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SHORT COMMUNICATION

Roberta Ceci
Stefania Sabatini
Guglielmo Duranti
Isabella Savini
Luciana Avigliano
Antonello Rossi

Acute, but not chronic, leptin treatment induces acyl-CoA oxidase in C2C12 myotubes

Abstract *Background* The product of the obesity gene (ob), leptin, has a well-recognized role in regulating energy homeostasis. During the period of weight maintenance, circulating leptin concentration reflects total body fat mass. On the other hand, overnutrition is accompanied by progressive hyperleptinemia. In overnourished animals, the elevation in circulating fatty acids results in increased uptake and excessive deposition of lipids within muscle cells. Consequently, triglicerydes overload seems to strongly correlate to the impairment of insulin signaling in skeletal muscle, the primary target for insulin stimulated glucose disposal. High levels of leptin in the course of fat storage may protect non-adipose tissues from lipid accumulation. *Aim of the study* Here, we aim to evaluate in vitro the relationship between leptin treatment and expression of acyl-CoA oxidase (ACOX), a peroxisomal key enzyme involved in fatty acid catabolism. We also evaluate the adaptive response of cells to a putative oxidative insult, resulting from H_2O_2 production. *Methods* The effects of increasing levels of leptin, at different times, were assessed on mouse C2C12 myotubes by semiquantitative PCR. Activation pathway was investigated by using extracellular signal-regulated kinase (ERK) and cytosolic phospholipase A_2 ($cPLA_2$) inhibitors. Cellular adaptive response to oxidative stress was evaluated by measuring glutathione concentration, oxidized/reduced glutathione ratio and the main antioxidant enzymatic activities. *Results* A 1.8-fold increase in ACOX mRNA expression was evident at 20 ng/ml leptin, a dose comparable to that found in hyperleptinemic subjects. The induction was dose-dependent, with an increase of 3-fold at 100 ng/ml; the ability of leptin to stimulate ACOX mRNA reached a maximum at 20 min and was lost in myotubes continuously exposed for more than 1 h. ACOX enzymatic activity followed mRNA changes: it was doubled after 1 h treatment and remained elevated for 24 h. ERK and $cPLA_2$ pathway is involved, since their inhibitors abrogated the ACOX mRNA induction. Myotubes counteract the resulting oxidative insult by catalase and glutathione peroxidase activation, thus removing H_2O_2 at the expenses of the reduced glutahione pool. *Conclusions* The present study shows that acute, but not chronic, leptin treatment of C2C12 myotubes induces ACOX expression. Peroxisomal fatty acid oxidation may work together with mitochondrial β-oxidation to remove excessive lipids from non-adipose tissues, during early stages of overnutrition and before development of leptin resistance.

Key words C2C12 myotubes – leptin – acyl CoA oxidase – antioxidant systems

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R. Ceci · S. Sabatini · G. Duranti
Dept. of Human Movement and Sport Sciences
IUSM-University of Sport and Movement Sciences
Rome, Italy

I. Savini · L. Avigliano · Dr. A. Rossi (✉)
Dept. of Experimental Medicine & Biochemical Sciences
University of Rome Tor Vergata
Via Montpellier 1
00133 Rome, Italy
Tel.: +39-6/7259-6466
Fax: +39-6/7259-6468
E-Mail: arossiit@yahoo.com

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Introduction

Leptin, a hormone mainly produced by the adipose tissue, is an important factor involved in a negative feedback loop regulating energy homeostasis [11]. While originally leptin action was linked to hypothalamus on appetite centers, it is now accepted that it stimulates a wide range of physiological responses also at peripheral level. Leptin, during a period of overfeeding, exerts protective effects against lipotoxicity in non-adipose tissues [20]. Skeletal muscle represents about 40% of total body mass and is a major sink for circulating glucose; furthermore, it takes up significant quantities of plasma fatty acids. An excessive deposition of lipids in this tissue seems to be correlated to the presence of insulin resistance [9]. Since this metabolic defect is widespread in western countries, we studied the mechanism(s) by which leptin helps lipid removal from muscle cells. Leptin acts on skeletal muscle stimulating fatty acid β-oxidation, through activation of the AMP-activated protein kinase (AMPK), thus avoiding triglycerides overloading [14]. Moreover, it has been recently reported that in mouse C2C12 muscle cells, leptin activates Peroxisome proliferator-activated receptors (PPARs), via ERK and $cPLA_2$ pathway [3]. PPARs are nuclear hormone receptors, whose activation brings about transcriptional induction of genes encoding for peroxisomal β-oxidation enzymes, including acyl-CoA oxidase (ACOX) [16]. Peroxisomal enzymes shorten very long-chain fatty acids, allowing their subsequent mitochondrial β-oxidation. Here, we investigated the target of the ERK-$cPLA_2$-PPARs pathway, by acute and chronic leptin supplementation in C2C12 muscle cells. Moreover, we evaluated if H_2O_2 generated by ACOX could alter the cellular redox state and induce antioxidant adaptive responses.

