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ALCOHOL AND INFLAMMATION

A STUDY OF EFFECTS OF ETHANOL ON ENDOTHELIAL AND EPITHELIAL CELL FUNCTIONS

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Bedjen, och eder skall varda givet; söken, och I skolen finna; klappen, och för eder skall varda upplåtet. (Matt. 7:7)

ABSTRACT

Although short–term alcohol drinking causes euphoric and stress relieving effects, alcohol abuse is a major risk factor for neurobehavioural, liver and muscle injury as well as reduced host defence with enhanced susceptibility to bacterial infections. Although such reductions also suggest potential use as an anti-inflammatory drug, well known side effects of ethanol limits its clinical use. Instead, an ethanol derivate, ethyl pyruvate (EtP), might serve as an alternative; it is not associated with cerebral intoxication, prolongs survival in a septic shock model and acts rapidly.

Endothelial and epithelial cells, the former at the interface between blood and tissues and the latter facing various bodily surfaces, are among the first cells to become exposed to ethanol following ingestion. In this thesis I explored their reactions to ethanol and EtP in the setting of an acute inflammatory reaction, e.g. after challenge with LPS, IL-1, TNFα or LTB_4 mimicking, for instance, sepsis.

In paper I, we observed that ethanol attenuated LPS triggered human umbilical vein endothelial cells (HUVEC) to release chemokines and myeloid growth factors such as IL-8, G-CSF, GM-CSF and SCF. Furthermore, exposure to ethanol reduced the adhesiveness of HUVEC for neutrophils, effects that were possibly linked to the reduced activation of the transcription factor NF-κB.

In paper II, we report that ethanol impaired G-CSF and IL-8 release from lung epithelial cells (EpC, A549) elicited by IL-1β or TNFα. Moreover, we found that ethanol interfered with translocation from cytosol to nucleus as well as gene activation of the p65 components of NF-κB, important for activation of many genes controlling pro-inflammatory systems.

In paper III, we compared effects of ethanol and EtP on LPS, IL-1β or TNFα stimulated reactions in and between neutrophils, HUVEC and EpC. EtP was several folds more potent than ethanol in decreasing cytokine release, surface expression of adhesion molecules and adhesion of neutrophils. Ethanol and EtP hampered IRAK-1, IκBα and p65 equally, whereas impaired translocation of p50 was dependant on stimulus use.

In paper IV, we report that LPS, IL-1β and TNFα, as well as LTB_4 itself induced the upregulation of expression of surface receptors for LTB_4, BLT, on HUVEC. In addition, treatment of HUVEC with LTB_4 caused increased release of both nitric oxide and MCP-1. Our data indicate that BLT receptors might have functional consequences during the early vascular responses to inflammation.

In paper V, we noted that ethanol and EtP abrogated stimulatory effects on HUVEC for neutrophil adhesion, calcium transients and nitric oxide generation when elicited by LTB_4. We also found that ethanol and EtP reduced BLT mRNA and protein expression.

Our results provide clues to understanding how ethanol and EtP affects pro-inflammatory responses of EC and EpC. Inhibition of chemokines and growth factors that promote myeloid cell development and maturation, may likewise contribute to the increased susceptibility to bacterial infections that is associated with alcohol abuse. Thus, these dual roles of ethanol and EtP might be used to modulate vascular responses to inflammation.

LIST OF PUBLICATIONS

I. **Anne-Sofie Jonsson** and J Palmblad. *Effects of ethanol and NFκB activation, production of myeloid growth factors, and adhesive events in human endothelial cells.* J Infect Dis. 2001;184:761-9.

II. **Anne-Sofie Johansson**, Liden J, Okret S, Palmblad J. *Effects of ethanol on cytokine generation and NFκB activity in human lung epithelial cell.* Biochem Pharmacol. 2005;70:545-51.

III. **Anne-Sofie Johansson** and Jan Palmblad. *Ethanol and ethyl pyruvate hamper adhesive and secretory reactions in human endothelial and lung epithelial cells – relation to NFκB*. Submitted.

IV. Oiu H, **Johansson AS**, Sjostrom M, Wan M, Schroder O, Palmblad J, Haeggstrom JZ. *Diffrential induction of BLT receptor expression on human endothelial cells by lipopolysaccharide, cytokines, and leukotriene B4.* Proc Natl Acad Sci. USA, 2006;103:6913-8.

V. **Johansson AS**, Wan M, Oiu H, Sjöström M, Haeggström JZ, Palmblad J. Effects of ethanol and ethyl pyruvate on expression of leukotriene B4 (BLT) receptors on human endothelial cells. Submitted.

LIST OF ABBREVIATIONS

AA	Arachidonic acid
BLT1	Leukotriene B_4 receptor 1
BLT2	Leukotriene B_4 receptor 2
$[Ca^{2+}]_i$	Intracellular calcium concentration
CD31	Platelet/endothelial cell adhesion molecule (PECAM)
^{51}Cr	Chromium 51
CSF	Colony stimulating factor
EC	Endothelial cells
ECGS	Endothelial cell growth supplement
ELISA	Enzyme-linked immunosorbent assay
EPC	Epithelial cells
ERK	Extracellular regulated kinase
ESAM	Endothelial cell-selective adhesion molecule
EtOH	Ethyl ethanol, alcohol, ethanol
EtP	Ethyl pyruvate
FITC	Fluoroscein isothiocyanate
fMLP	N-formyl-methionyl-leucyl-phenylalanine
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte macrophage colony-stimulating factor
GPCR	G-protein coupled receptors
HBSS	Hank's balanced salt solution
HUVEC	Human umbilical vein endothelial cells
ICAM	Intercellular cell adhesion molecule
IL	Interleukin
IκB	Inhibitor kappa B
IRAK	Interleukin-1 receptor associated kinase
JAM	Junctional adhesion molecule
JNK	c-jun N-terminal kinase
LPS	Lipopolysaccharide
LTB_4	Leukotriene B_4
M-CSF	Macrophage colony-stimulating factor
MCP-1	Monocyte chemotactic protein-1
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PDTC	Pyrrolidine dithiocarbamate
PMN	Polymorphonuclear neutrophil granulocyte
RT-PCR	Reverse transcription polymerase chain reaction
NF-κB	Nuclear factor kappa B
SCF	Stem cell factor
TLR	Toll-like receptor
TPO	Thrombopoietin
VCAM	Vascular cell adhesion molecule
VE-cadherin	Vascular endothelial cadherin
WPB	Weibel-Palade bodies

CONTENTS

1 NTRODUCTION

1.1 ALCOHOL AND SOCIETY

Alcohol is the most commonly used drug in today's society. Although modest and short–term alcohol drinking confers euphoric and stress relieving effects, numerous clinical and experimental studies have shown that excessive and chronic alcohol use is a major risk factor for neurobehavioral, pancreatic, hepatic and muscle diseases, as well as risk of fetal abnormalities during pregnancy [1] and enhanced susceptibility to bacterial infections [2]. Even though moderate alcohol consumption may have immune protective effects, excessive alcohol consumption is ruinous. In Sweden 3.5 % of all deaths are related to alcohol, and up to 10 % of a person's years of life lost [3]. Binge drinking (consuming more than 5 glasses a day) has increased over the last 10 years; above all in young women representing a growing challenge for welfare and health care.

1.2 INFLAMMATION

1.2.1 The acute inflammatory reaction

When facing invasion by disease-causing organisms (pathogens), we can call on an arsenal of defensive responses, the operation of which constitutes the acute inflammatory/immune reaction. The acute inflammatory reaction has two components: an innate response that is activated immediately after infection or injury, and an adaptive response that starts up only after a pathogen has been recognized by the innate system. The innate, non-adaptive, response is thought to have been developed early in evolution and is present in some form or other in most multicellular organisms.

Everyone has experienced the cardinal signs of the inflammatory response, i.e. redness, swelling, heat, pain and impairment of function. An important initiating event in the innate immunity is the recognition of pathogens. Cells of the innate immunity response recognize pathogen associated molecular patterns (PAMPs) common to entire classes of pathogens (bacteria, viruses and fungi). PAMPs are highly conserved structural components usually essential for survival and virulence. Examples of bacterial PAMPs are peptidoglycan (a part of the cell wall) and lipopolysaccharide (LPS; a component of the outer membrane of Gram-negative bacteria). The toll-like receptors (TLR) are a family of receptors involved in recognizing PAMP, so far 13 are known [4]. Interaction of a PAMP, e.g. LPS, with TLR-4 triggers cells to respond immediately; intra-cellular pathways activate the production of proinflammatory proteins that act on the vascular endothelial cells of venules, causing expression of adhesion molecules and increase in vascular permeability. Leukocytes, particularly polymorphonuclear neutrophil granulocytes (PMN), adhere to the endothelial cells and migrate out of vessels, attracted by chemokines generated by invading pathogens or by local tissue cells, where it can engulf, kill and digest microorganisms [5].

