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Apolipoprotein E in Macrophages and Hepatocytes Is Degraded via the Proteasomal Pathway

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Macrophage-derived apolipoprotein E (apoE) influences the susceptibility of the arterial wall to atherosclerosis. Previous studies have shown that production of apoE in these cells is regulated at a posttranscriptional level and is increased by inhibitors of proteasomal degradation. To further investigate this mechanism, we stably transfected RAW 264.7 macrophages and HepG2 cells with a construct overexpressing ubiquitin, the peptide targeting proteins to the proteasome, fused to an influenza virus hemagglutinin epitope tag. Ubiquitination of apoE was investigated by immunoprecipitation and Western blot analysis. In both cell types, apoE was ubiquitinated, and inhibition of proteasome function by lactacystin led to accumulation of ubiquitinated apoE. These studies provide strong evidence for proteasomal degradation of apoE in the two main cell types responsible for its production and indicate a possible new level of regulation of this important protein. © 2001 Academic Press

Key Words: **apolipoprotein E; atherosclerosis; hemagglutinin; HepG2 cell; macrophage; proteasome; ubiquitin.**

ApoE is a 34 kDa protein that is secreted mainly by the liver as a component of several classes of circulating lipoproteins (1). Its main function in this location is to act as a ligand allowing docking of triglycerides-rich lipoproteins to the apolioprotein B (apoB)/E receptor which is expressed on nearly all peripheral cells (1). However, apoE is also produced by tissue macrophages, including those located within atherosclerotic plaques (2). Recent studies have shown that the apoE produced by plaque macrophages may play an important role in removing excess cholesterol from the arterial wall and hence may protect against development or exacerbation of atherosclerosis (3).

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In previous studies, we (4) and others (5) have shown that a discrepancy exists between the rate of secretion of apoE from macrophages and the rate of apoE gene transcription as indicated by the level of apoE mRNA. The metabolism of apoE is complex, and may involve binding of newly-secreted apoE to proteoglycans on the cell surface (6) with subsequent reuptake and degradation in a lysosomal compartment (7). However, another explanation for the mRNA/secreted protein mismatch might lie in the proteasomal degradation of newly-synthesized apoE prior to secretion, as has been shown to be the case for apolipoprotein (a) (apo(a)) (8) and apoB (8, 9) in liver. Support for this hypothesis exists in the studies of Duan *et al.*, who studied the J-774 mouse macrophage cell line stably transfected to produce human apoE. Incubation of the stably transfected cells with lactacystin, a specific inhibitor of proteasomal degradation, led to accumulation of newly-synthesized apoE within the cell (10). However, Duan *et al.* failed to demonstrate that macrophages contained apoE tagged with ubiquitin, the universal intracellular signal which marks out proteins for proteasomal degradation. Furthermore, it is possible that apoE which is produced by stable transfection in a cell line which does not normally express apoE may be handled differently from constitutively produced apoE in native macrophages or, indeed, hepatocytes.

To clarify these issues, we studied the metabolism of apoE in human macrophages, in a mouse macrophage cell line which constitutively produces apoE (RAW 264.7 cells) and in the HepG2 hepatocyte cell line. In order to detect ubiquitination, the RAW 264.7 and HepG2 cells were stably transfected with a construct coding for ubiquitin fused to the a highly immunogenic influenza virus hemagglutinin epitope tag. Our data indicate that in macrophages and hepatocytes, apoE is ubiquitinated and that ubiquitinated apoE accumulates in cells treated with specific proteasomal inhibitors, indicating that this protein undergoes degradation in proteasomes.

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EXPERIMENTAL PROCEDURES

Human macrophage isolation and culture. Monocytes were isolated from the peripheral blood of healthy volunteers by cell separation and countercurrent elutriation and cultivated as previously described (4).

Cell culture of RAW 264.7 and HepG2 cells. RAW 264.7 (TIB-71) and HepG2 (HB-8065) cells were obtained from the American Type Culture Collection and cultured at 37°C in a humidified 5% CO_2, 95% air atmosphere in 75 cm^2 flasks containing DMEM supplemented with 10% (v/v) heat-inactivated fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine.

