Antioxidant properties of HDL in transgenic mice overexpressing human apolipoprotein A-II

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Abstract Transgenic mice overexpressing human apolipoprotein A-II (huapoA-II) display high VLDL and low HDL levels. To evaluate the antioxidant potential of huapoA-II enriched HDL, we measured the activities of paraoxonase (PON) and platelet-activating factor acetylhydrolase (PAF-AH). Both activities decreased up to 43% in the serum of transgenic mice compared with controls, varied in parallel to HDL levels, but decreased less than HDL levels. The major part of PON and PAF-AH was associated with HDL, except in fed high huapoA-II-expressing mice, in which 20% of PAF-AH and 9% of PON activities were associated with VLDL. PON mRNA levels in the liver, its major site of synthesis, were similar in transgenic and control animals, indicating normal enzyme synthesis. In transgenic mice, the basal oxidation of lipoproteins was not increased, whereas their VLDL were more susceptible to oxidation than VLDL of controls. Interestingly, HDL of transgenic mice protected VLDL from oxidation more efficiently than HDL of controls. In conclusion, the decrease in both PON and PAF-AH activities in huapoA-II transgenic mice is best explained by their lower plasma HDL levels. However, the unchanged basal lipoprotein oxidation in transgenic mice suggests that huapoA-II-rich HDL may maintain adequate antioxidant potential.—Boisfer, E., D. Stengel, D. Pastier, P. M. Laplaud, N. Dousset, E. Ninio, and A-D. Kalopissis. **Antioxidant properties of HDL in transgenic mice overexpressing human apolipoprotein A-II. *J. Lipid Res.* 2002.** 43: **732–741.**

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The strong negative correlation of plasma HDL levels with the risk of atherosclerosis is currently attributed to the role that HDL plays in mobilizing cholesterol from peripheral tissues for delivery to the liver for recycling (reverse cholesterol transport) (1) and their role in protecting LDL from oxidative damage (2). Indeed, minimally and extensively oxidized LDL can trigger various inflammatory processes leading to monocyte recruitment into the artery wall, foam cell formation, and promotion of proatherogenic events (3). However, direct evidence that oxidized LDL causes atherosclerosis is still lacking (4). The antioxidant properties of HDL may be related to enzymes associated with HDL in the circulation, namely paraoxonase (PON), platelet activating factor-acetylhydrolase (PAF-AH), and LCAT. PON is a calcium-dependent ester hydrolase that catalyzes the hydrolysis of organophosphates, aromatic carboxylic acid esters, and carbamates (5–7), but also of oxidized phospholipids including PAF (8, 9). However, the physiological substrate of PON is still not known. PAF-AH hydrolyzes the acetyl moiety in the *sn*-2 position of PAF, which is a proinflammatory molecule considered to play a role in the early events of atherosclerosis (10). PAF-AH also has the ability to hydrolyze oxidized phospholipids possessing short-chain acyl moieties in the *sn*-2 position, thereby protecting LDL from oxidative modification in humans (11, 12). All the same, lysophosphatidyl choline, the product of this reaction, may be pro-atherogenic. Although the primary role of LCAT is the esterification of cholesterol in HDL, LCAT can also hydrolyze oxidized polar phospholipids generated during lipoprotein oxidation (13). The latter action of LCAT does not necessitate apolipoprotein A-I (apoA-I) as a cofactor (14).

The protective effect of HDL has been generally linked to their apoA-I content, whereas the role of apoA-II is less well established. Genetically modified animals are a powerful tool to investigate the metabolic functions in vivo of human proteins. We therefore generated transgenic mice expressing human (hu)apoA-II, which forms a homodimer and is easily distinguished from endogenous

Abbreviations: apo, apolipoprotein; huAIItg, human apolipoprotein A-II transgenic; huapoA-II, human apolipoprotein A-II; mapoA-II, mouse apolipoprotein A-II; GGE, gradient gel electrophoresis; FPLC, fast protein liquid chromatography; PON, paraoxonase; PAF-AH, platelet activating factor acetylhydrolase; TBARS, thiobarbituric acid reactive substances.

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murine (m)apoA-II. Our huapoA-II transgenic mice display postprandial accumulation of triglyceride-rich lipoproteins and low plasma apoA-I and HDL levels (15), whereas LCAT activity with an exogenous substrate, which is representative of its mass, was diminished by 34% as compared with control mice (16). Of note, HDL transporting huapoA-II comprise two subpopulations of large and smaller particles, reminiscent of human HDL (16). Conversely, transgenic mice which overexpress mapoA-II display greater levels of plasma HDL which are a homogeneous population of large-sized particles with a high apoA-I content (17). Overexpression of mapoA-II has a pronounced atherogenic effect even on a chow diet (18), whereas huapoA-II overexpression has no effect on chow and is either protective on an atherogenic diet in two independently established transgenic lines with a moderate huapoA-II expression (19, 20) or proatherogenic in another line with a high huapoA-II expression (21). Thus, some structural and metabolic differences probably exist between huapoA-II and mapoA-II, so that the role of huapoA-II cannot be deduced from that of its mouse counterpart.

The present study focused on the impact of huapoA-II overexpression on the anti-oxidant properties of HDL. We sought to establish whether PON and PAF-AH activities were also decreased concomitantly to circulating HDL, the main carriers of these enzymes in mice (in humans, PAF-AH is essentially transported by LDL). To establish a link between HDL concentration and PON and PAF-AH activities, we took advantage of our recent finding that plasma HDL increase in transgenic and control mice after an overnight fast (16) and show that both enzyme activities varied in parallel to plasma HDL levels in all groups of mice. HuapoA-II transgenic mice consistently displayed decreased PON and PAF-AH activities compared with controls. However, both enzymes decreased less than HDL protein and lipid components, indicating that PON and PAF-AH were relatively preserved in HDL of transgenic mice. This finding may account for the similar basal oxidative states of lipoproteins of transgenic and control mice, whereas the triglyceride-rich VLDL of transgenic mice was more susceptible to oxidation. Finally, we show that HDL from transgenic animals protected VLDL more efficiently from lipid peroxidation than control HDL.

