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Lipoprotein lipase suppression in 3T3-L1 cells by an endotoxin-induced mediator from exudate cells

(hypertriglyceridemia/infection/shock/3T3-L1 adipocytes/macrophages)

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ABSTRACT **Conditioned medium from cultures of mouse peritoneal exudate cells incubated with endotoxin contains a mediator that markedly suppresses (>90%) lipoprotein lipase (triacylglycero-protein acylhydrolase, EC 3.1.1.34) activity in differentiating 3T3-L1 mouse preadipocytes. The effect is dependent upon the amount of mediator and is evident as early as 30 min after the addition of the mediator-containing medium to 3T3-L1 cell cultures. Neither endotoxin nor conditioned medium from cultures of exudate cells not exposed to endotoxin shows the presence of the mediator. Lysates of the exudate cells are also unable to suppress the lipase activity. Increasing the amount of insulin does not reverse this suppression, even at 1000 times the concentration used for standard experiments. The lipoprotein lipase suppression mediator present in the conditioned medium of endotoxin-treated exudate cells is heat labile and has an apparent molecular weight of at least 12,000. The mediator does not inhibit lipoprotein lipase activity directly nor does it affect the half-life of enzyme activity released in the medium. The present study demonstrates that endotoxin promotes the release of a mediator from exudate cells that suppresses the activity of lipoprotein lipase in 3T3-L1 preadipocytes.**

Several common physiological and biochemical derangements are seen in the mammalian host responding to a variety of invasive stimuli such as bacterial, viral, and protozoan infections. These responses include fever, leukocytosis, and increase in a class of plasma proteins known as "acute phase proteins" (1). Hyperlipidemia is another biochemical derangement observed in humans and animals responding to infection, tumor, or endotoxemia (2–7). Recently, a hypertriglyceridemia in rabbits infected with the protozoan parasite *Trypanosoma brucei* was described (8). In these rabbits, clearance of very low density lipoprotein triglyceride was impaired due to a decreased activity of lipoprotein lipase (triacylglycero-protein acylhydrolase, EC 3.1.1.34) in peripheral tissues. Utilizing endotoxemia as a model, we further investigated the mechanism by which the activity of lipoprotein lipase is depressed in animals responding to an invasive stimulus. In that *in vivo* study, we observed that the effect of endotoxin on adipose tissue lipoprotein lipase was mediated by a humoral factor secreted by peritoneal exudate cells (primarily macrophages) responding to endotoxin (9).

To further investigate the action(s) of the mediator, the well-defined 3T3-L1 "preadipocyte" model system was employed. 3T3-L1 preadipocytes, originally cloned from mouse embryo fibroblasts, differentiate in monolayer culture into cells having the biochemical and morphological characteristics of adipocytes (10–12). During adipocyte conversion, 3T3-L1 cells exhibit a coordinate rise in the enzymes of *de novo* fatty acid synthesis (13, 14) and triacylglycerol synthesis (15). Similarly, the activity of lipoprotein lipase, another key enzyme of lipid metabolism, rises 80- to 180-fold during adipose conversion (16–18). The activity of this enzyme is enhanced by the presence of insulin in the medium and appears to be similar to the lipoprotein lipase of adipose tissue (19, 20).

Utilizing cells of the 3T3-L1 preadipocyte cell line, we have found that addition of the mediator substance, derived from mouse peritoneal exudate cells exposed to endotoxin, suppresses the activity of lipoprotein lipase.

EXPERIMENTAL PROCEDURES

Materials. Endotoxin (lipopolysaccharide) from *Escherichia coli* 0127:B8 isolated by the method of Westphal (21) was purchased from Difco. Cell culture media and fetal calf serum were obtained from GIBCO. 3-Isobutyl-1-methylxanthine was from Aldrich, dexamethasone was from Sigma, and insulin was from Eli Lilly. Glycerol tri[9,10(n)-^{3}H]oleate was purchased from Amersham. Unlabeled glycerol trioleate (triolein) was from Nu Check Prep (Elysian, MN). Crystalline bovine serum albumin was from Calbiochem–Behring.

