Cardioprotective Effects of Cell Permeable NADPH oxidase inhibitors in Myocardial Ischemia/Reperfusion (I/R) Injury

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Introduction

In myocardial infarction, heart tissue becomes ischemic and completely deprived of oxygenated blood and its essential nutrients. Blood must quickly be restored to ischemic heart tissue to avoid irreversible cell death. The reperfusion of blood to previously ischemic areas can cause additional heart damage, which is referred to myocardial I/R injury. Reperfusion injury is closely related to an overproduction of reactive oxygen species (ROS) once oxygen is available during reperfusion. ROS can reduce vascular endothelial-derived nitric oxide (NO) bioavailability resulting in reduced flow to ischemic area because NO is a potent vasodilator[1]. ROS can also damage the mitochondria by opening mitochondrial permeability transition pore, leading to a reduction in ATP production and myocyte necrosis/apoptosis[2]. ROS can also damage proteins, lipids, and DNA and disrupt cell integrity. Furthermore, clinical trials suggest that nonselective antioxidants are not effective at attenuating reperfusion injury possibly because they do not specifically target the source of ROS. It has been proposed that NADPH oxidase, xanthine oxidase, uncoupled endothelial NO synthase (eNOS), and mitochondrial dysfunction can serve as ROS sources under I/R conditions. Moreover, it has been shown that an overproduction of superoxide (SO) by NADPH oxidase can cause dysfunctional modifications that can induce all other ROS sources, such as mitochondrial dysfunction and eNOS uncoupling[3]. So far, there are limited effective treatment strategies targeted at limiting reperfusion injury through inhibition of NADPH oxidase. In this study, selective peptide NADPH oxidase inhibitor, gp91 ds-tat[4], and a well-known NADPH oxidase inhibitor, apocynin[5], will be used to determine how such inhibition will effect myocardial I/R injury (see figure 1).

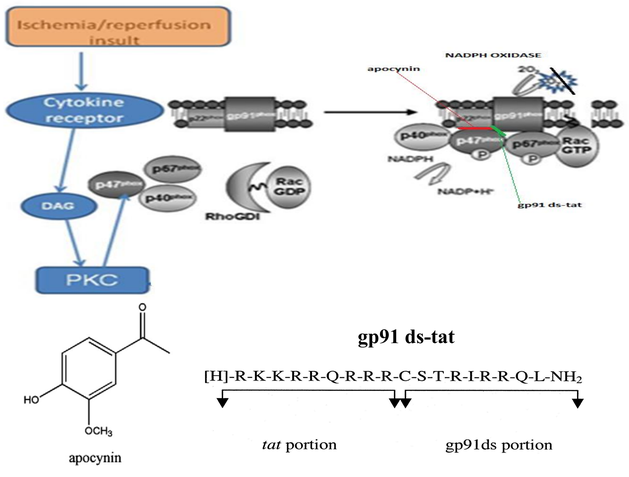

Figure 1. Apocynin and gp91 ds-tat inhibit NADPH oxidase subunit assembly and attenuate SO release under I/R conditions (upper panel). Structures of apocynin[6] and gp91 ds-tat[4] are shown in the bottom. Apocynin inhibits NADPH oxidase by inhibiting $p47^{phox}$ and $p22^{phox}$ assembly after forming diapocynin by peroxidase[5]. By contrast, gp91 ds-tat contains a docking sequence (ds) which prevents NADPH oxidase $p47^{phox}$ and gp91 assembly. The tat portion facilitates the peptide diffusion into the cell[4].

Hypothesis

We hypothesize that reducing ROS formation through inhibition of NADPH oxidase will attenuate myocardial I/R injury by limiting cardiac contractile and diastolic dysfunction associated with reduced infarct size and attenuated SO production in myocytes.

