

# Effects of Selective NADPH Oxidase Inhibitor on Real-Time Blood Nitric Oxide and Hydrogen Peroxide Release in Acute Hyperglycemia

M. Bertolet, M. Minni, T. Galbreath, R. Barsotti, L. Young, Q. Chen

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

## Introduction

Hyperglycemia (blood glucose  $\geq 5.5$  mM) and has been clearly linked to the development and progression of long-term microvascular, neurologic, and macrovascular complications in diabetic patients. Moreover, acute hyperglycemia such as during oral glucose tolerance tests and postprandially is also found to quickly and temporarily induce vascular endothelial dysfunction in non-diabetic subjects. Endothelial dysfunction is characterized by decreased endothelium-derived nitric oxide (NO) release and increased reactive oxygen species (ROS), such as superoxide (SO) and hydrogen peroxide ( $H_2O_2$ ). Vascular NO is produced by endothelial NO synthase (eNOS) by converting L-arginine to L-citrulline in the presence of an essential cofactor tetrahydrobiopterin ( $BH_4$ ). NO is a potent vasodilator and facilitates blood flow. It has been proposed that the hyperglycemia induces eNOS uncoupling resulting in a shift from NO to SO production when  $BH_4$  is oxidized to dihydrobiopterin ( $BH_2$ ), which can further initiate inflammation and lead to micro/macro vascular complications. Recently non-phagocytic nicotinamide adenine dinucleotide phosphate (NADPH) oxidase has been highlighted because it may be activated first to initiate the other ROS production sources causing eNOS uncoupling (Figure 1). NADPH oxidase is a multi-subunit enzyme that catalyzes SO production by the reduction of molecular oxygen by using NADPH as an electron donor. In addition to neutrophils, where it was originally discovered, NADPH oxidase is also present in endothelial cells, vascular smooth muscle cells, fibroblasts, and some other cells [1]. The physiological nonphagocytic NADPH oxidase derived SO is normally produced in low concentrations and has been implicated in the regulation of migration, activation, proliferation, vascular tone, and vascular cell growth [1]. Hyperglycemia may stimulate SO production through a Protein Kinase C (PKC)-dependent activation of NADPH oxidase in vascular endothelial cells [2]. The role of NADPH oxidase in acute hyperglycemia induced vascular dysfunction and oxidative stress remains unclear. Our lab recently established a measurement of blood NO and  $H_2O_2$  (i.e. indicator of ROS) in real time in an acutely induced hyperglycemia rat model. In this study, gp91ds-tat and apocynin, two NADPH oxidase inhibitors, will be utilized separately in this hyperglycemia rat model to explore the role of NADPH oxidase in hyperglycemia-induced blood NO/ $H_2O_2$  changes. gp91ds-tat peptide contains a docking sequence which is needed for p47phox binding to gp91phox. By contrast apocynin prevents p47phox binding to p22phox (Figure 2). Our lab found that gp91ds-tat and apocynin was effective in attenuating leukocyte-endothelial interactions in intravital microscopy (see abstract 86389) and showed to be cardioprotective in myocardial ischemia/reperfusion (I/R) injury model (see abstract 86592).

