Evidence for altered expression of the GTP-dependent regulatory proteins, G_s and G_i , in adipocytes from aged rats

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The α -subunits of G_i and G_s were quantified in adipocyte membranes from young (2-month) and older (18-month) rats by pertussis-toxin and cholera-toxin labelling respectively. Aging was associated with a 3-fold increase in G_i α -subunit, but only a 2-fold increase in one of the two G_s α -subunit species labelled. The findings may explain the altered sensitivity of adipocytes from aged rats to lipolytic and anti-lipolytic stimuli.

INTRODUCTION

Aging is associated with marked alterations in hormone-sensitivity (Sacktor, 1987). In adipose tissue there are changes in the sensitivity of lipolysis to both lipolytic and anti-lipolytic agents. Thus adipocytes from older rats are resistant to the lipolytic actions of catecholamines (Guidicelli & Pecquery, 1978; Dax *et al.*, 1981; Hoffman *et al.*, 1984b), but are hypersensitive to the anti-lipolytic actions of adenosine (Hoffman *et al.*, 1984a). Endogenous adenosine is thought to be important in regulation of lipolysis, in both man (Kather *et al.*, 1985) and rats (Ebert & Schwabe, 1973; Schwabe *et al.*, 1975).

Catecholamine stimulation, and adenosine inhibition of lipolysis, are mediated respectively via stimulation and inhibition of adenylate cyclase. Thus β -adrenergic receptors activate the enzyme, whereas A_1 adenosine receptors cause inhibition. These receptors regulate adenylate cyclase activity via the GTP-dependent regulatory proteins G_s and G_i , which mediate cyclase activation and inhibition respectively (for reviews, see Houslay, 1983; Gilman, 1987). Aging is associated with increased circulating catecholamine concentrations (Lakatta, 1987), but β -adrenergic-receptor binding has been found to be normal (Dax *et al.*, 1981), slightly decreased (Guidicelli & Pacquery, 1978), or even increased (Hoffman *et al.*, 1984b) in adipose tissue from older rats. Conversely, in adipocytes from aged rats, Hoffman *et al.* (1984a) found little or no change in adenosine receptors. These findings raise the question of whether the G-proteins are altered in aging. In this paper we show evidence that membranes from aged rat adipocytes have approx. 3 times more G_i than do those from young rats. We also demonstrate marked changes in other parameters related to regulation of lipolysis, which may be related to the metabolic changes that occur with aging.

METHODS

Animals

Male Sprague–Dawley rats, approx. 45 days old, were purchased from Harlan Sprague–Dawley (Houston, TX,

U.S.A.). They were given Purina rat chow and tap water *ad libitum* for either 17 months or approx. 2 weeks (young controls).

Isolation of adipocyte membranes

Adipocytes were isolated from epididymal fat-pads by the method of Rodbell (1964). The cells were homogenized, and a crude plasma-membrane fraction was isolated as previously described (Green, 1987). The membranes were suspended in 154 mM-NaCl/10 mM-MgCl₂/50 mM-Hepes, pH 7.6. Protein concentration was determined by the method of Bradford (1976), adjusted to 1 mg/ml, and the membranes were frozen at -70°C .

Toxin-catalysed ADP-ribosylation of G_s and G_i

The α -subunits of G_s and G_i were labelled as follows. Membranes (5 μg) were incubated for 1 h at 37°C in a final volume of 100 μl containing 50 mM-Hepes, 2.5 mM-MgCl₂, 20 mM-thymidine, 5 μCi of [³²P]NAD⁺, 1 mM-ATP, plus either 0.5 μg of pertussis toxin or 20 μg of cholera toxin and 0.1 mM-GTP (cholera and pertussis toxins were pre-activated by incubating with 25 mM-dithiothreitol for 30 min at room temperature immediately before use). The membranes were diluted with 2 ml of 154 mM-NaCl/10 mM-MgCl₂/50 mM-Hepes, pH 7.4, then collected by centrifugation (20000 g, 20 min) and analysed by SDS/PAGE (Laemmli, 1970) in 10% acrylamide gels. The gels were stained with Coomassie Blue and dried, and autoradiography was performed by using Kodak XAR-5 film at -70°C for 8–24 h.

