Original Contribution

Genetic modification of the manganese superoxide dismutase/glutathione peroxidase 1 pathway influences intracellular ROS generation in quiescent, but not contracting, skeletal muscle cells

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Abstract

Increased amounts of reactive oxygen species (ROS) are generated by skeletal muscle during contractile activity, but their intracellular source is unclear. The oxidation of 2′,7′-dichlorodihydrofluorescein (DCFH) was examined as an intracellular probe for reactive oxygen species in skeletal muscle myotubes derived from muscles of wild-type mice and mice that were heterozygous knockout for manganese superoxide dismutase ($Sod2^{+/-}$), homozygous knockout for glutathione peroxidase 1 ($GPx1^{-/-}$), or MnSOD transgenic overexpressors ($Sod2$-Tg). Myoblasts were stimulated to fuse and loaded with DCFH 5–7 days later. Intracellular DCF epifluorescence was measured and myotubes were electrically stimulated to contract for 15 min. Quiescent myotubes with decreased MnSOD or GPx1 showed a significant increase in the rate of DCFH oxidation whereas those with increased MnSOD did not differ from wild type. Following contractions, myotubes from all groups showed an equivalent increase in DCF fluorescence. Thus the oxidation of DCFH in quiescent skeletal muscle myotubes is influenced by the content of enzymes that regulate mitochondrial superoxide and hydrogen peroxide content. In contrast, the increase in DCFH oxidation following contractions was unaffected by reduced or enhanced MnSOD or absent GPx1, indicating that reactive oxygen species produced by contractions were predominantly generated by nonmitochondrial sources.
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Introduction

An increase in the levels of end-point indicators of the reactions of free radicals, reactive oxygen, and nitrogen species (ROS) has been reported to follow many different types of exercise [1,2], although the tissue and subcellular sources of the

ROS have not been extensively explored. Contracting skeletal muscle appears to be one major source for generation of ROS during exercise [3,4]. Most studies in skeletal muscle cite mitochondria as the major site for ROS generation but additional sites of generation are also recognized [5]. For example, the plasma membrane of skeletal muscle cells can be the source for generation of both nitric oxide (NO) and superoxide [6,7].

There have been few attempts to directly monitor ROS activity in contracting skeletal muscle cells. Silveira et al. [8] and Bejma et al. [9] analysed homogenised cultured skeletal muscle myotubes or intact muscle tissue immediately following contractions and reported increased oxidation of the ROS-sensitive fluorescent indicator, 2′,7′-dichlorodihydrofluorescein

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Abbreviations: D-PBS, Dulbecco's phosphate-buffered saline; DCFH, 2′,7′-dichlorodihydrofluorescein; DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate; GPx1, glutathione peroxidase 1; MnSOD, manganese-containing form of superoxide dismutase; ROS, reactive oxygen species; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
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(DCFH). This indicator has also been used as an intracellular probe to examine ROS activity directly by measurement of epifluorescence from strips of isolated diaphragm [10] and from skeletal muscle myotubes in culture [11]. Collectively, these studies confirm an increase in intracellular ROS activity during contractile activity in muscle cells, but provided no information on the sites or mechanisms of ROS generation.

During contractile activity a proportion of the oxygen utilized by skeletal muscle mitochondria has been proposed to form superoxide anion. This substance is a substrate of the mitochondrially located, manganese-containing form of superoxide dismutase (MnSOD, SOD2) for the generation of hydrogen peroxide. Within mitochondria one of the key enzymes responsible for degradation of hydrogen peroxide is glutathione peroxidase 1 (GPx1), [12]. Thus MnSOD and GPx1 enzymes are important regulators of ROS activity within mitochondria. Data from studies of genetically manipulated mice deficient in either enzyme indicate that each is important in protection against ROS-induced damage to tissues. Mice lacking GPx1 ($GPx1^{-/-}$) show evidence of increased mitochondrial oxidative damage [13] and increased susceptibility to toxicity following administration of ROS-generating agents [14]. Homozygous deletion of MnSOD ($Sod2^{-/-}$) is lethal with mice surviving 1–18 days [15,16], although heterozygous knockout mice with an approximately 50% reduction in MnSOD ($Sod2^{+/-}$) activity in all tissues survive without obvious phenotypic defects [14]. These $Sod2^{+/-}$ mice sustain increased mitochondrial oxidative damage and have reduced mitochondrial function compared with wild types. In contrast, tissues of mice in which MnSOD is overexpressed ($Sod2$-Tg) have been reported to be protected against injury induced by adriamycin or hyperoxia [17,18].

