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Original Contribution

REGULATION OF ANTIOXIDANT ENZYME GENE EXPRESSION IN RESPONSE TO OXIDATIVE STRESS AND DURING DIFFERENTIATION OF MOUSE SKELETAL MUSCLE

ALEXA A. FRANCO, RAANAN S. ODOM, and THOMAS A. RANDO

Department of Neurology and Neurological Sciences, Palo Alto Veterans Affairs Medical Center and Stanford University School of Medicine, Palo Alto, CA, USA

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Abstract—Various properties of skeletal muscle, including high metabolic activity and high levels of heme-containing proteins, render it particularly susceptible to free radical injury. Indeed, cellular injury from reactive oxygen species (ROS) has been implicated in many muscle disorders. Thus muscle cell survival is critically dependent on the ability of the cell to respond to periods of oxidative stress. To investigate this important homeostatic response, we studied the effect of oxidative challenges on the expression of genes encoding the antioxidant enzymes Cu,Zn-superoxide dismutase (CuZnSOD), Mn-superoxide dismutase (MnSOD), glutathione peroxidase (GPx), and catalase (CAT) in myotube cultures. Using Northern blot analysis, we found that treatment with the pro-oxidant paraquat resulted in time- and dose-dependent increases of transcript levels that were greatest for GPx and CAT (~4–5 fold). CuZnSOD and MnSOD transcripts were also increased, albeit more modestly (~2–3 fold). Transcript levels were also induced by treatment of the cells with two other pro-oxidants, menadione and H_2O_2, and correlated with the level of oxidative injury to the cells, measured as protein carbonyl group formation. Activities of all of the enzymes increased in response to the oxidative challenges, although the magnitudes of the increases were less robust than the increases of the respective transcript levels. In studying the effect of cellular differentiation on antioxidant gene expression and susceptibility to oxidative stress, we found that pro-oxidant treatment resulted in greater oxidative injury to differentiated myotubes than to undifferentiated myoblasts. Furthermore, the increased susceptibility of myotubes correlated with decreased antioxidant defenses—as muscle cells differentiated, both transcript and activity levels of antioxidant enzymes decreased. These data suggest that muscle cells regulate antioxidant defenses in response to oxidative stress and cellular differentiation.

Keywords—Antioxidant enzymes, Muscle, Oxidative stress, Free radicals, Differentiation, Superoxide dismutase, Glutathione peroxidase, Catalase

INTRODUCTION

Cellular injury from reactive oxygen species (ROS) has been implicated in a wide variety of muscle diseases and pathologic conditions [1]. There is a growing body of evidence that oxidative stress may contribute to the muscle necrosis seen in muscular dystrophies [2–6]. In muscle ischemia and muscle injury during extreme exercise, there is also necrotic degeneration of muscle fibers that has been attributed to the generation and toxic actions of ROS [7,8]. Animals that are deficient in vitamin E develop a necrotizing myopathy [9].

Certain properties of muscle may render it especially susceptible to free radical injury [10]. During contraction, there are rapid changes in energy supply and oxygen flux. The resulting alterations in electron flux through the mitochondrial respiratory chain and generation of ROS may predispose muscle to oxidative injury [1]. There is also a very high concentration of myoglobin in muscle, and such heme-containing proteins are known to confer greater sensitivity to free radical–induced damage by conversion of H_2O_2 to more reactive species [11,12]. As such, muscle must have a high capacity to

Address correspondence to: Thomas A. Rando, Department of Neurology and Neurological Sciences, Room A343, Stanford University School of Medicine, Stanford, CA 94305-5235, USA; Tel.: (650) 493-5000, ext. 63714; Fax: (650) 858-3935; E-Mail: rando@stanford.edu.

withstand oxidative challenges both acutely and chronically.

Increases in the levels of ROS, such as may occur during periods of oxidative stress, appear to be detected by redox-sensitive regulatory molecules in the cell, triggering homeostatic responses to prevent cellular injury [13]. Among those responses is the regulation of antioxidant enzymes (AOE), as the levels and balance of AOE modulate the susceptibility of the cell to oxidative injury [14]. The specific response appears to depend not only on the nature of the ROS perturbation, but also on the specific tissue being studied [15–18]. In muscle, variations in the activities of AOE have been reported under different pathologic conditions associated with free radical injury [19]. We recently found an induction in muscle of AOE mRNA levels just before the onset of the degenerative process in mice with a muscular dystrophy [6]. However, very little is known about the regulation of gene expression for the AOE in eukaryotic cells in response to oxidative stress in general, and in skeletal muscle cells in particular.

In this study, we examined the regulation of specific AOE (Cu,Zn-superoxide dismutase [CuZnSOD], Mn-superoxide dismutase [MnSOD], glutathione peroxidase [GPx], and catalase [CAT]) in response to oxidative stress in muscle cell cultures, primarily using differentiated myotube cultures to serve as a model for mature muscle in vivo. After exposure of myotubes to various pro-oxidants, there was an induction of AOE transcript and activity levels. The relative magnitudes of the changes depended on the specific oxidative challenge presented and the duration of that challenge. Studies of AOE during cellular differentiation revealed that transcript and activity levels decreased with differentiation, and this correlated with an increased susceptibility to oxidative injury of differentiated myotubes compared with undifferentiated myoblasts. These results demonstrate the regulation of antioxidant defenses in muscle in response to oxidative stress and during cellular differentiation.

MATERIALS AND METHODS

Cell culture

All experiments were carried out on cells of the C2C12 muscle cell line (ATCC # CRL1772). This cell line was obtained originally by the repeated passaging of primary muscle cell cultures derived from 2-month-old mice of the C3H strain [20]. Myoblasts were maintained in growth medium consisting of Dulbecco's modified Eagle's medium (BioWhittaker, Walkersville, MD, USA) supplemented with 10% fetal bovine serum (Sigma Chemicals, St. Louis, MO, USA), 10% horse serum (BioWhittaker), penicillin, and streptomycin. Myoblast cultures were induced to differentiate into myotubes by maintaining the cells in mitogen-poor differentiation medium (DM) consisting of Dulbecco's modified Eagle's medium supplemented with 2% horse serum, penicillin, and streptomycin with daily medium changes.

