Human apolipoprotein D overexpression in transgenic mice induces insulin resistance and alters lipid metabolism

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Submitted 28 August 2008; accepted in final form 10 January 2009

Do Carmo S, Fournier D, Mounier C, Rassart E. Human apolipoprotein D overexpression in transgenic mice induces insulin resistance and alters lipid metabolism. Am J Physiol Endocrinol Metab 296: E802–E811, 2009. First published January 27, 2009; doi:10.1152/ajpendo.90725.2008.—Apolipoprotein D (apoD), a widely expressed lipocalin, has the capacity to transport small hydrophobic molecules. Although it has been proposed that apoD may have multiple tissue-specific, physiological ligands and functions, these have yet to be identified. To gain insight in some of its functions, we generated transgenic mice overexpressing human apoD (H-apoD) under the control of neuron-specific promoters. In Thy-1/apoD and NSE/apoD mice, expression of H-apoD was strong in the nervous system although weakly detected in peripheral organs such as the liver and blood cells. These mice displayed not entirely anticipated metabolic defects. Although they are not obese and have normal lipid concentration in circulation, Thy-1/apoD and NSE/apoD mice are glucose intolerant, insulin resistant, and develop hepatic steatosis. The steatosis and its associated insulin resistance are correlated with impairments in hepatic lipogenesis. However, they are not strongly related with inflammation. This impaired insulin response is not caused by a decrease in circulating leptin or a modulation of adiponectin and resistin levels. These results suggest that variations in the levels and/or sites of apoD expression influence the lipid and glucose metabolism, consolidating apoD as a target for insulin-resistance-related disorders.

hepatic steatosis; inflammation; neuronal promoters

APOLIPOPROTEIN D (apoD) is a member of the lipocalin superfamily of small hydrophobic molecule transporters. apoD was first detected in 1963, in association with plasma lipoproteins (4). Since then, apoD was related with many physiological and pathological conditions and several potential ligands were identified: arachidonic acid (AA), progesterone, pregnenolone, bilirubin, cholesterol, and E-3-methyl-2-hexenoic acid (reviewed in Ref. 37).

apoD is widely expressed in vertebrates both during development and adulthood. In humans, it is poorly expressed in liver and intestines, the major sites of synthesis of other apolipoproteins. It is mainly expressed in the adrenal glands, kidneys, pancreas, placenta, spleen, lungs, ovaries, testes, brain, peripheral nerves, and cerebrospinal fluid (18). In the mouse, apoD is mostly expressed in the central nervous system (CNS; Ref. 43), mainly in glia but also in neurons. It is also found at high levels in adipose tissue and at lower levels in other tissues (10, 54). Therefore, it has been proposed that apoD may have multiple tissue-specific, physiological ligands and functions (37, 51).

Both the apoD transcript and/or protein are altered in a broad range of conditions. Those include cellular growth, differentiation, and stress response (16, 17, 27, 36); different types of cancer (51); several neurological disorders such as Alzheimer’s disease, stroke (46), schizophrenia (48), and Parkinson’s disease (32); and animal models of nervous system pathology (8, 47). apoD is also involved in diverse aspects of lipid metabolism. apoD gene polymorphisms affect plasma lipid levels (13) and can be used as a genetic marker for obesity, hyperinsulinemia, and noninsulin-dependent diabetes mellitus (5, 52). Furthermore, apoD may participate in lipid transfer and reverse cholesterol transport by directly binding cholesterol (34) or by its association with apoA-I, lecithin-cholesterol acyltransferase, and cholesteryl ester transfer protein in high-density lipoprotein (HDL) fractions (44). Because of its capacity to bind AA, apoD contributes to membrane phospholipid metabolism by stabilizing AA levels in cellular membranes (49). An involvement in lipid redistribution after peripheral nerve injury was also suggested (8). Moreover, apoD was documented as a liver X receptor (LXR)-responsive gene (24) and could play an important role in the modulation of the lipogenesis/lipolysis balance in adipocytes by transporting ligands for LXR or peroxisome proliferator-activated receptor (PPAR)-γ participating in LXR-dependent reverse cholesterol transport (24). Finally, apoD may play a role in the control of food intake and body weight by interacting specifically with the cytoplasmic portion of the long form of the leptin receptor Ob-Rb (26). Therefore, it is not surprising to find apoD modulation in conditions presenting a deregulation of lipid metabolism, such as Tangier disease (1), familial lecithin-cholesterol acyltransferase deficiency (2), and mutations in the apoA-I gene (12), or in conditions leading to lipid accumulation, such as type 2 diabetes (23) and mouse models of Niemann-Pick disease type C (54).

