Effects of litter removal on the lipolytic response and the regulatory components of the adenylate cyclase in adipocytes isolated from lactating rats

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The effects of litter removal on the status of different components of the hormone-sensitive adenylate cyclase system were analysed in plasma membranes of rat adipocytes. These effects were correlated with the decreased lipolytic response of adipose tissue. No changes in total number of A_1 adenosine receptors or their affinity were detected in response to litter removal. In contrast, β -adrenergic receptors showed a decrease (35%) in total number of receptors, without any significant change in their affinity. The status of α - G_s and α - G_i , the α -subunits of G proteins which mediate stimulation and inhibition respectively of adenylate cyclase, were probed by cholera- and pertussis-toxin-catalysed ADP-ribosylation respectively and by immunoblot. Associated with litter removal, decreases of 63% and 62% in the incorporation of [α - 32 P]ADP-ribose catalysed by cholera toxin and pertussis toxin into α - G_s and α - G_i respectively were detected. Immunoblotting using RM/1 (anti- α - G_s) and AS/7 (anti- α - G_i) antisera also showed decreases in the levels of α - G_s (52%) and α - G_i (55%) in adipocyte membranes from litter-removed rats compared with lactating rats. Alterations in the status of hormone-sensitive adenylate cyclase components, such as those described herein, may be biochemical mechanism(s) by which adipose tissue shows a decreased lipolytic response during recovery from lactation.

INTRODUCTION

Lactation brings about profound changes in the intermediary metabolism of the different tissues in order to supply precursors required for milk production (for reviews see [1,2]). In adipose tissue, marked decreases in the lipogenic capacity [3,4] as well as in the specific activities of several lipogenic enzymes [5–7] have been associated with lactation. The overall result consists of a net mobilization of lipid from adipose tissue to support milk production in mammary gland. Weaning is associated with the reversal of the above-mentioned changes, leading to rapid lipid storage in adipose tissue, whereas the mammary gland switches off its lipogenic capacity [6–8]. In addition to these changes in the intermediary metabolism, litter removal has been shown to decrease the lipolytic response of adipocytes [9]. This seems to fulfil the physiological role of adipose tissue during weaning in order to restore the triacylglycerol reserve, depleted during lactation. However, the mechanisms by which this modulation is exerted remain poorly understood.

The lipolytic response of adipose tissue is modulated by several physiological and pathological circumstances. The actions of catecholamines are potentiated by hyperthyroidism and blunted by hypothyroidism or adrenalectomy. In these cases, changes in the steady-state levels of the different components of the hormone-sensitive adenylate cyclase system (receptors, G-protein subunits, adenylate cyclase) have been reported [10–14].

In order to gain a greater insight in the biochemical basis of the modulation of adipose-tissue lipolytic responsiveness during the lactation–weaning transition, the status of different components of the hormone-sensitive adenylate cyclase system has been explored.

MATERIALS AND METHODS

Materials

All radiochemicals were purchased from New England Nuclear. Cholera toxin and pertussis toxin were purchased from

List Biological Laboratory (Campbell, CA, U.S.A.). All other reagents were purchased from standard commercial sources. RO 20-1724 was a gift from Dr. P. F. Sorter (Hoffman–La Roche, Nuttley, NJ, U.S.A.).

Animals

Female Wistar rats (10–15 days *post partum*) were used in all experiments as control lactating. Lactating/litter-removed refers to mid-lactating rats which had their litters removed for 48 h before the experiments.

Isolation of adipocytes and membranes

Adipocytes were obtained by collagenase digestion of parametrical adipose tissue by the method of Rodbell [15]. Highly purified plasma membranes were prepared by the method of McKeel & Jarret [16]. Adipocytes were homogenized and isolated by using buffers to which proteinase inhibitors [leupeptin (5 μ g/ml), aprotinin (5 μ g/ml) and freshly prepared phenylmethanesulphonyl fluoride (30 μ g/ml)] were added. In all cases adipocyte plasma membranes from lactating and lactating/litter-removed rats were prepared concurrently.

Glycerol release

Extracts from incubations were assayed for glycerol as described [17].

