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## Oleoyl-estrone inhibits lipogenic, but maintains thermogenic gene expression of brown adipose tissue in overweight rats

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**Short running title:** Oleoyl-estrone effects on overweight rat BAT

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### Synopsis

We intended to determine how brown adipose tissue (BAT) maintained thermogenesis under treatment with oleoyl-estrone (OE), a powerful slimming hormone that sheds off body lipid but maintaining the metabolic rate. Overweight male rats were subjected to daily gavages of 10 nmol/g OE or vehicle (controls) for 10 days. A pair-fed vehicle-receiving group (PF) was used to discount the effects attributable to energy availability limitation. Interscapular BAT weight, lipid, DNA, mRNA and the RT-PCR expression of lipid and energy metabolism genes for enzymes and regulatory proteins were measured. BAT weight and lipid decreased in OE and PF; the latter showing a marked reduction of tissue mRNA. Maintenance of perilipin gene expression in PF and OE rats despite the loss of lipid suggests the preservation of the vacuolar interactive surface, a critical factor for thermogenic responsiveness. OE (and to a lower extent PF) maintained the expression of genes controlling lipolysis and fatty acid oxidation, but markedly decreased those of lipogenic and acyl-glycerol synthesis. OE did not affect UCP1 (decreased in PF),  $\beta_3$  adrenergic receptors or hormone-sensitive lipase gene mRNAs, which may translate in maintaining a full thermogenic system potential. OE rats were able to maintain a less energetically stressed BAT (probably with the concurrence of glucose utilization) than PF animals. These changes were not paralleled in PF rats, which lower thermogenesis and glucose preservation resulted in a heavier toll on internal fat stores. Thus, the mechanism of action of OE is more complex and tissue-specific than previously assumed.

### Key words:

brown adipose tissue; energy metabolism; lipogenesis; oleoyl-estrone; thermogenesis; UCP1

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## Introduction

Oleoyl-estrone (OE) is a powerful slimming signal from adipose tissue that elicits both central and peripheral energy wasting effects [1,2], and has been postulated as a ponderostat signal [3]. OE decreases food intake without significant changes in energy expenditure [1]; the resulting energy gap is fulfilled essentially by lipid mobilization [1,3] in white adipose tissue (WAT). This lipid is mainly used by muscle, at least in obese rats [4], decreasing hyperlipidemia and inducing a marked drop in circulating cholesterol [5]. Under OE treatment, glycemia is maintained in the range of normalcy, but with lower insulin and leptin [5].

The marked WAT energy wasting induced by OE cannot be justified by increased catecholamine sensitivity [6], or increased stimulation of expression of key regulators of lipolytic pathways [4], but essentially by powerful inhibition of lipogenesis [7], which results in an unbalanced lipid equilibrium favoring the massive release of fatty acids and glycerol. This selective voiding of fat reserves is complemented by a lowered peripheral utilization of glucose [8], which reinforces the decreased food intake, partly induced by the inhibition of ghrelin expression in the stomach, and central effects on the brain [2] not mediated by neuropeptide Y. The relative availability of glucose can be attested by the accumulation of liver glycogen [1] in spite of the –partially– maintained hepatic conversion of glucose into triacylglycerols which, together with those coming from WAT increase the liver lipoprotein output in order to fuel the muscle and other peripheral tissues (unpublished data).

Brown adipose tissue (BAT) is a particular type of adipose tissue that contains all the basic metabolic machinery of WAT, complemented by a powerful oxidative mitochondrial apparatus and the unique presence of a functional UCP1 [9], as well as a tighter control of both blood flow and direct stimulation of cells by sympathetic nerves [10]. The apparent “quietness” of BAT during the energy turmoil elicited by OE may be the consequence of unchanged stimulation of thermogenesis, which concurs with energy wasting elsewhere. This peculiarity has been tested in the present study compared with a situation in which the limited energy intake (pair-feeding) theoretically exerts the same metabolic pressure on the rat body energy budget management, independently of OE treatment. The effects of OE are quite different in liver and WAT, but BAT could not be readily compared to neither model because of the unique function of this tissue in thermogenesis, at least in rodents.

