Resolution of mitochondrial oxidant stress improves aged-cardiovascular performance

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Background Senescence is a major factor that increases oxidative stress in mitochondria, which contributes toward the pathogenesis of heart disease. However, the effect of antioxidant therapy on cardiac mitochondria in aged-cardiac performance remains elusive.

Objectives We postulated that the mitochondrial targeting of superoxide scavenging would have benefits in the aged heart.

Methods and results Generation of superoxide in the mitochondria and nicotinamide adenine dinucleotide phosphate oxidase activity increased in the heart of old mice compared with that in young mice. In old mice treated with a mitochondria-targeted antioxidant MitoTEMPO (180 µg/kg/day, 28 days) co-infusion using a subcutaneously implanted minipump, levels of superoxide in the mitochondria and nicotinamide adenine dinucleotide phosphate oxidase activity as well as hydrogen peroxide decreased markedly in cardiomyocytes. Treatment with MitoTEMPO in old mice improved the systolic and diastolic function assessed by echocardiography. Endothelium-dependent vasodilation in isolated coronary arteries and endothelial nitric-oxide synthase phosphorylation were impaired in old mice compared with that in young mice and were improved by MitoTEMPO treatment. Mitochondria from the old mice myocardium showed lower rates of complex I-dependent and II-dependent respiration compared with that from young mice. Supplementation of MitoTEMPO in old mice improved the respiration rates and efficiency of ATP generation in mitochondria to a level similar to that of young mice.

Conclusion Resolution of oxidative stress in mitochondria by MitoTEMPO in old mice restored cardiac function and the capacity of coronary vasodilation to the same magnitude observed in young mice. An antioxidant strategy targeting mitochondria could have a therapeutic benefit in heart disease with senescence. *Coron Artery Dis* 28:33–43 Copyright © 2016 Wolters Kluwer Health, Inc. All rights reserved.

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Introduction

Epidemiological data from the Framingham Heart Study and the Baltimore Longitudinal Study on Aging showed that in healthy populations there is an age-dependent increase in the prevalence of declines in cardiac diastolic function and maximum left ventricular ejection fraction during exercise, assessed by echocardiography [1]. The occurrence of coronary artery disease increases with age, even in a population without other major risk factors [2,3]. In addition, the severity of tissue injury following acute and chronic ischemia increases with age [4]. Thus, aging is a major risk factor for heart disease and exacerbates coronary artery disease and heart failure with diabetes and hypertension. However, mechanisms of the age-associated decline in cardiac function are complicated. Although advancing age is an unmodifiable risk factor for heart disease, it might be possible to target specific molecular signals as an approach to limiting the age-related risk of heart disease.

The free radical theory of aging, first proposed by Harman more than 50 years ago, suggested that increased reactive oxygen species (ROS) generation underlies many features of aging [5]. According to the recent free radical theory of aging, ROS produced mainly in the mitochondria attack mitochondrial constituents, resulting in declines in cellular and organ function, which contribute toward disease progression [6].

Therefore, we carried out the present study to test the hypothesis that mitochondria-targeted antioxidant

therapy would be effective in the rejuvenation of cardiac performance. To gain insights into the role of mitochondrial superoxide in endothelial and left ventricular dysfunction, we examined the effect of treatment with a mitochondria-targeted superoxide scavenger, (2-(2,2,6,6-tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl) triphenylphosphonium chloride monohydrate (MitoTEMPO), in aged mice. Our data strongly suggest that mitochondrial superoxide plays a critical role in the age-associated decline of cardiac function and is thus a therapeutic target for coronary artery disease.

Materials and methods

Ethics statement
The investigations conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication, 8th ed., 2011). Our research protocol was approved by the Fukushima Medical University Animal Research Committee (permit number 27 058) and all animal experiments were conducted in accordance with the guidelines of the Fukushima Medical University Animal Research Committee. All efforts were made to minimize the suffering of animals.

Animals
Young (age: 8 weeks; body weight: 22.6 ± 2.6 g) and old (age: 100–112 weeks; body weight: 25.4 ± 2.8 g) male C57BL/6 mice were housed and bred in a room at $22 \pm 3°C$ with a relative humidity of $50 \pm 10\%$ and a 12-h light–dark cycle. The mice were provided food and water ad libitum.

Reagents
MitoTEMPO and MitoSOX were purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA). All other reagents were obtained from Sigma-Aldrich (St Louis, Missouri, USA). Methods are provided in the online data supplement, Supplemental digital content 1, *http://links.lww.com/MCA/A106*.

Animal experiments
The mice received a separate minipump for co-infusion of MitoTEMPO or saline. The mice were anesthetized by pentobarbital (50 mg per body, intraperitoneally) and an osmotic minipump (ALZET micro-osmotic pump, model 1002; DURECT Co., Cupertino, California, USA) was subcutaneously implanted. MitoTEMPO (180 μg/kg/day) and saline as a control for the old mice and saline for the young mice were infused continuously. Blood pressure was monitored using the tail-cuff method. Previously, we measured the systolic blood pressure and heart rate in mice administered MitoTEMPO (50, 180, and 500 μg/kg/day) with an osmotic minipump at 14 days after infusion. When treated with 500 μg/kg/day of MitoTEMPO, systolic blood pressure decreased over 5% ($P < 0.05$) compared with before treatment, which are

identical to the results of a previous report [7]. Left ventricular diastolic function (E/e') and heart rate did not change with or without administration of 50 μg/kg/day of MitoTEMPO; that is, the effect of MitoTEMPO is doubtful as the dose is too small. Therefore, we considered the suitable dose of MitoTEMPO to be 180 μg/kg/day. Twenty-eight days after surgery, the animals were killed with a lethal dose of pentobarbital and examinations of the coronary artery, cardiac myocytes, and aorta were performed.

Hemodynamic measurements
The systolic, mean, and diastolic blood pressures and the heart rate were measured using the tail-cuff method using a programmable sphygmomanometer (BP-98A-L; Softron, Tokyo, Japan) before and 28 days after treatment with MitoTEMPO [8].

Echocardiography
Transthoracic echocardiography was performed using the Vevo 2100 high-resolution in-vivo imaging system (Visual Sonics Inc., Toronto, Ontario, Canada) before and 28 days after pump implantation, as described previously [8].

