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G_i DOWN-REGULATION AND HETEROLOGOUS DESENSITIZATION IN ADIPOCYTES AFTER TREATMENT WITH THE α_2-AGONIST UK 14304

SLAVISA GASIC and ALLAN GREEN*

Division of Endocrinology, Department of Internal Medicine and Department of Pharmacology, The University of Texas Medical Branch, Galveston, TX 77555-1060, U.S.A.

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Abstract—Prolonged treatment of rat adipocytes with the A_1-adenosine receptor agonist [−]N^6-phenylisopropyl adenosine (PIA) or prostaglandin E_1 down-regulates G_i and induces heterologous desensitization. α_2-Adrenergic receptors also inhibit adenylyl cyclase through G_i, but whether α_2-receptors are present on rat adipocytes has been controversial. We have investigated the effects of the highly specific α_2-adrenergic agonist UK 14304 (5-bromo-6-[2-imidazolin-2-ylamino]-quinoxaline) on rat adipocytes. In previous studies on young rats, we were unable to demonstrate an effect of the α_2-agonist. We now report that, in cells isolated from older, more obese rats (300–400 g), UK 14304 inhibited lipolysis (measured as the rate of glycerol release) by ~40% (EC_{50} ~40 nM). To determine whether UK 14304 would induce heterologous desensitization, we incubated adipocytes with or without 1 μM UK 14304 for 4 days in primary culture. The cells were then washed, and the rate of lipolysis was determined during a 30-min incubation in the presence of various concentrations of PIA. The concentration–response curve for PIA-induced inhibition of lipolysis was shifted to the right, with the EC_{50} for UK 14304-treated cells about 2-fold higher than in the control cells. This finding demonstrates that the α_2-agonist can desensitize the response to PIA and indicates heterologous desensitization. To investigate the mechanism of this phenomenon, we isolated crude membrane fractions from the cells and analyzed them on Western blots using antibodies against $G_i\alpha1$, 2 and 3. In cells treated with UK 14304 for 4 days, $G_i1\alpha$ and $G_i2\alpha$ were down-regulated to about 15% of the control level, and $G_i3\alpha$ was decreased to 30% of control. We conclude that prolonged treatment of adipocytes with the α_2-agonist induces heterologous desensitization of lipolysis and causes down-regulation of G_i. The findings suggest that G-protein down-regulation is a mechanism for heterologous desensitization.

Key words: G-proteins; adipocytes; α_2-adrenergic agonists; lipolysis; down-regulation; adenylyl cyclase

Lipolysis in adipocytes is regulated by a number of hormones, including insulin, β-adrenergic and α_2-adrenergic agonists, adenosine and prostaglandins. Many of these hormones regulate lipolysis by either stimulating or inhibiting adenylyl cyclase through activation of G-proteins†, hence raising or lowering cellular cyclic AMP concentrations. Cyclic AMP activates protein kinase A, which phosphorylates and activates hormone-sensitive lipase, the rate-limiting enzyme for lipolysis [1, 2].

We recently demonstrated that prolonged treatment of adipocytes with certain agonists that inhibit lipolysis by inhibiting adenylyl cyclase can induce a sustained form of heterologous desensitization [3]. These agonists include PIA (a non-metabolizable A_1-adenosine receptor agonist) and PGE_1. That is, cells treated with PIA become desensitized to PIA (i.e. homologous desensitization), but also become desensitized to PGE_1 (heterologous desensitization). Conversely, adipocytes treated with PGE_1 become desensitized to both PGE_1 and PIA. Furthermore, we have established that the mechanism of this heterologous desensitization involves agonist-induced down-regulation of the various subtypes of G_i, the G-proteins that couple inhibitory receptors to adenylyl cyclase [3, 4]. Since these initial reports, it has become clear that agonist-induced down-regulation of G-proteins is an important mechanism for the regulation of cellular sensitivity to a number of stimuli (for review, see Ref. 5). However, it is also apparent that not all agonists that inhibit adenylyl cyclase through G_i will down-regulate G_i proteins. For example, nicotinic acid inhibits adenylyl cyclase and lipolysis [6], although it is not clear through which receptor this occurs. It is well established that the effect of nicotinic acid is transduced through G_i [7–9]. Despite this, prolonged treatment of adipocytes with nicotinic acid has no effect on cellular concentrations of G_i [3].

