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Loss of latent activity of liver microsomal membrane enzymes evoked by lipid peroxidation. Studies of nucleoside diphosphatase, glucose-6-phosphatase, and UDP glucuronyltransferase

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The effects of lipid peroxidation on latent microsomal enzyme activities were examined in NADPH-reduced microsomes from phenobarbital-pretreated male rats. Lipid peroxidation, stimulated by iron or carbon tetrachloride, was assayed as malondialdehyde formation. Independent of the stimulating agent of lipid peroxidation, latency of microsomal nucleoside diphosphatase activity remained unaffected up to microsomal peroxidation equivalent to the formation of about 12 nmol malondialdehyde/mg microsomal protein. However, above this threshold a close correlation was found between lipid peroxidation and loss of latent enzyme activity. The loss of latency evoked by lipid peroxidation was comparable to the loss of latency attainable by disrupting the microsomal membrane by detergent. Loss of latent enzyme activity produced by lipid peroxidation was also observed for microsomal glucose-6-phosphatase and UDPglucuronyltransferase. In contrast to nucleoside diphosphatase, however, both enzymes were inactivated by lipid peroxidation, as indicated by pronounced decreases of their activities in detergent-treated microsomes. According to the respective optimal oxygen partial pressure (p_{O_2}) for lipid peroxidation, the iron-mediated effects on enzyme activities were maximal at a p_{O_2} of 80 mmHg and the one mediated by carbon tetrachloride at a p_{O_2} of 5 mmHg. Under anaerobic conditions no alterations of enzyme activities were detected. These results demonstrate that loss of microsomal latency only occurs when peroxidation of the microsomal membrane has reached a certain extent, and that beyond this threshold lipid peroxidation leads to severe disintegration of the microsomal membrane resulting in a loss of its selective permeability, a damage which should be of pathological consequences for the liver cell. Because of its resistance against lipid peroxidation nucleoside diphosphatase is a well-suited intrinsic microsomal parameter to estimate this effect of lipid peroxidation on the microsomal membrane.

Introduction

Lipid peroxidation is a degradative process involving peroxidative decomposition of unsaturated fatty acids of membrane lipids [1,2]. It is stimulated in NADPH-reduced liver microsomes by the addition of transition metals such as iron, or haloalkanes like carbon tetrachloride (CCl_4) or halothane ($CF_3CHBrCl$). In the latter examples hypoxic oxygen partial pressures (p_{O_2}) are of crucial importance [3,4], while lipid peroxidation stimulated by ferrous iron increases with increasing p_{O_2} [4].

Lipid peroxidation is known to generate a

variety of products including aldehydes such as 4-hydroxynonenal and malondialdehyde [5,6], and it is assumed that pathological consequences may be mediated by reaction of these or other reactive products with essential cellular components. On the other hand, pathological consequences may also result merely from physical changes of the membrane due to lipid peroxidation.

In liver microsomes a quantitative index of the structural integrity of the microsomal membrane is the latent activity of microsomal enzymes such as nucleoside diphosphatase, glucose-6-phosphatase and UDPglucuronyltransferase, that are considered to be restrained from expressing their maximal activity by microsomal lipids [7,8]. In the present paper this intrinsic microsomal characteristic is used to study effects of NADPH/Fe^{3+}- and NADPH/CCl_4- stimulated lipid peroxidation on the microsomal membrane.

Materials and Methods

Materials. The non-ionic detergent Renex 690 was a gift from Atlas-Chemie (Essen, F.R.G.). CCl_4, $FeCl_3$, isocitrate, *p*-nitrophenol, and thiobarbituric acid were purchased from Merck (Darmstadt, F.R.G.). Inosine diphosphate, isocitrate dehydrogenase (EC 1.1.1.42), mannose 6-phosphate, and $NADP^+$ were from Boehringer (Mannheim, F.R.G.), 1,1,3,3-tetramethoxypropane from Fluka (Neu-Ulm, F.R.G.), and bovine serum albumin from Behring (Marburg, F.R.G.).

Inosine diphosphate was freed from inorganic phosphate by ion exchange chromatography (Dowex 1-4X, Bio-Rad, Munich, F.R.G.). All the other chemicals were of analytical grade and used without further purification.

