Trimetazidine, Administered at the Onset of Reperfusion, Ameliorates Myocardial Dysfunction and Injury by Activation of p38 Mitogen-Activated Protein Kinase and Akt Signaling

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ABSTRACT

Trimetazidine [1-(2,3,4-trimethoxybenzyl)piperazine; TMZ] is an anti-ischemic cardiac drug; however, its efficacy and mechanism of cardioprotection upon reperfusion are largely unknown. The objective of this study was to determine whether TMZ, given before reperfusion, could attenuate myocardial reperfusion injury. Ischemia/reperfusion (I/R) was induced in rat hearts by ligating the left anterior descending (LAD) coronary artery for 30 min followed by 48 h of reperfusion. TMZ (5 mg/kg b.wt.) was administered 5 min before reperfusion. The study used three experimental groups: control (–I/R; –TMZ), I/R (+I/R; –TMZ), and TMZ (+I/R; +TMZ). Echocardiography and EPR oximetry were used to assess cardiac function and oxygenation, respectively. The ejection fraction, which was significantly depressed in the I/R group (62 ± 5 versus $84 \pm 3\%$ in control), was restored to $72 \pm 3\%$ in the TMZ group. Myo-

cardial pO_2 in the TMZ group returned to baseline levels (~ 20 mm Hg) within 1 h of reperfusion, whereas the I/R group showed a significant hyperoxygenation even after 48 h of reperfusion. The infarct size was significantly reduced in the TMZ group (26 ± 3 versus $47 \pm 5\%$ in I/R). TMZ treatment significantly attenuated superoxide levels in the tissue. Tissue homogenates showed a significant increase in p38 and p-Akt and decrease in caspase-3 levels in the TMZ group. In summary, the results demonstrated that TMZ is cardioprotective when administered before reperfusion and that this protection appears to be mediated by activation of p38 mitogen-activated protein kinase and Akt signaling. The study emphasizes the importance of administering TMZ before reflow to prevent reperfusion-mediated cardiac injury and dysfunction.

Ischemic heart disease is the leading cause of mortality among both men and women in the United States, and in the world. Clinical interventions such as coronary angioplasty, coronary artery bypass graft surgery, or percutaneous transluminal coronary angioplasty are routinely used to reintroduction of blood flow to an ischemic region of the myocardium. Such interventions are unavoidably accompanied by an enzymatic cascade of reactions that result in damage to the myocardium, termed ischemia/reperfusion (I/R) injury.

Although the etiology of I/R injury is intricate, oxidative stress occurs due to an imbalance between free-radical production and the heart's ability to prevent the damage caused by free radicals. Numerous studies have shown that the generation of reactive oxygen species (ROS) in the oxygen-deprived tissue plays a crucial role in the cellular oxidative damage that happens during I/R (Zweier et al., 1989; Ambrosio et al., 1993; Griendling and FitzGerald, 2003). The generation of free radicals that occurs during I/R has been reported by several groups (Bolli et al., 1988; Zweier et al., 1989) and has revealed that ROS production peaks within the first few minutes of reperfusion. Free-radical scavengers (e.g., antioxidants) have been shown to protect the heart from oxidative damage resulting from the formation of ROS during an I/R episode (Ambrosio et al., 1987b).

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ABBREVIATIONS: I/R, ischemia/reperfusion; ROS, reactive oxygen species; TMZ, trimetazidine; MAPK, mitogen-activated protein kinase; PBS, phosphate-buffered saline; DHE, dihydroethidium; TTC, triphenyltetrazolium chloride; DAF-FM, 4-amino-5-methylamino-2',7'-difluorofluorescein; TTC, triphenyltetrazolium chloride; DEPMPO, 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide; EPR, electron paramagnetic resonance; LAD, left anterior descending; HE, hydroxyethidine; CK, creatine kinase; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; p-, phosphorylated; ERK, extracellular signal-regulated kinase.

Trimetazidine [1-(2,3,4-trimethoxybenzyl)piperazine; TMZ] is an anti-ischemic drug that modifies metabolic function without affecting the hemodynamic determinants of myocardial oxygen consumption (e.g., heart rate, systolic blood pressure, and rate-pressure product) (McClellan and Plosker, 1999). TMZ optimizes the cardiac metabolism by reducing fatty acid oxidation through the selective inhibition of mitochondrial 3-ketoacyl CoA thiolase. As a result, TMZ attenuates the adverse effects of free fatty acid-associated oxidative stress (Gambert et al., 2006), lessens oxygen demand by decreasing oxygen consumption (Monteiro et al., 2004), and improves mitochondrial metabolism and cardiac performance during ischemia (Kantor et al., 2000). TMZ has been reported to attenuate neutrophil activation, thereby protecting postischemic hearts from neutrophil-mediated injury (Tritto et al., 2005). At the cellular level, TMZ conserves ATP production and lowers intracellular acidosis and calcium overload, while maintaining cellular homeostasis (Kantor et al., 2000). It reduces oxidative damage to the mitochondria and protects the heart from I/R-induced damage arising from mitochondrial respiration (Guarnieri and Muscari, 1993). TMZ has also shown cytoprotective efficacy in several models of myocardial infarction (Harper et al., 1989; Pantos et al., 2005). In addition to its beneficial effects in the treatment of I/R injury, TMZ has been reported to provide modest protection to postischemic hearts by improving left ventricular function in patients with chronic coronary artery disease or ischemic cardiomyopathy and in patients experiencing acute periods of ischemia when undergoing percutaneous transluminal coronary angioplasty (McClellan and Plosker, 1999).

