Purification and partial characterization of lipoxygenase with dual catalytic activities from human term placenta

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Lipoxygenase possessing dual catalytic activities, i.e. dioxygenase and hydroperoxidase, was purified from the cytosols of term placentas from non-smoking women. Concanavalin A affinity chromatography followed by phenyl-Sepharose CL-4B chromatography resulted in the separation of one hydrophobic and one non-hydrophobic isoenzyme. The concanavalin A-purified enzyme was used in all subsequent experiments. The dioxygenase activity of the enzyme exhibited a $V_{\max}$ of 204.37 ± 17.66 nmol/min per mg of protein and a K_m of 0.79 mM for linoleic acid. The involvement of dioxygen in enzymic linoleic acid oxidation was confirmed by O_2 uptake studies. Arachidonic acid and linolenic acid also served as substrates for the dioxygenase activity. The placental lipoxygenase co-oxidized benzidine in the presence of linoleic acid (hydroperoxidase activity). Both the

dioxygenase and hydroperoxidase activities were significantly stimulated by Ca^{2+} (1–100 μ M), ATP (10–400 nM) and H_2O_2 (1–10 nM). Similarly, these two activities were inhibited by nordihydroguaiaretic acid, 5,8,11-eicosatriynoic acid, gossypol, esculetin, butylated hydroxyanisole and butylated hydroxytoluene. Boiled enzyme was without significant dioxygenase and hydroperoxidase activities. Pyrogallol, 3,3'-dimethoxybenzidine, 3,3',5,5'-tetramethylbenzidine, tetramethylphenylenediamine and 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) were also co-oxidized by the placental lipoxygenase. These results suggest that: (i) lipoxygenase from human term placenta exhibits both dioxygenase and hydroperoxidase activities, and (ii) this enzyme represents an important pathway for chemical oxidation in the placentas of non-smoking women.

INTRODUCTION

The lipoxygenases (linoleate:oxygen oxidoreductase; EC 1.13.11.12) comprise a family of non-haem iron-containing enzymes that catalyse the stereospecific dioxygenation of polyunsaturated fatty acids with a 1,4-*cis-cis*-pentadiene structure. Since its first isolation by Theorell et al. (1946), this enzyme has been purified from several plant and animal tissues, including those of the human.

Arachidonic acid (AA) metabolites are important in the physiology of female reproductive tissues. Normal levels of AA metabolites are required for uterine motility, implantation and maintenance of placental blood flow, and in the initiation and maintenance of parturition (Mitchell and Grzyboski, 1987; Romero et al., 1987). An imbalance in the metabolism of AA can adversely affect pregnancy and may result in retarded foetal growth or pre-eclampsia. Even though the presence of lipoxygenase capable of converting AA to several metabolites was shown *in vitro* using cultured human placental cells (Rose et al., 1987) or homogenates (Saeed and Mitchell, 1982), purification of the enzyme from this source has not been reported so far.

Lipoxygenase, in addition to its dioxygenase activity, exhibits oxygenase, leukotriene synthase and hydroperoxidase activities. Using lipoxygenase from soybean as a model, our laboratory has established a role for this enzyme in xenobiotic oxidation. Oxidation of hydrogen donors (Kulkarni and Cook, 1988a,b), epoxidation of benzo[a]pyrene-7,8-dihydrodiol (Byczkowski and Kulkarni, 1989) and aldrin (Naidu et al., 1991a), sulphoxidation

of thiobenzamide (Naidu and Kulkarni, 1991), desulphuration and dearylation of parathion (Naidu et al., 1991b), and oxidation of 2-aminofluorene (Roy and Kulkarni, 1991) have all been demonstrated. Similar to soybean lipoxygenase, xenobiotic-metabolizing capacity was also observed with lipoxygenases from rat tissues such as liver (Roy et al., 1990), lung (Kulkarni et al., 1991; Byczkowski and Kulkarni, 1992) and brain (Naidu et al., 1992).

The placenta is important for the developing foetus physiologically, pharmacologically and toxicologically, as it controls the microenvironment that modulates foetal development. Examples of foetal malformations in which bioactive intermediates of xenobiotics are implicated are many in the literature, and it is suggested that the placenta may be one of the sites of generation of reactive species of chemicals. Available information suggests that the classical mono-oxygenase (cytochrome *P*-450) involved in xenobiotic metabolism is undetectable in human placenta unless there is maternal induction by chemicals present in cigarette smoke (Juchau et al., 1987). There is no previous report on the existence of flavin-containing mono-oxygenases in the human placenta. In this connection, we have reported previously the presence of other constitutive, alternative pathways such as indanol dehydrogenase and peroxidase in the placentas of non-smoking women (Kulkarni et al., 1985; Nelson and Kulkarni, 1990; Joseph et al., 1992). In this paper we report the methodology for the purification of the human term placental lipoxygenase (HTPLx) and some of its biochemical characteristics, including hydroperoxidase-like activity towards xenobiotics.

