

ABSTRACT

The Role of PGC-1 α in Adipocyte Differentiation and Fat Mobilization

Calorie Restriction (CR) without malnutrition extends mean and maximal lifespan in multiple species, although the mechanism is unknown. CR has a dramatic effect on adipose tissue morphology, gene expression and energy metabolism. Transcription co-activator PGC-1 α is a key regulator of energy metabolism. CR influences components of the PGC-1 α pathway, resulting in PGC-1 α activation in adipose tissue. We set out to determine whether PGC-1 α plays a role in the regulation of adipocyte function.

Here we show the effect of manipulation of PGC-1 α activity on two parameters of adipocyte function; differentiation and fat mobilization. First, we show that pharmacological inhibition of PGC-1 α accelerates differentiation, and activation of PGC-1 α impairs differentiation. Second, pharmacological activation of PGC-1 α in mature adipocytes increases fat mobilization. Third, genetically increased levels of PGC-1 α also inhibit differentiation and fat storage. These data are consistent with involvement of PGC-1 α in the mechanism of adipose remodeling induced by CR.

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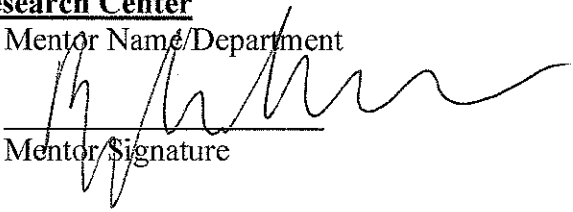


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The Role of PGC-1 α in Adipocyte Differentiation and Fat Mobilization

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ABSTRACT

Calorie Restriction (CR) without malnutrition extends mean and maximal lifespan in multiple species, although the mechanism is unknown. CR has a dramatic effect on adipose tissue morphology, gene expression and energy metabolism. The concept of adipose tissue as an endocrine organ has come into focus recently, raising the possibility that the whole-body beneficial effects of CR are initiated in adipose tissue. Transcription co-activator PGC-1 α is a key regulator of energy metabolism. CR influences components of the PGC-1 α pathway, resulting in PGC-1 α activation in adipose tissue. We set out to determine whether PGC-1 α plays a role in the regulation of adipocyte function.

Here we show the effect of manipulation of PGC-1 α activity on two parameters of adipocyte function; differentiation and fat mobilization. First, we show that pharmacological inhibition of PGC-1 α pathway accelerates differentiation, and activation of PGC-1 α pathway impairs differentiation. Second, pharmacological activation of PGC-1 α in mature adipocytes increases fat mobilization. Third, genetically increased levels of PGC-1 α also inhibit differentiation and fat storage. This study demonstrates that PGC-1 α plays a role in the processes of differentiation and fat mobilization in cultured adipocytes. These data are consistent with involvement of PGC-1 α in the mechanism of adipose remodeling induced by CR.

BACKGROUND

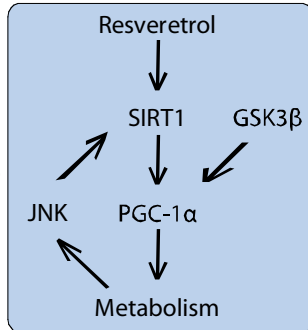
Calorie Restriction (CR) is the reduction of energy intake without malnutrition and is the most effective known way to slow aging and delay the onset of age-related disease in mammals (Anderson & Weindruch, 2007; Bordone & Guarente, 2005). The mechanism of CR is still unknown. CR has a dramatic effect on adipose tissue resulting in reduced adiposity, induction of metabolic pathways and reduction in inflammation (Anderson & Weindruch, 2006). Since adipose has recently been shown to be an endocrine organ, the changes seen in adipose by CR could be translated into whole body effects (Bordone &

Guarente, 2005). The PGC-1 α pathway regulates metabolism, and is a key factor in mitochondrial energy metabolism (Anderson & Weindruch, 2007; Orci, *et al.*, 2004; Puigserver & Spiegelman, 2003). CR alters activity of nutrient sensitive factors SIRT1, GSK3 β , and JNK, implicated in regulation of transcriptional co-activator peroxisome proliferator-activated receptor- γ (PPAR- γ) co-activator 1 α (PGC-1 α) (Fig. 1) (Anderson, *et al.*, 2008).