The question of whether α_2-adrenergic agonists can induce heterologous desensitization or down-regulation of G_i in adipocytes has not been addressed. Indeed, the question of whether rat adipocytes have α_2-adrenergic receptors has been controversial [10], and in a previous report we were unable to

* Corresponding author: Allan Green, Ph.D., Division of Endocrinology, Department of Internal Medicine, The University of Texas Medical Branch, 301 University Boulevard, Galveston, TX 77555-1060. Tel. (409) 772-1923; FAX (409) 772-8709.

† Abbreviations: G-protein, any member of a family of GTP-dependent regulatory proteins; α_i1, α_i2, α_i3, the α-subunits of G_i1, 2 and 3; α_s, the α-subunit of G_s; UK 14304, 5-bromo-6-[2-imidazolin-2-ylamino]-quinoxaline (an α_2-agonist); PIA, N^6-phenylisopropyl adenosine; PGE_1, prostaglandin E_1; and DPCPX, 8-cyclopentyl-1,3-dipropyl-xanthine.

demonstrate that UK 14304, a highly selective α_2-adrenoceptor agonist, has any effect on lipolysis [3]. However, Rebourcet *et al.* [11] reported that UK 14304 can inhibit lipolysis in rat adipocytes. These investigators used larger animals than we did. This fact, together with the observation that α_2-receptors increase with age in rabbits [12], prompted us to re-evaluate the effect of UK 14304 on lipolysis, and on concentrations of G_i subtypes, using larger animals than those in our previous reports.

In the present report, we confirmed that UK 14304 inhibits lipolysis in adipocytes, provided the animals are above a certain weight. More importantly, we demonstrated that prolonged treatment of adipocytes with UK 14304 down-regulates G_i and induces heterologous desensitization of lipolysis.

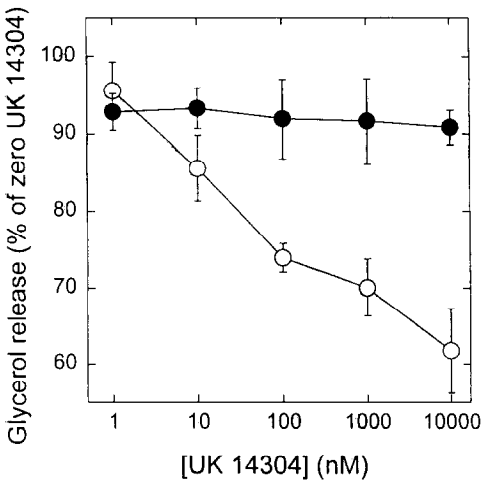

Fig. 1. Effect of UK 14304 on lipolysis in adipocytes. Adipocytes were incubated with adenosine deaminase, plus various concentrations of UK 14304 for 30 min. The rate of lipolysis (glycerol release) was measured as described in Materials and Methods. Values shown with closed circles were measured in the presence of 50 μM yohimbine. Results are means (± SEM) from three separate experiments. Values for glycerol release in the absence of UK 14304 were 250.2 ± 26.4 nmol/30 min/50 mg cells, and 220.8 ± 32.2 nmol/30 min/50 mg cells with 50 μM yohimbine. The rate of glycerol release in the absence of adenosine deaminase was 43.8 ± 7.7 nmol/30 min/50 mg cells.

MATERIALS AND METHODS

Materials. Collagenase was from Worthington (Freehold, NJ). BSA and fetal bovine serum were from Intergen (Purchase, NY). Reagents for electrophoresis and Western blotting were from Bio-Rad (Richmond, CA). UK 14304 was a gift from Dr. A. C. Bostock, Pfizer Central Research (Sandwich, U.K.). [^{3}H]DPCPX was from Amersham. Dulbecco's modified Eagle's medium was purchased from Gibco. Antisera SG1, I3B and CS1 were provided to us by Dr. Graeme Milligan (Molecular Pharmacology Group, Department of Biochemistry, University of Glasgow, U.K.). All other reagents were from Sigma (St. Louis, MO).