Radioligand-binding assays

(–)-[3 H]Dihydroalprenolol binding to the membranes was performed at 22 °C as described previously [11]. N^6 -[3 H]Cyclohexyladenosine binding was performed by the method of Bruns *et al.* [18] as described in [19]. Plasma membranes were always pretreated with adenosine deaminase (0.12 unit/mg of protein) for 30 min at 25 °C before [3 H]cyclohexyladenosine-binding assays to remove endogenous adenosine.

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Table 1. Effects of litter removal on glycerol release in adipocytes

Adipocytes $[(70-150) \times 10^3 \text{ cells}]$ were incubated in 1 ml of Krebs-Ringer phosphate (pH 7.4)/3 % BSA for 1 h at 37 °C in the absence and in the presence of phosphodiesterase inhibitor RO 20-1724 (0.1 mM) and in the presence of the indicated additions. Glycerol release was measured as indicated in the Materials and methods section. The results are expressed as nmol of glycerol released/ 10^6 cells and are means $\pm$ S.E.M. of at least four independent experiments performed in duplicate: * $P < 0.05$, significantly different from control lactating rats. Abbreviations: ADA, adenosine deaminase; PIA, N^6 -(phenylisopropyl)adenosine.

Additions	Glycerol release	
	Lactating	Lactating/ litter-removed
(a)		
–	< 50	< 50
Isoprenaline (0.1 μM)	1330 $\pm$ 105	777 $\pm$ 98*
Isoprenaline (1 μM)	1500 $\pm$ 225	1100 $\pm$ 111
Forskolin (10 μM)	1700 $\pm$ 125	1205 $\pm$ 107*
+ isoprenaline (0.1 μM)		
(b) + RO 20-1724		
–	< 50	< 50
Isoprenaline (1 μM)	925 $\pm$ 154	431 $\pm$ 86*
Forskolin (10 μM)	2327 $\pm$ 210	611 $\pm$ 114*
ADA (0.5 $\mu\text{g/ml}$)	602 $\pm$ 83	592 $\pm$ 94
ADA + isoprenaline (0.1 μM)	3156 $\pm$ 488	1601 $\pm$ 403*
ADA + isoprenaline (0.33 μM)	3010 $\pm$ 424	1793 $\pm$ 444*
ADA + isoprenaline (1 μM)	3040 $\pm$ 444	2184 $\pm$ 176*
ADA + forskolin (10 μM)	2761 $\pm$ 301	2053 $\pm$ 133
ADA + forskolin (10 μM)	1874 $\pm$ 288	1484 $\pm$ 239
+ PIA (100 nM)		

Cholera- and pertussis-toxin-catalysed ADP-ribosylations

Membranes (100 μg of membrane protein) were incubated in the presence of $[\alpha\text{-}^{32}\text{P}]\text{NAD}^+$ and cholera or pertussis toxin as described by Malbon *et al.* [20]. SDS/PAGE and autoradiography were carried out as described [20]. Toxins were pretreated by incubation with β -mercaptoethanol (20 mM) for 10 min at 37 °C. Quantification of the autoradiograms was carried out by laser-densitometric scanning with a Molecular Dynamic computer densitometer.

Immunoblotting

Membranes (30–50 μg of protein) were subjected to electrophoresis on 10 % polyacrylamide gels in the presence of SDS, transferred electrophoretically to nitrocellulose as described by Ros *et al.* [11] and blocked for 1 h with 5 % (w/v) non-fat skimmed milk in phosphate-buffered saline, pH 7.2 (PBS). The blots were probed with RM/1 (anti- $\alpha\text{-G}_s$) and AS/7 (anti- $\alpha\text{-G}_i$) antisera from Biotechnology Systems NEM Research Products (Du Pont de Nemours, Dreieich, Germany). These antisera recognize $\alpha\text{-G}_s$ [21,22] and both $\alpha\text{-G}_i$ -1 and $\alpha\text{-G}_i$ -2 [23,24] respectively. After washing with 3 % Tween 20/PBS, secondary goat anti-rabbit antiserum coupled to horseradish peroxidase was added (1:10000 in PBS), and specific bands corresponding to $\alpha\text{-G}_s$ and $\alpha\text{-G}_i$ subunits were detected by chemiluminiscent reaction, by using the ECL Western-blotting detection system from Amersham. Autoradiograms of the blots were quantified by laser-densitometric scanning with a Molecular Dynamic computer densitometer.

