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Attenuation of Myocardial Ischemia-reperfusion Injury by Trimetazidine Derivatives Functionalized with Antioxidant Properties

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Running title: Cardioprotective effect of trimetazidine derivatives

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Abbreviations

AAPH - Azobis-2-amidonopropane dihydrochloride

DEPMPO - 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide

DTPA - Diethylene-triamine-pentaacetate

EPR - Electron paramagnetic resonance

LVDP - Left-ventricular developed pressure

ROS - Reactive oxygen species

TTC - Triphenyltetrazolium chloride

TMZ - 1-(2,3,4-trimethoxybenzyl)piperazine

TMZ-NH - 4-(2,2,5,5-tetramethylpyrrolinyl-3-)-1-(2,3,4-trimethoxybenzyl)piperazine

TMZ-ΦNH - 4-(2,2,5,5-tetramethyl-4-phenylpyrrolinyl-3-)-1-(2,3,4-trimethoxybenzyl)piperazine

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Abstract

Trimetazidine (TMZ), an anti-ischemic metabolic drug, is used to treat chest pain (angina pectoris). We hypothesized that derivatives of TMZ with antioxidant functions may improve the cardiac dysfunction caused by ischemia/reperfusion (I/R) above that observed with TMZ alone. Isolated rat hearts, perfused with Krebs-Henseleit buffer according to the Langendorff method, were subjected to 30 min of global ischemia followed by 45 min of reperfusion. Trimetazidine, TMZ-NH (TMZ modified with a pyrroline moiety) or TMZ-ΦNH (TMZ-NH with a phenyl substitute) were infused (50 μM) for 1 min before the onset of ischemia. Untreated (control) hearts at the end of 45 min of reperfusion showed a significant decrease in the recovery of coronary flow (42%), left-ventricular-developed pressure (22%) and rate-pressure product (25%) as compared to pre-ischemic baseline values. The I/R hearts also showed markedly increased lactate dehydrogenase (LDH) and creatine kinase (CK) activities in the coronary effluent, significant myocardial infarction (46% of risk area), and activation of Akt, ERK, and p38 MAPK. Pretreatment of hearts with TMZ-NH or TMZ-ΦNH showed significantly enhanced the recovery of heart function and decreased infarct size. The I/R-induced activation of Akt was further enhanced by TMZ-ΦNH. The present study demonstrated that TMZ-NH and TMZ-ΦNH significantly protected hearts against I/R-mediated cardiac dysfunction and injury. The protective effect of the TMZ derivatives could be due to the combined effects of antioxidant and anti-ischemic activities as well as enhanced pro-survival Akt activity.

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Introduction

Reactive oxygen species (ROS) can cause oxidative damage to a variety of cellular components. ROS plays an important role in the etiology of myocardial ischemia-reperfusion (I/R) injury (Brown et al., 1988; Ambrosio et al., 1993). During ischemia, the coronary blood supply to the heart is reduced or stopped preventing oxygen, glucose and fatty acids from reaching the target tissue (Weiss et al., 2003). Ischemia inactivates oxidative phosphorylation, leading to loss of adenine nucleotides and cytochrome *c*, accumulation of free phosphate, fatty acids and lactic acid, increased cellular calcium, and a decrease in cellular pH (Dennis et al., 1991). Upon reperfusion, oxygen interacts with the damaged mitochondrial respiratory chain to produce a burst of ROS leading to I/R injury (Brown et al., 1988; Ambrosio et al., 1993) Apart from the mitochondrial respiratory chain, the activations of xanthine oxidase, arachidonic pathway and NADPH oxidase have also been reported to contribute to the generation of ROS during I/R (Kloner et al., 1989; Kukreja and Hess, 1992; Griendling et al., 1997). Recently, several studies have demonstrated the involvement of Akt and mitogen-activated protein kinases (MAPKs) in mediating intracellular signal-transduction events associated with stress conditions including I/R (Das et al., 1996; Omura et al., 1999). MAPKs, namely, p38 MAPK, ERK1/2 and JNK are shown to be activated in hearts subjected to I/R (Yue et al., 1998). The activation of Akt- and MAPK-signaling cascades have been shown to modulate oxidant-mediated tissue injury (Shimizu et al., 1998; Armstrong, 2004). Several pharmacological agents are shown to be cardioprotective by modulating Akt, p38 MAPK or ERK1/2 activities (Toth et al., 2003; Liu et al., 2004; Takada et al., 2004; Hausenloy et al., 2005; Khan et al., 2006).

Trimetazidine, 1-(2,3,4-trimethoxybenzyl)piperazine dihydrochloride (TMZ), is a metabolic anti-ischemic drug that exerts its beneficial effects without altering the hemodynamic function of the heart (Lopaschuk et al., 2003). TMZ acts by optimizing cardiac metabolism by reducing fatty acid oxidation through the selective inhibition of mitochondrial 3-ketoacyl CoA

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thiolase (3-KAT). As a result, TMZ decreases ischemic stress and improves cardiac performance during ischemia (Kantor et al., 2000). At the cellular level, TMZ preserves ATP production and reduces intracellular acidosis and calcium-overload, and thereby maintains the cellular homeostasis (Kantor et al., 2000). TMZ decreases oxidative damage to mitochondria and protects hearts from I/R-induced damage to mitochondrial respiration (Guarnieri and Muscari, 1993). TMZ also showed cytoprotective effect in several models of myocardial infarction (Harpery et al., 1989; Pantos et al., 2005). Recently it has been shown that TMZ protected the postischemic hearts by inhibiting the activation of neutrophils (Tritto et al., 2005).

To protect hearts from ROS-mediated myocardial reperfusion injury, in addition to that provided by TMZ, we have developed a class of compounds based on TMZ, but derivatized with heterocyclic nitroxide-precursor, 2,2,5,5-tetramethylpyrroline groups (Li et al., 2000; Shankar et al., 2000; Toth et al., 2003). The nitroxide-precursors can easily pass through the phospholipid bilayers and transform into nitroxides, and are thereby able to protect cells and tissues from extra-and intracellular oxidative damage (Krishna et al., 1996) Nitroxides, in general, have been shown to possess potential therapeutic values in a variety of diseases including myocardial I/R-injury (Gelvan et al., 1991; Samuni et al., 1991). Treatment of hearts with modified mexiletine molecules with a nitroxide-precursor, has been reported to protect against post-ischemic injury by up-regulating pro-survival Akt kinase activity (Toth et al., 2003). Since TMZ protects hearts from ischemic injury and the nitroxide precursor can protect hearts against reperfusion injury by scavenging ROS, we hypothesized that TMZ derivatized with a nitroxide-precursor group could exhibit an added beneficial effect in protecting hearts from ischemia-reperfusion injury. Therefore, in this study, the effectiveness of the derivatives of TMZ namely, 2,2,5,5-tetramethylpyrrolinyl trimetazidine (HO-2921, TMZ-NH) and 2,2,5,5-tetramethyl-4-phenyl-pyrrolinyl trimetazidine (HO-3630, TMZ-ΦNH) in protecting hearts against I/R-mediated injury was investigated using an isolated rat heart model. The results demonstrate that TMZ-NH and

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TMZ-ΦNH significantly improve the recovery of I/R-induced cardiac dysfunction and protects the hearts from I/R injury through its anti-ischemic and antioxidant properties and also possibly through the modulation of Akt activity by TMZ-ΦNH.