Microsomes. Male Wistar rats (180–200 g), fed on Altromin stock diet (Lage/Lippe, F.R.G.), were used for the preparation of the microsomal fraction [9]. The animals were allowed free access to food and water. They were pretreated with phenobarbital (0.1% sodium phenobarbital, w/v, dissolved in drinking water) for 4 days. Microsomal fractions were stored at 0°C and used within 1–4 h following preparation.

Incubations. Incubations were performed at 37°C as previously described [4]. The incubation medium consisted of $MgCl_2$/KCl/Tris-HCl buffer (6 mM/104 mM/50 mM; pH 7.4), microsomes (1.5–3.0 mg microsomal protein/ml), isocitrate (10 mM), isocitrate dehydrogenase (300 U/l), $NADP^+$(1 mM), and where indicated carbon tetrachloride (1 mM) or ferric iron (10 μM). The NADPH-regenerating system ensured a NADPH concentration of about 0.5 mM during the entire incubation period. Carbon tetrachloride was added as a solution of CCl_4-saturated buffer (5.2 mM [4]) and ferric iron as a freshly prepared solution of $FeCl_3$ (500 μM). During incubation 0.5 ml aliquots were taken for malondialdehyde determination and enzyme assays.

Assays. Malondialdehyde was determined with the thiobarbituric acid method [10] using 1,1,3,3-tetramethoxypropane as a standard and protein with the method of Lowry [11] using bovine serum albumin as a standard.

UDPglucuronyltransferase (EC 2.4.1.17) activity was measured according to Bock et al. [12] using *p*-nitrophenol as a substrate, glucose-6-phosphatase (EC 3.1.3.9) activity according to Nordlie and Arion [13] using mannose 6-phosphate as a substrate, and nucleoside diphosphatase (EC 3.6.1.6) activity according to Kuriyama [14] using inosine diphosphate as a substrate. Activities were measured at 37°C. P_i liberated by both phosphatases was determined enzymatically. Detergent-treated microsomes were prepared at 0°C supplementing 1 volume of microsomes (about 2 mg microsomal protein/ml) with 1 volume of 0.2% (w/v) Renex 690 in $MgCl_2$/KCl/Tris-HCl buffer (6 mM/104 mM/50 mM; pH 7.4) to give a detergent/microsomal protein ratio of about 1 and a final detergent concentration of 0.1%. The detergent-treated microsomes were kept on ice for 10 min before assays were performed.

Results

On incubation of NADPH-reduced microsomes with ferric iron at a p_{O_2} of 80 mmHg significant lipid peroxidation and following a lag phase a marked raise in the nucleoside diphosphatase activity of native (i.e. not detergent-treated) microsomes occurred (Fig. 1). Decreasing the p_{O_2} to 5 mmHg markedly diminished lipid peroxidation and also led to a less pronounced increase in the nucleoside diphosphatase activity of the native

