Mammalian 15-lipoxygenases have been suggested to be involved in cell differentiation and atherogenesis because of their capability of oxygenating polyenoic fatty acids esterified to biomembranes and lipoproteins. We investigated the interaction of the lipid-peroxidizing 15-lipoxygenase and the hydroperoxy lipid-reducing phospholipid hydroperoxide glutathione peroxidase during their reaction with biomembranes and lipoproteins and obtained the following results. 1) Lipoxygenase treatment of submitochondrial membranes led to the formation of hydroperoxyphosphatidyl ethanolamine and hydroperoxyphosphatidylcholine as indicated by high performance liquid chromatography with chemiluminescence detection. In 15-lipoxygenase-treated low density lipoprotein cholesterol hydroperoxylinolate was the major oxygenation product. 2) Phospholipid hydroperoxide glutathione peroxidase was capable of reducing the hydroperoxy lipids formed by the 15-lipoxygenase to their corresponding alcohols. 3) Preincubation of low density lipoprotein and submitochondrial membranes with the phospholipid hydroperoxide glutathione peroxidase completely prevented the lipoxygenase reaction. However, addition of exogenous hydroperoxy lipids restored the oxygenase activity. 4) Short-term incubations of the complex substrates with the 15-lipoxygenase led to a specific pattern of oxidation products which was rendered more unspecific at long-term incubation or at high substrate concentrations. If the phospholipid hydroperoxide glutathione peroxidase was present during the reaction, the specific product pattern was preserved. These data indicate that the phospholipid hydroperoxide glutathione peroxidase is capable of reducing hydroperoxy ester lipids formed by a 15-lipoxygenase, and that it may down-regulate the 15-lipoxygenase pathways in mammalian cells. The specificity of 15-lipoxygenase-derived hydroperoxy lipids depends on their immediate reduction to the corresponding alcohols preventing postcatalytic isomerization.

Enzymatic and nonenzymatic lipid peroxidation has been implicated in a variety of physiological and pathophysiological processes such as aging, cell differentiation, carcinogenesis, inflammation, hypoxia, atherogenesis, and others (1–8). The formation of lipid hydroperoxides within the bilayer of biomembranes alters the membrane structure (9, 10) and, thus, may lead to dysfunction of the cellular metabolism. Among the lipid-peroxidizing enzymes in mammalian cells, the 15-lipoxygenases (11, 12) and the closely related leukocyte-type 12-lipoxygenases (13, 14) are capable of oxidizing biomembranes and lipoproteins directly without the preceding action of ester lipid-hydrolyzing enzymes. Because of this property, 15-lipoxygenases may be considered as membrane-damaging noxae, and, hence, the control of the cellular 15-lipoxygenase activity appears to be of crucial cell-physiological relevance. While the cellular regulation of the 5-lipoxygenase pathway has been studied in detail (15–18), little is known on the regulation of the 15-lipoxygenases in mammalian cells. One general feature of the lipoxygenase reaction is the requirement for small amounts of hydroperoxy lipids acting as essential activators of the enzyme (19, 20). For this reason, the cellular "hydroperoxide tone" constitutes a possible site of regulation of lipoxygenase activity. In most mammalian cells, the steady-state concentration of hydroperoxides is rather low. In fact, even in cells exhibiting a high lipid-peroxidizing capacity, such as rabbit reticulocytes, mainly hydroxy fatty acids have been detected in the membrane lipids (21). A preferential cleavage of hydroperoxy lipids by synergistic action of phospholipases and peroxidase appears unlikely (22). Glutathione peroxidases are known for their capability of reducing organic and inorganic hydroperoxides in mammalian cells (23, 24). So far, 4 different selenoperoxidases have been identified (25) as translation products of 4 different genes (26). Among these enzymes, only the phospholipid hydroperoxide glutathione peroxidase (PH-GPx) is capable of reducing hydroperoxy lipids esterified to biomembranes (27) and lipoproteins (28, 29). Recently, a regulatory role of the PH-GPx has been reported for the 5-lipoxygenase in human leukocytes (30).

