Interactions between ROS and AMP kinase activity in the regulation of PGC-1α transcription in skeletal muscle cells

Isabella Irrcher, Vladimir Ljubicic, and David A. Hood

1School of Kinesiology and Health Science, 2Department of Biology, and 3Muscle Health Research Centre, York University, Toronto, Ontario, Canada

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THE FORMATION OF REACTIVE OXYGEN SPECIES (ROS) occurs during cellular respiration when approximately 1–3% of electrons in the electron transport chain are prematurely donated to oxygen, producing the free radical, superoxide (40). Superoxide is then rapidly dismutated into hydrogen peroxide (H₂O₂) by superoxide dismutase (MnSOD). H₂O₂ can be further reduced to water by catalase or glutathione peroxidase, or alternatively to the hydroxyl radical (·OH) in the presence of reduced copper or iron (12). The effects of superoxide and ·OH on DNA, protein, and/or lipid oxidation can cause irreversible cellular damage that has been associated with the underlying pathogenesis of a wide variety of diseases and also with degenerative processes associated with aging (7, 16). Recently, however, studies have also highlighted an important role for ROS at physiological concentrations in normal cell function. Indeed, ROS have been shown to mediate the activation of signaling cascades that are needed to regulate growth, differentiation, proliferation, and apoptosis (3, 16, 19, 23).

Mitochondria are the major source of ROS production (7). In turn, these organelles appear to respond to elevated ROS production by undergoing morphological and/or functional adaptations. For example, the application of exogenous ROS agents to normal cells promotes the elongation and branching complexity of the mitochondrial reticulum in fibroblast cells (26). The study of skin fibroblasts from patients with a deficiency in complex I of the mitochondrial electron transport chain further supports these findings (27). These morphological adaptations have been postulated to improve ATP delivery within cells and/or to initiate intramitochondrial signaling (5, 26, 27, 38).

ROS-mediated signals arising within mitochondria generate a retrograde response that is conveyed to the nucleus, causing the upregulation of nuclear genes encoding mitochondrial proteins and leading to the induction of mitochondrial biogenesis (10). Several recent studies have shown that ROS-mediated mitochondrial biogenesis is likely to occur via the upregulation and actions of nuclear respiratory factor-1 (NRF-1), the mitochondrial transcription factor Tfam, as well as the transcriptional coactivator peroxisome proliferator-activated receptor-γ (PPAR-γ) coactivator-1 protein-α (PGC-1α; 29, 32, 33, 39).

PGC-1α plays a central role in regulating the mitochondrial content within cells (35, 42). It is induced by stimuli such as thyroid hormone treatment, 5-aminomidazole-4-carboxamide-1-β-d-ribofuranoside (AICAR)-induced AMPK activation, as well as contractile activity in vivo and in vitro in skeletal muscle (6, 21, 34). More recently, it was demonstrated that PGC-1α gene expression is regulated by ROS, thereby establishing a potential link between ROS and the induction of mitochondrial biogenesis (39). The ROS signals that regulate PGC-1α transcription are not yet fully understood. However, several signaling kinases have been implicated in mediating PGC-1α transcriptional activation in response to various stimuli (1, 2, 14, 15, 20). The most important of these include CaMKIV (41), AMPK (22) and p38 (2). Their activation leads to the transcriptional regulation of the PGC-1α promoter via interactions between ROS and AMP kinase activity in the regulation of PGC-1α transcription in skeletal muscle cells.

