METALLOTHIONEIN (MT) is a highly conserved, low-molecular-weight, thiol-rich protein. The mammalian MT has 61 amino acids, including 20 cysteine residues but no aromatic amino acids or histidine or leucine (10). The basal level of MT in biological systems is very low, although it may vary with age and type of tissue. However, this protein is induced to a significant high level when the system is challenged by heavy metals, starvation, heat, inflammation, or other stress conditions (11). Because MT can both bind to and be induced by heavy metal ions, it is generally agreed that MT is somehow involved in metal metabolism and toxicity (27). Some early studies (1, 28, 29) have suggested that MT also plays a role in the scavenging of free radicals, which are produced under various stress conditions. Zinc-MT has been shown to scavenge hydroxyl radical in vitro and to be more effective than glutathione in preventing hydroxyl radical-induced DNA degradation (1).

The role of MT in cardiac protection against oxidative injury has been demonstrated with adriamycin (Adr), an important anticancer drug which causes heart damage. Preinduction of MT in mouse hearts by bismuth subnitrate significantly inhibited Adr-induced lipid peroxidation (24). Zinc, cadmium, cobalt, or mercury also induced MT production in the heart and suppressed the oxidative heart damage (24). In addition, pretreatment with these MT inducers was necessary to protect mice from lethal doses of Adr, their coadministration with the drug having no effect (19). More convincing evidence to demonstrate the importance of MT in cardiac protection against Adr toxicity was obtained from our recent studies using a transgenic mouse model, in which MT was overexpressed specifically in the heart (14). Adr-induced morphological changes in the myocardium and creatine kinase (CK) release from the heart were significantly inhibited in the MT-overexpressing transgenic mouse heart (14).

Ischemia-reperfusion causes depressed myocardial function and associated deleterious morphological alterations that lead to heart failure and cell death (3). Mechanisms by which this injury occurs are not well defined. Studies using antioxidants such as superoxide dismutase (SOD) and catalase suggest that oxidative stress and burst of free radical production are important mediators of the myocardial damage (8). The available evidence at present indicates that reperfusion arrhythmias and myocardial stunning result at least in part from oxygen radicals (4, 17). Myocardial infarction or cell death may also relate to oxygen radicals (26).

Because MT functions in protection against environmental toxic insults, particularly oxidative damage, in multiple organ systems including the heart, it is possible that MT also provides protection against ischemia-reperfusion injury in the heart. To test this hypothesis the present study was undertaken to determine whether elevation of MT in the heart of transgenic mice makes this organ resistant to ischemia-reperfusion injury.

MATERIALS AND METHODS

Animals and treatment. FVB mice, without regard to sex because our preliminary studies showed no significant difference in cardiac response to ischemia-reperfusion between sexes, were housed in quarters maintained at 22–24°C with a 12-h light-dark cycle. They were given free access to rodent chow and deionized water. Detailed descriptions for development and characterization of the cardiac MT-overexpressing transgenic mouse lines were reported previously (14). The transgenic founder mice were inbred with nontransgenic mice of the same strain. The resultant litters were analyzed by the PCR procedure using the genomic DNA isolated from 1-cm tail clippings from 3- to 4-wk-old mice. Transgenic positive mice (heterozygotes) and negative littermates were
then used for experiments. All animal procedures were approved by the Institutional Animal Care and Use Committee, which is certified by the American Association of Accreditation of Laboratory Animal Care.