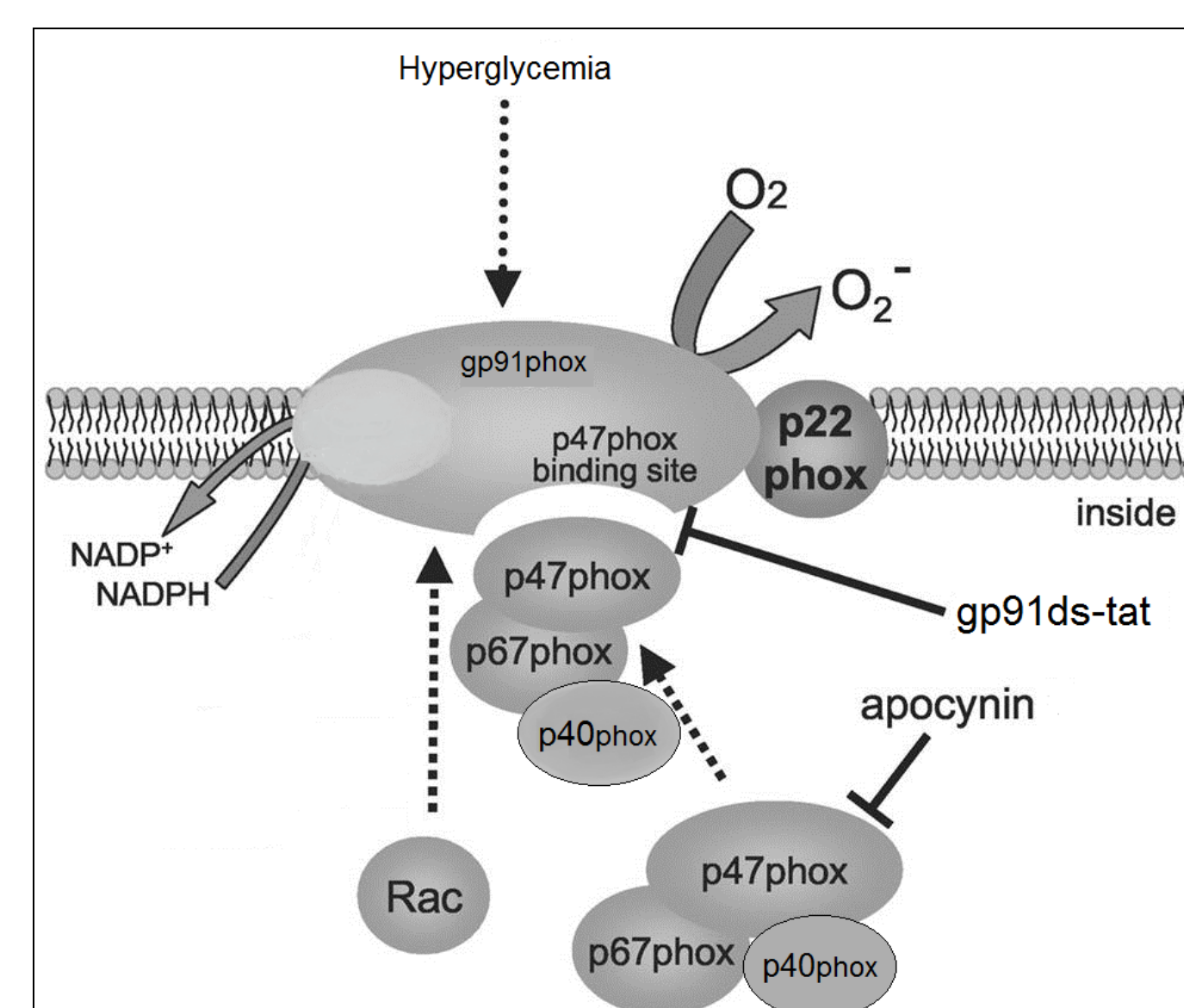


Figure 1. Apocynin and Gp91 ds-tat inhibit the assembly of catalytic subunits of NADPH oxidase. Adapted and modified from Brandes RP 2003 [3].

