Cardiomyopathy induced by doxorubicin (DOX) has long been a major impediment of clinical applications of this effective anticancer agent. Previous studies have shown that cardiac-specific metallothionein (MT)-overexpressing transgenic mice are highly resistant to DOX-induced cardiotoxicity. To investigate cellular and molecular mechanisms by which MT participates in this cytoprotection, transgenic mice containing high levels of cardiac MT and non-transgenic controls were treated intraperitoneally with DOX at a single dose of 15 mg/kg and sacrificed on the 4th day after treatment. Myocardial apoptosis was detected by a terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling assay and confirmed by electron microscopy of immunogold staining of apoptotic nuclei. Dual staining of cardiac &-sarcomeric actin using an immunohistochemical method further identified apoptotic myocytes. Apoptosis was significantly inhibited in the transgenic myocardium. The anti-apoptotic effect of MT was further revealed in primary cultures of neonatal mouse cardiomyocytes. Furthermore, DOX activated p38 mitogen-activated protein kinase (MAPK), which was critically involved in the apoptotic process, as demonstrated by inhibition of DOX-induced apoptosis by a p38-specific inhibitor, SB203580. Both DOX-induced p38 MAPK activation and apoptosis were dramatically inhibited in the transgenic cardiomyocytes. The results thus demonstrate that DOX induces apoptosis in cardiomyocytes both in vivo and in vitro and MT suppresses this effect through at least in part inhibition of p38 MAPK activation.

Suppression by Metallothionein of Doxorubicin-induced Cardiomyocyte Apoptosis through Inhibition of p38 Mitogen-activated Protein Kinases*

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In the present study, we employed the cardiac-specific MT-overexpressing transgenic mice and cardiomyocyte cultures isolated from these mice to determine the effect of MT on DOX-induced apoptosis. Furthermore, we examined whether activation of p38 mitogen-activated protein kinase (MAPK) is involved in the DOX-induced apoptosis and the effect of MT on this pathway. The results obtained demonstrate that DOX indeed induces apoptosis in the cardiomyocyte cultures and MT inhibition of the apoptotic effect is mediated at least in part by inhibition of p38 MAPK activation.

EXPERIMENTAL PROCEDURES

Materials—Eagle's minimum essential medium, fetal bovine serum and trypsin were purchased from Life Technologies, Inc. The biinchoninic acid (BCA) protein assay reagents were obtained from Pierce. DOX and monoclonal anti-a-sarcomeric actin antibody were obtained from Sigma. ApopTag in situ apoptosis detection kit (ST711 kit) was purchased from Oncor (Gaithersburg, MD). Annexin V-FTTC apoptosis detection kit (6693KT) was obtained from PharMingen (San Diego, CA). Biotinylated rabbit anti-mouse IgM, peroxidase-conjugated streptavidin, and alkaline phosphatase-conjugated streptavidin were purchased from Zymed Laboratories Inc. (San Francisco, CA). SB203580, a competitive inhibitor of p38 MAPK, was obtained from Calbiochem, La Jolla, CA. 109CdCl2 was the product of NEN Life Science Products, and 1,4-diaminobenzidine as the chromogen of new fuchsin was used to visualize the binding sites. Biotinylated rabbit anti-mouse IgM, Sigma) overnight at 4 °C, then with biotinylated rabbit anti-mouse IgM, followed by streptavidin-alkaline phosphatase incubation. A red color chromatographic of some of the tissue sections were processed for TUNEL assay to detect fragmented nuclei in the myocardium. An ApopTag in situ detection kit was used according to the manufacturer’s instruction. Briefly, the slides were pretreated with H2O2 and incubated with the reaction mixture containing TdT and digoxigenin-conjugated dUTP for 1 h at 37 °C. Labeled DNA was visualized with peroxidase-conjugated antidigoxigenin antibody (Fab alkaline phosphatase-conjugated. Rat mammary gland tissue provided in the kit was used as positive control. For negative control, TdT was routinely omitted from the reaction mixture.