Abnormal lipid metabolism contributes to several neurodegenerative disorders, including Alzheimer’s and Parkinson’s diseases. The involvement of apoD in both lipid metabolism and neurological disorders suggests a central role in neurodegeneration and/or repair. Still, the precise role of apoD remains undetermined. Recent reports (21, 41, 53) indicate a protective effect, as it counteracts aging and oxidative stress. In an attempt to reveal some aspects of the apoD function, we generated mice overexpressing human apoD (H-apoD) in the CNS. We chose to overexpress H-apoD because it allows its discrimination from the endogenous apoD. During the characterization process, we observed that H-apoD overexpression...
affects the metabolism of glucose and lipids of these mice in a promoter’s strength and expression pattern-dependent manner and also expression pattern. These defects correlate with impaired hepatic lipogenesis. However, they do not correlate with increased inflammation or altered adipokine signaling. It therefore appears that a modulation of apoD expression in specific tissues could alter the glucose and lipid metabolisms.

**MATERIALS AND METHODS**

**Animals.** All the experimental procedures were approved by the Animal Care and Use Committee of Université du Québec à Montréal. Animals were housed at 24 ± 1°C in a 12-h light-dark cycle and fed a standard rodent chow ad libitum (Charles River rodent chow #5075, St.-Hubert, QC, Canada) with free access to water. Experiments were carried out with 11- to 13-mo-old male animals, unless otherwise noted. Blood samples were collected for hematology and serum analysis. Tissues were collected, frozen in dry ice, and stored at −80°C. Sections of tissues were also frozen in Histoprep frozen tissue embedding medium (Fisher Scientific, Ottawa, ON, Canada) or fixed in 4% paraformaldehyde and embedded in paraffin for histological analysis.

**Generation of H-apoD transgenic mice.** The plasmid apoD/bovine growth hormone (BGH)/pBSSK generated in our laboratory and comprising the H-apoD coding sequence followed by the BGH polyadenylation signal in pBluescript II SK (Stratagene-VWR, Ville Mont-Royal, QC, Canada) was used for the transgene construction. Briefly, for the Thy-1-apoD construct, the promoter, the first exon, the first intron, and the 5′-noncoding region of the second exon of the human Thy-1 gene (~3.5 kb; generous gift from J. Silver, New York University Medical Center) were cloned upstream of the transgene coding sequence. The Thy-1 promoter/enhancer region (white box) of the H-apoD gene was constructed with the 5′-noncoding region of the rat Thy-1 gene (~1.8 kb; a generous gift from G. Sutcliffe; Scripps Research Institute, La Jolla, CA). Each transgene fragment was excised from its plasmid by digestion and prepared for microinjection by agarose gel electrophoresis and purified using QIAEX II Gel extraction kit (Qiagen, Mississauga, ON, Canada). Microinjection into the pronuclei of fertilized C57BL/6 × CBA zygotes was performed by standard procedures at the McGill Transgenic Facility (Montreal, QC, Canada). Transgenic animals were identified by PCR and Southern blot analysis of genomic DNA isolated from 3-wk-old mouse tail tissue biopsies. All mice were backcrossed into C57BL/6 genetic background for at least eight generations to ensure phenotype stability. Genotyping was performed by PCR using an apoD-specific primer (5′-CCC AAT CCT CCG GTG -CCC AAT CCT CCG GTG) and a BGH-specific primer (5′-GAA GGC ACA -GAA GGC ACA) corresponding to 1 (1c) or 10 (10c) copies of the transgenes. Copy standards (left) corresponding to 1 (1c) or 10 (10c) copies of the transgenes are used to estimate the transgene copy number.

**Southern blot analysis.** BamHI-digested genomic DNA was separated on a 0.8% agarose/TAE gel, denatured, transferred to Osmonics nylon transfer membrane (Fisher Scientific, Ottawa, ON, Canada), and ultraviolet fixed for 3 min. The membranes were hybridized with [α-32P]dCTP-labeled probe corresponding to each transgene, exposed to Bio-Rad Imaging Screen K and revealed with a PhosphorImager (Bio-Rad Molecular Imaging FX). Copy standards were prepared by mixing nontransgenic tail DNA with a known amount of transgene DNA.

**RNA extraction, Northern blot analysis, and semiquantitative RT-PCR.** Extraction of total RNA was performed with the TRIzol reagent (Invitrogen, Burlington, ON, Canada). Total RNA (10 µg) was separated on 1.5% (wt/vol) agarose-formaldehyde gels and blotted to a nylon membrane. The membranes were hybridized with [α-32P]dCTP-labeled H-apoD, mouse apoE, or mouse GAPDH cDNAs as described above. Total RNA was also reverse transcribed using Omniscript RT kit (Qiagen) and amplified with H-apoD, PPARα, PPARγ, fatty acid synthase (FAS), fatty acid-binding protein (LFABP), sterol regulatory element-binding protein-1c (SREBP-1c), or hypoxanthine-guanine phosphoribosyltransferase-specific primers (Table 1). Amplifications were carried out for 23 cycles for all genes. A control amplification of reverse transcription reaction without the addition of reverse transcriptase was used to ensure that there was no DNA contamination.

