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Transient Receptor potential Vanilloid type 1 contributes to modulate the nitric oxide pathway and oxidative stress in the isolated and perfused rat heart during ischemia and reperfusion.

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Abstract

The transient vanilloid receptor potential type 1 (TRPV1) regulates neuronal and vascular functions mediated by nitric oxide (NO) and by the calcitonin gene-related peptide (CGRP). Here we study the participation of TRPV1 in the regulation of myocardial injury caused by ischemia-reperfusion and in the regulation of NO, tetrahydrobiopterin (BH4), the cGMP pathway, CGRP, total antioxidant capacity (TAC), malondialdehyde (MDA) and phosphodiesterase-3 (PDE-3). Isolated hearts of Wistar rats were used (according to Langendorff) to study the effects of capsaicin (CS), capsazepine (CZ) and CZ+CS treatments. The hearts were divided into three subgroups; 1) perfusion, 2) ischemia and 3) ischemia-reperfusion. In all groups we studied cardiac work and levels of NO, cGMP, BH4, CGRP, TAC, MDA and PDE-3 in ventricular tissue. Western blots were used to determine the expressions of eNOS, iNOS and phosphorylated NOS (pNOS). Structural changes were determined by histological evaluation. CS prevented damage caused by ischemia-reperfusion by improving cardiac work and the levels of NO, cGMP, BH4, TAC and CGRP. TRPV1 and iNOS expression were increased under ischemic conditions, while eNOS and pNOS were not modified. We conclude that the activation of TRPV1 constitutes a therapeutic possibility to counteract the damage caused by ischemia and reperfusion by regulating the NO pathway through CGRP.

Key words: Ischemia reperfusion; TRPV1; cardioprotection.

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1. Introduction

Cardiovascular diseases are a serious health problem worldwide and they cause significant mortality. Many of the main risk factors for cardiovascular disease are directly related to lack of prevention, unhealthy nutrition, physical inactivity, dyslipidemia, hyperglycemia, high blood pressure, obesity, thrombosis, smoking and kidney dysfunction. Job stress in large cities and genetic load can also cause or magnify cardiovascular disease. [1-3] As a consequence of all these factors, diseases such as diabetes mellitus, heart failure, atherosclerosis and hypertension and generalized damage to organs and tissues are caused, that lead to acute myocardial infarction (AMI). During AMI, blood flow to the heart tissue is blocked damaging the cardiomyocites.

When perfusion is restored after AMI, there is damage by reperfusion that consists of the opening of the mitochondrial permeability transition pore, an increase in oxidative stress, cytosine activation, lipid peroxidation, and cell death due to necrosis or apoptosis. Moreover, the injury salvage kinase (RISK) and the survivor activating factor enhancement (SAFE) pathways are activated during reperfusion [4-9]

The Transient Receptor Potential Vanilloid type1 (TRPV1) is a non-selective cation channel that allows the passage of H^+, Na^+, Ca^{2+} y Mg^{2+} ions. [10] Although it was initially identified on sensory nerve fibers, it has been identified in other tissues and cells including trigeminal ganglia, dorsal root ganglia, neurons, urinary bladder, testis, adipocytes, smooth muscle cells, endothelial cells, pancreatic β-cells, liver, heart, skeletal muscle, and kidney cells with a high expression. In the

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cardiovascular system, TRPV1 is expressed in the ventricles, endothelial cells, vascular smooth muscle cells, and on sensory neurons innervating the myocardium. [11]

TRPV1 is activated by stimuli such as shear stress, changes in pH or temperature in the vasculature. Endogenously, these receptors are also activated by the anti-inflammatory responses and by anandamide which is a derivative of the metabolism of arachidonic acid and is an endogenous chemical activator of TRPV1 and also by cannabinoid receptors type 1 (CB1). In experimental research, agonists such as capsaicin (CS) which is the active principle in plants of the *Capsicum* genus and antagonists such as capsaizepine (CZ) are frequently employed to study the TRPV1 receptors. [12-14]

In the last two decades, TRPV1 has been proposed as a therapeutic possibility to reduce the effects produced by some pain-related diseases such as migraine or rheumatoid arthritis and also by cardiovascular diseases. [15-17] Since TRPV1 participates in the regulation of Ca^{2+} flux through the cell membrane and it also regulates the synthesis of NO, its role in some pathologies such as arterial hypertension, diabetes mellitus and ischemia-reperfusion injury are being studied. [11, 14, 18] When TRPV1 is activated, neuropeptides such as substance P and the calcitonin gene-related peptide (CGRP) are discharged from the perivascular sensory nerves that innervate the myocardium to provide its cardioprotective effects. [18,19]

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The purpose of this work was to demonstrate that the activation of TRPV1 at a systemic level generates cardioprotection, preventing damage by ischemia and reperfusion, through the control of oxidative stress. We also analyzed the levels of some cell damage biomarkers such as malondialdehyde (MDA) (which is the end product of lipoperoxidation) and phosphodiesterase-3 (PDE-3), which once activated induces the degradation of cGMP to guanosine monophosphate (GMP), inhibiting vascular smooth muscle relaxation. [20] Total antioxidant capacity (TAC) was also measured as a biomarker of oxidative stress levels in tissue. [21] Our hypothesis was that treatment with CS could activate TRPV1 in the heart under conditions of ischemia and reperfusion, restoring control of Ca^{2+} flow into cells and elevating the bioavailability of NO, thereby reducing ROS and enhancing mechanism that are cardioprotective.

2. Methods

Male Wistar rats of 300-350 g were used. They were provided by the Laboratory Animal Care of the National Institute of Cardiology “Ignacio Chávez” in México. All procedures for handling animals were approved by the Institutional Ethics Committee and in accordance with the National Rules for the care and handling of experimental animals (SAGARPA, NOM-062-ZOO-1999). The animals were kept under optimal conditions of temperature and light (12 h light/dark) with a standard diet (Lab diet 5012, PMI Nutrition International, Richmond, IN, USA) and water *ad libitum*. The experimental animals were randomly grouped as follows: 1.- Control; 2.- CS treatment (final dose of 20 mg/kg divided over 4 days); 3.- CZ treatment (final dose of 24 mg/kg divided over 4 days) and 4.- CZ+CS treatment for

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4 days. The CZ was applied first and an hour later the CS was injected. CS was applied at a lower dose (20 mg/kg) than that reported by Zhou *et al* [22] to avoid deletion of nerve endings and also because we performed a curve dose response in previous experiments with CS and CZ in isolated hearts [23]. The application of the drugs was through a s.c injection.

2.1. Reagents

The reagents used in this work were analytical grade (Sigma Chemical Co., St. Louis, MO, USA.). They included Capsaicin (8-methyl-N-vanillyl-6-nonenamide) agonist of TRPV1; Capsazepine N-(2-(4-chlorophenyl)ethyl)-1,3,4,5-tetrahydro-7,8-dihydroxy-2H-2-benzazepine-2-arbothioamide] antagonist of CS. CS and CZ were diluted separately in ethanol-water 2:1.

2.2. Determinations in the isolated and perfused heart according to the Langendorff technique

The experimental animals were anesthetized with sodium pentobarbital (60 mg/Kg of body weight) and heparin (1000 U/mL/Kg of body weight) was administered. Respiratory assistance was applied by means of a Palmer air pump and a cannula in the trachea inserted through a small incision.

The heart was exposed by a thoracotomy and removed to be placed in ice-cold Tyrode solution to arrest it and prevent ischemic preconditioning. Immediately, the heart was connected to the perfusion system through the ascending aorta. The heart was maintained in mechanical activity with Krebs-Henseleit solution with the following composition (mM): 120 NaCl, 23.4 $NaHCO_3$, 4.8 KCl, 1.2 KH_2PO_4, 0.86

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$MgSO_4$, 1.25 $CaCl_2$ and 11.0 of glucose (pH 7.4 and temperature 37 °C) through a constant retrograde perfusion (13 mL/min). The perfusion started with an adaptation period of 30 minutes (5 minutes with a flow (F) of 25 mL/min and 25 minutes with F of 13 mL/min). Heart rate (HR) was maintained at 312-324 beats per minute, using a Grass stimulator (U7, Grass Instruments Co., Quincy, Mass., USA).

