Mechanism of Accumulation of Cholesterol and Cholestanol in Tendons and the Role of Sterol 27-hydroxylase (CYP27A1)

Sara von Bahr, Tomas Movin, Nikos Papadogiannakis, Irina Pikuleva, Per Rönnow, Ulf Diczfalusy, Ingemar Björkhem

Objective—Tendon xanthomas are deposits of lipids and connective tissue commonly found in hypercholesterolemic patients. Macrophages are likely to be responsible for the lipid accumulation. Normolipidemic patients with the rare disease cerebrotendinous xanthomatosis, lacking the enzyme sterol 27-hydroxylase (CYP27A1), develop prominent xanthomas in tendons and brain containing both cholestanol and cholesterol, with a cholestanol:cholesterol ratio higher than that in the circulation. Because of its ability to convert cholesterol into polar metabolites that leave the cells faster, CYP27A1 has been suggested to be an antiatherogenic enzyme. The hypothesis was tested that tendons contain CYP27A1 that may be of importance for the normal efflux of both steroids.

Methods and Results—Western blotting and combined gas chromatography-mass spectrometry showed that human tendons contain significant amounts of CYP27A1 and its product, 27-hydroxycholesterol. Immunohistochemistry showed that CYP27A1 is present in macrophages and tenocytes. The tendons also contained cholestanol, with a cholestanol:cholesterol ratio slightly higher than that in the circulation. Recombinant human CYP27A1, and cultured macrophages containing this enzyme, had similar activity toward cholesterol and cholestanol. After loading of macrophages with labeled cholesterol and cholestanol, there was an efflux of these steroids in both unmetabolized and 27-oxygenated form, resulting in a significant cellular accumulation of cholestanol compared with cholesterol.

Conclusion—The results are consistent with the possibility that CYP27A1 is of importance for the efflux of both cholesterol and cholestanol from tendons. (Arterioscler Thromb Vasc Biol. 2002;22:1129-1135.)

Key Words: tendon xanthomas ■ cerebrotendinous xanthomatosis ■ atherosclerosis ■ sterol transport

Tendon xanthomas are deposits of lipid and connective tissue commonly found in patients with familial hypercholesterolemia.1,2 The size of the xanthomas seems to parallel the degree of hypercholesterolemia. Cholesterol and cholesteryl esters dominate among the lipid components with unesterifed cholesterol accumulating predominantly in the extracellular space and esterified cholesterol present both intra- and extracellularly. It has been clearly shown that most of the lipids in the tendons are derived from the circulation rather than from local synthesis.3,4 There are clear similarities between tendon xanthomas and atherosclerotic plaques.

However, the presence of tendon xanthomas is not always associated with hyperlipidemia. Patients with the rare metabolic disease cerebrotendinous xanthomatosis (CTX) are typically normolipidemic, but have prominent xanthomas in tendons and brain.5 The xanthomas in CTX patients contain a higher level of cholestanol than those in hyperlipidemic patients. The basic metabolic defect behind this disease is a lack of the cytochrome P-450 enzyme sterol 27-hydroxylase (CYP27A1). This enzyme is of importance for cholesterol homeostasis in human macrophages and endothelial cells.6–9 The enzyme is thus capable of oxidizing cholesterol into the more polar metabolites 27-hydroxycholesterol and cholestenoic acid (3β-hydroxy-5-cholestenoic acid) which are able to flux from the cells into the circulation. There is a net uptake of these steroids in the liver with a subsequent conversion into bile acids.6 This mechanism is antiatherogenic, and its absence may explain the premature atherosclerosis in CTX patients.

If the accumulation of cholesterol and cholestanol in the tendons of CTX patients is a direct consequence of the lack of CYP27A1, one would expect CYP27A1 to be normally present in tendons. Furthermore one would expect this enzyme to be active toward both cholesterol and cholestanol. In untreated CTX patients, there is an overproduction of cholestanol.5 At least part of this cholestanol is formed from...
bile acid, intermediates accumulate as a consequence of the CYP27A1 deficiency in bile acid synthesis.10 According to previous reports, the cholestanol:cholesterol ratio in tendons of patients with CTX is higher than that in the circulation.8 The reason for the selective trapping of cholestanol in the tendons is not known.

