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Cytokine Stimulation of Energy Expenditure through p38 MAP Kinase Activation of PPARγ Coactivator-1

Pere Puigserver,[1] James Rhee,[1] Jiandie Lin,[1] Zhidan Wu,[1] J. Cliff Yoon,[1] Chen-Yu Zhang,[2] Stefan Krauss,[2] Vamsi K. Mootha,[1] Bradford B. Lowell,[2] and Bruce M. Spiegelman[1,3]
[1] Dana-Farber Cancer Institute
Department of Cell Biology
[2]Division of Endocrinology
Department of Medicine
Beth Israel Deaconess Medical Center
Harvard Medical School
Boston, Massachusetts 02115

Summary

Cachexia is a chronic state of negative energy balance and muscle wasting that is a severe complication of cancer and chronic infection. While cytokines such as IL-1α, IL-1β, and TNFα can mediate cachectic states, how these molecules affect energy expenditure is unknown. We show here that many cytokines activate the transcriptional PPAR gamma coactivator-1 (PGC-1) through phosphorylation by p38 kinase, resulting in stabilization and activation of PGC-1 protein. Cytokine or lipopolysaccharide (LPS)-induced activation of PGC-1 in cultured muscle cells or muscle in vivo causes increased respiration and expression of genes linked to mitochondrial uncoupling and energy expenditure. These data illustrate a direct thermogenic action of cytokines and p38 MAP kinase through the transcriptional coactivator PGC-1.

Introduction

Cachexia is a chronic state of negative energy balance that is associated with a number of pathological states including chronic infection, cancer, and heart failure. Up to 50% of cancer patients are cachectic, and this severe complication can limit the ability of patients to tolerate therapy for their primary disease (Body, 1999; Nelson, 2000; Barber et al., 2000). Similarly, cachexia in patients chronically infected with agents such as HIV renders them more susceptible to other infections and less able to tolerate potentially beneficial therapies (Larkin, 1998; Evans et al., 1998). While cachectic patients are often also anorexic, cachexia is distinguished by two major features: loss of body weight that is not explainable by reduced food intake and preferential loss of muscle mass. Although the medical importance of cachexia is well established, the molecular mechanisms regulating this persistent state of inappropriately high metabolic rate is not.

A common feature of a variety of cachectic states is an elevation in circulating cytokine levels. As first identified by Cerami and colleagues, elevation in TNFα and other inflammatory cytokines is an important feature of cachexia induced by bacteria, HIV, a variety of cancers, and heart failure (Tracey et al., 1987; Tracey and Cerami, 1993). A causal role for these molecules such as TNFα, IL-1α, and IL-1β was shown by the fact that experimentally administered cytokines can induce a cachexia-like state. Conversely, neutralization of circulating cytokines can bring about an improvement in this persistent state of negative energy balance (Tracey et al., 1987). Despite strong evidence that cytokines are closely associated with cachexia, some of it now 20 yr old, there is little molecular mechanistic insight as to how they stimulate metabolic rates that are inappropriately high for the degree of food intake and physiological state.

Inflammatory cytokines are known to bind membrane surface receptors and trigger different signaling pathways that involve serine/threonine kinases such as IKB kinase (Mercurio and Manning, 1999; Karin, 1999) and stress activated kinases p38 MAPK and JNK (Davis, 2000; Tamura et al., 2000; Chang and Karin 2001). These protein kinases phosphorylate transcription factors either in the cytoplasm or in the nucleus and activate target gene transcription. Interestingly, activation of NFκB by cytokines has been shown to be involved in protein degradation in cultured muscle cells and has been proposed to be important for catabolism observed in cachexia, though no effects in energy expenditure have been reported (Guttridge et al., 2000). To date, no role for these stress-activated kinases has been demonstrated in activation of pathways linked to energy metabolism and mitochondrial respiration.

We have recently cloned a transcriptional coactivator, PPARγ coactivator (PGC)-1, that can function as a master regulator of oxidative metabolism. PGC-1 coactivates PPARγ as well as most other nuclear receptors; it also coactivates nuclear respiratory factor (NRF)-1, a key regulator of mitochondrial gene expression and mitochondrial biogenesis. PGC-1 is expressed in brown fat and skeletal muscle, key thermogenic tissues, as well as kidney, heart, liver, and brain. PGC-1 is induced when mice are exposed to cold, but only in skeletal muscle and brown fat. When PGC-1 is ectopically expressed in fat or muscle cells, an entire program of increased thermogenesis is activated, which includes mitochondrial biogenesis, increased respiration, mitochondrial uncoupling, and glucose uptake (Puigserver et al., 1998; Wu et al., 1999; Michael et al., 2001). Given the ability of PGC-1 to activate an entire program of adaptive thermogenesis in fat and muscle cells, we have asked whether PGC-1 might be the target of activation by one or more cytokines. We show here that many cytokines stimulate phosphorylation and activation of PGC-1 through p38 MAPK, resulting in increased respiration and energy expenditure in muscle cells. Transgenic expression of PGC-1 in mice caused increased respiration when they were injected with LPS. Taken together, our studies suggest a role for p38 MAPK and PGC-1 activation in hypermetabolic states where cytokine levels are elevated.

[3]Correspondence: bruce_spiegelman@dfci.harvard.edu

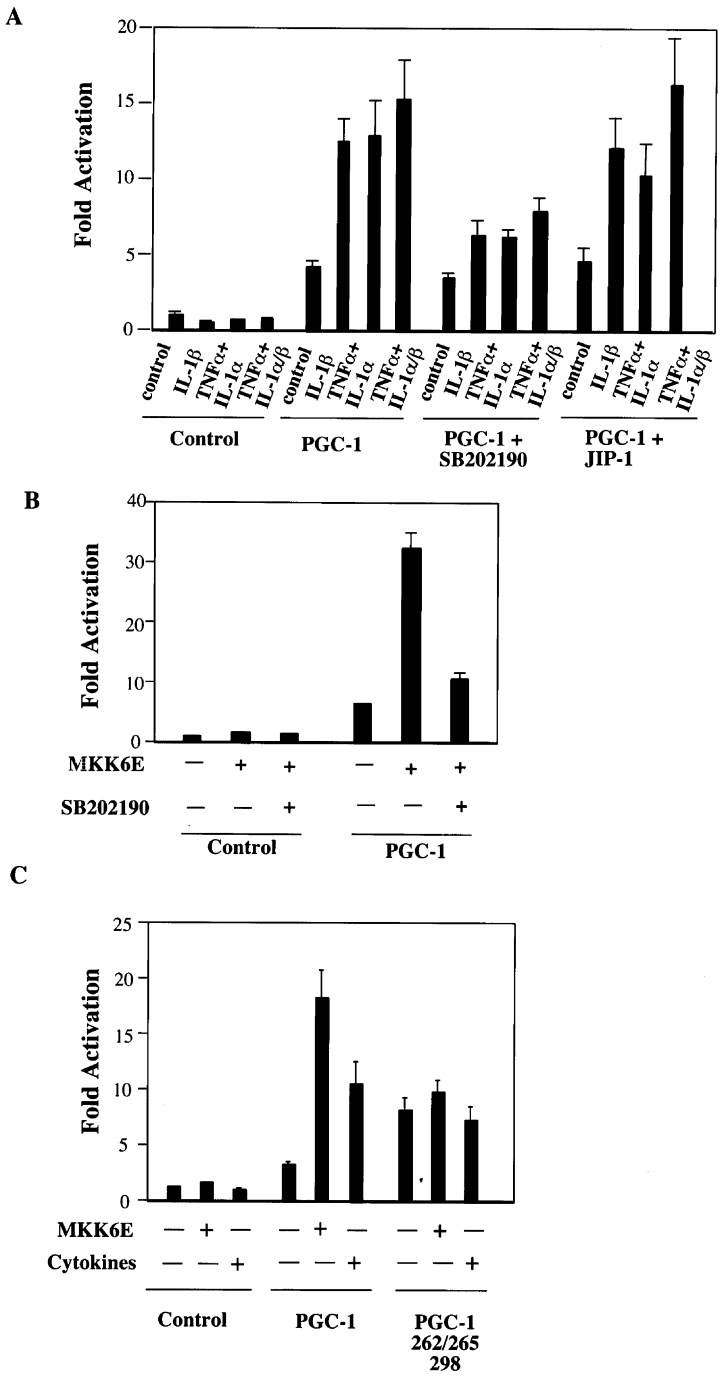

Figure 1. Cytokine-Induced Activation of PGC-1/NRF-1-Dependent Transcriptional Activity through p38 MAPK

