Zinc Inhibits Doxorubicin-Activated Calcineurin Signal Transduction Pathway in H9c2 Embryonic Rat Cardiac Cells

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Elevation of the zinc-binding protein metallothionein (MT) in the heart inhibits doxorubicin (DOX)-induced myocardial apoptosis and heart hypertrophy. Zinc release from MT in response to oxidative stress has been suggested as a mechanism of action of MT protection from DOX toxicity, and calcineurin is involved in the signaling pathways leading to myocardial apoptosis and heart hypertrophy. The present study was undertaken to determine if zinc can modulate the DOX-activated calcineurin signaling pathway. H9c2 cells were treated with 1 μ*M* DOX, and zinc release was monitored by a zinc ion–specific fluorophore, zinquin ethyl ester. Additionally, DOX-activated calcineurin signaling was detected by a calcineurin-dependent nuclear factor of activated T-cell reporter. DOX treatment induced an increase in intracellular labile zinc and activated calcineurin signaling. Pretreatment of H9c2 cells with a zinc-specific, membrane-permeable chelating agent, N,N,N′,N′-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN), inhibited the increase in intracellular labile zinc and increased the DOX-activated calcineurin signaling. Pretreatment of H9c2 cells with exogenously added zinc attenuated the DOX-activated calcineurin signaling in a dose-dependent manner. However, neither TPEN nor addition of exogenous zinc affected DOX-induced cellular hypertrophy or DOX-induced decrease in cell viability. Additionally, inhibition of DOX-induced calcineurin signaling with the calcineurin inhibitors cyclosporine A or tacrolimus (FK506) failed to restrict the DOX-induced decrease in cell viability. These results indicate that zinc suppresses DOX-induced calcineurin signaling in H9c2 cells; however, calcineurin signaling is not involved in the DOX-induced decrease in cell viability in H9c2 cells. (It had been shown previously that calcineurin is also not necessary for DOX-induced H9c2 cell hypertrophy.) Exp Biol Med 232:682–689, 2007

Key words: zinc; doxorubicin; metallothionein; calcineurin; hypertrophy; H9c2 cells

Introduction

Previous studies have shown that elevation of metallothionein (MT) inhibits doxorubicin (DOX)-induced cardiomyopathy, including myocardial apoptosis and heart hypertrophy (1–4). DOX is a valuable component of multiple chemotherapeutic regimens; however, severe cardiotoxicity limits its clinical use (5). The proposed mechanism for the cardiotoxic effect of DOX is the production of reactive oxygen species (ROS) during its intracellular metabolism (1, 4, 6, 7). DOX undergoes one-electron reduction through metabolic activation by flavin reductases, including NADPH-cytochrome P450 reductase, cytochrome b_5 reductase, NADH reductase, and xanthine oxidase, thereby generating DOX semiquinone free radical (8, 9). This semiquinone free radical intermediate is highly unstable and rapidly reacts with molecular oxygen to generate superoxide free radical with regeneration of intact DOX (10). Superoxide is quickly converted by superoxide dismutase to H_2O_2, which in turn is converted to hydroxyl radical *via* the Fenton reaction (10). Furthermore, hydroxyl radicals are also produced *via* a reaction between the DOX semiquinone and H_2O_2 (10, 11). These highly toxic ROS react with cellular molecules, including nucleic acids, proteins, and lipids, thereby causing cellular damage. In this context, many efforts have been made to increase myocardial antioxidant capacity as an approach to decrease the cardiotoxicity of DOX.

MT is a highly conserved, low–molecular-weight, thiol-rich protein. Mammalian MT consists of 61 amino acids, including 20 cysteine residues, but no aromatic amino acids, histidine, or leucine (12). That MT functions as a potent antioxidant has been demonstrated in both *in vitro* (13–15) and *in vivo* (1, 16, 17) studies. Recent insights into the antioxidant action of MT suggest that the protective

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function of MT may be related to the MT redox cycle (18). The cluster structure of zinc-MT (Zn-MT) provides a chemical basis by which the cysteine ligand can induce oxidoreductive properties (19). This structure allows for the thermodynamic stability of zinc in MT while permitting zinc to retain kinetic lability. The sulfur ligand confers redox activity on the Zn-MT complex and can be oxidized and reduced with concomitant release and binding of zinc in an oxidoreductive environment (18).

Zinc is an essential trace element that is involved in a variety of cellular functions including structural, catalytic/enzymatic, and regulatory processes. Zinc affects protein phosphorylation signaling and can modulate the activity of numerous protein kinases and phosphatases, including the calcium/calmodulin-dependent serine/threonine phosphatase calcineurin. (20–28). Calcineurin plays a pivotal role in the development of cardiac hypertrophy and has also been implicated in the process of programmed cell death (29, 30).

