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

Vijay Kumar Kutala, Mahmood Khan, Rajarsi Mandal, Latha P. Ganesan, Susheela Tridandapani, Tamas Kalai, Kalman Hideg, and Periannan Kuppusamy

Davis Heart and Lung Research Institute, Department of Internal Medicine, The Ohio State University, Columbus, Ohio (V.K.K., M.K., R.M., L.P.G., S.T., P.K.); and Institute of Organic and Medicinal Chemistry, University of Pécs, Pécs, Hungary (T.K., K.H.)

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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-4NH (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%) compared with pre-ischemic baseline values. The I/R hearts also showed markedly increased lactate dehydrogenase and creatine kinase activities in the coronary effluent, significant myocardial infarction (46% of risk area), and activation of Akt, extracellular signal-regulated kinase, and p38 mitogen-activated protein kinase. Pretreatment of hearts with TMZ-NH or TMZ-4NH significantly enhanced the recovery of heart function and decreased infarct size. The I/R-induced activation of Akt was further enhanced by TMZ-4NH. The present study demonstrated that TMZ-NH and TMZ-4NH 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.

Reactive oxygen species (ROS) can cause oxidative damage to a variety of cellular components. ROS play 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 initiates oxidative phosphorylation, leading to a 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 EGFR, ERK1/2, JNK1/2, p38, and ATM are activated in the heart during ischemia and reperfusion.

To further delineate the role of ROS in I/R injury, the involvement of ROS in the activation of Akt, extracellular signal-regulated kinase (ERK), and p38 mitogen-activated protein kinases was assessed. Reperfusion activated Akt, extracellular signal-regulated kinase, and p38 mitogen-activated protein kinases, and this activation was dependent on ROS generation. Pretreatment with TMZ-NH or TMZ-4NH significantly enhanced the recovery of heart function and decreased infarct size. The I/R-induced activation of Akt was further enhanced by TMZ-4NH. The present study demonstrated that TMZ-NH and TMZ-4NH 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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Abbreviations: ROS, reactive oxygen species; I/R, ischemia-reperfusion; MAPK, mitogen-activated protein kinase; ERK1/2, extracellular signal-regulated kinase 1/2; TMZ, 1-(2,3,4-trimethoxybenzyl)piperazine, trimetazidine; TMZ-NH, 4-(2,2,5,5-tetramethylpyrrolinyl-3)-1-(2,3,4-trimethoxybenzyl)piperazine, HO-2921; TMZ-4NH, 4-(2,2,5,5-tetramethyl-4-phenylpyrrolinyl-3)-1-(2,3,4-trimethoxybenzyl)piperazine, HO-3630; DHE, dihydroethidium; DEPMPO, 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide; CF, coronary flow; LVDP, left-ventricular developed pressure; HR, heart rate; LDH, lactate dehydrogenase; CK, creatine kinase; TTC, triphenyltetrazolium chloride; PBS, phosphate-buffered saline; EPR, electron paramagnetic resonance; HE, hydroethidium; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfonylphenyl)-5-(4-pyridyl)-1H-imidazole; H-2693, 2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrole-3-carboxylic acid [2-(2,6-dimethylphenoxy)-1-methyl]ethanamide.
p38 MAPK, extracellular signal-regulated kinase 1/2 (ERK1/2), and c-Jun NH2-terminal kinase, have been shown to be activated in hearts subjected to I/R (Yue et al., 1998). The activation of Akt- and MAPK-signaling cascades has been shown to modulate oxidant-mediated tissue injury (Shimizu et al., 1998; Armstrong, 2004). Several pharmacological agents have been 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 thiolase. 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 post-ischemic 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 thereby are 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 (Helvan et al., 1991; Samuni et al., 1991). Treatment of hearts with modified mexiletine molecules with a nitroxide precursor has been reported to protect against postischemic injury by up-regulating pro-survival Akt kinase activity (Toth et al., 2003). Because 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-tetramethylpyrroline trimetazidine (HO-2921, TMZ-NH) and 2,2,5,5-tetramethyl-4-phenyl-pyrroline 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 TMZ-ΦNH significantly improve the recovery of I/R-induced cardiac dysfunction and protect hearts from I/R injury through their anti-ischemic and antioxidant properties and also possibly through the modulation of Akt activity by TMZ-ΦNH.