(A) C2C12 cells were transfected with an NRF-1 response element/luciferase reporter gene (4X NRF-1 binding sites, four copies of nuclear respiratory factor-1 binding sequence) and the plasmids directing the expression of the pCMV-PGC-1 and pcDNA-JIP-1. Cultured medium was changed to DMEM with 0.5% bovine serum albumin for 24 hr before harvesting. IL-1β (1 ng/ml), TNFα (10 ng/ml), and IL-1α (2 ng/ml) were added to the cells 12 hr before analysis of luciferase activity. SB202190 (2 μM) was added to the cells 2 hr before the addition of cytokines. Luciferase activities were normalized to a co-transfected β-gal control.

(B) Activation of endogenous p38 MAPK induces PGC-1/NRF-1-dependent transcriptional activity. Cells were transfected as in (A), but using pcDNA3-MKK6E plasmid.

(C) Mutation of the p38 MAP kinase phosphorylation sites abolished the stimulation of the transcriptional activity of PGC-1/NRF-1 by cytokines and p38 MAPK. Error bars in (A), (B), and (C) indicate SEM of four to six independent experiments made in duplicate.

Results

Transcriptional Activity of PGC-1 Is Induced by Cytokines through the p38 MAPK Pathway

As previously shown, PGC-1 coactivates the transcriptional function of the endogenous mitochondrial regulator NRF-1 in C2C12 myoblasts (Wu et al., 1999) (Figure 1A). The addition of cytokines such as IL-1β and combinations of TNFα and IL-1α did not stimulate the transcriptional activity of endogenous NRF-1 without exogenous PGC-1; however, these cytokines stimulated the ability of PGC-1 to coactivate NRF-1 a further 4-fold (Figure 1A). TNFα alone had the same effect on transcription as in combination with other cytokines (data not shown). Inflammatory cytokines can activate two different major stress signaling pathways through nuclear translocation of p38 MAPK and JNK (Raingeaud et al., 1995, 1996; Chang and Karin, 2001). To determine if either of these kinases might be involved in the transcriptional activation of PGC-1, we used a specific inhibitor (SB 202190) and a constitutive activator (MKK6E) of the p38 MAPK, as well as a dominant-negative inhibitor of JNK (JIP-1). As shown in Figure 1A, inhibition of p38 MAPK greatly reduces the stimulatory effect of the cyto-

kines on PGC-1 to nearly control levels. Consistent with this, activation of p38 MAPK by its upstream activator MKK6E stimulates the coactivation effect of PGC-1 on the NRF-1 system, and this activation was suppressed by SB 202190 (Figure 1B). In contrast, inhibition of JNK by JIP-1 had no significant effect on the coactivation of NRF-1 by PGC-1 under the cytokine treatment (Figure 1A). These results clearly indicate that these cytokines stimulate the coactivation function of PGC-1 through NRF-1 and further suggest that it is mediated by the p38 MAPK pathway.

PGC-1 has been shown to have intrinsic transcriptional activity when fused to the yeast GAL4-DNA binding domain (DBD) (Puigserver et al., 1999; Knutti et al., 2000; Vega et al., 2000). To determine whether cytokines had a positive effect on PGC-1 itself, rather than on the PGC-1/NRF-1 unit, we performed similar studies using GAL4-DBD-PGC-1 in combination with a variety of cytokines. As shown in Figure 2A, many different cytokines including TNFα, IL-1α, and IL-1β activated GAL4-DBD-PGC-1 but not the GAL4-DBD alone. In data not shown, the GAL4-DBD fused to the PGC-1 activation domain (1–170) was also not activated by cytokines. In all cases, the activation shown on GAL4-DBD-PGC-1 was blocked by treatment of cells with the p38 inhibitor, SB 202190. Consistent with this, expression of MKK6E increased GAL4-DBD-PGC-1 transcriptional activity, and this was largely suppressed by the specific p38 MAPK inhibitor, SB 202190 (Figure 2B). That the p38 pathway, rather than the JNK pathway, was functioning here was also indicated by the fact that an upstream activator of JNK (MKK7) did not activate GAL4-DBD-PGC-1 (data not shown).

p38 MAPK Directly Phosphorylates PGC-1 on Residues Threonine 262, Serine 265, and Threonine 298

p38 MAPK has been shown to directly phosphorylate many different transcription factors such as MEF-2, ATF-2, Elk1, and CHOP (Raingeaud et al., 1996; Wang and Ron, 1996; Han et al., 1997), resulting in increases in transcriptional activity. Computer program analysis of the PGC-1 primary sequence using a peptide library-based searching algorithm (Yaffe et al., 2001), identified three clear consensus p38 MAPK phosphorylation sites, T262, S265, and T298 (Figure 3A). In vitro phosphorylation of recombinant-expressed PGC-1 by p38 MAPK followed by mass spectrometry indicated that these sites were indeed phosphorylated directly by p38 MAPK (data not shown). Furthermore, analysis of this in vitro phosphorylation with alanine substitution at these sites illustrates that the complete inhibition of phosphorylation required the mutation of all three residues (Figure 3B).

Phosphorylation of PGC-1 in BOSC cells transfected with the wild-type allele or different mutants of PGC-1 also show that p38 MAPK activation via MKK6E phosphorylates and causes an electrophoretic mobility shift in wild-type PGC-1 (Figure 3C). Complete suppression of this phosphorylation and shift could be observed only in the 262A/265A/298A triple mutant (PGC-1 3A) (Figure 3C). Of course, it is formally possible that mutation of one site could facilitate the phosphorylation of another site that is not phosphorylated in the wild-type protein. Phospho-specific antibodies will be required to directly address this possibility. Cytokines also induced the same phosphorylation and mobility shift. As expected, SB 202190 inhibition of p38 MAPK almost totally prevented PGC-1 phosphoryation by the cytokines (Figure 3D). These results indicate that p38 MAP kinase directly phosphorylates PGC-1 both in vitro and in cells at three amino acid residues.

Stabilization of PGC-1 Protein by Phosphorylation through p38 MAPK

Phosphorylation of PGC-1 could modify its inherent transcriptional activity, regulate its amount, or both. As shown in Figures 3C, 3D, and 4A, both MKK6E and the combination of TNFα and IL-1α increased the amount of PGC-1 protein. In addition, the triple mutant PGC-1 3A resistant to phosphorylation had an elevated level of expression in the control treatment, and this level was largely insensitive to further stimulation by MKK6E or cytokines. At first glance, it seems a bit paradoxical that the triple mutant had elevated PGC-1 levels relative to the wild-type protein; however, other examples, as with p53, have been observed where phosphorylation events stabilize the protein, and mutation of these serine/threonine sites to alanine leads to increased protein levels (Shieh et al., 1997; Sakaguchi et al., 2000). Presumably, these substitutions affect interactions with factors involved in protein turnover.