Coronary flow was regulated with a peristaltic pump (SAD22, Grass Instruments Co., Quincy, Mass., USA). Parameters such as left intraventricular pressure (LIVP) were recorded. It was obtained by means of a Grass hydropneumatic pressure transducer to which a catheter with a latex balloon was connected. The balloon was introduced through the mitral valve into the left ventricle and once inside the cavity, an internal pressure of 5-10 mmHg (diastolic pressure) was applied.

With another Grass hydropneumatic pressure transducer, the perfusion pressure (PP) was recorded and a range of 55-70 mmHg at the beginning of the experiment was considered as an inclusion criterion.

All parameters were recorded using a computer acquisition data system (Grass PolyView). Cardiac Work (CW) was calculated as HR x LIVP = CW. [4, 24]

Each experimental group (Control, CS, CZ and CZ+CS) was subjected to different conditions: 1.- Pe (perfusion) 30 min. 2.- Pe 30 min + I (Ischemia) 30 min, without reperfusion (R). 3.- Pe 30 min + I (30 min) + R 60 min. Global ischemia was induced by stopping coronary flow by turning off the perfusion pump. Left

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ventricular tissue samples were obtained from all experimental conditions and frozen at -70 °C until metabolite detection tests were performed.

2.3. Determination of NO, BH4, TAC, cGMP

2.3.1. Sample treatment

Ventricular tissue samples for determination of nitric oxide (NO), tetrahydrobiopterin (BH4), and total antioxidant capacity (TAC), were homogenized in 5mM phosphate buffer at pH 7.4 and were centrifuged at 16,000*g* for 15 min at 10 °C. (Sorvall SR70, Thermo Scientific Inc., Urbana, IL, USA). The supernatants were filtered with a 0.22 µm nitrocellulose filter (Millipore, Billerica, MA, USA), and they were reserved at 4-8 °C for later analysis.

For the determination of cGMP, the ventricular tissue was homogenized in a 100 mM phosphate buffer at 4 °C and a pH of 7.5 ± 0.05. Centrifugation (Sorvall SR70, Thermo Scientific Inc., Urbana, IL, USA) was performed at 16,000*g* for 15 min at 10 °C. The supernatant was diluted 1:10 with 0.05M NaOH and filtered with 0.22 µm filters (Millipore Billerica, MA, USA), and it was reserved at 4-8 °C for later analysis.

2.3.2. Sample analysis

2.3.2.1. Nitric oxide

The determination was made directly in the supernatant (obtained in point 2.3.1.) by a spectrophotometric method in the UV-Vis region (490nm) and a

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DW2000 spectrophotometer (SLM-Aminco, SLM Instruments Inc., Urbana Illinois, USA). [25]

2.3.2.2. Total antioxidant capacity

The determination was made directly in the supernatant (obtained in point 2.3.1.). A spectrophotometric method was used to TAC quantification. Samples were analyzed in UV-Vis region at a room temperature (Cary 4000, Varian Inc., Mulgrave, Victoria, Australia) at 490 nm. [26]

2.3.2.3. Tetrahydrobiopterin

The supernatant (obtained in point 2.3.1.) was diluted 1:10 with 0.01M NaOH before starting the analysis. The determination of BH4 levels in ventricular tissue was performed by capillary zone electrophoresis with UV-Vis detection by diode array using the methodology of Han. [27]

The sample was passed through a Sep-Pak Classic C18-NH2 cartridge (Waters, Urbana IL, USA) and directly analyzed with a P/ACETM MDQ (Beckman Coulter Inc., Fullerton, CA, USA) at 30 kV for 6 minutes and a wavelength of 230 nm at 10 °C using a running buffer (0.1 M Tris - 0.1 M boric acid - 2mM EDTA, pH 8.75). The samples were injected under hydropneumatic pressure of 0.5 psi/10 s.

2.3.2.4. Cyclic guanosin monophosphate

The evaluation of cGMP levels in ventricular tissue sample (obtained in point 2.3.1.) was performed by capillary zone electrophoresis (P/ACETM MDQ system; Beckman Coulter Inc., Fullerton, CA, USA). [28] The ventricular tissue homogenate

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filtrate was deproteinized with cold methanol in a 1:1 ratio. Centrifugation was performed at 16'000g for 10 min (Sorvall SR70, Thermo Scientific Inc., Urbana, IL, USA) and the supernatant was diluted 1:10 with 0.05M hydrochloric acid and filtered with a 0.22 µm nitrocellulose membrane (Millipore Billerica, MA, USA) and directly analyzed. The separation was carried out at -25 kV for 15 min at 190 nm using a running buffer (40 mM citric acid+0.8 mM CTAB at pH 4.4).

2.4. Determination of calcitonin gene related peptide

The CGRP analysis was carried out according to Seon *et al* method. The processed samples were purified by preparative reverse-phase HPLC (ACQUITY UPLC System, Waters Corporation, Barcelona, España) on a Waters RCM compact preparative cartridge Delta-Pak C 18 (300 Å; 25 3 100 mm/Waters, Barcelona, España) eluted at a flow rate of 8mL/min by a linear gradient of acetonitrile in 0.1% trifluoroacetic acid in water (5min; wash at 5% acetonitrile followed by a 5–60% linear gradient of acetonitrile at 0.5%/min). The purified sample was subjected to a HPLC analysis (ACQUITY UPLC System, Waters Corporation, Barcelona, España), using Lichrosorb C18 column (5 mm, 4.6x250 mm/Phenomenex, CA, USA), and lineal acetonitrile gradient (20-58%) at a flow rate of 0.75 mL/min, and detection to 220 nm at 10 °C. The concentration of the CGRP was determined by a standard curve of 0-100 fmol/mL. [29]

2.5. Damage indicators determinations (PDE-3 and MDA)

2.5.1. Phosphodiesterase 3

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To quantify PDE-3 levels in ventricular tissue [30], tissue was homogenized in cold buffer with 100 mM saccharose+20 mM HEPES+50 mM citrates at pH 5.6. Centrifugation was performed at 16,000*g* for 15 min at 10 °C (Sorvall SR70 centrifuge, Thermo Scientific Inc., Urbana, IL, USA). The supernatants were filtered with 22-µm nitrocellulose filters (Millipore Billerica, MA, USA); and the analysis was carried out directly. All samples were stored at -70 °C until the day of analysis.

The analysis of PDE-3 levels was carried out in ventricular tissue by capillary zone electrophoresis, using UV-Vis detection by diode array. The filtrate was deproteinized with cold methanol, cold trichloroacetic acid in a ratio of 10:1. Centrifugation was performed at 16'000g for 15 min at 10 °C (Sorvall SR70, Thermo Scientific Inc., Urbana, IL, USA). The filtering was carried out with a 0.22 µm nitrocellulose membrane (Millipore Billerica, MA, USA). It was subsequently diluted 1:10 with cold 0.1 M sodium hydroxide. Sep-Pak Classic C-18 cartridge (Waters, Urbana IL, USA)

The sample was passed through a 100 mM citrate buffer and pH 2.5. The analysis was performed directly with P/ACETM MDQ System (Beckman Coulter Inc., Fullerton, CA, USA). The separation was carried out at 20 kV for 30 min at 240 nm at 20 °C and a running buffer (100 mM boric acid at pH 2.8). Hydrodynamic pressure 0.5 psi/10s.

2.5.2. Malondialdehyde

The supernatant was diluted 1:10 with 0.1 M NaOH before starting the analysis. MDA was determined in ventricular tissue by capillary zone

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electrophoresis (P/ACETM MDQ System; Beckman Coulter Inc., Fullerton, CA, USA). The separation was carried out at -20 kV for 4 min at a wavelength of 267nm at 10 °C using a 100mM borate buffer+0.5 mM CTAB at pH 9.0. The samples were injected at a hydropneumatic pressure of 0.5 psi/10 s. [31]

2.6. eNOS, pNOS, iNOS and TRPV1 detection.

