BRIEF COMMUNICATIONS

Exposure to Fatty Acid Increases Human Low Density Lipoprotein Transfer across Cultured Endothelial Monolayers

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SUMMARY. Human low density lipoproteins radiolabeled with $^{125}$I transfer across confluent monolayers of cultured porcine pulmonary artery endothelial cells. The amount transferred was dependent on the low density lipoprotein concentration and was not saturable at concentrations up to 300 $\mu$g protein per 0.5 ml medium. Gel filtration showed that more than 90% of the $^{125}$I which crossed the endothelial monolayer remained associated with low density lipoproteins, indicating that appreciable amounts of lipoprotein were not degraded during the transfer process. When the endothelial monolayer was exposed for 24 hours to culture media supplemented with 100–300 $\mu$M fatty acid complexed with 100 $\mu$M albumin, the amount of low density lipoprotein subsequently transferred increased by 65% to 150%. The extent of the increase was dependent on the type of fatty acid added and its concentration. At 200 $\mu$M, albumin-bound oleic and linoleic acids increased low density lipoprotein transfer, whereas palmitic, linolenic, arachidonic, and eicosapentaenoic acids did not. These results are consistent with the hypothesis that exposure of the endothelium to elevated concentrations of fatty acid may allow excessive amounts of cholesterol-rich lipoproteins to enter the arterial intima. (Circ Res 57: 776–780, 1985)

ENDOTHELIAL injury may be involved in the pathogenesis of atherosclerosis (Ross and Harker, 1976; Renkin and Curry, 1982). Such injury might reduce the ability of the endothelium to limit the transfer of macromolecules, thereby allowing increased entry of cholesterol-rich lipoproteins into the arterial wall (Ross and Harker, 1976). Liberation of high amounts of fatty acid near the arterial surface during hydrolysis of lipoprotein triglycerides has been proposed as a mechanism for endothelial injury (Zilversmit, 1973). In support of this possibility, recent studies demonstrated that exposure of cultured endothelial monolayers to elevated concentrations of free fatty acid leads to phospholipid fatty acyl modifications, triglyceride accumulation, and an alteration in cell morphology (Denning et al., 1983). Subsequent studies utilizing porcine pulmonary artery endothelial monolayers grown on micropore filters in culture demonstrated that exposure of the cells to elevated concentrations of fatty acid increased the transfer of albumin across the endothelium (Hennig et al., 1984). As an extension of this work, we have examined the transfer of low density lipoproteins (LDL), which are atherogenic, across these cultured endothelial monolayers.

Methods

Porcine pulmonary artery endothelial cells were cultured in M-199 containing 10% fetal bovine serum (Hennig et al., 1984). Cultures were determined to be endothelial by uniform morphology and by quantitative determination of angiotensin-converting enzyme activity. Cells from passages 5–12 were plated on gelatin-impregnated polycarbonate filters (Nucleopore Corp., 13 mm in diameter and 0.8-$\mu$m pore size) glued to polystyrene chemotaxis chambers (ADAPS, Inc.). After 48 hours, the chemotaxis chambers—with attached filters and endothelial monolayers—were washed free of serum by gentle immersion in M-199 and incubated in control or fatty acid-supplemented media. The media were composed of M-199 enriched with vitamins, amino acids, 15 mM HEPES, 5% fetal bovine serum, 100 $\mu$M solution of crystalline fatty acid-free bovine albumin (Sigma Chemical Co.), and, in the case of most experimental cultures, 100–300 $\mu$M oleic acid (Nu Check Prep, 98% pure by gas-liquid chromatography). Other fatty acids were tested at a concentration of 200 $\mu$M. Fatty acids were dissolved in ethanol. After the addition of one or two drops of 1 N NaOH, the material was dried under high purity N$_2$, redissolved in a small amount of warm distilled water, and added to the medium containing albumin. The pH was adjusted immediately to 7.4. After a 24-hour incubation period, the chemotaxis chambers with...
attached endothelial monolayers were washed 3 times in M-199. The luminal compartment then was filled with M-199 containing 125I-low density lipoprotein (LDL), 125 or 250 µg LDL protein per 0.5 ml medium added to 0.57 cm² endothelium. After a 1-hour incubation, the luminal and abluminal media were sampled, and 125I-LDL radioactivity was determined in a Beckman 300 γ-counter.

LDL was prepared from human blood obtained from normal volunteers who were fasted for 12 hours. The LDL was isolated and washed by ultracentrifugation as previously described (Havel et al., 1955). The isolated LDL was purified further by gel filtration column chromatography (Mathur and Spector, 1976) using Bio-Gel A-5m with an operational range of 10,000–5,000,000 daltons (Bio-Rad Laboratories). The purity of LDL was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Hennig and Dupont, 1983). After the purified LDL was concentrated to approximately 5 mg protein/ml, it was iodinated with 125I by the modified iodine monochloride method (McFarlane, 1958; Bilheimer et al., 1972). This iodination method results in the incorporation of less than 4% of the radioactivity in LDL lipids, and not more than one 125I was incorporated per LDL molecule. There was no change in chromatographic elution pattern of LDL before or after iodination.