We have reported previously that TMZ and its antioxidant-modified derivatives administered 1 min before the induction of ischemia mitigated cardiac dysfunction and injury in isolated rat hearts (Kutala et al., 2006). In this study, we hypothesized that administration of TMZ before the onset of reperfusion could reduce the severity of I/R injury. The objective of the present study was to determine whether TMZ, given before reperfusion, could attenuate myocardial reperfusion injury in an in vivo rat model of myocardial I/R. The results showed that TMZ was cardioprotective when administered before reperfusion and that the protective appeared to be through activation of p38 mitogen-activated protein kinase (MAPK) and Akt signaling.

Materials and Methods

Chemicals. TMZ was synthesized as described previously (Kálai et al., 2006). The compound was freshly prepared in dimethyl sulfoxide and diluted with phosphate-buffered saline (PBS) before administration. Dihydroethidium (DHE), xanthine, xanthine oxidase, 2,2-azobis-2-amidinopropane dihydrochloride, diethylenetriaminepentaacetate, and triphenyltetrazolium chloride (TTC) were obtained from Sigma-Aldrich (St. Louis, MO). 4-Amino-5-methylamino-2,7-difluorofluorescein (DAF-FM) was obtained from Invitrogen (Carlsbad, CA). 5-(Diethoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide (DEPMPO) was obtained from Radical Vision (Jerome, Marseille, France). All other reagents were analytical grade or higher purchased from Sigma-Aldrich, unless otherwise mentioned.

Measurement of Superoxide and Alkylperoxyl Radicals by EPR Spectroscopy. The superoxide and alkylperoxyl radical-scavenging ability of TMZ was determined using EPR spectroscopy (Khan et al., 2007b; Mohan et al., 2009). A mixture of xanthine (0.2 mM) and xanthine oxidase (0.02 U/ml) in PBS, pH 7.4, was used to

generate superoxide radicals. Alkylperoxyl radicals were generated through the thermolytic fission of 25 mM 2,2-azobis-2-amidinopropane dihydrochloride in aerobic PBS solution at 37°C. All EPR measurements were performed in PBS, pH 7.4, containing 1 mM DEPMPO and 0.1 mM diethylenetriaminepentaacetate in the absence or presence of 1 mM TMZ. The superoxide and peroxyl radicals were detected as DEPMPO-OOH and DEPMPO-OOR adducts, respectively. The attenuation of DEPMPO adduct generation was quantified by double integration and expressed as percentage of untreated (without TMZ) levels.

Myocardial Ischemia/Reperfusion Injury In Vivo. Male Sprague-Dawley rats (300–350 g) were used. Rats were randomly divided into two groups of eight animals each: 1) I/R group (control, vehicle-treated) and 2) TMZ group (trimetazidine-treated I/R). Rats were anesthetized with ketamine (50 mg/kg i.p.) and xylazine (5 mg/kg i.p.) and maintained under anesthesia using 1.5 to 2.0% isoflurane mixed with air. Animals were given a single bolus intravenous injection of TMZ (5 mg/kg b.wt.) in 0.5 ml of saline. Myocardial infarction was created by ligating the left anterior descending (LAD) coronary artery, as described previously (Mohan et al., 2009). An oblique 12-mm incision was made 8 mm away from the left sternal border toward the left armpit. The chest cavity was opened with scissors by a small incision (10 mm in length) at the level of the fourth or fifth intercostal space, 3 to 4 mm from the left sternal border. The LAD coronary artery was visualized as a pulsating bright red spike, running through the midst of the heart wall from underneath the left atrium toward the apex. The LAD coronary artery was ligated 1 to 2 mm below the tip of the left auricle using a tapered needle and a 6-0 polypropylene suture. The suture was passed underneath the LAD coronary artery, and a double knot was made over the artery and PE-10 tubing to occlude the artery. Occlusion was confirmed by the sudden change in color (pale) of the anterior wall of the left ventricle. The chest cavity was closed by bringing together the fourth and fifth ribs with one 4-0 silk suture. The layers of muscle and skin were also closed with a 4-0 polypropylene suture. After ligation of LAD coronary artery, successful infarction was confirmed by an ST elevation on electrocardiograms that were recognized in all surgical groups of animals. TMZ (5 mg/kg b.wt.) was administered intravenously 5 min before reperfusion. The study used a single dose based on a recent report using a single bolus administration of 5 mg/kg b.wt. TMZ before coronary occlusion showed a significant reduction in infarct size (Argaud et al., 2005). After 30 min of ischemia, the flow was restored by releasing the ligation. After 60 min of reperfusion the chest was closed. After reinstallation of spontaneous respiration, animals were extubated and allowed to recover from the anesthesia. All the procedures were performed with the approval of the Institutional Animal Care and Use Committee of The Ohio State University and conformed to the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health publication 86-23).