DOX has previously been shown to increase calcineurin activity, and it activates the subsequent signaling pathway via the downstream transcriptional effector of calcineurin, nuclear factor of activated T cells (NFAT) (30, 31). DOX increases the accumulation of ROS in the cell, and ROS mobilize zinc from MT, thus making zinc available under oxidative stress conditions. It would therefore be interesting to know the effect of oxidative intracellular zinc mobilization on the calcineurin-NFAT signaling pathway. The present study was undertaken to determine if zinc (endogenously released and/or exogenously added) can modulate DOX-induced calcineurin-NFAT signaling.

Methods

Materials and Reagents. Doxorubicin hydrochloride (DOX), N,N,N′,N′-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN), zinc chloride ($ZnCl_2$), zinquin ethyl ester, cyclosporine A (CsA), and methylthiazolyldiphenyl-tetrazolium bromide were purchased from Sigma-Aldrich (St. Louis, MO). Tacrolimus (FK506) was purchased from PKC Pharmaceuticals, Inc. (Woburn, MA). All other reagents, which were at least analytic grade, were obtained from Sigma-Aldrich unless otherwise indicated.

Cell Culture and Treatment. Embryonic rat heart–derived cell line H9c2, obtained from the American Type Culture Collection (Manassas, VA), was cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA), L-glutamine (4 m*M*), 1.5 g/liter sodium bicarbonate, penicillin (100 units/ml), and streptomycin (100 μg/ml). The cells were maintained at 37°C under a water-saturated atmosphere of 95% ambient air and 5% CO_2. Stock cultures were passaged at 2- to 3-day intervals. The cells were cultured for 24 hrs prior to each experiment in DMEM containing 10% FBS.

For all experiments involving TPEN, $ZnCl_2$, CsA, or tacrolimus, H9c2 cells were pretreated for 2 hrs with TPEN, $ZnCl_2$, CsA, or tacrolimus prior to the addition of DOX. Cells were incubated with DOX at 37°C and 5% CO_2 in DMEM containing 10% FBS for 2 hrs for all experiments involving adenoviral NFAT-luciferase reporter (AdNFAT-luc) or measurements of protein content per cell and cell size. Following the 2-hr treatment, DOX-containing media were removed from the cells and fresh medium and TPEN or $ZnCl_2$ were added back to the cells when necessary for an additional 22 or 46 hrs. For measurements of cell viability, cells were incubated with DOX at 37°C and 5% CO_2 in DMEM containing 10% FBS for 24 or 48 hrs after pretreatment with TPEN, $ZnCl_2$, CsA, or tacrolimus.

Intracellular Zinc Staining with Zinquin. H9c2 cells were washed once with phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and then incubated with the zinc-specific fluorophore zinquin ethyl ester (20 μ*M*) in PBS containing 1% BSA for 15 mins at 37°C. DOX and TPEN were added at final concentrations of 1 μ*M* and 2.5 μ*M*, respectively, and cells were observed with a Nikon TE2000-S fluorescence microscope (Nikon Inc., Melville, NY).

Adenovirus Propagation and Infection. AdNFAT-luc was propagated and purified as previously described (31). H9c2 cells were infected at a multiplicity of infection of 100 plaque-forming units per cell in PBS supplemented with 2% FBS for 2 hrs at 37°C and 5% CO_2, followed by the addition of fresh medium containing FBS and incubation for an additional 22 hrs. The medium was replaced 24 hrs after infection with DMEM containing 10% FBS and incubated for an additional 24 hrs prior to treatment. Under these conditions, approximately 98% of cells were infected as assessed by infection with the same titer of Ad-GFP.

Luciferase Enzymatic Assay. Luciferase enzymatic activity in cell extracts was measured using the Luciferase Assay System (Promega, Madison, WI) according to the supplier's instructions. The light intensity was measured with a Sirius single-tube luminometer (Berthold Detection Systems, Oak Ridge, TN) over 10 secs and expressed as relative light units over 10 secs per μg of protein.

Measurement of Protein Content per Cell. Cells in 35-mm dishes were collected by trypsinization with trypsin-EDTA (Invitrogen) and washed twice in ice-cold PBS. Cells were then collected *via* centrifugation and lysed with 15 μl of cell lysis buffer (20 m*M* Tris-HCl [pH 7.5], 150 m*M* NaCl, 1 m*M* Na_2EDTA, 1 m*M* EGTA, 1% Triton, 2.5 m*M* sodium pyrophosphate, 1 m*M* β-glycerophosphate, 1 m*M* Na_3VO_4, 1 μg/ml leupeptin, and 1 m*M* phenylmethylsulfonyl fluoride) on ice for 30 mins. The suspension was centrifuged at 14,000 *g* for 10 mins at 4°C, and the supernatant was collected. Protein concentration in total cell lysate was measured using the Bradford assay (Bio-Rad, Hercules, CA) with BSA as a standard. Protein content per cell was determined by dividing the total amount of protein