Given that we observed that phosphorylation of PGC-1 by p38 MAPK was associated with an increase in the levels of this protein, we studied whether the effects were due to protein synthesis and/or degradation of PGC-1 and the PGC-1 3A mutant in the presence of MKK6E. Pulse-chase experiments were performed with wild-type and mutant PGC-1 3A, along with MKK6E. The data in Figure 4B clearly show that phosphorylation by p38 MAPK increased PGC-1 half-life from 2.28 to 6.27 hr. Consistently, with Figures 2C and 4A, the triple mutant protein had a half-life 3-fold longer than did wild-type PGC-1, but this half-life was not altered by p38 MAPK. Taken together, these results indicate that p38 MAPK, downstream of multiple cytokines, directly phosphorylates PGC-1 at three residues and increases the half-life of PGC-1 protein by inhibiting its degradation. The molecular mechanisms or events underlying this stabilization by either phosphorylation or triple mutation are under investigation.

Mutation of PGC-1 Phosphorylation Sites Abolishes Its Increased Transcriptional Activity by p38 MAPK

To directly determine the effects of phosphorylation on transcriptional activity of PGC-1, we performed transient transfection analysis using the NRF-1 system with wild-type and mutant PGC-1 proteins. As shown in Figure 1C, and consistent with Figures 3 and 4, the triple mutation of the phosphorylation sites rendered PGC-1 more active than the wild-type protein. However, the activation of PGC-1 by cytokines or MKK6E was completely abrogated by mutation of the three p38 MAPK phosphorylation sites of PGC-1. Importantly, these data also show that the triple PGC-1 mutant, though stable, is not as transcriptionally active as the phosphorylated

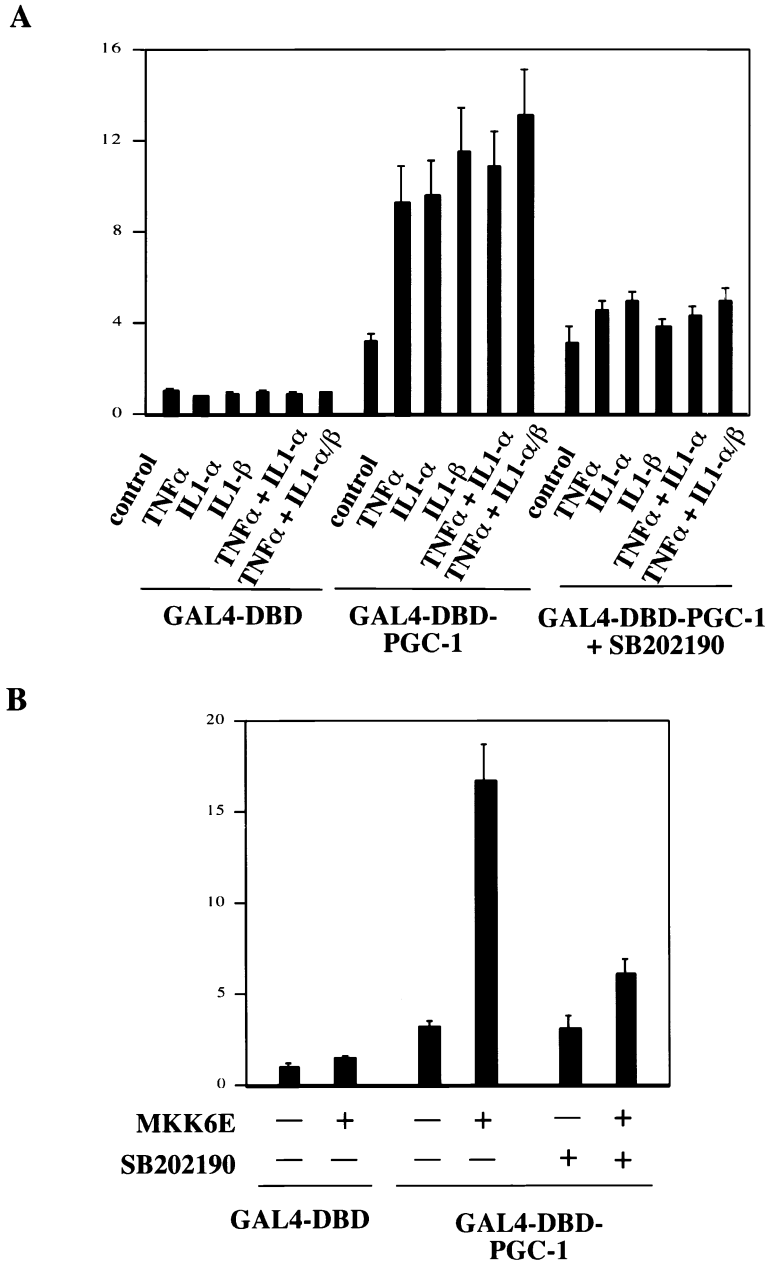

Figure 2. Intrinsic Transcriptional Activity of PGC-1 Is Activated by Cytokines

(A) C2C12 cells were transfected with a 5XUAS/luciferase reporter gene (5XUAS, five copies of upstream activation sequence) and plasmids directing the expression of GAL4-DBD or GAL4-DBD-PGC-1. Cells were harvested for analysis of luciferase activity 48 hr later. Cytokine treatment was performed as in Figure 1A.

(B) Activation of endogenous p38 MAPK induces GAL4-DBD-PGC-1 transcriptional activity. Cells were transfected as in (A), but using pcDNA3-MKK6E. Error bars in (A) and (B) indicate SEM of four to six independent experiments made in duplicate.

protein. These data demonstrate that these phosphorylation events are directly responsible for the cytokine and p38 MAPK activation of PGC-1 transcriptional function.

Phosphorylation of PGC-1 Increases Cellular Respiration

To investigate whether these cytokines and p38 MAPK phosphorylation events regulate cellular metabolic rates, we measured respiration in different cell lines expressing PGC-1 alleles and MKK6E. Cellular respiration is widely used as an accurate measure of energy expenditure (Rolfe and Brand, 1996; Stuart et al., 2001). Cultured C2C12 myotubes were used in these experiments because, although PGC-1 is highly expressed in muscle tissue in vivo (Michael et al., 2001), PGC-1 is almost absent in cultured muscle cells, presenting a very useful assay system. As shown in Figure 5A, cells expressing wild-type PGC-1 had 90% more total oxygen consumption than control cells. The triple PGC-1 mutant increased respiration 118% above control cells. MKK6E caused a further stimulation of oxygen consumption in wild-type PGC-1 expressing cells (to 140% above control), while MKK6E had no effect in the cells expressing the triple mutant form of PGC-1. Total oxygen consumption was also measured in C2C12 cell lines treated with cytokines. Again, only PGC-1-expressing cells showed a response to the cytokines (90% compared to control cells), and this increase was abolished by the p38 MAPK inhibitor (Figure 5A). These data indicate that phosphorylation of PGC-1 by p38 MAP kinase increases oxygen consumption and respiration in myotubes. These are

