Biochemical Pharmacology, Vol. 38, No. 8, pp. 1209–1216, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

SUBSTRATES FOR ARACHIDONIC ACID CO-OXIDATION WITH PEROXIDASE/HYDROGEN PEROXIDE

FURTHER EVIDENCE FOR RADICAL INTERMEDIATES

FRANK-M. LEHMANN, NORBERT BRETZ,* FRANZ V. BRUCHHAUSEN† and GOTTHARD WURM‡

Institut für Pharmakologie, Freie Universität Berlin, Thielallee 69/73, D-1000 Berlin 33; * Institut für Organische Chemie, Freie Universität Berlin, Takustr. 3, D-1000 Berlin 33; ‡ Institut für Pharmazie, Freie Universität Berlin, and Königin-Luise-Str. 2–4, D-1000 Berlin 33, Federal Republic of Germany

(*Received* 4 *April* 1988; *accepted* 31 *August* 1988)

Abstract—We tested the ability of a wide variety of organic compounds, including benzene and phenol derivatives, aromatic amines, pyrazoline derivatives and other non-steroidal anti-inflammatory drugs, to act as cosubstrates during the horseradish peroxidase/hydrogen peroxide-mediated oxygenation of arachidonic acid. Structural requirements for drug activation in our system proved to be an aromatic system and ring substitution by an easily oxidizable group. Complementary substituents modified drug activation. Among the phenol derivatives and aromatic amines we found the *meta*-substituted compounds to be significantly more effective than their *ortho*- and *para*-substituted analogues, indicating the involvement of radical intermediates in this type of reaction. The radical from 1-phenyl 3-methyl 2-pyrazolone(5) was detected by electron paramagnetic resonance spectroscopy. Kinetic studies on this radical were in good accordance with time-dependent measurement of arachidonic acid oxygenation.

The co-oxidation of suitable drugs during the action of PHS§ was first described by Marnett *et al.* [1]. Later, it was shown that cosubstrate oxidation occurred while prostaglandin G_2 was converted into prostaglandin H_2 by the hydroperoxidase component of the enzyme [2, 3]. These properties are shared by many other heme-containing unspecific peroxidases (e.g. HRP, lactoperoxidase, myeloperoxidase). Co-oxidation reactions catalyzed by peroxidases generate substrate-derived free radicals, which are considered to cause cytotoxic, mutagenic or carcinogenic effects. For the action of PHS this is well documented, e.g. in the case of acetaminophen [4, 5], *p*-aminophenol [6], aminopyrine [7, 8], benzidine [9], BW 755 C and phenidone [10], diethylstilbestrol [11], 1-naphthol [12], *p*-phenetidine [13] and 3,3′,5,5′-tetramethylbenzidine [14].

Recently, we discovered another possible consequence of peroxidase-mediated drug activation: AA oxygenation in the presence of suitable cosubstrates, so-called "reverse co-oxidation" [15]. Products of this reaction have been found to be HPETEs and the corresponding HETEs. This may be of physiological importance, considering the biological function of these compounds as potent mediators of allergy and inflammation.

The aim of this study was to present evidence for the cosubstrate-derived radical to be the causative agent in peroxidase-mediated AA oxygenation. Structural requirements were to be worked out for chemicals, acting as one-electron donors in this type of reaction, among them drugs, for which radical formation by peroxidases has not yet been described. Apparent inconsistency of our results with pharmacological properties of some of the tested drugs are discussed.

† To whom reprint requests should be addressed.

§ Abbreviations used: AA, arachidonic acid; EPR, electron paramagnetic resonance; HETE, hydroxyeicosatetraenoic acid; HPETE, hydroperoxyeicosatetraenoic acid; HPLC, high-pressure liquid chromatography; IR, infrared; NSAID, non-steroidal anti-inflammatory drug; PHS, prostaglandin H synthase; PMP, 1-phenyl 3-methyl 2-pyrazolone (5); TLC, thin layer chromatography.

MATERIALS AND METHODS

Chemicals. [1-^{14}C] AA (specific activity 50–59 Ci/mol = 1.85–2.18 TBq/mol) was purchased from Amersham Buchler (Braunschweig, F.R.G.) and from Du Pont de Nemours (Dreieich, F.R.G.). HRP (type II, 200 U/mg solid), lipoxygenase (type I from soybean, 150,000 U/mg solid), glutathione peroxidase (from bovine erythrocytes, 300–700 U/mg solid) and unlabelled AA (purity > 99%) were purchased from Sigma Chemicals (München, F.R.G.) and used without further purification.

H_2O_2 and all organic solvents were purchased from Merck (Darmstadt, F.R.G.). The tested phenol derivatives, aromatic amines, pyrazolines and other NSAIDs, the carcinogens and all other applied substances were of reagent grade and obtained from commercial sources.

