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Chronic Tempol treatment restores pharmacological preconditioning in the senescent rat heart

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Zhu J, Rebecchi MJ, Wang Q, Glass PS, Brink PR, Liu L. Chronic Tempol treatment restores pharmacological preconditioning in the senescent rat heart. Am J Physiol Heart Circ Physiol 304: H649–H659, 2013. First published December 28, 2012; doi:10.1152/ajpheart.00794.2012.—Cardioprotective effects of anesthetic preconditioning and cyclosporine A (CsA) are lost with aging. To extend our previous work and address a possible mechanism underlying age-related differences, we investigated the role of oxidative stress in the aging heart by treating senescent animals with the oxygen free radical scavenger Tempol. Old male Fischer 344 rats (22–24 mo) were randomly assigned to control or Tempol treatment groups for 2 or 4 wk (T×2wk and T×4wk, respectively). Rats received isoflurane 30 min before ischemia-reperfusion injury or CsA just before reperfusion. Myocardial infarction sizes were significantly reduced by isoflurane or CsA in the aged rats treated with Tempol (T×4wk) compared with old control rats. In other experiments, young (4–6 mo) and old rats underwent either chronic Tempol or vehicle treatment, and the levels of myocardial protein oxidative damage, antioxidant enzymes, mitochondrial Ca2+ uptake, cyclophilin D protein, and mitochondrial permeability transition pore opening times were measured. T×4wk significantly increased MnSOD enzyme activity, GSH-to-GSSH ratios, MnSOD protein level, mitochondrial Ca2+ uptake capacity, reduced protein nitrosylsine levels, and normalized cyclophilin D protein expression in the aged rat heart. T×4wk also significantly prolonged mitochondrial permeability transition pore opening times induced by reactive oxygen species in old cardiomyocytes. Our studies demonstrate that 4 wk of Tempol pretreatment restores anesthetic preconditioning and cardioprotection by CsA in the old rat and that this is associated with decreased oxidative stress and improved mitochondrial function. Our results point to a new protective strategy for the ischemic myocardium in the high-risk older population.

aging; oxidative stress; pharmacological preconditioning; antioxidant

CARDIOVASCULAR DISEASES are the major cause of death in the population above age 65 (6). Moreover, older patients are at higher risk of adverse cardiac events in the perioperative period (12). Clearly, improving our knowledge of ischemic injury and devising better cardioprotective strategies in the senescent myocardium are of paramount importance. Pharmacological preconditioning would seem a promising strategy to protect the heart, especially in the perioperative period. We and others previously have reported, however, that older animals fail to benefit from anesthetic preconditioning (APC), ischemic preconditioning, or pharmacological preconditioning including cyclosporine A (CsA) (34, 37, 42, 44). This could help explain the failure of preconditioning strategies to alter relevant outcomes in clinical studies (11, 48). Although aging is associated with oxidative stress, which has been implicated in the pathophysiology of cardiovascular diseases, as well as cardiac injury after episodes of ischemia-reperfusion (I/R), it is still unclear whether chronic oxidative stress and the damage it causes underlies the loss of pharmacological preconditioning with aging.

Mitochondrial dysfunction contributes to age-related changes in the heart by increasing the production of reactive oxygen species (ROS) (26), and this has been shown to reduce its tolerance to I/R injury, resulting in the opening of the mitochondrial permeability transition pore (mPTP) (33). Several studies have shown that various biomarkers of oxidative stress, such as protein nitration, increase with myocardial aging (2, 3), whereas antioxidant capacities decrease (38). Although the mechanism(s) underlying cardiac preconditioning have not yet been adequately elucidated, transient increases in ROS have been shown to be an early trigger for preconditioning, whereas high levels, as seen ROS in reperfusion, may lead to myocardial stunning, infarction, and apoptosis (10, 25, 30). In our previous studies, we observed that ROS levels appeared to be constitutively higher in the aged myocardium and that cardioprotection by APC or CsA is clearly lost in the senescent animals (34, 42). One potential explanation is that isoflurane-induced mitochondrial ROS production in the aged myocardium is not sufficient to trigger preconditioning. Another is that constitutively elevated levels of ROS may damage proteins, nucleic acids, and lipids in the aging heart that could lead to an accumulation of damaged mitochondria and a loss of the preconditioning response. In the present study we reestablish the reox balance in the aging heart using a well-known free radical scavenger and superoxide dismutase (SOD) mimetic Tempol to test whether pretreatment with this antioxidant could restore pharmacological preconditioning in the senescent heart and document the consequences of Tempol treatment for the aging myocardium. We hypothesize that a suitable antioxidant administered at a sufficiently high level for a sufficient time period would reverse the signs of oxidative damage and improve mitochondrial function. Furthermore, we hypothesize that this would reestablish sensitivity to pharmacological preconditioning in the aging heart.
MATERIALS AND METHODS

All experimental procedures and protocols used in this investigation were reviewed and approved by the Animal Care and Use Committee of SUNY Stony Brook. Furthermore, all procedures conformed to the “Guiding Principles in the Care and Use of Animals” of the American Physiological Society and were in accordance with the Guide for the Care and Use of Laboratory Animals.

