Regulation of prostanoid effects in whole blood: immediate-early anti-inflammatory effects of prostaglandin E_2

By

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Danh Phung

ABSTRACT

To understand the regulation of the pathophysiological processes, such as inflammation, thrombosis and atherosclerosis, it is very important to characterize the interactions between circulating cells and which molecules that contributes to and promote these interactions. As a part of this, the role of eicosanoids in cell-cell interactions in whole blood *ex vivo* and isolated blood cells are investigated. In this study, various inhibitors were used to regulate the amount of prostaglandins (and leukotrienes) and how these eicosanoids affect the activity- and expression level of tissue factor (TF), cytokines, enzymes, and receptors involved in the intercellular communication.

Before the inhibition study, time course experiments revealed that the incubation times between 1.5 h and 2 h was sufficient for further study of the parameters under investigation. In the inhibition study, whole blood samples were preincubated with different eicosanoid inhibitors, and then stimulated with LPS for ninety minutes prior to TF activity measurement and real-time PCR analysis of cytokine gene expressions.

Aspirin did not significantly enhance the lipopolysaccharide (LPS)-induced TF activity in whole blood, however a trend for enhance induction was indicated. Prostaglandin inhibitors enhanced the LPS-induced TF activity compared to a vehicle control, with significant effect for the microsomal prostaglandin E synthase (mPGES) inhibitor MF63. A dual cyclooxygenase-2/ 5-lipoxygenase (COX-2/ 5-LOX) inhibitor was used to investigate what happened if the leukotriene pathways were blocked in parallel with COX-2. And although not significant, an inhibitory effect of monocyte TF activity was seen for the weakest and strongest dose.

To measure the mRNA expression of the cytokines tumor necrosis factor (TNF-α), interleukin 1β (IL-1β), IL-8 and monocyte chemotactic protein-1 (MCP-1), real-time PCR analysis was performed. Aspirin was found to generally increase the gene expression of these cytokines. Moreover, significant enhancement of the IL-8 mRNA expression was found for all doses of aspirin, varying from 67% to 78% enhancement for 20 μM and 200 μM, respectively. For IL-1β and TNF-α mRNA expression was increased by 86% (2 μM) and 36% (200 μM), respectively. Like aspirin, the selective cyclooxygenase-1 (COX-1) inhibitor SC-560, increased mRNA expression of all cytokines. The most pronounced effects were observed for

IL-8 and IL-1β. The MCP-1 mRNA expression level was greatly enhanced, however due to testing a low number of individuals for this cytokine, the effect was not significant.
The enhancement of IL-8 expression was also seen after addition of the selective COX-2 inhibitor, CAY10404. With the exception of IL-8, the highest dose of COX-2 inhibitor caused an insignificant reduction in mRNA expression for all cytokines. The lowest dose of prostaglandin synthesis inhibitor CAY10526 resulted into most pronounced mRNA expression for TNF-α, IL-1β and IL-8. However, the inhibitor did not significantly enhance the LPS-induced TF mRNA expression. Samples with mPGES-1 inhibitor (MF63) showed a bi-phased expression for nearly all the genes. However, the middle dose of the inhibitor induced gene expression at a higher level than controls and other doses.

Flow cytometric analysis was carried out to investigate the platelet-leukocyte heteroconjugates in whole blood stimulated with LPS and LPS in combination with platelet activating factor (PAF). Whole blood samples were stimulated for 2 h. LPS-stimulated whole blood was found to increase platelet interactions with monocyte with approximately 50% (not significant). Addition of both LPS and PAF resulted in a 3-fold significant enhancement of the conjugate formation. The binding of platelets to granulocytes decreased when LPS was added but the aggregates were observed to slightly increase when stimulated with LPS and PAF in combination.

In conclusion, these experiments have demonstrated that inhibition of the PGE_2 synthesis enhanced LPS-induced TF activity in whole blood monocytes and expression of the chosen proinflammatory cytokines. In platelet-monocyte interactions, platelets bound easily to monocytes, while binding to granulocytes seemed to require stronger stimuli.

LIST of ABBREVIATIONS

5-lox	5-lipoxygenase
AA	Arachidonic acid
APC	Allophycocyanin
ASA	Acetylsalicylic acid, Aspirin, 2-(acetyloxy)-benzoic acid
BaCi	barium citrate
BSA	bovine serum albumin
CO cells	cells of the cumulus oophorus
COX-1/2	cyclooxygenase 1/2
COX	cyclooxygenase
$cPLA_2$	cytosolic phospholipase A_2
C_t	threshold cycle
CysLTs	cysteinyl leukotrienes receptors
DMF	dimethyl formamide
DMSO	dimethylsulfoxide
EDTA	ethylenediaminotetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
ETE	epoxyeicosatetraenoic acid
FACS	fluorescence-activated cell sorting
FITC	fluorescein isothiocyanate
FLAP	5-lipoxygenase-activating protein
FSC	forward scatter light
FVII/VIIa	factor VII/VIIa
GGLT	γ-glutamyl leukotrienase
GGT	γ-glutamyl transpeptidase
HETE	hydroxyeicosatetraenoic acid
HPETE	hydroperoxyeicosatetraenoic acid
HUVEC	human umbilical vein endothelial cells
HWB	human whole blood
IC_{50}	an indication of concentrations required to inhibit an enzyme by 50 percent
IFN	interferon
IL-1β	interleukin-1 beta
LPS	lipopolysaccharide
LRP-1	low-density lipoprotein-receptor-related protein-1
LTA_4/B_4	leukotriene A_4/B_4
LTA_4H	LTA_4 hydrolase

MAPEG	membrane-associated proteins in eicosanoids and glutathione metabolism
MAPK	mitogen-activted protein kinase
MCP-1	monocyte chemotactic protein 1
MF63	Phenanthrene imidazole 3
MFI	median fluorescent intensity
MIP-1α	macrophage inflammatory protein-1α
mPGES-1	microsomal PGE synthase-1
MRP1	multidrug resistance-associated protein
NK cells	natural killer cells
NSAIDs	non-steroidal anti-inflammatory drugs
PAF	platelet activating factor
PBS	phosphate buffered saline
PE	phycoerythrein
$PGE_2/G_2/H_2$	prostaglandin $E_2/G_2/H_2$
PGHS-1/2	prostaglandin-H endoperoxid synthase-1/2
PGHS	prostaglandin H synthase
PGI_2	prostaclyclin
PGIS	prostacyclin synthase
PGT	prostaglandin transporter
PMA	phorbol myristate acetate
pNa	para-nitroanilide
POA	preoptic area
PPAR-γ	peroxisomal proliferators-activated receptor- γ
PSGL-1	P-selectin glycoprotein 1
qPCR	quantitative real-time polymerase chain reaction
RANTES	regulated upon activation normal T cell expressed presumed secreted
RCS	rabbit aorta contracting substance
SSC	side scatter light
TBS	tris buffered saline
TF	tissue factor, thromboplastin
TFPI	tissue factor pathway inhibitor
TLR-4	toll-like receptor 4
TNF-α	tumor necrosis factor alpha
TxA_2/ B_2	thromboxane A_2/ B_2
TxS	thromboxane synthase
VSMC	vascular smooth muscle cell

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1. INTRODUCTION

The interactions between platelets and leukocytes in the circulation have a key role in physiological and pathophysiological processes. Inflammatory and thrombotic events control various cardiovascular syndromes. In patients with type I diabetes mellitus, acute myocardial infarction, stroke and sepsis accumulating levels of platelet-leukocyte conjugates are observed in the circulation (Furman *et al.*, 2001; Nijm *et al.*, 2005). Plasma from patients with hypercholesterolemia and smokers also contain high amount of platelet-leukocyte conjugates (Lehr *et al.*, 1997; Nijm *et al.*, 2005). Circulating larger platelet-leukocyte aggregates are often an indication of plaque instability and ongoing vascular thrombosis and inflammation (Freedman & Loscalzo, 2002). In addition, platelet-leukocyte aggregates interact with atherosclerotic lesions indicating that cell-cell interactions between platelets and leukocytes may modulate and sustain inflammatory events at the vascular wall (Huo *et al.*, 2003).

In ongoing inflammation, or when arterial wall is injured, cells located around this site will be activated. The activation lead to subsequent release of receptors and activator molecules, resulting in increased amounts of these molecules exposed on the cell surface. Following the exposure of, for example P-selectin on activated platelets, leukocytes expressing the P-selectin ligand, P-selectin glycoprotein-1 (PSGL-1), will bind stronger to platelets and thus also to the arterial wall (as reviewed in Weyrich *et al.*, 2006). These interactions send intracellular signals into the leukocytes, which in turn increases the expression of surface receptors such as CD11b/ CD18 (integrin $\alpha_M\beta_2$,Mac-1) and the monocytic tissue factor (TF, CD142). Furthermore, such cell-cell interactions take part in the initiation of the atherogenesis process at vascular injury spots (Nakagomi *et al.*, 2000). Thrombosis, atherosclerosis and sepsis are partly controlled through an inflammation process which starts in the circulation. The interactions between platelets, leukocytes and extracellular matrix are critical in the inflammation process as these interactions are sites for intercellular communication, transfer of information and formation of molecules which provide development and progression of the diseases (Lindemann *et al.*, 2001). The platelets then release various critical signal molecules, such as platelet activating factor (PAF) and P-selectin, which in turn stimulate biosynthesis of the leukocytes' secondary activator molecules. These molecules (PAF and P-selectin) interact and activate leukocytes to produce molecules such as interleukin 8 (IL-8), monocyte chemotactic protein 1 (MCP-1) and macrophage inflammatory protein 1 (MIP-1α). These molecules result in a further accumulation of leukocytes at the inflammatory point.

Together all these signal substances manifest the inflammation (Eilertsen & Østerud, 2005; Eilertsen & Østerud, 2004). It has earlier been indicated that leukotriene B_4 (LTB_4), prostaglandin E_2 (PGE_2), PAF and thromboxane A_2 (TxA_2) are important contributor molecules in upregulation and activation of TF (Eilertsen & Østerud, 2002; Eilertsen *et al.*, 2003; Østerud, 1992; Østerud *et al.*, 1992). However, these effects still remain to be fully investigated and some of the remaining questions will hopefully be answered through this work.

This thesis will give a brief introduction to blood coagulation and inflammation, including some background about bacterial lipopolysaccharide (LPS) as well as proinflammatory molecules and mediators such as tissue factor, arachidonic acid metabolites, cytokines, and enzymes. The aims of the studies are presented. Materials and methods used are presented including some theoretical background to the main methods/ techniques used in this study. Finally, this thesis will present various cellular activation responses to different inhibitors to regulate the amount of prostaglandins and leukotrienes, with respect to activity- and expression level of TF, cytokines, enzymes and intercellular communication.

1.1. Inflammation

The immune system is the body's major defence systems and is composed of many specialized cell types that collectively protect the body from bacterial, parasitic, fungal, viral infections, and from the growth of tumor cells. Inflammation is one of the first responses of the immune system to infection involving the recruitment of immune cells to the site of injury. An inflammatory reaction serves to establish a physical barrier against the spread of infection and to promote healing of any damaged tissue following the clearance of pathogens. Many cell types (granulocytes, monocytes and macrophages) and mediators (thromboxane, leukotrienes, PAF, interleukins and tumor necrosis factors) are involved in this process. Some mediators are pro-inflammatory (increasing inflammation), others are anti-inflammatory (decreasing inflammation).

Leukocyte such as T- and B-lymphocytes, granulocytes, monocytes, macrophages, natural killer (NK) cells and among other cells, and protein rich fluid are recruited from the circulation to fight infection in response to pro-inflammatory chemical mediators. The protein fluid mobilized to the injured tissue contains various proteins and materials to make foreign

substances easier to phagocytize. Exudate is the collective term of leukocytes and the protein rich fluid. They account for the swelling at the sight of damaged tissue. At the local site of injury, the pro-inflammatory products are released. The released products enhance neutrophils, T- and B cells, platelets and coagulation factors to produce anti-inflammatory mediators to counteract the local inflammation. The inflammatory process lasts until the damaged tissue has been removed and repaired.

1.2. Initiation of blood coagulation

The process of blood clotting and then subsequent dissolution of the clot, following repair of the damaged tissue is termed hemostasis. Upon vessel damage, four major events occur in a set order following the loss of vascular integrity: 1. vascular constriction, which limits the flow of blood to the area of injury, 2. platelets become activated by collagen and aggregate at the site of injury, forming a temporary, loose platelet plug, 3. to ensure stability of the initially loose platelet plug, a fibrin mesh (also called clot) forms and entraps the plug, 4. finally, the clot is dissolved in order for normal blood flow to resume. This dissolution of clot occurs through the action of plasmin.

When blood comes into contact with foreign matter like tissues or other surfaces, platelets and leukocytes agglutinate and blood becomes exposed to traces of TF. The exposed TF then initiates a reaction that leads to the conversion of prothrombin to thrombin in the presence of calcium ions, which converts fibrinogen into the fibrin strands of the blood clot. Destroyed tissue cells provide a second and major source of TF, which causes the blood to clot more rapidly at a wound site (Rapaport & Rao, 1995).

The formation of fibrin during haemostasis stems from local exposure of TF and the consequential activation of the coagulation cascade leading to the generation of thrombin at the site of injury. TF serves as a cellular receptor and cofactor for plasma factor VIIa (FVIIa), the enzyme initiating the coagulation protease cascade, leading ultimately to the generation of thrombin and fibrin (Eilertsen & Østerud, 2005) .

Coagulation is highly conserved throughout biology, and involves both a cellular (platelet) and a protein (coagulation factor) component. The system in humans has been the most extensively investigated and is therefore the best understood.

1.3. Introduction to tissue factor

Tissue factor, the protein component of tissue thromboplastin (Bach, 2006), also known as thromboplastin, coagulation Factor III and CD142, is a 47 kDa transmembrane glycoprotein. TF is a high-affinity, cell-surface receptor and essential cofactor for the serine protease factor VIIa (Bach *et al.*, 1986; Broze, 1982). The TF-VIIa complex activates factor X (FX) directly and indirectly via factor IXa (FIXa) generation, which lead to thrombin formation. The ability of TF to serve as a cofactor in the initiation of both the extrinsic and the intrinsic coagulation pathways underscores its critical role in coagulation (Rapaport & Rao, 1995).

Expressed TF has a large extracellular domain (219 residues), a hydrophobic transmembrane domain (23 residues) and a cytoplasmic carboxyterminal domain (22 residues) (Morrissey *et al.*, 1987; Spicer *et al.*, 1987), Figure 1.

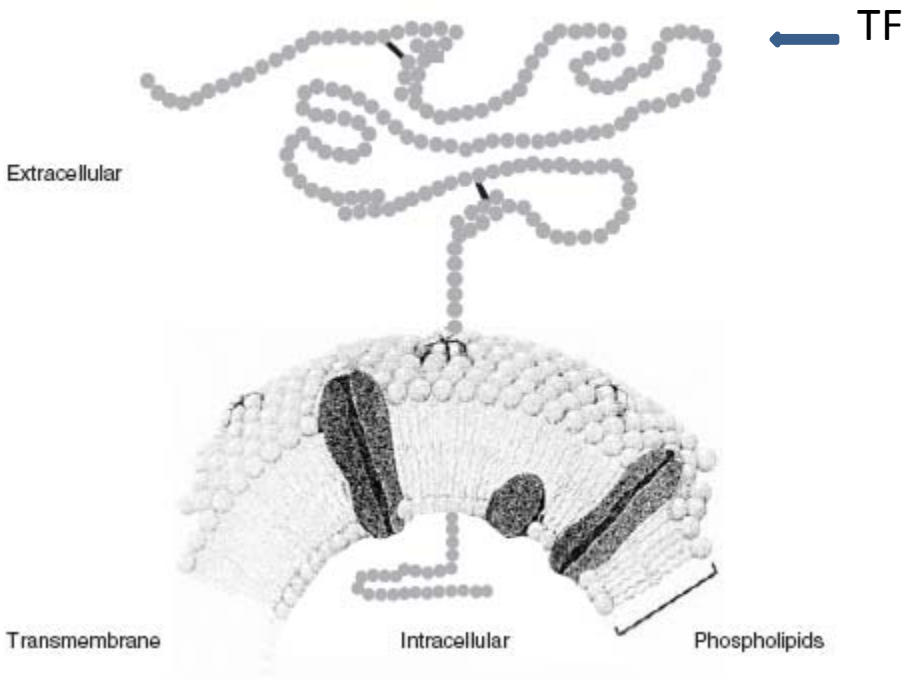

Figure 1. **Tissue factor model**. This TF model shows the extracellular part, which is located outside the cell and binds FVIIa, and transmembrane part, an intracellular domain, which is involved in the signaling function of TF. (Modified from Lwaleed *et al.*, 2007).

The structure of TF consists of three domains. Domain 1 of TF is located outside the cell and binds FVIIa. The binding of factor VIIa to TF occurs via protein-protein interactions by both molecules. Factor VIIa consists of several domains. One of these domains, the carboxylated GLA domain, binds in the presence of calcium to negatively charged phospholipids. Binding

of VIIa to negatively charged phospholipids greatly enhances the protein-protein binding of VIIa to TF. Domain 2 of TF crosses the hydrophobic membrane, and domain 3 is involved in the signaling function of TF.

TF is primarily located in the adventitia of blood vessels (the outermost part of arteria, i.e. fibroblast), and it is believed to come into contact with blood only after vascular damage (Drake *et al.*, 1989; Wilcox *et al.*, 1989). TF is a constituent of both the subendothelial layer of the vascular wall and the extravascular tissue. It thereby forms a protective lining around the blood vessels and is ready to activate blood coagulation if vascular integrity is compromised (Ryan *et al.*, 1992). Endothelial cells and blood monocytes (in contact with the bloodstream) do not constitutively express functional TF and do not have intracellular stores of TF (Lwaleed *et al.*, 2007). Functional (active) TF is not normally expressed by cells within the bloodstream except in trace amounts in circulating monocytes (Østerud *et al.*, 2007). However, gene transcription and subsequent protein expression can be induced in monocytes and macrophages by gram-negative bacterial lipopolysaccharide (endotoxin) or complement- and immune complexes (Amirkhosravi *et al.*, 1996; Østerud *et al.*, 1990; Roth, 1994).

1.3.1. Forms of tissue factor

There are three forms of TF which have been described, 1. free TF (includes a soluble, alternatively spliced TF in plasma resulting from alternative splicing of primary RNA transcripts (Bogdanov *et al.*, 2003; Bogdanov *et al.*, 2006)), 2. another soluble TF which is a truncated form of TF generated from a proteolytic cleavage at or near the linkage between the transmembrane and extracellular domains of the TF molecule, which forms protein fragments (Morrissey, 1991), and 3. membrane-bound TF, which includes cellular TF (found *in vivo* on fibroblasts, smooth muscle cells, monocytes, macrophages, and tumor cells) and lipid-vesicle-bound TF (found in urine or semen). “Blood-borne” TF is in addition to the two soluble forms, a full length plasma TF which circulates in association with cell-derived membrane microparticles and TF-bearing microparticles. This type of TF arises mainly from monocyte-macrophage membrane-lipid rafts or from regions of high raft content.

Cellular membrane-bound TF is found in surface-, encrypted- and intracellular pool. On the plasma membrane, TF resides mostly in a cryptic configuration. The release of TF often referred to as de-encryption, coincides with an increase in cell-surface phosphatidylserine, which results in apoptosis and necrosis (Lwaleed *et al.*, 2007).

It is believed that monocytes are the only circulating cells which synthesize and express TF upon stimulation. However, some studies have reported that neutrophils and platelets also may be able to synthesize and express TF (Giesen *et al.*, 1999). Studies from Østerud and colleagues have indicated that granulocytes and platelets in whole blood do not express significant amount of TF antigen or activity but may acquire expressed TF upon stimulation with LPS or LPS in combination with phorbol myristate acetate (PMA) or tumor necrosis factor alpha (TNF-α) (Østerud, 2000; Sovershaev *et al.*, 2007). The results from these studies suggest that the TF activity measured in granulocytes probably results from monocyte-derived TF-rich microparticles strongly bound to granulocytes (Østerud, 2000).

1.3.2. Regulation of tissue factor expression and activity

In addition to TF expression in adventitia of blood vessels, brain (astrocytes) (Eddleston *et al.*, 1993), lung (bronchiolar and alveolar cells), heart (cardiac monocytes) (Luther & Mackman, 2001), kidney (tubular cells), and placenta (trophoblasts) (Lwaleed *et al.*, 1999), TF is also found to be expressed in a number of embryonic cells including epithelial and smooth muscle cells (review, Siegbahn, 2000).

The human TF gene is located on chromosome 1. The TF promoters are organized in a way which allows the gene to be inducible- (monocyte, macrophage and endothelial cells) and constitutive (fibroblast and epithelial cells) regulated (as reviewed in Tremoli *et al.*, 1999). There are five putative transcription-factor sites, which are responsible for basal TF-gene expression, and two regulatory enhancers (proximal and distal) on the TF gene promoter (Moll *et al.*, 1995).

In vivo TF-FVII/FVIIa complex activity is regulated in an FXa-dependent manner by the TF-pathway inhibitor (TFPI). Although TFPI reacts directly with the TF-FVIIa complex, the efficiency of this reaction is significantly increased after TFPI binds FXa, which provide a feedback-inhibition pathway that limits coagulation activation. Moreover, TFPI down regulates cell-surface TF in monocytic cells through the low-density lipoprotein-receptor-related, protein-1 (LRP-1)-dependent pathway (Hamik *et al.*, 1999). This down- regulation is thought to be achieved via a mechanism involving the ability of TFPI to bridge TF-FVIIa complex to LRP-1 so that the multiprotein complex is internalized as an intact unit (review, Gonias *et al.*, 2004).