**Immunoblotting.** Tissues were homogenized in lysis buffer (50 mM Tris·HCl pH 7.3, 150 mM NaCl, 5 mM EDTA, 0.2% Triton X-100, and 10% Complete protease inhibitor; Roche, Mississauga, ON, Canada). After 30 min of incubation at 4°C, lysates were sonicated and cleared by centrifugation. The protein concentration was determined using a protein assay reagent (Bio-Rad Laboratories, Mississauga, ON, Canada). All extracts were stored at −80°C. For each sample, 10 µg of protein were loaded and separated on a 12% SDS-polyacrylamide gel. The proteins were then transferred to PVDF membranes blocked with 10% milk and incubated with the primary antibodies: H-apoD mouse monoclonal antibody (2B9; 1:100,000); GAPDH rabbit polyclonal antibody (Calbiochem, La Jolla, CA; 1:4,000). These primary antibodies were then detected with the appropriate horseradish peroxidase-conjugated secondary antibodies and visualized by chemiluminescence (Amersham ECL, GE-Healthcare, Baie d’Urfé, QC, Canada) and X-ray film.

**Blood analysis.** Blood hematology and serum biochemistry were carried out by the Diagnostic and Research Support Service of McGill University Animal Resources Center (Montreal, QC, Canada). Levels of cytokines and adipokines in serum were determined by SearchLight technology at the SearchLight Sample Testing Service of Pierce Biotechnology (Woburn, MA). Free fatty acids levels were measured using a colorimetric kit (MBL International, Woburn, MA).

**Competitive ELISA.** H-apoD in plasma was quantified by ELISA using the H-apoD monoclonal antibody 2B9 as previously described (46). Briefly, microtiter plates were coated with antigen (1 µg apoD/ml) in 5 mM glycine buffer (pH 9.2) and incubated overnight at 4°C.
The wells were washed with 0.25% PBS-Tween and saturated with 1% PBS-BSA for 1 h. A mixture that was previously incubated overnight and containing diluted plasma and 2B9 antibody in PBS-BSA 1% then replaced the saturation solution. After 2 h of incubation, wells were washed. Bound 2B9 antibody was detected by peroxidase-labeled anti-mouse IgG (KPL, Gaithersburg, MD) and revealed with 2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) substrate (KPL). Optical density was measured at 410 nm. All quantifications were performed in triplicate with 10 mice/genotype.

Liver lipid content. Fresh tissues were weighed in a precision balance, cut in small pieces, and dried in glass vials placed in bell jar with dessicant under vacuum for 24 h. Tissues were then reweighed to calculate dry tissue weight. Neutral lipids were extracted from tissues with diethyl ether for 24 h (2 extractions with 10 ml). Tissues were dried again for 24 h and reweighed to obtain the fat-free dry weight. Total lipid content was calculated as follows: (dry tissue weight – fat-free dry tissue weight) × 100/dry tissue weight. Total cholesterol (cholesterol and cholesteryl ester) and triglycerides were quantified using colorimetric kits according to the manufacturer’s instructions (Biovision Cedarlane, Burlington, ON, Canada).

Thiobarbituric acid reactive substance assay. The extent of oxidation in transgenic tissues was determined by the thiobarbituric acid reactive substance (TBARS) assay. Liver tissue was homogenized in cold PBS 1× (3 μl PBS per mg of tissue) in the presence of 5 mM butylated hydroxytoluene. A 12-μl aliquot of tissue extract was incubated with 390 μl of 0.2 M glycine-HCL, pH 3.6, and 250 μl of fresh TBA reagent (0.5% TBA, 0.5% SDS). Samples were incubated 15 min at 90°C and cooled on ice before a triplicate reading of the absorbance at 532 nm. Values were normalized by the lipid content of each sample.

Histology. The paraformaldehyde-fixed liver tissue was processed and embedded in paraffin and 5-μm-thick paraffin sections were stained with hematoxylin and eosin for histological analysis. To visualize lipids, frozen sections (10-μm thick) were stained with 0.5% Oil Red O in propylene glycol.

