ApoO, a Novel Apolipoprotein, Is an Original Glycoprotein
Up-regulated by Diabetes in Human Heart*

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Obesity is an independent risk factor for cardiac failure. Obesity promotes excessive deposition of fat in adipose and nonadipose tissues. Intramyocardial lipid overload is a relatively common finding in nonischemic heart failure, especially in obese and diabetic patients, and promotes lipoapoptosis that contributes to the alteration of cardiac function. Lipoprotein production has been proposed as a heart-protective mechanism through the unloading of surplus cellular lipids. We previously analyzed the heart transcriptome in a dog nutritional model of obesity, and we identified a new apolipoprotein, regulated by obesity in heart, which is the subject of this study. We detected this new protein in the following lipoproteins: high density lipoprotein, low density lipoprotein, and very low density lipoprotein. We designated it apolipoprotein O. Apolipoprotein O is a 198-amino acid protein that contains a 23-amino acid-long signal peptide. The apolipoprotein O gene is expressed in a set of human tissues. Confocal immunofluorescence microscopy colocalized apolipoprotein O and perilipins, a cellular marker of the lipid droplet. Chondroitinase ABC deglycosylation analysis or cell incubation with p-nitrophenyl-β-D-xyloside indicated that apolipoprotein O belongs to the proteoglycan family. Naringenin or CP-346086 treatments indicated that apolipoprotein O secretion requires microsomal triglyceride transfer protein activity. Apolipoprotein O gene expression is up-regulated in the human diabetic heart. Apolipoprotein O promoted cholesterol efflux from macrophage cells. To our knowledge, apolipoprotein O is the first chondroitin sulfate chain containing apolipoprotein. Apolipoprotein O may be involved in myocardium-protective mechanisms against lipid accumulation, or it may have specific properties mediated by its unique glycosylation pattern.

Obesity prevalence is reaching around 10–30% of the population in industrialized countries, and this rate is doubling every 10 years (1). Obesity is leading to enhanced mortality (2) and cardiovascular morbidity because of arterial hypertension and endocrine-metabolic abnormalities, such as type II diabetes or dyslipidemia, which are frequently seen in the X metabolic syndrome (3). Moreover, obesity was shown to be an independent factor for cardiovascular risk (4). A positive correlation was noticed between being overweight and cardiovascular mortality, even after adjusting for the other risk factors. Clearly, obesity is an independent risk factor for cardiac failure. It was shown that obese subjects of both genders had a doubled risk for cardiac failure (5). However, the mechanisms involved in the development of heart failure in obese persons are far from being elucidated because few studies have looked specifically at the cardiac abnormalities associated with obesity. Obesity is characterized at a late stage by left ventricular hypertrophy, which leads to a metabolic change (6). A well known feature of obesity is the excessive deposition of fat in both adipose and nonadipose tissues. Excessive lipid accumulation in nonadipose tissue has been observed in pancreatic β-cells and in hearts from obese rats (7, 8). In humans, fatty infiltration of the heart has been identified in ventricular and atrial myocardium (9–11). Recently, myocardial triglyceride accumulation has been positively correlated with body mass index and left ventricular mass (12). In addition, intramyocardial lipid overload was shown to be relatively common in nonischemic heart failure, especially in obese and diabetic patients (13).

Lipotoxicity and induction of lipoapoptosis have been described as a consequence of the accumulation of excessive lipids in nonadipose tissues (14, 15). These lipotoxic phenomena are responsible, at least in part, for the alterations of cardiac function (16). Thus, the heart has to be protected from excessive lipid accumulation when lipid influx overflows the energy needs and the oxidative abilities of the heart. Expression of apolipoprotein B was shown to protect the heart from triglyceride accumulation in transgenic mice (17). Therefore, lipoprotein production by the heart has been proposed as a protective mechanism to unload surplus cellular lipids (18–20).

We and others developed a dog nutritional model of obesity using a hypercaloric high fat diet (21, 22) in which we recently showed early and late specific gene regulations at the transcriptome level in heart from these animals (23, 24). Differentially expressed genes, when compared with regular fed animals, were categorized into groups involved in metabolism, cell signaling, ionic regulation, cell proliferation, protein synthesis, and tissue remodeling. In addition, we found a set of cDNAs encoding proteins with unknown functions. Analysis of the coding sequences identified a putative new apolipoprotein, reg-
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ultated by obesity in the heart, which is the subject of this study. Indeed, we found this protein in lipoproteins, thus we propose to name it apoO because the last discovered apolipoprotein had been named apoN (25).

EXPERIMENTAL PROCEDURES

Analysis of ApoO cDNA Nucleotide and Polypeptide Sequence
cDNA sequences of differentially expressed genes were searched against the GenBank™ nonredundant data base using NCBI Blast. Open reading frames were translated into polypeptide sequences and analyzed for potential subcellular localization using Psort (26) and SMART (27), which predict signal peptide and transmembrane sequences. Conserved domains and protein family homologies were tested by scanning against PROSITE and PHOSPHO_SITES with PrositeScan (Swiss Institute for Bioinformatics) and ProDom motif data base (28). In addition, peptide mass was calculated using the Peptide Mass program available on line. Multiple sequence alignments were done after the BLAST search of protein homology using the Protein Sequence Multiple Alignment program available on line. The predicted secondary structure of apoO was determined by computerized structure analysis of the deduced amino acid sequence from apoO using the nmpredict program (30).

