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Cholesterol oxidation in a heterogeneous system initiated by water-soluble radicals

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Generation of C-7 allylic cholesterol oxidation products was followed in a heterogeneous system with cholesterol solubilized in Tween-20 micelles in water. Initiation of oxidation by the water-soluble free radical initiator 2,2′-azobis(2-amidinopropan) dihydrochloride (AAPH) lead to accumulation of 7-ketocholesterol as the dominant oxysterol. The isomeric forms of 7-hydroxycholesterol which were also formed, although in minor amounts, were found to dehydrogenate to 7-ketocholesterol through a two-step radical reaction. 7α-hydroxycholesterol, which was found in higher concentration than 7β-hydroxycholesterol, was dehydrogenated in the oxidating system to 7-ketocholesterol at a rate approximately half of that for 7β-hydroxycholesterol. The higher rate of dehydrogenation of 7β-hydroxycholesterol may be explained by a more direct exposure of the β-surface of the sterol to the water phase and thus a more direct contact between the hydroxy group and the free radicals generated in the water phase. Copyright © 1996 Elsevier Science Ltd.

INTRODUCTION

7-Ketocholesterol (VI in Fig. 1) and the isomeric forms of 7-hydroxycholesterol (IV and V) seem to be the dominant species of cholesterol oxidation products in dairy products (Nielsen *et al.*, 1995). Both 7-ketocholesterol and the isomeric forms of 7-hydroxycholesterol are found to inhibit HMG-CoA reductase resulting in a insufficient synthesis of cholesterol and a general malfunction of the cell. The relatively high concentration of 7-ketocholesterol compared to other oxidation products found in dairy products (Nielsen *et al.*, 1995) makes the 7-ketocholesterol a useful indicator of cholesterol oxidation. The primary oxidation products, 7α-hydroperoxycholesterol (II) and 7β-hydroperoxycholesterol (III) have been shown to be reduced to 7α-hydroxycholesterol (IV) and 7β-hydroxycholesterol (V), respectively, or thermally dehydrated to 7-ketocholesterol (Smith *et al.*, 1973; Teng *et al.*, 1973). However, other mechanisms may also be operating in relation to further oxidation of the isomeric 7-hydroxycholesterol in order to account for the rather specific accumulation of 7-ketocholesterol. In order to simulate the conditions in a peroxidating biphase food system, we have undertaken an investigation in a model system of cholesterol or 7-hydroxycholesterol solubilized by Tween-20 in water in the presence of a water-soluble free radical initiator. A possible demonstration of a free-radical-initiated oxidation at the allylic C-7 of, not only cholesterol, but also of 7-hydroxycholesterol, would facilitate the development of rapid screening methods for cholesterol oxidation in dairy products.

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MATERIALS AND METHODS

Chemicals

Cholest-5-ene-3β-ol (cholesterol), 5,6α-epoxy-5α-cholestan-3β-ol (cholesterol-5α,6α-epoxide), 3β-hydroxy-cholest-5-en-7-one (7-ketocholesterol), cholest-5-ene-3β,7β-diol (7β-hydroxy-cholesterol), cholestane-3β,5α,6β-triol (cholestanetriol), all of more than 99% purity, and polyoxyethylenesorbitanmonolaurate (Tween 20), were purchased from Sigma (St. Louis, MO, USA). Cholest-5-ene-3β,7α-diol (7α-hydroxycholesterol) of more than 99% purity and 5,6β-epoxy-5β-cholestan-3β-ol (cholesterol-5β,6β-epoxide) were obtained from Steraloids Inc. (Wilton, NH, USA). The cholesterol-5β,6β-epoxide was purified by HPLC before use. 2,2′-Azobis(2-amidinopropan) dihydrochloride (AAPH) was purchased from Polyscience Inc. (Warrington, PA, USA). Methanol, hexane, heptane, acetone and 2-propanol, all of analytical grade, were from E. Merck (Darmstadt, Germany) and were used without further purification. $^{18}O_2$, 95–98% pure, for isotope labelling experiments