Cells were subjected to oxidative stress by the addition of pro-oxidants (paraquat [Sigma], menadione [Sigma], and H_2O_2 [Fisher Scientific, Pittsburgh, PA, USA]) to the daily medium changes. Paraquat generates superoxide intracellularly [21], menadione is a quinone compound that undergoes redox cycling intracellularly thus generating superoxide radical [22], and the toxicity of H_2O_2 is mostly the result of the generation of hydroxyl radical on reaction with metal ions in the Fenton reaction [23].

Northern blot analysis

Northern blot analysis was performed as previously described for skeletal muscle tissue samples [6]. Briefly, total RNA from muscle cell cultures was isolated in TRI reagent (Sigma) as described in the manufacturer's protocol. The homogenates were mixed with 1-bromo 3-chloropropane (Molecular Research Center, Cincinnati, OH, USA) then centrifuged at 14,000 $\times$ *g*. RNA was precipitated from the aqueous phase by the addition of isopropanol, washed in 75% ethanol, and dissolved in water. RNA content was determined by measuring absorbance at OD_{260}. RNA (15 μg) was separated by electrophoresis on a 1.2% agarose/formaldehyde gel and transferred onto a nylon membrane in 10 $\times$ sodium chloride/sodium citrate (SSC) (150-mM sodium chloride, 15-mM sodium citrate, pH 7.0). The membrane was hybridized with ^{32}P-labeled riboprobes for CuZnSOD or MnSOD, or to ^{32}P-labeled DNA probes for CAT, GPx, and glyceraldehyde phosphate dehydrogenase (GAPDH). Antisense probes were synthesized using ^{32}P-dUTP, linearized plasmid containing CuZnSOD or MnSOD, and appropriate T3 or T7 RNA polymerase following the methods outlined in the Stratagene (La Jolla, CA, USA) in vitro transcription kit. DNA probes were obtained by appropriate digestion to release the sequences of CAT (268 bp fragment), GPx (a pool of 650, 580, 300 bp fragments), or GAPDH (1.2 kb fragment). The ^{32}P-labeled antisense DNA probes were generated using the Amersham (Arlington Heights, IL, USA) random priming kit protocol with ^{32}P-dCTP. The hybridizations were performed for 0.5 (CuZnSOD) or 1 h (MnSOD, GPx, CAT, GAPDH) in ExpressHyb solution (Clontech, Palo Alto, CA, USA) at 68°C, then washed consecutively in 2 $\times$ SSC/0.1% sodium dodecyl sulfate (SDS) 1 $\times$ SSC/0.1% SDS (MnSOD, GPx, CAT, GAPDH), and 0.2 $\times$ SSC/0.1% SDS (CuZnSOD only) at

68°C for 20 min per wash. The membranes were exposed for 4–72 h, and the signal intensities were recorded using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA). CuZnSOD, MnSOD, GPx, and CAT signals were normalized to the level of GAPDH message for each sample.

The probes for MnSOD, GPx, and CAT recognized single mRNA species of 1, 1.2, and 2.4 kb, respectively, in mouse skeletal muscle [6]. The probe for CuZnSOD recognized two mRNA species of 0.4 and 0.6 kb which were poorly resolved, appearing as a single broad band (as in Fig. 1A). The intensity of this doublet was analyzed for quantitating CuZnSOD transcript levels.

Determination of carbonyl group formation by Western blot analysis

Cell extracts were isolated in a buffer consisting of 50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.5% DOC, 1 mM ethylenediaminetetraacetate, 1 mM ethyleneglycoltetraacetate, 5 μg/ml leupeptin, 5 μg/ml aprotinin, and 100 μg/ml PMSF. Insoluble material was removed by centrifugation (9000 $\times$ *g*, 10 min, 4°C), and supernatants were snap frozen in liquid nitrogen and stored at −80°C. For each sample, the protein content was determined using the Bio-Rad protein assay. To detect the presence of carbonyl groups [24], samples were reacted with 10 mM 2,4-dinitrophenylhydrazine in 6% SDS for 15 min at room temperature. The reaction was stopped by the addition of sample buffer containing 2 M Tris base, 30% glycerol, 19% β-mercaptoethanol. Derivitized samples (18 μg/lane) were loaded directly onto 10% polyacrylamide gels without boiling. Separated proteins were transferred to nitrocellulose membranes (0.45 μm; Schleicher and Schuell, Keene, NH, USA) and probed with a rabbit polyclonal antibody to the 2,4-dinitrophenyl moiety (D-9656, Sigma) at a 1:125 dilution in 5% powdered milk, 0.05% Tween, and 0.02% azide in phosphate buffered solution. The membrane was then incubated with an horseradish peroxidase–conjugated antirabbit secondary antibody (Amersham) at a 1:2000 dilution in phosphate buffered solution containing 0.05% Tween and 0.02% azide. Specific antibody binding was detected by an enhanced chemiluminescence system (Amersham) using exposure times between 5 and 15 min.

Antioxidant enzyme activity assays

For each assay, cells were isolated in assay buffer and lysed by a freeze-thaw cycle. The debris was removed by centrifugation at 8000 $\times$ *g* for 5 min at 4°C, and the protein content of each sample was determined using the Bio-Rad protein assay.

