Inhibition of hypoxia/reoxygenation-induced apoptosis in metallothionein-overexpressing cardiomyocytes

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Wang, Guang-Wu, Zhanxiang Zhou, Jon B. Klein, and Y. James Kang. Inhibition of hypoxia/reoxygenation-induced apoptosis in metallothionein-overexpressing cardiomyocytes. Am J Physiol Heart Circ Physiol 280: H2292–H2299, 2001.—To study possible mechanisms for metallothionein (MT) inhibition of ischemia-reperfusion-induced myocardial injury, cardiomyocytes isolated from MT-overexpressing transgenic neonatal mouse hearts and nontransgenic controls were subjected to 4 h of hypoxia (5% CO2-95% N2, glucose-free modified Tyrode’s solution) followed by 1 h of reoxygenation in MEM + 20% fetal bovine serum (FBS) (5% CO2-95% air), and cytochrome c-mediated caspase-3 activation apoptotic pathway was determined. Hypoxia/reoxygenation-induced apoptosis was significantly suppressed in MT-overexpressing cardiomyocytes, as measured by both terminal deoxynucleotidyl transferase-mediated deoxyuridine 5-triphosphate nick-end labeling and annexin V-FITC binding. In association with apoptosis, mitochondrial cytochrome c release, as determined by Western blot, was observed to occur in nontransgenic cardiomyocytes. Correspondingly, caspase-3 was activated as determined by laser confocal microscopic examination with the use of FITC-conjugated antibody against active caspase-3 and by enzymatic assay. The activation of this apoptotic pathway was significantly inhibited in MT-overexpressing cells, as evidenced by both suppression of cytochrome c release and inhibition of caspase-3 activation. The results demonstrate that MT suppresses hypoxia/reoxygenation-induced cardiomyocyte apoptosis through, at least in part, inhibition of cytochrome c-mediated caspase-3 activation.

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significantly improved the suppressed contractile force postischemia. The efflux of creatine kinase from these transgenic hearts was reduced by more than 50%. In addition, the zone of infarction induced by ischemia-reperfusion at the end of reperfusion was suppressed by ~40% in the transgenic heart. The results strongly indicate that MT provides protection against ischemia-reperfusion-induced heart injury (22).

Mechanisms for this MT cardioprotection are unclear. Myocardial damage induced by ischemia-reperfusion has been shown to be associated with apoptosis (11, 13). However, the signal transduction pathways by which ischemia-reperfusion leads to apoptosis remain largely unknown. Recent studies have shown that mitochondria play an important role in apoptosis (41). Mitochondrial cytochrome c release occurs under a variety of proapoptotic conditions (28, 34, 37, 51). Cytochrome c, through a series of cascade reactions, activates caspase-3, which leads to apoptosis (31, 35). Mitochondrial dysfunction is one of the more critical events associated with myocardial ischemia-reperfusion injury (10, 29). It is possible that MT protects the heart from ischemia-reperfusion injury through inhibiting the reactive oxygen species (ROS)-related mitochondrial cytochrome c release and caspase-3-activated apoptotic pathway.

This study was thus undertaken to investigate possible mechanisms by which MT functions in the cardioprotection against ischemia-reperfusion injury and focused on the effect of MT on myocardial apoptosis induced by hypoxia/reoxygenation with the use of primary cultures of neonatal mouse cardiomyocytes. We present evidence to demonstrate that hypoxia/reoxygenation induces apoptosis in nontransgenic cardiomyocytes, and this apoptotic effect was significantly suppressed in the MT-overexpressing transgenic cardiomyocytes. Moreover, mitochondrial cytochrome c release and caspase-3 activation induced by hypoxia/reoxygenation were inhibited in the MT-overexpressing transgenic cardiomyocytes. These results indicate that MT suppresses hypoxia/reoxygenation-induced cardiomyocyte apoptosis through the inhibition of a cytochrome c-mediated apoptotic pathway.

