Scand J Clin Lab Invest 1996; 56, Suppl 226: 9-17

Novel pathways for elimination of cholesterol by extrahepatic formation of side-chain oxidized oxysterols

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Diczfalusy U, Lund E, Lütjohann D, Björkhem I. Novel pathways for elimination of cholesterol by extrahepatic formation of side-chain oxidized oxysterols. Scand J Clin Lab Invest 1996; 56, Suppl 226: 9-17.

Recently, we described a new pathway whereby peripheral cells can eliminate intracellular cholesterol by conversion into the more polar oxysterols 27-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid. The latter steroids are easily excreted from the cells and transported to the liver for conversion into bile acids. Our attempts to evaluate the importance of this new mechanism are reviewed here and also our investigations on the possible presence of additional similar pathways for removal of extrahepatic cholesterol.

Human alveolar macrophages in culture were shown to have a high capacity to convert cholesterol into 27-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid and to excrete these steroids into the culture medium. Treatment of the macrophages with cyclosporin A, an inhibitor of sterol 27-hydroxylase, reduced the excretion of the 27-hydroxylated products by more than 90%, with a concomitant accumulation of intracellular cholesterol. The quantitative importance of the mechanism in relation to reverse cholesterol transport was investigated in ^{14}C-cholesterol labelled macrophages exposed to HDL. At very low concentrations of HDL, possibly similar to those present in tissues, the two pathways were about equally effective. At optimal concentrations of HDL, however, reverse cholesterol transport was about 10-fold more effective than the sterol 27-hydroxylase pathway.

The net uptake of 27-oxygenated steroids by the liver was measured in volunteers by comparison of the levels in the hepatic vein with those in a peripheral artery. Approximately 20 mg of 27-oxygenated oxysterols was taken up by the liver during 24 hours. Quantitative conversion of these oxysterols into bile acids would correspond to 4% of the total bile acid formation. It is evident that this new pathway contributes significantly to cholesterol elimination.

The possibility that the sterol 27-hydroxylase pathway is of importance for cholesterol homeostasis in the brain was investigated by measuring oxysterols in

the internal jugular vein and in an artery of healthy volunteers. There was no net flux of 27-hydroxycholesterol from the brain into the circulation. There was, however, a significant flux of 24-hydroxycholesterol, corresponding to elimination of about 4 mg cholesterol/24 hours. This flux is higher than the estimated rate of synthesis of cholesterol in the human brain.

To summarize, we have demonstrated two mechanisms for cholesterol elimination from extrahepatic cells by specific oxygenases capable of oxidizing the steroid side-chain. The efficiency of these mechanisms is based on the fact that side-chain hydroxylated cholesterol species are both translocated through lipophilic membranes and converted into bile acids at a much faster rate than cholesterol itself. The importance of the sterol 27-hydroxylase-mediated mechanism is illustrated by the fact that patients who lack this enzyme develop xanthomas and premature atherosclerosis in spite of normal levels of circulating cholesterol.

Key words: Atherosclerosis; cholesterol homeostasis; cytochrome P-450; reverse cholesterol transport; sterol 24-hydroxylase; sterol 27-hydroxylase.

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INTRODUCTION

Cholesterol homeostasis depends on a delicate balance between dietary intake and endogenous synthesis on the one hand and metabolism and excretion on the other. This balance is important for human health, and disturbances lead to a number of diseases, such as bile stones, stroke and coronary disease.

The liver is essential for both cholesterol uptake and disposal. A bidirectional flux of lipoproteins between the liver and peripheral cells maintains cholesterol homeostasis. While supply of cholesterol from the liver to peripheral tissues occurs by well-defined receptor-dependent endocytosis, the mechanism of the reverse cholesterol transport from peripheral tissues to the liver is less well defined.

We have recently described an additional pathway whereby peripheral cells can eliminate intracellular cholesterol [1]. Cholesterol is converted into the more polar oxysterols 27-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid, which are easily excreted from the cells and transported to the liver for conversion into bile acids. The enzyme sterol 27-hydroxylase, responsible for the conversion of cholesterol into the two oxysterols, is present in most or all peripheral tissues and was found to be particularly active in macrophages. Elimination of cholesterol may be critical in these cells since they can develop into foam cells under strong cholesterol pressure. The foam cells may develop further into advanced atherosclerotic lesions.