Preparation of cardiomyocyte
Cardiac myocytes were enzymatically isolated from the mouse heart using a modified Piper method [9–11]. In brief, after excision of the heart, the aorta was cannulated and the preparation was suspended in a perfusion apparatus. To rinse out residual blood and eliminate contraction, the left ventricle was initially perfused in a retrograde manner from the aorta at 37°C with oxygenated, calcium-free 3-(N-morpholino)propanesulfonic acid (MOPS) buffer [pH 7.40 (titrated with 5 mol/l NaOH)] that contained 8 mmol/l MOPS, 30 mmol/l taurine, 113 mmol/l NaCl, 4.7 mmol/l KCl, 0.6 mmol/l KH_2PO_4, 0.6 mmol/l Na_2HPO_4, 1.2 mmol/l $MgSO_4$, 0.032 mmol/l phenol red, 12 mmol/l $NaHCO_3$, and 10 mmol/l $KHCO_3$. After the cessation of contractile activity, the perfusion was switched to a buffer of the above constituents supplemented with 0.5 mg/ml type 2 collagenase (Worthington, Lakewood, New Jersey, USA) and 12.5 μmol/l $CaCl_2$. After perfusion of the heart for 20–25 min and the identification of isolated myocytes in the perfusate from the heart, the heart was detached from the perfusion apparatus and placed in a 'stop' solution containing the perfusion buffer with 1% BSA and 0.125 mmol/l $CaCl_2$. The heart tissue was minced into small pieces that were further titrated in stop buffer. After microscopic confirmation of the presence of myocytes, the cells were filtered and placed in a 10-ml conical tube. $CaCl_2$ was added in a series of three steps to arrive at a final concentration of 1.0 mmol/l. The cells were allowed to settle for 20 min and the supernatant was discarded. The cells were resuspended in the stop buffer with calcium and small aliquots were then used for cell

counts (by a hemocytometer) to enable dilution or concentration (by centrifugation) to a final concentration of 200 000 cells/ml.

Electrical stimulation of cardiac myocytes

Suspensions of enzymatically isolated cardiac myocytes were treated in 1.5 ml of MOPS buffer (pH 7.4) under no stimulation or electrical field stimulation at 600 bpm as described previously [9]. The duration of the electrical stimulations was 20 min in a closed chamber. We stimulated the suspension of cardiac myocytes in a handmade, closed, transparent, plastic chamber with paired platinum inner nets that faced each other. During electrical stimulation, we confirmed the beating of myocytes with an inverted microscope. When the sample had reached more than 15% damaged myocytes under continuous observation, we excluded it from the following experiment. The survival of the cardiac myocytes was determined using a 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay as described in the manufacturer's protocol (Cat. No. V-13154; Molecular Probes, Eugene, Oregon, USA). Briefly, the cells were incubated for 3 h in phenol red-free medium containing 0.5% of the yellow mitochondrial dye MTT-positive (MTT +). The amount of blue formazan dye generated from MTT + was proportional to the number of live cells. The MTT + reaction was terminated by the addition of dimethyl sulfoxide to the medium, followed by incubation for 10 min at 37°C. The absorbance was read at 540 nm using a spectrophotometer [12]. Moreover, we directly counted cardiac myocytes using the vital dye trypan blue. After electrical stimulation for each condition (nonstimulated or stimulated), cardiac myocytes were subjected to MitoSOX staining and mitochondria isolation, and the supernatant was used to measure the level of H_2O_2.

MitoSOX staining in cardiac myocytes

The production of superoxide anion radical (O_2^-) in the mitochondria of stimulated cardiac myocytes was measured by staining with MitoSOX (2 μmol/l) and myocytes were scanned using an Olympus IX71 inverted microscope (Olympus CCD, Tokyo, Japan). In isolated cardiac myocytes, O_2^- production was then measured in the 20-min electrically stimulated myocytes.

Mitochondrial isolation in cardiomyocyte

For some molecular studies, crude mitochondria were prepared using a mitochondrial extraction buffer (MitoSciences Inc., Eugene, Oregon, USA). For bioenergetics studies, mitochondria were isolated according to the protocol of Miyamoto *et al.* [13], with slight modifications. Briefly, after pacing, isolated myocytes were minced, rinsed, and homogenized in MSHE buffer (210 mmol/l mannitol, 70 mmol/l sucrose, 5 mmol/l HEPES, and 1 mmol/l EGTA) and 0.1% fatty acid-free

bovine serum albumin (pH 7.2). The homogenate was centrifuged at 27 000*g* for 10 min. The supernatant was collected as a cytosolic fraction. The pellet was supplemented with nargarse protease (1 mg/g wet weight tissue) for 4 min on ice and rehomogenized. The homogenate was centrifuged at 400*g* for 10 min to remove nuclei and debris. The supernatant was poured through cheesecloth. The pellet obtained was subjected to the same homogenization and centrifugation processes, and the supernatant was poured through cheesecloth. The resulting supernatant was combined and centrifuged at 13 000*g* for 10 min. The mitochondrial extract was finally resuspended in MSHE buffer. The protein concentration was determined using the BCA protein assay (Pierce, Rockford, Illinois, USA).

Measurement of superoxide in mitochondria

As a specific measure of mitochondrial superoxide formation, 2-hydroxyethidium levels were determined as described previously [14]. Briefly, the mitochondria were snap frozen and stored at −80°C after incubation with 50 mmol/l dihydroethidium for 20 min at 37°C. For analysis, samples were diluted with 50% acetonitrile, centrifuged, and the supernatant was subjected to ultraperformance liquid chromatography analysis (UPLC). Separation of 2-hydroxyethidium, ethidium, and dihydroethidium was performed using a Waters AQUITY UPLC H-class system with an AQUITY BEH C18 column (particle size: 1.7 μm, $\phi = 2.1 \times 50$ mm; Waters Corporation, Milford, Massachusetts, USA) at 40°C.

H_2O_2 generation in isolated cardiac myocytes

H_2O_2 concentrations in the MOPS buffer with isolated cardiac myocytes in both the nonstimulated and electrically stimulated states were measured using a free radical analyzer (Apollo 4000; WPI Co. Ltd, Sarasota, Florida, USA) as described previously [9].