1.2.2 Neutrophils

Neutrophils or PMN are produced in the bone marrow and released into the circulation upon maturation [6]. Neutrophils play a major role in orchestrating the inflammatory response, in resolution of inflammation, and in wound healing as well as in host defense through their capacity to phagocytose and destroy bacteria. They are the first to

emigrate from the bloodstream and assault microorganisms. To fulfill their mission neutrophils can kill microbes by two different mechanisms. One involves the production of toxic oxygen products and the other the release of antibacterial peptides from granules. Thus, neutrophils are armed with over 20 different potent "weapons". Granules are traditionally subdivided into two classes: primary or azurophilic granules containing e.g. myeloperoxidase, or secondary or specific granules containing e.g. alkaline phosphatase, defensins etc [7-9]. Granule components can be released extracellularly or intracellularly into the phagosomes that are formed when neutrophils engulf microorganisms [10, 11]. Activated neutrophils can also release other inflammatory mediators, e.g. interleukin-1β (IL1β) [12] tumour necrosis factor α (TNFα) [13], and arachidonic metabolites such as prostaglandins and leukotriene B_4 (LTB_4) [14-16]; and also growth factors that propagate the inflammatory phase of wound healing [17]. If neutrophils are excessively activated, toxic oxygen radicals and proteolytic products can injure the host's own tissues. During pathophysiological conditions neutrophils release their toxic chemicals, and then undergo apoptosis being recognized and engulfed by macrophages in a non-phlogistic way [18].

1.2.3 Endothelium

The endothelium is the continuous single-cell lining of the vascular system that forms an interphase between the blood and its components, on one side, and the tissues and organs, on the other. These cells were for a long time seen as a passive cellular barrier. However, venular endothelial cells are now known to have a major roll in the important inflammatory process of leukocyte trafficking from blood into tissue [19]. In the non-inflamed tissues, vascular endothelial cells regulate blood flow and fluidity, control vessel-wall permeability and restrict activation of circulating leukocytes and platelets. Nitric oxide is constitutively released and may contribute by suppressing the synthesis of pro-inflammatory proteins, but also with direct effects on leukocytes, preventing their activation [20].

Acute inflammation is a rapid process directed towards infectious microbes or injured tissues that involve local recruitment and activation of neutrophils. Efficient recruitment of leukocytes requires that endothelial cells are triggered. Endothelial cell activation may be divided into two responses, one rapid that is independent of gene activation (type I activation; Figure 1), and a second slower response that depends on gene expression, *de novo* mRNA and protein synthesis (type II activation; Figure 2) [21].

Type I activation is fast, completed in 1-20 min, and typically mediated by ligands (such as histamine and leukotrienes) that bind to heterotrimeric G-protein coupled receptor (GPCRs). Stimulation rapidly increase intracellular calcium ($[Ca^{2+}]_i$) levels by releasing calcium from endoplasmatic reticulum stores. $[Ca^{2+}]_i$ forms a complex with calmodulin that activates a number of systems, for instance nitric-oxide synthase (eNOS or called NOS3) to increase production of nitric oxide (NO), which is a potent vasodilator. The rise in $[Ca^{2+}]_i$ also has a major role in neutrophil recruitment; by activating actin filaments involved in exocytosis of Weibel-Palade bodies (WPB), bringing P-selectin, von Willebrand and tissue factor (TF) to the surface, by opening gaps between adjacent endothelial cells, PMN adherence and facilitating transmigration.

Most leukocytes appear to cross the vessel wall by passing between endothelial cells, but on occasion they may instead go through the endothelial cell body. Type I stimulation abates when the receptors are desensitized, thus preventing further stimulation and neutrophil extravasation [22].

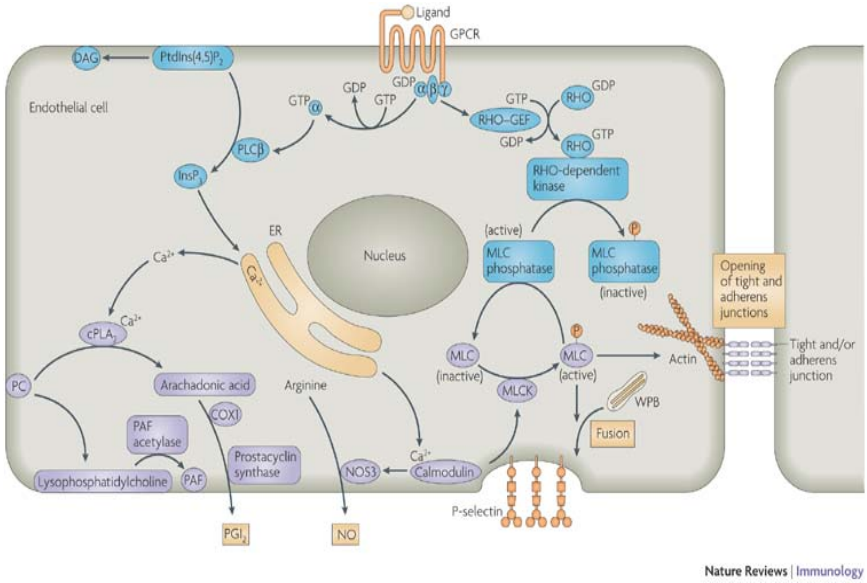

Figure 1. Type I rapid responses of endothelial cells. This is usually mediated through GPCRs, (such as leukotriene, thrombin, bradykinin receptors).

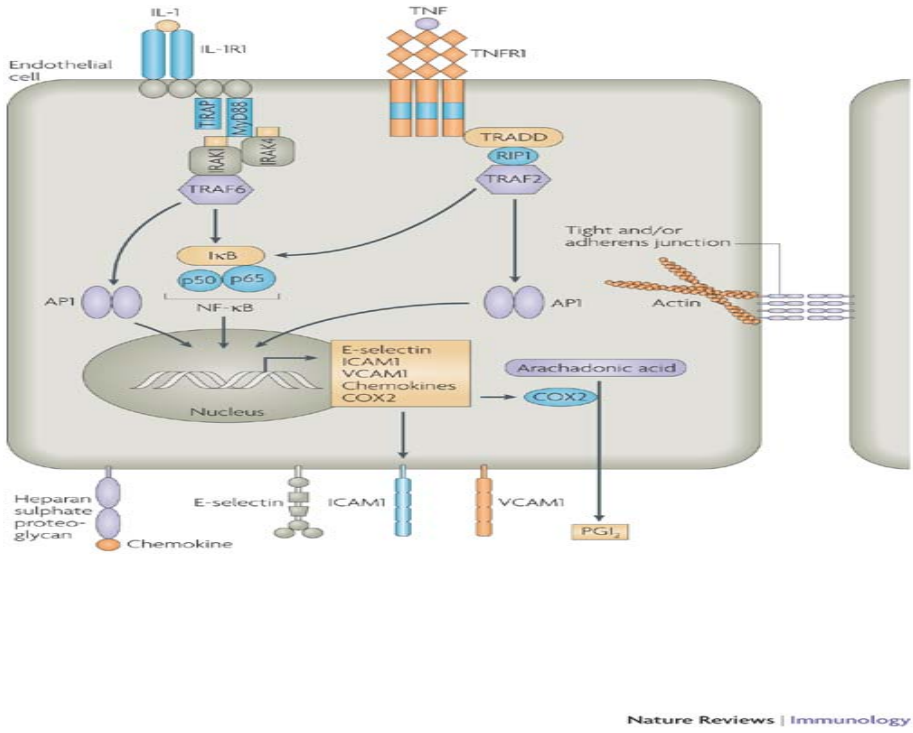

Figure 2. Type II slow responses of endothelial cells. This is mediated through gene activating pathways such as TNF, IL-1or Toll-like receptors.

Type II activation confers a more persistent activation of the endothelium. The prototypic mediators of this response are TNF and IL-1, principally released by activated leukocytes, or induced by LPS. In response to receptor ligation, signaling pathways are

induced in the endothelial cells, leading to synthesis of cytokines and adhesion molecules, starting the multistep process of leukocyte trafficking to an infected area [20].

1.2.4 Epithelium

The epithelium is a layer of cells that forms a tight barrier against fluids, air and other biological agents. Epithelial cells line the skin, digestive, reproductive, urinary and respiratory tracts, and is an important barrier against invading microorganisms. Furthermore, epithelial cells can produce antimicrobial peptides, important against a broad spectrum of bacteria, some enveloped viruses, and fungi. They may also play a role in the regulation of normal microflora [8].

Many epithelial cells are specialized for the movement of fluids, or the production of secretions. For example, the ciliated epithelium that lines the respiratory tract moves mucus up from the lungs and towards the throat. The mucus traps particles and pathogens and carries them away. The epithelial cells mediate diffusion across the membrane, and the capillaries in the underlying connective tissues provide the necessary oxygen and nutrients. It has become clear that some antibacterial proteins are expressed in epithelial cells during inflammation, e.g. LL-37, defensins, which constitute an important part of innate immunity [23].

