Chemically induced hypothyroidism produces elevated amounts of the α subunit of the inhibitory guanine nucleotide binding protein (G_i) and the β subunit common to all G-proteins

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Adipocytes of hypothyroid rats display an increased responsiveness to agents which function by inhibiting the production of cyclic AMP. Anti-peptide antisera which selectively recognise the α subunit of the inhibitory guanine nucleotide binding protein (G_i) detected a 40 kDa polypeptide in adipocyte plasma membranes of both euthyroid and hypothyroid rats. Amounts of the α subunit of G_i were elevated some 2-fold in the hypothyroid preparations in comparison with the euthyroid controls, when equal amounts of membrane protein of the two treatments were examined. As cells from the hypothyroid animals contained 2.7 times as much membrane protein as those from the control animals, the amounts of α subunit of G_i are elevated some 5.6-fold per cell in adipocytes of the hypothyroid animals compared with the euthyroid controls. Amounts of the 36 kDa β subunit of G-proteins were also elevated in plasma membranes of adipocytes of hypothyroid animals, in this case by some 50% when compared on a protein basis. These results provide direct evidence for alterations in the amounts of the subunits of G_i caused by the hypothyroid state.

INTRODUCTION

A family of heterotrimeric guanine nucleotide binding proteins (G-proteins) are involved in cellular signal transduction [1,2]. Information from receptors which inhibit the production of cyclic AMP is transduced to the catalytic moiety of adenylate cyclase via an inhibitory G-protein (G_i) [1,2]. The α subunit of G_i is a protein of 40 kDa and is a substrate for ADP-ribosylation catalysed by islet-activating protein, a toxin produced by *Bordetella pertussis*. After ADP-ribosylation, G_i is unable to mediate productive coupling between receptors and adenylate cyclase, as the α subunit can no longer dissociate from its associated β and γ subunits.

Pertussis-toxin-catalysed ADP-ribosylation has been frequently used as a marker for the presence of G_i , but it has recently become clear that G_i is not the sole substrate for this reaction. Transducin (TD_1), the G-protein of rod outer segments, which couples rhodopsin to a cyclic GMP phosphodiesterase [3] and G_o , a novel G-protein [4–6] of undefined function, are among other G-proteins which are also modified by this toxin. Unequivocal designation of a polypeptide solely on the basis of pertussis-toxin-catalysed ADP-ribosylation is thus not possible.

Receptors linked to inhibition of adenylate cyclase have previously been demonstrated to show increased responsiveness in adipocytes of hypothyroid rats in comparison with controls [7–9]. This was correlated with an increase in pertussis-toxin-catalysed ADP-ribosylation of an approx. 40 kDa polypeptide [9]. Here we

demonstrate directly, for the first time, that this represents an increase in amounts of the α subunit of G_i , by using anti-peptide antisera which specifically recognize this polypeptide. Using another selective antiserum, we also demonstrate for the first time that in hypothyroid animals there is a concomitant increase in the G-protein β subunit.

EXPERIMENTAL

Materials

The sources of all materials used in this study have previously been recorded [7,10–12], except that the horseradish-peroxidase-linked anti-rabbit IgG used as second antibody was from the Scottish Antibody Production Unit (Carlisle, Scotland, U.K.).

Antibody production and characterization

Production of the anti-peptide antisera AS6 and AS7 has previously been described [13,14]. Briefly, antisera were raised in rabbits against the C-terminal decapeptide of transducin (KENLKDCGLF), conjugated to keyhole-limpet haemocyanin with glutaraldehyde. Assessment of specificity of the antisera was conducted against G_i and G_o , which were purified from bovine brain as previously described [6]. These antisera recognize the α subunit of G_i strongly [14], but do not interact with the α subunit of G_o . In some cases antibodies against the α subunit of G_i were affinity-purified from these antisera by using a column of pertussis-toxin-sensitive G-proteins

Abbreviations used: G_i , the G-protein (guanine nucleotide binding protein) mediating inhibition of adenylate cyclase; G_o , a pertussis-toxin-sensitive G-protein of undefined function, with an α subunit of 39 kDa.

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Table 1. Calculated dimensions of white adipocytes of euthyroid and hypothyroid rats

Calculations were performed as described in the Experimental section and in [17]. Values are means $\pm$ S.E.M. Statistics were calculated by using Student's *t* test for unpaired samples: n.s., not significant.

