Short-term treatment with oleoyl-oestrone in liposomes (Merlin-2) strongly reduces the expression of the *ob* gene in young rats

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Young female rats of 160–180 g were implanted with osmotic minipumps releasing 3.0 $\mu\text{mol/day}$ per kg of oleoyl-oestrone in liposomes (Merlin-2) into the bloodstream for up to 14 days. Merlin-2 induced a loss of appetite in the first days, later recovered, and a decrease in body weight of 7%, which contrasts with the 15% increase in controls during the 2-week period. Neither plasma glucose nor urea was affected by treatment, but liver glycogen increased by 50% in 14 days. Insulin decreased slightly with Merlin-2 treatment. Plasma corticotropin (ACTH) and corticosterone showed a transient increase by day 6 of treatment. The expression of the *ob* gene in adipose tissue fell during the period studied to practically nil on day 14; circulating leptin levels decreased more than 70% from day 1 to day 14.

Oestrone levels increased from 0.3 nM (controls) to a maintained 40–60 nM level for the rest of the experiment. Oleoyl-oestrone levels first increased 4-fold, to decrease again to the initial levels on day 10, increasing later to 100-fold on day 14. The three phases observed in food intake, weight loss and oleoyl-oestrone levels match fairly well, which supports the direct involvement of oleoyl-oestrone in body-weight control. However, the control of oleoyl-oestrone levels seems to be mediated in part by corticosterone. The practical disappearance of leptin synthesis coincides with the massive accumulation of oleoyl-oestrone in plasma. The results presented suggest the involvement of oleoyl-oestrone in the main mechanisms of control of body weight and its regulation by glucocorticoids and leptin.

INTRODUCTION

The ponderostat model of body-weight control depends on a signalling compound informing the brain of the size of fat-tissue depots [1,2]. Several candidates have been postulated and tested [3–5]. The discovery of leptin, the product of the *ob* gene, renewed the hopes that such a signal could be a protein [6], but other, simpler, molecules, such as oleoyl-oestrone, have been proposed for this role [7]. In any case, it is clear that leptin participates in the maintenance of body weight [8], probably by counter-regulating the adipogenic action of insulin [9].

Circulating oleoyl-oestrone levels are directly correlated to the body-fat mass in humans (J. M. Fernández-Real, D. Sanchis, W. Ricart, F. Balada, R. Casamitjana, M. Fernández-Castañer, J. Soler, J. A. Fernández-López, X. Remesar and M. Alemany, unpublished work); oleoyl-oestrone is carried in the lipoprotein fraction and elicits the loss of body fat when injected into rats [7]. The slimming effects of oleoyl-oestrone are dose dependent and result in the loss of fat with a fair preservation of protein; even at large doses, the loss of weight tapers off after most of the fat has disappeared [7]. Oleoyl-oestrone also acts on genetically obese Zucker rats [10], in which leptin abundance is a direct consequence of defective leptin receptors [11,12]; this suggests that oleoyl-oestrone action does not require the action of leptin.

Here we study the effects of a 2-week treatment of oleoyl-oestrone in liposomes (Merlin-2) on the main hormones implicated in the process of body-weight control: insulin, glucocorticoids and leptin.

EXPERIMENTAL

Female Zucker lean rats (8 weeks old) from Charles River (Aubin-les-Elbeuf, France), weighing 160–180 g, were used. They were maintained under standard conditions (21 °C, 60–70%

relative humidity, lights on from 08.00 to 20.00), and were fed *ad libitum* standard rat chow pellets (B&K, Sant Vicent dels Horts, Spain). The rats were handled following the guidelines established by the European Community and the Governments of Catalonia and Spain. The rats were killed by decapitation at the end of the experiments.

Chemicals and standards from Sigma (St Louis, MO, U.S.A.) and solvents from Merck (Darmstadt, Germany) were of reagent quality. Oleoyl-oestrone was synthesized from oleoyl chloride and ³H-labelled oestrone (DuPont NEN, NJ, U.S.A.) in an anhydrous pyridine medium [7,13]. Oleoyl-oestrone was incorporated into liposomes [7]; the stable preparation, containing 20% lipid and 4.2 mM oleoyl-oestrone, was code-named Merlin-2. This preparation was used to fill osmotic minipumps (Alzet 2ML-2; Alza, Palo Alto, CA, U.S.A.), which were inserted under the skin of the back and connected via a short capillary tube (PE-10 polyethylene; Becton-Dickinson, Parsippany, NJ, U.S.A.) to the left jugular vein under diethyl ether anaesthesia. The minipumps released 5 $\mu\text{l/h}$ for 14 days (i.e. 500 nmol of oleoyl-oestrone/day) at a constant rate. The dose administered to the rats was, thus, 3.0 $\mu\text{mol/day}$ per kg of body weight. A group of control rats was included in the study, in which the minipumps contained only the liposome suspension, without oleoyl-oestrone. All rats were weighed daily.

