Tissue-specific posttranslational modification of rat apoE. Synthesis of sialated apoE forms by neonatal rat aortic smooth muscle cells

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Abstract We have studied the synthesis, modification, and secretion of rat apoE in primary cultures of neonatal aortic smooth muscle cells and adult rat hepatocytes. The cultures were pulsed with [^{35}S]methionine and the intracellular and secreted apoE were immunoprecipitated and analyzed by two-dimensional isoelectric focusing/polyacrylamide gel electrophoresis and autoradiography. A short pulse (10 min) showed the presence of a major unmodified apoE form in both cultures. This form comigrated on two-dimensional gels with the major rat plasma apoE isoprotein. A longer pulse (15–120 min) resulted in the progressive appearance of intracellularly modified apoE isoproteins in both cultures. The apoE secreted by aortic smooth muscle cells consisted exclusively of sialated apoE isoproteins that were sensitive to neuraminidase treatment. In contrast, the apoE secreted by primary cultures of adult rat hepatocytes, organ cultures of neonatal rat liver, as well as rat plasma apoE, contained several minor modified isoproteins. Nascent apoE secreted by the aortic smooth muscle cell cultures floats in the density range of 1.09 to 1.186 g/ml. We conclude that aortic smooth muscle cells can synthesize and secrete sialated apoE isoproteins associated with nascent lipoproteins floating in the high density lipoprotein region. — **Hussain, M. M., N. L. R. Bucher, B. Faris, C. Franzblau, and V. I. Zannis.** Tissue-specific posttranslational modification of rat apoE synthesis of sialated apoE forms by neonatal rat aortic smooth muscle cells. *J. Lipid Res.* 1988. **29:** 915–923.

Supplementary key words hepatocytes • apoE isoproteins • glycosylation • isoelectric focusing/polyacrylamide gel electrophoresis

ApoE is a constituent of several lipoprotein particles, e.g., VLDL, HDL, chylomicron remnants, and is thought to play an important role in cholesterol homeostasis. ApoE-containing lipoproteins are recognized by LDL (B/E) receptors, and contribute to the clearance of cholesterol from the circulation (1).

Rat plasma apolipoprotein E is a single polypeptide composed of 293 amino acids (2, 3), and is 69% homologous to human apoE (2–4). Its primary translation product contains an additional 18 amino acid signal peptide. Posttranslational glycosylation has been described for human and rat apoE (5–7). The lack of potential N-glycosylation sites for both proteins implies that the modification(s) involve O-glycosidic linkages (2–4).

Although the liver may be the major site of apoE synthesis, recent studies have shown that other peripheral tissues are capable of synthesizing apoE (8–12). The present study is a part of a project designed to investigate the biosynthesis of apolipoproteins in vascular tissue. Here we report that, under the experimental conditions used, rat aortic smooth muscle cells synthesize and secrete sialated apoE isoproteins that are associated with nascent lipoproteins.

EXPERIMENTAL PROCEDURES

Materials

Bovine serum albumin, ovalbumin, lysozyme, trypsin inhibitor, neuraminidase (*Clostridium perfringens*), collagenase type I, elastase type III, deoxycholate, Triton X-100, 2-mercaptoethanol, N,N,N',N',tetramethylenediamine, hydrogen peroxide (30% w/w), Tris, agarose, were purchased from Sigma. Ampholines pH 2.5, 5.8, and 3.5–10 were purchased from LKB Instruments, Inc., Rockville, MD. Nonidet P-40 was purchased from Particle Data Laboratories, Ltd. Acrylamide was obtained from Serva Fine Chemicals. Glycine, methanol, and glacial acetic acid were purchased from Fisher Scientific Co. Sodium dodecyl sulfate, bisacrylamide, ammonium persulfate, Coomassie brilliant blue, bromophenol blue, Bio-Lytes pH 4–6 were obtained from Bio-Rad. Urea (ultrapure

Abbreviations: VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins.

grade) was a product of Schwarz/Mann. [^{35}S]Methionine (1150 Ci/mmol) was obtained from New England Nuclear. X-ray film Cronex 4 was purchased from Du Pont Instruments, Wilmington, DE. Methionine-free Eagle's minimum essential medium (MEM) and glutamine were purchased from Gibco. IgGSorb was obtained from the Enzyme Centre, Boston, MA. Rabbit anti-rat apoHDL was a gift from Dr. S. Lux, Children's Hospital Corporation, Boston, MA. Rabbit anti-rat apoE was a gift from Dr. Weisgraber of Gladstone Foundation Laboratories, San Francisco, CA.