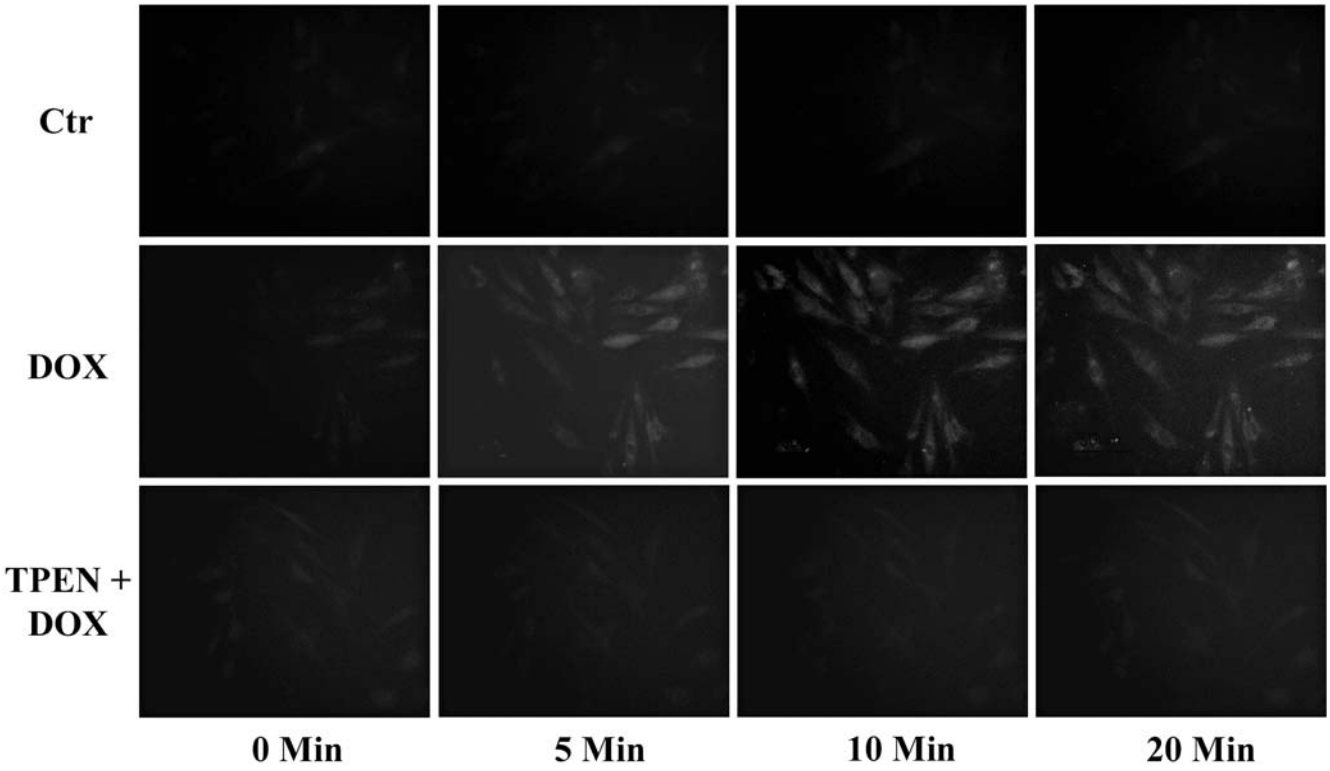

Figure 1. Detection of labile zinc by zinquin ethyl ester in H9c2 cells treated with DOX and TPEN. H9c2 cells in PBS containing 1% BSA were loaded with the zinc-specific fluorophore zinquin ethyl ester (20 μ*M*). Ctr = control cells; DOX = cells exposed to 1 μ*M* DOX; TPEN + DOX = cells pretreated with 2.5 μ*M* TPEN for 2 hrs prior to exposure to 1 μ*M* DOX. Photographs are representative of three independent experiments with consistent results.

by the cell number, which was determined using a hemocytometer.

Measurement of Cell Volume. Adherent cells in 35-mm dishes were detached via trypsinization, and images of rounded cells were acquired using a Nikon TE2000-S microscope with attached digital camera and ×20 lens. Measurements of cell diameters were made using Microsoft Office Document Imaging software, and cell volume was calculated using the equation for the volume of a sphere ($4/3[\pi r^3]$). The diameters of individual cells were measured, and one hundred cells per experimental group were measured randomly.

Microculture Tetrazolium (MTT) Assay. Cell viability was determined by an MTT assay. Cells were seeded at a density of 1.95×10^4 cells/well in 24-well cell culture plates. Following treatment, H9c2 cells were gently washed in warm PBS. Subsequently 450 μl of DMEM without phenol red or FBS and 50 μl of MTT solution (2 mg/ml MTT in PBS) were added to each well and incubated at 37°C for 4 hrs. The DMEM-MTT solution was then removed, and the formazan crystals were dissolved in 500 μl of 0.04 *N* HCl in isopropanol. The absorbance at 540 nm was subsequently measured. Percent viability was defined as the relative absorbance of treated versus untreated control cells.