To connect the observations that CR influences adipose tissue and also alters the activity of these metabolic regulators, we hypothesized that the changes in adipose by CR are caused by changes in the PGC-1 α metabolic pathway. We sought to explore the

involvement of the PGC-1 α pathway in the mechanism of adipose differentiation and fat mobilization.

Figure 1. The PGC-1 α pathway, including the metabolic regulators studied. PGC-1 α regulates metabolism and mitochondrial energy metabolism in adipose.



MATERIALS AND METHODS

Cell culture

NIH3T3-L1 cells (ATCC, Rockville, MD, USA) were cultured in ATCC low sodium bicarbonate media (1,500mg/L sodium bicarbonate) with 10% fetal bovine serum and antibiotics. To differentiate preadipocytes to lipid storing adipocytes, cells were grown to confluence and at two days post confluence (day 0) were induced with .5mM isobutylmethylxanthine (IBMX), 1 μ M Dexamethasone, and 2 μ M insulin in the media for two days. On day 2, media was changed and supplemented with 2 μ M insulin for two days. Subsequent media changes every two days until day 8 or 10 included no supplementation. On day 8 or 10, cells were fixed (10% formaldehyde, 10 minutes) and stained with Oil Red O for lipid accumulation visualization. Pictures were taken on a Carl Zeiss AxioVert200 microscope with a Zeiss AxioCam MRc5 (Carl Zeiss Light Microscopy, Göttingen, Germany) and AxioVision4 software. Transfection was performed with Lipofectamine (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions using pcDNA3.1 and pcDNA-PGC-1 α . Clones were isolated, and expression of PGC-1 α was confirmed by immunofluorescence. To determine response to inhibitors during differentiation, cells were incubated in Sirtinol [(Calbiochem, EMD/Merck, Darmstadt,

Germany), 10 μ M], Resveratrol [(Sigma, St Louis, MO, USA) 10 μ M], GSK3 β inhibitor VII (Calbiochem, 20 μ M), or JNK inhibitor II (Calbiochem, 25 μ M) from day 0 to day 8 of differentiation. To determine response to inhibitors post differentiation, cells were fully differentiated as described above and on day 8, preincubated with inhibitors in serum free DMEM for 24 hours and then incubated with inhibitors in 10% FBS media for an additional 24 hours. To determine response to epinephrine in the presence of inhibitors, cells were differentiated as described above, on day 8 preincubated with inhibitors in serum free DMEM for 24 hours, and then incubated with inhibitors in serum free media with epinephrine hydrochloride (Amphastar IMS, South El Monte, CA, USA, 10 μ M) for an additional 24 hours. Experiments were performed in triplicate, and compared to identically treated cells without inhibitor or epinephrine exposure. For immunofluorescence and live cell imaging studies, cells were grown on cell-culture-treated cover slips (Fisher Scientific, Thermo Fisher Scientific, Waltham, MA, USA).

Immunofluorescence and Live Cell Imaging

Levels of PGC-1 α were analyzed by immunofluorescence using standard techniques. Cells were grown, fixed (10% formaldehyde, 10 min) and incubated overnight in primary antibody. Proteins were visualized using fluorescent-tagged secondary antibodies (Vector Laboratories, Burlingame, CA, USA). For live imaging, cells were incubated in Mitotracker Red [(Molecular Probes, Invitrogen, Carlsbad, CA, USA), 100 nM] and Mitotracker Green (Invitrogen, 300nM) for 15 min in 10% FBS DMEM lacking phenol red. Images were captured using uniform exposure settings on a Leica DMLB microscope fitted for epi-fluorescence (Leica Microsystems, Wetzlar, Germany), with a Spot Insight Color camera (Diagnostic Instruments, Sterling Heights, MI, USA) and Spot 3.3.1 software.