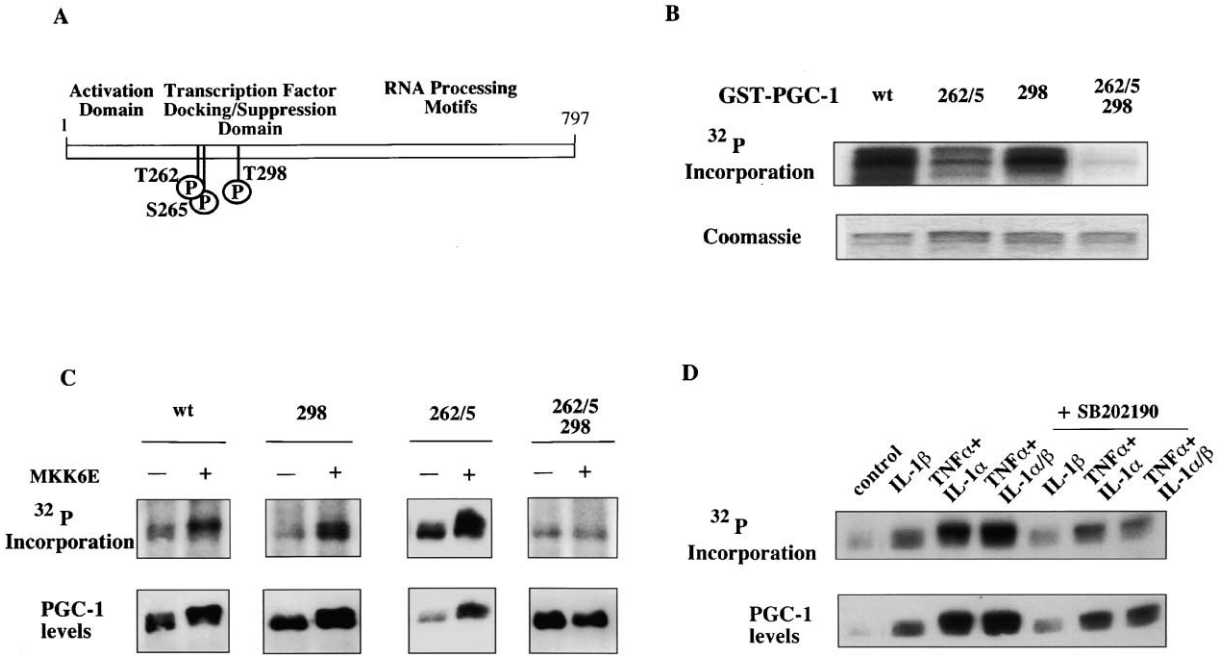

Figure 3. Phosphorylation of PGC-1 by p38 MAPK

(A) Schematic diagram of PGC-1. The region containing the p38 MAPK putative phosphorylation sites is indicated.

(B) In vitro phosphorylation of PGC-1 by p38 MAPK. Purified recombinant GST-PGC-1 fragment (amino acids 200–400) or the different mutants indicated were tested as substrates for recombinant activated p38 MAPK.

(C) In vivo phosphorylation of PGC-1 by activation of p38 MAPK. BOSC cells were transfected with pcDNA-Flag-PGC-1 (1–400) wild-type and the mutants indicated as well as pcDNA3 MKK6E. Cells were labeled for 4 hr with [^{32}P] phosphate, and PGC-1 protein was immunoprecipitated and analyzed by SDS-PAGE, protein blotting, and autoradiography. Protein levels were measured by probing with a polyclonal antibody against the N terminus of PGC-1.

(D) PGC-1 is phosphorylated by p38 MAPK in response to cytokines. BOSC cells were transfected with pcDNA-Flag PGC-1 (1–400) and were treated with the indicated cytokines or SB202190 (added 2 hr before the cytokines) for 4 hr during the [^{32}P] phosphate labeling. [^{32}P] phosphate incorporation and expression levels of PGC-1 protein were analyzed as in (C).

large changes in these parameters; mice exposed to cold for 3 weeks or injection of norepinephrine show increases in respiration between 60% and 200% (Doi and Kuroshima, 1982).

Oligomycin, an inhibitor of F1/F0 ATP synthase and respiration linked to ATP synthesis, allows measurements of uncoupled respiration. Uncoupled oxygen consumption was increased by MKK6E 123% above basal levels only in cells expressing PGC-1, but not in either the control or the cells containing the triple PGC-1 mutant (Figure 5B). Furthermore, only cells expressing wild-type PGC-1 increased uncoupled oxygen consumption

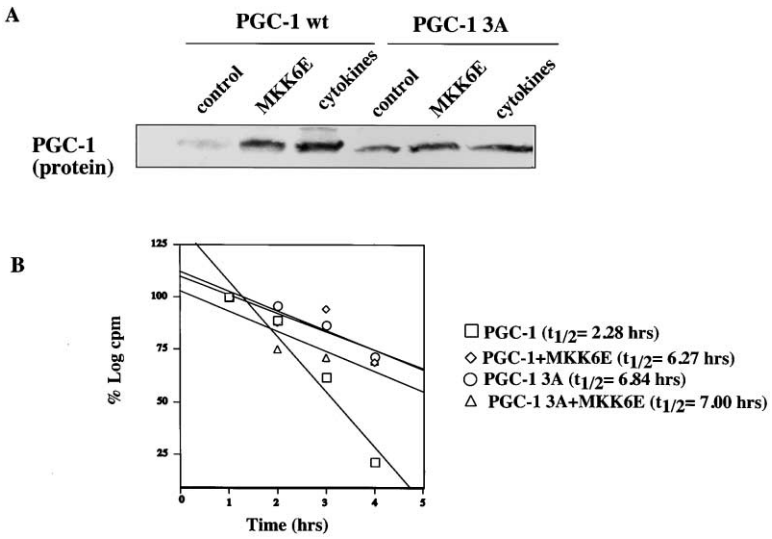

Figure 4. PGC-1 Protein Half-Life Is Increased by Activation of p38 MAPK

(A) Increased levels of PGC-1 protein by MKK6E and cytokines. BOSC cells were transfected with plasmids encoding PGC-1 wild-type and mutant as well as MKK6E. Whole-cell extracts were analyzed by Western blot analysis.

(B) Increased PGC-1 half-life by activation of p38 MAPK. Pulse-chase experiments were performed 48 hr after transfection and radiolabeled with [^{35}S]-methionine as described in Experimental Procedures. Experiments were performed three times in duplicate.

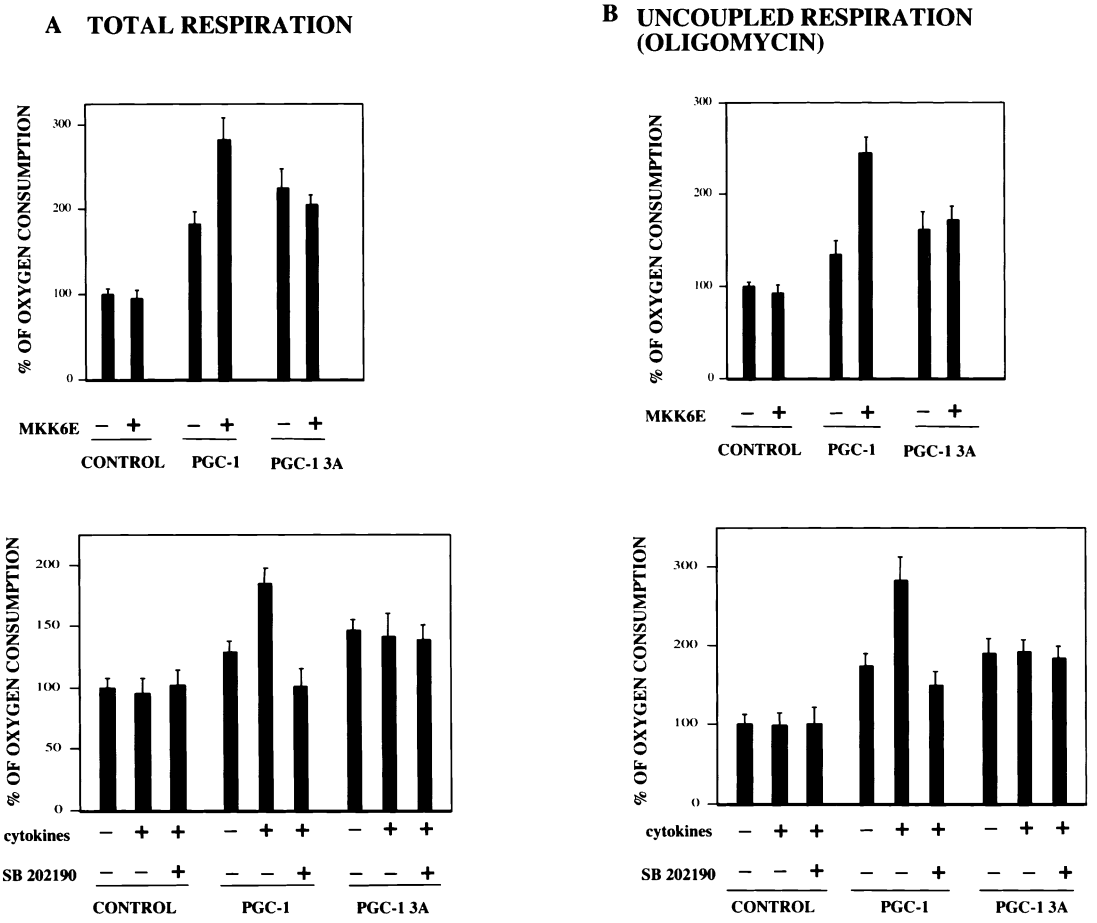

Figure 5. Cytokines and p38 MAPK Increase PGC-1-Dependent Activation of Oxygen Consumption In Muscle Cells