SOD enzyme activity was measured according to the method of Crapo et al. [25]. For each sample, aliquots containing 150-μg protein were added to the assay mixture containing 0.1-mM cytochrome *c*, 0.05-mM xanthine, and KCN (10 μM for total SOD activity, 3 mM for MnSOD activity) in phosphate buffer in a final volume of 3 ml. After blanking, xanthine oxidase was added to a final concentration of 0.6 U/ml, and the change in absorbance at OD_{550} was recorded at regular intervals over 4 min. Enzyme activity was determined by comparing samples to a standard curve of known SOD activity. CuZnSOD activity was calculated as the difference between total SOD activity and MnSOD activity.

GPx enzyme activity was measured according to the method described by Del Maestro and McDonald [26]. Briefly, aliquots of sample containing 140 μg of protein were added to the assay mixture of 1 U/ml glutathione reductase and 2 mM glutathione in 1 ml of phosphate buffer. Mixtures were preincubated at 37°C for 30 min. Subsequently, nicotinamide adenine dinucleotide phosphate and tert-butylhydroperoxide were added to final concentrations of 155 and 580 μM, respectively, and the change in absorbance at OD_{340} was recorded at regular intervals over 4 min. Enzyme units were defined as the amount of GPx required to oxidize 1 μmol nicotinamide adenine dinucleotide phosphate in 1 min at 25°C and calculated on the basis of a molar absorptivity for nicotinamide adenine dinucleotide phosphate at 340 nm of 6.22×10^{-6}.

CAT enzyme activity was measured by monitoring the decrease in H_2O_2 concentration spectrophotometrically over time [27]. For each sample, aliquots containing 130 μg protein were added to 50-mM phosphate buffer in a quartz cuvette. After zeroing, H_2O_2 was added to a final concentration of 10 mM in 0.9 ml, and the absorbance at OD_{240} was recorded at regular intervals over 4 min. The specific activity of each sample was calculated based on activity of pure CAT as described by Aebi [27].

Cell susceptibility

To assay the susceptibility of cultured cells to oxidative stress, we used the lactate dehydrogenase (LDH) assay as described previously [5], except that all assays were carried out on cells in 60-mm dishes. Myoblasts were maintained in growth medium; for myotube cultures, the medium was changed to DM 24 h after plating. Cells were maintained in DM for 4–5 d (myotube formation began by day 2) at which time pro-oxidant was added to the culture medium. After incubation with a pro-oxidant, the culture medium was aspirated and the cellular LDH activity was measured according to the manufacturer's instructions (Promega, Madison, WI,

USA). Cellular LDH was used as a measure of the number of surviving cells in each well. Cell death was calculated as a percentage, with 0% cell death being the value of cellular LDH in wells with no added toxin, and 100% cell death being the value of cellular LDH remaining after addition of 0.9% SDS to the culture medium just before the end of the incubation period. Thus for each cell population, there were internal positive and negative controls. In addition, cell death measurements using the LDH assay were also corroborated by microscopic inspection of cultures; relative changes in cell death as determined by the LDH assay always correlated with the appearance of more cell death visually. All assays were performed in triplicate.

Statistical analysis

Comparisons between paired samples were performed using paired Student's *t*-test; comparisons among samples in a group were carried out by analysis of variance. Differences were considered statistically significant at the $p < .05$ level.

RESULTS

When myotubes were subjected to treatment with the pro-oxidant paraquat, transcript levels for AOE were increased compared with untreated cells (Fig. 1A). The extent of induction differed among the transcripts with the greatest increases observed for GPx (approximately 5-fold) and CAT (approximately 4-fold). Smaller increases were seen for the CuZnSOD and MnSOD transcripts, but all of the changes were statistically significant compared with untreated cells (Fig. 1B). In the absence of oxidative challenge, none of the transcript levels changed over 72 h in control cultures. Based on the mechanism of toxicity of paraquat [21], we interpreted these changes in transcript levels as a cellular response to oxidative stress. To test this further, we used two other pro-oxidants, menadione and H_2O_2, to test for changes in AOE message levels. As shown in Table 1, treatment of myotube cultures with both menadione and H_2O_2 also resulted in significant increases of AOE message levels.

Because GPx message showed the most robust response to pro-oxidant treatment, we further examined the relationship between pro-oxidant treatment and AOE induction by focusing on the GPx response. Paraquat produced a statistically significant increase of GPx transcript within 24 h of exposure, and the levels increased further over the course of the next 48 h (Fig. 2A). During this time of exposure, there was evidence of cumulative oxidative injury to the cells as measured by protein