**MATERIALS AND METHODS**

**Cell cultures and treatments.** A new procedure was used for culturing neonatal mouse ventricular cardiomyocytes as described previously (47). The isolated cardiomyocytes were plated at a density of 1.0 × 10⁶ cells/mm² in fetal bovine serum (PBS)-MEM (MEM supplemented with 20% PBS, 100 U/ml penicillin, and 100 µg/ml streptomycin) at 37°C under a water-saturated atmosphere of 5% CO₂-95% air. The purity of cardiomyocyte cultures was monitored by staining with monoclonal antibody (MAb) to cardiac α-sarcomeric actin (clone No. AC-40) according to the manufacturer’s instruction (Sigma; St. Louis, MO) and was found to be 94 ± 5% when examined at 48 h after culturing. Cardiomyocytes cultured for 6 days were used for the experiments of hypoxia/reoxygenation. For hypoxia, the culture media were replaced by a modified Tyrode’s solution (in mM/l: 136.9 NaCl, 2.68 KCl, 8.1 Na₂HPO₄, 12 H₂O, 1.47 KH₂PO₄, 0.9 CaCl₂, and 0.49 MgCl₂·6 H₂O; pH 7.4), and a constant stream for 4 h of water-saturated 5% CO₂-95% N₂ was maintained over the cultures (<1% O₂). For reoxygenation, the solution was changed to PBS-MEM under a water-saturated atmosphere of 5% CO₂-95% air for 1 h.

**Cellular MT assay.** Total MT concentration was determined by a cadmium-hemoglobin affinity assay as described previously (9).

**Assay of lactate dehydrogenase leakage.** The activity of cytoplasmic lactate dehydrogenase (LDH) leakage into culture media was determined as described previously (48). After hypoxia/reoxygenation, 100 µl of media were collected, and the LDH activity was assayed in 2.4 ml of phosphate buffer (0.1 mol/l, pH 7.4) with 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 with the use of a spectrophotometer (model DU-650, Beckman Instruments; Columbia, MD).

**Detection of apoptosis.** Identification of cardiomyocyte apoptosis was performed by terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL) of fragmented nuclei assay and further confirmed by annexin V-FITC binding.

**TUNEL assay.** Cardiomyocytes plated on Lab-Tek chamber glass slides were washed with PBS and fixed in 1% paraformaldehyde for 10 min and postfixed in precooled ethanol-acetic acid (2:1) for another 5 min at ~20°C. After being washed with PBS, the cells were incubated with a TUNEL reaction buffer for 1 h at 37°C in a humidified chamber. As a positive control, cells were treated with DNase I (1.0 µg/ml, Sigma) for 10 min to introduce nicks in the genomic DNA. The percentage of cardiomyocytes with DNA nick-end labeling was determined by counting cells exhibiting brown nuclei among 1,000 nuclei in triplicate plates.

**Annexin V-FITC binding.** This assay was performed on cardiomyocytes plated on Lab-Tek II chambered glass slides. The cells were washed with a binding buffer and stained with FITC-conjugated annexin V (Immunotech) for 15 min. The samples were then optically sectioned with a Zeiss LSM510 confocal microscope equipped with an Axiovert 100-M microscope.

**Assay for mitochondrial cytochrome c release.** Cytochrome c release from mitochondrial into the cytosol was measured by Western blot analysis as described (25, 28). Cells were harvested by centrifugation at 1,000 g for 10 min at 4°C. The cell pellets were washed once with ice-cold PBS and resuspended with 5 volumes of buffer A (in mM/l: 20 HEPES-KOH, pH 7.5, 10 KCl, 1.5 MgCl₂, 1.0 sodium EDTA, 1.0 sodium EGTA, 1.0 dithiothreitol, 0.1 phenylmethylsulfonyl fluoride, and 250 sucrose), supplemented with protease inhibitors (in µg/ml: 10 pepstatin A, 10 leupeptin, and 10 aprotinin). The cells, after 15 min on ice, were homogenized with 10 strokes of a Teflon glass homogenizer. The nuclei and cell debris were removed by centrifugation at 1,000 g for 15 min at 4°C. The supernatants were centrifuged at 10,000 g for 15 min at 4°C, and the resulting mitochondria pellets were resuspended with the same buffer A as above. The supernatants of 10,000 g were further centrifuged at 100,000 g for 1 h at 4°C. The supernatant (S-100) and mitochondrial fractions were stored at ~80°C. The protein concentrations of mitochondria and S-100 were determined by the Bradford method (Bio-Rad; Hercules, CA). Proteins (25 µg) extracted from the cytosol and mitochondria were separated by 15% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk in Tween 20 in Tris-buffered saline and probed with purified mouse anti-cytochrome c antibody (7H8.2C12, Pharmingen; San Diego, CA). Blots were washed, incubated with goat anti-mouse IgG...
To examine the effect of MT on hypoxia/reoxygenation-induced apoptosis. As shown in Fig. 1C, numerous nontransgenic cardiomyocytes were shown to be positive for TUNEL. In contrast, the number of TUNEL-positive cells was significantly reduced in MT-overexpressing transgenic cardiomyocytes (Fig. 1D). Quantitative data showed that 26 ± 8% of cardiomyocytes were apoptotic in nontransgenic cardiomyocytes, whereas only 14 ± 5% transgenic cardiomyocytes underwent apoptosis (Fig. 1E). The basal levels of apoptosis in either transgenic or nontransgenic cardiomyocytes under normoxic conditions were ~4 ± 2%.

