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Endothelial Derived Vasorelaxation Is Impaired in Human APO A-I Transgenic Rabbits

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Endothelium-derived relaxing factor (nitric oxide: NO) may provide an endogenous defence against atherosclerosis which impairs endothelium-dependent vascular relaxation. Atherosclerosis development is inhibited in cholesterol fed human apo A-I transgenic rabbits (Duverger, N., ***Circulation,*** **1996, 94, 713–717). We investigated if endothelium-dependent vascular relaxation is modified in human apo A-I transgenic rabbits by testing in vitro endothelium-dependent receptor-dependent vascular relaxation to acetylcholine and endothelium-dependent receptor-independent vascular relaxation to A23187 of abdominal aorta, precontracted with phenylephrine, in human apo A-I transgenic rabbits (n=4) versus non transgenic littermates (n=4). Endothelium-independent vascular relaxation was investigated with sodium nitroprusside. Vascular precontraction to phenylephrine was significantly increased in human apo A-I transgenic rabbits ($p<0.05$) while endothelium-independent vascular relaxation to nitroprusside was similar between human apo A-I transgenic rabbits and control rabbits. Endothelium-dependent receptor-dependent and receptor-independent vascular relaxations were reduced in human apo A-I transgenic rabbits ($p<0.05$). Maximum endothelium-dependent receptor-dependent vascular relaxation was negatively correlated with HDL-cholesterol and total apo A-I (rabbit+human) plasma levels ($r=0.87$ and 0.86, $p=0.01$, respectively) but not with atherogenic plasma lipid (VLDL-cholesterol, LDL-cholesterol, VLDL+LDL cholesterol, triglycerides, apolipoprotein B) levels. These results suggest that the transgenesis of human apo A-I in rabbits impairs signal transduction of endothelial NO synthesis.**

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Atherosclerosis (1,2) and hypercholesterolaemia (increase in low-density (LDL) cholesterol levels) before the formation of atherosclerotic lesions (3,4) disturb the endothelium-dependent regulation of the vascular tone by labile liposoluble radical nitric oxide (NO). This defect predisposes to vasospasm and ischaemia, with anginal pain as a clinical manifestation. Furthermore, it is becoming clear that NO, in addition to regulating vasomotion, might also modulate the progression of atherosclerosis (5,6,7). In hypercholesterolemic rabbits, oral L-arginine supplementation (the substrate for NO synthase (NOS), the enzyme which catalyses the production of NO in vascular endothelial cells (8)) is associated with a marked reduction in aortic and coronary atherosclerosis (5,6).

Epidemiological studies in industrialized societies now indicate that a low high-density lipoprotein (HDL) cholesterol level, usually accompanied by elevated plasma triglycerides, is the most common abnormal lipoprotein phenotype associated with coronary heart disease susceptibility (9). Expression of human apolipoprotein A-I (apo A-I) (the major apolipoprotein of HDL) in mice (10) and in rabbits (11, 12) resulted in an increase in HDL cholesterol levels and in highly significant protection from the development of aortic atherosclerosis lesions in the transgenic animals fed a cholesterol rich diet. In spite of the strength of the association between high HDL cholesterol levels and atheroprotection, the basis of this association is not totally elucidated. The leading hypothesis, originally proposed by Glomset in 1968 (13), is called reverse cholesterol transport. This theory suggests that HDL regulate the movement of cholesterol from peripheral tissues, where it cannot be metabolized, to the liver for excretion. Nevertheless, HDL not only act on the so-called reverse cholesterol transport but also modify the biology of different cells such as platelets (14), renal proximal cells (15) and stimulate endothelin secretion by cultured endothelial cells (16, 17).

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As human apo A-I transgenic rabbits are protected against a cholesterol rich diet (12) and are also given oral L-arginine supplementation to inhibit atherosclerosis development (5,6), we tested the hypothesis that a relationship could exist in rabbits between the transgenesis of human apo A-I and the biological activity of NO. Therefore we measured endothelium-dependent vasorelaxation in human apo A-I transgenic rabbits and in non transgenic littermates.

