Attenuation of antioxidative capacity enhances reperfusion injury in aged rat myocardium after MI/R

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Liu, Peitan, Baohuan Xu, Thomas A. Cavalieri, and Carl E. Hock. Attenuation of antioxidative capacity enhances reperfusion injury in aged rat myocardium after MI/R. Am J Physiol Heart Circ Physiol 287: H2719–H2727, 2004. First published September 9, 2004; doi:10.1152/ajpheart.00317.2004.—Mortality due to ischemic cardiovascular diseases is significantly higher in elderly than in young adults. Myocardial ischemia-reperfusion (MI/R) can induce oxidative stress and an inflammatory response. We hypothesized that increased vulnerability of aged myocardium to reperfusion injury could be caused by decreased antioxidative capacity, rather than increased oxidant production, after MI/R. Aged (20-mo-old) and young (4-mo-old) male F344BN rats were subjected to 30 min of myocardial ischemia by ligation of the left main coronary artery followed by release of the ligature and 4 h of reperfusion. Four experimental groups were studied: young sham-operated rats, aged sham-operated rats, young rats subjected to MI/R, and aged rats subjected to MI/R. MI/R significantly increased infiltrated leukocyte number and myeloperoxidase (MPO) activity in perinecrotic areas of hearts of young rats compared with aged MI/R rats. These changes in infiltrated leukocyte number and MPO activity were associated with an increase in superoxide generation in perinecrotic areas from hearts of young rats compared with aged rats. Plasma levels of TNF-α and IL-1β were significantly higher in young than in aged MI/R rats. However, plasma 8-hydroxy-2′-deoxyguanosine levels and creatine kinase activity were increased in aged compared with young MI/R rats. Increased reperfusion damage in aged rats was associated with a significant decrease in plasma ratio of GSH to GSSG. Our results suggest that enhanced ischemia-reperfusion injury in aged rat hearts may be related to reduced antioxidative capacity, rather than increased reactive oxygen species production. These findings contribute to a better understanding of effects of aging on oxidative stress and inflammatory responses of the heart after MI/R.

reactive oxygen species; ratio of reduced glutathione to oxidized glutathione; copper/zinc superoxide dismutase; DNA oxidative damage; myocardial ischemia-reperfusion

ISCHEMIC CARDIOVASCULAR DISEASE is a common cause of death in the elderly (36, 39). The importance of oxidative stress and the cascade of inflammatory mediators in ischemic cardiovascular diseases have been recognized and vigorously investigated after the discovery of superoxide dismutase (SOD) in mammalian systems (31). An increased sensitivity of aged myocardium to oxidative stress results in accumulation of cellular and tissue damage, which may explain the high mortality due to myocardial ischemia-reperfusion (MI/R) in the elderly (1, 9, 15). Published data suggest that aging-related oxidative stress in the unstressed condition is mainly caused by increased production of reactive oxygen species (ROS) (9, 18).

However, it is unclear whether the aging process increases ROS production and/or decreases antioxidative capacity in the stressed condition, such as MI/R.

Oxidative stress plays a critical role in ischemia-reperfusion injury (1, 3, 23). Superoxide is a major initiator in the free radical cascade and is mainly catalyzed to cytotoxic H2O2 by SOD (31). ROS have the potential to directly injure cardiomyocytes and vascular cells (8, 9) and may trigger a cascade of inflammatory mediators through induction of cytokine expression (3, 13). Damage induced by ROS on intracellular and extracellular targets, such as membrane lipids, proteins, and DNA, clearly contributes to myocyte necrosis and/or apoptosis and organ dysfunction during MI/R (8, 33). Normally, ROS are quickly inactivated by the antioxidative system; therefore, the severity of oxidative tissue injury is determined by the balance between ROS production and the intrinsic antioxidative capacity of the tissue (23, 35). Aging may increase ROS production or enhance the toxic effects of ROS via impairment of antioxidative efficiency during MI/R (9, 15).