Animals Care and Tempol Administration

Male Fischer 344 rats of the following age groups, 3–5 mo (250–280 g) and 22–24 mo (420–480 g), were obtained from National Institute on Aging (Bethesda, MD). Animals were housed in the Division of Laboratory Animal Resources until the day of study. Each rat was randomized to receive or not receive a daily intraperitoneal injection of Tempol (125 mg·kg⁻¹·day⁻¹) dissolved in saline solution (125 mg/ml) each morning (9:00–10:00 AM) (18, 54). In the control groups, rats received intraperitoneal injection of saline solution (1 ml/kg) each day. The final dose of Tempol was administered 24 h before treatment with isoflurane or CsA, and I/R injury.

General Preparation and Surgery Protocol

Anesthesia was induced with an intraperitoneal injection of sodium thiobutabarbital (120–135 mg/kg; Sigma-Aldrich) with additional maintenance doses given as needed. Rats were tested for the absence of pedal reflexes throughout the experimental protocol to ensure adequate anesthesia. Heparin-filled (10 U/ml; APP Pharmaceuticals) catheters were inserted into the right jugular vein for fluid and drug administration. The right carotid artery was cannulated to measure arterial blood pressure. A tracheotomy was performed and the animal was ventilated using a Harvard Apparatus model 638 rat ventilator with an air and oxygen (1:1) mixture and 5 cmH₂O of positive end-expiratory pressure. Inspired oxygen concentrations were maintained at 50%, and end-tidal carbon dioxide concentration was maintained at 35–40 mmHg by adjusting the respiratory rate or tidal volume throughout the experiment. Arterial blood gas tension and acid-base status were monitored at regular intervals and maintained within a normal range (pH, 7.35–7.45; arterial partial pressure of CO₂, 30–40 mmHg; and arterial partial pressure of O₂, 90–150 mmHg). End-tidal concentrations of carbon dioxide and inspired oxygen concentrations were measured using a Puritan-Bennett 7800 infrared gas analyzer (Criticare Systems). Body temperature was maintained at 37.0 ± 0.2°C using a heating pad and radiant warmer. The surgery protocol followed our previous work (42). In brief, following surgery, to expose the anterior surface of the heart, a 6-0 prolene suture (Ethicon) was placed around the proximal left anterior descending coronary artery (LAD), and coronary artery occlusion was produced by clamping the snare onto the epicardial surface of the heart with a hemostat and was confirmed by the appearance of epicardial cyanosis. Reperfusion was achieved by loosening the snare and was verified by observing an epicardial hyperemic response. At the end of the experiment, the animal was euthanized with an overdose of sodium thiobutabarbital.

Our experimental design is illustrated in Fig. 1. Protocol A was designed for infarction size measurements. In this protocol, rats (n = 8 per treatment group) of age (22–24 mo) were randomly assigned to one of the groups. All animals underwent 30 min of LAD occlusion followed by 120 min of reperfusion. In the Tempol chronic treatment groups, Tempol (125 mg/kg) (Sigma-Aldrich) (45) was administrated daily via intraperitoneal injection for 2 or 4 wk, and 24 h after the final injection, animals underwent I/R injury. In the APC group, isoflurane 1.0 minimum alveolar concentration was administrated 30 min before ischemia. A 15-min rest period followed the discontinuation of the volatile anesthetic to allow the end-tidal isoflurane concentration to reach zero. In the CsA group, CsA (10 mg/kg) was dissolved in DMSO and administrated over 2 min intravenously 5 min before reperfusion. The dose of CsA was chosen based on our previous studies and those of others, comparing the effectiveness of CsA to protect the myocardium during I/R injury in young and old rats (23, 34). Protocol B was designed for measurements of protein oxidative damage, the levels of antioxidant enzymes, cyclophilin D (CypD) protein levels, and measurements of mitochondrial Ca²⁺ uptake studies. Protocol C was designed for reactive oxygen species induced mitochondrial permeability transition pore (mPTP) opening measurements in cardiomyocytes isolated from Tempol-treated or -untreated animals. CsA, cyclosporine A; ISO, isoflurane; TMRE, tetramethylrhodamine ethyl ester; O, Old; Y, young; TTC, triphenyltetrazolium chloride.
induced mPTP opening measurements in young and old cardiomyocytes isolated from Tempol treated (2 or 4 wk) or untreated animals (n = 5 rats per treatment group).

