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THE EFFECT OF CHRONIC FATTY ACID TREATMENT ON LIPOLYSIS IN 3T3-L1 ADIPOCYTES

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SUMMARY: Various saturated and unsaturated fatty acids were included in the culture medium to test their effects on lipolysis in 3T3-L1 adipocytes. Following prolonged incubation, only oleate was found to exert enhancing effect on basal and isoproterenol-stimulated lipolysis. The effect of oleate was concentration-dependent and was accompanied with increased intracellular cAMP content. Furthermore, the lipolytic response induced by isobutylmethylxanthine, forskolin or dibutyryl cAMP was also increased in adipocytes treated with oleate. Thus, it appears that in addition to an increased cAMP accumulation, a step distal to cAMP production in the cells may be involved in inducing enhanced lipolysis in 3T3-L1 adipocytes by prolonged exposure to oleate.

Patients with diabetes mellitus, either insulin-dependent or noninsulin-dependent type was frequently accompanied with increased plasma free fatty acid (FFA) concentrations (1-3) and enhanced lipolysis (4-7). Increased lipolytic activity may partly be due to insufficient insulin availability and enhanced circulating catecholamine levels as in insulin-dependent diabetes (8,9) or be due to resistance to insulin's antilipolytic action as observed in noninsulin-dependent diabetes (2,3). On the other hand, lipolysis in adipose tissue may be modulated by elevated plasma FFA. FFA has been implicated as a feed back regulator of lipolysis in adipocytes (10, 11).

In the present study, we have employed differentiated 3T3-L1 adipocytes, a well established *in vitro* cell model for adipocytes (12,13), to investigate whether lipolysis will be altered following prolonged exposure to fatty acids. The results indicated that in the presence of bovine serum albumin (BSA) at a concentration (4%) comparable to that in the plasma, among the tested fatty

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acids which included palmitate, oleate, linoleate, linoleneate and arachidonate, only oleate was found to exert enhancing effect on lipolysis.

EXPERIMENTAL PROCEDURES

Materials - Dexamethasone, 3-isobutyl-1-methylxanthine (IBMX), adenosine deaminase, (-)-isoproterenol, dibutyryl-cAMP, bovine serum albumin (BSA, fatty acid-free), saturated and unsaturated fatty acids were obtained from Sigma Chemicals, St. Louis. forskolin was purchased from Calbiochem-Behring, La Jolla, CA.

Cell Culture - 3T3-L1 cells, obtained from American Type Culture Collection, were grown in 6-well plates (Nunc) containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (GIBCO), glutamine, penicillin and streptomycin as described (14). The cultures were kept at 37°C in a humidified atmosphere of 10% CO_2, 90% air, and the medium was changed every 2 or 3 days.

Differentiation of 3T3-L1 Cells to Adipocytes - 3T3-L1 preadipocytes were differentiated to adipocytes as described previously by Carnicero(14) and Rubin, et al.(15) with modification. Briefly, 2 days after confluence (day 0), the medium was removed and fresh medium containing 0.5 mM IBMX and 0.25 uM dexamethasone was added. After another 3 days, the medium was replaced with fresh culture medium and the cultures were then maintained as described above. By day 8, more than 90% of the cells have differentiated into rounded cells with lipid droplets. Each well (dia. 35 mm) contained approx. 2 x 10^6 cells.

Chronic Fatty Acid Treatment of Differentiated Adipocytes - On day 8, the culture medium was replaced with the medium containing 4% fatty acid-free BSA (control) or the control medium also containing individual fatty acid. The fatty acid concentration in the control medium was estimated to be approx. 10 uM by the method of Noma, et al.(16). The medium containing fatty acid (fatty acid medium) was prepared as described earlier (17). The cells were then incubated for 6 days (or otherwise indicated) with medium change (control or fatty acid medium) every 2 days.