During reperfusion, mitochondria represent the major intramyocyte source of ROS production (32). The major extramyocyte source of ROS production is the activated leukocyte, although endothelial cells and other cell types can also produce ROS (17, 22). ROS can react with many cellular components and organelles, including lipid in the membrane and deoxyguanine in DNA. ROS-induced damage to the cell membrane promotes leakage of intracellular components, such as creatine kinase (CK), into extracellular space, and interaction of ROS with cellular DNA results in production of 8-hydroxy-2′-deoxyguanosine (8-OHdG), an oxidative adduct of deoxyguanine. Therefore, determination of plasma CK activity and the 8-OHdG adduct may be useful as a marker of reperfusion injury (10, 20, 34).

In the aged myocardium, many physiological changes occur that may alter the response to MI/R. Previous studies using heavy exercise, isolated blood vessels, and isolated, perfused hearts suggest that enhanced ROS production (2, 4, 9) and reduced antioxidative capacity (1, 6) may contribute to the enhanced susceptibility of the aged heart to oxidative injury. In the present study, we have used an in vivo model of MI/R in aged and young rats to clarify the influence of aging on the whole body response to MI/R.

MATERIALS AND METHODS

Materials

Young adult (4-mo-old) and senescent (20-mo-old) F344BN hybrid rats regularly monitored for genetic purity and health status were...
provided by the National Institute on Aging. To avoid the influence of gender on apoptosis signal transduction, male rats were used. The experimental protocols were conducted in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Medicine and Dentistry of New Jersey-School of Osteopathic Medicine Institutional Animal Care and Use Committee.

ELISA kits for assay of plasma levels of TNF-α and IL-1β were purchased from Biosource. A diagnostic kit for determining plasma CK activity was purchased from Beckman Coulter. A myeloperoxidase (MPO) enzyme-linked immunosorbsent assay kit was purchased from Bioxytech to assay MPO levels, and a GSH assay kit was purchased from Cayman (Ann Arbor, MI). The SOD-525 kit and glutathione reductase (GR)-340 kit (OxisResearch, Portland, OR) were used to detect Cu/Zn SOD and GR activities.

Monitoring ECG and mean arterial blood pressure. The method used to monitor mean arterial blood pressure and heart rate (HR) has been described previously (28). Briefly, rats were anesthetized by intramuscular injection of ketamine (100 mg/kg) and xylazine (7 mg/kg) and placed on the T-Pat (Raymar) to maintain body temperature at 36–37°C. The trachea was cannulated with a section of tubing (PE-240) to maintain the patent airway. A catheter (PE-50) was inserted into the left femoral artery and connected to Cardiomax III (Columbus Instruments) to monitor mean arterial blood pressure. ECG was monitored with Cardiomax III for confirming the ischemic changes and the alteration of HR.

Experimental MI/R. The surgical procedure used to achieve MI/R has been described previously (28). After measurement of preischemic data such as arterial blood pressure and ECG, a 3-cm skin incision was made over the left thorax, and the pectoral muscles were retracted to expose the ribs. A 1-0 silk ligature was placed loosely through the skin and underlying muscle in a modified purse-string suture to facilitate rapid closure of the chest wall. A thoracotomy was performed at the level of the fifth intercostal space. The heart was briefly everted from the thoracic cavity, and a 4-0 silk suture was secured around the left main coronary artery, 2–3 mm from its origin. To minimize damage to the coronary artery and, hence, maximize reperfusion, the suture was placed slightly deeper in the myocardium, and a 2- to 3-mm segment of 2-0 suture was placed parallel to the vessel within the ligature. One end of the slip-knot-tied coronary ligature was exteriorized around the chest wall to permit subsequent reperfusion. The heart was then repositioned in the thoracic cavity, air was evacuated from the thorax, and chest wall, muscles, and skin were rapidly closed by means of the previously placed purse-string suture. After the incision in the chest was closed, a tube connected to a rodent ventilator (model 683, Harvard) was inserted into the tracheal tube, and the animal was mechanically ventilated (10 ml/kg, 40 times/min) during the 4-h period of reperfusion. At the end of the period of occlusion of the coronary artery (30 min), the exteriorized end of the ligature was pulled free, allowing reperfusion of the ischemic myocardium for 4 h. Sham-operated control rats were subjected to the same surgical procedures, except the 4-0 silk suture was not tied. Rats were killed at the end of the reperfusion period (4 h). Rats were randomly divided into four groups: 1) young sham rats, 2) young rats subjected to MI/R, 3) aged sham rats, and 4) aged MI/R rats.

