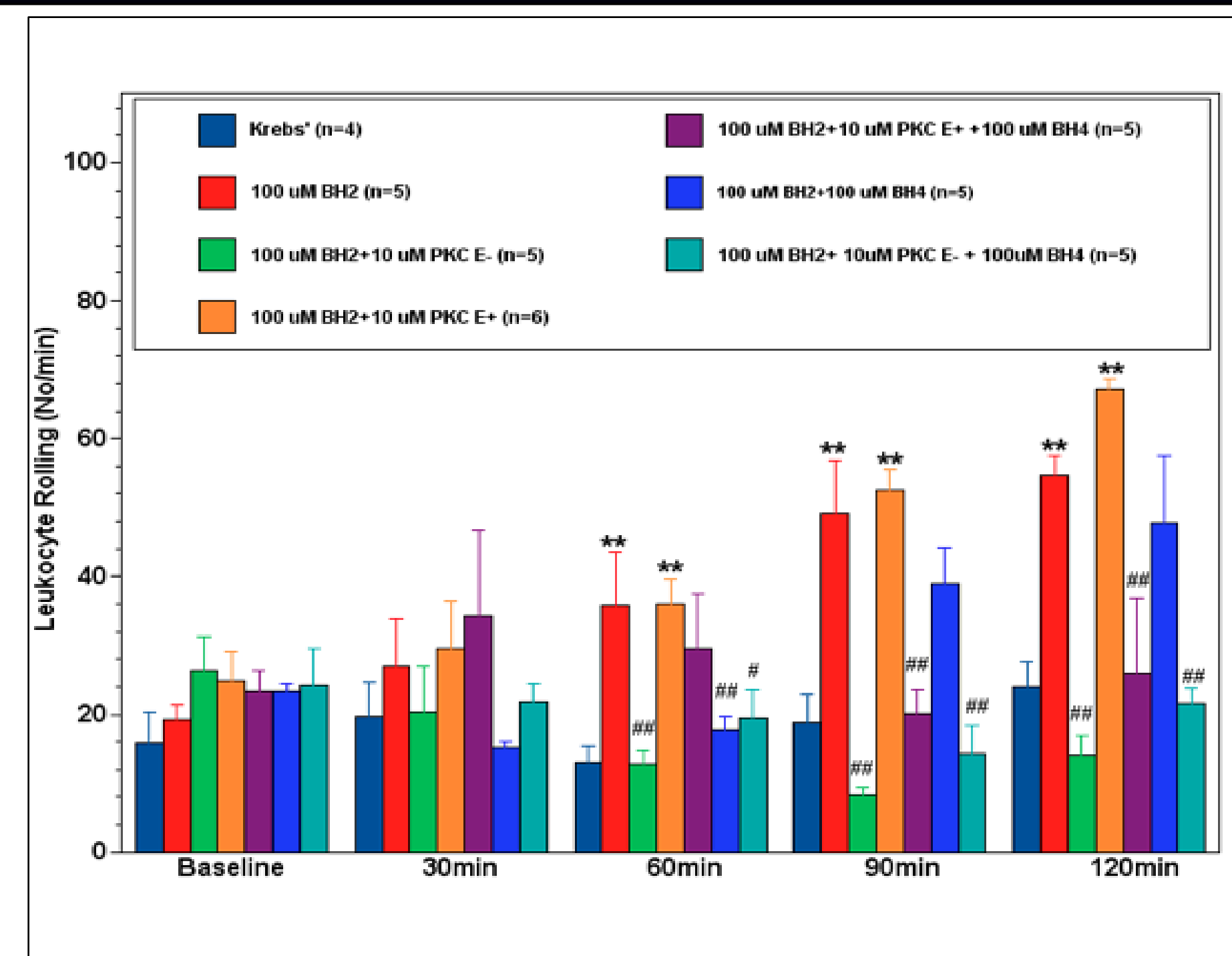
Results

*P<0.05, **P<0.01 from Krebs'; #P<0.05, ##P<0.01 from 100 μM BH_2

Figure 5. Leukocyte rolling among different experimental groups. 100 μM BH_2 and 100 μM BH_2 + 10 μM PKC ε+ significantly increased leukocyte rolling (**P<0.01 from Krebs') . The effect of BH_2 was significantly attenuated by the addition of 10μM PKC ε-, 100 μM BH_4 + 10 μM PKC ε-, and 100 μM BH_4 + PKC ε+ (##P<0.01 from 100 μM BH_2).

