The Unstirred Water Layer as a Site of Control of Apolipoprotein B Secretion*

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MATERIALS AND METHODS

A variety of seemingly unrelated stimuli affects the output of apolipoprotein B-100 (apoB) by cultured liver cells. These include cholesterol depletion and enrichment (2), oleic acid supplementation (2-4), insulin (4-6), and the viscosity of the culture medium (7). Similar stimuli affect hepatic output of apoB in vivo including inhibition of cholesterol biosynthesis by the administration of lovastatin (8-10) and cholesterol enrichment (11) or apoB in vivo including inhibition of cholesterol biosynthesis by the administration of lovastatin (8-10) and cholesterol enrichment (11) or synthetic substitutes (12). None of the stimuli examined to affect apoB mRNA, indicating that regulation is post-transcriptional (4).

To study mechanisms of regulation of apoB output, we used

the human hepatoma G2 (HepG2) line (3), which secretes apolipoprotein B-100 and is influenced by many of the factors in vitro listed above (2-4).

HepG2 cells grown to subconfluence in 35-mm wells were incubated overnight in a serum-free medium that sustains their usual rate of growth (1:1 mix of Waymouth's MD7051 and minimum essential medium supplemented with L-glutamine, 10^-4 M sodium selenite (GIBCO), and 0.2% fatty acid-free bovine serum albumin (Sigma)). Typically, this medium was supplemented with either 10 μM lovastatin (LS) or a combination of 10 μg of 25-hydroxycholesterol plus 50 μg of cholesterol (HCC). The next morning, L-[4,5-3H]leucine (120-190 Ci/mmol, Amersham Corp.) was added directly to the media already on the cells (75 μCi/ml). After a 100-min incubation at 37 °C, media were harvested, carrier LDL (210-280 μg of unlabeled apoB) was added to each sample, and lipoproteins were isolated by centrifugation (220,000 × g for 37 h at 4 °C, d = 1.21 g/ml). ApoB was purified from the lipoproteins by the method of Egusa et al. (13), with minor modifications, and [3H]apoB radioactivity was measured by scintillation counting. Total secreted [3H]-protein was measured by scintillation counting of the pellet obtained from precipitation of media with trichloroacetic acid (14). Total cellular protein was determined by a modified Lowry procedure (15). Recovery of labeled apoB and protein from media was assessed by yield of carrier apoB and bovine serum albumin masses and averaged 68.2 and 104.8%, respectively. Data for apoB output by the cells were expressed as the percentage of total labeled secreted protein that was labeled apoB (i.e., 100 times [3H]apoB radioactivity divided by [3H]-protein radioactivity).

Several modifications were introduced during the 100-min incubation of LS- and HCC-treated cells with labeled [3H]leucine. In some experiments, a non-immune rabbit IgG or a monospecific polyclonal rabbit anti-25-hydroxycholesterol IgG that blocks human and bovine LDL receptor binding (16) was added simultaneously with the [3H]leucine to achieve a final concentration of 50 μg of IgG/ml of medium. The anti-LDL receptor blocking antibody was the generous gift of Dr. Karl Seelig of the Gladstone Foundation Laboratories.

In other experiments, the width of the unstirred water layer adjacent to the cells was varied during the 100-min incubation by either shaking the cells or increasing the viscosity of the medium by adding gelatin. To widen the unstirred water layer, cells that had been treated with LS or HCC were given gelatin as a 7.2% solution in warmed media supplemented with LS or HCC added with the [3H]leucine directly to the media already on the cells. The final concentration of gelatin was 3.0% (7). Control cells, which were neither shaken nor given gelatin, and shaken cells also received [3H]leucine and extra media but without gelatin. To narrow the unstirred water layer, cells that had been treated with LS or HCC were shaken during the 100-min incubation by a miniorbital rotatory shaker (340 rpm, average radius = 1.5 mm) that had been placed on top of a rocking platform (16 cycles/min) (17). After the 100-min incubation period, secreted [3H]apoB and total secreted [3H]-protein were assayed as described above, except that the lipoprotein centrifugation was at 25 °C for 60 h.

Results are given as mean ± S.E. Statistical comparisons were performed by Student's two-tailed t test.

RESULTS AND DISCUSSION

We sought a cell culture system that would duplicate the
effects that lovastatin and hepatic cholesterol enrichment have on apoB secretion in vivo. Our initial studies with HepG2 cells indicated that 10 μM lovastatin (LS) lowered cellular apoB output to 85.8 ± 3.0% of control (t = 3, p < 0.01), while a combination of 10 μg of 25-hydroxycholesterol and 50 μg of cholesterol/ml (HCC) increased apoB output to 142.5 ± 3.7%
of control ($n = 3, p < 0.0001$). Cellular protein mass and total secretion of \(^{3}H\)-protein were not significantly different among the groups of cells. Our subsequent studies focused on the large difference in apoB output between LS and HCC cells.

Many of the factors that change apoB output by hepatocytes, including lovastatin and cholesterol, have been shown to produce reciprocal changes in cellular LDL receptors (2, 18, 19). Previous studies in vitro have examined the possibility of receptor-mediated re-uptake of secreted particles as an explanation for this inverse correlation. However, all have used the indirect technique of determining cellular uptake of exogenously supplied lipoproteins and have concluded that re-uptake does not occur (2, 5, 7, 20). In contrast to this approach, we directly blocked the uptake by HepG2 cells of their own secreted particles.

