

Apocynin Exerts Dose-Dependent Cardioprotective Effects by Attenuating Reactive Oxygen Species in Ischemia/Reperfusion

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Abstract

Ischemia/reperfusion results in cardiac contractile dysfunction and cell death partly due to increased reactive oxygen species and decreased endothelial-derived nitric oxide bioavailability. NADPH oxidase normally produces reactive oxygen species to facilitate cell signalling and differentiation; however, excessive release of such species following ischemia exacerbates cell death. Thus, administration of an NADPH oxidase inhibitor, apocynin, may preserve cardiac function and reduce infarct size following ischemia. Apocynin dose-dependently (40 μ M, 400 μ M and 1 mM) attenuated leukocyte superoxide release by $87 \pm 7\%$. Apocynin was also given to isolated perfused hearts after ischemia, with infarct size decreasing to $39 \pm 7\%$ (40 μ M), $28 \pm 4\%$ (400 μ M; $p < 0.01$) and $29 \pm 6\%$ (1 mM; $p < 0.01$), versus the control's $46 \pm 2\%$. This decrease correlated with improved final post-reperfusion left ventricular end-diastolic pressure, which decreased from $60 \pm 5\%$ in control hearts to $56 \pm 5\%$ (40 μ M), $43 \pm 4\%$ (400 μ M; $p < 0.01$) and $48 \pm 5\%$ (1 mM; $p < 0.05$), compared to baseline. Functionally, apocynin (13.7 mg/kg, I.V.) significantly reduced H_2O_2 by nearly four-fold and increased endothelial-derived nitric oxide bioavailability by nearly four-fold during reperfusion compared to controls ($p < 0.01$), which was confirmed in *in vivo* rat hind limb ischemia/reperfusion models. These results suggest that apocynin attenuates ischemia/reperfusion-induced cardiac contractile dysfunction and infarct size by inhibiting reactive oxygen species release from NADPH oxidase.

Keywords: NADPH oxidase; Apocynin; Myocardial ischemia/reperfusion; Hind limb ischemia/reperfusion; Nitric oxide; Hydrogen peroxide; Leukocyte superoxide release

Introduction

Coronary heart disease is the leading cause of death worldwide [1]. The most effective therapeutic intervention for reducing infarct size associated with myocardial ischemic injury is timely and effective reperfusion of blood flow back to the heart tissue by percutaneous coronary intervention. However, the reperfusion of blood itself can induce additional cardiomyocyte death that can account for up to 50% of the final infarct size [2]. Remarkably, after several decades of preclinical and clinical studies, there is still no effective therapy to limit reperfusion-induced tissue death and improve post-reperfusion cardiac function. Consequently, there is a great need to identify the mechanism(s) responsible for myocardial ischemia/reperfusion injury.

Ischemia/reperfusion injury is initiated in part by endothelial dysfunction, which occurs within 5 min of reperfusion. It is characterized by an increase in reactive oxygen species leading to endothelial nitric oxide synthase uncoupling, which in turn causes more reactive oxygen species production and limited endothelial-derived Nitric Oxide (NO) bioavailability [2-4]. The reduced NO bioavailability leads to compromised blood flow during reperfusion and increased leukocyte-endothelial interactions, which will exacerbate tissue damage. The time course of endothelial dysfunction in myocardial ischemia/reperfusion is similar to other vascular beds, such as hind limb ischemia/reperfusion and splanchnic ischemia/reperfusion [5,6]. The significance of this commonality is that compounds that attenuate endothelial dysfunction in the heart could be applied to other vascular beds such as pulmonary, kidney, liver, mesenteric and limb circulation. Therefore, this could be clinically applied to prevent organ damage in various ischemia/reperfusion injury settings, such as organ transplantation, circulatory shock, prolonged tourniquet use and coronary bypass/balloon angioplasty.

In order to understand endothelial dysfunction during ischemia/

reperfusion, we established an *in vivo* rat hindlimb ischemia/reperfusion injury model that directly measures reactive oxygen species levels in blood (i.e., hydrogen peroxide [H_2O_2]) and NO in real-time [4,7,8] from both femoral veins (with one limb subjected to ischemia/reperfusion and the other serving as a sham control). H_2O_2 is an indirect index of superoxide (SO) release because SO dismutase rapidly, within seconds, converts SO to H_2O_2 ; this process is enhanced under low pH conditions [9,10]. Our hind limb ischemia/reperfusion injury model is appropriate to measure H_2O_2 and NO blood levels during ischemia/reperfusion because the direct measurements are not feasible in the coronary circulation of the isolated perfused rat heart model. We found that an inverse relationship exists between blood H_2O_2 and NO levels in ischemia/reperfusion. Greater production of H_2O_2 and reduction of NO during reperfusion in hind limb ischemia/reperfusion closely correlates with a greater degree of post-reperfusion cardiac dysfunction in myocardial ischemia/reperfusion [4,7,8]. Therefore, quantifying blood reactive oxygen species release and NO levels in real-time during reperfusion is important to determine the initiating sources of reactive oxygen species in ischemia/reperfusion and to develop potential treatment strategies for ameliorating reperfusion injury.

