Dietary sodium chloride restriction enhances aortic wall lipid storage and raises plasma lipid concentration in LDL receptor knockout mice

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Abstract **This study aimed at measuring the influence of a low salt diet on the development of experimental atherosclerosis in moderately hyperlipidemic mice. Experiments were carried out on LDL receptor (LDLR) knockout (KO) mice, or apolipoprotein E (apoE) KO mice on a low sodium chloride diet (LSD) as compared with a normal salt diet (NSD). On LSD, the rise of the plasma concentrations of TG and nonesterified fatty acid (NEFA) was, respectively, 19% and 34% in LDLR KO mice, and 21% and 35% in apoE KO mice, and that of plasma cholesterol was limited to the LDLR KO group alone (15%). Probably due to the apoE KO severe hypercholesterolemia, the arterial inner-wall fat storage was not influenced by the diet salt content and was far more abundant in the apoE KO than in the LDLR KO mice. However, in the less severe hypercholesterolemia of the LDLR KO mice, lipid deposits on the LSD were greater than on the NSD. Arterial fat storage correlated with NEFA concentrations in the LDLR KO mice alone (n = 14, $P = 0.0065$).** **Thus, dietary sodium chloride restriction enhances aortic wall lipid storage in moderately hyperlipidemic mice.**—Catanozi, S., J. C. Rocha, M. Passarelli, M. L. Guzzo, C. Alves, L. N. S. Furukawa, V. S. Nunes, E. R. Nakandakare, J. C. Heimann, and E. C. R. Quintão. **Dietary sodium chloride restriction enhances aortic wall lipid storage and raises plasma lipid concentration in LDL receptor knockout mice.** ***J. Lipid Res.*** **2003.** 44: **727–732.**

Supplementary key words salt restriction • triacylglycerol • hypertriglyceridemia • hyperlipidemia • atherosclerosis

Epidemiological studies have shown that arterial hypertension often is associated with dyslipidemia, although the mechanisms that characterize this association are not completely understood (1). On the other hand, a severe restriction of the intake of sodium chloride has adverse side effects on glucose and lipid metabolism (2–5). When submitted to a low sodium chloride intake, an increase in plasma triacylglycerol (TG) and total cholesterol (TC) concentrations in nonobese normotensive subjects (4–7) has been reported, as well as an increase in fasting insulin concentration in hypertensive patients, in normotensive patients, and in nonobese normotensive subjects (2–4). In addition, increased plasma TC and TG concentrations were reported in hypertensive human subjects treated with the diuretic hydrochlorothiazide (8), a fact that might offset the beneficial effects of blood pressure lowering in the prevention of coronary heart disease (9, 10).

It is well known that hypercholesterolemia, hypertriglyceridemia, and low concentrations of HDL cholesterol are among the major risk factors contributing to premature coronary artery disease (11, 12). In this regard, although dietary salt restriction lowers blood pressure, its recommendation for the general population remains questionable because of possible deleterious effects on cardiovascular outcomes (13).

Previous work from our laboratory showed that Wistar rats fed a low salt diet (LSD) developed higher plasma concentrations of nonesterified fatty acid (NEFA), TG, and cholesterol, as compared with controls either on a normal-salt or on a high-salt intake (14). This fact has been explained by an impairment of the removal rate of TG-rich lipoproteins (LP) from plasma (14). However, in the plasma of Wistar rats and wild-type mice, HDL is the most abundant LP, whereas normal humans contain mostly LDL.

Present experiments were then carried out in mice models with a plasma LP profile closer to that found in

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Abbreviations: KO, knockout; LDLR, low density lipoprotein receptor; LP, lipoprotein; LSD, low sodium chloride diet; NSD, normal sodium chloride diet; TC, total cholesterol; U_KV, urinary potassium; $U_{Na}V$, urinary sodium.