MATERIALS AND METHODS

Transgenic mice

Transgenic mice were generated by microinjection into one-cell embryos of (C57BL/6J × CBA/2J) F1 female mice (IFFA-CREDO, Lyon) of the 3 kb genomic clone of the human apoA-II gene (−911/+2045), as previously described (15). The founder δ and λ (higher-expressing) mice have been backcrossed for at least eight generations to mouse strain C57BL/6J.

Animals

The animals were housed in animal rooms with alternating 12-h periods of light (7 AM–7 PM) and dark (7 PM–7 AM). All transgenic mice were hemizygous for the huapoA-II transgene and over 8 weeks of age. They were fed a chow diet (UAR, Villemoisson-sur-Orge), with free access to food and water, unless otherwise specified. The presence of the huapoA-II transgene was determined by PCR of tail-derived DNA and detection of huapoA-II in plasma by Western blot, using a rabbit anti-huapoA-II antiserum not recognizing mapoA-II (courtesy of Dr. A. Mazur, INRA, Theix). HuapoA-II was measured in plasma from all animals used in this study by immunonephelometry using an antibody specific for huapoA-II and not recognizing mapoA-II (Dade Behring Testkit OQBA09).

Blood was drawn from the retroorbital venous plexus between 9 and 11 AM without EDTA, because calcium is necessary for PON activity, and serum was stored at −80°C until assays. The same animals were used for measurements of PON and PAF-AH activities, first in the fed state and then 8 days later after an overnight 16 h fast. All assays were performed on individual male mice. PON and PAF-AH activities were also measured on lipoprotein fractions isolated by fast protein liquid column (FPLC) chromatography from pooled sera corresponding to 8 to 10 mice from each group.

Lipoprotein analysis

FPLC chromatography was performed on 0.2 ml freshly prepared pooled sera, using two Superose 6 columns operating in series. Sera and elution media were additioned with $CaCl_2$ (5 mM final concentration) to preserve PON activity. Elution rate was 0.4 ml/min. Fractions of 0.2 ml were collected into 96-well microplates and total cholesterol contents were determined.

Sequential ultracentrifugations of serum pools were performed at 100,000 *g* and 10°C to isolate VLDL and LDL (for 18 h at densities 1.006 and 1.063 *g*/ml, respectively), and HDL (for 40 h at density 1.21 *g*/ml). VLDL, LDL, and HDL were extensively dialyzed against PBS and used to measure oxidation. Protein (22), total cholesterol (Biotrol A01368), and phospholipid (BioMérieux PAP 150) were determined in lipoprotein fractions. The concentration of huapoA-II in the serum pools of transgenic mice before lipoprotein isolation was assayed by immunonephelometry.

The size distribution of HDL was analyzed by nondenaturing gradient gel electrophoresis (GGE) in 4–20% pre-cast polyacrylamide gels (BioRad). HDL was stained with Coomassie Brilliant Blue R250 (23). The same amount of HDL protein (5 μg) from each group of mice was loaded on the gels.

Apolipoprotein analysis

HDL apolipoproteins were analyzed by 15% SDS-PAGE (24) under non-reducing conditions in order to detect the dimeric form of huapoA-II. A constant amount of protein was loaded on each gel to compare relative HDL apolipoprotein contents between transgenic and control mice. HDL was subjected to nondenaturing GGE followed by protein transfer to nitrocellulose membranes (0.45 μm, BioRad). MapoA-I and mapoA-II were detected with specific rabbit antisera (Biodesign), and huapoA-II with the antiserum described above. Bands were visualized with an alkaline phosphatase substrate system (BioRad).

Enzyme activity assays

PON activity was determined in the presence of 2 mM $CaCl_2$ in serum and lipoprotein fractions by two different assays: *i*) using paraoxon (diethyl-p-nitrophenyl phosphate, Sigma) as substrate (25) and termed PON activity. The generation of p-nitrophenol was measured at 405 nm and 25°C in the presence of 6 μl of serum in a continuously recording spectrophotometer (Powerwave 200, Bio-Tek Instruments) during 6 min and the absorbance at 4 min was used for calculations of activity. PON activity was expressed as nmol p-nitrophenol/min/ml serum; *ii*) using phe-

nylacetate as substrate (26) and termed arylesterase activity. The arylesterase activity was measured in the presence of 10 μl lipoprotein fraction. The increase in absorbance at 270 nm was recorded during 6 min at 25°C and the absorbance at 4 min was used for calculations of activity. Arylesterase activity was expressed as nmol phenylacetate hydrolyzed/min/lipoprotein fraction. PAF-AH activity was measured with 2-[acetyl-^{3}H]PAF (NEN) as substrate, as previously described (27) and was expressed as nmol [^{3}H]acetate liberated/min/ml serum or lipoprotein fraction.

In vitro oxidation of lipoproteins

Oxidation of VLDL, LDL, and HDL was carried out by incubation at 37°C for 18 h with 5 μM $CuSO_4$ or PBS. Lipoprotein oxidation was determined by the measurement of thiobarbituric acid reactive substances (TBARS) and hydroperoxides. TBARS were determined by the phenylhydrazine-thiobarbituric acid assay as described by Yagi (28). Hydroperoxides were measured by the ferrous ion oxidation-xylenol orange assay, according to Jiang et al. (29). The degree of susceptibility of lipoproteins to oxidation was determined by subtracting the amount of TBARS and hydroperoxides formed during incubations with $CuSO_4$ from those measured during incubations with PBS (basal oxidation). Protection of VLDL from copper-induced oxidation was assessed by incubations of VLDL with 5 μM $CuSO_4$ or PBS, in the absence or presence of homologous and heterologous HDL, as indicated in the appropriate figure legend. Then, the incubation media were subjected to ultracentrifugation, VLDL and HDL were re-isolated, dialyzed extensively against PBS and TBARS, and hydroperoxides were measured in each lipoprotein fraction.