Animals. Male Sprague–Dawley rats, ~200 g, were purchased from Texas Animal Specialities (Houston, TX). They were kept for several weeks until their weight was 300–400 g.

Adipocyte isolation. Adipocytes were isolated from epididymal fat pads by the method of Rodbell [13] as previously described [3, 4]. Digestion was carried out at 37° with constant shaking (140 cycles/min) for 45 min. Cells were filtered through nylon mesh (1 mm) and washed three times with buffer containing 137 mM NaCl, 5 mM KCl, 4.2 mM $NaHCO_3$, 1.3 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.5 mM KH_2PO_4, 0.5 mM $MgSO_4$, 20 mM HEPES (pH 7.4), plus 1% BSA.

Lipolysis assay. Adipocytes were suspended at a 5% final concentration (w/v) in the above-described buffer supplemented with 5 mM glucose. In some experiments, adenosine deaminase (10 μg/mL) was included in the incubation medium to prevent accumulation of endogenously produced adenosine, which inhibits lipolysis. Cells were incubated at 37° in a final volume of 1 mL for 30 min with constant shaking. Preliminary experiments (not shown) established that the rate of lipolysis is linear with time for at least 45 min under these conditions. Four minutes before the incubation was finished, shaking was stopped to allow cells to float. Infranatant was transferred to another set of tubes and heated at 70° for 10 min to inactivate any enzymes released by the cells. Glycerol was assayed enzymatically, by the method of McGowan *et al.* [14], using a kit from Sigma.

Primary culture of adipocytes. After isolation, adipocytes were maintained in primary culture according to the method of Marshall and co-workers [15, 16], as described previously [3, 17]. Briefly, after isolation and digestion under sterile conditions, the cells were washed three times with Dulbecco's modified Eagle's medium supplemented with 2% fetal bovine serum, 20 mM HEPES (pH 7.4), 1% BSA and antibiotics. After that, adipocytes were resuspended in the same medium, supplemented with 1 μg/mL adenosine deaminase, and incubated at 37° for up to 4 days at 0.8% final concentration (1 g of cells per 120 mL medium).

A_1-adenosine receptor antagonist binding assay. A_1-adenosine receptor binding was measured with the antagonist radioligand [^{3}H]DPCPX [18]. Plasma membranes (50 μg) were incubated in a final volume of 250 μL containing 0.2 nM [^{3}H]DPCPX, 150 mM NaCl, 10 mM $MgCl_2$, 50 mM HEPES (pH 7.6), 10 μg/mL adenosine deaminase at 22° for 30 min. Non-specific binding was determined in the presence of a large excess (10 μM) of PIA. Reactions were stopped by rapid filtration through 25 mm diameter Whatman GF/B filters, followed by two washes with 4 mL of ice-cold buffer. The filters were counted in 5 mL of scintillation fluid.

Western immunoblotting of G-proteins. Crude plasma membrane fractions were prepared from the adipocytes as previously described [3, 4]. Protein concentrations of the samples were determined by the method of Bradford [19]. Membrane proteins were resolved on SDS–PAGE (12.5% acrylamide,

