Heparin releases newly synthesized cell surface-associated apolipoprotein E from HepG2 cells

Martha Lilly-Stauderman,* Thomas L. Brown,* Ambikaipakan Balasubramaniam,§ and Judith A. K. Harmony[1,*,†]

Developmental Biology Graduate Program* and Division of Lipoprotein Research, Department of Pharmacology and Cell Biophysics,† and Department of Surgery,§ College of Medicine, University of Cincinnati, Cincinnati, OH 45267-0575

Abstract Heparin significantly increased the amount of newly synthesized apolipoprotein E (apoE) released by HepG2 cells. Culturing cells in the presence of 10 μg/ml of heparin for 2–6 days caused a 1.3-fold (day 2) to 3-fold (day 6) increase of extracellular apoE without affecting the total amount of apoE synthesized by the cells. The amounts of apoA-I and apoB produced by HepG2 cells were unaffected by heparin. Surprisingly, short-term treatment with heparin (15–30 min) also increased extracellular apoE by 2- to 3-fold. In this situation, heparin exerted its effect on apoE post-translationally. Among glycosaminoglycans (GAGs), only heparan sulfate mimicked heparin at a concentration of 10 μg/ml; hyaluronic acid and the chondroitin sulfates were effective only at a higher concentration (100 μg/ml). Extracellular apoE was not increased by treating cells with anti-apoE antiserum or a heparin-binding peptide of apoE (amino acids 130–169). Removal of cell surface-associated GAGs by culturing cells in 4-methylumbelliferyl-β-D-xyloside ablated the effect of heparin on apoE. ApoE was released from cells by treatment with heparinases I and III, but not by chondroitinase ABC. The results provide evidence that a heparin-releasable pool of newly synthesized apoE is associated with cell surface GAGs that resemble heparin and/or heparan sulfate. **—Lilly-Stauderman, M., T. L. Brown, A. Balasubramaniam, and J. A. K. Harmony.** Heparin releases newly synthesized cell surface-associated apolipoprotein E from HepG2 cells. *J. Lipid Res.* 1993. **34:** 190–200.

Supplementary key words glycosaminoglycan • 4-methylumbelliferyl-β-D-xyloside • heparinase • heparan sulfate • extracellular matrix • lipoprotein

Apolipoprotein E (apoE) is a 34 kDa plasma glycoprotein that functions in lipoprotein and cholesterol metabolism (1). As a component of lipoprotein particles, it facilitates the transport of cholesterol from the intestine to the liver and from the liver to peripheral tissues. While apoE mRNA has been detected in almost every tissue tested, 65–75% of total plasma apoE is derived from the liver (2–4). Since many cell types, including macrophages, synthesize apoE, it is likely that apoE also functions in a local environment to facilitate lipid transfer from lipid-rich cells to lipid-poor cells. This model, first proposed to explain the redistribution of cholesterol during nerve injury and regeneration (5, 6), is applicable to tissue injury and repair in general, with apoE providing cholesterol to cells that require it for membrane biogenesis and proliferation.

One important aspect of tissue injury and repair is the contribution made by the extracellular matrix (ECM). The ECM serves as a supportive scaffold for cells, and also provides a reservoir of noncollagenous glycoproteins (e.g., fibronectin, vitronectin, laminin) that mediate cell adhesion and migration during growth and differentiation (7, 8). The glycosaminoglycan (GAG) components of the ECM can also regulate proliferation of cells such as smooth muscle cells (SMC) (8–11). Heparin not only inhibits SMC proliferation but also alters their secretory profile and, depending on the growth state of the cells, causes a dramatic and rapid accumulation of apoE in the culture medium (12, 13).

Glycosaminoglycans in the ECM can alter cell phenotype through regulation of gene expression (8). Heparin and heparan sulfate, the most abundant GAGs in the liver ECM, regulate the synthesis of specific liver proteins. Reid et al. (14–16) demonstrated that, in collaboration with certain hormones, heparin and heparan sulfate proteoglycans stimulate the synthesis of gap junction proteins and other tissue-specific proteins in primary hepatocytes

Abbreviations: apo, apolipoprotein; BSA, bovine serum albumin; ECM, extracellular matrix; EMEM, Eagle's minimal essential medium; FBS, fetal bovine serum; GAG, glycosaminoglycan; LDL, low density lipoproteins; SDS, sodium dodecyl sulfate; SMC, smooth muscle cells; HTGL, hepatic triglyceride lipase; FGF, fibroblast growth factor.

[1]To whom correspondence should be addressed.

and hepatoma cells. Busch et al. (17) found that heparin stimulates hepatic triglyceride lipase (HTGL) synthesis in HepG2 cells. These effects result from long-term exposure to heparin and involve both transcriptional and post-transcriptional regulatory mechanisms.

The existence of specific heparin binding domains in apoE (18–20) and the ability of heparin to augment apoE production in SMC suggest that heparin can influence apoE production in other cell types. Because *1)* heparin is abundant in liver ECM; *2)* the liver is the primary site of apoE synthesis; and *3)* heparin specifically regulates the synthesis of other liver-specific proteins, we investigated the possibility that heparin modulates the production of apoE by the liver. We used the human hepatoma cell line HepG2, which has been established as an appropriate model for studying hepatic apoE production. Zannis et al. (21–23) have characterized the synthesis, intracellular modification, and secretion of apoE by these cells. Using HepG2 cells, we found, unexpectedly, that heparin causes a rapid increase in the amount of apoE secreted. This effect of heparin on apoE occurs post-translationally, and we provide evidence that the heparin-sensitive pool of apoE is associated with the cell surface by interaction with a heparin-like GAG.