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humans than in rodents. For this purpose, we have submitted to a LSD apolipoprotein E (apoE) knockout (KO) and LDL-receptor KO (LDLR KO) mice that are known to display an elevated plasma concentration of LDL similar to that attained in mild hypercholesterolemia in humans. The hypercholesterolemia of LDLR KO mice is due to the slow clearance rates of VLDL, IDL, and LDL (15, 16). ApoE is a surface constituent of plasma cholesterol-rich LP that ascribes LP recognition and affinity to hepatic LP receptors (17). Compared with wild-types, apoE KO mice present plasma TG and cholesterol simultaneously elevated (18), resembling patients with apoE deficiency (19).

This study aims at measuring the degree of accumulation of lipids on the inner arterial wall, and at relating this process to variations of plasma lipids when normotensive hyperlipidemic mice are submitted to a low-salt diet.

MATERIALS AND METHODS

Materials

Enzymatic kits for TG and TC measurements were obtained respectively from Merck (Darmstadt, Germany) and Roche Diagnostics (Mannheim, Germany). Wako Chemicals supplied enzymatic kit for the NEFA measurement.

Experimental protocols

ApoE KO and LDLR KO mice were purchased from Jackson Laboratory (Bar Harbor, Maine). Animal experiments were performed in accordance with protocols approved by the Institutional Animal Care and Research Advisory Committee. A pelleted chow provided by Harlan Teklad (Madison, WI) was fed ad libitum to newly weaned 3-week-old male apoE KO and LDLR KO mice. The diet contained the following nutrients (g/100 g): casein (28.7), sucrose (31.3), corn starch (20.0), soybean oil (6.0), and minerals and vitamins. Added amounts of cellulose were substituted for sodium chloride. Animals were raised up to 3 months of age in conventional housing at 25°C on a 12 h light/12 h dark cycle.

Two bunches of apoE KO and two bunches of LDLR KO mice were randomly distributed into experimental groups that differed according to the levels of sodium chloride intake: low sodium chloride diet (LSD) (0.15%) and normal sodium diet (NSD) (1.27%). LSD groups were fed the minimum sodium chloride required for a normal rodent growth rate (20). Because experiments were time consuming and prolonged sample storage imposed technical constraints on some analyses, such as blood NEFA, urinary ions, and aortic histology, studies were carried out in batches of approximately six animals each. In order to avoid the interference of chylomicron TG on the measurements of plasma total TG, all determinations were carried out in blood samples drawn in the morning after a 12 h overnight fasting period; therefore, plasma TG must represent mainly the VLDL component. Plasma TG and TC concentrations measured after weaning disclosed that we were dealing with a homogeneous plasma lipid animal population. One week before the experiment, blood (140 μl) was drawn into capillaries via the tail vein for plasma TG, TC, and NEFA measurements, as well as plasma LP profile by fast protein liquid chromatography (FPLC) on a HR 10/30 Superose 6 column (Pharmacia Biotech, Uppsala, Sweden) using a constant flow of 0.5 ml/min of Tris-buffered saline (pH 7.2). Sixty fractions of 0.5 ml were automatically collected. TC was also determined in the fractions collected by an enzymatic assay in an automatic analyzer, Cobas (F. Hoffman-La Roche, Basileia, Switzerland). Due to the inconvenience of prolonged storage, NEFA measurements were carried out in the last experimental batch only. An FC280 flame spectrophotometer (CELM, SP, Brazil) was utilized for 8 h urinary sodium ($U_{Na}V$) and potassium (U_KV) measurements as control for the sodium chloride intake. Hematocrit was determined at the end of the experiment.

Histology

Under light ethyl-ether anesthesia, mice hearts and aortas were excised in the fresh state for histological evaluation. Aortas were transected in the aortic root segment, dissected, and exhaustively washed with a 0.9% NaCl cold solution under a stereoscope, and thereafter gradually submitted to liquid nitrogen and preserved in a tissue-freezing medium for frozen tissue specimens. Aortic arch proximal, medial, and distal segments (3 mm long each) were drawn along a 1 cm length adjacent to the aortic root. Three sets of 10 cryostat cross-sections (4 μm each) were serially obtained from each aortic segment at 1 mm intervals between each set. Ten sections of each segment were randomly chosen for histological analysis. Thirty aortic slice samples from each animal were microscopically analyzed. Each sample was then stained with Oil Red O and counterstained with hematoxylin, according to the modified method of Paigen et al. (21). The lipid deposits in the sections included the subendothelium and the media, and were quantified utilizing a light microscope connected to a video camera and a Leica (Cambridge, UK) Qwin Imaging software. The mean lipid deposit areas were calculated.