1.2.5 Adhesion cascade

Perhaps the most important function of the venular endothelial cell is, with respect to inflammation, the complex trafficking of leukocyte from the blood to infected tissue. This process involves a cascade of events referred to as capture and slow rolling, adhesion strengthening and spreading, intravascular crawling, and lastly transmigration [24]. Capture and rolling of leukocytes is mediated by interactions of selectins with their ligand. P-selectin and E-selectin is expressed by inflamed endothelial cells and L-selectins by most leukocytes [25]. Binding of neutrophils to E-selectin induces intergrin activation. The most relevant integrins that participate in rolling and mediate firm adhesion belong to the β_1-integrin and β_2-integrin subfamilies. Leukocyte activation and adhesion is rapidly triggered by chemokines or lipid chemoattractants that are mediated by the binding of leukocyte integrin to intercellular adhesion molecule 1 (ICAM-1) and vascular cell-adhesion molecule 1 (VCAM-1) expressed by endothelial cell during inflammation [26, 27]. This cell-to-cell signaling strengthens adhesion and induces conformational changes to promote leukocyte migration through endothelial junctions [28, 29]. Migration involves the release of endothelial-expressed vascular endothelial cadherin (VE-cadherin) [30] and is facilitated by intracellular membrane components containing a pool of platelet/endothelial-cell adhesion (PECAM) and other juntional molecules such as junctional adhesion molecule (JAM), CD99, ICAM-2 and endothelial cell-selective adhesion molecule (ESAM) with the intention to support neutrophils transmigration [31]. Transmigration through venular walls is one of the last steps in the process of neutrophil transmigration, leukocytes then need to migrate through the endothelial basement membrane and the pericyte sheath to get to the inflamed tissue (Figure 3) [24].

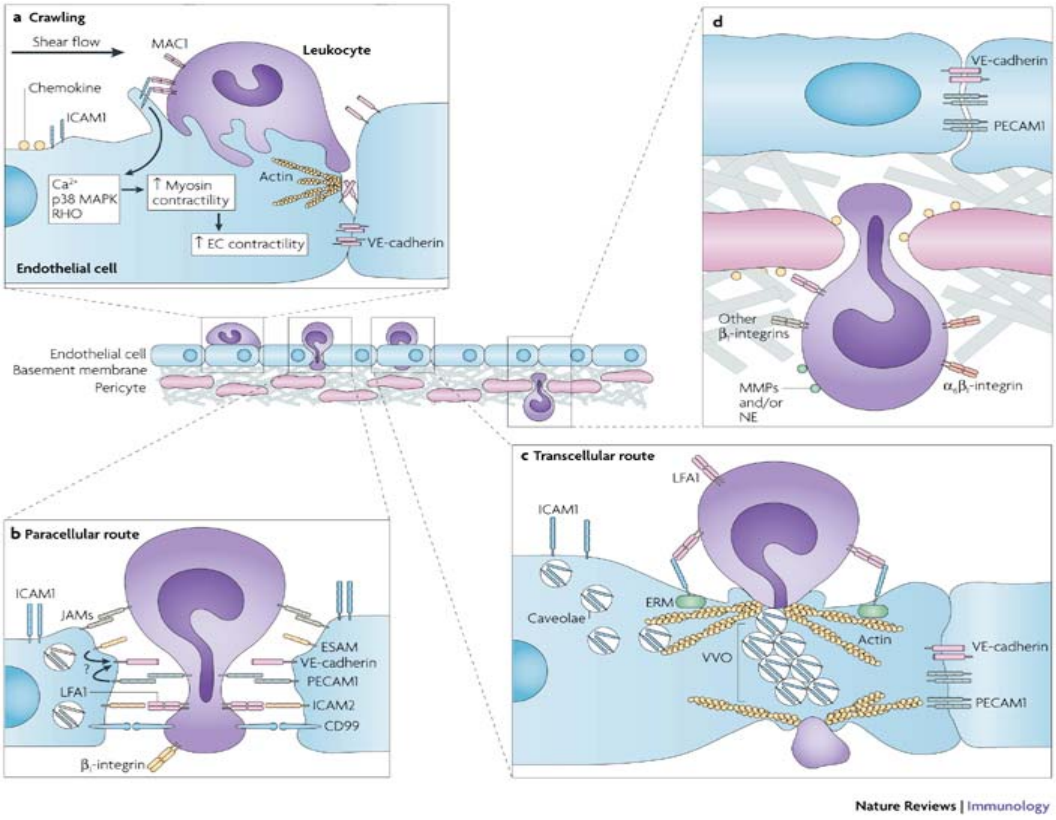

Figure 3. Diapedesis of neutrophils between or through endothelial cells.

1.2.6 Cytokines

Cytokines refer to a family of cellular proteins that regulate a broad range of inflammatory processes. Upon recognition of microbial molecules, immune cells respond by releasing TNFα and IL-1β, initiating the immune response. Several other cytokines are commonly associated with acute inflammation including interleukin 8 (IL-8) and monocyte chemoattractant protein 1 (MCP-1). IL-8, a potent neutrophil chemotaxin that promotes granule exocytosis, enhancement of respiratory burst, and up-regulation of membrane adhesion receptors on PMN, is expressed by activated cells [32].

MCP-1 is a chemoattractant and activator for monocytes/macrophages, but other cell types have been shown to respond include NK-cell, eosinophils, T-cells and neutrophils [33].

Various growth factors are required for the survival, proliferation, differentiation, and maturation of hematopoietic cells. During a host response, activated cells increase their release of growth factors. Colony-stimulating factors (CSF) are a family of growth factors with the ability to stimulate the formation of hematopoietic cells. Stem cell factor (SCF) and thrombopoietin (TPO) act during the early differentiation in the bone marrow to induce non-lymphoid blood cells. Granulocyte-macrophage CSF acts at later stage to promote the formation of the myeloid progenitor cells that later are divided and matured into granulocytes by granulocyte-colony stimulating factor (G-CSF) or to monocytes by monocyte colony stimulating factor (M-CSF) [34].

1.2.7 Leukotrienes

The leukotrienes (LTs) are a family of fatty acids derivat that play important roles in a variety of allergic and inflammatory reactions [35, 36]. These molecules are divided into two classes, the spasmogenic cysteinyl-leukotrienes (cys-LT) and LTB_4, which is a potent chemoattractant for leukocytes [15]. Because of these effects, LTB_4 is regarded as an important chemical mediator in a variety of acute and chronic inflammatory diseases and only recently, genetic and biochemical evidence strongly implicate LTB_4 as a mediator of vascular inflammation and arteriosclerosis [37-39]. LTB_4 is synthesized from arachidonic acid via the concerted action of 5-lipoxygenase, assisted by 5-lipoxygenase activating protein, and the terminal LTA_4 hydrolase [40].

LTB_4 signals primarily via a specific, high-affinity, G-protein coupled seven-transmembrane receptor, termed BLT_1 [41] or by the second, low-affinity BLT_2 [42]. BLT_1 and BLT_2 signal through G proteins and display different ligand affinity and specificity for LTB_4 and structurally related molecules [42, 43]. BLT_1 is found mainly in leukocytes [41, 44, 45], whereas BLT_2 is more common, present in almost all human tissues [42].

LTB_4 is very active in the microcirculation and promotes adhesion of leukocytes to the endothelium, followed by migration. The general theory is that effects of LTB_4 primarily originate from its action on the leukocyte, where chemoattractants induce upregulation of cell-adhesion molecules which can interact with their ligand receptors on endothelial cells [46, 47]. Of note, LTB4 has been regarded as a very fast agonist, where adherence effects are noted already after a few minutes. The responses have also been assumed to be short-lived, terminated after less than one hour.

1.3 ETHANOL AND ETHYL PYRUVATE IN INFLAMMATION

Suppression of innate immunity to infectious agents by ethanol was reported by Benjamin Rush as early as 1785. Sir William Osler remarked in his Principles and Practice of Medicine 1909 that Alcoholism is ”perhaps the most potent predisposing factor” to lobar pneumonia. Ethanol abuse impairs host defense against microorganisms [48] and alcohol-related infections becomes more prevalent and more severe in trauma victims [49]. Moreover ethanol exposure impairs the proliferative phase of wound healing [50].

The mechanisms for this impairment of host defense has traditionally been attributed to effects of ethanol on phagocytic cells, e.g. reduced neutrophil (PMN) adherence, chemotaxis, generation of superoxide ions and bactericidal functions [48, 51-55]. These reactions have been linked to the ability of ethanol to impair generation of phosphatidic acid and of calcium ion fluxes [56, 57] or interaction with signal coupling systems and generation of cytokines in phagocytes [58-62].

In addition to its impairment of phagocytic cell function, effects of ethanol on the number of such cells may contribute to the severity of alcohol-related infections. Thus, neutropenia (as well as thrombocytopenia), rather than the anticipated neutrophilia, may occur in alcoholics during infections [63-67]. Although the mechanisms of such actions remain unclear, a direct toxic effect of ethanol or its metabolites on myeloid precursor cells may be a contributing factor [66]. Administration of the myeloid lineage–specific growth factor G-CSF has thus been shown to ameliorate alcohol-associated infections [59, 68, 69]. This effect of G-CSF has been attributed to its ability not only to increase the production of mature neutrophils but also to enhance the bactericidal functions of these cells.

Many studies have demonstrated an association between alcoholism and pulmonary infections such as those caused by pneumococci and *Klebsiella*. The pulmonary epithelial cells represent an important part of local host defence. They are pivotal for emigration of leukocytes into the alveolar and bronchial tissues by means of adhesion and activation molecules. Acute intoxication as well as chronic ethanol consumption have been shown to affect the ability of the lung to generate and secrete some of the mediators evoked during inflammation [59, 70-74]. Exposure of the lungs to ethanol impairs bronchial ciliary motility and barrier functions [70, 75, 76].