Mitochondrial function analysis

An XF24 extracellular flux analyzer (Seahorse Bioscience, North Billerica, Massachusetts, USA) was used to measure intact isolated mitochondrial function/bioenergetics according to the protocol established by Rogers and colleagues [15,16], with modifications. First, a 24-well dual-analyte sensor cartridge was primed with calibration solution in a 37°C incubator without CO_2 overnight. Before the start of the experiment, the injection ports on the sensor cartridge were loaded with the appropriate mitochondrial substrates or inhibitors at 10× concentrations. The sensor cartridge was placed into the XF24 analyzer for automated calibration. During the calibration, isolated mitochondria were then seeded in an XF24 V7 cell-culture microplate (except for the background correction well). Following centrifugation of the plate at 2000*g* for 20 min at 4°C, MSHE (with the initial experimental conditions) was gently added to the wells containing the mitochondria and the plate was placed in a

37°C incubator without CO_2 for 5 min. The plate was then transferred to the XF24 analyzer and the experiment was initiated [17]. For the electron flow assay, the mitochondria (3 µg/well) were supplemented with electron flow initial medium containing 10 mmol/l pyruvate, 2 mmol/l malate, and 4 µmol/l carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP). Injections (10× concentration of either mitochondrial substrates or inhibitors) were administered as follows: 20 µmol/l rotenone, 100 mmol/l succinate, 20 µmol/l antimycin A, and 100 mmol/l ascorbate + 1 mmol/l tetramethyl-p-phenylenediamine. Typical mix, measure, and mix cycles for the electron flow assay were 30 s, 3 min, and 1 min, respectively. All data were analyzed using the XF software and displayed as 'point-to-point' oxygen consumption rates (pmol O_2/min/well). Mitochondria begin in a coupled state with substrate present, in this case, state 3 initiated with ADP (4 mmol/l), state 4 induced with the addition of oligomycin (2.5 µg/ml, state 4o), allowing respiratory control ratio (RCR, state 3/state 4o) to be assessed. Data are presented as the average of 3–5 wells/condition in each mouse.

Measurements of NADPH oxidase in myocardium
NADPH oxidase activity was quantified by lucigenin-enhanced chemiluminescence [18,19]. Briefly, NADPH (100 µmol/l) was added to the buffer containing myocardium (30 µg protein in 500 µl) and lucigenin was automatically injected at 5 µmol/l to avoid known artifacts when used at higher concentrations. NADPH oxidase activity was calculated by subtracting the basal values from those in the presence of NADPH.

Measurement of isolated coronary artery vasomotion
Left anterior descending arteries from the left ventricle [74 ± 8 (60–102) µm in diameter] of heparinized excised heart tissue were isolated using a microscope. Each artery and its surrounding ventricular muscle was excised, transferred to a temperature-controlled dissection dish (4°C) containing a physiological salt solution [PSS (mmol/l): 119 NaCl, 4.7 KCl, 1.17 $MgSO_4$, 1.6 $CaCl_2$, 1.18 NaH_2PO_4, 24 $NaHCO_3$, 0.026 EDTA, and 5.5 glucose], and dissected free of muscle tissue. The side branches were tied off using an 11-0 suture. The vessels were transferred to a lucite chamber, cannulated at both ends using micropipettes, and pressurized at 60 mmHg. The arteries were tied to each pipette using an 11-0 suture. The PSS, bubbled with 20% O_2, 5% CO_2, and 75% N_2, used to perfuse the vessels during the experiments was buffered to a pH of 7.4 at 37°C. The preparation was then transferred to the stage of an inverted microscope. To assess leaks, the pressure at zero flow was measured, which should be equal to that of the inflow reservoir pressure when there are no leaks. Any preparations showing leaks were discarded. The vessels were slowly warmed to 37°C and allowed to develop a spontaneous tone. First, the control inner diameter of the isolated coronary artery was measured at 37°C in a vessel chamber (2 ml) using an Olympus IX71 inverted microscope (Olympus CCD). The percentage change in diameter was calculated from the diameter change induced by an intervention as a percentage of the total amount of active tone (maximal diameter − baseline diameter). Vasodilative responses to increasing concentrations of acetylcholine with or without L-NAME and sodium nitroprusside were measured.

Evaluation of intracellular nitric oxide level in the aorta
To examine the production of nitric oxide (NO) in the aorta, the descending aorta was removed and rinsed with cold PSS with the following composition: 119 mmol/l NaCl, 4.7 mmol/l KCl, 1.17 mmol/l $MgSO_4$, 1.6 mmol/l $CaCl_2$, 1.18 mmol/l NaH_2PO_4, 24 mmol/l $NaHCO_3$, 0.026 mmol/l EDTA, and 5.5 mmol/l glucose. After the removal of any adhering connective tissue, the aorta was cut into several segments. Each segment was opened with fine scissors and pinned onto a piece of rubber with the endothelium facing up so that the endothelial cells on the inside surface of the aorta were exposed to the solution. After being fixed on the rubber, the segments of the aorta were placed into the PSS at 37°C and aerated for 2 h with a gas mixture containing 21% O_2, 5% CO_2, and 74% N_2. NO levels in the vessels were assessed using 4,5-diaminofluorescein [20,21]. The aortic segments were loaded with 5 µmol/l 4, 5-diaminofluorescein for 30 min at 37°C in HEPES buffer (pH 7.4). Once the loading was finished, the vessels were rinsed with HEPES buffer three times and placed in a chamber containing HEPES buffer maintained at 37°C with a water bath. L-arginine (100 µmol/l) was added to the chamber during the measurements to ensure adequate substrate availability for NOS. Fluorescent images were recorded using an Olympus IX71 inverted microscope (Olympus CCD) and analyzed for fluorescence intensity using fluorescein isothiocyanate excitation/emission spectra from digitized images and normalized to the vessel area (expressed as intensity/100 µm^2). All camera settings were maintained constant throughout the image analysis.

Western blotting
Cryostat sections (5 µm) from the aorta were co-incubated with eNOS rabbit polyclonal antibody and phospho-eNOS (Ser 1177) rabbit polyclonal antibody (Cell Signaling Technology, Danvers, Massachusetts, USA). The eNOS and the phospho-eNOS were quantified by western blotting assays [22].

Statistical analyses
All statistical analyses were carried out using StatView software (Abacus Concepts, Berkley, California, USA). We used a one-way ANOVA, followed by the Student–Neumann–Keuls post-hoc test to compare echocardiographic data. We also used a one-way ANOVA,

followed by Tukey's post-hoc test to determine differences among the different interventions for H_2O_2 concentration, O_2^- production, fluorescence intensity, and biochemical data. A two-way ANOVA for repeated measures, followed by Tukey's post-hoc test was used to determine differences in vasodilation resulting from the various interventions. The values are expressed as the mean $\pm$ SEM. Significance was set at P less than 0.05 for all experiments.

