RAPID COMMUNICATION

The Ultrastructural Changes of S-100 Protein Localization During Lipolysis in Adipocytes

An Immunoelectron-Microscopic Study

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To elucidate the changes of ultrastructural localization of S-100 protein during lipolysis in adipocytes, an immunoelectron-microscopic study was performed. Epididymal fat pads from Wistar rats were incubated in the buffer with or without 10 μM epinephrine. Before incubation with epinephrine, S-100 protein was found to be associated with closely packed polysomes, the membrane of microvesicles, plasma membranes, the outer membrane of mitochondria, and the pellicle around fat droplets. In the epinephrine-treated tissues, however, S-100 protein-positive polysomes decreased drastically. S-100 protein-positive microvesicles increased in number, lined up below the plasma membranes, and fused with the plasma membrane, frequently opening into the interstitium. These microvesicles were also found around the lipid droplets. These findings, together with those of a previous report on ultrastructural changes of adipocytes during lipolysis, suggest that S-100 protein molecules interact with free fatty acids (FFAs) on their hydrophobic portions on the membrane of microvesicle and then are translocated through the cytoplasm and discharged from the surface of plasma membranes with FFAs into the interstitium. That is, S-100 protein might serve as one of carrier proteins of FFAs in adipocytes. (Am J Pathol 1985, 121:185–191)

S-100 PROTEIN, an acidic Ca^{2+}-binding protein,[1] is present predominantly in the cytosol of glial cells.[2] It is also found as a membrane-bound form in the cells of the central nervous systems (CNS).[3] We have reported that adipose tissue and cartilage possess the same level of S-100 protein as CNS.[4,5] In addition, it is proved to be present immunohistochemically not only in adipocytes[6] but also in chondrocytes, melanocytes, and Langerhans cells of human skin, interstitial cells of pineal gland, stellate cells of hypophysis, and satellite cells of adrenal glands.[7] However, the biologic role of S-100 protein in these cells has not been elucidated. Recently, we obtained important clues clarifying the biologic significance of this protein in rat adipocytes[5,8–11]: 1) concentrations of S-100 protein in adipocytes are regulated hormonally through cyclic adenosine monophosphate (AMP)–dependent process in the presence of Ca^{2+}; 2) S-100 protein is released into the medium in the process of lipolysis; 3) the release of S-100 protein is enhanced by adrenocorticotropic hormone (ACTH) and the β-adrenergic effect of catecholamines, which are known to stimulate lipolysis in adipose tissue; and finally, 4) S-100 protein levels in adipose tissue were strikingly decreased by serial injections of epinephrine concomitant with a marked increase of the blood plasma S-100 protein level *in vivo*.[10,12]

From these biochemical findings, several problems arise from a morphologic point of view: 1) the precise ultrastructural localization of S-100 protein in adipo-

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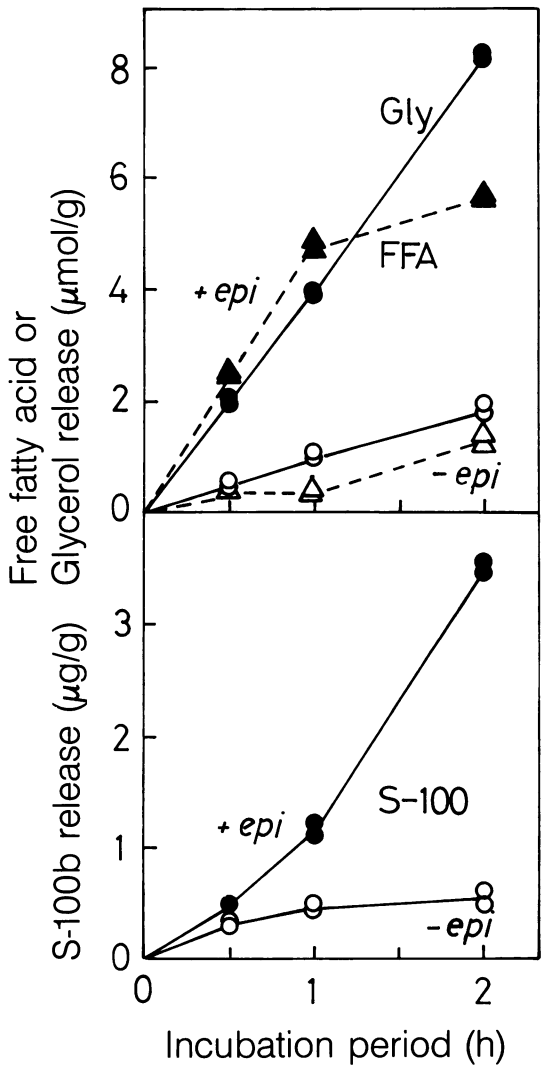

