Enzyme inhibition by secondary lipid autoxidation products from fish oil

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A series of secondary lipid autoxidation products (hydrocarbons, alcohols, aldehydes, ketones, and carboxylic acids), which have been found in oxidized fish oil, were tested for a possible effect on the in vitro activity of microsomal glucose 6-phosphatase. It was found that only those compounds that possess a carbon-carbon double bond conjugated to an oxo-group (aldehyde or ketone) exerted an appreciable inhibition. Among the homologous trans-*2-alkenals, the inhibitory effect increased with chain lengths from C5 to C9, and* trans-*2-nonenal was the most potent inhibitor of the compounds tested. This dependence on molecular size is not explainable solely by differences in partition coefficients between the incubation medium and the membrane. It is concluded that the inhibition of microsomal glucose 6-phosphatase by secondary autoxidation products requires covalent binding to the enzyme (probably to an SH-group) and, moreover, involves perturbation of the lipid phase of the membrane. The enzyme system may be of use in determining the presence of potentially toxic oxidation products in fish lipids.*

Keywords: glucose 6-phosphatase; *trans*-nonenal; microsomes; lipid peroxidation

Introduction

The growing interest in the nutritional and pharmacological significance of long-chain polyunsaturated fatty acids (PUFA) of marine origin[1] has concomitantly given rise to concern about the potential risk of an increased oxidative stress following ingestion of large amounts of fish oil.[2] Thus, autoxidation of highly unsaturated fatty acids leads to formation of compounds that, if absorbed in the intestine, may have adverse effects on cellular systems. Moreover, the intake of purified, non-oxidized polyunsaturated fatty acids results in an increased deposition and risk of in vivo peroxidation.[3–8]

Induced autoxidation in suspensions of microsomes has been used to study the initiation mechanisms and the possible involvement of enzyme systems (e.g., cytochrome P450, lipoxygenase, cyclooxygenase) in this process.[9–13] Also, the influence of induced autoxidation on the activity of some membrane-bound enzymes such as glucose 6-phosphatase has been investigated.[14–17] The formation of covalent complexes between microsomal structures and secondary oxidation products with free carbonyl groups is well established and has been proposed as an explanation of the cytotoxicity of 4-hydroxynonenal and other hydroxyalkenals.[18,19]

In the present paper, we describe an in vitro test system for the presence and potential toxicity of secondary autoxidation products with focus on substances identified in oxidized fish oil.[20,21] The system is based on glucose 6-phosphatase[22] from rat liver endoplasmic reticulum, an enzyme whose activity is modulated by covalent modification of important functional groups as well as by changes in the surrounding phospholipid phase. It was found that both of these phenomena probably play a role in the toxic effects of autoxidation products and that the α,β-unsaturated aldehydes and ketones were the only active compounds. Earlier findings regarding the inhibitory effect of 4-hydroxyalkenals are in accordance with the present results.

Methods and materials

Preparation of microsomes

Livers from laboratory rats that had been fed a standard pellet (Altromin 1324, Chr. Petersen, Ringsted, Denmark) diet were homogenized in a medium containing 250 mmol/

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L sucrose, 1 mmol/L Tris, and 0.1 mmol/L EDTA, pH, 7.0, in a Potter-Elvehjem homogenizer with a loose-fitting pestle. The homogenate was centrifuged (4° C, 15 min, g_{av} = 11,000g) in a Sorvall RC-5B centrifuge equipped with an SS-34 rotor (Du Pont Company, Wilmington, DE USA). The supernatant was further centrifuged (4° C, 65 min, g_{av} = 90,000g) in a Beckman L8-70M ultracentrifuge (70 TI rotor; Beckman Instruments, Palo Alto, CA USA) and the resultant pellet was resuspended in incubation buffer (50 mmol/L Tris, 50 mmol/L maleate, 150 mmol/L KCl; pH, 7.4) to a volume of 1 mL per g liver, making the protein concentration approximately 15–20 mg/mL. The preparation could be stored at −30° C for more than a month without measurable loss of glucose 6-phosphatase activity.