Although clinical trials suggest non-selective antioxidants do not effectively attenuate reperfusion injury, this may be due to lack of

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specifically targeting the source of oxidative stress. Four enzymatic sources principally contribute to oxidative stress (i.e., reactive oxygen species) during reperfusion: NADPH oxidase, xanthine oxidase, uncoupled eNOS, and the mitochondrial electron transport chain. NADPH oxidases comprise a family of enzymes that initiates and recruits the other three sources as follows [11]: 1. NADPH oxidase 2 (Nox2) naturally produces SO/H_2O_2 upon the assembly of the cytosolic subunits (i.e., rac, p67^{phox}, p47^{phox}, and p40^{phox}) with the membrane subunits (i.e., p22^{phox} and gp91^{phox}); 2. It is expressed in many cells in the heart, such as vascular endothelium and cardiac myocytes, and is the principle source of SO release in leukocytes; 3. SO/H_2O_2 release from activated NADPH oxidase can open mitochondrial ATP-dependent potassium channels and/or mitochondria permeability transition pores, which further lead to mitochondrial matrix alkalization, mitochondrial electron transport chain uncoupling, and SO production [12]; 4. Overproduction of SO/H_2O_2 will also promote the transformation of xanthine dehydrogenase into xanthine oxidase [13,14]; 5. SO can quench NO to form peroxynitrite anion, which in turn can promote eNOS uncoupling via the oxidation of the essential eNOS co-factor tetrahydrobiopterin (BH_4) to dihydrobiopterin (BH_2) [15]. It has been found *in vitro* that the increased ratio of BH_2 to BH_4 facilitates BH_2 binding at the eNOS oxygenase domain, allowing eNOS to use molecular oxygen to produce SO and, subsequently, H_2O_2 in blood [16]. 6. Reactive oxygen species release during reperfusion serves as a key initiating factor that triggers the release of a major inflammatory cytokine, tumor necrosis factor, alpha (TNF α), within the first 15 min of reperfusion [12,17-20] (Figure 1).

Consequently, inhibition of Nox2 may be an effective strategy to prevent the downstream sequelae of reperfusion injury and salvage heart tissue during myocardial infarction or organ transplantation. Previous studies show apocynin, an alkaloid derived from *Apocynum cannabinum*, is a selective Nox2 assembly inhibitor that may oxidize

the thiols in NADPH oxidase. Moreover, apocynin may be a prodrug and thus needs to be activated by peroxidase-mediated oxidation to diapocynin, its active oxidizing form, which is illustrated in Figure 2. Specifically, the effects of apocynin oxidize the cysteine-196 position of p47^{phox}, a cytosolic component of NADPH oxidase that is activated via phosphorylation by protein kinase C, which, in turn, inhibits the association of the p22^{phox} membrane subunit component of NADPH oxidase and, therefore inhibit Nox2 assembly and subsequent SO release, thereby decreased oxidative stress and tissue damage [21,22].

We hypothesize that administration of selective NADPH oxidase inhibitors, such as apocynin, will dose-dependently attenuate blood H_2O_2 and augment NO bioavailability in hindlimb ischemia/reperfusion, and will decrease SO release from leukocytes and reduce infarct size/area-at-risk in ischemia/reperfusion hearts, and thus improving post-reperfused cardiac function.

Methods

Animals

All animal procedures were approved by the Institutional Animal Care and Use Committee at PCOM for care and use of animals. Male Sprague Dawley (SD) rats (275-325 g; Ace Animals, Boyertown, PA)

Measurement of SO release from rat PMNs

The SO release from Poly Morphonuclear Leukocytes (PMNs) was measured spectrophotometrically (model 260 Gilford, Nova Biotech; El Cajon, CA) by the reduction of ferricytochrome c as previously described [23]. The PMNs (5×10^6) were resuspended in 450 μ l PBS and incubated with ferricytochrome c (100 μ M, Sigma Chemical) in a total volume of 900 μ l PBS in the presence or absence of varying concentrations of apocynin (MW = 166 g/mol; Sigma

