The role of leukotriene A_4 hydrolase/aminopeptidase in transcellular leukotriene B_4 synthesis in human epidermis

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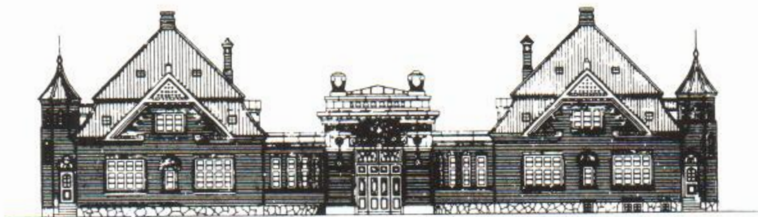

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PREFACE

This thesis is based upon studies carried out during my appointment as a research fellow at the Department of Dermatology, Marselisborg Hospital, University of Aarhus, Denmark from 1991 to 1994.

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This thesis is based upon the following previously published papers, which will be referred to by their roman numericals:

I. Iversen L, Fogh K, Ziboh VA, Kristensen P, Schmedes A, Kragballe K: Leukotriene B_4 formation during human neutrophil keratinocyte interactions: Evidence for transformation of leukotriene A_4 by putative keratinocyte leukotriene A_4 hydrolase. J Invest Dermatol 1993;100:293-298.

II. Iversen L, Ziboh VA, Shimizu T, Ohishi N, Rådmark O, Wetterholm A, Kragballe K: Identification and subcellular localization of leukotriene A_4 hydrolase activity in human epidermis. J Dermatol Sci 1994;7:191-201.

III. Iversen L, Kristensen P, Grøn B, Ziboh VA, Kragballe K: Human epidermis transforms exogenous leukotriene A_4 into peptide leukotrienes: possible role in transcellular metabolism. Arch Dermatol Res 1994;286:261-267.

IV. Iversen L, Kristensen P, Nissen JB, Merrick WC, Kragballe K: Purification and characterization of leukotriene A_4 hydrolase from human epidermis. FEBS Lett 1995;358:316-322.

V. Nissen JB, Iversen L, Kragballe K: Characterization of the aminopeptidase activity of epidermal leukotriene A_4 hydrolase against the opioid dynorphin fragment 1-7. Br J Dermatol 1995;133:742-749.

VI. Iversen L, Deleuran B, Hoberg AM, Kragballe K: LTA_4 hydrolase in human skin: decreased activity, but normal concentration in lesional psoriatic skin. Evidence for different LTA_4 hydrolase activity in human lymphocytes and human skin. Arch Dermatol Res 1996;288:217-224.

VII. Iversen L, Svendsen M, Kragballe K: Cyclosporin A down-regulates the LTA_4 hydrolase level in human keratinocyte cultures. Acta Derm Venerol 1996;76:424-428.

ABBREVIATIONS:

AA	arachidonic acid, eicosa-5,8,11,14-tetraenoic acid
BALF	bronchoalveolar lavage fluid
CO	cyclooxygenase
DTT	dithiothretiol
ECL	enhanced chemiluminescence
FLAP	5-lipoxygenase activating protein
GC-MS	gas chromatography-mass spectrometry
HETE	hydroxyeicosatetraenoic acid
IFN-γ	interferon gamma
Km	Michaelis constant
LO	lipoxygenase
LT	leukotriene
LTA_4	leukotriene A_4: 5(S)-trans-5,6-oxido-7,9trans-11,14-cis- eicosatetraenoic acid
LTB_4	leukotriene B_4: 5(S),12(R)-dihydroxy-8,10-trans-6,14- cis- eicosatetraenoic acid
LTC_4	leukotriene C_4: 5(S)-hydroxy-6(R)-S-glutathionyl-7,9- trans-11,14-cis-eicosatetraenoic acid
LTD_4	leukotriene D_4: 5(S)-hydroxy-6(R)-S-cysteinyl-glycyl-7,9- trans-11,14-cis-eicosatetraenoic acid
LTE_4	leukotriene E_4: 5(S)-hydroxy-6(R)-S-cysteinyl-7,9-trans- 11,14-cis-eicosatetraenoic acid
LX	lipoxin
MW	molecular weight
PG	prostaglandin
PL	phospholipase
PMN	polymorphonuclear cell
RIA	radioimmunoassay
RP-HPLC	reversed phase high performance liquid chromatography
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SRS-A	slow reacting substance of anaphylaxis
UV	ultraviolet

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1. GENERAL INTRODUCTION

1.1 History

In 1930 Kurzrok and Lieb reported that human semen caused both contraction and relaxation of the human uterus. Unrelated to this publication, Harkavy found in the same year that alcohol soluble extracts of sputum from patients with allergic asthma resulted in contractions of rabbit intestine in vitro. These two reports are regarded as the beginning of the area of research in arachidonic acid (AA) metabolism.

In the following decades new reports contributed to the growing interest in this field. In 1934-35 some bioactive substances isolated from human prostate and seminal vesicles were shown to lower blood pressure, and to activate the smooth muscles in the uterus (von Euler 1934, 1935). In 1936 von Euler termed these compounds prostaglandins (PGs).

In 1938 Feldberg and Kellaway showed that perfusates of dog and monkey lung treated with cobra venom contained a compound resulting in contraction of guinea-pig jejunum *in vitro.* The contraction was slow in onset but long lasting and therefore different from that of histamine which was known to cause a rapid contraction. The newly discovered compound was termed "slow reacting substance" (SRS) (Kellaway and Trethewie 1940). This name was later changed to "slow reacting substance of anaphylaxis" (SRS-A) (Brocklehurst 1960).

In the following decades these compounds were further characterized. The polyunsaturated fatty acid AA was shown to be the precursor of PGE_2 (Bergström et al. 1964, van Dorp et al. 1964) and SRS-A (Bach et al. 1977, Jakschik 1977). In 1979 SRS-A was shown to consist of three structurally different compounds (Murphy et al. 1979, Hammerström et al. 1979). These three compounds were termed leukotriene C_4, D_4 and E_4. The name leukotriene was given because the compounds were first described in leukocytes (leuko-) and because the leukotriene molecule has a common structural feature of a conjugated triene. At the end of the 1970s another extremely potent AA metabolite, leukotriene B_4 (LTB_4), was discovered (Borgeat and Samuelsson 1979 a,b).

1.2 Biosynthesis of leukotrienes

Arachidonic acid (eicosa-5,8,11,14-tetraenoic acid, 20:4 ω6) is a polyunsaturated fatty acid with 20 carbon atoms and 4 double bounds. Its precursor linoleic acid (18:2 ω6) is together with γ-linolenic acid (18:3 ω3) considered essential because the mammalian organism cannot introduce double bounds in the fatty acid structure closer to the ω-end than ω9 (Willis 1981). Polyunsaturated fatty acids including AA are incorporated in the phospholipids in the cell membrane mainly at the sn-2 position (Irvine 1982). AA is released from the cell membrane by the action of phospholipase A_2 (PLA_2) (Irvine 1982) or by a combined action of phospholipase C (PLC) and a diglyceride lipase (Hirata and Axelrod 1980). Different stimuli can cause activation of PLA_2 and PLC depending on the cell type. In platelets a rise in intracellular calcium was shown to cause increased PLA_2 activity (Van den Bosch 1980), and at least two different mechanisms, phosphorylation of PLC and a G-protein-mediated PLC activation, appear to regulate PLC activity (Honda and Shimizu 1993 review).

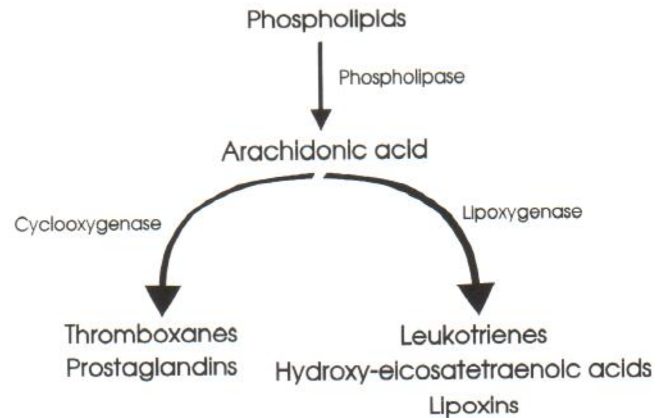

Fig. 1.: Major enzymatic pathways in the metabolism of arachidonic acid.

Once liberated from the cell membrane, AA can be further metabolized by the cyclooxygenase (CO) pathway resulting in the formation of prostaglandins (PGs) and thromboxanes (TXs), or by the lipoxygenase (LO) pathway resulting in leukotriene (LT), lipoxin (LX) and mono-hydroxyeicosatetraenoic acid (HETE) formation (see Fig 1). The name eicosanoids is often used as a synonym for AA metabolites, although it is a common name for all oxygenated metabolites derived from a 20 carbon fatty acid.

Figure 2 shows the metabolism of AA by the 5-LO path-

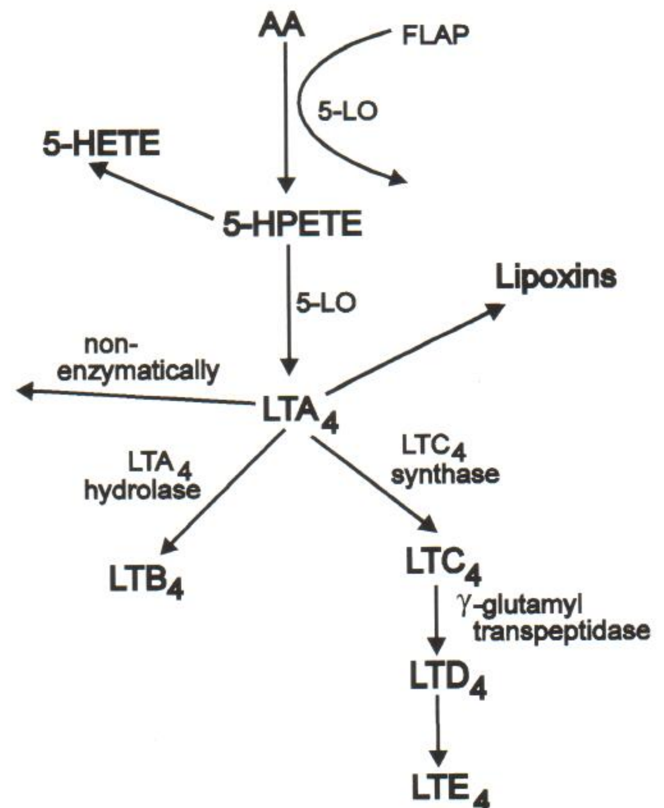

Fig. 2: Arachidonic acid metabolism by the 5-LO pathway.

way. Once AA is liberated from the phospholipids, the 5-LO is activated in the presence of ATP and Ca^{2+} (Shimizu et al. 1986, Rouzer et al. 1986) and translocated from the cytoplasm to the plasma membrane (Rouzer and Kargman 1988) by a Ca^{2+} dependent mechanism. Activated 5-LO is always membrane associated (Rouzer et al. 1986, Wong et al. 1988) and recently a novel membrane associated 5-LO activating protein (FLAP) has been described (Miller et al. 1990). So far all 5-LO expressing cells investigated have been shown to contain FLAP (Reid et al. 1990), and transfection experiments have demonstrated that both FLAP and 5-LO must be present in order to transform AA into 5-HPETE (Dixon et al. 1990). 5-HPETE is then further metabolized by the 5-LO into LTA_4 (Shimizu et al. 1984, 1986, Rouzer et al. 1986) or transformed, either enzymatically by a glutathione-dependent peroxidase or non-enzymatically into 5-HETE (Borgeat et al. 1976, for review Samuelsson and Funk 1989, Lewis et al. 1990). The transformation of AA into LTA_4 results in suicide inactivation of the 5-LO (Rouzer and Kargman 1988).