The tissue was homogenized in a mortar with liquid nitrogen and incubated 1 hour in lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 15 mM Imidazole, 10 % Glycerol, 100 mg/10 mL DOC, 1% Triton X-100) supplemented with a mixture of protease inhibitors (leupeptin, aprotinin, pepstatin, PMSF) (Sigma Chemical Co., St. Louis, Missouri, USA). Protein was determined by the Bradford method (Bio-Rad laboratories) [32]. 50 µg of the Ventricular tissue homogenate was mixed with 3X loading buffer (20% glycerol, 4% SDS, 0.02% bromophenol blue, 0.2% 2-mercaptoethanol, 125 mM Tris, pH 6.8) and heated for 5 min at 100 °C. SDS-PAGE to 8% was used to separate the proteins eNOS, pNOS, iNOS and TRPV1. Then the proteins were transferred to PVDF membrane (0.22 µm). The blots were blocked for 1h at room temperature using Tris saline buffer plus 0.01% Tween (TBS-T) and 5% non-fat dehydrated milk. Afterwards, membranes were incubated with primary antibodies overnight in a 1:1000 dilution, at 4 °C. The primary antibodies used were mouse anti-e-NOS, mouse anti-p-NOS, mouse i-NOS (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rabbit anti-TRPV1 (from Sigma-Aldrich, USA). TBS-T buffer was used to rinse the membranes four times and then membranes were incubated overnight at 4 °C with horseradish peroxidase conjugated secondary antibodies at a dilution of 1:10,000 (Santa Cruz

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Biotechnology, Santa Cruz, CA, USA). All blots were incubated as a control with the β-Actin antibody (sc-32251) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). A chemiluminescence assay (Clarity Western ECL Substrate, Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used for protein detection. X-ray films (AGFA, Ortho CP-GU, Agfa HealthCare NV, Mortsel, Belgium) were used to detect the emitted chemiluminescence. A GS-800 densitometer (including Quantity One software from Bio-Rad Laboratories, Inc.) was employed to acquire the images from each film. We expressed the values of each band density as arbitrary units (AU)

2.7. Histological sections

Another group of animals under the same experimental conditions had their hearts extracted and fixed in formaldehyde to make histological sections. Cross sections of the heart were obtained which included the heart tissue and the left and right ventricular chambers. The sections were washed in a 0.9% NaCl solution and fixed in 10% formaldehyde and pH 7.4. Once the tissue was perfectly fixed, hematoxylin-eosin markings were made.

The analysis of the marked histological sections was carried out with a Carl Zeiss light microscope (Carl Zeiss Axio Imager Z2, West Germany objective EC Plan-Neofluar 10x) and with an HP Z800 computer and an HP ZR30W screen. The photomicrographs were analyzed by densitometry using the SigmaScan Pro 5 Image Analysis software.

3. Statistical Analysis

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Results are expressed as mean ± standard error of the mean (SEM). Differences were considered statistically significant when $p \leq 0.05$. The different symbols in figures indicate significant differences. We applied a one-way analysis of variance (ANOVA) followed by a Tukey post hoc test using the SigmaPlot program version 11 (Jandel Scientific, San Jose, CA, USA).

4. Results

4.1. Cardiac work

The Langendorff´s model of the isolated heart allows for the study of the mechanical activity of the heart under three conditions: normal perfusion, ischemia and ischemia+reperfusion. Figure 1A shows that the cardiac work of the group without ischemia and reperfusion remained constant during the 120 minutes that the experiment lasts (approximately 23,000 mmHg/beats/min^{-1}, (black circles)). This curve was compared to that of another group of hearts in which a global ischemia of 30 minutes was induced and later, reperfusion was allowed for 60 minutes (Figure 1A, open circles). During reperfusion, the mechanical function of the heart was restored to an 80% but it did not reach 100%.

Figure 1B, shows the curve for the cardiac work of hearts obtained from rats after the CS treatment. These hearts were exposed to a period of global ischemia of 30 minutes. It is noteworthy that during reperfusion, the heart completely recovered its normal mechanical activity. The action of the CS treatment was compared in Figure 1C with that from a group of hearts obtained from rats treated

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with CZ and from the group of rats treated with CZ+CS. In both groups, cardiac work was decreased since the period prior to ischemia and it remained low during reperfusion, when compared with the group treated with CS.

4.2. NO, BH4 and cGMP

To explain the changes in cardiac work produced by treatments and by ischemia and reperfusion damage, we took ventricular tissue samples to evaluate important factors that participate in the NO pathway such as NO, BH4, and cGMP levels. Figure 2A shows that in the hearts of animals treated with CS and that were not subjected to ischemia, had higher levels of NO (7.6 ± 0.9 pmol/mg of protein) with respect to the control (3.3 ± 0.52 pmol/mg of protein). CS actions were significantly inhibited by CZ to 1.3 ± 0.15 and by CZ+CS to 1.4 ± 0.2 pmol/mg of protein. In hearts with global ischemia for 30 min, CS maintained NO values without significant changes with respect to the control hearts (2.8 ± 0.6 and 1.8 ± 0.8 pmol/mg of protein). Treatments with CZ and CZ+CS decreased the action of CS (to 0.3 ± 0.09 and to 0.9 ± 0.13 pmol/mg of protein respectively).

During reperfusion, CS maintained NO at the same level as in control hearts (3.6 ± 1.2 and 3 ± 0.17 pmol/mg of protein). These levels were decreased by CZ (to 0.4 ± 0.02 pmol/mg of protein). The CZ+CS combination did not induce significant changes.

We measured the BH4 levels in the heart. BH4 is an essential cofactor for NO synthesis due to its molecular coupling with eNOS. Figure 2B shows that the BH4 levels are modified in a similar way to those of NO (panel A) (3.8 ± 0.7

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pmol/mg of protein). In the hearts of CS group and that were perfused for 1 hour the BH4 levels were increased (8.7 ± 0.7 pmol/mg of protein). This effect was diminished by CZ (to 1.7 ± 0.2 pmol/mg of protein) and by CZ+CS (to 1.8 ± 0.3).

In the period of global ischemia, the BH4 levels in the group with CS were of 3.1 ± 0.4 pmol/mg of protein. They were significantly decreased in the groups treated with CZ and with CZ+CS (to 0.6 ± 0.1 and to 0.8 ± 0.1 pmol/mg of protein respectively). During reperfusion the levels of BH4 were significantly decrease with CZ (to 0.8 ± 0.1) and with CZ+CS (to 1.8 ± 0.3 pmol/mg of protein) with respect to the group with CS (8.6 ± 2 pmol/mg of protein).

cGMP plays a preponderant role for vessel relaxation when the NO pathway is activated. Figure 2C shows that the levels of cGMP in the controls hearts during perfusion and ischemia do not change significantly; however, there is a tendency to increase the levels of cGMP in the control hearts during reperfusion. In the of perfusion period the effect of CS was inhibited by CZ and by CZ+CS (to 0.192 ± 0.03 and to 0.196 ± 0.009 pmol/mg of protein respectively). During ischemia, cGMP levels in the heart decreased with CZ (to 0.051 ± 0.017) and with CZ+CS (to 0.22 ± 0.038 pmol/mg of protein respectively) with respect to the group with CS (0.483 ± 0.103 pmol/mg of protein). During reperfusion, cGMP levels decreased with CZ (to 0.076 ± 0.004 pmol/mg of protein) and with CZ+CS (to 0.22 ± 0.038) with respect to CS group (1.337 ± 0.42 pmol/mg of protein).

4.3. TAC and CGRP

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The activation of TRPV1 was reflected in an increase of TAC and CGRP levels (Figure 3). Figure 3A shows that during the perfusion period the TAC was significantly increase by the treatment with CS when compared to the control (0.021 ± 0.004 to 0.041 ± 0.003 mmol/mg of protein). This increase was inhibited by CZ (to 0.008 ± 0.0011) and by CZ+CS (to 0.005 ± 0.004 mmol/mg of protein).

In the ischemic period, the TAC (in the group without treatment and in the group with CS) remained at a level without significant differences with respect to the control of the perfusion period. The CZ and by CZ+CS significantly decreased its level (to 0.002 ± 0.0005 and 0.008 ± 0.0013 mmol/mg protein, respectively). A similar effect could be observed during reperfusion since the effect of CS (0.051 ± 0.012) was inhibited by CZ and by CZ+CS (0.003 ± 0.0001 and 0.008 ± 0.0013 mmol/mg of protein respectively).

CGRP is a neuropeptide that has recently been related to the vasodilator actions of NO. Figure 3B shows that in the perfusion period the CS significantly increased the levels of CGRP in comparison with the control (0.012 ± 0.002 to 0.042 ± 0.004). This effect was inhibited by CZ (0.004 ± 0.001) and by CZ+CS (0.003 ± 0.001 fmol/mg of protein). During ischemia, the CS kept the CGRP level very close to the baseline value (0.013 ± 0.001). This level was decreased by CZ (to 0.001 ± 0) and by CZ+CS (to 0.005 ± 0.001 fmol/mg of protein).