Materials and methods

Cell cultures

The mouse C2C12 myoblasts (American Type Culture Collection, Rockville, MD, USA) were grown and differentiated as already described [4]. After differentiation, cells were treated with leptin, with or without inhibitors, from 10 min to 48 h. For the 48 h treatment, the medium was replaced after 24 h and leptin readded. In order to exclude leptin degradation within 24 h treatments, we tested the medium collected from the cells (at 1 and 24 h) to treat new C2C12 cells. As biomarker we used ACOX mRNA induction, leptin was still able to elicit the same response.

The inhibitors used were: 25 μM $AACOCF_3$ (Calbiochem, San Diego, CA, USA), a cytosolic phospholipase A_2 ($cPLA_2$) inhibitor, added 60 min before leptin treatment and 30 μM PD-98059 (Biomol, Plymouth Meeting, PA, USA), an extracellular signal-regulated kinase (ERK) inhibitor, added during leptin (Sigma Chemical, St. Louis, MO, USA) treatment.

RT-PCR

Total RNA was isolated, reverse transcribed and PCR-amplified as described [4]. Amplified products were visualized by ethidium bromide staining. The primers used were: mACOX-forward 5′-TGACCCCAAGAC CCAAGAGTTC-3′ and mACOX-reverse 5′-AAAGTG GAAGGCATAGGCGG-3′. The amplification parameters were as follows: 94°C 40 s, 56°C 40 s and 72°C 40 s for 23 cycles.

Amplification of GAPDH mRNA was performed as loading control. The primers were: mGAPDH-forward 5′-TCGGTGTGAACGGATTTGGCCG-3′ and mGAPDH-reverse 5′-ATGCAGGGATGATGTTCTGGG CTGC-3′. The amplification parameters were as follows: 94°C 40 s, 56°C 40 s and 72°C 1 min for 25 cycles.

Glutathione content

Intracellular glutathione content was quantified according to Anderson [2]. GSSG was selectively measured in samples where GSH was masked by pretreatment with 2-vinylpyridine.

Enzymatic activities

Manganese (MnSOD) and copper/zinc (Cu,Zn SOD) superoxide dismutase activities were determined according to Crapo et al. [6]. The specific inhibition of Cu,Zn SOD by 9 mM potassium thiocyanate allows MnSOD determination by the same procedure. Catalase, glutathione peroxidase (GPx) and ACOX activities were determined according to Aebi [1], Del Maestro & McDonald [7] and Small et al. [19] respectively.

Statistics

Statistical analysis was conducted with the program KaleidaGraph 3.6 per Macintosh (Synergy Software, Reading, PA, USA). Comparisons between untreated and leptin-treated cells were evaluated by using ANOVA, followed by Bonferroni post hoc test.

Results and discussion

In this paper, we aim to show that ACOX represents one of the transcriptional outcomes of leptin's path-