Dual Staining of a-Sarcomeric Actin—After the TUNEL staining, some of the tissue sections were processed for a-sarcomeric actin localization with an immunoperoxidase method. Sections were incubated with monoclonal a-sarcomeric actin (clone 5C5, Isotype mouse IgM, Sigma) overnight at 4 °C, then with biotinylated rabbit anti-mouse IgM, followed by streptavidin-alkaline phosphatase incubation. A red color chromatographic of some of the tissue sections were processed for TUNEL assay to detect fragmented nuclei in the myocardium. The hearts were dissected from the mice 1 h after the DOX treatment.

Determination of DOX-induced Apoptosis in Vivo—The hearts removed from DOX- and saline-treated mice were fixed in 10% formalin in 0.1 M phosphate buffered saline, pH 7.3, for 24 h and embedded in paraffin. Tissue blocks were sectioned at a thickness of 5 µm and mounted on silanized slides.

TUNEL Assay—The slides obtained were processed for a TUNEL assay to detect fragmented nuclei in the myocardium. An ApopTag in situ detection kit was used according to the manufacturer’s instruction. Briefly, the slides were pretreated with H2O2 and incubated with the reaction mixture containing TdT and digoxigenin-conjugated dUTP for 1 h at 37 °C. Labeled DNA was visualized with peroxidase-conjugated antidigoxigenin antibody (Fab alkaline phosphatase-conjugated. Rat mammary gland tissue provided in the kit was used as positive control. For negative control, TdT was routinely omitted from the reaction mixture.

Cellular MT Concentration—Total MT was determined by a cadmium-hemoglobin affinity assay (20). MT concentrations in myocardial tissues were measured as described previously (3). MT concentrations in cultured myocytes were measured from cells that were cultured for 3 or 4 days and before being exposed to DOX. The cells were rinsed with 5 ml of cold PBS and centrifuged at 2000 x g for 10 min, and the pellet, 500 µl of 10 ml Tris-HCl was added. The cells were then pulse-sonicated on ice with a disemembrator (model 60, Fisher Scientific Inc.) at an output power of 8 for 15 s, and repeated three times with a 30-s interval. Following centrifugation at 10,000 x g for 15 min, 200 µl of supernatant was transferred to microtubes for MT analysis following the procedure described previously (20), and another 100 µl of supernatant was transferred to separate microtubes for total protein determination using the Pierce BCA protein assay reagents (21), using bovine serum albumin as the standard.

Electron Microscopic Examination of Apoptotic Myocytes—Heart tissue taken from the free wall of left ventricles was cut into about 1-mm3. The fragments were fixed in 3% glutaraldehyde and 1% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4, for 2 h at 4 °C and routinely osmicated in 1% osmium tetroxide. After dehydration with ethanol, the samples were embedded in LR White resin. Ultrathin sections were cut on a LKB ultratome and collected on gold grids. The fragmented DNA on the ultrathin sections were labeled basically with the ApopTag kit with some modulations. Briefly, the ultrathin sections were incubated with working solution of TdT at 37 °C with different concentrations (up to 10 times dilution) and incubation time (10–60 min). The reaction was terminated by rinsing in SSC for 15 min. After rinsing in immunogold buffer (0.01% v/v PBS with 1% bovine serum albumin, 0.1% Tween, and 0.1% NaN3, pH 8.2), the ultrathin sections were incubated with 10-nm gold conjugated sheep anti-digoxigenin (BIIBiological, United Kingdom) diluted 1:30 in immunogold buffer. The ultrathin sections were then rinsed in distilled water and counterstained with uranyl acetate and lead citrate. The labeled ultrathin sections were observed with a Hitachi transmission electron microscope. Negative controls were performed by omitting the TdT enzyme.

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incubated in an FITC-conjugated monoclonal anti-phospho-p38 antibody (Santa Cruz Biotechnology, Inc.) for 1 h. Controls for nonspecific antibody binding as well as peptide inhibition with relevant protein were performed. Samples were optically sectioned with the confocal microscopy system.