Materials and methods

Chemicals

Trimetazidine, 1-(2,3,4-trimethoxybenzyl)piperazine dihydrochloride (TMZ) and its pyrroline derivatives, TMZ-NH and TMZ-ΦNH (Figure 1) were synthesized as described (Kalai et al., 2006). Dihydroethidium (DHE) was purchased from Sigma (St. Louis, MO). 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide (DEPMPO) was purchased from Dojindo (Japan).

Isolated heart preparation

Sprague-Dawley rats (weight 300 - 350 g) were anesthetized with 60 mg/kg sodium pentobarbital and heparin (500 IU/kg), administered intraperitoneally. After a midline sternotomy, the hearts were rapidly excised and perfused retrogradely at a constant perfusion pressure of 80 mmHg with a modified Krebs solution containing NaCl (120 mM), $NaHCO_3$ (25 mM), $MgSO_4$ (1.2 mM), KH_2PO_4 (1.2 mM), $CaCl_2$ (1.2 mM) and glucose (11 mM). The perfusate buffer was saturated with a 95% O_2 and 5% CO_2 gas mixture at 37°C. A latex balloon was inserted in the left ventricle via left atrium and inflated with 0.4 ml of distilled water, sufficient to produce an end diastolic pressure of 8-12 mmHg. The contractile and hemodynamic functions of the heart were continuously monitored with a computer-based data acquisition system (PC PowerLab with Chart 5 software, ADI Instruments, Colorado Springs, CO). The following data were measured: coronary flow (CF), left ventricular systolic pressure (LVSP), left ventricular developed pressure (LVDP) and heart rate (HR). Rate pressure product (RPP) was calculated

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as LVDP x HR. The coronary flow rate was measured using a flow meter with an in-line probe (Transonic Systems Inc, Ithaca, NY).

I/R experimental protocol

Isolated rat hearts were perfused for 15 min to stabilize the functions and then subjected to 30-min of ischemia, followed by 45-min of reperfusion. The hearts were randomly divided into four groups of at least 6 hearts per group: (i) control group, received no treatment; (ii) TMZ (50 µM); (iii) TMZ-NH (50 µM) and (iv) TMZ-ΦNH (50 µM). The drugs (water soluble, dissolved in Krebs buffer) were infused through the side-arm at the rate of 1 ml/min for one min before ischemia. Coronary effluent was collected for the determination of lactate dehydrogenase (LDH) and creatine kinase (CK) activities, before ischemia, and then during reperfusion. Myocardial tissue was collected at the end of reperfusion. The tissue was quickly frozen in liquid nitrogen and stored at -80^{o}C until analysis. The hemodynamic measurements, biochemical assays, and infarct size determinations were done on the same hearts, whereas, the DHE fluorescence measurements and Western blot analyses of phosphorylation of Akt, ERK1/2 and p38 MAPK were done on different hearts.

LDH and CK assay

Myocardial tissue damage was assessed by determining the activities of LDH and CK in the coronary effluent collected before ischemia and at 1, 2, 3, 4, 5, 10, 15, 20, 25, 30 min of reperfusion. The activities of LDH and CK in the coronary effluents were determined using commercially available kit; (LDH, Sigma diagnostics and CK, Catachem Inc, USA). The rate of change in absorbance was determined by measuring on a Varian Cary 50 spectrophotometer at 340 nm for 5 min at 25^{o}C. The enzyme activities were calculated using the molar extinction coefficient of NADH (ε=6.22).

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Evaluation of myocardial infarct size

Measurement of the risk area and infarct size was performed by using triphenyltetrazolium chloride (TTC)-staining (Walker et al., 1993). TTC stains all living tissue brick red, while leaving the infarct area unstained (white). First, the hearts were frozen, stored at -20^{0}C for 30 min, and then sliced perpendicularly along the long axis from apex to base in 2-mm sections. Sections were then incubated for 20 min at 37^{o}C with 1% TTC in PBS (pH 7.4). The sections were then fixed in 10% formalin for 20 min and were digitally imaged using a Nikon microscope. The areas of infarct size (TTC-negative) and risk (TTC-positive) were determined by using MetaMorph software. The infarct size was expressed as a percentage of the risk area.

Measurement of superoxide generation

Superoxide generation in the heart tissue subjected to I/R was determined using dihydroethidium (DHE) fluorescence (Miller et al., 1998)**.** The cell-permeable DHE is oxidized to fluorescent ethidium by superoxide, which is then intercalated into DNA. Since it has been reported that the superoxide generation in the I/R heart occurs during the first 15 min of reperfusion, we measured the DHE fluorescence at 15 min of reperfusion. Hearts after 15 min of reperfusion were placed in cold PBS buffer and embedded in OCT for cryo-sectioning. Unfixed frozen segments were cut into 30 µm-thick sections and placed on glass slides. DHE (10 µM) was topically applied to each tissue section, which was then coverslipped and incubated in a light-protected chamber at 37^{0}C for 30 min. The fluorescent Images of the tissue sections were obtained using a fluorescence microscope with rhodamine filter. Fluorescence intensity, which positively correlates with the extent of superoxide generation, was determined in the myocardial tissue using MetaMorph software. The mean intensity of all fluorescence signals in a low-power field was compared between all the treated groups.

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In vitro studies

The superoxide and alkylperoxyl radical-scavenging properties of TMZ, TMZ-NH and TMZ-ΦNH were evaluated by using EPR spectroscopy. Xanthine (0.5 mM), xanthine oxidase (0.02 U/ml), in PBS, pH 7.4 was used to generate superoxide radicals. 2,2'-azobis-2-amidonopropane dihydrochloride (AAPH, 25 mM) in aerobic PBS solution at 37^{o}C was used to generate alkylperoxyl radicals (Niki, 1999)**.** The reaction mixture contained 0.1 mM diethylenetriaminepentaacetate (DTPA), 10 mM DEPMPO, PBS, (pH 7.4), in the presence and absence of 1 mM TMZ, TMZ-NH or TMZ-ΦNH. The superoxide and peroxyl radicals were detected as DEPMPO-OOH and DEPMPO-OOR adducts, respectively by X-band EPR spectroscopy.

Akt, p38 MAPK and ERK1/2 phosphorylation

Heart tissues were homogenized in a TN1 lysis buffer containing 50 mM Tris pH 8.0, 10 mM EDTA, 10 mM $Na_4P_2O_7$, 10 mM NaF, 1% Triton-X 100, 125 mM NaCl, 10 mM Na_3VO_4, and 10 µg/ml each aprotinin and leupeptin. 10 µg of protein from each sample was boiled in SDS sample buffer (60 mM Tris, pH 6.8, 2.3% SDS, 10% glycerol, 0.01% bromophenol blue and 1% 2-mercaptoethanol) for 5 min, separated by SDS-PAGE, transferred to nitrocellulose membranes, probed with the phospho-specific antibodies for Akt, ERK1/2 and p38 MAPK (1:1000 dilution, Cell Signaling, Beverly, MA), followed by horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). The filters were then developed by enhanced chemiluminescence (ECL). The same filters were re-probed with antibodies for total Akt, ERK1/2, and actin. The ECL signal was quantified using a scanner and a densitometry program (Scion Image). To quantify the phospho-specific signal in the activated samples, we first subtracted background, then normalized the signal to the amount of actin or total target protein in the lysate (Ganesan et al., 2004).

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Data analysis

The statistical significance of the results of the assays was evaluated by using ANOVA and Student's t-test. All the values are expressed as mean ± SD. A p value < 0.05 was considered significant.