Rats	Mean cell diameter (μm)	Mean cell surface area/cell (μm^2)	$10^{-6} \times$ No. of cells/mg of membrane protein	$10^{-9} \times$ Surface area (μm^2 /mg of membrane protein)
Euthyroid (<i>n</i> = 3)	28.6 ± 0.5	2585 ± 98	42.5 ± 6.8	109.1 ± 14.1
Hypothyroid (<i>n</i> = 4)	37.0 ± 1.2	4306 ± 274	15.9 ± 1.7	67.7 ± 6.4
	(<i>P</i> < 0.01)	(<i>P</i> < 0.02)	(<i>P</i> < 0.025)	(<i>P</i> < 0.1, n.s.)

(purified from bovine brain) coupled to CNBr-activated Sepharose 4B. Antibodies against the β subunit, which appears to be common to all members of the G-protein family, were affinity-purified from antiserum RV3 [12]. This antiserum contains antibodies against both the β subunit and the α subunit of G_o . The anti- β -subunit antibodies were selectively affinity-purified on a column of holomeric bovine transducin bound to Sepharose 4B, on which they were retained, whereas the anti- G_o - α -subunit antibodies were not, as they do not display affinity for the α subunit of transducin [12]. SDS/polyacrylamide-gel electrophoresis and immunoblotting were carried out as described previously [10-12]. For M_r estimation on immunoblots, prestained M_r markers (Bethesda Research Laboratories) were used. Quantitative immunoblotting of the G-protein subunits in white-adipocyte membranes against G-proteins purified from bovine brain [6] was performed as previously described [10,12]. The cross-reactivity of the antisera used in these studies with G-protein subunits from a range of species, including rat, has previously been demonstrated [12,14].

Data from the immunoblots were analysed by using a Bio-Rad Gel Scanner linked to an Olivetti M24 personal computer, as previously described in some detail [14,15].

Membrane preparation

Animals were treated to achieve the euthyroid and hypothyroid states as described previously [16]. White adipocytes were prepared as described, and crude and Percoll-purified plasma membranes isolated. Each individual cell preparation utilized the pooled cells from four to eight rats.

Dimensions of white adipocytes from euthyroid and hypothyroid rats (Table 1) were calculated as described by Jamal & Saggerson [17]. Briefly, the mean mass of a preparation of adipocytes was determined from the dry weight (heating at 70 °C to constant weight). DNA determinations were performed on portions of the same cell preparations. Assuming that the cells are spherical, have density 0.9 g/ml and, like all diploid mammalian cells, contain approx. 7.5 pg of DNA/cell [18], then mean diameter per cell and surface area can be calculated [18]. 5'-Nucleotidase was assayed in Percoll-purified membranes and in the initial cell homogenates [19], and was not different in the two treatments. This activity was then used as a reference to calculate the amount of plasma-membrane protein that can be obtained from a

Fig. 1. Antiserum AS6 recognizes the α subunit of G_i .

Different concentrations (0–676 ng) of a mixture of G_i and G_o , purified from bovine brain, were separated on a SDS/10%-polyacrylamide gel overnight at 50 V. The proteins were transferred to a nitrocellulose sheet, which was then blocked with 3% (w/v) gelatin. Antiserum AS6, diluted 1:100, was employed as first antibody and a horseradish-peroxidase-linked anti-rabbit IgG as second antibody. The blot was developed with *o*-dianisidine as substrate. Full details of the immunoblotting procedure are described in [12]. On the basis of Coomassie Blue staining and scanning of the G-protein preparation, the α subunit of G_i comprised 15% of the protein. Lane *a* contained 101 ng, *b* 75 ng, *c* 50 ng, *d* 25 ng and *e* 0 of the α subunit of G_i . Equivalent concentrations of the α subunit of G_o were not recognized by this antiserum or by antiserum AS7. The lack of cross-reactivity of these antisera with the α subunit of bovine brain G_o was confirmed by using antisera AS6, AS7 and RV3, as described for other selective antisera in [12]. Similar experiments with these antisera have been detailed in [26]. The positions of migration of the α subunit of G_i and of the common β subunit are indicated.

quantity of cells and hence to relate cell number to mg of purified membrane protein. This allowed estimations of the differences in cell size of the white adipocytes from the two treatments.

