Vol. 199, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1994 Pages 136-143

RISE IN CYTOSOLIC Ca^{2+} ABOLISHES IN PREADIPOSE CELLS THE EXPRESSION OF LIPOPROTEIN LIPASE STIMULATED BY GROWTH HORMONE

Sylvie BARCELLINI-COUGET, Georges VASSAUX, Raymond NEGREL, and Gérard AILHAUD*

Centre de Biochimie (UMR 134 CNRS), Université de Nice-Sophia Antipolis, Faculté des Sciences, Parc Valrose, 06108 Nice cédex 2 ,France

Received January 3, 1994

Lipoprotein lipase (**LPL**) is known to be an early marker of adipose cell differentiation. Growth hormone (**GH**) stimulates in preadipose Ob1771 cells the expression of LPL gene and this effect is mediated at least in part by *c-fos* protooncogene, the expression of which is transiently activated by a protein kinase C-dependent pathway (Barcellini-Couget *et al.*, Endocrinology, 1993, 132: 55-60). Since GH stimulates the formation of diacylglycerol from phosphatidylcholine independently of Ca^{2+} mobilization, the role of Ca^{2+} was studied in regard to LPL gene expression stimulated by GH. The results obtained in the presence of Ca^{2+} ionophores show that a rise in intracellular free Ca^{2+} abolishes this expression but has no effect on the expression of various adipose-related genes, including the transient expression of *c-fos* protooncogene. Therefore, the inability of preadipose cells to mobilize Ca^{2+} in response to GH, at an early stage of the differentiation process, appears as a prerequisite for the maximal expression of LPL.

Growth hormone has been shown to induce in adipose cells short, middle and long term effects on the expression of various differentiation-specific genes. In Ob1771 preadipose cells, which express early genes such as A2COL6/pOb24 and LPL genes, GH activates transiently the expression of *c-fos* gene by a protein kinase C (**PKC**) dependent pathway and modulates, at least in part by a PKC-dependent pathway, the expression of the LPL gene (1). The involvement of c-Fos protein in this PKC-dependent modulation of LPL gene expression has been recently demonstrated (2). One of the signalling pathways delineated for GH

* To whom correspondence should be addressed.

ABBREVIATIONS:
GH, growth hormone; **LPL**, lipoprotein lipase; **PKC**, protein kinase C; **DMEM**, Dulbecco's modified Eagle's medium; **CS**, calf serum; **PBS**, phosphate buffered saline; **GAPDH**, glyceraldehyde-3-phosphate dehydrogenase; **ALBP**, adipocyte lipid binding protein.

0006-291X/94 $5.00

action in preadipose Ob1771 cells is the formation of diacylglycerol associated with a phospholipase C-mediated hydrolysis of phosphatidylcholine (3) but not with that of phosphatidylinositol bis-phosphate as reported for canine kidney cells (4). In agreement with our previous results, the present study shows that GH is unable to induce any change in intracellular calcium concentration. To determine whether this formation of diacylglycerol, without any concomitant rise in intracellular Ca^{2+}, is of particular importance in regard to the expression of LPL gene and other differentiation-specific genes, we examined in preadipose cells the effects of calcium ionophores upon GH exposure. The results show that a rise in the intracellular Ca^{2+} concentration abolishes specifically the positive modulation of LPL gene expression induced by GH.

MATERIAL AND METHODS

Cell culture

Ob1771 cells were plated at an inoculation density of $2x10^3$ cells per cm^2 in Falcon 60-mm culture dishes and grown in standard medium, *i.e.* Dulbecco's modified Eagle's medium (**DMEM**) containing 200 units/ml of penicillin, 50 µg/ml streptomycin, 50 µM biotin and 17 µM pantothenate, supplemented with 8% (v/v) calf serum (**CS**). This medium was changed every two or three days. At confluence (day 0), which usually occurred within 5 days after seeding, cells were exposed to standard medium supplemented with 8% (v/v) CS and 17 nM insulin. This medium was changed daily or every other day . The emergence of LPL activity was observed between day 0 and day 2. The cells were used at day 4 or day 5. Unless otherwise stated, two to three hours before performing experiments, cells were washed with phosphate buffered saline (**PBS**) pH 7.4 and exposed to a standard medium containing 0.2% CS (v/v) and 17 nM insulin; this medium was termed incubation medium.

