The Role of NADPH Oxidase in Leukocyte-endothelial Interactions in Rat Mesenteric Postcapillary Venules

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Introduction

Inflammatory responses following vascular endothelial dysfunction have been considered critical and initial steps leading to the pathogenesis of many diseases, such as hypertension, ischemia/reperfusion (I/R) injury, and vascular complications in diabetes. Endothelial dysfunction is characterized by increase in reactive oxygen species (ROS) and decrease in endothelial-derived nitric oxide (NO). Under normal physiological conditions, endothelial-derived NO is produced via endothelial NO synthase (eNOS) and is a key component of vascular homeostasis responsible for regulating normal blood pressure, vasodilation, anti-inflammatory responses, and anti-coagulation properties. It has been shown that decrease in NO bioavailability by N^G-nitro-L-arginine-methyl-ester (L-NAME), a non-selective NOS inhibitor, can induce inflammatory responses (1). On the other hand, vascular endothelial NADPH oxidase produces ROS normally at small quantity for the purposes of signaling transduction and cellular regulation. However, during pathological conditions, NO bioavailability is decreased and leukocyte NADPH oxidase is activated via assembly of its subunits on the cell membrane resulting in overproduction of superoxide (SO) and subsequent hydrogen peroxide (H_2O_2) release and leading to oxidative stress. Furthermore, SO can directly react with NO to produce peroxynitrite ($ONOO^-$) and thereby decreases NO bioavailability, exacerbates endothelial dysfunction, and subsequently initiates leukocyte-endothelial interactions. Therefore, the recruitment of leukocytes will amplify the inflammatory responses and lead to tissue and organ damage. However, it is still unclear of the role of NADPH oxidase in leukocyte-endothelial under basal level and activated status during inflammatory responses. In this study, two NADPH oxidase inhibitors, apocynin and Gp91 ds-tat will be tested on basal and L-NAME induced leukocyte-endothelial interactions. Apocynin or Gp91 ds-tat, prevent the assembly of catalytic subunits of the enzyme and thereby inhibit SO release from NADPH oxidase as shown in figure 1 (2-4).

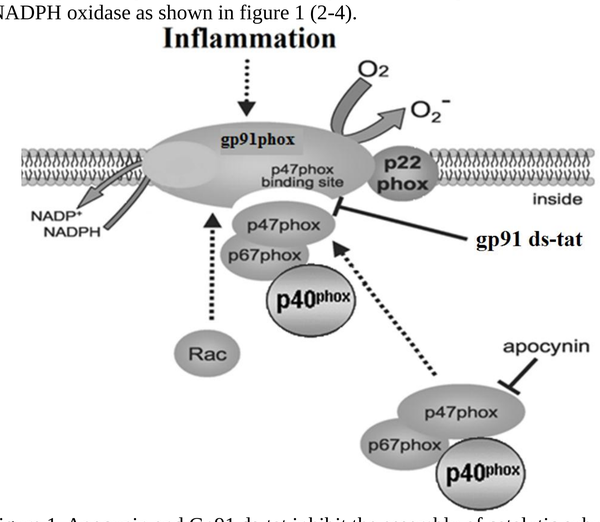

Figure 1. Apocynin and Gp91 ds-tat inhibit the assembly of catalytic subunits of NADPH oxidase. Apocynin inhibits the translocation of p47phox along with other cytosolic subunits (p67phox and p40phox) to the membranous subunits (gp91phox and p22phox). Gp91 ds-tat contains gp91phox docking sequence, which binds to p47phox and prevents the interaction of p47phox with gp91phox and thereby inhibits the assembly of NADPH oxidase. Adapted and modified from Brandes RP 2003 (2).

Hypothesis

We hypothesized that L-NAME treatment will induce leukocyte-endothelial interactions. Furthermore, apocynin will not influence basal leukocyte-endothelial interactions. Moreover, apocynin or Gp91ds-tat will dose-dependently attenuate L-NAME induced leukocyte-endothelial interactions.