Irrcher I, Ljubicic V, Hood DA. Interactions between ROS and AMP kinase activity in the regulation of PGC-1α transcription in skeletal muscle cells. Am J Physiol Cell Physiol 296: C116–C123, 2009. First published November 12, 2008; doi:10.1152/ajpcell.00267.2007.—Reactive oxygen species (ROS) play an important role in cellular function via the activation of signaling cascades. ROS have been shown to affect mitochondrial biogenesis, morphology, and function. Their beneficial effects are likely mediated via the upregulation of transcriptional regulators such as peroxisome proliferator-activated receptor-γ coactivator-1 protein-α (PGC-1α). However, the ROS signals that regulate PGC-1α transcription in skeletal muscle are not understood. Here we examined the effect of H₂O₂ on the regulation of PGC-1α expression, and its relationship to AMPK activation. We demonstrate that 24 h of exogenous H₂O₂ treatment increased PGC-1α promoter activity and mRNA expression. Both effects were blocked with the addition of N-acetylcysteine, a ROS scavenger. These effects were mediated, in part, via upstream stimulatory factor-1/Ebox DNA binding and involved 1) interactions with downstream sequences and 2) the activation of AMPK. Elevated ROS led to the activation of AMPK, likely via a decline in ATP levels. The activation of AMPK using 5-aminimidazole-4-carboxamide-1-β-d-ribofuranoside increased PGC-1α promoter activity and mRNA levels but reduced ROS production. Thus the net effect of AMPK activation on PGC-1α expression was a result of increased transcriptional activation, counterbalanced by reduced ROS production. The effects of H₂O₂ on PGC-1α expression differed depending on the level of ROS within the cell. Low levels of ROS result in reduced PGC-1α mRNA in the absence of an effect on PGC-1α promoter activation. In contrast, elevated levels of H₂O₂ induce PGC-1α transcription indirectly, via AMPK activation. These data identify unique interactions between ROS and AMPK activation on the expression of PGC-1α in muscle cells.

C₂C₁₂ cells; mitochondrial biogenesis; muscle gene expression; adenosine 5’-monophosphate kinase; upstream stimulatory factor-1; reactive oxygen species; peroxisome proliferator-activated receptor-γ coactivator-1 protein-α

Address for reprint requests and other correspondence: D. A. Hood, School of Kinesiology and Health Science, Rm. 302, Farquharson Life Sciences Bldg., York Univ., 4700 Keele St., Toronto, ON Canada M3J 1P3 (e-mail: dhood@yorku.ca).
demonstrate that the effect of ROS on PGC-1α mRNA expression via transcriptional activation of the PGC-1α promoter. We demonstrate that the effect of ROS on PGC-1α promoter activity occurs via an overlapping GATA/Ebox sequence that likely involves the actions of USF-1, an Ebox-binding transcription factor, and AMPK activation.

**METHODS**

**Chemicals and reagents.** Cell culture reagents, hydrogen peroxide (H2O2), and N-acetylcysteine (NAC) were purchased from Sigma (St. Louis, MO). Both compounds were resuspended in sterile DMEM before use. The dual luciferase assay system was from Promega (Madison, WI). Lipofectamine 2000, SuperscriptII first-strand cDNA synthesis kit, and 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (DCF) were obtained from Invitrogen (Burlington, Ontario, Canada). PCR primers were from Sigma Genosys (Toronto, Ontario, Canada). The AMPK antibodies were obtained from Cell Signaling Technology (Beverly, MD).

**Cell culture and treatment.** C2C12 muscle cells were cultured as previously described (21). Briefly, cells were maintained in DMEM containing 10% FBS and 1× antibiotic/antimycotic. When cells reached 90% confluence, they were switched to DMEM containing 5% heat-inactivated horse serum and 1× antibiotic/antimycotic and treated for 24 h with either vehicle or 300 μM H2O2 in the presence or absence of 20 mM NAC. For all experiments, cells were switched to differentiation medium and treated with indicated agents at the same time.

**Luciferase reporter assay and transient transfections.** Where indicated, C2C12 cells were cultured in six-well dishes and transiently transfected with 500 ng of the PGC-1α promoter plasmids. These PGC-1α promoter constructs have been reported previously (22) and include fragments of 2218, 1164, 851, 501, and 191 base pairs, linked to a luciferase reporter gene. Transfection efficiency of total RNA was normalized to Renilla luciferase activity (pRL-CMV; 5 ng/plate). Following treatments, cell extracts were prepared using 1× passive lysis buffer. Luciferase activity were measured using a Lumat LB9507 luminometer (EG&G Berthold) luminometer according to the manufacturer’s instructions.

**Western blot analysis.** Total protein was isolated from C2C12 cells as done previously (21). Briefly, total protein (20–40 μg) was electrophoresed through SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. The membranes were subsequently probed overnight with antibodies directed toward phospho-AMPK-α (1:400), or AMPK-α (1:1,000), washed 3× with PBS and Tween 20, incubated for 1 h at room temperature with the appropriate secondary antibodies conjugated to horseradish peroxidase, visualized with enhanced chemiluminescence, and quantified using SigmaScanPro (Jandel, San Rafael, CA).

