In lower organisms, increased expression of the NAD-dependent deacetylase Sir2 augments lifespan. The mechanism through which this life extension is mediated remains incompletely understood. Here we have examined the cellular effects of overexpression of SIRT1, the closest mammalian ortholog of Sir2. In PC12 cells, increased expression of the NAD-dependent deacetylase SIRT1 reduces cellular oxygen consumption by ~25%. We further demonstrate that SIRT1 overexpression can alter the transcriptional activity of the mitochondrial biogenesis coactivator PGC-1α. In addition, SIRT1 and PGC-1α directly interact and can be co-immunoprecipitated as a molecular complex. A single amino acid mutation in the putative ADP-riboyltransferase domain of SIRT1 inhibits the interaction of SIRT1 with PGC-1α but does not affect the interaction of SIRT1 with either p53 or Foxo3a. We further show that PGC-1α is acetylated in vivo. This acetylation is augmented by treatment with the SIRT1 inhibitor nicotinamide or by expression of the transcriptional coactivator p300. Finally, we demonstrate that SIRT1 catalyzes PGC-1α deacetylation both in vitro and in vivo. These results provide a direct link between the sirtuins, a family of proteins linked to lifespan determination and PGC-1α, a coactivator that regulates cellular metabolism.

The most widely held theory of aging suggests that mitochondrial oxygen consumption and resulting reactive oxygen species generation fuel the aging process (1). In lower organisms such as yeast and worms, increased expression of the enzyme Sir2, an NAD-dependent deacetylase, results in an increased life span (2, 3). Sir2 belongs to a growing family of enzymes collectively known as the sirtuins (4–7). The mechanism through which Sir2 extends lifespan is unclear, although its role in gene silencing has been commonly implicated (2, 8). The requirement for NAD in the deacetylase activity of Sir2 has led to the suggestion that enzymatic activity could be regulated by the concentration of NAD, the ratio of NAD/NADH, or by the intracellular concentration of nicotinamide (10–13). In particular, caloric restriction appears to augment lifespan in a wide range of species, and evidence suggests that the life extension induced by nutritional deprivation involves, and in some cases requires, Sir2 (11, 14, 15). Because the level of oxidized and reduced NAD may be altered under starved conditions, this has led to the hypothesis that Sir2 activity is augmented during caloric restriction by alterations in the NAD/NADH ratio (11). Nonetheless, considerable controversy exists as to whether starvation significantly alters the NAD/NADH ratio and whether or not this ratio does or does not physiologically regulate Sir2 activity (12, 16). In this report, we provide evidence for another direct link between sirtuins and metabolism. In particular we demonstrate a molecular interaction between SIRT1 and the transcriptional coactivator PGC-1α, a master regulator of metabolism and mitochondrial biogenesis (17).

EXPERIMENTAL PROCEDURES

Cell Lines and Plasmids—Clonal cell lines were established from PC12 cells obtained from ATCC (Rockville, MD). Cells were maintained in a basal growth medium consisting of Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 5% fetal calf serum. This media formulation contains high glucose (4.5 g/liter) but no exogenous pyruvate. HeLa cells (ATCC) were grown in the same medium. The wild type mouse SIRT1 cDNA was purchased from Upstate Biotechnology and subcloned into a HA1-tagged cDNA cloning vector (pCruzHA, Santa Cruz Biotechnology). A glycine to alanine site ADP-ribosyltransferase mutant at position 261 of SIRT1 was constructed by standard methods, and mutant and wild type constructs were confirmed by direct sequencing. This amino acid substitution has been previously demonstrated to abrogate essentially all of the ADP-riboyltransferase activity in yeast Sir2 protein (8). Clonal cell lines expressing wild type SIRT1 (WT), SIRT1G261A, or the empty HA-vector alone (Neo) were obtained by transfection and subsequent isolation by limiting dilutions in G418 selection. In stable clones that expressed wild type or mutant SIRT1, transgene expression was determined by Western blot analysis using an antibody recognizing the HA epitope (Santa Cruz Biotechnology). Total SIRT1 expression was determined by Western blot using an SIRT1-specific antibody (Upstate). Levels of SIRT1 mRNA were determined by reverse transcription-PCR analysis using the following SIRT1-specific primers: 5’-CCTGACTTCAGATCAAGAGACGGTA-3’.