The aim of this work is to review our attempts to evaluate the importance of this new mechanism for removal of extrahepatic cholesterol and compare it with reverse cholesterol transport. Part of this material was published very recently [2]. During our studies we discovered that the brain has a similar but not identical mechanism for transport of cholesterol over the blood-brain barrier. For experimental details the reader is referred to our previous publications [1, 2].

Sterol 27-hydroxylase activity is important for cholesterol homeostasis in human alveolar macrophages

In accordance with the results of our previous work [1], human alveolar macrophages, isolated from bronchoalveolar lavage fluid, had a high capacity to secrete 27-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid into the medium when cultured in the presence of 10% calf serum. The ratio between 27-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid varied between 0.1 and 0.5 in different experiments.

Macrophages isolated from eight patients secreted about 4 fmol/cell of the 27-oxygenated products during 24 hours of incubation. The cholesterol content in these macrophages was about 10 fmol/cell. Thus the oxidative mechanism has the potential to eliminate about 40% of the cell content of cholesterol in the cell in 24 hours (Fig. 1).

If this new mechanism is of importance for cholesterol homeostasis in macrophages, inhibition of the sterol 27-hydroxylase activity could be expected to lead to accumulation of intracellular cholesterol. This was also found to be the case. We have shown that cyclosporin A is an inhibitor of the enzyme [3] and when macrophages were cultured in the presence of 20 μmol/L cyclosporin, the flux of 27-oxygenated products decreased by more than 90%. This decrease was followed by an accumulation of cholesterol in the macrophages and the content of cholesterol increased from 10.5 ± 1.5 fmol/cell to 17.8 ± 3.2 fmol/cell (n=4). This accumulation of cholesterol should be compared with the flux of 27-oxygenated products which decreased from 6.1 ± 1.6 fmol/cell from the control macrophages to 0.4 ± 0.1 fmol/cell from the macrophages exposed to cyclosporin.

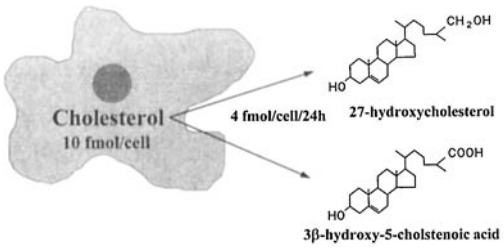

FIG. 1. Elimination of 27-oxygenated metabolites of cholesterol from cultured human macrophages.

Comparison between the sterol 27-hydroxylase mechanism and reverse cholesterol transport

Under the conditions employed, with fetal calf serum in the medium, there is a flux of cholesterol both from the medium into the cultured macrophages and from the cultured macrophages into the medium. To compare the flux of cholesterol from the cultured macrophages with the corresponding flux of 27-hydroxylated products, macrophages were preloaded with 4-^{14}C-cholesterol (Fig. 2). When such macrophages were cultured in a medium containing 10% fetal calf serum, there was a considerable flux of labelled cholesterol from the cells into the medium [2]. In two different experiments the amount of 27-oxygenated products was about 10% of that of cholesterol. In the absence of HDL-containing fetal calf serum the transport of both cholesterol and 27-oxygenated products was markedly reduced. Under these conditions, however, the amount of 27-oxygenated products excreted from the cells was similar to the amount of excreted cholesterol.

It should be emphasized that a 10-fold higher flux of cholesterol than of 27-oxygenated products from the macrophages does not mean that the former flux is 10-fold more important than the later under *in vivo* conditions. The 27-oxygenated products are rapidly transported to the liver and rapidly eliminated as bile acids. In contrast, cholesterol may recirculate or be taken up by other cells

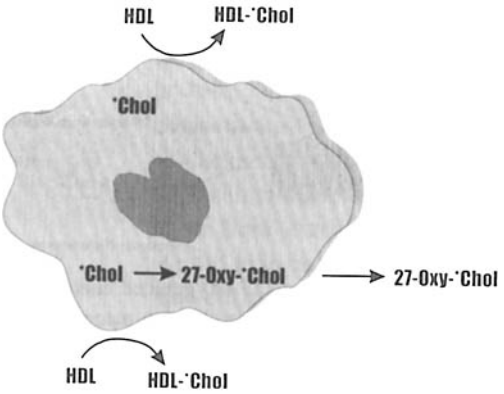

FIG. 2. Elimination of labelled cholesterol from human cultured macrophages by reverse cholesterol transport and by sterol 27-hydroxylase.

before it is ultimately eliminated as bile acids. It should also be emphasized that tissue macrophages like those studied here may never be exposed to levels of HDL as high as those used in the experiments with fetal calf serum.

