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Guanine-nucleotide-binding proteins G_i and G_s in fat-cells from normal, hypothyroid and obese human subjects

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In human adipocyte plasma membranes, pertussis toxin catalysed the ADP-ribosylation of an apparently single 40 kDa protein. The same protein was also observed in Western blots by using an antibody which identifies the *C*-terminal decapeptide of $G_i\alpha$ (α subunit of G_i). In analogous experiments, cholera toxin and an antibody raised against the *C*-terminal decapeptide of $G_s\alpha$ (α subunit of G_s) were used to identify two proteins of 42 and 45 kDa, the former of which was more prominent. A method was developed to estimate the relative amounts of G_i and G_s in crude adipocyte plasma membranes in a single immunoblot by using the two antisera. In animal models, changes in the amounts of G-proteins have been suggested to explain alterations in hormone-responsiveness in hypothyroidism and obesity. However, the amounts of G_i and G_s were unaltered in thyroidectomized papillary-carcinoma patients who had been without hormone substitution for 4 weeks. In adipocyte plasma membranes prepared from markedly obese subjects, the amounts of both $G_i\alpha$ and $G_s\alpha$ as calculated per mg of protein were decreased, but the G_i/G_s ratio remained unaltered in comparison with control subjects.

INTRODUCTION

The sensitivity of cells to hormones varies in different physiological and pathological conditions. In hypothyroidism, the response of fat-cells to stimulators of cyclic AMP accumulation and lipolysis, such as β-adrenergic agents, corticotropin and glucagon, is blunted (Debons & Schwartz, 1961; Armstrong *et al.*, 1974; Correze *et al.*, 1974; Ohisalo & Stouffer, 1979; Fredholm & Vernet, 1984; Wahrenberg *et al.*, 1986). On the other hand, it has been reported that the response to adenosine, which inhibits lipolysis, is increased in fat-cells from hypothyroid rats (Ohisalo & Stouffer, 1979; Chohan *et al.*, 1984; Malbon *et al.*, 1985; Saggerson, 1986; Woodward & Saggerson, 1986; Ohisalo *et al.*, 1987). However, two laboratories have reported unaltered responsiveness to adenosine in hypothyroidism (Fredholm & Vernet, 1984; Mills *et al.*, 1986). As for other inhibitors of lipolysis, the sensitivity of fat-cells from hypothyroid rats to prostaglandin E_1 and nicotinic acid has been reported to be increased (Saggerson, 1986), and the response of fat-cells from hyperthyroid humans to prostaglandin E_2 has been reported to be decreased (Richelsen *et al.*, 1986). The response of fat-cells from hypothyroid human patients to α-adrenergic inhibition of lipolysis has been reported to be unchanged (Wahrenberg *et al.*, 1986) or increased (Rosenquist *et al.*, 1971). Summing up, it has been well documented that fat-cells from hypothyroid rat and man have a blunted response to agents that stimulate cyclic AMP accumulation and lipolysis, and there is evidence that the response to inhibitory agents is enhanced, though the latter concept has not been accepted unanimously. Alterations in the amounts of cell-surface receptors to these agents cannot explain these changes (Chohan *et al.*, 1984; Wahrenberg *et al.*, 1986; Ros *et al.*, 1988), other than possibly in the case of prostaglandin E_2 (Richelsen *et al.*, 1986). The activity of adenylate cyclase and the intracellular effects of cyclic AMP also seem to be unchanged (Armstrong *et al.*, 1974), though there is one report that the stimulation of lipolysis by cyclic AMP is impaired (Mills *et al.*, 1986). By contrast with what is observed in hypothyroidism, in obesity the sensitivity of fat-cells to inhibitory agents is decreased, whereas that to stimulators of lipolysis is increased (Ohisalo *et al.*, 1986; Richelsen, 1988). These changes have been suggested to be due to changes in the amounts of G-proteins that transduce both activatory and inhibitory impulses from hormone receptors on the cell surface to adenylate cyclase (Malbon *et al.*, 1985; Milligan *et al.*, 1987; Richelsen, 1988; Ros *et al.*, 1988).