**Reverse transcription and PCR.** Total RNA from C2C12 cells was isolated using TRIzol reagent, following the manufacturer’s recommendations. The purity and concentration of total RNA was determined spectrophotometrically. Equal amounts (0.5 μg) of total RNA were reverse transcribed using Superscript II with oligo-18(dT) as primer, following the manufacturer’s recommendations. The forward (F) and reverse (R) sequence-specific primers to amplify PGC-1α [5′-GAC CAC AAA CGA TGA CCC TCC-3′ (F) and 5′-GCC TCC AAA GTC TCT AGG-3′ (R; 635 bp)]; MnSOD [5′-GAC CTG CCT TAC GAT TAC GG-3′ (F) and 5′-GAC CTT GCT CCT TAT TGA AGC-3′ (R; 385 bp)]; catalase [5′-TCT GCA GAT GCC TGT GAA CTG-3′ (F) and 5′-TAG TCA GGG TGG ATG CAT GCC GT-3′ (R; 357 bp)]; and S12 rRNA [5′-GGA AGG CAT AGC TGC TGG-3′ (F) and 5′-CCT GGA TGA CAT CCT TGG-3′ (R; 368 bp)] have been described elsewhere (17, 24, 30, 36). PCR reactions were carried out in a 50-μl volume containing 2 μl cDNA, 10 μl 5× GoTagDNA buffer, 0.2 mM dNTPs, 3 mM MgCl2, and 1 unit of Tag DNA polymerase. Total RNA samples were also tested without the addition of reverse transcriptase to verify the absence of genomic DNA contamination. All primer sets were initially denatured at 94°C for 3 min and had an annealing time at 72°C for 5 min following amplification. The PCR conditions for PGC-1α were as follows: denaturation at 94°C for 30 s, annealing at 69°C for 30 s, and extension at 72°C for 45 s for 29 cycles. MnSOD and catalase were amplified as follows: 94°C for 45 s, 58°C for 45 s, 72°C for 80 s for 28 and 25 cycles, respectively. S12 rRNA was amplified using the following conditions: denaturation at 94°C for 30 s, annealing at 54°C for 30 s, and extension at 72°C for 60 s for 23 cycles. Optimal cycle number was determined to obtain a PCR product within the linear range. PCR products were resolved on 1.8% agarose gels, scanned, and quantified with SigmaGel software (Jandel).

**Chromatin immunoprecipitation.** Chromatin immunoprecipitation (ChIP) assays were conducted using the ChIP-IT Express Kit (Active Motif, Carlsbad, CA) according to the manufacturer’s instructions. Briefly, cells in three 150-mm plates per condition were cross-linked for 10 min at room temperature. The reaction was stopped with the addition of Glycine Stop-Fix solution and then washed with ice-cold 1× PBS. Cells were pooled, pelleted, and then incubated on ice for 30 min in 1× lysis buffer supplemented with 100 mM PMSF and protease cocktail inhibitor mix. Cells were then transferred to an ice-cold dounce homogenizer and homogenized on ice for 40 strokes to aid in the release of nuclei. Following sonication (35 pulses, 20 s/pulse at 25–30% power) and centrifugation, sheared chromatin (12 μg) was incubated with magnetic-coupled Protein G, anti-USF-1, or IgG (as negative control) overnight at 4°C. An aliquot of chromatin that was not incubated with an antibody was used as the input control. Antibody-bound protein/DNA complexes were washed, eluted, and treated with proteinase K to digest proteins. The chromatin was then used in PCR analyses. The primers used to amplify the mouse PGC-1α promoter were as follows: F: 5′-AGC TGA TCT GAG CAG AGC AG-3′ and R: 5′-CTC AGG CTC ATG TGA CT-3′ generating a 543-bp product. PCR analyses were also performed with positive control primers (EF-1α) obtained from Active Motif. PCR products were resolved on 1.8% agarose gels containing ethidium bromide. Gels were scanned and quantified with SigmaGel software.