Echocardiography (M-Mode) for Cardiac Functional Analysis. Cardiac function was analyzed using echocardiography after 48 h of reperfusion. Rats were anesthetized using 1.5 to 2% isoflurane, and M-mode ultrasound images were acquired using a Vevo 2100 high-resolution ultrasonic rodent imaging system (Visualsonics; Toronto, ON, Canada).

Measurement of Myocardial pO₂ Using in Vivo EPR Oximetry. EPR oximetry was used to monitor myocardial tissue oxygenation (pO₂) at the baseline, during ischemia and after reperfusion as described previously (Khan et al., 2009a,b; Mohan et al., 2009). The principle of EPR oximetry is based on molecular oxygen-induced line width changes in the EPR spectrum of a paramagnetic probe. The probe is a microcrystal of stable, safe, and nontoxic paramagnetic material that can be permanently implanted in the tissue region of interest using a 25-gauge needle. Once implanted, the probe responds to pO₂ in the immediate surroundings, primarily at the crystal-tissue interface, enabling magnetic resonance-based noninvasive and repeated measurements of pO₂ for a prolonged period

from the site of implantation. The technology has been validated for measurements of pO_2 from single cells to whole organs (Pandian et al., 2003; Kutala et al., 2004; Khan et al., 2007a, 2009a,b; Mohan et al., 2009).

The myocardial pO_2 measurements in the present study were performed using an in vivo EPR spectrometer (Magnetech GmbH, Berlin, Germany) equipped with automatic coupling and tuning controls for measurements in intact beating hearts. Microcrystals of lithium octa-*n*-butoxy-naphthalocyanine (Pandian et al., 2003) were used as a probe for EPR oximetry. Rats under inhalation anesthesia (air containing 1.5–2.0% isoflurane) were implanted with the oxygen-sensing probe in the left ventricular midmyocardium. The animal was placed in a right lateral position with the chest open to the loop of a surface-coil resonator. EPR spectra were acquired as single 30-s duration scans. The instrument settings were as follows: microwave frequency, 1.2 GHz (L-band), incident microwave power, 4 mW; modulation amplitude, 180 mG, modulation frequency, 100 kHz; and receiver time constant, 0.2 s. The peak-to-peak width of the EPR spectrum was used to calculate pO_2 using a standard calibration curve (Pandian et al., 2003). Similarly, myocardial pO_2 was measured after 48 h of reperfusion.

Measurement of Superoxide by DHE. Rats were subjected to ischemia for 30 min and infused with saline (control) or TMZ (experimental) 5 min before reperfusion. The rats were sacrificed at 10 min after reperfusion, and the hearts were placed immediately in ice-cold PBS and then embedded in optimal cutting temperature medium for cryosectioning. Frozen heart sections were thawed, and superoxide generation in the heart tissue was determined using DHE fluorescence (Miller et al., 1998). The cell-permeable DHE is oxidized to fluorescent hydroxyethidine (HE) by superoxide, which is then intercalated into DNA. Because it is known that there is a burst of oxygen free radical generation in the early minutes of reperfusion in hearts subjected to I/R, we measured DHE fluorescence at 10 min of reperfusion (Khan et al., 2009b). The frozen segments from the heart tissue were cut into sections 6 μ m in thickness, which were then placed on glass slides. DHE (10 μ M) was topically applied to each tissue section. The slides were incubated in a light-protected chamber at 37°C for 30 min. The slides were then washed several times with PBS to remove nonspecific DHE staining, and a coverslip was applied using aqueous-mounting media. The images of the tissue sections were obtained using a fluorescence microscope (model TE 300; Nikon, Tokyo, Japan) equipped with a rhodamine filter (green excitation, 550 nm; red emission, 573 nm). Fluorescence intensity, which positively correlates with the amount of superoxide generated within the specimen, was quantified in the myocardial tissue samples using MetaMorph image analysis software (Molecular Devices, Sunnyvale, CA).