p38 MAPK Activity—An immune complex kinase assay using ATF2 as substrate was applied to measure the p38 MAPK activity, as described previously (22). Briefly, the hearts were homogenized with 1:9 (w/v) 10 mM Tris-HCl buffer (pH 7.4) and the homogenate was briefly centrifuged. The supernatant was treated with lysis buffer (20 mM Tris, pH 7.0, 1% Triton, 0.5% Nonidet P-40, 150 mM NaCl, 20 mM NaF, 0.2 mM NaVO3, 1.0 mM EDTA, 1 mM EGTA, and 1 mM phenylmethylsulfonyl fluoride) on ice and centrifuged at 15,000 g for 10 min at 4 °C. Samples thus obtained were incubated with anti-p38 antisera for 1 h at 4 °C. Protein A-Sepharose beads (15 μl of a 1:1 slurry in RIPA/EDTA) were added to the homogenate and incubated for 1 h at 4 °C to precipitate the immune complexes. Samples were centrifuged at 15,000 × g for 2 min and the beads were washed once in RIPA/EDTA and twice in PAN (10 mM PIPES, pH 7.0, 0.09 unit/ml aprotinin, and 100 mM NaCl). The kinase assay was initiated by the addition of 40 μl of PAN containing 30 μl [γ-32P]ATP (10 Ci/nmol) and 1 μg of recombinant fragment of ATF2 encoding amino acids. Reactions were incubated for 30 min at 30 °C, then terminated by the addition of 15 μl of 5× Laemmli’s SDS sample buffer. Samples were boiled, briefly centrifuged, and the products were resolved by 10% SDS-polyacrylamide gel electrophoresis. The incorporation of 32P was visualized by autoradiography and quantified by PhosphorImager (Molecular Dynamics Inc.).

Statistical Analysis—Data were analyzed initially by one-way analysis of variance. Scheffe’s F test was employed for further determination of the significance of differences. Differences between MT overexpressing transgenic cardiomyocytes and non-transgenic controls were considered significant at p < 0.05. The data are presented as mean ± S.D. values from triplicate samples for each treatment.

RESULTS

DOX-induced Myocardial Apoptosis in Vivo—Both cardiac-specific MT-overexpressing transgenic mice and non-transgenic littermates were treated intraperitoneally with DOX at a single dose of 15 mg/kg. Four days after the treatment, these animals were sacrificed, and the hearts were removed and subjected to measurement of MT concentrations and processing for detection of apoptosis by a TUNEL assay. The MT concentrations were 120.5 ± 6.8 and 5.6 ± 0.8 μg/g tissue in transgenic and non-transgenic hearts, respectively. As shown in Fig. 1, a significant number of cardiac cells in the DOX-treated non-transgenic myocardium were TUNEL-positive. In contrast, the number of TUNEL-positive cells was significantly reduced in the DOX-treated transgenic mouse hearts. To identify whether apoptosis determined by the TUNEL assay occurred in myocytes, a dual immunohistochemical staining for cardiac α-sarcomeric actin was performed. As also shown in Fig. 1, most of the TUNEL-positive cells were reactive to the monoclonal anti-α-sarcomeric actin antibody, demonstrating that these cells are of myocyte origin. To confirm the TUNEL result, an electron microscopic examination of the myocardium was performed. The results shown in Fig. 2 demonstrate that DOX induced dramatic morphological changes. Nuclear chromatin margination with many condensed pieces of coarse chromatin clumping was observed. A majority of cells displayed nuclear changes without obvious mitochondrial structure alterations (Fig. 2C), although mitochondrial cristae disappearance and membrane disruption occurred in some cells (Fig. 2C). However, in all events, the plasma membrane structure was preserved. These morphological changes demonstrate typical myocardial apoptosis. More importantly, immunogold staining of condensed nuclei provided further confirmation of cardiomyocyte apoptosis. Myocytes with these changes in the DOX-treated non-transgenic hearts were easily identified. In contrast to these findings, the chromatin was distributed...
homogeneously within the nucleus and mild mitochondrial morphological changes were seldom observed in the DOX-treated MT-overexpressing transgenic mouse heart. The immunogold TUNEL staining of myocytes in the transgenic myocardium was negative (Fig. 2F). Therefore, there were no severe morphological alterations or characteristic changes that indicate myocyte apoptosis in the DOX-treated MT-overexpressing mouse heart.

