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LEUKOTRIENE B_4 METABOLISM BY HEPATIC CYTOCHROME P-450

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Leukotriene B_4 (LTB) was found to be metabolized by suspensions of rat liver microsomes in the presence of NADPH and oxygen. The rate of LTB metabolism was also measured in reconstituted systems of both micelles and phospholipid vesicles containing cytochrome P-450-LM_2, NADPH cytochrome P-450 reductase, and cytochrome b_5. A 1 uM concentration of LTB was metabolized by rat hepatic microsomes at a rate of 4 pmol LTB/min/nmole P-450, and by vesicle and micelle reconstituted systems at 3 pmole/min/nmole P-450-LM_2. At this rate a 10 g rat liver exposed to 1 uM LTB can metabolize 30 ug per hour. In that the leukotrienes are pharmacologically active at nanomolar concentrations, hepatic metabolism may be an important pathway of leukotriene inactivation.

The leukotrienes are a family of highly pharmacologically active substances that were first described as the slow reacting substance of anaphylaxis (1). Leukotriene B_4 (LTB, 5,12-dihydroxy-6,8,10,14-eicosatetraenoic acid) is an enzymic oxidation product of arachidonic acid. At nanomolar concentrations it causes chemotaxis of macrophages (2), contraction of isolated guinea pig ileum (3), and reduction of peripheral blood flow (4). At pH 7 and 20°C it has a half-life of days in aqueous solution (5). It is important that mechanisms exist to remove leukotrienes from the blood so they do not accumulate and cause anaphylaxis. It has been shown that leukotrienes are taken up by hepatocytes and are excreted in the bile (6). In that it is known that most bond isomers and polyhydroxylated analogues of LTB are pharmacologically inactive (7), it is likely that hepatic metabolism such as hydroxylation by cytochrome P-450 would be an effective means of reducing the many effects of circulating leukotrienes.

MATERIALS AND METHODS

Microsomes were isolated from livers of untreated, unstarved Sprague-Dawley rats by homogenization in 0.25 M sucrose dissolved in 50 mM Tris HCl

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buffer pH 7.5 and sedimentation at 10^5g for 90 min. The microsome pellet was taken off the glycogen pellet, resuspended in 20 mM potassium phosphate buffer pH 7.5 with 20% glycerol and the suspension was used immediately.

Cytochrome P-450 LM_2 was isolated from livers of phenobarbital-treated rabbits (8) to a purity of 17 nmoles/mg of protein. NADPH-cytochrome P-450 reductase was purified by 2',5'-ADP-sepharose (9) to a specific activity of 40 umoles/min x mg of protein from the 0.3 M KCl eluate of DEAE-cellulose from the same preparation of cytochrome P-450. The same fraction also yielded cytochrome b_5 that was over 90% pure (10). Reconstitution of the purified proteins in vesicles was achieved by a modification of the slow cholate dialysis method previously described (11). Dioleoylphosphatidylcholine (DOPC), dimyristoylphosphatidylethanolamine (DMPE) and dimyristoylphosphatidylcholine (DMPC) were purified by HPLC on an Si 100 column with hexane/isopropanol/water 6:8:1.6 as eluting solvent. Then a mixture of 12.5 mg of each of these purified phospholipids were dissolved with 1 mg dithiothreitol (DTT) 1.6 mg NADPH-cytochrome P-450 reductase, 0.85 mg cytochrome b_5 and 5 mg cytochrome P-450 LM_2 in 0.3 M potassium phosphate buffer pH 7.5 with 20% glycerol and 3% sodium cholate. After allowing 2 h at 20°C for the formation of mixed micelles, the solution was dialyzed for 3 h at 25°C and then for 24 h at 15°C against 20 mM potassium phosphate buffer pH 7.5 with 0.1 mM EDTA, DTT and 20% glycerol and then for 17 h against the same buffer without DTT and EDTA. The yield of cytochrome P-450 in the vesicles was 77% and no cytochrome P-420 was detectable. Reconstitution of the purified proteins in dilauroylphosphatidylcholine (DLPC) micelles was done at the optimal molar protein-to-lipid ratio of 1:75 (12). The vesicular and the micellar preparations at the molar ratios of reductase/b_5/P-450 of 0.2:0.5:1 had activities toward benzphetamine of 16 and 11 nmoles CH_2O/min x cytochrome P-450.

The experiments with reconstituted vesicles and micelles as well as microsomes were performed in duplicate and were identical in total volume and content of cytochrome P-450. An aliquot of a solution of leukotriene B_4 (gift from Merck-Frosst) in MeOH was coated on the wall of a vial by removal of the solvent with N_2. Then 1 ml of a suspension of microsomes or vesicles was added and the tube was filled with O_2. The set of experiments that were carried out in the absence of O_2 required deoxygenation by a stream of N_2 followed by addition of 10 mM D-glucose, glucose oxidase (50 ug/ml) and catalase (10 ug/ml) and preincubation for 15 min at 20°C. The reactions were started by addition of a solution of an NADPH-generating system to result in a final concentration of 0.5 mM NADP, 5mM D-glucose-6-phosphate and 10 IU of glucose-6-phosphate dehydrogenase. The reactions were stopped by addition of 30 ul glacial acetic acid. The remaining LTB was extracted with 3 ml diethyl ether that had been distilled before use over $LiAlH_4$. The residue was taken up in 100 ul of the HPLC running solvent for analysis by HPLC. Separation was achieved with a 0.46 x 25 cm reverse-phase Spherosorb ODS column (Altex, Berkeley, CA) by elution with methanol/water/acetic acid/phosphoric acid/aqueous ammonia 630:310:1:0.5:1.2 pH 5.6. Relative peak heights were used to quantitate LTB metabolism. LTB was standardized by measuring its absorption at 270 nm and using an extinction coefficient of $4x10^4$ $M^{-1}cm^{-1}$.