Measurement of Lipolysis - Lipolysis was monitored by measuring glycerol released into the incubation mixture. Briefly, on the day of an experiment, the medium was removed and the adipocytes were incubated in serum-free DMEM for 2 h. The monolayer cells were then washed three times with a KRP buffer containing 128 mM NaCl, 4.7 mM KCl, 1.25 mM $CaCl_2$, 1.25 mM $MgSO_4$, and 10 mM sodium phosphate (pH 7.4). Ten minutes after the addition of adenosine deaminase (1 unit/ml) in 1 ml of KRH buffer (KRP buffer which also contained 50 mM Hepes and 4.2 % BSA, pH 7.4), test agents were added and the incubation was continued for another 10 min. The incubation mixture was aspirated and used for the assay of glycerol. In some experiments, as soon as the incubation mixture was aspirated, 0.4 ml of 10% trichloroacetic acid was added to the remaining cells which were used for the assay of cellular content of cAMP.

Assays - Glycerol was assayed enzymatically as described by Garland and Randle (18). Cyclic AMP was measured by a cAMP-binding protein assay (19) after being separated from trich-

loroacetic acid and other nucleotides by chromatography on a Dowex-50W resin (H^+-form, 200-400 mesh) as described earlier (20). Statistical differences were determined by Student's t-test.

RESULTS

Among the various fatty acids present in human serum, five representative ones including palmitate(16:0), oleate(18:1,ω-9), linoleate(18:2,ω-6), linoleneate(18:3,ω-3) and arachidonate (20:4,ω-6) were employed in this study to test their effects on lipolysis. The concentrations of these fatty acids used were chosen as to be close or higher than those reported in the serum of diabetic patients (21). As shown in Table 1, after prolonged exposure to various fatty acids in the presence of 4% BSA in the culture medium, only oleate was found to exert enhancing effect on lipolysis in 3T3-L1 adipocytes. Both basal and isoproterenol-stimulated lipolytic activities were increased. The effect of oleate was dependent on the incubation time; whereas the basal lipolysis was not influenced until 6 days' incubation, isoproterenol-stimulated lipolysis was apparently increased following 2 days' incubation (Fig. 1).

The enhancing effect of chronic oleate treatment on isoproterenol-induced lipolysis was concentration-dependent, with increasing effect of approx. 10%, 20%, and 50%, at 0.35 mM , 0.75 mM and 1.5 mM, respectively. At 0.2 mM of oleate, no effect was

Table 1. Effect of chronic fatty acid treatment on lipolysis in 3T3-L1 adipocytes

Medium	Glycerol Release (nmol/well)	
	Basal	Isop (1μM)
Control	61 ± 3	178 ± 3
16:0 (1.5mM)	59 ± 3	173 ± 4
18:1(ω-9)(1.5mM)	100 ± 4[a]	240 ± 12[a]
18:2(ω-6)(0.5mM)	56 ± 2	163 ± 6
18:3(ω-3)(0.2mM)	51 ± 4	164 ± 7
20:4(ω-6)(0.2mM)	59 ± 3	170 ± 4

[a] $p<0.01$ compared with corresponding control.

Differentiated adipocytes were maintained in control or fatty acid medium for 6 days and were tested for lipolysis in the absence (basal) or presence of 1 μM isoproterenol (Isop). Values are means ± S.E. (n=3).