MATERIALS AND METHODS

Animal model. Experiments were performed with New Zealand White rabbits transgenic for human apo A-I (line 20) (11, 12) and non transgenic littermates (controls) aged 12 months and fed with standard chow.

Solutions and drugs. The drug used were phenylephrine, acethylcholine, calcium ionophore A23187 and sodium nitroprusside (Sigma Chemical). Concentrated stock solutions were prepared with deionized water or 1% dimethyl sulfoxide (A23187).

Measurement of vascular reactivity. Abdominal aortic reactivity was studied in 2 male and 2 female human apo A-I transgenic rabbits and compared with sex matched control animals that had fasted overnight. Two rabbits were studied each day and the experimenter did not know which of the two rabbits was the transgenic. Rabbits were anesthetized with phenobarbital (30 mg/Kg IV). The abdominal aorta was removed and placed immediately in iced Krebs-Henseleit (KH) solution consisting of the following (in mmol/L): 118 NaCl; 4.6 KCl; 27.2 $NaHCO_3$; 1.2 $MgSO_4$; 1.75 $CaCl_2$; 0.03 Na_2EDTA and 11.1 d-glucose. This solution was aerated with 95% O_2-5% CO_2 (pH 7.4) and was maintained at 37°C. Intravenous heparin (1000 UI) was given before removal of the vessels to prevent coagulation. Vessel cleaning and dissection were performed in KH with the aid of a binocular magnifying-glass. Arteries were cut into rings of 3 to 5 mm in length. In half of the arteries, the endothelium was gently and mechanically removed with a small wooden applicator. Two stainless stell wires were introduced through the arterial lumen of the ring. One wire was fixed to the wall of the organ bath, while the other was connected to a force-displacement transducer (Radnoti glass technology, Monravia, CA). Each organ bath (Radnoti glass technology, Monravia, CA) contained 40 ml of KH. The baths were maintained at 37°C and bubbled continuously with 95% O_2 and 5% CO_2. Transducer outputs were amplified, recorded on a polygraph, digitized, and sampled by a microcomputer (Kenitec 486 DX_2 66). For each abdominal aorta, four rings were studied. To establish the resting tension for maximal force development, a series of preliminary experiments were performed on rubbed and unrubbed artery rings which were exposed repeatedly to 70 mM KCl. Basal tension was increased gradually until contractions were maximal. The optimal resting tension was found to be 8.0 g. The arteries were allowed to attain a steady level of tension for 60 minutes before testing. All the rings were then preconstricted with phenylephrine (10^{-9} to 3.10^{-4} mol/L). At the plateau of phenylephrine response, acetylcholine (10^{-9} to 3.10^{-5} mol/L) and calcium ionophore A23187 (10^{-9} to 3.10^{-6} mol/L) were added to assess endothelium-dependent responses. We also added the nonendothelium dependent dilator (sodium nitroprusside: 10^{-9} to 3.10^{-5} mol/L) to the bath with rubbed artery rings.

Serum lipids measurements. Serum lipids (cholesterol and triglycerides) were measured colorimetrically on a microtiter plate reader and with commercially available reagents (Boehringer Mannheim, FRG). To determine the distribution of cholesterol within different plasma lipoprotein fractions, 10 μl of serum was subjected to size fractionation on a Superose 6 column (Pharmacia LKB, Sweden). Total apo A-I (rabbit + human) was measured by immunonephelemetry using a mixture of 5 monoclonal antibodies (Diagnostics Pasteur Production) recognizing both human apo A-I and rabbit apo A-I while anti-human apo A-I antibodies raised in rabbit were used to determine human apo A-I specifically (Duverger 1995). Apo B was measured by immunonephelemetry using antibodies specific for rabbit apo B (Institut Pasteur, Lille, France).