EXPERIMENTAL PROCEDURES

Cell culture

The human hepatoma cell line HepG2 (24) was purchased from American Type Culture Collection. The cells were cultured at 37°C in a humidified 5% CO_2 atmosphere. Cells were plated in T25 flasks (Corning, Costar) at a density of 1×10^6 cells per flask and maintained in Eagle's minimal essential medium (EMEM, Whittaker) containing 10% fetal bovine serum (FBS) (Hazleton) and 4 mM L-glutamine (Sigma). For serum-free culture prior to metabolic labeling, cells were switched to EMEM supplemented with 4 mM L-glutamine, 10 μg/ml of transferrin, 5 μg/ml of catalase, 0.01 mM 2-mercaptoethanol, 0.5 μg/ml of linoleic acid, 1.13 mg/ml of bovine serum albumin (BSA), and 0.5 μg/ml of insulin.

Cells were labeled with [^{35}S]methionine (>1000 Ci/mmol, Amersham) in methionine-free EMEM (Flow Laboratories) supplemented as for serum-free culture. For pulse-chase experiments, the "chase" medium was serum-free medium supplemented with 150 μg/ml of methionine. For long-term treatment, heparin (porcine intestinal mucosa, Sigma) was added when the cells were plated, and fresh medium was added each day. Cells treated with 4-methylumbelliferyl-β-D-xyloside (β-D-xyloside) were plated in serum-supplemented medium and grown for 24 h. β-D-Xyloside was dissolved in DMSO (final concentration, 670 mM) and added to the culture medium. Cells were grown for 3 days and then switched to serum-free medium with β-D-xyloside for an additional 24 h prior to labeling with ^{35}S (also in the presence of β-D-xyloside). Total cell protein was determined, subsequent to cell lysis in 1 N NaOH, by the method of Lowry et al. (25).

Metabolic labeling

Cells were switched to serum-free medium 18–24 h prior to labeling. For long-term heparin treatment, heparin was included in this medium. Cells were then washed with, and subsequently incubated in, 2 ml of serum-free/methionine-free medium. For short-term treatment with heparin, other GAGs (Sigma), anti-apoE antiserum or apoE peptide, the additive was included in this medium. After 15 min at 37°C, 200 μCi of [^{35}S]methionine was added for 5 or 30 min, unless stated otherwise. For experiments with heparinase I, heparinase III, or chondroitinase ABC (Sigma), the enzyme was included in the serum-free/methionine medium, and the cells were labeled for 45 min. In pulse-chase experiments, cells were washed with chase medium and then incubated in 1.5 ml of chase medium. For experiments measuring the uptake of [^{35}S]apoE, cells were labeled for 30 min, and then incubated with chase medium for 30 min in the absence or presence of 10 μg/ml of heparin. To assay uptake, labeled chase medium was incubated with unlabeled cells grown in parallel. Culture media were collected and brought to a final concentration of 20 mM Tris-PO_4, pH 8, containing 5 mM Na_2EDTA, 0.5% deoxycholate, 0.5% Triton X-100, 1 mM benzamidine, 1 mM PMSF, 1 μM aprotinin, and 1 mg/ml of BSA. Cells were lysed on ice in 0.5% deoxycholate, 0.5% Triton X-100, 20 mM Tris-PO_4, pH 8, containing 5 mM Na_2EDTA, 2 mM benzamidine, 1 mM PMSF, and 1 μM aprotinin. The lysates were centrifuged at 500 *g* to pellet cell debris, and the supernatants were adjusted to a final concentration of 0.004% NaN_3, 20 mM Tris-PO_4, pH 8, 1 mg/ml of BSA, 1 mM Na_2EDTA, and protease inhibitors as above.

Quantitation of apoE

ApoE was immunoprecipitated from culture media and cell lysates, using rabbit antiserum to human apoE. For antiserum preparation, human plasma apoE, isoform E3/3, was purified, delipidated (26), and electrophoresed under reducing conditions on an 11% preparative SDS polyacrylamide gel (27). The protein was identified by staining with 1% Coomassie Blue R-250 in water. The band corresponding to apoE was excised; apoE was electroeluted in a Schleicher and Schuell Elutrap at 100 v for 18 h in 1X TBE (40 mM Tris, 40 mM boric acid, 0.5 mM Na_2EDTA). The purity of the protein was confirmed by amino acid analysis, amino-terminal sequencing, and 2-D gel electrophoresis (28). Female New Zealand White rabbits (4–6 kg) were initially injected with 50 μg of purified antigen in MPL-TDM emulsion (Ribi Immunochemical)

in 1 ml of saline. Subsequently, the rabbits were injected (25 μg, MPL-TDM emulsion, 1 ml saline) or bled on alternating weekly intervals. Antiserum was titered, and its specificity for apoE was confirmed by Western blotting and by immunoprecipitation of purified apoE. In some experiments, apoA-I and apoB were immunoprecipitated as controls, using sheep antisera to human apoA-I and human apoB (The Binding Site, Inc.).

Antiserum and GammaBind G Agarose (Genex), included as the secondary antibody, were added to cell lysates or culture media, and the mixtures were incubated overnight at 4°C in the presence of 0.1 mg/ml of human albumin. The precipitates were washed: *1)* twice in 20 mM Tris-PO_4, pH 8, containing 2 mM Na_2EDTA, 140 mM NaCl, 1 mg/ml of BSA, 0.5% deoxycholate, 0.5% Triton X-100, and protease inhibitors; *2)* twice in 100 mM Tris-PO_4, pH 8, containing 630 mM NaCl, 2 mM Na_2EDTA, and protease inhibitors; and *3)* twice in 100 mM Tris-PO_4, pH 8, containing 1 mM Na_2EDTA, 0.02% SDS, 150 mM NaCl, and protease inhibitors. The pellets were boiled in sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 40 μg/ml of bromophenol blue, 1% 2-mercaptoethanol) for 3 min, and the supernatants were analyzed on 11% acrylamide gels (apoE and apoA-I) or 3–17% gradient polyacrylamide gels (apoB). ^{35}S-Labeled apolipoproteins were visualized by fluorography, using Enlightning (DuPont). In some experiments, the bands were excised, soaked in 2 ml of H_2O_2 at 55°C overnight, and counted by liquid scintillation spectrometry in 20 ml of Scintiverse (Fisher). For some experiments, immunoprecipitated pellets were boiled in sample buffer without bromophenol blue and glycerol, and an aliquot of each supernatant was counted in 5 ml of Scintiverse. Total protein secretion was quantitated by precipitation of ^{35}S-labeled proteins with trichloroacetic acid. Determination of *P* values was done by the Student's *t* test.