The G-proteins are a family of heterotrimeric guanine-nucleotide-binding membrane proteins that are involved in transmembrane signalling. Information from hormone receptors that stimulate adenylate cyclase is mediated by G_s, whereas negative impulses are transduced to the cyclase by G_i. The α subunit of G_i ($G_i\alpha$) can be ADP-ribosylated by using a toxin from *Bordetella pertussis*. Similar ADP-ribosylation of the α subunit(s) of G_s ($G_s\alpha$) is catalysed by a toxin from *Vibrio cholerae* (see Gilman, 1987; Milligan, 1988). These toxins have been widely used for the identification of $G_i\alpha$ and $G_s\alpha$, though it has become clear that they are not specific for them (Gierschik *et al.*, 1986; Milligan & McKenzie, 1988). It has been shown that the amount of $G_i\alpha$ is increased in plasma membranes prepared from fat-cells of hypothyroid rats as compared with euthyroid control animals. This increase has been shown both by using pertussis-toxin-

Abbreviations used: G_s, G_i, stimulatory and inhibitory G-proteins; $G_s\alpha$, $G_i\alpha$, their α subunit.

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catalysed ADP-ribosylation and also, more recently, by specific antisera (Malbon *et al.*, 1985; Milligan *et al.*, 1987; Ros *et al.*, 1988). This could account for the altered sensitivity of hypothyroid-rat fat-cells to agents whose effects are transduced by G-proteins. Alterations in G-protein functions have also been described in fat-cells from genetically obese rats (Houslay *et al.*, 1988). Such studies have not been performed with fat-cells from obese human patients, but it is tempting to assume that alterations might exist, since changes in hormone-sensitivity have been observed (Ohisalo *et al.*, 1986; Richelsen, 1988).

The aim of the present study was to characterize human fat-cell $G_i\alpha$ and $G_s\alpha$, and to find out if their relative amounts are altered in fat-cells from hypothyroid and obese human subjects.

MATERIALS AND METHODS

Patients

Tissue samples were obtained from thyroidectomized papillary thyroid-carcinoma patients. These patients had been 4 weeks without any hormone-substitution therapy before a total-body iodine-uptake scan was performed to search for possible metastases. Before medication was stopped, the patients were treated only by tri-iodothyronine, which has a shorter half-life (2 days) than tetraiodothyronine. All hypothyroid patients had unmeasurably low tetraiodothyronine and tri-iodothyronine levels in plasma. Their serum thyrotropin levels averaged 68.6 munits/l (normal range 0.3–5). Detailed information about the patients and their age-, sex- and body-weight-matched controls is given in Table 1.

The obese subjects were otherwise healthy. Their body mass indexes [weight (kg) divided by square of height (m)] averaged 46 kg/m^2, as compared with the average value of 24.7 kg/m^2 of controls. Details are given in Table 2.

A written informed consent was obtained from all tissue donors. The project was approved by the ethical committees of the III Department of Medicine, Helsinki University Central Hospital, and the Invalid Foundation Hospital of Helsinki.

Table 1. Details of the hypothyroid tissue donors and corresponding controls (all female)

The numbers refer to Fig. 2: H, hypothyroid; E, euthyroid.

Patient	Age (years)	Body mass index (kg/m^2)	Relative $G_i\alpha/G_s\alpha$ ratio in fat-cells*
1H	42	23.6	0.65
2E	46	26.3	0.80
3H	70	27.6	0.53
4E	67	29.0	0.73
5H	43	25.3	0.61
6E	48	21.2	0.75
7H	61	33.2	0.65
8E	54	28.4	0.52

* Based on levels of $G_s\alpha_{42}$.

Table 2. Details of obese and normal-weight subjects (all female)

The numbers refer to those in Fig. 3.

Patient	Age (years)	Body mass index (kg/m^2)	Relative $G_i\alpha/G_s\alpha$ ratio in fat-cells*
1	46	26.3	0.68
2	43	40.1	0.77
3	27	23.6	0.66
4	26	47.4	0.50
5	48	21.2	1.00
6	41	50.0	0.62
7	24	27.5	0.49
8	38	46.5	0.92

* Based on signal from $G_s\alpha_{42}$.

