**RESEARCH ARTICLE**

**Diazoxide Modulates Cardiac Hypertrophy by Targeting H\(_2\)O\(_2\) Generation and Mitochondrial Superoxide Dismutase Activity**

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**Abstract:** Background: Cardiac hypertrophy involves marked wall thickening or chamber enlargement. If sustained, this condition will lead to dysfunctional mitochondria and oxidative stress. Mitochondria have ATP-sensitive K\(^+\) channels (mitoKATP) in the inner membrane that modulate the redox status of the cell.

Objective: We investigated the *in vivo* effects of mitoKATP opening on oxidative stress in isoproterenol-induced cardiac hypertrophy.

Methods: Cardiac hypertrophy was induced in Swiss mice treated intraperitoneally with isoproterenol (ISO - 30 mg/kg/day) for 8 days. From day 4, diazoxide (DZX - 5 mg/kg/day) was used in order to open mitoKATP (a clinically relevant therapy scheme) and 5-hydroxydecanoate (5HD - 5 mg/kg/day) or glibenclamide (GLI - 3 mg/kg/day) were used as mitoKATP blockers.

Results: Isoproterenol-treated mice had elevated heart weight/tibia length ratios (HW/TL). Additionally, hypertrophic hearts had elevated levels of carbonylated proteins and Thiobarbituric Acid Reactive Substances (TBARS), markers of protein and lipid oxidation. In contrast, mitoKATP opening with DZX avoided ISO effects on gross hypertrophic markers (HW/TL), carbonylated proteins and TBARS, in a manner reversed by 5HD and GLI. Moreover, DZX improved mitochondrial superoxide dismutase activity. This effect was also blocked by 5HD and GLI. Additionally, *ex vivo* treatment of isoproterenol-induced hypertrophic cardiac tissue with DZX decreased H\(_2\)O\(_2\) production in a manner sensitive to 5HD, indicating that this drug also acutely avoids oxidative stress.

Conclusion: Our results suggest that diazoxide blocks oxidative stress and reverses cardiac hypertrophy. This pharmacological intervention could be a potential therapeutic strategy to prevent oxidative stress associated with cardiac hypertrophy.

Keywords: Mitochondria, hypertrophy, oxidative stress, free radicals, antioxidants, hydrogen peroxide.

**1. INTRODUCTION**

Cardiac hypertrophy is a major contributing factor to sudden cardiac death and morbidity in the Western world [1]. The human heart responds to pressure or volume overload by developing hypertrophy [2], but maintenance of the insult results in progressive tissue deterioration and is an important risk factor for the development of heart failure [3]. Progression to heart failure is a result of the deregulation of many intracellular events, including changes in gene expression, contractile protein content [4], mitochondrial dysfunction [5, 6] and oxidative imbalance [7-11].

Oxidative stress is central in the development of cardiac hypertrophy. Several hypertrophy inducers have been shown to elicit increased reactive oxygen species (ROS) production [7-15]. Additionally, hypertrophy can be abrogated by antioxidants, confirming the relationship between oxidative stress and this process [7, 10, 11, 16]. Our recent studies pointed toward a simultaneous attenuation of the antioxidant enzymes catalase, superoxide dismutase (SOD) and glutathione peroxidase during hypertrophy [15, 17]. Interestingly, the opening of the mitochondrial ATP-sensitive potassium channel (mitoKATP) reversed the impairment of SOD activity [15] and glutathione peroxidase [17], while the inhi-
bition of SOD [18] or its cardiac specific knockout [19] triggers cardiac hypertrophy.