Results

Hemodynamic parameters

Hearts were perfused with 50 μM concentration of TMZ, TMZ-NH or TMZ-ΦNH for 1 min and subjected to 30 min of global ischemia followed by 45 min of reperfusion. Coronary flow (CF), left ventricular developed pressure (LVDP), and rate pressure product (RPP) were continuously measured prior to the start of global ischemia and during reperfusion. The functional recovery and CF data obtained during reperfusion were expressed as a percentage of their pre-ischemic baseline values, which were as follows: CF, 17±4 ml/min; LVDP, 124±18 mmHg; heart rate (HR), 274±24 bpm; To determine the effect of TMZ, TMZ-NH or TMZ-ΦNH on the contractile function of the perfused heart, a 50-μM dose of TMZ, TMZ-NH or TMZ-ΦNH was infused for 1 min through the side-arm on the perfusion line. A dose-response study revealed that TMZ-NH or TMZ-ΦNH at 50 μM was the most effective dose in protecting the heart from I/R-injury (data not shown). Hence all our experiments were performed using this dose. The HR, LVDP and CF were measured for 15 min after beginning the infusion of the drugs. The results indicated that there was a sudden drop in HR during infusion period in hearts infused with TMZ-ΦNH (Figure 2). The HR decreased by 37% at the end of 1 min infusion of TMZ-ΦNH, as compared to 4% and 6% in hearts perfused with TMZ and TMZ-NH, respectively. However, the HR recovered thereafter during continued perfusion with the normal perfusate. Similarly, LVDP measured during first few minutes of infusion of TMZ-NH or TMZ-ΦNH showed a decrease, but the values,

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however, returned quickly to the pre-infusion levels within 15 min (Figure 2). There was no significant change in CF in the hearts infused TMZ-NH or TMZ-ΦNH (data not shown).

Hearts were subjected to 30 min of global ischemia at 37^{o}C followed by 45 min of reperfusion. The drugs were infused at final concentration of 50 μM for 1 min prior to ischemia. Some representative tracings showing the change of left ventricular (LV) pressure in hearts subjected to I/R with and without TMZ, TMZ-NH or TMZ-ΦNH treatment are shown in Figure 3. The untreated control hearts subjected to 30 min of global ischemia followed by 45 min of reperfusion showed a significant decrease in CF (41%), LVDP (26%) and RPP (22%) as compared to pre-ischemic baseline values (Figure 4). Hearts treated with TMZ did not show any significant differences in the recovery of CF, LVDP and RPP compared to the control hearts. On the other hand, in hearts pretreated with TMZ-NH or TMZ-ΦNH, the recoveries of LVDP and RPP were significantly higher ($p < 0.001$) compared to the untreated hearts. The results suggested that TMZ-NH and TMZ-ΦNH, but not TMZ, protected hearts against I/R-induced dysfunction.

LDH and CK release

The LDH levels (measured as activity) in the coronary effluents of hearts treated with TMZ, TMZ-NH and TMZ-ΦNH were measured. In the untreated group of hearts subjected to I/R, the LDH activity increased as a function of reperfusion time (data not shown) with a maximum activity at 15 min of reperfusion (Table 1). On the other hand, the LDH activity was significantly less in hearts treated with TMZ, TMZ-NH or TMZ-ΦNH ($p < 0.001$ compared to control group). Similarly, the activity of CK in the coronary effluents from the control group of hearts was significantly elevated after I/R, and peaked at 15 min of reperfusion (Table 1). However, the effluents from hearts pretreated with TMZ, TMZ-NH or TMZ-ΦNH showed significantly less increase in the activity of CK ($p < 0.001$) at 15 min of reperfusion as compared to the untreated

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control group. The LDH and CK data indicated that TMZ, TMZ-NH and TMZ-ΦNH were effective in protecting hearts against I/R-induced release of LDH and CK enzymes.

Infarct size

TTC-staining of control hearts subjected to 30 min of ischemia and 120 min of reperfusion showed 43.0±5.0% infarct of risk area. On the other hand, the percent infarct sizes in hearts treated with TMZ (36.0±3.0; $p < 0.05$), TMZ-NH (19.0±4.0; $p < 0.001$) and TMZ-ΦNH (16.0±2.0, $p < 0.0001$) were significantly less as compared to the control group (Figure 5). The infarct data revealed that TMZ, TMZ-NH and TMZ-ϕNH resulted in the significant reduction of post-ischemic injury in the reperfused heart.

HE fluorescence

Reperfusion of ischemic myocardium is associated with the increased production of ROS, particularly superoxide radicals. To assess whether the cardioprotective effect of TMZ, TMZ-NH and TMZ-ΦNH could be attributed to a reduction in the production of ROS, superoxide production was determined in heart slices using HE fluorescence. The heart slices were stained with dihydroethidium (DHE) which was converted to the fluorescent hydroethidine (HE) by superoxide. Since the superoxide generation in the reperfused hearts typically occurs during the first 15 min of reperfusion, we performed the HE fluorescence measurements at 15 min of reperfusion. The fluorescence intensity of HE was significantly higher in untreated control hearts subjected to 30 min ischemia followed by 15 min of reperfusion (Figure 6). On the other hand, the fluorescence intensity in hearts pretreated with TMZ ($p < 0.01$), TMZ-ΦNH ($p < 0.001$) or TMZ-NH ($p < 0.001$) was significantly attenuated as compared to controls. The results demonstrated the generation of superoxide and its attenuation by TMZ, TMZ-NH or TMZ-ΦNH in the reperfused heart tissue.

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Scavenging of superoxide and peroxyl radical

To identify whether the molecules TMZ, TMZ-NH and TMZ-ΦNH are capable of scavenging superoxide and/or peroxyl radicals, *in vitro*, we used spin-trapping EPR spectroscopy. DEPMPO spin-trap was utilized for direct detection of exogenously generated superoxide and peroxyl radicals as DEPMPO-OOH and DEPMPO-OOR adducts, respectively. As shown in Figure 7, the formation of DEPMPO-OOH adduct by X/XO superoxide-generating system was significantly inhibited by TMZ-NH and TMZ-ΦNH ($p < 0.001$) as compared to control. Addition of SOD (200 U/ml) inhibited the EPR signal by >95% suggesting that the adduct formed was indeed from superoxide radicals. On the contrary, the compounds had no significant effect on the alkylperoxyl radicals, generated by thermal decomposition of AAPH in air saturated PBS, suggesting that TMZ, TMZ-NH, or TMZ-ΦNH has no peroxyl radical-scavenging property (data not shown). Thus, the results provide direct evidence that TMZ-NH and TMZ-ϕNH are scavengers of superoxide radicals.

Phosphorylation of p38 MAPK, ERK1/2 and Akt

In order to understand the underlying mechanism of biochemical pathways leading to the attenuation of postischemic reperfusion injury in the heart by the TMZ derivatives, we performed Western blot assays of the phosphorylation of p38 MAPK, ERK1/2, and Akt in the heart tissue homogenates. Hearts subjected to 30 min of ischemia followed by different periods (5 - 30 min) of reperfusion showed increased phosphorylation of p38 MAPK, ERK1/2, and Akt with a maximum increase at 10 min of reperfusion (data not shown). Hence, we performed all Western blot analyses on the treated hearts at 10 min of reperfusion. In hearts treated with TMZ, TMZ-NH, or TMZ-ΦNH, the phosphorylation of Akt and ERK1/2 during ischemia were significantly decreased ($p < 0.05$) compared to hearts not subjected to I/R (Figures 8). During ischemia, the phosphorylation of p38 MAPK was significantly increased in control hearts ($p < 0.001$ *versus*

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preischemic value) and this increase was not significantly altered by TMZ, TMZ-NH or TMZ-ΦNH treatments. The phosphorylations of Akt and ERK1/2 were significantly increased ($p < 0.001$ *versus* pre-ischemic or ischemic control) at 10 min of reperfusion. The phosphorylation of p38 MAPK was further enhanced in the reperfused hearts as compared to pre-ischemic or ischemic control ($p < 0.001$). TMZ, TMZ-NH and TMZ-ΦNH did not show any significant change on the phosphorylation of ERK1/2 and p38 MAPK. However, the phosphorylation of Akt in the hearts treated with TMZ-ΦNH, was markedly enhanced ($p < 0.001$ *versus* control I/R). Taken together, the Western blot analyses indicated markedly enhanced activation (phosphorylation) of Akt by TMZ-ΦNH in the post-ischemic hearts at 10 min of reperfusion.