Although these and other findings support the hypotesis that ethanol might be used as an inhibitor of inflammatory reactions, the entry of ethanol into the central nervous system, with ensuing intoxications symptoms, severely curtails the possibility of using ethanol in a clinical setting. However, ethanol was recently linked to pyruvate to form ethyl pyruvate (EtP), which has now been tested as an inhibitor of inflammatory reactions *in vitro* and in laboratory animals [77]. EtP is well tolerated and there appears to be no signs of ethanol intoxication. In these studies EtP afforded strong protection of delayed cerebral ischemic injury with a wide therapeutic window [78], improved hemodynamic stability in chronic endotoxemia [79], protected against or accelerate recovery from acute renal failure after the renal insult [80], and decreased local and distant organ injury in a murine model of necrotizing pancreatitis [81]. Moreover, delayed treatment with EtP conferred prolonged survival time in a lethal model of endotoxic shock, even if treatment begins after the early tumour necrosis factor response [82]. Some of the beneficial anti-inflammatory effects of ethyl pyruvate may be due to modification of p65, thereby inhibiting signaling via the NF-κB pathway [83].

2 METHODS

The methods used in this thesis are listed below as a reference to papers in which they appear, were a detailed description of the method can be found.

Method	Paper
Cell culturing HUVEC, A549	I-V
PMN isolation	I, III-V
Cell viability	I-V
ELISA	I-V
Cell-ELISA	I-V
Cell adhesion	I, III, V
RT-PCR	IV, V
Western blot	I, IV, V
Calcium mobilization	IV, V
Immunohistochemistry staining	I-V
Assessment of nitrite/NO	IV, V

3 AIMS OF THE PRESENT STUDY

The general aim of this study was to elucidate some of the mechanisms that are modulated by ethanol and ethyl pyruvate in immune response, especially in the vascular system.

Specific aims were:

Paper I

The aim of this study was to examine ethanol's effect on endothelial cells to produce IL-8 and growth factors, which are active in maturation, recruiting and binding of leukocytes.

Paper II

The aim was to investigate ethanol's ability to modulate gene activation and transcription of pro-inflammatory genes in epithelial cells.

Paper III

The plan was to compare the relative response of ethanol and ethyl pyruvate in neutrophils, endothelial and epithelial cells.

Paper IV

To identify and characterize the expression profile of BLT receptor in HUVECs and their functional response in induced inflammation.

Paper V

To explore how ethanol and ethyl pyruvate affects BLT receptor expression and there functional regulation.

4 RESULTS AND DISCUSSION

4.1 ETHANOL'S EFFECT ON LPS INDUCED ACTIVATION IN ENDOTHELIAL CELLS (PAPER I)

Ethanol abuse impairs host defense against microorganisms and many studies have demonstrated an association between alcoholism and neutropenia. The aim of this study was to examine ethanol's effect on endothelial cells to produce chemokines, adhesion molecules and myeloid growth factors, which are active in maturating, recruiting and binding leukocytes. Using an ELISA, we show that exposure of HUVECs to ethanol inhibited the generation of cytokines important for myeloid cell development and reduced the adhesiveness of HUVECs for neutrophils. Immunostaining and Immunoblot analysis of nuclear translocation of p50 and p65 showed that these effects are possibly linked to reduced activation of NF-κB in endothelial cells.

4.1.1 Generation of cytokines

With an ELISA we demonstrated that quiescent HUVEC released only small amounts of G-CSF, IL-8, GM-CSF, M-SCF, TPO and SCF into culture supernatant. Exposure of cells to ethanol alone did not affect the release of these cytokines.

G-CSF: Treatment of cells for 4 h with IL-1β or LPS induced a substantial increase in G-CSF released into the culture supernatant. Exposure of HUVECs to ethanol for 10 min before incubation with LPS resulted in a dose-dependent inhibition of G-CSF release. Likewise, ethanol reduced IL-1β induced G-CSF production. Most of the G-CSF produced by HUVECs was found to be released into the culture medium and ethanol affected only the LPS-induced increase in this portion of total cellular G-CSF; ethanol thus appeared to inhibit the synthesis of this cytokine by HUVECs.

IL-8: LPS induced an increase in the release of IL-8 by HUVECs. However, whereas 170 mM ethanol inhibited LPS-induced IL-8 release, ethanol at a concentration of 1.7 mM induced a small increase in LPS-stimulated IL-8 release.

GM-CSF: LPS also induced an increase of GM-CSF by HUVECs; this effect of LPS was inhibited in the presence of ethanol.

M-CSF: LPS approximately doubled the release of M-CSF by HUVECs. However, in contrast to its effects on G-CSF, GM-CSF, and IL-8 release, ethanol had no significant effect on LPS-induced M-CSF release.

SCF and TPO: incubation of cells with IL-1α for 24 h increased the concentration of SCF (but not TPO) in the culture supernatant. Ethanol inhibited the IL-1α–induced release of SCF.

4.1.2 Adhesiveness for neutrophils

Stimulation of HUVECs with LPS for 4 h induced a ~10-fold increase in the adhesion of neutrophils. Exposure of HUVEC monolayers to ethanol resulted in a dose-dependent inhibition of LPS-induced neutrophil adhesion.

4.1.3 Adhesion molecules

An adapted ELISA was used to measure the adhesion molecules expressed on the surface of HUVEC, both before and after treatment with ethanol and activation with

LPS. Stimulation of cells with LPS induced an increase in the abundance of expression of ICAM-1, VCAM-1 and E-selectin, however exposure to ethanol did not markedly effect the adhesion molecule expression.

4.1.4 NF-κB activation

Given that NF-κB mediates the transcriptional activation of the genes that encode pro-inflammatory cytokines, we investigated the possible role of this transcription factor in the ethanol-induced inhibition of the production of these cytokines by HUVECs. We, therefore, examined the effect of ethanol on the translocation of NF-κB from the cytoplasm to the nucleus.

We first examined the translocation of the p65 subunit of NF-κB by immunostaining. In quiescent HUVECs, p65 was detected in the cytoplasm but not in the nucleus. Conversely, LPS-stimulated cells exhibited pronounced staining for p65 in the nucleus, with minimal staining apparent in the cytoplasm, presumably reflecting translocation of p65 from the cytoplasm to the nucleus. Exposure of cells to ethanol inhibited the LPS-induced translocation of p65 to the nucleus. Immunostaining and densitometric analysis with antibodies to the p50 subunit of NF-κB showed an increased nuclear accumulation following LPS treatment. Ethanol also slightly reduced the LPS-induced translocation of p50 to the nucleus.

Immunoblot analysis also revealed that ethanol reduced the extent of these effects of LPS on the abundance of p65 in the cytoplasmic and nuclear fractions.

4.1.5 Cell viability (paper I-V)

HUVEC cell viability was assessed before experiments or after incubation. Less than 5 % of cells incubated for up to 4 h with cytokines or chemicals used herein exhibited an altered morphology or uptake of trypan blue, indicating that membrane integrity was not disrupted under the experimental conditions of the present study. To demonstrate that the apparent inhibition by ethanol in LPS stimulated HUVEC was not actually due to an ongoing apoptotic process, we assessed the extent of apoptosis in the cell cultures. Incubation of HUVECs for 4 h with 170 mM ethanol in the absence or presence of LPS did not increase the surface binding of annexin-V, suggesting that ethanol does not induce apoptosis in these cells. This conclusion was verified with the TUNEL assay to ensure that effects of ethanol on membrane fluidity and phospholipid expression did not interfere with annexin-V binding. The TUNEL assay failed to detect apoptosis in ethanol-treated cells. Moreover, HUVEC viability was tested by means of a [^{51}Cr] release assay [84] and neutrophil–mediated injury of endothelial cells. In our hands, ethanol concentrations used here displayed no cytotoxicity or neutrophil mediated injury of endothelial cells. We also assessed if exposure of HUVEC monolayer to 170 mM ethanol, together with agonist caused detachment of cells; this was not the case, as assessed by regular microscopic inspections of cell layers or by assessment of PECAM (CD31) or VE-cadherin expression. Therefore it is evident that the hampering effect of ethanol is not direct caused by apoptotic or necrotic effects.

4.1.6 Potential roles of ethanol in endothelium pathophysiology

Given that G-CSF promotes both neutrophil proliferation, maturation and survival, as well as facilitating various functional responses of mature neutrophils, the inhibition of G-CSF release by endothelial cells may contribute to the increased incidence and poorer outcome of bacterial diseases as well as to the neutropenia associated with such infections in individuals who abuse alcohol [59, 62, 64, 66, 67]. The inhibition by etha-

nol of GM-CSF and SCF release may similarly contribute to the increased susceptibility to bacterial infection that is associated with alcohol abuse. The inhibitory effect of ethanol of IL-8 production also might contribute to the impairment of host defenses by alcohol, given that this chemokine induces the release of mature neutrophils from the bone marrow as well as promotes neutrophil adhesion to endothelial cells and the subsequent migration and chemotaxis of these leukocytes to foci of infection [32]. Our observation that ethanol inhibited neutrophil adhesion to LPS-stimulated HUVECs is consistent with previous demonstrations that ethanol reduces the adherence of neutrophils to various surfaces [85]. Thus, exposure of endothelial cells to ethanol inhibited the generation of cytokines important for myeloid cell development and reduced the adhesiveness of HUVEC for neutrophils: effects that are possibly linked to the reduced activation of NF-κB.