Tissue and Lipoprotein Sources

All animal procedures were performed according to the French Ministry of Agriculture guidelines. After ethical committee approval, all patients included in the study gave their written consent for sample collection and molecular analysis prior to their inclusion. Patients were carefully selected by the physicians from the Department of Cardiology, Toulouse University Hospital, prior to cardiac surgery for coronary by-pass resulting from coronary disease. Six diabetic patients out of 17 (35%) were on insulin. A similar proportion of dyslipidemic patients was observed in diabetic (35%) and nondiabetic (31%) patients groups. Despite the same proportion (6/17), the insulin-treated patients group was not identical to the dyslipidemic patients groups. Indeed, we found this protein in lipoproteins, thus we propose to name it apoO because the last discovered apolipoprotein had been named apoN (25).

Preparation of RNA and Northern Blot Analysis

Total cellular RNA was isolated using TRIzol (Invitrogen) according to the manufacturer’s instructions. Twenty µg of total RNA were loaded per lane, and Northern blot analysis was performed using standard procedures. For human tissue expression analysis, the human endocrine system multiple tissue Northern blot (Clontech) that contains 2 µg of poly(A)+ mRNA per lane was used. A 246-bp dog apoO cDNA fragment (85% homologous to the nucleotides 173–389 of the human apoO cDNA, GenBank™ accession number AF061264; 85% homologous to nucleotides 173–389). Because My025 cDNA is shorter but 100% homologous to the putative protein MGC4825, gene card information for this protein was collected. Peptide mass was calculated using the Peptide Mass program available on line. Multiple sequence alignments were done after the BLAST search of protein homology using the Protein Sequence Multiple Alignment program available on line. The predicted secondary structure of apoO was determined by computerized structure analysis of the deduced amino acid sequence from apoO using the nmpredict program (30).

Real Time PCR Monitoring of mRNA Levels

Oligonucleotides were synthesized by Proligo and designed with Primer Express software. The sequences are as follows: MTP forward, 5’-TGTTCAGCAGTTGCCCTGGA-3’, and reverse, 5’-CATATTGTGAGCCGACATTTC-3’; apoO forward, 5’-ATATGAACTGGACCTGCACCA-3’, and reverse, 5’-AACTTGCAAAATGCCCATCTC-3’; apoO, forward, 5’-GTTTCGCAAGGACATCGCTTC-3’, and reverse, 5’-AAACCCCATTTGAACCACTCTT-3’; and 18 S, forward, 5’-CGCGCCTAGAGGTTGAAATCC-3’ and reverse, 5’-TGCTGACCTTGGTATGATTTA-3’. Real time PCR was performed with the SybrGreen master mix reagent in a GeneAmp 5700 apparatus (Applied Biosystems). Standard curve method was used for relative quantification of the PCR products, and gene expressions were normalized to 18 S RNA quantification. Real time PCR was statistically analyzed with SigmaStat software (SPSS).

Antigen Preparation and Rabbit Immunization

According to the human apoO cDNA sequence and the deduced amino acid sequence, two peptides were synthesized as follows: PR-16CK1, CYAAPKKSPPKNSVK; PR-16CK2, CNFQPNGVKNSPGTK. The peptides were coupled to key-hole limpet hemocyanin according to standard procedures. After checking for the absence of autoimmune antibodies, two rabbits were intradermally injected with antigen every 2 weeks for 6 weeks. The rabbit immune response was checked by enzyme-linked immunosorbent assay, and strong immunoreactivity against the peptides used for immunization was seen (data not shown).

Protein Extractions and Western Blot Analysis of Proteins

Human tissues were disrupted in mammalian cell lysis kit solution (Sigma) in the presence of mammalian protease inhibitors mix (Sigma), procedures were performed according to the manufacturer’s protocol. Sixty micrograms of protein were run

*The abbreviations used are: apo, apolipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; VLDL, very low density lipoprotein; MTP, microsomal triglyceride transfer protein; RTS, rapid translation system; CMM, canine pancreatic microsomal membrane; DMPC, dimyristoylphosphatidylcholine; PBS, phosphate-buffered saline; NBD, 22-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)).
on a 10% SDS-polyacrylamide gel and blotted onto 0.45-μm nitrocellulose membrane BA85 (Schleicher & Schuell). Multi-
Mark multicolored standard (Invitrogen) was used to deter-
mine the size of the proteins. For Western blot, nitrocellulose 
membranes were blocked for 2 h in TBS (10 mm Tris, pH 7.5, 
150 mm NaCl) with 0.1% Tween 20 and 5% nonfat dry milk. 
Antiserum incubation (1/3000 dilution) was performed in TBS/
Tween 0.1% for 2 h. After three washes in TBS/Tween 0.1%, an 
anti-rabbit IgG horseradish peroxidase conjugate (10-4 diluted) 
(Bio-Rad) was incubated for 2 h with the membrane in 
TBS/Tween 0.1%, 5% nonfat dry milk. Blots were washed three 
times in TWEEN/TBS, once with TBS, and then developed using 
Supersignal West Pico chemiluminescent substrate (Pierce), 
and signal detection was made using BioMax light film (East-
man Kodak).

Construction of Expression Vectors for Human ApoO

ApoO cDNA generated from reverse transcription of mRNA 
from human subcutaneous adipocytes was PCR-amplified 
using Taq polymerase (Invitrogen) and the following primers: 
forward, 5’-CAT GCC ATG GTC AAG GTA ATT CAG AGG 
TCC GTG GGG C-3’, and reverse, 5’-CC GCT CGA GCT 
TAG TTT CAG GTG AAT TCT TCA CAT TTC CTG G-3’. 
PCR products were then purified on Nucleospin columns 
(Macherey & Nagel), digested by the NcoI and Xhol enzymes, 
and ligated by T4 ligase (Invitrogen) to the NcoI and Xhol sites 
in the pQETrysmid plasmid expression vector (Qiagen) that 
harbors two promoters, which allowed for production of the 
recombinant protein in bacterial and mammalian cells. To 
generate pIVEX-apoO plasmid, apoO cDNA was amplified using 
the forward primers 5’-CAT GCC ATG GTC AAG GTA ATT 
CAG AGG TCC GTG GGG C-3’ and reverse 5’-CCC CCC 
GGG CTT AGT TCC AGG TGA ATT CAT TCC TCA CAT TCC CTG G-3’ and was further cloned in the NcoI/SmaI sites of 
pIVEX2.3 (Roche Applied Science) for cell-free coupled in vitro transcription/translation large scale protein production in a 
proteomaster instrument (Roche Applied Science) using the 
RTS 500 proteomaster kit (Roche Applied Science). The cloned 
cDNAs were verified by DNA sequencing using ABI PRISM® 
BigDye™ Terminator version 3.1 Ready reaction cycle 
sequencing kit (Applied Biosystems) and loaded on an ABI 
3100 capillary electrophoresis DNA sequencing instrument 
(Applied Biosystems).

