Metallothionein-overexpressing neonatal mouse cardiomyocytes are resistant to H$_2$O$_2$ toxicity

GUANG-WU WANG, DALE A. SCHUSCHKE, AND Y. JAMES KANG

Department of Medicine, Applied Microcirculation Research Center, and Department of Pharmacology and Toxicology, University of Louisville School of Medicine, Louisville, Kentucky 40292

Wang, Guang-Wu, Dale A. Schuschke, and Y. James Kang. Metallothionein-overexpressing neonatal mouse cardiomyocytes are resistant to H$_2$O$_2$ toxicity. Am. J. Physiol. 276 (Heart Circ. Physiol. 44): H167–H175, 1999.—To study cellular and molecular events of cardiac protection by metallothionein (MT) from oxidative injury, a primary neonatal cardiomyocyte culture was established from a specific cardiac MT-overexpressing transgenic mouse model. Ventricular cardiomyocytes were isolated from 1- to 3-day-old neonatal mice and cultured in an Eagle’s minimum essential medium supplemented with 20% fetal bovine serum under an atmosphere of 5% CO$_2$-95% air at 37°C. Forty-eight hours after plating was completed, the purity of such cultures was 95% myocytes, assessed by an immunocytochemical assay. Over 80% of the cardiomyocytes beat spontaneously on the first day of culture and synchronously in a confluent monolayer after the sixth day of culture. Cellular MT concentrations in the transgenic cardiomyocytes before culturing and on the sixth day postculturing were about seven- and twofold higher than nontransgenic controls, respectively. However, there were no significant differences in cell morphology, glutathione content, and antioxidant enzymatic activities between these two types of cardiomyocytes. When these cells were challenged by H$_2$O$_2$, the transgenic cardiomyocytes displayed a significant resistance to the toxic effect of this oxidant, as measured by cell viability, lactate dehydrogenase leakage, and morphological alterations. In addition, the transgenic cells were highly protected from H$_2$O$_2$-induced lipid peroxidation. These observations demonstrate that MT protects the cultured cardiomyocytes from H$_2$O$_2$ toxicity by preventing its interaction with macromolecules such as lipids, and this cultured primary neonatal mouse cardiomyocyte system provides a valuable tool to directly study cellular and molecular events of MT in cardiac protection against oxidative injury.

METALLOTHIONEIN (MT) is a highly conserved, low-molecular-weight, thiol-rich protein, which has been widely studied since its discovery in 1957 (18). Because MT can both bind to and be induced by heavy metals, there is general agreement that MT is somehow related to metal metabolism and toxicity (11). Recent studies have shown that MT also plays a role in the scavenging of free radicals (31). Zinc-MT has been shown to scavenge hydroxyl radicals in vitro and is more effective than glutathione (GSH) in preventing hydroxyl-radical-induced DNA degradation (1). In cultured HL-60 cells, MT was induced by zinc and was shown to protect the cells from H$_2$O$_2$ toxicity (26). Furthermore, the thiolate groups in the MT fraction reacted more preferentially with H$_2$O$_2$ than the other pools of sulfhydryl residues, which include those in GSH and high-molecular-weight proteins (26).

The role of MT in cardiac protection against oxidative injury has been demonstrated with doxorubicin, an important anticancer drug that causes heart damage. Preinduction of MT in the myocardium of mice by bismuth subnitrate significantly decreased the products of doxorubicin-induced lipid peroxidation (27). The decreased drug toxicity paralleled the level of cardiac MT. Importantly, pretreatment with bismuth subnitrate is necessary to protect mice against lethal doses of doxorubicin, its coadministration with the drug having no effect (20). Zinc, cadmium, cobalt, or mercury also induced MT production in the heart and suppressed the reactive oxygen-induced heart damage (27).

In our recent studies, we have produced a transgenic mouse model in which cardiac MT was specifically overexpressed (14). Using this unique experimental model, we demonstrated that doxorubicin-induced morphological changes in the myocardium and creatine kinase release from the heart were significantly inhibited in the MT-overexpressing transgenic mice (14). However, a study using transgenic mice in which MT was overexpressed in multiple organs, including the heart, has shown that MT did not provide protection against doxorubicin cardiotoxicity (4). This controversial result indicates that further studies to directly examine the effect of MT on cardiac protection against oxidative injury are necessary. To this end, a primary cardiomyocyte culture system would be desirable. It can offer two important advantages for the required studies: 1) experiments can be done with a homogeneous cell population, and 2) precise manipulation of the composition of the extracellular environment can be applied, being devoid of systemically metabolic, hormonal, and neuronal influences.

