Hepatic 7α-hydroxylation of cholesterol in ascorbate-deficient and ascorbate-supplemented guinea pigs

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Abstract Cholesterol 7α-hydroxylase activity was assayed in liver microsomes from guinea pigs supplemented with ascorbate and from guinea pigs in a state of ascorbate deficiency. A mass fragmentographic method was used by which the 7α-hydroxylation of endogenous cholesterol could be measured. The 7α-hydroxylation was markedly reduced in the ascorbate-deficient animals as compared to animals treated with ascorbate. Addition of ascorbate to the incubations did not increase this activity. 11- and 12-Hydroxylation of laurate as well as 25- and 26-hydroxylation of 5β-cholestane-3α,7α-diol were not significantly affected by the ascorbate status of animals. In the presence of excess NADPH-cytochrome P-450 reductase and a phospholipid, partially purified cytochrome P-450 from the microsomal fraction of liver of an ascorbate-deficient guinea pig had a much lower capacity to 7α-hydroxylate [4-^{14}C]cholesterol than a corresponding system containing cytochrome P-450 from liver of an ascorbate-supplemented guinea pig. It is suggested that ascorbate affects the synthesis or breakdown of the 7α-hydroxylating system, in particular the cytochrome P-450 component.

Supplementary key words 7α-hydroxylase · ascorbate deficiency · cytochrome P-450 · mass fragmentography

Guinea pigs with ascorbate deficiency have significantly higher serum and liver cholesterol levels than control animals (1). The reason for this has been sought in the biosynthesis as well as the catabolism of cholesterol. Evidence for an increased biosynthesis of cholesterol in the scorbutic state was presented by Guchait and Ganguli (2) who reported an increased incorporation of [^{14}C]acetate into cholesterol by preparations of the livers of scorbutic guinea pigs. In contrast, Weight et al. (3) reported a decreased incorporation of [^{14}C]acetate into cholesterol in liver homogenates of baboons deprived of ascorbate. Evidence for a decreased catabolism of cholesterol in the scorbutic state has been presented by Guchait, Guha and Ganguli (4) and by Ginter et al. (5). It was shown that the conversion of [4-^{14}C]cholesterol into bile acids was decreased and that [26-^{14}C]cholesterol was converted into $^{14}CO_2$ less efficiently in scorbutic animals than in control animals.

If ascorbate affects the overall conversion of cholesterol into bile acids it is likely that the effect concerns cholesterol 7α-hydroxylase, the rate-limiting enzyme in the degradation of cholesterol to bile acids. Two possibilities exist with respect to an effect of ascorbate on cholesterol 7α-hydroxylation: ascorbate might be a necessary factor for enzyme activity, or ascorbate might affect enzyme synthesis or breakdown. Recently, Kritchevsky, Tepper, and Story (6) showed that addition of ascorbate to the microsomal fraction of homogenates of livers of normal guinea pigs had no significant effect on cholesterol 7α-hydroxylase activity. This result, however, does not rule out the possibility that ascorbate is necessary for enzyme activity, since it is possible that a stimulatory effect of ascorbate can be demonstrated only if using microsomal fraction from homogenates of livers of scorbutic guinea pigs. In the present work it is shown for the first time that ascorbate deficiency is associated with a reduced cholesterol 7α-hydroxylase activity and evidence is presented that this effect is due to an effect of ascorbate on enzyme synthesis or breakdown. A mass fragmentographic method has been used that allows determination of 7α-hydroxylation of the endogenous cholesterol in the microsomal fraction (7, 8).

EXPERIMENTAL PROCEDURE

Materials

[4-^{14}C]Cholesterol (sp act 60 mCi/mmole) was obtained from the Radiochemical Centre, Amersham, England, and was purified by chromatography on a column of aluminum oxide, Grade III (Woelm, Eschwege, West Germany), prior to use. [1-^{14}C]-Laurate (sp act 3 mCi/mmole, Radiochemical Centre)

was purified by silicic acid chromatography prior to use (9). 5β-[7β-^{3}H]Cholestane-3α,7α-diol (sp act 1.6 mCi/mmole) was prepared as described previously (10). Tween 80 and cofactors were obtained from Sigma Chemical Co., St. Louis, Mo.).

Female guinea pigs were obtained from a local dealer. Their strain could not be identified. If kept on an ascorbate-free diet, the animals died after a few weeks (cf. ref. 11). Gall bladder bile was found to contain mainly chenodeoxycholic acid, small amounts of lithocholic acid, and only traces of cholic acid (12).

