Biochimica et Biophysica Acta 878 (1986) 194–199
Elsevier

BBA 52301

Oxygen concentration-dependent metabolism of leukotriene B_4 by hepatocyte monolayers

Josef Gut, Anita K. Costa and James R. Trudell *

Department of Anesthesia, Stanford University School of Medicine, Stanford, CA 94305 (U.S.A.)

(Received April 18th, 1986)

Key words: Leukotriene B_4; Hepatic clearance; Hypoxia; Cytochrome *P*-450; (Rat hepatocyte)

Leukotriene B_4 was found to be metabolized by rat hepatocyte monolayers at a rate that was linear with increasing substrate concentration from 74 to 740 nM leukotriene B_4. The rates of metabolism were dependent on the O_2 concentration and were 315, 213, 80, and 36 pmol leukotriene B_4 per min per nmol cytochrome *P*-450 at 20% (212 μM), 4% (42.5 μM), 2% (21.2 μM), and 1% (10.6 μM) O_2, respectively. The metabolic rate was not linear with respect to O_2 concentration; however, half maximal rate occurred at 4% O_2, an O_2 concentration found in the pericentral region of normally oxygenated liver. These results suggest that in vivo conditions of hypoxia or ischemia that lead to blood O_2 concentrations less than 4% may drastically decrease hepatic clearance of leukotriene B_4.

Introduction

Leukotrienes are a group of endogenous compounds active in hypersensitivity reactions and inflammation [1,2]. Leukotriene B_4, an enzymatic oxidation product of arachidonic acid, at nanomolar concentrations causes chemotaxis of macrophages [3], reduces peripheral blood flow [4], induces degranulation and release of lysosomal enzymes from human polymorphonuclear leukocytes [5], and acts as a calcium ionophore in liposomes [6]. In human polymorphonuclear leukocytes, leukotriene B_4 is rapidly converted to its pharmacologically less potent oxidation products 20-OH-leukotriene B_4 and 20-COOH-leukotriene B_4 by cytochrome *P*-450 isozymes [7]. However, little is known about the fate of leukotriene B_4 in hepatocytes. Whereas the peptide leukotrienes (i.e., leukotriene C_4) are taken up by hepatocytes and are rapidly excreted into the bile [8,9], no such mechanism is reported for leukotriene B_4. This laboratory has presented evidence for hepatic cytochrome *P*-450 dependent metabolism of leukotriene B_4 as a likely mechanism of removal of circulating leukotriene B_4 [10]. Recently, both ω and ω-1 hydroxylation of leukotriene B_4 by rat liver microsomes has been reported [11].

Hepatocyte monolayers are an excellent integrated experimental system in which to study metabolism of exogenous and endogenous substrates under varying O_2 concentrations. In intact perfused liver extracellular O_2 concentrations may range from 100 μM in periportal blood to as low as 1–5 μM in centrilobular blood of hypoxic livers [12]. In that the K_m for O_2 of various cytochrome *P*-450 isozymes ranges widely from 0.7–140 μM [12], reaction rates and even metabolite patterns [13–15] may drastically change upon reducing O_2 concentrations below 20% (212 μM), at which most metabolic studies are conducted and all the isozymes are saturated with O_2. Therefore, we

* To whom correspondence should be addressed.

0005-2760/86/$03.50

examined the metabolism by hepatocyte monolayers of nanomolar concentrations of the potent chemoattractant leukotriene B_4 under physiologically relevant O_2 tensions.

Materials and Methods

Synthetic leukotriene B_4 was generously provided by Dr. J. Rokach of Merck Frosst Canada Inc. Collagenase (Type IV) and prostaglandin B_1 were from Sigma St. Louis, MO. The cell culture media were obtained from Gibco, Chagrin Falls, OH. Amberlite XAD-7 was purchased from Alltech Associates, Deerfield, IL, and all HPLC-grade solvents were obtained from Baker Chemical Co., Phillipsburg, NJ.