Methods

Rat aortic smooth muscle cell cultures. Neonatal Sprague-Dawley rat smooth muscle cells were grown and isolated from the aortae of 2–3-day-old rats as described previously by Oakes et al. (13). Essentially, a collagenase (10 mg) elastase (2.5 mg; Sigma type III) mixture in 20 ml of medium without fetal calf serum was added to 20 aortae that were cut into small pieces. The digestion was carried out for 30–45 min with stirring at 37°C. The resulting cell suspensions were centrifuged at 400 *g* for 5 min, and each cell pellet was washed twice with Dulbecco's Modified Eagle's Medium containing 3.7 g/liter sodium bicarbonate, 20% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). Each cell pellet was then resuspended in 2–4 ml of fresh medium. Cells in primary culture were seeded at 5×10^5 cells/25 cm^2 tissue culture flask and maintained for 7 days in 5 ml of the medium described above. The cells were subcultivated (first passage) by trypsinization (0.05% trypsin–0.02% EDTA; Gibco) for 5–8 min at 37°C. They were subsequently seeded at 5×10^5 cells per 25 cm^2 flask or 35-mm Petri dish and maintained with 2–5 ml of medium.

Rat hepatocyte cultures. Hepatocytes were isolated from ad libitum fed 200-g Sprague-Dawley rats as previously described (14). Briefly, livers were perfused for 10 min with calcium-free Krebs-Ringer bicarbonate buffer, followed by calcium- and collagenase-containing buffer (15). Hepatocytes were then released into culture medium containing 5–10% dialyzed bovine serum, 200 mU of insulin, and 27 mM glucose, strained through nylon mesh, and washed several times by sedimentation and resuspension. The cells were counted in a hemocytometer, and after assessment of viability by dye exclusion, were plated in culture medium without serum at a density of 106×10^3 cells/cm^2 in Lux culture dishes. After a 1-hr attachment period, they were refed with culture medium without serum. The culture medium consisted of Waymouth's MAB 87/3 (16), modified by omission of thymidine and insulin, substitution of ornithine for arginine, reduction of glucose to 5.5 mM, and addition of hydrocortisone sodium succinate (4×10^{-6} M) and gentamycin (60 μg/ml). In some experiments the culture medium was Williams E with added dexamethasone (5×10^{-12} M) and gentamycin; the experimental outcome was unaffected by the change. The cultures were maintained at 37°C in an atmosphere of 5% CO_2/95% air for 24 hr, then used for labeling studies.

Immunoprecipitation of apolipoproteins from rat serum. For this precipitation we used anti-rat apoHDL antiserum. In control experiments, we found that this antiserum precipitated apoA-I, apoA-IV, apoE, and apoC-III from rat serum. The amount of antiserum required for optimum precipitation of these proteins from an aliquot of 50 μl of rat serum was determined as described (17). The immunoprecipitate was collected by centrifugation for 5 min in a microcentrifuge and was washed three times with normal saline. The immunoprecipitate was then dissolved in O'Farrell's lysis buffer (18), 9.5 M urea, 2% (w/v) Nonidet P-40, 2.1% Ampholine (1.2%, pH 5.8; 0.5%, pH 2.5–4; 0.4%, pH 4–6; and 5% (w/v) 2-mercaptoethanol, and analyzed by two-dimensional isoelectric focusing polyacrylamide gel electrophoresis (19).

Labeling of the primary cell cultures. Four- to 5-day-old (after first passage) aortic smooth muscle cell cultures or 18–24-hr-old hepatocyte cultures were washed twice with methionine-free Eagle's minimum essential medium supplemented with 2 mM glutamine and incubated in the same medium for the indicated time in the presence of 0.25 mCi of [^{35}S]methionine for various lengths of time.

After labeling, the medium was collected and the cells were lysed as described below. The cell lysate and the culture media were immunoprecipitated with anti-rat apoE or anti-rat HDL antibodies as described below and analyzed by two-dimensional isoelectric focusing/polyacrylamide gel electrophoresis and autoradiography (19, 20).

Neonatal rat liver organ cultures. Neonatal livers were obtained from 3-day-old rats and were promptly (within 15 min) used for organ cultures as described previously (19, 20). Within 15 min of killing, the specimens were dissected into pieces with a diameter of less than 0.5 mm.

Neuraminidase treatment. Medium obtained from cell cultures labeled for 18 to 24 hr was made to 0.1 M sodium acetate, pH 5.0, and treated with 2 units of neuraminidase (*Clostridium perfringens*) at 37°C for 2 hr. The reaction mixture was dialyzed against 10 mM sodium phosphate, pH 7.2, 85 mM NaCl, 5 mM KCl. It was then adjusted to a final concentration of 10 mM sodium phosphate, pH 7.2, 85 mM NaCl, 5 mM KCl, 0.25% deoxycholate, 0.5% Triton X-100, and 0.5% sodium dodecyl sulfate, and used for immunoprecipitation.