Protein determination

Protein determination was performed by the method of Lowry *et al.* [25], with BSA as the standard.

Data analysis

Data are presented as means $\pm$ S.E.M. for the numbers of independent experiments indicated. Significance between groups was determined by Student's *t* test.

RESULTS AND DISCUSSION

The lactation–weaning transition has been reported to cause a decrease in the lipolytic responsiveness of adipocytes [9]. In agreement with that report, Table 1(a) shows that isoprenaline- and forskolin-stimulated lipolysis, measured as glycerol release, was decreased in adipocytes isolated from rats which had their litters removed for 48 h. The fact that the plant diterpene forskolin, which directly activates the cyclase activity, brings about a decreased glycerol release in adipocytes suggests that a decrease in the adenylate cyclase activity may be at least one of the mechanisms responsible for the attenuated lipolytic response of adipocytes. This alteration in the lipolytic responsiveness of adipocytes might be explained, at least in part, by a decreased phosphodiesterase activity described by Aitchison *et al.* [26]. In order to study the lipolytic responsiveness of adipocytes, independently of phosphodiesterase status, measurements of glycerol release by adipocytes were also carried out in the presence of the specific phosphodiesterase inhibitor RO 20-1724. Table 1(b) shows that in the presence of this phosphodiesterase inhibitor, isoprenaline caused a lower lipolytic response, in terms

Fig. 1. Specific binding of $[\text{H}^3]$ dihydroalprenolol to membranes prepared from adipocytes from lactating and lactating/litter-removed rats

Freshly prepared plasma membranes from adipocytes isolated from lactating (●) and lactating/litter-removed (▲) rats were incubated at 22 °C for 20 min with increasing concentrations of radioligand. Specific binding was assayed as described in the Materials and methods section. The data in the plot are means of triplicate determinations (variance $< 10\%$) of an experiment representative of five separate experiments performed with as many individual adipocyte membrane preparations. Inset: Scatchard plot of these same data. Mean values $\pm$ S.E.M. of K_d were 7.6 ± 2.8 and 6 ± 2.5 (nM), and mean values of B_{max} were 480 ± 51 and $310 \pm 12^*$ (fmol/mg), for lactating and litter-removed rats respectively: * $(P < 0.05)$ significantly different from lactating rats.

Fig. 2. Polyacrylamide-gel analysis of radioactive products resulting from cholera-toxin-catalysed ADP-ribosylation of membranes of adipocytes isolated from lactating and lactating/litter-removed rats

Membranes (100 μ g of membrane protein) from lactating (L, $\square$) and lactating/litter-removed (LR, $\boxtimes$) rats were incubated with [α - 32 P]NAD⁺ in the presence of preactivated cholera toxin (100 μ g/ml) and then subjected to SDS/PAGE and autoradiography as described in the Materials and methods section. Autoradiographs were quantified by laser-densitometric scanning with a Molecular Dynamic computer densitometer, and expressed as percentages of values obtained in lactating rats (Panel a). The data are means $\pm$ S.E.M. of at least five separate experiments: * ($P < 0.05$) significantly different from lactating rats. Panel (b) shows staining with Coomassie Blue of an SDS/polyacrylamide gel (left) and its autoradiogram (right) from a representative experiment.

of glycerol release, in adipocytes isolated from rats which had their litter removed than in adipocytes isolated from lactating rats. This lower lipolytic response was observed at an isoprenaline concentration (1 μ M) high enough for us to assume that, in the presence of adenosine deaminase and RO-20-1724, the maximal response has been reached. These data suggest that other step(s) beside phosphodiesterase activity are responsible for the diminished lipolytic response of adipocytes from litter removed rats.