3T3-L1 Cell Culture. 3T3-L1 preadipocytes were cultured as described (13, 14) in Dulbecco's modified Eagle's medium (DME medium) containing 10% fetal calf serum. Differentiation leading to the adipocyte phenotype was induced by a modification (14) of the method of Rubin *et al.* (22). Two days after confluence, the medium was supplemented with 0.5 mM isobutylmethylxanthine, 1 μM dexamethasone, and 10 μg of insulin per ml. Forty-eight hours later, the medium containing isobutylmethylxanthine, dexamethasone, and insulin was withdrawn and replaced with medium containing insulin at a reduced concentration of 50 ng/ml.

Preparation of Peritoneal Exudative Cells and Mediator Substance. Peritoneal exudate cells were obtained by peritoneal lavage from C3H/HeN mice (25–33 g; Charles River Breeding Laboratories) that had been injected intraperitoneally with sterile Brewer's thioglycollate medium (Difco; 3 ml per mouse) 6 days prior to harvest. The exudate cells obtained by using this procedure are primarily macrophages, with some contaminating lymphocytes (23).

The cells (4×10^5 cells per cm^2) were incubated in serum-free RPMI 1640 medium for 3 hr, after which nonadherent cells were removed by washing three times with medium. Cells adhering to the dish were primarily macrophages. These cells were further incubated in serum-free RPMI 1640 medium in

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Abbreviations: DME medium, Dulbecco's modified Eagle's medium; U, lipoprotein lipase units.

the presence or absence of endotoxin at 5 μg/ml for 36 hr. The culture medium was removed after incubation and centrifuged at 1000 × *g* for 5 min at 4°C. The supernatant of conditioned medium obtained from cells exposed to endotoxin contained the mediator substance (9). No difference in activity was noted after storage of the conditioned medium for 1 month at −80°C.

Effect of Mediator on 3T3-L1 Cells. One hour after the culture medium was replaced with medium containing the reduced concentration of insulin, conditioned media from cultured exudate cells with or without added endotoxin were added to 3T3-L1 cell cultures. Incubation of the cells with the conditioned medium was carried out for up to 20 hr. At indicated times, the amount of lipoprotein lipase activity was measured in three compartments: (*i*) the activity in the medium; (*ii*) the activity released from the cells after incubation with heparin (this activity represents the enzyme associated with the outer surface of the cell membrane) (24); (*iii*) intracellular activity.

After the withdrawal of the medium, the dishes were rinsed once with fresh medium and the lipoprotein lipase associated with the cell membrane (24) was released by incubation for 1 hr in DME medium supplemented with herapin (10 units/ml) and insulin (50 ng/ml). After this medium had been removed, the dishes were rinsed with phosphate-buffered saline and the cells were scraped into 1 ml of 50 mM NH_3/NH_4Cl buffer (pH 8.1) containing heparin at 3 units/ml. The cell suspension was sonicated (on ice) for 15 sec and centrifuged at 500 × *g* for 5 min (17). The supernatant was assayed for lipoprotein lipase.

Lipoprotein lipase assays were performed within 30 min after the preparation of each sample in duplicate by the method of Nilsson-Ehle and Schotz (25) with minor modifications. Briefly, 75 μl of enzyme was mixed with 25 μl of substrate containing 22.6 mM [^{3}H]triolein (1.4 μCi/μmol; 1 Ci = 3.7×10^{10} becquerels), lecithin at 2.5 mg/ml, bovine serum albumin at 40 mg/ml, 33% (vol/vol) human serum, and 33% (vol/vol) glycerol in 0.27 M Tris·HCl (pH 8.1) and incubated at 37°C for 90 min. One milliunit (mU) of enzyme activity was defined as the release of 1 nmol of fatty acid per min. The lipase activity in all three compartments was inhibited >90% by addition of 1 M NaCl and >80% by omission of serum, which is the source of apolipoprotein C-II needed for enzymatic activity (16, 26, 27).

