Control of Secreted Protein Gene Expression and the Mammalian Secretome by the Metabolic Regulator PGC-1α

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Secreted proteins serve pivotal roles in the development of multicellular organisms, acting as structural matrix, extracellular enzymes, and signal molecules. However, how the secretome is regulated remains incompletely understood. Here we demonstrate, unexpectedly, that peroxisome proliferator-activated receptor γ coactivator 1-α (PGC-1α), a critical transcriptional co-activator of metabolic gene expression, functions to down-regulate the expression of diverse genes encoding secreted molecules and extracellular matrix components to modulate the secretome. Using cell lines, primary cells, and mice, we show that both endogenous and exogenous PGC-1α down-regulate the expression of numerous genes encoding secreted molecules. Mechanistically, results obtained using mRNA stability measurements as well as intronic RNA expression analysis are consistent with a transcriptional effect of PGC-1α on the expression of genes encoding secreted proteins. Interestingly, PGC-1α requires the central heat shock response regulator heat shock factor protein 1 (HSF1) to affect some of its targets, and both factors co-reside on several target genes encoding secreted molecules in cells. Finally, using a mass spectrometric analysis of secreted proteins, we demonstrate that PGC-1α modulates the secretome of mouse embryonic fibroblasts. Our results define a link between a key pathway controlling metabolic regulation and the regulation of the mammalian secretome.

Cells continuously respond to changing metabolic cues by regulating an elaborate network of transcription factors and co-activators that orchestrate the expression of a diverse array of target genes. PGC-1α is a critical metabolic transcriptional co-activator that binds to numerous transcription factors and boosts their ability to induce the expression of their target genes (1, 2). PGC-1α activity has been widely described as essential to promote oxidative metabolism and mitochondrial biogenesis (3). PGC-1α levels are elevated by an array of physiological stimuli in the tissues where it acts, including exercise in muscle, cold in brown adipose tissue, and fasting or diabetes in the liver (1, 2). In turn, PGC-1α possesses the ability to control tissue-specific programs such as thermogenesis in brown adipose tissue (4), fiber-type switching in skeletal muscle (5), and hepatic gluconeogenesis (6). The mechanism by which PGC-1α induces gene expression involves an N-terminal transcriptional activation domain; this domain interacts with several lysine acetyltransferase complexes including SRC-1, p300, and CREB-binding protein (CBP) (7). Although PGC-1α is well known to co-activate nuclear hormone receptors, it was also described as a co-activator of several non-nuclear hormone receptor transcription factors that include NRF1 and NRF2 (3). Mice with a deletion of the gene encoding PGC-1α display numerous phenotypes in several tissues (8). Although PGC-1α has been implicated in the regulation of numerous transcriptional programs, the full scope of PGC-1α binding partners and regulated targets is not fully elucidated.

More recently, experiments carried out mainly in mouse muscle suggested that PGC-1α may down-regulate the expression of certain inflammatory molecules. However, the mechanisms responsible for these effects remain poorly understood. Studies of mice with muscle-specific PGC-1α knock-out revealed transcriptional induction of a few markers, such as TNFα and IL-6, related to local or systemic inflammation (9, 10). Higher levels of TNFα and IL-6 mRNAs were detected in primary myotubes harboring a deletion of PGC-1α when compared with wild type myotubes. Furthermore, overexpression of PGC-1α in C2C12 myotubes was found to inhibit the expression of TNFα and IL-6 mRNAs (9). Conversely, other studies indicated that PGC-1α increases, rather than reduces, TNFα and IL-6 expression in skeletal muscle (11). In addition, PGC-1α muscle-specific knock-out mice have lower plasma TNFα levels and skeletal muscle TNFα mRNA levels following LPS treatment (11).

