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Endotoxin-Induced Alterations in Isolated Fat Cells: Effect on Norepinephrine-Stimulated Lipolysis and Cyclic 3′,5′-Adenosine Monophosphate Accumulation (37774)

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(Introduced by J. J. Spitzer)
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The underlying alterations in energy demand and tissue fuel supply associated with various forms of injury and shock have been the subject of a great deal of research in recent years (1). In this context the adipose tissue mass assumes special importance, since it represents usually at least 15% of body weight and it is the major storehouse to satisfy energy requirements.

In our earlier work we have investigated the changes in norepinephrine (NE)-stimulated lipolysis by isolated fat cells brought about by hemorrhagic (2) and endotoxic shock (3). Injection of *E. coli* endotoxin into dogs increased the NE-induced lipolytic response in SC, O and mesenteric (M) fat cells, as evidenced by increased FFA and G release (3). In an attempt to elucidate the mechanism whereby endotoxin renders the fat cells more metabolically responsive to NE, the interaction between endotoxin and fat cells *in vitro* was investigated. The present communication reports the results of these *in vitro* studies.

Materials and Methods. Fat cells were isolated by collagenase digestion of canine subcutaneous and omental adipose tissue according to the method of Rodbell (4). An aliquot of the washed cells was used as untreated control. The rest of the washed cells were exposed to *E. coli* endotoxin (Difco) in a concentration of 0.2 μg/0.5 ml of cell suspension for 10 min at 37° in a metabolic shaker. The 0.5 ml cell suspension corresponded to about 90–100 mg wet weight of the original adipose tissue. The cells were then washed three times and incubated in plastic, capped culture tubes, in Krebs–Ringer bicarbonate (KRB) buffer containing 4% bovine serum albumin, 5.5 μmoles/ml glucose, and 0.2 μg/ml NE. The gas phase for incubation was 95% O_2–5% CO_2. Incubation was carried out at 37° in a metabolic shaker for 2 hr. The untreated cells (designated as "before endotoxin" cell populations) were incubated under identical conditions. At the end of the incubation period aliquots of the medium were taken for FFA (5) and G analyses (6). Triglyceride (TG) content of each incubation tube was determined from the amount of total FFA after saponification. Cell diameters were determined in each experiment with the aid of a light microscope fitted with a calibrated ocular. The diameters of 100 cells from several fields were measured and the mean diameter, variance and the amount of TG per cell were calculated (7). By these means we were able to express our results not only relative to TG content, but also on the basis of cell number.

Assay of cAMP was based on the competitive binding of ^{3}H-labeled and unlabeled cAMP to a binding protein (8). Kits for the performance of the assay were purchased from Nuclear Dynamics, Inc., El Monte, CA.

When cells were assayed for cAMP content before and after endotoxin exposure *in*

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TABLE I. Effect of Endotoxin Treatment *in Vitro* on the NE-Induced Lipolytic Response of Isolated Fat Cells.[a]

	G released in 2 hr (μmoles)		FFA released in 2 hr (μmoles)	
	per mm TG	per 10^6 cells	per mm TG	per 10^6 cells
Before				
SC	1.13	0.159	2.33	0.328
O	1.16	0.171	2.35	0.346
After				
SC	3.60	0.506	6.21	0.877
O	4.88	0.716	9.97	1.47

[a]The cells were incubated for 10 min at 37° in a metabolic shaker. Dosage of endotoxin was 0.2 μg/0.5 ml cell suspension, corresponding to about 90–100 mg wet weight of the original adipose tissue.

vitro, 1.5 ml of Dole's solution and 1.0 ml heptane were added to 0.5 ml of cell suspension. The cells were homogenized in this mixture for 20 sec with a glass pestle and a motor driven homogenizer. The phases were allowed to separate. The top layer was taken off and used for TG determination, the lower phase was placed in a vacuum oven at room temperature for 20 min, then centrifuged at 5000 rpm for 10 min. The clear aqueous supernatants were extracted 5 times with 2 vol of ether after the addition of 0.1 ml of 1 *N* HCl. The extracts were then dried in a water bath at 40–45° under a stream of nitrogen, stored at −20° and later redissolved in 50 m*M* sodium acetate (pH 4), just prior to performing the binding assay.