RNA isolation and quantitative PCR

Total liver RNA was isolated using RNA Instapure kit (Eurogentec). RT was carried out with 1 μg RNA in a final volume of 30 μl, for 45 min at 42°C. The mRNAs of interest were normalized to the 18S RNA probe (ref. 4310893E, Applied Biosystems). The sequences used for PCR detection of murine PON1 mRNA were as follows: sense 5′-gg aat aaa aag ttt cga tcc cag-3′, antisense 5′-tcc aat agc agc tat atc gtt gat g-3′, and PON1 mRNA was quantified by 40 cycles of 95°C for 10 s, 55°C for 10 s, and 72°C for 10 s (Light Cycler, Roche). A standard curve was established by PCR using a mix of hepatic mRNA obtained by separate RTs from six individual control mice fasted overnight, with the following dilutions: 1:25, 1:50, 1:100, 1:200, and 1:400. For PON, the slope of the regression line was −3.456, with $r = 1.00$ and an error of 0.0407.

Statistical analysis

Results are given as mean ± SE and statistically significant differences were determined with Tukey's Multiple Comparison Tests after ANOVA, using GraphPad Prism.

RESULTS

PON and PAF-AH activities parallel variations of plasma HDL concentrations in control and huapoA-II transgenic mice.

Mice overexpressing huapoA-II displayed lower plasma HDL levels than control animals, as expected (15, 16). The overnight fast decreased the concentration of huapoA-II in transgenic mice and increased the plasma HDL level in control and transgenic mice (**Table 1**). The lipoprotein profiles obtained by FPLC chromatography clearly show consistently greater HDL peaks in fasted animals relative to fed animals of the same genotype (**Fig. 1**). HuapoA-II was the predominant HDL apolipoprotein in transgenic mice, especially in the fed state, whereas the amount of endogenous apoA-I decreased in parallel to the increase in huapoA-II (**Fig. 2A**). HDL from transgenic mice consisted of two subpopulations (Fig. 2B) transporting mainly huapoA-II (Fig. 2D). MapoA-I consistently associated with the larger HDL particles (Fig. 2C). Conversely, HDL from control mice displayed essentially a unimodal distribution (Fig. 2B) and contained mainly apoA-I (Fig. 2C) and little mapoA-II (Fig. 2D).

Serum PON and PAF-AH activities were lower in transgenic mice compared with controls in both fed and fasted states (**Table 2**). Interestingly, both enzyme activities displayed 160% to 210% increases in fasted compared with fed animals, irrespective of genotype. Of note, fasting stimulated enzyme activities in the same mouse, which was first studied under ad libitum feeding and then after an overnight fast. The assays in this study were performed in male mice, but similar results were obtained in female mice (not shown). On the other hand, the arylesterase activity of PON was also lower in transgenic mice (not shown), albeit differences were less marked than those of PON activity. This has also been described by other groups, and may be due to enzymes other than PON possessing arylesterase activity. Indeed, mice deficient in PON1 by homologous recombination still had 15% arylesterase activity (30). Interestingly, the ratio of PON and PAF-AH activities per mg HDL-protein was increased rather than decreased in transgenic mice compared with controls, suggesting that both enzymes were adequately transported by HDL particles enriched in huapoA-II.

TABLE 1. HDL composition and plasma human apo AII concentration in transgenic mice

Nutritional State	Genotype	HDL-Protein[a]	HDL-TC[a]	HDL-PL[a]	Human Apoa-II[a]
Fed	Controls	79.1 ± 7.4	24.7 ± 1.7	34.2 ± 3.3	—
	hAIItg-δ	47.7 ± 5.4	10.1 ± 1.3[d]	21.4 ± 4.2	49.9 ± 5.4
	hAIItg-λ	38.7 ± 7.6[c]	6.2 ± 1.1[d]	14.3 ± 2.9[b]	73.7 ± 8.3[h]
Fasted	Controls	113.6 ± 12.0	40.1 ± 5.1[g]	54.4 ± 9.0[e]	—
	hAIItg-δ	68.1 ± 6.1[b]	20.2 ± 1.4[d,e]	31.2 ± 1.4[b]	39.4 ± 2.9
	hAIItg-λ	48.2 ± 11.0[c]	17.1 ± 2.9[d,e]	29.8 ± 5.2[b]	38.1 ± 2.1[f]

[a] Results are expressed as mg/dl plasma and are the mean ± SE. Human apoA-II was measured in total plasma in the same three pools that served to prepare HDL by ultracentrifugation. Statistical significance of the results was calculated by Tukey's Multiple Comparison Test after ANOVA.

[b] $P < 0.05$, [c] $P < 0.01$, [d] $P < 0.001$ between transgenic and control mice in the same nutritional state.

[e] $P < 0.05$, [f] $P < 0.01$, [g] $P < 0.001$ between fasted versus fed mice of the same genotype.

[h] $P < 0.05$ between hAIItg-δ and hAIItg-λ mice in the same nutritional state.

TC, total cholesterol; PL, phospholipid.

