Free fatty acid release from endothelial cells

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Abstract Cultured bovine aortic endothelial cells that have been previously enriched with fatty acid are able to release free fatty acid (FFA) into the extracellular fluid. No stimulus other than the presence of albumin in the medium is needed to elicit the FFA release. Intracellular triglycerides appear to be the source of most of the FFA that is released. The released FFA is composed of a mixture of fatty acids, with the fatty acid used to enrich the cells contributing about half of the total. Under certain conditions sufficient fatty acid can be released to increase the FFA concentration of the extracellular fluid. Cells enriched initially with arachidonic acid released 1.7- to 2.9-times more FFA as compared to cells enriched with corresponding amounts of oleic acid. Neither prostaglandins nor lipoxygenase products contributed appreciably to the amount of FFA released from cells enriched with arachidonic acid. Porcine pulmonary artery endothelial cells also can release net amounts of FFA. These findings indicate that endothelial cells have the capacity to release fatty acid in the form of FFA. This process could possibly play a role in the transfer of fatty acids, particularly arachidonic acid, across the endothelium. — **Figard, P. H., D. P. Hejlik, T. L. Kaduce, L. L. Stoll, and A. A. Spector.** Free fatty acid release from endothelial cells. *J. Lipid Res.* 1986. **27:** 771–780.

Supplementary key words arachidonic acid • oleic acid • albumin • triglyceride

For many years adipocytes were thought to be the only cells capable of releasing net amounts of free fatty acid (FFA) into the extracellular fluid. Subsequent work demonstrated that the isolated perfused heart can also release fatty acid into the perfusate in the form of FFA (1). In this case, however, the fatty acid release is due to an exchange between FFA contained in the perfusate and fatty acid generated within the myocardium, and the process does not lead to any net increase in the FFA content of the extracellular fluid. Likewise, radioactive FFA release can occur in the intestine and lung (2, 3), but as in the case of the heart, this results from fatty acid exchange and does not produce any accumulation of FFA in the extracellular fluid. Ehrlich ascites tumor cells also release FFA (4), the fatty acid being derived from cellular phospholipids (5). Although only relatively small amounts of FFA are released, a net accumulation of FFA in the medium will occur if the Ehrlich cells are incubated with fatty acid-free albumin (4). When even a low concentration of FFA is present initially in the extracellular fluid, however, fatty acid uptake by the Ehrlich cell occurs so avidly that the overall effect is a reduction in medium FFA content. Therefore, as in the case of myocardium, intestine, and lung, Ehrlich cells probably do not release net amounts of FFA in their usual biologic environment (4).

Recently, mouse resident peritoneal macrophages and cultured J774 macrophages were observed to release FFA after they became enriched in triglycerides (6). The fatty acid was derived from the accumulated triglycerides, and the quantities of FFA released were large enough to suggest that a net increase in the fatty acid content would result even if the extracellular fluid contained physiologic amounts of FFA. Therefore, FFA release was postulated as a mechanism whereby macrophage foam cells are able to reduce their intracellular triglyceride content.

As in the case of macrophages, cultured bovine aortic endothelial cells accumulate considerable amounts of triglyceride if they are exposed to an excess of fatty acid (7). When these enriched endothelial cultures are transferred to a medium containing fatty acid-free albumin, the cellular triglyceride content gradually decreases. In the present study we have used the ^{63}Ni method (8) to determine whether FFA is released by the endothelial cultures under these conditions. Our findings indicate that bovine aortic endothelial cells and porcine pulmonary artery endothelial cells have the capacity to release relatively large amounts of FFA, sufficient to increase the FFA content of the medium in certain cases.

METHODS

Materials

Tissue culture supplies such as glutamine, BME vitamins, MEM nonessential amino acids, neomycin sulfate, and trypsin were obtained from Gibco (Grand Island, NY). Fetal bovine serum was provided by HyClone Laboratories (Logan, UT). [1-^{14}C]Arachidonic acid was purchased from Amersham Corporation (Arlington Heights, IL), and [9,10-^{3}H]oleic acid and $^{63}NiCl_2$ were from

Abbreviations: FFA, free fatty acid.

DuPont NEN Research Products (Boston, MA). Lipids were obtained from Nu-Chek-Prep (Elysian, MN), and fatty acid-free bovine albumin was from Miles Laboratories, Inc. (Naperville, IL). Silica gel thin-layer chromatography plates were from Alltech Associates, Inc. (Deerfield, IL). Fatty acids were > 98% pure as determined by collection of the eluted fractions and radioactivity assay following gas-liquid chromatography.

Tissue culture

Bovine endothelial cell cultures were obtained from the aorta and utilized up to passage 20 (9). Porcine endothelial cell cultures were obtained from the pulmonary artery and utilized between passages 5 and 12 (10). The cells were maintained on MEM medium supplemented with 10% fetal bovine serum, BME vitamins, MEM nonessential amino acids, 2 mM glutamine, 10 mM HEPES (pH 7.4) and 100 μg/ml neomycin sulfate in an atmosphere of 95% air and 5% CO_2. Seeding was performed following detachment of the cells from the flask with 0.25% trypsin and 0.2% EDTA in a solution containing 150 mM NaCl, 5 mM KCl, 8 mM Na_2HPO_4 and 2 mM KH_2PO_4, pH 7.4. The cells were suspended in maintenance medium and seeded at a concentration of 5–7 × 10^3 cells/cm^2 in T-25 Falcon flasks. Cultures were fed every 3 days and reached confluence within 5–7 days after seeding. Cell protein was measured by a modification (11) of the Lowry method.

Incubations

Fatty acids were added to fatty acid-free albumin dissolved in maintenance medium without serum (7). The pH was adjusted immediately to 7.4. The amount of added fatty acid varied in different experiments; the usual amount was between 50 and 300 μM fatty acid per 100 μM albumin.

Confluent monolayers were washed with Dulbecco's phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 1.5 mM KH_2PO_4, and 8.0 mM Na_2HPO_4, pH 7.4), and then incubated with the fatty acid-supplemented media to which 10% fetal bovine serum was added for 16 hr in a 5% CO_2 atmosphere. In some of the experiments, the medium contained radioactive fatty acid. After incubation, the medium was removed and the cultures were washed with the buffer solution, the same buffer containing 50 μM albumin, and again with the buffer alone. The cells were then incubated, usually for 6 hr, with serum-free maintenance medium containing, in most experiments, 100 μM fatty acid-free albumin. This medium was collected at the end of the incubation for measurement of FFA content (8).