Intraperitoneal glucose and insulin tolerance tests. For the glucose tolerance test, mice fasted overnight (12–15 h) were injected intraperitoneally with 2 g/kg body wt D-glucose (2 g/kg body wt). For the insulin tolerance test, mice fasted overnight (12–15 h) were injected intraperitoneally with 12% (v/v) Triton WR1339 (Tyloxapol; 5 ml/kg body wt; Sigma-Aldrich, Oakville, ON, Canada) diluted in PBS. Blood samples were taken before (time 0) and 30, 60, and 90 min after Triton injection. Glucose and insulin levels were measured from the saphen vein 0, 15, 30, 45, 60, 90, and 120 min after the injections. Blood glucose and serum insulin levels were measured with the Accu-Chek Advantage Glucose Monitor and test strips (Roche and the rat/mouse insulin ELISA kit (Linco/Millipore, Billerica, MA), respectively. The area under the curve in the intraperitoneal glucose tolerance test (IPGTT) was calculated by the trapezoid rule from the glucose measurements. Glucose levels in the insulin tolerance test (ITT) were expressed as percentages of initial blood glucose concentrations. The mice were allowed to recover for 2 wk between experiments.

Results are expressed as means ± SE. Statistical analysis was performed with Prism 5 software (GraphPad, San Diego, CA). The statistical significance from control values was determined by Student’s t-test. Values were considered to be significant at P < 0.05.

RESULTS

Generation of H-apoD Tg mice. We generated and used two transgenic mouse lines overexpressing H-apoD in neurons under the control of the Thy-1 (21) or the NSE promoters (Fig. 1A). We chose to overexpress the H-apoD because it allows its discrimination from the endogenous apoD and because the two proteins are highly homologous and are believed to achieve the same function both in humans and mice. For each transgene, three independent mouse lines that had integrated and were able to transmit the transgene were generated and analyzed. However, since the behavioral, molecular, biochemical, and general health characterization demonstrated similar phenotypes, only one line for each transgene was included in this study (Thy-1/apoD-555; NSE/apoD-182–4). The presence and the number of integrations of the transgene fragment and its transmission through the germline were first verified by Southern blot analysis. Thy-1/apoD and NSE/apoD integrated 8 and 12 copies of the corresponding transgene, respectively, as determined by densitometric comparison with copy standards (Fig. 1B). Subsequently, H-apoD Tg mice were identified by PCR genotyping. Only those mice where the apoD and BGH primers amplified a 0.6-kb band were considered transgenic (Fig. 1C).

Expression of H-apoD in H-apoD Tg mice. The expression of each transgene was then analyzed in different tissues. By Northern blot analysis, H-apoD was found expressed mainly in the nervous system, as expected (Fig. 2A). Immunoblotting on total brain extracts confirmed the presence of H-apoD protein in these tissues (Fig. 2B). As anticipated, in Thy-1/apoD mice, H-apoD expression is stronger and is expressed in all brain regions (22). The weaker expression in NSE/apoD mice (Fig. 2B) is explained by the strong expression of this gene in the gray but not in the white matter of the brain (19). H-apoD protein was also weakly detected in the liver with a slightly
stronger signal in Thy-1/apoD mice (Fig. 2B) but was undetectable in adipose tissue (data not shown). H-apoD protein was also detected in circulation in the plasma (Thy-1/apoD: 5.35 ± 0.93 µg/ml; NSE/apoD: 4.69 ± 0.71 µg/ml), as measured by ELISA.

Because of its greater sensitivity, semiquantitative RT-PCR was also used to analyze the transgene expression. Using H-apoD and internal control hypoxanthine-guanine phosphoribosyltransferase specific primers, we could determine that, in Thy-1/apoD mice, H-apoD is expressed in all the central nervous system regions tested but at lower levels in spinal cord and in midbrain. H-apoD expression was also detected in the thymus, heart, and liver and to a lower extent in the ovaries, testis, eyes, and blood cells. In NSE/apoD, H-apoD was detectable in all the nervous system except in the olfactory bulb with a stronger expression in the hippocampus, cerebellum, and spinal cord. It was also detected in blood cells, muscle, liver, and spleen. H-apoD expression was not observed in white adipose tissue from inguinal and mesenteric fat pads (data not shown). These expression patterns are in full agreement with the promoters used. Even though Thy-1 and NSE promoters were chosen to drive apoD expression into neurons, they are also expressed by other tissues. Thus human Thy-1 expression was also reported in fibroblasts, myofibroblasts, endothelial cells, smooth muscle cells, renal glomerular mesangial cells, NK cells, and a subset of CD34+ cells in the bone marrow, blood, and thymus (11, 29, 40). In the same way, a NSE expression was already reported in blood and marrow leukocytes (35). NSE expression in the skeletal muscle was unexpected and could be imputed on nerve fascicles running throughout the muscle (9).