To confirm the results obtained from the TUNEL assay, a more apoptotic-sensitive and early detection method, annexin V-FITC binding assay, was performed. The results presented in Fig. 2 showed that the number of annexin V-FITC-positive cells in nontransgenic cardiomyocyte cultures was much more than in...
transgenic cultures, in agreement with the result obtained from the TUNEL assay.

LDH leakage from cells into media was also determined. This assay measures cellular membrane damage. The results showed that there was no significant increase in LDH leakage from either MT-overexpressing transgenic cardiomyocytes or nontransgenic controls after hypoxia/reoxygenation (Fig. 3).

**Mitochondrial cytochrome c release and caspase-3 activation by hypoxia/reoxygenation.** Mitochondrial cytochrome c release and caspase-3 activation play important roles in apoptosis. We thus tested whether cytochrome c release and caspase-3 activation were involved in hypoxia/reoxygenation-induced apoptosis in cardiomyocytes. As shown in Fig. 4A, Western blot analysis revealed that most of the cellular cytochrome c in cardiomyocytes was found in mitochondria under normoxic conditions. Hypoxia/reoxygenation significantly increased cytosolic concentrations of cytochrome c with a concomitant decrease in the content in mitochondria. Quantitative data showed that hypoxia/reoxygenation reduced total mitochondrial cytochrome c by 61.9% in nontransgenic cardiomyocytes (Fig. 4B). This effect was suppressed in the MT-overexpressing transgenic cardiomyocytes: 26.6% of total cytochrome c was released from mitochondria.

We then sought to examine whether apoptosis due to hypoxia/reoxygenation in our experimental model was associated with caspase-3 activation. The activation of caspase-3 was examined by immunofluorescent confocal technique with the use of an antibody against the active caspase-3. This analysis revealed that hypoxia/reoxygenation markedly activated caspase-3 activity in nontransgenic cardiomyocytes. This elevation was significantly suppressed in the transgenic cells. To demonstrate the essentiality of caspase-3 in hypoxia/reoxygenation-induced apoptosis,
Ac-DEVD-cmk, an inhibitor of caspase-3, was used. Cells were treated with Ac-DEVD-cmk (1.0 mM) for 0.5 h before hypoxia/reoxygenation. This inhibitor efficiently suppressed caspase-3 activity (Fig. 7) and reduced the number of apoptotic cells (Fig. 8).

**DISCUSSION**

Our results indicate that MT suppresses hypoxia/reoxygenation-induced cardiomyocyte apoptosis through inhibition of cytochrome c-mediated caspase-3 activation pathway. The involvement of ROS in the pathogenesis of myocardial injury due to ischemia-reperfusion has long been recognized (8, 17, 30). Previous studies by Kang et al. (22) have demonstrated that elevation of MT in the heart made this organ highly resistant to ischemia-reperfusion injury. The results obtained from the present study thus provide an understanding of the mechanistic link between ROS-induced myocardial injury and MT protection.

Apoptosis is a critical cellular event involved in the pathogenesis of myocardial ischemia-reperfusion injury. However, investigation of the significance of the contribution of apoptosis to overall myocardial injury in vivo is technically difficult. Heterogeneity of cell types, influence of neurohormonal systems, undefined traverse time in apoptotic process in the myocardium, and the exacerbation of tissue damage caused by inflammation are among the different variables. In this context, primary cultures of cardiomyocytes provide advantages that overcome these complications. Therefore, in the present study, we used primary cultures of neonatal mouse cardiomyocytes to define the significance of apoptosis in cardiomyocyte loss due to hypoxia/reoxygenation, an in vitro model of ischemia-reperfusion.