Chemical analyses

FFA content was measured by the ^{63}Ni method (8). Fatty acids were extracted from the medium, and any phospholipids contained in the organic phase were removed by adsorption with activated silicic acid. A solution containing $^{63}NiCl_2 \cdot 6H_2O$ was added to form a ^{63}Ni-fatty acid complex. The complex was taken up in organic solvent and assayed for radioactivity by liquid scintillation spectrometry.

Triglycerides were measured by a micromodification of a spectrophotofluorometric method (12, 13). Lipids in the cells and release medium were extracted with chloroform–methanol 2:1 (v/v) (14). To determine incorporation of radioactivity into total cell lipids, aliquots of the chloroform extract were dried under N_2 and dissolved in 4 ml of Budget-Solve (Research Products International, Inc., Mount Prospect, IL). The distribution of radioactivity in the cellular lipid fractions was determined by spotting additional aliquots of the lipid extract on Silica Gel G plates (Analabs, North Haven, CT) along with a standard lipid mixture, and developing the chromatogram in hexane–ethyl ether–acetic acid–methanol 170:40:4:4 (v/v) (13). The plates were stained with I_2 and, after sublimation, bands corresponding to the different lipid fractions were scraped into scintillation vials containing 4 ml of Budget-Solve. All radioactivity measurements were made with a Beckman LS7000 liquid scintillation spectrometer. Quenching was monitored with the ^{137}Cs external standard.

For fatty acid analysis by gas–liquid chromatography, the lipid samples were dried under N_2, 1 ml of 14% BF_3 in CH_3OH was added, the samples were heated at 100°C for 10 min, and the methyl esters were extracted into *n*-heptane (13). The fatty acid methyl esters were separated by gas–liquid chromatography using a 2 mm × 1.9 m glass column packed with 10% SP2330 on 100/120 mesh Chromasorb W-AW and a Hewlett-Packard model 5700 gas chromatograph (7).

High performance liquid chromatography was carried out using a Beckman 332 gradient system equipped with a 4.6 × 250 mm Vydac column containing C18 reverse-phase 5-μm spherical packing and a gradient of acetonitrile and water adjusted to pH 3.4 with phosphoric acid (15). Radioactivity was assayed by mixing the column effluent with 3a70B scintillator solution (Research Products International, Inc.) at a 1:3 ratio and passing the mixture through an on-line Radiomatic Instruments Flo-one/Beta radioactive detector equipped with a 0.5-ml flow cell. Quenching was corrected, and the data were integrated using the computer and software of Radiomatic Instruments (Tampa, FL).

Total protein synthesis was measured by incubating the cultures for 1 hr in 1 ml of Dulbecco's phosphate-buffered saline solution containing 1 μCi of [4,5-^{3}H]leucine (147 Ci/mmol). After precipitation with 10% trichloroacetic acid and washing, the radioactivity was measured in a liquid scintillation spectrometer (16). Lactate dehydrogenase activity was assayed in 10–20 μl of incubation

medium by a modification of the method of Amador, Dorfman, and Wacker (17) in which the rate of NADH production was monitored fluorimetrically at 32°C. The assay mixture contained in a volume of 0.5 ml, 0.1 M glycine, pH 10, 20 mM sodium lactate, 1 mM NAD and 0.1 mg ovalbumin (Sando, G. N., unpublished observations).

RESULTS

FFA release

We find that when bovine aortic endothelial cells are loaded initially with an excess of fatty acid, they will release fatty acid in the form of FFA during a subsequent incubation. FFA release occurs even though the cells are not exposed to any chemical stimuli that mobilize stored fatty acid. The time dependence of the FFA release, measured as total FFA accumulation in the extracellular fluid by the ^{63}Ni method (8), is shown in **Fig. 1.** In this experiment, the endothelial cells were loaded by exposure to a medium containing serum and supplemental arachidonic acid and then washed with fatty acid-free albumin to remove any of the fatty acid that was loosely bound to the cells. When these cultures were incubated subsequently for up to 6 hr with 100 μM fatty acid-free bovine serum albumin, increasing amounts of FFA accumulated in the medium.

The amount of FFA released by the endothelial cultures was dependent on the quantity of albumin contained in the incubation medium. This is illustrated in **Fig. 2.** Some FFA was released even when the medium contained no protein. Increasing FFA release occurred as the albumin concentration was raised and, under these conditions, the maximum release occurred when the albumin concentration was 200 μM.

The amount of FFA released was also dependent on the quantity of supplemental arachidonic acid to which the endothelial cultures were initially exposed. As shown in **Fig. 3,** only a very small amount of FFA was released from cells that were not exposed to supplemental fatty acid during the first incubation. FFA release increased as the concentration of arachidonic acid in the loading medium was raised to 300 μM. This was the highest arachidonic acid concentration that the endothelial cultures could withstand when the medium contained 10% fetal bovine serum supplemented with 100 μM fatty acid-free albumin. Concomitant experiments with radioactive arachidonic acid under these conditions indicated that increasing amounts of fatty acid were incorporated by the endothelial cultures as the arachidonic acid concentration of the loading medium was raised.

Two approaches were utilized to assess whether exposure to these concentrations of arachidonic acid may have injured the endothelial cultures. The first experiment involved lactate dehydrogenase release to the medium.