## Materials and Methods

Adult male Wistar rats were made overweight by a limited period of cafeteria diet feeding, as previously described [11]. The rats, initially weighing  $355 \pm 5$  g, were kept under housing and feeding standard conditions. Three groups of 8 rats each were randomly selected: controls (C), oleoyl-estrone (OE) and pair fed (PF). All animals received every day an oral gavage of 0.2 mL of sunflower oil (7 kJ), which was supplemented in the OE group with 10 nmol/g oleoyl-estrone (OED, Barcelona, Spain). The controls and OE had free access to pellet food (maintenance chow, Panlab, Barcelona, Spain); and the PF were allowed every day only the amount of food consumed by the OE group; all rats had water available *ad libitum*. PF rats completely ate the food allotted each day. On day 10, the rats were killed and the interscapular BAT was rapidly excised, weighed, sampled, frozen and kept at  $-80^{\circ}\text{C}$  until processed.

The animals were kept, handled and killed following the procedures approved by the University of Barcelona Animal Welfare and Ethics Committee, in full compliment of the norms and procedures set forth by the European Union and the Governments of Spain and Catalonia.

Tissue samples were used for the estimation of total lipid by extraction with trichloromethane-methanol [12]. DNA was measured using a standard fluorimetric method with 3,5-diaminobenzoic acid (Sigma, St Louis MO USA), and bovine DNA (Sigma) as standard [13]. Tissue DNA content allowed the calculation of the approximate number of cells per g of tissue and in the whole interscapular BAT pad, based on the assumption that in mammals the cell DNA content is constant; we used the genomic DNA size data [14] for somatic rat cells (5.6 pg/cell). Mean cell weight was estimated from the number of cells and the organ weight.

Total tissue RNA was extracted using the Tripure reagent (Roche Applied Science, Indianapolis IN USA), and were quantified in a ND-100 spectrophotometer (Nanodrop Technologies, Wilmington DE

USA). RNA samples were reverse transcribed using the MMLV reverse transcriptase (Promega, Madison, WI USA) and oligo-dT primers. Tissue total mRNA was determined by using the poly-(A)mRNA detection system kit (Promega).

Real-time PCR (RT-PCR) amplification was carried out using 10  $\mu$ L amplification mixtures containing Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA USA), equivalent to 8 ng of reverse-transcribed RNA and 300 nM primers. Reactions were run on an ABI PRISM 7900 HT detection system (Applied Biosystems) using a fluorescent threshold manually set to OD 0.500 for all runs. The primers used for the estimation of gene expression in interscapular BAT are presented in Supplemental Table 1 of the Supplemental online material.

A semiquantitative approach for the estimation of the concentration of specific gene mRNAs per unit of tissue weight was used as previously described [15]. In any case, cyclophilin was used as charge control gene in all samples. The data have been presented as the amount of copies (femtomoles) found in the whole interscapular BAT mass, as an indication of the potentiality for the synthesis of these specific proteins in an organ which size changed with treatment. This may allow for comparisons between groups in a more direct way than simple comparison of relative expressions.

Statistical comparison between groups was established by using the unpaired Student's t test with a limit of significance of  $P < 0.05$  by applying the Prism-5 program (Graphpad Software, La Jolla CA USA).

