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Protection against reperfusion injury by 3′,4′-dihydroxyflavonol in rat isolated hearts involves inhibition of phospholamban and JNK2

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ABSTRACT

Background: Flavonols, including 3′,4′-dihydroxyflavonol (DiOHF), reduce myocardial ischemia and reperfusion (I/R) injury but their mechanism remains uncertain. To better understand the mechanism of the cardioprotective actions of flavonols we investigated the effect of DiOHF on cardiac function and the activation of protective and injurious signalling kinases after I/R in rat isolated hearts.
Methods: We assessed the effect of global ischemia (20 min) and reperfusion (5–30 min) on cardiac function and injury in rat isolated, perfused hearts in the absence or presence of DiOHF (10 μM) during reperfusion. Western blotting was used to assess changes in the phosphorylation state of kinases known to be involved in injury or protection.
Results: DiOHF improved cardiac contractility and reduced perfusion pressure and cell death in the isolated hearts. Phosphorylation of p38MAPK and CaMKII increased during ischemia with no further increase during reperfusion. Phosphorylation of other kinases increased during reperfusion. Phosphorylation of phospholamban (PLN) peaked at 5 min of reperfusion whereas phosphorylation of Akt, Erk, STAT3 and JNK2 was highest after 30 min. The presence of DiOHF during reperfusion significantly inhibited the activation of PLN and JNK without affecting phosphorylation of the protective kinases Erk1/2 and STAT3. Experiments *in vitro* demonstrated that DiOHF inhibited CaMKII by competing with ATP but not Ca^{2+}/calmodulin.
Conclusions: It is proposed that DiOHF confers protection against myocardial reperfusion injury by inhibiting CaMKII and subsequent PLN-induced leak of Ca^{2+} from the sarcoplasmic reticulum as well as by inhibiting JNK2 activation to reduce apoptosis.

1. Introduction

Increased oxidative stress and Ca^{2+} overload that occur during myocardial ischemia and reperfusion (I/R) may activate a wide range of signal transduction pathways to contribute to cell death or survival. In regard to cell death signalling pathways, increased oxidative stress and Ca^{2+} overload activates the multifunctional protein Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) [1]. The elevated intracellular Ca^{2+} concentration promotes Ca^{2+} binding to calmodulin and this calcified calmodulin then binds to CaMKII causing a conformational change and autophosphorylation [1]. Activated CaMKII will in turn activate downstream Ca^{2+}-related receptors including ryanodine receptors, sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) and phospholamban (PLN), the regulatory protein for SERCA2a on the sarcoplasmic reticulum [1]. Phosphorylation of PLN relieves an inhibition of SERCA activity resulting in the uptake of Ca^{2+} into the sarcoplasmic reticulum [2]. It is well known that CaMKII regulates myocardial excitation–contraction coupling under normal physiological conditions [1] however excessive CaMKII activation has been associated with various cardiac diseases including heart failure, cardiac hypertrophy and arrhythmias [1,3]. There is emerging evidence that CaMKII could also be a mediator of myocardial I/R injury [4,5].

Signalling pathways that have been implicated in cardioprotection include the mitogen-activated protein kinases (MAPKs) and phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) pathways [6,7], noting importantly that there may be marked species differences in these pathways [6]. The best-characterized MAPK subfamilies are extracellular signal-regulated kinases (Erk) 1/2, c-Jun N-terminal kinases (JNKs) and p38MAPK. Hausenloy and Yellon proposed that Akt and Erk1/2 form the reperfusion injury signalling kinase (RISK) pathway which is involved in protection against myocardial I/R injury [8,9]. Akt and Erk1/2 phosphorylate several targets including pro-apoptotic proteins such as the Bcl-2-associated death promoter (BAD) and glycogen synthase kinase (GSK)-3β leading to their inactivation. The inactivation of GSK-3β inhibits the opening of the mitochondrial permeability transition pore (mPTP) and prevents cell death [8]. Another pro-survival pathway which has been implicated during I/R is the survivor activating factor enhancement (SAFE) pathway [10] involving the activation of the tumor necrosis factor, Janus kinase (JAK) and signal transducer and

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activator of transcription (STAT) 3 to promote cell survival. While activation of Erk1/2 and Akt are pro-survival, extensive evidence *in vitro* and *in vivo* has supported a pro-injurious role of JNK and p38MAPK in myocardial I/R [11–14]. The relative activation of these kinases could influence the fate of cardiomyocytes to either undergo cell survival or death.

