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Selective inhibition of synthesis of enzymes for *de novo* fatty acid biosynthesis by an endotoxin-induced mediator from exudate cells

(acetyl-CoA carboxylase/fatty acid synthetase/3T3-L1 preadipocytes/shock/macrophage)

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ABSTRACT **An endotoxin-induced mediator from exudate cells markedly suppresses the activities of the key enzymes for *de novo* fatty acid biosynthesis—acetyl-CoA carboxylase [acetyl-CoA:carbon dioxide ligase (ADP-forming), EC 6.4.1.2] and fatty acid synthetase—in differentiating 3T3-L1 murine preadipocytes. The loss in activity, at least in part, appears to be due to a specific effect on the synthesis of the enzymes, as determined by a decreased incorporation of [^{35}S]methionine into immunoadsorbable acetyl-CoA carboxylase and fatty acid synthetase when the cells were exposed to the mediator. During this exposure, the radiolabeling of proteins with [^{35}S]methionine in a particulate fraction was decreased by nearly 50% with little change in the soluble protein fraction. Sodium dodecyl sulfate/polyacrylamide gel analysis of the labeled protein indicated no major disturbances of protein synthesis in general; however, the syntheses of specific proteins in both the soluble and particulate fractions were enhanced or depressed. The present study demonstrates that endotoxin promotes the release of a mediator from exudate cells that regulates key anabolic activities in adipose cells.**

One of the clinical hallmarks of animals with chronic infections or tumors is the presence of a catabolic state that can proceed to cachexia, shock, and death (1, 2). The biochemical basis for this phenomenon is not understood, but presumably once triggered it is of a universal nature. In order to gain insight into the mechanism of this process, we have been studying as a model system the hyperlipidemia that occurs with infection or endotoxemia (3–5). Previous studies have shown that this hyperlipidemia, which is due to the accumulation of very low density lipoproteins (VLDL) is the result of the loss of the enzyme lipoprotein lipase (3–5). We have recently demonstrated that the hyperlipidemia associated with endotoxemia is mediated by an endotoxin-induced macrophage product (4, 5), which markedly suppresses the activity of lipoprotein lipase. In attempting to elucidate the biochemical mechanism by which this occurs we have utilized the 3T3-L1 murine preadipocyte cell line (6). These cells, like adipocytes in general, utilize lipoprotein lipase as a means of hydrolyzing extracellular triacyglycerol to partial glycerides and free fatty acids. The liberated fatty acids are then taken up and reesterified for storage (7–12). When medium from cultured exudate cells that had been incubated with endotoxin was added to cultures of 3T3-L1 cells, a dramatic decrease (<90%) in lipoprotein lipase activity was observed (5). The loss of enzyme activity 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. Although the mechanism for the time-dependent suppression of lipoprotein lipase by the mediator is unknown, we presumed that it involves a step in the biosynthesis of the enzyme.

Reasoning that other anabolic activities of the 3T3-L1 cells might be inhibited by the mediator, we have studied two key enzymes for *de novo* fatty acid biosynthesis—acetyl-CoA carboxylase [acetyl-CoA:carbon dioxide ligase (ADP-forming), EC 6.4.1.2] and fatty acid synthetase. In the present communication, we present evidence that the synthesis of these enzymes is also inhibited by the addition of the macrophage mediator. These results implicate a larger role for the mediator(s) and point to the presence of a communication system between immune cells and energy storage cells of mammals. Presumably, during invasion the immune cells can function as an endocrine system and selectively mobilize energy supplies to combat the invasion.

EXPERIMENTAL PROCEDURES

Materials. Endotoxin (lipopolysaccharide) from *Escherichia coli* 0127:B8 isolated by the method of Westphal (13) 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. IgGsorb was from the Enzyme Center (Boston). L-[^{35}S]-Methionine (800–1,440 Ci/mmol; 1 Ci = 3.7×10^{10} Bq) was from Amersham. EN^{3}HANCE was obtained from New England Nuclear. Antiserum to fatty acid synthetase was kindly provided by Fasal Ahmad of the Papanicolaou Cancer Research Institute (Miami, FL).