These effects of PGC-1α on inflammatory gene expression have been initially speculated to involve regulation of reactive oxygen species levels by PGC-1α (12). Recent studies suggest that overexpression of PGC-1α can repress the transcriptional activity of NFκB in myotubes (13), contributing, at least partly, to the anti-inflammatory activity of PGC-1α. Our recent work uncovered a direct inhibitory effect of PGC-1α on the key regulator of the mammalian heat shock response, HSF1, resulting in down-regulation of various HSF1-dependent transcriptional programs (14).

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Here we show that PGC-1α can inhibit the expression of secreted protein and extracellular matrix (ECM) genes, including genes encoding extracellular proteases, molecules related to ECM organization, and cytokine-related molecules. By analyzing microarray data and expression data in several cell types, we demonstrate the ability of PGC-1α to down-regulate a variety of genes encoding secreted molecules, and we validate these observations at the mRNA level. We explore the possible mechanism of these effects, with data consistent with a transcriptional effect, and identify HSF1 as necessary for the effects of PGC-1α on some of its targets encoding secreted molecules. Importantly, using a mass spectrometric analysis of secreted molecules, we also establish an effect of PGC-1α on secreted and extracellular matrix molecules at the protein level. Thus, our data reveal a novel aspect of the activity of the critical metabolic regulator PGC-1α, and link it to regulation of the mammalian secretome.

Results

With the purpose of examining the effects of PGC-1α on gene expression in fibroblasts, we ectopically expressed PGC-1α in primary mouse embryonic fibroblasts (MEFs) using an adenoviral vector, followed by microarray analysis. As expected, the expression of PGC-1α elicited an induction of genes involved in energy metabolism, notably mitochondrial genes (supplemental Fig. S1). However, in addition to PGC-1α-activated genes, we noticed that many genes that were down-regulated by PGC-1α expression, with a cutoff of 2-fold change, 627 transcripts were down-regulated by PGC-1α expression, with a cutoff of 2-fold change, 627 transcripts were down-regulated by PGC-1α expression in MEF cells. Strikingly, a highly significant over-representation of genes encoding secreted and ECM proteins was found among the genes down-regulated by PGC-1α expression, with terms related to secretion and the ECM being the top terms identified as enriched (Fig. 1a). Notably, an over-representation of secreted and ECM genes was not identified among genes up-regulated by PGC-1α expression in this system (results not shown). Examination of the down-regulated genes revealed various genes encoding secreted molecules related to ECM organization, cytokine receptors, and proteinases, among others (Fig. 1b). The microarray data were validated by real-time PCR analysis showing PGC-1α-dependent down-regulation of many targets encoding secreted molecules such as extracellular matrix proteins (e.g. Col6a2 encoding collagen VI, Dpt encod-
ing dermatopontin, and Lama2 encoding laminin), cytokines (e.g. Ccl7 and Cxcl14), and proteinases (e.g. Mmp19 encoding matrix metalloproteinase 19) (Fig. 1c). As these results were obtained using ectopic expression of PGC-1α in MEFs, we next examined the effects of endogenous PGC-1α in these cells. For that purpose, we utilized WT and KO PGC-1α MEFs and compared the expression of several genes encoding secreted molecules previously identified in our analysis of MEFs with ectopic expression of PGC-1α. Importantly, real-time PCR analyses verified that endogenous PGC-1α in MEFs can affect many targets similarly to exogenous PGC-1α (Fig. 1d), whereas exogenous PGC-1α was expressed to a significantly higher level than endogenous PGC-1α (supplemental Fig. S2). The targets identified as regulated by endogenous PGC-1α in MEFs include ECM proteins (such as Postn encoding periostin), growth factors (such as Ptn encoding pleiotrophin), as well as the above discussed targets identified as regulated by exogenous PGC-1α (such as Mmp19, Lama2, Dpt, Cxcl14, Col6a2, and Ccl7). These results suggest that PGC-1α can regulate the expression of genes encoding secreted proteins in MEFs, which raised the question of whether PGC-1α can do so in other types of fibroblasts, as well as in human cells. To address this question, we ectopically expressed PGC-1α in the human hepatic stellate cell line LX2, and then examined the subsequent gene expression changes. Interestingly, upon examining the expression of relevant genes, we noted PGC-1α-dependent down-regulation of several targets (such as COL6A2, SEMA3C, and MMP19 among others) earlier identified in MEFs, as well as additional targets encoding secreted proteins identified in this cell type (such as MMP2, CTGF, and BMP4) (Fig. 1e). As PGC-1α is often studied in cell types other than fibroblasts, we wondered whether PGC-1α can exert similar effects in other relevant cell types.