Intracellular calcium measurement

The measurement of free cytosolic calcium variations was performed using Indo-1 as a calcium chelator (5). Confluent cells, usually maintained in serum-supplemented medium, were loaded with 6 µM Indo-1/AM at 37°C in the dark for 1 hour. Cells were then washed, trypsinized and collected by centrifugation. The cell pellet was resuspended in a saline buffer (140 mM NaCl, 5mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 10 mM glucose, 25 mM Hepes, pH 7.4) containing 0.1% bovine serum albumin. Various effectors, as indicated in the legend of figure 1, were added as small aliquots to this cell suspension at time zero. The analysis was performed on a ODAM Fluorescence Activated Cell Sorter as described by Ledbetter *et al.* (6). The ratio of fluorescence at 400 nm (Indo-1 complexed to Ca^{2+}) to fluorescence at 480 nm (free Indo-1) allows the evaluation of the changes in the cytosolic free Ca^{2+} concentration independently of the intracellular concentration of Indo-1. Data are given in arbitrary units.

Biochemical assays

LPL activity measurements were performed in duplicate as previously described using tri[9,10-^{3}H] oleylglycerol as substrate (7) and protein measurements as described by Lowry et *al.* (8).

RNA analysis

RNAs were prepared by the guanidinium thiocyanate technique as described by Chomczynski and Sacchi (9). For Northern blot analysis, 20 µg of total RNAs or 3-5 µg of poly(A)$^+$ RNAs were electrophoresed on 1.2% (w/v) agarose gel and transferred onto a Hybond-N membrane (Amersham). The membranes were hybridized with [^{32}P]labeled probes as previously described (10) and the autoradiographs were quantitated by densitometry with an LKB-XL laser photodensitometer. All measurements were performed within the linear response of the integrated peaks as a function of immobilized RNA and all results were normalized to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) signals.

Materials

Culture media were obtained from Gibco (Cergy-Pontoise, France). Recombinant human GH was a kind gift from Kabi Pharmacia (Stockholm, Sweden). [α-^{32}P]dCTP, glycerol tri-[9,10-^{3}H]oleate, nick translation kit and random priming kit were purchased from Amersham (France). Calf serum and all other chemicals were obtained from Sigma (France). Plasmid pfos-1 (American Type Culture Collection n°41040) was used for *c-fos* mRNA detection.

RESULTS

GH has been shown to stimulate the hydrolysis of phosphatidylcholine in Ob1771 cells, leading to the formation of diacylglycerol (3). As expected from these results, GH was unable to induce any change in the intracellular Ca^{2+} level at a concentration of 10 nM sufficient to saturate GH receptor sites (Fig. 1A) as well as within concentrations ranging from 0.1 to 40 nM (not shown). By contrast ionomycin (1 µM), used as a Ca^{2+} ionophore, induced a rapid and transient elevation of the intracellular free Ca^{2+} concentration (Fig. 1B). In the absence of GH, either 1 µM ionomycin or 2 µM A23187 had no effect on the basal cellular LPL activity of preadipose cells. It should be stressed that under these conditions,

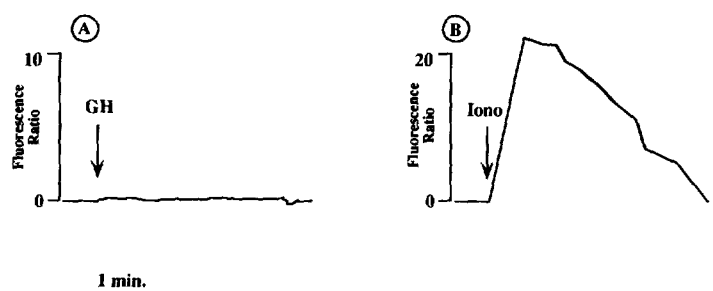

Figure 1. Comparative effects of ionomycin and GH on the free intracellular Ca^{2+} concentration in Ob1771 cells

Indo-1 preloaded Ob1771 cells were stimulated with 10 nM GH (A) or 1 µM ionomycin (B). The data presented are representative of three (A) and twelve (B) independent experiments performed on different series of cells. Time scale: 1 minute.

