POSTPRINT:

Life Sciences, 2005; 77: 2051-2058

Combined effects of oleoyl-estrone and a β_3-adrenergic agonist (CL316,243) on lipid stores of diet-induced overweight male Wistar rats

Raquel Ferrer-Lorente, Cristina Cabot, José-Antonio Fernández-López and Marià Alemany

Department of Nutrition and Food Science, Faculty of Biology, University of Barcelona. Barcelona, Spain

AUTHOR ADDRESSES

Raquel Ferrer-Lorente. Department of Nutrition and Food Science; Faculty of Biology, University of Barcelona; Av. Diagonal, 645; E-08028 Barcelona, Spain. e-mail: rferrer@bio.ub.es

Cristina Cabot. Department of Nutrition and Food Science; Faculty of Biology, University of Barcelona; Av. Diagonal, 645; E-08028 Barcelona, Spain. e-mail: ccabot@bio.ub.es

José-Antonio Fernández-López. Department of Nutrition and Food Science; Faculty of Biology, University of Barcelona; Av. Diagonal, 645; E-08028 Barcelona, Spain. e-mail: josfernandez@ub.edu

Marià Alemany (CORRESPONDING AUTHOR:) Department of Nutrition and Food Science; Faculty of Biology, University of Barcelona; Av. Diagonal, 645; E-08028 Barcelona, Spain; tel.: +34934034606; fax: +34934037064; e-mail: malemany@ub.edu

ABSTRACT

Oleoyl-estrone (OE) decreases appetite, induces adipose tissue wasting and resets the ponderostat setting, sparing glucose and protein. The β_3-adrenergic agonists increase energy expenditure and lipolysis. We studied the combination of both treatments to enhance fat mobilisation. Overweight male rats received oral OE for 10 days; they were compared with controls and rats receiving a β_3-adrenergic agonist, CL316,243 (B3A); another group received both OE and B3A. Serum 3-hydroxybutyrate, NEFA, triacylglycerols and glucose showed only slight changes in all groups vs. controls; OE-treated rats showed lower cholesterol. OE decreased food intake, and B3A increased energy expenditure. OE rats lost about 15 %, B3A 24 %, and those receiving both compounds lost 39 % of their initial total body energy. In all cases, most of this energy imbalance was accounted for by the loss of body lipid. The combined treatment of OE and B3A reduced food intake, nevertheless maintaining a high energy expenditure. The combination of a β_3-adrenergic agonist with OE may help compensate the short-lived effects of the agonist and enhance the lipid mobilization action of OE. The eventual combination of both compounds should be explored as a way to obtain faster and more effective ways to treat obesity.

KEY WORDS: obesity; oleoyl-estrone; CL316,243; β_3-adrenergic agonists

INTRODUCTION

Energy balance is maintained by compensating energy expenditure with energy intake; any alteration in that equilibrium affects body energy stores, mainly triacylglycerols. Most treatments of obesity and overweight rely either in decreasing energy intake or increasing energy expenditure, as a way to imbalance the energy equation and force the utilization of fat stores. This process is often hampered by neural and endocrine mechanisms that tend to restore the initial conditions. Oleoyl-estrone (OE) treatment has been found to alter the ponderostat setting (Adán et al., 1999) of rats, inducing the loss of body fat without concurring loss of protein and starvation-like changes in glucose handling (Sanchis et al., 1997), such as those encountered when food intake is reduced (Dietz and Wolfe, 1985). OE is known to decrease appetite (Sanchis et al., 1997; Adán et al., 1999), increase adipose tissue lipid mobilization (Grasa et al., 2001a) and maintain energy expenditure (Sanchis et al., 1996), or –at least– prevent the decrease that often parallels the decreases in energy intake (Shibata and Bukowiecki, 1987).