**ATP measurements.** Following treatments, cells were washed three times in ice-cold 1× PBS, trypsinized, and collected into 1.5-ml microtubes. A 100-μl aliquot from each sample was saved to normalize ATP levels to total protein concentrations. The remaining cells were centrifuged for 1 min at 14,000 g. Each supernatant fraction was resuspended in 80 μl of ice-cold 1× PBS and 10 μl of cold 70% HClO4, vortexed, and incubated on ice for 10 min. Following a 5-min spin (14,000 g) at 4°C, 110 μl of cold 2 M KOH was added to the supernatant, vortexed, and left on ice for 3 min, then centrifuged at 14,000 g. Extracts were stored at −80°C until used. ATP levels were measured using a Lumat LB9507 luminometer.

**DCF detection of ROS in intact cells.** C2C12 cells were plated in black 96-well dishes (Costar 3603) such that they were ~85% confluent the following day. The cells were then switched to differentiation medium and treated with either vehicle or AICAR for 24 h. Immediately following, cells were washed once with PBS and then incubated for 45 min with 100 μM DCF at 37°C in serum-free medium. Thereafter, cells were washed once with PBS, and the medium was replaced with differentiation medium. DCF fluorescence was measured using the Synergy HT reader at 485/20 (excitation) and 528/20 (emission) over a period of 30 min. Data are presented as relative fluorescence units/well.

**Statistical analyses.** All data are expressed as means ± SE. Where indicated, Student’s unpaired t-test or two-way ANOVA as followed by
Bonferroni posthoc tests were used to determine individual difference between conditions. Results were considered to be statistically significant if $P < 0.05$ was achieved.

**RESULTS**

**Effect of H$_2$O$_2$ treatment on PGC-1α mRNA expression and promoter activity.** PGC-1α mRNA expression was increased 1.4-fold ($P < 0.05$) over vehicle-treated cells following 24 h of H$_2$O$_2$ treatment. This H$_2$O$_2$-mediated increase, as well as basal PGC-1α mRNA expression, was inhibited by preincubation of the cells with NAC, a potent ROS inhibitor ($P < 0.05$, Fig. 1A). Indeed, NAC alone, which inhibits basal ROS production, significantly reduced PGC-1α mRNA expression (Fig. 4A). To establish a mechanism for the ROS-induced increase in PGC-1α mRNA, we assessed the effect of H$_2$O$_2$ treatment on the transcriptional activity of PGC-1α promoter constructs. Twenty-four hours of H$_2$O$_2$ treatment produced a threefold increase ($P < 0.001$) in the p851 reporter plasmid but was completely without effect on any other length of the PGC-1α promoter tested, up to 2.2 kb (Fig. 1B). These data suggest that the effect of H$_2$O$_2$ on PGC-1α mRNA expression occurs, in part, via the transcriptional activation of the PGC-1α promoter and requires DNA binding sites located between −473 and −823 bp upstream of the start site. Furthermore, the data also suggest the presence of possible repressors within the PGC-1α promoter, specifically in the region upstream of −823. As shown in Fig. 1C, the effect of H$_2$O$_2$ on p851 PGC-1α transcription was inhibited by pretreatment with NAC. This inhibition was not due to an effect of a change in medium osmolarity, because 20 mM sucrose or mannitol had no effect on promoter activity (data not shown). Furthermore, treatment with NAC alone had no effect on the p851 promoter activity (Fig. 4A).