**Materials and Methods**

**Chemicals.** TMZ and its pyrrole derivatives, TMZ-NH and TMZ-ΦNH (Fig. 1), were synthesized as described previously (Kalai et al., 2006). Dihydroethidium (DHE) was purchased from Sigma Chemical (St. Louis, MO). 5-(Diethoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide (DEPMPO) was purchased from Dojindo (Kumamoto, 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 i.p. After a midline sternotomy, the hearts were rapidly excised and perfused retrogradely at a constant perfusion pressure of 80 mm Hg with a modified Krebs’ solution containing NaCl (120 mM), NaHCO3 (25 mM), MgSO4 (1.2 mM), KH2PO4 (1.2 mM), CaCl2 (1.2 mM), and glucose (11 mM). The perfusate buffer was saturated with a 95% O2 and 5% CO2 gas mixture at 37°C. A latex balloon was inserted in the left ventricle via the left atrium and inflated with 0.4 ml of distilled water, sufficient to produce an end-diastolic pressure of 8 to 12 mm Hg. 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, left ventricular developed pressure (LVDP), and heart rate (HR). Rate pressure product (RPP) was calculated as LVDP × HR. The coronary flow rate was measured using a flowmeter 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 six hearts per group: 1) control group, received no treatment; 2) TMZ (50 μM); 3) TMZ-NH (50 μM), and 4) 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 1 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°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, and 30 min of reperfusion. The activities of LDH and CK in the coronary effluents were determined using a commercially available kit (Sigma Diagnostics, St. Louis, MO for LDH and Catachem Inc., Bridgeport, CT for CK). The rate of change in absorbance was determined by measurement on a Varian Cary 50 spectrophotometer at 340 nm for 5 min at 25°C. The enzyme activities were calculated using the molar extinction coefficient of NADH (ε = 6.22).

**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, leaving the infarct area unstained (white). First, the hearts were frozen, stored at −20°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°C with 1% TTC in PBS (pH 7.4). The sections were then fixed in 10% formalin for 60 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 DHE fluorescence (Miller et al., 1998). The cell-permeable DHE is oxidized to fluorescent ethidium by superoxide, which is then intercalated into DNA. Because it has been reported that 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 ice-cold PBS buffer and embedded in OCT for cryosectioning. Unfixed frozen sections were cut into 4-μm-thick sections and placed on glass slides. DHE (10 μM) was topically applied to each tissue section, which was then cover-slipped and incubated in a light-protected chamber at 37°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 among all of the treated groups.

**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 (25 mM) in aerobic PBS solution at 37°C was used to generate alkylperoxyl radicals (Niki, 1999). The reaction mixture contained 0.1 mM diethylenetriaminepentaacetic acid, 10 mM DEPMPO, and 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-OH 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 Na₂P₂O₇, 10 mM NaF, 1% Triton X-100, 125 mM NaCl, 10 mM Na₃VO₄, and 10 μg/ml each of aprotinin and leupeptin. Ten micromgs of protein from each sample was boiled in SDS sample buffer (60 mM Tris, pH 6.8, 2.3% SDS, 10% glycerol, 0.01% bromphenol blue, and 1% 2-mercaptoethanol) for 5 min, separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with the phosphospecific antibodies for Akt, ERK1/2, and actin. The enhanced chemiluminescence signal was quantified using a scanner and a densitometry program (Scion Image). To quantify the phosphospecific signal in the activated samples, we first subtracted background and then normalized the signal to the amount of actin or total target protein in the lysate (Ganesan et al., 2004).

**Data Analysis.** The statistical significance of the results of the assays was evaluated by using analysis of variance and Student’s t test. All of the values are expressed as means ± S.D. A p value < 0.05 was considered significant.

**Results**

**Hemodynamic Parameters.** Hearts were perfused with 50 μM concentrations of TMZ, TMZ-NH, or TMZ-ΦNH for 1 min and subjected to 30 min of global ischemia followed by 45 min of reperfusion. CF, LVDP, and RPP were continuously measured before the start of global ischemia and during reperfusion. The functional recovery and CF data obtained during reperfusion were expressed as a percentage of their preischemic baseline values, which were as follows: CF, 17 ± 4 ml/min; LVDP, 124 ± 18 mm Hg; and HR, 274 ± 24 beats/min. 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 of 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 (Fig. 2). The HR decreased by 37% at the end of 1 min infusion of TMZ-ΦNH, compared with 4 and 6% in hearts perfused with TMZ and TMZ-NH, respectively. However, the HR recovered thereafter during continued perfusion with the normal perfusate. Likewise, LVDP measured during first few minutes of infusion of TMZ-NH or TMZ-ΦNH showed a decrease; however, the values returned quickly to the preinfusion levels within 15 min (Fig. 2). There was no significant change in CF in the hearts infused with TMZ-NH or TMZ-ΦNH (data not shown).