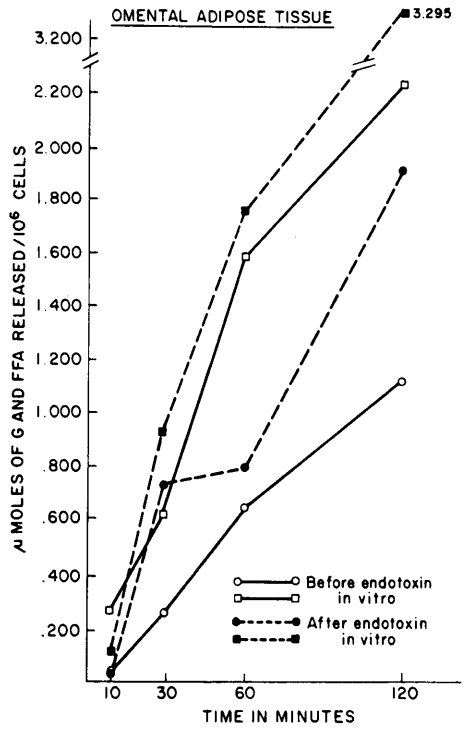

FIG. 1. Time course of G and FFA release by O adipocytes. Cells were incubated in the presence of 0.2 μg/ml. NE. (O—) G and (□—) FFA before endotoxin treatment *in vitro;* (●...) G and (■...) FFA after endotoxin treatment *in vitro.*

When cAMP was assayed in cells that were also studied with respect to their NE-induced lipolytic response, the procedure was as follows: At the end of the NE incubation period, the incubation tubes were placed on crushed ice to permit the fat cells to float to the top. The infranatant incubation medium was aspirated with a capillary pipet and used for FFA and G determinations. To the fat cells were added 0.3 ml KRB medium, 1.5 ml Dole's solution and 1.0 ml heptane. All subsequent steps were identical with those described for cells that were treated with endotoxin only.

Results. A. Effect of endotoxin treatment in vitro *on the NE-induced lipolytic response of isolated fat cells.* Endotoxins form aggregates easily and also complex with a number of other natural products. Therefore in assessing the effects of endotoxin on fat cells one cannot exclude the possibility of some direct interaction between the endotoxin molecule and portions of the cell membrane. In order to test this possibility, isolated fat cells were exposed to endotoxin *in vitro* as described in the Methods section. NE-induced lipolysis was then evaluated by the rate of G

and FFA release, and compared to rates obtained before endotoxin exposure. Results of a representative experiment are shown in Table I. Each value is the mean of duplicate incubations. The release of both G and FFA upon NE stimulation is enhanced by endotoxin. Cells isolated from both SC and O sites respond in this manner.

B. Time course of FFA and G release. Examples of the time course of G and FFA release by isolated O fat cells in the presence of 0.2 μg/ml NE are given in Fig. 1. These are data taken from one of the four experiments done in this group, and each point represents the mean of duplicate determinations. The release was linear for 2 hr of incubation. When fat cells pretreated with endotoxin *in vitro* were incubated for 30 min or longer, the release of both G and FFA was higher than before endotoxin treatment. Although intracellular cAMP was also measured at each time interval, no direct relationship was evident between the level of the cyclic nucleotide and the extent of lipolysis. Lipolysis proceeded in a linear fashion concurrently with lower levels of cAMP than were obtained shortly after NE application. This is in accordance with the reported formation and release of a hormone antagonist by rat adipocytes (9).

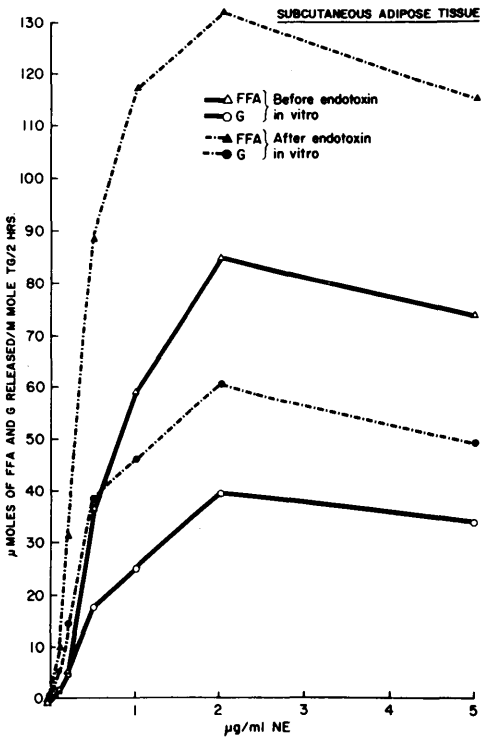

FIG. 2. NE dose–response curves with respect to G and FFA release. SC fat cells were incubated for 2 hr as described under Methods.

