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7 Lipoxygenases

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Introduction

Biotransformation of xenobiotics represents the key element in the understanding of the pharmacological efficacy of drugs and toxicity of pesticides, industrial chemicals, environmental contaminants/pollutants and other chemicals. Most, if not all, organic chemicals undergo oxidation in the body. Depending upon the chemical, oxidation may result in an increase or decrease in the biological response of the parent compound. The contribution of enzymes such as microsomal cytochrome P450 (P450), flavin-containing monooxygenase (MFMO), prostaglandin H synthase (PGS) and few others in the oxidation of xenobiotics is well documented. About twelve years ago, the potential importance of the lipoxygenase (LO) pathway in xenobiotic oxidation was recognised (Kulkarni and Cook 1988a,b). Several publications from different laboratories have now established LO as one of the major enzymes of xenobiotic oxidation. This chapter primarily focuses on the available literature on the subject. The discussion is intended to be provocative, to promote future research rather than be definitive as this area of investigation is just beginning to evolve.

Biochemistry and properties of lipoxygenases

Arachidonic acid, an essential polyunsaturated fatty acid, is oxidised in the body by three different enzymes, i.e. P450, LO and PGS (Figure 7.1). Functionally, these enzymes of arachidonic acid cascade differ from each other in the oxygenation process. P450 is a monooxygenase and inserts one of the two atoms of O_2 into arachidonic acid while LO, being a dioxygenase, incorporates both atoms of O_2 into arachidonic acid. PGS, on the other hand, is a bis-dioxygenase and adds four atoms of oxygen to arachidonic acid. The second difference resides in the catalytic centre of these enzymes. It is noteworthy that both P450 and PGS are microsomal haemoproteins while LOs are non-haemo iron proteins found mainly in the cytosol.

LOs are ubiquitous in aerobic organisms. They occur in many species of algae (Gerwick 1994), plants (Siedow 1991; Grechkin 1998), aquatic invertebrates (De

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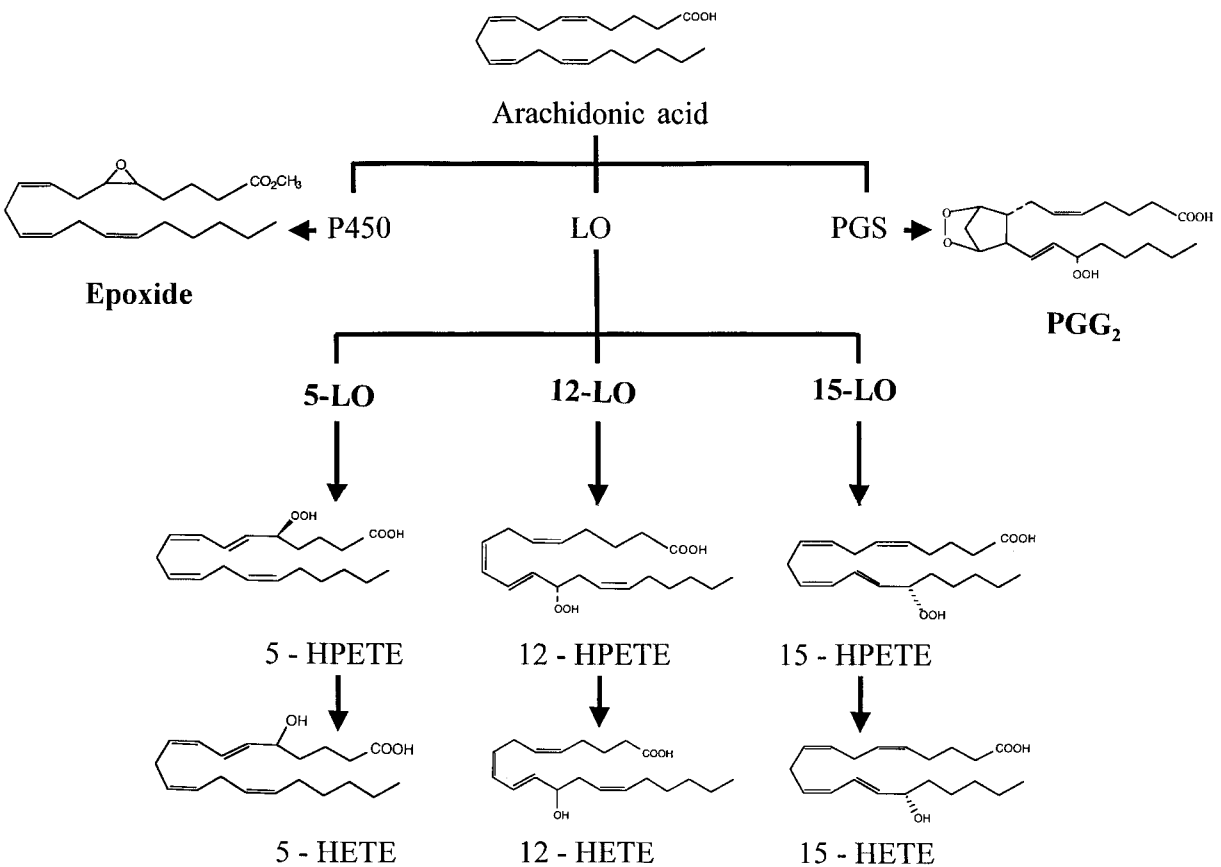

Figure 7.1 Metabolism of arachidonic acid by different pathways.

Petrocellis and Di Marzo 1994) and vertebrates (Yamamoto 1992). In mammals, a significant amount of LO activity occurs in blood cells and in many tissues (Table 7.1). Several excellent reviews on LO are available. For a more complete understanding of the LO system, the reader is encouraged to consult review(s) covering general information (Decker 1985; Schewe *et al.* 1986; Malle *et al.* 1987; Holtzman 1991, 1992; Gardner 1996) or specific aspects such as molecular biology and catalytic properties (Yamamoto 1992; Funk 1996; Kuhn and Thiele 1999), structure and function (Nelson and Seitz 1994; Gaffney 1996), regio- and stereo-chemistry (Lehmann 1994), 5-LO (Ford-Hutchinson *et al.* 1994; Bell and Harris 1999; Silverman and Drazen 1999; Steinhiber 1999), 12-LO (Yamamoto *et al.* 1997; Dailey and Imming 1999; Tang and Honn 1999) and the role in health and disease (Samuelsson *et al.* 1987; Konig *et al.* 1990).

DIOXYGENASE ACTIVITY

LOs catalyse regio- and stereo-specific dioxygenation of polyunsaturated fatty acids containing the 1(Z), 4(Z)-pentadiene system. The dioxygenation process involves hydrogen abstraction to produce a fatty acid radical ($L^{\bullet}$), radical rearrangement to form a conjugated diene and oxygen insertion to produce a fatty acid peroxyl radical intermediate ($LOO^{\bullet}$) before lipid hydroperoxide (LOOH) is released in the medium (Figure 7.2). Although the arachidonate molecule has several sites for oxygenation, LO selects one of them for the initial hydrogen abstraction. According to the current nomenclature, the classification of LOs is based on the positional specificity of arachidonate oxygenation. Mammalian cells contain at least three major types of LOs, i.e. 5-, 12- and 15-(S)LO. The dioxygenation of arachidonate by 5-LO yields 5-(*S*)hydroperoxy-6,8,11,14-(*E, Z, Z, Z*)-eicosatetraenoic acid (5-HPETE) while 12-LO and 15-LO convert arachidonate into cytotoxic 12-HPETE and 15-HPETE, respectively (Figure 7.1). Inside the cell, hydroperoxides are either reduced to the corresponding hydroxy derivatives, i.e. 5-HETE, 12-HETE and 15-HETE (Figure 7.1), or serve as intermediates in the genesis of a wide array of bioactive molecules such as leukotrienes (LTs), lipoxins, hepoxilins and other products by LO or other enzymes (Figure 7.1). Among plants, the most studied LOs are from soybean (SLO). Of the multiple known forms of SLO, L-1 is routinely used in xenobiotic oxidation studies. It is referred to hereafter simply as SLO.

The quantitative methods commonly used to assay the dioxygenase activity of LO include an estimation of oxygen uptake by means of a Clark electrode, spectrophotometric recording of conjugated diene formation absorbing at 234 nm and radiometry of ^{14}C or ^{3}H-arachidonic acid metabolites after their separation by TLC or HPLC. Haemoglobin (Hb), a common contaminant in the preparations of mammalian tissue LOs, poses a serious problem since it exhibits both pseudo-dioxygenase (Kuhn *et al.* 1981) and pseudo-peroxidase activities. A recently developed method using $ZnSO_4$ (Hover and Kulkarni 2000a) essentially eliminates this obstacle without affecting the dioxygenase and co-oxidase activities in the preparations of human term placental LO (HTPLO).

The positional specificity displayed by different LOs is not absolute as was once perceived. For example, SLO is a 15-LO with arachidonate but it generates

Table 7.1 Occurrence of lipoxygenase activity in mammalian tissues

Tissue	Species	LO	References
Heart	Rabbit	15-LO	Bailey *et al.* (1995)
Lymph node	Pig	12-LO	Shinjo *et al.* (1986)
Lung	Man	5-,12-,15-LO	Roy and Kulkarni, (1999)
Nasal polyp	Man	15-LO	Bioque *et al.* (1992)
Kidney	Man	5-LO	Stewart *et al.* (1997)
	Rat	5-,12-LO	Oyekan *et al.* (1997)
	Rabbit, Pig	LO	Stewart *et al.* (1993)
Urinary bladder	Pig	12-LO	Shinjo *et al.* (1986)
Liver	Man	5-,12-,15-LO	Roy and Kulkarni (1996b)
Gall bladder	Pig	12-LO	Shinjo *et al.* (1986)
Muscle (biceps femoris)	Pig	12-LO	Gata *et al.* (1996)
Dental pulp	Rat	12-, 15-LO	Doli *et al.* (1991)
Buccal cavity epithelial cells	Man	12-LO	Green (1989a)
Stomach (gastric mucosa)	Rat	5-,12-LO	Stein *et al.* (1991)
Small intestine	Pig	12-LO	Shinjo *et al.* (1986)
Intestinal epithelial cells	Rat	12-LO	Kamitani *et al.* (1999)
Caecum	Pig	12-LO	Shinjo *et al.* (1986)
Spleen	Pig	5-, 12-LO	Shinjo *et al.* (1986)
Colon, rectum	Man	15-LO	Ikawa *et al.* (1999)
Pancreatic cell lines	Man	5-, 12-LO	Ding *et al.* (1999)
Pacreatic islets	Rat	5-,12-,15-LO	Yamamoto *et al.* (1983)
Skin (keratinocytes)	Man	15-LO	Green (1989b)
Skin (epidermis)	Rat	12-,15-LO	Lomnitski *et al.* (1993)
Brain (different regions)	Many	5-,12-LO	Simmet and Peskar (1990)
	Pig	12-LO	Shinjo *et al.* (1986)
Ocular tissues	Man, Rabbit, Cynomolgus monkey, Rhesus monkey	5-, 12-LO	Kulkarni and Srinivasan (1989)
Thyroid gland	Pig	12-LO	Shinjo *et al.* (1986)
Thymus	Pig	12-LO	Shinjo *et al.* (1986)
Testis	Rat	12-LO	Shahin *et al.* (1978)
	Rat	15-LO	Lomnitski *et al.* (1993)
Prostate cancer cells	Man	5-LO	Myers and Ghosh (1999)
Vesicular gland	Sheep	LO	Portoghese *et al.* (1975)
	Bovine	5-,12-,15-LO	Halevy and Sklan (1987)
Leydig cells	Rat	LO	Mele *et al.* (1997)
Breast	Man	12-LO	Natarajan and Nadler (1998)
Preovulatory follicles (granulosa cells)	Man	5-,12-LO	Pridham *et al.* (1990)
Uterus	Man	12-LO	Flatman *et al.* (1986)
Placenta	Man	5-,12-,15-LO	Joseph *et al.* (1993)
Embryonic tissues	Man	5-,12-,15-LO	Joseph *et al.* (1994)
Foetal tissues	Man	5-,12-,15-LO	Datta and Kulkarni (1994a)
Endometrium	Man	12-LO	Ihno *et al.* (1993)
Decidua	Man	12-LO	Ihno *et al.* (1993)

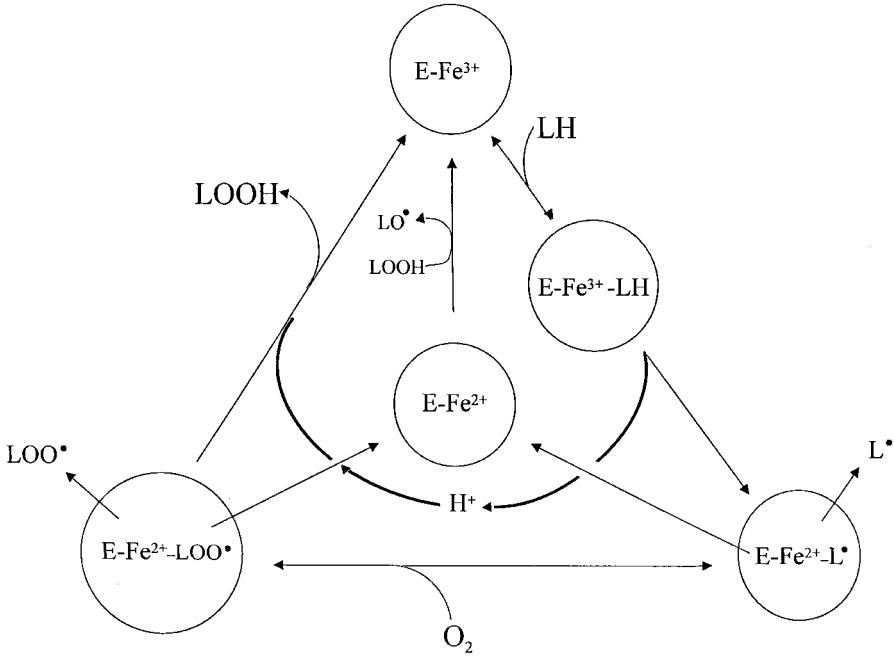

Figure 7.2 Catalytic cycle for the fatty acid peroxidation by lipoxygenase.

13-hydroperoxy octadecadienoic acid (13-HPOD) from linoleic acid. Some mammalian LOs also display similar dual positional specificity and can be classified as 12/15-LOs. The exact number of LOs in mammals is not known. Significant advances in genetic and molecular biology techniques have unveiled the existence of multiple forms of 12-LO and 15-LO (Kuhn and Thiele 1999; Yamamoto *et al.* 1999). It is certain that many more forms and subspecies will be discovered in the future. Human LOs have been purified and characterised in detail from different blood cells (Kuhn and Borngraber 1999; Yamamoto *et al.* 1999). Limited data also exist on the partial purification and properties of LOs from human lung (Roy and Kulkarni 1999), liver (Roy and Kulkarni 1996b), term placenta (Joseph *et al.* 1993), intrauterine conceptual tissues (Joseph *et al.* 1994; Datta *et al.* 1995), and other tissues.