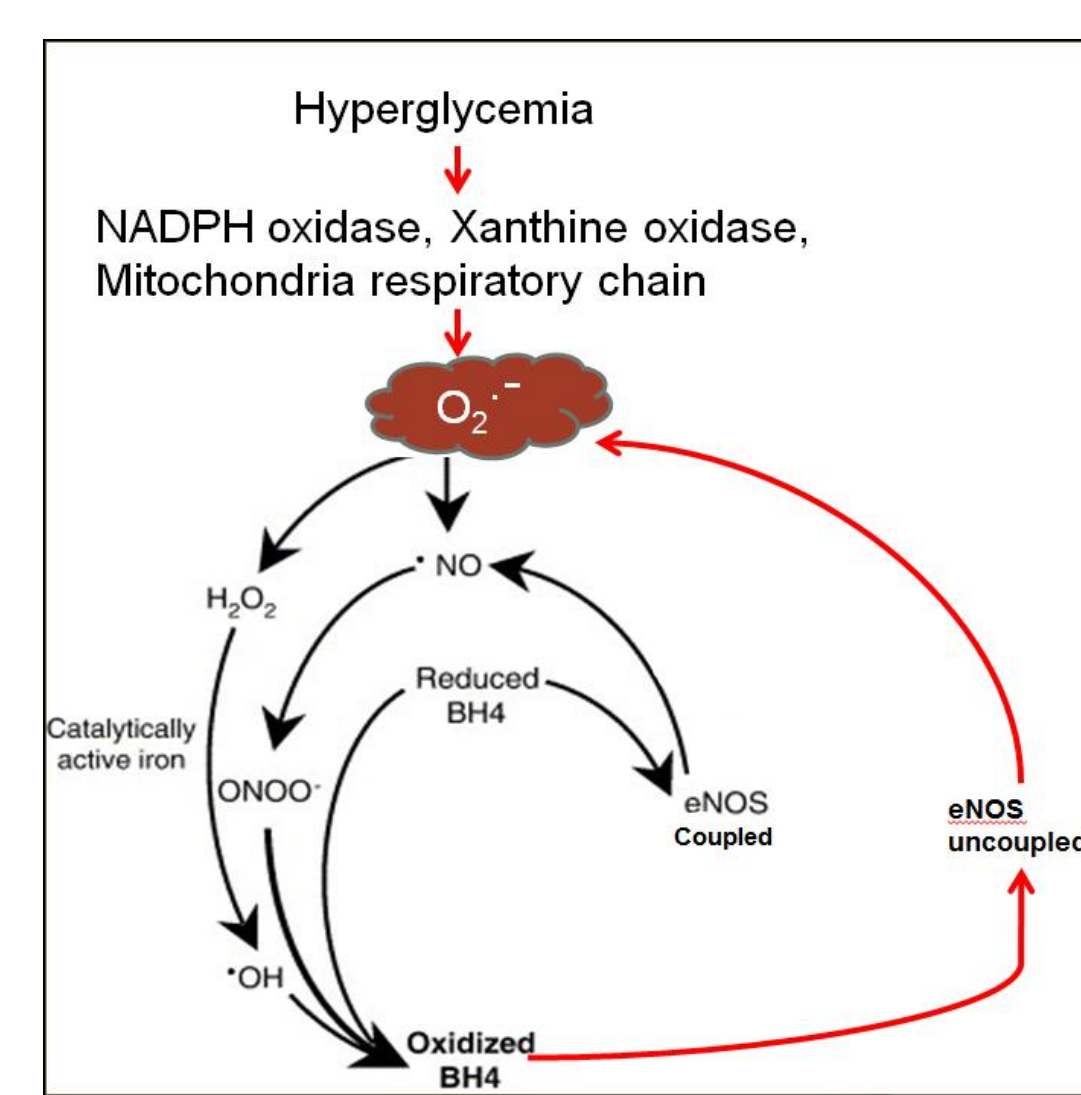


Figure 2. Oxidative stress under hyperglycemia. Modified from Wood et al., 2006. [4]

## Hypothesis

We hypothesized that acute hyperglycemia (200 mg/dL) would increase  $H_2O_2$  and decrease NO release in blood relative to saline control. By contrast, gp91ds-tat (RKKRRQRRR-CSTRIRRL-Amide, MW=2452 g/mol, 1.2 mg/kg, Genemed Synthesis Inc., San Antonio, Tx), a cell-permeable peptide that selectively inhibits NADPH oxidase assembly/activation, would attenuate acute hyperglycemia-induced vascular dysfunction. Furthermore apocynin (MW=166 g/mol, Sigma Chemicals), another type of NADPH oxidase inhibitor, should have similar effects on  $H_2O_2$  and NO blood levels as gp91ds-tat compared to hyperglycemia control, attenuating acute hyperglycemia-induced vascular dysfunction.