The aim of the current work was to examine the effect of genetic modification of the key regulatory proteins for mitochondrial ROS generation (MnSOD and GPx1) on intracellular ROS generation in contracting skeletal muscle cells. Cultured skeletal muscle myotubes, a model system to examine ROS activity in skeletal muscle cells [7,11], were studied both at rest and following electrical stimulation of contractions. Patterns of ROS activity in primary myotubes from mice that had reduced or enhanced MnSOD activity ($Sod2^{+/-}$ and $Sod2$-Tg) or reduced GPx1 activity ($GPx1^{-/-}$) were compared. Our hypothesis was that myotubes with reduced MnSOD or GPx1 activity would have increased intracellular DCFH oxidation at rest compared with wild-type cells and that this increase would be further exacerbated by contractile activity. For myotubes with enhanced MnSOD activity, we hypothesised that the DCFH oxidation both at rest and during contractile activity would be reduced in comparison with myotubes from wild-type mice.

Materials and methods

Mice

Myoblasts were derived from adult (4–8 months old) male C57BL/6 $Sod2^{+/-}$, $GPx1^{-/-}$, $Sod2$-Tg, and wild-type mice. Details regarding the generation and characterization of the knockout mouse models have been previously described [14,19,20]. The $Sod2^{+/-}$ mice were originally produced in the CD1 strain, and have been backcrossed onto the C57BL/6J genetic background for 14 generations (B6-Sod2[tm1Cje]). $GPx1^{-/-}$ mice used in this study were originally generated in the 129Sv/J strain, and were backcrossed for 10 generations onto the C57BL/6J background. The $Sod2$-Tg mice were originally described by Raineri et al. [21].

Cultures of skeletal muscle myotubes

Primary mouse myoblasts were prepared from hind leg muscles using a modification of the method of Rando and Blau [22]. Muscles were dissected away from nonmuscle tissue, minced, and then digested for 1 h at 37°C in DMEM (Sigma Chemical Co., Poole, Dorset, UK) containing filter-sterilized 0.1% (w/v) pronase solution (Calbiochem, San Diego, CA). The triturated digest was filtered and then myoblasts were separated on a Percoll gradient. Cells were expanded to 80% confluence on 0.1 % gelatin-coated tissue culture dishes in HAM F10 medium with 0.45% (w/v) glucose with 2 mM glutamine, 50 IU/ml penicillin, and 50 µg/ml streptomysin to which 20% fetal calf serum and 10 ng/ml basic fibroblast growth factor were added. Myoblast fusion was then induced using a differentiation medium of DMEM containing 2% horse serum (GIBCO, UK) with glucose, glutamine, penicillin, and streptomysin concentrations as above.

Loading of cells with 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA)

After 5–7 days of differentiation, the (multinuclear fused) myotubes were washed twice with warmed Dulbecco's phosphate buffered saline (D-PBS) (Sigma Chemical Co.) containing 0.1% glucose. A 10 µM DCFH-DA, Molecular Probes, Eugene, OR) in D-PBS was incubated with the cells for 30 min at 37°C. Cells were then washed with D-PBS and the media replaced with D-PBS alone. DCFH-DA is widely used as a relatively nonspecific intracellular probe for ROS [23]. DCFH-DA is nonpolar and crosses cell membranes readily and once within the cell it is hydrolysed by cytosolic hydrolases to DCFH. This compound reacts rapidly with hydrogen peroxide in the presence of peroxidases and less rapidly with some other ROS to form fluorescent dichlorofluorescein (DCF).

Microscopy and fluorescent imaging

The imaging system consisted of a Zeiss Axiovert 200M microscope equipped with ×10 and ×20 objectives and a 450–490 nm excitation, 515–565 nm emission filter (Filter Set 10), (Carl Zeiss GmbH, Germany). Images were acquired and analyzed using a computer-controlled Zeiss HRc charged–coupled device (CCD) camera (Carl Zeiss GmbH) and by Axiovision 3.0 image capture and analysis software