Methods

Intravital microscopy

Male Sprague-Dawley rats, weighing 275-325 g, were anesthetized with 60 mg/kg pentobarbital sodium intraperitoneally (i.p.) and maintained with 30 mg/kg pentobarbital sodium (i.p.). The left carotid was isolated and cannulated to monitor the mean arterial blood pressure (MABP). A loop of the ileal mesentery was exteriorized via midline laparotomy and superfused with physiological buffer (i.e. Krebs' buffer) or Krebs' buffer with different treatments (Table 1). After 30 min stabilization, Leukocyte-endothelial interactions within the mesenteric postcapillary venules were observed via intravital microscopy for 2 hr. After the experiment, mesenteric tissue was harvested for hematoxylin and eosin (H&E) staining to evaluate leukocyte adherence and transmigration. All data is represented as means ± standard error of the mean (SEM). The comparison of more than two groups was analyzed by ANOVA using post hoc analysis with the Bonferroni/Dunn test to detect differences among experimental groups with each aim. Probability values of <0.05 are considered to be statistically significant.

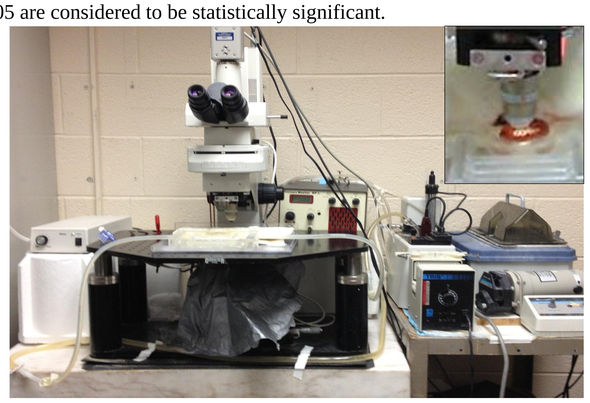

Figure 2. Experimental setup for intravital microscopy. Inserted picture upper right: exteriorized loop of mesenteric tissue undergoing superfusion of test solution.

Table 1. Experimental Groups

1.	Control (n=6)	superfusion of Krebs' buffer
2-3.	Apocynin (n=5-7)	superfusion of 400 µM/ 1000 µM in Krebs' buffer
4.	L-NAME (n=5)	superfusion of 50 µM L-NAME
5-7.	L-NAME+Apocynin (n=6-7)	superfusion of 50 µM L-NAME with 40 µM/ 400 µM/ 1000 µM apocynin
8-9	L-NAME+Gp91 ds-tat (n=4-6)	superfusion of 50 µM L-NAME with 5 µM/ 20 µM Gp91 ds-tat

Results

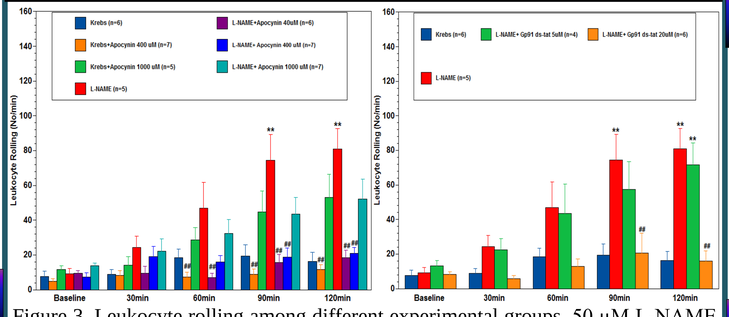

Figure 3. Leukocyte rolling among different experimental groups. 50 µM L-NAME significantly increased leukocyte rolling at T=90 min and T=120 min (**P<0.01 from Krebs'). The effect of L-NAME was significantly attenuated by the administration of 40 µM or 400 µM apocynin at T=60, 90, &120 min (left panel) and 20 µM Gp91 ds-tat at T=90 & 120min (right panel) (##P<0.01 from L-NAME).