The end product of the 5-LO activity is LTA_4, an unstable allylic intermediate (Rådmark et al. 1980 a,b,c) that can be further metabolized both enzymatically and non-enzymatically. Non-enzymatically, LTA_4 is metabolized into 5,6-Di-HETEs and 5,12-DiHETEs (Maycock et al. 1982). The epoxide hydrolase, LTA_4 hydrolase, catalyzes the transformation of LTA_4 into LTB_4 (Borgeat and Samuelsson 1979a, Rådmark et al. 1984), and this step has been shown to be the rate-limiting step in LTB_4 formation, at least in rat basophilic leukaemia cells (RBL-1) and human neutrophils (Jakschik and Kuo 1983, Sun and McGuire 1984). The catabolism of LTB_4 differs among tissues. In human polymorphonuclear leukocytes LTB_4 is metabolized via an ω-oxidation pathway into 20-hydroxy-LTB_4 (Soberman et al. 1987). Further oxidation of 20-hydroxy-LTB_4 by an aldehyde dehydrogenase leads to the formation of 20-carboxy-LTB_4 (Soberman et al. 1988). Thus, metabolism of LTB_4 by ω-oxidation has only been observed in human polymorphonuclear cells. In several other tissues and cell types including cultured human keratinocytes LTB_4 is metabolized into dihydro-LTB_4 (Wheelan et al. 1993, Yokomizo et al. 1995 review). Recently, a LTB_4-12-hydroxydehydrogenase has been identified as the initial step in the formation of dihydro-LTB_4 (Yokomizo et al. 1996).

LTA_4 may also be conjugated with glutathione by LTC_4 synthase to yield LTC_4 (Rådmark et al. 1980a, Bach et al. 1984). Successive cleavage by γ-glutamyl transferase (Orning and Hammarström 1980), and a dipeptidase (Lee et al. 1983) converts LTC_4 into LTD_4 and LTE_4. Together, LTC_4, LTD_4 and LTE_4 are termed peptide-leukotrienes.

LTA_4 is also the precursor of lipoxins, a recently discovered group of AA metabolites. They contain three hydroxyl groups and four conjugated double bounds (for review Dahlén and Serhan 1990). Lipoxins can be formed from LTA_4 by the 12-LO or the 15-LO (Serhan and Sheppard 1990, Edenius et al. 1990).

1.3 Biological activities of leukotrienes

Tables 1 and 2 show some of the *in vitro* and *in vivo* effects of leukotrienes. *In vitro* LTB_4 has several pro-inflammatory characteristics such as the ability to induce chemotaxis, chemokinesis and aggregation of leukocytes (Ford-Hutchinson et al. 1980, Palmer 1980, Palmblad et al. 1981), degranulation and superoxide generation (Serhan et al. 1982, Ford-Hutchinson 1990 review), leukocyte adhesion to endothelial cells (Gimbrone et al. 1984) and increased DNA synthesis in human keratinocytes (Kragballe et al. 1985). *In vivo* LTB_4 has been demonstrated to induce chemotaxis (Martin et al. 1989, Bisgaard et al. 1986), epidermal hyperproliferation (Chan et al. 1985, Baur et al. 1986), intra-epidermal microabscesses (Camp et al. 1984), weal and flare reaction in human skin (Camp et al. 1983a, Soter et al. 1983, Juhlin and Hammarström 1983) and increase in vascular permeability (Williams and Piper 1980, Peck et al. 1981, Soter et al. 1983, Camp et al. 1983a). Furthermore LTB_4 is extremely potent with *in vitro* effects in the concentration range of 10^{-8}-10^{-11} M (Ford-Hutchinson et al. 1980). The peptide-leukotrienes have been shown to increase DNA synthesis in human keratinocytes, to contract human bronchial and pulmonary artery and vein smooth muscle, to decrease coronary blood flow and to sup-

Table 1. Biological effects of leukotrienes *in vitro.*

Metabolite	Effect	Reference
LTB_4	Leukocyte chemotaxis, chemokinesis and aggregation	Ford-Hutchinson 1980, Palmer 1980, Palmblad 1981
LTB_4	Leukocyte/endothelium adhesion	Gimbrone 1984
LTB_4	Degranulation and superoxide generation	Ford-Hutchinson 1990 (Review), Serhan 1982
LTB_4	Suppression of T-lymphocyte activity	Ford-Hutchinson 1990 (Review)
LTB_4, LTC_4, LTD_4	Increased DNA synthesis in human keratinocytes	Kragballe 1985
LTC_4, LTD_4	Contraction of human bronchial and pulmonary artery and vein smooth muscle	Hanna 1981
LTC_4, LTD_4	Decrease in coronary blood flow Suppression of myocardial contraction	Burke 1982

Table 2. Biological effects of leukotrienes *in vivo.*

Metabolite	Effect	Reference
LTB_4	Chemotaxis	Martin 1989, Bisgaard 1986
LTB_4	Epidermal hyperproliferation	Chan 1985, Bauer 1986
LTB_4	Intra-epidermal microabscesses	Camp 1984
LTB_4, LTC_4, LTD_4	Weal and flare in human skin	Camp 1983a, Soter 1983, Juhlin 1983
LTB_4, LTC_4, LTD_4	Increase in vascular permeability	Williams 1980, Peck 1981, Soter 1983, Camp 1983a
LTC_4, LTD_4	Vasoconstriction in guinea pig skin Vasodilation in human skin	Williams 1980, Peck 1981, Bisgaard 1982
LTC_4, LTD_4	Bronchoconstriction	Weiss 1982, Drazen 1986 (Reveiw)
LTC_4	Affects pulmonary and cardiovascular dynamics	Smedegaard 1982

press myocardial contraction *in vitro. In vivo* the peptide-leukotrienes cause weal and flare reaction in human skin, an increase in vascular permeability, vasoconstriction in guinea pig skin, vasodilation in human skin and bronchoconstriction. Also, the peptide-leukotrienes are extremely potent, with LTC_4 as the most potent and LTE_4 as the least potent. Although less potent than LTC_4 and LTD_4, LTE_4 exerts a longer lasting smooth muscle contraction than LTC_4 and LTD_4 (Samhoun and Piper 1984). Because of these *in vitro* and *in vivo* effects, leukotrienes have been ascribed a central role in the pathogenesis of several inflammatory diseases such as asthma (Lam et al. 1988, Okubo et al. 1987, Barnes et al. 1995), adult respiratory distress syndrome (Matthay et al. 1984), allergic rhinitis (Bisgaard et al. 1985), gout (Rae et al. 1982), rheumatoid arthritis (Davidson et al. 1983, Griffiths et al. 1995), psoriasis arthritis (Kawana and Nishiyama 1995), inflammatory bowel diseases (Sharon and Stenson 1984, Peskar et al. 1986, Lauritsen et al. 1986, Shimizu et al. 1994) and inflammatory skin diseases such as psoriasis and atopic dermatitis (for review Fogh 1990).

1.4 Leukotrienes in psoriasis

Psoriasis is an inflammatory skin disease characterized in its most common form (psoriasis vulgaris) by rounded, well circumscribed, erythematous plaques covered with abundant scales. However, variants such as pustular psoriasis are also known. The histological features of the fully developed plaque are epidermal hyperproliferation and incomplete terminal epidermal differentiation. Furthermore, infiltration of activated T-lymphocytes, monocytes and neutrophils are seen in the dermis and epidermis, and in the papillary dermis an increased number of capillaries are seen.

Important properties of LTB_4 with respect to psoriasis are the ability to induce chemotaxis and degranulation of leukocytes *in vivo* and *in vitro*, to stimulate leukocyte adhesion to cultured endothelial cells, and together with LTC_4 and LTD_4 to stimulate DNA synthesis in cultured keratinocytes *in vitro.* Also, LTB_4 has been shown to induce skin inflammation and epidermal proliferation after topical application as well as after intradermal injection, and LTC_4 and LTD_4 have been shown to cause vasodilation in human skin (see Table 1 and 2 for references). Furthermore, LTB_4 has been determined in biologically active amounts in chronic plaques of psoriasis (Brain et al. 1984, Grabbe et al. 1984, Ruzicka et al. 1986, Fogh et al. 1987, Duell et al. 1988) as well as in acute guttate lesions (Fogh et al. 1989a). There is also evidence for the presence of LTC_4 and LTD_4 in psoriatic lesions (Brain et al. 1985). Furthermore, a significant increase in urinary LTE_4 has been found in psoriatic patients compared to healthy human volunteers (Fauler et al. 1992).

These results strongly indicate that LTs are involved in the pathophysiology of psoriasis. Although extensive work has been carried out in elucidating the role of LTs in psoriasis, there is still some controversy concerning the role of the keratinocytes in the formation of LTs. 5-LO activity has previously been shown in neutrophils (Borgeat and Samuelsson 1979a,b), eosinophils (Weller et al. 1983, Verhagen et al. 1984), monocytes (Goldyne et al. 1984, Williams et al. 1984), macrophages (Fels et al. 1982, Rouzer et al. 1982), mast cells (Peters et al. 1984, Freeland et al. 1988) and basophils (Warner et al. 1989). Although it has been reported that freshly isolated human epidermal cells (Grabbe et al. 1985, Rosenbach et al. 1985) and cultured mouse keratinocytes (Ziboh et al. 1984) can synthesize low quantities of LTB_4 as determined by HPLC (Ziboh) and by RIA and chemotactic activity (Grabbe and Rosenbach), others (Sola et al. 1992, I, II, III) have not been able to detect any 5-LO activity in human keratinocytes. However, recently it was reported that 5-LO gene expression as determined by 5-LO mRNA, and enzyme activity is induced in HaCat cells and to a lesser extent in cultured normal human keratinocytes when these cells are allowed to differentiate (Janssen-Timmen et al. 1995).

1.5 Purpose of the present study

The purpose of the present study was to further investigate the role of the human epidermis in leukotriene synthesis. Transcellular leukotriene synthesis, in which neutrophil derived LTA_4 was transformed into LTB_4 or peptide leukotrienes by

either cultured keratinocytes or human epidermis, was demonstrated. This finding prompted us to further investigate the epidermal LTA_4 hydrolase by purification and further characterization with respect to its hydrolase as well as its peptidase activity.

In the light of the biological actions of LTB_4 and the postulated role of LTB_4 in psoriasis, the epidermal LTA_4 hydrolase was further investigated in this disease. Finally, several compounds used in the treatment of psoriasis were investigated for their capacity to regulate the expression and/or the activity of the LTA_4 hydrolase in vitro.

