Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 4114–4119, April 1996
Medical Sciences

Centripetal cholesterol flux from extrahepatic organs to the liver is independent of the concentration of high density lipoprotein–cholesterol in plasma

(high density lipoprotein–cholesterol transport)

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Communicated by David L. Garbers, University of Texas Southwestern Medical Center, Dallas, TX, December 29, 1995 (received for review December 5, 1995)

ABSTRACT **High density lipoproteins (HDLs) play a role in two processes that include the amelioration of atheroma formation and the centripetal flow of cholesterol from the extrahepatic organs to the liver. This study tests the hypothesis that the flow of sterol from the peripheral organs to the liver is dependent upon circulating HDL concentrations. Transgenic C57BL/6 mice were used that expressed variable amounts of simian cholesteryl ester-transfer protein (CETP). The rate of centripetal cholesterol flux was quantitated as the sum of the rates of cholesterol synthesis and low density lipoprotein–cholesterol uptake in the extrahepatic tissues. Steady-state concentrations of cholesterol carried in HDL (HDL-C) varied from 59 to 15 mg/dl and those of apolipoprotein AI from 138 to 65 mg/dl between the control mice ($CETP^c$) and those maximally expressing the transfer protein ($CETP^+$). There was no difference in the size of the extrahepatic cholesterol pools in the $CETP^c$ and $CETP^+$ animals. Similarly, the rates of cholesterol synthesis (83 and 80 mg/day per kg, respectively) and cholesterol carried in low density lipoprotein uptake (4 and 3 mg/day per kg, respectively) were virtually identical in the two groups. Thus, under circumstances where the steady-state concentration of HDL-C varied 4-fold, the centripetal flux of cholesterol from the peripheral organs to the liver was essentially constant at ≈87 mg/day per kg. These studies demonstrate that neither the concentration of HDL-C or apolipoprotein AI nor the level of CETP activity dictates the magnitude of centripetal cholesterol flux from the extrahepatic organs to the liver, at least in the mouse.**

In all species, the major net flux of cholesterol in the body involves the movement of sterol from the extrahepatic organs to the liver. In a primate such as the cynomolgus monkey (and, very likely, the human), the magnitude of this flux equals 10–11 mg of sterol per day per kg of body weight (1). However, this flux increases dramatically in animals of lower body weight and higher basal metabolic activity, reaching approximately 100 mg/day per kg in small mammals such as the mouse (2). Since these peripheral organs take up little cholesterol carried in low density lipoproteins (LDL-C) from the plasma, most of this net flow of sterol consists of cholesterol that is newly synthesized in these same tissues (1, 3, 4). In the monkey and mouse, for example, *de novo* synthesis accounts for 77 and 95%, respectively, of the cholesterol involved in this centripetal sterol flux (1, 2). There is abundant experimental data supporting the concept that one of the major functions of high density lipoproteins (HDLs) is to facilitate this constant movement of cholesterol from the extrahepatic tissues to the liver (for reviews, see refs. 5 and 6). However, there is virtually no quantitative data dealing with the fundamental question of whether the magnitude of this centripetal flux is dictated by events within the cells of the peripheral organs or, alternatively, is determined by the concentration of HDL circulating in the plasma compartment.

In addition to this normal physiological function, the HDL particle has also been implicated as an important determinant of the pathological process of atheroma formation. The data that support this association are of two types. First, in epidemiological studies the concentration of plasma total cholesterol (in general) and of LDL-C (in particular) have been shown to vary directly with the incidence of coronary artery disease (7–9). In contrast, the concentration of cholesterol carried in HDL (HDL-C) varies inversely with such disease (10–13). Second, this role of HDL in preventing atherosclerosis has been demonstrated more directly in recent studies using a variety of transgenic mice. Lowering the concentration of HDL-C by overexpressing cholesteryl ester-transfer protein (CETP), for example, leads to more severe atherosclerosis in cholesterol-fed mice (14). Similarly, mice lacking apolipoprotein (apo)E develop striking atherosclerosis (15, 16), but this process is ameliorated by overexpressing apoAI and thereby raising the steady-state concentration of HDL-C (17, 18). Thus, these varied data strongly suggest that the severity of the atherosclerotic process is, in some manner, inversely related to the steady-state concentration of HDL-C.