Immunoprecipitation and immunoblot analysis. Immunoprecipitation was performed using a commercial kit (Roche Molecular Biochemicals, Mannheim, FRG) as follows. After preclearing with protein G-agarose the sample was incubated with goat anti-human apoE antibody (WAK-Chemie Medical GmbH, Bad Soden, FRG), goat anti-mouse apoE antibody (Santa Cruz Biotechnology, Inc., Heidelberg, FRG) or with mouse anti-hemagglutinin antibody (BAbCO, Richmond, USA) at 4°C on a rocking platform for 1 h. Protein G-agarose was added and the mixture was incubated overnight at 4°C. After several washes the immunoprecipitate was heated to 96°C for 5 min in Laemmli sample buffer containing 5% 2-mercaptoethanol and analyzed by SDS–PAGE on 4–15% gels or 10% gels. Proteins were transferred overnight at 4°C onto nitrocellulose or polyvinylidene difluoride membranes and probed either with goat anti-human apoE antibody (WAK-Chemie) which had been biotinylated by means of a biotinylation kit (Roche), with mouse monoclonal anti-hemagglutinin antibody (Roche), or with goat anti-mouse apoE antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The secondary reagents were streptavidin-biotin-POD-complex or appropriate peroxidase-conjugated secondary anti-IgG. Visualization was performed with the ECL Plus Western blotting detection system (Amersham Pharmacia Biotech AB, Uppsala, Sweden). The chemiluminescence signal was detected using MP Hyperfilms (Amersham).

Stable transfection of RAW 264.7 and HepG2 cells with a construct expressing hemagglutinin-tagged ubiquitin. A hemagglutinin-tagged ubiquitin (HA-ubiquitin) expression construct (pMT123), which was kindly provided by D. Bohmann, European Molecular Biology Laboratory, Heidelberg, FRG, was subcloned into the pcDNA3.1(−) mammalian expression vector (Invitrogen, Groningen, NL) using the DNA Ligation Kit v. II (Takara Biochemicals, Berkeley, CA). This vector was transformed into DH5α cells and positive clones were selected. Following isolation from these clones, 40 μg of plasmid DNA were added to 4×10^6 cells and electroporation was performed using a Gene Pulser II device (BioRad Laboratories, München, FRG) at 220 V and a capacitance of 975 μF. The cells were incubated for 10 min at room temperature and then transferred to ten 150 mm diameter culture plates. After 1 day of incubation, the medium was supplemented with 0.4 mg/ml and 1.0 mg/ml G418 sulfate for the RAW 264.7 and HepG2 cells, respectively. Clones resistant to G418 sulfate were selected after 14–18 days of incubation and tested by Western blot and immunofluorescence for HA-ubiquitin expression.

Indirect immunofluorescence microscopy. After washing twice with PBS and fixing with 4% (w/v) paraformaldehyde in PBS at 4°C overnight, the cells were washed twice with PBS and incubated at room temperature with 50 mM NH_4Cl in PBS for 10 min followed by permeablization using 0.1% Triton X-100 in PBS for 5 min. The cells were washed three times in PBS and blocked with 0.2% cold fish gelatin in PBS for 40 min at room temperature, after which they were incubated for 45 min with mouse anti-hemagglutinin antibody (HA.11, clone 16B12, BAbCO) in 0.2% gelatin in PBS. Finally, the cells were washed several times with PBS containing 0.2% gelatin, twice with PBS alone, and were then incubated for 30 min in a solution containing the fluorescently labeled secondary antibody (Molecular Probes Europe BV, Leiden, NL) in 0.2% gelatin in PBS. After washing, the cells were mounted onto slides using the ProLong Antifade Kit (Molecular Probes). The preparations were examined directly using an Axiophot II microscope (Carl Zeiss Jena GmbH, Oberkochen, FRG), a charged coupled-device camera, and KS-300 software (Kontron, Neufahrn, FRG).

Pulse-chase studies. Human macrophages were washed three times with methionine-free RPMI 1640 and pulsed for 15 min with 250 μCi/ml [^{35}S]methionine (ICN Biomedicals GmbH, Meckenheim, FRG) in methionine-free RPMI 1640. After the pulse period, cells were washed and chased for 30, 60, 120, and 240 min in RPMI 1640 with or without 10 μM lactacystin (Calbiochem-Novabiochem GmbH, Bad Soden, FRG). At each time point of the chase period, the cell-associated [^{35}S]apoE and [^{35}S]apoE in the media was quantitatively immunoprecipitated as described above. After electrophoresis and Western blotting, the nitrocellulose membranes were exposed to a BAS-IIIs phosphorimaging plate and detected using BAS1500 Bio-Imaging System (Fujifilm, Tokyo, Japan).