H-apoD overexpression has an effect on morphometric, serum, and hematological parameters. The two transgenic lines develop and breed normally. They have external phenotype and food and water consumption similar to wild-type (WT) mice at all ages (data not shown). However, some phenotypic differences appeared with aging. One-year-old Thy-1/apoD and NSE/apoD mice presented a liver significantly larger than WT mice (30 and 21%, respectively). This modification did not significantly affect the total body weight (Table 2). It is noteworthy that these transgenic mice express H-apoD in the liver (Fig. 2C). Serum parameters of nonfasting mice did reflect some of these differences (Table 3). Thus NSE/apoD mice presented increased alanine aminotransferase and aspartate aminotransferase levels, which are indicative of liver congestion or damage. These increases were not observed in

### Table 2. Morphometric parameters of H-apoD Tg mice and WT littermates

<table>
<thead>
<tr>
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<th>WT</th>
<th>Thy-1/apoD</th>
<th>NSE/apoD</th>
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</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>38.95±4.57</td>
<td>44.06±5.42</td>
<td>43.83±4.16</td>
</tr>
<tr>
<td>Body length, cm</td>
<td>9.86±0.40</td>
<td>10.11±0.36</td>
<td>10.00±0.18</td>
</tr>
<tr>
<td>Body mass index, g/cm²</td>
<td>0.40±0.03</td>
<td>0.43±0.05</td>
<td>0.44±0.04</td>
</tr>
<tr>
<td>Weights of tissue, g/g of body wt, ×100</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brain</td>
<td>1.07±0.08</td>
<td>0.97±0.08</td>
<td>0.96±0.07</td>
</tr>
<tr>
<td>Liver</td>
<td>4.03±0.58</td>
<td>5.24±0.68†</td>
<td>4.91±0.73*</td>
</tr>
<tr>
<td>Inguinal fat</td>
<td>4.87±1.54</td>
<td>4.40±0.68</td>
<td>5.19±0.90</td>
</tr>
<tr>
<td>Mesenteric fat</td>
<td>5.26±1.01</td>
<td>4.77±0.83</td>
<td>5.37±0.95</td>
</tr>
<tr>
<td>Epididymal fat</td>
<td>0.88±0.26</td>
<td>0.82±0.23</td>
<td>0.68±0.31</td>
</tr>
</tbody>
</table>

Data are means ± SE of 12 mice per group. WT, wild type; Tg, transgenic; NSE, neuron-specific enolase. *P < 0.05, †P < 0.01 vs. WT mice.
Thy-1/apoD mice, which also present a larger liver (Table 2).
However, the later showed a slight, although not significant,
increase in alkaline phosphatase, which also reflects liver
impairments in insulin resistance and steatosis, and the
modulation of leukocyte counts in H-apoD Tg mice demonstrated a significant
decrease of Akt phosphorylation at Ser473 in the liver and muscle
providing evidence that lipogenesis is stimulated in these mice (Fig. 5A).
In addition, nonfasting H-apoD Tg mice demonstrated a significant
decrease of Akt phosphorylation at Ser473 in the liver and muscle
cell count was also associated with an increased monocye count. Thy-1/apoD and
Thy-1/apoD mice also had an augmented platelet count.

**Table 3. Serum parameters in nonfasting H-apoD Tg mice and WT littermates**

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>Thy-1/apoD</th>
<th>NSE/apoD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total protein, g/l</td>
<td>52.20±4.09</td>
<td>52.33±5.16</td>
<td>55.40±11.61</td>
</tr>
<tr>
<td>Albumin, g/l</td>
<td>27.40±7.50</td>
<td>27.83±6.71</td>
<td>30.20±8.47</td>
</tr>
<tr>
<td>Glucose, mmol/l</td>
<td>10.90±0.75</td>
<td>12.52±3.44</td>
<td>11.28±2.95</td>
</tr>
<tr>
<td>Insulin, ng/ml</td>
<td>1.44±0.37</td>
<td>3.15±0.56</td>
<td>2.32±0.37</td>
</tr>
<tr>
<td>BUN, mmol/l</td>
<td>8.28±1.01</td>
<td>7.98±1.79</td>
<td>7.62±1.09</td>
</tr>
<tr>
<td>Creatinine, µmol/l</td>
<td>13.33±0.58</td>
<td>15.33±5.13</td>
<td>17.67±2.52*</td>
</tr>
<tr>
<td>Total bilirubin, µmol/l</td>
<td>2.92±0.94</td>
<td>2.82±1.48</td>
<td>3.56±1.36</td>
</tr>
<tr>
<td>ALT, U/l</td>
<td>39.00±1.00</td>
<td>38.00±2.65</td>
<td>72.00±26.29*</td>
</tr>
<tr>
<td>AST, U/l</td>
<td>86.00±20.88</td>
<td>78.67±2.08</td>
<td>173.67±65.26*</td>
</tr>
<tr>
<td>Alkaline phosphatase, U/l</td>
<td>85.43±47.51</td>
<td>102.67±27.01</td>
<td>94.00±49.12</td>
</tr>
<tr>
<td>Cholesterol, mmol/l</td>
<td>2.98±0.36</td>
<td>3.13±0.63</td>
<td>4.25±1.98</td>
</tr>
<tr>
<td>HDL, mmol/l</td>
<td>2.42±0.31</td>
<td>2.60±0.63</td>
<td>3.07±1.57</td>
</tr>
<tr>
<td>Triglycerides, mmol/l</td>
<td>1.15±0.31</td>
<td>1.71±0.89</td>
<td>1.14±0.50</td>
</tr>
<tr>
<td>Free fatty acids, mmol/l</td>
<td>0.63±0.38</td>
<td>0.65±0.16</td>
<td>0.69±0.28</td>
</tr>
</tbody>
</table>