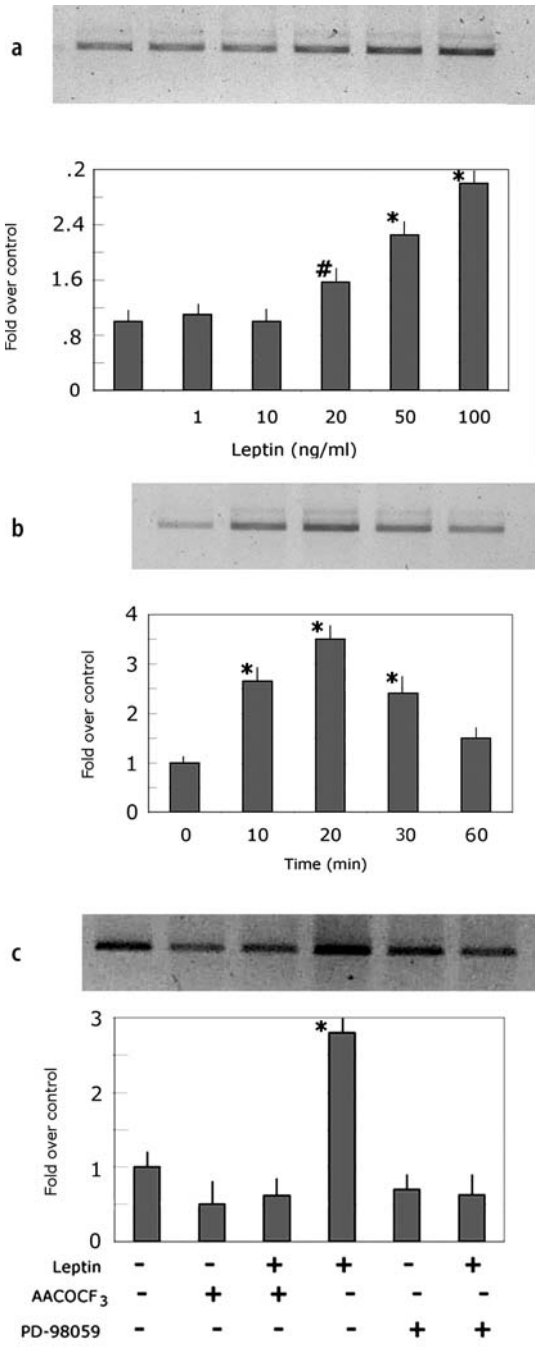

Fig. 1 (**A**) Stimulation of ACOX mRNA by leptin: dose-dependent effect. C2C12 myotubes were incubated with different concentrations of leptin for 20 min. (**B**) Time course of ACOX mRNA expression. C2C12 myotubes were treated with leptin (100 ng/ml) for the times indicated. (**C**) Inhibition of leptin-induced ACOX mRNA expression by PLA_2 inhibitor ($AACOCF_3$) and ERK inhibitor (PD-98059). C2C12 myotubes were treated with leptin (100 ng/ml, 20 min), alone or in combination with $AACOCF_3$ (25 μM, added 60 min before leptin) or PD-98059 (30 μM, added together with leptin). All samples were analyzed by RT-PCR and band intensities are represented as histograms. GAPDH were used as loading control. Intensities were evaluated as optical densities units and expressed as fold changes relative to the control (no leptin). In each panel, there is a representative gel with an histogram showing the mean ± S.E.M. of three separate experiments. They were analysed by ANOVA, followed by Bonferroni post hoc test $^{\#}P < 0.05$, $^{*}P < 0.001$

way. Leptin affects ACOX mRNA expression in a dose- and time-dependent manner. Induction of ACOX mRNA expression starts at 20 ng/ml (1.8 fold), a dose comparable to that found in hyperleptinemic subjects, and reaches a maximum level at 100 ng/ml, (Fig 1, panel A). Time-course analysis demonstrates that high levels of leptin causes the highest ACOX mRNA induction after 20 min, followed by a decrease to the basal levels within 1 h (Fig. 1, panel B). Time-course of the ACOX mRNA induction paralleles the reported $cPLA_2$ phosphorylation [3].

Since mouse ACOX promoter contains a PPARs response element at position –298, we investigated the pathway leading to PPARs activation [13]. As shown in Fig. 1 panel C, PD98059, an ERK inhibitor, and $AACOCF_3$, a $cPLA_2$ inhibitor, completely abrogates the mRNA induction due to leptin treatment, with time and dose previously found to be maximal (20 min, 100 ng/ml). Thus, leptin up-regulates the ACOX gene through activation of the ERK-$cPLA_2$ pathway.

The ACOX mRNA upregulation is followed by an increase in enzymatic activity, which doubles after 1 h, continues to increase up to 24 h and returns to basal levels after 48 h (Table 1).