Discussion

The present study clearly demonstrated that pre-ischemic treatment with TMZ-NH or TMZ-ΦNH provided marked protection of hearts against I/R-induced contractile dysfunction and tissue injury. The recovery of cardiac contractile function positively correlated with the prevention of tissue injury (infarction). The HE fluorescence and EPR spectroscopy results of the present study undoubtedly established that TMZ, TMZ-NH and TMZ-ΦNH treatments significantly lowered ROS generation in hearts subjected to I/R and the ability of TMZ-NH, and TMZ-ΦNH to scavenge superoxide *in vitro*. In addition, TMZ-ΦNH was observed to be involved in the activation of Akt signaling in the reperfused myocardium. Thus, the beneficial effect of the TMZ analogs appears to include, in addition to their anti-ischemic effect, antioxidant activity and activation of the pro-survival Akt enzyme.

Several studies have shown that ROS produced in the reperfused myocardium can cause oxidative stress-mediated injury, which is preventable, at least in part, by nitroxides and other antioxidants (Das et al., 1991; Gelvan et al., 1991; Shankar et al., 2000; Khan et al., 2006). The TMZ-NH and TMZ-ΦNH molecules contain five-membered pyrroline hetero-cycles that are expected to convert into corresponding nitroxides in tissues (Li et al., 2000; Shankar

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et al., 2000). In the present study, the I/R-induced superoxide generation in hearts was significantly attenuated by TMZ-NH or TMZ-ΦNH. This could be due to increased tissue concentrations of the nitroxide form of TMZ-NH or TMZ-ΦNH. Nitroxides, in general, have been known to possess significant antioxidant properties, and also, to provide membrane protection by site-targeted detoxification of ROS generated during reperfusion (Krishna et al., 1996; Goldstein et al., 2003). Several studies have established that pretreatment with five-membered pyrroline nitroxides protect hearts against reperfusion-mediated myocardial injury (Li et al., 2000; Shankar et al., 2000; Toth et al., 2003). Thus, the antioxidant action of TMZ-NH and TMZ-ΦNH against superoxide radicals can be attributed to their cardioprotection against I/R-injury.

Although TMZ showed significant protection against I/R-injury, the magnitudes of protection offered by TMZ-NH and TMZ-ΦNH were much higher than that of TMZ. This suggests that the observed protective effect is due to the attenuation of ischemic damage caused by the deprivation of oxygen (ischemic protection by TMZ) and the scavenging of ROS during reperfusion that otherwise lead to tissue damage during reperfusion. Both *in vitro* and *in vivo* studies have demonstrated that, during ischemia, TMZ limits intracellular acidosis, inhibits sodium and calcium accumulation, maintains intracellular ATP levels, reduces CK release, preserves mitochondrial function, and inhibits neutrophil infiltration (Kantor et al., 2000; Tritto et al., 2005). Although the present study revealed that TMZ did not scavenge superoxide radicals *in vitro,* the reperfusion-induced ROS generation was significantly attenuated by TMZ. This may be due to the indirect effect of TMZ on ROS production, e.g., inhibition/activation of enzymes. Our data confirm the results published previously by other investigators showing the reduction in the ROS production by TMZ during postischemic reperfusion, although there were no direct ROS-scavenging properties (Charlon et al., 1990; Tritto et al., 2005). Recently, it was demonstrated that TMZ reduced cardiac reperfusion injury by inhibiting neutrophil-mediated ROS production (Tritto et al., 2005).

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Recent studies have shown that a brief period of ischemia may protect the heart against subsequent ischemic episodes and reperfusion injury, possibly by the activation of pro-survival kinases such as Akt and ERK1/2 (Hausenloy and Yellon, 2004). On the other hand, activation of p38 MAPK during transient ischemia and reperfusion resulted in I/R injury (Ma et al., 1999; Sumida et al., 2005). Our current study showed a decrease in Akt and ERK1/2 phosphorylation during ischemia, but a marked increase in the phosphorylation of both kinases during reperfusion. Our current study also demonstrated an increase in phosphorylation of p38 MAPK during ischemia and a further increase during reperfusion. Studies have shown that ischemia alone, or I/R, activated p38 MAPK in cultured cardiomyocytes and in hearts, and the inhibition of p38 MAPK by a specific inhibitor, SB203580, reduced I/R-injury (Ma et al., 1999; Yue et al., 2000; Liu et al., 2004).

Earlier studies have demonstrated the activation of ERK1/2 and Akt and are cardioprotective (Ma et al., 1999; Toth et al., 2003; Liu et al., 2004; Hausenloy et al., 2005). It is likely that anti-ischemic agents such as TMZ and its derivatives may offer cardioprotection by modulating PI3K-Akt, p38 MAPK or ERK1/2 signaling pathways. The result of the present study indicated that TMZ, TMZ-NH or TMZ-ΦNH treatments did not show significant differences in the activation of p38 MAPK and ERK1/2 during ischemia and I/R. In support of this observation is a recent study by Pantos *et al,* showing that the cardioprotective effect of TMZ is not mediated through p38 MAPK and JNK signaling cascades (Pantos et al., 2005). Although the findings of the present study revealed that TMZ-NH or TMZ-ΦNH did not affect ERK1/2 and p38 MAPK pathways significantly, the ERK1/2 or p38 MAPK pathway in the protective action of TMZ-NH or TMZ-ΦNH is not ruled out. This needs to be addressed thoroughly. In our recent publication, we demonstrated that C-phycocyanin, a plant-based antioxidant, significantly protected the myocardial I/R-injury through the involvement of p38 MAPK and ERK1/2 signaling (Khan et al., 2006).

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Our data also demonstrated that the derivative with an aromatic substitute, TMZ-ΦNH, significantly enhanced the I/R-induced Akt activation. I/R itself can increase Akt-signaling as seen in the present study. TMZ-ΦNH treatment further increased the Akt activation independent of cardiac injury and exerted a protective role. Recently, Toth et al have demonstrated that, H-2693, a compound containing a secondary amine - nitroxide precursor, enhanced the I/R-induced activation of Akt and protected hearts from I/R-mediated injury (Toth et al., 2003). In another study, orthovanadate has been shown to have a protective role against I/R-injury in rat heart by increasing Akt activation (Takada et al., 2004).