## Results

At the end of the study, and related to their initial weights, control rats gained  $2.6 \pm 0.4$  % (NS vs. initial values), pair fed lost  $8.5 \pm 0.7$  % ( $P < 0.05$  vs. initial values and control's gains) and OE rats lost  $9.9 \pm 0.7$  % ( $P < 0.05$  vs. initial values and control's gains). Table 1 presents the nucleic acid, and cell contents of the interscapular BAT of overweight male rats treated with OE and compared with controls and a pair-fed group. OE treatment resulted in the loss of about 40 % of whole BAT lipids compared with controls, whereas PF rats lost 57 %, the differences being significant versus controls but not between OE and PF groups. Both OE and PF treatments resulted in the loss of about one third of the tissue mass, but with no parallel loss of cells. In any case, the RNA/DNA ratio decreased, which suggests a diminished cell function overall. However, the cells of the OE group maintained the same amount of mRNA per cell than controls; PF cell mRNA levels were about half of those of controls and OE. The loss of tissue weight and the maintenance of cell number resulted in smaller mean cell sizes (the results include both adipocytes and non-adipocyte cells) for OE and PF rats compared with controls.

Figure 1 shows the changes, induced either by pair-feeding or OE treatment, on the expression of a number of energy handling and control-related genes, presented as percentages of the corresponding control values. OE and pair-feeding induced a similar marked decrease in the expression of GLUT4 and hexokinase, and OE decreased that of NADPH-producing malic enzyme. PF raised the expression of phospho-enolpyruvate carboxykinase and pyruvate dehydrogenase kinase 4, both versus controls and OE rats. Supplemental Tables 2 and 3 show, in absolute terms, i.e. femtomols in the whole interscapular BAT mass, the expressions of the genes presented in Figure 1 in order to allow for more quantitative comparisons.

OE induced a marked decrease in the expression of lipogenic enzyme genes, a decrease that was less extensive in PF rats (which even showed an increased expression of acetyl-CoA carboxylase 2 versus controls). OE induced a dramatic decrease in the expression of stearoyl-CoA desaturase, which was unaffected in PF. Glycerol-phosphate acyl-transferase gene expression was decreased in both groups compared with controls. Fatty acid transport, translocation, and binding enzyme gene expressions were not altered by either treatment, the exception being a decreased expression of fatty acid transport protein by OE (unaffected by PF) and lower expression of fatty acid binding protein 3 in PF rats, which was not altered by OE. Beta-oxidation enzyme acyl-CoA dehydrogenase isoenzyme gene expressions were unaffected by OE and PF, except for decreased medium-chain dehydrogenase expression in PF rats.

With the exception of a considerable decrease in the expression of the adiponutrin gene (in both OE

and PF), gene expressions for lipases were unaffected by OE, and only lipoprotein lipase expression was enhanced by pair-feeding. Perilipin expression was unchanged in both OE and PF rats versus controls.

The expression of the UCP1 gene was decreased in pair-fed rats compared with controls, but this did not occur in those treated with OE. The expression of  $\beta_1$  adrenergic receptor gene was decreased in both OE and PF rats, but that of  $\beta_3$  was unchanged, and the  $\beta_2$  was considerably higher in PF rats compared with controls and OE rats. No differences were observed in the expression of phosphodiesterase in comparison to controls. Estrogen receptor  $\alpha$  gene more than doubled its expression in PF rats, but OE and controls showed similar values. Neither treatment changed the expression of thyroid hormone receptor gene, but both decreased the expression of thyroxine deiodinase.

OE decreased the expressions of the genes for PPAR $\gamma_1$ , and its coactivator 1 $\alpha$ , as well as the expression of phosphatase and tensin homolog, nuclear respiratory factor 1, ChREBP and the Spot 14/thyroid hormone-responsive protein genes, of which PF rats only followed suit in the last two factors; in PF rats the expression of the PPAR $\alpha$  gene was increased.