1.4. Lipopolysaccharide

Lipopolysaccharide from gram-negative bacteria induces production and exposure of TF on the surface of circulating monocytes. It is a much used study model for investigating the molecular reaction and interactions connected to TF and inflammation. Once LPS comes in contact with host cells (i.e. monocytes, macrophages or neutrophils) a cluster of proinflammatory responses are generated (Lund, 2004). LPS-stimulated monocytes also release prostanoids such as thromboxanes A_2 and prostaglandin E_2 which have a feedback on platelets.

The outer membrane of gram-negative bacteria is composed of a lipid and a polysaccharide (carbohydrate) joined by a covalent bond. The resulting macromolecule is known as lipopolysaccharide. LPS contribute greatly to the structural integrity of bacteria and protects the membrane from certain kinds of chemical attacks. LPS is also essential for bacterial growth and viability by participating in the physiological membrane function (Nikaido & Vaara, 1987).

1.4.1. LPS structure

Figure 2 shows a schematic representation of a gram-negative lipopolysaccharide.

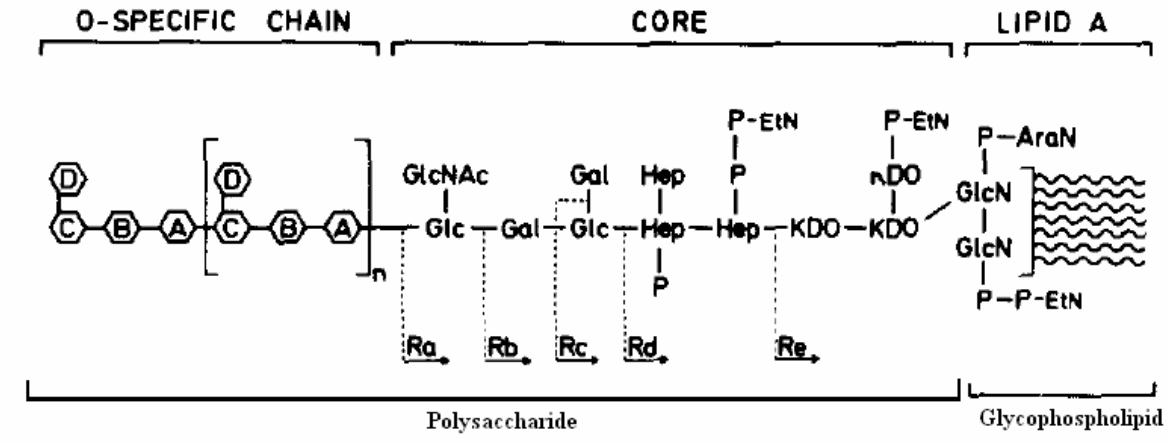

Figure 2. General structure of Gram-negative LPS. Capital letters represent sugar residues; Glc, $_D$-glucose; Gal, $_D$-galactose; GlcN, $_D$-glucosamine; GlcNAc, N-acetyl-$_D$-glucosamine; Hep, $_L$-glycero-$_D$-*manno*-heptose; KDO, 2-keto-3-deoxy-$_D$ -*manno*-ocotnate; AraN, 4-amino-$_L$-arabinose; P, phosphate; EtN, ethanolamine; Ra to Re, incomplete R form lipopolysaccharides. (Modified from Luderitz *et al.*, 1982).

Structurally, LPS can be divided into three separate regions, a lipid region, the lipid A, and a covalently bound hydrophilic heteropolysaccharide chain. The heteropolysaccharide chain is often branched and subdivided into the core and the O-specific chain (Luderitz *et al.*, 1982).

The **O-chains** are commonly highly hydrophilic and are made up by a sequence of identical oligosaccharides, the repeating units. It is the outermost part of the LPS molecule (Luderitz *et al.*, 1982). The average length of O-chains varies from one species to another. An SDS-polyacrylamide-gel electrophoresis investigation of the LPS of *Salmonella Typhimurium* revealed an average length of 7-10 repeating units, but a high degree of heterogeneity was seen in Palva & Mäkelä (1980). The O-polysaccharide part of the LPS is the major target by host antibodies and is thus referred to as the O-antigens (review, Erridge *et al.*, 2002; Wiese *et al.*, 1999).

The **core** structures express less diversity than O-chains. This section is composed of unusually sugars such as Kdo and Hep, in addition to glucose, galactose and glucosamine.

Lipid A is the highly hydrophobic lipid anchor, and is the toxic part of the LPS molecule, its structure is highly conserved among different bacterial groups. The structure is composed of a β-$_D$-glycosamine (GlcN)-(1-6)-α-$_D$-GlcN disaccharide backbone carrying two phosphoryl groups. Up to four acyl groups are attached by ester or amide linkage to this structure, which in turn can be substituted by more fatty acids to give LPS molecules with up to seven acyl chains (Luderitz *et al.*, 1982).

LPS strongly stimulates both the innate immune response and inflammation in humans and other mammals. It is a well known and frequently used inducer of TF expression on monocytes and of cytokine and eicosanoid production in *in vitro* models.

1.4.2. *Escherichia coli* LPS

The LPS of *E. coli* alone can be the causative agent of a number of diseases, including travelers' diarrhea and infantile diarrhoea. The human gut contains many grams of *E. coli* LPS. The release of large amounts of LPS into the blood stream (e.g. can occur following surgery), can lead to multiple organ failure, shock and potentially death (Erridge *et al.*, 2002). The high toxicity of *E. coli* LPS can be explained to a large extent by its structure (Figure 3). The hexa-acyl disphosphorylated lipid A with acyl chains C12-C14 is believed to provide

maximal activation of toll-like receptor 4 (TLR-4), and hence activation of monocytes. However, the optimum structure for cellular activation is not the same as the optimum structure for cell binding. Experiments revealed that the optimum structure for binding to cells is a bisphosphorylated disaccharide together with some fatty acids and in no particular arrangement. Structures that bind well to cells do not necessarily induce strong monokine release. Nevertheless, it is the number, nature and distribution of fatty acids, i.e. the acylation pattern of the lipid A which determines the endotoxic activity of these compounds (review, Rietschel *et al.*, 1994).

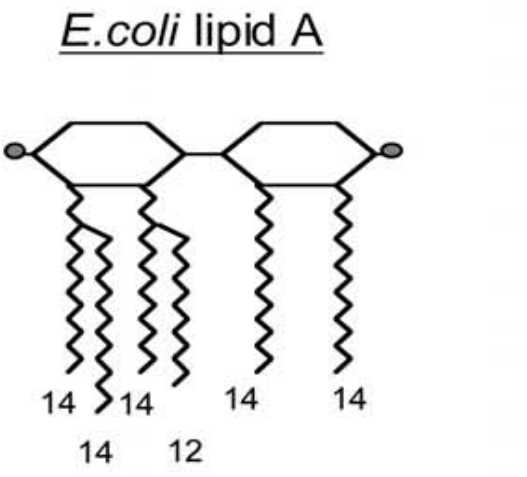

Figure 3. *E. coli* lipid A structure. Structure shown is in complete form (adapted from Erridge *et al.*, 2002). LPS used in the present study is from the *E.coli* 026:B6 strain.

1.5. Arachidonic acid and its metabolites (eicosanoids)

One of the most important classes of lipid mediators is known as eicosanoids. The word *eicosa* is Greek and means twenty; twenty carbon fatty acid derivatives. Eicosanoids are potent local hormones that are released by the cells to self-activate (autocrine) or activate nearboring cells (paracrine). These signaling are then rapidly inactivated.

In humans, the biosynthesis of eicosanoid starts with arachidonic acid (AA) oxygenation. The products of eicosanoids include prostanoids (prostaglandins, prostacyclins and thromboxanes), leukotrienes, and various epoxy, hydroxyl and hydroperoxy fatty acids [epoxyeicosatetraenoic acids (ETEs), hydroxyeicosatetraenoic acids (HETEs) and hydroperoxyeicosatetraenoic acids (HPETEs)] (Maclouf *et al.*, 1998; Samuelsson, 1979).

Arachidonic acid (5,8,11,14-eicosatetraenoic acid, C20:4, n-6), is a long chain polyunsaturated fatty acid and become released from the cell membrane by the action of phospholipase A_2 on membrane phospholipids. Metabolites of arachidonic acid (eicosanoids) have been implicated as mediators or modulators of a number of physiological functions and pathologic conditions, Figure 4 (Samuelsson, 1983). It is reported that eicosanoids can be generated in human skin and exert proinflammatory and immunoregulatory actions through their effects on blood vessels and inflammatory cells (Ruzicka, 1990). A number of skin diseases such as ultraviolet dermatitis (Miller *et al.*, 1994), atopic dermatitis (Ikai & Imamura, 1993), and urticaria (Sabroe & Greaves, 1997) are predicted to form due to the action of eicosanoids, since eicosanoids affect both blood vessels and inflammatory cells.

The rate limiting step for eicosanoid formation starts with esterification of the arachidonic acid at the *sn*-2 position of cell membrane phospholipids. Activated phospholipase A_2 catalyzes ester hydrolysis and the release of the free acid. Arachidonic acid can be subsequently transformed by several pathways, but these metabolic fates depend on the cell type and its specific complement of enzymes (Needleman *et al.*, 1986).

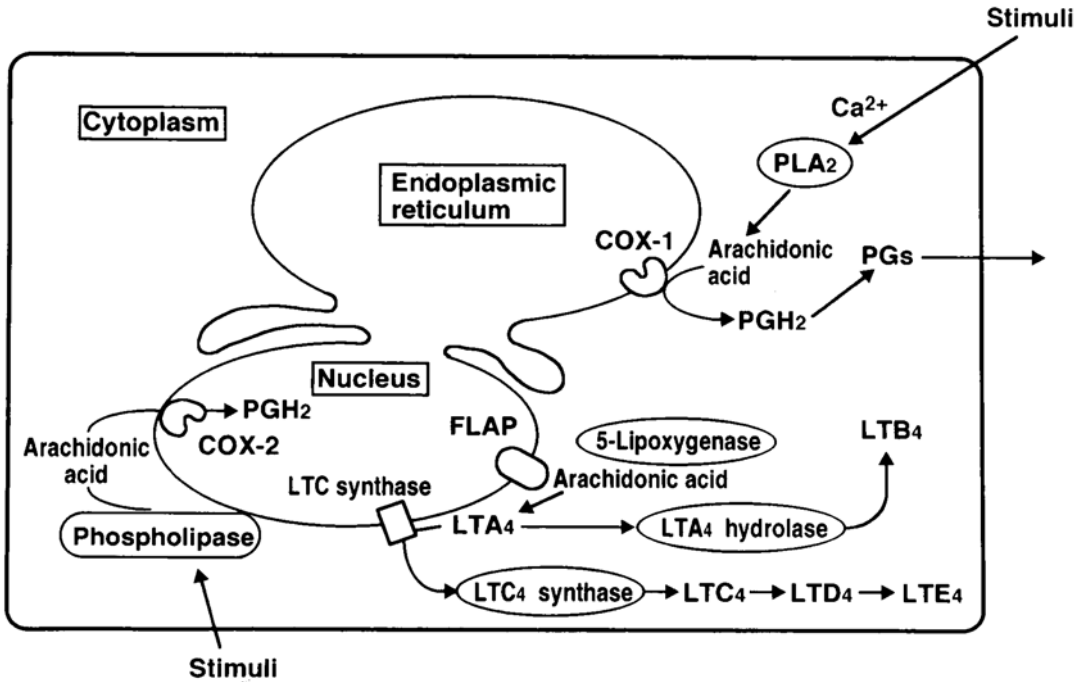

Figure 4. Eicosanoids mechanisms. Eicosanoids bind to individual receptor systems and transduce various signals in the cell (adapted from Ikai, 1999).

The arachidonic acid pathway constitutes one of the main mechanisms for the production of pain and inflammation, as well as controlling homeostatic function. The pathway produces different classes of end products (Figure 5):

1. The prostaglandins (from cyclooxygenase metabolism)
2. The prostacyclines PGI_2
3. Thromboxane TxA_2
4. The leukotrienes (from lipoxygenase metabolism)

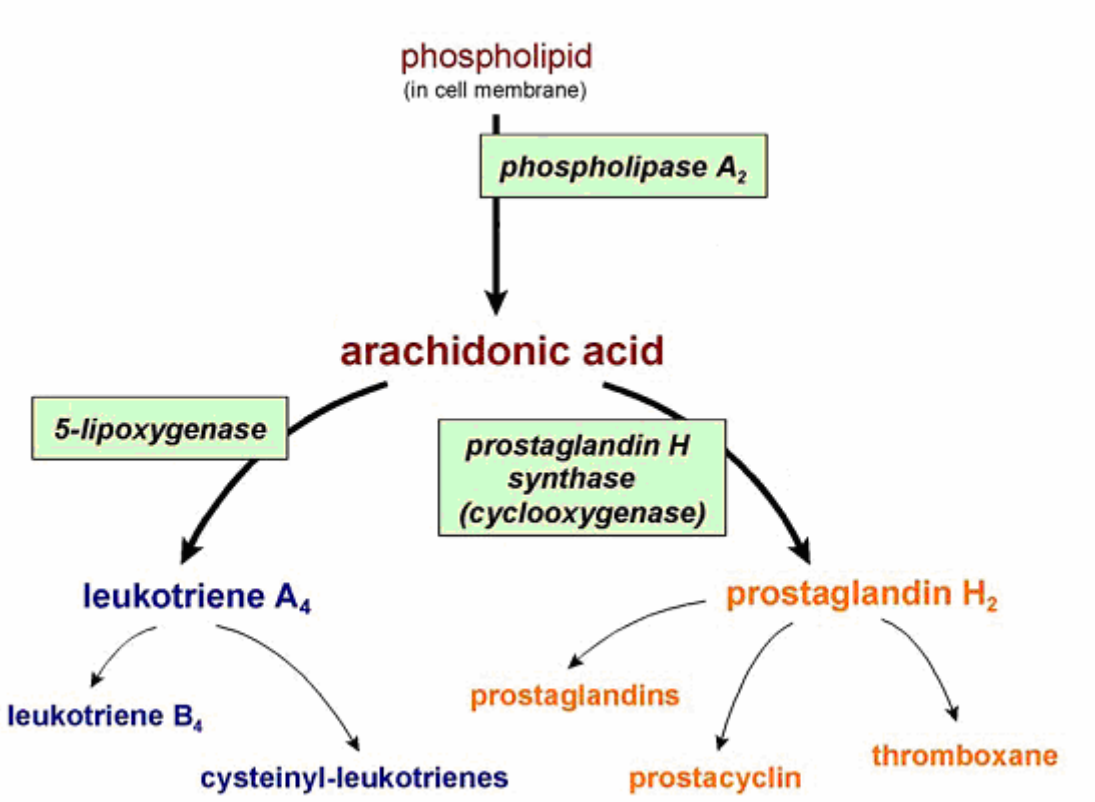

Figure 5. Biosynthesis of Eicosanoids from arachidonic acid. The metabolism of arachidonic acid is a highly branched pathway. Phospholipase A_2, prostaglandin H synthase, and 5-lipoxygenase are three important enzymes regulating eicosanoid synthesis. (Modified from Medical Center, 2004).

1.5.1. Biosynthesis of prostaglandins

The first enzyme unique to the synthesis of prostaglandins, prostacyclins and thromboxanes (Figure 5) is prostaglandin H synthase, or better known as cyclooxygenase (COX). The COX pathway involves a series of peroxidation and cyclizations of arachidonic acid, leading initially to unstable intermediates and prostaglandin G_2 and H_2 (PGG_2 and PGH_2) (review, Ikai, 1999). There are two COX isoforms, COX-1 and COX-2, each having specific catalytic, regulatory and tissue distribution properties. The former enzyme is responsible for basal,

constitutive prostaglandin synthesis, whereas COX-2 is important in various inflammatory and can be induced by cytokines, growth factors and tumor promoter (DeWitt, 1991).

The unstable intermediate, PGH_2, undergoes isomerization or reduction, and are converted to other products by their respective enzymes (Figure 6) (Urade *et al.*, 1995). Thromboxane synthase is found in platelets and macrophages, prostacyclin synthase in endothelial cells and PGF synthase in uterus, while two types of PGD synthase are found in brain and mast cells. Microsomal PGE synthase (mPGES), a member of the MAPEG (membrane-associated proteins in eicosanoid and glutathione metabolism) family, is responsible for the PGE_2 synthesis (Jakobsson *et al.*, 1999).

Prostaglandins act as autocrine and paracrine lipid mediators (i.e. they signal at or immediately adjacent to their site of synthesis). They are not stored but are synthesized *de novo* from activated cells. These cells are activated by mechanical trauma or by specific cytokines, or various inflammatory stimuli (Figure 6) (review, Funk, 2001).

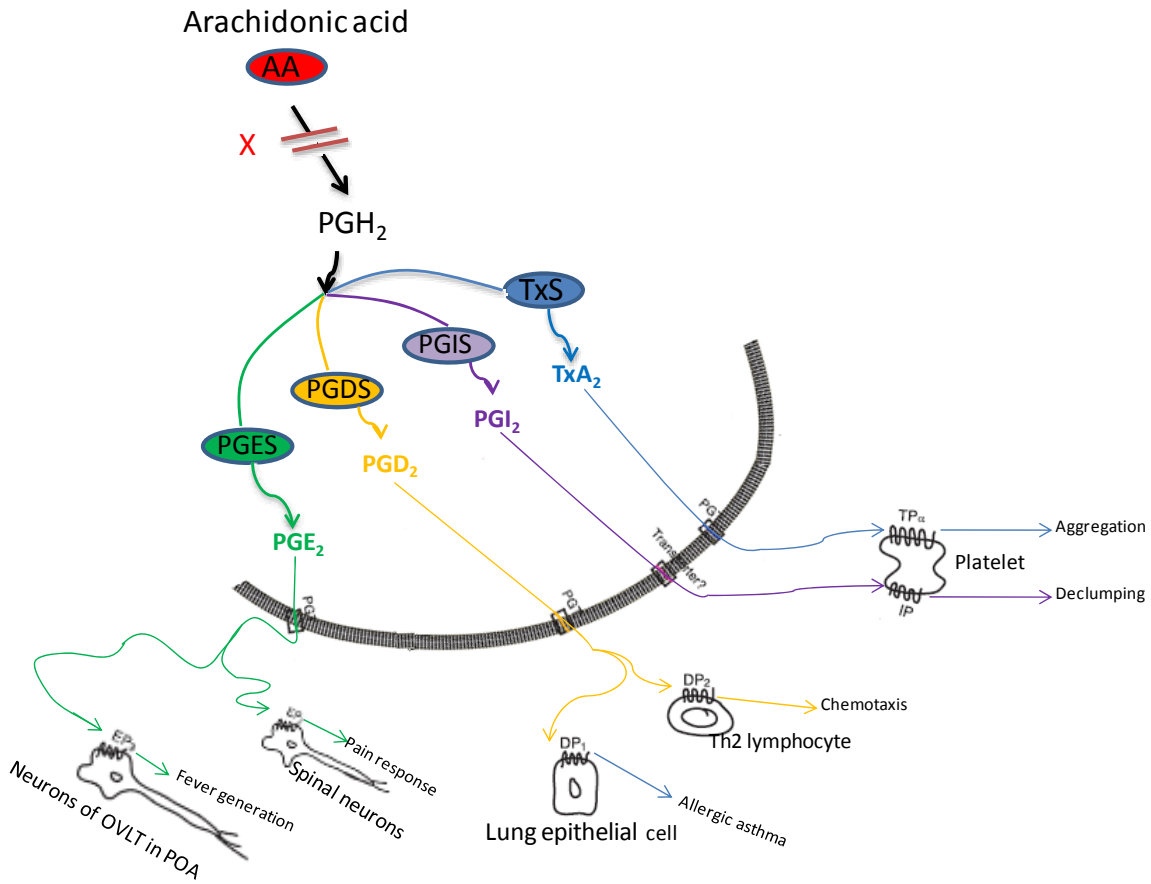

Figure 6. Prostaglandin synthesis and actions. Only a few of the many diverse activities of prostaglandins are shown here. Arachidonic acid release from membrane lipids and metabolism by COX-1 or COX-2 to the intermediate PGH_2. Formed prostaglandin products may undergo facilitated transport from the cell through a known prostaglandin transporter (PGT) or other carrier to exert autocrine or paracrine receptors EP_1, EP_3, DP_1, DP_2, IP, and TP_α on the cell type indicated. Prostaglandins could potentially enter the nucleus and activate nuclear hormone receptors such as PPAR-γ. PGES – PGE synthase; PGDS – PGD synthase; PGIS – prostacyclin synthase; TxS – thromboxane synthase. OVLT in POA – the organum vasculosa lamina terminalis at the midline of the preoptic area. X mark – site of inhibition by NSAIDs, celecoxib and rofecoxib. (Figure based on Funk, 2001).

1.5.1.1. Thromboxanes – biologically active compounds derived from prostaglandin endoperoxides

Thromboxane (Tx) is a member of the eicosanoids family and a potent vasoconstrictor (Samuelsson *et al.*, 1978), and causes both platelet aggregation (Hamberg *et al.*, 1975) and white blood cell adhesion (Spagnuolo *et al.*, 1980).There are two major thromboxanes, namely thromboxane A_2 and thromboxane B_2 (TxB_2). TxA_2 is a metabolitic oxidation product derived from arachidonic acid in cyclooxygenase and thromboxane synthase dependent reactions (Arita *et al.*, 1989; Hamberg *et al.*, 1975).