Methods

Isolated Rat Heart Preparation

Male Sprague Dawley (SD) rats (275-325g) were anesthetized intraperitoneally (i.p.) (pentobarbital sodium 60 mg/kg and 1,000U of sodium heparin). Hearts were rapidly excised and perfused with modified Krebs' buffer (in mmol/l: 17.0 dextrose, 120.0 NaCl, 25.0 $NaHCO_3$, 2.5 $CaCl_2$, 0.5 EDTA, 5.9 KCl, and 1.2 $MgCl_2$; maintained at 37°C, 80 mm Hg constant pressure, aerated with 95% O_2-5% CO_2, pH keep at 7.3-7.4) by langendorff preparation. Hearts were subjected to 15 minutes of baseline perfusion, 30 minutes of ischemia, and a 45 minute reperfusion period. 5ml of plasma (control), or plasma containing apocynin (166 g/mol, Sigma Chemicals; 40, 400 and 1000 µM) or gp91 ds-tat (MW=2452 g/mol, Genemed Synthesis Inc. San Antonio TX; 10, 40 and 80 µM) were injected during the first five minutes of reperfusion by a side arm line proximal to the heart inflow at a rate of 1 ml/min. Coronary flow and left ventricular developed pressure (LVDP), which is the left ventricular end-systolic pressure (LVESP) minus left ventricular end-diastolic pressure (LVEDP), the maximal and minimal rate of LVDP ($+dP/dt_{max}$ and $-dP/dt_{min}$), and heart rate were taken every 5 minutes during baseline and reperfusion using a flow meter (T106, Transonic Systems, Inc., Ithaca, NY) and pressure transducer (SPR-524, Millar Instruments, Inc., Houston, TX) respectively. Data was recording using a Powerlab Station acquisition system (ADInstruments, Grand Junction, CO). Sham hearts received no drug and experienced no ischemia. After 45 minutes of reperfusion, the left ventricle was isolated and cross sectioned into three pieces from apex to base. Two pieces were subjected to 1% triphenyltetrazolium chloride (TTC) staining for 15 min at 37°C to detect infarct size (viable stained red, infarct left unstained (white)). The third piece was frozen sectioned (8 µm) and subjected to dihydroethidium (DHE) staining for 2.5 min at room temp to fluoroscopically detect SO release for control and apocynin treated groups. Fluorescence intensity is expressed in arbitrary units and was quantitated by Image J.

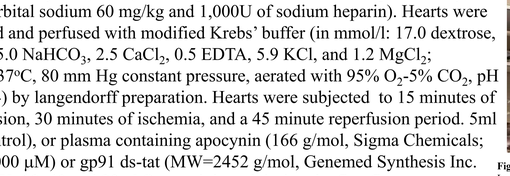

Figure 3. Langerdorff preparation

Statistical Analysis

All data in the text and figures are presented as means ± S.E.M, and analyzed by analysis of variance using post hoc analysis with the Student-Newman-Keuls test for the heart function, infarct size and SO data. Probability values of <0.05 are statistically significant.

Results

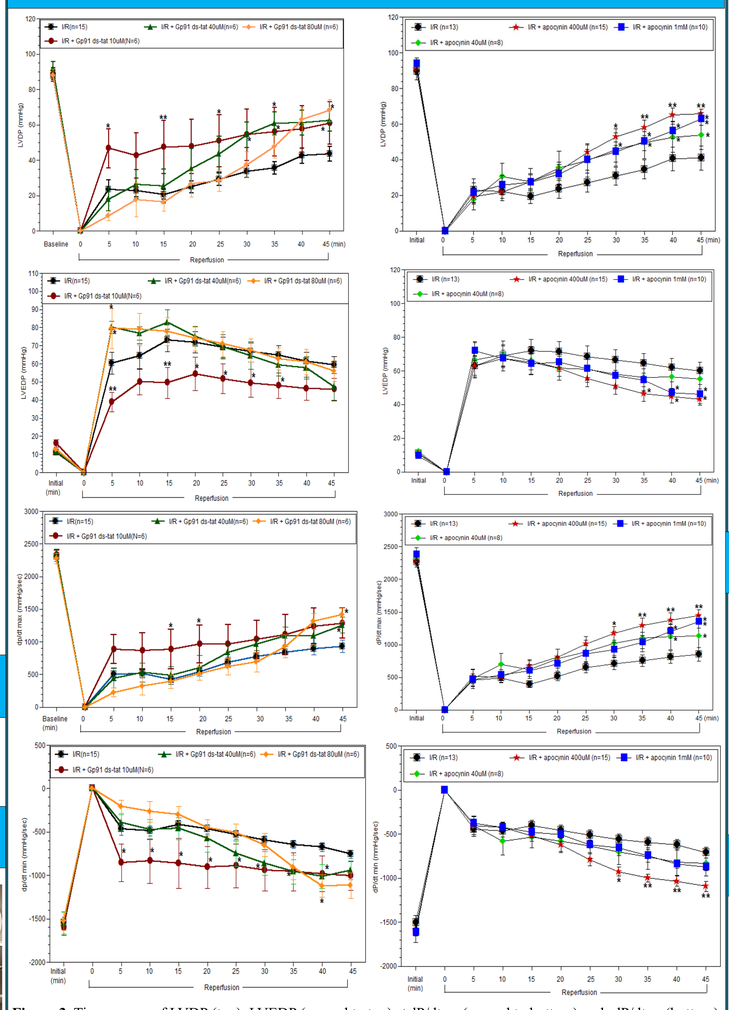

Figure 3. Time course of LVDP (top), LVEDP (second to top), $+dP/dt_{max}$ (second to bottom) and $-dP/dt_{min}$ (bottom) for gp91 ds-tat (left side) and apocynin (right side) treated I/R hearts. I/R hearts exhibited very compromised cardiac function during reperfusion. By contrast, I/R hearts treated with 10 µM gp91 ds-tat had significant restoration for all cardiac function indices. I/R hearts treated with 40 and 80 µM gp91 ds-tat had significant restoration for LVDP and $+dP/dt_{max}$ towards the end of reperfusion. Apocynin (40 µM to 1 mM) significantly restored all cardiac function variables dose-dependently compared to I/R control. *p<0.05,**p<0.01 vs. I/R control.