## Discussion

Under normal conditions, perilipin levels (and gene expression) are assumed to be related to the vacuole size (surface) [16]; and BAT contains multiple vacuoles per cell that facilitate the rapid mobilization of the fat. The lack of change of perilipin expression despite shrinking cell size due to the observed loss of fat (and maintenance of cell numbers) resulted in the following values for perilipin mRNA in fmol/g of tissue lipid:  $148 \pm 20$  (controls),  $309 \pm 22$  (PF), and  $207 \pm 17$  (OE), the differences of PF versus controls and OE being significant. Expression of perilipin in PF was actually increased when compared with total tissue mRNA, resulting in remarkably similar expressions in all three groups studied, irrespective of the much lower lipid content and smaller mean cell size of PF and OE. The data on fat and cell number indicate that the mean lipid vacuole size must be smaller in OE and PF rats than in controls in spite of the other differences in metabolism. However, unchanged perilipin expression, is probably correlated to perilipin levels' maintenance, and to unchanged vacuolar lining surface area, roughly proportional to perilipin content [16]; consequently, total surface area of vacuoles could not follow the vacuolar shrinking consequence of the loss of cell fat. This means that the total number of vacuoles must be increased to pack a lower volume of fat with a closely similar estimated surface area in PF and OE rats than in controls, thus preserving the cytoplasm/fat droplet interface area and consequently the responsiveness to hormonal stimulation. Unchanged expression of the hormone-sensitive lipase gene agrees with this interpretation. The data presented indirectly hint at the maintenance of the overall interactive surface of the BAT vacuole being an important factor in the thermogenic-response capability of the tissue.

The loss of fat (and thus postulated droplet fractionation) was more marked in PF than in OE rats, which somehow were able to maintain a much less energetically stressed tissue (probably with the concurrence of glucose utilization) than PF animals. This buffered effect of energy loss in OE rats is again exemplified by the marked difference in total tissue mRNA content between controls and OE rats on one side and PF on the other, which agrees –in BAT– with our previous findings that OE effects on energy balance are not mediated simply by a decreased dietary energy availability [17]. However, this lower mRNA content in PF rats is in full agreement with the decreased thermogenesis and BAT function [18] of food-deprived animals. Lowered mRNA levels are an index of decreased protein synthesis, in itself a normal mechanism of decreasing energy expenditure through slower protein turnover [19]. The loss of lipids in PF tends to be higher than in OE rats, the latter maintaining UCP levels and full mRNA (i.e. protein synthesis) complement, which means that the flow of energy substrates to and from BAT should be different for PF and OE rats. This is supported by the differences in lipoprotein lipase gene expression, but not by the other lipases and most fatty acid transportation / translocation enzyme gene expressions. It must be borne in mind, however, that the presence of the same amount of a given specific mRNA in PF rat's BAT represents a much higher "selective effort" of translation than in OE or control animals, which hints at the relative importance for the tissue of the maintenance of the corresponding path with respect to all others. This way we can

observe that lipase and  $\beta$ -oxidation operation, as well as 3C fragment preservation through activation of pyruvate dehydrogenase kinases are important paths that seem to deserve this special treatment in food restriction.

The effects of limited food availability and OE treatment on the cellularity, cell size and mass of lipid reserves, of BAT were apparently similar. The analysis of the expression of the main genes controlling lipid and energy metabolism seems to confirm this initial appreciation, but the effects induced by OE were deeper than those of simple food restriction (pair feeding), since lipogenesis gene expression was more markedly inhibited in OE than in PF rats.

The postulated reduction of NADPH synthesis in OE rats (malic enzyme) suggest a lower synthetic drive [20]; this is further stressed by a much decreased stearoyl-desaturase expression, not observed in PF rats, and the differentially more marked decrease in the expression of lipogenic enzyme genes in OE animals compared with both controls and PF. The marked decrease in adiponutrin expression in OE and PF supports a probable inhibition of lipogenesis, given the nature of adiponutrin as marker of lipid biosynthesis [21] in addition to reflecting decreased food intake.

Glucose uptake and phosphorylation seem to be inhibited in both OE and PF animals because of the decreased expressions of GLUT4 and hexokinase 2. However, 3-carbon fragment utilization through the pyruvate dehydrogenase pathway was probably increased in OE and decreased in PF rats (lower kinase 2 gene expression in OE and higher kinase 4 in PF).

