Hydrogen peroxide stimulates ubiquitin-conjugating activity and expression of genes for specific E2 and E3 proteins in skeletal muscle myotubes

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ELEVATED LEVELS of reactive oxygen species (ROS) have been linked to the muscle atrophy that occurs in various inflammatory diseases (24). Animal studies by Buck and Chojkier (3) support the concept that elevated ROS levels might promote muscle protein loss. These investigators observed that antioxidants, either α-tocopherol or BW775c, administered to mice can attenuate the muscle atrophy induced by tumor necrosis factor (TNF)-α, a cytokine that stimulates mitochondrial production of ROS (8, 15, 26). At the cellular level, a link between ROS and muscle protein loss was recently demonstrated by Gomes-Marcondes and Tisdale (7). They showed that incubation of C2C12 myotubes with 100 μM H2O2 for 24 h stimulates protein degradation and increases expression of the 20S proteasome α-subunit and E214K, a ubiquitin carrier protein. These findings suggest that ROS stimulate loss of muscle protein by upregulating elements of the ubiquitin-proteasome pathway.

In this pathway, ubiquitin conjugation to a protein substrate initiates degradation of the protein by the 26S proteasome complex. The ubiquitin-activating enzyme (E1 protein) activates ubiquitin, which is then transferred to a ubiquitin carrier protein (E2). The E2 protein interacts with a ubiquitin ligase (E3) to catalyze transfer of ubiquitin to a protein substrate, marking the substrate for proteasomal degradation as ubiquitin accumulates. Specificity of the system is attributed to the E2 and E3 proteins that target particular proteins for degradation (13). For example, in skeletal muscle E214K and E3α work together to transfer ubiquitin to protein substrates that contain basic and large hydrophobic NH2-terminal amino acids, termed the “N-end rule pathway” (14). Subsequent research identified additional E2 and E3 proteins that may contribute to muscle atrophy in catabolic conditions. These include the E2 protein UbcH2/E220K (16) and the E3 proteins atrogin1/MAFbx (2, 6) and MuRF1 (2). ROS effects on expression of the genes that code for these E2 and E3 proteins have not been established.

The current study addressed ROS effects on ubiquitin conjugation in muscle cells. Specifically, our experiments tested two hypotheses. The first hypothesis was that ROS stimulate ubiquitin-conjugating activity in skeletal muscle cells. The elevated levels of ubiquitin carrier protein previously measured in ROS-exposed muscle (7) could increase the rate at which ubiquitin is conjugated to protein substrates, i.e., ubiquitin-conjugating activity. To test this possibility, we exposed differentiated myotubes from the skeletal muscle-derived C2C12 cell line to H2O2 in cell culture. We subsequently measured the magnitude and time course of changes in integrated activity of the ubiquitin-conjugating pathway. The second hypothesis was that ROS upregulate expression of genes for E2 and E3 proteins that regulate ubiquitin conjugation during muscle wasting. Among the hundreds of E2 and E3 proteins expressed by mammalian cells, several have been identified as putative mediators of skeletal muscle catabolism. These include two E2 proteins (E214K,
UbcH2) and three E3 proteins (E3α, atrogin1/MAPbx, and MuRF1). We used Northern blot analysis to test for increased expression of these genes in C2C12 myotubes after H2O2 stimulation.

METHODS

Myogenic cell cultures. Myoblasts from the mouse skeletal muscle cell line C2C12 (American Type Culture Collection, Rockville, MD) were cultured as described previously (17). In brief, C2C12 cells were cultured in DMEM supplemented with 10% fetal calf serum and gentamicin at 37°C in the presence of 5% CO2. Myoblast differentiation was initiated by replacing the growth medium with differentiation medium, DMEM supplemented with 2% heat-inactivated horse serum. Differentiation activity, autoradiographs of the gel were analyzed for degradation of ubiquitinated proteins by the proteasome (Fig. 1) and the isopeptidase inhibitor ubiquitin aldehyde (isopeptidase removes conjugated ubiquitin from proteins). We used Northern blot analysis to test effects on ubiquitin-conjugating activity. Whole cell extracts of myotubes were made by three freeze-thaw cycles followed by 30-min incubation at 4°C in buffer C containing (in mM) 20 Tris-HCl pH 7.9, 420 NaCl, 1.5 MgCl2, 0.2 EDTA, 0.5 DTT, and 0.2 PMSF with 25% glycerol, 1 μg/ml leupeptin, and 2 μg/ml aprotinin. The extracts were dialyzed against a buffer containing 10 mM, Tris-HCl pH 7.6, 1 mM DTT, and 10% glycerol. 125I-labeled ubiquitin was prepared by iodination of ubiquitin (Sigma) with carrier-free Na125I (Amersham Pharmacia). Dialyzed extracts (~100 μg protein) were incubated with 125I-L-ubiquitin (~100,000 cpm) in a buffer containing 20 mM Tris-HCl, pH 7.6, 20 mM KCl, 5 mM MgCl2, 1 mM DTT, 10% glycerol, 30 μM MG-132 (to inhibit proteasome activity), 1 μM ubiquitin aldehyde (to inhibit the hydrolysis of ubiquitin conjugates by deubiquiti- nating isopeptidases), and 2 mM 5′-adenosyl-l-methyl- diphosphate (AMPPNP). AMPPNP is an ATP analog that provides energy for activation of ubiquitin by E1 but not for degradation of ubiquitinated proteins by the protease (10). The reaction mixtures (20 μl total volume) were incubated at 37°C for 60 min, after which the reaction was terminated by addition of 20 μl of 2× Laemmli sample buffer (12). The mixture was heated to 90°C for 3 min and separated on SDS-PAGE (15% gel). To quantify ubiquitin- nating activity, autoradiographs of the gel were analyzed by densitometry software (ImageQuant 5.2, Molecular Dynam- ics).

