Increased Concentrations of Proteins G_i1 and G_i2 in Adipocytes From Aged Rats Alter the Sensitivity of Adenylyl Cyclase to Inhibitory and Stimulatory Agonists

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We used a series of antipeptide antisera to estimate the relative amounts of G proteins in adipocytes from young lean versus aged obese Sprague-Dawley rats. Western blots were analyzed using antisera that recognize (1) the α-subunits of G_i1 and G_i2 (serum SG1), (2) the α-subunit of G_i3 (serum I3B) (3) two forms of the α-subunit of G_s (serum CS1), and (4) forms of the β-subunits common to all the G proteins (serum BN2). Adipocyte membranes from aged rats contained approximately fivefold to sixfold more α_i1 and α_i2 than those from young rats, but almost equal amounts of α_i3. Membranes from aged rats had a modestly higher (50%) amount of a 43-kd and normal amounts of a 47-kd form of α_s. Membranes from old rats also had approximately a threefold higher amount of β-subunits, consistent with increased concentrations of some of the G proteins but not others. Finally, the functional consequence of these differences in G proteins was investigated by measuring the effect of N^6-phenylisopropyl adenosine ([PIA] an A_1-adenosine receptor agonist) and isoproterenol on adenylyl cyclase activity. Adenylyl cyclase was more sensitive to inhibition by PIA in membranes from old rats than from young rats, but was less sensitive to stimulation by isoproterenol, suggesting that the differences we observed are in functionally active G proteins. These findings may account for the altered sensitivity of adipocytes from old rats to antilipolytic and lipolytic hormones.
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AGING IS ASSOCIATED WITH profound changes in the sensitivity of most tissues to a number of hormones (for review see Sacktor[1]). It has been reported that lipolysis in adipocytes from aged rats is less sensitive to stimulatory hormones, such as catecholamines working through β-adrenergic receptors, than in cells from young animals.[2-4] Conversely, adipocytes from aged rats are more sensitive to inhibitors of lipolysis, specifically adenosine and adenosine analogs.[5] These alterations in the sensitivity of adipose tissue to lipolytic and antilipolytic agents may result in a general decrease in the ability to mobilize fat, thus contributing to the tendency toward obesity that occurs with aging. Adenosine receptors appear to be normal in aged rats.[5,6] However, we have reported that adipocytes from aged Sprague-Dawley rats have a markedly higher amount of a 41-kd pertussis toxin substrate than cells from young rats.[6] This finding suggests that the amount of G_i (a major pertussis toxin substrate) is increased in the older animals. This could explain the higher sensitivity of the cells to adenosine, since A_1 adenosine receptors are thought to inhibit adenylyl cyclase via interaction with G_i.

In our previous studies, we were able to detect only one pertussis toxin substrate in adipocyte membranes. However, it is now clear that there are a number of substrates for pertussis toxin.[7] These include three forms of G_i, termed G_i1, G_i2, and G_i3. All are expressed in rat adipocytes, but G_i1 and G_i2 predominate.[8] Pertussis toxin can also adenosine diphosphate (ADP)-ribosylate G_o and transducin, but adipocytes do not appear to possess either of these.[8,9]

The experiments described in this report were performed to answer four questions. First, does the increase in pertussis toxin labeling in aged rats represent a true increase in the amount of protein, or is it related to an increase in the ability of the protein to serve as a substrate for the toxin? Second, which of the three "G_i-like" gene products is/are altered in adipocytes from aged rats? Third, does the increase in G_i have any functional significance with respect to regulation of adenylyl cyclase? Finally, in our previous report[6] we also found a modest increase in cholera toxin–catalyzed ADP-ribosylation of a 43-kd form of the α-subunit of G_s, raising the question of whether G_s is really increased in adipocytes from aged rats. To answer these questions, we have used a series of antisera that identify various components of both G_i and G_s.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats approximately 45 days old were purchased from Harlan Sprague-Dawley (Houston, TX). They were maintained on a 12-hour light-dark cycle and fed Purina rat chow (Ralston Purina, St Louis, MO) and tap water ad libitum for either 17 months (aged rats) or approximately 2 weeks (young controls). That is, at the time of study the aged rats were 18 to 19 months old and the young were about 8 to 9 weeks old.