RESULTS

For this study, we used a NIH3T3-L1 cell culture model. First, we established a fat storage timecourse during differentiation (as described above) over 8 days, with time points taken every two days, confirming that it takes 8 days to fully differentiate into fat storing adipocytes (Fig 2).

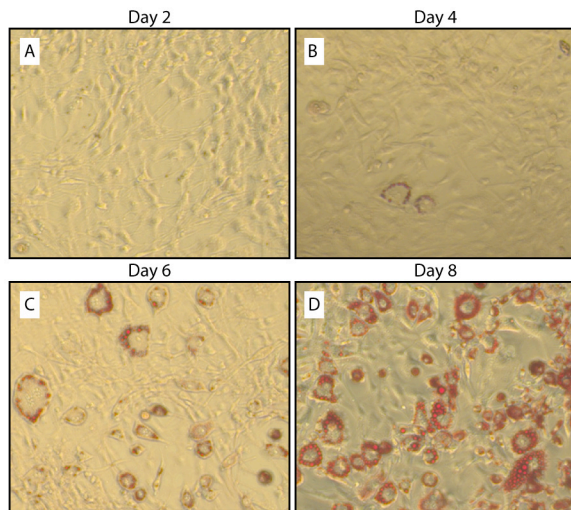


Figure 2. 8 day 3T3-L1 preadipocyte differentiation by induction with Dexamethasone, Isobutylmethylxanthine (IBMX), and Insulin. No fat accumulation seen in days 2 (A) or 4 (B), minor fat accumulation observed in day 6 (C), full differentiation observed by day 8 (D). All stained with Oil Red O.

Manipulation of PGC-1 α Pathway Alters Differentiation of Preadipocytes

Next, we took 3T3-L1 preadipocytes through 8-day differentiation in the presence of previously indicated concentrations of metabolic regulator inhibitors and activators to observe the effects of PGC-1 α pathway on fat storage during differentiation. Sirtinol SIRT1, a positive regulator of PGC-1 α activity. Thus, treatment with Sirtinol would be expected to reduce PGC-1 α activity. Resveratrol activates SIRT1 which, in turn, is expected to increase the activity of PGC-1 α . GSK3 β is a negative regulator of PGC-1 α , so when GSK3 β is inhibited, PGC-1 α activity increases. JNK is negatively regulated by CR in

adipose and this is associated with reduced fat storage.

Treatment of cells during differentiation with Sirtinol increased the amount of fat stored after 8 days. Resveratrol, GSK3 β inhibitor, and JNK inhibitor treatment all decreased the adipocytes' ability to store fat during differentiation (Fig. 3).

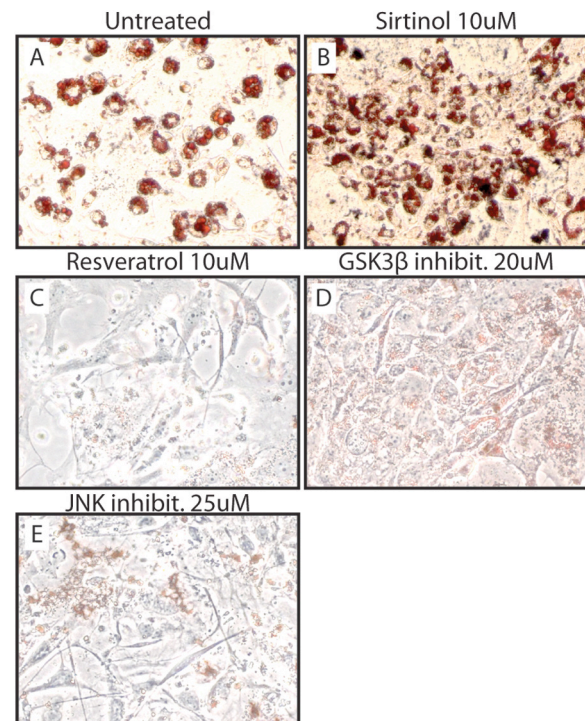


Figure 3. 8-day differentiation of 3T3-L1 preadipocytes incubated with specified concentrations of inhibitors in media during differentiation process from day 0 to day 8. (A) Untreated control, (B) treatment with 10uM Sirtinol, (C) treatment with 10uM Resveratrol, (D) treatment with 20uM GSK3 β inhibitor, (E) treatment with 25uM JNK inhibitor. All stained with Oil Red O.

