Inhibition of Copper-Zinc Superoxide Dismutase Induces Cell Growth, Hypertrophic Phenotype, and Apoptosis in Neonatal Rat Cardiac Myocytes In Vitro


Abstract—Oxidative stress has been implicated in the pathophysiology of myocardial failure. We tested the hypothesis that inhibition of endogenous antioxidant enzymes can regulate the phenotype of cardiac myocytes. Neonatal rat ventricular myocytes in vitro were exposed to diethyldithiocarbamic acid (DDC), an inhibitor of cytosolic (Cu, Zn) and extracellular superoxide dismutase (SOD). DDC inhibited SOD activity and increased intracellular superoxide in a concentration-dependent manner. A low concentration (1 μmol/L) of DDC stimulated myocyte growth, as demonstrated by increases in protein synthesis, cellular protein, prepro–atrial natriuretic peptide, and c-fos mRNAs and decreased sarcoplasmic reticulum Ca\(^2+\)ATPase mRNA. These actions were all inhibited by the superoxide scavenger Tiron (4,5-dihydroxy-1,3-benzene disulfonic acid). Higher concentrations of DDC (100 μmol/L) stimulated myocyte apoptosis, as evidenced by DNA laddering, characteristic nuclear morphology, in situ terminal deoxynucleotidyl transferase–mediated nick end-labeling (TUNEL), and increased bax mRNA expression. DDC-stimulated apoptosis was inhibited by the SOD/catalase mimetic EUK-8. The growth and apoptotic effects of DDC were mimicked by superoxide generation with xanthine plus xanthine oxidase. Thus, increased intracellular superoxide resulting from inhibition of SOD causes activation of a growth program and apoptosis in cardiac myocytes. These findings support a role for oxidative stress in the pathogenesis of myocardial remodeling and failure. (Circ Res. 1999;85:147-153.)

Key Words: superoxide dismutase ■ superoxide ■ myocyte ■ hypertrophy ■ apoptosis

Superoxide is a “foundation” radical that may lead to the formation of the reactive oxygen species hydrogen peroxide,\(^1\) hydroxyl radical,\(^2\) and peroxynitrite.\(^3\) Superoxide is produced intracellularly by electron leakage from mitochondria during oxidative phosphorylation and by activation of several cellular enzymes, including NADPH oxidases,\(^4\) cyclooxygenase, nitric oxide synthase,\(^5\) and xanthine oxidase. Antioxidant enzymes, including superoxide dismutase (SOD), catalase, and peroxidases protect cells by maintaining \(O_2^-\) and \(H_2O_2\) at low levels.\(^6\)

Studies in animal models suggest that a chronic increase in oxidative stress in the myocardium, possibly due to impairment of SOD and other antioxidant pathways, could contribute to myocardial remodeling and failure.\(^7,8\) Although the mechanism by which oxidative stress might cause myocardial remodeling is not clear, oxidative stress has been implicated as a mediator of both cell death\(^9\) and cell growth.\(^10,11\) Furthermore, Cheng et al\(^12\) showed that mechanical stretch of papillary muscle increased superoxide and caused apoptosis and that both effects were inhibited by nitric oxide, leading to the suggestion that superoxide mediates stretch-induced apoptosis in cardiac myocytes.

We hypothesized that an increase in superoxide due to inhibition of SOD would affect the growth and survival of cardiac myocytes. To test this thesis, Cu, Zn SOD was inhibited in a graded manner with the copper chelator diethyldithiocarbamic acid (DDC)\(^13\) in ventricular myocytes cultured from neonatal rats.