(A) MKK6E and cytokines increase total oxygen consumption in PGC-1-expressing cells. C2C12 muscle cells were infected with different retroviruses encoding pMSCV-vector, pMSCV-PGC-1, PGC-1 3A, and pMSCV-MKK6E. After appropriate neomycin and/or puromycin selection, cells were grown until confluence and differentiated with 2% horse serum for 4 days. Twenty-four hours before harvesting, cells were changed to serum-free medium containing 0.5% bovine serum albumin. SB 202190 (2 μM) was added 2 hr before the treatment with cytokines (at the same concentration as indicated in Experimental Procedures). Cytokines were added 20 hr prior to harvest. Oxygen consumption was measured as described in Experimental Procedures.

(B) MKK6E and cytokines increase oligomycin-dependent oxygen consumption in PGC-1 expressing cells through p38 MAP kinase. C2C12 muscle cells were generated and differentiated as in (A). The uncoupled respiration was determined in the presence of oligomycin (2.5 μg/ml). Results in (A) and (B) are normalized by protein content. Error bars in (A) and (B) indicate SEM of three to five independent experiments made in duplicate.

in response to cytokines; this elevation was inhibited completely by the p38 MAPK inhibitor, SB 202190. These results show directly that PGC-1 mediates the effect of cytokines in cellular respiration and also indicate that they work via p38 MAPK. Furthermore, a significant portion of the respiration induced by this pathway is mitochondrial-uncoupled respiration.

Phosphorylation of PGC-1 by p38 MAPK Increases Expression of Mitochondrial Genes Linked to Respiration

We investigated whether these increases in respiration were associated with changes in gene expression related to mitochondrial function. We have previously shown that PGC-1 increases expression of mitochondrial genes in muscle cells (Wu et al., 1999; Michael et al., 2001). As shown in Figure 6A, the expression of the PGC-1 alleles and MKK6E was identical in the different cell lines studied. Under the culture conditions used (serum-free) PGC-1 alone slightly induced (1.42-fold) the mRNA expression of cytochrome-c (Cyt-C), cytochrome c oxidase subunit II (COX II, 1.31-fold), and UCP-2 (1.52-fold). Importantly, mRNAs for several components of the respiratory chain such as cytochrome-c (2.9-fold), COX-II (2.1-fold), cytochrome c oxidase subunit IV (COX-IV, 1.51-fold), and β-ATP synthase subunit (2.85-fold) were elevated by MKK6E, but only in cells infected with PGC-1. MKK6E did not have any significant effect in control cells, once corrected for lane loading. Interestingly, MKK6E did not alter the expression of a known PGC-1 target gene like UCP-2 (Wu et al., 1999) but increased the expression of an alternative member of the uncoupling gene family, UCP-3 (increased 2.25-fold). Cells expressing the triple PGC-1 mutant had increased expression for some target genes of PGC-1 cytochrome-c (3.2-fold), COX-II (1.6-fold), COX-IV, (1.21-fold), and β-ATP synthase subunit (2.7-fold). However,

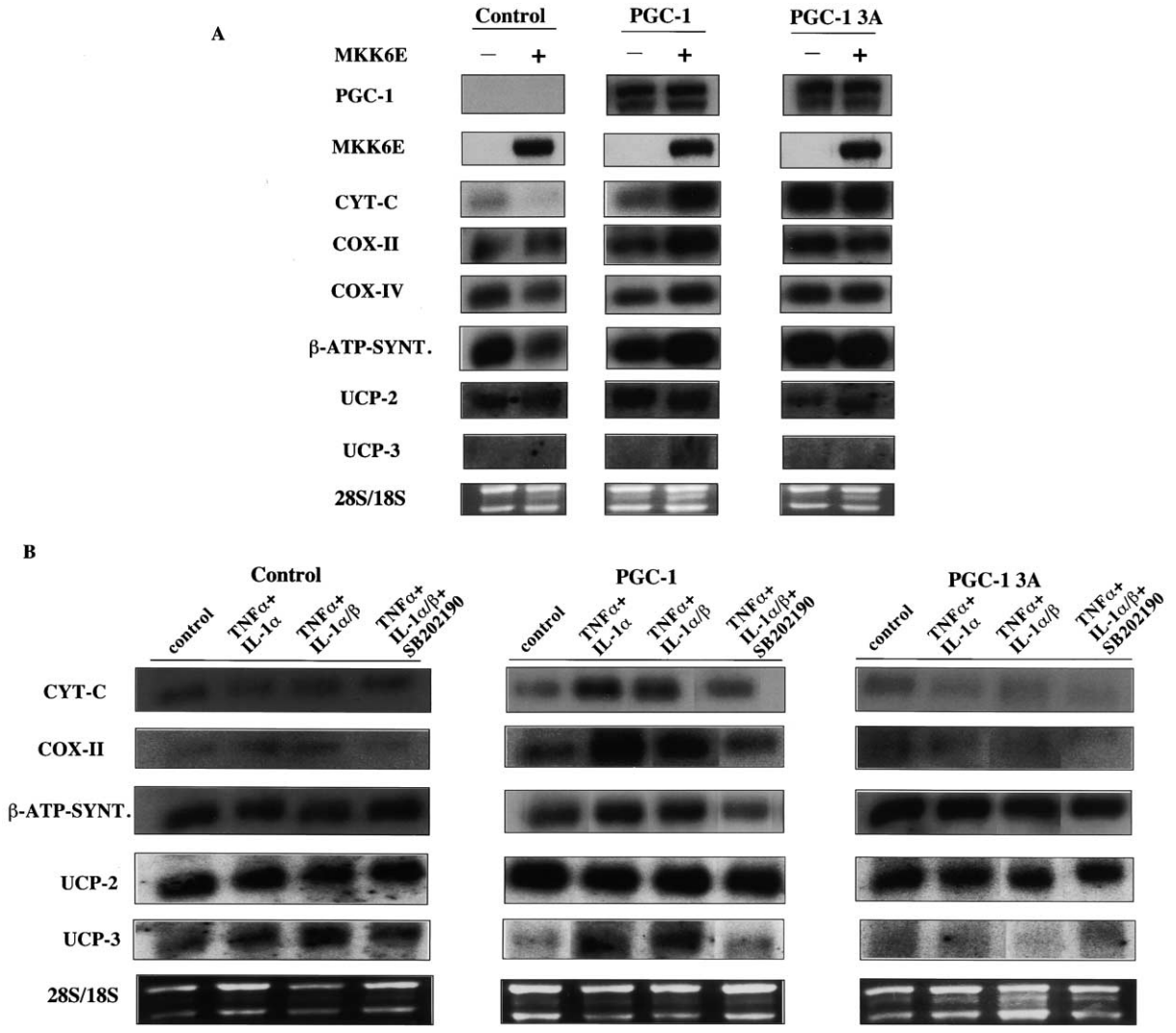

Figure 6. p38 MAPK and Cytokines Activate PGC-1-Targeted Mitochondrial Gene Expression in Muscle Cells

(A) Expression of MKK6E activates PGC-1-targeted gene expression. C2C12 cell lines were generated and treated as described in Figure 5 and Experimental Procedures. Total RNA was isolated and analyzed by Northern blot. Probes used for hybridization were PGC-1, MKK6E, cytochrome-c, COX II, COX IV, ATP-synthase (β-subunit), UCP-2, and UCP-3. Ribosomal RNA 28S and 18S were used as a loading control. (B) Cytokines increase mitochondrial gene expression through p38 MAPK in PGC-1-expressing cells. C2C12 muscle cells were differentiated and treated as in (A). Cytokine treatment was as in Figure 5. Total RNA was isolated and probed with the probes indicated.

the triple PGC-1 mutant did not affect the uncoupling proteins UCP-2 and UCP-3. In all cases, the triple mutant was resistant to the stimulatory effects of the MKK6E (Figure 6A).