In contrast to a strict requirement for a free fatty acid exhibited by the cyclooxygenase activity of PGS, the dioxygenase activity LOs displays a rather broad substrate specificity. Fatty acids containing two or more double bonds serve as substrates for LO. Certain LOs can utilise unusual substrates. For example, 5-LO from rat basophilic leukaemia cells oxygenates 5- and 6- fluoro- arachidonate (Nave *et al.* 1991) while LOs from different sources can accept 5-, 8-, 12-, 15-keto derivatives of arachidonate (Wiseman and Nichols 1988). Cholesterol esters of polyenoic fatty acids are oxygenated by purified rabbit reticulocyte 15-LO (Belkner *et al.* 1991). Similarly, 12-LO from porcine leukocytes can oxidise 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphocholine (Takahashi *et al.* 1993). More complex substrates such as

biomembranes are also attacked. For example, porcine 12-LO can oxygenate mitochondrial and endoplasmic membranes (Takahashi *et al.* 1993) while rabbit 15-LO oxygenates mitochondria and erythrocyte ghosts (Schnurr *et al.* 1995). Mounting evidence has established that the initiation of atherosclerosis is a free radical process that involves oxidative modification of low density lipoprotein (LDL) by 15-LO (Berliner and Heinecke 1996; Steinberg 1999; Yamashita *et al.* 1999). SLO can also mediate this reaction (Cathcart *et al.* 1991). Membrane-enclosed organelles, a defining characteristic of eukaryotic cells, are lost during maturation as well as differentiation of cells such as reticulocytes, central fibre cells of the eye lens and keratinocytes. Lipid peroxidation catalysed by 15-LO provides a precise mechanism by which this tightly regulated membrane degradation occurs in a timely manner with required specificity (Van Leyen *et al.* 1998).

Although most LOs are cytosolic proteins, debate continues regarding subcellular localisation of some LOs. With a rise in the cellular cytoplasmic calcium concentration, 5-LO migrates to the nuclear envelope. Earlier it appeared that a docking protein called 'Five Lipoxygenase Activating Protein (FLAP)' provides the assistance in the translocation and anchoring of 5-LO into nuclear membranes. Further studies have revealed that FLAP is a shuttling protein which transfers arachidonate to 5-LO and its expression is essential for LTS biosynthesis. This translocation phenomenon also occurs with 12-LO (Hagmann *et al.* 1993) and 15-LO (Brinckmann *et al.* 1998).

The addition of either Ca^{2+} or ATP results in a strong stimulation of dioxygenase activity of HTPLO (Joseph *et al.* 1993) and human liver LO (Roy and Kulkarni 1996b). A similar response is displayed by other LOs. Since iron in the catalytic centre of LOs exists mainly in the inactive Fe^{2+} state, an initial lag period of several seconds is commonly noted while assaying the dioxygenase activity. LOOH ($\sim$10 μM) shortens this lag period by converting the inactive Fe^{2+} into the active Fe^{3+} form. Similar spontaneous activation of both dioxygenase and co-oxidase activities of SLO (Kulkarni *et al.* 1989, 1990) and human liver LO (Roy and Kulkarni 1996b) occurs by nanomolar H_2O_2 in the presence of linoleic acid. High LOOH concentration usually causes autoinactivation of LOs.

CO-OXIDASE ACTIVITY

Besides dioxygenation of fatty acids, the same LO protein also oxidises xenobiotics (Kulkarni and Cook 1988a). The ability of LO to couple xenobiotic oxidation with lipid peroxyl radical or lipid hydroperoxide generation during peroxidation of polyunsaturated fatty acids is termed as the co-oxidase activity (Roy and Kulkarni 1996b). This aerobic reaction is called 'co-oxidation' and the xenobiotic oxidised is referred to as co-substrate. LOs are unique in that they synthesise themselves the oxidants (lipid peroxyl radical intermediates and/or hydroperoxides) needed for xenobiotic co-oxidation. Thus benzo[*a*]pyrene-7,8-dihydrodiol (BP-diol) epoxidation by SLO can be observed in the presence of either linoleic acid (Byczkowski and Kulkarni 1989), arachidonic acid (Hughes *et al.* 1989) or 15-HPETE (Hughes *et al.* 1989). Similarly, benzidine and other hydrogen donors are co-oxidised by SLO in the presence of either linoleic acid (Kulkarni and Cook 1988a) or via the peroxidase-like activity supported by H_2O_2 (Kulkarni and Cook 1988b). On the other hand, high rates of glutathione

(GSH) oxidation can only be observed in the presence of polyunsaturated fatty acids (Roy *et al*. 1995; Kulkarni and Sajan 1997, 1999) while the reaction is essentially absent in the presence of either H_2O_2 (Yang and Kulkarni 2000) or 13-HPOD (Roy *et al*. 1995). Thus, the term 'co-oxidase activity' implies LO-mediated xenobiotic oxidation supported by the fatty acid peroxyl radicals and/or lipid hydroperoxide while the terms 'pseudo-peroxidase, peroxidase-like activity or hydroperoxidase activity of LO' signify the reactions noted in the presence of lipid hydroperoxides, H_2O_2, or synthetic organic hydroperoxides.

Different approaches have been applied to detect free radical metabolites generated by LO during xenobiotic co-oxidation. The formation of a relatively stable free radical metabolite of the xenobiotic can be observed directly using spectrophotometry as in the case of aminopyrine (Perez-Gilabert *et al*. 1997; Yang and Kulkarni 1998) and phenothiazines (Perez-Gilabert *et al*. 1994a,b; Rajadhyaksha *et al*. 1999). Since most xenobiotic free radicals are unstable and survive for a very short period, indirect methods are used for their detection. For example, ESR studies are essential to detect the spin trap-adducts of relatively unstable phenoxyl radicals from various phenols (Van der Zee *et al*. 1989). In a few cases, free radicals undergo rapid dimerisation to stable metabolites which are amiable to spectrophotometry, as in the case of benzidine diimine formation from benzidine (Kulkarni and Cook 1988a,b) or HPLC to estimate azobis(biphenyl) formed from two 4-aminobiphenyl radicals (Datta *et al*. 1997). The quantitation of formaldehyde, resulting from the decomposition of nitrogen cation free radical, the initial metabolite, during *N*-demethylation of xenobiotics (Yang and Kulkarni 1998; Hu and Kulkarni 1998; Rajadhyaksha *et al*. 1999; Hover and Kulkarni 2000b,c) offers yet another indirect tool to evaluate the reactions involving free radicals generated by LOs.

Proposed mechanisms of xenobiotic co-oxidation

It is envisioned that at least four distinct mechanisms are involved in the oxidation of xenobiotics catalysed by LOs in the presence of either polyunsaturated fatty acids or hydroperoxides.

PEROXYL RADICAL-MEDIATED REACTIONS

The fatty acid peroxyl radicals ($LOO^{\bullet}$) serve as potent, direct acting oxidising agents. As shown in Figure 7.3, the hydrogen abstraction from a donor molecule represents one of the prominent reactions displayed by lipid peroxyl radicals. This process yields a free radical as the product of one electron oxidation of xenobiotic and lipid hydroperoxide. LO-dependent oxygenation of the acceptor molecules is another important attribute noted with peroxyl radicals. Peroxyl radicals are efficient epoxidising agents and they donate their terminal oxygen atoms to an aliphatic double bond to give rise to an epoxide (Figure 7.3) as noted in the LO-catalysed epoxidation of aldrin (Naidu *et al*. 1991a) and BP-diol (Byczkowski and Kulkarni 1989). In the case of sulphur-containing compounds, the LO-dependent oxygenation of a cosubstrate results in sulphoxidation as reported for thiobenzamide (Naidu and Kulkarni 1991) or

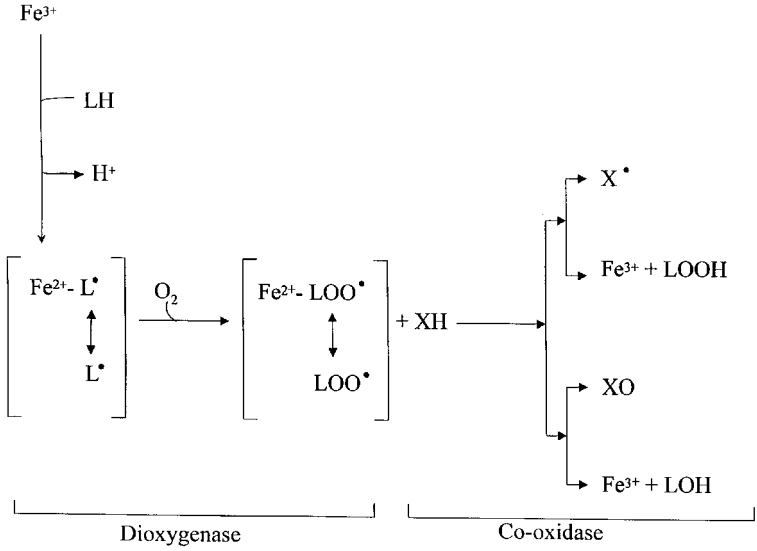

Figure 7.3 Peroxyl radical-dependent xenobiotic co-oxidation by lipoxygenase.

desulphuration (displacement of sulphur atom by oxygen atom), as in the case of parathion (Naidu *et al.* 1991b).

Multiple sources exist which supply $LOO^{\bullet}$ to the LO system (Figure 7.2).

(1) $LOO^{\bullet}$ are generated as intermediates during dioxygenation of fatty acids by LO before LOOH is released into the medium. This represents the primary source of $LOO^{\bullet}$ for xenobiotic oxidation.
(2) LO has been described as a leaky system. The hydrogen abstraction from a polyunsaturated fatty acid during dioxygenation by LO results in the formation of a fatty acid radical ($L^{\bullet}$). A portion of this $L^{\bullet}$ pool escapes into the medium where its reaction with O_2 yields $LOO^{\bullet}$.
(3) The third pathway of peroxyl radical generation originates from the decomposition of LOOH by LO under aerobic conditions (Hughes *et al.* 1989).

HYDROPEROXIDE-DEPENDENT REACTIONS

LOs oxidise various reducing agents, which are capable of reducing the Fe(III) enzyme to the Fe(II) form (Figure 7.4), in the presence of LOOH. The hydroperoxide-dependent xenobiotic oxidation is an indirect process that requires participation of LO in the hydrogen abstraction. As shown in Figure 7.4, hydroperoxide regenerates the active (Fe^{3+}) enzyme in the cycle and the xenobiotic undergoes a one electron oxidation by LO to a free radical. It is noteworthy that the reduction of hydroperoxide during LO-mediated xenobiotic co-oxidation does not yield the corresponding hydroxy derivative of fatty acid, as noted with PGS. Instead, the hydroperoxide undergoes a homolytic cleavage by LO to generate an alkoxy radical ($LO^{\bullet}$) and hydroxide ion (Van

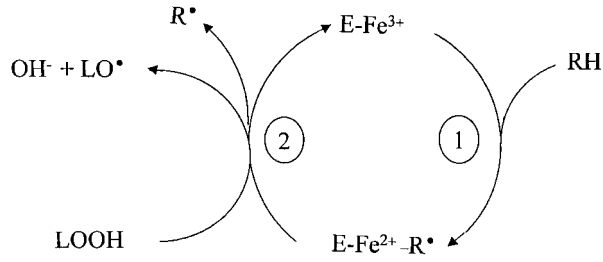

Figure 7.4 Lipid hydroperoxide-dependent xenobiotic co-oxidation by lipoxygenase.

der Zee *et al*. 1989; Cucurou *et al*. 1991). The studies conducted with SLO indicate that qualitatively, the enzyme lacks a strict specificity toward peroxide. Thus far, investigators have employed 5-HPETE (Cucurou *et al*. 1991), 15-HPETE (Hughes *et al*. 1989), and 13-HPOD (Mansuy *et al*. 1988; Riendeau *et al*. 1991; Chamulitrat *et al*. 1992) in studies on the LO-mediated xenobiotic oxidation. Kulkarni and Cook (1988b) were the first to demonstrate that H_2O_2 can be substituted for LOOH to support the co-oxidase activity of SLO. Subsequent reports have confirmed these observations for SLO (Perez-Gilabert *et al*. 1994a,b; Rosei *et al*. 1994; Datta *et al*. 1997) and extended to human tissue LO (Datta *et al*. 1997). A recent study from our laboratory (Hover and Kulkarni 2000d) has revealed that synthetic organic peroxides such as tert-butyl hydroperoxide or cumene hydroperoxide can also support hydroperoxidase activity of SLO. The experimental data on phenothiazine N-demethylation by SLO suggest that these hydroperoxides are as efficient as H_2O_2.

ELECTRON TRANSFER-DEPENDENT REACTIONS

In some cases, xenobiotic co-oxidation occurs indirectly. This involves generation of what is called a 'Shuttle Oxidant' from a good substrate by LO in the primary reaction which non-enzymatically oxidises another chemical. This is exemplified by the secondary hyperoxidation of benzidine (Hu and Kulkarni 2000) and L-dopa (Persad *et al*. 2000) by the primary phenothiazine cation radicals generated by SLO in the presence of H_2O_2 (Figure 7.5).

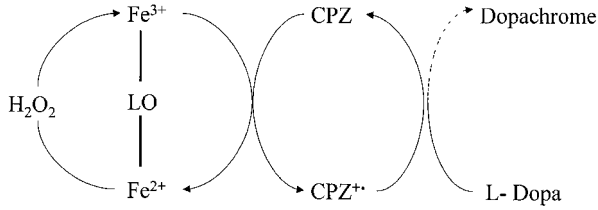

Figure 7.5 Lipoxygenase-mediated hyperoxidation of dopa in the presence of chlorpromazine.