On days 0 (untreated rats), 3, 6, 10 and 14 after the implantation of the minipumps, groups of five treated rats (and controls on day 14) were killed by decapitation, and their blood was recovered in dry heparinized beakers. Samples of visceral white adipose tissue (periovaric fat pads) as well as liver samples were immediately dissected and frozen in liquid nitrogen [14]. Blood was centrifuged to obtain plasma, which was then frozen. All samples were kept at –80 °C until processing.

Free oestrone was measured by direct radioimmunoanalysis with ³H-labelled oestrone (Amersham, Amersham, U.K.) in

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Table 1 Changes experienced in body weight and food intake of rats chronically treated with 3.0 μ mol/day per kg of oleoyl-oestrone in liposomes (Merlin-2)

The data are the means $\pm$ S.E.M. of five different animals per group. Data with different superscript letters are statistically different ($P < 0.05$; Student's t test). Food intake was measured over the 24 h before the other measurements on the indicated day. The day 0 value corresponds to that of undisturbed animals.

Parameter	Day ...	Untreated	Treated				Controls
		0	3	6	10	14	14
Initial body weight (g)		168 $\pm$ 2 ^A	169 $\pm$ 2 ^A	172 $\pm$ 2 ^A	168 $\pm$ 2 ^A	168 $\pm$ 3 ^A	163 $\pm$ 3 ^A
Final body weight (g)		168 $\pm$ 2 ^A	166 $\pm$ 2 ^{AB}	168 $\pm$ 2 ^A	164 $\pm$ 3 ^{AB}	160 $\pm$ 2 ^B	178 $\pm$ 3 ^C
Body weight change (g)		0.0 ^A	-2.5 $\pm$ 0.6 ^A	-4.1 $\pm$ 1.7 ^{AB}	-3.4 $\pm$ 1.1 ^A	-8.3 $\pm$ 1.8 ^B	+15.1 $\pm$ 2.3 ^C
Food intake (kJ/day)		190 $\pm$ 8 ^A	126 $\pm$ 4 ^B	134 $\pm$ 4 ^B	170 $\pm$ 5 ^{AC}	165 $\pm$ 6 ^C	193 $\pm$ 6 ^A

Table 2 Liver energy stores and plasma glucose and urea levels of rats treated chronically with oleoyl-oestrone in liposomes (Merlin-2)

The data are the means $\pm$ S.E.M. of five different animals per group. Data with different superscript letters are statistically different ($P < 0.05$; Student's t test). Glycogen levels are given in mmol of glycosyl residues/kg of tissue.

Parameter	Day ...	Untreated	Treated				Controls
		0	3	6	10	14	14
Plasma glucose (mM)		4.9 $\pm$ 0.6 ^A	6.9 $\pm$ 0.8 ^B	6.3 $\pm$ 0.3 ^{AB}	5.0 $\pm$ 0.5 ^{AB}	5.3 $\pm$ 0.4 ^{AB}	5.8 $\pm$ 0.2 ^{AB}
Plasma urea (mM)		5.0 $\pm$ 0.3 ^A	5.9 $\pm$ 0.6 ^A	4.9 $\pm$ 0.1 ^A	5.2 $\pm$ 0.2 ^B	5.8 $\pm$ 0.7 ^A	5.6 $\pm$ 0.3 ^A
Liver lipids (g/kg)		50 $\pm$ 4 ^A	54 $\pm$ 6 ^A	45 $\pm$ 2 ^A	51 $\pm$ 2 ^A	49 $\pm$ 4 ^A	52 $\pm$ 4 ^A
Liver glycogen (mmol/kg)		289 $\pm$ 27 ^{AB}	231 $\pm$ 26 ^A	359 $\pm$ 33 ^{BC}	381 $\pm$ 45 ^{BC}	435 $\pm$ 28 ^C	295 $\pm$ 18 ^{AB}