Identification of areas of infarction and perinecrotic areas. After the rat was euthanized, the left coronary artery of the heart was reocluded at the same position as before, and the heart was removed. A saline solution of 0.12% Evans blue dye was infused slowly via the aorta to verify the nonischemic zone of the myocardium, which stained dark blue. Then the heart was sliced into 1-mm-thick transverse sections, and the first section in the series was incubated in 1% triphenyltetrazolium chloride solution (TTC) in phosphate buffer (pH 7.4) at 37°C for 10 min and fixed in 3% formalin for ≥4 h. Viable tissue stains red, and infarcted tissue appears pale. Perinecrotic areas of heart tissues were obtained from other sections (without TTC staining) in the series by removal of the necrotic area (according to TTC staining of the 1st section) from the nonreperfused area (which did not stain with Evans blue dye).

ELISA for plasma TNF-α and IL-1β. Blood samples anticoagulated with 3.8% citrate solution were obtained at 90 min of reperfusion via an arterial catheter and centrifuged at 4,000 g for 10 min at 4°C. Plasma was prepared and stored in a −86°C freezer until ELISA. The procedure for assay of the cytokines was performed according to the manufacturer’s instructions. Duplicate samples and standards were included in each assay, and results are presented as picograms per milliliter of plasma.

Determination of plasma CK activity. Plasma CK activity is an index of MI/R injury (20). Plasma was obtained at 4 h of reperfusion and stored at −86°C until CK assay. Plasma CK activities were determined with a kit according to the manufacturer’s instructions, and results are presented as international units per liter.

Determination of circulating leukocytes. Citrate-anticoagulated blood samples were obtained from the carotid arterial catheter at the end of reperfusion. Fifty microliters of each sample were diluted 20-fold with 1% acetic acid solution to lyse red blood cells. Leukocytes were enumerated by light microscopy (Nikon Optiphot-2) using a hemocytometer.

Leukocyte infiltration into perinecrotic areas. Leukocytes that infiltrated perinecrotic areas of the heart were counted in hematoxylin-eosin-stained cryosections. The heart was rapidly removed at the end of reperfusion and placed on ice. After it was washed twice with PBS (pH 7.4, 4°C), the heart was cut horizontally in cross-section and placed in a tube containing 4% paraformaldehyde solution for 3 h at room temperature. Then the heart specimens were placed in a tube containing 20% sucrose for 8 h at room temperature and stored in a −86°C freezer for cryosection. Frozen samples of hearts were cryosectioned (7 μm thick) at −25°C using a cryostat (model CM 1850, Leica). After they were stained with hematoxylin and eosin, leukocytes that infiltrated perinecrotic areas of the heart were counted at 20 high-power (×200) fields (HPF) using a Nikon Optiphot-2 microscope.

MPO activity in perinecrotic areas of heart tissues. MPO is stored in the primary granules of neutrophils and released extracellularly via degranulation after activation of neutrophils. Extracellular MPO can be determined as an index of polymorphonuclear leukocyte infiltration in response to inflammation. An MPO enzyme immunoassay kit was used to assay MPO levels. Tissues from perinecrotic areas of the heart were snap frozen in liquid nitrogen and stored in a −86°C freezer until they were analyzed. A 20% (wt/vol) homogenate of perinecrotic areas of heart tissue in ice-cold PBS containing leupeptin (1 μg/ml), pepstatin A (1 μg/ml), and antipain (50 μg/ml) was prepared and centrifuged, and the supernatants were used for assay of MPO activity. A standard curve provided by the kit was used to calculate MPO levels, which are presented as nanograms per milligram of tissue.

Determination of superoxide generation. The method used to measure superoxide anion production has been described previously (28). Briefly, the hearts were removed at the end of reperfusion and washed twice with PBS (pH 7.4). Then pieces of perinecrotic areas of the heart tissue (80–170 mg) were incubated in Krebs-bicarbonate buffer (pH 7.4) consisting of (in mM) 118 NaCl, 4.7 KCl, 1.5 CaCl2, 25 NaHCO3, 1.1 MgSO4, 1.2 KH2PO4, and 5.6 glucose. Tissues were gassed with 95% O2-5% CO2 for 30 min and placed in plastic scintillation vials containing 0.25 mM lucigenin in 1 ml of Krebs-bicarbonate buffer (pH 7.4). The chemiluminescence elicited by superoxide in the presence of lucigenin was measured using a Beckman counter (model LS6001C).