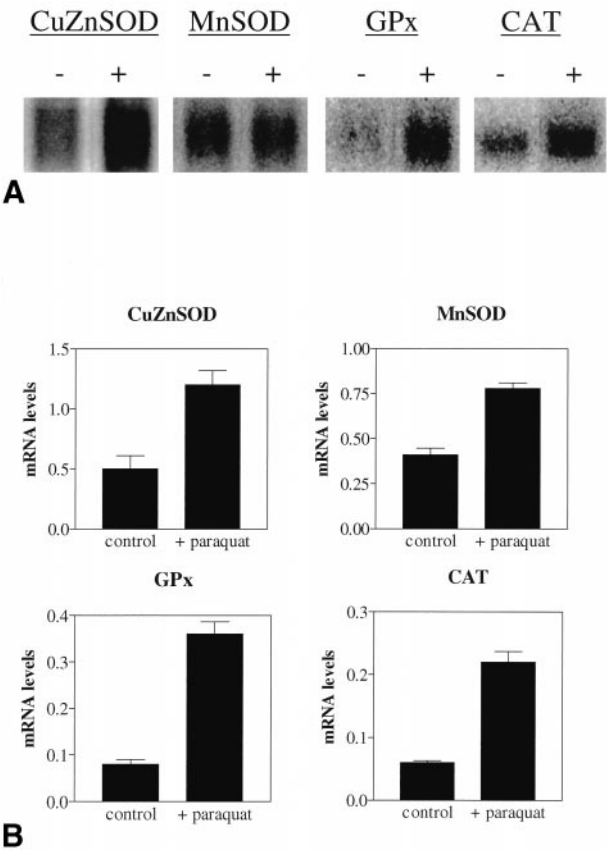

Fig. 1. Effect of oxidative stress on AOE mRNA levels. (A) Representative Northern blot of AOE mRNA levels from myotube cultures. Levels of transcripts of CuZnSOD, MnSOD, GPx, and CAT are shown 72 h after maintenance in DM alone (−) or in DM containing 0.1 mM paraquat (+). (B) Quantitative analysis of transcript levels. From experiments as shown in (A), transcript levels were quantified by signal intensity analysis using a PhosphorImager, normalized to the level of GAPDH transcript in the sample, and expressed as arbitrary units. Each point represents the value from four experiments, each performed in duplicate. The levels of GAPDH in each isolate did not change significantly either in absolute terms in relation to equal loading of RNA per lane, or in comparison with 18S ribosomal RNA in each lane. Data are expressed as mean ± SD. The differences between treated and control values for each AOE are statistically significant ($p < .05$).

oxidation (Fig. 2B). In control cultures, there was no increase in protein oxidation and no change in GPx transcript levels over 72 h. The induction of GPx message was dependent also on the dose of the oxidants. Figure 3A shows the dose–response relationship between GPx transcript level and concentration of either paraquat or H_2O_2. The induction of message levels correlated with the extent of cellular oxidative injury (Fig. 3B). Protein carbonyl group formation increased with increasing doses of both paraquat and H_2O_2 in dose ranges that resulted in elevated GPx transcript levels. Whereas there was evidence of cell death in cultures treated with the highest concentrations of pro-oxidants for the longest times, the induction of AOE transcripts was evident at

Table 1. Induction of AOE mRNA Levels in Response to Different Pro-oxidants

	Transcript level (fold increase over control)	
	Menadione treated	H_2O_2 treated
CuZnSOD	1.81 ± 0.28	2.03 ± 0.05
MnSOD	1.12 ± 0.18	1.85 ± 0.07
GPx	2.91 ± 0.22	2.79 ± 0.16
CAT	2.14 ± 0.18	2.60 ± 0.22

Myotube cultures were exposed to menadione or H_2O_2, and transcript levels for CuZnSOD, MnSOD, GPx, and CAT were determined as in Fig. 1. For each transcript, the data are presented as fold-increase over control, untreated cultures (mean ± SD). For these experiments, myotube cultures were exposed to 40 μM menadione for 24 h and to 2-mM H_2O_2 for 72 h. The increases are statistically significant ($p < .05$) for all values except for the increase in MnSOD transcript levels in response to menadione.

sublethal concentrations of all the pro-oxidants and at times before any evidence of cell death.

We examined the effect of pro-oxidant treatment on the activities of the antioxidant enzymes to determine if changes in enzyme activity paralleled changes in message levels. Although some investigators have found changes of AOE protein levels or enzyme activity to correlate well with mRNA levels, others have found discordant results [17,18,28]. Myotube cultures treated with paraquat were tested for CuZnSOD, MnSOD, GPx, and CAT enzyme activities. As with the Northern blot studies, the greatest increases were in GPx and CAT (Table 2). Although CuZnSOD and MnSOD activities showed only modest increases in pro-oxidant-treated cultures, the activities were significantly increased over controls. For all AOE, the elevations in enzyme activities were more modest than the increases in the respective transcript levels (Fig. 1).

Mature muscle tissue in vivo is composed primarily of differentiated myofibers. In addition, there is a small population of undifferentiated muscle precursor cells, or satellite cells, that are responsible for the regenerative

Table 2. Effect of Pro-oxidant Treatment on Enzyme Activities of AOE

	Enzyme activity (% increase over control)
CuZnSOD	134 ± 9.3%
MnSOD	114 ± 4.9%
GPx	206 ± 36%
CAT	284 ± 70%

Cultures that had been exposed to 0.1-mM paraquat for 3 d were assayed for CuZnSOD, MnSOD, GPx, and CAT enzyme activities. Data are expressed as percent increase over the activities in untreated cells (mean ± SD; average of four to six individual experiments, each with $n > 4$). All increases are statistically significant ($p < .05$) using a paired Student's *t*-test.