Miscellaneous. A lysate of mouse peritoneal exudate cells (3×10^6 cells per ml of RPMI 1640 medium) was prepared by sonicating the suspension on ice for 3 min. No intact cells were detectable by microscopic examination. The solution was filtered through a 0.45-μm-pore-diameter Millex membrane (Millipore). Previous studies showed that the mediator substance could be filtered without loss of activity.

Vacuum dialysis was carried out in dialysis tubing with molecular weight cut-off at 12,000–14,000. Samples of conditioned medium obtained from endotoxin-treated exudate cell cultures were placed under vacuum for 6 hr at 4°C with a 40% reduction in volume. Aliquots from inside and outside the bag were assayed for mediator activity.

RESULTS

Previous studies have shown that mouse peritoneal exudate cells secrete a mediator substance that suppresses lipoprotein lipase activity *in vivo* in the adipose tissue of the mouse (9). To test the effect of the mediator on the lipoprotein lipase activity of 3T3-L1 cells, conditioned medium obtained from mouse peritoneal exudate cells cultured in the presence or absence of endotoxin was added to 3T3-L1 cells in monolayer culture. After a 20-hr incubation at 37°C, lipoprotein lipase activity was assessed in three compartments: the culture medium, the cell surface (heparin-releasable lipase activity), and the intracellular fraction.

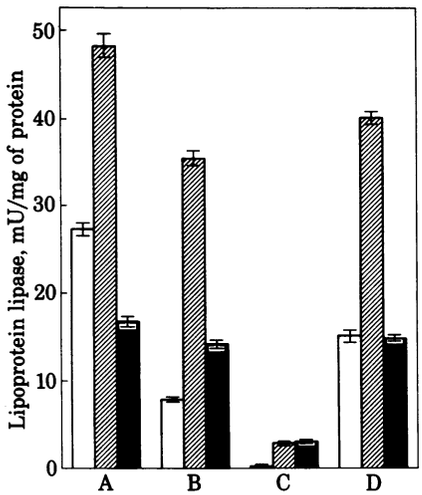

FIG. 1. Effect of conditioned medium from endotoxin-treated mouse peritoneal exudate cells on lipoprotein lipase activity of 3T3-L1 cells. One milliliter of either RPMI 1640 medium (*A*) or conditioned medium from exudate cells incubated in the absence (*B*) or presence of endotoxin (5 μg/ml) (*C*) or RPMI 1640 medium with endotoxin (22.5 μg/ml) (*D*) was added into a confluent culture of 3T3-L1 cells in 6.0-cm dishes containing 3.5 ml of DME medium. After 20 hr of incubation, lipoprotein lipase activity in the medium (open bars), in the cell surface (hatched bars), and in cell sap (solid bars) was measured. Data are expressed as mean ± SEM ($n = 4$).

As shown in Fig. 1 *A* and *C*, the addition of media containing the mediator substance from endotoxin-stimulated exudate cells markedly supressed the lipoprotein lipase activity in all three compartments. The enzyme activities in the medium, on the cell surface (heparin releasable), and in the intracellular compartment were 0.1%, 6%, and 18%, respectively, of that of the control cells incubated with the same amount of fresh RPMI 1640 medium. No difference in morphology or extent of adipocyte conversion was detected between cells in the experimental and control groups. At the beginning of the experiment, approximately 20% of the cells exhibited triglyceride accumulation in the cytoplasm; 20 hr later, approximately 50% of both the experimental and control cells had accumulated triglyceride.

The medium from the culture of exudate cells not treated with endotoxin had little effect on the lipoprotein lipase activity of 3T3-L1 cells. The medium from untreated exudate cells elicited some inhibition in the experiment shown (Fig. 1*B*), but in other experiments medium prepared identically had no inhibitory effect. Endotoxin itself also had a negligible inhibitory effect on lipoprotein lipase activity when the amount added was equivalent to that which might remain in the conditioned medium from endotoxin-treated exudate cells; a 19%, 9%, and 0% decrease was observed in medium, heparin-releasable, and intracellular compartments, respectively. The decrease was greater (45% in medium, 17% in heparin-releasable, and 11% in the cells) when larger amounts (4.5 times) of endotoxin were employed (Fig. 1*D*).