Figure 1—The fat pad pieces (100 mg) obtained from a single rat were incubated in duplicate without (*open symbols*) or with (*closed symbols*) 10 μM epinephrine as described in the text. After the indicated period of incubation, the incubation medium was withdrawn and centrifuged at 4 C at 10,000*g* for 20 minutes. Concentrations of S-100 protein and glycerol (*solid lines*) in the medium were assayed as described previously.[13] Concentrations of free fatty acids (*broken lines*) were estimated with palmitic acid as a standard. Results were expressed in micrograms or micromoles per gram wet tissue.

cytes, 2) the changes of ultrastructual localizations of S-100 protein in the process of lipolysis, and 3) the biologic role of S-100 protein in the process of lipolysis. To elucidate these problems, we employed the immunoelectron-microscopic technique, and discuss the role of S-100 protein during lipolysis in adipocytes by comparing our results with the previously reported morphologic changes of the adipocytes associated with lipid mobilization.[19-23] We also examined the effect of inhibitors for the cytoskeletal system or glycosylation in Golgi apparatus on the release of S-100 protein from adipocytes.

Materials and Methods

Epididymal fat pad pieces (100 mg) obtained from male Wistar rats were incubated at 37 C in 1 ml of Krebs-Ringer bicarbonate buffer (pH 7.4) equilibrated with a 5% CO_2, 95% O_2 gas phase containing 0.5% bovine serum albumin, 5 mM glucose with or without 10 μM epinephrine, and inhibitors for the assembly of microfilaments or microtubules and for glycosylation in Golgi apparatus (vinblastine, Sigma Chemical Co., St. Louis, Mo; colchicine, Nakarai Chemical Co., Osaka, Japan; cytochalasin B, Aldrich Chemical Co., Milwaukee, Wis, and monensin, Calbiochem-Behring Co., La Jolla). The incubation procedures and reagents used were described previously in detail.[9,11] Concentrations of S-100 protein in the soluble fraction of the tissue homogenate and in the incubation medium were determined by the sandwich enzyme immunoassay method as described previously.[13] Glycerol and free fatty acids (FFAs) were measured enzymatically as described by Garland and Randle[14] and Mizuno et al,[15] respectively.

The adipose tissues after incubation with or without epinephrine at 37 C for 30, 60, and 120 minutes were promptly fixed in periodate-lysine-4% paraformaldehyde (PLP)[16] at 4 C for 6 hours and washed in 0.01 M phosphate-buffered saline (PBS, pH 7.2) containing increased concentrations of sucrose. They were then embedded in OCT compound (Lab Tek, Naperville, Ill) and frozen in dry ice and ethanol.

Antiserum to bovine β subunit of S-100 protein was raised in New Zealand white rabbits as described previously,[13] and it was purified by immunoaffinity chromatography for the enzyme immunoassay and immunohistochemistry. The specificity to β subunit of S-100 protein was also documented in the previous report.[13] For the direct peroxidase-labeled antibody method, the Fab′ fragments of the monospecific antibody IgG were labeled with horseradish peroxidase (HRP) according to the maleimide method.[17]

For light microscopy, the direct peroxidase-labeled antibody method was employed with PLP-fixed, cryostat sections at 12 μ as described previously.[18] Sections were cut on a cryostat, placed on albumin-coated slides, and dried at room temperature. The sections were treated with 5 mM periodic acid solution to inactivate endogenous peroxidase, and they were then reacted with the HRP-labeled Fab′ fragments of the antisera. Control sections were reacted with the anti-β-subunit of S-100 protein antiserum absorbed by purified β subunit of S-100 protein or HRP-labeled Fab′ fragments of nonimmune rabbit IgG. After washing in PBS, the sections were reacted with 0.25% diaminobenzidine (DAB) solution containing 10 mM hydrogen peroxide and then counterstained with methyl green.