3T3-L1 Cell Culture. 3T3-L1 preadipocytes were cultured as previously described (14, 15) in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Differentiation leading to the adipocyte phenotype was induced by a modification (15) of the method of Rubin *et al.* (16). 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 decreased concentration of 50 ng/ml.

Preparation of Peritoneal Exudate 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

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prior to harvest. The exudate cells obtained by using this procedure are primarily macrophages, with some contaminating lymphocytes (17).

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 the presence or absence of 10 μg of endotoxin per ml. After 24 hr, the culture medium was removed and centrifuged at $1,000 \times g$ for 5 min at 4°C. The supernatant of conditioned medium obtained from cells exposed to endotoxin was assayed and found to contain the mediator substance that lowers lipoprotein lipase in 3T3-L1 cells (4). 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 decreased 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.

Labeling of Cellular Proteins. A 6-cm plate containing induced 3T3-L1 cells was washed twice with 5 ml of methionine-free medium and incubated for 1 hr with 2 ml of the same medium containing 0.5 mCi of L-[^{35}S]methionine, during which period the rate of [^{35}S]methionine incorporation into cellular protein was linear. The medium was removed, the cell monolayer was washed twice with phosphate-buffered saline, pH 7.4, and the soluble cytosolic proteins were released by the digitonin method of Mackall *et al.* (18). The remainder of the cell monolayer containing the membranous fraction was then scraped into 3.0 ml of 100 mM Hepes buffer, pH 7.5, containing 0.5% Nonidet P-40 nonionic detergent and 1 mM phenylmethylsulfonyl fluoride. After trituration with a Pasteur pipette, the suspension was centrifuged at $10,000 \times g$ for 10 min at 4°C, and the supernatant was saved.

[^{35}S]Methionine incorporation into acid-insoluble material was determined by adding 20 μl of digitonin or Nonidet P-40-released material to 0.5 ml of ice-cold 20% trichloroacetic acid with 25 μl of 0.5% bovine serum albumin added as carrier. After sitting at 4°C for 1 hr, the mixture was centrifuged at $2,000 \times g$ for 5 min. The pellet obtained was solubilized by incubation in 0.5 ml of 1 M NH_4OH at 37°C for 30 min. The protein was reprecipitated on addition of 5.0 ml of ice-cold 10% trichloroacetic acid and filtered on Whatman GF/C filters. The filters were washed with diethyl ether and the radioactivity present was determined.

Immunoadsorption and Electrophoresis. Aliquots of the [^{35}S]methionine-labeled proteins from the soluble (digitonin-released) fraction of the cell monolayer were made 1 mM in phenylmethylsulfonyl fluoride and 0.5% in Nonidet P-40 and then added to 5 μl of antiserum specific for acetyl-CoA carboxylase or fatty acid synthetase. After 2 hr at 25°C, 100 μl of 10% IgGsorb was added and the labeled enzymes were isolated from the mixture by the method of Student *et al.* (15). Polyacrylamide/$NaDodSO_4$ gels were run according to the method of Laemmli (19) and prepared for fluorography by use of EN^{3}HANCE according to the manufacturer's instructions.

Enzyme Assays. Acetyl-CoA carboxylase and fatty acid synthetase activities in the digitonin-released cytosolic fraction of the cell monolayers were assayed by the methods of Gregolin *et al.* (20) and Student *et al.* (15), respectively. When used for enzyme assays, the digitonin-released fraction did not contain phenylmethylsulfonyl fluoride.