For this paper we investigated the concerted action of the 15-lipoxygenase and the PH-GPx during the oxygenation of biomembranes and lipoproteins in reconstituted model systems. The data presented suggest that the PH-GPx may play
an important role as down-regulator of the cellular 15-lipoxygenase pathway and that the cell-protective action of this enzyme may in part be due to the suppression of the 15-lipoxygenase activity.

MATERIALS AND METHODS

Chemicals—The chemicals used were from the following sources: glutathione from Merck; 13S-hydroperoxy-2,11E-octadecadienoic acid (13S-HPODE) from Cascade; phospholipid standards, isoluminol (6-amino-2,3-dihydro-1,4-phthalazinedione), and microperoxidase (MP-11) from Sigma. All solvents used were of HPLC grade and purchased from Baker.

Preparations—The 15-lipoxygenase was purified to electrophoretic homogeneity from reticulocyte-rich blood cell suspensions of rabbits as reported before (12). A specific turnover rate of linoleic acid of about 10 s⁻¹ (0.13 microkatal/mg of protein) was determined for the enzyme preparations used. The specific activity with the complex substrates was roughly one order of magnitude lower than that with free polyenoic fatty acids. PH-GPx was prepared from pig hearts as described earlier (31). The enzyme activity was assayed with hydroperoxyphosphatidylcholine as substrate (32). The enzyme preparations used exhibited specific activities ranging between 50 and 200 nkat/ml (0.13 microkatal/mg of protein). Submitochondrial particles which constitute vesicles of the inner and outer mitochondrial membranes were prepared from beef heart mitochondria by ultrasound disintegration (33). Human low density lipoprotein (LDL) was obtained by sequential floating ultracentrifugation (34) and subsequent dialysis against isotonic saline containing 3 mM EDTA.

Assay Systems—The lipoxygenase activity with complex substrates was measured oxographically in a 1.6-ml oxigraphic chamber at 25 °C. Unless stated otherwise, the incubation mixture consisted of 0.1 mM Tris-HCl buffer, pH 7.4, containing either submitochondrial particles (1 mg of protein/ml) or human LDL (0.5 mg of protein/ml) as substrate. The reaction was started by addition of the pure 15-lipoxygenase (10 nanokatal/ml) in the presence or absence of 3 mM reduced glutathione and PH-GPx (0.4 nanokatal/ml). The reaction was stopped by the addition of 4 ml of ice-cold methanol to the reaction mixture. Subsequently, the total lipids were extracted (35), and the extracts were analyzed for hydroperoxy derivatives by reverse-phase HPLC (RP-HPLC) with simultaneous uv- and post-column chemiluminescence detection (36).

RESULTS

PH-GPx Reduces Hydroperoxy Ester Lipids in Biomembranes and LDL

Formed by the 15-Lipoxygenase—It has been reported before that the rabbit reticulocyte 15-lipoxygenase is capable of directly oxygenating polyenoic fatty acids esterified to biomembranes (38, 39) and lipoproteins (12) without the preceding action of a lipid-hydrolyzing enzyme. However, in those reports, the primary lipoxygenase products, the hydroperoxy ester lipids, have not been analyzed. Using HPLC with post-column chemiluminescence detection, we found hydroperoxy fatty acid moieties in the phosphatidylcholine, in the phosphatidylethanolamine, and, to a lesser extent, in the phosphatidylinositol fraction of the membrane lipids (Fig. 1A). No hydroperoxy lipids were found in control incubations with heat-denatured 15-lipoxygenase (trace II) or when the lipid extracts were reduced with either borohydride or triphenylphosphine (data not shown). With human LDL as substrate, cholesterol ester hydroperoxides were the major oxygenation products (Fig. 1B). Oxygenation of the cholesterol esters is restricted to the polyunsaturated fatty acid moiety since only very small amounts of oxysterols could be detected under our assay con-
The hydroperoxy lipids formed during 15-lipoxygenase-catalyzed reaction are suitable substrates for the PH-GPx. As indicated in Fig. 2, the chemiluminescence signal which indicated the presence of the hydroperoxy group disappeared after incubation of the lipoxygenase-treated complex substrates with the PH-GPx. However, the absorbance at 235 nm was retained suggesting that the conjugated diene chromophore was still present. These data suggest that PH-GPx reduced the hydroperoxy lipids to the corresponding alcohols which are not any more detectable in the chemiluminescence assay. For direct evidence of the formation of the hydroxy fatty acids, the lipid extracts of submitochondrial particles treated with the 15-lipoxygenase and PH-GPx were hydrolyzed and analyzed by SP-HPLC. As indicated in Fig. 3, 13S-hydroxy-9Z,11E-octadecadienoic acid was detected as major oxygenated fatty acid, whereas the corresponding hydroperoxide was not found.