Materials and Methods

Neonatal Rat Ventricular Myocytes (NRVMs)

NRVMs were prepared as previously described.\(^14\) Myocytes were plated at a density of 300 cells/mm\(^2\) on 24-well plates (Costar), 12-mm-diameter coverslips in 24-well plates, or 35- or 100-mm dishes (Falcon) for 24 hours in DMEM (GIBCO) containing 7% (vol/vol) heat-inactivated FBS (GIBCO) and 1% (vol/vol) penicillin-streptomycin (GIBCO). The culture medium was changed to serum-free DMEM for 24 hours before exposure to experimental treatments. DDC (Sigma), alone or in combination with the nonenzymatic superoxide scavenger Tiron (4,5-dihydroxy-1,3-benzene disulfonic acid, Sigma)\(^15\) or the SOD/catalase mimetic EUK-8 (Eukarion),\(^16\) was added for 24 hours or as indicated. In other

Received October 19, 1998; accepted April 8, 1999.
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147
experiments, the combination of xanthine (500 μmol/L; Sigma) plus xanthine oxidase (0.1 μM/mL; Boehringer Mannheim), referred to as DDC, was added to cells. Control cells were treated with DMEM alone.

**SOD Activity**

SOD activity was measured by the inhibition of pyrogallol auto-oxidation.¹⁷ NRVMs plated on 100-mm dishes were treated for 7 hours with DDC. The cells were trypsinized, centrifuged, and resuspended in the assay buffer containing 50 mmol/L Tris (pH 8.2) and 1 mmol/L diethyldithiophosphate. The cells were sonicated and centrifuged at 800g for 5 minutes. Protein concentration in the supernatant was determined by Bradford assay against a BSA standard (Bio-Rad protein assay dye reagent concentrate). Pyrogallol (Sigma) auto-oxidation was measured as the rate of change of the absorbance at 420 nm over 5 minutes of 200 μmol/L pyrogallol, diluted from acidic stock into assay buffer, in the presence of 1200 U/mL catalase (Sigma).

**Lucigenin-Enhanced Chemiluminescence**

DDC-enhanced production of superoxide was measured in NRVMs plated on 35-mm dishes. DDC (1 μmol/L) was added to the medium for 24 hours before superoxide measurements. The medium was removed and replaced with physiological buffer (in mmol/L: NaCl 119, HEPES 20, KCl 4.6, MgSO₄ 1, Na₂HPO₄ 0.15, KH₂PO₄ 0.4, NaHCO₃ 5, CaCl₂ 1.2, and glucose 11.1, pH 7.4). Lucigenin (100 μmol/L; bis-N-methylacridinium nitrate, Sigma) was added to the dishes and allowed to equilibrate for 10 minutes at 37°C. Luminescence, measured with a Turner 20/20 luminometer, was the quantity of formazan calculated as NBT reduction. Formazan was pelleted at 16 000 × g for 20 minutes and air-dried, and dissolved in 10 mmol/L Tris-HCl (pH 7.4), and 1 mmol/L EDTA (pH 8.0). The entire DNA sample for each treatment was electrophoresed on a 1.5% agarose gel with 40 mmol/L Tris-acetate and 2 mmol/L EDTA (pH 8.0). The DNA size markers (PCR Markers, Promega).

**Nitroblue Tetrazolium (NBT) Reduction**

NRVMs plated on 100-mm dishes were treated with DDC for 24 hours, and the medium was replaced with physiological buffer with NBT (100 μmol/L; Sigma) for 90 minutes. Cells were collected and centrifuged for 10 minutes at 12 000g, and the resulting pellet was resuspended in pyridine (100 μmol/L; Sigma). Formazan, the product of the reaction of superoxide with NBT, was extracted by heating the samples at 80°C for 90 minutes and was measured by absorbance at 540 nm. The quantity of formazan was calculated as NBT reduction = A × V × (T × n × e × l), where A is the absorbance value at 540 nm, V is the volume of solution, T is the time period of NBT incubation, n is the number of cells, e is the extinction coefficient of formazan (0.72 mmol/L⁻¹ · mm⁻¹), and l is the length of the light path. Nonspecific NBT reduction was determined by addition of euK-8 (100 μmol/L) at the time of DDC treatment. Superoxide levels are reported as Tiron-inhibited arbitrary units per minute.¹⁸

**Protein Synthesis**

NRVMs were plated on 24-well plates, and [³H]leucine incorporation was determined as previously described.¹⁴ To account for possible changes in cell number with experimental treatment, cell number was determined in parallel plates as described below, and [³H]leucine incorporation is reported as dpm/1000 cells.