In summary, our present studies have clearly documented that TMZ, TMZ-NH and TMZ-ΦNH administered 1 min before ischemia, are capable of protecting hearts against myocardial I/R-injury. The aromatic substitute, TMZ-ΦNH, showed a better recovery of the contractile function of the reperfused heart, significantly scavenged ROS, and protected the I/R-induced myocardial infarction compared to TMZ-NH and TMZ. This may be due to a possible bradicardiac activity of TMZ-ΦNH, as the pre-ischemic infusion of TMZ-ΦNH resulted in a 37% decrease in the heart rate during the first min of infusion as compared to a 6% and 4% decrease in hearts treated with TMZ-NH or TMZ. The TMZ-ΦNH analogue contains a lipophilic aromatic group and a hydrophilic amino group with the ability to scavenge oxygen radicals in the lipid-rich membrane as well as water-rich cytosolic areas. Also, the hearts treated with TMZ-NH or TMZ-ΦNH showed enhanced recovery of coronary flow, which may suggest the involvement of endothelial function, possibly by the enhanced release of nitric oxide during reperfusion. However, this is only a speculation and needs further investigation. Taken together, the results of the present study revealed protective effects of TMZ derivatives, TMZ-NH and TMZ-ΦNH against I/R-injury. The protective effects of the TMZ derivatives appear to stem from multiple mechanisms: radical-scavenging property (anti-oxidant activity) of the nitroxide-precursor function; anti-ischemic effect of the TMZ group; and pro-survival Akt activity of TMZ-ΦNH.

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Foot Notes

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Figure Legends

Fig. 1. Molecular structure of Trimetazidine (1-(2,3,4-trimethoxybenzyl)piperazine, TMZ) and its derivatives, TMZ-NH (2,2,5,5-tetramethylpyrrolinyl trimetazidine, HO-2921) and TMZ-ΦNH (2,2,5,5-tetramethyl-3-phenyl-pyrrolinyl trimetazidine, HO 3630).

Fig. 2. Effect of TMZ, TMZ-NH and TMZ-ΦNH on the contractile functions of perfused hearts. Hearts were infused with TMZ, TMZ-NH or TMZ-ΦNH (50 μM) for 1 min (shaded region) while the contractile functions, heart rate (HR) and developed pressure (LVDP) were measured continuously for 15 min. Values are expressed as mean ± SD (n=3). While TMZ had no significant effect, TMZ-NH and TMZ-ϕNH showed decreases in HR and LVDP which recovered on subsequent perfusion without the drug.

Fig.3. Representative tracing showing the left ventricular (LV) pressure in isolated hearts subjected to I/R with and without TMZ, TMZ-NH or TMZ-ΦNH treatment. Hearts were subjected to 30 min of global ischemia at 37^{o}C followed by reperfusion for up to 45 min. The drugs were infused at 50 μM final concentration for 1 min (indicated by up-arrow and vertical dotted line) prior to ischemia. .

Fig 4. Effect of Trimetazidine (TMZ), TMZ-NH and TMZ-ΦNH on the recovery of hemodynamic and contractile functions of heart subjected to ischemia-reperfusion. Hearts were pretreated with 50 μM TMZ, TMZ-NH or TMZ-ΦNH, 1 min before ischemia and subjected to 30 min of global ischemia at 37^{o}C followed by 45 min of reperfusion. Time course of coronary flow (CF), left ventricular developed pressure (LVDP) and rate pressure product (RPP). Right panels show the recovery of CF, LVDP and RPP at the end of 45 of reperfusion. Data represent the mean from 6 independent measurements (hearts). *$p < 0.001$ *versus* control group.

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Fig 5. Effect of TMZ, TMZ-NH and TMZ-ΦNH on I/R-induced myocardial infarction. Irreversible infarction was determined by treating the heart ventricular sections with 1% triphenyltetrazolium chloride (TTC) staining. Treatment protocol is same as shown in Fig 4, except that the reperfusion time was 120 min. (A) Representative photomicrograph of the infarct area showing white zones after TTC staining. (B) Percentage of infarct area from the total area of the sections determined by using Metamorph software. Values are expressed as mean ± SD (n=3). *p < 0.05 *versus* control (I/R); **p < 0.001 *versus* control (I/R). TMZ, TMZ-NH and TMZ-ΦNH significantly attenuated the infarct area induced by I/R.

Fig 6. Effect of TMZ, TMZ-NH and TMZ-ΦNH on I/R-induced superoxide generation. Superoxide generation in hearts was determined by hydroethidine fluorescence. Unfixed cryosections of hearts after reperfusion (15 min) were incubated with dihydroethidium (10 μM) at 37^{o}C in the dark for 30 min and then measured by fluorescence microscopy. A. Representative photographs from triplicate experiments are shown. B. Mean fluorescence intensity after deducting the base line values of pre-ischemic control hearts. Data are represented as mean ± SD. *p < 0.01 *versus* control (I/R); **p < 0.001 *versus* control (I/R).

Fig. 7. Scavenging of superoxide by TMZ, TMZ-NH and TMZ-ΦNH. The superoxide radicals were generated by the xanthine (0.5 mM)/XO (0.02 U/ml) system. The reaction mixture containing DTPA (0.1 mM), DEPMPO (10 mM) in air-saturated PBS, (pH 7.4) in presence of 1 mM TMZ, TMZ-NH and TMZ-ΦNH and measured after 15 min of incubation by EPR spectroscopy. Data represent mean ± SD (n=4), *p < 0.01 *versus* control.

Fig. 8. Effect of TMZ, TMZ-NH and TMZ-ΦNH on Akt, ERK1/2 and p38 MAPK phosphorylation

in hearts subjected to I/R. (A) The phosphorylation of Akt, ERK1/2 and p38 MAPK was determined by Western blot analyses in hearts subjected to ischemia (30 min) and after 10 min of reperfusion. (B) Quantitative analysis of phosphorylated Akt, ERK1/2 and p38 MAPK in hearts treated with TMZ, TMZ-NH and TMZ-ΦNH. Values are from 3 independent experiments and expressed as mean ± SD. *$p < 0.01$ *versus* pre-ischemic control; **$p < 0.001$ *versus* pre-ischemic control; #$p < 0.001$ *versus* control (I/R). TMZ-ΦNH treatment significantly enhances the Akt activity.

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Table 1. Lactate dehydrogenase (LDH) and creatine kinase (CK) activity measured in the heart effluents collected at 15 min of reperfusion.

Parameter	Control	TMZ	TMZ-NH	TMZ-ΦNH
CK (U/L)	70 ± 7	42 ± 6*	54 ± 6*	26 ± 8*
LDH (U/L)	28 ± 3	14 ± 3*	19 ± 2*	11 ± 5*

All values are mean ± SD (n=6). *p < 0.001 *versus* control

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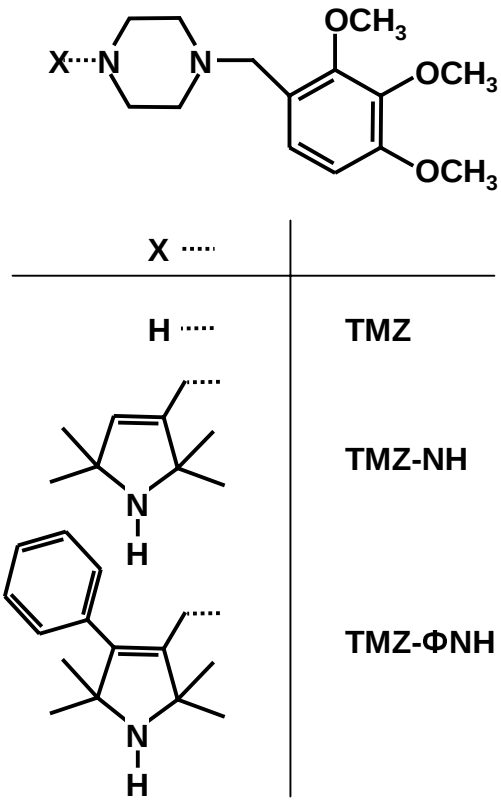