**Fig. 5.** H/R-induced caspase-3 activation. Laser confocal photograph of primary neonatal mouse cardiomyocytes maintained in cultures for 6 days and then exposed to H/R (original objective lens magnification ×40). A: nontransgenic controls; B: MT-transgenic controls; C: nontransgenic cardiomyocytes exposed to H/R; D: MT-transgenic cardiomyocytes exposed to H/R.

**Fig. 6.** Inhibition of MT on caspase-3 activities induced by H/R. Cardiomyocytes were maintained in cultures for 6 days and then exposed to H/R. Activity of caspase-3 was measured by using a caspase-3 colorimetric protease assay kit. Values represent means ± SD from triplicate samples for each treatment. *Significantly different from nontransgenic control for the corresponding group (P < 0.05).

**Fig. 7.** Effect of Ac-DEVD-cmk on the activity of caspase-3 induced by H/R. Cardiomyocytes were maintained in cultures for 6 days and then treated with Ac-DEVD-cmk (1 μmol/l) for 0.5 h before H/R. Values represent means ± SD from triplicate samples for each treatment. *Significantly different from nontransgenic control for the corresponding group (P < 0.05).

**Fig. 8.** Effect of Ac-DEVD-cmk on H/R-induced apoptosis, as measured by annexin V-FITC binding. Laser confocal photograph of primary neonatal mouse cardiomyocytes were maintained in cultures for 6 days and then treated with Ac-DEVD-cmk (1 μmol/l) for 0.5 h before H/R (original objective lens magnification, ×63). A: nontransgenic controls; B: nontransgenic cardiomyocytes treated with Ac-DEVD-cmk only. C: nontransgenic cardiomyocytes exposed to H/R. D: nontransgenic cardiomyocytes treated with Ac-DEVD-cmk (1 μmol/l) for 0.5 h and then exposed to H/R.
A TUNEL assay was used to identify cardiomyocytes containing fragmented nuclei, an indication of apoptosis. Numerous TUNEL-positive cells were found in the nontransgenic cardiomyocyte cultures under hypoxia/reoxygenation. The number of TUNEL-positive cells was significantly reduced in the transgenic cell cultures. The TUNEL assay identifies breaks in double-stranded DNA, which are seen in apoptosis. However, this DNA break may also occur late in the terminal evolution of cell necrosis. The complexity of measuring apoptosis in cardiomyocytes thus involves the difficulty of distinguishing apoptosis from necrosis. For this reason, we used another method, annexin V-FITC binding assay, a more apoptosis-specific and early detection procedure. The result obtained from this experiment further revealed that hypoxia/reoxygenation induced apoptosis and MT inhibited this apoptotic process.

To further confirm the results obtained from the TUNEL and annexin V-binding assays and to elucidate the important contribution of apoptosis to total cell death, cellular LDH leakage was determined. This assay generally measures changes in cell membrane permeability. Although the integrity of the cell membrane changes in the late phase of the apoptotic process, this change occurs post-DNA cleavage. However, in the necrotic process, increased permeability in cell membrane is an early event. The measurement of LDH leakage revealed that under the hypoxia/reoxygenation treatment employed in our model, neither transgenic nor nontransgenic cardiomyocytes underwent significant changes in cell membrane permeability. However, it should be noted that studies using adult rat cardiomyocytes have shown that hypoxia for 48 h followed by reoxygenation for 3 h significantly increased LDH leakage (50). The observed insignificant LDH leakage in the present study may relate to the short-term treatment (hypoxia for 4 h and reoxygenation for 1 h) and a higher resistance of the neonatal cells to overt detrimental environments in general (2, 52). On the other hand, this study demonstrates that apoptosis was a predominate model of cell death during the early response to hypoxia/reoxygenation in our model.

Overall, the results obtained from this study demonstrate that hypoxia/reoxygenation indeed causes apoptosis in primary cultures of neonatal mouse cardiomyocytes. This apoptotic process would make a significant contribution to the overall cell loss under the experimental conditions. Importantly, MT significantly suppressed hypoxia/reoxygenation-induced apoptosis.