The animals were divided into two groups with five animals in each group. One group (group 1) was fed ordinary guinea pig pellets (ascorbate content 450 mg/kg) with free access to vegetables. To the animals in this group additional ascorbate, 2 × 20 mg in 20 g/100 ml sucrose, was given by stomach tube. The other group (group 2) was fed ascorbate-free pellets (Astra-Ewos, Södertälje, Sweden). When the animals stopped thriving (2–3 weeks with diet), 0.5 mg of ascorbate in sucrose was given orally every second day. With this dose, the animals maintained their weights but signs and symptoms of ascorbate deficiency were obvious, especially in regard to the condition of fur and teeth. The animals could be kept on this regimen for at least four months. At the time of preparation of liver homogenates (generally after about 2 months on diet and ascorbate supplementation) serum ascorbate (13) was 2.21 ± 0.18 and 0.11 ± 0.05 (mean ± SEM) mg/100 ml in groups 1 and 2, respectively. Control animals that were given guinea pig pellets and had free access to vegetables had serum ascorbate concentrations of 1.30 ± 0.30 mg/100 ml.

It should be pointed out that addition of ascorbic acid alone to the ascorbate-deficient diet (450 mg/kg diet) brought the deficient animals back to the normal state as judged from increase of body weight. The increase in weight of ascorbate-supplemented animals was parallel to that of control animals. During the period of observation they increased in weight from 250–350 g to 850–959 g. The weight of animals in group 2 remained at 250–350 g.

Preparation of cytochrome P-450

A crude cytochrome P-450 fraction containing cholesterol 7α-hydroxylase activity and laurate hydroxylase activity was prepared according to Lu et al. (14) as described previously (15). The cytochrome P-450 preparations from an ascorbate-treated guinea pig and an ascorbate-deficient guinea pig contained 1.5 and 2.6 nmoles of cytochrome P-450 per mg microsomal protein, respectively, when assayed according to Omura and Sato (16).

Incubation procedures

For incubations of cholesterol, liver homogenates (20%, w/v) were prepared in 0.25% sucrose containing 0.001 M EDTA (8). The homogenate was centrifuged at 20,000 *g* for 15 min. The microsomal fraction was isolated by centrifugation of the 20,000 *g* supernatant fluid for 1 hr. The protein content was of the same order of magnitude in the microsomal fraction from ascorbate-deficient animals and ascorbate-treated animals, (4 mg/ml and 5 mg/ml, respectively), when determined according to Lowry et al. (17).

The cholesterol content in the microsomal fraction from the ascorbate-deficient animals and the ascorbate-treated animals was 72 ± 4 μg/ml and 53 ± 5 μg/ml (mean ± SEM, n = 5) respectively, as determined by gas–liquid chromatography of the trimethylsilyl ether of cholesterol from an ether extract of saponified microsomal fractions. The cytochrome P-450 content of the microsomal fraction from the ascorbate-deficient animals and the ascorbate-treated animals was 0.39 ± 0.08 and 0.75 ± 0.11 nmoles/mg microsomal protein, respectively. The microsomal fraction was suspended in 0.1 M potassium phosphate buffer, pH 7.0, containing 0.028 M nicotinamide, in a volume corresponding to that of the 20,000 *g* supernatant fluid from which it had been isolated. [4-^{14}C]Cholesterol, 10 μg, was added in 0.5 ml of a suspension with Tween 80 (8). This suspension was prepared by dissolving the appropriate amount of [4-^{14}C]cholesterol in 0.1 ml of methanol and adding 3 mg of Tween 80 dissolved in 0.1 ml of methanol. The methanol was evaporated under a stream of nitrogen and 0.5 ml of the above mentioned phosphate buffer was added.

In incubations with partially purified cytochrome P-450, the microsomal fraction was substituted with 5 nmoles of cytochrome P-450 together with 1000 units of NADPH-cytochrome P-450 reductase and 50 μg of a mixture of mono- and dilauroylglycero-3-phosphorylcholine (14). The NADPH-cytochrome P-450 reductase fraction had been isolated from the liver of phenobarbital-treated rats (14, 15) and contained 500 units per mg of protein. Incubations were carried out for 20 min with 3 ml of microsomal fraction and 3 μmoles of NADPH in a total volume of 5 ml of buffer. Incubations were terminated by the addition of 20 volumes of chloroform–methanol 2:1 (v/v). After filtration, 0.2 volumes of 0.9% (w/v) sodium chloride solution were

added. The chloroform phase was collected and evaporated.