Several studies have shown that 3′,4′-dihydroxyflavonol (DiOHF) protects against myocardial I/R injury *in vivo* and *in vitro* [15–18]. The administration of DiOHF, or its water soluble pro-drug NP202, reduced infarct size and improved post-ischemic myocardial function in anesthetized sheep [15,16]. Although the protective action of DiOHF has been known for almost a decade, the precise mechanism of its cardioprotective action remains unclear. In this study, we aimed to explore the effect of DiOHF on the change in kinase activation, especially in early reperfusion. In this study we investigated the effect of DiOHF on activation of injurious and protective kinases during reperfusion.

2. Methods

Full details of materials and methods and experimental protocols are provided in Supplementary Materials. This investigation conforms with the National Health and Medical Research Council of Australia code of practice for the care and use of animals for scientific purposes. All the procedures involved in this project were approved by the RMIT University Animal Ethics Committee.

2.1. Langendorff heart preparation

Adult male Sprague–Dawley rats (250–300 g) were anesthetized with sodium pentobarbitone 325 mg/kg i.p. and the heart was excised and perfused with Krebs' buffer bubbled with 95% O_2 and 5% CO_2 at pH 7.4 and 37 °C at a constant flow of ~12 mL/min to generate a perfusion pressure of 62 ± 5 mm Hg.

2.2. DiOHF treatment protocol

In I/R-treated groups, hearts were equilibrated for 30 min followed by 20 min global ischemia. Hearts were then reperfused for either 5 or 30 min with Krebs' buffer in the presence of 0.5% dimethyl sulfoxide (DMSO), the vehicle control for DiOHF (S1), or in the presence of 10 μM DiOHF. We have previously demonstrated that this concentration of DiOHF exerts effective antioxidant activity [19] and significantly reduces ischemia/reperfusion injury and improves contractility in rat isolated hearts [20]. A separate set of control experiments were carried out where rat isolated hearts were perfused continuously with Krebs' buffer for 80 min without any further intervention (S2).

2.3. Protein extraction and Western blot

Wester blotting was used to measure p^{Ser473}-Akt, Akt, $p^{Thr202/Tyr204}$-Erk 1/2, Erk 1/2, p^{Tyr705}-STAT3, STAT3, $p^{Thr183/Tyr185}$-JNK, JNK, $p^{Thr180/Tyr182}$-p38 MAPK, p38 MAPK, $p^{Thr286/287}$-CaMKII, CaMKII $p^{Ser16/Thr17}$-PLN, and PLN in the left ventricle.

2.4. Lactate dehydrogenase assay

The amount of lactate dehydrogenase (LDH) in the effluent sample was measured by the rate of reduction in the absorbance value during conversion of nicotinamide adenine dinucleotide (NADH) with sodium pyruvate to its oxidized form (NAD^+) at 340 nm.

2.5. TUNEL assay

Detection of apoptosis by TUNEL labelling was performed using the CardioTACS™ reagent kit according to the manufacturer's instructions.

2.6. Cell culture and treatment

Rat cardiac myoblasts (H9C2) were maintained in Dulbecco's Modified Eagle's Media (DMEM) containing L-glutamine and 4.5 g/L D-glucose supplemented with 10% (*v*/*v*) fetal calf serum (FCS) and 100 U/mL penicillin–streptomycin (P/S) (growth DMEM). Cells were pre-treated with DiOHF 60 min prior to the application of stress stimuli. Following pre-treatment with DiOHF, cells were stimulated with either ionomycin (5 μM, 1 min) or H_2O_2 (1 mM, 60 min).

2.7. In vitro kinase assay with ADP-Glo™

The ADP-Glo™ Kinase assay kit was used according to the manufacturer's (Promega) protocol. Luminescent signals were then measured on a POLARStar Optima microtitre platereader (BMG Labtech) to measure ADP levels and kinase activity. DiOHF was pre-incubated with CAMKIIδ for 15 min before the addition of Ca^{2+}/calmodulin or the peptide substrate.