Analysis of cell lysate and culture media for apoE synthesis. The cell cultures were washed and lysed in a solution containing 10 mM sodium phosphate, pH 7.2, 85 mM NaCl, 5 mM KCl, 0.5% deoxycholate, and 1% Triton X-100. The cell lysate was diluted 1:1 with a solution of 10 mM sodium phosphate, pH 7.2, 85 mM NaCl, 5 mM KCl, 1% sodium dodecyl sulfate, and centrifuged for 5 min in a

microcentrifuge; the supernatant was retained. The cell media were centrifuged in a microfuge to remove cell debris and were adjusted to a final concentration of 10 mM sodium phosphate, pH 7.2, 85 mM NaCl, 5 mM KCl, 0.25% deoxycholate, 0.5% Triton X-100, and 0.5% sodium dodecyl sulfate.

An aliquot of 10 μl of anti-rat HDL or anti-rat apoE was added to 1 ml of cell lysate or culture medium prepared as described above and the mixture was incubated at 4°C overnight. Next, a 100-μl aliquot of 10% IgGSorb suspension was added followed by an additional hour of incubation at 4°C. The mixture was centrifuged for 30 sec in a microcentrifuge. The pellet was washed once by resuspension and 30 sec centrifugation in a buffer containing 10 mM sodium phosphate, pH 7.2, 85 mM NaCl, 5 mM KCl, 0.5% deoxycholate, 1.0% Triton X-100, 1.0% sodium dodecyl sulfate, and 1 mg/ml bovine serum albumin, then four times in the same buffer without albumin. The immunoprecipitates obtained from the cell lysates and the culture media were dissolved in O'Farrell's lysis buffer (18) and analyzed by two-dimensional isoelectric focusing/polyacrylamide gel electrophoresis and autoradiography.

Quantification of apoE isoproteins. To quantify the apoE isoproteins, the apoE isoprotein spots were excised from one- or two-dimensional polyacrylamide gels and solubilized in 2.5 ml of 30% (w/v) H_2O_2 at 60°C in a scintillation vial. The solubilized acrylamide was then mixed with 15 ml of scintillation fluid and counted in an LKB scintillation counter.

Density gradient ultracentrifugation. Smooth muscle cells from one 150-mm flask were grown in 4 ml of methionine-free minimum essential medium containing [^{35}S]methionine for 6 hr. The culture medium was collected and analyzed by discontinuous density gradient ultracentrifugation (21). For this analysis, a 2.25-ml aliquot of the culture medium was adjusted to a density of 1.21 g/ml with potassium bromide, placed in a cellulose nitrate tube, and overlaid sequentially with 1.75 ml of potassium bromide solution of d 1.15 g/ml, 3 ml each of potassium bromide solutions of d 1.063 g/ml and d 1.019 g/ml, and then normal saline. The tubes were then centrifuged in a Beckman SW41 rotor at 35,000 rpm for 22 hr. After centrifugation, 12 one-ml fractions were collected from the top of the tube with the Haake/Buchler Fraction Collector, dialyzed, lyophilized, and suspended in O'Farrell's lysis buffer (18). Two-thirds volume of each fraction was analyzed by one-dimensional SDS polyacrylamide gradient gel electrophoresis and one-third was analyzed by two-dimensional isoelectric focusing/polyacrylamide gel electrophoresis. The density of contents of a blank tube was determined by a refractometer.

Gradient SDS polyacrylamide gel electrophoresis. Gradient SDS polyacrylamide gel electrophoresis was performed on vertical slab gels of 17 × 20.5 cm with a thickness of 1.5 mm. The separating gel consisted of linear 4–18% acrylamide (acrylamide to bisacrylamide ratio 2.92% to 0.08%) gradient in 0.375 M Tris-HCl buffer, pH 8.8, containing 0.1% SDS, 0.017% ammonium persulfate, and 0.1% TEMED. The stacking gel was comprised of 2.92% acrylamide and 0.08% bisacrylamide in 0.125 Tris-HCl, pH 6.8, containing 0.1% SDS, 0.03% ammonium persulfate, and 0.2% TEMED. Electrophoresis was performed at a constant voltage of 50 volts for 1 hr and then at 110 volts until the marker dye reached the bottom of the separating gel.

After electrophoresis, the gels were fixed in 50% methanol and 10% acetic acid for 1 hr and stained in a solution of 0.25% Coomassie brilliant blue, 50% methanol, and 10% acetic acid for 30–45 min. Destaining was accomplished by sequential exposure of the gel for a period of ½ to 2 hr to 100 ml of fixing solution (10% acetic acid, 50% methanol) followed by 200 ml of water until proper stain background was achieved. The gels were dried using a slab gel dryer and then exposed to Cronex-4 film.