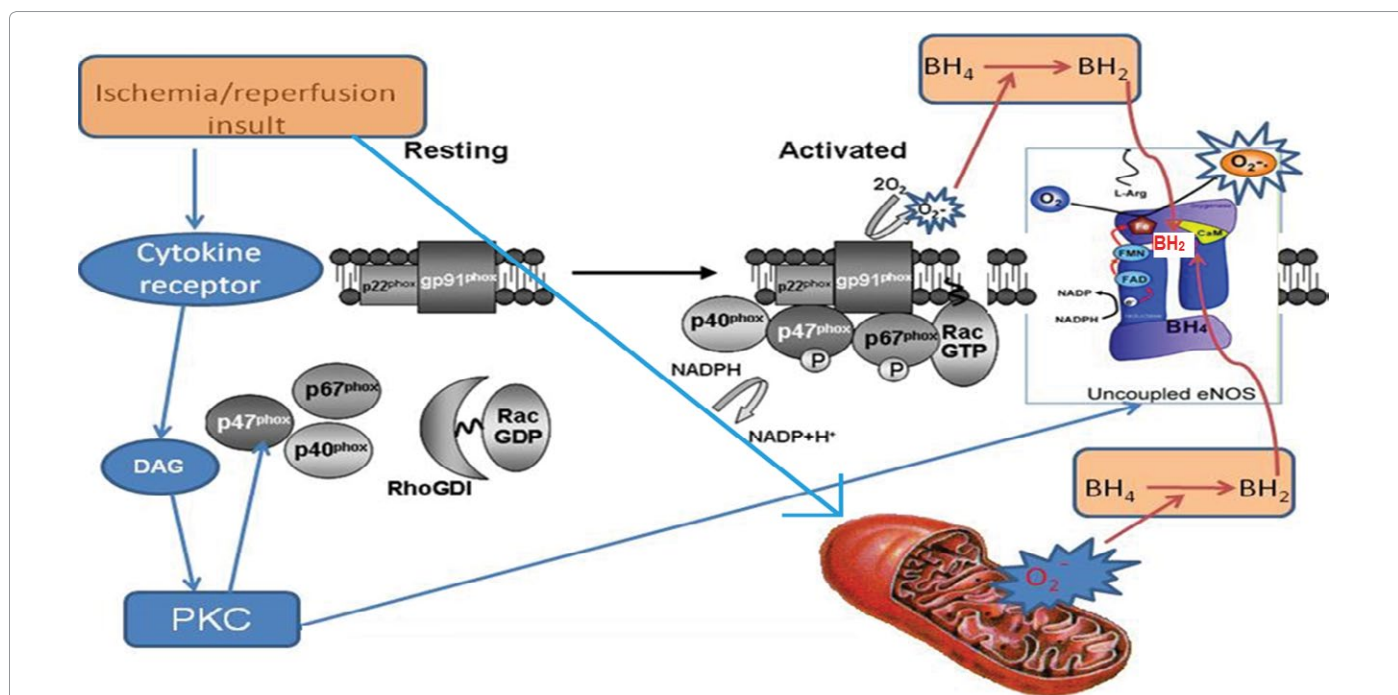


Figure 1: Ischemia/reperfusion insults leads to mitochondria damage releasing O_2^- to initiate the oxidation process of BH_4 to BH_2 causing the uncoupling of eNOS. Meanwhile cytokine mediated receptor stimulation activates protein kinase C which in turn phosphorylates NADPH and eNOS to produce O_2^- that also leads to the increase of oxidation of BH_4 to BH_2 causing more uncoupling of eNOS. Collectively these sources of reactive oxygen species reduce the amount of nitric oxide available. Modified from Wilkinson et al. and Schmidt and Alp [15,38].

Chemical), 40 μM , 400 μM , or 1 mM, respectively, for 15 min at 37°C in spectrophotometric cells. The PMNs were stimulated with 1 μM N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP, MW = 437 g/mol; Calbiochem) in a final reaction volume of 1.0 ml. Absorbance at 550 nm was measured every 30 sec for up to 90 sec for fMLP (peak response), and the change in absorbance (SO release) from PMNs was determined relative to time 0. The dose-response curve of apocynin on PMN SO release was used to determine a dose range that may be effective in myocardial ischemia/reperfusion experiments.

Isolated rat heart preparation

SD rats were injected with pentobarbital sodium 60 mg/kg and sodium heparin (1,000 U) Intra peritoneally (I.P.); after which the hearts were rapidly excised. The plasma was collected from the abdominal aorta of the same rat from which the heart was isolated for each cardiac perfusion experiment in order to be used infusion at the beginning of reperfusion [24]. The isolated rat heart was cannulated via the aorta onto a perfusion needle and immersed in a water-jacketed reservoir that contained 160 ml of Krebs' buffer. The hearts were perfused with a 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 at a constant pressure of 80 mmHg and aerated with 95% O_2 -5% CO_2 to keep the pH at 7.3-7.4. A side arm in the perfusion line proximal to the heart inflow cannula allowed infusion of plasma (control hearts) or plasma containing apocynin (40 μM , 400 μM and 1 mM). Coronary flow was monitored by a flowmeter (T106, Transonic Systems, Inc., Ithaca, NY). Left ventricular developed pressure, which is the left ventricular end-systolic pressure minus left ventricular end-diastolic pressure, the maximal and minimal rate of left ventricular developed pressure (+ $\text{dP}/\text{dt}_{\text{max}}$ and - $\text{dP}/\text{dt}_{\text{min}}$, respectively) and heart rate were monitored using a pressure transducer (SPR-524, Millar Instruments, Inc., Houston, TX) positioned in the left ventricular cavity and recorded using a Powerlab Station acquisition system (ADInstruments, Grand Junction, CO). Coronary flow, left ventricular developed pressure, + $\text{dP}/\text{dt}_{\text{max}}$ - $\text{dP}/\text{dt}_{\text{min}}$ and heart rate were measured every 5 min for 15 min to obtain baseline measurements. After baseline, global ischemia was induced for 30 min by stopping the flow of Krebs' buffer. Hearts were infused at the beginning of reperfusion with plasma or plasma containing apocynin at a rate of 1 ml/min for 5 min. Data continued to be recorded every 5 min for the 45 min reperfusion. At the end of the experiment, the infarct area and area-at-risk (i.e. entire heart) was visualized and measured by treating the tissue with 1% 2,3,5-triphenyltetazolium (TTC), as previously described [25].