OTHER MECHANISMS

In a few cases, xenobiotics are directly oxidised by purified LO in the absence of exogenous unsaturated fatty acid or hydroperoxide. For a long time, it has been known that acetylenic fatty acids such as 5,8,11-eicosatriynoic acid (ETI) and 5,8,11,14-eicosatetraynoic acid (ETYA) serve as suicide substrates and are directly oxidised by LO. The other reported examples of xenobiotic co-oxidation in the absence of fatty acid or lipid peroxide include the SLO-mediated oxidative conversion of hexanal phenylhydrazone into its a-azo hydroperoxide (Galey *et al*. 1988), 2-[(4′-hydroxy-3′-methoxy)-phenoxy]-4-(4″-hydroxy-3″-methoxy-phenyl)-8-hydroxy-6-oxo-3-oxabicyclo [3.3.0]-7-octene formation from curcumin (Schneider *et al*. 1998), the hydroperoxide generation from alkenes (Novak 1999) and several others. Additionally, it has been shown that the SLO-generated peroxyl radicals of LDL can co-oxidise α-tocopherol and probucol to their respective phenoxyl radicals, in the absence of exogenous fatty acid (Kalyanaraman *et al*. 1992). SLO can utilise tissue microsomes as a source of oxidisable lipid substrate during xenobiotic co-oxidation. Thus, the addition of increasing amount of SLO to incubates containing either human (Smith *et al*. 1995) or mouse (Rioux and Castonguay 1998) lung microsomes can oxidise 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) to keto aldehyde and keto alcohol and the metabolite production is suppressed by nordihydroguaiaretic acid (NDGA), an inhibitor of LO (Smith *et al*. 1995).

FATE OF XENOBIOTIC FREE RADICALS

The fate of the primary free radicals generated during one-electron oxidation of reducing cosubstrate by LO depends on the nature of the radical and the surrounding environment. They may (1) undergo coupling reactions to yield dimers, or oligomers; (2) undergo further oxidation to yield two electron oxidation products (e.g. aminopyrine or benzidine); (3) serve as a shuttle oxidant and react with another compound to generate secondary metabolite(s) as shown for the phenothiazine-benzidine combination, (4) react with endogenous or exogenous thiol and trigger thiol pumping, i.e. reduction of a free radical back to the parent chemical and the generation of $GS^{\bullet}$ or (5) react with macromolecules to produce protein, DNA, or RNA adducts.

Chemicals oxidized

ENDOGENOUS OR PHYSIOLOGICALLY RELEVANT CHEMICALS

A number of endogenous chemicals are oxidised by LO (Table 7.2). In the presence of linoleic acid, SLO oxidises both NADH and NADPH at the rate of ~350 nmoles/min/mg protein (Roy *et al*. 1994). The blockade of the reduction of ferricytochrome-C or nitroblue tetrazolium during LO-mediated NDA(P)H oxidation by superoxide dismutase suggests the generation of superoxide anions (Figure 7.6). The noted apparent K_m values of 13 μM and 117 μM for NADH and NADPH, respectively, are within physiological range. O′ Donnell and Kuhn (1997) have recently described that at near physiological concentration, both NADH and NADPH are co-oxidised by 15-LO

Table 7.2 Oxidation of naturally occurring chemicals via lipoxygenase pathway

Chemical	Enzyme	Cofactor	References
Glutathione	SLO	PUFAs	Roy *et al.* (1995)
	SLO	LA	Kulkarni and Sajan, (1997)
	HTPLO	LA	Kulkarni and Sajan, (1999)
Proline	SLO	LA	Byczkowski *et al.* (1991)
NADH	SLO	PUFAs	Roy *et al.* (1994)
NADPH	SLO	PUFAs	Roy *et al.* (1994)
Ascorbic acid	SLO	LA,AA	Roy and Kulkarni (1996a)
Dopa	SLO	H_2O_2	Rosei *et al.* (1994)
α-Methyl dopa	SLO	H_2O_2	Rosei *et al.* (1994)
Dopamine	SLO	H_2O_2	Rosei *et al.* (1994)
N-Acetyl dopamine	SLO	H_2O_2	Rosei *et al.* (1994)
Noradrenaline	SLO	H_2O_2	Rosei *et al.* (1994)
Adrenaline (Epinephrine)	SLO	H_2O_2	Rosei *et al.* (1994)
DL-α-Tocopherol	SLO	13-HPOD	Mansuy *et al.* (1988)
Vit.E derivatives (10)	SLO	13-HPOD	Cucurou *et al.* (1991)
5,6-Dihydroxyindole	SLO	H_2O_2	Blarzino *et al.* (1999)
5,6-Dihydroxyindole-2-carboxylic acid	SLO	H_2O_2	Blarzino *et al.* (1999)
β-Carotene	SLO	LA	Wu *et al.* (1999)
	Potato 5-LO	LA	Aziz *et al.* (1999)
Retinol	SLO	LA	Waldmann and Schreier (1995)
β-Ionone	SLO	LA	Waldmann and Schreier (1995)
4-Hydroxy-β-ionone	SLO	LA	Waldmann and Schreier (1995)

SLO, Soybean lipoxygenase-1; HTPLO, Human term placental lipoxygenase; PUFAs, Polyunsaturated fatty acids; LA, Linoleic acid; AA, Arachidonic acid.

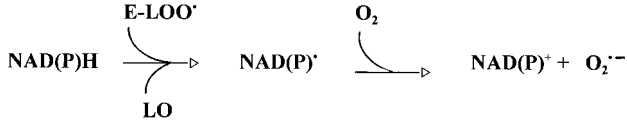

Figure 7.6 NAD(P)H oxidation and superoxide anion generation by lipoxygenase.

purified from rabbit reticulocytes in the presence of linoleic acid. The reaction was not observed with 13-HPOD.

Reduced glutathione (GSH) is the most abundant non-protein intracellular thiol. In mammals, GSH regulates 5-and 15-LO activities in human polymorphonuclear leukocytes and lymphocytes (Hatzelmann and Ullrich 1987; Claesson *et al.* 1992; Jakobsson *et al.* 1992). It is noteworthy that GSH is an extremely poor substrate for horseradish peroxidase (Harman *et al.* 1986) and is not oxidised by purified PGS in the presence of arachidonate (Eling *et al.* 1986). In fact, the authors reported that GSH itself inhibits the cyclooxygenase activity of the enzyme. In contrast to these reports, it is interesting to note that GSH serves as an excellent substrate for SLO in the presence of polyunsaturated fatty acids (Roy *et al.* 1995; Kulkarni and Sajan 1997). It is accompanied by a low rate of superoxide anion formation (Roy *et al.* 1995). The reaction is directly linked with the fatty acid peroxyl radical generation by LO. Negligible GSH oxidation by SLO occurs in the presence of 13-HPOD or H_2O_2 (Roy

et al. 1995; Yang and Kulkarni 2000). Human tissue LO (e.g. HTPLO) also displays this ability (Kulkarni and Sajan 1999). The reaction occurs at pH 7.4 at a significant rate in the presence of physiologically relevant concentrations of fatty acid and GSH (Kulkarni and Sajan, 1999). The spectrophotometric assay of dioxygenase activity of SLO revealed that the addition of increasing concentration of GSH to the incubation media results in a proportional decline in 13-HPOD accumulation (Roy *et al*. 1995). However, oxygen consumption under identical conditions proceeds unaltered. This suggests that the dioxygenase activity of SLO is not inhibited by GSH, and the fatty acid peroxyl radicals are diverted away from hydroperoxide formation. The final fatty acid metabolites resulting from the reaction were not identified in this study. Considering the data on NAD(P)H and GSH oxidation and concurrent reactive oxygen species production, it is tempting to speculate that LO plays a significant contributory role in the genesis of cellular oxidative stress.

Ascorbic acid, an essential dietary vitamin, serves as a readily available reducing agent in mammalian cells. Ascorbate is easily oxidised by SLO in the presence of linoleic or arachidonic acid but not in the reaction media supplemented with either 13-HPOD or H_2O_2 (Roy and Kulkarni 1996a). This suggests that ascorbate is not a substrate for the hydroperoxidase activity of LO. The lack of 13-HPOD-dependent ascorbate oxidation has also been reported for potato tuber 5-LO (Cucurou *et al*. 1991). The absence of superoxide generation suggests that fatty acid peroxyl radicals co-oxidise ascorbate to free radicals which disproportionate to yield dehydro-L-ascorbate (Roy and Kulkarni 1996a). The ascorbate addition markedly decreases the apparent rate of LO-mediated xenobiotic oxidation due either to a competitive inhibition and/or reduction of xenobiotic free radical back to the parent compound. Interestingly, at low concentrations, ascorbate itself apparently does not reduce the iron in the LO to inactivate dioxygenase activity. On the other hand, several phenols and arylamines enhance the basal rate of ascorbate oxidation in the linoleic acid-coupled reactions mediated by SLO (Roy and Kulkarni 1996a). It is interesting to note that the palmitate ester of ascorbate serves as a substrate for the hydroperoxidase activity of SLO and potato tuber 5-LO (Cucurou *et al*. 1991).

Other physiologically relevant compounds oxidised via the LO pathway (Table 7.2) include noradrenaline, *N*-acetyldopamine, adrenaline, and dopa to the corresponding melanin pigments (Rosei *et al*. 1994). Both 5-*S*-cysteinyl-dopa and 5-S-cysteinyl dopamine ultimately give rise to pheomelanin (Mosca *et al*. 1996). Catecholic tetrahydroisoquinolines such as salsolinol, tetrahydropapaveroline, laudanosoline and apomorphine are easily oxidised by SLO in the presence of H_2O_2 to their respective melanins (Mosca *et al*. 1998). Linoleic acid supports oxidation of dopamine, serotonin and norepinephrine by rat brain cytosolic LO (Byczkowski *et al*. 1992). Additionally, hydroxylation of proline by SLO in the presence of linoleic acid is also known (Byczkowski *et al*. 1991).

ENVIRONMENTAL CHEMICALS

Polycyclic aromatic hydrocarbons (PAHs) are a class of ubiquitous environmental pollutants, many of which are mutagenic, carcinogenic and teratogenic. The prototype of this class of compounds is benzo[*a*]pyrene (BP) that requires double epoxidation to

produce the ultimate toxicant, the diolepoxide (Figure 7.7). Although the role played by P450 in the initial epoxidation of the aromatic system is well accepted, it now appears that the LO pathway may be more important in the final epoxidation step. Sevanian and Peterson (1989) reported arachidonate- or 13-HPOD- dependent BP-caused cytotoxicity and mutagenesis in Chinese hamster lung fibroblasts (V79 cells). Although V79 cells possess both PGS and LO activities, nordihydroguaiaretic acid (NDGA) but not indomethacin, the inhibitor of PGS, exhibited the inhibitory response. This suggests an involvement of LO pathway in the induction of BP toxicity. Both P450 and PGS may not be involved in the BP activation by human or rat colonic mucosal microsomes since an addition of arachidonate, linoleate or their hydroperoxides enhanced the process 4-5 fold whereas NADPH had no effect (Craven and DeRubertis 1980; Craven *et al*. 1983). Nemoto and Takayama (1984) studied the covalent binding of BP to proteins following activation by microsomal and cytosolic enzymes in rat liver and lung. With microsomes, linoleic acid was more effective than arachidonate, whereas, with cytosol linoleic acid, linolenic acid and arachidonate but not oleic acid supported the reaction; arachidonate being the best cofactor. Indomethacin did not inhibit BP activation but NDGA and quercetin significantly reduced the binding. The linoleic acid-dependent binding with liver and lung microsomes was >3 and >16-fold greater respectively than that after incubation with NADPH. The authors opined that LO plays a dominant role in BP activation in liver and lung. In line with this postulate, Adriaenssens *et al*. (1983) did not observe a decrease in covalent binding of BP to DNA by indomethacin in mice. Aspirin, also an inhibitor of PGS, did not alter the number of pulmonary adenomas in mice treated with BP suggesting that PGS does not activate this carcinogen *in vivo*. Byczkowski and Kulkarni (1992) studied the LO-mediated co-oxidation of [^{14}C]BP in rat lung cytosol using linoleic acid. The oxidation yielded 1,6-dione, 3,6-dione and 6,12-dione of BP, with the 6,12-dione production being predominant. The quinones derived from BP may either bind covalently to tissue macromolecules or further undergo redox reactions.

Scharping *et al*. (1992) studied epoxidation of BP-7,8-dihydrodiol (BP-diol), the proximal carcinogen, in human liver microsomes and by SLO in the presence and absence of arachidonate. The reaction was inhibited by NDGA. The SLO-mediated reaction was augmented by the addition of phenylbutazone. Apparently, phenylbutazone is oxidised by LO to a carbon-centred free radical which traps molecular oxygen to form a peroxyl radical that effects epoxidation of BP-diol. The crucial direct evidence that purified LO can mediate the final BP bioactivation step is available. Byczkowski and Kulkarni (1989) were the first to demonstrate that BP-diol can be activated by SLO in the presence of linoleic acid. BP-*trans*-anti-7,8,9,10-tetrahydrotetrol, the product of hydrolytic breakdown of ultimate mutagenic BP-anti-7,8-dihydrodiol-9,10-epoxide was detected as the major metabolite by radiometry combined with HPLC. The peroxy radical derived from linoleic acid during dioxygenation by SLO was reported to be responsible for the epoxidation of BP-diol. Subsequently, Hughes *et al*. (1989) confirmed these findings and noted that the SLO-catalysed BP-diol epoxidation can also occur in the presence of arachidonate, γ-linolenic acid and 15-HPETE. Based on oxygen consumption studies, it was postulated that 15-HPETE-is reduced to an alkoxyl radical and hydroxyl anion. The radical rearranges to an allylic epoxyl radical which reacts with molecular oxygen to form a peroxyl radical that