![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), whereas the contractile functions, HR and LVDP were measured continuously for 15 min. Values are expressed as means ± S.D. (n = 3). Although TMZ had no significant effect, TMZ-NH and TMZ-ΦNH showed decreases in HR and LVDP, which recovered on subsequent perfusion without the drug.](image)
Hearts were subjected to 30 min of global ischemia at 37°C followed by 45 min of reperfusion. The drugs were infused at final concentration of 50 μM for 1 min before ischemia. Some representative tracings showing the change of left ventricular pressure in hearts subjected to I/R with and without TMZ, TMZ-NH, or TMZ-ΦNH treatment are shown in Fig. 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%) compared with preischemic baseline values (Fig. 4). Hearts treated with TMZ did not show any significant differences in the recovery of CF, LVDP, and RPP compared with 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 with 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 maximal 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 with the control group). Likewise, 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 a significantly smaller increase in the activity of CK (p < 0.001) at 15 min of reperfusion compared with the untreated control group. The LDH and CK data indicated that TMZ, TMZ-NH, and TMZ-ΦNH were effective in protecting hearts against the 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 the risk area. On the other hand, the percentage 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 compared with the control group (Fig. 5). The infarct data revealed that TMZ, TMZ-NH, and TMZ-ΦNH resulted in the significant reduction of postischemic 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 HE by superoxide. Because the superoxide generation in the reperfused hearts typically occurs during the first 15 min of reperfusion, we performed HE fluorescence measurements at 15 min of reperfusion. The fluorescence intensity of HE was significantly higher in untreated control hearts subjected to 30 min of ischemia followed by 15 min of reperfusion (Fig. 6). On the other hand, the fluorescence intensity in hearts pretreated with TMZ (p < 0.01), TMZ-NH (p < 0.001), or TMZ-ΦNH (p < 0.001) was significantly attenuated compared with controls. The results demonstrated the generation of superoxide and its attenuation by TMZ, TMZ-NH, or TMZ-ΦNH in the reperfused heart tissue.

**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 used for direct detection of exogenously generated superoxide and peroxyl radicals as DEPMPO-OOH and DEPMPO-OOR adducts, respectively. As shown in Fig. 7, the formation of DEPMPO-OOH adduct by a xanthine/xanthine oxide superoxide-generating system was significantly inhibited by TMZ-NH and TMZ-ΦNH (p < 0.001) compared with control. The 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 2,2’-azobis-2-amidopropyl dihydrochloride 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. 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 maximal 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 was significantly decreased (p < 0.05) compared with hearts not subjected to I/R (Fig. 8). During ischemia, the phosphorylation of p38 MAPK was significantly increased in control hearts (p < 0.001 versus 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 preischemic or ischemic control) at 10 min of reperfusion. The phosphorylation of p38 MAPK was further enhanced in the reperfused hearts compared with preischemic or ischemic controls (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 postischemic hearts at 10 min of reperfusion.

Discussion

The present study clearly demonstrated that preischemic 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 heterocycles that are expected to convert into corresponding nitroxides in tissues (Li et al., 2000; Shankar 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 reduc-
Fig. 8. Effect of TMZ, TMZ-NH, and TMZ-\(\phi\)NH on Akt, ERK1/2, and p38 MAPK phosphorylation in hearts subjected to I/R. Top panel, 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. Bottom panels, quantitative analysis of phosphorylated Akt, ERK1/2, and p38 MAPK in hearts treated with TMZ, TMZ-NH, and TMZ-\(\phi\)NH. Values are from three independent experiments and are expressed as means \(\pm\) S.D. \(* p < 0.01\) versus preischemic control; \(* * p < 0.001\) versus control (I/R). TMZ-\(\phi\)NH treatment significantly enhances Akt activity. a.u., arbitrary unit.

Our data also demonstrated that the derivative with an aromatic substitute, TMZ-\(\phi\)NH, significantly enhanced the I/R-induced Akt activation. I/R itself can increase Akt-signaling as seen in the present study. TMZ-\(\phi\)NH treatment further increased the Akt activation independent of cardiac injury and exerted a protective role. Recently, Toth et al. (2003) 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. 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-\(\phi\)NH administered 1 min before ischemia are capable of protecting hearts against myocardial I/R injury. The aromatic substitute, TMZ-\(\phi\)NH, showed a better recovery of the contractile function of the reperfused heart, significantly scavenged ROS, and protected the I/R-induced myocardial infarction compared with TMZ-NH and TMZ. This may be due to a possible bradycardic activity of TMZ-\(\phi\)NH, as the preischemic infusion of TMZ-\(\phi\)NH resulted in a 37% decrease in the heart rate during the first minute of infusion compared with 6 and 4% decreases in hearts treated with TMZ-NH or TMZ. The TMZ-\(\phi\)NH analog 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. In addition, the hearts treated with TMZ-NH or TMZ-\(\phi\)NH 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 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 (antioxidant 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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Address correspondence to: Dr. Periannan Kuppasamy, Davis Heart and Lung Research Institute, The Ohio State University, 420 W. 12th Ave., Room 114, Columbus, OH 43210. E-mail: kuppasamy.1@osu.edu