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Concurrent up-regulation of guanine-nucleotide-binding proteins $G_i1\alpha$, $G_i2\alpha$ and $G_i3\alpha$ in adipocytes of hypothyroid rats

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Rat white adipocytes express three distinct 'G_i-like' guanine-nucleotide-binding proteins (G-proteins) [Mitchell, Griffiths, Saggerson, Houslay, Knowler & Milligan (1989) Biochem. J. **262**, 403–408]. We have previously noted elevated levels of G_i in membranes of adipocytes from hypothyroid rats [Milligan, Spiegel, Unson & Saggerson (1987) Biochem. J. **247**, 223–227]. Using a series of anti-peptide antisera able to discriminate between the individual gene products we have examined levels of each G_i-like G-protein in adipocyte membranes of hypothyroid rats compared with euthyroid controls. We demonstrate that up-regulation of G_i in adipocytes of hypothyroid rats is not restricted to a single subtype of G_i but that each of $G_i1\alpha$, $G_i2\alpha$ and $G_i3\alpha$ is present at markedly higher levels compared with euthyroid animals. In contrast, levels of both the 45 and 42 kDa forms of $G_s\alpha$ were not altered substantially in the hypothyroid state.

INTRODUCTION

Members of a family of heterotrimeric guanine-nucleotide-binding proteins (G-proteins) are involved in the transmission of information from agonist-activated receptors to effector systems which are either enzymes involved in the generation of intracellular second messengers or ion channels [1,2]. Each G-protein consists of individual α, β and γ subunits. Although genetic diversity is known to occur at the level of both β and γ subunits [3,4], it is the identity of the α subunit which defines the nature of each individual G-protein [5]. To date, at least ten separate α subunits have been identified by the isolation of corresponding cDNAs, and it is likely that further species are expressed [6,7].

One sub-division of the G-protein family is based on whether or not the α subunit of the G-protein is a substrate for pertussis toxin-catalysed ADP-ribosylation [5]. Of the currently identified G-proteins, six (G_i1, G_i2, G_i3, G_o, and transducins TD1 and TD2) have a cysteine residue four amino acids from the *C*-terminus of the α subunit, and it is this residue that acts as the acceptor for ADP-ribosylation catalysed by pertussis toxin. This covalent modification is sufficient to prevent productive interactions between receptors and these G-proteins. As pertussis toxin treatment of cells or tissues produces attenuation of receptor-mediated inhibition of adenylate cyclase, then one or more of the pertussis toxin-sensitive G-proteins must function as the inhibitory G-protein of the adenylate cyclase cascade, i.e. G_i. Strong evidence now exists, at least in human platelets [8] and neuroblastoma × glioma hybrid NG108-15 cells [9], to indicate that $G_i\alpha$ is the product of the $G_i2\alpha$ gene.

Receptors linked to inhibition of adenylate cyclase have previously been demonstrated to show increased responsiveness in adipocytes of hypothyroid rats and man in comparison with controls [10–12]. This can be correlated both with increased pertussis toxin-catalysed [^{32}P]ADP-ribosylation of an approx. 40 kDa polypeptide [13] and with increased immunoreactivity detected by an antiserum which is able to identify the α subunits of both G_i1 (41 kDa) and G_i2 (40 kDa) [14]. However, in the experiments to date these two G-proteins could not be separated by the electrophoresis conditions utilized. We have recently defined conditions which allow the resolution of these two G-proteins, and with a series of antisera selective for the various pertussis toxin-sensitive G-proteins we have demonstrated the expression of each of G_i1, G_i2 and G_i3 in rat white adipocytes [15].

In this report we demonstrate that the increased pertussis toxin-catalysed [^{32}P]ADP-ribosylation noted in membranes of adipocytes from hypothyroid rats results from increased levels of each of G_i1, G_i2 and G_i3.

MATERIALS AND METHODS

The sources of all materials used in this study have been reported previously [14–16].