Results

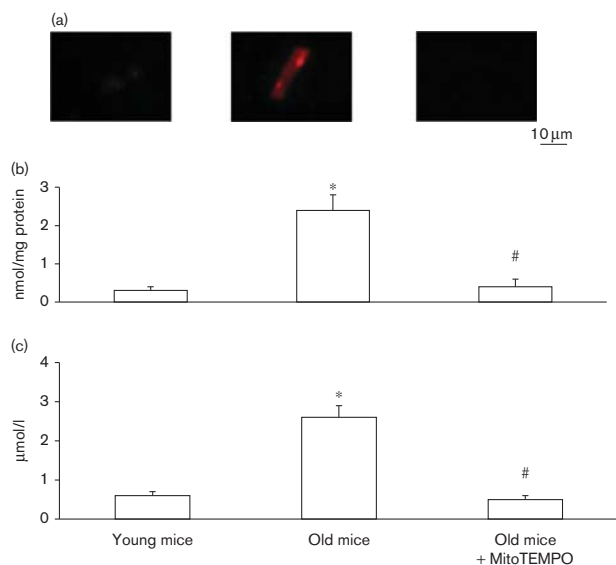### Aging and oxidative stress in the mitochondria of cardiomyocytes

Because the increase in mitochondrial ROS is assumed to be a major cause of cardiovascular function decline, initially, we measured the level of superoxide and H_2O_2 in the cardiomyocytes of old mice. To monitor mitochondrial O_2^- levels in isolated cardiomyocytes, we used the mitochondria-specific fluorescent probe MitoSOX. As expected, electrical stimulation increased the density of MitoSOX fluorescence in cardiomyocytes of old mice compared with those of young mice, and this result was consistent with the result of the UPLC analysis of the O_2^-

Fig. 1

Effect of MitoTEMPO on mitochondrial O_2^- and H_2O_2 in isolated cardiomyocytes. Mitochondrial O_2^- was measured in electrically stimulated cardiomyocytes of young, old, and MitoTEMPO-treated old mice using the fluorescent probe MitoSOX (a). A specific measure of mitochondrial O_2^- level was determined using the UPLC method (b). H_2O_2 concentration in MOPS buffer with isolated cardiomyocytes after electrical stimulation was measured using a free radical analyzer (c). Generation of mitochondrial O_2^- and H_2O_2 in cardiomyocytes was greater in the old mice than in the young mice. Treatment with MitoTEMPO is effective in decreasing O_2^- and H_2O_2 in cardiomyocytes. The data are expressed as the means $\pm$ SEM; $n = 14$, each. *$P < 0.01$ versus young mice, #$P < 0.01$ versus old mice. MOPS, 3-(N-morpholino)propanesulfonic acid; UPLC, ultra-performance liquid chromatography analysis.

in isolated mitochondria of cardiomyocytes (Fig. 1a and b). Supplementation of MitoTEMPO decreased the level of O_2^- in the cardiomyocyte mitochondria of old mice and also decreased the MitoSOX density. In addition, the level of H_2O_2 in electrically stimulated cardiomyocytes increased in old mice, which was reduced by MitoTEMPO treatment (Fig. 1c). Therefore, we confirmed that aging increases ROS in cardiomyocytes, and MitoTEMPO is effective in scavenging oxidative stress in cardiomyocytes.

Aging and mitochondrial respiration

Scavenging mitochondrial O_2^- by MitoTEMPO could improve the age-related decline of mitochondrial function. We measured the oxygen consumption rate in isolated mitochondria respiration from cardiomyocytes using an XF assay [15]. In the presence of FCCP as an uncoupler, mitochondria from the old mice showed lower respiration rates, compared with that of the young mice, with successive exposure to the following substrates or inhibitors: pyruvate + malate ($\approx 27\%$; complex I-dependent respiration; $P < 0.05$; $n = 14$ pairs; 3–5 wells/condition) (Fig. 2a), rotenone + succinate ($\approx 24\%$; complex II-dependent respiration; $P < 0.05$; $n = 14$ pairs; 3–5 wells/condition) (Fig. 2b), and antimycin + tetramethyl-p-phenylenediamine + ascorbate ($\approx 12\%$; $n = 14$ pairs; 3–5 wells/condition) (Fig. 2c). MitoTEMPO treatment of the old mice *in vivo* normalized the respiration rates of the subsequently isolated mitochondria to a level similar to that found in the young mice. Respiratory control ratio (RCR, state 3/state 4o) is an index of how coupled the electron transport chain is to ATP production. Using succinate as a substrate, we measured the RCR values in mitochondria of cardiomyocytes. The RCR values in mitochondria from old mice (3.02 ± 0.28, $n = 14$) were lower than those from young mice (4.24 ± 0.36, $n = 14$, $P < 0.05$). Supplementation of MitoTEMPO in old mice improved the RCR values (4.16 ± 0.38, $n = 14$) similar to those of young mice. These results indicate that treatment of MitoTEMPO improved the efficiency of ATP generation in aged mitochondria.

Left ventricular function

Cardiac aging in mice mimics human cardiac aging, as documented by previous epidemiological studies showing an age-dependent decline of diastolic dysfunction and worsening myocardial performance [23]. We examined the scavenging effect of mitochondrial oxidative stress in left ventricular performance using echocardiography. Aging decreased the diastolic function (E/e') and left ventricular ejection fraction in mice, which were restored with MitoTEMPO treatment (Fig. 3a and b). These results suggest that scavenging oxidative stress in mitochondria exerts a therapeutic effect of preserving cardiac performance in the failing heart.