Hind limb ischemia/reperfusion *in vivo* procedure

We measured blood H_2O_2 /NO release in real-time from sensors placed in the femoral veins of male SD rats anesthetized with sodium pentobarbital (60 mg/kg, I.P. for induction and 30 mg/kg IP for maintenance). Thereafter, one limb was subjected to ischemia/reperfusion, while the other limb was used as a non-ischemic sham control in the same rat. The H_2O_2 or NO microsensors (100 μm , World Precision Instruments [WPI] Inc., Sarasota, FL) were connected to a free radical analyzer (Apollo 4000, WPI Inc.) and inserted into a 24 gauge catheter placed inside each femoral vein, as previously described by Perkins et al. [8] and reviewed by Young et al. [26]. Ischemia in one hind limb was induced by clamping the femoral artery/vein for 20 min followed by 45 min of reperfusion. Apocynin (13.7 mg/kg prepared in saline; ~ 1 mM in blood) or saline (non-drug control group) was administered at the beginning of reperfusion as a bolus via the jugular vein. We continuously recorded and collected the H_2O_2 or NO release data at 5 min intervals during a 15 min baseline, 20 min

ischemia and 45 min reperfusion. The changes in H_2O_2 or NO release (in pA) during ischemia/reperfusion are expressed as relative change to baseline (initial) readings. Thereafter, the values were converted to the concentration of H_2O_2 (μM) or NO (nM) after correction to the respective calibration curves. The values recorded from the femoral vein of the ischemia/reperfusion limb were subtracted from the values of the sham limb to yield a net difference and were recorded every 5 min continuously on time course graphs.

Statistical analysis

All data in the text and figures are presented as means \pm S.E.M. The data were analyzed by analysis of variance using post hoc analysis with the Student-Newman-Keuls test for PMN SO release and cardiac function/infarct size/area-at-risk and Student's t-test for the hindlimb ischemia/reperfusion data. Probability values of ≤ 0.05 are considered to be statistically significant.

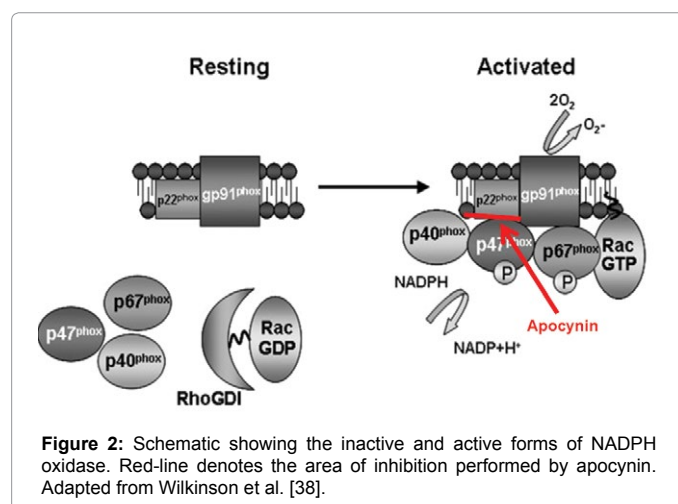
Results

Apocynin dose-dependently decreased fMLP-induced PMN SO release

PMNs release SO principally by activating NADPH oxidase. fMLP peptide activates PMN chemotactic receptor to generate the second messengers Ca^{++} and DAG that activates protein kinase C isoforms to phosphorylate serine residues on p47^{phox} to promote NADPH oxidase assembly at the PMN membrane to generate SO release (Figure 1). We found that apocynin exhibited dose-dependent reduction in fMLP-stimulated PMN SO release (Figure 3). Apocynin at 40 μM had no effect on PMN SO, whereas 400 μM and 1 mM significantly inhibited this response by 64 ± 7 and $87 \pm 7\%$, respectively. The data generated from this assay was used to test a similar dose range of apocynin in the isolated rat heart ischemia/reperfusion experiments.

Apocynin attenuated post-reperfused cardiac dysfunction and infarct size and area-at-risk

Apocynin given at reperfusion significantly does-dependently attenuated ischemia/reperfusion induced cardiac contractile dysfunction and improved post-reperfused left ventricular developed pressure to $60 \pm 6\%$ (40 μM ; $p < 0.05$) and $74 \pm 3.6\%$ (400 μM ; $p < 0.01$) and $66 \pm 3.6\%$ (1 mM; $p < 0.01$) of baseline at 45 min post-reperfusion compared to control ischemia/reperfusion hearts that only recovered to $47 \pm 5\%$ of baseline values for left ventricular developed pressure (Table 1). Apocynin (400 μM and 1 mM) significantly reduced post-