Incubation with test substance

Stock solutions (1–2 mmol/L) of test substances in incubation buffer were prepared in 100 mL measuring flasks. Four volumes of stock solution, diluted with buffer to the appropriate concentration, were mixed with one volume of microsomes and gently shaken for 15 min at 37° C in sealed bottles.

In experiments where a partially purified enzyme preparation was substituted for microsomes, 0.3 U/mL was used in the incubation mixture.

Enzyme assay

500 μL assay buffer (50 mmol/L maleate, 4.6 mmol/L EDTA, 2.3 mmol/L NaF; pH = 6.0) were mixed with 100 μL 200 mmol/L glucose 6-phosphate (pH, 6.0) in a 15 mL polycarbonate tube (Sorvall, Norwalk, CT USA). The enzymatic reaction (at 37° C) was started by adding 100 μL incubation mixture and terminated after an appropriate time (usually 20 min) by addition of 1.3 mL 100 g/L trichloroacetic acid. The precipitated protein was settled by centrifugation at 12,000 rpm for 10 min in a Sorvall SM-24 rotor (4° C) and 1,000 μL was taken for phosphate analysis. Blank assays were performed by addition of acid prior to enzyme.

Determination of phosphate

The amount of phosphate released in the assay was determined essentially as described by Fiske and Subbarow.[23] One thousand μL sample was mixed with 2,000 μL water, 1,000 μL "molybdate reagent" (1.36 vol concentrated sulfuric acid, 3 vol water, 5 vol 50 g/L hexaammonium molybdate; diluted to 10 vol with water) and 1,000 μL "reducing reagent" [30 g/L sodium disulfite, 10 g/L 4-(methylamino)-phenol sulfate (Photo-Rex, Merck, Darmstadt, Germany)]. Absorbance at 660 nm was read after no less than 40 min.

Determination of protein

The protein content of the microsomal preparations was estimated by the method of Lowry et al.[24] with bovine serum albumin as standard.

Reagents

The test substances *(Table 1)* were purchased from Aldrich-Chemie, Heindenheim, Germany, in the highest grade available (typically 93–99%) and used without further purification. Glucose 6-phosphate (analytical grade) and a crude preparation of partially purified glucose 6-phosphatase were obtained from Sigma Chemical Company, St. Louis, MO USA. Buffer reagents were of the highest quality commercially available.

Table 1 List of the substances tested

	a	b
Butanal	n.r.	n.r.
Pentanal	360	1,070
Trans-2-pentenal	930	660
1-Penten-3-one	240	n.r.
1-Pentanol	n.r.	4,100
Trans-2-hexenal	540	n.r.
Trans-2-hexene	n.r.	n.r.
Trans-2-hexenoic acid	n.r.	n.r.
Trans-2-hexen-1-ol	n.r.	n.r.
Trans,trans-2,4-hexadienal	100	n.r.
Heptanal	410	420
Trans-2-heptenal	140	400
Cis-4-heptenal	n.r.	2,000
Trans,trans-2,4-heptadienal	2,180	2,200
3-Heptanone	8,530	n.r.
Octanal	240	2,750
Trans-2-octenal	280	320
Nonanal	500	520
Trans-2-nonenal	n.r.	380
2-Nonanone	620	530
Decanal	640	70
Trans,trans-2,4-decadienal	n.r.	50
Pentadecane	520	n.r.

[a]Content (ng/g) of a non-refined menhaden oil.[20]
[b]Content (ng/g) of a grade 1 cod liver oil.[21]
N.R., not reported.

The homogeneity of the stock solutions of some of the aldehydes and ketones, which are only slightly soluble in the incubation buffer, was tested by direct absorption spectrometry at 295 nm and by thin layer chromatography of dinitrophenyl-hydrazone derivatives.