A number of specific β_3-adrenergic agonists have been developed to stimulate brown adipose tissue thermogenesis (Lipworth, 1966). In general, these agonists increase energy expenditure (Himms-Hagen et al., 1994; Atgié et al., 1998), but their effects are shortly counteracted by glucocorticoids (Fève et al., 1992; Onai et al., 1995), reducing their long-term potential for the treatment of obesity (Fernández-López et al., 2002).

Since the metabolic effects of β_3-adrenergic agonists (increasing energy expenditure, increasing lipolysis) are only partially overlapping (lipolysis) with those of OE (decreasing appetite, resetting the ponderostat, inducing adipose tissue wasting), we studied the possibility of their combined administration on energy balance, in order to magnify the decrease of fat stores.

MATERIALS AND METHODS

Male Wistar rats (Harlan-Interfauna, Sant Feliu de Codines, Spain) of 45 days were used. The rats were maintained at 21-22ºC, 60-50 % relative humidity, and 12h light/dark cycle (on from 08.00) in 3-rat cages, and were fed for five weeks a reduced cafeteria diet (Balada et al., 1997) ad libitum. At the end of this phase, the animals were already overweight (i.e. their weight was at least 15 % higher than the weight corresponding to animals of the same stock and age fed the standard pellet diet). The rats were re-conditioned during an additional week with standard rat chow ad libitum (maintenance chow, Panlab, Barcelona, Spain) as sole food. They were used in the ensuing experiment at this point, when their age was 90 days, and they weighed 330-390 g.

The experimental setup and procedures were approved by the Ethics Committee of the University of Barcelona. All animal handling procedures were carried out following the guidelines established by the EU, and the Spanish and Catalan Governments.

All animals received a daily gavage of 0.2 mL sunflower oil at the beginning of the light cycle, and were maintained under standard housing conditions, with full access to water and food pellets; their weight and food consumption were recorded daily. Four groups of six animals were randomly selected: a) Controls; b) oleoyl-estrone: OE; c) β_3-adrenergic agonist CL316,243 (B3A): B3; and d) oleoyl-estrone and β_3-adrenergic agonist: OE-B3. Groups B3 and OE-B3 were implanted on day 0 subcutaneously in the back with an osmotic Alzet minupump (type 2002, Alzet, Palo Alto CA, USA) under isoflurane anaesthesia; the minipumps were loaded with B3A (β_3 -adrenergic agonist CL316,243; Sigma, St Louis, MO USA) dissolved in saline. The minipumps released B3A at a rate of 0.5 μL/h in the subcutaneous space, at a dose of 1 mg/kg and day. The rats in the OE and OE-B3 groups received a gavage containing OE (OED, Barcelona, Spain) in oil, at a daily dose of 10 μmol/kg. The gavage of rats in the control and B3 groups contained only oil.

The treatments continued for 10 days. At the end of the experiment, the rats were killed by decapitation. Blood was let to clot; the serum was stored at -80ºC until processed. The rats were dissected; the stomach and intestinal contents were removed; the remaining carcass (including the unused blood and packed blood cells) were sealed in polyethylene bags, autoclaved, and thoroughly homogenized (Grasa et al., 2001a). The rat paste was used for the estimation of lipid (Folch et al., 1957), and energy content using an adiabatic bomb calorimeter (C-7000 Ika, Heitersheim, Germany). Paste composition was related to in vivo weight correcting by digestive canal contents. The percentage body composition of controls was used to estimate the absolute lipid and energy content of the rats at the beginning of the experiment, by applying it to their known initial weights. The measured body weight and composition of the rats at the end of the study were used to determine the changes in body size and composition occurred during the 10 days of treatment.

Energy intake was estimated from the food consumed, that contained a metabolisable energy of 13.3 kJ/g. Energy accretion was the difference between estimated energy on day 0 and the measured energy content (bomb calorimeter) on day 10. Mean energy expenditure was estimated as the difference between energy intake and energy accretion.