Antibody production and characterization

All antisera used in this study have previously been described [9,15,17]. In synopsis, antisera were produced in New Zealand White rabbits against glutaraldehyde conjugates of keyhole-limpet haemocyanin (Calbiochem) and synthetic peptides which represent sections of the primary sequence of the various pertussis toxin-sensitive G-proteins. Antiserum SG1 was raised against the *C*-terminal decapeptide of the α subunit of TD1 (KENLKDCGLF). This antiserum identifies both $G_i1\alpha$ and $G_i2\alpha$, in addition to the forms of transducin, as both $G_i1\alpha$ and $G_i2\alpha$ differ from TD1α in this region by only a single conservative amino acid substitution. This antiserum does not identify $G_i3\alpha$ [15]. Antiserum I1C was raised against a peptide (LDRIAQPNYI) which is equivalent to amino acids 159–168 of the α subunit of G_i1. This antiserum is specific for $G_i1\alpha$ [15]. Antiserum I3B was raised against a peptide (KNNLKECGLY) which corresponds to the *C*-terminal decapeptide of $G_i3\alpha$. This antiserum does not cross-react with either $G_i1\alpha$ or $G_i2\alpha$. It does however show weak cross-reactivity with $G_o\alpha$ [18]. The expression of $G_o\alpha$ in rat white adipocytes is, however, below current levels of detection [15]. Antiserum CS1 was generated against a peptide (RMHLRQYELL) which corresponds to the *C*-terminal decapeptide of forms of $G_s\alpha$ [17].

Abbreviations used: G-protein, guanine-nucleotide-binding protein; G_i and G_s, inhibitory and stimulatory G-proteins respectively; PBS, phosphate-buffered saline; NP40, Nonidet P40.

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Immunological analysis

SDS/PAGE was performed with membranes from white adipocytes from control and hypothyroid rats using either of two distinct protocols. When immunoblotting was to be performed with an antiserum which is specific for a single G-protein α subunit, then electrophoresis was performed in gels containing 10% (w/v) acrylamide and 0.27% (w/v) bisacrylamide. In cases in which resolution of $G_i1\alpha$ and $G_i2\alpha$ was to be achieved, the gels contained 12.5% (w/v) acrylamide and 0.063% (w/v) bisacrylamide [15]. Proteins were subsequently transferred to nitrocellulose (Schleicher and Schuell) and blocked for 3 h in 5% non-fat skimmed milk (Marvel) in phosphate-buffered saline (PBS; 136 mM-NaCl, 2.7 mM-KCl, 80.5 mM-Na_2HPO_4 and 14.7 mM-KH_2PO_4) pH 7.5. Primary antiserum was added in 1% Marvel in PBS containing 0.2% Nonidet P40 (NP40) and incubated overnight. The primary antiserum was then removed and the blot was washed extensively with PBS containing 0.2% NP40. Secondary antiserum [donkey anti-(rabbit IgG) coupled to horseradish peroxidase (Scottish Antibody Production Unit, Wishaw, Scotland)] was added (1:200 dilution in PBS containing 0.2% NP40) and incubated with the nitrocellulose for 2 h. The antiserum was then removed and, following extensive washing of the blot with PBS containing 0.2% NP40 and finally with PBS alone, the blot was developed using *o*-dianisidine as the substrate for horseradish peroxidase as previously described [15]. In a number of cases the developed immunoblots were subsequently treated with a ^{125}I-labelled donkey anti-(rabbit IgG) (Amersham International) (0.1 μCi/ml) in 1% Marvel/PBS/0.2% NP40 for 2 h. After extensive washing with PBS/0.2% NP40 and then PBS, the blot was air-dried and the coloured bands were excised and radioactivity was assessed in a γ-radiation counter. In such cases preliminary experiments were performed to assess the range of linearity of the assay for each antiserum. These involved immunoblotting and subsequent treatment with ^{125}I-labelled anti-(rabbit IgG) of various amounts of white adipocyte plasma membranes as described above (Fig. 1). Amounts of membranes used to assess the effects of the hypothyroid state on levels of the various G-proteins were, in all cases, within the linear region. Except in cases where particular antisera identify two G-protein α subunits equally (CS1 in the identification of the 45 and 42 kDa forms of $G_s\alpha$ and SG1 in SDS/PAGE conditions which can resolve the α subunits of G_i1 and G_i2), the counting of ^{125}I-anti-(rabbit IgG)-labelled immunoblots cannot be used to assess the relative amounts of the different G-proteins expressed in adipocytes, as binding of the radiolabelled probe will be dependent upon the titre and affinity of each primary antiserum.

All immunoblotting experiments were performed at least three times with equivalent results. Statistical analyses were performed using Student's *t* test for unpaired samples.