In the present work, the following hypotheses were tested: 1) CYP27A1 is present in human tendon; 2) CYP27A1 is active toward cholestanol; 3) the flux of 27-hydroxylated metabolites of cholestanol from cells is lower than the corresponding flux of 27-oxygenated metabolites of cholesterol.

Methods

Materials
All organic solvents used were of gas chromatography or high-performance liquid chromatography grade. Human CYP27A1,11 as well as adrenodoxin and adrenodoxin reductase, was prepared as described previously. Polyclonal peptide antibodies toward CYP27A1 were a generous gift from Dr David Russell, University of Texas Southwestern Medical Center, Dallas.12

In some experiments, polyclonal antibodies toward the whole CYP27A1 protein were used. 4,4'-Labeled cholesterol was obtained from New England Corp and had a specific radioactivity of 0.3 x 10^6 cpm/µg. 1,2'-H-Labeled cholestanol was synthesized from 1,2'-H-labeled cholesterol (obtained from the same source as above) by hydrogenation and purification by thin-layer chromatography as described previously13 and diluted with unlabeled cholestanol to a specific radioactivity of 0.3 x 10^6 cpm/µg before use.

Achilles tendons were obtained at autopsy of 9 subjects (7 men) with a median age of 57 (range 28 to 63) years. These subjects had diabetes mellitus, and two had a history of alcoholism. Whether or not some of these subjects have had achillodynia or tendon disease is not known. The autopsy specimens were immediately frozen to −20°C or −70°C after a median post-mortem time of 41 (range 25 to 72) hours.

Tendon biopsies (n=6) were obtained from male patients with a median age of 41 (range 35 to 56) years, treated surgically for Achilles tendinosis.14 Macroscopically, the tendons were characterized by a slight dull grey discoloration with altered fiber structure. Specimens were excised from the macroscopically abnormal-looking areas. Each specimen was 10 to 15 mm long, 2 to 6 mm wide, and 3 to 8 mm thick and immediately frozen to −20°C.

Massive Achilles tendon xanthomas were obtained from a male familial hypercholesterolemia patient, 41 years old. At the time of the operation, the level of plasma cholesterol was 9.4 mmol/L.

Histopathologic Examination

Biopsy tissue from the hypercholesterolemic patient (see above) was obtained for histopathologic examination from left and right tendons, as well as from the macroscopically recognizable xanthomatous areas. All the biopsy specimens were routinely processed, embedded in paraffin and stained with hematoxylin and eosin for standard morphological evaluation.

Immunohistochemistry for CYP27A1 was performed on frozen tissue from xanthoma areas (left and right), by using a polyclonal antibody (rabbit anti-CYP27A1) according to standard procedures, with peroxidase as substrate. Additionally, all paraffin-embedded sections were stained with an anti-CD68 antibody (Dakopats) to demonstrate the macrophage/histiocyte origin of xanthoma cells.

Preparation of Lipid Extracts From Tendons and Xanthoma

The tissue was submerged in liquid nitrogen for 20 minutes and shaken for 30 seconds (Micro-Dismembrator II, B-Braun) until completely powdered. The powder was dissolved in chloroform:methanol 2:1, vol/vol, 1 ml/0.01g of tissue, homogenized (Ultra-Turrax, Janke&Kunkel), and then boiled for 1 hour.

Analysis of Cholesterol and Oxyesters

Cholesterol and oxysterols in serum and in lipid extracts from tissues, incubation media, and macrophages were quantitated by isotopic dilution, mass spectrometry and use of deuterium-labeled internal standards as described previously.15,16

Medium and cells were extracted with 6 volumes of chloroform: methanol 2:1, vol/vol, evaporated to dryness and dissolved in 0.5 mL of chloroform. The 1H/14C-ratio was determined before separation. The separation of the substrate (ie, the neutral sterols, 1H-cholesterol, and 14C-cholesterol) from the products (ie, the oxysterols, 1H-27-hydroxycholesterol and 14C-27-hydroxycholesterol, and the acids 1H-cholestanolic acid and 14C-cholestenolic acid) was performed as follows: the sample was added to a Bond Elute NH2-column (Varian) preconditioned with 4 mL of hexane. Neutral sterols and oxysterols were eluted with 4 mL of chloroform:2-propanol 2:1 for further separation, and the acids were eluted with 4 mL of 2% acetic acid in ether. The 1H/14C-ratio in the acid phase was determined before evaporation, methylation, silylation, and analysis on gas chromatography-mass spectrometry (GC-MS) as previously described.15,16