Many of the specific processes involved in the centripetal flow of sterol from the interior of each extrahepatic cell to the liver are now known. In nearly all of these tissues, most cholesterol comes from continuous synthesis on the endoplasmic reticulum (1, 3, 4, 19, 20), and this unesterified sterol may then move to the cell membrane by vesicular transport through the Golgi apparatus (21). Indeed, direct observations have shown progressive sterol enrichment of the stacked cisternae between the endoplasmic reticulum and the plasma membrane (22). This cholesterol is then presumably inserted into the plasma membrane where it is concentrated in structures like caveolae and glycosphingolipid-enriched complexes (23–25). In the presence of an appropriate acceptor such as apoAI or apoE, cholesterol readily desorbs from the external leaflet of the plasma membrane and is esterified to cholesteryl ester by the enzyme lecithin/cholesterol acyltransferase (6, 26–28). This esterified cholesterol is then cleared into the liver after being transferred to apoB-containing lipoproteins by CETP or may be taken up directly by a HDL receptor (5, 6).

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Abbreviations: LDL, low density lipoprotein; HDL, high density lipoprotein; LDL-C, cholesterol carried in LDL; HDL-C, cholesterol carried in HDL; CETP, cholesteryl ester-transfer protein; apo, apolipoprotein.

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While there now seems to be little question that the pathological process of atheroma formation is critically related to the steady-state concentration of HDL-C, there is little data on how this concentration affects the physiological process of centripetal cholesterol flux. The present studies were specifically designed to explore the fundamental question of whether the magnitude of this flux, and, therefore, cholesterol homeostasis in every extrahepatic cell is determined by events within each of these cells or, alternatively, is dictated by the steady-state concentration of HDL-C in the plasma space. To evaluate this question the net flux of cholesterol from the extrahepatic tissues to the liver was measured under circumstances where the steady-state concentrations of apoAI and HDL-C in the vascular space were varied by expressing increasing activities of CETP in different groups of mice.

MATERIALS AND METHODS

Animals and Diets. The mice used in these experiments were the male descendants of C57BL/6 animals with normal, essentially zero, levels of CETP ($CETP^c$) or similar mice that expressed high levels of cynomolgus monkey CETP ($CETP^+$) (29, 30). A few experiments were also done using two other groups of animals that expressed intermediate levels of this transfer protein and were designated $CETP^{34}$ and $CETP^{45}$. Before the experiments, the animals were maintained on a lowfat, ground diet (Wayne Lab Blox; Allied Mills, Chicago) which, by direct analysis, contained 0.24 mg of cholesterol and 50 mg of total lipid per g of diet. It was experimentally determined that the $CETP^c$ and $CETP^+$ animals ate similar amounts of this diet (4.9 ± 0.4 and 4.6 ± 0.1 g/day, respectively) and absorbed similar percentages of the dietary cholesterol (53 ± 3% and 51 ± 4%, respectively) (2, 31).

Plasma and Tissue Cholesterol Concentrations, ApoAI Concentrations, and CETP Activity. The concentration of cholesterol in the various plasma lipoprotein fractions was measured as described (2, 31). The major organs were removed, weighed, and saponified. The remaining carcass, which was composed primarily of skeleton, muscle, and adipose tissue, was also saponified and the content of total cholesterol determined. The levels of apoAI and CETP in the plasma of these animals were determined as previously described (29, 32).