2. METHODS FOR ANALYSIS OF LEUKOTRIENES

In this chapter only the more general methods will be described. Methods used for purification of the LTA_4 hydrolase and for quantification of the LTA_4 hydrolase will be discussed in later chapters.

2.1 Tissue preparation

In vitro studies of keratinocytes were carried out using skin-biopsies or keratinocyte cell cultures. Skin biopsies were obtained using a keratome and by inducing a suction blister. Keratomed biopsies were from psoriatic patients and normal human volunteers as well as from excess skin from plastic surgery (Sauder et al. 1982, Kragballe et al. 1987, II, IV-VI). The disadvantages of this technique are the use of local anesthetics such as lidocaine and that 10-20 % of the biopsy is dermis (Duell et al. 1988, Fogh et al. 1987). Another technique is the suction blister method (Kiistala and Mustakallio 1964, II, III). This method separates the epidermis from the dermis between the basal layer and the basement membrane without major cell damage (Kiistala and Mustakallio 1967) or impairment of viability (Ingemansson-Nordqvist et al. 1967). This technique leaves no scar and does not require local anesthetics. The major disadvantage of this technique is that eicosanoid synthesis may be activated during the 90-120 minutes it takes to induce the blister.

When keratinocyte cell cultures are used, pure keratinocytes are obtained and large numbers of keratinocytes can be grown. However, it must be kept in mind that there is a down regulation of some LOs during culturing of keratinocytes (Kondoh et al. 1985). Furthermore, it is very important to define the culture conditions because the AA metabolism by human keratinocytes depends upon the maturational stage (Henneicke-von Zepelin et al. 1991, Janssen-Timmen et al. 1995).

2.2 Lipid extraction and fast phase-extraction

After incubation has been carried out, LO products are extracted and further purified before being analyzed by HPLC. At this step an internal standard can be added in order to determine recovery during the purification procedures. An internal standard must be structurally related to the compounds of interest and its localization during the purification procedures must be known. When LTB_4 was determined, tritiated eicosanoids were added (Fogh et al. 1987, I, II, V-VII) and in study III ^3H- LTD_4 was added. When small peptides were investigated, bestatin was used as an internal standard (V). Bestatin is eluted in a separate chromatographic peak in our HPLC system and furthermore, bestatin is an inhibitor of the LTA_4 hydrolase (Orning et al. 1991b, I, II, III).

After depletion of proteins and cellular debris by addition of methanol followed by centrifugation, the crude lipid extract is left in the supernatant. This fraction can either be analyzed directly by HPLC or further purified by fast phase extraction using column chromatography (Luderer et al. 1983, Fogh et al. 1987). Extraction and purification of peptide-leukotrienes is difficult because they adhere to glass and plastic surfaces. Therefore, only siliconized glassware was used in study III. Furthermore, peptide-leukotrienes are difficult to extract in acidic and aqueous media, and they are very sensitive to alterations of the organic component (Clancy and Hugli 1983) as well as the pH (Powell 1985a) of the mobile phase.

2.3 High Performance Liquid Chromatography (HPLC)

In HPLC the molecules are separated in a column containing small coated particles (the solid phase), and a liquid (the mobile phase) is led through the column under pressure. The eluent is monitored by a UV-detector, determining the UV-absorption at a chosen wavelength as a function of time after the sample has been applied to the column. In Reversed Phase (RP)-HPLC the solid phase is non-polar, typically consisting of carbon chains. RP-HPLC with a column containing 18-carbon atoms in a chain (C-18) is typically used in the separation of LTs, utilizing the different polarities of the AA metabolites. In RP-HPLC the mobile phase is more polar than the solid phase leading to the elution of the more polar metabolites first, followed by less polar metabolites.

RP-HPLC has particularly been used for the analysis of LTs (Borgeat and Samuelsson 1979a,b, Powell 1985a,b, de Laclos et al. 1984, I-VII), but straight-phase (SP)-HPLC can also be used (Camp et al. 1983b, Brain et al. 1984, Powell 1985c).

LTs are also quantified by HPLC combined with UV-detection using authentic standards to construct a calibration curve. Integrated areas obtained during analysis of biological samples are then converted to amounts by comparison with areas obtained from the authentic standards. This method of quantification requires that no other compounds with absorption at the determined wavelength co-elute with the compound of interest.

Separation of LTB_4: For separation of LTB_4 a Hypersil C-18 column was used in studies I-II and IV-VII. The mobile phase consisted of a mixture of methanol/water/acetic acid (70:30:0.01) (v/v). A mobile phase consisting of methanol, water and acetic acid has previously been used to separate LTB_4 (Borgeat and Samuelsson 1979a, Rådmark et al. 1980b, Kumlin and Dahlén 1990, Fogh et al. 1992). The flow rate was 0.80 ml/min for the first 10 min and then raised to 1.3 ml/min for the following 20 min. LTB_4 was detected by UV-detection at 270 nm because LTB_4 has the characteristic triene structure with maximal absorbance at 270 nm (I). It has been proposed that non-enzymatically generated 5(S),12(S)-di-HETE co-elutes with LTB_4 on RP-HPLC when a methanol containing

mobile phase is used (Borgeat 1984). However, in our system there was no non-enzymatically formed product co-eluting with LTB_4 when LTA_4 was incubated with or without inactivated keratinocytes/epidermis (I and II), nor were there any compounds when the incubation was carried out with AA (own unpublished data).

Separation of peptide-leukotrienes: LTC_4, LTD_4 and LTE_4 were also separated by RP-HPLC using a mixture of acetonitrile/methanol/water/acetic acid as mobile phase (Brain et al. 1985, Miyamoto et al. 1987, Edenius et al. 1988, III). In III an UltraTech sphere C-18 column was used and the mobile phase consisted of acetonitrile/methanol/water/acetic acid (29:19:52:1 by vol). The pH was adjusted to 5.6 with NaOH. With a flow rate of 1.4 ml/min the relevant LTs were separated.

Separation of amino acids and small peptides: For RP-HPLC separation of small peptides such as dynorphin and enkephalin, a mobile phase of acetonitrile, water and triflouroacetic acid (TFA) is common (Tan and Yu 1980, Goldstein et al. 1991, Griffin et al. 1992, Bathon et al. 1992). In V a Sperisorb C-18 column was used. The elution program was 3% solvent A (0.1% TFA in acetonitrile) and 97 % solvent B (0.1% TFA in water) from 0-1 min, and then changed to 62% solvent A and 38% solvent B over the next 39 min with a flow rate of 0.6 ml/min. UV-detection was carried out at 215 nm.

2.4 Identification of leukotrienes

Identification of an unknown compound only by comparing the retention time in the HPLC chromatogram with the retention time of a known authentic standard is unreliable. Therefore, the following methods were used in this study to identify a chromatographic peak:

UV-absorption scan: Comparison of the retention times in the RP-HPLC combined with a UV-scan of the collected fraction is an important method for identifying LTs. LTs contain a conjugated triene structure which results in a characteristic UV-absorption scan with a triplet absorption (Sweeney et al. 1987, I). However, HPLC separation before the UV-scan is necessary because other compounds will interfere with the absorption of LTs. LTB_4 has maximal absorption at 270 nm with shoulders at 260 nm and 280 nm, whereas LTC_4 has maximal absorption 280 nm with shoulders at 270 nm and 292 nm (I and III).

Radioimmunoassay (RIA): RIA is useful for both quantitation and identification of leukotrienes (I, II). Furthermore, RIA is very sensitive (picomolar concentration whereas HPLC needs nanomolar concentration). However, if cross reacting compounds are present in the unknown sample, both quantitation and identification will be unreliable. Therefore, HPLC separation prior to RIA is essential to minimize cross reactivity. RIA is widely used for determination of LTs (Salmon et al. 1982a,b, Ruzicka et al. 1986, Fogh et al. 1987, 1988a, 1989a,b, I and II).

Mass spectrometry (MS): MS is often used in combination with gas chromatography (GC). GC is performed in order to separate different compounds which then in the same analysis can be identified by MS. GC-MS can be used for identification as well as quantitation (Ewing 1985), and GC-MS is regarded as the most reliable technique for identification of unknown compounds (Woollard and Mallet 1984, Steffenrud et al. 1986). However, GC-MS is not suitable for a routine assay because it is very time consuming and expensive. GC-MS or MS alone has been used for identification of LO products such as mono-HETEs (Camp et al. 1983b, Cunningham et al. 1985, Fogh et al. 1988b, Iversen et al. 1991) and for identification of LTB_4 (Mathews et al. 1988, VI).

Enzymatic transformation: Collection of a chromatographic peak followed by incubation with an enzyme which transforms the collected compound into another known compound with a different HPLC elution profile is a suitable method for identification. This method has been used for identification of LTC_4 (Pace-Asciak et al. 1985, III) which is transformed to LTD_4 by γ-glutamyl-transpeptidase (Fig 2).

3. CELLULAR INTERACTION IN LEUKOTRIENE FORMATION

3.1 Introduction

In 1986 Marcus defined three types of cell-cell interaction in the eicosanoid pathway (Table 3). In type I of cell-cell interaction different cells metabolize a common precursor. Type I can be subdivided in type IA and IB depending on whether or not the acceptor cell is able to synthesize the precursor itself. An example of type IA cell-cell interaction is the transformation of platelet and alveolar macrophage derived AA into LTB_4 by neutrophils (Marcus et al. 1982 and Grimminger et al. 1991). Another example is the transformation of neutrophil derived LTA_4 into LTC_4 by mast cells (Dahinden et al. 1985). In type IB the acceptor cell is not able to form the precursor. Examples of this type are the transformation of neutrophil derived LTA_4 into LTB_4 by cultured keratinocytes (Sola et al. 1992 and I), epidermis (II) and erythrocytes (McGee and Fitzpatrick 1986).

Table 3. Classification of cell-cell interactions in eicosanoid synthesis.

Type I:	Different cells metabolize a common precursor.
IA:	The acceptor cell is capable of synthesizing the precursor.
IB:	The acceptor cell is unable to synthesize the precursor.
Type II:	Eicosanoids from one cell are metabolized by the acceptor cell to a new product which neither cell can synthesize alone.
IIA:	Both cell types are activated.
IIB:	Only one cell-type is activated.
Type III:	An eicosanoid from a donor cell acts as an agonist or as an inhibitor for the acceptor cell.

In cell-cell interaction of type II, eicosanoids from one cell are metabolized by a second cell to a new product which none of the cells can synthesize alone. Also, type II is subdivided in A and B depending on whether both cell-types or only one cell-type are activated. If activated neutrophils and activated platelets are co-incubated, platelet derived 12-HETE and neutrophil derived 5-HETE are transformed into 5(S),12(S)-DiHETE by neutrophils and platelets respectively (Marcus et al. 1982). However, if only the platelets are activated, platelet derived 12-HETE is hydroxylated to 12(S),20-DiHETE instead of 5(S),12(S)-DiHETE (Marcus et al. 1984). Other examples of type II cell-cell interaction are the formation of lipoxins after co-incubation of neutrophils with either platelets (Serhan and Sheppard 1990, Edenius et al. 1994), nasal polyps, or bronchial tissue (Edenius et al. 1990). Lipoxin formation can be of both type IIA and B depending on the cell types involved.