Cell culture and incubation at varying O_2 concentrations

Hepatocytes were prepared from fed adolescent male Sprague-Dawley rats as described [16,17] and were allowed to attach to collagen-coated, gas-permeable, 60 mm Lux Permanox contur dishes in 2 ml of M-199 in 5% CO_2/95% air at 37°C for 24 h. At this stage, the confluent monolayers were washed and then pre-incubated with 2 ml of Earle's medium at the appropriate O_2 concentrations for 2 h in order to achieve full equilibrium of the gaseous with the aqueous phase as well as of the aqueous phase with the cytoplasm of the cells. The normoxic group was placed in a standard incubator with 5% CO_2/95% air at 37°C, while the hypoxic groups were incubated in 1, 2 or 4% O_2/5% CO_2/balance N_2. After the 2 h preincubation, leukotriene B_4 (stored in liquid nitrogen as a 100 ng/μl solution in CH_3OH) was added as a 1 ng/μl solution prepared in Earle's medium, dishes were swirled thoroughly and incubated. Additions to the hypoxic dishes were made via an airtight plastic glove box built into the door of the incubator in order to maintain hypoxia throughout the experiment.

Extraction and analysis of leukotriene B_4

At the end of the incubation, 3 ml of CH_3OH containing 50 ng prostaglandin B_1 as internal standard were added to each dish, monolayers were scraped off, and the material was transferred to test tubes containing 100 μl 1 N acetic acid followed by centrifugation at $2000 \times g$ for 5 min at room temperature. Supernatants were diluted to 15 ml with distilled H_2O and passed through 1 ml columns of Amberlite XAD-7. After sample application, columns were washed with 3 ml of 65% CH_3OH/35% H_2O (v/v) followed by extraction with 90% CH_3OH/10% H_2O (v/v). The extracts were concentrated under a stream of N_2, re-suspended in 60 μl of the initial mobile phase [18], and an aliquot (50 μl) of each was applied to HPLC. Separation was achieved on an Ultrasphere ODS HPLC column (C-18, 0.46×25 cm, 5 μm, Altex) using a gradient consisting of methanol/acetonitrile/H_2O as described [18] at a flow rate of 0.8 ml/min. Relative peak heights were used to quantify leukotriene B_4 and the internal standard prostaglandin B_1 in individual chromatograms. A standard curve of 50 ng prostaglandin B_1 with varying leukotriene B_4 amounts was constructed and used to calculate the amounts of leukotriene B_4 metabolized. The recovery of prostaglandin B_1 was typically $82 \pm 5\%$ ($n = 10$) and that of leukotriene B_4 90% $\pm$ 3% ($n = 8$). Retention times were 16.5 min for prostaglandin B_1 and 18.7 min for leukotriene B_4.

Other methods

24 h after the initial preparation, cells from 12 dishes were harvested, microsomes were prepared, and cytochrome *P*-450 was estimated according to Ref. 19 using an $\hat{E}$ of 91 $mM^{-1} \cdot cm^{-1}$. Protein was determined by the Bio-Rad assay with bovine serum albumin as a standard. The number of cells per plate was calculated after counting five fields of cells on a Nikon Diaphot inverted microscope.

Results

When rat hepatocyte monolayers were incubated with leukotriene B_4 at nanomolar concentrations, a time-dependent disappearance of leukotriene B_4, reflecting metabolism of that substrate, was observed (Fig. 1). With an initial concentration of 148 nM leukotriene B_4, the time course of its metabolism was strongly dependent on the O_2 concentrations used during the experiments. Within 15 min, 60% of the leukotriene B_4 was metabolized at 20% O_2, whereas at 1% and 2% O_2, only 46 and 54%, respectively, were metabo-