Thromboxane is named for its role in clot formation (thrombosis). Hamberg *et al.* demonstrated in 1975 a substance which was former known as rabbit aorta contracting substance (RCS). This substance was a chemically unstable metabolite of prostaglandin endoperoxides and was predominantly produced in platelets, they named it thromboxane A_2 (Hamberg *et al.*, 1975). TxA_2 is rather unstable and is rapidly hydrolyzed into the almost inactive and stable thromboxane B_2 with a very short half-time in physiological solutions (ca. 30 sec) (Arita *et al.*, 1989). TxA_2 is a potent inducer of platelet aggregation and a constrictor of vascular and respiratory smooth muscles (Svensson *et al.*, 1976; Svensson *et al.*, 1977). It has been postulated to be a mediator contributing to the pathophysiology of a variety of disease processes, such as thrombosis, atheroscleoris and myocardial ischemia (Arita *et al.*, 1989). TxA_2 is released in substantial amount by activated platelets, and has also been demonstrated to be the principal product of the COX pathway in monocytes (Caughey *et al.*, 1997). The production of TxA_2 is dependent on COX. The COX-1 inhibitors such as aspirin will decrease the production and redirect AA metabolism to other products through the

lipoxygenase pathways. Caughey *et al*. found in 1997 that if thromboxane production is inhibited in stimulated monocytes, both TNF-α and IL-1β productions decrease (Caughey *et al.*, 1997). However, the same group proposed later that TxA_2, through the activation of the mitogen-activated protein kinase (MAPK) pathways helps upregulate regulate COX-2 synthesis and prostacyclin synthesis as demonstrated in human umbilical vein endothelial cells (HUVEC) (Caughey *et al.*, 2001).

1.5.1.2. Cyclooxygenases

Prostaglandin endoperoxide H synthases-1 and 2 (PGHS-1 and 2) catalyze the committed step in prostanoid synthesis (Smith & DeWitt, 1996; Smith *et al.*, 1996). The generic names for these isozymes are cyclooxygenase-1 and 2 (COX-1 and COX-2). The COX enzymes are monotopically inserted in the endoplasmic reticulum (ER) and nuclear membrane with the substrate binding pocket precisely orientated to take up released arachidonic acid (Smith *et al.*, 2000). These two isozymes have very similar active site structures, catalytic mechanisms, products and kinetics. However, there are structural differences between them which are of important for pharmacological and biological consequences. First, the active site of COX-2 is larger and more accommodating than that of COX-1. Second, although the gross kinetic properties of the enzymes are nearly identical, COX-1, but not COX-2, exhibits negative allosterism at low arachidonate concentrations. This difference may permit COX-2 to compete more effectively for newly released arachidonate when the isozymes are expressed in the same cell (Smith *et al.*, 2000).

COX-1 is a constitutive enzyme whose expression is regulated developmentally. This enzyme produces prostaglandins in the ER, which exit cells and signal through cell surface G protein linked receptors (as reviewed in DeWitt & Smith, 1995). Prostaglandins produced by COX-1 are thought to mediate physiological responses to circulating hormones that require constant or rapid modulation or both. COX-1 is expected to be expressed at higher concentrations in tissues and in cells where prostaglandins have specialized signaling functions, such as kidney, stomach, platelets and vascular endothelium (DeWitt & Smith, 1995).

COX-2 is an inducible enzyme that is normally absent from cells, but is expressed in response to growth factors, tumor promoters or cytokines. In contrast with COX-1 which forms products on the ER, COX-2 produces prostaglandins within or on the nuclear envelope (Morita *et al.*, 1995). One aspect of COX-2 function that is more clearly defined is its role in

inflammation. It is expressed at sites of inflammation and in monocytes and macrophages stimulated with lipopolysaccharide or interleukin-1 (IL-1). The expression of COX-2 is inhibited by anti-inflammatory glucocorticoids, both *in vivo* and *in vitro*, and by anti-inflammatory cytokines such as IL-4 and IL-10 (DeWitt & Smith, 1995).

These two enzymes are of particular interest because they are the major targets of nonsteroidal anti-inflammatory drugs (NSAIDs) including aspirin and ibuprofen and the COX-2 inhibitors Celebrex and Vioxx. They are also involved in a range of pathologies that for COX-1 includes thrombosis (Patrignani *et al.*, 1994; Patrono, 1994), and for COX-2 includes inflammation, pain, and fever (Riendeau *et al.*, 1997; Zhang *et al.*, 1997), various cancers, and Alzheimer's disease (McGeer & McGeer, 1999).

1.5.2. Biosynthesis of leukotrienes

In contrast to its metabolism in the presence of PGHS, arachidonic acid may face different fates in other cell environments. Leukotrienes are made predominantly by inflammatory cells, such as polymorphonuclear leukocytes, macrophages and mast cells (Funk, 2001).
The lipooxygenase pathway begins with the oxygenation of arachidonic acid to 5-, 12-, or 15-hydroperoxyeicosatetraenoic acids. 5-lipoxygenase (5-LOX) is a soluble, non-heme-iron protein and, like PGHS, is expressed as distinct isoforms. The enzyme is located in the nucleus in some cell types and the cytosol of others, and catalyzes the first two steps of the 5-lipoxygenase pathway (Ford-Hutchinson *et al.*, 1994). The first step is the conversion of free arachidonic acid to 5-HPETE. Then 5-HPETE is converted to LTA_4 with the concerted efforts of 5-lipoxygenase-activating protein (FLAP). This labile intermediate is the substrate for various enzymatic reactions that form a number of biologically important compounds.

Depending on the cellular context, there are two subsequent metabolic routes from LTA_4 that lead to the generation of bioactive eicosanoids (Figure 4 and Figure 5). The first one involves LTA_4 hydrolase (LTA_4H), which make a hydrolytic attack of LTA_4 and yields LTB_4, a potent neutrophil chemoattractant and stimulator of leukocyte adhesion to endothelial cells (Peters-Golden & Brock, 2001; Samuelsson, 1983). The second route for LTA_4 metabolism involves conjugation with glutathione by the enzyme LTC_4 synthase to produce LTC_4. LTC_4 is further metabolized through the enzymatic cleavage of a glutamic acid to produce LTD_4 (Figure 4). LTD_4 in turn can be converted to LTE_4. These three latter leukotrienes comprise the cysteinyl

leukotrienes, or an entity described as "slow-reacting substance of anaphylaxis" for its slow and sustained smooth muscle contracting abilities (Figure 5) (Samuelsson, 1983).

The mechanism of leukotrienes actions are shown in Figure 7 below. The B-LT_1 receptor that binds LTB_4 with high-affinity elicits a toxin-sensitive G_i linked chemotatic response and stimulates neutrophil secretion. Interestingly, two subtypes of cysteinyl leukotrienes receptors, $CysLT_1$ and $CysLT_2$, mediate the actions of LTC_4 and LTD_4. $CysLT_1$ is found on airway smooth muscle cells and vascular endothelial cells, while $CysLT_2$ is detected in spleen, Purkinje fibers of the heart and discrete regions of the adrenal gland (Funk, 2001).

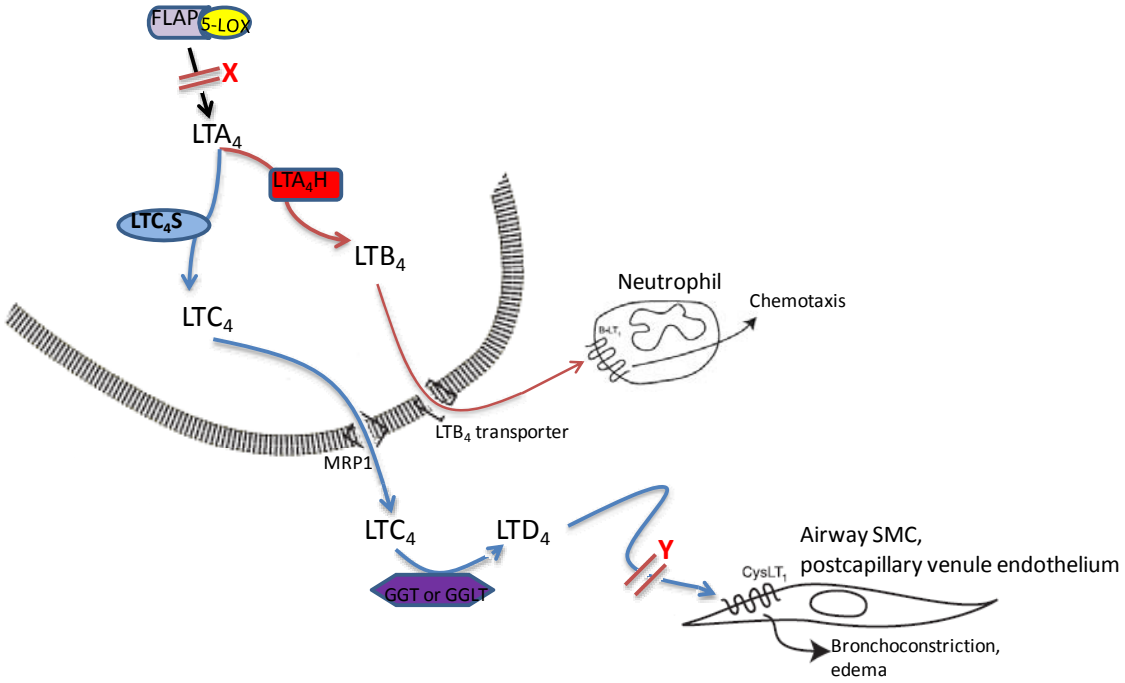

Figure 7. Leukotrienes synthesis and actions. Upon cellular activation of a mast cell or macrophage, a cascade of cell activation events leading to leukotrienes biosynthesis occurs. The FLAP protein acts as a transfer protein and facilitates presentation to 5-LOX for conversion to LTA_4. 5-LOX activity is inhibited by zileuton (Zyflo) (X mark). LTA_4 is further metabolized into LTB_4 by LTA_4 hydrolase. An uncloned transporter facilitates the efflux of LTB_4 out of the cell, where it can act on neutrophils to elicit chemotaxis or to evoke other functions. LTA_4 can also be converted to LTC_4, by LTC_4 synthase. The multidrug resistance-associated protein (MRP1) facilitate transfer of LTC_4 out of the cell, where it is metabolized by extracellular-localized γ-glutamyl transpeptidase (GGT) or γ-glutamyl leukotrienase (GGLT) to LTD_4, LTD_4 activate CysLT1 receptors to cause bronchoconstriction and edema. Drugs in e.g asthma block this binding step (Y mark). (Figure based on Funk, 2001).

1.5.3. Anti-inflammatory drugs affecting arachidonic acid metabolism

The development of anti-inflammatory pharmacological agents has been rapid and competitive. Non-steroidal anti-inflammatory agents (e.g. aspirin), act by inhibiting the COX enzymes in cells of the tissues at the inflamed site. The result is a block in the conversion of arachidonic acid, which is liberated from membrane phospholipids, into prostaglandins, prostacyclin, and thromboxane. The link between aspirin and prostaglandins were established in 1971. Aspirin inhibits the cyclooxygenase activity of prostaglandin H synthase by acetylating the hydroxyl group of a unique serine residue, this inhibition is irreversible (Medical Center, 2004) thus new protein synthesis is required for the recovery of prostaglandin synthesis.

Most NSAIDs (e.g. ibuprofen, celecoxib, flurbiprofen, indomethacin) act by different mechanisms. For instance, ibuprofen is a reversible inhibitor of PGHS activity, while indomethacin exhibits a complex, time-dependent inhibition.

Steroidal anti-inflammatory drugs are natural and synthetic steroids with glucocorticosteroid agonist characteristics. Examples of these drugs are beclomethasone, budesonide and dexamethasone. Corticosteroids bind to cytosolic or nuclear receptors and regulate the transcription of target genes. It is likely that corticosteroids carry out the inhibition of phospholipase A_2. This inhibition will prevent the mobilization of arachidonic acid and other pro-inflammatory eicosanoid precursor fatty acids. As a result, the synthesis of all eicosanoids will be blocked.

Aspirin – COX- 1/2 inhibitor (Cayman Chemical Company)

Aspirin, also known as acetylsalicylic acid (ASA), is widely used as an analgesic, antipyretic and anti-inflammatory agent. Aspirin is a potent inhibitor of prostaglandin synthesis (Vane, 1971). The compound acetylates serine-529 and serine-516 in the active site of the COX-1 and COX-2 enzymes, respectively. This deactivates the COX enzymes in an irreversible manner (Loll *et al.*, 1995). Aspirin is 170-fold more potent in inhibiting COX-1 than COX-2 (Vane *et al.*, 1998), and low-dose aspirin is expected to inhibit cyclooxygenase-1 only (Macchi *et al.*, 2006), whereas aspirin at higher doses is anti-inflammatory due to inhibition of COX-2. The antithrombotic effect of aspirin is primarily a result of and prevent thromboxane A_2 platelet activation by interfering with the biosynthesis of thromboxanes A_2 and inhibition of TxA_2-dependent platelet aggregation (Macchi *et al.*, 2006). The IC_{50} values for ovine

COX-1 and -2 are 0.75 mM and 1.25 mM, respectively, but concentrations of aspirin used in this study ranged from 2 μM as the lowest to 200 μM.

CAY10404 – selective COX-2 inhibitor (Cayman Chemical Company)

COX-2 was proved to be upregulated by bacterial lipopolysaccharide, cytokines, growth factors, and tumor promoters, which distinguished it from the primarily constitutively expressed COX-1 (Xie *et al.*, 1991). COX-2 selective inhibitors provide a unique example of accelerated drug discovery and development.

CAY10404 is one of the most selective inhibitors of COX-2, with a selectivity index (SI; SI = IC_{50} COX-1/IC_{50} COX-2) of > 500,000 (Cayman Chemical Company).

The IC_{50} of COX-2 is < 1 nM. Concentrations used in this study were 50 nM as the lowest to 5 μM.

CAY10416 – dual COX-2/ 5-LOX inhibitor (Cayman Chemical Company)

CAY10416 is a dual COX-2/ 5-LOX inhibitor. It is a potential therapeutic agent for inflammatory diseases and prostate cancer with IC_{50} values of 50 and 3 nM, respectively (Barbey *et al.*, 2002).

SC-560 – selective COX-1 inhibitor (Cayman Chemical Company)

SC-560 is a member of the diaryl heterocycle class of cyclooxygenase inhibitors which includes celecoxib (Celebrex) and rofecoxib (Vioxx), which are selective COX-2 inhibitors. Unlike these inhibitors, SC-560 is a selective inhibitor of COX-1. Using the human recombinant enzymes, the IC_{50} value for the compound with respect to COX-1 is 9 nM. However, concentrations used in this study were from 50 nM to 5 μM.

CAY10526 – mPGES-1/ COX-2 inhibitor (Cayman Chemical Company)

Prostaglandin E_2 is synthesized at sites of inflammation and plays an important role in different inflammatory diseases. It acts as a mediator of pain and inflammation and promotes bone destruction. The increased synthesis of PGE_2 during inflammation can be accounted for by increased expression of both COX-2 and mPGES-1 (Claveau *et al.*, 2003).

CAY10526 is an inhibitor of PGE_2 production through the selective modulation of mPGES-1 expression. It dose-dependently inhibits PGE_2 production in LPS-stimulated RAW 264.7 cells with an IC_{50} value of 1.8 μM without any effect on COX-2 expression (Guerrero *et al.*, 2007). Doses used in the study were from 100 nM to 10 μM as the highest concentration.

MF63 – selective mPGES-1 inhibitor (Merck)

Phenanthrene imidazole 3 (MF63) has been identified as a new potent, selective, and orally active mPGES-1 inhibitor. The compound is the first reported mPGES-1 inhibitor to demonstrate potency in a human whole blood (HWB) assay. When freshly collected blood was stimulated with LPS, the compound selectively inhibited the production of PGE_2 with an IC_{50} of 1.3 μM with no concomitant TxB_2 inhibition. This HWB IC_{50} is comparable to the ones of marketed coxibs (Riendeau *et al.*, 2001). Doses used in the study were from 200 nM to 20 μM as the highest concentration.

1.6. Cytokines

Infection, cancer, and inflammation triggers production of immunological mediators termed cytokines. Cytokines are a group of proteins and peptides used as signaling compounds by organisms. These chemical signals are similar to hormones and neurotransmitters, which allow one cell to communicate with another. Cytokines have autocrine or paracrine effects that have the ability to affect several target cells through membrane receptors inducing gene activation and protein synthesis. Cytokines often promote (inflammatory) or inhibit (anti-inflammatory) the synthesis of other cytokines, which in turn forms complex cytokine networks. Monocytes/ macrophages are one of the major sources of cytokine production in the body. The over 50 cytokines reported are clustered into several classes, such as interleukins (IL), tumor necrosis factors, interferons (IFN) and chemokines (Boulay *et al.*, 2003; Langer *et al.*, 2004). Among the proinflammatory cytokines, tumor necrosis factor α, interleukin-1β, IL-6 and IL-8 have been implicated as the primary endogeneous mediators of inflammation.

1.6.1. TNF-α

After LPS infusion, TNF-α is the first cytokine to be detected in the circulation. TNF-α is an extremely potent peptide cytokine which serves as an endogenous mediator of inflammatory, immune and host defence functions. It is a product of monocytes during monocyte activation. TNF-α is also reported to be produced by other cell types, such as granulocytes, eosinophils, mast cells, T-lymphocytes, NK-cells and tumor cells, in response to certain stimuli (Jirro *et al.*, 1995). The term "tumor necrosis factor" refers to its ability to suppress certain tumor cells in the defense system of man (Waage *et al.*, 1987). Among other effects, this pivotal mediator of inflammation also activates leukocytes, enhances adherence of neutrophils and monocytes

to endothelium, and triggers local production of other proinflammatory cytokines (Tracey & Cerami, 1994). Moreover, the role of TNF-α in septic shock, cancer, rheumatoid arthritis, malaria, and other afflictions have been extensively investigated (Tracey & Cerami, 1994).

1.6.2. Monocyte chemotactic protein- 1

MCP-1 is a CC chemokine essential for monocyte recruitment in *in vivo* models of inflammation. MCP-1 induces the recruitment of other leukocytes such as T lymphocytes, eosinophils and basophils. It is mainly expressed by macrophages in response to cytokines such as IL-6, TNF-α and IL-1β. Upon stimulation, it can also be produced by a variety of cells and tissues, such as fibroblasts, endothelial cells or certain tumor cells. Adhesion of human monocytes to P-selectin, the most rapidly expressed endothelial tethering factor, increased the secretion of MCP-1 and TNF-α from leukocytes stimulated with PAF (Weyrich *et al.*, 1995). Due to its target cell specificity, MCP-1 was postulated to play a pathogenic role in a variety of diseases characterized by mononuclear cell infiltration, including atherosclerosis, rheumatoid arthritis and allergic responses (Carr *et al.*, 1994). Elevated levels of MCP-1 have also been found in connection with osseous inflammation (Graves *et al.*, 1999) and Alzheimer's disease (AD) as well as myocardial ischemia and viral infections (Mateo *et al.*, 2000).

1.6.3. IL-1β

Following an LPS stimulation, IL-1 release is typically observed to occur shortly after TNF-α liberation (DeForge *et al.*, 1992). Interleukin-1 is one of the first described cytokines, and is seen to be expressed by white blood cells (leukocytes) as a mean of communication. In mid 1980s scientist confirmed that IL-1 was actually composed of two distinct proteins, now called IL-1α and IL-1β, both pro-inflammatory cytokines are involved in immune defence against infection. These two molecules are produced by monocytes, macrophages, fibroblasts, keratinocytes, endothelial cells and other cell types. They are potent modulators of the acute phase, inflammatory, and immune responses (Dinarello & Savage, 1989).

IL-1β biosynthesis is complex and regulated at multiple levels. The blood monocyte is found to be the best characterized cell to produce high levels of IL-1. However, there is controversy regarding the release of IL-1 by macrophages. Suttles and colleagues (1990) and Beuscher et al. (1990) observed release of both IL–1α and IL–1β from LPS-stimulated macrophages. On

the contrary, other groups have reported that fresh human monocytes release IL-1β, but cultured monocytes and macrophages do not (Burchett *et al.*, 1988; Wewers & Herzyk, 1989). Furthermore, the role of IL-1β in pathogenesis of estrogen-dependent cancers is found to be implicated in protumorigenic insults, cell proliferation, angiogenesis and cell adhesion. The IL-1βs stimulatory or inhibitory paracrine and/ or autocrine signals regulating the growth of estrogen-dependent tumors are concentration dependent.

1.6.4. IL-8

Interleukin 8 or neutrophil activating protein is a cytokine that functions as a neutrophil chemoattractant/ activator and as a lymphocyte chemoattractant. It is produced in response to LPS-stimulated monocytes, and in a variety of cell types (i.e. endothelial cells, fibroblasts, keratinocytes, lymphocytes), in response to inflammatory stimuli such as TNF-α and IL-1β (Yoshimura *et al.*, 1987). IL-8 induces shape change, chemotaxis, release of granule contents, up-regulation of adhesion proteins, formation of bioactive lipids, and respiratory burst. IL-8 is expressed at high levels particularly in keratinocytes in skin diseases such as psoriasis and palmoplantar pustulosis (Gillitzer *et al.*, 1991). It is also present in the synovial fluid of patients with inflammatory joint diseases.

This chemokine has gained considerable attention because of its ability to attract and activate leukocytes and its undisputed role as mediators of inflammation.

2. AIMS of STUDY

The aim of this thesis was to study the roles of prostaglandins by regulating their amount using different specific inhibitors. How these lipid mediators directly and indirectly influence the TF activity- and expression level (mRNA) of TF and proinflammatory cytokines involved in the intercellular communication important for inflammation, thrombosis, atherosclerosis and sepsis was investigated in whole blood.

Furthermore, interactions between leukocyte subpopulations (of which monocytes and granulocytes) and platelets were examined using flow cytometric analysis. Whole blood stimulated with LPS and LPS and PAF in combination was analyzed for the amount of adherent platelets in the granulocyte and monocyte windows.

3. MATERIALS and METHODS

3.1. REAGENTS

Table 1. Reagents and solutions.