Transendothelial electrical resistance was measured by determining the current that passed across the monolayer when a 0.3-mV potential difference was produced across the monolayer by an automatic current voltage clamp (University of Iowa Bioengineering Dept.). The initial resistance was measured on the first day in a medium containing only M-199. Subsequently, the monolayers were placed in culture with M-199 containing 5% fetal bovine serum supplemented with either 100 µM fatty acid-free albumin or this concentration of albumin and 200 µM oleic acid. After 24 hours of incubation, the transendothelial electrical resistance was determined again in a medium containing only M-199.

**Results**

125I-LDL transferred across the confluent monolayer cultures of porcine pulmonary artery endothelial cells. As shown in Figure 1, the amount of LDL that passed across the endothelial monolayer during a 1-hour incubation was dependent on the concentration of LDL initially present in the upper chamber. There was no indication of saturability at LDL concentrations of up to 300 µg protein/ml. Exposure to LDL for 1 hour did not enhance the transfer of bovine albumin across the endothelial cell monolayer (8.7 ± 0.6 nmol albumin, control; 7.9 ± 0.5, 125 µg LDL protein; 7.3 ± 0.4, 250 µg LDL protein), indicating that LDL itself does not increase the transfer of macromolecules across these cultures.

Figure 2 shows gel filtration chromatograms of the 125I-LDL. Before traversing the endothelial monolayer (Fig 2, top), about 98% of the radioactivity eluted with the LDL peak, and only 2% was present in the low molecular weight region where phenol red eluted. This radioactivity profile of 125I-LDL was unaltered when it passed through filters without cells; more than 97% was recovered as 125I-LDL. The bottom of Figure 2, which shows a chromatogram of 125I-LDL which passed through an endothelial monolayer, demonstrates that 90 ± 2% (n = 4) of the radioactivity still eluted with the LDL peak. Treatment of the endothelium with fatty acid did not appreciably affect the distribution of the LDL radioactivity that passed through the monolayer. Although these findings indicate that some degradation was associated with LDL passage across the monolayer, most of the material that was transferred remained as LDL.

Figure 3 shows that the amount of 125I-LDL transfer was dependent on the oleic acid concentration to which the cultures were initially exposed. In these experiments, the cultures were exposed to medium containing oleic acid bound to albumin for 24 hours, and this medium then was removed. Subsequently, 125I-LDL transfer was measured during 1 hour of incubation in M-199 containing 125 µg LDL-protein per 0.5 ml medium. Additional experiments indicated that, although more LDL transfer occurred when the LDL concentration was raised from 125 µg to 250 µg LDL protein (2.4 ± 0.3 µg LDL protein to 4.4 ± 0.3 µg), the enhancement produced by prior exposure to 300 µM oleic acid persisted when the LDL concentration was 250 µg protein/ml (8.7 ± 0.9 µg LDL protein).

Other albumin-bound fatty acids were tested for their effect on LDL transfer across the endothelium. Figure 4 shows the amount of LDL transfer after endothelial cell monolayers were exposed to 200 µM...
Figure 2. Distribution of 125I-LDL radioactivity. After transfer experiments, samples were applied on Bio-Gel A-5m columns, and 1-ml fractions were eluted and assayed for radioactivity. The Bio-Gel A-5m column was equilibrated in a buffer containing 0.15 M NaCl and 0.3 mM EDTA, pH 7.4. Before application to the column, 0.1 ml of purified, unlabeled LDL was added to the sample. The 125I-LDL eluted with the LDL peak, measured by absorbance at 280 nm. The top shows a chromatogram of 125I-LDL from the upper chamber of a flask after a 1-hour incubation with endothelium. Only about 2% of the radioactivity eluted with the phenol red peak, fractions 28 to 32. The bottom half shows a chromatogram of 125I-LDL from the lower chamber after 1 hour, this material having passed across the endothelial monolayer. About 90% of the applied radioactivity eluted as LDL, and 10% eluted with the phenol red peak.

Figure 3. Effect of oleic acid exposure on the subsequent transfer of 125I-LDL across cultured porcine pulmonary artery endothelial monolayers. Before 125I-LDL transfer was measured, cells were incubated for 24 hours in M-199 containing 5% fetal bovine serum and 100 μM albumin with 0, 100, or 300 μM oleic acid. These media were removed and, after washing, 125I-LDL transfer was measured as indicated in Figure 1. Each value is the mean ± se, n = 6. The data were analyzed by Student’s t-test.

of fatty acids that differed in carbon number and degree of unsaturation. Linoleic acid was as effective as oleic acid in increasing LDL transfer across the endothelial monolayer during a subsequent 1-hour incubation. By contrast, exposure to palmitic, linolenic, arachidonic, or eicosapentaenoic acids did not increase LDL transfer. Similar differences between linoleic and linolenic acids were observed for transendothelial albumin transfer (data not shown). The amount of albumin transferred was 2.11 ± 0.17 nmol/hr (n = 6 for this and the other groups) when the endothelial cultures were incubated for an initial 24-hour period without supplemental fatty acids. Exposure for 24 hours to a medium containing 200 μM linoleic acid increased albumin transfer during a subsequent 1-hour incubation to 3.37 ± 0.38 nmol/hr. However, there was no increase in albumin transfer (2.09 ± 0.38 nmol/hr) when the cultures were exposed initially to 200 μM linolenic acid.