Protein determination. Protein was determined using the BCA Protein Assay (Pierce, Rockford, IL) using bovine serum albumin as standard.

RESULTS

Lactacystin Promotes Intracellular Accumulation of ApoE in Human Macrophages

To investigate the possible proteasomal degradation of apoE in human macrophages, 14-day-old cells were incubated for 2 h in the presence of 10 μM lactacystin. The amount of intracellular apoE was examined by immunoprecipitation and enzyme-linked immunoassay. Addition of lactacystin was associated with a 10% increase in the amount of intracellular apoE (Fig. 1). The time kinetics of this accumulation were investigated by means of pulse-chase experiments with radiolabelled methionine. These experiments showed that the lactacystin-induced accumulation of apoE was maximal after 30 min but was maintained for at least 4 h, indicating that proteasomal degradation of apoE begins immediately after its synthesis (Fig. 2). The lower apoE band in the figure shows nonsialylated apoE, a form that is present only in cells and that does not appear in the cell culture medium.

Use of a Hemagglutinin–Ubiquitin Construct to Improve Detection of Ubiquitinated Proteins

Ubiquitin is a poorly immunogenic protein, a factor which hinders investigation of proteasomal degradation. To circumvent this problem, we utilized the construct shown in Fig. 3, which comprised a DNA sequence encoding eight ubiquitin molecules, each of which contained at its NH_2-terminus an epitope tag consisting of hemagglutinin from the influenza virus, under the control of the strong cytomegalovirus promoter (modified from (11)). This construct was introduced by stable transfection into the mouse RAW 264.7 macrophage and the human HepG2 hepatic cell lines. The success of the stable transfection was confirmed by

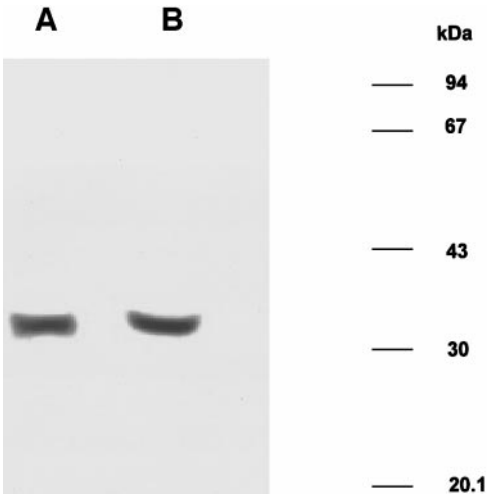

FIG. 1. Lactacystin promotes intracellular accumulation of apoE in human monocyte-derived macrophages. Human 14-day-old macrophages were incubated for 2 h in RPMI 1640 cell culture medium with (lane B) or without (lane A) addition of 10 μM lactacystin. The cells were then harvested and immunoprecipitation of 1.2 mg of cell protein was performed with an antibody against human apoE. The immunoprecipitate was loaded onto an SDS–PAGE gel. Following Western blot analysis, detection was by means of a biotinylated anti-human apoE antibody, a streptavidin-biotin-horseradish peroxidase complex, and chemiluminescence. Quantitative scanning of the image (inset) showed a 10% increase in apoE in the lactacystin-treated cells.

indirect immunofluorescence microscopy and Western blot analysis using a mouse monoclonal antibody directed against the hemagglutinin epitope tag.

Ubiquitination of ApoB in HepG2 Cells Stably Transfected with a Hemagglutinin–Ubiquitin Construct

In order to investigate if our strategy of stable transfection with a ubiquitin–hemagglutinin construct improved detection of ubiquitinated proteins, immunoprecipitation of lysates from non-transfected and transfected HepG2 cells, with or without treatment with lactacystin, a specific inhibitor of proteasomal degradation (12, 13) was performed using an antibody directed against human apoB (Roche Molecular Biochemicals, Mannheim, FRG), a protein which is known to undergo ubiquitination (9). Detection was by means of an anti-hemagglutinin antibody (Fig. 4). Immunoprecipitation of albumin, a protein which does not undergo ubiquitination (8), served as a negative control. In the lysates from the nonlactacystin-treated transfected cells, but particularly in those from the lactacystin-treated transfected cultures, a smear of high-molecular weight (>200 kD) was detected representing intact and partially degraded ubiquitinated apoB. No signals were detected either in the transfected cells precipitated with an antibody directed against human albumin (DAKO Diagnostika GmbH, Hamburg, FRG), or in the nontransfected HepG2 cells. Thus stable transfection with our hemagglutinin–ubiquitin construct allowed us to clearly detect ubiquitinated proteins.