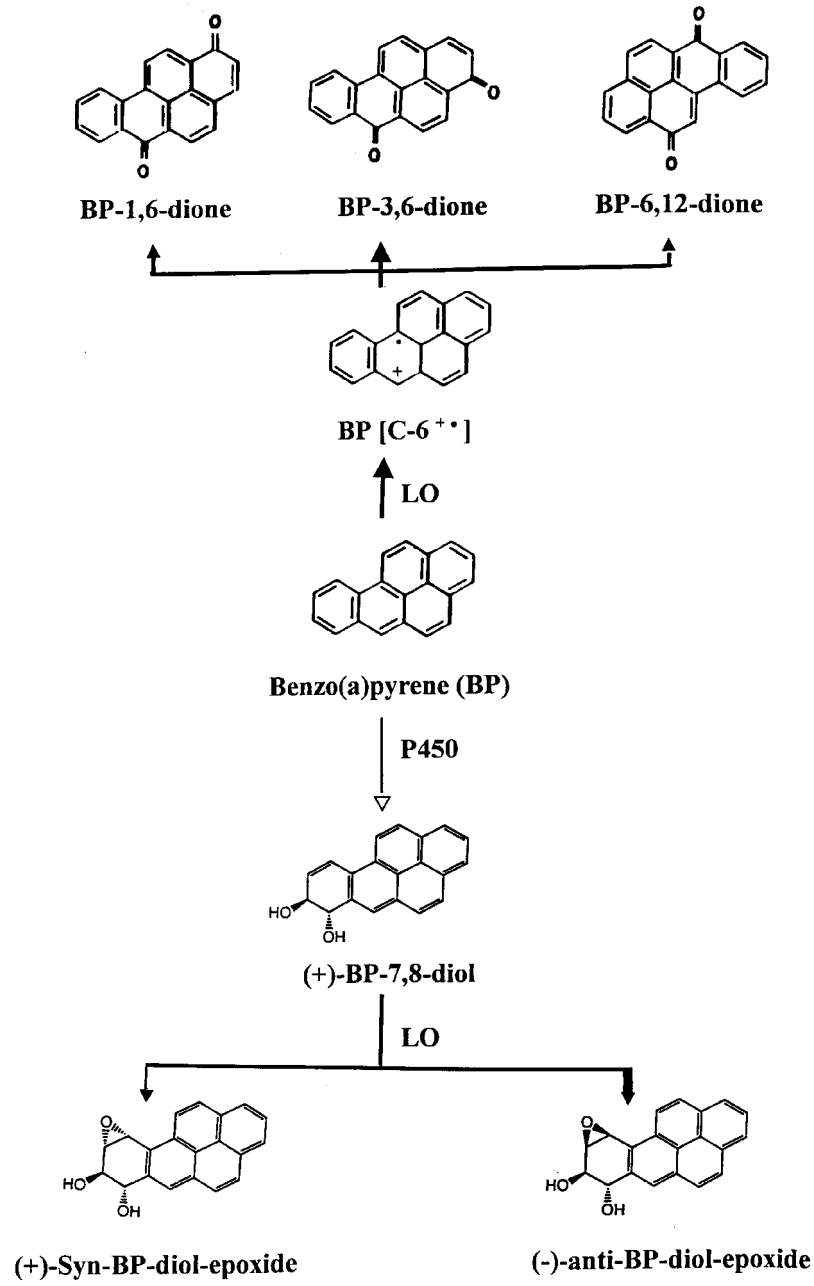

Figure 7.7 Lipoxygenase-mediated oxidation of benzo[*a*]pyrene.

serves as the oxidant for the epoxidation reaction. The attempts to detect 15-HPETE-derived peroxyl radical by ESR were unsuccessful, but the formation of two carbon-centred radicals either at C11 or C13, the precursors of this species, was noted. Although the fatty acid metabolites were not analysed, a simultaneous formation of epoxy-hydroxy, dihydroxy and trihydroxy fatty acids was proposed (Hughes *et al*. 1989). These observations with SLO raise a question whether LO-mediated BP-diol bioactivation occurs in human lung. The possibility is strongly indicated since LO capable of xenobiotic oxidation occurs in human (Roy and Kulkarni 1999) and animal lungs (Nemoto and Takayama 1984, Kulkarni *et al*. 1992). Indomethacin does not inhibit arachidonate-dependent BP-diol epoxidation by either hamster trachea or human bronchus explant in culture (Reed *et al.* 1984). This provides compelling evidence that the activation proceeds via the LO pathway and not by PGS. Joseph *et al*. (1994) have documented that purified LO from human intrauterine conceptual tissues and HTPLO can easily epoxidise radioactive BP-diol to several metabolites. The production of BP-*trans*-anti-tetrol by LO was about eight-times above the control value noted with boiled enzyme preparations. These results explain the report (Manchester *et al*. 1988) on the detection of DNA adducts of BP in human placentas of non-smokers which lack both P450 and PGS activities (Kulkarni 1996, 2000).

A potent tobacco-specific carcinogen, NNK, induces lung tumours in animals and is a suspected human carcinogen. Smith *et al*. (1995) opined that P450 is enzymes are only partially responsible for the activation of NNK in human lung microsomes. They noted an increased formation of keto aldehyde and keto alcohol by the addition of exogenous arachidonate and the inhibition of their formation by NDGA, thus suggesting an involvement of the LO pathway in the NNK oxidation by human lung microsomes. The observed NDGA-caused modest decline in the rate of keto alcohol and keto aldehyde production by patas monkey lung microsomes are in harmony with this postulate (Smith *et al*. 1997). More recently, Rioux and Castonguay (1998) and Castonguay *et al*. (1998) have provided the necessary direct evidence that purified SLO mediates NNK oxidation by a-carbon hydroxylation and *N*-oxidation.

Occupational exposure to arylamines such as 4-aminobiphenyl (4-ABP) is known to be associated with an elevated risk of bladder cancer. 4-ABP also occurs in environmental tobacco smoke. Interestingly, the concentration of 4-ABP is 30 times greater in the sidestream smoke than in the mainstream smoke. Thus, under certain conditions, non-smokers can be exposed to relatively high levels of 4-ABP. Several reports have documented an increased risk of haematopoietic malignancies and lung cancers in adults to be related to smoking by the mother during pregnancy. It is widely accepted that oxidative metabolism of 4-ABP is essential to exert its carcinogenic effect. Datta *et al*. (1997) have demonstrated that both SLO and HTPLO can oxidise 4-ABP. HPLC and mass spectroscopy were used to isolate and identify the metabolite, respectively. The major metabolite 4,4-azobis(biphenyl), was reported to arise via a mechanism involving an initial one-electron oxidation of 4-ABP to a free radical species. When the specific activity data were normalised on the basis of units of dioxygenase activity, HTPLO was found to be twice as efficient as SLO in the 4-ABP oxidation. These results explain, at least partly, the reported 4-ABP-adducts in the non-smokers' placentas, a tissue which is essentially deficient in both P450 and PGS capable of xenobiotic oxidation (Kulkarni 1996, 2001).

Although the use of benzidine is banned in the USA, benzidine-based dyes are still being used in many countries in textile and other industries. Therefore, cancer incidence and mortality due to exposure to aromatic amines are still important public health issues on a global scale. Besides uroepithelial cancers, few isolated reports have implicated human exposure to benzidine and other arylamines in the cancers of other organs such as pancreas (Anderson *et al*. 1997), kidneys and liver (Morikawa *et al*. 1997). In rats, mice and hamsters, benzidine causes hepatocarcinogenicity (Whysner *et al*. 1996). Benzidine undergoes bioconversion to electrophiles that covalently bind to DNA, induce mutations and initiate carcinogenesis. Benzidine is a poor substrate for the NADPH-dependent oxidation by rat hepatic P450 and MFMO and it does not generate protein-bound and water-soluble metabolites (Zenser *et al*. 1979). Prior acetylation seems to be involved. Although PGS-catalysed bioactivation of benzidine explains bladder carcinogenicity, it can not explain hepatocarcinogenicity because PGS occurs in negligible amounts in the liver (Smith and Marnett 1991). Benzidine serves as an excellent substrate for co-oxidation by SLO in the presence of either linoleic acid or H_2O_2 (Kulkarni and Cook 1988a,b; Kulkarni *et al*. 1989; Hu and Kulkarni 2000). Initial one-electron oxidation of benzidine yields a nitrogen-centred cation radical which may either undergo a second one-electron oxidation to produce benzidine di-imine or disproportionate to produce parent diamine and benzidine di-imine. Both the cation radical and the di-imine are electrophilic derivatives of benzidine capable of covalent binding to macromolecules. Similar to SLO, extensive benzidine oxidation to reactive benzidine di-imine can be observed with animal LO isolated from lung (Kulkarni *et al*. 1992), brain (Naidu *et al*. 1992), liver (Roy and Kulkarni 1994) and embryo (Roy *et al*. 1993). The purified LO from human term placenta (Joseph *et al*. 1993), conceptual tissues (Datta *et al*. 1995), adult lung (Roy and Kulkarni 1999) and liver (Roy and Kulkarni 1996b) also catalyse this reaction at a high rate.

2-Aminofluorene (2-AF) is a potent carcinogen and teratogen to which humans may be exposed from environmental, industrial and dietary sources. Metabolic activation is obligatory for 2-AF toxicity. 2-AF activation by P450 and MFMO plays some role in hepatocarcinogenesis. However, extrahepatic tissues such as mammary gland and Zymbal gland, which contain very low levels of these enzymes, are also targets for 2-AF-induced cancer. Since 2-AF is a poor substrate for PGS, its bioactivation via the LO pathway was examined (Roy and Kulkarni 1991). In the presence of linoleic acid, SLO catalysed oxidation of 2-AF at the rate of 521 nmol/min/nmol of SLO. Arachidonate, linolenic acid, and *cis*-11,14-eicosadienoic acid were less than half as effective as linoleate. The experiments conducted with radioactive substrate revealed the generation of electrophilic 2-AF intermediates by SLO which bind covalently in significant amounts to either bovine serum albumin or calf thymus DNA. SLO-mediated 2-AF bioactivation can be blocked by ETI, ETYA, NDGA, gossypol, and esculetin, the classical LO inhibitors, and by butylated hydroxyanisole (BHA) and butylated hydroxytolyne (BHT) clearly suggesting that LO may serve as an alternate pathway to P450 in 2-AF bioactivation in mammalian extrahepatic tissues.

Aflatoxin B_1 (AFB), a substituted coumarin produced as a secondary metabolite by *Aspergillus flavus* and *A. parasiticus*, is a known contaminant in various food products. AFB is a potent hepatocarcinogen and teratogen in humans. It is believed that a

requisite event in AFB toxicity is prior oxidation at the 8,9-vinyl ether bond to form the AFB-8,9-epoxide, an electrophilic species which is the ultimate carcinogenic metabolite. Linoleic acid, linolenic acid and arachidonic acid strongly inhibit NADPH-dependent hepatic microsomal AFB activation (Firozi *et al*. 1986; Ho *et al*. 1992). This leads to a logical question as to whether P450 serves as the sole catalyst. PGS can epoxidise AFB but cannot explain hepatocarcinogenicity since liver contains biologically non-significant levels of this enzyme (Smith and Marnett 1991). Earlier, Amstad and Cerruti (1983) reported that the binding of AFB metabolites to DNA in mouse embryo fibroblasts is inhibited by inhibitors of LO. Purified SLO can mediate AFB activation (Liu and Massey 1992). A recent study by Roy and Kulkarni (1997) has provided the necessary evidence that partially purified adult human liver LO can epoxidise AFB in the presence of polyunsaturated fatty acids. Thus, LO clearly represents an additional or alternate pathway of AFB bioactivation in the human liver. AFB contamination occurs in grain dust and handling of grains in large quantity can result in a significant pulmonary exposure to AFB. Available data indicate that AFB epoxidation can be effected by human lung cytosolic LO (Donnelly *et al*. 1996) as well as by the cytosolic LO from guinea pig lungs and kidney (Liu and Massey 1992). Similarly, the experimental data on AFB bioactivation collected for the purified LO from human intrauterine conceptual tissues and HTPLO (Datta and Kulkarni 1994a) explain, at least partly, the developmental toxicity associated with the exposure to this fungal alkaloid during pregnancy.

The colon carcinogen 1,2-dimethylhydrazine is activated by P450 to the proximate carcinogen methylazoxymethanol. Either NAD-dependent oxidation or fatty acid supported co-oxidation releases the final methylating agent and formaldehyde. Craven *et al*. (1985) reported that rat colonic mucosal LO, but not P450, mediates this reaction in the presence of arachidonate, linoleate and 15-HPETE.

INDUSTRIAL CHEMICALS

Styrene is a high-volume industrial chemical used in the manufacture of plastics, resins, synthetic rubber and insulators. As in the past, a high potential of worker exposure to low levels of styrene monomer can be anticipated in the future. Many metabolic studies have established that prior styrene oxidation to styrene-7,8-oxide is essential to observe toxicity, since it binds covalently to cellular macromolecules. P450, haemoglobin, myoglobin, and horseradish peroxidase have been shown to mediate this epoxidation reaction. For reasons unclear at present, PGS does not catalyse styrene epoxidation (Stock *et al*. 1986). On the other hand, Belvedere *et al.* (1983) observed that the LO pathway serves as an additional route for epoxidation of styrene in the presence of arachidonic acid. The rate of the SLO-mediated reaction was about 4-fold greater than that noted with the NADPH-dependent P450-mediated hepatic microsomal reaction. The peroxyl radicals of arachidonate or those derived from hydroperoxide degradation are expected to mediate this reaction.

Acrylonitrile (ACN) is another important industrial chemical widely used in the manufacture of plastics, rubber, and acrylic fibres. Residues of ACN are found in water, food, and cigarette smoke. ACN is carcinogenic in the rat but the potential carcinogenic risk to humans is uncertain. The mechanism by which ACN initiates

tumour formation is unknown; however, the available evidence suggests that metabolic activation is required. ACN undergoes epoxidation to produce reactive 2-cyanoethylene oxide (CEO) and ultimately cytotoxic cyanide. The proposal that P450 is responsible for bioactivation of ACN seems inconsistent in view of the following:

(1) The covalent binding of ACN to brain, lung and liver microsomes from the untreated rats is not significantly increased by the addition of NADPH (Roberts *et al*. 1989).
(2) In the physiologically based dosimetry study, Gargas *et al.* (1995) concluded that an extrapolation of *in vitro* data to the whole animal does not agree since the V_{max} for ACN epoxidation by rat liver P450 accounts for <8% of the *in vivo* estimate for the CEO formation.
(3) In animals, ACN carcinogenicity is also observed in Zymbal' s gland, stomach, brain and uterus that contain very low or undetectable levels of P450. The detailed study conducted by Roy and Kulkarni (1999) revealed that LO pathway may be involved. SLO and partially purified human lung LO preparations, which predominantly contain 15-LO, and smaller amounts of 5-LO and 12-LO, were found to be capable of extensive ACN metabolism to CEO and cyanide in the presence of polyunsaturated fatty acids (Figure 7.8). Among the fatty acids tested, linoleic acid was most effective in supporting epoxidation of ACN to CEO by the human lung LO. Interestingly, the human lung enzyme was an approximately sixfold better catalyst than SLO in converting ACN to cyanide. Significant covalent binding of the radioactivity derived from [^{14}C]-ACN to bovine serum albumin and calf thymus DNA occurred when the reaction media contained either active SLO or human lung LO. These reactions were strongly inhibited by NDGA and the antioxidant butylated hydroxytoluene (BHT). The experimental evidence clearly suggests that the LO pathway may be of toxicological relevance in the ACN bioactivation in humans.