A possible explanation for the decreased activity of lipoprotein lipase described above is a direct inhibitory effect of the mediator on the enzyme. This was examined by incubating medium from 3T3-L1 cell cultures which contained lipoprotein lipase with conditioned medium from cultures of endotoxin-treated exudate cells. As shown in Fig. 2, the enzyme activity was not inhibited by the mediator (103% of the control) at the time of mixing, and the rate of decay of enzyme activity was the same in the experimental group and the control group. Endotoxin also had no effect on the activity of lipoprotein lipase.

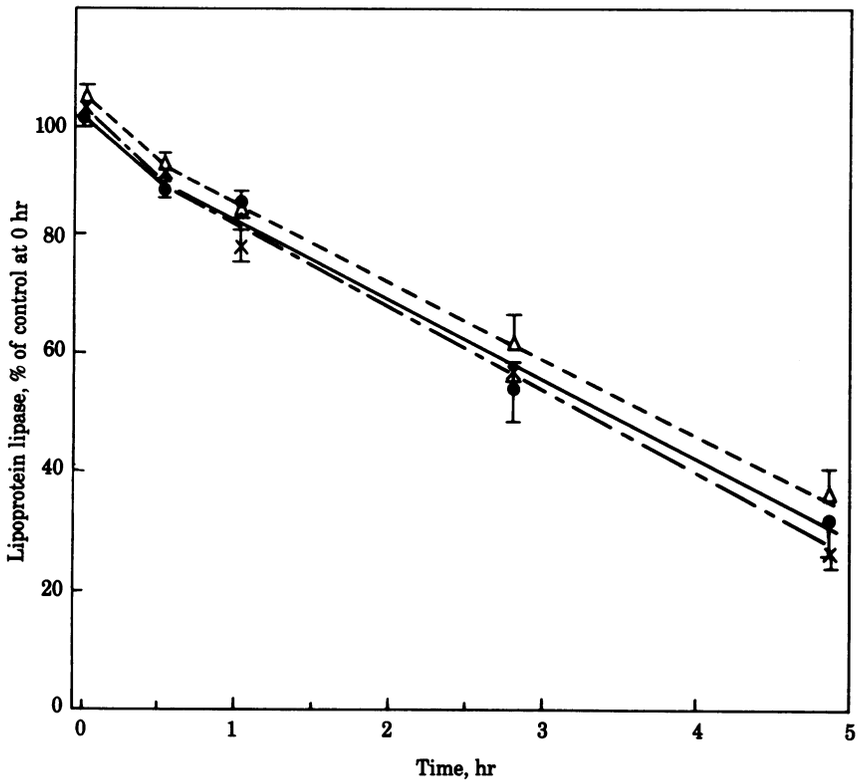

FIG. 2. Effect of lipoprotein lipase suppression mediator on lipoprotein lipase activity. One milliliter of medium from 3T3-L1 cells containing lipoprotein lipase was mixed with 200 μl of fresh RPMI 1640 medium (●), conditioned medium from endotoxin-treated exudate cells (△), or RPMI 1640 medium with endotoxin (5 μg/ml) (×). Lipoprotein lipase activity was measured immediately and at the indicated times. The data represent the mean ± SEM (n = 6).

These results imply that the mediator depresses lipoprotein lipase activity in 3T3-L1 cells by inhibiting the intracellular synthesis or processing of the enzyme.

The relationship between the amount of mediator substance and lipoprotein lipase activity of 3T3-L1 cells was examined by incubating the cells with increasing amounts of the conditioned medium from endotoxin-treated exudate cells for 20 hr at 37°C (Fig. 3). Ten microliters of conditioned medium added to 1.5 ml of culture medium was sufficient to cause a substantial decrease in lipoprotein lipase activity—i.e., 57% decrease in the medium, 40% decrease in the heparin-releasable compartment, and 8% decrease in the cells. Enzyme activity was further depressed by increasing the amount of mediator-containing medium. When 250 μl was added, a decrease of greater than 95% was observed in all three compartments. The amount of mediator present in conditioned medium varied somewhat from preparation to preparation.