RESULTS

Antisera AS6 (Fig. 1) and AS7 reacted strongly with a range of concentrations of the α subunit of G_i which had

been purified from bovine brain, but not with equivalent concentrations of the α subunit of G_o (results not shown, but see [14]). Antiserum AS6, and antibodies affinity-purified from this antiserum, recognized a 40 kDa protein, corresponding in mobility to the α subunit of purified bovine brain G_i , in Percoll-purified plasma membranes of white adipocytes of both euthyroid and hypothyroid rats. No other proteins within the plasma-membrane preparation were stained, even when the crude antiserum was used. In a number of separate preparations (four hypothyroid and three euthyroid), when equal amounts of Percoll-purified plasma membranes from adipocytes of rats in the two treatments were immunoblotted with antiserum AS6, concentrations of the α subunit of G_i were increased in the hypothyroid preparations by some $110 \pm 15\%$ (mean $\pm$ s.d., $n = 4$; $P < 0.001$) in comparison with the euthyroid animals (Fig. 2). Quantification of the α subunit of G_i in Percoll-purified plasma membranes of white adipocytes was performed by including standard curves consisting of known amounts of purified bovine brain G_i on the immunoblots (see, e.g., Fig. 1). Amounts of the α subunit of G_i in the membranes of euthyroid animals were calculated to be some 25 pmol/mg of protein. Adipocytes isolated from the hypothyroid rats contain 2.7 times as much Percoll-purified plasma membrane per cell as do those from control animals (Table 1), thus calculations based on these parameters show there to be a 5.6-fold increase in G_i per cell in the hypothyroid animals compared with the euthyroid controls. Coomassie Blue staining of SDS/polyacrylamide gels of the Percoll-purified adipocyte plasma membranes showed no gross changes in protein pattern between the two treatments (Fig. 3).

Fig. 2. Detection of the α subunit of G_i in plasma membranes of adipocytes of euthyroid and hypothyroid rats

Samples (50 μ g) of Percoll-purified plasma membranes of adipocytes of individual animals, euthyroid (E) or hypothyroid (H), were separated by SDS/polyacrylamide-gel electrophoresis and immunoblotted by using a 1:100 dilution of antiserum AS6 as first antibody. Further steps in the detection were as detailed in Fig. 1. Abbreviation: D. F., dye front.

To investigate whether the increase in α subunit of G_i was accompanied by corresponding increases in the β subunit, we used anti- β -subunit antibodies which were affinity-purified from antiserum RV3. In immunoblots of the same Percoll-purified adipocyte plasma-membrane preparations, the anti- β -subunit antibodies stained a single band of 36 kDa in preparations from both euthyroid and hypothyroid animals. As with the α subunit of G_i , there was an increase in amounts of the β subunit, in this case by $54 \pm 16\%$ (mean $\pm$ s.d., $n = 4$; $P < 0.01$), in the hypothyroid animals (Fig. 4). Quantitative immunoblotting against standards of the β subunit, which is purified along with the α subunits of G_i and G_o from bovine brain, indicated that β subunit, in Percoll-purified plasma membranes of euthyroid-rat white adipocytes, was present at some 32 pmol/mg of protein. Similar calculations to those used for the α subunit of G_i indicated there to be a 4.2-fold increase in amounts of the β subunit per cell in the hypothyroid preparation in comparison with the euthyroid control.

DISCUSSION

Lipolysis in adipose tissue can be stimulated by catecholamines, corticotropin and glucagon and inhibited by adenosine, nicotinic acid and the E-series prostaglandins [7]. It has previously been shown that treatments which induce a hypothyroid state modify the lipolytic responses to these agents [7-9]. Hypothyroidism

Fig. 3. Coomassie Blue staining of Percoll-purified plasma membranes

Samples (50 μ g) of Percoll-purified plasma membranes of adipocytes of euthyroid (E) and hypothyroid (H) rats were electrophoresed on a SDS/10%-polyacrylamide gel and stained with 0.1% Coomassie Blue.

Fig. 4. Detection of the β subunit of G-proteins in adipocytes of euthyroid and hypothyroid rats

Samples (100 μ g) of Percoll-purified plasma membranes from adipocytes of euthyroid (E) or hypothyroid (H) rats were resolved on a SDS10%-polyacrylamide gel. The proteins were transferred to nitrocellulose and blotted with a 1:100 dilution of anti- β -subunit antibodies, which were affinity purified from antiserum RV3 as first antibody. Further steps in the detection protocol are described in Fig. 1. Lanes a, b and c contain respectively 255, 170 and 85 ng of the β subunit of G-protein which was purified from bovine brain.

decreases responsiveness to the agents which stimulate adenylate cyclase, whereas responses to the hormones which inhibit adenylate cyclase are increased. One potential mechanism of this alteration in responsiveness is that in the hypothyroid state there are higher concentrations of G_i .