Myocardial Infarction Size Experiment

Myocardial infarction size was measured as previously described (42). Briefly, at the end of each experiment, the LAD was reoccluded and patent blue dye was injected intravenously to stain the normal region of the left ventricle. The heart was rapidly excised, and six 1- to 2-mm cross sections of the left ventricle were obtained using a scalpel. Both surfaces of the six sections were then scanned using an Epson 3200 photo scanner and analyzed using two-dimensional planimetry within MatLab software (MathWorks, Natick, MA) to determine the total weight of the areas at risk (AAR). The ischemic area was reported as AAR divided by the ventricular weight. Within 5 min of preparation, the scanned regions were incubated at 37°C for 15 min in 1% 2,3,5-triphenyltetrazolium chloride in 0.1 M phosphate buffer adjusted to a pH of 7.4. This process left the infarct area white. Each slice was weighed, and the slices were then rescanned on both sides. The total area of infarct was determined by two-dimensional planimetry as described above. Infarction size was expressed as the area of infarct weight divided by the total AAR weight and multiplied by 100 to determine the percent infarct (Infarct/AAR).

Heart Tissue Collection and Total Protein Extraction

Euthanasia was induced with an intraperitoneal injection of sodium thiobutabarbital (200 mg/kg, Sigma-Aldrich). The left ventricular samples were homogenized using a Polytron homogenizer (Kine- matica, Bohemia, NY) in ice-cold lysis buffer containing (in mmol/l) 20 Tris·HCl (pH 7.4), 150 NaCl, 1 EDTA, 1 NaVO4, 1 NaF, 2.5 NaN3, 1 DTT, and 1 PMSF, as well as 1% Nonidet P-40 and 0.1% SDS and a complete proteinase inhibitor cocktail (1 tablet per 10 ml, Sigma-Aldrich, St. Louis, MO). The samples were then centrifuged at 13,000 g for 30 min at 4°C to isolate total soluble protein. The clarified supernatant was used to quantify protein expression. Protein concentrations were determined using the BCA protein assay kit (Pierce, Rockford, IL). The samples were then centrifuged at 10,000 g for 15 min at 4°C, the supernatants were deproteinized using freshly made metaphosphoric acid (10% wt/vol). The samples were stored at -20°C until analyzed for GSH. On the day of the assay, all samples were added 50 μl of 4 mol/l triethanolamine for each milliliter of supernatant to adjust the pH. For total GSH measurement, 50-μl sample was added to 150 μl of freshly prepared assay cocktail (according to manufacturer’s instructions). After incubation for 25 min at room temperature, total GSH was then determined by absorbance at 405 nm. The total GSH amounts were calculated by comparing sample values against the standard curve. For GSSG assay, 10 μl of 1 mol/l 2-vinylpyridine were added to each milliliter of sample and incubated at room temperature for 60 min to remove GSH. The remaining GSSG in the sample was then quantified by the total GSH assay. GSH was obtained by subtracting GSSG from total GSH; the levels of GSH and GSSG were expressed as micromole per gram wet tissue. The ratio of GSH to GSSG was used to indicate the redox status of the tissue.

SOD Activity Assay

Total heart tissue SOD (Cu/Zn-, Mn-, and Fe-SOD) activity was measured by using a SOD assay kit (Cayman Chemical). Briefly, 100 mg fresh tissue was homogenized in 1 ml of cold HEPES buffer containing (in mmol/l) 20 HEPES, 1 EGTA, 210 mannitol, and 70 sucrose (pH 7.2) and was centrifuged at 1,500 g for 5 min at 4°C. The 1,500-g supernatant was assayed for total SOD activity (cytosolic and mitochondrial). To measure mitochondrial SOD activity, the 1,500-g supernatants were centrifuged at 10,000 g for 15 min at 4°C and the mitochondrial pellet was homogenized in cold HEPES buffer. All the samples were stored at -80°C till assay. On the day of assay, for total SOD assay, 10-μl samples were added to 200-μl diluted radical detector (according to manufacturer’s instructions); for MnSOD activity measurement, 1 mmol/l potassium cyanide was added to the mitochondrial samples to inhibit both Cu/ZnSOD and extracellular SOD. Then 10-μl samples were added to 200-μl diluted radical detector. Assays were initiated by adding 20 μl of diluted xanthine oxidase to each reaction. After the samples were incubated on a shaker for 20 min at room temperature, SOD was determined by absorbance at 450 nm. The SOD activities of the samples were calculated by using the equation obtained from the standard curve substituting the linearized rate for each sample. One unit is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. The SOD activity is expressed as units per milligram protein.