Cell Culture and Transfection

3T3-F442A, COS-1, and HepG2 cells were cultured under conditions as described previously (32, 33). Because HepG2 
cells produced large amounts of apoO as well as other apoli-
ipoproteins like apoB and because this cell line was already used in 
studies dealing with MTP inhibitor effects on apolipoprotein 
secretions (34, 35), we used this cell line to evaluate the effect of 
drugs on apoO secretion and glycosylation. For transfection 
experiments, COS cells were transfected with expression 
pQETrysmid plasmids encoding human apoO (15 μg of 
DNA) using the calcium phosphate precipitation technique, 
essentially as described (36), and 48 h after transfection, the 
cells were lysed, and total protein was assayed by Western blot.

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Plasmids pQETri were electrotransformed into Escherichia coli 
DH5α.

Confocal Microscopy Immunofluorescence Analysis

Anti-ApoO Antibody Fluorescent Labeling—IgGs of anti-
apoO serum were purified by Biologic LP low pressure liquid 
chromatography system (Bio-Rad) with protein A-Sepharose 4 
Fast Flow column (Amersham Biosciences). Binding buffer was 
50 mM Tris, pH 7, and elution buffer was 0.1 M glycine, pH 3, 
flow rate was 1.5 ml/min. Elution fractions containing IgG were 
concentrated using a Centricon filter device with YM-3 mem-
brane (Millipore) to a final concentration of 1 mg/ml. These 
IgG were labeled with FluoroLink-Antibody Cy5 labeling kit 
(Amersham Biosciences), following the manufacturer’s 
instructions.

Fluorescence Immunodetection—H9c2 cells were seeded 
on Falcon culture slides (BD Biosciences). Subconfluent cells 
were washed twice in phosphate-buffered saline (PBS) and 
fixed in PBS containing 4% formaldehyde for 15 min at room 
temperature followed by 5 min at 20 °C. Cells were then 
then washed five times in PBS and incubated for 20 min at 37 °C 
in protein block serum-free buffer before being incubated for 90 
min at 37 °C with a guinea pig anti-perilipin serum (Tebu-bio) 
diluted to 1/200 in protein block serum-free buffer. Cells were 
then washed once with PBS and incubated for 60 min at 37 °C 
with a fluorescein isothiocyanate-labeled goat anti-guinea pig 
immunoglobulin secondary antibody (1/200) in protein block 
serum-free buffer. For apoO detection, secondary incubation 
was unnecessary because it was labeled with Cy5 (see above). 
Cells were washed five times with PBS and covered with a few 
drops of fluorescent mounting medium and coverslips before 
being analyzed on a Zeiss LSM 510 confocal microscope (Carl 
Zeiss).

Evaluation of ApoO Secretion

MTP Inhibitor Treatment—HepG2 cells were grown in 
6-well plates, to 80% confluence in Dulbecco’s modified Eagle’s 
medium containing 10% fetal bovine serum, penicillin (100 
units/ml), and streptomycin (100 μg/ml). Medium was 
renewed every 2 days. Cells were incubated in a gassed, humid-
fied incubator (5% CO2 at 37 °C). HepG2 cells were treated 
with several dilutions of MTP inhibitors, naringenin (Sigma) or 
CP-346086 (Pfizer) or vehicle as a control and further diluted in 
culture media for 24 h. Conditioned media proteins were pre-
ceptitated with cold acetone. One volume of media with 4 vol-
umes of cold acetone were kept overnight at −20 °C. This mix-
ture was centrifuged for 15 min at 11,000 × g and at 4 °C, and 
the pellet was air-dried and resuspended in denaturing buffer 
for Western blot analysis. ApoO and apoB in media were 
detected by immunoblotting in 10% SDS-PAGE with rabbit 
anti-apoO antisera and goat anti-apoB (Chemicon), diluted 
1/3000E each. Secondary antibody were goat anti-rabbit IgG 
horseradish peroxidase conjugate (Bio-Rad) and donkey anti-
 goat IgG horseradish peroxidase conjugate (Santa Cruz Bio-
technologies), respectively; 1/10,000E dilutions were used for 
each antibody. ApoO and apoB bands were quantified by den-
sitometry with Image J software.
Naringenin Treatment—Naringenin was first dissolved in Me2SO (Me2SO concentration in cell culture media was under 0.2%). HepG2 cells were treated with 0, 10, 50, 100, or 200 μM naringenin.

CP-346086 Treatment—CP-346086 was first dissolved in Me2SO (Me2SO concentration in cell culture media was under 0.1%). HepG2 cells were treated by 0, 5, 10, 50, or 100 nM CP-346086; Me2SO was added in control dishes at maximal concentrations.