## Methods

Male Sprague-Dawley rats (275 to 325g, Charles River, Springfield, MA) were anesthetized with 60 mg/kg of pentobarbital sodium with 1000 unit heparin via intraperitoneal (i.p.) injections. The jugular vein is catheterized in order to infuse intravenously with saline, 20% D-glucose, 20% D-glucose with 1.2 mg/kg gp91ds-tat, or 20% D-glucose with 14 mg/kg apocynin (see figure 3). The continuous infusion of 20% D-glucose solution is to maintain hyperglycemia at 200 mg/dL for about 180 min. gp91ds-tat and apocynin will be added to 20% glucose to reach approximately 20  $\mu$ M and 1 mM in blood, respectively. Both femoral veins will be exposed and catheterized in order to place the calibrated NO and  $H_2O_2$  microsensors (100 $\mu$ m, WPI Inc., Sarasota, FL) at random into each femoral vein (see figure 4). These microsensors will then be connected to the Apollo 4000 free radical analyzer (WPI Inc., Sarasota, FL) to measure for blood NO and  $H_2O_2$  levels in real-time. NO,  $H_2O_2$ , and glucose levels will then be recorded at baseline and every 20 minutes intervals throughout 180 minutes infusion period. The changes of blood NO and  $H_2O_2$  levels will be expressed as the relative change to the baseline. Blood NO and  $H_2O_2$  recording in picoAmps pA will be converted to the concentration (nM for NO and  $\mu$ M for  $H_2O_2$ ) according to the corresponding calibration curve. All the data are represented as a mean  $\pm$  SEM. The data was then analyzed by ANOVA using post hoc analysis with the Student Newman Keuls.  $P < 0.05$  was considered as significant.



Figure 3 – The catheterization of the jugular vein



Figure 4 – Nitric oxide and hydrogen peroxide sensors in the femoral veins.