Our previous work that characterized the AICAR-mediated regulation of the PGC-1α promoter highlighted the importance of an overlapping GATA/Ebox sequence within the −473 and −823 region of the PGC-1α promoter (22). Recent observations (13) have suggested that H$_2$O$_2$ could mediate some of its effects via the activation of AMPK. Thus, we next examined the potential role of the overlapping GATA/Ebox in mediating the effect of H$_2$O$_2$ on the PGC-1α promoter. Mutation of the Ebox within the p851 PGC-1α reporter plasmid decreased the activity of this promoter length by 2.2-fold ($P < 0.05$), illustrating that this Ebox element contributes to basal PGC-1α transcription in untreated cells (Fig. 2A). H$_2$O$_2$ treatment induced a significant 3.1-fold increase ($P < 0.05$) in the transcriptional activation of the intact p851 PGC-1α promoter. In contrast, H$_2$O$_2$ tended to increase the promoter activity of p851ΔEbox construct, but this increase was less (≈2.5-fold) and was not statistically different from the activity of the p851ΔEbox construct in the absence of H$_2$O$_2$. Thus, these data indicate that the Ebox mediates a significant portion of the H$_2$O$_2$ effect on PGC-1α transcriptional activity, but that it may not represent the only response element responsible for the H$_2$O$_2$ effect. We next performed ChIP analyses to determine the identity of the Ebox binding protein that may be responsible for mediating the effect of H$_2$O$_2$ on PGC-1α promoter activity. As shown in Fig. 2B, the Ebox binding protein USF-1 is associated with the PGC-1α promoter in vehicle-treated cells. Following 24 h of H$_2$O$_2$ treatment, the amount of USF-1 associated with the PGC-1α promoter, corrected for nonspecific EF-1α binding, was increased by twofold, suggesting that USF-1 is involved in both basal and in H$_2$O$_2$-mediated PGC-1α transcription. This increase in binding likely represents recruitment of preexisting USF-1, since no change in USF-1 protein was detected following treatment with H$_2$O$_2$ (unpublished observations). We next evaluated whether the −823 to −473 region alone was sufficient to confer transcription responsiveness to H$_2$O$_2$. We did this by subcloning this fragment upstream of a TATA-driven minimal promoter. As shown in Fig. 2C, the activity of the minimal pGL4.23 promoter increased 3.5-fold ($P < 0.05$) on insertion of the −823 to −473 region. However, the administration of H$_2$O$_2$ did not
further enhance this activity. This suggests that the observed increase in the activity of the p851 length of the PGC-1α/H2O2 promoter brought about by H2O2 requires additional transcription factor binding sites downstream of position 473.

**Effect of H2O2 on AMPK activation.** We next sought to elucidate some of the signaling mechanisms that may be involved in mediating the effect of H2O2 on PGC-1α gene transcription. We hypothesized that AMPK may play a role since oxidative stress has been previously shown to activate AMPK (11, 13, 25). In agreement with those observations, treatment of C2C12 skeletal muscle cells with H2O2 increased AMPK phosphorylation by 3.4-fold (P < 0.05) above control levels, which was attenuated in the presence of NAC (Fig. 3A). This H2O2-mediated activation and attenuation by NAC were likely mediated, in part, by the decline in ATP levels, which fell 35% (P < 0.05) below control in H2O2-treated cells, and were rescued in the presence NAC (Fig. 3B). Therefore, these data suggest that the H2O2 effect on PGC-1α promoter activity and mRNA level may be mediated by the phosphorylation of AMPK. Alternatively, since it has also been shown that AMPK activation increases the transcriptional activity of the PGC-1α promoter (22), the possibility also exists that AMPK activation could stimulate the production of ROS and this, in turn, could activate the PGC-1α promoter. To test these hypotheses, we treated cells with 1 mM AICAR for 24 h in the presence or absence of NAC to determine whether scavenging ROS could block the AICAR-mediated effect on AMPK.

**Effect of ROS production on AMPK phosphorylation and gene expression.** As shown in Fig. 4A, preincubation of myoblasts with the ROS scavenger NAC reduced steady-state expression of p851 luciferase constructs and harvested for the measurement of luciferase activities. p851 and p851ΔEbox PGC-1α promoter activity (corrected for total protein) in vehicle- or H2O2-treated cells is shown (n = 3). *P < 0.05 vs. p851 vehicle-treated control. B: chromatin immunoprecipitation analysis (ChIP) was performed using an antibody against upstream stimulatory factor-1 (USF-1) or IgG (used as negative control). Primers flanking the overlapping Ebox/GATA sites were used to analyze USF-1 binding to the PGC-1α promoter. PCR analysis was also performed from the same samples using control primers from the EF-1α promoter. The representative image from this control reaction was obtained from different parts of the same gel. Quantification of the effect of H2O2 on USF-1/Ebox binding in vivo (right) indicates a 2-fold increase in binding (n = 10). *P < 0.05 vs. vehicle-treated cells. EF-1α binding was used to correct for differences in loading. AU, arbitrary scanner units. C: region between −473 and −823 was cloned into the pGL4.23 minimal promoter vector as previously described (22). Cells were transfected with indicated luciferase constructs and harvested for the measurement of luciferase activities. H2O2-induced transcriptional regulation of this region was assessed (n = 3). *P < 0.05 vs. pGL4.23 vehicle-treated control.

