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

C. Woodworth Parker, Kyle D. Bartol, Kerry-Anne Perkins, Qian Chen, Lindon H. Young

Department of Pathology, Microbiology, Immunology & Forensic Medicine, Philadelphia College of Osteopathic Medicine

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 (H_2O_2) 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 Gö 6983, a broad-spectrum protein kinase C inhibitor that can inhibit NADPH oxidase activity, has attenuated blood H_2O_2 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 (Fig. 2). Specifically, apocynin blocks the Cys196 residue interaction between endothelial NADPH oxidase subunits $p47^{phox}$ and $p22^{phox}$ (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).

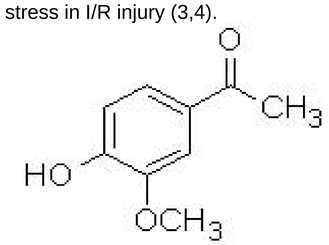

Figure 1. Apocynin (acetovallinone) molecular structure. Molar Mass: 166.2. $T_{1/2}$ of hours. Sigma Chemicals.

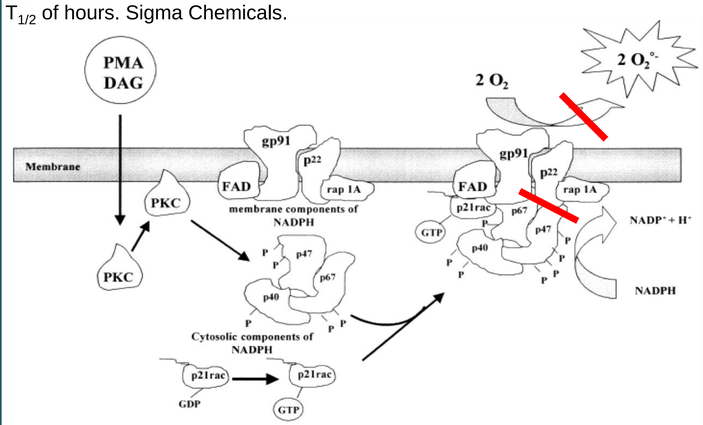

Figure 2. Apocynin inhibits endothelial NADPH oxidase subunits $p47^{phox}$ and $p22^{phox}$ assembly, preventing the production of SO. Adapted from Morena M et al. 2002 (5).

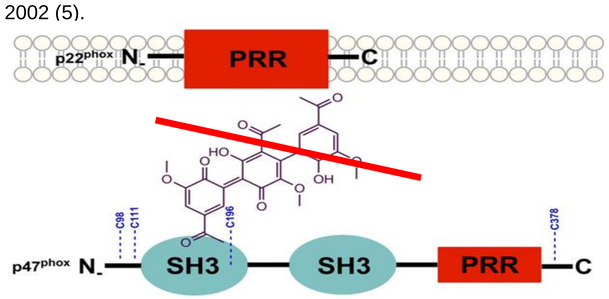

Figure 3. Apocynin interrupts the Cys196 interaction between NADPH oxidase subunits $p47^{phox}$ and $p22^{phox}$, preventing enzyme assembly and SO generation. Adapted from Mora-Pale et al, 2012 (4).

Hypothesis

We hypothesize that the femoral I/R vein will show an increase in blood H_2O_2 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 ($T_{1/2}$ 3h), is given at the beginning of reperfusion we predict a decrease in H_2O_2 release and an increase in endothelial-derived NO bioavailability compared to saline control group.

Methods

We measured H_2O_2 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 H_2O_2 or NO microsensors (100 um, WPI inc.) were connected to a free radical 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 mM in blood) or saline (for non-drug control group) was administered through a juglar vein cannulation at the beginning of reperfusion. Experimental groups were compared with student t-test.

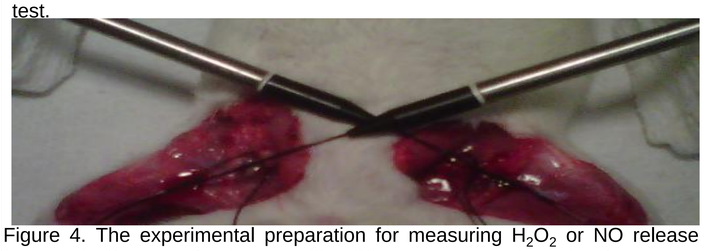

Figure 4. The experimental preparation for measuring H_2O_2 or NO release from male Sprague-Dawley rats (275-325 grams, Ace Animals, Boyertown, PA) femoral veins.