Samples and cell and plasma-membrane preparations

The fat samples were taken from the lower abdominal region under local anaesthesia or under general anaesthesia at the beginning of routine operations (gastroplasties in the case of two obese patients). Fat-cells were prepared by a modification of the method of Rodbell (1964). The samples were first cut into pieces with a razor blade. The pieces were incubated in a solution containing 0.5 mg of collagenase/ml of 4 mM-glucose/125 mM-NaCl/5 mM-KCl/2.5 mM-$MgCl_2$/1 mM-$CaCl_2$/1 mM-KH_2PO_4/25 mM-Tris/2% (w/v) bovine serum albumin at pH 7.4. After 60 min of constant shaking at 2 Hz at 37 °C, the cells were washed three times with the same buffer without collagenase. Crude adipocyte plasma membranes were then prepared as described by Mills *et al.* (1986).

Toxin-catalysed ADP-ribosylation

Cholera and pertussis toxins were activated in 50 mM-dithiothreitol at room temperature for 1 h immediately before use. Membranes (approx. 20 μg of protein) were then labelled in the presence of the activated toxin and [^{32}P]NAD$^+$ as described by Milligan & McKenzie (1988). After toxin labelling, the membrane proteins were subjected to SDS/polyacrylamide (10%, w/v)-gel electrophoresis and then blotted on to nitrocellulose paper as described below. After staining with antisera, autoradiography was performed.

Western blots

Usually, 15 μg samples of membrane protein were run on SDS/polyacrylamide-gel electrophoresis in a 1.5 mm-thick 10% gel in a Protean Microphor set. Prestained molecular-mass standards were run in parallel. The proteins were electrophoretically transferred to nitrocellulose paper (100 V for 80 min). After blocking with 3% (w/v) gelatin in Tris-buffered saline (0.5 M-NaCl/20 mM-Tris/HCl, pH 7.6) for 2 h at 37 °C, the paper was incubated with the first antibody in 1% gelatin in Tris-buffered saline overnight. The bound antibody was detected by using *o*-dianisidine and horseradish peroxidase coupled to goat anti-rabbit IgG. Details of this procedure have been reported previously (Milligan *et al.*, 1987). Estimations of the relative ratios of $G_i\alpha$ to $G_s\alpha$ were performed by densitometric scanning of a photographic positive with a Helena Flur-Vis Autoscanner.

Antisera

Antiserum CS-1 was produced in a New Zealand White rabbit by using a conjugate of keyhole limpet haemocyanin and a synthetic peptide (RMHLRQYELL), which corresponds to the *C*-terminal decapeptide of forms of $G_s\alpha$. Conditions for coupling and use of the conjugate as antigen were as described by Goldsmith *et al.* (1987). Antiserum SG-1 was raised in a similar fashion, except that the synthetic peptide used (KENLKDCGLF) corresponds to the *C*-terminal decapeptide of rod transducin. This sequence differs in only a single conservative substitution from the equivalent region of $G_i\alpha$ and, as for other antisera we have generated against this conjugate (Goldsmith *et al.*, 1987; Milligan *et al.*, 1987), this antiserum identifies forms of $G_i\alpha$. As transducin is limited in distribution to tissues that contain photoreceptors, this antiserum can be used to identify $G_i\alpha$ in all other tissues.

Protein assay

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard.

Reagents

Pertussis toxin was kindly given by Dr. L. Irons from PHLS Laboratories, Porton Down, Salisbury, Wiltshire, U.K. Cholera toxin was purchased from Sigma. [^{32}P]NAD$^+$ was from New England Nuclear. Goat anti-rabbit IgG–horseradish peroxidase conjugate was from Bio-Rad or the Scottish Antibody Production Unit, Wishaw, Scotland, U.K. Prestained molecular-mass standards were purchased from Bio-Rad and from BRL. The apparent molecular masses of these standards were determined by comparing them with non-stained regular Bio-Rad low-molecular-mass standards.

