The regulation of adenylate cyclase in adipocyte plasma membrane from genetically obese (ob/ob) mice

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Summary. Adenylate cyclase activity was determined using a purified plasma membrane fraction prepared from isolated adipocytes by a Percoll gradient centrifugation procedure. The activation of adenylate cyclase by isoprenaline was reduced by approximately 50% in membranes from obese mice, although no reduction in the sensitivity of the response was apparent. Basal and fluoride- and 5'-guanylyl-imidodiphosphate-stimulated activities were similar in both groups. However, two defects in the obese system were identified which

suggested that an alteration in the properties of the regulatory protein may account for the impaired hormonal activation. The specific binding of ^3H -dihydroalprenolol to membranes from obese animals also was decreased, suggesting a reduction in the number of β -adrenergic receptors.

Key words: Obese mice, adipocyte plasma membranes, adenylate cyclase, isoprenaline, guanine nucleotides, β -receptors.

The stimulation of adenylate cyclase by β -adrenergic agonists is impaired in white adipose tissue from the genetically obese (ob/ob) mouse [1–5]. This mechanism is just one of a range of membrane-related processes which have been shown to be defective in the ob/ob mouse; others include brown fat thermogenesis [6], hepatic ($\text{Na}^+ + \text{K}^+$) ATPase [7] and glucose transport [8]. We have suggested that these defects may be related to lipid compositional changes in the membranes of ob/ob mice [9, 10].

The hormonal stimulation of cyclic AMP production involves an ordered series of interactions between three distinct membrane proteins, the β -receptor, the regulatory protein, and the catalytic unit of adenylate cyclase [11]. Previous studies using relatively crude membrane preparations have suggested that a defect in the coupling process between the hormone receptor and the regulatory protein may be the basis for impaired isoprenaline stimulation of adenylate cyclase in membranes from ob/ob animals [3–5]. In this study we have used a purified epididymal adipocyte plasma membrane fraction to compare the regulation of adenylate cyclase and β -receptor binding in membranes from lean and obese mice.

Materials and methods

Materials

[2-8- ^3H] Adenosine 3',5' cyclic phosphate (spec. act. 40 Ci/mmol) and [1-propyl-2,3- ^3H] dihydroalprenolol (spec. act. 52 Ci/mmol) were ob-

tained from Amersham International, Bucks, UK. Collagenase (Type I) was from Worthington Biochemicals, New Jersey, USA. Percoll was from Pharmacia Fine Chemicals, London, UK. Creatine phosphate, creatine kinase, cyclic AMP, (–) isoprenaline, 5'-guanylyl-imidodiphosphate (Gpp(NH)p), GTP and bacitracin were from Sigma, Poole, Dorset, UK and Synperonic NXP was from Cargo Fleet Chemicals, Middlesbrough, UK.

Animals

Lean (+/?) and obese (ob/ob) mice (non-defined genetic background, but originating from the Aston strain, Birmingham University, Birmingham, UK), aged 8–10 weeks and bred in the Department's animal facility, were used in the experiments. The housing temperature was 24°C with a constant light-dark cycle (07.00–07.00 h).

Preparations of purified adipocyte plasma membranes

Animals were killed by cervical dislocation and the epididymal fat pads removed. Fat pads from ten lean or six obese animals were pooled and adipocytes were prepared by the collagenase digestion procedure described by Rodbell [12], using 1 mg/ml collagenase and incubating at 37°C with gentle shaking. The isolated adipocytes were broken by homogenisation at 25°C in four volumes of buffer A (Tris-HCl (10 mmol/l), sucrose (0.25 mol/l), EDTA (1 mmol/l), pH 7.4 at 4°C) containing bacitracin (1 mg/ml). Six up and down strokes of a loose fitting pestle were employed at speed setting 2.5 (~2,500 rev/min) on a Tri-R-Stir-R tissue homogenizer (Tri-R Instruments, Rockville Centre, New York, USA). The crude homogenate was defatted by centrifugation at room temperature at 2,000 rev/min for 30 s in a bench centrifuge. All subsequent operations were at 0–4°C. The defatted homogenate was centrifuged at 16,000 g for 15 min in the 8 × 50 fixed angle rotor of an MSE Hi-Spin 21 centrifuge (MSE Scientific Instruments, Crawley, Sussex, UK). The supernatant was discarded and the 16,000 g pellet resuspended in 5 ml buffer A.