We continuously recorded the H_2O_2 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 H_2O_2 or NO release during I/R (in pA) were expressed as relative change to baseline H_2O_2 (uM) or NO (nM) after correction to the calibration curve of H_2O_2 or NO microsensors.

Results

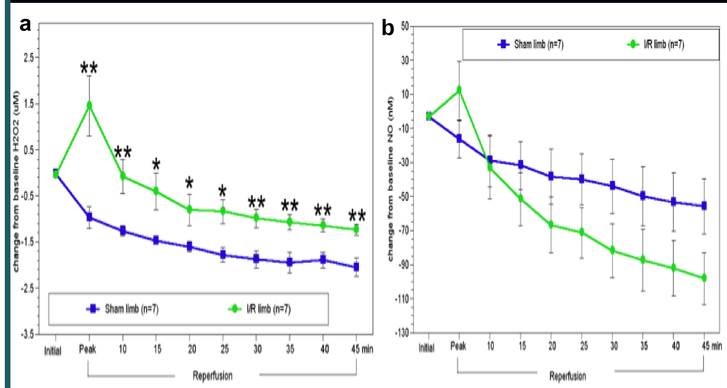

Figure 5. The time course of change in H_2O_2 (uM) or NO (nM) release from femoral veins during reperfusion in the saline control group.
(a) There was a significant increase in H_2O_2 release from I/R veins compared to sham veins during reperfusion (* p<0.05, **p<0.01 from sham).
(b) There was no significant difference in NO release from I/R veins compared to sham during reperfusion (* p<0.05, **p<0.01 from sham).

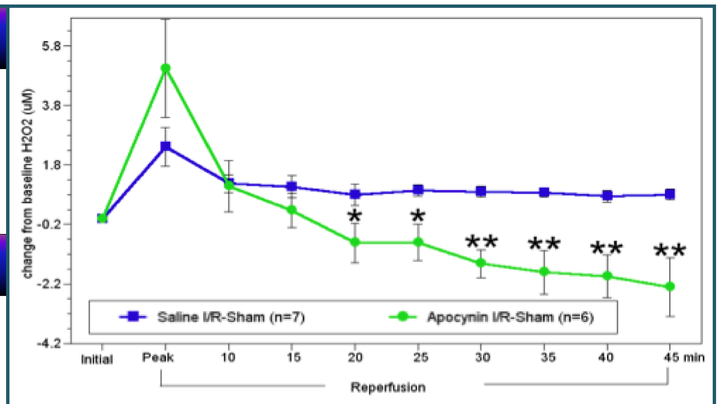

Figure 6. Comparison of the relative difference in H_2O_2 release between I/R and sham femoral veins during reperfusion. There was a significant decrease in H_2O_2 release in the apocynin-treated group compared to saline from 20 minutes to 45 minutes of reperfusion (* p<0.05, **p<0.01 from saline).

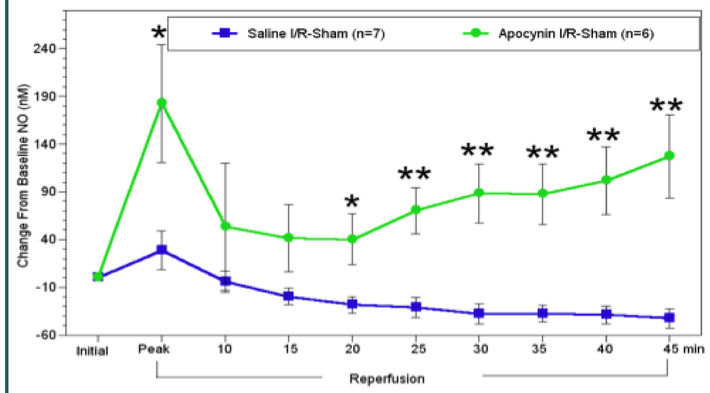

Figure 7. Comparison of the relative difference in NO bioavailability between I/R and sham femoral veins during reperfusion. There was a significant increase in NO release in the apocynin-treated group compared to saline from 20 minutes to 45 minutes of reperfusion (* p<0.05, **p<0.01 from saline).

Conclusions

When apocynin is given at the beginning of reperfusion, there is a significant reduction of blood H_2O_2 and a significant increase in endothelial-derived NO bioavailablity compared to the saline group. Apocynin has reportedly been successful in preventing Cys196 interaction between the endothelial NADPH oxidase subunits $p47^{phox}$ and $p22^{phox}$, 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 H_2O_2 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

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5. Morena, M., Jean-Paul Cristol, et al. (2002). "Oxidative stress in hemodialysis patients: Is NADPH oxidase complex the culprit?" Kidney International **61**, S109–S114.