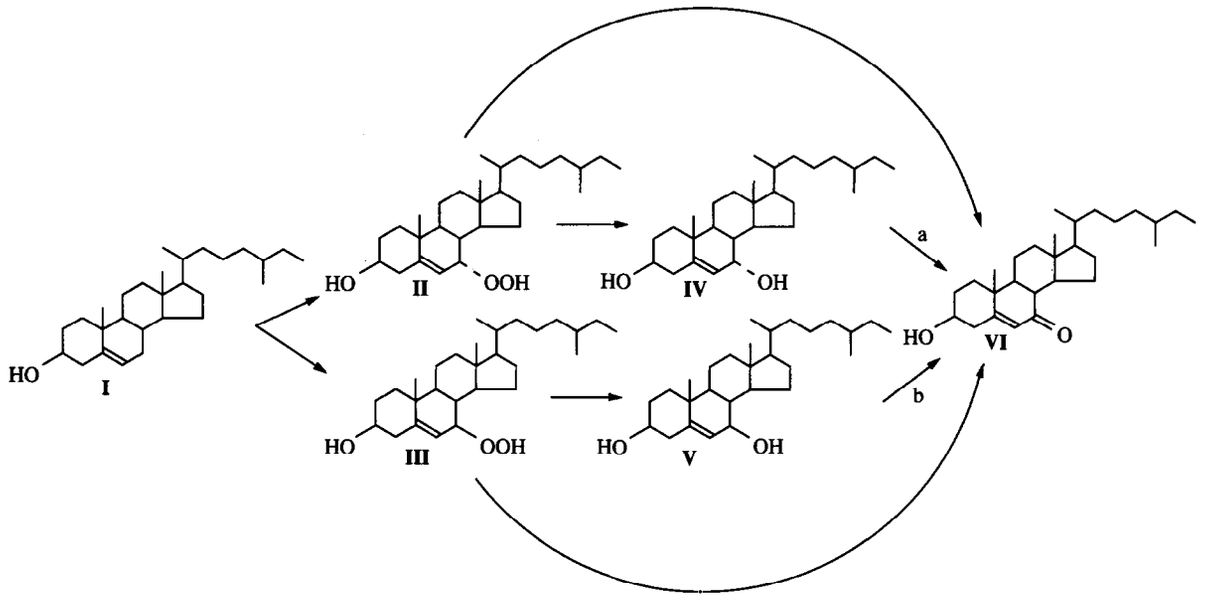

Fig. 1. I, Cholesterol oxidation normally initiates at the allylic C-7; II, 7α-hydroperoxycholesterol; III, 7β-hydroperoxycholesterol; IV, 7α-hydroxycholesterol; V, 7β-hydroxycholesterol; and VI, 7-ketocholesterol.

was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Prior to GC separation, the oxysterols were derivatized by Sylon BTZ (N,O-bis(trimethylsilyl)-acetamide–trimethylchlorosilane–trimethylsilylimidazole, 3:2:3)from Supelco Inc.(Bellefonte, PA, USA).

Analytical equipment

An HP series 1050 liquid chromatographic system with HP 1047 refractive index detector was used for preparative HPLC separation with a LiChrospher 100 CN 5 μm 250×4 mm column (Hewlett-Packard Co., Palo Alto, CA, USA). Separation of the oxysterols and quantification was executed on an HP 5890 series II gas chromatograph with a flame ionization detector (Hewlett-Packard Co.). The analysis of the isotope experiment with $^{18}O_2$ and identification of the cholesterol oxidation products was verified using a HP 5890A gas chromatograph (Hewlett-Packard Co.) connected with a VG70-250SE mass spectrometer (VG Analytical, Manchester, UK) using a Chrompack CP-SIL 5CB (25 m × 0.25 mm × 0.25 μm) obtained from Chrompack (Middelburg, The Netherlands).

Heterogeneous system

Cholesterol, 7β-hydroxycholesterol and 7α-hydroxycholesterol were separately dissolved in heptan–2-propanol (95:5 v/v). Tween-20 was dissolved in methanol and AAPH was dissolved in phosphate buffer at pH 5.85. The appropriate sterol and Tween-20 solutions were mixed gently in a reaction flask and the mixture was evaporated to dryness with N_2. The residue was dissolved in a small amount of phosphate buffer and shaken gently to generate a heterogeneous system. The AAPH solution was added, and the mixture was sonicated for 1 min. The final concentrations in the heterogeneous water system were: cholesterol, 230 μM; 7β- and 7α-hydroxycholesterol, 22.5 μM; Tween-20, 1.63 mM; AAPH, 11 mM. The reaction flask was placed in a thermostatted water bath at 37°C. N_2 was used to degas the solvent.