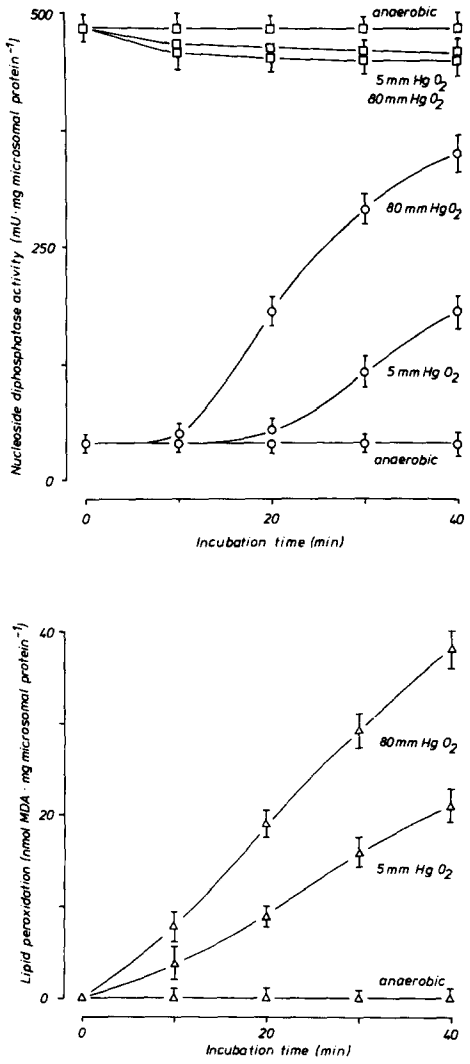

Fig. 1. Time-courses of nucleoside diphosphatase activity and lipid peroxidation in microsomes incubated with NADPH/Fe^{3+} under various p_{O_2}. Microsomes (about 1.5–3.0 mg microsomal protein per ml) from phenobarbital-pretreated male rats were incubated at 37°C in a medium containing $MgCl_2$/KCl/Tris-HCl buffer (6 mM/104 mM/50 mM; pH 7.4), NADPH (about 0.5 mM, regenerating system), and $FeCl_3$ (10 μM). Steady state p_{O_2} was maintained constant by an oxystat system. During incubation aliquots were taken for malondialdehyde (MDA) measurements and for further enzyme assay. Nucleoside diphosphatase activity was determined either after pretreatment of the incubated microsomes with Renex 690 (detergent-treated microsomes, □) or without further treatment (native microsomes, ○). Lipid peroxidation (△) was estimated by the amounts of malondialdehyde formed. Vertical bars denote S.E. of the mean for at least five separate incubations.

microsomes. In microsomes disrupted by addition of detergent prior to enzyme assay (detergent-treated microsomes) no significant alterations in the nucleoside diphosphatase activity were observed at both p_{O_2} (5 and 80 mmHg) studied. Under anaerobic conditions (Fig. 1), in the presence of NADPH alone (data not shown), and in the absence of cofactor (Table I) no formation of malondialdehyde was detected and no alteration of enzyme activity of the native and detergent-

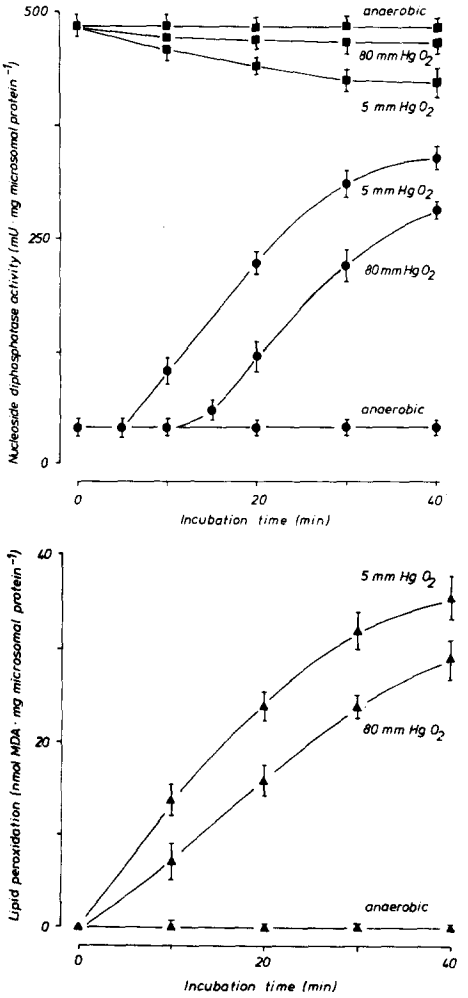

Fig. 2. Time-courses of nucleoside diphosphatase activity and lipid peroxidation in microsomes incubated with NADPH/CCl_4 under various p_{O_2}. Carbon tetrachloride concentration was 1 mM; ■ nucleoside diphosphatase activity of detergent-treated microsomes, ● nucleoside diphosphatase activity of native microsomes, ▲ lipid peroxidation. Further experimental details are as in Fig. 1. MDA, malondialdehyde.

treated microsomes occurred.

Addition of carbon tetrachloride to NADPH-reduced microsomes was of comparable effect on lipid peroxidation and nucleoside diphosphatase activity of native and detergent-treated microsomes as the addition of ferric iron, except for one marked difference (Fig. 2): in the presence of NADPH/CCl_4 the most pronounced effects on malondialdehyde formation and enzyme activity were observed at a p_{O_2} of 5 mmHg, in line with the maximum of the NADPH/CCl_4-induced lipid peroxidation at hypoxic p_{O_2} [4]. Under anaerobic conditions again no lipid peroxidation and no alteration in enzyme activity occurred. The latter rules out the possibility that in the case of NADPH/CCl_4 the observed effects on nucleoside diphosphatase activity were directly produced by reductively formed CCl_4-metabolites without mediation of lipid peroxidation. The relationship between lipid peroxidation, induced by either NADPH/Fe^{3+} or NADPH/CCl_4, and the increase in nucleoside diphosphatase activity of the native microsomes is demonstrated in more detail in Fig. 3. Independent of the inducing agent above a value of about 12 nmol malondialdehyde per mg microsomal protein a close correlation existed between lipid peroxidation and the increase of nucleoside diphosphatase activity. Taking into consideration the slight inactivation of nucleoside diphosphatase produced by lipid peroxidation, as indicated by the slight loss of nucleoside diphosphatase activity of the detergent-treated micro-