Abbreviations used: AA, arachidonic acid; HTPLx, human term placental lipoxygenase; Con A, concanavalin A; NDGA, nordihydroguaiaretic acid; BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; DMBD, 3,3'-dimethoxybenzidine; TMBD, 3,3',5,5'-tetramethylbenzidine; ABTS, 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid); ETI, 5,8,11-eicosatriynoic acid; LA, linoleic acid; LAOOH, LA hydroperoxide.

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MATERIALS AND METHODS

Chemicals

Linoleic acid (LA) (free acid, 99% pure), benzidine (99.9% pure), concanavalin A (Con A)-Sephacryl CL-4B, phenyl-Sepharose CL-4B, Sephacryl S-200, methyl α -D-mannoside, ethylene glycol, ATP, CaCl_2 , hydrogen peroxide (30%, v/v), nordihydroguaiaretic acid (NDGA), gossypol, esculetin, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), 3,3'-dimethoxybenzidine (DMBD), 3,3',5,5'-tetramethylbenzidine (TMBD), tetramethylphenylenediamine (TMPD), 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), pyrogallol and standard proteins for gel filtration chromatography were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. AA, linolenic acid and 5,8,11-eicosatriynoic acid (ETI) were purchased from Calbiochem. Polyacrylamide gels and buffer strips used for electrophoresis were purchased from Pharmacia LKB. All other chemicals and reagents used were of analytical grade.

Human term placentas

Human term placentas obtained from healthy women with no known history of smoking, drug or alcohol abuse, or pathological/physiological problems, were used in this study. Placentas obtained from a local hospital immediately after caesarian section were kept on ice and all subsequent manipulations were performed at 4 °C. The use of human term placentas in this *in vitro* study was approved by the University of South Florida's Institutional Review Board governing the policies and procedures of experiments involving human subjects.

Affinity chromatography

Placental villus tissue was teased and rinsed with copious amounts of ice-cold 10 mM Tris buffer (pH 6.5) to remove as much of the contaminating blood as possible. The tissue was homogenized in 50 mM Tris buffer (pH 6.5) using a Waring blender, and the resulting homogenate was sonicated for 30 s. The different subcellular fractions, i.e. mitochondria (10000 g pellet), microsomes (100000 g pellet) and cytosol (100000 g supernatant), were prepared by differential centrifugation. The cytosol, which possessed the majority of the dioxygenase activity (see Table 1), was applied at a flow rate of 15 ml/h onto a Con A-Sepharose column (20 cm $\times$ 2.6 cm) pre-equilibrated with at least 10 bed volumes of 50 mM Tris buffer (pH 6.5). After sample application, the column was washed with the equilibrating buffer until the protein absorbance of the effluent at 280 nm had returned to the baseline value. Subsequently, HTPLx bound to the column was eluted with the equilibrating buffer containing 0.5 M methyl α -D-mannoside and fractions of 2 ml were collected.

The fraction were assayed for dioxygenase activity with LA as the substrate, as well as for protein absorbance at 280 nm. Those fractions exhibiting activities within a distinct peak were pooled and dialysed against 50 mM Tris buffer (pH 6.5) overnight, with two changes of buffer. The dialysate was centrifuged at 2500 g for 10 min to remove the precipitated proteins and suspended particles. The resulting clear supernatant was used either directly or after concentration for various studies, including hydrophobic chromatography. The enzyme was concentrated by immersing the dialysis bag containing HTPLx in dry sucrose at 4 °C for 2–4 h.

Hydrophobic chromatography

The Con A-purified HTPLx was applied on a phenyl-Sepharose

CL-4B column (10 cm $\times$ 1 cm) pre-equilibrated with 50 mM Tris buffer. After sample application the column was washed with the equilibrating buffer until the protein absorbance at 280 nm had returned to the baseline value. Subsequently the HTPLx activity that was bound to the column was eluted using the equilibrating buffer containing ethylene glycol (70%, v/v). Throughout the hydrophobic chromatography, the flow rate of the column was maintained at 12 ml/h. Fractions of 2 ml were collected during the sample application and the subsequent washing of the column; this fraction size was reduced to 1 ml during elution with ethylene glycol. The fractions were assayed for dioxygenase activity using LA as the substrate, as well as for protein absorbance at 280 nm.

Gel electrophoresis

PAGE of the cytosolic purified (untreated) HTPLx was performed on a 12.5% polyacrylamide gel using a Phastsystem electrophoresis instrument (Pharmacia LKB) according to the procedure described in the Owner's Manual (file no. 111).

Molecular mass determination

The apparent molecular mass of the Con A-purified HTPLx was determined by gel filtration on a Sephacryl S-200 column. The column (80 cm $\times$ 2.6 cm) was pre-equilibrated with 50 mM Tris buffer (pH 6.5) and calibrated with Blue Dextran (2000 kDa) to estimate the void volume (V_0), and with the following protein standards: BSA (66 kDa); phospholipase D (112.5 kDa), alcohol dehydrogenase (150 kDa) and catalase (250 kDa). The elution volumes (V_e) of the standard proteins were determined from the absorbance at 280 nm, while that of HTPLx was determined by measuring the dioxygenase activity.