4.2 ETHANOL'S EFFECT ON IL-1β AND TNFα INDUCED ACTIVATION IN EPITHELIAL CELLS (PAPER II)

Alcohol abuse is associated with enhanced risk of pulmonary infections, particularly with Klebsiella sp. The excretion of a small (5-10 %) but consistent proportion of alcohol is through the lungs. So, we wanted to assess if ethanol reduced generation of cytokines, as well as translocation and gene activation by NF-κB in alveolar lung epithelial cells. In this study, we used a κB binding and luciferase-coupled construct, transfected in to A549 cells to detect p65 activity.

4.2.1 Generation of cytokines

During 24 hours, quiescent A549 cells secreted only relative small amounts of G-, M-CSF and IL-8 into the culture supernatant. Exposure of cells to ethanol alone did not affect the release of these cytokines.

G-CSF: Now, when A549 cells were stimulated for 24 h to produce G-CSF, IL-1β caused a massive, i.e. a 290-fold, increment of G-CSF secretion. Similar to endothelial cells, TNFα induced considerably lower G-CSF secretion: a 36-fold increase of G-CSF release into the supernatants was observed after 24 h of incubation of A549 cells. Exposure of A549 cells to ethanol before incubation with IL-1β resulted in a dose-dependent inhibition of G-CSF release. In contrast, the observed TNFα-induced G-CSF release was not consistently inhibited by ethanol.

M-CSF secretion was induced by IL-1β and by TNFα, upon which ethanol had no inhibitory effect. This is in agreement with previously published reports in endothelial cells, and points to a difference in the effects of ethanol on the stimulus-response coupling for these myeloid cytokines. It also emphasizes that effects of ethanol are not unspecific.

IL-8: IL-1β as well as TNFα caused approximately 10-fold increase of the release of this chemokine. Ethanol impaired the IL-1β and TNFα-stimulated IL-8 secretion in epithelial cells.

4.2.2 NF-κB activity

Given the present results of how ethanol affected cytokine secretion in A549 cells, we asked whether ethanol influenced the transcriptional activity of NF-κB. For this purpose, we used a luciferase reporter gene under the control of three defined NF-κB

binding sites. When A549 cells were treated with IL-1β or TNFα, for 24 h we observed that both agonists caused activation of the gene. When A549 cells had been pretreated with ethanol prior to the agonist, these responses were reduced in a dose dependent way.

Since ethanol exerted inhibitory effects on the reporter gene activity, it might be argued that this can reflect unspecific effects of the alcohol. We, then, sought to resolve this possibility by using a constitutively activated reporter gene system, the RSV-LUC construct. When ethanol was added to this system no change in the luciferase activity was observed after 24 h, suggesting that ethanol did not exert a general negative effect on transcriptional reporter gene activity.

Next, we compared the ratios of cytoplasm to nuclei staining for the endogenous NF-κB component p65 in IL-1β or TNFα-stimulated A549 cells and analyzed whether treatment with ethanol changed the ratios. The results show that IL-1β and TNFα caused relocation of p65 from the cytosol to the nucleus after 1 h, respectively, and that treatment with ethanol impaired this translocation when IL-1β was used as stimuli. In contrast, ethanol did not affect TNFα-induced translocation.

4.2.3 Potential roles of ethanol in lung epithelium pathophysiology

It is obvious that epithelial cells are not a passive barrier, but plays a highly active role in the inflammatory process by means of production of various mediators (such as leukotrienes, cytokines/chemokines) and expression of adhesion molecules. Thus, G-CSF and IL-8, present systemically or locally, are not only stimuli of neutrophil delivery systems from the bone marrow, of functional responses of mature cells, but they also promote emigration of inflammatory cells to the alveolar and bronchial tissues. Neutrophil and monocyte interactions with epithelial cells may partake in an amplification process, where IL-8 and other cytokines are generated, which in turn recruit more inflammatory cells, prolong their life-span and aid in the maturation of monocytes into macrophages. Thus, this study shows that ethanol impaired various parts of the first line of host defense to inflammatory stimuli, represented by the reactivity of epithelial cells. The findings of reduced secretion of G-CSF and IL-8 by ethanol may partly explain why alcohol abuse is associated with a higher incidence and worse outcome of bacterial diseases particularly in the respiratory system.

4.3 DIFFERENCES IN INDUCED ACTIVATION (PAPER III)

In the two previous studies we reported that ethanol reduces the generation of myeloid growth factors and IL-8 in human endothelial cells, interactions with neutrophils and nuclear translocation of NF-κB components [86]. Similar findings, including effects of ethanol on gene expression of NF-κB, were observed in human lung epithelial cells [87]. These observations extend the spectrum of ethanol effects to the vessel wall and lung barrier system. The interactions of endo- and epithelial cells is vital for inflammatory and defence reactions in the lung, e.g. in the adult respiratory distress syndrome as well as pneumonia. Ethanol was recently linked to pyruvate to form ethyl pyruvate (EtP), which has been shown to inhibit inflammatory reactions. Here, we assessed if ethanol and ethyl pyruvate were able to modulate a common pathway for signals generated by binding of LPS or IL-1β to their receptors, namely translocation of IRAK-1 and the NF-κB complex in HUVEC. We focused here on IL-1β as the stimulus and comparisons were made with TNFα-stimulation.

4.3.1 Generation of cytokines

G-CSF: When HUVECs and A549, epithelial cells were stimulated for 4 h to produce G-CSF by means of IL-1β, a massive, i.e. a hundredfold, increment of G-CSF secretion was observed; we found that ethanol as well as EtP treatment reduced this G-CSF release remarkably. TNFα induced considerably lower G-CSF secretion and no effect of Ethanol was noted. However, treatment with EtP reduced the amount of G-CSF released.

IL-8: In HUVECs and in A549 cells, IL-1β stimulation for 4 h caused a substantial increase in concentration of IL-8 in supernatants. However, whereas ethanol did not alter IL-8 release from HUVECs, IL-8 release from A549 was significantly inhibited. Likewise, TNFα induced IL-8 release was not decreased by ethanol treatment in HUVECs, but in A549 cells. EtP treatment repressed IL-8 release from both cell types independent of agonists.

4.3.2 Adhesiveness for polymorphonuclear neutrophils (PMN)

IL-1β induced increased neutrophil adherence to HUVECs. The reaction to ethanol treatment in IL-1β activated cells was negligible. When PMN adherence to A549 cells was analyzed we observed that IL-1β increased PMN adherence and that ethanol treatment augmented it.

TNFα stimulated adhesiveness to HUVEC were not altered with ethanol treatment. However, in A549 cells ethanol treatment enhanced TNFα induced PMN adherence. When EtP was administrated before the challenge of agonists, we observed a dose-dependent decrease in PMN adhesiveness to HUVEC as well as to A549 cells. Treatment of PMN with ethanol and EtP decreased the N-formyl-metionyl-leucyl-phenyl-alanine (fMLP) induced adhesiveness against plastic.

4.3.3 Adhesion molecules

ICAM-1: In HUVEC and in A549 cells, activation with IL-1β for 4 hours conferred an up-regulation in ICAM-1 expression; EtP treatment caused a dose-dependent decrease, whereas ethanol slightly decreased ICAM-1 expression in A549 cells, but not in HUVEC.

Pretreatment of HUVECs and A549 cells with ethanol and EtP significantly reduced TNFα induced expression of ICAM-1.

VCAM-1: In HUVEC, the surface expression of VCAM-1 was not modulated with ethanol treatment. In A549 cells, ethanol and EtP caused a reduction of VCAM-1 surface expression.

E-selectin: When HUVEC had been treated with ethanol prior to addition of agonists, E-selectin expression was not significantly affected. However, EtP diminished E-selectin expression induced by IL-β, LPS as well as TNFα. E-selectin was not expressed at all by A549 epithelial cells.

4.3.4 IRAK activation

The translocation of IRAK-1 from the cytosol to the nucleus after stimulation with IL-1β, also described by Böl et al. [88], was induced after 15 min. with IL-β and LPS. Ethanol or EtP treatment hampered the IL-1β and the LPS induced translocation of IRAK-1. However, IRAK-1 translocation was not induced with TNFα and thus no

effects were seen with ethanol treatment, as assessed by the staining technique and densitometric analyses.

4.3.5 NF-κB activation

As reported previously, LPS induced a significant translocation of p65 from the cytosol to the nucleus of HUVEC after 1 h that was inhibited by 46 % after ethanol treatment prior to LPS stimulation (Paper I). Moreover, ethanol impaired gene activation by means of the NF-κB system in A549 cells (Paper II). Here, this effect was corroborated and extended to find that ethanol did not block p65 translocation induced by IL-1β in HUVEC, but did so in A549 cells. Ethanol treatment did not inhibit TNFα induced p65 translocation in HUVECs or in A549 cells. We observed that EtP treatment inhibited LPS induced p65 translocation, but not IL-1β or TNFα induced.