For that purpose, and as PGC-1α activity is well characterized in brown fat, we first utilized previously established PGC-1α KO and WT brown pre-adipocyte cell lines (15). The lines were differentiated in vitro into mature brown adipocytes, and RNA was extracted for microarray analysis. Importantly, in this system as well, a highly significant over-representation of genes encoding secreted and ECM molecules was found among the genes down-regulated dependent on PGC-1α, with terms related to secretion and the ECM being the top terms identified as enriched (Fig. 2a). An over-representation of secreted and ECM genes was not identified among genes up-regulated by PGC-1α in this system (results not shown). With a cutoff of 2-fold change, 398 transcripts were down-regulated by PGC-1α in this system out of about 25,000 genes on the array. Importantly, re-analysis of publicly available data derived using these cells (GSE5041) confirms the results of our gene annotation enrichment analysis (supplemental Fig. S3). Examination of the down-regulated genes revealed several genes encoding secreted molecules previously identified as down-regulated by PGC-1α in MEFs, as well as additional genes (Fig. 1b). Real-time PCR analyses validated our microarray data by establishing PGC-1α-dependent down-regulation of many of the targets encoding secreted molecules previously identified in fibroblasts (such as Col6a2, Vcan, Ang, and Mmp2 among others), as well as additional targets in these cells (Fig. 2c).

PGC-1α is also known to act in liver cells. To examine PGC-1α effects on genes encoding secreted molecules in liver-derived cells, we utilized the hepatocellular carcinoma cell line HepG2. Ectopic expression of PGC-1α in these cells resulted in down-regulation of the expression of several genes encoding secreted molecules as determined by real-time PCR. These genes included genes identified earlier in this study such as CTGF, ANG, and TGFBR3, as well as others (Fig. 2d).

Finally, we examined the potential effect of PGC-1α on the expression of genes encoding secreted molecules in two muscle-related systems. First, we utilized the muscle myocyte cell line C2C12 that was differentiated in vitro into mature myotubes. Ectopic expression of PGC-1α in these cells also resulted in down-regulation of several secreted protein encoding genes (Fig. 2e), including earlier identified genes such as Col6a2, Vcan, and Ccl7 (Fig. 2d), as well as others. To extend these observations to a more physiological context, we utilized a mouse model with muscle-specific PGC-1α ectopic expression that is widely used as a model for exercise-related PGC-1α effects (7, 11). Comparing WT and transgenic mice revealed PGC-1α-dependent inhibition of the expression of several genes encoding secreted proteins in this system as well (Fig. 2f).

To explore possible mechanisms responsible for the effect of PGC-1α on genes encoding secreted molecules, we first measured the mRNA stability of relevant mRNAs in PGC-1α WT or KO MEF cells. Thus, by using actinomycin D treatment to block transcription in these cells, we were able to determine the half-lives of several relevant mRNAs. Importantly, PGC-1α expression in these cells did not result in a significant decrease in mRNAs of several genes encoding secreted molecules, and in fact somewhat increased the stability of several relevant mRNAs (Fig. 3a). Therefore, effects on mRNA stability of the genes examined cannot explain the observed down-regulation of those mRNAs in the presence of PGC-1α.

As intronic RNA expression is believed to reflect transcription rates in cells (16, 17), we examined the expression of intronic RNA from several genes encoding secreted molecules in PGC-1α WT or KO MEF cells. Importantly, PGC-1α elicited down-regulation of intronic RNA expression from several genes encoding secreted proteins (Fig. 3b), consistent with an effect on transcription of those genes.