Extraction and enrichment of oxysterols

After incubation, samples were transferred to a −35°C solution of heptan–2-propanol which stopped further generation of radicals. The mixture was shaken vigorously for 1 min and the oxysterols were extracted. To increase resolution of oxysterols, unoxidized cholesterol was separated from the mixture prior to quantification by GC–FID. The preparative HPLC separation of cholesterol and oxysterols was performed by a CN-column with hexan–2-propanol (95:5 v/v) as mobile phase and a flow rate of 0.4 ml/min.

Quantification of oxysterols

Prior to the separation step, the effluent oxysterol solution from the HPLC was dried in a vacuum evaporator. The oxysterols were derivatized to their trimethylsilyl (TMS) derivatives by dissolution in Sylon BTZ and heating at 60°C for 60 min. Analysis of the derivatized oxysterols was carried out by gas chromatography using an HP-Ultra1 column and helium as carrier gas. Injection was performed in splitless mode at an injector temperature of 300°C and a flow of 1.2 ml/min, which was held for 1 min. The flow was changed to 0.6 ml/min for the rest of the run. Initial column temperature was 50°C, which was held for 1 min. The temperature was raised to 270°C at a rate of 30°C/min. and further changed at a rate of 0.5°C/min to 285°C which was held for 0.5 min. The detection was performed by a flame ionization detector. Verification of the formed oxysterols and check for the incorporation of $^{18}O_2$ were performed using GC–MS recording full spectra.

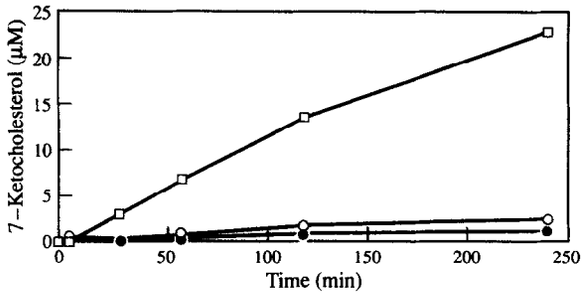

Fig. 2. Formation of C-7 allylic oxysterols in the heterogeneous water-based food model system with cholesterol (230 μM) oxidizing after initiation by AAPH. (○) 7α-hydroxycholesterol; (●) 7β-hydroxycholesterol; and (□) 7-ketocholesterol. At the end of the experiment approximately 5% of the cholesterol was oxidized.

RESULTS

Oxidation of cholesterol by water-soluble radicals in a heterogeneous system

Two hundred μM cholesterol was oxidized in a heterogeneous system of phosphate buffer (pH 5.85), Tween-20 and AAPH. Besides 7-ketocholesterol and the isomeric forms of 7-hydroxycholesterol, cholesterol-5α,6α-epoxide and cholesterol-5β,6β-epoxide were found in low concentrations. Figure 2 shows the accumulation of allylic oxidation products during incubation with AAPH. Accumulation of 7α-hydroxycholesterol was higher than the accumulation of 7β-hydroxycholesterol and, at the end of the incubation period, the ratio 7α/7β-hydroxycholesterol was 2.2. Moreover the dominant oxidation product was 7-ketocholesterol which was formed in much higher concentration than the 7-hydroxycholesterol.

Dehydrogenation of 7α- and 7β-hydroxycholesterol to 7-ketocholesterol

7α- and 7β-hydroxycholesterol were separately incubated with AAPH in a heterogeneous solution, and the generated products were identified and quantified by HPLC. The only detected product by FID-detection was 7-ketocholesterol, a result confirmed by mass spectrometry. Figure 3 shows the concentrations of 7-ketocholesterol generated from the isomeric 7-hydroxycholesterols in the presence of AAPH. For the present reaction conditions 7β-hydroxycholesterol was transformed to 7-ketocholesterol at a rate of 0.069 μmol/min and 7α-hydroxycholesterol was transformed to 7-ketocholesterol at a rate of 0.038 μmol/min. Dehydrogenation of 7-hydroxycholesterol to 7-ketocholesterol in the absence of a free radical initiator could not be detected, as may be seen from the results in Fig. 4 for the more labile β-isomer. When the oxygen pressure in the solution was reduced by degassing with N_2, it was found that the 7β-hydroxycholesterol was dehydrogenated to 7-ketocholesterol at a lower rate, as may be seen from Fig. 5, and it could be concluded that oxygen was participating in the dehydrogenation of 7-hydroxycholesterol to 7-ketocholesterol. To further investigate the role of free oxygen dissolved in the heterogeneous system on the conversion, the system with 7β-hydroxycholesterol was saturated with $^{18}O_2$. The mass spectrum of 7-ketocholesterol produced under these conditions was identical to that of 7-ketocholesterol produced without $^{18}O_2$. Thus it can be concluded that free oxygen is not incorporated during the dehydrogenation process.