Statistical analysis. Contractile response elicited by PE is expressed as a proportion of KCl induced constriction. Relaxations to the vasodilator agents are expressed as percentages of the initial contraction to the PE. To analyse contractile and vasodilatation responses we determined the Emax and the EC_{50} (drug concentration producing 50% of the maximal vasoconstrictor response). All data is expressed as mean +/− SD. Aortic abdominal condtriction and relaxation and lipid levels of human apo A-I transgenic rabbits and control rabbits were compared by the Mann-Whitney test. Correlation coefficients were calculated between maximal aortic abdominal relaxation and lipids. $p<0.05$ was acknowledged as significant.

RESULTS

Aortic Abdominal Motricity

Contractile response to phenylephrine (PE). Contractile responses to PE are illustrated in Figure 1. The contractile response to PE was significantly increased in rings with endothelium from transgenic human apo A-I as compared with vessels from the control group (apo A-I: Emax=253.5±14.0%, versus control: Emax=177.0±7.1%; p=0.03). Sensitivity to PE was not different between human apo A-I transgenic rabbits for human and control rabbits (apo A-I: EC_{50} = 5.9 ± $3.3.10^{-7}$ mol/L versus control: EC_{50} = 3.31 ± $1.3.10^{-7}$ mol/L; p=NS). After endothelial denudation, contractile response to PE in both control and human apo A-I were similar.

Endothelium-dependent and -independent relaxation. The maximal endothelium-dependent receptor-dependent relaxation elicited by acetylcholine was significantly attenuated in the transgenic group for human apo A-I (apo A-I: Emax=26.8±9.5%, versus control: Emax=68.6±3.3%; p=0.03). (Figure 2A). Similarly, endothelium-dependent receptor-independent relaxation induced by A23187 was significantly decreased in the transgenic group for human apo A-I (Figure 2B). However, for the highest dose, Emax were not stastistically different (apo A-I: Emax=57.4±2.2%, versus control: Emax=80.9±7.6%; p=NS). In contrast, cGMP-mediated endothelium-independent relaxation was not altered, yielding superimposable concentration-response curves for the sodium nitroprusside-induced relaxation from control and transgenic groups for human apo A-I (Figure 2C).

Serum lipids measurements. The serum levels of total lipids and the cholesterol of lipoprotein fractions in control and transgenic rabbits are presented in table 1. The levels of total cholesterol, triglycerides and HDL cholesterol tended to be higher in the transgenic rabbits, but due to the small number of animals and the large dispersion of these lipidic values, the differences

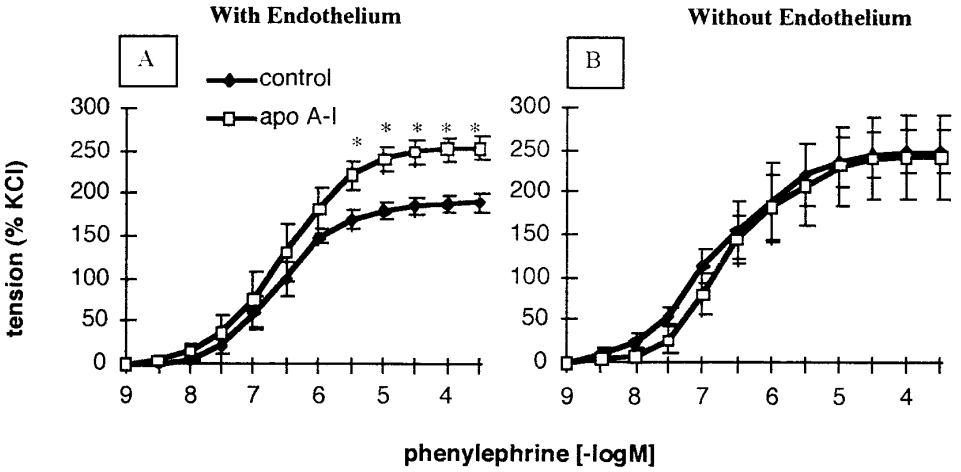

FIG. 1. Vasoconstrictor dose–response curves for PE in abdominal aorta for both human apo A-I transgenic rabbits and in control rabbits. The effect of PE was determined in rings with intact endothelium (A, n=4) and in rings from which endothelium was removed (B, n=4). All contractions are expressed as percentages of maximal contraction to potassium. Standard error bars are shown. An asterisk denotes a value that differs significantly between human apo A-I transgenic and control rabbits ($p<0.05$).