Isolation of Adipocyte Membranes

Rats were killed by cervical dislocation, and adipocytes were isolated from epididymal fat pads by the method of Rodbell.[10] The cells were homogenized, and a crude plasma membrane fraction was isolated as previously described.[11] The membranes were suspended in 154 mmol/L NaCl, 10 mmol/L $MgCl_2$, and 50 mmol/L HEPES, pH 7.6. The protein concentration of the membrane suspensions was determined by the method of Bradford,[12] and the membranes were frozen at −70°C.

Antisera

The production and characterization of the antisera used in these studies have been described in detail elsewhere.[8,13] Briefly,

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each was raised in a rabbit, against a synthetic decapeptide corresponding to a sequence in the antigen of interest, coupled to keyhole-limpet hemocyanin. Antiserum SG1 was raised against a synthetic peptide (KENLKDCGLF) corresponding to the C-terminal 10 amino acids of transducin (which is absent from adipocytes). This antiserum recognizes the α-subunit of G_i1 and G_i2 equally, since in the C-terminal region these two molecules are identical and differ from transducin in only one amino acid. Antiserum I3B was raised against a peptide (KNNLKECGLY) corresponding to the C-terminal region of α_i3. Antiserum CS1 was raised against a peptide (RMHLRQYELL) corresponding to the C-terminal decapeptide of all forms of α_s. Finally, antiserum BN2 was raised against a synthetic peptide corresponding to the *N*-terminal 10 amino acids of the β-1 subunit of G proteins (MSELDQLRQE) and recognizes all known forms of β-subunits.

Immunological Quantification of G Proteins

Membranes prepared as described above were diluted to 2 mg/mL and then further diluted with an equal volume of 2×-concentrated Laemmli sample buffer[14] and heated for 5 minutes at 95°C. The membranes were resolved on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (12.5% acrylamide, 0.06% bisacrylamide) and then electrophoretically transferred to nitrocellulose. The gels were loaded with 25 μg protein per lane, except that when blots were to be probed with antiserum I3B 150-μg samples were loaded. Nonspecific protein-binding sites on the nitrocellulose membranes were blocked for 2 hours with 5% dried skim milk in Tris-buffered saline ([TBS] consisting of 20 mmol/L Tris hydrochloride, 500 mmol/L NaCl, pH 7.5) and then washed twice in TBS containing 0.2% Nonidet P-40. Following further washes with TBS, nitrocellulose membranes were incubated with primary antiserum (diluted 1:200 in 1% dried milk in TBS) overnight. The membranes were washed again as described above, incubated with a second antibody (goat antirabbit IgG coupled to alkaline phosphatase, diluted 1:3,000 in 1% dried milk/TBS) for 1 hour and then subjected to the same series of washes, and finally developed with 5-bromo-4-chloro-3-indoyl phosphate and Nitro Blue Tetrazolium using the instructions provided by BioRad (Hercules, CA).

In some experiments (see Results) the bands were quantified with ^{125}I-labeled protein A as follows: The developed blots were washed several times with distilled water, blocked for a further 15 minutes with 5% dried skim milk in TBS, and then washed once with 0.2% Nonidet P-40 in TBS, followed by three washes in TBS. The blots were then incubated with ^{125}I-labeled protein A (0.5 μCi/mL, in 1% dried milk/TBS) for 2 hours, and then washed extensively with TBS over a 1-hour period and allowed to dry. The visible bands were carefully excised and counted in a gamma counter. Equivalent-size pieces of nitrocellulose were cut from parts of the blot with no visible bands to allow subtraction of background.

