Vol. 102, No. 2, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 30, 1981 Pages 577-587

DEVELOPMENT OF LIPOLYTIC RESPONSE TO ISOPROTERENOL DURING ADIPOSE CONVERSION OF OB17 PREADIPOCYTE CELLS

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Received June 16,1981

SUMMARY : Responsiveness of ob17 cells to lipolytic agents was examined by cAMP* production and by fatty acid release in the presence of isoproterenol. EC_{50} values for cAMP production increased from 70 nM (growth phase) to 120 nM (resting phase), with a maximal response at 2.5 µM. Maximal responsiveness was already present in subconfluent cells, was magnified 3 fold when the cells attained the confluent state and subsequently decreased as a function of time in culture. In contrast early confluent ob17 cells, containing triglycerides prelabeled in their fatty acid moiety, did not show any significant lipolytic response at 2.5 µM isoproterenol. A full lipolytic response was observed in differentiated ob17 cells, indicating that the most critical event during adipose conversion for lipolysis occurs at a step beyond the β-adrenergic-adenyl cyclase coupling. Chronic exposure of post-confluent ob17 cells to insulin led to a diminished maximal responsiveness to isoproterenol, suggesting the establishment of an alteration on a *long term basis* to the action of β-agonists.

INTRODUCTION

The adipose conversion of preadipocyte cell lines and of preadipocyte precursors has been well documented (1-4). Ob17 preadipocytes isolated from the epididymal fat pad of C57BL/6J ob/ob mouse were previously shown to respond, when differentiated, to physiological concentrations of epinephrine and corticotropin by mobilization of triglycerides (3). They differ from 3T3-L1 fibroblasts with regard to i) down-regulation of insulin receptors and decreased sensitivity to insulin by chronic exposure to the hormone ii) responsiveness to triiodothyronine with amplification of the

* Abbreviations : cAMP, adenosine-3',5'-monophosphate ; BSA, bovine serum albumin ; KRB, Krebs Ringer Bicarbonate buffer ; IBMX, isobutylmethylxanthine ; EDTA, ethylenediaminetetracetic acid ; FCS, fetal calf serum.

0006-291X/81/180577-11$01.00/0

differentiation program (J. Gharbi-Chihi et al., submitted for publication). Among events taking place when murine 3T3-L1 fibroblasts enter the differentiation program was a magnification of the hormonal response to isoproterenol and the acquisition of a sensitivity to corticotropin (5), determined for both compounds at a single maximal concentration by cAMP production and adenylate cyclase activities.

As described in this report undifferentiated ob17 cells, in contrast to undifferentiated 3T3-L1 cells, possess already a sensitivity and a maximal responsiveness to isoproterenol quite comparable to those observed in differentiated ob17 cells. However the latter cells will only develop a full lipolytic response, determined by fatty acid release, during adipose conversion. Our results indicate also that ob17 cells chronically exposed to insulin develop on a *long-term basis* an altered response to the action of β-adrenergics.

MATERIALS AND METHODS

Cell culture : Methods of cell growth, cell numbering and determination of cell protein content were as previously described (3). Where indicated, 170 nM insulin was added at confluence to standard medium (changed every other day), with inclusion of biotin (8 µg/ml) and pantothenate (4 µg/ml).

Determination of cAMP content : All experiments regarding the stimulatory effect of β-adrenergics on cAMP production were performed 24 h to 36 h after changing the medium of cells present as a monolayer in 35 mm diameter culture dishes. When insulin was included, cells were first treated in order to remove the hormone (6). Control experiments, carried out on cells not chronically exposed to insulin, showed no effect of this treatment on the production of cAMP. Cells were then preincubated for 5 min at 37°C in 1 ml of KRB buffer pH 7.6 containing 1% delipidized BSA. The medium was aspirated and replaced by the same medium containing a phosphodiesterase inhibitor (RO 201724, IBMX, Theophylline), in the presence or in the absence of isoproterenol.

Unless otherwise stated, after 3 min of incubation, the medium was removed and the cells rinsed twice with 2 ml of cold KRB buffer. Intracellular cAMP was extracted twice with 250 µl of 5% trichloracetic acid. The acid supernatant (8 000 g, 4 min) containing cAMP was stored at -20°C. After thawing, trichloracetic acid was removed with 4x8 volumes of diethyl oxide. cAMP content was assayed according to a modification of the original procedure described by Gilman (7), using a commercial kit (TRK 432 - Radiochemical Centre, Amersham). The sensitivity ranged from 0.2 to 16

picomol of cAMP per assay. Within a single series of cells, determinations for each condition were performed in duplicate on at least 2 culture dishes (basal and stimulated) treated separately. Mean values are reported ; they did not differ by more than 10%. Variability between mean values of identical experiments using two different serie of cells did not exceed 18%.