Incubation and extraction procedure. The production of HPETEs and HETEs from AA was determined in an assay system containing K_2HPO_4 buffer (0.1 M, pH 8.0), [1-^{14}C]AA (2.5 μM), HRP (10 μg), H_2O_2 (10^{-2}–10^{-6} M) in the presence or absence of various cosubstrates (2×10^{-3}–10^{-5} M), the pH being carefully adjusted to 8.0. Incubation (20 min, 37°), reaction stop and selective extraction of non-oxidized AA were carried out in a sample volume of 300 μl according to Knippel *et al.* [16]. The labelled

Table 1. Amounts of HPETEs and HETEs generated in a donor/HRP/H_2O_2-system depending on the chemical structure of added benzene and phenol derivatives.

Substance	Yield in % of AA x	±SD	Structure	
Benzene	—	—	1:	H
Phenol	34	11.3	1:	OH
Anisole	—	—	1:	OCH_3
Pyrocatechol			2:	
Resorcinol	29	4.5	3:	OH
Hydroquinone			4:	
o-	37	4.2	2:	
m-Cresol	51	9.0	3:	CH_3
p-	40	7.5	4:	
p-Ethylhenol	71	2.0	4:	C_2H_5
p-Propylphenol	77	4.1	4:	C_3H_7
o-	—	—	2:	
m-Aminophenol	—	—	3:	NH_2
p-	—	—	4:	
o-	19	4.9	2:	
m-Acetaminophen	72	10.6	3:	$NHCOCH_3$
p-	37	5.0	4:	
o-	47	2.1	2:	
m-Nitrophenol	72	7.9	3:	NO_2
p-	27	5.2	4:	
o-	35	6.0	2:	
m-Hydroxydiphenyl	73	13.5	3:	C_6H_5
p-	48	9.1	4:	
2,6-Dimethylphenol	29	3.0	2:	CH_3
			6:	
2,6-Di-tert.-butylphenol	—	—	2:	C_4H_9
			6:	
Salicylic acid	—	—	2:	
m-Hydroxybenzoic acid	15	5.7	3:	COOH
p-	37	14.0	4:	
Protocatechuic acid	—	—	2:	OH
			4:	COOH
Vanillic acid	35	9.1	2:	OCH_3
			4:	COOH
3,5-Dihydrobenzoic acid	—	—	3:	OH
			5:	COOH
Diflunisal	24	3.7	2:	COOH
			4:	$C_6H_4F_2$
Diethylstilbestrol	21	2.9	4:	$C_2(C_2H_5)_2C_6H_4$

Final concentrations in a total sample volume of 300 µl were: HRP 10 µg, H_2O_2 2–4 mM, donor 1 mM, [1-^{14}C]AA 2.5 µM in 0.1 M phosphate buffer, pH 8.0. Mean values (x) and standard deviations (SD) are calculated from at least five experiments.

products of AA were quantified by liquid scintillation counting.

Identification of oxygenated AA products by TLC. After different incubation periods (2–20 min) and temperatures (25° or 37°), the reaction was terminated and AA and its oxygenated products were extracted by adding 2.5 vol. of ice-cooled ethyl acetate three times. All subsequent steps were carried out in the cold (0–4°) to protect the hydroperoxides from autoxidation. The organic layers were pooled and the solvent evaporated under nitrogen stream. The residue was redissolved in 60 µl benzene/ethanol (1:1, v/v) and applied to 20 × 20 cm precoated silica gel glass plates (0.5 mm, impregnated with fluorescent indicator F 254, Merck, Darmstadt, F.R.G.) with Hamilton microsyringes using a Linomat III-applicator (Camag, Berlin, F.R.G.). Solvent systems were diethylether/n-hexane/acetic acid (60:40:1, v/v/v) or chloroform/methanol/acetic acid/water (80:8:1:0.8, v/v/v/v) according to Salmon *et al.* [17]. The radioactive zones were detected using a Berthold LB 2723 scanner (Wildbad, F.R.G.). Labelled lipoxygenase products were used as authentic standards and prepared as follows: [1-^{14}C]15-HPETE was obtained by incubating soybean lipoxy-

Table 2. Amounts of HPETEs and HETEs generated in a donor/HRP/H_2O_2-system depending on the chemical structure of added aromatic amines and related compounds. For further details see legend to Table 1

