EICOSANOIDS, ENDOTOXINS AND LIVER DISEASE

EICOSANOIDS, ENDOTOXINS AND LIVER DISEASE

EICOSANOIDEN, ENDOTOXINEN EN LEVERZIEKTEN

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Voor Eugenie, Koen en Mieke

Voor mijn ouders.

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ABBREVIATIONS

AA	arachidonic acid
COP	cyclooxygenase products
EFA	essential fatty acid
5-HETE	(5S)-5-hydroxy-6,8,11,14-eicosatetraenoic acid
15-HETE	(15S)-15-hydroxyperoxy-5,8,11,13-eicosatetraenoic acid
HHT	(12S)-12-hydroxy-5,8,10-heptadecatrienoic acid
5-HPETE	(5S)-5-hydroperoxy-6,8,11,14 eicosatetraenoic acid
6-keto PGF_{1alpha}	6-keto prostaglandin F_{1alpha}
LTA_4	leukotriene A_4
LTB_4	leukotriene B_4
LTC_4	leukotriene C_4
LTD_4	leukotriene D_4
LTE_4	leukotriene E_4
LTF_4	leukotriene F_4
LTs	leukotrienes
NSAIDs	non steroidal anti inflammatory drugs
PGD_2	prostaglandin D_2
PGDH	15-hydroxy prostaglandin dehydrogenase
PGE_2	prostaglandin E_2
PGF_{2alpha}	prostaglandin F_{2alpha}
PGG_2	prostaglandin G_2
PGH_2	prostaglandin H_2
PGI_2	prostacyclin
PGs	prostaglandins
PLA_2	phospholipase A_2
PLC	phospholipase C
RES	reticulo endothelial system
SRS-A	slow reacting substance of Anaphylaxis
TXA_2	thromboxane A_2
TXB_2	thromboxane B_2

CHAPTER 1

INTRODUCTION

Endotoxins are cell wall lipopolysacharides of gram negative bacteria. The gut contains large numbers of bacteria and is generally accepted to be a large reservoir of endotoxins. In the normal state absorbed endotoxins are rapidly removed from the portal blood by especially the reticulo-endothelial cells of the liver. In patients with liver disease there is a diminished function of the reticulo-endothelial system, resulting in a raised frequency of systemic endotoxemia. Systemic endotoxemia in liver disease, as measured by the Limulus lysate test, correlates with a higher frequency of clotting disorders, renal failure and a high mortality rate.

Interaction of endotoxins with macrophages results in the liberation of mediators such as lysozyme and eicosanoids, and these may possibly be responsible for the effects of endotoxin. Eicosanoids is the collective name for prostaglandins and leukotrienes. Eicosanoids are very potent mediators, which are thought to play a role in the pathogenesis of several diseases. For example, thromboxane A_2, a potent platelet aggregator and vasoconstrictor, has been implicated in vascular diseases. Administration of endotoxins to experimental animals results in raised levels of thromboxane B_2, the stable metabolite of thromboxane A_2,

prostaglandin E_2, prostaglandin F_{2alpha} and 6 keto prostaglandin F_{1alpha}. The raised thromboxane B_2 levels are associated with shock, renal failure and a high mortality rate. As some of the circulatory and laboratory changes seen in patients with liver disease are similar to the effects of thromboxane A_2, it is conceivable that complications attributable to endotoxemia in cirrhosis might be mediated by eicosanoids.

The aim of this study was to investigate the possible role of eicosanoids in the complications of liver disease, and to answer the following specific questions:

1. Are plasma thromboxane B_2 levels raised in liver disease ?
2. Do raised plasma thromboxane B_2 levels correlate with the severity of liver disease ?
3. Is thromboxane involved in the hemodynamic changes in liver disease ?
4. What is the site of production and elimination of thromboxane B_2 in liver disease ?
5. Are raised plasma endotoxin levels correlated with raised plasma thromboxane B_2 in liver disease ?
6. Is it possible that portal hypertension and ethanol enhance the permeability of the intestine to endotoxins ?
7. Do human peritoneal macrophages derived from patients with liver disease produce eicosanoids ?
8. Is the ability to produce eicosanoids species and tissue dependent ?

CHAPTER 2

PROSTAGLANDINS AND LEUKOTRIENES

2.1 INTRODUCTION

Eicosanoids is the collective name for prostaglandins (PGs) and leukotrienes (LTs). Eicosanoids have received much attention during the last decade and are now thought to play an important role in the pathogenesis of several diseases. For example eicosanoids are involved in asthma (1,2), rheumatoid arthritis and other inflammatory diseases (3,4), cardiovascular diseases (5), renal diseases (6) and cancer (7,8). Synthetic prostaglandins have been used therapeutically in obstetrics, pediatrics and vascular diseases (9,10,11).
The field of PGs and LTs is relatively new and complex and as a great part of this dissertation will deal with the eicosanoids, it is necessary to provide an introduction on the pathophysiology of these compounds. This section is not meant to cover all aspects of the eicosanoids but to summarize some characteristic properties of these extremely fascinating compounds.

2.2 HISTORY

In 1930 Kurzrok and Lieb found that fresh human semen produced contractions of the human uterus (12). Goldblatt and von Euler

discovered that lipophilic compounds were the active components responsible for these uterus contractions (13,14). Von Euler named these lipophilic compounds prostaglandins, because they were thought to be produced by the prostate (15). Later it became clear that the majority of PGs production in semen was produced by seminal vesicles. It was only in 1957 that Bergström et al isolated the first PGs, prostaglandin E (PGE) and prostaglandin F_{2alpha} (16). In 1964 two independent groups of investigators, namely van Dorp et al from the Unilever Research Laboratory, Vlaardingen, and Bergström et al from Sweden, showed that PGs were derived from essential fatty acids (17,18).

In 1973 the unstable endoperoxides PGG_2 and PGH_2 were isolated by Nugteren and Hazelhof (19) and Hamberg and Samuelsson (20).

Thromboxane A_2, initially described in platelets, but later in many other tissues, was found in 1975 (21). In 1976 Vane and associates isolated prostacyclin (PGI_2) (22). Borgeat and Samuelsson first described in 1979 the generation of several dihydroxy fatty acids by isolated polymorphonuclear leucocytes, following incubation with arachidonic acid (23). These substances were called leukotrienes, referring to their first established origin (leucocytes) and the presence of a certain chemical structure in their molecules, i.e. three conjugated double bonds (triene) typical for all these compounds (24). It became clear that slow reacting substance A (SRS-A) is a mixture of several peptidoleukotrienes (LTC_4, LTD_4, LTE_4) (25). For their discoveries concerning prostaglandins and related biologically active substances, Bergström, Samuelsson and Vane received the Nobel prize in 1982.

2.3 PRECURSORS OF PROSTAGLANDINS AND LEUKOTRIENES

The eicosanoids are formed from essential fatty acids (EFAs) having 20 carbon atoms and three to five methylene-interrupted, unsaturated bonds, all in the cis configuration (26,27). The essentiality of dietary fat was first demonstrated in 1929 by Burr and Burr, who observed poor growth and skin lesions in rats raised on a fat-free diet (28). These symptoms could be prevented by the addition of linoleic acid to the diet (29,30).

There are two fundamental fatty acids, linoleic acid and alpha-linolenic acid, from which all other EFAs are metabolically derived (27,30) (Fig.1). Linoleic acid and alpha-linolenic acid cannot be synthesized by higher animals and need to be present in food (26). Linoleic acid is the major dietary EFA in humans (26,27,30). The structure of any EFA is fully described by three numbers, for example linoleic acid is C18:2ω6; 18 is the number of carbon atoms in the chain, 2 is the number of double bonds and ω6 means the number of carbon atoms between the terminal unsaturated bond and the methyl end of the fatty acid.

Linoleic acid, which is present in organ meats such as liver, milk products and vegetable seed oils, such as sunflower oil and safflower oil, is desaturated and elongated to the important arachidonic acid (C20:4 ω6), via gamma-linolenic acid (C18:3 ω6) and dihomo gamma-linolenic acid (C20:3ω6) (26).

Gamma-linolenic acid is especially found in evening primrose oil. Arachidonic acid (AA) is present in human milk, and in meat, some seaweeds and shrimps (26). The average daily intake of arachidonic acid

is 100-190 mg/day which is more than enough for PGs production, which requires 1 mg/day (26). The other fundamental fatty acid, alpha-linolenic acid is present in sea food and is converted to eicosapentanoic acid (C20:5ω3) (26,27,30).

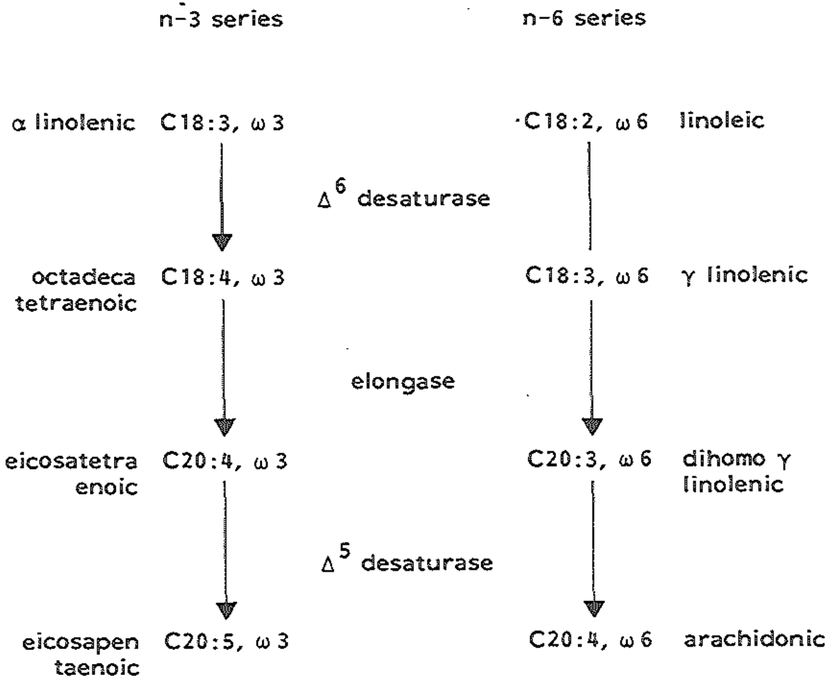

Fig.1. The conversion of alpha linolenic acid and linoleic acid to the precursors of the eicosanoids.

The enzymes involved in the desaturation and elongation of EFA are influenced by several factors, such as hormones, nutrition and ethanol (31,32,33). For example saturated fatty acids in the diet increase EFA requirement due to competition for the enzymes necessary for elongation and desaturation (27).

During the last years much work has been done with EFA deficient diets (4,26,30,34,35,36). EFA deficient diets give rise to complex changes in animals and humans including changes in skin morphology and permeability, pulmonary function, liver morphology, immunity and resistance to infections (30). In humans EFA deficiency occurred in patients receiving long term parenteral nutrition, before intravenous fat infusions were recommended (30). Infants treated with fat free parenteral nutrition for several months showed decreased growth rate, a typical skin rash and diminished resistance to infection (30). Some of these symptoms could be treated by giving linoleic acid or prostaglandins (30). Not only linoleic acid but possibly also alpha-linolenic acid is important in the diet to maintain health (30). The normal requirement of EFA in the diet is thought to be about 4% of the dietary energy (27).

Dihomo-gamma-linolenic acid, arachidonic acid and eicosapentaenoic acid are the precursors of the PGs and the LTs (3,29) (Fig.2). Dihomo-gamma-linolenic acid, with three double bonds, gives rise to the 1 series of prostaglandins as denoted by the suffix 1 (e.g. PGE_1) and LTs of the 3 series; arachidonic acid, with four double bonds, is the parent compound of the PGs of the 2 series (e.g. PGE_2)and 4 series of LTs (e.g. LTA_4); and from eicosapentaenoic acid with five double bonds the prostaglandins of the 3 series (e.g. PGE_3) and LTs of the 5 series (e.g. LTA_5) are formed (3,24,29) (Fig.2).

The EFAs are found in esterified form in several lipid pools. The bulk of EFAs in mammalian cells are esterified in the fatty acyl chains of phospholipids in membranes (26,27). To a lesser degree the EFAs are present in cholesterol esters and triglycerides (26,27). Phospholipids

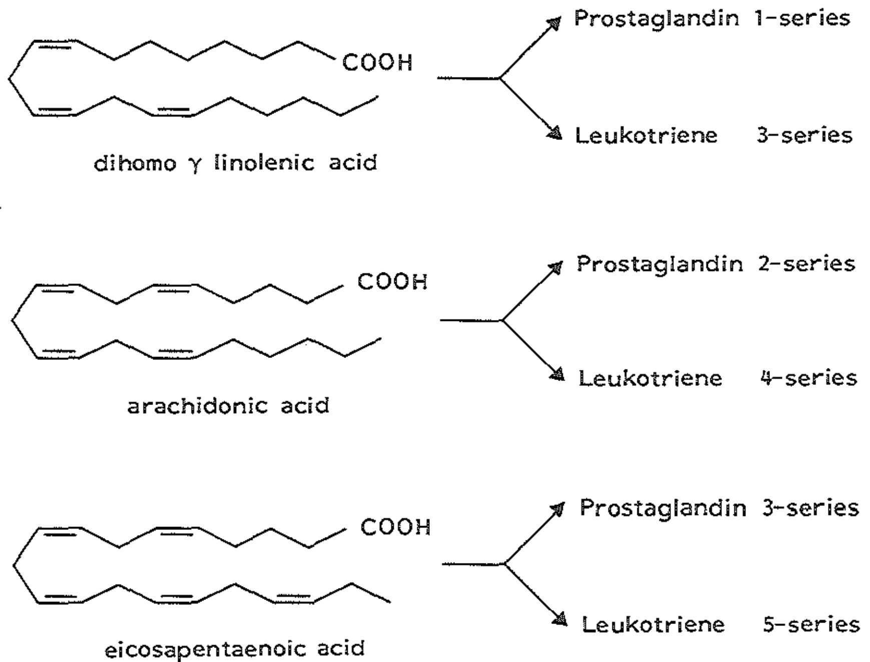

Fig.2. Conversion of the three essential fatty acids to respectively the prostaglandins and leukotrienes.

have a glyceryl backbone to which various groups are attached by ester linkages. Saturated fatty acids are linked mostly to position alpha and unsaturated fatty acids to position bêta. The $alpha_1$ position is esterified to phosphoric acid and to either choline, ethanolamine, serine or inositol giving phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol (37,38). Because EFAs have a predilection for the bêta position of phospholipids, arachidonic acid, the most abundant EFA in mammalian cells, is usually esterified to position ß (26,27). The phospholipids

phosphatidylethonalamine phosphatidylserine and phosphatidyl inositol are thought to be rich in AA in position B. Phosphatidyl choline contains only a small amount of AA (4). Despite intensive investigation it is still not clear which phospholipid fraction contribute to most of the AA liberated.

2.4 PHOSPHOLIPASES

As eicosanoids are not stored in cells, biosynthesis must immediately precede release. Conversion of AA into eicosanoids does not take place unless AA is first released from phospholipids. As soon as the eicosanoids are formed they cannot reincorporate into the phospholipids (3,26). Phospholipids are attacked by several phospholipases (Fig.3).

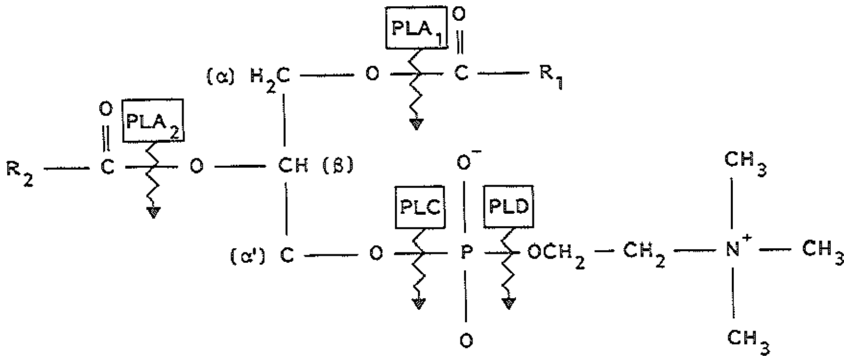

Fig.3. Sites of action of the phospholipases (PL).

Phospholipase A_1 liberates the fatty acid in position alpha and phospholipase A_2 (PLA_2) the mainly unsaturated fatty acid in position (37,38). Phospholipase C hydrolyses the glyceryl-phosphate bond to

liberate a phosphorylated base and a diglyceride. Phospholipase D cleaves the base from the phosphatide to yield phosphatidic acid (37,38,39) (Fig.3).

Because AA is mainly localised at position , PLA_2 is the main phospholipase for eicosanoid synthesis (3,37). In platelets however, phospholipase C (PLC) and a diglyceride lipase are possibly also responsible for AA release (40). Intracellular calcium is thought to be a major activator of PLA_2 and is therefore probably a major controlling factor in arachidonate liberation. However not everyone is convinced of the central role of calcium (41).

Various stimuli other than calcium can activate phospholipases, e.g. mechanical disruption of membranes, immunological stimuli, bradykinine, several hormones such as prolactin, non-enzymatic proteins and endotoxins (37,41).

Phospholipase interaction with phospholipids not only results in the release of EFAs but also in lysophosphatides (37). Lysophosphatides might be modified by acyltransferases to yield phospholipids or might be further degraded by lysophospholipases resulting in hydrophilic glycerophosphonyl esters of the polar lipid head groups which are readily lost from the hydrophobic membranes. Lysophosphatides have well documented cytotoxic properties, which are responsible for several pathological states such as in pancreatitis, where lysolecithin contributes to several complications (42,43).

Besides the obviously important role of phospholipases in the release of the precursors of eicosanoids it must be born in mind that there may be other pathways which lead to the liberation of these precursors (37).

In this way the complex system regulating phospholipids can be disturbed at several stages. For more detailed information of this system the reader is referred to the excellent reviews of Horrobin, van den Bosch and Irvine (26,38,39,41).

2.5 SYNTHESIS OF EICOSANOIDS

2.5.1 INTRODUCTION

The EFAs, AA (C20:4,ω6), dihomo-gamma-linolenic acid (C20:3,ω6) and eicosapentaenoic acid (20:5,ω3), released from phospholipids by phospholipase A_2 can be transformed by at least two enzyme systems. One pathway is the conversion of these EFAs by the enzyme system cyclooxygenase to prostaglandins (PGs) and thromboxanes (TXA), the cyclooxygenase products (COP) (44). The other pathway is mediated by the enzyme 5-lipoxygenase to yield leukotrienes (LTs) (23). The PGs are those of the 1, 2 and 3 series and of the LTs those of the 3, 4 and 5 series (27,29). The biological importance of the 1 series of PGs is not clear at the moment (26). The COP of the 3 series have received great attention because of the possible relation between a high intake of alpha-linolenic acid and a low incidence of cardiovascular disease (45). Eskimos have a high dietary intake of long-chain 3 polyunsaturated fatty acids from seafood and these fatty acids are thought to play an important role in the low incidence of cardiovascular disease (45,46). The explanation of the protective effect of C20:5ω3 formed from linolenic acid might be the absence of a proaggretory TXA_3, while PGI_3 as antiaggretory agent is as active as

PGI_2 (47,48). However Hornstra et al could not confirm the protective role of eicosapentaenoic acid in the prevention of thrombosis and atherogenesis in experimental animals, so the protective role of COP of the 3 series is not certain (49). Because AA is the most abundant EFA in mammals the conversion of this EFA to its eicosanoids will be mainly discussed in the following part of this chapter.

2.5.2 CYCLOOXYGENASE PATHWAY

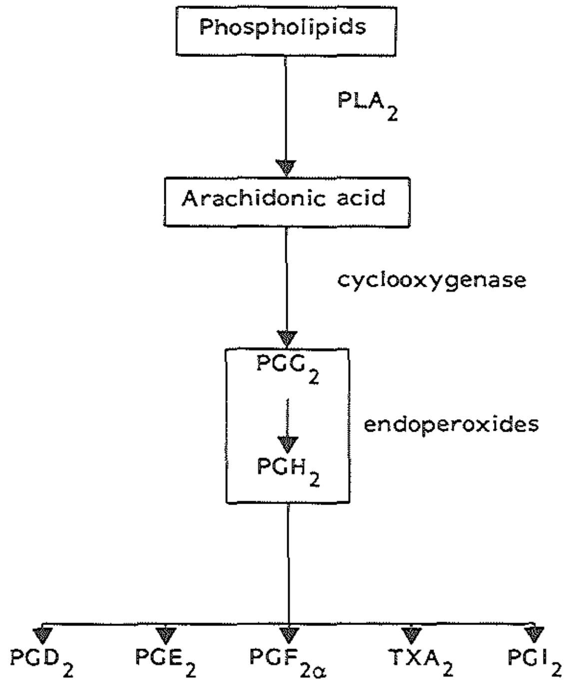

Fig.4. The conversion of arachidonic acid by the cyclooxygenase pathway.

After the liberation of AA from the phospholipids, it can be converted by the membrane bound enzyme, cyclooxygenase, to the endoperoxides, prostaglandin G_2 (PGG_2) and prostaglandin H_2 (PGH_2) (3,44) (Fig.4). The endoperoxides PGG_2 and PGH_2 are unstable with a half life of approximately 5 minutes (3,29). PGH_2 is converted within the cell both non-enzymatically and enzymatically to the prostaglandins E_2 (PGE_2), prostaglandins F_{2alpha} (PGF_{2alpha}), prostaglandin D_2 (PGD_2), prostacyclin (PGI_2), thromboxane A_2 (TXA_2), malondialdehyde (MDA) and 12 hydroxy 5, 8, 10 heptadecatrienoic acid (HHT) (3,4,29) (Fig.5).

The properties of the different products will be described in relation to different organ systems. Some general properties will be briefly mentioned here. PGE_2 is thought to play a role in hyperalgesia and fever, and gives rise to dilatation of the vascular smooth muscle and the bronchial tree (2,50,51) and is an inhibitor of several functions of the macrophages (52[a],53[a]). PGF_{2alpha} is a vasoconstrictor and can constrict the bronchial tree (2). PGE_2 and PGF_{2alpha} induce relaxation of the longitudinal smooth muscles in the gastrointestinal tract (GIT) whereas PGE_2 relaxes and PGF_{2alpha} constricts the circular muscles of the GIT (52). PGI_2, PGE_2 and PGF_{2alpha} play an important role in gastrointestinal diseases (52). PGD_2 is produced by a number of different cells; at the moment only some effects are yet known such as bronchoconstriction, potentiation of the effects of histamine in the inflammatory action and inhibition of platelet aggregation (2,53,54,55). TXA_2 is synthesized from PGH_2 by the enzyme thromboxane synthetase. TXA_2 is a very potent platelet aggregator and also constricts vessels and airways (3,53,56). In vivo the half life of TXA_2 is 30 seconds and it is converted into the biological inactive

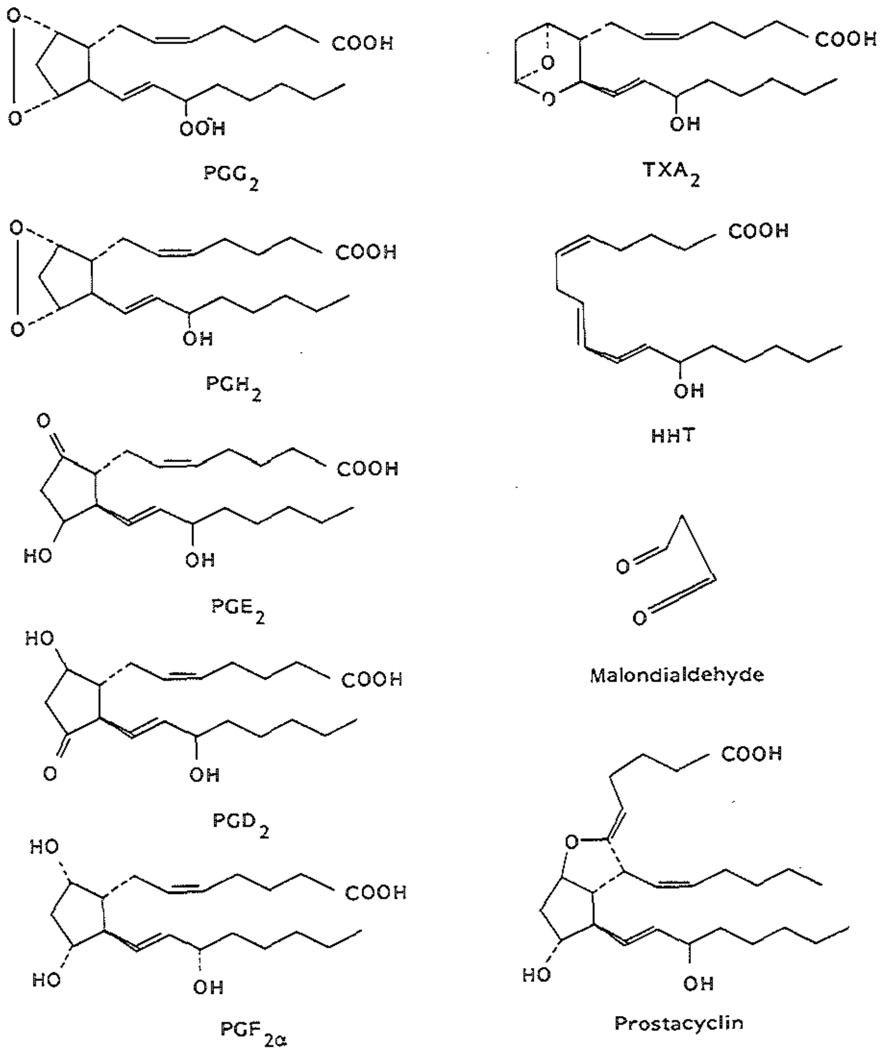

Fig.5. Structure of cyclooxygenase products of arachidonic acid.

metabolite TXB_2 (56). TXA_2 can be produced by platelets, leucocytes, macrophages, mast cells, and human fibroblasts (3,56). PGI_2 has a biological action which is exactly opposite to that of TXA_2, causing vasodilatation and inhibition of platelet aggregation (3,56,57). Prostacyclin has a half life of 2 to 3 minutes and degrades spontaneously to 6 oxoprostaglandin F_{1alpha} (6 oxo- PGF_{1alpha}), which is biologically less active (3,56,57). PGI_2 is the main product of all arteries and veins and can be produced by heart, lung, stomach, kidney and uterus (3,56). It is thought that an imbalance in TXA_2/PGI_2 ratio provides an explanation for some pathological conditions such as in arterial thrombosis, myocardial infarction, diabetes, thrombotic thrombocytopenic purpura (56).