To determine whether exposure to fatty acids affected the paracellular pathway across the endothelium, we measured the transendothelial electrical resistance of monolayers exposed for 24 hours to 200 μM oleic acid bound to 100 μM albumin. The initial electrical resistance of the control monolayers (4.91 ± 0.26 ohm·cm², n = 11) and those subsequently exposed to oleic acid (4.90 ± 0.29 ohm·cm², n = 10) was the same. After exposure for 24 hours to 100 μM albumin, the electrical resistance of the control cultures did not change significantly (4.54 ± 0.16 ohm·cm²), whereas those exposed to oleic acid bound to albumin exhibited a 30% decrease in electrical resistance (3.40 ± 0.32 ohm·cm²).

Discussion

These results support the hypothesis that exposure to elevated concentrations of fatty acid can increase the passage of lipoproteins through the endothelial monolayer and thereby possibly contrib-
tions, and hence, high molar ratios, occur during
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tions indicate that oleic and linoleic acid, even when
bound to plasma albumin, increase LDL transfer
across cultured endothelial monolayers. Since this
effect was not produced by exposure to highly
polyunsaturated fatty acids such as linolenic, arach-
idonic, and eicosapentaenoic acids, it is unlikely that
the greater LDL transfer is caused by peroxidative
injury. This is consistent with our previous findings
that the incorporation of labeled leucine into total
LDL transfer was measured, cells were washed, 125I-LDL transfer was measured during a 1-hour incubation. Each bar represents the mean ± se n = 4.

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the greater LDL transfer is caused by peroxidative
injury. This is consistent with our previous findings
that the incorporation of labeled leucine into total
cell protein is not affected by incubation of the
cultures with 300 μM oleic acid, suggesting that there
is no change in endothelial cell viability due to fatty
acid exposure (Hennig et al., 1984). Furthermore,
the fatty acid effect on albumin transfer was found
to be totally reversible at 100 μM oleic acid and
partially reversible at 300 μM (Hennig et al., 1984).
Even though the free fatty acid concentrations pro-
ducing the increased LDL transfer are high from the
physiological standpoint, they are still reasonable,
relative to values that can occur in humans. Plasma
free fatty acid concentrations can range from 180–
1,650 μM (Fredrickson and Gordon, 1958), the usual
value in the basal state being about 300–500 μM
(Spector, 1975). Assuming a normal albumin concen-
tration of 600 μM, the molar ratio of free fatty
acid to albumin in human plasma can vary between
0.3 and 2.8. High plasma free fatty acid concentra-
tions, and hence, high molar ratios, occur during
severe stress (Knizta et al., 1978; Batt and Topping,
1979), uncontrolled diabetes (Galton et al., 1975;
Hall et al., 1979), starvation (Fredrickson and Gor-
don, 1958; Sawin and Willand, 1970), and after
prolonged exercise (Havel et al., 1963). In addition,
it is possible that the local concentration of fatty acid
generated at the endothelial surface during hydrol-
ysis of lipoprotein triglycerides may exceed these
levels.

Porcine pulmonary artery endothelial cultures
were used in this study. Although atherosclerosis
usually is localized to systemic arteries, pulmonary
atherosclerosis occurs in disease states where the
pulmonary circulation is exposed to systemic blood
pressures (Moore et al., 1982). Therefore, even
though these cultures are not derived from a more
prevalent site of atherogenesis, they probably can
provide some basic insight into mechanisms in-
volved in the atherosclerotic process.

LDL transfer across the endothelial monolayer in
this system is concentration dependent but not sat-
urable. Since the LDL concentrations tested are far
above those reported to saturate LDL receptor bind-
ing in other systems (Goldstein and Brown, 1974;
von Hinsburgh et al., 1983; Baker et al., 1984), it is
likely that most or all of the LDL transfer is not
receptor-mediated. There is evidence that intact LDL
can cross normal endothelium in vivo and enter the
subendothelial region of an artery (Walton, 1975;
Bratzler et al., 1977; Kurozumii et al., 1983). In
agreement with these findings, some LDL can be
recovered from the arterial wall when labeled LDL
is injected into humans (Scott and Hawley, 1970).
Recently, an increased and selective accumulation
of LDL has been observed in a damaged arterial
wall (Roberts et al., 1983). Our data suggest that
exposure of endothelium to a high concentration of
fatty acid might also facilitate the entry of LDL into
the arterial wall. The conductance measurements
indicate an alteration of the paracellular transfer
pathway. Although we have not actually demon-
strated that LDL penetrated the paracellular path-
way, this is a potential mechanism for the enhanced
transendothelial transfer of LDL produced by fatty
acid exposure. Such a pathological mechanism
would provide a potential link between fatty acid
elevations and atherogenesis, as has been postulated
previously (Zilversmit, 1973, 1976; Ross and Harker,
1976).

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