Fig. 2
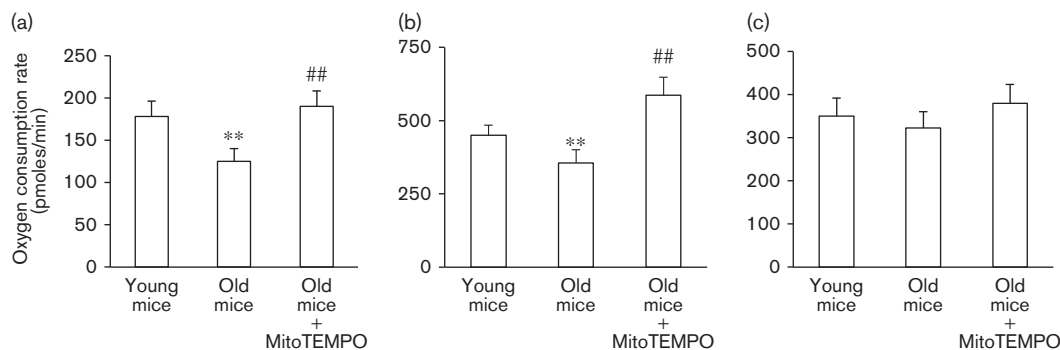
Respiration profile from isolated mitochondria in cardiomyocytes. In the presence of a submaximal dose of carbonyl cyanide p-trifluoromethoxyphenylhydrazone, the old mice showed low respiration rates with successive exposure to the following substrates or inhibitors: pyruvate + malate (complex I-dependent respiration; $n = 14$ pairs; 3–5 wells/condition) (a), rotenone + succinate (complex II-dependent respiration; $n = 14$ pairs; 3–5 wells/condition) (b), antimycin + tetramethyl-p-phenylenediamine + ascorbate ($n = 14$ pairs; 3–5 well/condition) (c) compared with the young mice. MitoTEMPO treatment of the old mice normalized the respiration rates to a level similar to that of the young mice. The data are expressed as the means ± SEM. **$P < 0.05$ versus young mice, ##$P < 0.05$ versus old mice.

Vasodilation

This study showed that treatment with MitoTEMPO decreased mitochondria-dependent H_2O_2 production in aged mice. H_2O_2 is freely diffusible and can stimulate extra-mitochondrial NADPH oxidase through a c-Src-mediated mechanism [7,24]. We therefore tested the hypothesis that the inhibition of mitochondrial H_2O_2 by MitoTEMPO would decrease the activity of NADPH oxidase, reduce cellular O_2^-, and improve NO production. To examine this speculation, we measured the NO generation induced by L-arginine and eNOS phosphorylation in the aorta. Decreased NO generation (Fig. 4a) and eNOS phosphorylation in old mice were improved by treatment with MitoTEMPO (Fig. 4b). In addition, increased activity of NADPH oxidase in the myocardium of aged mice recovered to the same level as in young mice (Fig. 5). Increased vascular O_2^- production has been implicated in the pathogenesis of endothelial dysfunction and hypertension [25,26]. From our results, we speculated that decreasing oxidative stress and recovering eNOS function by MitoTEMPO treatment could improve endothelial function and decrease blood pressure by relaxing vessels in aged mice. Co-infusion of MitoTEMPO in aged mice not only improved endothelial function, as evidenced by measurements of acetylcholine-induced vasodilation, and altered the endothelium-independent response to sodium nitroprusside (Fig. 6a and b), but also decreased blood pressure (Fig. 7). Our findings indicate that targeting mitochondrial ROS with an agent such as MitoTEMPO might therefore be effective in treating human coronary vasospasm, in which endothelial dysfunction contributes toward pathogenesis or several other hypertension-related diseases.

Discussion

Aging is the major factor in the decline of cardiovascular function [1]. Our results indicate that oxidative stress plays a pivotal role in the age-associated decrease of cardiovascular efficiency, providing direct evidence for the role of mitochondria ROS in the aging of this vital organ. Several lines of evidence support this conclusion. In aged mice, we found a decline in left ventricular function, concomitant with an increase in ROS in the mitochondria of cardiomyocytes and attenuated vascular endothelial function, including difficulty of efficient ATP generation in mitochondria. Administration of the mitochondrial-targeted antioxidant MitoTEMPO restored cardiovascular function in aged mice to the same level as in young mice, suggesting that an increase in ROS in the mitochondria plays a crucial role in age-associated endothelial dysfunction and in the decline of myocardial function.

In this study, we also characterized mitochondrial bioenergetics in young and old mice and evaluated the metabolic profile after treatment with MitoTEMPO. During uncoupling with FCCP, the oxygen consumption rate in the mitochondria of aged mice decreased significantly compared with that of young mice. Chronic administration of MitoTEMPO prevented this decrease. Therefore, MitoTEMPO induced a protective effect in the mitochondria of aged mice by normalizing the maximum respiration rates to the levels observed in the young mice. In addition, MitoTEMPO restored the RCR value, which reflects efficiency of energy with ATP generation in myocardium. Diastolic dysfunction is well documented in human cardiac aging. Impaired myocardial relaxation caused by a functional decline in calcium handling proteins and increased myocardial stiffness related to cardiac fibrosis and hypertrophy are