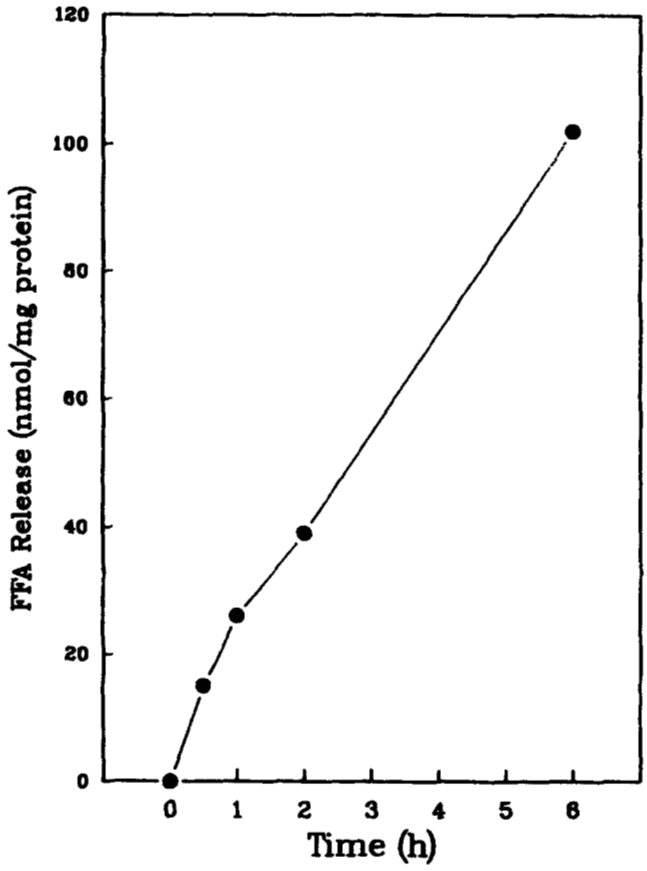

Fig. 1. Time dependence of FFA release from bovine aortic endothelial cells. Confluent endothelial cultures were enriched with fatty acid by incubation for 16 hr at 37°C in a 5% CO_2 atmosphere with a medium containing 200 μM arachidonic acid, 100 μM fatty acid-free bovine serum albumin, and 10% fetal bovine serum. After removal of the medium, the cultures were washed with buffer, including a wash with 50 μM albumin to remove any loosely attached FFA. The cultures then were incubated at 37°C in a 5% CO_2 atmosphere with a medium containing 100 μM fatty acid-free bovine serum albumin, and the FFA contained in the medium was measured by the ^{63}Ni assay (8). Each point is the average of values obtained from duplicate assays on each of two separate cultures from the same passage. The differences between the values were less than 10%.

Cultures incubated for 16 hr with a medium containing 10% fetal bovine serum and 100 μM albumin released 12.8 units/ml lactate dehydrogenase, while those incubated with this medium containing 200 or 250 μM arachidonic acid released 11.3 and 12.6 units/ml, respectively. When these cultures were washed and then incubated for an additional 5 hr in a medium containing 10% fetal bovine serum, the control cultures released 9.0 units/ml of lactate dehydrogenase, whereas those initially exposed to the media containing arachidonic acid released 8.0 and 9.5 units/ml, respectively. In a second experiment, cultures were incubated with either the control medium or the media supplemented with arachidonic acid for 16 hr, washed, and then incubated for an additional 1 hr with [4,5-^{3}H]leucine. Radioactivity incorporated into cellular protein was measured following precipitation with tri-

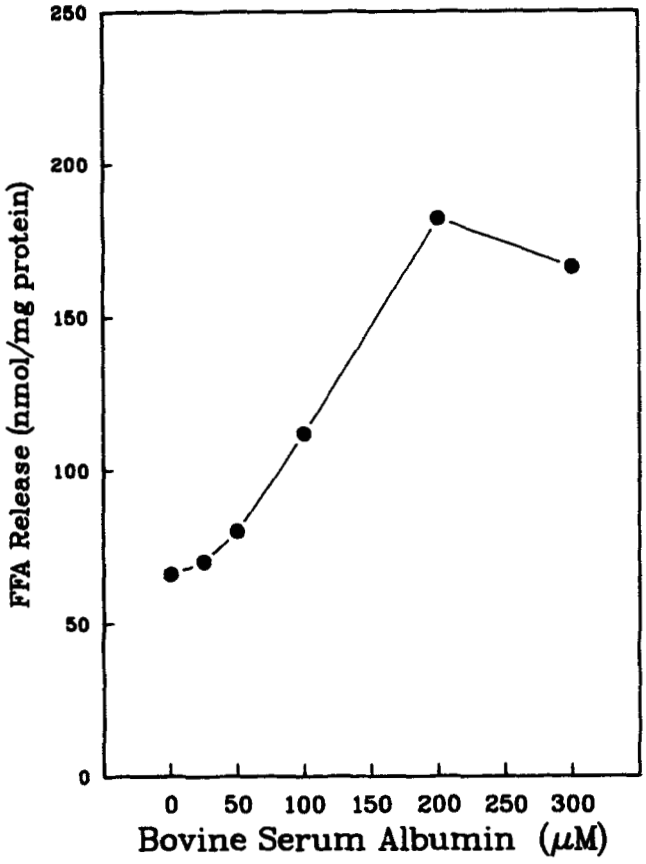

Fig. 2. Effect of albumin concentration on the amount of FFA released by bovine aortic endothelial cells. The conditions are the same as those described in Fig. 1, except that the time of release was 6 hr and the release medium contained 0 to 300 μM fatty acid-free bovine serum albumin.

chloroacetic acid. The control cultures incorporated 18,800 ± 700 dpm, while those initially incubated with 200 or 250 μM arachidonic acid incorporated 21,200 ± 1100 and 19,200 ± 800 dpm (n = 4), respectively. Therefore, based on lactate dehydrogenase release and total protein synthesis, these concentrations of arachidonic acid were not overly toxic to the endothelial cultures.

Net release in the presence of extracellular FFA

In the above studies where the endothelial cells were able to increase the FFA content of the extracellular fluid, the release medium initially contained no fatty acid. Even when the plasma FFA concentration is extremely low under physiologic conditions, however, the molar ratio of FFA to albumin is at least 0.25 (18). Previous isotopic studies demonstrated that endothelial cultures can readily take up fatty acid even when it is present in very small amounts (4, 19). Therefore, the question remained as to whether the endothelial cultures would still release a net amount of fatty acid if the extracellular fluid initially contained any FFA. To deal with this question, measurements of the net change in extracellular FFA content were made during incubation of the cultures with media that initially contained supplemental fatty acid in addition to albumin.

In the experiment shown in **Fig. 4,** endothelial cultures initially loaded by incubation with serum containing supplemental arachidonic acid were subsequently incubated with media containing 100 μM fatty acid-free albumin and 0 to 300 μM fatty acid. The extracellular fatty acid was either arachidonic acid or oleic acid; generally similar results were obtained in both cases. When no fatty acid was added to the medium, the cultures released 240 nmol of FFA in 6 hr. Net FFA release also occurred when either 50 or 100 μM fatty acid was initially present, the amount becoming less as the extracellular fatty acid concentration increased. When the extracellular fatty acid concentration was either 200 or 300 μM, equivalent to a fatty acid to albumin molar ratio of 2 and 3, respectively, a net release of FFA did not occur. Instead, there was a considerable net uptake of fatty acid by the endothelial cultures.