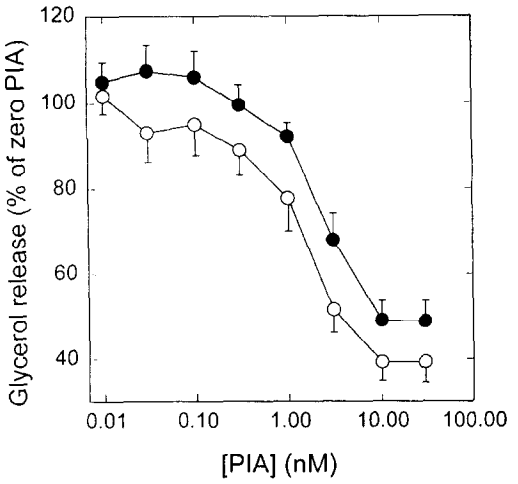

Fig. 2. Inhibition of lipolysis with PIA in UK 14304-treated cells. Adipocytes were incubated for 4 days in primary culture without (○) or with (●) 1 μM UK 14304. The cells were washed to remove UK 14304, and rates of lipolysis were measured over a 30-min period in the presence of adenosine deaminase plus various concentrations of PIA, as indicated. The results are means (± SEM) for six separate experiments. Absolute values for glycerol release in the absence of PIA were 202 ± 15 nmol/30 min/50 mg cells in the controls, and 204 ± 13 in the UK 14304-treated cells. The EC_{50} values were calculated for each individual experiment. The shift in the concentration–response curve was significant ($P < 0.05$), using a paired *t*-test for EC_{50} values for control and treated cells.

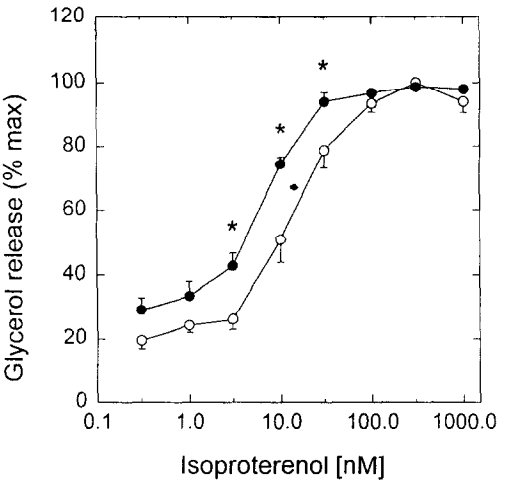

Fig. 3. Stimulation of lipolysis by isoproterenol. Adipocytes were incubated in primary culture for 4 days without (○) or with (●) 1 μM UK 14304, and then washed. The cells were then incubated with various concentrations of isoproterenol (in the absence of adenosine deaminase) as indicated, and the rate of lipolysis was measured over 30 min. The results shown are means (± SEM) of four separate experiments. Differences between the means were tested with a paired *t*-test and statistically significant points ($P < 0.05$) are marked with an asterisk (*). Maximal glycerol release was 241.5 ± 65.1 nmol/30 min/50 mg cells for the control group, and 300.9 ± 45.8 nmol/30 min/50 mg cells for adipocytes incubated with UK 14304 in primary culture.

0.06% bisacrylamide) and electrophoretically transferred from the gel to the nitrocellulose. The gels were loaded with 40 μg of protein/lane. As described in more detail in Ref. 4, specific $G_i\alpha$ proteins were probed with a polyclonal antibody, SG1, which binds to both $G_i\alpha1$ and $G_i\alpha2$, and I3B, which binds to $G_i\alpha3$; forms of $G_s\alpha$ were probed with antiserum CS1. Bands were visualized using goat anti-rabbit secondary antibody coupled to alkaline-phosphatase. The density of the bands was measured with a laser densitometer.

RESULTS

The effect of UK 14304 on lipolysis in freshly isolated adipocytes is shown in Fig. 1. In this experiment, lipolysis was stimulated by including adenosine deaminase (10 μg/mL) in the incubation medium, which resulted in more than a 5-fold increase in the rate of lipolysis compared with the basal, non-stimulated rate. UK 14304 inhibited lipolysis with an EC_{50} of ~40 nM. Maximal inhibition was ~40%. Higher concentrations of UK 14304 (up to 100 μM) did not cause further inhibition (not shown). The effect of UK 14304 was blocked by 50 μM yohimbine, an α_2-antagonist, which is consistent with an α_2-adrenergic receptor-mediated response. In these experiments, animals at least 12 weeks old and weighing an average of 340 g were used. UK 14304 had no effect on lipolysis in adipocytes isolated from younger animals (6 to 8-weeks-old, weighing 200–220 g; data not shown). The inhibitory effect of UK 14304 on lipolysis was not very pronounced, and it appeared to be much less potent and efficacious than other inhibitors of lipolysis that act on adenosine A_1-receptors, like PIA, or on prostaglandin receptors, like PGE_1. Effects of prolonged exposure to the α_2-agonist were investigated by incubating adipocytes in primary culture for 4 days with or without UK 14304 (1 μM). After this prolonged incubation period, cells were washed to remove UK 14304, and rates of lipolysis were measured in the presence of adenosine deaminase and the non-hydrolyzable analog of adenosine, PIA, which inhibits lipolysis through A_1-adenosine receptors. The concentration–response curve for inhibition of lipolysis by PIA was shifted to the right, with the EC_{50} increased about 2-fold in UK 14304-treated cells (Fig. 2) compared with the control. The shift in the concentration–response curve demonstrates a loss of sensitivity to PIA-induced inhibition of lipolysis, that is, heterologous desensitization.