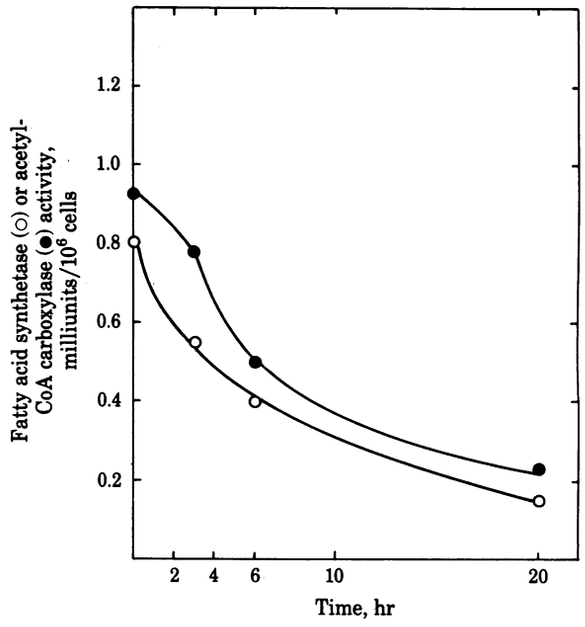

FIG. 1. Effect of conditioned medium from endotoxin-treated mouse peritoneal exudate cells on the activities of acetyl-CoA carboxylase and fatty acid synthetase in 3T3-L1 cells. Three hundred microliters of conditioned medium was added to cultures of 3T3-L1 cells (4.2×10^6 cells per dish) in 6-cm dishes containing 3.5 ml of Dulbecco's modified Eagle's medium and 10% fetal calf serum. After the indicated times of incubation, the enzymatic activities of acetyl-CoA carboxylase (○) and fatty acid synthetase (●) in a digitonin-releasable cytosolic fraction of the cells were assessed.

RESULTS

Effect of Mediator on Acetyl-CoA Carboxylase and Fatty Acid Synthetase. To examine the effect of the mediator substance on the activities of acetyl-CoA carboxylase and fatty acid

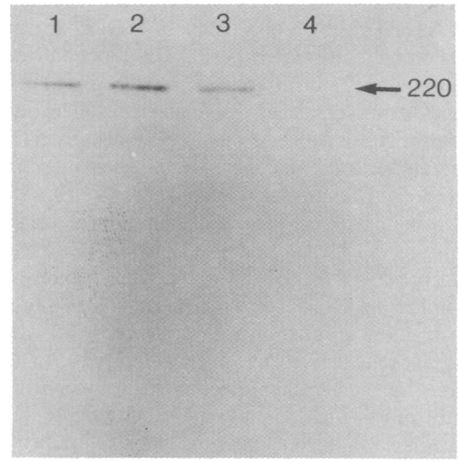

FIG. 2. Effect of mediator that suppresses the synthesis of acetyl-CoA carboxylase. After exposure of the 3T3-L1 cells to the mediator (300 μl of conditioned medium), the cells were pulse-labeled with 0.5 mCi of [^{35}S]methionine for 1 hr. Cytosolic fractions were obtained by digitonin treatment of the monolayer. Aliquots of the cytosolic fractions (2×10^5 cpm for all determinations) were incubated with anti-acetyl-CoA carboxylase and the immunoadsorbable material was isolated and analyzed by electrophoresis in a 7.5% acrylamide/0.1% $NaDodSO_4$ gel. The autoradiogram is shown. Lane 1, control without exposure to mediator; lanes 2, 3, and 4, exposure of the cells to the mediator for 3, 6, and 20 hr, respectively. Molecular weight $\times 10^{-3}$ is indicated on the right.

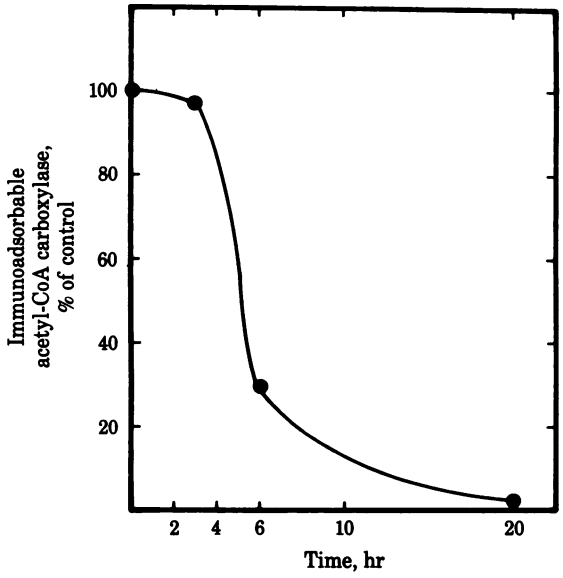

FIG. 3. Results of a densitometric scan of the autoradiogram shown in Fig. 2, indicating percent of immunoadsorbable acetyl-CoA carboxylase remaining relative to control, after exposure to the mediator.