Cholesterol Synthesis in the Extrahepatic Organs *in Vivo*. Cholesterol synthesis was measured *in vivo* at the mid-dark phase of the light cycle as previously outlined (2, 33, 34). The rates of sterol synthesis in each organ in the live animal were then calculated as the nmol of [^{3}H]water incorporated into digitonin-precipitable sterols per g of tissue or per whole organ (nmol/h per g or organ). The rate of cholesterol synthesis in the whole animal was determined as the sum of the rates of synthesis in all of the organs and carcass.

LDL-C Transport into the Extrahepatic Tissues *in Vivo*. All LDL-C transport measurements were undertaken using homologous LDL preparations because lipoproteins from other species give transport rates that vary from those obtained with mouse LDL (2). Mouse plasma was harvested from both male and female LDL receptor knockout animals that had been maintained on a low cholesterol diet (2), and an LDL fraction was isolated between the densities of 1.020 and 1.055 g/ml. The lipoproteins in this fraction were labeled with either ^{125}I-tyramine cellobiose or ^{131}I (1, 35). These radiolabeled lipoprotein fractions were then applied to a slurry gel composed of Geon–Pevikon and electrophoresed to separate the LDL from the small amounts of apoE-containing HDL present in the preparations (36, 37). All preparations used were determined to be essentially free of apoE-containing HDL on agarose gels (CIBA–Corning Diagnostics, Oberlin, OH). Rates of tissue LDL-C clearance were determined *in vivo* as previously described using a primed/continuous infusion of ^{125}I-tyramine cellobiose-labeled LDL that was administered through an intravenous catheter secured in an external jugular vein. Clearance rates in each organ were expressed as the μl of plasma cleared of its LDL content per h per g of tissue or per whole organ (μl/h per g or per organ).

Calculations. In these studies, the rate of net centripetal cholesterol flux from the peripheral organs to the liver was calculated as the sum of the rates of *de novo* sterol synthesis and LDL-C acquisition in these organs. Since the concentration of cholesterol in each of the tissues did not vary with time, the rate of net cholesterol movement out of these extrahepatic organs must have equaled the rate of acquisition from synthesis and LDL-C uptake. Only data from the adrenal gland and testes were excluded from these calculations, because it was assumed that the cholesterol acquired by these two organs was converted to steroid hormones and excreted in the urine and bile. For the purposes of these calculations, the rates of [^{3}H]water incorporation into digitonin-precipitable sterols per h per g were converted to mg of cholesterol synthesized per day per kg of body weight using the observation that 1.45 carbon atoms are incorporated into digitonin-precipitable sterols for each ^{3}H atom (33, 34, 38). Similarly, the rates of LDL-C uptake in this same group of tissues were calculated by multiplying the LDL clearance rates times the concentration of LDL-C in the plasma. These latter rates are also expressed as mg of cholesterol acquired per day per kg body weight. All data are presented as mean values ± 1 SEM. The Student's unpaired *t* test was used to compare various sets of data where appropriate.

RESULTS

Most studies were undertaken using either control animals that had virtually no CETP activity or mice that expressed high levels of this transfer protein. The characteristics of these two groups at approximately 3 months of age are shown in Table 1. $CETP^c$ and $CETP^+$ mice had nearly identical weights of the liver, small bowel, and whole animal even though the control group had undetectable levels of CETP whereas the $CETP^+$ group had 7.7 units. This high level of CETP was associated with lower concentrations of plasma apoAI (65 vs. 138 mg/dl), plasma total cholesterol (36 vs. 73 mg/dl), and, particularly, HDL-C (15 vs. 59 mg/dl) as has been previously described (29, 30, 32).