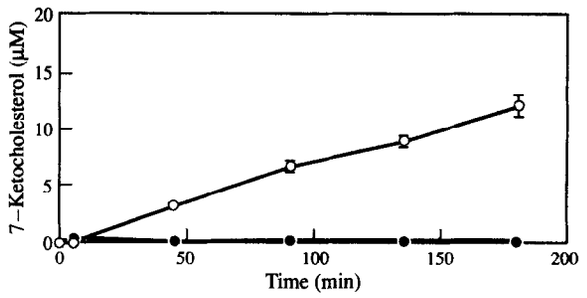

Fig. 4. Generation of 7-ketocholesterol from 7β-hydroxycholesterol in a heterogeneous aqueous system exposed to (○) AAPH ($n=3$) or without (●) AAPH.

DISCUSSION

As may be seen from Fig. 2, 7-ketocholesterol accumulated as the dominating oxysterol when oxidation of

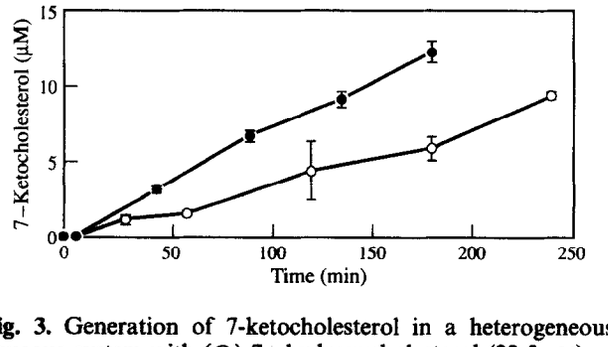

Fig. 3. Generation of 7-ketocholesterol in a heterogeneous aqueous system with (○) 7α-hydroxycholesterol (22.5 μM) or (●) 7β-hydroxycholesterol (22.5 μM). The oxysterols were exposed to AAPH. Average ± SD ($n=3$).

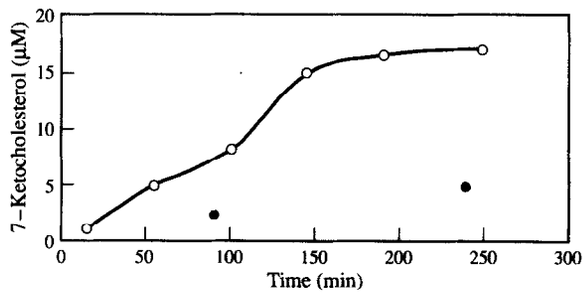

Fig. 5. Generation of 7-ketocholesterol from 7β-hydroxycholesterol in a heterogeneous aqueous system exposed to AAPH, (○) saturated by O_2 or (●) degassed with N_2.

cholesterol was initiated by water-soluble carbon-centred radicals in a heterogeneous water-based food model system. This observation is in accordance with the results found for various dairy products, for which 7-ketocholesterol appears to be an indicator of cholesterol oxidation (Nielsen *et al.*, 1995). It was further found that 7α-hydroxycholesterol is formed in a higher concentration than 7β-hydroxycholesterol. Based on this observation alone, it is not possible to conclude whether cholesterol is oxidized more easily to 7α-hydroxycholesterol than to 7β-hydroxycholesterol or whether 7α-hydroperoxycholesterol, as a reaction intermediate, is dehydrated to 7-ketocholesterol with a lower rate than 7β-hydroperoxycholesterol, cf. Fig. 1. From the results of Fig. 3 it may, however, be seen that both 7α- and 7β-hydroxycholesterol are dehydrogenated to 7-ketocholesterol when exposed to a carbon-centred radical in the two-phase system. 7β-hydroxycholesterol is, moreover, dehydrogenated to 7-ketocholesterol at a higher rate (0.069 μmol/min for the present conditions) compared to dehydrogenation of 7α-hydroxycholesterol (0.038 μmol/min for the present conditions). It is accordingly supposed that the higher concentration of 7α-hydroxycholesterol compared to 7β-hydroxycholesterol during cholesterol oxidation is kinetically controlled; one possible explanation is higher reactivity of the β-isomer in the presence of water-soluble radicals. The observation that the isomeric forms of 7-hydroxycholesterol are also precursors for 7-ketocholesterol in cholesterol oxidation is important for future use of 7-ketocholesterol as a general indicator of cholesterol oxidation. Direct transformation of the hydroxy forms has not been previously reported, but enzymatic dehydrogenation of 7α-hydroxycholesterol to 7-ketocholesterol has been observed in a microsomal fraction of liver (Bjorkhem *et al.*, 1968; Cantfort, 1972).