To determine whether treatment of adipocytes with UK 14304 alters adenosine receptor binding, we used an A_1-adenosine receptor antagonist, [^{3}H]-DPCPX. The results indicate that DPCPX binding to plasma membranes prepared from control adipocytes did not differ significantly from adipocytes treated with UK 14304 (13.2 ± 0.8 fmol/mg protein

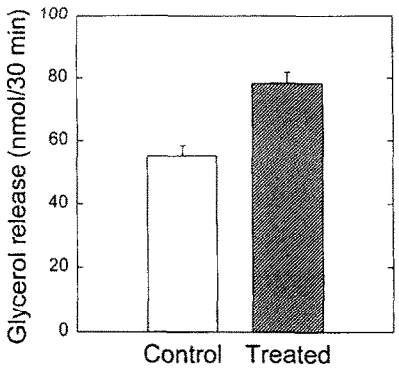

Fig. 4. Increase in basal level of lipolysis in UK 14304-treated adipocytes. The basal rate of lipolysis (non-stimulated, i.e. no isoproterenol or adenosine deaminase) was measured in adipocytes that had been incubated in primary culture for 4 days without or with 1 μM UK 14304, as indicated. Data are the means (± SEM) of four separate experiments performed on different days. The increase in lipolysis in treated cells compared with the controls was highly statistically significant ($P < 0.001$).

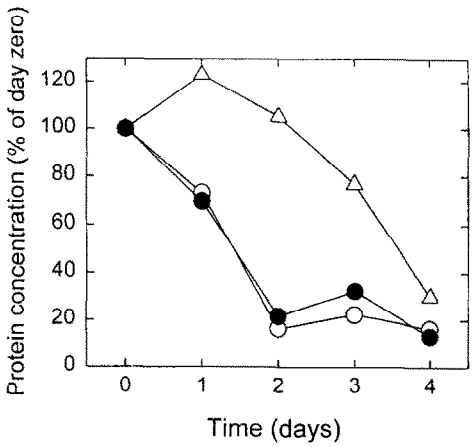

Fig. 5. Immunological quantification of G_i proteins. Adipocytes were incubated with 1 μM UK 14304 for 0–4 days, as indicated. The cells were washed, and membranes were resolved on SDS–PAGE, blotted to nitrocellulose, and developed with specific antibodies as described in Materials and Methods. The bands were quantified with a laser densitometer. The figure shows relative concentrations of each $G_i\alpha$ subtype compared with controls. Key: $G_i\alpha1$ (●), $G_i\alpha2$ (○), and $G_i\alpha3$ (△). Results represent means of two separate experiments.

in controls vs 15.8 ± 4.5 fmol/mg protein in treated). Therefore, the heterologous desensitization in response to UK 14304 is not due to altered adenosine-receptor binding.

In another series of experiments, rat adipocytes were incubated in primary culture for 4 days with 1 μM UK 14304 and washed, and the effect of the β-adrenergic agonist isoproterenol on the rate of lipolysis was measured. Isoproterenol caused a marked stimulation of lipolysis in both control and treated cells (Fig. 3). The concentration–response curve for the treated cells was shifted to the left, with an EC_{50} of about 7 nM, compared with 11.5 nM for the control cells. This indicates increased sensitivity of adipocytes treated with UK 14304 for stimulation of lipolysis.