Characterization of lipoygenase

The dioxygenase activity of HTPLx was partially characterized. The use of LA as the substrate was preferred over AA, because of: (i) the relatively high instability of AA due to auto-oxidation, and (ii) the fact that AA can also be peroxidized by prostaglandin synthetase, while LA can be used as substrate only by lipoygenase. Details of the preparation of LA were as described earlier (Kulkarni et al., 1991). In all of the assays, both sample and reference cuvettes contained an identical reaction medium except that the enzyme was present only in the sample cuvette. The reaction was initiated by mixing LA and enzyme in 50 mM Tris buffer, pH 6.5 (37 °C), in a total reaction volume of 1 ml. The rate of LA oxidation by HTPLx (dioxygenase activity) was followed spectrophotometrically as an increase in the absorbance at 234 nm, and the enzyme activity was calculated using a molar absorption coefficient of $25 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ for the LA hydroperoxide (LAOOH), a conjugated diene product (Kulkarni and Cook, 1988a). The oxidation of AA and of linolenic acid catalysed by HTPLx was also studied under conditions similar to those used for LA.

The LA-dependent hydroperoxidase activity of HTPLx was characterized by using benzidine as the co-substrate (Kulkarni and Cook, 1988b). Benzidine co-oxidation was followed spectrophotometrically as an increase in absorbance at 425 nm due to the formation of benzidinedi-imine. An ϵ_{425} of $6.25 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ was used to calculate the amount of benzidinedi-imine produced.

Oxygen uptake studies

O_2 uptake during the oxidation of LA by HTPLx was monitored using a Clark electrode on a YSI model 5300 biological oxygen

monitor under assay conditions identical to those used in the spectrophotometric studies, except that the final volume of the reaction mixture was 2 ml.

Stimulation and inhibition studies

Stimulation of the dioxygenase and hydroperoxidase activities of the purified HTPLx by Ca^{2+} , ATP and H_2O_2 and its inhibition by lipoxygenase inhibitors (NDGA, ETI, gossypol, esculetin and antioxidants such as BHA and BHT) were investigated spectrophotometrically. In each case, the indicated concentration of the stimulator or the inhibitor (for details, see Figures 6 and 7) was pre-incubated at 37 °C with the enzyme in 50 mM Tris buffer, pH 6.5, for 1 min, and the reaction was started by the addition of LA alone (dioxygenase) or LA plus benzidine (hydroperoxidase). The increase in absorbance was monitored at 234 nm for dioxygenase activity and at 425 nm for hydroperoxidase activity.

Oxidation of xenobiotics

Oxidation of the selected xenobiotics was examined under conditions (final reaction mixture 1.0 ml) similar to those employed for benzidine as described previously (Strohm and Kulkarni, 1986; Kulkarni and Cook, 1988b; Joseph et al., 1992).

Protein estimation

The amount of protein present in the enzyme samples was estimated by the method of Bradford (1976), using BSA fraction V as the standard protein.

RESULTS

Subcellular distribution of placental lipoxygenase

The data presented in Table 1 show that virtually all fractions, i.e. mitochondria, microsomes and cytosol, possessed detectable lipoxygenase activity. However, the majority of the activity (about 80 %) was associated with the cytosol, and therefore this fraction was used to purify HTPLx.

Purification of lipoxygenase

A summary of the results of the HTPLx purification experiments is presented in Table 2. Con A-Sepharose chromatography (Figure 1) resulted in the successful separation of HTPLx from the contaminating proteins, mainly haemoglobin, present in the cytosol. It was noticed that a majority (> 95 %) of the haemoglobin passed out unadsorbed during sample application. The remaining haemoglobin and a few other contaminating proteins were removed during washing of the column with the equilibrating buffer. The inability of haemoglobin to bind to the Con A-Sepharose column was tested in a separate experiment using purified human haemoglobin. It was found that haemoglobin does not exhibit affinity for Con A and fails to bind to the column [the results are not presented here, since they were identical to those reported previously by Nelson and Kulkarni (1990)]. HTPLx bound to the column was eluted as a single peak with 50 mM Tris buffer containing 0.5 M methyl α -D-mannoside.

Dialysis of the Con A-purified HTPLx resulted in the precipitation of some protein, which was removed by centrifuging the samples at 3000 rev./min for 5 min. Assay of the precipitated protein after resuspension in the buffer showed that it possessed very little lipoxygenase activity, whereas more than 95 % of the activity was associated with the clear supernatant. This also resulted in a small increase in the specific (dioxygenase) activity of the enzyme in the supernatant

fraction from 178.43 ± 11.69 nmol/min per mg of protein to 213.54 ± 20.36 nmol/min per mg of protein. The clear supernatant was used either directly or after concentration in all the experiments.