Ventricular cardiomyocytes isolated from neonatal animals such as rats have been used in studies of the cellular and molecular aspects of cardiac alterations induced by oxidative stress, such as ischemia and reperfusion (7, 10, 13, 24, 30, 35). The advantages of using neonatal cardiomyocyte cultures include 1) they are easy to be cultured relative to adult cardiomyocytes (35), and 2) the phenotype of cultured neonatal cardiomyocytes is highly stable (36). For example, their contractile profile during hypoxia-reoxygenation is highly compatible with that of in situ hearts during ischemia-reperfusion, whereas the phenotypical change

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of isolated adult cardiomyocytes is quite different from that of in situ hearts (36). Surprisingly, such an experimental procedure for culturing ventricular cardiomyocytes from the neonatal mouse heart has not been described. This procedure would be very valuable for studying molecular mechanisms of cardiac manipulation using the mouse model. Particularly, mouse genetics and its gene regulation have been much better understood, and more transgenic mouse models for cardiac research are continually available. Establishment of a neonatal mouse cardiomyocyte culture system would thus greatly complement the use of transgenic mice in cardiological studies. Therefore, this study was undertaken to develop an experimental procedure for culturing ventricular cardiomyocytes from neonatal mice. A primary cardiomyocyte culture system was established and characterized from a specific cardiac MT-overexpressing transgenic neonatal mouse model. When these cells were challenged by \( \text{H}_2\text{O}_2 \), a significant resistance to the toxic effect of this oxidant was observed.

### MATERIALS AND METHODS

#### Animals

FVB mice obtained from the University of Louisville Research Resources Center were housed in the animal quarters maintained at 22°C with a 12:12-h light-dark cycle. They were given free access to rodent chow and deionized water. Transgenic mice overexpressing MT specifically in the heart were produced from the FVB strain. Detailed descriptions for development and characterization of these transgenic mouse lines were reported previously (14). These animals were identified by a pigment marker (dark eye and fur) at birth. The resultant transgenic litters were produced from a specific strain. The resultant transgenic mouse model. When these cells were challenged by \( \text{H}_2\text{O}_2 \), a significant resistance to the toxic effect of this oxidant was observed.

#### Materials

- **MEM** and **DMEM** without phenol red, fetal bovine serum (FBS), and trypsin were purchased from Gibco (Life Technologies, Grand Island, NY). The biocinchonic acid (BCA) protein assay reagents were obtained from Pierce (Rockford, IL).
- 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma (St. Louis, MO). All other chemicals were purchased from either Fisher, Sigma, or Aldrich (Milwaukee, WI).

#### Neonatal Mouse Primary Cardiomyocyte Culture

A new procedure for culturing ventricular cardiomyocytes from neonatal mouse was established by modifications of the methods used for neonatal rat (8, 17) and fetal mouse (9, 21) cardiomyocyte cultures. One- to three-day-old neonatal transgenic or nontransgenic mice were euthanized by cervical dislocation. Hearts from the mice were removed aseptically, retaining the ventricles only, and kept in Hanks’ balanced salt solution (HBSS) without \( \text{Ca}^{2+} \) and \( \text{Mg}^{2+} \). After centrifugation at 10,000 \( g \), the cells were resuspended in the HBSS-MEM (MEM supplemented with 20% FBS (vol/vol), 100 U/ml penicillin, and 100 \( \mu \)g/ml streptomycin). To exclude nonmuscle cells, the isolated cells were first plated in tissue culture dishes at 37°C for 2 h under a water-saturated atmosphere of 5% \( \text{CO}_2 \)-95% \( \text{air} \) (as a 2-h preculture control) based on the observation that nonmuscle cells attach to the substrata more rapidly (25). The suspended cells were then collected and plated at a density of \( 1.0 \times 10^5 \) cells/cm² and incubated under the same conditions as above.