Cardiac Parameters	Control I/R (n = 13)	I/R + apocynin 40 μ M (n = 9)	I/R + apocynin 400 μ M (n = 15)	I/R + apocynin 1 mM (n = 10)
Initial LVDP (mmHg)	87 \pm 3.7	92 \pm 3.1	92 \pm 3.1	94 \pm 3.4
Final LVDP (mmHg)	41 \pm 4.6	55 \pm 6*	68 \pm 3.4**	62 \pm 3.4**
Initial LVESP (mmHg)	98 \pm 3.9	105 \pm 3.2	103 \pm 3.5	104 \pm 3.8
Final LVESP (mmHg)	101 \pm 5.8	111 \pm 6.4	111 \pm 4	111 \pm 4.6
Initial LVEDP (mmHg)	11 \pm 0.6	14 \pm 0.7	11 \pm 0.8	10 \pm 0.9
Final LVEDP (mmHg)	60 \pm 5.2	56 \pm 4.8	43 \pm 3.8**	48 \pm 4.9*
Initial + dP/dt _{max} (mmHg/sec)	2336 \pm 73	2331 \pm 56	2284 \pm 69	2385 \pm 101
Final + dP/dt _{max} (mmHg/sec)	882 \pm 104	1121 \pm 135	1446 \pm 89**	1342 \pm 95**
Initial dP/dt _{min} (mmHg/sec)	-1554 \pm 72	-1585 \pm 45	-1585 \pm 68	-1617 \pm 112
Final dP/dt _{min} (mmHg/sec)	-745 \pm 57	-793 \pm 99	-1091 \pm 58**	-928 \pm 45
Initial coronary flow (ml/min)	16.5 \pm 1.3	19.1 \pm 0.9	21 \pm 1.3	17 \pm 1.5
Final coronary flow (ml/min)	7.8 \pm 0.57	10.5 \pm 1.1	11.8 \pm 1.4	8.9 \pm 0.84
Initial Heart Rate (BPM)	279 \pm 14.3	283 \pm 16.5	273 \pm 6.7	273 \pm 11.1
Final Heart Rate (BPM)	239 \pm 16.9	259 \pm 13.1	245 \pm 8.5	239 \pm 9.6

*p < 0.05, **p < 0.01 versus control I/R final cardiac function parameters

I/R = Ischemia/Reperfusion

LVDP = Left Ventricular Developed Pressure

LVESP = Left Ventricular End-Systolic Pressure

LVEDP = Left Ventricular End-Diastolic Pressure

Table 1: Cardiac function parameters among different experimental groups.

reperfused left ventricular end-diastolic pressure compared to control ischemia/reperfusion values by 17 \pm 3.8 (p < 0.01) and 12 \pm 4.9 mmHg (p < 0.05). Whereas, apocynin 40 μ M did not significantly affect this parameter compared to ischemia/reperfusion controls (Table 1). Similarly, apocynin exhibited a dose-dependent effect on reducing infarct size/area-at-risk, as illustrated in Figure 4.

Other cardiac function parameters are illustrated in Table 1. Of interest, dose-dependent effects of apocynin were also observed for + dP/dt_{max}. Specifically, apocynin improved post-reperfused + dP/dt_{max} by 48 \pm 6% (40 μ M), 63 \pm 4% (400 μ M; p < 0.01), and 56 \pm 4% (1 mM; p < 0.01) at 45 min reperfusion compared to control ischemia/reperfusion hearts that only recovered to 38 \pm 4%. Sham hearts (n = 6) were not subjected to ischemia/reperfusion and maintained cardiac function parameters and flow by > 90% and had < 5% infarct size/area-at-risk throughout the experimental time-course (data not shown).

Apocynin reduced blood H₂O₂ levels

Apocynin given I.V. (13.7 mg/kg) at the beginning of reperfusion decreased H₂O₂ in hind limb ischemia/reperfusion models compared to saline controls (Figure 5). Specifically, in saline control trials, H₂O₂ peaked up to 2.5 μ M during the first 5 min of reperfusion. Thereafter, the levels of H₂O₂ dropped to 1 μ M in blood, a concentration that was maintained throughout the reperfusion period. By contrast, apocynin-treated animals H₂O₂ had an increase to nearly 5 μ M during the first five minutes of reperfusion; however, the level decreased, up to 4-fold, compared to saline control trials throughout the experimental time course. Of note, the decreased H₂O₂ levels were significantly different compared to saline controls between 20 and 45 min.

Apocynin increased blood NO levels

Apocynin given I.V. (13.7 mg/kg) at the beginning of reperfusion increased NO in hind limb ischemia/reperfusion models compared to saline controls (Figure 6). Blood NO levels in saline controls a brief transiently increased to ~ 30 nM during the first 5 min of reperfusion; however, the levels decreased to approximately 50 nM, compared to baseline, at the end of the experimental time course. Conversely, apocynin-treated animals' blood NO levels significantly increased to ~ 180 nM, as compared to baseline, during the first 5 min of

reperfusion. These levels quickly dropped to ~ 50 nM between 10 and 20 min post-reperfusion, but at 20 min post-reperfusion began to significantly rise to ~ 130 nM throughout the remainder of the reperfusion time period (p < 0.05 at 20 min and p < 0.01 from 25-45 min).