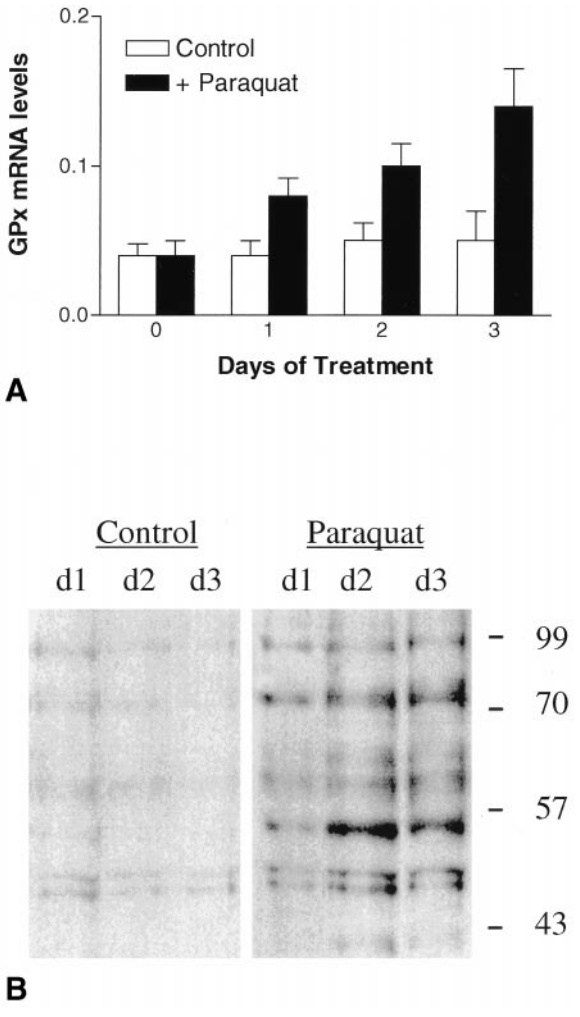

Fig. 2. Time dependence of oxidative injury and cellular response. (A) Time-dependent increase of GPx message level in response to oxidative stress. Myotube cultures were either maintained in DM alone (open bars) or maintained in DM containing 0.1 mM paraquat (filled bars). RNA was isolated at different times, and the levels of GPx transcripts were determined as described in Fig. 1. The level of GPx transcript was significantly elevated 24 h after initiation of treatment and increased further over the next 48 h. As in Fig. 1, values are normalized to GAPDH and expressed as arbitrary units. GPx message levels of control cells remained constant, with no significant change over 3 d. (B) Time-dependent increase of cellular oxidative injury. From cultures treated as in (A), total protein was isolated and cellular oxidative injury was assessed as carbonyl group formation by Western blot analysis (see Materials and Methods for details). There was an increase in the level of protein carbonyl group formation in treated compared with control cultures, and the oxidative injury increased over the duration of exposure to paraquat. In control cultures, there was no detectable increase in carbonyl group formation with time in culture.

capacity of the tissue [29]. In response to tissue damage, these satellite cells activate and begin proliferating as myoblasts, and it is these mononucleated cells that can be derived and propagated in vitro. Satellite cells in vivo are relatively resistant to metabolic and toxic challenges compared with differentiated myofibers [30,31].

We compared the sensitivity of myoblast and myo-

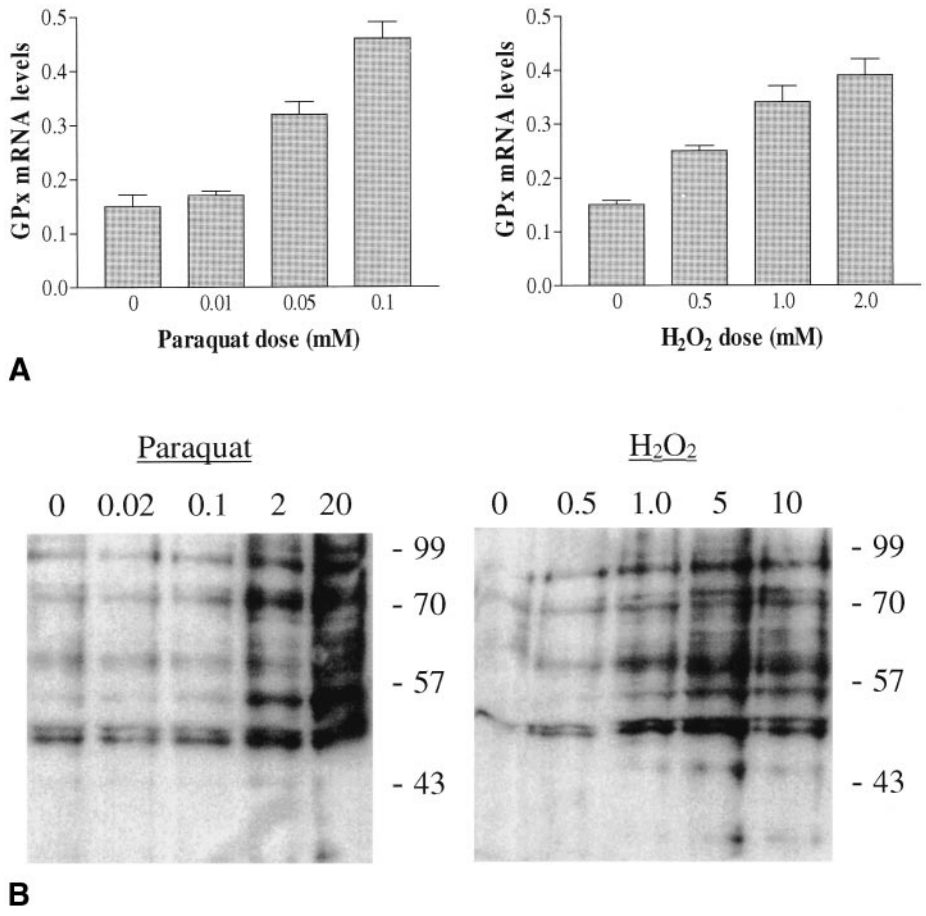

Fig. 3. Dose dependence of oxidative injury and cellular response. (A) Dose-dependent increase in GPx transcript levels in response to oxidative stress. Myotube cultures were treated with different concentrations of either paraquat or H_2O_2 as indicated. After 72 h, RNA was isolated and Northern blot analysis was performed to measure the levels of GPx transcript. As in Fig. 1, values are normalized to GAPDH and expressed as arbitrary units. Statistically significant increases over control (untreated) values were obtained for paraquat concentrations $\geq$ 0.05 mM and H_2O_2 concentrations $\geq$ 0.5 mM ($p < .05$). (B) Dose-dependent increases in cellular oxidative injury. In parallel cultures to those shown in (A), total protein was isolated 24 h after beginning treatment with the pro-oxidants and analyzed for protein carbonyl formation by Western blot analysis. There was a dose-related increase in carbonyl group formation in response to both paraquat and H_2O_2.

tube cultures to oxidative injury to determine if there was also a differential susceptibility between undifferentiated and differentiated cells in vitro. Actively proliferating myoblast cultures and differentiated myotube cultures (Fig. 4A) were exposed to toxic concentrations of paraquat, and the extent of cell death was compared. We found that differentiated myotubes were indeed more sensitive to oxidative injury than were undifferentiated myoblasts (Fig. 4B). This increased susceptibility correlated with increased protein oxidation in myotubes compared with myoblast cultures when the two populations were subjected to sublethal concentration of the pro-oxidant (Fig. 4C).