#### Immunocytochemical Staining

Myocyte purity was monitored by staining with an antibody to cardiac \( \alpha \)-sarcomeric actin according to the manufacturer’s instructions (Sigma). Briefly, myocytes were plated on plastic four-chamber culture slides at \( 1.0 \times 10^5 \) cells/cm². The cells were fixed with 50% methanol and 50% acetone at -20°C and then incubated with an antigen-specific primary antibody [mouse monoclonal anti-\( \alpha \)-sarcomeric-actin antibody (1:500 diluted)]. After a brief wash, the myocytes were incubated with biotinylated secondary antibody (goat antimouse IgM, \( \mu \)-chain specific). At the addition of an ExtrAvidin peroxidase reagent, a stable avidin-biotin complex is formed with the bound biotinylated antibody. The sites of antibody deposition were visualized by the addition of freshly prepared substrate that contains \( \text{H}_2\text{O}_2 \) and the chromogen 3-amin-9-ethyl-carbazole. Nuclei were stained with Mayer’s hematoxylin. Myocytes were visualized under a light microscope (Zeiss, Germany).

#### Morphological Observations

Morphological observations of the cultured cardiomyocytes were performed with an inverted phase-contrast microscope (Zeiss). Photomicrographs were taken at 70-fold magnification using a 35-mm Cannon camera attached to the microscope.

#### Cellular MT Concentration

Total MT was determined by the cadmium-hemoglobin affinity assay (5). Cells were harvested after 2 h of preculture and on the sixth day of postculturing. The cells were rinsed with 5 ml cold PBS and centrifuged at 2,000 g for 10 min, and 500 \( \mu \)l of 10 mM Tris·HCl were added to the pellet. The cells were then pulse-sonicated on ice with a Fisher Sonic Dismembrator (model 60, Fisher Scientific) at an output power of 8 for 15 s and repeated for three times with a 30-s interval. After centrifugation at 10,000 g for 15 min, 200 \( \mu \)l of supernatant were transferred to microtubes for MT analysis, and another 100 \( \mu \)l of supernatant were transferred to separate microtubes for total protein concentration determination using the Pierce BCA protein assay reagents as described by Smith et al. (28), using bovine serum albumin (BSA) as the standard.
Cellular GSH Concentration

Total GSH was determined by a 5,5′-dithiobis(2-nitrobenzoic acid)-GSSG reductase recycling assay described by Tietze (32). After we harvested the cells at the second hour of preculture and on the sixth day of postculture, we rinsed the cells with 5 ml cold PBS, centrifuged the cells at 2,500 g for 10 min, and added 500 µl of 5% sulfosalicylic acid to the pellet. The cells were sonicated for exactly 2 s, transferred to microtubes, and centrifuged at 1,000 g for 20 min. Supernatants were then collected for GSH assay, and 0.5 ml of 0.1 N NaOH was added to the pellet for protein content determination.

Cellular Enzymatic Assays

Cellular enzyme extracts were prepared as previously described (12). The media were removed from the dishes, and the cells were rinsed with cold PBS, harvested with trypsin-EDTA (0.1% trypsin, 0.53 mM EDTA-Na4), and resuspended in cold PBS containing 0.1% BSA. After centrifugation at 1,000 g for 15 min, the supernatant was discarded, and cells were resuspended in 5 ml cold PBS without BSA and then centrifuged at 1,000 g for 15 min. Cells were resuspended in respective enzyme buffer and lysed by sonication with the model 60 Fisher Sonic Dismembrator at an output power of 8 for 15 s and repeated for three times with a 30-s interval. After centrifugation at 12,000 g for 45 min, the supernatant was collected for respective enzyme assay and protein analysis.

Catalase. The enzyme activity was determined by the method described by Aebi (2). The assay buffer was 50 mM KH2PO4-50 mM NaH2PO4 (1:1.5, pH 7.0). In a cuvette, a 1.0-ml sample was mixed with 1.0 ml of the buffer. The reaction was initiated by adding 1.0 ml of 30 mM H2O2, and the change at 340 nm was monitored at 25°C for 1.0 min. Enzyme activity was calculated as described by Nelson and Kiesow (22). Specific activity is expressed as moles of H2O2 per minute times milligram of protein.