Two-dimensional isoelectric focusing/polyacrylamide gel electrophoresis. Two-dimensional equilibrium isoelectric focusing/polyacrylamide gel electrophoresis was performed according to the method of O'Farrell (18) with previously described modifications (19, 20). The ampholytes used were Bio-Lyte pH 5–8 (1.5%), Bio-Lyte pH 2.5–4 (0.2%), and Bio-Lyte pH 4–6 (0.5%). For the second dimension, the focused cylindrical gels were thawed quickly and placed on slab gels. The slab gels (19.5 × 20.5 cm with a thickness of 0.5 mm) consisted of the separating gel (13.7% acrylamide and 0.37% bisacrylamide) and the stacking gel (4.4% acrylamide and 0.12% bisacrylamide). Electrophoresis was performed at a constant voltage. After electrophoresis, the gels were processed as above.

RESULTS

Synthesis of apolipoproteins by primary cultures of rat aortic smooth muscle cells and rat hepatocytes

To identify the apolipoproteins synthesized by primary cultures of rat aortic smooth muscle cells and rat hepatocytes, the cultures were labeled with [^{35}S]methionine for 4 hr and the apolipoproteins were immunoprecipitated with anti-rat HDL or anti-rat apoE as explained in Experimental Procedures. In control experiments the anti-rat HDL precipitated apoA-I, apoA-IV, apoCs, and apoE from rat plasma (data not shown). This analysis showed that primary cultures of aortic rat smooth muscle cells synthesize and secrete a series of isoproteins that could be precipitated both with anti-rat HDL and anti-rat apoE (**Fig. 1A;** see also Fig. 2K). These proteins were subsequently identified as sialated apoE isoproteins. No proteins corresponding to apoA-IV,

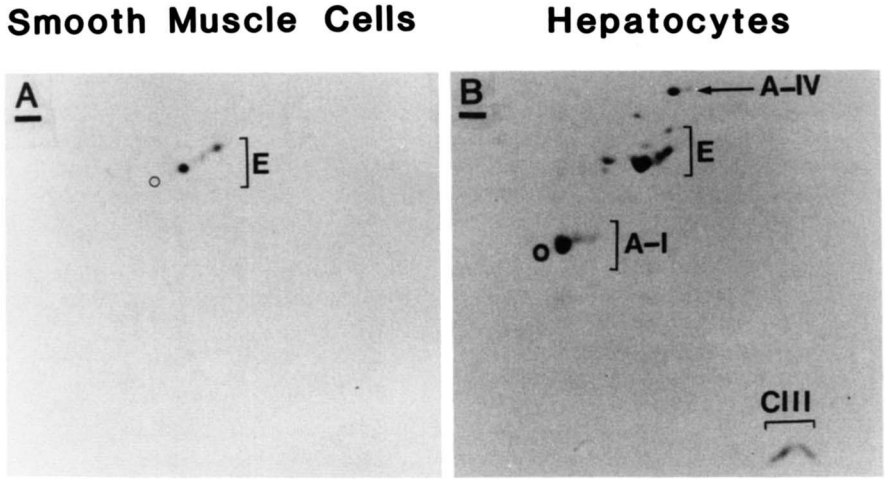

Fig. 1. Analysis of the culture medium of primary cultures of aortic smooth muscle cells (panel A) and rat hepatocytes (panel B) for apolipoprotein synthesis. The cultures were grown in medium containing [^{35}S]methionine for 4 hr and the secreted apolipoproteins were immunoprecipitated with anti-rat HDL from the culture medium and analyzed by two-dimensional isoelectric focusing/polyacrylamide gel electrophoresis and autoradiography as explained in Experimental Procedures. This figure shows the autoradiogram obtained from this analysis. The positions of rat apoproteins are indicated. The symbol (○) in panels A and B indicates the position of the major plasma apoE and apoA-I isoprotein, respectively.

apoA-I, and apoC-III could be immunoprecipitated from the aortic smooth muscle media using anti-rat HDL antiserum. Similar analyses showed that primary cultures of rat hepatocytes secrete into the culture medium apoA-I, apoA-IV, apoE, and apoC-III which comigrate on two-dimensional gels with their plasma counterparts (Fig. 1B). All the above apolipoproteins can also be isolated by ultracentrifugation of the culture medium (data not shown).

Intracellular modification and secretion of apoE synthesized by rat aortic smooth muscle cells and rat hepatocytes

The time course of intracellular apoE modification and secretion was assessed by labeling experiments of smooth muscle cells and hepatocyte cultures. **Fig. 2** shows a typical labeling experiment. In this and other similar experiments, primary cultures of rat aortic smooth muscle cells and hepatocytes were pulsed with [^{35}S]methionine. The intracellular and extracellular apoE was immunoprecipitated with anti-rat apoHDL and analyzed by two-dimensional isoelectric focusing/polyacrylamide gel electrophoresis and autoradiography. In some cases, the apoE isoproteins were excised from the gels, solubilized, and counted.