Peptide synthesis

The peptide Thr-Glu-Glu-Leu-Arg-Val-Arg-Leu-Ala-Ser-His-Leu-Arg-Lys-Leu-Arg-Lys-Arg-Leu-Leu-Arg-Asp-Ala-Asp-Asp-Leu-Gln-Lys-Cys-Leu-Ala-Val-Tyr-Gln-Ala-Gly-Ala-Arg-Glu-Gly, representing amino acids 130–169 of apoE, was synthesized as described in Diccianni et al. (29).

RESULTS

Initially, HepG2 cells were grown for 6 days in the absence or presence of heparin, as previous studies (14–17) have shown that the effects of heparin on liver cells require long exposures. ApoE secretion was assayed each day by pulse-labeling and quantitating [^{35}S]apoE in the culture medium by immunoprecipitation with an apoE-specific rabbit antiserum. Pre-immune serum did not precipitate apoE, and heparin did not enhance the immunoprecipitation of apoE from control cell medium (data not shown).

Heparin increased the amount of extracellular [^{35}S]apoE at each time point (**Fig. 1A, B**). The optimum heparin concentration was 5–10 μg/ml. In contrast, 1 μg/ml of heparin had no effect, and there was no increased effect above 10 μg/ml (data not shown). Heparin exerted an increasingly greater effect with increasing time of cell culture. After 2 days, 10 μg/ml of heparin caused a 1.3-fold increase in extracellular apoE; after 6 days, heparin increased extracellular apoE by 3-fold. Exposure of cells to 10 μg/ml of heparin for 24 h had no effect on apoE mass in the cell medium (19.5 ± 0.7 μg/ml for control cells vs. 19.0 ± 1.4 μg/ml for heparin-treated cells, as determined by ELISA).

The heparin-induced increase in extracellular [^{35}S]apoE produced by HepG2 cells did not reflect an influence of heparin on cell growth or protein synthesis. Heparin, at concentrations ≤10 μg/ml, had little effect on the proliferation of HepG2 cells (**Fig. 2A**). Concentrations of heparin as high as 500 μg/ml also failed to influence proliferation

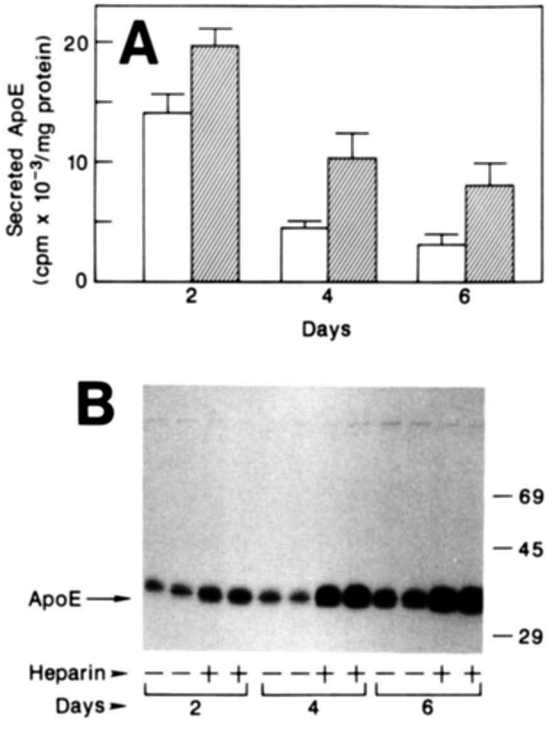

Fig. 1. Long-term treatment of HepG2 cells with heparin causes an increase of extracellular apoE. HepG2 cells were cultured for 6 days without or with heparin. Each day a group of cells was labeled for 30 min with [^{35}S]methionine. ApoE was immunoprecipitated from culture medium, analyzed by gel electrophoresis, and quantitated by elution of the gel bands. A) Quantitative data from cells treated with 10 μg/ml of heparin. Open bars: control cells; striped bars: heparin treated cells. B) Fluorograph of apoE isolated from cells treated with 10 μg/ml of heparin. Molecular weight markers (kDa) are indicated.

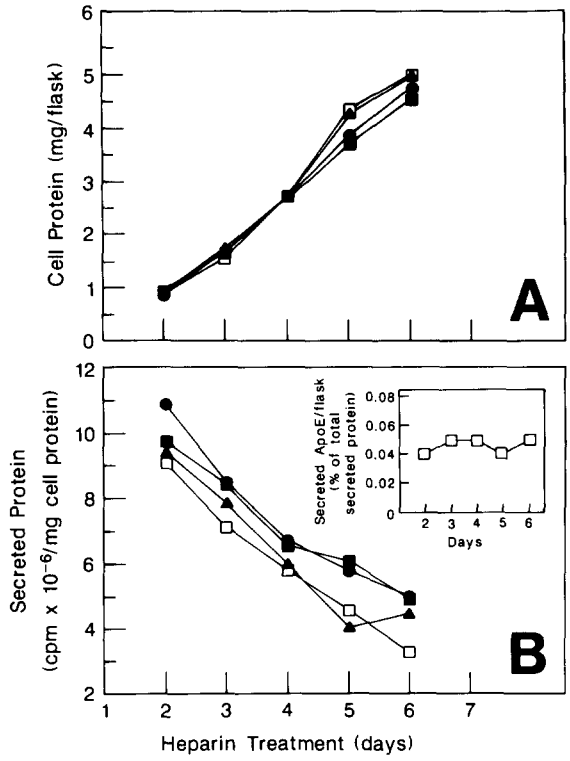

Fig. 2. Heparin does not cause general stimulation of cell growth or protein secretion. Cells were cultured without or with heparin, as described in Experimental Procedures. A) At each timepoint, cells were washed in phosphate-buffered saline, and total cell protein was determined. B) At each timepoint, cells in a parallel group of flasks were labeled, as described in the Fig. 1 legend. Protein was precipitated from an equal volume of each medium fraction to determine total secreted protein. Heparin concentrations: (□–□) 0 μg/ml; (▲–▲) 1 μg/ml; (●–●) 5 μg/ml; (■–■) 10 μg/ml. Insert: At each time point, [^{35}S]apoE was immunoprecipitated from medium of untreated cells, quantitated, as described in the Fig. 1 legend, and compared to total secreted ^{35}S-labeled protein.