Compound	*Supplier*	*Catalogue no/ note*
Sterile saline	Invitrogen, USA	
Sterile distilled water	Millipore	Ultra Pure Distilled water
LPS	Difco Laboratories, Detroit, USA	Lipopolysaccharide B *E.coli* 026:B6
Lymphoprep	Axis-shield POC AS, Oslo, Norway	Lot 01C2482552
Fragmin	Pfizer	
DMSO	Sigma chemical co, USA	
PAF	Sigma- Aldrich, inc. USA	
Tempus solution	Applied Biosystems, USA	
95% or better alcohol	Arcus kjemi AS, Norway	
FACS™ Lysing Solution	BD Biosciences, San Jose, Ca, USA	
Anti-CD14 – APC	BD Biosciences, San Jose, Ca, USA	Fluorescent dyes conjugated with antibodies
Anti-CD16 – PE	BD Biosciences PharMingen	
Anti-CD62P (P-sel) – FITC	BD Biosciences PharMingen	
Anti-CD42a - FITC	Serotec, UK	

Table 2. Kits used in this thesis.

Kit	*Supplier*	*Used for*
5 Prime PerfectPure RNA Blood kit	5 Prime GmbH, Germany	RNA isolation
Vacuette Tempus Blood RNA Tube	Applied Biosystems, USA	
High-Capacity cDNA Reverse Transcription Kits	Applied Biosystems, USA	cDNA synthesis
TaqMan® Fast Universal PCR Mastermix (2X)	Applied Biosystems, USA	Real time PCR

Table 3. Instruments used in this study.

Instruments	*Supplier*
Spectrophotometer, Thermo max microplate reader	Molecular Devices
7900 HT Fast Real-Time PCR systems	Applied Biosystems, Singapore
Nanodrop, NanoDrop® ND-1000	Saveen Werner (NanoDrop Technologies, USA)
Centrifuges, Megafuge 1.0	Heraeus Sepatech
Table centrifuge, MiniSpin eppendorf	VWR™ International
Cold centrifuge, Minifuge RF	Heraeus Sepatech
Horizontal shaker, G24 Environmental incubator shaker	New Brunswick Scientific Co. Inc, Edison, N.J, USA
Heating device (for cDNA synthesis)	Grant, Dan Meszansky AS
FACSAria™ Cell Sorter	BD Biosciences, USA
APT. line® CB CO_2 Incubators	BINDER GmbH, Tuttlingen, Germany

3.1.1. Preparation of reagents

LPS, with a stock concentration of 50 µg/ mL, was diluted 1:100 with sterile saline before addition to the samples. This gave a final concentration of 5 ng/ mL.

PAF stock (in DMSO) concentration was 10 mM. 4 µL of the working (5 mM) dilution (diluted in sterile saline) was added to 2 mL blood. This gave final concentration 10 µM.

FACS lysis solution mix was prepared by 1:10 dilution of the 10X FACS lysing solution concentrate.

Phosphate buffer saline (PBS), pH 7.2, was made by diluting 10X stock PBS solution with destilled H_2O. 10X PBS was composed of 207.72 g Na_2HPO_4 x $12H_2O$, 26.52 g NaH_2PO_4 x $2H_2O$, and 39.739 g sodium chloride.

Aspirin: COX-1 (COX-2) inhibitor (Cayman Chemical), Mw 180.2

Aspirin was supplied as a crystalline solid. Aspirin is soluble in organic solvents such as ethanol, DMSO, and dimethyl formaide (DMF). Preparation of 200 mM aspirin stock solution (41 mg/mL): 20 mg aspirin was solved in 555 µL DMSO. The aspirin-stock was frozen in 10 µL aliquots. To make the working solution, 10

µL aliquot was further diluted 1:10 with ice-cold saline, which gave a working concentration of 20 mM. Other concentrations were made from this solution.

SC-560: selective COX-1 inhibitor (Cayman Chemical), Mw 352.7

SC-560 was supplied as a crystalline solid, and the compound is soluble in organic solvents such as ethanol, DMSO, and DMF. Ten microliter 5 mM aliquots of SC-560 were prepared by solving 1 mg of the chemical with 567 µL DMSO. The working solution with a concentration of 500 µM was made by adding 90 µL ice-cold saline to the 10 µL freeze-stock.

CAY10404: selective COX-2 inhibitor (Cayman Chemical), Mw 367.4

CAY10404 was supplied as a crystalline solid and it is soluble in organic solvents such as ethanol, DMSO, and DMF. 1 mg of CAY10404 was solved in 544 µL DMSO, and the solution was frozen in 10 µL 5 mM aliquots. To make the working solution, 10 µL aliquot was diluted 1:10 with ice-cold saline, which gave a working solution of 500 µM. Further dilutions were made from this working solution.

CAY10416: dual COX-2/ 5-LOX inhibitor (Cayman Chemical), Mw 536.6

CAY10416 was supplied as a crystalline solid. It is soluble in organic solvents such as ethanol, DMSO, and DMF and a 5 mM solution of the compound was prepared by solving 1 mg of CAY10416 was solved into 372 µL DMSO and frozen in 10 µL 5 mM aliquots at -20°C. A 500 µM working solution was made by adding 90 µL ice-cold saline to 10 µL aliquot freeze-stock.

CAY10526: mPGES-1/COX-2 inhibitor (Cayman Chemical), Mw 311.1

CAY10526 was supplied as a crystalline solid. The compound is soluble in organic solvents such as ethanol, DMSO and DMF. One miligram CAY10526 was solved in 321 µL DMSO and froze down in 10 µL 10 mM aliquots at -20°C. The working solution of 1 mM was made by adding 90 µL ice-cold saline to the 10 µL freeze-stock.

MF63: selective and orally active mPGES-1 inhibitor (Merck), Mw 415.

MF63 was kindly provided by Merck. Ten microliter 20 mM aliquots were frozen down at -20°C. The working solution of 2 mM was made by adding 90 µL ice-cold saline to the 10 µL freeze-stock.

3.2. METHODS

3.2.1. Blood sampling

Venous blood was drawn from an anticubital vein in the forearm of healthy volunteers using a plastic syringe (BD Plastipak™, Spain) and a 19 gauge x 1.5" needle (BD Microlance 3™, Ireland). For all analyses blood was obtained between 9:00 and 10:00 a.m. immediately transferred into polycarbonate tubes (Nalge Nunc International, NY, USA) containing fragmin to a final concentration of 10 μL/ mL blood, and mixed by gentle inversion. The samples were kept at room temperature for immediate use as indicated. All blood donors have been healthy volunteers recruited at the university campus.

3.2.2. Whole blood assay

Anticoagulated (fragmin, 10 μL/ mL blood) blood was aliquoted into sterile cell culture (6- or 12 wells) plates. For 6-wells plates 2 mL blood aliquots were used and for 12-wells plates 1 mL aliquots were used. After addition of the respective stimuli all samples were incubated at 37°C in a 5% CO_2 atmosphere at constant rotation as indicated under each experiment part. All assays were performed in duplicates.

3.2.2.1. LPS time response: stimulation of whole blood – effects on mRNA expression of selected proinflammatory genes

First, an LPS time response study was performed in order to determine the optimal incubation time for each proinflammatory gene/ assay to be investigated in the present study. As shown in table 4, whole blood was incubated with LPS (5 ng/ mL) or saline (as control) for the indicated incubation times (0 h, 0.5 h, 1 h, 1.5 h, 2 h, 4 h, 8 h, 12 h and 24 h). LPS was diluted in sterile saline to a final concentration of 5 ng/ mL from stock preparations. All samples were mixed gently and incubated at 37°C. The reactions were stopped by transferring the blood samples into polycarbonate tubes containing 2 mL Tempus solution. The blood-Tempus mixtures were vigorously shaken (10-15 s) and stored at -20°C until RNA isolation, see section **3.2.5**, and subsequent real time PCR analysis, as described in section **3.2.8**.

Table 4. Incubation setup for LPS time response experiments.

Volume blood in mL	Volume reagent added in µL	Incubation time (hour)
990 990	+ 10 LPS (5 ng/mL) + 10 saline	0
990 990	+ 10 LPS (5 ng/mL) + 10 saline	0.5
990 990	+ 10 LPS (5 ng/mL) + 10 saline	1
990 990	+ 10 LPS (5 ng/mL) + 10 saline	1.5
990 990	+ 10 LPS (5 ng/mL) + 10 saline	2
990 990	+ 10 LPS (5 ng/ml) + 10 saline	4
990 990	+ 10 LPS (5 ng/mL) + 10 saline	8
990 990	+ 10 LPS (5 ng/mL) + 10 saline	12
990 990	+ 10 LPS (5 ng/mL) + 10 saline	24

3.2.2.2. LPS time response: stimulation of whole blood – effects on TF activity

Samples were prepared as in section **3.2.2.1**, however, the reactions were stopped by adding 100 µL of 2% EDTA. Subsequently, 1 mL ice cold saline was added to each sample, and the isolation of mononuclear cells was carried out, as described in section **3.2.3**. Samples were subsequently subjected to tissue factor activity measurement, see section **3.2.4**.

3.2.2.3. Eicosanoid inhibition studies – effects on TF activity and mRNA expression of selected proinflammaotry genes

In this part of the experiment, inhibitors of the arachidonic acid metabolism were used to explore the effects inhibition of eicosanoids in general and specifically of prostaglandins, on TF activity and proinflammatory gene expression. Whole blood samples were preincubated with the respective inhibitors (see tables 5, 6, 7, 8, 9 and 10) for 15 min. at 37°C, and subsequently incubated with LPS (5 ng/ mL) for another ninety minutes. After incubations the samples were prepared as described in sections **3.2.2.1** and **3.2.2.2**.

Table 5. Setup for aspirin (COX-1/2 inhibitor) and subsequent LPS-stimulation of whole blood samples.

			15 min preincubation	+	90 min incubation
2 wells[1]	980 µL blood	+	10 µL DMSO:saline (1:10)	+	10 µL saline
2 wells	980 µL blood	+	10 µL aspirin (200 µM final)	+	10 µL saline
2 wells	980 µL blood	+	10 µL DMSO:saline (1:10)	+	10 µL LPS (final: 5 ng/ mL)
2 wells	980 µL blood	+	10 µL aspirin (200 µM final)	+	10 µL LPS (final: 5 ng/ mL)
2 wells	980 µL blood	+	10 µL aspirin (20 µM final)	+	10 µL LPS (final: 5 ng/ mL)
2 wells	980 µL blood	+	10 µL aspirin (2 µM final)	+	10 µL LPS (final: 5 ng/ mL)

Table 6. CAY10404 (selective COX-2 inhibitor) and subsequent LPS-stimulation of whole blood samples.

			15 min preincubation	+	90 min incubation
2 wells	980 µL blood	+	10 µL DMSO:saline (1:10)	+	10 µL saline
2 wells	980 µL blood	+	10 µL CAY10404 (5 µM final)	+	10 µL saline
2 wells	980 µL blood	+	10 µL DMSO:saline (1:10)	+	10 µL LPS (final: 5 ng/ mL)
2 wells	980 µL blood	+	10 µL CAY10404 (5 µM final)	+	10 µL LPS (final: 5 ng/ mL)
2 wells	980 µL blood	+	10 µL CAY10404 (500 nM final)	+	10 µL LPS (final: 5 ng/ mL)
2 wells	980 µL blood	+	10 µL CAY10404 (50 nM final)	+	10 µL LPS (final: 5 ng/ mL)

Table 7. CAY10416 (dual COX-2/5-LOX inhibitor) and subsequent LPS-stimulation of whole blood samples.

			15 min preincubation	+	90 min incubation
2 wells	980 µL blood	+	10 µL DMSO:saline (1:10)	+	10 µL saline
2 wells	980 µL blood	+	10 µL CAY10416 (5 µM final)	+	10 µL saline
2 wells	980 µL blood	+	10 µL DMSO:saline (1:10)	+	10 µL LPS (final: 5 ng/ mL)
2 wells	980 µL blood	+	10 µL CAY10416 (5 µM final)	+	10 µL LPS (final: 5 ng/ mL)
2 wells	980 µL blood	+	10 µL CAY10416 (500 nM final)	+	10 µL LPS (final: 5 ng/ mL)
2 wells	980 µL blood	+	10 µL CAY10416 (50 nM final)	+	10 µL LPS (final: 5 ng/ mL)

Table 8. SC-560 (selective COX-1 inhibitor) and subsequent LPS-stimulation of whole blood samples.

			15 min preincubation	+	90 min incubation
2 wells	980 µL blood	+	10 µL DMSO:saline (1:10)	+	10 µL saline
2 wells	980 µL blood	+	10 µL SC-560 (5 µM final)	+	10 µL saline
2 wells	980 µL blood	+	10 µL DMSO:saline (1:10)	+	10 µL LPS (final: 5 ng/ mL)
2 wells	980 µL blood	+	10 µL SC-560 (5 µM final)	+	10 µL LPS (final: 5 ng/ mL)
2 wells	980 µL blood	+	10 µL SC-560 (500 nM final)	+	10 µL LPS (final: 5 ng/ mL)
2 wells	980 µL blood	+	10 µL SC-560 (50 nM final)	+	10 µL LPS (final: 5 ng/ mL)

[1] Per assay

Table 9. CAY10526 (mPGES-1/COX-2 inhibitor) and subsequent LPS-stimulation of whole blood samples.

			15 min preincubation	+ 90 min incubation
2 wells	980 µL blood	+	10 µL DMSO:saline (1:10)	+ 10 µL saline
2 wells	980 µL blood	+	10 µL CAY10526 (10 µM final)	+ 10 µL saline
2 wells	980 µL blood	+	10 µL DMSO:saline (1:10)	+ 10 µL LPS (final: 5 ng/ mL)
2 wells	980 µL blood	+	10 µL CAY10526 (10 µM final)	+ 10 µL LPS (final: 5 ng/ mL)
2 wells	980 µL blood	+	10 µL CAY10526 (1 µM final)	+ 10 µL LPS (final: 5 ng/ mL)
2 wells	980 µL blood	+	10 µL CAY10526 (100 nM final)	+ 10 µL LPS (final: 5 ng/ mL)

Table 10. MF63 (selective mPGES-1 inhibitor) and subsequent LPS-stimulation of whole blood samples.

			15 min preincubation	+ 90 min incubation
2 wells	980 µL blood	+	10 µL DMSO:saline (1:10)	+ 10 µL saline
2 wells	980 µL blood	+	10 µL MF63 (20 µM final)	+ 10 µL saline
2 wells	980 µL blood	+	10 µL DMSO:saline (1:10)	+ 10 µL LPS (final: 5 ng/ mL)
2 wells	980 µL blood	+	10 µL MF63 (20 µM final)	+ 10 µL LPS (final: 5 ng/ mL)
2 wells	980 µL blood	+	10 µL MF63 (2 µM final)	+ 10 µL LPS (final: 5 ng/ mL)
2 wells	980 µL blood	+	10 µL MF63 (0.2 µM final)	+ 10 µL LPS (final: 5 ng/ mL)

3.2.3. Isolation of mononuclear cells for TF activity measurements

1 mL of the incubated blood samples (after addition of EDTA) was mixed with 1 mL saline (1:1 ratio). This mixture of blood-saline was carefully applied on top of 1.7 mL lymphoprep in a new TT tube. The tube was centrifuged at 415 x g for 15 minutes, and the mononuclear band was collected and transferred to a new tube containing 4 mL 0.15 M NaCl and washed by centrifugation at 1450 x g for 10 min, this washing step was repeated twice with equal volume of saline each time. The supernatant was pipetted off and the pellet of mononuclear cells from 1 mL blood was resuspended in 200 µL sterile saline and frozen at -20°C until testing for tissue factor activity.

3.2.4. Quantification of tissue factor activity

Tissue factor activity was measured from isolated mononuclear cells based on the ability of TF to accelerate FX activation mediated by FVIIa, using bovine FV and barium citrate (BaCi) eluate (containing FVII, FX, and FII). A tissue factor standard curve was created through a serial dilution using ½ TBS-BSA buffer (0.025 mol/L Tris-HCl, 0.075 mol/L NaCl, 1 mg/mL BSA, pH 7.5). Resting samples (without LPS stimulation) were diluted 1:20 with the ½ TBS-BSA buffer and samples with incubated with LPS were diluted 1:100 with the same buffer. Substrate Th-1 dissolved in sterile distilled water (2.5 mmol/L) was mixed in a 1:1 ratio with 0.1 mol/L Tris-HCl, pH 9.5. 60 µL of preincubated mixture, composed of 5 µL cephalin, 100

µL bovine factor V, 50 µL BaCi eluate, in 4 mL ½ TBS-BSA buffer, was mixed with 15 µL of mononuclear cell sample or the TF standards. Following the addition of 15 µL of 50 mM $CaCl_2$, the mixture was incubated for four minutes at 37°C. The thrombin generated was then quantified by adding 30 µL of Th-1 substrate and the mixture was incubated for an additional four minutes. The reaction was then stopped with 120 µL of 50% acetic acid. The TF activity is directly proportional to the amount of the coloured product para-nitroanilide dihydroacetate (pNa) produced, which can be measured spectrophotometrically at 405 nm.

3.2.5. RNA ISOLATION (PerfectPure RNA Blood Kit, 5 Prime GmbH, Germany)

The frozen blood-Tempus mixtures were put at room temperature for approximately twenty minutes prior to RNA isolation.

Sample homogenization

For isolation, the blood-Tempus samples were transferred to 50 mL conical tubes containing 1.5 mL of 95% ethanol (or higher), high-speed vortexed for 2 minutes, followed by centrifugation at 5800 x g for 60 min. at 4°C. The supernatant was poured off and the tubes blotted on clean absorbent papers for 2 min. 300 µL Lysis Solution was added to each tube and vortexed for 1 min. on high speed to dissolve the RNA pellet.

RNA binding and wash 1

The entire lysate was pipetted onto a purification column and centrifuged at 13400 rpm for 1 min. The purification column was then transferred to a new collection tube, 400 µL of Wash 1 Solution was added and centrifuged for 2 min. at 13400 rpm.

DNase treatment

The purification column was transferred to a new collection tube, 50 µL of DNase Solution was added to the entire column and it was incubated at room temperature for 15 min. 200 µL of DNase Wash Solution was added to the column, and centrifuged at 13400 rpm for 1 min. before addition of another 200 µL of DNase Wash Solution, and the column was centrifuged again for 1 min. at 13400 rpm.

Wash 2

The purification column was transferred to a new collection tube. To the column, 200 µL of Wash 2 Solution was added, and it was centrifuged for 1 min. at 13400 rpm. This step was repeated with 2 min. centrifugation.

RNA elution

Finally, the purification column was carefully transferred to a new tube. 50 µL of Elution Solution was added and the tube was incubated at room temperature for 3 min. 1 minute centrifugation at 13400 rpm was carried out after the incubation. The purification column was discarded, and the collection tube containing the purified RNA was immediately put on ice. RNA was stored at -70°C.

3.2.6. RNA concentration measurements

RNA concentrations were determined using a NanoDrop® ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies, North Carolina, USA).

3.2.7. cDNA synthesis

The High-Capacity cDNA Reverse Transcription Kit (ABI) was used for cDNA synthesis. The kit uses the random primer scheme for initiating cDNA synthesis. Random primers ensure that the first strand synthesis occurs efficiently with all species of RNA molecules present, including mRNA and rRNA. Reverse transcription of RNA was carried out in a 20 µL final volume using 500 ng total RNA. The measured RNA concentration values were calculated and 2X RT Master mixture was prepared as shown in table 11 below.

Table 11. Making the 2X Reverse Transcription (RT) Master Mix for cDNA synthesis.

Reagents		+ RT	- RT
	á µL	**x15 samples**	**x5 µL samples**
10X RT buffer	2	30	10
25X dNTP mix (100 mM)	0.8	12	4
10X RT random primers	2	30	10
MultiSribe™ RT	1	15	5 dH_2O
RNase free dH_2O	4.2	63	21
	10 µL	150 µL	50 µL

Referring to the table, the volume of components needed to prepare one sample is indicated to the left, while the required number of reactions, for instance 15 totally, were calculated. This calculation also included additional reactions to provide excess volume for the loss that

occurred during reagent transfers. Samples without Reverse Transcriptase (-RT) were also prepared, and functioned as no amplification control during real-time PCR performance.
10 µL of 2X RT mastermix was added into each tube containing 10 µL of RNA sample. The content was mixed by pipetting up and down before the tubes were centrifuged shortly (1-2 s) to spin down the contents and to eliminate any air bubbles. The tubes were placed on ice until loading on the thermal cycler as shown in table 12.

Table 12. Thermal cycler condition.

	Step 1	**Step 2**	**Step 3**	**Step 4**
Temperature	25 °C	37 °C	85 °C	4 °C
Time	10 min	120 min	5 sec	∞
Event	Binding of random primers to RNA	cDNA synthesis	Inactivation of enzymes	

After the thermal cycling 60 µL dH_2O was added to each tube (1:4 dilution). The tubes were stored at -20 °C, if not immediately used for real time PCR.

3.2.8. REAL TIME PCR

Polymerase chain reaction generates copies of DNA/ cDNA template and is the most sensitive technique for detection of often-rare mRNA targets (cDNA) (Bustin & Nolan, 2004). Real-time PCR is the continuous collection of fluorescent signal from one or more polymerase chain reactions over a range of cycles. It is the only truly quantitative method available. It offers rapid turn-around as no post PCR processing is necessary and since closed systems are used, the risk of contamination is reduced/ abolished. In quantitative real-time PCR fluorescent signals from each reaction is transformed into a numerical value representing each sample. In this study, real-time PCR analysis was performed with a 7900 HT Fast Real-Time PCR system from Applied Biosystems, and by using hydrolysis/ TaqMan® probes.