The transition from hypertrophy to heart failure leads to mitochondrial damage [6]. Biochemically, mitochondrial damage will disrupt ATP production by lowering the inner membrane potential and ATP synthase activity, which is responsible for 90% of all intracellular ATP [20]. Additionally, an impaired electron transport chain (mainly complex I and III) leads to enhanced electron leakage to molecular oxygen [21, 22], generating superoxide anions and ultimately causing oxidative stress. Normally, intramitochondrial superoxide anion concentration are very low due to the antioxidant enzyme manganese superoxide dismutase (MnSOD). This enzyme specifically converts superoxide to hydrogen peroxide (H₂O₂) [23]. Interestingly, knockout mice for this enzyme specifically converts superoxide to hydrogen peroxide (H₂O₂) [23]. Interestingly, knockout mice for MnSOD or lacking one allele of the gene have enlarged hearts, indicating that MnSOD is important to prevent the development of cardiac hypertrophy [24, 25].

The goal of the present work was to investigate whether stimulating mitochondrial potassium flux through mitochondrial KATP opening had antihypertrophic effects by improving H₂O₂ release, mitochondrial SOD activity, decreasing protein and lipid oxidation as part of its mechanism of action. Our findings suggest that diazoxide protects against cardiac hypertrophy through attenuation of oxidative stress.

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 25-30 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. 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) or Glibenclamide (3 mg/kg/day - GLI group) as used elsewhere [15, 17]. 5-Hydroxydecanoate or Glibenclamide was administered 20 minutes before diazoxide in order to inhibit mitochondrial KATP. A solution containing saline (0.9%) and DMSO (2%) was administered to the control group.

2.3. Sample Preparation and Mitochondrial Isolation

Mitochondria were isolated by differential centrifugation. Briefly, mouse hearts were 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. This homogenate was used for the Thiobarbituric Acid Reactive Substances (TBARS) detection assay. 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 10 min. The supernatant was used for the protein carbonyl assay. The mitochondrial pellet was resuspended in a minimal amount of buffer (100 μL) and used for manganese superoxide dismutase assays.

2.4. Manganese Superoxide Dismutase Activity (MnSOD)

Mitochondria were subjected to three fast freeze-thaw cycles in hypotonic buffer (25 mM K₂PO₄ pH 7.2, 5 mM MgCl₂), pH 7.2, before the assay. Mitochondria were then added to a reaction medium containing 0.1 mM EDTA, 13 mM L-methionine, and 75 mM nitro blue tetrazolium (NBT) in potassium phosphate buffer (pH 7.8). The reaction was initiated by the addition of 2 μM riboflavin and exposed uniformly to an unfiltered white light for 10 minutes. The developed blue color due to NBT reduction was measured at 560 nm. The samples were pre-incubated for 20 minutes with 5 mM sodium cyanide in order to inhibit residual Cu/ZnSOD and thus measure only MnSOD activity. SOD activity was expressed as U/mg of protein. One unit is the amount of enzyme required to inhibit the reduction of NBT by 50%.

2.5. Protein Carbonyl Detection

Protein carbonyl content was measured using a method described elsewhere [26]. Briefly, cardiac extract supernatant was placed in glass tubes, then 4 mL of 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 2.5 N HCl were added. The blank sample was prepared using 4 mL of 2.5 N HCl. Tubes were mixed by vortex and incubated for 1 h at room temperature. Samples were vortexed every 15 min, then 5 mL of trichloroacetic acid (20%) was added, and tubes were incubated on ice for 10 min followed by centrifugation (12000 x g) for 10 min. The pellet was washed twice with ethanol-ethyl acetate (v/v). The final precipitate was dissolved in 2 mL of 6 M guanidine hydrochloride solution and incubated for 10 min at 37°C. The absorbance of the sample was measured at 370 nm. The carbonyl content was calculated based on the molar extinction coefficient of DNPH (ε=2.2×10⁴ cm⁻¹ M⁻¹) and expressed as nanomoles per milligram protein.