Adenylyl Cyclase Assay

Adenylyl cyclase assays were performed using 20 μg membranes in a total volume of 100 μL containing the following: 20 mmol/L Tris hydrochloride, 5 mmol/L magnesium acetate, 0.1% bovine serum albumin, 2 mmol/L cyclic adenosine monophosphate (cAMP), 2 mmol/L creatine phosphate, 25 U/mL creatine phosphokinase, 10 μg/mL adenosine deaminase, 50 mmol/L adenosine triphosphate, 0.2 μCi [α-^{32}P]adenosine triphosphate, pH 7.6, plus other additions as indicated in the text and figure legends. The reactions were stopped after 10 minutes by addition of 20 μL 2-mol/L HCl containing approximately 1,500 cpm [^{3}H]cAMP (to allow calculation of recovery of [^{32}P]cAMP). [^{32}P]cAMP was purified by the single-column method of Alvarez and Daniels[15] and counted in a liquid scintillation counter. Recovery of [^{32}P]cAMP was typically 75% to 80% by this technique.

Statistical Analysis

The statistical significance of differences between young and old rats was determined by Student's *t* test. Representative experiments are shown in the figures. Each experiment was performed at least three times using membranes isolated from separate groups of rats.

RESULTS

Figure 1 shows representative Western blots of adipocyte membranes from young and old Sprague-Dawley rats. The young (8- to 9-week-old) animals weighed 220 ± 8 g, and the old animals (18 to 19 months) weighed 647 ± 23 g. In each of the panels (a) to (d), the first lane was loaded with membranes from a young rat and the second lane with membranes from an old animal.

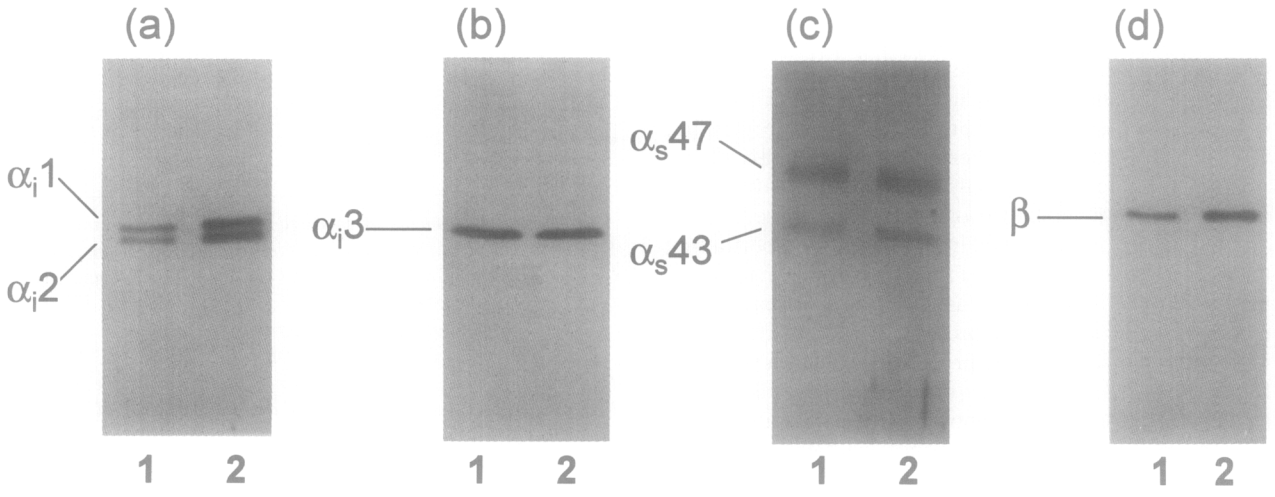

Fig 1. Quantification of G proteins in adipocytes from aged rats. Membranes isolated from a young rat (*lanes 1*) and an old rat (*lanes 2*) were separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose. The blots were probed with antisera SG1 (a), I3B (b), CS1 (c), or BN2 (d) to detect the α-subunits of G_i1, G_i2, G_i3, and G_s and the β-subunits, as indicated.