dried diethyl ether plasma extracts [15], using specific anti-oestrone antibodies (E-3135; Sigma, St. Louis, MO, U.S.A.). Plasma samples were processed as follows for the measurement of total oestrone: 75 μ l of plasma was extracted with 1 ml of trichloromethane/methanol (2:1, by vol.), washed with 4 mM MgCl₂ [16] and centrifuged. The lower organic phase was dried and 1 ml of hydroalcoholic KOH was added, and the samples were then left for 20 min at 85 °C in order to complete their saponification. The samples were then twice extracted with ethyl ether, and the organic phases were combined, brought to pH 7 with 100 mM KH₂PO₄, centrifuged and dried. The residue was resuspended in 200 μ l of ethanol and used for the measurement of oestrone using the same standard oestrone radioimmunoassay method.

Plasma samples were also used for the measurement of insulin [17] using a rat insulin kit (Amersham), corticosterone with labelled hormone from Amersham and polyclonal antibodies from Sigma (C-8784), as well as corticotropin (ACTH) using a specific kit (07-106101; ICN, Costa Mesa, CA, U.S.A.). Blood samples were also used to determine the concentrations of glucose [18] and urea, the latter with a ready-use kit (B 8035; Menarini, Firenze, Italy). Liver samples were used for the extraction, purification [19] and determination of its glycogen content [20].

Adipose-tissue fat pads were used to obtain total RNA with the Tri Pure isolation reagent (1667 165; Boehringer Mannheim, Mannheim, Germany) and then to determine the proportion of *ob* gene mRNA compared with total RNA using appropriate oligonucleotides through Northern-blot transfer [21]. Fixed oligonucleotides were detected by chemiluminescence, signals being revealed by exposure to X-ray film, which was scanned and quantified with a laser scanner (Seiko Epson 67 8500; Seiko, Nagano, Japan), using the Phoretix (Newcastle upon Tyne, U.K.) software.

Two additional groups of rats with minipumps implanted (five controls and five with oleoyl-oestrone) were killed on day 14, and their blood plasma was used for the measurement of plasma leptin using a specific rat leptin radioimmunoassay kit (RL-83K; Linco, St. Charles, MO, U.S.A.).

The statistical significance of differences between groups was assessed by Student's t test.

RESULTS

Table 1 shows that loss of weight was related to the duration of Merlin-2 treatment; rats receiving oleoyl-oestrone lost about 8.3 % of their initial weight in 2 weeks, in contrast with the 15.1 % gain observed in the controls. The weight change attributable to Merlin-2 treatment was, thus, 23.4 % of the initial weight in 14 days. Food intake was strongly limited at the beginning of the treatment, but later recovered to practically the initial values.

No changes were observed in plasma urea levels and there was little variation in glucose concentration (Table 2). Liver lipid levels did not change, but glycogen stores increased significantly with treatment; after 14 days they were 50 % higher than in controls.

Insulin levels decreased with Merlin-2 treatment, but the extent of the decrease was small compared with the levels in control groups (Table 3). Corticotropin levels increased by day 6, to decrease again to control values. Corticosterone levels closely followed the corticotropin pattern, with marked peaks on day 6 and day 10. The expression of the *ob* gene was markedly altered by Merlin-2 treatment, reaching negligible levels on day 14 (Figure 1). Circulating leptin levels on day 14 were 4.60 $\pm$ 0.36 ng/ml in the liposome-receiving controls and 1.29 $\pm$ 0.42 ng/ml for oleoyl-oestrone-treated rats ($P < 0.05$).

Plasma oestrone levels rose by two orders of magnitude with

Table 3 Plasma hormone levels and white-adipose-tissue *ob* gene expression in rats chronically treated with oleoyl-oestrone in liposomes (Merlin 2)

The data are the means $\pm$ S.E.M. of five different animals per group. Data with different superscript letters are statistically different ($P < 0.05$; Student's *t* test). The expression of the *ob* gene is given in arbitrary units (a.u.; % of the values of the controls).