Lipolytic response assayed by fatty acid release : Cell prelabeling was initiated 4 days before performing lipolysis measurements. Ob17 cells (60 mm diameter culture dishes ; 4 ml total medium) were maintained for 2 days in 10% FCS supplemented with 170 nM insulin and [^{14}C]palmitate (57 mCi/mmol ; 0.3 µCi per dish). Then medium was changed without addition of labeled palmitate. 24 hours later the medium was replaced by one not containing insulin in order to remove any residual hormone. Lipolysis experiments were performed in 6 ml Dulbecco's modified Eagle's medium with inclusion of 1% delipidized and dialyzed BSA at 37°C in a humid atmosphere containing 5% CO_2. After 30 min preincubation, 2.5 µM isoproterenol were added or not. 0.5 ml aliquots were removed for counting at different time intervals. Control experiments showed that released radioactivity was almost entirely due (80%) to unesterified fatty acids. Analysis of labeled lipids before and after isoproterenol stimulation was performed according to Samuel et al. (8).

Materials : Dulbecco's modified Eagle's medium was purchased from Gibco (catalogue number H21), FCS was a product of Seromed. Phentolamine mesylate was obtained from Ciba, RO 201724 (4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone) from Hoffman-Laroche and Co.. 1-[^{14}C]palmitic acid was purcharsed from Amersham. All other compounds were from Sigma.

RESULTS

As shown in Table 1, differentiated ob17 cells are able to respond to isoproterenol by a potent increase in the production of cAMP. Inclusion of a phosphodiesterase inhibitor was needed since i) when absent, the basal level was too low to be determined accurately, ii) when present, any phosphodiesterase activity sensitive to these inhibitors would not interfere with cAMP accumulation. Among inhibitors tested at maximal concentrations, the data indicate that RO 201724 led to the highest levels of cAMP produced both under basal and stimulatory conditions. The stimulation factors were similar in all cases. Data of Table 1 do show also that after stimulation i) cAMP released into the medium is not proportionnal to that present inside, ii) most of cAMP produced above basal levels is present inside the cells (92%), iii) in any case the proportion of cAMP recovered extracellularily does not exceed 15% of total cAMP

Table 1 : Production of cAMP by ob17 cells under basal and stimulatory conditions

INHIBITOR	cAMP PRODUCED (pmol/3 min/mg protein)							
	CELLS				MEDIUM			
	BASAL	STIMULATED	STIMULATED MINUS BASAL	STIMULATION FACTOR	BASAL	STIMULATED	STIMULATED MINUS BASAL	STIMULATION FACTOR
Experiment 1								
RO201724 (0.25 mM)	67	721	654	10.8	54 (44.6)	111 (13.3)	57	2.1
Experiment 2								
RO201724 (0.25 mM)	167	653	486	3.9	72 (30)	117 (15)	45 (8.5)	1.6
Experiment 3								
Theophylline (1mM)	14.5	52.5	38.1	3.6				
IBMX (0.25 mM)	53	231	178	4.4				
RO201724 (0.25 mM)	75	334	259	4.5				

Pre-confluent cells (day 2, experiment 1) and post-confluent cells (day 8 and 16, experiments 2 and 3 respectively) were treated as described in "Materials and Methods", in the absence (basal) or in the presence (stimulated) of 2.5 µM isoproterenol. The amounts of cAMP present in the medium (experiments 1 and 2) and in the cells were determined as described in "Materials and Methods". Numbers are the mean of at least triplicate dishes treated separately ; their values did not differ by more than 8%.

Numbers in parentheses correspond to the percentage of cAMP recovered in the extracellular medium, by taking total cAMP (intra and extracellular) as 100% under basal or stimulatory conditions respectively.

