

Mitochondrial ATP-sensitive K⁺ channels mediate the antioxidative influence of diosgenin on myocardial reperfusion injury in rat hearts

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Abstract. The contribution of reactive oxygen species and oxidative stress in the pathogenesis of ischemia-reperfusion (I/R) injury has been supported by many studies. The effect of diosgenin on oxidative stress induced by I/R injury was evaluated in this study. Rat hearts were subjected to 30 minutes of global ischemia followed by 90 minutes of reperfusion. 5-hydroxydecanoate (5-HD) was used before administration of diosgenin and before ischemia. The activities of myocardial creatine kinase (CK), malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GPX) were measured. Administration of diosgenin before ischemia significantly lowered CK and MDA levels as compared with control group ($p < 0.05$) and increased GPX ($p < 0.05$) and SOD ($p < 0.01$) activities in comparison with control group. Pre-administration of 5-HD significantly attenuated the protective effects of diosgenin. In conclusion, opening of mitochondrial ATP-sensitive K⁺ channels and attenuating of oxidative stress can be suggested as underlying mechanisms for cardioprotective effect of diosgenin in I/R injury.

Key words: Diosgenin — Oxidative stress — Mito K_{ATP} channels — I/R injury

Abbreviations: 5-HD, 5-hydroxydecanoate; CK, creatine kinase; GPX, glutathione peroxidase; I/R injury, ischemia/reperfusion injury; LDH, lactate dehydrogenase; MDA, malondialdehyde; mitoK_{ATP} channel, mitochondrial ATP-sensitive potassium channel; MPTP, mitochondrial permeability transition pore; NOS, nitric oxide synthase; ROS, reactive oxygen species; SOD, superoxide dismutase; TBARS, thiobarbituric acid-reactive substances.

Introduction

Ischemic heart diseases are one of the major causes of death worldwide. Thrombolytic therapies and primary percutaneous intervention are common therapies used for restoring the blood flow of ischemic myocardium and to reduce the infarcted zone size. This process that is called reperfusion can itself induce more cell damage and cardiomyocyte death, known as ischemia-reperfusion (I/R) injury (Derek et al.

2013). That's why reperfusion is called as double-edged sword (Braunwald and Kloner 1985). At first minutes of myocardial reperfusion a mass of oxidative stress is produced by different sources (Hearse et al. 1973; Zweier et al. 1987). When molecular oxygen is reintroduced to the previously ischemic myocardium, it goes under repetitive reduction that induces superoxide radicals. Potent free radicals like superoxide anions, hydroxyl radicals and peroxynitrite that are generated at first minutes of reperfusion have an essential role in inducing I/R injury (Verma et al. 2002; Derek et al. 2013).

Reactive oxygen species (ROS) induces I/R injury by opening mitochondrial permeability transition pore (MPTP), acting like a chemoattractant for neutrophils and inducing dysfunction of sarcoplasmic reticulum. All

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of these cause intracellular Ca^{2+} overload, cell membrane damage caused by lipid peroxidation, enzymatic denaturation and also direct oxidative damages to DNA (Derek et al. 2013). Pharmacologic as well as ischemic preconditioning are the therapeutic ways to reduce lethal outcomes of I/R injury (Loubani and Galinanes 2002). Cardioprotection can be triggered by mitochondrial ATP-sensitive potassium ($\text{mitoK}_{\text{ATP}}$) channels (Vanden Hoek et al. 2000; Facundo et al. 2006a). While antagonists of these channels remove the positive effect of ischemic preconditioning, it indicates these channels as mediators of preconditioning (Facundo et al. 2006a). Potassium transport by this channel and its consequent removal by K/H exchanger alter the inner membrane potential and stimulate the respiratory chain (Kowaltowski et al. 2001). Improvement of energy metabolism is the result of $\text{mitoK}_{\text{ATP}}$ channels opening, alongside that inner membrane potential changes reduce Ca^{2+} uptake by mitochondrion during ischemia and prevents oxidative stress during reperfusion (Vanden Hoek et al. 2000; Belisle and Kowaltowski 2002; Facundo et al. 2006a, 2006b). These events together are responsible for cardioprotective effects of $\text{mitoK}_{\text{ATP}}$ channels.