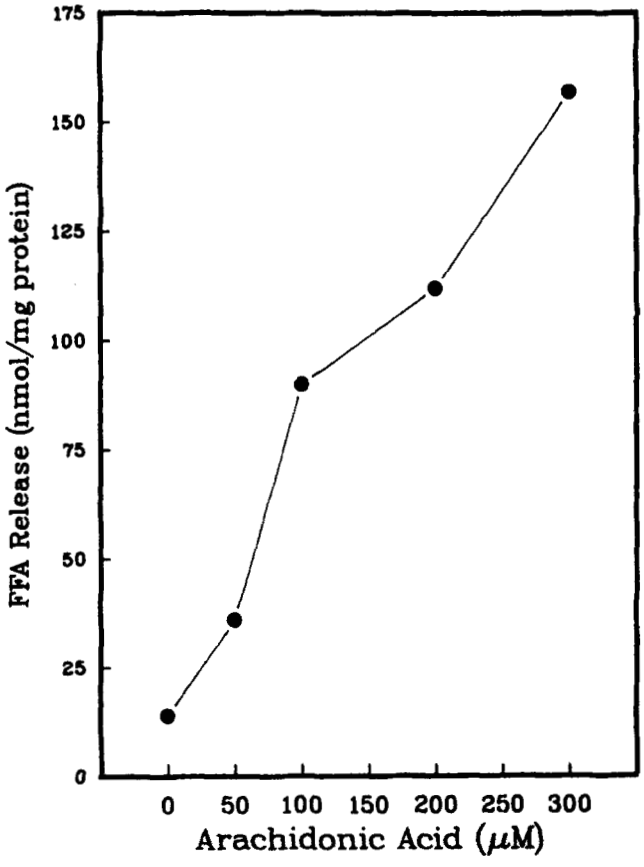

Fig. 3. Dependence of the amount of FFA release from bovine aortic endothelial cells on the concentration of arachidonic acid to which the cells initially were exposed. The conditions are the same as described in Fig. 1, except that the arachidonic acid concentration in the loading medium was varied from 0 to 300 μM. The release medium contained 100 μM fatty acid-free bovine serum albumin, and the time of release was 6 hr.

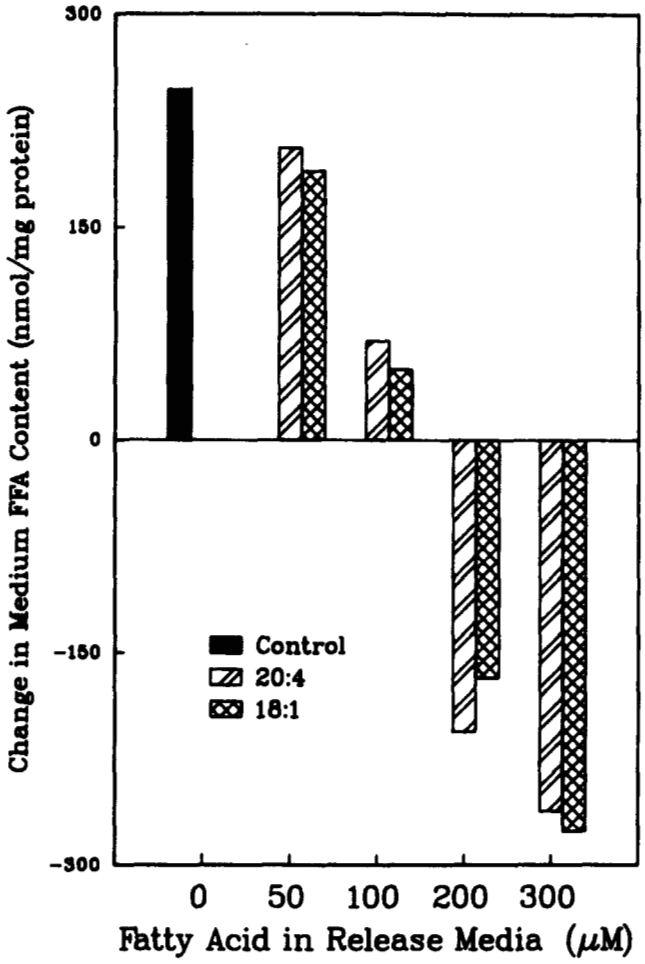

Fig. 4. Net changes in the FFA content of the medium as a result of incubation with bovine aortic endothelial cells previously enriched with arachidonic acid. The conditions were the same as those described in Fig. 1, except that the cultures were loaded with 250 μM arachidonic acid. A 6-hr incubation was employed to measure the net change in medium FFA content. These media contained 100 μM fatty acid-free bovine serum albumin and 0 to 300 μM of either arachidonic or oleic acid. Each bar is the average of values obtained from duplicate assays on each of two separate cultures; these differed by less than 10%.

Another experiment was done under identical conditions except that the cultures were initially loaded by incubation with serum containing supplemental oleic acid. The results are shown in **Fig. 5.** A net release of FFA occurred when these cells were incubated with 100 μM fatty acid-free albumin, but the amount was only 35% as much as from the cultures initially loaded with arachidonic acid. No appreciable net change in medium FFA content was observed when 50 μM fatty acid was present in the extracellular fluid. With 100 μM fatty acid, there also was no net change in the medium FFA content in the presence of arachidonic acid, but a net uptake of fatty acid by the cells occurred when the medium contained oleic acid. At higher concentrations of both fatty acids, a net uptake of fatty acid by the cells occurred; this was considerably larger when oleic acid was available as compared with arachidonic acid.

Taken together, these results indicate that endothelial cells loaded with either supplemental oleic or arachidonic acid have the capacity to release net quantities of FFA. When the cells are loaded with arachidonic acid, they are able to release net amounts of FFA even when the molar ratio of FFA to albumin in the medium is 0.5 to 1.0. However, the cells initially loaded with oleic acid shift from releasing to accumulating net amounts of fatty acid at substantially lower extracellular FFA concentrations.