To test whether the increased susceptibility of myotubes to oxidative stress correlated with reduced antioxidant defenses, we examined the expression of antioxidant genes during cellular differentiation. Myoblast cultures were induced to differentiate, and the levels of CuZnSOD, MnSOD, GPx, and CAT messages were assessed during the process of myogenic differentiation. Myoblast fusion was evident within 24 h of switching the cells to DM, and during this time the transcript levels for all AOE decreased (Fig. 5). Maturation of the differentiated cells over the next 72 h did not result in a further significant reduction in AOE message levels.

We then examined the levels of AOE activities during differentiation (Fig. 6). There were important differences between changes in transcript levels and changes in enzyme activity levels. First, although both GPx and CAT activities decreased, there was no significant change in the activities of either CuZnSOD or MnSOD. This differential response may indeed lead to a greater

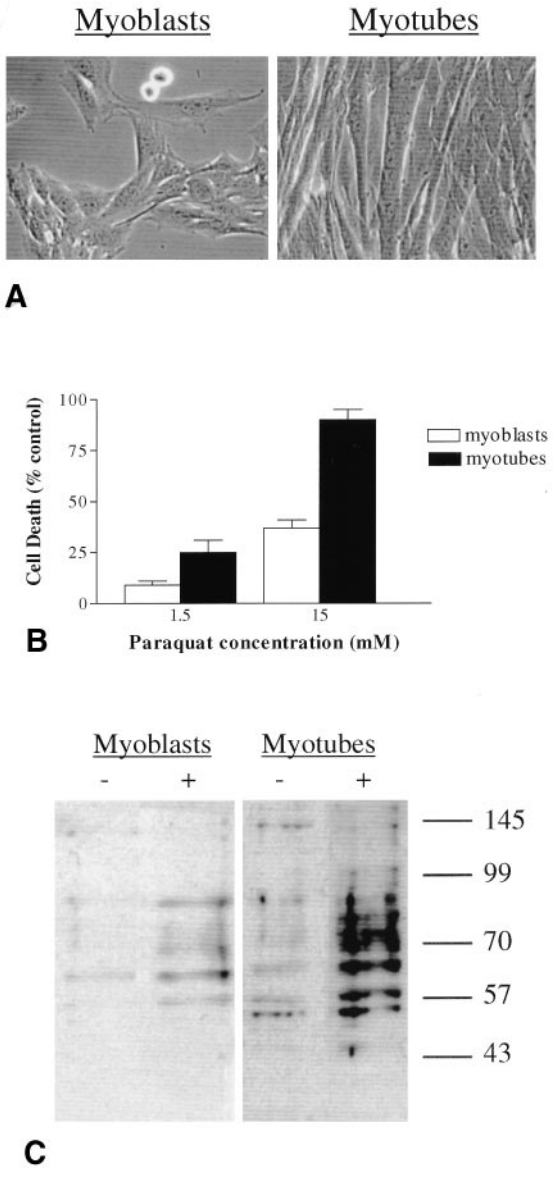

Fig. 4. Differentiated muscle cells are more susceptible to oxidative injury. (A) Photomicrographs of undifferentiated myoblast and differentiated myotube cultures. The panel on the left shows proliferating myoblasts maintained in growth medium. The panel on the right shows multinucleated myotubes 3 d after the medium was changed to DM. (B) Susceptibility of myoblasts and myotubes to oxidant-induced cell death. Myoblast and myotube cultures were subjected to oxidative stress by the addition of 1.5 and 15 mM paraquat to the culture medium. After 48 h, the extent of cell death was determined using the LDH assay. At both concentrations, myotubes exhibited greater sensitivity to the toxin; there was more cell death in the myotube cultures than in the myoblast cultures. Data are expressed as mean ± SD ($n = 5$). The differences between the two cell populations were statistically significant at each concentration ($p < .05$). (C) Susceptibility of myoblasts and myotubes to oxidative injury. Consistent with the finding of increased susceptibility shown in (B), myotube cultures exhibited higher levels of protein oxidation upon paraquat treatment than did myoblast cultures. Total protein was isolated from untreated cells (−) or cells treated with 1.5 mM paraquat for 8 h (+), and carbonyl group formation was assessed by Western blot analysis. Although there was a dramatic increase in protein oxidation in the myotube cultures, there was little increase in the myoblast cultures exposed to the same conditions.

increase in the susceptibility to oxidative stress than if all of the enzyme activities had decreased in proportion (see Discussion). Second, whereas both GPx and CAT transcripts had declined significantly and reached new steady state levels within 24 h of the induction of differentiation, the corresponding enzyme activity levels showed no significant change over the first 24 h. Activity levels for GPx and CAT declined to new steady state levels by 3 d after the induction of differentiation, and there was no significant change over the next several days. Therefore, the sensitivity of the differentiated cells to oxidative challenges (Fig. 4B) correlated with a reduction of the antioxidant capacity of myotubes compared with myoblasts.

DISCUSSION

The results of the studies in this report demonstrate that there is an increase in expression of specific antioxidant genes in muscle exposed to oxidative stress. The greatest inductions were in the levels of GPx and CAT mRNA; CuZnSOD and MnSOD messages showed more modest increases. These increases were dependent on both the time of exposure to and dose of the pro-oxidants and correlated with increases in cellular damage resulting from the oxidant treatments.

Differential responses of these genes to oxidative stress have been demonstrated in other tissues. Ho et al. [17] reported that expression of both MnSOD and GPx increased in rat lung under conditions of oxidative stress, with little change in CuZnSOD or CAT. Yoshioka et al. [32] determined the expression pattern of rat glomerular cells exposed to H_2O_2 and found that MnSOD, but not CuZnSOD, was induced. Shull et al. [16] showed the greatest induction of CAT in response to H_2O_2 in lung epithelial cells and smaller increases in GPx and MnSOD, whereas only MnSOD mRNA was increased in response to xanthine/xanthine oxidase treatment. In studies of rat hepatic cells, Rohrdanz and Kahl [18] showed that H_2O_2 treatment led to increases of MnSOD and CAT messages, but not CuZnSOD. Thus it appears that the response of these genes to oxidant stress differ in a tissue-specific manner.