Substance	Yield in % of AA x	±SD		Structure
Aniline	32	8.4	1:	NH_2
N-Methylaniline	79	10.1	1:	$NHCH_3$
N,N-Dimethylaniline	—	—	1:	$N(CH_3)_2$
o-	35	2.6	2:	
m-Toluidine	45	5.0	3:	CH_3
p-	7	0.8	4:	
2,6-Dimethylaniline	28	3.7	2:	
			6:	CH_3
3,5-Dimethylaniline	52	8.2	3:	
			5:	CH_3
o-	19	3.9	2:	
m-Phenylenediamine	26	1.8	3:	NH_2
p-	10	0.6	4:	
N,N-Dimethyl-p-phenylenediamine	16	3.4	4:	$N(CH_3)_2$
N,N,N',N'.Tetramethyl-p-phenylenediamine	—	—	1:	
			4:	$N(CH_3)_2$
o-	8	0.9	2:	
m-Phenetidine	33	2.7	3:	OC_2H_5
p-	9	1.4	4:	
o-	—	—	2:	
m-Acetophenetidine	—	—	3:	OC_2H_5
p-	—	—	4:	
o-	—	—	2:	
m-Nitroaniline	—	—	3:	NO_2
p-	—	—	4:	
Anthranilic acid	21	2.1	2:	
m-Aminobenzoic acid	28	3.9	3:	COOH
p-	37	2.6	4:	
Mefenamic acid	43	1.7	1:	$NH[C_6H_4(CH_3)_2]$
			4:	C_6H_4COOH
Flufenamic acid	18	0.9	1:	$NH(C_6H_4CF_3)$
			4:	C_6H_4COOH
Benzidine	40	7.4	4:	$C_6H_4(C_6H_4NH_2)$
N,N,N',N'-Tetramethylbenzidine			1:	$N(CH_3)_2$
	—	—	4:	$C_6H_4[C_6H_4N(CH_3)_2]$

genase (2 μg) and [1-^{14}C]AA in K_2HPO_4 buffer (0.1 M, pH 8.0) in a total sample volume of 300 μl [18]. An incubation time of 2 min was sufficient to yield quantitative conversion of AA into the single product, which was extracted as described above. To prepare [1-^{14}C]15-HETE, the preceding steps were followed by the addition of glutathione peroxidase (3 μg) and reduced glutathione (10^{-4} M) and another incubation period of 5 min before the extraction procedure was carried out.

Radical detection by EPR. Spectra were recorded on an EPR spectrometer (Bruker 200 END console, X-band spectrometer, AEG 20 × magnet) and a variable temperature accessory. Measurements were carried out under anaerobic conditions. The solutions were freed from oxygen by gassing with highly purified nitrogen before use, and the sample was set under argon atmosphere. The reaction was started by adding HRP anaerobically, and the capillary tube was placed immediately into the EPR cavity. The neutral radical of PMP was produced chemically by adding KOH/$[K_3(FeCN)_6]$ to a solution of the substance in oxygen-free toluene.

Determination of hydrogen bonding by IR-spectroscopy. Spectra of hydroxybenzoic acids were recorded on a Perkin–Elmer 1420 ratio-recording IR spectrometer in KBr solution.

Measurement of oxygen uptake. AA-dependent oxygen consumption was determined using a Biometer equipped with a Clark electrode (B. Braun, Melsungen, F.R.G.). Reaction was started by adding HRP. Concentrations were: HRP 70 μg, H_2O_2 2 mM, PMP 1 mM, AA 10^{-3}–10^{-5} M in a sample volume of 2.5 ml.

RESULTS

A total of more than 100 compounds of different

Table 3. Amounts of HPETES and HETEs generated in a donor/HRP/H_2O_2-system depending on the chemical structure of added acid NSAIDs. For further details see legend to Table 1

Substance	Yield in % of AA x	$\pm$SD
Acetylsalicylic acid	—	—
Benoxaprofen	—	—
Carprofen	—	—
Diclofenac	—	—
Diflunisal	24	3.2
Flufenamic acid	18	0.9
Indometacin	—	—
Mefenamic acid	43	1.7
Naproxen	—	—
Piroxicam	—	—
Tiaprofenic acid	—	—

chemical origin was tested concerning their suitability for HRP-mediated activation, resulting in AA oxygenation. Discrepancies between our study and that of Baumann *et al.* [15] concerning the yields of reaction products in the case of some tested drugs were due to deviations in pH after alkaline solubilization procedures, which were not completely compensated in their study. For reasons of a better overview the (repeated) earlier data and our new results are jointly summarized in the tables. Blanks with omission of cosubstrates yielded only small amounts (2–8%) of fatty acid-derived products. The lack of either enzyme or H_2O_2 completely inhibited AA conversion. Comparison of the applied phenol derivatives led to the following findings (Table 1):

(1) Benzene did not undergo peroxidase-mediated oxidation, phenol, however, was activated in our system. Esterification of the free hydroxyl group or transformation to an ether (e.g. anisole) abolished AA oxygenation.
(2) A carboxylic function neighbouring the free hydroxyl group inhibited peroxidase-mediated activation of the phenol derivatives to reactive intermediates (e.g. salicylic acid). This was in contrast to the results obtained with *m*- and *p*-hydroxybenzoic acids. Dihydroxybenzoic acids, independent of their sterical structure, remained inactive in our system.
(3) Whereas the peroxidase activated 2,6-dimethylphenol, the bulky substituents of 2,6-di-tert. butylphenol abolished AA oxygenation.
(4) Increasing length of the side chain corresponded with enhanced potency of the phenol derivatives concerning AA oxygenation (*p*-cresol < *p*-ethylphenol < *p*-propylphenol).
(5) *Meta*-substitution resulted in a remarkably higher cooxidative potency of the mono-substituted phenol derivative than did *o*- and *p*-substitution (e.g. diphenols, acetaminophens, hydroxydiphenyls).
(6) Aminophenols, although being compounds of which radical formation during the action of peroxidases is well documented [6, 19], did not cause AA oxygenation in our system (Table 1). This was, however, observed using their *N*-acetylated (acetaminophens) and *O*-ethylated (phenetidines) derivatives. Substitution of both the free hydroxyl group and the free amino group to a tertiary amine (acetophenetidines) completely inhibited fatty acid conversion.
(7) The carcinogen, diethylstilbestrol, which can be regarded as phenol derivative with stilbenediol structure, also initiated AA oxygenation after being activated by HRP/H_2O_2.