Plasma 8-OHdG assay. A competitive ELISA kit was used for quantitative measurement of plasma 8-OHdG, which serves as an index of oxidative damage to DNA. Citrate-anticoagulated blood samples were obtained from the carotid arterial catheter at the end of reperfusion. After centrifugation, the plasma was obtained, and deproteinized by addition of 0.1% metaphosphoric acid (1:1 vol/vol),...
recentrifuged at 3,000 g for 5 min at 4°C. The supernatant was placed in a – 86°C freezer before measurement of 8-OHdG. The assay was performed according to the manufacturer’s instructions.

**Plasma GSSG and GSH + GSSG assays.** The principle of this procedure is based on the fact that the sulfhydryl group of GSH reacts with 5,5′-dithiobis-2-nitrobenzoic acid and produces a yellow 5-thio-

2-nitrobenzoic acid (26, 27). Citrate-anticoagulated blood samples were obtained from the carotid arterial catheter at the end of reperfusion. Blood samples were prepared with or without 2-vinylpyridine (2-VP), which was obtained, deproteinized by addition of 0.1% metaphosphoric acid (1:1 vol/vol), and recentrifuged at 3,000 g for 5 min at 4°C. Supernatants were placed in a – 86°C freezer before measurement of GSH + GSSG (supernatants without 2-VP) and GSSG (supernatants with 2-VP). The assay was performed according to the manufacturer’s instructions.

**Cytosol Cu/Zn SOD activity assay.** The principle of this procedure is based on SOD-mediated increase in the rate of autoxidation of 5,6-dihydrorhodamine-3,3-dihexyloxacarbocyanine (Hex-Fluorene in aqueous alkaline solution) to yield a chromophore with maximum absorbance at 525 nm. At the end of the reperfusion, the hearts were rapidly removed and washed twice in ice-cold PBS (pH 7.4) with heparin (10 U/ml). A 50% (wt/vol) homogenate of perinecrotic areas of the myocardium in PBS containing PMSF (Sigma, St. Louis, MO; 30 μl with 1 g tissue) was prepared and centrifuged at 4,000 g. Diluted supernatant was used for determination of cytosol Cu/Zn SOD activity.

**Cytosol GR activity assay.** Assay of GR activity is based on oxidation of NADPH to NADP+ catalyzed by a limiting concentration of GR. At the end of the reperfusion, the hearts were rapidly removed and washed twice in ice-cold PBS (pH 7.4) with heparin (10 U/ml). A 50% (wt/vol) homogenate of perinecrotic areas of the myocardium in PBS containing EDTA was prepared and centrifuged at 5,000 g for 15 min at 4°C. The supernatant was stored in a – 86°C freezer. The supernatant was thawed and transfected (200 μl) by pipette, along with 400 μl of GSSG solution (provided by the kit), to a cuvette. The cuvette was placed in a spectrophotometer, and 400 μl of NADPH solution (provided by the kit) were added and mixed. Then the absorbance at 340 nm (A340) was recorded for 5 min, and GR activity was calculated as follows: GR activity (μM/ml) = A340/min/ (6.22 × 10⁻³ ml/μmol).

