THE LEUKOTRIENES

Action on lung parenchymal strips
and the formation by macrophages

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DE LEUKOTRIENEN

Werkingsmechanisme op long parenchymweefsel en de vorming door macrofagen

PROEFSCHRIFT

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Let there be praise
Let there be joy in our hearts
Sing to the Lord
Give Him the glory
The peace that He gives none can equal
His love, it knows no end
In our weakness, His strength will defend us
When His praise is on our tongue

Sandi Patti

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CONTENTS

ABBREVIATIONS

AA	Arachidonic acid
AcN	Acetonitrile
cAMP	cyclic Adenosine monophosphate
5,12-diHETE	5(S)-12(S)- dihydroxy 6,8,10,14 eicosa tetra enoic acid
EDTA	Ethylene diamino tetra acetic acid
EFA(D)	Essential fatty acid (deficient)
FMLP	N-Formyl-methionyl leucyl phenylalanine
GSH	glutathione (reduced)
HAc	acetic acid
5-HETE	5-hydroxy-6,8,11,14-eicosa tetraenoic acid
12-HETE	12-hydroxy-5,8,10,14-eicosa tetraenoic acid
15-HETE	15-hydroxy-5,8,11,13-eicosa tetraenoic acid
HHT	12(S)-hydroxy-5,8,10-heptadeca trienoic acid
HPETE	hydroperoxy eicosa tetra enoic acid
HPLC	High performance liquid chromatography
IBD	Inflammatory bowel disease
IC_{50}	fifty percent inhibitory concentration
$6kPGF_{1\alpha}$	6-keto-Prostaglandine $F_{1\alpha}$
LT	Leukotriene(s)
LTB_4	Leukotriene B_4
LTC_4	Leukotriene C_4
LTD_4	Leukotriene D_4
LTE_4	Leukotriene E_4
MØ	Macrophage
NDGA	Nor dihydro guai aretic acid
NSAID	Non steroidal anti-inflammatory drug(s)
PAF	Platelet-activating factor
PMNL	Poly morpho nuclear leucocyte
PG	Prostaglandin(s)
PGD_2	Prostaglandin D_2
PGE_2	Prostaglandin E_2
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PLA_2	Phospholipase A_2
RIA	Radio immuno assay
RP-HPLC	reverse phase high performance liquid chromatography
SRS-A	Slow-reacting substance of anaphylaxis
TxA_2	Thromboxane A_2
TxB_2	Thromboxane B_2

CHAPTER 1.

INTRODUCTION

1.1 SCOPE OF THE STUDY

In this thesis the history, biosynthetic pathways and biological activities of leukotrienes is being reviewed. Their contractile effects on lung parenchymal strips and their formation by macrophages are investigated. In addition the action of specific synthesis inhibitors is determined. These two approaches were chosen , firstly to investigate the mechanism of action of these potent bronchoconstrictive agents and secondly to determine the capacity of cells to generate the whole cascade of arachidonic acid products, to which leukotrienes belong. Leukotrienes belong to the so-called eicosanoids , which also comprise the group of prostaglandins and mono-hydroxy eicosatetraenoic acids. The results presented in chapters 3 and 4, show that the three main groups of eicosanoids have different biological effects and are able to interact with each other.

1.1.1 *Mode of action of leukotrienes on lung parenchymal strips*

It has been shown that the guinea pig lung parenchyma is particularly sensitive to the bronchoconstrictive effect of leukotrienes. During contractions induced by these compounds, another eicosanoid , thromboxane A_2, is formed. This substance has been shown to be both a potent vasoconstrictor and bronchoconstrictor. The decrease in contractile activity induced by specific synthesis inhibitors and beta adrenergic

agents was investigated and the effects of their antagonists were determined. The release of thromboxane A_2 following to contractions with phospholipase A_2 was studied in guinea pig lung and compared with the effects on human and porcine parenchyma. In addition, it was the aim of these studies to further investigate the mechanisms of action of the leukotrienes, in particular in so far as their role in respiratory diseases is concerned.

1.1.2 *Formation of leukotrienes and other eicosanoids by chopped tissue and macrophages*

The next step was to further define the role of the formation of arachidonic acid metabolites as measured in chopped human lung tissue. The development of chromatographic methods made it possible to separate a number of structurally closely related compounds in tissue extracts and incubation media. Starting-point of this part of the investigations was the idea that a relationship existed between the biological response and the formation in the tissue of one or more compounds belonging to the group of the eicosanoids.

Subsequently, a number of experiments are described in which the production of leukotrienes and other eicosanoids by particularly human macrophages, obtained from ascites of patients with liver cirrhosis, was determined.

Arachidonic acid derived products play an important role in the regulation of the inflammatory process. Finally therefore the use of specific synthesis inhibitors in relation to the participation of leukotrienes in the pathogenesis of asthma and related inflammatory diseases was investigated.

CHAPTER 2

EICOSANOIDS

2.1 INTRODUCTION

The voluminous literature on prostaglandin and leukotriene research explains the growing interest in the biological activity of compounds of the family of eicosanoids. These substances are generated from unsaturated fatty acids by tissues and cells. Their discovery and the development of their chemistry has led to advances in pharmacology and clinical medicine. Although the structure and chemistry of eicosanoids are now well established, it is still difficult to assign specific biological functions to each of these substances. The lack of a clear-cut functional definition is attributable to several factors.

Firstly, tissues can synthesize several eicosanoids or respond to more than one. Secondly the response of a tissue to eicosanoids may reflect the type and number of receptors that it contains for only one specific prostaglandin or leukotriene. Thirdly, because a number of biological active eicosanoids are inactivated rapidly after they are synthesized, they cannot always be detected by the physiological parameters used as indicators of their effect. Finally eicosanoids are involved in many cell to cell interactions as mediators, in which other derivatives of arachidonic acid can cause opposite effects.

The properties and potencies of biologically active eicosanoids on a specific tissue prove to be wide ranging. Since the elucidation of the structure of leukotrienes, a large number of different actions has

been found at physiological concentrations. This suggests that leukotrienes, like prostaglandins, may be of important biological significance in most tissues of the body.

Eicosanoids as potent naturally occurring biological modulators are becoming prominent in cardiovascular diseases [Moncada,1982; Berkowitz *et al.*, 1984], dermatology [Kragballe and Voorhees,1983], cancer [Hohn *et al.*, 1983], asthma [Weiss *et al.*,1982] and inflammatory diseases [Bonta and Parnham, 1982]. In this chapter current knowledge of the biochemistry and physiology of eicosanoids will be discussed, with particular emphasis on the role of leukotrienes on lung parenchyma and their formation by macrophages.

2.2 HISTORY

In 1930 Kurzok and Lieb, two New York gynecologists, discovered the muscle-stimulating actions of seminal fluid on the human uterus. Subsequently, Goldblatt in England [1935] and Von Euler in Sweden [1936] independently described some of the actions of seminal plasma on smooth muscle. Von Euler, who believed that the myometrial-contracting activity originated from the prostate, called the active substance "prostaglandin". Bergström and Sjövall [1957] established that prostaglandin was a mixture of biologically active compounds. It was not until 1959, however, that Eliasson discovered that the seminal vesicles were the origin of prostaglandins.

Working with acidified lipid extracts from sheep seminal vesicular glands, Bergström [1964] demonstrated that the active principle was present in a fraction containing unsaturated hydroxy acids. Monitoring of

these products was until then performed by biological assay systems. Independently of each other, Bergström [1964] and Van Dorp [1964] performed studies, in which ^{3}H-labelled arachidonic acid was incubated with homogenates from sheep seminal vesicles, which led to the new and important concept that prostaglandins are derived from essential fatty acids, constituents of membrane phopholipids.

The next significant biological step was the demonstration by Vane *et al.*, [1971], that aspirin and other nonsteroidal anti-inflammatory compounds inhibited cyclo-oxygenase, the enzyme that catalyzes the transformation of arachidonate to prostaglandins. Just before this finding, Piper and Vane [1969] observed the release of a substance during anaphylaxis on guinea pig lung, which had a contractile effect on rabbit aorta. This rabbit aorta contracting substance (RCS) was not a classical prostaglandin such as E_2, although aspirin and indomethacin inhibited its formation. Shortly thereafter [1973] Vargaftig and Zirinis reported RCS formation by platelets during aggregation, after addition of arachidonic acid. It has taken another two years before this substance was named as thromboxane A_2, at that time believed to be the most potent vasoconstrictor and platelet aggregator known [Hamberg *et al.*,1975]. This short-lived intermediate is formed via biologically active, unstable endoperoxides, which are intermediates in the transformation of arachidonic acid to prostaglandins [Hamberg and Samuelsson,1973].

The next breakthrough in prostanoid research was published in 1976 by Moncada and co-workers. They observed that prostacyclin (the so-called PGX), formed by endothelial cells, prevented platelet aggregation and had strong vasodilator properties.

The discovery of leukotrienes and related compounds goes parallel to this

50-years history of prostaglandin research.

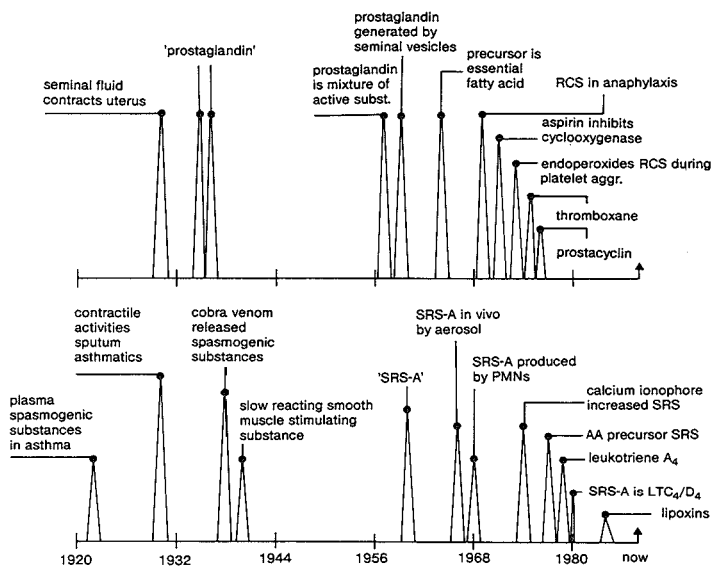
Spasmogenic substances in the plasma of patients suffering from asthma were discovered in 1922. They were also described in 1930 by Harkavy in a study performed with ethanol extracts of sputum of patients before, during and after an asthmatic attack. The dissolved residue from patients during an attack caused contractions of both the cat jejunum and the rabbit duodenum.

Some years after this finding Feldberg and Kellaway [1938] investigated the spasmogenic substances released after cobra venom infusion into guinea pig lungs, and compared their effects to those of histamine. Shortly thereafter Kellaway and Trethewie [1940] discovered the presence of a slow reacting smooth muscle stimulating substance (SRS-C) during anaphylaxis. The name slow-reacting substance of anaphylaxis (SRS-A) was introduced by Brocklehurst in 1960. Orange and Austen described in the late sixties a number of characteristics of SRS-A. The first experiments in which SRS-A preparations were administered to asthmatic subjects by aerosol date from 1966 by Herxheimer and Stresemann.

Pharmacological investigations proved that mast cells were responsible for the release of histamine, whereas SRS-A was predominantly produced by leucocytes [Orange and Austen,1968]. In 1974 Back and Brasher discovered that challenge of rat peritoneal cells with the calcium ionophore A23187, in the presence of calcium, resulted in the generation of SRS. Incorporation of large amounts of cysteine increased the SRS production. This observation led to structural work, using new methods such as mass spectrometry, to obtain highly purified SRS-A from biological extracts [Blackwell *et al.*,1980]. Although it became clear that SRS-A did not belong to the group of prostaglandins, it still was believed that arachidonic acid could be the precursor of SRS-A [Jakschik *et al.*,1977]. It was the group of Samuelsson who finally, in collaboration with Borgeat,

suggested that dihydroxy acids, with functional groups on the 5 and 12 positions, were derived from a hypothetical epoxy-eicosatetraenoic acid intermediate, which they named leukotriene A_4 [Borgeat and Samuelsson,1979].

This was the first step leading to the discovery of a whole cascade of biologically active substances, called leukotrienes [Samuelsson *et al.*,1980]. Subsequent work showed that SRS-A was in fact a combination of at least 2 of these leukotrienes (LTs). In 1984 Serhan *et al.* isolated another class of metabolites of arachidonic acid, the trihydroxy eicosatetraenoic acids or lipoxins. These are not derived from the same intermediate as leukotrienes, but through a different lipoxygenase pathway. The biological importance of these group of substances is still unknown. During the last few years a number of LT-derivatives have been introduced, which will be discussed later in the section Biosynthesis of leukotrienes.



Highlights in prostaglandin and leukotriene research

2.3 PRECURSORS OF EICOSANOIDS

Eicosanoids are formed from essential fatty acids and are present throughout the body. The prostaglandins (PGs), leukotrienes (LTs) and related compounds are called eicosanoids, because they are derived from 20-carbon containing essential fatty acids that include three, four or five double bonds. The substrate for the biosynthesis of eicosanoids, arachidonic acid (AA), is structarally a part of phospholipid, triglycerids and cholesterol (Fig.1.). The essentiality of unsaturated dietary fatty acids was first observed by Burr and Burr in 1929. The clinical and biochemical consequences of deficiences of these fatty acids, could be reversed by feeding linoleic acid to mammals. In figure 2 the conversion of linoleic acid to the precursor of the eicosanoids is shown. In this thesis only the formation of metabolites derived from AA and their effects were investigated, and for that reason, only the pathways involved will be discussed in detail. Linoleic acid is desaturated and elongated via gamma-linolenic acid and dihomogammalinolenic acid to AA. The structure of each essential fatty acid (EFA) is fully described by three distinguishing marks in the generally accepted nomenclature.

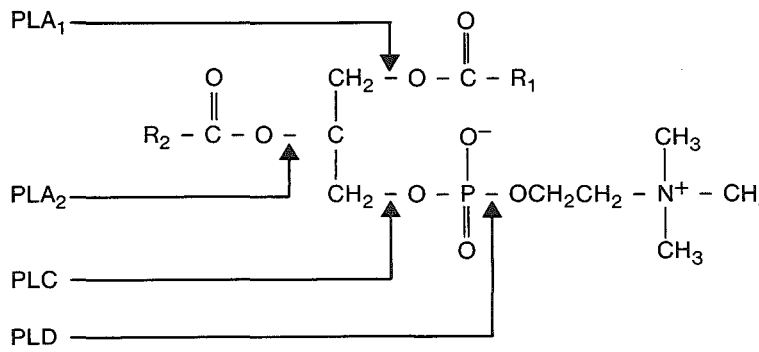


Fig.1 Differential action of phospholipases

The notation of AA is C20 : 4 w 6, in which 20 is the number of carbon atoms in the chain, 4 is the number of the double bonds and w 6 means the number of carbon atoms between the terminal unsaturated bond and the final methyl group of the EFA.

Linoleic acid is the major dietary EFA in humans [Burr and Burr,1929], and is present in vegetable seed oils and milk products. Linoleic acid cannot be synthesized by man and has to be present in the daily food intake [Vincent *et al.*,1975]. Gamma-linolenic acid is found in evening primrose oil. The minimum daily intake of AA in rats needed to obtain sufficient prostaglandin formation by cells, is 1 mg/day [Horrobin,1983].

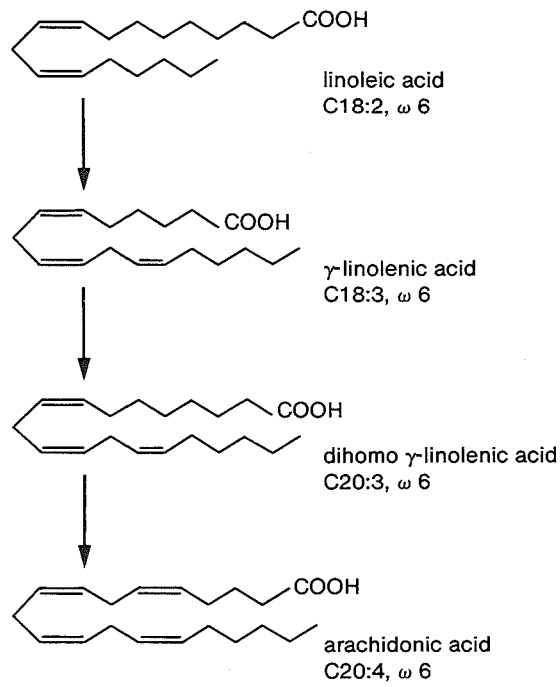


Fig.2 Conversion of linoleic acid to arachidonic acid, the precursor of eicosanoids

The amount of linoleic acid in the diet is correlated with the concentration of AA metabolites, the PGs, in the urine [Nugteren *et al.*,1980]. Much work has been done with EFA deficient diets in experimental models [Kort,1987], rat blood platelets [Vincent and Zijlstra, 1976, 1978] and man to investigate the significance of EFA in disease [Yamanaka,1981]. Pitfalls of the use of EFA deficient rats for the study of the role of prostaglandins in pathophysiology was reviewed by Parnham *et al.* [1979].

Slight increases in the linoleic acid content of the diet led to the production of appreciable amounts of endogenous PGs in platelets. In conclusion it was found that, when a triene / tetraene (20:3(n-9) / 20:4(n-6)) -ratio of 6 or more was achieved, a high level of deficiency was present. EFA deficiency exerted an anti-inflammatory effect in both acute and chronic inflammation [Bonta and Parnham,1982].

Recently, macrophage fatty acid metabolism and function in EFA deficient mice were described [Lefkowith *et al.*,1987]. EFA deficiency was shown to decrease phosphatidylinositol in peritoneal macrophages. After stimulation with zymosan, control macrophages synthesized LTC_4 and LTB_4, whereas EFA deficient macrophages synthesized markedly less LTC_4 and also metabolized 20:3(n-9) to LTC_3. EFA deficient macrophages synthesized minimal amounts of LTB_4. The prostaglandin production was only minimally affected by EFA deficiency. The lack in leukotriene formation by EFAD macrophages could be responsible for the anti-inflammatory and protective effect of the deficiency state.

2.4 BIOSYNTHESIS OF EICOSANOIDS

2.4.1 *Phospholipases*

Eicosanoids are synthesized in the cell membrane from AA, which is released from membrane phospholipid and other lipid esters by the action of a phospholipase. (Fig.1.) Phospholipase can be activated by a variety of stimuli, and its activation is evidently the initial and rate-limiting step in eicosanoid biosynthesis [Lands,1979]. In response to this stimulus, phospholipase A_2 (PLA_2) or a combination of phospholipase C (PLC) and diglyceride lipase, catalyze the cleavage of an esterified eicosanoid precursor from the 2 position of specific glycerophospholipids [Blackwell and Flower,1983]. Arachidonic acid can also be released either from diacylglycerol or from phosphatidic acid. This could be one reason that the so-called selective inhibitors of PLA_2, are insufficiently selective to discriminate between the direct PLA_2-dependent pathway and this indirect one [Lapetina *et al.*,1981; Hofmann *et al.*,1982].

Arachidonic acid can be activated by several distinct enzyme systems. PLA_2 activity is dependent on the availability of intracellular Ca^{2+}, intracellualar cAMP content [Van den Bosch,1980] and the activity of lipocortin. This has been shown in several celltypes.

Lipocortin is a glucocorticosteroid induced polypeptide. It has been isolated from macrophages (MØ), neutrophils and renomedullary interstitial cells [Blackwell *et al.*,1982; Hirata *et al.*,1980; Cloix *et al.*,1983]. Both PLA_2 and PLC are inhibited by lipocortin. Enhanced eicosanoid production was observed in several tissues and in macrophages of adrenalectomized rats, probably due to a diminished formation or inactivation of lipocortin. [Vincent *et al.*,1986].

Cell activation appears to be dependent on the turnover of phosphatidyl inositol, mediated by PLC, which results in the elevation of Ca^{2+} into the cell and activation of PLA_2 and protein kinase C and consequently the release of eicosanoids (the indirect pathway [Lapetina *et al.*,1981]).

The inactivation of lipocortin through phosphorylation is carried out by a tyrosine kinase which is activated by stimulating agents. Tyrosine kinase itself is regulated by phosphorylation by serine kinases. The Ca^{2+} dependent protein kinase C activates it and the cAMP dependent protein kinase A, deactivates tyrosine kinase [Hirata *et al.*,1984]. Receptor mediated, PLC dependent reactions can be mimicked by the Ca^{2+} ionophore A23187. In fig.3 interactions between Ca^{2+}, cAMP and PLA_2 are presented.

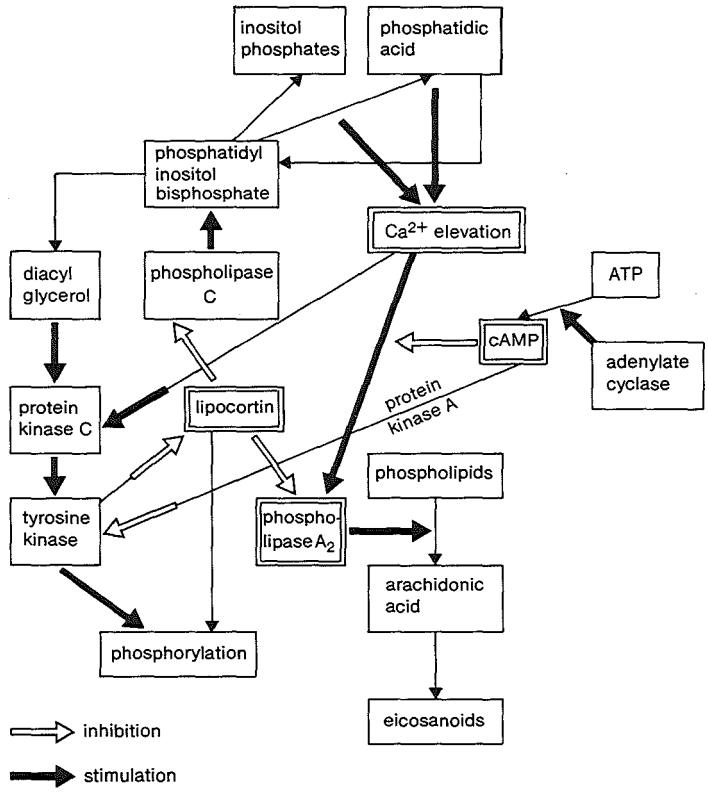


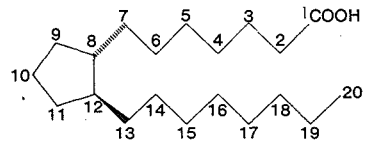
Fig.3 Interactions between lipocortin, Ca^{2+}-elevation, cAMP and PLA_2.

In this scheme [acc. to Schenkelaars,1985], a summary is given, comprising the PLA_2 dependent and independent AA release, the inhibition of the PLA_2 dependent AA release by lipocortin and the inactivation of lipocortin by phosphorylation through tyrosine kinase. Following this scheme both an increased cAMP level and an enhanced lipocortin concentration will result in a diminished eicosanoid production. An increased Ca^{2+} elevation, on the contrary, stimulates eicosanoid formation.

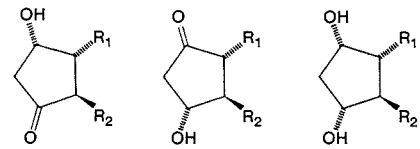
2.4.2 *Prostaglandins*

Prostaglandins are cyclic fatty acids that contain 20 carbon atoms similar to the basic carbon skeleton of the hypothetical parent compound, prostanoic acic (Figure 4.). A prostaglandin (PG) consists of a

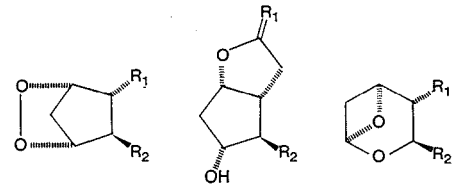
Fig.4



The hypothetical parent compound, prostanoic acid



Ring structures of three primary prostaglandines.



The cyclic endoperoxides, prostacyclin and thromboxane A

cyclopentane ring and two aliphatic side chains. The synthesis of PGs from AA is proceded by the oxygenation and cyclization of the pentane ring by cyclooxygenase, leading to an unstable C_{15} hydroperoxy, C_9 C_{11} endoperoxide, prostaglandin G_2 (PGG_2). Endoperoxides, which are chemically unstable, form the intermediate substrates for tissue-specific synthesis of a variety of biologically active PGs [Flower,1978]. Each PG is designated by a letter and a subscript, indicating the nature of the cyclopentane substituents and the number of double bonds, being dependent upon the precursor. In this case, dienoic acid is synthesized from AA, mentioned as the 2-series (Figure 5). Another cyclooxygenase product is

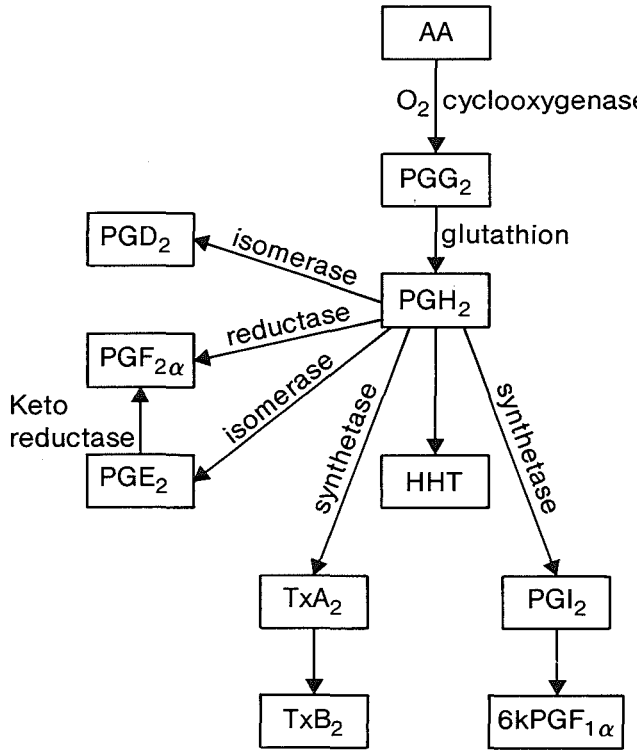


Fig.5 Formation of prostaglandins, prostacyclin and thromboxane

thromboxane A_2 (TxA_2), formed by an enzyme and first isolated from human platelets. TxA_2 has a very short chemical half-life ($T_{½}$=30 seconds at 37°C and pH 7.5), and it breaks down non enzymatically into the stable thromboxane B_2 (TxB_2). Formation of thromboxane A_2 is inhibited by aspirin and other non-steroidal anti-inflammatory compounds, via the inactivation of cyclooxygenase [Vane,1971]. In comparison to other cyclooxygenase inhibitors (indomethacin ,ibuprofen) , the inactivation by aspirin is markedly prolonged.

Considerable effort has been made to discover selective inhibitors of thromboxane synthetase. This enzyme is inhibited by imidazole and imidazole derivatives such as dazoxiben [Moncada,1977] and by other compounds such as nicotinic acid [Vincent and Zijlstra,1978].

Another route of metabolism of PGG_2 leads to prostacyclin (PGI_2), which is also an unstable compound ($T_{½}$=3 minutes at 37°C and pH 7.5), formed by an enzyme, prostacyclin synthetase. PGI_2 was first discovered in vascular tissue and differs chemically from other PGs in that it has a double-ring structure, which includes a furane ring. It is hydrolyzed non enzymatically into the stable compound, 6-keto-prostaglandin $F_{1\alpha}$ ($PGF_{1\alpha}$) [Moncada,1976].

The prostaglandins of the E_2, D_2 and $F_{2\alpha}$ type are usually referred to as the primary PGs, although they are products of the same intermediates PGG_2 and PGH_2. These prostaglandins are the so-called stable prostaglandins. They are however rapidly converted into biological inactive compounds Two main pathways exist for the catabolism of PGs. By the enzyme prostaglandin 15-hydroxydehydrogenase, PGs are oxidized to the corresponding ketones and further reduced to obtain 13,14-dihydro,15-keto-PGs. These metabolites are excreted via the urine [Lands,1979].

Recently PGD_2 was found to be stereospecifically converted into the

compound 9α,11β-PGF_2 by a human lung and liver cytosolic NADPH-dependent 11-ketoreductase [Seibert *et al.*,1987]. This substance is a potent bronchoconstrictor and is released into bronchoalveolar lavage fluid after allergen stimulation in patients with allergic asthma. 9α,11β-PGF_2 is poorly inactivated in situ. This led to the conclusion that this particular compound may participate, along with other mediators, in the pulmonary response in humans. The biological activities of PGs and TxA_2 are summarized in table 1.

TABLE 1

SOME BIOLOGICAL ACTIVITIES OF PROSTAGLANDINS

(C = contraction; R = relaxation; + = stimulates; − = inhibits; ? = unknown)

Effect on cell or tissue	D_2	E_2	$F_{2\alpha}$	I_2	TxA_2
smooth muscle vascular	R	R	C	R	C
respiratory	R	R	C	R	C
gastrointestinal	?	C/R	C	C	?
platelet aggregation	−	−	?	−	+
provoke edema	?	+	+	+	?
fever	?	+	?	?	?
pain	?	+	−	+	?
chemotactic activity	?	+(?)	+	−	+(?)

2.4.3 *Leukotrienes*

As already mentioned the leukotrienes (LTs) are generated through mobilization of arachidonic acid from phospholipids, mediated by a phospholipase. From arachidonic acid as a precursor, LTs of the 4-series, compounds with 4 double bonds are synthesized [Borgeat and Samuelsson,1979]. Leukotrienes are formed via the enzyme 5-lipoxygenase through an intermediate, the leukotriene A_4. This is the most important lipoxygenase generating these biologically active substances [Samuelsson,1980]. In fig.6 the metabolism of AA via the 5-lipoxygenase through 5-hydroperoxyeicosatetraenoic acid (5-HPETE) and LTA_4 is shown.

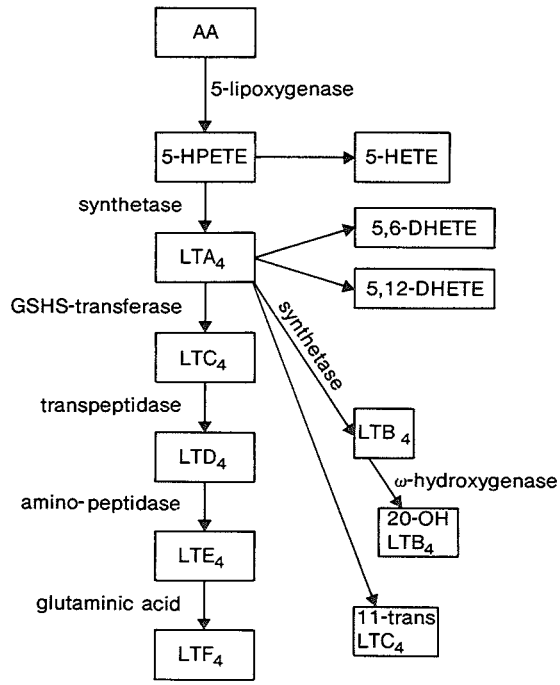


Fig.6 Formation of leukotrienes and other 5-lipoxygenase products

The enzymatic conversion of the subsequent LTs by the conjugation with glutathion leads to the cysteine-containing sulfidopeptide leukotrienes (fig.7).

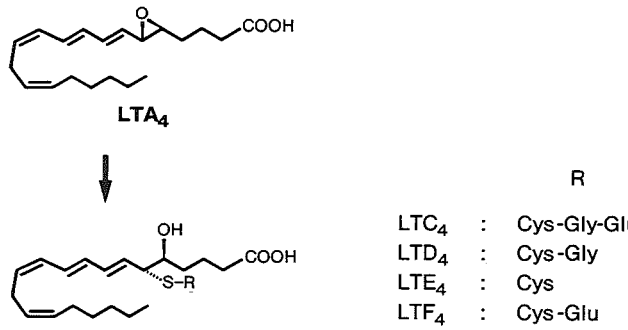


Fig.7 Substitution of amino acids on the 5-lipoxygenase intermediate, leukotriene A_4, to obtain sulfidopeptide leukotrienes

The potencies of LTs are 100-1000 times that of histamine in the contractile reponse of the guinea pig ileum [Piper and Samhoun,1983]. Intravenous infusion of LTC_4, LTD_4 and LTE_4 caused a dose dependent bronchoconstriction in unanaesthesized mechanically ventilated guinea pigs [Drazen *et al.*,1980]. This constriction can be partly blocked by the cyclooxygenase inhibitor indomethacin, suggesting that bronchoconstriction is mediated via PGs [Leitch,1984]. This mechanism of action is described in detail in the results presented in chapter 3. Constriction of coronary arteries was observed in some cases, predominantly in experimental models [Feuerstein,1985]. In rat peritoneal macrophages lysosomal enzyme release was activated by LTC_4 [Schenkelaars and Bonta,1983] and PGE_2 release increased, whereas the formation of TxB_2 was decreased dose-dependently. The increased release of cyclooxygenase products by LTC_4 is in agreement with the observations with guinea pig lung parenchyma. A summary of biological activities is given in table 2.

TABLE 2

SOME BIOLOGICAL ACTIVITIES OF LEUKOTRIENES

(C = contraction; + = stimulates; 0 = no effect; ? = unknown)

Effect on cell or tissue	LTB_4	LTC_4	LTD_4	LTE_4
smooth muscle vascular	?	C	C	0(?)
respiratory	C	C	C	C
chemotactic activity	+	0	0	0
vascular permeability >> edema	+	+	+	+
degranulation	+	+	+	0

The production of leukotrienes of the C,D,E and F-type has been investigated with exogenous AA in human cells. Besides the polymorphonuclear cells [Borgeat and Samuelsson,1979], production was also observed in human peritoneal macrophages [Ouwendijk,1985], human alveolar macrophages [Fels *et al.*,1982], eosinophils [Borgeat *et al*,1984; Henderson *et al.*,1984] and mast cells [Peters *et al.*,1984]. LTB_4 is predominantly generated from neutrophils [Palmer and Salmon,1983] and furthermore by PMNLs, macrophages and mast cells [Bray,1984].

In aspirin-sensitive asthmatic patients total cell counts from broncho alveolar lavage were increased, and the number of the eosinophils was highly increased [Godard *et al.*,1982]. Increased cell activity is reflected in the release of lysosomal enzymes, and in increased leukotriene production, similar to that observed during phagocytosis [Rouzer *et al.*,1980]. By the action of the very potent chemotactic agent LTB_4, granulocytes adhere to blood vessels and leukocytes migrate to inflammatory regions.

2.4.4 *Other lipoxygenase products*

A number of enzymes has been discovered that peroxidize arachidonic acid in different positions. The most important of these is the 5-lipoxygenase. The action of this enzyme results in the formation of leukotrienes (see 2.4.3). Hydro-peroxy eicosatetraenoic acids (HPETEs) are relatively unstable and are reduced enzymatically or non-enzymatically to the corresponding hydroxy- eicosa tetra enoic acids. HPETEs are not cyclic compounds; their structure consists of the parent fatty acid with a hydro-peroxy substituent. The position of this group is designated by a numerical prefix, indicating the carbon position of the substitution. The first enzyme was originally found in lung and platelets [Nugteren,1977], and is responsible for the formation of lipid peroxides, such as 12-hydroxy- eicosatetraenoic acid (12-HETE). In figure 8 the 11-,12-, and the 15-lipoxygenase pathways are given. In fig.6 it was shown that besides the di-HETE which is LTB_4, other di-hydroxy- eicosa tetra enoic

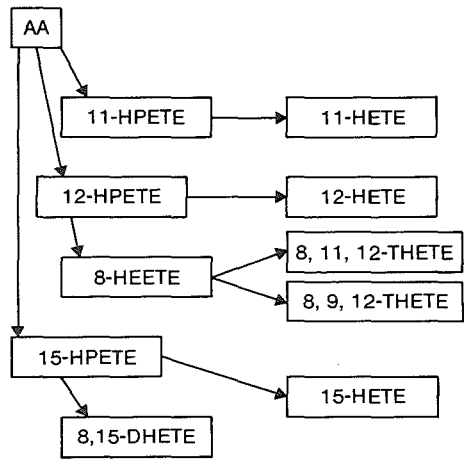


Fig.8 Formation of mono- and di-hydroxy tetraenoic acids and tri-hydroxy trienoic acids via other lipoxygenases

acids were formed. A degradation product of 15-HPETE is also a di-HETE, the 8,15-DHETE. Via the 12-HPETE route a hydroxy epoxy eicosa tri enoic acid is formed, from which two tri-HETEs proceed.