Thus, during differentiation, we see that pharmacologically decreased PGC-1 α activity results in increased fat storage, and pharmacologically increased PGC-1 α activity results in decreased fat storage.

Manipulation of PGC-1 α Pathway Influences Fat Storage Maintenance

Next, we studied fat storage maintenance by PGC-1 α in fully differentiated 3T3-L1 adipocytes. After differentiation, the cells

were treated with inhibitors and activators of the PGC-1 α pathway. We observed after treatment that Sirtinol increased fat storage in mature adipocytes, Resveratrol and GSK3 β inhibition decreased fat storage after differentiation, and JNK inhibition had no visible change in fat storage maintenance from control (Fig. 4, "Untreated" column). The Resveratrol observation is in concordance with previously published studies (Picard, *et al.*, 2004).

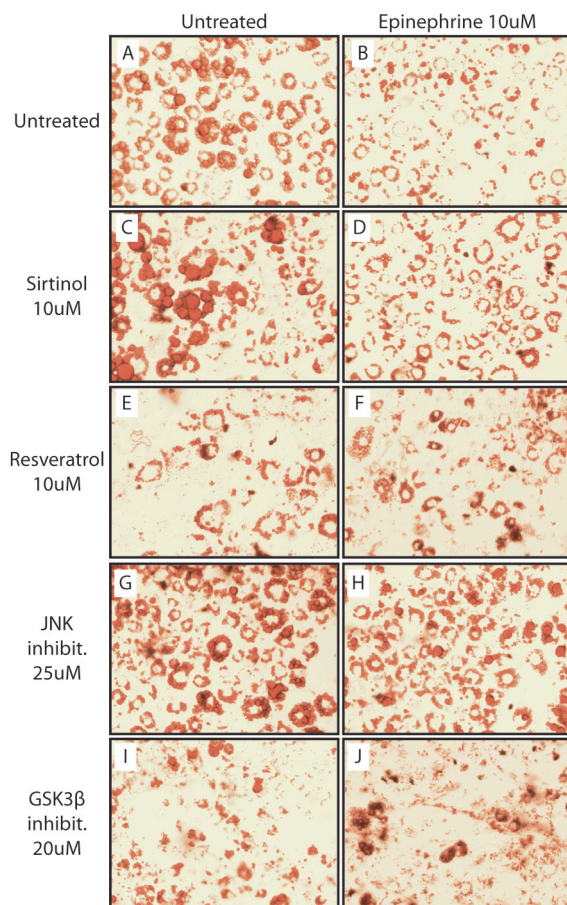


Figure 4. Normal 8 day differentiation of 3T3-L1 preadipocytes, followed by 24 hour preincubation with inhibitors in serum-free DMEM, then 24 hour incubation with inhibitors in serum-free DMEM with either 10uM epinephrine (B,D,F,H,J) or without (A,C,E,G,I). All stained with Oil Red O.

This shows that pharmacological manipulation of the PGC-1 α pathway to decrease activity leads to increased fat storage in fully differentiated adipocytes. In contrast, increased PGC-1 α pathway activity increase

leads to decreased fat storage after differentiation. Unlike the other components of the PGC-1 α pathway, JNK is not required for maintenance of fat stores in mature adipocytes.