Figure 1a shows a Western blot probed with antiserum SG1. This serum recognizes two bands, approximately 40 and 41 kd. These are the α-subunits of G_i1 and G_i2, respectively. The two are labeled equally because the sequences of these proteins are identical in the region used for generation of this antiserum. As can be seen, there is markedly more intense labeling of both α_i1 and α_i2 in membranes from aged rats, indicating that the amount of each of these G proteins increases with age. The blot in Fig 1b was probed with antiserum I3B, which recognizes a 41-kd band, the α-subunit of G_i3. In contrast to the markedly more intense labeling of α_i1 and α_i2 in the older rats, there was little or no difference in the labeling of α_i3 between young and old animals.

The relative amounts of α-subunits of G_s in young and old animals were determined with antiserum CS1 (Fig 1c). This antiserum detected two forms of α_s in adipocyte membranes, approximately 43 and 47 kd. There was no difference in the intensity of labeling of the 47-kd band, but there appeared to be slightly more labeling of the 43-kd band in membranes from older rats. This was confirmed by further experiments (see below).

G protein β-subunits were quantified with antiserum BN2 (Fig 1d). Membranes from aged rats had more intense labeling of this 36-kd protein than those from young animals, which is consistent with the finding of increased concentrations of G_i (see Discussion).

To ensure that the differences we observed are not due to a gross difference in the population of membranes isolated from young versus aged rats, a gel was run containing membranes from five different young and five old animals, and then stained with Coomassie blue. There were only minor differences in the overall pattern of bands in the two groups (not shown). Furthermore, we[6] have reported that both adenylyl cyclase activity (maximally stimulated with forskolin and manganese) and A_1-adenosine receptor binding are equal between membranes from young and old rats (also see below). Together, these findings suggest that the differences we observed are due to differences in specific proteins rather than to a difference in the membrane populations isolated from the two groups of animals.

To determine the relative amounts of G proteins in young and old animals more quantitatively, blots developed as before were incubated with ^{125}I-labeled protein A as described earlier. The visible bands were carefully excised and counted for ^{125}I. The technique was first validated by establishing that the number of counts per minute was linear with the amount of adipocyte membrane protein in both young and old rats, up to at least 40 μg membrane protein (data not shown).

Western blots of individual membrane preparations from six young and six old rats were probed with the various antisera as before and then quantified with ^{125}I–protein A as described above (Fig 2). This confirmed the findings shown in Fig 1, and demonstrated that α_i1 and α_i2 were increased approximately fivefold to sixfold in old as compared with young rats. There was no statistically significant difference in α_i3 between the young and old. The 47-kd form of α_s was labeled approximately equally in the young and old, but there was a slight (~50%) statistically significant increase in labeling of the 43-kd form of α_s in membranes from old rats. Consistent with the marked