GSH peroxidase. The enzyme activity was determined by the method described by Flohe and Gunzler (6). The assay buffer was 0.1 M KH2PO4 (pH 7.0) containing 1.0 mM EDTA. In the assay tubes, 400 µl of buffer containing 2 mM sodium azide, 100 µl GSH (10 mM), 100 µl GSH reductase (2.4 U/ml), and 20 µl samples were incubated for 10 min in a 37°C water bath, and then 100 µl of NADPH (1.5 mM) were added, and the reactive mixture was transferred to cuvettes. Absorbance at 340 nm was monitored at 37°C for 3 min before 100 µl of prewarmed H2O2 (1.5 mM) were added, followed by an additional 5 min of monitoring under the same conditions. Specific activity is expressed as nanomoles of NAPDPH per minute times milligram of protein.

GSH reductase. The enzyme activity was determined by the method described by Carlberg and Mannervik (3). The assay buffer was 0.2 M KH2PO4 (pH 7.0) containing 2 mM EDTA. In a 1.0-ml cuvette, 0.5 ml buffer, 200 µl distilled and deionized H2O, 50 µl GSSG (20 mM), and 50 µl NADPH (2 mM) were combined. After the addition of the 200-µl sample, the change in absorbance at 340 nm was monitored at 37°C for 2 min. Specific activity is expressed as nanomoles of NAPDPH per minute times milligram of protein.

Superoxide dismutase (SOD). Total SOD activities were determined by the method described by Spitz and Oberley (29) with some modifications. The following compounds were added to a 1.0-ml cuvette: 50 mM potassium phosphate buffer, 1.0 mM diethyl enetriamine nponenta-acetic acid, 1.0 U catalase, 5.6 × 10−5 M nitro blue tetrazolium, 0.1 mM xanthine, 0.05 mM BCS, 0.13 mg/ml BSA, 5 mM NaCN, and 50 µl xanthine oxidase (0.02 U/ml). The production of formazan blue was monitored at 560 nm. Bovine liver Cu-Zn-SOD was used as the standard. Specific activity is expressed as units per milligram of protein.

Determination of H2O2 Cytotoxicity

The cytotoxicity of H2O2 was determined by examining morphological alterations of the cardiomyocytes, measuring lactate dehydrogenase (LDH) release from the cells, and monitoring cell viability. To observe cell morphological alterations, 6-day-old cultures were treated with 25, 50, 100, 250, and 500 µM H2O2. At the time of H2O2 exposure, the PBS MEM was removed and replaced with fresh serum-free MEM containing the desired concentrations of H2O2. Toxicities were evaluated 30, 60, and 120 min after the primary cell cultures were treated with H2O2. A Zeiss inverted phase-contrast microscope was used to observe cell morphology by the methods previously described by Melchert et al. (19). Photomicrographs were taken at a ×70 magnification using a 35-mm Canon camera attached to the microscope. Morphological alterations were classified as 1) pseudopodia: extension or retraction of the cell membrane; 2) vacuoles: the appearance of clear inclusion bodies of cytoplasmic materials; or 3) granules: the appearance of dark granular materials. Gross cellular morphological alterations were evaluated with a grading scale: NC, no obvious changes; +, minimal alterations; ++, intermediate alterations, and +++ extensive alterations.

The activity of cytoplasmic enzyme LDH that was released into the culture media was determined by the method of Wroblewski and LaDue (34). On the sixth day of culturing, cardiomyocytes were treated with the same concentrations of H2O2 and followed the same protocol as described for the morphological examination. After 60 min of treatment with H2O2, a 100-µl sample from the culture media was collected and the LDH activity was assayed in 2.4 ml of phosphate buffer (0.1 M, pH 7.4) with 100 µl of Na pyruvate (2.5 mg/ml phosphate buffer) and 100 µl of NADH (2.5 mg/ml phosphate buffer). The rate of NADH oxidation was determined by following the decrease in absorbance at 340 nm at 25°C using a spectrophotometer.