2.8. Cellular CaMKII autonomous activity assay

Endogenous CaMKII activity from cell lysates was assayed immediately following cell lysis. The reaction mixtures consisted of 20 μg of cell lysate, 131 μM Autocamtide 2 peptide substrate (KKALRRQETVDAL) (Merck Millipore), stress reaction buffer (SRB) (50 mM PIPES, pH 7.2, 10 mM $MgCl_2$, 0.1% (*w*/*v*) BSA), either 25 μM KN-93 or 1 mM $CaCl_2$ with 300 nM calmodulin or 1 mM ethylene glycol tetraacetic acid (EGTA).

2.9. Statistical analysis

Myocardial function was expressed as the percentage change from the pre-ischemic value. All results are expressed as group mean ± standard error of mean (SEM), with the number of independent experiments denoted as 'n'. Data analysis was performed using Graphpad Prism® (version 6.0, USA). Myocardial function and time point LDH data were analysed using 2-way ANOVA with Sidak's multiple comparison test. Area-under-the-curve (AUC) data was analysed using Student's unpaired *t*-test. All Western blot data, total LDH assay and quantitative data for CardioTACS™ assay were analysed using 1-way ANOVA with Tukey's multiple comparison test. In all cases, $p < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of DiOHF on post-ischemic myocardial function, LDH release and apoptosis

DiOHF treatment during reperfusion significantly improved myocardial function. The recovery of LV + dP / dt tended to increase in DiOHF-treated hearts (although not significantly) compared to its vehicle control (Supplementary Fig. 1A) whereas LV – dP / dt was significantly greater in DiOHF-treated hearts compared to its vehicle control (Supplementary Fig. 1B). DiOHF treatment significantly reduced LDH release and also reduced the increased number of apoptotic cardiac cells (Supplementary Fig. 2C–F).

3.2. Effects of DiOHF on kinase activation during 5 min reperfusion

In vehicle-treated hearts, the phosphorylation of Erk1/2 at 5 min of reperfusion was not different from sham hearts (Fig. 3A). DiOHF during reperfusion increased the phosphorylation of Erk1/2 compared to sham. Phosphorylation of JNK1/2 and p38MAPK in vehicle-treated hearts at 5 min of reperfusion was increased compared to sham while DiOHF treatment had no effect on the I/R-induced increase in phosphorylation of either protein (Fig. 2A/C). In vehicle-treated hearts, the phosphorylation of Akt at 5 min of reperfusion was not different from sham hearts while DiOHF during reperfusion increased the phosphorylation of Akt compared to sham (Supplementary Fig. 6A). In contrast, the phosphorylation of STAT3 was not changed by either I/R or DiOHF treatment (Supplementary Fig. 6C). Phosphorylation of CaMKII was comparable in all groups (Fig. 3A) while phosphorylation of PLN at 5 min of reperfusion was enhanced but this effect was abolished by DiOHF treatment (Fig. 3C).

3.3. Effects of DiOHF treatment on kinase activation 30 min after reperfusion

At 30 min reperfusion, phosphorylation of Erk2 was significantly increased in vehicle-treated hearts ($p < 0.05$) while DiOHF had no effect on the I/R-induced increased phosphorylation of Erk2 (Fig. 1B). By contrast, the phosphorylation of Erk1 at 30 min reperfusion was comparable in all groups. The phosphorylation of p38MAPK and JNK2 at 30 min of reperfusion was significantly increased in vehicle-treated hearts compared to sham (Fig. 2B/D). I/R-induced increased phosphorylation of p38MAPK was not affected by DiOHF treatment while DiOHF treatment significantly reduced the I/R-induced increased phosphorylation of JNK2. The phosphorylation of JNK1 was comparable in all groups. At 30 min reperfusion, the phosphorylation of Akt was increased in the vehicle-treated group (Supplementary Fig. 6B), however DiOHF treatment significantly attenuated the I/R-induced increase in phosphorylation of Akt. In vehicle-treated hearts, phosphorylation of STAT3 at 30 min of reperfusion was