2.5.3 TRANSPORT AND METABOLISM

COP do not diffuse freely across cell membranes, so a transport system is required to remove them from the circulation. Transport mechanisms for PGE_2 and PGF_{2alpha} have been described and are very fast (29,57). The metabolism of COP leads to a marked loss of biological activity of these substances. The inactivation of COP in vivo is rapid: for example a single passage through the pulmonary circulation inactivates more than 90% of the circulating PGE or PGF_{2alpha} (58). Inactivation of COP has been described in lungs, liver, spleen, kidneys and blood vessels (29,44). The enzyme system, 15-hydroxy-prostaglandin dehydrogenase (PGDH) initiates the breakdown of the prostaglandins of the E and F series by removing the hydrogen at the hydroxyl group at the C15 position. They are subsequently reduced at carbon 13 by prostaglandin

reductase (44,2,58,59). This leads to the biologically inactive prostaglandin metabolite 13,4-dihydro-15-keto PG during the metabolism of the E and F type. Metabolism continues in the liver, with bêta oxidation and oxidation of the methyl and carboxyl chain, resulting in 5,11 diketo-7- hydroxy tetra nor prosta-1,16 dioic acids (44,58,59). In contrast to other COP, PGI_2 is not metabolished by the lung but is degraded rapidly in the liver (29,57). For more detailed information of COP metabolism the reader is referred to the recent reviews (29,44,58,59).

2.5.4 LIPOXYGENASE PATHWAY

AA can be transformed by the lipoxygenase pathway to leukotrienes (23,24), 5-hydroxy eicosa 6,8,11,14 tetraenoic acid (5 HETE), 11-,12-, and 15 HETE (60,61,62). The most important pathway appears however to be the 5 lipoxygenase pathway, resulting in a family of biologically active substances named leukotrienes (61,62). After activation of the enzyme 5 lipoxygenase, AA is converted into the unstable peroxide 5 hydroperoxyeicosatetraenoic acid (5-HPETE). This product can spontaneously be hydrolysed to 5-hydroxy-6-trans 8,11,16, cis eicosatetraenoic acid (5 HETE) or converted by a dehydratase to the unstable epoxide 5,6 oxido 7,9-trans-11,14- cis eicosatetraenoic acid, leukotriene A_4 (LTA_4) (61,63). LTA_4 in turn can be converted enzymatically by an epoxide hydroxylase into 5(S), 12(R)-dihydroxy-6,14-cis 8,10 trans eicosatetraenoic acid, leukotriene B_4 (LTB_4) (64). LTA_4 can be conjugated with glutathione by a glutathionyl-S-transferase to form 5(S)-hydroxy-6(R)-S-glutathionyl

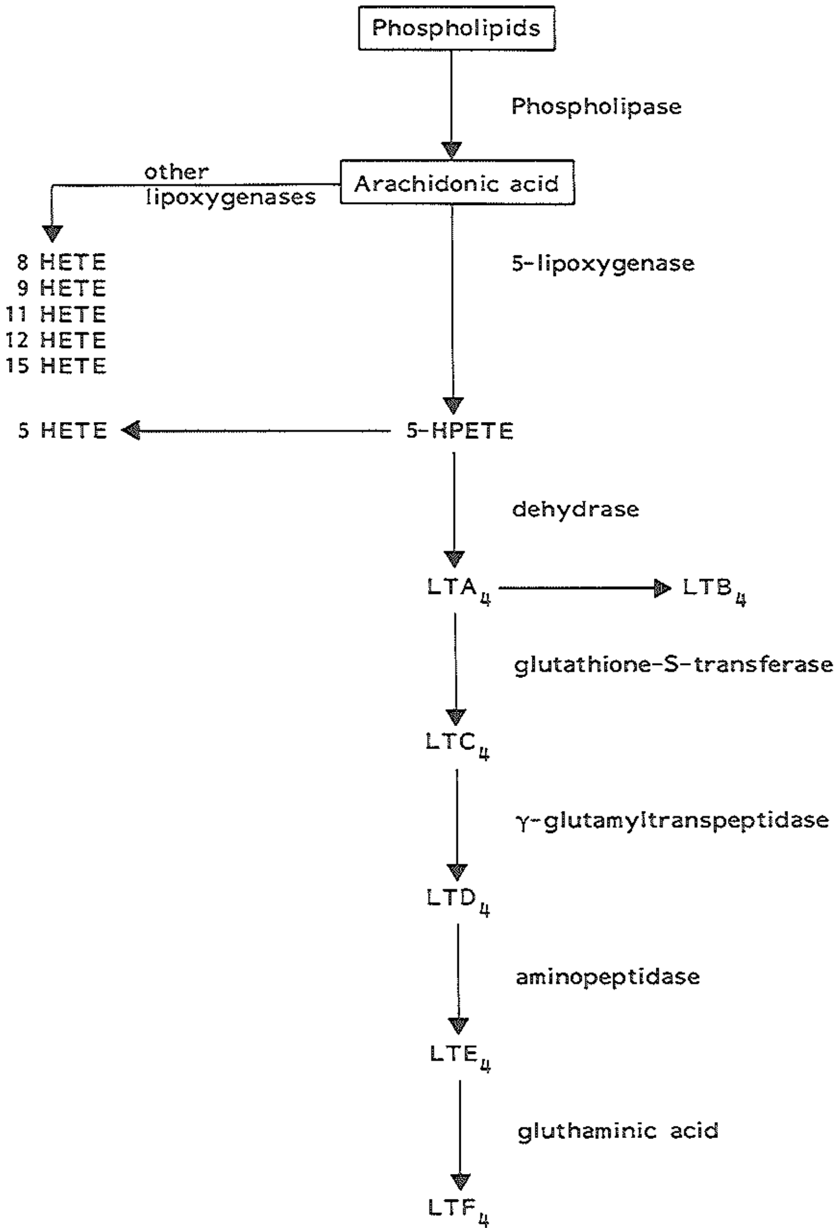

Fig.6. The conversion of arachidonic acid by the lipoxygenase pathway.

7,9 trans 11,14 cis eicosatetraenoic acid, leukotriene C_4 (LTC_4). LTC_4 is further metabolized by peptides to generate leukotrienes D_4 and E_4 and F_4 (61,62,63,64,65) (Fig.6 and Fig.7).

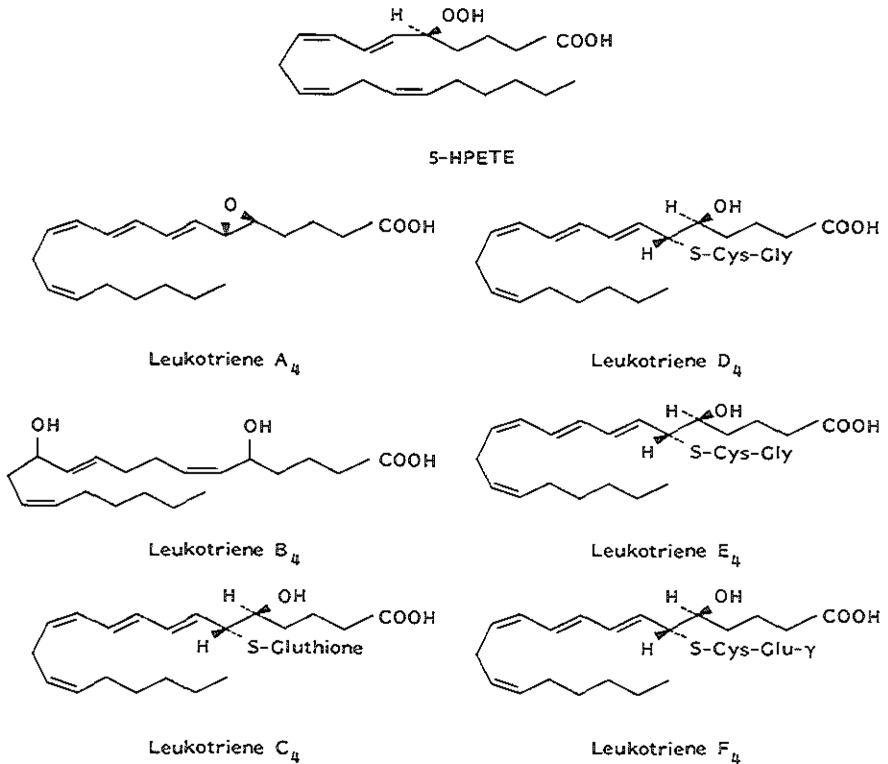

Fig.7. Structure of leukotrienes formed from arachidonic acid.

Just as in the cyclooxygenase pathway, AA is the most important substrate for leukotrienes. However dihomo-gamma linolenic acid (20:3,ω6) and eicosapentaenoic acid (20:5,ω3) too, are substrates for respectively LTs of the 3 and 5 series. There is evidence that these

LTs have qualitatively the same biological properties as the LTs of the 4 series. Slow reacting substance (SRS), an important mediator in asthma and other types of immediate hypersensitivity reactions, is a mixture of LTC_4, LTD_4 and LTE_4. SRS-A is thought to be released together with other mediators (for example histamine and chemotactic factors) after interaction between immunoglobulin E(IgE) molecules bound to membrane receptors and antigens such as pollen (65).

The cysteine containing LTs (LTC_4, LTD_4 and LTE_4) are hundreds to thousands of times more active than histamine in contracting the guinea pig ileum (62). Intravenous administration of LTC_4, LTD_4 and LTE_4 give bronchoconstriction which can be blocked by the cyclooxygenase blocker indomethacine, suggesting that the bronchoconstriction is mediated by TXA_2 and PGs (62,65). However LTC_4 and LTD_4 given as aerosal in guinea pig and man results in bronchoconstriction lasting for 30 minutes, which cannot be blocked by cyclooxygenase blockers (62,65). These LTs are 600-900 times more active as bronchoconstrictors than histamine (62). In vivo LTC_4, LTD_4 and LTE_4 give a short lived contraction of the arterioles, followed by a dose dependent and reversible leakage of the macromolecules, especially in the post capillary venules. They also cause constriction of the coronary arteries and large arteries (62,66,67). It must be born in mind that these experiments have been done mainly in animals and not in humans and that the effects of LTs are species dependent (67). Thus the exact role of LTs and its cardiovascular effects in men is not yet clear. The most potent LTs is LTD_4 followed by LTC_4. LTE_4 has the lowest biological activity.

LTC_4 and LTD_4 can be produced by human polymorphonuclears, human lung and peritoneal macrophages, eosinophils, mast cells and possibly

Kupffer cells (62,68).

LTB_4 is a very potent chemotactic factor resulting in adherence of granulocytes to blood vessels and migration of leukocytes to areas of inflammation (64).

LTB_4 can be produced by polymorphonuclears, monocytes, lymfocytes, macrophages and mast cells (64,68). In several diseases elevated levels of LTS have been described, such as in asthma, psoriasis, rheumatoid arthritis, gout and inflammatory bowel disease (62,70).

2.6 EICOSANOIDS AND OBSTETRICS AND GYNECOLOGY

Prostaglandins are involved at multiple levels in fertility regulation. They play an important role in the follicular rupture process (71), in subprimate species they control corpus luteum function (72), they are active stimulators of uterine muscle contractility (73,74) and regulate the onset of labor (9).

Dysmenorrhea includes a variety of signs and symptoms such as abdominal cramping, mood changes, headache, edema and frequently excessive menstrual flow. The disorder is a major cause of work loss by the female population. PGs and mainly PGF_{2alpha}, which is a potent constrictor of the uterus myometrium, are thought to play a major role in this disorder (75,76). The non steroidal anti inflammatory drugs (NSAIDs) block the cyclooxygenase pathway and are accepted as drugs of choice for treating dysmenorrhea (77,78) and menorrhagia (79). Besides PGF_{2alpha}, PGI_2 is also involved in dysmenorrhea (80).

PGF_{2alpha} regulates ovarian periodicity in cows, sheep and guinea pigs, but not in the human (81). This effect of PGF_{2alpha} is of economical

importance because estrus can be synchronized by exogenous administration of PGF_{2alpha}, allowing simultaneous insemination of groups of animals (81).

In pregnant women there is a decline in progesteron production and an increase in PGs and oxytocine concentration during the last 24 hours before delivery (9,74). The PGs and oxytocin induce cyclic uterine contractions. The importance of PGs for the delivery is supported by the use of NSAIDs to prolong gestation and lengthen parturation (82). However the therapeutic use of PGs for induction of labor at term has not been utilized to a great extent, because of the side effects such as uterine hyperstimulation, gastrointestinal complaints and the risk of intrauterine closure of the ductus Botalli (74). Administration of PGs can induce labor at any time during the pregnancy. An analogue of PGF_{2alpha} , 15 methyl PGF_{2alpha} has been used succesfully to induce abortion in the first and second trimesters of pregnancy (9,83). The abortion after intraamniotic injections of hypertonic saline is also probably caused by an interaction of oxytoxine and PGF_{2alpha} on the uterus (84). In addition to COP, leukotrienes may induce uterus contractions (62).

2.7 EICOSANOIDS AND THE LUNG

In the lung prostaglandins are readily formed and metabolized. As mentioned before, PGDH activity, which initiates the breakdown of PGE and PGF series, is high in the lung (2,3). The main COP in the lung are PGI_2 and TXA_2. PGI_2 dilates the pulmonary vascular bed and airways (2,85), whereas TXA_2 constricts the pulmonary vascular bed and airways

(85). The pulmonary complications of endotoxins, such as the early pulmonary vasoconstriction, are thought to be mediated by TXA_2 (86). PGD_2 and PGF_{2alpha} are also pulmonary vasoconstrictors and broncho constrictors in man (85). PGE_2 has weak dilatory effects on bronchial muscles. However in human allergic airway diseases, the LTs are considered to play a more important role than the COP, airway constriction in asthmatics being mediated by histamine and Slow Reacting Substance A (SRS-A) (87).

The characterisation of SRS-A as LTC_4 by HPLC by Murphy et al was a large step forward in the research on asthma (25). At present LTC_4, LTD_4 and LTE_4 are thought to be collectively responsible for the biological activity of SRSA (88). After administration of LTC_4 or LTD_4 as aerosols to humans, a slow-onset, long-lasting bronchoconstriction occurs, which is not reversed by pretreating the subjects with cyclooxygenase blockers (89). In contrast to the hyperreactive response to histamine in asthmatic patients, there is a similar bronchoconstrictor response to LTD_4 aerosols in asthmatics and normal subjects (89).

LTs are hundred times more active than histamine in contracting human respiratory smooth muscles (65) and, in general, peripheral airways are more sensitive than central airways.

Recently Creticos et al described the in vivo release of LTs in allergic patients. They found that intranasally administrated pollen grains in ragweed sensitive patients released in a dose-dependent fashion LTC, LTD and LTE in the nasal fluid, which correlated with the clinical response in these patients. Non-allergic patients had neither symptoms nor LTs release (90). Lung fragments obtained from patients

with extrinsic asthma generate large amounts of LTC_4, LTD_4 and LTE_4 when challenged with the relevant antigen in vitro (91).
Besides bronchoconstriction, LTC_4 and LTD_4 increase the release of mucus from human airways in vitro, constrict the pulmonary vascular bed, and increase vascular permeability (62).
The possible pathogenic role of LTs in lung diseases is further sustained by the presence of LTs in sputum from patients with chronic bronchitis, emphysema and cystic fibrosis (65). Further proof of the important role of LTs in the pathophysiology in allergic diseases, such as asthma, will, however, only be forthcoming if the use of selective LTs inhibitors alleviates the symptoms in these patients.

2.8 EICOSANOIDS AND THE VASCULAR SYSTEM

The eicosanoids have extensive effects on vascular tissue and platelets in vitro. They have been suggested to play a role in atherosclerosis (5), thrombosis (29,93), myocardial ischemia (46,92), transient ischemic attacks (TIAs) (5,93) and the vascular problems of diabetes mellitus (94). In these diseases interactions between platelet and vessel walls are considered to be responsible for eicosanoids production.
The main cyclooxygenase product of platelets is TXA_2, a potent vasoconstrictor and platelet aggregator. In addition platelets synthesize 12-L-hydroxy 5,8,10 heptadecatrenoic acid (HHT), malondialdehyde (MDA) and only small amounts of PGD_2, PGE_2 and PGF_{2alpha} (4,29,95). Of the lipoxygenase products the monohydroxy acids 12 and 15-HETE are mainly formed by platelets, which induce chemotaxis

of polymorphonuclear leucocytes (95).

TXA_2 synthesis by platelets can be stimulated by adenosine diphosphate (ADP), epinephrine, dilute collagen and low doses of thrombin, which activate membrane phospholipase, resulting in TXA_2 formation. TXA_2 in turn causes release of granules, resulting in platelet aggregation (5,95). Platelet aggregation is however not solely dependent on TXA_2 synthesis, for it can be induced by thrombin and collagen independently of arachidonate metabolites (5,95).

TXA_2 synthesis can be blocked by PGE_2, PGD_2, PGE_1 and PGI_2. PGI_2 is the most potent inhibitor of platelet aggregation, and acts by raising the levels of cAMP in the platelets which block TXA_2 production.

In contrast to platelets where TXA_2 is the main COP, in the vessel wall the main COP is PGI_2 which has opposite effects to TXA_2, namely vasodilation and inhibition of platelet aggregation (57,59). Besides PGI_2 the vessel wall can produce PGE_2, PGF_{2alpha}, PGD_2 and the lipoxygenase product 12 HETE (92). The ability of the vessel wall to synthesize PGI_2 is greatest at the intimal surface and progressively decreases toward the adventitia (97). PGI_2 production by endothelial cells is stimulated by angiotensin II, bradykinin, histamine and thrombin (5,95). Under certain circumstances the endoperoxides (PGG_2 and PGH_2) derived from platelets can be utilized by vascular endothelial cells for biosynthesis of PGI_2, a phenomenon called endoperoxide steal (98).

In view of the biological activity of TXA_2 and PGI_2 vascular diseases could be due to a dysbalance between TXA_2/PGI_2 production. The knowledge of the vascular effects of the lipoxygenase products are at the moment fragmentary and the pathophysiological role of these

substances in vascular diseases is a subject of much research. Some general properties of LTs will be mentioned, which are mostly derived from experiments with animals and not from human studies.

Leukotrienes C_4, D_4 and E_4 induce coronary, renal and systemic arteriolar constriction and augmentation of the permeability to macromolecules in postcapillary venules (66,67,99). At the moment there is no consensus about the effects of LTs on the blood pressure (95,99,100,101). The blood pressure reaction to LTs may be a species- and a vessel wall dependent (95,100,101).

The adventitia, also most abundant in macrophages and mast cells, is the main layer of the vascular wall to produce LTs. LTB_4 has no direct vascular effects but gives adhesion of polymorphonuclear leucocytes to the endothelial lining of blood vessels (66,102).

In atherosclerosis cholesterol is a clear risk factor. Under special circumstances cholesterol enhances TXA_2 production and inhibits PGI_2 production (5,46).

Thus atherosclerosis in man might be partly due to a disturbance of TXA_2/PGI_2 production.

In myocardial ischemia and acute myocardial infarction, raised coronary sinus TXB_2 levels (46), and in angina pectoris, a reduction in prostacyclin platelet receptors have been described in man (103). The difficulty of these studies is that they do not show whether the changes in eicosanoids concentrations are a primary or secondary phenomenon. However because of the possible adverse effects of a dysbalance of TXA_2/PGI_2, many clinical trials have been carried out with COP blockers in the prevention of myocardial infarction. If all trials are pooled it appears that acetylsalicylic acid is of modest

benefit, reducing deaths by 16% and recurrent infarction by 21% (104). The disappointing results of NSAIDs in the prevention of myocardial infarction might be partly due to the high salicylate doses used in these studies. It must be born in mind that the high doses of NSAIDs used in these studies not only block TXA_2, but also PGI_2 production (104,105). Further studies using low dose aspirin are needed to prove or disprove beneficial effects of these drugs in vascular diseases (104,106). The improved patency of aorta-coronary bypass by low dose aspirin is promising evidence for prevention of vascular diseases by low dose aspirin (105). The intracoronary administration of PGI_2 in patients with acute myocardial infarction has been used in attempts to recanalize the obstructed coronaries, however not without side effects (107). In patients with peripheral vascular diseases prostacyclin infusions appear to be beneficial (96).

Clinical trials with NSAIDs in patients with transient ischemic attacks (TIAs) (5,108) aorto coronary vein grafts (106) and lower extremity bypass grafts (109) have given some positive results, but at the moment more controlled trials are needed to prove the beneficial effects of these drugs (104,105,106,109).

Another therapeutic tool for ischemic vascular diseases might be dietary manipulation. As mentioned before, Greenland Eskimos, whose diet consists largely of fish, have a low incidence of myocardial infarction and increased tendency to bleed. Fish contains especially eicosapentaenoic acid, the precursor of the 3 series of prostaglandins and thromboxane. TXA_3, in contrast to TXA_2 which is derived from arachidonic acid, does not have platelet aggregating activity, while PGI_3 has the same vasodilator and platelet anti-aggregating effects as

PGI_2. This results in a diminished vasoconstriction and platelet aggregation in these patients. Humans fed a Western diet supplemented with cod liver oil had markedly changed membrane phospholipids which were associated with less reactive platelets and lower blood pressure (47). Another important feature in patients with coronary heart diseases is the lower arachidonic and eicosapentaenoic acid levels in phospholipids and the low levels of dihomo-gamma linolenic acid and linoleic acid in adipose tissue in these patients (110,111). These studies and the finding that 3 polyunsaturated fatty acid enriched diets in animals are associated with a reduction in size and sequelae of cerebral and myocardial infarctions should be a stimulus to further investigation to the role of diets in the prevention of vascular diseases.

In several other diseases eicosanoids are thought to be important. For example the complications in diabetes mellitus may be partly due to the raised TXA_2 and decreased PGI_2 production (94) and in thrombotic thrombocytopenic purpura, characterized by formation of microvascular thrombo-emboli, a deficiency of PGI_2 generation has been suggested (112,113). Raised plasma and urinary TXB_2 levels have been described in patients with borderline and manifest essential hypertension (114). Renal function and dysfunction might be partly due to changes in prostaglandin homeostasis (6,115).