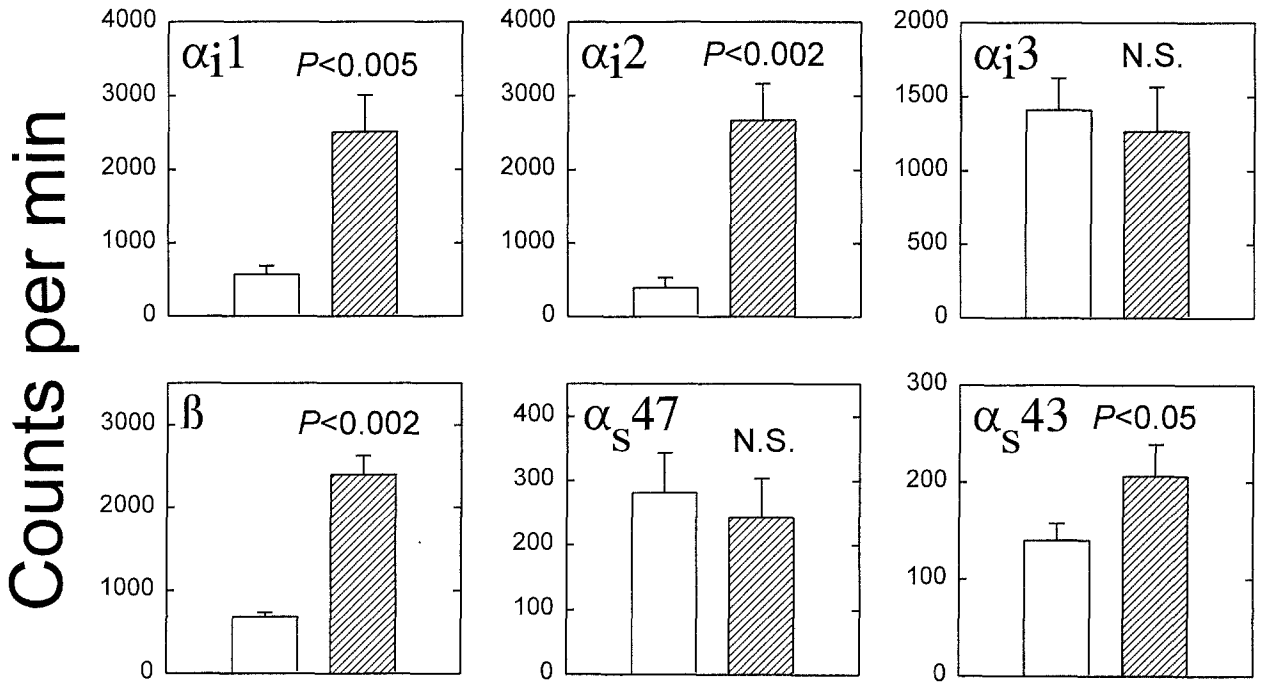

Fig 2. Relative concentrations of G protein subunits in adipocyte membranes. Blots containing individual membrane preparations from six young and six old rats were probed with the various antisera as described in Fig 1. After development, blots were further incubated with ^{125}I-labeled protein A and then washed. The bands were excised and counted for radioactivity. (□) Young rats; (▨) aged rats. Data are the mean ± SEM (n = 6). *P* values were obtained by Student's *t* test.

increase in G_i α-subunits, there was approximately a three-fold increase in the labeling of β-subunits in old rats. These experiments were also performed using membranes from a separate group of three young and three old rats, but instead quantified by laser densitometry of the blots. This technique produced data similar to those obtained with the ^{125}I-labeled protein A technique (data not shown).

The functional consequence of altered G protein concentrations was investigated by determining the effect of an A_1-adenosine receptor agonist, N^6-phenylisopropyl adenosine (PIA), on adenylyl cyclase activity. PIA is a potent inhibitor of adenylyl cyclase, and we have reported that downregulation of forms of G_i decreases the sensitivity of cyclase to inhibition by this agonist.[16] The effect of PIA on adenylyl cyclase activity in membranes from young and old rats is illustrated in Fig 3. The experiment was performed in the presence of a maximally effective concentration of isoproterenol (50 μmol/L) to stimulate adenylyl cyclase. There was no difference in isoproterenol-stimulated cyclase between membranes from old and young animals (not shown). PIA inhibited adenylyl cyclase activity in membranes from young animals by approximately 35%, which is typical for the inhibition of cyclase reported by us[16] and others.[17] However, in membranes from old animals PIA was a more potent inhibitor of adenylyl cyclase, with a maximal inhibition of approximately 55%. Therefore, the increased concentrations of α_i1 and α_i2 are associated with increased sensitivity of adenylyl cyclase to PIA.

The basal activity of adenylyl cyclase was lower in membranes from old rats than in those from young animals (Fig 3b), as we have reported before.[6] This may reflect an increased tonic inhibition of adenylyl cyclase secondary to the increase in forms of G_i. Furthermore, the sensitivity of membranes from old animals to low concentrations of isoproterenol was lower than in membranes from young animals. Thus, the increased concentration of G_i1 and/or G_i2 may alter the sensitivity of adenylyl cyclase to both stimulatory and inhibitory agonists.