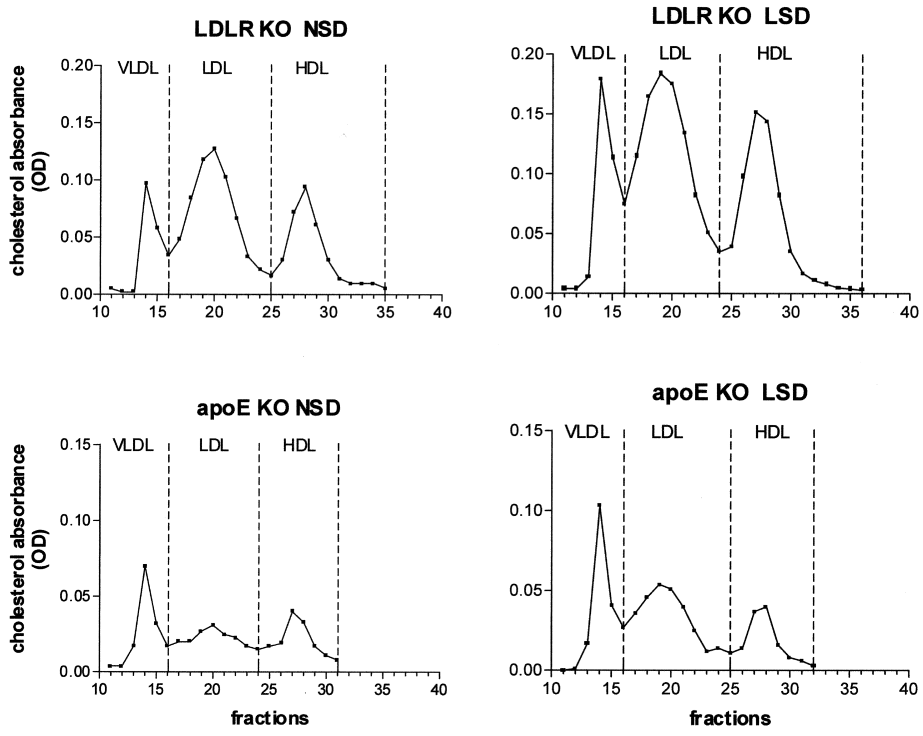
TABLE 1. Plasma triacylglycerol, nonesterified fatty acid, cholesterol levels, body weight, and hematocrit of apoE knockout and low density lipoprotein-receptor knockout mice

	apoE KO		LDLR KO	
	NSD	LSD	NSD	LSD
		mg/dl		
Triacylglycerol	119 ± 17 (13)	144 ± 29[a] (19)	146 ± 32[b] (18)	174 ± 42[a] (23)
		mEq/l		
NEFA	1.60 ± 0.63 (9)	2.19 ± 0.55[a] (10)	1.98 ± 0.66 (12)	2.66 ± 0.75[a] (8)
		mg/dl		
Cholesterol	386 ± 102 (13)	374 ± 62 (19)	273 ± 48[b] (18)	316 ± 72[a] (23)
		g		
Body weight	20.9 ± 3.5 (13)	22.0 ± 2.0 (19)	21.1 ± 1.6 (18)	21.7 ± 2.4 (23)
		%		
Hematocrit	47 ± 3 (13)	46 ± 3 (19)	46 ± 3 (18)	47 ± 3 (23)

Apo E, apolipoprotein E; KO, knockout; LDLR, LDL-receptor; LSD, low sodium chloride diet; NEFA, nonesterified fatty acid; NSD, normal sodium chloride diet. Mice were submitted either to a low sodium chloride diet or to a normal sodium chloride diet. Twelve-week-old apoE KO or LDLR KO mice fed ad libitum during the previous 9 weeks on a LSD or on a NSD. Data are expressed as mean ± SD; (n), number of animals. Statistical comparison by Student's *t*-test.