were not significant between the two groups. On the other hand VLDL-cholesterol, LDL cholesterol and apo B levels were identical in both groups. Rabbit apo A-I was non-significantly lower in the transgenic group while total apo A-I (rabbit apo A-I + human apo A-I) was significantly higher in the transgenic group ($p<0.01$). There were strong negative correlations between the maximal endothelium dependent receptor dependent vasorelaxation and HDL cholesterol and total apo A-I levels ($r=0.87$ and 0.86 respectively, $p<0.01$) but there was no correlation between maximal endothelium-dependent receptor-dependent vascular relaxation and total cholesterol, VLDL-cholesterol, LDL-cholesterol, VLDL+LDL-cholesterol, triglycerides and apo B levels (table 2).

DISCUSSION

Endothelium dependent vascular relaxation is mediated by NO release and is inhibited in humans and in rabbits by high LDL-cholesterol levels and atherosclerosis (1, 3). Overproduction of endogeneous NO induced by L-arginine oral supplementation in cholesterol fed rabbits inhibits atherosclerosis development (5, 6) and atherogenesis is also inhibited in human apo A-I transgenic rabbits submitted to a cholesterol rich diet (12). Duverger et al. (12) reported that this atheroprotection is partly mediated through an improvement in reverse cholesterol transport. Our goal was to study endothelium-dependent vasorelaxation dependent on NO release in human apo A-I transgenic rabbits and we made the unexpected observation that this vascular relaxation was dramatically impaired in these animals. This data is highly surprising because to our knowledge it has never been reported in humans and animals that high HDL cholesterol and apo A-I levels are associated with in vivo impaired endothelial vascular relaxation. Whereas, it has undoubtingly been established that oxidized-LDL impairs ex-vivo endothelium-dependent arterial relaxation by their lysolecithin content (18,19), only one publication reports that HDL inhibits ex-vivo endothelium-dependent relaxation through their phospholipid content (20). We speculate that at least 4 biological mechanisms may be involved in the inhibition of endothelium-dependent vascular relaxation in human apo A-I transgenic rabbits:

-1. The human apo A-I transgene could be inserted near the endothelial (e) NOS gene in the genome of the rabbits and could inhibit the expression of this enzyme. Although this hypothesis is valid, the probality of such an aleatory gene insertion is very low.

-2. Nitroprusside-mediated endothelium-independent vasorelaxation is not altered in the human apo A-I transgenic rabbit. This indicates that the relaxing mechanism of NO- dependent vascular smooth muscle cells relaxing is not altered in human apo A-I transgenic rabbits. We can speculate on the direct action of high levels of HDL and human apo A-I on NO synthesis in human apo A-I transgenic rabbits. Shaul et al. (21) reported that eNOS is localized in the plasmalemmal microdomains implicated in signal transduction called caveolae and Ju et al. (22) demonstrated that direct interaction of endothelial eNOS and caveolin-1 inhibits synthase activity. On the other hand, caveolae represent a major site of HDL3-induced cellular cholesterol efflux (23) and HDL3 interact with glycosyl phosphatidylinositol-anchored proteins in caveolae (24). Furthermore, HDL3 induce a signal that involves glycosylphosphatidylinositol-anchored proteins (25) and Feron et al. (26) reported that the muscarinic cholinergic ago-

