Leptin controls the fate of fatty acids in isolated rat white adipocytes

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Abstract

Leptin directly increases the rate of exogenous glucose and fatty acids oxidation in isolated adipocytes. However, the effects of leptin on fatty acid metabolism in white adipose tissue have not been examined in detail. Here, we report that in adipocytes incubated for 6 h in the presence of leptin (10 ng/ml), the insulin-stimulated *de novo* fatty acid synthesis was inhibited by 36% ($P < 0.05$), while the exogenous oxidation of acetic and oleic acids was increased by 50% and 76% respectively. Interestingly, leptin did not alter the oxidation of intracellular fatty acids. Leptin-incubated cells presented a 16-fold increase in the incorporation of oleic acid into triglyceride (TG) and a 123% increase in the intracellular TG hydrolysis (as measured by free fatty acids release). Fatty acid–TG cycling was not

affected by leptin. By employing fatty acids radiolabeled with ³H and ¹⁴C, we could determine the concomitant influx of fatty acids (incorporation of fatty acids into TG) and efflux of fatty acids (intracellular fatty acids oxidation and free fatty acids release) in the incubated cells. Leptin increased by 30% the net efflux of fatty acids from adipocytes. We conclude that leptin directly inhibits *de novo* synthesis of fatty acids and increases the release and oxidation of fatty acids in isolated rat adipocytes. These direct energy-dissipating effects of leptin may play an important role in reducing accumulation of fatty acids into TG of rat adipose cells.

Journal of Endocrinology (2002) **175**, 735–744

Introduction

Leptin has been implicated in body weight regulation through its central effects on the hypothalamus (Friedman & Halaas 1998). However, the presence of the leptin receptor (OB-R) in peripheral tissues intimately involved in the intermediary metabolism, such as skeletal muscle, adipocytes, pancreatic islets and liver (Luoh *et al.* 1997), indicate that these tissues are also targets for the action of leptin. In the early pair feeding studies, it was demonstrated that leptin has weight reducing effects in excess of those mediated through its central nervous system action (Levin *et al.* 1996). Subsequently, Barzilai *et al.* (1999) observed that animals previously treated with leptin present an increase in glucose uptake and glycogen synthesis in peripheral tissues during insulinemic clamp as compared with food-restricted littermates.

We have previously demonstrated that leptin increases glucose uptake and decarboxylation and glycogen synthesis in incubated soleus muscle (Ceddia *et al.* 1999c). Leptin also inhibits glycogenolysis and gluconeogenesis in isolated hepatocytes (Ceddia *et al.* 1999a) and modulates insulin secretion by perfused pancreatic islets (Ceddia *et al.* 1999b). Chronic exposure (15 h culture) of isolated adipocytes to leptin has been reported to increase basal and

insulin-induced glucose decarboxylation and to inhibit insulin-stimulated glucose uptake and incorporation into lipids (Müller *et al.* 1997, Ceddia *et al.* 1998). Leptin seems to up-regulate Krebs' cycle activity in these cells, as could be concluded by an increase in acetic acid oxidation and citrate synthase maximal activity (Ceddia *et al.* 2000).

In spite of the increasing interest in the direct actions of leptin on adipocytes, its possible effects on the metabolism of fatty acids in these cells have not been fully characterized. Previous studies suggest that, at least in rodents, these actions do occur and might play an important role in the overall weight reducing effect of leptin. Evidence for a paracrine/autocrine effect of leptin on lipolysis (i.e. glycerol release) of adipose tissue has been obtained from *in vivo* (Frühbeck *et al.* 1998) and *in vitro* (Frühbeck *et al.* 1997) studies. Zhou *et al.* (1999) demonstrated that adenovirus-induced hyperleptinemia leads to an increase in the expression of enzymes of fatty acid oxidation, such as acyl-CoA oxidase (ACO) and carnitine palmitoyl transferase 1 (CPT), accompanied by a complete depletion of triglyceride (TG) in adipose tissues without a concomitant increase in the concentration of circulating free fatty acids (FFA) (Shimabukuro *et al.* 1997, Zhou *et al.* 1999). Such a phenomenon was attributed to a putative increase in intracellular fatty acid oxidation produced by leptin

(Shimabukuro *et al.* 1997). The same research group reported the disappearance of ectopic denervated adipose tissue using the same hyperleptinemic animal model, suggesting that leptin has a direct effect on these cells (Wang *et al.* 1999c). Furthermore, in incubated isolated adipocytes, leptin up-regulates the expression of ACO, CPT, uncoupling protein 2 (UCP-2) and peroxisome proliferator activated receptor alpha (PPAR- α), all of which are involved in lipid oxidation (Wang *et al.* 1999a, Ceddia *et al.* 2000).