Measurement of Nitric Oxide Using DAF-FM. The NO produced in I/R hearts was measured by fluorescence microscopy using DAF-FM diacetate. DAF-FM diacetate is cell-permeable and passively diffuses across cellular membranes. Inside cells, DAF-FM diacetate is deacetylated by intracellular esterases to DAF-FM, which reacts with NO to form fluorescent benzotriazole. Hearts, after 30 min of ischemia and 10 min of reperfusion, were placed in an ice-cold PBS buffer and embedded in optimal cutting temperature compound for cryosectioning. The frozen tissues were cut into 6- μ m-thick sections and incubated with 20- μ M DAF-FM diacetate for 1 h at 37°C. Images of the tissue sections were obtained using a fluorescence microscope (model TE 300; Nikon) with a fluorescein isothiocyanate filter (blue excitation, 495 nm; green emission, 510 nm). The fluorescence intensity, which positively correlated with the amount of NO generation, was quantitatively determined using the MetaMorph image analysis software (Molecular Devices, Sunnyvale, CA).

Determination of Plasma Creatine Kinase. The plasma concentration of creatine kinase (CK) was determined in rats subjected to 30 min of ischemia followed by 48 h of reperfusion. Rats were anesthetized using pentobarbital sodium (50 mg/kg b.wt.). Approximately 1 ml of blood was collected from the abdominal aorta using a

22-gauge needle. The blood samples were centrifuged, and the plasma was stored at -80°C until the analyses were performed. Plasma CK levels in the circulation was determined using commercially available kits obtained from Catamount (Bridgeport, CT) according to the manufacturer's instructions.

Measurement of Myocardial Infarct Size. After 48 h of reperfusion, the rats were sacrificed, and their hearts were washed with PBS and perfused with Krebs' buffer. The LAD coronary artery was ligated once again, and 0.2 ml of Evans blue dye (2%) was infused retrogradely from the aorta. The heart was frozen at -20°C for 10 min and then cut into four transverse slices. The slices were then incubated at 37°C for 10 min with 1.5% TTC to determine the infarct area and the area at risk. Photographs were taken using dissecting microscope (Nikon). The left ventricular area, area at risk, and infarct area were quantified by computerized planimetry using MetaVue image analysis software (Molecular Devices). The area of myocardial tissue showing white color was defined as infarct, and the region in red was defined as the area at risk. Infarct size was expressed as a percentage of the area at risk.

Assessment of Apoptosis by TUNEL Assay. Tissue sections were mounted on poly(L-lysine)-coated slides. The cryopreserved sections (6 μ m in thickness) were fixed in freshly prepared 4% paraformaldehyde for 20 min at room temperature. The slides were allowed to sit for 30 min in PBS and washed twice with fresh PBS. The samples were then incubated with permeabilization solution (0.1% Triton X-100 and 0.1% sodium citrate, freshly prepared) for 2 min on ice. DNA strand breaks were detected using the terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay. The reagents were procured from Roche Diagnostics (Indianapolis, IN). In brief, sections were covered with 50 μ l of TUNEL reaction mixture and incubated in this solution for 60 min at 37°C in a humidified chamber. After rinsing in PBS, the sections were mounted with Vectashield HardSet Mounting Medium with 4,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA) and visualized using a fluorescence microscope. The apoptotic index (the percentage of TUNEL-positive cardiomyocytes relative to total nuclei) was then calculated from at least three slides, five fields/slide with a nucleus in the ischemic area using MetaMorph image analysis software (Molecular Devices).

Western Blot Analysis. Determination of molecular signaling cascades involved after ischemia/reperfusion injury was determined by Western blot analysis in cardiac tissues homogenates. Western blots for p-Akt, p-ERK1/2, p-p38, Bcl-2, cytochrome *c*, and caspase-3 signaling were performed with the tissue homogenates prepared from the anterior wall of the left ventricles of rats from pre-I/R, I/R, and TMZ groups. After the treatment period, I/R and TMZ rats were anesthetized and sacrificed at 48 h of reperfusion. Control rats (non-I/R; $n = 4$) were also used. The hearts were rapidly excised, rinsed in ice-cold PBS, pH 7.4, containing 500 U/ml heparin to remove red blood cells and clots, frozen in liquid nitrogen, and stored at -80°C until analysis. Heart tissues were homogenized in TN1 lysis buffer containing 50 mM Tris, pH 8.0, 10 mM EDTA, 10 mM NaF, 1% Triton X-100, 125 mM NaCl, 1 mM Na_3VO_4 , and 1% protease inhibitor (Sigma-Aldrich). The tissue homogenate was incubated for 60 min on ice, followed by microcentrifugation at 10,000g for 15 min at 4°C. Aliquots of 75 μ g of protein from each sample were boiled in Laemmli buffer (Bio-Rad Laboratories, Hercules, CA) containing 1% 2-mercaptoethanol for 5 min. The protein was separated by SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membrane, and probed with primary antibodies for Bcl-2, Akt, and phospho-Akt (Ser 473) (Cell Signaling Technology Inc., Danvers, MA). The membranes were incubated overnight at 4°C with the primary antibodies, followed by incubation with horseradish peroxidase-conjugated secondary antibodies (GE Healthcare, Little Chalfont, Buckinghamshire, UK) for 1 h. The membranes were then developed using an enhanced chemiluminescence detection system (ECL Advanced kit; GE Healthcare). The same membranes were then reprobed for glyceraldehyde-3-phosphate dehydrogenase. The

protein intensities were quantified by an image scanning densitometer (Scion Corporation, Frederick, MD). To quantify the phospho-specific signal in activated proteins, we first subtracted the background and then normalized the signal to the amount of glyceraldehyde-3-phosphate dehydrogenase or total target protein in the tissue homogenate (Selvendiran et al., 2007; Mohan et al., 2009; Wisel et al., 2009). Data are expressed as percentage of the expression in the control group.