Cell viability was determined by a short-term microculture MTT assay (33). On 96-well microplates, 2.5 × 104 cells/well were incubated in 100 µl of culture media for 48 h. The cells were then exposed to different concentrations of H2O2 for 60 min. The media containing H2O2 were replaced by fresh media without H2O2, and the cells were incubated for another 48 h. The media were then removed again and replaced by 90 µl of DMEM (containing no phenol red or FBS) and 10 µl of MTT solution (2 mg/ml phosphate buffer) for 4 h. After the DMEM and MTT solutions were removed, the remaining formazan blue crystals were dissolved in 75 µl/well of a 0.04 N HCl-isopropyl alcohol mixture. Absorbance at 540 nm was measured using a Bio-Tek Instruments model EL 311 microplate reader.

Measurement of Lipid Hydroperoxide

Lipid peroxidation is traditionally quantified by measuring malondialdehyde and 4-hydroxy nonenal. These assays are nonspecific and often lead to misestimation of lipid peroxidation. A new Cayman Chemical (Ann Arbor, MI) lipid hydroperoxide assay kit measuring the hydroperoxide concentration directly utilizing the redox reactions with ferrous ions was therefore used. Cardiomyocytes were cultured for 6 days and exposed to different concentrations of H2O2 for 60 min. The cells were then harvested for determination of lipid hydroperoxide concentrations. The extraction procedure and the measurement of the extracted lipid hydroperoxides followed the instruction provided by Cayman Chemical.
Statistical Analysis

Data were analyzed initially by one-way ANOVA. Scheffé's F-test was employed for further determination of the significance of differences. Differences between MT-overexpressing transgenic cardiomyocytes and nontransgenic controls were considered significant at $P < 0.05$. The data are presented as means ± SD values from triplicate cultures for each treatment.

RESULTS

Primary Cardiomyocyte Culture

Both transgenic and nontransgenic neonatal mouse cardiomyocytes were isolated and cultured separately. It was noted that the animal age should not be more than 3 days old because cardiomyocytes obtained from neonatal mice older than 3 days did not attach to the dishes well (data not shown). After separation from nonmuscle cells by preculturing for 2 h, the cardiomyocytes were replated at a density of $1.0 \times 10^5$ cells/cm$^2$ and incubated under the condition described in the MATERIALS AND METHODS. Twenty-four hours postculturing, almost all the cardiomyocytes attached and spread on the substrata of the dishes and beat spontaneously. The culture media were changed at this time and subsequently changed every 3 days. Forty-eight hours after culturing was completed, the myocyte purity was determined by the immunocytochemical assay described in the MATERIALS AND METHODS. The result showed that the myocyte purity averaged 94 ± 5%. The contractile profile of the cardiomyocytes was observed on day 1, 2, 3, 4, 5, 6, 9, and 12 after culturing. The spontaneous beating of these cells continued, and a synchronous beating was observed on the sixth day when a confluent monolayer was formed and thereafter. The morphological characteristics of the cultured cardiomyocytes are shown in Fig. 1. There are no
Table 1. MT concentrations in neonatal mouse heart and isolated cardiomyocytes

<table>
<thead>
<tr>
<th>MT Concentration</th>
<th>Control</th>
<th>MT-TG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart tissue, µg/mg protein</td>
<td>0.49 ± 0.06</td>
<td>20.93 ± 2.80*</td>
</tr>
<tr>
<td>Precultured cardiomyocytes, µg/mg protein</td>
<td>0.45 ± 0.19</td>
<td>3.45 ± 0.38*</td>
</tr>
<tr>
<td>Cultured cardiomyocytes, µg/mg protein</td>
<td>0.44 ± 0.09</td>
<td>1.01 ± 0.13*</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = 3–6 mice. *P < 0.05. MT, metallothionein; TG, transgenic mouse hearts.