The biological effects of (mono)HETEs on the vascular system appear not to be of significant importance. 5-HETE produces slow contractions of isolated human bronchial smooth muscle, with a potency comparable to that of histamine. 15-HETE is less potent than histamine, whereas 12-HETE is inactive. 5-HPETE and 5-HETE may be required for the release of histamine from basophils, and they promote the secretion of histamine and other substances from mast cells [Leitch,1984]. The effects of these substances on gastrointestinal smooth muscle have not been well studied.

Chemotactic activities of HETEs have been established, but proved to be less active than LTB_4. Cunningham *et al* have suggested that mono hydroxy derivatives of AA may contribute to the inflammatory process, observed in psoriasis. The AA-induced aggregation of platelets and the secretion of cyclooxygenase products was inhibited by 12-HPETE [Aharony *et al*.,1982]. 15-HETE proved to be a potent inhibitor of prostacyclin formation in human arterial segments [Yamaja and Stuart,1986] and also stimulates the LTB_4 and 5-HETE formation in mast cells [Vanderhoek *et al*.,1982]. The 12-lipoxygenase pathway is possibly inhibited by 15-HETE [Fletcher-Cieutat *et al*.,1985]. It has been suggested that HETEs, like the LTs C_4 and D_4, increase the mucus secretion and vasopermeability of the lungs [Johnson *et al*,1985].

2.5 EICOSANOIDS AND THE LUNG

Three criteria must be followed in order to prove that eicosanoids are indeed involved in allergic and other inflammatory respiratory diseases. Firstly it must be shown that eicosanoids are generated in disease or during an asthma attack; secondly the administration of synthetic eicosanoids must cause symptoms which are comparable to those of the disease under study and finally intervention with selective inhibitors of the biosynthesis of eicosanoids must have beneficial effects.

In vivo formation. Three animal models have been developed in which an anaphylactic response can be associated with the production of leukotrienes, i.e. guinea pigs [Ritchie *et al.*,1981], greyhound dogs [Hirshman *et al.*,1982] and sheep [Abraham, 1987]. After anaphylactic reactions, human lung tissue generates large amounts of leukotrienes [Drazen and Austen,1987]. Plasma levels of eicosanoids are enhanced during an asthma attack [Keppler *et al.*, 1985]

Intravenous administration of LTC_4, LTD_4 and LTE_4 causes bronchoconstriction in unanaesthesized guinea pigs [Drazen *et al.*,1980]. Aerosol administration of LTC_4 and LTD_4 to normal man results in dose-dependent bronchoconstriction [Weiss *et al.*,1982], with the effect persisting for at least 30 min. These LTs are active as bronchoconstrictors at concentrations 600-9500 times lower than histamine.

Intervention. The therapeutic implications for asthma are considerable, and competitive inhibitors or a specific 5-lipoxygenase inhibitor, suitable for administration in man, could be major additions to the pharmacotherapy of this disease. FPL 55712, a relatively specific

antagonist of SRS-A (LTs C_4 and D_4) inhibits the contractile effects of SRS-A in guinea-pig [Drazen *et al.*,1979] and human lungs [Ghelani *et al.*,1980]. At present there have been no reports of the use of a leukotriene receptor antagonist useful in man.

The SRS-A induced bronchoconstriction in guinea pigs differs from that seen after infusion of histamine, bradykinin or $PGF_{2\alpha}$ in that SRS-A has a preferential effect on the pulmonary compliance, and a prominant action in the pulmonary periphery, with a prolonged duration of action.

It has been shown that injection of SRS-A into the pulmonary artery of isolated perfused guinea pig lungs results in the release of prostaglandins and thromboxane [Engineer *et al.*,1978]. These observations suggest that some of the contractile effects of SRS-A in these species may be due to bronchoconstrictor cyclo-oxygenase products synthesized in response to SRS-A.

The *in vitro* effects on the contractile response of superfused guinea pig lung strips of LTC_4, LTD_4 and LTE_4 are substantially inhibited by indomethacin [Piper and Samhoun,1981]. The enhanced plasma concentrations of thromboxane seen in guinea pigs after leukotriene infusion [Omini *et al.*,1981], may be generated by the lung or platelets.

LTC_4 and LTD_4 have been shown to increase the release of mucus from human airways *in vitro* [Maron *et al.*,1982] and to induce a dose-related increase of glucoprotein secretion by human bronchial mucosa [Coles *et al.*,1983]. The presence of SRS-A or leukotrienes in the sputum of patients with chronic bronchitis and emphysema may also indicate a pathogenic role for these substances [Hirschman *et al.*,1982]. Conversion into the 11-trans-leukotriene occurs spontaneously with 100-fold loss of biological activity [Lewis *et al.*,1980].

Implications in the pathogenesis of allergic diseases. Since sulphidopeptide leukotrienes can be released from human mast cells and alveolar macrophages, these products are likely to be implicated in the pathogenesis of asthma, where their properties as bronchoconstrictors, secretagogues and permeability-enhancing agents can make a major contribution to the known clinical manifestations of this disease. Chemotaxis of inflammatory cells by LTB_4 may contribute to the late phase response to antigen challenge in man.

In Chapter 3 the biological activities of LTs on lung parenchymal strips and the effects by antagonists and synthesis inhibitors are presented in detail. In Chapter 4 the formation of eicosanoids by chopped lung tissue and macrophages are studied; in the last paragraph the effects of a substance, found to inhibit specifically the 5-lipoxygenase, are summarized.

2.6 EICOSANOIDS AND THE LIVER

The investigations on chemical structure, metabolism and biological actions of the leukotrienes have contributed to a better understanding of some liver diseases and therapeutic interventions. The liver plays an important role in the metabolism of eicosanoids.

In the group of prostaglandins PGI_2 is converted by the lung in the biologically inactive substance 6-keto-$PGF_{1\alpha}$, which in turn is metabolized to 6-keto-PGE_1. This substance is a potent vasodilator and inhibitor of platelet aggregation [Wong *et al.*, 1981; Lewis *et al.*,1984]. PGD_2 is converted stereospecifically via $PGF_{2\alpha}$ to the compound $9\alpha,11\beta$-PGF_2 by a 11-ketoreductase [Liston and Roberts,1985]. This substance is also a inhibitor of platelet aggregation, and in contrast with 6-keto-PGE_1 a potent vasoconstrictor.

A possible role of the liver in the degradation of biologically active leukotrienes was suggested by studies on its metabolism after intravenous administration. It was found that LTs were taken up rapidly by hepatocytes and that sulphidopeptide LTs were excreted in the bile [Uehara *et al.*,1983; Hagmann *et al.*,1984]. Renal failure is associated with insufficient degradation of cysteinyl containing LTs by the liver.

Liver cells can also generate eicosanoids. The most important producer cells of leukotrienes are macrophages, monocytes, neutrophils, eosinophils and mast cells. Recently also Kupffer cells were shown to release LT-material upon stimulation [Decker,1985; Ouwendijk,1985]. Hepatocytes, the main cell type in the liver, can generate thromboxane when stimulated with vasopressin [Chong *et al.*,1983]. Hepatocytes and Kuppfer cells mainly generate PGD_2 and $PGF_{2\alpha}$ [Kuiper *et al.*,1987].

The major action of LTB_4 after administration *in vivo* is to recruit

leukocytes. This chemotactic property is responsible for the effect of LTB_4 as an important mediator of inflammatory cell infiltration. The immunoregulatory effects of LTB_4 include inhibition of the proliferation of T-lymphocytes [Keppler *et al.*, 1985] , but also stimulation of the activity of natural cytotoxic cells [Rola-Pleszczynski *et al.*,1983]

Endotoxin is a potent trigger for cysteinyl containing LT production *in vivo* [Hagmann *et al.*,1984]. It is an example of a hepatotoxin which initially interacts with Kupffer cells and other mononuclear phagocytes and subsequently leads to inflammatory cell infiltration and hepatocellular necrosis.

Drugs interfering with LT synthesis or action prevent or reduce the increase in 5-lipoxygenase activity in plasma, resulting from hepatocyte injury. Protective action was exerted by diethylcarbamazine which inhibits LTA_4 synthesis and also by FPL 55712 which is a receptor antagonist for cysteinyl containing LTs [Bach,1984]. Protection was also achieved by pretreatment with BW 755C, which is both a lipoxygenase and a cyclooxygenase inhibitor [Higgs *et al.*,1984]. BW 755C reduced leukocyte migration and edema provoked with carrageenin, whereas low-dose indomethacin did not prevent leukocyte migration, indicating that LTs were responsible. Dexamethasone, which antagonizes arachidonate liberation by the synthesis and release of the anti-phospholipase protein, lipocortin, had the same effect as BW 755C [DiRosa *et al.*,1984]. Flavonoids, some of which are used in clinical and experimental hepatoprotection, are potent inhibitors of the 5-lipoxygenase pathway [Yoshimoto *et al.*,1983].

Inhibitors of leukotriene synthesis or action, protect against lethal endotoxin shock in experimental models [Hagmann *et al.*,1984]. The degradation and elimination of the LTs C_4, D_4 and E_4 seems to be important after severe trauma and tissue injury and in pancreatitis [Keppler *et al.*,1985].

The kidney serves as an alternative organ for elimination of LTs in man, as well as a target organ for these LTs causing renal vasoconstriction and decreased glomerular filtration rate [Badr *et al.*,1984]. Inhibition of PG synthesis resulted in a hepatorenal syndrome in cirrhotic patients with ascites. Selective thromboxane synthetase inhibitors did not improve renal functions in these patients, suggesting that LTs may be the predominant vasoconstrictors in this syndrome [Zipser *et al.*,1984].

Stimulated peritoneal macrophages derived from ascites of experimental animals and patients with ascites due to liver cirrhosis, chronic ambulatory peritoneal dialysis and alveolar lavages can produce leukotrienes such as LTB_4, LTC_4 and LTD_4 and prostanoids 6-keto-$F_{1\alpha}$, E_2 $F_{2\alpha}$ and TxB_2 [Feuerstein *et al.*,1981; Adolfs *et al.*,1985; Ouwendijk,1985; Schenkelaars,1985; Steinhauer *et al.*,1985; Laviolette *et al.*,1986].

2.7 EPILOGUE

In fig.9 the structural formulas of some of the eicosanoids, of which the biological effects, the synthesis by cells and amounts in body fluids were determined, are given.

Besides the structural differences, these stable eicosanoids have varying biological activities and are synthesized in different amounts in different organisms or following different stimuli.

The balance of these substances and their vaso- and bronchoconstrictive actions (TxA_2 / TxB_2, PGD_2 and LTC_4), vaso- and bronchodilative actions (PGI_2 / $6kPGF_{1\alpha}$) and chemotactive activities (LTB_4 and 15-HETE), and also the interactions between several eicosanoids, could be of importance in the regulation of the diseases described above.

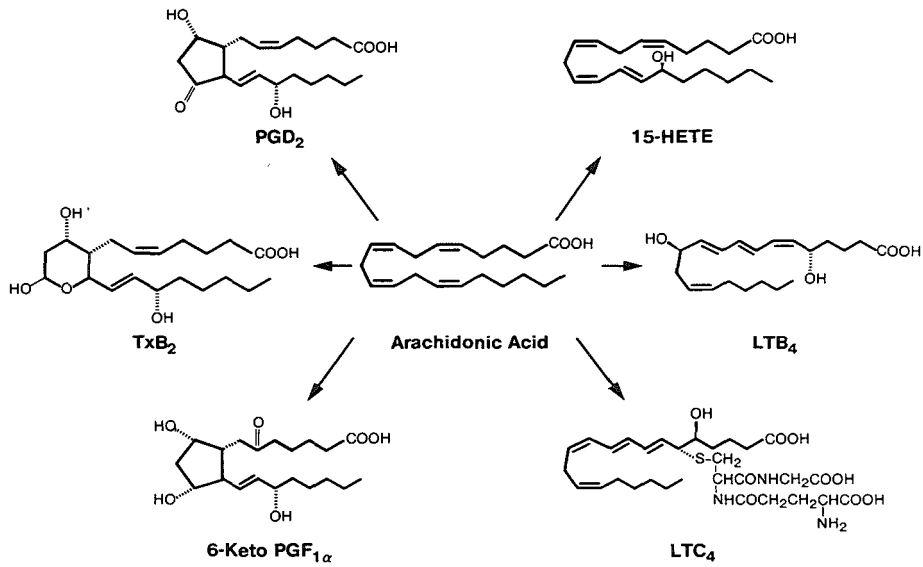
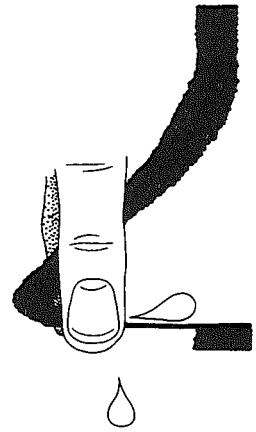


Fig.9 Structural formulas of the most occurring eicosanoids, discussed in the next part of this thesis.



CHAPTER 3

ACTIONS ON LUNG PARENCHYMA

3.1 INTRODUCTION

The actions of leukotrienes have been studied on human, porcine and guinea pig lung parenchymal tissues. Both the direct effect of the LT and the secondary effect via synthesis of biologically active substances were investigated.

The effects obtained were compaired with the actions of thromboxane A_2, generated from aggregated platelets and a thromboxane A_2-mimetic. The contractions induced by phospholipase A_2 were compared with those obtained after application of LTC_4 or LTD_4.

The influence of a β-adrenoceptor agonist and a β-adrenoceptor antagonist on the contractile activities and release reaction induced by LTs were studied.

For the assessment of the effect of leukotrienes on tissue a superfusion cascade was used. Eicosanoid production was measured by radioimmunoassay.

3.1.1 *Superfusion method*

In an anesthesized ventilated guinea pig the ***in vivo*** effects of substances on bronchial smooth muscle tissue can be investigated according to the method of Konzett-Rössler. This method is fast, but complicated and poorly reproducible.

A very sensitive method is the *in vitro* superfusion cascade, in which lung parenchymal strips are used. This bioassay is suitable both to examine the contractile effects induced by unknown substances and to determine the concentrations of biologically active substances in samples.

The guinea pig lung parenchymal tissue is very sensitive to leukotrienes in comparison to other smooth muscle contracting agents. In the experiments described the contraction inducing substances were added to the system directly, whereas antagonists and synthesis inhibitors were added to the superfusion buffer and preincubated before application of the constrictive agent under study.

Guinea pigs (male and female, 400-600 g) were killed by a blow on the head and exsanguinated. Lungs, the heart and trachea were taken out and washed in Krebs Henseleit buffer. Piglets (male and female, 15-20 kg) were anaesthesized with low-dose nembutal and exsanguinated; outer parts of the lungs were used for experiments. Human lung parenchyma was obtained from lobectomy because of tumours. Strips of about 40x3x3 mm were prepared.

In figure 10 the basic set-up of the superfusion cascade system is presented. In the upper baths lung parenchymal strips are mounted ("donor"-strip). The release of vasoconstrictive substances from lung parenchymal strips by leukotrienes and other agents was measured on rabbit mesenteric artery ("measurement"-strip), which was mounted in a bath underneath. This artery is highly sensitive to thromboxane A_2 [Zijlstra and Vincent,1981].

The contractile activity was standardized before and after the experiment with angiotensin II and/or the thromboxane A_2 mimetic U-44069.

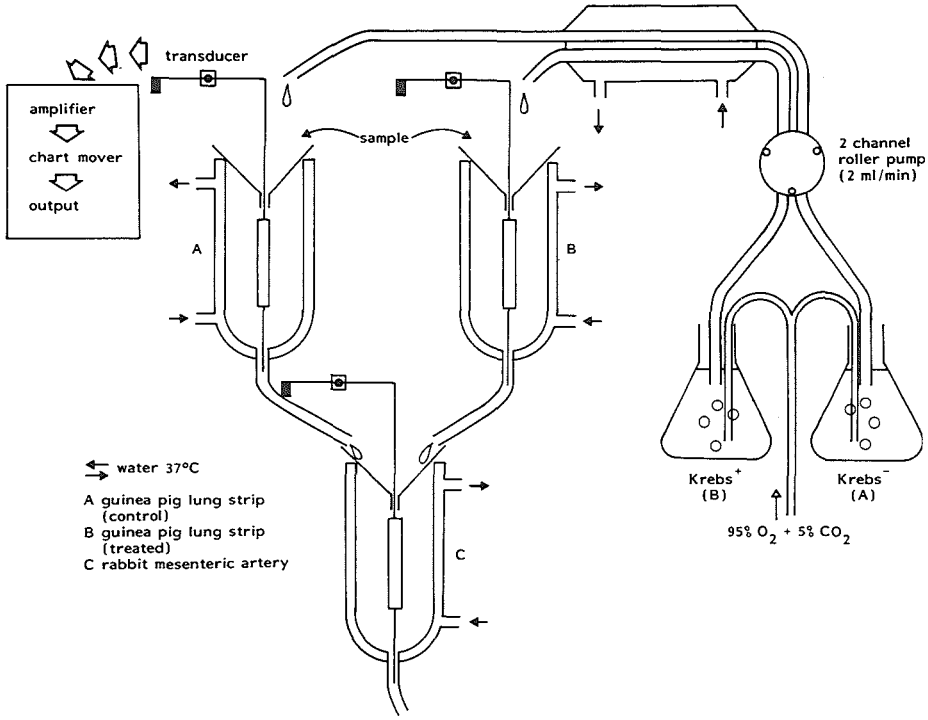


Fig.10. Basic set-up of a two-channel superfusion cascade system.

Contractions were registered isotonically using a Harvard smooth muscle transducer and employing an isotonic load of 0.5 g which stretched the preparations to their optimal length as found in preliminary experiments.

The strip was superfused with Krebs-Henseleit buffer containing NaCl 118mM, KCl 4.7mM, $CaCl_2$ 2.5mM, $MgSO_4$ 1.18mM, KH_2PO_4 1.18mM, $NaHCO_3$ 25mM nd glucose 5.6mM. The perfusion rate was 1.6 ml/min at 37°C. The superfusion fluid of the cascade system was oxygenated with 95% O_2 and 5% CO_2. Initially no antagonists were added. Constriction inducing substances (LTs, TxA_2 or PLA_2) were added and the resulting contraction and release of constricting agents were measured. After contraction was abolished and base-line noise achieved, antagonists or synthesis

inhibitors were added to the superfusion system. The reduction in contraction and release of constricting agents were registered after application of the same concentration of constriction inducing substance. Data were expressed as percentages of the first application.

3.1.2 *Radio immuno assay*

Radioimmunoassays of eicosanoids have been especially designed for research purposes. They combine the use of a highly specific tracer of eicosanoid, an antiserum which is specific for the eicosanoid under study and a standard.

This provides a rapid, simple and sensitive method for the determination of several eicosanoids ***in vitro*** over a range of 0 -1000 pg/tube.

The basic principle of this radioimmunoassay is competitive protein binding between unlabelled eicosanoid and a fixed quantity of the tritiated compound for a fixed number of antibody binding sites. The amount of radioactive antigen bound by the antibody, is inversely proportional to the concentration of added non-radioactive ligand. The measurement of antibody-bound radioactivity enables the amount of unlabelled eicosanoids in the sample to be determined. Separation of the antibody-antigen complexes from free antigen is achieved by adsorption of the free tracer onto dextran-coated charcoal, followed by centrifugation. The supernatant containing the antigen-antibody complexes is decanted into a counting vial and a scintillation cocktail added. The radioactivity is measured in a beta counter. The concentration is then calculated from a standard (dose-response) curve.

Samples collected after the addition of leukotrienes or other lung parenchyma constricting substances were used for the determination of stable thromboxane B_2. Kits ready for use were obtained from New England

Nuclear (Boston). Antibody was purchased from l'Institut Pasteur (Paris, France) and ^{3}H-TxB_2 from Amersham. TxB_2 was a gift of Dr Pike (Upjohn, Kalamazoo, USA). Cross reactivities for other cyclooxygenase products were all less than 0.2% at 50% displacement. Cross reactivities for lipoxygenase products were not determined.

3.2.1

THE EFFECTS OF LEUKOTRIENES, THROMBOXANE A_2 AND PHOSPHOLIPASE A_2 ON HUMAN, PORCINE AND GUINEA PIG LUNG PARENCHYMA

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The effects of leukotrienes, thromboxane A_2 and phospholipase A_2 on human, porcine and guinea pig lung parenchyma

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Abstract

A comparison was made of the contractions, induced by LTD_4, histamine and phospholipase A_2 in parenchymal strips of guinea pig (GPLP), porcine and human lung in a cascade superfusion system. The effects of LTD_4 and phospholipase A_2 on the release of TxA_2 in these tissues and of TxA_2, 5-HT and acetylcholine on the GPLP were also determined.

In the GPLP strip, the LTC_4-induced contractions are due for ± 80% to the release of TxA_2 and for ± 20% to the direct effect of LTC_4.

The guinea pig tissue displayed the highest sentivity towards all substances, except to the contraction induced by histamine, which was most effective in the porcine tissue. Low activities were found in the human tissue in all tests. The reason for these effects may be a difference in activities or number of cell types which participate in the reactions leading to the contractions.

Introduction

It is well known that the biologically active leukotrienes[2] induce slow contractions in smooth muscles. The effects of the LT on peripheral and central airway functions of pulmonary tissue have also been described [1–3]. Recently, PIPER and SAMHOUN [4] reported that LTC_4 and LTD_4 induced TxA_2 release from guinea pig isolated perfused lungs. In our experiments this finding was confirmed and extended. It was demonstrated that after repeated application of LTC_4 a considerable tachyphylaxic occurs [5].

In previous work we reported that isoprenaline reverses the LTC_4-induced contraction of the GPLP and inhibits the TxA_2 release. These effects were inhibited by the β-adrenoceptor antagonist sotalol [6]. Isoprenaline has clinical importance in anaphylactic pulmonary conditions. In this study a comparison is made between the contractile activities and TxA_2 release of human, porcine and guinea pig lung parenchymal strips after the application of LTC_4, LTD_4, histamine and PLA_2.

Methods

Collection of tissue samples

Male and female guinea pigs of 400–600 g (CPB-TNO, Zeist, The Netherlands) were killed by a blow on the head and exsanguinated. The lungs were removed and kept in Krebs' buffer until use.

Male and female pigs of 20–25 kg (8–10 weeks, MDF raised piglets, random bred, CDI Lelystad, The Netherlands) were anaesthesized with nembutal (30 mg/kg) and exsanguinated.

Human lung parenchymal strips were obtained from three infants and three adults.

Infants: All were born with an affection of the heart and died of a ventricle septum defect. Medication consisted of furosemide (Lasix®) and digoxin.

Child 1, 3.5 years, girl. Child 2, 2 months, boy. Child 3, 3 months, girl.

Adults: Lobectomy was performed on account of tumours. The premedication consisted of 0.25 mg atropine and 10 mg Opial®. During the operation the following agents were administered; Fentanyl® (0.5 mg), pancuronium-bromide (Pavulon®, 6 mg) and thiopental (Pentotal®, 150 mg) and 3 g cephalothin (Keflin®).

Patient 1, man of 72 years; medication: euphyline. Patient 2, man of 67 years, diabetes mellitus; 36 U insulin/d. Patient 3, woman of 49 years; no medication.

Bioassay of lung parenchyma

Strips were prepared from the largest lobes of guinea pig lungs (40 × 3 × 3 mm, approx. 300 mg) and from the outer parts of human and porcine lungs. In a superfusion cascade system the contractions of a strip of lung parenchyma were measured. A rabbit mesenteric artery placed under the lung strip served to bioassay the thromboxane A_2 release [7] (Fig. 1). The strips were superfused

[1] To whom correspondence should be sent.

[2] The following abbreviations are used: LT = leukotriene, TxA_2 = thromboxane A_2, TxB_2 = thromboxane B_2, PLA_2 = phospholipase A_2, GPLP = guinea pig lung parenchyma, SRS-A = Slow Reacting Substance-A.

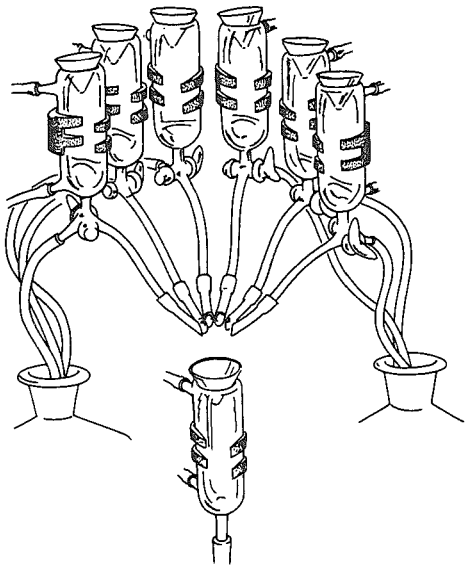

Figure 1
Superfusion cascade to determine the contractile effects of leukotrienes and other substances on the lung parenchymal strips and the release of TxA_2 by means of a rabbit mesenteric artery (RbMA). Each bath is superfused with its own buffer; a three-way cock is used to superfuse the underplaced RbMA or discard the superfusion fluid.

with oxygenated Krebs' buffer without antagonists (1.6 ml/min at 37°C). Contractions were measured isotonically with a Harvard smooth muscle transducer.

Biosynthesis of TxA_2

TxA_2 was generated by rat blood platelets in which aggregation was induced by collagen. Hepinarized blood was taken by heart puncture, centrifuged and the supernatant platelet-rich plasma (PRP) was collected. 200 μl PRP was preincubated at 37°C under stirring in a Payton aggregometer. 10 μg arachidonic acid and 8 μg collagen suspension were added. The sample was collected when 50% aggregation occurs. A sample of 100 μl was applied to the cascade after 30 seconds. This sample was collected and the amount of total (stable) TxB_2 determined by radioimmunoassay.

Materials

All reagents used were of analytical grade (Merck Darmstadt). Leukotrienes C_4 and D_4 were gifts of Dr J. Rokach (Merck Frosst Labs, Pointe Claire, Canada) and FPL 55712 of Mr Sheard (Fisons, Loughborough, UK). Phospholipase A_2 (from hog pancreas) was obtained from Boehringer Mannheim, histamine from Brocacef (The Netherlands), serotonin from Merck and acetylcholine from O.P.G., Utrecht, The Netherlands. Arachidonic acid and collagen (insoluble, from achilles tendon) were purchased from Sigma Co. Radioimmunoassay of stable TxB_2 was carried out with kits of New England Nuclear, Boston.

Results

In our experiments the contractile activities of LTC_4 and LTD_4 were of the same magnitude. In agreement with other observations, we found that the LT were 70–100 times more potent than histamine on the GPLP strip, based on the molar weight (Fig. 2). TxA_2, generated by aggregating rat platelets, was slightly less potent than LTD_4. Figure 3 shows the effect of indomethacin on LTC_4-induced contraction.

Cumulative doses of LTC_4 were added to the GPLP. Thereafter, indomethacin was superfused (30 min, 3 μM) and again 50 ng LTC_4 was added. The contraction is diminished by 45%, which is caused by the total inhibition of TxA_2 release. However, it appears that after indomethacin, the dose of 50 ng LTC_4 causes a contraction equieffective to that of 4–6 ng LTC_4 before indomethacin treatment (an inhibition of approx. 80%). The part of the contraction which is still present after indomethacin is due to the effect of LTC_4 itself. This can be suppressed by the addition of the SRS-A antagonist FPL 55712 in a dose of 10 μg/ml.

Figure 4 gives a comparison of the effects of LTD_4 and histamine added to LP strips of different species on both the contraction and the LTD_4-induced release of TxA_2. 50 ng had no effect on the human LP strip, whereas that on the porcine LP strip was small. This correlates with the TxA_2 release. The effect of histamine was different. The porcine LP strip was more sensitive to histamine than the GPLP strip, whereas the human LP strip was almost insensitive to high doses of histamine.

In other experiments, the effect of TxA_2 either applied directly to or released from these tissues was determined.

In Fig. 5 tracings of porcine and human LP strip are shown after the addition of LTD_4, TxA_2 and PLA_2. The effect of LTD_4 is (again) very small. Exogenous TxA_2 (expressed as ng stable TxB_2) contracts the porcine LP strip but had less effect on the human LP strip even in a four times higher dose.

In Table 1 a summary is given of the TxA_2 release, measured by radioimmunoassay, carried out in experiments comparable to those described in Figs 4 and 5. It is clear from the table that irrespective of the use of LTD_4 or PLA_2,

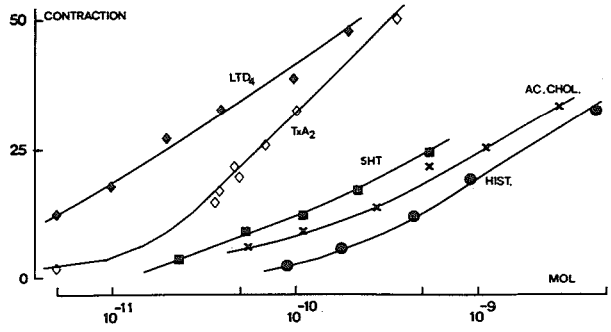

Figure 2
Dose-response curves of LTD_4, TxA_2, 5-hydroxytryptamine (5-HT) acetylcholine (AC.CHOL.) and histamine (HIST.) on superfused guinea pig lung parenchyma. The contractions are expressed as mg force.

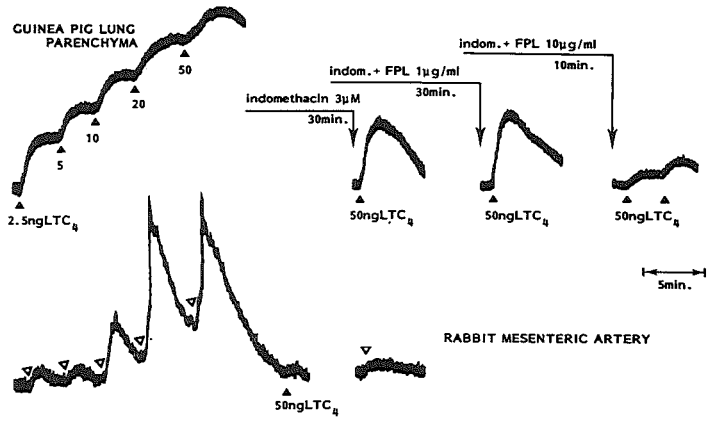

Figure 3
Recordings of the contractile activity of LTC_4 (50 ng) on the GPLP strip and of the TxA_2 release measured by means of an underplaced rabbit mesenteric artery. After the cumulative additions of 2.5, 5, 10, 20 and 50 ng LTC_4, indomethacin was superfused (final conc. 3 μM), and again 50 ng LTC_4 was added. Thereafter, FPL 55712 (1 and 10 μg/ml) was added followed by 50 ng LTC_4. A representative experiment is shown ($n = 4$).

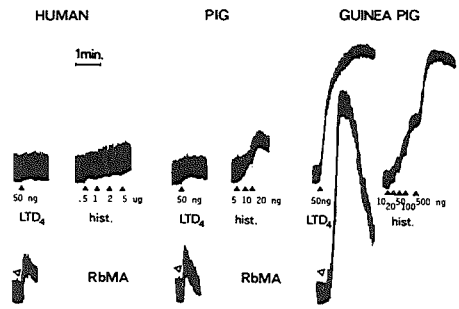

Figure 4
The effects of LTD_4 on the contraction of human (child 3 years), porcine and guinea pig LP strip, compared to histamine. The LTD_4-induced release of TxA_2 was determined by means of an underplaced rabbit mesenteric artery (RbMA).

both the human and porcine LP strip have a much smaller capacity to release TxA_2 than does the lung tissue of guinea pigs.

Discussion

The results of the experiments presented here indicate that TxA_2 released by the LT induces approximately 80% of the contraction of the GPLP strip. The remaining 20% should be considered as a direct effect of the LT. This confirms earlier data on the role of TxA_2 in the LT-induced contraction [4]. It has also been shown that SRS-A, of which LTC_4 and LTD_4 are constituents, releases TxA_2 from the lungs [8].

When exogenous TxA_2 is added to the lung tissue contraction also occurs. Considerable amounts of TxA_2 are formed when PLA_2 is added

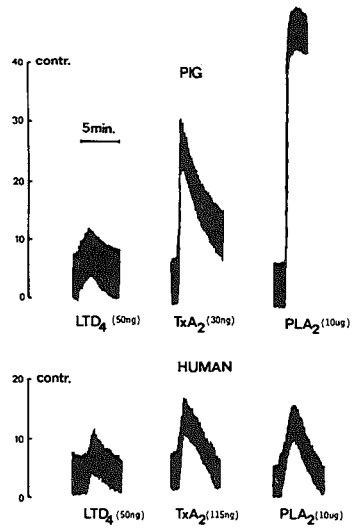

Figure 5
Contractile activities of LTD_4, TxA_2 and PLA_2 on human (patient No. 3) and porcine LP strips. Contractions are expressed as mg force.

to the lung strip. These effects are, to a certain extent, similar to those of the LT. This may indicate that the LT activate a PLA_2. It has been shown that mepacrine, which inhibits PLA_2, also inhibits the effects of the LT on lung tissue [9]. It is unlikely that the addition of LT to the tissue inhibits the formation of endogenous LT and in this way changes the sensitivity. We found that after labelling of the strips with 1-^{14}C-arachidonic acid, no effect was observed of the addition of LT on the formation of lipoxygenase products. The formation of TxB_2 was increased under these circumstances (unpublished experiments).

The effects of LTD_4, TxA_2, histamine and PLA_2 on the different tissues are summarized as follows:

Contraction to LTD_4	GPLP $\gg$ porcine LP $\geqslant$ human LP
Release of TxA_2 after LTD_4	GPLP $\gg$ porcine LP $\geqslant$ human LP
Contraction to TxA_2	GPLP $\gg$ porcine LP > human LP
Release of TxA_2 after PLA_2	GPLP $\gg$ porcine LP > human LP
Contraction to histamine	porcine LP > GPLP $\gg$ human LP

The differences may be related to a number of factors, such as the amounts or the activities of

Table 1

Species	TxA_2 production (ng TxB_2)	
	LTD_4 (50 ng)	PLA_2 (100 μg)
Human	0.7 ± 0.16 (6)	4 ± 1.0 (6)
Porcine	0.5 ± 0.14 (6)	11 ± 0.6 (6)
Guinea pig	21 ± 2.1 (14)	151 ± 9.5 (5)

certain types of cells present in these tissues. At present it is not known in which type of cells TxA_2 is formed.

The results presented here indicate that the LT have only a small contractile activity on human lung tissue compared to that of the guinea pig. This raises the question of the importance of the guinea pig lung as a model for the investigation of human anaphylactic conditions. It has been shown that SRS-A is formed in human lung cells after immunological challenge [10]. This would indicate that this substance plays a role in human respiratory disease.

The effect of LTD_4 is of the order of 100 times that of histamine on the human lung. SIROIS et al. [11] found that human LP strips were about as sensitive to LT as the guinea pig tissue. HEDQVIST [12] reported that the human lung is 1000 times more sensitive to LT than to histamine. Whether the presence of tumours has an effect on the sensitivity of these tissues is unknown.

It is not very likely that anaesthesia or previous medication has an effect on the response of the tissues, although this cannot be completely ruled out. We found that the contractions of lung strips from guinea pigs treated *in vivo* with nembutal were not different from the controls. As the strips are superfused for a long time before the measurements are made, the substances used in the medications or their metabolites are most probably removed from the tissues.

In summary, the results presented here indicate that considerable differences occur in the

sensitivity of lung tissue of different origin towards the LT and histamine.

Acknowledgments

Leukotrienes C_4 and D_4 were obtained through the courtesy of Dr J. Rokach (Merck Frosst Labs, Canada). Pig lungs were obtained from Dr L.P. Jager (CDI, Lelystad, The Netherlands). Parts of human lungs were obtained from Dr P.A. Frederiksz (Thorax Centre) and Dr C.E. Essed (Dept. of Pathology). We wish to thank Dr R.J.T. Ouwendijk (Dept. of Internal Medicine) for help and discussions.

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3.2.2

SEPARATION OF THE TWO COMPONENTS OF THE CONTRACTILE ACTIVITY OF LEUKOTRIENE C_4 ON THE GUINEA PIG LUNG PARENCHYMAL STRIP

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PROSTAGLANDINS LEUKOTRIENES AND MEDICINE

Prostaglandins Leukotrienes and Medicine 11: 385-389, 1983

SEPARATION OF THE TWO COMPONENTS OF THE CONTRACTILE ACTIVITY OF LEUKOTRIENE C_4 ON THE GUINEA PIG LUNG PARENCHYMAL STRIP

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ABSTRACT

The contractions, induced by LTC_4 in the guinea pig lung parenchymal strip are composed of two parts, one of which is due to the effect of the released TxA_2 and the other one to the direct action of LTC_4. After the addition of indomethacin, the TxA_2 release is totally inhibited, but the contractions only partly. It is shown that the release of TxA_2 only occurs during the initial period of the contractions. This indicates that the direct action of LTC_4 represents the slow acting component in the contraction.