Discussion

Summary of findings

The major findings of this study are as follows: 1. Apocynin dose-dependently attenuated fMLP-induced leukocyte SO release by 64 \pm 7% and 87 \pm 7% at 400 μ M and 1mM, compared to untreated controls (p < 0.01); 2. In isolated perfused hearts, apocynin attenuated infarct size/area-at-risk by 39 \pm 7% (40 μ M), 28 \pm 4% (400 μ M; p < 0.01) and 29 \pm 6% (1 mM; p < 0.01), compared to control ischemia/reperfusion hearts that had an infarct size/area-at-risk of 46 \pm 2%; 3. Apocynin decreased left ventricular end-diastolic pressure to 56 \pm 5% (40 μ M), 43 \pm 4% (400 μ M; p < 0.01) and 48 \pm 5% (1 mM; p < 0.05) compared

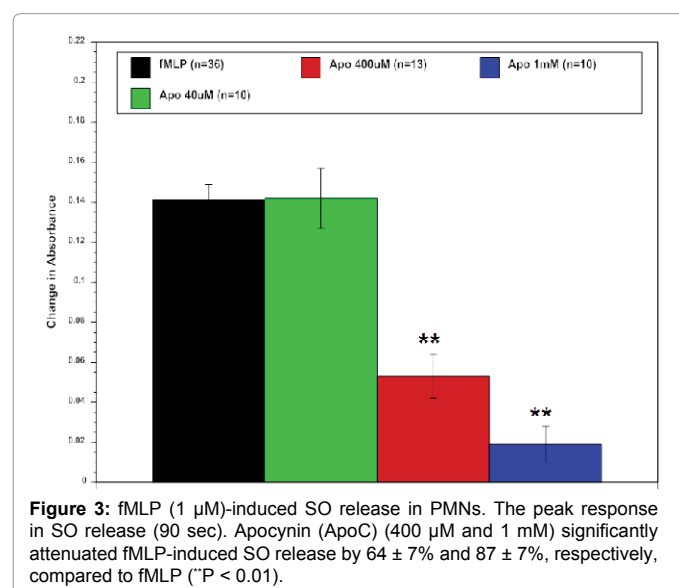


Figure 3: fMLP (1 μ M)-induced SO release in PMNs. The peak response in SO release (90 sec). Apocynin (ApoC) (400 μ M and 1 mM) significantly attenuated fMLP-induced SO release by 64 \pm 7% and 87 \pm 7%, respectively, compared to fMLP (**P < 0.01).

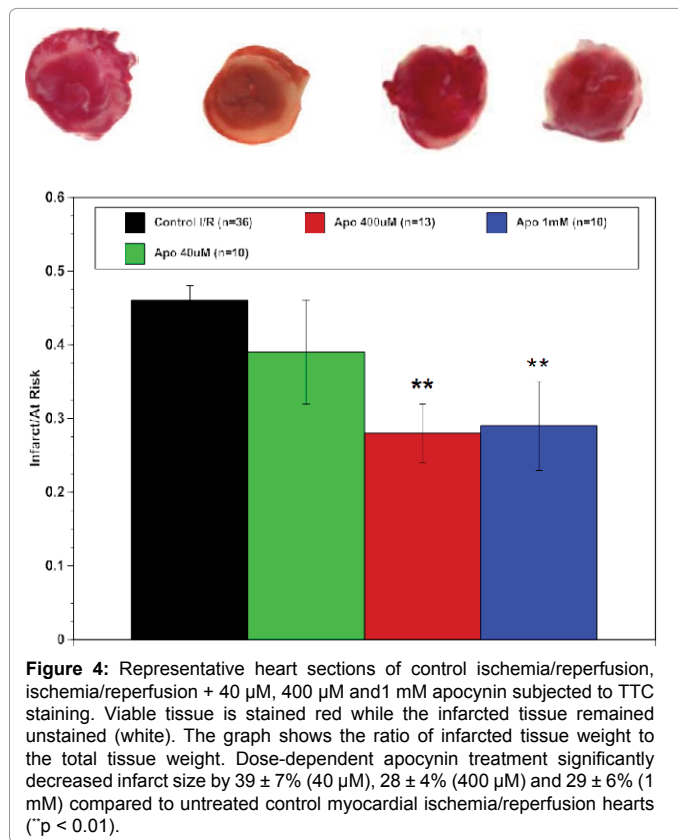


Figure 4: Representative heart sections of control ischemia/reperfusion, ischemia/reperfusion + 40 μM, 400 μM and 1 mM apocynin subjected to TTC staining. Viable tissue is stained red while the infarcted tissue remained unstained (white). The graph shows the ratio of infarcted tissue weight to the total tissue weight. Dose-dependent apocynin treatment significantly decreased infarct size by 39 ± 7% (40 μM), 28 ± 4% (400 μM) and 29 ± 6% (1 mM) compared to untreated control myocardial ischemia/reperfusion hearts (*p < 0.01).