As our previous study revealed an inhibitory effect of PGC-1α on HSF1-mediated gene expression in the context of the heat shock response and beyond (14), we were interested to examine whether HSF1 might be involved in the down-regulation of genes encoding secreted proteins observed in this study. To that end, we employed WT and KO Hsf1 MEFs. First, we examined whether HSF1 may by itself regulate the expression of several genes encoding secreted molecules identified in this study. Interestingly, some of the previously identified genes were differentially expressed between WT and KO Hsf1 MEFs, showing HSF1-dependent induction of those genes in MEFs (Fig. 3c). To explore the HSF1 dependence of the effects of PGC-1α on those genes, either WT or KO Hsf1 MEFs were infected with an adenovirus encoding PGC-1α, and the expression of several relevant genes was monitored by real-time PCR. Intriguingly, some relevant targets, such as Dpt, Col6a2, and Mmp19, but not Egfl6, Mfap5, and C2, were down-regulated by
PGC-1α more efficiently in the WT Hsf1 MEFs (Fig. 3d), consistent with an involvement of HSF1 in this down-regulation. To examine whether PGC-1α may be physically associated with its gene targets in cells, we carried out chromatin immunoprecipitation studies. Chromatin immunoprecipitation studies in MEFs were unsuccessful on either up-regulated or down-regulated targets, possibly because of the significantly lower levels of PGC-1α in these cells when compared with cells derived from muscle, liver, or brown adipose. Thus, we performed chromatin immunoprecipitation studies in HepG2 cells, revealing the association of both HSF1 and PGC-1α with genes identified (Fig. 2d) as down-regulated by PGC-1α in this cell type (Fig. 3, e and f).

Although our studies focused on the effects of PGC-1α on mRNA expression of genes encoding secreted molecules, we were interested to further investigate the possible effects of PGC-1α on secreted proteins themselves, at a global level. For that purpose, we collected supernatants from either PGC-1α WT or KO MEFs and subjected them to proteomic analyses using mass spectrometry. Extensive proteomic analyses were performed on the supernatants from PGC-1α WT and KO MEFs from three replicates, and the respective amounts of identified proteins in these different samples were quantified by a label-free approach. The quality and reproducibility of the quantification were high as illustrated in the label-free quantification-intensity correlation diagrams, with replicates having a Pearson’s correlation coefficient of above 0.91 (supplemental Fig. 4). Qualitative analyses, evaluating the presence of proteins identified in at least two of the three replicates, showed that the majority of proteins were common to both PGC-1α WT and KO MEF supernatants, but also that each cell type contained a restricted set of unique proteins (Fig. 4, a and b). Volcano plot representation of the relative abundance of identified proteins in supernatants from PGC-1α WT or KO MEFs revealed proteins

FIGURE 2. Regulation of genes encoding secreted molecules by PGC-1α in brown adipocytes, HepG2 cells, and muscle. a, gene annotation enrichment analysis of genes down-regulated 2-fold or more in PGC-1α WT brown adipocytes when compared with PGC-1α KO brown adipocytes. b, heat map representing mRNA expression of genes down-regulated 2-fold or more in PGC-1α WT brown adipocytes when compared with PGC-1α KO brown adipocytes. All genes shown are at least 2-fold down-regulated by PGC-1α expression. c, mRNA expression of selected genes down-regulated 2-fold or more in PGC-1α WT brown adipocytes when compared with PGC-1α KO brown adipocytes as determined by real-time PCR. Statistical significance was calculated using Student’s t test. *, p < 0.05; **, p < 0.01. d, mRNA expression of selected genes affected by adenoviral expression of PGC-1α in HepG2 cells (P) relative to control cells (V) as determined by real-time PCR. Statistical significance was calculated using Student’s t test. *, p < 0.05; **, p < 0.01. e, mRNA expression in gastrocnemius muscle of WT mice and mice expressing a PGC-1α transgene under a muscle-specific promoter (Tg). Values represent averages of data from 5–6 mice. Statistical significance was calculated using Student’s t test, p < 0.05. Error bars indicate ± S.E.
significantly ($p < 0.05$) up- or down-regulated and more than 25% dependent on PGC-1α. (Fig. 4b). In this analysis, 63 proteins out of 976 proteins detected were down-regulated dependently on PGC-1α. Gene Ontology term enrichment analysis of the proteins down-regulated by PGC-1α revealed enrichment for terms related to ECM and secreted proteins (Fig. 4c). Importantly, several targets (such as COL6A2, CCL7, and LUM among others) previously identified as down-regulated by PGC-1α in these cells at the mRNA level were also identified as down-regulated by PGC-1α in our mass spectrometric analysis (Fig. 4d). Finally, Western blotting analyses confirmed the results from the mass spectrometric analysis by demonstrating several secreted proteins down-regulated by PGC-1α in MEF supernatants (Fig. 4e).