Data are means ± SE of 6 mice per group. ALT, alanine aminotransferase; AST, aspartate aminotransferase; HDL, high-density lipoprotein; BUN, blood urea nitrogen. *P < 0.05, †P < 0.01, ‡P < 0.001 vs. WT mice.

H-apoD Tg mice show glucose intolerance and insulin resistance. The increased fed plasmatic insulin levels in H-apoD Tg mice (Table 3) can be related to abnormal glucose homeostasis, and we examined this by IPGTT (Fig. 3). After intraperitoneal glucose injection to fasting mice, the serum glucose was raised significantly in Thy-1/apoD mice and to a lesser extent in NSE/apoD mice, compared with WT littermates. The hyperglycemia was sustained up to 120 min in Thy-1/apoD but was no longer observed at 90 min in NSE/apoD mice (Fig. 3A). The integrated net rise in glucose levels above the fasting levels, calculated as the area under the IPGTT curve (Fig. 3B), further demonstrated the glucose intolerance in H-apoD Tg mice. These two transgenic lines also showed increased insulin levels during IPGTT at 30 min after glucose administration (Fig. 3C). The increased insulin levels of these two transgenic lines during IPGTT (Fig. 3C) suggest that glucose intolerance must be due to the resistance of tissues to the insulin action. The presence of insulin resistance in Thy-1/apoD and, less importantly, in NSE/apoD mice was confirmed by the reduced decline of blood glucose after the administration of insulin in ITT (Fig. 3D).

H-apoD Tg mice develop hepatic steatosis. Since Thy-1/apoD and NSE/apoD mice exhibited hepatomegaly (Table 2) and altered levels of indicators of hepatic function (Table 3), liver histology was examined. As shown by hematoxylin-eosin and Oil Red O staining, Thy-1/apoD and NSE/apoD livers showed severe steatosis (Fig. 4, B, C, E, and F). The steatosis was further confirmed by the quantification of the liver lipid content (Fig. 4G). This steatosis corresponded to an increase in triglycerides but not in total cholesterol levels (Fig. 4, I-J).

However, the lipid peroxidation levels, measured as TBARS, were similar in WT and H-apoD Tg mice, when normalized by the total lipid content (Fig. 4H). Lipid accumulation in muscle was also examined. A mild muscle steatosis appeared in Thy-1/apoD and NSE/apoD mice (data not shown).

Mechanisms of insulin resistance and steatosis. To investigate the mechanisms underlying lipid accumulation, impaired glucose tolerance, and insulin sensitivity in H-apoD Tg mice, we examined the effect of H-apoD overexpression on lipid metabolism components in the liver of mice fed ad libitum. Hepatic steatosis can be caused by a decrease in lipid secretion. Apolipoprotein E (apoE) deficiency was already associated with the development of hepatic steatosis because of impairment in VLDL-triglyceride secretion (30). Only NSE/apoD mice showed differences in this process with a 30% decrease of apoE transcription (Fig. 5A). The rate of plasma triglyceride accumulation in fasting mice after intravenous injection of Triton WR1339 was also measured. The VLDL-triglyceride secretion rate, calculated from the slope of each individual lines, was not statistically different (Fig. 5G).

Mice with hepatic steatosis also exhibited significantly increased expression of LFABP, FAS, PPARγ, and SREBP-1c, providing evidence that lipogenesis is stimulated in these mice (Fig. 5, B-E). PPARα, which is involved in the lipid-β oxidation pathway, was also stimulated in these mice (Fig. 5F).

Am J Physiol Endocrinol Metab website). The circulating levels of the adipokines leptin, adiponectin, and resistin were also measured. Only leptin showed variations in its levels with a twofold increase in NSE/apoD mice (Supplemental Fig. S1).

**DISCUSSION**

Since its isolation from HDL particles in 1976 (28), apoD was reported associated with many processes. However, the precise physiological function(s) of this protein still remains unclear. Some beneficial roles were assigned to apoD. apoD is
thought to be involved in the regeneration and reinnervation processes after nervous tissue injury (8, 31, 38, 47). In addition, apoD has been described as a marker of differentiation and good prognosis in cancer (14). It also plays a protective role in stress situations (21, 41, 53). Paradoxically, apoD was also associated with neuronal death (20), oxidative stress (3), and tumor progression (39).

