

The Course of Enzymatically Induced Lipid Peroxidation in Homogenized Porcine Kidney Tissue

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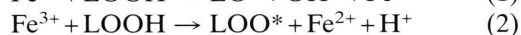
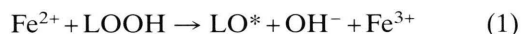
Cell Damage, Activation of Enzymes, Lipoxxygenase, Hydroxy Fatty Acids

Homogenization of mammalian tissue – exemplified by porcine kidney – causes enzymatically induced lipid peroxidation (LPO) processes proven by measuring the amounts of the typical lipid peroxidation products 9- and 13-hydroxy-octadecadienoic acid (HODE) either after homogenization in aqueous (activation of enzymes) or an organic (inactivated enzymes) solvent. A kinetic study revealed that the level of the 9- and 13-isomer reached maximum values 6 hours after tissue injury. Within one day the amount of these primary oxidation products was reduced fast, indicating that they undergo degradation in their biological environment. In contrast, the level of 10-hydroxy-octadecanoic acid – obviously derived from LPO of oleic acid – increased continuously even after one day. These observations reflect that the generation and degradation of hydroperoxides occurs at different rates which might be of interest in pathological processes connected with tissue injury, e.g. myocardial infarction.

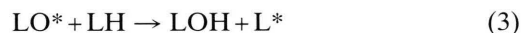
Introduction

Injury of plant cells induces degradation of membrane phospholipids and triacylglycerides by activation of hydrolases and lipoxxygenases (Galliard, 1975). The latter attack polyunsaturated fatty acids (PUFAs) possessing a *cis*-1,4-pentadiene structural element (Hamberg, 1971), e.g. linoleic and arachidonic acid. Lipoxxygenases transform double allylically activated methylene groups to hydroperoxides in a stereospecific reaction by incorporation of oxygen forming a conjugated diene system (Veldink, Vliegenhart and Boldingh, 1977; Kühn, Belkner and Wiesner, 1990). Hydroperoxides of unsaturated fatty acids suffer easily

degradation in presence of bivalent metal ions generating either in a Fenton like reaction alkoxy-radicals (1) or by interaction with Fe^{3+} ions peroxy-radicals (2) (Gardner, 1991).



Iron ions are probably liberated after cell damaging processes: cell injury activates besides esterases and lipoxxygenases other degrading enzymes, e.g. proteases. These may remove the shielding protein cover of iron containing proteins (Aust, 1989). LO^* - and LOO^* -radicals may start a non-enzymatic chain reaction (3), (4) in the course of which more and more hydroperoxides are produced.



These reactions are neither regio- nor stereospecific since any double allylically activated CH_2 -group is attacked with about equal probability regardless whether located in a free fatty acid or a conjugated derivative. The generated radicals do not only attack double allylically activated methylene groups but also monoallylically activated ones, however to a much lower extent. As a consequence, hydroperoxides of oleic acid (Frankel,

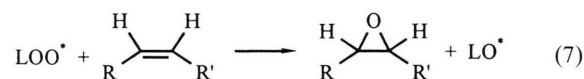
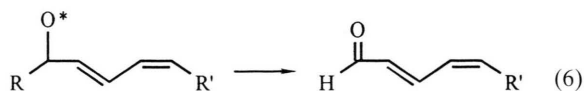
Abbreviations: AcOH, acetic acid; BHT, 2,6-bis-*tert*-butyl-*p*-hydroxy toluene; GC/MS, gas chromatography / mass spectrometry; HETE, hydroxy eicosatetraenoic acid; HNDE, hydroxy nonadecadienoic acid; HODE, hydroxy octadecadienoic acid; LOH, hydroxy fatty acid; LOOH, hydroperoxy fatty acid; LPO, lipid peroxidation; LOX, lipoxxygenase; MDA, malondialdehyde; MSTFA, N-methyl-N-trimethylsilyl-trifluoroacetamide; PUFA, polyunsaturated fatty acid; TMSO, trimethylsilyloxy-group.

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1984) and of cholesterol are also generated. Besides this, LO*-radicals (Esterbauer, 1985) are cleaved to form aldehydic compounds (5), (6) while ROO*-radicals may react with nearby located double bonds of other molecules by epoxidation (7) (Meyer and Spiteller, 1993).



Probably some of the generated compounds serve as biological signals, e.g. after attack of insects or fungi (Kato, 1984) the plants start to enlarge cell walls and to produce phytoalexins (Kuc and Rush, 1985).