Recently, it has been proposed that leptin at supra-physiological concentrations is unable to cross the blood-brain barrier so as to further increase its central nervous system levels and action (Wang *et al.* 1999b). Nevertheless, fat mass is still reduced in animals made hyperleptinemic by adenovirus infection. According to these studies, there must be a mechanism by which leptin prevents fat accumulation independently of the hypothalamus (Wang *et al.* 1999b). The elucidation of these mechanisms might be important for the future use of exogenous leptin to treat human obesity, a condition where central resistance to leptin is thought to occur (Friedman & Halaas 1998).

White adipose tissue is estimated to store 95% of total body TG and plays a central role in the development of obesity (Coppack *et al.* 1994). White fat cells express leptin receptors and are metabolically responsive to this hormone. Therefore, the aim of the present study was to investigate the effects of leptin on fatty acid metabolism in isolated adipocytes. The following parameters were measured in incubated cells in the presence of leptin: *de novo* fatty acid synthesis from glucose, comparison of long chain (oleic acid) and short chain (acetic acid) exogenous fatty acid oxidation, incorporation of oleic acid into TG, fatty acid-TG cycling, intracellular fatty acid oxidation and TG hydrolysis. The net influx and efflux of fatty acids were also determined. Evidence for a direct peripheral effect of leptin on adipose tissue reducing fat accumulation is provided.

Materials and Methods

Experimental animals

Male lean albino rats (Wistar strain) weighing 150–180 g were maintained on a 12 h light/12 h darkness cycle at 22 °C and fed a standard laboratory chow which was available *ad libitum*. Ethics approval was granted for these studies by the Institute of Biomedical Science, Animal Experimental Committee, University of São Paulo.

Cell isolation and washing procedures

White adipocytes were isolated as previously described (Rodbell 1964). Briefly, rats were killed by cervical dislocation, the epididymal fat pads were removed under

sterile conditions and pooled. Isolated cells were obtained by shaking (150 orbitals per min) finely minced tissues (2–4 g) in 4 oz sterile polypropylene containers at 37 °C for 15–30 min in 10 ml DMEM containing Hepes (25 mM), type II collagenase (1.25 mg/ml) and 4% BSA. Cells were then filtered through nylon mesh and washed three times in Krebs-Ringer sodium bicarbonate buffer (KRB). Cell density (volume of cells/volume of cell suspension) was determined as previously described (DiGirolamo *et al.* 1971).

Cell incubation

After washing, 1 ml cell suspension (10% lipocrit, $4\text{--}5 \times 10^5$ cells) was incubated for 6 h, at 37 °C, in 20 ml plastic scintillation flasks in KRB saturated with carbogen (O₂/CO₂, 95/5%), pH 7.4, containing defatted BSA (1.0–1.5%) and specific additions according to the metabolic parameter to be determined (Table 1). Leptin 10 ng/ml (Amgen Inc., Thousand Oaks, CA, USA) and/or insulin 10 000 µU/ml (Sigma Chemical Co., St Louis, MO, USA) were added where indicated. In all experiments where oleic acid was used (radiolabeled or not), it had previously been complexed with defatted BSA (2:1 ratio) by overnight incubation and vigorous shaking at 37 °C (Ceddia *et al.* 2000).

De novo fatty acid synthesis

After the incubation period, 0.3 ml H₂SO₄ (4 M) was added to the incubation medium and lipids were extracted with isopropanol/n-heptane/H₂SO₄ (4:1:0.25, v/v/v) as previously described (Dole & Meimertz 1960). The lipid phase was separated and dried, resuspended in ethanolic KOH solution (1 ml saturated KOH per 100 ml 95% ethanol) and saponified for 1 h at 37 °C with vigorous shaking. Lipids were then re-extracted with hexane. The radioactivity present in this fraction was determined and represents the incorporation of [U-¹⁴C]D-glucose into the fatty acid moiety of TG (Rodbell 1964).

[1-¹⁴C]Acetic and [1-¹⁴C]oleic acid oxidation

The 20 ml scintillation flasks for incubation of adipocytes had a centered-isolated well containing a loosely folded piece of filter paper moistened with 0.2 ml 2-phenylethylamine/methanol (1:1, v/v). After the 6-h incubation period, the medium was acidified with 0.3 ml H₂SO₄ (4 M), the flasks were maintained sealed and were incubated for a further 60 min. At the end of this period, the radioactivity trapped in the filter paper was determined and represents the conversion of [1-¹⁴C]acetic acid/[1-¹⁴C]oleic acid into ¹⁴CO₂ (Ceddia *et al.* 2000).