During reperfusion, there was an important and significant increase of CGRP in the CS group with respect to the control (0.009 ± 0.002 to 0.04 ± 0.007 fmol/mg of protein) taking into account that the damage due to ischemia and

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reperfusion had already occurred. CS-stimulated CGRP production was inhibited by CZ (to 0.003 ± 0.001) and CZ+CS (to 0.003 ± 0.001 fmol/mg of protein).

4.4. MDA and PDE3 levels

The activation of TRPV1 was reflected by alterations in the levels MDA and PDE-3. The levels of MDA and PDE-3 with the CS treatment remained approximately at the same as in their respective controls during perfusion, ischemia and reperfusion. In the perfusion period PDE-3 was significantly increased in the hearts of rats treated with CZ (to 0.173 ± 0.013) and to a lesser extent but maintaining a high level with CZ+CS (to 0.086 ± 0.007 pmol/mg of protein) with regard to CS group (0.13 ± 0.007).

During ischemia, PDE-3 levels were increased when compared to hearts in the perfusion period without treatment (0.025 ± 0.003 to 0.117 ± 0.013 pmol/mg of protein). CS regulated PDE-3 levels and kept them at 0.055 ± 0.016 pmol/mg of protein. With respect to CS group, the PDE-3 increased with CZ (to 0.173 ± 0.013) and with CZ+CS (to 0.193 ± 0.021 pmol/mg of protein). With the restoration of coronary flow, there was a tendency to reestablish baseline PDE-3 values, except with the CZ+CS treatment, with which the level remained significantly elevated when compared to the CS group.

4.5. Lipoperoxidation

The lipoperoxidation biomarker MDA maintained the same basal levels during the perfusion period in the control (0.002 ± 0.0001) and in the CS group (0.0015 ± 0.0001). MDA significantly increased with CZ (to 0.0.0112 ± 0.003) and

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with CZ+CS (to 0.0087 ± 0.002 pmol/mg of protein). In the ischemia period, CS maintained a low level of MDA (0.0056 ± 0.001) when compared to its control (0.0087 ± 0.0025), the CZ treatment (0.0219 ± 0.003) and CZ+CS treatment (0.027 ± 0.005 pmol/mg of protein).

This same tendency was observed in reperfusion. In contrast, treatments with CZ and with CZ+CS raised MDA levels (to 0.021 ± 0.002 and to 0.011 ± 0.002 pmol/mg of protein) in comparison with the CS group (0.002 ± 0.0004).

4.6. Nitric oxide synthase

Figure 4. shows that there are no important modifications under any of the experimental conditions in the expression of total eNOS. Panel A shows the relative density of eNOS in control hearts with perfusion of 30 and 120 min, ischemic hearts, and hearts with ischemia and reperfusion. Panels B, C and D also show the relative density of eNOS under the same conditions as in panel A but in hearts of rats treated with CS, CZ and CZ+CS respectively.

Figure 5 shows the results for the levels of phosphorylated NOS (pNOS) (panel A). In control conditions, pNOS tends to decrease after two hours of perfusion and during ischemia. There is a significant decrease in the relative density of pNOS in the ischemia-reperfusion condition. Treatments with CS or CZ did not show significant changes. Surprisingly, treatment with CZ+CS shows an increase in the relative density of pNOS during ischemia and during reperfusion.

Panel B shows that the levels of iNOS had no modifications in the hearts of animals without treatment and under perfusion conditions of 30 and 120 min, as

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well as during ischemia and reperfusion. Treatment with CS (panel B-2) decreased the levels of iNOS at 30 and 120 min of perfusion, with respect to the hearts of animals without treatment (B-1). The levels of iNOS increased significantly during ischemia and during reperfusion with respect to its control (perfusion 30 min) with the treatment with CS. Treatment with CZ (panel B-3) did not generate changes in the levels of iNOS, which were maintained at the same level as that observed in hearts of animals without treatment. With the combined treatment CZ+CS (panel B-4), the levels of iNOS significantly increased during ischemia and decreased with reperfusion.

4.7. TRPV1 levels and histological changes

Figure 6-A (control group) shows that the levels of TRPV1 increased with the perfusion time (120min) and with ischemia and reperfusion. Treatment with CS (6-B) significantly increased the levels of TRPV1 during ischemia and they remained at baseline levels during reperfusion. Treatments with CZ and with the combination CZ+CS did not show changes in the levels of TRPV1

The histological sections (Figure 7) do not show important modifications in the conformation of the cardiac tissue. However, a tendency to damage was observed in the tissue with ischemia and reperfusion. This tendency to damage was prevented in the groups treated with CS and with CS+CZ.

5. Discussion

In this paper we tested the participation of TRPV1 in the possible restoration of cardiac function in ischemia and reperfusion. We explored how TRPV1

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participates in the regulation of mechanical work and some components of nitric oxide pathway in the heart and on oxidative stress. We also explored the expression of iNOS, eNOS, pNOS and TRPV1 and analyzed by fluoresce the changes in cardiac tissue on each experimental condition. Damage that occurs to the myocardium during ischemia is correlated to changes in temperature, increases in pH, decreases in mitochondrial ATP and retention of lactic acid, among others. Then, the ardioprotective pathways such as NO and protein kinase G (PKG) are activated. [33] It is possible to restore cardiac function and decrease the size of the infarct in AMI conditions as proven by the studies made by Jennings *et al* in 1960, (on cardiac damage due to ischemia and reperfusion) [34,35] and the studies by Murry *et al* in 1986 (on pre-conditioning and post-ischemic conditioning). The restoration depends on the time of exposure to ischemia. [8, 36]. The role of TRPV1 in cardioprotection has been studied in different preconditioning, postconditioning and remote preconditioning models [37-39] but the mechanism of transmission of signals that are involved in cardioprotection have not yet been elucidated.

Preclinical studies have revealed that the efficacy of dietary CS is greater at low doses and it can modulate processes such as adipocyte browning or the activation of metabolic modulators, including AMP protein kinase (AMPK), peroxisome proliferator-activated receptor α (PPARα), uncoupling protein 1 (UCP1), USP2 and glucagon-like peptide 1 (GLP-1). [40] Moreover, CS blocks damage by anoxia/reoxygenation in cardiomyocytes by regulating mitochondrial function, preventing the opening of the mitochondrial pore and the release of

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cytochrome-C. Under these conditions, the reduction and control of reactive oxygen species (ROS) is promoted. [41] We also used as an antagonist CZ and as expected, CZ, produced damage since the pre-ischemic period and CZ inhibited the actions of CS during reperfusion.

In this paper we found that after exposure to global heart ischemia for 30 min, the stunned heart does not return to its normal mechanical activity with reperfusion. (Fig. 1A) This allowed us to compare and demonstrate that the activation of TRPV1 at the systemic level by the administration of CS reversed the ischemic and reperfusion damage in the isolated and perfused heart. (Figure 1B). We found that with CZ the heart loses 40% of its mechanical activity. Treatment with CZ+CS also generated a significant decrease in cardiac work. This affectation by both of these treatments is adding to ischemic and reperfusion damage. (Fig. 1C) Therefore, CS actions were inhibited by CZ and part of the explanation to this phenomenon is that CZ inhibits the flow of Ca^{2+} and modifies NO levels. It is possible that alteration in NO levels and Ca^{2+} flux is decisive to decrease the mechanical work. (Figure 1C). It is possible that in the CZ+CS treatment there is a greater molecular influence of CS. (Figure 5)

With the increase in the levels of NO, BH4 (Figure 2 A y B) and CGRP (Figure 3 B) in the heart due to activation of TRPV1 before ischemia-reperfusion found in this study, we can affirm that these factors are part of a myocardial protection pathway. In addition, these variables were not modified in ischemia and reperfusion and adequate levels of these factors were maintained. A reflection of this improvement is that the TAC was modified accordingly (Figure 3 A).