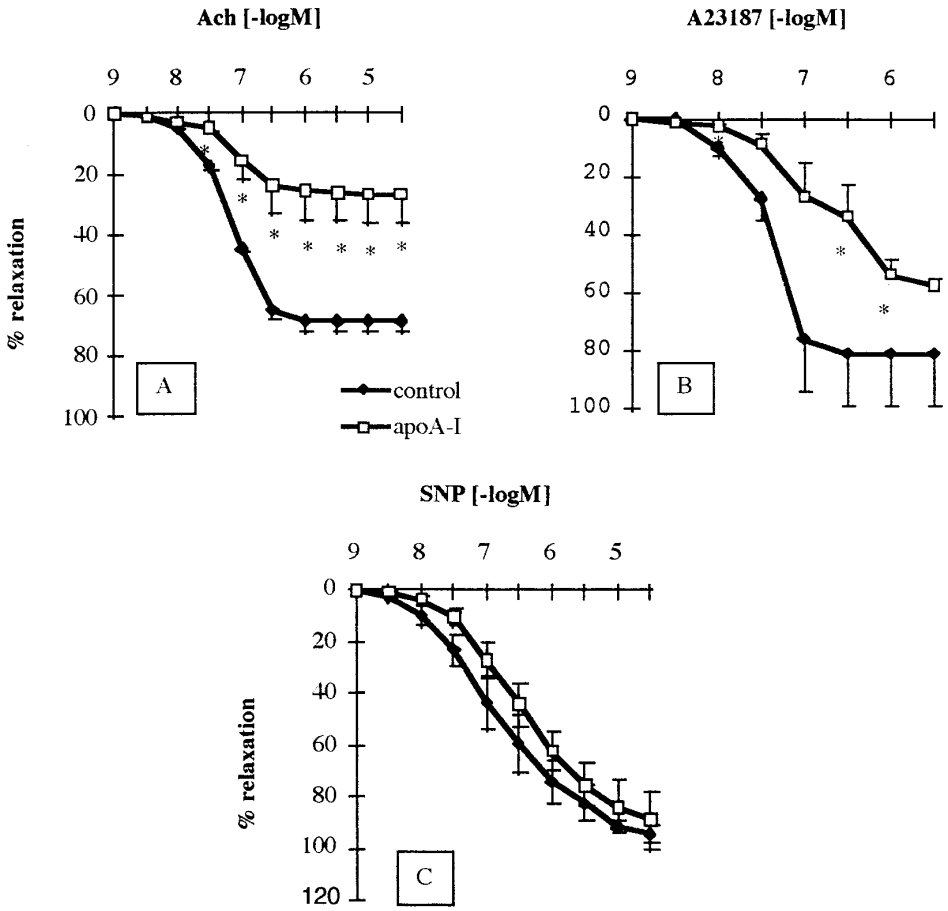

FIG. 2. Vasodilator dose–response curves showing percentage relaxation elicited by increasing doses of acetylcholine (Ach, A), calcium ionophore A23187 (B) and sodium nitroprusside (SNP, C) in the abdominal aorta of human apo A-I transgenic and control rabbits. Standard error bars are shown, n=4 for each group. Although endothelium-dependent receptor-dependent and -independent relaxation is altered in transgenic for human apo A-I, endothelium-independent relaxation elicited by SNP did not differ between the two groups. An asterisk denotes a value that differs significantly between human apo A-I transgenic and control rabbits ($p<0.05$).

nist carbachol promotes the translocation of the muscarinic acetylcholine receptor to caveolae where eNOS is localized in cardiac myocytes. We can therefore suppose that high concentrations of human apo A-I containing HDL in transgenic rabbits invade caveolae modifying the muscarinic receptor working and inhibiting eNOS activity. The sensitivity of the aortae of transgenic rabbits to the lower doses of A23187 that induce receptor-independent endothelium-dependent vasorelaxation was reduced but the level of the relaxation induced by the higher concentrations (3.10^{-6} mol/L) was not different from controls. This could indicate that higher doses of A23187 are necessary in human apo A-I transgenic rabbits than in control rabbits to induce endothelial cell calcium entrance or that higher intracellular calcium concentrations are necessary to stimulate eNOS in transgenic rabbits. The sensitivity of transgenic rabbits to acetylcholine to induce receptor-dependent endothelial-dependent vascular relaxation is dramatically reduced and the maximal acetylcholine-dependent relaxation is highly decreased in transgenic rabbits in comparison with controls. Conjointly along with putative eNOS activity inhibition in transgenic rabbits (lower sensitivity to calcium . . .), one can imagine alterations in endothelial cell muscarinic receptor activation. For example, the recruitment of the muscarinic receptors could be decreased in the caveolae of endothelial cells in transgenic rabbits due to the locally high concentration of HDL bound to the binding protein anchored in the caveolae (24). For this reason the working of the muscarinic receptors could also be altered and the transduction of the signal could be degraded, or the accessibility of acetylcholine to the ligand binding domain of these receptors could be diminished.