There is a net flux of 27-oxygenated metabolites of cholesterol from extrahepatic cells to the liver

The present mechanism for removal of cholesterol is not restricted to macrophages. We [1] and others [4] have shown that also endothelial cells have a capacity to secrete 27-oxygenated cholesterol metabolites into the medium. In view of the broad tissue distribution of the sterol 27-hydroxylase, it seems likely that other types of cells may also utilize this mechanism for cholesterol elimination.

If the mechanism is of general importance, one would expect the presence of 27-oxygenated cholesterol metabolites in the circulation and a net flux of these compounds to the liver, where they are known to be converted into bile acids (for a review, see ref. 5). In addition to 27-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid, there are significant amounts of 3β,7α-dihydroxy-5-cholestenoic acid and 7α-hydroxy-3-oxo-4-cholestenoic acid in the peripheral circulation [6]. The latter compounds may also be formed in nonhepatic cells [7].

Fig. 3 summarizes the results of the measurements of the 4 different 27-oxygenated products in the hepatic vein and a peripheral artery in six healthy volunteers [2]. The lowest concentrations of the four products were always found in the hepatic vein, indicating an uptake of the compounds in the splanchnic area. The total uptake of the four 27-oxygenated compounds was 26 ± 4 mg/24 h.

For reasons of comparison we also measured the concentration of other oxysterols in the two vessels. There was no significant uptake of any of these oxysterols in the liver.

Theoretically, elimination of 27-oxygenated metabolites in the splanchnic region may occur in the liver and/or the intestine. If the intestine is involved, there should be lower levels in the portal vein than in a peripheral artery or vein. In a separate experiment it was shown that the uptake of the 27-oxygenated metabolites in the intestine was less than 4 mg of 27-oxygenated metabolites per 24 hours [2]. Thus the uptake of 27-oxygenated metabolites in the liver should be about 22 mg/24 hours.

To summarize, it is evident that there is a significant flux of 27-oxygenated metabolites from extrahepatic tissues to the liver (Fig. 4). In the liver these 27-oxygenated metabolites are known to be converted into bile acids. If about 20 mg 27-oxygenated products are taken up by the liver during 24 hours and are converted into bile acids, it would correspond to about 4% of the total bile acid formation.

This pathway to bile acids is thus an alternative to the two major pathways to bile acids [5] starting with a 7α-hydroxylation or a 27-hydroxylation of cholesterol in the liver (Fig. 4). The importance of the present pathway involving extrahepatic 27-hydroxylation