**Effect of MT on DOX-induced Apoptosis in Cultured Cardiomyocytes**—Neonatal mouse cardiomyocytes after being cultured on 8-well glass coverslips for 72 h were treated with DOX at final concentrations of 0.1, 0.5, or 1.0 μM for 6 h. A time-course study was also performed by exposing the cells to 1.0 μM for different times. The same TUNEL assay used for detecting apoptosis in vivo was applied to identify apoptotic cells in cultures. As shown in Fig. 3, a small number of cells underwent apoptosis spontaneously and substantial numbers of cells were TUNEL-positive in the DOX-treated non-transgenic myocyte cultures. A concentration-dependent apoptotic effect of DOX was observed. After a 6-h exposure, DOX at its final concentrations above 0.1 μM in the cultures significantly induced apoptosis. About one-third of the total populations of cultured cardiomyocytes underwent the apoptotic process in the cultures containing 1.0 μM DOX. The time course study revealed that 1 h after the cultures were treated with 1.0 μM DOX, some cells traversed the apoptotic process, but the numbers of these cells were not statistically significantly different from those of spontaneous apoptotic cells. The number of apoptotic cells was significantly increased 3 h after the treatment and continued to increase as the exposure time increased. Both of the concentration- and time-dependent apoptotic effects were dramatically inhibited in the transgenic myocytes, about 50% inhibition was observed (Fig. 3). To confirm the results obtained from the TUNEL assay, a more apoptotic-sensitive and early detection method, annexin V-FITC staining and confocal microscopy, was used. The results presented in Fig. 4 show the time-course studies of the cells exposed to 1.0 μM DOX. The annexin V-positive cells were detected as early as 30 min after DOX exposure. The number of annexin V-FITC-positive cells in the DOX-treated non-transgenic myocyte cultures at any of the time points was much greater than in the DOX-treated transgenic cell cultures, which was in agreement with the results obtained from the TUNEL assay.

**Effect of MT on DOX-activated p38 MAPK Pathway**—MAPKs, such as p38, have been shown to be involved in oxidative stress-induced apoptosis in cardiomyocytes (23). Because DOX-induced cardiotoxicity is mediated at least in part by reactive oxygen species and DOX induces lipid peroxidation in the cultured neonatal mouse cardiomyocytes (4), it is interesting to determine whether DOX also activates the p38 MAPK pathway in the myocytes. We employed a rather sensitive detection method to determine DOX-induced activation of p38 MAPK. This method uses an FITC-conjugated monoclonal anti-phospho-p38 antibody to recognize the activated form of p38 MAPK (phospho-p38). As shown in Fig. 5, DOX indeed activated p38 MAPK in the non-transgenic cardiomyocytes. This activation was time-dependent. The earliest detection of p38...
MAPK activation was 5 min after the cells were treated with 1.0 \( \mu M \) DOX. A remarkable activation was observed at 20 min, and a maximum one was achieved 30 min after the treatment. Surprisingly, it was observed that the FITC/confocal microscopy-detected p38 MAPK activation in the DOX-treated transgenic myocytes was almost completely undetectable (Fig. 5).

To demonstrate the effect of MT on DOX-induced p38 MAPK activation in vivo, an immune complex kinase assay using ATF-2 as substrate was performed. Both transgenic and non-transgenic control mice were treated with DOX for 12 h before the hearts were collected for the assay. The result shown in Fig. 6 demonstrates that DOX significantly increased p38 MAPK activity in vivo and MT almost completely suppressed this activation.

**Inhibition of DOX-induced Apoptosis by a p38-specific Inhibitor, SB203580**—The results obtained above suggest that p38 MAPK is a mediator of DOX-induced apoptosis and MT inhibits the apoptotic effect through inhibition of p38 MAPK activation. To demonstrate this possibility, we employed a pyridinyl imidazole compound, SB203580, which has been shown to act as a specific inhibitor of p38 MAPK \( \alpha \) and \( \beta \) isoforms (24, 25), but not \( \gamma \) and \( \delta \) isoforms (26–28), through competition with ATP for the same binding site on the kinase (29). Cardiomyocytes cultured on glass coverslips for 72 h were exposed to 10 \( \mu M \) SB203580 for 20 min before exposed to 1.0 \( \mu M \) of DOX. Six hours after DOX treatment, the cells were washed and fixed on the slips. The aforementioned TUNEL assay was then applied to identify apoptotic cells. As shown in Fig. 7, SB203580 itself...
did not cause any TUNEL-detectable alterations in the DOX-untreated myocyte cultures, but significantly reduced the number of TUNEL-positive cells in the DOX-treated cultures. The extent of this inhibition was comparable with that observed in the MT-overexpressing transgenic cardiomyocytes (Fig. 3).