Results

A variety of aldehydes and related substances, most of which have been identified as lipid autoxidation products in fish oils *(Table 1)*, were obtained as pure chemicals and tested for inhibitory effect on microsomal glucose 6-phosphatase. In the test system, the microsomes were incubated with the potential inhibitor prior to assay of enzymatic activity to obtain optimal protein and inhibitor concentrations. The reaction between enzyme and inhibitor was rather slow even with *trans*-2-nonenal, the most potent of the substances tested *(Figure 1)*. The slow inactivation without added inhibitor was first order, but the reaction with aldehyde was apparently biphasic. This behavior was also seen with the shorter-chain alkenals, *trans*-2-heptenal and *trans*-2-hexenal. The initial rate of inhibition, as well as the time span in which the primary phase of the process is dominating, depended on inhibitor type and concentration. Fifteen minutes of incubation was, however, a satisfactory choice that balanced the need for an appreciable response with an optimal concentration dependence.

The substances listed in *Table 1* possess several functional parts: the alkenals, as an example, contain an aldehyde group, a *trans*-C=C double bond, and a hydrophobic aliphatic chain. The importance of the

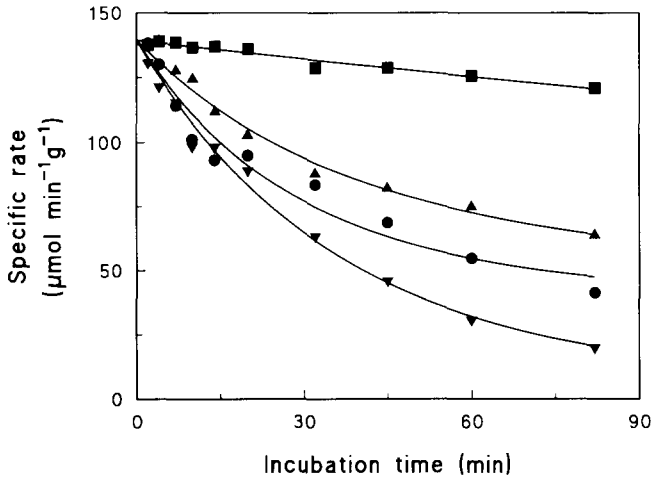

Figure 1 Time course of the inhibition of glucose 6-phosphatase. Microsomes were incubated with *trans*-2-nonenal at 37° C for the time indicated after which the rate of glucose 6-phosphate hydrolysis was determined. The concentration of aldehyde during incubation was: ■, no aldehyde; ▲, 0.3 mmol/L; ●, 0.6 mmol/L; ▼, 1.0 mmol/L.

various functional moieties with regard to inhibitory effect on microsomal glucose 6-phosphatase was tested by comparing several compounds that differed only in one part of the molecule. *Figure 2* shows the effect of four mono-unsaturated C6-compounds with various degree of oxidation. It was found that an oxo-group (aldehyde or ketone, as seen from other experiments) was necessary for inhibition. In the concentration range tested, neither the alcohol nor the acid affected the enzyme activity to an appreciable extent.

The presence of an oxo-group alone was, however, not sufficient as to achieve a marked inhibition. Conjugation with a C=C double bond was apparently required. In *Figure 3,* the effects of several C7-aldehydes, differing in degree of saturation and position of the double bond, were compared. It is seen from the figure that only the α,β-conjugated aldehydes acted as inhibitors. The mono-unsaturated compound was much more potent than was the bis-unsaturated aldehyde, despite the larger degree of conjugation in the latter. This behavior may be due to sterical hindrance, as the dienal molecule possesses a rather rigid planar part.

All the substances listed in *Table 1* fit into the same picture: only those containing a C=C double bond conjugated to an oxo-group were inhibitors of microsomal glucose 6-phosphatase.