Blood serum was used for the measurement of glucose (Trinder kit, Sigma, St Louis MO USA), non-esterified fatty acids (NEFA kit, Wako Chemicals, Neuss, Germany), 3-hydroxybutyrate (kit 907979, Roche, Mannheim, Germany), total triacylglycerols (kit 11528, Biosystems, Barcelona, Spain), total cholesterol (Cholesterol reagent easy, Menarini, Firenze Italy), as well as insulin (SRI-13K, Linco, St Charles, MO USA).

Insulin resistance was evaluated using the formulas for the homeostasis model assessment (i.e. the HOMA score) (Bonora et al., 2000; Matthews et al., 2000); this index has been found to be highly correlated in humans with insulin resistance assessed by the euglycemic hyperinsulinemic clamp technique, and is widely used in clinical studies.

Statistical comparisons between groups were established by two-way ANOVA.

RESULTS

Table 1 shows the changes in body weight and composition, and food intake of the four experimental groups. The rats receiving OE alone or combined with B3A decreased significantly their body weight when compared with controls, the maximal loss corresponding to the OE-B3 group, which lost c. 1 % of body weight per day. The energy content and body lipid of the groups OE, B3 and OE-B3 were lower than those of controls. Controls increased their lipid content by only 0.6±5.5 % of the initial lipid; the OE rats lost 17.5±%, the loss of the B3 group was 44.1±% and the combined OE-B3 lost 63.2±3.2 %. The loss of lipid in the OE-B3 group was close to the sum of the lipid lost by the OE and B3 groups.

Food intake of the B3 group was similar to that of controls, however, both groups receiving OE decreased food intake by almost one third.

Table 2 presents the energy balances of the four experimental groups. Energy expenditure was similar in the OE rats and controls, but increased by 47-48 % in the rats receiving B3A: B3 and OE-B3. Energy accretion was not different from 0 in the controls; in the OE group, the rats lost about 15 % of their energy in 10 days, in the B3 lost 24 % and the OE-B3 lost 39 % (Figure 1). The lipid loss accounted for most of the negative energy accretion of the OE, B3 and OE-B3 groups, and completely justified the small energy increase of controls.

Blood glucose slightly increased in OE rats, but decreased in the B3 and B3-OE animals compared with controls (Table 3). B3A treatment induced a small increase in circulating ketone bodies, an effect not observed in OE-treated rats. In spite of the dramatic changes in lipid-energy content of the rats treated with OE and/or B3A, no significant changes were observed in triacylglycerol or non-esterified fatty acids. Cholesterol levels were unaffected by B3A, but in both groups receiving OE, cholesterol levels decreased significantly.

Insulin levels decreased in all treated animals, the decrease being maximal in the rats receiving B3A; similarly, insulin resistance decreased in all the treated groups according to the HOMA scores, the decrease being significant for the groups receiving B3A.

DISCUSSION

The marked lipid mobilizing effects of OE and B3A were clearly observed in the OE and B3 groups, but in the OE-B3 group, the effects appeared to be additive. The combined treatment with OE and B3A reduced food intake, and maintained a high level of energy expenditure; as a consequence, the loss of body energy was dramatic, more than one third of all the body energy in just 10 days. The loss of energy was accounted for by the loss of body lipid. The effects on body weight were less marked, since the loss of energy was limited to

fat, which occupies only a limited space and packs little water in the adipose tissue.

