Metallothionein Inhibits Doxorubicin-Induced Mitochondrial Cytochrome c Release and Caspase-3 Activation in Cardiomyocytes

GUANG-WU WANG, JON B. KLEIN, and Y. JAMES KANG

Departments of Medicine (G.W.W., J.B.K., Y.J.K.) and Pharmacology and Toxicology (Y.J.K.), University of Louisville, Louisville, Kentucky; Veterans Affairs Medical Center (J.B.K.), Louisville, Kentucky; and Jewish Hospital Heart and Lung Institute (Y.J.K.), Louisville, Kentucky

Received November 2, 2000; accepted April 11, 2001

ABSTRACT

Previous studies using transgenic mice in which metallothionein (MT) was overexpressed only in the heart have demonstrated that MT protects from oxidative cardiac injury induced by doxorubicin (DOX), an important anticancer agent. MT cardioprotection is associated with its antiapoptotic effect. The present study was undertaken to test the hypothesis that MT suppresses DOX-induced apoptosis through inhibition of mitochondrial cytochrome c release and caspase-3 activation. Primary cultures of cardiomyocytes isolated from the hearts of transgenic neonatal mice and nontransgenic controls were treated with DOX at a clinically relevant concentration (1.0 μM) for varying time periods. Apoptosis was detected in nontransgenic cardiomyocyte cultures by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling and Annexin V-fluorescein isothiocyanate binding. This apoptotic effect was significantly suppressed in the MT-overexpressing transgenic cardiomyocytes. Western blot analysis revealed that DOX caused mitochondrial cytochrome c release. Furthermore, caspase-3 activation was observed. The activation of this apoptotic pathway by DOX was dramatically inhibited in the MT-overexpressing cardiomyocytes. To elucidate the role of reactive oxygen species (ROS) in the activation of the cytochrome c-mediated caspase-3 activation pathway, the intracellular levels of ROS and their localization were detected by fluorescent confocal microscopy. Mitochondrial ROS concentrations were dramatically elevated by DOX in nontransgenic cardiomyocytes. This elevation was completely inhibited almost in the MT-overexpressing cardiomyocytes. Thus, these results demonstrate that MT suppresses DOX-induced apoptosis in cardiomyocytes through, at least in part, inhibition of the cytochrome c-mediated apoptotic pathway.

This work was supported in part by National Institutes of Health Grants CA68125 and HL59225, an Established Investigator Award (9640091 N) from the American Heart Association National Center, a research grant from the Jewish Hospital Foundation, Louisville, KY (J.B.K.), and a research grant from National Institutes of Health Grant HL66358 (J.B.K.). Y.J.K. is a University scholar of the University of Louisville. This work was presented in part at the 39th Annual Meeting of the Society of Toxicology held in Philadelphia, Pennsylvania, March 19–23, 2000.

ABBREVIATIONS: MT, metallothionein; DOX, doxorubicin; MAPK, mitogen-activated protein kinase; ROS, reactive oxygen species; DCFDA, dichlorofluorescein diacetate; DCF, dichlorofluorescein; FITC, fluorescein isothiocyanate; CMXRos, Mito Tracker Red CMXRos; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; PBS, phosphate-buffered saline; LDH, lactate dehydrogenase; TG, thioglycerol; CHAPS, 3-(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.
include mitochondrial cytochrome c release, followed by a formation of cytochrome c and dATP-dependent apoptotic protease-activating factor-1/caspase-9 complex, and eventually activation of caspase-3 (Liu et al., 1996; Li et al., 1997). The induction of apoptosis is then associated with caspase-3-mediated cleavage of poly (ADP-ribose) polymerase, protein kinase C δ, and other proteins (Datta et al., 1996; Tafani et al., 2000; Tian et al., 2000). The role of the cytochrome c-mediated apoptotic pathway in DOX-induced apoptosis in cultured cells, including cardiomyocytes, has not been examined. We believe that this pathway would contribute significantly to myocardial cell death induced by DOX and MT by inhibiting oxidative stress, which would inhibit mitochondrial cytochrome c release, leading to inhibition of caspase-3 activation and suppression of apoptosis.