synthetase enzymes, 3T3-L1 cells were exposed to conditioned medium from mouse peritoneal exudate cells cultured in the presence of endotoxin. After incubation of the 3T3-L1 cells with the mediator for 3, 6, and 20 hr, acetyl-CoA carboxylase and fatty acid synthetase activities were determined in a digitonin-released cytosolic fraction of the cells (Fig. 1). The activities of both enzymes decreased over the 20-hr period to approximately 25% of the initial values. Incubation of the 3T3-L1 cells in the endotoxin or conditioned medium from exudate cells cultured in the absence of endotoxin did not significantly alter the activities of these enzymes (data not shown).

To determine if the loss in activity of the two enzymes was a result of a direct effect on protein synthesis, 3T3-L1 cells were incubated with conditioned medium from cultures of endotoxin-treated exudate cells for 3, 6, and 20 hr. During the final

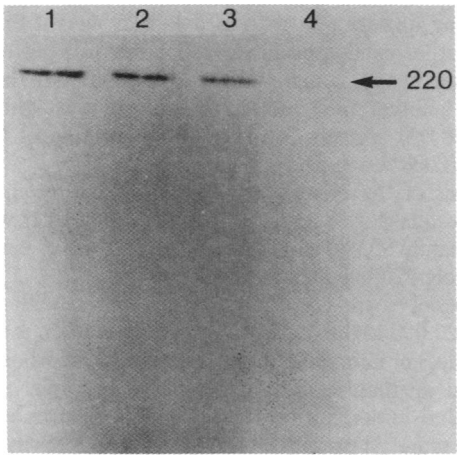

FIG. 4. Effect of a mediator that suppresses the synthesis of fatty acid synthetase. Experimental design is identical to that described in the legend to Fig. 2. An autoradiogram of a 7.5% acrylamide/0.1% $NaDodSO_4$ gel analysis of immunoadsorbable fatty acid synthetase is shown. Lane 1, control without exposure to mediator; lanes 2, 3, and 4, exposure of the cells to the mediator for 3, 6, and 20 hr, respectively. Molecular weight $\times 10^{-3}$ is indicated on the right. Note that fatty acid synthetase and acetyl-CoA carboxylase have nearly identical molecular weights.

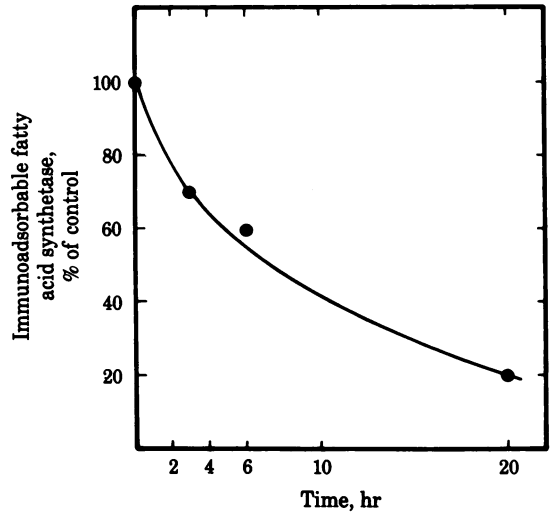

FIG. 5. Results of a densitometric scan of the autoradiogram shown in Fig. 4, indicating percent of immunoadsorbable fatty acid synthetase remaining relative to control, after exposure to the mediator.