2.6. Thiobarbituric Acid Reactive Substances (TBARS)

The concentration of TBARS in tissues, an index of lipid peroxidation, was determined spectrophotometrically. Briefly, an aliquot of 0.3 ml of tissue extract was mixed with 600 μl of 0.6% thiobarbituric acid solution and 600 ul of 10% trichloroacetic acid solution. Then the samples were vortexed, incubated for 15 min at 95°C and cooled on ice for 5 min. The samples were then centrifuged at 2,500 g for 5 min. The resulting supernatant was read using a spectrophotometer at 540 nm. TBARS were calculated using a molar extinction coefficient for malondialdehyde of 1.56 x 10⁵ M⁻¹ cm⁻¹ and expressed as μmol of TBARS per milligram protein.
2.7. H$_2$O$_2$ Measurements

H$_2$O$_2$ production was measured from blocks of cardiac tissue harvested from control and isoproterenol-treated mice. Both hearts of control and ISO groups were cut into 20 mg blocks and incubated with Amplex red (50 μmol/L) and horseradish peroxidase (1 U/mL) at 37°C for 30 min, pH 7.4. In order to open the mitoKATP, we treated the blocks of cardiac tissue with DZX (30 μM) 15 minutes before and during the procedure. SHD (150 μM) was given to the blocks of tissue in the presence of DZX (SHD group). Control samples were treated with DMSO. Left ventricles were incubated (protected from light) in Krebs-Hepes buffer containing (in mM) 118 NaCl, 25 NaHCO$_3$, 1.2 KH$_2$PO$_4$, 4.7 KCl, 1.2 MgSO$_4$, 1.25 CaCl$_2$, 10 glucose, and 10 Hepes. After incubation period, the tubes were centrifuged at 5000 g for 2 minutes, 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$_2$O$_2$ released was calculated in μmol/20 mg tissue using a calibration curve created using H$_2$O$_2$ standards and expressed as relative to DMSO group.

2.8. Protein Content

Total protein content in each sample was estimated using the Bradford method using bovine serum albumin as a standard.

2.9. Statistical Analysis

Statistical analysis was conducted using Graphpad Prism software. Data are presented as mean ± S.E.M. Student’s t test or one-way analysis of variance followed by a Tukey’s post hoc test was performed where indicated. p < 0.05 was considered statistically significant.

3. RESULTS

3.1. Diazoxide Blocks Cardiac Hypertrophy In vivo

In order to investigate the impact of mitoKATP opening on isoproterenol-induced cardiac hypertrophy in vivo, we used isoproterenol (a beta adrenergic agonist drug) to induce cardiac hypertrophy. After 8 days of treatment with isoproterenol, mice had larger hearts, as indicated by elevated heart weight/tibia length (HW/TL Fig. 1). Opening of the mitoKATP using diazoxide (a mitoKATP opener) significantly prevented this. Additionally, co-treatment with 5-hydroxydecanoate or glibenclamide (mitoKATP blockers) reversed this beneficial effect (Fig. 1).

3.2. Diazoxide Inhibits Cardiac Oxidative Damage During Isoproterenol-Induced Hypertrophy

We have previously shown that mitoKATP opening (using diazoxide) prevents ROS production and antioxidant enzyme activity repression during cardiac hypertrophy [15, 17]. To get further insight into the ability of mitoKATP to avoid cellular oxidative damage, we examined protein oxidative damage (carbonyl protein) and lipid peroxidation (TBARS levels). Isoproterenol infusion significantly increased both protein carbonyl (Fig. 2A) and TBARS levels (Fig. 2B) after 8 days of treatment, indicating oxidative stress in hypertrophic hearts. Strikingly, mitoKATP opening (using diazoxide) significantly attenuated the increased protein carbonyl (Fig. 2A) and TBARS levels (Fig. 2B) during isoproterenol-induced cardiac hypertrophy. As expected, mitoKATP inhibition using 5-hydroxydecanoate and glibenclamide exacerbated the accumulation of both oxidative stress indicators during isoproterenol-induced hypertrophy.