(data not shown). At concentrations of 5 and 10 μg/ml, heparin caused only a slight increase in total secreted protein (Fig. 2B). The amount of apoE secreted per cell decreased with time, as did the amount of total secreted protein. The ratio of secreted [^{35}S]apoE/total secreted protein remained relatively constant over 6 days (Fig. 2B, insert).

Because heparin causes a rapid increase of extracellular [^{35}S]apoE in SMC (12, 13), we evaluated the temporal requirements for heparin's effect on apoE produced by HepG2 cells. The stimulatory effect of heparin on [^{35}S]apoE was rapidly reversible. It was ablated by removing heparin during the 30-min labeling period after 6 days of heparin treatment (data not shown), suggesting a rapid response to heparin. To confirm that the effect of heparin occurs rapidly, its effect on cells grown in the absence of heparin was evaluated. HepG2 cells were cultured for 4 days without heparin. On day 4, heparin (10 μg/ml) was added to the cells 15 min prior to [^{35}S]methionine, and the cells were labeled for 30 min in the presence of heparin. Heparin increased the amount of extracellular [^{35}S]apoE by 2- to 3-fold (**Fig. 3,** open bars). Because the effect of long-term heparin treatment was readily reversible, and because an equivalent increase of apoE resulted from short-term exposure to heparin (Fig. 1 vs. Fig. 3), we focused on the mechanism of this rapid release of [^{35}S]apoE by heparin.

We first asked whether heparin increases extracellular [^{35}S]apoE by stabilizing the protein in the medium. We assayed the turnover of extracellular [^{35}S]apoE in the presence and absence of heparin. ^{35}S-labeled proteins, produced by HepG2 cells in the presence or absence of heparin, were incubated with unlabeled cells grown in parallel. Uptake of [^{35}S]apoE was measured by immunoprecipitation of medium and cell lysate fractions. The results in **Fig. 4** show that over time the amounts of [^{35}S]apoE that remain in the medium or become associated with the cells are virtually identical in the absence and presence of heparin, demonstrating that heparin does not influence the turnover of apoE. We also monitored the amount of [^{35}S]apoE that adheres nonspecifically to a T25 culture flask and found this value to be identical in the absence or presence of heparin (data not shown).

We tested the specificity of the heparin effect. Heparin had no effect on the amount of secreted [^{35}S]apoA-I, an apolipoprotein that does not bind heparin (Fig. 3, striped bars). Likewise, heparin had no effect on secreted

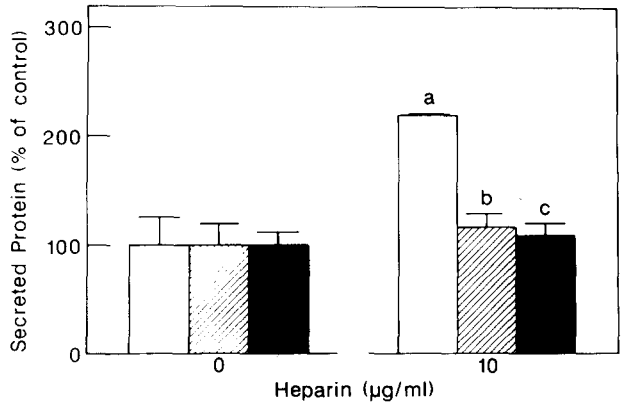

Fig. 3. Short-term treatment of HepG2 cells with heparin increases extracellular apoE but not apoA-I or apoB. Cells were cultured for 4 days without heparin. On day 4, heparin (10 μg/ml) was added to cells 15 min prior to labeling, and the cells were labeled with [^{35}S]methionine. The labeling period was 30 min to compare apoE with apoA-I and 2 h to compare apoE with apoB. Secreted apoE and apoA-I were quantitated by immunoprecipitation and subsequent counting of boiled supernatants, while apoB was quantitated by immunoprecipitation, gel electrophoresis, and elution of gel bands, as described in Experimental Procedures. Percent of control was determined for each data point by the formula: cpm secreted apoX/average control cpm for apoX, where X = E, A-I, or B and control refers to cells not exposed to heparin. Open bars: apoE; striped bars: apoA-I; solid bars: apoB; [a] indicates $P = 0.013$; [b] indicates $P = 0.405$; [c] indicates $P = 0.536$.

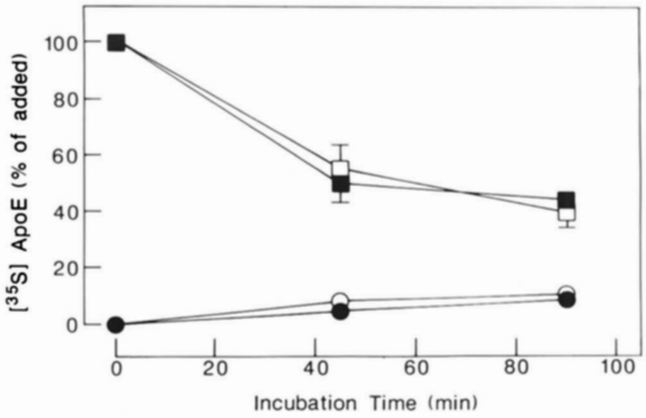

Fig. 4. Heparin does not interrupt the turnover of [^{35}S]apoE. Cells were cultured without heparin. On day 4, cells were labeled for 30 min with [^{35}S]methionine, and then chased with cold methionine for 30 min in the absence or presence of 10 μg/ml of heparin as described in Experimental Procedures. ^{35}S-labeled chase medium was removed from the cells and immediately added to unlabeled cells grown in parallel. The uptake of [^{35}S]apoE was measured by immunoprecipitation of medium and cell lysate fractions at each timepoint. The immunoprecipitates were quantitated by gel electrophoresis and elution of gel bands as described. The amount of [^{35}S]apoE that was cell-associated or present in the medium at each timepoint was expressed as a percent of the total cpm of [^{35}S]apoE added to the unlabeled cells at time zero. (□—□) cell medium without heparin; (■—■) cell medium with heparin; (○—○) cell lysate without heparin; (●—●) cell lysate with heparin.