Fig. 3
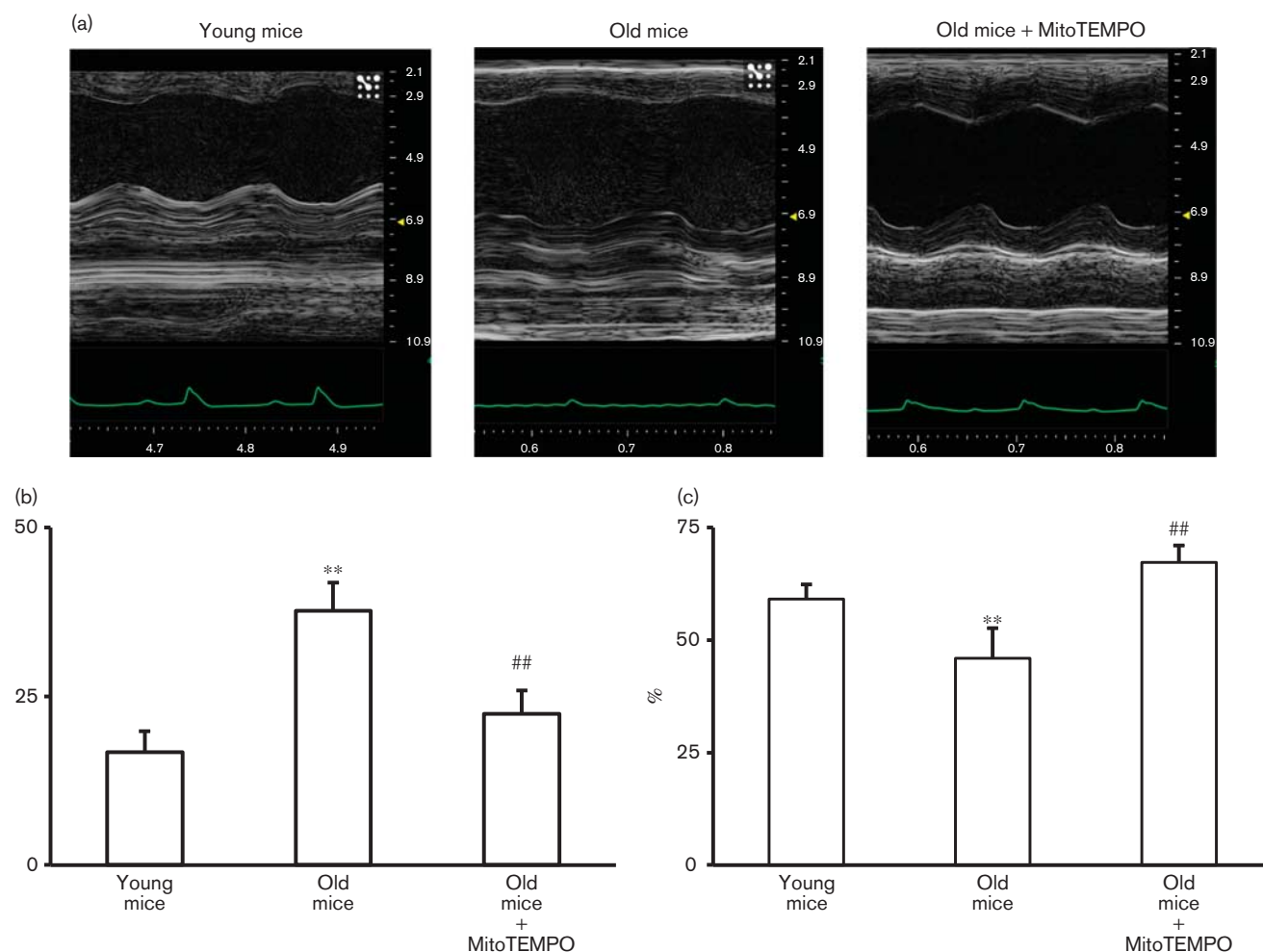
Representative M-mode echocardiograms of the left ventricle (a), E/e' (b), and ejection fraction (c) in young, old, and MitoTEMPO-treated old mice. In the old mice, diastolic function (E/e') and ejection fraction in the left ventricle were decreased compared with those of the young mice, and these decreases were recovered by treatment with MitoTEMPO. The data are expressed as the means $\pm$ SEM; $n = 14$, each. **$P < 0.05$ versus young mice, ##$P < 0.05$ versus old mice.

common causes of diastolic dysfunction in old mice [27]. Dai *et al.* [23] reported that a decrease in SERCA2 protein levels is the strongest predictor of diastolic dysfunction in aged mice and the overexpression of mitochondrial catalase, a hydrogen peroxide scavenger, restored cardiac dysfunction in aged mice with preservation of SERCA2. In our experiment, the level of hydrogen peroxide increased in cardiomyocytes concomitant with superoxide overproduction in aged mice. Treatment with MitoTEMPO decreased the level of hydrogen peroxide and increased SOD activity (Supplementary Fig., Supplemental digital content 1, *http://links.lww.com/MCA/A106*); that is, we speculate that MitoTEMPO has a beneficial effect in preserving SERCA2 activity, similar to a mitochondrial catalase that contributes toward the restoration of cardiac diastolic function, in addition to improving the energy performance of the electron transport complex in the mitochondria.

Bioavailable NO production in the aorta in response to L-arginine, acetylcholine-induced coronary artery dilation, and eNOS phosphorylation were reduced in aged mice, as found in our previous report showing that eNOS activity was reduced and that thiol oxidation appeared in the coronary artery of overoxidatively stressed mice [28]. Protein thiols can undergo thiol oxidation, a reversible protein modification involved in cellular signaling and adaptation. Under oxidative stress, thiol oxidation, such as S-glutathionylation in cysteine residues of eNOS, occurs through a thiol-disulfide exchange with glutathione-S-S-glutathione, which can only occur when the cellular glutathione (GSH)/glutathione-S-S-glutathione ratio is low, as shown in old mice [29–31], or when a reaction of oxidant-induced protein thiol radicals occurs with reduced