Determination of ApoO Glycosylation

Deglycosylation of ApoO by Chondroitinase ABC—Chondroitinase ABC (EC 4.2.2.4) (Sigma C-2905) digestions were performed according to Sigma protocol. Briefly, 60 μg of total serum proteins were incubated at 37 °C for at least 2 h in 50 mM Tris, 60 mM sodium acetate, 0.02% (w/v) bovine serum albumin, and 0.1 unit/ml chondroitinase ABC.

p-Nitrophenyl-β-D-xyloside Treatment—HepG2 cells were treated for 24 h by 0, 0.5, 1, 2, or 4 mM p-nitrophenyl-β-D-xyloside (Sigma) diluted in culture media. p-Nitrophenyl-β-D-xyloside was solubilized in methanol (methanol concentration in cell culture media was under 0.3%). Cells were scraped and lysed in mammalian cell lysis kit reagent (Sigma) following the manufacturer’s instructions. Protein quantities were measured by DC protein assay (Bio-Rad). 30 μg of HepG2 proteins were immunoblotted in 10% SDS-PAGE with rabbit anti-apoO antibody (1/3000 dilution), and then goat anti-rabbit IgG horseradish peroxidase conjugate (1/10,000 dilution) was incubated with the membrane for 2 h. ApoO signal was quantified by densitometry with Image J software.

In Vitro Glycosylation of ApoO

ApoO was produced by in vitro transcription/translation using rabbit reticulocyte lysate systems (Promega), and pIVEX-apoO plasmid, combined with several doses of canine pancreatic microsomal membranes (Promega), according to the manufacturer’s instructions. [35S]Methionine was incorporated into apoO during the translation. 6 μl of each sample were applied to a 10% SDS-PAGE, and digital imaging of the detected radioactive apoO was performed using a Typhoon 9400 (Amer sham Biosciences).

ApoO Stimulation of Cholesterol Efflux

J774 mouse macrophages (ECACC) were grown in 6-well plates up to 80% confluence, in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, glutamine (2 mM), penicillin (100 units/ml), and streptomycin (100 μg/ml). Medium was renewed every 2 days. Cells were incubated in a gassed, humidified incubator (5% CO2 at 37 °C). J774 cells were incubated for 24 h with 5 μM NBD-cholesterol (Invitrogen), a fluorescent analogue of cholesterol, diluted in culture media. NBD-Cholesterol was first dissolved in pure ethanol (ethanol concentration in cell culture media was under 0.2%). Then cells were washed with culture medium and incubated for 12 h in culture medium with 0.2% bovine serum albumin fatty acid-free (Sigma) and 0.3 mM cAMP (Roche Applied Science). Cells were washed with culture medium. Reference wells were extracted with mammalian cell lysis kit (Sigma) following the manufacturer’s instructions. These wells provided base-line (time 0) values for total NBD-cholesterol content. Others wells were incubated for 4 h without or with recombinant apoA1 or recombinant apoO (see below) diluted at 10 μg/ml in culture media. Cellular cholesterol efflux was quantified by measuring the release of cellular NBD-cholesterol into the medium as a function of time. 50-μl aliquots of the incubation medium were centrifuged for 2 min at 100 × g to pellet any floating cells. Fluorescence was read with Fluoroskan ascent FL (Labsystems), set at excitation 470 nm and emission 530 nm. The percentage of cholesterol efflux was calculated by dividing the amount of fluorescence released into the medium by the total fluorescence in the cells at time 0 (37).

Production and Purification of Recombinant Apolipoproteins

Production and Purification of Recombinant ApoA1—The plasmid pET30-apoA1 (38) was transfected into Rosetta E. coli cells (Novagen). Cells were grown on Luria-Bertani (LB) medium with 30 μg/ml kanamycin (Euromedex) and 34 μg/ml chloramphenicol (Sigma) at 37 °C with shaking at 300 rpm. A 200-ml culture was grown until A600 nm reached 0.6. This culture was induced by 1 mM isopropyl β-D-thiogalactoside final (Invitrogen) and was grown for 4 h. Cells were pelleted at 5,500 × g for 20 min at 4 °C, frozen in liquid nitrogen, and resuspended in binding buffer (50 mM NaH2PO4, 300 mM NaCl, 5 mM imidazole, pH 8). Cells were lysed on ice by sonication, and insoluble proteins were pelleted by centrifugation at 5,500 × g for 30 min at 4 °C. Supernatant contained soluble apoA1 His-tagged. Recombinant apoA1 was purified using nickel-nitritolactriatic acid-agarose (Qiagen) under native conditions following the manufacturer’s recommendations with a Biologic LP low pressure liquid chromatography system (Bio-Rad). Wash buffer was binding buffer with 250 mM imidazole (Sigma) and elution buffer was the same buffer with 250 mM imidazole, flow rate was 1 ml/min. Elution fractions containing apoA1 were dialyzed in PBS at 4 °C overnight.

Production and Purification of Recombinant ApoO—ApoO was produced by cell-free coupled in vitro transcription/translation system in a proteomaster instrument (Roche Applied Science) using the RTS 500 proteomaster kit (Roche Applied Science) and pIVEX2.3-apoO vector. Recombinant apoO His-tagged was purified using nickel-nitritolactriatic acid-agarose in batch upon an artificial chaperone-assisted refolding method (39). Purification protocol was performed as published previously (40). Briefly, 100 mM NaH2PO4, 10 mM Tris-HCl 8 M urea (Sigma), pH 8, was used as binding buffer, and successive wash buffers were binding buffer supplemented with 20 mM imidazole, pH 6.3, then with 10 mM β-mercaptoethanol (Sigma), and then with buffer A (20 mM Tris-HCl, 100 mM NaCl, pH 8), including 0.1% Triton X-100 (Sigma), and then buffer A supplemented with 5 mM β-cyclodextrin (Sigma) and buffer A supplemented with 500 mM NaCl. Elution buffer was buffer A, including 500 mM imidazole. ApoO-containing elution fractions were dialyzed against PBS at 4 °C overnight.