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Regarding the role of TRPV1 during ischemia, its expression was significantly increased (Figure 6). Therefore, we think that the activation of TRPV1 generated a possible preventive state corresponding to myocardial protection by eliminating ischemic and reperfusion damage. Thus, the question that arises is how can CS generate this cardioprotective state? One possibility is that the activation TRPV1 improves Ca^{2+} regulation, which is one of its well-known functional characteristics. It is also possible that it could also induce a mitochondrial protective state (this is more than evident since the mitochondria are involved as effector organelles in cardioprotection (as Bøtker *et al* say). [8]

Since the expression of TRPV1 and the levels of CGRP in cardiac tissue increased during ischemia and during reperfusion while PDE-3 and MDA decreased, we propose that CGRP may be one of the mediators of the cardioprotection observed in this study. The fact that TRPV1 is expressed during ischemia allows us to speculate that TRPV1 inhibits the opening of the mitochondrial transition pore and regulates Ca^{2+} flux which leads to its possible participation in the control of the cell damage pathways that are activated during ischemia. [42] We are therefore working on the design of a study at the level of the mitochondria of the heart to elucidate the cardioprotective actions of TRPV1. Although the TRPV1 protein has been mainly visualized in neuronal cells, there are studies that show its presence in the mouse heart (for example). TRPV1 has been detected in mitochondrial fractions. [16]

Due to an imbalance between the levels of NO and the cofactors for its synthesis such as BH4, there is a change in the levels of cGMP and an increase in

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ROS. Under these conditions, the levels of biomarkers of cell damage increase. In this study we also analyzed the modifications in the levels of PDE-3 and MDA by the pharmacological activation or inhibition of TRPV1. The levels of both biomarkers increased in response to ischemia and reperfusion. However, treatment with CS decreased and controlled their levels. In other words, the activation of TRPV1 synchronized the NO pathway reactions that were altered by the ischemia and reperfusion events.

In our analysis, we did not find modifications in the expression of eNOS (Figure 4) with any of the treatments or with perfusion or ischemia and therefore, there was no effect on the constitutive state of the enzyme. However, when we analyzed the expression of pNOS, we found that the activation of TRPV1 generated some differences. For example, the pNOS levels decreased in hearts with each treatment with respect to the hearts of animals without treatment. Interestingly, there is a tendency of a recovery of relative density in reperfusion in CS treated group.

Due to the modifications observed in the relative density (Figure 5) of the iNOS of the CS group, we propose that the activation of TRPV1 is regulating this enzyme (because the increases observed during ischemia and during reperfusion were not different from those observed in ischemia and reperfusion of the control groups and with CZ or in the ischemia of the CZ+CS group). We do not have an explanation for the modification in the reperfusion of the CZ+CS group. It may be that the action of CS is more evident with the combination treatment. However, more observations in this regard are needed.

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On the other hand, we were surprised that the relative density of TRPV1 increased with ischemia and reperfusion in the hearts of untreated animals. It is possible that it is part of a preventive response mechanism to the damage that is being generated.

As we know, if NO levels in the heart are altered, either due to disease or due to ischemia and reperfusion as is in our study. Oxidative stress is increased and damage signals are activated. In healthy animals, this occurs even with the application of drugs in high doses. An imbalance in NO levels reduces its bioavailability because it rapidly interacts with ROS, causing an enzymatic uncoupling between eNOS and BH4, therefore producing inhibition in the formation cGMP. Activation of TRPV1 with CS maintains the increased levels of cGMP during ischemia. It is possible that the increased cGMP functions as part of the mechanism for preventing damage and of the reparation processes. In reperfusion, TRPV1 levels return to normal values because, as we have seen, this receptor is regulated by stimuli from its environment such as shear stress, changes in temperature and pH that occur in reperfusion. With CS, the regulation of the NO pathway is restored and the damaged pathways are repaired.

With respect to the histological studies of cardiac tissue, we did not find important changes. Since the global ischemia was of only 30 min and this may be a short time to cause histological changes. This correlates with cardiac work, since with ischemia and reperfusion damage, the heart affects its mechanical activity by only a 20 or 25%. There were also variations in NO levels that did not affect the

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mechanical activity of the heart or TAC, as can be observed in hearts of healthy animals with CS treatment that were perfused for one hour.

Therefore, when the proper functioning of the heart is compromised, as in ischemia and reperfusion, the activation of TRPV1 may protect the heart by regulating oxidative stress, the NO pathway and by controlling the flow of Ca^{2+} to prevent damage to the heart tissue. With this evidence, the possibility exists that TRPV1 is related to the mechanisms necessary to keep mitochondrial function in synchrony.

6. Conclusion

We can affirm that the activation of TRPV1 participates in the correction and control of alterations in the levels of NO, cGMP, BH4 and CGRP caused by ischemia reperfusion damage. The damage is manifested by the modifications on the mechanical activity of the heart. TRPV1 also controls the levels of MDA and PDE-3 in cardiac tissue. Our conclusion is that TRPV1 is involved in the regulation of the NO pathway and on the production of reactive oxygen species, through a mechanism that may be mediated by CGRP.

Conflicts of Interest

The authors declare no conflict of interests.

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Vicente Castrejón Téllez and Leonardo del Valle Mondragón share the first authorship of this paper.

Author contributions

Vicente Castrejón Téllez participated in design of the experimental protocol and treatment and analysis of samples for expression of metabolites.

Leonardo del Valle Mondragón applied the different measurement and analysis techniques of tissue samples.

Israel Pérez Torres participated in the treatment of samples for tissue cuts. The analysis of the marked histological sections and analysis of the results.

Verónica Guarner Lans and Gustavo Pastelín Hernández participated in the review and correction of the work.

Angelica Ruíz Ramírez participated in analysis of samples to determine protein expression and statistical analysis.

Elvira Varela López and Julieta Anabell Diáz Juárez performed isolated and perfused heart experiments according to Langendorff.

Álvaro Vargas González contributed with animal care, control and the application of different treatments.

Victor Hugo Oidor Chan contributed with design of the experimental protocol and analysis results.

Raúl Martínez Memije and Pedro Flores Chávez participated in the technical and interpretation needs with the Langendorff system.

Juan Carlos Torres Narváez contributed in design of the experimental protocol and the writing of the article.

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Figure captions

Figure 1. Cardiac work in isolated and perfused hearts with perfusion, global ischemia and reperfusion periods of rats without treatment and after treatment with CS, CZ and combination CZ+CS. Values are the mean ± SE (*n* = 10).

* Without I-R *vs* I-R; ** I-R *vs* CS+I-R; γ CS+I-R *vs* CZ+I-R; γγ CS+I-R *vs* CZ+CS+I-R.

Figure 2. Levels in cardiac tissue of cofactors for NO synthesis including cGMP, BH4 and NO. Values are the mean ± SE (*n* = 5).

*C *vs* CS; **γ** CS *vs* CZ; **β** CS *vs* CZ+CS.

Figure 3. Cardiac CGRP levels as an indicator of TRPV1 activation and TAC levels as an indicator of modifications in oxidative stress. Levels of tissue damage indicators PDE3 and MDA. Values are the mean ± SE (*n* = 5).

*C vs CS; γ CS vs CZ; β CS vs CZ+CS.

Figure 4. eNOS expression in isolated hearts from control and treated animals with CS, CZ and CZ+CS and exposed to different perfusion times (30 min and 120 min), ischemia (30 min) and ischemia-reperfusion (60 min). Values are the mean ± SE (*n* = 5).

Figure 5. pNOS (A) and iNOS (B) expression in isolated hearts of rats treated with CS, CZ and CZ+CS under conditions of perfusion (30 and 120 min), ischemia (30 min) and reperfusion (60 min). Values are the mean ± SE (*n* = 5).

A) * C; 30min *vs* I/R, A) #CZ+CS (30min vs I/R).
B) *CS (30min *vs* Isq); **CS (30min *vs* I/R); #CZ+CS (30 min *vs* Isq);. ##CZ+CS (Isq *vs* I/R)

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Figure 6. TRPV1 in isolated hearts of rats treated with CS, CZ and CZ+CS under conditions of perfusion (30 and 120 min), ischemia (30 min) and reperfusion (60 min). Values are the mean ± SE (*n* = 5).

*C (30min *vs* 120min); *CS (30min *vs* Isq); **CS (Isq vs I/R).

Figure 7. Heart tissue photomicrograph analysis in isolated hearts of rats treated with CS, CZ and CZ+CS under conditions of perfusion (30 min), ischemia (30 min) and reperfusion (60 min). Values are the mean ± SE (*n* = 5). The tissue was processed according to conventional histological procedures, and histological sections were made and stained by hematoxylin-eosin stain at 10x.