2.9 EICOSANOIDS AND THE GASTROINTESTINAL TRACT

In the gastrointestinal tract (GIT) eicosanoids are involved at several levels. They play a role in GIT motility (52,116), gastric acid

secretion (52,117), cytoprotection (52), peptic ulcer disease (118), diarrhea (52,116) and inflammatory bowel disease (119).

Prostaglandins of E and F series affect smooth muscles of the GE tract. In general, longitudinal smooth muscles are contracted by PGE and PGF_{2alpha} whereas PGE relax and PGF_{2alpha} contract the circular smooth muscles of the GIT (120). PGD_2 and PGI_2 have minimal actions on both muscle layers of the GIT, whereas TXA_2 contracts both smooth muscle layers (64,121).

In vitro LTB_4 contracts the smooth muscles of guinea pig duodenum, ileum and rat stomach probably due to stimulation of the cyclooxygenase pathway (64). LTC_4, LTD_4 and LTE_4 induce a potent smooth muscle contraction of the guinea pig ileum and rat stomach strip; these reactions are independent of the cyclooxygenase pathway suggesting a direct effect of LTs on ileum and stomach (62). The PG production is most abundant in the stomach and small intestines and least in the colon (116,117). For example the average basal gastric luminal output of PGE_2 is 20 ng. per 15 minutes in healthy subjects (117). The mucosa has the greatest ability to synthesize PGs of the GIT wall layer (117). The effect of PGs on the GIT motility is dependent on the organ. In the esophagus PGs only affect the lower esophageal sphincter (LES) and not the upper esophageal sphincter or the motility of the body of the esophagus (52,116). Administration of PGE_1 or PGE_2 orally or intravenously causes relaxation of the LES, whereas PGF_{2alpha} and probably TXA_2, give contraction of the LES (52,122). Indomethacin, a cyclooxygenase blocker, increases the LES pressure in humans, suggesting a net inhibitory role by PGs on LES pressure (116,122). In contrast to the intestines, PGs do not have a marked effect on gastric

motility in man (52). PGI_2 might be an exception in view of the inhibitory effect on gastric emptying seen in animals. In the small intestine PGE and PGF_{2alpha} augment gut propulsion, whereas PGI_2 prolongs small intestinal transit time (123). The therapeutic use of PGE derivates in peptic ulcer disease and obstetrics revealed a disturbing side effect, namely diarrhea. The diarrhea after administration is due to an enhanced motility of especially the small intestine and accumulation of fluid in the small intestine, called enteropooling (52,124). The importance of eicosanoids for motility is further sustained by the observation that NSAIDs diminish the basal motility of the rabbit small intestine (125). The successful administration of PGs to animals to reverse postoperative ileus may lead to the evaluation of these compounds in patients with post-operative ileus (126). These findings suggest that PGs and probably LTs are involved in the physiological and pathological motility of the GIT. The stomach is the most interesting target organ of PGs in the GIT. PGs block gastric acid secretion, provide cytoprotection against several drugs and compounds, and promote healing or prevent the development of gastric and duodenal ulcers. In experimental animals and humans PGA, PGE_2, PGE_1 and PGI_2 inhibit gastric secretion by reducing volume, acid secretion and pepsin output in vitro and in vivo (52,117,127). PGF_{2alpha} has no antisecretory effect (116). The stimulatory effect of histamine, pentagastrin and food on gastric acid secretion can be totally abolished by PGs (52). The naturally occurring PGs have only in high concentrations an effect on gastric acid secretion (127). This might be due to the rapid metabolism of PGs to inactive metabolites by stomach tissue, liver and lung. Several analogs of PGE have been

synthesized which can be administrated orally, and they have been found to be 50 to 100 times as potent as PGE_2 in inhibiting gastric acid secretion (117). Most of these PGs have a methyl group attached to carbon 15 or 16, e.g. 15(R) - 15 methyl PGE_2 (Arbaprostil^R, Upjohn, Michigan) 15(S), 15 methyl PGE_2 and 16, 16 dimethyl PGE_2. These compounds cannot be inactivated by the 15 PGDH and the reductase enzym system, which is the reason for the better and longer lasting inhibition of gastric acid secretion than that given by naturally occuring PGs (128). In a dose of 1.7 ug./kg. of 16, 16 dimethyl PGE_2 there is almost total inhibition of acid secretion, whereas doses of 1 mg. of PGE_2 are needed to provide the same antisecretory effect as 16, 16 dimethyl PGE_2 in man (117,127). The exact mechanism whereby PGs block gastric acid secretion is still not clear.

PGs protect the gastroduodenal mucosa in at least two different ways. Firstly PGs protect the gastric mucosa by inhibiting gastric acid secretion and secondly they have protective actions on gastric mucosa independent of gastric acid inhibition, a phenomenon called cytoprotection (52,129). Inhibition of gastric acid secretion is a general property of several anti-ulcer drugs such as the histamine 2 receptor blockers, cimetidine and ranitidine, PGs, anticholinergic agents and antacids (52). However cytoprotection of the gastric mucosa by PGs against damage by several drugs and necrotizing agents is an unique property of PGs which has not been described for H_2 blockers, antacids or anticholinergic agents (52,117,129). Cytoprotection can be divided in direct cytoprotection and adaptive cytoprotection. Direct cytoprotection can be produced by the administration of PGs, which protect against gastric injury by NSAIDs or necrotizing agents (52).

Adaptive cytoprotection is the mechanism whereby mild irritants elicit endogenous formation of PGs, which protect the gastrointestinal epithelium against several necrotizing agents (52). The NSAIDs block the endogenous formation of PGs, resulting in gastric erosions, acute upper gastrointestinal hemorrhage and an increase in fecal blood loss in healthy subjects (130,131,132). These drugs are frequently used in patients with rheumatic diseases and the gastrointestinal side effects such as ulcerations of stomach and small intestine are the most common reasons to withdraw the drugs in these patients. The NSAID-induced lesions can be prevented by antisecretory agents such as anticholinergic agents, antihistaminics, antacids and PGs (52). The prevention of such lesions by these compounds are a direct consequence of the antisecretory activity (129). However administration of PGs in concentrations which have no antisecretory effect and even PGs devoid of gastric antisecretory activity still prevent NSAIDs lesions, in contrast to low doses of antacids, antihistaminics or anticholinergic drugs (52,133,134,135). Severe gastric necrosis produced by strong irritants such as absolute ethanol, thermal injury, hypertonic solutions of HCl, NaOH and NaCl and taurocholate are totally prevented by pretreating the animals with PGs. Robert called this property a direct cytoprotective effect of PGs by an as yet unknown mechanism (52). One possible explanation for this cytoprotective effect might be the increased mucus production after PGs administration (136).

The protective effect of mild irritants is mediated by endogenously PGs production of the gastric mucosa. This effect of mild irritants can be totally abolished by indomethacin (52,135,137).

In peptic ulcer disease there is no absolute deficiency of PGs but

rather a diminished PGs synthesis after duodenal acid load (138). Because of the antisecretory and cytoprotective properties of PGs for gastric mucosa, several double-blind, multicentre trials have been done to investigate the effectiveness of PGs in healing peptic ulcers (117,118). Both gastric and duodenal ulcers healed significantly faster after treatment with PGE_2 in comparison with placebo (116,117,118). However studies comparing PGs with antihistaminics ranitidine and cimetidine need to be done to define the place of PGs in peptic ulcer disease. Sucralfate, an aluminium salt of sucrose octasulfate, is an effective drug in treating and preventing peptic ulcer disease, and this effect is PGs dependent (139).

In the small intestine PGs have, as mentioned before, effects on motility, cytoprotection and on secretion of water and electrolytes. (52). Enteropooling can be induced by PGF_{2alpha} and PGE_2 in animals and men (52,119,140). High doses of PGE_2 cause a secretory diarrhea, whereas PGI_2 and PGD_2 have an anti-enteropooling activity (52). PGs have been implicated in diarrhea due to exotoxin-producing bacteria, inflammatory bowel disease, radiation enteritis, some endocrinologic diseases and some laxatives (52,116,119,141). In Crohn's disease and colitis ulcerosa raised levels of PGs, 12HETE, 15HETE and LTB_4 have been found in luminal secretion, stools and mucosa (119,142,143,144). Some investigators have claimed that 5 amino salicylic acid (5 ASA), a product of sulfasalazine, exerted it's effects by blocking the cyclooxygenase pathway. Recent studies, however, show that 5 ASA not only blocks the cyclooxygenase but also the lipoxygenase pathway (145). This might be the explanation why NSAIDs which block only the cyclooxygenase pathway are not effective in inflammatory bowel disease

(146). The results of numerous studies suggest important roles of PGs and LTs in the GIT. More studies, however, need to be done to show that the changes in PGs and LTs in disease are not merely epiphenomena.

2.10 EICOSANOIDS AND THE LIVER

The liver plays an important role in the metabolism of eicosanoids (58,147). The liver is the main organ for metabolism of PGI_2, which cannot be inactivated by the lung (148). The hepatic metabolism of PGI_2 results in the formation of several biologically inactive lipids and one biologically active product named 6-keto-PGE_1, 6-keto-PGE_1 is a vasodilator and potent inhibitor of platelet aggregation (148). In the liver PGD_2 can be converted to $PGF_{2\ alpha}$ by prostaglandin 11-keto reductase (149,150). Thus the liver is able to convert a potent inhibitor of platelet aggregation to a potent vasoconstrictor without effect on platelet aggregation. Knowledge of LTs metabolism is fragmentary. Recently Uehara et al reported that intravenously administrated LTs are rapidly taken up by hepatocytes and excreted in the bile (147). These findings suggest that the liver has a role in the removal of biologically active eicosanoids from the circulation. Hepatic injury might impair this process and lead to prolonged and or augmented actions of eicosanoids in various organs. It might also be possible that eicosanoids taken up by the liver may contribute to the development of hepatic diseases (151).

The liver not only metabolizes but also synthesizes several eicosanoids such as PGE_2, PGF_{2alpha}, PGD_2, TXA_2, PGI_2 and probably leukotrienes (152,153,154,68). Isolated liver cells can synthesize a scala of

eicosanoids. For example hepatocytes stimulated by vasopression produce TXB_2 (155) and Kupffer cells can produce PGE_2, PGD_2, PGF_{2alpha}, PGI_2 and TXA_2 and leukotrienes after stimulation with endotoxins, calcium ionophore or zymosan (68,156,157). Phospholipase A_2 can enhance TXB_2 and 6-keto-PGF_{1alpha} release from the isolated perfused rabbit liver (154). Liver disease might therefore influence not only metabolism but also synthesis of eicosanoids. Some of the deleterious effects of liver disease might be mediated by eicosanoids, whereas other eicosanoids could protect the liver against several toxins. PGI_2 has been suggested to be involved in the development of portosystemic collateral circulation in rats with portal hypertension, to enhance hepatic blood flow in man and dogs and it is also a very potent stimulus for renin release (158,159,160).

The 16,16 dimethyl PGE_2 analogues have been succesfully used to protect the liver against several hepatotoxic drugs such as alpha napthylisothiocyanate (161), bromobenzene (162), galactosamine (163), carbon tetrachloride (164). Severe liver damage by ischemia can be prevented by PG administration (165). Besides the protective effects of these prostaglandins, another COP namely TXA_2 is thought to be the cause of several complications in liver disease (115,166). In patients with alcoholic liver disease we found raised plasma TXB_2 levels, which were associated with higher levels of serum urea, a more disturbed liver function as measured by an increase in alkaline phosphatase and gamma glutamyl transpeptidase and a decrease in antiplasmin and antithrombin III levels (166). In the hepatorenal syndrome raised urinary TXB_2 and lowered PGE_2 levels have been found (115). These two observations suggest a potential role for TXA_2 for the complications in

these cirrhotics. The raised levels of TXB_2 in liver disease might be secondary due to the high frequency of endotoxemia in these patients (166,167). In vitro and in vivo studies have shown that the deleterious effects of endotoxemia are mediated by several eicosanoids (168,169, 170). Endotoxins, preferentially taken up by Kupffer cells, induce eicosanoids production which then give rise to several complications seen in patients with liver disease (156,166). Non-selective or selective blocking of the cyclooxygenase pathway gave an undeniably better survival in experimental animals with endotoxemia and probably septic patients (168,170,171,172). Even endotoxin-induced liver damage can be prevented by NSAIDs (173). Based on these findings it seems reasonable to suggest a trial with NSAIDs in cirrhosis. However, inhibition of TXA_2 production by the administration of NSAIDs, which not only block TXA_2 but all COP, has been shown to cause renal failure in patients with liver disease (174,175,176). This is in agreement with the suggestion that some of the COP might be beneficial in liver disease (174,176,177). The use of selective TXA_2 synthetase blockers such as dazoxiben in cirrhotics with portal hypertension and hepato-renal syndrome was unfortunately not followed by changes in systemic, pulmonary and portal pressures nor was there an improvement of the renal function in the hepato renal syndrome (178,179). Further work needs to be done to unravel the exact role of TXA_2 in the complications of liver cirrhosis.

One possible explanation for the failure of TXB_2 blockade in these patients might be that the raised TXB_2 levels are a secondary phenomenon. Leukotrienes have been shown to stimulate the release of TXA_2 in vitro and in vivo (180,181). It has been shown that especially

leukotrienes C_4 and D_4 give a potent vasoconstriction of the isolated perfused rat kidney and decreased splanchnic blood flow (100,182). Administration of LTC_4 to isolated hypoxic hepatocytes result in death of approximately 95% of the hepatocytes, suggesting a direct toxic effect of LTC_4 on hepatocytes (151). Halothane induced hepatic damage is possibly mediated by leukotrienes.

The maintenance of renal function by eicosanoids and the use of NSAIDs in liver disease have been extensively studied (174,175,176,177). Patients with cirrhosis and ascites frequently develop a decrease of renal blood flow and glomerular filtration rate, secondary to an active renal vasoconstriction (183). This change can even result in functional renal failure called the hepatorenal syndrome, which is characterized by a marked reduction in renal blood flow without structural abnormalities of the kidney, suggesting a circulating agent as the cause (184). This proposition is further sustained by the fact that kidneys from a patient with the hepatorenal syndrome can be successfully transplanted in a patient with normal liver. Conversely, the kidney in a patient with the hepatorenal syndrome resumes function if the patient undergoes successful liver transplantation (184). It has been postulated that renal dysfunction in cirrhotics is caused by a dysbalance between vasoconstrictive agents such as TXA_2, angiotensin II, and the sympathetic nervous activity and vasodilating agents such as PGE_2 (177). Several reports provide strong evidence that especially PGE_2, but also PGI_2 play an important role in maintaining renal function in cirrhotics (175,176,177,185). These vasodilatory PGs are thought to protect the renal function of cirrhotics from the powerful vasoconstrictors such as angiotensin II, symphatic nervous activity and TXA_2 and probably

leukotrienes (177,178,186). Raised urinary PGE_2 have been found in cirrhotics with ascites without renal failure and there is a direct correlation between reduced urinary PGE_2 levels and the severity of the renal failure in patients with cirrhosis (177). Boyer et al. first described the deleterious effects of NSAID administration in cirrhotic patients (174). Reduced effective renal plasma flow and creatinine clearance could be abolished by the administration of PGA_1 (174). Later, several other workers found that the use of NSAIDs in patients with severe liver disease resulted in decreased renal blood flow, decreased creatinine clearance, reduced urinary volume and sodium excretion. The NSAIDs enhance the pressor sensitivity to angiotensin II (177). These effects are paralled by the reduction of PGE_2 (176). Several authors therefore conclude that NSAIDs should not be employed in patients with severe liver disease (175,177). Another interesting finding is that the renal response of furosemide in cirrhotics is mediated by PGE_2 and that administration of furosemide protect these patients from developing renal insufficiency after administration of NSAIDs, probably due to enhanced renal PGs synthesis stimulated by furosemide (176).

Ascites is a frequently encountered complication in cirrhotics. Stimulated peritoneal macrophages derived from ascites of experimental animals and patients with ascites due to liver cirrhosis can produce several leukotrienes such as LTB_4, LTC_4 and LTD_4 and in minor degree TXB_2 and 6-keto-PGF_{1alpha} (68,69,187,188,189). Thus several complications, such as renal dysfunction, shock, coagulation disorders in liver cirrhosis and especially those that occur after the insertion of a peritoneal jugular shunt might be (partly) mediated by eicosanoids

from the ascitic fluid which enters the systemic circulation (68,69,139, 190).

References: see page 163.

CHAPTER 3

ENDOTOXINS

3.1 GENERAL ASPECTS

Endotoxins are cell wall lipopolysaccharides of gram negative bacteria with variable amounts of proteins and lipids (192,193). The lipopolysaccharide complex can be divided to three major parts: the O-polysaccharide, the R-core and lipid A (fig.1) (194).

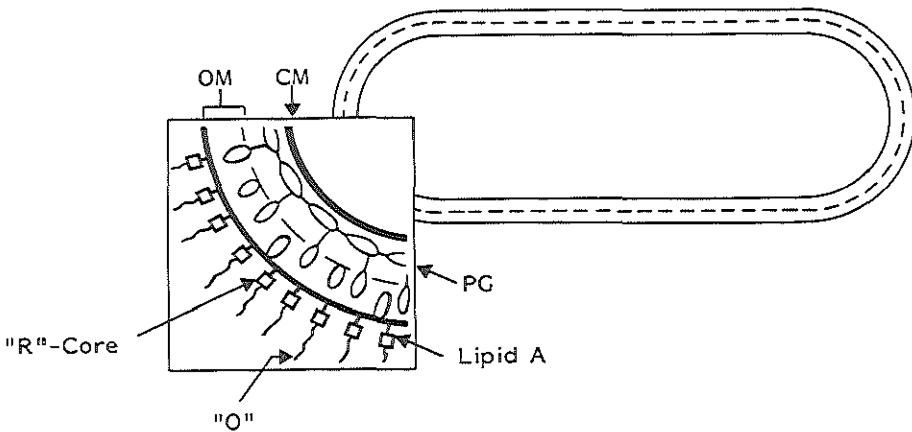

Fig.1. Simplified scheme of a cross-section of the cell wall of a gram-negative bacterium. The wall consists of three layers: cytoplasmic membrane (CM), a peptidoglycane layer (PG) and the outer membrane (OM) with lipopolysaccharide which contains 3 parts: lipid A, R-core and the O-polysaccharide.

The O-polysaccharide is chemically unique for each type of bacterium. The R-core is the middle part of the lipopolysaccharide molecule and links the O-polysaccharide with lipid A (192). Lipid A is remarkably similar for a broad spectrum of gram-negative bacteria and is responsible for most of the biological effects of endotoxins (192,194). The gut is generally accepted to be a large reservoir of endotoxin producing gram negative bacteria. Small amounts of endotoxins are continuously taken up from the gut lumen and reach the liver with the portal blood. In the normal state, they are rapidly removed from the circulation by the Reticulo Endothelial System (RES), especially by the Kupffer cells (195). After phagocytosis a variety of beneficial and toxic mediators are released from the macrophages (192,195,196).

Endotoxins are involved in several diseases. For example a major factor complicating the treatment of gram-negative bacteraemia is the release from bacterial cell walls of endotoxins. For this reason these substances have been implicated in the pathogenesis of gram negative sepsis and shock (197).

The clinical relevance of endotoxins in gram negative bacterial shock is sustained by two recent studies using antibodies to endotoxins in septic patients (198,199). Ziegler et al prepared human anti-serum by vaccinating healthy men with heat-killed Escherichia Coli J5, only containing a Core-Lipid A complex, which is very similar for most bacteria strains. They found a better survival in the 103 patients, receiving antiserum, compared to the control group (198). In the other study freeze dried human plasma rich in anti lipopolysaccharide immunoglobulin was used in fourteen septic shock patients and reduced the mortality and morbidity rate in the treated group (199). These

studies indicate that treatment with anti lipopolysaccharide might be beneficial in patients, where endotoxemia is suspected to be responsible for complications (198,199,200).

A major problem interfering with prospective clinical studies is the difficulty in detecting endotoxins in biological samples. Endotoxins can be measured by the Limulus Amebocyte Lysate (LAL) test introduced by Levin et al (192,201).

The principle of the LAL test for the detection of endotoxins is an endotoxin induced gelation of a clottable protein present in the blood amebocytes from Limulus polyphemus (201). For the detection of endotoxins in blood special precautions are needed, because of the presence of an inhibitor in human blood which interferes with the LAL coagulation (192). The limulus test with the aid of chromogenic substrate has been used in recent years and seems to be sensitive (detection limit 10 pg/ml), quantitative and reproducible (202,203). Radioimmunoassay (RIA) or ELISA (enzyme-linked-immunoabsorbent assay) have been developed but are not generally available.

3.2 THE BIOLOGICAL EFFECTS OF ENDOTOXINS

The biological effects of endotoxins are numerous and have recently been reviewed (192,193,195,204). Some of the effects are listed in table I. It must be born in mind that the biological effects of endotoxins are species and concentration dependent. Rats for example are rather insensitive whereas men and rabbits are highly sensitive to endotoxins (193,205).

Table I. BIOLOGICAL EFFECTS OF ENDOTOXINS

- Fever
- Complement activation
- Activation of clotting system
- Metabolic and hormonal changes
- Immunological reactions
- Thrombocytopenia and leucopenia
- Hypotension and shock
- Renal dysfunction
- Liver damage
- Release of eicosanoids
- Positive Limulus Amebocyte Lysate test

Fever is a dose-dependent reaction after endotoxin administration and has been used as an in vivo test for the detection of pyrogens (193,197). The fever reaction is mediated by an endogenous pyrogen, interleukin I, released from several cells such as leucocytes, monocytes, Kupffer cells and macrophages (195,197,206). Endogenous pyrogen exerts its thermoregulatory effects by acting on the thermoregulatory center of the anterior hypothalamus producing a change in the specific body thermostatic setting (197).

In patients with sepsis due to gram negative bacteria diffuse intravascular coagulation is frequently seen. Endotoxins activate factor XII (Hageman factor) resulting in activation of the intrinsic pathway and activate the extrinsic pathway by the release of tissue factors of monocytes (193,197,207,208). Endothelial cells are damaged

and even torn off from their support resulting in a widespread source of activation of the clotting cascade.
Thrombocytopenia after endotoxin administration is caused by platelet aggregation and the release of several active mediators such as platelet factor 3, ADP, serotonine, histamine and TXA_2 (192,208). The massive microthrombosis induced by endotoxins appears to reflect a state of intravascular inflammation, leading to complement activation, leucocyte sequestration with release of lysosomal enzymes and other mediators and activation of the clotting and fibrinolytic system (207,209). Endotoxins give an initial leukopenia followed by leucocytosis. The leucopenia is due to sticking of the leucocytes to the vessel walls (193). Plasma withdrawn one hour after endotoxin injection significantly enhances the adhesiveness of leucocytes from control animals, suggesting a circulating substance as the cause for the leucopenia (192). An important pathological effect after endotoxin administration is the hemodynamic changes such as systemic hypotension, diminished cardiac output, lowered systemic vascular resistance, renal insufficiency, splanchnic vasoconstriction and increase in pulmonary vascular permeability and pulmonary artery pressure (168,169,195,210, 211). The hemodynamic changes are thought to be due to interaction of endotoxins with leucocytes, platelets, and the complement system resulting in the release of vasoactive substances such as eicosanoids, catecholamines, myocardial depressant factor, kinine, serotonin and histamine (212,213). For example histamine is released rapidly after endotoxin injection and is thought to play a role in the early vascular and hemodynamic changes induced by endotoxins (195).
Endotoxemia in septic patients and liver cirrhosis is correlated with

renal dysfunction (195,214). Renal function can be disturbed, independently of diffuse intravascular coagulation and systemic hypotension. Endotoxemia results in renal vasoconstriction, decreased urinary sodium secretion and even oliguria (171,195,211,214).