INTRODUCTION

The formation of a substance in immediate type hypersensitivity reactions which contracts smooth muscle more slowly than histamine was described more than 40 years ago. This compound was called Slow Reacting Substance of Anaphylaxis (SRS-A). The action of this compound can not be blocked by a H_1 antagonist (1). More recently, it was shown that SRS-A contains a mixture of leukotrienes (LT). In SRS-A of the rat, LTC_4, LTD_4 and LTE_4 are present (2). LTC_4 and LTD_4 induce long lasting contractions in the guinea pig lung parenchymal (GPLP) strip. During the contraction, thromboxane A_2 (TxA_2) is released (3). In the contraction induced by LTB_4, the same effect occurs (4).

The action of LTC_4 and LTD_4 consists of two parts, one of which represents the effect of the released TxA_2 and the other one the direct action of the LT. In the experiments described here, the contribution of these two components to both the rapid onset and the prolonged action of the LTC_4 induced contraction in the GPLP strip were investigated.

METHODS

The contractions of the GPLP strip were measured in a superfused organ cascade system. The preparation of the lung strip has been described before (5). Under the lung strip, a rabbit mesenteric artery (RbMA) is placed, which is used in the bioassay of TxA_2 (6). The strips were superfused with oxygenated Krebs buffer without antagonists (1.6 ml/min at 37°C). The buffer solution had the following ionic composition: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.18, KH_2PO_4 1.18, $NaHCO_3$ 25 and glucose 5.6 mM. The contractions were measured isotonically with a Harvard smooth muscle transducer.
The effluent of the GPLP strip was collected and the amount of TxB_2, the metabolite of TxA_2 measured by radioimmunoassay using a rabbit anti-TxB_2 serum (obtained from NEN).

RESULTS AND DISCUSSION

A dose-response curve for the effect of LTC_4 on the GPLP strip was determined. Such curves were routinely contructed at the beginning of every experiment. An example is shown in Fig. 1. A small decrease in contraction corresponds to a much larger decrease in the dose. When e.g. a maximal contraction is reached after 100 ng LTC_4, a contraction of 60% is obtained after 10 ng LTC_4. When indomethacin (3μM) is added to the superfusion fluid, the contraction induced by LTC_4 is partly, and the TxA_2 release, as measured by the RbMA, totally inhibited (Fig. 2). When TxB_2 is determined by RIA, the same result is obtained. Samples of the superfusion fluid were collected from the baseline to the top of the peak. The release of TxA_2, measured as TxB_2 was only 3.5 ± 0.8% , compared to the first addition of LTC_4 before indomethacin was added (n=4). Under these circumstances, the two components of the contractile activity of LTC_4 are separated. LTC_4 has no effect on the RbMA. After the inhibition of the TxA_2 release by indomethacin, the contraction is diminished by ± 20%, which corresponds to a decrease of 70% in the equieffective dose of LTC_4, as shown in Fig. 1. This indicates that TxA_2 is the main component in the induction of the contraction. The time required by the superfusion fluid to reach the lower placed tissue accounts for the lag period between the two curves. After a few minutes, the TxA_2 release is finished, whereas the tissue continues to contract (Fig. 3). The same results are obtained when the amount of TxB_2 is determined at regular time intervals in the effluent of the GPLP strip. At the end of a three minute period, only a small amount of TxB_2 is still present in the effluent (Fig. 4).
These results indicate, that in the action of LTC_4 on the GPLP strip, the slow reacting component is due to the direct effect of LTC_4. The TxA_2 release plays a role in the onset of the contraction.

It has not yet been investigated, whether the contractions, induced by LTC_4 in other smooth muscles also consist of two different components.

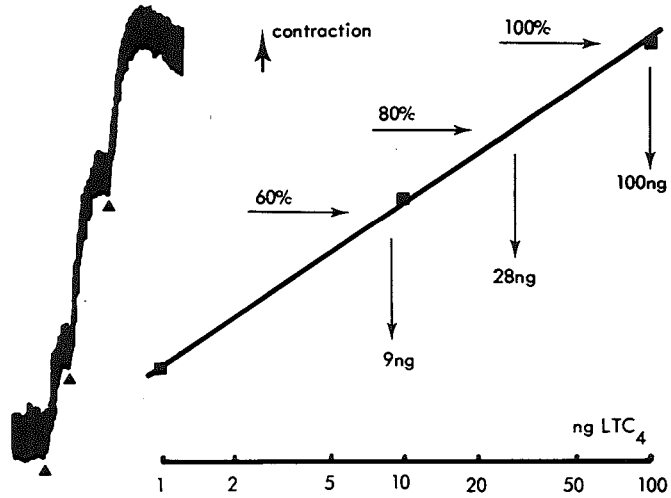

Figure 1. Log dose-response curve of LTC_4 on the GPLP strip.

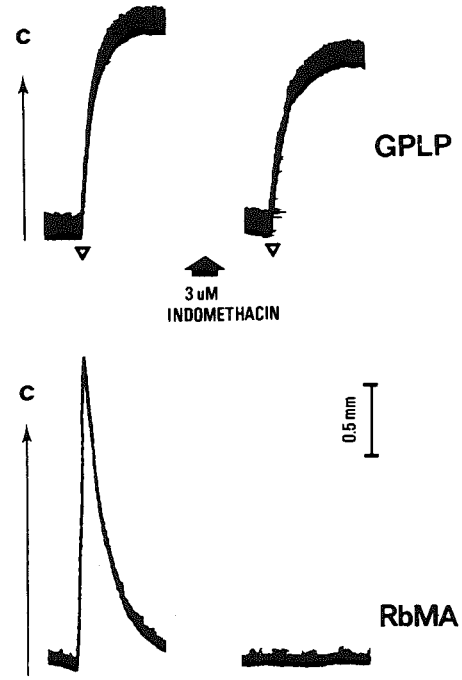

Figure 2. Recordings of the contractile activity of 50 ng LTC_4 on the GPLP strip and the release of TxA_2-measured on a RbMA. After the first addition of LTC_4, indomethacin was superfused. (final conc. 3 μM).

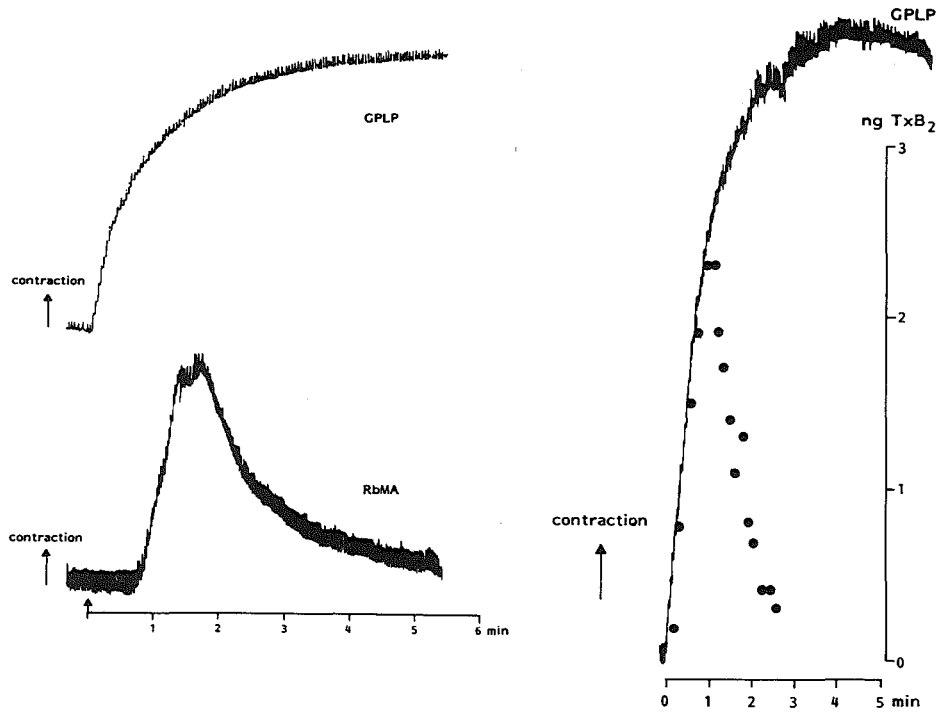

Figure 3. The effect of 50 ng LTC_4 on the GPLP strip and that of the released TxA_2 on an underplaced RbMA. The lag time of the superfusion fluid between the two tissues is approx. 30 seconds.

Figure 4. The formation of TxA_2, measured as TxB_2, during the contraction induced by 50 ng LTC_4 in the GPLP strip. Fractions of 10 seconds were collected and the TxB_2 content determined.

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Dr. J. Rokach, Merck Frosst Lab, Pointe Claire/Dorval, Quebec, Canada is thanked for a gift of LTC_4.

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3.2.3

TACHYPHYLAXIS OF LEUKOTRIENE C_4-INDUCED RELEASE OF THROMBOXANE A_2 FROM THE GUINEA PIG LUNG PARENCHYMA AND ISOPROTERENOL INHIBITION OF THIS RELEASE

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Tachyphylaxis of Leukotriene C_4-Induced Release of Thromboxane A_2 from Guinea Pig Lung Parenchyma and Isoproterenol Inhibition of This Release

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Leukotrienes C_4 and D_4 (LTC_4, LTD_4) are 5-lipoxygenase-generated metabolites of arachidonic acid. Furthermore, it is now an accepted view that LTC_4 and LTD_4 belong to constituents of slow reacting substances of anaphylaxis (SRS-A). Indeed, the pharmacological profiles of LTC_4 and LTD_4 on guinea pig lung parenchyma (GPLP) are strikingly similar to that of SRS-A (8). Isoproterenol, which yields clinical relief in anaphylactic pulmonary conditions, was found to reverse the LTC_4-induced contraction of GPLP (4). The constricting activities of LTC_4 and LTD_4 on GPLP were reported to be associated with the release of thromboxane A_2 (TxA_2), and evidence was presented that on GPLP the leukotriene-induced contractions are, in fact, largely mediated through release of TxA_2 (6). We examined the influence of isoproterenol on the LTC_4-induced release of TxA_2 from GPLP and observed that this event is markedly inhibited by isoproterenol. During these studies, we also found that the LTC_4-induced release of TxA_2 is liable to marked tachyphylaxis. Our observation with isoproterenol has been reported in a preliminary communication (8).

METHODS

Male and female guinea pigs weighing 250 to 300 g were killed by a blow on the head and exsanguinated. The lungs were removed and kept in Krebs solution until use. In a superfusion cascade organ system, a strip of GPLP (7) measured the LTC_4-induced contraction, and a rabbit mesenteric artery (RMA) placed under it served to bioassay the amount of TxA_2 released (1). The contractile activity of the RMA was standardized with angiotensin II, as described earlier (9). The contractions were measured isotonically with a Harvard heart/smooth muscle transducer. Occasionally the effluent of the GPLP was collected to measure the stable metabolite thromboxane B_2 (TxB_2) by radioimmu-

noassay using a rabbit anti-TxB_2 serum (New England Nuclear, Boston). After 16 hr incubation with the antiserum, the free antigen was adsorbed on charcoal, and, after centrifugation, the radioactivity of the supernatant was measured by scintillation counting.

Two batches of LTC_4 were used during this work. The batches were supplied as 200 μg/ml in distilled water. The materials were stored at −20°C. Under such conditions, a certain decay occurs, which is reflected in diminished biological activity. Thus, with the older batch, 50 ng was necessary to contract the GPLP, whereas with a fresh batch 7.5 ng was sufficient.

RESULTS

We did not find an appreciable difference between the contractile activities of LTC_4 and LTD_4 on GPLP. In agreement with other observations (4), we found these leukotrienes to be much more active than histamine. However, TxA_2 proved only slightly less potent than LTC_4 or LTD_4 in contracting GPLP. A representative experiment in which LTD_4 was used in comparison with a variety of nonleukotriene mediators is shown in Fig. 1.

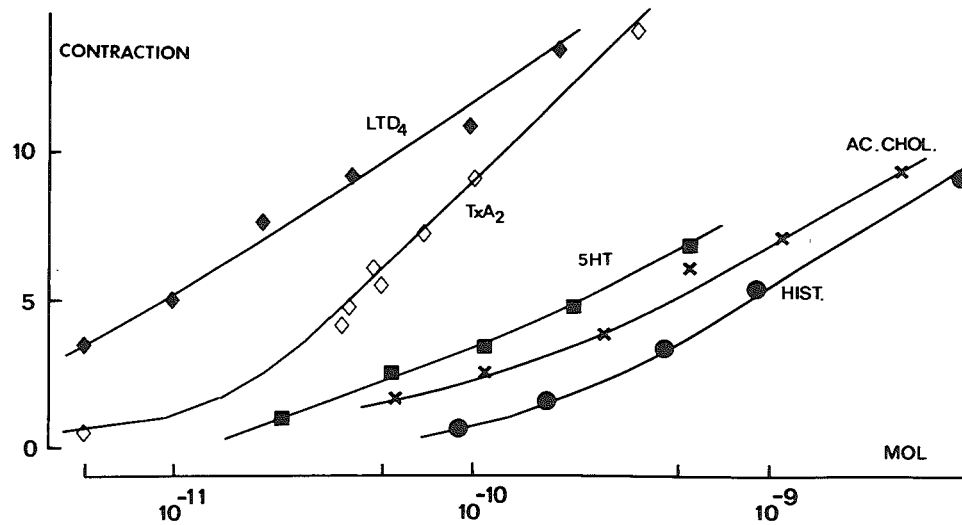

FIG. 1. Dose–response curves of leukotriene D_4 (LTD_4), thromboxane A_2 (TxA_2), 5-hydroxytryptamine (5-HT), acetylcholine (AC.CHOL.), and histamine (HIST.) on superfused guinea pig lung parenchyma. TxA_2 was generated from collagen-aggregated rat platelets which were preloaded with arachidonic acid. Immediately after the addition of collagen to the platelet-rich plasma, different amounts of this plasma were applied to the superfusate. TxB_2 in the plasma was determined by radioimmunoassay, and the amounts of TxA_2 added to the superfusate were retrospectively calculated. The contractions are expressed as millimeters (mm) on the recording chart.

After addition of LTC_4 to GPLP, a release of TxA_2, measured as contraction of RMA, occurs (Fig. 2). Also, the same figure shows that the contractile activity of GPLP slightly declines after several additions of LTC_4. However, the decline in the amount of TxA_2 released is very marked, again as apparent in Fig. 2.

In view of the tachyphylaxis, slight with GPLP contraction but pronounced with TxA_2 release, in the experiments that were aimed at determining the influence of isoproterenol, the effects of LTC_4 have been expressed in percentages of the response to the initial administration of LTC_4. Table 1 shows that isoproterenol counteracted the LTC_4-induced contraction of GPLP and markedly inhibited the release of TxA_2 caused by LTC_4. Both effects of isoproterenol were prevented in the presence of the β-adrenoceptor antagonist sotalol. The values in Table 1 do not include the results of the experiments in which the bioassay of TxA_2 was replaced by radioimmunoassay of TxB_2. In the effluent

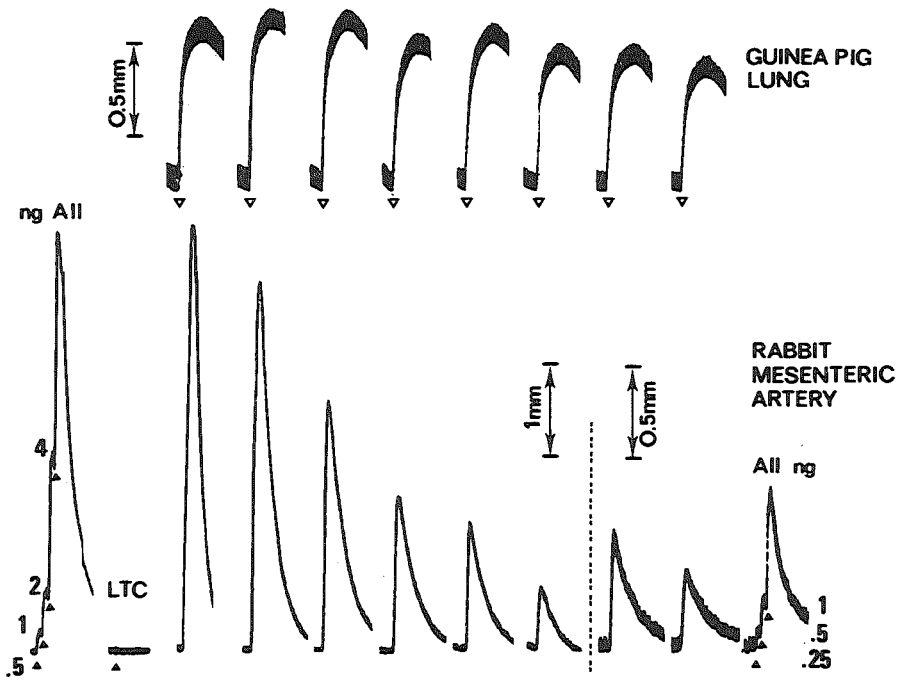

FIG. 2. Effects of repeated additions of LTC_4 (50 ng) to the superfusate of guinea pig lung parenchyma (GPLP) under which a rabbit mesenteric artery (RMA) was placed in a cascade. Before starting the additions of LTC_4 (*empty triangles*) to the GPLP, the contractile activity of the RMA was standardized with additions of angiotensin II (AII, *black triangles*); furthermore, a single dose of LTC_4 was added directly to the RMA. Note that RMA, which is sensitive to TxA_2, is unresponsive to LTC_4. *Vertical arrows* indicate the magnitude of the real contractions of the organs. The *dotted vertical line* shows that the sensitivity of the recorder was increased. At the end of the experiment, the contractile activity of the RMA was again tested with AII. Note that RMA retained its sensitivity to contracting material. Accordingly, the decay in contractions following the additions of LTC_4 to GPLP is caused by diminished release of TxA_2.

TABLE 1. *Effects of isoproterenol and sotalol on LTC_4-induced responses of guinea pig lung parenchyma*[a]

Parameters measured and drugs in the perfusate	Responses (%) after additions of LTC_4 (50 ng) (response to first addition = 100%)	
	Second	Third
GPLP contraction		
Vehicle	92 ± 2.4 (4)	86 ± 2.4 (4)
Isoproterenol, 10^{-6} M	28 ± 4 (7)	N.T.
Isoproterenol, 10^{-6} M + sotalol, 2×10^{-6} M	N.T.	68 ± 14 (3)
TxA_2 release		
Vehicle	66 ± 6 (7)	45 ± 5.3 (7)
Isoproterenol, 10^{-6} M	16 ± 5.3 (4)	N.T.
Isoproterenol, 10^{-6} M + sotalol 2×10^{-6} M	N.T.	53 ± 9 (3)

[a] TxA_2 release was bioassayed on rabbit mesenteric artery which was in a cascade way under the guinea pig lung parenchyma. The values are means ± SE. Numbers of observations are shown in parentheses. N.T., not tested. The table is compiled from the data originally published in ref. 8.

of GPLP untreated with isoproterenol, after the first application of LTC_4 (7.5 ng), the amount of TxB_2 was 15 ng, and following the second application of LTC_4, the measured TxB_2 was 11.5 ng. In another GPLP, the initial application of LTC_4 caused the release of 20 ng of TxB_2. In the same GPLP, but in the presence of isoproterenol, 10^{-6} M, in the superfusion fluid, the second dose of LTC_4 caused the release of only 1.4 ng of TxB_2. Following the addition of sotalol, 2×10^{-6} M, but still in the presence of isoproterenol, the third dose of LTC_4 induced the appearance of 5.7 ng of TxB_2. Thus, the measurements of TxB_2 confirmed the observation that LTC_4-induced release of TxA_2 is inhibited by isoproterenol and that sotalol reverses this effect.

DISCUSSION

The tachyphylaxis of the LTC_4-induced release of TxA_2 has not been reported by others who showed that the contractile effect of LTC_4 on GPLP is predominantly mediated by the release of TxA_2 (6). The reason for the tachyphylaxis could be the depletion of a pool of arachidonic acid which is used in the formation of cyclooxygenase products. However, the evidence for this obvious assumption still needs to be established. Another unanswered question is which cell population in the LTC_4-treated GPLP serves as a source of the released TxA_2. Leukotriene C_4 and LTD_4 have been reported to promote the release of various products

of cyclooxygenase with peritoneal macrophages of rats (3), but as yet, such studies have not been carried out with other isolated cell populations.

With regard to the isoproterenol inhibition of the TxA_2 release caused by LTC_4, the sotalol reversal of this effect of isoproterenol clearly shows the participation of β-adrenoceptors in preventing the release. In several tissues, stimulation of β-adrenoceptors leads to activation of adenylate cyclase. In human platelets, an increased activity of adenylate cyclase followed by subsequent elevation of intracellular cyclic AMP results in diminished availability of arachidonic acid to cyclooxygenase and in decreased release of TxA_2 (5). Whether a similar sequence of events occurs in the GPLP cannot be concluded from the present experiments, although this possibility is quite conceivable. However, TxA_2 release inhibition by β-adrenoceptor agonists, at least in dog platelets, is not necessarily mediated through elevation of cyclic AMP (2).

Finally, it should be kept in mind, that TxA_2 release by LTC_4 has not been shown with lung parenchyma of other species than the guinea pig (6). Therefore, the relevance of the present finding with isoproterenol on GPLP to inhibition of LTC_4-induced pulmonary events in other species still needs to be established.

ACKNOWLEDGMENT

The LTC_4 and LTD_4 were obtained through the courtesy of Dr. J. Rokach (Merck Frosst, Pointe Claire, Canada).

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3.2.4

ISOPRENALINE INHIBITS THE LEUKOTRIENE C_4-INDUCED RELEASE OF THROMBOXANE A_2 FROM GUINEA PIG LUNG PARENCHYMA

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European Journal of Pharmacology, 76 (1981) 297–298
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Rapid communication

ISOPRENALINE INHIBITS THE LEUKOTRIENE C_4-INDUCED RELEASE OF THROMBOXANE A_2 FROM GUINEA PIG LUNG PARENCHYMA

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Leukotrienes C_4 and D_4 (LTC_4, LTD_4) are endogenous metabolites of arachidonic acid by the lipoxygenase pathway and the pharmacological profiles of these substances on guinea-pig lung parenchyma (GPLP) are strikingly similar to that of slow reacting substance of anaphylaxis (SRS-A) (Hedquist et al., 1980). Both LTC_4 and LTD_4 were shown to release thromboxane A_2 (TXA_2) from GPLP; the TXA_2 release might account for at least part of the contraction of GPLP upon exposure to LTs (Piper and Samhoun, 1981). Isoprenaline reverses the LTC_4-induced contraction of GPLP (Hedquist et al., 1980). We now report that isoprenaline inhibits the LTC_4-induced release of TXA_2 from GLP and that the β-adrenoceptor antagonist sotalol counteracts this inhibition.

In a superfusion cascade organ system a strip of GPLP (Sirois et al., 1981) measured the LTC_4-induced contraction and rabbit mesenteric artery placed under served to bioassay the amount of TXA_2 released (Bunting et al., 1976). In a separate experiment the effluent of the GPLP was collected to measure the stable metabolite thromboxane B_2 (TXB_2) by radioimmunoassay using a rabbit anti-TXB_2 serum (New England Nuclear, Boston). Two batches of synthetic LTC_4, obtained through courtesy of Dr. J. Rokach (Merck Frosst, Pointe Claire, Canada), were used.

Following repeated application of LTC_4 to the superfusion fluid the contractile response of the GPLP became liable to slight tachyphylaxis which, however, was more pronounced with TXA_2 release. In the following results, obtained with 50 ng LTC_4, the first figure indicates the contractions of the GPLP, the second TXA_2 release and both figures are percentages (means $\pm$S.E.M.) of the responses after the initial administration of LTC_4. Following the second application of LTC_4, these were $92 \pm 2.4\%$ ($n = 4$) and $66 \pm 6\%$ ($n = 7$), and after the third dose $86 \pm 2.4\%$ ($n = 4$) and $45 \pm 5.3\%$ ($n = 7$), respectively. In the presence of isoprenaline 10^{-6} M the responses to the second dose of LTC_4 were 28 ± 4.0 ($n = 7$) and $16 \pm 5.3\%$ ($n = 4$). In the same experiment upon addition of sotalol 2×10^{-6} M, but still in the presence of isoprenaline, the third dose of LTC_4 caused 80, 82, 43% and 62, 44% responses (individual values).

Fig. 1 illustrates a similar experiment in which a new batch of LTC_4 was used and the bioassay of TXA_2 was replaced by radioimmunoassay of the stable metabolite TXB_2. The 7.5 ng dose of LTC_4 caused the GPLP to contract and to release TXB_2, the latter event being liable to tachyphylaxis. Isoprenaline relaxed the GPLP and markedly counteracted both effects of LTC_4. Sotalol reversed the effects of isoprenaline. This experiment confirms that TXA_2 release is inhibited by isoprenaline, as described above in the text.

Sotalol reversal of the isoprenaline effect clearly shows the participation of β-adrenoceptors in the inhibition of TXA_2 release. In several tissues stimulation of β-adrenoceptors leads to activation of adenylate cyclase. In platelets, an increased activity of adenylate cyclase and subsequent elevation of intracellular cyclic AMP results in a decreased release of TXA_2 (Minkes et al., 1977). Whether a similar sequence of events occurs in the present experiments cannot be concluded, although this possibility is quite conceivable. Irre-

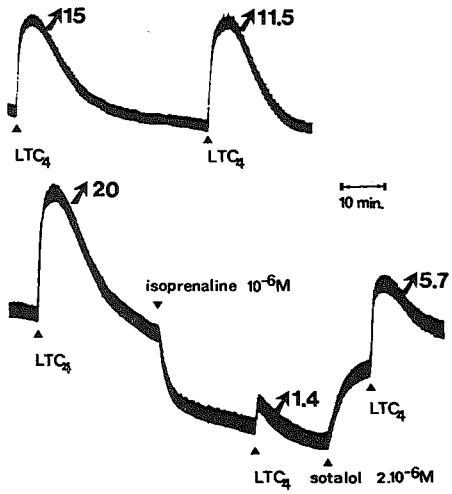

Fig. 1. Effect of isoprenaline and sotalol on LTC_4-induced TXB_2 release from an contractile response of GPLP. The dose of LTC_4 added to the superfusion fluid was in each case 7.5 ng. Oblique arrows indicate the moment of collecting the effluent in which TXB_2 was determined. Figures near the arrows show the amount of TXB_2 (ng). These are mean values of triplicate measurements. The upper record shows the responses of a control GPLP, the lower record shows the responses of a GPLP under influence of the β-adrenoceptor agonist and antagonist.

spective of the mechanism, to our knowledge this report is the first which demonstrates isoprenaline inhibition of LTC_4-induced release of TXA_2. SRS-A is implicated in immediate hypersensitivity reactions leading to perturbations of pulmonary mechanics, furthermore SRS-A consists of LTC_4 and LTD_4, the constricting activity of which on lung parenchyma and bronchi is at least partially mediated through release of TXA_2 (Hedquist et al., 1980; Piper and Samhoun, 1980). Therefore the present finding, provided that its in vivo relevance can be established, may shed new light on the question of the efficacy of isoprenaline in relieving anaphylactic pulmonary conditions.

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3.2.5

SOTALOL POTENTIATES THE LEUKOTRIENE C_4-INDUCED CONTRACTIONS AND THROMBOXANE A_2 RELEASE OF GUINEA PIG LUNG PARENCHYMAL STRIPS

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SOTALOL POTENTIATES THE LEUKOTRIENE C_4-INDUCED CONTRACTIONS AND THROMBOXANE A_2 RELEASE OF GUINEA PIG LUNG PARENCHYMAL STRIPS

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Abstract—1. The leukotriene C_4 (LTC_4)-induced contraction and thromboxane A_2 (TxA_2) release of the guinea pig lung parenchymal (GPLP) strip are both inhibited by the β_2-adrenergic agent salbutamol. The effect of LTC_4 is restored to nearly normal by the β-adrenergic antagonist sotalol.

2. The latter substance alone also induces a contraction in the GPLP strip and potentiates the contractions and the TxA_2 release of LTC_4. During the sotalol-induced contractions, no TxA_2 release occurs. An antihistaminic, mepyramine had no effect on the sotalol-induced contraction.

3. When sotalol is added repeatedly to a GPLP strip, only the first time a contraction occurs.

INTRODUCTION

The leukotrienes (LTs) are a group of substances with several biological activities, which are formed from arachidonic acid by the lipoxygenase pathway. Some of these compounds have a potent bronchoconstrictor action. It has been shown that the guinea pig lung parenchyma (GPLP) is particularly sensitive to this effect (Piper and Samhoun, 1982; Sirois *et al.*, 1981). During the contractions, induced by LTC_4 and LTD_4, thromboxane A_2 (TxA_2) is formed. This accounts for a part of the contraction, whereas another part is due to a direct effect of the LT (Piper and Samhoun, 1982; Zijlstra *et al.*, 1983a). Both the LTC_4-induced contraction and the TxA_2 release are inhibited by isoproterenol (Zijlstra *et al.*, 1981). This action is possibly related to the increase in cyclic AMP, induced by the β-adrenergic agent. An inhibition is also observed after the addition of IBMX (3-isobutyl-1-methyl-xanthine), a phosphodiesterase inhibitor to the system (Zijlstra *et al.*, 1983c).

In the experiments presented here, the effect of a substance with β_2-adrenergic activity, salbutamol was determined on the LTC_4-induced contraction and TxA_2 release in the GPLP strip. This action is inhibited by sotalol, a β-adrenergic antagonist. Sotalol also induces a contraction itself and potentiates the effects of LTC_4. A relationship may exist between these results and the induction, by β-adrenergic antagonists of bronchospasms in asthmatic and normal subjects (McLagen and Ney, 1979).

METHODS

Male and female guinea pigs of 250–300 g were used. The animals were killed by a blow on the head and exsanguinated. The lungs were removed and kept in Krebs' solution (ionic composition: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.18, KH_2PO_4 1.18, $NaHCO_3$ 25 and glucose 5.6 mM). The strips of lung parenchyma were prepared from the largest lobes as has been described before (Zijlstra *et al.*, 1984). Strips of tissues were superfused with oxygenated (95% O_2 + 5% CO_2) Krebs solution. The contractions were measured isotonically with a Harvard heart/smooth muscle transducer. The perfusion rate was 2 ml/min at 37°C. Samples were collected after the addition of the substances to the GPLP strip. The amount of TxB_2 formed was measured by radioimmunoassay (Zijlstra *et al.*, 1984).

The Student's *T*-test was used in the statistical analysis.

MATERIALS

The leukotrienes C_4 and D_4 were a gift of Dr J. Rokach, Merck Frosst Labs, Pointe Claire, Quebec, Canada. Salbutamol was a gift of Glaxo. Sotalol was obtained from Bristol. In the radioimmunoassay of TxB_2, the antibody used was obtained from l'Institut Pasteur, Paris and [^{3}H]TxB_2 from New England Nuclear, Boston. All chemicals used were analytical grade.

RESULTS

The contraction, induced by 200 ng LTC_4 is nearly totally inhibited when the system is perfused with 1 μM salbutamol. When the perfusion is continued with both salbutamol and 6 μM sotalol, this effect is reversed. The amounts of TxA_2 released (determined as TxB_2) follow the same tendency (Fig. 1 and Table 1). In some of these experiments, it was found that the LTC_4-induced contraction was larger after the administration of both salbutamol and sotalol than when LTC_4 was added alone. For this reason, the effect of sotalol on the LTC_4-induced contractions was further investigated.

Table 1. Effect of salbutamol and sotalol on the contractions and TxA_2 release, induced by 500 ng LTC_4 in the GPLP strip. *$P < 0.01$ compared with salbutamol

		Response after addition of LTC_4 (%) compared with the first addition (= 100%)
Contraction	Salbutamol 1 μM	25 ± 6.4
	Salbutamol 1 μM + Sotalol 6 μM	105 ± 19.2*
TxA_2 release	Salbutamol 1 μM	25 ± 11.7
	Salbutamol 1 μM + Sotalol 6 μM	91 ± 31.7*

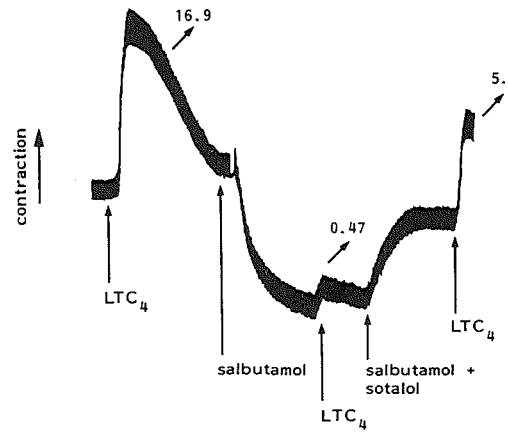

Fig. 1. The effect of LTC_4 (200 ng), salbutamol (1 μM) and sotalol (3.2 μM) on the GPLP strip. The tissue is perfused with salbutamol, followed by salbutamol + sotalol. The numbers indicate the ng TxB_2 formed during the perfusion. A representative experiment is given ($n = 11$).

When a GPLP strip was superfused with 2 μM sotalol, a contraction occurred. The effect of LTC_4 was increased both in so far as the contraction and the TxA_2 release are concerned. The sotalol-induced contraction was not dependent on the formation of TxA_2 (Fig. 2).

The effects of sotalol on the LTC_4-induced contractions and TxA_4 release are represented in Table 2. When LTC_4 is added for the second time, a tachyphylaxis occurs. For this reason, the values obtained are lower than those after the first addition.

The effect of the repeated additions of sotalol to

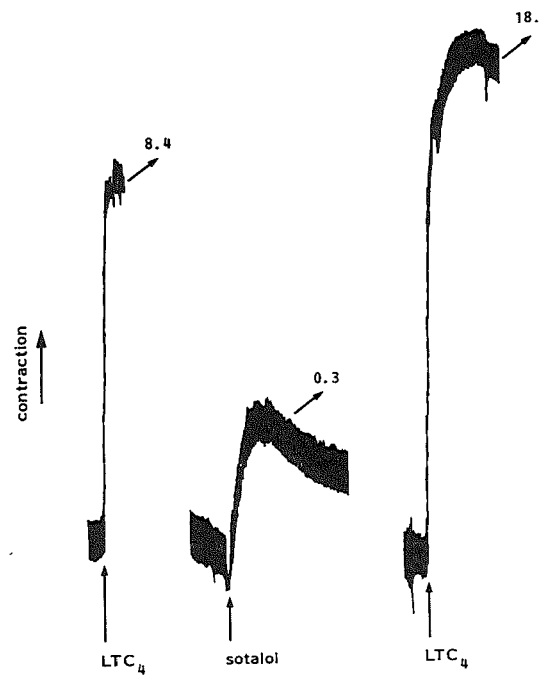

Fig. 2. The effect of LTC_4 (500 ng) and sotalol (2 μM) on the GPLP strip. The tissue is perfused with sotalol. Numbers indicate ng TxB_2 ($n = 13$).

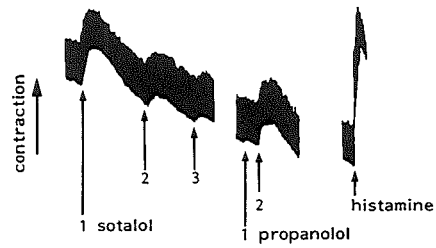

Fig. 3. The effect of the repeated addition of 10 μg sotalol, of 10 (1) and 50 μg (2) propranolol and of 100 ng histamine on the GPLP strip ($n = 3$).

the GPLP strip was also measured. A contraction only occurred after the first addition of 10 μg, not after the second and third one. Propranolol in doses of 10 and 50 μg also induced a contraction in the GPLP strip. For comparison, the effect of 100 ng histamine is given (Fig. 3). The effect of sotalol was not inhibited by the perfusion of the GPLP strip with 0.14 μM mepyramine.

DISCUSSION

Substances with β_2-adrenergic activity induce relaxation in bronchial tissue. This effect is used in the treatment of a number of respiratory diseases. In the experiments represented here, the action of a substance with β_2-adrenergic activity, salbutamol has been determined. This compound inhibited both the contraction and the TxA_2 release, induced by LTC_4 in the GPLP strip. After the addition of sotalol to the system, the effect of LTC_4 was partly restored to normal (Fig. 1). With isoprenaline, which has both β_1 and β_2 adrenergic activity, a similar effect occurred (Zijlstra *et al.*, 1981).