The third type of cell-cell interaction occurs when an eicosanoid from a donor cell acts as an agonist or antagonist for the acceptor cell. The inhibitory effect of 12-HETE on prostacyclin formation (Hadjiagapiou and Spector 1986) and the inhibitory effect of 15-HETE on LTB_4 formation (Vanderhoek et al. 1980) are examples of this type of interaction.

3.2 Cellular interaction in LTB_4 formation

Cell-cell interaction in LTB_4 formation, also termed transcellular LTB_4 synthesis, has been described for several cell types (Table 4). Type IB has most commonly been described for transcellular LTB_4 synthesis. The intermediate, LTA_4, is released from either neutrophils or monocytes and then further metabolized in a cell type which is not able to form LTA_4 (Fig 3) (I, II). In the cases with AA as the intermediate (Table 4), the mechanism of cell-cell interaction is best described as type IA.

The first detection of LTA_4 hydrolase outside leukocytes was not in another cell type, but in blood plasma (Fitzpatrick et al. 1983). Later several different cell types and bronchoalveolar lavage fluid (BALF) were added to the list of fluids and cells that contain LTA_4 hydrolase activity without expressing 5-LO activity (Table 4). Transcellular LTB_4 synthesis was therefore speculated to occur by the mechanism illustrated in Figure 3. In 1985 Dahinden et al. demonstrated the release of LTA_4 from stimulated neutrophils. This provided the evidence for transcellular LTB_4 synthesis between 5-LO containing cells and cells only containing the LTA_4 hydrolase.

In study I co-incubation of human neutrophils and cultured keratinocytes resulted in a 73% increase in LTB_4 formation compared to incubation of neutrophils alone. Furthermore, incubation of cultured keratinocytes with exogenous LTA_4 resulted in formation of LTB_4. Transfer of AA from the keratinocytes to the neutrophils was found not to take place. Also, the incubation of neutrophils with the medium from stimulated keratinocytes failed to increase LTB_4 formation, indicating that the increased LTB_4 formation in the co-incubation experiments was not caused by the release of soluble factors from the keratinocytes. Taken together, these results indicated that the increased LTB_4 formation was due to keratinocyte transformation of neutrophil derived LTA_4 into LTB_4. In study II transcellular LTB_4 synthesis was also shown with suction blister raised normal human epidermis. The epi-

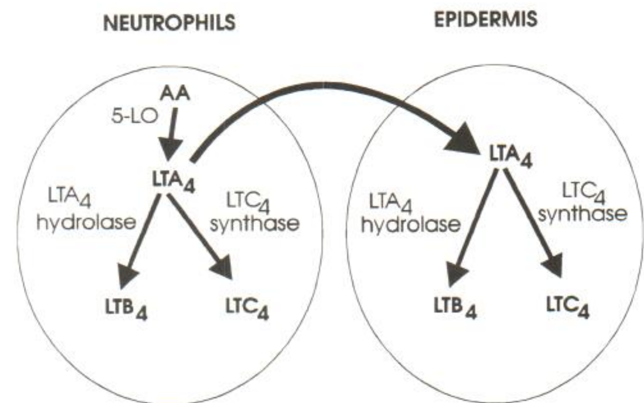

Fig. 3: Schematic presentation of transcellular leukotriene synthesis.

Table 4. Transcellular LTB4 synthesis.

Donor	Intermediate	Acceptor	Type of interaction	Reference
Neutrophils	LTA_4	Keratinocytes	IB	Solà 1992, I
Neutrophils	LTA_4	Epidermis	IB	II
Neutrophils	LTA_4	Erythrocytes	IB	Mcgee 1986
Neutrophils	LTA_4	Endothelial cells	IB	Claesson 1988
Neutrophils	LTA_4	Alveolar macrophages	IB	Grimminger 1991
Neutrophils	LTA_4	Alveolar epithelial cells	IB	Grimminger 1992
Neutrophils	LTA_4	Transformed airway epithelial cells	IB	Zhou 1995
Neutrophils	LTA_4	Broncho-alveolar-lavage fluid	IB	Munafo 1994
Neutrophils	LTA_4	Glomerular endothelial cells	IB	Brady 1995
Monocytes	LTA_4	Endothelial cells	IB	Claesson 1991b
Monocytes	LTA_4	Lymphocytes (Raji cell line)	IB	Claesson 1991a
Monocytes	LTA_4	Lymphocytes	IB	Jakobsson 1991
Alveolar macrophages	AA	Neutrophils	IA	Grimminger 1991
Platelets	AA	Neutrophils	IA	Marcus 1982

dermal LTA_4 hydrolase activity was localized to the cytoplasm by subfractionation, and the epidermal LTA_4 hydrolase was detected by Western blot analysis using an affinity purified anti-LTA_4 hydrolase antibody (II).

The examples of transcellular LTA_4 metabolism in I, II and other studies (Table 4) are all *in vitro* findings. The non-physiological calcium ionophore A23187 has been used to stimulate the leukocytes in most *in vitro* studies. The extracellular release of LTA_4 might, therefore, reflect an overflow of LTA_4 rather than a physiological process. However, transcellular lipoxin and leukotriene formation between neutrophils and platelets with LTA_4 as the intermediate has been reported after receptor mediated stimulation (Fiore and Serhan 1990, Maclouf et al. 1990). Furthermore, at an inflammatory site, multiple stimuli are present, which may lead to a synergistic stimulatory effect. Another critical question regarding the *in vivo* relevance of this observation is the instability of LTA_4 in aqueous media at physiological pH (Maycock et al. 1982). However, LTA_4 is markedly stabilized by albumin (Maycock et al. 1982) and phospholipids (Fiore and Serhan 1989), which might be of importance *in vivo*. Furthermore, cell activation leads to a closer cell-cell contact because of expression of adhesion molecules (Lasky 1992). It is therefore of interest that blocking the leukocyte adhesion molecules with monoclonal antibodies leads to a decreased leukotriene synthesis after co-incubation of granulocytes and glomerular endothelial cells (Brady and Serhan 1992).

Transcellular metabolism of LTA_4 is of interest because LTA_4 hydrolase has previously been shown as the rate limiting step in LTB_4 formation (Jakschik and Kou 1983, Sun and McGuire 1984). Transcellular LTA_4 metabolism may, therefore, result in an increased LTB_4 formation at an inflammatory site.

3.3 Cellular interaction in LTC_4 formation

Similar to transcellular LTB_4 synthesis, transcellular LTC_4 synthesis has only been shown to occur *in vitro*. In Table 5 it is seen that the dominating mechanism of cell-cell interaction is type IB with LTA_4 as the intermediate. The only exception is the neutrophil/mast cell interaction, which both have an active 5-LO (Peters et al. 1984, Freeland et al. 1988). Tanscellular LTC_4 synthesis in the skin was demonstrated to occur via type IB of cell-cell interaction (III). Co-incubation of suction blister lifted epidermis and neutrophils resulted in a marked increase (90%) in LTC_4 formation when compared to neutrophils and epidermis alone. Furthermore, suction blister epidermis and keratomed skin were demonstrated to transform LTA_4 into LTC_4 and LTB_4. Interestingly, cultured keratinocytes did not form LTC_4, but only LTB_4 when incubated with LTA_4. This might be explained by changes in the eicosanoid generating properties in cultured keratinocytes, which has previously been reported by Rosenbach et al. (1990).

LTA_4 is transformed into LTC_4 by a LTC_4 synthase (Fig 2 and 3), which in mouse mastocytoma cells has been shown as a highly specific, membrane bound glutathione-S-transferase (GST) (Söderström et al. 1988). The LTC_4 synthase has been purified >10000 fold from U937 cells (Nicholson et al. 1992). In the skin a specific LTC_4 synthase has, however, never been shown. Several isoforms of GST with activity towards LTA_4 have been identified in human and rodent skin (Del Boccio et al. 1987, Raza et al. 1991), and human, rat and mouse skin has been demonstrated to transform LTA_4-methyl ester into LTC_4-methyl ester (Agarwal et al. 1992). No stimulus is needed to activate the LTC_4 synthase. The addition of substrate and reduced glutathione alone results in a conjugation yielding LTC_4 (III).

Whether the human skin is capable of further transforming LTC_4 into LTD_4 and LTE_4 is still not known. However, in study III two out of six experiments with incubation of human skin and LTA_4 resulted in a chromatographic peak, coeluting with authentic LTD_4 and LTE_4 after 30-60 min of incubation. Furthermore, incubations longer than 20 min resulted in continuous decrease in the LTC_4 content (III), indicating that LTC_4 is further metabolized in the human skin. This is of interest because psoriatic patients have an increase in urinary LTE_4 compared to healthy volunteers (Fauler et al. 1992). It is likely that this is secondary to increased skin formation of LTC_4. Because LTD_4 and LTE_4 in study III were identified by HPLC, further studies are needed to determine the capacity of human epidermis to metabolize LTC_4 into LTE_4.

As reviewed in Chapter 1 leukotrienes are believed to play an important role in the pathogenesis of psoriasis and atopic dermatitis. The demonstration of transcellular leukotriene synthesis in the epidermis is therefore of considerable interest. In inflammatory skin diseases neutrophils migrate into the epidermis where they are in close contact with the keratinocytes, and the release of LTA_4 into the extracellular space has previously been shown with activated neutrophils (Dahinden et al. 1985). It is, therefore, possible that the epidermis plays a more active role in the generation of pro-inflamma-

Table 5. Transcellular LTC_4 synthesis.

Donor	Intermediate	Acceptor	Type of interaction	Reference
Neutrophils	LTA_4	Epidermis	IB	III
Neutrophils	LTA_4	Mast cells	IA	Dahinden 1985
Neutrophils	LTA_4	Endothelial cells	IB	Feinmark 1986, Claesson 1988
Neutrophils	LTA_4	Platelets	IB	Maclouf 1988, Edenius 1988
Neutrophils	LTA_4	Smooth muscle cells	IB	Feinmark 1987
Monocytes	LTA_4	Platelets	IB	Bigby 1989
Kupffer cells	LTA_4	Hepatocytes	IB	Fukai 1993

tory mediators in inflammatory skin diseases than previously believed.

4. PURIFICATION AND CHARACTERIZATION OF LTA_4 HYDROLASE

4.1 Introduction

LTA_4 hydrolase was first purified from human leukocytes (Rådmark et al. 1984). Since then, it has been purified from several sources including guinea pig lung (Bito et al. 1989), guinea pig liver (Haeggström et al. 1988), rat neutrophils (Evans et al. 1985a), the B-lymphocytic cell line Raji (Odlander et al. 1991), human lung (Ohishi et al. 1987, 1990a), human cultured airway epithelial cell (Bigby et al. 1994), human neutrophils (IV), human erythrocytes (McGee and Fitzpatrick 1985), human cultured keratinocytes (IV) and human epidermis (IV).