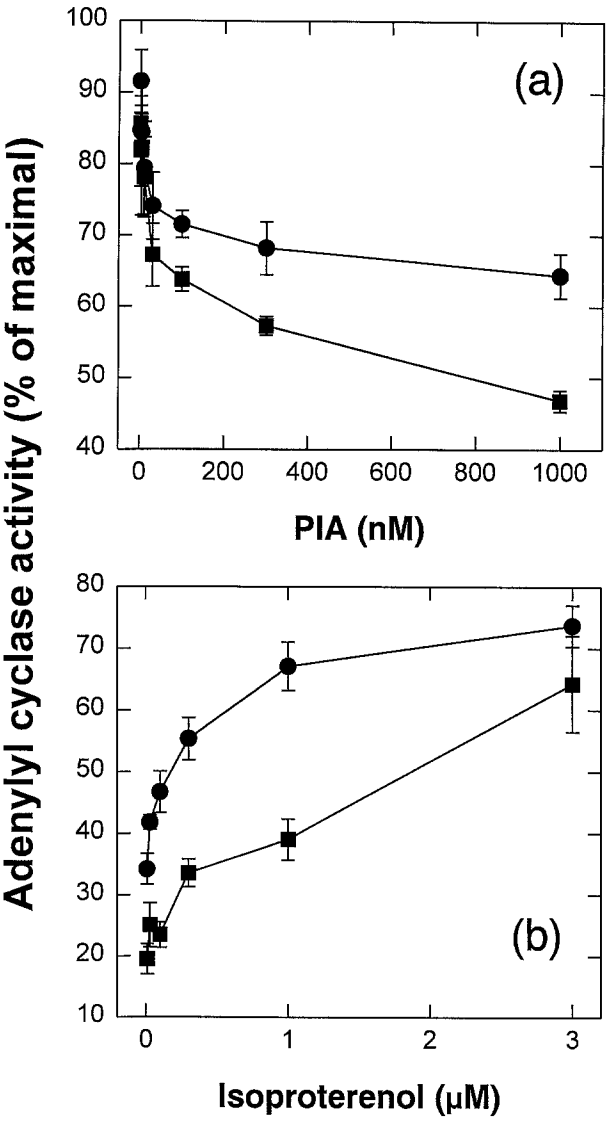

Fig 3. Effect of PIA and isoproterenol on adenylyl cyclase. Four young rats and four old rats were killed, and adipocyte plasma membranes were isolated separately from each animal. Each preparation was assayed in duplicate for adenylyl cyclase activity. (a) Assays were performed in the presence of 50 μmol/L isoproterenol, plus PIA as indicated. (b) Effect of the indicated concentrations of isoproterenol was determined. All data are expressed as a percentage of the activity measured in the presence of 50 μmol/L isoproterenol, which did not differ between membranes from young and old rats. Data are the mean ± SEM (n = 4). (●) Young rats; (■) old rats.

DISCUSSION

The findings presented in this report demonstrate that adipocyte membranes from aged (18-month-old) Sprague-Dawley rats contain markedly higher concentrations of G_i1 and G_i2 than those from young (2-month-old) rats. At the same time, there was no difference in G_i3 and only a modest increase in the amount of one form of G_s in the aged animals. These G proteins are all heterotrimeric molecules consisting of an α-, β-, and γ-subunit. It is believed that the nature of each G protein is determined by its α-subunit and that they all share a common pool of β- and γ-subunits, which are normally tightly bound together.

Several studies have demonstrated that adipocytes from aged Sprague-Dawley rats are markedly more sensitive to antilipolytic agents, in particular adenosine, than those from young rats, but that there is little or no difference in adenosine receptors.[5,6] In this report, we demonstrate that adenylyl cyclase is more sensitive to PIA in membranes from old rats than from young. Furthermore, membranes from older animals are less sensitive to stimulation by isoproterenol as compared with membranes from young animals. With no difference in either maximal adenylyl cyclase activities or adenosine receptors, it seems probable that the higher concentration of G_i1 and/or G_i2 is the explanation for the increased sensitivity to antilipolytic agents, and perhaps also for the decreased sensitivity to lipolytic compounds that has been reported.[2-4]