PH-GPx Down-regulates 15-Lipoxygenase Activity—Lipoxygenases are known to require small amounts of hydroperoxy fatty acids as activators for their catalytic action (19, 20). This product activation is expressed by a kinetic lag phase which can be abolished by addition of exogenous hydroperoxides (40). For the soybean lipoxygenase, it has been shown that stoichiometric amounts of hydroperoxy fatty acids lead to an oxidation of the ferrous non-heme iron to its ferric form (41, 42). The activation process involves this oxidation but presumably also the subsequent reaction of the ferric lipoxygenase with an additional hydroperoxy fatty acid molecule (43). If this concept of hydroperoxide activation is applicable for the interaction of the rabbit 15-lipoxygenase with complex substrates, pretreatment of submitochondrial particles with PH-GPx should prevent the lipoxygenase reaction. The lipoxygenase-catalyzed oxygenation of submitochondrial membranes can be measured oxygraphically (Fig. 4). If the membranes were preincubated with PH-GPx prior to the addition of the 15-lipoxygenase, no oxygen uptake was measured. This complete inhibition of the reaction was not due to an inactivation of the enzyme since aliquots of the incubation mixture still exhibited linoleate oxygenase activity which we measured routinely in the presence of 5 μM 13-HPODE (data not shown). Moreover, addition of exogenous 13S-hydroperoxy-9Z,11E-octadecadienoic acid (13S-HPODE) to PH-GPx-treated membranes restored the lipoxygenase activity (Fig. 4). When PH-GPx and 15-lipoxygenase were added simultaneously to the assay mixture, only a partial inhibition of the membrane oxygenation was observed. For independent evidence for the inhibition of the lipoxygenase by PH-GPx, we analyzed the oxygenated fatty acids formed during the reaction after borohydride reduction of the hydroperoxides formed and alkaline hydrolysis of the lipid extracts. The HPLC chromatograms (inset to Fig. 4) confirm the oxygraphic finding that preincubation of complex substrates with PH-GPx inhibited the lipoxygenase reaction.

PH-GPx Preserves the Specificity of the Oxygenation Products—Lipoxygenase-catalyzed lipid peroxidation differs from
its nonenzymatic counterpart by the specific pattern of oxygenation products. However, under certain reaction conditions, such as a high concentration of submitochondrial membranes (38), a more unspecific product pattern was detected. This unspecificity was prevented when radical scavengers such as butylated hydroxytoluene were added to the incubation mixture (38). These data suggested that free radical-mediated secondary oxygenation reactions and/or hydroperoxide isomerization, which involves the formation of radical intermediates (44), may be responsible for the unspecificity. In contrast to hydroxypropanoic lipids, the corresponding hydroxy compounds are not capable of inducing secondary lipid peroxidation and do not undergo isomerization. Thus, immediate reduction of the lipoxygenase-derived hydroperoxy lipids by PH-GPx was presumed to give rise to a specific product pattern even under experimental conditions under which an unspecific product pattern was observed if PH-GPx was not present. From Table I, it can be seen that incubation of the 15-lipoxygenase with human LDL for 40 min led to a specific product pattern as indicated by both the high 13-HODE/9-HODE ratio and the high S/R ratio of the major oxygenation product 13-HODE. When the incubation was continued for 24 h, a much more unspecific product pattern was observed. However, when the long-term incubation was carried out in the presence of PH-GPx, a highly specific product pattern was detected. Similar results were obtained with submitochondrial particles. In agreement with earlier observations (38), we found that at high substrate concentrations (20 mg of membrane protein/ml) an unspecific pattern of oxygenation products was formed by the lipoxygenase. In the presence of PH-GPx, however, a highly specific product pattern was analyzed. These data indicate that immediate reduction of the hydroxypropanoic lipids formed by the lipoxygenase appears to be essential for preserving the product specificity.