Northern blot analysis. Total RNA was isolated from C2C12 myotubes by use of the RNAzol reagent (TEL TEST). Twelve micrograms of each sample was separated by agarose gel electrophoresis modified from the procedure described by Liu and Chou (18). Briefly, RNA samples were denatured by heating to 65°C for 3 min in a sample loading buffer containing 1× Tris-boric acid-EDTA (TBE), 6.5% Ficoll, 0.005% bromphenol blue, 0.025% xylene cyanol ff, and 3.5 M urea (final concentrations). The samples were immediately chilled on ice and separated with 1% native agarose gel at 8 V/cm. RNA was then blotted and ultraviolet cross-linked to a Gene- Screen membrane (NEN Life Science Products, Boston, MA). Prehybridization (4 h) and hybridization (16 h) were carried out in ULTRAhyb buffer (Amion) at 42°C. Hybridization probes were full-length human coding sequences of the specific genes except that the polyubiquitin probe was of mouse origin. The probes were prepared from isolated total RNA with a one-step RT-PCR kit (Promega, Madison, WI) and were labeled with [α-32P]dCTP (3,000 Ci/mmol, Amesham Pharmacía, Arlington Heights, IL) by using the random primer method. After hybridization, each membrane was washed and exposed to X-ray film. Levels of mRNA were quantified by analyzing autoradiographs with densitometry software (ImageQuant).

Electrophoresis mobility shift assay. Nuclear extracts of C2C12 myotubes were prepared as described previously (17) with buffer C modified by the addition (in mM) of 30 sodium pyrophosphate, 5 Na2VO4, 2 iodoacet acid, 50 NaCl, 50 NaF, and 5 ZnCl2. Electrophoresis mobility shift assay (EMSA) was carried out as previously described (17). Briefly, the binding assay buffer contained (in mM) 5 Tris-HCl, pH 7.5, 100 NaCl, 0.3 DTT, and 5 MgCl2 with 10% glycerol, 2 μg of bovine serum albumin, 0.2% NP-40, and 1 μg of poly(dI-dC)-poly(dI- dC). A DNA probe containing the NF-κB consensus binding site (5′-AGTTGAGGGGACTTTCCCAGGG-3′; consensus binding site underlined) was labeled with [α-32P]dATP (3,000 Ci/mmol, Amersham Pharmacia, Arlington Heights, IL) with the Klenow fragment. After 20-min preincubation of 10 μg of nuclear extract, 1 ng (10,000–15,000 cpm) of labeled probe was added and incubation was continued for 30 min on ice; the reaction mixtures were resolved on 4.5% polyacrylamide gels. Protein concentration of the nuclear extracts was determined by the Bradford method (Bio-Rad protein assay kit).

Statistical analysis. Densitometry data sets were tested for normality and equal variance with commercial software (SigmaStat, SPSS Science, Chicago, IL). Data were then evaluated with one-way analysis of variance combined with Tukey’s multiple-comparison test (29). Differences between groups were considered significant at the P < 0.05 level. Values are reported as means ± SE.

RESULTS

H2O2 and ubiquitin-conjugating activity. As shown in Fig. 1, myotubes possess a basal level of ATP-dependent ubiquitin-conjugating activity that is detectable before H2O2 stimulation. This activity remains unaltered 1 h after exposure to 100 μM H2O2 but becomes elevated within 4 h (Fig. 1) and remains elevated for at least 24 h (data not shown). Control studies confirmed that formation of ubiquitin conjugates requires inclusion of the ATP analog AMPPNP (Fig. 1) and the isopeptidase inhibitor ubiquitin aldehyde (isopeptidase removes conjugated ubiquitin from proteins; data not shown). The ability of H2O2 to stimulate ubiquitin-conjugating activity was abolished by preincubating myotubes with 20 μg/ml cycloheximide, a protein synthesis inhibitor, or 1 μg/ml actinomycin D, an RNA synthesis inhibitor (Fig. 2). Activity also appeared to be increased by cycloheximide alone. The direct effect of H2O2 on ubiquitin-conjugating activity was assessed with a cell-free system. Whole cell extracts were incubated with 40, 100, or 250 μM H2O2 for 1 h and then were tested for ubiquitin-conjugating activity; H2O2 preconditioning had no effect (data not shown). Together, these findings suggested that H2O2 effects on ubiquitin-conjugating activity are likely to be regulated at the transcriptional level.