Therefore, the present study was undertaken to test the hypothesis that MT inhibits DOX-induced ROS accumulation in the myocardium, leading to inhibition of mitochondrial cytochrome c release and suppression of caspase-3 activation. To accomplish this, we used primary cultures of cardiomyocytes isolated from the MT-overexpressing transgenic neonatal mouse hearts and the controls. This experimental model has been proven to be a valuable tool in studying the cellular and molecular mechanisms of the effect of MT on DOX-induced myocardial injury (Wang et al., 1999), including apoptosis (Kang et al., 2000).

Materials and Methods

Chemicals

Anti-cytochrome c (7H8.2C12) and anti-caspase-3 (active form) antibodies were purchased from PharMingen (San Diego, CA). Horseradish peroxidase-conjugated secondary antibody (anti-mouse IgG) was obtained from Sigma (St. Louis, MO). An ApoTag in situ apoptosis detection kit (S7001 kit) was purchased from Oncor (Gaithersburg, MD). An Annexin V-FITC apoptosis detection kit (6693K) was obtained from PharMingen. Carbonyl-dichlorodihydrofluorescein diacetate (carboxy-H₂DCFDA) and Mito Tracker Red CMXRs (CMXRos) were purchased from Molecular Probes (Eugene, OR). All other reagents were obtained from Sigma and were at least analytic grade.

Neonatal Mouse Ventricular Cardiomyocyte Culturing

Transgenic mice in which MT was overexpressed only in the heart were produced as described previously (Kang et al., 1997). These animals were maintained at the animal quarters of the University of Louisville at 22°C with a 12-h light/dark cycle. They had free access to rodent chow and tap water. The transgenic founder mice were bred with nontransgenic mice of the same strain. Transgenic littermates were identified at birth by a pigment marker (dark eye and fur). The pigment transgene was coinjected with the MT transgene into the early embryo when the transgenic mice were produced. Both, transgenic positive and negative neonatal mouse ventricular cardiomyocytes were isolated as described previously and cultured in minimal essential medium/20% fetal bovine serum (Wang et al., 1999). The purity of the cultures (i.e., the percentage of cardiomyocytes in the cultures) was 94 ± 5%, as described previously (Wang et al., 1999). 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 (Frederick, MD).

Determination of Cellular MT Concentration

Total MT was determined by a cadmium-hemoglobin affinity assay (Eaton and Cherian, 1991), as described previously (Wang and Kang, 1999).

Assays for Detecting Apoptotic Cells

Identification of apoptotic cardiomyocytes was performed by terminal deoxynucleotidyl transferase-mediated deoxyuridine nick-end labeling (TUNEL) assay and further confirmed by Annexin V-FITC binding.

TUNEL Assay. Cardiomyocytes plated on Lab-Tek II chamber glass slide system (Nalge Nunc, Naperville, IL) 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 washing with PBS, the cells were incubated with the reaction mixture of TUNEL for 1.0 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 1000 nuclei in triplicate plates.

Annexin V-FITC Binding. This assay was performed on cardiomyocytes that had been plated on Lab-Tek chambered glass slides (Nalge Nunc). The cells were washed with a binding buffer and stained with FITC-conjugated Annexin V (BD Pharmingen, San Diego, CA) for 15 min. The samples were then sectioned optically with a Zeiss LSM510 confocal microscope equipped with an Axiovert 100 M microscope (Carl Zeiss, Inc., Thornwood, NY).