Incorporation of [1-¹⁴C]oleic acid into TG

After the 6-h incubation period, 0.3 ml H₂SO₄ (4 M) was added to the incubation medium and lipids were extracted

Table 1 Additions to the Krebs–Ringer sodium bicarbonate buffer (KRB) for the incubation of adipocytes. Cells were isolated as described in Materials and Methods and incubated for 6 h in KRB containing cold and radiolabeled substrates according to the metabolic measurement to be performed

	Glucose (mM)	[U- ¹⁴ C]D-glucose (μCi/ml)	Acetic acid (mM)	[1- ¹⁴ C]acetic acid (μCi/ml)	Oleic acid (mM)	[1- ¹⁴ C]oleic acid (μCi/ml)	³ H ₂ O (mCi/ml)	[9, 10- ³ H (N)]oleic acid (μCi/ml)
Metabolic measurements								
De novo fatty acid synthesis	5.6	0.5	—	—	—	—	—	—
Acetic acid oxidation	—	—	2	0.2	—	—	—	—
Oleic acid oxidation	—	—	—	—	0.9	0.3	—	—
Oleic acid incorporation into TG	0, 5.6	—	—	—	0.9	0.3	—	—
Fatty acid–TG cycling	5.6	—	—	—	—	—	2	—
Intracellular fatty acids metabolism	5.6	—	—	—	—	—	—	—
Fatty acid fluxes	—	—	—	—	0.9	—	—	0.6

with isopropanol/n-heptane/H₂SO₄ (4:1:0.25, v/v/v) as previously described (Dole & Meirneitz 1960). The lipid phase was separated and dried, resuspended in chloroform/methanol (2:1, v/v) and submitted to thin layer chromatography for lipid fractions separation. The mobile phase consisted of a hexane/diethyl ether/acetic acid solution (70:30:1, v/v/v) (Homem de Bitencourt & Curi 1998). The radioactivity present in the TG fraction of each sample was then determined.

Fatty acid–TG cycling

After the incubation period, 0.3 ml H₂SO₄ (4 M) was added to the incubation medium and lipids were extracted with an isopropanol/n-heptane/H₂SO₄ (4:1:0.25, v/v/v) mixture as previously described (Dole & Meirneitz 1960). The lipid phase was separated and dried, resuspended in ethanolic KOH solution (1 ml saturated KOH per 100 ml 95% ethanol) and saponified for 1 h at 37 °C with vigorous shaking. Lipids were then re-extracted with hexane. The radioactivity present in the ethanolic and hexane phases was determined and represents the ³H-glycerol moiety of TG and the ³H-fatty acid moiety of TG synthesized from glucose respectively (Rodbell 1964). It was assumed, for the calculation of TG and fatty acid synthesis, that the specific activity of water in the adipocyte was identical to the one in the incubation flask and that each glycerol incorporated into TG contained 3.3 atoms of tritium and that each fatty acid incorporated into TG contained 13.3 atoms of tritium (Jungas 1968). The rate of incorporation of free fatty acids into TG is three times the rate of incorporation of glycerol phosphate into TG; this results from the fact that three fatty acids are esterified to one glycerol phosphate molecule to form one TG molecule. Fatty acids incorporated into TG were either newly synthesized from glucose (and thus are ³H-radiolabeled) or derived from intracellular TG breakdown (and thus are not ³H-radiolabeled and represent fatty acid–TG cycling). Therefore, fatty acid–TG cycling can be calculated by the following equation (Brooks *et al.* 1982, Tagliaferro *et al.* 1990):

$$\text{Cycling} = [3 \times (^3\text{H-glycerol synthesis})] - [(^3\text{H-fatty acid synthesis})]$$

Radiolabeling of adipocytes

For the study of intracellular fatty acids metabolism and fluxes, cells were radiolabeled with [1-¹⁴C]oleic acid before the 6-h incubation period. After isolation and washing, cells were incubated for 1 h, at 37 °C, in 50 ml sterile polypropylene tubes, with cells floating on top (forming a thin cell layer) of 25 ml DMEM containing Hepes (20 mM), fetal bovine serum (5%), glucose (8.7 mM), penicillin/streptomycin (20 U/ml/20 mg/ml), and [1-¹⁴C]oleic acid (100 μCi/ml) previously complexed

with defatted BSA (1.5%). After the radiolabeling period, cells were washed three times with KRB and incubated as described in a previous section (Cell incubation).

An aliquot of these cells was separated before incubation and submitted to lipid extraction with isopropanol/*n*-heptane/H₂SO₄ (4:1:0.25, v/v/v). Thin layer chromatography was performed and the radioactivity present in each lipid fraction was determined (Dole & Meirneitz 1960, Homen de Bitencourt & Curi 1998). The majority of [1-¹⁴C]oleic acid was incorporated into triglyceride (70%). Polar lipids (mono and diacylglycerol) accounted for 20% of the intracellular radiolabeled lipids. The remaining 10% of the [1-¹⁴C]oleic acid incorporated was found in the free fatty acid and phospholipid fractions. Therefore, medium contamination with unwashed [1-¹⁴C]oleic acid was assumed to be negligible.