Measurement of A_1 adenosine receptors

Adenosine receptors were quantified by binding of ¹²⁵I-HPIA, and A_1 -adenosine-receptor agonist (Munshi & Baer, 1982; Schwabe *et al.*, 1982; Linden, 1984), to the crude membrane preparations as previously described (Green, 1987). Briefly, membranes (20 μg of protein) were incubated with adenosine deaminase (10 $\mu\text{g}/\text{ml}$) and 0.2 nM-¹²⁵I-HPIA. After 2.5 h at 37°C the membranes were precipitated with poly(ethylene glycol) and counted for radioactivity in a γ -radiation counter. Non-specific binding was determined in the presence of 10 μM -

Abbreviations used: PAGE, polyacrylamide-gel electrophoresis; HPIA, (–)-N⁶-p-hydroxyphenylisopropyladenosine.

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HPIA, and was typically about 10% of total binding (1% of total radioactivity). All data have been corrected for non-specific binding.

Enzyme assays

Adenylate cyclase assays were performed with 100 μ g of membranes for measurement of basal activity, or 25 μ g for measurement of forskolin/ Mn^{2+} -stimulated rates. Assays contained 0.5 mM-ATP, 10 μ g of adenosine deaminase/ml, 0.5 mM-3-isobutyl-1-methylxanthine, 50 mM-Hepes, pH 7.4, 5 mM- MgCl_2 , 1 mg of bovine serum albumin/ml plus, as indicated, 100 μ M-forskolin and 10 mM- MnCl_2 . After 20 min at 37 °C the assays were terminated by boiling for 3 min. The samples were centrifuged (2000 g, 10 min), and cyclic AMP formation was measured in the supernatants by using a competitive protein-binding assay (Amersham). 5'-Nucleotidase activity was determined by measuring conversion of [^3H]AMP into [^3H]adenosine (Newby *et al.*, 1975). Sodium β -glycerophosphate (20 mM) was used to inhibit non-specific phosphatases (Stanley *et al.*, 1980).

Except where otherwise indicated, all values are means $\pm$ S.D. of duplicate determinations performed on five animals. Statistical comparisons were performed by Student's *t* test.

RESULTS

Characteristics of the animals

The young rats in these experiments (approx. 2 months) weighed 226 ± 25 g and the old rats (approx. 18 months) weighed 618 ± 75 g. The mass of epididymal adipose tissue was 1.50 ± 0.29 g and 13.8 ± 3.2 g in the young and old animals respectively. Adipocyte membranes were isolated from five young and five old rats. The membranes (70 μ g samples) were analysed by SDS/PAGE and stained with Coomassie Blue. The overall protein composition of the membranes from young and old rats was comparable (results not shown).

Toxin-labelling experiments

Under the conditions employed, pertussis toxin labelled a single protein, of approx. 41 kDa, thought to be the α -subunit of G_i (Okajima & Ui, 1984; Katada *et al.*, 1986b). The intensity of labelling of this band was markedly greater in the membranes from the older rats (Fig. 1a). The labelled bands were cut from the gels and counted for ^{32}P radioactivity. This revealed an approx. 3-fold increase in ADP-ribosylation of this band (Fig. 1b). These findings suggest that membranes from adipocytes of aged rats possess markedly higher amounts of G_i than do those from young animals.

Unlike pertussis toxin, which labelled only one band, cholera toxin labelled two proteins in adipocyte membranes, of approx. 43 and 47 kDa (Fig. 2). This has been reported by others, and it is thought that the two bands represent variants of the α -subunit of G_s (Parsons & Stiles, 1987). The 24 kDa band is the A-subunit of cholera toxin. Labelling of the 43 kDa band was increased slightly in the aged rats compared with the young, but that of the 47 kDa band was not altered. This was confirmed by excising the bands and counting for ^{32}P (Fig. 2b); the 43 kDa band had an approx. 2-fold increase in labelling, whereas labelling of the 47 kDa band was not significantly different in the old or young rats.

Fig. 1. Pertussis-toxin labelling of membranes from young and old rats

Membranes were incubated with pertussis toxin and [^{32}P]NAD $^{+}$, and then analysed by SDS/PAGE. Panel (a) shows an autoradiogram of the gel. Lanes 1–5 contained 5 μ g of membranes from young rats, and lanes 6–10 contained equal amounts of membranes from old rats. Lane 11 contained membranes incubated with [^{32}P]NAD $^{+}$ but no pertussis toxin. Panel (b): the 41 kDa band was excised and counted for ^{32}P radioactivity. Data are means $\pm$ S.D. for five young and five old rats.

Fig. 2. Cholera-toxin labelling of adipocyte membranes

Fat-cell membranes were incubated with cholera toxin and [^{32}P]NAD $^{+}$. Panel (a) shows the autoradiogram (lanes as in Fig. 1), and panel (b) shows ^{32}P in the 43 and 47 kDa bands (N.S., not significant).

