Original Paper

Resistin Regulates Fatty Acid β Oxidation by Suppressing Expression of Peroxisome Proliferator Activator Receptor Gamma-Coactivator 1 α (PGC-1 α)

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Key Words

Fatty acid β oxidation • Resistin • PGC-1 α • CREB

Abstract

Background/Aims: Abnormal fatty acid β oxidation has been associated with obesity and type 2 diabetes. Resistin is an adipokine that has been considered as a potential factor in obesity-mediated insulin resistance and type 2 diabetes. However, the effect of resistin on fatty acid β oxidation needs to be elucidated. **Methods:** We detected the effects of resistin on the expression of fatty acid oxidation (FAO) transcriptional regulatory genes, the fatty acid transport gene, and mitochondrial β -oxidation genes using real-time PCR. The rate of FAO was measured using ¹⁴C-palmitate. Immunofluorescence assay and western blot analysis were used to explore the underlying molecular mechanisms. **Results:** Resistin leads to a reduction in expression of the FAO transcriptional regulatory genes ERR α and NOR1, the fatty acid transport gene CD36, and the mitochondrial β -oxidation genes CPT1, MCAD, and ACO. Importantly, treatment with resistin led to a reduction in the rate of cellular fatty acid oxidation. In addition, treatment with resistin reduced phosphorylation of acetyl CoA carboxylase (ACC) (inhibitory). Mechanistically, resistin inhibited the activation of CREB, resulting in suppression of PGC-1 α . Importantly, overexpressing PGC-1 α can rescue the inhibitory effects of resistin on fatty acid β oxidation. **Conclusions:** Activating the transcriptional activity of CREB using small molecular chemicals is a potential pharmacological strategy for preventing the inhibitory effects of resistin on fatty acid β oxidation.

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Introduction

Fatty acid metabolism is the central structural and functional component of lipid metabolism. In mammal cells, fatty acids can be rapidly oxidized by peroxisomes and mitochondria to maintain cellular bioenergetic homeostasis [1]. Abnormal fatty acid oxidation has been associated with obesity and type 2 diabetes. It is becoming increasingly important to understand the mechanisms involved in mitochondrial fatty acid oxidation. Fatty acid oxidation rates are controlled at multiple levels and involve both transcriptional and non-transcriptional components. At the transcriptional level, several hormone nuclear receptors regulate the transcription of genes encoding for fatty acid oxidation enzymes, including PPAR γ and ERR α [2]. The assembly of co-activators, such as peroxisome proliferator activator receptor gamma-coactivator 1 α (PGC-1 α), plays an essential role in enhancing the ability of these hormone nuclear receptors to drive transcriptional programs [3]. The expression of PGC-1 α is controlled by the transcription factor CREB [4].

Multiple lines of evidence have shown that in obesity, insulin sensitivity is dramatically affected by changes in factors secreted by adipose tissue, such as the release of inflammatory cytokines. Resistin is a cysteine-rich 12.5-kDa polypeptide adipokine secreted by adipose tissue in rodents and by macrophages in humans [5; 6]. Resistin has been considered as a potential factor in obesity-mediated insulin resistance and type 2 diabetes. It can also promote the inflammatory reaction [7]. Resistin expression was found to be increased in both 3T3-L1 cells and primary pre-adipocytes derived from Zucker obese rats during the process of differentiation and maturation [8]. Results of *in vitro* experiments show that lipid content is significantly decreased in 3T3-L1 cells transfected with anti-resistin small interfering RNA (siRNA) after adipocyte differentiation, suggesting that resistin knockdown induces suppression of lipid production [9]. However, the underlying mechanism by which resistin regulates lipid metabolism remains unknown. In this study, we report that treatment with resistin inhibits lipid β oxidation by suppressing the expression of PGC-1 α .

Materials and Methods

Cell culture, treatment, and transfection

Murine C2C12 cells were maintained in Dulbecco's modified low glucose Eagle (DMEM) medium with 10% fetal bovine serum (FBS) (v/v) plus 4.0 mM glutamine and 1% antibiotics (penicillin and streptomycin). When cells were grown to 70–80% confluence, the medium was switched to DMEM containing 2% horse serum (GIBCO, Grand Island, NY, USA) for 72 h to induce the differentiation of myoblasts into myotubes. Then, cells were treated with resistin at concentrations of 5 or 10 ng/ml for 24 h. To activate CREB, C2C12 cells were treated with resistin at the concentration of 10 ng/ml in the presence or absence of 100 μ M cAMP for 24 h. C2C12 cells were then transduced with the PGC-1 α lentivirus system (Applied Biological Materials, Canada) to overexpress PGC-1 α . Briefly, 2×10^5 cells were seeded into each well of a 6-well plate and cultured for 24 h. Polybrene was mixed with complete media at a final concentration of 8 μ g/ml. Then, 2 ml of the polybrene-media-mix containing a positive GFP control virus or a target virus was added to each well with a multiplicity of infection (MOI) of 100:1. After 12 h, the cultured medium was replaced with normal complete medium. C2C12 cells were transfected with pcDNA3.1 (vector control) and ERR α construct (Addgene, USA) using lipofectamine 2000 (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. Adenoviruses harboring wild-type NOR1 (Ad-NOR1) and GFP (Ad-GFP) were used to infected C2C12 cells as previously described [10].