3.3. Diazoxide Prevents Mitochondrial Superoxide Dismutase Dysfunction

Mitochondrial superoxide dismutase (MnSOD) uses manganese ion as a cofactor and is only expressed in the mitochondrial matrix. In order to identify if cardiac mitochondria from treated mice had improved oxidative balance, we tested MnSOD activity during cardiac hypertrophy with or without DZX treatment. Isolated cardiac mitochondria from mice treated with isoproterenol had decreased MnSOD activity (Fig. 3). Consistent with decreased protein and lipid oxidation (Fig. 2), we observed increased MnSOD activity (mitochondrial major antioxidant) after mitoKATP opening using diazoxide. This effect was reversed by mitoKATP blockers 5-hydroxydecanoate and glibenclamide (Fig. 3). Thus, we propose that preservation of mitochondrial antioxidant capacity is part of the mechanism for long-term diazoxide protection against hypertrophy.
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Fig. (2). Diazoxide avoids lipid and protein oxidation during cardiac hypertrophy in vivo. A, Bar graph showing quantitative analysis of protein carbonyl levels 8 days after isoproterenol (ISO), diazoxide (DZX), or 5-hydroxydecanoate (5-HD)/Glibenclamide (GLI) treatments. B, TBARS levels 8 days after isoproterenol (ISO), diazoxide (DZX), or 5-hydroxydecanoate (5-HD)/Glibenclamide (GLI) treatments. n = 5 per group. * P<0.05, **P<0.01.

3.4. Diazoxide Avoids Cardiac H₂O₂ Release During Cardiac Hypertrophy

On the basis of previous reports showing that DZX protects cardiac tissue from hypertrophic stimuli, we asked whether DZX could acutely avoid cardiac H₂O₂ formation/release from hypertrophic hearts. Blocks of isoproterenol-induced hypertrophic hearts along with their controls were acutely treated with DZX or DZX plus 5HD as depicted in Fig. (4A). Hypertrophic tissue released significantly more H₂O₂; this was reduced by DZX incubation. Consistent with the idea of mitoKATP involvement in DZX-prevented H₂O₂ release, we observed increased levels of H₂O₂ in 5HD-treated samples. Importantly, both treatments had no significant effects on control samples (Fig. 4B). In order to illustrate the robustness of our detection of H₂O₂ released from cardiac blocks, we detected H₂O₂ from ex-vivo treated (30 minutes, 37 °C) cardiac samples with rotenone (an inhibitor of mitochondrial complex I) or with antimycin A (an inhibitor of mitochondrial complex III). As expected, both inhibitors increased H₂O₂ release from cardiac samples (Fig. S1).

Taken together, these results indicate that mitoKATP opening exerts anti-hypertrophic effects in part by improving H₂O₂ release and mitochondrial SOD activity, ultimately avoiding oxidative damage.

4. DISCUSSION

The results of the present study support an increasing body of literature which demonstrates that cardiac hypertrophy induces oxidative imbalance [5, 7-9, 12, 14, 15, 17] and mitochondrial damage [6, 17]. Here, we induced cardiac hypertrophy by treating mice with isoproterenol and evaluated the impact of the mitoKATP opening by treating mice...
previously confirmed and advanced these observations by demonstrating that mitoKATP inhibits cardiac hypertrophy by preventing loss of glutathione peroxidase and protecting mitochondria against Ca$^{2+}$-induced swelling [17]. We have also demonstrated the protective effect of diazoxide on superoxide dismutase levels [15]. The present study extends these observations by showing that mitoKATP exerts antihypertrophic effects in a manner associated to the maintenance of intramitochondrial redox balance and by lowering damage to lipids and proteins.

A notable effect of diazoxide was the reduction of oxidized protein (carbonyl proteins) and lipid (malondialdehyde) levels. This may reflect a favorable intracellular oxidant balance and provides a plausible molecular mechanism for the antihypertrophic effects of diazoxide. Indeed, ROS are important contributors toward cardiac hypertrophy [7, 9, 11, 15, 29] and increased ROS production favors cardiac hypertrophy. These enhanced oxidants would contribute toward the formation and accumulation of damaged lipids and proteins. Interestingly, treating mice with antioxidants (that would favor less accumulation of oxidized proteins and lipids) blocks cardiac hypertrophy in vivo [11].