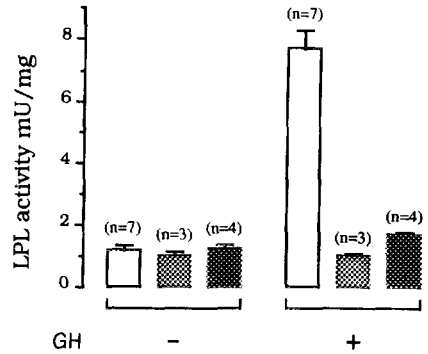

Figure 2. Inhibitory effect of calcium ionophores on the expression of LPL activity upon stimulation by growth hormone
Cells were exposed to the incubation medium in the absence (-) or in the presence (+) of 4 nM GH. Various calcium ionophores were also added (▩, ■) or not (❑): 2 μM A23187 (▩) or 1 μM ionomycin (■). After 15 hours, LPL activities were determined from duplicate dishes. The values are the means ± S.E.M obtained from 3 to 7 (n) independent series of cells.

no cytotoxic effect of calcium ionophores could be observed and no lactate dehydrogenase release from the cells could be detected.

We had previously reported that GH exposure led to a potent increase in the transcription rate of LPL gene, and in parallel to that in the content of LPL mRNA and LPL protein as well as LPL activity (1). As shown in Figure 2, calcium ionophores abolished the positive effect of GH on LPL gene expression. The same phenomenon occurred in the absence of insulin as well as when the incubation medium was deprived of external Ca^{2+} (not shown), indicating that this inhibition involves intracellular events which appear independent of insulin but clearly dependent upon a rise in intracellular Ca^{2+}.

The results of Figure 3 show that the increase in both LPL mRNA content and LPL activity was prevented in a parallel manner in preadipose cells exposed simultaneously to calcium ionophores and GH as compared to cells exposed to GH only. Once the GH-treated cells expressed LPL activity at a high level, inclusion of calcium ionophores in the incubation medium resulted in a time-dependent decrease of LPL activity to the value determined in cells exposed continuously to ionophores in the presence of GH (Fig.4). The half-life of this decrease could be estimated to ~ 2 hours; a parallel decrease in the LPL mRNA content was also observed (not shown). This half-life was higher than that of LPL activity determined in cycloheximide-treated Ob1771 cells ($t_{1/2}$ ~ 35 min) but similar to that of LPL mRNA and LPL activity following GH removal from GH-pretreated cells (1, 7, 11). It is of interest to note that the activity of LPL released constitutively from the cells decreased in parallel to the cellular LPL activity (not shown), suggesting that no distinct effect of calcium ionophores was taking place on the constitutive secretion of LPL. This effect of calcium

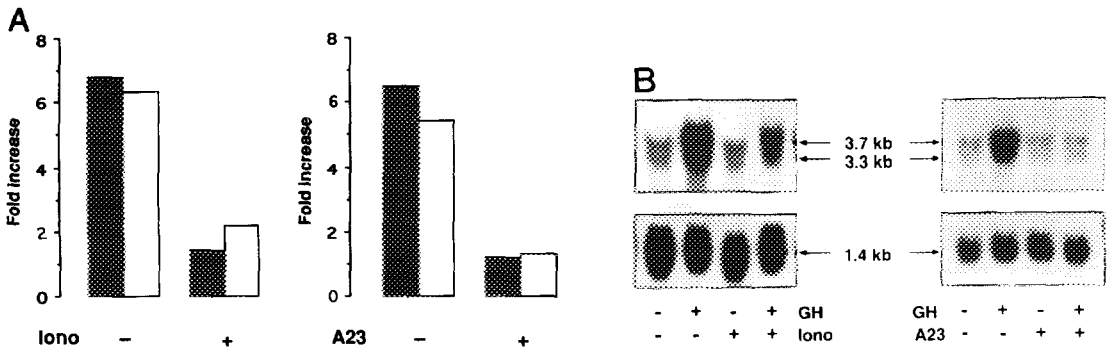

Figure 3. Comparative effect of calcium ionophores on the GH-promoted increase of LPL mRNA content and activity

A: Cells were maintained in the incubation medium in the absence or in the presence of 4 nM GH, supplemented or not with calcium ionophores (Iono: 1 µM Ionomycin; A23: 2 µM A23187) during 15 hours. Total RNAs were isolated from three pooled 60-mm dishes, electrophoresed and probed for LPL mRNAs (3.3 and 3.7 kb species) (empty bars). Cellular LPL activity was determined from triplicate dishes (black bars). The results are expressed as fold increase above the values obtained with cells not exposed to GH.