Data Analysis. The statistical significance of the results was evaluated using one-way analysis of variance followed by a Student's *t* test. The values were expressed as mean $\pm$ S.D. A *p* value of <0.05 was considered significant.

Results

Scavenging of Free Radicals by TMZ. DEPMPO spin trap (1 mM) was used for direct detection of superoxide and peroxy radicals as DEPMPO-OOH and DEPMPO-OOR adducts, respectively, using EPR spectroscopy. Figure 1 shows the scavenging effect of TMZ against these radicals. TMZ (1 mM) challenged against 1 mM DEPMPO decreased the intensity of the DEPMPO-OOH spectrum by more than 20%. Likewise, 1 mM TMZ decreased peroxy radical adduct formation by more than 50%. The results showed that TMZ significantly reduced the superoxide and peroxy radical levels in vitro.

Effect of TMZ on Cardiac Functional Recovery in the Reperfused Heart. The experimental protocol is shown in Fig. 2. Cardiac function was measured in rats after 48 h of reperfusion by ultrasound M-mode echocardiography (Fig. 3A). The baseline cardiac function in noninfarct control rats was $84 \pm 2\%$. In untreated I/R group of rats, the cardiac function was significantly reduced ($62 \pm 5\%$) after 48 h of reperfusion

Fig. 1. Free radical-scavenging efficacy of TMZ. Superoxide (O_2^-) and alkylperoxyl ($ROO^\bullet$) radicals were generated in the presence of 1 mM TMZ and detected by EPR spectroscopy using 1 mM DEPMPO as a spin-trapping/competing agent. Quantification (right) and EPR spectra (left) show a reduction in EPR signal intensity corresponding to superoxide and alkylperoxyl radicals. The bar graphs represent mean $\pm$ S.D. from five independent experiments.

Fig. 2. Experimental design. In the I/R group, rats underwent 30 min of LAD coronary artery ligation. TMZ (5 mg/kg b.wt.) or vehicle (saline) was administered intravenously at 5 min before the onset of reperfusion. After myocardial pO_2 measurements after 60 min of reperfusion, the chest cavity was closed and the animals were allowed to recover. Echocardiography and oximetry were performed after 48 h of reperfusion, after which the animals were sacrificed for further analysis as indicated.

compared with the baseline value. However, in the TMZ, the cardiac functional recovery was significantly enhanced ($74 \pm 3\%$) after 48 h of reperfusion compared with the I/R group at the same period. The results from this study clearly indicated that TMZ treatment enhanced cardiac functional recovery.

Effect of TMZ on Myocardial Oxygenation in the Reperfused Heart. Rat hearts were implanted with an oxygen-sensing microcrystalline probe in the left ventricular midmyocardium, the expected site of I/R after LAD coronary artery ligation and reperfusion (Mohan et al., 2009). We then performed continuous pO_2 measurements in the heart at the baseline, during ischemia, and after reperfusion. Figure 4 shows the myocardial tissue pO_2 changes during the 30 min of ischemia followed by 1 and 48 h after reperfusion in both I/R and TMZ groups. The basal (pre-I/R) levels of myocardial pO_2 were in the range of 18 to 21 mm Hg, and there was no significant difference between the two I/R groups. The myocardial pO_2 dropped sharply and remained approximately 2 mm Hg during the entire 30 min of ischemia in the I/R group. The mean pO_2 value in the TMZ group was slightly above that of the I/R group during ischemia; however, there was no significant difference between the two groups. A rapid increase in pO_2 , leading to marked hyperoxygenation, was observed in the I/R group at 1 h, and it continued to persist to 48 h after reperfusion. In contrast, the mean pO_2 in TMZ-treated hearts returned to near-normal levels. In the I/R group, the mean pO_2 value remained elevated at 48 h after reperfusion, whereas TMZ-treated hearts showed a significant attenuation of hyperoxygenation by comparison.

Effect of TMZ on the Release of Creatine Kinase from the Reperfused Heart. The key events after I/R injury to the heart is associated with cessation of contractile activity, alteration of membrane integrity, and leakage of key enzymes such as CK into the plasma. The levels of CK were high in I/R hearts (Fig. 5A). However, in hearts treated with TMZ, there was a significant decrease in CK levels in serum collected after 48 h of reperfusion.