To the incubations in which the mass of 5-cholestene-3β,7α-diol was measured, 0.6 μg of 5-[3α,4β,7β-$^2H_3$]cholestene-3β,7α-diol was added just prior to addition of the chloroform–methanol mixture to the incubations mixture (cf. 7, 8). 5-[3α,4β,7β-$^2H_3$]Cholestene-3β,7α-diol was prepared from 4-cholestene-3-one by equilibration in *tert*-butanol and potassium *tert*-butoxide followed by addition of [1-^{2}H]acetic acid and reduction with sodium borodeuteride (18). [3α,4β-$^2H_2$]Cholesterol was converted into 5-[3α,4β,7β-$^2H_3$]cholestene-3β,7α-diol by oxidation with chromic acid followed by reduction with lithium aluminum deuteride (7). The material contained mainly trideuterated molecules and was equivalent to the 5-[24,25,7β-$^2H_3$]cholestene-3β,7α-diol used in previous work (7).

For incubations with [1-^{14}C]laurate and 5β-[7β-^{3}H]cholestane-3α,7α-diol, liver homogenates (20%, w/v) and microsomal fractions were prepared as above, with the exception that a modified Bucher medium (19) was used as homogenizing medium and incubation medium. [1-^{14}C]Laurate, 50 μg, dissolved in 50 μl of acetone, was incubated with 1 ml of microsomal suspension and medium for 15 min at 37°C (9). The incubations were terminated by addition of ethanol. The incubation mixtures were diluted with water, acidified, and extracted with ether. 5β-[7β-^{3}H]Cholestane-3α,7α-diol, 250 μg, dissolved in 50 μl of acetone, was incubated under the same conditions as [1-^{14}C]laurate, with the exception that the incubation time was 20 min (10), the incubations were terminated and extracted as were the incubations with cholesterol.

The conditions used for assay of all hydroxylase activities were optimal. Thus the conversion was linear with incubation time and, with the exception of incubations with cholesterol, also with amount of microsomal fraction.

Analysis of incubations

The residue of the chloroform extract of incubations with [4-^{14}C]cholesterol was subjected to thin-layer chromatography with benzene–ethyl acetate 2:3 (v/v), as solvent. The thin-layer chromatography was carried out immediately after evaporation in order to minimize autoxidation. The conversion of labeled cholesterol was measured by scanning the chromatoplates with a radioscanner (Berthold, Wildbad, West Germany). This method of assay did not allow accurate determination of a conversion less than about 0.05% (cf. Results).

The silica gel zones corresponding to cholesterol and 5-cholestene-3β,7α-diol were collected, eluted with methanol, and converted into trimethylsilyl ethers. The mass of 5-cholestene-3β,7α-diol was determined by mass fragmentography as described previously (7, 8) using an LKB 9000 instrument equipped with a MID-unit (multiple ion detector) and a 1.5% SE-30 column. The first channel of the MID-unit was focused on the ion at *m/e* 456 (M − 90 of the trimethylsilyl ether of unlabeled 5-cholestene-3β,7α-diol) and the second channel on the ion at *m/e* 459 (M − 90 of the trimethylsilyl ether of trideutero-5-cholestene-3β,7α-diol). The amplification used for both channels was 300×.

The residue of the ether extract of the incubations with [1-^{14}C]laurate was subjected to thin-layer chromatography using diethyl ether–hexane–acetic acid 2:18:1 (v/v/v) as solvent. The extent of conversion into hydroxylated products was assayed by thin-layer radioscanning as above. Under the chromatographic conditions used, the 11- and 12-hydroxylated products of laurate were obtained in one zone. The ratio between the methyl esters of the 11- and 12-hydroxylated products of laurate was determined with a Barber-Colman radio-gas chromatograph equipped with a 3% QF-1 column (9).