A short pulse (10 min) showed the presence of one major unmodified apoE isoprotein designated E3 (Fig. 1, A and B). This isoform was designated apoE3 because it is the major plasma form. This is analogous to the designation of E3 for the major human plasma form. This form coincided on two-dimensional gels with the major rat plasma apoE isoproteins. A longer pulse (15–45 min) resulted in the progressive appearance of new intracellularly modified apoE isoproteins in both cultures (Fig. 1, C through F). The apoE secreted by the aortic smooth muscle cells consisted of three distinct modified apoE isoproteins that differed from the major unmodified apoE isoprotein by one, two, and three charges, respectively; they were designated as $E3_{s1}$, $E3_{s2}$, and $E3_{s3}$. This isoprotein pattern was similar regardless of the antibody (anti-rat HDL or anti-rat apoE) used for immunoprecipitation (compare Fig. 2, I and K). The relative concentrations of these isoproteins were $E3_{s1}$ = 59%, $E3_{s2}$ = 14%, and $E3_{s3}$ = 27% (Fig. 1, G and I). In contrast, the apoE secreted by primary cultures of rat hepatocytes consisted mainly of one major unmodified form designated apoE3 and several minor apoEs. The major unmodified apoE3 form and a minor isoprotein designated $apoE3_{s1}$ comigrated on two-dimensional gels with the corresponding smooth muscle cell apoE isoproteins. The other minor apoE isoproteins differed from apoE3 by isoelectric point and/or molecular weight (Fig. 2, H and J). The isoprotein patterns were similar when apoE was immunoprecipitated with either anti-rat HDL or anti-rat apoE (compare Fig. 2, J and L). To assess possible age-dependent differences in the posttranslational modification of hepatic apoE, we studied the synthesis and modification of this protein by organ cultures of neonatal (3-day-old) liver. This analysis showed that apoE isoproteins secreted by these cultures have similar features and overlap on two-dimensional gels with the apoE synthesized by the