As previously noted one study reported that HDL inhibit endothelium-dependent vascular relaxation through their phospholipid content (20). Phospholipid levels are increased in human apo A-I transgenic rabbits although there is no difference in the lipid and

TABLE 1

Lipids and Apolipoproteins in Human apo A-I Transgenic Rabbits and Nontransgenic Littermates (Control Rabbits)

	Transgenic rabbits	Control rabbits	p
Total cholesterol (*mg/dl*)	102 ± 43	51 ± 24	0,16
Triglycerides (*mg/dl*)	136 ± 39	82 ± 28	0,13
VLDL-cholesterol (*mg/dl*)	2 ± 1	21 ± 29	0,36
LDL-cholesterol (*mg/dl*)	28 ± 33	17 ± 18	0,62
HDL-cholesterol (*mg/dl*)	52 ± 19	32 ± 7	0,20
Rabbit apo A-I (*mg/dl*)	17 ± 22	37 ± 8	0,25
Human apo A-I (*mg/dl*)	84 ± 37	—	—
Total apo A-I (*mg/dl*)	101 ± 15	37 ± 8	0,01
Apo B (*mg/dl*)	95 ± 90	43 ± 23	0,46

protein composition of HDL between transgenic and control rabbits (11). Therefore, it is possible that high concentrations of phospholipids are susceptible to be transformed into molecules such as lysolecitin capable of inhibiting endothelium-dependent vascular relaxation (18,19). Howerver, data concerning the putative role of HDL in inhibiting endothelium-dependent vascular relaxation is conflicting since Plane et al. (27) reported that the ex-vivo inhibition of relaxation by oxidized-LDL was prevented by the presence of HDL and suggested that this prevention is consistent with the inhibition by HDL of inhibitory factors, different from lysolecithin, that are present in oxidized-LDL. In 1994, Galle et al. (28) also reported that HDL inhibit the oxidized-LDL induced inhibition of endothelium-dependent vasodilation.