Parameter	Day ...	Untreated	Treated				Controls
		0	3	6	10	14	14
Plasma insulin (nM)		0.44 $\pm$ 0.09 ^{AB}	0.38 $\pm$ 0.04 ^A	0.25 $\pm$ 0.01 ^{BC}	0.21 $\pm$ 0.02 ^C	0.25 $\pm$ 0.02 ^{BC}	0.28 $\pm$ 0.01 ^B
Plasma corticotropin (pM)		18.3 $\pm$ 3.3 ^{AB}	12.3 $\pm$ 1.8 ^A	37.1 $\pm$ 8.0 ^B	17.0 $\pm$ 2.0 ^{AB}	14.8 $\pm$ 2.7 ^A	23.4 $\pm$ 10.1 ^B
Plasma corticosterone (nM)		367 $\pm$ 65 ^{AC}	307 $\pm$ 45 ^{AC}	703 $\pm$ 55 ^B	507 $\pm$ 70 ^{AB}	280 $\pm$ 51 ^C	410 $\pm$ 87 ^{ABC}
White adipose tissue <i>ob</i> mRNA (a.u.)		99 $\pm$ 11 ^A	84 $\pm$ 26 ^A	79 $\pm$ 30 ^A	36 $\pm$ 14 ^B	7 $\pm$ 4 ^B	100 $\pm$ 14 ^B
Plasma oestrone (nM)		0.3 $\pm$ 0.1 ^A	69 $\pm$ 24 ^B	40 $\pm$ 12 ^B	53 $\pm$ 21 ^B	49 $\pm$ 11 ^B	0.3 $\pm$ 0.1 ^B
Plasma fatty-acyl oestrone (nM)		242 $\pm$ 99 ^A	1142 $\pm$ 580 ^A	417 $\pm$ 177 ^A	430 $\pm$ 154 ^A	19500 $\pm$ 5300 ^B	195 $\pm$ 27 ^A

Figure 1 Northern blot of *ob* gene expression in white adipose tissue of lean Zucker rats chronically treated with oleoyl-oestrone in liposomes

Upper panel: *ob* mRNA presence in cell extracts of rats on days (d) 0, 3, 6, 10 and 14 of treatment. C represents a positive control (untreated rat white adipose tissue), and L represents the liver of untreated rats (negative control). Each run corresponds to 20 μ g of tissue RNA from a single representative rat. Lower panel: 18 S ribosomal RNA controls for the above runs.

the Merlin-2 treatment, reaching a value in the 40–70 nM range, which was then maintained throughout the treatment (Table 3). In contrast, oleoyl-oestrone levels changed considerably during its constant infusion, showing three distinct phases: (1) increase from days 0 to 3, when the levels of oleoyl-oestrone were four times the basal values; (2) transient decrease from day 3 to about the control levels by day 10; and (3) 100-fold increase from day 10 to 14. However, the variability of data was considerable, and only the values on day 14 were statistically different from all the others.

Assuming a blood volume of 5% of body weight, the total free oestrone present in the rat blood under basal conditions was less than 3 pmol, rising to about 400 pmol under treatment. The minipump released that amount of esterified oestrone in less than 1 min. In the case of oleoyl-oestrone, the plasma pool was in the range of 2 nmol, rising to the maximum peak (on day 14) of 165 μ mol, i.e. the amount released in less than 8 h.

DISCUSSION

The effects of the Merlin-2 preparation (Table 1) on the body weight of young rats were similar to those reported under similar conditions and dose [7]. They showed that the loss of body weight is due to a decrease in energy intake coupled with maintained energy expenditure, the difference being covered by internal fat stores [7]. Little change was observed on plasma parameters with treatment (Table 2). Blood glucose was maintained despite sustained energy drainage and changes in insulin (Table 3). Nevertheless, the additional accumulation of liver glycogen (with unchanged liver fat stores) is difficult to explain with maintained circulating levels of glucose and lowered insulin, and hints at a high energy surplus in the liver at the expense of

extended lipolysis in peripheral adipose tissue. This ample glycogen availability contrasts with the reduced food intake and the obvious negative energy budget resulting in weight loss. On the whole, these results demonstrate/suggest that oleoyl-oestrone could influence glycogen deposition without the mediation of insulin.

The overall effects induced by the constant Merlin-2 infusion are clear and repeatedly shown in rat models [7,10], but the mechanism by which these effects are exerted remains unexplained. Our study shows that the slimming effects of oleoyl-oestrone were not uniform during the period studied, since the maximal effects were observed in the first and the last days of treatment, with a fairly stable phase between days 3 and 10. The peak of corticotropin and corticosterone coinciding with this period (Table 3) hints at the involvement of the hypothalamus–hypophysis–adrenals axis in the control of the process, as occurs in most of the mechanisms controlling body weight [22–24].