To gain insight into some aspects of the apoD function, we generated two transgenic mice lines overexpressing H-apoD. Here, we demonstrate that overexpression of H-apoD driven by neuronal promoters in mice results in an interesting, although not entirely anticipated, alteration of the glucose/insulin metabolism. More precisely, the presence of H-apoD amplifies the insulin resistance that occurs with aging, as 3-mo-old mice have similar insulin levels independently of genotype. This was first suggested by elevated insulin plasma concentration in nonfasting 1-yr-old mice and explored by IPGTT and ITT. The results show that, although H-apoD Tg mice are not obese and present normal food intake and normal lipid levels in circulation, they develop glucose intolerance and insulin resistance with aging. This insulin resistance is associated with lipid accumulation in the liver, where it causes hepatomegaly, as evidenced by histology and abnormal liver function parameters in serum. A strong connection between lipid accumulation in liver and insulin resistance was already reported (25).

Table 4. Hematological parameters in H-apoD Tg mice and WT littermates

<table>
<thead>
<tr>
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</tr>
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<tbody>
<tr>
<td>Hemoglobin, g/l</td>
<td>123.50 ± 9.19</td>
<td>123.50 ± 13.44</td>
<td>133.00 ± 7.07</td>
</tr>
<tr>
<td>RBCs, ×10^12/l</td>
<td>8.07 ± 0.18</td>
<td>8.31 ± 1.01</td>
<td>8.28 ± 0.14</td>
</tr>
<tr>
<td>WBCs, ×10^9/l</td>
<td>3.75 ± 0.21</td>
<td>4.75 ± 0.35</td>
<td>6.65 ± 1.06</td>
</tr>
<tr>
<td>Neutrophils, ×10^9/l</td>
<td>0.42 ± 0.34</td>
<td>0.87 ± 0.33</td>
<td>0.93 ± 0.05</td>
</tr>
<tr>
<td>Lymphocytes, ×10^9/l</td>
<td>3.27 ± 0.16</td>
<td>3.59 ± 0.30</td>
<td>4.99 ± 0.37</td>
</tr>
<tr>
<td>Monocytes, ×10^9/l</td>
<td>0.02 ± 0.03</td>
<td>0.05 ± 0.06</td>
<td>0.11 ± 0.06</td>
</tr>
<tr>
<td>Eosinophils, ×10^9/l</td>
<td>0.04 ± 0.06</td>
<td>0.26 ± 0.22</td>
<td>0.64 ± 0.57</td>
</tr>
<tr>
<td>Platelets, ×10^12/l</td>
<td>747.00 ± 241.83</td>
<td>1,139.00 ± 53.74</td>
<td>1,203.00 ± 205.06</td>
</tr>
</tbody>
</table>

Data are means ± SE of 6 mice per group. RBC, red blood cells; WBC, white blood cells. *P < 0.05, **P < 0.01, ***P < 0.001 vs. WT mice; ***P < 0.01, ****P < 0.001 vs. Thy-1/apoD mice.
The slight H-apoD expression in the liver might contribute to fat, predominantly triglycerides, accumulation, and insulin resistance. Being a secreted protein, and expressed namely by blood cells, H-apoD was found circulating in the plasma and thus it could also account for lipid accumulation in the liver. Accumulation of lipids in the liver is generally multifactorial. One possible mechanism is the trapping of AA, a well-known apoD ligand, or other fatty acids by H-apoD, therefore creating an imbalance favoring fat accumulation vs. its elimination by the liver. In addition, given that polyunsaturated fatty acids can inhibit SREBP-1c expression (55), the trapping of fatty acids by H-apoD could support the activation of SREBP-1c expression induced by insulin resistance and thus favor lipid accumulation in the liver. Another potential source of lipid accumulation is an increase in the hepatic lipid uptake, but this seems unlikely as the circulating free fatty acids,
triglycerides, cholesterol, and HDL levels are similar in all mice. Still, we cannot rule out an increased transport of lipids towards the liver by the circulating H-apoD because of the potential apoD participation in lipid transfer and reverse cholesterol transport (34, 44). A defect in lipid oxidation can also be involved in lipid accumulation. However, since the levels of PPARγ, known to control the expression of many genes involved in lipid oxidation, are increased in Thy-1/apoD and NSE/apoD mice as explored by semiquantitative RT-PCR. Gene levels were normalized by HPRT expression. For each graph, the H-apoD Tg values were normalized by the WT values, which was given an arbitrary value of 1. A–F: values are means ± SE of 6 mice per group. *P < 0.05, **P < 0.01, ***P < 0.001 vs. WT mice. G: plasma triglyceride production rates were measured in fasting H-apoD Tg and WT mice after Triton WR1339 injection. Data represent mean triglyceride concentrations (in mM) ± SE; n = 5 per group. The VLDL-triglyceride secretion rate, calculated from the slope of each individual line, was not statistically different (WT: 53.04 ± 5.69; Thy-1/apoD: 39.0 ± 2.47; and NSE/apoD: 45.0 ± 4.64 µmol·kg⁻¹·h⁻¹).
tion between H-apoD expression in the liver and liver steatosis is not clear and requires further examination.