Table 1. Marker enzyme activities in crude membranes (16,000 g pellet) and purified adipocyte plasma membranes from lean and obese mice

Enzyme	Lean mice			Obese mice		
	Specific activity	Purification (-fold)	Recovery (%)	Specific activity	Purification (-fold)	Recovery (%)
5'-Nucleotidase (nmol · min ⁻¹ · mg ⁻¹)						
Crude	0.52 ± 0.15	2.5	57.0 ± 3.0	0.11 ± 0.03	3	60.1 ± 7.2
Purified	1.28 ± 0.33			0.33 ± 0.07		
Succinate-cytochrome reductase (μmol · min ⁻¹ · mg ⁻¹)						
Crude	0.202 ± 0.036	0.18	5.8 ± 1.3	0.167 ± 0.008	0.39	5.8 ± 1.8
Purified	0.037 ± 0.006			0.066 ± 0.007		

Values represent mean ± SEM of three determinations

This crude plasma membrane fraction was further purified by Percoll gradient centrifugation as described by Belsham et al. [13]. Percoll was mixed with buffer A and a buffer containing Tris-HCl (20 mmol/l), sucrose (2 mol/l) and EDTA (2 mmol/l) in the proportions 7:32:1 (by vol). The membrane suspension was dispersed in 40 ml of this Percoll solution, and the mixture centrifuged at 16,000 g for 20 min in the fixed angle rotor. The Percoll solution forms a density gradient during centrifugation and the purified plasma membrane fraction collects just below the surface of the gradient. This fraction was removed in a volume of ~5 ml, washed by diluting to 45 ml with buffer A and harvested by centrifugation at 30,000 g for 20 min in the fixed angle rotor. The pellet was resuspended in buffer A to give a final concentration of 1–2 mg/ml and stored at –70 °C under nitrogen.

Assay of adenylate cyclase activity

Adenylate cyclase activity was assayed as described by Houslay et al. [14], except that the regenerating system consisted of creatine phosphate (20 mmol/l) and creatine kinase (20 U/ml). Aliquots of the supernatant were assayed for cyclic AMP by the adrenal receptor assay of Brown et al. [15].

Assay of ³H-dihydroalprenolol binding to adipocyte plasma membranes

Aliquots of purified plasma membrane (150–200 μg) were incubated with ³H-dihydroalprenolol (1–10 nmol/l) in a reaction mixture (1 ml) containing Tris-HCl (50 mmol/l), pH 7.8, and MgCl₂ (20 mmol/l), either in the presence or absence of a 1,000-fold excess of (–) isoprenaline (100 μmol/l). The tubes were incubated for 30 min at 30 °C. The reaction was terminated by vacuum filtration on Whatman GF/C glass fibre filters and the filters were washed with 12 ml of ice-cold Tris-MgCl₂ buffer. Filtration and washing took <10 s. The filters were placed in scintillation vials and the radioactivity eluted with 0.5 ml, 2% sodium dodecyl sulphate for 1 h and counted in 5 ml Tritosint scintillation fluid (15 l xylene; 7.5 l synperonic NXP; 90 g PPO; 6.75 g dimethyl-POPPOP).

Enzyme assays

5'-nucleotidase (E. C. 3.1.3.5) was assayed as described by Avruch and Wallach [16]. Succinate-cytochrome c reductase was estimated as described by Tisdale [17].

Protein determinations

Protein was determined by the method of Lowry et al. [18] or Bradford [19], using bovine serum albumin as standard.

Statistical analysis was by the Student's t-test.