## Results

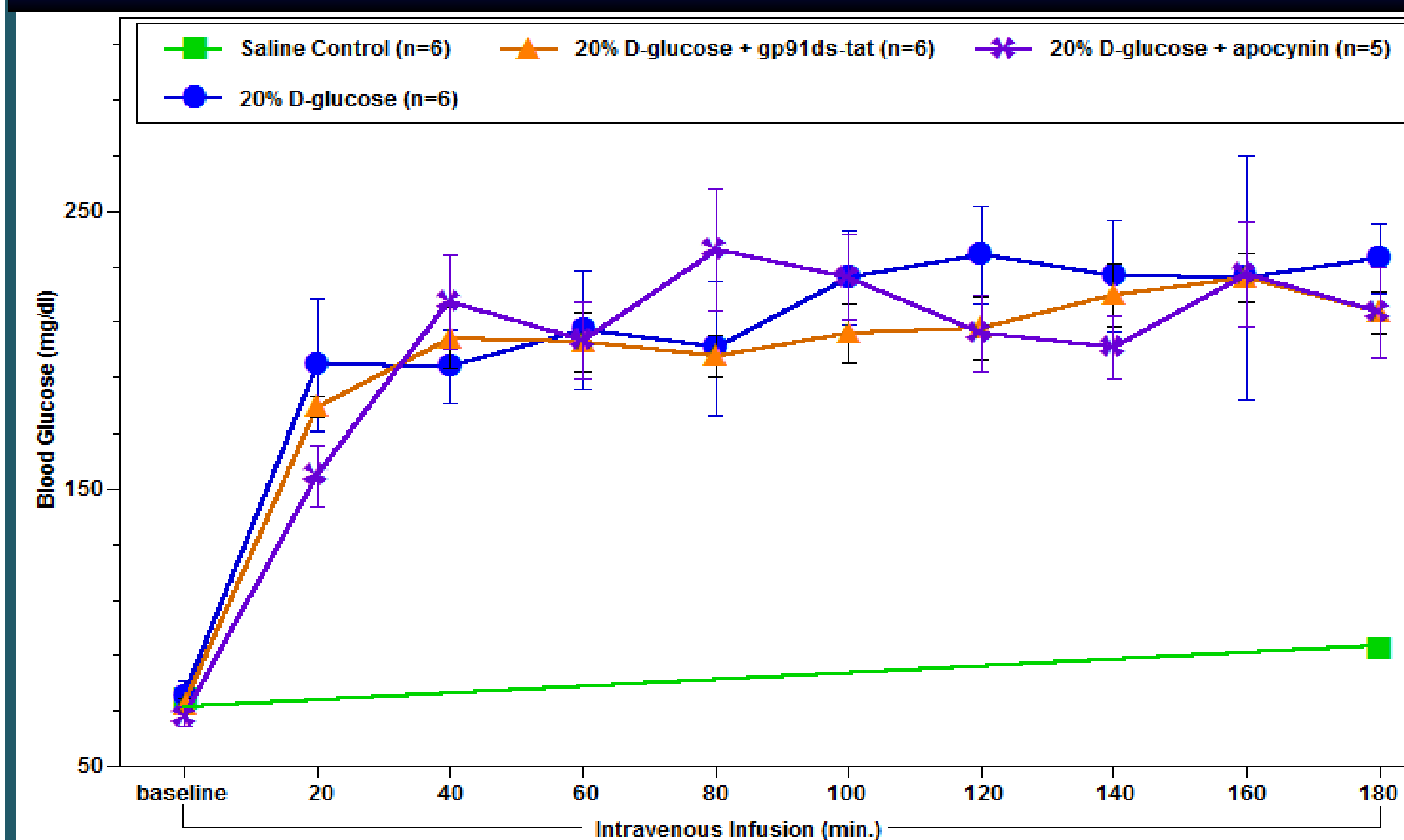


Figure 5. Blood glucose level with time between saline and 20% glucose infusion groups.