**Fig. 2. Effect of H2O2 on PGC-1α promoter activity.** A: cells were transfected with indicated PGC-1α luciferase constructs and harvested for the measurement of luciferase activities. p851 and p851ΔEbox PGC-1α promoter activity (corrected for total protein) in vehicle- or H2O2-treated cells is shown (n = 3). *P < 0.05 vs. p851 vehicle-treated control. B: chromatin immunoprecipitation analysis (ChIP) was performed using an antibody against upstream stimulatory factor-1 (USF-1) or IgG (used as negative control). Primers flanking the overlapping Ebox/GATA sites were used to analyze USF-1 binding to the PGC-1α promoter. PCR analysis was also performed from the same samples using control primers from the EF-1α promoter. The representative image from this control reaction was obtained from different parts of the same gel. Quantification of the effect of H2O2 on USF-1/Ebox binding in vivo (right) indicates a 2-fold increase in binding (n = 10). *P < 0.05 vs. vehicle-treated cells. EF-1α binding was used to correct for differences in loading. AU, arbitrary scanner units. C: region between −473 and −823 was cloned into the pGL4.23 minimal promoter vector as previously described (22). Cells were transfected with indicated luciferase constructs and harvested for the measurement of luciferase activities. H2O2-induced transcriptional regulation of this region was assessed (n = 3). *P < 0.05 vs. pGL4.23 vehicle-treated control.
PGC-1α mRNA levels. This occurred in the absence of AMPK phosphorylation (Figs. 3A and 4C) or a drop in ATP levels (Fig. 3B), supporting a direct effect of ROS on PGC-1α mRNA levels. As expected on the basis of our previous work (22), incubation of the cells with AICAR produced a 3.5-fold increase ($P < 0.05$) in AMPK phosphorylation (Fig. 4C), along with a similar increase in PGC-1α mRNA expression ($P < 0.05$; Fig. 4A). Unexpectedly, we observed that the induction of PGC-1α mRNA expression by AICAR was partially attenuated by NAC, without affecting AMPK phosphorylation (Fig. 4C). Indeed, the effect of AICAR remained 1.8-fold ($P < 0.05$) above vehicle-treated myoblasts, but it was reduced ($P < 0.05$) compared with the effect of AICAR in the absence of NAC. This suggests that the effect of AICAR on PGC-1α mRNA expression is mediated, in part, by an influence of AICAR on ROS balance within the cell. To assess this, we measured ROS production in intact myoblasts following 24 h of AICAR treatment. In contrast to our expectations, AICAR produced a small but significant 15% reduction ($P < 0.05$) in ROS production (Fig. 4B). This occurred without an effect on the mRNA expression of antioxidant enzymes catalase and MnSOD. While it remains to be seen whether this lack of change was also evident at the protein level or whether other antioxidant enzymes are changed by H$_2$O$_2$, the unaltered levels of these antioxidant mRNAs suggest that H$_2$O$_2$ did not affect antioxidant capacity. This implies that AICAR may have had a direct effect on the cellular production of ROS. Whether this is mediated by an influence on mitochondrial ROS production, or via other ROS-producing pathways, remains to be determined. It should also be noted that the effect of NAC on PGC-1α mRNA expression occurred in the absence of change in PGC-1α promoter activity (Fig. 4A, inset). This suggests the possibility that the antioxidant properties of NAC serve to destabilize PGC-1α mRNA, leading to enhanced PGC-1α mRNA decay.

**DISCUSSION**

ROS have commonly been associated with irreversible cellular damage, disease, and the degenerative processes associated with aging. However, their recent implication in the promotion of cellular growth, differentiation, proliferation, and apoptosis underscores the importance of ROS in the overall maintenance of cellular homeostasis. Both the positive and negative effects of ROS have been attributed to mitochondria, in view of the fact that these organelles are the major source of ROS and are also responsible for generating many of the cellular events that promote cell death and survival.