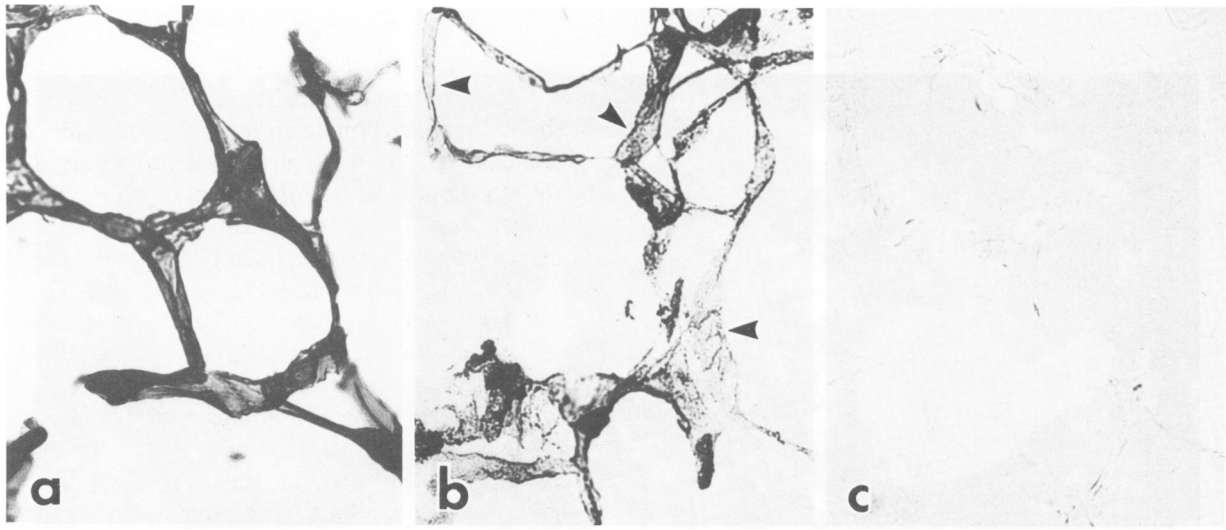

Figure 2—Light micrographs of adipocytes from epididymal fat pads reacted with HRP-labeled anti-β-subunit of S-100 protein. Before incubation with epinephrine (**a**), reaction products of HRP, indicating the site of S-100 protein, are present in the cytoplasm in a diffuse pattern. In adipocytes after 2-hour incubation with epinephrine (**b**), the immunoreactivity for S-100 protein is markedly reduced in the cytoplasm (*arrowheads*). A control section shows uniformly negative staining for S-100 protein (**c**). (×380)

In the immunoelectron-microscopic study, the direct peroxidase-labeled antibody method was employed with sections adjacent to those used for light microscopy as described previously.[18] The sections, after being treated with the HRP-labeled antibody, were postfixed in 0.5% glutaraldehyde in PBS and incubated sequentially in 0.25% DAB solution for 20 minutes and 0.25% DAB solution containing 10 mM hydrogen peroxide for 10 minutes. Control sections were treated with the HRP-labeled antibody absorbed by purified β subunit of S-100 protein or HRP-labeled Fab′ fragments of nonimmune rabbit IgG. The sections were washed, fixed in 2% osmium tetroxide in phosphate buffer (pH 7.6), dehydrated in graded alcohol, and embedded in Epon 812. Ultrathin sections were viewed with a Hitachi H-600 electron microscope. Counterstaining was omitted.

Results

When the fat pad pieces were incubated with epinephrine, S-100 protein, together with glycerol and FFA, was released into the medium in a time-dependent fashion. But the rates of the release were low in the control incubation without epinephrine (Figure 1). Contents of S-100 protein in the adipose tissue were strikingly decreased to about 47% that of the control by the 2-hour epinephrine treatment as described previously.[9,11] The control and epinephrine-treated adipose tissues contained 5.75 and 2.71 μg S-100 protein/g tissue, respectively.