Hydrophobic chromatography (Figure 2) of the affinity-purified HTPLx resulted in its separation into two isoenzymes, one hydrophobic and the other non-hydrophobic. Approx. 70 % of the Con A-purified HTPLx was found to be non-hydrophobic and passed out of the column unadsorbed during sample application and subsequent washing of the column. The hydro-

Table 1 Subcellular distribution of HTPLx (dioxygenase) activity

A total of 100 g of tissue was used for homogenization. Activity was determined using LA as the substrate, as described in the Materials and methods section. Values represent means $\pm$ S.E.M. of four different placentas.

Fraction	Specific activity (nmol/min per mg of protein)	Total activity (μ mol/min)	Distribution (%)
Homogenate	40.86 ± 5.66	1495.01	100
Mitochondria (10 000 g pellet)	11.11 ± 2.60	204.36	13.67
Microsomes (100 000 g pellet)	8.31 ± 2.26	90.35	6.04
Cytosol (100 000 g supernatant)	679.46 ± 51.49	1200.30	80.29

Table 2 Purification of HTPLx (dioxygenase) activity

Dioxygenase activity towards LA was assayed as described in the Materials and methods section. Values represent means $\pm$ S.E.M. of four separate experiments.

Step	Total activity (nmol/min)	Specific activity (nmol/min per mg of protein)	Recovery (%)
Con A-Sepharose	6828.77 ± 1027.51	178.43 ± 11.69	100
Dialysis and centrifugation	6510.90 ± 868.92	213.54 ± 20.36	95.34
Phenyl-Sepharose (effluent + wash)	5010.64 ± 637.83	246.57 ± 24.14	73.38
Phenyl-Sepharose (eluate)	991.07 ± 186.60	174.11 ± 19.64	14.51

Figure 1 Elution profile of HTPLx from a Con A-Sepharose CL-4B column

Figure 2 Elution profile of HTPLx from a phenyl-Sepharose CL-4B column

A, addition of Con A-purified lipoxigenase; B, addition of 70% ethylene glycol.

Figure 3 PAGE of purified HTPLx

Lane 1, Purified human haemoglobin; lane 2, hydrophobic HTPLx; lane 3, non-hydrophobic HTPLx; lane 4, Con A-purified HTPLx; lane 5, placental cytosol.

Table 3 Effect of temperature on the dioxigenase and hydroperoxidase activities of placental lipoxigenase

Activities were determined as described in the Materials and methods section. Values represent means $\pm$ S.E.M. of four different enzyme preparations.

Temperature	Activity (nmol/min per mg of protein)	
	Dioxigenase	Hydroperoxidase
Room temp. (25 °C)	146.59 $\pm$ 16.34	13.87 $\pm$ 1.86
37 °C	202.92 $\pm$ 11.93	21.17 $\pm$ 1.81
Boiled enzyme	7.38 $\pm$ 1.74	0.82 $\pm$ 0.50

phobic component of the Con A-purified HTPLx, which was bound to the column, was eluted when Tris buffer containing 70% ethylene glycol was applied to the column. The non-hydrophobic isoenzyme was a 50% better catalyst of LA dioxigenation (246.57 ± 24.14 nmol of LAOOH/min per mg of protein) than the hydrophobic form (174.11 ± 19.64 nmol of LAOOH/min per mg of protein). The Con A-purified HTPLx preparation and the two isoenzymes all exhibited very similar

Figure 4 Effect of experimental conditions on the dioxigenase activity of HTPLx

(a) pH, (b) enzyme protein concentration, (c) LA concentration.

hydroperoxidase activities (approx. 20 nmol of benzidinedimine/min per mg of protein).

Attempts to concentrate the purified enzyme sample using commercial ultrafiltration membranes (model 52 Amicon cell and a PM-10 Diaflo membrane, CX 30 membrane etc.) were unsuccessful and resulted in the inactivation of HTPLx. However, immersing the dialysis bag containing the enzyme in solid sucrose for 2–4 h (two or three changes of dry solid sucrose) was found to be effective in concentrating the enzyme without any significant loss of activity. The purified HTPLx was stable for several days at 0–4 °C without any significant loss in activity.

Gel electrophoresis

PAGE (Figure 3) showed that Con A-Sepharose chromatography resulted in a substantial purification of HTPLx. A comparison of the electrophoretic patterns of proteins suggested that the purified HTPLx was completely free of haemoglobin, which was a major contaminant protein present in the placental

Table 4 Oxygen uptake during the oxidation of LA by HTPLx

Assays were performed as described in the Materials and methods section. Values represent mean $\pm$ S.E.M. of three different enzyme preparations.