In order to investigate the effects of cytokines on gene expression, similar experiments were performed treating PGC-1 expressing cell lines with cytokines. As shown in Figure 6B, combination of cytokines such as TNFα + IL-1α induced the same repertoire of mitochondrial genes as MKK6E (cytochrome-c [1.95-fold], COX-II [3.2-fold], β-ATP synthase subunit [1.5-fold], and UCP-3 [2.2-fold]). The increased expression of these genes was abolished by the treatment with the SB 202190 inhibitor, indicating that these stimulatory effects were mediated by the p38 MAPK pathway. These data indicate that activation of the p38 MAP kinase pathway increases gene expression associated with mitochondrial respiration. In particular, activation of this signaling pathway is able to specifically induce UCP-3 in a PGC-1-dependent manner. In addition, the fact that the triple mutant of PGC-1 has similar stability as the phosphorylated wild-type protein but does not activate some of the known PGC-1 targets (e.g., uncoupling proteins) indicates that these phosphorylations must regulate functions in addition to its stability.

Lipopolysaccharide Induction of Respiration and Mitochondrial Genes in Transgenic Mice Expressing PGC-1

To investigate the role of PGC-1 in mediating cachexia-like effects in vivo, we utilized transgenic mice that express PGC-1 from the muscle creatine kinase (MCK) promoter. Several strains of mice were created; in these experiments we utilized a strain that has only a modest elevation in PGC-1 expression (2.3-fold) (Figure 7A). Importantly, this strain also has no alteration in respiration

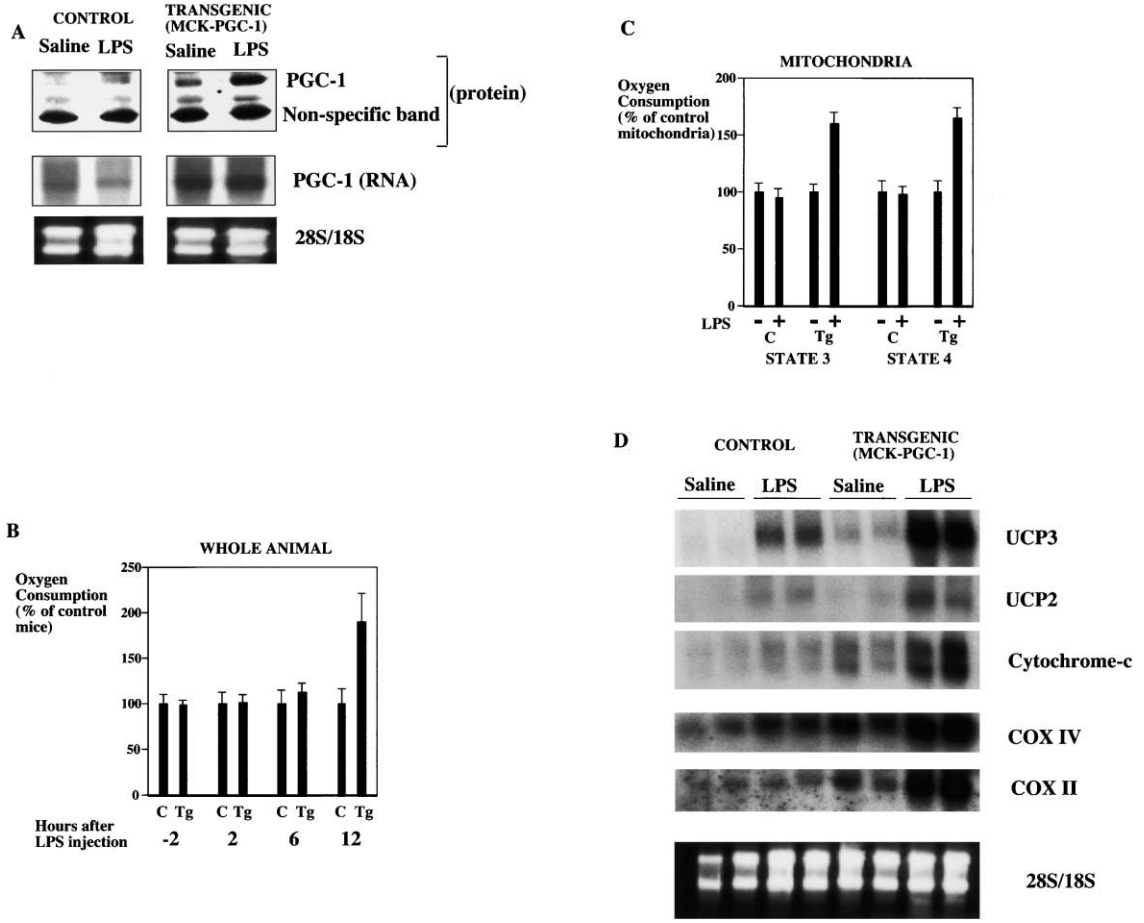

Figure 7. Transgenic Mice Expressing PGC-1 in Skeletal Muscle Have Elevated Sensitivity to LPS-Induced Respiration

(A) Stimulation of PGC-1 protein levels by LPS. Mice transgenic for PGC-1 were generated as described in Experimental Procedures. Control and PGC-1-transgenic mice were fasted and treated with either saline or LPS (4 mg/kg). Skeletal muscle was homogeneized and nuclear extracts were prepared as described (Mora and Pessin, 200). Nuclear protein extracts were analyzed by SDS-PAGE, immunoblotting, and probing with a polyclonal PGC-1 antibody. RNA was isolated an analyzed by Northern blot using a PGC-1 probe.

(B) Transgenic mice expressing PGC-1 have elevated oxygen consumption after injection of LPS. Animals were age-matched and randomly divided into the experimental groups described in (A). Oxygen consumption was recorded (Experimental Procedures) and corrected by body weight. C, control; Tg, transgenic.

(C) Mitochondrial respiration is increased in transgenic mice expressing PGC-1. Mice used in (B) were sacrificed and skeletal muscle was dissected. Mitochondria was isolated as described in Experimental Procedures. Oxygen consumption was measured in a Clark-type oxygen electrode, and state 3 and state 4 respirations were recorded. Results were normalized by amount of mitochondrial protein.

(D) Mitochondrial gene expression is augmented in transgenic mice expressing PGC-1. Skeletal muscle from mice used in (C) was isolated and analyzed by Northern blot using probes described in Figure 6.

in the basal state (−2 hr, in Figure 7B). To challenge these mice, we utilized a short-term model of cachexia, injection of lipopolysaccharide (LPS), an active component of bacterial endotoxin. This agent induces multiple cytokines and also brings about fever, hypermetabolism, and anorectic responses characteristic of bacterial infection (Parillo, 1993). When these mice were injected with 4 mg/kg of LPS, a relatively low dose, control animals showed little respiratory response. The transgenic mice, however, showed a doubling of respiration at the 12 hr time point. Importantly, LPS injection caused no change in PGC-1 mRNA levels in control or the transgenic mice but did stimulate an increase in PGC-1 protein levels (Figure 7A); the ability of LPS to alter PGC-1 protein levels without altering mRNA amount is consistent with the data presented above from cultured muscle cells (Figures 3 and 4).