As has been mentioned before, in some cases the effect of LTC_4 was increased when, after salbutamol perfusion, sotalol was given. This was unexpected as a tachyphylaxis occurs in both the contraction and the TxA_2 release when LTC_4 is added repeatedly to the GPLP strip (Zijlstra *et al.*, 1983b). For this reason, the effect of sotalol on the GPLP strip was further investigated. The results indicate, that sotalol induces a contraction itself and enhances both this effect and the TxA_2 release induced by LTC_4. The latter is not an additive effect as no TxA_2 release occurs with sotalol alone (Fig. 2).

After one addition of 10 μg sotalol, a contraction occurs. The second and third additions of the same dose only have a small effect. This may indicate that the receptors for this substance are nearly completely occupied after this dose. Propranolol also induces contractions.

Table 2. Effect of sotalol on the contraction and TxB_2 release induced by 500 ng LTC_4 in the GPLP strip. The controls are lower after the second addition because of the tachyphylaxis. $^*P < 0.01$, $^{**}P < 0.001$ compared with controls

		Response after addition of LTC_4 (%) compared with the first addition (=100%)
Contraction	Control	90 ± 2.2 ($n = 10$) (second add.)
	Sotalol 2 μM	124 ± 8.2 ($n = 13$)*
TxB_2 release	Control	61 ± 3.6 ($n = 6$) (second add.)
	Sotalol 2 μM	178 ± 24.0 ($n = 13$)**

Recently, the propranolol-induced bronchoconstriction was ascribed to the release of histamine (Terpstra *et al.*, 1981). This cannot explain our results, as the superfusion of the system with mepyramine had no influence on the contraction induced by sotalol. A potentiating effect of propranolol and practolol on the LTC_4-induced bronchoconstriction was demonstrated in *in vivo* experiments in the guinea pig. There was no effect *in vitro*. This action was not mediated by a serotonergic, cholinergic or α-adrenergic mechanism (Bongrani *et al.*, 1983).

The bronchoconstrictive action of β-adrenoceptor blockers *in vivo* is in general attributed to the inhibition of the relaxing effect of circulating adrenaline. It is unlikely, that sotalol acts through β_2-adrenergic blockade in an *in vitro* system, which is constantly superfused. Small amounts of adrenaline, with β_2 activity which may have been present in the beginning of the experiment most probably have been removed.

In the contractions, induced by LTC_4, 2 different components are present: one is due to the action of the released TxA_2 and the other one to a direct effect of LTC_4 (Piper and Samhoun, 1982). The release of TxA_2 only lasts for a few minutes, whereas the direct effect has a much longer duration (Zijlstra *et al.*, 1983a). TxA_2 is formed from arachidonic acid, which is released from the phospholipids of the tissue, either by the action of a phospholipase A_2, or by phospholipase C. It has been shown that the action of LTC_4 is inhibited by chloroquine (Zijlstra *et al.*, 1983c). This substance is an inhibitor of phospholipases A_2 and C (Matsuzawa and Hostetler, 1980). The relaxation, induced by salbutamol can most probably be ascribed to an activation of the adenylate cyclase in the lung parenchymal tissue. An increase in cyclic AMP inhibits the release of arachidonic acid in platelets (Lapetina *et al.*, 1981). A similar effect may occur in lung tissue.

It is a point of interest to consider, whether the effects described here can also occur in asthmatic patients as a result of treatment with a β_2-adrenoceptor blocker. As the conditions *in vivo* are, of course, different from the experiments described and also because guinea pig lung tissue is more sensitive to the effect of LTC_4 than human lung (Zijlstra *et al.*, 1984), a comparison is difficult to make. It has been reported that human alveolar macrophages, when activated, form 10 ng $LTC_4/10^6$ cells (Fels *et al.*, 1982) and human mast cells 25 ng/10^6 cell (Lewis and Austen, 1984). 50 ng LTC_4 induces a contraction in human lung strips (Zijlstra *et al.*, 1984). These figures indicate that human lungs can produce amounts of this substance that have a contractile action on this tissue.

In summary, the results presented here, show that sotalol inhibits the effect of salbutamol on the LTC_4-induced contractions in the GPLP strip. Sotalol itself also contracts the GPLP strip and potentiates the effect of LTC_4. These actions are not mediated by the release of TxA_2 or histamine.

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3.3 SUMMARY

The mechanisms of action, the potency in comparison to other biologically active sbstances and the effects of antagonists and synthesis inhibitors of the leukotriene C_4 and D_4-induced contractions of the guinea pig lung parenchymal (GPLP) strip were investigated. In conclusion it was found that

-the contractions of the GPLP are composed of two parts, one of which is due to the immediate effect of the released thromboxane A_2 and the other one to the slow acting, long lasting direct effect of LT itself [3.2.2].

-both the contractile response and the release of thromboxane A_2 in the GPLP was at least 40 times higher than those obtained in human and porcine lung parenchymal strips [3.2.1].

-LTC_4 and LTD_4 are much more active on GPLP than histamine, serotonine or acetylcholine [3.2.3 or 3.2.1].

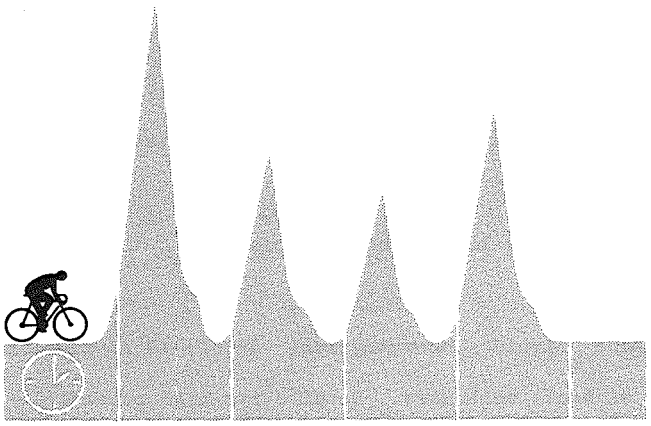
-after repeated application of LTs to the GPLP both the contraction and the TxA_2 release became liable to tachyphylaxis, most pronounced in the release reaction [3.2.4 and 3.2.3].

-TxA_2 generated from aggregated platelets was slightly less potent than LTs C_4 and D_4. Phospholipase A_2 induced contraction and TxA_2 release were subject to fast tachyphylaxis [3.2.1].

-although inhibition of the TxA_2 release by indomethacin was complete, approximately 80% of the contractile activity after LT application tc the GPLP remained [3.2.2].

-physiological antagonism to the LTC_4-induced contractions and the TxA_2 release was obtained after the addition of the β_1,β_2-adrenoceptor agonist isoprenaline [3.2.4] and the β_2-adrenoceptor agonist salbutamol [3.2.5].

-sotalol reversed the effect of β-adrenoceptor agonists. This β-adrenergic antagonist caused a small contraction in the GPLP strip and potentiated those induced by LTC_4, in the absence of an agonist [3.2.5].



CHAPTER 4

MEASUREMENT OF LEUKOTRIENE SYNTHESIS WITH HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

4.1 INTRODUCTION

In the previous chapter the biological activities of leukotrienes were examined on lung parenchymal strips. In this chapter the synthetic capacity of lung tissue and macrophages from lungs and other origins to generate eicosanoids are described and compared with their occurrence both in healthy and diseased tissues or cells.

The inhibition of eicosanoid production by specific synthesis inhibitors was examined. In lung tissue the formation of eicosanoids was measured by high performance liquid chromatography and radioimmunoassay.

In vitro incubation with ^{14}C-arachidonic acid of macrophages, isolated from ascitic fluid of patients with alcoholic liver cirrhosis or dialysates from patients with kidney insufficiency, made it possible to investigate the whole cascade of eicosanoids synthesized.

4.1.1 *High performance liquid chromatography*

High-performance liquid chromatography (HPLC) is an ideal technique for the analysis of small molecules. Despite its high potential demonstrated during the last two decades, liquid chromatography frequently confronts us with a selectivity or sensitivity of the LC system which is insufficient for an accurate and precise quantitation of eicosanoids eluted from the column. Pre- or post-column derivatization, sample clean-up and sample

concentration improved the result only to a limited degree. One of the problems of preconcentration techniques is that not only the compounds of interest will be concentrated, but also the interfering compounds. Hence the selectivity is reduced. In the area of eicosanoid-research this problem can be avoided by using a radiolabelled precursor, which allows the radioactive determination of the metabolites in the extract of the incubation sample, without interference from non-labelled compounds.

Elucidation of the pathways of arachidonic acid (AA) metabolism in different tissues and cells has been made possible by the separation and detection of the metabolites of AA labelled with ^{14}C. HPLC using a single 25 cm x 4.6 mm i.d. column packed with 5μm ODS-silica and a tetrahydrofuran/methanol/water or acetonitrile/water solvent system is used to separate the major metabolites of the lipoxygenase and cyclooxygenase pathways [Tracey,1986].

Prostaglandins can be separated from thromboxane, leukotrienes and hydroxyeicosatetraenoic acids using reversed phase (RP) systems and gradient elution. RP chromatography is so named because it behaves in a way which is opposite to normal or adsorption chromatography, in which interaction occurs between the absorbent and the solute and solvent molecules in solution. There is a competition between the solute and solvent molecules for adsorption sites on the solid surface. Separation is effected since different molecules are absorbed and displaced differently. Solutes are eluted in the order of increasing polarity and retention decreases with increasing solvent polarity.

In RP chromatography the stationary phase is silica which is chemically bounded to an alkylsilyl compound. This results in a non-polar, hydrophobic surface. Solute retention is due to hydrophobic interactions between the solutes and the hydrocarbonaceous stationary phase surface.

Polar mobile phases, usually water-miscible organic solvents, are used for elution. Solutes are eluted in the order of decreasing polarity (=increasing hydrophobicity), whereas increasing the polar (aqueous) component of the mobile phase increases the retention of the solutes. Packing material of the RP columns is usually of the C_{18} type in which octadecylsilyl (ODS) groups are bonded to the silica surface.

Detection of prostaglandins in naturally-occurring concentrations presents some problems. The amounts are usually so low that they can not be measured without radiolabelling or derivatization with a chromophore which will enhance the UV absorption or fluorescence of the compound. Detection of amounts in the order of micrograms by UV absorption is possible at 192 nm without derivatization. Improved peak shape is obtained at low pH (2-3), while the elution order for prostaglandins is not pH dependent. The use of methanol instead of acetonitrile reverses the elution order of $PGF_{2\alpha}$ and PGE_2, whereas PGE_2 and PGD_2 are not separated . In general retention time increases with the number of double bonds and hydroxy groups. The longer the column the better the separation of *cis/trans* and diastereoisomers [Green *et al.*,1978].

Leukotrienes are pH dependent. At higher pH they will have shorter retention times, whereas at lower pH LTs are unstable. Measurement of LTs by HPLC is much easier than that of PGs, because of the conjugated triple bond which makes them detectable at the nanogramlevel using the UV absorption at 270 and 280 nm. Separation of LTs is performed on columns packed with 5 μm Nucleosil C_{18} using solvent systems containing methanol / water, at a pH of 5.5. Reducing this pH to 4 considerably enlarges the retention times of the sulphidopeptide LTs. Addition of tetrahydrofuran to the solvent system allows separation of diastereoisomers of mainly LTB_4. Detection down to 2 ng per peak can be obtained. This method is as

sensitive as the normally used bioassays with guinea pig lung parenchymal strips or guinea pig ileum, and has the advantage that it is a quantitive and not merely a qualitative method.

In fig.11 the basic set-up for on-line and off-line detection of radiolabel is presented. The system is controlled by the HP 1084 computer, which enables the start of both the fraction collector and the beta flow detector. By the splitter a certain percentage of the HPLC-eluent is divided and further examined. After collection of samples by the fraction cutter, liquid scintillator is added and samples are counted in a tritium-carbon scintillation counter. Data obtained are calculated and plotted by the PDP 11/70 computer (the "off-line" method).

Loss of resolution depends on the fraction volume, whereas the sensitivity depends both on the background and the number of counts

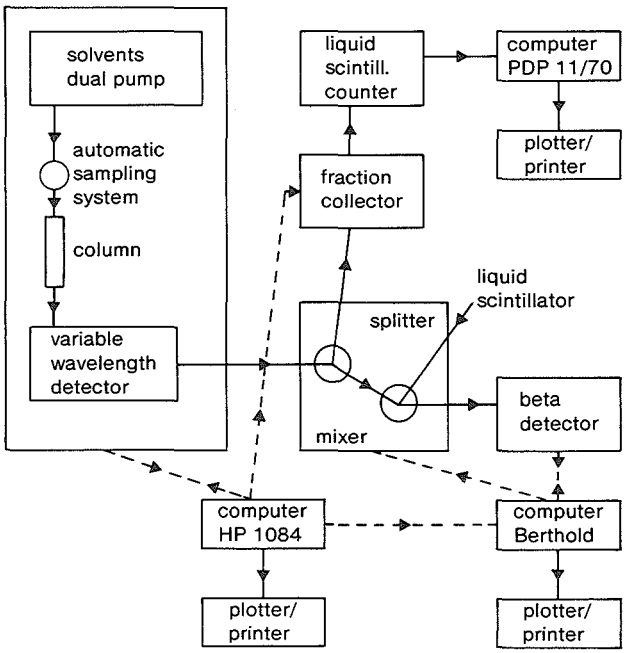


Fig.11 Basic set-up for on-line and off-line radiometric detection in column liquid chromatography.

collected. The recently introduced "on-line" method seems to offer some promise for low-level radioactivity detection in liquid chromatography [Veltkamp *et al*,1987]. This dual label system is flexible in storing raw data, or entire chromatograms without losing the chromatographic properties and the possibility to reintroduce parameters, zones of interest and signal smoothing. All these operations enable automation of the entire separation and detection process, controlled by a computer. In conclusion, this set-up presents an output consisting of chromatograms of non-labelled LTs and HETEs, plotted data of dual labelled PGs, LTs and HETEs (on-line and/or off-line) and the possibility to collect samples for further identification or quantitation (RIA, bioassay, GC-MS).

4.1.2 *Eicosanoid formation by cells*

Whereas prostaglandins can be synthesized by most cells, the lipoxygenase products leukotrienes and hydroxy-eicosatetraenoic acids are formed by a limited number of cell types. Producer cells for LTs are mainly macrophages, monocytes, neutrophils, eosinophils, and mast cells [table 3]. In body fluids LTs were demonstrated in bile after endotoxin-induced shock and in plasma, sputum and nasal secretions after allergen challenge.

Various substances and triggers stimulate the generation and release of LTC_4 and/or LTB_4. These include chemotactic peptides, such as F-Met-Leu-Phe (FMLP), platelet-activating factor (PAF), IgE directed antigens, a Ca^{2+} ionophore such as A23187, phagocytic stimuli such as opsonized zymosan, bacterial endotoxin, phospholipase A_2, mechanical or thermal tissue injury. In the studies reported in this chapter the Ca^{2+} ionophore A23187 was used as a trigger of cells to generate leukotrienes.

TABLE 3

CELLULAR SOURCES AND IN VIVO OCCURRENCE OF LEUKOTRIENES IN MAN

(+, ++, +++ relative amounts)

Stimulated cells/tissues	**pept. LTs**	**LTB_4**
Eosinophils	+++	+
Neutrophils	+	+++
Monocytes	++	+++
Macrophages	+	+++
Lymphocytes		
Mast cells	++	+
Basophils	+	+
Psoriatic skin		+
Body fluids		
Bile	+++	
Urine	+	
Blood plasma asthma	+	
Synovial fluid		+
Sputum cystic fibrosis	+	+
Nasal secretes (allergic)	++	+
Tear fluid (allergic)	+	
Pulmonary fluid (hypertens.)	+	
Seminal fluid	+	

[Data compilated from Keppler et al., 1985]

4.1.3 *^{14}C-Arachidonic acid labeling of cells or tissues*

Lung tissue. Macroscopically normal, tumor free parts of human lung tissue were used for incubation with arachidonic acid. The pleura was removed and parenchymal tissue dissected free from major blood vessels and bronchi. Approx.10 g was chopped with a McIlwain tissue chopper in slices of 0.5x0.5 mm. This fraction was washed and erythrocytes were removed by lysation. Incubation was performed in 20 ml of Krebs-Henseleit buffer with a pH of 7.45. 1 μCi ^{14}C-arachidonic acid per gram tissue was added.

Macrophages. Dialysis fluid of patients with continuous ambulatory peritoneal dialysis was collected at different phases of peritonitis and cells were isolated by centrifugation. Ascites from patients with an

alcoholic liver cirrhosis was cleaned over a macro gauze and cells isolated after centrifugation. Macrophages were separated from granulocytes and erythrocytes on Lymphoprep^{R}. Incubation was performed in 10 ml buffer with 1 μCi ^{14}C-AA per ca. 10^{6} macrophages.

Incubation and work-up procedure. In fig.12 a flow-sheet is presented containing the most important steps in the work-up procedure of the analyses of eicosanoid formation by cells. From the 5th step onwards the procedure can also be applied with tissue incubations. In short, incubations were carried out in a waterbath at 37°C, gassed continuously with 95% O_2/5% CO_2. Labelled precursor, glutathion and Ca-ionophore A23187 were added. At the end of the incubation ^{3}H-labelled PGs, LTs and HEs were added in known amounts to calculate recoveries (step 7). Eicosanoids were extracted from the incubation medium by application to SepPak cartridges, and after the evaporation of the eluate and filtration, injected into the HPLC column (step 13).

HPLC. Prostaglandins were separated on a Zorbax C_8 column using a solvent system containing acetonitrile /benzene /water /acetic acid, (24 : 0.2 : 76 : 0.1), with a flow rate of 2 ml/min at 37°C. Lipoxygenase products were separated on HPLC by a Nucleosil $5C_{18}$ column using as eluens tetrahydrofuran /methanol /0.1% EDTA in water /acetic acid, (25 : 30 : 45 : 0.1, adjusted to pH 5.5), with a flow rate of 0.9 ml/min at 37°C.

Materials. Synthetic leukotrienes were generous gifts from Dr. J.Rokach (Merck Frosst Canada Inc.). Ethanol soluble Ca-ionophore A23187 was obtained from Hoechst. Labelled compounds were purchased from the Radiochemical Centre Amersham (U.K.). Antibodies for determinations of the endogenous formation of prostaglandins and thromboxane were obtained from l'Institut Pasteur, anti- 6-keto-$PGF_{1\alpha}$ and anti- HETEs from Seragen and anti- LTB_4 from Wellcome. Prepacked HPLC columns were from Chrompack.

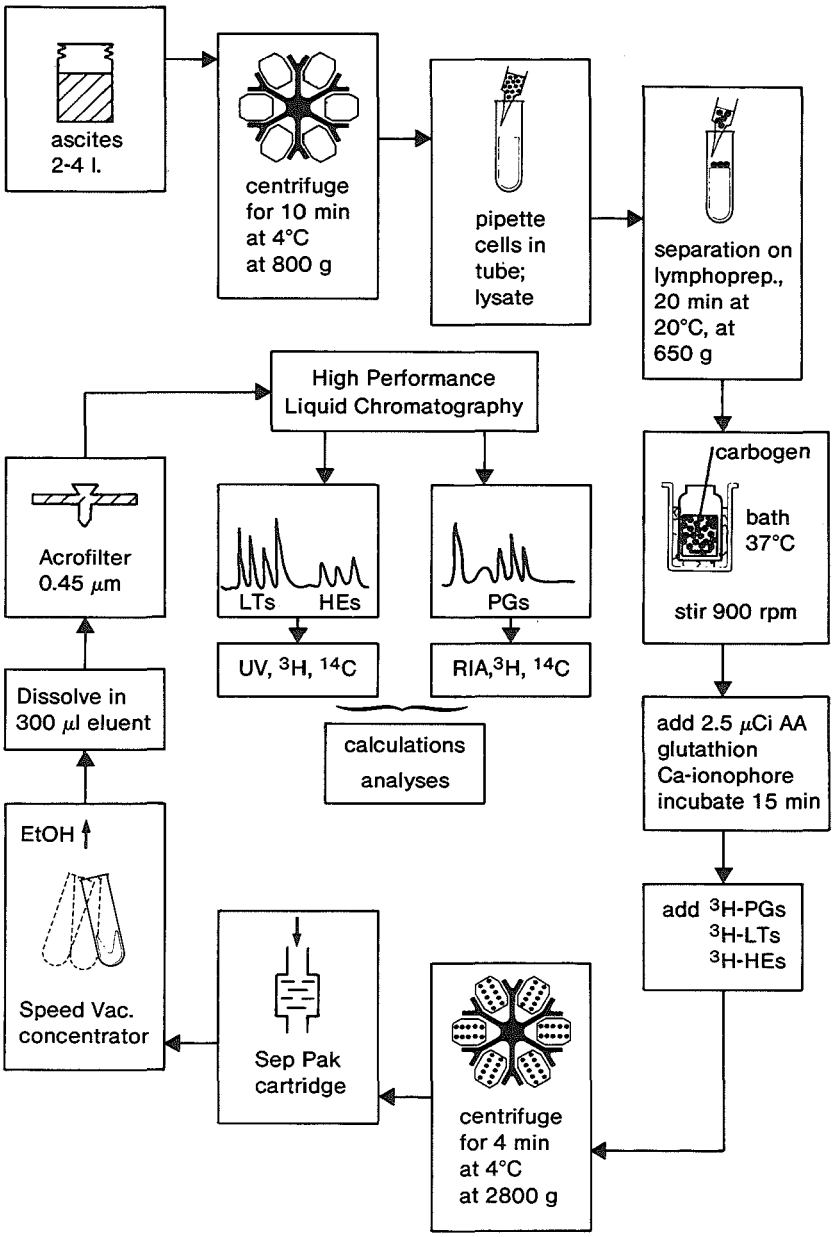


Fig.12 Flowsheet to determine exogenous formation of eicosanoids

4.2.1

DETERMINATION OF LEUKOTRIENES AND PROSTAGLANDINS IN ^{14}C-ARACHIDONIC ACID LABELLED HUMAN TISSUE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND RADIOIMMUNOASSAY

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DETERMINATION OF LEUKOTRIENES AND PROSTAGLANDINS IN [^{14}C] ARACHIDONIC ACID LABELLED HUMAN LUNG TISSUE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND RADIOIMMUNOASSAY

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SUMMARY

A liquid chromatographic method for the determination of ^{14}C-labelled prostaglandins, leukotrienes and other lipoxygenase products formed by human lung tissue is described. In this paper we report our problems identifying these substances when ^{3}H- or ^{14}C-labelled compounds are compared with measurements of the mass by absorption or radioimmunoassay. Furthermore, some preliminary results of [^{14}C]arachidonic acid labelled human lung tissue, stimulated by the Ca-ionophore A23187, show that, of the lipoxygenase products, mostly leukotriene B_4 like compounds are formed and less leukotriene C_4, E_4 and D_4. Relatively large amounts of hydroxyeicosatetraenoic acids are present. The main cyclooxygenase products are thromboxane B_2, 6-ketoprostaglandin $F_{1\alpha}$ and prostaglandin D_2.

INTRODUCTION

The biologically active leukotrienes (LTs) are formed from arachidonic acid (AA). Several of these substances induce slow contractions in smooth muscles. The effects of the LTs on peripheral and central airway functions of pulmonary tissue have also been described [1]. Furthermore, it has been reported that the addition of leukotriene C_4 (LTC_4) and D_4 (LTD_4) induces thromboxane A_2 (TxA_2) release from guinea pig isolated perfused lungs [2, 3]. Both the contraction of lung parenchymal strips and the TxA_2 release could be inhibited by the β-adrenoceptor agonist isoprenaline [4]. In previous work, a comparison was made between the contractile activities and TxA_2 release of human, porcine and guinea pig lung parenchymal strips after the application of LTC_4 and LTD_4 [5]. The results indicate that both the contractile activity of LTs on human lung strips and the TxA_2 release were rather low in com-

parison with the guinea pig lung strip. In cultured endothelial cells from human umbilical vein, however, LTC_4 promotes prostacyclin synthesis [6]. Recently, it has become evident that human alveolar macrophages produce leukotriene B_4 (LTB_4) [7], and it was also shown that human peritoneal macrophages synthesize LTB_4 and LTC_4 [8]. Further, Dahlén et al. [9] showed that allergen challenge of chopped human lung tissue elicits contraction that correlates with the release of both LTC_4, LTD_4 and leukotriene E_4 (LTE_4) and prostaglandins.

In this paper we describe an extraction procedure and high-performance liquid chromatographic (HPLC) techniques for the separation of cyclooxygenase and lipoxygenase products with comparatively high recoveries and discuss some of the problems concerned with the identification of these substances formed from [^{14}C]AA-labelled and Ca-ionophore-triggered chopped human lung tissue.

EXPERIMENTAL

Apparatus

A 1082B high-performance liquid chromatograph (Hewlett-Packard) was used, consisting of double-head pump, temperature-controlled column compartment, variable-volume injector and variable-wavelength detector. The Superrac fraction collector (LKB, Sweden) was connected to this apparatus and used as an automatic sampling system. Radioactivity in the labelled fractions was counted in a 3255 Tricarb liquid scintillation counter (Packard, Brussels, Belgium).

Chemicals

LTB_4, LTC_4 and LTD_4 were gifts of Dr. J. Rokach (Merck Frosst, Canada). Ca-ionophore A23187 was obtained from Hoechst (Calbiochem-Behring, U.S.A.), reduced glutathione from ICN (Cleveland, OH, U.S.A.) and prostaglandins D_2 (PGD_2), E_2 (PGE_2) and $F_{2\alpha}$ ($PGF_{2\alpha}$) from Sigma (U.S.A.). 6-Keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) and thromboxane B_2 (TxB_2) were gifts of Dr. J.B. Smith (Philadelphia, PA, U.S.A.). Siliclad® was obtained from Clay Adams (Becton Dickinson, U.S.A.). Tetrahydrofuran, methanol, acetonitrile, benzene and acetic acid were all of analytical grade from E. Merck (Darmstadt, F.R.G.). Picofluor-15 (Packard) was used as premixed scintillation cocktail.

Radiochemicals

[1-^{14}C]AA, 5-D-[5,6,8,9,11,12,14,15-^{3}H(n)]hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE), 12-L-[5,6,8,9,11,12,14,15-^{3}H(n)]HETE and 15-L-[5,6,8,9,11,12,14,15-^{3}H(n)]HETE were purchased from New England Nuclear. All other radiolabelled compounds mentioned below were obtained from the Radiochemical Centre (Amersham, U.K.): [5,6,8,9,11,12,14,15-^{3}H(n)]LTB_4, [14,15-^{3}H(n)]LTC_4, [14,15-^{3}H(n)]LTD_4, 6-keto-[5,8,9,11,12,14,15-^{3}H(n)]-$PGF_{1\alpha}$, [5,6,8,9,11,12,14,15-^{3}H(n)]TxB_2, [5,6,8,11,12,14,15-^{3}H(n)]PGE_2, [5,6,8,11,12,14,15-^{3}H(n)]$PGF_{2\alpha}$, [5,6,8,9,12,14,15-^{3}H(n)]PGD_2, [1-^{14}C]PGE_2 and [1-^{14}C]$PGF_{2\alpha}$. The purity of the radiochemicals was shown to be greater than 97%.

Materials

Sep-Pak C_{18} and Sep-Pak silica cartridges and HPLC-solvent filters HA (0.45 μm) and FH (0.5 μm) were obtained from Waters Assoc.; prepacked HPLC columns Nucleosil $5C_{18}$ and Zorbax $BPtmC_8$ (each 250 × 4.6 mm) were from Chrompack (Middelburg, The Netherlands).

Antisera

Anti-TxB_2, anti-$PGF_{2\alpha}$, anti-PGE_2 and anti-PGD_2 were obtained from l'Institut Pasteur (Paris, France); anti-6-keto-$PGF_{1\alpha}$ was obtained from Seragen (Boston, MA, U.S.A.). Cross-reactivities are given in Table I.

TABLE I

CROSS-REACTIVITIES OF COMMERCIALLY OBTAINED ANTIBODIES

Data are expressed in per cent at 50% B/B_0 (B = bound).

Compound	Antibody				
	6-Keto-$PGF_{1\alpha}$	TxB_2	$PGF_{2\alpha}$	PGE_2	PGD_2
6-Keto-$PGF_{1\alpha}$	100	—	0.04	<0.01	0.01
$PGF_{1\alpha}$	7.8	—	12.0	0.01	0.01
$PGF_{2\alpha}$	2.2	0.1	100	0.11	0.04
6-Keto-PGE_1	6.8	—	—	0.16	—
PGE_1	0.7	—	0.03	10.7	0.01
PGE_2	0.6	0.1	0.03	100	0.01
PGA_1	<0.01	—	<0.01	0.04	—
PGA_2	<0.01	0.1	<0.01	0.3	—
PGD_2	<0.01	0.2	3.0	<0.01	100
PGD_1	—	—	<0.4	<0.01	78
TxB_2	<0.01	100	<0.01	<0.01	1.2
13,14-Dihydro-15-keto-PGE_2	<0.01	0.1	—	0.6	0.01
13,14-Dihydro-15-keto-$PGF_{2\alpha}$	<0.01	0.1	<0.01	<0.01	0.01
13,14-Dihydro-PGE_2	—	—	—	2.1	—
6-Keto-PGE_2	—	0.1	<0.01	13.2	—

Human lung tissue

Human lung tissues were obtained from adults [5]. Lobectomy was performed on account of tumours. The premedication consisted of 0.25 mg of atropine and 10 mg of Opial®. During the operation, the following agents were administered: Fentanyl® (0.5 mg), pancuronium bromide (Pavulon®, 6 mg), thiopental (Pentotal®, 150 mg) and 3 g of cephalothin (Keflin®). Parenchymal tissue of the outer parts was used in our experiments.

Method

Human lung tissue (10 g) from which the lung membrane had been removed was cut into slices. The slices were chopped in a McIlwain tissue chopper and divided in portions of 0.5 × 0.5 mm. The whole fraction was washed three times with Krebs–Henseleit buffer (20 ml of buffer, 5 min, 400 *g*) in a polypropylene 50 ml tube (Falcon®). Then 20 ml of Krebs–Henseleit buffer were added and the tube was placed in a water-bath of 37°C on a magnetic stirrer (900 rpm). Through a thin pipette, the sample was continuously gassed with a

mixture of 95% O_2 + 5% CO_2. Thereafter, 10 μCi of [1-^{14}C]AA (55 mCi/mmol), glutathione (final concentration 2 m*M*) and 100 μg of Ca-ionophore A23187 (dissolved in 100 μl of ethanol) were added. At the end of the 10-min incubation, [^{3}H]LTs and [^{3}H]PGs were added and the homogenate was centrifuged (10 min, 1400 *g*, 4°C). The pellet was washed once, and the combined supernatant centrifuged (90 min, 30,000 *g* average, 4°C) to separate the cells and small particles. The clear incubation supernatant was then applied to a Sep-Pak C_{18} cartridge and the effluent was placed on a Sep-Pak silica cartridge. (The C_{18} cartridge was prewashed with 10 ml of methanol and 10 ml of distilled water; the silica cartridge was prewashed with 10 ml of methanol and 100 ml of water [10].) The sample was eluted with 2.5 ml of methanol on each column; these eluates were combined and evaporated to dryness with a gentle stream of nitrogen at 40°C. Thereafter, the dried sample was dissolved in 1 ml of solvent A (tetrahydrofuran—methanol—water—acetic acid, 25:30:45:0.1, v/v, adjusted to pH 5.5 with ammonium hydroxide), filtered and kept in a siliconized micro-vial.

Chromatographic system

Reversed-phase HPLC of LTs and other lipoxygenase products was carried out on a Nucleosil 5 C_{18} column, using solvent system A. Mobile phases were filtered by vacuum filtering through a Millipore filter and degassed with helium [11, 12]. The flow-rate was 0.9 ml/min and the absorption was measured at 280 nm. Prior to use, the system was washed with approx. 15 ml of water, thereafter with approx. 30 ml of a 2% (w/v) EDTA solution in water, and rewashed with water [10]. The column was equilibrated with the mobile phase A at an oven temperature of 37°C. Fractions were collected for scintillation counting. After each run (90 min) the column was rinsed for at least 30 min because of contamination with Ca-ionophore, which elutes after approx. 115 min.

Reversed-phase HPLC of PGs was performed on a Zorbax C_8 column. This solvent system (B) contained acetonitrile—benzene—water—acetic acid (24:0.2:0.1:76, v/v). The flow-rate of this eluent was 2.0 ml/min. From the contents of each collected fraction, 50 μl were taken and kept at 4°C for the radioimmunoassay (RIA) of the PGs. The main fraction was immediately used for ^{14}C and ^{3}H counting. The column was rinsed with acetonitrile for 30 min after each sample to elute the lipoxygenase products.

Radioimmunoassay of prostaglandins

Reagents were equilibrated to room temperature before use. After the addition of standards (range 0—500 pg) and diluted samples, [^{3}H]PG and antibody were added. At the end of the incubation (2 h at room temperature and 18 h at 4°C), charcoal suspension was added. The tubes were allowed to stand for 15 min at 4°C and centrifuged for 10 min at 1400 *g*. The supernatants were decanted and mixed with 6 ml of scintillation fluid. The risk of cross-reactions was negligible when RIA was performed after HPLC (cross-reactivities and specifications are given in Tables I and II).

TABLE II

SPECIFICATIONS OF VARIABLE CONDITIONS IN THE RIAs

Immunogen	Amount of added tracer		Bound/total (%)	Non-specific binding (%)
	dpm	pg		
6-Keto-$PGF_{1\alpha}$	16,000	18.4	39.7	9.2
TxB_2	15,000	18.5	57.9	1.9
$PGF_{2\alpha}$	8,500	9.2	17.0	4.5
PGE_2	12,500	13.0	40.1	3.9
PGD_2	8,500	10.7	34.3	7.4

Quantitative evaluation

The settings for double-labelled scintillation counting were such that there was no spillover of radioactivity of ^3H into the ^{14}C channel. Calculations of dpm were carried out using quenched standard sets by a computer (Digital, PDP 11/70). For daily analysis, a plotting system was programmed in order to obtain data of total counts covering the peak areas. Amounts calculated in dpm of both channels were plotted as separate chromatograms.

The data obtained from the RIAs were linearly plotted as the ln (mass) against the negative (—) ln $\{\%B/(100 - \%B)\}$ (where B=bound). The linearity was tested by means of a variance analysis.

RESULTS AND DISCUSSION

Fig. 1 shows a representative chromatogram (one of five experiments) of LTs and other lipoxygenase products, after [^{14}C]AA labelling of the lung tissue. The upper part of the figure represents the mass, measured by absorption at 280 nm. It is evident that substances are present with the same retention times (t_R) as LTs, indicated by C, D and B. The major compound is LTB_4, whereas LTC_4 is hardly detectable because of the strong tailing effect, caused by substances with identical chromatographical properties to some of the phospholipids, running on the front. Based on the retention time, peak 1 is tri-HETE, and peak 2 is 6-*trans*-LTB_4 + 12-*epi*,6-*trans*-LTB_4, according to Verhagen et al. [12].

The lower curve of the HPLC separation gives the plotted ^{14}C-labelled fractions. Peak 1 covers both the above-mentioned tri-HETE and a substance with the same t_R as LTC_4. Prostaglandins, however, nearly cochromatograph with LTC_4, so that a not unimportant part of peak 1 is due to the presence of cyclooxygenase products. The compound indicated by H is most probably HHT (12-OH-5,8,10-heptadecatrienoic acid). The identification of this peak was based upon the following observation: washed rat platelets were labelled with [^{14}C]AA as described before [13], aggregated with collagen, extracted as described above and applied to HPLC for further analysis. Our earlier observations on platelet aggregation indicate that the main compounds formed are, respectively, 12-HETE, HHT and TxB_2. A similar result was obtained by Luderer et al. [14]. As in our system, TxB_2 cochromatographs with LTC_4 and the retention time of 12-HETE appeared to be approx. 60 min; the peak at

26 min is likely to be HHT. In the example shown in Fig. 1, relatively large amounts of HETEs are formed.