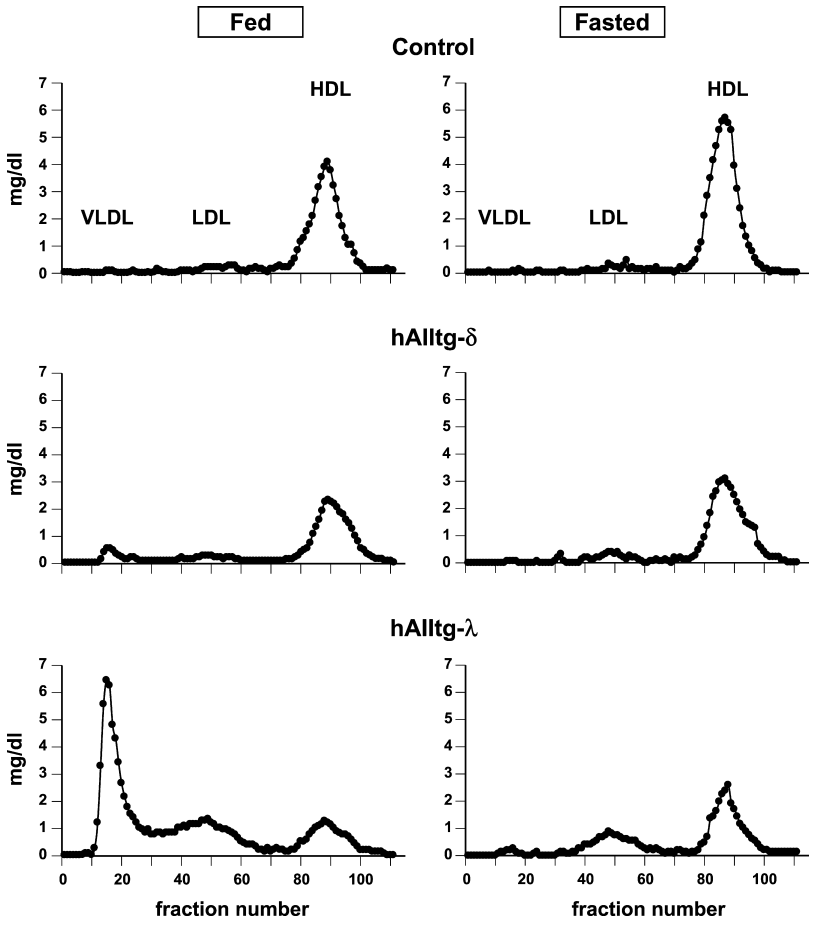

Fig. 1. Fast protein liquid chromatography (FPLC) profiles of plasma lipoproteins from control and human apolipoprotein A-II (huapoA-II) transgenic mice. FPLC chromatography was performed using two Superose 6 columns operating in series. Elution rate was 0.4 ml/min. Total cholesterol was measured in each fraction and is expressed in mg/dl.

To establish whether differences in PON activity among transgenic and control mice were due to differences in PON transcriptional level, we determined PON1 mRNA amounts in the liver, its main site of synthesis (31). The relative levels of PON mRNA to 18S were comparable among all groups of mice (Table 2), suggesting that PON synthesis was not decreased in huapoA-II transgenic mice.

Distribution of PON and PAF-AH activities among plasma lipoproteins

In mice, PON and PAF-AH are associated with HDL. Because mice with high expression of huapoA-II have considerably less HDL particles in the circulation but have high VLDL levels in the postprandial state (as shown in Fig. 1), it was of interest to determine whether the two enzymes were partly carried by apoB containing lipoproteins (LP-B). Lipoproteins were fractionated by FPLC, because ultracentrifugation may displace the enzymes from the lipoproteins, and we measured PAF-AH and arylesterase activities in these fractions. The arylesterase activity of PON was measured instead of the paraoxonase activity due to higher sensitivity.

PAF-AH activity was essentially present in the larger HDL of fed control and transgenic mice and increased after an overnight fast (**Fig. 3**). In the high-expressing λ mice, approximately 20% of the total PAF-AH activity were present in VLDL in the fed but not in the fasted state. Arylesterase activity presented two distinct peaks in the HDL region of all groups of animals. In both fed and fasted control mice, the major arylesterase peak was in HDL and was followed by a second smaller peak probably corresponding to small HDL particles. Such small HDL was not detected by the total cholesterol assay in individual FPLC fractions (Fig. 1) and by GGE, probably because their concentration is very low. In δ mice, the two arylesterase peaks were apparently related to the two HDL populations shown in Fig. 2: in the fed state, large and small HDL were roughly equally present, and so were the two arylesterase peaks. Conversely, in the fasted state large HDL particles were predominant, and the arylesterase peak was greater in this fraction. In λ mice, arylesterase activity was greater in the smaller HDL particles, which predominated under both nutritional conditions. In addition, 9% of total arylesterase activity was associated with VLDL from fed λ mice. We verified that neither PON nor PAF-AH was present as free proteins in the circulation, since these activities could not be detected in fractions between 25 and 40.