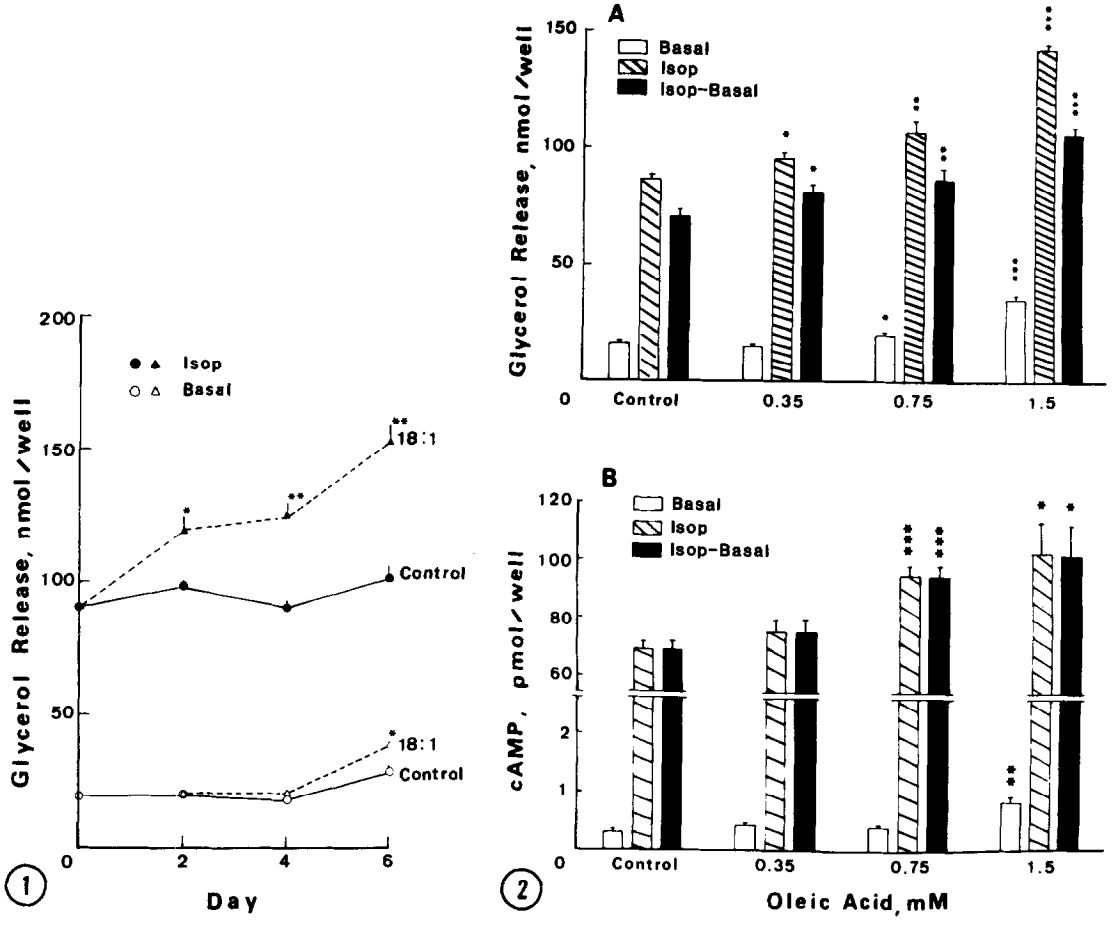

Fig.1. Time-dependent effect of oleate treatment on lipolysis in 3T3-L1 adipocytes. After 3T3-L1 adipocytes were incubated in control or oleate (18:1, 1.5 mM) medium for various days, basal and isoproterenol(Isop)-stimulated lipolysis were measured. Values are means ± S.E. (n=3). *$p<0.05$, **$p<0.01$.

Fig.2. (A)Concentration-dependent effect of oleate treatment on lipolysis in 3T3-L1 adipocytes. Differentiated 3T3-L1 cells were maintained in control (containing 4% fatty acid-free BSA) medium or oleate media containing various concentrations of oleic acid for 6 days and lipolysis was measured in the absence (basal) or presence of 1 μM isoproterenol(Isop). Values are means ± S.E.(n=3). *$p<0.05$, **$p<0.02$, ***$p<0.001$. (B)Cellular cAMP accumulation in control or oleate-treated 3T3-L1 adipocytes in the absence (basal) or presence of 1 μM isoproterenol(Isop) as described in legend to Fig. 2A. *$p<0.05$, **$p<0.01$.

detected. The basal lipolytic response of adipocytes, on the other hand, was not increased until the concentration of oleate reached 0.75 mM (Fig. 2A). Parallel experiments indicated that cellular accumulation of cAMP induced by isoproterenol was also increased with increasing concentrations of oleate. The increase in basal cAMP level, however, was not evident until the concentration of oleate reached 1.5 mM (Fig. 2B).