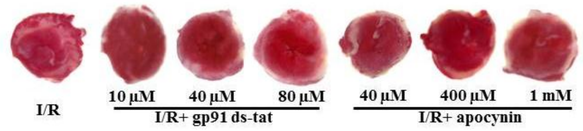

Figure 3. The representative TTC stained heart cross sections from I/R control, gp91 ds-tat or apocycnin treated I/R hearts. Infarct tissue is unstained (white). Viable is stained red.

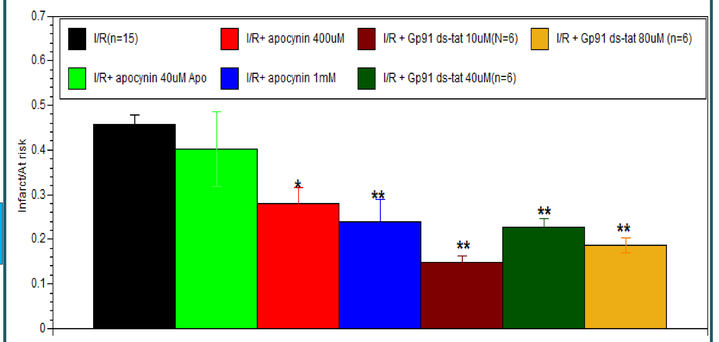

Figure 4. The graph shows the ratio of infarcted tissue weight to the total tissue weight. Apocynin treatment significantly decreased infarct size dose-dependently compared to untreated control myocardial I/R. Similarly, gp91 ds-tat significantly reduced infarct size without a dose-dependent manner compared to untreated control myocardial I/R. (*p<0.05, ** p<0.01).

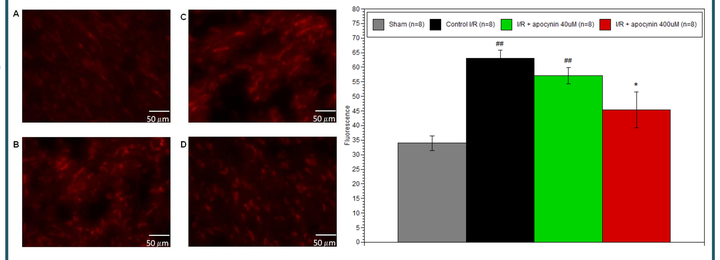

Figure 5. The representative DHE staining images (40X) among sham (A), control I/R (B), I/R+apocynin 40 µM (C), and I/R+apocynin 400 µM (D) (left panel). I/R control and low-dose apocynin (40 µM), exhibited a significant increase in SO release compared to sham hearts (##p<0.01). The graph also shows that apocynin (400 µM) significantly reduced SO release dose-dependently compared to control I/R hearts (*p<0.05).

Conclusions

This study showed that both NADPH oxidase inhibitors, gp91 ds-tat and apocynin significantly improved post-reperfused cardiac function associated with reduction of infarct size. When given at reperfusion, apocynin exerted the cardioprotective effects dose-dependently associated with decreased myocyte SO release. The lag time during reperfusion may be due to apocynin conversion to diapocynin by tissue peroxidase in order to inhibit NADPH oxidase assembly. By contrast, all three gp91 ds-tat concentrations significantly restored post-reperfused cardiac function and reduced infarct size, suggesting that these effects are not dose-dependent in this concentration range (i.e. 10-80 µM). This study indicates that NADPH oxidase, especially in vascular endothelium and myocytes, is a significant source of ROS in myocardial I/R injury. Therefore, both NADPH oxidase inhibitors may be potential agents to reduce SO production and mitigate reperfusion induced heart damage.

References

1. Young LH, Balin BJ, Weis MT. Gö 6983: A fast acting protein kinase C inhibitor that attenuates myocardial ischemia/reperfusion injury. Cardiovasc Drug Rev. 2005;23(3):255-72.
2. Szeto HH. Mitochondria-targeted peptide antioxidants: Novel neuroprotective agents. AAPS Journal. 2006;8(3):521-31.
3. Schramm A, Matusik P, Osmenda G, Guzik TJ. Targeting NADPH oxidases in vascular pharmacology. Vascul Pharmacol. 2012;56(5-6):216-31.
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. Circ Res. 2001;89(5):408-14.
5. Mora-Pale M, Joon Kwon S, Linhardt RJ, Dordick JS. Trimer hydroxylated quinone derived from apocynin targets cysteine residues of p47 phox preventing the activation of human vascular NADPH oxidase. Free Radic Biol Med. 2012;52(5):962-9.
6. Petrônio MS, Zeraik ML, Da Fonseca LM, Ximenes VF. Apocynin: Chemical and biophysical properties of a NADPH oxidase inhibitor. Molecules. 2013;18(3):2821-39.