The results presented indicate a fully additive effect of OE and the β_3-adrenergic agonist. The increase of energy expenditure elicited by B3A (Yoshida et al., 1996) was maintained, since OE does not significantly affect energy expenditure (Sanchis et al., 1996) and, contrarily to B3A (Lipworth, 1996), do not increase thermogenesis (Cabot et al., 2001). On the other side, most β_3-adrenergic agonists do not affect food intake, but OE decreases the ingestion of food (Sanchis et al., 1996), in part by maintaining glycaemia through the sparing of glucose (Grasa et al., 2001a). Nevertheless, both OE and B3A induce the massive mobilization of lipid from adipose tissue (Atgié et al. 1997; Carpéné et al. 1998; Remesar et al., 2002). In the case of the β_3-adrenergic agonists this mobilization, largely due to a direct stimulation of lipolysis (Atgié et al. 1997; Carpéné et al. 1998), supplies lipid to fuel thermogenesis (Himms-Hagen et al., 1994; Atgié et al., 1997). The β_3-adrenergic agonists also enhance glucose disposal (de Souza et al., 1997), although lipid mobilisation elicited by OE provides energy for the maintenance of metabolic activity and muscle function under limited energy availability (Blay et al., 2002), sparing glucose and protein (Sanchis et al. 1997). In addition, OE promotes adipocyte apoptosis (Remesar et al., 2002), and maintains energy and glucose homeostasis (Grasa et al., 2001a) increasing insulin sensitivity (Grasa et al., 2001b), processes that facilitate the selective disposal of stored fat. Blood glucose was lower in all groups receiving B3A, which hints at OE not being sufficiently effective in maintaining glycemia to compensate for the enhancement of glucose utilization elicited by the adrenergic agonist.

The rat model used here: mildly overweight adult males, is adequate for the study of fat mobilisation in comparison with younger normal-weight rats, since the accumulation of some fat during the cafeteria diet period allows for a more potent and continued loss of fat, and also because their increased insulin resistance, makes them closer to the condition of overweight humans (Carey et al., 1996).

The effects of OE were practically superimposed to those of B3A, as can be ascertained by changes in the insulin resistance HOMA score. OE continued exerting its homoeostatic maintenance of energy and glucose availability even under the strain of a pharmacologically-elicited increase in energy expenditure.

The results presented, suggest that the combination of a β_3-adrenergic agonist with OE may help compensate for the usually short-lived effects of the agonist and enhance the lipid mobilisation action of OE. The eventual combination of both compounds should be explored as a way to obtain faster and more effective ways to treat obesity.

ACKNOWLEDGEMENTS

Funding by grants 01/1300 and 01/1309 from the Fondo de Investigaciones Sanitarias from the Government of Spain (including FEDER funds from the European Union) is gratefully acknowledged.

REFERENCES

Adán, C., Cabot, C., Esteve, M., Grasa, M.M., Massanés, R., Vilà, R., Estruch, J., Fernández-López, J.A., Remesar, X. and Alemany, M., 1999. Oleoyl-estrone treatment affects the ponderostat setting differently in lean and obese Zucker rats. International Journal of Obesity 22: 366-373.

Atgié, C., d'Allaire, F. and Bukowiecki, L.J., 1997. Role of β_1- and β_3-adrenoceptors in the regulation of lipolysis and thermogenesis in rat brown adipocytes. American Journal of Physiology 273: C1136-C1142.

Atgié, C., Faintrenie, G., Carpéné, C., Bukowiecki, L.J. and Géloën, A., 1998. Effects of chronic treatment with noradrenaline or a specific β_3-adrenergic agonist, CL 316,243, on energy expenditure and epididymal adipocyte lipolytic activity in rat. Comparative Biochemistry and Physiology A 119A: 629-636.

Balada, F., Sanchis, D., Virgili, J., Grasa, M.M., Monserrat, C., Fernández-López, J.A., Remesar, X. and Alemany, .M, 1997. Effect of the slimming agent oleoyl-estrone in liposomes (Merlin-2) on the body weight of rats fed a cafeteria diet. Archives of Physiology and Biochemistry 105: 487-495.

Blay, M., Peinado-Onsurbe, J., Grasa, M.M., Díaz-Silva, M., Fernández-López, J.A., Remesar, X. and Alemany, M., 2002. Effect of oral oleoyl-estrone treatment on plasma lipoproteins and tissue lipase and lipase activities of Zucker lean and obese female rats. International Journal of Obesity 26: 618-626.