**DISCUSSION**

Among the glutathione peroxidases described so far, the PH-GPx has the highest capability of reducing membrane-
bound hydroperoxy lipids as compared with free organic and anorganic hydroperoxides (27). Measurement of PH-GPx is usually carried out by monitoring the disappearance of NADH consumed during the glutathione reductase reaction which is coupled to the glutathione-dependent hydroperoxide reduction (32). However, at high concentrations of biomembranes, this photometric assay system is not applicable because of the turbidity of the sample. In such cases, the HPLC analysis of the hydroperoxy phospholipids by chemiluminescence detection described here may be used to test for PH-GPx activity.

The 15-lipoxygenases constitute the major lipoygenase sub-type capable of oxidizing membrane phospholipids without the preceding action of lipid-cleaving enzymes (11, 38). It may be suggested that the concerted action of both 15-lipoxygenase and PH-GPx regulate the hydroperoxide tone of the membranes. Usually, the steady-state concentrations of hydroperoxy lipids in mammalian cells and in particular in the biomembranes are rather low. We investigated several mammalian cells (erythrocytes, liver cells, monocytes, adipose tissue, U937 cells, several endothelial cell lines) for the occurrence of oxygenated lipids in the membranes and found less than 0.01% of the polyenoic fatty acids to be present as oxygenated derivatives.2 The actual hydroperoxide concentration should even be lower and thus usually escapes detection by the currently available analytical methods.

Our data on the concerted action of 15-lipoxygenase and PH-GPx indicate that the 15-lipoxygenase reaction is influenced by the PH-GPx in two different ways. (i) The 15-lipoxygenase activity is down-regulated probably by reducing the hydroperoxy lipids necessary as activator for the lipoxygenase reaction from the incubation mixture. In the case of preincubation of the substrate with PH-GPx, which may lead to a complete disappearance of the activator, the lipoxygenase activity is completely abolished. This effect may be of physiological relevance since the cellular hydroperoxide tone of the membranes appears to be crucial for the activity of cellular lipoxygenases (45). In the absence of hydroperoxide activator, the lipoxygenase should remain enzymatically inactive even if the enzyme protein is expressed at relatively high levels. The in vitro activation of the soybean lipoxygenase by hydroperoxy linoleic acid has been studied in detail (41–43). In contrast, only limited information is available on the mechanism of hydroperoxide oxidation of mammalian lipoxygenases during the oxygenation of biomembranes. Our data indicate that depletion of membranes from hydroperoxy lipids by reducing them to their corresponding hydroxy derivatives leads to a complete inhibition of the lipoxygenase activity. In cells expressing high levels of PH-GPx, the 15-lipoxygenase pathways should be inhibited simply by the fact that the concentration of hydroperoxide activator is too low. These cells may express the lipoxygenase protein at a high level but the enzyme remains catalytically silent. In fact, there are several reports in the literature indicating that cellular 15-lipoxygenase activity can only be measured after activation or disruption of the cells (46, 47). Several authors suggest that the enzyme may be present in a cryptic form and becomes liberated upon cell activation. However, so far, no experimental evidence has been presented that the 15-lipoxygenase is sequestered in such a way. In light of our findings, the lack of hydroperoxide activation may also be considered as a reason for functional masking of the 15-lipoxygenase in cells. It is tempting to speculate that cell activation may lead to an increase in hydroperoxide-forming processes which in turn may act as 15-lipoxygenase activator. Work is in progress to address this issue on the cellular level. (ii) PH-GPx preserves the specificity of the product pattern of the lipoxygenase reaction which was shown to be rather unspecific under certain experimental conditions. The PH-GPx prevents hydroperoxide-induced secondary lipid peroxidation or peroxide rearrangement simply by reducing the specific hydroperoxy lipids formed by the lipoxygenase to their corresponding alcohols. These data suggest that the formation of unspecific oxygenation products during the lipoxygenase reaction appears to be a post-catalytic phenomenon (secondary reactions) rather than a syn-catalytic one (dissociation of free radicals from the enzyme during its catalytic cycle).