Langendorff-perfused mouse heart. The MT transgene-positive and -negative mice (7 wk old) were brought to the Langendorff perfusion operative room by animal care personnel; the experimental operator was unaware of the treatment and transgenic status of these animals. The animals were anesthetized by intraperitoneal injection of pentobarbital sodium (150 mg/kg) coadministered with 100 IU of heparin. The heart was isolated according to a described procedure (2). To reduce injury to the heart, modifications of the procedure were made as will be described below. Once the mouse was deeply anesthetized, the chest was opened and the descending aorta was separated in situ and cannulated with a 20-gauge flanged stainless steel cannula, which was connected to a peristaltic pump (Rainin Rabbit Plus, Woburn, MA) set at a flow rate of 500 µl/min. An incision was made in the inferior vena cava to allow retrograde perfusion of the heart. The flow rate was then accelerated to 3 ml/min. The innominate artery, common carotid artery, and left subclavian artery were tightly sutured and disconnected. The heart was then removed and trimmed while being continuously perfused. The cannula was attached to a fixed arm of the perfusion rig. The perfusion buffer contained (in mmol/l) 120 NaCl, 28 NaHCO3, 4.63 KCl, 1.17 KH2PO4, 1.25 MgCl2, 1.25 CaCl2, and 8 glucose. The solution was adjusted to pH 7.4, prefiltered to particle size of <0.22 µm, and equilibrated with 95% O2-5% CO2.

The temperature of the heart was maintained at 37°C by warming the perfusion buffer by means of a water-jacketed chamber and coil. After 30 min of equilibration, the heart was made ischemic by turning off the buffer flow for 50 min. During ischemia the temperature of the heart was maintained by lowering the heart into an organ bath containing the same perfusion buffer at 37°C. The heart was then reperfused with the perfusion buffer (3 ml/min) for 60 min. Mechanical activity was measured throughout each perfusion experiment. Briefly, apicobasal displacement was obtained by attaching a strain gauge transducer (FT.03, Grass Instruments, W. Warwick, RI) to the heart apex. The transducer was positioned to yield an initial resting tension of 0.3 g. Mechanical activity was recorded on a polygraph (model TA 2000, Gould, Cleveland, OH). Both heart contractile force and heart rate were recorded. The contractile force was expressed as a percentage of developed contractile force (obtained at any time point as a percentage of the force measured during the preischemia period).

CK analysis. CK release from the heart was measured in the effluent buffer. Samples were collected during the last minute of preischemia, and at 1, 2, 3, 5, 10, 20, 30, and 60 min of reperfusion. CK activity was determined by the spectrophotometric method of Oliver (21) by using a CK kit from Sigma Chemical. CK release was then expressed as activity (IU) per minute per gram wet weight of the heart.

Assessment of amount of infarction. After reperfusion for 90 min postischemia, the heart was lowered into the organ bath and a 10% (wt/vol) triphenyltetrazolium in phosphate buffer (88 mM Na2HPO4 and 1.8 mM NaH2PO4) was infused into the coronary vasculature through the side-arm of the aortic cannula. Tetrazolium was infused until the surface of the heart was discolored (~1 ml). Then the heart was preserved in 10% Formalin for 10 h. The fixed heart was cut into five slices. The slices were oriented caudal surface upward and compressed, together with a calibration grid, between two Plexiglas plates separated by a 0.57-mm space. The basal surface of each slice was photographed. Infarction size from gross photographs of the tetrazolium-stained slices was measured by projecting 0.57-mm transparencies of the basal surface of each slice on a screen at a magnification of ×10 and measuring the stained and unstained areas by use of a planimeter. The procedure of this measurement described previously (15) was closely followed.

Measurement of tissue MT. Total tissue MT concentrations were determined by the cadmium-hemoglobin affinity assay (6). Briefly, tissues were homogenized in four volumes of 10 mM Tris-HCl buffer, pH 7.4, at 4°C. After centrifugation of the homogenate at 10,000 g for 15 min, 200 µl of supernatant were transferred to microtubes for MT analysis as described previously (6). MT concentration in the heart was expressed as micromgram per gram tissue.

Statistical analysis. All values are expressed as means ± SD (n = 5). A repeated-measurements and multiple-comparisons method described by Ludbrook (16) was applied to analyze the data presented in Figs. 1 and 2. Multiple vertical pairwise contrasts with the Dunn-Sidak adjustment were used to make the comparisons within the same time point. The effect of MT transgene on baseline heart contractile force and contraction rate was examined by two-way ANOVA between time 0 and time 30 during the 30-min preischemia equilibration period. Student’s two-tailed, paired t-test was used to analyze the data presented in Fig. 3. P < 0.01 was considered significant.