Table III lists the recoveries (mean ± S.E.M.) of ^{3}H-labelled standards added to tissue samples. It is remarkable that the overall recoveries of HETEs are low, especially of 5-HETE. The chromatogram given at the bottom of Fig. 1

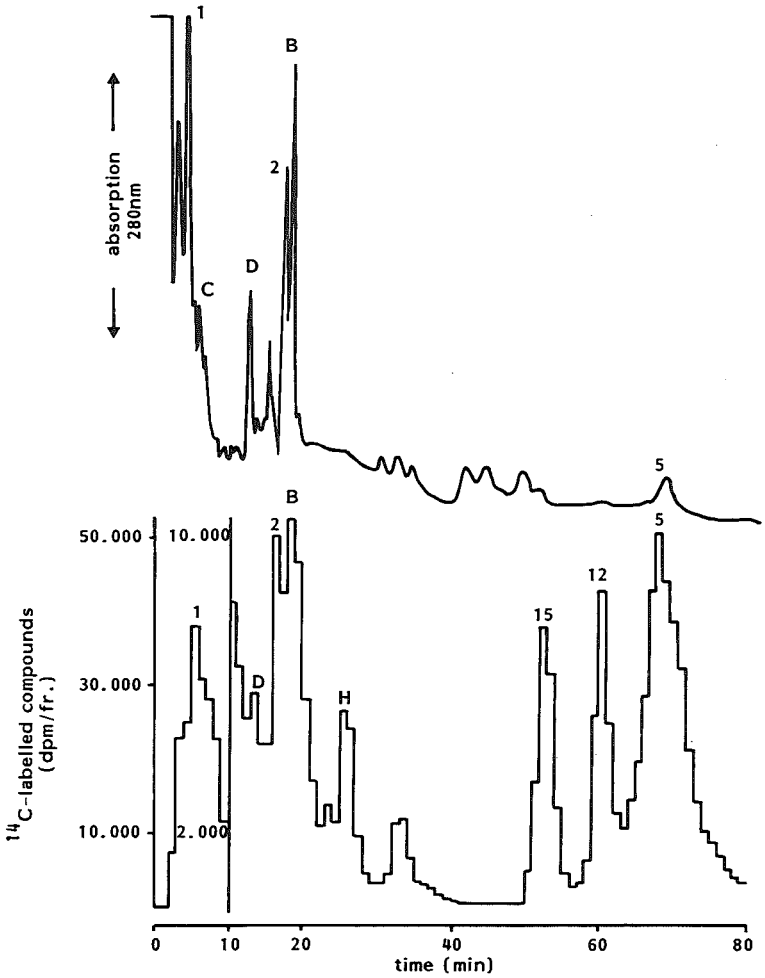

Fig. 1. Chromatograms of LTs and HETEs, synthesized by chopped human lung tissue, after [^{14}C]AA loading, in the presence of glutathione (2 m*M*) and Ca-ionophore A23187 (approx. 10 μM). The incubation medium was processed through Sep-Pak C_{18} and silica cartridges as described in Experimental and the methanol fractions after evaporation to dryness were subjected to HPLC in solvent system A. A Chrompack Nucleosil $5C_{18}$ column (250 × 4.6 mm) was used. Mobile phase: tetrahydrofuran—methanol—water—acetic acid (25:30:45:0.1) adjusted to pH 5.5 with ammonium hydroxide. Flow-rate 0.9 ml/min. The chromatogram at the bottom, representing radioactivity in fractions of one per min, is corrected for delay time between the absorption cell and the fraction collector. Peaks: C = LTC_4, D = LTD_4-like, B = LTB_4, 15 = 15-HETE, 12 = 12-HETE and 5 = 5-HETE. Based on retention times, 1 = tri-HETE, 2 = 6-*trans*-LTB_4 + 12-*epi*,6-*trans*-LTB_4.

TABLE III

RECOVERIES OF TRITIATED LTs, HETEs, AND PGs, MEASURED BY HPLC

Data were obtained after the extraction procedure as described in the methods section. Values are given as the mean ± S.E.M.

	Recovery (%)	*n*
LTC_4	59 ± 5.0	3
LTD_4-like	86 ± 1.5	3
LTB_4	70 ± 5.7	3
15-HETE	34 ± 1.1	3
12-HETE	34 ± 0.9	3
5-HETE	18 ± 0.6	3
6-Keto-$PGF_{1\alpha}$	64 ± 2.9	8
TxB_2	86 ± 2.7	8
PGF_2	44 ± 1.8	8
PGE_2	73 ± 2.9	8
PGD_2	59 ± 2.6	8

is not corrected for recoveries listed in Table III; in this case the plotted quantity of 5-HETE should be three times higher compared to the leukotrienes.

Furthermore, there is a pronounced difference in recovery of certain PGs obtained from HPLC separations as shown in Fig. 2. Fig. 2A shows the internal PG standards (^3H), fig. 2B the ^{14}C-labelled PGs formed from exogenous AA. The small peak 2 shows the immunoreactivity of 6-keto-$PGF_{1\alpha}$, and the wide peak 5 that of TxB_2. However, peak 6 (PGF-immunoreactive), peak 7 (PGE-immunoreactive) and peak 8 (PGD-immunoreactive) do not have the same t_R as the added ^3H standards. The longer the t_R, the greater the delay between ^3H-labelled and unlabelled material.

Determination of the amounts present in the sample by the different RIAs is represented in Fig. 2C. In this case, the immunoreactivities of the PGs mentioned before have the same t_R as the ^{14}C-labelled compounds. Others have noticed that a difference exists between the t_R of PGs labelled with ^3H or ^{14}C [15]. This might be due to the isotope effect. Four double bounds are labelled with ^3H whereas ^{14}C only is labelled on the 1-position. Increase of the mass plays an unimportant role, because spheric occlusion occurs at molecular weights of 2000 and higher (MW_{PG} approx. 350). It would appear that labelling with ^3H makes these substances more hydrophilic. Fig. 3 confirms the findings presented in Fig. 2. Commercially available ^3H- and ^{14}C-labelled PGE_2 and $PGF_{2\alpha}$ have a difference in retention times of approx. 5%. This could be a reason of errors in selecting the wrong fractions for RIA when ^3H-labelled standards are used as markers.

A second observation that needs further explanation is the difference that occurs in the specific activities of the PGs. This can be calculated from the data presented in Fig. 2. The pattern of ^{14}C-labelled and RIA-determined amounts is not identical. In this case, there is even a difference by a factor of 4 between the highest and the lowest specific activity (expressed as dpm/ng, covering the whole peak area). Peaks 3 and 4 in Fig. 2B are unknown. 13,14-Dihydro-15-keto-PGs run after 80 min, so that differences are not caused by these compounds.

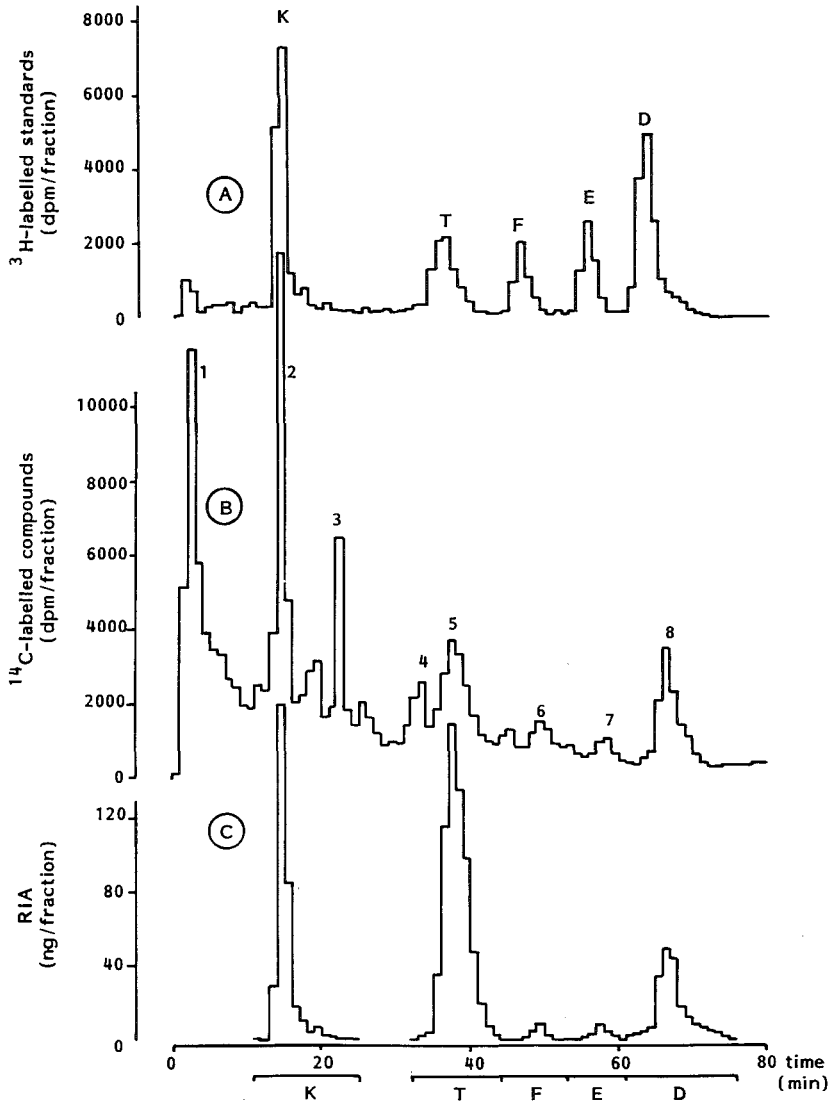

Fig. 2. Reversed-phase HPLC separation of PGs in the same sample as used in Fig. 1. A Chrompack Zorbax BP5 column was used; the solvent system contained acetonitrile—benzene—water—acetic acid (24:0.2:76:0.1); the flow-rate was 2 ml/min. One fraction per min was collected and divided as described in the text. (A) Chromatogram of [^{3}H]PG standards. Peaks: K = 6-keto-$PGF_{1\alpha}$, T = TxB_2, F = $PGF_{2\alpha}$, E = PGE_2 and D = PGD_2. (B) Chromatogram of ^{14}C-labelled compounds. Identification of peaks 1—8 is discussed in the results section. (C) Measurement of the fractions in (A) by RIA.

Fig. 4 shows the chromatograms of an experiment in which ^{3}H-labelled LTs were used as markers, without the addition of [^{14}C]AA to the tissue. Several problems arise when the LTs in the tissue are identified with the use of ^{3}H-labelled substances. This difficulty is due to the fact that in LTC_4 and LTD_4

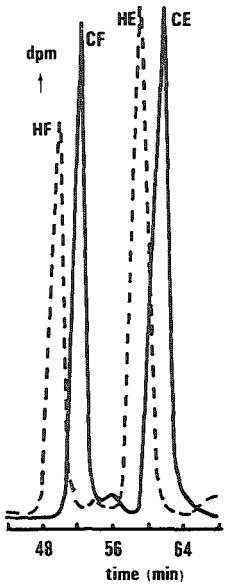

Fig. 3. Chromatogram of collected fractions (two per min) after the injection of ^{3}H- and ^{14}C-labelled $PGF_{2\alpha}$ and PGE_2. The chromatographic conditions were the same as described in Fig. 2. This chromatogram was plotted by means of a computer-programmed XY printer. Peaks: HF = [^{3}H]$PGF_{2\alpha}$, CF = [^{14}C]$PGF_{2\alpha}$, HE = [^{3}H]PGE_2, CE = [^{14}C]PGE_2.

one double bound is ^{3}H-labelled, and in LTB_4 this amount is four. The latter is comparable with the labelled PGs (see section on radiochemicals). In Fig. 4A, peaks 2 and 5 have approximately the same t_R as in Fig. 4B. The delay is only ± 2%. However, the difference is much greater between peak 6 of Fig. 4A and peak 8 of Fig. 4B. Batch 9 of the [^{3}H]LTB_4 used in the experiment as shown in Fig. 4A proved to be a racemic mixture of 6-*trans*-LTB_4 and 12-*epi*,6-*trans*-LTB_4. We thereafter injected both batch 9 and the newly prepared batch 10 directly into the HPLC system. The t_R of the first peak was the same as the one obtained in Fig. 4A after extraction procedures. The second peak (batch 10) had a delay of approx. 1 min. Compared to Fig. 4B, these two peaks were shifted 8% t_R. Peaks 3, 5 and 7+8 were collected in order to compare the biological activity of the so far unknown compound 5. The fractions were evaporated to dryness, dissolved in Krebs' buffer and added to a guinea pig lung parenchymal strip, as described before [5]. Expressed in factors of potency, the biological activities were respectively: peak 3 (LTC_4-like), 20; peak 5, 5; peak 7+8 (LTB_4-like), 1. The unknown peak 5 could be LTE_4 on the basis of its t_R [9, 16] and activity [9, 17, 18].

Finally, we determined the presence of glutamine and glycine in the hydrolysed fraction, compared to standards of LTC_4 (containing cysteine—glycine—glutamine) and LTD_4 (containing cysteine—glycine), following the method as described earlier [19]. In this fraction 5, a relatively small amount of glycine was present. We concluded from these data that this compound may be LTE_4*.

*During the preparation of the manuscript, synthetic LTE_4 was obtained. It shows the same t_R as the compound described here.

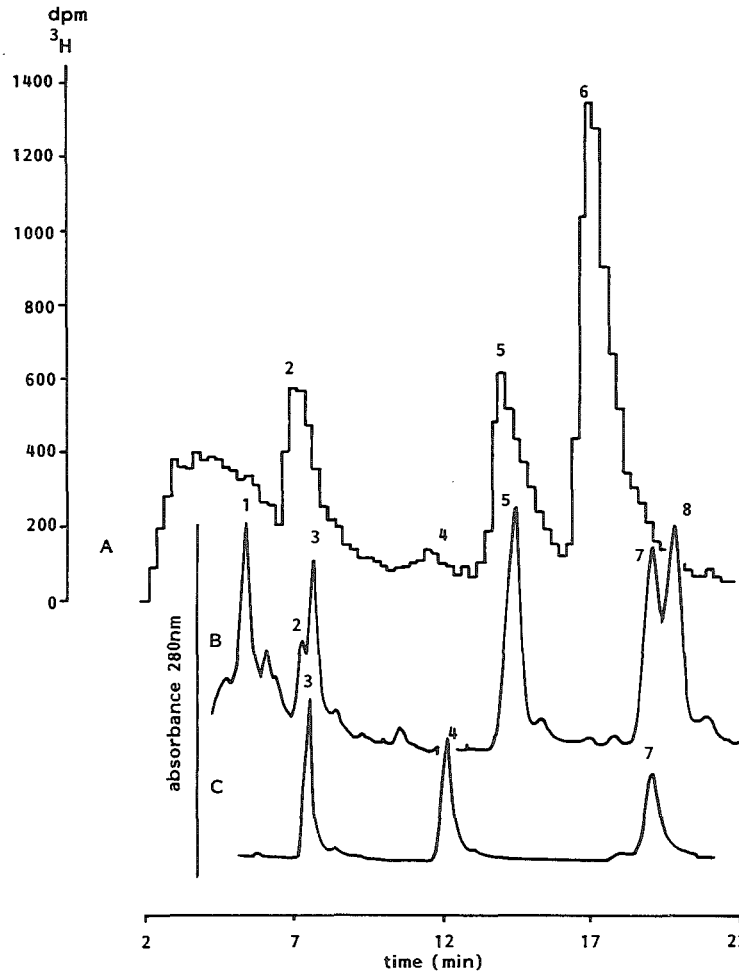

Fig. 4. Reversed-phase HPLC separation of LTC_4, LTD_4 and LTB_4. (A) ^{3}H-Labelled LTs were added to a non-labelled incubation medium of chopped human lung tissue, triggered with Ca-ionophore as described in Experimental. The numbers 2, 5 and 6 indicate the ^{3}H-labelled LTC_4, LTD_4 and LTB_4 (batch 9), respectively. (B) Measurement of the absorbance at 280 nm of the same tissue extract as mentioned in (A). (C) Chromatogram of synthetic LTC_4 (3), LTD_4 (4) and LTB_4 (7, a degradation product?), directly applied to the reversed-phase HPLC system.

When [^{3}H]LTD_4 was injected directly onto the HPLC column, the t_R was the same as peak 4 in Fig. 4A.

CONCLUSIONS

Whole human lung tissue, stimulated with Ca-ionophore A23187, produced under the conditions described, large amounts of LTB_4-like compounds and less LTC_4 and LTD_4. The major PG-like substances were immunoreactive with TxB_2, 6-keto-$PGF_{1\alpha}$ and PGD_2. The role of these different compounds has not yet been established. The formation of comparatively high amounts of TxB_2

and PGD_2 in antigen-challenged human lung tissue has been observed [20]. TxA_2 has bronchoconstrictor activity. The differences observed in the specific activities of the PGs may indicate that these substances are not formed from the same AA pool. Identification of AA metabolites could give problems when tritiated standards are used, due to the decrease of retention times. Whether recently available tritiated LTs are suitable for receptor binding studies and other specific interactions is doubtful as long as it remains uncertain that these compounds are pure LTs and not chemically degraded products.

The conversion of LTC_4 in LTD_4 by γ-glutamyltranspeptidase is inhibited by serine–borate complex [21]. Although in receptor binding studies in guinea pig lung, [^{3}H]LTD_4 was not metabolized to LTE_4 [22], this bioconversion was demonstrated in experiments with guinea pig ileum. In the presence of L-cysteine, the conversion of LTD_4 to LTE_4 was largely inhibited [23]. In future work, the effect of a 5-lipoxygenase inhibitor on the formation of PGs will be investigated, as well as the differences in AA metabolism of lung tissue obtained from asthmatics and non-asthmatics [24].

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We appreciate the assistance of Mr. T. van Immerzeel (Department of Computing Systems) and Mr. W.F. Clotscher (Department of Biochemistry) for the development of computer programs. Dr. D. Fekkes is thanked for determinations of glycine and Mrs. B.H.M. Busscher-Lauw for typing the manuscript, Dr. J. Rokach (Merck Frosst Labs., Canada) for gifts of leukotrienes and Dr. J.B. Smith (Philadelphia, PA, U.S.A.) for standards of TxB_2 and 6-keto-$PGF_{1\alpha}$. Without the help of Dr. P.A. Frederikz (Thoraxcentre, Academic Hospital Dijkzigt) from whom parts of human lungs were obtained, our investigations would not have been possible.

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4.2.2

FORMATION OF PROSTAGLANDINS AND LEUKOTRIENES BY HUMAN LUNG TISSUE IN VITRO AFTER ACTIVATION BY THE CALCIUM IONOPHORE A23187

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Formation of prostaglandins and leukotrienes by human lung tissue *in vitro* after activation by the calcium ionophore A23187

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Abstract. The formation of metabolites of arachidonic acid by the cyclo-oxygenase and lipoxygenase pathways were determined in human lung tissue, obtained from surgery. In this measurement the chopped tissue was incubated with the calcium ionophore A23187. Formation of metabolites from [1-^{14}C] arachidonic acid was also determined. The metabolites were extracted, separated by HPLC and identified by measurement of the absorption spectrum at 280 nm, radioactivity, biological activity and by radioimmunoassay.

6-keto-prostaglandin $F_{1\alpha}$ (6-ketoPGF$_{1\alpha}$), the metabolite of prostacyclin, is the cyclo-oxygenase product present in the highest amount (400 ± 49 ng g^{-1}), followed by PGD_2 (162 ± 59 ng g^{-1}) thromboxane B_2 (102 ± 32 ng g^{-1}) PGE_2 (104 ± 46 ng g^{-1}) and $PGF_{2\alpha}$ (58 ± 26 ng g^{-1}). The amounts of the lipoxygenase products are: leukotriene B_4 (LTB_4), 163 ± 100 ng g^{-1}; LTC_4, 63 ± 31 ng g^{-1} and LTE_4 121 ± 34 ng g^{-1}. From [1-^{14}C] arachidonic acid higher amounts of the cyclo-oxygenase than of the lipoxygenase products were formed, with the exception of PGE_2.

The effects of several of these substances on the contraction of human small airway smooth muscle were measured. The contractions, induced by equivalent amounts of LTC_4 and a synthetic analogue of thromboxane T × A_2 were approximately one hundred times those induced by PGD_2, $PGF_{2\alpha}$ and histamine.

These results suggest that thromboxane A_2 and LTC_4 are the most important arachidonic acid metabolites that induce bronchoconstriction in the human lung.

Keywords. Prostaglandins, leukotrienes, thromboxane A_2, human lung tissue, human small airway smooth muscle.

Introduction

The metabolites formed from arachidonic acid by the cyclo-oxygenase and lipoxygenase pathway have numerous effects on the contractility and other properties of lung tissue. Some of these actions resemble those that occur during asthma and related diseases. For this reason the possible role of prostaglandins and leukotrienes as mediators of the pathological changes occurring in these diseases seems of interest. When lung tissue is stimulated by an immunological reaction, phospholipase C and A_2 are activated, resulting in the release of arachidonic acid and the formation of the metabolites. Inhalation of PGE_2 induces relaxation of bronchial smooth muscle in asthma patients. $PGF_{2\alpha}$ induces a bronchoconstriction, both in normal and asthmatic patients. The latter are particularly sensitive to this compound [1]. Recently, it has been demonstrated that PGD_2 also has a potent bronchoconstrictive effect in humans [2]. TxA_2 has a similar activity [3]. All these compounds are formed by lung tissue after the induction of an anaphylactic reaction [4].

It is likely that one or more of the substances formed through the lipoxygenase pathway also participate in the pathogenesis of asthma and related diseases. The effects of LTB_4 are the induction of vascular permeability and enhanced granulocyte adherence and migration. This results in peripheral neutropenia and leucocyte accumulation [5]. LTC_4 and LTD_4 induce bronchoconstriction, reduce the clearance of mucus and increase vascular permeability [7].

The formation of PG and LT by human lung tissue, after activation by the calcium ionophore A23187, was determined in these experiments. This is a measure of the formation from arachidonic acid present in the tissues. The formation after labelling the tissue with [1-^{14}C] arachidonic acid was also determined.

Patients and methods

Patients

Lung tissue was obtained from ten patients (aged 53–75) undergoing surgery on account of bronchial carcinoma. The following drugs were administered: in premedication, atropine and Opial® and during the operation, Fentanyl, pancuronium bromide, thiopental and cephalothin.

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Methods

Preparation of lung tissue. Immediately after surgery, a macroscopically normal part of the tissue that was not connected with the tumour was dissected free from major blood vessels and bronchi. Tissue (8–10 g) was chopped and erythrocytes removed after lysis by washing. After centrifugation, 20 ml Krebs buffer was added to the pellet and the mixture gassed with carbogen (95% O_2+5% CO_2). One microcurie [1-^{14}C] arachidonic acid per gram, 100 μl glutathione (final concentration 1 mM) and 100 μl A23187 (final concentration 10 μM) were added. After 10-min incubation, 100 μl serine (final concentration 0·25 mM) were added and after 15-min incubation 50 μl of a mixture of [^{3}H]LT and [^{3}H]PG. Thereafter, the suspension was centrifuged at 1600 × *g*. The supernatant was decanted. Twenty millilitres Krebs buffer was added to the pellet and again centrifuged at 1600 × *g*. The supernatants were centrifuged for 1 h at 30 000 × *g* at 4°C. The clear supernatant was then applied to a SEP-PAK C_{18} and a SEP-PAK silica cartridge. The cartridges had been prewashed with methanol and water. Prior to the elution, the cartridges were washed with 5 ml water and then the samples eluted with 5 ml methanol. The elutates were combined and dried in a stream of N_2 at 40°C. The dried sample was then dissolved in 1 ml of solvent A (tetrahydrofuran-methanol-0·1% EDTA in water–acetic acid, 25:30:45:0·1 v/v, adjusted to pH 5·5), filtered and kept in a polypylene micro vial. Reversed phase HPLC of LTs was performed on a Nucleosil $5C_{18}$ column using solvent system A and for PGs on a Zorbax C_8 column with solvent system B (acetonitrile–benzene–water–acetic acid, 24:0·2:76:0·1 v/v) The separation and identification of the fractions have been described before [8].

In the identification of the substances present in the lung extract obtained after separation by HPLC, the following methods were used: (i) the absorption at 280, 270 and 234 nm, (ii) the measurement of the radioactivity, (iii) the determination of the quantity by RIA, (iv) the determination of the biological activity, (v) the measurement of the absorption spectrum at different wave lengths. In this way both the formation from endogenous and exogenous arachidonic acid has been determined.

Chemicals. LTB_4, LTC_4, LTD_4 and LTE_4 were gifts from Dr J. Rokach (Merck Frosst, Canada). PGD_2, PGE_2, $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and TxB_2 were obtained from Sigma, U.S.A., the thromboxane mimetic U-44069 (9, 11 didioxy, 9, 11-epoxy methano prostaglandin $F_{2\alpha}$) from Upjohn, U.S.A. and methacholine hydrobromide and histamine hydrochloride from Janssen Pharmaceuticals, Belgium. Siliclad® was obtained from Clay Adams (Beckton Dickinson, U.S.A.). Tetrahydrofuran, methanol, acetonitrile, benzene and acetic acid were all of analytical grade and obtained from E. Merck, F.R.G. Picofluor was used as a premixed scintillation cocktail and was obtained from Packard, Belgium.

Radiochemicals. [1-^{14}C] arachidonic acid (specific activity 55 mCi mmol^{-1}) was purchased from New England Nuclear. The following compounds were obtained from the Radiochemical Centre (Amersham, U.K.): [5,6,8,9,11,12,14,15-^{3}H(*n*)] LTB_4 (210 Ci mmol^{-1}), [14,15-^{3}H(*n*)] LTC_4 (36 Ci mmol^{-1}), [14,15-^{3}H(*n*)] LTD_4 (42 Ci mmol^{-1}), 6-keto-[5,8,9,11,12,14,15-^{3}H(*n*)] $PGF_{1\alpha}$ (150 Ci mmol^{-1}), [5,6,8,9,11,12,14,15-^{3}H(*n*)] TxB_2 (177 Ci mmol^{-1}), [5,6,8,11,12,14,15-^{3}H(*n*)] PGE_2 (160 Ci mmol^{-1}), [5,6,8,11,12,14,15-^{3}H] $PGF_{2\alpha}$ (160 Ci mmol^{-1}) and [5,6,8,9,12,14,15-^{3}H(*n*)] PGD_2 (184 Ci mmol^{-1}). The purity of the radiochemicals was shown to be greater than 97%.

Materials. SEP-PAK C_{18} and silica cartridges and HPLC-solvent filters HA (0·45 μm) and FH (0·5 m) were obtained from Waters Associated and prepacked HPLC columns Nucleosil $5C_{18}$ and Zorbax $BPtmC_{18}$ (each 250 × 4·6 mm) from Chrompack (Middleburg, The Netherlands). HPLC micro vials were obtained from Weichmann Plastics, Switzerland.

Anti-sera. Anti-TxB_2, anti-$PGF_{2\alpha}$, anti-PGE_2 and anti-PGD_2 were obtained from l'Institut Pasteur (Paris, France); anti-6-keto-$PGF_{1\alpha}$ from Seragen (Boston, U.S.A.) and anti-LTB_4 from Wellcome. The cross-reactivities of the PG have been reported earlier [8]. Those of LTB_4 are given in Table 1.

Radioimmunoassay. The determination of the PG has been described before [8]. The method of Salmon was used in the determination of LTB_4 [9].

Bioassay. Biological activities of leukotrienes formed by human lung tissue and separated by HPLC were measured on a guinea-pig lung parenchymal strip [3]. Samples of fractions were dried under nitrogen, dissolved in Krebs buffer and applied to a strip. The contractile response was measured and compared with the amounts calculated by UV-monitoring of the HPLC fractions.

Table 1. Cross-reactivities of the LTB_4-antibody. Data are expressed in per cent at 50% B/B_0 (B = bound)

LTB_4	100
LTC_4	0·03
LTD_4	0·03
5-HETE	0·03
12-HETE	2·0
15-HETE	<0·03
PG	<0·03
Arachidonic acid	<0·03

Measurement of human small airway smooth muscle contractions. This method has been described earlier [10]. In brief, bronchioles are dissected free from parenchyma and blood vessels and extensively washed to remove anaesthetics. Six bronchiolar strips were mounted in 10-ml double-jacketed organ baths containing aerated buffer at 37°C. The contractile responses were measured isotonically using Penny & Giles transducers. Cumulative concentration–response curves were prepared by adding agonists in small volumes. The concentration of the agonist that produced 50% of the maximal contraction was determined (EC_{50}).

Results

In the lung tissue, after incubation with A23187, extraction and separation of the lipoxygenase products, LTB_4, LTC_4 and LTE_4 were present (see Fig. 1.). The other two peaks have not yet been identified; they may represent hydroxylated derivatives, such as 20-OH LTB_4. This represents the formation from arachidonic acid present in the tissue. In another experiment, the tissue was incubated with [1-^{14}C] arachidonic acid and A23187 and the lipoxygenase products determined. In the determination of the PG, the substances formed after incubation with either A23187 or [1-^{14}C] arachidonic acid and A23187 were separated. The result is shown in Fig. 2. The metabolite of prostacyclin (6-keto-$PGF_{1\alpha}$) and PGD_2 are present in the highest amounts. The formation of the eicosanoids is given in Table 2. Only the production of LTC_4 from arachidonic acid present in the tissue is given, not the one from the labelled substance. This is due to the overlapping of the curves for PG and LTC_4 in the chromatogram of the labelled compounds in our system.

In order to compare the bronchoconstrictive activity of the substances with the amounts formed by the lung tissue, the effects of LTC_4, the thromboxane analogue U-44069, PGD_2, $PGF_{2\alpha}$, histamine and methacholine on the contraction of human small airway smooth

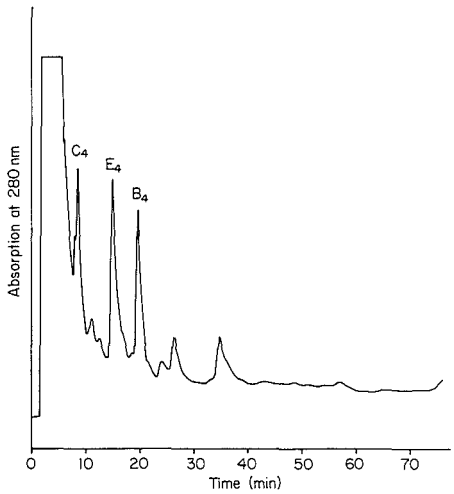

Figure 1. Chromatogram of LT, synthesized from chopped human lung tissue, after incubation with the calcium ionophore A23187 (approximately 10 μM) in the presence of glutathione (1 mM). Separation was obtained by means of a Nucleosil $5C_{18}$ column. Mobile phase: tetrahydrofuran–methanol–acetic acid–0·1% (w/v) EDTA in water, pH 5·5 (25:30:0·1:45). Flow rate 0·9 ml min^{-1}; oven temperature 37°C; absorption: 0·06 AUFS—at $t=0$, 280 nm; at $t=15$, 270 nm; at $t=30$, 234 nm.

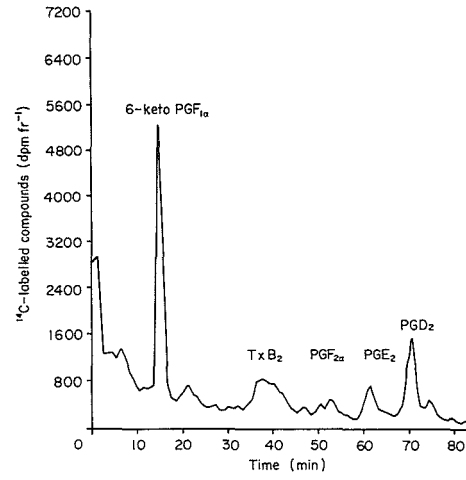

Figure 2. Reversed-phase HPLC separation of [1-^{14}C] PG. A Zorbax BP5 column was used; the solvent system contained acetonitrile–benzene–water–acetic acid (24:0·2:76:0·1); the flow rate was 2 ml min^{-1}, oven temperature 37°C. One fraction per minute was collected, counted and plotted.

Table 2. Formation of arachidonic acid metabolites in human lung tissue activated by A23187

	Formation from arachidonic acid in the tissue (ngg^{-1})	Formation from added [1-^{14}C] arachidonic acid (ngg^{-1})
$6kPGF_{1\alpha}$	400±49	44±8·5
TxB_2	102±32	15±1·9
$PGF_{2\alpha}$	58±26	11±4·0
PGE_2	104±46	6±1·2
PGD_2	162±59	36±15
LTB_4	163±100	10±3·3
LTC_4	63±31	ND
LTE_4	121±34	5±1·4

The values are expressed as means±SEM.
Patients were non-smokers and had no chronic obstructive pulmonary disease, $n=7$.

In order to compare the bronchoconstrictive activity of the substances with the amounts formed by the lung tissue, the effects of LTC_4, the thromboxane analogue U-44069, PGD_2, $PGF_{2\alpha}$, histamine and methacholine on the contraction of human small airway smooth

Table 3. EC_{50} values for LTC_4, the thromboxane mimetic U-44069, PGD_2, $PGF_{2\alpha}$, histamine and methacholine, obtained from the curves of Fig. 3.

Agonist	EC_{50}	SEM
LTC_4	$5{\cdot}89\times10^{-9}$	$\pm 0{\cdot}47\times10^{-9}$
U-44069	$1{\cdot}23\times10^{-8}$	$\pm 0{\cdot}17\times10^{-8}$
PGD_2	$4{\cdot}01\times10^{-6}$	$\pm 0{\cdot}42\times10^{-6}$
$PGF_{2\alpha}$	$1{\cdot}35\times10^{-5}$	$\pm 1{\cdot}06\times10^{-5}$
Methacholine	$1{\cdot}45\times10^{-6}$	$\pm 0{\cdot}30\times10^{-6}$
Histamine	$9{\cdot}80\times10^{-7}$	$\pm 4{\cdot}48\times10^{-7}$

Results expressed as means ± SEM.

muscle were compared (Table 3). The contractions are expressed as a percentage of the maximum value of methacholine. LTC_4 and the thromboxane analogue U-44069 are one hundred times more potent than PGD_2, $PGF_{2\alpha}$ and histamine.

Discussion

The order of the amounts of the cyclo-oxygenase products formed in the lung tissue for the endogenous production is: 6-keto-$PGF_{1\alpha}$, the metabolite of prostacyclin, PGD_2, PGE_2, TxB_2, $PGF_{2\alpha}$. It is conceivable that, when lungs are activated *in vivo* by an immunological process, the tonus of the bronchial smooth muscle depends, insofar as the cyclo-oxygenase products are concerned, on the relative amount of PGE_2, with a relaxing effect on one side; and of PGD_2, $PGF_{2\alpha}$ and TxB_2, which act as bronchoconstrictors on the other side. LTC_4 and LTE_4 also induce bronchoconstriction and LTB_4 to a much lesser extent. The effect of LTE_4 is lower than that of LTC_4, as measured on the guinea-pig parenchymal lung strip [11]. In the chromatogram of Fig. 1, no peak representing LTD_4 is present. In guinea-pig lung LTD_4 is formed. The reason for the absence of the formation in human lung is not known.

The activity of these substances has also been tested on human small airway smooth muscle. The TxA_2 mimetic U-44069 and LTC_4 have similar effects and are approximately one hundred times more potent than PGD_2, $PGF_{2\alpha}$ and histamine. An example of the contractions obtained is shown in Fig. 3. In other experiments it has been demonstrated that 115 ng TxA_2, formed by aggregating blood platelets, contract a human lung strip [3]. This is of the same order of magnitude as the amount formed per gram of lung tissue. These results indicate that the contributions of PGD_2 and $PGF_{2\alpha}$ are of minor importance. Eventually, other activities than bronchoconstriction by these substances could play a role.

The amount of LTC_4 formed in the tissue activated by the ionophore is sufficient to induce contractions in the human lung strip. A 50% contraction is obtained with approximately 40 ng (Fig. 3). In this experiment the effect of exogenously added compounds is measured. It was shown that the effect of TxA_2 formed inside the tissue is considerably higher than that of the exogenously added compound [12]. For this reason the contractile effects of the PG and LT formed in the lung are most probably higher than those indicated in Fig. 3.

In the experiments reported here, the overall effect of lung tissue on PG and LT synthesis after calcium ionophore-induced activation is measured. It has been shown that several types of cells contribute to the formation of the PG and LT. Activated human lung mast cells form mainly PGD_2, LTC_4 and LTB_4 [13]. In a fraction containing monocytes and macrophages TxB_2, $PGF_{2\alpha}$ and immunoreactive PGE were formed [14]. Sautebin *et al.* consider the macrophages as the

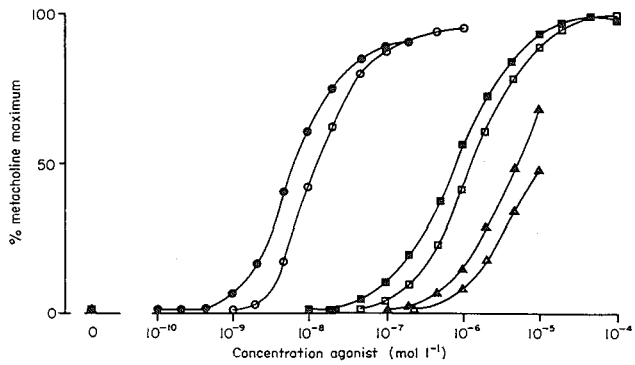

Figure 3. Contraction of human small airway smooth muscle. Mean concentration–response curves for LTC_4 (●), the thromboxane mimetic U-44069 (○), PGD_2 (△), $PGF_{2\alpha}$ (▲), histamine (■) and metacholine (□). The results are expressed as a percentage of the maximal contraction induced by metacholine.

most important source of LT in human lung tissue [15]. In our own experiments, LTD_4 and LTB_4 were formed in approximately equal amounts in human pulmonary macrophages isolated from human lungs after activation by the A23187 (unpublished).