Analysis of Mitochondrial Cytochrome c Release

Cytochrome c release from mitochondria into the cytosol was measured by Western blot analysis, as described previously (Liu et al., 1996; Kim et al., 1997). Cells were harvested by centrifugation at 1,000g for 10 min at 4°C. The cell pellets were washed once with ice-cold PBS and resuspended with 5 volumes of buffer A (20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl₂, 1.0 mM sodium EDTA, 1.0 mM sodium EGTA, 1.0 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 250 mM sucrose), supplemented with protease inhibitors (10 µg/ml pepstatin A, 10 µg/ml leupeptin, 10 µg/ml aprotinin). After sitting on ice for 15 min, the cells were homogenized with 10 strokes of a Teflon glass homogenizer. The nuclei and cell debris were removed by centrifugation at 1,000g for 15 min at 4°C. The supernatants were centrifuged at 10,000g for 15 min at 4°C, and the resulting mitochondrial fractions were resuspended with buffer A. The supernatants created at 10,000g were further centrifuged at 100,000g for 1 h at 4°C. The supernatants (S-100) and mitochondrial fractions were stored at −80°C. Activities of lactate dehydrogenase and citrate synthase in both the cytosolic and mitochondrial fractions were determined, as described below, to examine cross-contamination between the two fractions. The protein concentrations of mitochondria and S-100 were determined by the Bradford method (Bradford, 1976). Proteins (25 µg) extracted from the cytosol and mitochondria were separated by 15% SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.01% Tween 20 and probed with purified mouse anti-cytochrome c monoclonal antibody (7H8.2C12, PharMingen). Blots were washed, incubated with goat anti-mouse IgG conjugated to horseradish peroxidase, and developed by incubation with enhanced chemiluminescence Western blot detection reagents (Amersham Pharmacia Biotech, Inc., Piscataway, NJ). Signal intensities of the proteins in Western blot were determined by a densitometric analysis (Personal Densitometer SI, Molecular Dynamics, Sunnyvale, CA).

Assay of Lactate Dehydrogenase Activity. The activity of lactate dehydrogenase (LDH) in the cytosolic and mitochondrial fractions was determined as described previously (Weider et al., 1991).
After isolation, 100 µL of each fraction 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 of phosphate buffer). The rate of NADH oxidation was determined by following the decrease in absorbance at 340 nm at 25°C using a spectrophotometer (model DU-650; Beckman Coulter, Fullerton, CA). The activity was expressed as units per milligram of protein.

**Citrate Synthase Activity.** The activity of citrate synthase in the cytosolic and mitochondrial fractions was determined as described previously (Serre, 1969). After isolation, 50 µL of each fraction was collected, and the citrate synthase activity was assayed in 100 µL of 5.5'-dithiobis(2-nitrobenzoic acid) (1 mM), 30 µL of acetyl-CoA (10 mM), and 770 µL of H₂O. The citrate synthase reaction was started by adding 50 µL of oxaloacetate. The linear rates were obtained for at least 3 min at 25°C using a spectrophotometer. The activity was expressed as units per milligram of protein.

**Assay for Caspase-3 Activation**

Activation of caspase-3 was detected by laser confocal microscopy, using polyclonal antibody against active caspase-3 (PharMingen). Cardiomyocytes that had been plated on Lab-Tek II chambered glass slides were washed with PBS and fixed and permeated with ice-cold methanol/acetone (1:1) for 10 min at −20°C. The cells were then incubated with 20% nonimmune goat whole serum in PBS for 30 min at 37°C with primary antibody (1:4000 dilution in PBS containing 10% goat whole serum) for 1 h at 37°C and goat anti-rabbit IgG conjugated to FITC (1:1000), respectively. The samples were then observed under laser confocal microscopy.

Caspase-3 activity was determined by using a caspase-3 colorimetric protease assay kit (Chemicon International, Temecula, CA). The assay was based on spectrophotometric detection at 405 nm of the chromophore p-nitroanilide (pNA) after cleavage from the labeled substrate DEVD-pNA by caspase-3. Cells were harvested by centrifugation at 1000 g for 10 min at 4°C. The cell pellets were washed once with ice-cold PBS and resuspended in extract buffer containing 25 mM HEPES, pH 7.5, 5 mM EDTA, 2 mM dithiothreitol, 0.1% CHAPS, 1.0 mM phenylmethylsulfonyl fluoride, and 10 µg/mL apro tinin. After sitting on ice for 20 min, the suspension was forced through a 25-gauge needle 10 times to break cells. The homogenate was centrifuged at 10,000g for 30 min at 4°C. The supernatants were stored at −80°C for assays. The protein concentration was determined by the Bradford method. For enzyme assay, a 96-well microplate was equilibrated to 37°C for 10 min. The cell lysates (50 µg of protein in 50 µL) and 2× reaction buffer (50 mM HEPES, pH 7.5, 100 mM NaCl, 1.0 mM EDTA, 10 mM dithiothreitol, 0.1% CHAPS, 10% glycerol) were added to each well and incubated for 10 min at 37°C before adding the substrate (200 µM DEVD-pNA). The absorbance at 405 nm was read using a microtiter reader (EL311s; Bio Tek Instruments, Inc., Winooski, VT) and recorded at 10-min intervals for 2 h.