Assay conditions			O ₂ uptake (ng of O ₂ /min per mg of protein)
[LA] (mM)	Enzyme (μ g)	[NDGA] (μ M)	
0.38	200		40.35 $\pm$ 5.03
0.75	200		80.53 $\pm$ 10.08
1.5	200		154.89 $\pm$ 10.92
3.0	200		275.10 $\pm$ 13.58
6.0	200		331.22 $\pm$ 23.05
12.0	200		191.93 $\pm$ 12.62
3	25		16.66 $\pm$ 1.94
3	50		92.70 $\pm$ 15.89
3	100		252.22 $\pm$ 34.14
3	200		563.74 $\pm$ 40.09
3	400		664.86 $\pm$ 22.76
3	800		696.05 $\pm$ 13.65
3	200	0.13	275.10 $\pm$ 13.58
3	200	0.25	209.08 $\pm$ 17.54
3	200	0.5	160.06 $\pm$ 14.52
3	200	1	104.54 $\pm$ 13.76
3	200	2	82.53 $\pm$ 12.51
3	200	4	35.08 $\pm$ 6.21

cytosol. The purified HTPLx showed the presence of major and minor protein staining bands (Figure 3, lane 4). These two protein staining bands corresponded to the hydrophobic and

non-hydrophobic isoenzymes (Figure 3, lanes 2 and 3) which were separated by hydrophobic chromatography using phenyl-Sepharose CL-4B.

Molecular mass determination

Using the calibration curve prepared for the gel filtration of the standard proteins on a Sephacryl S-200 column, the apparent molecular mass determined for the Con A-purified HTPLx was 158 kDa.

Characterization of lipoxygenase activity

The oxidation of LA by the Con A-purified HTPLx exhibited characteristics typical of an enzyme-catalysed reaction. The accumulation of LAOOH in the reaction medium was time-dependent and proceeded with a high initial velocity. A lag phase, characteristic of several lipoxygenases, was not observed during this reaction. The rate of the reaction was significantly higher at 37 °C than at room temperature (Table 3). No LA oxidation occurred when the enzyme was omitted from the reaction medium. Boiling of the enzyme at 100 °C for 5 min resulted in almost complete inactivation.

The rate of HTPLx-catalysed LAOOH production was maximum at pH 6.5 (Figure 4a) and declined markedly in the alkaline pH range. Product formation increased with an increase in the enzyme protein concentration in the reaction medium up to 125 μ g/ml (Figure 4b). A slight increase in the specific activity was observed at protein concentrations above 75 μ g/ml. the dioxygenase activity exhibited dependence on the concentration of LA, and the maximum rate of oxidation was observed at

Figure 5 Effect of experimental conditions on the hydroperoxidase activity of HTPLx

(a) pH, (b) enzyme protein concentration, (c) LA concentration, (d) benzidine concentration.

Figure 6 Stimulatory effects of Ca^{2+} (a), H_2O_2 (b) and ATP (c) on the dioxxygenase activity of HTPLx

The inset in (c) shows the stimulation of dioxxygenase activity by ATP at lower concentrations.

3.0 mM LA (Figure 4c). Kinetic analysis of the data yielded a K_m of 0.79 mM for LA and a V_{max} of 204.37 ± 17.66 nmoles/min per mg of protein. Higher LA concentrations resulted in a decline in the specific activity of the enzyme, possibly due to substrate inhibition.

The results of oxygen uptake studies (Table 4) confirm the involvement of a true dioxxygenase activity during the oxidation of LA catalysed by HTPLx. The amount of oxygen consumed during the catalytic oxidation of LA was dependent upon the amounts of enzyme protein and LA in the reaction medium. Also, the specific lipoxygenase inhibitor NDGA caused a dose-dependent inhibition of oxygen uptake. In addition to LA, other fatty acids such as AA and linolenic acid (3 mM each) also served as substrates for the dioxxygenase activity of HTPLx when tested

Figure 7 Stimulatory effects of Ca^{2+} (a), H_2O_2 (b) and ATP (c) on the hydroperoxidase activity of HTPLx

The inset in (c) shows the stimulation of hydroperoxidase activity by lower concentrations of ATP.

under identical conditions. However, the rates of oxidation were several-fold lower for AA and linolenic acid (21.65 ± 3.47 and 14 ± 3.21 nmoles/min per mg of protein respectively).

The hydroperoxidase activity of HTPLx was characterized using benzidine as a model substrate. LA-dependent benzidine co-oxidation by HTPLx also exhibited characteristics typical of an enzyme-catalysed reaction. The enzymic conversion of benzidine to benzinedi-imine by HTPLx proceeded at a significantly higher rate at 37 °C than at room temperature (Table 3). Heat denaturation of the enzyme (100 °C for 5 min) resulted in almost complete blockage of the co-oxidation. The maximum rate of benzinedi-imine formation occurred at pH 6.5 (Figure 5a). The rate of benzidine co-oxidation increased proportionally with the amount of the enzyme protein up to 200 μg/ml; higher

Table 5 IC_{50} values of different chemicals for the dioxygenase and hydroperoxidase activities of HTPLx

Assays were performed as described in the Materials and methods section. Control activities of dioxygenase and hydroperoxidase were 218.71 ± 18.96 and 19.76 ± 3.36 nmol/min per mg of protein respectively. Values represent means $\pm$ S.E.M. of four different enzyme preparations.