(Carl Zeiss Vision, GmbH, Germany) for quantification of changes in emission fluorescence. This software allows fluorescence measurements to be made from user-defined areas of the microscope field; in this case the fluorescence measurements were localised to selected areas of the myotubes avoiding the nuclei. DCF fluorescence was measured from user-defined segments of individual myotubes and from multiple (4) myotubes in each well. In order to control for variability in the amount of DCFH loaded into myotubes, data were normalised for the total amount of oxidisable DCFH in each measurement area as previously described [11]. In brief, following collection of experimental DCF fluorescence data, myotubes were exposed to a maximum dose of UV from the microscope lamp at the excitation wavelength for 3 min during which florescence was measured every 5 s. This procedure rapidly photo-oxidised all available DCFH and was documented by the measured fluorescence reaching a plateau. This plateau of fluorescence was considered to represent the total amount of DCFH available for oxidation and all fluorescence data are presented as a proportion of this total for individual areas of myotubes.

Stimulation of myotube contractions

After 15 min at rest, the myotubes were stimulated using platinum electrodes as previously reported [4,7]. Stimulation was performed with trains of alternating square wave pulses of 2 ms in duration for 0.5 s repeated every 5 s at 50 Hz and 30 V/well. Total stimulation time was 15 min. Following stimulation, the cells remained at rest for 15 min. Nonstimulated cells in D-PBS acted as controls.

Analysis of MnSOD content and GPx1 activity in myotubes

At 7 days following differentiation, separate cultures of myotubes were harvested and sonicated in 1% SDS containing 1 mM iodoacetimide, 1 mM benzithonium chloride, and 5.7 mM phenylmethylsulfonyl fluoride and 5 mM EGTA (Sigma Co.). Following sonication, cellular debris was removed by centrifugation, and samples were stored at −70°C until analysis. Protein content of samples was determined by using the bicinchoninic acid method (Sigma Co.). Twenty five micrograms of total cellular protein was separated on SDS-PAGE followed by Western blotting. The content of MnSOD was analysed by using a rabbit polyclonal antibody obtained from Bioquote (Cat. No. SOD-110). Bands were visualised using a Biorad Chemi-Doc System (Bio-Rad, Hercules, CA).

To measure GPx1 activity of myotubes, cell pellets were harvested and sonicated in 50 mM Tris, pH 7.5, 5 mM EDTA, and 1 mM dithiothreitol (DTT; Sigma Chemical Co.) Following sonication, cellular debris was removed by centrifugation, and samples were stored at −70°C until analysis. GPx1 activity was determined using an indirect assay that links GPx1-mediated oxidation of reduced glutathione (GSH) with the recycled reduction of oxidised glutathione (GSSG) to GSH by glutathione reductase using NADPH as a reductant [24].

Statistics

Data are presented as mean + SE of values for 5–7 wells for each experiment. Data were initially analyzed by analysis of variance followed by modified Student's t test. Data were considered significant at $p < 0.05$.

Results

Characterization of myotubes

The myotubes derived from the different strains of mice were examined at 5–7 days of differentiation when all appeared morphologically similar. Western blot analysis of MnSOD protein showed the anticipated changes in protein content (Fig. 1A). The MnSOD content in the myotubes from the $Sod2^{+/-}$ mice was 30–40% of the levels in myotubes from wild-type mice and increased by 3- to 4-fold in the myotubes from $Sod2$-Tg mice compared with those from wild-type mice. MnSOD contents of the myotubes from $GPx1^{-/-}$ mice were not different from those derived from wild-type mice. GPx1 activities were undetectable in the myotubes from $GPx1^{-/-}$ mice compared with myotubes from wild-type mice (GPx1 activity: 278 ± 133 nmol/min/mg protein), but were unchanged in myotubes derived from
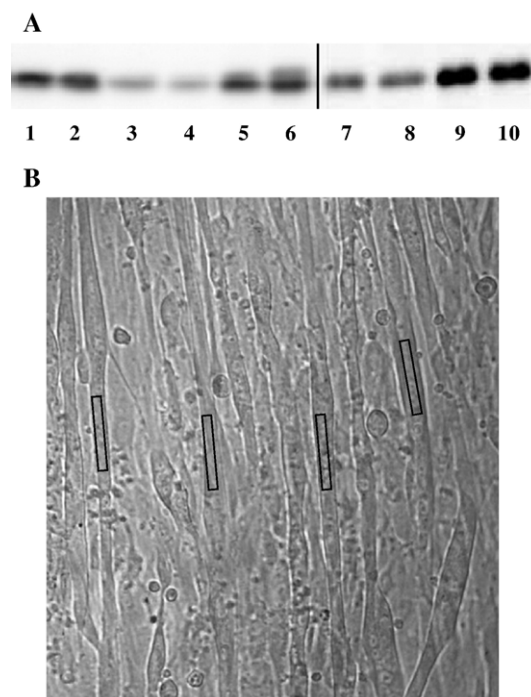
A