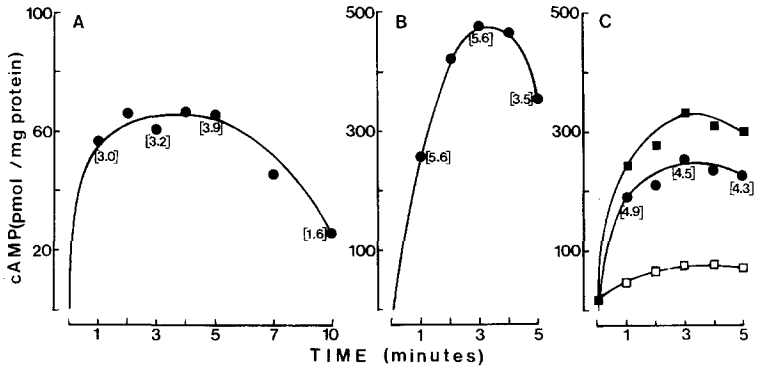

Fig. 1: Kinetics of cAMP production stimulated by isoproterenol in ob17 cells

Ob17 cells were maintained after confluence under standard conditions without insulin. cAMP production was determined on 2 day post-confluent cells (A and B) and on 17 day post-confluent cells (C) in the presence of 1 mM theophylline (A) or of 0.25 mM RO 201724 (B and C). The values reported in curves A and B correspond to stimulated (2.5 μM isoproterenol) minus basal (no isoproterenol) values. In Fig.C are presented the curves obtained in the presence of 2.5 μM isoproterenol (■——■), in its absence (□——□) and the difference stimulated minus basal (●——●). Each value corresponds to the mean of duplicate dishes treated separately under each condition. Numbers in parentheses correspond to the values of the stimulation factor.

Therefore, all determinations of cAMP content were performed on the cells after elimination of the incubation medium.

The curves of Fig. 1 illustrate the fact that, whatever the phosphodiesterase inhibitor used, the stimulation factor remains constant as a function of time up to 3 min and then decreases more or less slowly. The production of cAMP is maximal at 3 min under all conditions. All subsequent experiments were performed by measuring cAMP accumulation in cells maintained for 3 min at 37°C in the presence of 0.25 mM RO 201724, with or without isoproterenol.

Dose-response curves of cAMP production as a function of isoproterenol concentrations (Fig. 2A) were obtained on subconfluent (2 days before confluence) and post-confluent cells (day 17). The concentrations of half-maximal effects (EC_{50}) for isoproterenol were about 60-70 nM for growing cells and 100-120 nM for resting cells,

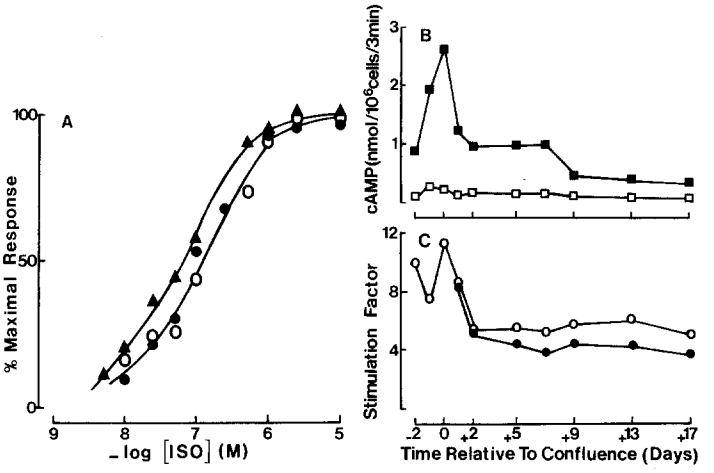

Fig. 2: Dose-response curves and maximal responsiveness to isoproterenol as a function of adipose conversion

Ob17 cells were grown under standard conditions (▲) and maintained post-confluence without (○, □, ■) or with 170 nM insulin (●). Stimulation experiments were performed for 3 min in the presence of 0.25 mM RO 201724. In Fig. 2A, each point represents the mean of at least duplicate dishes treated separately in two different series of cells ; 100% maximal response corresponds to 860 pmol/3 min/10⁶ cells for growing cells (▲) and to 211 and 269 pmol/3 min/10⁶ cells for cells maintained 17 days post-confluence in the presence (●) or in the absence (○) of added insulin. Basal levels were substracted in each case. In Fig. 2B values of cAMP production were obtained either in the absence (□——□) or in the presence of 2.5 μM isoproterenol (■——■). Values of stimulation factor reported in Fig. 2C were calculated by using data from Fig. 2B. Each point of the curves of Figs. 2B and C corresponds to the mean value from two distinct series of cells. In each series assays were performed on triplicate dishes at any given time.

while maximal effects were attained in each case at 2.5 μM isoproterenol. This change in sensitivity was small - 2 fold - but highly reproducible in different series of cell cultures. In contrast no change in EC_{50} values was observed when ob17 cells were maintained during adipose conversion in the absence or in the presence of insulin. Altogether, these results do indicate no dramatic variations in the sensitivity to isoproterenol responsiveness during maturation to adipose cells. They indicate also that chronic exposure to insulin of differentiating cells does not influence their sensitivity to respond to a β-agonist. As expected, isoproterenol should exert its effect through binding to β-adrenergic receptors : the stimu-

lation of cAMP production by 100 nM isoproterenol was abolished after incubation of resting ob17 cells with β-antagonists (10 μM alprenolol or 10 μM propranolol), but not with a typical α-antagonist (100 μM phentolamine) (not shown). These experiments do not exclude the existence of α-adrenergic receptors, although the latter have not been reported so far to be present in mouse adipocytes (9).