3.2.8.1. Real time PCR efficiency/ "standard curve"

The assay efficiency is calculated from the slope of the plot of C_t versus log dilution series ("number of amplicon") by the formula:

$$\text{Efficiency} = [10^{(-1/\text{slope})}] - 1$$

Efficiency is primarily an indication of how well the PCR reaction has proceeded. An assay can have an apparently acceptable efficiency of 95-100%. A 100% efficiency would have a slope of -3.32, which equals to a detection between 33.3-36.5 cycles, a y-intercept between 33 and 37 cycles and an r^2 of 1.00 (Adams, 2006).

Procedure

To make a standard curve, 5 µL of each sample with +RT was used to make a cDNA pool. From this cDNA pool, 15 µL was taken out and diluted as shown in table 13 below.

Table 13. Standard curve dilution for real time PCR

	Dilution	
1	1:2	15 µl of pooled samples + 15 µl dH_2O
2	1:5	10 µl (of 1) + 40 µl dH_2O
3	1:5	10 µl (of 2) + 40 µl dH_2O
4	1:5	10 µl (of 3) + 40 µl dH_2O
5	1:5	10 µl (of 4) + 40 µl dH_2O

Standards were run in triplicate.

3.2.8.2. Primers and probes

There are currently several techniques to perform quantitative real time PCR. However, in this thesis, the hydrolysis probes or Taqman method has been applied. The probe is an oligonucleotide with both a reporter fluorescent dye and a quencher dye attached. The Taqman assay utilizes the 5'-nuclease activity of the DNA polymerase to hydrolyze a hybridization probe bound to its target amplicon. The method is more sensitive and therefore able to detect lower transcript levels than i.e. SYBR green.

Procedure

The primers (forward primer: FP, reverse primer: RP) and probes of the genes used in this study are shown in table 14. Cyclophilin A was run as a reference gene, while TF, TNF-α, IL-1β, IL-8, and MCP-1 as target gene. Probe modifications are also indicated as shown in the sequence.

Table 14. Primers and probes used in real-time PCR analysis.

Gene	Primers or probe	Sequence written 5' to 3'	Final concentration (nM)
Cyclophilin A (reference gene)	FP	GTACTATTAGCCATGGTCAACCCC	200
	RP	CAGTCAAAGGAGACGCGGCC	200
	Probe	6-FAM-CGTCGACGGCGAGCCCTTG-BHQ-1	200
TF	FP	CCCCAGAGTTCACACCTTACCT	200
	RP	CACTTTTGTTCCCACCTGTTCA	200
	Probe	6-FAM-AGACAAACCTCGGACAGCCAACAATCCA-BHQ-1	100
TNF-α	FP	CCCAGGGACCTCTCTCTAATC	400
	RP	ATGGGCTACAGGCTTGTCACT	400
	Probe	6-FAM-TGGCCCCAGGCAGTCAGATCATC-EDQ	200
MCP-1	FP	AGTCTCTGCCGCCCTTCT	400
	RP	GTGACTGGGGCATTGATTG	400
	Probe	GCCTGCTG (Roche Applied Science Universal Probe #40)	100
IL-1β	FP	CTGTCCTGCGTGTTGAAAGA	400
	RP	TTGGGTAATTTTTGGGATCTACA	400
	probe	AGCTGGAG (Roche Applied Science Universal Probe #78)	100
IL-8	FP	GTTTTTGAAGAGGGCTGAGAATTC	400
	RP	CATGAAGTGTTGAAGTAGATTTGCTTG	400
	Probe	6-FAM-ATCCAAGAATCAGTGAAGATGCCAGTGAAACT-BHQ	200

3.2.8.3. Real-time quantitative PCR (qRT-PCR) analysis

In general, two types of quantification strategies can be performed in qRT-PCR. The levels of expressed genes may be measured by an 'absolute' quantification or by a relative real-time qRT-PCR (Pfaffl, 2004). The 'absolute' quantification approach relates the PCR signal to a known input copy number, using a calibration curve (standard curve). Calibration curves can be derived from diluted PCR products, recombinant DNA or RNA or linearized plasmids. The reliability of such an absolute real-time RT-PCR assay depends on the condition of 'identical' amplification efficiencies for both the native mRNA target and the target RNA or DNA used in the calibration curve (Pfaffl, 2001; Souaze *et al.*, 1996). In our study, relative quantification was performed.

Procedure

Before running real time PCR analysis, the samples were further diluted 1:2 (in addition to 1:4 dilution in part **3.2.7,** cDNA synthesis) with dH_2O. These samples were placed on ice and a Master mixture was made according to table 15. Appendix 1 listed various amounts of primers and probes used in this thesis.

Table 15. Mastermix table for real time PCR

Reagents	**á µL**		**x 80 µL**	
2X Mastermix	10		800	
Forward primer	X	these volumes were	80 x X	volumes subsequently
Reverse primer	Y	adjusted according to	80 x Y	adjusted to the
Probe	Z	concentration such that	80 x Z	column to the left.
dH_2O	Δ	they made up 6 µL	80 x Δ	
	16 µL		µL	

The real-time PCR reactions were performed on a clear 96-well plate (MicroAmp™ Fast Optical 96-well Reaction Plate with Barcode (0.1 mL), Applied Biosystems, Foster city, CA, USA) using adhesive seal as cover. 16 µL mastermix was added into each well reserved for sample, standards or control, and 4 µL of each sample was added into sample-wells, in duplicate, while standards were run in triplicate. For water sample (negative control), 16 µL of mastermix and 4 µL of dH_2O were added into a separate well on the sample plate. The whole plate was quickly centrifuged (11 s) and run on a real time PCR instrument (7900 HT Fast Real-Time PCR system, Applied Biosystems). Samples were heated for 20 s at 95°C and then subjected to 40 cycles of denaturation at 95°C for 1 s and annealing and elongation at 60°C for 20 s. In addition to negative control, no amplification control (RT- minus) was included in every assay.

3.2.8.4. Relative gene expression

Relative quantification measures the relative change in mRNA expression levels. It determines the changes in steady state mRNA levels of a gene across multiple samples and expresses it relative to the levels of another RNA. Relative quantification does not require a calibration curve or standards with known concentrations and the reference can be any transcript, as long as its sequence is known (invited review, Bustin, 2002). The units used to express relative quantities are irrelevant (Pfaffl, 2006).

To achieve optimal relative expression results, appropriate normalization strategies are required to control for experimental error. A control gene/ reference gene that is expressed at a constant level is used to normalize the gene expression results for variable template amount or template quality and to control the cDNA synthesis. Reference genes are the most common method for normalizing qRT-PCR data. This strategy targets RNAs that are universally and constitutively expressed, and whose expression does not differ between the experimental and control groups, to report any variation that occurs due to experimental error. The relative expression of a target gene to another gene, mostly a reference gene, can be calculated on the basis of "delta C_t"(ΔC_t, normalization to a reference gene) and "delta delta C_t" ($\Delta\Delta$ C_t, normalization to control sample) values, with following equation

$$R = \frac{2^{[C_t \text{ cyclophilin A} - C_t \text{ target gene}]}}{2^{[C_t \text{ cyclophilin A} - C_t \text{ target gene}]}} \quad \begin{matrix} \text{Treated sample} \\ \text{Control sample} \end{matrix}$$

Procedure

In this study, the relative expression of each target genes were quantified by first normalizing to the reference gene (cyclophilin A, ΔC_t) and then to the control samples ($\Delta\Delta C_t$).

3.2.9. FLOW CYTOMETRY

Flow cytometer is an instrument that illuminates cells (or other particles) as they flow individually in front of a light source and then detects and correlates the signals from those cells that result from the illumination (Givan, 2004). Each particle will scatter the emitted light, and this scattered light can be detected and stored in a data file for subsequent analysis. In most cases, particles without background (auto-) fluorescence have to be stained with fluorescent dyes or fluorophores during preparation to make non-fluorescent compounds "visible" to the cytometer. The fluorescent dyes can be conjugated to antibodies, and then the

fluorescence from a cell will be a readout for the amount of protein/ antigen (on cell surface/ in cytoplasm or nucleus), to which the antibody has bound to.

Procedure

The fragminized blood was distributed to three wells in a 6-wells plate, so that each contained 2 mL of blood. Three different samples were studied:

1. Controls: blood with vehicle control (saline)
2. LPS-stimulated samples
3. Samples stimulated with LPS+PAF

Both LPS and PAF were diluted from stock preparations using a serial dilution by using sterile saline to a final concentration of 5 ng/ mL and 10 µM in blood, respectively. The samples were incubated for two hours at 37°C in a 5% CO_2 atmosphere under constant rotation.

Table 16. Setup for flow cytometry experiments.

Well no	Incubation at 37°C for 120 min.
1	1980 µL blood + 20 µL saline
2	1980 µL blood + 20 µL LPS (final: 5 ng/ mL)
3	1976 µL blood + 4 µL PAF (final: 10 µM) + 20 µL LPS (final: 5 ng/ mL)

After two hours of incubation, 100 µL aliquots were taken from each well, as shown in table 17 below.

Table 17. Aliquots and antibodies preparation.

Well no	Stimulation	100 µL aliquots	µL antibody added
1	Saline (control)	Unstained	
		CD14 – APC	5
		CD16 – PE	5
		CD62P (P-sel) – FITC	10
		CD42a – FITC	10
		CD14 + CD16 + CD62P	5 + 5 + 10
		CD14 + CD16 + CD42a	5 + 5 + 10
2	LPS	CD14 + CD16 + CD62P	5 + 5 + 10
		CD14 + CD16 + CD42a	5 + 5 + 10
3	LPS+PAF	CD14 + CD16 + CD62P	5 + 5 + 10
		CD14 + CD16 + CD42a	5 + 5 + 10

For well number 1, seven 100 µL aliquots were made, while two aliquots á 100 µL were prepared for each of well number 2 and 3, TT tubes were used in this step.

Antibodies. The following murine monoclonal antibodies from BD Biosciences, USA and Serotec, UK, were used: allophycocyanin (APC)-conjugated anti-CD14 (5 µL per test) to identify monocytes, granulocytes were identified by using phycoerythrein (PE)-conjugated anti-CD16 (5 µL per test), fluorescein isothiocyanate (FITC)-conjugated anti-CD62P (10 µL per test) to identify expression of

P- selectin and FITC-conjugated anti-CD42a (10 μL per test) to identify the (glycoprotein) gpIX part of the gpIb-IX complex found on the human platelets.

Table 18. Antibodies for flow cytometric analysis.

Antibody	Specificity	Supplier	Conjugate
Anti-CD14	Monocytes marker	BD Biosciences, San Jose, Ca, USA	APC
Anti-CD16	Granulocytes marker (also on 5-10% monocytes)	BD Biosciences PharMingen	PE
Anti-CD62P	P-selectin, α-granule marker	BD Biosciences PharMingen	FITC
Anti-CD42a	gpIX part of the vWf receptor complex on human platelets	Serotec, UK	FITC

Preparation of whole blood samples for flow cytometry. The 100 microliter aliquots were taken as shown in table 17. The aliquots were incubated with antibodies for 30 min. in the dark. Four of the samples were incubated with only one antibody while for the rest in three antibodies (volume added as given above) were used combination. One aliquot was left unstained and incubated without any antibody. After incubation, two milliliters of FACS lysis solution mix was added to each tube, for lysis of red blood cells, and incubated at room temperature in the dark for 11 minutes. All the tubes were so spun down at 1450 x g for 10 min. The resulted supernatant was removed and the tubes were washed once by filling with PBS and spun down at 1450 x g for 10 min. The supernatant was removed and the pellet dissolved in 400 μL PBS. The tubes were placed on ice and protected from light until flow cytometric analysis could be performed.

3.2.9.1. Flow cytometric analysis

All information that a flow cytometer reveals about a cell comes from the time when cell is passing through the laser beam. The intersection point between the laser beam and the flowing cells is called the “analysis point” or “interrogation point”. The flow cytometer used in this thesis is a FACSAria^{TM} Cell Sorter from BD Biosciences. The cells are analysed as they travel one by one in a moving fluid stream past fixed focused laser beam from a built-in, air-cooled, Coherent Sapphire solid state laser and He-Ne laser, and an optional violet diode laser. The laser light is scattered in all directions as the cell passes through a laser beam. The scattered light that is measured at 0.7° to 10° is called forward scatter and at about 90° side scatter. Up to twelve parameters can be measured for each cell: forward scatter light (FSC), side scatter light (SSC) and an octagon containing six photomultiplier tubes (PMTs) and two trigons containing two PMTs each. These characteristics, pertaining to how the cell scatters the laser light and emits the fluorescence, provide the information about the cell size (FSC),

internal complexity or granularity (SSC), and relative fluorescence intensity. This information is collected and transmitted to a computer.

Procedure

The samples were immediately analyzed after preparation, in a FACSAria™ flow cytometer equipped with the FACSDiVa digital data system and software (BD Biosciences). The fluorescence intensity was controlled daily with the FACSDiVa according to the manufacturer's instructions. The fluorescence compensation was manually adjusted with unstained whole blood, whole blood labeled with FITC-conjugated anti-CD62P (and anti-CD42a), whole blood labeled with PE-conjugated anti-CD16 and whole blood labeled with APC-conjugated anti-CD14. Light scatter and fluorescence data were obtained with gain settings in the logarithmic mode from calculated events collected from each sample. The saved files were then exported from the flow cytometer data system and imported into another FACSDiVa data system software for analyzing.

Leukocyte subpopulations, especially granulocytes, monocytes (and lymphocytes), and the population of platelets were identified by means of their light scatter characteristics, enclosed in electronic gates and separately analyzed for fluorescence intensity from different bound fluorochrome-labeled antibodies, Figure 8.

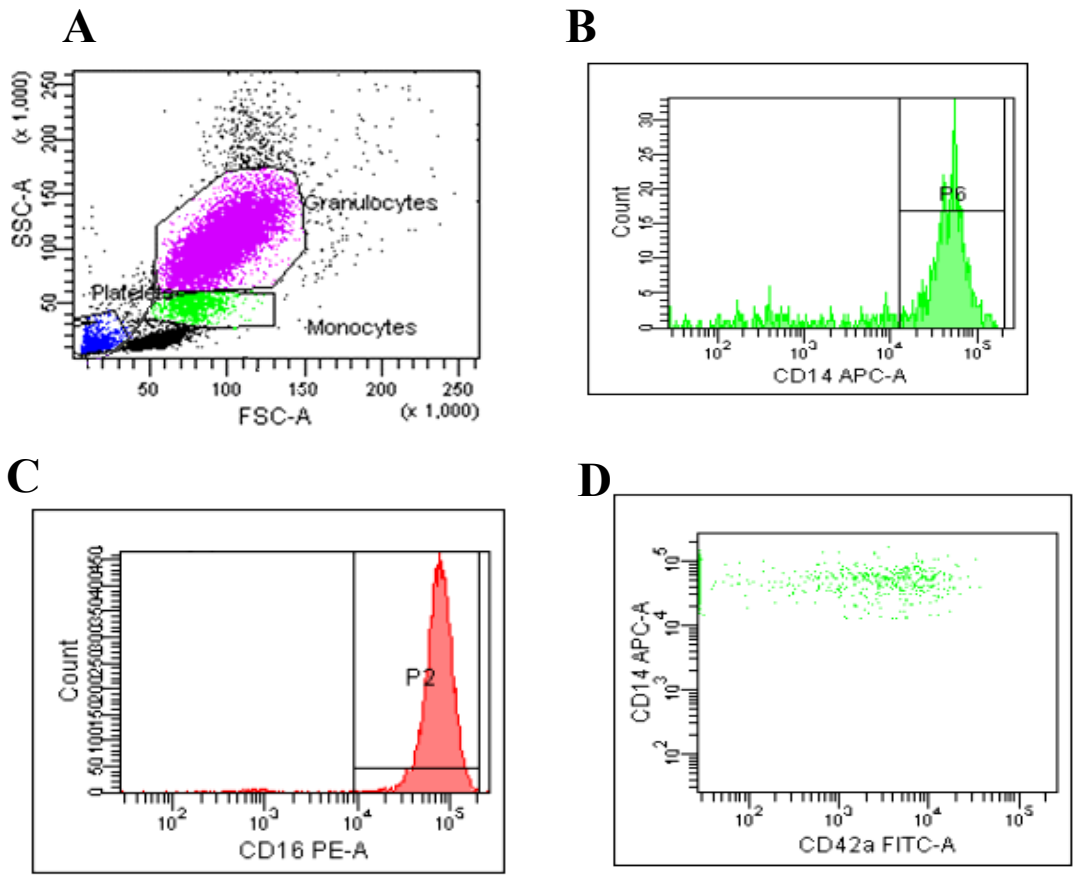

Figure 8. Platelet-monocyte aggregates in whole blood analysis by flow cytometry. **A.** A plot of FSC vs. SSC characteristics from cells obtained from hemolysed whole blood indicates that regions can be drawn around cells with different scatter characteristics. Distinct populations of granulocytes (purple), monocytes (green), lymphocytes (black) and platelets (blue), are separated by gating. Cell populations were determined by immunostaining of whole blood with PE-conjugated anti-CD16 (a marker of granulocytes, purple), monocytes were labelled with CD14-APC and platelets with CD42a-FITC and defined by the MFI values during flow cytometrical analysis**. B.** A histogram was created over the gated CD14-bright monocytes population. The monocytefraction contains approximately 90% CD14-positive (CD14+) cells. Upon gating to P6, 100% CD14+ is observed. **C**. Granulocytefraction from the scatterplot contains nearly 100% CD16+ cells. Narrowing the population by gating to P2 resulted into 100% CD16+. **D.** A plot of CD14-bright monocytes against CD42a was used to determine the amount of monocytes positive for CD42a (platelet-monocyte aggregates). The platelets were not further gated but calculated directly from platelet population as gated in the scatterplot graph. FSC = forward scatter, SSC = side scatter, APC = allophycocyanin, PE = phycoerythrin, MFI = median fluorescence intensity.

3.3. Statistical analysis

Results are reported as mean ± SEM, however, the flow cytometry results were expressed as median. Statistical analyses were performed using the SPSS 15. Paired-samples t-test was used to test for significance of the observed effects. Significance was considered as $p<0.05$.

4. RESULTS

4.1. Time course of TF activity in response to LPS stimulation

Since the purpose of this thesis was based on previous results obtained when studying the effects of aspirin and prostaglandin E_2 upon LPS-induced TF activity, a characterization of the time effects of the LPS-stimulation of whole blood was performed. As shown in Figure 9, maximum TF activity in response to LPS stimulation peaked between 1.5 and 4 hours of incubation, however adjusting for controls TF activity peaked at 2 h (see table 19). As expected, samples stimulated with LPS showed a much higher TF activity when compared with non-stimulated control. TF activity was 1.8-fold higher in the 2 h incubated LPS-stimulated samples compared to 1.5 h ($p<0.05$), however when adjusted for background TF activity, there were no significant differences between the 1.5 h, 2 h and 4 h time points.

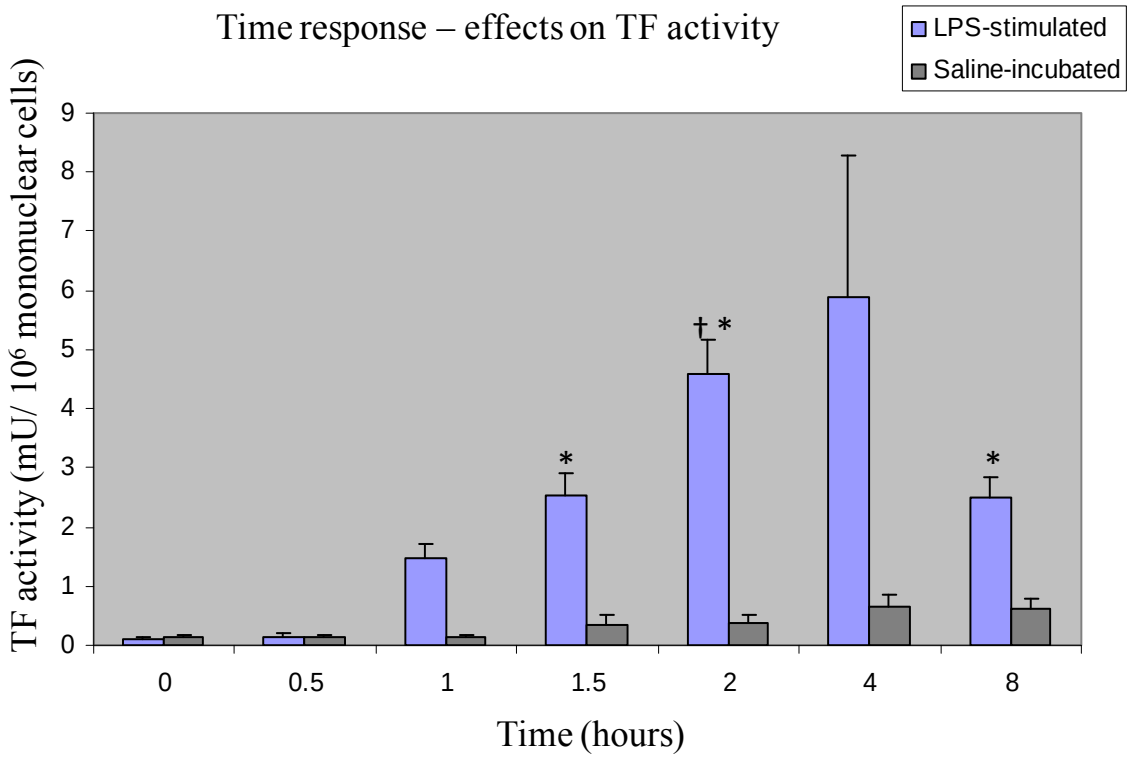

Figure 9. LPS time response – effects on TF activity. Whole blood was stimulated with LPS for the indicated incubation time. Saline was used as control samples. * $p < 0.05$ vs. saline-incubated, † $p<0.05$ vs. LPS-stimulated 1.5 h. Paired-samples t-test. n = 3.