**Protein Content**

Total protein content was determined in 24-well plates by Bradford assay against a BSA standard. Cell number was determined in parallel plates as described below, and protein values are reported as μg protein/1000 cells.

**Northern Hybridization**

NRVMs plated on 100-mm dishes were treated for 24 hours or as indicated. Total RNA isolations and Northern hybridizations with ³²P-labeled full-length cDNA of rat prepro–atrial natriuretic peptide (ANP), rabbit sarcoplasmic-endoplasmic reticulum Ca²⁺ ATPase (SERCA2), rat c-fos, or rat bax²⁰ were performed essentially as previously described.¹⁴ Blots were exposed to a phosphor screen for 2 to 3 hours and quantified with a phosphor imager (GS-363, Bio-Rad) or exposed to XOMAT-AR film (Kodak, Rochester, NY) overnight and quantified with an imaging densitometer (GS-700, Bio-Rad) using Molecular Analyst software (Bio-Rad). mRNA levels were normalized to 18S RNA determined by reprobing blots with a ³²P-labeled oligonucleotide complementary to 18S RNA.

**Mitogen-Activated Protein Kinase Activity**

NRVMs plated on 100-mm dishes were treated as indicated. The activities of the extracellular signal–regulated kinases (ERK)-1/ERK2 (p44/p42 mitogen-activated protein kinase) were measured as phosphorylation of myelin basic protein by immunoprecipitated ERK1/ERK2.²¹

**Cell Number and Membrane Integrity**

Cell number was determined in 24-well plates treated with DDC or XOO, with or without Tiron for 24 hours. An aliquot of the medium (including floating cells) was counted with a hemacytometer (Hausser). The number of adherent cells was determined by trypsinization (GIBCO) and subsequent counting with a hemacytometer. Cell membrane integrity was determined in NRVMs plated on 100-mm dishes treated with DDC or XOO, with or without EUK-8, for 24 hours. The medium, containing floating cells, was removed and collected. Adherent cells were trypsinized and pooled with the corresponding floating cells. The cells were pelleted at 730g for 5 minutes and resuspended in PBS containing 0.04% (wt/vol) trypan blue (Sigma). An aliquot of the cell suspension, containing both adherent and floating cells, was counted with a hemacytometer, and the percentage of cells excluding dye was calculated.

**DNA Laddering**

NRVMs plated on 100-mm dishes were treated for 24 hours as indicated. The medium, containing floating cells, was removed and collected. Adherent cells were trypsinized and pooled with the corresponding floating cells. The cells were pelleted at 730g for 5 minutes, washed once with PBS, and then lysed on ice in buffer containing 10 mmol/L EDTA (pH 8.0), 10 mmol/L Tris-HCl (pH 7.4), and 0.5% (v/v) Triton X-100 for 30 minutes. Lysed cells were centrifuged at 16 000g for 20 minutes, and the supernatants were treated with 0.4 mg/mL RNase A for 30 minutes at 37°C, followed by 0.4 mg/mL proteinase K for 30 minutes at 37°C. The DNA was precipitated overnight in 1 mol/L NaCl and 50% isopropanol. The DNA was pelleted at 16 000g for 20 minutes, air-dried, and dissolved in 10 mmol/L Tris-HCl (pH 8.0) and 1 mmol/L EDTA (pH 8.0). The entire DNA sample for each treatment was electrophoresed on a 1.5% agarose gel with 40 mmol/L Tris-acetate and 2 mmol/L EDTA (pH 8.5). The DNA ladders were visualized with ethidium bromide and UV light, and DNA band sizes were estimated using DNA size markers (PCR Markers, Promega).