The potentiality to dispose of internal fat stores remained unchanged in all three groups, as was  $\beta$ -oxidation (showing only a decrease in the medium-chain acyl-CoA dehydrogenase gene expression of PF rats). Possible incorporation of lipid from lipoproteins was unchanged in OE rats, since the decreased activity found in WAT [5] was not paralleled in BAT by the expression (unchanged) of lipoprotein lipase. In PF rats, lipoprotein lipase expression actually increased, which suggests that circulating lipids may continue to be taken up for energy supply under food restriction [22]. The differential expression of fatty acid binding protein 3 [23] and fatty acid transport protein 1 genes [24] in PF and OE rats may be directly related to this different potential use of circulating lipoprotein lipid, since the latter is closely related to fatty acid transport into the cell [25].

The raised expression of the PPAR $\alpha$  gene in PF versus controls, and its lack of differences in OE rats agree with maintained lipolytic activity of OE rats. The more lipogenic PPAR $\gamma$  [26] expression was –again– strongly inhibited by OE, since PPAR $\gamma$  activation increases both lipid and adipose tissue masses [27].

Lipid synthesis is partially controlled in adipose tissue by the insulin signaling cascade [28] through modulation of SREBP1c [29] and ChREBP [30]. Both factors have been found to play a key role in the OE-stimulated liver lipogenesis [7], which is in part fueled by glucose in spite of the generalized body lipid mobilization and decreased food intake. In the present study, however, SREBP1c remained unchanged in both PF and OE rats, even when the Spot 14/thyroid hormone-responsive protein, closely related to SREBP1c action [31] –and also a marker of lipogenesis– was inhibited, as was that of ChREBP. These results suggest that OE modulation of lipid metabolism did not proceed along the same paths in liver and BAT.

The possibly most far-reaching “metabolic” difference, however, between PF and OE animals was the relative maintenance of the expression of UCP1 in OE, compared with its significant fall in PF. This finding is in agreement with the known maintenance of energy expenditure under OE treatment [1], that contrasts with its decrease under conditions of starvation or food restriction.

The maintenance of unchanged adrenergic path ( $\beta_3$  receptors, phosphodiesterase, hormone sensitive lipase and maintained vacuolar surface area) and UCP-1 ensures the full operativity of the thermogenic response to eventual adrenergic stimuli in OE rats. These arrangements are less complete or, probably, efficient in pair-fed animals. Decreased adrenergic receptor expression is often a consequence of saving strategies under conditions of limited energy availability; the differential expression of UCP1 in OE and PF rats contrasts with the lack of change in  $\beta_3$  adrenergic receptors that control UCP-1 expression [32]. Reduced expression of UCP1 in PF rats may be related to the effects of estrogen signaling, which reduces the sensitivity to catecholamine stimulation in BAT [33] as a way to decrease thermogenic responsiveness. However, the less marked increase of estrogen receptor

expression in OE rats may be either a consequence of increased circulating estrogen of OE rats [34] that may help downregulate the receptor's expression, or another way of maintaining untouched the ability of the BAT thermogenic system to respond to hormonal signals.

The data presented on gene expression suggest that in PF rats, the BAT energy needs are low; and thus a limited glucose availability (very low 3C fragment conversion into acetyl-CoA), was translated into decreased fat stores despite external lipid utilization. However, in the OE rats, this same overall energy scarcity was compounded by the need to fuel thermogenesis, which poses an additional burden to BAT, and can explain why glucose utilization (in spite of assumedly limited entry) may be increased, and why lipid synthesis is strongly limited in contrast with  $\beta$ -oxidation, only slightly decreased. The success of this OE-oriented strategy is proven by both the little change in metabolic orientation, maintenance of thermogenesis and lower overall loss of lipid. Since the main difference in PF and OE rats seems to lie on the regulation of pyruvate dehydrogenase (and a stronger inhibition of lipid synthesis in OE) through differential gene expression, it can be speculated that the continuous availability of glucose in OE rats [5,8] may be a key factor in the preservation of part of BAT lipid and the full operativity of thermogenesis.