The eluate containing neutral sterols and oxysterols was evaporated, dissolved in 1 mL of toluene and added to a Isolute silica column (International Sorbent Technology) preconditioned with 2 mL of hexane. The neutral sterols were eluted with 8 mL 0.5% 2-propanol in hexane, and the oxysterols were eluted with 5 mL of 30% 2-propanol in hexane. The 1H/14C-ratio in both the neutral sterol phase and oxysterol phase was determined before evaporation, silylation, and analysis on GC-MS as previously described.15,16

Western Blotting

For immunoblotting, aliquots of the homogenates were diluted with sample buffer and subjected to SDS-PAGE (10% gels). Approximately 10 µg of total protein was loaded in each well. The samples were transferred to a nitro-cellulose membrane, which was blocked with 1% bovine serum albumin in Tris buffered saline with 0.1% tween (TBS-T), washed with TBS-T, and incubated with the primary antibody for 1.5 hours followed by wash with TBS-T and incubation with peroxidase-conjugated goat anti-rabbit IgG for 1 hour. The blots were visualized with a chemiluminescence-kit (Amersham), and we scanned to compare the intensities of the bands. The primary antibody used in the Western blotting of CYP27A1 was a rabbit polyclonal antibody, kindly provided by Dr. David Russell, or a rabbit antibody toward the whole CYP27A1 protein.

Experiments With Recombinant Human CYP27A1
The human CYP27A1 and the cofactors (adrenodoxin and adrenodoxin reductase) were incubated with NADPH and substrate (cholesterol and/or cholestanol under the conditions essentially as described previously).15 In a 1-mL reaction mixture, 1.5 nmol of adrenodoxin and adrenodoxin reductase, 150 pmol of CYP27A1 and 0.8 mg of NADPH were added, yielding a total conversion of cholesterol into 27-hyroxysterols of ~5% with a turnover number of ~0.3 nmol -min⁻¹-µmol⁻¹ CYP27A1. The reaction mixture was extracted with chloroform:methanol 2:1, vol/vol, as above and analyzed.

Studies on Primary Human Alveolar Macrophages

Primary human alveolar macrophages were isolated from bronchoalveolar lavage fluid obtained at the Lung Clinic. The fluid was centrifuged at 90g, and the pellet was dissolved in medium (Minimal Essential Medium with 1% Penicillin/Streptomycin and 3% ultrosol, all from Gibco) and washed. Cells were seeded at a density of 1 to 2 x 10⁶ per 50 mm dish, and 3 mL of medium was added. After 1 to 5 hours, the medium was changed, and nonattached cells were discarded. After 12 to 24 hours, the medium was changed, and the incubation was started. 1H-Cholesterol, 7.5 µg, (corresponding to 2.3 x 10⁶ cpm) and 5 µg of 14C-cholesterol (corresponding to
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Figure 1. Western blotting of sterol 27-hydroxylase in tendons. Lane 1, recombinant human CYP27A1. Lanes 2 and 3, tendon samples.

1.3×10⁸ cpm were dissolved in 10 µL of ethanol and added to each dish together with medium. After 24 hours, the medium was collected and analyzed. In some dishes, the cells were harvested and analyzed after 24 hours of incubation; the remaining dishes were cultured for an additional 24 or 48 hours. Medium was changed every 24 hours and analyzed.

Radio–High-Performance Liquid Chromatography and Combined GC-MS

A Packard (Meriden) instrument equipped with a YMC-Pack ODS-A column, 250×4.6 mm, was used with methanol-water 85:15 (vol/vol) as mobile phase. The combined GC-MS was performed with a Hewlett-Packard 5890 Series II instrument equipped with an HP 5890 column (25 m×200 µm internal diameter×0.33 µm film thickness). The initial temperature was 180°C followed by an increase of 20°C/min up to 250°C and then 5°C/min up to 300°C.

Ethical Aspects

Permission to use the tendon materials and the human alveolar macrophages for the above research had been obtained from the local ethics committee at Huddinge University Hospital.

