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Diazoxide prevents reactive oxygen species and mitochondrial damage, leading to anti-hypertrophic effects

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ABSTRACT

Pathological cardiac hypertrophy is characterized by wall thickening or chamber enlargement of the heart in response to pressure or volume overload, respectively. This condition will, initially, improve the organ contractile function, but if sustained will render dysfunctional mitochondria and oxidative stress. Mitochondrial ATP-sensitive K^+ channels (mitoKATP) modulate the redox status of the cell and protect against several cardiac insults. Here, we tested the hypothesis that mitoKATP opening (using diazoxide) will avoid isoproterenol-induced cardiac hypertrophy *in vivo* by decreasing reactive oxygen species (ROS) production and mitochondrial Ca^{2+}-induced swelling. To induce cardiac hypertrophy, Swiss mice were treated intraperitoneally with isoproterenol (30 mg/kg/day) for 8 days. Diazoxide (5 mg/kg/day) was used to open mitoKATP and 5-hydroxydecanoate (5 mg/kg/day) was administrated as a mitoKATP blocker. Isoproterenol-treated mice had elevated heart weight/tibia length ratios and increased myocyte cross-sectional areas. Additionally, hypertrophic hearts produced higher levels of H_2O_2 and had lower glutathione peroxidase activity. In contrast, mitoKATP opening with diazoxide blocked all isoproterenol effects in a manner reversed by 5-hydroxydecanoate. Isolated mitochondria from Isoproterenol-induced hypertrophic hearts had increased susceptibility to Ca^{2+}-induced swelling secondary to mitochondrial permeability transition pore opening. MitokATP opening was accompanied by lower Ca^{2+}-induced mitochondrial swelling, an effect blocked by 5-hydroxydecanoate. Our results suggest that mitoKATP opening negatively regulates cardiac hypertrophy by avoiding oxidative impairment and mitochondrial damage.

1. Introduction

Cardiac hypertrophy is initially an adaptive response in which the cardiac tissue grows and changes to accommodate hemodynamic overload [1]. Even though the human heart is a dynamic organ, the maintenance of the hemodynamic insult results in progressive tissue deterioration and is an important risk factor for the development of heart failure [2]. Cardiac hypertrophy is also a major contributing factor to sudden cardiac death and morbidity in the Western world [3].

There is increasing evidence for the involvement of oxidative imbalance in cardiac hypertrophy and heart failure [4–13]. Whatever the mechanism for increased oxidative imbalance, finding new tools to prevent it would be desirable. The first evidence for the role of oxidative imbalance in cardiac hypertrophy emerged from a study reporting that angiotensin II induced reactive oxygen species (ROS) generation in cardiac myocytes [9]. This hypothesis was confirmed by other groups showing that hypertrophy can be abrogated by antioxidants [4,5,12,13]. Conversely, ROS are capable of inducing hypertrophic features in cardiomyocytes [14,15]. In this line, we have recently shown that cardiac hypertrophy suppressed the activity of antioxidant enzymes catalase and superoxide dismutase. Additionally, the opening of the mitochondrial ATP-sensitive potassium channel (mitoKATP) reversed the impairment of superoxide dismutase activity [16].

The transition of hypertrophy to heart failure is accompanied by

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mitochondrial damage [17]. Perturbed mitochondrial function would impair the ability of this organelle to produce ATP efficiently. It is important to point out that mitochondria produce 90% of all intracellular ATP [18]. Additionally, cardiac Ca^{2+} participates as an obligatory signaling molecule resulting in increased calcium-dependent activation of intracellular hypertrophic factors [19]. Interestingly, stimulating mitochondrial potassium flux, through mitoKATP opening triggers cardioprotection by decreasing mitochondrial [20] and cytosolic [21] Ca^{2+} accumulation. Cardiac hypertrophy induces mitochondrial Ca^{2+} overload and oxidative imbalance triggering the opening of the mitochondrial permeability transition pore (mPTP) [22].

The aims of the present study were to investigate whether the opening of the mitoKATP, using diazoxide, triggers anti-hypertrophic effects by changing the cellular redox status and to determine if diazoxide avoids Ca^{2+}-induced mPTP opening during cardiac hypertrophy as part of its mechanism of action. Our findings suggest that diazoxide may protect against cardiac hypertrophy through attenuation of ROS and mPTP formation.

2. Materials and methods

2.1. Animals

All animals were used in compliance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. The protocol was approved by the institutional Animal Experimentation Ethics Committee. All mice used were 60-day-old Swiss Male weighting between 25 and 30 g.