The rate at which lipoprotein lipase activity declines after the addition of the mediator was also investigated. Conditioned medium containing the mediator was added at the indicated times (Fig. 4) and lipoprotein lipase activity was measured. A reduction of lipase activity was apparent as early as 30 min after

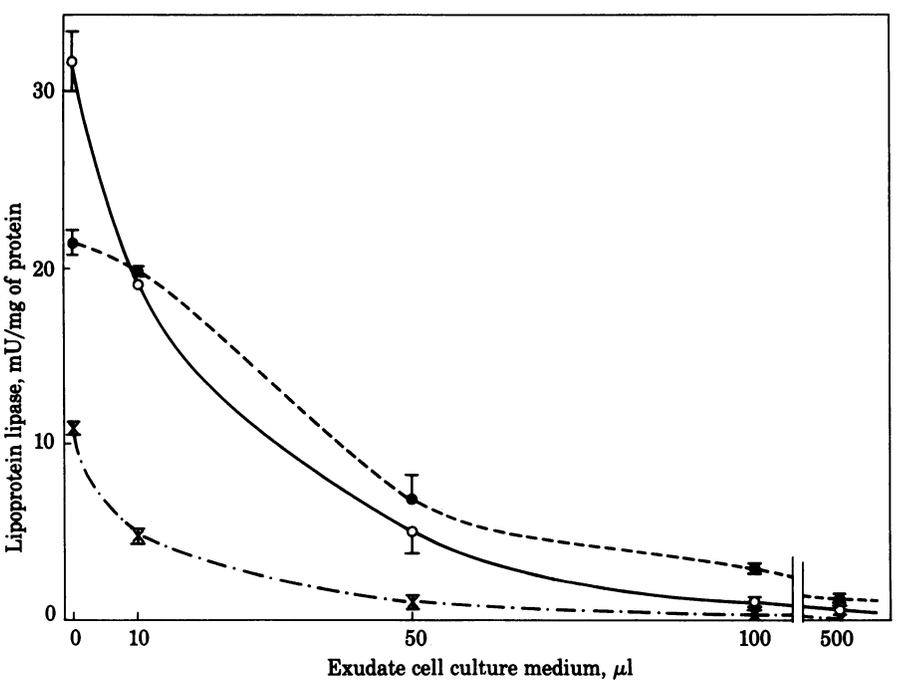

FIG. 3. Dependence of lipoprotein lipase activity on the concentration of lipoprotein lipase suppression mediator. Ten to 1000 μl of conditioned medium from endotoxin-treated exudate cells was added to 3T3-L1 cell cultures in 3.5-cm dishes with 1.5 ml of DME medium. Conditioned medium added was supplemented with fresh DME to make the final volume of medium 2.5 ml in all dishes. The lipoprotein lipase activity in the medium (×), on the cell surface (○), and in cell sap (●), was assessed after 20-hr incubation. The data represent mean ± SEM (n = 4).

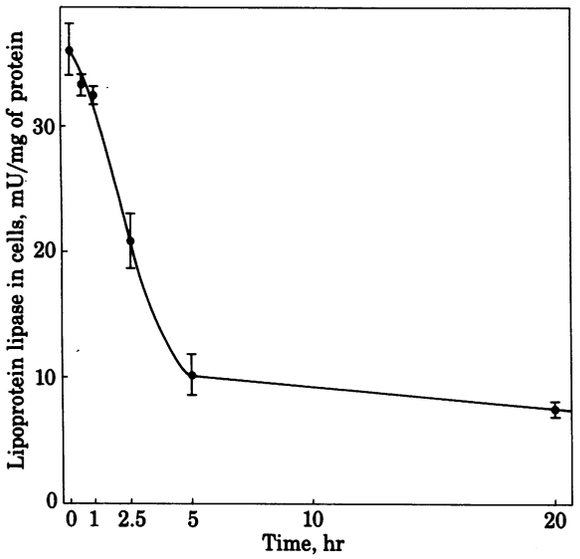

FIG. 4. Time course of the effect of the mediator. Conditioned medium (100 μl) from endotoxin-treated exudate cell culture was added to 3T3-L1 cell cultures in 3.5-cm dishes with 1.5 ml of DME medium at indicated times prior to the assay for the intracellular lipoprotein lipase activity. Data represent mean ± SEM (n = 6).

addition to 3T3-L1 cells. Approximately half of the intracellular enzyme activity was lost after 2.5 hr. After 5 hr of incubation with the mediator, a maximal effect was observed. The amount of enzyme activity in the medium and that on the cell surface were also observed to decrease with a similar time course (data not shown).