[a] LSD × NSD, $P < 0.05$.

[b] apoE KO × LDLR KO, in NSD group, $P < 0.05$.



	NSD			LSD		
	VLDL	LDL	HDL	VLDL	LDL	HDL
LDLR KO		n = 12			n = 10	
	13.0 (5.6 - 26.9)	52.8 (42.8 - 56.4)	32.8 (25.7 - 48.4)	16.8 (9.1 - 31.9)	50.0 (43.7 - 55.5)	32.4 (18 - 41.9)
ApoE KO		n = 6			n = 12	
	29.7 (16.3 - 40.3)	40.7 (34.3 - 61.4)	27.9 (16.9 - 46.0)	30.1 (20.5 - 41.2)	44.6 (38.9 - 51.9)	25.9 (19.9 - 28.7)

Fig. 1. Lipoprotein (LP) profiles as measured by fast protein liquid chromatography (FPLC) analyses in both groups of animals [LDL-receptor knockout (KO) and apolipoprotein E KO] on a low sodium chloride diet (LSD) and on a normal sodium chloride diet (NSD). n, number of analyses. The lower panel shows that LSD, compared with NSD, did not modify the median cholesterol percent distribution and range in the LP fractions. Data were calculated as area under the VLDL, LDL, and HDL peaks of the FPLC profile.

Statistical analyses

Data are expressed as mean ± SD. Student's *t*-test was used for statistical comparison of the data. Univariate analysis to examine the potential effects of the plasma TC, TG, and NEFA on accumulation of arterial wall lipids was performed by Spearman's analysis. Data evaluation was calculated using GraphPad Prism (GraphPad Software Inc., San Diego, CA). Results of the statistical tests were considered significant at the 95% confidence level ($P < 0.05$).

RESULTS

On NSD, the plasma concentration of NEFA was similar in LDLR KO and apoE KO mice, whereas plasma TG was higher in LDLR KO than in apoE KO, and cholesterol was higher in apoE KO than in LDLR KO (**Table 1**). On the LSD, cholesterol concentration was not modified in the apoE KO group, whereas the plasma TG and NEFA increased. In the LDLR KO mice, on the other hand, compared with NSD, LSD elicited a rise on the plasma concentrations of cholesterol, TG, and NEFA. Despite of the rise of plasma lipids, alterations in the LP profiles as measured by FPLC analyses were not observed in both groups of animals as a consequence of the LSD (**Fig. 1**). Furthermore, the rise of plasma lipids could neither be explained by variations in plasma volumes nor in caloric intake, respectively, because LSD and NSD animals did not differ according to their hematocrit and body weight. Similarity of body weight was observed in both diets in the two experimental mice groups.

According to the corresponding diets, the $U_{Na}V$ data were significantly lower in the LSD than in the NSD groups, and the U_KV data were similar in the LSD and in the NSD groups (**Table 2**). Therefore, dietary sodium

TABLE 2. Urinary sodium and potassium excretion of apoE KO or LDLR KO mice submitted either to LSD or to NSD

	apoE KO		LDLR KO	
	NSD Group	LSD Group	NSD Group	LSD Group
	mEq/8 h			
$U_{Na}V$	0.036 ± 0.020 (9)	0.010 ± 0.008[a] (13)	0.036 ± 0.015 (8)	0.009 ± 0.005[a] (12)
U_KV	0.013 ± 0.007 (9)	0.023 ± 0.014 (13)	0.017 ± 0.006 (8)	0.024 ± 0.015 (12)

Eight hour urinary sodium ($U_{Na}V$) and potassium (U_KV) excretion was determined in apolipoprotein E (apoE) or in LDLR KO mice fed ad libitum either on a LSD or on a NSD during previous 9 weeks. Data are expressed as mean ± SD; (n), number of animals. Statistical comparison by Student's *t*-test: LSD × NSD.