Centrifugation has been the initial step in all studies with LTA_4 hydrolase purification (Rådmark et al. 1984, Evans et al. 1985a, McGee and Fitzpatrick 1985, Ohishi et al. 1987, Haeggström et al. 1988, Bito et al. 1989, Ohishi et al. 1990a, Odlander et al. 1991, Bigby et al. 1994, IV). In most studies centrifugation was followed by ammonium sulphate precipitation with collection of either the 40-80% fraction (Rådmark et al. 1984, Evans et al. 1985a, Haeggström et al. 1988, Odlander et al. 1991, Bigby et al. 1994) or the 40-70% fraction (Ohishi et al. 1987, Bito et al. 1989, Ohishi et al. 1990a, IV). For further purification, different columns taking the advantage of anion exchange chromatography (Rådmark et al. 1984, Evans et al. 1985a, McGee and Fitzpatrick 1985, Ohishi et al. 1987, Haeggström et al. 1988, Bito et al. 1989, Ohishi et al. 1990a, Odlander et al. 1991, Bigby et al. 1994, IV), hydrophobic interaction chromatography (Ohishi et al. 1987, Bito et al. 1989, Ohishi et al. 1990a, Odlander et al. 1991, IV), gel filtration (Rådmark et al. 1984, Evans et al. 1985a, Ohishi et al. 1987, Haeggström et al. 1988, Ohishi et al. 1990a, Odlander et al. 1991, Bigby et al. 1994), chromatofocusing (Rådmark et al. 1984, McGee and Fitzpatrick 1985, Haeggström et al. 1988, Bito et al. 1989, IV) or a hydroxyapatite column (Ohishi et al. 1987, Haeggström et al. 1988, Ohishi et al. 1990a) have been used.

The highest degree of LTA_4 hydrolase purification was a 1419 fold purification from Raji cells (Odlander et al. 1991). In contrast, only a 109 fold purification was obtained for rat neutrophil LTA_4 hydrolase (Evans et al. 1985a).

4.2. Purification of the LTA_4 hydrolase from human epidermis and human cultured keratinocytes

In study IV the human epidermal and human cultured keratinocyte derived LTA_4 hydrolase was purified. For comparison the LTA_4 hydrolase was also purified from human neutrophils. After obtaining the 100,000 x g supernatant ammonium sulphate precipitation, anion exchange chromatography, hydrophobic interaction chromatography and chromatofocusing was carried out. These steps resulted in a 853 fold purification of the LTA_4 hydrolase from neutrophils. However, only a 150 fold and 200 fold purification was obtained from human epidermis and human cultured keratinocytes respectively. A new method was therefore developed. After ammonium sulphate precipitation followed by anion exchange chromatography, an affinity chromatography column was introduced. This novel column consisted of bestatin coupled to Sepharose and took the advantage of bestatin as a LTA_4 hydrolase inhibitor (Orning et al. 1991b, Evans and Kargman 1992, I, II, III). One problem in making an affinity column is to avoid steric hindrance for the interaction between the ligand and the protein while binding the ligand to the matrix. Furthermore, the binding to the matrix should be stable during the chromatography. As it will be discussed below, the LTA_4 hydrolase has some similarities with some aminopeptidases and it also contains aminopeptidase activity. Bestatin is a potent inhibitor of several aminopeptidases (Suda et al. 1976, Rich et al. 1984). It has been suggested that bestatin mimics the tetrahedral intermediate of hydrolysis using the C-2 OH to form a complex with the active site Zn^{2+} of these aminopeptidases (Nishizawa et al. 1977) as indicated in Fig 4. It was therefore speculated that bestatin could be coupled to the AH-Sepharose through its carboxy group (Fig 4) resulting in an amide bound. By this way of coupling, the hydroxyl group would be kept free to interact with the Zn^{2+} and steric hindrance should be avoided because AH-Sepharose contains a very flexible 6 carbon spacer arm.

In principle, elution of a protein from an affinity column can be carried out in three ways: by increasing the salt concentration in the buffer, by changing the temperature, or by affinity elution by inclusion of a free ligand in the buffer (Scopes 1987). In study IV the LTA_4 hydrolase was eluted from the bestatin column by a stepwise increase in the salt concentration. After the bestatin column, the hydrolase containing fraction was subjected to hydrophobic chromatography and finally again affinity chromatography on the bestatin column. These purification steps increased the purification of the epidermal LTA_4 hydrolase from 150-fold to 396-fold.

The bestatin column might also be useful in the separation of active and inactive LTA_4 hydrolase. When the LTA_4 hydrolase transforms LTA_4 into LTB_4, the enzyme undergoes suicide inactivation by covalent binding of LTA_4 to the enzyme (thoroughly discussed below). Bestatin has been shown to inhibit this covalent coupling of LTA_4 (Evans and Kargman

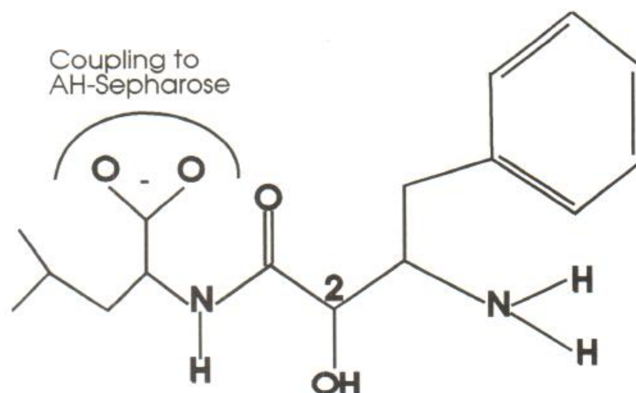

Fig. 4: Bestatin [(2S, 3R)-3-amino-2-hydroxy-4-phenylbutanoyl-L-leucin]. The C2 of bestatin and Zn^{2+} of the hydrolase are indicated. Also, the region for coupling to AH-Sepharose is indicated.

1992). It is, therefore, possible that inactivated LTA_4 hydrolase, in contrast to active LTA_4 hydrolase, will pass through the bestatin column without binding.

4.3 Characterization of the LTA_4 hydrolase

Immunological studies as well as activity determinations have detected LTA_4 hydrolase in almost all cells and tissues studied in rat (Medina et al. 1988), guinea pig (Izumi et al. 1986, Shimizu et al. 1990, Ohishi et al. 1990b) and man (Fu et al. 1989), including mammalian plasma (Fitzpatrick et al. 1983) and BALF (Munafo et al. 1994). The only exception is rat heart (Medina et al. 1988). The epidermis was never investigated in these studies, and it was not until 1994 that LTA_4 hydrolase was first shown in human epidermis (Ikai et al. 1994, II).

It has been proposed that different isoforms of the enzyme may exist (Samuelsson and Funk 1989) and the results in study VI support this idea. In peripheral lymphocytes a significantly higher enzyme activity (per mg enzyme) was found compared to human epidermis (VI). Furthermore, Odlander et al. (1991) have demonstrated two catalytically divergent forms of the LTA_4 hydrolase in the human B-lymphocytic cell line Raji. One form obeyed Michaelis Menten kinetics and had a catalytic activity which correlated with human leukocyte LTA_4 hydrolase. The other form had a higher catalytic activity and did not conform to Michaelis Menten kinetics. Also, there were differences in the heat-inactivation pattern of the two LTA_4 hydrolase forms. The presence of two catalytically different forms of LTA_4 hydrolase in a lymphocytic cell line may explain the higher activity found in human peripheral lymphocytes compared to human epidermis in VI. Bigby et al. (1994) compared purified human airway epithelial cell LTA_4 hydrolase with neutrophil LTA_4 hydrolase. Their findings suggested that airway epithelial cells and neutrophils have structurally and functionally related LTA_4 hydrolase, although not identical.

There are other indications of LTA_4 hydrolase isoforms. Haeggström et al. (1988) showed that guinea pig liver LTA_4 hydrolase elutes as two peaks in chromatofocusing differing by 0.4 in their pI if not pre-treated with dithiothreitol (DTT), and Bito et al. (1989) have shown two catalytically active forms of guinea pig lung LTA_4 hydrolase with a pI of either 5.7 or 5.4 depending on the presence or absence of DTT in the buffer during the purification procedures. Furthermore, human LTA_4 hydrolase have been cloned and expressed in cultured Spodoptera frugiperda insect cells (Gierse et al. 1993). Two major isoforms with pIs of 5.3 and 5.1 were isolated, and NH_2-terminal sequence analysis showed that the two isoforms differed by an NH_2-terminal blocking group. Although these results demonstrate the presence of isoforms within the same cell, this was not found in human epidermis and cultured keratinocytes, in which the LTA_4 hydrolase eluted as one peak in chromatofocusing when investigated in the presence of DTT (IV). The pI of the LTA_4 hydrolase in human epidermis was between 5.1 and 5.4, which is in accordance with what has been observed in other human tissues (reviewed in IV).

LTA_4 hydrolase is a cytoplasmic enzyme (Rådmark et al. 1984, Evans et al. 1985a, II), which in most studies has been determined to have a molecular weight (MW) of approximately 70,000 DA (reviewed in IV). In human erythrocytes the MW was approximately 54,000 DA (McGee and Fitzpatrick 1985), although this might represent a breakdown product as indicated by Orning et al. (1990). As judged from Western blot analysis erythrocyte LTA_4 hydrolase MW has recently been determined by Rådmark and Haeggström (1990a) to be 70,000 DA. Electrospray ionization mass spectrometry has indicated that the MW of pure recombinant LTA_4 hydrolase is $69{,}399 \pm 4$ DA, while calculations from human cDNA have revealed a MW of 69,154 DA (IV). It is therefore likely that the lower MW found in human erythrocytes represents a degradation product rather than an isoform of the LTA_4 hydrolase.

Kinetic studies of the human epidermal LTA_4 hydrolase has demonstrated its adaptation to Michaelis Menten kinetics (II). This is in accordance with the findings in most cell types (Rådmark et al. 1984). However, the LTA_4 hydrolase from transformed human airway epithelial cells (BEAS-2B) (Bigby 1992) and an isoform of the LTA_4 hydrolase in the Raji cell line did not obey this kinetic. In the human epidermis the Michaelis constant K_m was 6 μM and the V_{max} was 300 pmol LTB_4/ mg protein/ min. Large variations in the K_m and the V_{max} have been found in different studies (Odlander et al. 1991, Ohishi et al. 1990a, Rådmark et al. 1984, II). Correct analysis of these constants is difficult because of the instability of the substrate (Maycock et al. 1982), and because the LTA_4 hydrolase undergoes suicide inactivation when it transforms LTA_4 into LTB_4 (to be discussed below). Ohishi et al. (1990a) have demonstrated a 10-fold increase in the V_{max} by changing the incubation time from 1 min to 10 sec. Furthermore, the V_{max} is expressed per mg protein and it therefore depends on the purity of the enzyme in the sample analyzed. This could explain the great variation from study to study.

The amino acid composition of the epidermal and keratinocyte derived LTA_4 hydrolase was determined in IV. Neutrophils were included in IV to allow a direct comparison with a well characterized cell type. Separation by SDS-PAGE was carried out in order to obtain a high purity of the sample before determination of the amino acid composition. The gel was blotted to an immobilion-P transfer membrane, and the band containing the LTA_4 hydrolase was then subjected to automated amino acid analysis. There were only minor differences in the LTA_4 hydrolase amino acid composition between human epidermis, keratinocytes and neutrophils. Also, comparison with other human tissues and cell types (IV) and guinea pig lung (Bito et al. 1989) and guinea pig liver (Haeggström et al. 1988) revealed only minor differences.