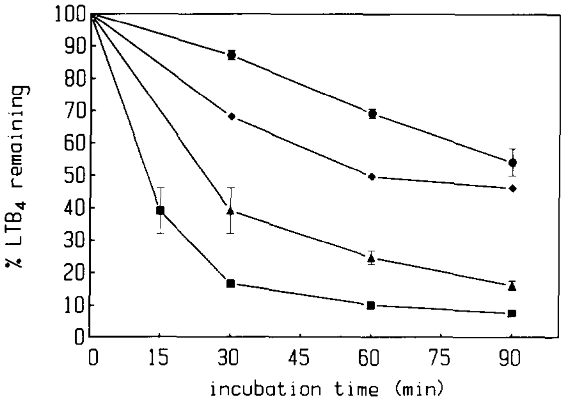

Fig. 1. Time course of O_2 concentration-dependent metabolism by hepatocyte monolayers of leukotriene B_4 (LTB_4). Individual culture dishes were kept at 20% (■), 4% (▲), 2% (◆) and 1% (●) O_2, incubated with 100 ng leukotriene B_4 each and assayed for the amount of leukotriene B_4 remaining at the given time intervals. Figures represent mean ± S.D. ($n = 2$) of a typical experiment. For certain values, error bars lie within margins of symbols and are therefore not drawn (see also Fig. 2).

lized within 90 min (Fig. 1). The results in Fig. 1 are all from an experiment performed on a set of monolayers of hepatocytes prepared from a single rat. Cytochrome *P*-450 and protein determinations were made on the same set of monolayers as were used in the experiments. Preliminary experiments showed that the variation among replicate samples was small, as can be seen by the standard errors in Fig. 1. However, due to differences such as the pharmacological history of the rat and the number of hepatocytes that attached per cm^2 of culture dish, there was considerable variability between monolayers from various preparations of hepatocytes. For example, in four experiments on monolayers of hepatocytes from different rats conducted at 20% O_2 for 30 min, the leukotriene B_4 remaining ranged from 16.5 to 37.6%. Nevertheless, in each of these other experiments the pattern seen in the family of curves of metabolic rate versus O_2 concentration was identical to that of Fig. 1. Therefore, Fig. 1 is presented as typical of four experiments rather than the less precise mean of all values.

The low O_2 concentrations used in the incubations had no effect on cell viability; more than 90% excluded Trypan blue at the end of an 180 min incubation at either 1 or 2% O_2. In order to test for an irreversible inhibition of metabolism by hypoxia, we incubated hepatocytes at 2% O_2 for 210 min, re-oxygenated the cells with 20% O_2, added 148 nM leukotriene B_4, and observed disappearance rates identical to those found in cells incubated only in 20% O_2. A progressive self-inactivation of the enzymes metabolizing leukotriene B_4 with time can be excluded, since re-addition of an equal amount of leukotriene B_4 (i.e., 148 nM) 30 min after the start of an experiment resulted in the same reaction rate as that observed at the beginning of the experiment at both 20% and 2% O_2 (data not shown).

We thought it important to probe for a low K_m/high affinity enzyme for leukotriene B_4, since one would expect the circulating leukotriene B_4 concentration in blood to be in the nanomolar concentration range. We examined the concentration dependency of leukotriene B_4 metabolism between 74 and 740 nM leukotriene B_4 at 20% and 2% O_2. As shown in Fig. 2, no saturation of the reaction with respect to leukotriene B_4 concentration could be observed. Instead, a linear relationship between reaction rate and leukotriene B_4 concentration exists with a 3–4-fold lower capacity of

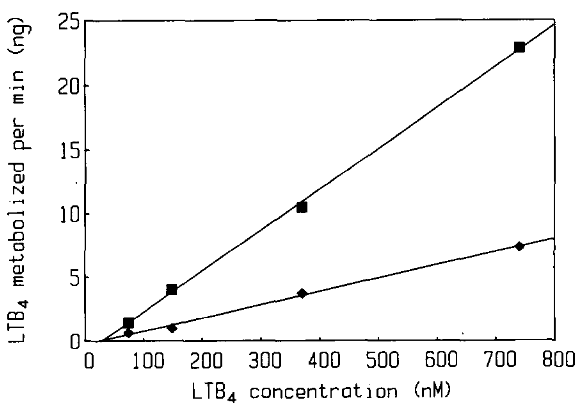

Fig. 2. Leukotriene B_4 (LTB_4) concentration-dependent metabolism by hepatocyte monolayers. Culture dishes were incubated with 74, 148, 370 and 740 nM leukotriene B_4 each for 15 and 30 min at 20% (■) and 2% O_2 (◆), respectively. The amount of leukotriene B_4 metabolized per dish was determined as described. Values are mean ± S.D. ($n = 2$) and were fit by linear regression lines using non-averaged values.

hepatocytes for leukotriene B_4 metabolism at 2% O_2 than at 20% at each of the tested substrate concentrations.