B: Northern-blot analysis of LPL mRNAs and GAPDH mRNA from cells incubated for 15 hours as above (same series of cells). The arrows indicate the 3.3 and 3.7 kb specie of LPL mRNAs and the 1.4 kb specie of GAPDH mRNA.

ionophores on the expression of LPL appeared rather specific since the content of A2COL6/pOb24 mRNA, which has been characterized like LPL as an early-differentiation marker (12), remained unchanged whereas glycerol-3-phosphate dehydrogenase mRNA and adipocyte lipid binding protein (**ALBP**) mRNA, two late differentiation-specific markers, could not be detected at that stage.

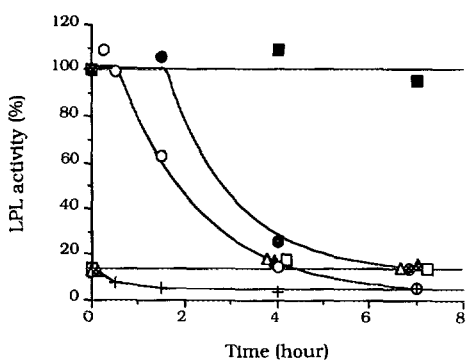

Figure 4. Inhibition of LPL activity following calcium ionophore addition to growth hormone pre-treated cells

At day 5 post-confluence, Ob1771 cells were exposed to the incubation medium in the presence (▲, ■, ●, ○) or in the absence (□, Δ, +) of 4 nM GH, with (□, ■, ○, ●, +) or without (Δ, ▲) 2 µM A23187. Fifteen hours later, either GH was removed (●) or 2 µM A23187 were added (○, +) or not (■, □, ▲, Δ) (time zero). At the indicated times, triplicate dishes were used for the determination of LPL activity. The results are expressed as percent of the value obtained at time zero for GH pre-treated cells.

Taken together, the above results suggested that calcium ionophores were effective at a pre-translational level. Since we had previously shown that c-Fos protein played a role in the regulation of LPL gene expression at a transcriptional level (2), it was of interest to see whether an increase of cytosolic calcium was also preventing the induction of *c-fos* mRNA and subsequently that of c-Fos protein. The results of Figure 5 indicate clearly that this is not the case since the GH-induced expression of *c-fos* gene still occurred and is actually slightly enhanced as a function of time in the presence of 2 μM A23187.

DISCUSSION

The above results show that calcium ionophores abolish the positive modulation of LPL gene expression by GH. Since *c-fos* mRNA is present under these conditions (Fig.5) and since the expression of c-Fos protein follows that of *c-fos* mRNA in preadipose cells (2), it is likely that c-Fos protein is synthetized in the presence of calcium ionophores. In spite of that, under these conditions, no effect of GH on the expression of LPL is observed. In addition to the well-known phosphorylation of c-Fos protein involving protein kinase A and C activities (13), it is possible that the activity of c-Fos protein, as a *trans*-acting factor regulating positively the expression of LPL gene, depends upon its degree of phosphorylation *via* Ca^{2+}-dependent processes.

Alternatively, a dominant and negative Ca^{2+}-dependent regulation of LPL gene expression cannot be ruled out. In favor of this hypothesis is the fact that, in differentiated Ob1771 cells, and in spite of a lack of GH modulation of LPL already expressed at very high levels, an inhibitory effect of Ca^{2+} ionophores is still

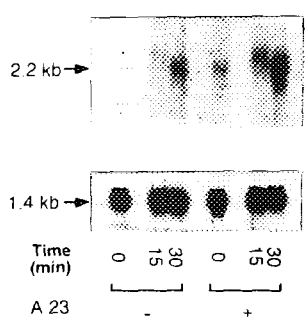

Figure 5. Effect of A23187 on the GH-promoted activation of *c-fos* gene expression

Five-day post-confluent Ob1771 cells maintained in the incubation medium alone (time zero) were exposed for 15 or 30 minutes to 4 nM GH, in the absence (-) or in the presence (+) of 2 μM A23187 (A23). Poly(A)$^+$ RNAs were prepared from three pooled dishes in each experiment, electrophoresed and probed for *c-fos* (2.2 kb) and GAPDH (1.4 kb) mRNAs. At time zero, before exposure to the various effectors, the basal level of *c-fos* mRNA expression varies within the same series of cells from different plates (compare lanes 1 and 4).

observed (not shown). In that respect, it is interesting to note that a putative Ca^{2+}-response element of unknown function has been described within the promoter region of the human LPL gene (14).