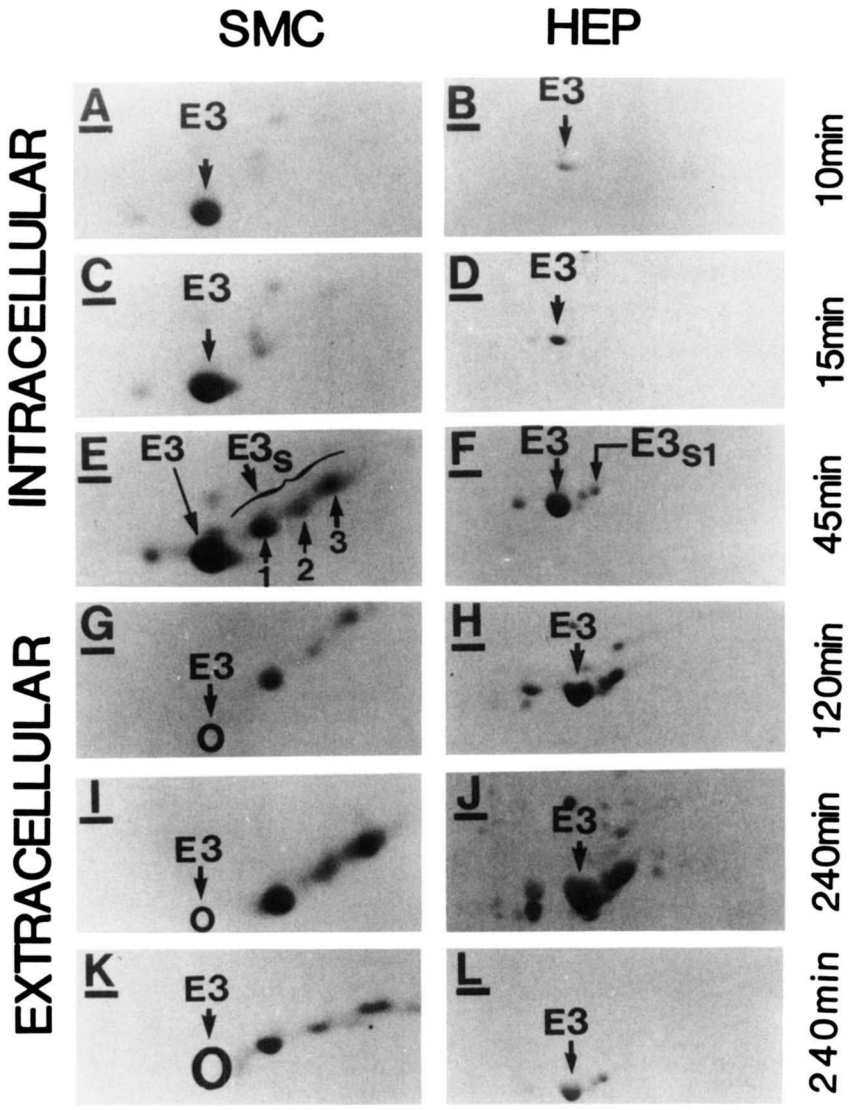

Fig. 2. Time course of intracellular modification of rat apoE synthesized by rat aortic smooth muscle cells (SMC) and hepatocytes (HEP). Cell cultures grown in 35-mm Petri dishes were pulsed continuously with 0.25 mCi/ml [^{35}S]methionine for the indicated times. The cell lysate and the culture medium were collected, immunoprecipitated (A–J with anti-rat HDL, K and L with anti-rat apoE) and analyzed by two-dimensional isoelectric focusing/polyacrylamide gel electrophoresis and autoradiography as explained in Experimental Procedures. This figure shows the autoradiogram obtained from this analysis. Only regions of the autoradiogram corresponding to apoE are shown. E3 is the unmodified precursor. $E3_{s1}$, $E3_{s2}$, and $E3_{s3}$ are the sialated apoE isoproteins. Note that different isoforms appear intracellularly in both cultures after 15 min. Only the modified apoE forms appear in the culture medium (panels G, I, and K) of aortic smooth muscle cells, whereas unmodified and modified forms of apoE appear in the culture medium of rat hepatocytes. The symbol (O) in panels G, I, and K indicates the position of the major plasma apoE isoprotein.

primary cultures of adult rat hepatocytes (data not shown). These findings suggest that the posttranslational modification of hepatic apoE is not dependent on the age of the hepatocytes. Thus the complete sialation of apoE secreted by aortic smooth muscle cells is a distinct feature of these cells. The type(s) of posttranslational modification of apoE produced by different cell types or tissues remains to be determined.

Treatment of secreted apoE with neuraminidase

To identify the nature of apoE modification, the secreted apoE was treated with neuraminidase. The smooth muscle cells and hepatocytes were labeled for 18 hr with [^{35}S]methionine and the labeled medium was treated with neuraminidase (**Fig. 3, A and B**). This treatment resulted in loss of all the acidic isoproteins of smooth

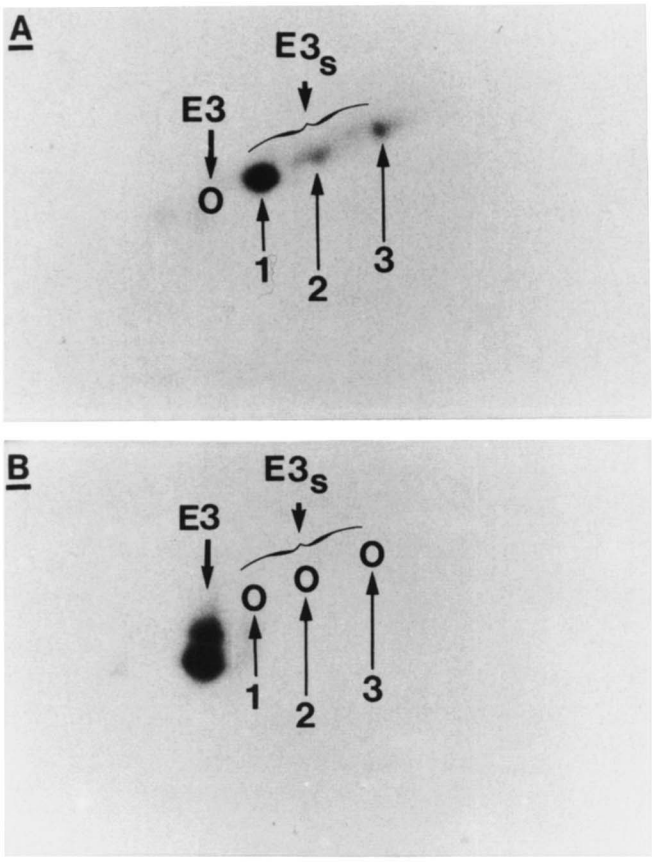

Fig. 3. Effect of neuraminidase treatment on the isoprotein composition of nascent rat apoE primary cultures of aortic smooth muscle cells. The cell cultures were labeled with [^{35}S]methionine for 18 hr. The culture medium was treated with *C. perfringens* neuraminidase, immunoprecipitated, and analyzed by two-dimensional isoelectric focusing/polyacrylamide gel electrophoresis and autoradiography as explained in Experimental Procedures and Fig. 1. This figure shows the autoradiogram obtained from the analysis of aortic smooth muscle cell cultures (panels A and B). The sample in panel A was not treated with neuraminidase whereas panel B was. Note that in the aortic smooth muscle cell cultures, neuraminidase converts the sialo (apoE3$_s$) forms to asialo (apoE3) forms (panels A and B).

muscle cell apoE, resulting in two populations of apoE with the same pI but different molecular weight. One form comigrated on two-dimensional gels with the unmodified apoE3 whereas the other had slightly higher molecular weight. Treatment of the hepatocyte medium with neuraminidase resulted in the loss of the acidic isoprotein apoE3$_{s1}$ (data not shown) and is in accordance with the finding of Reardon et al. (22).