RESULTS

Human fat-cell membrane proteins were [^{32}P]ADP-ribosylated with pertussis toxin and [^{32}P]NAD$^+$, separated by SDS/polyacrylamide-gel electrophoresis, blotted on to nitrocellulose paper and stained with antiserum SG-1 (100 μl/20 ml of 1% gelatin in Tris-buffered saline), which was raised in rabbits against the *C*-terminal decapeptide of the subunit of transducin and identifies forms of $G_i\alpha$. The stained blot and an autoradiograph of the same blot are shown in Fig. 1(*a*). Both techniques detected the same 40 kDa protein, which therefore is the α subunit of G_i. A similar blot of membranes ADP-ribosylated by cholera toxin and stained by antiserum CS-1 (150 μl/20 ml of 1% gelatin in Tris-buffered saline), raised against the *C*-terminal decapeptide of $G_s\alpha$, is shown in Fig. 1(*b*). Both techniques showed two bands, of 42 and 45 kDa, of which the former was more prevalent. These proteins are therefore two forms of $G_s\alpha$. In experiments depicted in Fig. 1(*c*), human adipocyte plasma membranes were first labelled by cholera toxin as described above. The proteins were separated by SDS/polyacrylamide-gel electrophoresis and blotted on to nitrocellulose. Fig. 1(*c*) shows an autoradiograph of the blot and a photograph of the same

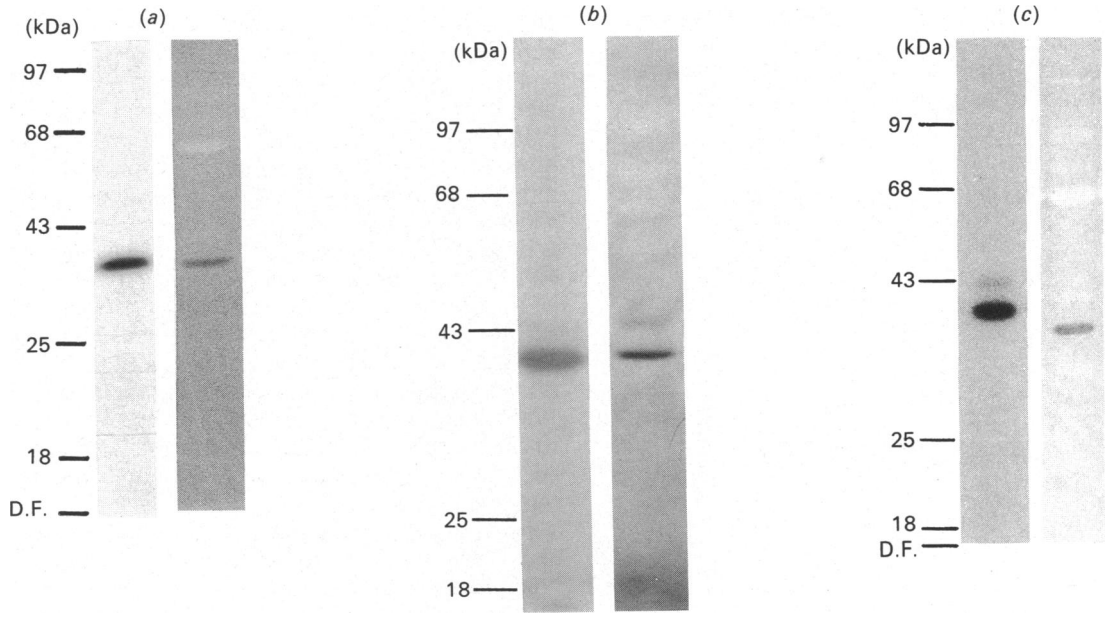

Fig. 1. $G_i\alpha$ and $G_s\alpha$ in human adipocyte plasma membranes

(*a*) Detection of $G_i\alpha$ in human adipocyte plasma membranes by pertussis toxin and antiserum SG-1. Human adipocyte plasma membranes (15 μg) were ADP-ribosylated by pertussis toxin as described in the text. The sample was precipitated by trichloroacetic acid, dissolved in Laemmli (1970) buffer and separated by SDS/polyacrylamide-gel electrophoresis. The proteins were transferred to nitrocellulose paper by electroblotting, and the paper was stained by antiserum SG-1 as described in the text. Right: immunoblotting; left: autoradiograph of the same paper. (*b*) Detection of $G_s\alpha$ in human adipocyte plasma membranes by cholera-toxin labelling and by antiserum CS-1. The experiment was done analogously to that described in Fig. 1(*a*). See the text for details. (*c*) Detection of $G_i\alpha$ and $G_s\alpha$ of human adipocyte plasma membranes in a single blot. Plasma membranes (20 μg) were labelled by cholera toxin and [^{32}P]NAD$^+$. After SDS/polyacrylamide-gel electrophoresis and electroblotting, the blot was autoradiographed (left lane) and stained with antiserum SG-1 (right lane) as described in the text. Abbreviation: D.F., dye front.