Among the inhibitory active 2-alkenals, the potency increased with increasing chain length *(Figure 4).* This finding may reflect differences in partition coefficient between the aqueous phase and the lipid phase around the enzyme. Perturbation of the membrane may, however, also play a part in inhibition, as glucose 6-phosphatase is known to be sensitive to even small changes in its environment. In accordance with this, several of the active inhibitors affected the kinetic constants of the enzyme in qualitatively different ways.[25]

The possibility of using a commercially available partially purified glucose 6-phosphatase in the test system was also considered. This preparation is very unstable at elevated temperatures[26] *(Table 2),* but it could be used at 22° C. The preparation was inhibited by the same substances as was the microsomal activity, but to a lesser degree when compared with microsomes incubated for the same time interval at 37° C, a consequence of the temperature dependence on rate of inhibition. Besides, the lack of a membrane phase may be of consequence for the inhibitory mechanism.[25] For that reason, and because of the need for incubating the partially purified enzyme at a suboptimal temper-

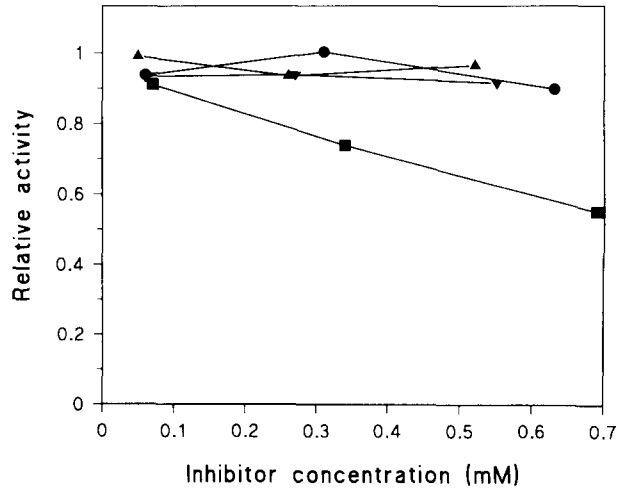

Figure 2 Effect of some C6-compounds on the glucose 6-phosphatase activity. Microsomes were incubated at 37° C for 15 min with various concentrations of 2-hexene (●), 2-hexen-1-ol (▼), 2-hexenal (■), or 2-hexenoic acid (▲), all in the *trans*-configuration, prior to assay. The activities are expressed relative to that of a control incubated with medium only.

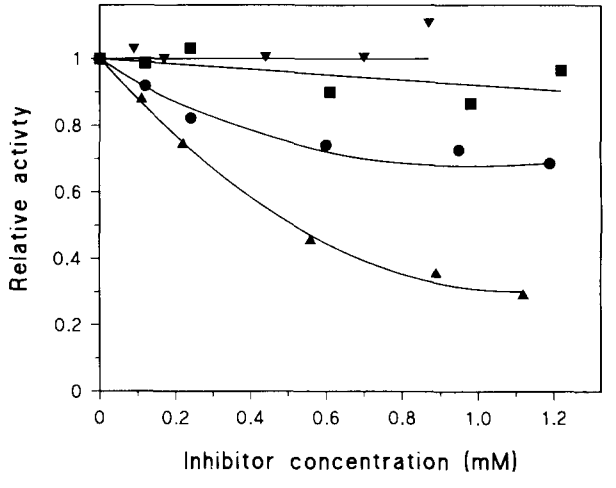

Figure 3 Effect of the double bond location on inhibitory potency. Microsomes were incubated at 37° C for 15 min with various concentrations of heptanal (■), *trans*-2-heptenal (▲), *cis*-4-heptenal (▼), or *trans,trans*-2,4-heptadienal (●) prior to assay of the glucose 6-phosphatase activity. The activities are expressed relative to that of a control incubated with medium only.