Since the majority of [1-¹⁴C]oleic acid was incorporated into TG, the radioactivity present in CO₂ or medium's free fatty acid measured during the subsequent incubation was considered to be mainly derived from intracellular TG. Previous studies have provided evidence that, at least during fasting, intracellular TGs are metabolized in a non-specific pattern; i.e. the five most abundant fatty acids in adipose tissue (palmitic, palmitoleic, stearic, oleic and linoleic) are mobilized from TG in the same proportion as their original content in TG molecules (Stein & Stein 1962). Therefore, the metabolism of intracellular [1-¹⁴C]oleic acid incorporated into TG was assumed to be representative of the metabolism of all fatty acids esterified to TG inside the cell.

Intracellular fatty acid metabolism

Previously radiolabeled adipocytes (average of 140 000 c.p.m./4–5 × 10⁵ cells/flask) were incubated for 6 h in 20 ml scintillation flasks with the additions to the medium as described in Table 1. After the 6-h incubation period, the medium was acidified with 0.3 ml H₂SO₄ (4 M), the flasks were maintained sealed and incubated for a further 60 min. At the end of this period, the radioactivity trapped in the filter paper was determined. For the analysis of intracellular lipid hydrolysis (i.e. TG hydrolysis, mainly), adipocytes from the same pool were incubated for 6 h in plastic test tubes and periodically agitated. At the end of the incubation period, the tubes were centrifuged so as to separate the cells from the incubation medium. An aliquot of the supernatant was separated for lipid extraction and thin layer chromatographic analysis (as described above). The radioactivity present in the free fatty acid fraction was determined and represents TG hydrolyzed and released to the incubation medium. Non-specific free fatty acid released from the cells (due to cell lysis or unwashed [1-¹⁴C]oleic acid contamination) was determined and discounted for each sample by rapid centrifugation and lipid extraction from the medium at time zero of incubation.

Fatty acid fluxes

Incubation of adipocytes was performed as described for the study of intracellular fatty acids metabolism, except for the absence of glucose in the incubation medium and the addition of cold (0.9 mM) and labeled (0.6 µCi/ml) [9, 10-³H (N)]oleic acid (Table 1). This procedure allowed us to study the influx of [³H]oleic acid and efflux of [¹⁴C]oleic acid in the same cell pool. After the 6-h incubation period, the incorporation of ¹⁴C radioactivity into ¹⁴CO₂ and into medium free fatty acids was determined, as well as the incorporation of [9, 10-³H (N)]oleic acid into TG, as described in the previous sections.

For the calculation of fatty acid influx and efflux of the adipocyte in absolute values (nmol/10⁵ cells), the specific activity of [1-¹⁴C]oleic acid in the intracellular compartment was determined. Since [¹⁴C]oleic acid was mainly incorporated into TG, ¹⁴C radioactivity present in medium free fatty acids and CO₂ was assumed to be derived 100% from intracellular TG. The intracellular [¹⁴C]oleic acid incorporated was considered to be representative of the whole fatty acid moiety of intracellular TG. As discussed in the section above, adipocytes seem to mobilize intracellular TG in a non-specific pattern (Stein & Stein 1962). Therefore, the metabolism of incorporated [¹⁴C]oleic acid reflects all intracellular TG's fatty acids mobilized from control and leptin-treated cells. The specific activity of [1-¹⁴C]oleic acid (i.e. ¹⁴C-fatty acids) in the intracellular compartment of the adipocytes was determined as follows: an aliquot of the cell's lipid extract obtained before incubation was separated, dried, resuspended in reaction medium and the total TG content was measured using Merck's Ecoline 25 Triglyceride kit (Merck, Brazil) for plasmatic determinations (McGowan *et al.* 1983). Since each TG molecule contains three fatty acids, multiplying the intracellular TG concentration by 3 assessed the intracellular amount of fatty acids esterified to TG. This value was used for the estimation of intracellular dilution of [¹⁴C]oleic acid and, therefore, for the calculation of its specific activity in the intracellular milieu. The flux of fatty acids was then determined by the following equations:

Fatty acid influx =
incorporation of [9, 10-³H (N)]oleic acid into TG

Fatty acid efflux = (incorporation of [1-¹⁴C]oleic acid equivalents into ¹⁴CO₂) + (incorporation of [1-¹⁴C]oleic acid equivalents into medium free fatty acids)

Statistical analysis

Statistical analysis was performed by one-way ANOVA with Tukey-Kramer multiple comparison test or Student's unpaired *t*-test. The level of significance was set at *P* < 0.05.

Figure 1 Effect of insulin, leptin and leptin plus insulin on *de novo* fatty acid synthesis from glucose in adipocytes. Control=no leptin/no insulin; Ins 10000=insulin 10 000 μ U/ml; Lep 10=leptin 10 ng/ml; L10 I10000=leptin 10 ng/ml plus insulin 10 000 μ U/ml. Data are expressed as means $\pm$ S.E.M. of 10 determinations; * $P<0.05$ vs Ins 10000.