The concentration of cholesterol in each of the extrahepatic organs was essentially constant in these animals between the

Table 1. Characteristics of the two groups of mice used in these experiments

	Type of mouse	
	$CETP^c$	$CETP^+$
Animal weight, g	28 ± 0.4	26 ± 0.4
Liver weight, g	1.41 ± 0.03	1.26 ± 0.02
Small bowel weight, g	1.14 ± 0.03	1.08 ± 0.05
Peripheral tissues weight, g	25 ± 0.4	24 ± 0.4
Plasma CETP activity, units	0	7.7 ± 0.2
Plasma apoAI, mg/dl	138 ± 6	65 ± 4
Plasma total cholesterol, mg/dl	73 ± 3	36 ± 1
Plasma CM/VLDL-C, mg/dl	7 ± 1	15 ± 1
Plasma LDL-C, mg/dl	7 ± 1	7 ± 1
Plasma HDL-C, mg/dl	59 ± 3	15 ± 1

Animals were fed diets containing a constant, low quantity of cholesterol each day and were then used for these experiments at about 3 mo of age. This table shows the characteristics of the experimental groups of control mice ($CETP^c$) and animals with elevated levels of CETP ($CETP^+$) with respect to body and organ weights, plasma CETP activities and the concentrations of cholesterol in various lipoprotein fractions. The term "peripheral tissues" refers to all organs in the animal except the liver and small bowel. For the purposes of these experiments, the plasma lipoproteins from each animal were separated into classes having a density of <1.020 (CM/VLDL-C), 1.020–1.063 (LDL-C), and >1.063 (HDL-C) g/ml. Mean values ± 1 SEM are shown.

ages of 2 and 4 months. Fig. 1 summarizes these concentrations in the 3-month-old mice used in all subsequent experiments. In the control animals, the adrenal gland and brain had the highest concentrations of cholesterol (20.1 ± 1.6 and 15.0 ± 0.3 mg/g, respectively), whereas the concentration in all of the other peripheral organs was <5.0 mg/g. In the CETP$^+$ mice the concentration of cholesterol was virtually identical to the control mice in all of the extrahepatic organs except the adrenal gland, where it equaled only 6.6 ± 0.9 mg/g. This lower level of tissue sterol presumably reflected the lower concentration of circulating HDL-C in these animals because the rodent is known to have a HDL-specific cholesterol uptake process in the adrenal gland (39–41). While not shown in Fig. 1, the concentration of hepatic cholesterol was also the same in the CETP^c (2.1 ± 0.1 mg/g) and CETP$^+$ (2.2 ± 0.1 mg/g) mice. From these concentration values and the weights of the individual organs, it was calculated that the total extrahepatic cholesterol pool equaled 1.84 ± 0.04 and 1.73 ± 0.04 g per kg body weight, respectively, in the control and CETP$^+$ animals. However, the sterol in the adrenal gland and testes presumably was largely used for hormone synthesis and was not being transported to the liver. When these two small subpools of sterol were subtracted from the total peripheral pool, the mass of the cholesterol in the remaining extrahepatic organs equaled 1.82 ± 0.04 and 1.70 ± 0.04 g per kg body weight, respectively. Thus, the steady-state pool of cholesterol in the extrahepatic organs contributing sterol to the liver was essentially the same in the presence and absence of CETP activity in the plasma.

Since the most important source for cholesterol turnover in these extrahepatic pools was *de novo* sterol synthesis, the rates of [^{3}H]water incorporation into digitonin-precipitable sterols were next measured *in vivo* in these same organs. As summarized in Fig. 2*A*, the highest rates of synthesis per g of tissue in the extrahepatic organs of the CETP^c animals were found in the adrenal gland, small bowel, stomach, and colon. Lesser rates were seen in the other organs. A similar profile of synthetic activity was found in the CETP$^+$ mice, although in a few tissues the rate of synthesis was either significantly ($P <$ 0.05) higher—e.g., small bowel and colon—or significantly lower—e.g., spleen and lung—than in the control mice. However, when the rates of cholesterol synthesis were calculated in the whole organs (Fig. 2*B*) these small differences essentially disappeared. Clearly the small bowel, colon, skin, and carcass were the major sites for sterol synthesis and there were no consistent differences seen in these rates in the CETP^c and CETP$^+$ animals. From these data, the rates of [^{3}H]water incorporation into sterols *in vivo* in all of the extrahepatic organs, again excluding the adrenal gland and testes, were determined to equal 2941 ± 201 and 2647 ± 185 nmol/h in