We also examined the effect of ethanol and EtP on translocation of NF-κB subunit p50, observing that exposure to ethanol conferred a decreased amount of p50 in nucleus compared to cells stimulated with IL-1β only. Likewise, when LPS was used to stimulate HUVEC there was a decrease in p50 translocation after ethanol or EtP treatment. Neither ethanol nor EtP had effect on TNFα induced p50 translocation.

4.3.6 IκBα modulation

Finally, we analyzed IκBα in HUVEC exposed to ethanol or EtP and stimulated with agonist. This inhibitory factor is believed to be phosphorylated, ubiquitinated and degraded upon cell activation and to release NF-κB. We observed staining for IκBα in the nucleus and in the cytosol after stimulation with LPS. Cellular staining then declined, presumably reflecting degradation of IκBα. Exposure of cells to ethanol or EtP prior to LPS for 45 min adjusted the IκBα staining in HUVEC. The same was noted when EtP was administered before TNFα; that was not observed by means of IL-1β.

4.3.7 Different roles in vein endothelium and lung epithelium

Here, we compared effects of ethanol and EtP on reactions in and between HUVEC, lung epithelial (A549) cells and neutrophils *in vitro*. This study shows that ethanol as well as EtP is highly efficient molecules to disrupt the adherence process of and between neutrophils, endothelial and lung epithelial cells. Our rationale for studying neutrophils, endothelial and epithelial cells is that these are among the first to be engaged in an inflammatory reaction, particularly in the lung.

Three main conclusions can be drawn from our study.

The first is that ethanol and EtP displayed rather similar effects. Given these similarities it is likely that the major target for both drugs is located down stream of surface receptors.

The second point is that effects of ethanol and EtP effects differed depending on the agonist used. Thus, effects elicited by LPS were clearly susceptible to ethanol and EtP, whereas effects by IL-1β and TNFα were far less influenced. This suggests specific targets in the signaling pathways for instance variable effects of ethanol/EtP on receptors for these agonists.

The third point is that HUVEC and A549 cells reacted rather similarly to treatment with ethanol and EtP in the limited sets of experiments performed here.

Thus, it is clear that ethanol and EtP have effects on release, translocation and degradation of components of the NF-κB system that are complex and which may be specific to concentration, stimulus and cell type.

4.4 PROINFLAMMATORY STIMULI INDUCE BLT RECEPTORS (PAPER IV)

LTB_4 is found in inflammatory exudates and tissues in many inflammatory conditions, and several lines of evidence indicate that leukotrienes are involved in vascular inflammation, in particular arteriosclerosis [38].

In this study we used semiquantitative RT-PCR and Western blot to assess the mRNA and protein expression of BLT receptors in HUVECs. We analyzed mobilization of cytosolic $[Ca^{2+}]_i$, and MCP-1 and nitrite/NO generation to detect functional activity.

4.4.1 Effects of LPS on BLT receptors.

Treatment of HUVEC with LPS led to a rapid (within 1 h) increase in the levels of BLT_1 mRNA to amounts that were approx. 12 times above baseline. This increase persisted at the same level after 2 h of incubation. In contrast, LPS did not have any significant impact on the BLT_2 mRNA levels. As assessed by Western blot, BLT_1 protein increased 3 times relative to the untreated control.

When the functional consequences of receptor activation were assessed by intracellular calcium fluxes, no or minimal $[Ca^{2+}]_i$ response was observed when quiescent HUVECs were stimulated with LTB_4. However, when HUVEC had been exposed to LPS for 2-4 h, increases of $[Ca^{2+}]_i$ fluxes were seen in response to LTB_4. The LTB_4-induced $[Ca^{2+}]_i$ reply was completely blocked by pretreatment with the selective BLT_1 inhibitor CP-105696. The weaker BLT_1 antagonist U75302 influenced the LTB_4 response marginally. BLT_2 antagonist, LY-255283 inhibited only a small part of the response.

4.4.2 Effects of TNFα and IL-1β on BLT receptors.

Treatment of HUVEC with TNFα led to a rapid increase in the amounts of BLT_2 mRNA, which were ~5 times the controls after 30 min and increased further to 6 times after 2 h. above the levels in untreated cells. Treatment with TNFα also led to a weak increase in BLT_1 mRNA, which peaked after 60 min (2 times). This slight increase did not lead to a significant increase in the amounts of BLT_1 protein as assessed by Western blot. $[Ca^{2+}]_i$ analyses showed that pre-incubation with TNFα induced a rapid and enhanced $[Ca^{2+}]_i$ LTB_4 response. This effect were completely abolished by addition of a selective BLT_2 antagonist, LY-255283, prior to adding LTB_4.

Treatment of HUVEC with IL-1β increased the levels of both BLT_1 and BLT_2 receptor mRNA. In Western blot analysis, the levels of BLT_1 protein were increased. IL-1β exposure increased the LTB_4 induced $[Ca^{2+}]_i$ response compared to un-exposed cells.

4.4.3 Effects of LTB_4 (ligand) on BLT receptors.

The natural ligand LTB_4 also induced BLT_1, but not BLT_2, mRNA and protein expression in a time-dependent manner. Following stimulation of HUVEC with LTB_4, BLT_1 mRNA increased 3 times and stayed elevated for at least 2 h. The effects of LTB_4 on BLT_1 mRNA expression appeared specific, since LTC_4, LTD_4, and 5-HETE were without significant effects. In addition, Western blot showed a corresponding increase of BLT_1 protein compared with untreated HUVECs. We also tested the appraisal effect of $[Ca^{2+}]_i$ responses after preincubation with LTB_4 itself and exposed HUVEC to the ligand for 3-6 h. Upon a second stimulation with LTB_4 enhanced $[Ca^{2+}]_i$ responses were observed.

4.4.4 Nitrite and MCP-1 release

In these experiments we focused on supplementary physiological effects of the upregulation of BLT_1 by LPS or LTB_4. To this end, we treated HUVEC for 3 hours with either LPS, LTB_4 or HBSS alone to up-regulate BLT receptors, then removed media, added new media and LTB_4. As shown in Paper IV, Figure 7, nitrite diffusion increased in HBSS treated samples that were stimulated by LTB_4 for15 min. Treatment with LPS, followed by LTB_4, enhanced nitrite release.

In similarly designed experiments on MCP-1 release, we used LTB_4 treatment for 3 h in order to upregulate BLT_1. Compared to controls first treated with HBSS alone and then with LTB_4, samples treated with LTB_4 during both time periods showed enhanced cytokine release, suggesting that priming with LTB_4 enhanced the subsequent LTB_4 response (Figure 7). When CP 105696 was present, most of the subsequent response to a second LTB_4 challenge was abolished.

4.4.5 Potential roles of BLT and LTB_4

LTB_4 is a classical chemoattractant agent that also promotes neutrophil adhesion and diapedesis through the endothelial cell barrier, a key sequence of events during vascular inflammatory responses and host-defense. Treatment of HUVECs with LPS and certain other cytokines leads to differential induction of BLT receptors. We have shown that these induced receptors are functionally active and capable of transmitting LTB_4 signals via $[Ca^{2+}]_i$ rises, which in turn may lead to activation of the constitutive isoform of eNOS controlled by intracellular calcium-calmodulin and increased diffusion of NO, a potent vasodilator that can increase blood flow. This concept is further supported by our findings that HUVEC can promote generation MCP-1 a powerful chemoattractant for monocytes, neutrophils and T-cells [33, 89].

The principal findings in this study is that endothelial cells can respond to pro-inflammatory stimuli with increased expression of leukotriene B_4 receptors, which will further amplify the inflammatory effects of LTB_4.

4.5 ETHANOL'S EFFECT ON LTB_4 LIGAND ACTIVATION (PAPER V)

Ethanol has effects on vascular responses in inflammation, and BLTs and LTB_4 ligand are present in inflammatory cells. So, we sought to analyze the effects of ethanol and EtP on direct stimulation with LTB_4 for functional responses in HUVEC. To this end, we assessed adhesion of neutrophils, expression of ICAM-1 and generation of nitric oxide (NO).

4.5.1 Adhesiveness for polymorphonuclear neutrophil (PMN)

Initial activation of HUVEC by LTB_4 confered increased adherence of neutrophils to the HUVEC. Recently, we reported that this reaction of HUVEC is biphasic, with a first, smaller peak occurring at 5-15 min that can inhibited with a BLT receptor blocker [90]. The second phase of adherence commences after 3 hours and is observed over 6 hours, then it declines; that response, being several-fold higher than that occurring after 15 min, is comparable in magnitude to that induced by LPS [90]. Here, we tested primarily if the second, pronounced peak was affected by the presence of ethanol or EtP. As shown in paper V, Figure 1, after 5 h of exposure, ethanol and EtP impaired this adhesive response.

4.5.2 Generation of adhesion

Stimulation with LPS, TNFα and IL-1β increased ICAM-1 surface expression. In addition, incubation with LTB_4 increased ICAM-1 expression. But, when HUVECs were exposed to ethanol or EtP prior to adding LTB_4, ICAM-1 expression was not significantly affected. Treatments of HUVEC with LTB_4, ethanol or EtP conferred no changes of PECAM (CD31) and VE-cadherin expression compared to baseline.