Several pathways that lead to apoptosis have been identified. Among these, the mitochondrial cytochrome c release and caspase-3 activation pathway has been demonstrated to be activated by ROS (12, 45, 54). MT is a free radical scavenger (1, 7, 20, 36, 39, 42, 43), and recent studies have shown that MT has antiapoptotic action (23, 24, 26, 44). In our previous studies, we observed that Adr-induced apoptosis was suppressed in MT-overexpressing cardiomyocytes both in vivo and in vitro (23), and this protection was related to subcellular MT localization (53). Therefore, our effort in the present study was focused on determining the effect of MT on hypoxia/reoxygenation-induced activation of cytochrome c-mediated apoptotic pathway. The release of cytochrome c from mitochondria into cytosol is a critical initiation step in ROS-triggered apoptosis (12, 54).

Cytochrome c aggregates with apoptotic protease-activating factor-1 (Apaf-1, another factor released from mitochondria under oxidative stress), procaspase-9, and dATP and subsequently activates caspase-9 (27). Activated caspase-9 in turn activates caspase-3 (14, 34, 35). Detection of changes in cytochrome c concentrations between mitochondria and cytosol by Western blot showed that mitochondrial cytochrome c was decreased in the nontransgenic cardiomyocytes under hypoxia/reoxygenation. This change was suppressed in the MT-overexpressing cardiomyocytes. Corresponding to this alteration, caspase-3 was also activated in the nontransgenic cardiomyocytes. But this activation was almost completely suppressed in the MT-transgenic cells. To elucidate the significance of the caspase-3 activation in the hypoxia/reoxygenation-induced apoptotic pathway, Ac-DEVD-cmk, an inhibitor of caspase-3, was used. This inhibitor efficiently suppressed caspase-3 activity and reduced the number of apoptotic cells.

These results thus show that MT attenuates hypoxia/reoxygenation-induced apoptosis in cardiomyocytes by inhibiting mitochondrial cytochrome c release and subsequent caspase-3 activation. A direct interaction between MT and ROS or RNS has been demonstrated in cell-free experiments (7, 43), but has not been demonstrated in vivo. However, we have demonstrated that lipid peroxide levels induced by ROS were dramatically decreased by MT both in cultured cardiomyocytes (46, 47) and in the heart of intact animals (23, 49). In this context, if ROS serve as a signal for mitochondrial cytochrome c release, MT, by interacting with ROS, would eliminate this signal.

Although the results demonstrate that MT inhibits hypoxia/reoxygenation-induced cardiomyocyte apoptosis through inhibition of oxidant-activated apoptotic pathways, there are several limitations associated with this study. First, although oxygen deprivation is involved in ischemia in vivo, many other factors and their interactions with oxygen deprivation as discussed above make the causes of cell death far more complicated. Therefore, the observation that MT protects from hypoxia/reoxygenation-induced apoptosis in the cultured cardiomyocytes cannot be simply extrapolated to myocardial injury by ischemia-reperfusion in vivo. Second, although apoptosis was a predominant mode of cell death under the experimental conditions, this acute and simple factor-induced cell death may not represent what would occur under the condition of in vivo ischemia-reperfusion, in which both apoptosis and necrosis have been shown to occur (13, 19). Third, neonatal cardiomyocytes, due to their morphological immaturity and genetic program differences from adult cardiomyocytes would respond to environmental stimuli differently relative to adult cells. For instance, neonatal cardiomyocytes are more resistant to various environments and, therefore, there are much easier to
maintain in cultures than adult cells (2, 4). Finally, the mechanisms for the antiapoptotic effect of MT in vivo are more complicated. For instance, we have observed that MT suppressed dietary copper deficiency-induced myocardial apoptosis through a mechanism that involves inhibition of atrial natriuretic peptide production and its apoptotic effect (24). These limitations suggest that further in vivo studies to investigate mechanisms by which MT protects the heart from ischemia-reperfusion injury are necessary.

Regardless of these limitations, it is important to note that MT is highly inducible under a wide variety of stress conditions including oxidative stress. 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 subnitrate (33), isoproterenol (6), and tumor necrosis factor-α (38). Therefore, the basis for developing pharmaceutical agents to increase MT concentration in the heart already exists. Exploring the potential of MT in protection against ischemia-reperfusion injury would likely result in novel approaches to this clinical problem and could positively influence clinical outcomes.

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