**In Situ Terminal Deoxynucleotidyl Transferase–Mediated Nick End-Labeling of DNA Strand Breaks (TUNEL)**

NRVMs plated on glass coverslips in 24-well dishes were treated for 24 hours as indicated. Cells were fixed in 3.7% formaldehyde for 30 minutes at room temperature and then permeabilized in 0.1% (vol/vol) Triton X-100 and 0.1% (wt/vol) sodium citrate for 30 minutes at 4°C. Coverslips were exposed to the TUNEL reaction mixture containing terminal deoxynucleotidyl transferase and fluorescein-labeled dUTP (In Situ Cell Death Detection Kit—Fluorescein, Boehringer Mannheim) for 1 hour in a humidified 37°C incubator, washed, and counterstained with Hoechst 33342 for 10
minutes at room temperature. Coverslips were mounted onto glass slides and visualized with an epifluorescent microscope. At least 100 total nuclei (Hoechst stained) were counted from each coverslip, and the number of TUNEL-positive cells for each field was determined. Slides were scored in a blinded fashion.

**Statistical Analysis**

All data are presented as mean±SEM. Statistical analysis used the Student *t* test or 1-way ANOVA with Bonferroni correction, as appropriate. A *P* value ≤0.05 was considered significant.

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**Results**

**DDC Inhibits SOD and Increases Superoxide Levels in a Concentration-Related Manner**

The addition of DDC to cultures for 7 hours inhibited SOD activity in a concentration-dependent manner, reducing total cellular activity by 33±10% and 63±5% at 1 μmol/L and 1 mmol/L, respectively (Figure 1A). Exposure to DDC (1 μmol/L, 24 hours) increased superoxide levels by 162±13% as measured by lucigenin-enhanced chemiluminescence (Figure 1B) and by 43±5% as measured by NBT reduction (Figure 1C), which confirms that inhibition of Cu, Zn SOD causes a modest increase in superoxide levels.

**Inhibition of SOD Stimulates Myocyte Growth**

Exposure to DDC for 24 hours increased protein synthesis, as measured by [3H]leucine incorporation, in a concentration-dependent manner with a maximum increase of 109±34% at 1 μmol/L (Figure 2A). Concomitant addition of the superoxide scavenger Tiron (100 μmol/L) with DDC (1 μmol/L) abolished the effect of DDC. DDC (24 hours) likewise
increased cellular protein content in a concentration-dependent manner, and this effect was abolished by Tiron (Figure 2B). DDC (1 μmol/L) caused induction of c-fos mRNA (Figure 3A) and activation of ERK1/ERK2 (Figure 3B).

The effects of SOD inhibition were mimicked by addition of XXO for 24 hours, which caused increases in [3H]leucine incorporation (88±22%; n=7; P<0.002) and protein content (92±9%; n=7; P<0.001) that were abolished by Tiron.

**Effect of SOD Inhibition on Myocyte Phenotype**

DDC (1 μmol/L, 24 hours) increased ANP mRNA by 194±58% and decreased SERCA2 mRNA by 34±5% (Figure 4). Both effects of DDC were inhibited by Tiron (100 μmol/L).

**Effect of SOD Inhibition on Cell Membrane Integrity and Apoptosis**

Exposure to DDC for 24 hours at concentrations up to 1 μmol/L had no effect on the membrane integrity of adherent or floating myocytes, as evidenced by the ability to exclude trypan blue dye (control cells, 85±6%; DDC-treated cells, 87±5%; n=3; P=NS), which indicated that DDC did not cause cell necrosis. At DDC concentrations >1 μmol/L, there was a decrease in the number of adherent cells (data not shown). XXO had no effect on membrane integrity (control cells, 85±6%; XXO-treated cells, 80±12%; n=3; P=NS).