Responsiveness to isoproterenol was examined during adipose conversion at 2.5 μM isoproterenol in growing and differentiating cells (Figs. 2B and C). Maximal responsiveness rose sharply within a couple of days before confluence, peaked at confluence, decreased rapidly within 2 days and more slowly thereafter. Although not apparent due to the scale used in Fig. 2B, both stimulated *and* basal levels of cAMP production in ob17 cells decreased quite regularly with time in culture.

Chronic exposure of resting cells to insulin led to a small but reproducible difference in the values of the stimulation factor observed for ob17 cells, which decreased 18-28% (Fig. 2C). Since in these experiments adipose conversion was 30 to 50% in the absence of insulin and 50 to 70% in its presence, it should not be possible a priori to exclude the fact that this difference was due only to adipose conversion per se. This is not likely to be the case since the stimulation factor, obtained with treated or with non-treated cells, remained at constant values from day 5 to day 17, time during which adipose conversion proceeded at a high rate.

A different picture emerged by examination of the full lipolytic response as a function of adipose conversion (Fig. 3). Experimental conditions of cell prelabeling were delineated in order that a significant proportion of [^{14}C] palmitate was recovered into triglycerides *even* at day 3 where lipid accumulation is normally weak as compared to that observed between day 6 and 10 and thereafter. At

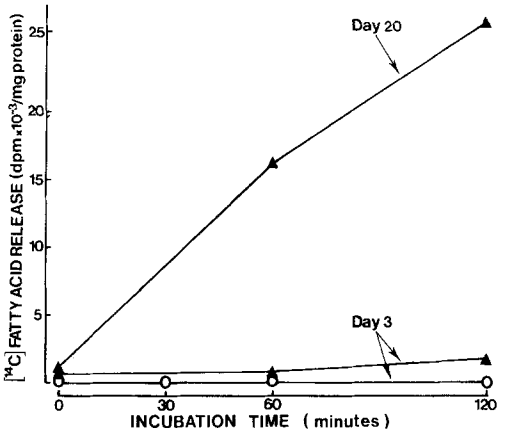

Fig. 3: Lipolytic response of differentiating ob17 cells in the presence of 2.5 µM isoproterenol

The total radioactivity recovered into lipids at day 3 corresponds to 247 500 dpm ± 10 600 dpm per mg of protein (12.1% triglycerides, 2.8% diglycerides, 38.7% unesterified fatty acids and 45.5% polar lipids) and at day 20 to 151 700 dpm ± 9 500 dpm per mg of protein (81.8% triglycerides, 1.5% diglycerides, 8.9% unesterified fatty acids, 7.2% polar lipids). Values of basal lipolysis were substracted in each case and correspond at day 3 to 8 300 and 5 000 dpm per mg per hour in experiments A (▲) and B (○) respectively, and at day 20 to 3 100 dpm per mg of protein per hour in experiment A. Each point is representative of the mean of triplicate dishes ; their values did not differ by more than 8%.

day 3, in the early phase of cell differentiation, no significant fatty acid release could be observed. In contrast fully differentiated cells, that is at day 20, developped a potent lipolytic response which corresponds to a 24% decrease in the radioactivity initially present into labeled triglycerides. These results are in agreement with previous data which showed that the whole machinery needed for a full lipolytic response was already present in 14 day post-confluent ob17 cells (3).

DISCUSSION

Assay conditions of cAMP production were defined in order to minimize the activity of phosphodiesterase(s) likely to be present in differentiating adipose cells.

Theophylline is less potent than IBMX and especially less than RO201724, which would suggest that the main effect of theophylline in ob17 cells proceeds indirectly, as demonstrated in isolated mature adipocytes, through binding to adenosine receptors and not directly by inhibiting phosphodiesterase(s) (10). However, even with RO 201724, the kinetics of cAMP production shows a maximum and then decreases ; this observation would be in favor of the presence of phosphodiesterase(s) insensitive to this inhibitor.