PESTICIDES

Pesticides represent a group of potentially dangerous chemicals of diverse structures deliberately distributed in our ecosystem. Few *in vitro* studies have documented the LO-mediated oxidative metabolism of pesticides (Table 7.3). Low levels of residues of now abandoned organochlorine insecticides still occur in human tissues, milk and the food we consume (Kulkarni and Mitra 1990). Scientific interest is further amplified since some of these pesticides are suspected of being endocrine disruptors. Epoxidation of aldrin to dieldrin is a model reaction noted with P450 and PGS. The demonstration that SLO (Naidu *et al.* 1991a) can easily oxidise aldrin to dieldrin clearly implicates LO as an additional pathway for this epoxidation. The rate of SLO-mediated reaction was 8-20-fold greater than different isoforms of P450. Parathion, an organophosphorous insecticide, is oxidised by SLO (Figure 7.9) (Naidu *et al.* 1991b). The fatty acid peroxyl radicals and to some extent those derived from the13-HPOD decomposition were proposed to donate oxygen to parathion to produce initially a sulphur-oxygen intermediate. The breakdown of this unstable intermediate releases the desulphuration product, paraoxon, and dearylation metabolites, *p*-nitrophenol

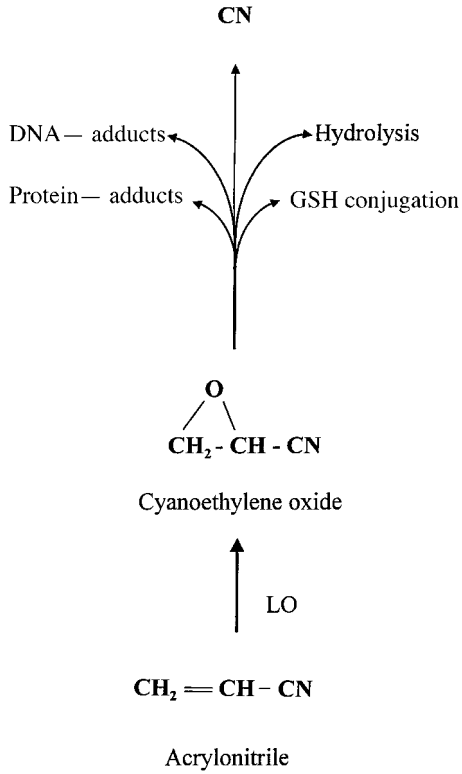

Figure 7.8 Lipoxygenase-mediated oxidative metabolism of acrylonitrile.

and diethyl phosphate or diethyl phosphorothioic acid. The displacement of a sulphur atom by oxygen (desulphuration) results in the bioactivation of parathion since paraoxon is a very potent anticholinesterase. Again, the rate of paraoxon formation by SLO was up to 20 times greater than the P450-dependent reaction. It is noteworthy that cytosolic rat brain LO also yields similar results (Naidu *et al.* 1991b). Recently Hu and Kulkarni (1998) have documented that several pesticides undergo SLO-catalysed N-demethylation to release formaldehyde in the presence of H_2O_2 (Table 7.3). The highest specific activity was noted with aminocarb. A significant suppression of the formaldehyde production by GSH, DTT (dithiothreitol), BHT, and BHA suggests a free radical nature of the aminocarb *N*-demethylation. HTPLO can also efficiently catalyse this reaction in the presence of linoleic acid (Hover and Kulkarni 2000c).

DRUGS

Aminopyrine is extensively used by many investigators as a prototype xenobiotic in the mechanistic studies on *N*-dealkylation catalysed by P450, PGS and horseradish peroxidase. According to Agundez *et al.* (1995), *in vivo* metabolism of aminopyrine in

Table 7.3 Oxidation of pesticides by lipoxygenase

Pesticide	Enzyme	Cofactor	Reaction	Reference
Aldrin	SLO	Linoleic acid	Epoxidation	Naidu *et al.* (1991a)
Parathion	SLO	Linoleic acid	Desulphuration, Dearylation	Naidu *et al.* (1991b)
Aminocarb	SLO	H_2O_2	*N*-demethylation	Hu and Kulkarni (1998)
	SLO, HTPLO	Linoleic acid	*N*-Demethylation	Hover and Kulkarni (2000c)
Zectran	SLO	H_2O_2	*N*-Demethylation	Hu and Kulkarni (1998)
	SLO, HTPLO	Linoleic acid	*N*-Demethylation	Hover and Kulkarni (2000c)
Dicrotophos	SLO	H_2O_2	*N*-Demethylation	Hu and Kulkarni (1998)
	SLO, HTPLO	Linoleic acid	*N*-Demethylation	Hover and Kulkarni (2000c)
Chlordimeform	SLO	H_2O_2	*N*-Demethylation	Hu and Kulkarni (1998)
	SLO, HTPLO	Linoleic acid	*N*-Demethylation	Hover and Kulkarni (2000c)
Famphur	SLO	H_2O_2	*N*-Demethylation	Hu and Kulkarni (1998)
Formetanate	SLO	H_2O_2	*N*-Demethylation	Hu and Kulkarni (1998)
Pirimicarb	SLO	H_2O_2	*N*-Demethylation	Hu and Kulkarni (1998)
Tetramethiuram	SLO	H_2O_2	*N*-Demethylation	Hu and Kulkarni (1998)

SLO, soybean lipoxygenase; HTPLO, Human term placental lipoxygenase

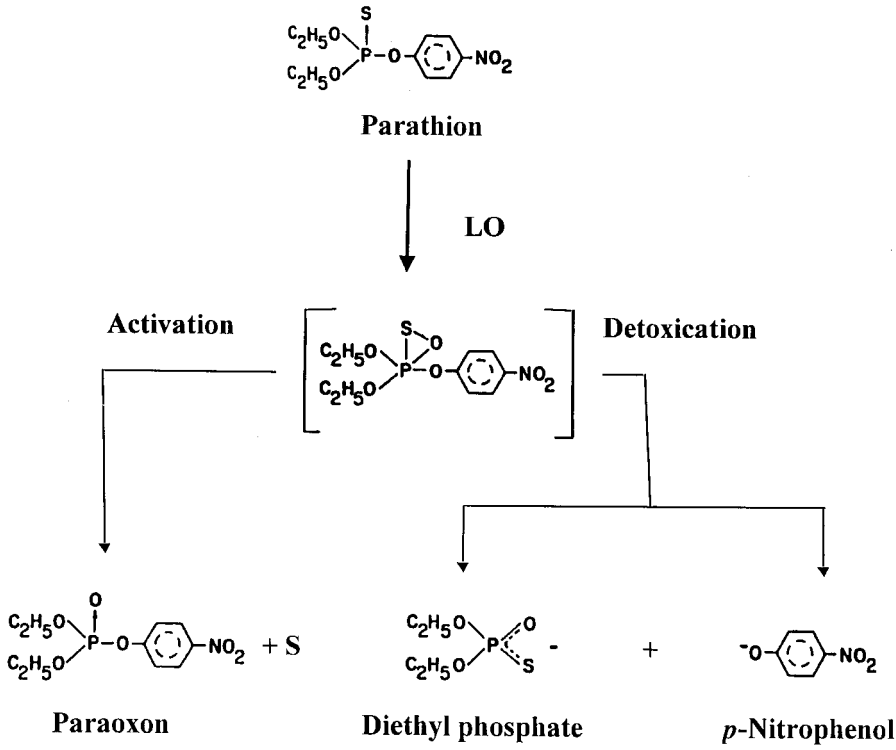

Figure 7.9 Oxidative metabolism of parathion by lipoxygenase.

humans exhibits a ~200-fold variation and this is not related to gender, intake of caffeine or alcohol, or P450 polymorphism. Since LO activity occurs in the liver and many other tissues, a hypothesis that this pathway may be involved was tested using SLO in the presence of H_2O_2 (Perez-Gilabert *et al.* 1997; Yang and Kulkarni 1998). As compared to P450 and PGS, aminopyrine *N*-demethylation by SLO occurs at a much higher rate (823 nmol/min/mg protein) (Yang and Kulkarni 1998). The formaldehyde production is strongly inhibited by NDGA and gossypol, clearly implicating LO as the catalyst. A nitrogen-centred free radical cation, the expected initial one-electron oxidation product, can be observed by spectrophotometry (Perez-Gilabert *et al.* 1997; Yang and Kulkarni 1998). The rate of accumulation of this radical species and formaldehyde depends on pH, the amount of the enzyme, and the concentration of aminopyrine and H_2O_2. Ascorbate, GSH, and DTT markedly suppress the radical formation supporting the contention that a free radical mechanism is involved in the *N*-dealkylation of aminopyrine via the LO pathway (Figure 7.10). It was postulated that the cation radical is further converted to an iminium cation either by deprotonation or hydrogen atom abstraction. Subsequent hydrolysis of the iminium cation yields monomethylamine and formaldehyde (Perez-Gilabert *et al.* 1997; Yang and Kulkarni 1998).

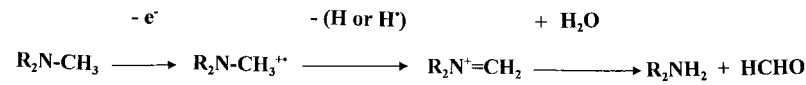

Figure 7.10 Mechanism for *N*-demethylation of xenobiotics by lipoxygenase.

Tricyclic antidepressants, such as imipramine and closely related compounds, represent a group of drugs widely used today in the USA and other countries for the treatment of major depression. Their remarkable efficacy in alleviating depression is well established. However, few side-effects such as cardiovascular toxicity and species-specific teratogenicity in animals have been noted. Imipramine, a prototype of this class, undergoes extensive metabolism in the body. However, it is not clear which enzyme(s) is responsible for its oxidation. The published data on the role played by P450 are not convincing in view of the facts that (1) the expected increase in the rate of imipramine oxidation by brain or liver microsomes is not observed in rats pretreated with P450 inducers such as phenobarbital and β-naphthoflavone; (2) imipramine behaves as a P450 inhibitor and inactivates P450 since reactive intermediates generated during its oxidation quickly form stable complexes with P450 and accumulate in microsomes with time; (3) a large inter-individual variation occurs in the blood levels of imipramine in patients. The role of MFMO in imipramine oxidation is also debatable since the purified enzyme does not catalyse this reaction unless detergent is added. Since LO activity occurs in the liver of rat (Roy and Kulkarni 1994) and human (Roy and Kulkarni 1996b, 1997) and in the rat brain (Byczkowski *et al.* 1992; Naidu *et al.* 1992), its involvement in imipramine oxidation can be suspected. An exploration of this hypothesis revealed that imipramine is an excellent substrate for dealkylation by SLO in the presence of H_2O_2 (Hu *et al.* 1999). Desipramine, the expected product of imipramine mono *N*-demethylation, was identified by HPLC. When incubated separately, desipramine also yielded formaldehyde, suggesting that imipramine undergoes double dealkylation by SLO. Under identical conditions, not all the antidepressants tested are equally oxidised. The most rapid oxidation (~210 nmol/min/mg SLO) was observed with trimipramine while desipramine, clomipramine, diltiazem, amitriptyline and doxepin exhibited a relatively low oxidation rate (10 nmol/min/mg SLO) (Table 7.4).

Currently, phenothiazines enjoy wide acceptance as relatively safe and efficacious drugs for the treatment of psychotic illnesses. Other therapeutic applications include their use as tranquilisers, sedatives, antiemetics, antimicrobials, etc. Some reports point out alterations in the endocrine function and untoward effects on the cardiovascular and reproductive systems associated with acute exposure. Phenothiazines undergo extensive oxidative metabolism in the body. It is generally accepted that oxidation of phenothiazines to relatively stable nitrogen-centred cation radicals is the first essential step responsible for their biological activity. In each case, the cation radical serves as an intermediate that yields the phenothiazine sulphoxide and other metabolites. The ability of P450, haemoglobin, myeloperoxidase and horseradish peroxidase to metabolise phenothiazines is well established. Recently, Perez-Gilabert *et al.* (1994a,b) identified an involvement of a non-haem iron protein-dependent pathway. The authors reported the formation of cation-free radical from phenothiazines by SLO in the presence of H_2O_2. Besides SLO, purified HTPLO also generates cation radicals from

Table 7.4 Oxidation of drugs by lipoxygenase

Drug	Reaction	Enzyme	Cofactor	Reference
Aminopyrine	*N*-Demethylation	SLO	H_2O_2	Perez-Gilabert *et al.* (1997)
		SLO	H_2O_2	Yang and Kulkarni (1998)
Chlorpromazine	*N*-Demethylation	HTPLO, SLO	Linoleic acid	Hover and Kulkarni (2000c)
		SLO	H_2O_2	Rajadhyaksha *et al.* (1999)
		HTPLO, SLO	CHP, TBHP	Hover and Kulkarni (2000d)
Promazine	*N*-Demethylation	HTPLO, SLO	Linoleic acid	Hover and Kulkarni (2000c)
		SLO	H_2O_2	Rajadhyaksha *et al.* (1999)
		HTPLO, SLO	CHP, TBHP	Hover and Kulkarni (2000d)
Promethazine	*N*-Demethylation	HTPLO, SLO	Linoleic acid	Hover and Kulkarni (2000c)
		SLO	H_2O_2	Rajadhyaksha *et al.* (1999)
		HTPLO, SLO	CHP, TBHP	Hover and Kulkarni (2000d)
Triflupromazine	*N*-Demethylation	SLO	H_2O_2	Rajadhyaksha *et al.* (1999)
Trimeprazine	*N*-Demethylation	HTPLO, SLO	Linoleic acid	Hover and Kulkarni (2000c)
		SLO	H_2O_2	Rajadhyaksha *et al.* (1999)
		HTPLO, SLO	CHP, TBHP	Hover and Kulkarni (2000d)
Trifluoperazine	*N*-Demethylation	SLO	H_2O_2	Rajadhyaksha et al. (1999)
Imipramine	*N*-Demethylation	SLO	H_2O_2	Hu *et al.* (1999)
Desipramine	*N*-Demethylation	SLO	H_2O_2	Hu *et al.* (1999)
Trimipramine	*N*-Demethylation	SLO	H_2O_2	Hu *et al.* (1999)
Clomipramine	*N*-Demethylation	SLO	H_2O_2	Hu *et al.* (1999)
Amitriptyline	*N*-Demethylation	SLO	H_2O_2	Hu *et al.* (1999)
Diltiazem	*N*-Demethylation	SLO	H_2O_2	Hu *et al.* (1999)
Doxepin	*N*-Demethylation	SLO	H_2O_2	Hu *et al.* (1999)
Phenytoin	Oxidation	SLO	Linoleic acid	Yu and Wells (1995)
Diethylstilboestrol	Oxidation	SLO	H_2O_2	Nunez-Delicado *et al.* (1997b)
Isoproterenol	Oxidation	SLO	H_2O_2	Rosie *et al.* (1994)
Oxyphenbutazone	Hydroxylation	SLO	Linoleic acid	Portoghese *et al.* (1975)
Phenidone	Oxidation	SLO, 5-LO	13-HPOD	Cucurou *et al.* (1991)

SLO, soybean lipoxygenase-1; HTPLO, human term placental lipoxygenase; CHP, cumene hydroperoxide; TBHP, tert-butyl hydroperoxide; 13-HPOD, 13-hydroperoxy octadecadienoic acid.

phenothiazines (Rajadhyaksha *et al.* 1999). Phenothiazines are easily demethylated releasing formaldehyde in the H_2O_2 supplemented incubation media containing either SLO or HTPLO (Table 7.4) (Rajadhyaksha *et al.* 1999). Among the phenothiazines tested, promazine is oxidised by SLO at the highest rate (828 nmol/min/mg protein) while the slowest oxidation rate is exhibited by trifluprom azine (12 nmol/min/mg protein). A similar structure–activity response was displayed by HTPLO. Cumene hydroperoxide and tert-butyl hydroperoxide also support the reaction (Table 7.4) with equal or better efficiency (Hover and Kulkarni 2000d). In this case, promethazine appears to be a better substrate than chlorpromazine (CPZ). Additional experimental evidence (Hover and Kulkarni 2000c) revealed that although H_2O_2 and synthetic hydroperoxides are better cofactors, polyunsaturated fatty acids can also support *N*-dealkylation of phenothiazines by SLO and HTPLO. Linoleic acid is up to eight times more efficient in supporting *N*-dealkylation of CPZ than either γ-linolenic acid or arachidonic acid. Although CPZ, promazine, promethazine and trimeprazine are oxidised in the linoleic acid-supported HTPLO-catalysed reaction (Table 7.4), promethazine seems to be the best substrate while promazine, the most resistant.