DDC (100 μmol/L, 24 hours) increased DNA laddering (Figure 5), and this effect was inhibited by concomitant treatment with the SOD/catalase mimetic EUK-8 (10 μmol/L). Likewise, DDC (100 μmol/L, 24 hours) caused an apparent increase in the fraction of cells with nuclear condensation and fragmentation as visualized with the fluorescent DNA dye Hoechst 33342 (Figure 6A).

In control myocytes, 6±2% of nuclei were TUNEL-positive. Treatment with DDC (100 μmol/L, 24 hours) increased the percentage of TUNEL-positive nuclei 3.8±2.2-fold (Figures 6B and 7). EUK-8 (10 μmol/L) alone had no effect on the number of TUNEL-positive nuclei but prevented completely the DDC-stimulated increase (Figure 7). DDC concentrations >1 μmol/L caused cell detachment, which may be a stimulus for apoptosis known as anoikis. To avoid the possibility that apoptosis was due to detachment, per se, TUNEL staining was assessed only in the adherent cells. XXO likewise increased DNA laddering (data not shown) and increased the number of TUNEL-positive nuclei 4.9±1.2-fold (n=3; P=0.014).

DDC (24 hours) increased the expression of bax mRNA in a concentration-dependent manner (Figure 8). The level of bax mRNA was not affected at DDC concentrations of 1 μmol/L or less but was increased at 10 μmol/L and maximal at 100 μmol/L. XXO likewise increased bax 52±13% (n=5; P<0.001).

**Discussion**

The major new finding of this study is that a sustained subnecrotic increase in superoxide level caused by partial inhibition of SOD has profound effects on the growth,
phenotype, and death of cardiac myocytes. These effects were related to the concentration of DDC. At low concentrations (1 μmol/L), DDC stimulated cell growth, induced a fetal gene program, and activated growth-signaling pathways involving c-fos and ERK1/ERK2. At a higher concentration (100 μmol/L), DDC stimulated apoptosis and increased expression of bax mRNA. DDC-stimulated growth and apoptosis were mimicked by superoxide generation with X XO and prevented by a superoxide scavenger or an SOD/catalase-mimetic, which supports the conclusion that the observed effects were due to increased superoxide levels caused by SOD inhibition.

**Oxidative Stress Stimulates Myocyte Growth**

The growth effect of DDC is comparable in magnitude with that observed with several other stimuli such as norepinephrine, interleukin-1β, or endothelin in neonatal rat cardiac myocytes. Coincident with cell growth, there was increased expression of ANP mRNA, which is typical of myocardial hypertrophy, and decreased expression of SERCA2 mRNA, which may be observed with myocardial hypertrophy. Oxidative stress is emerging as a growth signal in other cell types. In vascular smooth muscle cells, the hypertrophic effect of angiotensin depends on increased production of superoxide anion via NADH oxidase. A similar role was suggested in fibroblasts, in which increases in superoxide anion and oxidative stress have been implicated in mediating the growth effects of stimuli acting through ras.

**Oxidative Stress Stimulates Apoptosis**

Oxidative stress can cause necrotic cell death characterized by a loss of membrane integrity. However, DDC concentrations up to 1 mmol/L did not impair cell membrane integrity, which suggests that apoptosis occurred at a level of oxidative stress that was subnecrotic. Apoptosis was assessed by changes in nuclear morphology and increases in DNA laddering and in situ TUNEL staining. It should be noted that although DNA laddering is specific for apoptosis, it is not quantitative, and conversely, that TUNEL staining is quantitative but not specific for apoptosis.

**Figure 5.** Inhibition of SOD stimulates DNA laddering. Myocytes were incubated with DDC (100 μmol/L), the SOD/catalase-mimetic EUK-8 (10 μmol/L), or both for 24 hours. Low molecular weight DNA was extracted and visualized by gel electrophoresis and ethidium bromide staining. The figure is representative of 4 similar experiments.