B

Fig. 1. (A) Western blot analysis for MnSOD protein. Lanes 1, 2, 7, and 8: myotubes from wild-type mice; lanes 3 and 4: myotubes from $Sod2^{+/-}$ mice; lanes 5 and 6: myotubes from $GPx1^{-/-}$ mice; lanes 9 and 10: myotubes from $Sod2$-Tg mice. Note lanes 1–6 and 7–10 are from different Western blots. (B) Example light micrograph of myotubes from wild-type mice showing the areas for measurement of epifluorescence in segments of 4 myotubes.

muscles of mice with modified MnSOD content in comparison with wild type.

DCF fluorescence from quiescent myotubes

DCF fluorescence was measured from distinct regions of at least 4 myotubes in each culture well as shown in Fig. 1B. Background measurements of fluorescence from areas of the well where myotubes were not present were also undertaken. The net DCF fluorescence (i.e., minus background readings) at 15-min intervals was used to calculate the rate of change of DCF fluorescence over three 15-min periods. The data are presented as a proportion of the total DCF available in that area of the myotube [11]. The rate of change of DCF fluorescence in the resting primary skeletal muscle myotubes from the four groups of mice over 45 min is presented in Table 1. At rest, myotubes from mice with decreased MnSOD or lacking GPx1 showed a significantly increased rate of DCFH fluorescence in comparison with myotubes from wild-type mice. The rate of change of DCF fluorescence observed from myotubes with increased MnSOD did not differ significantly from myotubes from wild-type mice. The DCF fluorescence from none of the groups changed significantly over the period of the study (45 min).

DCF fluorescence from electrically stimulated myotubes

The effect of a 15-min period of electrical stimulation of contraction in myotubes from wild-type mice is shown in Fig. 2 in comparison with the rate of change in DCF formation in quiescent myotubes from wild-type cells. Stimulation induced an approximate doubling of the rate of DCF fluorescence in myotubes that declined to levels similar to that of control, nonstimulated cells over the 15-min period following the end of contractions. Nonstimulated cells showed a tendency to increase the rate of DCF fluorescence throughout the 45 min of study, but this increase was not statistically significant. In any field of myotubes, 10–20% of the cells do not contract on electrical stimulation. These myotubes appear to load well with DCFH, but are apparently inexcitable by electrical stimulation. As a further control experiment, we examined DCF fluorescence in these cells before, during, and after the period of electrical stimulation of the well. Data shown in Fig. 2 demonstrate that these electrically stimulated, but noncontracting, myotubes did not show an increase in the rate of DCF
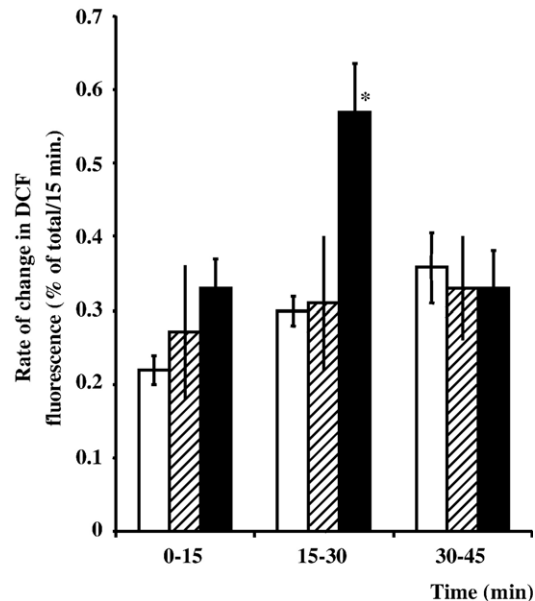

Fig. 2. Rate of change in DCF fluoresecence in quiescent myotubes from wild-type mice ($\square$), electrically stimulated myotubes that did not contract ($\boxbackslash$), and electrically stimulated myotubes that visibly contracted ($\blacksquare$). Electrical stimulation occurred during the period 15–30 min. Data are presented as mean ± SE. *$p < 0.05$ compared with initial values for the same myotubes, nonstimulated myotubes at the same time point, and stimulated but noncontracting myotubes at the same time point.

formation during electrical stimulation in contrast to contracting cells.