**Discussion**

PGC-1α is a central transcriptional co-activator involved in the regulation of metabolic genes in a variety of tissues. More recent studies have suggested that PGC-1α can exert some inhibitory effects on gene expression in specific contexts. In this study, we uncover an unexpected link between PGC-1α, a key metabolic co-activator, and a transcriptional program controlling the expression of numerous secreted proteins. Although PGC-1α has been previously shown to regulate spe-
ACCELERATED COMMUNICATION: *PGC-1α Regulates Mammalian Secretome*

A. Venn diagram showing the number of proteins qualitatively identified by mass spectrometry in PGC-1α KO and WT MEFs. B, quantitative analysis of proteins present in PGC-1α KO MEFs when compared with PGC-1α WT MEFs is shown as a volcano plot. Data represent averages of three replicates (proteins with hits in a minimum of two replicates in at least one group, KO or WT, were included). Significant hits ($p < 0.05$ calculated by applying Student’s $t$ test using false discovery rate with 5% cut off, and in addition at least 25% difference between PGC-1α KO and WT MEFs) are marked in purple. All other hits are marked in gray. C, gene annotation enrichment analysis of terms enriched among proteins down-regulated at least 25% with $p < 0.05$ (calculated as in panel b above) dependently on PGC-1α. D, heat map representing secreted protein expression down-regulated by PGC-1α in the mass spectrometric analysis. E, Western blotting analysis of secreted proteins down-regulated by PGC-1α in MEFs. IB, immunoblot.

Specific secreted factors (e.g. to inhibit IL-6 (9, 10)), our study reveals a significant inhibitory effect of PGC-1α on numerous secreted factors related to the extracellular matrix and beyond. This effect is manifested in several cell types examined in our study, although it was most notable in fibroblasts and cultured brown adipocytes in which genes encoding secreted molecules were highly enriched among genes down-regulated by PGC-1α. Interestingly, although many genes encoding secreted molecules were similarly down-regulated in several cell types, some genes encoding secreted proteins appeared to be cell type-specific. We extended our gene expression data to a mass spectrometric analysis, confirming an effect of PGC-1α on several secreted molecules at the protein level as well. Intriguingly, some proteins seem to be up-regulated dependent on PGC-1α in our mass spectrometric analysis. Although this effect may well be indirect, it is noteworthy that PGC-1α down-regulates the expression of several secreted proteinases (Fig. 1b), possibly stabilizing their targets.

Mechanistically, we show that the expression of some of the genes regulated by PGC-1α in this study is dependent upon the heat shock response master regulator HSF1 in fibroblasts. Recent work demonstrated that HSF1 can regulate several relevant transcriptional programs in cancer-associated fibroblasts (18). Indeed, our data suggest that the ability of PGC-1α to down-regulate some of its targets in fibroblasts relies on intact HSF1 (Fig. 3d). However, not all targets were dependent on HSF1 under the conditions used, and potential additional pathways may be involved in mediating the inhibitory effect of PGC-1α on genes encoding secreted proteins. Interestingly, some genes regulated by PGC-1α are jointly bound by PGC-1α and HSF1 as determined by chromatin immunoprecipitation studies (Fig. 3, e and f), suggesting direct regulation. However, PGC-1α was not detected on several other genes tested, possibly suggesting an indirect mechanism of regulation.