H2O2 and E2/E3 gene expression. We then evaluated H2O2 effects on the expression of genes that regulate ubiquitin-conjugating activity during muscle wasting. Changes in mRNA levels were determined by Northern
Because the polyubiquitin gene is generally upregulated by stimuli that increase ubiquitin-conjugating activity, we first examined H$_2$O$_2$ effects on polyubiquitin expression. As shown in Fig. 3, C$_2$C$_{12}$ myotubes incubated with 100 μM H$_2$O$_2$ exhibited a 19% increase in polyubiquitin mRNA after 6 h. We subsequently evaluated mRNA levels for E2 and E3 proteins that are reported to regulate muscle catabolism. Each E2 and E3 had a detectable level of constitutive expression, but only a subset responded to H$_2$O$_2$ stimulation. We observed a 29% increase in E2$_{14k}$ mRNA levels 6 h after H$_2$O$_2$ stimulation (Fig. 4), which is consistent with the findings of Gomes-Marcondes and Tisdale (7). In contrast, there was no change in UbcH2/E2$_{20k}$ mRNA levels during this period (data not shown).

Fig. 1. H$_2$O$_2$ stimulates ubiquitin-conjugating activity. C$_2$C$_{12}$ myotubes were incubated with 100 μM H$_2$O$_2$ for indicated period. Whole cell extracts prepared from harvested myotubes were incubated with $^{125}$I-labeled ubiquitin ($^{125}$I-Ub). A: ubiquitin conjugation to muscle proteins as visualized by autoradiography of SDS-PAGE used to separate the proteins; film depicts composite image obtained from a single film generated in 1 of 3 experiments; conjugation after H$_2$O$_2$ exposure [optical density (OD) = 172% control at both 4 and 6 h] illustrates responses seen in each of 3 experiments. AMPPNP, 5'-adenylylimidodiphosphate. B: average data as quantified by densitometry. The overall $P$ value shown was obtained by ANOVA; *difference from control ($P < 0.05$) determined by Tukey's multiple-comparison test.

Fig. 2. Cycloheximide and actinomycin D block H$_2$O$_2$ effects on ubiquitin-conjugating activity. C$_2$C$_{12}$ myotubes were preincubated with 20 μg/ml of cycloheximide or 1 μg/ml of actinomycin D for 30 min before incubation with or without 100 μM H$_2$O$_2$ for 6 h. Autoradiograph depicts a composite image obtained from a single film illustrating $^{125}$I-ubiquitin conjugation into muscle proteins.

Fig. 3. H$_2$O$_2$ upregulates the polyubiquitin gene. C$_2$C$_{12}$ myotubes were incubated with 100 μM H$_2$O$_2$ for indicated period. Total RNA was isolated for Northern blot analysis with full-length cDNA probes. A: autoradiograph from 1 of 3 experiments; polyubiquitin mRNA increased 6 h after H$_2$O$_2$ exposure (OD = 130% time 0). B: photograph of corresponding ethidium bromide-stained agarose gel (loading control). C: averaged data quantified by densitometry; means ± SE are shown. ANOVA indicated an overall difference among groups ($P < 0.05$); *difference from time 0 as analyzed by Tukey's multiple-comparison test ($P < 0.05$).
shown) and H$_2$O$_2$ did not alter E3 expression (data not shown). However, other E3 genes responded to H$_2$O$_2$ stimulation. Atrogin1/MAFbx expression was increased 42% after 3-h incubation (Fig. 5), and MuRF1 expression was increased 46% after 6 h (Fig. 6). These observations confirm that H$_2$O$_2$ upregulates genes that regulate ubiquitin conjugation and suggest that this transcriptional response involves a subset of ROS-responsive genes.

We were puzzled by the failure of UbcH2 to respond to H$_2$O$_2$ because TNF-$\alpha$ appears to upregulate the UbcH2 gene via ROS-dependent NF-$\kappa$B activation (15, 16). We therefore conducted follow-up studies to test the effect of H$_2$O$_2$ exposure on NF-$\kappa$B activity. As illustrated in Fig. 7, incubation with H$_2$O$_2$ increased NF-$\kappa$B translocation to myotube nuclei, which confirmed prior reports (16). However, this response was dose dependent and relatively weak. H$_2$O$_2$ concentrations $>$100 $\mu$M were required to evoke detectable NF-$\kappa$B activation, and the magnitude of this response was less than the NF-$\kappa$B activation stimulated by 6 ng/ml TNF-$\alpha$. These data suggest that in prior experiences (above) 100 $\mu$M H$_2$O$_2$ did not activate NF-$\kappa$B and therefore did not upregulate UbcH2 expression.