hour of incubation the cells were exposed to a pulse of [^{35}S]-methionine. After the pulse, [^{35}S]methionine-labeled acetyl-CoA carboxylase and fatty acid synthetase were isolated from the digitonin-releasable cytosolic fractions by immunoadsorption. Identification was accomplished by $NaDodSO_4$/polyacrylamide gel electrophoresis and fluorography (Figs. 2 and 4). The decreased incorporation of [^{35}S]methionine into immunoadsorbable acetyl-CoA carboxylase and fatty acid synthetase with respect to time after exposure to the mediator is readily observed. Densitometric scanning of the autoradiograms (Figs. 3 and 5) indicated that, after 20 hr of exposure to the mediator, the amounts of [^{35}S]methionine incorporated into fatty acid synthetase and acetyl CoA carboxylase were decreased by 80% and 95%, respectively. These results are consistent with the

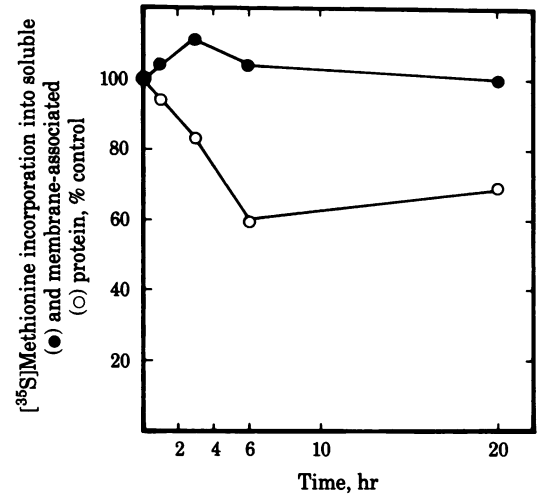

FIG. 6. Effect of the mediator on [^{35}S]methionine incorporation into protein. 3T3-L1 cells were incubated with 300 μl of conditioned medium from endotoxin-treated mouse peritoneal exudate cells for the appropriate period and protein was pulse-labeled with 0.5 mCi of [^{35}S]methionine for 1 hr. Soluble proteins were obtained by digitonin treatment of the cells; the remainder of the monolayer was then extracted with Nonidet P-40 and a membrane protein fraction was obtained. Incorporation of [^{35}S]methionine into acid-precipitable material was determined. The incorporation of radioactivity into soluble protein (●) or membrane protein (○) after exposure of the cells to the mediator for the indicated time is shown.

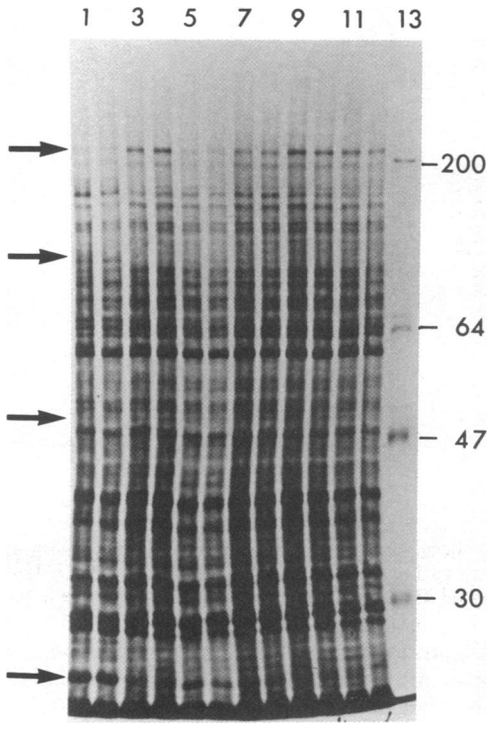

FIG. 7. Effect of mediator on protein synthesis in the cytosolic fraction of the cells. Autoradiogram of a 7.5% acrylamide/0.1% $NaDodSO_4$ gel analysis of [^{35}S]methionine-labeled cytosolic protein after exposure of the cells to the mediator. 3T3-L1 cells were pulse-labeled and the soluble protein was obtained by digitonin treatment. Aliquots (2×10^5 cpm) of the cytosolic fraction for each time point were applied to the gel and electrophoresed. All time points are shown in duplicate. Lanes 1 and 2, exposure of the cells to the mediator for 20 hr; lanes 3 and 4, 20 hr of exposure to conditioned medium from mouse peritoneal exudate cells *not* exposed to endotoxin; lanes 5 and 6, 6 hr of exposure; lanes 7 and 8, 3 hr of exposure; lanes 9 and 10, 1 hr of exposure to the mediator; lanes 11 and 12, control without exposure to mediator; lane 13, protein standards; molecular weights $\times 10^{-3}$ are indicated.