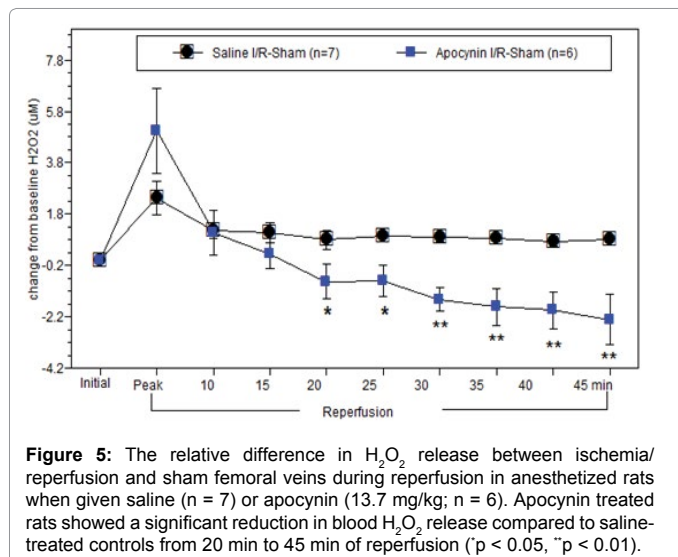


Figure 5: The relative difference in H₂O₂ release between ischemia/reperfusion and sham femoral veins during reperfusion in anesthetized rats when given saline (n = 7) or apocynin (13.7 mg/kg; n = 6). Apocynin treated rats showed a significant reduction in blood H₂O₂ release compared to saline-treated controls from 20 min to 45 min of reperfusion (*p < 0.05, **p < 0.01).

to control ischemia/reperfusion hearts that were 60 ± 5%; 4. Apocynin significantly decreased blood H₂O₂ levels and increased blood NO by four-fold compared to saline controls at the end of reperfusion (both, p < 0.01). These results suggest that apocynin exerted cardio protective effects by inhibiting reactive oxygen species release generated by NADPH oxidase during ischemia/reperfusion.

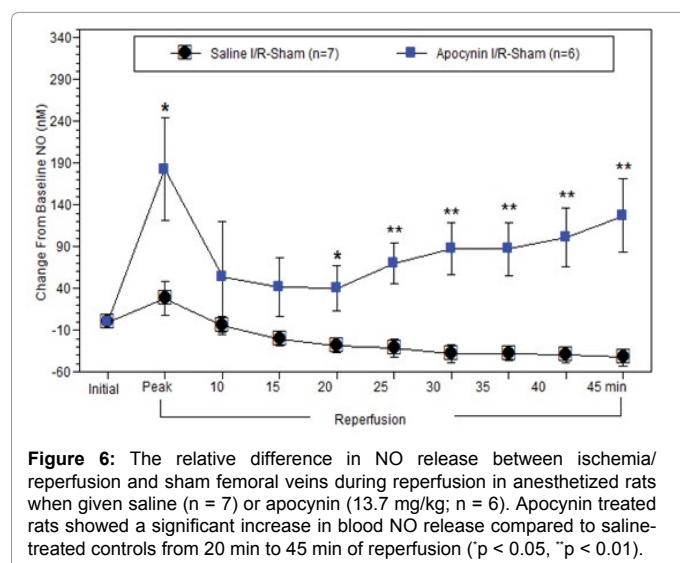
The role of NADPH oxidase and oxidative stress during ischemia/reperfusion

NADPH oxidase activity is increased in myocardial ischemia/

reperfusion in part through activation of TNFα receptors that in turn activate protein kinase C [20,27,28]. Activated protein kinase C isoforms (e.g., alpha, beta II and zeta) phosphorylate serine residues on cytosolic p47^{phox}, which is primarily expressed in leukocytes and vascular endothelium, to promote assembly and activation of NADPH oxidase [27,29] leading to the production of SO (Figure 1). It has been indicated that NADPH oxidase activation is the initial source of SO, that in part mediates mitochondrial potassium ATP channel opening and release of reactive oxygen species during angiotensin II induced preconditioning [30]. It is well-known that transient mitochondrial potassium ATP channel opening is important to induce preconditioning in the heart to reduce infarct size [31]. However, prolonged opening of this mitochondrial channel throughout prolonged ischemia (i.e., 30 min) and during reperfusion does not reduce infarct size compared to untreated I/R controls [32]. Consequently, prolonged opening of mitochondria potassium ATP channels during ischemia and reperfusion may contribute to oxidative stress during ischemia/reperfusion and in turn further oxidize BH₄ to BH₂ to promote eNOS uncoupling and further reactive oxygen species release (Figure 1).

Regarding NADPH oxidase and eNOS uncoupling, activation of Protein kinase C beta II isoform is mostly responsible for NADPH oxidase activation and inhibition of eNOS activity [23,33]. Our previous studies found that a selective PKC beta II peptide inhibitor significantly attenuated oxidative stress in hind limb I/R and restored post-reperfusion cardiac function in isolated I/R hearts [23,33,34]. Given that increased uncoupled eNOS activity facilitates oxidative stress, the antioxidant and cardioprotective effects of Protein kinase C beta II inhibitor most likely are principally due to inhibition on NADPH oxidase effects which in turn would reduce oxidation of BH₄ to BH₂ and therefore attenuate uncoupled eNOS activity. Therefore, understanding vascular/cardiac NADPH oxidase during reperfusion following prolonged ischemia may disclose the triggering source of oxidative stress in reperfusion injury (Figure 1). Our *in vivo* and *ex vivo* hind limb and myocardial ischemia/reperfusion models will largely exclude the contribution of polymorphonuclear leukocyte NADPH oxidase because they are recruited into the reperfusion site after a few hours of reperfusion.