3.3 ENDOTOXINS AND THE LIVER

In the normal state endotoxins are rapidly removed from the portal blood by the Kupffer cells (204). By means of the Limulus Lysate Assay, endotoxins could be detected in portal blood in almost 50% of 120 patients who underwent abdominal surgery. In only three patients endotoxins could be detected in portal and systemic circulation (193). The accumulation of endotoxins in the systemic circulation may take place in case of insufficiency of the Kupffer cell and or spill over of the portal blood into the inferior vena cava in liver disease (193,204). Recently McCuskey et al investigated the relative species sensitivity of E. Coli endotoxin in guinea pig, hamster, mouse and rat, and found a strong correlation between the sensitivity to endotoxins and the number of Kupffer cells. The guinea pigs, with the highest number of Kupffer cells, were very sensitive to endotoxins, whereas rats with the lowest number of Kupffer cells had the lowest sensitivity to endotoxins (205). These findings suggest that the Kupffer cells are not only the principal site for the removal of circulating endotoxins but also the source of toxic mediators that participate in the host response to endotoxins. Endotoxins have direct hepatotoxic effects resulting in a diminished hepatic microcirculation, due to swelling of Kupffer and endothelial cells and the adhesion of leucocytes and platelets to the

sinusoidal walls, swelling and vacuolisation of hepatocytes with cell necrosis and cholestasis (215).

There are at least four experimental models that show that endotoxins contribute to liver injury. In choline deficient rats the presence of intestinal bacteria was found to be crucial for the development of liver cirrhosis (216). The hepatic injury caused by CCl_4, D-galactosamine and frog virus 3 can be prevented by pretreatment of the animals with either polymixin B, which disrupt the cell walls of gram negative bacteria and binds endotoxins or colectomy (216,217,218, 219). Besides a direct hepatotoxic effect of endotoxins, liver disease is often associated with a diminished clearance of endotoxins resulting in enhanced systemic toxic effects, such as coagulation disorders, hypotension and renal failure (204). Several authors have described the presence of endotoxemia in liver disease (192,193,195,204). Van Vliet and co-workers found, for example, in 36% of the patients with acute hepatitis and in 46% of the patients with liver cirrhosis a positive Limulus amoebocyte lysate test. The patients with systemic endotoxemia had a lower platelet count, a more disturbed renal and liver function, and a higher mortality rate (193). These findings confirmed those of several other authors (195,204).

Many studies have found a correlation between endotoxemia and impaired renal function in liver disease (183,184,195,204,220,221). The hepatorenal syndrome might be directly or indirectly due to systemic endotoxemia (184,204,214,220). In fulminant hepatic failure endotoxemia has been shown to be related to renal failure and intravascular coagulation (221). In patients with liver disease several immunological abnormalities including hypergammaglobulinemia, decreased delayed type

hypersensitivity and a disturbed function of monocytes have been found (193,222). In lipopolysaccharide stimulated monocytes derived from patients with liver disease increased interleukin I release was found suggesting that the immunoregulatory function of monocytes is disordered in liver disease (222). Systemic endotoxemia is found in patients with a diminished RES function. Acute and chronic ethanol administration impairs RES function. Within the first 24 hours of acute alcohol intoxication, the clearance rate of micro aggregated human serum albumin was 1.5 to 2 - fold less than in a healthy control group (223). Acute and chronic ethanol intoxication suppress DNA synthesis in the liver of rats resulting in inhibition of hepatic regeneration (224,225, 226). The suppression of ethanol on hepatic regeneration can be prevented by pretreatment with 16,16 dimethyl prostaglandin E_2 (226). Which again subscribe that NSAIDs must be used with caution in liver disease (175,226).

Administration of ethanol results in histologic changes of the small intestine and enhanced permeability for several macromolecules by the gut (227,228). As endotoxins are macromolecules, ethanol might affect endotoxin absorption of the gut. However the permeability of the intestine to endotoxins is not increased after chronic administration of ethanol in the rat (227). In patients with liver disease intestinal bacterial overgrowth have been found, so an increased absorption of endotoxins from the gut is not ruled out (229,230,231).

Recently Bode et al examined in aspirates from the jejunum of 27 chronic alcoholics and 13 hospitalized control patients without liver disease the types and numbers of bacteria. In the alcoholic group they obtained significant more anaerobic and aerobic bacteria than in the

control group, suggesting that the bacterial overgrowth in alcoholics might contribute to functional and or morphological abnormalities of the small intestine and systemic endotoxemia (229).

We recently confirmed these findings with the aid of breath test detecting bile acid deconjugation (232,233). In the presence of bacterial colonization of the small bowel, bile acids are deconjugated by anaerobic bacteria. The deconjugation of these bile acids can detected by means of a breath test. Bacteria in the bowel split off 1-^{14}C-glycine from (1-^{14}C-glyco)cholic acid which is further broken down to $^{14}CO_2$ which can be measured in the expired air. In a number of small intestinal disorders characterized by stagnation or incomplete absorption of bile acids an abnormal breath test occurs. Patients with a bacterial overgrowth have a peak $^{14}CO_2$ excretion before 4 hours (234,235). We were interested if patients with liver cirrhosis had a disturbed breath test as a measure of bacterial overgrowth and examined 7 patients with alcoholic liver cirrhosis. In five out of seven patients we found a late peak after 4 hours. As alcoholics have a diminished gastrointestinal motility (236), this peak might be due to bacterial overgrowth in these patients.

3.4 ENDOTOXINS AND ENDOGENOUS MEDIATORS

Interaction of endotoxins with macrophages results in the liberation of several mediators, such as procoagulant substances, collagenase, colony stimulating factors, complement components, lysozyme factors, substances cytotoxic or cytolytic for tumour cells, histamine and eicosanoids (192,196). One mediator released after endotoxin

administration is histamine, which has been suggested to play a role in galactosamine induced liver cirrhosis by causing edema of the colon which promotes increased absorption of endotoxins (195). Indeed cimetidine, a H_2 blocker protects against CCl_4 damage in rats (204,237). This protection is not necessarily an antihistamine effect of cimetidine but could be due to interference with CCl_4 metabolism. In a pilot study in collaboration with Dr. J. Keyser from Groningen we measured urine histamine metabolites (238) in ten patients with liver cirrhosis and found raised levels of methylhistamine in five out of ten patients (unpublished results). Further study on the role of histamine in the pathogenesis of the complications in liver cirrhosis are needed before conclusions can be drawn.

There is increasing evidence that eicosanoids are involved in endotoxin-induced injury in several species (table II) (168,169,171, 239,240,241).

Table II. IN VITRO AND IN VIVO RELEASE OF EICOSANOIDS BY ENDOTOXINS

PGE_2

PGF_{2alpha}

PGI_2

TXB_2

LTB_4

LTC_4

For example the endotoxin effects such as fever, shock, diffuse intra- vascular coagulation, renal failure, adult respiratory distress

syndrome (ARDS), thrombocytopenia and leucopenia are all possibly mediated by eicosanoids.

Endotoxin induced fever is the result of the effect of Interleukin I on eicosanoids production in the thermoregulatory center in the brain, where interleukin I initiates an abrupt increase in the synthesis of PGE_2 in the anterior hypothalamus. PGE_2 raise the thermostat set point in the hypothalamus. Besides the raised PGE_2 production in the hypothalamus, interleukin I induces a dramatic increase in PGE_2 production in muscles (197,206). The beneficial effects of antipyretics on fever and associated myalgias are the result of blocking the cyclooxygenase pathway (197).

After endotoxin administration raised levels of PGE_2, PGF_{2alpha} and especially TXB_2 and 6 keto PGF_{1alpha} are found and these are thought to be the most important mediators of the cyclooxygenase pathway in endotoxin shock (243). Endotoxins induce raised plasma TXB_2 levels in several species including cats, mini pigs, baboons, rats and sheep (168,169,240). Immediately after endotoxin administration there is a sharp rise in plasma TXB_2 levels followed by a prolonged rise of 6 keto PGF_{1alpha}, PGF_{2alpha} and PGE_2 (243). Several NSAIDs such as indomethacin salicylate and ibuprofen improve hemodynamics and survival in experimental endotoxin shock (240,243). The importance of TXB_2 in endotoxin shock is sustained by the development of a selective TXA_2 synthetase inhibitor, imidazole, which gives a 24 hour survival rate of 80% compared to 8% in the non treated group and lessened the severity of coagulapathies in endotoxin shock (168,172). Additional evidence, that prostaglandins are detrimental in endotoxic shock has been obtained in essential fatty acid deficient rats. These rats can only release small

amounts of prostaglandins derived from arachidonic acid and appear to be less susceptible to endotoxic shock, indicated by a markedly reduced mortality rate (243,241).

Reines et al measured plasma TXB_2 levels in twelve patients with septic shock. In eight patients dying with septic shock the plasma TXB_2 levels were ten times higher than in the four survivors (170). Recently we measured plasma TXB_2 levels in seven patients with gram negative septic shock. In six patients intermittently raised plasma TXB_2 levels were found, which were associated with a more disturbed renal function (to be published). These studies raise the possibility that thromboxanes may be involved in the pathophysiology of sepsis.

However Fletcher et al recently found that imidazole in contrast to the use of NSAIDs did not improve survival of rats with a gram negative sepsis even though thromboxane synthesis was inhibited (244). Their findings suggest that thromboxane mediates some events early in sepsis but is not important in the irreversibility of the shock state in this experiment. One possible explanation of the failure of imidazole in septic shock might be the involvement of leukotrienes. This hypothesis is sustained by the finding that endotoxic shock in rabbits results in raised levels of phospholipase A_2, which release the precursor AA, the substrate for PGs and LTs (245, and see section PLA_2).

The ARDS is a major complication in septic patients (197). Several investigations suggest that endotoxins play a major role in the pathophysiology of ARDS. In intact animals endotoxins provoke broncho constriction, pulmonary hypertension and increased pulmonary vascular permeability leading to edema and respiratory failure. Sequestration of leucocytes and platelets in the lung, as well as the generation of COP

have all been implicated as important factors in the lung injury. The endotoxin induced bronchoconstriction can be reversed by the use of cyclooxygenase blockers. The endotoxin induced lung vasoconstriction and platelet sequestration is mediated by thromboxanes and can be abolished by selective TXA_2 synthetase blockers (86,210,246,247). The increased vascular permeability leading to pulmonary edema can be abolished by high doses of corticosteroids, but not by NSAIDs or selective TXA_2 synthetase blockers, suggesting that COP do not mediate the increase of vascular permeability after endotoxins (86,210). Leukotrienes C_4 and D_4 can increase pulmonary vascular resistance (see section lung and eicosanoids). Large increases of 5HETE, approximatedly coincident with the onset of increased permeability in the lung indicate that lipoxygenase products, such as leukotrienes, could play a role in mediating the increased lung vascular permeability after endotoxin (86).

The myocardial depressent factor, which reduces myocardial contractility, constricts the splanchnic arteries and impairs RES phagocytosis is elevated after endotoxin administration (213). The thromboxane synthetase inhibitor, imidazole, has been found to be effectively in preventing the formation of myocardial depressent factor in endotoxin shock (213).

The induced adhesion of leucocytes to the vascular wall by endotoxins is probably due to leukotriene B_4 production (102,215).

Endotoxins inhibit the hepatobiliary elimination of leukotriene D_4 and this might facilitate liver damage (248). The endotoxin induced hypoglycemia is, in part, mediated by PGE_2 (249,250).

Human and rat peritoneal macrophages and Kupffer cells produce a scala

of eicosanoids, such as PGE_2, TXB_2, 6-keto-PGF_{1alpha}, PGF_{2alpha}, LTC_4, LTB_4 and LTD_4, after incubation with calcium ionophore or endotoxins (68,69,156,157,251,252).

In conclusion several biological effects of endotoxins are mediated by eicosanoids and can be prevented by using blockers of the eicosanoids pathway. The deleterious effect of endotoxemia in liver disease, such as diffuse intravascular coagulation, hypotension, renal failure, hepatorenal syndrome, leucopenia, fever, high mortality rate and liver damage might be mediated by eicosanoids.

References: see page 163.

CHAPTER 4

RAISED PLASMA THROMBOXANE B_2 LEVELS IN ALCOHOLIC LIVER DISEASE

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4.1 ABSTRACT

In experimental animals endotoxin administration causes increased levels of thromboxane B_2 and prostaglandins. Liver cirrhosis is often complicated by endotoxemia. In sixteen patients with alcoholic liver cirrhosis, we measured plasma thromboxane B_2 levels. In twelve patients we found on one or more occasions raised plasma thromboxane B_2 levels. Raised plasma thromboxane B_2 levels were associated with significantly higher serum levels of urea, alkaline phosphatase, gamma glutamyl transpeptidase and lower antiplasmin and antithrombin III levels. It is possible that some of the complications in patients with alcoholic liver cirrhosis are mediated by thromboxanes.

4.2 INTRODUCTION

Endotoxins are bacterial cell wall lipopolysaccharides of gram negative bacteria (1). Administration of endotoxins to experimental animals results in raised plasma thromboxane B_2 (TXB_2), the stable metabolite of thromboxane A_2, levels (2) and is associated with shock, decreased renal blood flow, pulmonary hypertension and a high mortality rate (3,4,5). Some of these effects of endotoxin administration can be blocked by pretreating the animals with a thromboxane A_2 (TXA_2) synthetase inhibitor (6,7,8).

Severe liver disease is associated with a high frequency of endotoxemia (9,10). The circulatory and laboratory changes seen in these patients (11,12) are similar to the effect of TXA_2. We therefore measured plasma levels of TXB_2 in sixteen patients with liver cirrhosis to determine whether raised plasma TXB_2 levels were associated with other complications.

4.3 PATIENTS AND METHODS

Sixteen patients with alcoholic liver cirrhosis (14 men, 2 women, aged 31-76 yrs) were studied. One or more of the following complications were present: ascites, jaundice, encephalopathy and bleeding oesophageal varices.

On two or more occasions peripheral venous blood samples (10 ml) were collected under resting conditions in polypropylene tubes containing 20 ul of heparin (5000 U/ml, Thromboliquine[R], Organon, Oss, The Netherlands) and 50 ul indomethacin (0,1 mg/ml in 0.1 M phosphate

buffer pH 8).

At the same time blood was taken for liver and renal function tests, platelet count, Normotest^R (a measure of the levels of clotting factor II, VII, X (Nyegaard Pharmacie B.V., Haarlem, The Netherlands)), antithrombin III, antiplasmin and fibrinogen levels. Normal values for plasma TXB_2 levels were obtained from blood samples taken from 17 controls (aged 22-78 yrs) on two occasions. The controls did not have a history of liver disease and had not received non-steroidal anti inflammatory drugs (NSAIDs) or corticosteroids during the previous month.

The blood samples taken for plasma TXB_2 determination were centrifuged immediately at 1400 g for 10 mins and the plasma stored at -20°C until assay. Two ml of plasma was applied to a Sep-pak $C_{18}{}^R$ cartridge (Waters Ass. Inc., The Netherlands) which had previously been washed with 10 ml of absolute ethanol and 10 ml of distilled water.

The column was rinsed with 2 ml of distilled water and the prostaglandin-like compounds eluted with 2 ml of absolute ethanol. 200 ul aliquots were dried under a stream of nitrogen at 40°C in a radioimmunoassay tube and redissolved in assay buffer. Radioimmuno assay kits were obtained from New England Nuclear.

Statistical analysis: Results of laboratory tests at different times in the same patient were compared by the paired two-tailed Wilcoxon test.

4.4 RESULTS

In twelve of sixteen patients with alcoholic liver cirrhosis we found raised plasma TXB_2 levels (200-1000 pg/ml) on one or more occasions.

Table I. Plasma thromboxane B_2 levels in relation to some other laboratory findings in patients with liver cirrhosis.

laboratory test	normal value	group 1 (4)	group 2 (12)	
		TXB_2 always normal	during normal TXB_2 period	during raised TXB_2 period
platelet count $10^9/l$	120 - 320	151 ± 40	128 ± 18	140 ± 26
serum creatinin umol/l	60 - 110	83 ± 8	85 ± 7	90 ± 8
serum urea mmol/l	2,5 - 8,0	6,0 ± 1,0	6,6 ± 1,0	8,0 ± 2,0*
serum alk.phosph U/l	18 - 45	48 ± 8	58 ± 10	71 ± 10*
serum g.g.t. U/l	5 - 25	86 ± 23	68 ± 15	95 ± 24**
plasma Normotest %	65 - 110	59 ± 7	49 ± 7	44 ± 5
plasma antithrombin III	80 - 120	63 ± 5	60 ± 7	50 ± 8**
plasma antiplasmin %	85 - 120	72 ± 5	69 ± 6	59 ± 7**

The laboratory findings are given as mean± SEM. The platelet count, serum creatinin, urea, alkaline phosphatase (alk.phosph.) and gamma glutamyl trans-peptidase (g.g.t) are expressed in SI units, the levels of clotting factors as a percentage of a normal standard. The results of the laboratory tests in patients in group 2 during periods with raised plasma TXB_2 levels (<200 pg/ml) are compared with results during periods with normal TXB_2 levels by the paired two-tailed Wilcoxon test (* $p<0,01$, ** $p<0,05$).

The mean plasma TXB_2 level in 17 healthy individuals was 118 pg/ml (range 25-190).

Raised plasma TXB_2 levels were associated with a disturbed renal function as measured by the serum urea level, a disturbed liver function as measured by raised plasma alkaline phosphatase and gamma glutamyl transpeptidase level and disturbed clotting mechanism as measured by lower antiplasmin and antithrombin III levels, compared to the values obtained by the same twelve patients at times when plasma TXB_2 levels were normal (table I). There were no significant differences in platelet count, leucocyte count and fibrinogen levels between the periods of raised and normal plasma TXB_2 levels. In the four patients with repeatedly normal plasma TXB_2 levels mean platelet count, mean renal function and liver function tests and mean clotting factors were less abnormal than in the patients with raised plasma TXB_2 levels.

Two patients with raised plasma TXB_2 levels died, one after two and one after eight days. All four patients with normal plasma TXB_2 levels survived. The mean duration of hospitalisation in the group with raised plasma TXB_2 levels was 33 days (range 2-66), compared to sixteen days in the group with normal plasma TXB_2 levels.

4.5 DISCUSSION

In this study we have shown that twelve out of sixteen patients with complicated alcoholic liver cirrhosis had raised plasma TXB_2 levels. Recently Zipser reported increased urinary TXB_2 levels in patients with the hepatorenal syndrome (13), which is in agreement with our findings.

The raised plasma TXB_2 levels are associated with changes in plasma concentration of clotting factors and deterioration of liver and renal function tests, features which we have previously shown to be correlated with endotoxemia in such patients (11).

TXB_2 is a stable metabolite of TXA_2, which is a potent platelet aggregating and vasoactive substance (2). The raised plasma TXB_2 levels in these patients are probably due to increased production of TXA_2. Thromboxanes have been proposed as mediators of some endotoxin effects. Thus the increased plasma TXB_2 levels are possibly an effect of endotoxemia in these patients.

In experimental animals NSAIDs and corticosteroids, which block the production of prostaglandins, diminish the effects of endotoxin administration (14-18). In mice with multiple liver granulomas extensive liver parenchymal cell damage following endotoxin administration could be prevented by pretreatment with NSAIDs or corticosteroids (19). In rats there is a marked elevation of plasma TXB_2 levels after intravenous administration of endotoxins (3,6). Pretreatment of these animals with a TXA_2 synthetase inhibitor gives a 24 hour survival rate of 80% compared to 8% in the non-pretreated group (6). NSAIDs are also effective in several diseases with raised prostaglandin levels, such as rheumatoid arthritis (20,21), Barrter's syndrome (22) and systemic mastocytosis (23).

Although it seems reasonable to suggest a trial of NSAIDs in cirrhosis, the administration of such drugs to patients with liver cirrhosis, especially those with ascites, has been shown to cause renal failure (24,25) and treatment of patients with low levels of clotting factors, as in liver cirrhosis, may give rise to bleeding problems (26).

Some authors have found raised plasma prostaglandin E (PGE) levels in the hepatorenal syndrome (27) and elevated urinary PGE_2 levels in liver cirrhosis with ascites (28) and suggested that the raised PGE levels play a supportive role in maintaining renal function in patients with liver disease (28). Others have found a relationship between urinary prostaglandins E_2 and F_{2alpha} and sodium excretion in various stages of chronic liver disease (29). The proposed beneficial effect of prostaglandins on renal blood flow in cirrhosis provides an explanation for the deterioration of renal function after NSAIDs administration in patients with severe liver disease.

Thus although we tentatively conclude that some of the complications of cirrhosis may be mediated by thromboxanes, the clinical experience with NSAID suggests that some of the other arachidonic acid metabolites have a beneficial function in these patients. As NSAID block the enzyme cyclooxygenase, they reduce the formation not only of thromboxanes, but also of the beneficially-acting prostaglandins. The recent development of specific thromboxane synthetase blockers (30,31) has potential value as a means of determining the role of thromboxane in the complications of liver cirrhosis.

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CHAPTER 5

SITES OF THROMBOXANE B_2 PRODUCTION AND REMOVAL IN LIVER CIRRHOSIS.

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5.1 ABSTRACT

In patients with alcoholic liver cirrhosis raised plasma thromboxane B_2 levels are found. Thromboxane B_2 is the stable metabolite of thromboxane A_2, a potent vasoconstrictor. To determine the site of production and removal of thromboxane B_2 and to investigate the hemodynamic consequences of raised plasma thromboxane B_2 levels, we measured plasma thromboxane B_2 levels during hepatic vein catheterisation in 3 patients with alcoholic cirrhosis and 2 patients with cryptogenic liver cirrhosis. In four patients there was a net thromboxane B_2 production by the splanchnic area and in all patients there was a removal of thromboxane B_2 by the lungs and kidneys. Following dazoxiben, a thromboxane synthetase blocker, thromboxane B_2 levels fell by 50-70% from their original value. The fall of thromboxane B_2 levels was not associated with consistent changes in systemic and pulmonary pressures, cardiac index, hepatic vein pressures or hepatic blood flow. The extraction fraction of indocyanine green decreased after dazoxiben; probably due to interference of indocyanine green uptake by the liver by dazoxiben. The lack of circulatory changes following a decrease in thromboxane levels suggests that the raised plasma thromboxane B_2 levels in cirrhosis might be a secondary phenomenon.

5.2 INTRODUCTION

Prostaglandins and other arachidonate metabolites may be involved in the circulatory changes in liver cirrhosis. Prostaglandins, especially prostaglandin E_2, are thought to play a role in maintaining renal function in cirrhosis (1-7); non-steroidal anti-inflammatory drugs which block prostaglandin synthesis have been shown to reduce renal blood flow (1,7) and block the effects of furosemide in cirrhotics (4,5,7). Other arachidonate metabolites which have received attention as possible vasoactive mediators of the circulatory changes in liver disease are thromboxane and prostacyclin. Recently Hamilton et al (8) found an increased production of prostacyclin (PGI_2) in the portal vein in experimental portal hypertension. These workers postulated that PGI_2, which is a vasodilator, might be involved in the development of a collateral circulation.

Thromboxane A_2, a potent vasoconstrictor and platelet aggregant, is rapidly metabolised to the stable compound, thromboxane B_2 (TXB_2) (9). We recently showed that plasma TXB_2 levels are intermittently raised in patients with alcoholic liver disease (10). The levels of TXB_2 found in these patients were similar to levels shown to be associated with vasoconstriction in rabbits (11,12). The raised TXB_2 levels were associated with higher levels of serum urea and a more disturbed liver function as measured by an increase in alkaline phosphatase and gamma glutamyl transpeptidase and a decrease in antiplasmin and antithrombin III levels. Raised urinary TXB_2 levels have also been described in patients with the hepatorenal syndrome (6). These findings suggest that in addition to the possible beneficial effects of prostaglandins in

liver disease, another arachidonate metabolite - thromboxane A_2 - might be contributing to the complications of liver cirrhosis.

In order to determine the site of thromboxane production in liver cirrhosis, we measured hepatic venous TXB_2 levels during hepatic vein catheterisation. The catheterisation permitted sampling in both right atrium and renal vein, which meant that we could also determine the site of TXB_2 elimination. In an attempt to study the hemodynamic effects of the raised thromboxane levels, we administered dazoxiben, a specific thromboxane synthetase blocker, during the catheterisation procedure and followed the changes in TXB_2 levels and various parameters of the systemic, pulmonary and portal circulation.

5.3 PATIENTS AND METHODS

Five consecutive patients with cirrhosis of the liver in whom hepatic vein catheterisation was to be performed as part of the diagnostic work-up of portal hypertension were asked to participate in the study. The aims and procedures of the study were explained to the patients, who all gave informed consent. The protocol was approved by the medical ethics committee of the University Hospital-Rotterdam. Three of the patients had an alcoholic cirrhosis, the other two had a cryptogenic cirrhosis. The diagnosis of cirrhosis was based in all patients on laparoscopy and liver biopsy. Parenchymal liver function was assessed by serum albumin, bilirubin and Normotest (Nyegaard, Oslo, Norway), which is a measure of the clotting factors II, VII, IX and X. Serum creatinine was used as a measure of renal function (Table I).