Statistical Analysis. Data are presented as mean ± SD. Experiments involving a 2×2 factorial experimental design were analyzed by two-way analysis of variance (ANOVA). After a significant interaction was detected by the two-way ANOVA, the significance of the main effects was further determined. The level of significance was considered at $P < 0.05$.

Results

Mobilization of Endogenous Zinc by Doxorubicin Is Inhibited by the Zinc-Chelating Agent TPEN. To determine whether or not DOX induces mobilization of endogenous zinc in the H9c2 rat cardiac cell line, H9c2 cells were incubated with the zinc ion–specific fluorophore zinquin ethyl ester for 15 mins prior to and throughout treatment with 1 μ*M* DOX. Following DOX treatment, intracellular zinc was rapidly mobilized (within 5 mins) in H9c2 cells and gradually increased to saturation within 20 mins as visualized *via* fluorescence microscopy using zinquin as the zinc indicator (Fig. 1). Addition of the membrane-permeable, zinc-chelating agent TPEN quenched the zinquin fluorescence in DOX-treated cells, indicating zinc specificity (data not shown). Furthermore, pretreatment of H9c2 cells with TPEN inhibited the DOX-induced increase in zinquin fluorescence (Fig. 1).

Chelation of DOX-Mobilized Zinc with TPEN Increases DOX-Induced Calcineurin-NFAT Signal-

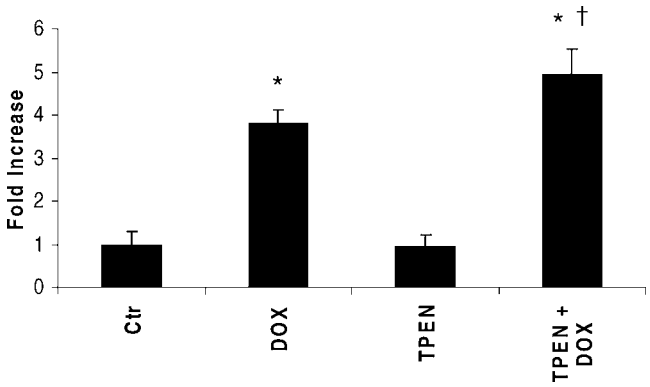

Figure 2. AdNFAT-luc reporter activity in H9c2 cells treated with DOX and/or TPEN. AdNFAT-luc–infected H9c2 cells in DMEM containing 10% FBS were pretreated with 2.5 μ*M* TPEN for 2 hrs prior to 2-hr treatment with 1 μ*M* DOX. Cells were then allowed to recover in fresh medium containing 10% FBS and TPEN for an additional 22 hrs. NFAT reporter activity was determined *via* the luciferase enzymatic assay as described in Materials and Methods. The data are mean ± SD of triplicates from one experiment representative of two. * = significantly different from corresponding control group ($P < 0.05$); † = significantly different from DOX group ($P < 0.05$).

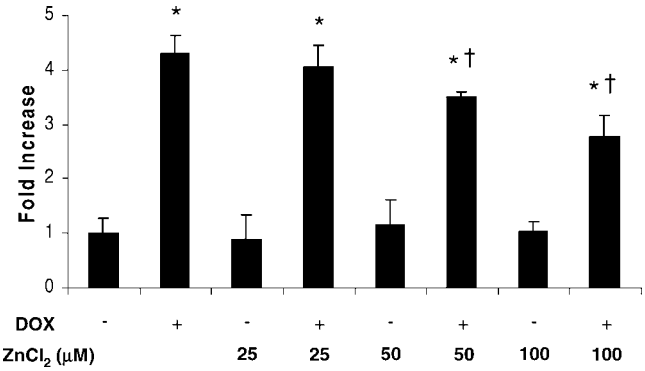

Figure 3. AdNFAT-luc reporter activity in H9c2 cells treated with DOX and/or $ZnCl_2$. AdNFAT-luc–infected H9c2 cells in DMEM containing 10% FBS were pretreated with $ZnCl_2$ (25, 50, or 100 μ*M*) for 2 hrs prior to 2-hr treatment with 1 μ*M* DOX. Cells were then allowed to recover in fresh medium containing 10% FBS and $ZnCl_2$ for an additional 22 hrs. NFAT reporter activity was determined *via* the luciferase enzymatic assay as described in Materials and Methods. The data are mean ± SD of triplicates from one experiment representative of two. * = significantly different from corresponding control group ($P < 0.05$); † = significantly different from DOX group ($P < 0.05$).

ing. DOX has previously been shown to activate calcineurin-NFAT signaling in H9c2 rat cardiac muscle cells (30, 31). To determine if DOX-induced calcineurin-NFAT signaling is modulated by endogenous cellular zinc, H9c2 rat cardiac cells were infected with AdNFAT-luc. AdNFAT-luc–infected H9c2 cells were treated with 2.5 μ*M* TPEN for 2 hrs prior to and throughout treatment with 1 μ*M* DOX. After the 2-hr DOX treatment, the cells were allowed to recover in fresh medium containing TPEN for an additional 22 hrs. In the presence of TPEN, DOX-induced NFAT reporter activity was increased in comparison with AdNFAT-luc–infected cells treated with DOX alone (Fig. 2). Treatment of AdNFAT-luc–infected H9c2 cells with TPEN alone, however, did not affect NFAT reporter activity (Fig. 2).