Membrane preparations

Male Sprague–Dawley rats were fed a low-iodine diet with 6-n-propyl-2-thiouracil in the drinking water to achieve the hypothyroid state essentially as described in [16], except that the treatment commenced at age 3 weeks and was maintained until the rats were 7 weeks old. At time of death hypothyroid rats weighed 137±8 g and age-matched euthyroid controls were 248±11 g (means±S.D., $n = 40$ in each case). Epididymal white adipocytes were prepared and Percoll-purified plasma membranes were isolated [16]. Each individual preparation utilized the pooled cells from 5 rats. Dimensions of white adipocytes were calculated as described in [14]. As found previously [14], induction of hypothyroidism did not significantly alter the relationship between the abundance of plasma membrane total protein and plasma membrane surface area [3.7±1.1 and 3.0±1.7 mg/m^2 in the euthyroid and hypothyroid cases respectively (means±S.D., $n = 8$ in each case)]. However, by contrast with our previous study [14] in which 9-week-old animals were used, induction of hypothyroidism also did not significantly alter the calculated amount of plasma membrane protein per cell [28±6 pg and 24±13 pg in the euthyroid and hypothyroid cases respectively (means±S.D., $n = 8$)]. All changes described below therefore occurred relative to membrane surface area and per cell as well as relative to plasma membrane protein.

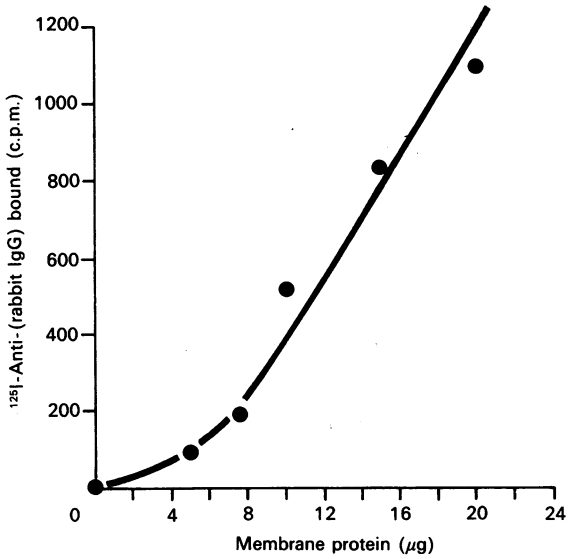

Fig. 1. Quantification using ^{125}I-anti-(rabbit IgG) overlay of horseradish-peroxidase substrate-developed immunoblots

Various amounts of white adipocyte membranes from euthyroid rats were resolved by SDS/PAGE (10% acrylamide, 0.27% bisacrylamide) and immunoblotted using antiserum SG1 as primary reagent. Following treatment with a horseradish peroxidase-linked anti-(rabbit IgG) and colorimetric development, the immunoblot was treated with ^{125}I-anti-(rabbit IgG) (0.1 μCi/ml) as described in the Materials and methods section, and the coloured bands were excised and counted for radioactivity. Areas of the nitrocellulose of equivalent size which generated no colorimetric signal were counted as blanks. Similar results were obtained with membranes from hypothyroid animals.

RESULTS

Following SDS/PAGE [10% (w/v) acrylamide/0.27% (w/v) bisacrylamide], plasma membranes isolated from white adipocytes of both euthyroid and hypothyroid rats were immunoblotted with an antiserum (SG1) which identifies $G_i1\alpha$ and $G_i2\alpha$ equally. This demonstrated a marked increase in levels of the combination of these two polypeptides in the hypothyroid samples. Assessment of the binding of ^{125}I-anti-(rabbit IgG) to these immunoblots demonstrated there to be a significant 2.9-fold increase (hypothyroid, 442±220 c.p.m., mean ±S.D., $n = 8$; euthyroid, 154±29 c.p.m., mean±S.D., $n = 8$; $P < 0.01$).

Immunoblotting of the same membranes with antiserum SG1 after SDS/PAGE under conditions able to resolve the α subunits of G_i1 and G_i2 indicated that levels of each of polypeptide [$G_i1\alpha$ (41 kDa), $G_i2\alpha$ (40 kDa)] were markedly elevated in adipocytes from the hypothyroid rats (Fig. 2). Treatment of the developed immunoblots with ^{125}I-anti-(rabbit IgG) showed that the increase in the levels of $G_i1\alpha$ was 1.6-fold (hypothyroid, 185±29 c.p.m., mean±S.D., $n = 4$; euthyroid, 114±39 c.p.m., mean±S.D., $n = 4$). These values represented a statistically significant difference between euthyroid and hypothyroid samples ($P = 0.03$). The increase in levels of immunoreactive $G_i2\alpha$ was some 2.2-fold