Inhibitor	IC_{50} (μ M* or nM†)	
	Dioxygenase	Hydroperoxidase
NDGA	$0.62 \pm 0.04^*$	$0.48 \pm 0.03^*$
Esculetin	$18.75 \pm 2.40^*$	$22.31 \pm 3.15^*$
Gossypol	$7.69 \pm 0.94^*$	$9.62 \pm 1.07^*$
ETI	$57.50 \pm 6.90^*$	$51.96 \pm 5.62^*$
BHA	$2.40 \pm 0.26^\dagger$	$2.21 \pm 0.18^\dagger$
BHT	$2.49 \pm 0.34^\dagger$	$2.00 \pm 0.32^\dagger$

Table 6 Co-oxidation of xenobiotics by purified HTPLx

Assays were performed as described in the Materials and methods section. Values represent means $\pm$ S.E.M. of four different enzyme preparations.

Chemical	Rate of oxidation (nmol/min per mg of protein)
DMBD	26.54 ± 6.19
TMPD	56.35 ± 9.35
TMBD	23.95 ± 5.29
ABTS	3.36 ± 0.92
Pyrogallol	13.70 ± 2.92
Guaiacol	0.92 ± 0.15
Benzidine	21.17 ± 1.81

concentrations resulted in a decline in enzyme activity (Figure 5b). The rate of benzidine oxidation increased with LA concentration up to 6 mM (Figure 5c).

Stimulation and inhibition studies

The results of experiments on the stimulation of the dioxygenase and LA-dependent hydroperoxidase activities of HTPLx by Ca^{2+} , H_2O_2 and ATP are presented in Figures 6(a)–(c) and 7(a)–(c). Ca^{2+} at concentrations up to 75μ M resulted in a marked stimulation of both dioxygenase and hydroperoxidase activities. A maximal stimulation of about 1.5-fold was observed in both activities at 10μ M Ca^{2+} (Figures 6a and 7a). H_2O_2 at nanomolar concentrations caused a greater than 2-fold increase in dioxygenase activity and co-oxidation of benzidine. The maximum stimulation in both the activities occurred at 3 nM H_2O_2 (Figures 6b and 7b). ATP was also stimulatory at concentrations ranging between 0.25 and 400μ M, with the maximum stimulation being at 25μ M (Figures 6c and 7c) for the production of both LAOOH and benzidinedi-imine. Higher concentrations of ATP ($> 800 \mu$ M) were inhibitory to both activities (results not shown). No additive effect was observed when the three stimulating factors were added simultaneously in combinations of either two or three.

NDGA inhibited the dioxygenase and hydroperoxidase activities of HTPLx in a dose-dependent manner, with IC_{50} values of $0.62 \pm 0.04 \mu$ M and $0.48 \pm 0.03 \mu$ M respectively (Table 5). In addition to NDGA, all of the other chemicals tested (gossypol,

esculetin, ETI, BHA and BHT) also resulted in varying degrees of dose-dependent inhibition of the dioxygenase and hydroperoxidase activities of HTPLx (Table 5).

Oxidation of xenobiotics

Studies on the oxidation of xenobiotics by HTPLx employing LA as the co-substrate showed positive results with all six chemicals tested (Table 6). The highest rate of oxidation occurred with TMPD while guaiacol, the most commonly used peroxidase substrate, showed the lowest rate of LA-dependent co-oxidation.

DISCUSSION

This paper represents the first report on the purification and characterization of a lipoyxygenase from human term placenta that possesses dual catalytic activities, i.e. dioxygenase and hydroperoxidase. Although it was possible to purify the HTPLx into two electrophoretically homogeneous isoenzymes, the Con A-purified HTPLx (a mixture of both the hydrophobic and non-hydrophobic isoenzymes) was used in all experiments. This was necessary mainly because of the availability of only a small quantity of the hydrophobic isoenzyme at the time of this study. It would be interesting to investigate the biochemical similarities/differences between the two isoenzymes as well as their relative contributions to the xenobiotic metabolism in the human placenta.

The pseudolipoyxygenase activity of haemoglobin is well known (Kuhn et al., 1981), and because of the high contamination of the placental subcellular organelles by haemoglobin it is difficult to accurately quantify and attribute the HTPLx activity to any particular cellular organelle. Therefore the data presented in Table 1 on HTPLx activity represent, to some extent, an overestimate of the true activity. More precise immuno-histochemical studies are required to establish the definitive subcellular localization of HTPLx. These results, although tentative, suggested that HTPLx primarily resides in the cytosol (Table 1). This observation is in accordance with reports on lipoyxygenases isolated from other mammalian tissues (Narumiya et al., 1981; Ochi et al., 1983; Nichols and Vanderhoek, 1991). It is also possible that sonication of the placental homogenate causes some solubilization of the membrane-associated lipoyxygenase (if any), making cytosol the most suitable starting material for HTPLx purification.