**Assay for ROS Accumulation in Cardiomyocytes**

Detection of ROS accumulation in cardiomyocytes was done by a carboxy-H₂DCFDA staining method as described previously (Karbowski et al., 1999). This assay is based on the principle that the nonpolar, nonionic H₂DCFDA crosses cell membranes and is enzymatically hydrolyzed by intracellular esterases to nonfluorescent H₂DCF (LeBel et al., 1992; Garland and Halestrap, 1997). In the presence of ROS, H₂DCF is rapidly oxidized to become highly fluorescent DCF. Both nontransgenic and transgenic cells were treated with DOX for varying times. At each time point, cells treated with saline were used as the control. At the end of DOX or saline treatment (performed simultaneously), all the cells were incubated for 1.0 h at 37°C with 5 µM carboxy-H₂DCFDA dissolved in the culture medium. To confirm the specificity of the DCFDA reaction with ROS, a control experiment in which the cells were treated with saline only, saline with DCFDA, DOX only, or DOX with DCFDA, was performed. A time course response of the cells was followed. DCFDA was added 1 h before the cells were processed for ROS analysis. The result showed that there was no detectable fluorescence in the cells treated with DCFDA without DOX. To determine the compartmentalized accumulation of ROS, mitochondria were visualized by a cell-permeable, mitochondria-specific fluorescent dye, CMXRos. This provides a dual staining if ROS is accumulated in the mitochondria. The cells were incubated with CMXRos at a final concentration of 500 nM for 30 min after incubating with the carboxy-H₂DCFDA for 30 min. The culturing coverslips were then fixed with a fixative containing 2% glutaraldehyde and 2% formaldehyde dissolved in PBS and analyzed under a confocal laser microscope using FITC/rhodamine barrier filters with excitation settings of 543 and 488 nm, respectively. The laser intensities (50%) and photodetector gains (720) were held constant to allow comparisons of relative fluorescence intensities of cells between the transgenic and nontransgenic cardiomyocytes.

**Results**

**Effect of MT on DOX-Induced Apoptosis.** MT-overexpressing transgenic cardiomyocytes and nontransgenic controls were exposed to 1.0 µM DOX for varying times after culturing for 6 days. The total cellular MT concentrations were 1.01 ± 0.13 and 0.44 ± 0.09 µg/mg of protein in transgenic and nontransgenic cardiomyocytes, respectively. As

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<th>Non-TG DOX</th>
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<th>MT-TG</th>
<th>MT-TG DOX</th>
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<tr>
<td>Apoptotic Cell (%)</td>
<td>2.52±0.52</td>
<td>23.95±3.51</td>
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Fig. 1. Detection of apoptotic cells by TUNEL assay. Primary neonatal mouse cardiomyocytes maintained in cultures for 6 days and then exposed to DOX at a final concentration of 1.0 µM for 6 h (original magnification, 260×). Results of quantitative analysis are presented under the microphotograph. a, significantly different from respective controls; b, significantly different from nontransgenic/DOX-treated cell cultures (p < 0.05).
shown in Fig. 1, a small number of cells underwent apoptosis spontaneously, and a substantial number of cells were apoptotic in the DOX-treated nontransgenic cardiomyocyte cultures, as determined by the TUNEL assay. Quantitative data showed that about one-fourth of the total populations of cultured cardiomyocytes underwent apoptosis. This apoptotic effect of DOX was dramatically inhibited in the MT-overexpressing myocytes, about 50% inhibition was observed. To confirm the results from the TUNEL assay, a more apoptotic sensitive and early phase 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 the DOX-treated nontransgenic cardiomyocyte cultures was much more than in the transgenic cultures, which confirms the results obtained from the TUNEL assay.