Under optimal conditions the production of cAMP after stimulation in differentiating cells is within the range of 300-2600 pmol/ 3 min/10^6 cells. These levels are similar to those obtained on mature adipocytes isolated from ob/ob mouse (11), or on differentiated 3T3-L1 (5) and on human adipocyte precursors (12). Our data show clearly that responsiveness to isoproterenol is already present in undifferentiated cells as previously observed in 3T3-L1 cells (5). However, in contrast to the latter cell line, the magnification in β-adrenergic responsiveness was observed as a function of development only during the period of time where the cells reached confluence and decreased rapidly thereafter. The factors responsible for this transient phenomenon are not known. An event linked to intercellular contacts is likely to be excluded, since parallel experiments performed on early confluent and late confluent cells - present either as monolayers or dissociated after EDTA treatment - led to identical results. An increase in the proportion of cAMP released after stimulation in the extracellular medium of post-confluent cells could also be excluded (not shown). An increase in differentiating cells of the production of adenosine or of adenosine-like compounds can also be ruled out, since control experiments in the presence of theophylline gave a similar decrease of the stimulation factor observed after confluence. Among other alternatives, it is

possible that the appearance or the activation of phosphodiesterase(s) insensitive to inhibitors and modulated by long-term exposure of ob17 cells to insulin are involved, since a "low Km" phosphodiesterase activity was shown to be expressed during adipose conversion of 3T3-L1 cells (13).

The development of a full lipolytic response is not apparent at day 3, in contrast to the appearance of lipoprotein lipase and monoglyceride lipase activities already induced within a couple of days post-confluence (14). It is likely that, in both differentiated ob17 and 3T3-L1 cells (15), this complete response is a consequence of the appearance of hormone-sensitive lipase. If it were so, the role of monoglyceride lipase would be more to operate sequentially with lipoprotein lipase than to be active intracellularily with hormone-sensitive lipase.

It is worth noticing that long-term exposure of post-confluent ob17 cells to insulin does not bring any change in their sensitivity to isoproterenol but leads to a reproducible decrease in their maximal responsiveness to the β-agonist. This altered response could also be due to an induction of a phosphodiesterase insensitive to the inhibitors used in this study. Although extrapolation to the situation in vivo remains to be shown, in addition to its well-known short-term antilipolytic effects, it might be significant that insulin is capable to induce on a long-term basis an attenuation to the action of β-adrenergics on cultured adipose cells.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. P. Grimaldi for helpful discussion, Miss B. Wdziekonski for expert technical assistance and Mrs. G. Oillaux for skilful typing. This work was supported by grants from "Centre National de la Recherche Scientifique" (LP 7300) and from "Institut National de la Santé et de la Recherche Médicale" (ATP 58-78-90).

REFERENCES

1. Green, H. (1978) Differentiation and development, Miami Winter Symposium (Ahmad, F., Russel, T.R., Schultz, J. and Werner, R., Eds). 15, 13-36, Academic Press New York.
2. Green, H. (1980) in "Obesity : cellular and molecular aspects". Eds. INSERM Vol. 87, 15-24.
3. Négrel, R., Grimaldi, P., and Ailhaud, G. (1978) Proc.Natl.Acad.Sci.USA 75, 6054-6058.
4. Roncari, D.A.K., and Van, R.L.R. (1980) in "Obesity : cellular and molecular aspects". Eds. INSERM Vol. 87, 65-88.
5. Rubin, C.S., Lai, E., and Rosen, O.M. (1977) J.Biol.Chem. 252, 3554-3557.
6. Grimaldi, P., Négrel, R., Vincent, J.P., and Ailhaud, G. (1979) J.Biol.Chem. 254, 6849-6852.
7. Gilman, A.G. (1970) Proc.Natl.Acad.Sci.USA 67, 305-312.
8. Samuel, D., Paris, S., and Ailhaud, G. (1976) Eur.J.Biochem. 64, 583-595.
9. Lafontan, M., and Berlan, M. (1981) Trends in Pharmacol. Sci. 2, 126-129.
10. Fain, J.N., and Shepherd, R.E. (1979) in "Advances in experimental medicine and biology" (Klacklo, D.M., Anderson, R.R., and Heimberg, M. Eds.) 111, 43-77, Plenum Press, New-York and London.
11. Shepherd, R.E., Malbon, C.C., Smith, C.J., and Fain, J.N. (1977) J.Biol.Chem. 252, 7243-7248.
12. Roncari, D.A.K., Wang, H., and Desai, K.S. (1980) Can.J.Biochem. 58, 201-205.
13. Murray, T., and Russel, T.R. (1980) Eur.J.Biochem. 107, 217-224.
14. Murphy, M., Négrel, R., and G. Ailhaud (1981) 664, 240-248.
15. Kawanura, M., Jensen, D.F., Wancewicz, E.V., Joy, L.L., Khoo, J.C., and Steinberg, D. (1981) Proc.Natl.Acad.Sci.US. 78, 732-736.