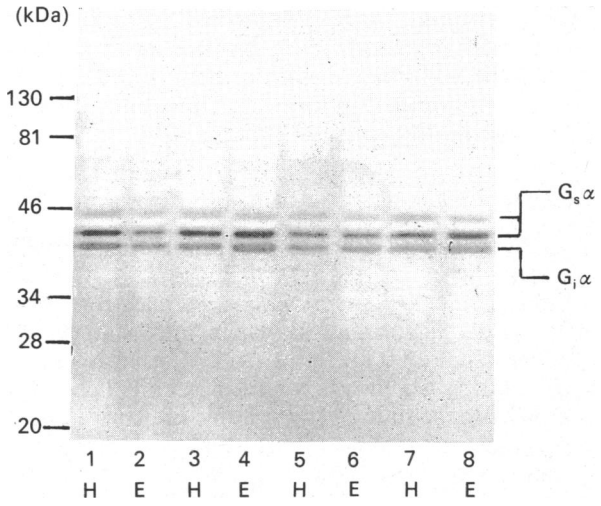

Fig. 2. Detection of $G_i\alpha$ and $G_s\alpha$ in fat-cells from hypothyroid (H) and euthyroid (E) human subjects

Samples (15 μg) of crude fat-cell membrane protein were separated by SDS/polyacrylamide-gel electrophoresis and immunoblotted as described in the text. The numbers refer to Table 1.

blot after staining with antiserum SG-1. It is obvious that $G_i\alpha$ and $G_s\alpha$ can be resolved and observed in the same blot. In experiments described below, $G_i\alpha$ and $G_s\alpha$ were stained on the same blot with a mixture of 30 μl of SG-1 and 150 μl of CS-1 in 20 ml of 1% gelatin in Tris-buffered saline. This ratio was chosen to give bands of about equal intensity. The amount of CS-1 had to be higher because of lower antibody titre and possibly because the amount of $G_s\alpha$ is lower than that of $G_i\alpha$.

Fig. 2 shows a Western blot of fat-cell membranes from four hypothyroid patients and age-, sex- and body-weight-matched controls. There were no differences in the amounts of $G_i\alpha$ and $G_s\alpha$ in absolute or relative terms. The G_i/G_s ratio of the hypothyroid patients was 0.61 ± 0.02, as compared with 0.70 ± 0.05 in the control group (arbitrary units; means $\pm$ S.E.M.). Several runs were performed with the same samples, with similar results. The linearity of the staining was confirmed in several such experiments by running a double amount of one of the samples. In additional experiments, two more such pairs were compared, with similar results (results not shown).

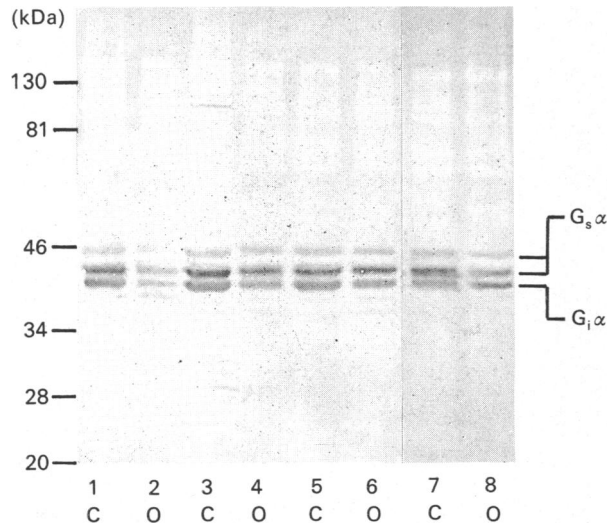

Fig. 3. Detection of $G_i\alpha$ and $G_s\alpha$ in adipocyte plasma membranes prepared from obese (O) and normal-weight (C, control) subjects

The experiment was done as that in Fig. 2. Details of the tissue donors are given in Table 2. The numbers refer to Table 2.