The aromatic amines and related compounds examined showed similar characteristics during co-oxidation reactions (Table 2). In addition, we found:

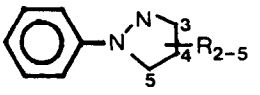
(1) a free amino group enabled organic molecules to act as electron donors during HRP-mediated cooxidation of AA (e.g. aniline);
(2) secondary amines increased the extent of AA oxygenation (e.g. *N*-methylaniline);
(3) tertiary amines abolished fatty acid conversion in our system (e.g. *N,N*-dimethylaniline);
(4) the carcinogen, benzidine, a primary diamine, was activated to yield AA oxygenation, whereas *N,N,N',N'*-tetramethylbenzidine, a tertiary diamine, remained inactive.

The acidic NSAIDs tested included single representatives possessing co-oxidative potency towards AA (Table 3). Flufenamic and mefenamic acid, both of which are anthranilic acid derivatives, served as electron donors during peroxidase-mediated reactions. Diflunisal, a salicylic acid derivative, also showed co-oxidative potency, whereas its parent compound remained inactive. To elucidate this discrepancy, C = O valence vibrations of both were compared using IR spectroscopy (spectra not shown). Salicylic acid presented C = O valence vibrations of a low wave number, indicating distinct hydrogen bonding between the carboxylic function and the neighbouring hydroxyl group (υ_{CO} = 1658 cm^{-1}). The same result was obtained with 5-acetamidosalicylic acid. Diflunisal, however, showed IR properties (υ_{CO} = 1681 cm^{-1}) similar to *p*-hydroxybenzoic acid. In addition, C = O valence vibrations for 3,4-dihydroxybenzoic acid (protocatechuic acid, υ_{CO} = 1678 cm^{-1}) and 3,5-dihydroxybenzoic acid (υ_{CO} = 1691 cm^{-1}) were determined, both of which were inactive in our system.

Among the pyrazoline derivatives several NSAIDs proved to be potent cosubstrates for peroxidase-mediated AA oxygenation (Table 4). While phenidone and PMP yielded highest amounts of co-oxidation products, antipyrine and 4-aminoantipyrine were inactive in our system.

In order to obtain further evidence for the involvement of cosubstrate-derived radical intermediates in AA oxygenation, we used EPR spectroscopy. In the case of PMP, which showed excellent reactivity and to our knowledge was not yet studied using this technique, we were successful. PMP addition to the mixture produced a well resolved 1:1:1 three-line signal with a *g*-value of 2.0008 and a coupling constant of a_N = 0.67 mT (Fig. 1). To confirm the electrochemical structure of the radical we made experiments in enzyme-free systems under protonating, dehydrating and neutral conditions. Only the oxidation of PMP by potassium hexocyanoferrate

Table 4. Amounts of HPETEs and HETEs generated in a donor/HRP/H_2O_2-system depending on the chemical structure of added 1-phenyl pyrazoline derivatives. For further details see legend to Table 1



Substance	Yield in % of AA x	±SD	Structure	
Phenidone	82	10.7	2:	H
			3:	=O
			4:	H_2
			5:	H_2
1-Phenyl-3-methyl-2-pyrazolone (5)	83	11.4	2:	H
			3:	CH_3
			4:	H
			5:	=O
Antipyrine	—	—	2:	CH_3
			3:	CH_3
			4:	H
			5:	=O
4-Aminoantipyrine	—	—	2:	CH_3
			3:	CH_3
			4:	NH_2
			5:	=O
Aminopyrine	17	1.8	2:	CH_3
			3:	CH_3
			4:	$N(CH_3)_2$
			5:	=O
Phenylbutazone	68	9.0	2:	C_6H_5
			3:	ONa
			4:	$(CH_2)_3CH_3$
			5:	=O
Oxyphenbutazone	84	12.4	2:	C_6H_4OH
			3:	=O
			4:	$(CH_2)_3CH_3$
			5:	=O
Sulfinpyrazone			2:	C_6H_5
			3:	=O
			4:	$(CH_2)_2SOC_6H_5$
			5:	=O
BW 755 C	24	3.9	1:	$C_6H_4CF_3$
			2:	H
			3:	NH_2
			4:	H
			5:	H_2