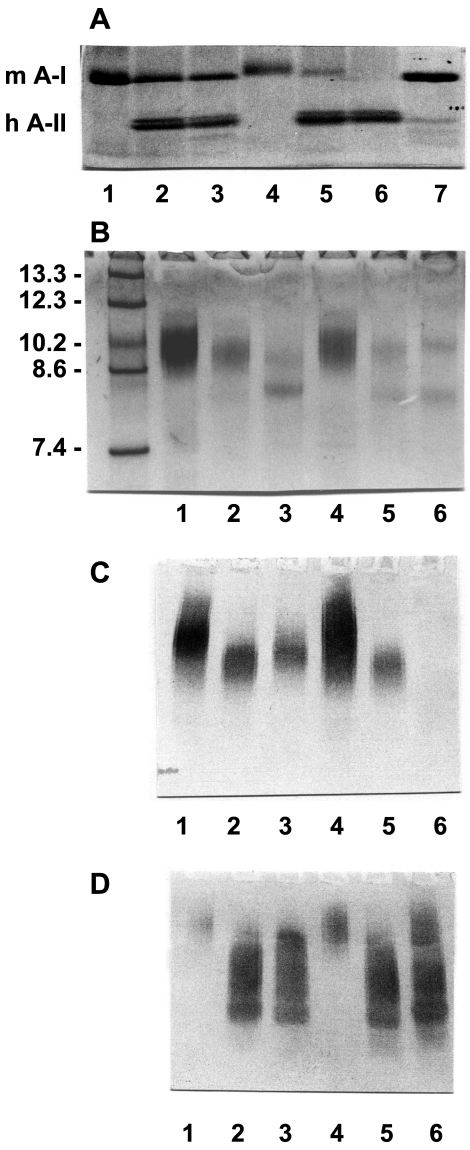

Fig. 2. Characterization of HDL size distribution and apoA-I and huapoA-II contents. A: Fifteen percent SDS-PAGE of HDL apolipoproteins, with 6 μg HDL-protein loaded on the gels to compare relative apolipoprotein contents among transgenic and control mice. B: Non denaturing gradient gel electrophoresis (GGE) of HDL (6 μg protein/lane) was performed on 4–20% gradient gels and lipoproteins were stained with Coomassie brilliant blue. On the left are shown the High Molecular Weight Standards (Pharmacia-Amersham). C: Non denaturing GGE of HDL as in B, followed by immunoblotting with rabbit antiserum directed against mapoA-I. D: Non denaturing GGE of HDL as in B, followed by immunoblotting with two rabbit antisera, one directed against huapoA-II and the other directed against mapoA-II. A–D, lanes 1–3: HDL from fasted control, human apoA-II transgenic (huAIItg)-δ and huAIItg-λ mice, respectively; lanes 4–6: HDL from fed control, huAIItg-δ and huAIItg-λ mice, respectively; lane 7: human HDL (A).

Susceptibility of lipoproteins to oxidation

To determine whether the lower PON and PAF-AH activities in transgenic mice would render LP-B more susceptible to oxidation, we isolated lipoproteins of control and transgenic mice by ultracentrifugation and measured the production of TBARS and hydroperoxides before (basal oxidation) and after peroxidative stress (**Table 3**). The basal oxidation was highest in VLDL relatively to LDL and HDL, irrespective of genotype, and was similar among the same lipoprotein fractions of control and transgenic mice. On the other hand, significantly more TBARS and hydroperoxides were produced under peroxidative stress in VLDL particles of δ and λ mice, even when expressed per mg VLDL protein. Thus, VLDL of huapoA-II transgenic mice displayed a greater susceptibility to oxidation, which was not related to their higher plasma concentration. On the contrary, the susceptibility to oxidation of LDL and HDL was the same among control and transgenic mice.

Protection of VLDL from oxidation by HDL

To assess whether HDL from transgenic mice could protect VLDL against copper-induced oxidation, VLDL from control, δ, and λ mice was incubated with homologous or heterologous HDL. We have chosen a protein ratio of VLDL to HDL of 1:5 (w/w) as an approximation of a particle to particle ratio. **Figure 4** illustrates the changes in TBARS formation in VLDL in the absence and presence of HDL. HDL isolated from all groups of mice protected VLDL from copper-induced oxidation. Of note, the protection afforded by homologous or heterologous HDL was lowest for VLDL of control mice, which displayed the smaller susceptibility to oxidation and greatest for VLDL of both transgenic lines, which were more susceptible to oxidation. Interestingly, HDL of both transgenic lines was more protective than control HDL. The amounts of TBARS in HDL did not increase after incubation with VLDL (not shown). Similar results were obtained when hydroperoxides were measured under the same conditions (not shown).

DISCUSSION

The present study focused on establishing the antioxidant potential of HDL from transgenic mice overexpressing huapoA-II, because these HDL circulate in low amounts and huapoA-II is their major apolipoprotein (15, 16). We show for the first time that the activities of two antiinflammatory enzymes, PON and PAF-AH, were decreased in huapoA-II transgenic mice, in parallel to the lower plasma HDL levels, as compared with control mice. However, the activities of both enzymes decreased to a lesser extent than HDL protein and lipid components, suggesting that HDL of transgenic mice is an efficient carrier of PON and PAF-AH. The basal oxidation of VLDL was similar among transgenic and control mice, whereas VLDL of transgenic mice was more susceptible to oxidative stress than control VLDL, possibly because of greater triglyceride content. Interestingly, HDL of transgenic mice protected VLDL more efficiently from oxidative stress than HDL from controls. Thus, huapoA-II-rich HDL may provide in vivo an adequate protection of VLDL against oxidation.

We sought to determine whether the decrease in PON and PAF-AH activities in huapoA-II transgenic mice originated from increased catabolism due to the 2-fold lower

TABLE 2. PON and PAF-AH activities and hepatic relative mRNA amounts of PON in control and hAII-transgenic mice

Nutritional State	Genotype	PON[a]	PAF-AH[a]	mRNA PON/18S[b]
		nmol/ml/min	*nmol/ml/min*	*% fasted controls*
Fed	Controls	136 ± 5 (10)	140 ± 10 (10)	103.3 ± 17.3
	hAIItg-δ	101 ± 4 (9)[e]	112 ± 17 (8)	98.3 ± 12.1
	hAIItg-λ	78 ± 15 (9)[d]	86 ± 12 (9)[d]	93.8 ± 20.6
Fasted	Controls	215 ± 8 (10)[h]	253 ± 13 (10)[h]	100
	hAIItg-δ	138 ± 16 (9)[e,f]	198 ± 24 (9)[c,g]	79.4 ± 13.7
	hAIItg-λ	124 ± 24 (9)[d]	181 ± 28 (9)[c,g]	85.8 ± 21.9

[a] Blood was withdrawn from the retro-orbital plexus of eight to 10 individual control and transgenic mice, first in the fed state and then, 2 weeks later, after an overnight fast. PON and PAF-AH activities were measured in serum as described in Materials and Methods.