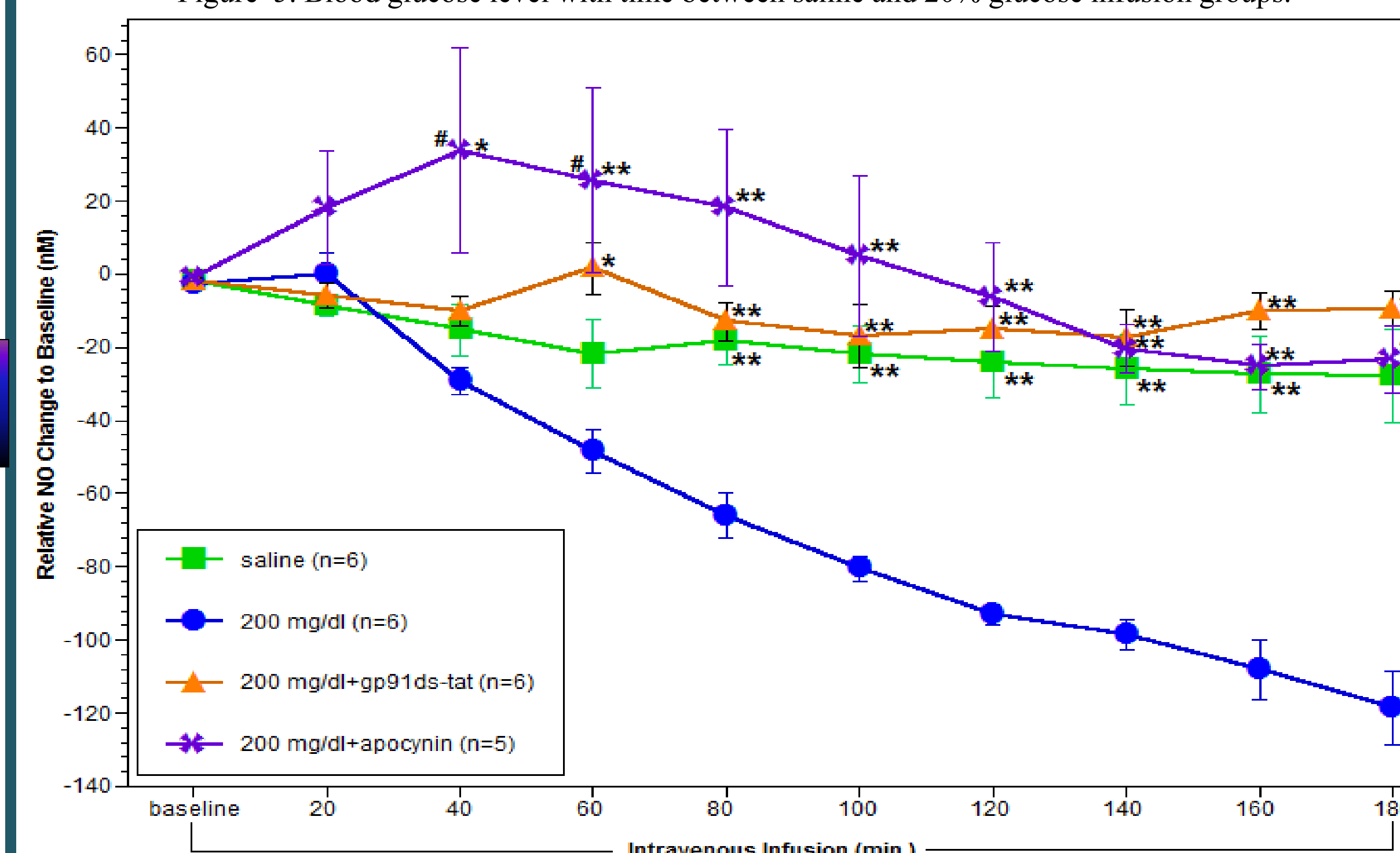


Figure 6. The comparison of blood NO levels relative to baseline among saline, 20% D-glucose, 20% D-glucose with gp91ds-tat, and 20% D-glucose with apocynin (\* $p < 0.05$ , \*\* $p < 0.01$  vs Glucose, #  $p < 0.05$ , ##  $p < 0.01$  vs Saline).