**Determination of mRNA expression of TNF-α and IL-1β by RT-PCR.** The RT-PCR method has been described previously (28). Pieces of perinecrotic areas of heart tissues were homogenized with a Polytron homogenizer in RNA Stat-60 reagent (Tel-Test). Total RNA was extracted with chloroform, and the pellet was dissolved in diethyl pyrocarbonate-H2O (Sigma). Total RNA concentration was determined by spectrophotometric analysis at 260 nm, and 4 μg of total RNA were reverse transcribed into cDNA in 30 μl of reaction mixture containing Superscript II (GIBCO BRL, Gaithersburg, MD), dNTP, and oligo(dT)12-18 primers. The cDNA was amplified using specific primers with a DNA thermal cycler (model 480, Perkin-Elmer). The amplification mixture contained 1 μl of 15 μM forward primer, 1 μl of 15 μM reverse primer, 5 μl of 10× buffer, 1.5 μl of 50 mM MgCl₂, 5 μl of reverse-transcribed cDNA samples, and 1 μl of Taq polymerase. Primers were designed from the published cDNA sequences using the Oligo Primer Design Program. The cDNA was amplified after determination of the optimal number of amplification cycles within the exponential amplification phase for each primer set. After amplification, the sample (10 μl) was separated on a 2% agarose gel containing 0.003% ethidium bromide (0.3 μg/ml), and bands were visualized and photographed using ultraviolet transillumination. Quantitative assessment of gene expression was performed using the Image Master VDS program (Pharmacia Biotech) and normalized by assessment of GAPDH expression. The number of PCR cycles for TNF-α, IL-1β, and GAPDH was 38, 33, and 20, respectively. The designed primer sequences are as follows: 5′ ACCACAGAAGATGCTGAAGTTG3′ (sense) and 5′ AT- GATCCTGAAAGGAGCAAGG3′ (antisense) for TNF-α, 5′ CTCTTTGAGAATGTTCAACAG3′ (sense) and 5′ AAG- AAGTTGGTCTGCTTCATC3′ (antisense) for IL-1β, and 5′ GGTGAAGGTCGGTGCAACGGATT 3′ (sense) and 5′ GAT- GCCAAATGTGCTAGGAC3′ (antisense) for GAPDH.

**Statistical analysis.** Statistical significance for multigroup comparisons was determined using ANOVA (Sigma Stat, Jandel Scientific). For comparison of multiple groups, data were tested using two-way ANOVA. When measurements from the same rat were tested at different times, a two-way repeated-measures ANOVA was used. If a significant F value was obtained, group means were compared by paired and independent t-tests, in which Bonferroni’s adjustment was used to control for the family-wise error rate. All tests for significance were conducted at Bonferroni’s adjusted 0.05 level (2-tailed test).

**RESULTS**

**Alteration of Mean Arterial Blood Pressure and HR**

Time-dependent alteration of mean arterial blood pressure and HR among the experimental groups is shown in Fig. 1.

![Fig. 1. Differences in mean arterial blood pressure (A) and heart rate (B) among the experimental groups. Values are means ± SE. *P < 0.05 compared with young rats subjected to myocardial ischemia-reperfusion (MI/R). **P < 0.05 vs. aged MI/R. ***P < 0.05 vs. aged sham.](http://ajpheart.physiology.org/)

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MI/R induced a significant decrease in mean arterial blood pressure in aged rats at 2 and 4 h of reperfusion compared with young MI/R rats. Moreover, a significant decrease in shear rate of aged MI/R rats was observed before ischemia and during MI/R compared with young MI/R rats.

**Inflammatory Responses Induced by MI/R**

Inflammatory responses induced by MI/R are necessary for host defense and tissue repair; however, overwhelming inflammatory responses may be life-threatening. In the present study, we investigated the early events of acute inflammatory responses after MI/R.

**Circulating leukocyte count.** An increase in circulating leukocytes is an early manifestation of acute inflammation. We counted the alteration in circulating leukocytes at 4 h of reperfusion. MI/R induced a significant increase in circulating leukocytes in aged and young MI/R rats compared with aged and young sham control rats. Moreover, a significant increase in circulating leukocytes was observed in young compared with aged MI/R rats: 9,957 ± 414 vs. 7,150 ± 754 cells/mm³ (Fig. 2).

**Infiltrated leukocyte count and MPO activity in perinecrotic areas of the heart.** The process of leukocyte extravasation is an interaction of leukocytes and endothelium mediated by adhesion molecules and chemokines. There were a few leukocytes in the aged and young sham control rats; however, MI/R significantly increased leukocyte infiltration into the perinecrotic areas of the hearts of young MI/R rats compared with aged MI/R rats: 512 ± 66 vs. 277 ± 32 cells/HPF. Moreover, MPO activity in the perinecrotic areas of the heart of young rats was markedly increased compared with aged MI/R rats: 39 ± 2.6 vs. 23 ± 1.8 ng/g tissue (Fig. 3).