Phenytoin is an efficacious anticonvulsant and a known human teratogen. The SLO-mediated arachidonate- or linoleate-dependent oxidation to free radical species is accompanied by covalent binding to proteins which can be suppressed by LO inhibitors such as NDGA, quercetin, BW755C, and ETYA (Kubow and Wells 1988; Yu and Wells 1995). Cyclophosphamide is a prodrug whose toxicity and therapeutic efficacy depends on its metabolic activation. Kanekal and Kehrer (1994) observed co-oxidation of cyclophosphamide with SLO and 15-LO from rabbit reticulocytes in the presence of linoleic acid. This results in the generation of unstable tautomer of 4-hydroxy intermediate, an active metabolite, which breaks down to release acrolein phosphoramide mustard in the incubation medium.

Diethystilboestrol (DES), a human transplacental carcinogen, is widely used in livestock and in few other therapeutic applications. It is oxidised by P450, and serves as an excellent reducing substrate for various peroxidases including PGS. DES-quinone, one of the metabolites of DES, binds to DNA and is presumed to be the ultimate toxicant. Although DES-quinone formation by human tissue LO has not yet been reported, Nunez-Delicado *et al.* (1997b) noted that SLO is capable of the initial one-electron oxidation of DES to DES semiquinone in the presence of H_2O_2. Subsequently, dismutation of two molecules of DES semiquinone yields one molecule each of DES-quionone and DES. Isoproterenol, a β-adrenoreceptor agonist, is widely used as a bronchodilator in the treatment for asthma. This two-electron donor compound serves as a co-substrate for the hydroperoxidase activity of SLO in the presence of H_2O_2 and is easily oxidised to a final stable product, aminochrome (Rosei *et al.* 1994; Nunez-Delicado *et al.* 1996). A sequential generation of *o*-quinone and leukaminochrome as intermediates was postulated in the aminochrome production (Nunez-Delicado *et al.* 1996).

OTHER CHEMICALS

Several model chemicals, drugs and other chemicals of diverse structures are also known to be oxidised via the LO pathway (Table 7.5).

Table 7.5 Oxidation of other compounds via lipoxygenase pathway

Chemical	Enzyme (source)	Cofactor	References
N-Methyl aniline	SLO and HTPLO	H_2O_2	Hover and Kulkarni (2000b)
N,*N*-Dimethyl aniline	SLO and HTPLO	H_2O_2	Hover and Kulkarni (2000b)
N,*N*,*N*′,*N*′-Tetramethyl benzidine	SLO and HTPLO	H_2O_2	Hover and Kulkarni (2000b)
N,*N*-Dimethyl-p-phelylenediamine	SLO and HTPLO	H_2O_2	Hover and Kulkarni (2000b)
N,*N*-Dimethyl-3-nitroaniline	SLO and HTPLO	H_2O_2	Hover and Kulkarni (2000b)
N,*N*-Dimethyl-p-toluidine	SLO and HTPLO	H_2O_2	Hover and Kulkarni (2000b)
Phenol	SLO	13-HPOD	Mansuy *et al.* (1988)
p-Aminophenol	SLO, Potato 5-LO	13-HPOD	Cucurou *et al.* (1991)
1,3-Dihydroxybenzene (Resorcinol)	SLO, Potato 5-LO	13-HPOD	Cucurou *et al.* (1991)
1,2-Dihydroxybenzene(pyrocatechol)	SLO, Potato 5-LO	13-HPOD	Cucurou *et al.* (1991)
Guaiacol (O-methoxyphenol)	SLO	13-HPOD	Streckert and Stan (1975)
	SLO, Potato 5-LO	13-HPOD	Cucurou *et al.* (1991)
	SLO	H_2O_2	Kulkarni and Cook (1988a,b)
	SLO	H_2O_2	Fontana *et al.* (1997)
1,2,3-Trihydroxybenzene (Pyrogallol)	SLO	H_2O_2	Kulkarni and Cook (1988a,b)
	SLO	H_2O_2	Fontana *et al.* (1997)
4-Methyl catechol	SLO	H_2O_2	Nunez-Delicado *et al.* (1999)
4-tert-Butyl catechol	SLO	H_2O_2	Nunez-Delicado *et al.* (1999)
4-tert-Octyl catechol	SLO	H_2O_2	Nunez-Delicado *et al.* (1999)
Trolox C	SLO	H_2O_2	Nunez Delicado *et al.* (1997a)
2,2,5,7,8-Pentamethylchroman-6-ol	SLO	H_2O_2	Nunez-Delicado *et al.*(1997c)
Hydrocaffeic acid	SLO	H_2O_2	Mosca *et al.* (1996)
Salsolinol	SLO	H_2O_2	Mosca *et al.* (1996)

continued overleaf

Table 7.5 (*continued*)

Chemical	Enzyme (source)	Cofactor	References
Tetrahydropapaveroline	SLO	H_2O_2	Mosca *et al.* (1996)
Dopa methyl ester	SLO	H_2O_2	Mosca *et al.* (1996)
Thiobenzamide	SLO	Linoleic acid	Naidu and Kulkarni (1991)
ABTS	SLO	H_2O_2	Kulkarni and Cook (1988a,b)
	SLO	H_2O_2	Fontana *et al.* (1997)
Scopoletin	SLO	H_2O_2	Fontana *et al.* (1997)
Homovanillic acid	SLO	H_2O_2	Fontana *et al.* (1997)
Pentadienols (8 chemicals)	SLO	–	Zhang and Kyler (1989)
N-Phenyllinoleamide	Mouse macrophage	–	Bioque *et al.* (1995)
N,N,N′,N′-Tetramethyl-1,4-phenylenediamine	SLO	Linoleic acid	Kulkarni and Cook (1988a)
	SLO	H_2O_2	Kulkarni and Cook (1988b)
3,3′,5,5′-Tetramethylbenzidine	SLO	Linoleic acid	Kulkarni and Cook (1988a)
	SLO	H_2O_2	Kulkarni and Cook (1988b)
3,3′-Dimethoxybenzidine	SLO	Linoleic acid	Kulkarni and Cook (1988a)
	SLO	H_2O_2	Kulkarni and Cook (1988b)
p-Phenylenediamine	SLO	Linoleic acid	Kulkarni and Cook (1988a)
	SLO	H_2O_2	Kulkarni and Cook (1988b)
4-Hydroxy-*β*-ionone	SLO	Linoleic acid	Waldmann and Schrieber (1995)
trans-4-Hydroxy-2-nonenal	SLO	PUFA, H_2O_2	
		9-,13-HPOD	Chen and Chung (1996)
3Z-Nonenal	SLO	13-HPOD	Gardner and Grove (1998)
Pinolenic acid	15-LO	–	Kuklev *et al.* (1993)
Crocin	SLO	Linoleic acid	Spaapen *et al.* (1980)
2-Bromorthanolamine	Rat medullary interstitial cells	–	Grieve *et al.* (1990)

SLO, soybean lipoxygenase-1; HTPLO, human term placental lipoxygenase; 13-HPOD, 13-hydroperoxy octadecadienoic acid; ABTS, 2,2-azinobis(3-ethylbenzothiazoline-6-sulphonic acid).

Drug–chemical interactions

Unintentional human exposure to multiple chemicals from the working or living environment is unavoidable and common. Also, under certain medical situations, some patients simultaneously consume multiple drugs. The major concern over such concurrent exposure to chemicals is an exaggerated or diminished biological response, which is always difficult to predict or understand. To provide a logical explanation, an assessment of the metabolic fate of each component of the mixture has been a focal point of many investigations. At least three examples of drug–chemical interactions involving LO can be cited. Hu and Kulkarni (2000) tested a hypothesis that under certain conditions, the primary free radical metabolites of efficient substrates for LO may stimulate the generation of reactive species from other chemicals in the secondary reaction. The evidence presented indicates that SLO-generated CPZ cation radical ($CPZ^{+\bullet}$) serves as a shuttle oxidant which is capable of simultaneous oxidation of several other chemicals. Such a metabolic interaction results in a 42-fold stimulation of benzidine diimine formation from benzidine by SLO in the H_2O_2 supplemented incubation media. Seven other phenothiazines also display similar phenomenon and stimulate benzidine oxidation, albeit to a lesser extent. $CPZ^{+\bullet}$ also stimulates the oxidation of tetramethyl benzidine, o-dianisidine, guaiacol, pyrogallol, phenylenediamine, and tetramethyl phenylenediamine (Table 7.6). The highest degree of $CPZ^{+\bullet}$-caused stimulation (94-fold) was noted in the Wursters Blue radical formation from tetramethyl phenylenediamine while the least degree of enhancement was noted with guaiacol oxidation by SLO.

L-Dopa deficiency in brain neurones is a hallmark of Parkinsonism. Although levodopa is the therapeutic agent commonly used to treat the disease, a combination of CPZ and L-dopa is contraindicated. Many investigators have invoked a receptor mechanism to explain this undesirable drug–drug interaction. However, the results of the metabolic study (Persad *et al.* 2000) conducted with SLO may provide an alternate explanation since it was observed that the oxidation of dopa to dopachrome is stimulated ~25-fold by $CPZ^{+\bullet}$ generated by SLO. *In vivo*, dopa undergoes decarboxylation to produce dopamine. However, *in vitro*, $CPZ^{+\bullet}$ also accelerates dopamine

Table 7.6 Stimulation of xenobiotic oxidation by soybean lipoxygenase in the presence of chlorpromazine

Xenobiotic	Final concentration (μM)	Fold stimulation	References
Benzidine	10	42	Hu and Kulkarni (2000)
Tetramethyl benzidine	25	8	Hu and Kulkarni (2000)
o-Dianisidine	25	2	Hu and Kulkarni (2000)
Guaiacol	100	4	Hu and Kulkarni (2000)
Pyrogallol	10	24	Hu and Kulkarni (2000)
Phenylenediamine	25	25	Hu and Kulkarni (2000)
Tetramethyl phenylenediamine	50	94	Hu and Kulkarni(2000)
L-Dopa	1000	25	Persad *et al.* (2000)

The reaction media contained 200 nM SLO, 1.0 mM chlorpromazine and 1.0 mM H_2O_2 and the indicated concentration of the test xenobiotic.

oxidation significantly. Thus, it appears that the hyperoxidation of both dopa and dopamine via the LO pathway may contribute, at least in part, to the exhaustion of cellular reserves and thereby may impart dopamine deficiency as observed in the brains of the Parkinsonism patients. An enhancement of H_2O_2-dependent SLO-catalysed oxidation of dopa, 5-S-cysteinyl-dopa and 5-S-cysteinyl dopamine by catechols such as NDGA, salsolinol, tetrahydropapaveroline, dopa methyl ester, hydrocaffeic acid, and caffeic acid (Mosca *et al.* 1996) represents another example of metabolic modulation by LO. Dopa was ineffective while NDGA-caused enhancement in the initial rate of oxidation was five-fold with dopa, 14-fold with 5-*S*-cysteinyl-dopa and 2-fold with 5-S-cysteinyl dopamine. The authors (Mosca *et al.* 1996) proposed that the mechanism of enhancement involves redox cycling of catechols. Thus, the quinones produced by SLO rapidly interact with the test substrate causing its non-enzymatic oxidation.

Modulation of lipoxygenase activity

The anecdotal reports support the notion that LO is an inducible enzyme. For example, Coffey *et al.* (1996) reported that effect of 2-h human exposures to 0.4 ppm ozone results in a 8-fold increase in the bronchoalveolar lavage fluid LTC_4 content. Exposure of rabbits to phosgene results in a 10-fold increase in the synthesis of LO-generated arachidonate metabolites in lungs (Guo *et al.* 1990). The lung vascular injury caused by intraperitoneal injection of endotoxin precipitates into high levels of lung 5-HETE and LTC_4 in rats (Chang *et al.* 1989). Bailey *et al.* (1993) reported that in rabbits fed an atherogenic diet containing 1% cholesterol for 14 weeks, 15-LO levels in heart, aortic adventitia, and lung, but not liver, are increased up to 100-fold. The subcutaneous treatment of rabbits for 5-6 days with the haemolytic agent, phenylhydrazine, results in >1000-fold increase in 15-LO levels in heart, lung and aorta (Bailey *et al.* 1993, 1995). Similar results were obtained when severe anaemia was induced in rats by frequent bleeding (Bailey *et al.* 1996). Infusion of the oxidant tert-butyl hydroperoxide in isolated rabbit lung elevates LTB_4, C_4, D_4, and E_4 production by 2 to 3-fold (Farrukh *et al.* 1988). A significant rise in 15-HETE occurs when human bronchial epithelial cells are exposed to toluene diisocyanate (Mattoli *et al.* 1990). Acrolein exposure to cultured bovine tracheal epithelial cells leads to elevation of 12-HETE and 15-HETE production (Doupnik and Leikauf 1990).

An exposure of A/J mice to NNK for 7 weeks in the drinking water causes a >2-fold increase in the plasma concentration of LTB_4 (Castonguay *et al.* 1998; Rioux and Castonguay 1998) and this effect can be blocked by the co-treatment with the LO inhibitor, A-79175. The authors proposed that NNK-derived intermediates induce the expression of 5-LO (Castonguay *et al.* 1998). Feeding oxidised palm oil to rats for 3 days results in more than 2-fold increase in the liver LO activity (Pereira and Das 1991). A significant increase in the production of 15-HETE and LTB_4 was reported in the livers of rats treated with the diabetogen, streptozotocin by Rosello-Catafau *et al.* (1994). A recent report indicates that the streptozotocin-induced experimental diabetes is also associated with an increase in the renal LO activity (Stewart *et al.* 1998).