The decreased isoprenaline-stimulated glycerol release in adipocytes from rats which had their litter been removed was observed both in the absence and in the presence of adenosine deaminase (Table 1), suggesting that neither the amount of adenosine released by adipocytes nor the number of A₁ adenosine receptors is altered by litter removal in adipocytes. Alternatively, the amount of adenosine released and the A₁ adenosine receptor number might change in opposite directions. Nevertheless, radioligand binding studies with the A₁ adenosine agonist N⁶-cyclohexyladenosine were carried out. Plasma membranes from adipocytes of lactating rats showed $B_{\max} = 150 \pm 27$ fmol/mg of protein, with $K_d = 3 \pm 0.5$ nM. No significant differences were found between these parameters and those in plasma membrane isolated from rats which had their litter removed.

Stimulation of β -adrenergic receptors by catecholamines represents the first step in the transmembrane signalling system which mediates stimulation of adenylate cyclase and finally lipolysis in adipocytes. Any alteration in the status of β -adrenergic receptors might be a mechanism by which the lipolytic response is modulated. In order to study this possibility, radioligand binding of the β -antagonist [3 H]dihydroalprenolol to adipocyte plasma membranes at equilibrium was carried out. As shown in Fig. 1, adipocyte plasma membranes from rats which had their litter removed showed a decrease (35%) in total number of receptors compared with membranes isolated from lactating rats. No significant change was observed in receptor affinity between these two circumstances. The decrease in the total number of β -adrenergic receptors, together with the above-

mentioned decrease in the adenylate cyclase activity, may explain the diminished β -adrenergic-agonist-stimulated lipolytic response of adipocytes during the lactation/weaning transition.

G-proteins are now a firmly established class of important signal-transducing molecules. Given that signal transduction between β -adrenergic receptors and adenylate cyclase is mediated by G_s, the effects of litter removal on the status of α -G_s have been investigated, by using cholera-toxin-catalysed ADP-ribosylation in the presence of [α - 32 P]NAD⁺, and by immunoblot using RM/1 antiserum, which recognizes α -G_s, as described previously [23,24]. Cholera-toxin-catalysed ADP-ribosylation has been used on several occasions to establish the relative amounts of α -G_s in plasma membranes [11,20,27]. In agreement with the reports quoted above, cholera toxin catalysed the ADP-ribosylation of two major peptides of 42 kDa and 46–48 kDa in plasma membranes isolated from rat adipocytes (Fig. 2). Ribosylation in each case was quantified as described in the Materials and methods section. Fig. 2 shows that litter removal caused a significant decrease in this parameter in both of the cholera-toxin substrates. In agreement with these data, a decrease in the steady-state levels of α -G_s was also detected by immunoblot (Fig. 3) in plasma membranes. In contrast, the patterns of plasma-membrane proteins (Fig. 2), made visible by Coomassie Blue, were not altered.

Adenylate cyclase is also regulated by an inhibitory G-protein-mediated pathway. The steady-state levels of α -G_i, the α -subunit of the G-protein that mediates inhibition of the adenylate cyclase, have been analysed by both pertussis-toxin-catalysed ADP-ribosylation (Fig. 4) and immunoblotting (Fig. 5). A band of 39–40 kDa was detected by both approaches. As adipocytes have been shown to express α -G_i-1, α -G_i-2 and α -G_i-3 [28] and AS/7 antiserum recognizes α -G_i-1 and α -G_i-2 [28], the 39–40 kDa band may express a combination of the above-mentioned α -G_i subunits. Figs. 4 and 5 show that litter removal brings about a decrease in the steady-state levels of α -G_i subunits in adipocyte plasma membranes, detected by pertussis-toxin-catalysed ADP-ribosylation and by immunoblot, respectively. Given that, when

Fig. 3. Effect of litter removal on the steady-state levels of α -G_s of rat adipocyte membranes

Membranes (35–50 μ g of membrane protein) isolated from lactating (L, $\square$) and lactating/litter-removed (LR, $\boxtimes$) rats were subjected to SDS/PAGE and then to immunoblotting using RM/1 antiserum and horseradish-peroxidase-coupled goat anti-(rabbit IgG) as second antibody after transfer to nitrocellulose. Bands corresponding to α -G_s subunits were made visible by a chemiluminescent reaction using a ECL Western-blotting detection system from Amersham. Quantification of the bands was carried out as described in the Materials and methods section. Inset shows a representative autoradiogram of the blot corresponding to α -G_s subunits. *($P < 0.05$) significantly different from control lactating rats.