4.5.3 Diffusion of nitrite

In previous study we observed that LTB_4 induced nitric oxide (NO) release from HUVEC (Paper IV). Here, we found that when HUVEC were treated with ethanol or EtP followed by LTB_4 stimulation, the NO release was hampered.

4.5.4 Leukotriene receptors

Since ethanol and EtP impaired LTB_4-induced functional responses we hypothesized that this effect might be due to interference with the dynamic regulation of BLT. Hence, we assessed the expression of BLT_1 and BLT_2 as well as the effects on the upregulation of BLT_1 by LPS. Treatment of HUVEC with LPS led to increase in the levels of BLT_1 mRNA and protein expression, but not of BLT_2. However, when HUVEC were preincubated with ethanol or EtP prior to LPS stimulation, a marked reduction of BLT_1 mRNA and protein was observed. However, the up-regulation of BLT_1 induced by LTB_4 itself was not affected.

4.5.5 Calcium flux

Here, we asked if the reductions of BLT_1 mRNA and protein, as described above for ethanol and EtP treatment, would translate into a reduced $[Ca^{2+}]_i$ response, when LTB_4 was used as stimulus. Indeed, that was observed, approximately half of the response remained. Since it was not known if ethanol, added prior to LPS, would also interact with receptor-ligand interactions or on downstream signaling pathways for LPS, we tested the $[Ca^{2+}]_i$ response to another fatty acid, arachidonic acid (20:4 ω6, AA), the obligate precursor for LTB_4. Arachidonic acid is believed to confer $[Ca^{2+}]_i$ response via binding to one of the free fatty acid surface receptors, FFAR1 (or GPR40) [91, 92]. Here, when HUVEC had been treated with HBSS or LPS for 3 h and then stimulated with AA (at 40 μmol/L), $[Ca^{2+}]_i$ responses were identical; thus FFAR1 activity was not upregulated by LPS, showing the specificity of LPS treatment for BLT. Notwithstand-

ing, when ethanol had been added prior to LPS there was a significant delay in the AA peak response. As a second control for specificity we assayed thrombin induced calcium transients. LPS did not up-regulate thrombin-mediated $[Ca^{2+}]_i$ responses and ethanol had no influence on this response.

To further see if ethanol also affected the ligand binding of LTB_4 to BLT_1 we examined $[Ca^{2+}]_i$ transients in HUVEC first treated with LPS for 3 h, then exposed to ethanol or EtP for 15 min, just prior to LTB_4 stimulation. As shown in paper V, Figure 5 (right panel), ethanol and EtP totally abolished the intracellular calcium response elicited by LTB_4. Thus, not only did ethanol and EtP reduce mRNA and protein for BLT_1 but also the ability of the receptor to mediate signals to a pivotal second messenger system.

4.5.6 Potential roles of ethanol's effects on BLT

These findings implicate that ethanol has functional effect on leukotriene receptors, effects that might modulate the response to vascular injury.

Endothelial cells, strategically located at the vessel wall interphase between the blood and stroma, react to exposure to LTB_4 by promoting adhesion and transmigration of neutrophils. Here we show that ethanol and EtP can reduce the LTB_4 induced late hyperadhesiveness of endothelial cells as well as generation of nitrite, a potent vasodilator. This might lead to dawdling blood flow and hampered leukocyte recruitment. One can then imagine a scenario in which ethanol or EtP can affect endothelial cells respond to pro-inflammatory stimuli with decreased expression of leukotriene B_4 receptors, which presumably will further diminish the functional effects of LTB_4. Thus, based on the here presented results it is tempting to assume that EtP might be uses in vascular inflammatory disorders, especially in acute situations.

5 DISCUSSION

Our results provide clues to how some inflammatory functions and mechanisms are affected by ethanol and ethyl pyruvate, mechanisms that can be related to reduced neutrophils and epithelial and endothelial cell activation and recruitment seen in infected alcoholics.

5.1 RATIONALES FOR ALCOHOL CONCENTRATIONS AND FOR HUVEC

Although peripheral venous blood ethanol concentrations exceeding 0.5 per cent ethanol only rarely seen in clinical and legal practice [93], it is very probable that regional epithelial and endothelial cells encounter much higher ethanol concentrations. For example, during ingestion of wine or strong liquor, oral, esophageal, gastric and enteral epithelial cell are exposed to high 10-40 % ethanol levels. Likewise, the ethanol concentration in portal vein blood, prior to metabolism of hepatic alcohol dehydrogenases [94], is likely to be manifold higher than in any conventional peripheral blood alcohol test. Thus, the concentration of ethanol used in our study is relevant for human conditions.

To compose a relevant model system for addressing specific issues of endothelial vascular biology *in vitro* employed cells should display as many characteristics of *in vivo* cells as possible. In a study of Unger *et al*, commercially available cells expressed only a few of these desired characteristics [95]. However, HUVECs freshly obtained from human umbilical veins expressed such markers up to 4th passage. HUVECs ability to respond to, for instance thrombin or LTB_4 decline after an increasing number of passages [96].

5.2 MODERATE, BINGE AND CHRONIC ALCOHOL INTAKE IN RELATION TO EXPERIMENTAL MODELS

According to the J-shaped curve of all-cause mortality for humans, ethanol in low doses seems to give health benefits, but higher amounts of ethanol worsen the outcome and negatively affects the body's organ [2].

The effects of acute and chronic ethanol intake on the immune system may differ; in that the acute intake usually down-regulates the inflammatory response while the chronic might be associated with enhanced responses [97, 98].

The effect of ethanol on leukocyte-endothelial cell interaction may not only vary depending on the type of exposure (acute vs chronic) but also on whether an infection (or another pro-inflammatory condition) is present or not. Alcohol administration in absence of an inflammatory challenge has been reported to be associated with enhanced leukocyte recruitment to endothelial cells [99, 100], whereas we and others did not observe that ethanol alone induced such endothelial cell activation [48, 86, 100, 101]). Our present findings are consistent with previous reports showing that acute alcohol administration appears to suppress leukocyte-endothelial cell host response induced by inflammatory stimuli [101, 102]. This might be of consequence for the enhanced infection susceptibility of alcoholics, particularly for pulmonary infections by Klebsiella species [103].

5.3 ETHANOL METABOLISM IN ENDOTHELIAL CELLS

One might ask if it is ethanol *per se* that give rise to here observed effects. Ethanol is metabolized mainly by alcohol dehydrogenase (ADH), or, during chronic or excessive alcohol consumption, also by a microsomal ethanol oxidazing system (MEOS) involving cytochrome P450, notably CYP2E1. However, since HUVEC do not express ADH, and almost no CYP2E1 it is very unlikely that acetaldehyde, the major metabolite of ethanol *in vivo*, mediated the *in vitro* effects observed here, whereas thrombocytopenia in alcohol abusers has been attributed to *in vivo* acetaldehyde formation [66].

5.4 ETHANOL AND HOST DEFENCE

The inhibitory effect of ethanol and EtP of IL-8 and G-CSF production contribute to the impairment of host defenses given that these factors promote neutrophil proliferation, maturation release from the bone marrow and survival, as well as they facilitate various functional responses of mature neutrophils and promote neutrophil adhesion to endothelial cells and the subsequent migration and chemotaxis of leukocytes to foci of infection. Additionally, ethanol-associated neutropenia, together with effects on epithelial cells and other host defense barriers contribute to the increased incidence and poorer outcome of bacterial diseases in individuals who abuse alcohol [49, 59, 63, 67, 104, 105].

5.5 ETHANOL, ETHYL PYRUVATE, LEUKOTRIENE AND CELL SIGNALING

The finding of LTB_4 as an inducer of late and robust pro-inflammatory endothelial reactions is a novel concept that we will develop further in the future. Here, this phenomenon is additionally supported by our findings that LTB_4 stimulation of HUVEC appears to promote generation of nitrite [106]. We show here that treatment of endothelial cells with ethanol or EtP before LTB_4 significantly suppressed the late and sustained NO generation. This might further attenuate inflammatory reactions and host defence against intracellular pathogens, such as Mycobacterium tuberculosis and Klebsiella pneumoniae in the lung [107-109].

When LPS was used as the BLT_1 inducer, ethanol and EtP decreased this receptor expression. In contrast, LTB_4 induced BLT_1 levels was not affected with ethanol or EtP, pointing to specific regulations in this pathway. This is consistent with another study showing that BLT_1 up-regulation is dependant on NF-κB [110] and this seems to be reflected by the decrease of BLT_1 levels after ethanol and EtP treatments. One explanation for the resistance of LTB_4 induced BLT_1 up-regulation might be that LTB_4 signals through the MAP kinase pathway and not through NF-κB [111]. We reported that ethanol and EtP hamper LPS and IL-1β -induced NF-κB translocation but not TNFα induced. TNFα can bind to two different receptors, TNFR1 and TNFR2 to induce responses that are characteristic to acute inflammation via MAP kinase and NF-κB pathway, but TNFα can also signal through an epithelial/endothelial tyrosine kinase (ETK) and thereby promote migration [112]. Collectively, these results point to effects of ethanol and EtP on the NF-κB pathway.