resulted in a spectrum, which was identical to that obtained by HRP/H_2O_2 with $g = 2.0045$ and $a_N =$ 0.67 mT (spectrum not shown). These data indicate that the radical from PMP is a neutral aminyl radical. Time-dependent recording with a constant magnetic field setting (338.96 mT) resulted in a specific radical kinetic (Fig. 2), which gave us the possibility to prove its causative action in AA oxygenation. Following a resting state of about 2 min, during which no signal was observed, the radical formed rapidly and decayed with a half-life of about 1.75 min. Absence of either enzyme, H_2O_2 or cosubstrate, as well as the use of heat-denatured enzyme did not produce any radical signal. The lag period preceding radical formation appeared to be dependent on H_2O_2 concentration at constant temperature conditions: increasing amounts of peroxides lengthened and decreasing amounts shortened the lag period. These findings were interpreted as inhibitory effects of high peroxide levels on the enzyme. Comparison of the particular kinetic of the PMP radical, ascertained by EPR-spectroscopy, with time-dependent oxygen uptake in a system containing HRP, H_2O_2, PMP and AA revealed corresponding results (Fig. 3): depending on the molarity of added H_2O_2 to the reaction sample, the onset of oxygen uptake also followed a certain lag period, during which no oxygen consumption was detectable and which increased with higher H_2O_2 concentration. A cosubstrate hydroperoxide ratio of 0.5 proved to result in maximal oxygen uptake at a fixed AA concentration of 400 μM, H_2O_2 concentrations of lower than 200 μM or above 10 mM abolished oxygen consumption with PMP kept at 1 mM. Omission of either PMP or AA

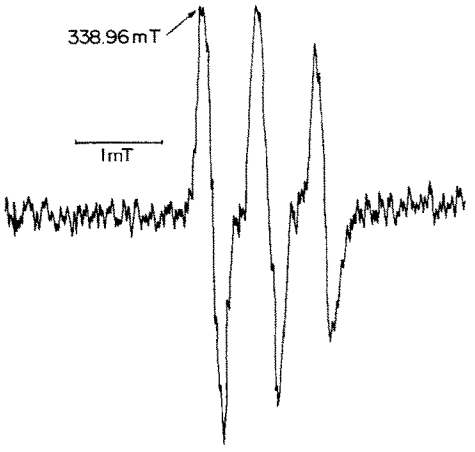


Fig. 1. EPR spectrum of the radical generated from PMP by HRP and H_2O_2. Concentrations in a total sample volume of 300 μl: HRP 4 μg, H_2O_2 1.2 mM, PMP 1 mM in 0.05 M phosphate buffer, pH 8, coupling constant a_N = 0.67 mT, g-value = 2.0008. Instrumental settings: Temperature 298 °K, microwave power 0.63 mW, modulation amplitude 0.1 mT, receiver gain 1.6×10^{-6}, time constant 0.2 s, scan range 10 mT, scan time 500 sec.

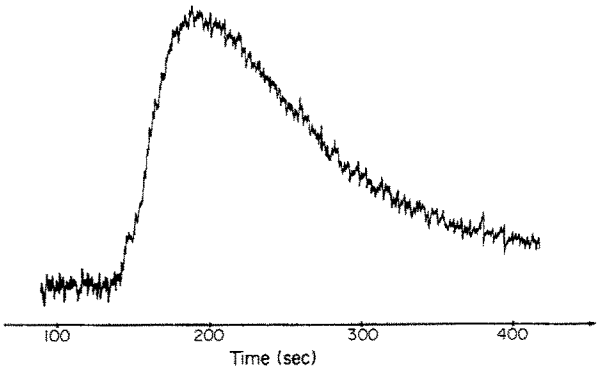


Fig. 2. Kinetics of the radical generated from PMP by HRP and H_2O_2. Concentrations and instrumental settings were as in Fig. 1. Field frequency was locked at 338.96 mT.

did not lead to any measurable reaction by using this method.

It has to be mentioned that fundamental experimental differences exist between the two methods using EPR-spectroscopy and the Clark oxygen electrode (e.g. absence of oxygen in the first, oxygen-enriched and rapidly stirred solutions in the latter case), which can account for discrepancies found for the lag periods, measured under otherwise comparable conditions. Nevertheless, our results support the postulated radical mechanism, in which first the neutral PMP radical reacts with AA, prior to oxygen uptake. The PMP radical itself does not react with molecular oxygen in a measurable amount so that the formation of a PMP peroxyradical as triggering mechanism for AA conversion can be excluded.

The oxygenated products were separated from the fatty acid precursor by TLC. Depending on the cosubstrate added, large amounts of radiolabelled compounds were formed, which were more hydrophilic than AA and whose major portion cochromatographed with 15-HPETE and 15-HETE. To exclude adduct formation between AA and the cosubstrate, fluorescence indicator-impregnated silica gel plates were used to facilitate the detection of aromatic structures. In no case did the fluorescence-quenched areas representing the cosubstrate or its oxidized derivatives comigrate with the radiolabelled zones detected by the TLC-scanner. After detection, the radiolabelled products were scraped off and their UV spectra recorded in aqueous solution. Comparison with spectra derived from the pure cosubstrates revealed no similarities, indicating that no adduct-formation took place.