The rapid decrease in lipoprotein lipase activity might reflect a competition with insulin, because removal of insulin has been shown to lead to a rapid decline in lipoprotein lipase activity in 3T3-L1 cells (20). Accordingly, an attempt was made to reverse the suppressive effect of the mediator by increasing the concentration of insulin in the medium. The effect on lipoprotein lipase activity of incubating 3T3-L1 cells with media containing insulin at various concentrations (50 ng/ml to 50 μg/ml) and mediator was assessed. As shown in Table 1, the inhibitory effect of the mediator on enzyme activity was not changed with increasing insulin concentrations. Even at an insulin concentration 1000 times greater (50 μg/ml) than that of standard conditions (50 ng/ml), the inhibition was not reversed.

Preliminary characterization of the mediator (Table 2) revealed that all of the activity was retained during vacuum dialysis with membranes having a 12,000 dalton pore cut-off. This

Table 1. Effect of insulin on the mediator suppression of lipoprotein lipase in 3T3-L1 cells

Insulin, μg	Mediator*	Lipoprotein lipase†, mU/mg protein		
		In medium	Cell surface	Intracellular
0.050	+	0.3 ± 0.2	1.0 ± 0.2	1.3 ± 0.2
	−	25.2 ± 2.9	68.5 ± 5.4	8.8 ± 0.3
1	+	0.2 ± 0.1	1.6 ± 0.2	1.9 ± 0.2
	−	22.0 ± 2.3	54.9 ± 3.6	8.2 ± 0.8
50	+	0.3 ± 0.2	0.9 ± 0.2	0.8 ± 0.1
	−	34.0 ± 5.9	68.2 ± 9.5	11.2 ± 1.2

* 3T3-L1 cells were incubated with 100 μl of mediator in 3.5-cm plates containing 1.5 ml of DME medium supplemented with insulin at the indicated levels.
† Lipoprotein lipase activity was assessed after a 20-hr incubation. Data are expressed as mean ± SEM (n = 6).

Table 2. Effect of dialysis on lipoprotein lipase suppression mediator

Experiment*	Lipoprotein lipase†, mU/mg protein		
	In medium	Cell surface	Intracellular
1. Control medium	59.2 ± 2.9	68.9 ± 1.3	36.5 ± 1.2
2. Media containing lipoprotein lipase suppression mediator	3.0 ± 0.4	22.4 ± 0.6	29.7 ± 0.8
3. Same as 2, inside dialysis membrane after dialysis	0.5 ± 0.2	3.4 ± 0.4	16.5 ± 0.4
4. Same as 2, outside dialysis membrane after dialysis	59.9 ± 1.0	71.8 ± 1.7	38.3 ± 0.5

* For each sample, 150 μl was added to 1.5 ml of medium in 3.5-cm plates of 3T3-L1 cell culture.
† The enzyme assays were carried out after a 20-hr incubation. Results are mean ± SEM (n = 6).

suggests that the mediator has a molecular mass greater than 12,000 daltons. The stability of the mediator to heat was assessed by treating the conditioned medium containing the mediator at 100°C for 15 min (Table 3). The inhibitory effect of the mediator on the lipoprotein lipase was abolished by this treatment.

To determine whether the mediator is an intracellular constituent of nontreated exudate cells, exudate cells were sonicated and the extract was assayed for mediator (Table 3). This extract had no measurable mediator. This suggests that the mediator is not a normal intracellular substance of exudate cells but is synthesized or processed in these cells after stimulation by endotoxin.