5.6 DYNAMIC ORGANIZATION

One intriguing finding is that ethanol and EtP rapidly reduce endothelial pro-inflammatory reactions several hours after induction. This is of obvious clinical relevance, in settings of sepsis and other acute vascular injuries. These effects were not dose-dependent; therefore this modulation might be explained by posttranscriptional regulation. Recently, Banan *et al* showed that ethanol have the ability to induce dynamic rearrangement and involve instability of the subunit components of actin network [113]. Likewise, ethanol and EtP might interfere with the dynamic organization of the actin cytoskeleton in endothelial and epithelial cells. Similar results have been reported for TLR4, indicating that ethanol acts on redistribution of components of the TLR4 complex within the lipid rafts and that this is related to changes in actin cytoskeleton rearrangement [114].

5.7 SPECIFIC INTERACTIONS OF ETHANOL

Ethanol binds to highly specific molecules. Phosphatidylethanol, a direct ethanol metabolite of phosphatidylcholin, has much lower biological activity than the normally produced phosphatidic acid (PA) [57]. This leads to reduced activation of calcium channels in HUVEC, lower intracellular Ca^{2+} levels and reduced cellular activation [115]. Thus, ethanol is specifically interacting with calcium-dependent cellular reactions.

Moreover, Maiya *et al* showed that ethanol binds to a novel site in the dopamine transporter [116]. This suggests that ethanol binds preferentially to highly defined and recognizable molecules.

These examples show that ethanol is not a general cell function disrupting agent, but has discrete effects. That is further illustrated in this thesis, since, for example, G-CSF but not M-CSF release from HUVEC was inhibited. Another example is that ethanol had no effect on adhesion molecules on HUVEC or on A549 cells, yet inhibited PMN adhesion, presumably by effects on other adhesion steps that may or may not depend on IL-8 exposure on the cell surface. A third example of the specificity of ethanol is that it inhibited binding of p65 to a reporter gene but had no effect of the luciferase signal in the constitutive activated reporter system.

5.8 COMPARISONS OF ETHANOL AND ETHYL PYRUVATE

In this thesis it is clear that EtP is associated with more and more pronounced effects than ethanol on a molar basis. As discussed previously, this opens up for further evaluations of EtP in clinical settings. It also suggests that it is the ethyl moiety of EtP that is the essential molecular part and not the pyruvate group. That is further underlined by our findings that sodium pyruvate had virtually no effects on the here used systems.

6 CONCLUDING REMARKS

According to these studies, ethanol appears to attenuate the pathogen-derived pro-inflammatory gene activation and cytokine cascade. Our results provide clues to the mechanisms for reduced neutrophil and endothelial cell activation and recruitment seen in infected alcoholics.

The three first studies show that ethanol is a specific inhibitor. Thus, it is clear that ethanol and EtP have effects on release, translocation and degradation of components of the NF-κB system that are complex and which may be specific to concentration, stimulus and cell type.

The two last papers demonstrate that the rapid communication between cells induced by LTB_4 is attenuated by ethanol and EtP, likewise the later, long lasting response. That finding might be relevant for the resolution of inflammation, sometimes referred to as the katabasis phase of inflammation.

The most important result in this study indicates that ethanol and ethyl pyruvate can affect endothelial cells responses to pro-inflammatory stimuli with decreased expression of essential receptors, cytokines and growth factors, which presumably will further diminish the functional responsiveness of host response.

In conclusion, our findings regarding effects of ethanol and ethyl pyruvate on *in vitro* responses of two important cells of the inflammatory response might have implications for the development of new therapeutic strategies aiming at reducing the impact of inflammatory diseases that is a leading cause of mortality throughout the world.

7 POPULÄRVETENSKAPLIG SAMMANFATTNING

Mina studier har visat att alkohol hämmar delar av det medfödda immunförsvaret på ett specifikt sätt. Det är särskilt de celler som bildar frontlinjen mot inträngande bakterier och svampar, endotel- och epitelceller samt en sorts vita blodkroppar som heter neutrofiler, som påverkas så att de inte kan bilda viktiga signalsubstanser och tillväxtfaktorer eller samverka med varandra lika bra. Detta kan vara en förklaring till varför stort alkoholintag leder till ökad benägenhet att ådra sig infektioner (sår såväl som lunginflammation) samt utveckla brist på vita blodkroppar. Samtidigt pekar mina resultat på möjligheten att använda alkohol som läkemedel mot alltför kraftig inflammation, något som ses bl.a. vid svår allmän blodförgiftning (sepsis).

P.g.a. de många biverkningarna har etanol ingen plats bland dagens antiinflammatoriska medel. Men när etanol binds till pyruvat (en nedbrytningsprodukt av socker) bildas etylpyruvat (EtP). Detta medel testas nu i intensivvårdssituationen vid svår blodförgiftning eftersom djurförsök visat att dödligheten kan minskas påtagligt och biverkningarna är få. Jag har testat EtP parallellt med etanol och funnit att EtP är en kraftigare hämmare, som dessutom hämmar fler cellulära reaktioner och än av etanol.

Innerväggarna i blodbanorna är beklädda med endotelceller (EC). På samma sätt är lungornas luftblåsor klädda med epitelceller (EpC). Dessa celler kan odlas i provrör under speciella förhållanden. När EC behandlas med etanol eller EtP minskar såväl vidhäftning av neutrofiler som ECs och EpCs produktion av faktorer som påverkar tillströmning av neutrofiler från benmärgen. Därpå visade vi att det är inbindningen av ett signalprotein, transkriptionsfaktor κBs bindning till gener i cellkärnans DNA, som hämmas av etanol och EtP; detta leder till sämre produktion av ämnen viktiga för attraktion av neutrofiler till infektionsstället. Slutligen visade vi att etanol och EtP får cellernas mottagarsystem för signaler från andra celler att bli ineffektiva.

Sammantaget blir resultatet att etanol försämrar framkomligheten för neutrofiler, ett av kroppens naturliga bakterieförsvar, vilket kan vara orsaken till att hög alkohol konsumtion ger ökad risk för infektioner och sämre sårläknings förmåga.

I vår studie är det först vid höga koncentrationer som etanol har en hämmande effekt. Sådana höga koncentrationer uppnås visserligen lätt i mun och mage, där celler kan utsättas för upp till 50 % etanol. I blodet som leds från tarmar till levern (portablodet) förekommer sannolikt koncentrationer >1 %, som celler inom hela detta område utsätts för när alkoholen sugs upp från tarmen. Dock är koncentrationen av etanol väsentligt lägre i resten av blodbanan och i utandningsluften.

Sammanfattningsvis visar min avhandling på mekanismer varför alkoholmissbruk kan leda till ökad infektionsbenägenhet men även att dess derivat etylpyruvat i framtiden kan få en viss medicinsk användning t.ex. vid sepsis el. vid autoimmun sjukdom.

8 SAMMANFATTNING

I den här avhandlingen har jag sett att alkohol kan ha effekter på immunförsvaret. Alkoholen har en försvagande effekt på de förlopp som dödar bakterier och för bort gammal och skadad cellvävnad. Jag har kartlagt värdefulla mekanismer för infektionssvaret som påverkas av alkohol. Bland det som identifierats är att alkohol påverkar genaktivering och produktionen av vissa proteiner.

Alkohol (etanol) är det äldsta och mest använda av alla berusningsmedel. Alkohol är ett bedövningsmedel som kan verka lindrande och avslappnande. Stora mängder alkohol innebär risker för skador i hjärnan och nervsystem, lever, muskler, samt i infektionsförsvaret.

Alkoholisters benägenhet att ådra sig infektioner (såväl i sår som lungpneumonier) samt utveckla brist på vita blodkroppar (neutropeni) i samband med infektioner var startpunkt för forskningen.

I samband med infektion eller skada binder sig de vita blodkropparna till innerväggarna i blodbanorna för att komma ut till det skadade området. Väggarna i blodbanorna är beklädda med celler som kallas endotelceller. Jag har odlat dessa celler i provrör, behandlat dem med alkohol och sett att de vita blodkropparna minskar sin inbindning till endotelcellerna. Det visade sig att minskningen berodde på att alkoholen reducerar vissa proteiner som påverkar tillväxt och utmognad av vita blodkroppar från bl. a. benmärgen.

I mina försök kunde jag även visa att det är en transkriptionsfaktors bindning till genen i cellkärnans DNA som hindras. Hämningen leder till att ämnen som är viktiga för att locka vita blodkroppar till t.ex. ett sår producerades i mindre mängd. En annan viktig funktion som påverkas av alkohol är blodflödet som möjliggör framkomsten av vita blodkroppar och bakterieavdödning.

Sammantaget blir konsekvensen att alkohol förklenar kroppens naturliga försvar, vilket kan förklara varför hög alkoholkonsumtion kan leda till ökad risk för infektioner och sämre sårläkningsförmåga.

I min studie är det först vid höga koncentrationer som alkohol har en hämmande effekt. Sådana höga koncentrationer uppnås visserligen lätt i mun och mage, medan alkoholkoncentrationen är väsentligt lägre i resten av blodbanan och i utandningsluften. I blodbanorna som leder från tarmarna till levern (portablodet) däremot förekommer sannolikt höga koncentrationer av alkohol.

Alkohol betraktas inte längre som läkemedel, även om det i framtiden åter kan få en viss medicinsk användning t.ex. vid blodförgiftning.

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