On the other hand, when examined at a fixed leukotriene B_4 concentration (i.e., 148 nM), a plot of leukotriene B_4 metabolism against O_2 concentrations revealed saturation (Fig. 3). However, a double reciprocal analysis of these data exhibited a complex relationship between leukotriene B_4 metabolism and its dependency on O_2. This finding is to be expected in the case of studies performed in intact cells, rather than with isolated enzymes, when the many energetic and metabolic changes induced by hypoxia are taken into account [20,21]. For example, not only do the various isozymes of cytochrome *P*-450 have different K_m values for O_2, but also the concentration of NADPH available to the isozymes is altered by hypoxia. Therefore, since the requirements for simple Michaelis-Menten behavior are not fulfilled, a P_{50} value for O_2, as described by Jones and Mason [20], rather than a K_mO_2, was estimated and found to be close to 4% O_2 (Fig. 3).

After counting the cell number in each individual dish, we isolated the microsomes from the combined monolayers of 12 culture dishes kept for 24 h at 20% O_2. We obtained average values of $1.63 \cdot 10^6$ cells/plate, 8.25 mg protein/plate, 46.25 μg microsomal protein/plate, 38.4 pmol cytochrome *P*-450/plate, 0.8 nmol cytochrome *P*-450/mg microsomal protein, $2.29 \cdot 10^{-17}$ mol cytochrome *P*-450/cell. Assuming that overall leukotriene B_4 metabolism in these hepatocytes is mainly due to microsomal cytochrome *P*-450, we calculated the initial rates of leukotriene B_4 metabolism (Table I). A drastic drop in reaction rates at 1% and 2% O_2 as compared to that at 4% was observed. Increasing the O_2 concentration from 4% to normoxic then resulted in only a slight further increase of the reaction rate. The observed reaction rate of 1275 pmol leukotriene B_4/mg protein per min at 740 nm leukotriene B_4 and 20% O_2 compares well with that obtained with a post-mitochondrial pellet from sonicated polymorphonuclear leukocytes (i.e., 708 pmol/mg protein per min at 1 μM leukotriene B_4) [7].

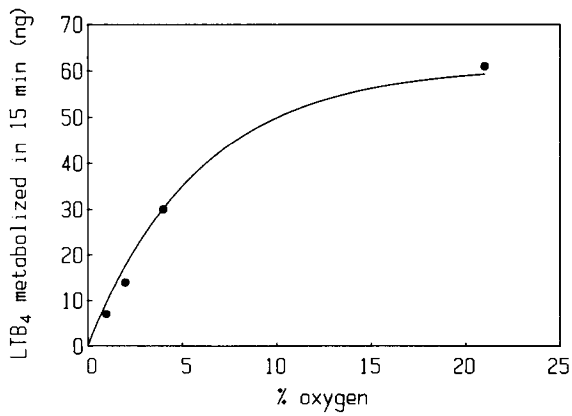

Fig. 3. O_2 concentration dependency of rates of leukotriene B_4 (LTB_4) metabolism. The amount of leukotriene B_4 metabolized by individual monolayer dishes within 15 min at 20, 4, 2 and 1% O_2 was calculated from Fig. 1 by interpolation of the value of the curves at 15 min in order to minimize effects of substrate depletion. These values were then plotted against the O_2 concentration, their propagated S.D. is $< \pm 6$ ng. The solid curve is a plot of an exponential association equation of the form $Y = A\ (1 - e^{-Bx})$ where $A = 61$ and $B = 0.17$.

TABLE I

RATES OF DISAPPEARANCE OF LEUKOTRIENE B_4 AS CATALYZED BY PRIMARY HEPATOCYTE MONOLAYERS AT VARYING O_2 CONCENTRATIONS

Culture dishes were incubated at varying O_2 concentrations in the presence of 148 nM leukotriene B_4. The amount of leukotriene B_4 metabolized was determined at 15 min and reaction rates are calculated based on microsomal cytochrome *P*-450 and microsomal protein present per dish.