The marked and progressive inhibition of *ob* gene (leptin) expression in the white adipose tissue of treated rats was further compounded by the strong decrease in adipose-tissue mass caused by Merlin-2 treatment [7]; it may be assumed that on day 14 the production of leptin is almost nil. It may be speculated that the decrease in *ob* gene expression may be a consequence of strongly diminished adipose tissue, but it may be also the direct consequence of oleoyl-oestrone administration.

The high but maintained levels of oestrone underscore the ability of the rat to dispose of excess oestrone, in spite of a large overload in comparison with its circulating pool (Table 2). The remarkable stability of circulating oleoyl-oestrone in normal untreated rats, which is directly related to fat mass, contrasts with the enormous changes found in its plasma levels under the continuous pharmacological infusion of this compound (Table 3). The transient (but not significant) decrease in oleoyl-oestrone levels observed from days 3 to 10 may represent what probably is a safety mechanism able to process high loads of oleoyl-oestrone. Even more, the changes experienced by oleoyl-oestrone levels hint at the superposition of at least two counter-regulatory mechanisms preventing its action: first, the rise in oleoyl-oestrone observed on day 3 triggered a response by the hypothalamus–hypophysis–adrenals axis, raising corticotropin and then corticosterone levels, which resulted in a slowing of weight loss, decrease of oleoyl-oestrone levels (in spite of constant infusion) and recovery of appetite. In addition, insulin levels fell slightly and leptin mRNA followed suit. This situation was maintained for a time, but the constant infusion of oleoyl-oestrone finally interrupted it by reducing the corticosteroid activity to basal values (Table 3). At this point, however, excess oleoyl-oestrone accumulated in the plasma. It affected weight loss but not appetite, which

lends further support to the hypothesis that different pathways may be involved in the two actions.

The three phases observed, mainly in oleoyl-oestrone levels and food intake, matched fairly well, which supports the physiological involvement of oleoyl-oestrone in the control of these processes. However, the control of oleoyl-oestrone levels is probably mediated by corticosterone but not only by that hormone, since with comparable levels of the corticosteroid (e.g. days 3 and 14), the ability of the rat overall to split oleoyl-oestrone was radically different. This effect could not be attributed either to insulin or to free oestrone, since they maintain fairly stabilized levels (albeit grossly elevated in the case of oestrone) (Table 3). The only other deep change observed was in leptin expression, resulting in a marked decrease of circulating leptin. The practical disappearance of leptin synthesis coincides with the breakup of the *status quo* of phase 2 and the accumulation of oleoyl-oestrone in plasma to pharmacological levels. Nevertheless, the results can be examined from another point of view, since the administration of oleoyl-oestrone decreases *ob* gene expression, but this effect is probably induced synergically with the fall of energy or insulin [21,25,26]; from the data on blood glucose and liver lipid and glycogen it is not clear that the wasting of adipose tissue proceeds in parallel with an overall lack of energy availability, and thus this interpretation may not be fully applicable. Thus the fall in *ob* gene (leptin) expression may be the consequence of the increased lipolysis-induced accumulation of free fatty acids, which inhibit leptin expression [27]. Increased fatty acid levels could also help explain the maintenance of circulating glucose levels, since they increase liver glucose synthesis [28] and lower glucose disposal by peripheral tissues such as muscle [29].

The inhibitory effect on *ob* gene expression is maximal (day 14) when fully active lipolysis has already carried away most of the original adipose-tissue fat mass. The lower adipocyte size and energy availability may further help limit leptin production.

Another main conclusion derived from this study is the good maintenance of plasma glucose levels in spite of accumulation of glycogen by the liver. It may reflect a situation in which at least one of the traits that characterize insulin resistance (the lack of ability to control the liver glucose output) is suppressed. This change is paralleled by high oleoyl-oestrone levels and, significantly, by practically nil leptin production, which agrees with the postulate that leptin induces insulin resistance [9,30].

The results presented here support the physiological involvement of oleoyl-oestrone in the mechanism of control of body weight and hint at the complexity of its regulation by corticoids and leptin. Further work is needed to unravel the closely knit imbrication of mechanisms designed to minimize the losses of body fat reserves.