Figure 1

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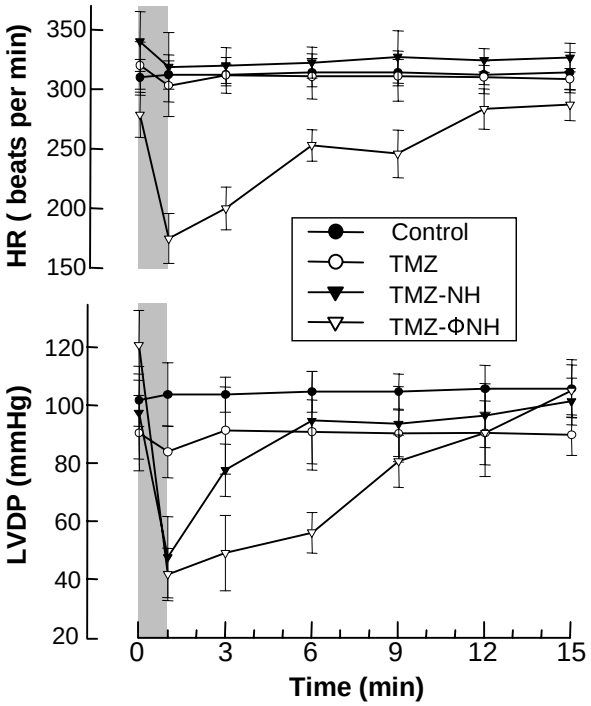

Figure 2

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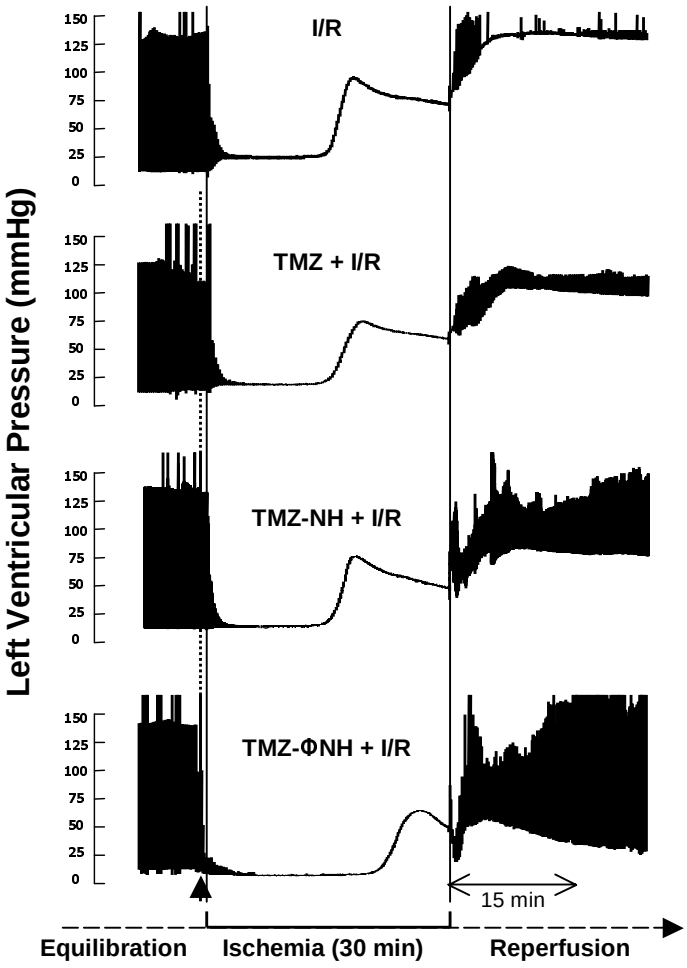

Figure 3

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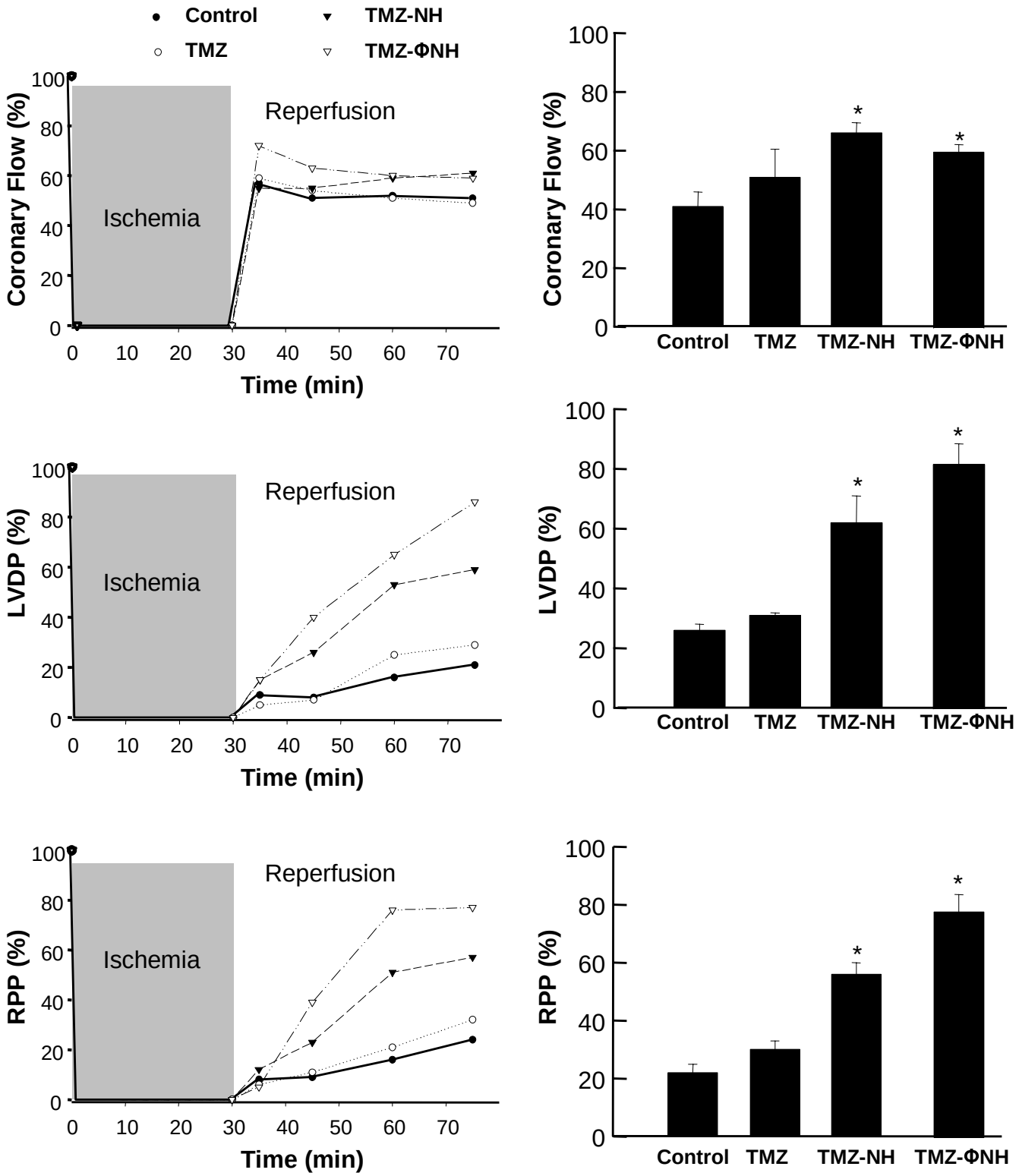