Turbidimetric Clearing Assay

The ability of apoO to reorganize DMPC multilamellar liposome into apoO-DMPC discoidal complexes was determined...
by the decrease in turbidity (clearing) of DMPC liposome solution. Multilamellar DMPC liposomes were prepared using 10 mg of DMPC (Avanti Polar Lipids) as published previously (41). Kinetics of apolipoprotein and lipid association was followed by the decrease in absorbance at 325 nm that accompanies the transformation of the multilamellar liposomes into discoidal lipoprotein particles (42). The kinetics was performed at 24 °C on an agitated multiwell plate loaded in a Labsystems iEMS Reader MF. The reaction was performed at a 1/1 lipid to protein molar ratio using 17 μl of DMPC (2 mg/ml) in a 200-μl final volume in 20 mM Tris, pH 7.5.

**Statistical Analysis**

All results were depicted as means ± S.E. Multiple comparisons were analyzed using analysis of variance followed by the ad hoc post hoc test using SigmaStat 8 software (SPSS). Single comparisons were performed using unpaired Student’s t test with a value of \( p \leq 0.05 \) considered as significant.

**RESULTS**

Identification of ApoO as a Novel Apolipoprotein Conserved among Species—Sequence analysis of differentially expressed genes in obese hypertensive dog heart provided a set of 11 cDNA sequences encoding new protein sequences (23). One of these cDNA sequences encoded an uncharacterized polypeptide chain containing a signal peptide. Therefore, attention was given to this cDNA sequence to determine whether it encoded a new secreted protein and a new plasmatic marker of disease. Analysis of this cDNA sequence with RPS-Blast identified the polypeptide motif pfam01442 homologous to the amino acids 40–83 of a new protein, MGC4825 (Fig. 1A). The pfam01442 motif contains a 22-residue repeat, which forms a pair of \( \alpha \)-helices that is a signature of the apolipoprotein A1, A-IV, and E family. The observation and detection of this new protein in lipoproteins (see below) led us to call this protein apoO. Chromosomal localization of apoO was found at Xp22.11 in Entrez.
Gene (NCBI). This chromosomal location is a locus related to X-linked mental retardation (43) and the sex-reversing locus (44). The apoO gene was precisely localized by sequencing at 36294 and 73.5%, respectively (Fig. 1A). Sequence multiple alignment with other vertebrate species ranging from mouse to Xenopus displayed a strong conservation of the protein sequence. Indeed, HomoloGene cDNA sequence homology analysis to humanapoO with several orthologues found apoO conserved in sequence homology analysis to human apoO with several orthologues from various species is shown in Fig. 1A.

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Incubation with nonimmune serum or conjugate alone did not reveal any band (Fig. 4A and data not shown). Serum from immunized rabbit revealed one intense 55-kDa band in human auricle that was even stronger in human serum. This 55-kDa band was present at similar amounts in HepG2 cell lysates but was weak in mouse P19 differentiated cardiomycocytes. We observed a faint smaller band in human auricle that was also observed with nonimmune sera, and it was considered to be nonspecific (Fig. 4A).

To check for the specificity of our anti-apoO antibody, we used in vitro (RTS) produced apoO as a competitor in Western blot detection of apoO. Incubation of RTS transcription/translation reagents with apoO antibody did not affect the Western blot except for the very low amounts of loaded apoO (Fig. 4B, panel B). Preincubation with 1 and 10 μl of RTS produced apoO progressively and specifically abolished the detection of apoO either in human serum or when recombinant protein was loaded.

ApoO was also detected in mouse, human, and dog sera (Fig. 4C). Our anti-apoO antibody detected the rat apoO in sera (data not shown). Because we had found expression of the apoO gene in adipose tissue, we checked whether apoO was indeed secreted by mouse 3T3-F442A adipocytes. Western blot analysis of 3T3-F442A differentiated adipocytes conditioned media showed a single 55-kDa band (Fig. 4D). This band had a weak intensity at early adipocyte differentiation stage, and a much stronger intensity was observed at day 10 of differentiation. This observation was in accordance with the apoO gene expression checked by Northern blot analysis of mRNA from these cells (data not shown). Thus, a molecular mass of 55 kDa was not in accordance with the predicted molecular weight of apoO according to the cDNA coding sequence, and several experiments were undertaken to elucidate this feature.

Production of Recombinant ApoO in Bacterial and COS-1 Cells Indicates Cleavage of the Signal Peptide—Western blot analysis of the recombinant protein produced either in E. coli DH5α or COS-1 cells displayed a molecular mass of 22 and 20 kDa, respectively (Fig. 5). Because prokaryotic cells cannot mature eukaryotic proteins, this molecular mass difference suggests that the apoO signal peptide is cleaved in mammalian cells to generate the 20-kDa band.

The 55-kDa ApoO Complex Contains Sugars—Because the molecular weight for apoO observed by Western blot analysis

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displayed a strong discrepancy with the predicted size, we tested several enzymes and chemical treatments to attempt to decipher this phenomenon. Chondroitinase ABC digestion of human sera released a 20-kDa band as expected for apoO (Fig. 6A). Incubation buffer pH variations, use of increased amount of enzyme, or extended digestion time of human sera by chondroitinase ABC did not improve the digestion yield (data not shown). Chondroitinase ABC treatment showed that chondroitin sulfate glycans (GAG) are involved in the detected complex. Because GAGs are O-linked to Ser first through a xylose subunit (46), we investigated whether p-nitrophenyl-β-D-xyloside, a “primer” compound that competes with the extension enzymes and blocks extension of GAG chains from the initial xylose on the polypeptide chain (47), would have any effect on the immunodetection pattern. We observed a dose-responsive increase in the release of a 20-kDa form of apoO when HepG2 cells were treated with this compound (Fig. 6B). To provide further evidence of apoO glycosylation, an in vitro transcription/translation system coupled to an in vitro glycosylation system (CMM) was used. 22-kDa apoO was produced by transcription/translation system. Gradual addition of CMM led to a lower production of apoO as usually observed (note from Promega) and to a dose-dependent generation of the 55-kDa apoO in presence of CMM. Treatment of TNT in in vitro glycosylation reactions with p-nitrophenyl-β-D-xyloside and digestion of the reaction product with chondroitinase ABC led to the apparition of a 22-kDa band that is in accordance with the molecular weight of the nonglycosylated apoO (Fig. 6C).