Apocynin is a selective NADPH oxidase inhibitor that works by blocking the association of p47^{phox} with the membrane p22^{phox} in endothelial cells and leukocytes [14,35] (Figure 1). Apocynin is considered to be a prodrug that is converted to diapocynin by endothelial peroxidase and leukocyte myeloperoxidase which then oxidizes cysteine-196 on p47^{phox} and thereby inhibits NADPH oxidase assembly and activation [22]. P47^{phox} is highly expressed in myeloid and vascular smooth muscle/endothelial cells [10]. Therefore, the primary target for apocynin would be attenuation of vascular NADPH oxidase during ischemia and early reperfusion since only resident PMNs would be present during this time. Mozaffari et al. [13] reported that apocynin (100 μM) given during pre-ischemia improved + dP/dt_{max} and + dP/dt_{min} and decreased infarct size/area-at-risk compared to untreated controls in isolated perfused rat hearts subjected to 40 min regional ischemia followed by 2 hour reperfusion [13]. However, their study did not measure SO or H₂O₂ release in the post-reperfusion tissue to assess the level of reactive oxygen species attenuation induced by apocynin to attribute a mechanism of action. Moreover, they did not give apocynin exclusively during reperfusion since pretreatment is not always a clinical option. Therefore, it is of high clinical importance to determine the degree of reactive oxygen species inhibition from NADPH oxidase and its relative contribution during the reperfusion phase of ischemia/reperfusion injury. Another study by Jiao et al. [36],



reported that apocynin reduced SO in cardiac ischemia/reperfusion tissue, which was associated with a decrease in infarct size/area-at-risk compared to controls in mice subjected to regional ischemia for 30 min followed by 24 hour reperfusion and administered apocynin I.V. (5 mg/kg) 10 min prior to reperfusion. However, their study did not assess cardiac function or infarct size/area-at-risk, which may be associated with the decreased SO release [36]. Similarly, another study by Gao et al. [28] also performed regional ischemia in mice for 30 min followed by 90 min reperfusion and found that acetylcholine (ACh)-induced vasodilatation in isolated coronary arterioles was improved in mice given apocynin I.V. (25 mg/kg) prior to reperfusion. This effect was associated with decreased SO production [28]. Moreover, their study attributed the increased NADPH oxidase activity to increased TNF α released from vascular smooth muscle in myocardial ischemia/reperfusion tissue. However, like the Jiao et al. study, Gao et al. study did not assess cardiac function or infarct size/area-at-risk.

Interestingly, apocynin at 1 mM did not improve cardiac function, infarct size or area-at-risk compared to the 400 μ M dose. It is possible that apocynin exerted its maximal effect at 400 μ M in the isolated perfused heart study. In contrast, the SO release from leukocytes was inhibited to a greater extent at 1 mM compared to 400 μ M, suggesting that vascular and cardiac myocyte NADPH oxidase may have reached its maximal inhibition at 400 μ M. It is also possible that the 1 mM dose may have started to exert some paradoxical oxidative stress in the heart tissue.

According to Riganti et al. [37], apocynin induced oxidative stress in a dose-dependent way in glial cells *in vitro*. Furthermore, high concentration apocynin incubated in cultured glial cells showed an increase in oxidative stress via measurements of lipid peroxidation, H₂O₂, and cytotoxic level. It should be emphasized that the concentration of apocynin used in the hindlimb ischemia/reperfusion model (i.e., 13.7 mg/kg) approximated to a concentration of about 1 mM in blood. However, given that apocynin is a small molecular compound that could be cleared by the kidneys, it is plausible that the effective dose was less in the blood over time since it took about 20 minutes for apocynin to exert significant effects on blood H₂O₂ levels and NO levels. Of interest, it should be noted that the initial increase in blood H₂O₂ and NO levels observed in both saline and apocynin-treated animals is consistent with previous studies showing an initial increase in these levels, and may, in part, be due to the initiation of

blood flow contacting the respective microsensors [4,7,8]. Subsequent measurements after this initial burst may more accurately represent the relative increase or decrease of the biomolecule being measured (i.e., H₂O₂ or NO).

Conclusion

In summary, apocynin exerted cardio protective effects on post-refused cardiac function that paralleled a decrease in infarct size/area-at-risk. Moreover, the reduction of blood H₂O₂ release and increased NO bioavailability in the hind limb ischemia/reperfusion model suggests that apocynin exerted its cardio protective effects via the inhibition of SO release. Collectively, this study suggests that NADPH oxidase significantly contributes to reactive oxygen species production during ischemia/reperfusion and that inhibiting this production during reperfusion is effective to attenuate oxidative stress and subsequent cardiac cell death and cardiac contractile dysfunction. However, it is possible that the results of apocynin may in part be due to scavenging H₂O₂ in addition to selective inhibition of NADPH oxidase as suggested by Johnson et al. [35].

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