Results

Demonstration of CYP27A1 Enzyme and Product in Human Tendons and a Xanthoma

CYP27A1 could be demonstrated in all autopsy and biopsy tendon material analyzed by the Western blotting technique (Figure 1). Only one band with the expected molecular weight of ~54 000 could be seen in the blots when using the peptide antibodies. In some of the samples, bands corresponding to higher molecular weights could also be seen when using the antibodies toward intact CYP27A1.

27-Hydroxycholesterol, the primary product of CYP27A1 activity toward cholesterol, was present in extracts from all the tendons examined as well as in the extract of the xanthoma (Table). The ratio between 27-hydroxycholesterol and cholesterol was ~25-fold higher than that in the circulation. There were no significant differences between the levels of 27-hydroxycholesterol in biopsy and autopsy tendon materials. Interestingly, there was a high degree of correlation between levels of 27-hydroxycholesterol and total cholesterol in the tendons (R²=0.96).

The levels of cholesterol were not different in the two types of tendon materials. When analyzing the degree of esterification in the autopsy and biopsy preparations, there was a difference, however. The ratio between free and total cholesterol was found to be 0.39±0.08 in the autopsy material and 0.66±0.06 in the biopsy material. The level of cholesterol was ~6-fold higher in the xanthoma as compared with the tendons, although the ratio between 27-hydroxycholesterol and cholesterol was similar.

For reasons of comparison, the levels of cholestanol were also measured in the tendons. These levels were ~0.5% of those of cholesterol. The cholestanol:cholesterol ratio was higher than the corresponding ratio in the circulation, indicating a relative accumulation of cholestanol. No correlation was observed between the levels of cholestanol and levels of 27-hydroxycholesterol or cholesterol. In two cases, the degree of esterification of cholestanol was measured and found to be almost identical to that of cholesterol in the same material.

Cellular Location of CYP27A1 in Tendons and Xanthomas

Tendon tissue from the hypercholesterolemic patient, obtained from the non xanthomatous areas, revealed routine morphological changes of diffuse infiltration by foamy, lipid-laden xanthoma cells, disarray and abnormalities in collagen fiber structure, and focal chronic inflammation. Sections from xanthomatous areas showed classic tendinous xanthomas, with multiple nodules of foamy xanthoma cells admixed with lymphocytes and occasional neutrophil polymorphs. Multinucleated giant cells were also seen. The xanthoma cells were, as expected, positive for CD68 antigen. Immunohistochemistry for CYP27A1 demonstrated that the

| Content of Cholesterol, Cholestanol and 27-Hydroxycholesterol in Tendons |
|-----------------------------|-----------------|----------------|----------------|
| Steroid                     | Tendons         | Xanthoma       | Circulation    |
| Cholesterol, µg/mg tissue (n=13) | 7±1             | 40             |                |
| Cholestanol, ng/mg tissue (n=14) | 15±3            | 144            |                |
| Cholestanol/cholesterol, ng/µg (n=12) | 5±2          | 4              | <2*           |
| 27-Hydroxycholesterol, ng/mg tissue (n=8) | 9±1        | 135            |                |
| 27-Hydroxycholesterol/cholesterol, ng/µg (n=6) | 2.5±0.1 | 3.4            | <0.1†, 0.09‡   |

* A group of 10 healthy volunteers all had a ratio <2. Koopman et al. have reported a ratio of 0.8–2.1 for healthy subjects.
† A group of 111 healthy volunteers had a ratio 0.068±0.003 (A. Babiker, I. Björkhem, unpublished observation, 2000).
‡ The hypercholesterolemic patient with xanthomas.
majority of immunoreactive cells were macrophages (Figure 2). However, significant staining was also observed in tenocyte-like cells, although the staining was less intense compared with macrophages, and only a minority of the tenocyte population stained positive with the CYP27A1 antibody.

Demonstration of Activity of CYP27A1 Toward Cholestanol
Cultured human macrophages were shown to have a significant 27-hydroxylase activity toward cholestanol added to the incubation medium. Thus, both 27-hydroxycholestanol (Figure 3) and 3β-hydroxy-5α-cholestanolic acid (Figure 4) could be identified as products by combined GC-MS.