RNA isolation and real-time PCR

Whole RNA was isolated from cultured cells using Trizol (Invitrogen, USA) according to the manufacturer's instructions. Then, 1 μ g of total mRNA was used to synthesize cDNA by using an iScript CDNA synthesis kit (Bio-Rad, USA). Real-time PCR was carried out by a StepOne Plus Real-time PCR System using TaqMan gene expression assays (Applied Biosystems, USA). Gene expression was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using the $\Delta\Delta$ Ct method. The following primers were used in this study:

mouse ERR α : 5'-TTCGGCGACTGCAAGCTC-3' and
5'-CACAGCCTCAGCATCTTCAATG-3';
CD36: 5'-ATGGGCTGTGATCGGAAGT-3' and
5'-GTCTTCCCAATAAGCATGTCTCC-3';
CPT1: 5'-CTCCTGGAAGAAACGCCTTATT-3'
and 5'-CACCTTGCAGTAGTTGGAACC-3';
ACO: 5'-GCCCAGGTGAAGCCTGATGGA-3' and
5'-GACTGGTGCCTCACAGCGTG-3';
NOR1: 5'-TCTTCTTAGGCCAGTCTCTG-3' and
5'-CAGTGTGGCTTTTGGTC-3';
MCAD: 5'-TTGGTGACGGAGCTGGTTTCA-3' and
5'-CATTGCCATTTCAGCCAGCA-3';
GAPDH: 5'-GTGCCGCTGGAGAAACCT-3' and
5'-TGAAGTCGACGAGACAACC-3'.

Western blot analysis

Cell lysates were made using cell lysis buffer (Cell Signaling, USA). Twenty μ g of total protein extract was subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and were electrotransferred onto Immobilon-P membranes (Millipore, USA). Transferred membranes were blocked in TBS solution containing 10% non-fat dry milk and 0.5% Tween-20 for 2 h at room temperature (RT). Then, the membranes were probed with the indicated primary antibodies for 3 h at RT followed by incubation with secondary antibodies for 2 h at RT [11]. The blots were developed with Immobilon Western Chemiluminescent HRP Substrate (Millipore).

This study used rabbit monoclonal antibody anti-PGC-1 α (1:1000, #ab54481, Abcam, USA); Rabbit monoclonal antibody anti- β -actin (1: 8000, #ab16039, Abcam, USA); Rabbit polyclonal antibody against ACC (1:2000, #3662, Cell Signaling Technology, USA); Rabbit monoclonal antibody against P-ACC (Ser79) (1:1000, #3662, Cell Signaling Technology, USA); Mouse monoclonal antibody against Phospho-AMPK (Thr172) (1:1000, #50081, Cell Signaling Technology, USA); Rabbit monoclonal antibody against AMPK (1: 2000, #2532, Cell Signaling Technology, USA); Rabbit monoclonal antibody against CREB (1:2000, #9197, Cell Signaling Technology, USA); Rabbit monoclonal antibody against P-CREB (Ser133) (1:1000, #9198, Cell Signaling Technology, USA); Horse radish peroxidase (HRP)-linked anti-rabbit IgG antibody (1: 1000, #7074, Cell Signaling Technologies, USA); Horse radish peroxidase (HRP)-linked anti-mouse IgG antibody (1: 1000, #7076, Cell Signaling Technologies, USA).

Determination of fatty acid oxidation rate

The rate of fatty acid oxidation was measured by 14 C-palmitate in differentiated C2C12 myotubes. The key to this method is detecting altered FAO, commonly obtained by quantifying 14 CO $_2$, a radioactive end product of the oxidation reaction. Briefly, 14 C-labeled CO $_2$ produced during the incubation period was trapped with a Whatman paper wetted with 100 μ l of phenylethylamine-methanol (1:1). Finally, radioactivity was recorded using a scintillation counter.

Immunocytochemistry

C2C12 cells were washed in PBS, pH 7.4, and then fixed in 4% para-formaldehyde (PFA) at RT for 15 min. Then, cells were permeabilized with 0.4% Triton X-100 and blocked with 15% (v/v) FBS in PBST. Cells were probed with anti-PGC-1 α primary antibody (Invitrogen, USA) in PBS with 5% BSA for 2 h at RT, followed by incubation with an Alexa-594 conjugated secondary antibody for 1 h at RT. Cells were counterstained in Vectashield mounting medium (Vector Laboratories, USA).