Fig. 4
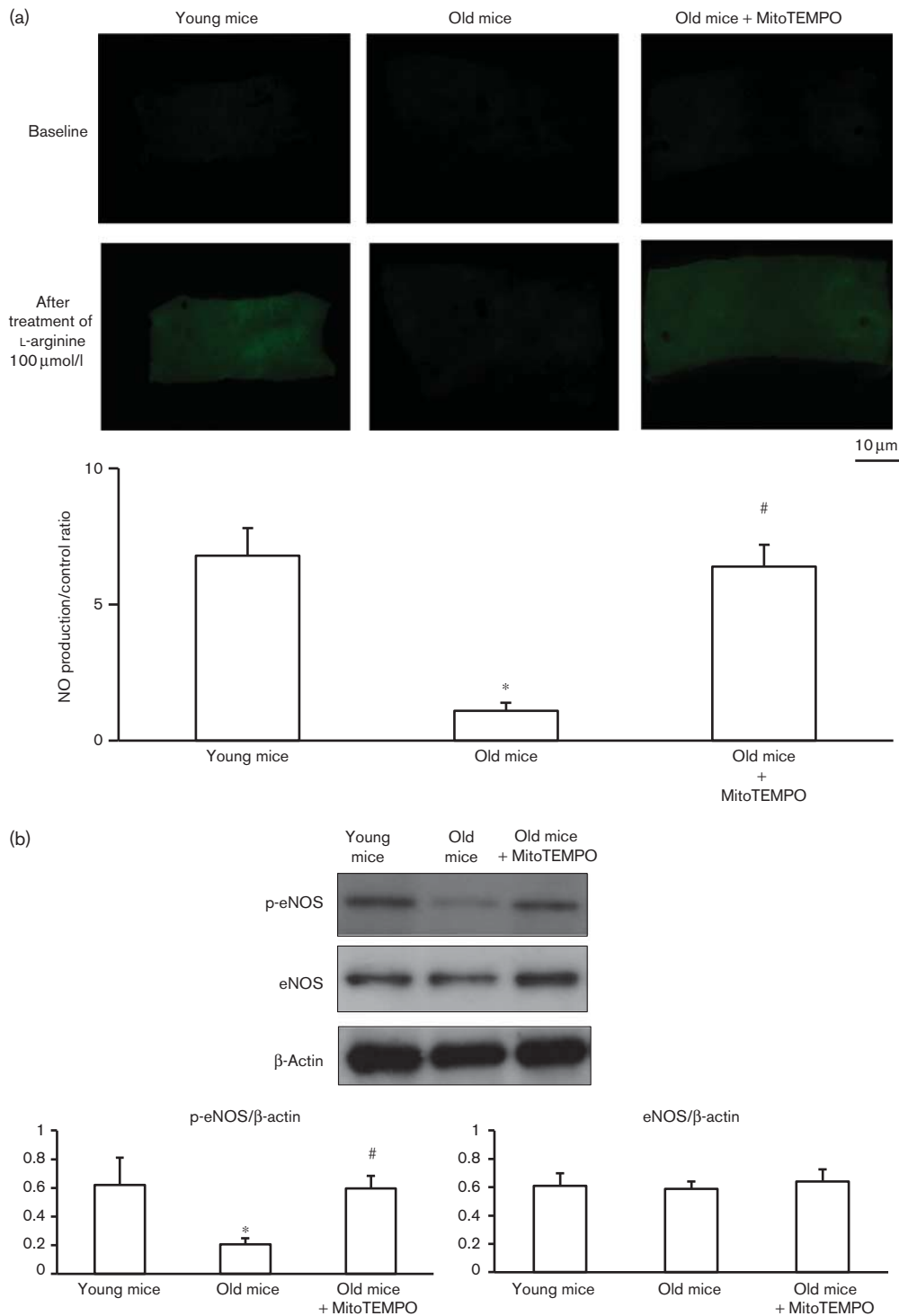
Nitic oxide (NO) production and endothelial nitric-oxide synthase (eNOS) phosphorylation in the aorta. Immunofluorescent staining of NO induced by L-arginine from an aortic strip, stained for DAF-2DA (a, upper panel). Quantification of NO production in response to L-arginine (a, lower panel). NO production was impaired in the aortic tissue of old mice. Treatment with MitoTEMPO recovered NO production in the aorta of the old mice. The expression of eNOS and phospho-eNOS protein in the aorta was quantified using a western blotting assay. The phosphorylation of eNOS was suppressed in the aorta of the old mice compared with that of the young mice and this suppression was recovered by treatment with MitoTEMPO (b). The data are expressed as the means $\pm$ SEM; $n = 14$ for each experimental group. $*P < 0.01$ versus young mice, $^{\#}P < 0.01$ versus old mice.
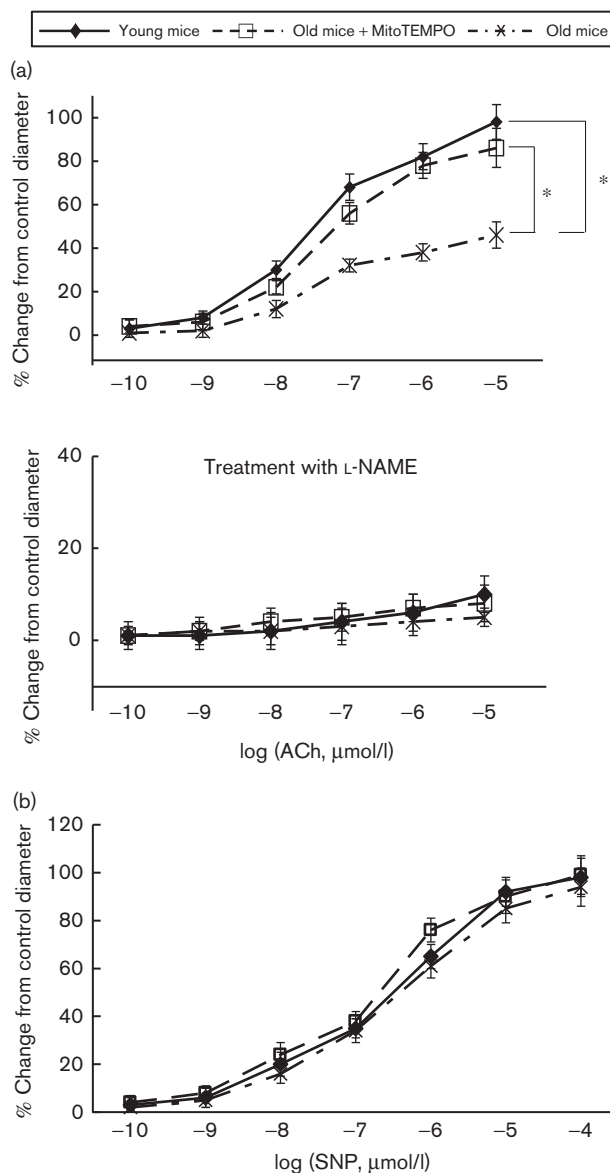
Fig. 5
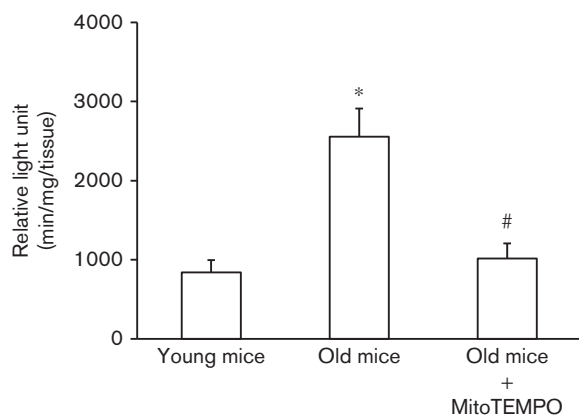
Effect of MitoTEMPO on the activity of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in the myocardium. The NADPH oxidase activity was greater in the old mice than in the young mice and this difference decreased with MitoTEMPO treatment. The data are expressed as the means ± SEM; $n = 14$ for each experimental group. *$P < 0.01$ versus young mice, #$P < 0.01$ versus old mice.