Anti-LDL receptor blocking antibody (16) abolished most of the difference in apoB output between LS and HCC cells (Fig. 1). In the presence of non-immune rabbit IgG, the output of apoB from LS cells was 45.0 ± 2.6% less than the output from HCC cells. In the presence of polyclonal rabbit anti-LDL receptor-blocking antibody, the output from LS cells was only 19.3 ± 2.6% less than the output from HCC cells. These results indicate that most, if not all, of the difference in apoB output between LS and HCC cells depends on the presence of unblocked receptors. The difference in output was reduced because the blocking antibody was associated with a large increase in the absolute output from the LS cells (Fig. 1), suggesting substantial re-uptake of secreted apoB. The HCC cells showed a small but statistically insignificant rise in apoB output in the presence of blocking antibody (Fig. 1), suggesting minimal re-uptake.

To reconcile our data with the prior literature demonstrating no re-uptake of secreted particles, we repeated a published experiment using exogenous lipoproteins (20). Nascent lipoproteins that contained \(^{3}H\)apoB were prepared by incubating a set of HepG2 cells with \(^{3}H\)leucine. Unincorporated \(^{3}H\)leucine was then removed from the culture medium by extensive dialysis. Aliquots of this conditioned dialyzed medium were placed directly, without further processing, onto a set of unlabeled HepG2 cells for a second incubation. The content of \(^{3}H\)apoB in the conditioned dialyzed medium before and after the second incubation was assayed by ultracentrifugation and isopropyl alcohol precipitation (13). As previously reported (20), there was no statistically significant loss of labeled apoB from the media during the second incubation. Note that similar results have been reported for plasma lipoproteins; only a small fraction of the total amount typically incubated for several hours with a monolayer of cultured cells is taken up and degraded (see, for example, p. 246 of Ref. 21).

Although this type of experiment seems to disprove significant re-uptake of newly secreted lipoproteins, it actually examines events only in the bulk fluid phase of the incubation media. Ongoing secretion of \(^{3}H\)apoB could, however, be plausibly associated with a high local concentration of \(^{3}H\)apoB in the unstirred water layer adjacent to the cultured cells. Local accumulation of secreted particles would facilitate receptor-mediated re-uptake.

To test the involvement of the unstirred water layer in the regulation of apoB secretion by HepG2 cells, we sought to vary the width of this layer (17). To narrow the unstirred water layer, HepG2 cells preincubated with LS or HCC were shaken during the labeling period (Fig. 2). Shaking increased the apparent secretion rate of \(^{3}H\)apoB from LS cells by 35.5 ± 3.1% ($n = 4, p < 0.0005$) compared with unshaken LS cells. Shaking produced only a 4.9% (not significant) rise in the output from HCC cells, again suggesting that minimal re-uptake occurs in these cells under ordinary conditions (cf. Fig. 1).

To widen the unstirred water layer, we supplemented the media with 3% gelatin during the labeling period (7). Gelatin reduced the net output of apoB from LS and HCC cells (Fig. 2; cf. Ref. 7). This result suggests that severely restricted...
diffusion of nascent particles allows substantial re-uptake by both sets of cells. An ability of HCC cells to take up their own secreted particles is consistent with the observation that HepG2 cells, unlike fibroblasts, only incompletely suppress their LDL receptors after supplementation with cholesterol or 25-hydroxysterol (22, 23). Uptake independent of LDL receptors (24, 25) may also play a role. Neither shaking nor gelatin affected the secretion of total 3H-protein (Fig. 3). Gelatin was previously shown to have no effect on the secretion of albumin (7).

Overall, our data indicate that there is a significant involvement of the unstirred water layer in the regulation of apoB output by cultured hepatocytes. This involvement is likely to be important in vivo because the unstirred water layer around hepatocytes in the liver is anatomically protected in the space of Disse, a fluid filled gap between hepatocytes and vascular endothelial cells.

Although substantial re-uptake of newly secreted lipoproteins has generally been discounted (2, 5, 7, 20), there are several reports that nascent particles are, in fact, taken up by cells. Nascent very low density lipoprotein from one set of cells is rapidly taken up by a second set of perfused livers (26). Nascent hepatic very low density lipoprotein also stimulates cholesterol esterification in cultured macrophages (27).

Regulation of hepatic apoB output by local re-uptake suggests that "reverse" transport of cholesterol from peripheral tissues to the liver by high density lipoproteins (37, 38) or synthetic substitutes (39) may not necessarily be anti-atherogenic. Cholesterol delivery may provoke suppression of hepatic LDL receptors (1), which would reduce re-uptake of nascent lipoproteins and thereby enhance total hepatic output of atherogenic apoB-rich particles (cf. Refs. 11 and 12). To be salutary, "reverse" transport may require concomitant cholesterol depletion of the liver.

In summary, our results indicate that the unstirred water layer, though not internal to the cell, is nonetheless an integral metabolic part. It is analogous to the front porch of a house.

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Fig. 3. Effect of varying the width of the unstirred water layer on the total 3H-protein output from HepG2 cells treated with LS or HCC (HC&C). Data are from the cells described in Fig. 2. Means ± S.E. are shown, n = 3. Outputs of total 3H-protein into the media were not significantly affected by shaking or gelatin.
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