RESULTS

Suppression of ischemia–reperfusion injuries in MT-overexpressing transgenic mouse heart. As reported before (14), MT was overexpressed specifically in the heart of transgenic mice. The cardiac MT concentrations in the transgenic mice used for this study were 55.7 ± 6.2 µg/tissue, ~10-fold higher than that in the nontransgenic control mice (5.9 ± 0.5 µg/tissue). The
contractile force data for isolated hearts subjected to Langendorff ischemia-reperfusion are shown in Fig. 1. There was no significant difference in the developed contractile force between the transgenic and control hearts during the 30-min equilibration period, 0.55 ± 0.08 and 0.56 ± 0.06 g, respectively. Myocardial contractile force for each mouse heart during ischemia and reperfusion was expressed as the percentage of the developed contractile force just before ischemia. Developed contractile force at the beginning of ischemia in both groups increased and then fell to zero by 10 min of ischemia. There was no significant difference in tension between transgene-positive and transgene-negative hearts during ischemia. The hearts from the transgene-positive mice showed significantly better postischemic recovery of the suppressed contractile force (P < 0.01). The changes in the heart rate between transgene-positive and -negative hearts were not significantly different (data not shown).

CK activity in the collected perfusion effluent samples was measured. This activity from the samples during the preischemic period was undetectable in either group. On reperfusion postischemia a high CK activity was detected in the effluent collected from the transgene-negative mouse hearts (Fig. 2). A marked reduction in the CK activity in the effluent collected from the transgenic mouse hearts was observed, especially during the first 5 min of reperfusion. The peak values of CK activity in the effluent samples were 0.62 ± 0.12 and 0.16 ± 0.05 IU·min⁻¹·g tissue⁻¹ (P < 0.01) for the transgene-negative and -positive hearts, respectively.

Reduction in myocardial infarct size in MT transgene-positive heart. The gross slices from both experimental groups showed that infarct and noninfarct areas were clearly discernible. The staining patterns of slices from transgene-positive and transgene-negative hearts subjected to 50 min of ischemia and 90 min of reperfusion were compared. Large continuous infarct zones were observed in the endocardial area that were connected to epicardial infarction zones in the transgene-negative mouse heart. However, only small scattered infarct zones were observed in the endocardial area in the transgene-positive heart. Analysis of slices from all the hearts indicated that the total volume of myocardial infarction was significantly greater in the transgene-negative hearts than in the transgene-positive hearts (Fig. 3).

DISCUSSION

The results obtained from this study demonstrate for the first time that MT provides myocardial protection from ischemia-reperfusion injury. Myocardial ischemia occurred when the perfusion flow rate of the Langendorff-perfused heart was zero. The myocardial oxygen demand therefore exceeded oxygen supply under this condition. This situation resulted in cell injury as shown by the high CK activity measured in the effluent immediately after reperfusion and the repression of the contractile force. Reperfusion of the ischemic myocardium restores oxygen and also produces another form of myocardial damage, termed “reperfusion injury” (5). In the present study we have observed that MT functions in protection against both ischemia- and reperfusion-induced damage. It improved the recovery of the suppressed contractile force postischemia and inhibited CK release from the ischemic myocardium on reperfusion. It also reduced the size of the infarction zone produced by the cycle of ischemia and reperfusion.