[^{35}S]apoB, an apolipoprotein that does bind heparin (Fig. 3, solid bars). When other GAGs (10 μg/ml) were substituted for heparin, heparan sulfate caused a significant increase in extracellular [^{35}S]apoE (**Fig. 5**). Hyaluronic acid and the chondroitin sulfates had no significant effect; these GAGs increased extracellular [^{35}S]apoE only when present at the much higher concentration of 100 μg/ml (data not shown). To test further the specificity of the heparin effect, the amounts of extracellular [^{35}S]apoE obtained by incubating cells with anti-apoE antiserum or a synthetic peptide (amino acids 130–169) containing a heparin-binding domain (amino acids 142–147) of apoE were measured (**Fig. 6**). Anti-apoE antiserum, incubated with cells in an amount equivalent to that used for immunoprecipitation, did not increase [^{35}S]apoE in the culture medium. Likewise, addition of 5 μg/ml (Fig. 6) or 35 μg/ml (data not shown) of $apoE_{130-169}$ did not increase extracellular [^{35}S]apoE.

The rapid and reversible nature of heparin's effect on [^{35}S]apoE suggests a post-translational mechanism. In this event, heparin should not be required during apoE's synthesis to increase extracellular apoE. To test this possibility, cells were labeled without heparin for 5 to 30 min, and heparin was added with excess unlabeled methionine during the chase period. Heparin significantly increased extracellular [^{35}S]apoE after a 5-min pulse-label with a 10-min chase (**Fig. 7**). In fact, at each subsequent time point, heparin caused an increase in [^{35}S]apoE. Similar results were obtained after a 30-min label (data not shown). The total amount of [^{35}S]apoE produced by the cells (intracellular + extracellular) was not significantly affected by heparin. The amount of [^{35}S]apoE (cpm × 10^{-3}/T25 flask) in control cells after 20, 45, and 120 min of chase was 93.7 ± 1.04, 118.2 ± 10.5, and 85.9 ± 10.1, respectively. The corresponding values for heparin-treated cells were 77.3 ± 5.1 (P = 0.02 compared to control),

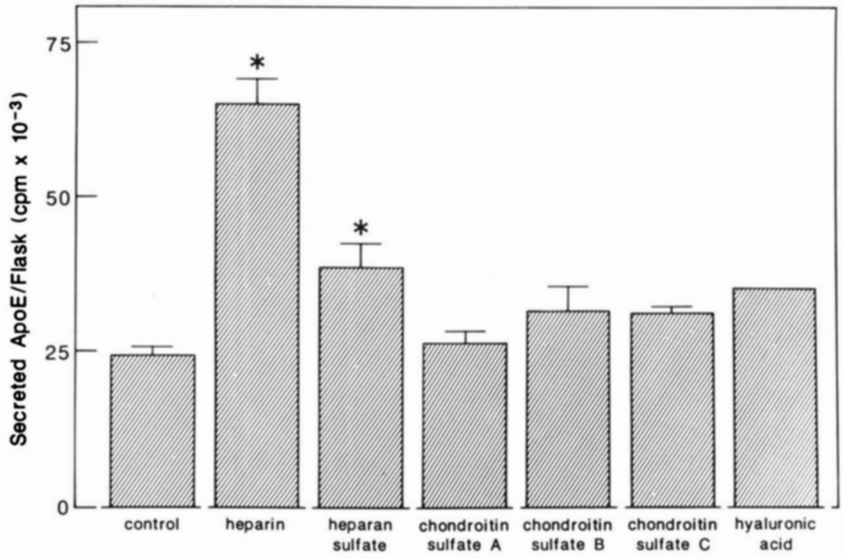

Fig. 5. Extracellular apoE is increased by heparin and heparan sulfate and not by other GAGs. Cells were cultured for 4 days without GAGs. On day 4, the indicated GAGs (10 μg/ml) were added to cells 15 min prior to labeling, and the cells were labeled for 30 min with [^{35}S]methionine. Secreted apoE was quantitated by immunoprecipitation and subsequent counting of boiled supernatants, as described in Experimental Procedures; * indicates $P < 0.01$ as compared to control value.

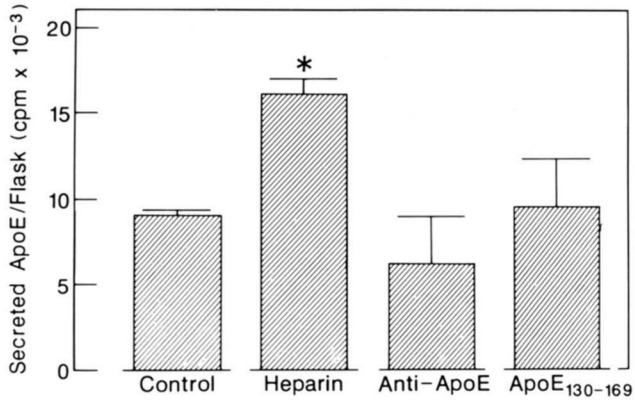

Fig. 6. ApoE is not released into the cell medium by anti-apoE antiserum or a heparin-binding apoE peptide. Cells were cultured for 4 days without heparin. On day 4, heparin (10 μg/ml), anti-apoE antiserum (anti-apoE, 100 μl/ml) or apoE peptide 130–169 ($apoE_{130-169}$, 5 μg/ml) were added to cells 15 min prior to labeling, and the cells were labeled for 30 min with [^{35}S]methionine. ApoE was immunoprecipitated from culture medium, analyzed by gel electrophoresis, and quantitated by elution of the gel bands. Additional antiserum was not included in the immunoprecipitation of medium from cells labeled in the presence of antiserum; • indicates $P < 0.01$.