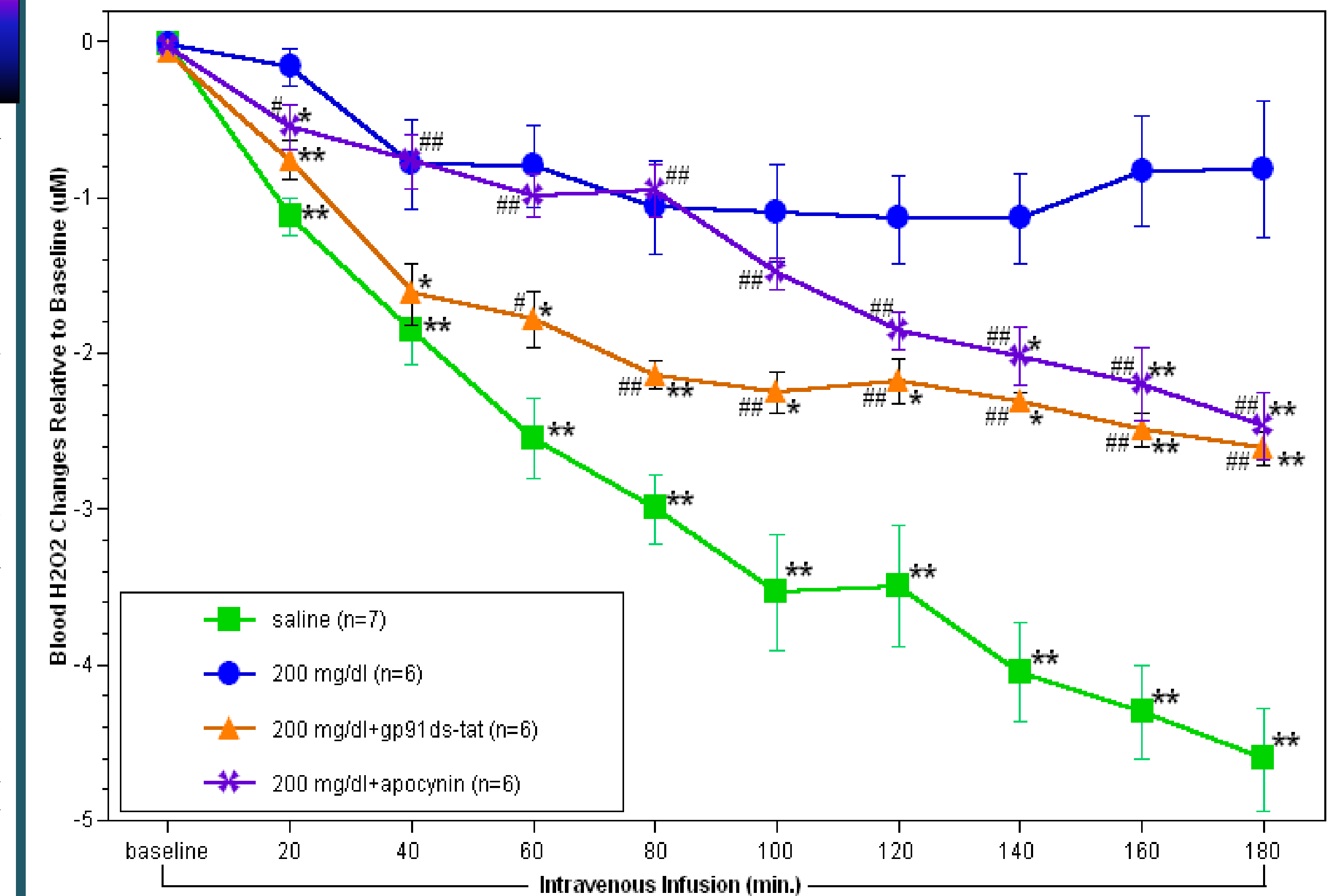


Figure 7. The comparison of blood  $H_2O_2$  levels relative to baseline among saline, 20% D-glucose, 20% D-glucose with gp91ds-tat, and 20% D-glucose with apocynin (\* $p < 0.05$ , \*\* $p < 0.01$  vs Glucose, #  $p < 0.05$ , ##  $p < 0.01$  vs Saline).