Agents derived from plants are notably used in traditional and modern medicine and also can be used in treatment of heart disease because of their low toxic nature and less side effects. Diosgenin (3β -hydroxyl-5-spirostene) is a phytoestrogen, a plant based estrogen, which can be found in Wild Yam roots (*Dioscorea villosa*), Fenugreek (*Trigonella foenum graecum*) and Soybean (*Glycin max*) (Raju and Mehta et al. 2009). In traditional medicine diosgenin is used as an anti-hypercholesterolemic, anti-hypertriacylglycerolemic, anti-diabetic and anti-hyperglycemic agent (Jnarez-Oropeza et al. 1987; McAnuff et al. 2005; Son et al. 2007). Cardioprotective effects of diosgenin were observed in myocardial infarction induced by isoproterenol to its antioxidant and membrane-stabilizing effects (Jayachandran et al. 2009). In previous study, we found that this agent reduces the myocardial reperfusion injury through its capacity to inhibit the production of inflammatory cytokines in rat (Ebrahimi et al. 2014). Hypolipidemic and antioxidative effects of diosgenin was observed in rats fed with high fat and high cholesterol diet (Gong et al. 2010). Diosgenin also reduces intracellular ROS levels and apoptosis induced by H_2O_2 in human vein endothelial cells (Gong et al. 2010). Anti-thrombotic effect has also been attributed to diosgenin (Corbiere et al. 2003; Gong et al. 2011).

According to antioxidant and cardioprotective potentials of diosgenin, in this study we have aimed to examine its effect on oxidative stress caused by ischemia-reperfusion injury and to further understand the mechanism by which it induces its cardioprotective effect; is $\text{mitoK}_{\text{ATP}}$ channel involved in this process?

Materials and Methods

Animals

Thirty male Wistar rats, weighted 250–300 g, were used in this study. Animals were housed in a room with a temperature of 22–24°C, free access to water and food and with a natural cycle of darkness and brightness. All the stages of experiment as well as animals care and handling were in accordance with guidelines of animal care committee of Tabriz University of Medical Science.

Isolated heart protocol

Animals were anesthetized intraperitoneally with a mixture of ketamine (60 mg/kg) and xylosine (10 mg/kg) and heparinized with sodium heparin (500 IU), and then hearts were excised quickly and mounted on the Langendorff perfusion apparatus. Thereafter, the aorta was cannulated and hearts were perfused with a Krebs-Henseliet (K-H) solution containing (in mM): NaHCO_3 25, KCl 4.7, NaCl 118, glucose 11.1, CaCl_2 2.5, KH_2PO_4 1.2, MgSO_4 1.2. The buffer solution was equilibrated with 95% O_2 and 5% CO_2 at bath temperature of 37°C, pH 7.4 and under constant perfusion pressure of 75 mmHg delivered to the aortic cannula.

Ischemia-reperfusion protocol

After 15 min (the stabilization period of hearts activity), all hearts were subjected to 30 min of global ischemia followed by 90 min of reperfusion. Ischemia and reperfusion was induced by occluding and reopening of the aortic flow (Ghyasi et al. 2011). Chemicals were administered for 20 min, after 15 min of stabilization period and before ischemic insult in different groups as discussed below.

Experimental design

Rat hearts were randomly divided into 5 groups ($n = 6$), as following:

1. Control group: isolated hearts were subjected to 30 min of ischemia followed by 90 min of reperfusion (30 I/90 R) with a normal K-H solution.
2. Cremophor-EL (EL-C) group: 30 I/90 R, perfusion with a K-H solution containing 0.1% Cremophor-EL (the solvent of diosgenin) for 20 min before ischemia.
3. Diosgenin (Diosg) group: 30 I/90 R, perfusion with a K-H solution containing 0.1 nM diosgenin (the most protective dose according to the dose-response study that had been done before) for 20 min before ischemia.
4. 5-hydroxydecanoate (5-HD) group: 30 I/90 R, perfusion with a K-H solution containing 100 μM 5-HD

(selective $\text{mitoK}_{\text{ATP}}$ channel blocker) for 20 min before ischemia.

5. Diosgenin + 5-HD (Diosg+5-HD) group: 30 I/90 R, perfusion with a K-H solution containing 100 μM 5-HD for 5 min and then 0.1 nM diosgenin + 100 μM 5-HD for 20 min before ischemia.