[b] Livers from six animals from each group were used for measurements of PON mRNA levels, which are presented as a ratio to 18S. Results are expressed as mean ± SE, with the number of animals used indicated in parentheses. Statistical significance of the results was calculated as in Table 1.

[c] $P < 0.05$, [d] $P < 0.01$, [e] $P < 0.001$ between transgenic and control mice in the same nutritional state.

[f] $P < 0.05$, [g] $P < 0.01$, [h] $P < 0.001$ between fasted versus fed mice of the same genotype.

plasma HDL, their main carrier (15, 16). Indeed, hepatic PON1 mRNA levels were similar among all groups of mice, indicating that PON synthesis was unaffected in transgenic mice. To establish whether the HDL concentration was a key determinant for PON and PAF-AH activities in the circulation, we measured both activities under two experimental conditions: *i*) in the fed state, *ii*) after an overnight fast, when HDL is increased in all groups of mice (16). In both the fed and fasted states, plasma HDL levels were lower (by at least 60%) and PON and PAF-AH activities were also lower (up to 43%) in transgenic relative to the corresponding control mice. On the other hand, fasting increased plasma HDL and the activities of both enzymes in transgenic and control mice alike. Thus, PON and PAF-AH apparently depend on HDL for transport in mice, as previously shown in LCAT knockout mice (32) and in C57BL/6 mice fed on an atherogenic diet (33).

An interesting finding of the present study was that PON and PAF-AH activities of huapoA-II transgenic mice decreased less than HDL protein and lipid components, similarly to LCAT activity (16). Thus, huapoA-II rich HDL efficiently carry all three antioxidant enzymes. Conversely, PON activity was 50% lower when expressed per mg HDL-protein in transgenic mice overexpressing mapoA-II (34). On the other hand, it has been reported that PON may be associated with apoA-I in HDL (5, 6). Expression of the huapoA-I transgene in the C57BL/6 background or in the C57BL/6 and apoE$^{-/-}$ background resulted in increased PON and PAF-AH activities, in parallel to the increase in huapoA-I (35). However, in the present study the decrease in apoA-I was considerably greater than that of the antioxidant enzymes in both transgenic lines. Thus, our results indicate that PON and PAF-AH associate with HDL containing huapoA-II despite the low amounts of apoA-I, especially in the higher expressing λ mice.

Recent studies have reported that PAF may also be processed by PON and LCAT, which inactivates PAF by transacylation (36). Human PON in HDL hydrolyzes PAF, raising the possibility that, in HDL, PON rather than PAF-AH represents the main anti-oxidant activity (37). Two arguments favor the hypothesis that PAF-AH and PON are distinct enzymes, at least in mice: *i*) in the present study, PAF-AH activity eluted in a single peak in the HDL region, whereas PON eluted in two peaks corresponding to large and small HDL; *ii*) PAF-AH activity was unchanged in PON1 knockout mice (30).

PON and PAF-AH have been implicated in the protective role of HDL against atherosclerosis. The presence of purified paraoxonase (but not heat-inactivated paraoxonase) in the incubation medium of LDL decreased the formation of lipid peroxides in the presence of $CuCl_2$ (38). VLDL containing PAF-AH neither stimulated monocyte chemotaxis nor their adhesion to endothelial cells, contrary to PAF-AH depleted VLDL (39). A positive correlation between PON mRNA levels and the ability of HDL to protect LDL against oxidation was established in inbred mouse strains differing in susceptibility to diet-induced aortic fatty streak lesions (40). Transgenic mice overexpressing mapoA-II had higher plasma HDL levels, but developed more fatty streak lesions than control mice on a chow diet (34). Interestingly, PON activity was reduced 2-fold in HDL from mapoA-II transgenic mice, whereas PAF-AH activity was not altered. PON1-knockout mice fed on an atherogenic diet developed larger aortic atherosclerotic lesions than control mice (30). Finally, mice deficient in both PON1 and apoE displayed increased levels of oxidized phospholipids in LP-B and developed significantly larger atherosclerotic lesions than apoE deficient mice (41). Adenoviral gene transfer of human PAF-AH in apoE knockout mice reduced βVLDL-induced ex vivo macrophage adhesion and in vivo macrophage homing (42). In the same mouse model, a 77% decrease of injury-induced neointima formation and a 42% decrease of atherosclerotic lesions were achieved after PAF-AH gene transfer (43). In humans, the relationship between the activities of antioxidant enzymes and atherosclerotic risk is less well established.

The antioxidant properties of HDL have also been attributed to their main apoA-I and apoA-II, while it was