The facts that HTPLx bound to the Con A-Sepharose column and that 0.5 M methyl α -D-mannoside was required for its elution suggest that HTPLx is a glycoprotein. Earlier, Rapoport et al. (1979) reported that rabbit reticulocyte lipoyxygenase is a glycoprotein containing $4.6 \pm 1.2\%$ neutral sugars. The chemical composition of the carbohydrate moiety of HTPLx is unknown at present, and further studies are needed to firmly establish the glycoprotein nature of this enzyme. In the present study, the chemical identity of the end products of fatty acid dioxygenation by the purified enzyme was not determined. Earlier, Saeed and Mitchell (1982) reported the formation of several metabolites following incubation of human placental homogenates with [14 C]AA. The major metabolites were identified as 5- and 12-hydroperoxy-AA. Therefore a possibility exists that the hydrophobic and non-hydrophobic isoenzymes of HTPLx that are separated by phenyl-Sepharose CL-4B chromatography may represent 5- and 12-lipoyxygenases. During extensive dialysis of the Con A-purified HTPLx, some contaminant protein was found to precipitate in the dialysis bag, facilitating further purification of the enzyme. Attempts to concentrate the purified enzyme using commercially available ultrafiltration membranes

resulted in the inactivation of HTPLx. Schewe et al. (1986) have reported similar inactivation of rabbit reticulocyte lipoxigenase during concentration, which was attributed to the adsorption of the enzyme protein to the commonly used commercial ultra-filtration membranes. However, immersing a dialysis bag containing the enzyme in dry sucrose was found to be a suitable alternative method for concentrating HTPLx without any loss of activity.

Although the chemical identity of the final reaction product(s) was not established in this study, the increase in absorbance at 234 nm due to the formation of conjugated diene(s) accompanied by a concurrent O_2 uptake reflects the oxidation of LA by the dioxygenase activity of HTPLx. The fact that no benzidine oxidation was observed when LA was absent from the system (Figure 5c) suggests that the co-oxidation of benzidine was dependent on the generation of the oxidant during or immediately following the dioxygenase reaction. The dependence of both dioxygenase and hydroperoxidase reactions on the buffer pH and on the concentrations of both enzyme protein and substrates (LA and benzidine), as well as the thermal inactivation of both reactions, clearly suggests their enzymic nature. Furthermore, the inhibition of both activities by NDGA, gossypol, esculetin and ETI (all specific lipoxigenase inhibitors) confirms that the enzyme involved in these reactions is truly a lipoxigenase. The inhibition of the dioxygenase and hydroperoxidase activities of HTPLx by the antioxidants BHA and BHT provides evidence for the involvement of free radicals during both the oxidation of LA and benzidine co-oxidation by HTPLx.

Lipoxigenases in mammalian tissues are regulated by complex mechanisms. Several of the purified mammalian lipoxigenases exhibit a unique requirement for certain stimulatory factors. As found in the present study, a general requirement for stimulatory factors such as Ca^{2+} and ATP has been reported for the maximum expression of lipoxigenase activity purified from various mammalian tissues (Jakschik et al., 1980; Parker and Aykent, 1982; Rouzer and Samuelsson, 1990). Most of these reports document that both Ca^{2+} and ATP at millimolar concentrations (2 mM) were effective in stimulating the lipoxigenase activity maximally. In contrast to these reports, we observed that the concentrations of Ca^{2+} and ATP required for the maximum stimulation of HTPLx were several orders of magnitude lower. Similar to our results, stimulation at physiologically attainable concentrations of Ca^{2+} has been reported for the 5-lipoxigenase of MC-9 mast cells and human leucocytes (Bryant et al., 1986; Puustinen et al., 1988), for 5-lipoxigenase purified from guinea pig peritoneal polymorphonuclear leucocytes (Ochi et al., 1983), and for the 15-lipoxigenase of human polymorphonuclear leucocytes (Nichols and Vanderhoek, 1991). Similar to our results, Sigal et al. (1988) also observed inhibition of AA 15-lipoxigenase activity in the human eosinophil-enriched leucocytes by millimolar concentrations of ATP.

The actual biochemical mechanism underlying the stimulation of lipoxigenase by the various factors has not been fully elucidated. Parker and Aykent (1982), from their studies on the regulation of 5-lipoxigenase activity of rat basophilic leukaemia-1 cells, concluded that the native enzyme (a monomer of 90000 Da) is inactive and that addition of calcium (about 0.5 M) results in the production of a protein dimer (180000 Da) which is active. Whether such a dimerization occurs with HTPLx needs to be evaluated.