Bonora, E., Saggiani, F., Targher, G., Zenere, M.B., Alberiche, M., Monauni, T., Bonadonna, R.C. and Muggeo, M., 2000. Homeostasis model assessment closely mirrors the glucose clamp technique in the assessment of insulin sensitivity. Studies in subjects with various degrees of glucose tolerance and insulin sensitivity. Diabetes Care 23: 57-63.

Cabot, C., Grasa, M.M., Fernández-López, J.A., Remesar, X. and Alemany, M., 2001. Effect of oleoyl-estrone treatment on the expression of β1- β2- and β3-adrenoreceptors in rat adipose tissues. Molecular and Cellular Biochemsitry 221: 109-115.

Carey, D.G., Jenkins, A.B., Campbell, L.V., Freund, J. and Chisholm, D.J., 1996. Abdominal fat and insulin resistance in normal and overweight women. Direct measurements reveal a strong relationship in subjects at both low and high risk of NIDDM. Diabetes 45: 633-638.

Carpéné, C., Bousquet-Mélou, A., Galitzky, J., Berlan, M. and Lafontan, M., 1998. Lipolytic effects of β_1-, β_2-, and β_3-adrenergic agonists in white adipose tissue of mammals. Annals of the New York Academy of Sciences 839: 186-189.

de Souza, C.J., Hirshman, M.F. and Horton, E.S., 1997. CL-316,243, a β_3-specific adrenoceptor agonist, enhances insulin-stimulated glucose disposal in nonobese rats. Diabetes 46: 1257-1263.

Dietz, W.H. and Wolfe, R.R., 1985. Interrelationships of glucose and protein-metabolism in obese adolescents during short-term hypocaloric dietary therapy. American Journal of Clinical Nutrition 42:380-390.

Fernández-López, J.A., Remesar, X., Foz, M. and Alemany, M., 2002. Pharmacological approaches for the treatment of obesity. Drugs 62: 915-944

Fève, B., Baude, B., Krief, S., Strosberg, A.D., Pairault, J. and Emorine, L.J., 1992. Inhibition by dexamethasone of β3-adrenergic receptor responsiveness in 3T3-F442A adipocytes: evidence for a transcriptional mechanism. Journal of Biological Chemistry 267: 15909-15915.

Folch, J., Lees, M. and Sloane-Stanley, G.H., 1957. A simple method for the isolation and purification of total lipids from animal tissues. Journal of Biological Chemistry 232: 497-509.

Grasa, M.M., Cabot, C., Esteve, M., Yubero, P., Massanés, R.M., Blay, M., Vilà, R., López-Martí, J.,

Fernández-López, J.A., Remesar, X. and Alemany, M., 2001a. Daily oral oleoyl-estrone gavage induces a dose-dependent loss of fat in Wistar rats. Obesity Research 9: 202-209.

Grasa, M.M., Esteve, M., Massanés, R.M., Yubero, P., Blay, M., López-Martí, J.,Cabot, C., Vilà, R., Fernández-López, J.A., Remesar, X. and Alemany, M., 2001b. Oral gavage of oleoyl-estrone has a stronger effect on body weight in male obese Zucker rats than in female. Diabetes Obesity and Metabolism 3: 203-208.

Himms-Hagen ,J., Cui, J.Y., Danforth, E., Taatjes, D.J., Lang, S.S., Waters, B.L. and Claus, T.H., 1994. Effect of Cl-316,243, a thermogenic β_3-agonist, on energy- balance and brown and white adipose tissues in rats. American Journal of Physiology 266: R1371-R1382.

Lipworth, B.J., 1996. Clinical pharmacology of β_3-adrenoceptors. Br. J. Clin. Pharmacol. 42: 291-300.

Matthews, D.R., Hosker, J.P., Rudenski, A.S., Naylor, B.A., Teacher, D.F. and Turner, R.C., 1985. Homeostasis model assessment: insulin resistance and B cell function from fasting plasma glucose and insulin concentration in man. Diabetologia 28: 412-419.