**Effect of MT on DOX-Induced Mitochondrial Cytochrome c Release.** Cytochrome c contents in the mitochondria and cytosol after DOX treatment were measured by a Western blot assay. To ensure there was no cross-contamination between the cytosolic and mitochondrial fractions caused by the isolation procedure, activities of LDH and citrate synthase in each fraction were measured. The LDH activities were 0.294 and 0.015 U/mg of protein in the cytosolic and the mitochondrial fraction, respectively. The activities of citrate synthase were 0.717 and 0.004 U/mg of protein in the mitochondrial and the cytosolic fraction, respectively. There was no difference in the distribution of these enzyme activities between nontransgenic and MT-overexpressing transgenic cells. Therefore, there was no accountable cross-contamination between the cytosolic and mitochondrial fractions. DOX significantly increased cytosolic concentrations of cytochrome c with a concomitant decrease in mitochondria, as shown in Fig. 3A. Quantitative data showed that cytosolic cytochrome c concentrations were increased from 20 to 52% of the total cellular concentration in the DOX-treated nontransgenic cardiomyocytes. This effect was suppressed sig-
nificantly in the MT-overexpressing transgenic cardiomyocytes (Fig. 3B). The DOX-induced mitochondrial cytochrome c release was inhibited by about 75% in the MT-overexpressing transgenic cardiomyocytes (Fig. 3, A and B).

**Inhibition by MT of DOX-Induced Caspase-3 Activation.** The activation of caspase-3 was examined by immunofluorescent confocal microscopy using an anti-active caspase-3 antibody. This analysis revealed that DOX caused a marked activation of caspase-3 in nontransgenic cardiomyocytes. This activation was blocked in the MT-overexpressing transgenic cells (Fig. 4). The result obtained from the immunofluorescent confocal microscopic study was further confirmed by the analysis of caspase-3 activity. As shown in Fig. 5, DOX increased caspase-3 activity in nontransgenic cardiomyocytes. This elevation was suppressed significantly in the transgenic cells. To demonstrate the essentiality of caspase-3 in DOX-induced apoptosis, Ac-DEVD-cmk, an inhibitor of caspase-3, was used. Cells were treated with Ac-DEVD-cmk (1.0 μM) for 30 min before exposure to DOX. This inhibitor efficiently suppressed caspase-3 activity (Fig. 6) and reduced the number of apoptotic cells (Fig. 7).

**Effect of MT on DOX-Induced ROS Accumulation.** Oxidative stress triggers mitochondrial cytochrome c release, leading to apoptosis (Zhuang et al., 1999), and DOX is known to generate ROS during its intracellular metabolism. We therefore examined whether there is a correlation between accumulation of ROS produced by DOX and cytochrome c-mediated apoptosis. A nonfluorescent dye, H₂-DCFDA, has been shown to enter the cell passively and to form a fluorescent DCF in the presence of ROS, which is a reporter of ROS generation at the level of a single cell (Karbowski et al., 1999). In this study, we used a carboxy-H₂-DCFDA, an analog of H₂-DCFDA that has an enhanced retention inside the cell due to two negative charges at physiological pH. The results presented in Fig. 8 show that the ROS level was elevated remarkably in nontransgenic cardiomyocytes. This

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**Fig. 4.** Detection of caspase-3 activation by confocal microscopy. Cardiomyocytes were maintained in cultures for 6 days and then exposed to 1.0 μM DOX for 3 h. Caspase-3 activation was detected by an anti-active caspase-3 antibody and observed with a laser confocal microscope (original objective lens magnification, 63×).

**Fig. 5.** Enzymatic assay of caspase-3 activity. Cardiomyocytes were maintained in cultures for 6 days and then exposed to 1.0 μM DOX for 6 h. Activity of caspase-3 was measured by using a caspase-3 colorimetric protease assay kit. Values represent mean ± S.D. from triplicate samples for each experiment. a, significantly different from the corresponding controls; b, significantly different from nontransgenic/DOX-treated cells (p < 0.05).