The findings extend our previous report on G proteins in aged rats.[6] Our previous study was performed using toxin-labeling techniques, which measure the ability of G proteins to serve as substrates for the toxins rather than measuring

the relative amount of protein. The ability of G proteins to serve as substrates for toxins can easily be altered, since it is known that holomeric forms of pertussis toxin–sensitive G proteins are better substrates than dissociated α-subunits, whereas the opposite is true for the cholera toxin–catalyzed ADP-ribosylation of $G_s\alpha$.[7] Furthermore, the changes we saw previously could conceivably have been due to differences in NAD^+ hydrolase activity, which could alter the availability of [^{32}P]NAD for labeling. In our previous report, we were also unable to determine which of the G_i α-subunits were altered because our gel conditions failed to resolve them. We now have found by Western blotting using specific antisera that adipocyte membranes from aged rats have markedly increased concentrations of α_i1 and α_i2, but that there is little or no change in α_i3. Since we have now quantified these G proteins immunologically, this demonstrates that the increase is indeed due to increased amounts of protein rather than to changes in their ability to serve as substrates for the toxins.

There are quantitative differences between our current and previous reports. First, the increase in G_i between young and old rats was greater by Western blot analysis (fivefold to sixfold) than by the pertussis toxin–labeling technique (~threefold). Some of this might be accounted for by the fact that our previous technique measured the sum of the three forms of α_i, since our gel conditions failed to resolve them. However, G_i3 is expressed very little in adipocytes in relation to G_i1 and G_i2,[8] so this is unlikely to be the full explanation for the difference. Second, using the cholera toxin–labeling technique, we saw approximately twice as much of the 43-kd form of α_s in the old membranes. This appears to have been an overestimation, since the Western blotting technique, which is quantitatively more reliable for reasons discussed above, demonstrated only about a 50% increase in this form of G_s.

An alternative explanation for the findings might be that there is a major difference in the population of membranes from aged rats as compared with the young. This is unlikely for several reasons. First, Coomassie blue–stained gels from the two groups appear very similar. Second, in our previous report[6] we found that adenylyl cyclase activities, maximally stimulated with a combination of forskolin and manganese, were equal in the two groups of animals. Similarly, isoproterenol-stimulated adenylyl cyclase was equal in the young and old animals (Fig 3). Finally, A_1-adenosine receptor binding was not altered in the aged rats.[6] Together with the observations that G_s is altered only slightly in the older animals, G_i3 is not altered at all, and two of the three forms of G_i are markedly increased, it seems likely that our findings represent genuine alterations in specific regulatory proteins.

It is not yet established which of the proteins G_i1, G_i2, and G_i3 is/are functional G_i, ie, which is/are responsible for inhibition of adenylyl cyclase. Recent experiments have demonstrated that δ-opioid receptor–mediated inhibition of adenylyl cyclase in neuroblastoma × glioma hybrid NG108-15 cells is transduced specifically by G_i2.[18] Similarly, α_2-adrenergic inhibition of adenylyl cyclase in platelet membranes is mediated by G_i2.[19] Recently, Moxham et al[20] have reported a transgenic mouse model in which expression of $G_i2\alpha$ was selectively blocked by expression of antisense RNA in certain tissues, particularly liver and adipose tissue. Among other things, they found that PIA did not inhibit adenylyl cyclase in liver plasma membranes from these animals, supporting the essential role of α_i2 in the inhibition of cyclase. Interestingly, the biggest change we observed was in α_i2, which was present in more than sixfold-higher levels in membranes from aged rats. Assuming that G_i2 is the major G protein involved in the inhibition of adenylyl cyclase in adipocytes, this would suggest that inhibitory receptors are much more tightly coupled to adenylyl cyclase in adipocytes from aged rats.

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 observed phenomena are a result of aging or of obesity. A recent study of G proteins in adipose tissue from humans suggests that any change in G proteins specifically due to obesity is minor.[21] Thus, it will be important to determine whether the changes we observed are present in age-matched obese rodents or in weight-matched aged animals. Whatever the cause, it is clear that adipose tissue from older rats has a markedly different hormonal sensitivity to that from young animals. The current observations provide a potential mechanism for these changes. Such a mechanism could also be involved in the alterations in hormone sensitivity that occur in other pathological and/or physiological conditions.

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