The residue of the chloroform extract of the incubations with 5β-[7β-^{3}H]cholestane-3α,7α-diol was subjected to thin-layer chromatography using ethyl acetate as solvent. The extent of conversion into the hydroxylated products was assayed by thin-layer radioscanning as above. The 25- and 26-hydroxylated products were obtained in the same chromatographic zone. The ratio between the 25- and the 26-hydroxylated products was determined by radio–gas–liquid chromatography of the trimethylsilyl ether using the same instrument as above equipped with a 1.5% SE-30 column (cf. 9, 20). 5β-Cholestane-3α,7α,25-triol has not been previously identified as a product of 5β-cholestane-3α,7α-diol in incubations with homogenate of guinea pig liver (cf. ref. 21). The identity of 5β-cholestane-3α,7α,25-triol was confirmed by mass fragmentography of the trimethylsilyl ether. By this technique it was shown that the compound had the same retention time on gas–liquid chromatography and the same characteristic peaks in the mass spectrum (*m/e* 546 (M − 90), *m/e* 456 (M − 2 × 90), *m/e* 131) as the trimethylsilyl ether of 5β-cholestane-3α,7α,25-triol isolated from incubations of 5β-cholestane,3α,7α-diol with microsomal fraction of human liver homogenate (20) and as recently reported for trimethylsilyl ether of synthetic 5β-cholestane-3α,7α,-25-triol (22). The peak at *m/e* 131 is characteristic for

steroids with a trimethylsilyl function in the 25-position (23).

RESULTS

The extent of conversion of a trace amount of [4-^{14}C]cholesterol into [4-^{14}C]5-cholestene-3β,7α-diol by microsomal fraction from five ascorbate-deficient guinea pigs was less than 0.05%. The corresponding conversion obtained with microsomal fractions from five guinea pigs treated with ascorbate varied from 0.15% to 0.69% (mean ± SEM = 0.28 ± 0.10%). The extent of conversion of endogenous cholesterol into 5-cholestene-3β,7α-diol, as determined by mass fragmentography, was more than ten times higher in incubations with microsomal fraction from guinea pigs treated with ascorbate than in those from the ascorbate-deficient guinea pigs (**Table 1**). In two incubations with microsomal fractions from ascorbate-treated guinea pigs and in two incubations with microsomal fractions from ascorbate-deficient guinea pigs, ascorbate was added to the incubation mixture to give a final concentration of 0.4 mmole/l. The extent of conversion of cholesterol was the same in these incubations as in the corresponding control incubations to which no ascorbate had been added.

11- and 12-Hydroxylation of lauric acid as well as 25- and 26-hydroxylation of 5β-cholestane-3α,7α-diol were about the same in microsomal fractions from ascorbate-treated guinea pigs and ascorbate-deficient guinea pigs (Table 1).

TABLE 1. Hydroxylations catalyzed by microsomal fraction of homogenate of livers from ascorbate-deficient guinea pigs and guinea pigs treated with ascorbate

	Ascorbate-deficient Guinea Pigs	Ascorbate-treated Guinea Pigs	
	nmoles/mg protein/min		t^a
7α-Hydroxylation of endogenous microsomal cholesterol (n = 5)	0.002 ± 0.001[b]	0.030 ± 0.009	3.09[c]
11-Hydroxylation of lauric acid (n = 4)	0.48 ± 0.05	0.59 ± 0.03	1.89
12-Hydroxylation of lauric acid (n = 4)	0.27 ± 0.06	0.35 ± 0.02	1.26
25-Hydroxylation of 5β-cholestane-3α,7α-diol (n = 4)	0.033 ± 0.002	0.032 ± 0.007	0.14
26-Hydroxylation of 5β-cholestane-3α,7α-diol (n = 4)	0.014 ± 0.002	0.015 ± 0.003	0.28

[a] t refers to independent variable of Student's t distribution.
[b] mean ± SEM.
[c] $P < 0.02$.

TABLE 2. Hydroxylations catalyzed by partially purified cytochrome P-450 fraction from an ascorbate-deficient guinea pig and a guinea pig treated with ascorbate in the presence of excess NADPH cytochrome P-450 reductase (1000 units) and a phospholipid (50 μg)

	Ascorbate-deficient Guinea Pig	Ascorbate-treated Guinea Pigs
	pmoles/nmole cytochrome P-450/min	
7α-Hydroxylation of exogenous [4-^{14}C] cholesterol	≤0.1	0.6
11- and 12-Hydroxylation of laurate	110	60

Table 2 summarizes experiments with a partially purified cytochrome P-450 fraction from a deficient guinea pig and an ascorbate-treated guinea pig, respectively. The activity towards laurate as well as cholesterol per nmole cytochrome P-450 was much less than in the original microsomal fraction (Table 1). Significant 7α-hydroxylation was only observed with exogenous 4-^{14}C-labeled cholesterol and the endogenous cholesterol present in the fraction was not hydroxylated. The small amounts of product obtained in the incubations with laurate prevented separation into 11- and 12-hydroxylaurate. The capacity to hydroxylate laurate was about twice higher with cytochrome P-450 from the ascorbate-deficient guinea pig than with cytochrome P-450 from the ascorbate-treated guinea pig. The capacity to 7α-hydroxylate [4-^{14}C]cholesterol was, however, considerably less with cytochrome P-450 from the ascorbate-deficient guinea pig than with cytochrome P-450 from the ascorbate-treated guinea pig.