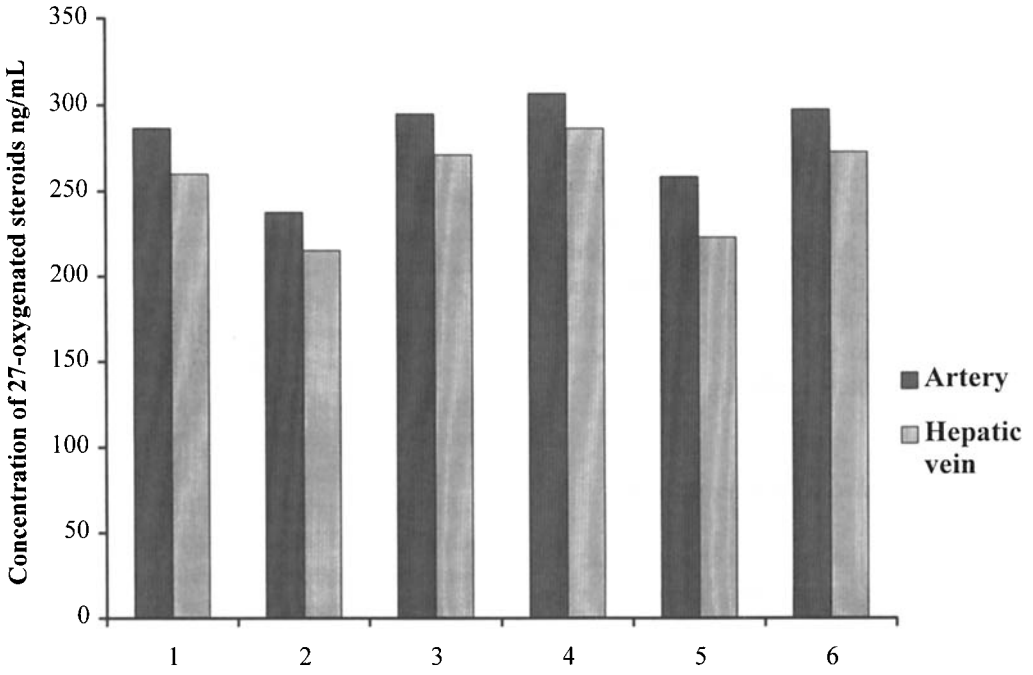

FIG. 3. Hepatic uptake of 27-oxygenated steroids in six healthy volunteers.

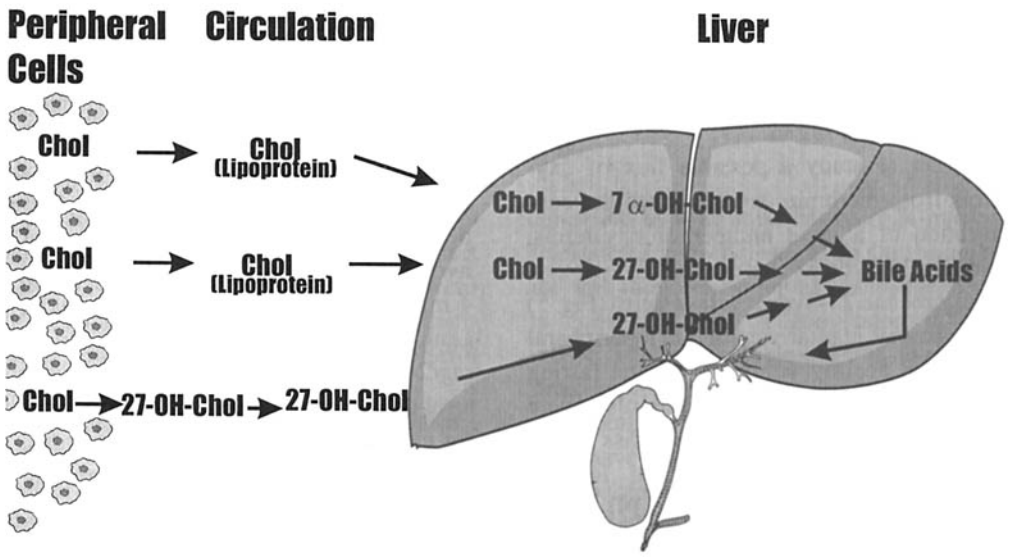

FIG. 4. Flux of 27-oxygenated steroids from different cells and tissues to the liver.

of cholesterol may conceivably be higher in patients with liver disease and a down-regulated cholesterol 7α-hydroxylase.

In the above calculations it is assumed that the levels of the different 27-oxygenated products are relatively stable and that there is little or no diurnal variation. In a separate study we found that there is very little diurnal variation of the different 27-oxygenated metabolites, with coefficients of variation varying between 6% and 16% when measuring at 4 hour intervals during 24 hours [2].

Flux of 24-hydroxycholesterol from the brain

Theoretically, the new mechanism can be expected to be particularly important when there is little or no reverse cholesterol transport. Owing to the effective blood-brain barrier this is the situation in the brain. The brain is the most cholesterol-rich organ in the body and there must be some mechanism for removal of excess cholesterol. We have previously described the presence of a sterol 27-hydroxylase activity in the brain [8] and thus it appeared possible that the above mechanism could be of importance for removal of cholesterol also in this organ.