Results

Effect of insulin, leptin and insulin plus leptin on de novo fatty acid synthesis

In the presence of 10 ng/ml leptin, no significant alteration was observed in the basal incorporation of [U - 14 C]D-glucose into the fatty acid moiety of TG in isolated adipocytes. Insulin at a supraphysiological concentration (10 000 μ U/ml) elicited a 117% increase in *de novo* lipogenesis. However, this effect of insulin was significantly reduced by 36% when the adipocytes were incubated in the presence of both insulin and leptin (Fig. 1).

Effect of leptin on [1 - 14 C]acetic and [1 - 14 C]oleic acid oxidation

Leptin (10 ng/ml) increased [1 - 14 C]acetic acid oxidation by 50% in isolated adipocytes (Fig. 2A). Under the same incubation conditions (i.e. in the absence of glucose or insulin in the incubation medium), leptin stimulated exogenous oleic acid oxidation to a greater extent than that

observed for the short chain fatty acid, reaching up to 76% higher values of [1 - 14 C]oleic acid conversion to 14 CO $_2$ as compared with control (Fig. 2B).

Effect of glucose, leptin and insulin on [1 - 14 C]oleic acid incorporation into TG

As expected, the addition of glucose (5.6 mM) to the incubation medium led to a 2- to 6-fold increase in the incorporation of [1 - 14 C]oleic acid into TG in both the control and the stimulated groups (insulin 10 000 μ U/ml or leptin 10 ng/ml). In the absence of glucose, insulin (10 000 μ U/ml) elicited an 11-fold increase in TG synthesis as compared with control. Leptin (10 ng/ml), however, up-regulated the flux of exogenous FFA to TG to a greater extent than insulin, promoting TG synthesis values 17-fold higher than the unstimulated group. In the presence of glucose (5.6 mM), the stimulating effects of insulin and leptin were maintained: insulin (10 000 μ U/ml) and leptin (10 ng/ml) enhanced the incorporation of [1 - 14 C]oleic acid into TG by 11 and 7 times respectively (Fig. 3).

Effect of insulin, leptin and insulin plus leptin on fatty acid-TG cycling

As previously described (Brooks *et al.* 1982), insulin (10 000 μ U/ml) did not alter fatty acid-TG cycling in adipocytes. No effect of leptin (10 ng/ml) or insulin plus leptin could be detected on this metabolic parameter (Table 2).

Effect of leptin on intracellular fatty acid metabolism

Experiments were carried out in the presence of glucose (5.6 mM) and in the absence of oleic acid in the incubation medium. Under these conditions, leptin (10 ng/ml) was unable to modify the oxidation of fatty acids arising from the hydrolysis of intracellular TG in isolated

Figure 2 Effect of leptin on (A) [1 - 14 C]acetic acid oxidation and (B) [1 - 14 C]oleic acid oxidation in adipocytes. Control=no leptin/no insulin; Lep 10=leptin 10 ng/ml. Data are expressed as means $\pm$ S.E.M. of 10 determinations. * $P<0.05$ compared with Control.

Figure 3 Effect of insulin and leptin on [1-¹⁴C]oleic acid incorporation into TG in adipocytes in the absence or presence of glucose (5.6 mM). Control=no leptin/no insulin; Ins 10000=insulin 10 000 µU/ml; Lep 10=leptin 10 ng/ml. Data are expressed as means ± S.E.M. of 4 determinations. **P*<0.05 compared with respective Control groups. #*P*<0.05 compared with respective Ins 10000 groups.

Table 2 Effect of insulin, leptin and leptin plus insulin on fatty acid–TG cycling in adipocytes. Control=no leptin/no insulin. Data are expressed as means ± S.E.M. of 10 determinations

Group	Fatty acid–TG cycling (nmol/10 ⁵ cells)
Control	63.94 ± 5.37
Insulin (10 000 µU/ml)	49.28 ± 11.96
Leptin (10 ng/ml)	55.06 ± 4.56
Leptin (10 mg/ml)+insulin (10 000 µU/ml)	55.25 ± 2.70

adipocytes (Fig. 4A). However, it is noteworthy that leptin increased by 123% the release of free fatty acids to the incubation medium, reflecting a stimulation of TG hydrolysis (Fig. 4B).

Effect of leptin on fatty acid fluxes

Experiments were carried out in the absence of glucose and in the presence of oleic acid (0.9 mM) in the incubation medium. Under these conditions, no differences were observed in the oxidation of intracellular fatty acids between the control and the leptin-treated groups (Fig. 5B). Leptin (10 ng/ml) stimulated free fatty acid release to the incubation medium by 38% (Fig. 5C). As expected leptin also raised – by 2.8-fold – the incorporation of exogenous [9, 10-³H (N)]oleic acid into TG in these cells (Fig. 5A). Under these incubation conditions, the efflux of fatty acids exceeded the influx in both control and leptin-treated cells. However, leptin (10 ng/ml) exerted a more pronounced efflux- than influx-stimulating effect (considering absolute values, in nmol/10⁵ cells of fatty acids metabolized), thus resulting in higher net fatty acid efflux as compared with control (145.6 nmol/10⁵ cells versus 111.6 nmol/10⁵ cells respectively) (Fig. 5D).