Adipocytes before incubation with epinephrine or after 2-hour incubation without epinephrine exhibited striking diffuse staining for S-100 protein in the cytoplasm (Figure 2a), whereas adipocytes after 2-hour incubation with epinephrine showed marked reduction of the immunoreactivity for S-100 protein (Figure 2b). Control sections showed uniformly negative results (Figure 2c).

Immunoelectron-microscopically, before incubation with epinephrine (Figure 3a and 3b), S-100 protein in adipocytes was found to be associated with closely packed polysomes and associated with ribosomes on the rough endoplastic reticulum in a granular pattern. S-100 protein was also localized on the membrane of microvesicles, plasma membranes, the outer membrane of mitochondria, and the pellicle around fat droplets. However, S-100 protein was not demonstrated in the microvesicles, mitochondrial matrix, the cisternae of the rough endoplasmic reticulum, Golgi apparatus, or cytoplasmic vesicles. S-100 protein was detected in the nuclei of almost all adipocytes in a granular pattern. A control section showed negative staining for S-100 protein (Figure 3c). After 2-hour incubation with epinephrine, the number of S-100-protein–positive polysomes decreased drastically (Figure 4a). S-100 protein-positive pellicles around the lipid droplets became irregular and some of them were negative for S-100 protein (Figure 4a). Numerous S-100-protein–positive microvesicles were observed in the cytoplasm. They were linked together with the inner surface of the plasma membranes and fused with each other or the plasma membranes, which resulted in the formation of microvesicular invaginations (Figure 4a and 4b). The nuclear S-100 protein was scarcely changed by the treat-