While liberation of enzymes by plant injury is rather well known, reports of similar reactions of mammalian cells are rare: *Tappel* reported in 1960 that lipid peroxides are readily formed whenever tissue homogenates were exposed to air (Zalkin and Tappel, 1960). A few years later, *Wills* investigated the LPO of organ homogenates. He observed in agreement with the results of *Tappel* a fast production of peroxides by homogenation of tissue especially derived from liver and kidney (Wills, 1966). *Bergers et al.* investigated LPO production in homogenized skin samples under various conditions (Bergers and Verhagen, 1986). They found enhanced amounts of hydroperoxides, but nearly none after thermal treatment of the samples explaining this fact by postulating an inhibition of phospholipase A₂ responsible for liberation of fatty acids. We have been able to support these results by subjecting a porcine liver to homogenation before and after thermal treatment. The identification of reaction products by GC/MS proved the involvement of hydroperoxy fatty acids (Herold and Spiteller, 1996). In this paper we report on kinetic studies of hydroperoxide formation in porcine kidney tissue.

Materials and Methods

MSTFA was obtained from Macherey and Nagel (Düren, Germany), all other chemicals were purchased from Fluka (Neu Ulm, Germany) or Merck (Darmstadt, Germany). All solvents were distilled before use.

HPLC was performed on a Beckmann System 112 coupled with a System Gold Scanning UV Detector Module 167. Reverse-phase HPLC was carried out on a Spherisorb ODS II column (250 mm x 8 mm, 5 µm particle size, Bischoff, Germany). A solvent system of CH₃OH / H₂O / AcOH 950:50:1 (v:v:v) at a flow rate of 4 ml/min. was used and the absorbance at 234 nm was recorded.

Compounds were identified by GC/MS-analysis carried out on a Hewlett Packard 5890 Series II GC equipped with a fused silica DB-5 capillary column (J&W Scientific, 30 m, Ø = 0.3 mm, 2 ml/min. H₂) and a double focussing Finnigan MAT 95 mass-spectrometer (70eV) fitted with inversed Nier-Johnson geometry and EI/CI ion source. The temperature program increased from 80 °C at a rate of 3 °C/min. up to 280 °C.

Tissue incubation and extraction procedure

A porcine kidney was removed from the renal capsule immediately after slaughtering and divided in halves. One part was homogenized for 2 min in the 1.5 fold amount of bidistilled water in a Waring blender sustaining enzymatic activity followed by stirring at room temperature under aerobical conditions. Aliquots containing 20 g tissue were withdrawn in time intervals of 0, 3, 6 and 24 hours. In a control experiment the other part of the same kidney was homogenized in a mixture of organic solvents CHCl₃ / CH₃OH 1:2 (v:v) to exclude enzyme activation. All homogenates, those in which enzymatic oxidation was either suppressed by extraction in organic solvents or retained by homogenization in bidistilled water were treated exactly in the same way.

The aliquots were extracted in a one-phase system consisting of CHCl₃ / CH₃OH / H₂O 1:2:0.8 (v:v:v) following the procedure of Bligh and Dyer (Bligh and Dyer, 1959). The water already present in the tissue should be considered as a ternary component of this system. Since tissue already has a water content rather than 80 per cent, the separation in two layers was achieved by addition of

CHCl_3 and a 10%-aqueous KCl solution in equal amounts. Then a methanolic solution of BHT was injected (50 $\mu\text{g}/\text{ml}$) to prevent further oxidation and 20 μl of 14-hydroxy-10,12-nonadecadienoic acid (5% HNDE in CH_3OH) was added as internal standard.

After centrifugation at $1500\times g$ for 15 minutes, the chloroform-layer was collected and the solvent removed in a vacuum rotary film evaporator. In a preliminary experiment, the aqueous phase was separated and investigated to ensure that no highly oxidized, water soluble lipids may have escaped into this phase. Since polyoxidized fatty acids were not detected by RP-HPLC and GC/MS analysis the aqueous phase was rejected in later experiments. The lipids obtained from the organic layer were redissolved in 20 ml CHCl_3 , 40 ml CH_3OH and 16 ml bidest. H_2O . Then 1 ml of a 10%-aqueous SnCl_2 -solution in 0.5 ml HCl was added (Frei, Yamamoto, Niclas and Ames, 1988) to convert unstable hydroperoxides to the corresponding hydroxy fatty acids and the mixture was stirred for 60 min. at room temperature. Separation of the phases was achieved by addition of 20 ml CHCl_3 and 20 ml H_2O . The organic layer was washed with 30 ml water and dried under reduced pressure. The extracted lipid fraction was stored on dry ice overnight under argon.