Further separation of positional isomers (e.g. 5-, 8-, 9-, 11-, 12- and 15-HETE), which do not differ concerning their relative R_f-values in our system, was recently carried out by Just [20] by two-dimensional TLC and reversed-phase HPLC. He also used HRP/H_2O_2 as enzymatic source for cosubstrate-triggered AA oxygenation and, in the cases of p-cresol, mefenamic acid, paracetamol, phenylbutazone and PMP as cosubstrates, he found a mixture of all possible hydroxyeicosatetraenoic acids, among them 5- and 15-HETE.

DISCUSSION

The hydroperoxide-supported generation of substrate-derived free radical intermediates by HRP has been studied in detail. It has been shown that the reaction proceeds via a stepwise mechanism, during which two molecules of cosubstrate (XH) are oxidized per molecule hydroperoxide (ROOH) [21]. Referring to the following scheme [Eqns (1)–(3)], compound I represents the two-, compound II the one-electron oxidation state of the enzyme:

$$\text{HRP} + \text{ROOH} \rightarrow \text{comp. I} + \text{ROH} \quad (1)$$
$$\text{comp. I} + \text{XH} \rightarrow \text{comp. II} + \text{X}^* \quad (2)$$
$$\text{comp. II} + \text{XH} \rightarrow \text{HRP} + \text{H}_2\text{O}_2 + \text{X}^* \quad (3)$$

During the action of PHS, enzyme species were detected showing similar spectral properties with compound I and II of HRP, suggesting the same electron transfer mechanism via ferryl-oxo-complexes during cooxidation reactions [22]. It has been described that different types of radicals are generated by peroxidases, which can be carbon-centered, as in the case of phenylbutazone [23], oxygen-centered as the phenoxy radical of acetaminophen [5], sulfur-centered like the thiyl radicals derived from glutathione [24] or nitrogen-centered as it occurs in the case of the aminopyrine cation radical [8]. Our experiments using PMP as cosubstrate for HRP/H_2O_2 demonstrate the formation of a neutral aminyl radical, which has not yet been described. Experimental data concerning redox potentials of the one-electron oxidation steps to the radical states of organic molecules are hardly available and subsequent reactions of these radicals difficult to foresee: a wide variety of possible actions is described, among them disproportionation, di- and polymerization, formation of charge-transfer-com-

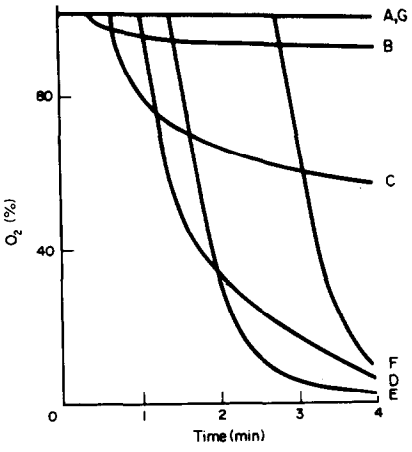


Fig. 3. Time curves of oxygen consumption. The total sample volume of 2 ml contained: HRP (70 μg), AA (400 μM), PMP (1 mM), and different concentrations of H_2O_2 (A: no H_2O_2, B: 250 μM, C: 500 μM, D: 1 mM, E: 2 mM, F: 4 mM, G: 10 mM).

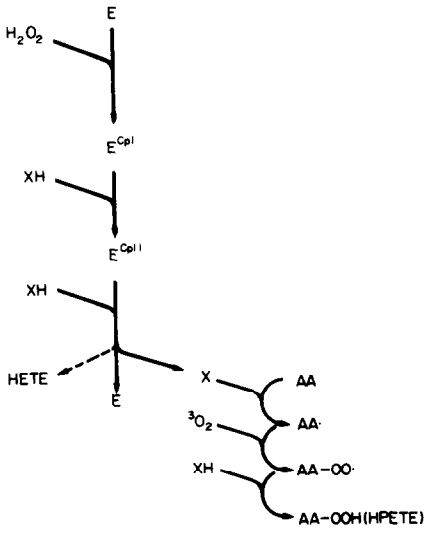


Fig. 4. Hypothetical mechanism for AA oxygenation caused by free radical intermediates generated from suitable cosubstrates in a peroxidase/H_2O_2-containing system. AA* = AA radical; AA-OO* = AA peroxy radical; E = native enzyme; E^{CpI} = peroxidase compound I; E^{CpII} = peroxidase compound II; XH = cosubstrates; X* = co-substrate-derived free radical.

plexes, adduct formation with macromolecules like proteins or DNA and with glutathione and other radical-chain reaction cascades. Our experiments dealing with the peroxidation of 20:4 unsaturated fatty acid triggered by enzymatically formed organic radicals, revealed some structural requirements for the reactivity of those species towards AA: regarding the tested phenol- and anilin-derivatives, an easily oxidizable group bound to the aromatic system proved to be essential (non-substituted benzene was inactive).