**DISCUSSION**

Significant efforts have been placed on investigating the biochemical mechanisms by which DOX induces cardiotoxicity (1). It is widely accepted that cardiac toxicity of DOX is mediated by reactive oxygen species (ROS) (2). However, a comprehensive understanding of the subsequent consequences of ROS generation is lacking. In particular, cellular events and molecular mechanisms that lead to ROS-mediated cardiotoxicity have not been documented. A hypothesis that has been derived mainly from non-cardiac tissue and tumor studies is that DOX induces cardiomyocyte apoptosis. However, it is quite difficult to test this hypothesis. Cells undergoing apoptosis traverse the process from minutes to hours and days depending on different organ systems. The apoptotic cells are quickly removed by macrophages and other surrounding cells. Therefore, this dynamic process in vivo presents an extreme challenge for estimating the significance of apoptosis in cell loss due to exogenous and endogenous triggers. Furthermore, the duration of the apoptotic process is not known for cardiomyocytes. This adds more difficulties for the quantitative analysis of apoptosis in cardiomyocytes under DOX treatment. Above all, controversy exists regarding whether DOX induces apoptosis in cardiomyocytes (16, 18). This has to be addressed first.

In the aforementioned study using spontaneously hypertensive rats, the animals were treated chronically with DOX at 1 mg/kg/week for 6–12 weeks. Apoptosis was evaluated by morphological changes examined through electron microscope and

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**FIG. 4. Detection of apoptosis in cardiomyocyte cultures by annexin V-FITC staining.** Cells were treated with 1.0 μM DOX for varying time periods as indicated. Procedures for annexin V-FITC staining and confocal microscopic examination of positive cells were described under “Experimental Procedures.”

**FIG. 5. Detection of activation p38 MAPK by the FITC/confocal microscopy.** Cells were treated with DOX at 1.0 μM for varying times as indicated, then processed for detection of p38 MAPK activation as described under “Experimental Procedures.”
observed to occur in the cardiac endothelial and interstitial dendritic cells and macrophages, but not in myocytes (16). In the present study, a TUNEL assay was used to identify fragmented nuclei in the myocardium of mice treated with a single dose of DOX at 15 mg/kg for 4 days. Numerous TUNEL-positive cells were identified in the DOX-treated non-transgenic myocardium. To determine whether the TUNEL staining occurred in the myocytes, we used an anti-cardiac a-sarcomeric actin antibody to dually stain the TUNEL-stained myocardium tissue. a-Sarcomeric actin is a specific protein present in the cardiomyocytes. This staining thus identifies cells of myocyte origin. The result obtained showed that significant numbers of cells were dually stained by the TUNEL and the anti-a-sarcomeric actin antibody, demonstrating that DOX-induced DNA fragmentation did occur in the cardiomyocytes.

The TUNEL assay identifies DNA strand breaks, which are seen in apoptosis. However, DNA strand breaks may also occur late in the terminal evolution of cell necrosis. The complexity of measuring apoptosis in myocardium thus involves the difficulty of distinguishing apoptosis from necrosis. A fundamental difference between the two mechanisms of myocyte cell death is the morphological alteration of the cell. The nuclear modification of the apoptotic cells is accompanied by a preservation of the cytoplasmic structures of the cell. In contrast, immediate loss of plasma membrane integrity occurs in the necrotic cells. This distinction thus far has made the electron microscopic evaluation of morphological changes a most reliable tool for the determination of apoptosis. We thus used electron microscope to examine cardiomyocyte apoptosis. In the DOX-treated non-transgenic myocardium, a number of typical apoptotic myocytes were identified, although necrotic myocytes were also found. In addition to the ultrastructural examination, the immunogold staining of apoptotic nuclei in the myocyte provided further convincing evidence of DOX-induced apoptosis in cardiomyocytes. Both apoptotic and necrotic myocytes were rarely found in the DOX-treated transgenic myocardium: the same result as that obtained in the saline-treated controls.