Table 1. Adenylate cyclase, adenosine-receptor binding and 5'-nucleotidase in membranes from young and old rats

Adipocyte membranes were isolated from young and old rats, and assays were performed as described in the Methods section. Abbreviation: N.S., not significant.

	Young rats	Old rats	P
Adenylate cyclase activity (pmol/min per mg of protein)			
Basal	27 ± 3	13 ± 2	< 0.001
+ Forskolin/Mn ²⁺	993 ± 242	953 ± 119	N.S.
¹²⁵ I-HPIA binding (fmol/mg of protein)	167 ± 40	134 ± 8	N.S.
5'-Nucleotidase V _{max} (nmol/min per mg)	18.4 ± 6.1	4.6 ± 2.4	< 0.001
K _m (μM)	16.3 ± 3.0	18.0 ± 3.1	N.S.

Adenylate cyclase activities

Since there appeared to be changes in both G_s and G_i in membranes from aged rats, we also evaluated the catalytic subunit of adenylate cyclase. Quantification of the catalytic subunit is difficult, because changes in the G-proteins may be responsible for altered activity. However, adenylate cyclase activity measured in the presence of forskolin and Mn²⁺ ions is probably a good index of the catalytic subunit (Seamon & Daly, 1981; Daly, 1984; Ho & Shi, 1984). There was no difference in forskolin/Mn²⁺-stimulated adenylate cyclase activities, suggesting that the catalytic subunit is not altered in fat-cell membranes from old rats. Interestingly, the non-stimulated adenylate cyclase activity was about 50% lower in the membranes from aged rats. This difference probably reflects the alterations that we observed in the G-proteins.

Adenosine receptors

A₁ adenosine receptors were quantified by measuring binding of ¹²⁵I-HPIA to the membranes (Table 1). As reported by Hoffman *et al.* (1984a), when expressed per mg of protein, there was no difference between binding to membranes from young and old rats. This confirms that the changes in hormone- and adenosine-sensitivity of lipolysis observed in aging are not related to changes in the adenosine receptor. However, it is possible that differences in adenosine formation are involved. The activity of 5'-nucleotidase, a membrane-bound enzyme involved in adenosine production, is about 75% lower in membranes from the older rats (Table 1). This decreased activity was due to a lower V_{max}, with no change in K_m.

DISCUSSION

We have found a marked increase in ADP-ribosylation of a pertussis-toxin substrate in fat-cell membranes from older (18-month) rats, as compared with young (2-month) animals. Although it is now recognized that pertussis toxin can ADP-ribosylate a number of G-protein α-subunits (Iyengar *et al.*, 1987), under the conditions of our assay a single protein, of 41 kDa, was labelled. This is generally recognized to be the α-subunit

of G_i (Okajima & Ui, 1984; Katada *et al.*, 1986a). This finding may explain previous observations on adipose tissue from older rats. Thus it has been reported that lipolysis is resistant to the stimulatory effects of catecholamines, but hypersensitive to inhibition by adenosine (Guidicelli & Pecquery, 1978; Dax *et al.*, 1981; Hoffman *et al.*, 1984b). Similarly, the cyclic AMP response to these agents is abnormal in membranes from older rats (Dax *et al.*, 1981; Hoffman *et al.*, 1984a). Conversely, the respective receptors are only marginally altered, or not altered at all, as compared with young animals (Guidicelli & Pecquery, 1978; Dax *et al.*, 1981; Hoffman *et al.*, 1984a,b). Thus our finding that G_i is markedly increased may explain this apparent defect in coupling of receptors to adenylate cyclase.

An interesting observation is that the 43 kDa cholera-toxin substrate, which is thought to be a species of G_s α-subunit (Parsons & Stiles, 1987), is also increased in adipocytes from older animals, although not to the same extent as G_i. Since adenylate cyclase is resistant to catecholamines in fat-cells from older animals, this suggests either that the 43 kDa subunit is of minor importance or, more likely, that the large increase in the inhibitory G_i more than outweighs the increase in G_s. This is supported by the observation that basal adenylate cyclase activity, which presumably reflects the overall net status of the G-proteins, is decreased in the older animals.

A problem with any study on aged rats is that the animals also become obese as they age. Thus it is difficult to be certain of whether the phenomena observed are a result of aging or of obesity. Whatever the cause, it is clear that adipose tissue from older rats has markedly different hormone-sensitivity from that from young animals. The current observations provide a potential mechanism for these changes. Such a mechanism could be involved in the alterations in hormone-sensitivity that occur in many pathological and/or physiological conditions.

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