Myocardial damage induced by ischemia-reperfusion has been proposed to be caused at least in part by the generation of reactive oxygen species (18). However, direct evidence to support the role of free radicals in

![Fig. 2. Creatine kinase (CK) activity in effluent buffer of hearts isolated from MT-TG mice and nontransgenic controls. CK was measured at last minute of preischemic period (0 min) and during 60-min of reperfusion. Each point represents mean ± SD of 5 animals. *P < 0.01.](#)

![Fig. 3. Comparison of myocardial infarct size measured from nontransgenic controls and MT-TG mouse hearts. Myocardial infarction caused by 50 min of global ischemia and 90-min reperfusion was delineated by tetrazolium staining as described in MATERIALS AND METHODS. Data presented are means ± SD values from 5 mouse hearts of each group. *P < 0.01.](#)
this myocardial injury has not been obtained. Most supporting findings have been the cardioprotective effects of agents capable of inducing antioxidants such as glutathione peroxidase (23) and SOD (7) and from the beneficial effects of supplementing antioxidants in vivo or in vitro (22). It is difficult to interpret these experimental findings because the inducers may not necessarily affect the status of only one or two antioxidant systems. If antioxidants are supplemented in vivo, it is impossible to maintain constant plasma antioxidant concentrations and to accurately predict the target tissue concentrations. Importantly, high-molecular-weight antioxidants such as SOD, glutathione peroxidase, catalase, and MT are unlikely to be transported into intracellular compartments. To overcome the shortcomings of these earlier studies, we produced the unique transgenic mouse model in which MT was specifically overexpressed in the heart. As described previously (14), MT was constitutively overexpressed only in the heart of the transgenic mice. Other antioxidant systems were found to be unaffected in the MT-overexpressing heart.

In our previous studies we have produced transgenic mice in which cardiac catalase was overexpressed (13). Catalase, like MT, was found to suppress ischemia-reperfusion-induced myocardial functional and morphological alterations (15). However, the required level at which catalase effectively functions in this cardioprotection was much higher (60- to 100-fold higher than normal) than that of MT (~10-fold in the present study) to provide the same extent of protection. Under physiological conditions, catalase is highly localized in peroxisomes and reacts with hydrogen peroxide to produce water and molecular oxygen. Although we do not know the subcellular localization of the catalase expressed from the transgene, we have observed increased numbers of peroxisomes in the catalase overexpressing transgenic myocardium (13). This would suggest that a significant amount of the elevated catalase was contained in peroxisomes. This localization thus diminishes the reaction of catalase with extraperoxisomal hydrogen peroxide. In contrast, the cytosolic localization of MT would make it more readily available to react with reactive oxygen species generated under oxidative stress. In vivo studies have shown that MT is the most effective and potent scavenger of hydroxyl radicals (1, 28, 29), which are the most potent of the reactive oxygen species. In agreement with these in vitro findings, the results obtained from the present study strongly suggest that MT is more potent than catalase in protection against myocardial oxidative injuries.

The results obtained from this study would have implications in the setting of cardiac surgery and heart transplantation. It also would be of significance in other cardiac diseases related to oxidative stress. In the United States acute myocardial infarction is the most common single cause of death in humans. The treatment of this condition has been significantly improved by procedures allowing rapid return of blood flow to jeopardized myocardium (9). However, if a prolonged coronary occlusion results in severe myocardial infarction, the efficacy of these procedures significantly diminishes (9, 11). Therefore, any intervention that could delay the onset of acute infarction would benefit current therapies.

MT may provide an alternative approach to this clinical problem. MT induction in the heart has been shown in bismuth-treated mice (19) and the elevated level was high enough to render the heart resistant to Adr-induced oxidative injury. It is possible that bismuth-elevated MT concentrations also protect the heart from ischemia-reperfusion injury. Alternatively, gene therapy approach may be eventually applied to the heart, and MT would be such a candidate.

The results obtained from the present study clearly define the role of MT in cardiac protection against ischemia-reperfusion injury. The regulation of MT expression has been well studied, and several agents have been identified to selectively elevate MT levels in the heart, such as bismuth nitrate (19), isoproterenol (20), and tumor necrosis factor-α (25). Therefore, the basis for developing pharmaceutical agents to increase MT concentration in the heart has already existed. Exploring the potential for MT protection against cardiac ischemia-reperfusion injury would therefore likely result in novel approaches to myocardial ischemic disease and would eventually be beneficial to the patient.

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