C. NE dose response curves in vivo *and* in vitro. On the hormone–receptor interaction level, the enhanced lipolytic response to NE stimulation following endotoxin treatment (*in vivo* or *in vitro*) could theoretically result from an improved affinity of the receptor(s) for NE or from the unmasking of receptor sites previously not exposed.

Preliminary dose–response studies have indicated that the concentration of NE we customarily use for incubation (*i.e.*, 0.2 μg/ml) is far from saturating (10). We therefore undertook to investigate the lipolytic response as a function of increasing NE concentration (up to saturation) before and after endotoxin treatment *in vitro*. Figure 2 shows representative data from one of the five dose–response experiments. As can be seen, at each dose level, even at saturation, the NE-induced G and FFA release is higher after endotoxin treatment than before. The concentration of NE at which half maximal stimulation occurs shows no consistent changes due to endotoxin.

D. Effect of endotoxin treatment in vitro *on cAMP content.* Exposure of isolated SC and O fat cells to endotoxin *in vitro* (as described in the Methods section) resulted in statistically significant increases in cAMP content. Figure 3 shows the mean increases from control values ± standard error of the mean in this nucleotide, exhibited by SC and O adipocytes. The mean increase is about threefold compared to the untreated control level.

E. Effect of graded doses of NE on cAMP. Stimulation of lipolysis by graded doses of NE in fat cells was also accompanied by increasing levels of intracellular cAMP. Table II shows the results of one of the four experiments. The cells were incubated for 2 hr, and each value is the mean of duplicate determinations. Even in the absence of NE, the endotoxin-treated cells had a higher

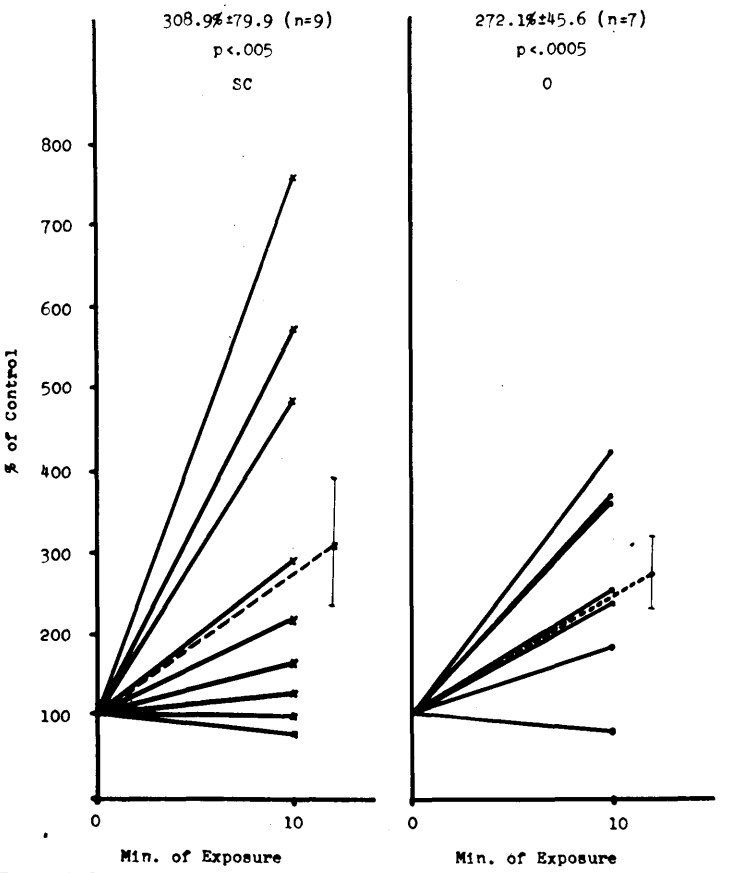

FIG. 3. Intracellular cAMP increase in SC and 0 fat cells after exposure to endotoxin *in vitro*.

cAMP content than normal cells, as also shown under Sect. D. At each concentration of NE the cells pretreated with endotoxin responded with even higher levels of cAMP.

Discussion. The reactivity of fat cells can be influenced by digestion with various bacterial products. Enhanced binding of ^{125}I-insulin to fat cells results from digestion with phospholipase C from *C. welchii*. Concurrently, phospholipase C destroys the capacity of these cells to increase the rate of glucose oxidation in response to insulin. Digestion of fat cells with neuraminidase from *C. perfringens* results in a profound loss in the capacity of these cells to increase the rate of glucose transport in response to insulin (11, 12).