Table I. Clinical and biochemical characteristics of five patients undergoing catheterisation.

Patient no.	1	2	3	4	5
Cirrhosis	Alc	Alc	Alc	Crypt	Crypt
Age (yr)	61	57	60	59	79
Sex	M	M	F	M	F
BW (kg)	84	87	67	65	56
Ascites	+	+	+	+	-
Bilirubin (mg/dl)	2.6	2.4	0.58	0.7	0.7
Normotest (%)	35	37	74	72	96
Albumin (g/l)	24	27	40	32	39
Creatinine (mg/dl)	0.9	1.0	1.0	0.9	1.0

Right sided heart catheterisation was performed via the femoral vein by the Seldinger technique, using a Swan-Ganz thermodilution catheter. Pressures were measured by a strain gauge transducer and cardiac index by thermodilution in triplicate. Pressures were measured in the right atrium (RA), pulmonary artery in free (PA) and wedged (PCW) position and hepatic vein in free (FHV) and wedged (WHV) position and are expressed in mmHg as mean value of the triplicate observations. The WHVP was confirmed by monitoring the pressure during expansion of the catheter balloon and by the absence of reflux of injected contrast medium. The hepatic venous pressure gradient is the WHVP minus the FHVP.

Arterial pressures were monitored by a strain gauge transducer via a catheter in the femoral artery. Another catheter was placed in the renal vein to allow blood sampling. Renal vein catheterisation was not performed in patient 4 due to technical difficulties.

Estimated hepatic blood flow was determined by the indocyanine green (ICG) continuous infusion method (13). Blood was taken from the RA, renal vein (RV), hepatic vein (HV) and artery (A) for determination of oxygen saturation (SaO2) and plasma TXB_2 levels. The initial 3 ml of blood was discarded, 3 ml of blood was collected in a 3 ml plastic syringe containing 20 ul of heparin (Thromboliquine 5000 U/ml, Organon, Oss, The Netherlands) and the SaO2 were measured immediately. Blood for plasma TXB_2 was collected in polypropylene tubes containing 20 ul of heparin (5000 U/ml) and 50 ul of indomethacin (0.1 mg/ml in 0.1 M phosphate buffer pH 8.0). The plasma TXB_2 was measured by RIA, after applying the plasma to a Sep-Pak $C_{18}^{(R)}$ cartridge (Waters Ass. Inc. The Netherlands) (10). The overall recovery of (^{3}H) TXB_2 added to plasma was 85-90%, and the purity of the sample was found to be greater than 95% on HPLC. The cross reactivity of the TXB_2 antibody with other prostaglandins was as follows: TXB_2 100%, prostaglandin E_2 0.2%, prostaglandin A_2 0.2%, prostaglandin F_2 0.2% and 6-keto-PGF_{1alpha} 0.2%. Normal values for plasma TXB_2 levels, < 200 pg/ml, were taken from our previously described study (10). Blood was taken from the various sites within 60 seconds by rapidly moving the catheter from HV to RA. Systemic vascular resistance and pulmonary vascular resistance (expressed per m^2 body surface area) were calculated from the mean arterial or pulmonary arterial pressures and the cardiac index. After baseline measurements at 0 and 15 minutes, the patients were given 100

mg of dazoxiben as a bolus injection intravenously and the hemodynamic measurements and blood sampling performed 15, 30 and 45 minutes later. Net uptake or release of TXB_2 by the splanchnic area, kidneys or lungs was demonstrated by comparing HV, RV or RA TXB_2 levels with arterial TXB_2 levels. We made an estimate of TXB_2 production in the splanchnic area, before the administration of dazoxiben, by multiplying the net HV-A difference by the estimated hepatic blood flow. In a similar fashion we calculated net pulmonary TXB2 uptake, before dazoxiben, by multiplying the RA-A difference by the cardiac output.

5.4 RESULTS

All patients had portal hypertension as judged by a raised hepatic venous pressure gradient (HVPG) (Table II). The normal HVPG using this method is 4 mmHg (14). Before administration of dazoxiben the three patients with alcoholic liver cirrhosis had raised plasma TXB_2 levels in RA, HV and A, as opposed to the two patients with cryptogenic cirrhosis, although one of these had a plasma TXB_2 level at the upper limit of the normal range (Table III). Before the administration of dazoxiben there was evidence of a net TXB_2 production in the splanchnic area in four patients (Table IV), the exception being the patient with cryptogenic cirrhosis and the lowest initial TXB_2 level. Total TXB_2 production in the splanchnic area was high in the alcoholic cirrhotics. Before dazoxiben there was a positive RA-A and A-RV, difference of plasma TXB_2 levels (Table IV), suggesting that the kidneys and lungs eliminate TXB_2 from plasma. Following the administration of dazoxiben

Table II. Values expressed as mean pressures before (pre) and the mean value of the pressures measured at 15', 30', 45' after dazoxiben (post) intravenously in patients with cirrhosis.

Patient no.		1		2		3		4		5	
		pre	post	pre	post	pre	post	pre	post	pre	post
Right atrium,	mmHg	2	3	7	7	2	1	0	7	0	0
A. Pulmonalis,	mmHg	11	14	15	14	18	16	9	14	11	12
Wedge pulmonalis,	mmHg	10	9	11	10	7	5	3	5	6	6
Artery,	mmHg	71	71	78	70	114	110	112	113	71	73
Heart rate,	min	85	92	80	79	79	83	86	82	68	68
Cardiac index,	$1/min/M^2$	4.3	3.98	5.58	5.26	3.4	3.68	3.65	4.17	3.16	3.13
SVR,	$dyne.sec/cm^5.M^2$	1282	1014	966	993	2632	2334	2452	2031	1795	1867
PVR,	$dyne.sec/cm^5.M^2$	18.6	106	57	61	258	225	131	178	126	140
Hepatic vein free,	mmHg	9	9	10	10	4	4	9	8	1	3
Wedge hepatic,	mmHg	19	19	34	35	20	20	25	23	14	14
HVPG,	mmHg	10	10	24	25	16	16	16	15	13	11

SVR = systemic vascular resistance

PVR = pulmonary vascular resistance

HVPG = hepatic venous pressure gradient.

Table III. Plasma TXB_2 levels (pg/ml) before (pre) and 15', 30' and 45' after (post) dazoxiben.

Patient no.	1		2		3		4		5	
	pre	post	pre	post	pre	post	pre	post	pre	post
Hepatic vein	656	125	611	161	410	142	192	85	106	59
Right atrium	585	116	413	167	325	186	183	85	129	67
Artery	368	143	328	165	250	170	162	64	115	99
Renal vein	190	104	190	127	187	144	-[a]	-[a]	71	64

[a] not measured due to technical difficulties.

Table IV. Arterio-venous differences in TXB_2 concentrations and calculated net TXB_2 production by the liver and net TXB_2 removal by the lung.

Patient	1	2	3	4	5
HV - A	288	283	160	30	-9
RA - A	217	85	75	21	14
A - RV	178	138	63	-[a]	44
TXB_2 production by liver.ng/min.	173	131	128	17	0
TXB_2 elimination by lung. ng/min.	1801	995	435	128	70

[a] RV samples not measured due to technical difficulties.

Table V. Estimated hepatic blood flow (EHBF), ml/min and extraction fraction (EF) of indocyanine green before (pre) and after (post) dazoxiben.

	EHBF (ml/min)		EF (%)	
	pre	post	pre	post
Patient 1	601	603	38	29
Patient 2	463	928	27	20
Patient 3	799	727	51	46
Patient 4	565	571	37	32
Patient 5	1081	895	43	39

plasma TXB_2 levels dropped in all patients (Table III). Mean plasma TXB_2 levels in hepatic vein from all patients dropped after 15' to 58% (range 23-78), at 30' to 72% (range 44-84%) and at 45' to 59% (range 40-80%) from the original values. In figure 1 the changes in plasma TXB_2 levels in patient 2 after the intravenous administration of 100 mg dazoxiben are shown.

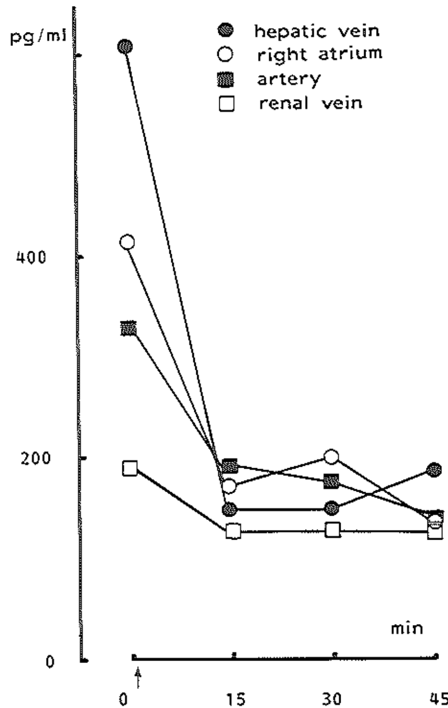

Fig. 1. Plasma TXB_2 levels (pg/ml) before (0) and 15-, 30-, and 45 min. after administration of dazoxiben 100 mg intravenously in patient 2.

Following the injection of dazoxiben no significant changes occurred in RA, PA, PCW, FHV, WHV or A pressures (Table II). No consistent changes were seen in cardiac index, systemic or pulmonary vascular resistance

after dazoxiben (Table II). Estimated hepatic blood flow (EHBF) did not change after dazoxiben (Table V). In all patients, however, the extraction fraction of ICG by the liver declined after dazoxiben administration (Table V).

There were no significant changes in SaO2 after dazoxiben in RA, HV, RV, A.

5.5 DISCUSSION

Raised peripheral plasma TXB_2 levels were found in the three patients with alcoholic liver cirrhosis. This is in agreement with our previous findings (10). In four of five patients with cirrhosis we found the highest TXB_2 levels in hepatic vein, which points to a net production of TXB_2 by the splanchnic area, possibly by the liver. The patient without a net TXB_2 production had a compensated cryptogenic liver cirrhosis with the best liver function and the highest EHBF of all patients (Table I, Table V). The origin of the TXB_2 in the splanchnic area is uncertain. Chong et al. (15) found TXB_2 synthesis by isolated hamster hepatocytes after exposure to vasopressin, demonstrating that hepatocytes are capable of producing TXB_2. Kupffer cells are a possible alternative source for the TXB_2 production.

The splanchnic area is not the only site of increased production of TXB_2 in liver cirrhosis, the calculated net production rates of TXB_2 by the splanchnic area being lower than the calculated net removal rate of TXB_2 by the lungs. As we did not measure renal blood flow it was not possible to calculate the relative contribution of the kidney to the removal of TXB_2 from the plasma.

The pathophysiologic implications of the raised plasma TXB_2 levels and increased production rates in alcoholic liver cirrhosis are still not clear.

Recently Zipser et al. found raised urinary TXB_2 and lower PGE_2 levels in cirrhotics with the hepatorenal syndrome but not in patients with cirrhosis and normal renal function. Plasma TXB_2 levels were however not measured in this study (6).

In our investigation the decrease in plasma TXB_2 induced by dazoxiben, a thromboxane synthetase blocker (16,17), was not followed by changes in systemic, pulmonary or portal pressures and was not associated with changes in cardiac index or EHBF. In a small number of patients with the hepatorenal syndrome given either 400 mg or 600 mg of dazoxiben daily for four days Zipser et al (Zipser et al, Clinical Research 32:288A,1984, abstract) observed a mean reduction in urinary TXB_2 by 54%, however without consistent improvement of renal function.

One possible explanation for the failure of TXB_2 blockade to result in hemodynamic changes in patients with liver cirrhosis is that the raised plasma TXB2 levels in these patients are a secondary phenomenon. Leukotrienes are candidates as stimulators of TXA_2 production. Leukotrienes are arachidonic acid products of the 5 lipoxygenase pathway (18). Leukotrienes C4 and D4 are potent bronchoconstrictors (19) and cause a short lived vasoconstriction followed by extravasation of macromolecules (20). Leukotrienes have been shown to stimulate the release of TXA_2 in vitro and in vivo (21,22). Recently Rosenthal et al. found that leukotrienes C4 and D4 gave a potent vasoconstriction of the isolated perfused rat kidney, which could be blocked by a specific blocker of the leukotriene synthesis (FPL 55712) but not by a specific

blocker of thromboxane synthesis (23).

An interesting finding was that although the EHBF did not change, in all patients the EF for ICG decreased after administration of dazoxiben (Table V). The decrease in EF could be due to increased transhepatic shunting of blood, but this does not seem likely as we did not find changes in SaO2 in hepatic venous blood after dazoxiben. Another possibility is that dazoxiben interferes with ICG uptake by the liver. Dazoxiben contains an imidazole ring, as does cimetidine (16,24). The administration of cimetidine results in a lowered extraction fraction of ICG, probably due to interference of cimetidine with the uptake of ICG by the liver (Jackson EJ. N Eng J Med 335:99,1981, correspondence, 25).

In conclusion, the raised plasma TXB_2 levels found in alcoholic cirrhosis are in part derived from a net production in the splanchnic area. TXB_2 is removed from the circulation by the lungs and the kidneys. Dazoxiben causes an acute drop in TXB_2 production and in plasma TXB_2 levels, but this is not associated with hemodynamic changes, suggesting that thromboxanes do not play an important role in the circulatory complications of liver cirrhosis. Further studies on other arachidonic metabolites, especially leukotrienes, are needed to unravel the cause of the overproduction of TXB_2 in liver cirrhosis.

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CHAPTER 6

USE OF THE CHROMOGENIC LIMULUS ASSAY FOR THE DETECTION OF ENDOTOXEMIA IN SEPTIC PATIENTS OR IN CIRRHOTICS

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6.1 ABSTRACT

Endotoxins are thought to be responsible for a number of manifestations of gram negative sepsis and for several complications of liver disease. In experimental animals the administration of endotoxins results in raised levels of thromboxanes. Raised plasma thromboxane levels have been found in patients with liver cirrhosis and patients with sepsis.
We performed a study to determine if there was a correlation between endotoxin levels measured by a Limulus Lysate assay with the aid of a chromogenic substrate and plasma thromboxane B_2 levels. The study was performed in 6 patients with a gram negative sepsis and 12 patients with decompensated alcoholic liver cirrhosis. In only 3 of the 6 patients with sepsis and in 8 of the 12 patients with cirrhosis did we find endotoxin levels above the upper limit of normal for the assay of 12 pg/ml. In agreement with earlier work plasma TXB_2 levels were raised in these patients. There was no correlation between endotoxin and thromboxane B_2 levels. The lack of correlation could in part be ascribed to a considerable variability in the Limulus Lysate chromogenic assay.
We conclude that the Limulus Lysate assay for endotoxin using a chromogenic substrate, as performed in this study, is unsuitable for clinical research due to variability of the standard curves and the lack of sensitivity of the assay. More reliable and more sensitive diagnostic tools for endotoxins are needed to evaluate the role of endotoxins in patients with liver disease or with sepsis.

6.2 INTRODUCTION

Endotoxins from gram-negative bacteria play an important role in the manifestations of gram-negative sepsis and probably liver disease (1,2). The gut is a large reservoir of endotoxin producing gram-negative bacteria (2,3). In the normal state absorbed endotoxins are rapidly removed from the portal blood by the Reticulo Endothelial System (RES), especially by the Kupffer cells (2). Detection of endotoxins was originally based on their in-vivo effects, e.g. pyrogenic effects in rabbits injected with the material to be tested (4).

After a more detailed chemical survey of the active component of endotoxins it became clear that lipid A is responsible for the in-vivo reactions (5). This resulted in a search for a more sensitive and simpler analysis. At that time a very remote but interesting biological observation concerning the life cycle of the horseshoe crab revealed a clue for possible analysis (6,7). Horseshow crabs frequently die in shallow water due to gram-negative sepsis and intravasal coagulation. This led Levin and Bang to the idea of using the amebocytes of this crab for a clotting gelation test for the determination of endotoxins (7). This test subsequently was used for clinical work (2,3,8).

Various modifications and additions to this principle have been developed. The major one is the use of the "artificial" chromogenic substrates measuring peptidase activity giving not only a qualitative but also a quantitative result in the presence of endotoxins (9,10).

Several authors have described the presence of endotoxemia in liver disease and sepsis (1,2,3,8,11). In the past we found a positive

Limulus amebocyte lysate (LAL) gelation test in 46% of patients with liver cirrhosis (8).

In experimental animals endotoxin administration results in raised levels of several prostaglandins. Especially thromboxane B_2 (TXB_2) and 6 keto-PGF_{1alpha} are thought to be the most important prostaglandins playing a role in endotoxin shock (12,13,14). In patients with sepsis and liver disease raised plasma thromboxane B_2 levels have been found (14,15). It is thought that the raised plasma TXB_2 levels are due to endotoxemia in these patients.

The aim of our study was to investigate the correlation between endotoxemia, measured by the generally accepted endotoxin assay with the aid of a chromogenic substrate S2423 (Kabi Vitrum, Amsterdam, The Netherlands) and the plasma TXB_2 levels as measured by radioimmuno assay (RIA).

6.3 PATIENTS AND METHODS

Twelve patients with alcoholic liver cirrhosis (8 men, 4 women), age 40-76 yrs, and six patients (2 men, 4 women), age 29-70 yrs, with a gram-negative septicaemia proven by positive blood cultures were studied. All patients with sepsis had a systolic pressure below 100 mmHg and renal dysfunction. Four patients had a positive blood culture with either Pseudomonas Aeraginosa, Klebsiella, Enterococcus and Escherichia Coli. Two patients had Bacillus anitratum and Bacteriodes in their blood cultures. Venous blood samples were collected on one or more occasions.

For the endotoxin determinations 5 ml blood was taken by venopuncture with a pyrogen-free needle and syringe containing 50 ul of heparin (5000 IU/ml, Thromboliquine^{R}, Organon, Oss, The Netherlands). The blood was immediately transferred to a pyrogen-free plastic tube on ice and centrifuged at 1200 xg for 10 mins. The plasma was stored in pyrogen-free plastic tubes at -20°C until assay. Immediately after blood sampling for endotoxin assay blood samples (10 ml) were collected for TXB_2 assay in polypropylene tubes containing 20 ul of heparin (5000 IU/ml Thromboliquine^{R}, Organon, Oss, The Netherlands) and 50 ul indomethacin (0.1 ng/ml in 0.1 mM phosphate buffer pH 8).

Blood samples for plasma TXB_2 determination were centrifuged immediately at 1400 g for 10 mins and the plasma stored at -20°C until assay.

Endotoxins in plasma were measured according to the method described by Thomas et al (9,10). Some adjustments and modifications have since been made to optimize the results. These will be mentioned when appropriate. The heparin solution for blood collection was tested for endotoxin contamination. Plasma samples were diluted 10 times with pyrogen-free water and heated for 10 mins at 75°C. A plasma pool was constituted from twenty plasma batches to serve as reference plasma pool. The endotoxin assays were carried out generally according to the procedure suggested by the company. The exact procedure is as follows. A standard curve with known endotoxin concentration was constructed as follows. The endotoxin standard (2 ng) was solubilished in 1,0 ml of water and mixed vigorously for 3 mins. The endotoxin stock solution was diluted twenty times. The standard curve was made according to the instruction of the company. These standards were inactivated for 10 mins at 75°C

and left to cool. The LAL was solubilished in the appropriate amounts of water. Of the test sample or standard (both heat inactivated) 50 ul was equilibrated for 3-5 mins at 37°C, mixed with 50 ul LAL and incubated at 37°C for 20 mins. To the mixture 100 ul of the substrate buffer solution containing 1.1 mM S2423 (Kabi Vitrum, Amsterdam, The Netherlands) in 2,5 mM Tris pH 9 was added. After 10 mins the reaction was stopped by the addition of 400 ul of 50% acetic acid. A blank was made of every plasma sample. The extinction was read at 405 nm. A calibration line was constructed and unknown samples were calculated using this line. Plasma TXB_2 levels were measured by RIA as described earlier (15).

6.4 RESULTS

The reproducibility of the endotoxin assay was investigated by making a set of calibration lines using twenty individually plasma samples and a single endotoxin stock standard. The resulting calibration lines varied considerably from 5.1 to 10.1 (n = 20).

Values for endotoxin levels in eighteen healthy controls were found to be 6,3 ± 2,8 pg/ml (mean ± SD) and for TXB_2 a median level of 70 pg/ml (range 25-225).

In the 69 plasma samples of the twelve patients with liver cirrhosis we found an endotoxin level of 10,2 ± 4,2 pg/ml (mean ± SD) and for TXB_2 a median value of 228 pg/ml (range 55 to 1521) (fig.1). Eight of the twelve patients with liver cirrhosis had intermittently endotoxin levels above 12 pg/ml. There was no correlation between plasma endotoxin and TXB_2 levels.

In six patients with sepsis the endotoxin and respectively TXB_2 levels were 8.9 ± 4.4 pg/ml (mean ± SD) and 326 pg/ml (median; range 60 to 1386) (fig.I). Only three of the patients had on one or more occasions an endotoxin level above 12 pg/ml.

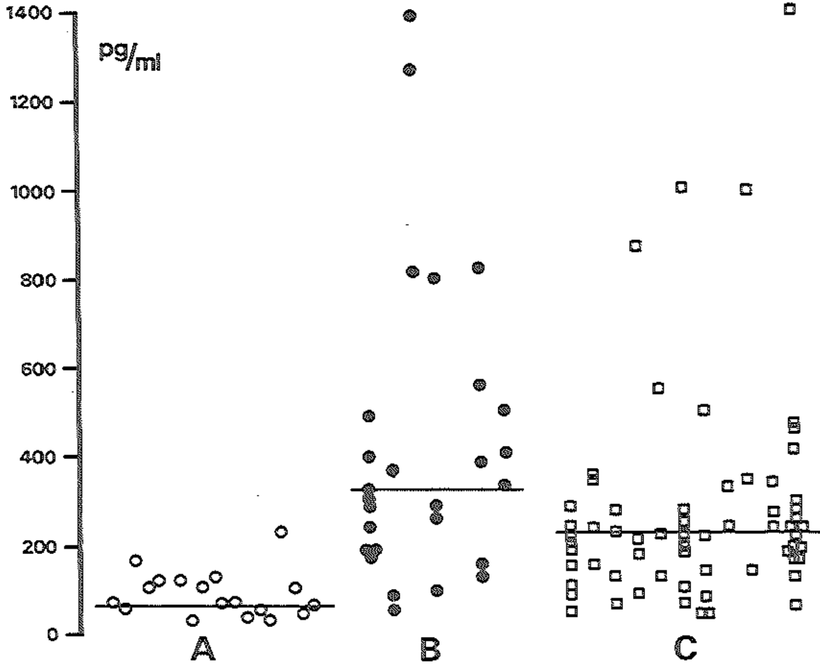

Fig.I. Plasma TXB_2 levels on one or more occasions in controls (A), septic patients (B) and cirrhotics (C).
Points on the same vertical line represent the same patient.

6.5 DISCUSSION

In eight of the twelve patients with liver cirrhosis and in three of the six patients with sepsis we found endotoxin levels above 95%

confidence limit for normals of 12 pg/ml. One possible explanation of the negative results in septic patients might be due that blood cultures become positive when less than 100 microorganism per ml are present, whereas a positive LAL test requires more than 1000 microorganism per ml (16,17). As is in agreement with earlier work we found raised plasma TXB_2 levels in patients with liver cirrhosis and sepsis (14,15,18). There was however no correlation between endotoxin levels and thromboxane B_2. This might be due to the failure of the limulus assay to detect endotoxemia even at levels giving symptoms - as was the case in the septic patients, to a lack of sensitivity of the assay - i.e. an overlap between endotoxemic patients and healthy controls, or to time related factors.

The variability of the standard curves of the endotoxin assay and the poor sensitivity makes this test unsuitable for clinical research. Despite the poor results of this test we do think that endotoxins play a role in liver disease and sepsis. However to unravel the role of endotoxins more reliable diagnostic tools than the chromogenic endotoxin assay are needed. In the future sensitive radioimmuno assays or monoclonal antibodies to lipid A might help us to determine the role of endotoxins in liver disease.