Onai, T., Kilroy, G., York, D.A. and Bray, G.A., 1995. Regulation of β3-adrenergic receptor mRNA by sympathetic nerves and glucocorticoids in BAT of Zucker obese rats. American Journal of Physiology 269: R519-R526.

Remesar, X., Fernández-López, J.A., Blay, M.T., Savall, P., Salas, A., Díaz-Silva, M., Esteve, M., GRASA, M.M. and Alemany, M., 2002. Effect of oral oleoyl-estrone on adipose tissue composition in male rats International Journal of Obesity 26: 1092-1102.

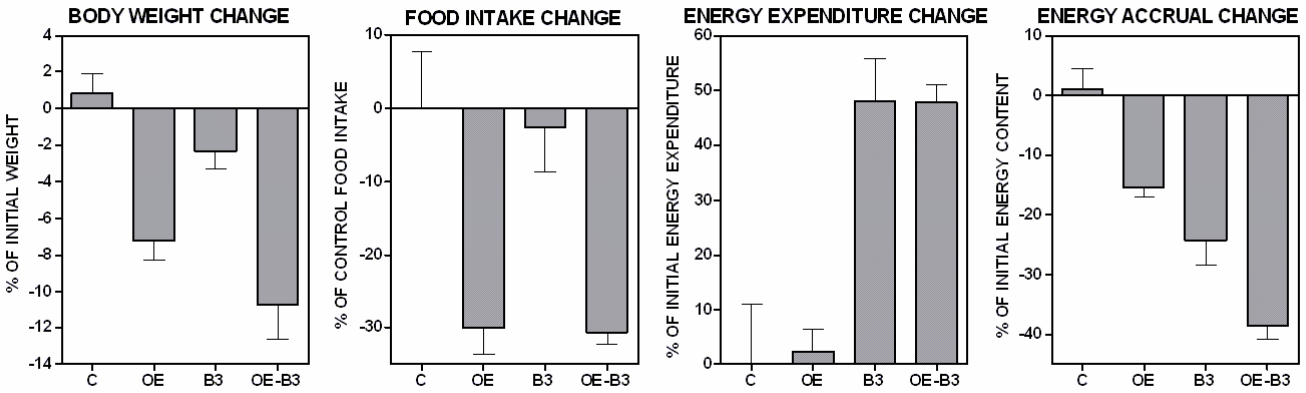
Sanchis, D., Balada, F., Grasa, M.M., Virgili, J., Peinado, J., Monserrat, C., Fernández-López, J.A., Remesar, X. and Alemany, M., 1996. Oleoyl-estrone induces the loss of body fat in rats. International Journal of Obesity 20: 588-594.

Sanchis, D., Balada, F., Picó, C., Grasa, M.M., Virgili, J., Farrerons, C., Palou, A., Fernández-López, J.A., Remesar, X. and Alemany, M., 1997. Rats receiving the slimming agent oleoyl-estrone in liposomes (Merlin-2. decrease food intake but maintain thermogenesis. Archives of Physiology and Biochemistry 105: 663-672.

Shibata, H. and Bukowiecki, L.J., 1987. Regulatory alterations of daily energy expenditure induced by fasting or overfeeding in unrestrained rats. Journal of Applied Physiology 63: 465-470.

Yoshida, T., Umekawa, T., Sakane, N., Yoshimoto, K. and Kondo, M., 1996. Effect of CL316,243, a highly specific β_3-adrenoceptor agonist, on sympathetic nervous system activity in mice. Metabolism 45: 787-791.