Enrichment of free acids by RP-HPLC

Aliquots of 25 mg raw lipid were dissolved in a mixture of $\text{CH}_3\text{OH}/\text{H}_2\text{O}/\text{AcOH}$ 85:15:0.1 (v:v:v). These solutions were transferred to a SepPak C_{18} cartridge (Waters). The extraction was carried out by use of a vacuum chamber (Supelco) with 10 ml of the same eluent in order to separate free fatty acids from cholesterol and esterified components. After removal of the solvent, the residue was dissolved in 50 μl CH_3OH and injected onto a 250 mm $\times$ 8 mm Spherisorb ODS II column. Reversed-phase HPLC was performed with $\text{CH}_3\text{OH}/\text{H}_2\text{O}/\text{AcOH}$ 950:50:1 (v:v:v) at a flow rate of 4 ml/min. The fraction containing hydroxy fatty acids was recognized by the typical UV absorption-maximum at 234 nm indicating a conjugated diene system (Ingram and Brash, 1988). This fraction – eluting between 4 and 8 minutes – was collected. Finally, the solvents were removed under reduced pressure.

Saponification

The determination of membrane-bound fatty acids required an additional degradation step preceding the enrichment of PUFAs by RP-HPLC: The lipid samples were dissolved in a solution of 20 ml 1M methanolic potassium hydroxide and stirred at 40 °C for 3 hours. After cooling to room temperature, 40 ml H_2O were added to the mixture. Long-chain alcohols and sterol derivatives were removed by thoroughly extraction with 60 ml diethylether (Christie, 1982). Half concentrated HCl was added to the aqueous layer containing the potassium salts of the saponified fatty acids to reach a pH of 2. The acidic compounds were obtained by extraction with 3x30 ml hexane and the organic solvent was removed in a rotary evaporator.

Hydrogenation and derivatization

Saturated trimethylsilylated methylates of hydroxy acids are characterized by intense α -cleavage fragments in their mass spectra (Eglington, Hunnemann and McCormick, 1968; Lehmann, Stephan and Fürstenberger 1992), while those of unsaturated methylates are less informative. Therefore quantification by measuring the ion currents of the α -fragments required hydrogenation of double bonds. The fraction showing high UV-absorption at 234 nm was hydrogenated by bubbling H_2 for 5 minutes through an ethyl acetate solution using a PtO_2 -catalyst (Frank, Wiegand and Remmer, 1984). After filtration through a disposable syringe filter (Waters, Dyna Gard, 0.45 μm /PP) the solvent was removed. The residue was dissolved in etheric diazomethane solution to convert hydroxy fatty acids in their methyl esters. Then the hydroxy functions were trimethylsilylated by addition of 20 μl MSTFA (room temperature, 12 h).

Quantification was achieved by gaschromatography / mass spectrometry single ion monitoring of the peaks resulting from α -cleavage adjacent to the $>\text{CH-OTMS}$ groups.

Results

Two halves of a porcine kidney removed immediately after slaughtering were either homogenized in water to maintain enzyme activity or en-

zymes were inactivated by homogenization in organic solvents, respectively by preceding thermal treatment (Herold and Spiteller, 1996). Separation of fats and free fatty acids was achieved by a Bligh and Dyer extraction with chloroform/methanol. The residue of the chloroform layer was further separated by RP-HPLC in a fraction of oxidized and non-oxidized fatty acids: Lipid hydroperoxides and derived hydroxy acids due to biological reductase activity (Lindstrom and Aust, 1984) possess a conjugated diene system. These dienes show a characteristic UV absorption at 234 nm allowing their detection and separation from non-oxidized fatty acids. In contrast to insignificant UV-absorption of aliquots withdrawn after LOX inhibition, the level of conjugated dienes in samples with sustaining enzyme activity was elevated up to 12 times within a period of 6 hours.

The advantage of this method compared to usual MDA determination is based on complete measurement of all compounds with diene structure while in former investigations using adduct formation between the final degradation product malondialdehyde (MDA) and thiobarbituric acid (Ohkawa, Ohishi and Yagi, 1979) lipid hydroperoxides of linoleic acid and derived hydroxy acids escaped detection, since their oxidation products generate MDA only in traces (Janero, 1990). Hydroxy fatty acids with a conjugated diene system were methylated and trimethylsilylated (Eglington, Hunnemann and McCormick, 1968; Wheelan, Zirolli and Murphy, 1995) to ensure GC separation. Compounds were identified by MS. Quantification was achieved by single ion monitoring of characteristic α -cleavage fragments containing the OTMS- and the methoxycarboxyl group indicating a time-depending distribution pattern of the different isomers (Fig. 1).