Origin of the released FFA

Concomitant studies were done with endothelial cultures labeled with [1-^{14}C]arachidonic acid in an attempt to determine the origin of the released FFA. The results are shown in **Fig. 6.** After the labeling period and wash with albumin, only 4% of the radioactivity contained in the cells was in the form of FFA. When these cells were incubated subsequently with 100 μM fatty acid-free albumin for 4 hr, the longest time tested, 23% of the radio-

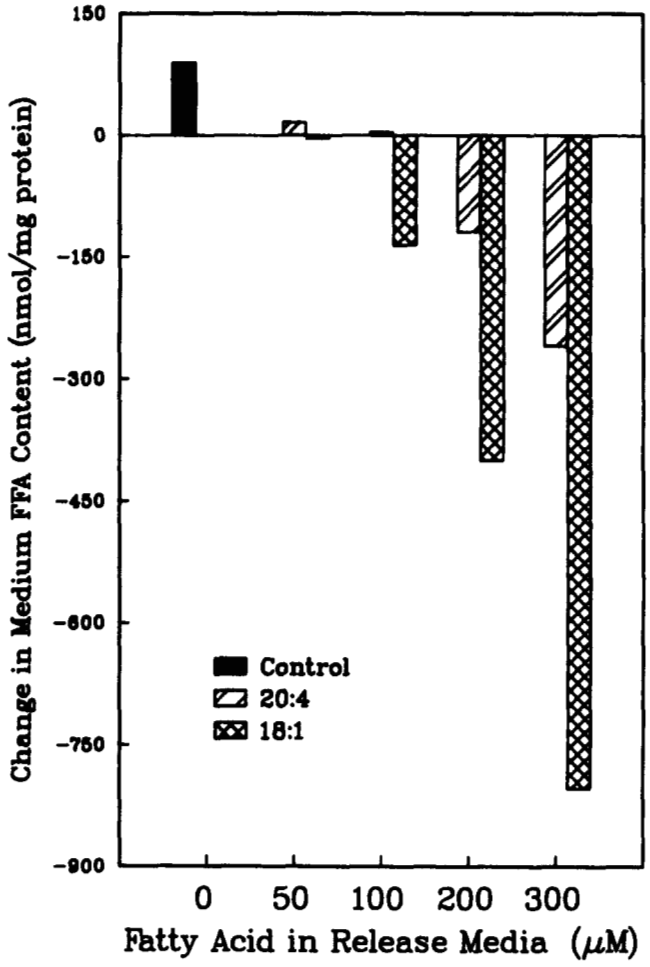

Fig. 5. Net changes in the FFA content of the medium as a result of incubation with bovine aortic endothelial cells enriched with oleic acid. The conditions are the same as described in Fig. 4, except that the cultures were loaded with 250 μM oleic acid.

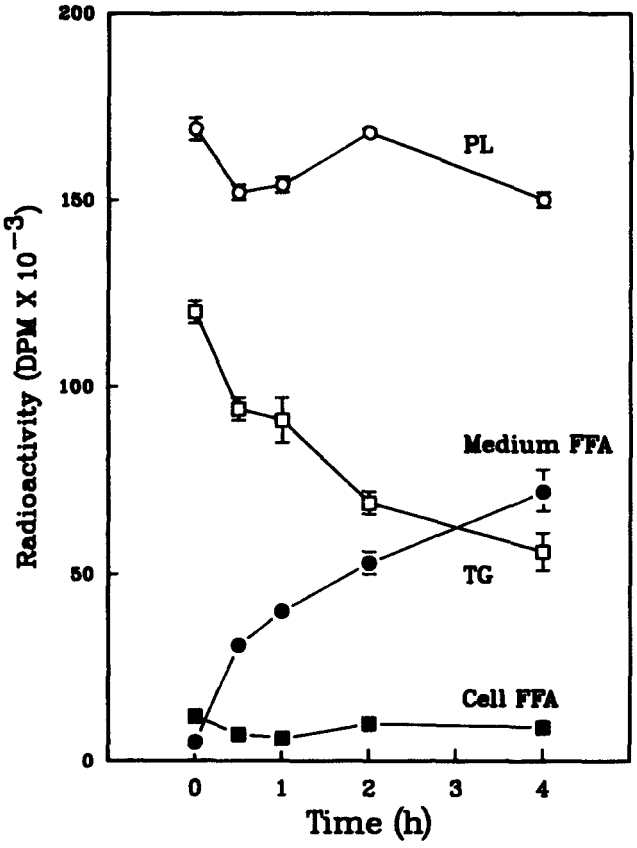

Fig. 6. Changes in cell lipid radioactivity and accumulation of radioactive FFA in the medium during incubation of bovine aortic endothelial cells labeled with [1-^{14}C]arachidonic acid. The conditions were the same as those described in Fig. 1, except that the cultures were loaded with 50 μM arachidonic acid containing 0.5 μCi [1-^{14}C]arachidonic acid. The lipids were separated by thin-layer chromatography. Each point is the mean ± SE of values obtained from three separate cultures, each from the same passage and incubated at the same time. The SE is omitted in those cases where the bars are too small to be visible.

activity initially present in the cells was released to the medium in the form of FFA. Therefore, most of the released FFA had to be derived from intracellular fatty acid initially contained in esterified form. After loading, 56% of the radioactivity was present in phospholipids and 40% in triglycerides. During the 4 hr incubation, there was very little change in phospholipid radioactivity, the amount present after the 4 hr incubation being only 12% less than the initial value. By contrast, there was a continuous decline in triglyceride radioactivity, and the amount after 4 hr was 52% smaller than the initial value. At the end of the 4 hr incubation, 68,000 dpm were released into the medium in the form of FFA. The decrease in triglyceride radioactivity over the 4 hr period was 63,000 dpm, whereas the decline in phospholipid radioactivity was only 20,000 dpm. These findings suggest that triglycerides probably are the main source of the FFA released by the endothelial cultures.

In a similar experiment done with [9,10-^{3}H]oleic acid, the endothelial cells released 12% of their initial radioactivity content in the form of FFA during a 4 hr incubation with 100 μM fatty acid-free albumin, amounting to 58,000 dpm (data not shown). There was a similar decline in cell triglyceride radioactivity, no decrease in cell phospholipid radioactivity, and the cell FFA radioactivity declined by only 10,000 dpm. Therefore, the isotopic results with oleic acid-loaded cells also indicate that triglycerides are the main source of the released FFA.