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CHAPTER 7

ENDOTOXIN ABSORPTION FROM THE RAT INTESTINE: THE EFFECT OF COLITIS, PORTAL HYPERTENSION AND ALCOHOL

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7.1 SUMMARY

Increased endotoxin absorption from the intestine, due to changes in mucosal permeability, might contribute to endotoxemia in patients with liver disease. We studied the rate of absorption of ^{51}Chromium-labelled Escherichia coli endotoxin from loops of intestine of male Wistar rats. Portal hypertension was created by incomplete ligation of the portal vein in one group of rats. Ethanol was administered to two groups, either in the drinking water (15% w/v) or in a liquid diet to provide 36% of the energy for a period of three weeks. Ulcerative colitis was created in another group by brief exposure of the colon to dilute acetic acid. Endotoxin absorption was also studied in vitro in everted intestinal sacs. Endotoxin absorption was very low (mean 0.60, range 0-2.83 ug/10 cm intestine/2 hrs) from both small and large intestine of control animals. Neither ethanol administration nor portal hypertension increased endotoxin absorption, whereas the absorption rates from the colon were raised in rats with colitis (mean 5.83, range 0.10-17.30 mcg/10 cm colon per 2 hrs). Endotoxin absorption was found to be much higher from everted sacs than from the intestinal loops in vivo.

We conclude that the intact intestinal mucosa forms an efficient barrier to intraluminal endotoxin. The permeability of the intestine to endotoxins is not increased by portal hypertension or by the administration of ethanol in rats. Everted intestinal sacs are not suitable models for studying the process of endotoxin absorption.

7.2 INTRODUCTION

Endotoxins are lipopolysaccharide components of the cell wall of Gram-negative bacteria. Systemic endotoxemia has been implicated in several complications of liver disease, and endotoxins have been shown to potentiate the effects of hepatotoxins (1). Systemic endotoxemia, as demonstrated by the limulus lysate test (1,2) or radioimmunoassay (3) is thought to be due to failure of the reticuloendothelial system of the liver to clear the portal blood of endotoxins derived from intestinal bacteria (4). The mechanism of intestinal absorption of endotoxins is not clear. Using everted gut sacs, Nolan and co-workers have found a saturable transport mechanism for endotoxin absorption from the rat small intestine (5). Endotoxins are macromolecules, and it is possible that permeability changes induced by disease or damage of the intestinal mucosa might affect endotoxin absorption. Increased endotoxin absorption has indeed been demonstrated in hemorrhagic shock (6,7,8), a situation in which damage to the mucosa is likely (9).

We decided to examine various factors which could possibly affect endotoxin absorption in patients with liver disease. We therefore studied the effects of alcohol administration at two dosage levels, portal hypertension following partial ligation of the portal vein and experimental colitis on the absorption of ^{51}Chromium-radiolabelled endotoxin from the rat intestine. To obviate the possible damaging effects of inversion of the intestine, we studied the absorption from isolated loops in vivo rather than everted gut sacs in vitro. In a single experiment the rate of absorption from everted sacs was compared with that from isolated loops in vivo.

7.3 MATERIALS AND METHODS

Male Wistar rats weighing between 200 and 250 grams, obtained from T.N.O., Zeist, were used for the experiments. Portal hypertension was created by placing 2 incomplete ligatures around the portal vein as described previously (9). Briefly the portal vein is dissected free during light ether anaesthesia, a catheter with an external diameter of 1 mm placed alongside, and two ligatures are tied around both catheter and portal vein with 4-0 silk. The catheter is then withdrawn. This leads to a presinusoidal portal hypertension with a raised portal pressure and splenomegaly (10). Ethanol was administered either in the drinking water (150 g ethanol, 5 g glucose/L) or in a liquid diet in which ethanol provided 36% of the total energy (11). The rats were kept on the diet for 3 weeks prior to the absorption studies.

Experimental colitis was provoked by the method of MacPherson and Pfeiffer (12). Under light ether anaesthesia 1 ml of 10% acetic acid is instilled in the colon by a rectal canula. After 60 seconds the acetic acid is removed by 3 successive washings with distilled water. Within 48 hrs all rats developed a persistent bloody diarrhoea. The absorption studies were performed 10 days later. Two groups of colitic rats were formed, one for histological examination and one for the absorption studies. Three control groups were used - two (one for jejunum, one for colon studies) on normal laboratory chow, one on a liquid diet without ethanol (11). Six to eight animals were used per group.

Absorption studies: under ether anaesthesia a laparotomy was performed and a jejunal loop immediately distal to the duodenum was selected and two loose ligatures placed approximately 15 cms apart. A catheter with

an external diameter of 1 mm was introduced into the lumen by a small incision proximal to the first ligature, passed through the ligature into the loop and then both ligatures were tightened and the catheter fixed to the proximal jejunum by means of a third ligature (Fig.1).

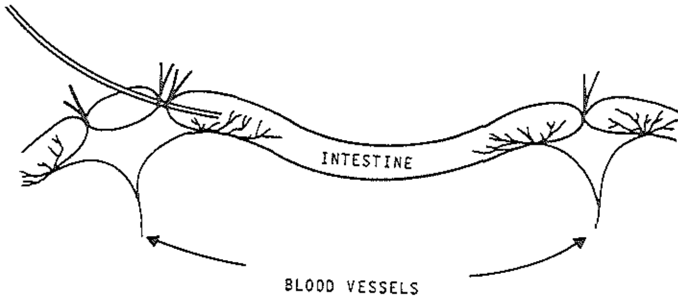

Fig.1 A loop of intestine is cannulated as shown here, care being taken not to damage the blood vessels supplying the intestinal segment.

Two ml of ^{51}Cr-labelled endotoxin solution was introduced into the loop by the catheter, followed by 0.25 ml of air, and the catheter sealed. The abdominal wall was closed and the animals were allowed to regain consciousness. Two hours later the animals were stunned and exsanguinated by cardiac puncture. The isolated loop was removed intact, as were the lungs, kidneys, spleen and liver. A standard small weight was attached to one end of the intestinal loop and its length measured. The organs, blood and the loop were placed in separate test tubes and the radioactivity counted in a gamma counter (Automatic Gamma

System model 1185, Nuclear Chicago). The radioactivity in blood and organs was expressed as a percentage of the total activity in these organs plus the activity of the isolated loop. During preliminary experiments, the rest of the carcass was also counted, but this consistently failed to show any increase in counts over the background activity. Absorption was calculated as mcg endotoxin/10 cm bowel length/2 hrs. In the absorption studies on the colon, the colon was gently flushed with 0.154 M NaCl until clean, and the last 8 cms of the colon used for the isolated loop experiments.

Everted sacs were prepared from another group of rats as described by Nolan et al (5). Two segments of small intestine approximately 15 cm long were removed from the anaesthetized rat and placed in a solution of 0.154 M NaCl and 0.0045 M KCl at 4°C. The intestine was everted over a glass rod, one end tied off and 0.75 ml of intestinal solution (0.147 M NaCl, 0.1 mM CaCl 2, 0.04 M glucose, 0.004 M tris-HCl, pH 7.2, 320 mOsmol/kg) introduced by a small syringe. The second ligature was then placed 10 cm from the first. The filled, everted sac was placed in an Erlenmeyer flask containing 4.5 ml intestinal solution which contained ^{51}Cr-labelled endotoxin in a concentration of 2 mg/ml and shaken gently in a water bath at 37°C for 2 hrs. Oxygen was passed into each flask by means of a catheter. The whole procedure from abdominal incision to start of incubation lasted less than 15 mins. Following incubation the solution was removed from within the sac by means of a gauge needle and the radioactivity in both internal and bathing fluids measured in a gamma counter. Absorption is expressed as mcg endotoxin absorbed/10 cm bowel length/2 hrs.

Endotoxin (E. Coli 0127:B8, Difco) was labelled with ^{51}Cr as described

by Braude (13) with some modifications (14). The labelled product was checked by chromatography and solubility experiments to exclude the presence of free ^{51}Chromate and by passage through a 0.22 um bacterial filter to exclude aggregate formation (14).

Statistical analysis: Differences in absorption between the various groups were compared by the Mann-Whitney U test.

7.4 RESULTS

Endotoxin absorption in control animals was low from both small and large intestine (less than 2.83 mcg/10 cm/2 hrs, table 1). The portal hypertensive rats had larger spleens (607 ± 79 mg, mean ± SD) than control animals (405 ± 50 mg, p 0.01) and histological examination revealed splenic congestion but no thrombosis of the portal veins. Portal hypertension was not associated with an increased absorption of endotoxin (table 1). Neither of the two groups which received alcohol had an increased rate of endotoxin absorption from the small intestine (table 1).

On microscopical examination, the colons of the rats which had been pretreated with acetic acid showed also ulcers which sometimes penetrated to the muscularis mucosae. The remaining mucosa was oedematous and contained a dense inflammatory infiltrate of lymhpocytes plasma cells and granulocytes which extended to the serosa in some areas. A purulent exsudate was seen within the lumen. Inflammatory infiltrates were also found in the portal areas of the liver in these animals, and Councilman bodies were observed. Colitis was associated with significantly greater absorption of endotoxin from the colon than

occurred in control animals (table 1).

Table 1. TOTAL ABSORPTION OF ENDOTOXIN FROM INTESTINE

	mean	range
Jejunum		
controls	0.60	0.02- 2.83
pH	0.05	0.00- 0.15
alcohol 1	0.06	0.00- 0.21
alcohol 2	0.05	0.00- 0.13
liquid diet controls	0.46	0.00- 1.80
everted sacs	11.4	3.5 -26.9*
Colon		
controls	0.33	0.00- 1.27
ulcerative colitis	5.82	0.10-17.30*

*p 0.05 vs. controls

ug/10cm/2hrs.

Endotoxin absorption was much higher in vitro in the everted sacs than under any of the situations studied in vivo using isolated loops (mean 11.4 mcg, range 3.5-26.9, vs. 0.60, range 0.02-2.83 in vivo, p 0.001). Histology of the everted sacs showed some damage to the mucosal cells, especially those at the tips of the villi, but without obvious ischemic changes.

7.5 DISCUSSION

The very low rates of absorption of endotoxin from the normal rat intestine in vivo, contrasts with the findings in vitro using isolated everted gut sacs. The range of absorption we found using everted sacs corresponds to the rate of absorption found by Nolan et al (5). The histological signs of mucosal damage in everted sacs do not support the concept that absorption studies under these circumstances represent the situation of the intact mucosa. The damage to the mucosa is probably due to manipulation and disruption of the vascular supply, and the metabolic status of such preparations is poor (15). A good oxygen supply is essential for the integrety of the mucosa (4). It therefore seems justified to conclude that the everted sac is not a suitable model system for the absorption of macromolecules by the intestine.
The low rates of absorption in vivo suggest that an intact mucosa forms a highly effective barrier to endotoxin absorption in the rat. The colon and small intestine seem to be equally (im)permeable to endotoxins. Gross damage to the colon as seen with acetic acid-induced ulcerative colitis does result in an increased rate of endotoxin absorption. This finding, and histological changes in the liver of these animals, are compatible with the theory that increased endotoxin absorption might be one of the main causative factors of the hepatic lesions seen in patients with inflammatory bowel disease.
Acute alcohol administration increases the transferal of horseradish peroxidase across the intestinal mucosa (16). We have not studied acute alcohol administration, but chronic alcohol consumption at two dosage levels failed to increase the intestinal permeability to endotoxins in

these experiments. Portal hypertension also failed to induce an increased rate of endotoxin absorption. These findings do not exclude the possibility of an increased rate of endotoxin in patients with liver disease, in whom several potentially damaging factors, such as protein or phosphate deficiency (17,18), intestinal bacterial overgrowth (19,20), portal hypertension and relative intestinal ischemia, and chronic alcohol consumption may be present simultaneously. Possible differences in susceptibility to alcohol damage and changes in portal blood flow in various species should also be considered.

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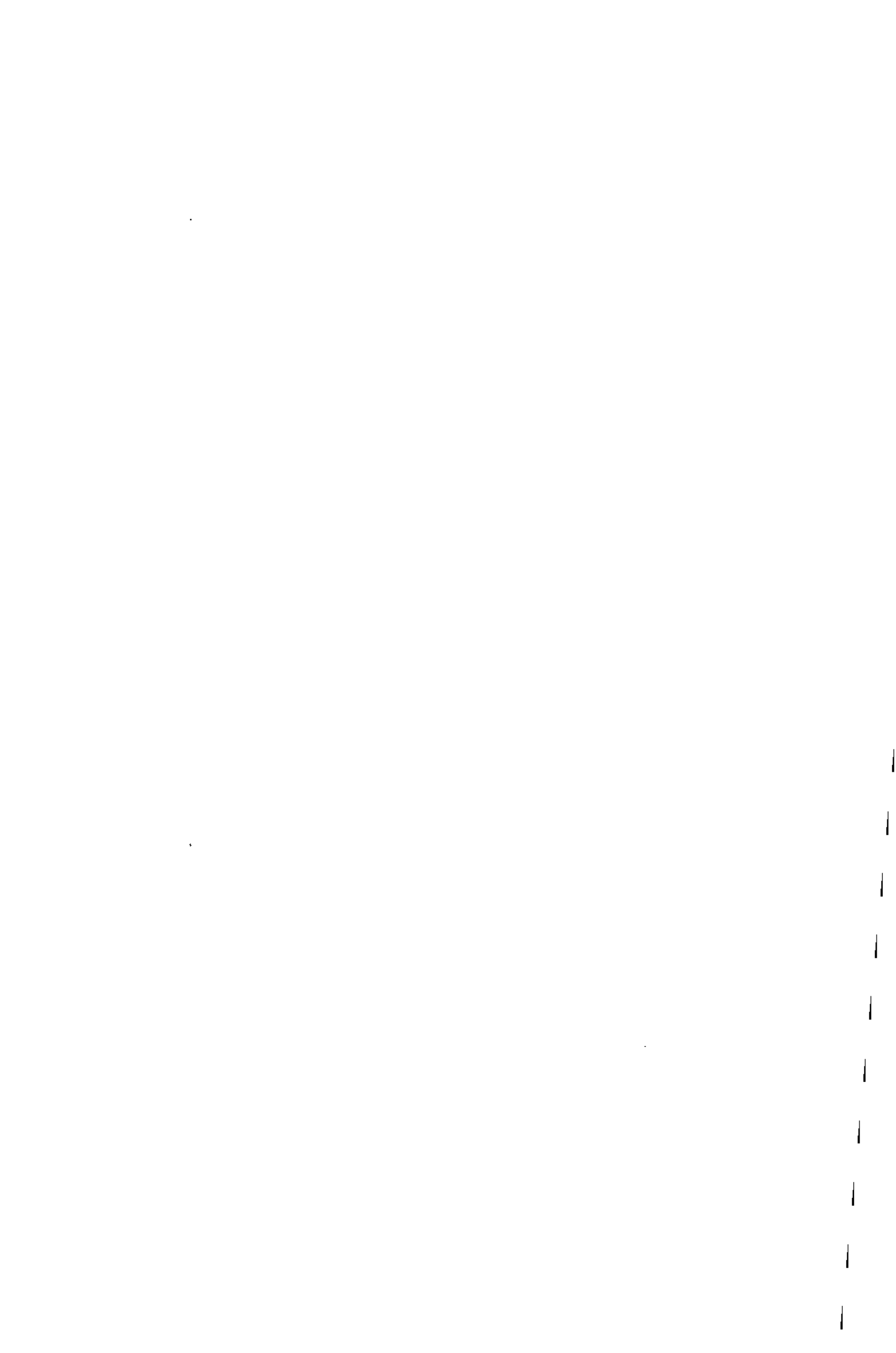

CHAPTER 8

PRODUCTION OF LEUKOTRIENES AND PROSTAGLANDINS BY HUMAN ASCITES CELLS

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8.1 ABSTRACT

Ascites was collected from six patients with liver cirrhosis and the cells isolated. These cells, mainly macrophages, were labelled with ^{14}C arachidonic acid and stimulated with the calcium ionophore A23187. The metabolites formed were separated by HPLC. The main substances formed by the ascites cells were leukotriene B_4, 5-hydroxy-6,8,11,14 eicosatetraenoic acid and leukotriene C_4. Smaller amounts of thromboxane B_2, 12-hydroxy-5,8,10 heptodecatrienoic acid and 6-keto-prostaglandin F_{1alpha} were isolated. Human peritoneal macrophages are therefore capable of producing leukotrienes and prostaglandins. Production of these substances might play a role in some of the complications of patients with liver cirrhosis and ascites.

8.2 INTRODUCTION

Eicosanoids (prostaglandins, thromboxanes and leukotrienes) are potent mediators which have been reported to be released by both rodent and human peritoneal and alveolar macrophages (1,2,3,4). The peritoneal macrophages used in the reported studies were obtained by washing the peritoneal cavity with exogenous fluids. Du and co-workers studied human peritoneal macrophages from patients undergoing chronic ambulatory peritoneal dialysis (3). In such patients concentrated glucose solutions are infused into the peritoneal cavity by means of an indwelling catheter. This procedure could conceivably influence macrophage function.

We were interested whether peritoneal macrophages present in ascitic fluid of patients with portal hypertension were also capable of producing leukotrienes and prostaglandins. We isolated peritoneal macrophages from six patients with ascites due to liver cirrhosis and determined the production of arachidonate metabolites by these cells by means of high pressure liquid chromatography (HPLC) and radioimmunoassay (RIA).

8.3 MATERIALS AND METHODS

8.3.1 Patients

Six patients with ascites were investigated. Four patients had an alcoholic liver cirrhosis, one patient a chronic active hepatitis and one patient sclerosing cholangitis complicated by a bacterial peritonitis caused by enterobacter and E. Coli. One liter of ascites

was collected from each patient under sterile conditions and centrifuged immediately. The cells were isolated by centrifugation, stained by May- Grünwald-Giemsma method and examined microscopically and found to comprise less than 5% granulocytes.

8.3.2 Materials and chemicals

Leukotrienes B_4, C_4, D_4 and E_4 were gifts of Dr. J. Rokach (Merck Frosst Canada Inc.), Ca ionophore A23187 was obtained from Hoechst (Calbiochem-Behring Inc. USA) prostaglandins D_2, E_2 and F_{2alpha} from Sigma Chem. Comp.(USA), 6-keto PGF_{1alpha} and thromboxane B_2 were gifts of Dr. J.B. Smith (Philadelphia, USA). $1\text{-}^{14}C$ arachidonic acid, 5-D-(5,6,8,9,11,12,14,15-^3H(n))-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE), 12-L-(5,6,8,9,11,12,14,15-^3H(n))-HETE and 15-L-(5,6,8,9,11, 12,14,15-^3H(n))-HETE were purchased from New England Nuclear. All other radiolabelled compounds mentioned below were obtained from the Radiochemical Centre of Amersham (U.K.): (5,6,8,9,11,12,14,15-^3H(n))-leukotriene B_4, (14,15-^3H(n))-leukotriene C_4, (14,15,^3H(n)) leukotriene D_4, 6 keto (5,8,9,11,12,14,15-^3H(n)) prostaglandin F_{1alpha} (5,6,8,9,11,12, 14,15-^3H(n)) thromboxane B_2, (5,6,8,11,12,14,15-^3H(n)) prostaglandin E_2 (5,6,8,11,12,14,15-^3H(n)), prostaglandin F_{2alpha}(5,6,8,9,12,14,15-^3H(n)) prostaglandin D_2. Antibodies to TXB_2, PGF_{2alpha} and PGE_2 were obtained from Institute Pasteur (Paris, France); anti-6-keto-PGF_{1alpha} was obtained from Seragen (Boston, USA).

8.3.3 Chromatographic system

RP-HPLC of leukotrienes and other lipoxygenase products were carried out on a Nucleosil 5 C18 column (5). The solvents system (A) was tetrahydrofuran/methanol/water/acetic acid (25/30/45/0.1), adjusted to pH 5.5 with ammoniumhydroxide. Mobile phases were filtered by vacuum

filtering through a millipore filter and degassed with helium (6,7). 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 in water solution, and rewashed with water (8). The column was equilibrated with the mobile phase (A) at an oventemperature of 37°C. Fractions were collected for scintillation counting. After each run (90 min) the column was rinsed for at least 60 minutes, because of contamination with Ca.ionophore and ^{14}C-AA which run respectively after 115 and 140 min. RP-HPLC of prostaglandins was performed on a Zorbax C8 column. This solvent system (B) contained acetonitrile/benzene/water/acetic acid (24/0.2/0.1/76). The flow rate of this eluent was 2.0 ml/min. Fractions were collected for scintillation counting. The column was rinsed with acetonitrile for 30 min after each sample to elute the lipoxygenase products (5).

8.3.4 Methods

Cells were spun down in 500 ml flasks for 10 min. at 800 x g. The pellets were combined in a polypropylene 50 ml tube (Falcon^{R}) and centrifuged for 5 min. at 250 x g. The supernatant was decanted, and the pellet washed with 20 ml of distilled water during 10 seconds and 2 ml NaCl solution (18.9%, w/v) added. The cells were spun down again (5 min. at 250 x g) and washed until the erythrocytes were removed.

The pellet, containing white cells, was suspended in 10 ml of Krebs-Henseleit buffer. A sample was taken for counting and differentiation of the cells. Cell differentiations, based on morphology, resulted in 70-80% macrophages, maximally 15% lymphocytes

and less than 5% granulocytes. Attempts to purify the macrophages population further resulted in increasing numbers of non-viable cells. 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, 5 uCi (1-^{14}C) arachidonic acid (55 mCi/mmol) was added, glutathione (final conc. 2 mmol/l) and Ca-ionophore A23187 (1 umol/l). At the end of the 10 min. incubation, ^{3}H-LTs and ^{3}H-GPs were added and the homogenate spun down (10 min., 1400 x g, 4°C). The pellet was washed once, and the combined supernatants were then applied to a Seppak C18 cartridge and the effluent was placed on a Seppak silica cartridge. (The C18 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 (8). The sample was eluted with 2.5 ml 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, filtered and kept in a siliconized HPLC micro vial. Volumes of 200 ul were injected on the column and chromatographed using a 1082B high performance liquid chromatograph (Hewlett Packard). Fractions were collected with a Superrac fractioncollector (LKB Sweden) and counted in a 3255 Tricarb liquid scintillation counter (Packard, Brussels, Belgium (5).

8.3.5 Quantitative evaluation

The settings for double labelled scintillation counting were such that there was no spillover of radioactivity of ^{3}H into the ^{14}C channel. Dpm calculations were carried out, using quenched standard sets. 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. Radioimmunoassay was used to confirm the identity of eicosanoid peaks in high performance liquid chromatograms.

8.4 RESULTS

Figure 1 shows a representative chromatogram (patient I) of LTs and other lipoxygenase products, after ^{14}C-arachidonic acid labelling of the cells. The upper part of the figure represents the mass, measured by absorption at 280 nm. Only LTC_4-like and LTB_4-like compounds are present in detectable amounts. The curve in between gives plotted ^{14}C-labelled fractions. Prostaglandins cochromatograph with LTC_4, so that a not unimportant part of this peak is due to the presence of cyclooxygenase products. HHT was characterized by means of experiments with platelets in which predominantly TXB_2, HHT and 12-HETE is synthesized (9).