Fig. 1 illustrates the predominant presence of free hydroxy fatty acids derived from linoleate (9-/10-/13-HODE) compared to arachidonate (5-/15-HETE) in samples withdrawn from the homogenization mixture in aqueous solution (retained enzyme activity) after the indicated time intervals. This predominance reflects that hydroperoxides after a primary enzymatic induction of lipid peroxidation are nearly exclusively generated by non enzymatic LPO. Radicals formed in this process attack all activated CH_2 -groups with nearly equal probability. Since linoleic acid is much more abun-

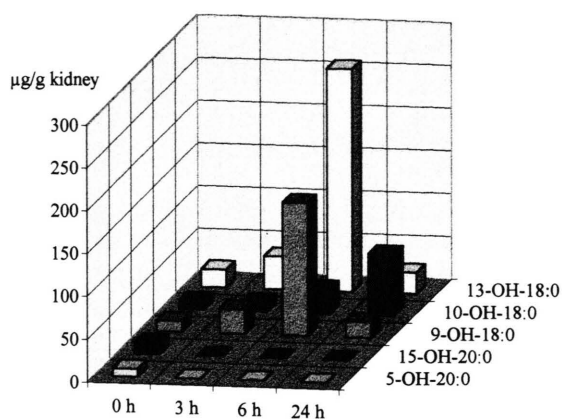


Fig. 1. Amount of the positional isomers of free hydroxy fatty acids in renal tissue (after hydrogenation, methylation and trimethylsilylation; mean values obtained from 5 samples).

dant than arachidonic acid, the oxidation products of the first are dominant. In addition arachidonic acid contains a double bond system which is certainly easier attacked by oxygen, respectively faster degraded than that of linoleic acid derivatives. These deductions are corroborated by the observation that 5- and 15-HETE was detected in samples withdrawn immediately after homogenization (0h) in amounts comparable with those derived from linoleate, while in later extracted samples the ratio changed in favour of linoleic derived hydroxy acids (see Fig. 1). As a consequence, the steady state concentrations of hydroxy acids derived from arachidonate in later extractions showed lower levels than of samples removed from the homogenate immediately after cell disruption. In contrast, the levels of the 9- and 13-hydroxy isomers of linoleic acid increased steadily to maximum values six hours after cell injury, indicating that the equilibrium between formation and degradation of oxidation products is reached much later than that of arachidonic acid. The prevalence of isomers oxidized in position 9 and 13 decreases in later phases of the oxidation period in favour of the 10-hydroxy isomer. This conspicuous enhancement of 10-hydroxy octadecanoic acid after 24 hours needs discussion: As detected by Hudson oleic acid suffers LPO to 10-hydroxy octadecenoic acid in biological media. (Hudson, Mackenzie and Joblin, 1996). In the course of hydrogenation 10-hydroxy-octadecenoate is transformed to 10-hy-

droxy-octadecanoic acid. Thus, its occurrence indicates that in non-enzymatic LPO processes even monoallylically activated CH_2 -groups are attacked slowly. Since biological degradation of hydroxy-octadecenoic acids is more difficult than that of HODEs, the hydroxy octadecenoic acid accumulates in later phases of the experiment.

The total amount of all hydroxy fatty acids was also recorded. The values remained constantly low after blocking of lipoxygenases indicating that lipoxygenases are necessary to induce LPO processes (Fig. 2).

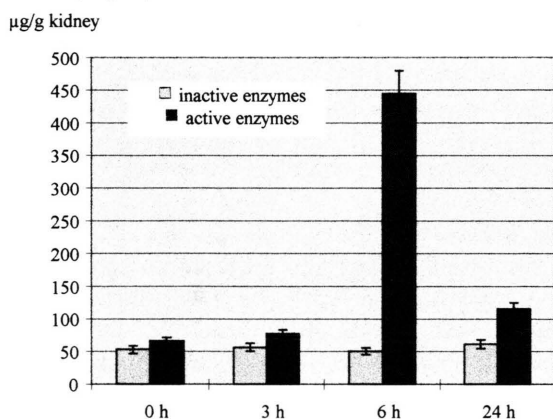


Fig. 2. Total amount of the different positional isomers of hydroxy fatty acids (after hydrogenation, methylation and trimethylsilylation) depending on enzymatic activity.