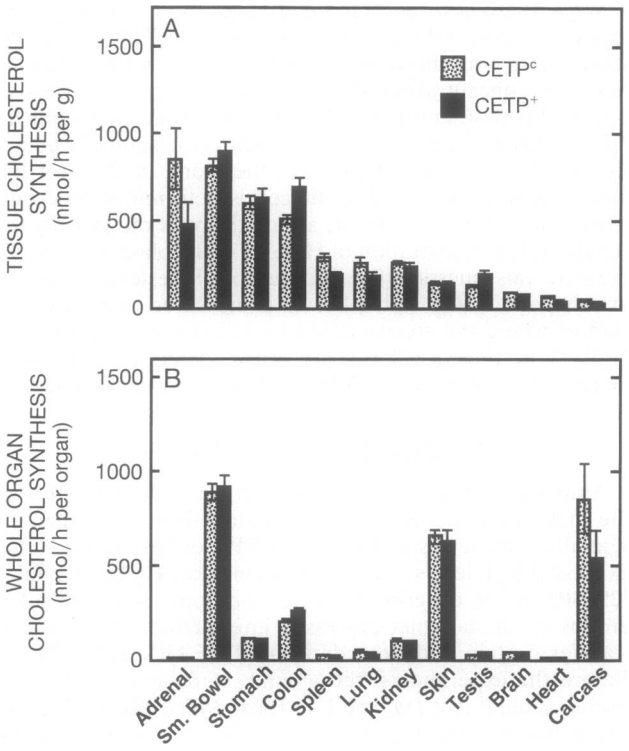

FIG. 2. Rates of cholesterol synthesis in the extrahepatic tissues measured *in vivo* in the control animals (CETP^c) and in animals overexpressing CETP (CETP$^+$). (*A*) Rates of [^{3}H]water incorporation into sterols per h per g of each organ. (*B*) These rates of synthesis have been multiplied by the respective organ weights to give the rates of cholesterol synthesis per organ. Each value represents the mean ± 1 SEM for measurements in 10 animals in each group.

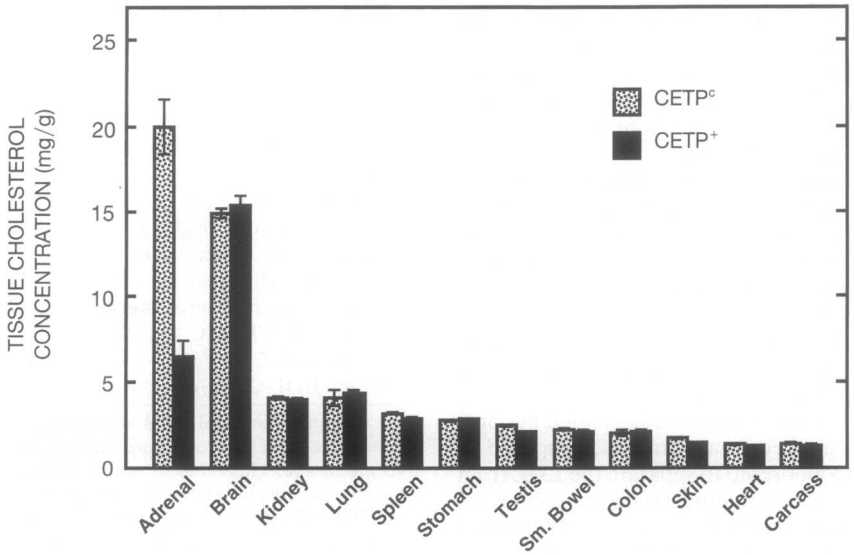

FIG. 1. Concentration of cholesterol in the extrahepatic organs of the CETP^c and CETP$^+$ mice. Each organ was dissected from the animals and the concentration of total cholesterol per g wet weight of tissue was determined. The tissue designated "carcass" represents the residual tissues after the dissections were completed and consisted primarily of bone, muscle, and adipose tissue. Mean ± 1 SEM are shown for 7 CETP^c animals and 10 CETP$^+$ mice.