Isolation of Cardiomyocytes

Ventricular myocytes were obtained by enzymatic dissociation as previously described (43, 61). Briefly, rats were injected intraperitoneally with heparin (3,000 U/kg) to inhibit blood coagulation. Thirty minutes later, rats were euthanized by an overdose of sodium thiobu- tabarbital (200 mg ip, Sigma-Aldrich), and the hearts, with major blood vessels attached, were removed. The aorta of each animal was cannulated and heart was perfused ex vivo for 15–20 min with a Ca2+-free Tyrode solution, containing (in mmol/l) 130 NaCl, 5.4 KCl, 10 HEPES, 1 MgCl2, 0.33 NaH2PO4, 5.5 glucose (pH 7.40 gassed with 100% O2 5%, at 37 ± 1°C). Collagenase type 2 (331 U/mg, Worthington; 0.4 and 0.6 mg/ml for young and old rat hearts) and protease type XIV (0.45 U/ml, Sigma-Aldrich) were added. After perfusion with the enzyme solution, the ventricles were separated from the atria and minced in Krebs solution containing (in mmol/l) 100 K-gluta- m ate, 10 K-aspartate, 25 KCl, 10 KH2PO4, 2 MgSO4, 5 creatine, 20 taurine, 0.5 EGTA, 5 HEPES, and 20 glucose (pH 7.2, adjusted with KOH). The supernatant was then decanted and filtered through a 225-μm polyethylene mesh to remove large particles. The isolated cardiomyocytes were suspended in 1.0 mmol/l Ca2+ Tyrode solution. Only quiescent, rod-shaped myocytes with visible striations and no
visible membrane damage were used in our experiments. As in previous work, a decrease in myocytes yield with age was observed (15). Following isolation, ventricular myocytes were then plated in Tyrode solution onto laminin- precoated cover glass-bottomed petri dishes (In Vitro Scientific, Avon, CT). Myocytes were allowed to recover for 1 h in Krebs solution and were used for experiments within 4 h.

Mitochondria Isolation and Measurements of Mitochondria Ca\(^{2+}\) Concentration Uptake

Mitochondria were isolated from the left ventricle by differential centrifugation. In brief, fresh tissue samples were homogenized using a Polytron homogenizer (Kinematica) in ice-cold sucrose buffer containing (in mmol/l) 300 sucrose, 5 TES, and 0.2 EGTA (pH 7.2 with KOH). The homogenate was centrifuged at 800 g for 5 min at room temperature. Mitochondria were then centrifuged at 8,000 g for 10 min and then the supernatant was centrifuged at 8,000 g for 15 min. The mitochondria pellets were washed twice with ice-cold sucrose buffer.

Mitochondrial free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{i}\)) was measured using the fluorescent Ca\(^{2+}\) indicator indocarbocyanine (Indo-1 AM, 17, 57). Isolated mitochondria (about 5 mg/ml protein) were loaded with 5 μM indo-1 AM (Invitrogen, Carlsbad, CA) for 30 min at room temperature. Mitochondria were then centrifuged at 8,000 g for 10 min and resuspended with indo-1 free homogenizing buffer without EGTA. Spectrofluorometry (ILC Technology, Sunnyvale, CA) was used for the detection of indo-1 fluorescence intensity. Indo-1 was excited at 350 nm, and the emitted fluorescence at 385 nm and 465 nm was recorded every 5 s. [Ca\(^{2+}\)]\(_{i}\) was expressed as the indo-1 (385 nm-to-465 nm) fluorescence ratio. CaCl\(_2\) (10 μmol/l) was added every 100 s. Indo-1 fluorescence intensity ratio changes were expressed as a percentage of baseline values.

mPTP Opening in Isolated Cardiomyocytes

To measure mPTP opening time, freshly isolated cardiomyocytes were loaded with tetramethylrhodamine ethyl ester (TMRE; 100 nmol/l) for 25 min at room temperature. Cardiomyocytes were then placed in a glass-bottomed petri dish on the stage of the laser-scanning confocal microscope (Fluoview FV1000, Olympus, Tokyo, Japan), equipped with a 60× oil immersion objective lens. For TMRE fluorescence, cells were scanned at 543-nm. The emitted fluorescence was collected at 590 nm. TMRE generates ROS within mitochondria during laser illumination, which leads to an opening of mPTP as previously described (61). In brief, regions of the myocyte (30 × 30 μm\(^2\)) were subjected to laser-induced localized production of ROS. Increasing ROS induces mPTP opening that collapses mitochondrial membrane potential which can be visualized with the same dye. Each region of interest was scanned at 3-s intervals, and the pixel dwell time was 2 μs. The image sequences (each 512 × 512 pixels) were used to record changes in signal throughout. For all photosensitization experiments, all settings of the confocal microscope (laser power, confocal pinhole, and pixel dwell time) were identical to ensure comparability between experiments. Images were analyzed using NIH ImageJ 1.45. Background was calculated by subtracting a cell-free area. After background subtraction, images were corrected for photobleaching by normalization to a predetermined monoeponential decay function. This decay was calculated from the differences of intensities averaged over the whole recording in the absence of mPTP opening. The peak corrected signal was normalized to 100%, and the nadir to 0%. Time required for a 50% decrease in TMRE fluorescence emission signal is defined as the time to mPTP opening (t\(_{\text{mPTP}}\)) (50). Cardiomyocytes were isolated from at least five individual animals from each age group, and the results of at least five cells from each animal in each treatment group were analyzed.