Addition of Exogenous Zinc Attenuates DOX-Induced Calcineurin-NFAT Signaling. The finding that TPEN treatment increased DOX-induced calcineurin-NFAT signaling in H9c2 cells suggests that zinc inhibits calcineurin-NFAT signaling. We next sought to determine if addition of exogenous zinc could have a similar effect on calcineurin-NFAT signaling. AdNFAT-luc–infected H9c2 cells were treated with increasing concentrations of $ZnCl_2$ for 2 hrs prior to and throughout treatment with 1 μ*M* DOX. After the 2-hr DOX treatment, the cells were allowed to recover in fresh medium containing $ZnCl_2$ for an additional 22 hrs. In the presence of $ZnCl_2$, DOX-induced NFAT reporter activity was attenuated in a dose-dependent manner in comparison with AdNFAT-luc–infected cells treated with DOX alone (Fig. 3). Treatment of AdNFAT-luc–infected H9c2 cells with $ZnCl_2$ alone did not affect NFAT reporter activity (Fig. 3).

Zinc Does Not Protect Against DOX-Induced Cellular Hypertrophy. DOX treatment has previously been shown to induce cellular hypertrophy in H9c2 cells (31). Since calcineurin has been shown to be essential and sufficient to induce heart hypertrophy (32), we next sought to determine if zinc can inhibit DOX-induced hypertrophy in H9c2 cells. We exposed H9c2 cells to 2.5 μ*M* TPEN or to increasing concentrations of $ZnCl_2$ (25, 50, or 100 μ*M*) for 2 hrs prior to and throughout treatment with 1 μ*M* DOX. After the 2-hr DOX treatment, the cells were allowed to recover in fresh medium containing TPEN or $ZnCl_2$ for an additional 46 hrs (48 hrs total). While the cell volume and protein content per cell following treatment with 1 μ*M* DOX were significantly increased, treatment with TPEN or $ZnCl_2$ (25–100 μ*M*) did not affect the DOX-induced hypertrophic response in H9c2 cells (Fig. 4). These results indicate that zinc alone does not inhibit DOX-induced cellular hypertrophy.

Zinc Does Not Protect Against the DOX-Induced Decrease in Cell Viability. Zinc is generally thought to be cytoprotective and can suppress major pathways leading to apoptosis (33). We next sought to determine if zinc can inhibit the DOX-induced decrease in cell viability in H9c2 cells. H9c2 cells were pretreated with 2.5 μ*M* TPEN or with increasing concentrations of $ZnCl_2$ (25, 50, or 100 μ*M*) for 2 hrs. The cells were subsequently exposed to 1 μ*M* DOX for 24 or 48 hrs in the presence of TPEN or $ZnCl_2$, and cell viability was analyzed by an MTT assay. Exposure of H9c2 cells to DOX resulted in a time-dependent decrease in cell viability. Pretreatment with TPEN or $ZnCl_2$ (25–100 μ*M*) did not affect the DOX-induced decrease in cell viability (Fig. 5). Additionally, treatment of H9c2 cells with TPEN or $ZnCl_2$ did not affect cell viability, indicating that TPEN or $ZnCl_2$ (25–100 μ*M*) are not cytotoxic to H9c2 cells (Fig. 5). These results