In order to study a possible flux of oxysterols from the brain, we measured the concentration of a great number of oxysterols, including 24-hydroxycholesterol, 25-hydroxycholesterol and 27-hydroxycholesterol (Fig. 5) in the internal jugular vein and in a peripheral artery of 8 healthy volunteers in the fasting basal state [18]. The oxysterols were measured by isotope dilution mass spectrometry with deuterium labelled internal standards as previously described [9]. There was no net flux of 27-oxygenated steroids from the brain, and if anything there was a slight uptake. Among all the oxysterols tested, there was only one, namely 24-hydroxycholesterol, that was transported from

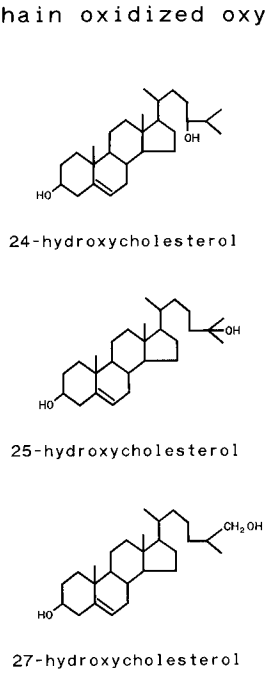

FIG. 5. Structure of 24-, 25-, and 27-hydroxycholesterol.

the brain into the circulation. Thus the levels of 24-hydroxycholesterol in serum samples from the internal jugular vein and the brachial artery showed significant differences ($p < 0.02$, two-tailed paired t-test) with higher levels in the internal jugular vein (66 ± 12 ng/mg) than in the brachial artery (59 ± 8 ng/mL). It was calculated that the flux of 24-hydroxycholesterol from the brain was about 4 mg/24 h. Since cholesterol synthesis in the adult brain of primates is maximally a few mg/24 h [10], the flux of 24-hydroxycholesterol demonstrated here may be of importance for cholesterol homeostasis in this organ.

Presence of 24-hydroxycholesterol in the brain has been reported previously [11], and in older literature 24-hydroxycholesterol has been denoted "cerebrosterol". Presence of a microsomal enzyme capable of hydroxylating cholesterol in the 24-position has also been reported [12]. The capacity of the latter

enzyme is very low, however, and is barely detectable with the methodology used.

In order to evaluate the importance of the flux of 24-hydroxycholesterol we measured the content of 24-hydroxycholesterol in the brain and in different organs (autopsy samples from two corpses) [18]. The isotope dilution mass spectrometric method was used for these measurements [9]. In accordance with previous work [11-13] the content of 24-hydroxycholesterol was relatively high in the brain (9-15 ng/mg wet weight) and in the adrenals (about 3 ng/mg). In all other organs the concentration of this steroid was very low, only 0.01-0.2 ng/mg wet weight. This information, coupled with the demonstrated flux of 24-hydroxycholesterol from the brain, is compatible with the contention that a major portion of the 24-hydroxycholesterol in the circulation originates from the brain.

In order to further study the flux of 24-hydroxycholesterol from the brain we used a rat exposed to $^{18}O_2$ in the inhalation atmosphere for 210 minutes [18]. As previously reported [14,15] this technique can be used to study *in vitro* formation of oxysterols formed by oxygen-dependent mixed function oxidases. If an oxysterol is formed by such an enzyme during the exposure with $^{18}O_2$ there will be incorporation of one atom of ^{18}O in the molecule. As shown in Fig. 6, 24-hydroxycholesterol isolated from the brain of this rat had an incorporation of 11% ^{18}O in the molecule, whereas 24-hydroxycholesterol isolated from the circulation had an ^{18}O content of 9%. This is compatible with a flux of 24-hydroxycholesterol from the brain into the circulation.

Information about the quantitative importance of the enzymatic mechanism for removal of brain cholesterol can be obtained from the present $^{18}O_2$-experiment. Under the conditions employed, about 11% of the total pool of 24-hydroxycholesterol in the brain of the rat was replaced with newly synthesized material during the 210 minutes exposure.