Statistical Analysis

Results are expressed as means ± SD. Two-way ANOVA followed by Tukey post hoc test was performed on baseline hemodynamics, myocardial infarction size. Western blot analysis, antioxidant enzymes measurements, mitochondrial Ca\(^{2+}\) uptake results, and cardiomyocyte TMRE data. Analysis of variance for repeated measures was used to test for time-related differences in hemodynamics within groups. Differences of P < 0.05 were considered significant. Analyses were performed on Sigmastat [version 3.5, Systat Software (SSI), San Jose, CA].

RESULTS

One hundred thirty-five animals were instrumented to obtain 132 successful experiments, a single old rat was excluded because of intractable ventricular arrhythmias, and two young animals were excluded because of severe bleeding. The ages and body weights were similar within old and young age groups.

Systemic Hemodynamics

No differences in the baseline hemodynamics were observed among experimental (protocol A) groups (Table 1). Isoflurane (ISO), however, significantly (P < 0.05) decreased the mean arterial blood pressure and heart rate when compared with each animal’s respective baseline periods, as previously observed (11). There were no differences in mean arterial blood pressures and heart rates during the memory period (washout) or coronary artery occlusion when compared with their respective baseline period. In protocol A, all mean blood pressures significantly (P < 0.05) decreased during the first hour of reperfusion and decreased ~40–50% below the baseline during the second hour of reperfusion.

Myocardial Infarction Size

Myocardial infarction sizes were significantly reduced by Tempol at 4-wk treatment follow by APC or CsA in the aged rats (OT×4wk + ISO = 35.9 ± 11.4%; and OT×4wk + CsA = 39.3 ± 6.6%) compared with either old controls or to old control animals subjected to APC or CsA (OC = 55.4 ± 10.9%; OISO = 56.3 ± 12.7%; and OCsA = 51.1 ± 10.4%; P < 0.05). Interestingly, no significant differences were observed between old animals treated with Tempol for 2 wk or 2-wk Tempol treatment follow by APC or CsA (OT×2wk + ISO = 54.8 ± 7.9%; OT×2wk + CsA = 51.3 ± 6.5%; and OT×2wk + CsA = 50.5 ± 6.2%). By itself, Tempol 4-wk treatment (OT×4wk = 52.6 ± 12.6%) showed no effect on myocardial infarction size compared with the old control group (P > 0.05) (Fig. 2A). There were no significant differences in AAR among all experimental groups (Fig. 2B).

Tempol Effects on Myocardial Oxidative Stress in the Aged Rats

Glutathione redox system. To assess the effects of Tempol treatment on the myocardial redox state, GSH and GSSG were measured in myocardium harvested from experimental rats. Table 2 presents the effect of Tempol on the levels of the nonenzymatic antioxidants GSH, GSSG, and the ratios of GSH-to-GSSG in the myocardium of young and old rats. In untreated senescent rats, the myocardial levels of GSH were decreased (P < 0.05) and the levels of GSSG were increased (P < 0.001), resulting in a lower GSH-to-GSSG ratio (P < 0.05) compared with young control animals. The administration of Tempol decreased (P < 0.001) the levels of GSSG in...
Table 1. Hemodynamics

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Values are means ± SD; n = 8 in each group. Heart rate (HR) and mean arterial blood pressure (MAP) were monitored during isoflurane preconditioning (ISO) or cyclosporine A administration (CsA) and subsequent occlusion coronary artery (OCC) and reperfusion. C, control; O, old; T, Tempol; Y, young. *P < 0.05, significantly different from baseline within the same group.

both age groups. Interestingly, Tempol 2- or 4-wk treatment shifted the levels of GSSG and GSH/GSSG in the old animals toward those levels observed in young control rats. Although there were no significant differences in GSH levels between young and old rats after Tempol treatment, an increased ratio of GSH-to-GSSG was observed after treatment with Tempol in both age groups.

**SOD enzymatic activity.** Total SOD activity, which includes Cu/ZnSOD and MnSOD activities, and MnSOD activity itself were measured in myocardial tissues harvested from animals in each group. Both total SOD and MnSOD activities were significantly lower (P < 0.001) in old control rats compared with young control rats. Tempol treatment (2 and 4 wk) significantly elevated both total SOD and MnSOD activities in old myocardium (P < 0.05) (Table 3).

**Western blot analysis for myocardial CuZnSOD and MnSOD protein.** There were no significant differences between age groups in the expression of myocardial CuZnSOD protein after Tempol treatment (Fig. 3A). However, Tempol 2- or 4-wk treatment significantly increased the levels of myocardial MnSOD protein expression in old animals compared with old control rats (OT×2wk = 88.1 ± 21.3%; OT×4wk = 89.4 ± 15.6%; and OSC = 59.0 ± 10.3%, P < 0.05, Fig. 3B). MnSOD protein expression levels were lower in old control rats compared with young control rats (P < 0.05). Western blot analysis for myocardial for nitrotyrosine protein. Nitrotyrosine is a marker of oxidative stress, as this adduct is formed from reaction of tyrosine residues with peroxynitrite which is formed from nitric oxide (NO) and superoxide anion (51). The levels of cardiac nitrotyrosine protein expression were elevated in the old control rats (OSC = 245.6 ± 24.3%, P < 0.001) compared with the young control group, which is in agreement with another’s report (59). Chronic treatment with Tempol for 2 or 4 wk significantly decreased myocardial nitrotyrosine protein expression levels [OT×2wk = 144.2 ± 28.2%; and OT×4wk = 126.0 ± 20.6%, P < 0.001 compared with old control rats (Fig. 3C)].