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1 The abbreviations used are: HA, hemagglutinin; WT, wild type; GST, glutathione S-transferase.
and 5'-CTGATTAAAAATGTCTCCACGAAACAG-3'. Primers for β-actin were provided by the manufacturer (Clontech). Measurement of cytochrome oxidase subunit II expression was also determined by Western blot expression using a commercially available antibody (Santa Cruz Biotechnology). Human SIRT3 was cloned by standard PCR-based methods and verified by direct sequencing.

**Oxygen Consumption**—Oxygen consumption was measured using a fiber optic oxygen monitor (Instech). All measurements of oxygen consumption were performed in intact cells. Cells were plated in 15-cm dishes and allowed to grow for 7 days prior to trypanosization and subsequent oxygen determination. Oxygen consumption was performed in basal growth medium (see above), because we observed that measured oxygen consumption varied substantially depending on the extracellular supply of glucose, pyruvate, and serum. For analysis we used 20 individual control cell lines (Neo), 23 SIRT1-overexpressing cells, and 7 clones of SIRT1G261A. Each cell was measured by either duplicate or triplicate determinations.

**PGC-1α Transcriptional Activity and Protein-Protein Interaction**—For determination of PGC-1α transcriptional activity we transfected PC12 cells with a previously described construct encoding full-length PGC-1α fused to the GAL-4 DNA binding domain (18). This construct (0.4 μg) was co-transfected with increasing amount of wild type SIRT1 (0.1–0.4 μg) or a similar amount of the ADP-ribosyltransferase-inactive mutant. A GAL-4-dependent luciferase reporter (0.2 μg) and an internal Renilla control (0.05 μg) were also transfected into each dish. Twenty-four hours after transfection, cells were harvested and PGC-1α transcriptional activity was analyzed using the dual luciferase reporter assay (Promega). Results are expressed in arbitrary units from a single experiment performed in triplicate and are representative of at least three similar experiments.

To assess the interaction of PGC-1α and SIRT1, cells were transfected with wild type HA-SIRT1 and Myc-PGC-1α alone or in combination. These experiments were performed in HeLa cells, because these cells resulted in substantially higher transgene expression. Twenty-four hours after transfection, extracts were prepared in Lysis buffer (19), and 1 mg of total cell extract was immunoprecipitated with an anti-HA antibody (BabCO). The immunoprecipitates were resolved by SDS-PAGE, and the co-immunoprecipitated Myc-PGC-1α was visualized following Western blot analysis using an anti-Myc antibody (Santa Cruz Biotechnology). Reciprocal immunoprecipitations were also performed in an analogous fashion by first immunoprecipitating 1 mg of protein lysate with a Myc-epitope antibody followed by Western blotting with an anti-HA antibody.

To analyze the interaction of wild type SIRT1 and SIRT1G261A with FoxO3a and p53, we immunoprecipitated cell lysates as above except that cells were previously transfected with expression vectors encoding either wild type p53 or epitope-tagged Myc-FoxO3a. These constructs have been previously described (20). Co-immunoprecipitated p53 was visualized following Western blot analysis using an anti-p53 antibody (FL-393; Santa Cruz Biotechnology).