Several ROS-mediated signals elicit favorable adaptations that manifest within or outside the mitochondrial compartment. For example, intramitochondrial signaling affects mitochondrial morphology and function that may improve ATP delivery, whereas the extramitochondrial effects of ROS have been shown to lead to biogenesis of the organelle (10, 23, 26–28). This latter effect of ROS on biogenesis likely occurs via the induction of a variety of nuclear genes encoding mitochondrial proteins, as well as important transcriptional regulators including NRF-1, Tfam, and PGC-1α (29, 32, 33, 39).

It has recently been demonstrated that ROS can increase the expression of PGC-1α in 10T1/2 cells, and that the lack of the coactivator in knockout animals is correlated with elevated...
ROS-induced damage in the brain (39). These findings establish a potential link between ROS, PGC-1α, the induction of mitochondrial biogenesis, and antioxidant capacity. However, the ROS-mediated signals that regulate PGC-1α expression in skeletal muscle are not yet fully understood. To gain further insight into the mechanisms of the ROS-mediated increases in PGC-1α expression, we treated skeletal muscle cells with an exogenous ROS agent and assessed its effects on PGC-1α transcription and gene expression. In addition to this, we sought to elucidate some of the signaling mechanisms that may be responsible for mediating the effects of ROS.

In this study we demonstrate that 24 h of H2O2 treatment resulted in the induction of PGC-1α mRNA expression that was prevented by pretreatment of the myoblasts with NAC, a potent antioxidant. This supports the findings of existing studies using various models of oxidative stress (9, 37, 39). Interestingly, since NAC treatment alone decreased PGC-1α mRNA expression in non-H2O2-treated cells, these results also demonstrate that basal ROS levels are important in controlling PGC-1α mRNA expression. Thus, endogenously produced ROS, at least within skeletal muscle cells, may be important for the maintenance of PGC-1α expression levels within a normal physiological range.

The increase in PGC-1α mRNA expression with H2O2 treatment coincided with an increase in the activity of the p851 PGC-1α promoter construct. This finding supports the existing literature that suggests that the induction of PGC-1α mRNA expression is controlled, at least in part, via transcription (1, 2, 14, 15, 20). Since this H2O2-induced transcriptional activity was also prevented with the use of NAC, our results indicate that the effect of ROS on PGC-1α promoter activity required the binding of transcription factors residing between −473 and −823 bp upstream of the transcriptional start site. However, the activity of this promoter region in response to ROS relies heavily on the regions proximal to the transcription start site. This was revealed when we removed this region from the context of the downstream promoter regions and tested its activity for responsiveness to H2O2. Although this region was sufficient to transcriptionally activate a minimal promoter, it was not responsive to ROS. This confirms the need for transcription factors that bind downstream of −473 to mediate the full transcriptional effect brought about by H2O2. In support of this, it was recently reported that H2O2 can also mediate its effect on the PGC-1α promoter via the activation of CREB (39). Although we failed to observe an increase in the activity of the p191 PGC-1α promoter construct that houses the binding site for CREB, we have observed increases in CREB phosphorylation in response to H2O2 treatment that could be abolished with NAC pretreatment (I. Ircher and D. A. Hood, unpublished observations).

Within the −473/−823 region, our data suggest that the Ebox found at positions −494 and −489 plays an important regulatory role in PGC-1α transcription. This is based on the fact that the mutation of an Ebox within this region decreased basal PGC-1α promoter activity and inhibited the H2O2 effect. The identity of the Ebox binding protein as USF-1 was established using ChIP analyses, and thus the data suggest that USF-1 may be a target of ROS-induced signaling. Indeed, precedents for the involvement of USF-1 in oxidative stress-mediated changes in gene expression have been previously established (4, 31). In theory, an interaction between USF-1 and CREB, either directly or indirectly via the interaction with CREB-recruited coactivators, could promote the ROS effect on PGC-1α promoter activity leading to the ultimate upregulation of PGC-1α mRNA.