2.2. Hypertrophy induction

The animals were treated daily (for the first four days) with intraperitoneal (i.p.) injections of saline (0.9% – control group) or isoproterenol (30 mg/kg/day – ISO group). From that point on, all drugs were administered with a solution containing saline (0.9%) and DMSO (2%) and mice receiving isoproterenol were randomly divided into 3 groups and treated for four more days. In summary, one group received isoproterenol alone (30 mg/kg/day – ISO group), another received isoproterenol (30 mg/kg/day) plus diazoxide (5 mg/kg/day – DZX group) and the third group received isoproterenol (30 mg/kg/day) plus diazoxide (5 mg/kg/day) plus 5-hydroxydecanoate (5 mg/kg/day – 5-HD group). 5-hydroxydecanoate was administered 20 min before diazoxide in order to inhibit the mitoKATP. A solution containing saline (0.9%) and DMSO (2%) was administered to the control group. A summary of the treatment is depicted on Table 1.

2.3. Histology and image analysis

Hearts were collected and rapidly fixed in 10% buffered formalin, and subsequently embedded in paraffin. Serial 5-μm heart sections from each group were cut transversely and stained with H&E for histological analysis. Only myocytes cut transversely with both a visible nucleus and unbroken cellular membrane were used for cross-sectional area measurement. We used the Image J software (from National Institutes of Health) to trace the outer border of the myocytes.

2.4. H_2O_2 measurements

H_2O_2 production was measured using Amplex Red (Molecular Probes) according to the manufacturer's instructions. In brief, left ventricles were cut into blocks (50 mg) and incubated (protected from light) with Amplex Red (50 μmol/L) and horseradish peroxidase (1 U/mL) for 30 min at 37 °C in Krebs-Hepes buffer containing (in mM) 118 NaCl, 25 $NaHCO_3$, 1.2 KH_2PO_4, 4.7 KCl, 1.2 $MgSO_4$, 1.25 $CaCl_2$, 10 glucose, and 10 Hepes, pH 7.4. The tubes were centrifuged at 5000*g* for 2 min, the supernatant was transferred to a cuvette, and the absorbance measured at 560 nm. Background absorbance was determined by incubating Amplex red and horseradish peroxidase without the sample. H_2O_2 release was calculated using H_2O_2 standards to create a calibration curve (Fig. 2a) and expressed as μmol/50 mg tissue.

2.5. Glutathione peroxidase activity assay

The left ventricle tissue was homogenized in ice-cold buffer containing 10 mM Tris, 1 mM EDTA and 20% Sucrose, pH 7.4 at 4 °C. The homogenates were centrifuged at 12,000*g* for 30 min at 4 °C. The resulting supernatant was used. Glutathione peroxidase activity was determined by the decrease in NADPH concentrations. We used 1 mM cumene hydroperoxide (Sigma Aldrich) as a substrate. First the cellular extract was added to a buffer containing 50 mM potassium phosphate, 0.5 mM EDTA, 0.2 mM NADPH (Sigma Aldrich),1 mM GSH (Sigma Aldrich) and 0.2 U/mL Glutathione Reductase purified for *S. cerevisiae* (Sigma Aldrich), pH 7.0. After 5 min incubation, cumene hydroperoxide was added to initiate the reaction. We followed the disappearance of the co-substrate NADPH at 340 nm for 5 min. Glutathione peroxidase activity is expressed as Units/mg protein.

2.6. Mitochondrial isolation

Mitochondria were isolated by differential centrifugation. Briefly, mice hearts were removed and immediately washed in ice-cold buffer containing 300 mM sucrose, 10 mM K^+ Hepes buffer, pH 7.2, and 1 mM K^+ EGTA. The tissue was minced finely and then homogenized manually. Nuclei and cellular residues were pelleted by centrifugation at 600*g* for 5 min. In order to obtain the mitochondrial pellet the supernatant was recentrifuged at 9000*g* for 8 min. This pellet was washed to eliminate contaminating blood and resuspended in a minimal amount of buffer (~100 μL). Samples were kept over ice and used within 1 h of isolation, to ensure

Table 1
Treatment protocol.

Group	Treatment
Control	Saline (0.9%) for the first 4 days
	Saline (0.9%) and DMSO (2%) for the following 4 days
ISO	30 mg/kg/day isoproterenol in saline (0.9%) for the first 4 days
	30 mg/kg/day isoproterenol in saline (0.9%) and DMSO (2%) for the following 4 days
DZX	30 mg/kg/day isoproterenol in saline (0.9%) for the first 4 days
	30 mg/kg/day isoproterenol and 5 mg/kg/day diazoxide in saline (0.9%) and DMSO (2%) for the following 4 days
5-HD	30 mg/kg/day isoproterenol in saline (0.9%) for the first 4 days.
	30 mg/kg/day isoproterenol plus 5 mg/kg/day diazoxide plus 5 mg/kg/day 5-hydroxydecanoate in saline (0.9%) and DMSO (2%) for the following 4 days.