Table 2. GSH concentrations and antioxidant enzymatic activities in MT-overexpressing transgenic cardiomyocytes and nontransgenic controls

<table>
<thead>
<tr>
<th></th>
<th>Preculture</th>
<th>6-Day Culture</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>MT-TG</td>
</tr>
<tr>
<td>GSH, nmol/mg protein</td>
<td>0.40 ± 0.05</td>
<td>0.45 ± 0.22</td>
</tr>
<tr>
<td>GSH peroxidase, nmol NADPH⋅min⁻¹⋅mg protein⁻¹</td>
<td>24.08 ± 5.82</td>
<td>28.35 ± 4.29</td>
</tr>
<tr>
<td>GSH reductase, nmol NADPH⋅min⁻¹⋅mg protein⁻¹</td>
<td>16.72 ± 3.39</td>
<td>14.25 ± 3.12</td>
</tr>
<tr>
<td>Catalase, µmol H₂O₂⋅min⁻¹⋅mg protein⁻¹</td>
<td>69.33 ± 40.27</td>
<td>85.56 ± 19.66</td>
</tr>
<tr>
<td>SOD (total), U/mg protein</td>
<td>3.43 ± 1.35</td>
<td>3.76 ± 1.46</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = 3–6 mice. GSH, glutathione; SOD, superoxide dismutase. *P < 0.05.

Cytotoxicity of H₂O₂

Both transgenic and nontransgenic cardiomyocytes were challenged by H₂O₂. The cytotoxicities of this oxidant in these two types of cells were compared. Morphological alterations, LDH leakage, and cell viability were measured as the determination of cytotoxicity. As shown in Fig. 2, a dramatic difference in the morphological changes induced by H₂O₂ between the transgenic and the nontransgenic cardiomyocytes was observed. H₂O₂ induced both concentration- and time-dependent injurious effects on both transgenic and nontransgenic cells. However, the damage to the nontransgenic cells was much more severe than the damage to the transgenic cells (Table 3).

LDH leakage was measured to indicate alterations in the integrity of the sarcolemmal membrane. This measurement was done by assessing the LDH activities in media collected from both transgenic and nontransgenic cardiomyocyte cultures at different time points. The remaining LDH activities in the cells were also measured using the cell homogenates collected from the cultures on the ninth day postculturing. There are no significant differences in the LDH activities measured in the media collected from the cultures between the two types of cells at any time point (data not shown). The LDH activities that remained in the cells are not significantly different either, being 237.9 ± 70.2 and 226.5 ± 58.8 U/mg protein in the transgenic cardiomyocytes and in the nontransgenic controls, respectively. When these cells were challenged by H₂O₂, a significant difference in the LDH activity released into media was observed between the transgenic and the nontransgenic cardiomyocytes. Again, the transgenic cells were much more resistant to the toxicity of H₂O₂ (Fig. 3).
again displayed much more resistance to the toxic effect (Fig. 4).

Inhibition of MT on H₂O₂-Induced Lipid Peroxidation

We tested whether the observed protection by MT elevation against H₂O₂ cytotoxicity could result from its interception in H₂O₂ reaction with macromolecules, a proposed mechanism of action of MT. The lipid hydroperoxide concentrations in the cells were estimated as shown in Table 4. There was no significant difference in cellular lipid hydroperoxide concentrations between these two types of cardiomyocytes untreated with H₂O₂. After these cells were treated with different concentrations of H₂O₂ for 60 min, a dose-dependent increase in lipid hydroperoxide concentrations was observed in both cell types. However, this H₂O₂ effect was significantly attenuated in the transgenic cardiomyocytes, especially at low concentrations of H₂O₂ (<50 µM). The H₂O₂-induced lipid peroxidation was completely inhibited.

DISCUSSION

Recent studies (15–17) have demonstrated that MT functions in cytoprotection against oxidative injury. In studying the role of MT in cardiac protection against reactive oxygen species-induced damage, we have produced transgenic mouse lines in which MT was specifically overexpressed in the heart (14). Using this experimental model, we have demonstrated that the MT-overexpressing mouse heart was highly resistant to doxorubicin-induced oxidative injury (14). A study by others, however, has shown that MT overexpression did not protect the heart from doxorubicin toxicity (4). Several speculations could be derived from these controversial results. Elevation of cardiac MT in the transgenic mouse heart may not reach an effective level in
However, the study using bismuth subnitrate showed a compatible level of MT induced in the heart, yet, at this level the heart was effectively protected from doxorubicin toxicity (20). Other speculations have been proposed (4). A critical factor that may affect the experimental outcome is the cell type to which MT was overexpressed. None of the above studies has addressed this issue.