108.1 ± 17 ($P = 0.52$) and 107.05 ± 15.7 ($P = 0.18$), respectively.

The rapidity of the heparin effect combined with its selectivity is consistent with the association of apoE with a heparin-like GAG on the HepG2 cell surface. Therefore, cells were treated with GAG-specific lyases to determine whether removal of cell surface GAGs releases [^{35}S]apoE into the cell medium. Heparinase III (heparitinase) and chondroitinase ABC, which preferentially cleave heparan sulfate (30) and chondroitin sulfates (31), respectively, were substituted for heparin during the labeling period, and extracellular [^{35}S]apoE was quantitated. Heparinase III significantly increased extracellular [^{35}S]apoE, albeit to a lesser extent than heparin (**Table 1**). Chondroitinase ABC was much less effective. Heparinase I, which preferentially cleaves heparin but displays low activity toward heparan sulfate (30), increased [^{35}S]apoE to the same extent as did heparinase III, but was ineffective if boiled prior to incubation with cells (data not shown).

If apoE is associated with a membrane GAG, reduction of GAG synthesis should reduce the extent of the heparin effect on [^{35}S]apoE. Cells were treated for 4 days with 4-methylumbelliferyl-β-D-xyloside (β-D-xyloside), a compound that can substitute for the protein core moiety during proteoglycan synthesis, significantly reducing the appearance of proteoglycan at the cell surface (32–34). Subsequently, the cells were labeled with [^{35}S]methionine in the absence or presence of heparin, and [^{35}S]apoE was quantitated in the cell medium. Incubating cells for 4 days with 1 mM β-D-xyloside significantly reduced the amount of [^{35}S]apoE releasable by heparin (**Fig. 8**). Concentrations of β-D-xyloside < 1 mM were less effective, and treatment with 1 mM β-D-xyloside for < 4 days had no effect on apoE (data not shown). β-D-Xyloside (1 mM) had no effect on the growth of HepG2 cells, as determined by measuring total cell protein (2.73 ± 0.15 mg protein/flask for control cells vs. 2.57 ± 0.12 mg protein/flask for xyloside-treated cells). DMSO, used to dissolve β-D-xyloside, had no effect on cell growth or secretion of [^{35}S]apoE.

DISCUSSION

Short-term treatment of HepG2 cells with heparin increases extracellular [^{35}S]apoE by 2- to 3-fold. Heparan sulfate mimics heparin, but other GAGs are ineffective, except at high concentrations. Among apolipoproteins, this effect may be specific for apoE as extracellular [^{35}S]apoA-I and [^{35}S]apoB are not increased by heparin. The rapidity and reversibility of heparin's effect on apoE indicate that heparin acts post-translationally, but the mechanism does not involve stabilization of apoE in the cell medium. Culturing cells with β-D-xyloside ablates the effect of heparin. Treating cells with heparinase III releases [^{35}S]apoE into the cell medium. [^{35}S]ApoE is not released by incubating cells with anti-apoE antiserum or a heparin-binding peptide of apoE. Thus, our results provide good evidence that heparin displaces a pool of apoE that is bound to the cell surface by interaction with heparin-like GAGs. This pool of apoE appears to be comprised of newly synthesized protein as heparin does not

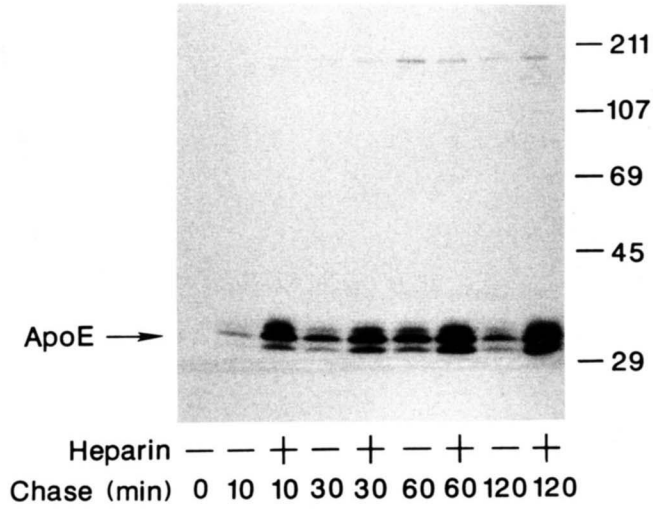

Fig. 7. Heparin stimulates the increase of apoE by a post-translational mechanism. Cells were cultured without heparin for 3 days, labeled without heparin for 5 min with [^{35}S]methionine, and chased in the absence or presence of heparin (10 μg/ml) for 0–120 min. ApoE was immunoprecipitated from the culture medium and analyzed by gel electrophoresis and fluorography, as described in Experimental Procedures. Molecular mass markers (kDa) are indicated.

TABLE 1. Extracellular apoE is increased by treatment of cells with heparinase III

Heparin	Heparinase III	Chondroitinase ABC	ApoE Secreted/Flask
10 μg/ml	*U/ml*	*U/ml*	*cpm* × 10^{-3}
–	–	–	32 ± 5.8
+	–	–	106 ± 17[a]
–	0.1	–	45 ± 5.5[a]
–	1.0	–	54 ± 4.9[a]
–	–	0.1	42 ± 6.8
–	–	1.0	43 ± 8.6[b]

Cells were cultured for 4 days, then labeled with [^{35}S]methionine for 45 min in the presence of 10 μg/ml heparin or 0–1 U/ml enzyme. ApoE was quantitated in the culture medium by immunoprecipitation and subsequent counting of boiled supernatants, as described. Data are expressed as average ± standard deviation.