The basal (non-stimulated) rates of lipolysis in adipocytes incubated with UK 14304 were increased almost 43% ($P < 0.001$) compared with those measured in control adipocytes (Fig. 4). Under normal conditions, lipolysis is tonically inhibited, so this increase in basal rate could be attributed to up-regulation of the stimulatory pathway or to down-regulation of the inhibitory pathway that controls lipolysis.

To examine the possible mechanisms for heterologous desensitization, we measured the relative amounts of G-proteins involved in the signaling pathway for lipolysis inhibition. Adipocytes were incubated with 1 μM UK 14304 for 4 days in primary culture, and a crude membrane fraction was isolated, resolved on SDS–PAGE, and blotted onto nitrocellulose. Specific antibodies were used to probe for $G_i1\alpha$, $G_i2\alpha$ and $G_i3\alpha$, and the bands were quantified with a laser densitometer as in our previous reports [3, 4]. All three proteins were down-regulated (Fig. 5); the decrease in both $G_i1\alpha$ and $G_i2\alpha$ was quite marked from the first day, and they were both down to about 15% of the control value by 2 days. Down-regulation of $G_i3\alpha$ was less pronounced; it was clear by day 3 and reached ~30% by day 4.

To determine whether treatment with UK 14304 changes the concentration of stimulatory G-proteins, we probed the blots with a $G_s\alpha$-specific antibody, which binds to the various splice-variants of this G-protein. We could detect no difference in the concentration of either the 43-kDa or the 47-kDa form of $G_s\alpha$ (Fig. 6).

DISCUSSION

We have found effects of the highly selective α_2-adrenergic agonist UK 14304 on both lipolysis and concentrations of G-proteins in rat adipocytes. First, in freshly isolated adipocytes, UK 14304 inhibited lipolysis moderately (by ~40%). Compared with other inhibitors of rat adipose tissue lipolysis, like adenosine or PGE_1, UK 14304 is both less potent and efficacious. However, UK 14304 is more potent though less efficacious than nicotinic acid [3]. To see an effect of UK 14304, it was necessary to use older, more obese animals than those in our previous studies [3, 4]. UK 14304 did not have any inhibitory effect on adipocytes isolated from younger animals. Unfortunately, the ability of UK 14304 to inhibit lipolysis decreases during the time in culture (as does the ability of PIA and PGE_1 to inhibit lipolysis). Even in fresh cells, the inhibitory effect is modest, and so we were unable to demonstrate a consistent effect after the primary culture step. Therefore, although UK 14304 induces heterologous desensitization, we cannot say with certainty that it induces homologous desensitization.

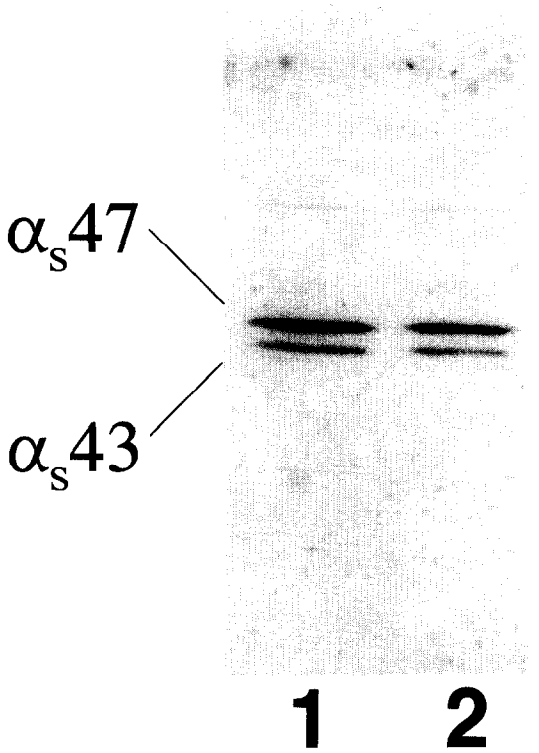

Fig. 6. Western blot of $G_s\alpha$ proteins. Plasma membranes obtained from adipocytes incubated in primary culture for 4 days without (lane 1) or with (lane 2) 1 μM UK 14304 were resolved on 12.5% SDS–PAGE and blotted; $G_s\alpha$ was detected with CS1 antibody, as described in Materials and Methods. Laser densitometry of this, and two other experiments, showed that UK 14304 did not alter concentrations of either the 43- or the 47-kDa form of $G_s\alpha$.