**ApoO Is Present in Human Lipoproteins and Transforms Multilamellar Liposomes into Discoidal Lipoprotein Particles**—Western blot analysis revealed a single 55-kDa band in human lipoprotein fractions. ApoO was found mostly in the nonlipoprotein fractions and in HDL3 and HDL2 (data not shown) but was also present at weak concentrations in LDL nonlipoprotein fractions and in HDL3 and HDL2 (data not shown). Western blot analysis revealed a single 55-kDa band in human lipoprotein fractions. Avocado and potato were transfected either into E. coli/DH5α or in mammalian COS-1 cells.

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**Figure 5.** Production of recombinant apoO in E. coli and in mammalian cells. pQE-tri-apoO expression vector that allows for protein expression in E. coli, insect, and mammalian cells was transfected either into E. coli/DH5α or in mammalian COS-1 cells.

**Figure 4.** A, Western blot analysis of apoO protein in mouse cardiomyocyte differentiated P19 cellular extracts, human serum, human auricle, and human hepatoma cells (HepG2). 30 μg of each proteins sample were loaded per lane. B, competition of anti-apoO antibody with recombinant apoO (RTS-apoO) analyzed by Western blot analysis. 10 μl of anti-apoO antisera (corresponding to 1/3000 dilution in Western blot) were incubated for 30 min at room temperature with mock (A), 10 μl of RTS (B), 1 μl of RTS-apoO (C), or 10 μl of RTS-apoO (D). These mixtures were used for detection of apoO as primary antibody in Western blot. Lane 1, 0.1 μl of RTS-apoO; lane 2, 0.3 μl of RTS-apoO; lane 3, 1 μl of RTS-apoO; lane 4, 30 μg of human serum proteins; lane 5, 60 μg of human serum proteins; and lane 6, 90 μg of human serum proteins. C, Western blot detection of apoO in sera from mouse, human, and dog. ApoO was also detected in the rat (not shown). 30 μg of sera proteins were analyzed. D, Western blot detection of secreted apoO during adipocyte differentiation of the mouse 3T3-F442A preadipocyte cell line at days 0, 7, and 10 (D0, D7, D10). 60 μg of each proteins sample were loaded per lane.
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A

kDa

55

20

20-kDa apoO level (% of total apoO)

B

C

kDa

55

22

CMM (μl) 0 0.3 1.5 3 6

FIGURE 6. Glycosylation analysis of apoO. A, Western blot analysis of chondroitinase ABC digestion. T, treated with chondroitinases ABC; C, control. 60 μg of total human serum proteins were digested and analyzed. B, inhibition of apoO glycosylations by p-nitrophenyl-β-D-xyloside. HepG2 cells were treated for 24 h by only methanol or by 0, 0.5, 1, 2, or 4 mM of p-nitrophenyl-β-D-xyloside diluted in methanol. ApoO in cell extracts was detected by immunoblotting and quantified by densitometry. 20-kDa apoO relative quantification was expressed in percentage of total apoO (55 + 20-kDa forms). Values are means of triplicate ± S.D. **, p < 0.005; ***, p < 0.001 versus control; §§, p < 0.005; §§§, p < 0.001 versus methanol; #, p < 0.05; ##, p < 0.005 versus 0.5 mM. C, in vitro glycosylation of apoO. In vitro transcription/translation of apoO was performed with different doses of CMM, which is an in vitro glycosylation system (lanes 1–4). This experiment was repeated in the presence of 4 mM of p-nitrophenyl-β-D-xyloside (lane 5). The apoO product obtained with microsomes was treated at 37 °C with 0.5 units of chondroitinases ABC for 3 h (lane 6). Sample were applied to SDS-PAGE separation, and dried gel was exposed in a PhosphorImager cassette and visualized by scanning in a Typhoon 9400 apparatus.

ApoO Promotes Cholesterol Efflux—ApoA1 is known to stimulate cholesterol efflux from mouse macrophage J774 cells (37). Using a fluorescent analog of cholesterol, NBD-cholesterol (49), we observed that apoO stimulates cholesterol efflux from J774 cells as efficiently as apoA1 (Fig. 11).

Discussion

In our previous obese heart transcriptome analysis (23, 24), we had identified a set of 63 differentially expressed genes, and 11 cDNA sequences representative of mRNAs coding for new proteins of unknown functions were found to be differentially expressed in atria after 9 weeks of high fat diet (23). One cDNA that was already present in expressed cDNA data bases was found to encode for a potentially new putative secreted protein. Computer analysis of the polypeptide sequences for this new protein further identified sequence homology to a signature motif found in the A1/A4/E apolipoprotein family. We effectively detected this new protein in human sera and in human lipoproteins by Western blot analysis. According to current apolipoprotein nomenclature and because it is detected in lipoproteins, we named it apolipoprotein O (apoO). Recombinant apoO was specifically detected with our apoO antibodies in crude cell lysates and in cell-free systems (RTS, Roche Applied Science). Western blot competition analysis with recombinant apoO abolished the detection of apoO in human sera (Fig. 4B). Therefore, we can conclude that our rabbit antibodies specifically detect apoO. ApoO is present in several lipoprotein subtypes (HDL, LDL, and VLDL), which is also observed for some other apolipoproteins such as apoC (50) and apoE (51). ApoO is probably an exchangeable apolipoprotein because it is found on HDL, LDL, and VLDL, and its secretion is inhibited by MTP inhibitors. Moreover, apoO in the presence of liposome formed discoidal lipoprotein that is a feature of exchangeable apolipoproteins like apoA1 (52), apoE (53), or apoLp-III (41). However, apoO was mainly present in HDL and in a much lower amount in LDL and VLDL. ApoO secretion is MTP-dependent, like apoB secretion. It is likely that apoO is secreted as a VLDL-associated protein, which would be transferred to HDL (data not shown).