Metabolic Regulators Play a Distinct Role in the Response to Epinephrine

To study the effects of PGC-1 α pathway manipulation on fat mobilization in 3T3-L1 adipocytes, we fully differentiated the preadipocytes, pretreated with inhibitors, and treated with inhibitors and epinephrine as described previously. Epinephrine is an activator of β -adrenergic receptors, which causes the release of lipid stores from the cell. We observed that epinephrine treatment mobilized fat in all metabolic regulator treatment types with the exception of the JNK inhibitor treated cells (Fig. 4). Because no difference is observed in fat mobilization by epinephrine between PGC-1 α activators and inhibitors, it suggests that fat mobilization by epinephrine works independently of the upstream factors in the PGC-1 α pathway. Also, since JNK inhibited cells were observed to maintain control level fat when treated with epinephrine, JNK is suggested again to have a role in fat mobilization in response to epinephrine.

Increased levels of PGC-1 α inhibit fat storage in adipocytes

Prompted by the findings in the inhibitor studies, we set out to study effects of directly changing PGC-1 α levels in 3T3-L1 preadipocytes. We transfected 3T3-L1 preadipocytes to add in extra copies of PGC-1 α , and isolated clones as described previously. To confirm increased levels of PGC-1 α in these transfected cells, we measured the level of PGC-1 α protein present in the cells by immunofluorescence (Fig. 5). Immunofluorescence treatment detects PGC-1 α protein, indicated by accumulation of fluorescent signal that present as brighter color under the fluorescent microscope.

Immunofluorescence confirmed the presence of increased PGC-1 α protein in the transfected cells.

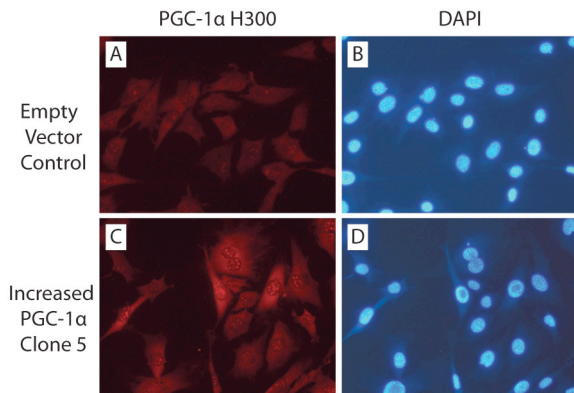


Figure 5. Immunofluorescence of undifferentiated 3T3-L1 preadipocytes previously untransfected (A, B) or transfected with pcPGC-1 α (C, D). Treated with PGC-1 α H300 primary antibody (A, C), and DAPI (B, D).

Additional copies of PGC-1 α cause an increase in mitochondrial membrane potential (Anderson, *et al.*, 2008). To confirm increased PGC-1 α activity, we determined mitochondrial membrane potential in transfected cells (Fig. 6).

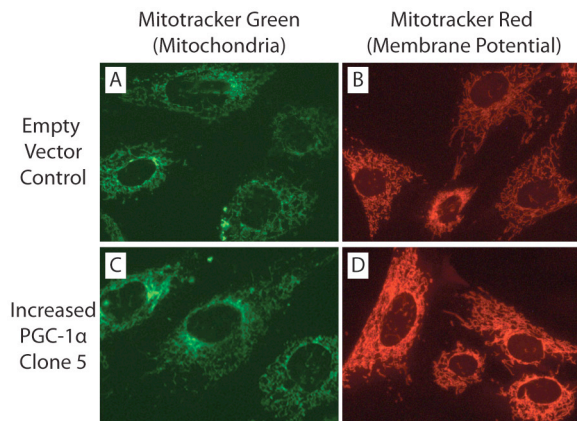


Figure 6. Mitotracker™ live cell culture dyes on undifferentiated 3T3-L1 preadipocytes previously untransfected (A, B) or transfected with pcPGC-1 α (C, D). Mitotracker™ Green dye detects mitochondria within the cells (A, C). Mitotracker™ Red dye detects mitochondrial membrane potential within the cells (B, D).

Mitotracker Green, an indicator of mitochondrial number, shows no increase between PGC-1 α clones and the empty vector

control preadipocytes (Fig. 6, “Mitotracker Green” column). Mitotracker Red shows increased staining indicative of increased mitochondrial membrane potential (Fig. 6, “Mitotracker Red” column). Both the Mitotracker Green and Mitotracker Red findings are consistent with previously published data generated in an increased PGC-1 α transfected fibroblast model (Anderson, *et al.*, 2008).