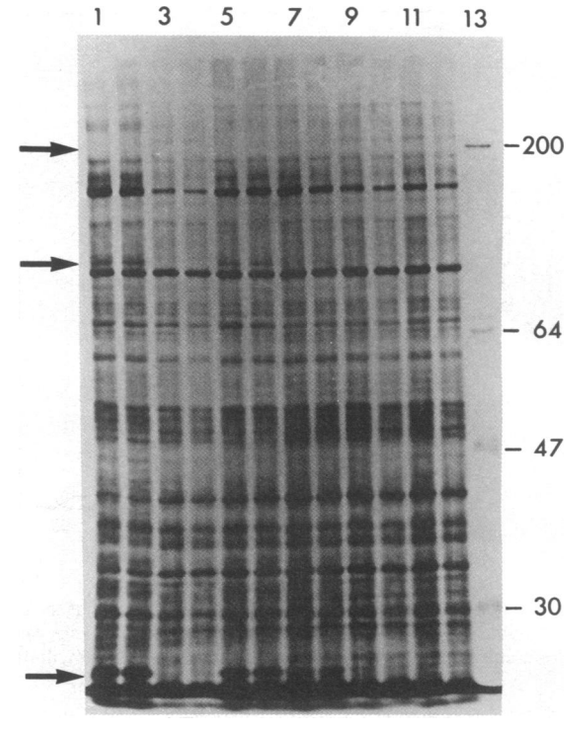

FIG. 8. Effect of mediator on protein synthesis in the membrane fraction of the cells. Autoradiogram of a 7.5% acrylamide/0.1% $NaDodSO_4$ gel analysis of [^{35}S]methionine-labeled membrane protein after exposure of the cells to the mediator. Experimental design was identical to that described in the legend to Fig. 7. Membrane proteins were obtained by Nonidet P-40 extraction. All time points are shown in duplicate. Lanes 1 and 2, exposure of the cells to the mediator for 20 hr; lanes 3 and 4, 20 hr of exposure of the cells to conditioned medium from mouse peritoneal exudate cells not exposed to endotoxin; lanes 5 and 6, 6 hr of exposure; lanes 7 and 8, 3 hr of exposure; lanes 9 and 10, 1 hr of exposure; lanes 11 and 12, control without exposure to mediator; lane 13, protein standards; molecular weights $\times 10^{-3}$ are indicated.

concept that the mediator depresses the activity of acetyl-CoA carboxylase and fatty acid synthetase by interfering with the synthesis of the enzyme.

Effect of Mediator on Protein Synthesis in General. The observed effect on acetyl-CoA carboxylase and fatty acid synthetase could be explained by a general inhibition of protein synthesis by the mediator. To examine this possibility, the effect of mediator on amino acid incorporation into protein was investigated. 3T3-L1 cells were incubated for various periods of time with conditioned medium obtained from mouse peritoneal exudate cells cultured in the presence of endotoxin. [^{35}S]Methionine incorporation into soluble and membrane-associated protein was determined after 1, 3, 6, and 20 hr of exposure of the cells to the added factor. When 3T3-L1 cells were exposed to conditioned medium from mouse peritoneal exudate cells that were cultured in the absence of endotoxin, no effect on [^{35}S]methionine incorporation into acid insoluble protein was observed. However, as seen in Fig. 6, [^{35}S]methionine incorporation into trichloroacetic acid-precipitable material in the soluble fraction (digitonin-releasable protein) increased approximately 10% in the first 3 hr, with no further change observed. A 50% decrease was observed for label incorporation into acid-insoluble material in the membrane fraction (Nonidet P-40-solubilized protein). [^{35}S]Methionine-labeled proteins were analyzed by $NaDodSO_4$ gel electrophoresis after exposure to the mediator. The autoradiograms of the soluble proteins obtained on digitonin treatment and those solubilized by Nonidet P-40 treatment of the 3T3-L1 cells are shown in Figs. 7 and 8. Closer inspection of Fig. 7 reveals the gradual disappearance with time, after the addition of the mediator, of a band corresponding to a protein with a molecular weight of 220,000, while another band appears corresponding to a protein of molecular weight approximately 18,000. In addition to these major changes, another new protein of molecular weight approximately 80,000 appears, while a second protein of molecular weight 50,000 disappears.