Table 19. LPS time response – effects on TF activity. LPS- stimulated samples.

Time (h)	TF activity (mU/10^6 mnc) (mean ± SEM)	TF activity fold induction vs. saline control
0	0.11 ± 0.01	1.00 ± 0.38
0.5	0.13 ± 0.07	1.44 ± 1.47
1	1.47 ± 0.24	12.19 ± 5.05
1.5	2.53 ± 0.37	12.01 ± 6.45
2	4.60 ± 0.58	17.21 ± 8.22
4	5.90 ± 2.37	8.18 ± 2.30
8	2.51 ± 0.32	5.26 ± 1.80

4.2. Time course of individual gene expression in response to LPS stimulation

The resulted mRNA expression is relative to zero hour and normalized to the reference gene cyclophilin A. Cyclophilin A has previously been demonstrated to be the most stably expressed of common reference genes in LPS-stimulated whole blood in our lab.

TF mRNA expression is shown in Figure 10, and table 20 (LPS-stimulated values). Maximum TF mRNA expression was observed between 1 h to 2 h. Between 2 h and 4 h a significant reduction (-56%, $p<0.05$) in LPS-induced TF mRNA was evident. Further incubation reduced the TF mRNA event more and this was paralleled by an increase in background TF mRNA levels. This indicates that whole blood samples are gradually activated without any added stimulation during prolonged incubation.

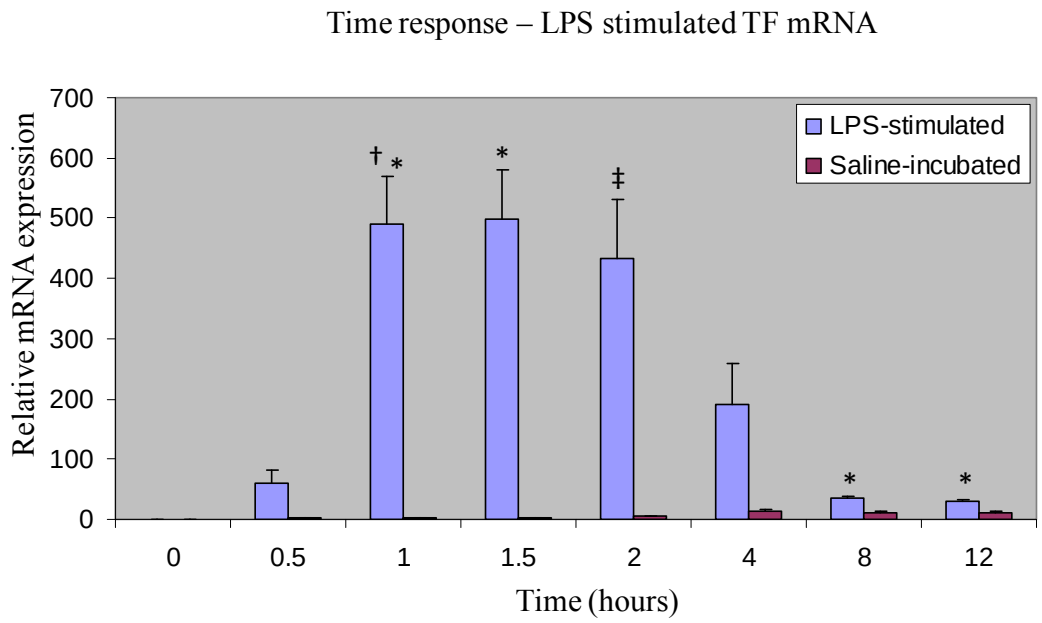

Figure 10. Tissue factor mRNA expression. Stimulated samples were incubated with 5 ng/ mL LPS. Whole blood incubated with saline was used as control. * $p<0.05$ vs. saline-incubated. † $p<0.05$ vs. LPS-stimulated 0.5 h, ‡ $p<0.05$ vs. LPS-stimulated 4 h. Paired-samples t-test. n = 3.

Table 20. Tissue factor mRNA expression. LPS-stimulated samples.

Incubation time (h)	Tissue factor relative mRNA expression
0	1.0 ± 0
0.5	61.3 ± 21.1
1	491 ± 78.2
1.5	498± 81.1
2	434 ± 98.3
4	191 ± 67.4
8	35.1 ± 2.4
12	28.6 ± 4.0

TNF-α mRNA expression is shown in Figure 11 and table 21 (LPS-stimulated values). In response to LPS, monocytes release the pleiotropic cytokine TNF-α. The TNF-α expression is extremely rapidly activated and maximum TNF-α is evident as early as 30 min after addition of LPS to the samples. The TNF-α mRNA concentration stays at this level until 2 h after LPS, whereas the background TNF-α mRNA levels gradually increases (not significantly). Between 4 h and 8 h stimulation, the expression decreased with approximately 68% ($p<0.05$).

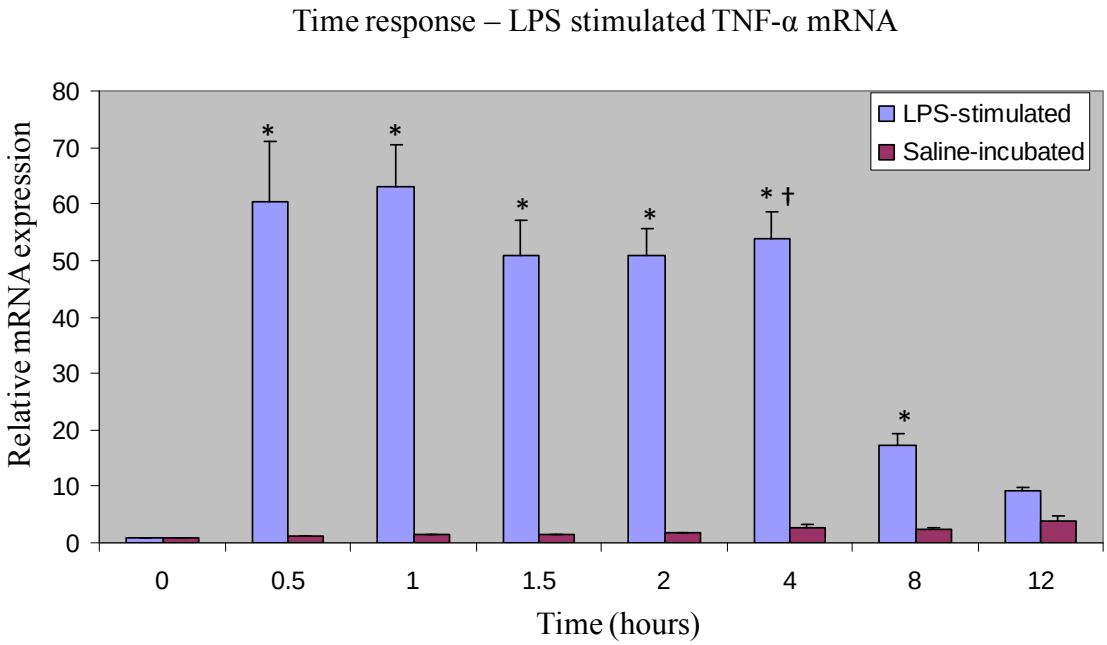

Figure 11. TNF-α mRNA expression. The figure shows mRNA levels for both LPS-stimulated and control (saline) samples. * $p<0.05$ vs. saline-incubated. † $p<0.05$ vs. LPS-stimulated 8 h. Paired-samples t-test. n = 3.

Table 21. TNF-α mRNA expression. LPS- stimulated samples.

Incubation time (h)	TNF-α relative mRNA expression
0	1 ± 0
0.5	60.50 ± 10.7
1	63 ± 7.4
1.5	50.8 ± 6.4
2	51 ± 4.8
4	53.7 ± 4.8
8	17.1 ± 2.3
12	9.3 ± 0.4

LPS-induced **IL-1 beta mRNA** expression was found to peak around 1.5 h and 2 h stimulation, although not significant, there is nearly 3% increment with half an hour longer incubation between these time points. The background (saline controls) IL-1β levels were very low compared to the levels obtained by LPS-stimulation (7-fold induction for 1.5 h, $p<0.05$, 5-fold for 2 h).

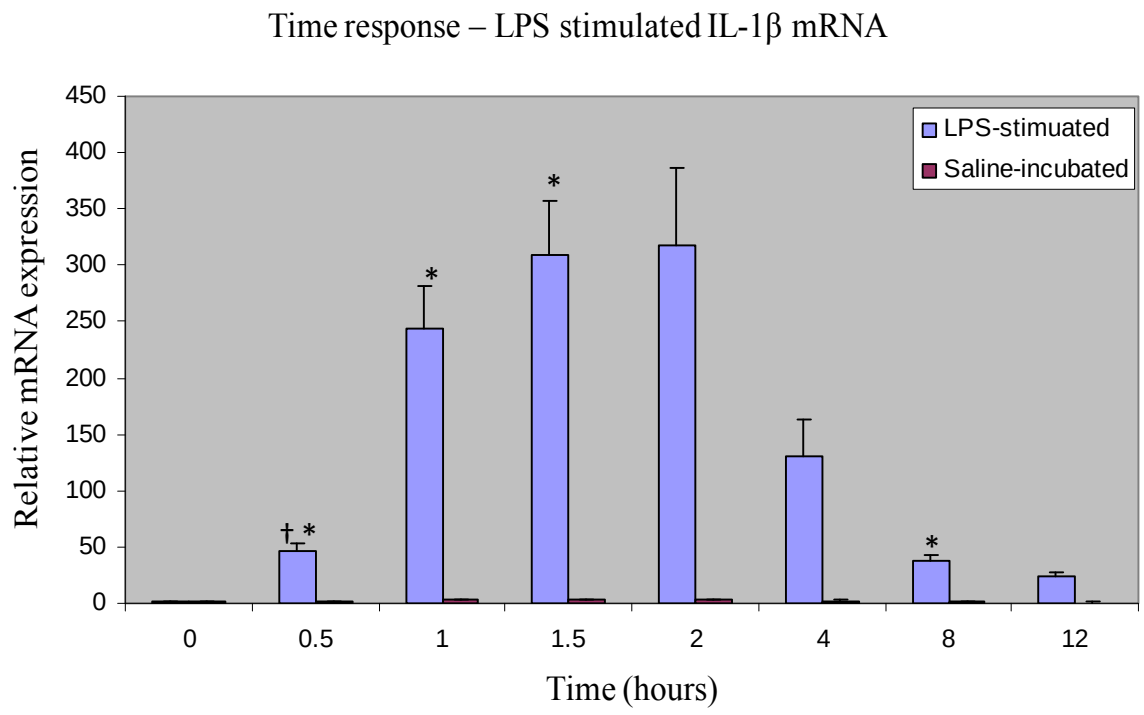

Figure 12. IL-1β mRNA expression. Fragminized whole blood stimulated with 5 ng/ mL LPS incubated as indicated. Saline samples were used as control. * $p<0.05$ vs. saline-incubated. † $p<0.05$ vs. LPS-stimulated 0 h. Paired-samples t-test. n = 3.

Table 22. IL-1 beta mRNA expression. LPS- stimulated samples.

Incubation time (h)	IL-1 beta relative mRNA expression
0	1 ± 0
0.5	46.3 ± 6.5
1	243.5 ± 37.5
1.5	309.3 ± 48.3
2	318.5 ± 67.7
4	131 ± 32.8
8	38.0 ± 4.2
12	23.5 ± 4.5

Figure 13 shows that a biphasic effect is observed for **IL-8 mRNA** expression in LPS-stimulated whole blood. Also IL-8 has a primary peak observed after 1.5-2 h of LPS-stimulation, however, a secondary peak is observed after prolonged incubation. This secondary production of IL-8 mRNA is a result of increased levels of TNF-α (and other cytokines such as IL-6) which also will stimulate synthesis of IL-8 in the stimulated blood. It is also important to note that IL-8 is a sensitive marker which tells a lot about the activation state of the samples, and it can be observed from the figure that the background levels for IL-8 mRNA increased a lot after 1-2 h incubation. The mRNA levels reached after 12 h LPS-stimulation is not significantly higher compared to the levels obtained in the first peak (1.5 h or 2 h). It is further observed that there is a significant increment, approximately 94%, for stimulated samples incubated from 1 to 1.5 h ($p<0.05$), LPS-stimulated and saline-incubated samples also show obvious differences at this incubation period ($p<0.05$).

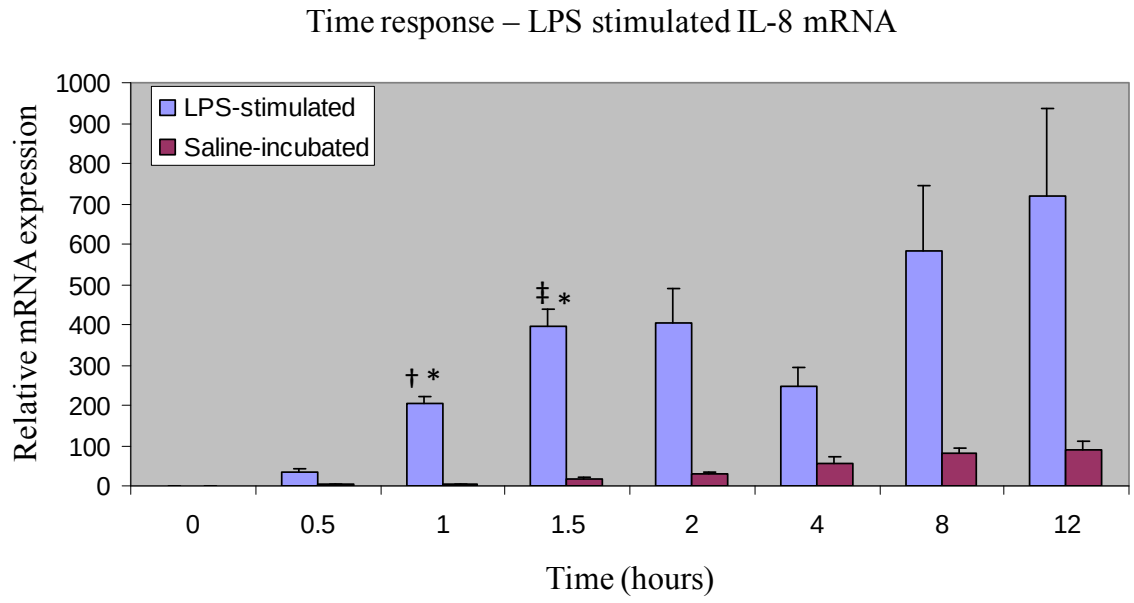

Figure 13. IL-8 mRNA expression. Figure shows the bi-phased gene expression of IL-8 for samples stimulated with LPS. Samples incubated with saline were used as control. * $p<0.05$ vs. saline-incubated, † $p<0.05$ vs. LPS-stimulated 0.5 h, ‡ $p<0.05$ vs. LPS-stimulated 1 h.
Paired-samples t-test. n = 3.

Table 23. IL-8 mRNA expression. LPS- stimulated samples.

Incubation time (h)	IL-8 relative mRNA expression
0	1 ± 0
0.5	33.4 ± 8.5
1	202.8 ± 18.5
1.5	393.7 ± 43.9
2	403 ± 88.2
4	247.8 ± 47.60
8	582.3 ± 162.50
12	721.2 ± 215.3

MCP-1 mRNA expression is shown in Figure 14. The LPS-induced MCP-1 expression peaked at 8 h at an mRNA level 35-fold higher when compared to 1.5 h, but this effect was not significant. It is important to consider the enormous background-induced expression of MCP-1 mRNA.

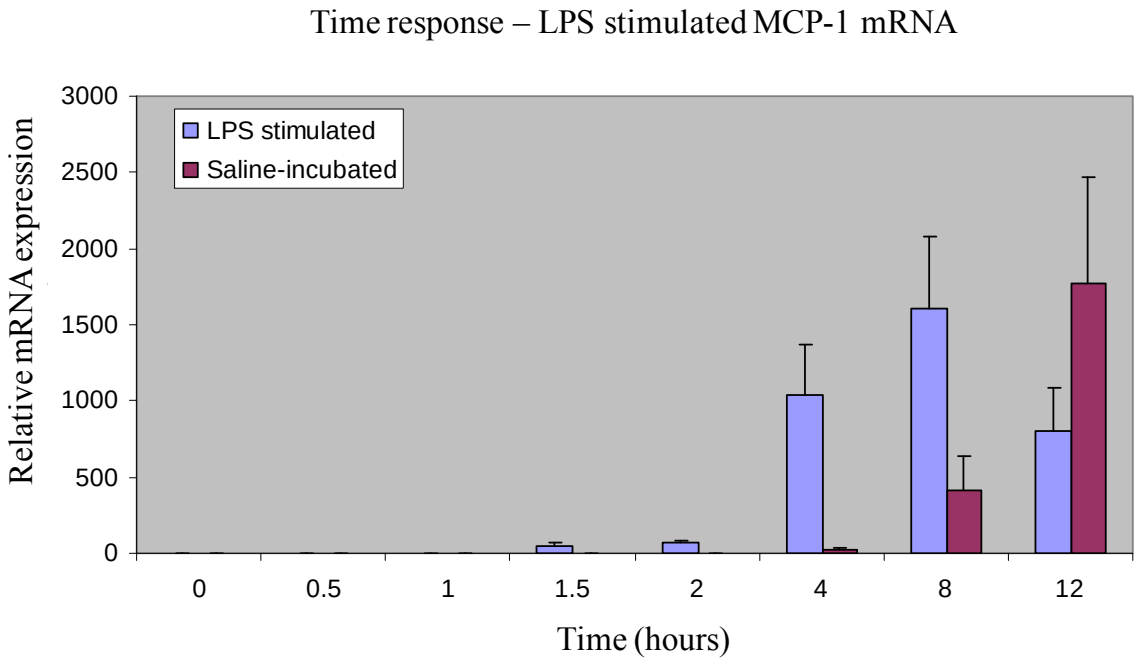

Figure 14. MCP-1 mRNA expression. 5 ng/ mL LPS was added to whole blood and incubated as shown. Saline was used as control samples. Paired-samples t-test. n = 3.

Table 24. MCP-1 mRNA expression. LPS- stimulated samples.

Incubation time (h)	MCP-1 relative mRNA expression
0	1 ± 0
0.5	3.1 ± 0.6
1	4.8 ± 0.95
1.5	45.9 ± 24.2
2	67.0 ± 15.9
4	1045.1 ± 326.5
8	1603 ± 478.0
12	806.5 ± 275.0

Summary of the LPS-induce time responses. The time response study is an indication on how long samples should be incubated. It is observed that incubation around 1.5 h to 2 h should be sufficient for further work.

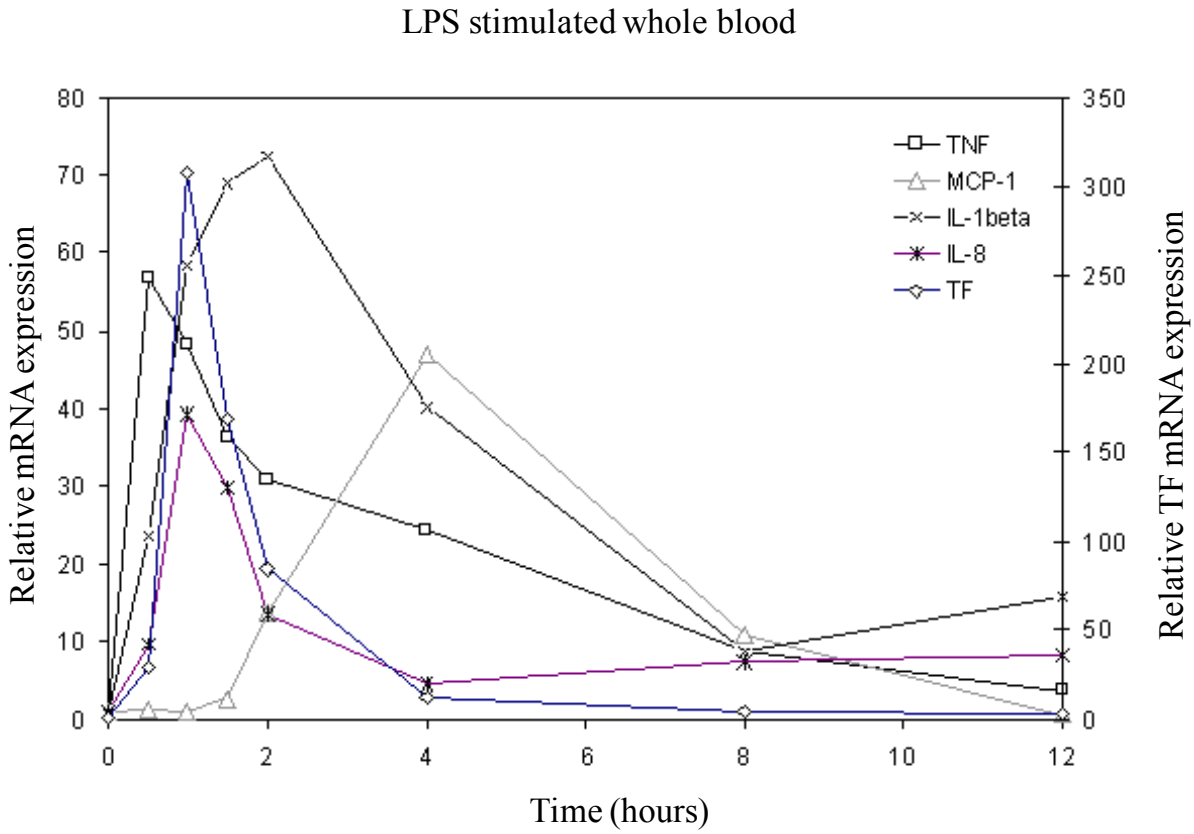

Figure 15. Summary of the LPS-induced time responses. In the figure, the relative mRNA levels are normalized to the reference gene and related to the mRNA expression in the saline controls. As shown, 90 min incubation is relatively optimal for most of the genes.