[a] $P \leq 0.005$ as compared to untreated cells.

[b] $P = 0.016$ as compared to value for 1 U of heparinase III.

affect the total amount of apoE as mass in medium conditioned for 24 h by HepG2 cells.

The effect of heparin on nascent secreted apoE is rapid, reminiscent of the rapid release by heparin of HTGL and lipoprotein lipase from the endothelium (35–38). In contrast, heparin regulation of HTGL and gap junction protein synthesis in liver cells requires long-term treatment. Although released [^{35}S]apoE increases after 1–6 days of heparin exposure, an increase of the same magnitude occurs when HepG2 cells are exposed to heparin for only 15 min. In pulse-chase analysis, addition of heparin only during the chase period after [^{35}S]apoE has been synthesized increases extracellular [^{35}S]apoE without affecting the total amount of apoE made by the cells. Thus, in the short term, heparin can increase extracellular [^{35}S]apoE by a post-translational mechanism. The increase of [^{35}S]apoE after long-term heparin treatment may occur by this same mechanism, or it may involve a combination of transcriptional and post-transcriptional mechanisms such as those reported by Busch et al. (17) who observed a 1.5-fold increase in apoE mRNA after 44 h of exposure of HepG2 cells to heparin. Our experiments do not address this distinction.

Our results suggest that heparin increases extracellular [^{35}S]apoE by rapid release of the protein from interaction with cell surface GAGs. The release of apoE by heparin is virtually abolished by β-D-xyloside, a compound which, in various cell types, substitutes for the protein core moiety in proteoglycan synthesis (32–34), resulting in a deficiency of cell surface proteoglycans and release of GAG chains, bound to β-D-xyloside, into the cell medium. ApoE likely binds a heparin or heparan sulfate-like GAG, based on the preferential release of apoE by these GAGs relative to that by hyaluronic acid and the chondroitin sulfates. In addition, [^{35}S]apoE is released by treating cells with the heparin/heparan sulfate-specific lyases, heparinases I and III, but not by chondroitinase ABC. The release of apoE by both heparinases suggests that apoE interacts with a heterogeneous population of GAGs. None of the enzymes is as effective as heparin in increasing extracellular apoE, possibly because of chemical heterogeneity of the GAG anchors, or their differential accessibility to the enzymes, or both.

Heparin-releasable [^{35}S]apoE can become associated with cell surface GAGs by one of two mechanisms. Nascent apoE can be first secreted, then become associated with cell surface GAGs. Alternatively, apoE can be linked to GAGs intracellularly, then transported to the cell surface as an apoE-GAG complex. Our data suggest the latter. If apoE were secreted before binding to GAGs, removal of the GAGs by β-D-xyloside prior to ^{35}S-labeling should divert the [^{35}S]apoE into the cell medium, causing an increase of extracellular apoE comparable to that seen with heparin treatment. To the contrary, β-D-xyloside-treated cells secrete the same amount of [^{35}S]apoE as untreated cells. In addition, if apoE were secreted before GAG interaction, this interaction should be interrupted by extracellular molecules that can bind to apoE or the GAG, diverting this pool of apoE into the cell medium. Incubation with the heparin-binding $apoE_{130-169}$ peptide or anti-apoE antiserum does not increase extracellular [^{35}S]apoE, indicating that extracellular molecules cannot interrupt the apoE-GAG interaction. The data are consistent with a model in which the heparin-releasable pool of nascent apoE is first bound to a GAG inside the cell and then directed to the cell surface.

Direct demonstration of this alternative pathway for secretion is difficult because most of the apoE synthesized by HepG2 cells remains inside the cell. Pulse-chase experiments show that ~70–80% of the total [^{35}S]apoE remains inside the cell during a 2-h chase. This observation

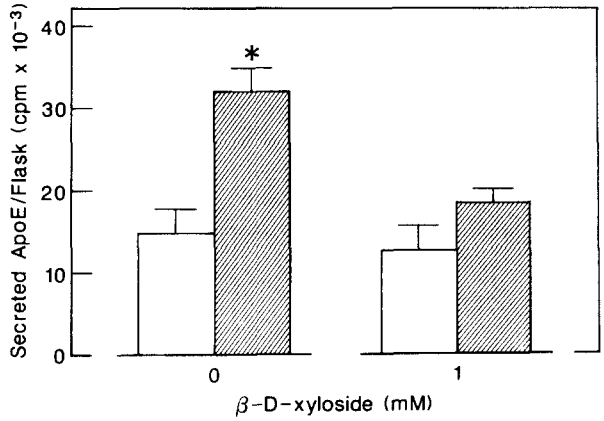

Fig. 8. Heparin-releasable apoE is diminished by treatment of cells with β-D-xyloside. Cells were treated with β-D-xyloside and apoE was quantitated by immunoprecipitation and subsequent counting of boiled supernatants, as described in Experimental Procedures. Open bars: control cells; striped bars: heparin treated cells; * indicates $P < 0.001$.

is in contrast to recent data by Fazio et al. (39), but is consistent with the data of Zannis, vander Spek, and Silverman (23) showing that a significant amount of the ^{35}S-labeled apoE generated during a 20-min pulse remains associated with the cell after 2–4 h of chase. The apoE being trafficked to the cell surface is a small portion of the total apoE and is difficult to track. Also, the cell surface GAGs with which apoE becomes associated may undergo very slow turnover. This is evident during long-term heparin treatment, where the pool of heparin-releasable [^{35}S]apoE becomes larger with each day of treatment (Fig. 1A). Consistent with this observation is the fact that 4 days of β-D-xyloside exposure is required to remove cell-surface GAGs and deplete the heparin-releasable pool of [^{35}S]apoE.