α_2-Adrenergic receptors are important mediators of lipolysis inhibition in many mammalian species, including humans [20–22]. Their expression depends on adipocyte size and seems to be a marker for adipocyte hypertrophy [23]. There is also a specific regional distribution of α_2-adrenergic receptors in regard to the adipose tissue depot [24]. It is well established that age is important in the development of adipose tissue α_2-adrenergic responsiveness in other species like hamsters and rabbits [12, 25]. A functional consequence of this phenomenon is decreased sensitivity to stimulation of lipolysis by agents like epinephrine and glucagon [26]. Whether obesity is caused by up-regulation of antilipolytic cell-signaling systems or this up-regulation is merely a byproduct of obesity is another unresolved question.

To examine the effect of prolonged exposure of adipocytes to UK 14304, we incubated cells in primary culture together with the drug for 4 days. The cells were washed on day 4, and the rates of lipolysis were measured with PIA, another inhibitor of lipolysis that acts through A_1-adenosine receptors. Adipocytes treated with UK 14304, compared with control cells, were less sensitive to the inhibitory action of PIA. That is, UK 14304 induced heterologous desensitization. UK 14304 did not affect binding of an A_1-adenosine receptor radioligand ([^{3}H]DPCPX), thus demonstrating that the heterologous desensitization to PIA that we have observed is not due to an effect of the α_2-agonist on the adenosine receptor. α_2-Mediated responses can be desensitized by agonist treatment in a variety of cell types [27–29]. α_2-Adrenergic receptors undergo subtype selective down-regulation that involves receptor phosphorylation and sequestration [27]. These phenomena can account for homologous desensitization, but considerably less is known about the mechanisms of heterologous desensitization.

Adipocytes treated for prolonged periods with UK 14304 showed increased sensitivity to the lipolytic action of isoproterenol. This increased sensitivity for stimulation of lipolysis may be due to down-regulation of the inhibitory pathway, because we did not detect an increase in $G_s\alpha$. Furthermore, the basal rate of lipolysis in adipocytes treated with UK 14304 was higher, which is consistent with a decrease in the inhibitory component for regulation of lipolysis. Under basal conditions, the rate of lipolysis is very low, inhibited mostly by endogenously produced adenosine that acts on A_1-adenosine receptors coupled through G_i to inhibition of adenylyl cyclase [6, 30]. As results of the adenosine receptor binding experiment show, UK 14304 did not down-regulate adenosine receptors, so it is likely that down-regulation of G_i is also responsible for this increased basal rate of lipolysis.

In previous reports [3, 4], we have demonstrated G_i down-regulation and heterologous desensitization in response to prolonged treatment with PIA and PGE_1. UK 14304 appears to have effects similar to these other agonists, although the magnitude of the heterologous desensitization is less pronounced. The reason for this quantitative difference is not clear, but may be due to the differences in G_i subunit expression in adipocytes from the older, more obese animals used in these studies as compared with the younger, lean animals used in our previous studies. Adipocytes from older animals have markedly higher concentrations of both G_i1 and G_i2 than those from young animals [31, 32]. Therefore, the more modest heterologous desensitization seen after treatment of adipocytes from older animals with UK 14304 may be because they still have fairly high concentrations of G-proteins, even after 80% down-regulation. The coupling of receptors that inhibit lipolysis through different G_i subtypes to adenylyl cyclase may also differ.

In summary, we have demonstrated that prolonged incubation of rat adipocytes with the α_2-adrenergic agonist UK 14304 causes heterologous desensitization of lipolysis to inhibition by the A_1-adenosine receptor agonist PIA. Further experiments showed that UK 14304 also results in down-regulation of G_i proteins, without changes in the level of G_s. The data therefore suggest that this form of heterologous desensitization is caused by G-protein down-regulation.

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