O_2 (%)	leukotriene B_4 metabolized	
	pmol/mg protein per min	pmol/nmol cyt. *P*-450 per min
20	255	315
4	172	213
2	64	80
1	29	36

Discussion

In this study, we demonstrate that metabolism of leukotriene B_4 in rat hepatocyte monolayers is dependent on O_2 concentration. As opposed to leukotriene B_4 metabolism in human polymorphonuclear leukocytes, where 20-OH-leukotriene B_4 is the major metabolite [7], in the liver one would expect a variety of metabolites because leukotriene B_4 could serve as a substrate for a number of hepatic cytochrome *P*-450 isozymes. In addition, there may be alternative routes of degradation [22] in analogy to arachidonic acid as a substrate for hepatic microsomes [23]. Recently, it has been shown that both 20-OH- and 19-OH-leukotriene B_4 arise from leukotriene B_4 in the presence of rat hepatic microsomes with K_m values of 14 and 53 μM, respectively [11]. In addition, a metabolic fraction corresponding to 15% of the initial leukotriene B_4, 18-carboxy-19,20-dinor-leukotriene B_4, as well as a minor component tentatively identified as 3-hydroxy-leukotriene B_4, have been described very recently [24]. Moreover, earlier studies in our laboratory of metabolism of nanomolar concentrations of leukotriene B_4 by rat liver microsomes revealed that ω-hydroxylation products alone could not account for total leukotriene B_4 metabolism [10]. Indeed, the well-known production of malondialdehyde as well as small fragments such as pentane during lipid peroxidation demonstrates the complexity of the metabolic pathways of fatty acid-derived substrates [11,23]. Therefore, throughout this study, we examined the disappearance of leukotriene B_4, rather than the appearance of metabolites.

The finding that leukotriene B_4 metabolism does not reach saturation even at 740 nM demonstrates that the normoxic liver has the capacity to deal with instantaneous pulses of leukotriene B_4 formation and/or systemic releases that far exceed the 1–10 nM concentrations that have been measured in blood. However, although metabolic rates are normally measured under 100 or 20% O_2 (1060 or 212 μM O_2), pericentral blood in a normoxic liver is approximately 42 μM O_2 and may be less than 1–5 μM O_2 in a severely hypoxic liver [12,26]. Therefore, hepatic hypoxia may result in failure to remove circulating leukotriene B_4 from the blood. Under hypoxia, too, reductive metabolism of the hepatotoxins CCl_4 and halothane results in the generation of free radicals [26,27]. Besides binding to cellular macromolecules [28], those free radicals could initiate a sequence of lipid peroxidative processes. In fact, at low O_2 concentrations, CCl_4 and halothane markedly increase the extent of lipid peroxidation in isolated hepatocytes and in rat liver microsomes [29,30]. Among the primary products of cellular fatty acid peroxidation, one would expect hydroperoxy derivatives of arachidonic acid which are possible precursors of a number of icosanoids. We have previously shown that generation of 1-chloro-2,2,2-trifluoroethyl free radicals by ultraviolet radiation of halothane and their addition to arachidonic acid in the presence of oxygen results in formation of 5-hydroperoxyeicosatetraenoic acid (5-HPETE) [31]. In that cytochrome *P*-450 can catalyze the transformation of 5-HPETE to leukotriene B_4 under anoxic conditions [32], one must consider a presumably delicate equilibrium between generation and inactivation of leukotriene B_4 in the liver, the distortion of which by hypoxia may have deleterious effects on many cell functions and may ultimately lead to liver necrosis.

Acknowledgements

We thank Dr. J. Rokach of Merck-Frosst Canada for generously providing synthetic LTB_4, Mr. T. Schieble, Mr. D. Heffel and Ms. K. Loper for help in hepatocyte preparation and Ms. A. Stevens for editing this manuscript. This work was supported by a grant from The National Institute of Occupational Safety and Health, No. OH00978. J. Gut is a recipient of stipend No. 83,297.0.85 from the Swiss National Science Foundation.