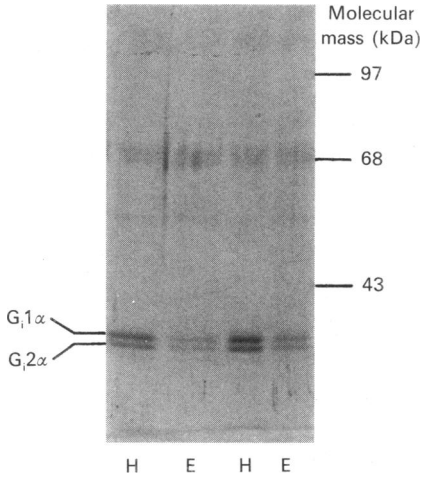

Fig. 2. Increased levels of the α subunits of both G_i1 and G_i2 in adipocyte plasma membranes from hypothyroid rats: immunoblotting with antiserum SG1

Plasma membranes (7 μg) from individual preparations of white adipocytes of hypothyroid (H) and euthyroid (E) rats were separated by SDS/PAGE as described in the Materials and methods section and immunoblotted with antiserum SG1 (1:200 dilution) as first antibody and a horseradish peroxidase-linked donkey anti-(rabbit IgG) as second antibody following transfer to nitrocellulose. The blot was developed using *o*-dianisidine as substrate. Under these gel conditions the α subunits of G_i1 and G_i2 are resolved [15]. This immunoblot was subsequently treated with ^{125}I-anti-(rabbit IgG) for quantitative purposes (see the text for details).

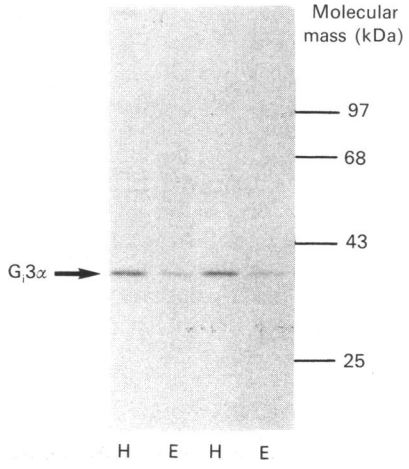

Fig. 3. Increased levels of $G_i3\alpha$ in white adipocytes from hypothyroid rats

Plasma membranes (10 μg) of white adipocytes from both hypothyroid (H) and euthyroid (E) rats were treated as in Fig. 2, except that the primary antiserum used was I3B (1:200 dilution) (raised against a synthetic peptide corresponding to amino acids 345–354 of $G_i3\alpha$).

(hypothyroid, 151 ± 26 c.p.m., mean ± S.D., $n = 4$; euthyroid, 69 ± 9 c.p.m., mean ± S.D., $n = 4$). These values were also significantly different ($P < 0.01$).

Independent confirmation of increased levels (1.9-fold) of $G_i1\alpha$ in plasma membranes of white adipocytes from hypothyroid rats was obtained from immunoblotting experiments using antiserum I1C, which is specific for this polypeptide. After treatment of these developed immunoblots with ^{125}I-anti-(rabbit IgG), the hypothyroid samples contained 408 ± 42 c.p.m. (mean ± S.D., $n = 4$) and the euthyroid samples contained 211 ± 69 c.p.m. (mean ± S.D., $n = 4$). This represented a significant difference ($P < 0.01$). We have recently demonstrated that $G_i3\alpha$ is also expressed in rat white adipocytes [15]. Immunoblotting of white adipocyte plasma membranes with an antiserum (I3B) able to identify this polypeptide demonstrated increases in levels of $G_i3\alpha$ in hypothyroid animals in comparison with the euthyroid controls (Fig. 3). This increase was by some 1.9-fold (hypothyroid, 50 ± 7 c.p.m., mean ± S.D., $n = 4$; euthyroid, 27 ± 12 c.p.m., mean ± S.D., $n = 4$) and was significant ($P = 0.02$). Relative levels of $G_s\alpha$ were assessed using antiserum CS1, which identifies the *C*-terminal decapeptide of all forms of $G_s\alpha$. In contrast with the increased levels of the 'G_i-like' G-proteins, the amounts of each of the 42 and 45 kDa forms of $G_s\alpha$, although showing a trend towards slightly decreased levels on ^{125}I-anti-(rabbit IgG) treatment of the immunoblots [42 kDa; hypothyroid, 196 ± 5 c.p.m; euthyroid, 221 ± 55 c.p.m. (means ± S.D., $n = 4$); 45 kDa: hypothyroid, 258 ± 8 c.p.m.; euthyroid, 301 ± 51 c.p.m. (means ± S.D., $n = 4$)] were not altered significantly (42 kDa, $P = 0.4$; 45 kDa, $P = 0.15$) in membranes from hypothyroid rats compared with euthyroid animals.