The N-terminal amino acid sequence in human LTA_4 hydrolase (Rådmark et al. 1984, Ohishi et al. 1987) has been compared to guinea pig derived LTA_4 hydrolase (Haeggström et al. 1988, Bito et al. 1989). Considerable homology was found. Only two amino acids of 20 were different between the two species. Furthermore, cDNA encoding for LTA_4 hydrolase has been isolated from human placenta (Funk et al. 1987), human spleen (Minami et al. 1987), mouse spleen (Medina et al. 1991) and rat mesangial cells (Makita et

al. 1992). Human LTA_4 hydrolase cDNA has been expressed in Escherichia Coli (Minami et al. 1988) and in cultured Spodoptera frugiperda insect cells (Gierse et al. 1993), and the mouse LTA_4 hydrolase cDNA has been expressed in Escherichia Coli (Medina et al. 1991). A protein of 610 amino acids has been deduced from the isolated cDNA clones except for the rat mesangial cell clone which only contained 609 amino acids.

The optimal pH for the epidermal LTA_4 hydrolase was slightly alkaline with a pH between 7.5 and 9.0, which is similar to other cell types (reviewed in IV). The LTA_4 hydrolase is independent of co-factors, and no activation of the enzyme is needed to transform LTA_4 into LTB_4. A single report (McColl et al. 1987) has suggested that the LTA_4 hydrolase activity is regulated by a protein kinase C-dependent phosphorylation. However, this has been questioned by Samuelsson and Funk (1989) because unpublished data have shown that the LTA_4 hydrolase is not a good substrate for protein kinase C.

LTA_4 has been used as the substrate in all kinetic studies of the epidermal LTA_4 hydrolase in II and IV because it is known as the best substrate for the enzyme. Several other possible substrates, mostly eicosanoid-epoxides and also some xenobiotics, have been tested (reviewed by Rådmark and Haeggström 1990a). While LTA_5 was shown to be hydrolyzed by the enzyme with approximately 40% velocity compared to LTA_4 (Nathaniel et al. 1985), LTA_3 did not act as a substrate (Evans et al. 1985b).

Another interesting characteristic of the epidermal LTA_4 hydrolase is the suicide inactivation of the enzyme when it transforms LTA_4 into LTB_4, demonstrated in study IV and VI. This has been demonstrated in several cell types (McGee and Fitzpatrick 1985, Ohishi et al. 1987, Haeggström et al. 1988), with transformed human airway epithelial cells (BEAS-2B) as an exception (Bigby 1992). Suicide inactivation means that the LTA_4 hydrolase is subject to substrate inactivation. Covalent binding of ^3H labeled LTA_4 to the enzyme has been determined (Evans et al. 1985b, Orning et al. 1990) and may account for the irreversible inactivation. Furthermore, Orning et al. (1992b) have shown the MW of pure recombinant LTA_4 hydrolase to 69.399 $\pm$ 4 by electrospray ionization mass spectrometry, whereas covalent modification leads to a MW of 69.717 $\pm$ 4 indicating a 1:1 stoichiometry of LTA_4:enzyme.

The data regarding pI, MW, amino acid composition, pH optimum and the kinetic data obtained in study IV revealed a close resemblance between the epidermal LTA_4 hydrolase and the LTA_4 hydrolase found in almost all other cell types. Therefore, inflamed human epidermis may provide a good *in vivo* model for the study of transcellular leukotriene synthesis.

5. PEPTIDASE ACTIVITY OF THE LTA_4 HYDROLASE

5.1 Introduction

The presence of the LTA_4 hydrolase in cells not containing the 5-LO indicates a role different from that of converting LTA_4 into LTB_4. After the LTA_4 hydrolase was cloned and its primary structure elucidated (Funk et al. 1987, Minami et al. 1987), the enzyme was shown to have some sequence homology with certain zinc metalloenzymes, e.g. aminopeptidase M and thermolysins from various sources (Malfroy et al. 1989, Toh et al. 1990, Vallee and Auld 1990). Accordingly, purified human LTA_4 hydrolase was shown to contain 1 mol of zinc per mol of enzyme as determined by atomic absorption spectrometry (Haeggström et al. 1990a), and soon after the enzyme was characterized as a zinc metalloenzyme with both peptidase and hydrolase activity (Haeggström et al. 1990a,b, Minami et al. 1990). From the sequence homology with other zinc hydrolases, His-295, His-299 and Glu-318 were proposed to be the zinc-binding ligands in LTA_4 hydrolase (Malfroy et al. 1989, Vallee and Auld 1990). This was soon after confirmed by Medina et al. (1991) by site-directed mutagenesis combined with zinc analysis and activity determination of mutated recombinant mouse and human LTA_4 hydrolase. Furthermore, site-directed mutagenesis of Glu-296 has suggested the glutamic acid residue at 296 to be essential for the peptidase activity of the LTA_4 hydrolase, while the side chain of Glu or Gln is required for LTA_4 hydrolase activity (Wetterholm et al. 1992b, Minami et al. 1992, Izumi et al. 1993, Shimizu et al. 1993). Recently, a second catalytic region important for the LTA_4 hydrolase activity has been demonstrated encompassing the amino acid residues 365-385 (Mueller et al. 1995). From these studies it was suggested that both enzyme activities are exerted via separate, but closely related active sites (Orning et al. 1994). However, anion exchange chromatography of human airway epithelial cells revealed that LTA_4 hydrolase and aminopeptidase activity did not co-elute, indicating that LTA_4 hydrolase isoforms not exhibiting aminopeptidase activity may exist (Bigby et al. 1994).

Peptidase activity was first demonstrated against the synthetic substrate alanine-4-nitroanilide and leucine-4-nitroanilide (Haeggström et al. 1990b). These substrates are degraded to nitroaniline, which can be assayed spectrophotometrically at 405 nm (Minami et al. 1990, Orning et al. 1991a,b, Wetterholm et al. 1992a,b). The method has also been used as a competition assay in which a potential substrate or a potential inhibitor is incubated with the enzyme and l-amino acid-p-nitroanilide (Orning et al. 1991a,b, Griffin et al. 1992, Ollmann et al. 1995, V). The presence of an inhibitor or a substrate for the enzyme will decrease the hydrolysis of l-amino acid-p-nitroanilide to p-nitroaniline. A more direct way to determine whether a compound is substrate for the enzyme is to measure the substrate and the end products after incubation with the enzyme (Griffin et al. 1992, Orning et al. 1992a, 1994, V).

5.2 Incubation conditions used for determination of peptidase activity of the epidermal LTA_4 hydrolase

The incubation medium used in study V contained albumin (1 mg/ml) because albumin has been shown to stimulate the peptidase activity of the LTA_4 hydrolase up to 12-fold depending on the substrate (Orning and Fitzpatrick 1992a). No effect of albumin was seen on the transformation of LTA_4 into LTB_4 (Orning and Fitzpatrick 1992a). In studies I-IV and VI-VII, albumin was added because it stabilizes the unstable epoxide

LTA_4 (Maycock et al. 1982). Also NaCl (100 mM) was added to the incubation medium in V. The chloride ion stimulates the peptidase activity of the LTA_4 hydrolase, whereas the hydrolase part is unaffected (Wetterholm and Haeggström 1992a, Orning and Fitzpatrick 1992a). Cl^- concentrations above 200 mM can, however, decrease the enzyme activity (Orning and Fitzpatrick 1992a). The LTA_4 hydrolase has been described as a zinc metalloenzyme, and zinc ion is essential for both aminopeptidase activity as well as hydrolase activity (Haeggström et al. 1990a,b, Medina et al. 1991). Cobalt can substitute for zinc in both enzyme activities and may even result in increased peptidase activity (Haeggström et al. 1990b). Therefore, 20 μM $CoCl_2$ was added to the incubation medium in study V. Finally, incubations were carried out at 37 °C at pH 7.0. The pH optimum for the peptidase activity has been demonstrated to depend on the presence of albumin (Orning and Fitzpatrick 1992a). The optimum pH of the non-activated enzyme occurred at pH 8.0, whereas catalysis was most efficient with a pH between 7.0 and 7.4 when albumin was added (Orning et al. 1992a, 1994). The optimum temperature for aminopeptidase activity of the LTA_4 hydrolase is 37 °C (Wetterholm and Haeggström 1992a).

5.3 Characterization of the peptidase activity of the epidermal LTA_4 hydrolase

The original studies demonstrating peptidase activity of the LTA_4 hydrolase used the synthetic substrate l-amino acid-p-nitroanilide (Haeggström et al. 1990b, Minami et al. 1990). Later, naturally occurring substrates have been tested (Orning and Fitzpatrick 1992a, Griffin et al. 1992, V). In study V the aminopeptidase activity of the epidermal LTA_4 hydrolase was investigated. Dynorphin fragment 1-7 and to some extent also angiotensin I and II, histamine, kallidine and bradykinin were shown to inhibit the hydrolysis of l-alanine-p-nitroanilide indicating that these compounds might act as substrates for the LTA_4 hydrolase. In contrast, substance P did not affect the LTA_4 hydrolase activity which is in accordance with a previous report (Griffin et al. 1992).

The aminopeptidase activity against dynorphin fragment 1-7 of the epidermal LTA_4 hydrolase was further investigated in V. Purified epidermal LTA_4 hydrolase was incubated with dynorphin fragment 1-7, and the degradation determined as the increase in tyrosine formation. The LTA_4 hydrolase cleaved the tyr^1-bond of dynorphin fragment 1-7 which is of interest, because the N-terminal tyrosine is necessary for the binding of opioids to their receptors (Dua et al. 1985, Schwartz et al. 1981, review). For the epidermal LTA_4 hydrolase, a K_m of 5.3 nmol and a V_{max} of 8.8 nmol/min/mg protein was found for the degradation of dynorphin fragment 1-7, and for the neutrophil LTA_4 hydrolase, the K_m and V_{max} was 2.5 nmol and 6.6 nmol/min/mg protein respectively. It is difficult to compare these results with values for other substrates obtained in other studies (Orning et al. 1992a,b, 1994, Griffin et al. 1992,V) because of the different enzyme preparations used in the different studies.

As demonstrated in IV and VI and discussed in Chapter 4, the LTA_4 hydrolase undergoes suicide inactivation when it transforms LTA_4 into LTB_4. This inactivation is probably caused by covalent binding of LTA_4 to the enzyme. Thus, in V it was not only the hydrolase activity of the enzyme that was inactivated. Also the ability of the enzyme to cleave amide bonds was reduced (Haeggström et al. 1990b, Minami et al. 1990, V). In contrast, activation of the peptidase part does not result in inactivation of the peptidase activity (Haeggström et al. 1990b, Minami et al. 1990,V) nor the hydrolase activity (V). Because LTA_4 also inhibits the peptidase part of the enzyme, it was used as a specific inhibitor of the LTA_4 hydrolase/ peptidase in V. Pre-incubation of the enzyme preparation with LTA_4 almost abolished the formation of tyrosine after incubation with dynorphin fragment 1-7, indicating that LTA_4 hydrolase was the only aminopeptidase with activity against dynorphin fragment 1-7 present in the enzyme preparation (V). Finally, study V revealed no differences in the aminopeptidase activity of the epidermal and neutrophil derived LTA_4 hydrolase.