References

1 Samuelsson, B. (1983) Science 220, 568–575
2 Hammarstrom, S. (1983) Annu. Rev. Biochem. 52, 355–377
3 Hafstrom, I., Palmblad, J., Halmsten, C.L., Radmark, O. and Samuelsson, B. (1981) FEBS Lett. 130, 146–148
4 Williams, T.J. and Piper, P.J. (1980) Prostaglandins 19, 779–783
5 Palmblad, J., Malmsten, C.L., Uden, A.-M., Radmark, O., Engstedt, L. and Samuelsson, B. (1981) Blood 58, 658–661
6 Serhan, C.N., Fridovich, J., Goetzl, E.J., Dunham, P.B. and Weissmann, G. (1982) J. Biol. Chem. 257, 4746–4752
7 Shak, S. and Goldstein, I.M. (1985) J. Clin. Invest. 76, 1218–1228
8 Uehara, N., Ormstad, K., Orning, L. and Hammarstrom, S. (1983) Biochim. Biophys. Acta 732, 69–74
9 Hagmann, W., Denzlinger, C. and Keppler, D. (1985) FEBS Lett. 180, 309–313
10 Bösterling, B. and Trudell, J.R. (1983) Biochem. Biophys. Res. Commun. 114, 850–854
11 Newton, J.F., Eckardt, R., Bender, P.E., Leonard, T. and Straub, K. (1985) Biochem. Biophys. Res. Commun. 128, 733–738

12 Jones, P. (1981) Biochem. Pharmacol. 30, 1019–1023
13 Erickson, R.R., Yu-Drent, P. and Holtzman, J.L. (1982) J. Pharmacol. Exp. Ther. 220, 35–38
14 Takahara, E., Ohta, S. and Hirobe, M. (1986) Biochem. Pharmacol. 35, 541–544
15 Jones, D.P. (1984) Biochem. Pharmacol. 33, 413–417
16 Bissell, D.M. (1980) Ann. N.Y. Acad. Sci. 341, 85–98
17 Trudell, J.R., Bendix, M.. and Bösterling, B. (1984) Biochim. Biophys. Acta 803, 338–341
18 Shak, S. and Goldstein, I.M. (1984) Biochem. Biophys. Res. Commun. 123, 475–481
19 Omura, T. and Sato, R. (1964) J. Biol. Chem. 239, 2370–2378
20 Jones, D.P. and Mason, H.S. (1978) J. Biol. Chem. 253, 4874–4880
21 Aw, T.Y. and Jones, D.P. (1982) J. Biol. Chem. 257, 8997–9004
22 Henderson, W.R. and Klebanoff, S.J. (1983) Biochem. Biophys. Res. Commun. 110, 266–272
23 Capdevila, S., Snyder, G. and Falck, J.R. (1985) in Microsomes and Drug Oxidations (Boobis, A.R., Caldwell, J., DeMatteis, F. and Elcombe, C.R., eds.), pp. 84–94, Taylor & Francis, London
24 Harper, T.W., Garrity, M.J. and Murphy, R.C. (1986) J. Biol. Chem. 261, 5414–5418
25 Ji, S., Lemasters, J.J. and Thurman, R.G. (1980) Pharmacol. Biochem. Behav. 13, 41–45
26 Trudell, J.R., Bösterling, B. and Trevor, A.J. (1982) Proc. Natl. Acad. Sci. USA 79, 2678–2682
27 Trudell, J.R., Bösterling, B. and Trevor, A.J. (1982) Mol. Pharmacol. 21, 710–717
28 Wolff, S.P., Garner, A. and Dean, R.T. (1986) Trends Biochem. Sci. 11, 23–31
29 De Groot, H. and Noll, T. (1986) Biochem. Pharmacol. 35, 15–19
30 De Groot, H. and Noll, T. (1985) Anesthesiology 62, 44–48
31 Bösterling, B. and Trudell, J.R. (1984) Anesthesiology 60, 209–213
32 Bösterling, B. and Trudell, J.R. (1983) Biochem. Biophys. Res. Commun. 115, 995–1001