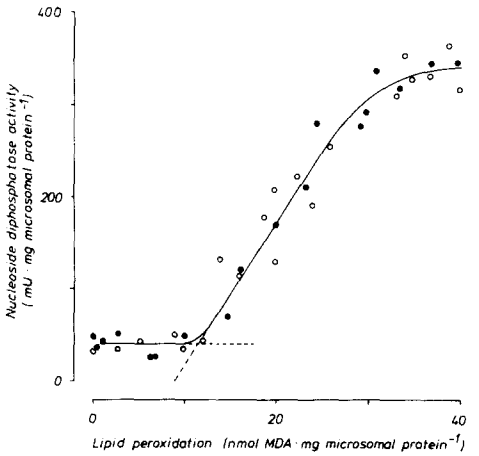

Fig. 3. Effect of lipid peroxidation induced by NADPH/Fe^{3+} (open symbols) or NADPH/CCl_4 (filled symbols) on nucleoside diphosphatase activity of native microsomes. Experimental details are as in Figs. 1 and 2. MDA, malondialdehyde.

TABLE I

EFFECT OF LIPID PEROXIDATION INDUCED BY NADPH/Fe^{3+} OR NADPH/CCl_4 ON THE LATENCY OF MICROSOMAL ENZYME ACTIVITIES

Microsomes were incubated for 40 min in the presence of NADPH/Fe^{3+} at a p_{O_2} of 80 mmHg and in the presence of NADPH/CCl_4 at a p_{O_2} of 5 mmHg. Control values were obtained in the absence of cofactor. Further experimental details are as in Figs. 1 and 2. Latency was calculated according to Arion [15] as

$$\left(1 - \frac{\text{activity of native microsomes}}{\text{activity of detergent-treated microsomes}}\right) \cdot 100$$

Microsomal enzyme	Additions	Residual activity of detergent-treated microsomes (mU/mg microsomal protein)	Residual activity of native microsomes		Apparent latency (%)
			mU/mg microsomal protein	% activity of detergent-treated microsomes	
Nucleoside diphosphatase	control	482 ± 8	43 ± 3	9	91
	NADPH/Fe^{3+}	447 ±11	352 ±22	79	21
	NADPH/CCl_4	418 ±14	338 ±20	81	19
Glucose-6-phosphatase	control	41.3± 2.4	2.8± 0.2	7	93
	NADPH/Fe^{3+}	8.6± 3.8	6.7± 2.1	78	22
	NADPH/CCl_4	9.4± 3.5	6.9± 2.4	73	27
UDPglucuronyl-transferase	control	12.4± 1.2	0.8± 0.2	6	94
	NADPH/Fe^{3+}	6.9± 1.6	4.4± 0.5	64	36
	NADPH/CCl_4	7.3± 1.4	3.8± 0.6	52	48

somes at high levels of lipid peroxidation (Figs. 1 and 2), the nucleoside diphosphatase activity of the native microsomes came near to the activity of the detergent-treated microsomes demonstrating an almost complete loss of latency (Table I).