**Tempol Effects on Mitochondria [Ca2+]M Uptake**

Various studies have suggested that mitochondrial Ca2+ handling is disrupted by aging that could contribute to cell death because mitochondria play an important role buffering cytosolic Ca2+ concentration (4, 36, 46). Importantly, reduced mitochondrial Ca2+ uptake in aging may contribute to subsequent tissue injury. In our study, mitochondria free Ca2+ levels were measured using indo-1 fluorescence in mitochondria isolated from myocardium harvested from young or old control or Tempol-treated animals. Fig. 4A shows free Ca2+ levels in mitochondria in different age and treatment groups responding to increments in buffer Ca2+. When compared with young control rats, old rat mitochondria responses to Ca2+ are attenuated. Tempol treatment (2 and 4 wk) improved Ca2+ responses in the mitochondria from aged rats. After 4 wk of treatment with Tempol, Ca2+ responses in the old mitochondria returned to those observed in mitochondria from young control rats (Fig. 4B).

**Tempol Effects on the mPTP Component/Regulator CypD**

There were significant increases in CypD levels in the aged rat hearts (OSC = 188.4 ± 35.2%, P < 0.001) compared with the young control rats. This result is in agreement with Figueira’s recent report (13). Tempol treatment (4 wk) reduced cardiac CypD expression levels in senescent animals (OT×4wk = 108.2 ± 37.6%, P < 0.001), but 2 wk of treatment was ineffective (OT×2wk = 160.5 ± 24.2%, P > 0.05). In contrast, CypD levels were unchanged in young animals after 2 or 4 wk of Tempol treatment (Fig. 5).
Inhibition of mPTP opening appears to be the key step in preconditioning-induced cardioprotection (7). Figure 6A shows a typical recording of TMRE fluorescence obtained from a 30 × 30-μm² region in isolated cardiomyocytes, as assessed by laser-scanning confocal microscopy. ROS were rapidly produced from laser excitation of TMRE. The present results are consistent with previous work from our laboratory, showing decreased t_mPTP with old rats (OSC = 174.0 ± 58.5 s; and YSC = 290.7 ± 52.4 s, P < 0.001). After 4 wk of Tempol treatment, t_mPTP was substantially prolonged in the old cardiomyocytes in the setting of oxidative stress (OT×4wk = 348.6 ± 93.5 s, compared with the old control group, P < 0.001). These results could suggest an increase in the ROS threshold required to induce mPTP opening. There were no significant differences in the young animals treated with 2 or 4 wk of Tempol. The data are summarized in Fig. 6C.

**DISCUSSION**

Myocardial aging is associated with high levels of ROS, mitochondrial dysfunction, and altered responsiveness of the heart to I/R injury. We and others have shown that pharmacological and ischemic preconditioning are attenuated in the aging heart. The molecular bases for the latter deficiencies have not been elucidated. The novel finding of the present study is that chronic treatment with Tempol restored pharmacological preconditioning in the senescent animal. Our results showed that 4 wk of Tempol pretreatment restored the sensitivity to APC and cardioprotection by CsA as reflected in a 40% reduction in myocardial infarct size in the aged rats, whereas the vehicle-pretreated old rats showed no benefit from APC or CsA. Interestingly, there was no reduction in myocardial infarct size in the aged rat receiving Tempol alone for 2 wk, or 4 wk in the absence of preconditioning. These findings contribute toward understanding the mechanisms that underlie the loss of pharmacological preconditioning in the elderly and may lead to the development of new therapeutic strategies at high-risk elderly population.

Tempol, a stable nitroxyl free radical that permeates biological membranes and functions as an intracellular scavenger of superoxide anions and other reactive radicals, has been previously used to reduce oxidative stress in older animals (58), as well as spontaneously hypertensive rats (55, 56). A recent study has shown that 3 wk treatment with Tempol normalizes arterial superoxide production, reverses endothelial dysfunction, and reduces oxidative stress in old mice (14).

GSH is essential to antioxidant defenses and plays a crucial role in coordinating the natural antioxidant defense mechanisms in living organisms. GSH is capable of donating reduced electrons to other GSH molecules to produce the oxidized form GSSG. Decreases in the levels of GSH and increases in the levels of GSSG in normal aging have been well documented and are consistent with increasing oxidative stress in older animals (35, 52). Lowered GSH levels may result from increased use of GSH by antioxidant enzymes such as glutathione peroxidase, which scavenge H₂O₂. Thus GSH content as well as the GSH-to-GSSG ratio are useful indexes of oxidative stress (5). Our study showed that the loss of pharmacological preconditioning in the elderly may contribute toward understanding the mechanisms that underlie the development of new therapeutic strategies at high-risk elderly population.