Tissue processing and homogenate preparation

At the end of experiments, left ventricles of hearts were dissected, weighed and rapidly frozen in liquid nitrogen. For antioxidant activities measurement, cardiac homogenates were prepared as described by Rothermel et al. (2000). In brief, fifty milligrams of ventricle muscle were homogenized in 2 ml of ice-cold lysis buffer (1 mM KH_2PO_4 , 1 mM KCL, 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM NaF, 1 mM Na_3VO_4 , and 0.1% Triton X-100). The homogenates were centrifuged at 1000 rpm for 10 min at 4°C. The supernatant containing the cytoplasmic protein fraction was collected and a protease inhibitor cocktail (104 mM AEBSF, 0.08 mM aprotinin, 2 mM leupeptin, 4 mM bestatin A, and 1.4 mM E-64) (Sigma-Aldrich, St Louis, MO) was added to it and stored at -80°C until use. Protein concentration of supernatant was estimated using Bradford technique (Bradford 1976).

Measurement of creatine kinase

For evaluating the effect of diosgenin on the activity of creatine kinase (CK) as an indicator of tissue damage, the coronary effluent in early reperfusion phase was collected and the CK activity in the coronary effluent was measured by an automatic biochemistry analyzer using a commercially available kit according to the manufacturer's instructions. The values were normalized to whole volumes of the effluent in each heart and the absorbance of the solution was detected at 340 nm by spectrophotometry. The results were reported in U/l.

Lipid peroxidation measurement

In this study, malondialdehyde (MDA) has been considered as a main indicator of lipid peroxidation. Lipid peroxidation is usually analyzed by measuring thiobarbituric acid-reactive substances (TBARS) in homogenates, as previously described by Draper and Hadley (1990). Briefly, the samples (250 μl) were mixed with 1 ml 10% trichloroacetic acid (TCA) and 1 ml of 0.67% thiobarbituric acid. Afterward, the samples were heated in a boiling water bath for 15 min and then n-butyl-alcohol (2:1 v/v) were added to the solution. After centrifugation (800 \times g, 5 min), TBARS were determined from the absorbance at 535 nm, using a spectrophotometer (Pharmacia Biotech, UK).

Enzymatic antioxidant measurement

Superoxide dismutase activity

Superoxide dismutase (SOD) activity was determined using a RANSOD kit (Randox Crumlin, UK) according to Delmas-Beauvieux et al. (1995). In this method, xanthine and xanthine oxidase were used to generate superoxide radicals that react with 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride (ITN) to form a red formazan dye. Concentrations of substrates were 0.05 mmol/l for xanthine and 0.025 mmol/l for ITN. SOD activity was measured by the degree of inhibition of this reaction. After calculating the percent of inhibition by using related formula, SOD activity value was calculated by comparing with the standard curve. SOD activity was measured at 505 nm by a spectrophotometer and the values were expressed as U/mg protein.

Glutathione peroxidase activity

Glutathione peroxidase (GPX) activity was determined using a RANSEL kit (Randox Crumlin, UK) according to the method of Paglia and Valentine (1967). GPX catalyzes the oxidation of glutathione (at a concentration of 4 mmol/l) by cumene hydroperoxide. In the presence of glutathione reductase (at a concentration \geq 0.5 units/l) and 0.28 mmol/l of NADPH, oxidized glutathione is immediately converted to the reduced form with concomitant oxidation of NADPH to NAD^+ . The decrease in absorbance at 340 nm (at 37°C) was measured using a spectrophotometer (Pharmacia Biotech, England), and then GPX concentration was calculated.

Statistical analysis

All values were expressed as means \pm SEM. The between-group parameters were analyzed using one-way ANOVA followed by Tukey's post-hoc test. Differences were considered statistically significant when $p < 0.05$.

Results

The effect of diosgenin and blocking of $\text{mitoK}_{\text{ATP}}$ channel on CK release in I/R hearts

The CK release into coronary effluent was measured as an indicator of I/R injury. There was no significant difference in CK release between control and EL-C groups (Figure 1). Administration of diosgenin significantly decreased the CK release into coronary effluent during reperfusion as compared with those of control and EL-C groups ($p < 0.05$). Adding 5-HD (as a $\text{mitoK}_{\text{ATP}}$ channel blocker) removed the

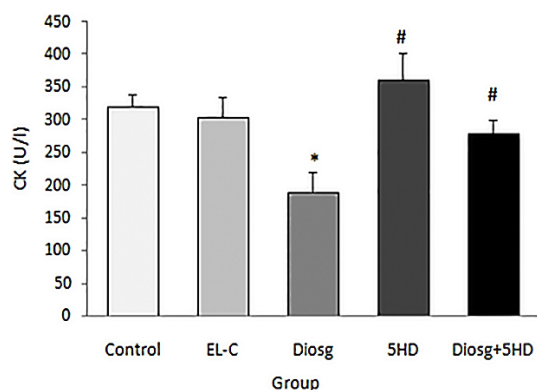


Figure 1. The levels of creatine kinase (CK) release into coronary effluent of I/R hearts in experimental groups. * $p < 0.05$ vs. control group; # $p < 0.05$ vs. Diosg group. Groups: EL-C, cremophor-EL group (0.1%); Diosg, diosgenin group (0.1 nM); 5-HD: 5-hydroxydecanoate group (100 μ M); Diosg+5-HD, Diosgenin + 5-HD group. For more details see Materials and Methods. I/R, ischemia/reperfusion.