To investigate whether this increased respiration was related to mitochondrial function, we isolated mitochondria from control and transgenic mice at the 12 hr time point and analyzed respiration in vitro. Respiration was measured under conditions of ADP excess (state 3, coupled respiration) and ADP depletion (state 4, uncoupled respiration). As shown in Figure 7C, mitochondria from the transgenic mice showed similar respiration in the basal state. However, LPS injection led to increased respiration, per unit of mitochondrial protein, in both the coupled and uncoupled states.

mRNA analysis indicated that LPS injection caused a modest increase in mRNAs for UCP-3, UCP-2, cytochrome-c, and COX-IV in control mice. However, these changes were consistently greater in the transgenic mice. Notably, UCP-3 was induced 6-fold in the transgenic mice compared to 2.5-fold in the controls (Figure 7D).

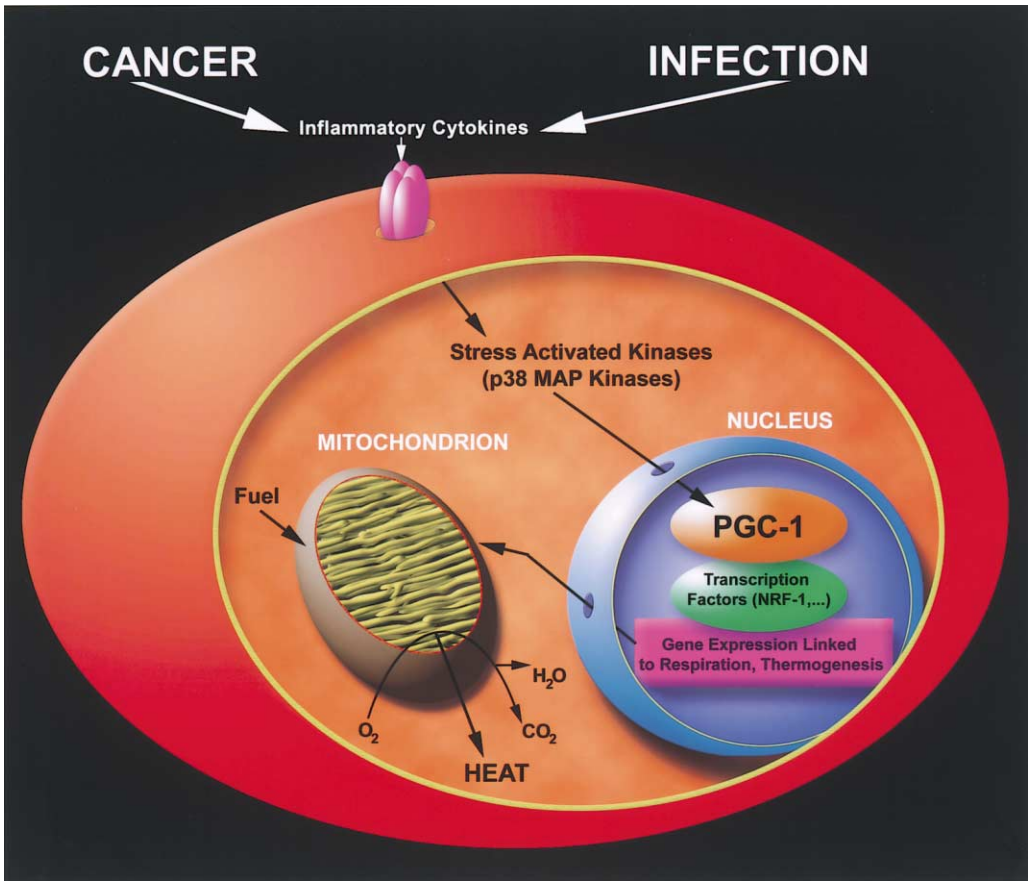

Figure 8. Schematic Representation of the Regulation of PGC-1 by Cachectic Stimuli through p38 MAPK Pathway and Its Effects on Mitochondrial Gene Expression and Respiration

These data clearly demonstrate that a mild elevation of PGC-1 levels in muscle greatly sensitizes mice to the respiratory and hypermetabolic effects of LPS and indicate that these effects correlate with greatly activated mitochondrial function.

Discussion

Many cytokines have been previously shown to affect the differentiation status of adipocytes and muscle cells, and these activities may play a significant role in the cachectic state (Brun et al., 1997; Naya and Olson, 1999). However, the essence of cachexia is a chronic state of negative energy balance, with respiration and energy expenditure set at a level that is inappropriately high for the nutritional status of the animal or patient. Fat, and especially muscle, catabolism follows from negative energy balance. To date, there have been essentially no pathways elucidated whereby cytokines can increase respiration and energy expenditure. The data presented here is summarized in Figure 8 and illustrate that multiple cytokines, working via p38 MAPK, stimulate the phosphorylation and activation of the thermogenic transcriptional coactivator PGC-1. Once phosphorylated, PGC-1 becomes both more stable to degradation and more active transcriptionally. This leads to gene activation events associated with mitochondrial respiration and uncoupling. Thus, PGC-1 provides a link between cytokines and increased energy expenditure and respiration.

It is important to note that PGC-1 is expressed in normal skeletal muscle tissue; the deficiency of this protein observed in the common cultured muscle cell lines provided us with a quasigenetic system in which to investigate the role of PGC-1 in mediating the effects of cytokines in cellular respiration. The deficiency of PGC-1 from the cultured muscle cells also likely explains why previously studies failed to observe an effect of cytokines in the regulation of thermogenesis (Lee et al., 1987; Zentella et al., 1993). Catabolism of muscle is also part of the cachectic state in vivo. Whether this is also stimulated by PGC-1, possibly working through other transcription factors described to be targeted by cytokines such as NFκB (Guttridge et al., 2000), or whether it comes about as a secondary consequence of increased energy expenditure remains to be determined.

The p38 MAPK-mediated phosphorylation of PGC-1 increases the half-life of this protein and probably accounts for much of the increased levels of this coactivator under cytokine treatment. Presumably, this triple phosphorylation alters the interaction of PGC-1 with the protein degradation machinery. It is also likely that the triple alanine replacement, necessary to block phosphorylation, similarly alters the interaction of PGC-1 with the destruction machinery. However, while control of

protein stability is likely to be an important function for the p38-mediated activation, it is quite clear that there must be additional positive effects of these modifications. The triple mutant allele of PGC-1 has equal stability and accumulates to similar high levels as the phosphorylated wild-type protein, but it is not as active in stimulating transcription, respiration, and inducing the expression of certain genes such as the uncoupling proteins 2 and 3.

It is interesting that these phosphorylations occur in a region previously shown to play an important regulatory role in PGC-1. Earlier work has illustrated that this region (amino acids from 200 to 403) mediates a repressive effect in transcription and also serves to dock several transcription factors, including many nuclear receptors and NRF-1. The docking of these transcription factors causes a conformational change that accelerates the binding of other transcriptional effector proteins into this complex, including CBP/p300 and SRC-1 (Puigserver, et al., 1999). Whether the p38 MAPK-mediated phosphorylations affect transcription factor docking or the recruitment of other coactivator proteins to a PGC-1 complex remains to be determined.

One interesting aspect of the p38-modified PGC-1 is that it has some qualitatively different activities compared to the wild-type protein. In particular, phosphorylated PGC-1 activates UCP-3, a gene not targeted by this coactivator when not modified by p38 MAPK. Conversely, UCP-2 is induced by unmodified PGC-1, but is not induced (indeed, is slightly decreased) by the p38-activated protein. Since UCP-3 has been shown to have positive effects in systemic energy expenditure in vivo, at least when overexpressed in transgenic mice (Clapham et al., 2000), it is tempting to suggest that this protein may play a role in the uncoupled respiration observed here.

The effects of PGC-1 activation on respiration and gene expression linked to mitochondrial function observed in cultured muscle cells could also be observed in transgenic mice expressing PGC-1 in muscle after injection of LPS. Ectopic expression of PGC-1 in transgenic mice clearly sensitized to the LPS increasing oxygen consumption in the whole animals and mitochondria from skeletal muscle was also increased to a greater extent in transgenic than in control mice. These results also provide in vivo evidence for the involvement of PGC-1 in LPS-induced hypermetabolic state, a model for bacterially induced cachexia.