The relative rate of conversion of cholesterol and cholestanol by CYP27A1 could not be evaluated in the above experiments due to the fact that the amount of endogenous intracellular cholesterol available for the enzyme is difficult to measure. When the cultured human macrophages were exposed to a mixture of 3H-labeled cholestanol and 14C-labeled cholesterol the conversion into 27-oxygenated products was about the same from the two steroids. To specifically measure the capacity of CYP27A1 to convert the two substrates, human recombinant CYP27A1 was incubated with adrenodoxin reductase, adrenodoxin, NADPH and a 1:1 mixture of cholesterol and cholestanol. In these experiments, the degree of conversion of the two steroids into the 27-oxygenated products was about the same. In one typical experiment, the degree of conversion of cholesterol was 4% and conversion of cholestanol 5%. The total turnover in this experiment was ≈0.3 3 nmol · min⁻¹ · nmol⁻¹ CYP27A1.

Demonstration of Flux of Cholesterol and Cholestanol and Their 27-Oxygenated Metabolites From Cultured Human Macrophages
Primary human alveolar macrophages were cultured with a mixture of 3H-labeled cholestanol and 14C-labeled cholesterol.
to study differences in uptake and efflux of the two steroids. Figure 5 shows the results of these experiments, performed with 3 different preparations of macrophages. After exposure of the macrophages for 24 hours to the above steroid mixture, the macrophages had taken up 30% to 50% of the labeled steroids, with a significant decrease in the ratio of $^3$H to $^{14}$C, from 1.8 to 1.2. This is consistent with a slower uptake of cholestanol than of cholesterol. After 48 and 72 hours culture of the steroid-loaded macrophages, the ratio between cholestanol and cholesterol increased significantly, from 1.2 to 1.9 (Figure 5), indicating a slower efflux of cholestanol from the cells. As shown in Figure 5, the $^3$H/$^{14}$C ratio of the material fluxing from the cells into the medium was lower than that retained in the cells. Approximately 90% of the radioactive steroids fluxing from the cells consisted of cholesterol and cholestanol and 10% of the 27-oxygenated products of these steroids (see Reference 5). The $^3$H/$^{14}$C ratio of the 27-oxygenated products (27-hydroxycholesterol and 27-hydroxycholestanol) fluxing from the cells to the medium was about similar to that of the unmetabolized steroids. The difference in $^3$H/$^{14}$C-ratio between the cholestanol/cholesterol fraction and the 27-hydroxycholesterol/27-hydroxycholesterol fraction in the medium thus varied between −4% and +20% in the different experiments with a mean of +10%. This difference was however not statistically significant.

**Discussion**

**Accumulation of Cholesterol in Tendons and Xanthomas**

Tenocytes are the dominating type of cells in normal tendons. Although macrophages are considerably less frequent, they may be responsible for most of the accumulation of lipids in tendons, particularly as they are more frequent in xanthomas than in normal tendons. We have previously shown that macrophages from various tissues contain CYP27A1, and this enzyme was also detected in macrophages from tendons and tendon xanthomas. Tenocytes were also shown to contain CYP27A1, although the content was considerably lower than in macrophages. In preliminary experiments, we have isolated primary tenocytes from a tendon xanthoma and cultured them under standardized conditions. Their capacity to excrete 27-oxygenated products into the medium was similar to that previously observed for cultured fibroblasts (results not shown).

As CYP27A1 has the potential to promote eflux of cholesterol by converting it into 27-hydroxycholesterol and cholestenolic acid, the lack of CYP27A1 may explain the development of cholesterol-rich xanthomas in the tendons and brain of patients with CTX. In hypercholesterolemic patients with xanthomas, there may be an imbalance between the capacity of the CYP27A1-mechanism and the influx of cholesterol from the circulation. It is evident that the classical mechanism for reverse cholesterol transport may also be of importance.

The high ratio between 27-hydroxycholesterol and cholesterol in tendons and xanthomas, more than 20 times higher than that in the circulation, is noteworthy and may reflect a very active defense mechanism toward accumulation of cholesterol. A similarly high ratio has been reported in atheromas. There was a significant correlation between the levels of the two steroids.