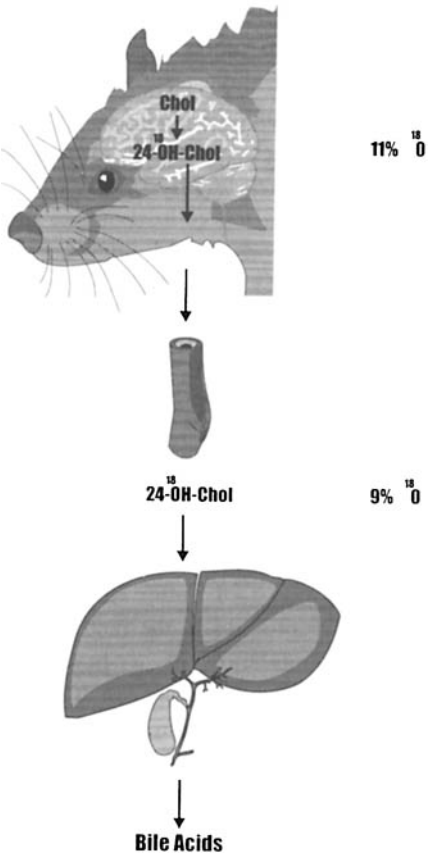

FIG. 6. Flux of ^{18}O-labelled 24-hydroxycholesterol from the brain of a rat exposed to $^{18}O_2$-atmosphere [14].

Since the over-all ratio between 24-hydroxycholesterol and cholesterol in the brain of a rat was found to be about 1:250, the finding is consistent with a conversion of about 0.01% of all brain cholesterol into 24-hydroxycholesterol per hour. Since the equilibration of the enzyme system with $^{18}O_2$ may have been 60% rather than 100% under the conditions employed, the rate of conversion may have been even higher. This degree of conversion is of the same magnitude as that described for the brain microsomal 24-hydroxylase *in vitro* [12]. Theoretically, the half life for elimination of all cholesterol

present in rat brain by this mechanism would then range between 6 and 10 months. This elimination may be compared with the estimated half-life of cholesterol in slices of rat brain of about 6 months [16]. Most probably the half life of cholesterol is considerably longer in the human brain.

CONCLUDING REMARKS

It has been demonstrated that introduction of a hydroxyl group in the side-chain of cholesterol increases the rate of transfer of cholesterol through lipophilic membranes by up to three orders of magnitude [17]. The side-chain hydroxylated cholesterol species are rapidly transported to the liver by lipoproteins, mainly LDL and HDL. In the absence of lipoproteins in the medium, there is some accumulation of 27-hydroxycholesterol in cultured macrophages. The major 27-oxygenated product in macrophages, 3β-hydroxy-5-cholestenoic acid, appears, however, to be transported from the macrophages also in the absence of lipoproteins in the culture medium. In the liver all the side-chain oxygenated compounds are converted into bile acids at a rate much faster than cholesterol itself.

It is of interest to compare this oxidative strategy for removal of cellular cholesterol with reverse cholesterol transport (Fig. 2). The latter seems to be considerably more effective when optimal amounts of the critical lipoprotein are present. In tissues, however, the availability of lipoproteins may be restricted and the relative importance of the oxidative mechanism may be greater. In this connection it is notable that we have found that the critical sterol 27-hydroxylase is induced severalfold when monocytes isolated from the circulation are differentiated into macrophages.

The mitochondrial sterol 27-hydroxylase is a very old enzyme from an evolutionary point of view. The presence of this oxidative system in most or all cells is puzzling and it is possible that the importance of it might have been greater at an earlier stage in the evolution. Because of compensatory mechanisms it is possible to survive a lack of the enzyme. In addition to a reduced rate of synthesis of bile acids, such a lack leads, however, to development of xanthomas, dementia and premature atherosclerosis. The fact that xanthomas and atheromas are developing in spite of normal circulating levels of cholesterol points to the importance of the oxidative mechanism for cholesterol removal from the cells. At the present state of knowledge the sterol 24-hydroxylase in the brain has not been characterized at a molecular level and the consequences of a lack of this enzyme activity are not known.

We have given two examples of oxidative alternatives to the classical reverse cholesterol transport mechanism for removal of intracellular cholesterol. Further work is needed to establish the relative importance of these mechanisms for cholesterol homeostasis under different conditions.

ACKNOWLEDGMENTS

This work was supported by a grant from the Swedish Medical Research Council, The Swedish Heart-Lung Foundation and by the Foundation of Marianne and Marcus Wallenberg.