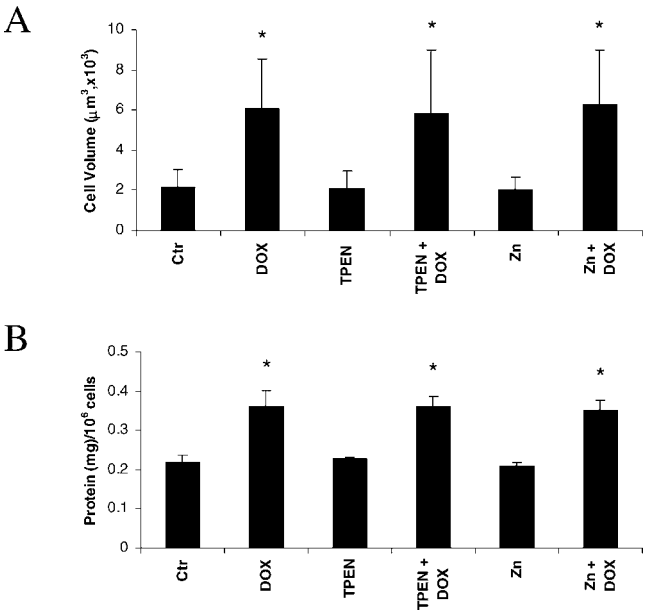

Figure 4. Effect of TPEN and zinc on DOX-induced H9c2 cell hypertrophy. H9c2 cells in DMEM containing 10% FBS were pretreated with 2.5 μ*M* TPEN or 100 μ*M* $ZnCl_2$ for 2 hrs. The cells were treated with 1 μ*M* DOX for 2 hrs in the presence of TPEN or $ZnCl_2$ and then placed in fresh medium containing 10% FBS and TPEN or $ZnCl_2$ for an additional 46 hrs. Cell volume (A) and protein content per cell (B) were measured as described in Materials and Methods. The data are mean ± SD of 100 cells per experimental group (A) or triplicates from one experiment representative of three (B). * = significantly different from corresponding control group ($P < 0.05$).

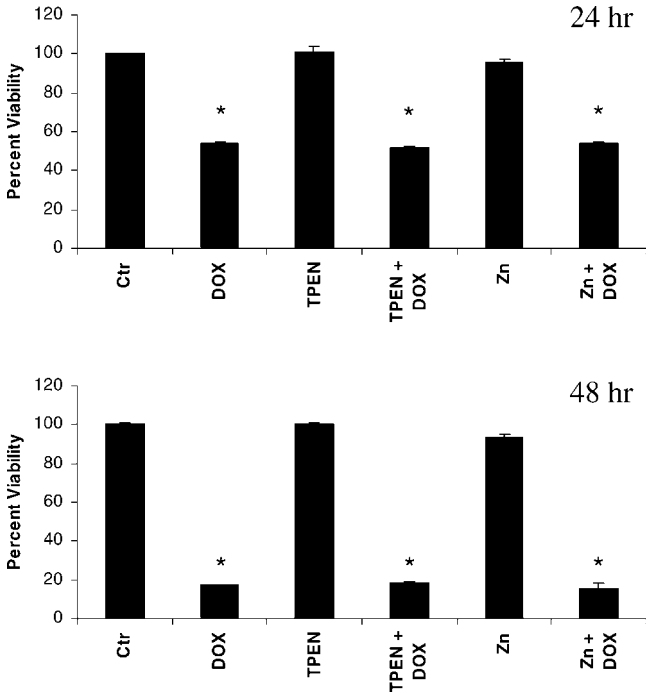

Figure 5. Effect of TPEN and zinc on DOX-induced H9c2 cell viability. H9c2 cells in DMEM containing 10% FBS were pretreated with 2.5 μ*M* TPEN or 100 μ*M* $ZnCl_2$ for 2 hrs. The cells were then treated with 1 μ*M* DOX for 24 or 48 hrs in the presence of TPEN or $ZnCl_2$. Cell viability was measured using an MTT assay as described in Materials and Methods. The data are means ± SD of triplicates from one experiment representative of three. * = significantly different from corresponding control group ($P < 0.05$).

indicate that zinc does not inhibit the DOX-induced decrease in H9c2 cell viability.

Recently calcineurin-NFAT signaling has been implicated in DOX-induced apoptosis in H9c2 cells (30). To determine if calcineurin-NFAT signaling is involved in the DOX-induced decrease in cell viability, H9c2 cells were treated with the calcineurin inhibitor CsA or tacrolimus for 2 hrs prior to and throughout treatment with 1 μ*M* DOX. These inhibitors have previously been shown to inhibit DOX-induced calcineurin-NFAT signaling in H9c2 cells (31). Pretreatment with 100 n*M* CsA or 1 μ*M* tacrolimus did not affect the DOX-induced decrease in cell viability (Fig. 6). These results indicate that although calcineurin is activated by DOX treatment, it is not involved in the DOX-induced decrease in cell viability in H9c2 cells.

Discussion

The results obtained from the present study demonstrate that DOX treatment of H9c2 cells induces an increase in intracellular labile zinc as detected by the zinc-specific fluorophore zinquin and also leads to the activation of calcineurin-NFAT signaling. Pretreatment of H9c2 cells with the zinc-specific, membrane-permeable chelating agent TPEN inhibits the DOX-induced increase in intracellular labile zinc and potentiates DOX-activated calcineurin-NFAT signaling. Addition of exogenous zinc prior to DOX treatment attenuates DOX-activated calcineurin-NFAT signaling in a dose-dependent manner. Collectively, these results indicate that zinc can suppress calcineurin-NFAT signaling in H9c2 cells. Further findings from the present study demonstrate that zinc alone does not inhibit either DOX-induced cellular hypertrophy or a DOX-induced decrease in cell viability as neither endogenous zinc chelation with TPEN nor addition of exogenous zinc affected either of these cellular processes. Inhibiting calcineurin-NFAT signaling with the calcineurin inhibitors CsA or tacrolimus did not affect the DOX-induced decrease in cell viability, indicating that although calcineurin is activated by DOX treatment, it is not necessary for DOX-induced cell death in H9c2 cells.