A number of observations indicate that a relationship may exist between the formation of LT in the lung and the pathophysiological changes occurring in some types of asthma, e.g. the one sensitive to aspirin, and related allergic diseases. Dahlen *et al.* observed long-lasting contractions induced by LTC_4, LTD_4 and LTE_4 after allergenic challenge in bronchi from asthmatics [16]. After an allergic reaction, increased amounts of LTC_4 were found in the tears of patients with allergic conjunctivitis [6]. Furthermore, after allergenic challenge of patients with rhinitis, LTC_4, LTD_4 and LTE_4 are present in the nasal fluid [17]. During asthmatic attacks the decrease in mucociliary clearance can be blocked by the LT antagonist FPL 55712 [18]. LTB_4 is chemotactic and may induce the accumulation of eosinophils occurring in bronchoconstriction [19]. These cells can produce comparatively large amounts of LTC_4 [20].

Our results indicate that TxA_2 and LTC_4 are the most important arachidonic acid metabolites in the induction of bronchoconstriction of the human lung. It would be interesting to compare the formation of these compounds in the lungs of normal and asthmatic patients. The pharmacological properties, and also results of clinical tests of a number of synthesis blockers and receptor antagonists of the leukotrienes, have been discussed recently [21]. Their role in the treatment of asthma is a subject for further investigation. Other substances with potent bronchoconstrictive properties, e.g. the platelet activating factor, could also be involved.

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4.2.3

PULMONARY ALVEOLAR PROTEINOSIS: DETERMINATION OF PROSTAGLANDINS AND LEUKOTRIENES IN LAVAGE FLUID

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Lung

Pulmonary Alveolar Proteinosis: Determination of Prostaglandins and Leukotrienes in Lavage Fluid

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Abstract. In bronchoalveolar lavage fluid from a patient with pulmonary alveolar proteinosis, leukotrienes and prostaglandins were measured on 4 occasions during 10 months. Large amounts of leukotriene-C_4-like substances (10–25 nmol) and oxygenated products of leukotriene B_4 were detected. These substances were characterized by the measurement of absorption spectra in high-performance liquid chromatography, biological activities by means of guinea pig lung parenchymal strips, and immunoreactivities in radioimmunoassay. The cyclooxygenase products of arachidonic acid were present in lower amounts (40–500 pmol).

Our results indicate that arachidonic acid metabolites are mainly present in the first fraction of the lavage fluid. These substances may have been formed by alveolar macrophages.

Key words: Leukotrienes—Prostaglandins—Pulmonary alveolar proteinosis—Lavage fluid.

Introduction

In pulmonary alveolar proteinosis (PAP), proteinlike material is accumulated in the alveoli. This leads to impaired gas exchange in the alveoli and a decreased lung capacity. Surfactant, consisting in part of dipalmitoyl phosphatidylcholine, is present in the accumulated material [14]. Several investigators indicate that the impaired breakdown of the surfactant is 1 of the main factors in the pathologic changes. This effect is attributed in part to a diminished function of the pulmonary macrophages [6]. It has been demonstrated that phospholipase

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A_2 is present in the pulmonary secretion of patients with PAP, [15]. This enzyme plays a role both in the formation and breakdown of the surfactant [7]. Phospolipase A_2 releases arachidonic acid (AA) from phospholipids. AA is transformed by both cyclooxygenase and lipoxygenase into prostaglandins and leukotrienes, respectively. Several of these substances have effects on lung function. Bronchoconstriction is induced by thromboxane A_2, prostaglandins $F_{2\alpha}$ and D_2 [5], and the leukotrienes LTC_4 and LTD_4 [1]. The latter substances also increase mucus secretion [11] and the vasopermeability of the lungs [17]. In the experiments presented here, prostaglandins and leukotrienes were measured in the lavage fluid of a patient with PAP. In the identification of the substances the following methods were used [22]: absorption at 280 and 270 nm and quantitation with external standards, determination of the quantity by radioimmunoassay (RIA), determination of the biological activity, and measurement of the absorption spectrum.

Case Report

Case History

An 11-year-old girl had complained of a dry cough, fatigue, and dyspnea on exertion. Auscultation revealed no abnormalities, while respiratory frequency was 60 per minute. The chest roentgenogram showed a fine, diffuse infiltrate most pronounced in the upper lobes. Lung function tests revealed a forced vital capacity (FVC) of 700 ml (26% of expected) at admission, which rapidly decreased to 460 ml. The blood gas levels were as follows: PaO_2 = 6.8 KPa; SaO_2 = 84%; $PaCO_2$ = 5.2 KPa. Treatment with prednisone induced a worsening of the symptoms. Lung biopsy revealed a typical picture of alveolar proteinosis.

Bronchoalveolar Lavage

The first lavage was performed using cardiopulmonary bypass. With the patient under general anesthesia a balloon-cuffed endotracheal tube was inserted through the mouth. The lungs were ventilated with 60% oxygen, while both femoral veins and the right femoral artery were cannulated and cardiopulmonary bypass established with a 2.5 m^2 Kolobow spiral membrane oxygenator (Sci-Med. Inc., Minneapolis, MN). When a flow of approximately 2.5 L/min was achieved, ventilation of the lungs was stopped. Lavage of both lungs was started after establishment of adequate arterial blood gases. The lungs were filled with 500 ml saline at 37°C to 40 cmH_2O pressure and emptied to a negative pressure of 10 cmH_2O. Each cycle of filling and drainage required approximately 6 min. The sequence was repeated until lavage with 20 L was accomplished.

The next lavages were performed ventilating 1 lung. A balloon-cuffed endotracheal tube was inserted through the mouth into the right main bronchus and

the right lung was ventilated with 60% oxygen. A 10 Fr suction catheter was inserted through the mouth into the left main bronchus. Lavage of the left lung was performed in the same way using aliquots of 100 ml saline. After 2 weeks lavage of the other lung was performed. During each procedure lavage with 7 L saline could be accomplished.

The patient improved symptomatically after the procedures.

Materials and Methods

Apparatus

A 1082 B high-performance liquid chromatograph (HPLC, Hewlett Packard) was used, consisting of variable wavelength detector and scanning mode. The Superrac fraction collector (LKB, Sweden) was connected to this apparatus. Radioactivity was measured in a 3255 Tricarb scintillation counter (Packard, Brussels, Belgium).

Chemicals

LTB_4, LTC_4, LTD_4, and LTE_4 were gifts of Dr. J. Rokach (Merck Frosst Canada). Standards of 20-OH-LTB_4 were not available. Prostaglandins and thromboxane B_2 were obtained from Sigma (USA). Tetrahydrofuran, methanol, and acetic acid were all of analytical grade and obtained from Merck (Darmstadt, FRG). Picofluor-15 (Packard, Brussels) was used as premixed scintillation cocktail. ^{3}H-LTB_4, ^{3}H-LTD_4, ^{3}H-6-keto-$PGF_{1\alpha}$, ^{3}H-PGE_2, ^{3}H-$PGF_{2\alpha}$, and ^{3}H-TxB_2 were purchased from the Radiochemical Centre (Amersham, UK). The purity of the radiochemicals was shown to be greater than 97%. PGE_2, $PGF_{2\alpha}$ and TxB_2 antibodies were obtained from l'Institut Pasteur (Paris, France); anti-6-keto-$PGF_{1\alpha}$ from Seragen (Boston, MA), and anti-LTB_4 from the Wellcome Foundation Ltd.

Materials

Sep-Pak C_{18} and Sep-Pak silica cartridges and filters HA (0.45 μm) and FH (0.5 μm) were obtained from Waters Assoc. and a prepacked Nucleosil $5C_{18}$ HPLC column (250 × 4.6 mm) from Chrompack (Middelburg, The Netherlands).

Sample Preparation

Fifty ml samples of lavage fluid were used. ^{3}H-LTD_4 was added as an internal standard (approx. 10,000 cpm per sample), to calculate the recoveries after extraction. The fluid was applied to a Sep-Pak C_{18} and silica column, pretreated as described before [22], eluted with methanol and evaporated to dryness under nitrogen. The extract was dissolved in 300 μl HPLC eluent, centrifuged (10 min, 1400 × g), and portions of 50 μl were injected on the HPLC column.

Chromatographic System

Reversed phase-HPLC (RP-HPLC) of LTs was carried out on a Nucleosil $5C_{18}$ column, using a solvent system consisting of tetrahydrofuran–methanol 0.1% (w/v) EDTA solution in water–acetic acid, 25 : 30 : 45 : 0.1 (v/v), adjusted to pH 5.5 with ammonium hydroxide [18, 22]. Mobile phases

Table 1. Cross-reactivities for radioimmunoassays of prostaglandins (% at 50% B/B_0)

	6-keto-$PGF_{1\alpha}$	$PGF_{2\alpha}$	PGE_2	TxB_2
6-keto-$PGF_{1\alpha}$	100	0.04	<0.01	<0.1
$PGF_{2\alpha}$	2.2	100	0.11	0.1
PGE_2	0.6	0.03	100	0.1
TxB_2	<0.01	<0.01	<0.01	100
$PGF_{1\alpha}$	7.8	12.0	0.01	<0.1
6-keto-PGE_1	6.8	<0.01	0.16	<0.1
PGE_1	0.7	0.03	6.5	<0.1
PDG_2	<0.01	3.0	<0.01	0.2
PGD_1	<0.01	0.4	<0.01	<0.1
Dihydro-$PGF_{2\alpha}$	<0.01	3.0	<0.01	<0.1
Keto-PGE_2	<0.01	<0.01	13.2	<0.1
Dihydro-PGE_2	<0.01	<0.01	2.1	<0.1

were filtered and degassed with helium. Oven temperature was adjusted to 37°C, the flow rate at 0.9 ml/min, and the absorption was measured at 280 nm [22].

Radioimmunoassay of Prostaglandins

Ten ml of lavage fluid was applied to a Sep-Pak C_{18} column and eluted with methanol. A sample of 200 µl was brought into a RIA tube and evaporated to dryness. The extract was dissolved in RIA buffer and ^{3}H-PG and antibody were added. At the end of the incubation (2 h at room temperature and overnigt at 4°C), charcoal suspension was added. The tubes were allowed to stand for 15 min at 4°C and centrifuged. The supernatants were decanted and counted. Cross-reactivities of PG-like substances are listed in Table 1.

Radioimmunoassay of Leukotriene B_4

RP-HPLC of extracts was performed as described above. Two fractions per minute were collected and 25 µl portions were used for RIA determination of LTB_4 immunoreactive substances. The detection range of the LTB_4 RIA was 10–1000 pg. Cross-reactivities of LTC_4, LTD_4, LTE_4, and other lipoxygenase products were less than 2%.

Biological Activity

The biological activity of fractions was tested with a superfused guinea pig lung parenchymal strip, as described before [21]. The detection level of this method was shown to be greater than 0.5 ng LTC_4 or LTD_4.

Results

Figure 1A shows a representative chromatogram of leukotrienes (LTs) in an extract of the first lavage fluid. Portions of 1 L were collected and frozen at −20°C without nitrogen for 3 months. The first portion contained large amounts

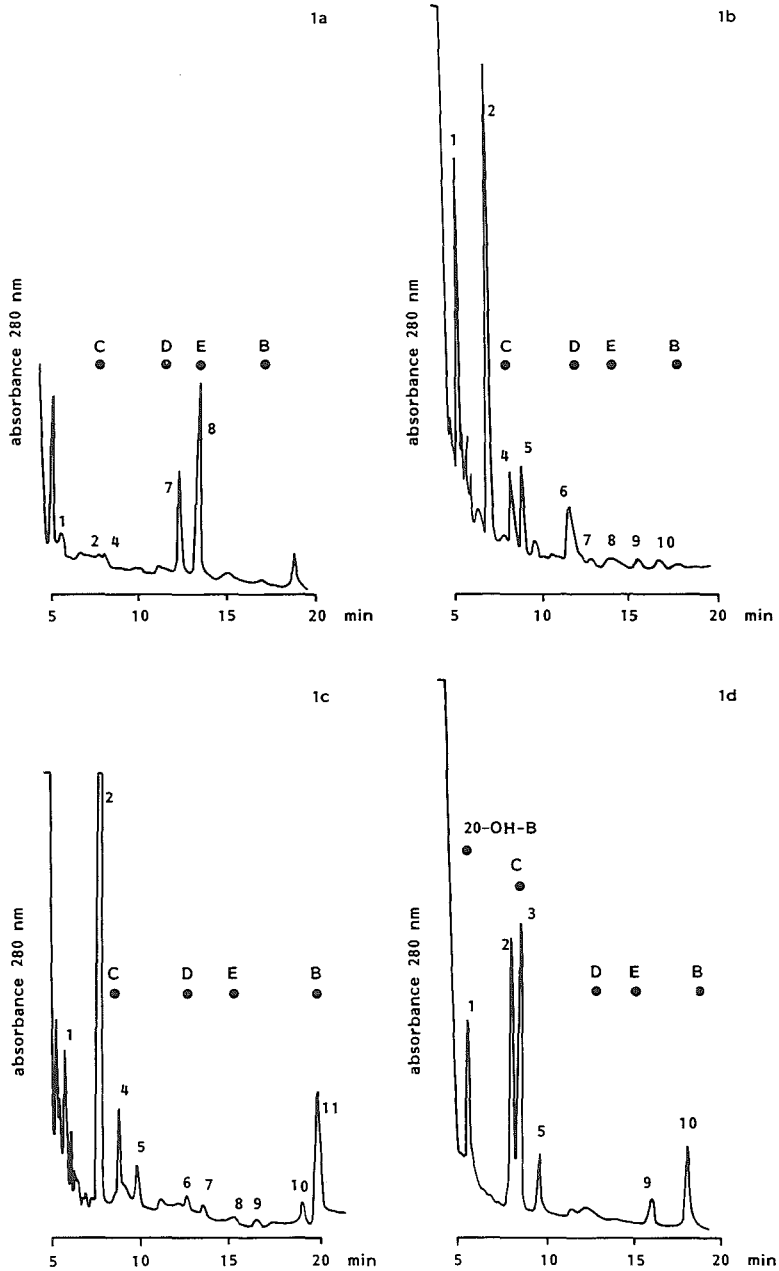

Fig. 1. **a** Reversed-phase HPLC separation of leukotrienes in an extract of the PAP fluid of the first lavage, measured 3 months after BAL. Conditions as mentioned in the methods section. A portion of 50 ml of the total volume of 1000 ml was used for further investigations (data in Table 2, I). **b** Chromatogram of LTs, measured 2 days after BAL, in the PAP fluid of the second lavage. (volume 500 ml) The HPLC conditions were the same as in **a** (data in Table 2, II). **c** Chromatogram of LTs, measured 2 days after BAL, in the PAP fluid of the third lavage. The fluid was collected in portions of 50 ml. This represents the first portion of 50 ml (Fig. 2, fraction 1). **d** Chromatogram of LTs, measured on the day of BAL, of the fourth lavage. This represents the first 100 ml of PAP lavage fluid. Peaks: *1*, 20-hydroxy-LTB_4; *2*, LT^a; *3*, LTC_4; *4*, LT^b; *5*, unknown; *6*, LTD_4; *7*, 11-*trans*-LTD_4; *8*, LTE_4; *9*, 11-*trans*-LTE_4; *10*, 12-*epi*,6*trans*-LTB_4 + 6-*trans*-LTB_4; *11*, LTB_4. *C*, *D*, *E*, *B*, retention times of synthetic leukotrienes C_4, D_4, E_4, and B_4

Table 2. Leukotriene and prostaglandin contents in PAP BALF in 4 collections (ng of the first L)

	I	II	III	IV
Date of lavage (d/m)	0402	1305	2305	2810
Leukotrienes (by HPLC)				
C_4, D_4 and E_4-like	12.100	8.000	5.600	32.300
Day of HPLC after BAL	96	2	2	0
Prostaglandins (by RIA)				
6-keto-$PGF_{1\alpha}$	68	68	40	ND
$PGF_{2\alpha}$	52	16	21	ND
PGE_2	68	124	30	ND
TxB_2	170	ND	14	ND

I, lavage of the right and left lungs filled with 500 ml fractions of saline of which 1 L was collected for LT/PG determinations (Fig. 1a: 50 ml extracted and measured on HPLC)

II, as I; 2 fractions of 500 ml were collected for experiments (Fig. 1b)

III, lavage of the left lung was performed using aliquots of 100 ml saline. 50 ml fractions were collected (Figs, 1c and Fig. 2), thereafter, 1 L fractions (Fig. 3)

IV, as III; 100 ml fractions were collected (Fig. 1d)

ND, not determined

of proteins. After centrifugation LTs were measured by HPLC. Relatively large amounts were detected, predominantly LTD_4-like substances and LTE_4. The amounts of LTs and PGs in this portion are given in Table 2 (I).

In a second experiment LTs were determined in fluid obtained from the second BAL. In this case HPLC was carried out 2 days after collection. The volume of the collected portion was 500 ml. Figure 1B represents a RP-HPLC separation of LTs in an extract of this portion. A large unidentified peak is present between 6 min and that of synthetic LTC_4. The 2 small peaks at retention time t_R 8.5 and t_R 9 could be isomers of LTC_4 (further indicated as LT^a and LT^b). The peak at t_R 12 corresponds to the retention time of LTD_4. The amounts of LTs and PGs are given in Table 2 (II).

Data from the third BAL, carried out 15 weeks after the first, are shown in Figures 1C, 2, and 3. HPLC was performed 2 days after collection. For this study first portions of 50 ml (Fig. 2) and thereafter of 1000 ml (Fig. 3) were collected during lavage.

The chromatogram of the first 50 ml in Fig. 1C has nearly the same pattern as that of Fig. 1B. The only difference is the amount of LTB_4-like substance, which is not present in Fig. 1B. The large peak before LTC_4 in Fig. 1B (t_R 5.5 min) could be 20-OH-LTB_4 [16]. In Figure 1C, LTB_4 most probably has been partially metabolized to this substance.

In the second portion of 50 ml BALF, the amount of the LTC_4-like substance is decreased, whereas in the third portion only a relatively small amount of LTC_4-like substance is detected. The LT and PG contents in 6 portions of 50 ml BALF are shown in Figure 2. In the first and second portion a higher level of

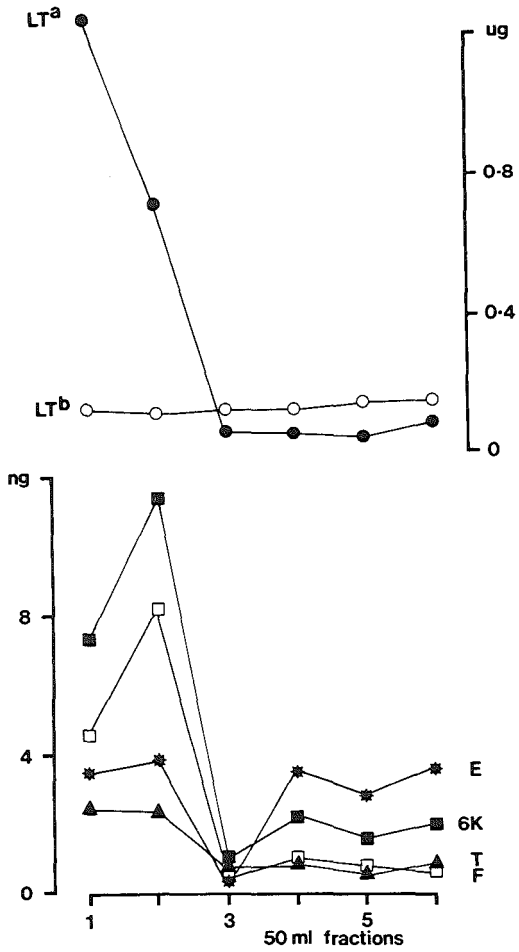

Fig. 2. Amounts of LTs (upper part, measured by HPLC) and PGs (lower part, measured by RIA) of PAP BALF from the same collection as in Figure 1C (third lavage). Lavage fluid was collected in portions of 50 ml. *LT*^{a}, peak before synthetic LTC_4; *LT*^{b}, peak after LTC_4; *E*: PGE_2; *6K*, 6-keto-$PGF_{1\alpha}$; *T*, thromboxane B_2; *F*, $PGF_{2\alpha}$. Leukotrienes were measured by HPLC, the prostaglandins and thromboxane B_2 by radioimmunoassay

LT^a and LT^b, 6-keto-$PGF_{1\alpha}$, and $PGF_{2\alpha}$ is observed. Figure 3 presents these arachidonic acid metabolites in the 1 L portions collected after the 50 ml portions. These data indicate that whereas metabolites are present during the whole lavage, their amount is enhanced in the first 100 ml of lavage fluid (see Fig. 2). The amounts of LTs and PGs are given in Table 2 (III).

The biological activities of the 2 main LT peaks in Fig. 1C were investigated by means of a guinea pig lung parenchymal strip. Approximately 10 ng of each compound was used, calculated on the basis of synthetic LTC_4. The potency ratios proved to be $LTC_4/LT^a/LT^b = 1 : 0.05 : 0.025$.

In Figure 1D the results of the fourth BAL lavage are shown. The first 100 ml of PAP lavage fluid was taken, extracted, and measured by HPLC on the day of BAL. This chromatogram shows 2 main peaks, LT^a and LTC_4. Furthermore an unknown substance at t_R 9.5, a trace of 11-*trans*-LTE_4 and LTB_4-

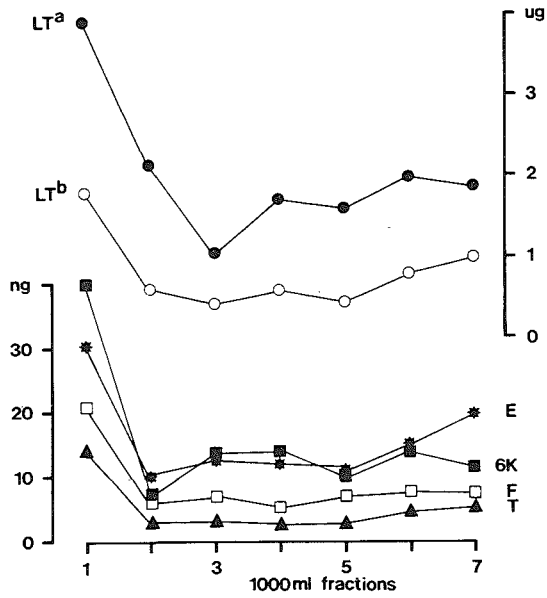

Fig. 3. Presence of LTs (upper part, by HPLC) and PGs (lower part, by RIA) in portions of 1 L PAP BALF collected after the 50 ml portions presented in Figure 2. Portion number 1 covers these 50 ml samples

derivatives (such as 12-*epi*,6*trans*-LTB_4) were observed. LTB_4 was also biologically converted into 20-OH-LTB_4.

The two LT-peaks at t_R 8 in Figure 1D proved to have biological activities, 5–10% of that of synthetic LTC_4. Amounts of LTs and PGs are given in Table 2 (IV).

In the PAP fluid LTB_4 immunoreactive substances were observed at t_R 5.5, (20-OH-LTB_4), t_R 17 (LTB_4 isomers), and t_R 20 (LTB_4). Scans were made of the absorption of the LT^a, LT^b, LTC_4, and LTE_4 peaks. The E_{max} values were 278, 272, 270, and 288 nm, respectively. Absorption maxima of synthetic LTC_4, LTD_4 and LTE_4 were 280 nm and 272 nm for LTB_4-derivatives. The E_{max} values of synthetic 11-*trans*-LTs and other derivatives could not be determined.

Discussion

These results indicate that considerable amounts of LTC_4-like substances are present in the BALF of a patient with PAP. Two peaks were observed, 1 with a slightly shorter and another with a longer retention time of LTC_4 (LT^a and LT^b). The fact that when the BALF was kept frozen for longer periods mainly LTE_4 isomers were found indicates that LT^a and LT^b were most probably precursors of this compound.

An attempt was made to obtain further information on the origin of the LT-like substances by examining the types of cells in the lavage fluid. Cell differentiations were made of the different portions described in Figure 1C. Only the first sample contained fragments of macrophages and monocytes. No eosino-

phils or neutrophils were observed. It has been reported that alveolar macrophages produce LTB_4 [4]. In human lung parenchyma after allergen challenge, LTC_4, D_4, and E_4 are formed [3]. The origin of the LTs in the experiments described here is not yet clear. In Figures 1 and 2, a peak is present with a retention time of 5–5.5 min, the same as that of 20-OH-LTB_4, the ω-oxidation product of LTB_4. LTB_4 immunoreactivity was found in the fraction with the same t_R. This indicates that LTB_4 has also been formed, possibly in the alveolar macrophages.

The nature of the substances designated as LT^a and LT^b has not yet been defined. LT^a is not a *tri*-HETE because these substances chromatograph at elution times between 9 and 10 min. In Figure 1 this could be the peaks indicated by 5. It has been shown that LTC_4 is converted spontaneously into the 11-*trans*-isomer [9, 19] and that conversion into other oxidation products such as 15-OH-LTC_3 [12] can also occur. During this transformation the biological activity may decrease by 90–99% [10]. LT^b, with 5% of the activity of LTC_4, is possibly 11-*trans*-LTC_4, according to data provided by Dr. J. Rokach.

It is of interest to speculate to what extent the substances present in the lavage fluid can contribute to the pathologic changes in the disease state. In the first liter of lavage fluid, amounts of LTD_4 and LTE_4-like substances on the order of 10–25 nmol were present. It seems likely that these amounts are sufficient to induce bronchoconstriction. It has been demonstrated that aerosols of LTD_4 induce bronchoconstriction in humans when 10 nmol is inhaled [1]. Of this amount, only 5% was deposited in the lungs. A pronounced decrease in airflow, mainly in the small airways, was found after inhalation of less than 0.5 nmol. As the biological activities of LT^a and LT^b are 5% and 2.5% of that of LTC_4, bronchoconstriction could be induced by the amounts present.

In comparison with the LTs, the PGs are present in much lower amounts in the lavage fluid. Therefore, it is unlikely that these compounds play a role in the pathologic effects on the lung described above, since the biological activities of PGs and TxA_2 are only a few percent of those of the LTs. The PGs present in the fluid could be a secondary effect of LTs, because LTs can release PGs from lung tissue [13, 21].

The results of HPLC on the BAL in a normal would be of great importance in determining the possible significance of these findings, but volunteers for BAL under the above described circumstances are not available. A comparison can be made with data obtained from BALFs of 6 patients with sarcoidosis undergoing lavage for evaluation of the respiratory tract [23 and unpublished data]. In that study BAL was performed with a fiberoptic bronchoscope in wedge position in the right middle lobe. Premedication consisted of thiazinamium and local anesthesia of the upper respiratory tract with lidocaine spray. Fifty ml of 0.9% sterile saline solution at room temperature was inserted in the suction part during slow inspiration. The lungs were emptied to a negative pressure of 10–15 cmH_2O and collected in a specimen trap. This procedure was repeated three times. The mean of the total amounts of LT-like substances recovered from these BALFs was 260 ± 30 ng. A comparison between the PAP BALFs presented in Figure 2 and Table 2 (III) and these data would result in a

10-fold increase of LTs. However, care must be taken because of the different techniques used and the absence of real controls.

Sahu et al. measured fatty acids in the lavage fluid of PAP patients. AA was not detectable [14]. It could be that this substances had been released from the phospholipids by the phospholipase present in the protein-containing secretions and transformed into the LTs.

The relationship between the presence of LTs in the lavage fluid and the occurrence of symptoms has not yet been established. Although the amounts of LTs are large enough to induce bronchoconstriction, there is no evidence that this occurs in these patients. It is possible that other biological effects of LTs play a role in the pathologic changes. LTC_4 and LTD_4 induce mucus secretion and enhanced vascular permeability [11, 17]. One of or both of these effects could lead to the accumulation of proteins in the lung. If this is shown to be the case, it would be of interest to investigate the effects of lipoxygenase inhibitors [2, 8] or LT antagonists [20] on the course of the disease.

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4.2.4

FORMATION OF LEUKOTRIENE B_4, 20-HYDROXY LEUKOTRIENE B_4 AND OTHER ARACHIDONIC ACID METABOLITES DURING PERITONITIS IN PATIENTS WITH AMBULATORY PERITONEAL DIALYSIS

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FORMATION OF LEUKOTRIENE B_4, 20-HYDROXY LEUKOTRIENE B_4 AND OTHER ARACHIDONIC ACID METABOLITES BY MACROPHAGES DURING PERITONITIS IN PATIENTS WITH CONTINUOUS AMBULATORY PERITONEAL DIALYSIS

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ABSTRACT

Macrophages, isolated from dialysis fluid of three patients with continuous ambulatory peritoneal dialysis (CAPD) at different times during peritonitis were labelled with ^{14}C-arachidonic acid and stimulated with the calcium ionophore A23187. The main metabolites formed by 5-lipoxygenase activity were leukotriene B_4 (LTB_4) and 5-hydroxy-6,9,11,14-eicosatetraenoic acid (5-HETE). Smaller amounts of cyclooxygenase metabolites were present and also a major compound with an elution time between 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) and thromboxane B_2 (TxB_2). This substance was isolated, analyzed by GC-MS and identified as 20-hydroxy-leukotriene B_4 (20-OH-LTB_4). This indicates that human peritoneal macrophages obtained from CAPD not only produce leukotrienes and prostaglandins, but also the ω-hydroxylase product of LTB_4, which has been demonstrated to be present in polymorphonuclear leucocytes. The activity of this enzyme was not correlated with the severity of the peritonitis.

INTRODUCTION

Continuous ambulatory peritoneal dialysis (CAPD) has become increasingly popular in the treatment of end-stage renal disease (ESRD). Approx. 15% of all patients in the Netherlands treated for ESRD are on CAPD therapy. However, despite this increase in numbers, the major complication of this treatment is the frequent development of peritonitis. The mean peritonitis incidence still is one episode every 7.5 patient months (107 patients in 1440 patient months) (1).

In macrophages (Mφ) present in different tissues, arachidonic acid (AA) is metabolized by both cyclooxygenase and lipoxygenase pathways. It has been reported that human peritoneal Mφ produce prostacyclin (PGI_2), thromboxane A_2 (TxA_2), 5-HETE, LTB_4 and leukotriene C_4 (LTC_4) after activation (2,3). In inflamed tissue, considerable amounts of Mφ are present and it is likely that the mediators secreted by the Mφ contribute to the inflammatory changes occurring. Whereas the role of PGE_2 in several parameters of the inflammatory process such as vasodilatation and increased vascular permeability is well documented, much less is known about the other PGs, TxA_2 and the LTs and HETEs. The *in vivo* effects of LTB_4 include chemotaxis for leukocytes and the induction of enhanced granulocyte sticking and vascular permeability. This makes it likely that this substance also participates in the inflammatory process.

In the experiments described here, the eicosanoid production from [1-^{14}C] AA in Mφ was determined at different times in the dialysis fluid during a peritonitis of three patients with CAPD. The inflammation was due to bacterial infection. The production of LTB_4, 20-OH-LTB_4, 5-HETE, PG and TxB_2 was measured in the course of the peritonitis. The presence of 20-OH-LTB_4 indicates that metabolism of LTB_4 occurs in the Mφ. It has been shown recently, that the enzyme catalyzing this conversion, leukotriene B_4 ω-hydroxylase is present in human granulocytes (4).
The results obtained in the present study further demonstrate that during the inflammatory process production of AA metabolites by the peritoneal Mφ is diminished.

MATERIALS AND METHODS

Patients

Peritoneal fluid was obtained from 3 male patients. Peritonitis was diagnosed by the presence of cloudy dialysate and a white cell estimate in the dialysate of more than 100 per ml.
Patient R.: age 75, nephrosclerosis; cultures revealed E.coli. Patient B.: age 62, diabetic nephropathy; cultures revealed staphylococcus epidermialis. Patient deB.: age 67, as patient B.

Chemicals

Lymphoprep™ (Ficoll-Isopaque) was obtained from Nyegaard , Oslo, Norway. Ca-ionophore A23187 was obtained from Hoechst (Calbiochem -Behring Inc. U.S.A.), prostaglandins D_2, E_2, $F_{2\alpha}$, 6-keto-$PGF_{1\alpha}$, 6-keto-PGE_1 and thromboxane B_2 from Sigma Chem. Comp. (USA). Leukotrienes B_4, C_4, D_4 and E_4 were gifts of Dr. J. Rokach, Merck Frosst Canada Inc. [1-^{14}C] arachidonic acid, specific activity 60 mCi/mmol,
5 -D- {5,6,8,9,11,12,14,15-^{3}H(n)} -hydroxy- 6,9,11,14-eicosatetraenoic acid (5-HETE), 12-L-{5,6,8,9,11,12,14,15-^{3}H(n)}-HETE, 15-L-{5,6,8,9,11,12,14,15-^{3}H(n)}-HETE, {5,6,8,9,12,14,15-^{3}H(n)} -leukotriene B_4, {14,15-^{3}H(n)} -leukotriene D_4, 6-keto-{5,8,9,11,12,14,15-^{3}H(n)} -prostaglandin $F_{1\alpha}$, {5,6,8,9,11,12,14,15-^{3}H(n)} thromboxane B_2, {5,6,8,11,12,14,15-^{3}H(n)} prostaglandin E_2, {5,6,8,11,12,14,15-^{3}H(n)} prostaglandin $F_{2\alpha}$, {5,6,8,11,12,14,15-^{3}H(n)} prostaglandin D_2 were obtained from the Radiochemical Centre of Amersham (U.K.). Sep-Pak cartridges, HPLC-solvent filters and Millex-Sample filters were purchased from Millipore. All chemicals were analytical grade (Merck Darmstadt). The derivatization reagent, N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) was obtained from Pierce Chemical Co., Rockford, Illinois, U.S.A.

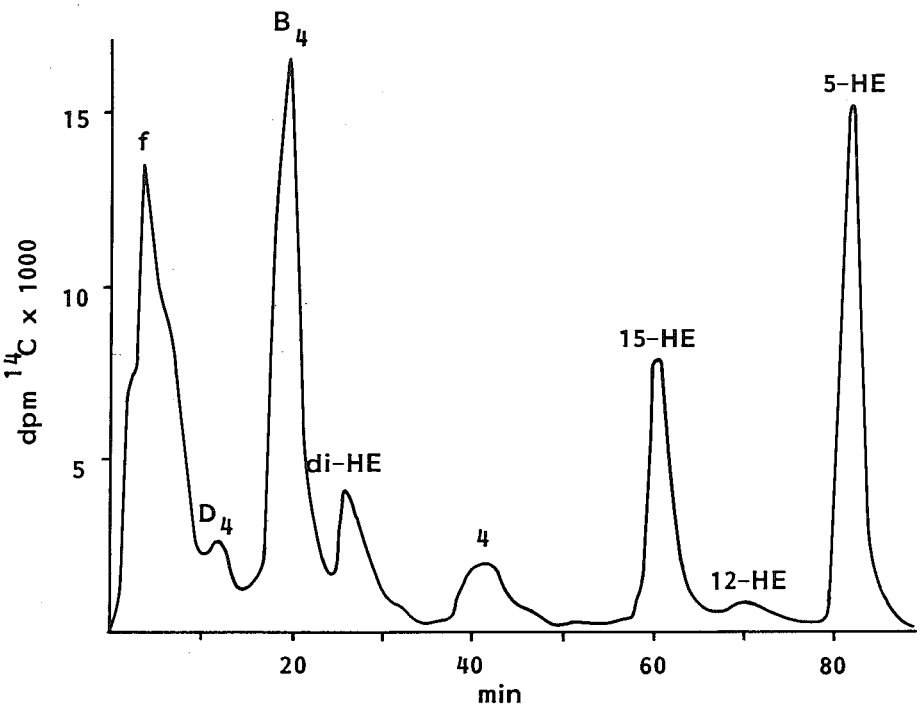


Fig. 1. RP-HPLC separation of leukotrienes and other lipoxygenase products in [1-^{14}C]-A loaded human peritoneal macrophages (patient R., the first day of peritonitis, time 12-18). In this chromatogram the crude data are shown, not corrected for recoveries. A Nucleosil 5C_{18} column was used. Other conditions are described in the section HPLC-analysis (a).
Identification of peaks: f = front, including all prostaglandins; D_4 = LTD_4; B_4 = LTB_4; di-HETE = 5,12-di-HETE; 4 = unknown compound(s); 15-HE = 15-HETE; 12-HE = 12-HETE and 5-HE = 5-HETE.