TABLE II. cAMP Content of Subcutaneous Fat Cells After Incubation in the Presence of Increasing Concentrations of NE, Before and After Exposure to Endotoxin *in vitro*.[a]

NE (μg/ml)	cAMP content/10^6 cells (pmoles)	
	Before	After
0	0.165	0.361
0.2	0.833	7.08
0.5	0.755	35.23
1.0	0.978	29.19
2.0	3.71	45.68
5.0	5.57	11.16

[a]The conditions of endotoxin exposure were the same as described in Table I.

Results of the studies reported here show that following endotoxin exposure *in vitro*, canine fat cells release more G and FFA upon NE stimulation than what is evoked by the same dose of NE under control conditions. Such an alteration in a vital metabolic activity of the cell is very likely to be indicative—at least partially—of some changes brought about by endotoxin at the site of the biochemical communications junction, the

cell membrane. In recent years several mitochondrial functions have been shown to be altered by endotoxin (13), suggesting changes in the mitochondrial membrane.

Three approaches have been employed to study the mechanism of the increased NE-induced lipolytic response due to endotoxin treatment. One approach has been at the hormone–receptor interaction level. The NE dose–response studies established that even the maximal lipolytic response was increased after endotoxin exposure, without a consistent change in the affinity of the receptor for NE (*i.e.*, the dose at which the magnitude of the lipolytic response was half maximal). The interpretation of such data is consistent with the availability of additional receptor sites. The concept of previously unavailable receptor sites being uncovered by endotoxin is supported by the compatibility of the NE dose–response curves with respect to G and FFA release with the Clark-Stetton model (14, 15). Plotting velocity of response against hormone concentration, gives a hyperbolic form, which resembles a real dose–response curve for NE acting on G and FFA release by adipocytes (Fig. 2) before and after treatment of the cells with endotoxin.

Perturbation of certain lipid and/or protein components of the membrane by endotoxin could account for the availability of receptor sites previously unexposed. The relationship between binding and activation by NE may be changed also by endotoxin. It is conceivable that under control conditions (before endotoxin treatment) not all of the specific binding sites for NE are "coupled" to the adenylate cyclase system. Exposure to endotoxin may increase the number of binding sites that are functionally involved in the activation process.

In the second approach the effect of endotoxin treatment on the level of intracellular cAMP was evaluated. The statistically significant increases in cAMP content of both SC and O cells could be due to activation of adenylate cyclase and/or inhibition of phosphodiesterase. The former is more likely, since cyclase is membrane bound, whereas phosphodiesterase is primarily found in the microsomal fraction, although present in the cell membrane to some extent also, especially in the brain. Endotoxin is likely to interact with the plasma membrane of these cells rather than the intracellular contents.

The possibility of indirect activation of adenylate cyclase by endotoxin due to removal of Ca^{2+} — a key component (16) —from the cell membrane must be considered also. Changes in Ca^{2+} binding alter many of the physical properties of the membrane (17). In addition to changes in lipoprotein structure, endotoxin may also cause alterations in ionic distribution within the membrane. Calcium is known to inhibit cyclase (17) and is implicated in the activation of phosphodiesterase. Binding of Ca^{2+} by endotoxin, which has been reported (18), and which also promotes the influx of Na^{+} (17), may contribute to the accumulation of cAMP by derepression of cyclase and inactivation of diesterase.

Prostaglandins have emerged as endogenous modulators of hormone-induced lipolysis in adipose tissue (19). It has been shown that in order for prostaglandins to act, they need an intact fat cell membrane. In rat fat cell particles, when no response to PGE_1 can be obtained, adenylate cyclase can still be activated by epinephrine and inhibited by propanolol (20). Interaction with endotoxin may result in such alterations in the plasma membrane that the prostaglandin effect is no longer possible, thus an inhibitory influence on lipolysis is removed.

As a third approach, the relationship between intracellular cAMP level and lipolysis was explored. Assay of cAMP levels in homogenates of rat epididymal fat pads previously exposed to epinephrine has established before that addition of a lipolytic hormone enhances the level of this cyclic nucleotide (21). Increasing concentrations of NE resulted in increasing levels of cAMP in SC cells, as shown in Table II. After endotoxin treatment the cells started out with a higher cAMP level. Subsequent NE stimulation further elevated the level of the cyclic nucleotide. Beyond an intermediate dose of NE, however, no additional increases in cAMP content were elicited.