CK lowering effect of diosgenin and significantly increased the CK activity compared to diosgenin group.

The effect of diosgenin and blocking of mitoK_{ATP} channel on MDA activity in I/R hearts

Preconditioning with diosgenin significantly reduced MDA activity in diosgenin-treated hearts in comparison with control group ($p < 0.05$). Adding 5-HD abolished diosgenin effect on MDA activity. There was no significant difference in MDA activity between Diosg+5HD and control groups (Figure 2).

The effect of diosgenin and blocking of mitoK_{ATP} channel on GPX activity in I/R hearts

Administration of diosgenin before ischemia significantly increased GPX activity in comparison with those of control group ($p < 0.05$). Blocking mitoK_{ATP} channels using 5-HD decreased GPX activity toward control values and thus attenuated the protective effect of diosgenin on GPX activity (Figure 3). There were no significant changes between control, EL-C, 5HD, and Diosg+5HD groups.

The effect of diosgenin and blocking of mitoK_{ATP} channel on superoxide dismutase (SOD) activity in I/R hearts

Pretreatment of I/R hearts with diosgenin increased the activity of SOD in comparison with control group ($p < 0.01$). The effect of diosgenin on SOD activity in the presence of 5-HD was reduced (Figure 4), and the SOD activity in

5HD group was significantly less than those of Diosg group ($p < 0.05$).

Discussion

In order to evaluate the effect of diosgenin on oxidative stress in I/R model of injury in rats, the myocardial activity of MDA as a major index for lipid peroxidation, and the activities of SOD and GPX, as the first line of defense against oxidative stress were measured. Diosgenin increased the activity of these enzymes; it reveals the antioxidative role of this agent in I/R injury. In addition, the activity of CK as an indicator of cell damage was decreased by diosgenin, accompanied with reduced MDA activity. These observations show that diosgenin induces its cardioprotective effect in this I/R injury model by its antioxidative activity.

Enhanced activity of antioxidant system and reduction in lipid peroxidation levels were observed in the study planned for evaluation of the protective effect of diosgenin on hepatocytes of rats with chronic renal failure (Manivannan et al. 2013). In rats fed with high cholesterol diet, diosgenin increased the total SOD activity in plasma and liver, GPX activity in erythrocytes, and catalase activity in erythrocytes and liver tissue (Son et al. 2007). Dioscin, another member of saponin family similar to diosgenin, protected the H₉C₂ cells from I/R injury by modulation of mitochondrial apoptotic pathway and attenuation of oxidative stress, it had reduced cell death and LDH level, prevented cytochrome *c* release from mitochondria, declined pro-apoptotic protein Bax level but anti-apoptotic protein Bcl-2 level was increased (Qin et al. 2014). Dioscin also increased SOD activity and decreased intracellular ROS and MDA levels (Qin et al. 2014). Similarly, cardioprotective effect of diosgenin was

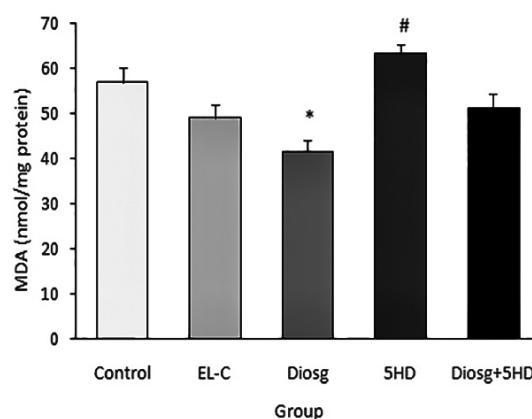


Figure 2. The levels of malondialdehyde (MDA) in I/R hearts in experimental groups. * $p < 0.05$ vs. control group; # $p < 0.05$ vs. Diosg group. For abbreviations see Fig. 1.

observed in isoproterenol-induced myocardial infarction; groups treated with diosgenin had lower levels of CK-MB, lipid peroxidation and lysosomal hydrolysis activity (Jayachandran et al. 2009). On the other hand, diosgenin reduced the apoptosis level induced by H₂O₂ in human vein endothelial cells by increasing SOD, GPX and NOS activity and by reducing free radicals levels (Gong et al. 2010).