Previous studies have shown that adipocytes from hypothyroid animals show increased amounts of a pertussis-toxin substrate of 40 kDa. Pertussis-toxin-catalysed ADP-ribosylation of a protein of this approximate size is frequently taken as an indication of the presence of G_i . However, it is now appreciated that G_i is not the sole substrate for pertussis toxin, and that transducin and G_o , as well as other less well characterized G-proteins, can also be modified [3–6]. Whereas the distribution of transducin is restricted to photoreceptor-containing tissues, G_i is present in many tissues, including the pre-adipocyte cell line NIH 3T3-L1 [20], and may be mistaken for G_i when ADP-ribosylation is used as an assay. A number of other potential problems exist in the use of pertussis-toxin-catalysed ADP-ribosylation as a means of detecting and quantifying G_i in tissues from different sources. Firstly, it is difficult to ensure that ADP-ribosylations carried out *in vitro* have modified all the available G-protein substrate. This is particularly so because endogenous NADases are able to hydrolyse this substrate of the reaction rapidly [21,22]. Also the holomeric forms of pertussis-toxin substrates are more efficiently modified by the toxin than are the individual α subunits [5]; therefore increased amounts of the β subunit would be expected to produce more rapid labelling of a constant pool of available G_i α subunit and hence, under non-equilibrium conditions, suggest an increase in amounts of the α subunit. A more conclusive means of identification of G_i α subunit is thus required. Antisera which specifically recognize particular species of G-proteins offer such a means of identification and have

been used to distinguish between distributions and populations of G_i and G_o [12,23].

Here we utilize anti-peptide antisera which recognize the α subunit of G_i , but not G_o , to demonstrate conclusively that amounts of the α subunit of G_i are substantially increased after treatments which induce a hypothyroid state. We also demonstrate that these treatments concurrently increase amounts of the β subunit of G-proteins. This might be expected if regulation of the expression of the different subunits of G-proteins is co-ordinated. Amounts of G_i α subunit are shown to increase more than 100% in hypothyroid animals in comparison with controls, whereas the β subunit increases by some 50%. As the anti- β -subunit antibodies used in this study recognize the β subunit, which co-purifies with a range of different G-protein α subunits [12], then it is not surprising that the relative increases measured for G_i α subunit and the β subunit are not the same. Quantification of the immunoblots by using purified G-proteins [23] from bovine brain indicate that the α subunit of G_i increases by some 27 pmol/mg of protein in the plasma-membrane fraction of white adipocytes of hypothyroid rats in comparison with euthyroid animals. In comparison, the β subunit was estimated to increase by some 18 pmol/mg of protein. Although it is often stated that there is a single β subunit which interacts with the range of G-protein α subunits, this may be an oversimplification. A doublet of proteins, of 36 and 35 kDa, is frequently observed to co-purify with G-protein α subunits [4,11]. Although the anti- β -subunit antibodies used in the present study recognize the upper polypeptide of this doublet, we cannot be sure that they also recognize the lower band, as the two migrate very closely together. It is thus possible that we are underestimating the total pool of β subunits present in the white-adipocyte plasma membrane. This may explain the small differences in absolute amounts in the alterations of the concentrations of the α subunit of G_i and the β subunit affected by the hypothyroid state. Coomassie Blue staining of SDS/polyacrylamide gels of plasma membranes of euthyroid and hypothyroid rats show no gross alterations in protein pattern, suggesting that the alterations in concentration of the α subunit of G_i and the β subunit are a specific response to the hypothyroid state. The enhanced responsiveness of ligands which inhibit adenylate cyclase, observed in the hypothyroid state, may be mediated by the increase in the subunits of G_i .

We can offer no information on whether the increased amounts of G-protein subunits measured in the hypothyroid state represent an increase in synthesis *de novo* or a decreased rate of turnover. This question may be addressed by probing relevant mRNA amounts with oligonucleotide probes constructed by using information from the recent isolation of cDNAs corresponding to G-protein subunits [24,25].

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