the CETP^c and CETP$^+$ animals, respectively. Taking into consideration both the weight of each animal and the C/^{3}H incorporation ratio, these values established that the absolute rate of cholesterol synthesis in the extrahepatic compartment equaled 83 ± 4 mg/day per kg in the control mice and 80 ± 5 mg/day per kg in the CETP-expressing animals. Thus, each day the CETP^c and CETP$^+$ mice synthesized an amount of cholesterol equal to only 4.5 and 4.6%, respectively, of the steady-state pool of cholesterol present in these same extrahepatic organs.

The second process responsible for cholesterol acquisition in the extrahepatic tissues is the uptake of cholesterol carried in LDL. Using a primed/continuous infusion technique, the rates of LDL clearance were next measured. As shown in Fig. 3*A*, of all of the extrahepatic tissues only the adrenal gland manifested a fairly high rate of LDL clearance (115 ± 14 μl/h per g) in the CETP^c animals, and this rate was nearly 3-fold higher (300 ± 101 μl/h per g) in the CETP$^+$ mice. In contrast to this endocrine tissue, however, the rates of LDL clearance in all of the remaining extrahepatic organs were much lower and essentially the same in the two groups of animals. When organ weights were taken into consideration, as shown in Fig. 3*B*, it was apparent that very little LDL transport occurred into any extrahepatic organ. After subtracting the small amounts of LDL cleared by the adrenal gland and testes, LDL clearance in all of the remaining extrahepatic organs equaled only 79 ± 5 μl/h in the control animals and 90 ± 14 μl/h in the CETP$^+$ mice. From these values and the concentration of LDL-C found in the plasma (Table 1), the rates of acquisition of cholesterol by the tissues of the extrahepatic compartment were calculated to equal only 4 ± 0.3 mg/day per kg in the control mice and 3 ± 0.5 mg/day per kg in the mice expressing CETP. Thus, each day the CETP^c and CETP$^+$ animals took up into their extrahepatic tissue compartments an amount of LDL-C equal to only 0.2% of the steady-state cholesterol pool present in these organs.

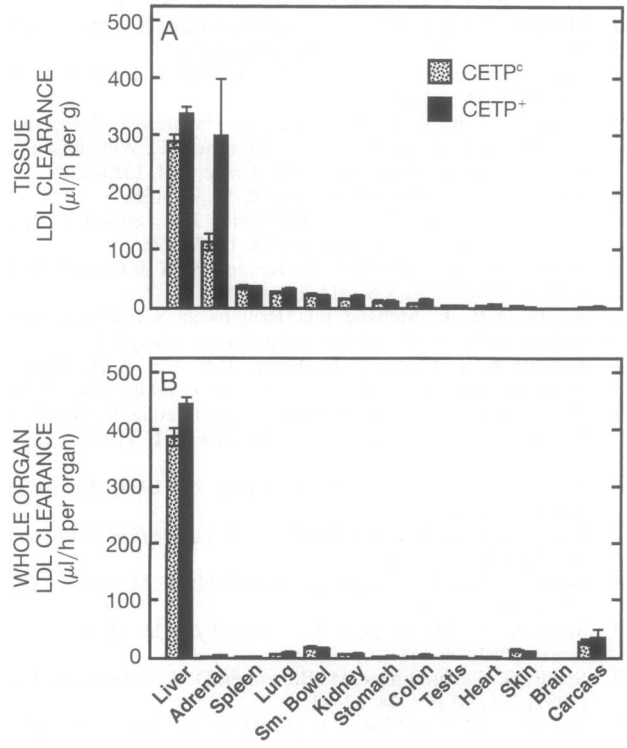

FIG. 3. Rates of homologous LDL uptake in the extrahepatic tissues. (*A*) Rate of LDL clearance per g of each organ. These values represent the μl of plasma cleared entirely of their LDL-C content per h per g wet weight of tissue. (*B*) Rate of LDL clearance by each of the major organs in the two groups of mice. Data represent means ± 1 SEM for measurements made in four CETP^c mice and in three CETP$^+$ animals.