Perchellet and Perchellet (1989) reported that daily application of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) results in up to 4-fold increase in the lipid hydroper-

oxide levels in mouse skin and the effect is blocked by the LO inhibitors but not by the cyclooxygenase inhibitors. According to Jiang *et al.* (1994), a maximal induction of mouse epidermal 8-LO results 24 h after TPA application. The oral treatment of mice with TMK688, a LO inhibitor, not only inhibits LO induction but also protects mice from skin carcinogenesis induced by 7,12-dimethylbenz[*a*]anthracene and TPA plus BP (Jiang *et al.* 1994). In human erythroleukaemia cells, the membrane fraction contains about 90% of the total cellular 12-LO activity and the pretreatment of the cells for 3 days with TPA causes a marked, time-dependent increase in membrane-bound 12-LO activity and protein (Hagmann *et al.* 1993). Phorbol 12-myristate 13-acetate also causes a significant increase in the expression of microsomal 12-LO activity and mRNA in human epidermoid carcinoma A431 cells (Liaw *et al.* 1998). Dexamethasone treatment has been found to result in a selective upregulation of 5-LO in human mast cells (Colamorea *et al.* 1999) and in DMSO-stimulated HL-60 cells (Zaitsu *et al.* 1998). The increased expression of 5-LO and FLAP in human monocytes and THP-1 cells can be noted with the dexamethasone treatment (Riddick *et al.* 1997). Reports describing an increase in the LT production following an exposure to TPA and Ca^{2+} ionophore A23187 in isolated perfused rat liver (Hagmann *et al.* 1989) or to ethanol by rat hepatocytes (Peres *et al.* 1984) in primary culture are also available. Bioque *et al.* (1992) reported that a 2-hour exposure of mouse peritoneal macrophages to *N*-phenyllinoleamide increases the LO-mediated metabolism of exogenous arachidonate to 12-HETE. According to Brungs *et al.* (1994), vitamin D_3 addition to culture medium causes a 4-fold induction of 5-LO mRNA, a 14-fold increase in 5-LO protein and a 38-fold upregulation of 5-LO activity of intact HL-60 cells after differentiation in the presence of DMSO and serum protein for 4 days. Hamasaki and Miyazaki (1991) observed that overnight incubation of the calcium-ionophore-stimulated RBL-1 cells with retinoic acid enhances LTC_4 production by >28-fold and LTD_4 by >7-fold. However, the authors concluded that the induction of LTC_4 synthase, but not 5-LO was responsible for these results.

Lipoxygenase inhibition

Several hundred chemicals have been synthesised and screened during the past few years as selective inhibitors for 5-, 12-, or 15-LO. Many reviews covering this subject are available (Batt 1992; Negro *et al.* 1997; Cornicelli and Trivedi 1999). Unfortunately, there is no universally accepted unified approach to evaluate the relative potency of different chemicals to cause inhibition of LOs. The absence of a standardised protocol has resulted in a wide variation in reports in terms of the enzyme source, assay procedure and end points measured to establish the potency of candidate compounds.

To establish the role of a specific enzyme in xenobiotic oxidation *in vivo*, most investigators, use so-called 'selective inhibitors'. However, a serious problem of non-specificity, often ignored by many, exists. Thus, for example, SKF-525 and metyrapone, which are considered as selective inhibitors of P450, also inhibit SLO with respective IC_{50} values of 40 and 150 μM (Pretus *et al.* 1985). Indomethacin and several non-steroidal anti-inflammatory drugs (NSAID), are widely believed as the specific PGS inhibitors, but they cause a significant denaturation of rat hepatic

microsomal P450 (Falzon *et al.* 1986). Siegel *et al.* (1980b) have reported that aspirin, indomethacin, sodium salicylate, phenylbutazone, ibuprofen, naproxen and sulindac, the PGS inhibitors, can also inhibit the LO in rat neutrophils from carrageenan pleural exuate. The reports are not consistent with indomethacin. Thus, indomethacin causes a three-fold increase in LTB_4 formation (Docherty and Wilson 1987) in the calcium ionophore stimulated human neutrophils along with an increase in the 5-, 12-, and 15-HETE formation. Indomethacin blocks the LO pathway in hamster isolated lungs (Uotila *et al.* 1981). Apparently, indomethacin, aspirin and salicylate inhibit the conversion of 12-HPETE to 12-HETE in the LO preparations of human blood platelets (Siegel *et al.* 1979, 1980a). According to Sircar *et al.* (1983), indomethacin and other anti-inflammatory drugs inhibit SLO while Marcinkiewicz *et al.* (1985) observed no decrease in the rate of oxygen consumption during arachidonate peroxidation by SLO. Indomethacin caused $\sim$ 50% inhibition of covalent binding of activated phenytoin by SLO (Yu and Wells 1995) while it enhanced significantly the binding of arachidonate-dependent activated BP in the cytosol of rat liver and lung (Nemoto and Takayama 1984). Acetylenic fatty acids, e.g. ETI and ETYA, are widely accepted as dual inhibitors of PGS and LO pathways. However, they can also effectively inhibit different glutathione *S*-transferases (Datta and Kulkarni 1994b). NDGA, a commonly used LO inhibitor also inhibits PGS (Smith and Marnett 1991). Considering these reports, it is imperative that investigators exercise extreme caution while interpreting the results and drawing conclusions. Using this approach, it is nearly impossible to establish the role played by LO in xenobiotic oxidation *in vivo*.

Despite numerous difficulties (see above), continued efforts in this area have led to the discovery of some potent LO inhibitors which exhibit high potency and isoform selectivity. According to Suzuki *et al.* (1997), compound YT-18 (2,3-dihydro-2,4,6,7-tetramethyl-2-[(4-phenyl-1-piperazinyl)methyl]-5-benzofuranamine) selectively inhibits 5-LO from human and porcine leukocytes and RBL cells but has almost no effect on 12-, 15-LO and cyclooxygenase-1 and -2. Gorins *et al.* (1996) tested a series of (carboxyalkyl)benzyl propargyl ethers as inhibitors of 12-LO from porcine leukocyte cytosol. The most potent acetylenic (carboxyalkyl)benzyl ethers did not inhibit human platelet 12-LO, human neutrophil 5-LO, rabbit reticulocyte 15-LO or soybean 15-LO. Some examples of so called selective LO inhibitors are shown in Figure 7.11.

Since a large number of chemicals inhibit LO activity, several mechanisms have been proposed to explain their mode of action. Thus a chemical may inhibit LO activity by serving as an antioxidant, iron chelator, substrate analogue, FLAP inhibitor, blocker of LO induction etc. However, the discussion here is limited to those inhibitors which are co-oxidised in the process (Table 7.7). The phenolic antioxidants BHT and BHA, which break free radical chain reactions, also block xenobiotic metabolism by different LOs. The ESR data reported by Kagan *et al.* (1990) demonstrate that seven BHT homologues undergo one-electron oxidation by SLO in the presence of linoleic acid to generate free radical species. It was proposed that phenoxyl radicals are formed from an interaction of phenols with the SLO-derived lipid peroxyl radicals. According to Schilderman *et al.* (1993), linoleate-supported metabolism of 2-tert-butyl(1,4)hydroquinone, a demethylated metabolite of antioxidant BHA, by SLO involves a two-electron oxidation process that directly yields 2-tert-butyl(1,4)paraquinone without semiquinone radical or oxygen radical formation.

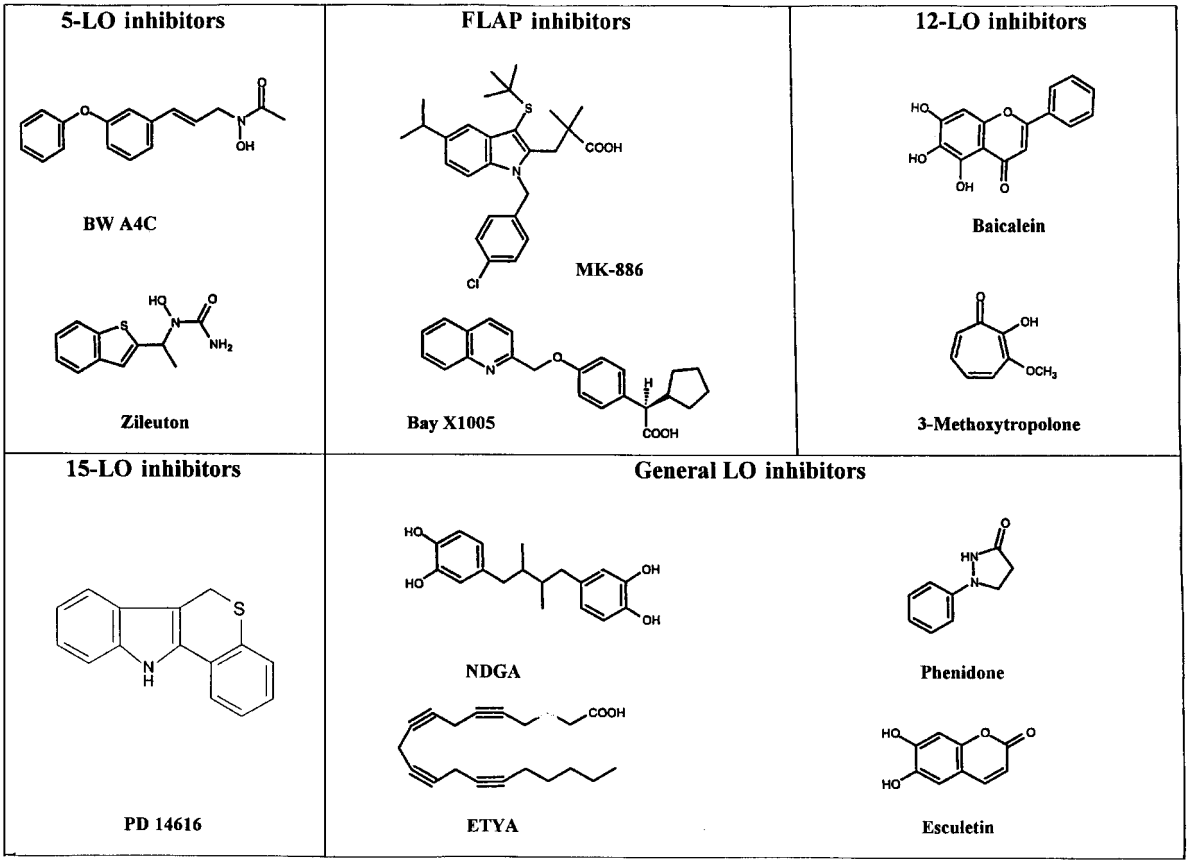

Figure 7.11 Some inhibitors of lipoxygenase.

Table 7.7 Oxidation of inhibitors and related compounds by lipoxygenase

Chemical	Enzyme	Co-factor	References
N-(4-Chlorophenyl)-*N*-hydroxy-*N*′-(3-chlorophenyl)urea (CPHU)	SLO	LA, 13-HPOD	Chamulitrat *et al.* (1992)
	SLO	13-HPOD	Falgueyret *et al.* (1992)
	SLO	13-HPOD	Desmarais *et al.* (1994)
N-[(E)-3-(3-Phenoxyphenyl)prop-2-enyl]acetohydroxamic acid (BWA4C)	5-LO	13-HPOD	Riendeau *et al.* (1991)
	SLO	LA, 13-HPOD	Chamulitrat *et al.* (1992)
	SLO, 5-,12-LO	13-HPOD	Falgueyret *et al.* (1992)
N(1-Benzo[*b*]thien-2-ylethyl)-N-hydroxyurea (Zileuton)	SLO	LA, 13-HPOD	Chamulitrat *et al.* (1992)
	SLO, 5-,12-LO	13-HPOD	Falgueyret *et al.* (1992)
Dimehtylhydroxylamine	SLO	LA, 13-HPOD	Chamulitrat *et al.* (1992)
Isopropylhydroxylamine	SLO	LA, 13-HPOD	Chamulitrat *et al.* (1992)
Cyclohexylhydroxylamine	SLO	LA, 13-HPOD	Chamulitrat *et al.* (1992)
Desferal	SLO	LA, 13-HPOD	Chamulitrat *et al.* (1992)
N-Hydroxyurea	SLO	LA, 13-HPOD	Chamulitrat *et al.* (1992)
5-Hydroxy-2-phenethyl-2,3-dihydrobenzofuran	5-LO	13-HPOD	Riendeau *et al.* (1991)
5-Hydroxy-2-phenethyl-6-(3-phenoxypropyl)-2,3-dihydrobenzofuran	5-LO	13-HPOD	Riendeau *et al.* (1991)
7-Chloro-4-hydroxy-2-(4-methoxyphenyl)-methyl-3-methylbenzofuran	5-LO	13-HPOD	Riendeau *et al.* (1991)
7-Chloro-4-hydroxy-2-[(4-methoxyphenyl)-methyl]-3-methyl-5-propylbenzofuran	5-LO	13-HPOD	Riendeau *et al.* (1991)
N-(4-Chlorophenyl)-N-hydroxy-N′-(3-chlorophenyl)urea	5-LO	13-HPOD	Riendeau *et al.* (1991)
N-(3,4-Dichlorophenyl)-N-hydroxy-N′-(3-chlorophenyl)urea	5-LO	13-HPOD	Riendeau *et al.* (1991)
N-Methyl-2-[4-(2,4,6-trimethylphenyl)phenyl]propenehydroxamic acid	5-LO	13-HPOD	Riendeau *et al.* (1991)

N-(1-benzo[*b*]thien-2-ylethyl)-*N*-hydroxyurea (A-64077)	5-LO	13-HPOD	Riendeau *et al.* (1991)
Phenidone	SLO	13-HPOD	Mansuy *et al.* (1988)
BW 755C	SLO	13-HPOD	Mansuy *et al.* (1988)
BW755C	SLO	15-HPETE	Reynolds (1988)
BW540C	SLO	15-HPETE	Reynolds (1988)
BW A4C	SLO	15-HPETE	Reynolds (1988)
BW A137C	SLO	15-HPETE	Reynolds (1988)
1-Phenyl-3-amino-2-pyrazoline	SLO	13-HPOD	Mansuy *et al.* (1988)
Nordihydroguaiaretic acid (NDGA)	SLO	13-HPOD	Mansuy *et al.* (1988)
	SLO	H_2O_2	Mosca *et al.* (1996)
Phenylhydrazine	SLO	13-HPOD	Mansuy *et al.* (1988)
2-Hydrazinopyridine	SLO	13-HPOD	Mansuy *et al.* (1988)
N-Alkylhydroxylamines (7 compounds)	SLO	LA, 13-HPOD	Clapp *et al.* (1985)
(E)Hexanal phenylhydrazone	SLO	LA	Galey *et al.* (1988)
2,2,5,7,8-Pentamethylchroman-6-ol	SLO	H_2O_2	Nunez-Delicado *et al.* (1997c)
N-Hydroxyamphetamine	SLO	13-HPOD	Mansuy *et al.* (1988)
N-Methylbenzaldehyde-4′-bromophenylhydrazone	SLO	13-HPOD	Mansuy *et al.* (1988)
N′-Phenylbenzoylhydrazine	SLO	13-HPOD	Mansuy *et al.* (1988)
Caffeic acid	SLO	H_2O_2	Mosca *et al.* (1996)

SLO, soybean lipoxygenase; LA, Linoleic acid, 13-HPOD, 13-hydroperoxy-9,11-octadecadienoic acid.