Pair-feeding changed little the expression of growth and differentiation factors compared with controls, in deep contrast with OE-treated rats. The expression of the phosphatase and tensin homolog, implicated in cell survival [35], as well as the PPAR $\gamma$  coactivator 1 $\alpha$  [36] and the nuclear respiratory factor 1 genes [37] related to mitochondriogenesis, were inhibited by OE, but not by pair feeding. However, neither the vascular endothelial growth factor A, an angiogenic factor [38], or the mitochondriogenic transcriptional factor A [39] gene expressions were altered by OE (nor by PF), suggesting little changes in tissue architecture in parallel to the loss of tissue mass and fat (but not cells). In any case, the effects of OE were more marked than those of pair-feeding. The better maintenance of cell development factors' expression by PF rats disagree with the decrease in UCP expression, presumably an index of thermogenic capability. The cell changes cannot be either attributed directly to changed thyroid receptor regulation, in spite of the important thyroidal control of BAT development and function. The decrease in thyroxine deiodinase expression may be a direct consequence of energy scarcity [40].

In spite of some differences in the control of tissue control factors, the limits set to the synthesis of fat, management of its stores, use of glucose, and maintenance of thermogenic potential conform the bulk of changes induced by OE on BAT. These changes were not paralleled in PF rats, which used a lower thermogenesis and glucose-preservation strategy that takes a heavier toll on internal fat stores. The effects of OE on BAT energy metabolism are, thus, fairly different from those observed in liver, which suggests that the mechanism of action of OE is more complex and tissue-specific than previously assumed.

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**Legend to Figure**

Figure 1 - Specific mRNA for enzyme and regulatory protein expression in the interscapular brown adipose tissue mass of overweight male Wistar rats treated with OE or pair fed to these.

The values represent the content of specific gene mRNA in the whole IBAT mass, and are expressed as percentages of the controls' values (presented in Supplemental Tables 2 and 3), and are the mean  $\pm$  sem of 8 different animals. Pair-fed: white columns, OE: gray columns. Statistical significance of the differences between groups: \* =  $P < 0.05$  versus the control group (100 %); ° =  $P < 0.05$  between OE and PF groups.