Statistical analysis

All data are presented as means $\pm$ SD. Statistical comparisons were performed using one-way analysis of variance (ANOVA), and $P < 0.05$ was considered statistically significant.

Results

Firstly, we tested the ability of resistin to modulate expression of genes that promote fatty acid oxidation. Our results indicate that expression of the fatty acid oxidation transcriptional regulatory genes *ERR α* and *NOR1* is reduced by treatment with resistin in a dose-dependent manner (Fig. 1A). In addition, it was shown that treatment with resistin also reduced expression of the fatty acid transport gene *CD36* (Fig. 1B). Importantly, resistin treatment caused complete inhibition of the expression of all three mitochondrial β -oxidation genes: *CPT1*, *MCAD*, and *ACO* (Fig. 1C). Interestingly, overexpression of *ERR α* (Fig. 1D) or *NOR1* (Fig. 1E) prevented resistin-induced reduction of *CPT1*, *MCAD*, and *ACO*, thereby suggesting the involvement of *ERR α* and *NOR1* in regulating the effects of resistin on these genes.

We then measured the complete rate of fatty acid oxidation in the presence and absence of resistin. As shown in Fig. 2, our results demonstrate that the rate of cellular fatty acid oxidation is indeed markedly reduced following treatment with resistin as measured by radioactive CO_2 derived from radiolabeled ^{14}C -palmitate. *PGC-1 α* is a main activator of fatty acid oxidation and a regulator of these target genes. Thus, we examined the effects of resistin on the expression of *PGC-1 α* . After incubation with resistin, the expression of *PGC-1 α* was reduced at both the mRNA (Fig. 3A) and protein levels (Fig. 3B), suggesting that resistin suppresses expression of *PGC-1 α* at the transcriptional level. Immunofluorescence results confirmed this result, as shown in Fig. 3C.

Fig. 1. Resistin reduces expression of genes linked to fatty acid oxidation in cultured muscle cells in a dose-dependent manner. Real-time PCR results reveal that treatment with resistin reduces (A) fatty acid oxidation transcriptional regulatory genes *ERR α* and *NOR1*; (B) fatty acid transport gene *CD36*; (C) all three mitochondrial β -oxidation genes *CPT1*, *MCAD*, *ACO*. Cells were treated with resistin at concentrations of 5 or 10 ng/ml for 24 h; (D) Overexpression of *ERR α* prevented resistin (10 ng/ml)-induced reduction of *CPT1*, *MCAD*, and *ACO*; (E) Overexpression of *NOR1* prevented resistin (10 ng/ml)-induced reduction of *CPT1*, *MCAD*, and *ACO* (*, $P < 0.001$ vs. untreated control; #, $P < 0.001$ vs. resistin treatment group $n = 4-6$, ANOVA).

Fig. 2. Resistin reduces the rate of fatty acid oxidation in C2C12 myotubes. Cells were treated with resistin at concentrations of 5 or 10 ng/ml for 24 h. The rate of fatty acid oxidation was measured by ^{14}C -palmitate in differentiated C2C12 myotubes (*, $P < 0.01$ vs. control group).

Fig. 3. Resistin inhibits the expression of PGC-1 α . (A) mRNA levels of PGC-1 α were determined by real-time PCR (*, $P < 0.01$ vs. nontreated control, $n = 4$); (B) Protein levels of PGC-1 α were determined by western blot analysis (*, $P < 0.01$ vs. nontreated control, $n = 4$); (C) Representative immunofluorescence staining of PGC-1 α (*, $P < 0.01$ vs. nontreated control, $n = 4$).

Under conditions that require energy, adenosine monophosphate-activated protein kinase (AMPK) plays an important role as a regulator by enhancing phosphorylation of acetyl CoA carboxylase (ACC) (inhibitory), decreasing synthesis of malonyl-CoA and increasing fatty acid oxidation [12].

It has been reported that the effects of PGC-1 α on lipid metabolism might be partially mediated by the AMPK/ACC-dependent pathway [13]. We next investigated whether ACC is involved in this process. Indeed, phosphorylated ACC (inhibited) was significantly reduced by resistin in a dose-dependent manner. However, neither total/phosphorylated AMPK nor total ACC was changed in response to resistin treatment (Fig. 4).

Expression of PGC-1 α is regulated by the transcription factor CREB at the transcriptional level. We then investigated the effects of resistin on CREB phosphorylation at Ser133. Our results indicate that resistin inhibits phosphorylation of CREB at Ser133 (Fig. 5A). Importantly, treatment with cAMP, a CREB activator that works through the PKA pathway, attenuated the inhibitory effect of resistin on PGC-1 α expression (Fig. 5B).