Vitamin A derivatives are free radical scavenger antioxidants and serve as LO inhibitors. Hypervitaminosis-A has been linked with birth defects in humans and animals. The parent retinoid *per se* may not be teratogenic, and bioactivation is required. Lomnitski *et al.* (1993) found a significant linear correlation between SLO-2 inhibition and fatty acid peroxyl radical-dependent bleaching of β-carotene. Wu *et al.* (1999) tentatively identified apocarotenal, epoxycarotenal, apocarotenone and epoxycarotenone among several metabolites of β-carotene generated by SLO and pea LO. Retinol displays a high affinity towards LO and behaves as a competitive inhibitor of the enzyme (Lomnitski *et al.* 1993). According to Datta and Kulkarni (1996), all-*trans*-retinol acetate is an excellent substrate for co-oxidation for SLO and HTPLO in the presence of linoleic acid. It was proposed that the peroxyl radical of linoleic acid generated by LO attacks the π-electrons of the C═C bond and produces 5,6-epoxide of all-*trans*-retinol acetate. The formation of the 5,6-epoxy metabolite has been noted during the SLO-catalysed co-oxidation of all-*trans*-retinoic acid (Matsui *et al.* 1994), retinol, and β-ionone (Waldmann and Schreier 1995). All-trans-retinol acetate is also co-oxidised by the purified LO from chickpeas (Sanz *et al.* 1992). Vitamin E (α-tocopherol) and its seven analogues serve as substrates for the hydroperoxidase activity of SLO and potato tuber 5-LO in the presence of 13-HPOD (Cucurou *et al.* 1991). However, the oxidation rates exhibit some LO-specific differences. Trolox C, a phenolic antioxidant, was found to undergo an H_2O_2-supported multistep oxidation process catalysed by SLO (Nunez-Delicado *et al.* 1997a). The authors proposed that the one-electron oxidation generates the phenoxyl radicals from Trolox-C which, following dismutation, results in the formation cross-conjugated ketodiene. Hydrolysis of the ketodiene finally yields Trolox-C quinone. However, Trolox-C oxidation is not supported by 13-HPOD with either SLO or potato tuber 5-LO (Cucurou *et al.* 1991).

Reynolds (1988) demonstrated that SLO is rapidly inactivated when incubated with arachidonate and either NDGA, the aminopyrazolines BW 755C or BW 540C or the acetohydroxamic acid derivatives BW A4C and BW A137C. 15-HPETE was as effective as arachidonate in promoting inactivation, but linoleic acid and 13-HPOD were much less effective. SLO inhibition was linked to the pseudoperoxidase activity of the enzyme (Reynolds 1988). Using a spectrophotometric assay, Riendeau *et al.* (1991) examined the ability of purified 5-LO from porcine leukocytes to degrade 13-HPOD in the presence of derivatives of diphenyl-*N*-hydroxybenzofurans, 4-hydroxybenzofurans and 5-hydroxydihydrobenzofurans (Table 7.7). A strong stimulation of pseudoperoxidase reaction could be detected only with very effective inhibitors of LTB_4 biosynthesis by human leukocytes. The results indicated that *N*-hydroxyurea and benzofuranol derivatives can function as reducing agents for the enzyme. *N*-(4-Chlorophenyl)-*N*-hydroxy-N′-(3-chlorophenyl)urea (CPHU) serves as a reducing agent and stimulates 13-HPOD-supported hydroperoxidase activity of the recombinant human 5-LO, porcine leukocyte 12-LO and SLO (Falgueyret *et al.* 1992; Desmarais *et al.* 1994). RP-HPLC data support the proposal put forth by the authors that nitroxide radical is formed during the SLO-mediated metabolism of CPHU in the presence of 13-HPOD. ESR studies conducted by Chamulitrat *et al.* (1992) provided the direct evidence that NOH of the hydroxamate group of CPHU, *N*–[(E)-3-(3-phenoxyphenyl)prop-2-enyl]acetohydroxamic acid (BW A4C) and *N*-(1-benzo(b) thien-2-ylethyl)-*N*-hydroxyurea (Zileuton) is oxidised by SLO to form their corres-

ponding nitroxides when incubated in the presence of linoleic acid. In addition, the authors provided ESR evidence and documented the formation of expected nitroxide metabolites as the one-electron oxidation products of dimethylhydroxylamine, isopropylhydroxylamine, desferal, *N*-hydroxyurea and cyclohexylhydroxylamine in the incubation media containing SLO and 13-HPOD. *N*-hydroxyurea is an animal teratogen while zileuton, a 5-LO inhibitor is a drug for the treatment of asthma. As an 5-LO inhibitor, diphenyl disulphide was a thousand-fold more potent than diethyldisulphide and it is noteworthy that glutathione, a typical reducing thiol, is almost inactive in inhibiting this enzyme even at 80 mM (Egan and Gale 1985). Other chemical classes reported as LO inhibitors include *n*-alcohols and *n*-alkylthiols (Kuninori *et al*. 1992), flavonoids (Yoshimoto *et al*. 1983; Kim *et al*. 1998; You *et al*. 1999) and many others. Some of these chemicals are expected to be oxidised by LO.

Lipoxygenase-mediated glutathione conjugation of xenobiotics

The formation of GSH conjugate, a result of an interaction between an electrophile of either endo- or exo-biotic origin and the sulphydryl group in the GSH molecule, is one of the most common reactions encountered in the human body. Currently, many believe that glutathione transferase (GST) is the only pathway responsible for an enzymatic generation of thioethers from xenobiotics in different mammalian tissues. Kulkarni and Sajan (1997) were first to report GSH conjugation of ethacrynic acid (EA), a diuretic drug, by SLO in the presence of arachidonic acid, linoleic acid and γ-linolenic acid. Spectrophotometric, TLC, HPLC, radiometry and MS analyses of the reaction media indicated that both SLO and GST produce an identical adduct, i.e. EA-SG. The rate of EA-SG formation was up to 1650-fold greater than that observed with different purified isozymes of mammalian GSTs. A recent study (Kulkarni and Sajan 1999) has further established that human tissue LO (HTPLO) is also capable of extensive EA-SG formation. A marked blockade of EA-SG formation by NDGA, ETI, esculetin and gossypol clearly implicates LO involvement in the reaction. The observations that the reaction is significantly suppressed by BHT, BHA and spin traps suggest the free radical nature of the reaction. Two possible mechanisms of EA-SG formation were proposed. As shown in Figure 7.12, it is envisioned that GSH is first oxidised by LO to $GS^{\bullet}$. In the second step, the $GS^{\bullet}$ directly attacks the C═C bond in the EA molecule to generate a carbon-centred radical of EA which reacts with another molecule of GSH to finally yield EA-SG. According to the second mechanism, EA is presumed to be first oxidised by LO to $EA^{\bullet}$. A spontaneous interaction of this cation radical with GSH is expected to result in EA-SG. Noteworthy is the fact that the reaction occurs at a significant rate under the physiologically relevant concentrations of GSH and fatty acid, and pH. These results strongly suggest that *in vivo* thioether formation from certain chemicals may occur via the LO pathway.

Another example of GSH conjugation of xenobiotics via LO pathway includes *p*-aminophenol (PAP). Although the mechanism(s) responsible for PAP nephrotoxicity is not yet established, oxidative metabolism and subsequent conjugation with GSH are believed to be the key steps involved. For several reasons, many investigators have dismissed the involvement of microsomal P450 and PGS in the PAP bioactivation process. On the other hand, the ESR study conducted with SLO has shown the

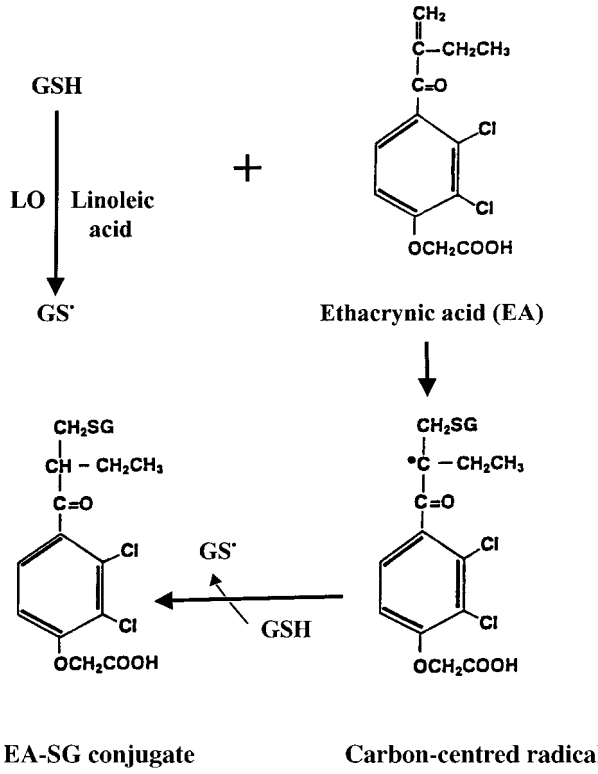

Figure 7.12 Conjugation of ethacrynic acid with glutathione by lipoxygenase.

formation of a short-lived 4-aminophenoxyl radical as the initial one-electron oxidation product of PAP in the reaction media supplemented with linoleic acid (Van der Zee *et al.* 1989). Furthermore, one-electron oxidation of PAP by SLO also occurs in the presence of 13-HPOD (Mansuy *et al.* 1988; Cucurou *et al.* 1991). Recently, Yang and Kulkarni (2000) noted SLO-mediated formation of GSH conjugates from PAP in the presence of H_2O_2. The LO inhibitors and free radical scavengers markedly decreased the rate of SLO-mediated GS-PAP formation. Since LO activity occurs both in the livers of rats (Roy and Kulkarni 1994) and humans (Roy and Kulkarni 1996b, 1997), and in human kidney (Oyekan *et al.* 1997; Stewart *et al.* 1997), a role for this pathway in nephrotoxicity of PAP is expected.

In vivo evidence

Gathering *in vivo* evidence to document LO involvement in xenobiotic oxidation has been troublesome. Currently, the use of so-called 'selective inhibitors' is popular, considering the non-selectivity of enzyme inhibitors (discussed above), the conclusions reached may be debatable. Despite this, the results of many studies suggest that

LO pathway plays a contributory role in xenobiotic oxidation *in vivo*. Castonguay *et al.* (1998) and Rioux and Castonguay (1998) investigated the preventive efficacy of PGS and 5-LO inhibitors against NNK carcinogenesis in female A/J mice. A-79175, the 5-LO inhibitor, was found to be a stronger inhibitor of lung tumourigenesis than the PGS inhibitor aspirin. Both lung tumour multiplicity and incidence were inhibited by A-79175. MK-886, which binds to FLAP and inhibits 5-LO also decreased the mean tumour volume (Rioux and Castonguay 1998). The authors proposed that besides P450, LO activates NNK (Castonguay *et al*. 1998; Rioux and Castonguay 1998). The incubation of 82-132 and LM2 murine lung tumour cells with MK-886 and A-79715 decreased NNK-caused cell proliferation in a concentration-dependent manner (Rioux and Castonguay 1998). The authors opined that an inhibition of NNK activation by LO may be the mechanism responsible for the observed effects. NDGA pretreatment diminishes the bladder toxicity of cyclophosphamide in male ICR mice (Frasier and Kehrer 1993). A significant decrease in the excretion of acrolein equivalents into urine was observed during the first 6 h following cyclophosphamide dosing. Although these results suggest that the LO pathway may be involved, the authors concluded that the protective action of NDGA and indomethacin is not due to interference with the metabolism of cyclophosphamide. An inhibition of the LO pathway also prevents DMBA-caused cancer of skin (Katiyar *et al*. 1992; Jiang *et al*. 1994) and mammary gland (Noguchi *et al*. 1993; Kitagawa and Noguchi 1994) suggesting *in vivo* carcinogen bioactivation by LO.

It has been documented that the preparations of rodent embryos (Vanderhoek and Klein 1988; Roy *et al*. 1993) and human intrauterine conceptual tissues during early gestation period (Joseph *et al*. 1994; Datta and Kulkarni 1994a; Datta *et al*. 1995) possess LO activity capable of xenobiotic oxidation. Yu and Wells (1995) observed that pretreatment of CD-1 mice with ETYA, a dual PGS and LO inhibitor, results in a dose-related decrease in the incidence of phenytoin-induced foetal cleft palates and resorptions. This reduction in phenytoin teratogenicity was considerably greater than that previously reported for acetylsalicylic acid, which inhibits PGS. Thus, these results provide strong evidence that LO pathway may be more important in the bioactivation of phenytoin *in vivo*.

Conclusions

Although the available information reviewed here reflects a promising start in the understanding of the role played by the LO pathway in xenobiotic oxidation, a lot needs to be accomplished. Few examples document oxidation, epoxidation, desulphuration, dearylation, sulphoxidation and dealkylation of xenobiotics; however, much remains to be investigated as regards the spectrum of reactions catalysed by different LOs. At present, the data are too sparse to establish clearly *in vivo* xenobiotic oxidation via the LO pathway. More data are needed to establish the relative significance of LO-mediated co-oxidation of chemicals in the light of other competing pathways. Although it appears that the use of inhibitors to selectively block the oxidative pathways of xenobiotic oxidation may finally turn out to be futile, an exploration of stereochemical differences in the metabolite generation may be beneficial. Exploitation of cell culture techniques and *ex-vivo* models for organs

culture represents another fruitful approach that would provide a good start in this direction. Inducibility of LO and LO-mediated GSH conjugation of xenobiotics represent important areas which deserve serious attention as they may explain some of the toxicological puzzles. Finally, one should bear in mind that although laboratory animal data are useful for the advancement of science, one should not forget the critical need for human data since the science of toxicology is meant to serve human interests and not those of the rats.

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