We suggest that, during the first step of overfeeding, high levels of leptin have a protective effect preventing lipid accumulation in muscle, through activation of both AMP kinase [14] and ACOX. The latter is the rate-limiting enzyme in peroxisomal oxidation and its activation can contribute to lower the intra-myocellular lipid accumulation, by shortening long chain fatty acids, thereby directing them into the oxidative pathway. The short-lived effect on ACOX expression could be due to a desensitization of

Table 1 Effect of leptin supplementation on antioxidant systems

	0	1 h	24 h	48 h
Cu–Zn SOD[a]	2.4 ± 0.2	2.3 ± 0.2	2.4 ± 0.2	2.4 ± 0.2
Mn SOD[a]	2.0 ± 0.2	2.1 ± 0.2	2.1 ± 0.2	2.2 ± 0.2
Catalase[b]	1.7 ± 0.1	3.4 ± 0.15*	2.8 ± 0.12#	1.6 ± 0.1
GPx[c]	0.9 ± 0.04	0.36 ± 0.03#	1.9 ± 0.1*	0.8 ± 0.03
ACOX[d]	4.1 ± 0.4	7.9 ± 0.5#	10 ± 0.9#	3.9 ± 0.4
Glutathione[e]	8.5 ± 0.5	8.0 ± 0.5	4.1 ± 0.3#	7.0 ± 0.4
GSSG/GSH (%)	14.8 ± 0.9	13.8 ± 0.9	24.8 ± 1.0#	14.0 ± 0.8

[a]U/mg of protein. One unit of SOD activity is defined as the amount of the enzyme required to inhibit the rate of reduction of cytochrome c by 50% in a coupled system with xanthine and xanthine oxidase
[b]One unit of Catalase activity is defined as the amount of the enzyme decomposing 1 mmol of H_2O_2 per min
[c]mU/mg of protein. One unit of GPx activity is defined as the amount of the enzyme catalyzing the oxidation by tert-butylhydroperoxide of 1 mmol of GSH per min
[d]U/mg of protein. One unit of ACOX activity is defined as the amount of the enzyme required to oxidize 1 nmole of leuco-2,7-dichlorofluorescein per min
[e]nmol/mg of protein
Data are means ± S.E.M. of three independent experiments, each performed in triplicate. Data were analysed by ANOVA with Bonferroni post hoc test $^{\#}P < 0.05$, $^{*}P < 0.001$

leptin receptor. Desensitization of leptin receptor by chronic leptin stimulation has been recently reported in human embryonic kidney and rat myotubes [8, 10] trough rapid induction of suppressor of cytochine-3 (SOCS3) protein expression. High SOCS3 protein levels are found in muscle cells of type-2 diabetic patients [17] and in adipocytes of obese rats [18], suggesting its involvement in leptin resistance observed in these subjects.

Differently to mRNA induction, protein activity persists at higher levels for at least 24 h allowing lipids removal within muscle cells. After 24 h of leptin treatment both ACOX expression and activity are back to basal level. Therefore we suppose that overfeeding state, could result in a permanent desensitization of leptin receptor leading to leptin resistance in obese subjects.

Increasing fatty acid oxidation and ACOX activity can lead to reactive oxygen species generation, and H_2O_2 generated in peroxisomes can leakage into the cytoplasm [15], therefore altering the cellular redox state. We found that myotubes are able to counteract the oxidative insult, by increasing antioxidant defense systems. In early steps of leptin supplementation, catalase activity doubles (Table 1), accordingly with the presence of a PPAR responsive element in the catalase promoter [12]. At the same time, GPx decreases, as previously reported in liver of rats treated with hypolipidemic drugs [5]. However, after 24 h an enhanced GPx activity is observed. Late activation of GPx (Table 1) helps to remove H_2O_2 at the expenses of GSH, which represents the most versatile antioxidant molecule. Indeed, Table 1 shows that the GSSG-to-GSH ratio increased by about 2-fold after 24 h. These results reflect a coordinated adaptive response to leptin-mediated H_2O_2 generation; during all the time, ACOX activity is elevated and muscle cells coped with oxidative stress. Leptin does not affect Cu–Zn SOD or Mn SOD activities at any time of supplementation, indicating that these enzymes are not involved in the adaptive response to peroxisomal fatty acid oxidation. When the exposure to leptin is prolonged up to 48 h, the phenomenon is fully abrogated, since all the examined parameters are comparable to untreated cells.

Alltogether, these data suggest that early stages of hyperleptinemia observed during overnutrition protect peripheral tissues from lipid accumulation, increasing fatty acid oxidation and carrying out an adaptive response to increased oxidative stress.

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