*CS; **CZ+CS; p=0.06; p=0.004.

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FIGURE 1

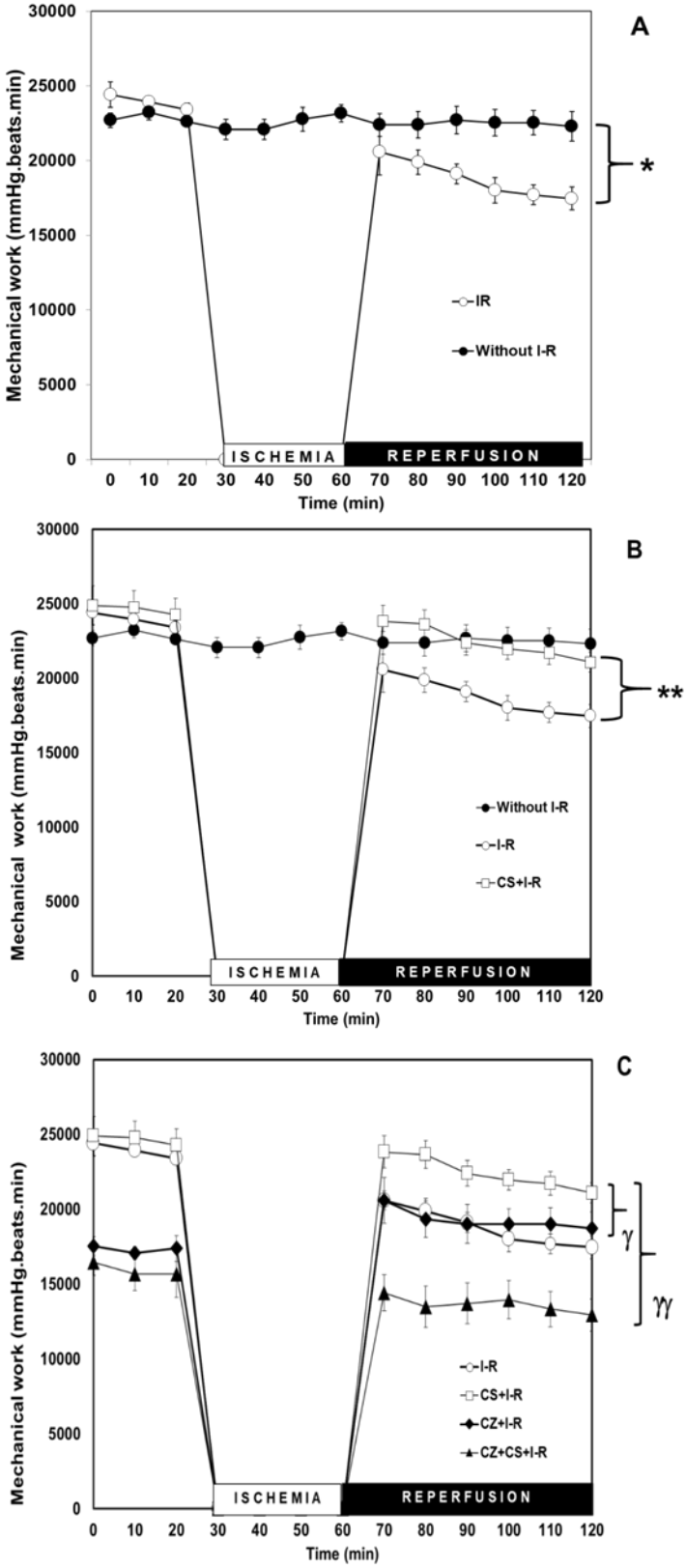

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FIGURE 2

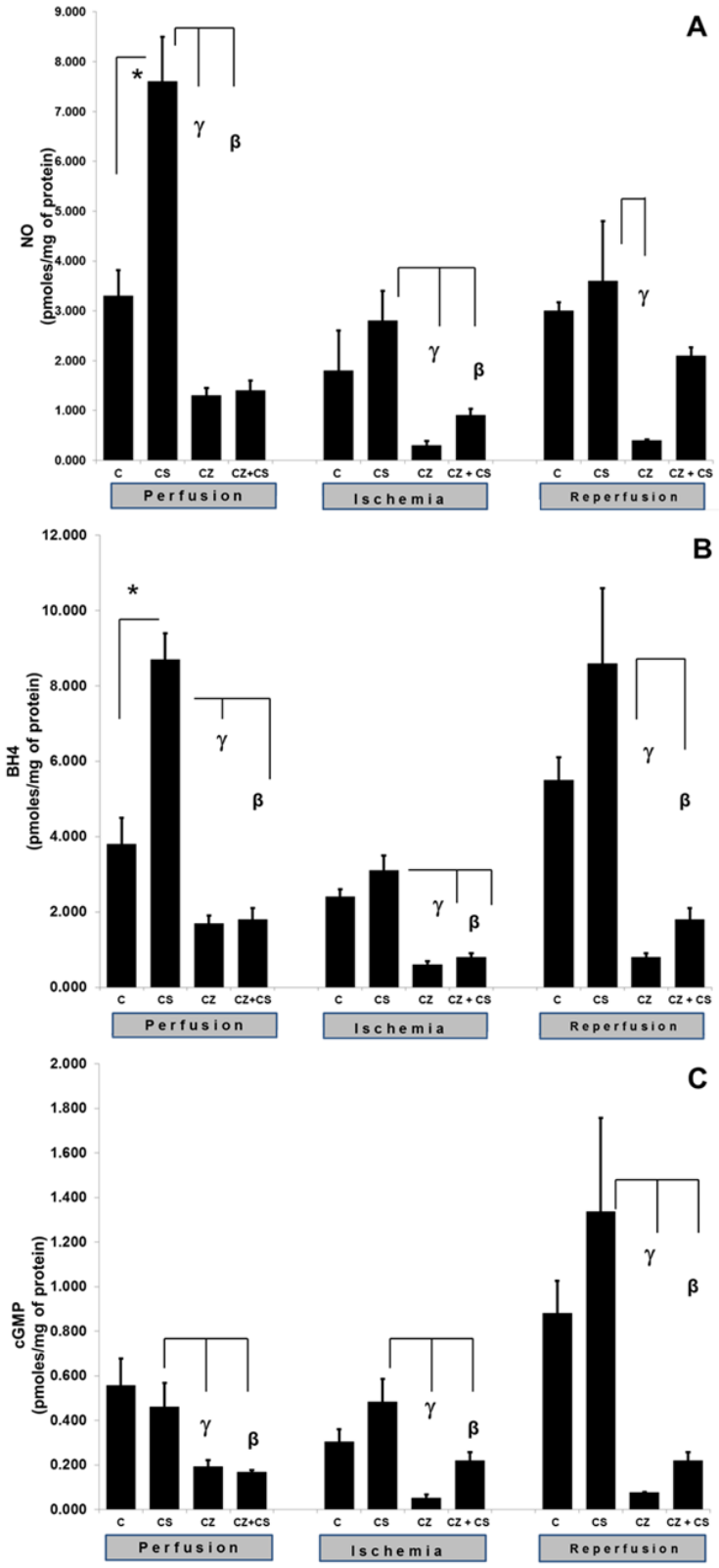