Because the concentration of cholesterol in the organs of the extrahepatic compartment did not change, the rate of centripetal cholesterol flux from the peripheral tissues to the liver could be calculated as the sum of the rates of sterol synthesis and LDL-C uptake in these organs. These rates, in turn, could be plotted as a function of the steady-state concentration of HDL-C found in the same animals, as shown in Fig. 4. This figure shows data derived from the CETP^c and CETP$^+$ animals and, in addition, shows two other sets of similar measurements made in mice expressing intermediate levels of CETP (CETP^{34} and CETP^{45}). As is apparent, the steady-state concentration of HDL-C in these four groups of mice varied from 59 ± 3 mg/dl in the control animals to 15 ± 1 mg/dl in the CETP$^+$ mice. However, the rates of centripetal cholesterol flux from the extrahepatic compartment to the liver equaled 87 ± 5 (CETP^c), 90 ± 6 (CETP^{45}), 88 ± 5 (CETP^{34}), and 83 ± 5 (CETP$^+$) mg/day per kg in these four experimental groups. Clearly there was no significant change in the rate of centripetal cholesterol flux from the extrahepatic organs under circumstances where there were major alterations in the concentration of plasma CETP activity and a 4-fold variation in the plasma HDL-C concentration.

DISCUSSION

These studies provide strong evidence that the molecular processes that are involved in the regulation of cholesterol movement from the interior of cells in the extrahepatic organs to the liver are very different from those that are associated with interaction of the HDL particle with the arterial wall. In the mouse model used in these experiments, lowering the steady-state concentration of HDL-C by expressing CETP resulted in more severe atherosclerosis (14), yet there was a change in the rate of neither cholesterol synthesis (Fig. 2) nor LDL-C uptake (Fig. 3) in the extrahepatic organs nor an alteration in the flow of sterol from these tissues to the liver (Fig. 4). A similar situation has been reported in another mouse model where deletion of apoE was also associated with reduced concentrations of circulating HDL-C and atheroma formation (16, 42), but again, the flow of cholesterol from the extrahepatic organs to the liver was essentially the same in the control mice (84 ± 3 mg/day per kg) and in the homozygous knockout animals (81 ± 2 mg/day per kg) (43). Thus, in both experimental settings, the physiological process of centripetal cholesterol flux was independent of the steady-state HDL-C level, while the magnitude of the pathological process of atheroma formation varied inversely with this concentration.

It is conceivable that alterations of the steady-state HDL-C concentration brought about by manipulation of a more proximal step in the pathway of cholesterol movement through the plasma would have yielded different results. ApoAI, for example, is apparently the immediate acceptor for sterol desorbed from the peripheral cell membranes (5, 6), and it is theoretically possible, therefore, that the magnitude of centripetal cholesterol flux would vary directly with the apoAI production rate. However, if this were the case, then sterol synthesis in the extrahepatic organs would necessarily have to increase as the production rate increased to maintain tissue cholesterol levels in these cells constant. Under these conditions, there would be an increase in net cholesterol delivery to the liver that was proportional to the increased apoAI production and this, in turn, would lead to suppression of hepatic cholesterol synthesis, suppression of hepatic LDL receptor activity and an increase in net biliary cholesterol secretion. Thus, the steady-state concentration of LDL-C and the rate of whole animal cholesterol excretion would vary directly with the steady-state concentration of HDL-C. Such associations have not been