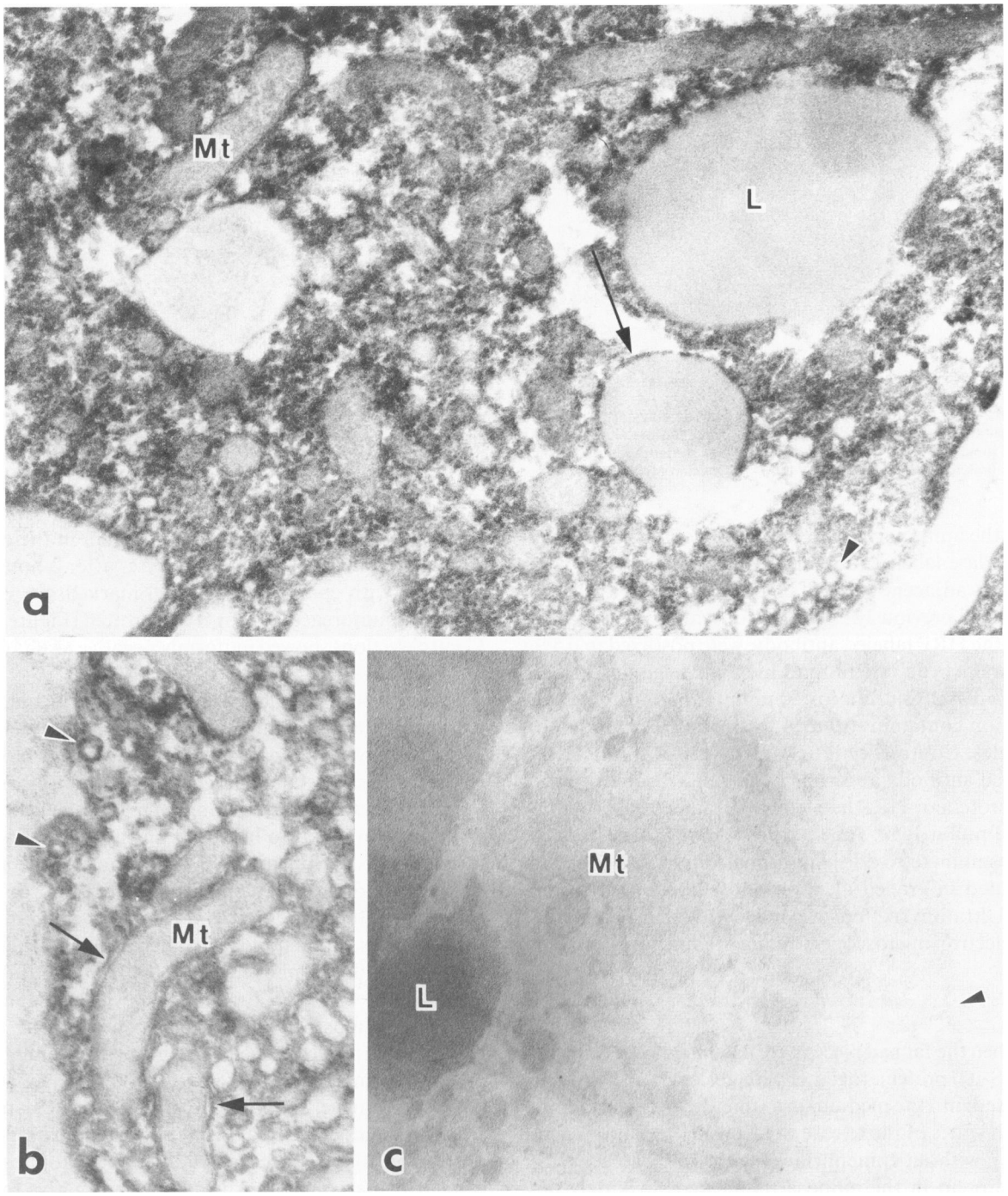

Figure 3—Immunoelectron micrographs of adipocytes before incubation with epinephrine. S-100 protein is found to be associated with closely packed polysomes and ribosomes on the rough endoplasmic reticulum in a granular pattern (**a** and **b**). S-100 protein is also localized on the membrane of microvesicles (**a** and **b**, *arrowheads*), the plasma membrane, the outer membrane of mitochondria (*Mt*) (**b**, *arrows*), and the pellicle around fat droplets (*L*) (**a**, *arrow*). However, S-100 protein is not demonstrated in the microvesicles, mitochondrial matrix (*Mt*), cisternae of the rough endoplasmic reticulum, Golgi apparatus, or cytoplasmic vesicles. In a control staining with the antibody absorbed by the purified β subunit of S-100 protein (**c**), the immunoreaction is not found in the cytoplasm, the pellicle around lipid droplets (*L*), the outer membrane of mitochondria (*Mt*), the plasma membrane, or the membrane of microvesicles (*arrowhead*). (**a**, ×38,000; **b**, ×40,000; **c**, ×70,000)