Analysis of the Nonidet P-40-solubilized proteins showed similar results (Fig. 8). Protein bands of molecular weights of approximately 80,000 and 30,000 appeared, while bands of approximately 220,000 and 50,000 disappeared.

The loss of a band corresponding to a protein with molecular weight 220,000 in the digitonin-releasable protein is consistent with the loss of immunoadsorbable acetyl-CoA carboxylase and fatty acid synthetase. The enzymes have similar molecular weights and under the conditions of this electrophoresis migrate the same. At present it is not possible to identify the other protein bands with known enzymes or proteins.

DISCUSSION

In our previous work, we demonstrated that a mediator substance, produced by exudate cells that had been incubated with endotoxin, suppressed the activity of lipoprotein lipase in both adipose tissue and 3T3-L1 cells, preventing the uptake and storage of extracellular triglyceride (4, 5). As described in this

report, the addition of medium containing this mediator to cultures of differentiating 3T3-L1 cells leads also to a dramatic loss in the activities of the key enzymes essential for *de novo* fatty acid biosynthesis. The time frame for loss of activity for both acetyl-CoA carboxylase and fatty acid synthetase is quite similar to that observed for lipoprotein lipase. By inhibiting the uptake and synthesis of fatty acids, the mediator(s) in effect switches the fat cell from an anabolic storage mode to a catabolic supply mode.

The mediator appears to decrease enzymatic activity by suppressing the synthesis of the enzymes. The effect on protein synthesis appears to be quite specific because there are no gross perturbations of the protein patterns observed on the autoradiograms (Figs. 7 and 8). It is conceivable that the loss of activity of these enzymes could be due to an accelerated rate of protein degradation. However, in half-life determinations, a value of 24 hr was obtained for fatty acid synthetase both in the presence and in the absence of mediator (data not shown), which is nearly identical to that previously reported (15). This supports our hypothesis that the loss of activity is due primarily to an effect on synthesis. In response to the mediator, the synthesis of several proteins is inhibited or induced. It was possible by immunoprecipitation to identify fatty acid synthetase and acetyl-CoA carboxylase (molecular weights 220,000) as two proteins whose synthesis is inhibited by the mediator. The identification of the other proteins that are modulated by the mediator is not possible at present, although lipoprotein lipase is a potential candidate for the molecular weight 50,000 protein that disappears. The nature of proteins that are induced in response to the mediator and the mechanism for the modulation of specific protein synthesis are deserving of further investigations.

Whether the mediator responsible for regulating the synthesis of acetyl-CoA carboxylase and fatty acid synthetase is the same as the mediator that suppresses the activity of lipoprotein lipase is not known. The relationship of these mediator(s) to the leukocyte factor that has been reported to stimulate mobilization of amino acids from muscle to the liver is of considerable interest because this factor also imparts a catabolic state on the tissue. Isolation of the mediator that decreases lipoprotein lipase activity and studies with the purified mediator should help to clarify these questions.

The mediators that we have described in the present and previous papers constitute part of a communication system between the immune system and the energy storage tissues of the body. It can be hypothesized that in response to various types of invasion—e.g., bacteria, protozoa, tumor—the immune system warns the energy storage tissue—e.g., adipose tissue, muscle, liver—of the impending need for energy to combat the invasion. These target cells respond by switching from a storage to a supply mode. If the invasion is of short duration, the animal can quickly recover and replenish the stores; however, if the invasion is of a chronic nature, complete depletion, cachexia, and death can result.

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