DISCUSSION

Lipolysis is inhibited in adipose tissue by agents such as adenosine and the E-series prostaglandins which function by inhibiting the production of cyclic AMP. In the hypothyroid state, the effectiveness of these anti-lipolytic agents is increased [10–12]. One potential mechanism which has been widely discussed for this enhanced effectiveness would be increased levels of the inhibitory G-protein of the adenylate cyclase cascade (G_i). A number of previous studies have demonstrated the potential up-regulation of G_i in plasma membranes of adipocytes from hypothyroid rats in comparison with euthyroid controls. Such studies have used either, or both, of two approaches. The first of these utilizes the ability of pertussis toxin to catalyse a [^{32}P]NAD-dependent [^{32}P]ADP-ribosylation of G_i. Although such studies have shown higher levels of incorporation of radioactivity into an approx. 40 kDa polypeptide, there are two reasons why these results cannot provide definitive evidence for upregulation of G_i. First, pertussis toxin-catalysed ADP-ribosylation of G-protein α subunits will be modified, at least kinetically if not in maximal level, by the concentration of available β/γ subunit. As we and others have previously noted the presence of higher levels of G-protein β subunit in plasma membranes of white adipocytes from hypothyroid compared with euthyroid rats (see [14] for example), then it must be anticipated that the pertussis toxin-sensitive G-proteins in membranes from the hypothyroid animals will act as more effective substrates for pertussis toxin-catalysed ADP-ribosylation than those in membranes from euthyroid animals. It is thus possible that an artifactual increase in pertussis toxin-catalysed incorporation of radioactivity could be noted. Secondly, although it has been demonstrated that G_i2 mediates receptor-driven inhibition of adenylate cyclase in both human platelets [8] and neuroblastoma × glioma hybrid NG108-15 cells [9], it is not known whether G_i2 functions as G_i in adipocyte membranes. This is of particular relevance, as G_i1, the function of which remains undefined, is expressed in adipocytes but not in platelets or NG108-15 cells [8,9]. Pertussis toxin-catalysed ADP-ribosylation of adipocyte membranes will label both G_i1 and G_i2, which are present in approximately equimolar amounts in adipocyte membranes [15], as well as G_i3. Previous studies on alterations in concentration of G_i in hypothyroidism have used SDS/PAGE conditions which are unable to resolve the forms of G_i. Thus it has not been possible to assess if the true G_i was up-regulated or not.

The second approach has been to utilize antisera against the α

Table 1. G-protein subunits in white adipocyte membranes from hypothyroid rats

Results, which represent means ± S.D., are derived from between four and eight individual membrane preparations from different pools of animals. *Significantly different from euthyroid controls ($P < 0.05$).

G-protein subunit	Level (% of euthyroid controls)
$G_i1\alpha$	193 ± 17*
$G_i2\alpha$	219 ± 17*
$G_i3\alpha$	185 ± 24*
$G_s\alpha42$	89 ± 25
$G_s\alpha44$	87 ± 17

subunit of G_i. As noted above, three separate G_i-like G-proteins, G_i1, G_i2 and G_i3, have been identified. Further, a fourth G-protein, G_o, which is also a pertussis toxin substrate, has been shown to be expressed in a range of systems [18,19]. However, prior to this report no studies on the enhanced degree of pertussis toxin-catalysed ADP-ribosylation observed in membranes of hypothyroid adipocytes have used a range of antisera able to identify selectively all of the various G-proteins to determine directly the molecular species involved.