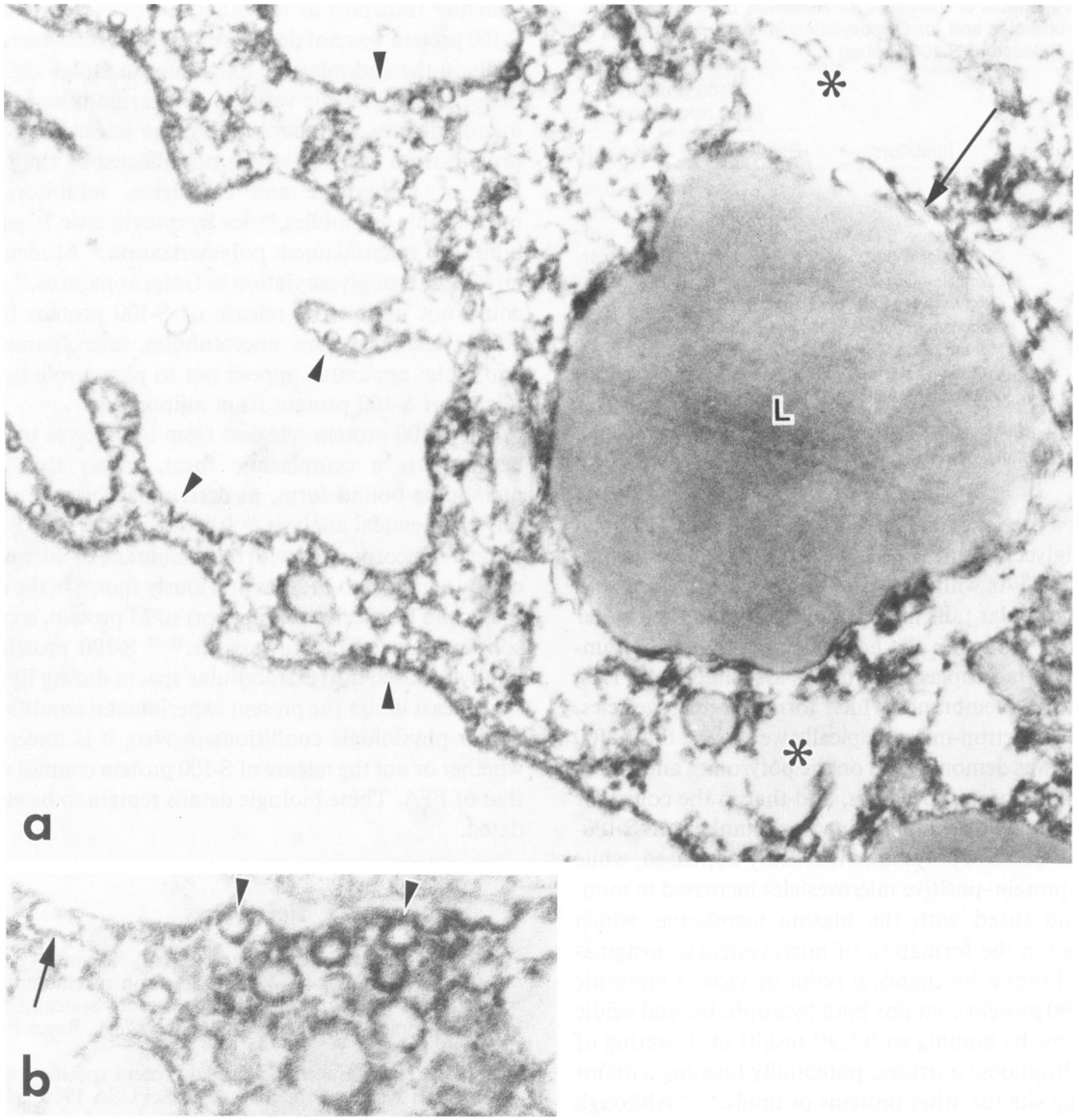

Figure 4—Immunoelectron micrographs of adipocytes after 2-hour incubation with epinephrine. The number of S-100-protein–positive polysomes are markedly reduced (**a**, *). Numerous S-100-protein–positive microvesicles are present beneath the plasma membrane (**a**, *arrowheads*), frequently fusing with each other (**b**, *arrow*) or with the plasma membrane, and opening into the interstitium (**b**, *arrowheads*). The pellicles around lipid droplets are irregular, and some of them became negative for S-100 protein (**a**, *arrow*). (**a**, ×37,000; **b**, ×75,000)

ment with epinephrine. On the other hand, in adipocytes incubated in the medium without epinephrine, the mode of the ultrastructural localization of S-100 protein was similar to that before incubation as described above.

The possible role of microtubules, microfilaments, and Golgi apparatus in the transcellular transport or release mechanism of S-100 protein was examined under the same conditions, and the results are summarized in Table 1. At the concentrations sufficient to inhibit the vesicular transport or the glycosylation of secretory proteins, vinblastine, colchicine, cytochalasin B, and monensin did not significantly inhibit the release of S-100 protein from adipocytes in the absence or presence of epinephrine.