We then took these transfected clones with extra copies of PGC-1 α through full 8-10 day differentiation to study the fat storage phenotype presented by these adipocytes when differentiated (Fig. 8).

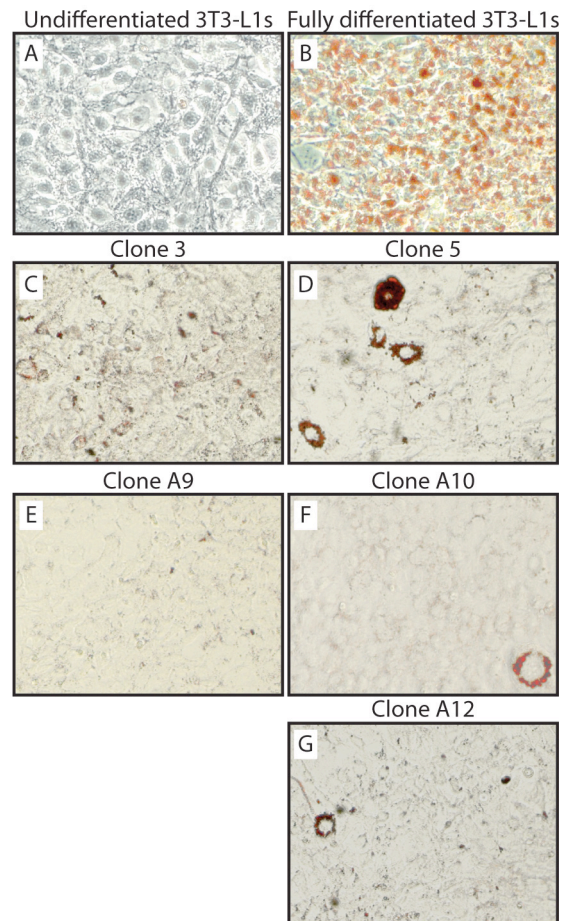


Figure 7. Normal 8-10 day differentiation in untransfected vs. pcPGC-1 α transfected 3T3-L1 preadipocytes. Undifferentiated, untransfected confluent 3T3-L1 preadipocyte negative control (A). Fully 8 day differentiated untransfected 3T3-L1 preadipocyte positive control (B). pcPGC-1 α transfected ‘clones’ fully differentiated for 8 days (E, G) or 10 days (C, D, F). All stained with Oil Red O.

Once treated for differentiation and stained with Oil Red O, the adipocytes with extra copies of PGC-1 α had reduced oil red staining, indicative of decreased levels of lipid storage, like untransfected differentiated 3T3-L1 adipocytes (Fig. 7). With the exception of a few outlying cells in some frames, the increased PGC-1 α cells showed a decrease in fat storage during differentiation, concordant with the pharmacological study findings above.