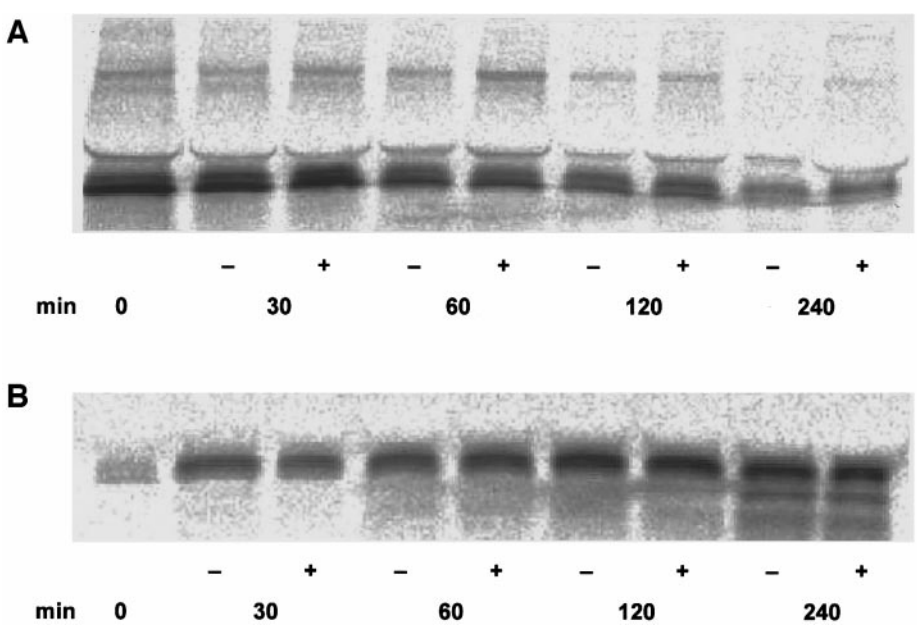

FIG. 2. The lactacystin-induced accumulation of apoE in human monocyte-derived macrophages is maximal at 30 min but is maintained for at least 4 h. Fourteen-day-old human macrophages were pulsed for 15 min with ^{35}S-labelled methionine and incubated for the times shown in RPMI 1640 cell culture medium with (+) or without (−) addition of 10 μM lactacystin. The cells were then harvested and immunoprecipitation of cell lysates containing 1.5 mg total cell protein (A) and corresponding media (B) was performed with anti-human apoE antibody. Following Western blot analysis, detection was by radiography.

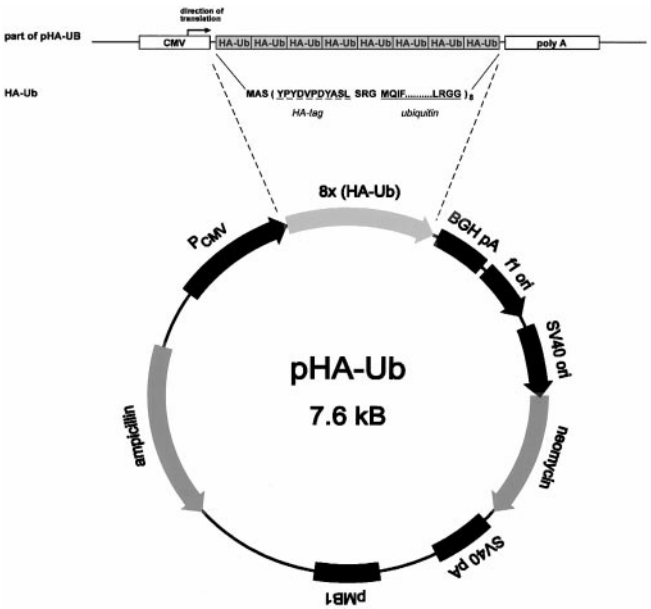

FIG. 3. Structure of the vector expressing hemagglutinin-tagged ubiquitin. The vector was used for stable transfection of murine RAW 264.7 macrophages and HepG2 cells.