There was no clear relation between the levels of CYP27A1 and absolute or relative levels of 27-hydroxycholesterol in the tendons. Some of the tendons were obtained at autopsy from subjects without any known tendon diseases and some from subjects treated surgically for long-standing achillodynia. It has been shown that tendons from patients with achillodynia have some changes in the fiber structure and arrangement and that they contain increased levels of interfibrillar glycosaminoglycan. However, the biochemical findings (cholesterol and cholestanol accumulation, CYP27A1 content) were not different in the two types of tendons. The only difference observed was that the degree of cholesterol esterification was higher in healthy tendons than in achillodynia tendons. In this respect, it is interesting that achillodynia patients have higher levels of extracellular glycosaminoglycans than controls and that unesterified cholesterol accumulates preferentially in the extracellular space. The possibility must be considered that there is a coupling between presence of free cholesterol and density of extracellular glycosaminoglycans.

**Accumulation of Cholestanol**

There is an overproduction of cholestanol in CTX patients, and xanthomas from these patients are known to contain cholestanol in addition to cholesterol. Because it was clearly shown here that also cholestanol is a substrate for CYP27A1, part of the explanation for the accumulation of cholestanol in tendon and brain xanthomas of CTX patients may be the lack of the CYP27A1-dependent mechanism for eflux of the steroids.

In general, the cholestanol/cholesterol ratio is higher in tendons than in the circulation of CTX-patients. It has been reported that cholestanol accounts for up to 17% of total sterols in tuberous xanthoma from CTX patients with much lower elevations of cholestanol in the circulation. In the present study, the ratio between cholestanol and cholesterol was found to be somewhat higher in normal tendons than in the circulation (Table). This is consistent with the slight accumulation of cholestanol in relation to cholesterol observed in the present experiments with cultured macrophages.
The selective accumulation of cholestanol may be due to either increased uptake or decreased efflux. Lower efflux of cholestanol could be due to enhanced stability of cholestanol esters compared with cholesterol esters. Similar degrees of esterification of cholesterol and cholestanol were found in the tendons used in our study.

Mechanisms for Uptake and Efflux of Sterols From Alveolar Macrophages

In the present work, human alveolar macrophages were used as model cells for investigating the uptake and efflux of cholesterol and cholestanol. The reason for using these cells is that they have a higher CYP27A1 activity than any other cell type hitherto studied. Under the conditions used here, these cells have a very low Acyl CoA:cholesterol O-acyltransferase (ACAT) activity, and little or no cholesterol esters are formed during the culture period. When the macrophages were exposed to a mixture of cholesterol and cholestanol, there was a preferential uptake of cholesterol. There was however also a preferential efflux of cholesterol from the cells, both in the form of unmetabolized cholesterol and 27-oxygenated metabolites. The net result of these two processes is preferential retention of cholesterol in the cells. The preferential efflux of 27-hydroxycholesterol as compared with 27-hydroxycholesterol is not due to the substrate specificity of CYP27A1, because the activity of recombinant human CYP27A1 was very similar for cholesterol and cholestanol. In accordance with previous studies, the efflux of the 27-oxygenated metabolites was only ~10% of that of the unmetabolized steroids. From a quantitative point of view, the flux of unmetabolized steroids is thus dominating. However, it should be pointed out that the unmetabolized cholesterol has a much longer half-life in the body than the oxygenated metabolites and may be taken up again by macrophages or other cells.

It is interesting that the preference for efflux of cholesterol was similar for unmetabolized steroids and 27-oxygenated steroids. This finding is consistent with a common pool of steroids exposed to the CYP27A1 enzyme and those available for direct efflux. The efflux of unmetabolized steroids from the human macrophages may be dependent on ABCAl or ABCG1 transporters.18–21 Whether or not these transporters have a preference for cholesterol as compared with cholestanol is not known and was not studied here.

Does 27-Hydroxycholesterol Have a Dual Role?

At the present time, the possibility must be considered that 27-hydroxycholesterol may have a dual role in the transport of cholesterol from tendons—a direct role as a transport form of cholesterol and a more indirect role as a stimulator of efflux of cholesterol via the ABC transporters. These transporters have been shown to be responsive to the nuclear receptor LXR. Very recently, some experimental evidence was presented that 27-hydroxycholesterol may be an activator of LXR. If this is true, production of 27-hydroxycholesterol may be a driving force for efflux of cholesterol (and most probably also of cholestanol) by the ABC transporters. Our demonstration that there is a relatively high production of 27-hydroxycholesterol in the tendons is of interest in relation to this hypothesis. Whether or not 27-hydroxycholesterol is an activator of LXR is however controversial at this time, and according to two other reports, 27-hydroxycholesterol does not efficiently activate LXR.23,24

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References


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