The data did not reveal any simple, direct correlation between cAMP content and G or FFA release. This is not totally unexpected

since the exact relationship between cyclase and hormone receptors as well as information transfer to other enzymes and their substrates are as yet undefined.

The mere presence of a high level of cAMP in the fat cell is not necessarily indicative of equally high levels in all intracellular compartments, some of which may be more crucial or the final expression of the lipolytic response than others. Butcher *et al.* (21) have found that when cAMP reached an elevated level in epididymal fat pads, one or more components of the system involved in FFA release had become saturated, and the cyclic compound was no longer a limiting factor on the rate of FFA release. In the studies reported here it was found that the enormously elevated cAMP levels (up to 30–40-fold) at graded doses of NE after endotoxin action did not induce a correspondingly high FFA or G release. One interpre tation of these data would be that cAMP is less effective in endotoxin-treated cells possibly due to some other effect of NE—which is part of the total response of the cell to this hormone—being blocked by endotoxin. Further studies are in progress to resolve these alternatives.

Summary. This study was designed to investigate the mechanism of interaction between endotoxin and isolated canine fat cells. Exposure to endotoxin *in vitro* elicited a higher NE-stimulated lipolytic response than that obtained from normal cells. The release of G and FFA in both normal and endotoxin-treated cells was linear for 2 hr.

NE dose–response studies revealed increased G and FFA release at each dose level including saturation, after endotoxin treatment, without consistent changes in the dose at which half-maximal response occurred. The NE dose–response curves were compatible with the Clark-Stetton model of hormone action. Thus the data are consistent with the interpretation of a greater number of receptor—hormone complexes being involved after exposure to endotoxin. Endotoxin-treated fat cells of both SC and O origin had a significantly higher cAMP level, which was further elevated by subsequent NE stimulation, manyfold above the level of normal cells.

The results suggest that exposure of isolated fat cells to endotoxin *in vitro* causes alterations in the lipoprotein components as well as in the ionic distribution within the fat cell membrane.

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1. Kovach, A. G. B., Stoner, H. B., and Spitzer, J. J., eds. "Advances in Experimental Medicine and Biology," Vol. 33. Plenum, New York (1973).

2. Storck, R., and Spitzer, J. A., Physiologist **15**, 276 (1972).

3. Spitzer, J. A., Kovach, A. G. B., Rosell, S., Sandor, P., Spitzer, J. J., and Storck, R., *in* "Advances in Experimental Medicine and Biology" (A. G.B. Kovach, H. B. Stoner and J. J. Spitzer, eds.), Vol. 33, p. 337. Plenum, New York (1973).

4. Rodbell, M., J. Biol. Chem. **239**, 375 (1964).

5. Dole, V. P., and Meinertz, H., J. Biol. Chem. **235**, 2595 (1960).

6. Laurell, S., and Tibbling, G., Clin. Chim. Acta **13**, 317 (1966).

7. Hirsch, J., and Gallian, E., J. Lipid Res. **9**, 110 (1968).

8. Gilman, A. G., Proc. Nat. Acad. Sci. USA **67**, 305 (1970).

9. Ho, R. J., and Sutherland, E. W., J. Biol. Chem. **246**, 6822 (1971).

10. Spitzer, J. A., Kovach, A. G. B., Sandor, P., Spitzer, J. J., and Storck, R., unpublished data.

11. Cuatrecasas, P., J. Biol. Chem. **246**, 6532 (1971).

12. Cuatrecasas, P., and Illiano, G., J. Biol. Chem. **246**, 4938 (1971).

13. Mela, L., Bacalzo, L., and Miller, L. D., Amer. J. Physiol. **220**, 571 (1971).

14. Goodman, D. S., Science **124**, 365 (1956).

15. Rudman, D., Garcia, L. A., Brown, S. J., Malkin, M. I., and Perl, W., J. Lipid Res. **5**, 28 (1964).

16. Manery, J. F., Fed. Proc., Fed. Amer. Soc. Exp. Biol. **25**, 1804 (1966).

17. Rasmussen, H., Science **170**, 404 (1970).

18. Skarnes, R. C., J. Bacteriol. **95**, 2031 (1968).

19. Illiano, G., and Cuatrecasas, P., Nature (London) New Biol. **234**, 72 (1971).

20. Lewis, G. P., and Matthews, J., J. Physiol. (London) **202**, 95P (1969).

21. Butcher, R. W., Ho, R. J., Meng, H. C., and Sutherland, E. W., J. Biol. Chem **240**, 4515 (1965).

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