ApoE Is Ubiquitinated in RAW 264.7 Macrophages

To investigate the stably transfected macrophage cell line for ubiquitination of apoE, a portion of the nontransfected and transfected cells was treated with lactacystin, and the cell lysates were immunoprecipitated using an antibody directed against mouse apoE and detected with an anti-hemagglutinin antibody. Conversely, lysates from both lactacystin-treated and untreated cells were immunoprecipitated with an anti-hemagglutinin antibody and detected with an anti-mouse apoE antibody (Fig. 5). In the cell lysates which were immunoprecipitated with an anti-apoE antibody and detected with an anti-hemagglutinin antibody, treatment of the cells with lactacystin led to the appearance of a smear of >60 kDa in size representing ubiquitinated apoE; no ubiquitinated apoE was detectable in the nonlactacystin-treated cells. A band of 50 kDa was seen in both the transfected and the nontransfected control cells which probably represents IgG heavy chain. In cell lysates which were immunoprecipitated with an anti-hemagglutinin antibody and detected with an anti-apoE antibody, transfected cells showed a smear of >40 kDa, which was greatly increased in intensity by incubation of the cells with lactacystin. In this series of experiments also, a band of 50 kDa was visible representing IgG heavy chain. It is to be expected that precipitation with anti-apoE and detection with anti-hemagglutinin detects larger species than precipitation with anti-hemagglutinin and detection with anti-apoE. In the former case, those

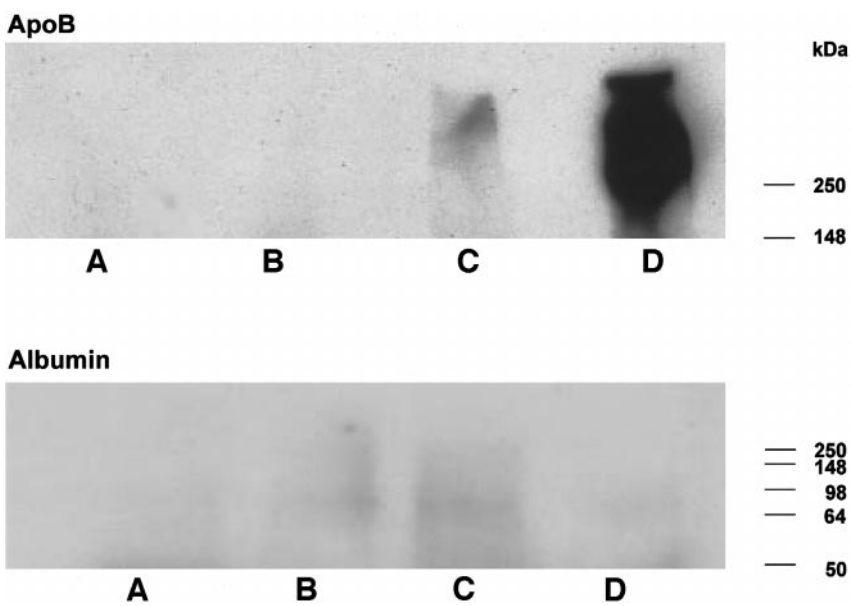

FIG. 4. Addition of lactacystin promotes accumulation of ubiquitinated apolipoprotein B, but not of albumin in HepG2 cells stably transfected with a hemagglutinin-ubiquitin construct. HepG2 cells were stably transfected with an expression construct coding for hemagglutinin-tagged ubiquitin and incubated for 1 h in DMEM cell culture medium with or without addition of 10 μM lactacystin. The cells were then harvested, immunoprecipitation of 2 mg of cell protein was performed with an antibody against human apolipoprotein B or human albumin as shown. Following Western blot analysis, detection was by means of a mouse monoclonal anti-hemagglutinin antibody. The secondary antibody used was horseradish peroxidase-labelled anti-mouse IgG. The image was developed using a chemiluminescence detection system. (A) Nontransfected HepG2 cells incubated without lactacystin; (B) Nontransfected HepG2 cells incubated in the presence of lactacystin; (C) Transfected HepG2 cells incubated without lactacystin; (D) Transfected HepG2 cells incubated in the presence of lactacystin.