The enhanced levels of hydroxy isomers of linoleate and arachidonate should be reflected in decreasing amounts of membrane-bound unsaturated fatty acids after tissue injury. Therefore in another set of experiments the content of membrane-bound lipids was determined to investigate the susceptibility of PUFAs integrated in membranes to oxidative attack. Saponification was carried out with raw lipid samples and the obtained free acids were subjected without hydrogenation after trimethylsilylation of the methylesters to a gaschromatographic analysis. The separated products were identified by MS. As expected, a time depending decrease of the different membrane-bound non-oxidized PUFAs was observed. Remarkably the ratio among the different PUFAs remained constant within a time interval of 24 hours (Fig. 3). No evidence of oxidation products in the phospholipids and neutral lipids was observed, pointing to their fast hydrolysis after oxidation by enzymes.

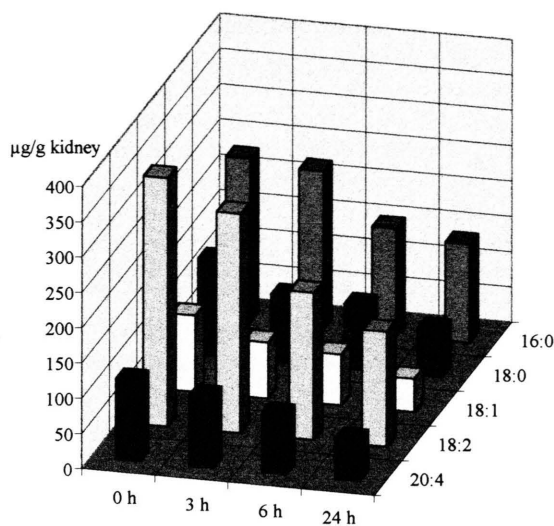


Fig. 3. Total amount of membrane-bound fatty acids detected as methylesters after hydrolysis.

15-LOX was reported to attack conjugated eicosatetraenoic acids in membrane lipids (Kühn, Schewe, Rapoport and Brash, 1988). Since we were unable to isolate any oxidized lipids in the fraction of membrane-bound fatty acids, such lipoxygenases are either not present in porcine kidney tissue or hydroperoxides generated by 15-LOX suffer a fast inactivation or degradation so that they are not detectable.

Discussion

The data presented in this paper confirm that injury of mammalian tissue results in an activation of hydrolases and lipoxygenases as a consequence of cellular injury. This is in agreement with similar reactions observed in plants infested by fungi (Kato *et al.*, 1984; Bostock *et al.*, 1992). Several reperfusion experiments on myocardial infarcted tissue proved an increasing activity of lipases (Grune *et al.*, 1993). The consequence of enzyme activation processes is lipid peroxidation. The generated products are decomposed obviously by bivalent metal ions liberated by proteases (Aust, 1989) – also activated by cell damage – which probably remove the peptide cover of metal containing proteins. As a consequence, the labile oxygen-oxygen bond of hydroperoxides is cleaved resulting in a generation of radicals. These induce a non-enzymatic LPO recognizable by generation

of products which are not only derived from PUFAs but also from oleic acid. Alkoxy radicals may be cleaved to aldehydes (Gardner, 1991) and peroxy radicals may attack PUFAs and other compounds containing double bonds to form epoxides. These may serve as second messengers to induce in surrounding – not yet injured – cells the activation of proteins generating protective compounds.

Since cell damaging processes occur in many diseases connected with inflammation, it is not astonishing that high amounts of oxidized products are generated in the course of mechanical tissue injury, e.g. burnt injury (Hiramatsu *et al.*, 1984; Wooliscroft *et al.* 1990), transplantations (Holter *et al.*, 1990) as well as rheuma (Halliwell, 1993; Jira, Spiteller and Richter, 1997) or atherosclerosis (Steinberg, 1988).

ω -3 fatty acids are ascribed protecting properties against vascular diseases connected with cell injury. Cell damage in turn generates enzymatic LPO products which undergo further non enzymatic steps as discussed above. The radicals formed in these non-enzymatic LPO processes are able to attack any polyunsaturated fatty acid independent of the location of double bonds. Thus, hy-

droperoxides of linolenic acid (Mlakar and Spiteller, 1994) and other ω -3 fatty acids are generated equally easy as those of ω -6 fatty acids in non-enzymatic induced oxidation reactions. Therefore the protecting properties of ω -3 fatty acids are certainly not based on differences in oxidation probability.

In feeding experiments carried out to prove these properties, a diet rich in fish or vegetable oils was fed containing high amounts of ω -3 fatty acids (Sanders and Rohanai, 1983). Fish and vegetables are also rich sources of furan fatty acids found to be potent scavengers of peroxy radicals producing dioxenoic compounds (Batna and Spiteller, 1994). Therefore we suspect that the protecting properties ascribed to ω -3 fatty acids might be caused by accompanying compounds, e.g. furan fatty acids.

Acknowledgements

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