Table 2. Lipolysis in response to isobutylmethylxanthine(IBMX), forskolin and dibutyryl cAMP(dB-cAMP) in 3T3-L1 adipocytes maintained in control or oleate medium for 6 days

	Glycerol Release (nmol/well)	
	Control	18:1(1.5 mM)
Basal	35 ± 3	50 ± 3[a]
IBMX (0.5 mM)	117 ± 4	145 ± 6[b]
Forskolin (50 μM)	118 ± 2	141 ± 3[b]
dB-cAMP (10 mM)	127 ± 2	147 ± 4[b]

[a] $p<0.05$, [b] $p<0.02$ compared with corresponding control.
Values are means ± S.E. (n=3)

Dose-response curve of isoproterenol-induced lipolysis in oleate-treated adipocytes indicated that while the glycerol release in response to isoproterenol was increased, the EC_{50} (2 nM) for isoproterenol was not changed (data not shown). Further studies with lipolysis induced by IBMX, forskolin and dibutyryl cAMP, agents acting at various intracellular sites along the cAMP cascade, showed that the lipolytic responses to all these agents were increased in adipocytes after prolonged exposure to oleate (Table 2).

DISCUSSION

The results of this study have demonstrated that long-term exposure to certain fatty acid, i.e. oleic acid, may alter the lipolytic response of adipocytes to catecholamines. At a concentration as low as 0.35 mM, the enhancing effect of oleate on lipolysis is already evident. If the *in vitro* cell model 3T3-L1 adipocytes may resemble the *in vivo* adipocytes of the adipose tissue in the human body, the findings of this study may imply that, although under normal condition the plasma concentration of oleate is below 0.2 mM, under certain pathogenic conditions such as uncontrolled diabetes mellitus, Reye's syndrome, acute pancreatitus, which are manifested with elevated plasma FFA concentrations(22), the lipolytic response of adipose tissue may be further positively regulated by its product, oleic acid. As a consequence, higher serum insulin concentration may be needed to inhibit lipolysis. Indeed, reduced insulin suppression of

lipolysis has been reported in noninsulin-dependent diabetes mellitus(2,3).

The enhancing effect on lipolysis by FFA seems to be specific for oleate, a monounsaturated fatty acid belonging to ω-9 series. Saturated or polyunsaturated fatty acids of ω-3 or ω-6 series did not show similar enhancing effect on lipolysis. Thus, it is of interest to find out whether other ω-9 fatty acids with higher degree of unsaturation, i.e. 20:2 (ω-9), or monounsaturated fatty acid other than ω-9 series, i.e. 16:1(ω-7), may show any enhancing effect on lipolysis.

Although both basal and isoproterenol-induced lipolysis were influenced by prolonged exposure of adipocytes to oleate, they seemed to be affected differentially. Neither time course (Fig. 1) nor dose-response(Fig. 2) experiments indicated that basal and isoproterenol-stimulated lipolysis were parallelly altered.

At present, the mechanism of influencing lipolysis by long-term exposure of adipocytes to oleate is not certain. On the one hand, oleate may be incorporated into the phospholipid in the plasma membranes such that activities of certain membrane proteins or enzymes would be altered by their lipid environments. Adenylate cyclase or G-proteins, for example, might be influenced in a way that increased accumulation of cellular cAMP was observed in adipocytes exposed to oleate. On the other hand, the finding that dibutyryl cAMP-induced lipolysis was also increased in adipocytes seemed to suggest that a step distal to cAMP production may also be involved. Either cAMP-dependent protein kinase or hormone-sensitive triglyceride lipase may be a good candidate. Since these enzymes are not membrane-bound, a mechanism different from the one mentioned above may be involved for oleate to exert its effect.

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