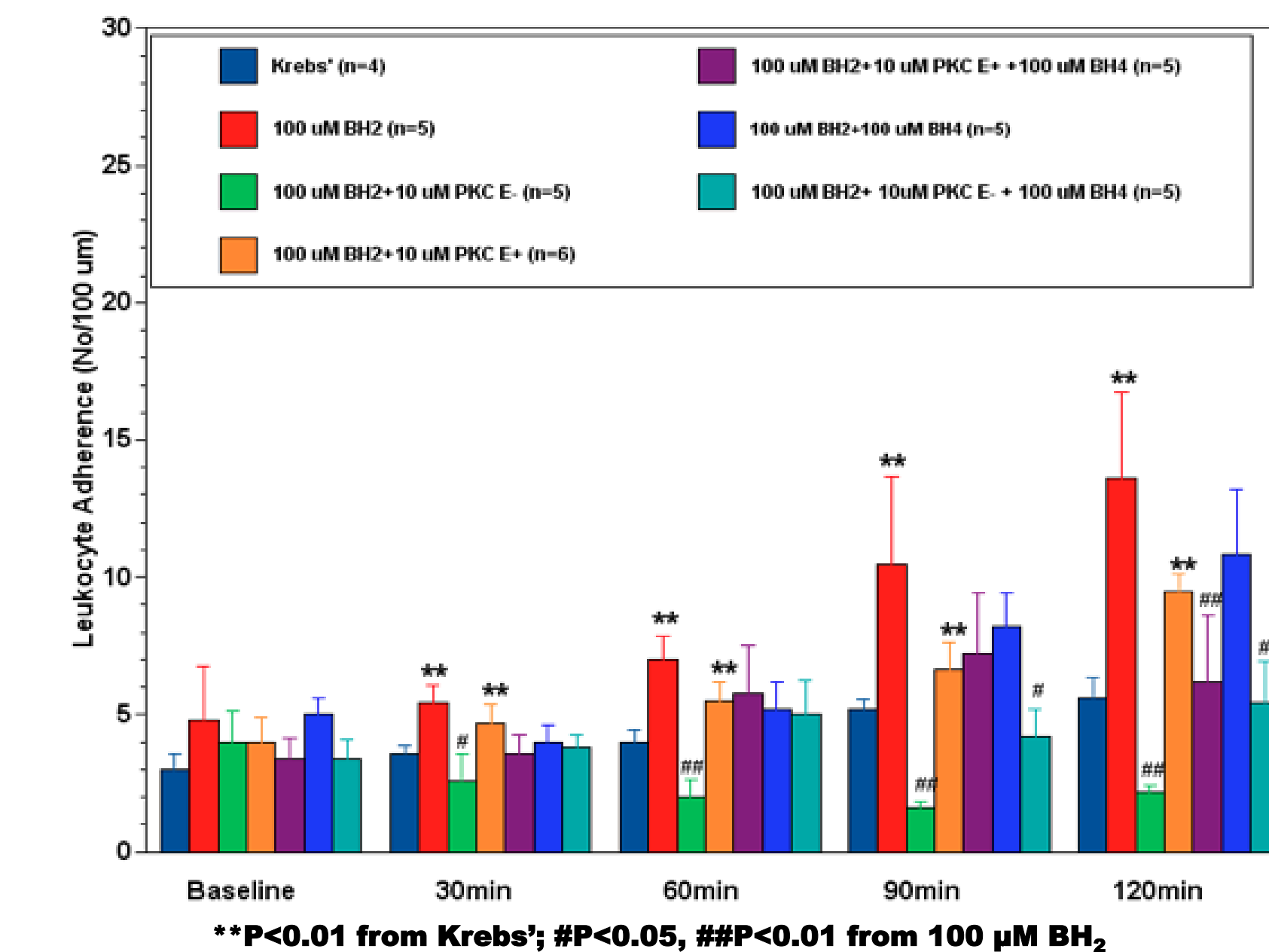**P<0.01 from Krebs'; #P<0.05, ##P<0.01 from 100 μM BH_2

Figure 6. Leukocyte adherence among different experimental groups. 100 μM BH_2 and 100 μM BH_2 + 10 μM PKC ε+ significantly increased leukocyte adherence (**P<0.01 from Krebs') . The effect of BH_2 was significantly attenuated by the addition of 10μM PKC ε-, 100 μM BH_4 + 10 μM PKC ε-, and 100 μM BH_4 + PKC ε+ (##P<0.01 from 100 μM BH_2).