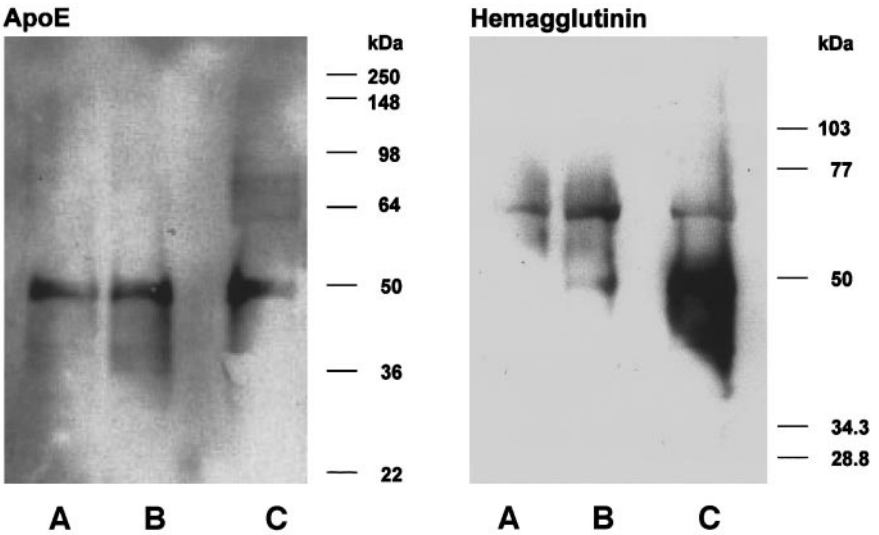

FIG. 5. ApoE is ubiquitinated in RAW 264.7 murine macrophages and addition of lactacystin promotes accumulation of ubiquitinated apoE. Cells of the murine RAW 264.7 cell line were stably transfected with an expression construct coding for hemagglutinin-tagged ubiquitin and incubated for 1 h in DMEM cell culture medium with or without addition of 10 μM lactacystin. Immunoprecipitation of 0.4 mg cell protein was performed with a goat polyclonal antibody against mouse apoE (left panel), or with a mouse monoclonal antibody against hemagglutinin (right panel). Following Western blot analysis, detection was by means of an anti-hemagglutinin antibody (left panel) or anti-mouse apoE antibody (right panel). The secondary antibody used was horseradish peroxidase-labelled anti-mouse IgG (left panel) or horseradish peroxidase-labelled anti-goat IgG (right panel). The image was developed using a chemiluminescence detection reagent. (A) Nontransfected RAW 264.7 cells (control) incubated in the presence of lactacystin; (B) Transfected RAW 264.7 cells incubated without lactacystin; (C) Transfected RAW 264.7 cells incubated in the presence of lactacystin.

apoE molecules which contain multiple ubiquitin-hemagglutinin tags (and which are therefore larger in size) bind more anti-hemagglutinin antibody and are preferentially displayed, while in the latter case the anti-hemagglutinin antibody efficiently precipitates even those apoE molecules which contain only one ubiquitin-hemagglutinin tag; however since each precipitated molecule contains only one apoE, all are displayed with equal intensity by the polyclonal anti-apoE antibody.

ApoE Is Ubiquitinated in HepG2 Cells

In direct analogy to the experiments in macrophages, a portion of the control and transfected HepG2 cells was treated with lactacystin and cell lysates were immunoprecipitated using an antibody directed against apoE and detected with an anti-hemagglutinin antibody (Fig. 6). As with the mouse macrophages, a smear of >70 kDa in size was detected in the lactacystin-treated HepG2 cells, indicating accumulation of ubiquitinated apoE.

DISCUSSION

Eukaryotes contain a highly conserved multienzyme system which covalently links a small 76-residue protein to a variety of intracellular proteins that bear degradation signals recognized by this system (14). Ubiquitin-dependent pathways are central in many biological processes including cell differentiation, cell cycle, apoptosis, signal transduction, DNA repair, transmembrane, and vesicular transport and stress responses (15–20). Our studies provide for the first time clear evidence of a role for the proteasome in the intracellular degradation of apoE and extend those of Duan *et al.* who showed that intracellular degradation of macrophage apoE could be inhibited by proteasomal inhibitors but who did not detect ubiquitinated apoE within the cell (10).