The higher rate of dehydrogenation of 7β-hydroxycholesterol compared to 7α-hydroxycholesterol to yield 7-ketocholesterol may be explained by the more exposed β-surfaces of the sterol molecule giving better contact with the water phase. While the two hydroxy groups in the α-isomer cannot be exposed simultaneously to water, the β-surface of the sterol allows the diols to face both the hydroxy groups to the water-soluble radicals.

7β-Hydroxycholesterol is not dehydrogenated to 7-ketocholesterol in the absence of AAPH as may be seen from Fig. 4. This indicates that carbon-centred radicals are involved at some stage in the reaction sequence from 7β-hydroxycholesterol to 7-ketocholesterol. Experiments in deoxygenated systems clearly showed a decreased rate for dehydrogenation of 7β-hydroxycholesterol to 7-ketocholesterol. Dissolved oxygen is accordingly also important for the dehydrogenation reaction, but an experiment with $^{18}O_2$ showed that dissolved oxygen is not incorporated in the formed 7-ketocholesterol.

Depending on the oxygen pressure, the carbon-centred radicals formed by thermal cleavage of AAPH

$$\text{AAPH} \rightarrow \text{R}\cdot + \text{R}\cdot + \text{N}_2 \qquad (1)$$

may recombine in a solvent cage

$$\text{R}\cdot + \text{R}\cdot \rightarrow \text{RR(non-radical product)} \qquad (2)$$

or react in a diffusion-controlled reaction with oxygen

$$\text{R}\cdot + \text{O}_2 \rightarrow \text{ROO}\cdot \qquad (3)$$

The peroxyl radical may abstract an hydrogen from C-7

$$\text{ROO}\cdot + \text{H} - \text{C(7)} - \text{OH} \rightarrow \text{ROOH} + \cdot\text{C(7)} - \text{OH} \qquad (4)$$

The hydroperoxy compound may be cleaved at the phase-boundary of the micelles

$$\text{ROOH} \rightarrow \text{RO}\cdot + \cdot\text{OH} \qquad (5)$$

and the hydroxyl radical will in a concerted reaction abstract the hydrogen from the 7-hydroxy group

$$\cdot\text{C(7)} - \text{OH} + \cdot\text{OH} \rightarrow \text{C(7)} = \text{O} + \text{H}_2\text{O} \qquad (6)$$

Although speculative, the suggested mechanisms do account for the observations that dissolved oxygen is increasing the rate of conversion without being incorporated into the 7-ketocholesterol. An intermediate formation of 7-hydroperoxycholesterol is expected to result in incorporation of dissolved oxygen in the final product, 7-ketocholesterol. An alternative mechanism could involve a reaction sequence with two free radicals with an enol as reaction intermediate.

In conclusion, the model study of the oxidation of cholesterol to the allylic oxidation products showed that 7-ketocholesterol was not only a product of dehydration of the isomeric forms of 7-hydroperoxycholesterol, but also a product of dehydrogenation of the isomeric forms of 7-hydroxycholesterol. Accordingly lipid peroxyl radicals formed in foods or in biological systems appear to be at least partly responsible for the generation of 7-ketocholesterol from the isomeric forms of 7-hydroxycholesterol. However, when lipid peroxyl radicals, rather than radicals generated in the aqueous phase, are involved in the formation of 7-ketocholesterol, the α-surface of the sterol exposed to the interior and apolar environment of the heterogeneous system, is more vulnerable to radical attack compared to the β-surface, and the ratio between 7α-hydroxycholesterol and 7β-hydroxycholesterol is expected to change in favour of the β-isomer.

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