The effect of 15 min of stimulation on the rate of change in DCF fluorescence for all groups of cells is shown in Fig. 3A and these data have been recalculated to show the net effect of the electrical stimulation of contraction on the rate of change in DCF fluorescence in Fig. 3B. All groups showed a significant, but equivalent increase in DCF fluorescence following electrical stimulation, with no significant differences in the net effect of stimulation between the groups. Following the end of stimulation the rate of change in DCF fluorescence declined to prestimulation levels in myotubes from wild-type, $Sod2^{+/-}$, and $GPx1^{-/-}$ mice, but remained above baseline levels in the myotubes from $Sod2$-Tg mice.

Discussion

Cell morphology

Myoblasts obtained from skeletal muscle of $Sod2^{+/-}$, $GPx1^{-/-}$, $Sod2$-Tg, and wild-type mice proliferated in culture and, following addition of differentiation medium, all formed myotubes. There were some variations in the efficiency of proliferation and fusion that will be reported in a separate study (Csete et al., in preparation), but myotubes were analyzed at an approximately equivalent stage of maturation rather than at precisely the same time point following differentiation. Myotubes from all of the wild-type and genetically manipulated mice showed some spontaneous twitching prior to stimulation with no clear differences between the different strains.

Table 1
Rate of change in DCF fluorescence (% of total/15 min) from quiescent myotubes

	0–15 min	15–30 min	30–45 min
Wild type	0.23 ± 0.02	0.31 ± 0.02	0.36 ± 0.05
MnSOD$^{+/-}$	0.51 ± 0.09 *	0.58 ± 0.16	0.76 ± 0.14
GPx1$^{-/-}$	0.45 ± 0.04*	0.38 ± 0.09	0.38 ± 0.09
MnSOD-Tg	0.32 ± 0.03	0.25 ± 0.02	0.44 ± 0.04

* $p < 0.05$ compared with myotubes from wild-type mice at the same time point.
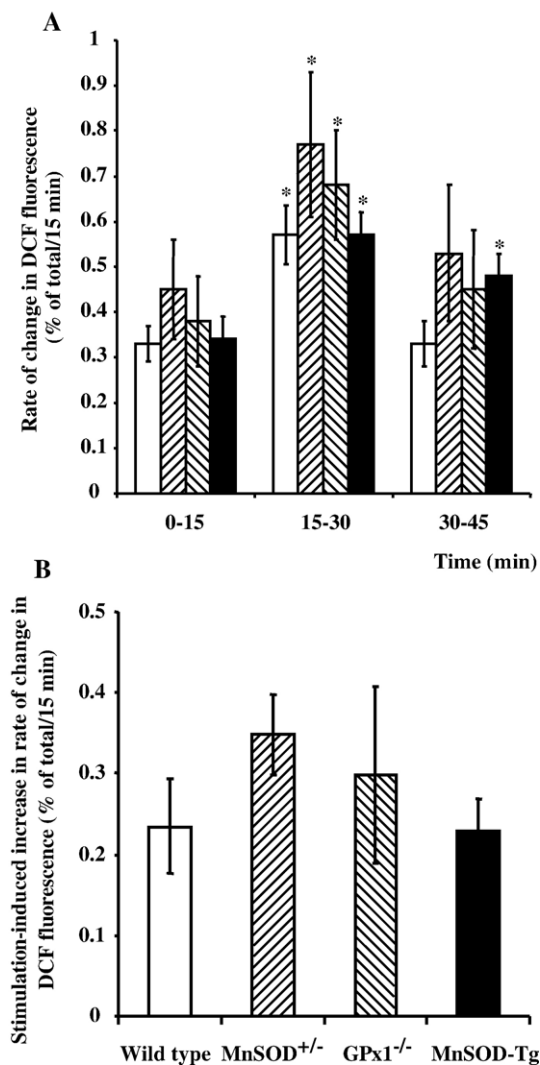
Fig. 3. (A) Rate of change in DCF fluoresecence in myotubes derived from skeletal muscle of wild-type (□), $Sod2^{+/-}$ (▨), $GPx1^{-/-}$ (▧), and $Sod2$-Tg (■) mice stimulated during the period 15–30 min. $*p < 0.05$ compared with values during the period 0–15 min (i.e., prior to stimulation) for the same group. (B) Stimulation-induced increase in the rate of change in DCF fluorescence from myotubes derived from skeletal muscle of wild-type, $Sod2^{+/-}$, $GPx1^{-/-}$, and $Sod2$-Tg mice.