To further assess the possibility that the released FFA may be derived primarily from intracellular triglycerides, additional experiments were done in which the FFA release measured by the ^{63}Ni method was compared with the decline in the cellular triglyceride content. The results are shown in **Table 1.** Before loading, the triglyceride content of the cultures was 21 ± 3 nmol/mg cell protein. This increased by 76% after loading with oleic acid, and by 104% after loading with arachidonic acid. In both cases the cellular triglyceride content decreased to the preloading value after a 6 hr incubation with 100 μM fatty acid-free albumin. This was associated with an increase in the FFA content of the medium. Assuming that 3 nmol of fatty acid was obtained from each nmol of triglyceride that was consumed, the decrease in triglyceride content can account for all of the FFA released by the cultures loaded with oleic acid. In the case of the cells initially loaded with arachidonic acid, the triglyceride decrease can account for 87% of the FFA that was released.

TABLE 1. Comparison of FFA release with changes in the triglyceride content of bovine aortic endothelial cells

Parameter	Amount	
	Arachidonic Acid[a]	Oleic Acid[a]
	nmol/mg cell protein	
Cell triglyceride after loading	43 ± 3	37 ± 3
Cell triglyceride after incubation[b]	18 ± 4	20 ± 1
Calculated reduction in triglyceride content	25	17
Calculated fatty acid equivalents from cell triglyceride hydrolysis[c]	75	51
FFA release to medium[d]	85 ± 6	49 ± 5

[a] Fatty acid supplement to which the cells were exposed during an initial 16-hr incubation at 37°C in a 5% CO_2 atmosphere. The media contained 200 μM of the supplemental fatty acid, 100 μM bovine albumin, and 10% fetal bovine serum. Prior to loading, the cells contained 21 ± 3 nmol of triglyceride.

[b] The incubation was for 6 hr at 37°C in a 5% CO_2 atmosphere with a medium containing 100 μM fatty acid-free albumin.

[c] It is assumed that each nmol decrease in triglyceride gives rise to 3 nmol fatty acid.

[d] Medium FFA content after the 6 hr incubation as measured by the ^{63}Ni method (8). Each value is the mean ± SE of assays from three separate cultures.

Composition of the released FFA

The composition of the FFA released by the endothelial cells was assayed by gas-liquid chromatography. Bovine endothelial cultures were supplemented initially with either 150 μM arachidonic acid or 150 μM oleic acid bound to 100 μM albumin in the presence of 10% fetal bovine serum. After removal of this medium and washing, the cultures were incubated for 6 hr with 100 μM fatty acid-free albumin. Analysis by gas-liquid chromatography indicated that when the cultures were supplemented with arachidonic acid, 46% of the FFA released to the medium was in the form of 20:4.[1] In addition, 4% was recovered as 22:4, the elongation product of 20:4 that is formed by endothelial cultures (19). The remainder of the released FFA consisted of 2% 14:0, 20% 16:0, and 11% 18:0, 16% 18:1 and 2% 18:2. When the cultures were supplemented with oleic acid, 54% of the released FFA was in the form of 18:1 and the remainder consisted mostly of 16:0 and 18:0. Therefore, the released FFA is composed of a mixture of fatty acids, with the fatty acid used to supplement the cultures contributing only about half of the total.

FFA release by porcine pulmonary artery endothelial cells

To determine whether the capacity to release FFA is a special property of bovine aortic endothelial cells, some of the studies were repeated with cultured porcine pulmonary artery endothelial cells. When these cells were loaded with [1-^{14}C]arachidonic acid, 24% of the radioactivity was released in the form of FFA during a subsequent 4 hr incubation with 100 μM fatty acid-free albumin. Additional studies done with the ^{63}Ni method demonstrated that the porcine cells, loaded with either arachidonic or oleic acid, were also capable of releasing net amounts of FFA. The results are shown in **Table 2.** As in the case of the bovine aortic endothelial cells, the net release to fatty acid-free albumin was considerably larger when the cultures were incubated initially with serum containing supplemental arachidonic acid. When these cells were incubated in a medium containing albumin and 100 μM of arachidonic acid, no net change in medium FFA content occurred. By contrast, when cells incubated initially with serum containing supplemental oleic acid were then incubated in a release medium containing albumin and 100 μM oleic acid, a considerable net uptake of FFA was observed.

Assay for eicosanoid formation

Cultured endothelial cells produce prostaglandins and other eicosanoids when they are incubated with arachidonic acid (20-24). Therefore, it was necessary to determine whether eicosanoids contributed substantially to the fatty acid release from the cultures that were enriched with arachidonic acid. Bovine aortic endothelial cells were loaded with 150 μM arachidonic acid containing 1 μCi of [1-^{14}C]arachidonic acid. After removal of this medium and washing, the cultures were incubated for 6 hr in a medium containing 100 μM fatty acid-free albumin. A high performance liquid chromatogram of the lipid radioactivity released into the medium revealed that 93% remained as arachidonic acid and less than 1% was converted into eicosanoids. These findings indicate that eicosanoids do not contribute substantially to the amount of fatty acid that is released spontaneously from endothelial cells.

TABLE 2. Changes in medium FFA content during incubation with porcine pulmonary artery endothelial cultures

Fatty Acid Supplement[a]	Addition to Release Medium[b]	Change in Medium FFA Content[c]
		nmol
Arachidonic	None	+ 54 ± 5
Oleic	None	+ 21 ± 4
Arachidonic	100 μM Arachidonic acid	+ 2 ± 11
Oleic	100 μM Oleic acid	− 39 ± 6[d]

[a]The cultures were supplemented with fatty acid as described in Table 1.
[b]This medium contained 100 μM fatty acid-free bovine serum albumin.
[c]The fatty acid-loaded cells were incubated for 6 hr, and the medium FFA content was measured by the ^{63}Ni method (8). Each value is the mean ± SE of measurements from three separate cultures.
[d]In this case there was a net uptake of FFA from the medium.

DISCUSSION

These findings demonstrate that when cultured endothelial cells are enriched with fatty acid, they are capable of releasing enough fatty acid to raise the FFA content of the extracellular fluid under certain conditions. The FFA that is released appears to be derived primarily from intracellular triglycerides. As in the case of macrophages which also can release net quantities of FFA (6), no stimulus is required to initiate the release process, other than the presence of albumin which acts as the FFA acceptor (18). FFA release was observed with two different types of arterial endothelial cells, each cultured from a different species. This suggests that the capacity to release FFA may be a rather general property of endothelium derived from large vessels.