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FIGURE 3

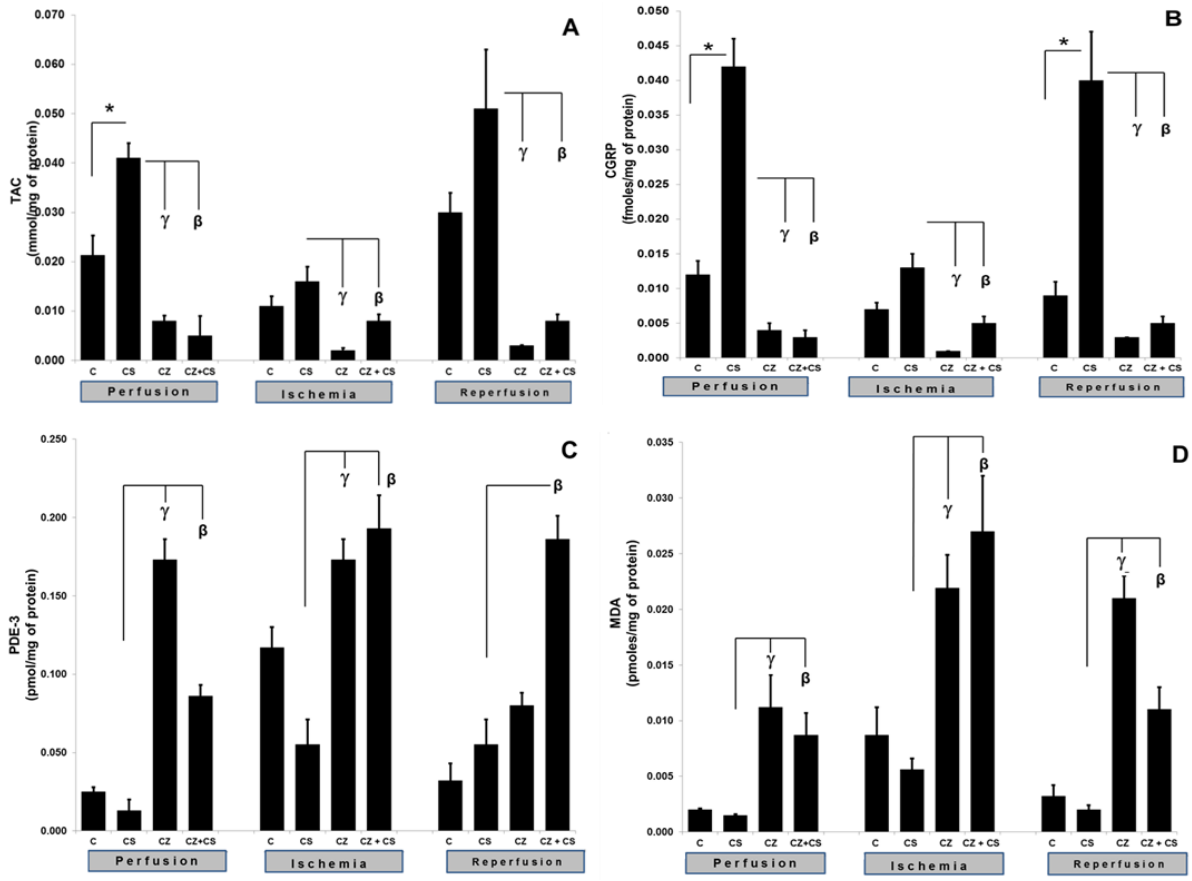

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FIGURE 4

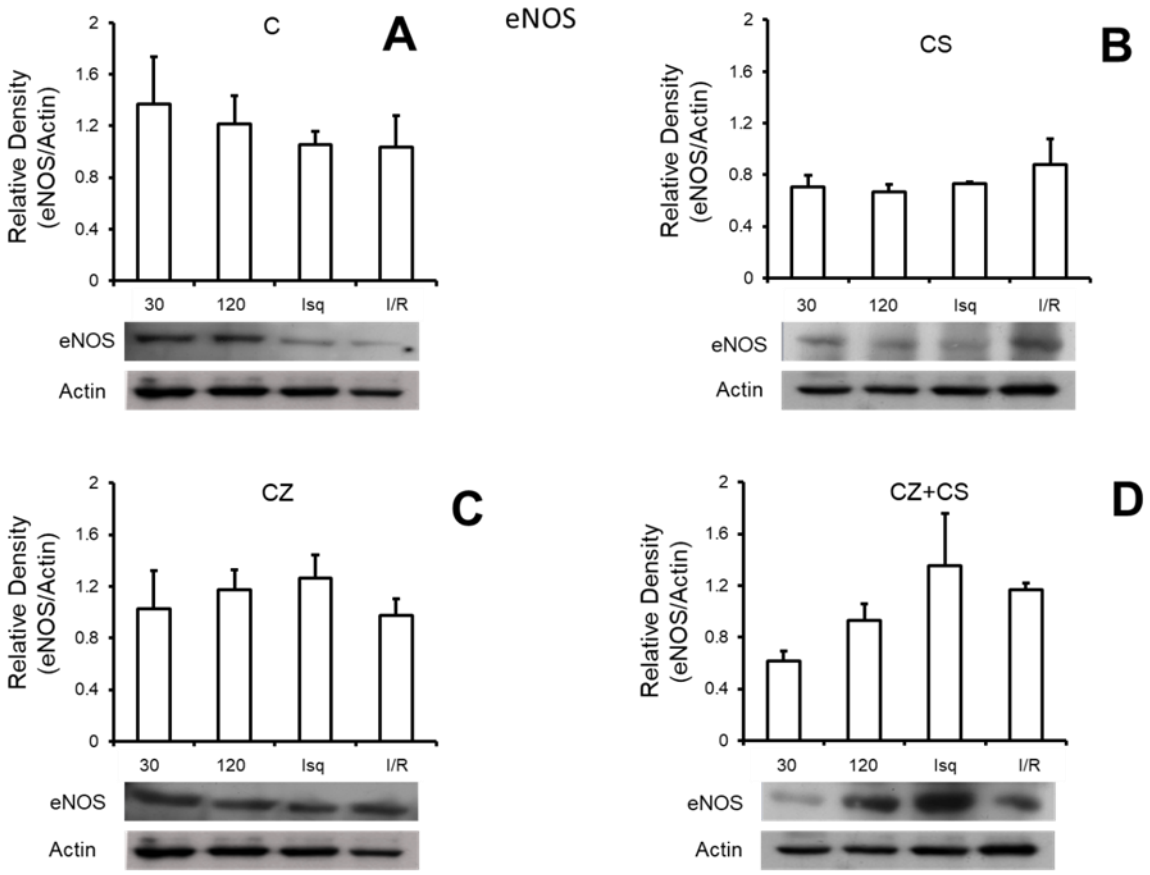

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FIGURE 5