The effects of PGC-1α described herein may be relevant to an additional family member of PGC-1α, PGC-1β. Future studies will examine possible effects of PGC-1β on the expression of genes encoding secreted proteins. Likewise, the extent of the effects mediated by endogenous PGC-1α in this study may be partly reduced by the presence of endogenous PGC-1β such that knocking down PGC-1β may reveal a more pronounced effect or additional genes regulated by PGC-1 family members.

The potential biological implications of our findings will be the subject of future studies. Conceivably, the changes in secreted gene expression described herein impinge on processes controlled by secreted factors already suggested to be regulated by PGC-1α, such as IL-6 and TNFα. Alternatively, the PGC-1α-regulated factors we detail in this work may be involved in modulating other pathways and processes controlled by the mammalian secretome, such as inflammation, immunity, or fibrosis.

As studies unveil the importance of secretome proteins in tissue homeostasis and systemic regulation (19), our analysis reveals a yet unexplored connection between a key pathway controlling metabolic gene expression and regulation of the secretome, thereby defining a novel aspect in the activity of the key metabolic co-activator protein PGC-1α.
Experimental Procedures

Cell Culture and Treatments—LX2 cells were a kind gift from Dr. Scott Friedman, Mount Sinai Hospital, and were grown in Eagle’s minimum essential medium containing 10% FBS and antibiotics. Immortalized brown preadipocytes were a kind gift from Dr. Bruce Spiegelman, Harvard Medical School, and were grown in DMEM containing 10% FBS and antibiotics. Immortalized brown preadipocytes were differentiated as described (15). C2C12 cells (ATCC CRL-1772) were grown in DMEM containing 20% FBS and antibiotics, and then differentiated into myotubes in DMEM supplemented with 2% donor equine serum and 1 μM insulin for 7 days. Primary PGC-1α WT and KO MEFs were a kind gift from Dr. Daniel Kelly, Medical College of Wisconsin, and were maintained in DMEM containing 10% FBS and antibiotics. HSF1 WT and KO immortalized MEFs were a kind gift from Dr. Ivor J. Benjamin, Medical College of Wisconsin, and were maintained in DMEM containing 10% FBS and antibiotics. Adenoviruses were generated and produced in HEK293 cells as described previously (20) and then added directly to the culture medium where indicated. Actinomycin D was from Sigma (A1410) and was used at 2 μg/ml in culture medium for 18 h.

Gene Expression Analysis—RNA was extracted using the RNeasy kit from Qiagen. RNA was reverse-transcribed using the Superscript III First Strand Synthesis Kit from Invitrogen. Real-time PCR was performed on an Applied Biosystems 7300 Real-Time PCR System using QuantiTect SYBR Green Master Mix. Complete primer information is available as supplemental Table 1. Microarray analysis was performed using the Affymetrix GeneChip Gene Arrays according to the manufacturer’s instructions. The data expressed as CEL files were normalized by the robust multiarray average (RMA) method with the Expression Console software (Affymetrix), and then deposited in the GEO database under accession number GSE87100. Gene annotation enrichment analysis was performed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) with the Benjamini-Hochberg procedure to control for false discovery rate.

Antibodies—Antibodies used were fibronectin 1 (Abcam a6328), periostin (R&D Systems MAB3548), and pleiotrophin (Max Planck Institute of Biochemistry). Full mass spectrometry data are available as supplemental Table 2.

Author Contributions—N. M. performed and analyzed the experiments. N. M. and R. G. R. reviewed the results and approved the final version of the manuscript.

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