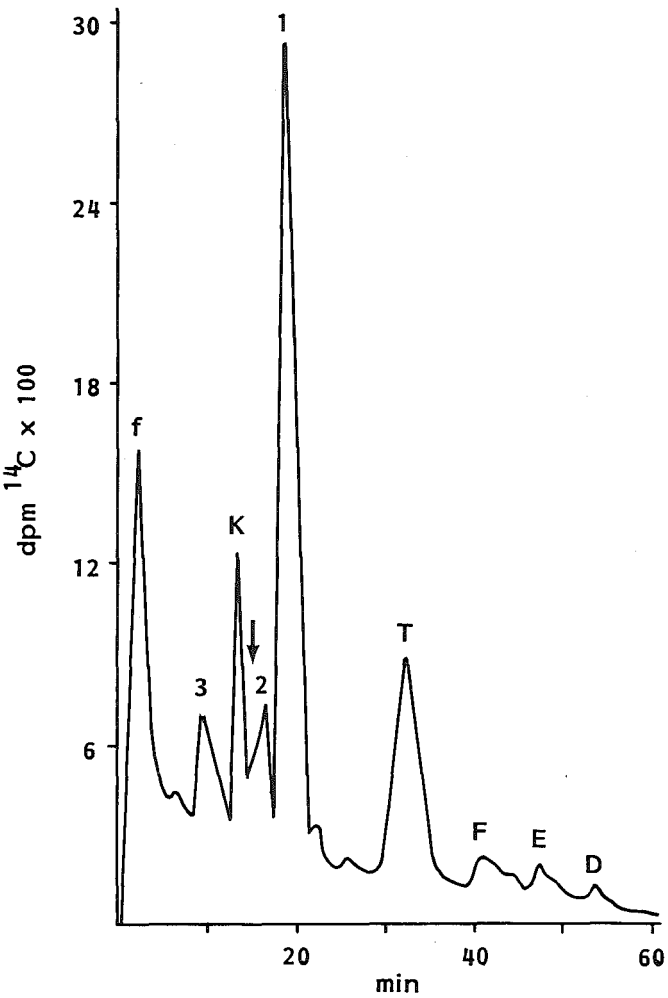


Fig. 2. RP-HPLC separation of prostaglandins in [1-^{14}C]-AA loaded human peritoneal macrophages (same sample as separated for LTs and HEs in fig.1) , on the first day of peritonitis. A Zorbax BP5 column was used. Conditions as mentioned in section HPLC-analysis (b). Peaks: f = front; k = 6-keto-$PGF_{1\alpha}$; 1, 2 and 3 = unknown compounds of which 1 was identified as 20-OH-LTB_4; T = TxB_2; F, E and D = prostaglandins $F_{2\alpha}$, E_2 and D_2. Arrow indicates t_R of 6-keto-PGE_1 (standard).

HPLC-analysis

a. Reversed phase HPLC of leukotrienes and other lipoxygenase products was carried out on a Nucleosil $5C_{18}$ column (5). The solvent system (A) was tetrahydrofuran/methanol/0.1% EDTA (w/v) in water/acetic acid (25:30:45:0.1), adjusted to pH 5.5 with ammonium hydroxyde. The flow rate was 0.9 ml/min and UV absorption was measured at 280 nm for LTC_4, D_4, and E_4, at 270 nm for LTB_4 and di-HETE, at 234 nm for 15-HETE, 12-HETE and 5-HETE. Prior to use, the column was equilibrated with the mobile phase A at 37°C. One minute fractions were collected for scintillation counting. After each run (84 min) the column was rinsed for 30 min with methanol (flow rate 2 ml/min) to prevent contamination with Ca-ionophore and [1-^{14}C]-AA.

b. RP-HPLC of prostaglandins and 20-OH-LTB_4 was performed on a Zorbax C8 BP5 column (5). The solvent system used for RP-HPLC (B) consisted of acetonitrile/benzene/water/acetic acid (24:0.2:0.1:76). The flow rate of this eluent was 2 ml/min. UV absorption of 20-OH-LTB_4 was monitored at 280 nm. One minute fractions were collected for scintillation counting. The column was rinsed with acetonitrile/benzene (99:1) for 30 min after each analysis to elute the monohydroxy lipoxygenase products.

Collection of cells, incubation and sample work-up procedures

The cells were obtained from bags with dialysis fluid starting with three fast changing bags. Thereafter a bag was collected every 4 hours during daytime and one bag overnight. These collections were performed for two days. Thereafter two or three bags were combined daily. The patients were followed during the first 4 days of the peritonitis and a control was taken approx. 5 weeks after recovery.

The cells were spun down in 500 ml flasks at 800xg for 10 min. The resuspended cells were purified on 20 ml Lymphoprep (650xg, 4°C, 20 min). The purity of separated Mφ proved to be greater than 90% on day 2, based on Giemsa-May-Grünwald staining. The other cells were characterized as granulocytes (approx. 5%) and lymphocytes (approx. 5%). In the incubations $30\text{-}40\text{x}10^6$ Mφ were suspended in 10 ml of Krebs-Henseleit buffer, using a Falcon^R tube. The tube was placed in a waterbath of 37°C and gassed continuously with a mixture of 95% O_2 + 5% CO_2 through a thin plastic pipette. After 5 min preincubation 2.5 µCi [1-^{14}C] arachidonic acid (60 mCi/mol), glutathion (final conc. 2 mM) and Ca-ionophore A23187 (in 50 µl of ethanol, final conc. 10 µM) were added. At the end of the 10 min incubation, ^{3}H-HETEs, ^{3}H-LTs and ^{3}H-PGs were added (approx. 50.000 dpm per metabolite, corresponding to 425, 320 and 50 pg resp.) and the homogenate centrifuged (10 min, 1400xg, 4°C). Pellets were washed once, and supernatants were applied to a couple of Sep-Pak cartridges (C_{18} and Silica)(4). Eluates were dried in a Savant Speed Vac concentrator at 40°C. Samples were dissolved in 400 µl of solvent (A), filtered through a Millex 0.2 µm and kept in plastic HPLC micro vials until measurement. An aliquot of 100 µl was injected into the HPLC columns and chromatographed using a 1082 Hewlett Packard HPLC system. Fractions were collected with a Superrac fraction collector (LKB, Sweden) for further analysis. The chromatographic method and the identification of the peaks have been described earlier (5).

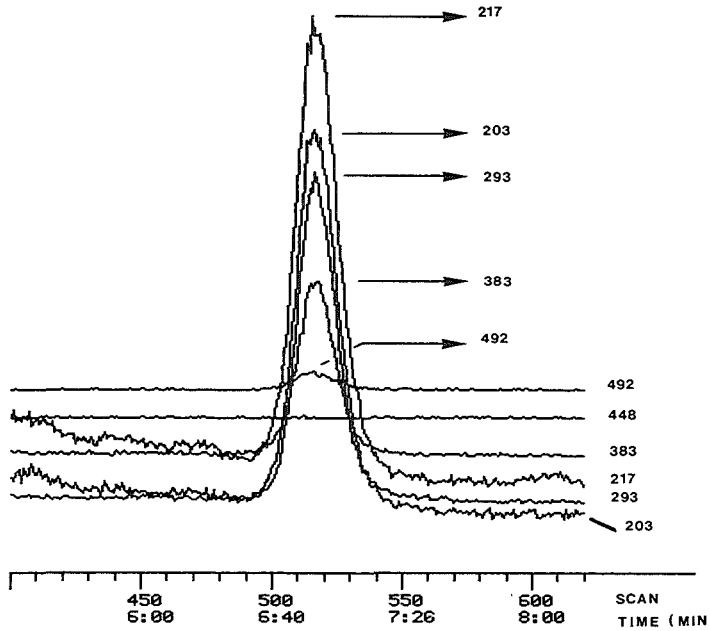


Fig. 3. Selected ion monitoring analysis obtained on material of peak 1 (Fig. 2) after derivatization into the methyl ester, trimethylsilyl (TMS)-ether. Ions selected for the analysis were at m/z 203, 217, 293, 383, 448 and 492 (see Materials and Methods.

GC-MS.

The material eluted in peak 1 (as shown in fig. 2) during RP-HPLC analysis was derivatized into the methyl ester, trimethylsilyl ether. The methyl ester was prepared with an ethereal diazomethane solution. Trimethylsilylation was done with 30 μl of a mixture of BSTFA/pyridine (2:1) for 1h at room temperature. Immediately before GC/MS analysis, the sample was taken to dryness under N_2 and redissolved in 10 μl of n-hexane.

GC/MS analysis was carried out on a Finnigan 4000 instrument, equipped with an Incos 2000 data system, using the jet separator as GC/MS interface and with the mass spectrometer operated in the electron impact ionization mode and in the selected ion monitoring detection mode. GC was performed on a 2m x 2mm internal diameter glass column, packed with 1% OV-1 coated on Chromosorb WHP 100-200 mesh, which was run isothermally at 230°C. Helium was used as carrier gas at a flow rate of 25 ml/min. Injector and GC/MS interface temperatures were 250 and 230°C respectively. The MS conditions were: electron energy, 70 eV; emission current, 0.3 mA; ion source temperature, 230°C. Selected ion monitoring was carried out at specific m/z values for the 20-OH-LTB_4 and 20-COOH-LTB_4 derivatives (6), i.e. at m/z 492

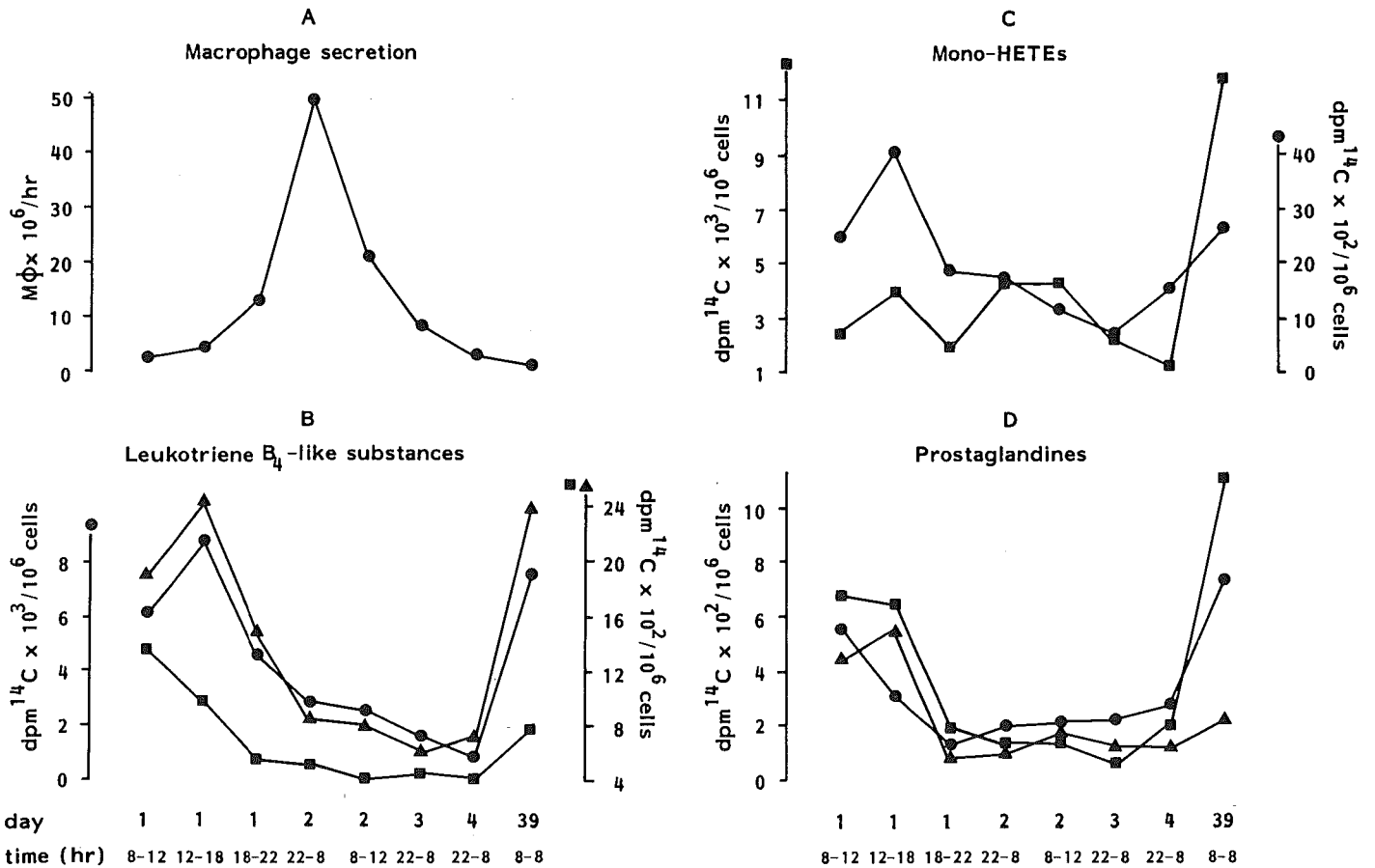
[$M^{+\cdot}$(20-OH-LTB_4-der.)-TMSOH], m/z 448 [$M^{+\cdot}$(20-COOH-LTB_4-der.)-TMSOH], m/z 383 [$M^{+\cdot}$-CH_2-CH=CH(CH_2)$_5$-OTMS] or [$M^{+\cdot}$-CH_2-CH=CH-(CH_2)$_5$-$COOH_3$], m/z 293 [383-TMSOH], m/z 217 [TMSO-CH=CH-CH=$^+$OTMS] and m/z 203 [$TMSO^+$=CH-(CH_2)$_3$-$COOH_3$].

This mode of mass spectral analysis was selected because of the small amount of material available (± 500 ng) and because of the greater sensitivity of this technique compared to the conventional scanning mode.

RESULTS

In the Mφ, isolated from the dialysis fluid of patients with CAPD at different times during the peritonitis, the substances formed from [1-^{14}C] AA after activation with Ca-ionophore A23187 have been characterized. The main metabolites formed by 5-lipoxygenase activity were LTB_4 and 5-HETE (fig.1), less 15-HETE and a compound characterized as 5-(S),12-(S)-dihydroxy-6,8,10,14-eicosatetraenoic acid (M.Claeys *et al*, in publication). In the chromatogram of the HPLC analysis aimed at detecting cyclooxygenase metabolites TxB_2, $PGF_{2\alpha}$, PGE_2 and PGD_2 were present and a major peak with an elution time between 6kP$GF_{1\alpha}$ and TxB_2 was observed (Fig. 2). This substance was isolated and analyzed by GC-MS. The peak (1) was identified as 20-OH-LTB_4 by selected ion monitoring at specific m/z values (Fig. 3). The peak areas obtained for the signals at m/z 203, 217, 293 and 383 are comparable, which is in agreement with literature data, whereas the signal at m/z 492 confirms the presence of 20-OH-LTB_4 (6). The RP-HPLC behavior of 20-OH-LTB_4 is further in agreement with data reported by *Powell* for polymorphonuclear leukocytes (7).
The formation of the above mentioned substances was determined in the cells in the dialysis fluid at different times during the peritonitis. After four days of treatment the symptoms of the disease had disappeared. A sample taken at day 39, when the patients were recovered, was considered as a control (Fig. 4). Recoveries of the main metabolites present were of the same order of magnitude as described earlier (5) and approx. 62, 50, 20, 75, 81 and 70 % for LTB_4, 15-HETE, 5-HETE, 6kP$GF_{1\alpha}$, TxB_2 and PGE_2 respectively. The recovery factor of LTB_4 was used for calculation of the total amount of 5,12-diHETE and that of 6kP$GF_{1\alpha}$ for 20-OH-LTB_4. The final results, corrected for recoveries, indicate that the amount of radioactivity incorporated in LTB_4 and 5-HETE is approximately ten times higher than that in the PG and TxB_2. In the course of the peritonitis, the amount of all substances formed diminishes 60-80% after the first day, when results are expressed per 10^6 cells. There is an increase in the number of Mφ in the dialysis fluid, reaching a peak at day two (4 → 50 x 10^6 Mφ/hr). The formation of the metabolites has returned to the original level at day 39, with the exception of PGE_2.

Legend to fig. 4. Representative data of the formation of eicosanoids by human peritoneal Mφ during and after peritonitis Patient R., as fig.1 and fig.2). Sampling times are indicated by the number of the day and the duration in hours. Explanation of figures: part B. ▲ = 5,12-di-HETE; ● = LTB_4; ■ = 20-OH-LTB_4; part C. ● = 15-HETE; ■ = 5-HETE; part D. ■ = 6-keto-P$GF_{1\alpha}$; ● = TxB_2 and ▲ = PGE_2. (n=3)


A
Macrophage secretion
Mϕx 10^6/hr
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40
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Mono-HETEs
dpm 14C x 10^3/10^6 cells
11
9
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dpm 14C x 10^2/10^6 cells
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Leukotriene B4 -like substances
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DISCUSSION

The results indicate, that human peritoneal Mφ have the capacity to metabolize AA by the cyclooxygenase and lipoxygenase pathways. The higher incorporation of radioactivity into LTB_4, 5-HETE and 5,12-diHETE (approx. 35, 30 and 11 % of total metabolites formed) demonstrates the relative importance of 5-lipoxygenase activity. Earlier, we determined the eicosanoids produced by human ascites cells, mainly Mφ, in patients with liver cirrhosis (3). In these cells the main substances formed were LTB_4 and 5-HETE, each representing approximately 30%. In both cases the PG and TxB_2 were produced in smaller quantities. The formation of 20-OH LTB_4, which was not observed in cirrhosis, indicates, that the enzyme LTB_4 ω-hydroxylase which has been demonstrated in polymorphonuclear leucocytes (4), is also present in human CAPD Mφ, after activation. However, the formation of this substance by the small amounts of neutrophils available, or by neutrophil-macrophage interaction can not be excluded. The formation of this metabolite of LTB_4 is not dependent on the inflammation, as it also occurs in the controls, at day 39.

In Fig. 4, the curves obtained for the different metabolites indicate that during the inflammation the amounts produced per 10^6 cells decrease considerably and return to normal in the controls, with the exception of PGE_2. The amounts produced are inversely proportional to the number of Mφ in the dialysis fluid. Several explanations can be given for this effect. First, a depletion of precursor pools may occur during peritonitis. This is not very likely, as the amounts formed in the beginning of the peritonitis are approximately the same as those in the controls and as there is no particular reason for depletion.

Second, the newly formed Mφ may have a lower capacity to synthesize eicosanoids. The reason for the decrease could be that during the peritonitis the production of corticosteroids in the adrenal is probably enhanced. This will lead to a higher level of circulating cortisol and a diminished eicosanoid production. Glucocorticosteroids induce the formation of a peptide, lipocortin with phospholipase A_2 inhibitory activity in Mφ and neutrophils (8). This will in turn result in an inhibition of eicosanoid formation. *Flower et al.* have shown that when rats are treated with dexamethasone, lipocortin is formed in the peritoneal fluid (9). This result is supported by the observations that dexamethason inhibits the TxB_2 and LTB_4 production in human peritoneal Mφ (10). A similar effect could occur in the dialysis fluid of the CAPD patients. It is possible, that a relationship exists with the decline in cyclic AMP content in peritoneal Mφ of the dialysis fluid of CAPD patients shown by *Adolfs et al.* In these experiments, a decrease in PG and TxB_2 was also observed during the inflammation (11).

In the Mφ, part of the LTB_4 (approx. 12 %) is oxidized to 20-OH-LTB_4, which has been shown to contain to a great extent some of the biological activities of LTB_4 (12). Whether a relationship exists with the peritonitis is unknowm. In order to obtain more complete information on the effects of the inflammation, measurements of the endogenous eicosanoid production, and of the amounts present in the peritoneal fluid, would be of great interest.

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4.2.5

DIFFERENTIAL EFFECTS OF MALOTILATE ON 5-, 12-, AND 15-LIPOXYGENASE IN HUMAN PERITONEAL MACROPHAGES

Submitted for publication

Rapid Communication

DIFFERENTIAL EFFECTS OF MALOTILATE ON 5-, 12- AND 15-LIPOXYGENASE IN HUMAN ASCITES CELLS

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Malotilate (diisopropyl-1,3-dithiol-2-ylidene malonate) is a new drug which protects against acute liver damage induced by a variety of toxins and reduces hepatic fibrosis in experimental cirrhosis. It is mechanism of action in liver injury is not clearly understood, although in high concentrations it suppresses collagen formation by cultured fibroblasts [Ryle and Dumont,1987]. Macrophages may play a central role in inflammation and their products are thought to be involved in liver damage and fibrosis in experimental hepatitis. Human peritoneal macrophages produce mainly lipoxygenase and smaller amounts of cyclooxygenase products after stimulation by the calcium ionophore A23187 [Ouwendijk et al.,1985], and are suitable target cells to test the effects of antiinflammatory agents on eicosanoid production.

Macrophages were isolated from ascitic fluid of patients with alcoholic liver cirrhosis by centrifugation and sedimentation on Lymphoprep (Nycomed, Norway), and labelled with [1-^{14}C] arachidonic acid (AA, 60mCi/mmol, Amersham) to determine exogenous formation of eicosanoids [Zijlstra et al.,1987]. Approximately 20.10^6 macrophages /10ml Krebs-Henseleit buffer per sample were preincubated with malotilate for 2 min. at 37°C while stirring gently, glutathione and ^{14}C AA were added and after 3 min the cells stimulated with calcium ionophore A23187. After a further 10 min incubation [^{3}H]-labelled standards were added, and the incubation fluids passed through SepPak cartridges. Eluates were separated on HPLC using a Chromsep Nucleosil $5C_{18}$ column and a solvent system consisting of tetrahydrofuran / methanol / 0.1% EDTA in water / acetic acid (25:30:45:0.1, pH 5.5) for determination of lipoxygenase products (Leukotrienes (LTs) and monohydroxy eicosatetraenoic acids (HETEs)). Prostaglandins (PGs) were measured on a Zorbax C_8 column (Dupont) and the solvent system acetonitrile / benzene / water / acetic acid (24:0.2:76:0.1). Endogenous formation was measured in samples containing 10^6 macrophages /1ml Krebs-Henseleit buffer, without addition of AA following the same incubation procedure. Radioimmunoassay of PGs, HETEs and LTB_4 was performed in 50, 25 and 10 µl resp., using specific antisera from Seragen and Amersham. ^{3}H-Labelled eicosanoids were obtained from Amersham.

The main substances formed by untreated macrophages *in vitro* after stimulation were 5-HETE and LTB_4, and smaller amounts of $6kPGF_{1\alpha}$, TxB_2, 15- and 12-HETE (table 1). After incubation with malotilate, a concentration dependent decrease occurred in the 5-lipoxygenase products 5-HETE and LTB_4, both in the formation of exogenous and endogenous metabolites.

The mean IC_{50} of malotilate on the 5-lipoxygenase was 4.7 µM. 15- and 12-HETE were not inhibited and these products tended to increase. There was a decrease in the cyclooxygenase products 6-keto-$PGF_{1\alpha}$, TxB_2 and PGD_2, which could possibly be related to the increase of 15-HPETE and 12-HPETE respectively [Vanderhoek et al.,1982]. The formation of prostaglandins $F_{2\alpha}$ and E_2 was not significantly influenced by malotilate. This finding was confirmed with data obtained after labelling of sheep seminal vesicles with ^{14}C-AA, a source of predominantly PGs E_2, F_{2a}, D_2 and 6-keto-$F_{1\alpha}$. Formation of HPETEs was inhibited by addition of ascorbic acid, to eliminate the inhibitory effects of these metabolites. Malotilate did not decrease the PG-formation, in contrast to indomethacin.

These effects of malotilate on eicosanoid formation differ from those of known lipoxygenase inhibitors such as BW 755C (weak 5-lipoxygenase and cyclooxygenase inhibitor, strong 15-lipoxygenase inhibitor), nordihydroguiaretic acid (not specific lipoxygenase inhibitor, weak cyclooxygenase inhibitor) and ketoconazole (weak 5-lipoxygenase inhibitor, 12-lipoxygenase not affected and 15-lipoxygenase increased) [data summarized in table 2 ; Beetens *et al.*,1986]. The differential effects of malotilate on the 5-, 12- and 15-lipoxygenases and also on the formation of the compounds of the cycloxygenase, have not previously been reported. The suppression of leukotriene production *in vitro* occurred at concentrations found following normal therapeutic doses *in vivo*. Inhibition of the production of the chemotactic substance LTB_4 and the vasoconstrictive TxA_2 provide a possible explanation for the beneficial effects of this drug on liver necrosis and liver fibrosis.

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TABLE 1 THE EFFECT OF MALOTILATE ON THE METABOLISM OF ARACHIDONIC ACID BY HUMAN PERITONEAL MACROPHAGES

	EXOGENOUS FORMATION				ENDOGENOUS FORMATION			
	controls	percentage change vs. controls			controls	percentage change vs. controls		
	dpm. 100/2.10^7 MΦ	0.85 μM	8.5 μM	85 μM	pg/10^6 MΦ	3.4 μM	34 μM	340μM
LTB_4	1290 ±155	−23 ±4	− 79 ±6	− 99 ±1	59700 ±6800	−21 ±2	−77 ±18	− 98 ±1
5-HETE	2170 ±83	− 1 ±8	− 73 ±7	− 94 ±3	74700 ±950	−47 ±5	−80 ±9	− 99 ±1
15-HETE	775 ±190	+15 ±11	+125 ±56	+172 ±99	2050 ±575	+45 ±8	+68 ±26	+209 ±60
12–HETE	114 ±26	+ 1 ±19	+ 59 ±43	+133 ±66	520 ±101	−14 ±8	+14 ±43	+111 ±61
6-keto-$PGF_{1\alpha}$	232 ±67	−12 ±5	− 34 ±4	− 70 ±13	2960 ±820	−24 ±6	−56 ±8	− 62 ±9
TxB_2	300 ±118	− 2 ±10	− 24 ±8	− 75 ±7	2600 ±400	− 6 ±6	−31 ±13	− 80 ±3
$PGF_{2\alpha}$	72 ±30	− 6 ±11	+ 21 ±3	+ 17 ±18	340 ±120	+ 8 ±17	+28 ±17	+ 40 ±5
PGD_2	72 ±41	− 4 ±11	− 34 ±3	− 48 ±5	260 ±60	−15 ±27	−37 ±12	− 87 ±8
PGE_2	88 ±52	+32 ±15	− 11 ±7	− 8 ±18	N.D.	N.D.	N.D.	N.D.

Each value represents the mean ± SEM of 4 separate determinations. N.D. = not detectable.

Table 2. Summary of the inhibition of the oxygenation reactions in several *in vitro* systems by different drugs ($IC_{50}\mu M$)

	5-LO	12-LO	15-LO	CO
NDGA	1	26	1	15
Malotilate	5	+	++	90
Nafazatrom	10	+	9	>100
Phenidone	16	+	3	>100
Ketoconazole	28	ni	+	ni
BW 755C	36	>100	1	21

LO: lipoxygenase CO: cyclooxygenase
+ ++: stimulation ni: not inhibited

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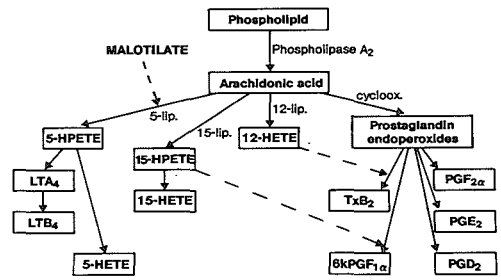

4.3 COMPARISON OF EICOSANOID FORMATION IN MACROPHAGES OF HUMAN, RAT AND GUINEA PIG FROM DIFFERENT ORIGIN.

In this paragraph results from incubations of macrophages with labelled arachidonic acid from different species and origin will be summarized. Data were obtained from investigations by research institutes both in the Erasmus University of Rotterdam and abroad.

The labelling of cells, measurements by HPLC and the calculation and interpretation of data were performed as described in section 4.1.3. Differences exist in the methods of separation and purification of the cells.

Liver Kupffer cells (KC) were isolated by perfusion and incubation of tissue with pronase and collagenase and kept for 24 h at 37°C in Eagles medium to recover from the isolation procedure [Kuiper *et al.*,1987].

Peritoneal rat macrophages (Per.MØ) were obtained from animals that had been injected with 50 ml of sterile physiological saline [Vincent *et al.*, 1986].

Human peritoneal macrophages were isolated from ascitic fluid of patients with alcoholic liver cirrhosis by centrifugation and sedimentation on Ficoll-Paque [Ouwendijk *et al.*,1985].

Human alveolar macrophages (Alv.MØ) were obtained by lavage of one side of the lungs with 200 ml of saline [Schenkelaars *et al.*,1987].

Guinea pig and rat alveolar macrophages were harvested with 100 ml saline injected via an incision in the trachea [Bachelet *et al.*,1987; Klein *et al.*,1987].

Rat testis macrophages (TMØ) were obtained after leakage of the interstitial fluid and adherence on petri dishes [Dreteler *et al.*,1987].

In table 4 the formation of exogenous arachidonic acid metabolites is given as means in percent of total formation. In this summary the distribution of metabolites is reviewed, considering that Kupffer cells represent liver macrophages.

The data show that the capacity to generate products of the lipoxygenase pathway or the cyclooxygenase pathway (the lip/cyclo ratio) is dependent on the origin of the macrophage.

Alveolar macrophages. In the guinea pig only the production of eicosanoids by alveolar macrophages was investigated. It is clearly shown that thromboxane synthetase is the most common enzyme activated after stimulation by Ca-ionophore A23187. The marked formation of TxB_2 (and HHT) in contrast to the absence of lipoxygenase products could be related to the TxA_2-induced contractions of parenchymal strips of this species.

Alveolar macrophages of rat and human origin predominantly formed 5-lipoxygenase products and less 12-HETE. A small amount of thromboxane as the most important cyclooxygenase product was measured. A marked difference between these species was the formation of LTC_4-like substances in human alveolar macrophages. This is in agreement with findings by other investigators [Fels *et al.*,1982].

Peritoneal macrophages. A significant difference between rat and human peritoneal macrophages was observed. The profile of eicosanoid formation in human peritoneal macrophages was almost identical with that of human alveolar macrophages, with the exception of the formation of LTC_4. In comparison with these findings, rat peritoneal macrophages preferentially formed $6kPGF_{1\alpha}$ and TxB_2 [Schenkelaars and Bonta,1983], as well as mono- and di-hydroxy-eicosatetraenoic acids.

Kupffer cells. In rat Kupffer cells the formation of large amounts of PGD_2 was noticable. The importance of this finding in view of its further metabolism to the vasoactive substance $9\alpha,11\beta$-PGF_2 has already been discussed in paragraph 2.6. The lack of production of 5-lipoxygenase substances is comparable with that of the rat peritoneal macrophages. In human Kupffer cells a large PGD_2 production was not present. A base-line level of cyclooxygenase products was seen, with the exception of TxB_2. Lipoxygenase products were formed, however these cells failed to produce leukotrienes.

Testis macrophages. Finally one would expect that macrophages obtained from interstitial fluid would preferentially generate prostaglandins, as interstitial fluid generally contains these substances. Surprising however was that the substance formed in the largest amount was 12-HETE. In macrophages of other origin, except rat Kupffer cells, the 5- and 15-HETE were dominant.

Table 4. Eicosanoid formation from A23187 stimulated ^{14}C-AA loaded cells

	Rat				Guinea Pig	Human		
	Alv. MΦ	Per. MΦ	KC	TMΦ	Alv. MΦ	Alv. MΦ	Per. MΦ	KC
LTC_4/D_4	-	-	-	-	-	7	-	-
LTB_4	26	3	-	1	2	44	40	1
15-HETE	5	3	5	7	1	$\leq$ 1	5	25
12-HETE	20	14	10	40	1	8	2	14
5-HETE	34	-	2	4	-	14	30	14
di-HETE	8	22	-	3	-	3	1	-
HHT	2	13	10	7	54	8	8	7
$6kPGF_{1\alpha}$	-	21	$\leq$ 1	10	-	$\leq$ 1	4	7
TxB_2	2	17	10	5	40	7	6	16
$PGF_{2\alpha}$	$\leq$ 1	3	5	12	1	5	2	5
PGE_2	$\leq$ 1	1	2	6	1	$\leq$ 1	1	7
PGD_2	$\leq$ 1	3	55	5	-	$\leq$ 1	1	4
lip./cyclo ratio	13/1	1/1.4	1/4.9	1.2/1	1/24	3.4/1	3.5/1	1.2/1

4.4 SUMMARY

The formation of prostaglandins, leukotrienes and other lipoxygenase products was determined in human lung tissue and in human peritoneal macrophages after prelabelling with ^{14}C-arachidonic acid. Separation was performed by HPLC and further quantitation by RIA. A comparison was made between macrophages of different origin and from different species. Eicosanoid levels were measured in human alveolar lavage fluid. In conclusion it was found that:

-in chopped human lung tissue after stimulation with Ca-ionophore A23187 eicosanoids are present in nanogram amounts per gram tissue [4.2.1].

-after stimulation with A23187 mostly LTB_4-like compounds were generated and also leukotrienes C_4, D_4 and E_4. Large amounts of HETEs were also present [4.2.1] and [4.2.2].

-the main cyclooxygenase products were 6-keto-$PGF_{1\alpha}$, TxB_2 and PGD_2 [4.2.2].

-specific activities (dpm/ng) derived from data of 7 non smokers varied both in cyclooxygenase and lipoxygenase products [4.2.2].

-eicosanoid production by macrophages isolated from dialysis fluid of patients decreased considerably during inflammation (peritonitis) and returned to normal in clinically stable periods [4.2.4].

-LTB_4 was further metabolized in these macrophages by the enzyme omega-hydroxylase to 20-OH-LTB_4 [4.2.4].

-malotilate, an anti-fibrotic substance, inhibited the 5-lipoxygenase, whereas both the 12- and the 15-lipoxygenase pathways were stimulated, followed by a possible inhibition of two main cycloxygenase products, TxB_2 and 6-keto-$PGF_{1\alpha}$ [4.2.5].

-large amounts of LTC_4-like substances and oxygenated products of LTB_4 were detected in lung lavage fluids of a patient with pulmonary alveolar proteinosis [4.2.3].

-the ratio lipoxygenase/cyclooxygenase products, generated from ^{14}C-arachidonic acid labelled macrophages after stimulation by the Ca-ionophore A23187, is origin and species dependent [4.3].

CHAPTER 5

GENERAL DISCUSSION

The purpose of the experiments described in chapter 3 of this thesis was to study the biological effects of leukotrienes on lung parenchyma, and the influence here upon of pharmacological substances. Secondly the profile of eicosanoids generated by different cells and different tissues was determined (chapter 4). Furthermore the role of synthesis inhibitors and blockers in inflammatory processes and diseases was studied .

5.1 ACTIONS ON LUNG PARENCHYMA

In this part the bronchoconstrictive effects of eicosanoids and other agents on human and guinea pig lung parenchyma and airway smooth muscle are discussed.

5.1.1 *Human lung.*

Both the contractile response of human lung parenchymal strips after the application of LTs and the release of TxA_2 were shown to be considerably lower than those obtained on the GPLP [3.2.1]. From our studies on the contractile effect of several substances on human small airway smooth muscle, we concluded that on an equimolar base this tissue tended to be less sensitive.

The thromboxane mimetic U-44069 and LTC_4 were still 100-times more active than histamine or methacholine on human smooth muscle [4.2.2]. Measurements of AA-metabolites in chopped human lung tissue after stimulation with the calcium ionophore A23187 suggested that PGD_2 was

formed in larger amounts than TxB_2. Recently it was shown, that this PG is metabolized to 9α,11β-PGF_2 in the lungs after one passage [Seibert *et al.*,1987]. The substance proved to be equipotent to PGD_2 and $PGF_{2\alpha}$ in bronchoconstrictive effect. However the results presented in table 3 of paragraph 4.2.2 indicate a minor role for these PGs in the contraction of the lung, having an EC_{50} one thousend times higher than that of LTC_4. The contractile activity of TxA_2 is of the same order of magnitude as that of LTC_4 or LTD_4 in guinea pig lung strips [3.2.3] and human lung parenchyma [4.2.2]. A comparison of the amounts produced by chopped human lung tissue and the effects on the lung strips indicates that it could play a role in the induction of bronchoconstriction *in vivo*.