The nature of the connection between steatosis and insulin resistance remains unclear. The accumulation of lipid metabolites such as ceramides, diacylglycerol, or long-chain acyl-CoA, inside skeletal muscle and liver results in the downregulation of insulin signaling and therefore in insulin resistance (42).

Several studies (42) also suggest a link between oxidative stress and insulin resistance. Indeed, intracellular accumulation of fatty acid metabolites contributes to the production of reactive oxygen species. Reactive oxygen species contribute to hepatic insulin resistance through the activation of PKCs. They also attack polyunsaturated fatty acids and initiate lipid peroxidation. However, although H-apoD Tg mice featured hepatic steatosis, their lipid peroxidation levels were not increased. This suggests, on the one hand, that oxidative stress is not the cause of insulin resistance and, on the other, that H-apoD prevents the oxidative stress caused by lipid accumulation. apoD was already reported to play a protective role in oxidative stress situations (21, 41, 53).

In addition, insulin resistance in humans is associated with chronic low-grade inflammation (33, 50). Whether inflammation causes insulin resistance or is an epiphenomenon of fat accumulation is still unknown. In this perspective, increased leukocyte counts in Thy-1/apoD and NSE/apoD mice predicted higher serum cytokines levels. The absence of upregulation of most of the cytokines tested would be in agreement with previous studies (7, 15, 16) suggesting that apoD may have anti-inflammatory properties, as its expression is upregulated in response to inflammatory stimuli. However, the presence of inflammation cannot be completely excluded.

It is also noteworthy that H-apoD is primarily expressed in neurons and that H-apoD levels in the brain are directly correlated with the incidence of insulin resistance, as Thy-1/ apoD mice are more insulin resistant than NSE/apoD mice. H-apoD could interfere directly with leptin or insulin receptors in the arcuate, paraventricular, and other hypothalamic nuclei. apoD is involved in the leptin receptor signal transduction pathway that controls appetite and body fat accumulation. It interacts specifically with the cytoplasmic portion of the long form of the leptin receptor Ob-Rb. In hypothalamic, apoD transcription is stimulated by dietary fat and correlates positively with adipose tissue mass and circulating leptin levels. However, this positive association is lost in mice carrying a mutant leptin or Ob-Rb gene, in which levels of hypothalamic apoD mRNA are reduced compared with those of WT mice (26). H-apoD overexpression in the arcuate or paraventricular could cause a partial desensibilization of the leptin receptor leading to a leptin resistance and further to an insulin resistance. However, as H-apoD Tg mice do not show increased weight gain or hyperphagia, a downregulation of the leptin pathway seems unlikely. Still, the presence of H-apoD in neurons could interfere with the central regulation of insulin sensitivity.

Apart from lipid accumulation, H-apoD Tg mice display other insulin-resistance-associated features such as abnormal hematomal parameters. Therefore, in H-apoD Tg mice, insulin resistance is associated with abnormal leukocyte and platelet concentration in blood. Hematomal abnormalities are often associated with insulin resistance in humans, and an increased leukocyte count, particularly T cells, was associated with the progression of atherosclerosis (45). It was also reported that human platelets have insulin receptors that participate in the regulation of platelet function. Insulin resistance, and its associated hyperinsulinemia, promotes platelet activation, thus increasing the risk of cardiovascular disease (6).

In conclusion, these results pinpoint the importance of a complex regulation of apoD levels and sites of expression, which influence apoD function and its impact on glucose and lipid metabolism. Consequently, these results bring new evidence for apoD as a potential target for glucose and lipid metabolism related disorders. However, the molecular basis for the role of apoD in steatosis and insulin resistance, as well as its contribution to other components of the metabolism control, such as energy expenditure and response to high-fat diet, have yet to be defined.

ACKNOWLEDGMENTS

We thank Diego Sanchez, Maria D. Ganfornin, and Louise Brissette for helpful discussions. We also thank Marie Trudel for advice concerning the transgenic mice.

GRANTS

This work was supported by a Canadian Institutes for Health Research Grant (MOP-156777). D. Fournier was supported by Fonds pour la Formation des Chercheurs et l’Aide à la Recherche and Natural Sciences and Engineering Research Council studentships. S. Do Carmo was supported by Fonds de la Recherche en Santé du Québec, Natural Sciences and Engineering Research Council, and Université du Québec à Montréal studentships.

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