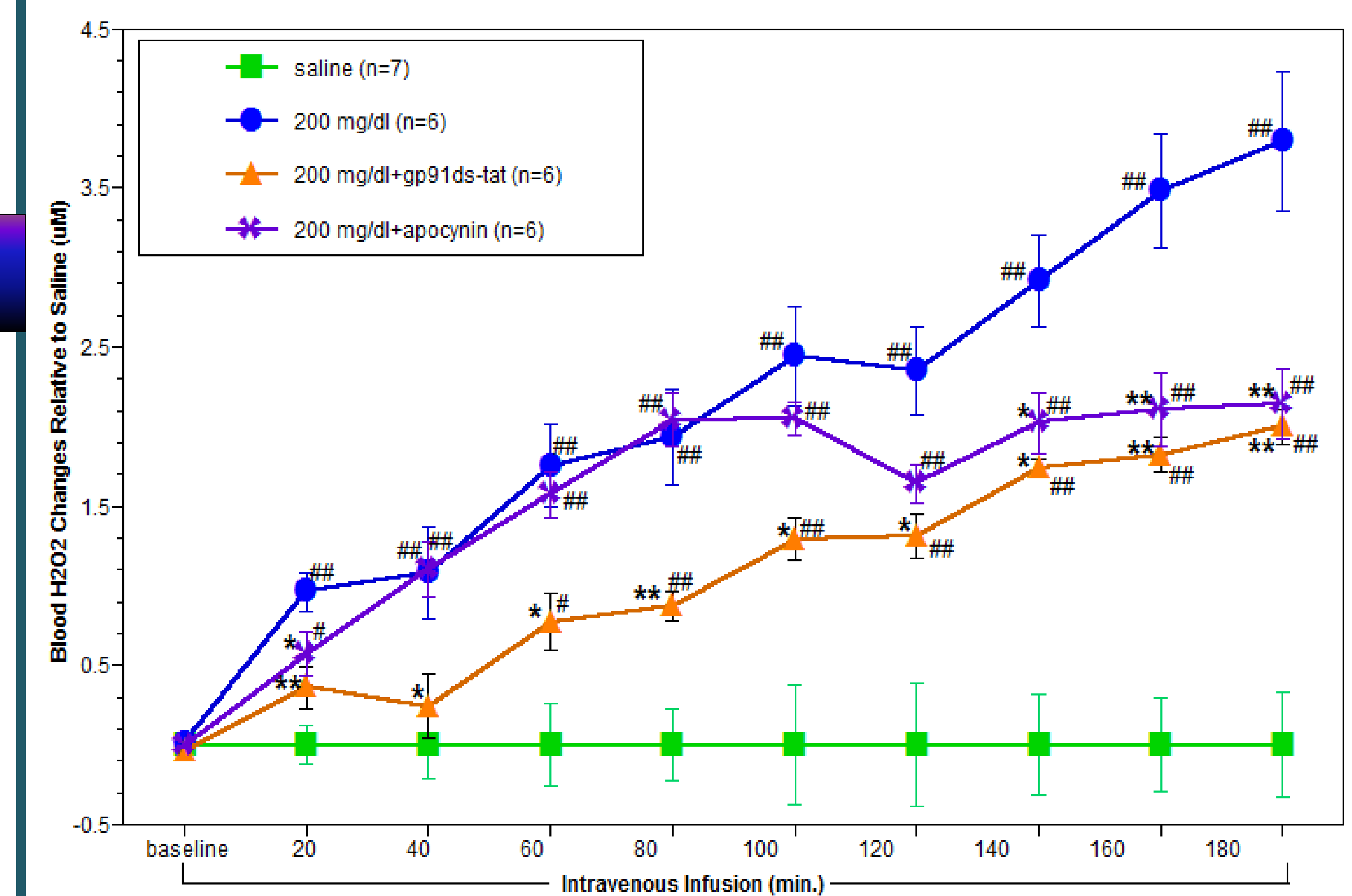


Figure 8. The comparison of blood  $H_2O_2$  levels relative to saline among 20% D-glucose, 20% D-glucose with gp91ds-tat, and 20% D-glucose with apocynin (\* $p < 0.05$ , \*\* $p < 0.01$  vs Glucose, #  $p < 0.05$ , ##  $p < 0.01$  vs Saline).

## Conclusions

We found that acute hyperglycemia significantly reduced blood NO compared to saline control. The addition of gp91ds-tat or apocynin with hyperglycemia significantly improved blood NO levels, similar to saline control. Meanwhile we found acute hyperglycemia maintained a higher level of  $H_2O_2$  in blood compared to saline control. By contrast, gp91ds-tat or apocynin with hyperglycemia reduced blood  $H_2O_2$  levels significantly compared to hyperglycemia. These results suggest that NADPH oxidase is a significant source of ROS overproduction and vascular endothelial dysfunction under acute hyperglycemic conditions, and that supplementation with gp91ds-tat or apocynin may be beneficial to attenuate hyperglycemia induced vascular endothelial dysfunction. Moreover, blood  $H_2O_2$  levels in gp91ds-tat or apocynin treated groups were still significantly higher compared to that in saline groups. This may indicate that other sources of ROS exist such as in mitochondria, which will be further investigated.

## References

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