Figure 4

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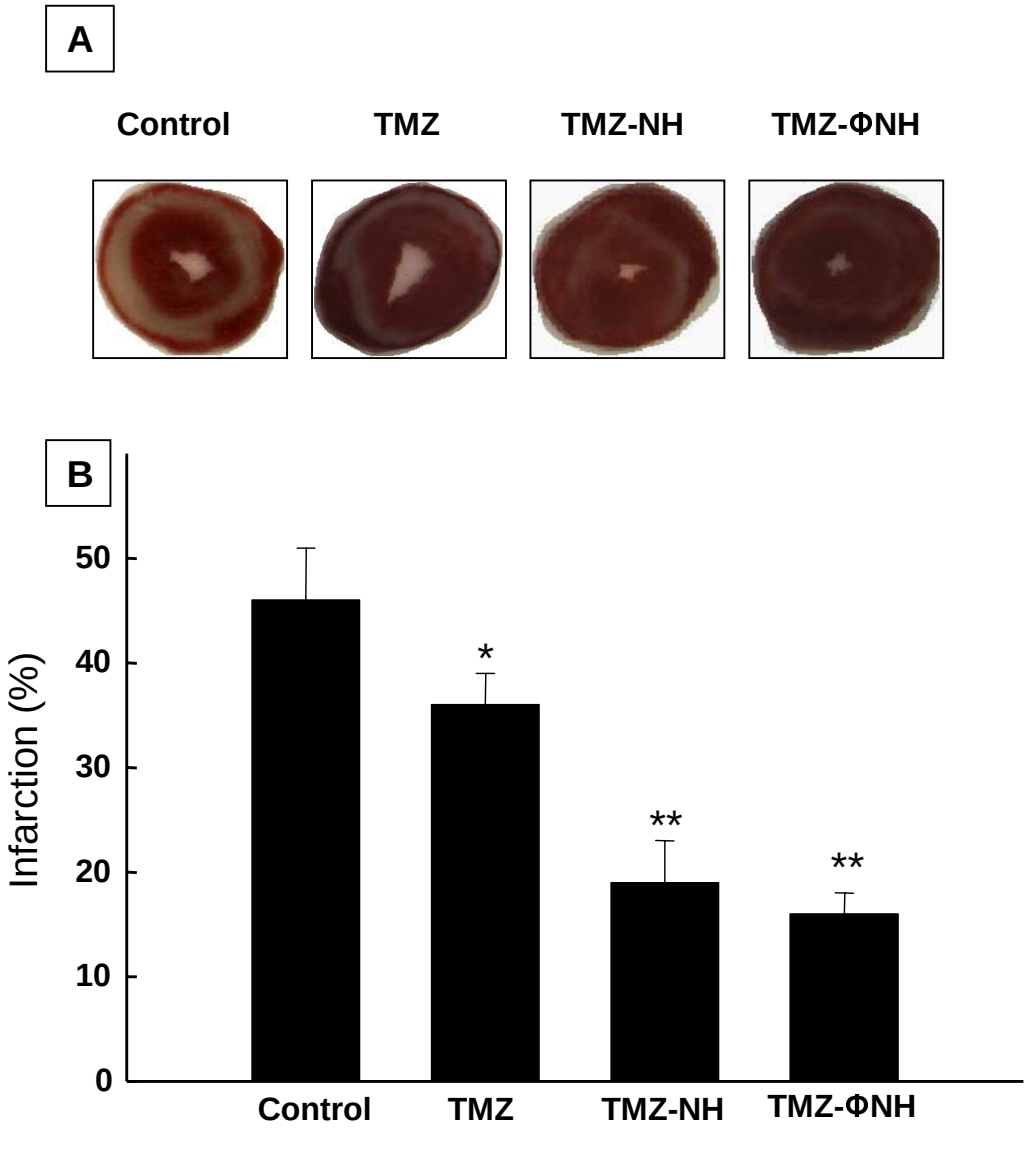

Figure 5

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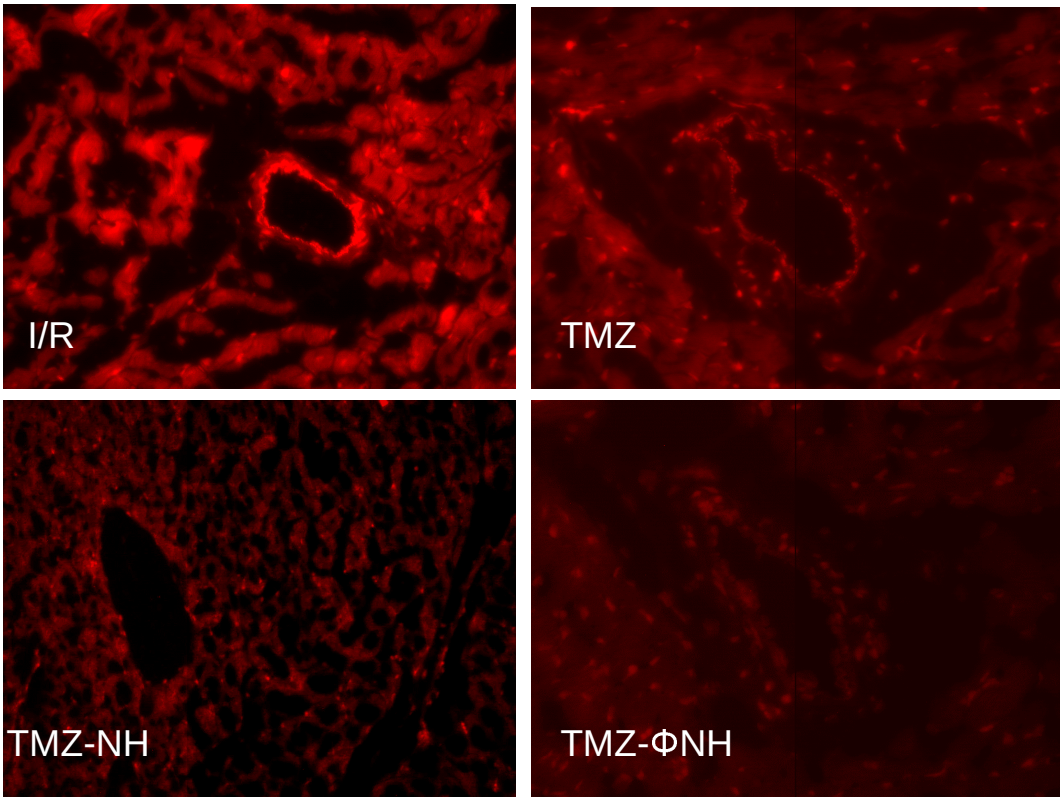