Using such a series of specific anti-peptide antisera, we have recently demonstrated that rat white adipose tissue expresses each of $G_i1\alpha$, $G_i2\alpha$ and $G_i3\alpha$, but that $G_o\alpha$ is essentially undetectable [15]. Herein we demonstrate that the α subunits of each of G_i1, G_i2 and G_i3 are present in increased amounts in membranes of white adipocytes isolated from hypothyroid rats compared with euthyroid controls (Table 1). As such, if G_i2 is the true G_i of the adenylate cyclase cascade in adipocytes, then such an increase in levels would be consistent with the increased effectiveness of anti-lipolytic hormones. Unfortunately, the elevated levels of each G_i-like G-protein and the fact that all three are present at approximately twice the levels found in adipocytes from euthyroid animals does not hint at which one might function as G_i in adipocytes. We noted an overall increase in the hypothyroid samples of 2.9-fold in levels of a combination of $G_i1\alpha$ and $G_i2\alpha$ when the immunoblotting experiments were performed with antiserum SG1 in conditions unable to resolve these two polypeptides (see the Results section). This is a greater increase than that noted when relative levels of the two polypeptides were assessed with the same antiserum in conditions able to resolve these polypeptides ($G_i1\alpha$, 1.6-fold; $G_i2\alpha$, 2.2-fold). This discrepancy is likely to be due to the large standard deviation [S.D. was 50% of the mean (see the Results section)] in the samples from the hypothyroid animals in the experiments in which G_i1 and G_i2 were not resolved. The large standard deviation was due to very high levels of G_i in two of the eight samples analysed, both of which indicated an approx. 5-fold increase in levels compared with the euthyroid samples. When these two samples were excluded from the analysis then the increase was 2.2-fold, and the S.D. fell to 35% of the mean.

Ros *et al.* [20] have recently reported that levels of two pertussis toxin-sensitive G-proteins, $G_i\alpha41$ and $G_o\alpha39$, are increased by some 50 and 70% respectively in fat cell membranes from hypothyroid rats. Although our data would support the view that thyroid status alters the steady-state levels of at least two distinct pertussis toxin-sensitive G-proteins, we cannot agree with the molecular identification of these. It is unclear if the $G_i\alpha41$ of Ros *et al.* [20] corresponds to $G_i1\alpha$, $G_i2\alpha$ or a combination thereof, and we have been unable to detect substantial expression of authentic $G_o\alpha$ ($G_o\alpha39$) in rat white adipocytes [15]. A similar inability to detect $G_o\alpha$ in rat adipocytes has been noted by Hinsch *et al.* [21]. Interestingly, Rapiejko *et al.* [22] have noted no increase in mRNA coding for $G_i2\alpha$ in adipose tissue of hypothyroid rats, whilst recording an increase of some 50% in the total levels of $G_i1\alpha$ and $G_i2\alpha$ protein by immunoblotting with an antiserum (AS7) [23] which, like the antiserum (SG1) used in these studies, identifies these two polypeptides equivalently. Complex regulation of steady-state levels of receptors and G-proteins has been noted such that, in various situations, each of transcriptional, translational and post-translational controls can be seen to operate [24], and it may be that such results suggest a decrease in the rate of turnover of the protein in hypothyroidism rather than an increase in the rate of transcription of the relevant gene. Little is known about the rate of turnover of G-protein subunits, but this is likely to be a fruitful area for future research.

Our failure to observe any significant change in the abundance of either form of $G_s\alpha$ is interesting in view of the observations that elevation of the fat-cell cyclic AMP content [25] or stimulation of lipolysis [11] can be impaired in hypothyroidism. This change is relatively slight when the adenylate cyclase/lipolysis system is stimulated through either the corticotropin or the glucagon receptor [11], but is severe when stimulation is via the β-adrenoceptor [11,25]. The implication therefore is that this lesion is proximal to G_s, and it has been proposed [25] that hypothyroidism selectively affects the ability of the β-adrenoceptor to act productively in this system without changing β-adrenoceptor abundance or the function of G_s. Our finding is not at variance with these conclusions. A simplistic interpretation of data which demonstrate both increased responsiveness of anti-lipolytic agents and elevated levels of forms of $G_i\alpha$ in adipocytes of hypothyroid rats would be that the increased levels of G_i produce the enhanced effectiveness of anti-lipolytic hormones. This, however, is unlikely to provide a complete answer. In human studies, although the general consensus is that the effectiveness of lipolytic agents on adipocytes is blunted, results with anti-lipolytic agents have been somewhat variable [26,27]. Moreover, alterations in the absolute levels of G_i or in the ratio of G_i to G_s have not been noted in membranes from adipocytes of hypothyroid patients [28]. It is of course possible that subtle alterations in G-protein levels in the human hypothyroid patients are masked by the genetic variability of the population. If $G_i1\alpha$ and $G_i3\alpha$ are involved in signalling cascades in adipocytes other than the inhibition of adenylate cyclase, then, potentially, the upregulation of these polypeptides in the rat hypothyroidism model might produce greater sensitivity of responsiveness of these cascades. It has been reported, for example, that α-adrenergic generation of inositol phosphates in rat adipocytes is sensitive to pertussis toxin treatment [29]. It would be of interest to know if this signalling cascade is also altered in hypothyroidism.

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