Association of nascent apoE with lipoprotein particles

We have examined the ability of nascent apoE secreted by the aortic smooth muscle cells to associate with lipoprotein particles by density gradient ultracentrifugation as explained in Methods. This analysis showed that nascent apoE floats in the density range of 1.09–1.186 g/ml (**Fig. 4A**). The relative concentration of apoE isoproteins isolated by ultracentrifugation is similar to that obtained by immunoprecipitation of apoE.

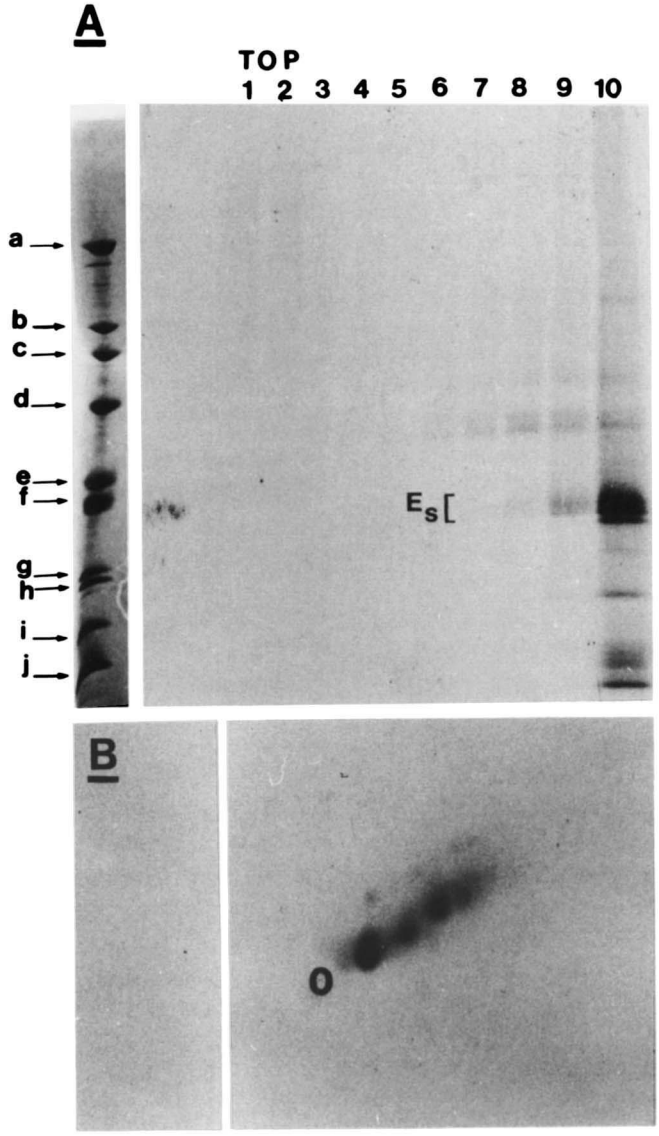

Fig. 4. Analysis of the nascent apoE secreted by primary cultures of aortic smooth muscle cells by density gradient ultracentrifugation. Aortic smooth muscle cell cultures were labeled with [^{35}S]methionine and analyzed by density gradient ultracentrifugation as explained in Experimental Procedures. Twelve fractions were collected and the top ten fractions were analyzed by one- or two-dimensional gel electrophoresis and autoradiography. Panel A shows one-dimensional analyses of 0.75-ml fractions following dialysis and lyophilization. The first lane contains protein molecular weight markers as follows: a) myosin, 200K; b) beta-galactosidase, 116.25K; c) phosphorylase B, 92.5K; d) bovine serum albumin, 66.2K; e) ovalbumin, 45K; f) aldolase, 40K; g) human apoA-I, 28K; h) bovine chymotrypsinogen, 25.7K; i) trypsin inhibitor, 19K; j) lysozyme, 14.7K. The density of the fractions 1–10 (in g/ml) are as follows: 1, 1.005; 2, 1.012; 3, 1.016; 4, 1.028; 5, 1.044; 6, 1.062; 7, 1.09; 8, 1.124; 9, 1.152; and 10, 1.186. Panel B shows two-dimensional analysis of 0.25-ml aliquots of pooled fractions 7–10 following dialysis and lyophylization. Note that the isoprotein composition of apoE obtained by ultracentrifugation is similar to that obtained by immunoprecipitation (compare Figs. 2I and 4B).

DISCUSSION

Rat plasma apolipoprotein E is a single polypeptide composed of 293 amino acids of known sequence (2, 3). Synthesis of this protein has been demonstrated in a variety of rat tissues including cells of central and peripheral nervous system (8–12, 23, 24). It is believed that, similar to its human counterpart, rat apoE directs the catabolism of lipoprotein particles by hepatic and extrahepatic (B/E) receptors (1, 25, 26), and through this action it helps maintain cellular cholesterol homeostasis.