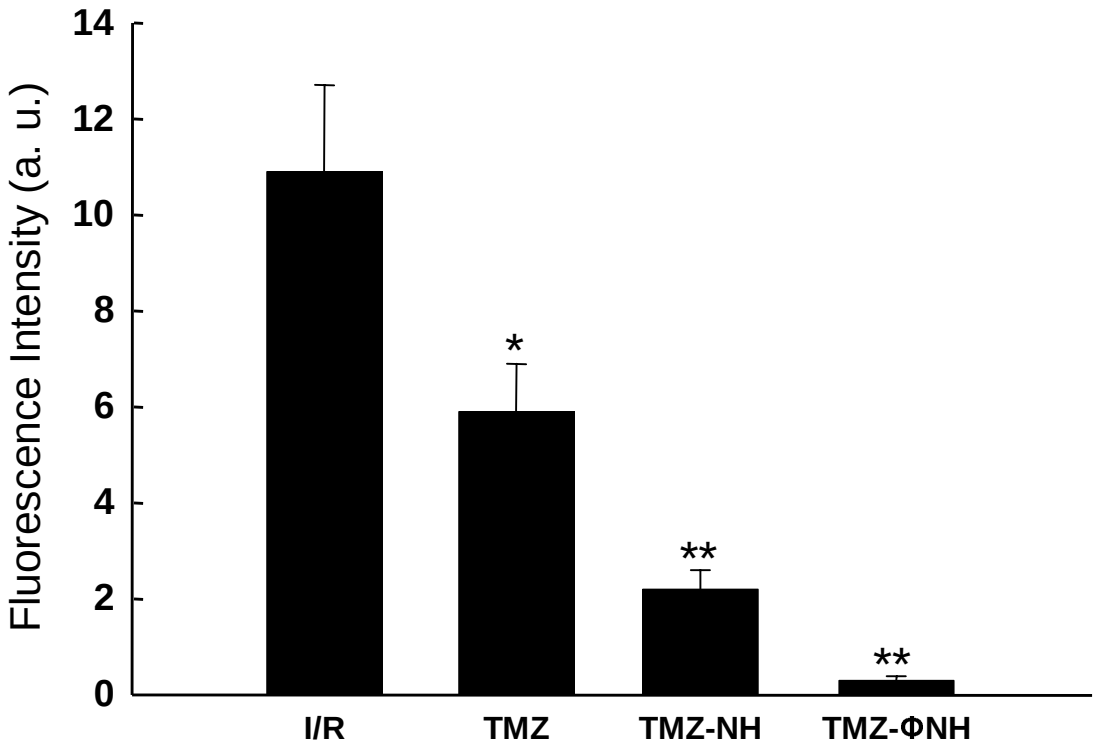

Figure 6

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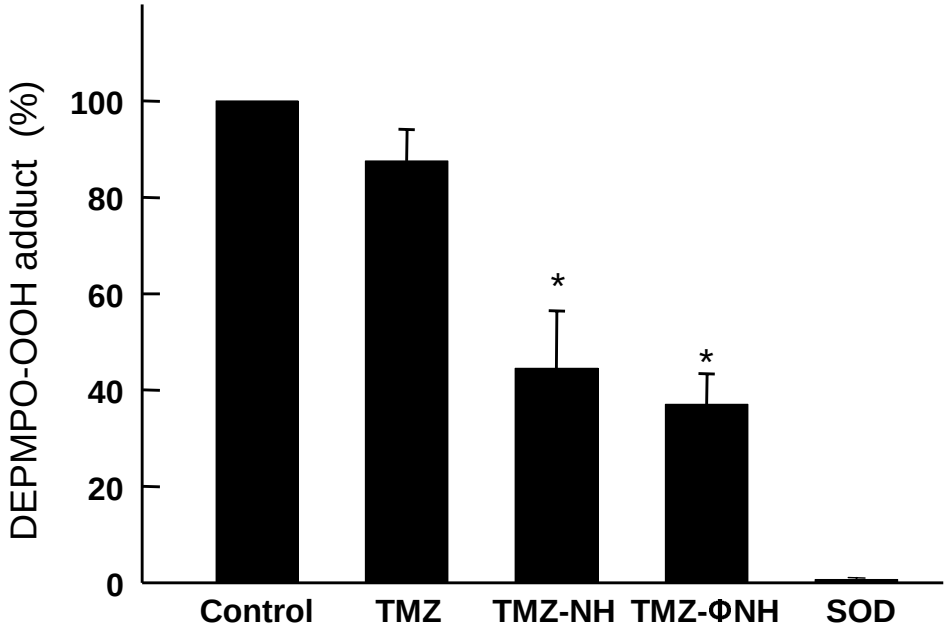

Figure 7

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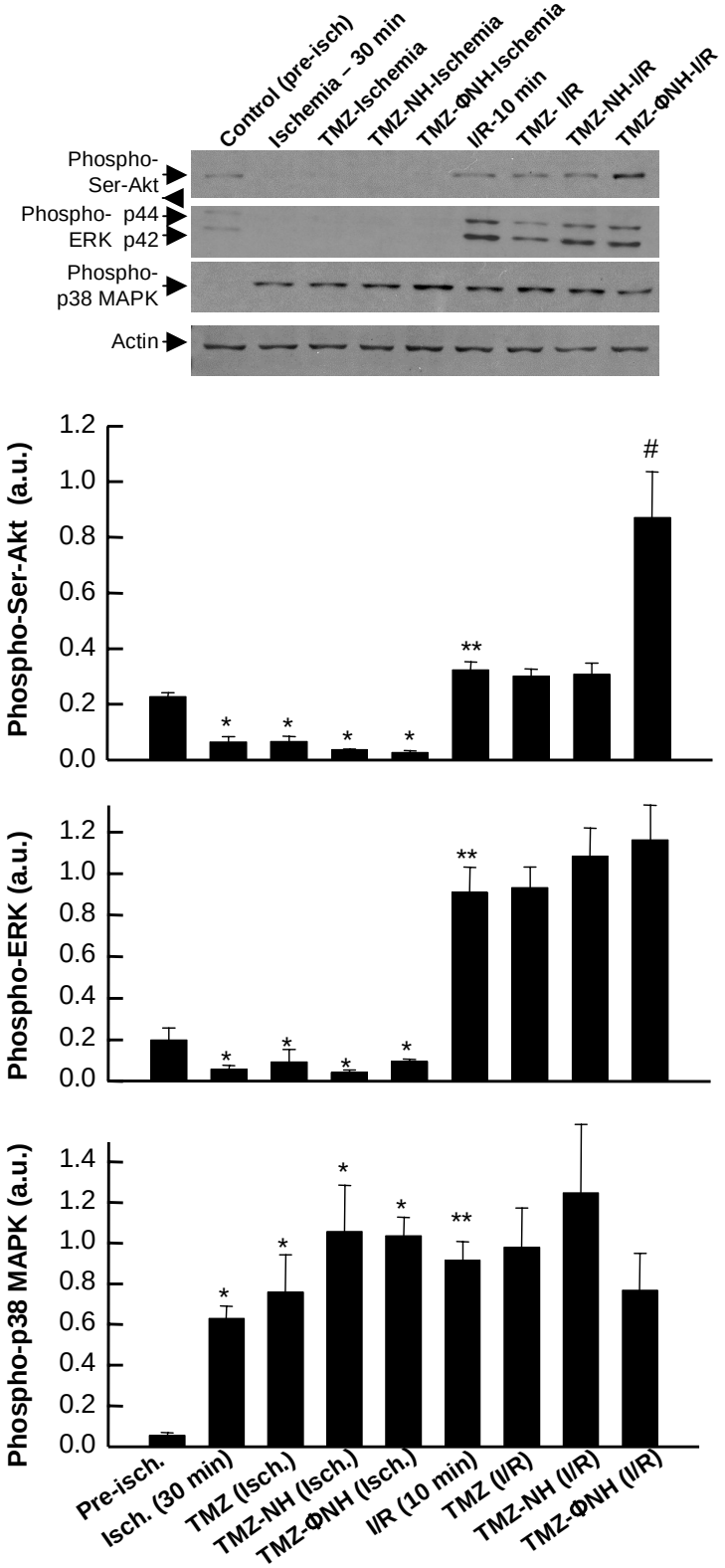

Figure 8