Discussion

Treatment of mice with leptin alters the morphology and function of white adipose tissue, resulting in dramatic body fat reduction. Such changes include atrophy of adipocytes, shrinkage of intracellular lipid droplets, increased number of mitochondria and, under higher circulating leptin concentrations, complete disappearance of TG and even of the white adipose tissue itself (Sarmiento *et al.* 1997, Shimabukuro *et al.* 1997). Two possible direct mechanisms of leptin action on adipocytes may explain these *in vivo* observations: decreased TG synthesis (either *de novo* fatty acid synthesis and/or fatty acid esterification into TG) and increased TG breakdown (either fatty acid oxidation and/or fatty acid exportation to non-adipose cells).

In the present study, leptin (10 ng/ml) was found to decrease insulin-stimulated *de novo* fatty acid synthesis

Figure 4 Effect of leptin on (A) intracellular ¹⁴C-fatty acid oxidation and (B) intracellular ¹⁴C-TG hydrolysis in adipocytes. Control=no leptin/no insulin; Lep 10=leptin 10 ng/ml. Data are expressed as means ± S.E.M. of 5 determinations. CPM index represents the relation between sample c.p.m./intracellular TG c.p.m. × 1000. **P*<0.05 compared with Control.

Figure 5 Effect of leptin on (A) [9, 10-³H (N)]oleic acid incorporation into TG, (B) intracellular ¹⁴C-fatty acid oxidation, (C) intracellular ¹⁴C-TG hydrolysis and (D) fatty acid flux in adipocytes. Control=no leptin/no insulin; Lep 10=leptin 10 ng/ml. Data are expressed as means $\pm$ S.E.M. of 5 determinations. * $P < 0.05$ compared with Control.

from glucose in adipocytes *in vitro*. These results are in accordance with earlier findings that adipocytes cultivated for 15 h with leptin present an impairment of insulin-stimulated glucose transport and formation of glycogen and TG (Müller *et al.* 1997, Ceddia *et al.* 1998). In these latter studies, however, no distinction was made as to whether leptin inhibited the synthesis of glycerol or of the fatty acid moieties of TG, since only the incorporation of glucose into total lipids was determined (Ceddia *et al.* 1998). Our findings demonstrate that leptin down-regulates *de novo* fatty acid synthesis, which depends on the rate limiting enzymes acetyl-CoA carboxylase (ACC) and fatty acid

synthase (FAS). These enzymes may be down-regulated by leptin, since it decreases the incorporation of acetic acid into lipids (Ceddia *et al.* 2000). Acetic acid is converted into acetyl-CoA by acetyl-CoA synthase, and from then on to malonyl-CoA and fatty acids by ACC and FAS respectively. Leptin has also been shown to inhibit ACC and FAS mRNA expression in adipocytes *in vivo* (Zhou *et al.* 1999) and *in vitro* (Wang *et al.* 1999a) and this could be the mechanism by which *de novo* lipogenesis was decreased in the present study. Furthermore, it has also been reported previously that malic enzyme and ATP-citrate lyase, which are also important enzymes for fatty

acid synthesis, are down-regulated by leptin in isolated adipocytes (Fukuda & Iritani 1999, Ceddia *et al.* 2000).

In our study, leptin stimulated acetic acid and oleic acid oxidation by 50% and 76% respectively. Acetic acid is a short chain fatty acid and is metabolized to CO_2 by its conversion to acetyl-CoA followed by entry into the Krebs' cycle. The oxidation pathway of oleic acid, a long chain fatty acid, is different, since it must first be activated in the cytosol, transported across the mitochondrial membrane (via CPT) and then converted to CO_2 by the enzymes of fatty acid oxidation and the Krebs' cycle (Greville & Tubbs 1968). Previous studies have demonstrated that leptin stimulates Krebs' cycle activity in adipocytes and skeletal muscle, leading to increased glucose and fatty acid oxidation in these tissues (Muio *et al.* 1997, Ceddia *et al.* 1999c, 2000). Such effects have been attributed to a possible increase in UCP expression/activity caused by leptin, which in turn would decrease the intracellular ATP/ADP and NADH/NAD⁺ ratios activating the enzymes of the Krebs' cycle (Ceddia *et al.* 1999c). In fact, it has been reported that leptin raises the expression of UCP-2 and the activity of citrate synthase in isolated adipocytes (Ceddia *et al.* 2000). The results of the present study, however, suggest that it is not only the Krebs' cycle activity that is stimulated by leptin, because the increment found in oleic acid oxidation (76%) exceeded the one of acetic acid oxidation (50%) under the same incubation conditions. Therefore, leptin may also activate CPT and/or the enzymes of long chain fatty acid oxidation (such as ACO) in the isolated cells as well. Up-regulation of CPT and ACO expression in adipocytes has been reported following *in vivo* leptin treatment (Zhou *et al.* 1999). Furthermore, CPT activity can be allosterically down-regulated by malonyl-CoA (McGarry 1998). Since leptin inhibits the fatty acid synthesis pathway, the intracellular malonyl-CoA concentration could be lower in these cells, leading to increased CPT activity and higher long-chain fatty acid oxidation rates.