As noted in the introduction, two other proteins involved in lipid metabolism, apo(a) and apoB, also undergo ubiquitination (8, 9, 21–27). In the case of apo(a), ubiquitination and proteasomal degradation appear primarily to fulfil the function of quality control of proteins leaving the endoplasmatic reticulum, but also influence plasma levels of lipoprotein (a), an atherogenic lipoprotein which contains apo(a) covalently linked to apoB (8). By contrast, the main role of ubiquitination of apoB in the liver appears not to lie in quality control of this protein, but rather in the physiological regulation of the amount of apoB secreted by the cell (9). In addition, Du *et al.* have shown that the normally rapid proteasomal degradation of apoB53 in Chinese hamster ovary cells was reversibly blocked by transfection with the enzyme cholesterol 7α-hydroxylase via a sterol-sensitive ubiquitin conjugation step in the endoplasmatic reticulum (26).

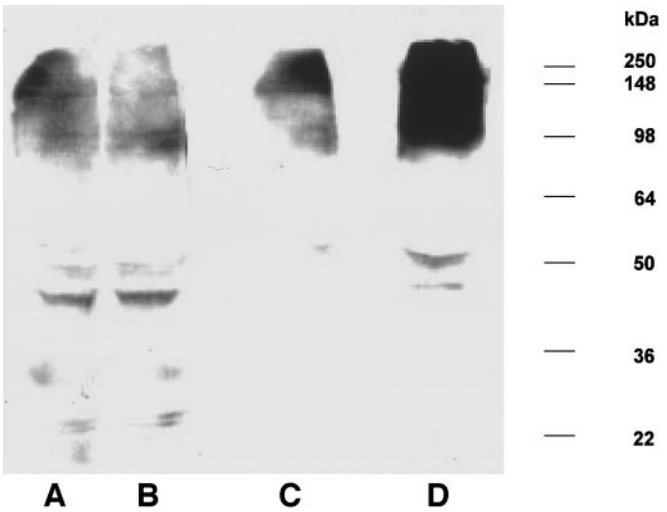

FIG. 6. ApoE is ubiquitinated in cells of the HepG2 hepatocyte cell line. Addition of lactacystin promotes accumulation of ubiquitinated apoE. HepG2 cells were stably transfected with an expression construct coding for hemagglutinin-tagged ubiquitin and incubated for 1 h in DMEM cell culture medium with or without addition of 10 μM lactacystin. Immunoprecipitation of 1.4 mg cell protein was performed with an antibody against human apoE. Following Western blot analysis, detection was by means of an anti-hemagglutinin antibody. The secondary antibody used was horseradish peroxidase-labelled anti-mouse IgG. The image was developed using a chemiluminescence detection reagent. (A) Non-transfected HepG2 cells incubated without lacatacystin; (B) Non-transfected HepG2 cells incubated in the presence of lactacystin; (C) Transfected HepG2 cells incubated without lactacystin; (D) Transfected HepG2 cells incubated in the presence of lactacystin.

In contrast to apoB, only a small portion of the apoE within the cell appears to undergo ubiquitination under the experimental conditions investigated by us. This is consistent with a primary function of ubiquitination of apoE in quality control. However this may not be its only role. We have shown that a discrepancy exists between the level of apoE mRNA within macrophages and the secretion of apoE by these cells, and that the degree of mismatch is influenced by the apoE phenotype (4). This phenomenon may at least partly due to phenotype-dependent differences in the ubiquitination of apoE in macrophages. It is also possible that the ubiquitination of apoE in macrophages, in analogy to that of apoB in hepatocytes, is influenced by cellular sterol metabolism. In macrophages, intracellular degradation of apoE is reduced, and secretion of apoE is increased, by loading with sterols (10, 28) or by modulating intracellular sterol transport (29), and this degradation is reduced by proteasome inhibitors (10). Proteasomal degradation of apoE in hepatocytes may of course also be subject to regulation by sterols. The investigation of these hypotheses is the subject of ongoing studies in our laboratory.

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