Effect of TMZ on Myocardial Infarct Size. The measurement of myocardial infarct area is one of the important parameters used to assess I/R-induced myocardial damage. The percentage of myocardial infarct area was measured using TTC and Evans blue staining in rat hearts subjected to I/R injury (Fig. 5B). We did not observe any significant change in the area at risk between the I/R and TMZ groups (Fig. 5C). However, the infarct size in the I/R group was $45.0 \pm 4.0\%$ of at risk area. Infarct area was significantly

Fig. 3. Effect of TMZ on cardiac functional recovery in the reperfused heart. A, representative ultrasound echocardiography (M-mode) images and data collected from rat hearts 48 h after reperfusion. B, ejection fraction. TMZ-treated rats showed significant improvements in the recovery of ejection fraction and fractional shortening compared with the I/R group ($n = 6$).

Fig. 4. Effect of TMZ on myocardial pO₂ in the reperfused rat heart. Myocardial tissue pO₂ values (mean $\pm$ S.D.; $n = 6$ animals/group) measured by EPR oximetry at 30 min of ischemia, followed by 1 and 48 h of reperfusion in the I/R and TMZ group of hearts. The “baseline” data were obtained from hearts before induction of ischemia. The results show an oxygen overshoot (hyperoxygenation) during reperfusion in the I/R group. However, in TMZ-treated hearts, the pO₂ values remained at near normal levels.

decreased in hearts treated with TMZ ($24 \pm 7\%$) compared with the I/R group (Fig. 5D).

Effect of TMZ on Superoxide Levels in the Reperfused Heart. We determined the effect of TMZ on the superoxide generation in the hearts subjected to 30 min of ischemia followed by 10 min of reperfusion using HE fluorescence staining (Fig. 6A). The HE fluorescence intensity was higher in the I/R group compared with the control group (Fig. 6B). In TMZ-treated hearts, the HE intensity was significantly decreased compared with the I/R group. The results clearly showed the attenuation of superoxide level in the early minutes of reperfusion after TMZ treatment.

Effect of TMZ on Nitric Oxide Levels in the Reperfused Heart. The NO levels in the hearts at 10 min of reperfusion was significantly higher in TMZ-group, compared with the I/R group (Fig. 6B). The results suggested

that TMZ enhanced tissue NO level during the early phase of reperfusion.

Effect of TMZ on the Apoptosis of Cardiomyocytes in the Reperfused Heart. Apoptosis is one of the most important events involved in I/R injury. Apoptotic cell death typically occurs via activation of caspases that cleave DNA and other components. Cardiac apoptosis was evaluated by TUNEL assay (Fig. 7A). Increased TUNEL-positive nuclei were found in the epicardial regions of the I/R group of hearts after 48 h of reperfusion (Fig. 7B). The TMZ group of hearts had a significant reduction in TUNEL-positive nuclei in infarct and peri-infarct regions of the heart.

Effect of TMZ on the Signaling Molecules in the Reperfused Heart. To further elucidate the underlying molecular mechanism involved in signal transduction responsible for cardioprotection by TMZ during and after I/R injury, we performed Western blot analysis of heart homogenates to determine the expression levels of several antiapoptotic or survival proteins (Akt, phospho-Akt, p38 MAPK, phospho-p38 MAPK, ERK1/2, phospho-ERK1/2, and Bcl-2) and an apoptosis-promoting protein (caspase-3 and cytochrome *c*). TMZ significantly enhanced the expression of phospho-Akt and phospho-p38 MAPK (Fig. 8). Concurrently, there was a significant reduction in caspase-3 expression. There was no significant difference in the levels of cytochrome *c*, Bcl-2, and phospho-ERK1/2 between the two groups. The Western blot analyses indicated that TMZ-treated hearts had an enhanced activation of p-Akt and p38 MAPK and decreased caspase-3 expression, leading to cardioprotection in the hearts at 48 h of reperfusion.

Discussion

The results of the present study provided important new information that administration of TMZ a few minutes before reperfusion attenuates myocardial injury and dysfunction in an in vivo experimental model of ischemia/reperfusion. This

Fig. 5. Effect of TMZ on myocardial injury and infarct size. **A**, CK levels in the serum collected from the rats at 48 h of reperfusion. The mean CK level was significantly less in the TMZ group compared with the I/R group of rats (mean $\pm$ S.D.; $n = 6$ animals/group). *, $p < 0.05$ versus I/R group. **B**, myocardial infarct size, determined using TTC staining. Representative images show TTC-stained sections of rat hearts from the I/R and TMZ groups at 48 h of reperfusion. Evans blue dye was used to quantify the area at risk (red). The infarct region is shown by white color. **C**, area at risk (AAR). **D**, infarct area (IA). Data represent mean $\pm$ S.D. ($n = 4$). *, $p < 0.05$ versus I/R group.

information has important relevance to the administration of TMZ in the clinic, either for treating stable angina or as an anti-ischemic agent before any surgical procedures that are expected to cause acute ischemic episodes. The clinical significance of our finding is that TMZ is also protective of the myocardium against postischemic injury and dysfunction caused by burst of oxygen free radicals. The results clearly demonstrated the amelioration of superoxide generation, hyperoxygenation, and apoptosis of cardiomyocytes, leading to significant decrease in myocardial infarction and greater recovery of cardiac function. Analyses of vital signaling proteins that are involved in I/R revealed the activation of pro-survival proteins, such as p38 MAPK and Akt, and inhibition of apoptotic caspase-3 by TMZ treatment.