When muscle cells were exposed to free radical–generating compounds, the induction of AOE genes coincided with increases in protein oxidation (Figs. 2 and 3), supporting the postulated relationship between oxidative stress and regulation of AOE. The mechanisms of regulation of gene expression by ROS have been well established in prokaryotic systems [33], and parallel mechanisms are being elucidated in eukaryotic organisms. ROS have been shown to stimulate kinase cascades in signal transduction pathways [34–36] and to activate or inhibit specific transcription

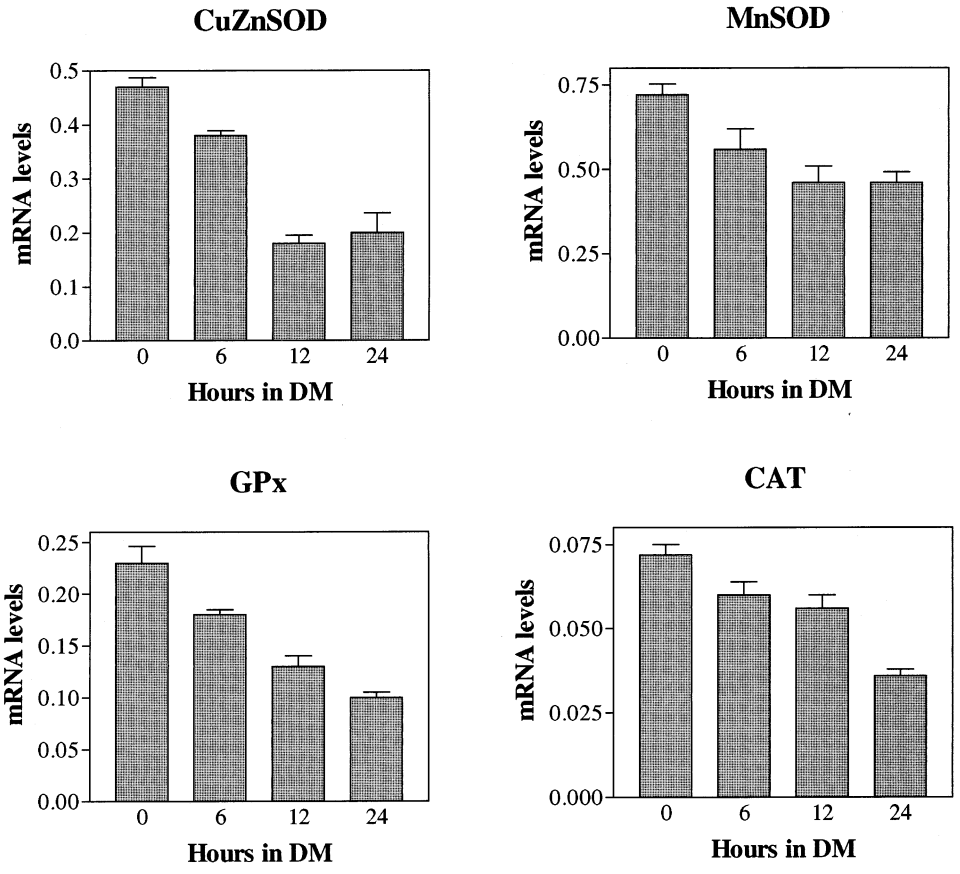

Fig. 5. Decreases in AOE expression during differentiation. Northern blot analysis of CuZnSOD, MnSOD, GPx, and CAT mRNA from myogenic cell cultures 0, 6, 12, and 24 h after initiation of differentiation. Data are normalized to the levels of GAPDH, which did not change during differentiation (determined as described in the legend for Fig. 1). Data are expressed as mean ± SD and represent the results of four separate determinations, each performed in duplicate. The decreases in transcript levels were significant for all AOE within 6 h of changing to DM.

factors [37]. Among the transcription factors that have been shown to be responsive to the redox state of the cell, the most widely studied are NFκB and AP-1 [37,38]. In this regard, it is noteworthy that NFκB and AP-1 consensus sites, as well as other redox-sensitive sites, have been identified in the regulatory regions of all of the AOE genes [39–45]. The balance between positive and negative regulatory factors is likely to determine the specific patterns of antioxidant gene induction in response to different oxidative stimuli and in different tissues. To the extent that NFκB may be a critical mediator of redox-sensitive transcriptional regulation in muscle as in other tissues, it is noteworthy that there is activation and increased DNA binding of NFκB in muscle in response to treatment with pro-oxidants [46,47].

Changes in antioxidant enzyme activities in muscle exposed to oxidative stress did not uniformly parallel the changes in the corresponding mRNA levels. Whereas GPx and CAT transcript levels were elevated 4–5-fold in response to paraquat treatment (Fig. 1), the respective enzyme activities were increased only 2–3-fold (Table 2). Similarly, increases in CuZnSOD and MnSOD activities were less robust than the increases in their message levels. Some studies have revealed increases in enzyme activity that follow increases in mRNA levels (although not quantitatively) [32,48]. However, others have found no changes or only minimal changes in AOE activities even when message levels are elevated [17,18,28] or increases in activities without changes in message levels [49]. Clearly, translational and post-translational regulation of antioxidant enzymes is important in determining changes in the levels of activity in response to oxidative stress. Ho et al. [17] suggested that the absence of an increase in protein level or enzyme activity in the setting of increase mRNA levels may result from a decrease in translational efficiency in cells under oxidative stress. The increase in message level would thus be necessary simply to prevent a reduction in AOE activity.