As mentioned before, the oxidation of either [¹⁴C]oleate or [¹⁴C]acetate added to the incubation medium was significantly increased in adipocytes exposed to leptin. However, when cells were allowed to incorporate [¹⁴C]oleate into TG and then were subsequently incubated with leptin, despite the increase in the rate of lipolysis, no difference was found for ¹⁴CO₂ production between leptin-incubated adipocytes and controls. One possible explanation is that specific radioactivity of the intracellular TG pool of the adipocytes was too low and the experimental method would not be sensitive enough to detect a possible increase in ¹⁴CO₂ production induced by leptin. However, it seems unlikely that the methodology used would fail to detect changes in intracellular fatty acid oxidation while being able to identify an increase in TG hydrolysis induced by leptin, since the amount of radioactivity recovered as ¹⁴C-free fatty acids in the incubation medium (which represents TG hydrolysis) in the control

group was only three times higher than the radioactivity recovered as ¹⁴CO₂ (c.p.m. index/10⁵ cells of 0.71 ± 0.16 versus 0.23 ± 0.03 respectively) (Fig. 4). Based on these results, it seems that fatty acids entering isolated adipocytes are metabolized differently from fatty acids derived from the hydrolysis of the intracellular pool of TG in leptin-incubated cells. Differential intracellular free fatty acid mobilization in adipocytes has previously been described by Edens *et al.* (1990). These authors have shown that fatty acids arising from intracellular TG hydrolysis must first leave the adipocyte and then re-enter the cell in order to be re-esterified into TG. In fact, in the last decades, several authors have provided evidence for the existence of metabolically distinct free fatty acid pools inside the adipose cell (Dole 1961, Vaughan *et al.* 1964). According to the results herein presented, leptin seems to favor exogenous fatty acid oxidation, while intracellular fatty acids derived from TG hydrolysis are preferentially released to the extracellular compartment. This could be a mechanism by which leptin improves fatty acid exportation from adipocytes to other tissues, such as skeletal muscle and liver, where a substantially higher amount of this substrate may be oxidized.

According to our data, leptin increased TG synthesis from oleic acid to an extent comparable with that of supra-physiological levels of insulin. At first, it seemed paradoxical that leptin, often considered an anti-obesity hormone, would favor TG synthesis in isolated adipocytes. We subsequently tested if the increased TG synthesis from oleic acid induced by leptin could be a result of the stimulation of fatty acid-TG cycling. Substrate cycling exists when two opposing non-equilibrium reactions are simultaneously active. They contribute to energy expenditure (through facultative thermogenesis), because energy utilization is required to drive both reactions (Tagliaferro *et al.* 1990). Cycling is also thought to increase sensitivity of controlling substrate flux through a metabolic pathway (Tagliaferro *et al.* 1990). In adipocytes, glucagon and noradrenaline stimulate fatty acid-TG cycling (Brooks *et al.* 1982). By this mechanism, intracellular fatty acids derived from TG hydrolysis are re-esterified into TG in a futile reaction, resulting in ATP consumption. If cells with an accelerated fatty acid-TG cycling were exposed to exogenous [1-¹⁴C]-labeled fatty acid, an increase in its incorporation into TG would be expected to occur, since both TG synthesis and breakdown reactions would be active. However, according to our results, leptin failed to up-regulate fatty acid-TG cycling.

Since stimulation of fatty acid-TG cycling could not account for the increase in the incorporation of [1-¹⁴C]oleic acid into TG induced by leptin treatment of adipocytes, we investigated whether leptin altered fatty acid fluxes through these cells, i.e. leptin stimulated fatty acid incorporation into TG but also enhanced TG breakdown (fatty acid oxidation and/or exportation) to a greater extent, contributing therefore to an adipose reducing