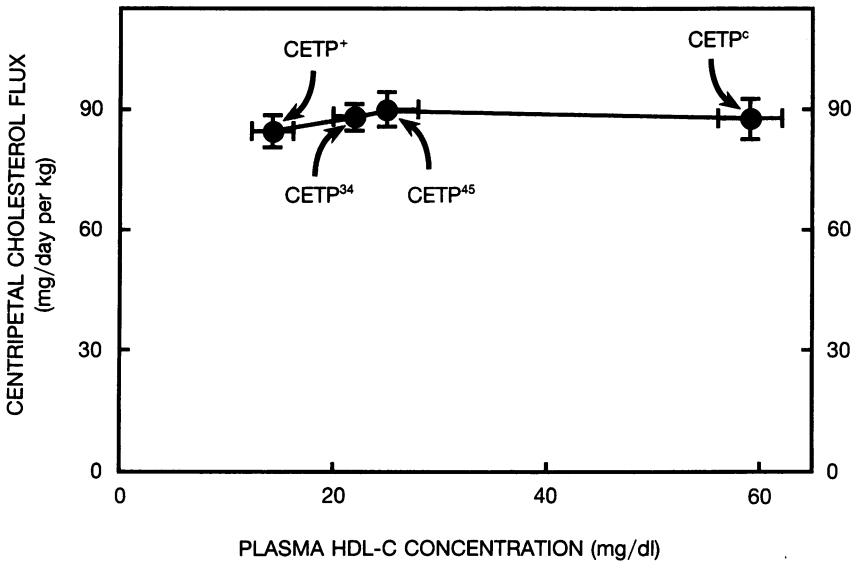

FIG. 4. Rates of centripetal cholesterol flux from the extrahepatic tissues to the liver. These rates equal the sum of the rates of cholesterol synthesis and LDL-C uptake in all extrahepatic organs, except the endocrine glands, and are given as the mg of cholesterol that move to the liver each day per kg body weight. The data represent means ± 1 SEM for 14 $CETP^{+}$, 10 $CETP^{34}$, 10 $CETP^{45}$, and 13 $CETP^{c}$ animals.

observed in either animal or human studies. Nevertheless, it will be important in future experiments to examine the role of apoAI production in net centripetal cholesterol flux.

If, as these findings suggest, the magnitude of net sterol flux from the extrahepatic organs is independent of events in the vascular space, then the rate-determining step for this process must reside within the cells of the tissues themselves. This possibility is supported by observations in a third mouse model. In the normal animal where >90% of LDL-C is removed from the plasma by the liver, little LDL-C normally reaches the peripheral organs (Fig. 3). However, when the LDL receptor activity responsible for this hepatic clearance is deleted, the rate of LDL-C uptake by receptor-independent mechanisms in the peripheral organs increases from 5 mg/day per kg to 25 mg/day per kg. Under these conditions, the centripetal flux of cholesterol from these organs to the liver does increase, from 106 mg/day per kg in the control mice to 133 mg/day per kg in the receptor negative animals (2). While this extreme example suggests that some process within the cells of the extrahepatic organs does dictate overall centripetal flux, under more physiological conditions it is not known whether the velocity of this process is determined by rates of vesicular transport or some component of plasma membrane cholesterol translocation. The elucidation of which of these steps is involved is fundamental to understanding cholesterol homeostasis in these cells.

Finally, two other conclusions should be emphasized. First, the molecular events whereby HDL-C ameliorates atheroma formation in the arterial wall are, presumably, very different from those specific steps involved in maintenance of physiological cholesterol balance in the cells of the extrahepatic tissues. It is not appropriate, therefore, to link these two processes together and describe them under the single term "reverse cholesterol transport". Second, if suppression of CETP activity, with the attendant elevation of HDL-C concentrations, inhibits atheroma formation without adversely affecting sterol movement from the peripheral organs to the liver, then this finding strengthens the theoretical basis for using inhibitors of CETP in humans to alter the rate of atheroma formation.

We thank Brian Darnell, Jeffrey Graven, Stephen Ostermann, Trista Prasifka, and Monti Schneiderman for their excellent technical assistance and Merikay Presley for preparation of the manuscript. This work was supported by Grant HL 09610 from the National Institutes of Health and by a grant from the Moss Heart Fund.

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