**Fig. 6.** Effect of Ac-DEVD-cmk on the activity of caspase-3 induced by DOX. Nontransgenic cardiomyocytes were maintained in cultures for 6 days and then exposed to Ac-DEVD-cmk (1.0 μM) 30 min before exposure to 1.0 μM DOX for 6 h. Values represent mean ± S.D. from triplicate samples for each experiment. a, significantly different from respective controls; b, significantly different from DOX-treated cells (p < 0.05).
Discussion

The results obtained from the study show that MT at a level that can be induced by chemical inducers in the heart significantly inhibits DOX-induced apoptosis in the cardiomyocytes. This inhibition correlates with the inhibitory effect of MT on DOX-induced mitochondrial cytochrome c release and caspase-3 activation. The caspase-3 activation was critically involved in the DOX-induced myocardial apoptosis as demonstrated by the result obtained from the experiment using the caspase-3 inhibitor Ac-DEVD-cmk. Moreover, MT effectively blocked ROS accumulation in the DOX-treated cardiomyocytes. Many reports have demonstrated that ROS induces apoptosis in vivo and in vitro by activating the cytochrome c-dependent caspase-3 activation pathway. Therefore, the present study demonstrates that MT suppresses DOX-induced myocardial apoptosis through, at least in part, inhibition of the mitochondrial cytochrome c release-mediated apoptotic pathway, which is triggered by the increased levels of ROS generated by DOX. Thus, the mechanism of action of MT probably is the inhibition of ROS accumulation in the cardiomyocytes.

The concentration of DOX used in the present study is clinically relevant. A range of 0.1 to 1.0 μM DOX accumulated in the myocardium has been shown to occur between 15 and 60 min after treatment of Syrian hamsters with DOX at a single i.v. dose of 5 mg/kg (Egorin et al., 1974). The same observation was also made in a study using a rat model 30 min after treatment with DOX by i.v. infusion at 16 mg/kg (Lee et al., 1995). Therefore, these results indicated that cardiomyocytes exposed to in vivo pharmacologically comparable levels of DOX undergo apoptosis. We have shown that this mode of cell death contributes remarkably to the total loss of cardiomyocytes in the DOX-treated myocardium (Kang et al., 2000).

Molecular and cellular mechanisms by which DOX induces cardiomyocyte apoptosis include the activation of the mitochondrial pathway, which involves the release of cytochrome c from the mitochondria. This release leads to the activation of caspase-3, a key enzyme in the execution phase of apoptosis. MT appears to inhibit this process, thereby preventing the activation of caspase-3 and thus protecting the cardiomyocytes from apoptosis.

Fig. 7. Effect of Ac-DEVD-cmk on DOX-induced apoptosis in nontransgenic cardiomyocytes by Annexin V-FITC staining. Primary nontransgenic cardiomyocytes were maintained in cultures for 6 days and then treated with Ac-DEVD-cmk (1.0 μM) 30 min before exposure to 1.0 μM DOX for 6 h (original objective lens magnification, 63×).

Fig. 8. Assay of ROS accumulation in cardiomyocytes. Both nontransgenic and transgenic cardiomyocytes were maintained in cultures for 6 days and then exposed to 1.0 μM DOX for different times as indicated. Carboxyl-H$_2$-DCFDA (for labeling ROS) and CMXRos (for mitochondrial staining) were added to the cultures for 60 and 30 min before the end of the treatment, respectively. A, cells stained by CMXRos for mitochondria; B, cells stained by carboxyl-H$_2$-DCFDA for ROS only; C, cells dually stained. Yellow color indicates ROS accumulation in mitochondria. Both nontransgenic and transgenic control cells were treated with saline for the same time course as indicated. The results obtained at different time points from the controls were essentially the same as those labeled for time “0”. Thus, the results for controls were not shown in the figure (original objective lens magnification, 63×).
myocardial injury have not been fully documented. Although it is widely accepted that the cardiac toxicity of DOX is mediated by ROS (Blum and Carter, 1974), a comprehensive understanding of the consequence of ROS generation is lacking. Two major aspects of recent progress in cardiac research have made further investigation of DOX cardiotoxicity possible. The first is the production of cardiac-specific antioxidant-overexpressing transgenic mice (Kang et al., 1996, 1997). These unique experimental models provide valuable tools to dissect cellular events that lead to DOX cardiotoxicity (Kang, 1999). The second is the establishment of a primary neonatal mouse ventricular cardiomyocyte culturing procedure (Wang et al., 1999). The relatively homogeneous cardiomyocyte populations in cultures make the myocyte-specific studies feasible.