Furthermore electrical stimulation of the cultures produced visible contractions in more than 80% of the myotubes in each cell culture well as previously reported for immortalised myotubes [7]. The myotubes remained adhered to the plates during and after the contraction protocols and retained their morphology throughout with no gross evidence of cell damage after the contraction protocol as previously reported for immortalised myotubes [7].

Myotubes showed similar changes in MnSOD content to those reported for skeletal muscles of the $Sod2^{+/-}$ and $Sod2$-Tg mice. GPx1 activity could not be detected in myotubes from the $GPx1^{-/-}$ mice and showed considerable variability in the myotubes from wild-type mice and those from mice with modified MnSOD content. This variability did not show any consistent changes between myotubes from the modified or wild-type mice.

DCF fluorescence from quiescent myotubes

The specific ROS that are able to oxidise DCFH in cells and hence contribute to DCF fluorescence has been the subjects of several studies (e.g., [23,25–28]). Most data suggest that DCFH reacts rapidly with hydrogen peroxide in the presence of peroxidases and with some other ROS with different sensitivities. In a review of this area, Zuo and Clanton [23] reported that DCFH oxidation was very sensitive to peroxynitrite in addition to hydrogen peroxide (in the presence of peroxidase), less sensitive to nitric oxide, hydroxyl radical, ferrous iron, and cytochrome c, and less and virtually unreactive with superoxide. Murrant et al. [25] reported that DCF fluorescence from quiescent diaphragm muscle fibers originates from reaction of DCFH with NO and ROS in approximately equivalent amounts. Several authors have concluded that DCFH oxidation may provide a comprehensive indicator of a broad range of oxidising reactions [23,25,26]. Our previous work using cultured immortalised myotubes indicated that DCF fluorescence from both quiescent and electrically stimulated myotubes was substantially decreased by treatment with the intracellular superoxide scavenger, Tiron [11]. In the current study no attempt was made to localise measurements of DCF fluorescence to specific areas of the cell (other than to avoid the nuclei) and hence the measurements primarily reflect cytosolic DCF fluorescence.

The differences in rate of change in DCF fluorescence from myotubes at rest are consistent with the hypothesis that the activity of cytosolic ROS activity is dependent upon mitochondrial ROS generation. $Sod2^{+/-}$ mice have evidence of increased superoxide-mediated oxidation of mitochondrial components [14] indicating an elevated intramitochondrial level of superoxide and this appears to have influenced cytosolic DCFH oxidation. Simple diffusion of superoxide from the mitochondria across the mitochondrial membrane to the cytosol seems unlikely, but the protonated form may cross membranes and there is evidence that superoxide crosses mitochondrial and potentially other membranes through anion channels [29,30]. Thus we hypothesise that the decreased mitochondrial MnSOD activity was sufficient to increase the transfer of superoxide (or potentially other substances generated due to a high local superoxide activity such as peroxynitrite) across the mitochondrial membrane to the cytosol where any increased superoxide could lead to an increase in hydrogen peroxide (by CuZnSOD activity) or peroxynitrite by reaction with nitric oxide and hence to increased DCFH oxidation.

GPx1 appears to be an important component of the mitochondrial system for detoxification of hydrogen peroxide although peroxiredoxins also play a role in this process [31]. In myotubes derived from skeletal muscle of $GPx1^{-/-}$ mice, it is anticipated that there is a reduced clearance of mitochondrial hydrogen peroxide and some previous data indicate that cells from $GPx1^{-/-}$ mice have an increased susceptibility to hydrogen peroxide exposure [32]. Hydrogen peroxide can freely diffuse across the mitochondrial membrane to oxidise cytosolic DCFH and our data are compatible with the $GPx1^{-/-}$ knockout leading to an increased transfer of hydrogen peroxide

from mitochondria to the myotube cytosol and producing increased oxidation of DCFH.

Myotubes from *Sod2-Tg* mice showed no significant change in DCF fluorescence in comparison with myotubes from wild-type mice at rest. Our conclusion from these data is that MnSOD activity is not limiting in myotubes at rest and that the protein is present in excess at normal levels of expression. Thus overexpression of the transgene did not influence resting mitochondrial superoxide or hydrogen peroxide sufficiently to modify the oxidation of cytosolic DCFH.