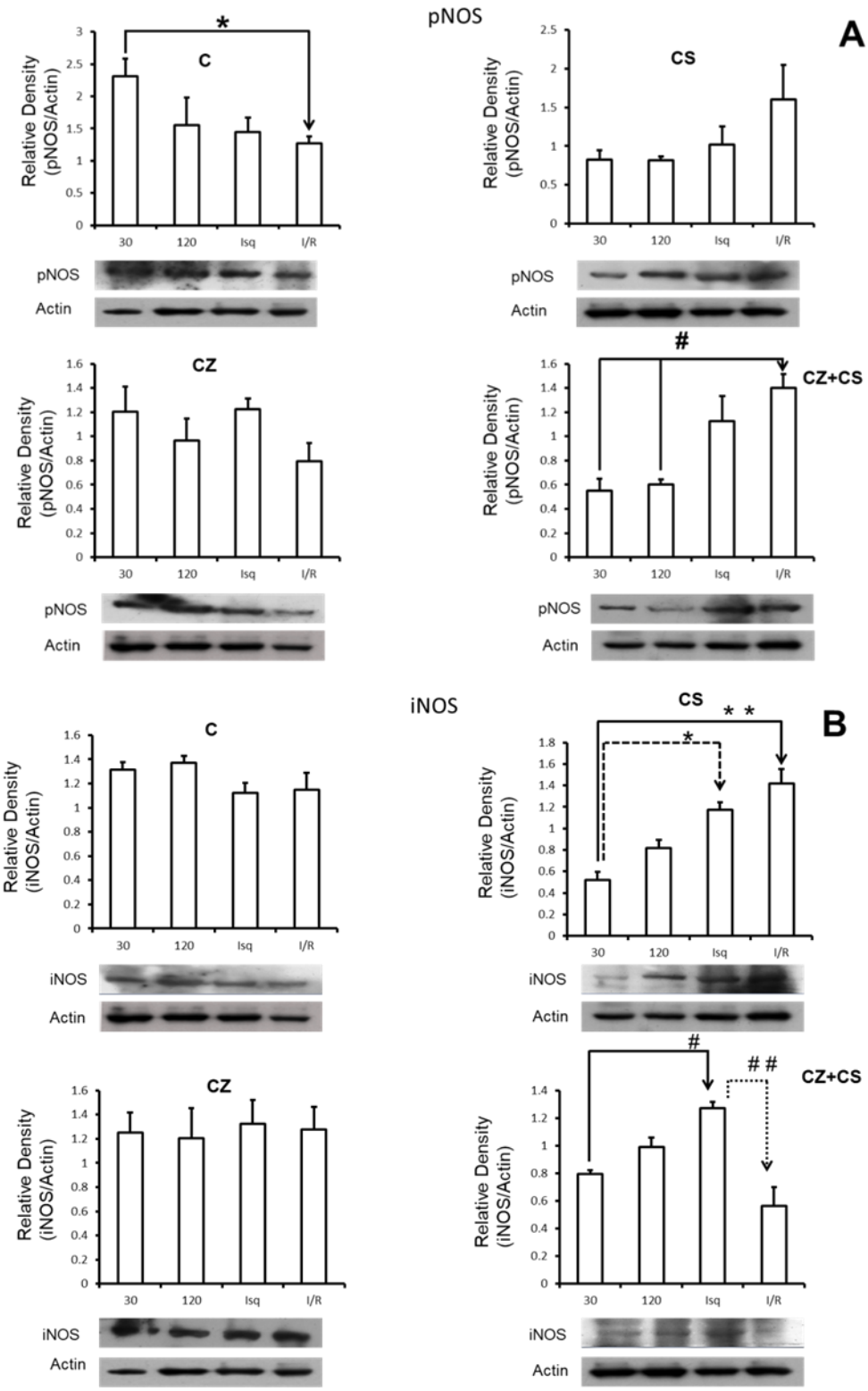

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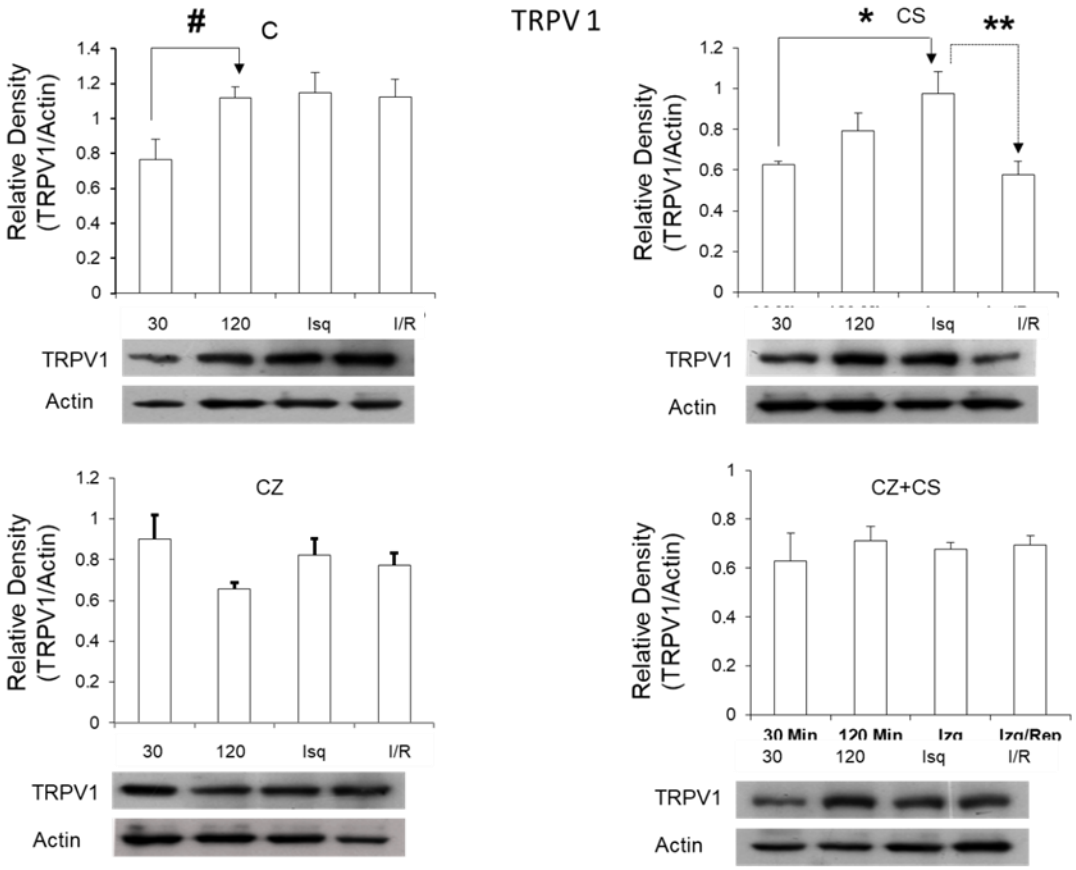
FIGURE 6



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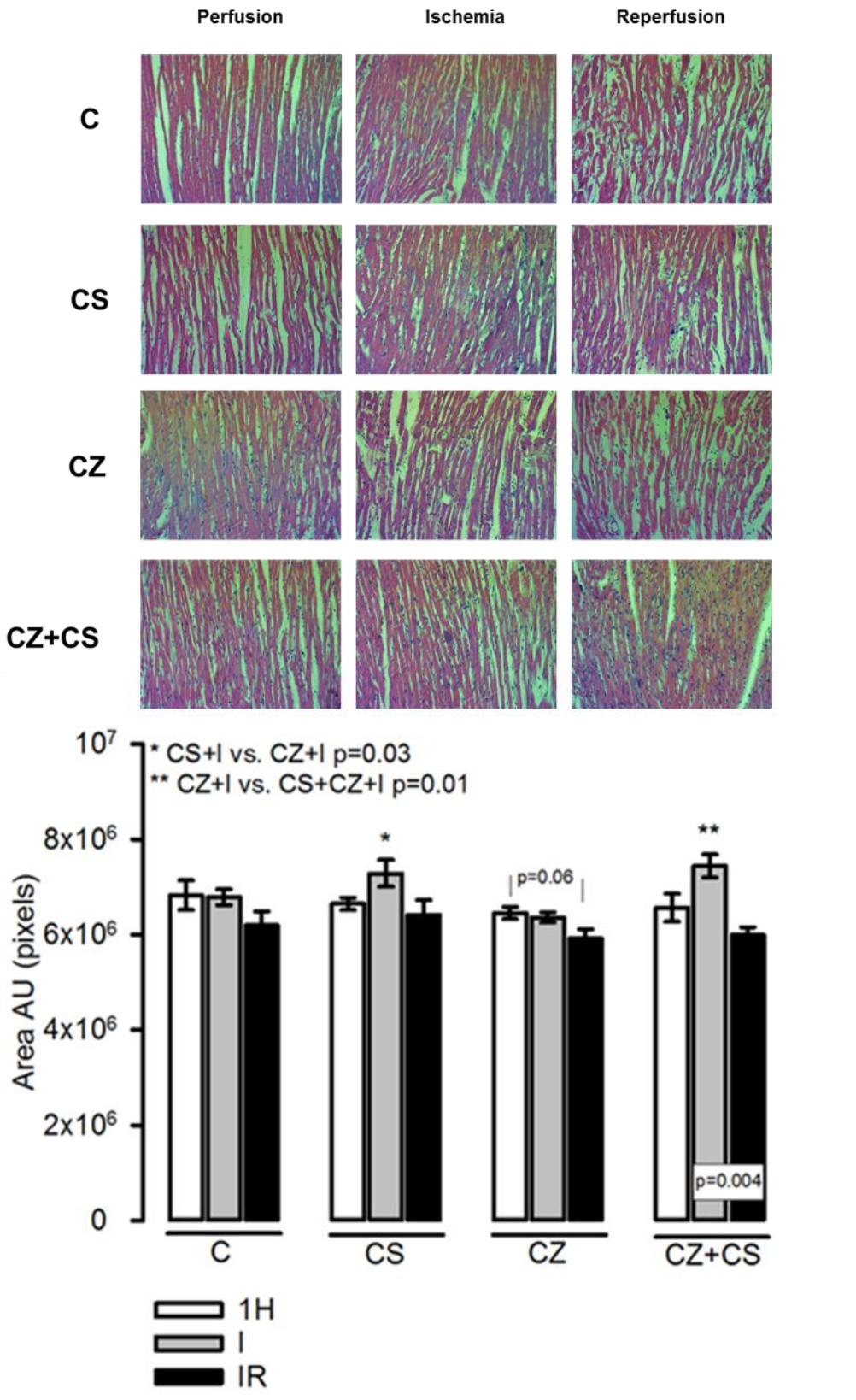
FIGURE 7