Results

Analysis of the purified plasma membrane preparations

It was necessary to establish that the Percoll gradient procedure afforded comparable purification of plasma membranes from lean and obese mice. Table 1 shows details of the activities of plasma membrane 5'-nucleotidase and mitochondrial succinate-cytochrome c reductase marker enzymes in both the crude 16,000 g pellet, obtained after disruption of the isolated adipocytes, and the purified fraction. 5'-nucleotidase activity was consistently lower in the membrane fractions obtained from obese animals, confirming a previous report [2]. However, on the basis of 5'-nucleotidase activity, the Percoll gradient procedure resulted in a similar level of purification and recovery of plasma membranes from lean and obese animals. Analysis of succinate cytochrome c reductase activity indicated a slightly higher level of mitochondrial contamination in the plasma membrane fraction from obese animals, but the recovery of activity in this fraction was low.

A comparison of the stimulation of adenylate cyclase activity by various agonists in membranes from lean and obese animals

The response of adenylate cyclase in purified adipocyte plasma membranes from lean and obese mice to various agonists is shown in Table 2. Basal activity was similar in membranes from lean and obese mice, as was the degree of stimulation by NaF (fourfold) and Gpp(NH)p (fourfold). Isoprenaline gave a significantly lower stimulation of adenylate cyclase activity in 'obese' membranes; 50 μmol/l isoprenaline produced a fivefold stimulation in 'lean', but only a threefold stimulation in 'obese' membranes. The dose-response curves for the stimulation of adenylate cyclase by isoprenaline in membranes from lean and obese animals are illustrated in Figure 1. Although the stimulation elicited by isoprenaline was lower in the 'obese', the concentrations of isoprenaline required to produce half-maximal and

Table 2. The effect of various agonists on adenylate cyclase activity in adipocyte plasma membrane from lean and obese mice

Agonist	Adenylate cyclase activity (pmol·min ⁻¹ ·mg ⁻¹)	
	Lean mice	Obese mice
Basal (n = 12)	153 ± 22	156 ± 25
Sodium fluoride (15 mmol/l) (n = 9)	874 ± 182	878 ± 165
Isoprenaline (50 µmol/l) (n = 16)	978 ± 174	538 ± 98 ^a
Gpp(NH)p (100 µmol/l) (n = 8)	879 ± 225	814 ± 198

Values represent mean ± SEM. ^a $p < 0.01$ significant difference between preparations from lean and obese mice

maximal stimulation were the same in 'lean' and 'obese' membranes, being 5 and 50 µmol/l, respectively.

In the presence of Gpp(NH)p (100 µmol/l) the sensitivity of adenylate cyclase to isoprenaline was increased in membranes from both lean and obese animals; the concentration required to obtain half-maximal activity being reduced to 1.5 µmol/l in both cases. The presence of GTP did not affect the sensitivity to isoprenaline in membranes from lean animals, but reduced the sensitivity to isoprenaline in the obese, the concentration required to obtain half-maximal activity being increased to 15 µmol/l.

Dose-response curves for the stimulation of adenylate cyclase by guanine nucleotides are illustrated in Figure 2. For stimulation by Gpp(NH)p the maximum response was similar in both groups and the concentrations required to produce half-maximal and maximal stimulation were the same in lean and obese mice (1 and 10 µmol/l respectively). The response of adenylate cyclase to GTP was lower than the response to the non-hydrolyzable Gpp(NH)p in both 'lean' and 'obese' groups. In membranes from lean mice, the concentration of GTP required to obtain half-maximal stimulation was 0.5 µmol/l with maximal stimulation at 3 µmol/l, whereas in 'obese' membranes the dose-response curve was shifted to the right with no clear maximum stimulation even at 100 µmol/l GTP.