with diazoxide. Treatment with diazoxide for 8 days inhibited isoproterenol-induced hypertrophy [15]. Cardiac hypertrophy is likely to be caused (or is a consequence) of increased oxidative stress. Therefore, decreasing oxidative stress might be a powerful way to preserve the cardiac tissue. Consistent with this hypothesis, we observed that diazoxide-treated mice had lower levels of TBARS and protein carbonyls, two indicators of oxidative imbalance. Our data also suggest that mitoKATP opening is important for redox balance by maintaining the levels of manganese-SOD and ultimately suggest that protecting mitochondria by mitoKATP opening can be a valuable tool to avoid cardiac hypertrophy-associated oxidative stress.

The first evidence for the effect of mitoKATP against cardiac hypertrophy emerged in 2004 when Xia and colleagues [27] showed that diazoxide inhibited phenylephrine-induced hypertrophy in isolated rat cardiomyocytes by modulating a Na-H exchanger isoform. Moreover, mitoKATP opening has also been associated with the antihypertrophic effect of K-opioid receptor activation [28]. We have previously confirmed and advanced these observations by...
H2O2 production [17]. Here we extended this observation that protein carbonylation and protein dysfunction is still poorly understood in cardiac hypertrophy. These protein modification seem to accumulate in the presence of severe oxidative imbalance and lead to protein dysfunction [31]. Consistent with this idea, we have previously shown that cardiac hypertrophy leads to a redox imbalance with higher protein carbonyls, whereas treatment with diazoxide helped to maintain protein carbonyl levels similar to controls. After the establishment of the hypertrophic phenotype, it is difficult to establish whether carbonyl protein formation is the cause or the result of cardiac hypertrophy. Indeed, the relationship between protein carbonylation and protein dysfunction is still poorly understood in cardiac hypertrophy. These protein modification seem to accumulate in the presence of severe oxidative imbalance and lead to protein dysfunction [31].

Another important finding reported here is the ability of diazoxide to prevent MnSOD loss during isoproterenol-induced cardiac hypertrophy. MnSOD transforms superoxide anions to hydrogen peroxide (H2O2) and is located in the mitochondrial matrix. Interestingly, cardiomyocytes are densely packed with mitochondria to meet their high energy demand, making this protein an attractive target to avoid mitochondrial/cellular oxidative stress under pathological conditions. In humans, mutations of mitochondrial antioxidants, including MnSOD, increase the risk for cardiovascular diseases [33]. Cardiac dysfunction can be triggered by relatively small reductions in MnSOD activity [25], which suggests that MnSOD is essential for normal heart function. Additionally, hypertrophic hearts from mice [34] and human failing myocardium presented diminished levels of MnSOD protein and activity. Our data show that diazoxide treatment prevents the suppression of MnSOD activity and this effect might contribute toward the anti-hypertrophic effect of diazoxide. These results suggest that pharmacological protection of MnSOD by diazoxide could be an effective therapeutic strategy to prevent the pathological growth and oxidative damage of hypertrophied hearts.

CONCLUSION

In conclusion, our findings support the idea that opening of the mitoKATP by diazoxide avoids the development of cardiac hypertrophy by targeting ROS generation, preventing loss of MnSOD activity and avoiding protein/lipid oxidation. This pharmacological intervention could be a potential therapeutic strategy to prevent oxidative stress associated with cardiac hypertrophy.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was approved by the Animal Experimentation Ethics Committee from Universidade Federal do Cariri, Barbalha, Ceará, Brazil (protocol number 01/2014).

HUMAN AND ANIMAL RIGHTS

No humans were involved in this study. All animal procedures followed were performed in accordance with the standards set forth in the 8th Edition of Guide for the Care and Use of Laboratory Animals (https://olaw.nih.gov/sites/default/files/Guide-for-the-Care-and-Use-of-Laboratory-Animals.pdf) published by the National Academy of Sciences, The National Academies Press, Washington DC, USA.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

All data generated or analysed during this study are included in this published article and its supplementary information files.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's website along with the published article.
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