RESULTS AND DISCUSSION

Leukotriene B_4 (LTB) was found to be metabolized in suspensions of rat liver microsomes. The process was dependent on NADPH and oxygen. Each incubation was followed by extraction and analysis by HPLC. A typical HPLC chromatogram is shown in Fig. 1. The decrease of LTB as a function of incubation time is shown in Fig. 2.

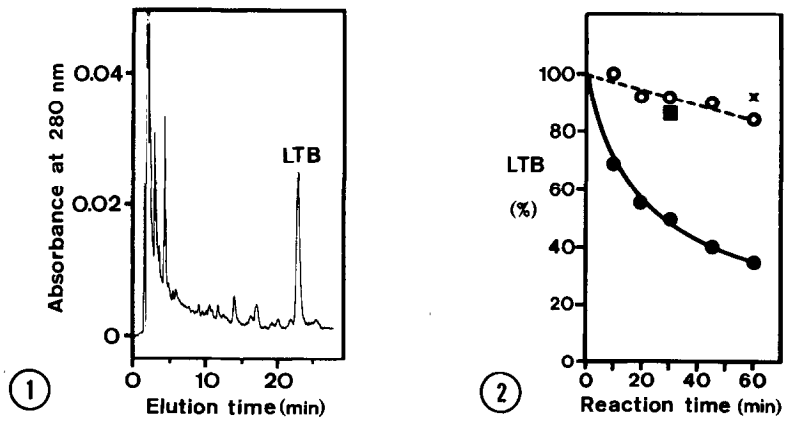

Fig. 1. High performance liquid chromatogram of an ether extract of a rat liver microsome suspension aerobically incubated for 30 min at 30°C with 200 ng of LTB and an NADPH-generating system.

Fig. 2. Time dependence of LTB metabolism as measured by the decrease of the ether-extractable amount of LTB after aerobic incubation (●) in a microsome suspension with NADPH at 30°C. As a control the same aliquot of an LTB solution was added to a suspension after incubation, followed by immediate acidification and extraction (○). The amount of LTB was essentially unchanged when incubated in the absence of O_2 (■) or microsomes.

The involvement of cytochrome P-450 was demonstrated by complete inhibition of metabolism in the presence of CO and by the requirement of cytochrome P-450 in reconstitution experiments. LTB was metabolized by cytochrome P-450-LM_2, cytochrome P-450 reductase and cytochrome b_5 co-reconstituted in either vesicles or micelles. LM_2 was chosen because of its demonstrated ability to interact with various fatty acids, even those that are chemically modified (13) and because of its capability to hydroxylate them (14). However, compared to microsomes, the rate of LTB metabolism was found to be about 30% less per nmol P-450. The rates of N-demethylation of benzphetamine in the reconstituted systems were optimal (12) for the molar ratios of the three proteins with reductase being the limiting factor, and were 10 to 15 times as high per nmol P-450 as in microsomes. Therefore, the lower rate of LTB metabolism by reconstituted cytochrome P-450-LM_2 means that at least one uninduced form of cytochrome P-450 in rat liver microsomes is involved in LTB metabolism.

In a study of prostaglandin metabolism by Vatsis et al (15), it was shown that P-450 LM_4 possesses considerably higher activities than P-450 LM_2.

Furthermore, the presence of cytochrome b_5 was essential for metabolism by P-450 LM_2. This is an interesting observation from the standpoint of a structure-function relationship because both leukotrienes and prostaglandins might be metabolized in a similar way in that they are both derivatives of arachidonic acid with a negatively charged carboxylic acid function that might be involved in the binding to cytochrome P-450 (13). In view of the hydrophobicity of most substrates, it is surprising that dihydroxylated fatty acids can act as substrates for P-450 in microsomes where they have to compete with a multitude of lipid-derived products (13).

Prostaglandins and leukotrienes are produced in very low quantities and are highly active at concentrations of nanograms per ml. Therefore, we chose to study the metabolism of LTB at submicromolar concentrations. Such a concentration is far below a concentration at which LTB would saturate cytochrome P-450 as a substrate. In fact,.prostaglandin hydroxylation by cytochrome P-450 was found to reach substrate saturation at millimolar concentrations (15). If one extrapolates the prostaglandin data to the lower concentration occurring in vivo, the rate of hydroxylation of prostaglandin is similar to the one we found for LTB_4.

At the low LTB_4 concentrations that we used, the rate of metabolism is first-order with respect to the LTB_4. At a LTB_4 concentration of 1 uM, the rate of metabolism would be 420 pmole/h x nmol P-450. At this rate about 30-50 ug LTB would be expected to be metabolized per hour by a whole 10 g rat liver. Bond isomers and polyhydroxy analogues of LTB_4 have been shown to be much less active (7). Therefore hepatic metabolism may constitute an important mechanism for reduction of leukotriene activity.

Some toxic mechanisms, such as metabolism of halocarbons, may lead to destruction of cytochrome P-450 as well as to production of leukotrienes. We have shown (unpublished data) that halothane ($CF_3CHBrCl$) free radicals initiate the formation of 5-hydroperoxyeicosatetraenoic acid which is thought to be a precursor of leukotrienes (1,7). It is known that metabolism of halocarbons results in inactivation of cytochrome P-450 (16). Therefore, it

is possible that halocarbon metabolism increases leukotriene production while the amount of cytochrome P-450 decreases, resulting in a net accumulation of the leukotrienes. This could have significant cellular and pathological consequences due to the involvement of leukotrienes in macrophage activation and alterations in microcirculation.

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