Elevation of the transition metal–binding protein MT inhibits DOX-induced cardiomyopathy, including myocardial apoptosis and heart hypertrophy (1–4). MT protection against oxidative stress has previously been demonstrated in both *in vitro* (13–15) and *in vivo* (1, 16, 17) models. The recent recognition of the redox regulation of MT offered an insightful perspective on this biologic function (18, 34). In mammals, MT predominantly binds to the redox inert transition metal zinc in a thermodynamically stable manner (18). Within MT, zinc ions are bound by 20 cysteine

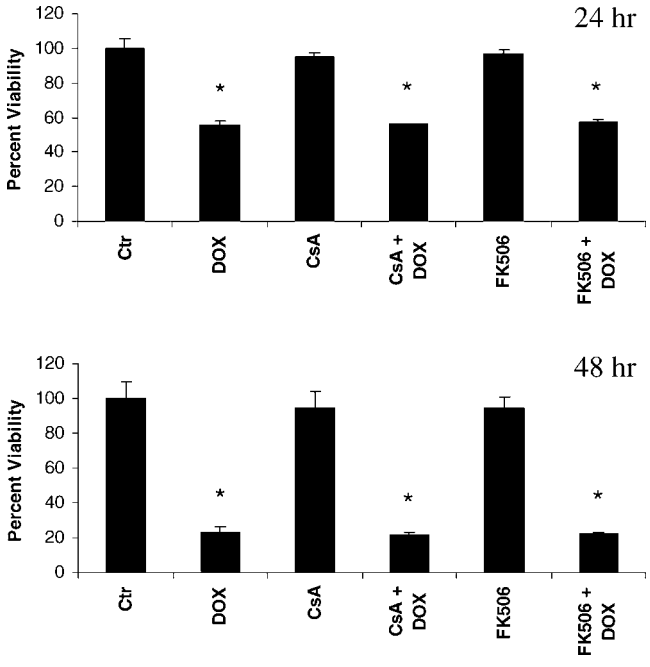

Figure 6. Effect of CsA or tacrolimus on DOX-induced H9c2 cell viability. H9c2 cells in DMEM containing 10% FBS were pretreated with 100 n*M* CsA or 1 μ*M* tacrolimus for 2 hrs. The cells were then treated with 1 μ*M* DOX for 24 or 48 hrs in the presence of the inhibitors. Cell viability was measured using an MTT assay as described in Materials and Methods. The data are mean ± SD of triplicates from one experiment representative of three. * = significantly different from corresponding control group ($P < 0.05$).

residues in two zinc/thiolate clusters (34). This cluster structure of Zn-MT provides a chemical basis by which the cysteine ligand can induce oxidoreductive properties, thereby permitting zinc to retain kinetic lability (19). Oxidation or reduction of the thiolate ligands of MT allows for the concomitant release or binding of zinc in an oxidoreductive environment (18, 34). The recognition of this redox cycle of MT has led to the notion of MT as a transducer of redox signals into zinc signals (34). Zinc release from MT under oxidative stress conditions has previously been demonstrated in multiple cell types (18, 35, 36). The release of zinc from MT under oxidative conditions makes zinc available for the functional demands of other molecules, including metalloproteins and metal-dependent transcription factors, thereby allowing for the modulation of cellular processes including signal transduction pathways (18, 34). In the present study, we demonstrated that DOX treatment induced an increase in intracellular labile zinc in H9c2 cells, suggesting that zinc is available for the modulation of subsequent DOX-induced cellular processes.

The redox regulation of zinc mobilization from MT suggests that MT protection against oxidative stress may be related to zinc. In recent years a role for zinc in the modulation of cellular signaling pathways has come to light. Zinc has been shown to modulate extracellular signal recognition, second messenger metabolism, and activities of a multitude of transcription factors (23). Moreover, zinc has been shown to affect protein phosphorylation, and it can affect the activity of numerous protein kinases and phosphatases including mitogen activated protein kinase (MAPK), protein kinase C, protein tyrosine phosphatases, calcium/calmodulin-dependent protein kinase 2, and p70S6 kinase (20–27). The activity of the calcium/calmodulin-dependent serine/threonine phosphatase calcineurin has also been shown to be modulated by zinc; however, the results have been conflicting. Krinks *et al*. (37) reported that zinc stimulated the phosphatase activity of calcineurin in a cell-free system. More recently, however, physiologic concentrations of zinc were shown to inhibit nickel-stimulated calcineurin activity in a cell-free system (28). Furthermore, a study by the same group found that zinc suppressed the calcineurin-dependent production of interleukin 2 by concanavalin A in Jurkat T cells, although no direct effect of zinc on calcineurin activity or calcineurin signaling was observed (38). These findings, however, led the authors to postulate that calcineurin activity may be modulated *in vivo via* regulation of intracellular zinc concentration (38). The results presented here demonstrate that both endogenously released zinc and exogenously added zinc can suppress DOX-induced calcineurin-NFAT signaling in H9c2 cells.