**Superoxide generation in perinecrotic areas of the heart.** Superoxide generation was measured in perinecrotic areas of heart tissue sections obtained at 4 h of reperfusion with a chemiluminescence count elicited by superoxide in the presence of lucigenin. MI/R induced a significant increase in superoxide generation in young and aged MI/R rats compared with young and aged sham control rats. Moreover, there was a significant increase in young MI/R rats compared with aged MI/R rats: 1,524 ± 212 vs. 1,166 ± 173 counts per minute/mg tissue (Fig. 4).

**Plasma Levels of 8-OHdG and CK Activity**

The antioxidative system consists of enzymes, such as SOD and catalase, small molecules (e.g., GSH), and some large molecules. SOD and GSH play a critical role in the antioxidative system.

**Cytosol GR activities.** GR probably contributes to antioxidant defense, inasmuch as it catalyzes the reduction of GSSG to GSH. We determined GR activities in perinecrotic areas of heart tissue at 4 h of reperfusion. After MI/R, GR activity was increased to a similar extent in young and aged rats: 43.4 ± 3.1 and 39.9 ± 3.7 mU/mg protein, respectively. However, this increase in GR activity after MI/R in young rats was not statistically different from that in aged rats.

**Cytosol Cu/Zn SOD activities.** Superoxide is a major mediator in the free radical cascade and is mainly catalyzed to H₂O₂ by SOD. Cu/Zn SOD is present predominantly in the cytosol. We measured Cu/Zn SOD in perinecrotic areas of the heart after 4 h of reperfusion. Our results indicate an increase in Cu/Zn SOD activity in young and aged MI/R rats: 1,203 ± 202 and 997 ± 180 U/mg protein, respectively. However, this difference between young and aged MI/R rats did not reach statistical significance (Fig. 8).

**DISCUSSION**

Epidemiologically, ischemic cardiovascular disease is the major cause of death in the elderly (36, 39). Biomedical
research is focused on identifying reasons for the higher prevalence of cardiovascular disease in the elderly. Ischemia can rapidly deplete myocardial cell pools of ATP and cause cell necrosis. Reperfusion of the ischemic tissue is necessary to increase tissue levels of ATP and to improve heart function. Although blood flow is restored, tissue injury may be enhanced by the acute inflammatory response and increased oxidative stress induced by reperfusion (12a, 17). The inflammatory response involves the delivery of leukocytes to the site of injury. The leukocytes, initially predominantly neutrophils, adhere to the endothelium via adhesion molecules, transmigrate across the endothelium, and migrate to the reperfused tissue.
postischemic tissue under the influence of chemotactic agents (7, 14). Chemoattractants and some cytokines, such as TNF-α and IL-1β, affect this process by modulating the surface expression of adhesion molecules. After extravasation, leukocytes release ROS (13, 23), which can cause endothelial cell damage and injury to a variety of cell types. ROS are detoxified by tissue and circulating antioxidant systems. The net effect of ROS on tissue injury depends on the balance between the antioxidative capacity of the tissue and the ROS production. Despite the potential for reperfusion-induced injury, substantial evidence suggests that reperfusion enhances cardiac repair and improves patient survival. However, the effects of aging on oxidative stress and the inflammatory response induced by MI/R are unclear.

In the present study, a rat model of MI/R was used to investigate the effects of aging on inflammatory responses and oxidative stress. Our results indicate that MI/R significantly increased leukocyte infiltration and MPO activity. These increases were associated with a significant increase in superoxide generation in perinecrotic areas of hearts of young compared with aged MI/R rats. Moreover, plasma levels of TNF-α and IL-1β were also significantly increased in young compared with aged rats after MI/R. In contrast, plasma 8-OHdG levels and CK activity were significantly augmented in aged compared with young rats, suggesting enhanced DNA damage and tissue injury in aged rat hearts after MI/R. The enhanced tissue damage in aged rats was associated with decreased Cu/Zn SOD and GR activities in perinecrotic areas of the heart and a significant reduction in the ratio of plasma GSH to GSSG in aged compared with young MI/R rats.