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REFERENCES

- 1 Faust, I. M. (1981) in *The Body Weight Regulatory System: Normal and Disturbed Mechanism* (Cioffi, L. A., James, W. P. T. and Van Itallie, T. B., eds.), pp. 39–43, Raven Press, New York
- 2 Alemany, M. (1989) *Prog. Food Nutr. Sci.* **13**, 45–66
- 3 Wirtshafter, D. and Davis, J. D. (1977) *Science* **198**, 1271–1274
- 4 Greenway, F. L., Bray, G. A. and Heber, D. (1980) *Clin. Res.* **28**, A16
- 5 Porte, D. and Woods, S. C. (1981) *Diabetologia* **20**, 274–280
- 6 Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L. and Friedman, J. M. (1994) *Nature (London)* **372**, 425–431
- 7 Sanchis, D., Balada, F., Grasa, M. M., Virgili, J., Peinado, J., Monserrat, C., Fernández-López, J. A., Remesar, X. and Alemany, M. (1996) *Int. J. Obesity* **20**, 588–594
- 8 Caro, J. F., Sinha, M. K., Kolaczynski, J. W., Zhang, P. L. and Considine, R. V. (1996) *Diabetes* **45**, 1455–1462
- 9 Remesar, X., Rafecas, I., Fernández-López, J. A. and Alemany, M. (1997) *FEBS Lett.* **402**, 9–12
- 10 Balada, F., Sanchis, D., Grasa, M. M., Virgili, J., Estruch, J., Fernández-López, J. A., Remesar, X. and Alemany, M. (1996) *Int. J. Obesity* **20** (suppl. 4), 30
- 11 Iida, M., Murakami, T., Ishida, K., Mizuno, A., Kuwajima, M. and Shima, K. (1996) *Biochem. Biophys. Res. Commun.* **222**, 19–26
- 12 Iida, M., Murakami, T., Ishida, K., Mizuno, A., Kuwajima, M. and Shima, K. (1996) *Biochem. Biophys. Res. Commun.* **224**, 597–604
- 13 Mellon-Nussbaum, S. H., Ponticorvo, L., Schatz, F. and Hochberg, R. S. (1982) *J. Biol. Chem.* **257**, 5678–5684
- 14 Ardévol, A., Cañas, X., Remesar, X. and Alemany, M. (1993) *J. Biochem. Biophys. Meth.* **27**, 77–86
- 15 Zumoff, B., Strain, G. W., Kream, J., O'Connor, J., Levin, J. and Fukushima, D. K. (1981) *Metabolism* **30**, 1011–1014
- 16 Folch, J., Lees, M. and Sloane-Stanley, G. H. (1957) *J. Biol. Chem.* **232**, 497–509
- 17 Heding, L. G. (1972) *Diabetologia* **8**, 260–266
- 18 Trinder, P. (1969) *Ann. Clin. Biochem.* **6**, 24–27
- 19 Good, C. A., Kramer, H. and Somogyi, M. (1933) *J. Biol. Chem.* **100**, 485–491
- 20 Serafini, M. T. and Alemany, M. (1987) *J. Biochem. Biophys. Meth.* **15**, 33–39
- 21 Trayhurn, P., Thomas, M. E. A., Duncan, J. D. and Rayner, V. D. (1995) *FEBS Lett.* **368**, 488–490
- 22 Plotsky, P. M., Thiruvikraman, K. V., Watts, A. G. and Hauger, R. L. (1992) *Endocrinology* **130**, 1931–1941
- 23 Streeten, D. H. P. (1993) *J. Clin. Endocrinol. Metab.* **77**, 339–340
- 24 Björntorp, P. (1995) *Metabolism* **44** Suppl. 3, 21–23
- 25 Mizuno, T. M., Bergen, H., Funabashi, T., Kleopoulos, S. P., Zhong, Y. G., Bauman, W. A. and Mobbs, C. V. (1996) *Proc. Natl. Acad. Sci. U.S.A.* **93**, 3434–3438
- 26 Sivitz, W. I., Bailey, H. L. and Donohoue, P. (1996) *Biochem. Biophys. Res. Commun.* **220**, 520–525
- 27 Rentsch, J. and Chiesi, M. (1996) *FEBS Lett.* **379**, 55–59
- 28 Reaven, G. M. (1988) *Diabetes* **37**, 1595–1607
- 29 Roden, M., Price, T. B., Perseghin, G., Petersen, K. F., Rothman, D. L., Cline, G. W. and Shulman, G. I. (1996) *J. Clin. Invest.* **97**, 2859–2865
- 30 Cohen, B., Novick, D. and Rubinstein, M. (1996) *Science* **274**, 1185–1188