**PGC-1α Acetylation**—To assess the level of PGC-1α acetylation, HeLa cells were transfected with a Myc-PGC-1α expression vector and where indicated, an expression vector encoding p300. Acetylation status was determined by immunoprecipitation of 2 mg of protein lysate in lysis buffer (19) using an antibody directed against the Myc-epitope tag. Levels of acetylated PGC-1α were subsequently assessed with an anti-acetyl lysine antibody (Cell Signaling). Where indicated, 10 mM nicotinamide and 5 mM sodium butyrate were included in the culture medium (see above). The acetylation reaction was performed in 20 mM Tris, pH 8.0, for 1 h at 30 °C in the presence or absence of the indicated concentration of NAD or nicotinamide. After incubation with 1 mg of GST-SIRT1, the deacetylation reaction was performed in 20 mM Tris, pH 8.0, for 1 h at 30 °C in the presence or absence of the indicated concentration of NAD or nicotinamide. After incubation with GST-SIRT1 the level of acetylated PGC-1α was determined as above by Western blot analysis followed by detection using an anti-acetyl lysine antibody.

**RESULTS**

To assess whether SIRT1 influenced cellular metabolic rate, we sought to mimic the conditions previously demonstrated to extend life span in lower organisms. In both yeast and worms, simple overexpression of Sir2 results in an increase in life span (2, 3). We therefore established stable PC12 cell lines that constitutively expressed increased levels of SIRT1. On average SIRT1 mRNA expression and protein (data not shown) were increased ~3-fold in these cell lines (Fig. 1A). We also simultaneously established cell lines that expressed a mutant of SIRT1 harboring a glycine to alanine substitution at amino acid position 261 of the protein (SIRT1G261A). This amino acid is in a highly conserved domain required for the ADP-ribosyltransferase activity of yeast Sir2 but not its deacetylase activity (8). To avoid the confounding issue of clonal variation, we established 20 individual control clones expressing G418 resistance alone (Neo), n = 20 individual clones, wild type SIRT1 (WT, n = 23 individual clones), or an ADP-ribosyltransferase domain mutant of SIRT1 (G261A, n = 7 individual clones). Measurements of individual clones were made in duplicate or triplicate and mean ± S.E. of all clones are shown. C, oxygen consumption of control PC12 cells before or after the addition of the electron transport inhibitor antimycin A. The arrow indicates the addition of 80 nM antimycin A and demonstrates that essentially all measured oxygen consumption derives from mitochondrial metabolism. Similar results were obtained in SIRT1-overexpressing cells. D, expression of subunit II of cytochrome oxidase (COX II) in four random control cell lines (Neo) and four random SIRT1 wild type overexpressing cell lines (WT).

![Fig. 1. SIRT1 regulates oxygen consumption.](image-url)