Previous studies have demonstrated a regulation of

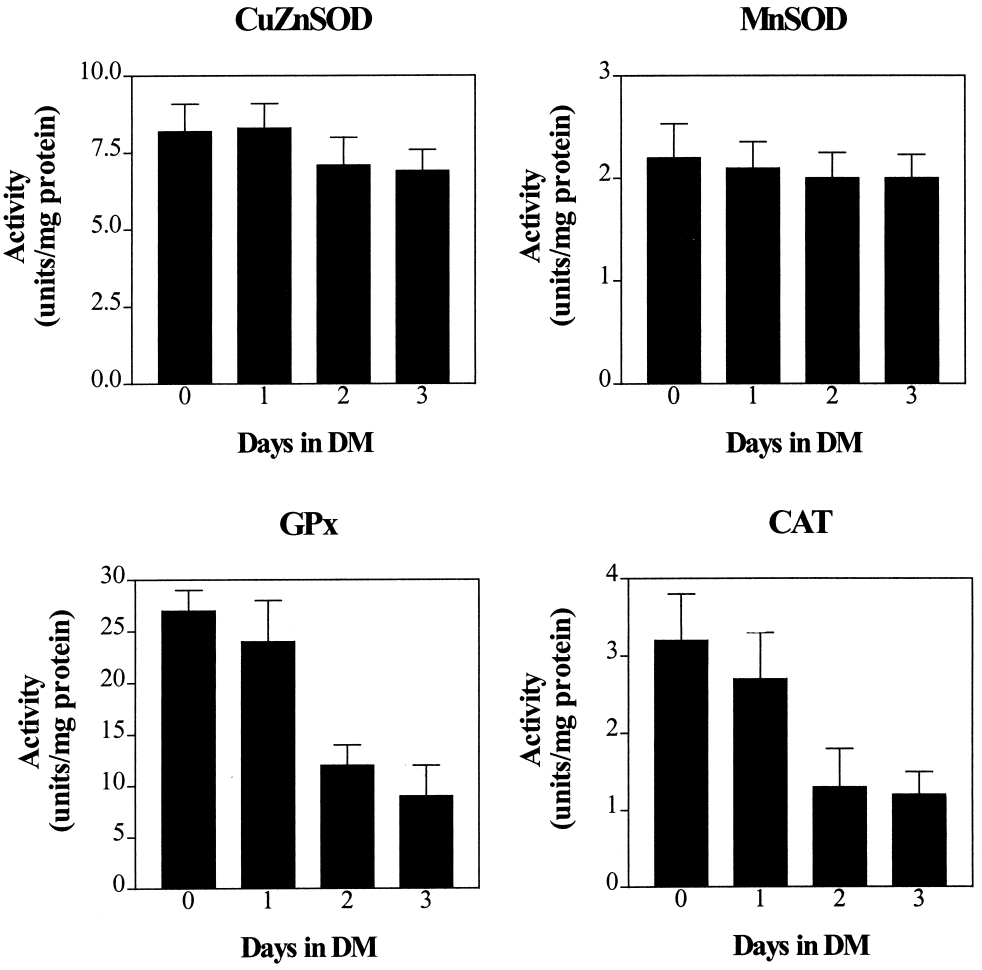

Fig. 6. Regulation in AOE activity during differentiation. Enzyme activities for CuZnSOD, MnSOD, GPx, and CAT were determined at different times after switching myoblasts to DM. All data are compared with the levels in myoblasts in growth medium (day 0). Data are expressed as mean ± SD and show the results of four separate experiments, each performed in triplicate. The levels of activity of CuZnSOD and MnSOD did not change significantly on differentiation. GPx and CAT activity levels decreased significantly within 48 h after the induction of differentiation.

antioxidant defense mechanisms during cellular differentiation [50,51]. During myogenic differentiation, there was a decrease in transcript levels for AOE (Fig. 5) and a decrease in activity levels for both GPx and CAT (Fig. 6). With regard to the possibility that NFκB and AP-1 are involved in the regulation of AOE gene expression, it is noteworthy that both are downregulated during myocyte differentiation [52]. As with the response to oxidant stress, the regulation of enzyme activity levels during differentiation involves more than just transcriptional regulation. The delay in the decline of activities relative to transcripts for GPx and CAT could be the result of post-transcriptional regulatory mechanisms or simply to the stability of the proteins themselves. The specific pattern of changes of the different AOE activities is interesting in light of the finding of an increased susceptibility of the differentiated cells to oxidant injury. The relative decrease in GPx and CAT activities compared with the SOD activity is just the differential pattern than has been shown to render cells particularly susceptible to oxidative stress [4,53–56]. This adds to the growing body of literature demonstrating that the levels of expression of AOE as well as the balance among them are critical determinants of the susceptibility of cells to oxidative injury [4,14,54].

Within individual muscles, there are subsets of myofibers that can be readily distinguished by their biochemical and physiologic properties. Studies of AOE in different fiber types have revealed intrinsic differences in basal activities [57,58]. It therefore should come as no surprise that subsets of myofibers within a muscle are differentially affected by pathologic conditions, such as muscular dystrophies, in which there is evidence of oxidative stress playing a pathogenetic role [2,4–6]. It has

been suggested that the differential effects may be the result of intrinsic differences in basal or inducible antioxidant defense capacities [59]. A better understanding of the mechanisms of regulation of antioxidant defenses in muscle may further explain the phenotypic variability among muscles in disorders in which ROS play a pathogenetic role.

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ABBREVIATIONS

AOE—antioxidant enzymes
CAT—catalase
CuZnSOD—Cu,Zn-superoxide dismutase
DM—differentiation medium
GAPDH—glyceraldehyde phosphate dehydrogenase
GPx—glutathione peroxidase
LDH—lactate dehydrogenase
MnSOD—Mn-superoxide dismutase
ROS—reactive oxygen species
SOD—superoxide dismutase
SSC—sodium chloride/sodium citrate