In search of potential signaling mechanisms to account for the H2O2-mediated increase in PGC-1α promoter activity, we focused on AMPK since it has been shown that AICAR-induced AMPK activation targets the same elements within the PGC-1α promoter (22), AMPK is activated by H2O2 (13), and activation of this enzyme constitutes an important signal for the induction of mitochondrial biogenesis in skeletal muscle (8, 21, 43). Here we demonstrate that H2O2 treatment leads to the phosphorylation and activation of AMPK. This is likely mediated via the ROS-induced decrease in ATP levels, since NAC prevented the decline in ATP levels in cells treated with H2O2 and coincidentally attenuated AMPK activation. This establishes the possibility that the observed effects of ROS are mediated indirectly, via AMPK activation.

A further examination of the relationship between ROS, AMPK activation, and PGC-1α gene expression led us to evaluate whether the previously observed AICAR-induced activation of AMPK, as well as increases in PGC-1α promoter activity and mRNA expression, was related to the production of ROS. On the basis of previous studies in pancreatic cells in
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which it was shown that AICAR promotes ROS production (11, 25), we hypothesized that AMPK activation by AICAR would increase the production of ROS. This, in turn, would activate PGC-1α transcription by the mechanisms described above, resulting in increased mRNA expression in skeletal muscle cells. In testing this hypothesis, we observed that the induction of PGC-1α mRNA expression by AICAR was not accounted for by increases in cellular ROS production because 1) myoblast ROS production was reduced by AICAR treatment; 2) the increase in PGC-1α mRNA expression by AICAR was not completely abolished by the pretreatment with NAC, a ROS scavenger; and 3) AICAR-induced AMPK activation was unchanged in cells that had been pretreated with NAC. Taken together, these data support the argument that at least two separate mechanisms are involved in increasing PGC-1α mRNA expression in response to AICAR, one involving AMPK activation, and the other counteracted by changes in ROS levels within the cell.

Our data suggest that the physiological expression of the PGC-1α gene requires an optimal concentration of ROS. We demonstrated this by reducing the cellular concentration of ROS with the addition of a NAC, which, in turn, significantly reduced PGC-1α mRNA expression. Since this effect was observed in the absence of a change in AMPK activation, we attribute the reduction in basal PGC-1α expression to an AMPK-independent mechanism. Furthermore, as the reduction in PGC-1α mRNA expression occurred in the absence of a change in promoter activity, it is conceivable that the reduction in ROS produced by NAC enhanced PGC-1α mRNA decay. Thus the basal level of ROS within skeletal muscle cells may be responsible for maintaining the expression of PGC-1α via alterations in mRNA stability and not via constitutive activity of the PGC-1α promoter. We speculate that when ROS become elevated, the stability of PGC-1α mRNA is restored, and activation of AMP kinase occurs. This stimulates the binding of USF-1 to an Ebox within the PGC-1α promoter, increases transcription, and results in the induction of PGC-1α mRNA expression (Fig. 5). Thus, an important effect of AICAR and AMPK activation on PGC-1α gene expression is via increases in promoter activity. This effect would be even greater if it was not counterbalanced by the modest effect of AICAR in decreasing ROS production. The effect of NAC serves to illustrate how decreases in ROS production attenuate the levels of PGC-1α mRNA, in the absence of an effect on promoter activity.

In summary, our data establish that H$_2$O$_2$ can mediate its effects on PGC-1α mRNA expression via the transcriptional activation of the PGC-1α promoter. The mechanism underlying this increase can be accounted for, in part, by an overlapping GATA/Ebox sequence that binds USF-1, an Ebox binding transcription factor. We also establish that AMPK activation is involved in the transcriptional responses elicited by elevated levels of H$_2$O$_2$, but that this does not account for the effect of H$_2$O$_2$ produced under basal conditions. Our results also implicate a novel ROS-mediated pathway that could underlie the induction of PGC-1α in skeletal muscle in response to mitochondrial biogenesis-inducing stimuli. A more thorough understanding of the transcriptional mechanisms that regulate PGC-1α expression in skeletal muscle in response to oxidative stress has potential therapeutic value, since ROS may underlie several pathologies that involve skeletal muscle and mitochondrial dysfunction. Careful titration of ROS levels within the cell may therefore lie at the crux between the manifestation of disease and cell death, the induction of mitochondrial biogenesis, and cellular metabolic health.

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