Identifying cellular events and signal pathways leading to myocardial injury are essential for understanding mechanisms of DOX cardiotoxicity. Suppressing critical cellular events and blocking signal transduction pathways potentially lead to the inhibition of DOX-induced myocardial injury. However, the most effective approach would be inhibiting the trigger event that leads to cascade reactions, thereby resulting ultimately in damage. Our results identified that mitochondrial cytochrome c release, caspase-3 activation, and apoptosis comprise a pathway of critical importance that leads to DOX-induced myocardial cell death. In previous studies (Kang et al., 2000), we have demonstrated that p38 MAPK activation and apoptosis is another important pathway leading to DOX-induced cell death. Specific inhibitors for p38 MAPK (Kang et al., 2000) and for caspase-3 indeed reduced the number of DOX-induced apoptotic cells. However, MT efficiently blocked both pathways, along with inhibiting significantly DOX-induced apoptosis. Taken together, these results suggest that the p38 MAPK activation and the mitochondrial cytochrome c release may be triggered by a common upstream trigger, which probably is the upstream trigger that leads to apoptosis.

There is a correlation between increased MT concentrations and decreased accumulation of ROS in mitochondria. In previous studies, we have demonstrated that MT is not localized in mitochondria in the transgenic myocardium (Zhou and Kang, 2000). However, DOX-induced mitochondrial damage was suppressed significantly in the MT-overexpressing transgenic myocardium (Kang, 1999; Kang et al., 2000; Zhou and Kang, 2000). The result that DOX caused increased accumulation of ROS in mitochondria correlated well with DOX-induced mitochondrial damage. However, is the decreased accumulation of ROS in mitochondria increased cytosolic MT concentrations? If directly related, what is the mechanism of action of MT? To answer these questions is a rather difficult and important undertaking in our future studies.

Although the results obtained from this study suggest MT protection of myocardial cells from DOX-induced apoptosis through inhibition of oxidative stress, other possible mechanisms cannot be excluded. For example, MT is a zinc-binding protein, and under oxidative stress conditions, zinc is released from MT (Jacob et al., 1998). In cytoprotection, zinc has been shown to function against apoptosis by inhibiting caspase-3 activities (Aiuchi et al., 1998) and by regulating other signal transduction pathways (Meerarani et al., 2000). By activating transcription factors, zinc also up-regulates expression of genes that are involved in cytoprotection (Vallee and Falchuk, 1993). These scenarios need to be investigated in future studies.

Myocardial oxidative injury is a common cause of heart diseases. Apoptosis is a critical cellular process involved in oxidative heart injury. To rescue myocardial cells that are under oxidative stress, inhibitors for caspase have been investigated extensively to explore their potential to block apoptosis induced by oxidative stress (Islam et al., 2000; Tafani et al., 2000; Zisterer et al., 2000). However, the results obtained from the studies presented here and those reported previously (Kang et al., 2000) indicate that if the trigger event persists, the caspase inhibitor approach may not be efficient enough to block myocardial apoptosis because of multiple pathways. On the other hand, enhancement of MT expression would be a better approach to achieve the goal because MT may interact directly with the trigger event.

Acknowledgments

We thank Donald Mosley, Kristie Lock, and Angela Mitchell for technical assistance.

References

Li P, Nijihawan D, Bushidjardio I, Sinivasula SM, Ahmad M, Alnernrri ES and Wang...


Address correspondence to: Dr. Y. James Kang, Department of Medicine, University of Louisville School of Medicine, 511 S. Floyd St., MDR 530, Louisville, KY 40292. E-mail: yjkang01@athena.louisville.edu