Opioid peptides such as enkephalins (Griffin et al. 1992, Orning and Fitzpatrick 1992a) and dynorphin fragments (Griffin et al. 1992) have previously been demonstrated as substrates for LTA_4 hydrolase, and also LTD_4 can act as a substrate for recombinant LTA_4 hydrolase (Minami et al. 1990, own unpublished results). Recently it has been reported that tripeptides, preferentially with arginine at the amino end, are even better substrates than LTA_4 for recombinant LTA_4 hydrolase (Orning et al. 1994). When determining the binding affinity (K_m) and the turnover (k_{cat}), several peptides were found to have a k_{cat}/K_m ratio that exceed by 10-fold the k_{cat}/K_m ratio for LTA_4 (Orning et al. 1994). Together with the results obtained in study V, these data suggest a role for the LTA_4 hydrolase/ aminopeptidase in the metabolism of opioid peptides which might be important in inflammatory skin diseases and in modulation of pain.

5.4 Role in psoriasis

Several neuropeptides have been detected in mammalian skin (Weihe and Hartschuh 1988, Brain and Williams 1988). Furthermore, clinical and experimental evidence have indicated a role of peripheral nerves and neuropeptides in the pathogenesis of psoriasis (Farber et al. 1990, Pincelli et al. 1992). Surgical denervation (Farber et al. 1990), dermabrasion (Gold and Roenigk 1987) and skin injury (Eyre and Krueger 1982) have been reported to induce local remission of psoriasis, possibly as a consequence of peripheral nerve damage. Also, treatment with topical capsaicin, which depletes primary-sensory nerves of neuropeptides, has been shown to improve psoriasis (Bernstein et al. 1986). Recently some neuropeptides have been reported to modulate LTB_4 mitogenicity toward cultured human keratinocytes (Rabier et al. 1993), probably through modulation of the cyclic AMP cascade (Takahashi et al. 1993). The synthesis and metabolism of neuropeptides may, therefore, be of importance in psoriasis.

Because of its peptidase activity, the LTA_4 hydrolase may have a role in the degradation of opioid peptides and other neuropeptides. In normal skin the peptidase activity of the enzyme may even be its main function, because no substrates are available for the hydrolase part of the enzyme. However, the transformation of leukocyte derived LTA_4 into LTB_4 re-

sults in inactivation of the LTA_4 hydrolase/aminopeptidase activity. Therefore, the catabolism of opioid peptides and other neuropeptides may be inhibited by inflammatory processes which may contribute to the maintenance of the inflammatory reaction. Furthermore, LTA_4 hydrolase may play a role in modulation of pain because it synthesizes LTB_4, which is known to be hyperalgesic (for review see Ford-Hutchinson 1990), and at the same time it degrades analgesic opioids.

6. THE *IN VIVO* ROLE OF LTA_4 HYDROLASE

6.1 Introduction

Transcellular leukotriene formation has been shown *in vitro* with several different cell types. However, the *in vivo* role of this mechanism has not yet been thoroughly evaluated. The LTA_4 hydrolase activity has been determined in inflammatory skin diseases (Okano-Mitani et al. 1993). In this study the LTA_4 hydrolase activity was measured in the cytoplasmic fraction of peripheral polymorphonuclear leukocytes (PMN) of patients with atopic dermatitis. Patients were classified in three groups, "severe", "moderate" and "mild", and compared to normal controls. Severe atopic dermatitis patients showed higher LTA_4 hydrolase activity than all other groups. In the same study immunohistochemical analysis of LTA_4 hydrolase did not show any difference between involved and uninvolved epidermis. The LTA_4 hydrolase activity has also been determined in the BALF from active smokers and non-smokers (Munafo et al. 1994). A significantly higher LTA_4 hydrolase activity was found in the BALF from active smokers than from non-smokers, and it was suggested that LTA_4 hydrolase in the BALF might contribute to the inflammatory response in tobacco-related lung diseases. In study VI the contribution of epidermal LTA_4 hydrolase to transcellular leukotriene synthesis was investigated in psoriasis.

6.2 Methods for quantifying LTA_4 hydrolase

In most studies LTA_4 hydrolase has been investigated by either immuno-reactivity or activity determination (Munafo et al. 1994, Okano-Mitani et al. 1993, Medina et al. 1988, Izumi et al. 1986). Quantitation of the LTA_4 hydrolase has only been performed in a few studies (Fu et al. 1989, Ohishi et al. 1990b, VI). Fu et al. used RIA to quantify LTA_4 hydrolase in different normal human tissues. The LTA_4 hydrolase was quantified in the 10,000 x g supernatant without further separation. This technique requires a very specific antibody to avoid cross-reactivity. In studies VI and VII the cytoplasmic fraction was separated by SDS-PAGE (9% polyacryl amide) and then transferred to a nitrocellulose membrane before subjection to immunoblot analysis. The immuno-reactive bands were visualized by exposure to a hyperfilm-ECL after incubation with ECL-Western blotting detection reagents. The amount of LTA_4 hydrolase was determined by densitometric analysis at 570 nm comparing the unknown samples to a standard series of recombinant LTA_4 hydrolase. By determining the activity of the LTA_4 hydrolase in the same samples, it was possible to express the activity per mg LTA_4 hydrolase (Ohishi et al. 1990b, VI). This allowed a comparison of the enzyme activity in different tissues. Quantitation of the LTA_4 hydrolase in normal guinea pig tissues has been carried out by an almost identical method (Ohishi et al. 1990b).

6.3 LTA_4 hydrolase and transcellular LTB_4 synthesis in psoriasis

Study VI is the first study dealing with the role of LTA_4 hydrolase and transcellular LTB_4 formation in the epidermis in psoriasis. The activity of the LTA_4 hydrolase was shown to be significantly decreased in involved psoriatic skin compared to matched samples from uninvolved psoriatic skin. Furthermore, transformation of LTA_4 into LTB_4 resulted in inactivation of epidermal LTA_4 hydrolase (IV,VI). Because the epidermis itself is not able to form LTA_4 (Sola et al. 1992, I, II), these results are compatible with the idea that the decreased LTA_4 hydrolase activity in involved psoriatic skin reflects transcellular LTB_4 formation *in vivo*. The activity of the LTA_4 hydrolase in normal human skin was found to be 0.12 nmol/min/mg protein in study VI compared with 0.16 nmol/min/mg protein in another study (Ikai et al. 1994). These values are approximately 10-fold lower than the values found in different guinea pig tissues (Ohishi et al. 1990b). These differences may be explained by differences in the incubation time. Ohishi et al. incubated for 10 sec, whereas 1 min incubations were carried out by Ikai et al. and in VI. It has previously been demonstrated that shortening of the incubation time from 1 min to 10 sec results in a 10-fold increase in V_{max} (Ohishi et al. 1990a).

The LTA_4 hydrolase content was normal in involved as well as uninvolved psoriatic skin (VI). Compared to other human tissues (Fu et al. 1989), the LTA_4 hydrolase content in the epidermis is rather high (VI). In VI the hydrolase content was measured in the 100,000 x g supernatant whereas the 10,000 x g supernatant was used as a reference in the study by Fu et al., which may explain the observed difference. In different guinea pig tissues the LTA_4 hydrolase content was found to vary between 1.6 and 6.5 μg/mg protein in the 100,000 x g supernatant (Ohishi et al. 1990b).

Immunohistochemical staining of involved psoriatic skin has demonstrated that LTA_4 hydrolase is predominantly present in the basal and spinous layers (VI). Also the infiltrating leukocytes were positive for the LTA_4 hydrolase. This localization is not different from normal skin nor uninvolved psoriatic skin (Ikai et al. 1994, VI).

Taken together the data presented in study VI strongly support the idea of transcellular leukotriene synthesis as an important mechanism for synthesis of proinflammatory compounds in inflammatory skin diseases.

7. REGULATION OF THE LTA_4 HYDROLASE

7.1 Introduction

The demonstration of transcellular metabolism as a putative mechanism for LTB_4 formation in the skin together with the indication of suicide inactivation of LTA_4 hydrolase in psoriatic skin suggested an important role of the epidermal LTA_4 hydrolase in the pathogenesis of psoriasis. This prompted us to investigate several anti-psoriatic drugs for

their capability to regulate the level and activity of LTA_4 hydrolase in cultured human keratinocytes (VII). Investigations were carried out with cultured keratinocytes grown for either 24 or 72 hours with different potential modulators of the LTA_4 hydrolase level or activity. Regulation of the LTB_4 forming capacity may be mediated by a direct inhibition of the LTA_4 hydrolase or by regulation of either the synthesis or the degradation of the LTA_4 hydrolase. Therefore, the activity and the amount of LTA_4 hydrolase was determined.

7.2 Regulation of the keratinocyte LTA_4 hydrolase activity

In study VII cyclosporin A caused a dose dependent decrease in the amount of LTA_4 hydrolase in cultured keratinocytes after incubation for 72 hours. The decrease in LTA_4 hydrolase level was paralleled by a significant decrease in LTB_4 formation. This cyclosporin A mediated dose dependent down regulation of the LTA_4 hydrolase in cultured keratinocytes was exerted at concentrations similar to those determined in the skin during treatment of psoriasis (Fisher et al. 1988) and may therefore in part be responsible for its anti-psoriatic effect. The anti-psoriatic effect of cyclosporin A is usually believed to be due to its immunosuppression (Wong et al. 1993, review). Thus, in study VII an anti-inflammatory effect was demonstrated in concentrations similar to those resulting in immunosuppression. Very recently, Hamasaki et al. (1995) demonstrated that cyclosporin A inhibits leukotriene production in intact rat basophilic leukemia-1 cells. In this study it was concluded that the inhibitory effects of cyclosporin A on leukotriene synthesis are attributable to a modulatory effect on one of the intracellular events that includes the activation of the 5-LO, and not due to a down regulation of the LTA_4 hydrolase. However, Hamasaki et al. only incubated the cells with cyclosporin A for 10 min, and the effects after 72 hours incubation found in study VII was therefore not seen in this study.

The synthetic compound RP 64699 A was shown to inhibit the activity of the keratinocyte derived LTA_4 hydrolase after preincubation for 10 minutes (VII). No regulatory effect of 1,25-OH-vitamin D3, retinoic acid, eicosatrienoic acid, dexamethasone, IFN-γ and methotrexate was seen in study VII as determined by the level and the activity of keratinocyte derived LTA_4 hydrolase. In previous studies, eicosatrienoic acid and eicosapentaenoic acid have been demonstrated to inhibit LTB_4 synthesis by inhibition of the LTA_4 hydrolase (Prescott 1984, James et al. 1993, Sperling et al. 1993). This inhibition is probably due to formation of the 5-LO products, LTA_3 and LTA_5, which have previously been demonstrated to inhibit LTA_4 hydrolase activity (Evans et al. 1985b, Nathaniel et al. 1985, Rådmark and Haeggström 1990a). Therefore, inhibition by these fatty acids in cells like keratinocytes not expressing 5-LO activity is not to be expected. In patients with rheumatoid arthritis, methotrexate has been suggested to inhibit the LTA_4 hydrolase (Sperling et al. 1990, Leroux et al. 1992). Rådmark et al. (1990b) have also demonstrated a decrease in LTA_4 hydrolase mRNA in differentiating HL-60 cells incubated with dexamethasone, and this inhibition was reflected by diminished LTB_4 synthesis after incubation with LTA_4. Also, a divalent cation like zinc has been shown to inhibit the activity of the LTA_4 hydrolase (Wetterholm et al. 1994). Furthermore, several synthetic compounds (Yuan et al. 1993, Labaudinière et al. 1992, Wetterholm et al. 1995) including bestatin (Orning et al. 1991b, I, II) and captopril (Orning et al. 1991a, I, II) have been demonstrated to inhibit LTA_4 hydrolase activity, and the effect of bestatin and captopril have even been demonstrated *in vivo* (Muskardin et al. 1994, Shindo 1994). Recently IL-4 and IL-13 have been shown to suppress LTA_4 hydrolase mRNA as well as LTB_4 formation in human monocytes, whereas no effects were seen in glomerular mesangial cells (Montero et al. 1995).