To this end and to examine directly the effect of MT overexpression on oxidative injury in cardiomyocytes, we established the neonatal mouse cardiomyocyte culture system in the present study. We have successfully modified current experimental procedures used for other animals toward establishing the neonatal mouse cardiomyocyte culture. The cell culture has been obtained as expected. The cardiomyocyte purity reached about 95%, providing homogeneous populations for our studies. In addition, the MT-overexpressing transgenic cardiomyocytes displayed the same characteristics as the nontransgenic cells, except the MT concentrations in the cells were different. This provides a pair of well-controlled experimental system to study the antioxidant function of MT in vitro.

It was unexpected to observe that MT concentrations in the isolated transgenic cardiomyocytes were dramatically increased by 10.22 ± 0.33. This observation is consistent with the findings of previous studies using bismuth subnitrate (4). However, the study using bismuth subnitrate showed a compatible level of MT induced in the heart, yet, at this level the heart was effectively protected from doxorubicin toxicity (20). Other speculations have been proposed (4). A critical factor that may affect the experimental outcome is the cell type to which MT was overexpressed. None of the above studies has addressed this issue.

Table 3. Morphological alterations of neonatal cardiomyocyte cultures upon exposure to varying concentrations of H$_2$O$_2$

<table>
<thead>
<tr>
<th>H$_2$O$_2$ (µM)</th>
<th>Pseudopodia</th>
<th>Vacuoles</th>
<th>Granule</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control MT-TG</td>
<td>Control MT-TG</td>
<td>Control MT-TG</td>
<td></td>
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<tr>
<td>Untreated control</td>
<td>NC NC NC NC NC NC NC</td>
<td></td>
<td></td>
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<tr>
<td>30 min</td>
<td>NC NC NC NC NC NC NC</td>
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<td>60 min</td>
<td>NC NC NC NC NC NC NC</td>
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<tr>
<td>120 min</td>
<td>NC NC NC NC NC NC NC</td>
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<tr>
<td>25 µM H$_2$O$_2$</td>
<td>++ ++ ++ ++ ++ ++</td>
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<tr>
<td>30 min</td>
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<tr>
<td>60 min</td>
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<tr>
<td>120 min</td>
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<tr>
<td>100 µM H$_2$O$_2$</td>
<td>++ ++ ++ ++ ++ ++</td>
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<tr>
<td>30 min</td>
<td>++ ++ ++ ++ ++ ++</td>
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<tr>
<td>60 min</td>
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<td>120 min</td>
<td>++ ++ ++ ++ ++ ++</td>
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<tr>
<td>250 µM H$_2$O$_2$</td>
<td>++ ++ ++ ++ ++ ++</td>
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<tr>
<td>120 min</td>
<td>++ ++ ++ ++ ++ ++</td>
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</table>

Fig. 3. Release of lactate dehydrogenase (LDH) in primary MT-overexpressing transgenic (MT-TG) cardiomyocyte and nontransgenic control cultures after exposure to varying concentrations of H$_2$O$_2$ for 60 min. Values are means ± SD from triplicate dishes for each treatment concentration. *Significantly different from nontransgenic control for the corresponding concentration (P < 0.05).

Table 4. H$_2$O$_2$-induced lipid peroxidation as estimated by lipid hydroperoxide concentrations in MT-overexpressing transgenic cardiomyocytes and nontransgenic controls

<table>
<thead>
<tr>
<th>H$_2$O$_2$, µM</th>
<th>Lipid Hydroperoxide, nmol/mg protein</th>
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</thead>
<tbody>
<tr>
<td>Control MT-TG</td>
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<tr>
<td>0</td>
<td>2.35 ± 0.36</td>
</tr>
<tr>
<td>25</td>
<td>6.41 ± 0.32</td>
</tr>
<tr>
<td>50</td>
<td>6.85 ± 0.31</td>
</tr>
<tr>
<td>100</td>
<td>10.37 ± 1.60</td>
</tr>
<tr>
<td>250</td>
<td>13.96 ± 2.31</td>
</tr>
<tr>
<td>500</td>
<td>14.80 ± 0.56</td>
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</table>