DISCUSSION

Under various in vivo conditions Ginter et al. (5) found that the biosynthesis of bile acids in scorbutic guinea pigs was reduced by a factor of about two and Guchait et al. (4) found a reduction in cholesterol catabolism in scorbutic guinea pigs which was approximately 10-fold. The ascorbate-deficient guinea pigs used in the present investigation had an elevated concentration of cholesterol in the liver and a much lower cholesterol 7α-hydroxylase activity (about 15 times) than guinea pigs treated with ascorbate. It may be mentioned that according to preliminary experiments, there seems to be little or no difference in capacity to 7α-hydroxylate cholesterol between guinea pigs on ordinary guinea pig diet and guinea pigs further supplemented with ascorbate. It is reasonable to assume that the increased cholesterol concentration in the liver of

the deficient animals at least in part was due to the reduced capacity to 7α-hydroxylate cholesterol. It cannot be completely excluded, however, that the increased hepatic cholesterol could be due to effects on biliary secretion of cholesterol or due to increased mobilization.

The effect of ascorbate on the 7α-hydroxylation of cholesterol is not an effect on the enzyme activity per se since addition of ascorbate to the incubation mixture had no effect on the rate of 7α-hydroxylation, regardless of whether microsomal fraction from deficient guinea pigs or microsomal fraction from guinea pigs treated with ascorbate were used. The decreased cholesterol catabolism in ascorbate-depleted animals might therefore be due to changes in the rate of synthesis or breakdown of the cholesterol 7α-hydroxylating system. The cholesterol 7α-hydroxylating system consists of cytochrome P-450 and NADPH cytochrome P-450 reductase, and it was recently possible to demonstrate 7α-hydroxylation of cholesterol in a reconstituted system from rat liver consisting of partially purified cytochrome P-450 and NADPH-cytochrome P-450 reductase together with a phospholipid (15). The species of cytochrome P-450 involved in 7α-hydroxylation of cholesterol is different from the bulk of cytochrome P-450 in the liver and seems to be responsible for most of the specific properties of the system (15, 24, 25).

It is well established that the bulk of cytochrome P-450 in the liver is decreased in the scorbutic state (26) and in accordance with this the concentration of cytochrome P-450 in the microsomal fraction of the ascorbate-deficient guinea pigs used in the present work was only about half of that of the control animals. It was recently reported that NADPH-cytochrome P-450 reductase also is reduced by a factor of about two in the scorbutic state (27). The relatively small reduction of the bulk of cytochrome P-450 cannot explain the marked reduction in cholesterol 7α-hydroxylase activity.

It could be calculated that the activity per nmole of cytochrome P-450 was reduced more than 5-fold in the deficient guinea pigs. The difference in cholesterol 7α-hydroxylase activity per nmole of cytochrome P-450 also was retained after partial purification of the cytochrome P-450 component from the two different microsomal fractions. In these experiments a great excess of NADPH cytochrome P-450 reductase was added and the concentration of cytochrome P-450 alone should be rate limiting in the system (cf. 15). The cytochrome P-450 fraction had little or no activity towards the endogenous cholesterol remaining in the system after the purification. This is in accordance with the previous work with a reconstituted system from rat liver microsomes in which it was shown that there was a preferential hydroxylation of exogenous cholesterol (15).

In contrast to the marked reduction in cholesterol 7α-hydroxylase activity, other cytochrome P-450 dependent hydroxylations studied in the present work, including 11- and 12-hydroxylation of laurate and 25- and 26-hydroxylation of 5β-cholestane-3α,7α-diol, were affected only to a small extent by the scorbutic state. In the reconstituted system laurate hydroxylase activity per nmole of cytochrome P-450 was even higher with cytochrome P-450 from the ascorbate-deficient animal than with cytochrome P-450 from the ascorbate supplemented guinea pig.

The most reasonable explanation for the above findings appears to be that the ascorbate-deficient state is associated with a more or less selective reduction of the specific type of cytochrome P-450 involved in 7α-hydroxylation of cholesterol. Whether this reduction is due to an effect of ascorbate on synthesis or degradation of the cytochrome P-450 remains to be established.

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