REFERENCES

1. Björkhem I, Andersson O, Diczfalusy U, Sevastik B, Xiu R-J, Duan C, Lund E. Atherosclerosis and sterol 27-hydroxylase: Evidence for a role of this enzyme in elimination of cholesterol from human macrophages. Proc Natl Acad Sci USA 1994;91: 8592-6.

2. Lund E, Andersson O, Zhang J, Babiker A, Ahlborg G, Diczfalusy U, Einarsson K, Sjövall J, Björkhem I. Importance of a novel oxidative mechanism for elimination of intracellular cholesterol in humans. Arterioscler Thromb Vasc Biol 1996;16:208-12.
3. Dahlbäck H, Björkhem I, Princen H. Selective inhibition of mitochondrial 27-hydroxylation of bile acid intermediates and 25-hydroxylation of vitamin D by cyclosporin A. Biochem J 1993;293:203-6.
4. Reiss AB, Martin KO, Javitt NB, Martin DW, Grossi EA, Galloway AC. Sterol 27-hydroxylase: high levels of activity in vascular endothelium. J Lipid Res 1994;35: 1026-30.
5. Björkhem I. Mechanism of degradation of the steroid side chain in the formation of bile acids. J Lipid Res 1992;33:455-71.
6. Axelsson M, Mörk B, Sjövall J. Occurrence of 3β-hydroxy-5-cholestenoic acid, 3β,7α-dihydroxy-5-cholestenoic acid and 7α-hydroxy-3-oxo-4-cholestenoic acid as normal constituents in human blood. J Lipid Res 1988;29:629-41.
7. Zhang J, Larsson O, Sjövall J. 7α-hydroxylation of 27-hydroxycholesterol and 25-hydroxycholesterol in human fibroblast cultures. 13th International Bile Acid Meeting, Falk Symposium no. 80. San Diego, Calif; 1994:86-90.
8. Pedersen J, Oftebro H, Björkhem I. Reconstitution of C-27 steroid 26-hydroxylase activity from bovine brain mitochondria. Biochem Int 1989;18:615-22.
9. Dzeletovic S, Breuer O, Lund E, Diczfalusy U. Determination of cholesterol oxidation products in human plasma by isotope dilution-mass spectrometry. Anal Biochem 1995;225:73-80.
10. Spady D, Dietschy JM. Sterol synthesis *in vivo* in 18 tissues of the squirrel monkey, guinea pig, rabbit, hamster and rat. J Lipid Res 1983;24:303-15.
11. Ercoli A, DeRuggieri P. The constitution of cerebrosterol, a hydroxysterol isolated from horse brain. Gazz Chim Ital 1953;83:73-86.
12. Dhar AK, Teng JI, Smith LL. Biosynthesis of cerebrosterol by bovine cerebral cortical microsomes. J Neurochem 1973;21:51-60.
13. Prasad VV, Ponticorvo L, Lieberman S. Identification of 24-hydroxycholesterol in bovine adrenals in both free and esterified forms and in bovine brains as its sulfate ester. J Steroid Biochem 1984;21:733-6.
14. Björkhem I, Lewenhaupt A. Preferential utilization of newly synthesized cholesterol as substrate for bile acid biosynthesis. J Biol Chem 1979;254:5252-6.
15. Breuer O, Björkhem I. Use of an $^{18}O_2$ inhalation technique and mass isotopomer distribution analysis to study oxygenation of cholesterol in rat. J Biol Chem 1995;270: 20278-84.
16. Andersson M, Elmberger PG, Edlund C, Kristensson K, Dallner G. Rate of cholesterol, dolichol and dolichol-P biosynthesis in rat brain slices. FEBS Lett 1990;269:15-8.
17. Lange Y, Ye J, Strebel F. Movement of 25-hydroxycholesterol from the plasma membrane to the rough endoplasmic reticulum in cultured hepatoma cells. J Lipid Res 1995; 36:1092-7.
18. Lütjohann D, Brever D, Ahlborg G, Nennesmo I, Sidén Å, Diczfalusy U, Björkhem I. Cholesterol homeostasis in human brain: Evidence for an age-dependent flux of 24S-hydroxycholesterol from the brain into the circulation. Proc Natl Acad Sci USA 1996; 93: in press.