*Fig. 1. SIRT1 regulates oxygen consumption. A, reverse transcription PCR analysis of SIRT1 mRNA expression in control cell lines (Neo) or four random cell lines expressing wild type SIRT1 (WT). B, levels of β-actin are included as a control. C, measurement of oxygen consumption in control PC12 cells expressing G418 resistance alone (Neo, n = 20 individual clones), wild type SIRT1 (WT, n = 23 individual clones), or an ADP-ribosyltransferase domain mutant of SIRT1 (G261A, n = 7 individual clones). Measurements of individual clones were made in duplicate or triplicate and mean ± S.E. of all clones are shown. C, oxygen consumption of control PC12 cells before or after the addition of the electron transport inhibitor antimycin A. The arrow indicates the addition of 80 nM antimycin A and demonstrates that essentially all measured oxygen consumption derives from mitochondrial metabolism. Similar results were obtained in SIRT1-overexpressing cells. D, expression of subunit II of cytochrome oxidase (COX II) in four random control cell lines (Neo) and four random SIRT1 wild type overexpressing cell lines (WT).*
PGC-1α we employed a previously described fusion construct of full-length PGC-1α fused to the DNA binding domain of GAL4 (18). This construct was transfected into PC12 cells along with a luciferase reporter construct under the control of tandem GAL4 DNA binding elements. As noted in Fig. 2, expression of wild type SIRT1 but not the SIRT1G261A mutant reduced the transcriptional activity of a GAL4-PGC-1α fusion protein. Based on these observations, we next sought to determine whether both PGC-1α and SIRT1 were capable of direct interaction. We transiently expressed epitope-tagged forms of wild type SIRT1 or SIRT1G261A along with PGC-1α in HeLa cells. As demonstrated in Fig. 3A, immunoprecipitation of wild type HA-tagged SIRT1 co-immunoprecipitated PGC-1α. In contrast to the association of wild type protein with PGC-1α, similar analysis of SIRT1G261A resulted in significantly reduced levels of co-immunoprecipitated PGC-1α (Fig. 3B). This disparity was also evident when the reciprocal immunoprecipitation was performed. As shown in Fig. 3C, immunoprecipitation of PGC-1α from lysates revealed an association with wild type SIRT1 but not with SIRT1G261A. Previous results have demonstrated that SIRT1 can interact with both p53 (21–23) and with members of the Forkhead transcription factor family, including Foxo3a (24–27). Interestingly, both of these protein partners of SIRT1 have been implicated in the aging process (28, 29). To date the region of SIRT1 required for these various interactions has not been defined. We therefore sought to test whether the reduced strength of interaction between PGC-1α and the SIRT1G261A mutant was also observed with other known protein partners. As shown in Fig. 4, the interaction of the SIRT1G261A mutant with both p53 (Fig. 4A) and with Foxo3a (Fig. 4B) was qualitatively very similar to wild type SIRT1. These results suggest the SIRT1/PGC-1α interaction differs structurally from the interaction with either the tumor suppressor p53 or with the transcription factor Foxo3a. Given that SIRT1 has been shown to deacetylate numerous transcriptional regulators, including p53 (21–23), forkhead proteins (24–27), and NF-κB (30), we next wondered whether PGC-1α might also be a deacetylation target of SIRT1. Using an antibody that recognized acetyl-lysine, we were able to detect low levels of acetylated PGC-1α in cells (Fig. 5A). Treatment with nicotinamide, a known inhibitor of sirtuins (13, 31), resulted in a substantial increase in PGC-1α acetylation. Previous studies have demonstrated that the transcriptional co-activator p300 can directly bind PGC-1α a (32). The interaction of PGC-1α and p300 has been documented to result in a conformational change in PGC-1α that increases transcriptional activity (32). Given that p300 also possesses histone acetyltransferase activity, we wondered whether co-expression with PGC-1α would alter acetylation. As noted in Fig. 5B, the expression of p300 dramatically increased PGC-1α acetylation. Indeed, the level of PGC-1α acetylation showed a mark correlation to the amount of transfected p300 (Fig. 5C). To assess whether PGC-1α could be deacetylated by SIRT1 in vitro, we immunoprecipitated PGC-1α from lysates of cells co-transfected with both a PGC-1α and p300 expression vector. To these immunoprecipitates we added purified recombinant SIRT1. As demonstrated in Fig. 6, recombinant SIRT1 in the presence of NAD but not nicotinamide deacetylated PGC-1α. Addition of NAD did not reduce PGC-1α acetylation in the absence of SIRT1 (data not shown). Finally, we asked whether SIRT1 could stimulate similar deacetylation in vivo. Cells were therefore transfected with
SIRT1 Regulates PGC-1α

**DISCUSSION**

Our results demonstrate that SIRT1 and PGC-1α physically interact and that SIRT1 can regulate PGC-1α acetylation both in vitro and in vivo. In the context of a GAL4 fusion protein, SIRT1 appears to inhibit the transcriptional activity of PGC-1α. Similarly, the effect of stable overexpression of SIRT1 is to reduce the level of oxygen consumption. Given that oxygen consumption is linked to the generation of reactive oxygen species and reactive oxygen species levels correlate with lifespan (1), these results may have important implications for how sirtuins regulate aging.