ROS are known as important mediators of I/R injury. There is also a reverse relation between ROS generation and mitoK_{ATP} channel activation. MitoK_{ATP} channels act as sensors of ROS and reduces generation of mitochondrial free radicals and ROS release in response to local increase of oxidant level and changes in mitochondrial redox state (Ferranti et al. 2003; Heberty et al. 2007). Taking together ROS release acts as a stimulator of mitoK_{ATP} channel and in turn it reduces ROS generation and induces protection from I/R injury. However, high level of ROS could inactivate mitoK_{ATP} channels. In this study, the administration of 5-HD removed the protective effects of diosgenin on I/R hearts. It presents that diosgenin induces its cardioprotective and anti-oxidative effects by opening of mitoK_{ATP} channels and blocking these channels reverses diosgenin effects.

The other explanation for cardioprotective effect of diosgenin is its ROS scavenging ability; decreasing ROS level by diosgenin may activate K⁺ channel and induce cardioprotective effect. In both ways, mitoK_{ATP} channels are involved in the cardioprotective effect of diosgenin. Earlier investigations have revealed that diosgenin can increase NOS activity (Gong et al. 2010). There is also a link between NO and mitoK_{ATP} channel. NO is indicated as an endogenous mitoK_{ATP} channel opener and cardioprotective effect of NO can be removed by ROS generation (Sasaaki et al. 2000; Derek et al. 2013).

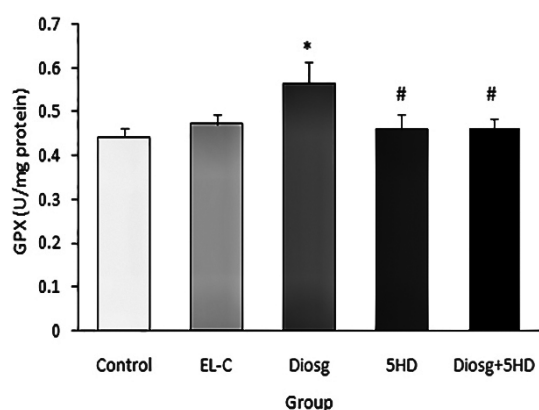


Figure 3. The levels of glutathione peroxidase (GPX) in I/R hearts in experimental groups. * $p < 0.05$ vs. control group; # $p < 0.05$ vs. Diosg group. For abbreviations see Fig. 1.

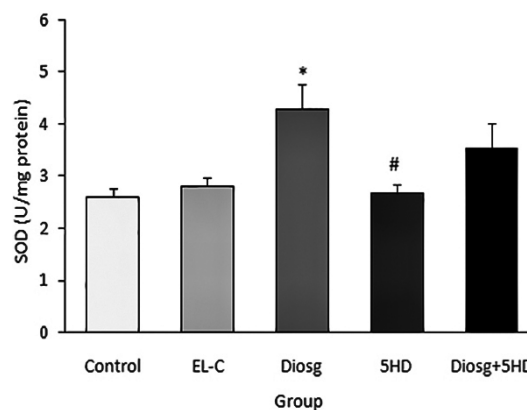


Figure 4. The levels of superoxide dismutase (SOD) in I/R hearts in experimental groups. * $p < 0.05$ vs. control group; # $p < 0.05$ vs. Diosg group. For abbreviations see Fig. 1.

In conclusion, administration of diosgenin reduced CK activity (as an indicator of cardiac tissue damage) and MDA level (as an indicator of lipid peroxidation). It also increased the activity of anti-oxidant enzymes GPX and SOD. All these findings reveal the anti-oxidative stress capability of diosgenin. 5-HD (as a blocker of mitoK_{ATP} channel) reversed the cardioprotective effects of diosgenin; this indicates that diosgenin induces its protective effects on I/R injury somewhat by opening the mitoK_{ATP} channel and decreasing the generation of reactive oxygen species. Taken together, diosgenin can be used in pharmacological preconditioning for attenuating the harmful effects of I/R injury.

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Conflict of interest. The authors declare none conflict of interest in relation to this article.

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