PGC-1 represents a novel molecular target that directly link cytokines to the stimulation of energy expenditure and respiration. An inappropriately high rate of energy dissipation is characteristic of the cachectic state, and it could be therapeutically useful to inhibit this uncontrolled respiration. Taken together, the studies presented here suggest a possible anticachectic benefit for the inhibition of p38 or PGC-1 activity in muscle tissues.

Experimental Procedures

Cell Culture and Treatments

C2C12 cells were maintained and differentiated as described in Wu et al. (1999). BOSC cells were maintained in DMEM containing 10% cosmic calf serum (CCS, Hyclone, Logan, UT). Treatment with cytokines were performed after changing culture medium to 0.5% BSA in DMEM. Cytokines used in the different experiments were mouse or human TNFα (10 ng/ml, R&D, Minneapolis, MN), human IL-1α, and IL-1β (2 ng/ml, Roche, Indianapolis, IN). SB 202190 (2μM, Calbiotech, San Diego, CA) was added to the cells 2 hr before the addition of cytokines.

Transcriptional Activation Assays

C2C12 were transiently transfected using FuGENE (Roche) at a confluence between 80% and 90%. After overnight transfection, cell culture was changed to 0.5% BSA in DMEM for 24 hr. Cytokines at the concentration indicated above were added 12 hr before harvesting with luciferase lysis buffer (Pharmingen, San Diego, CA). Aliquots were used to measure β-galactosidase (as a transfection control) and luciferase activities. 4X NRF-1 and 5XUAS luciferase reporter plasmids, and pCMV-PGC-1 and GAL4-DBD-PGC-1 plasmids have been described previously described in Puigserver et al. (1999). pcDNA-MKK6E and pcDNA-JIP1 were a gift from Dr. R. Davis. Mutant pCMV-PGC-1 3A and Flag-PGC-1 3A were generated by in vitro mutagenesis (Stratagene, La Jolla, CA) and sequenced.

In Vitro and In Vivo Phosphorylation Analysis

GST-PGC-1 fragment (200–400) wild-type and mutants (generated by in vitro mutagenesis, Stratagene) were expressed in bacteria (BL21 strain, Novagen, Madison, WI) and purified using glutathione agarose beads. Recombinant proteins were used as a substrate for in vitro phosphorylation reaction with activated p38 MAPK using manufacturer's intructions (Upstate Biotechonology, Lake Placid, NY). After phosphorylation reaction, glutathione beads were extensively washed and analyzed by SDS-PAGE and autoradiography. Protein levels were monitored by Coomassie blue staining. For in vivo phosphorylation, BOSC cells were transfected at confluence using Fugene, with pcDNA-Flag PGC-1 and mutants, as well as pcDNA-MKK6E. Cells were treated as above and labeled for 4 hr with [^{32}P] phosphate and cytokines and/or SB202190 inhibitor. Cells were harvested for immunoprecipitation with agarose beads linked to flag antibody (Sigma, Saint Louis, MO). Immunoprecipates were solved by SDS-PAGE and transferred to PVDF and autoradiography. PGC-1 protein levels were analyzed by Western blot.

Retroviral Constructs and Infection Conditions

C2C12 cells were infected with retrovirus containing pMSCV-neomycin (PGC-1) and/or pMSCV-puromycin (MKK6E) made by using the viral packaging system (Clontech, Palo Alto, CA). Following puromycin and neomycin selection, infected cell lines were grown to confluence in DMEM with 10% CCS. To induce differentiation cells were cultured in DMEM containing 2% heat-inactivated horse calf serum (Hyclone).

Oxygen Consumption Measurements

Retrovirally infected C2C12 were differentiated and treated with cytokines. Four days after differentiation, cells were washed with room temperature PBS and briefly tripsinized from the plates. After centrifugation, cells were resuspended with PBS and transferred to a 1 ml Clark-type oxygen electrode chamber. After recording the basal respiration rate, the uncoupled respiration was measured in the presence of the ATP synthase inhibitor, oligomycin (2.5 μg/ml). Rates of oxygen consumption were normalized by protein content determined using the BCA kit (Pierce, Rockford, IL).

Mitochondrial oxygen consumption was measured in mitochondria isolated from skeletal muscle from wild-type and PGC-1 transgenic mice. Mitochondria were isolated essentially as described (Vidal-Puig et al., 2000), using a medium containing 250 mM sucrose, 5 mM Tris, and 2 mM EGTA (pH 7.4) at 4°C. Mitochondrial respiration was measured in a Clark-type oxygen electrode at 37°C. To this end, mitochondria (0.1–0.5 mg of protein) were incubated in assay buffer containing 120 mM KCl, 5 mM KH2PO4, 5mM malate, 10 mM glutamate, 3% (w/v) defatted BSA, 1 mM EGTA, and 3 mM HEPES (pH 7.2). State 3 was induced by adding 300 μM ADP.

Oxygen consumption was measured in wild-type and PGC-1 transgenic mice with either saline or LPS (4 mg/Kg). Oxygen consumption was recorded using the OXYMAX System 4.93 (Columbus Instruments, Columbus, OH), with a settling time of 100 s, a measur-

ing time of 50 s, and with reference as room air. The animals were placed in four chambers at room temperature.

Immunoprecipitation and Western Blot Analysis

Immunoprecipitation was performed for in vivo phosphorylation and metabolic labeling with [^{35}S]-methionine. Cells with different flag-tagged PGC-1 plasmids (Puigserver, et al., 1999) were transfected in BOSC cells with FuGENE. Cells were treated as described above and total whole-cell extract was performed as in Wu et al. (1999). Approximately 800 μg of protein was incubated with agarose beads linked to an antibody against flag (Sigma). After overnight incubation, immunoprecipitates were washed three times, resolved by SDS-PAGE, and transferred to PVDF membrane (Millipore, Bedford, MA). Western blots were perfomed using a PGC-1 polyclonal antibody made against the N terminus of the protein (Puigserver et al., 1999) and developed using the ECL method (Amersham, Piscataway, NJ).

Nuclear extracts from skeletal muscle tissue were prepared as described (Mora and Pessin, 2000), and 300 μg of nuclear protein was used to run SDS-PAGE; immunoblotting was performed as above.

Pulse-Chase Experiments

BOSC cells were transfected with plasmids encoding Flag PGC-1 and PGC-1 3A. Pulse-chase experiments were performed 48 hr after transfection. Before the pulse, cells were incubated with DMEM without methionine/cysteine for 1 hr. Cells were radiolabeled with [^{35}S] methionine in DMEM and 0.5% BSA for 1 hr and harvested at the time points indicated after chasing with nonradioactive culture medium. Cell lysates using RIPA buffer were immunoprecipated as described above. After autoradiography, quantitation of the bands was performed using the ImageQuant program (Molecular Dynamics, Piscataway, NJ).

Northern Blot Analysis

Total RNA was isolated from cultured C2C12 cells by Trizol (GIBCO) extraction, and 20 μg of RNA was analyzed by electrophoresis, transferred, and hybridized with specific cDNAs probes as in Wu et al. (1999). After autoradiography, quantitation of the bands was performed using the ImageQuant program (Molecular Dynamics), and fold inductions were corrected by the loading control (28S/18S).

Animal Experiments

To generate transgenic mice expressing PGC-1 in the muscle, a cDNA encoding PGC-1 full length (Lehman et al., 2001) was cloned into an expression vector containing the MCK promoter. The expression pattern and characteristics of this vector have been described (Bruning et al., 1998). The MCK-PGC-1 plasmid was purified and injected into fertilized oocytes derived from C57BL6 mice, and oocytes were transferred into the oviducts of pseudopregnant mice.

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