Discussion

Ultrastructural changes during lypolysis in adipocytes have been intensely studied.[19–23] In morphologic analysis, it is widely accepted that the number of microvesicles and fat droplets increase and numerous microvesicular invaginations of the plasma membrane are present in the process of lipolysis.[19–23] Williamson[19] has pro-

Table 1—Effects of Inhibitors for Assembly of Microfilaments or Microtubules and for Glycosylation in Golgi Apparatus on the Release of S-100 Protein

Epinephrine	Inhibitors	S-100 protein (μg/2 hr/g tissue) Remaining	Released
–	–	5.75 ± 0.73	0.422 ± 0.076
10 μM	–	2.71 ± 0.98	3.52 ± 0.30
–	Cytochalasin B (21 μM)	5.56 ± 0.47	0.486 ± 0.073
10 μM	Cytochalasin B (21 μM)	2.30 ± 0.17	3.03 ± 0.37
–	Colchicine (10 μM)	6.83 ± 0.48	0.402 ± 0.118
10 μM	Colchicine (10 μM)	1.81 ± 0.12	3.59 ± 0.37
–	Monensin (5 μM)	6.04 ± 0.66	0.373 ± 0.096
10 μM	Monensin (5 μM)	1.55 ± 0.66	3.67 ± 0.50
–	Vinblastine (10 μM)	5.14 ± 0.99	0.438 ± 0.078
10 μM	Vinblastine (10 μM)	1.36 ± 0.43	3.99 ± 0.50

The fat pad fragments were incubated at 37 C for 2 hours with or without the additions. All values represent the mean ± SD of four assays.

posed a model of FFA egress in the process of lipolysis: triglycerides at the surface of droplets are hydrolyzed to FFAs which are oriented at the interface with their nonpolar tails in the lipid phase and their polar carboxyl groups in the aqueous phase, and then unknown intracytoplasmic proteins associate with FFAs, producing membranes which form the microvesicles. Immunoelectron-microscopically we showed that S-100 protein was demonstrated on the polysomes and on the membrane of microvesicles, and that in the course of incubation with epinephrine the number of S-100-protein-positive polysomes markedly decreased, while S-100-protein-positive microvesicles increased in number and fused with the plasma membrane, which resulted in the formation of microvesicular invaginations. From a biochemical point of view, a molecule of S-100 protein contains both hydrophobic and acidic portions. Its binding with Ca^{2+} results in clustering of its hydrophobic portions, potentially forming a major binding site for other proteins or lipids.[24,25] Although S-100-protein–FFA complexes are not yet directly proved by these morphologic or biochemical methods, the immunoelectron-microscopic observations reported here, the biochemical characterization of S-100 protein, and the morphologic model of FFA egression proposed by Williamson[19] allow a model for the role of S-100 protein to be proposed. In the course of lipolysis by epinephrine, S-100 protein molecules interact with FFA on their hydrophobic clusters, which results in S-100-protein–FFA complexes in the presence of Ca^{2+}. These complexes are microvesicles which are translocated through the cytoplasm and discharged into the interstitium from the surface of the plasma membrane. This suggests that S-100 protein might serve as a carrier protein for FFA in the process of lipolysis in adipocytes.

The translocation and release of S-100-protein–FFA complexes appear to involve the microvesicles but not vesicular transport as for other serum proteins, since S-100 protein was not demonstrated immunocytochemically in the endoplasmic reticulum, in Golgi apparatus or in cytoplasmic vesicles. Concerning the role of microfilaments or microtubules, the release of S-100 protein from adipocytes was not affected by the presence of vinblastine and colchicine, inhibitors of microtubule assemblies,[26] nor by cytochalasin B, an inhibitor of microfilament polymerization.[27] Monensin, an inhibitor of glycosylation in Golgi apparatus,[28] also could not inhibit the release of S-100 protein from adipocytes. Therefore, microtubules, microfilaments, and Golgi apparatus appear not to play a role in the release of S-100 protein from adipocytes.

The S-100 protein released from adipocytes by epinephrine is a cytoplasmic form, rather than the membrane-bound form, as determined by our previous biochemical analysis.[10] It is speculated that S-100 protein is incorporated with the membrane of microvesicles by coupling to FFA, as previously shown in the synthesis and transcellular transport of M protein, a cytosolic protein of paramyxovirus.[29,30] S-100 protein is finally released into extracellular spaces during lipolysis, at least under the present experimental conditions. Under physiologic conditions *in vivo*, it is uncertain whether or not the release of S-100 protein couples with that of FFA. These biologic details remain to be elucidated.

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