Northern blot analysis displayed a minor 0.9-kb band and a major 1.1-kb band that were in accordance with the cDNA sequences present for proteins Myo25 and MGC4825 in data bases. Sequence analysis of the 1.1-kb mRNA encoding apoO predicted a molecular mass of 22 kDa, including a 2-kDa signal peptide that could be cleaved. Surprisingly, a higher molecular size (55 kDa) signal was detected by Western blot of sera, cell culture extracts, and tissues samples. This feature could be explained by the possible association of apoO in multimers, by covalent linkage to other polypeptide chains by sugar bridges, as observed in the inter-α-inhibitor family of human plasmonic proteins (54), or by heavy glycosylation of the protein (52). We found we could remove the sugar components of the 55-kDa complex with chondroitinase ABC digestion, and we detected a 20-kDa band as predicted. This 20-kDa band corresponds to the translated MGC4825 polypeptide size. We did not observe a smaller band of 18-kDa that could be predicted by Myo25

ApoO Gene Expression Is Up-regulated in Diabetic Patients and Correlates with MTP and ApoB Gene Expression—Real time PCR analysis of apoO gene expression in 54 human right appendage biopsies displayed a 60% up-regulation of apoO gene expression in diabetic patients (Fig. 10A). Interestingly, we observed correlation of expression of apoB and apoO (R = 0.57; p = 2.35 × 10⁻⁵) (Fig. 10B). In addition, MTP gene expression was found to correlate with apoO expression (R = 0.656; p = 2 × 10⁻⁷) (Fig. 10C) in human heart.

ApoO is known to stimulate cholesterol efflux from mouse macrophage J774 cells (37). Using a fluorescent analog of cholesterol, NBD-cholesterol (49), we observed that apoO stimulates cholesterol efflux from J774 cells as efficiently as apoA1 (Fig. 11).
sequence. It is possible that only one form is translated or stable. Moreover, the 0.9-kb band may be generated by known apoO pseudogene transcription (55).

Indeed, NetOGlyc 2.0 software (56) predicted multiple glycosylation sites for apoO. Digestion with other enzymes, such as endoglycosidase H, O-glycosidase, peptide:N-glycosidase, sialidase A, neuraminidase, and hyaluronidase, had no effect on the complex, which is in accordance with the specific structure of chondroitin sulfate-linked glycosylation. In addition, treatments with strong reducers (β-mercaptoethanol, NaSCN) had no effect on the 55-kDa band (data not shown). A 5-min room

FIGURE 7. ApoO is present in different lipoprotein subclasses and forms discoidal lipoprotein. A, lipoprotein subspecies, nonlipoprotein fraction (NLF), HDL, LDL, and VLDL were isolated from normolipidemic plasma by sequential ultracentrifugation. Each class of lipoprotein was then analyzed by Western blotting. 30 μg of each proteins sample were loaded per lane. B, DMPC turbidity at 325 nm. DMPC liposomes and recombinant apoO or recombinant apoA1 were combined at a 1:1 molar ratio and incubated at 24 °C.

FIGURE 8. Immunocolocalization of apoO and perilipins in H9c2 cardiomyocytes. A, confocal fluorescence detection of apoO; B, confocal fluorescence detection of perilipin; C, merge of the apoO and perilipin detection (magnification, ×250).
temperature incubation of sera proteins with an NaOH concentration ranging from 0 to 100 mM, known to remove classical O-glycosylation (57), led to progressive and partial release of the 20-kDa protein, but overnight incubation destroyed most of the protein (data not shown). Thus, we used chondroitinase digestion of apoO to free the 20-kDa apoO. Chondroitinase digestion was not 100% efficient, and we could get at best 50% digestion of the 55-kDa apoO complex (Fig. 6A). Moreover, only chondroitinase ABC released apoO. Because increased amounts of enzyme or extended digestion times did not improve the digestion yield, we cannot exclude that a fraction of apoO is held by other covalent links with other molecules. The isolation by ultrafiltration of the 20-kDa apoO from the 55-kDa apoO, in a mixture of sera proteins, was unsuccessful at a preparative scale because of poor recovery (data not shown). Finally, by using freshly collected human sera, we were able to detect a minor 20-kDa band that represented free apoO (data not shown). It is likely that apoO is unstable in the free 20-kDa form as proposed by protparam software and as observed with our NaOH deglycosylation assay. However, we were able to induce the 20-kDa form of apoO production by treating the HepG2 cells with p-nitrophenyl-β-D-xyloside (Fig. 6B), a chemical that blocks the coupling of the chondroitin chain to the core protein because the first incorporated sugar is xylose (46). Because chondroitinase treatment of sera and incubation of the cells with p-nitrophenyl-β-D-xyloside both led to the release of the 20-kDa apoO form, we can conclude the 55-kDa complex contains apoO and is originally O-glycosylated with chondroitin sulfate chains. To provide more data about apoO glycosylations, apoO was produced in an in vitro transcription/translation system associated with canine microsomes that provide in vitro glycosylations. This strategy was already performed for the study of apolipoprotein M (58). A 22-kDa apoO was produced by the transcription/translation system and was glycosylated to a 55-kDa form in dose-dependent manner (Fig. 6C). By addition of p-nitrophenyl-β-D-xyloside in the reaction media, the 22-kDa apoO was incompletely glycosylated to generate the 55-kDa product. Digestion of this 55-kDa complex by chondroitinases ABC led to the appearance of the 22-kDa apoO. Therefore, the 22-kDa apoO alone is sufficient to generate a 55-kDa complex containing chondroitin sulfate chains.