An interesting aspect of human and rat plasma apoE is that both are composed of numerous isoproteins (27, 28). Studies with the human apoE have established that the more acidic and higher molecular weight apoE isoproteins result from posttranslational modification of this protein with O-linked carbohydrate chains containing sialic acid (27). Furthermore, it was shown that human apoE is secreted in the form of di-, tetra-, and hexasialated forms and is subsequently desialated in plasma (7, 29).

The present study shows that, similar to its human counterpart, apoE synthesized by rat aortic smooth muscle cells is modified intracellularly with carbohydrate chains containing 1, 2, and 3 sialic acid residues and is secreted exclusively in the modified form. The primary sequence of rat apoE shows absence of N-linked glycosylation sites (2, 3). Thus, the present data imply that apoE synthesized by aortic smooth muscle cells contains one or more O-linked carbohydrate chains containing sialic acid residues. The relative abundance of the modified apoE isoproteins secreted into the culture medium is $E3_{s1}$ = 59%, $E3_{s2}$ = 14%, $E3_{s3}$ = 27%. The rat aortic smooth muscle cell system permitted us to address the question of whether the apoE is secreted in the form of a lipoprotein particle or in the lipid-free form. The density gradient centrifugation experiments did show that lipoprotein particles containing nascent apoE float in the density range of 1.09–1.186 g/ml. Furthermore, the isoprotein profile of apoE isolated by ultracentrifugation was similar to that obtained by immunoprecipitation. This latter observation suggests that different degrees of modification did not affect the lipid binding properties of the nascent apoE isoproteins. These studies clearly indicate that smooth muscle cells derived from rat aorta are capable of synthesizing apoE which associates with lipoprotein particles floating in the HDL region.

Labeling of the rat hepatocyte cultures has shown that following synthesis the hepatic apoE undergoes only limited sialation producing the $apoE3_{s1}$ form. Other modified apoE isoproteins differing in isoelectric point and/or molecular weight appear intracellularly and in the culture medium after the cultures were labeled for 45 min. The unmodified as well as the modified apoE forms are secreted into the culture medium. The ratio of the secreted unmodified to the modified apoE forms has been estimated as 2/1. The sialated apoE form was estimated to be less than 10% of the total apoE. These findings are consistent with modifications reported recently for hepatic and plasma rat apoE (22, 28).

An interesting and unexpected finding in this study was that the intracellular modification of the hepatic rat apoE is drastically different from that observed in the cultures of aortic smooth muscle cells. These studies showed that the newly synthesized hepatic apoE consists of one major unmodified isoprotein (apoE3) which comigrates on two-dimensional gels with its plasma counterpart.

The unmodified apoE3 isoprotein and the monosialated $apoE3_{s1}$ isoprotein synthesized by rat hepatocytes overlap on two-dimensional gels with the corresponding isoproteins synthesized by aortic smooth muscle cells. These data suggest, but do not prove, that these two proteins are the products of the same gene.

The modification of secretory glycoproteins like apoE occurs in an intracellular membrane system which consists of the endoplasmic reticulum (ER) and the Golgi complex. In addition, transfer vesicle and secretory vesicles are involved in the movement of the modified proteins from ER to Golgi and their secretion to the extracellular space (30, 31). Carbohydrate chains can be linked either to the asparagine residues of the protein chains through N-linked glycosidic bonds or to serine or threonine residues through O-linked glycosidic bonds (30–33). The findings that both rat aortic smooth muscle cultures and hepatocyte cultures can modify apoE3 to $apoE3_{s1}$ suggest that the glycosylation sites and the modifying machinery exist in both systems. However, other factors relevant to the intracellular movement of the proteins and their association with lipid components may affect the extent of apoE glycosylation.

It has been suggested that the synthesis of apoE by peripheral tissues could probably provide a mechanism for cholesterol redistribution between the tissues (34, 35), or transport to liver for excretion (8). The physiological significance of protein glycosylation has been studied in several systems. There is evidence that oligosaccharide moieties can serve as signals for protein clearance from serum (36), for directing lysosomal enzymes to their target organelles (37), for biological function of glycoproteins (38–40), or for regulation of the cell cycle (41–44). The different posttranslational modifications observed between the rat smooth muscle cell and hepatocyte cultures may impart different biological functions to lipoprotein particles. Thus, liver may recognize desialated apoE, and secretion of sialated apoE by the smooth muscle cells may be a mechanism to combat excessive accumulation of lipids in the aortic tissue. Further studies are required to understand the control mechanisms for apolipoprotein synthesis by these cells and the role, if any, of endogenous lipoprotein synthesis in the well-being of the blood vessel. It would be interesting to know the effects of

vascular injury and/or disease, such as atherosclerosis and hypertension, on the expression of lipoprotein synthesis by vascular smooth muscle cells.

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