**Figure 6.** Effects of DDC (100 μmol/L, 24 hours) on nuclear morphology, as assessed by staining with Hoechst 33342 (A), and DNA fragmentation, as assessed by TUNEL staining (B). Arrows identify condensed or fragmented nuclei (A) or TUNEL-positive cells (B). The figure is representative of 3 similar experiments. Bar=100 μm.

**Figure 7.** Inhibition of SOD increases TUNEL-positive myocytes. Cells were treated for 24 hours with DDC (100 μmol/L), EUK-8 (10 μmol/L), or both and stained as per Figure 6. Shown is the mean percentage of Hoechst 33342-stained nuclei that are TUNEL-positive from 3 to 4 experiments. *P<0.05 vs control; #P<0.05 vs DDC alone. Data are mean±SEM.
Prior observations have implicated superoxide as a mechanism of cardiac myocyte apoptosis. Cheng et al.12 showed that stretch causes myocyte apoptosis associated with increased levels of reactive oxygen species and that scavenging superoxide with a nitric oxide donor reduced the extent of apoptosis. Sawyer et al.28 implicated superoxide as a mechanism for anthracycline-stimulated myocyte apoptosis. Likewise, Dhalla et al.8 showed that vitamin E is cardioprotective in pressure overload–induced hypertrophy, which appears to involve myocyte apoptosis.29

Mechanism of Action of DDC

DDC inactivates Cu, Zn SOD, and extracellular SOD by chelating the copper ion at the active sites13 and has been shown to inhibit SOD activity in rat cardiac myocytes.22 Of note, chronic dietary deficiency of copper causes a characteristic cardiomyopathy that is associated with myocyte hypertrophy, ventricular dilation,30 and decreased Cu, Zn SOD activity31 and is ameliorated by antioxidants.32 Dithiocarbamates such as DDC may have other actions relevant to the redox state of a cell and its response to oxidative stress. As thiols, they can auto-oxidize to form superoxide and other reactive oxygen species,33 and alternatively, at high concentrations they may act as reducing agents.34 Dithiocarbamates may also inhibit the activation of nuclear factor-κB.34 However, the ability of the superoxide scavenger Tiron15 and the SOD/catalase mimetic EUK-816 to inhibit DDC-stimulated myocyte growth and apoptosis strongly supports a mechanistic role for superoxide anion in these experiments.

It is unclear whether DDC-stimulated hypertrophy and apoptosis are mediated by superoxide anion or other downstream reactive oxygen species, such as H₂O₂, produced by the spontaneous dismutation of superoxide anion. Relatively high concentrations of H₂O₂ can activate ERK1/ERK2 in neonatal rat cardiac myocytes.35 Both Euk-8, which has SOD and catalase activities, and Tiron, which scavenges superoxide, would be expected to decrease H₂O₂ levels.

Implications

These observations were made in vitro in myocytes from neonatal rats, and therefore, they may not reflect events in adult myocytes in vivo. Nevertheless, the effects demonstrated here by inhibition of SOD suggest that oxidative stress may be an important mediator of myocyte growth, functional phenotype, and apoptosis. Oxidative stress may be increased in the myocardium of patients with heart failure.36 Therefore, our findings suggest that a decrease in SOD activity could contribute to pathologic myocardial remodeling in humans and, conversely, that antioxidants might attenuate the development of myocardial failure.

Acknowledgments

This work was supported by NIH grants HL42539, HL52320, and HL61639 (to W.S.C.); HL55425 (to P.J.P.); and HL07224 (to D.A.S.); and by a Grant-in-Aid from the American Heart Association (to P.J.P.); a Grant-in-Aid from the American Heart Association, Massachusetts Affiliate (to D.B.S.); and a Merit Grant from the Department of Veterans Affairs (to K.S.).

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Figure 8. DDC stimulates bax mRNA in a concentration-dependent manner. A, Representative Northern hybridization. B, Mean±SEM from 5 experiments, normalized to 18S rRNA. *P<0.05 vs control.


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Circ Res. 1999;85:147-153
doi: 10.1161/01.RES.85.2.147

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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