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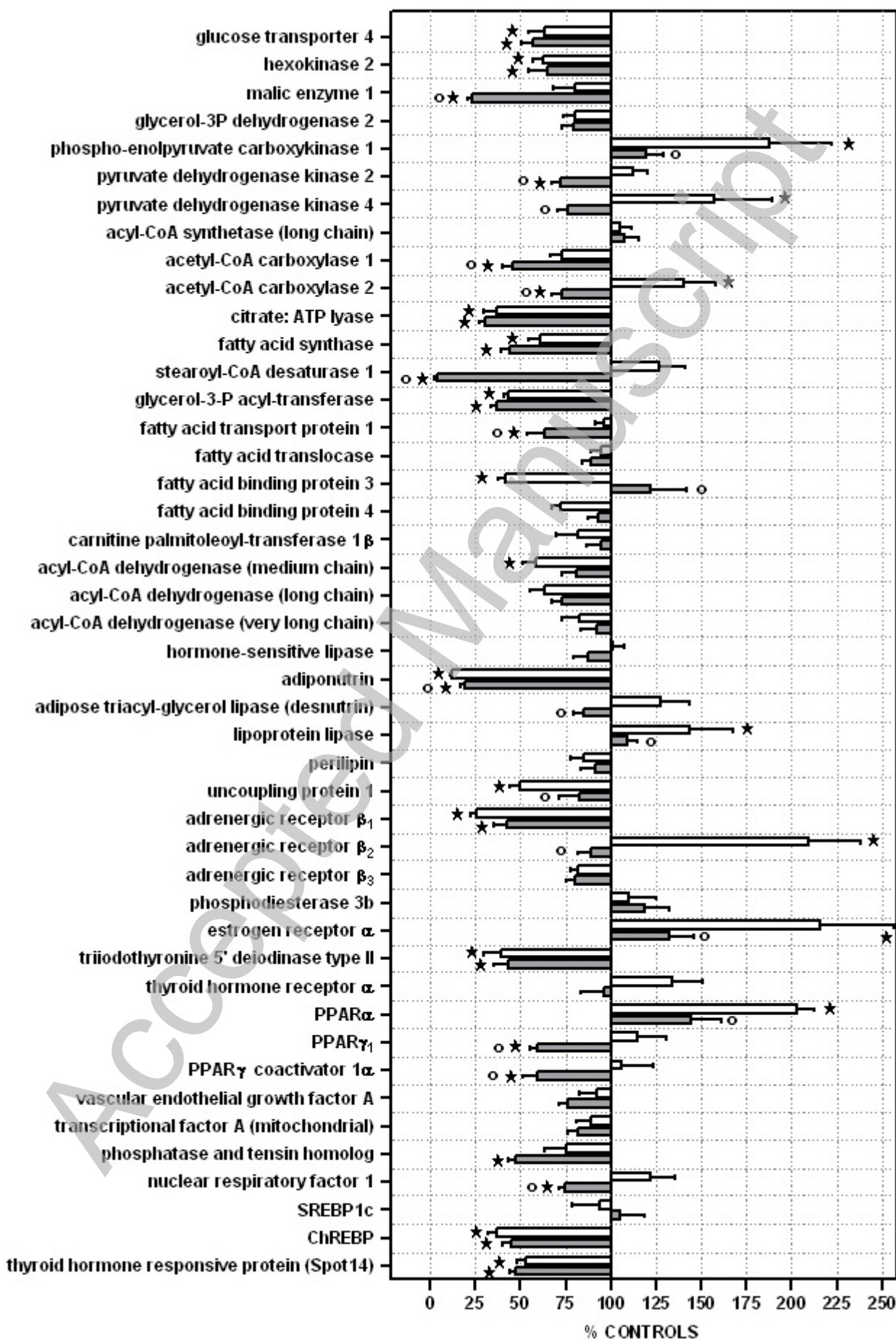
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Table 1 - Nucleic acid content and cellularity in interscapular brown adipose tissue of OE-treated and pair-fed male overweight rats.

IBAT parameter	units	control	pair-fed	OE
tissue weight	mg	551±30	363±29*	379±21*
lipid content	mg/g tissue	569±34	374±64*	497±19
	mg/BAT site	314±18	136±20*	188±16*
DNA content	µg/g tissue	1.57±0.12	2.68±0.12*	2.54±0.12
	mg/BAT site	0.87±0.06	0.94±0.06	0.95±0.06
total RNA content	mg/g tissue	1.05±0.02	1.29±0.04* <sup>o</sup>	1.17±0.03*
	mg/BAT site	0.58±0.03	0.46±0.03*	0.44±0.02*
RNA/DNA ratio	-	0.70±0.06	0.49±0.01*	0.47±0.02*
number of cells	x10 <sup>6</sup> cells/BAT site	155±13	168±9	170±11
mean cell size	ng	3.72±0.30	2.15±0.10*	2.25±0.11*
total mRNA content	µg/g tissue	20.7±2.3	14.5±1.9* <sup>o</sup>	28.3±2.6*
	fg/cell	70.7±8.4	32.3±3.3* <sup>o</sup>	62.5±6.7

The data are the mean±sem of 8 different animals. References of parameter to BAT represent the absolute content in the whole interscapular site BAT mass. Significance of the differences between groups: \* = p<0.05 vs. controls; <sup>o</sup> = p<0.05 of pair-fed vs OE-treated.

Figure 1



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