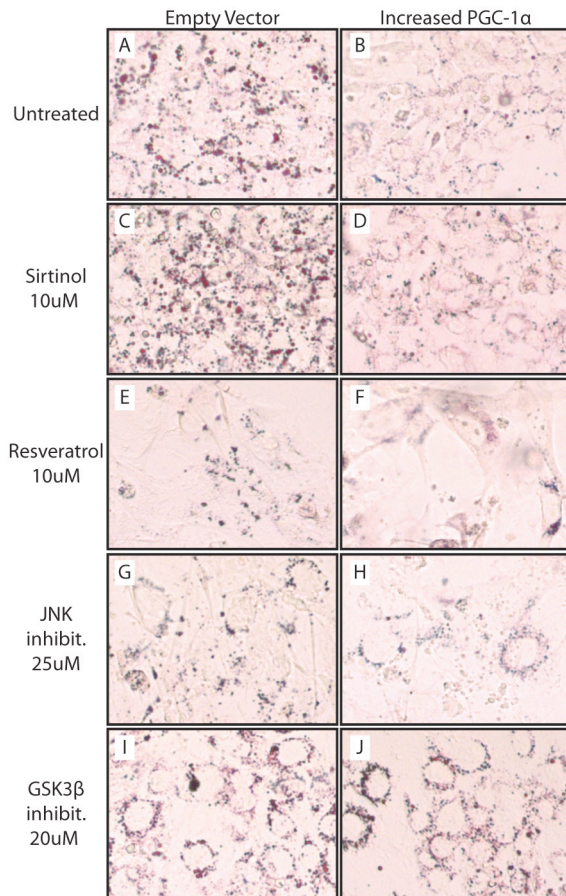


Figure 8. 8-day differentiation of 3T3-L1 preadipocytes incubated with inhibitors in media during differentiation process from day 0 to day 8. Untransfected Empty Vector Controls (A, C, E, G, I) vs. pcPGC-1 α transfected adipocytes (B, D, F, H, J). All stained with Oil Red O.

Finally, to determine if the overexpressor phenotype could be rescued, we compared the increased PGC-1 α transfected cells against empty vector cells under treatment of the different metabolic regulator inhibitors and activators during differentiation. The empty

vector cells have the same phenotype as the original untransfected 3T3-L1 adipocytes studied previously (Fig. 8, "Empty Vector" column). In cells with additional copies of PGC-1 α (Fig. 8), the untreated and Sirtinol treated cells showed reduced fat storage during differentiation compared to the controls. The Resveratrol treated and JNK inhibited cells showed no more fat storage reduction from controls because the controls were already inhibited from storing fat during differentiation by the respective metabolic regulator treatments. In contrast, treatment with GSK3 β abrogates the PGC-1 α phenotype, suggesting a partial rescue by GSK3 β in increased PGC-1 α adipocytes. These data suggest that GSK3 β has PGC-1 α dependent and PGC-1 α independent effects on differentiation.

DISCUSSION

In this study, we have demonstrated that PGC-1 α negatively regulates fat storage in adipocytes. During differentiation, manipulations that cause increased PGC-1 α activity resulted in reduced fat accumulation. Decreased PGC-1 α pathway activity resulted in increased fat accumulation during differentiation. In mature adipocytes, inhibition of PGC-1 α led to increased fat accumulation. Resveratrol and GSK3 β inhibition resulted in mobilization of fat, whereas JNK inhibition resulted in no change in fat level maintenance, indicating that JNK is not involved in the maintenance of fat stores. However, when we treated the cells with epinephrine to mobilize fat, all cells released fat except those treated with JNK inhibitor. This finding suggests that JNK could be necessary for fat mobilization.

We have also confirmed that our model PGC-1 α pathway is conserved in adipocytes. Increased copies of PGC-1 α show the same phenotype of less fat storage during differentiation as that identified in the inhibitor study. Factors downstream of PGC-1 α in the pathway, would be expected to

overcome the PGC-1 α phenotype. If we observe a partial phenotype rescue, it would imply that the factor operates in one or more other pathways with the same target in addition to its role in the PGC-1 α pathway. Our results suggest that SIRT1 is upstream of PGC-1 α and GSK3 β is parallel to PGC-1 α . The PGC-1 α pathway scheme is in accordance with our results.

We have identified a novel role for PGC-1 α in differentiation and fat storage in cultured 3T3-L1 adipocytes. Manipulation of factors that regulate PGC-1 α reveals distinct roles in accrual of fat and in maintaining the fat store. These data are consistent with the PGC-1 α pathway being involved in the adipose remodeling seen in CR animals.

While many of the phenotypes of aging are instantly recognizable, the underlying causes are poorly understood, in particular in the context of age-associated disease. With age, fat is redistributed in the body and is associated with deregulated adipose function. Adipose tissue dysfunction has been implicated in systemic inflammation and disease onset (Higami, *et al.*, 2006). Advances in the study of adipose regulation could expand knowledge about and solutions to aging-related disease states.

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