*P<0.05, **P<0.01 from Krebs'; #P<0.05, ##P<0.01 from 100 μM BH_2
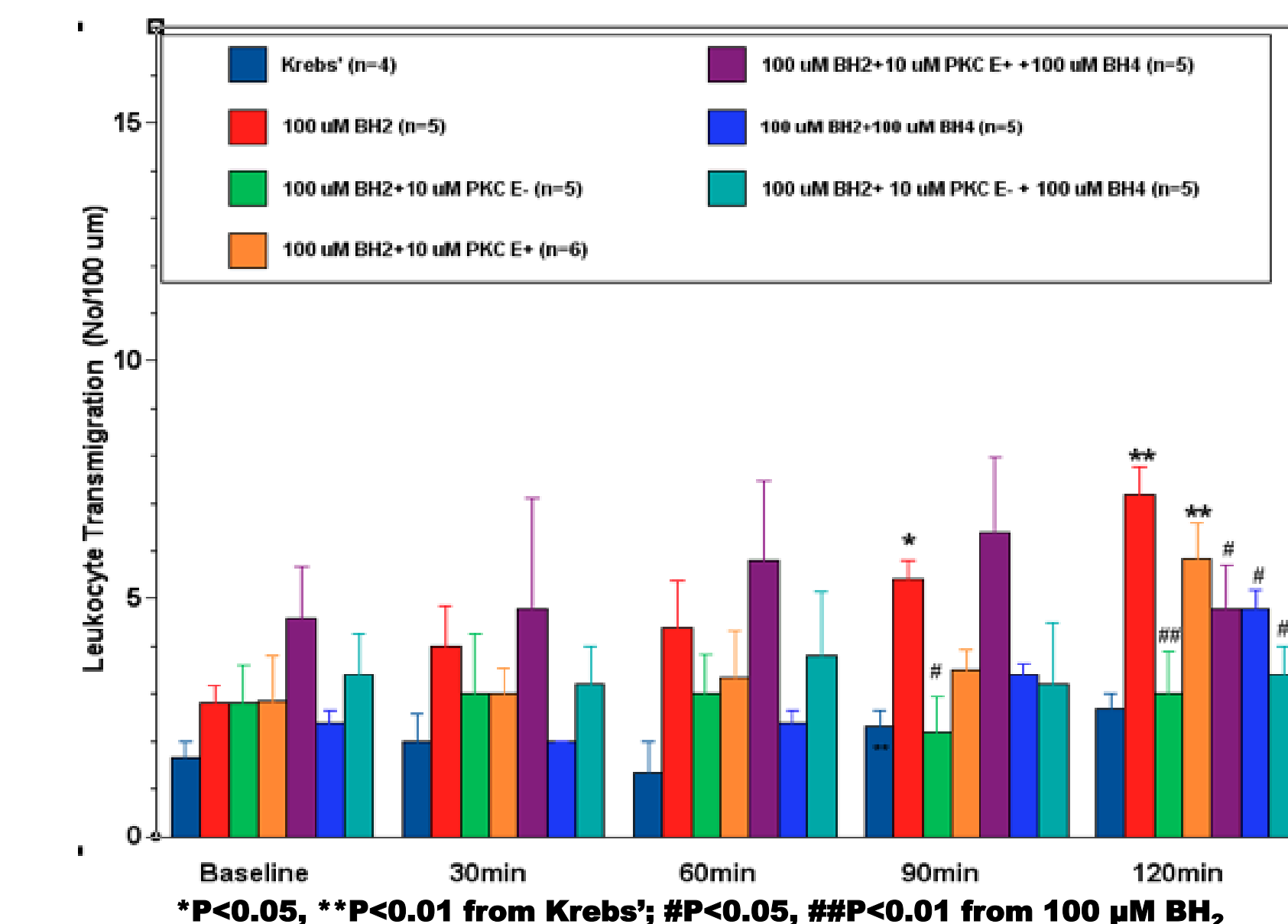
Figure 7. Leukocyte transmigration among different experimental groups. 100 μM BH_2 and 100 μM BH_2 + 10 μM PKC ε+ significantly increased leukocyte transmigration (*P<0.05, **P<0.01 from Krebs') . The effect of BH_2 was significantly attenuated by the addition of 10μM PKC ε-, 100 μM BH_4 + 10 μM PKC ε-, and 100 μM BH_4 + PKC ε+ (# P<0.05, ##P<0.01 from 100 μM BH_2).

Conclusions

We found that BH_2 significantly increased leukocyte rolling, adherence, and transmigration when compared to Krebs' control (P<0.05), and this effect was similar with BH_2 + PKC ε+ (P<0.05). Whereas, BH_2 + PKC ε- (P<0.01) significantly attenuated all three types of BH_2-induced inflammation . The data suggest that eNOS uncoupling may be an important mechanism mediating inflammation-induced vascular injury, and that inhibiting uncoupled eNOS activity with PKC ε- may attenuate oxidative stress and restore vascular endothelial function. However, we found that BH_4 + PKC ε+ significantly reduced BH_2 -induced inflammation. This data suggest that PKC ε+ can exert anti-inflammatory effects when the BH_4/BH_2 ratio is increased to promote eNOS coupling. This study outlines the importance that the BH_2/BH_4 ratio determines whether eNOS is in the uncoupled or coupled state. Moreover, inhibiting uncoupled eNOS activity with PKC ε- or increasing coupled eNOS activity with PKC ε+ can both exert anti-inflammatory effects. This idea provides indirect evidence as a potential strategy for attenuating endothelial dysfunction-induced inflammatory responses in various vascular diseases.

References

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