Additional substituents considerably altered the oxidative potency of the tested compound towards AA. Electrochemical interactions (e.g. hydrogen bonding) between the free hydroxyl groups and neighbouring substituents prevented peroxidase-mediated drug activation. This proved to be the case with salicylic acid in IR spectroscopic studies. Steric hindrance by bulky substituents had similar effects.

Neither cosubstrates known to generate long-living radicals such as 2,6-di-tert. butylphenol, nor chemicals generating extremely short-living radicals (like the aminophenols) yielded fatty acid conversion worth mentioning. The exceptional position of the *m*-substituted phenol derivatives and aromatic amines must be interpreted in an analogous manner. While the semiquinone radicals of the *o*- and *p*-dihydroxybenzene (pyrocatechol, hydroquinone) spontaneously disproportionate into corresponding diphenols and quinones, this reaction pathway is not possible for the *m*-semiquinone radical. Hence, we concluded that, in our system, the radical intermediate (and not the quinone) causes AA oxygenation. Differences concerning the efficacy of the tested position isomers should be due to the properties of the corresponding radicals. They might either be caused by a higher reactivity of the *m*-substituted radical or by an earlier radical chain break-off in the case of the *o*- and *p*-substituted radicals.

Whereas these findings only indicate that most probably cosubstrate-derived radicals are responsible for the observed fatty acid oxygenation, evidence for this hypothesis was found only in the case of PMP. Although the specific kinetic of the aminyl radical has not yet been sufficiently clarified, it seems to be a promising model to study the effects of peroxidase-mediated drug-derived radicals on biological structures without being obliged to use extensive experimental efforts such as fast-flow EPR-techniques. Further experiments on this field will follow. Our results support the conception of Baumann *et al.* [15], who suggested a radical chain reaction cascade, during which the free radical reacts with the fatty acid under hydrogen abstraction (Fig. 4), while slightly correcting some of their results. The following steps are supposed to imitate a classical lipoxygenase reaction. This includes the formation of a peroxy radical with triplet oxygen, electron shift generating a conjugated diene and simultaneous hydration to the hydroperoxide, whereby another cosubstrate-derived radical is produced.

Products of AA oxygenation, mediated by peroxidases in the presence of suitable drugs, were found to be HPETEs and HETEs, among them the biologically highly active 5- and 15-isomers. These findings, which have in the meantime been supported by Just [20], are in accordance with Fridovich and Porter [25], who identified 5- and 15-HPETE besides lower amounts of 8-, 9-, 11- and 12-isomers after incubating AA in a hydroxyl radical-generating system. Bösterling and Trudell [26] also described preferential formation of 5- and 15-HPETE from AA caused by physically generated halothan radicals *in vitro*.

The anti-inflammatory effects of some of the drugs

tested (known as NSAIDs) and the suggested eicosanoid formation after their enzymatic activation seem to be contradictory and explanations are difficult to provide. In addition, the plant-derived origin of the model enzyme HRP limits possible conclusions concerning the physiological relevance of our results. On the other hand, preliminary studies dealing with lactoperoxidase, myeloperoxidase and PHS revealed similar findings suggesting this mechanism to play a role in mammalian tissue. Intensive further studies on this field remain to be made. Peroxidase-mediated drug activation resulting in the formation of potent eicosanoids from AA may serve as one reason for otherwise inexplicable side-effects of those drugs, possibly including the well-known allergizing potency of substances containing the so-called para-groups.

Acknowledgements—The authors wish to thank Mrs Ellen Mahlow for excellent technical assistance, and Mrs Rosemarie Krüger for typing the manuscript. This work was supported by a grant of the Deutsche Forschungsgemeinschaft given to F. v. B.