FIGURE 1



LEGEND TO FIGURE

Figure 1. Percent changes in body weight, food intake, energy expenditure and energy content of rats treated with a β_3-adrenergic agonist (CL316,243) and OE

The data are the mean ± SEM of six animals per group. Statistical differences between groups (two-way ANOVA): the columns show the P values for the significance of the effect of OE, and the β_3-adrenergic agonist; in all cases, the P for interaction between both agents was not significant. NS = not significant (P>0.05)

	P values	
	OE	β_3-agonist
body weight	<0.001	0.023
food intake	<0.001	NS
energy expenditure	NS	<0.001
energy accretion	<0.001	<0.001

Table 1 Body weight and composition of rats treated with a β_3-adrenergic agonist (CL316,243) and OE

parameter	units	Control d10	OE d10	B3 d10	OE-B3 d10	OE	B3A
initial body weight (d 0)	g	367 ± 14	356 ± 10	346 ± 14	351 ± 12	NS	NS
final body weight (d10)	g	370 ± 13	330 ± 9	338 ± 16	313 ± 4	0.018	NS
body energy content	kJ/g	11.73 ± 0.49	10.63 ± 0.07	8.95 ± 0.46	7.86 ± 0.30	0.010	<0.001
final body energy	MJ	4.35 ± 0.29	3.51 ± 0.08	3.51 ± 0.12	2.47 ± 0.12	0.016	<0.001
body lipid	% BW	17.2 ± 1.1	15.3 ± 0.7	9.9 ± 1.3	7.1 ± 0.6	0.025	<0.001
final body lipid pool	g	63.8 ± 5.0	50.4 ± 1.3	33.0 ± 3.3	22.2 ± 2.0	0.005	<0.001
food intake	g/d	16.6 ± 1.3	11.6 ± 0.6	16.2 ± 1.0	11.5 ± 0.3	<0.001	NS

The data are the mean ± sem of six animals per group. Statistical differences between groups (two-way ANOVA): the columns show the P values for the significance of the effect of OE and B3A; in all cases, the P for interaction between both agents was not significant. NS = not significant (P>0.05)

Table 2 Energy balance of rats treated with a β_3-adrenergic agonist (CL316,243) and OE

parameter	units	Control d10	OE d10	B3 d10	OE-B3 d10
energy intake	W	2.54 ± 0.20	1.78 ± 0.09	2.39 ± 0.18	1.76 ± 0.04
energy accretion	mW	52 ± 174	-771 ± 68	-1212 ± 206	-1919 ± 120
lipid contribution to energy accretion	%	109 ± 36	68 ± 8	97 ± 7	88 ± 6
energy expenditure	W	2.49 ± 0.28	2.55 ± 0.10	3..69 ± 0.19	3.68 ± 0.09

The data are the mean ± sem of six animals per group. Statistical analysis of the differences (in experimental data only) between groups (two-way ANOVA): effect of OE: $P<0.01$ for energy intake and energy accretion; effect of B3A; $P<0.01$ for energy accretion; the P for interaction between both agents was not significant except for lipid contribution to energy accretion ($P=0.002$)

Table 3 Serum composition of rats treated with a β_3-adrenergic agonist (CL316,243) and OE

parameter	units	Control d10	OE d10	B3 d10	OE-B3 d10	OE	B3A
glucose	mM	8.23 ± 0.13	8.68 ± 0.17	7.49 ± 0.08	7.84 ± 0.18	0.020	<0.001
triacylglycerol	mM	1.79 ± 0.09	1.45 ± 0.20	1.17 ± 0.31	1.25 ± 0.21	NS	NS
3-hydroxybutyrate	µM	127 ± 6	100 ± 13	148 ±15	131 ± 2	NS	0.034
non-esterified fatty acids	µM	420 ± 33	449 ± 47	350 ± 11	371 ± 36	NS	NS
total cholesterol	mM	2.26 ± 0.16	1.55 ± 0.06	2.34 ± 0.19	1.61 ± 0.12	<0.001	NS
insulin	pM	561 ± 60	384 ± 49	290 ± 50	327 ± 28	NS	0.008
HOMA score		28.6 ± 3.1	20.8 ± 2.9	13.4 ± 2.3	15.9 ± 1.5	NS	0.003

The data are the mean ± sem of six animals per group. Statistical differences between groups (two-way ANOVA): the columns show the P values for the significance of the effect of OE and B3A; in all cases, the P for interaction between both agents was not significant. NS = not significant (P>0.05).