The effect of isoprenaline on the dose-response curves to guanine nucleotides was investigated. Figures 3 and 4 show comparisons of dose-response curves to Gpp(NH)p and GTP in the presence and absence of isoprenaline (50 µmol/l). In the presence of isoprenaline, the stimulation of adenylate cyclase elicited by Gpp(NH)p was lower over the whole concentration range in membrane from obese animals (Fig. 3). The response to Gpp(NH)p in the presence of isoprenaline was approximately additive in both cases. However, isoprenaline did not alter the concentrations of Gpp(NH)p required to obtain half-maximal or maximal stimulation in 'lean' or 'obese' preparations (1 and 10 µmol/l respectively). The stimulation of adenylate cyclase by GTP in the presence of isoprenaline was lower also in membranes from obese animals (Fig. 4). In both 'lean' and 'obese' preparations, the presence of isoprenaline

Fig. 1. Dose-response curves for the stimulation of adenylate cyclase by isoprenaline in adipocyte plasma membranes from lean (●) and obese (○) mice. The results are expressed as a percentage of the maximum isoprenaline-stimulated adenylate cyclase activity obtained in lean animals and represent the mean ± SEM of four paired experiments with different membrane preparations. Maximum isoprenaline-stimulated activity in lean mice was 855 ± 162 pmol·mg⁻¹·min⁻¹ **Fig. 2.** Dose-response curves for the stimulation of adenylate cyclase activity by Gpp(NH)p (●) and GTP (○) in adipocyte plasma membrane from lean and obese mice. The results are expressed as a percentage of the maximum Gpp(NH)p stimulated adenylate cyclase activity obtain in lean animals and represent the mean ± SEM of three experiments with different membrane preparations. Maximum Gpp(NH)p-stimulated activity in lean mice was 866 ± 147 pmol·mg⁻¹·min⁻¹

Fig. 3. Dose-response curves for the stimulation of adenylate cyclase by Gpp(NH)p in the presence and absence of isoprenaline in adipocyte plasma membrane from lean and obese mice. Gpp(NH)p alone (○); Gpp(NH)p + isoprenaline (50 μmol/l) (●). The results are expressed as a percentage of the maximum Gpp(NH)p + isoprenaline stimulated adenylate cyclase activity obtained in lean animals and represent mean ± SEM of three paired experiments with different membrane preparations. Maximum Gpp(NH)p + isoprenaline stimulated activity in lean mice was $1,500 \pm 254$ pmol · mg⁻¹ · min⁻¹.

and GTP together resulted in a synergistic stimulation of adenylate cyclase. In membranes from lean animals, isoprenaline did not alter the concentration of GTP required for half-maximal or maximal stimulation (0.5 and 3 μmol/l respectively). By contrast, in obese animals isoprenaline caused a leftward shift in the dose-response curve to GTP; the concentrations required for half-maximal and maximal stimulation being 1.2 and 3 μmol/l respectively. However, even in the presence of isoprenaline, the concentration of GTP required to give half-maximal stimulation was still higher in 'obese' than in 'lean' membranes.

Fig. 4. Dose-response curves for the stimulation of adenylate cyclase by GTP in the presence and absence of isoprenaline in adipocyte plasma membranes from lean and obese mice. GTP alone (○); GTP + isoprenaline (50 μmol/l) (●). The results are expressed as a percentage of the maximum GTP + isoprenaline stimulated adenylate cyclase activity obtained in lean animals and represent mean ± SEM of three paired experiments with different membrane preparations. Maximum GTP + isoprenaline stimulated activity in lean mice was $1,100 \pm 100$ pmol · mg⁻¹ · min⁻¹.

Time course for the stimulation of adenylate cyclase activity by Gpp(NH)p

The time courses for stimulation of adenylate cyclase activity by Gpp(NH)p in the presence and absence of isoprenaline are shown in Figure 5. In adipocyte plasma membranes from lean animals, stimulation by Gpp(NH)p alone was preceded by a lag period of approximately 2 min; however, with membranes from obese animals, the lag period was increased to approximately 5 min. Following the lag period, the Gpp(NH)p-activated rate was similar in both cases. The presence of

isoprenaline reduced the lag period prior to activation in membranes from both lean and obese animals to approximately 1 and 2 min, respectively. Following the reduced lag period, the presence of isoprenaline resulted in a 100% increase in the adenylate cyclase activity in membranes from lean mice, but only a 20% increase in the activity in membranes from obese mice.