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**Effects of Tempol Pretreatment on mPTP Opening Times**

Table 2. Effects of 2- or 4-wk Tempol treatment on the levels of GSH, GSSG, and ratios of GSH-to-GSSG in the ventricles of young and aged rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>YC treatment</th>
<th>YT×2wk</th>
<th>YT×4wk</th>
<th>OC</th>
<th>OT×2wk</th>
<th>OT×4wk</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH, μmol/g wet tissue</td>
<td>1.11 ± 0.22</td>
<td>1.11 ± 0.24</td>
<td>1.05 ± 0.08</td>
<td>0.85 ± 0.14*</td>
<td>0.95 ± 0.10#</td>
<td>0.97 ± 0.17</td>
</tr>
<tr>
<td>GSSG, μmol/g wet tissue</td>
<td>0.21 ± 0.06</td>
<td>0.12 ± 0.04*</td>
<td>0.12 ± 0.05*</td>
<td>0.35 ± 0.07#</td>
<td>0.20 ± 0.04*#</td>
<td>0.19 ± 0.04*#</td>
</tr>
<tr>
<td>GSH/GSSG</td>
<td>5.92 ± 2.97</td>
<td>9.58 ± 2.89*</td>
<td>9.43 ± 2.44*</td>
<td>2.48 ± 0.59#</td>
<td>5.04 ± 1.43*#</td>
<td>5.18 ± 1.30*#</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = 5 in each group. *P < 0.05, significant compared with control within the same age group; #P < 0.05, significant compared to the same Tempol treatment group in different age groups.
young rats. When Tempol was administrated to aged rats for 2 or 4 wk, the GSH-to-GSSG ratio was significantly increased and the levels of GSSG were significantly decreased compared to aged untreated rats. These results suggest that Tempol, through its free radical scavenging properties, lowered the myocardial ROS levels, thereby increasing the GSH-to-GSSG ratio.

Manganese-containing SOD (MnSOD) is an essential primary antioxidant enzyme that converts superoxide radical to hydrogen peroxide and molecular oxygen within the mitochondrial matrix. It is well known that during normal aging there is reduced SOD activity (32) and enzyme expression levels (14, 52). In our study, 2 or 4 wk of Tempol treatment in the aged rat significantly increased myocardial MnSOD enzyme activity and protein expression, whereas Tempol had no significant effect on the levels of myocardial MnSOD in the young, which were already at higher levels compared with the untreated older animals. Nitrotyrosine detected by Western blot analysis was substantially reduced in Tempol-treated animals. This adduct is produced by posttranslational nitration of tyrosine residues, primarily by peroxynitrite, a by-product of the reaction between superoxide anion and NO and is a signature of NO-primarily by peroxynitrite, a by-product of the reaction between superoxide anion and NO and is a signature of NO-

Oxidative stress has been implicated in the reperfusion injury after myocardial infarction, and aging exacerbates this process. One possible mechanism by which Tempol modifies mitochondrial ROS production is enhanced mitochondrial biogenesis associated with amplified antioxidant mechanisms. Another possible mechanism is age alteration in the levels or activities of mPTP components after Tempol treatment. As has been amply demonstrated, the major determinant of cardiomyocyte death following an episode of I/R injury is the formation and opening of mPTP (19). Although the molecular composition of mPTP is still unclear, the current view of the mPTP is that it includes adenine nucleotide translocator, voltage-dependent anion channel, and CypD, which form a modulated pore (21). There is general agreement that the mitochondrial protein CypD is a critical regulator of mPTP opening. CypD is targeted by the immunosuppressant drug CsA, which interacts with CypD and inhibits the opening of the mPTP (1, 9). CsA has been shown to have cardioprotective effects in both experimental and clinical studies (20, 47). However, our previous work has shown that cardioprotection by CsA is lost in senescent animals (34). Studies have demonstrated that those CypD-deficient mice are remarkably protected from I/R injury and resistant to mPTP opening (1, 41). A recent study also showed that pretreatment with CypD-small interfering RNA was effective against I/R injury after 30 min of global ischemia (28). Our results showed 4 wk, but not 2 wk, of Tempol treatment significantly lowered CypD expression level in the senescent myocardium. Although 2 wk of Tempol treatment normalized redox balance in aged myocardium, it did not restore APC or the cardioprotective effects by CsA. We suggest that replacement of key mitochondrial components and the normalizing of expression of critical mPTP regulatory proteins are likely to

Table 3. Effects of 2- or 4-wk TEMPOL treatment on the levels of total SOD and MnSOD activities in the ventricles of young and aged rats