**DISCUSSION**

This study contributes to our understanding of the cellular and molecular mechanisms by which ROS might promote skeletal muscle catabolism. The current data provide the first direct evidence that H$_2$O$_2$ increases ubiquitin-conjugating activity in skeletal muscle cells. The data further demonstrate that H$_2$O$_2$ exposure can alter the expression of genes that regulate this pathway, including the polyubiquitin gene and genes for key E2 and E3 proteins. The biological significance of these observations is briefly outlined below.

**ROS and muscle catabolism.** Like all eukaryotic cells, mammalian skeletal muscle cells continuously generate ROS, which are detectable in the cytosol (23) and are released into the extracellular space (25). The physiological level of H$_2$O$_2$ in mammalian tissue is believed to be in the range of several micromoles per
that were incubated with H$_2$O$_2$ in cell culture but not in whole cell extracts directly exposed to H$_2$O$_2$. These findings argued against a posttranslational mechanism of action for H$_2$O$_2$.

A transcriptional mechanism seemed more likely. Gomes-Marcondes and Tisdale (7) had already shown that E2$_{14k}$ protein levels are elevated 24 h after H$_2$O$_2$ exposure. Their finding suggested that H$_2$O$_2$ might stimulate ubiquitin-conjugating activity by increasing the expression of one or more regulatory enzymes in the pathway. Consistent with this model, we found a 4-h lag between H$_2$O$_2$ exposure and the subsequent increase in ubiquitin-conjugating activity. Moreover, this increase could be blocked by cycloheximide or actinomycin D. These findings suggested that H$_2$O$_2$ stimulates ubiquitin conjugation by altering muscle gene expression.

**Transcriptional regulation of the process.** Subsequent experiments determined that H$_2$O$_2$ upregulates genes that are known to affect ubiquitin conjugation during muscle atrophy. These include the polyubiquitin gene. Ubiquitin availability is not generally considered a rate-limiting factor in ubiquitin conjugation.
However, expression often is upregulated when ubiquitin-conjugating activity is elevated (5, 19–21). This may reflect a compensatory response that maintains adequate ubiquitin supplies under conditions of increased utilization.

Among the regulatory enzymes upregulated by H₂O₂, E₂₁₄k is a critical regulator of ubiquitin conjugation via the N-end rule (14). E₂₁₄k is thought to interact with E₃α to mediate muscle wasting in various catabolic states (13). The E3 proteins atrogin1/MAFbx and MuRF1 also appear to play important roles in muscle atrophy. Recent evidence implicates both of these E3 proteins in experimental models of catabolism that include fasting, diabetes, cancer, renal failure, hindlimb suspension, immobilization, and denervation (2, 6).

The timing of these responses is of interest. The mRNA for atrogin1/MAFbx was elevated at 3 h, much earlier than the 6-h response time exhibited by other H₂O₂-responsive genes, suggesting a distinct mechanism of regulation for atrogin1/MAFbx expression. These transcriptional events also appear to have persistent effects on cell function. Twenty-four hours after H₂O₂ exposure, Gomes–Marcondes and Tisdale (7) measured elevated E₂₁₄k levels and we observed continuing elevation of ubiquitin-conjugating activity.

Subsequent experiments tested H₂O₂ effects on NF-κB, a redox-sensitive transcription factor that regulates UbcH2/E₂₂₀K expression (16). We found no evidence that 100 μM H₂O₂ stimulates NF-κB activation under the current experimental conditions. This explains the failure of UbcH2/E₂₂₀K mRNA to increase in myotubes after H₂O₂ exposure. Furthermore, these results suggest that NF-κB is not an essential regulator of the genes that did respond to H₂O₂, including polyubiquitin, E₂₁₄k, atrogin1/MAFbx, and MuRF1.

In conclusion, H₂O₂ exposure appears to promote protein degradation in skeletal muscle via the ubiquitin-proteasome pathway. Results of the current study suggest that muscle catabolism is due, at least in part, to ROS effects on the multienzyme process by which ubiquitin is conjugated to protein substrates. Oxidative signaling appears to activate a subset of redox-sensitive genes that code for ubiquitin and regulatory E2 and E3 proteins. Upregulation of these gene products increases the rate of ubiquitin conjugation within skeletal muscle, a persistent response that accelerates the targeting of muscle proteins for degradation by the 26S proteasome.

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REFERENCES