We previously reported (Kulkarni et al., 1989, 1990) that H_2O_2 at nanomolar concentration stimulates the LA-dependent dioxygenase and hydroperoxidase activities of soybean lipoxigenase. More recently, H_2O_2 was also found to stimulate rat brain lipoxigenase (Byczkowski et al., 1992). The stimulatory effect of

H_2O_2 on soybean lipoxigenase was postulated to be due to the transformation of the enzyme to an active state, possibly by the oxidation of the ferrous iron present in the inactive enzyme. All the three stimulatory factors employed in the present study, i.e. calcium, ATP and H_2O_2 , resulted in an increase in the velocity of the reaction, and their stimulatory effects were not additive. It is believed that several factors in addition to a change in the redox state of iron may be involved in the activation of the enzyme. The possibility of a change in the conformation of the polypeptide chains or in the substrate binding site cannot be ruled out at present. A marked stimulation of HTPLx by several endogenous chemicals also suggests that this enzyme is controlled by a complex multicomponent regulatory system, similar to the lipoxigenases isolated and purified from other mammalian tissues (Jakschik et al., 1980; Parker and Aykent, 1982; Rouzer and Samuelsson, 1990; Nichols and Vanderhoek, 1991).

Xenobiotic metabolism in organs other than liver is currently poorly understood, primarily due to a lower abundance of the classical xenobiotic-metabolizing enzymes in the extrahepatic organs. A complete understanding of the xenobiotic-metabolizing capacity of the placenta is important toxicologically, as the *in situ* bioactivation of lipophilic transplacentally active chemicals can lead to developmental toxicity either directly after translocation or indirectly via placental dysfunction. Although bioactivation of chemicals catalysed by cytochrome P-450-dependent mono-oxygenases has been demonstrated in human placenta by some investigators and questioned by others (Juchau et al., 1987), induction of these enzymes by polycyclic aromatic hydrocarbons and other chemicals present in cigarette smoke seems to be a pre-requisite to observe these reactions. In contrast to this, the presence of a constitutive peroxidase enzyme system capable of bioactivation of several xenobiotics has been reported in the placentas of non-smokers (Nelson and Kulkarni, 1990; Joseph et al., 1992; Kulkarni et al., 1992). The fact that all six chemicals tested in this study underwent LA-dependent co-oxidation catalysed by the HTPLx suggests that, in addition to peroxidase activity, LA-dependent co-oxidation catalysed by lipoxigenase is also an important constitutive xenobiotic-oxidizing pathway present in the placentas of non-smoking women.

Peroxidase contamination is a common problem associated with lipoxigenase purification. Several authors have demonstrated co-purification of a peroxidase activity along with that of lipoxigenase (Eriksson and Svensson, 1970; Johns et al., 1973). Flurkey et al. (1978) successfully separated contaminating peroxidase from affinity-purified soybean lipoxigenase by employing hydrophobic chromatography. Since we reported earlier that the human term placenta possesses a peroxidase activity that can also be purified by the chromatography procedures described here (Nelson and Kulkarni, 1990; Joseph et al., 1993), a question arises about the origin of the peroxidase activity observed in the HTPLx preparations used in this study. However, possible contamination of HTPLx with an extraneous peroxidase is unlikely, for the following reasons: (i) the placental peroxidase is a membrane-bound protein, whereas lipoxigenase is mainly, if not exclusively, cytosolic; (ii) guaiacol was a relatively poor substrate for the purified lipoxigenase using LA as the co-substrate, whereas this commonly used chemical serves as an excellent substrate for the placental peroxidase; (iii) H_2O_2 at concentrations above 10 nM inhibited HTPLx activity, whereas 300–400 μ M H_2O_2 is required to observe maximal activity of the membrane-bound peroxidase; and (iv) the placental peroxidase cannot oxidize LA or catalyse fatty acid-dependent xenobiotic oxidation (Nelson and Kulkarni, 1990). The observed similarities in various biochemical characteristics, such as heat inactivation,

inhibition by lipoxygenase inhibitors, stimulation by Ca^{2+} , ATP and H_2O_2 , co-elution of both the dioxygenase and hydroperoxidase activities from the Con A- and phenyl-sepharose CL-4B columns and the electrophoretic homogeneity (single band after PAGE) of the purified enzyme, strongly suggest that both the dioxygenase and hydroperoxidase activities observed for HTPLx are indeed associated with a single protein.

To date there has been no previous report on polyunsaturated fatty acid-coupled co-oxidation of xenobiotics by purified human tissue lipoxygenases. Lipoxygenases have been purified mainly from human blood cells, but their availability in only small amounts coupled with the instability of the purified enzyme impose a serious limitation on their use in such experiments. In contrast, human placenta not only represent a healthy human tissue that can be obtained fresh in large quantities with relative ease, but is also a rich source of lipoxygenase. In view of this, further in-depth investigations on the characterization of the two HTPLx isoenzymes, the biochemistry of fatty acid dioxygenation, xenobiotic co-oxidation and other aspects are clearly warranted.

This study was supported in part by the Council for Tobacco Research Inc., U.S.A.

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