In contrast to these inhibitors, auranofin and IFN-γ have been suggested to stimulate the LTA_4 hydrolase activity (Betts et al. 1990, Renkonen and Ustinov 1990, Montero et al. 1995). Endogenous regulation of the LTA_4 hydrolase activity by a protein kinase C-dependent phosphorylation has also been suggested (McColl et al. 1987), although this has recently been questioned by Samuelsson and Funk (1989).

Taken together these results demonstrate that several different compounds can modulate the activity as well as the amount of LTA_4 hydrolase.

8. CONCLUSIONS AND FUTURE STUDIES

8.1 Conclusions

This thesis investigates the transcellular leukotriene synthesis in the epidermis and the role of the epidermal LTA_4 hydrolase in LTB_4 synthesis in psoriasis. Together with the results from the literature, our results provide the basis for the following conclusions:

1: Cultured human keratinocytes and normal human epidermis have a large capacity to transform neutrophil derived LTA_4 into LTB_4 as well as LTC_4, suggesting that the human epidermis may play an important role in the synthesis of LTB_4 and peptide-leukotrienes during skin inflammation *in vivo*.

2: An affinity column with bestatin coupled to AH-Sepharose was developed to purify LTA_4 hydrolase. This method was effective in the purification of LTA_4 hydrolase. Bestatin inhibits the covalent coupling between LTA_4 and LTA_4 hydrolase. Therefore, this column may also be useful in the separation of active and inactivated LTA_4 hydrolase.

3: The LTA_4 hydrolase in human epidermis was demonstrated by
- I: purification of the enzyme using anion exchange chromatography, affinity chromatography and hydrophobic chromatography,
- II: Western blot analysis using an affinity purified antibody,
- III: immunohistochemical staining, and
- IV: activity determination of the enzyme.

The epidermal LTA_4 hydrolase has a close resemblance to the LTA_4 hydrolase found in other cell types except airway epithelial cells in which the enzyme does not undergo suicide inactivation when transforming LTA_4 into LTB_4 and human lymphocytes in which a higher enzyme activity

was shown. Because of the close resemblance to other tissues, the human epidermis provides an excellent *in vivo* model for studying transcellular LTB_4 synthesis.

4: The epidermal LTA_4 hydrolase has an aminopeptidase activity resulting in inactivation of small peptides, in particular opioid peptides. As a result of suicide inactivation of the LTA_4 hydrolase, the aminopeptidase activity is abolished after the transformation of LTA_4 into LTB_4. In inflammatory skin diseases in which LTB_4 is produced, the catabolism of small peptides may, therefore, be inhibited, leading to sustained biological effects of these opioid peptides.

5: The LTA_4 hydrolase content is similar in normal and involved and uninvolved psoriatic skin. In contrast, the enzyme activity is significantly decreased in involved psoriatic skin compared to matched uninvolved psoriatic skin. Because suicide inactivation of the LTA_4 hydrolase takes place when LTA_4 is transformed into LTB_4, this observation is compatible with the idea that transcellular LTB_4 formation occurs in inflammatory skin diseases.

6: Several compounds including bestatin, captopril and RP 64699 A have been demonstrated to inhibit the LTA_4 hydrolase activity. Furthermore, we have shown that cyclosporin A causes a down regulation of the amount of LTA_4 hydrolase in keratinocyte cultures. Therefore, cyclosporin A may have anti-inflammatory effects in addition to its immunosuppression.

8.2 Future studies

As described in this thesis transcellular leukotriene synthesis can take place in the human epidermis by transformation of neutrophil derived LTA_4. However, the importance of this phenomenon *in vivo* still remains to be demonstrated. In future studies the presence of an active and an inactive form of the LTA_4 hydrolase in inflammatory skin should be determined. Because LTA_4 cannot be formed by the epidermis itself, the presence of these two forms would suggest that transcellular leukotriene synthesis takes place *in vivo*. Separation of the two forms on the bestatin column developed in IV or demonstration of two different MWs by electrospray ionization mass spectrometry of the LTA_4 hydrolase should be suitable methods. Alternatively two dimensional gel electrophoresis could be used to separate the two forms of LTA_4 hydrolase.

Since no substrate is available for the hydrolase part of the LTA_4 hydrolase in normal skin, future studies should further elucidate the role of the peptidase activity of the enzyme. The presence of opioid peptides in normal and inflammatory skin and their role in the development of skin inflammation should be investigated. Furthermore, other small peptides present in the skin should be investigated for their potential to act as substrates for the epidermal LTA_4 hydrolase.

Finally, further characterization of endogenous regulators of the LTA_4 hydrolase activity should be carried out, and in view of the LTA_4 hydrolase as a potential pharmacological target for treatment of inflammatory disorders, new exogenous inhibitors should be developed. For this purpose a more skillful characterization of the catalytical mechanism(s) may be required. Also, the importance of the inhibitory effect of cyclosporin A on the expression of the LTA_4 hydrolase should be investigated in inflammatory diseases.

9. DANISH SUMMARY

Denne afhandling beskæftiger sig med betydningen af transcellulær leukotrien syntese med speciel vægt på den epidermale leukotrien A_4 (LTA_4) hydrolases rolle ved inflammatoriske tilstande i huden. Leukotriener af 4-serien dannes ud fra den flerumættede fedtsyre, arachidonsyre (AA). 5-lipoxygenasen (5-LO) omdanner AA til LTA_4. Via LTA_4 hydrolasen omdannes LTA_4 til LTB_4. Alternativt kan LTA_4 omdannes til LTC_4 via LTC_4 syntasen. Huden har ikke nogen målelig 5-LO aktivitet, men kan bidrage til leukotrien dannelsen via transcellulær leukotrien syntese. Ved transcellulær leukotrien syntese forståes, at syntesen foregår via enzymer i to forskellige celler. I dette studie blev LTA_4 hydrolase aktivitet påvist i human epidermis, og hudens LTA_4 hydrolase blev oprenset og yderligere karakteriseret med hensyn til hydrolase og aminopeptidase aktivitet. Desuden blev LTA_4 hydrolasens rolle ved psoriasis undersøgt, og endelig blev forskellige stoffer screenet for deres evne til at regulere mængden og/eller aktiviteten af LTA_4 hydrolasen i humane keratinocytter *in vitro*.

Følgende konklusioner kan drages ud fra de beskrevne undersøgelser:

1: Kulturer af normale humane keratinocytter og suction blister isoleret epidermis kan omdanne LTA_4 syntetiseret i neutrofile granulocytter til LTB_4. Yderligere omdanner epidermis LTA_4 til LTC_4. Dette indikerer, at epidermis spiller en vigtig aktiv rolle i syntesen af LTB_4 og peptid-leukotriener (LTC_4, LTD_4 og LTE_4) *in vivo*.

2: En affinitetssøjle med bestatin koblet til AH-Sepharose er effektiv i oprensningen af LTA_4 hydrolasen. Da LTA_4 hydrolasen under omdannelsen af LTA_4 til LTB_4 inaktiveres som følge af kovalent binding af LTA_4 til enzymet, og da bestatin hæmmer kovalent binding mellem LTA_4 og LTA_4 hydrolasen, kan denne søjle muligvis bruges til at adskille aktiv og inaktiv LTA_4 hydrolase.

3: LTA_4 hydrolasen er tilstede i human epidermis, og den epidermale LTA_4 hydrolase har stor lighed med LTA_4 hydrolasen i næsten alle andre celle typer med undtagelse af luftvejsepithelceller, hvor enzymet ikke inaktiveres efter omdannelse af LTA_4 til LTB_4, og humane lymfocytter, hvor enzymet har en højere aktivitet. Hud udgør derfor en god *in vivo* model for yderligere studier af transcellulær LTB_4 syntese.

4: Hudens LTA_4 hydrolase indeholder også aminopeptidase aktivitet, som resulterer i inaktivering af forskellige små peptider inklusiv opioid peptider. Som et resultat af den inaktivering, der sker af LTA_4 hydrolasen efter omdannelse af LTA_4 til LTB_4, hæmmes også aminopeptidase aktiviteten. Derfor er kataboliseringen af forskellige små peptider også hæmmet i inflammatoriske hudsygdomme, hvor LTB_4 dannes. Denne enzym-inaktivering medfører muligvis en forlænget biologisk aktivitet af disse peptider.

5: Mængden af LTA_4 hydrolase i normal såvel som involveret og ikke-involveret psoriasis hud er ens. I modsætning hertil er enzymaktiviteten signifikant nedsat i involveret psoriasis hud sammenlignet med parret ikke-involveret psoriasis hud. På grund af den inaktivering, der sker af LTA_4 hydrolasen, når LTA_4 omdannes til LTB_4, er hæmningen af LTA_4 hydrolase aktiviteten i involveret psoriasis hud i overensstemmelse med antagelsen, at transcellulær LTB_4 syntese foregår i huden ved inflammatoriske hudsygdomme.

6: Adskillige stoffer såsom bestatin, captopril og RP64699 A er vist at hæmme LTA_4 hydrolase aktiviteten, men kun cyclosporin A nedregulerer mængden af LTA_4 hydrolase i keratinocyt kulturer. Derfor har cyclosporin A muligvis en anti-inflammatorisk virkning foruden den velbeskrevne immunosuppressive virkning.

Afhandlingen viser således, at transcellulær leukotriensyntese med omdannelse af LTA_4 syntetiseret i neutrofile granulocytter kan finde sted i huden. Det er derfor muligt, at hudens LTA_4 hydrolase spiller en både aktiv og central rolle i den inflammatoriske reaktion ved inflammatoriske hud lidelser såsom psoriasis. I normal hud er der intet substrat (LTA_4) tilgængelig for LTA_4 hydrolasen, hvorfor den dominerende aktivitet må formodes at være enzymets aminopeptidase aktivitet. Under normale forhold er der således en given balance mellem enzymets hydrolase og peptidase aktivitet. Denne balance kan ved forskellige sygdoms tilstande forstyrres som følge af transcellulær leukotrien syntese og således være en medvirkende årsag til det inflammatoriske respons. Den endelige afklaring af den transcellulære leukotrien synteses betydning i inflammatoriske hudsygdomme må afvente udviklingen af potente og specifikke LTA_4 hydrolase hæmmere, som kan appliceres lokalt på huden.

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