effect. Our first approach to this problem was to assess whether leptin stimulated intracellular TG breakdown. We performed experiments where isolated adipocytes had their intracellular TG previously labeled with [$1\text{-}^{14}\text{C}$]oleic acid and were then incubated with leptin. At 10 ng/ml, leptin did not increase the intracellular fatty acid oxidation, as previously mentioned. However, these cells presented an increase in free fatty acids release to the incubation medium. These results are in accordance with previous studies that evidenced a lipolytic effect of leptin in isolated adipocytes (Frühbeck *et al.* 1997, Wang *et al.* 1999a). Although free fatty acids release was not measured in those experiments, adipocytes incubated with leptin showed a higher glycerol release rate, which is compatible with an increase in TG hydrolysis observed in the present study. We then incubated previously ^{14}C -labeled adipocytes in a medium containing [$9, 10\text{-}^3\text{H}$ (N)]oleic acid in the presence and absence of leptin so as to measure, concomitantly and in absolute values, the amount of fatty acids incorporated into intracellular TG and the amount of intracellular fatty acids released as CO_2 or free fatty acids (fatty acid fluxes). The net fatty acid efflux of adipocytes incubated with leptin exceeded that of controls in 34 nmols/ 10^5 cells (30%). Therefore, although leptin increases the incorporation of oleic acid into intracellular TG, it increases intracellular TG breakdown and free fatty acids release to a greater extent, promoting a higher net fatty acid efflux than that which is observed in the control group.

Animals made hyperleptinemic by adenovirus infection have their adipose tissue depleted by a mechanism independent of the central nervous system (Wang *et al.* 1999b). Since no elevation in the circulating concentration of free fatty acids of these animals is observed, it has been assumed that leptin induces intracellular fatty acid oxidation (Shimabukuro *et al.* 1997). Furthermore, isolated white adipocytes incubated with 20 ng/ml leptin present an increase in glycerol release to the incubation medium without a proportional elevation in release of free fatty acids, indicating that probably the fatty acids are oxidized inside the adipocyte following TG hydrolysis (Wang *et al.* 1999a). It should be noted, however, that the oxidation of intracellular lipids in adipocytes stimulated by leptin has never been directly evaluated. Our results indicate that leptin does enhance fatty acid oxidation in white adipocytes. However, contrary to what has been inferred by *in vivo* observations and glycerol release experiments (Shimabukuro *et al.* 1997, Wang *et al.* 1999a), this effect is only observed with fatty acids taken up from the extracellular milieu. On the other hand, leptin modulates fatty acid fluxes in the white adipose tissue, leading to TG hydrolysis and release of fatty acids to the extracellular space. By extrapolating our *in vitro* data to *in vivo* situations, we could postulate that the main direct effect of leptin on fatty acid metabolism of white adipose cells is to enhance TG breakdown and offer free fatty acids to other tissues

where they can be oxidized at higher rates, rather than stimulating its degradation inside the adipocyte, which is a mitochondria-poor cell type. The fact that rodents do not present a rise in the levels of circulating free fatty acids when exposed to hyperleptinemia (Shimabukuro *et al.* 1997) does not rule out the fatty acids flux shifting effects of leptin. In fact, these animals have higher levels of expression of enzymes of fatty acid oxidation in non-adipose cells (Zhou *et al.* 1997) and no alterations in the circulating free fatty acid concentrations should be expected if these tissues could oxidize increasing amounts of circulating fatty acids in a similar proportion to their increased release from the adipose tissue. Muoio *et al.* (1997) have recently demonstrated that skeletal muscles incubated with leptin present a higher exogenous fatty acid oxidation rate, which corroborates the idea of increased lipid oxidation in non-adipose cells stimulated by leptin. Furthermore, in a recent trial involving nine patients with lipodystrophy, who often present severe deficiency of adipose cells, hypoleptinemia, hypertriglyceridemia, reduced insulin sensitivity and increased liver volume due to steatohepatitis, leptin replacement significantly reduced food intake, body weight, serum TG levels and insulin resistance. Liver volume was also lowered in the leptin-treated patients, possibly because of reduction of local TG stores. From the analysis of one patient, the authors suggest that the effects of leptin on TG metabolism are independent of decreased food intake (Oral *et al.* 2002). These results are consistent with the hypothesis that leptin induces TG breakdown in non-adipose cells, including the liver.

We propose therefore that, *in vivo*, leptin alters fatty acid flux in adipocytes, stimulating both TG synthesis and breakdown. As a result, free fatty acids are exported to non-adipose cells (such as skeletal muscle and liver), where they can be oxidized at a higher rate. *De novo* lipogenesis is inhibited by leptin, whereas exogenous fatty acid oxidation is enhanced. This peripheral mechanism of action described herein could contribute to the putative physiological role of leptin in lean animals preventing obesity development.

Acknowledgements

The authors are indebted to the technical assistance of J R Mendonça, G de Souza and C K Miyasaka. We would like to thank Amgen Inc., Thousand Oaks, CA, USA for kindly supplying leptin. This study was supported by FAPESP, CNPq, CAPES, and PRONEX (168/97).

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Received in final form 12 August 2002

Accepted 14 August 2002