4.3. Manipulation of prostaglandin metabolism – effects on monocyte TF activity in whole blood

We analyzed the effect of manipulation of the prostaglandin metabolism by inhibiting single enzymes involved in the prostaglandin synthesis, COX-1, COX-2, and mPGES. The tissue factor activity after treatment with different inhibitors is shown in table 25 below. The COX-1 inhibitors (SC-560 and aspirin) did not significantly influence the LPS-induced TF activity in our experiments. However, there was a trend for enhancement of the LPS-induced TF activity for both of these inhibitors. This effect was quite pronounced in the highest LPS responders, whereas there was no effect of the COX-1 inhibitors in the individuals with the lowest inducing effect from LPS. Treatment with the more specific prostaglandin synthesis inhibitors, CAY10526, also resulted in an insignificant trend for enhanced TF activity, whereas the specific prostaglandin E synthase (mPGES) inhibitor MF63, resulted in a significant enhancement of the LPS-induced TF activity (0.2 μM MF63, +41%, $p<0.05$ and 20 μM MF63, +107%, $p<0.05$). Also the specific COX-2 inhibitor CAY10404 had no significant effect on the TF activity. However, the trend was towards an enhancement of the activity. To investigate the link over to the leukotrienes pathways, a dual COX-2/ 5-LOX inhibitor (CAY10416) was tested. This compound did not result in any significant effects, however there was a small trend towards inhibition of the TF activity.

Table 25. The effects of manipulating the prostaglandin metabolism on TF activity. Samples were pre-incubated with different doses of the inhibitors, and subsequently stimulated with 5 ng/ mL LPS. * $p<0.05$ vs. vehicle control. Paired-samples t-test. n = 6, [a]n = 4. Values are mean ± SEM.

Sample	Target	TF activity ($mU/10^6$)
LPS/ Vehicle control (DMSO:saline (1:10))		**5.30 ± 1.34**
Aspirin, 2 μM + LPS	COX-1 (COX-2)	6.54 ± 1.97
Aspirin, 20 μM + LPS	COX-1 (COX-2)	8.50 ± 3.38
Aspirin, 200 μM + LPS	COX-1 (COX-2)	8.92 ± 4.65
SC-560, 50 nM + LPS	COX-1, selective	7.30 ± 2.81
SC-560, 500 nM + LPS	COX-1, selective	8.98 ± 2.86
SC-560, 5 μM + LPS	COX-1, selective	6.84 ± 2.15
CAY10404, 50 nM + LPS	COX-2, selective	8.60 ± 2.74
CAY10404, 500 nM + LPS	COX-2, selective	6.97 ± 2.03
CAY10404, 5 μM + LPS	COX-2, selective	9.48 ± 3.37
CAY10526, 100 nM + LPS	Inhibits PGE_2 synthesis	6.72 ± 1.67
CAY10526, 1 μM + LPS	Inhibits PGE_2 synthesis	10.18 ± 3.35
CAY10526, 10 μM + LPS	Inhibits PGE_2 synthesis	6.82 ± 2.55
MF63, 0.2 μM + LPS	mPGES	7.48 ± 2.07*
MF63, 2 μM + LPS	mPGES	8.63 ± 2.25
MF63, 20 μM + LPS	mPGES	10.95 ± 3.23*
LPS/ Vehicle control (DMSO:saline (1:10))		**19.65±8.49**
CAY10416, 50 nM + LPS	COX-2/5-LOX	16.45 ± 4.37[a]
CAY10416, 500 nM + LPS	COX-2/5-LOX	19.95 ± 6.14[a]
CAY10416, 5 μM + LPS	COX-2/5-LOX	15.58 ± 3.94[a]

4.4. Manipulation of prostaglandin metabolism, eicosanoid inhibition studies – effects on individual cytokine mRNA expression in response to LPS stimulation

mRNA expression levels of the various cytokine samples are all normalized first to the reference gene, cyclophilin A, and then to the control samples.

4.4.1. Effect of aspirin on LPS-induced expression of proinflammatory genes in whole blood

Whole blood samples incubated with aspirin is shown in Figure 16 below. In general, preincubation of the blood with aspirin prior to addition of LPS, resulted in enhanced mRNA expression of TF and the chosen cytokines. The effects of aspirin are similar for all the genes analyzed. For both IL-1β and IL-8, preincubation with aspirin resulted in a significant enhancement of the mRNA expression, whereas for TF and MCP-1, the enhancing effects were not significant. For IL-8, all doses of aspirin resulted in significant increase in mRNA expression, varying from 67% to 78% enhancement for 20 μM and 200 μM, respectively. The lowest dose resulted in over 100% enhancement of the mRNA expression for this cytokine. A very similar aspirin response was observed for IL-1β. But for this cytokine only the lowest aspirin-dose reached significant levels (+86%, $p<0.05$).

For TNF-α, the aspirin response was less pronounced compared to IL-8 and IL-1β and the response was significant only for the 200 μM concentration (+36%, $p<0.05$).

For tissue factor mRNA expression, the aspirin seemed to have an enhancing effect, however this was not significant for any aspirin concentration. This was also the case when analyzing the aspirin effect on LPS-induced MCP-1 expression. Note that only 2 individuals were used for the MCP-1 mRNA expression analyses, and if the numbers had been increased also MCP-1 would have become significant.

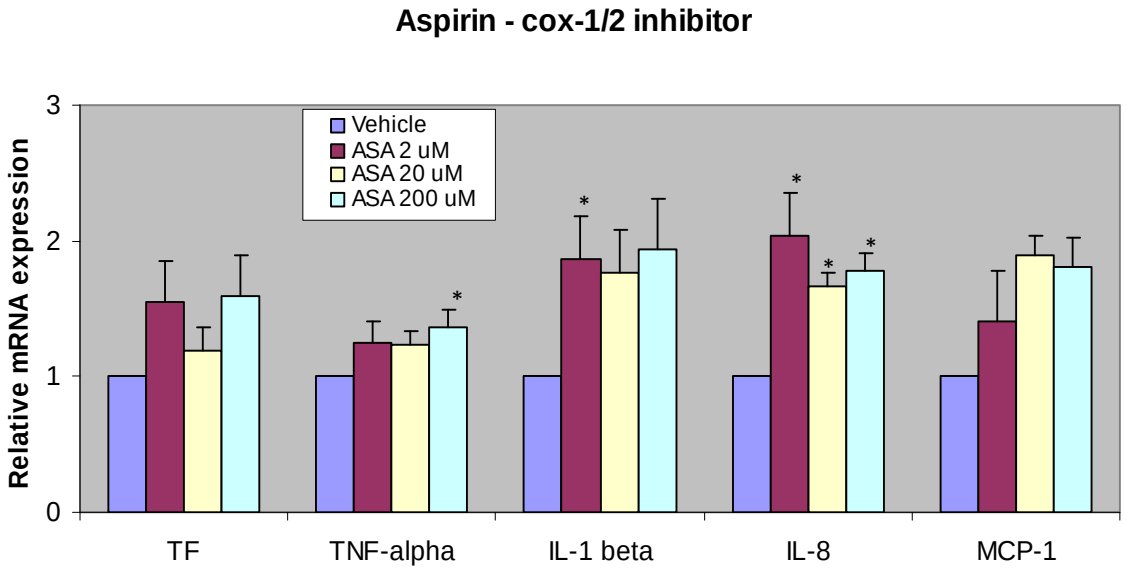

Figure 16. Aspirin as COX-1/2 inhibitor. Whole blood samples were first preincubated with various doses of the inhibitor, then stimulated with LPS for 90 min. Vehicle sample (DMSO and saline) was stimulated with LPS. * $p<0.05$ vs. vehicle sample. Paired-samples t-test. n = 6, n = 2 (MCP-1).

4.4.2. The effect of the selective COX-1 inhibitor SC-560 on LPS-induced expression of proinflammatory genes

As aspirin, also the COX-1 inhibitor SC-560, generally increased whole blood cytokine mRNA expression in response to LPS. Furthermore, like aspirin, the most pronounced effects of SC-560 were observed for IL-8 and IL-1β, but TF mRNA was also enhanced by this inhibitor. And for both cytokines a pronounced effect were observed for the lowest inhibitor concentration, on the other hand, a significant enhancement of the LPS-induced cytokine mRNA expression were evident (+37% and 38%, respectively, for IL-1β and IL-8). For TNF-α mRNA expression, the effect of SC-560 was less pronounced when compared to aspirin, and no significant effect was observed for this cytokine. SC-560 increased the LPS-induced MCP-1 expression levels, however due to the low parallels (n), this effect was not significant.

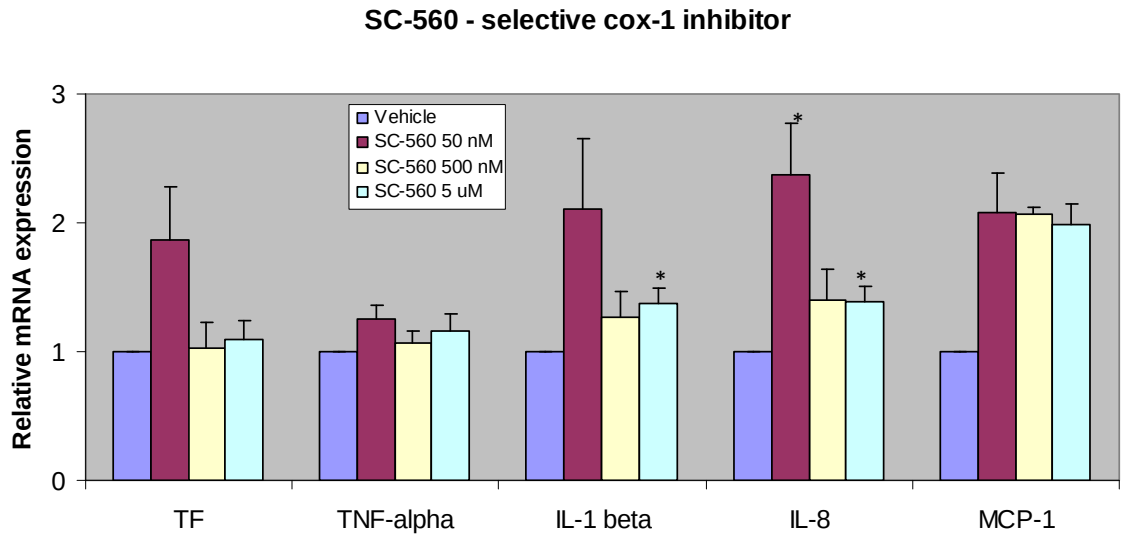

Figure 17. Selective COX-1 inhibitor on cytokines gene expressions. Whole blood samples were first preincubated with various doses of the inhibitor, then stimulated with LPS for 90 min. Vehicle sample (DMSO and saline) was stimulated with LPS. * $p<0.05$ vs. vehicle sample.
Paired-samples t-test. n = 6, n = 2 (MCP-1).

4.4.3. The effect of the selective COX-2 inhibitor CAY10404 on LPS-induced expression of proinflammatory genes

The effects of the inhibition of the inducible COX-2, was not as pronounced as inhibition of COX-1, however also CAY10404 resulted in enhancement of the LPS-induced expression of IL-8. For none of the other cytokines did the effects of the COX-2 inhibitor reach any significant levels. Contrary to the COX-1 inhibitors, the highest concentration seemed to cause a non-significant reduction in the mRNA expression for all cytokines except IL-8.

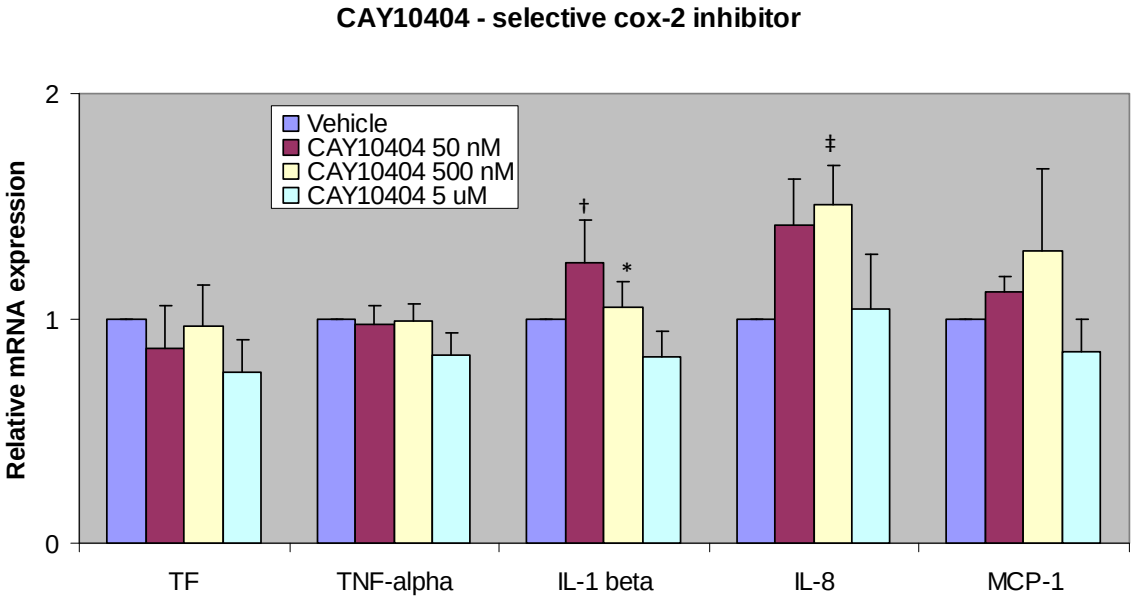

Figure 18. CAY10404 – selective COX-2 inhibitor. Whole blood samples were first preincubated with various doses of the inhibitor, then stimulated with LPS for 90 min. Vehicle sample (DMSO and saline) was stimulated with LPS. * $p<0.05$ vs. CAY10404 5 µM, † $p<0.05$ vs. CAY10404 5 µM, ‡ $p<0.05$ vs. vehicle sample. Paired-samples t-test. n = 6, n = 2 (MCP-1).

4.4.4. The effect of prostaglandin synthesis inhibitor CAY10526 on LPS-induced expression of proinflammatory genes

Inhibition of the prostaglandin synthesis using CAY10526 also resulted in a dose-related increase in cytokine mRNA expression in response to LPS. However, for TNF-α, IL-1β, and IL-8, this effect was most pronounced at the lower inhibitor concentrations whereas the effect was lower at higher inhibitor concentrations, and the highest concentration of CAY10526 did not have any significant effect for the cytokines tested. The strongest effect of the inhibitor was evident for IL-1β mRNA expression (apart from MCP-1: +80% MCP-1 mRNA expression using 100 nM inhibitor, n = 2, $p<0.05$) and the relative IL-1β mRNA levels were increased by 80% ($p<0.05$) and 90% ($p<0.05$) using inhibitor concentrations of 1 μM and 100 nM, respectively. For TNF-α and IL-8, inhibition of the prostaglandin synthesis result in an enhancement of LPS-induced mRNA expression of +37% and + 73% (100 nM inhibitor, TNF-α and IL-8, respectively). The mPGES-1/ COX-2 inhibitor did not significantly influence LPS-induced TF mRNA expression.

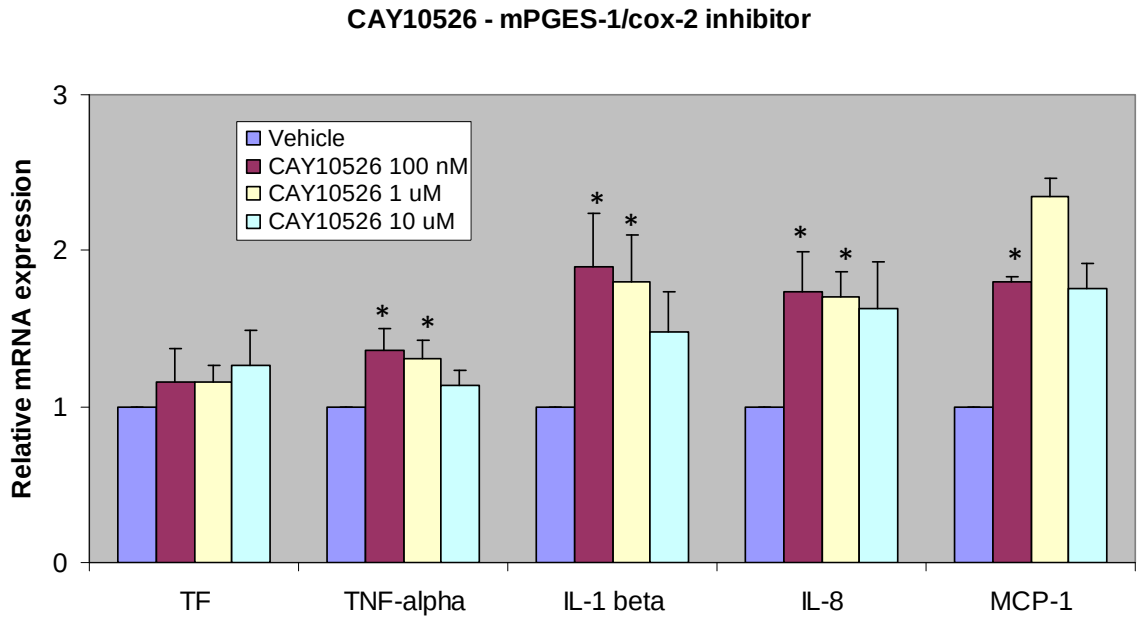

Figure 19. mPGES-1/cox-2 inhibitor. Whole blood samples were first preincubated with various doses of the inhibitor, then stimulated with LPS for 90 min. Vehicle sample (DMSO and saline) was stimulated with LPS. * $p<0.05$ vs. vehicle control. Paired-samples t-test. n = 6, n = 2 (MCP-1).

4.4.5. The effect of selective prostaglandin synthesis inhibitor MF63 on LPS-induced expression of proinflammatory genes

Samples with mPGES-1 inhibitor (MF63) show both augmentation and inhibiting effect of TF mRNA expression, where the highest doses resulted into an approximately 11% increase. A bi-phased expression is seen for nearly all the genes. It is further observed that the middle dose of the inhibitor seemed to induce gene expressions at a higher level than controls and other doses.

Although not significant, high increases in expression of MCP-1 were also found for strongest- and weakest doses, in addition to the most pronounced effect of the middle dose. Similar effects were observed for TNF-α, IL-1β and IL-8 gene expression, but with a slightly weaker effect.

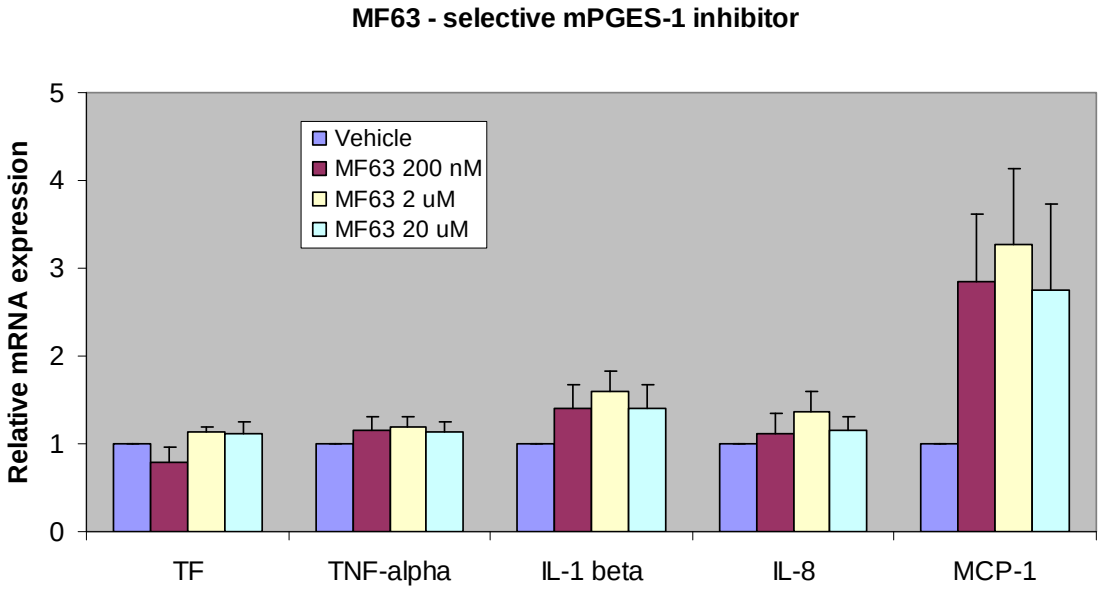

Figure 20. Selective mPGES-1 inhibitor. Samples were preincubated with the inhibitor then subsequent to LPS stimulation. Vehicle sample composed of DMSO and saline, then further stimulated with LPS. Paired-samples t-test. n = 6, n = 2 (MCP-1)

4.5. Antibody identification of leukocyte subpopulations

Leukocyte subpopulations (granulocytes and monocytes) interacting with platelets were examined using the FACSAria™. Differential countings for granulocyte-, monocyte- and platelet population were performed based on the FSC/SSC gating. PE-conjugated anti-CD16 was used to identify the granulocyte population, APC-conjugated anti-CD14 address to the monocyte population, and identification of platelet population was done using FITC-conjugated anti-CD42a. In whole blood samples stimulated with LPS, a significant increase was observed for the surface expression of the granulocyte- and monocyte markers CD16 and CD14 ($p<0.05$), as depicted in Figure 21. No further increase was observed in these markers when blood was stimulated with LPS and PAF in combination. The amount of the platelet specific marker CD42a did not change when blood was stimulated with either LPS alone or in combination with PAF.