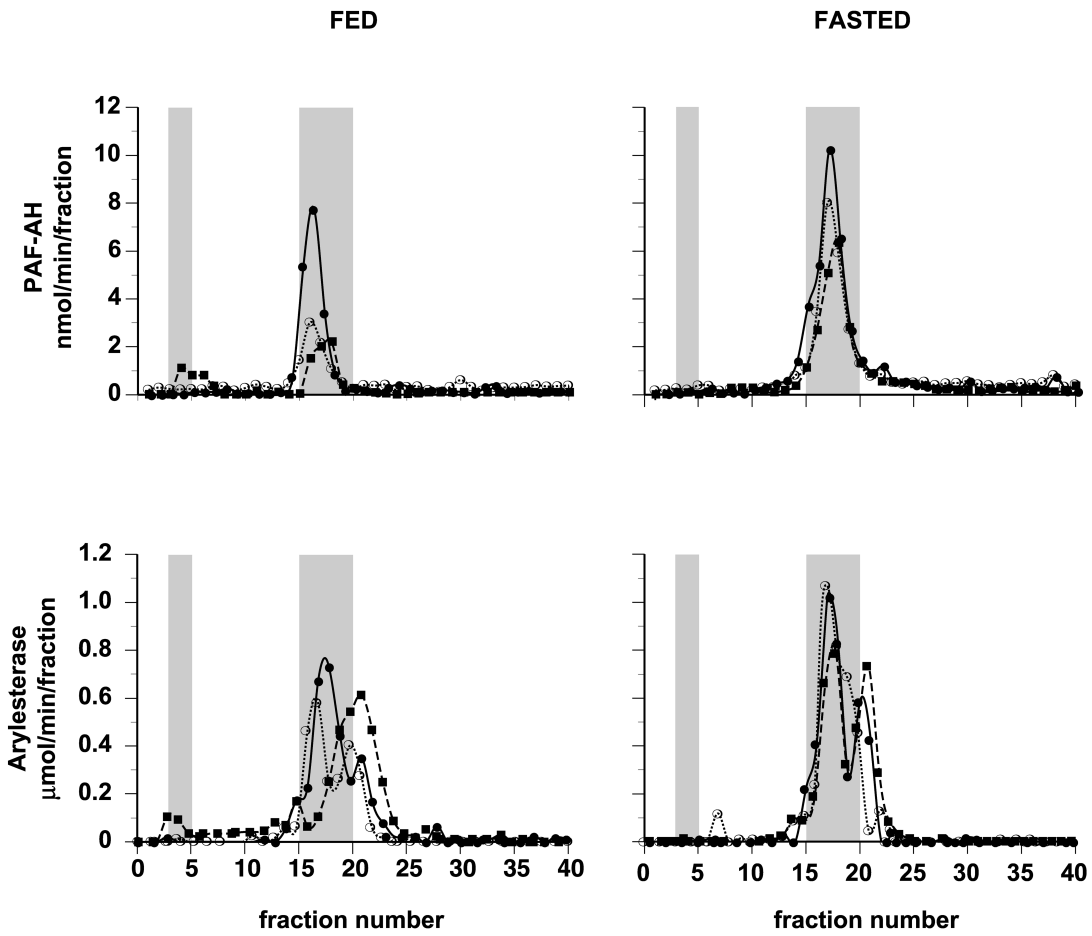

Fig. 3. Distribution of platelet-activating factor acetylhydrolase and arylesterase activities among lipoprotein fractions of control and huapoA-II transgenic mice. Sera were fractionated by FPLC as described in Materials and Methods. Sera and elution media were additioned with $CaCl_2$ (5 mM final concentration) to preserve arylesterase activity. PAF-AH and arylesterase were assayed in every five consecutive 0.2 ml-fractions pooled together. The gray bar between pooled fractions three and five delineates the VLDL fraction and the gray bar between pooled fractions 15 and 20 delineates the HDL fraction. The column was calibrated with serum from control mice, by measurement of total cholesterol in all fractions. closed circle, control mice; open circle, huapoA-II δ mice; closed square, huapoA-II λ mice.

shown that HDL protects LDL from oxidative damage independently of PON (44). Purified huapoA-I, and especially huapoA-II, effectively protected against LDL oxidation. Human HDL_2 and HDL_3 reduced phosphatidylcholine hydroperoxides to the corresponding hydroxides independently of PON and LCAT activities (45). This reducing activity was also expressed by reconstituted particles containing huapoA-I or huapoA-II only, as well as isolated huapoA-I. The authors showed that the oxidation of specific methionine residues in huapoA-I and huapoA-II plays a significant role in the 2-electron reduction of hydroperoxides of cholesteryl esters and phosphatidylcholine of HDL. Interestingly, the reduction of phosphatidylcholine hydroperoxides by huapoA-I was inhibited by anti-huapoA-I antibodies (46).

Ultimately, the antioxidant properties of HDL have to be assessed at the vessel wall, where expression of adhesion molecules at the surface of endothelial cells may lead to monocyte recruitment into the arterial intima, monocyte transformation to macrophages, and initiation of atherosclerosis. Baker et al. (47) demonstrated the ability of reconstituted HDL (rHDL) to inhibit cytokine-induced expression of vascular cell adhesion molecule-1 (VCAM-1) in endothelial cells. Spherical rHDL with huapoA-I and rHDL with huapoA-II inhibited VCAM-1 expression to similar degrees when compared at equivalent particle molarities. Expression of the huapoA-I transgene in apoE knockout mice increased HDL cholesterol and greatly diminished fatty streak lesion formation (48). The huapoA-I transgene inhibited foam cell formation at a stage following lipid deposition, endothelial activation, and monocyte adherence without appreciable increases in serum PON in 2-month-old animals. However, serum PON activity decreased in apoE knockout mice after 3 months of age, coincident with increases in aortic lesion area and serum lipid peroxidation (9). The recent review of experimental data on the role of antioxidants in the treatment or prevention of atherosclerosis in humans and animal models highlights the need to link biochemical changes in the arterial wall more directly to the oxidation theory of atherosclerosis (49).

To determine whether the lower PON and PAF-AH activities in our huapoA-II transgenic mice modified the oxidative

TABLE 3. Basal oxidative state and susceptibility to oxidation of VLDL, LDL, and HDL of control and hAII-transgenic mice

		VLDL		LDL		HDL	
	Genotype	TBARS[c]	Hydroperoxides[c]	TBARS[c]	Hydroperoxides[c]	TBARS[c]	Hydroperoxides[c]
Oxidative state[a]	Controls	135.5 ± 4.5	335.5 ± 15.5	3.3 ± 2.0	113.7 ± 52.3	4.2 ± 2.0	15.2 ± 7.3
	hAIItg-δ	108.3 ± 23.7	426.0 ± 65.8	6.3 ± 1.4	155.5 ± 54.4	2.8 ± 1.6	15.2 ± 3.8
	hAIItg-λ	101.0 ± 39.5	572.7 ± 99.7	15.0 ± 11.1	175.7 ± 80.5	3.2 ± 2.0	13.4 ± 2.8
Susceptibility	Controls	447.5 ± 7.5	475.0 ± 45.0	153.9 ± 20.4	143.1 ± 20.8	35.4 ± 9.3	21.3 ± 4.3
to oxidation[b]	hAIItg-δ	678.0 ± 13.9[e]	1701 ± 42.1[f]	193.8 ± 10.2	131.0 ± 27.4	18.2 ± 3.9	24.3 ± 2.7
	hAIItg-λ	884.3 ± 43.2[f,g]	1816 ± 63.6[d]	194.3 ± 27.7	179.7 ± 21.4	18.0 ± 6.2	24.7 ± 1.5

All animals were fed ad libitum. Lipoproteins were prepared from plasma pools of eight to 12 mice.