In agreement with the production of DAG triggered by GH in preadipocytes with no release of inositol phosphates (3), no change in the concentration of cytosolic calcium can be detected upon GH exposure. Therefore, in preadipose cells, this phenomenon will favor a positive modulation of LPL gene expression by GH. As a consequence, an increased flux of fatty acids entering preadipose cells from triacylglycerol-rich lipoprotein particles can be expected. Fatty acids, which do not modify intracellular Ca^{2+} concentration (G. Vassaux *et al.*, unpublished experiments), are in turn required as transcriptional regulators to activate late differentiation-related genes such as ALBP and acyl-CoA synthetase genes (15-17). In conclusion, it is suggested that a sustained accumulation of cytosolic calcium would preclude not only the full expression of LPL activity but also subsequent molecular events linked to fatty acid entry.

ACKNOWLEDGMENTS

The authors are grateful to Dr. D. Gaillard for very helpful discussion. The authors wish to thank Drs. M. Schotz and Y. Kirchgessner (Los Angeles, CA) for providing us with the mouse LPL cDNA probe. Thanks are also due to Dr. P. Fort (Montpellier, France) for the kind gift of pR GAPDH-13 probe and to Dr. A. Skottner-Lundin (Kabi Pharmacia, Stockholm, Sweden) for the generous gift of recombinant human GH. The secretarial assistance of G. Oillaux and the photographic work of J. Sayegh and C. Cibre are gratefully acknowledged.
This work has been supported by C.N.R.S. (UMR 134), Rhône Poulenc Rorer (France) and Kabi Pharmacia (Sweden).

REFERENCES

1. PRADINES-FIGUERES A., BARCELLINI-COUGET S., DANI C., VANNIER C. and AILHAUD G. (1990) J. Lipid Res. 31, 1283-1291
2. BARCELLINI-COUGET S., PRADINES-FIGUERES A., ROUX P., DANI C. and AILHAUD G. (1993) Endocrinology, 132, 53-60
3. CATALIOTO R.M., AILHAUD G. and NEGREL R. (1990) Biochem. Biophys. Res. Commun. 173, 840-848
4. ROGERS S.A. and HAMMERMAN M.R. (1989) Proc. Natl. Acad Sci. USA 86, 6363-6366
5. GRYNKIEWICZ G., POENIE M. and TSIEN R.Y. (1985) J. Biol. Chem. 260, 3440-3450
6. LEDBETTER J.A., JUNE C.H., GROSMAIRE L.S. and RABINOVITCH P.S. (1987) Proc. Natl. Acad Sci. USA 84, 1384-1388
7. VANNIER C., AMRI E.Z., ETIENNE J., NEGREL R. and AILHAUD G. (1985) J. Biol. Chem. 260, 4424-4431
8. LOWRY O.H., ROSEBROUGH N.J., FARR A.L. and RANDALL R.J. (1951) J. Biol. Chem. 193, 265-275
9. CHOMCZYNSKI P. and SACCHI N. (1987) Anal. Biochem. 162, 156-159

10. DOGLIO A., DANI C., GRIMALDI P. and AILHAUD G. (1986) Biochem J. 238, 115-122
11. VANNIER C. and AILHAUD G. (1986) Biochim. Biophys. Acta. 875, 324-333
12. DANI C., DOGLIO A., AMRI E.Z., BARDON S., FORT P., BERTRAND B., GRIMALDI P. and AILHAUD G. (1989) J. Biol. Chem. 264, 10119-10125
13. MEEK D.W. and STREET A.J. (1992) Biochem J. 287, 1-15
14. DEEB S.S. and PENG R. (1989) Biochemistry 28, 4131-4135
15. AMRI E.Z., BERTRAND B., AILHAUD G. and GRIMALDI P. (1991) J. Lipid Res. 32, 1449-1456
16. AMRI E.Z., AILHAUD G. and GRIMALDI P. (1991) J. Lipid Res. 32, 1457-1463
17. GRIMALDI P., KNOBEL S.M., WHITESELL R.R. and ABUMRAD N.A. (1992) Proc. Natl. Acad. Sci. USA 89, 10930-10934