In unstimulated cells, NFAT resides in the cytoplasm in a hyperphosphorylated state (29). Nuclear localization and transcriptional activity of NFAT is directly induced by calcineurin-mediated dephosphorylation of a nuclear localization signal in the N-terminus of NFAT (29). Therefore, analysis of NFAT transcriptional activity is a functional assessment of calcineurin activity and the most reliable means of evaluating calcineurin activation (39). Given that zinc has been shown to inhibit nickel-stimulated calcineurin activity in a cell-free system (28), it would be easy to prematurely conclude that zinc inhibits DOX-induced calcineurin activity in H9c2 cells. However, the activation profile of NFAT is also modulated by numerous kinases that promote rephosphorylation of NFAT and thereby modulate the nuclear localization and transcriptional activity of NFAT (29, 39). In fact, zinc has been shown to induce marked activation of two of these kinases, c-Jun N-terminal kinase and P38 MAPK, in human bronchial epithelial cells (21), although the concentration of zinc used was significantly higher (500 μ*M*) than that in the current study. The potential effects of zinc on the signaling pathways upstream of calcineurin activation also need to be taken into consideration. For example, zinc has previously been shown to inhibit the uridine 5′-triphosphate–evoked increase in intracellular calcium concentration in rat PC12 cells (40). A similar inhibition in H9c2 cells could potentially lead to the suppression of DOX-induced calcineurin-NFAT signaling observed in the current study. Therefore, a careful evaluation of the multitude of factors that affect calcineurin activation and subsequent downstream NFAT signaling will be necessary to determine the mechanism whereby zinc

inhibits DOX-induced calcineurin-NFAT signaling in H9c2 cells.

Calcineurin-NFAT signaling has previously been implicated in DOX-induced apoptosis in H9c2 cells via increased transcription and translation of Fas ligand (FasL) and subsequent activation of the caspase 8 and caspase 3 apoptotic signaling cascades (30). However, the present study showed that inhibition of calcineurin-NFAT signaling with zinc does not inhibit the DOX-induced decrease in cell viability. Zinc is generally thought to be cytoprotective and can suppress major pathways leading to apoptosis (33). In particular, it is well known that zinc can inhibit the activation of caspase 3 and thereby prevent caspase 3–dependent apoptosis (33). Inhibition of DOX-induced calcineurin-NFAT signaling and subsequent FasL expression by zinc might represent a possible mechanism of zinc inhibition of caspase 3 activation and would therefore be expected to inhibit DOX-induced cell death. Such a response was not identified in this study. In a previous study from our laboratory, zinc inhibited DOX-induced activation of caspase 3 in HeLa cells; however, DOX-induced apoptosis or overall cell death was not suppressed, suggesting that DOX-induced caspase 3 activation may not play a major role in apoptotic cell death (41). Similarly, a more recent study indicated that although caspase 3 is activated following DOX treatment in H9c2 cells, this activation is not an essential step in the promotion of cell death in these cells (42). Thus, the failure of zinc to inhibit the DOX-induced decrease in cell viability in H9c2 cells should not be so unexpected. The failure of the calcineurin inhibitors CsA or tacrolimus to inhibit the DOX-induced decrease in cell viability in H9c2 cells lends further evidence that calcineurin-NFAT signaling and subsequent activation of caspase 3 are not essential in DOX-induced cell death. Results obtained from this study also demonstrate that zinc does not inhibit DOX-induced cellular hypertrophy. These results are in accordance with our earlier study, that calcineurin is activated by DOX treatment but is not necessary for DOX-induced hypertrophy in H9c2 cells (31). Rather, the phosphoinositide-3 kinase-Akt signaling pathway appears to be more critically involved in DOX-induced hypertrophy (31).

The finding of the current study, that zinc alone does not inhibit DOX-induced cellular hypertrophy or DOX-induced cell death, can be interpreted along with our recent finding that MT is required to transfer zinc to some zinc-binding proteins such as mitochondrial aconitase (43). Such an interaction would retain zinc as an effector of cardioprotection and ensure the specificity of zinc transfer, thereby allowing for the appropriate regulation of the function of the zinc-binding protein in response to changes in the intracellular environment (18). Such direct transfer of zinc from MT may lead to modification of signaling pathways or of specific proteins under oxidative stress, thereby resulting in cardioprotection against oxidative damage (44). However, zinc alone may not be able to modulate or activate these proteins under oxidative stress conditions so that protection may not be apparent.

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