[a] $P < 0.001$.

chloride restriction brought about differences in the concentrations of plasma lipids.

Using a quantitative assay in which Oil Red O was utilized to stain lipids present in the inner arterial wall (intima plus media), lipid deposits were present in all KO animals (**Table 3**). In the LDLR KO mice, the mean area of lipids per aorta was significantly higher in LSD than in NSD animals. The apoE KO mice had a greater mean aortic lipid area than the LDLR KO mice; however, there were no differences between apoE KO LSD and NSD mice.

DISCUSSION

Confirming a previous work on Wistar rats from this laboratory (14), the present study utilizing LSD on LDLR KO and on apoE KO mice also demonstrates an elevation of the plasma concentrations of TG and NEFA, respectively, 19% and 34% in LDLR KO, and 21% and 35% in apoE KO mice, although the rising of plasma cholesterol was limited to the LDLR KO group (15%). On normal diets, as compared with the wildtype, KO animals are known to have higher plasma concentrations of LDL and TG resembling those found in humans. In addition, because VLDL, IDL, or LDL clearances are simultaneously impaired (16, 18, 22, 23) in these KO animals, their sensitivity to raise the plasma lipid concentrations on the LSD is anticipated. LSD aggravated the hypercholesterolemia of LDLR KO, but did not modify the more severe hypercholesterolemia of the apoE KO mice that already was present on NSD. A likely explanation for the worsening of the hyperlipidemia on LSD is drawn from our previous study, where an impaired removal of TG-containing LP was demonstrated (14); a finding ascribed to a state of insulin resistance in LSD rats (24). VLDL and LDL particles normally compete for common liver receptors that recognize apoB and apoE belonging to these LPs (25). LDLR KO mice lack high-affinity LDL receptors but retain low-affinity LDL receptors. Consequently, an impairment of the VLDL removal rate is likely to compete with the rate of uptake of LDL particles by the liver low-affinity LDL receptors, and both types of particles may then simultaneously rise in plasma on the LSD. On the contrary, in apoE KO mice the rate of liver removal of all particles (VLDL and LDL that normally contain apoE) may have already been severely impaired on a NSD (22), and consequently is less likely to worsen on the LSD.

Although the biochemical mechanism of the LSD-induced insulin resistance is presently unknown, its consequence includes the lowering of the activity of the enzyme LP lipase and the faster release rate of NEFA from the adipose tissue. In this regard, there is an inverse relationship between salt intake and the sympathetic nervous system activity (26, 27). Accordingly, an increase of the latter together with the plasma norepinephrine concentration has been reported in experimental normotensive animals and humans submitted to LSD. In addition, the higher concentration of plasma angiotensin II in LSD animals stimulates the release of norepinephrine from sympathetic nerve terminals, as well as of epinephrine from the adrenal medulla (28, 29) that contribute to an insulin resistance state (30–32). This sympathetic overactivity elicits a reduction of the blood flow in peripheral tissues due to vasoconstriction, which includes the precapillary arteriolar sphincters in adipose tissue, and, simultaneously, enhances the adipose tissue lipolysis rate leading to an increase in the plasma NEFA concentration (30, 31, 33). The latter by itself suppresses the LP lipase activity (34).

Whatever mechanisms elicited elevated plasma NEFA

TABLE 3. Aortic lipid stained deposits in apoE KO or in LDLR KO mice submitted either to LSD or to NSD

	apoE KO		LDLR KO	
	NSD Group	LSD Group	NSD Group	LSD Group
Inner arterial wall area	1423 ± 231 (6)	1438 ± 283 (6)	323 ± 66 (7)	797 ± 116[a] (7)

Aortic lipid stained deposits were measured in 3-month-old apoE KO or in LDLR KO mice fed ad libitum either on LSD or on a NSD. Lipid staining deposits represent arterial section area per animal (μm^2 mean ± SD) as identified by Oil Red O on the inner aortic wall (intima plus media). Animal number (n). Statistical comparison by Student's *t*-test: LSD × NSD.