We overexpressed PGC-1 α in C2C12 cells using lentiviral PGC-1 α construct, with the null as control. Successful overexpression of PGC-1 α was confirmed by real-time PCR at the mRNA

Fig. 4. Resistin reduced the phosphorylation (inhibitory) of ACC. (A) Phosphorylated ACC and total ACC were determined by western blot analysis (*, $P < 0.01$ vs. nontreated control, $n = 4$); (B) Phosphorylated AMPK and total AMPK were determined by western blot analysis.

Fig. 5. Resistin inhibits the expression of PGC-1 α , mediated by CREB. (A) Phosphorylated levels of CREB (*, $P < 0.01$ vs. nontreated control, $n = 4$); (B) cAMP attenuates the effects of resistin on PGC-1 α expression (*, $P < 0.01$ vs. nontreated control, $n = 4$).

Fig. 6. Overexpressing PGC-1 α rescues the inhibitory effects of resistin on fatty acid β oxidation. (A) Successful overexpression of PGC-1 α at the mRNA level; (B) Successful overexpression of PGC-1 α at the protein level; (C) Fatty acid oxidation transcriptional regulatory genes ERR α and NOR1 (*, $P < 0.01$ vs. nontreated control; #, $P < \text{Resistin treated group}$, $n = 4$); (D) Fatty acid transport genes CD36; (E) All three mitochondrial β -oxidation genes CPT1, MCAD, ACO; (F) The rate of fatty acid oxidation measured by ^{14}C -palmitate (*, $P < 0.01$ vs. nontreated control; #, $P < \text{Resistin treated group}$, $n = 4$).

level (Fig. 6A) and western blot analysis at the protein level (Fig. 6B). Our results indicate that overexpression of PGC-1 α mitigated the reduction in expression of the transcriptional regulatory genes ERR α and NOR1 induced by resistin (Fig. 6C).

We also found that overexpression of PGC-1 α prevented resistin-induced reduction of the fatty acid transport gene CD36 (Fig. 6D) as well as the three mitochondrial β -oxidation genes CPT1, MCAD, and ACO (Fig. 6E). More importantly, overexpression of PGC-1 α rescued the

reduction in the rate of cellular fatty acid oxidation (Fig. 6F) induced by resistin, suggesting that PGC-1 α plays an essential role in the inhibitory effect of resistin on lipid β oxidation.

Discussion

In this study, we found a new function of resistin by demonstrating that it can reduce the rate of fatty acid

β oxidation. Mechanistically, administration of resistin inhibits activation of CREB, which in turn leads to suppression of PGC-1 α transcription, a main regulator in lipid metabolism, which can activate expression of the fatty acid oxidation transcriptional regulatory genes ERR α and NOR1, as well as expression of the fatty acid transport gene CD36 and the three mitochondrial β -oxidation genes CPT1, MCAD, ACO. Importantly, overexpression of PGC-1 α can rescue the inhibitory effects of resistin on fatty acid β oxidation. A schematic representation of the underlying mechanism is shown in Fig. 7.

Increasing evidence has demonstrated that circulating resistin levels are correlated with both type 2 diabetes and metabolic syndrome [14]. However, the effects of resistin on lipid metabolism are still controversial. A recent study showed that suppression of resistin expression resulted in a significant decrease in intracellular lipid content transfection of anti-resistin small hairpin RNA expression plasmid DNA into 3T3-L1 cells for a more effective RNA interference effect on resistin gene expression [9]. This is consistent with the findings of our current report. In addition, there are several studies indicating an inconsistent effect of resistin on accumulation of lipid droplets in 3T3-L1 cells [15]. Interestingly, Kim and colleagues reported resistin to be a key mediator of fatty acid import through a pathway involving increased phosphorylation of PI3K in adipocytes [16]. Our study showed that resistin can inhibit lipid β oxidation and explored a potential mechanism by which resistin regulates lipid metabolism. The increase of PGC-1 α activity has been considered as a valid therapeutic target in the amelioration of several metabolic diseases [17]. Moreover, *in vivo* studies have shown that specific overexpression of PGC-1 α in skeletal muscle tissue provides strong beneficial effects and protects against age-associated diseases [18]. Importantly, we discovered that the inactivation of the transcriptional factor CREB is involved in the transcription suppression of PGC-1 α by resistin, suggesting that activating the transcriptional activity of CREB using small molecular chemicals is a potential pharmacological strategy to prevent the inhibitory effects of resistin on fatty acid β oxidation.

Acknowledgements

This study was supported by National Natural Science Foundation of China F (81660466, 81360398) and Foundation of Yunnan Provincial Bureau of Health (2016NS224), in part by Yunnan Health Training Project of High Level Talents (H-201642).

Fig. 7. Schematic drawing of the effects of resistin on fatty acid β oxidation in C2C12 cells.

Disclosure Statement

None of the authors of this study have any conflict of interest that they need to disclose.

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