Therefore, the results obtained from all of these carefully designed in vivo experiments demonstrate that DOX indeed induced apoptosis in the cardiomyocytes and that MT inhibited the apoptotic effect of DOX. The issues that remain to be resolved are whether this apoptotic process significantly contributes to the overall cardiotoxicity, and more importantly, what are possible pathways that lead to DOX-induced cardiomyocyte apoptosis. We then took advantage of the primary cultures of neonatal mouse cardiomyocytes to address these two important questions.

Both time-course and concentration-dependent studies using cultured cardiac cells demonstrated that apoptosis did significantly contribute to the total loss of the cells after these cultures were treated with DOX. As much as one-third of the total population of cardiac cells in cultures were TUNEL-positive under the treatment with 1.0 μM DOX for 6 h and about two-thirds after 12-h treatment with the same concentration of DOX. The results obtained from the TUNEL assay were confirmed by the annexin V-FITC detection of the early stage of apoptosis. Because the purity of the cultures was about 95% myocytes, as determined by an immunocytochemical assay using an anti-cardiac a-sarcomeric actin antibody (19), the majority of the apoptotic cells then represents cardiomyocytes. On the other hand, the range of DOX concentrations (0.1–1.0 μM) used to treat these cultured cells were within the levels accumulated in the myocardium of Syrian hamsters between 15 and 60 min after DOX treatment in vivo at a single intravenous

![](Image) FIG. 6. Representative autoradiogram showing 32P incorporation into the p38 substrate ATF-2–110 in DOX-treated myocardial tissues and controls. The experimental procedure was described under “Experimental Procedures.” The positive control was obtained by treating non-transgenic mice with 0.5 μg/kg tumor necrosis factor-a for 12 h. Quantitative analysis by PhosphorImaging and the arbitrary data thus obtained show the intensity of p38 MAPK activation by DOX and the inhibitory effect of MT. Superscript a indicates significant difference from their corresponding controls (p < 0.01), and superscript b indicates significant difference from non-transgenic mouse samples (p < 0.01).

![](Image) FIG. 7. Effect of SB203580 on DOX-induced apoptosis in cultured cardiomyocytes. Cells were treated with SB203580 at a final concentration of 10 μM (C and D) 20 min before exposed to 1.0 μM DOX (B and D). Control cells were treated with the same volume of saline only (A). Six hours after DOX treatment, the cultures were processed for the TUNEL assay. Original magnification, ×260.
dose of 5 mg/kg (30) and in the myocardium of rats 30 min after intravenous infusion of DOX at 16 mg/kg (31). Therefore, these results obtained from the cultured neonatal mouse cardiomyocytes clearly demonstrated that cardiomyocytes exposed to in vivo pharmacologically comparable exposure levels of DOX under go apoptosis and that this mode of cell death would contribute remarkably to the total loss of cardiomyocytes in the DOX-treated myocardium. It has been known that the phenotype of cultured neonatal cardiomyocytes is highly stable (32). For example, their contractile profile during hypoxia-reoxygenation is highly comparable with that of in situ hearts subjected to ischemia-reperfusion (32). It is thus important to stress that apoptosis would play an important role in the loss of myocardial function due to DOX treatment.

The p38 MAPK is a subfamily of the MAPK superfamily and is stress-responsive. This subfamily consists of p38α, p38β, p38γ, and p38δ (24–29). Recent studies have identified that the p38 MAPK is an important group of signaling molecules that mediate environmental stress responses in various cell types (33, 34–37). In non-cardiac cells, p38 MAPK has been implicated in gene expression, morphological changes, and cell death in response to endotoxin, cytokines, physical stress, and chemical insults (33–35). In cardiac cells, it has been reported that p38 MAPK is associated with the onset of apoptosis in ischemia-reperfusion-treated hearts (36, 37). In particular, transfection experiments using primary cultures of neonatal rat cardiomyocytes have shown that p38α is critically involved in myocyte apoptosis (23). In any event, the common observation is that p38 MAPK activation is associated with accumulation of reactive oxygen species generated under stress conditions. Therefore, in the present study, we focused on the possible role of p38 MAPK in mediating DOX-induced apoptosis.

