SUPPLEMENTARY MATERIAL

Mitochondrial ATP Sensitive Potassium Channel Opening Inhibits Isoproterenol-Induced Cardiac Hypertrophy by Preventing Oxidative Damage

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Short running title: Diazoxide Protects Against Isoproterenol-Induced Cardiac Hypertrophy
Supplementary Methods

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. Male 60-day-old Swiss mice weighting between 25-30 g were kept at 23 ± 2ºC with a 12 hour light/dark cycle and ad libitum access to a standard diet (Purina do Brasil Ltd., São Paulo, SP, Brazil) and water. Mice were anesthetized with pentobarbital (50 mg/kg of body weight) before being sacrificed by decapitation immediately after.

Measurement of Cardiac Hypertrophy – On day 9 after the beginning of the treatment protocol, mice were injected with sodium pentobarbital (50 mg/kg body weight), weighed, and sacrificed by decapitation. Hearts were removed, washed in cold saline phosphate buffer to remove excess blood, and weighed. Tibias were excised, dissected and their lengths were measured. Cardiac hypertrophy was expressed both as the ratio of heart weight (mg) to body weight (g) and heart weight (mg) to tibia length (mm). Left ventricles were dissected and used for biochemical analysis.

Sample Preparation – The heart tissue (100 mg of tissue) was minced into small pieces using scissors, washed twice with ice-cold PBS and homogenized with a precooled glass potter (40 strokes) in 6 mL of ice-cold buffer composed of 10 mM tris-HCl, 1 mM EDTA, and 20% sucrose, pH 7.4 at 4ºC. The homogenates were kept at 4ºC for 10 min before being centrifuged at 12,000 g for 30 minutes at 4ºC. The resultant supernatant was stored immediately at -80ºC and used for biochemical assays.

Catalase Activity – Catalase activity was measured as described by Aebi¹. Briefly, the supernatants of homogenized samples were added to the reaction media containing 50 mM H₂O₂ in 100 mM of phosphate buffer (pH 7.4). Changes in absorbance at 240 nm were recorded for 10 minutes. Catalase activity was calculated as milliunits of catalase per milligram of protein.
**Superoxide Dismutase Activity** – SOD activity was measured as described previously². The tissue homogenate supernatant was added to a reaction media 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. 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%.

**Protein Thiol Content** - Oxidative damage to proteins is inversely correlated with the protein thiol content. Thiols in proteins were measured through the reduction of DTNB, generating a yellow product (TNB) measured at 412 nm³. The homogenate supernatants (prepared as described above) were incubated in the dark for 30 minutes at room temperature with 0.2 mM DTNB (prepared in PBS plus 1 mM EDTA). The protein thiol content was calculated based on the molar extinction coefficient of TNB (14500 M⁻¹cm⁻¹) and reported as micromoles of TNB per milligram protein. The reaction with DTNB with no protein and submitted to the same incubation served as the blank.

**Glutathione Levels** – The levels of glutathione was determined using a method based on a reaction of DTNB with some modifications⁴. This reaction produces a yellow product that was detected at 412 nm. An aliquot (0.5 mL) of heart homogenate was added to 3 mL of freshly prepared 4% sulfosalicylic acid dihydrate in H₂O (Sigma Aldrich) to precipitate proteins. After homogenization, the mixture was centrifuged at 1,600 x g for 15 minutes. The resulting supernatant was added to DTNB (2 mM). After 10 minutes the absorbance was measured at 412 nm. The experiment was conducted in absence of light. Total glutathione was calculated using a standard curve with purified glutathione. Total GSH content was expressed as micrograms per mg protein.

**Protein detection** – The amount of protein in each sample was estimated using the biuret method with bovine serum albumin as a standard.
Figure S1: DZX prevents cardiac hypertrophy in a Glibenclamide-sensitive manner. A, Treatment scheme for mice injected with Isoproterenol (ISO), diazoxide (DZX) and glibenclamide (Gli). For the first four days, the mice received daily intraperitoneal (i.p.) injections of saline (0.9% - control group) or 30 mg/kg/day isoproterenol. From that point on, mice that received isoproterenol were randomly divided into 3 groups and treated with...
isoproterenol alone (30 mg/kg/day, ISO group), isoproterenol (30 mg/kg/day) plus diazoxide (5 mg/kg/day, DZX group) or isoproterenol (30 mg/kg/day) plus diazoxide (5 mg/kg/day) plus glibenclamide (Gli, 3 mg/kg/day, Gli group) for four more days. From day 5 on, all drugs were administered in a solution containing saline (0.9%) and DMSO (2%). A solution containing saline (0.9%) and DMSO (2%) was also administered to the control group. B. Heart weight/Tibia length ratio following 8 days isoproterenol (ISO), diazoxide (DZX) and glibenclamide (Gli) treatments. C. Heart weight/body weight ratio after 8 days on isoproterenol (ISO), diazoxide (DZX) and glibenclamide (Gli) treatments. * P<0.05, ** P<0.01, *** P<0.001.
Figure S2: Cardiac growth is preserved after DZX or 5-HD treatment in the absence of isoproterenol. A, Treatment scheme for mice injected with diazoxide (DZX) and 5-hydroxydecanoate (5-HD). Control mice received daily intraperitoneal (i.p.) injections of saline (0.9%) and DMSO (2%). Some mice received diazoxide (DZX, 5 mg/kg/day, DZX group) or 5-hydroxydecanoate (5-HD, 5 mg/kg/day, 5-HD group). Both drugs were diluted in saline (0.9%) plus DMSO (2%). B, Heart weight/Tibia length ratio 5 days following diazoxide or 5-hydroxydecanoate treatments.
References