Besides oxidative damage, ROS also exert a direct inhibitory effect on myocardial function in vivo and play a critical role in the pathogenesis of myocardial stunning (5, 16). In the present study, ROS production was lower in perinecrotic areas of hearts of aged than of young MI/R rats. Furthermore, recent publications from our laboratory indicate that myocardial function is significantly decreased in aged compared with young MI/R rats (28). This decrease in myocardial function in aged rats after MI/R may involve other mechanisms, such as decreased antioxidative capacity and/or increased cardiomyocyte apoptosis (11, 15, 28, 30). A significant decrease in mean arterial blood pressure was observed at 2 and 4 h of reperfusion in aged compared with young MI/R rats. The decreased mean arterial blood pressure may result in a reduction in coronary perfusion pressure and an increased myocardial loss by necrosis and/or apoptosis in aged rats. These changes in blood pressure may impact on the extent of reperfusion, although this cannot be directly assessed and is, thus, a limitation of this work. Moreover, aged rats exhibited a marked reduction in HR at baseline, during ischemia, and during reperfusion compared with young MI/R rats. The decrease in HR in aged rats would not be expected to enhance cardiac injury; however, this remains a subject for future studies.

Elevation in total leukocyte count, e.g., leukocytosis, is a common feature of acute inflammatory reactions. Leukocytosis is possibly caused by the proliferation of leukocyte precursors in the bone marrow and an acceleration of cell release from the bone marrow induced by TNF-α and IL-1β. TNF-α and IL-1β
are the proinflammatory cytokines released early during MI/R, and they play an important role in activation of transcriptional factors, such as activator protein-1 and nuclear factor-κB (14). Activation of transcriptional factors results in an upregulation of the gene expression of adhesion molecules, resulting in the subsequent accumulation of activated neutrophils in the reperfused tissue (7, 12a, 22). Moreover, the expression of antioxidant enzymes is adaptively upregulated by cytokines (e.g., TNF-α and IL-1β) during oxidative stress. Results of the present studies indicate that MI/R induced an increase in Cu/Zn SOD and GR activities in young and aged rats, which illustrates that aging does not abolish this adaptation. However, the increase in Cu/Zn SOD and GR activities in aged MI/R rats is less than that observed in young MI/R rats. Attenuation of antioxidative capacity in aged myocardium after MI/R may be due to the diminished cell signaling (i.e., TNF-α and IL-1β) at the transcriptional level. This is consistent with the report of Edwards et al. (12), who observed an age-related impairment of specific inducible pathways in response to oxidative stress in hearts of aged mice. Specifically, they report that paraquat induced a number of early-response genes, at the transcriptional level.

Fig. 6. A: IL-1β mRNA expression in perinecrotic areas of myocardium at 4 h of reperfusion analyzed by RT-PCR and gel electrophoresis analysis. B: quantified results of RT-PCR of IL-1β by an Image Master VDS program. C: plasma IL-1β at 90 min of reperfusion. Values are means ± SE. *P < 0.05 vs. young and aged sham. #P < 0.05 vs. aged MI/R.

Fig. 7. A: plasma total glutathione (GSH + GSSG) and GSSG in blood samples obtained at 4 h of reperfusion. Plasma was prepared with and without GSH scavenger (2-vinylpyridine). B: glutathione reductase (GR) activity in peri-ischemic areas of hearts. Values are means ± SE. *P < 0.05 vs. young and aged sham. #P < 0.05 vs. aged MI/R.

Fig. 8. Cu/Zn SOD activity in supernatant of perinecrotic areas prepared from hearts obtained at 4 h of reperfusion. Values are means ± SE. *P < 0.05 vs. young and aged sham.
of H2O2 to H2O (8, 26, 27). During this process, GSH becomes equivalents for the glutathione peroxidase-catalyzed reduction is a major antioxidant in human tissues, providing reducing tripeptide (γ-glutamylcysteinylglycine) with a free thiol group, \( \text{H}_2\text{O}_2 \) is, in turn, converted to H2O by GSH. GSH, a major antioxidant in human tissues, providing reducing equivalents for the glutathione peroxidase-catalyzed reduction of H2O2 to H2O (8, 26, 27). During this process, GSH becomes GSSG. GSSG is then recycled to GSH by GR and NADPH. When tissue that has a reduced antioxidant capacity is exposed to increased oxidative stress, the ratio of GSH to GSSG will decrease (11, 26). The ratio of GSH to GSSG is a useful indicator of host antioxidant capacity. In the present studies, the ratio of plasma GSH to GSSG is 1.45 ± 0.17 and 2.69 ± 0.31 in aged and young MI/R rats, respectively. Attenuation of the ratio of plasma GSH to GSSG in aged MI/R rats is primarily caused by a decrease in plasma GSH (Fig. 7), whereas reduction of GR activity in the perinecrotic area of the heart is not significantly different between aged and young MI/R rats. A possible explanation for the observed decrease in the ratio of plasma GSH to GSSG in aged MI/R rats, at a time when GR activities were not markedly decreased, may be related to an age-associated decline in GSH content that is mainly due to downregulation of the synthetic gene expression of glutamate cysteine ligase (29, 38).