Although the endothelial cells enriched with oleic acid were able to effect a net increase in medium FFA content when fatty acid-free albumin was present, no net release was observed when the albumin contained as little as 0.5 mol fatty acid (Fig. 5). At higher external fatty acid con-

[1]The fatty acids are abbreviated as number of carbon atoms: number of double bonds.

centrations, a net uptake by the cells occurred. Under ordinary conditions plasma albumin contains 0.25 to 1.5 mol of fatty acid (18). Therefore, one would predict that in many physiologic situations, there would not be a net efflux of FFA from the endothelium even when the cells are enriched with fatty acid and have a high triglyceride content. In the special situation where the cells build up an excess of stored fatty acid and the extracellular fluid has a relatively low concentration of FFA, however, our results demonstrate that a net efflux of FFA out of the endothelial cell is possible. Whether this actually might occur in vivo remains open to question for several reasons. To elicit easily measurable quantities of FFA release, the cultures usually were loaded by exposure to 200 or 250 μM fatty acid bound to 100 μM albumin. This is a higher ratio of FFA to albumin than ordinarily occurs in human plasma (18), and the use of these conditions probably led to greater intracellular triglyceride accumulation than might be expected in vivo. Yet, as shown in Fig. 3, net release of FFA was observed even when the cultures were loaded with 50 or 100 μM arachidonic acid, so the possibility that net FFA efflux can occur under some conditions in vivo cannot be excluded. Another uncertainty regarding physiologic relevance concerns the fact that, except for the small amount of fatty acid present in the serum, the cells were exposed to only a single fatty acid instead of a fatty acid mixture during the loading phase. Because of these uncertainties, other approaches will be required before it is possible to say whether endothelial cells actually are able to release net amounts of FFA in vivo.

The FFA released from the endothelial cells is composed of a mixture of fatty acids, with the particular fatty acid used as a supplement contributing about half of the total. This is consistent with our previous observation that while the triglycerides that accumulate during loading are enriched with the fatty acid supplement, they too are composed of a mixture of fatty acids (7). It is possible that the additional fatty acids are derived from the serum contained in the loading medium. Alternatively, they may be derived from the cellular phospholipids because, like the triglycerides, the phospholipids also become enriched with the supplemented fatty acid (19, 23, 24). In the case of phospholipids, however, the enrichment results from a replacement of existing fatty acyl groups, and there is no increase in the phospholipid content of the cells (25, 26). When the triglycerides that accumulate in the endothelial cells are subsequently hydrolyzed, many of the fatty acids that are generated apparently are available for release into the medium.

Larger amounts of total FFA were released from the cells enriched with arachidonic acid as compared to oleic acid. A possible explanation is that the cells exposed to arachidonic acid accumulated more triglyceride. In the one set of experiments where we actually measured cellular triglyceride content (Table 1), the bovine aortic endothelial cultures incubated with supplemental arachidonic acid accumulated 16% more triglyceride. More extensive previous measurements indicated, however, that 60 to 120% more triglyceride accumulates when these cultures are incubated with supplemental arachidonic acid as compared to an equivalent amount of oleic acid (7). Incubation of the endothelial cultures with these concentrations of arachidonic acid did not increase lactate dehydrogenase release or reduce protein synthesis. This suggests that the greater FFA release from the cultures enriched with arachidonic acid is not due to a toxic effect of arachidonic acid on the cells. However, prostaglandin I_2 is formed when endothelial cultures are incubated with arachidonic acid (24). It is possible that prostaglandin I_2 or a related eicosanoid may have exerted a pharmacological effect on the cells and that this is responsible for the quantitative differences between the cells enriched with arachidonic and oleic acids.

The amount of arachidonic acid spontaneously released to albumin is far greater than that occurring when endothelial cultures are stimulated to produce prostaglandins, even when the cultures are similarly enriched with arachidonic acid (24). Based on total FFA releases of 80 to 240 nmol/mg of cell protein (Figs. 1–4) and an estimate of 45% arachidonic acid, we calculate that a 25-cm^2 monolayer culture of enriched endothelial cells, equivalent to about 300–400 μg of cell protein, is capable of releasing between 10 and 45 nmol of arachidonic acid in 6 hr. When incubated with these concentrations of extracellular arachidonic acid, endothelial cultures of this size produce 400 to 800 pmol of prostaglandin I_2 (24), which accounts for about 75% of the total eicosanoid output (27). This amounts to only 1–3% of the spontaneous arachidonic acid release and explains why appreciable amounts of eicosanoids were not detected in the release medium by high performance liquid chromatography.

When endothelial cells are stimulated to produce prostaglandins, some intracellular arachidonic acid also is released as FFA. The amount of arachidonic acid release increases when the medium contains fatty acid-free albumin (Figard, P. H., and A. A. Spector, unpublished observations). The quantity, however, is similar to the amount of prostaglandins formed, and is in the range of 0.5 to 1 nmol in a 25-cm^2 monolayer culture. While this amount of FFA is considerably smaller than the spontaneous release over 6 hr from cultures previously enriched with arachidonic acid, stimulated release occurs much more rapidly and is complete within 5–20 min (16).

Although there is at present no indication as to what functional role endothelial FFA release might subserve, several possibilities seem worthy of consideration. One is that FFA release could be part of a mechanism whereby excess fatty acid is cleared from the arterial wall. In such a process, the endothelial cells would take up and temporarily store the excess fatty acid in the form of triglycer-

ide (7, 27, 28). This triglyceride would be subsequently hydrolyzed and the fatty acid cleared through release to albumin, which would then bind the fatty acid and remove it through the circulation. An alternative possibility is that endothelial FFA release is part of the mechanism whereby fatty acids are transferred from the blood into tissues. Although evidence exists that fatty acids cross the endothelium by an energy-independent, lateral diffusion process (29, 30), it is conceivable that an intracellular triglyceride intermediate might be involved in situations where relatively large amounts of fatty acid were available. Both of these postulated functions imply that the FFA release process might have polarity, for release would be expected to occur at the luminal surface in the case of a clearance mechanism, as opposed to the interstitial surface in an uptake mechanism. It is now possible to test for such polarity because endothelial cells can be grown on a micropore filter under conditions where tight junctions will form (10). Finally, the possibility that the FFA release process might be related to supplying substrate for eicosanoid synthesis by other cells, either in the vessel wall or the blood, should be considered because endothelial FFA efflux appears to operate especially effectively with arachidonic acid.