<table>
<thead>
<tr>
<th></th>
<th>YC</th>
<th>YT×2wk</th>
<th>YT×4wk</th>
<th>OC</th>
<th>OT×2wk</th>
<th>OT×4wk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total SOD, U/mg protein</td>
<td>16.97 ± 2.75</td>
<td>16.48 ± 3.47</td>
<td>18.50 ± 2.92</td>
<td>9.67 ± 3.13#</td>
<td>13.03 ± 2.59*</td>
<td>15.28 ± 2.42*</td>
</tr>
<tr>
<td>MnSOD2, U/mg protein</td>
<td>6.33 ± 1.71</td>
<td>6.64 ± 1.37</td>
<td>8.04 ± 1.76</td>
<td>2.35 ± 1.38#</td>
<td>4.74 ± 1.23*#</td>
<td>5.15 ± 1.13*#</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = 5 in each group. *P < 0.05, significant compared with control within the same age group; #P < 0.05, significant compared with the same Tempol treatment group in different age groups.
account for the prolonged treatment time necessary to restore preconditioning.

It is well known that transient increases in ROS have been shown to be an early trigger for preconditioning (29, 40). Studies showed that ROS scavenger abrogated myocardial preconditioning when administered during the preconditioning period (22, 24, 31). However, in our study, the timing of the last Tempol dose is a full 24 h before preconditioning and I/R injury. Our results suggest that chronic Tempol treatment increases antioxidant capacity and thereby increases the myocardial defenses against oxidative stress and so may reverse the cumulative mitochondrial damage caused by aging. Whether that is sufficient to account for restoration of pharmacological preconditioning is unclear. It is plausible that increased antioxidant reserve itself could improve the response to I/R injury; however, no reduction of infarction size was seen with Tempol treatment alone in the older animals. Moreover, the decrease in GSSG levels and increased MnSOD expression and activity were maximal by 2 wk, a time at which Tempol treatment had not yet restored cardioprotection by APC or CsA. Therefore, the restoration of APC and CsA sensitivity likely involved other, slower processes. One possibility that fits a 4-wk time frame is the turnover of mitochondria which have an estimated half-life of between 16 and 18 days for young and senescent myocardium, respectively (39).
Clinical Implications

There is substantial experimental evidence that ischemic preconditioning and APC reduce myocardial I/R injury in various animal models (29, 40). Recent research has been focused on the possible implementation of this finding in patient care. Age dependence of preconditioning response is highly relevant to clinical utility, because the majority of patients that could benefit from adjunctive cardioprotection are in the older age range. Several studies have addressed the potential clinical implication of cardioprotection by volatile anesthetics in patients undergoing coronary artery surgery, looking at cellular enzyme release and myocardial function (16, 27, 49, 60). Other clinical preconditioning studies, however, have shown no significant cardioprotective effects in terms of either better preservation of myocardial function or less postoperative myocardial damage (11, 48). This underscores the possibility that the clinical preconditioning protocol, patient age, and disease condition may be critical to its putative efficacy. An understanding of the apparent lack of efficacy of preconditioning will be critical to devising more effective cardioprotective strategies in the at-risk elderly population, particularly in the perioperative period. Our results may point to novel interventional strategies that restore the benefits of preconditioning particularly in older patients. Pretreatment with Tempol or related antioxidants for up to 4 wk before a scheduled surgery procedure could be part of a simple cardioprotective strategy that restores the protective effects of inhaled agents or other cardioprotective drugs, such as the clinically useful CsA, and thereby attenuates the risk of fatal myocardial infarction in the elderly during the perioperative period.

Study Limitations

First, only a few of the principal components of the myocardial antioxidant system were measured. For example, we did not measure other antioxidant enzymes activities after Tempol treatment such as catalase and glutathione peroxidase. Second, myocardial ROS levels were not measured after Tempol treatment so as to rule out the contribution of ROS generated by Tempol. Third, in our in vitro study, we used a cellular model of oxidative stress to study induced mPTP opening. This model simulates ROS production during the reperfusion of ischemic myocardium and may not include other contributors to mPTP opening in cardiomyocytes during reperfusion, particularly the increased influx of Ca$^{2+}$ that occurs following I/R injury and may or may not be affected by Tempol pretreatment. Finally, we measured the free intramitochondrial Ca$^{2+}$ only, and other measures that specify influx, efflux, and internal storage capacity would be useful.

In conclusion, 4 wk of Tempol treatment restores APC and cardioprotection by CsA in the aged heart. This is associated with decreased oxidative stress, improved antioxidant reserve, and reduced CypD levels in the aged myocardium. This study raises the possibility that a multiweek free radical scavenging strategy may protect newly formed mitochondria against oxidative stress, thereby restoring pharmacological cardioprotection in the high-risk elderly patient.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

J.Z., M.J.R., Q.W., P.S.G., P.R.B., and L.L. conceived and design of research; J.Z. performed experiments; J.Z., M.J.R., and L.L. analyzed data; J.Z., M.J.R., Q.W., and L.L. interpreted results of experiments; J.Z. prepared figures; M.J.R. and L.L. edited and revised manuscript; M.J.R., Q.W., P.S.G., P.R.B., and L.L. approved final version of manuscript; L.L. drafted manuscript.

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