In fat-cell membranes from the four massively obese subjects described in Table 2, the amounts of both G_i (131 ± 17 versus 224 ± 22 arbitrary units/mg of protein; means $\pm$ S.E.M.) and G_s (198 ± 35 versus 324 ± 25 arbitrary units/mg of protein; means $\pm$ S.E.M.) were lower than in membranes of fat-cells prepared from age- and sex-matched controls (Table 2 and Fig. 3). However, the G_i/G_s ratio was the same in both groups (0.71 ± 0.09 and 0.70 ± 0.08 arbitrary units in the normal-weight and obese groups respectively; means $\pm$ S.E.M.). Similar results were obtained with two additional similar pairs of patients (results not shown).

DISCUSSION

Given the known heterogeneity of G-proteins as substrates of pertussis toxin (Jones & Reed, 1987), immunological identification is necessary to confirm the molecular identity of a pertussis-toxin substrate (Milligan, 1988). The presence of the α subunit of G_i in human fat-cell membranes was demonstrated both by [^{32}P]ADP-ribosylation catalysed by pertussis toxin and immunologically, by the use of an antipeptide antiserum which identifies the extreme *C*-terminus of this polypeptide.

Two separate forms of $G_s\alpha$ were identified in these membranes by using either cholera-toxin-catalysed [^{32}P]ADP-ribosylation or an anti-peptide antiserum directed against the *C*-terminal decapeptide of $G_s\alpha$. The individual forms of $G_s\alpha$ are generated by differential splicing of mRNA precursors transcribed from a single gene (Bray *et al.*, 1986). In human fat-cell membranes the 42 kDa form predominated over the 45 kDa form. This is in contrast with rat adipocyte membranes, which express higher levels of the larger form (F. Mitchell & G. Milligan, unpublished work). G_s can function to activate dihydropyridine-sensitive Ca^{2+} channels (Yatani *et al.*, 1988), as well as adenylate cyclase, but there is no reason to believe that the individual forms of G_s display different specificities for either receptor or effector species (Graziano *et al.*, 1987).

Previous studies in rats have suggested that altered levels of the G-proteins (G_i, G_s), which modulate adenylate cyclase activity, might be responsible for the altered sensitivity of fat-cell lipolysis to various hormones in experimentally induced hypothyroidism (Milligan *et al.*, 1987; Ros *et al.*, 1988). In the present study we have attempted to assess whether equivalent alterations in G-protein levels or in the relative ratios of G_i to G_s could be noted in fat-cells obtained from normal human subjects or from subjects who had undergone a thyroidectomy and were not currently receiving hormone-replacement therapy. In contrast with the animal models which have previously been used, we noted no obvious alterations in levels of either $G_i\alpha$ or $G_s\alpha$ which could be

correlated with thyroid function. Lack of thyroid hormones was total in the hypothyroid subjects (see the Materials and methods section), although this state had lasted for only 4 weeks. It is possible that more long-standing hypothyroidism would effect an alteration in G-protein levels in human fat-cells. Obviously, the larger inherent genetic diversity of the population used in these studies might mask some inherent trend, but assessment of the relative ratios of $G_i\alpha$ to $G_s\alpha$ by densitometric scanning of immunoblots from experiments in which mixtures of both anti-$G_s\alpha$ and anti-$G_i\alpha$ antisera were used to identify concurrently these two G-proteins indicated the presence of similar ratios of these polypeptides in all of the subjects studied (Table 1).

In contrast with what is observed in hypothyroidism, fat-cells isolated from obese subjects demonstrate decreased sensitivity in the control of lipolysis to hormones which function via interaction with G_i (Ohisalo *et al.*, 1986; Richelsen, 1988). Levels of both $G_i\alpha$ and $G_s\alpha$ were substantially lower in relation to overall protein concentration in fat-cell membranes derived from obese subjects compared with those from normal subjects. However, no specific trend was noted in the $G_i\alpha/G_s\alpha$ ratio within the two populations (Table 2).

From these studies it would appear that specific alterations in the ratio of the concentrations of G_i and G_s cannot explain the observed alterations in hormonal responsiveness in fat-cells of either hypothyroid or obese human subjects. Further studies on the functionality of expressed G-proteins will now be required, as it has previously been noted that a non-functional form of G_i is present in membranes of hepatocytes from obese, but not lean, Zucker rats. The obese animals display tissue insulin-resistance but their lean littermates do not (Houslay *et al.*, 1988).

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