Under the treatment with DOX that significantly induced myocyte apoptosis in the cultures, p38 MAPK was dramatically activated. That p38 MAPK was involved at least in part in the DOX-induced myocyte apoptosis was demonstrated by two important observations. First, the time-course analysis revealed that p38 MAPK activation preceded the onset of apoptosis, as demonstrated by the data presented in Figs. 4 and 5. The sensitive and early apoptosis detection method of annexin V-FITC detected the onset of myocyte apoptosis as early as 30 min after DOX treatment, while the early detection of p38 MAPK activation by the sensitive FITC-conjugated anti-phospho-p38 antibody and confocal microscopy was 20 min after DOX treatment. Second, application of SB203580, a specific inhibitor of p38 MAPK, significantly inhibited DOX-induced myocyte apoptosis. Because SB203580 acts as a specific inhibitor of p38α and p38β, but not p38γ and p38δ, the involvement of the former specific isoforms of p38 MAPK in the DOX-induced myocyte apoptosis is implicated. Recent studies have identified that the p38α is specifically involved in apoptosis of neonatal rat cardiomyocytes in primary cultures and p38β mediates hypertrophy of these cells (23). Further studies are required to determine which specific isoform(s) of p38 MAPK are essential in the signal transduction pathway of the DOX-induced apoptosis.

Another important novel observation in the present study is that MT inhibited both apoptosis and p38 MAPK activation by DOX in cardiomyocytes. Although DOX-induced apoptosis was partially inhibited (by 50%), the activation of p38 MAPK as detected by the fluorescent confocal microscopy was almost completely blocked in the MT-overexpressing transgenic myocytes. The extent of the inhibition of apoptosis in the myocytes was the same as that observed from the SB203580-treated non-transgenic cardiomyocytes exposed to DOX. Taken together, these observations suggest that MT suppresses DOX-induced apoptosis through inhibition of p38 MAPK activation. Importantly, the same inhibition by MT of p38 MAPK activation by DOX was also observed in vivo, which correlates with the observation that MT significantly inhibited DOX-induced myocardial apoptosis. The inhibitory action of MT in the DOX-induced apoptotic pathway is likely mediated by reducing the accumulation of reactive oxygen species, because MT inhibits DOX-induced lipid peroxidation in these cells (4).

The pathways by which DOX stimulates the formation of reactive oxygen species have been extensively studied. One is the formation of a DOX-iron complex (38). This complex spontaneously reacts to generate hydrogen peroxide and hydroxyl radicals, which cause oxidative damage (38, 39). Dextrazoxane (ICRF-187, ADR 529) reacts directly with the DOX-iron complex to promote the opening of its amide ring with a simultaneous transfer of the iron from DOX to the carboxylamine generated by the ring opening (40). This compound has been studied both experimentally and clinically for its potential as a cardioprotective agent (41). Limited protection against DOX cardiotoxicity with this agent has been observed, but it has never been sufficient (42). Most likely, this is due to other important pathways of reactive oxygen species generation by DOX.

The flavin reductases, including cytochrome P-450 reductase, cytochrome b₅, reductase, NADH dehydrogenase, and xanthine oxidase, all have the capacity to reduce DOX to DOX semiquinone free radical (43). In the presence of oxygen, the DOX semiquinone reacts rapidly to reduce the oxygen to superoxide, with regeneration of intact DOX. The superoxide is then converted to hydrogen peroxide, which is in turn converted to hydroxyl radical. The DOX semiquinone also reacts with hydrogen peroxide to yield hydroxyl radicals. A recent study has demonstrated another pathway, in which DOX binds to the endothelial isoform of nitric-oxide synthase and undergoes reduction mediated by that endothelial isoform to become the semiquinone radical (44). As a consequence, superoxide formation is enhanced and nitric oxide production is decreased. This may lead to generation of peroxynitrite and hydrogen peroxide, both of which are further converted to hydroxyl radicals. Neither of these two pathways of generation of reactive oxygen species by DOX is sensitive to the action of iron chelators.

Based on the results obtained from the present study and our previous observations, we speculate that MT may be useful in the prevention of DOX-induced cardiotoxicity. In particular, MT is highly inducible under a wide diversity 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 (11), isoproterenol (45), and tumor necrosis factor-α (46). Therefore, the basis for developing pharmaceutical agents to increase MT concentration in the heart already exists. Exploring the potential for MT to protect against DOX cardiotoxicity would likely result in novel approaches to this clinical problem and could positively influence clinical outcomes.

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