Values are means ± SD; n = 3 mice. *P < 0.05.
cally different from that observed in the neonatal heart. The MT concentrations in the transgenic cells were ~5% of that in the neonatal heart, when they were measured right after the cardiomyocytes were separated from nonmuscle cells. Because of the purity of the isolated cardiomyocytes, it is clear that the MT concentrations measured using the cell homogenate represented the levels in the cardiomyocytes. What would cause this dramatic difference? It seems from the results obtained here that MT synthesized in the cells may be transported out of the cell. This export process seems proportional to the intracellular concentrations or the cells may retain a threshold level of intracellular MT. Above this level this protein would be surpassed to other cells or extracellular matrix. The observation obtained from the 6-day cultures may further support this speculation. On the sixth day of culturing, the MT concentrations in the transgenic cells were much lower than the concentrations in the precultured cells. This may suggest that the cells would retain the threshold level by exporting the overproduced MT, although decreased synthesis of MT in these cells cannot be excluded. This phenomenon has not been reported before. We are currently using this cultured neonatal cardiomyocyte model to examine the cellular hemostasis of MT. On the other hand, the result clearly demonstrated that MT was indeed overexpressed in the cardiomyocytes.

The discrepancy in MT concentrations between the isolated cardiomyocytes before culturing and on the sixth day of postculturing suggests that some biochemical alterations may occur in the cardiomyocytes during the culturing process. Because of the importance of other antioxidant systems in cardioprotection against oxidative injury, we then compared the activities of other antioxidant systems in the cardiomyocytes before culturing and on the sixth day of postculturing. It was found that cellular GSH concentrations were significantly elevated in the 6-day cultures, although there was no significant difference between transgenic and nontransgenic cardiomyocytes at either stage of culturing. Another significant change associated with culturing time was the catalase activity that was increased in the 6-day cultures of both transgenic and nontransgenic cells. The significance and cause of these changes related to culturing time are not currently understood.

When these cultured cardiomyocytes were exposed to H₂O₂, the transgenic cells were highly resistant to the toxicity of this oxidant. Detoxification of H₂O₂ in the cells involves catalase, glutathione peroxidase, glutathione reductase, and GSH. The results showed that although catalase and GSH were increased as the culturing time elapsed, there were no significant differences in any of these antioxidant activities between the transgenic and nontransgenic cardiomyocytes. It clearly demonstrates that the difference in cellular responses to H₂O₂ between the two types of cells did not result from the status of these antioxidant systems. Studies with HL-60 cells demonstrate that the sulfhydryl groups in MT directly react with H₂O₂ (26). This reaction, therefore, intercepts the injurious action of H₂O₂ on cells. This direct reaction between MT and H₂O₂ is also indicated in the present study. H₂O₂ is converted to a hydroxyl radical, which, in turn, reacts with macromolecules, including DNA, proteins, and lipids, and the ultimate cell damage. Measurement of the products of lipid peroxidation often provides indication of the early events of reactive oxygen reaction with macromolecules. The results presented in the present study clearly demonstrate that MT indeed inhibits H₂O₂-induced lipid peroxidation in the cardiomyocytes. This indicates that the protective action of MT is mediated at least partially by its direct reaction with H₂O₂, as reported (26), although MT also efficiently protects from other reactive oxygen species-induced injury (1, 31). Therefore, the differential responses to H₂O₂ toxicity between the two types of cells most likely resulted from the MT action.

In summary, we successfully modified an experimental procedure used for other animals for culturing cardiomyocytes from neonatal mice. The MT-overexpressing transgenic neonatal mouse cardiomyocytes thereby established have shown to share the same characteristics with that of nontransgenic controls, except the MT concentrations were much higher in the transgenic cardiomyocytes and decreased along with the elapsed time of culturing. The transgenic cardiomyocytes were highly resistant to H₂O₂ toxicity, most likely resulting from a direct reaction between the elevated MT and H₂O₂. This culture system thus provides a valuable tool to study cellular and molecular mechanisms by which MT functions in cardiac protection against oxidative injury. In addition, the experimental procedure for culturing neonatal mouse cardiomyocytes described in this study provides a complementary tool to the future studies using transgenic mice.

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Address for reprint requests: Y. J. Kang, Dept. of Medicine, Univ. of Louisville School of Medicine, 530 S. Jackson St., Louisville, KY 40202.

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REFERENCES