Crisz et al. (9) reported that superoxide generation in isolated arterioles was significantly higher in healthy aged than in young rats. However, Boucher et al. (6) and Abete et al. (1) reported evidence that the age-related intolerance to ischemia may stem from impaired antioxidant capacity, rather than increased oxidant generation. These reports tend to agree with our findings of worsened oxidant damage, despite reduced superoxide generation, in aged hearts after MI/R. On the other hand, our data do not show a difference in absolute levels or induction of SOD or GR in aged compared with young hearts. Thus the present data might reflect a greater sensitivity to oxidant damage in aged tissues. Other studies support an age-related increase in oxidant injury, rather than a decline in oxidant status, in older hearts after heavy exercise (2) and enhanced oxidant generation with age (4). The latter tends to oppose the viewpoint that antioxidant capacity is of key importance. Thus some controversy remains, because studies generally support increased oxidant injury with age, yet they support unchanged or reduced antioxidant capacity. These controversial results may be interpreted as follows: many experimental variables affect ROS generation and antioxidative capacity, including the different experimental settings (model of ischemia-reperfusion or other treatment, in vivo and in vitro models), the different periods of ischemia and reperfusion, and ages of the animals. In an in vivo model of MI/R, levels of oxidative stress largely depend on the severity of ischemia and the period of reperfusion, because the major components of inflammation and the sequence of leukocyte extravasation are modulated by many cytokines and adhesion molecules, each of which has its time-dependent expression.

In the present studies, the levels of plasma 8-OHdG significantly increased in aged compared with young rats after MI/R. The major repair pathway for oxidative damage of DNA is the base excision and release of the oxidized base, such as 8-OHdG, into the cytosol. 8-OHdG is a water-soluble compound that eventually diffuses into the plasma (34, 37). An increase in oxidative damage to myocardium not only worsens the myocardial dysfunction, it also enhances myocyte apoptosis (5, 28, 30).

There are limitations to the interpretation of data from the present study. First, activated leukocytes are the major source for generation of extramyocyte ROS, whereas the mitochondria are the source for generation of intramyocyte ROS. The effect of aging on mitochondrial superoxide production requires further study. Second, the intracellular antioxidant systems, including intracellular GSH and mitochondrial Mn SOD, may also play an important role in protection of tissue from oxidative injury. Results of the present studies indicate that oxidative stress may be caused by a deficiency of extramyocyte antioxidant(s) in aged MI/R rats. Future studies should include the effects of aging on intramyocyte antioxidant systems. Finally, recent work from our laboratory indicates that aging increased the ratio of Bax to Bcl-2 and enhanced myocyte apoptosis in the rat model of MI/R (21). Control mechanisms for the apoptosis-signaling pathway and its relation to oxidative damage of DNA require further study.

![Fig. 9. Plasma 8-hydroxy-2′-deoxyguanosine (8-OHdG) levels (A) and creatine kinase (CK) activity (B). MI/R significantly increased plasma 8-OHdG levels and CK activity at 4 h of reperfusion in aged rats vs. young rats. Values are means ± SE. *P < 0.05 vs. young and aged sham. *P < 0.05 vs. aged MI/R.](http://ajpheart.physiology.org/)
The constantly increasing aging population in this century requires a substantial investment and increase in knowledge to effectively prevent and manage common age-related disease. Our results indicate that enhanced tissue damage in the aged myocardium is caused by an attenuation of antioxidative capacity, rather than an increase in ROS production, after MI/R. These findings indicate that antioxidative capacity plays a critical role in protection of the myocardium from reperfusion injury and suggest a new concept for therapeutic intervention in patients with ischemic heart disease, particularly the elderly.

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