[a] Oxidative state was measured after incubations of lipoproteins with PBS, as described in Materials and Methods and represents basal oxidation.

[b] Susceptibility to oxidation was assessed as the difference of TBARS and hydroperoxides measured after incubations of lipoproteins with $CuSO_4$ or with PBS.

[c] TBARS and hydroperoxides are expressed as nmol/mg protein of each lipoprotein fraction. Results are the mean ± SE for three separate lipoprotein preparations by ultracentrifugation. Statistical significance of the results was calculated as in Table 1.

[d] $P < 0.05$, [e] $P < 0.01$, [f] $P < 0.001$ between transgenic and control mice.

[g] $P < 0.01$ between hAIItg-δ and hAIItg-λ transgenic mice.

state of LP-B, we measured TBARS and hydroperoxides under basal conditions and following oxidative stress. The basal oxidation of VLDL and LDL was comparable among transgenic and control mice, despite the long residence time of VLDL in the circulation of transgenic mice (15). Conversely, VLDL and LDL accumulating in plasma of apoE deficient mice displayed an increased basal oxidative state and a greater susceptibility to oxidation relative to lipoproteins of control animals (50). On the other hand, VLDL of our fed huapoA-II transgenic mice displayed a 2–3-fold greater oxidizability, probably due to their enrichment in triglyceride, comparably with VLDL of apoE knockout mice (50).

To establish the antioxidant potential of HDL from huapoA-II transgenic mice, which have decreased PON and PAF-AH activities but transport high huapoA-II amounts displaying antioxidant activities (45), we compared protection of VLDL from oxidative stress by HDL of control and transgenic mice. An intriguing finding was that HDL from transgenic mice protected VLDL from oxidation more efficiently than control HDL. Interestingly, VLDL from transgenic mice, which were more susceptible to copper-induced oxidation than control VLDL, were more protected from oxidation by all types of HDL. Mackness et al. (51) reported similar findings by comparing the ability of HDL to protect LDL from oxidation. LDL prepared from different subjects exhibited different susceptibilities to oxidation; the individual LDL fractions most susceptible to oxidation were those that were more protected by HDL.

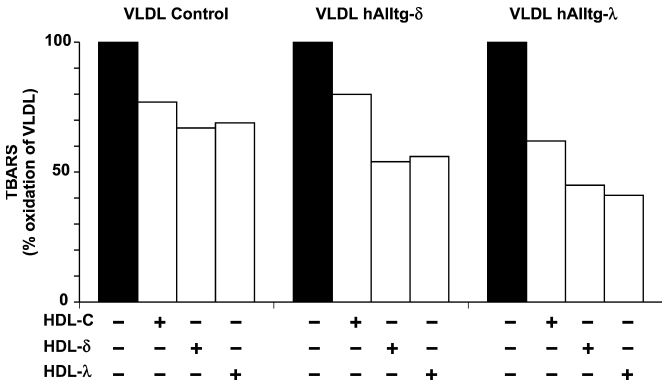

Fig. 4. Protection of VLDL from oxidative stress by HDL. VLDL of control and transgenic mice were incubated in the absence or presence of homologous or heterologous HDL. Thiobarbituric acid reactive substances (TBARS) values represent the difference between incubations with 5 μM $CuSO_4$ and PBS, as described in Materials and Methods. TBARS in VLDL incubated without HDL were taken as 100%. Two separate experiments with different VLDL and HDL preparations were performed and one representative experiment is shown. The TBARS values of VLDL from control, δ, and λ transgenic mice were: 450.5, 690.3, and 900.7 nmol/mg VLDL-protein, respectively. HDL-C, HDL-δ, and HDL-λ are HDL prepared from control and transgenic lines δ and λ, respectively.

Considering that the antiatherogenic properties of HDL result from the combined effects of their main apolipoproteins, A-I and A-II, and of associated antioxidant enzymes, the question now arises as to the anti- or pro-atherogenic properties of HDL of our huapoA-II transgenic mice. On the one hand, the decreases in plasma HDL and apoA-I concentrations and in PON, PAF-AH, and LCAT activities may be proatherogenic. On the other hand, these HDL contain high amounts of huapoA-II, which may have appreciable antioxidant capacities (45, 47) and transport adequate amounts of PON and PAF-AH on a particle basis. This study showed that huapoA-II rich HDL efficiently protected VLDL against copper-induced oxidation. The net result of these various effects of huapoA-II overexpression on cardiovascular risk cannot be evaluated at present. Contradictory results have been reported on aortic fatty streak formation in mice expressing huapoA-II. In transgenic lines with moderate huapoA-II expression, a protective effect has been observed after a challenge with an atherogenic diet (19, 20). On the contrary, a high huapoA-II expression in independently established transgenic lines resulted in greater lesion formation than in control C57BL/6 mice after feeding with the atherogenic diet (21). In humans, epidemiologic studies have linked the

atheroprotective effect of HDL either to apoA-I alone (52) or to apoA-I and apoA-II (53). Clearly, more studies are needed to evaluate the pro- or anti-atherogenic role of huapoA-II under different physiological settings.

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