The binding of ^3H -dihydroalprenolol to adipocyte plasma membranes

The specific binding of the labelled β -adrenergic ligand, ^3H -dihydroalprenolol, to adipocyte plasma membranes was investigated using isoprenaline (100 $\mu\text{mol/l}$) as the displacing ligand. Figure 6 shows Scatchard analysis of ^3H -dihydroalprenolol binding over the 0–6 nmol range. The results suggest the presence of a binding site with a dissociation constant (K_d) of approximately 10 nmol/l in membranes from both lean and obese mice, but indicate a reduction in the number of binding sites (B_{max}) of approximately 30% in membranes from obese animals (120 fmol/mg protein in lean versus 80 fmol/mg protein in obese). Because of the large number of animals required to prepare the purified membrane fraction for use in binding studies, repeat experiments were not possible.

Discussion

We have used a purified plasma membrane fraction prepared from isolated adipocytes to investigate the cause of the defect in isoprenaline stimulated adenylate cyclase activity observed in obese mice. This contrasts with previous studies in which relatively impure membrane preparations were used [1, 3, 5, 9]. Our results indicate that basal adenylate cyclase activity is similar in lean and obese mice, confirming a previous study in which a purified membrane preparation was also used [2]. These results contrast with studies using either crude membrane fractions [3, 5, 9] or fat cell ghosts [4] in which lower basal activity was reported. Despite these discrepancies, an impaired response of adenylate cyclase to β -agonists in membranes from obese animals has been reported universally [1–5, 9]. This observation was confirmed in our studies in which the maximum stimulation of activity by isoprenaline was reduced by approximately 50% in obese mice.

Present evidence indicates a collision coupling mechanism for the hormonal activation of adenylate cyclase in the presence of guanine nucleotides [11, 20]. Binding of hormone to its receptor initiates a transitory coupling between the hormone-receptor complex and the regulatory protein which induces a conformational change and activation of the regulatory protein by the exchange of GDP for GTP. The activated regulatory protein then interacts with the catalytic unit which is in turn activated to catalyze the conversion of ATP to cy-

Fig. 5. Time courses for the stimulation of adenylate cyclase by Gpp(NH)p in the presence and absence of isoprenaline in adipocyte plasma membranes from lean and obese mice. Gpp(NH)p alone (100 $\mu\text{mol/l}$) (○) Gpp(NH)p + isoprenaline (50 $\mu\text{mol/l}$) (●). The data given represent one of two similar experiments

Fig. 6. Scatchard analysis of specific ^3H -dihydroalprenolol (DHAP) binding to adipocyte plasma membranes from lean (●) and obese (○) mice. Adipocyte plasma membranes (180 μg) were incubated with ^3H -dihydroalprenolol as described in the Methods section. Data points represent the mean value obtained from triplicate determinations. The dissociation constants (K_d) and maximal number of binding sites (B_{max}) were calculated by regression analysis. Correlation coefficients were 0.94 and 0.7 for binding of ^3H -dihydroalprenolol to membranes from lean and obese animals respectively

clic AMP. The rate-limiting step may be either the dissociation of GDP from the regulatory protein [21] or a slow conformational change in the regulatory protein from an inactive to an active form [22]. Fluoride ions stimulate adenylate cyclase by causing an interaction between the regulatory and catalytic components without any exchange of GDP for GTP [23]. In this study, adenylate cyclase activation by fluoride was similar in membranes from lean and obese mice, suggesting that both the coupling between the regulatory and catalytic components and the number of catalytic units was normal in obese animals.