This feature is typical of a glycoprotein family called proteoglycans. Proteoglycans were originally thought to be present only in cartilage but have been found in the extracellular matrix...
FIGURE 10. A, regulation of apoO expression in human heart. 37 nondiabetic human biopsies and 17 biopsies from diabetic patients were analyzed. Groups were appraised for other risk factors such as hypertension, ischemic heart diseases, and dyslipidemia. Values are means ± S.D. **, p = 0.0038. B, correlation of apoO expression with apoB expression. C, correlation of apoO expression with MTP. 54 biopsies were studied in this analysis.
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FIGURE 11. Effect of apoO on cholesterol efflux. J774 cells were labeled with 5 μg/ml NBD-cholesterol, equilibrated with bovine serum albumin and cAMP, and incubated with 10 μg/ml apoA1 or apoO. At each time point, fluorescence was read in an aliquot of medium. Values are means ± S.D. from triplicate wells. *, p < 0.05; **, p < 0.005, apoA1 or apoO versus without apo at the same time. There is no significant difference between apoA1 and apoO effect.

of many tissues. Such a high molecular mass increase because of glycosylation was also observed for a proteoglycan called decorin, and its polypeptidic chain has a molecular mass of 42 kDa, whereas the complete proteoglycan has an apparent mass of 103 kDa (52).

ApoB and apoH are also glycosylated but with mannose chains instead (59, 60). To our knowledge apoO is the first apolipoprotein that harbors such a post-translational modification. Therefore, we postulate that this original feature will provide specific properties and functions to apoO. Indeed, chondroitin sulfate proteoglycan from the arterial wall has been shown to interact with lipoproteins (61); therefore, apoO may be involved in lipoprotein interactions, and free apoO (i.e. not incorporated into lipoproteins) could compete with the binding to the arterial wall and prevent arteriosclerosis. In addition, lipoprotein lipase binds to proteoglycans (62). Thus, lipoprotein lipase could be bound to lipoproteins with the involvement of the chondroitin sulfate chain of apoO. In addition, the chondroitin sulfate chain may help targeting of the apoO-containing HDL to the liver (63). Moreover, chondroitin sulfate chains are known to interact with chemokines (64), and one could speculate on the possible role for apoO as a carrier for chemokines.

To our knowledge, few circulating chondroitin-containing proteoglycans have been described. Once could cite the liver-secreted inter-α-inhibitor family (54), serglycin that is secreted by monocytes (65), C1q inhibitor (66), and endocan produced by endothelial cells that regulates the hepatocyte growth factor/scatter factor-mediated proliferation of human embryonic kidney mitogenic activity (67).

Because mouse apoO cDNA sequence is present in data bases, we designed the peptidic sequences used for rabbit immunization to generate antibodies that will also detect mouse apoO protein. Accordingly, we also observed a weak signal in mouse cardiomyocyte differentiated P19 cells and the 3T3-F442A mouse preadipocyte cell line that was higher in differentiated adipocytes. Therefore, we can propose that the lipid content of the cells may regulate the expression of the apoO gene. In accordance with this proposition, MTP gene expression was shown to be regulated by the myocardial lipidic content (68), and we found a strong correlation between apoO and MTP gene expression (Fig. 10C). Interestingly, there was also correlation between apoO and apoB expression (Fig. 10B). MTP activity was shown to be important for apolipoprotein secretion (48, 69). Naringenin and CP-346086, MTP inhibitors that were known to inhibit secretion of apoB (34, 35), also inhibited secretion of apoO (Fig. 9, A and B). Therefore, apoB and apoO are probably secreted through the same pathway. In addition, mRNA encoding for apoO was also found to be highly expressed in adipose tissue and also at a lower level in adrenal cortex and adrenal medulla (Fig. 3). It is well known that these tissues are lipid-rich and a source of hormones. Indeed, experiments in the mouse 3T3-F442A preadipocyte cell line showed that apoO mRNA levels are higher in differentiated adipocyte cells (data not shown). Increased levels of apoO mRNA were directly linked to the enhanced secretion of the protein in the media (Fig. 4D). In addition, we found colocalization of apoO and perilipin in H9c2 cardiomyocytes (Fig. 8) that indicated apoO has an affinity for lipids and/or is strongly hydrophobic and may be involved in cellular lipid trafficking. Moreover, hearts in diabetics are known to accumulate lipid species (8, 70). We have observed an up-regulation of apoO expression in the human diabetic heart (Fig. 10A). These observations are in favor of an up-regulation of apoO expression mediated by lipid accumulation in cells. ApoO has an apoA1/A4/E family domain and is mainly detected in HDL. ApoO stimulates cholesterol efflux from mouse macrophage J774 cells (37). The ability of apoO to stimulate cholesterol efflux was investigated in mouse macrophage J774 cells. ApoO promotes cholesterol efflux as efficiently as apoA1 (Fig. 11). This is the first functional assay validated for this new apoO. This feature leads us to propose first that apoO produced in heart could participate to the myocardium lipid homeostasis and second that apoO may also be involved in reverse cholesterol transport and could contribute to the anti-atherogenic effects of HDL (71). Therefore, we propose that apoO could be involved in protective mechanisms against lipoapoptosis (72). Indeed, the heart has been shown to synthesize and secrete apoB-containing lipoproteins (73). This feature raised a number of
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