The chromatogram given at the bottom of figure 1 shows the added ^3H-labelled substances. In table 1 the recoveries are listed. Data in the figures are not corrected for these recoveries. In figure 2 chromatograms of prostaglandins, obtained from the same sample as mentioned in figure 1 are represented. In this case only the labelled compounds are shown. The upper part gives the formation of 6-keto-prostaglandin F_{1alpha} and thromboxane B_2, the lower part the markers added to the mixture. ^3H-labelled compounds are shifted to the left. The longer the retention time, the greater the delay between the different labelled compounds of the same substance. Probably labelling of 4 double bounds with ^3H makes these substances more hydrofilic than

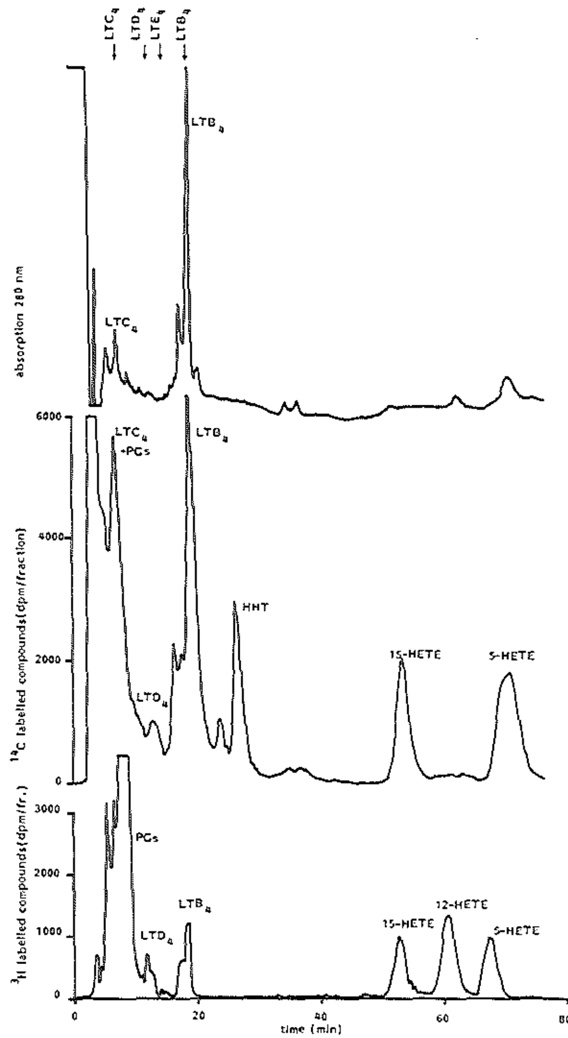

Fig.1. Chromatograms of lipoxygenase products, synthesized by human ascites cells (patient Ia), after ^{14}C arachidonic acid loading, in the presence of glutathion (2mmol/l) and Ca-ionophore A23187 (1 umol/l). Top: absorption (arrows indicate retention times of synthetic LTs); middle: ^{14}C-labeled metabolites; bottom: ^{3}H-labeled standards.

Table 1. 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_{1alpha}	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

these labelled with ^{14}C at the first C-atom (5).

In tables 2 and 3 the amounts of labelled AA metabolites are given as percentages of total formed metabolites. Table 2 lists the lipoxygenase and table 3 the cyclooxygenase products. By measuring the absorption of the LT at 280 nm, the ratio LTC_4/LTB_4 was determined. The amount of radioactivity of LTC_4 was calculated with this ratio from that of LTB_4 (5). Amounts are expressed as percentages of total formed metabolites synthesized per 10^8 viable cells.

Table 2. LIPOXYGENASE PRODUCTS BY STIMULATED ASCITES CELLS OF SIX PATIENTS WITH LIVER CIRRHOSIS.

	mean ± SD	individual patients					
		I	II	III	IV	V	VI
number of cells x 10^8		0.54	1.45	2.41	0.39	0.50	1.04
% viability		91	89	93	98	87	46
LTB_4	31.5 ± 8.3*	40.8*	24	43	24.4	29	27.5
LTC_4	6.3 ± 3.1	4	9	6.4	9	8.1	1.3
LTD_4	1.7 ± 1.8	0.6	1	1.4	5.3	1.0	0.6
5-HETE	30 ± 16.1	22.5	25	31	34	9.6	58
12-HETE	2.3 ± 2.9	0.3	1.5	8	2.2	1.0	0.9
15-HETE	5.5 ± 4.3	8.4	3.2	1.1	12.5	5.3	2.2

* results expressed as % of total formed metabolites.

Table 3. CYCLOOXYGENASE PRODUCTS BY STIMULATED ASCITES CELLS OF SIX PATIENTS WITH LIVER CIRRHOSIS.

	mean ± SD	individual patients					
		I	II	III	IV	V	VI
number of cells x 10^8		0.54	1.45	2.41	0.39	0.50	1.04
% viability		91	89	93	98	87	46
TXB_2	6.0 ± 4.6*	8*	11	2.5	2.1	11	1.1
6-keto-PGF_{1alpha}	4.1 ± 2.8	3.5	3.7	1.9	4.1	9.5	2
HHT	9 ± 7.8	9.6	18	2.8	2.9	19	1.9
PGF_2	1.7 ± 0.9	1.3	1.4	0.6	1.6	3.4	1.9
PGE_2	0.8 ± 0.3	0.5	0.9	0.3	1	1.1	1
PGD_2	1.0 ± 0.5	0.4	1.1	0.5	0.9	1.4	1.5

* results expressed as % of total formed metabolites.

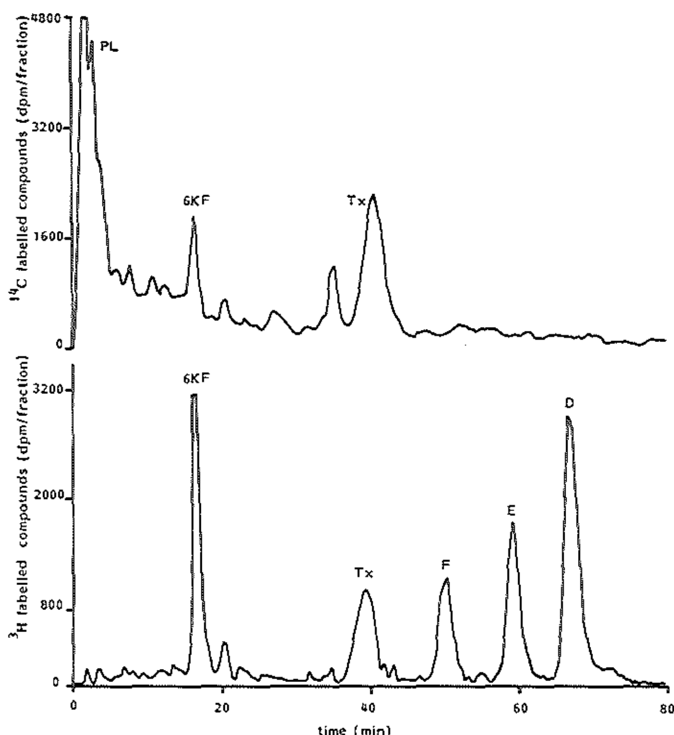

Fig.2. HPLC separation of prostaglandins in the same sample as in Fig.1. 6KF = 6-keto PGF_{1alpha}, Tx = TxB_2, F = PGF_{2alpha}, E = PGE_2, D = PGD_2.

8.5 DISCUSSION

In this study of leukotriene and prostaglandin production by ascites cells, incubated with arachidonic acid and calcium ionophore, 77.5% of the arachidonic acid was converted to lipoxygenase products and 22.5% to cyclooxygenase products. The main constituents of the lipoxygenase pathway were LTB_4, 5-HETE and to a lesser degree LTC_4 and LTD_4. The

main cyclooxygenase products were TXB_2, HHT and 6-keto-PGF_{1alpha}. Human alveolar macrophages have been reported to produce mainly LTB_4 (4). Peritoneal macrophages derived from dialysate bags mainly produce LTB_4 and LTC_4 (3). However the ascitic fluid in these latter patients is artificial and contains high glucose concentrations. The ascites cells used in our study were derived from a less artificial environment. The in vivo effects of LTB_4 are increased vascular permeability, enhanced granulocyte sticking and emigration, resulting in peripheral neutropenia and leukocyte accumulation (10). In humans LTB_4 has been found in the synovial fluid of patients with rheumatoid arthritis (11), gouty effusions (12), psoriatic skin (13), inflammed bowel mucosa (14) and in sputum from patients with cystic fibrosis (15). 5-HETE has a weak LTB_4-like activity in vivo (10). LTC_4 and LTD_4 can contract smooth muscles, causing bronchoconstriction, constriction of the large arteries and the coronary arteries, and also cause dilatation of the microcirculatory vessels and extravasation of macromolecules from human capillaries (16). In patients 1 and 2 a LeVeen peritoneal jugular shunt (17) was inserted subsequently to treat the ascites. In both patients the shunts occluded. One possible explanation is that LTB_4 production by ascites cells causes cell aggregation and clotting.

The role of prostaglandins and thromboxanes in the pathophysiology of liver failure has been subject of increasing interest during the recent years. There is circumstancial evidence that prostaglandin E_2 (PGE_2) helps maintain the renal blood flow and function in cirrhosis by counteracting the effects of vasoconstrictive substances (18). Plasma and urinary thromboxane B_2 (TXB_2) levels have been reported to be raised in alcoholic liver disease and in the hepatorenal syndrome

(19,20). TXB_2 is a stable metabolite of thromboxane A_2 (TXA_2), a potent vasoconstrictor and potent aggregant and it is therefore possible that TXA_2 is involved in the pathogenesis of some of the complications of cirrhosis such as the hepatorenal syndrome.

Our finding that peritoneal macrophages in liver cirrhosis are capable of producing leukotrienes suggests that the role played by the lipoxygenase products of arachidonate may be a fruitfull field for further study in liver disease.

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CHAPTER 9

COMPARISON OF THE PRODUCTION OF EICOSANOIDS BY HUMAN AND RAT PERITONEAL MACROPHAGES AND RAT KUPFFER CELLS.

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9.1 ABSTRACT

Human and rat peritoneal macrophages and rat Kupffer cells were labelled with ^{14}C arachidonic acid and stimulated with the calcium ionophore A23187. The metabolites formed were separated by high pressure liquid chromatography (HPLC). Human peritoneal macrophages formed especially leukotriene B_4, 5-hydroxy-6,8,11,14 eicosatetraenoic acid and small amounts of leukotriene C_4 and thromboxane B_2, 12-hydroxy-5,8,10 heptadecatrienoic acid and 6 keto-prostaglandin F_{1alpha}, whereas rat peritoneal macrophages mainly produced cyclooxygenase products and in particular thromboxane B_2 and 12-hydroxy-5,8,10 heptadecatrienoic acid. Rat Kupffer cells synthesized mainly cyclooxygenase products such as prostaglandin F_{2alpha}, prostaglandin D_2 and prostaglandin E_2. These results indicate that the profile of eicosanoids production by macrophages is dependent both on the species and on the tissue from which the macrophage is derived.

9.2 INTRODUCTION

It has been reported that macrophages of different origin can synthesize both prostaglandins (PGS) and leukotrienes (LTS), when activated by several substances (1,2,3). We recently investigated the formation of these substances in cells obtained from the ascitic fluid of patients with liver cirrhosis. The isolated cells, mainly macrophages, were labelled with ^{14}C arachidonic acid (AA) and stimulated with Ca ionophore A23187. In these experiments, AA was mainly converted to lipoxygenase products (77%), of which leukotriene B_4 (LTB_4) and 5-hydroxy-6,8,11,14 eicosatetraenoic acid (5-HETE) were present in the highest amounts. The most important cyclooxygenase products were 6-keto-PGF_{1alpha} and thromboxane B_2 (TXB_2) (3).

We were interested if there were species and tissue differences in the production of eicosanoids. For this reason we compared the eicosanoids produced by human ascites cells, rat peritoneal macrophages and rat Kupffer cells.

9.3 PATIENTS AND METHODS

9.3.1 Patients. Nine patients with ascites were investigated. Seven patients had an alcoholic liver cirrhosis, one patient a chronic active hepatitis and one patient a sclerosing cholangitis complicated by a bacterial peritonitis caused by Enterobacter and E. Coli. From each patient two liters of ascites were collected under sterile conditions in pyrogen free disposable plastic bags and

centrifuged immediately. After resuspending the cells, erythrocytes were lysed 2 or 3 times; thereafter the cells were washed once with Krebs buffer and finally taken in 10 ml of Krebs buffer. A sample was taken for cell differentiation and stained by May-Grünwald-Giemsa method and examined microscopically as described earlier (3) and found to consist of 70-80% macrophages, maximally 15% lymphocytes and less than 5-15% granulocytes.

9.3.2 Rat Kupffer cells and peritoneal macrophages. Kupffer cells (liver macrophages) and peritoneal macrophages were obtained from 3-month-old female BN/BiRij rats weighing 135-150 g. Sinusoidal liver cells were isolated by perfusion and incubation of rat liver tissue with pronase and collagenase, as described earlier. The crude sinusoidal liver cell suspension was freed from erythrocytes and cell debris by density centrifugation in a single layer Nycodenz gradient (4). Purified Kupffer cells were obtained by centrifugal elutration of the sinusoidal liver cells (5). The Kupffer cells were suspended in 10-15 ml Dulbecco's modification of Eagles (DME) medium containing 20% (v/v) new born calf serum, penicillin (100 units/ml) and streptomycin (100 ug/ml). The cells were then incubated at 37°C under 95% O_2-5% CO_2 in a sterile 50 ml Falcon tube for 24hours, to allow the cells to recover from the isolation procedure. The cells were harvested by centrifugation, washed and resuspended in ice-cold DME medium, before use.

Peritoneal macrophages were obtained from animals that had been injected with either sterile thioglycollate (Merck) 4 days previously or 10 ml sterile physiological saline 1 day previously. The cells were harvested by a single peritoneal lavage with 50 ml

physiological saline.

9.3.3 Materials. Leukotrienes B_4, C_4, D_4 and E_4 were gifts of Dr. J. Rokach (Merck Frosst Canada Inc.), Ca ionophore A23187 was obtained from Hoechst (Calbiochem-Behring Inc. USA), prostaglandins D_2, E_2 and F_{2alhpa}, 6-keto PGF_{1alpha} and thromboxane B_2 from Sigma Chem. Comp. (USA), (1-^{14}C)arachidonic acid, 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 of Amersham (U.K.): (5,6,8,9,11,12,14,15-^{3}H(n))-leukotriene B_4, (14,15-^{3}H(n))-leukotriene C_4, (14,15-^{3}H(n))-leukotriene D_4, 6-keto-(5,8,9,11,12,14,15 -^{3}H(n)) prostaglandin F_{1alpha}, (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_{2alpha},(5,6,8,9,12,14,15-^{3}H(n)) prostaglandinD_2. Antibodies to TXB_2, PGF_{2alpha} and PGE_2 were obtained from Institut Pasteur (Paris, France); anti-6-keto-PGF_{1alpha} was obtained from Seragen (Boston, USA).

Sep-Pak C_{18} and silica cartridges and HPLC filters HA (0.45 um), FH (0.5 um) and Millex (0.45 uM) were obtained from Waters/Millipore (The Netherlands).

Prepacked HPLC columns Nucleosil $5C_{18}$ and Zorbax C_8 were obtained from Chrompack (Middelburg, The Netherlands).

9.3.4 Chromatographic system. Reversed phase-HPLC (RP-HPLC) of leukotrienes and other lipoxygenase products was carried out on a Nucleosil 5 C_{18} column (7). The solvent system (A) was: tetrahydrofuran/methanol/0.1% (w/v) EDTA solution in water/acetic

acid (25/30/45/0.1), adjusted to pH 5.5 with ammoniumhydroxide (7,8). Mobile phases were filtered by vacuum filtering through a millipore filter and degassed with helium. The flow rate was 0.9 ml/min and the absorption was measured at 280 nm. 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 (80 min) the column was rinsed with methanol for at least 30 minutes, because of contamination with Ca. ionophore and ^{14}C-AA which elute after 115 and 140 min respectively in system A. RP-HPLC of prostaglandins was performed on a Zorbax C_8 column. The solvent system (B) contained acetonitrile/benzene/water/acetic acid (24/0.2/76/0.1). The flow rate of this eluent was 2 ml/min. Fractions were collected for scintillation counting. The column was rinsed with acetonitrile for 30 min after each sample to elute the lipoxygenase products (7).

9.4 METHODS

The pellets of human peritoneal macrophages, rat peritoneal macrophages and rat Kupffer cells were suspended in 10 ml Krebs-Henseleit buffer. The tubes were placed in a water bath of 37°C. The samples were continuously gassed through a thin pipette with a mixture of 95% O_2 and 5% CO_2. Firstly, 2.5 uCi (1-^{14}C) arachidonic acid (55 mCi/mol), thereafter, gluthathione (final concentration (2 mmol/l)) and calcium ionophore A23187 (10 umol/l) were added. At the end of the 10 min incubation, serine (5 mmol/l), cysteine (10 mmol/l), ^{3}H-LTs and ^{3}H-PGs were added and the

homogenates spun down (10 min, 1400 x g, 4°C). Serine and cysteine were added to prevent breakdown of respectively LTC_4 to D_4 and D_4 to E_4. The pellets were washed once, and the combined supernatants of each cell type were then applied to a couple of Seppak C_{18} and silica cartridges. (The cartridges were prewashed with 10 ml of ethanol and 10 ml of distilled water (8)). The samples were eluted with 5 ml ethanol on each column; these eluates were combined and evaporated to dryness with a gentle stream of nitrogen at 37°C. Thereafter, the dried samples were dissolved in 0.5 ml of solvent A, centrifuged and purified by a Millex-filter and kept in a HPLC micro vial (Weichmann, Switzerland). Volumes of 100 ul were injected on the column and chromatographed using a 1082B HPL chromatograph (Hewlett Packard). Fractions were collected with a Superrac fraction collector (LKB Sweden) and counted in a 3255 Tricarb liquid scintillation counter (Packard, Brussels, Belgium).

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. Dpm calculations were carried out, using quenched standard sets. 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.

9.5 RESULTS

Figure I shows a representative chromatogram of prostaglandins produced from ^{14}C-labelled arachidonic acid. The upper one

represents the rat ascites cells (70% macrophages) and the lower part the rat Kupffer cells. ^{3}H labelled prostaglandins were used as references.

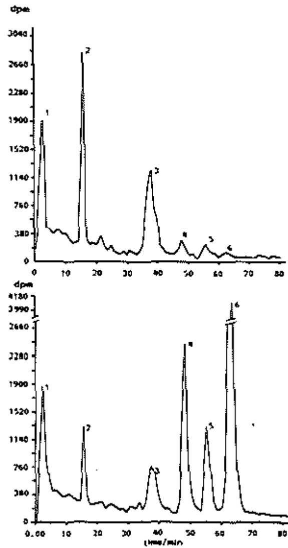

Fig.I: RP-HPLC chromatograms of prostaglandins produced from ^{14}C arachidonic acid by rat Kupffer cells (upper) and ascites cells (70% macrophages) (at the bottom).
Identification of the peaks: 1: front, 2: 6-keto-PGF_{1alpha}, 3: TXB_2, 4: PGF_{2alpha}, 5: PGE_2, 6: PGD_2.

Figure II shows a RP-HPLC chromatogram of leukotrienes produced from ^{14}C-labelled arachidonic acid by human ascites cells (70% macrophages). The upper curve shows the absorption of leukotrienes at 280 nm. Synthetic LTC_4, LTD_4, LTE_4 and LTB_4 were used as

references. The chromatogram at the bottom shows the radioactivity of all compounds. ^{3}H labelled leukotrienes and HETES were used as references.

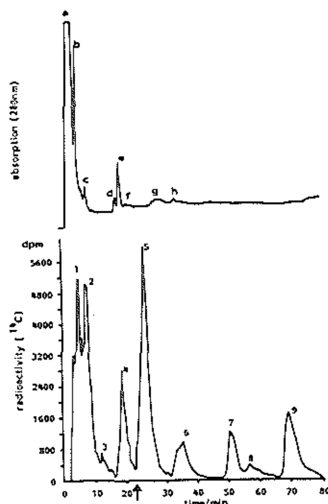

Fig.II.: RP-HPLC chromatograms of leukotrienes produced from ^{14}C arachidonic acid by ascites cells (70% macrophages). The upper one shows the absorption of the leukotrienes at 280 nm. The chromatogram at the bottom shows the radioactivity of all the ^{14}C-labelled compounds.

Identification of the peaks: a: front, b: -oxidation products of LTB_4, c: LTC_4, d: epi, 6 t-LTB_4 and 6 t-LTB_4, e: LTB_4, f: 12 epi, 6 t, 8 c-LTB_4, g/h: di-HETE's.

1: front, 2: PGs and LTC_4, 3: LTD_4, 4: LTB_4 and LTB_4-like substances (see d and f), 5/6: di-HETE's, 7: 15-HETE, 8: 12-HETE, 9: 5-HETE.

In figure III and IV the percentage (mean ± SD) of the total formed metabolites of respectively the cyclooxygenase and lipoxygenase products of the human, rat peritoneal macrophages and rat Kupffer cells are shown as block diagrams. The rat peritoneal macrophages stimulated with either saline or thioglycollate did not show significant differences of eicosanoids production. The rat Kupffer cells produced small amounts of LTC_4.

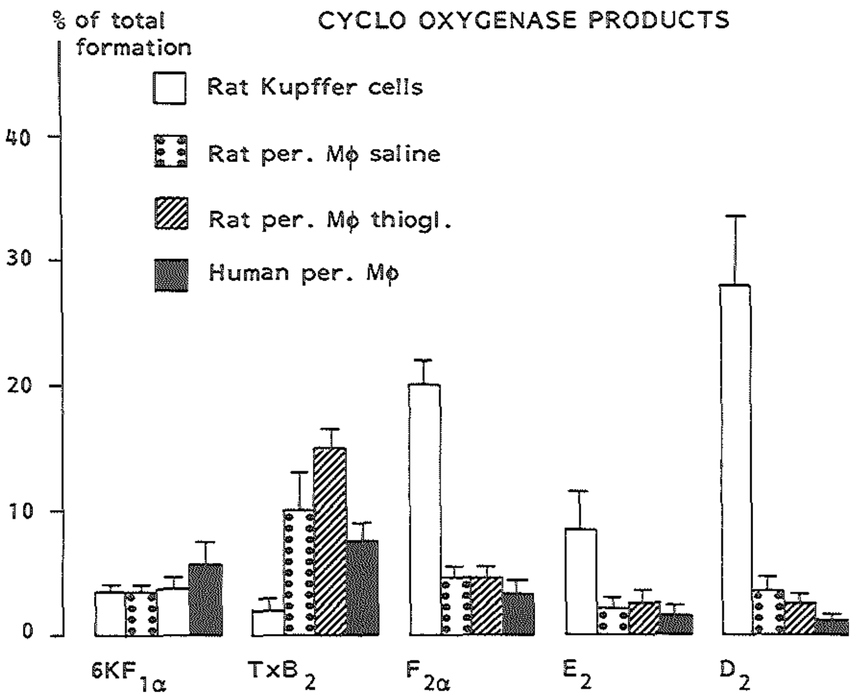

Fig. III: Graph of total formed cyclooxygenase products expressed as percentages (mean ± SEM) of rat Kupffer cells, rat peritoneal macrophages after stimulation with saline or thioglycollate and human peritoneal macrophages.

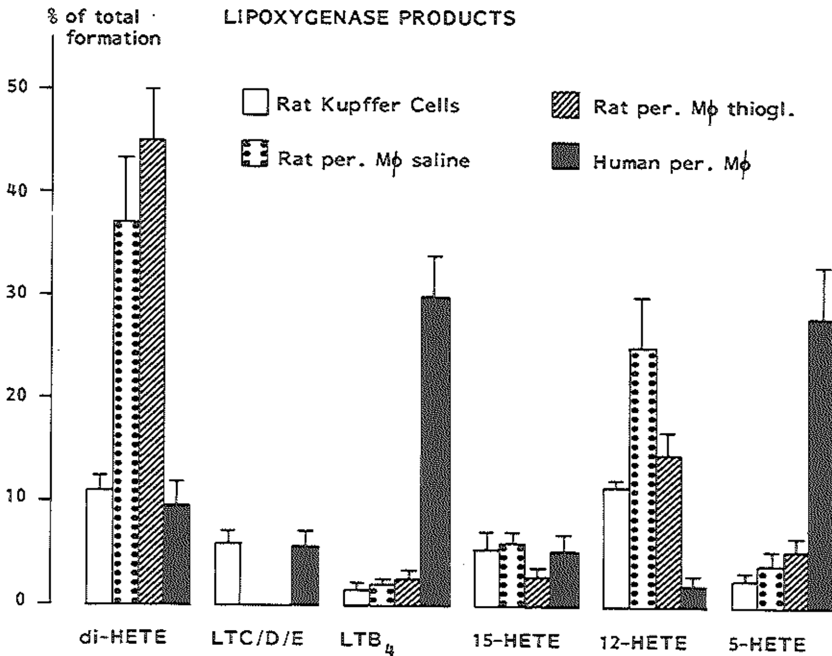

Fig. IV: Graph of total formed lipoxygenase products as percentages (mean ± SEM) of rat Kupffer cells, rat peritoneal macrophages after stimulation with saline or thioglycollate and human peritoneal macrophages.