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REFERENCES

1. Evans, J. R. 1964. Cellular transport of long chain fatty acids. *Can. J. Biochem.* **42:** 955–969.
2. Porte, D., Jr., and C. Entenman. 1965. Fatty acid metabolism in segments of rat intestine. *Am. J. Physiol.* **208:** 607–614.
3. Nasr, K., and H. O. Heinemann. 1965. Lipid synthesis by rabbit lung tissue in vitro. *Am. J. Physiol.* **208:** 118–121.
4. Spector, A. A., and D. Steinberg. 1966. Release of free fatty acids from Ehrlich ascites tumor cells. *J. Lipid Res.* **7:** 649–656.
5. Spector, A. A., and D. Steinberg. 1967. Turnover and utilization of esterified fatty acids in Ehrlich ascites tumor cells. *J. Biol. Chem.* **242:** 3057–3062.
6. von Hodenberg, E., J. C. Khoo, D. Jensen, J. L. Witztum, and D. Steinberg. 1984. Mobilization of stored triglycerides from macrophages as free fatty acids. *Arteriosclerosis.* **4:** 630–635.
7. Denning, G. M., P. H. Figard, T. L. Kaduce, and A. A. Spector. 1983. Role of triglycerides in endothelial cell arachidonic acid metabolism. *J. Lipid Res.* **24:** 993–1001.
8. Ho, R. J. 1970. Radiochemical assay of long-chain fatty acids using ^{63}Ni as tracer. *Anal. Biochem.* **36:** 105–113.
9. Goldsmith, J. C., C. T. Jafvert, P. Lollar, W. G. Owen, and J. C. Hoak. 1981. Prostacyclin release from cultured ex vivo bovine vascular endothelium. Studies with thrombin, arachidonic acid and ionophore A23187. *Lab. Invest.* **45:** 191–197.
10. Hennig, B., D. M. Shasby, A. B. Fulton, and A. A. Spector. 1984. Exposure to free fatty acid increases the transfer of albumin across cultured endothelial monolayers. *Arteriosclerosis.* **4:** 489–497.
11. Lees, M. B., and S. Paxman. 1972. Modification of the Lowry procedure for the analysis of proteolipid protein. *Anal. Biochem.* **47:** 184–192.
12. Kessler, G., and H. Lederer. 1966. Fluorometric measurement of triglycerides. *In* Automation in Analytical Chemistry. L. T. Skeggs, Jr., editor. Technicon Symposium. Mediad, New York. 341–344.
13. Spector, A. A., R. E. Kiser, G. M. Denning, S-W. Koh, and L. E. DeBault. 1979. Modification of the fatty acid composition of cultured human fibroblasts. *J. Lipid Res.* **20:** 536–547.
14. Folch, J. M., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226:** 497–509.
15. Eling, T., B. Tainer, A. Ally, and R. Warnock. 1982. Separation of arachidonic acid metabolites by high-pressure liquid chromatography. *Methods Enzymol.* **86:** 511–517.
16. Spector, A. A., A. M. Scanu, T. L. Kaduce, P. H. Figard, G. M. Fless, and R. L. Czervionke. 1985. Effect of human plasma lipoproteins on prostacyclin production by cultured endothelial cells. *J. Lipid Res.* **26:** 288–297.
17. Amador, E., L. E. Dorfman, and W. E. C. Wacker. 1963. Serum lactate dehydrogenase activity: an analytical assessment of current assays. *Clin. Chem.* **9:** 391–399.
18. Spector, A. A. 1975. Fatty acid binding to plasma albumin. *J. Lipid Res.* **16:** 165–179.
19. Spector, A. A., T. L. Kaduce. J. C. Hoak, and G. L. Fry. 1981. Utilization of arachidonic and linoleic acids by cultured human endothelial cells. *J. Clin. Invest.* **68:** 1003–1011.
20. Marcus, A. J., B. B. Weksler, and E. A. Jaffe. 1978. Enzymatic conversion of prostaglandin endoperoxide H_2 and arachidonic acid to prostacyclin by cultured human endothelial cells. *J. Biol. Chem.* **253:** 7138–7141.
21. Gorman, R. R., T. D. Oglesby, G. L. Bundy, and N. K. Hopkins. 1985. Evidence for 15-HETE synthesis by human umbilical vein endothelial cells. *Circulation.* **72:** 708–712.
22. Johnson, A. R., G. Revtyak, and W. B. Campbell. 1985. Arachidonic acid metabolites and endothelial injury: studies with cultures of human endothelial cells. *Federation Proc.* **44:** 19–24.
23. Kaduce, T. L., A. A. Spector, and R. S. Bar. 1982. Linoleic acid metabolism and prostaglandin production by cultured bovine pulmonary artery endothelial cells. *Arteriosclerosis.* **2:** 380–389.
24. Spector, A. A., T. L. Kaduce, J. C. Hoak, and R. L. Czervionke. 1983. Arachidonic acid availability and prostacyclin production by cultured human endothelial cells. *Arteriosclerosis.* **3:** 323–331.
25. Spector, A. A., J. C. Hoak, G. L. Fry, L. L. Stoll, C. T. Tanke, and T. L. Kaduce. 1981. Essential fatty acid availability and prostacyclin production by cultured human endothelial cells. *Prog. Lipid Res.* **20:** 471–477.
26. Spector, A. A., and M. A. Yorek. 1985. Membrane lipid composition and cellular function. *J. Lipid Res.* **26:** 1015–1035.
27. Spector, A. A., T. L. Kaduce, P. H. Figard, K. C. Norton, J. C. Hoak, and R. L. Czervionke. 1983. Eicosapentaenoic acid and prostacyclin production by cultured human

endothelial cells. *J. Lipid Res.* **24:** 1595–1604.
28. Sivarajan, M., E. R. Hall, K. K. Wu, M. E. Rafelson, and C. Manner. 1984. Regulation of intracellular arachidonate in normal and stressed endothelial cells. *Biochim. Biophys. Acta.* **795:** 271–276.
29. Stein, O., and Y. Stein. 1968. Lipid synthesis, intracellular transport and storage. III. Electron microscopic radioautographic study of the rat heart perfused with tritiated oleic acid. *J. Cell Biol.* **36:** 63–77.
30. Scow, R. O., E. J. Blanchette-Mackie, and L. C. Smith. 1980. Transport of lipid across capillary endothelium. *Federation Proc.* **9:** 2610–2617.