The *in vitro* EPR spectroscopic studies established the antioxidant efficacy of TMZ in scavenging superoxide and peroxyl radicals. This suggests that TMZ is capable of attenuating the burst of superoxide production that occurs immediately upon reperfusion. Although superoxide is a free radical and classified as ROS, it is not very reactive and by itself may not cause oxidative damage to biological molecules. However, the byproducts of superoxide generated by self-mediated or superoxide dismutase-mediated dismutation, namely hydrogen peroxide and its reaction product with reduced metals, in particular, hydroxyl radicals, are potentially cytotoxic. Thus, superoxide may merely serve as an initiator

of a cascade of reactive oxygen species in the cellular milieu. The hydroxyl radical is the most deleterious of all ROS. It is nonspecific and can abstract a hydrogen atom from membrane lipids (polyunsaturated fatty acid) to give a carbon-centered alkyl radical. The reaction of carbon-centered radicals with molecular oxygen leads to peroxyl radicals, which are capable of initiating a chain reaction leading to massive lipid peroxidation and membrane damage. Because the peroxyl radicals are longer lived intermediates than the superoxide anion, it is also important to eliminate these peroxyl radicals to prevent membrane damage. The ability of TMZ to scavenge peroxyl radicals better than superoxide renders an added/complementary protection of cellular constituents against superoxide and its byproducts. The peroxyl radical-scavenging ability of TMZ (Fig. 1) might ensure a comprehensive obliteration of the reactive oxygen free radicals in the reperfused heart.

Likewise, under *in vivo* conditions, administration of TMZ just before the onset of reperfusion decreased hydroethidine fluorescence, which is a marker for superoxide radicals. The diminished level of superoxide in the reperfused tissue may also attenuate the deleterious peroxynitrite formation and enhance the bioavailability of NO (Paolocci et al., 2001; Khan et al., 2009b) as we have observed in the present study (Fig. 6). Thus, this study confirms the antioxidant effect of TMZ in attenuating ROS generation at reperfusion, thereby abrogating reperfusion-induced myocardial damage.

To our knowledge, this is the first report in which the *in vivo* myocardial oxygen concentration in the heart was quantified after TMZ administration. The results demonstrated that TMZ ameliorates the I/R-induced oxygen overshoot (hyperoxygenation) that we have routinely observed in rodent hearts (Zhao et al., 2005; Khan et al., 2009b; Mohan et al., 2009). The myocardial oxygenation measured at the ischemic region was elevated as much as 36% at 1 h after reflow, and it remained significantly elevated even after 48 h of reperfusion. The hyperoxygenation may be due to contractile “stunning,” which is a reversible loss of contractility known to occur immediately up on reperfusion and last for several hours to days (Bolli and Marbán, 1999). During this time, the myocardium receives adequate oxygen supply, but it does not fully use the oxygen because of depressed contractility (myocardial work) that is not immediately restored to preischemic levels. This condition may lead to a paradoxical hyperoxia due to lack of oxygen use. Ambrosio et al. (1987a) have observed an overshoot of cardiac phosphocreatine concentration in rabbit hearts upon postischemic reflow. The overshoot effect was attributed to a decrease in phosphocreatine use leading to an imbalance between supply and rate of use of the high-energy phosphate metabolic reserve in the stunned heart (Ambrosio et al., 1987a). In the present study, the absence of hyperoxygenation in the TMZ-treated hearts at 1 and 48 h of reperfusion could be associated with an increased recovery of contractility and attenuation of myocardial injury. In addition, the hyperoxygenation at reperfusion might indicate a decrease in oxygen consumption by the injured tissue. In addition, the increased NO production in TMZ-treated hearts at 10 min of reperfusion did not correlate or attribute to any changes in tissue oxygenation as reported in our previous study (Khan et al., 2009b). It is likely that the increased level of NO in the reperfused heart could be due to the decreased level of superoxide, which is capable of con-

Fig. 6. Effect of TMZ on the tissue levels of superoxide and nitric oxide in the infarct hearts. Superoxide and nitric oxide levels in the excised heart tissue were determined at 10 min after reperfusion by fluorescence microscopy using DHE and DAF-FM, respectively. A, representative images (at 200 $\times$ magnification) of superoxide and nitric oxide from tissue sections obtained from control, I/R (untreated), and TMZ-treated hearts. B, mean fluorescence intensity for superoxide and nitric oxide. Data represent mean $\pm$ S.D. ($n = 3$). *, $p < 0.05$ versus I/R group.

verting free NO into peroxynitrite. Therefore, it is reasonable to assume that the decreased hyperoxygenation in TMZ-treated hearts is more due to restoration of contractility in the reperfused myocardium. Further studies are needed to delineate the mechanisms responsible for the changes in myocardial oxygenation up on reflow.