DCF fluorescence from electrically stimulated myotubes

Myotubes from wild-type mice showed an approximate doubling in the rate of DCF formation in response to electrical stimulated contractions. This modest rise in oxidation is similar to that previously reported from electrically stimulated, immortalised *H-2k^b* myotubes in culture [11], but differs from what would be anticipated from other current literature in this area. Several groups maintain that increased ROS generation during contractile activity is directly related to the elevated oxygen consumption that occurs with increased mitochondrial activity [1,33,34]. The pattern of demanding contractile activity imposed on the cells here would be predicted to cause a considerable increase in the skeletal muscle oxygen utilisation, but the modest increase in DCFH oxidation seen in contracting myotubes from wild-type mice is not consistent with the hypothesis that ROS generation is skeletal muscle cells is directly related to oxygen consumption. In order to exclude the possibility that the increased DCF fluorescence observed during electrically stimulated contractile activity was due to direct oxidation of the DCFH by the electrical stimulation, we examined the DCF fluorescence in the 10–20% of myotubes in wells that do not contract on electrical stimulation. These myotubes load well with DCFH, but despite repeated exposure to the electrical stimulation are not excitable and do not contract. These noncontracting cells showed the same pattern of DCF fluorescence as quiescent nonstimulated cells, indicating that contractile activity was necessary for the increase in DCF formation observed.

Use of the myotubes derived from *Sod2^{+/−}*, *GPx1^{−/−}*, and *Sod2-Tg* mice provides an alternative approach for examining the roles of mitochondrial vs nonmitochondrial ROS generation during contractile activity. If mitochondrial generation of superoxide due to increased oxygen utilisation is the ultimate source of the ROS generated during contractile activity, we hypothesised that the myotubes with reduced MnSOD or GPx1 activity would show relatively enhanced ROS activity during contractions, while the myotubes with increased MnSOD activity would show a less marked increase following contractions. In contrast to the data obtained at rest, no differences in the stimulation-induced change in the rate of increase in DCF formation were seen between the myotubes from genetically modified mice and those from wild-type mice (Fig. 3). Thus these data provide no support for the hypothesis that, in skeletal muscle myotubes, the increase in cellular ROS

activity that occurs following contractions is related to increased generation of superoxide and hydrogen peroxide within mitochondria. In contrast, the data can be interpreted as indicating that nonmitochondrial source(s) play the major role in increasing cytosolic ROS activity during contractile activity in muscle cells. A number of nonmitochondrial sources for ROS generation have been recognised in skeletal muscle cells including plasma membrane sites for generation of superoxide [7] and nitric oxide [6,7], sarcoplasmic reticulum-localised NAD(P)H oxidase activity [35], and phospholipase A2-dependent processes [36,37].

Interestingly the rate of DCFH oxidation in myotubes from MnSOD-Tg mice did not decline to prestimulation values following the end of the contraction protocol in contrast to those from wild-type, *Sod2^{+/−}*, and *GPx1^{−/−}* mice. The implication of this is that the increased MnSOD activity continued to induce a significant increase in cytosolic hydrogen peroxide following contractions, although potential mechanisms are not immediately apparent.

Although DCFH is a valuable tool for examining ROS activity in cells in situ, intracellular DCFH must compete with cytosolic antioxidant enzyme systems (e.g., CuZnSOD and catalase) and with potential scavengers of ROS (e.g., glutathione). Thus rapid changes in these competing pathways might additionally modify the amount of ROS available for reaction with DCFH. We have some evidence that a similar contraction protocol to that used here induces a rapid fall in the skeletal muscle glutathione content in mice in vivo [38] and hence there remains a possibility that the contraction-induced changes in DCF fluorescence reflect a decrease in muscle ROS scavenging potential rather than a rise in ROS generation. It is also feasible that the rate of intracellular DCFH oxidation may be influenced by a change in intracellular peroxidase activity, but no data in support of this have been presented.

In summary therefore our data indicate that the oxidation of DCFH in resting skeletal muscle myotubes is influenced by changes in enzymes that regulate mitochondrial ROS and hence may be modified by changes in mitochondrial superoxide and/or hydrogen peroxide. In contrast the contraction-induced increase in DCFH oxidation is unaffected by these processes and hence is likely to derive from ROS generated by nonmitochondrial sources.

Acknowledgments

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