In guinea pig isolated trachea, application of $PGF_{2\alpha}$ to the segments stimulated the generation of lipoxygenase products. The release of lipoxygenase-mediated LT-like substances differed in the mode of action from that of histamine and LTD_4 [Shikada *et al.*,1987]. A comparison of the responses of isolated human airways, obtained from a single asthmatic patient at thoracotomy, was made to the responses of airways of non-asthmatics *in vitro*. In asthma bronchiolar strips exhibited an increased airway smooth muscle contractility in response to histamine, methacholine and LTC_4 [de Jongste *et al.*, 1987a]. In bronchiolar strips of controls the contractile responses after repeated exposure to LTC_4 did not induce tachyphylaxis [de Jongste *et al.*, 1987b]. It was hypothesized that a high endogenous LT synthesis would lead to desensitisation of airway smooth muscle. It was however found that the sensitivity and contractility of airway smooth muscle to LTC_4 were not diminished in lungs in which large amounts of LTs are synthesized.

5.1.2 *Guinea pig lung parenchyma (GPLP).*

In the experiments presented in chapter 3 the mechanisms of leukotriene-induced contractions of lung parenchyma were scrutinized. The most important finding was the ***thromboxane-release related action*** of leukotrienes [3.2.4 and 3.2.3]. The release of TxA_2 through LTC_4 and LTD_4-induced contractions of guinea pig lung parenchymal tissue was observed by others in the same year [Piper and Samhoun, 1981].

Our first results of thromboxane A_2 formation were obtained by using a superfused rabbit mesenteric artery, measuring the release on a system by which contraction-inducing substances released from lung parenchymal strips or platelets were identified. Usually this method proved to be sufficiently sensitive [Zijlstra and Vincent, 1981]. When specific antisera against TxB_2 became available, a comparison was made between the results of the bioassay and the radioimmunoassay. It turned out that the latter method allowed a better separation of the two components of the contractile activity of LTC_4 on the guinea pig lung parenchymal strip.

It was shown that the release of TxA_2 occurred during the initial period of the contractions [3.2.2]. Both this finding and the contraction obtained after addition of the cyclooxygenase inhibitor indomethacin indicated that the direct action of LTC_4 represents the long-lasting slow-acting component in the contraction.

Tachyphylaxis. Repeated applications of the same dose of LTC_4 or LTD_4 to the GPLP lead to a marked diminution of the thromboxane release. There was only a slight decline in the contractile activity. After 8 or more applications of the same dose LTC_4 or D_4 approximately 80% of the initial contractile activity was still present but no more TxA_2 was formed. This is in agreement with experiments in which the thromboxane synthesis was totally inhibited [Zijlstra *et al.*,1983a].

The rapid loss of effect upon repeated applications of LTs was reported

by a number of authors [Leitch, 1984; Feuerstein, 1985; Bach, 1984], but only observed in human cerebral arteries *in vitro* [Tagari *et al.*,1983]. *In vivo*, however, frequent injection of LTD_4 into the intact pig coronary circulation did not result in tachyphylaxis [Feuerstein, 1985].

The fact that other investigators did not observe the phenomenon of tachyphylaxis in LT-induced contractions of the GPLP [Piper and Samhoun, 1982], can possibly be explained by the fact that they did not use a radioimmunoassay and failed to standardize the superfused artery model before and after the experiment with angiotensin II or the thromboxane A_2 mimetic U-44069. Using the contraction of the mesenteric artery as a measure of TxA_2 released from the GPLP, the area under the contraction curve should be taken for calculations. From other observations it was proven that an adequate relationship existed between the area and the quan tity of TxB_2 obtained by radioimmunoassay [Zijlstra and Vincent, 1983].

Application of TxA_2 generated by aggregated rat platelets to a GPLP caused a pronounced contraction [Zijlstra and Vincent,1984]. The amounts of added TxA_2 were measured by RIA of TxB_2. The amount of TxA_2 released from the GPLP due to LT-induced contraction was about 5-times lower than that of externally applied TxA_2 required to induce a contraction of the same order of magnitude. The same occurred when phospholipase A_2 was administered to the GPLP in the superfusion bath. Other experiments indicate that TxA_2, released by either PLA_2 or LTC_4/D_4 originates from different sources of arachidonic acid [Zijlstra *et al.*, 1983a]. After the depletion of TxA_2, induced by repeated applications of LTC_4 a pronounced formation of TxB_2 was still seen after the administration of a single dose of PLA_2 and which gave also a contraction. Inverse application of the two contraction inducing substances resulted in the same phenomenon [Zijlstra and Vincent, 1985a].

5.2 EICOSANOIDS IN BODY FLUIDS

Besides the determination of the profiles of eicosanoid synthesis of cells involved in inflammatory processes and chronic diseases, measurements of the concentration of eicosanoids in tissue fluids could give more insight in the relative contribution of these substances to their ultimate effect. In the next paragraph results of measurements of eicosanoids in lung lavage fluid and in mucus of ulcerative colitis are discussed.

5.2.1 *Lung lavage fluid.*

In chapter 4, table 3, it was summarized that LTs are present in body fluids after allergen challenge or in inflammatory processes. These data indicate the importance of specific inhibitors of leukotriene synthesis. In paragraph 4.2.3 the results of measurements of LTs in broncho- alveolar lavage fluid of a patient with pulmonary alveolar proteinosis were discussed. In view of the relatively large amount of sulphidopeptide LTs present, we expected smooth muscle constriction. This was however not the case. We have no explanation for this finding. The capacity of LTC_4 and LTD_4 to increase vascular permeability could be the reason of the accumulation of proteins in the lung.

5.2.2 *Eicosanoid production in inflammatory diseases.*

To determine the influence of inflammation and topical treatment with 5-aminosalicylic acid or prednisolone on arachidonic acid metabolism *in vivo*, a study on the release of PGE_2 and LTB_4 from the rectal lumen in patients with distally located ***ulcerative colitis*** was performed [Lauritsen *et al.*, 1986]. Luminal concentrations of these eicosanoids were

positively correlated to disease activity and decreased in the prednisolone treated patients [Lauritsen *et al.*, 1987].

In our laboratory PGs, LTs and HETEs were measured in mucus of freshly recovered morning stools of a patient with an exacerbation of ulcerative proctocolitis who was not treated with drugs [Zijlstra and van Blankenstein, 1987]. 15-HETE was present in extremely high amounts compared to those of LTB_4, TxB_2 and 6-keto-$PGF_{1\alpha}$ (1070, 20, 9 and 4 ng/g mucus resp.). Comparatively small amounts of sulphidopeptide LTs were measured (< 1 ng/g mucus).

Also incubates with ^3H-labelled AA from jejunal mucosal biopsies from patients with gluten-sensitive enteropathy, generated extremely high amounts of 15-HETE [Krilis *et al.*, 1986].

Studies in experimental colitis established a relationship between LTB_4 and LTC_4 and the severity of the inflammation [Zipser *et al.*, 1986]. The pro-ulcerogenic action of LTC_4 has been demonstrated in dogs [Whittle *et al.*, 1986]. Furthermore LTC_4 increases mucus secretion [Johnson *et* al., 1983].

Both the LTs and HETEs are synthesized by macrophages, eosinophils and neutrophils. In our studies mucus samples contained small numbers of these cells. Normally considerable amounts of macrophages are present in inflamed tissue and it is likely that the mediators secreted contribute to the changes occurring.

The presence of LTB_4 was demonstrated in synovial fluids and tissue of patients with rheumatoid arthritis [Keppler *et al.*,1985]. This indicates that LTB_4 is a likely stimulator of cellular infiltration in acute phase of the inflammatory process in rheuma. Interactions between PGs, LTs and HETEs occur after the initial activation of the process by immunological or chemical triggers.

5.3 EICOSANOID PRODUCTION BY MACROPHAGES

In this part of the discussion the formation of eicosanoids by cells in human lung and macrophages during peritoneal inflammation and the differences between species and origin in the profile of eicosanoid formation of macrophages, are described.

5.3.1 *Cells in human lung.*

Human mast cells predominantly form PGD_2 (55%) and the leukotrienes C_4 (20%) and B_4-isomers (14%) after labelling with ^{14}C-AA and activation with anti-IgE or ionophore A23187 [Peters *et al.*, 1984].

Macrophages (MØ) were considered as the most important source of LT in human lung tissue. In our own experiments human alveolar macrophages generated mainly LTB_4 (44%) whereas LTC_4/D_4 and TxB_2 were synthetized in limited quantities (7%) [4.3].

Accumulation of eosinophils after application of the chemotactic agents LTB_4 and 5-HETE could lead to a comparatively large production of LTC_4 [Verhagen *et al.*, 1984; Weller *et al.*, 1983]. The formation of LTC_4 could in turn result in the release of TxA_2.

In asthmatics the eosinophil count is significantly higher [Godard *et al.*, 1982]. Accumulation of these cells and stimulation of the LTC_4 production and subsequent generation of thromboxane and prostaglandins could in combination result in an excessive bronchoconstriction. Eosinophils from patients with eosinophilia, including asthmatics, have an increased capacity to release PAF (platelet activating factor), as compared to those from normal individuals [Lee *et al.*, 1984]. PAF stimulates leukotriene and prostaglandin formation in lungs and various

cell systems [Rola-Pleszczynski, 1985]. Recently it was found that PAF induces LTC_4 synthesis in purified human eosinophils as well [Bruynzeel *et al.*, 1987].

5.3.2 *Peritoneal macrophages.*

The generation of arachidonic acid metabolites by peritoneal macrophages was studied in patients with end-stage renal disease undergoing continuous ambulatory peritoneal dialysis (CAPD) during infection-free periods and during bacterial peritonitis [4.2.4]. The most important metabolites generated by macrophages isolated from the CAPD fluid were LTB_4 and 5-HETE (each 30%). 15-HETE and 20-OH-LTB_4 were synthetized in small amounts (each 5%). Approximately 20% of the metabolized AA yielded cyclooxygenase products.

During inflammation the amounts produced diminish considerably but return to baseline after cure in the control period. A likely explanation is, that the production of corticosteroids in the adrenal might be enhanced during peritonitis, which leads to a higher level of circulating cortisol and hence a diminished eicosanoid production. Also the induction of lipocortin with its phospholipase A_2 inhibitory activity will result in an inhibition of the eicosanoid formation. Furthermore it has been shown that during peritonitis a decline in cAMP content of peritoneal macrophages correlates with a decrease in PGs and TxB_2 synthesis [Adolfs *et al.*, 1985].

The prostacyclin metabolite 6-keto-$PGF_{1\alpha}$ was found to be the major prostanoid generated by human peritoneal mesothelium. The ratio of the vasodilating PGs and their metabolites to the vasoconstricting PGs and their metabolites increased considerably during peritoneal inflammation [Steinhauer *et al.*, 1985]. This may be responsible for the increased

peritoneal blood flow during peritonitis. The loss of proteins in peritonitis was also considered to be caused by eicosanoids.

The results of our studies [4.2.4] indicate that, although the formation of eicosanoids per unit of macrophages decreased during peritonitis, the extremely high influx of cells induced an inbalance between the chemotactic, vasoconstrictive and vasodilator agents and their interactions. In all probability the losses of proteins are caused by lipoxygenase products [Marom *et al.*,1982], [4.2.3]. *In vivo* inhibition of these products by specific synthesis inhibitors could give more insight in this problem.

In human peritoneal macrophages obtained from ascites of patients with liver cirrhosis, the amounts of exogenous TxB_2 are six times higher than those of the classic PGs [Ouwendijk *et al.*, 1985; paragraph 4.2.5].

In elicited populations of rat peritoneal macrophages an increase of prostaglandin E_2 production was observed after stimulation by LTC_4 [Schenkelaars and Bonta, 1983]. In these experiments approximately equal amounts of endogenous PGE_2 and TxB_2 were measured. In resident rat peritoneal macrophages the amounts of exogenous TxB_2 were approx. 15 times higher than those of PGE_2 [Vincent and Zijlstra, 1986].

5.3.3 *Macrophages from several species and origin.*

In paragraph 4.3 a summary is given of the synthetic properties of macrophages obtained from several species and origin to generate eicosanoids. The lipoxygenase/cyclooxygenase ratio presents in one view the contribution of LTs, considering human macrophages.

Differences were present in the synthesis of peptide LTs. In the rat the testis macrophages formed mainly 12-HETE, but this was not true in macrophages from other origin. Peritoneal macrophages differ from

alveolar macrophages with regard to the formation of cyclooxygenase products [5.3.1]. Remarkable is the generation of PGD_2 by rat liver macrophages or Kupffer cells. This was in contrast to findings in man in whom 54% of AA was metabolized to mono-HETEs.

Considering the origin of peritoneal macrophages in liver cirrhosis the diversion of 12- and 15-HETE formation into LTB_4 formation is of interest. Moreover, a marked difference between peritoneal macrophages from ascites fluid of liver cirrhosis and those from CAPD was not observed in our results.

The noticable generation of TxB_2 (and HHT) in alveolar macrophages of the guinea pig could be related to the TxA_2 related bronchoconstriction in these animals. In conclusion all types of macrophages have the capacity to produce eicosanoids, but the profile of eicosanoid production is dependent both on the tissue and on the species from which the macrophage is derived.

Changing profiles of eicosanoids may be produced by human macrophages in different diseases. As discussed in paragraph 5.3.1 peritoneal macrophages from patients undergoing continuous ambulatory peritoneal dialysis, synthetize almost the same pattern of eicosanoids as peritoneal macrophages derived from patients with ascites due to liver disease.

5.4 PRECURSORS.

It was hypothetized that deficiencies of precursors or enzymes or an inbalance in phospholipid composition, could lead to subpopulations of macrophages which generate either less or more different types of eicosanoids in different amounts. In view of our findings that possibly

two pools of arachidonic acid are involved in the LT- or PLA_2- induced contractions of the guinea pig lung parenchyma [Zijlstra and Vincent, 1985a], and taking into account the differences in the generation of PGs, LTs or HETEs in macrophages of different species and origin, one would expect that the phospholipid metabolism is the rate-limiting factor.

Incorporation of ^{14}C-AA into alveolar macrophages from healthy subjects (HS), allergic asthmatics (AS) and chronic bronchitis patients (CB) revealed more labelling of phospholipids in macrophages from AS and CB than in those from HS [Damon *et al*, 1987]. An increase in the incorporation into PI and PE was seen, from which the authors conclude to the existence of two different sources of AA release, one for inflammatory macrophages and another for quiescent cells.

In guinea pig lungs linoleic acid-derived products can also be synthesized by non-stimulated alveolar macrophages [Engels *et al*, 1986]. These compounds, 9-hydroxy-10,12-octadecadienoic acid (9-HODE) and 13-hydroxy-9,11-octadecadienoic acid (13-HODE) are not synthesized via exogenous arachidonic acid, or when a stimulus is used to liberate AA from membrane lipids. The role of these substances as mediators in inflammatory processes has not yet been established.

Recently it was shown that during inflammation the arachidonic acid composition of phospholipids in colonic mucosa is increased in patients with active ulcerative colites [Nishida *et al.*,1987]. Furthermore a positive relationship existed between the cell density of the inflammatory infiltrate and the arachidonic acid composition. Unfortunately the investigators failed to measure the endogenous formation of eicosanoids by inflammatory cells.

Incorporation into the tissues and metabolism of ^{14}C-AA after i.v. administration to rats was investigated [Zijlstra and Vincent, 1985b]. A

considerable incorporation occurred into perispinal fat, liver, heart muscle, kidney and adrenal. In the heart the composition of the phospholipids was determined. 44% of the radioactivity was incorporated into phosphatidylcholine (PC) and 7 and 10% into phosphatidylinositol (PI) and phosphatidylethanolamine (PE).

In aggregated rat platelets it was observed that the rapid formation of TxA_2 was correlated with a decline in PI and PE. The slow formation of 12-HETE, on the contrary, was inversely proportional to a decline in PC. A preliminary conclusion based on these data would be that cyclooxygenase products are generated from PI and PE, whereas PC is the precursor of lipoxygenase products. This could imply that the rate-limiting factor was the incorporation of the exogenous AA into the phospholipids [Vincent and Zijlstra, unpublished].

In platelets of patients indicated as deficient in 12-lipoxygenase, incubations in the presence of ^{14}C-AA lead to the rapid formation of TxB_2 only. Administration of aspirin ***in vivo***, however, resulted in a total inhibition of TxB_2, and the formation of 12-HETE [de Vetten ***et al***, 1987].

Recently results of investigations on PG synthesis and release by subpopulations of rat alveolar macrophages were published [Chandler and Fulmer, 1987]. The results demonstrated that density-defined alveolar macrophages are heterogenous in ability to synthesize and release PGs, which is dependent on the stimuli applied. These findings suggest that there may be a specific role for individual macrophage subpopulations in the control and/or modulation of the pulmonary inflammatory and immune processes. Investigations with subpopulations of macrophages obtained from CAPD before and during peritonitis could confirm this hypothesis.

5.5 PHARMACOLOGICAL INTERVENTION

In the last part of the general discussion the influence of β-adrenoceptor agonists and antagonists, the role of cyclic AMP, leukotriene antagonists and synthesis inhibitors are subjects of discussion.

5.5.1 *Beta-adrenergic agonists.*

Adrenergic substances stimulate the β_2-receptor of smooth muscle cells. This leads to an increase in cyclic-AMP and subsequently to relaxation. In experiments described in paragraphs 3.2.4, 3.2.3 and 3.2.5 the effects of β-adrenoceptor agonists and antagonists in LT-induced contractions of the guinea pig lung parenchyma were investigated. Isoprenaline reversed the LTC_4-induced contraction of the GPLP and inhibited the release of TxA_2 from the GPLP. The β-adrenoceptor antagonist sotalol counteracted these effects. The specific β_2-agonist salbutamol mimicked the results obtained by isoprenaline, whereas the phosphodiesterase inhibitor isobutyl-methyl-xanthine (IBMX) had an identical activity [Zijlstra *et al.*, 1983a].

In platelets, activation of adenylate cyclase and subsequent enhancement of intracellular cyclic-AMP results in a decrease in the release of TxA_2 [Minkes *et al.*, 1977]. Adrenergic substances stimulate the β_2-receptor of the smooth muscle cells. This leads to an increase in cyclic AMP, inhibition of the secretion of mediators and consequently relaxation of smooth muscle.

5.5.2 *Leukotriene antagonists.*

The anti-bronchoconstrictive effect of corticosteroids can possibly be attributed to the inhibition of phospholipase A_2 and the subsequent decrease in formation of eicosanoids. Inhibition of the release of

mediators from the mast cell by cromoglycate can also result in a reduction of smooth muscle constriction upon challenge with triggers. The limited effects of histamine antagonists indicated that histamine might not play a dominant role in many processes leading to bronchoconstriction. Therefore studies on the effect of LT synthesis inhibitors and antagonists became interesting.

In paragraph 3.2.1 the effect of FPL 55712, an antagonist of SRS-A, on the LT-induced contraction of the GPLP is described. The remaining direct contractile effect of LTC_4 after the addition of indomethacin to the superfusion buffer, was almost completely abolished. The clinical and therapeutic usefulness of FPL 55712 in naturally occurring diseases of man, e.g. allergic asthma, will depend upon the final results arising from the use of FPL 55712 as an aerosol in experimental models. The short biological half-life ($T_{½}$ = 0.6 min in guinea pig i.v.) is one of the problems to be solved [Chand, 1979].

Another recently introduced pharmacological antagonist of LT-induced bronchoconstriction is SKF 88046 [Weichmann *et al.*, 1984]. The results obtained by the administration of this compound demonstrated that its effect is based on an indirect, Tx-dependent inhibition of LTD_4 in the guinea pig, presumably via thromboxane antagonism.

5.5.3 *Lipoxygenase inhibitors.*

Much effort was made to obtain a specific 5-lipoxygenase inhibitor. If leukotrienes are ultimately established as major mediators of distinct disorders, selective leukotriene synthesis inhibitors may offer an advantage as a therapy in major aspects of allergic airway disease and inflammatory processes.

The ability of retinoids to inhibit the generation of chemotactic LTB_4

can be considered as an explanation of the activity of such compounds as inhibitors of inflammatory cell accumulation in diseases such as psoriasis [Bray, 1984]. Another non-specific lipoxygenase inhibitor is caffeic acid. It inhibits both the leukotriene synthesis and the 12-HPETE synthesis, whereas platelet aggregation and the biosynthesis of thromboxane tended to decrease [Koshihara *et al.*, 1984].

During the last few years a number of drugs were described which inhibit lipoxygenase pathways. A summary is given by Beetens *et al.* [1986], who tested the inhibition of the oxygenation reactions in several *in-vitro* systems. The relative potency of the various compounds is nor-dihydro-guaiaretic acid (NDGA) > nafazatrom > phenidone > ketoconazole > BW-755C, for the 5-lipoxygenase pathway. However, none of these compounds specifically inhibited this particular pathway. Differences occurred in the dosages necessary to obtain inhibition of the 12- and 15-lipoxygenase pathways, and in the ability to influence parts of the cyclooxygenase pathway.

Recently more selective inhibitors of 5-lipoxygenase were introduced. AA-861 dose-dependently inhibited LTB_4 and LTC_4 generation by human polymorphonuclear leukocytes with IC_{50} values of 0.3 μM and 0.01 μM respectively, being about 1000 times more potent than BW-755C [Mita *et al.*,1986]. Auranofin (AF) is a newly introduced oral gold compound, having antirheumatic properties, with differential inhibitory effects on LTB_4 and LTC_4 formation by human leukocytes [Honda *et al.*,1987]. IC_{50} values of this compound are extremely higher than those of AF, being 100 μM and 14 μM respectively.

5.5.4 ***Malotilate.***

As presented in paragraph 4.2.5, our results with malotilate suggest a new mechanism of action for this compound as a specific 5-lipoxygenase inhibitor. Malotilate has been shown to protect against injury of the liver, chronic hepatitis, alcohol intoxication and fibrotic liver. It inhibits collagenous fibre formation under ordinary cell culture conditions. Because macrophages are involved in liver damage and fibrosis, the effect of malotilate on the synthesis of arachidonic acid metabolites was investigated. The scope of the initial study was to establish whether the ***in vivo*** effects of malotilate are related to the modulation of inflammatory modulators, such as the eicosanoids.

In the ***in vitro*** set-up macrophages isolated from ascitic fluid of patients with alcoholic liver cirrhosis were incubated with ^{14}C-AA and triggered with calcium ionophore A23187. Separation and quantitation was performed on HPLC. In a second series of experiments the endogenous formation of eicosanoids, triggered with ionophore, was measured with RIA. Both the exogenous and endogenous formation of the 5-lipoxygenase products LTB_4 and 5-HETE was inhibited, whereas the 12- and 15-HETE tended to increase. The formation of the cyclooxygenase products TxB_2, 6-keto-$PGF_{1\alpha}$ and PGD_2 was also inhibited, but at a IC_{50} being 10 times higher than that of 5-lipoxygenase.

As mentioned before the inhibitory effect of the hydroperoxides on the thromboxane synthetase and prostacyclin synthetase was described by Vanderhoek. This finding was confirmed by experiments performed with sheep seminal vesicles, in the presence of ^{14}C-AA and malotilate, in which only cyclooxygenase compounds were synthetized. The formation of hydroperoxides was inhibited by the addition of ascorbic acid to the

medium. No inhibitory effect of malotilate was observed, in contrast to the inhibition of cyclooxygenase products by indomethacin (IC_{50} 6 μM). Malotilate was less potent than NDGA (IC_{50} resp. 4.8 and 1.4 μM) on 5-lipoxygenase, but appeared to have no direct inhibitory effect on the other lipoxygenase pathways. This behaviour was more or less identical to that of ketoconazole.

The development of specific 5-lipoxygenase inhibitors opens new perspectives for the study of the role of leukotrienes in disease processes.

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SUMMARY

Since the discovery of the leukotrienes it has become clear that these substances, and in particular the sulphidopeptide leukotrienes, are involved in asthmatic and inflammatory processes. The aim of the investigations described in this thesis was to gain more insight into the biological effects of leukotrienes on lung parenchymal tissue and the formation of these substances by (inflammatory) cells and tissues. The mechanism of action and metabolism was investigated in the presence of agonists, antagonists, blockers and synthesis inhibitors.

Contractions of the guinea pig lung parenchymal strip (GPLP), induced by the leukotrienes C_4 or D_4 are biphasic. The initial part is due to the fast release of thromboxane A_2 (TxA_2), whereas the slow long-lasting effect is caused by the direct action of LT itself. Repeated application of LTC_4 to parenchymal tissue results in the development of tachyphylaxis. It was found, however, that the tachyphylaxis to the induced formation of TxA_2 was greater than that to the contractile response. The addition of β-adrenoceptor agonists, such as isoprenaline and salbutamol, preferentially inhibited TxA_2 production with less effect on the contraction of this tissue. Indications that this effect was initiated via an increase of cellular cAMP were obtained by an investigation in which the phosphodiesterase inhibitor IBMX was used, with identical effects as the β-adrenoceptor agonists. Addition of a β-adrenoceptor antagonist, such as sotalol and propranolol, to the superfusion system reversed both the inhibition of the contraction and the release of TxA_2. There was no marked difference observed between the $\beta_{1,2}$-agonist and the specific β_2-agonist.

The release of TxA_2 and the contractile effects induced after application of LTs or phospholipase A_2 (PLA_2) to human and porcine lung parenchymal strips were considerably less than found with the GPLP. Application of LTs and PLA_2 to the GPLP in distinct order indicated that two or more pools of arachidonic acid are present which generate TxA_2. Addition of indomethacin, a specific cyclooxygenase inhibitor, to the superfusion system, totally inhibited the LT-induced TxA_2 release, whereas only a small part of the contraction was abolished. FPL 55712, a SRS-A antagonist nearly completely counteracted the LT-induced contraction of the GPLP. The constrictive activities of LTC_4 and a thromboxane A_2 mimetic appeared to be much more potent than those induced by other eicosanoids and bronchoconstrictive agents (histamine, acetylcholine and 5-hydroxytryptamine) in both the GPLP and human small airway smooth muscle.

The introduction of high performance liquid chromatography increased the possibilities to measure prostaglandins, leukotrienes and other arachidonic acid metabolites in body fluids or cell incubates. In addition the availability of specific antisera for these substances made it possible to characterize and quantitize these metabolites in femtomoles. Work-up procedures for fluids and extracts were considerably simplified by the introduction of disposable cartridge systems. Finally the enormous development in computing controlled systems contributed for a large part to the progress of these measurements.

Levels of sulphidopeptide LTs were measured in lung lavage fluids and LTB_4 and 15- and 12-HETE in mucus of a patient with ulcerative procto colitis. In ^{14}C-arachidonic acid loaded chopped human lung tissue mostly LTB_4-like substances were formed and less leukotrienes C_4,D_4 and E_4, whereas large amounts of mono hydroxy eicosatetraenoic acids were

generated. PGD_2 appeared to be the most common cyclooxygenase product. Differences were observed between the amounts formed from endogenous and exogenous by applied arachidonic acid.

Macrophages isolated from fluids of patients with peritonitis and liver cirrhosis generated mainly the 5-lipoxygenase products LTB_4 and 5-HETE, depending on the severity of the inflammation. The cyclooxygenase products 6-keto-$PGF_{1\alpha}$ and TxB_2 were the most important prostaglandin-like substances. Malotilate, an anti-fibrotic substance, selectively inhibited the 5-lipoxygenase, whereas both the 12- and the 15-lipoxygenase pathways were stimulated. The effects of malotilate on eicosanoid production differ from those of known lipoxygenase inhibitors. Such differential effects have not previously been reported. The inhibition of the 5-lipoxygenase pathway, and thus of the production of LTB_4, which plays a major role in the regulation of the immune response through numerous and varied effects on several cell types, and the inhibition of the formation of vasoconstrictive TxA_2, provide possibilities for the beneficial effects of this drug in inflammatory diseases.

SAMENVATTING

Sinds de ontdekking van leukotrienen is het duidelijk geworden dat deze stoffen, en met name de sulfido peptide houdende leukotrienen, zijn betrokken bij asthmatische en ontstekingsprocessen. De onderzoekingen die in dit proefschrift zijn gebundeld omvatten twee hoofdonderwerpen, namelijk de biologische effekten van leukotrienen op long parenchym weefsel en voorts de vorming van deze stoffen door (ontstekings) cellen en weefsel. Het werkingsmechanisme en de omzetting naar andere produkten werden bestudeerd in tegenwoordigheid van farmaca die de effekten blokkeren of de synthese remmen.

Contracties van de cavia long parenchym strip (GPLP), opgewekt door de leukotrienen C_4 en D_4 zijn bifasisch in de wijze waarop ze tot stand komen. Het eerste traject van de contractie wordt veroorzaakt door de snelle afgifte van thromboxane A_2 (TxA_2), terwijl het langzame aanhoudende effekt teweeg wordt gebracht door de direkte werking van LT zelf. Herhaalde toediening van LTC_4 aan parenchym weefsel heeft een uitputting van het systeem tot gevolg. Er werd echter gevonden dat er een grotere uitputting van het gevormde TxA_2 optrad dan wat werd waargenomen aan teruglopende respons van de contractie. Toevoeging van de β-adrenerge stoffen isoprenaline en salbutamol aan de superfusiebuffer hadden met name een grote remmende invloed op de TxA_2 synthese terwijl het samentrekkende effekt van LT slechts ten dele werd gereduceerd. Aanwijzingen dat dit effekt tot stand werd gebracht via een toename van het cellulaire cyclisch AMP werden ondermeer bevestigd door experimenten waarin de fosfodiesterase remmer IBMX werd gebruikt, waarbij vergelijkbare resultaten werden verkregen. Als vervolgens een β-

adrenoceptor antagonist aan het systeem werd toegevoegd, zoals sotalol en propranolol, dan bleken zowel het TxA_2 vormend als het contractieve effekt reversibel te zijn. Opmerkelijke verschillen tussen een $\beta_{1,2}$-agonist en een specifieke β_2-agonist werden niet waargenomen.

Het vrijkomen van TxA_2 en de contractie geinduceerd door toediening van LTs of fosfolipase A_2 (PLA_2) aan humane en varkens long strips vertoonden reakties die slechts een paar procent bedroegen ten opzichte van de verkregen effekten met de cavia long strip. Toediening van LTs en PLA_2 aan de GPLP in verschillende volgorde gaven aan dat er sprake kan zijn van twee of meer opslagplaatsen van arachidonzuur in de cel waaruit de vorming van TxA_2 tot stand komt na aktivatie. Onder invloed van aan het superfusiesysteem toegevoegd indomethacine, een cyclooxygenase remmer, wordt een totale inhibitie van de LT geinduceerde TxA_2 vorming bewerkstelligd, hoewel slechts een gedeeltelijke afname van de longstripcontractie verkregen wordt. De SRS-A antagonist FPL 55712 bleek de contractie van de GPLP door LT totaal te blokkeren. De constrictieve aktiviteiten van LTC_4 en een op thromboxane A_2 lijkende stof vertoonden een veel grotere aktiviteit dan andere arachidonzuur metabolieten en in het lichaam voorkomende bronchoconstrictieve stoffen, bij zowel de GPLP als stripjes van humane luchtwegen.

De introduktie van de hoge druk vloeistof chromatografie vergrootte de mogelijkheden om prostaglandinen, leukotrienen en andere arachidonzuur metabolieten te meten in lichaamsvloeistoffen of inkubatiemedia van cellen. Bovendien maakte de beschikbaarheid van specifieke antisera voor deze substanties het mogelijk om ze te karakterizeren en hoeveelheden te bepalen in de femtomolen range. Opwerkingsprocedures voor vloeistoffen en extracten werden aanzienlijk vereenvoudigd door de invoering van wergwerp zuiveringssystemen. Tot slot heeft de enorme ontwikkeling in de

computergestuurde systemen voor een belangrijk deel bijgedragen in de voortgang van meetmethoden.

Zodoende konden sulfidopeptide houdende leukotrienen ook gemeten worden in long spoelingen van patienten en was het technisch uitvoerbaar LTB_4 en 15- en 12-HETE in darmslijm van een patient met een ulceratieve procto colitis te meten. Het vormend vermogen van cellen om na het verzadigen met ^{14}C-arachidonzuur metabolieten te vormen werd zo eenvoudig vast te stellen. Humaan long weefsel bleek voornamelijk LTB_4-achtige verbindingen te vormen en beduidend minder van de leukotrienen C_4, D_4 en E_4, hoewel niet onaanzienlijke hoeveelheden monohydroxy verbindingen van arachidonzuur werden gevormd. Het prostaglandine D_2 bleek de voornaamste cyclooxygenase component te zijn. Duidelijke verschillen werden vastgesteld tussen de endogene en exogene gevormde hoeveelheden.

Macrofagen verkregen na isolatie uit vloeistoffen van patienten met een buikontsteking en leveraandoeningen zijn in staat voornamelijk en in grote mate verbindingen van de 5-lipoxygenase route te vormen, de produkten LTB_4 en 5-HETE, afhankelijk van de ernst van de ontsteking. De cyclooxygenase produkten 6-keto-$PGF_{1\alpha}$ en TxB_2 waren daarnaast de meest voorkomende prostaglandine achtige metabolieten. Malotilate, een stof met anti fibrotische werking, blijkt zeer selektief de 5-lipoxygenase route te remmen, terwijl zowel de 12- als de 15-lipoxygenase routes niet geremd doch gestimuleerd worden, resulterend in een neveneffekt waarbij de vorming van genoemde cyclooxygenase verbindingen licht geremd worden. Deze effekten van Malotilate op de arachidonzuur produkten verschillen van die welke bekend zijn van als zodanig aangeduide lipoxygenase remmers. Dergelijke verschillen in effekten zijn nog niet eerder waargenomen. De remming van de 5-lipoxygenase route, en daarmee de vorming van bijvoorbeeld LTB_4, welke een belangrijke rol vervult in de

regulering van het immuunsysteem via talrijke en gevarieerde effekten op verschillende cellen, en bovendien de remming van de bloedvatvernauwende stof TxA_2, geven mogelijkheden om dit farmacon op termijn te gebruiken als substantie bij allerlei onstekingsprocessen.

NAWOORD

Dit proefschrift was niet tot stand gekomen zonder de hulp van velen, die met woord, geschrift of het aanbieden van patiëntenmateriaal bijgedragen geleverd hebben, dat veelal heeft geleid tot publikaties, waaruit voor een gedeelte dit boekje is samengesteld.

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CURRICULUM VITAE

De schrijver van dit proefschrift werd geboren op 2 januari 1953 te Rotterdam-Overschie. Na ter plaatse de lagere- en middelbare school doorlopen te hebben, werd aanvankelijk een opleiding gevolgd aan de HTS (studierichting elektronica) te Rotterdam, doch wegens langdurige ziekte afgebroken en omgezet in een HBO-opleiding tot chemisch analist aan het Van 't Hoff Instituut te Rotterdam, van 1971 tot 1975. Op 23 oktober 1973 werd een aanstelling als analist verkregen aan de Medische Faculteit te Rotterdam binnen het instituut Farmacologie (beheerder: prof. dr. I.L. Bonta) en toegevoegd als assistent aan dr. J.E. Vincent.

Diverse cursussen op het gebied van de radiochemie werden gevolgd, afgesloten met de C-akte stralingsbescherming in 1978.

In de zeventiger jaren werd onderzoek gedaan naar mediatoren bij de aggregatie van bloedplaatjes en ontstekingsprocessen. Hierbij werd gebruik gemaakt van de superfusie cascade methode en in later stadium van radioimmunoassays (RIA's).

Het vormingspatroon van radioaktieve arachidonzuurmetabolieten werd onderzocht in verschillende organen van proefdieren, gebruik makend van dunnelaagchromatografie, toegespitst op essentiële vetzuurdeficiëntie.

Na de ontdekking van leukotriënen werd in 1980 begonnen met een *in vitro* model van de cavia long strip waarbij biologische aktiviteiten geregistreerd en gevormde metabolieten gemeten konden worden, in samenwerking met prof.dr. I.L. Bonta.

De snelle opkomst van de HPLC leidde in 1983 tot het ontwikkelen van geschikte scheidingsmethoden van alle mogelijke produkten van arachidonzuur. In combinatie met de reeds genoemde RIA's en superfusie methode werd zo de mogelijkheid gecreëerd een compleet beeld te krijgen van in het lichaam aanwezige hoeveelheden, vormingspatronen en biologische aktiviteiten van eicosanoiden.

Deze ongekende mogelijkheden hebben geleid tot vele vruchtbare samenwerkingsverbanden binnen de facultaire gemeenschap, met andere universiteiten en op internationaal nivo met de farmaceutische industrie. Het totaal aantal publikaties waarvan hij (mede)auteur is hebben de 60 reeds overschreden. Een 10-tal presentaties van eigen werk werd gehouden.

Enkele onderzoeken hebben een bijdrage geleverd in het tot stand komen van dissertaties van arts-assistenten. Daarnaast zijn diverse keuzepraktikanten, studentassistenten, assistenten-in-opleiding en stagiaires begeleid in het uitvoeren van wetenschappelijk onderzoek.

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