Cardiomyocyte apoptosis is induced during ischemia as well as at reperfusion (Gottlieb et al., 1994; Fliss and Gattlinger, 1996). In the present study, we observed TUNEL-positive nuclei in the ischemic region and along the borders of the ischemic region in untreated hearts subjected to I/R. In comparison, TMZ-treated hearts had a significant attenuation of TUNEL-positive nuclei (Fig. 7). The TUNEL assay data clearly demonstrated that TMZ administration before reperfusion prevents cardiomyocyte apoptosis. This was further supported by a significant decrease in serum CK and myocardial infarct size in TMZ-treated hearts.

Several studies have demonstrated the involvement of Akt and MAPKs in mediating intracellular signal transduction

events associated with the oxidative stress conditions that occur during I/R (Omura et al., 1999). MAPKs, namely, p38 MAPK, ERK1/2, and c-Jun NH₂-terminal kinase are activated in the I/R hearts and modulate oxidant-mediated tissue injury (Armstrong, 2004). Several cardioprotective pharmacological agents are known to mitigate the I/R-mediated oxidative stress through modulation of Akt, p38 MAPK, or ERK1/2 activities (Toth et al., 2003; Liu et al., 2004; Takada et al., 2004; Khan et al., 2006). Our results revealed an increased activation of phospho-p38 MAPK in the hearts treated with TMZ at reperfusion. In addition, we observed a significant increase in the prosurvival phospho-Akt protein level but no significant difference in ERK1/2 or Bcl-2 levels. Previous studies have demonstrated the activation of ERK1/2 and Akt in the reperfused myocardium to be cardioprotective (Ma et al., 1999; Liu et al., 2004). We have reported previously using isolated rat hearts subjected to global ischemia-reperfusion that TMZ administered before induction of ischemia did not show any change in the activa-

Fig. 7. Evaluation of cellular apoptosis by TUNEL assay. Effect of TMZ on the apoptosis in heart tissues subjected to I/R. A, apoptosis (green) was imaged in infarct heart tissues at 48 h after reperfusion. B, increased TUNEL-positive nuclei were seen in the I/R group, whereas TMZ-treated hearts exhibited a significant decrease in TUNEL-positive nuclei (mean $\pm$ S.D.; $n = 4$ hearts/group). *, $p < 0.05$ versus I/R group. C, overlay of TUNEL-positive (green) and 4,6-diamidino-2-phenylindole (DAPI; blue) images from expanded regions as indicated.

tion of ERK1/2, suggesting that TMZ has no effect on ERK1/2 activation in I/R injury (Kutala et al., 2006). It should be noted that caspase-3 level, although was significantly decreased by TMZ, was still substantially elevated in the TMZ group. However, the absence of any significant change in the cytochrome *c* level would imply that the mitochondrial function was preserved in TMZ-treated I/R hearts.

Trimetazidine has been in clinical use for more than 20 years. It is an effective treatment for stable angina and a potential drug for treating systolic dysfunction in cardiac

failure patients. 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). TMZ has a positive influence on I/R injury, endothelial dysfunction, and prognosis in patients with coronary artery disease. The present study revealed that TMZ, in addition to its above-mentioned therapeutic potential, could attenuate reperfusion-induced

Fig. 8. Western blot analysis. Western blot analysis results for p38, p-p38, Akt, p-Akt, ERK1/2, p-ERK1/2, Bcl-2, and cytochrome *c* in heart tissue homogenates obtained 48 h after ischemia/reperfusion injury (data represent mean $\pm$ S.D.; $n = 4$ hearts/group). *, $p < 0.05$ versus I/R group. The results show that TMZ treatment significantly enhanced the expression of p-p38 and p-Akt, and decreased caspase-3 expression.

ROS generation by its inhibitory as well as radical-scavenging properties. The ROS-scavenging ability of TMZ in the present study is further augmented by its ROS-inhibiting functions such as the inhibition of neutrophil-mediated ROS production (Duilio et al., 2001; Tritto et al., 2005). Our future studies will evaluate the potential of the antioxidant-conjugated forms of TMZ, which have been developed previously (Kutala et al., 2006), for protection against I/R-mediated cardiac dysfunction and injury under in vivo settings.

In conclusion, the present study underlines the potential clinical significance of administering TMZ before the onset of reperfusion, which may be valuable for patients undergoing coronary angioplasty or percutaneous coronary interventions.

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