The effects of NADPH oxidase inhibitor apocynin on real-time blood nitric oxide and hydrogen peroxide release in femoral artery/vein ischemia and reperfusion injury

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Introduction
Vascular endothelial dysfunction can initiate oxidative stress, i.e. superoxide (SO) overproduction, during ischemia/reperfusion (I/R). Endothelial dysfunction is characterized by an increase of oxidative stress, leading to an increase in blood hydrogen peroxide (H2O2) and a decrease in endothelial-derived nitric oxide (NO) bioavailability. Leukocyte NADPH oxidase is well studied; however, endothelial NADPH oxidase contributing to I/R injury is not well characterized. Previous studies using GO 6983, a broad-spectrum protein kinase C inhibitor that can inhibit NADPH oxidase activity, have attenuated blood H2O2 levels during femoral I/R in vivo (1,2). A selective NADPH oxidase inhibitor, apocynin (Fig. 1), interrupts the intracellular assembly of the enzyme by preventing the translocation to the cell membrane (2). Specifically, apocynin blocks the Cyss196 residue interaction between endothelial NADPH oxidase subunits p47phox and p22phox (Fig. 3). This in turn inhibits SO release from endothelial NADPH oxidase, which further enhances endothelial-derived NO release both of which may reduce oxidative stress in I/R injury (3,4).

Figure 1. Apocynin (acetovallorine) molecular structure. Molar Mass: 166.2. T1/2 of hours. Sigma Chemicals.

Hypothesis
We hypothesize that the femoral I/R veins will show an increase in blood H2O2 release compared to its counterpart sham femoral vein. Whereas, a decrease in NO release is expected in the femoral I/R vein compared to the sham vein of saline control animals. When the direct NADPH oxidase inhibitor, apocynin (T=3h), is given at the beginning of reperfusion we predict a decrease in H2O2 release and an increase in endothelial-derived NO bioavailability compared to saline control group.

Methods
We measured H2O2 or NO release in real-time from femoral veins of the anesthetized rat: one limb subjected to I/R while the other was used as a non-ischemic sham control. The H2O2 or NO microsensors (100 µm, WPI Inc.) were connected to a flow through sensor analyzer (Apollo 4000, WPI Inc.) and were inserted into a catheter placed inside each femoral vein. Ischemia of femoral circulation in one limb was induced by clamping the femoral artery/vein for 20 minutes followed by 45 minutes of reperfusion. Apocynin (13.7 mg/kg, diluted in saline ~ 1 mL in blood) or saline (for non-drug control group) was administered through a jugular vein cannulation at the beginning of reperfusion. Experimental groups were compared with student t-test.

Figure 6. Comparison of the relative difference in H2O2 release between I/R and sham femoral veins during reperfusion. There was a significant decrease in H2O2 release in the apocynin-treated group compared to saline from 20 minutes to 45 minutes of reperfusion (* p<0.05, **p<0.01 from saline).

Figure 7. Comparison of the relative difference in NO bioavailability between I/R and sham femoral veins during reperfusion. There was a significant increase in NO release in the apocynin-treated group compared to saline from 20 minutes to 45 minutes of reperfusion (* p<0.05, **p<0.01 from saline).

Results
We continuously recorded the H2O2 or NO release and collected the data at 5 minute intervals during a 15 minute baseline period, 20 minute ischemia and 45 minute reperfusion. The changes in H2O2 or NO release during I/R (in pm) were expressed as relative change to baseline NO (μM) or NO (μM) after correction to the calibration curve of H2O2 or NO microsensors.

Figure 8. The experimental preparation for measuring H2O2 or NO release from male Sprague-Dawley rats (275-325 grams, Ace Animals, Boyertown, PA) femoral veins.

Conclusions
When apocynin is given at the beginning of reperfusion, there is a significant reduction of blood H2O2 and a significant increase in endothelial-derived NO bioavailability compared to the saline group. Apocynin has reportedly been successful in preventing Cyss196 interaction between the endothelial NADPH oxidase subunits p47phox and p22phox, which is necessary for NADPH oxidase assembly at the cell membrane. By preventing NADPH oxidase assembly under I/R conditions, SO production should decrease, thus leading to a decrease in H2O2 and an increase of endothelial-derived NO bioavailability. This inhibition appears to be consistent with our findings, suggesting that endothelial NADPH oxidase is a major contributor to oxidative stress in this model of I/R injury since only resident leukocytes are present during this time course.

References