9.6 DISCUSSION

The results presented here indicate that the capacity of the three different types of macrophages used in this study to produce eicosanoids is dependent both on the species and on the tissue from which the cells were derived. The human peritoneal macrophages, rat

peritoneal macrophages after saline and thioglycollate and rat Kupffer cells converted arachidonic acid respectively for 29%, 61%, 74% and 73% to cyclooxygenase products and for 71%, 37%, 25% and 27% to lipoxygenase products.

Besides the differences of these three cell-types in the total lipoxygenase and cyclooxygenase products, the individual eicosanoids also show great differences. Human peritoneal macrophages mainly synthesize LTB_4, 5-HETE and to a lesser degree LTC_4 and LTD_4. The main cyclooxygenase products are TXB_2 and 6-keto PGF_{1alpha} and only trace amounts of prostaglandin E_2 (PGE_2), prostaglandin F_{2alpha} (PGF_{2alpha}) and prostaglandin D_2 (PGD_2) were formed.

Human peritoneal macrophages derived from dialysate bags of patients undergoing continuous ambulatory peritoneal dialysis, synthesize almost the same pattern of eicosanoids as those described in this study (1,2).

In contrast to the human peritoneal macrophages, rat peritoneal macrophages converted arachidonic acid almost exclusively to TXB_2 and in minor degree to 6-keto PGF_{1alpha} and PGF_{2alpha}; only small amounts of leukotrienes being formed. The high TXB_2 production of these cells is in agreement with earlier work (9).

The individual eicosanoids produced by Kupffer cells are totally different from those by rat peritoneal macrophages. Kupffer cells mainly synthesize PGF_{2alpha}, PGD_2, PGE_2 and only trace amounts of TXB_2 and 6-keto PGF_{1alpha} and leukotrienes.

Macrophages play a central role in inflammation, being both producers and targets of inflammatory mediators. It is therefore not surprising that much work has been performed on macrophage function

in experimental animals.

The capacity of the three different types of macrophages used in our study to produce eicosanoids is dependent on species and on the site from with the macrophage is derived. Another study reported different responses of rabbit hepatic versus alveolar macrophages to endotoxins (10). Studies in experimental animals therefore cannot always be extrapolated to sick humans. The species and tissue difference of eicosanoids production might influence the reaction to different stimulations of these cells.

Rats are much less sensitive to endotoxins than guinea pigs or humans. This has been attributed to the number of Kupffer cells in the liver, as there is a correlation between increasing number of Kupffer cells and increasing sensitivity to endotoxins (11). However species differences in the pattern of eicosanoids synthesized may also play a role in differing reactions to endotoxins.

It is possible that varying profiles of eicosanoids may be produced by human peritoneal macrophages in different diseases. For example a cyclic variation in number and activity of the peritoneal macrophages has been found in fluid obtained from the peritoneal cavity of healthy women at laparoscopy (12). Although peritoneal macrophages from patients undergoing continuous ambulatory peritoneal dialysis produce almost the same eicosanoids as the peritoneal macrophages derived from patients with ascites due to liver disease (3,13), recently differences between peritoneal macrophages derived from healthy volunteers and patients on CAPD have been described (14).

Further studies on the influence of tissue of origin and disease status on eicosanoid production by macrophages in humans are needed.

9.7 REFERENCES

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CHAPTER 10

DISCUSSION

In chapter 1 the aims of the study are formulated. This chapter contains a discussion about the results outlined in chapter 4 to 9 in the light of these goals. Eicosanoids (prostaglandins and leukotrienes) are potent mediators and play a role in several diseases (1,3,56). The most important precursor of the eicosanoids is arachidonic acid (3,24,29).
Administration of endotoxins results in raised levels of several prostaglandins such as PGE_2, PGF_{2alpha}, TXB_2 and 6-keto-PGF_{1alpha} (168,169,243). The main substance is TXB_2, which is a potent platelet aggregator and vasoconstrictor (3,56). Several NSAIDs, such as indomethacin, salicylate and ibuprofen improve hemodynamics and survival in experimental endotoxin shock (240,241,243). As endotoxemia occurs in liver disease, it is conceivable that several of the manifestations of decompensated liver disease are due to endotoxins and are mediated by eicosanoids (192,193,195,204).
The first two questions to be answered were whether thromboxane levels are raised in liver disease and whether this correlate with the severity of liver disease.
In twelve out of sixteen patients with alcoholic liver cirrhosis we found raised plasma TXB_2 levels, as measured by RIA, on one or more occasions. Raised plasma TXB_2 levels were associated with a disturbed renal function as measured by the serum urea level, a disturbed liver

function as measured by raised alkaline phosphatase and gamma glutamyl transpeptidase levels and disturbed clotting mechanism as measured by lower antiplasmin and antithrombin III levels, compared to the values obtained by the same twelve patients at times when plasma TXB_2 levels were normal. There were no significant differences in platelet count, leucocyte count and fibrinogen levels between the periods of raised and normal plasma TXB_2 levels. In the four patients with repeatedly normal plasma TXB_2 levels the mean platelet count, mean renal function and liver function tests and mean clotting factors were less abnormal than in the patients with raised plasma TXB_2 levels. Two patients with raised plasma TXB_2 levels died. The mean duration of hospitalisation in the group with raised plasma TXB_2 levels was 33 days compared to 16 days in the group with normal plasma TXB_2 levels.

To unravel questions 3 and 4, namely whether raised plasma thromboxane levels are involved in the hemodynamic changes and to investigate the site of TXB_2 production and elimination in liver disease, a catheterisation study was performed. In four out of five patients with liver cirrhosis we found a net TXB_2 production by the splanchnic area and in all patients there was a net TXB_2 removal by the lungs and kidneys. However the administration of dazoxiben, a thromboxane synthetase blocker, although reducing plasma TXB_2 to normal levels did not result in changes in systemic and hepatic pressures or hepatic blood flow.

These two studies show that raised plasma TXB_2 levels are present in patients with liver disease. These findings are further sustained by the findings of Zipser who found raised levels of urinary TXB_2 in the hepatorenal syndrome (115). The lack of circulatory changes following a

decrease in plasma TXB_2 levels suggest that the raised plasma TXB_2 levels in cirrhotics might be a secondary phenomenon or that the effects of chronically raised TXB_2 levels might not be easily reversible. In pulmonary hypertension it has been postulated that secondary changes in the vessels might be the reason that many therapeutic trials with various drugs are not succesfull (253,254). This might also be the explanation for the failure of dazoxiben to lower portal pressure. Prolonged treatment with dazoxiben in early portal hypertension needs to be studied to unravel the role of thromboxane in portal hypertension.

Based on these findings it would seem reasonable to suggest a trial with NSAIDs to lower the production of TXB_2 in liver cirrhosis. However the administration of such drugs have been shown to result in renal failure and to give rise to bleeding problems in these patients (174,175,176,255). NSAIDs block the whole cyclooxygenase pathway, including the production of PGE_2 and PGF_{2alpha}. In contrast to TXB_2, PGE_2 seems to play a supportive role in maintaining renal function in cirrhotics (174,176,177). The postulated beneficial effect of some prostaglandins on renal blood flow in cirrhotics, provides an explanation for the deterioration of renal function after NSAID administration.

Although we tentatively conclude that some of the complications of cirrhosis may be mediated by thromboxanes, the clinical experience with NSAIDs suggests that some of the other cyclooxygenase products have a beneficial function in these patients.

As we postulated that the raised plasma TXB_2 levels were mediated by endotoxins in cirrhotics we were interested if raised endotoxin levels

were correlated with raised TXB_2 levels.

In six patients with gram negative septicemia proved by positive blood cultures and in twelve patients with liver cirrhosis we measured endotoxin levels by a Limulus lysate endotoxin assay with the aid of a chromogenic substrate and plasma TXB_2 levels on one or more occasions (202,203).

In eight of the twelve patients with liver cirrhosis and in three of the six patients with sepsis we found endotoxin levels above the 95% confidence limit for normals of 12 pg/ml. However the variability of the standard curves of the endotoxin assay and the lack of sensitivity of the assay were considerable.

In agreement with earlier work we found raised plasma TXB_2 levels in patients with liver cirrhosis and patients with sepsis (166,170,178). There was however no correlation between endotoxin and TXB_2 levels.

This might be due to the failure of the limulus assay to detect endotoxemia even at levels giving symptoms, as was the case in the septic patients, due to a lack of sensitivity of the assay, or to time related factors. More studies need to be done to unravel the role of endotoxins with specific assays to detect endotoxemia in patients.

Monoclonal antibodies to lipid A, which is remarkably identical for a broad spectrum of gram-negative bacteria and which is responsible for most of the biological effects of endotoxins, might be helpfull in the future.

We tried to obtain antibodies to lipid A by immunizing rabbits with lipid A-coated bacteria as described by others (256,257). The presence of anti lipid A antibodies was detected by a passive hemolysis test.

We could detect high levels of anti lipid A antibodies in the serum of

the rabbits, however further testing revealed some cross reactions with whole endotoxins which meant that the anti-lipid-A antibodies were not suitable for clinical research. Others have encountered similar problems in attempting to develop an antibody against lipid A (A. Brouwer, J.P. Nolan, personal communication). In the future more specific antibodies to lipid A might help unravel the role of endotoxins in several diseases.

Endotoxemia in liver disease could be due to decreased removal from the circulation by the RES or to increased absorption from the gut. Question six was the possible contribution of portal hypertension and ethanol on the permeability of the intestine to endotoxins. Neither portal hypertension nor ethanol in low and high concentrations increased the permeability of the intestine to endotoxins in rats. These findings do not exclude the possibility of increased endotoxin absorption in patients with liver cirrhosis in whom several potentially damaging factors, such as portal hypertension and relative intestinal ischemia, protein or phosphate deficiency and chronic alcohol consumption may be present simultaneously.

Another possibility we considered was that alcoholism could lead to bacterial overgrowth of the small intestine, with concomittant mucosal damage and increased endotoxin absorption. We studied seven patients with alcoholic cirrhosis with the aid of a breath test, detecting bile acid deconjugation by intestinal bacteria (232,233). In five out of seven patients we found a late peak after 4 hours. As alcoholics have a diminished gastrointestinal motility this peak might be due to bacterial overgrowth in these patients. Bode et al found significant more anaerobic and aerobic bacteria in the jejunum of patients with

alcoholic liver disease. Bacterial overgrowth of the small intestine, which has a much larger absorptive surface than the colon and the integrity of which is damaged by bacterial overgrowth, might therefore contribute to higher levels of circulating endotoxins in these patients (229).

The next question was to investigate the production of eicosanoids by human peritoneal macrophages derived from patients with liver cirrhosis, as little was known about eicosanoid production by macrophages in this disease. The isolated ascites cells (mainly macrophages) of six patients with cirrhosis produce mainly lipoxygenase products, such as LTB_4, 5-HETE and LTC_4 and smaller amounts of the cyclooxygenase pathway, namely TXB_2, HHT and 6-keto-PGF_{1alpha}. Thus human peritoneal macrophages, when stimulated are capable of producing leukotrienes. In view of the similarity between leukotriene effects in animals and a number of manifestations of alcoholic liver cirrhosis, further studies on the role of LTs in liver disease would be of great interest. Such studies are, however, as yet not possible due to the problems inherent in leukotriene detection.

Endotoxin administration has diverging effects in different species and the effects are also influenced by the route of administration (e.g. intravenous v.s. intraportal).

To investigate the possible species and tissue dependency of the ability of macrophages to produce eicosanoids, we compared the production of eicosanoids by human ascites cells, rat ascites and rat Kupffer cells. In contrast to the human peritoneal macrophages rat peritoneal macrophages converted arachidonic acid mainly to COP and especially TXB_2 whereas rat Kupffer cells converted arachidonic acid to

COP but especially to PGF_{2alpha}, PGD_2, and PGE_2 and only trace amounts of TXB_2. These two studies with macrophages show us that macrophages can produce eicosanoids but that the type of eicosanoid produced is dependent both on the tissue and on the species used.

The possibility that endotoxin effects are in part mediated by leukotrienes, and the similarity of many leukotriene effects to changes seen in especially alcoholic liver disease opens interesting perspectives for further research on the interaction between eicosanoids in liver disease.

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SUMMARY

Endotoxins are cell wall lipopolysaccharides of gram negative bacteria. In the normal state they are absorbed from the bowel and removed and detoxified by the Kupffer cells. Systemic endotoxemia is found in liver disease and is at least in part due to a diminished reticuloendothelial system (RES) function. Endotoxemia results in disorders of the clotting mechanism, renal dysfunction and a high mortality rate. In animals, endotoxins induce the liberation of eicosanoids. Eicosanoids is the generic name of prostaglandins (PGs) and leukotrienes (LTs). Endotoxin administration to experimental animals results in raised levels of thromboxane B_2 (TXB_2), PGE_2, PGF_{2alpha} and 6 keto PGF_{1alpha}. Pretreatment of the animals with selective thromboxane synthetase- or prostaglandin blockers improved survival after endotoxin administration. The aims of the investigation described in this thesis were to determine if raised plasma TXB_2 levels were to be found in these patients with liver disease and if they could be correlated with the complications.

In addition a study was performed to investigate the ability of human macrophages, derived from patients with liver disease, to synthesize eicosanoids. In view of the marked differences in sensitivity to endotoxins between species and the postulated importance of the Kupffer cell for removal of endotoxins on the species and site dependence of eicosanoid production by macrophages was investigated.

In chapter 2 a review of the literature on eicosanoids is given. It deals with the precursors, such as linoleic acid, gamma linolenic acid and arachidonic acid, of the eicosanoids, the cyclooxygenase pathway, which converts arachidonic acid to prostaglandins and thromboxanes and the lipoxygenase pathway which results in leukotrienes. The importance of eicosanoids in obstetrics and gynecology, for the lung, the vascular system, the gastro-intestinal tract and finally the liver are discussed.

In chapter 3 a summary is given of the biological effects of endotoxins such as fever, hypotension, renal dysfunction and clotting disorders. The direct and indirect endotoxin toxicity in liver disease and the release of mediators, such as histamine and eicosanoids in liver disease are described.

Plasma TXB_2 levels of sixteen patients with alcoholic liver disease are reported in chapter 4. In twelve patients raised plasma TXB_2 levels were found on one or more occasions. The raised plasma TXB_2 levels were associated with significantly higher serum levels of urea, alkaline phosphatase and gamma glutamyl transpeptidase and lower antiplasmin and antithrombin levels. This study suggests that some of the complications in patients with alcoholic liver disease may be mediated by thromboxanes.

In chapter 5 is shown that there is a net TXB_2 production by the splanchnic area and that TXB_2 is removed by the lungs and the kidneys in patients with liver cirrhosis. Blocking TXB_2 production by a selective thromboxane synthetase blocker however did not result in circulatory changes in these patients.

In chapter 6 a report is given of the rather disappointing results

of our attempts to detect and to quantitite circulating endotoxins in cirrhotics and in patients with sepsis. We found that the chromogenic limulus assay, although highly specific and sensitive for endotoxins in protein-free solutions, failed to detect endotoxemia in all patients with bacteraemia. Variability in the test itself and the presence of inhibitors in plasma are probably responsible.

In chapter 7 the effect of colitis, portal hypertension and alcohol on endotoxin absorption from the rat intestine are described. Increased endotoxin absorption from the intestine, due to changes in mucosal permeability, might contribute to endotoxemia in liver disease.

The absorption of ^{51}Chromium labelled Escherichia Coli endotoxin from loops of intestine of rats were studied. Neither portal hypertension nor ethanol administration in two concentrations increased endotoxin absorption in the rat. However the absorption rates from the colon were raised in rats with colitis. These findings do not exclude the possibility of an increased absorption rate of endotoxin in patients with liver disease in whom several potentially damaging factors might be present simultaneously.

In chapter 8 the ability of peritoneal macrophages derived from ascites of six patients with liver cirrhosis to produce eicosanoids was investigated. The main substances formed by the ascites cells were LTB_4, 5HETE and LTC_4, whereas smaller amounts of TXB_2, HHT and 6 keto PGF_{1alpha} were synthesized.

In chapter 9 an investigation of the species and site differences in the ability of macrophages to produce eicosanoids was made. Rat

Kupffer cells synthesize mainly PGF_{2alpha}, PGD_2 and PGE_2 whereas rat peritoneal macrophages mainly produce TXB_2, HHT and 12HETE.

The human peritoneal macrophages in contrast to the rat Kupffer cells and rat peritoneal macrophages, converted arachidonic acid to lipoxygenase products such as LTB_4, 5HETE and LTC_4 and only smaller amounts of TXB_2, HHT and 6 keto PGF_{1alpha} were formed.

In chapter 10 the results of the studies described in this thesis are evaluated in the light of the aims set out in chapter 1.

SAMENVATTING

Endotoxinen zijn celwandbestanddelen van gram negatieve bacteriën. Na toediening aan proefdieren of aan de mens veroorzaken endotoxinen koorts, circulatiestoornissen (o.a. shock, verminderde nierdoorbloeding) en veranderingen in leucocyten- en thrombocytenaantal en -funktie. Endotoxinen komen voor in de darm. Geabsorbeerde endotoxinen worden uit de circulatie genomen door de Kupffer cellen. Bij patienten met leverziekten bestaat er een verminderde werking van het reticulo-endotheliale systeem, waardoor systemische endotoxinemie juist bij deze patienten vaak voorkomt. Bij proefdieren induceren endotoxinen vrijlating van eicosanoïden. Eicosanoïden is de naam voor prostaglandinen (PG) en leukotriënen (LT). Toediening van endotoxinen aan proefdieren geeft een verhoogde plasmaspiegel van thromboxaan B_2 (TXB_2), PGE_2, PGF_{2alpha} en 6-keto-PGF_{1alpha}. Voorbehandeling van dieren met een selektieve thromboxane - of prostaglandine synthetase remmer verbetert de overleving na toediening van endotoxinen.

Het doel van de in dit proefschrift beschreven studie was te onderzoeken of patienten met leverziekten verhoogde plasma TXB_2 spiegels hebben en een eventueel verband tussen de verhoogde plasma TXB_2 spiegels en de komplikaties van leverziekten na te gaan. Tevens werd onderzocht of menselijke macrophagen, verkregen van patienten met leverziekten, in staat waren eicosanoïden te produceren.

In het licht van de verschillen in gevoeligheid voor endotoxinen tussen verschillende diersoorten en de gepostuleerde rol van de

Kupffer cel bij het verwijderen van endotoxinen, werd een studie gedaan naar de afhankelijkheid van soort en plaats van macrophagen voor produktie van eicosanoïden.

In hoofdstuk 2 wordt een overzicht gegeven van de literatuur over eicosanoïden. De voorlopers van eicosanoïden, zoals linolzuur, gamma linoleenzuur en arachidonzuur, cyclooxygenase, die arachidonzuur omzet in prostaglandinen en thromboxanen, en lipoxygenase die arachidonzuur omzet in leukotriënes, worden besproken. Verder wordt ingegaan op de betekenis van eicosanoïden voor de longen, het vasculaire systeem, het maag-darm-stelsel en de lever, alsook in de gynaecologie en verloskunde.

In hoofdstuk 3 wordt een samenvatting van de biologische effekten van endotoxinen gegeven. De direkte en indirekte schade die endotoxines zou kunnen veroorzaken bij leverziekten, en de afgifte van mediatoren - o.a. histamine en eicosanoïden - worden beschreven.

Hoofdstuk 4 behandelt de resultaten van plasma TXB_2 bepalingen bij 16 patienten met alcoholische levercirrose. Bij 12 patienten werden op 1 of meer tijdstippen verhoogde plasma TXB_2 spiegels gevonden. Deze verhoogde plasma TXB_2 waarden gingen gepaard met significant hoge waarden voor serum ureum, alkalische fosfatase en gamma glutamyl transpeptidase en lagere antiplasmine en antithrombine 3 spiegels. Uit dit onderzoek wordt geconcludeerd dat enkele komplikaties bij patienten met alcoholische leverziekten gemedieerd zouden kunnen worden door thromboxanen.

In hoofdstuk 5 wordt aangetoond dat er een netto TXB_2 produktie is door het splanchnicusgebied, en dat bij patienten met levercirrose TXB_2 door de longen en door de nieren wordt verwijderd. Remming van

TXB_2 produktie door toediening van een selektieve thromboxaan synthetase remmer (dazoxiben) bracht echter geen verandering in de bloedsomloop bij deze patienten teweeg.

In hoofdstuk 6 worden de tegenvallende resultaten beschreven van een onderzoek naar de mogelijkheid om circulerende endotoxinen bij patienten met cirrose en sepsis te kunnen aantonen met een chromogeen lymulus lysaat test. Ondanks de hoge specificiteit en sensitiviteit van deze test voor endotoxinen in eiwitvrije oplossingen lukte het niet bij alle patienten met bacteriëmieën endotoxinemie aan te tonen. De variabiliteit van de test zelf en de aanwezigheid van plasma inhibitoren zijn waarschijnlijk hiervoor verantwoordelijk.

In hoofdstuk 7 wordt het effekt van colitis, portale hypertensie en alcohol op de absorptie van endotoxinen uit de darm van de rat beschreven. Verhoogde endotoxinenabsorptie uit de darm tengevolge van verandering in de doorlaatbaarheid van de mucosa zou kunnnen leiden tot endotoxinemie o.a. bij leverziekten. De absorptie van ^{51}Cr-gemerkte Escherichia coli endotoxinen uit darmlissen van ratten werd bestudeerd. Zowel portale hypertensie als alcoholtoediening in 2 concentraties gaf geen verhoging van endotoxinenopname bij de rat. Bij de ratten met colitis werd wel een verhoogde absorptie uit het colon gevonden. Echter deze resultaten sluiten de mogelijkheid van een verhoogde absorptie van endotoxinen bij patienten met chronische leverziekten niet uit, daar er meerdere faktoren gelijktijdig aanwezig kunnen zijn die een toegenomen absorptie van endotoxinen veroorzaken.

In hoofdstuk 8 wordt aangetoond dat macrophagen, verkregen van ascites van patienten met levercirrose, eicosanoïden kunnen produceren. Ascitescellen vormen voornamelijk LTB_4, 5-HETE en LTC_4,

en kleinere hoeveelheden TXB_2, HHT en 6 keto PGF_{1alpha}.
In hoofdstuk 9 wordt een onderzoek naar de effekten van diersoort en plaats van afkomst van de macrophaag op eicosanoïdenproduktie beschreven. De rat Kupffercellen maken voornamelijk PGF_{2alpha}, PGD_2 en PGE_2, terwijl rat peritoneale macrophagen voornamelijk TXB_2, HHT en 12-HETE vormen. Menselijke peritoneale macrophagen zetten arachidonzuur voornamelijk om in lipoxygenaseprodukten, zoals LTB_4, 5-HETE en LTC_4, en kleine hoeveelheden TXB_2, HHT en 6-keto-PGF_{1alpha}, dit in tegenstelling tot rat Kupffercellen en rat peritoneale macrophagen.
In hoofdstuk 10 worden in het kort de resultaten van de verschillende onderzoekingen geëvalueerd in het licht van de doelstellingen vermeld in hoofdstuk 1.

VERANTWOORDING

Dit proefschrift werd bewerkt op de afdeling Inwendige Geneeskunde II in samenwerking met de afdeling Farmacologie van het Academisch Ziekenhuis Rotterdam-Dijkzigt.

Velen ben ik dank verschuldigd bij de totstandkoming van dit proefschrift, enkelen wil ik hier met name noemen.

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Ook de hulp van Anja van de Broek bij de laboratorium bepalingen dient

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CURRICULUM VITAE

De schrijver van dit proefschrift werd geboren op 29 december 1952 te Delft. Na de MULO-B opleiding werd in 1972 het eindexamen HBS-B afgelegd aan de Stedelijke Scholengemeenschap "Hugo Grotius" te Delft.

In dat zelfde jaar werd een aanvang gemaakt met de studie in de geneeskunde aan de Rijksuniversiteit te Leiden, alwaar in 1977 het doctoraal examen en in januari 1979 het artsexamen werden afgelegd.

Op 1 april 1979 werd de opleiding tot internist begonnen aan de Afdeling Inwendige Geneeskunde II, onder leiding van prof.dr. M. Frenkel.

Op 1 december 1984 werd hij als internist ingeschreven in het specialistenregister.

Sinds februari 1985 is hij in deze funktie werkzaam in het Ikazia Ziekenhuis te Rotterdam.