Fig. 5. Effects of litter removal on the steady-state levels of α -G_i of rat adipocyte membranes

Membranes (35–50 μ g of membrane protein) isolated from lactating (L, $\square$) and lactating/litter-removed (LR, $\boxtimes$) rats were subjected to SDS/PAGE and then to immunoblotting using AS/7 antiserum and horseradish-peroxidase-coupled goat anti-(rabbit IgG) as second antibody after transfer to nitrocellulose. Bands corresponding to α -G_i subunits were made visible by a chemiluminescent reaction using a ECL Western-blotting detection system from Amersham. Quantification of the bands was carried out as described in the Materials and methods section. Inset shows a representative autoradiogram of the blot corresponding to α -G_i subunits. *($P < 0.05$) significantly different from control lactating rats.

Fig. 4. Polyacrylamide-gel analysis of radioactive products resulting from pertussis-toxin-catalysed ADP-ribosylation of membranes of adipocytes isolated from control lactating and lactating/litter-removed rats

Membranes (100 μ g of membrane protein) from lactating (L, $\square$) and lactating/litter-removed (LR, $\boxtimes$) rats were incubated with [α -³²P]NAD⁺ in the presence of preactivated pertussis toxin (10 μ g/ml) and then subjected to SDS/PAGE and autoradiography as described in the Materials and methods section. Autoradiographs were quantified by laser-densitometric scanning with a Molecular Dynamic computer densitometer and expressed as percentages of values obtained in lactating rats (panel a). The data are means $\pm$ S.E.M. of at least five separate experiments: *($P < 0.05$) significantly different from lactating. Panel (b) shows staining with Coomassie Blue of an SDS/polyacrylamide gel (left) and its autoradiogram (right) from a representative experiment.

adenosine deaminase is added, attenuation of the differences of lipolytic responsiveness is observed (Table 1), an impaired response to adenosine in litter-removed rats appears to be involved. This would be in agreement with the decrease in G_i described above. Nevertheless, when the effects of N⁶-(phenylisopropyl)adenosine expressed as a percentage of forskolin-stimulated lipolysis were analysed (Table 1), no significant differences were found between lactating and litter-removed rats. However, the decrease in glycerol release caused by N⁶-(phenylisopropyl)adenosine in litter-removed rats was lower than in lactating rats. These results agree with those of Vernon & Finley [9]. These data suggest a key role of the elements of the inhibitory pathway of adenylate cyclase beyond agonist-receptor interaction during recovery of lactation.

Steady-state levels of β -adrenergic receptors, adenylate cyclase and G-protein subunits have been shown to be altered in several physiological and pathological circumstances in adipocytes. Hypothyroidism is associated with an increase in the steady-state levels of α -G_i, α -G_o and the β -subunits of G-proteins [11,12,20]. Diabetes has been reported to alter the expression and phosphorylation state of α -G_i [29]. On the other hand, aging and obesity have also been associated with alterations in G-protein subunit expression [27,30]. These modifications at the level of signal transduction may well reflect mechanisms by which the lipolytic response of adipose tissue, among others, is regulated in different physiological circumstances. In the present report, the effects of litter removal on the status of several components of the hormone-sensitive adenylate cyclase have been explored. A decrease in the adenylate cyclase activity, defined in terms of its activation by the plant diterpene forskolin, as well as a decrease in the number of β -adrenergic receptors and the relative steady-state levels of α -G_s have been associated with 48 h litter removal.

These changes can explain, at least in part, the diminished lipolytic response of adipocytes. Nevertheless other regulatory mechanisms, such as the reported changes in the activity of phosphodiesterase [26], may also contribute to modulate the lipolytic responsiveness of adipocytes during the lactation-weaning transition.

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