In atherosclerotic lesions, a functional 15-lipoxygenase is expressed (48, 49). In the time course of plaque formation in cholesterol-fed rabbits, specific lipoxygenase products were detected after 12 weeks of feeding (50). After longer feeding periods, these products were superimposed by large amounts of nonspecific oxygenation products originating from nonenzymatic lipid peroxidation. Our data suggest that the specificity of lipoxygenase products is preserved over long time periods only if the reducing capacity of the cells is high enough to quickly reduce the hydroperoxy lipids formed by the lipoxygenase. Such a situation may be found in early stages of plaque development. At later stages, the oxidizing processes may prevail, and hydroperoxides may accumulate and may undergo isomerization and/or provoke nonenzymatic lipid peroxidation leading to the more unspecific product pattern as detected advanced lesions (51–53).

The data reported here support the assumption that the intracellular action of the PH-GPx may down-regulate the cellular 15-lipoxygenase pathways and that a balanced concerted interaction of both enzymes is a precondition for the formation of specific 15-lipoxygenase products in mammalian tissues.

---

2 H. Kühn, unpublished data.

---

### Table 1

| Substrate | PH-GPx | Incubation period | Substrate concentration | 13-HODE/9-HODE | S/R ratio of 13-HODE |
|-----------|--------|-------------------|------------------------|----------------|---------------------|
| LDL       | –      | 40 min            | 0.5                    | 96/4           | 85/15               |
| LDL       | +      | 40 min            | 0.5                    | 96/4           | 91/9                |
| LDL       | –      | 24 h              | 1                      | 61/39          | 59/41               |
| LDL       | +      | 24 h              | 1                      | 96/4           | 91/9                |
| SMP       | –      | 15 min            | 1                      | 81/19          | 81/19               |
| SMP       | +      | 15 min            | 1                      | 95/5           | 90/10               |
| SMP       | –      | 15 min            | 20                     | 61/39          | 60/40               |
| SMP       | +      | 15 min            | 20                     | 92/8           | 91/9                |

The data reported here support the assumption that the intracellular action of the PH-GPx may down-regulate the cellular 15-lipoxygenase pathways and that a balanced concerted interaction of both enzymes is a precondition for the formation of specific 15-lipoxygenase products in mammalian tissues.
The Selenoenzyme Phospholipid Hydroperoxide Glutathione Peroxidase Controls the Activity of the 15-Lipoxygenase with Complex Substrates and Preserves the Specificity of the Oxygenation Products

Kerstin Schnurr, Jutta Belkner, Fulvio Ursini, Tankred Schewe and Hartmut Kühn

J. Biol. Chem. 1996, 271:4653-4658.
doi: 10.1074/jbc.271.9.4653

Access the most updated version of this article at http://www.jbc.org/content/271/9/4653

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 48 references, 14 of which can be accessed free at http://www.jbc.org/content/271/9/4653.full.html#ref-list-1