-3. We have previously indicated that it has clearly been established that oxidized-LDL inhibit ex-vivo endothelium-dependent vascular relaxation probably through their lysolecithin content (18, 19). In cholesterol fed rabbits dietary antioxidants preserve endothelium-dependent vessel relaxation (29) and in humans an antioxidant therapy improves endothelium-dependent coronary vasomotion (30). Furthermore, in patients treated with lipid-lowering agents, the coronary vasodilator response to acetylcholine is related to the susceptibility of LDL to oxidation. These findings suggest that oxidative stress is an important determinant of coronary endothelial dysfunctioning to be observed in patients with hypercholesterolemia (31). A higher level of AB-MDA-LDL has been observed in human apo A-I transgenic rabbits in a following protocol (32) carried out on 13 controls and 18 transgenic rabbits to study atherosclerosis development during a cholesterol rich diet. AB-MDA-LDL is a marker of LDL oxidation and higher serum levels of these autoantibodies have been reported to be proportional to the progression of carotid atherosclerosis in humans (33) and in the serum of apo E-deficient mice depicted as models of lipoprotein oxidation in atherogenesis (34). In our study the VLDL- and the LDL-cholesterol levels were not higher in the human apo A-I transgenic rabbits but the triglyceride concentrations tended to be increased in these animals. This increase has been confirmed in the following protocol (32) where we also note an increase in phospholipid concentrations in transgenic animals. In this protocol, the increase in phospholipid concentration is largely linked to the threefold higher level of HDL in transgenics, but the increase in triglyceride is partly due to increases in HDL-triglyceride and in non-HDL triglyceride (VLDL+LDL). The LDL of diabetic patients with higher serum triglyceride and phospholipid concentrations are more susceptible to lipid oxidation than the LDL of control patients (35) and the plasma of these patients contains higher concentrations of AB-MDA-LDL than the plasma of controls (36). It can therefore be supposed that the intensity of in vivo LDL oxidation is higher in human apo A-I transgenic rabbits than in controls because of a higher triglyceride concentration in the non-HDL fraction (VLDL+LDL). This would lead to the formation of more oxidized immunogenic LDL and to the production of more AB-MDA-LDL. Furthermore, it has been proposed that in vivo, cell-derived NO can inhibit LDL oxidation (37, 38). In our study, assuming that the basal endothelial NO release is reduced in human apo A-I transgenic rabbits, as suggested by the increase in the contractile response to phenylephrine, one can suggest that LDL are less protected against in vivo oxidation. The resulting higher oxidized-LDL concentrations in the artery wall of human apo A-I transgenic rabbits would impair endothelium-dependent vascular relaxation. This putative increase in oxidized-LDL in transgenic rabbits would be sufficiently low not to promote atherosclerosis development because aortic atherosclerosis has only been observed in cholesterol fed transgenic rabbits and not in animals on a chow diet (12), and we did not observe macroscopic atheroma on aortae in our study. Nevertheless, it has clearly been demonstrated

TABLE 2

Correlation between Maximum Endothelium-Dependent Receptor-Dependent Aortic Relaxation and Plasma Lipids and Apolipoprotein Levels in Pooled Human apo A-I Transgenic Rabbits and Control Rabbits

	r
Total cholesterol	−0.51
Triglycerides	−0.53
VLDL-cholesterol	−0.11
LDL-cholesterol	−0.19
VLDL- + LDL-cholesterol	−0.16
HDL-cholesterol	−0.87*
Total apo A-I	−0.86*
Apo B	−0.60

* $p < 0.05$

that any alteration in lipid metabolism can induce endothelium-dependent vascular relaxation alteration in the absence of atherosclerosis development (39).

-4. Hu et al. (16) and Martin-Nizart et al. (17) have reported that HDL and oxidized HDL highly stimulate endothelin secretion in cultured endothelial cells. Furthermore, Hu et al. (16) have shown that human apo A-I increased endothelin secretion as potently as HDL in these cutured cells. Endothelin is the highest vasoconstrictor peptide isolated up to date(40). We may speculate that a high circulating concentration of human apo A-I in human apo A-I transgenic rabbits could induce the endothelium-dependent endothelin secretion that would result in impairment in endothelium-dependent vasorelaxation. On the other hand oxidized LDL also stimulate endothelin secretion in cultured endothelial cells (41,42). Assuming as suggested above that oxidized LDL would accumulate (insufficiently to induce atheroma) in the vascular sub-endothelium of human apo A-I transgenic rabbits, we may put forward that these oxidized LDL would stimulate endothelin secretion that would alter endothelium-dependent vascular relaxation.

In conclusion, we have observed that endothelium-dependent vascular relaxation is impaired in human apo A-I transgenic rabbits but the biological mechanism of this inhibition remains unexplained. This inhibition is surprising inasmuch as these transgenic rabbits are protected against a cholesterol rich diet inducing atherosclerosis development (12). In order to determine if over-expression of human apo A-I in other species than rabbits also inhibits endothelium-dependent vascular relaxation we plan to study vasorelaxation in human apo A-I transgenic mice. Furthermore, we will also investigate the functional relationships between HDL binding proteins, eNOS and muscarinic receptors in the caveolae of endothelial cells.

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