However, this investigation has identified two clear defects in the adenylate cyclase system of white adipocytes of obese animals in the absence of isoprenaline, which suggests that an alteration in the properties of the regulatory protein may contribute towards the impaired hormonal activation. Firstly, the sensitivity of adenylate cyclase to stimulation by GTP was reduced in obese animals, although the sensitivity to non-hydrolyzable Gpp(NH)p was normal. These results conflict with those of Begin-Heick [5], who found no differential sensitivity of the enzyme to GTP and Gpp(NH)p in 'obese' preparations in the presence or absence of isoprenaline. However, a crude membrane preparation was used in those studies, and no maximal response to GTP alone was observed in either 'lean' or 'obese' membranes. An enhanced GTPase activity or an increased availability of GTP for hydrolysis may be responsible for the impaired response in obese mice, since hydrolysis of GTP bound to the regulatory protein is associated with inactivation of the catalytic unit. However, alternative explanations, such as a defect in the guanine nucleotide binding site on the regulatory protein cannot be ruled out, particularly in the light of a second observation, that the lag period preceding activation by Gpp(NH)p alone was prolonged in obese mice, suggesting that the rate of binding of guanine nucleotides to the regulatory protein is slower in the obese state.

The ^3H -dihydroalprenolol binding studies indicated a reduction in specific binding to adipocyte plasma membranes from obese mice. However, two observations suggest that it is unlikely that a 30% reduction in β -receptor number is the only cause of the lack of potentiation of the response to guanine nucleotides by isoprenaline in obese animals. Firstly, the dose-response curve to isoprenaline in membranes from obese animals showed a decrease in maximum response. A reduction in receptor number normally would be associated with a similar maximum response but a decrease in sensitivity; no significant rightward shift in the dose-response curve to isoprenaline in obese animals was apparent in these experiments, either with isoprenaline alone, or in the presence of Gpp(NH)p. Secondly, according to the collision coupling hypothesis [20], in the presence of guanine nucleotides a single occupied receptor can interact with and activate several regulatory subunits. Therefore, in the presence of the irreversible activator

Gpp(NH)p, a reduced receptor number would result in a similar final extent of activation, although the rate of activation would be slower. The results of the time course study shown in Figure 5 indicate that this is not the case. Another possibility to explain the impaired potentiation of the response to guanine nucleotides by isoprenaline is a defect in the interaction between the hormone-receptor complex and the regulatory protein. Such a defect may be related directly to the alterations in guanine nucleotide binding or GTPase activity described previously, or to a more general change, for example, in the conformation of the regulatory protein.

The presence of Gpp(NH)p increased the sensitivity of adenylate cyclase to isoprenaline in both groups of mice; however, GTP had no effect on isoprenaline sensitivity in the lean, but caused a reduction in sensitivity in the obese animals. A reduction in the sensitivity of the adenylate cyclase system to isoprenaline in the presence of GTP has been described in other systems [24, 25]. Nahorski has shown that in the presence of GTP the affinity of isoprenaline binding to β_2 -receptors is reduced, but the affinity of binding to β_1 -receptors is unchanged [26]. Begin-Heick has shown that the β -receptors in adipocytes of obese animals are of the β_2 -subtype rather than the β_1 -subtype [27]. Thus, the reduction in sensitivity to isoprenaline in the presence of GTP in the obese animal may reflect this difference in β -receptor subtype.

Hormone-stimulated adenylate cyclase is sensitive to membrane fluidity and lipid composition [28] both at the level of hormone-receptor binding [29] and in the interactions between hormone-receptor, regulatory protein and catalytic unit [30–35]. Previous studies in this laboratory have shown that adipocyte plasma membranes from obese mice are characterized by an increase in membrane fluidity and by major alterations of membrane phospholipid fatty acyl composition which were particularly prominent in phosphatidylethanolamine [9, 10]. When membrane fluidity was reduced in obese mice by housing at 34°C an increase in the response of adenylate cyclase to isoprenaline was observed [9]. It is thus possible that the regulatory changes in the adenylate cyclase system which are observed in the obese mouse are secondary to the changes in membrane lipid composition.

Acknowledgements. This work was supported by a grant from the Medical Research Council. RRF is a Drummond Fellow.

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Received: 19 July 1983
and in revised form: 10 March 1984

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