DISCUSSION

Previous studies revealed a decrease in lipoprotein lipase activity of adipose tissue of mice treated with endotoxin or a mediator present in the supernatant of exudate cells incubated with endotoxin (9). As described above, the addition of media containing this mediator to 3T3-L1 cell cultures *in vitro* also leads to a dramatic decrease in lipoprotein lipase activity in three components: that found in the medium, on the cell surface, and inside the cells. The loss of enzyme activity in all compartments was dependent on time and concentration of the mediator and was not the result of a direct inhibition or destruction of the enzyme by the mediator. In addition, the cells retained normal

Table 3. Presence of lipoprotein lipase suppression mediator in media after heating and in sonicated exudate cells.

Experiment	Lipoprotein lipase*, mU/mg protein		
	In medium	Cell surface	Intracellular
1. Control medium	47.6 ± 1.3	75.0 ± 3.1	34.8 ± 1.2
2. Medium containing mediator	1.0 ± 0.4	7.2 ± 1.4	7.9 ± 1.0
3. Heat treatment of medium containing mediator†	58.8 ± 7.2	80.9 ± 7.9	37.2 ± 3.1
4. Sonicate of exudate cells‡	55.3 ± 1.8	75.0 ± 1.5	35.5 ± 9.6

* The enzyme activity from 3T3-L1 cells was measured after a 20-hr incubation. Results are mean ± SEM (n = 4).
† Medium containing lipoprotein lipase suppression mediator was heated at 100°C for 5 min.
‡ Peritoneal exudate cells (9×10^6 cells) were harvested and sonicated in 3 ml of RPMI 1640 medium.

viability and structure, and they continued to accumulate cytoplasmic triglycerides by endogenous lipid synthesis to the same extent as control cultures. That the mediator from the endotoxin-stimulated macrophages was responsible for the suppression of lipoprotein lipase activity was evidenced by the lack of a significant effect of added media from macrophages not stimulated with endotoxin or of endotoxin itself added to the cultures of 3T3-L1 cells.

The mechanism for the time-dependent suppression of lipoprotein lipase in 3T3-L1 cells by the mediator remains to be elucidated, but it presumably involves synthesis of the enzyme, reflected by the fact that lipoprotein lipase is being replaced continually. Because of its lability, the half-life of enzyme activity is approximately 25 min (16). Of interest is the fact that a similar decline of lipoprotein lipase activity in 3T3-L1 cells after the removal of insulin from the culture medium has been described (20). Insulin concentrations up to 1000 times the usual concentration, however, were not capable of reversing the suppression of lipoprotein lipase in response to the mediator. Although this observation argues against a direct competition of the mediator with insulin, interaction of the mediator with insulin receptors or a subsequent prevention of insulin action by some other mechanism remains a strong possibility. Insulin resistance commonly observed in infection, injury, or shock (28–30) suggests an important interplay between insulin and the expression of lipoprotein lipase activity.

The lipoprotein lipase suppression mediator found in the media of exudate cells after endotoxin stimulation is either synthesized or processed, because no mediator activity was detectable in extract of sonicated macrophages. Preliminary studies show the mediator to be heat labile, with an apparent molecular weight greater than 12,000. This suggests that the mediator is a protein, but the possibility of a protein-bound small molecule cannot be ruled out. Recently, several factors have been reported to be released from exudate cells in response to endotoxin. These factors promote the induction of fever, serum amyloid A protein, and granulocytosis (31–33). Further studies will be needed to define and compare the mediator described in this report with these other factors.

The 3T3-L1 tissue culture system described above offers a valuable means of studying the biochemical response of cells to endotoxin and other inducers of shock, as well as a means of quantifying the amount of lipoprotein lipase suppression mediator in biological fluids. This should aid in the isolation of the mediator and in the investigation of its relationship to other concomitants of shock. In addition, if the lipoprotein lipase suppression mediator is responsible for some of the deleterious effects of shock such as the shutdown of various organ systems, then, the 3T3-L1 cell system might prove extremely useful for the screening of pharmacological agents that could prevent these clinical sequelae of shock.

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