THE REVERSIBLE INHIBITION OF β-GLUCURONIDASE BY ORGANIC PEROXIDES

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SUMMARY

Contrary to previously published results, β-glucuronidase is not inhibited by cholesterol and retinol, but by the hydroperoxides formed during the autoxidation of these and other lipids. 25-Hydroperoxycholest-5-en-3 β-ol, isolated from aged cholesterol preparations, and various alkyl hydroperoxides are highly inhibitory. β-Glucuronidase was inhibited 50% by 1.2 μM linoleic acid hydroperoxide. Dilution of the enzyme-inhibitor mixture reverses the inhibition thus ruling out oxidation of essential groups on the enzyme by the peroxides. The results of this study show that the relationship of cholesterol to atherosclerosis is not explained by its action on β-glucuronidase as had been previously suggested.

The inhibition of β-glucuronidase (β-D-glucuronide glucuronohydrolase, EC 3. 2.1.31) by cholesterol, retinol and a variety of other lipids has been reported by Tappel and Dillard (1) and Romeo and Panfili (2). Since these were the first reports of specific effects of cholesterol on an enzyme, this finding was of considerable importance. However, in attempting to investigate the mechanism of this inhibition, we have found that, contrary to results obtained by others, pure cholesterol has no inhibitory activity. It was subsequently discovered that the inhibitor could be generated by exposing pure cholesterol to ultraviolet light. This observation coupled with the fact that all inhibitory activity could be destroyed by treatment with sodium borohydride, iodide, or sulfite, strongly suggested that a peroxide was the active ingredient. The finding that products of various autoxidized lipids, as well as numerous organic peroxides, inhibit bovine liver β-glucuronidase is reported in this communication.

MATERIALS AND METHODS

Autoxidized cholesterol was obtained from a sample of commercial cholesterol which had been stored at room temperature in an amber-colored bottle for several years. 25-Hydroperoxycholest-5-en-3 β-ol was isolated from autoxidized cholesterol and identified by the methods of Van Lier and Smith (3). The compound, recrystallized from benzene, had a melting point of 165-167° and gave the expected carbon and hydrogen analyses. The identification was confirmed by reducing the hydroperoxide with sodium borohydride to give cholest-5-en-3β ,25-diol [m.p. 180-181.5° (lit. value 180°)] which was then reacted with acetic anhydride in pyridine to give the 3 β-acetoxy derivative [m.p. 140-141° (lit. value 140-141°)]. 7 α-Hydroperoxycholest-5-en-3β -ol and 3 β-hydroxy-cholest-6-en-5 α-hydroperoxide were prepared by the methods of Schenck and Neumuller (4).

Linoleic acid was purified by partition between petroleum ether and 75 per cent aqueous methanol (5). Linoleic acid hydroperoxide was isolated from linoleic acid that had been previously autoxidized eight hours at 50° by the method of Frankel, et al. (6). The analyses by titration, iodometry (7), and ultraviolet light absorption (5) agreed within 95 per cent. After thin-layer chromatography on silica gel with petroleum ether: diethyl ether: glacial acetic acid (60:40:1, v/v) as the solvent system, only one spot (R_f = 0.24) was obtained upon spraying with peroxide reagent (8) or 50 per cent sulfuric acid indicating that breakdown products such as aldehydes were absent.

All-trans-retinol, obtained from the Sigma Chemical Company, was purified by preparative thin-layer chromatography on silica gel with benzene: methanol (90:10, v/v) as the solvent. The retinol (R_f = 0.3) was identified and quantitated by its ultraviolet light absorption.

Oleic acid was used as obtained from the Sigma Chemical Company. Olive oil, obtained from the Fisher Scientific Company, and peanut oil (a household product) were used without further purification. Autoxidation of the oleic acid and the oils was carried out at 100° for 12 hours. Retinol was autoxidized in visible light at room temperature for 12 hours.

Cumene hydroperoxide was purified by vacuum distillation. The remainder of the organic peroxides were used as obtained from the Hercules Powder Company or the Lucidol Division of the Penwalt Corporation.

β-Glucuronidase activity was assayed with phenolphthalein glucuronide as the substrate. The assay solution contained 66 mM sodium acetate buffer, pH 5.0, 1 mM phenolphthalein-β-D-glucuronide (Pierce Chemical Company), 100 ug of bovine

Table I

Inhibition of β-Glucuronidase by Various Lipids

Compound	Concentration μM	Inhibition %
Cholesterol	415	0
Autoxidized cholesterol	135	50
25-Hydroperoxycholest-5-en-3 β-ol	20	40
7 α-Hydroperoxycholest-5-en-3 β-ol	476	50
3 β-Hydroxy-cholest-6-en-5 α-hydroperoxide	476	8
Retinol	344	0
Autoxidized retinol	92	50
Oleic acid	715	0
Autoxidized oleic acid	67	50
Linoleic acid	770	50
Linoleic acid hydroperoxide	1.2	50
	mg/ml	
Olive oil	0.2	0
Autoxidized olive oil	0.2	8
Peanut oil	0.2	0
Autoxidized peanut oil	0.05	50

The inhibition was determined at a series of inhibitor concentrations and the level required to give 50 per cent inhibition was obtained by interpolation. Inhibitions of less than 50 per cent correspond to the highest concentration at which the inhibitor could be tested due to limits of solubility.

serum albumin, 30 μg of β-glucuronidase (Sigma Chemical Company, Type B-3), and the inhibitor dissolved in 10 μl of ethanol. The enzyme was added last and the mixture was incubated for 15 minutes at 37°. The total reaction volume was 0.25 ml. The reaction was stopped by the addition of 1.0 ml of alkalinizing reagent which consisted of 0.133 M glycine and 0.088 M Na_2CO_3 adjusted to pH 10.7 with NaOH. The amount of phenolphthalein released was found by determining the optical density at 550 nm. The control contained 10 μl of ethanol.