Association of apoE with hepatic cell surface GAGs is consistent with published data. By immunocytochemistry, heparan sulfate proteoglycans are localized to the sinusoidal domain of the rat hepatocyte plasmalemma (40). ApoE exists on the surface of liver cells in this same region (41). By immunogold labeling of rat liver slices, there is intense apoE staining at the sinusoidal front of parenchymal cells, in the adjacent space of Disse and on microvilli projecting into the space of Disse. Staining is variably associated with lipoprotein particles, suggesting that much lipid-deficient apoE is present. HepG2 cells are therefore a reasonable model for investigating the functional significance of cell surface apoE, even though heparan sulfate produced by hepatoma cells is undersulfated and secreted more heavily into the extracellular medium compared to normal liver cells (16).

The biological function of membrane GAG-associated apoE is unknown, but there are potential roles for the apoE-GAG interaction in cell and tissue maintenance where the interaction of apoE with GAGs may establish or maintain the differentiated state. Evidence for this possibility is provided by SMC, the proliferation of which is inhibited by heparin. Proliferating SMC do not secrete apoE (13), although apoE is secreted after 1 (12) to 2 (13) h of heparin exposure. Cells that are growth-arrested by serum deprivation produce apoE, and the amount of extracellular apoE is dramatically increased by 2 h of heparin treatment. The rapid response of apoE to heparin suggests that it is bound to the cell surface. Growth arrest of SMC by serum deprivation or heparin treatment also increases apoE mRNA. Thus, in SMC, heparin can increase apoE by transcriptional and post-secretional mechanisms, and the production of apoE is correlated with reduced proliferation and increased differentiation. ApoE, rather than heparin itself, may be responsible for growth inhibition. It is relevant that apoE can suppress proliferation of T lymphocytes (42, 43).

If apoE plays a regulatory role in cell proliferation or differentiation, it may do so by interacting with a cell surface GAG, thereby transmitting negative signals or blocking binding of a factor that transmits positive signals. Heparan sulfate plays a critical role in the action of known growth factors. Sakaguchi et al. (44) defined two high-affinity receptors for acidic fibroblast growth factor (FGF), one of which is a heparan sulfate proteoglycan. Also, basic FGF must first interact with a soluble or surface-bound heparan sulfate proteoglycan before it can bind its high-affinity receptor (45), and in fact, interaction with heparan sulfate is required for its biological activity (46).

The interaction between heparin/heparan sulfate and apoE may be important during liver development or regeneration. Panduro et al. (47) showed that apoE mRNA decreases dramatically upon liver damage or partial removal, and does not return to normal until after the peak of cell proliferation associated with regeneration. Thus, as with SMC, apoE is associated with the nonproliferative (differentiated) state. Similarly, during rat liver development, apoE mRNA levels are low in utero, but increase after birth in the adult liver (48). Hepatic ECM GAGs change during growth and development. Heparan sulfate is the major GAG species in adult rat liver, but chondroitin sulfate predominates during hepatic growth (49). Because apoE interacts differently with these two GAGs, its function in liver development may be regulated by changes in ECM GAGs.

Cell surface heparan sulfate may, alternatively, facilitate apoE's role in lipid acquisition or transport. In particular, the localization of apoE on the hepatic cell surface may provide a reservoir of apoE available for the uptake of incoming apoE-deficient lipoproteins. Previous studies, such as those by Mokuno et al. (50), have shown enhanced uptake by HepG2 cells of VLDL enriched in apoE. A recent study by Evans et al. (51) provides evidence that apoE secreted by the HepG2 cells is the ligand responsible for uptake by HepG2 cells of VLDL obtained from patients with type IV hypertriglyceridemia. HepG2 cells appear to produce a constant amount of apoE. In fact, Fazio et al. (39) showed that lipogenic factors that cause a 5-fold increase in apoB production by HepG2 cells have no effect on apoE. These investigators also found that secreted apoE can associate with exogenous chylomicron remnants, demonstrating the exchangeability of apoE between lipoprotein particles. The heparin-sensitive pool of hepatic cell-surface apoE that we have identified may supply a constant amount of ligand for the uptake of apoE-deficient lipoproteins. In this regard, Oswald et al. (52) showed that the uptake of apoE-supplemented triglyceride emulsions by hepatocytes is inhibited by heparin and heparan sulfate and by heparanase; hyaluronic acid and the chondroitin sulfates are ineffective. Heparanase has only a slight effect on the uptake of low density lipoproteins (LDL), indicating that heparan sulfate-mediated uptake of apoE-associated lipids does not require LDL receptors. Thus, heparan sulfate may participate in the uptake of apoE-associated lipid by liver

cells, and perhaps by lipid-poor cells, in general. Other precedents exist for the localization of key factors in lipid uptake at their sites of action. Lipoprotein lipase is localized at the endothelium by interaction with heparan sulfate, enabling fatty acids released from lipoproteins to be readily internalized (53–55). A similar cell-surface localization is presumed for HTGL (56, 57). Cholesterol esterase and triglyceride lipase are also bound to membrane-associated heparin in the intestinal brush border, facilitating uptake of dietary lipid into the small intestine (58, 59).

Finally, our results identify a potential mechanism by which apoE can be increased rapidly in a local environment. Heparin is released from mast cells (8) into the ECM during tissue damage. The increase of heparin in the immediate environment can displace apoE from cell surface GAGs, so that apoE also increases. Such an increase in apoE is likely to be important in tissue damage and repair where lipid redistribution is required for membrane biogenesis.

In summary, we have demonstrated that brief treatment of HepG2 cells with heparin causes a rapid increase of extracellular nascent apoE. The mechanism of this effect is post-translational and apparently involves release of apoE from its interaction with cell surface heparin/heparan sulfate-like GAGs. We suggest that the interaction of apoE with cell surface GAGs is important in lipid transport and/or in tissue growth and differentiation, and provides a readily available pool of apoE that is susceptible to changes in ECM composition in vivo.

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