Fig. 6

Isolated coronary artery responses to acetylcholine and sodium nitroprusside. In isolated pressurized coronary arteries, we measured the vasodilative response induced by a cumulative dose of acetylcholine (ACh, $1 \times 10^{-10 \sim -5}$ µmol/l) with or without NOS inhibitor, L-NAME (a), and sodium nitroprusside (SNP, $1 \times 10^{-10 \sim -5}$ µmol/l) (b). ACh-induced vasodilation was decreased in the old mice compared with that in the young mice, and the vasodilation recovered to a level equal to that of the young mice with MitoTEMPO treatment. SNP-induced vasodilation did not differ among the three groups. The data are expressed as the means ± SEM; $n = 14$, each. *$P < 0.01$.

glutathione [32]. Recently, it has been reported that S-glutathionylation of eNOS reversibly decreases NOS activity with an increase in superoxide generation primarily from reductase. In this state, two highly conserved cysteine residues are identified as sites of S-glutathionylation and are found to be critical for redox regulation of eNOS function [33]. GSH synthase inhibition without oxidation by buthionine-(S,R)-sulfoximine has been reported to have no effect on NO bioactivity [34]. However, thiol depletion *in vivo* considerably reduces NO generation from eNOS [35–37]. The decrease in NO bioactivity with GSH depletion under oxidant stress has been reported [38], and thiol oxidizing agents, such as diamide, decreased both the GSH level and the NO bioactivity [34]. Therefore, we speculate that decreases in GSH and total thiols, including cysteine residues in the eNOS domain by oxidative stress, might contribute toward the impairment of NO generation in aged mice and that scavenging oxidant stress by MitoTEMPO is effective for recovery of thiols.

Previously, we reported that NADPH oxidase is a major source of superoxide in the vasculature and that superoxide from this enzyme serves as an important physiological redox-signaling molecule participating in the regulation of vascular function associated with a novel Ca^{2+}-signaling pathway [28,39]. Doughan *et al.* [40] reported that the inhibition of NADPH oxidase activity by apocynin prevented mitochondrial impairment and attenuated mitochondrial superoxide production in the vascular endothelium. Moreover, it has been reported that mitochondrial superoxide stimulates extramitochondrial NADPH oxidase activity in a feedforward manner [7]. In the present study, treatment with
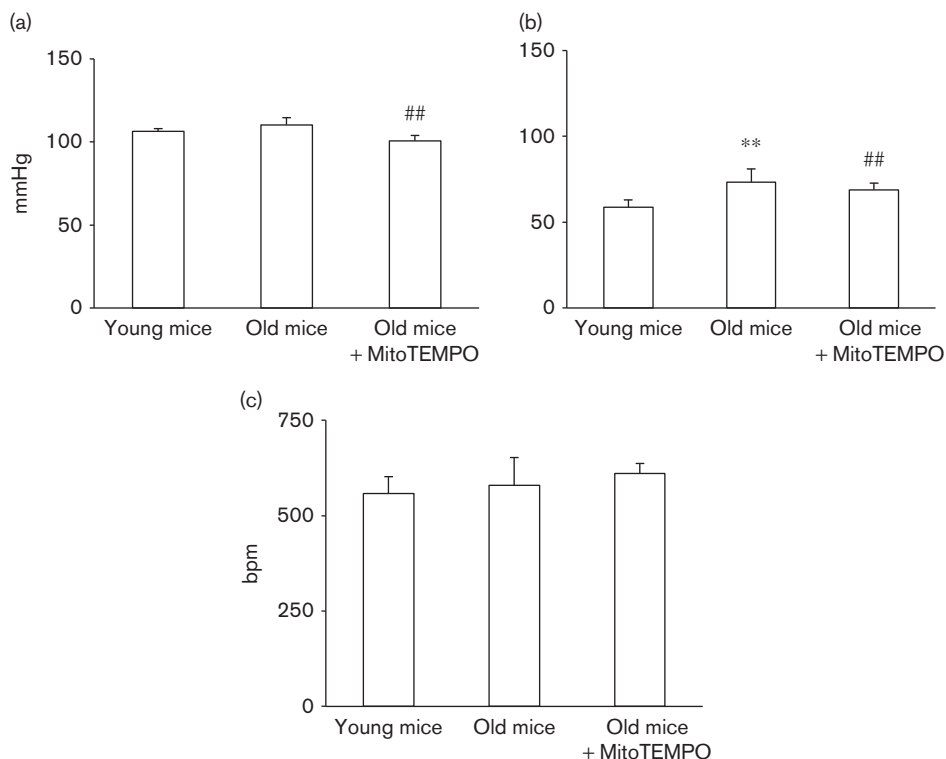
MitoTEMPO decreased NADPH oxidase activity in aged mice. In addition, MitoTEMPO is effective in decreasing the level of H_2O_2, which might stimulate NADPH oxidase activity [24]. Taken together, we speculate that the interplay between mitochondrial and NADPH oxidase-derived superoxide constitutes a

Fig. 7

Change in hemodynamics with MitoTEMPO treatment. With MitoTEMPO administration, the increase in systolic (a) and diastolic (b) blood pressure in the old mice decreased to a level equal to that of the young mice. Heart rate did not differ among the three groups (c). The data are expressed as the means $\pm$ SEM; $n = 14$, each. **$P < 0.05$ versus young mice, ##$P < 0.05$ versus old mice.

vicious cycle in which the NADPH oxidase increases mitochondrial ROS, which further activates the cytoplasmic NADPH oxidase and increases cellular superoxide production, decreasing NO$^\bullet$ bioavailability and uncoupling eNOS; however, further study is needed [41].

We speculate that the scavenge of ROS by mitoTEMPO recovered the eNOS phosphorylation and improved endothelial function in the coronary artery, as evidenced by measurements of endothelium-dependent relaxation induced by acetylcholine, and results in the recovery of arterial stiffness, as shown by the decrease in blood pressure in aged mice. Moreover, it has been reported that MitoTEMPO is effective in decreasing systolic blood pressure after hypertension is established [7]. This result suggests the possibility that a scavenger of mitochondrial ROS would be an antihypertensive agent and an improved agent of vascular endothelial function, although further study is needed to clarify the mechanism of the effect of MitoTEMPO on blood pressure.

There is increasing evidence to support the concept that mitochondria play an important role in aging and various age-related cardiovascular diseases. However, the question of whether the oxidative stress in mitochondria is a cause or a result of various cardiovascular diseases in elderly patients is far from resolved. A better understanding of cardiac senescence and mitochondrial oxidative stress, the availability of pharmacological agents, and the development of various aging models with impaired mitochondrial function would aid in resolving this question. In addition, the scavenging activity of superoxide decreased with aging (Supplementary Fig, Supplemental digital content 1, *http://links.lww.com/MCA/A106*); therefore, the increase in the ROS level in aging might be because of less reduction as well as more generation, although further study is needed. In the context of our study, we conclude that the resolution of oxidative stress in mitochondria rescues the age-related decline of cardiac performance with improvement in myocardial energy metabolism and coronary circulation.

Acknowledgements

Conflicts of interest

There are no conflicts of interest.

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