Our original impetus for these experiments was based on an observation derived from mitochondrial proteomics analysis that demonstrated a coordinate regulation of SIRT1 and mitochondrial gene expression (33). Given that PGC-1α plays a central role in mitochondrial gene expression, we hypothesized that PGC-1α and SIRT1 might physically or functionally interact. Our results support this conjecture. It should be noted however that recent evidence suggests that SIRT1 and p300 can also interact (24, 34). In addition, as previously mentioned, PGC-1α and p300 also bind to each other (32). This situation is analogous to the interactions described between SIRT1 and other protein targets. For instance, SIRT1 had been shown to separately bind to both p53 and Foxo3a (21–27). In addition, evidence suggests that p53 and Foxo3a can bind to each other (20, 25). Thus as described above, SIRT1 may be an important aspect for a number of distinct transcriptional complexes potentially acting as a scaffold to tether various members of the complex together. In addition, given that NAD regulates the activity of SIRT1, it is possible that SIRT1 functions as bridge coordinating metabolic status with transcription of key target genes.

We cannot at this point determine whether SIRT1 deacetylation of PGC-1α is the sole means by which SIRT1 overexpression regulates oxygen consumption in cells. Clearly, as described above, SIRT1 has numerous cellular targets, including other potential regulators of metabolism such as the Forkhead family of transcription factors. In addition, two other observations suggest that the relationship between SIRT1 and oxygen consumption is complex. First, although SIRT1G261A is not as efficient in deacetylating PGC-1α as wild type SIRT1, the mutant still appears relatively robust in this activity (see Fig. 7A). As such, one might have expected that if the level of PGC-1α acetylation was the only factor regulating oxygen consumption, than the increase in expression of SIRT1G261A would also lower respiration. As noted in Fig. 1 this does not appear to be the case. These results suggest that there is not a perfect concordance between PGC-1α acetylation status and measured oxygen consumption. It should be noted that some of the observed differences between Fig. 1B and 7A might relate to specific experimental variables such as the presence or absence of coexpressed p300 or the difference between stable and transient expression of SIRT1. Alternatively, these results perhaps suggest that a stable SIRT1-PGC-1α interaction may have important cellular effects that are independent of the deacetylation reaction. The second caveat concerning the effects of SIRT1 and oxygen consumption is that our preliminary results suggest that knockdown of SIRT1 by siRNA also leads to a...
reduction of oxygen consumption. These results suggest that the relationship between SIRT1 expression and oxygen consumption may be more bell-shaped than linear.

The role of PGC-1α in oxygen consumption is undoubtedly complex. Part of this complexity may result from the realization that cellular respiration integrates the number of mitochondria, the degree of mitochondrial coupling, and the level and type of metabolic supply. Interestingly, PGC-1α can regulate each of these parameters (17). Although forced overexpression of PGC-1α in cells and organs has been shown to increase mitochondrial content, there is a pronounced tissue-specific component to the subsequent changes in cellular respiration. For instance, in liver PGC-1α stimulates an increase in uncoupled respiration (35), whereas in heart the respirations are largely coupled (36). The results obtained from the recently described PGC-1α knock-out mouse are even less straightforward. For instance, total respiration of the PGC-1α−/− mouse is elevated compared with wild type littersmates, whereas isolated hepatocytes demonstrated a decrease in mitochondrial respiration (37). The authors speculated that this discordance might relate to a central hyperactivity observed in the PGC-1α−/− animals, although it also remains possible that tissue-specific effects account for these differences.

In summary, our results suggest that SIRT1 and PGC-1α form a stable complex and that SIRT1 regulates the activity and acetylation status of PGC-1α. Given that overexpression of Sir2 can extend life in both yeast and worms (2, 3), our observations that SIRT1 overexpression reduces oxygen consumption in PC12 cells suggests a potential new mechanism for the anti-aging effects of the sirtuins. Similarly, given the diverse role for PGC-1α in regulating metabolism, the physical interaction of this coactivator with an NAD-dependent enzyme suggests a way of coordinating metabolic decisions with metabolic status. Further elucidation of this interaction in different tissue types and in the context of a whole organism should therefore provide significant insight into cellular energetics, metabolism, and organismal aging.

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