[a] $P < 0.001$.

TABLE 4. Univariate regression analysis between aortic lipid stained deposits and plasma cholesterol, triacylglycerol, and NEFA concentrations on both diets (LSD and NSD) in LDLR KO (n = 14) and apoE KO (n = 12) mice

Variables	*r*	*P*
	LDLR KO	
Cholesterol	0.283	0.326
Triacylglycerol	0.416	0.139
NEFA	0.688[a]	0.006
	apoE KO	
Cholesterol	0.087	0.783
Triacylglycerol	−0.385	0.218
NEFA	0.224	0.485

Spearman's correlations were carried out considering the inner arterial wall (intima plus media) lipid staining areas and plasma cholesterol, triacylglycerol, and NEFA concentrations.

[a] Significant correlation.

concentration in the present report and in the previous study (14), two consequences are anticipated. In the liver, more NEFA is incorporated into TG and delivered into plasma as VLDL (35), however, a faster rate of VLDL-TG production was not observed on LSD in rats (14). The likely possibility is that an impairment of the LP lipase activity due to the increased plasma NEFA concentration in response to insulin resistance (36–38) slows down the plasma TG fractional removal rate.

The possibility that corticosterone might stimulate the release of NEFA from the adipose tissue due to increased activity of the hormone sensitive lipase (39, 40) was ruled out because a low, not high, concentration of corticosterone was present in the rat plasma on LSD (14). Accordingly, another study showed that the plasma corticosterone level in rats on salt restriction was not different from that of control animals (41). A likely explanation for a low corticosterone level is that the angiotensin-converting enzyme-dependent rate of conversion of corticosterone into aldosterone is favored on LSD (41).

In this experiment, only the proximal aorta has been selected to measure lipid deposits, since this arterial segment is among the first sites to develop premature atherosclerotic lesions (42). The present investigation has shown that the higher plasma concentrations of NEFA, TG, and TC in the LDLR KO mice submitted to the LSD coincide with greater degree of arterial lipid deposit. However, when each plasma lipid parameter and independent animal group on both diets are considered, univariate analysis failed to show a correlation between either plasma TC or TG and the intensity of the arterial lipid deposits. This may have occurred because apoE KO mice display very high concentrations of cholesterol that could have masked any independent effect on the arterial fat deposit brought about by other parameters (TG and NEFA). On the other hand, in the LDLR KO animals, NEFA alone relates to the degree of arterial wall lipid storage (**Table 4**; $n = 14$, $P = 0.0065$). Interestingly, the LDLR KO group, where plasma TC and TG increased on the LSD, failed to show a univariate correlation between these plasma lipids and the arterial lipid area. This observation suggests that on the LSD, as compared with TG and TC, NEFA plays a primary role in the development of premature arterial lipid deposits, most likely reflecting a state of insulin resistance as previously reported in this circumstance (24).

Our experimental model indicates that a low-salt diet elicits premature arterial lipid storage in hyperlipidemic normotensive animals. This finding leads to the speculation that LSD might have even a greater impact on the development of atherosclerosis of hypertensive than of normotensive animals. However, in a recent work on hypertensive rats sensitive to dietary salt (Dahl salt sensitive), the maintenance of a normal blood pressure level on LSD simultaneously increased the animal survival rate and diminished the degree of coronary lesion (43). This was observed both in normolipidemic as well as in severely hyperlipidemic Dahl rat carriers of the CETP gene. In other words, the development of hypertension brought about premature atherosclerosis that was independent of the plasma lipid concentration. Consequently, the low-salt-induced arterial lipid accumulation in our study may be limited to the normotensive hyperlipidemic animals and needs confirmation in hypertensive hyperlipidemic mice. Besides confirming other studies where plasma TC and TG concentration rose in hypertensive human subjects treated with the diuretic hydrochlorothiazide (8), the present experimental investigation may contribute to explain some controversial studies regarding the effectiveness of diuretics in the prevention of coronary heart disease in humans (9, 10).

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