RESULTS AND DISCUSSION

Table I shows the inhibitory effect of various lipids on β-glucuronidase. Of the cholesterol derivatives, it appears that the 25-hydroperoxide is much more accessible to the enzyme than either the 7 α- or 5 α-hydroperoxides. 25-Hydroperoxycholesterol is very insoluble in the assay mixture. Therefore, higher concentrations could not be tested. The alcohols derived by reduction of the cholesterol hydroperoxides are not inhibitory. As shown by preparative thin-layer chromatography, autoxidized cholesterol contains numerous unidentified peroxides which inhibit β -glucuronidase. Retinol and oleic acid are inhibitory only upon being subjected to autoxidation conditions. Although we were unable to prepare linoleic acid devoid of inhibitory activity, completely pure linoleic acid most likely would not be inhibitory in view of the results obtained with oleic acid. The extent of inhibition obtained with autoxidized vegetable oils appears to depend on the content of unsaturated fatty acid components. We were unable to obtain any inhibition by serum cholesterol added either as the serum solution or after extraction with isopropanol. It is possible that the artificial serum standards used by Tappel and Dillard (1) contained autoxidized cholesterol.

Appropriate control experiments showed that the peroxides do not affect the development of color by phenolphthalein.

A variety of peroxides, including acyl-peroxides (Table II), also show inhibitory activity, but di-tert-butyl peroxide is completely inactive. The lipid solubility of the peroxides may be important in the inhibition mechanism since the more water-soluble t-butyl hydroperoxide and hydrogen peroxide are much less inhibitory

Table II

Inhibition of β-Glucuronidase by Organic Peroxides

Compound	Concentration μM	Inhibition %
Diisopropyl benzene hydroperoxide	43.7	50
Benzoyl peroxide	52.5	50
p-Menthane hydroperoxide	71.6	50
Cumene hydroperoxide	115	50
2,5-Dimethyl-hexane-2,5-dihydroperoxide	442	50
t-Butyl perbenzoate	734	27
t-Butyl hydroperoxide	2260	50
Hydrogen peroxide	14100	15
Di-tert-butyl peroxide	4520	0

All peroxide concentrations are based on iodometric analysis (9) with the exception of di-tert-butyl peroxide concentration which was established by weight.

than the compounds that are less water-soluble. However, only a portion of the inhibitor needs to be lipophilic since the relatively water-soluble linoleic acid hydroperoxide was the most inhibitory compound tested.

Tappel and Dillard (1) noted that the inhibition of β-glucuronidase was reversible upon dilution. Similarly, we have found that the cumene hydroperoxide inhibition of β-glucuronidase is reversed by dilution of the reaction mixture (Table III). Therefore, the interaction of the peroxide and the enzyme probably does not result in protein aggregation (10) or sulfhydryl oxidation (11, 5) as has been described by other investigators. The involvement of aldehyde products of autoxidation as described by Chio and Tappel (12) has been ruled out by the reversible nature of the inhibition as well as by the use of pure hydroperoxides. The exact mechanism of inhibition is now under investigation.

β-Glucuronidase plays a role in the catabolism of mucopolysaccharides (13)

Table III

Reversibility of Cumene Hydroperoxide Inhibition of β-Glucuronidase on Dilution

	Pre-Assay Mixture		Assay Mixture			
Experiment	Enzyme μg	Inhibitor μM	Enzyme μg	Inhibitor μM	O.D.$_{550}$	Inhibition %
1	40	0	40	0	0.637	
2	4.0	0	4.0	0	0.067	
3	40	160	40	148	0.325	49.0
4	4.0	160	4.0	148	0.032	52.3
5	40	16.0	40	14.8	0.575	9.7
6	4.0	16.0	4.0	14.8	0.062	8.1
7	40	160	4.0	14.8	0.062	8.1

The pre-assay mixture contained 66 mM sodium acetate buffer, pH 5.0, 100 μg of bovine serum albumin, the indicated amount of β-glucuronidase, and the indicated amount of inhibitor (in 10 μl of ethanol) in a final volume of 0.25 ml. The mixture was allowed to stand ten minutes at room temperature after addition of the inhibitor. In experiments 1 to 6, 20 μl of 12.5 mM phenolphthalein glucuronide was added to the pre-assay mixture to yield the assay mixture which was incubated at 37° for 15 minutes. In the last experiment, 25 μl of the pre-assay mixture was withdrawn and diluted ten fold in a solution containing the same concentrations of buffer, ethanol and bovine serum albumin. Substrate was added and the mixture incubated as above.

and therefore could be involved in the development of atherosclerosis as has been implied by the work of other investigators (14). It has been postulated (1) that the primary effect of cholesterol in the development of arterial lesions is on β-glucuronidase. As shown in the present communication, this effect would require the formation of the hydroperoxide or peroxide. However, cholesterol hydroperoxides are formed very slowly and it is much more likely that if any type of lipid has an effect on β-glucuronidase it is the much more easily autoxidizable unsaturated fatty acids.

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