In contrast to nucleoside diphosphatase, two further latent microsomal enzymes, glucose-6-phosphatase and UDPglucuronyltransferase, exhibited pronounced decreases in their activities in the detergent-treated microsomes following incubation of the NADPH-reduced microsomes with either ferric iron or carbon tetrachloride, while in the native microsomes only small increases in the activities of glucose-6-phosphatase and UDPglucuronyltransferase were observed (Table I); the latter in the case of UDPglucuronyltransferase in agreement with previous findings [16,17]. These alterations in enzyme activities also exhibited a close correlation with lipid peroxidation. So under anaerobic conditions no alterations occurred and maximal effects were observed at a p_{O_2} of 80 mmHg in the presence of NADPH/Fe^{3+} and at a p_{O_2} of 5 mmHg in the presence of NADPH/CCl_4. In these experiments glucose-6-phosphatase activity was measured using mannose 6-phosphate as a substrate. With its physiological substrate glucose 6-phosphate, glucose-6-phosphatase reveals only slight latency, possibly because of the presence of a specific glucose 6-phosphate transporter within the microsomal membrane that facilitates access of glucose 6-phosphate, but not of mannose 6-phosphate, to the active site of the enzyme [15]. The loss of mannose-6-phosphatase activity of the detergent-treated microsomes presumably reflects the known loss of glucose-6-phosphatase activity of native microsomes following peroxidation [16,18,19] and points to a peroxidatively induced inactivation of these enzymes. Presumably, the inactivation of glucose-6-phosphatase and UDPglucuronyltransferase is the reason for the relatively small increase in the activities of both enzymes in the native microsomes. Since, however, following microsomal peroxidations the activities of the native microsomes approaches the activities of the detergent-treated microsomes for both glucose-6-phosphatase and UDPglucuronyltransferase, a marked loss of latency may also be concluded (Table I).

Discussion

Both glucose-6-phosphatase and UDPglucuronyltransferase are integral microsomal enzymes, the activity of which largely depends on an intact lipid bilayer. Hence, their inactivation may already point to a marked loss of integrity of the microsomal membrane. On the other hand, both enzymes may also be inactivated by reactive breakdown products of the peroxidative decomposition of unsaturated fatty acids of membrane lipids, independent of alterations of the physical state of the microsomal membrane. For example, Benedetti et al. [6] could demonstrate that 4-hydroxyalkenals such as 4-hydroxynonenal are capable of inactivating glucose-6-phosphatase possibly by reacting with essential thiol groups. Deficiency in essential thiol groups attackable by reactive breakdown products of lipid peroxidation might also explain the resistance of nucleoside diphosphatase against lipid peroxidation. Moreover, in contrast to glucose-6-phosphatase and UDPglucuronyltransferase, nucleoside diphosphatase is not as tightly associated with the microsomal membrane [7,14]; a possible further explanation for its stability against lipid peroxidation. Because of this stability nucleoside diphosphatase is well suited to determine quantitatively the effect of lipid peroxidation on microsomal latency. Since with increasing lipid peroxidation an almost complete loss of latency was observed (Table I), a marked disintegration of the microsomal membrane can be concluded. Interestingly, taking into consideration their inactivation, from glucose-6-phosphatase and UDPglucuronyltransferase a similar effect of lipid peroxidation on microsomal integrity can be estimated (Table I) even though in view of the marked inactivations of these enzymes exact quantifications of changes in their latencies may be unjustified.

Latency is often considered to demonstrate that the active site of an enzyme is orientated towards the microsomal lumen [7,8]. Thus, loss of latency indicates an increased permeability of the microsomal membrane to charged substrates due to peroxidative alterations of the microsomal membrane. This interpretation is supported by experiments in lysosomes [20] and liposomes [21] where increases in permeability were observed following

lipid peroxidation. Similar to our experiments (Fig. 3), loss of barrier function in liposomes only occurred following a lag indicating that the peroxidatively induced alterations of the microsomal membrane must reach a certain extent and possibly involve both leaflets of the microsomal bilayer before a diminution of selective permeability occurs.

Loss of latency already started at levels of lipid peroxidation (about 12 nmol malondialdehyde per mg microsomal protein, Fig. 3) at which the electron microscope indicated no signs of membrane damage and where in the microsomal membrane the contents of unsaturated fatty acids were diminished between about 60% (22:6 and 20:4 fatty acids) and 10% (18:2 fatty acids) [16]. However, at levels of lipid peroxidation (about 25 nmol malondialdehyde per mg microsomal protein) where loss of latency is almost fully expressed, morphologic alterations became visible.

Loss of barrier function of the endoplasmic reticulum membrane may be of pathological consequences for the liver cell and an increased permeability for charged molecules of low molecular weight may also account for the inactivation of the endoplasmic reticulum calcium pump [22,23] which is possibly a decisive event in carbon tetrachloride hepatotoxicity.

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