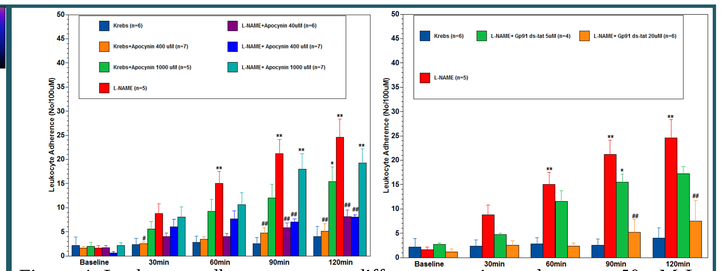

Figure 4. Leukocyte adherence among different experimental groups. 50 µM L-NAME significantly increased leukocyte adherence at T=60, 90, and 120 min (**P<0.01 from Krebs'). The effect of L-NAME was significantly attenuated by the administration of 40 µM or 400 µM apocynin at T=60, 90, &120 min (left panel) and 20 µM Gp91 ds-tat at T=90 & 120 min (right panel) (##P<0.01 from L-NAME).

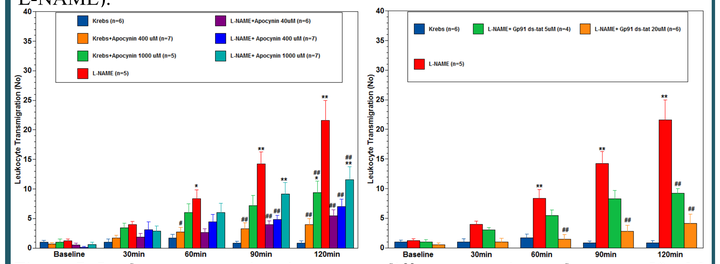

Figure 5. Leukocyte transmigration among different experimental groups. 50 µM L-NAME significantly increased leukocyte transmigration at T=60, 90, and 120 min (**P<0.01 from Krebs'). The effect of L-NAME was significantly attenuated by the administration of 40 µM or 400 µM apocynin at T=60, 90, &120 min (left panel) and 20 µM Gp91 ds-tat at T=90 & 120 min (right panel) (##P<0.01 from L-NAME).

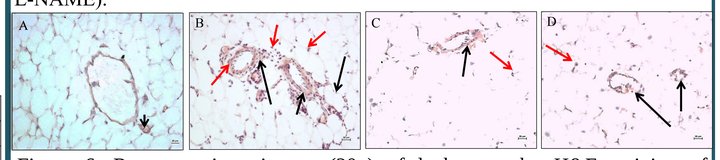

Figure 6. Representative pictures (20x) of leukocytes by H&E staining of mesenteric tissue from Krebs' control (A), L-NAME (B), L-NAME+ 400 µM apocynin (C), L-NAME+ 20 µM Gp91 ds-tat (D). L-NAME treatment exhibited a marked increase in leukocyte vascular adherence and transmigration compared to Krebs' buffer. By contrast, apocynin or Gp91 ds-tat exhibited a marked decrease in adherent and transmigrated leukocytes compared to L-NAME. Black arrow indicates adherence and red arrow indicate transmigration (scale bar: 20 µm).

Conclusions

L-NAME treatment significantly increased leukocyte-endothelial interactions compared to the Krebs' buffer control ($p<0.01$). Apocynin had less influence on basal leukocyte-endothelial interactions. However, apocynin or Gp91 ds-tat dose-dependently attenuated L-NAME induced leukocyte-endothelial interactions with time ($p<0.01$). High dose of apocynin (1000 µM) suggested that apocynin exerted inflammatory responses independent of inhibition of NADPH oxidase. The results suggested that inhibiting the assembly of NAPDH oxidase is an important mechanism to attenuate leukocyte-endothelial interactions induced by endothelial dysfunction. Therefore, NADPH oxidase inhibitors may be beneficial to mitigate the pathogenesis of inflammatory-mediated vascular diseases.

References

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4. Rey FE, Cifuentes ME, Kiarash A, Quinn MT, Pagano PJ. Novel competitive inhibitor of NAD(P)H oxidase assembly attenuates vascular O2 - and systolic blood pressure in mice. *Circulation Research*. 2001;89(5):408-414.