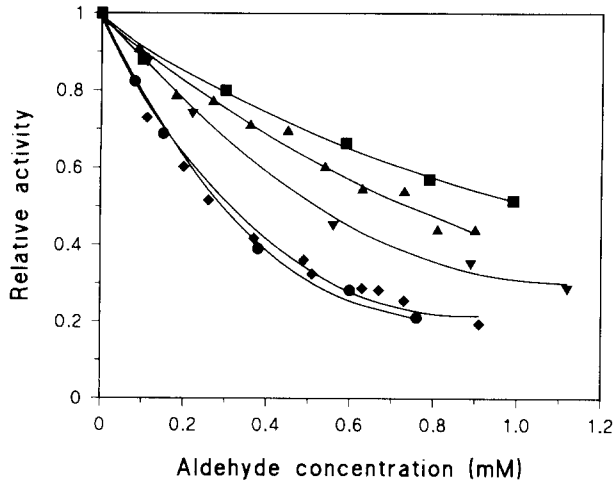

Figure 4 Effect of chain length on inhibitory potency. Microsomes were incubated at 37° C for 15 min with various concentrations of a *trans*-2-alkenal prior to assay of the glucose 6-phosphatase activity. The aldehydes were: pentenal (■), hexenal (▲), heptenal (▼), octenal (●), and nonenal (◆). The activities are expressed relative to that of a control incubated with medium only.

ature, the microsomal activity was found more appropriate for the test system.

Discussion

The presence of a $-CH=CH-CO-$ structure in all the inhibitory active components tested points to a vinylogous addition as the mechanism of binding of inhibitor to the enzyme. An analogous mechanism has been postulated as responsible for binding to protein SH-groups of some other secondary lipid autoxidation products of similar structure, namely the 4-hydroxyalkenals.[19] Glucose 6-phosphatase possesses an SH-group that modifies the kinetic parameters of the enzyme when blocked by mercapto group reactive reagents.[27,28] This group may well be involved in inhibition by the oxidation products, as thiols are known to prefer addition to the β-carbon in unsaturated conjugated carbonyls.[29] An observed competition between alkenal and the mercapto group reactive reagent *p*-hydroxymercuribenzoate further substantiates this suggestion.[25]

However, binding to the SH-group is by itself, although necessary, not the only event that causes inhibition. The influence of carbon chain length on the inhibitory potency must have another explanation. Based on the present findings and other experiments,[25] it is suggested that a membrane perturbation is also involved. This agrees well with the known sensitivity of glucose 6-phosphatase to its phospholipid environment.[30] It is concluded that covalent binding, as well as membrane perturbation, play a part in the toxicity of some secondary lipid autoxidation products.

The sensitive, but rather stable, microsomal glucose 6-phosphatase system is thus useful in a test for occurrence of potentially toxic lipid autoxidation products. Also, this system with an active SH-group may be used as a model for studying in vivo reactions

Table 2 Enzyme inhibition and degradation rates[a]

Enzyme	Aldehyde	Rate of decrease in activity during incubation (10^{-2} min^{-1}) 0° C	22° C	37° C
Partially purified	−	0.2	0.4	5.1
	+	0.3	2.9	21.5
Microsomal	−	0.0	0.1	0.2
	+	0.2	3.5	4.1

[a]Enzyme was incubated in buffer with or without 0.56 mmol/L *trans*-2-nonenal according to the procedure described except that the incubation temperature was as indicated and either microsomes or the partially purified preparation was used. The values reported are the initial rates of decrease in activity relative to the initial activity (standard assay at 37° C in all cases).

between the secondary autoxidation products and cellular systems. It seems that the most important substances in this context are α,β-mono-unsaturated carbonyls. The concentration of added inhibitor necessary for a measurable effect is, however, several orders of magnitude higher than the value normally found in a microsomal suspension where the substance is generated in situ by induced lipid oxidation.[31,32] Likewise, it is much higher than the level in fish oil *(Table 1)*. To make the test system applicable to analysis of oxidation products in PUFA-rich food, a step involving isolation of volatile compounds, therefore, has to be included in the procedure. The demand for a relatively high concentration of added inhibitor is probably created by a limited access to the surroundings of the enzyme, because autoxidation products generated in the microsomal membrane in situ are effective in much lower amounts.[32] Attempts to lower that barrier are currently in progress.

Abbreviations

g_{av} centrifugal force at average radius
PUFA polyunsaturated fatty acid

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