REFERENCES

1. Marnett LJ, Wlodawer P and Samuelsson B, Light emission during the action of prostaglandin synthetase. *Biochem Biophys Res Commun* **60**: 1286–1294, 1974.
2. Egan WE, Gale PH, Baptista EM and Kuehl FA, Mechanism of prostaglandin hydroperoxidase cooxygenation reactions. *Progr Lip Res* **20**: 173–178, 1981.
3. Baumann J, v Bruchhausen F and Wurm G, Decreasing inhibitory potency of prostaglandin synthetase inhibition during their cooxidative metabolism. Studies on aminophenols, pyrazolone derivatives and 1,3-diphenylisobenzofuran. *Pharmacol* **27**: 267–280, 1983.
4. Nelson SD, Dahlin DC, Rauckman EJ and Rosen GM, Peroxidase-mediated formation of reactive metabolites of acetaminophen. *Mol Pharmacol* **20**: 195–199, 1981.
5. West PR, Harman LS, Josephy PD and Mason RP, Acetaminophen: enzymatic formation of a transient phenoxyl free radical. *Biochem Pharmacol* **33**: 2933–2936, 1984.
6. Josephy PD, Eling TE and Mason RP, Oxidation of *p*-aminophenol catalyzed by horseradish peroxidase and prostaglandin synthase. *Mol Pharmacol* **23**: 461–466, 1983.
7. Renneberg R, Damerau W, Jung C, Ebert B and Scheller F, Study of H_2O_2 supported N-demethylations catalyzed by cytochrome P-450 and horseradish peroxidase. *Biochem Biophys Res Commun* **113**: 332–339, 1983.
8. Eling TE, Mason RP and Sivarajah K, The formation of aminopyrine cation radical by the peroxidase activity of prostaglandin H synthase and subsequent reactions of the radical. *J Biol Chem* **260**: 1601–1607, 1985.
9. Josephy PD, Eling TE and Mason RP, An electron spin resonance study of the activation of benzidine by peroxidases. *Mol Pharmacol* **23**: 766–770, 1983.
10. Marnett LJ, Siedlik PH and Fung LWM, Oxidation of phenidone and BW 755 C by prostaglandin endoperoxide synthetase. *J Biol Chem* **257**: 6957–6964, 1982.
11. Ross D, Melhorn RJ, Moldéus P and Smith MT, Metabolism of diethylstilbestrol by horseradish peroxidase and prostaglandin H synthase. Generation of a free radical intermediate and its interaction with glutathione. *J Biol Chem* **260**: 16210–16214, 1985.
12. Doherty MDA, Wilson I, Wardman P, Basra J, Patterson LH and Cohen GM, Peroxidase activation of 1-naphthol to naphthoxy or naphthoxy-derived radicals and their reaction with glutathione, *Chem Biol Interact* **58**: 199–215, 1986.
13. Moldéus P, Ross D and Larsson R, Co-oxidations of xenobiotics. *Biochem Soc Trans* **13**: 847–850, 1985.
14. Josephy PD, Eling TE and Mason RP, The horseradish peroxidase-catalyzed oxidation of 3,5,3′,5′-tetramethyl benzidine. Free radical and charge-transfer complex intermediates. *J Biol Chem* **257**: 3669–3675, 1982.
15. Baumann J, Just I and Wurm G, Oxidation of suitable drugs by horseradish peroxidase/hydrogen peroxide results in the oxygenation of arachidonic acid to form hydroperoxyeicosatetraenoic acids in a reverse cooxidation process. *Agents Actions* **17**: 392–394, 1985.
16. Knippel I, Baumann J, v Bruchhausen F and Wurm G, Interactions of sulfhydryl agents and soybean lipoxygenase inhibitors. *Biochem Pharmacol* **30**: 1677–1684, 1981.
17. Salmon JA and Flower RJ, Extraction and thin-layer chromatography of arachidonic acid metabolites. In: *Methods in Enzymology* (Eds. Lands WEM and Smith WL), Vol. 86, pp. 477–493. Academic Press, New York, 1982.
18. Funk MO, Isaac R and Porter NA, Preparation and purification of lipid hydroperoxides from arachidonic and gamma-linolenic acids. *Lipids* **11**: 113–117, 1976.
19. Subrahmanyam VV, McGirr LG and O'Brien PJ, Glutathione oxidation during peroxidase-catalysed drug metabolism. *Chem-Biol Interactions* **61**: 45–59, 1987.
20. Just I, Die Entstehung von Arachidonsäure-Oxygenierungsprodukten in Gegenwart von Peroxidasen und Fremdstoffen. Inauguraldiss. Med. Fachbereiche Freie Universität Berlin, 1988.
21. Chance B, Powers L, Ching Y, Poulos T, Yamazaki I and Paul KG, X-Ray absorption studies of intermediates in peroxidase activity. *Arch Biochem Biophys* **235**: 596–611, 1984.
22. Lambeir A-M, Markey CM, Dunford HB and Marnett LJ, Spectral properties of the higher oxidation states of prostaglandin H synthase. *J Biol Chem* **260**: 14894–14896, 1985.
23. Egan RW, Gale PH, Vanden-Heuvel WJ, Baptista E and Kuehl FA, Mechanism of oxygen transfer by prostaglandin hydroperoxidase. *J Biol Chem* **255**: 323–326, 1980.
24. Eling TE, Curtis JF, Harman LS and Mason RP, Oxidation of glutathione to its thiyl free radical metabolite by prostaglandin H synthase. A potential endogenous substrate for the hydroperoxidase. *J Biol Chem* **261**: 5023–5028, 1986.
25. Fridovich SE and Porter NA, Oxidation of arachidonic acid in micelles by superoxide and hydrogen peroxide. *J Biol Chem* **256**: 260–265, 1981.
26. Bösterling B and Trudell JR, Production of 5- and 15-hydroperoxyeicosatetraenoic acid from arachidonic acid by halothane free radicals generated by UV-irradiation. *Anaesthesiol* **60**: 209–213, 1984.