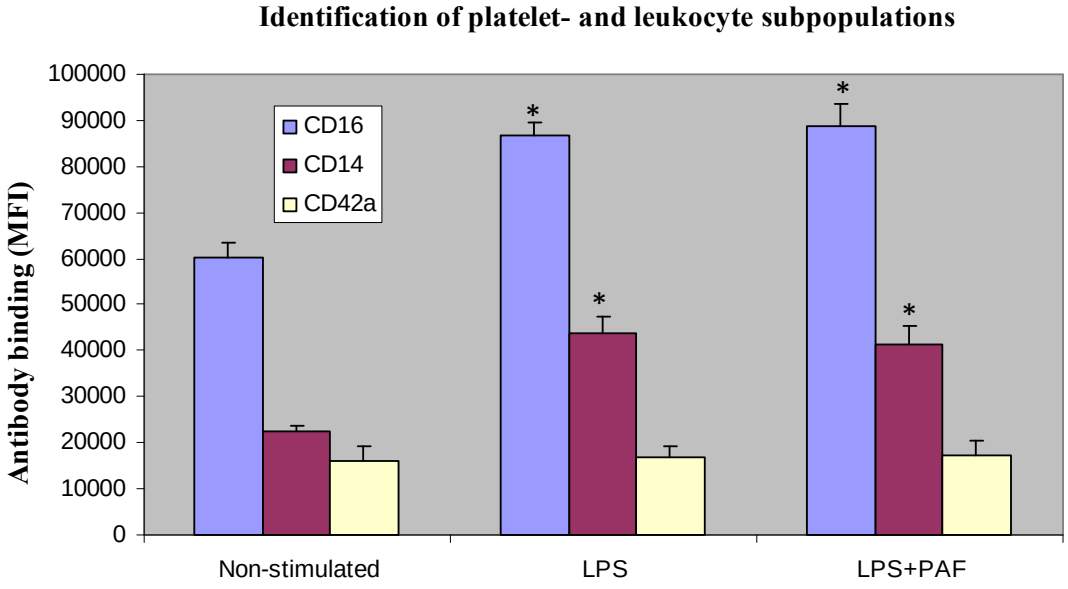

Figure 21. Electronic gate based on the FSC/SSC gating separated the different leukocyte subpopulations and platelet population. Samples were run as non-stimulated, LPS-stimulated and stimulated with LPS and PAF in combination. Anti-CD16 marker for granulocyte-, anti-CD14 marker for monocyte- and anti-CD42a marker for platelet population. * $p<0.05$ vs. non-stimulated. Paired-samples t-test. n = 4.

4.5.1. Quantification of adherent platelets in leukocytes

Platelet-leukocyte heteroconjugates were defined as monocytes positive for CD42a. Platelet (CD42a) interactions with monocyte increased (approximately 50%, not significant) upon LPS stimulation (Figure 22). Addition of PAF in addition to LPS to the samples further enhanced the conjugate formation and a 3-fold significant increase in platelet-monocyte aggregates were observed ($p<0.05$), when compared to LPS. Compared to the non-stimulated control samples, the increase after LPS+PAF stimulation was over 6-fold but not significant due to interindividual variance in the conjugate formation.

Ex vivo LPS stimulation of fragminized whole blood demonstrated that the binding of platelet to granulocytes decreased when compared to non-stimulated samples. However, the platelet-granulocyte aggregates slightly increased when stimulated with both LPS and PAF.

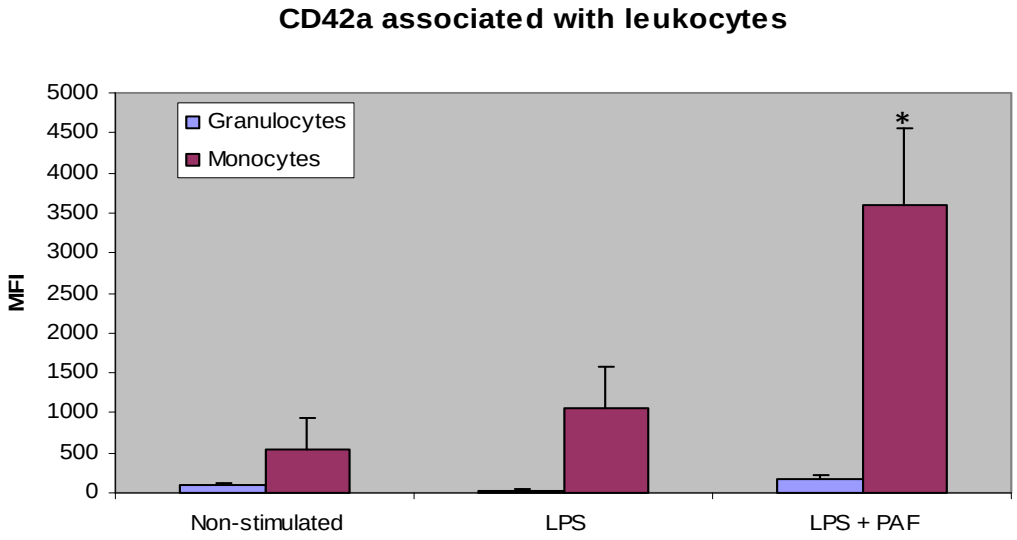

Figure 22. Platelet-leukocyte aggregates. Non-stimulated (saline)- and stimulated samples were incubated for 120 min. at 37°C, to prior flow cytometry preparation and analysis. * $p<0.05$ vs. LPS. Paired-samples t-test. n = 4.

It has been reported that platelet-leukocyte aggregates form mainly via platelet-expressed P-selectin and its leukocyte receptors, PSGL-1 and CD15 (Lehr *et al.*, 1994). The amount of P-selectin associated with leukocytes was measured using anti-CD62P. When compared to non-stimulated samples, CD62P associated with monocytes increased non-significantly after both LPS and LPS+PAF stimulation (+24% and 47.7%, respectively) (Figure 23). A marked decrease in P-selectin-granulocyte aggregates are seen for samples stimulated with LPS and PAF (-23%, $p<0.05$).

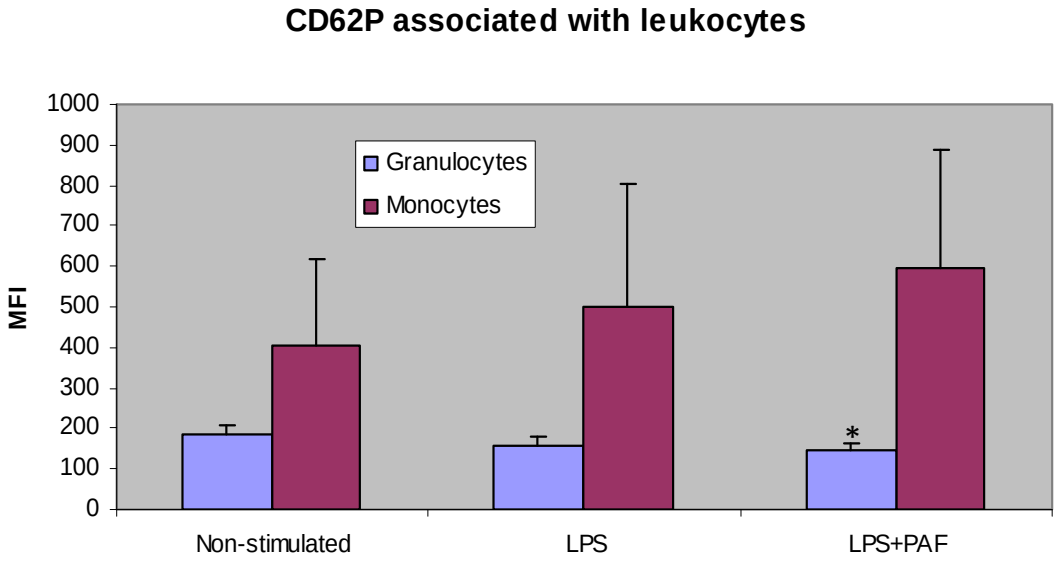

Figure 23. P-selectin (CD62P) associated with leukocyte subpopulations. Non-stimulated (saline)- and stimulated samples were incubated for 120 min. at 37°C, prior to flow cytometry preparation and analysis. * $p<0.05$ vs. non-stimulated. Paired-samples t-test. n = 4.

The amount of platelets bound to the leukocyte subpopulations was quantified and compared with the total amount of platelets. As expected, most of the detected CD42a was found in the platelet population (Figure 24). Stimulation of the samples resulted in a small release of CD42a from the platelet surface. For the LPS-stimulated samples a minor non-significant reduction of CD42a present in the platelet window was observed (-2.5%), whereas samples stimulated with both LPS and PAF revealed a marked decrease of CD42a (-13.3%, $p<0.05$). When the relative amount of CD42a in the platelets window dropped, this part of the CD42a was found in the monocyte window. After stimulation with LPS and LPS and PAF in combination, an increase of monocyte associated CD42a of 3% and 13% ($p<0.05$), respectively, was seen. The percentage of CD42a associated with the granulocyte population was very low.

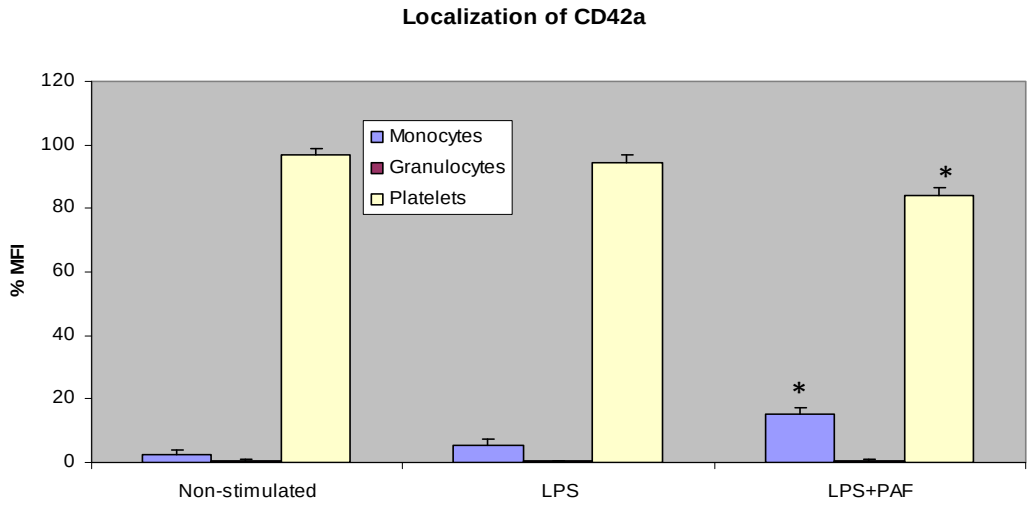

Figure 24. Localization of CD42a. Detected amount of the platelet marker CD42a in the platelet, monocyte and the granulocyte window. * $p<0.05$ vs. non-stimulated or LPS.

The relative amount of CD62P (P-selectin) in the platelet, monocyte and granulocyte windows was also quantified. In the non-stimulated situation, the amount of CD62P associated with the monocytes and granulocytes (approximately 40% in both populations) were higher compared to the platelet surface associated CD62P (ca. 20%) (Figure 25). After stimulation with LPS and LPS+PAF, the relative amount of CD62P-positive granulocytes was reduced (-5% LPS-stimulated and -14% for LPS+PAF, $p<0.05$). The CD62P was slightly increased on both monocytes and platelets upon stimulation with both LPS and LPS in combination with PAF. For the platelets, the relative increase in CD62P-positive cells increased with 3% (LPS) and 9% (LPS+PAF).

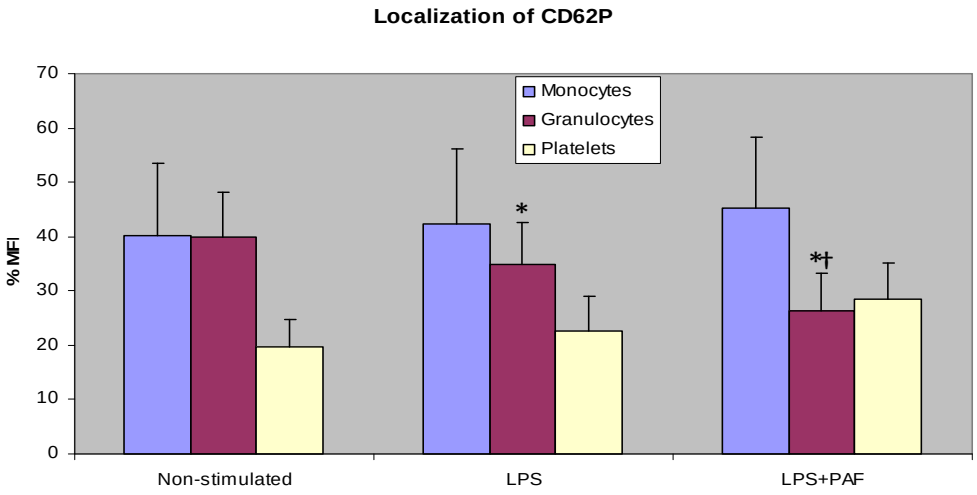

Figure 25. Localization of CD62P. P-selectin is detected as CD62P-positive in various population. * $p<0.05$ vs. LPS or LPS+PAF. † $p<0.05$ vs. LPS.

5. DISCUSSION

The whole blood system is a convenient test system, as it is readily available and reduces artifacts due to handling and isolation of cells. Furthermore, all the circulating blood components are present, and the different cell types are maintained at their *in vivo* state and may interact with each other. Since interindividual variations in blood cell number and blood component concentrations are preserved, experimental data might have a large variation, which is also useful in a clinical setting.

The results obtained from TF activity measurement, real-time PCR analysis and flow cytometry indicated that the applied methods were suitable to carry out the objectives of this study. The flow cytometry procedure showed that rapid information on platelet-leukoycte conjugate formation in whole blood can be obtained. However, improvement of the results would be to include a higher number of test persons.

5.1. Time course in response to LPS-stimulation

The time course TF activity in response to LPS-stimulation has previously been investigated in a study examining how different anticoagulants (EDTA, citrate, heparin, hirudin) modify blood cell behavior (Engstad *et al.*, 1997). When hirudin or heparin was used as anticoagulants a peak was reached after two hours of incubation. After a transient drop in activity it further increases up to 6 h. It was further observed that there was significantly increase from 1 to 2 h of incubation. In our study, LPS-induced TF activity increased up to a plateau level reached at 2 h. No significant increase was obtained when incubating the blood for 4 h, and at 8 h the LPS response had started to drop. At 4 h stimulation there was a huge interindividual variation in our study as the activity was very high in some individuals and had started to drop in other individuals. Increasing the incubation time from 1.5 h (and 1 h) to 2 h resulted in a significant enhancement of the LPS-induced TF activity. The observation that TF activity decreased after reaching the peak has also been observed in previously studies of Østerud's group (Østerud, personal communication). This indicates that it takes several hours before the blood status normalizes after being challenged with a single LPS-administration.

For the TF activity to increase significantly, the blood cells have to synthesize more of the TF protein. In addition, it has been indicated that potential (intracellular) pools of encrypted TF may be decrypted (or made bioavailable) in a process involving platelets (Østerud, 2001). When using isolated peripheral blood monocytes or adherent monocytes, other authors have indicated that TF activity reaches maximum after 3 h (Ramani *et al.*, 1993), 7 h (van der Logt *et al.*, 1994) and 10 h (Meszaros *et al.*, 1994). These observations demonstrate how different monocytes behave when they are isolated, extensively manipulated, and deprived for their natural environment. In addition, an adequate LPS response towards low-concentration LPS, as used in our study, is dependent of the presence of soluble serum proteins such as CD14 and LPS-binding protein to function optimally (Meszaros *et al.*, 1994).

In our study, the LPS concentration used (5 ng/ mL), was in agreement with Engstad *et al.* (1997). The LPS response also varied between batches and this is routinely checked in our laboratory. Using higher LPS concentrations also result in a completely different situation as the soluble plasma proteins no longer are necessary to present LPS to toll-like receptor 4, the cellular receptor which mediates the LPS-response.

The mRNA expression is generally more rapidly compared with the protein synthesis dependent TF activity, and TNF-α gene expression was observed to reach its maximum plateau level as quickly as half an hour after the addition of LPS. This rapid increase in the mRNA levels indicates that the cells are quickly signaled to begin transcribing the TNF-α gene. This observation is consistent with studies using human whole blood and murine macrophages of (Dedrick & Conlon, 1995) and DeFranco's group (Weinstein *et al.*, 1991), respectively. In our study, TNF-α mRNA levels remained high for several hours (>4 h).
TF mRNA expression had a later onset compared to TNF-α mRNA, and maximum TF mRNA expression was observed after 1 h stimulation. TF mRNA expression seemed to decline more rapidly compared to TNF-α, as TF mRNA levels had started to drop after 4 h stimulation. Yet, the kinetics of the TF mRNA expression seems to correspond well with the observed kinetics for LPS-induced TF activity.

The expression of IL-1β followed very similar kinetic pattern as TF mRNA expression. This might indicate that they have common properties as LPS-induced inflammatory genes in monocytes. The work of (Dedrick & Conlon, 1995) demonstrated that the TNF-α mRNA levels declined after 60 min, with the concentration used of LPS 1 ng/ mL, the expression was

maintained only if more LPS was added, so it is speculated that the declining of expression is caused by the weakening of the LPS concentration. In addition, LPS-induced genes, such as TNF-α, IL-1β and TF, quickly degrade once the transcription is stopped due to their very unstable mRNAs (Taniguchi, 1988). Furthermore, Zuckerman and colleagues showed that prolonged stimulation by LPS for at least 6 to 8 h, causes tolerance towards LPS, leading to reduced ability to induce cytokine synthesis both *in vivo* and *in vitro* (Zuckerman *et al.*, 1989). In our studies it is probable that the low LPS levels used will be neutralized during the first hours of incubation time, otherwise one might speculate that since our experiments were carried out up to twelve hours, tolerance might have an effect on the results. It is worth noting that the amount of LPS used will have an influence on the results. However, our study used a concentration of 5 ng/ mL, which is low-to-normal level in the whole blood system. Since binding and internalization of LPS by monocytes are greatly affected by the LPS concentration, these results might indicate that that expression of genes requires a cell to accumulate a critical amount of LPS (Gallay *et al.*, 1993).

A bi-phased expression was observed for IL-8 mRNA. This observation is also consistent with the study of DeForge and Remick in 1991 (DeForge & Remick, 1991). This complex pattern of the gene expression may be explained by that the stimulus for IL-8 release provided by LPS itself is self-limiting, and the early mediators such as TNF and IL-1 released by LPS stimulation, serves as a prolonged signal further enhancing IL-8 mRNA expression (DeForge *et al.*, 1992). The IL-8 release in turn can attract neutrophils and lymphocytes to tissue site of inflammation. Activated platelets may also induce IL-8 mRNA expression in this whole blood model (Weyrich *et al.*, 1996).

In our experiments, MCP-1 mRNA expression was observed to be late, and the maximum expression was observed at 8 h of incubation. However, at this time point also saline started to induce MCP-1 mRNA expression. When adjusting for the background MCP-1 expression, the optimal incubation time seemed to be 4 hours for analyzing MCP-1 mRNA expression. And it seems obvious that MCP-1 mRNA expression analyzed after 1.5-2 h LPS stimulation is not the optimal conditions. Studies of Weyrich et al. on activated platelets with ELISA observed that monocytes did not release MCP-1 under basal conditions, and when monocytes were incubated without stimulation, little or no secretion of MCP-1 was seen (Weyrich *et al.*, 1996). However, Weyrich et al. observed that addition of activated platelets (or RANTES (regulated upon activation normal T cell expressed presumed secreted) released from activated platelets)

to monocyte induce expression of MCP-1 and this may explain why we observed the MCP-1 mRNA also started to accumulate in the saline controls after 8-12 h of incubation as this might cause some activation of the platelets (Weyrich *et al.*, 1996).

5.2. Eicosanoids inhibition studies

Aspirin – COX-1/2 inhibitor. Pretreatment of human blood *ex vivo* with various doses of aspirin were shown to result in a dose-related enhancement (not significant) of LPS-induced TF activity. In agreement with this observation, a trend for enhanced LPS-induced gene expression of all the proinflammatory genes under investigation was demonstrated after pretreatment with aspirin. However, this enhancing effect was significant only for IL-8, IL-1β and TNF-α. These observations confirm a previous study demonstrating that *ex vivo* LPS-induced TF activity was increased when healthy volunteers took 300 mg of aspirin 1 h before blood donation (Østerud *et al.*, 1992).

In low concentration aspirin only inhibits COX-1, however when higher doses are used aspirin will inhibit the inducible COX-2 as well. Aspirin is 170-fold more potent in inhibiting COX-1 than COX-2 (Vane *et al.*, 1998). It has previously been demonstrated that exogenously added PGE_2 inhibits LPS-induced TF activity (Østerud *et al.*, 1992), which may indicate that inhibition of PGE_2 synthesis should enhance LPS-induced TF activity. Matsumoto *et al*. (1997) reported that when arachidonic acid was added to resting rat peritoneal macrophages, TxA_2 was produced immediately by COX-1, while production of PGE_2 was delayed and dependent on the induction of COX-2. After COX-2 had been induced by LPS, exogenously added arachidonate resulted in no additional TxA_2 synthesis, but large stimulation of PGE_2 synthesis (Matsumoto *et al.*, 1997). It is difficult to speculate about how this situation is when COX-1 is inhibited by aspirin, but it has been demonstrated that aspirin inhibits both TxA_2 and PGE_2 synthesis in LPS-stimulated whole blood (Østerud *et al.*, 1992). Alternatively, when the cyclooxygenase pathway is inhibited by aspirin, more arachidonic acid becomes available as a substrate for the lipooxygenase pathway which is known to contribute to the LPS-induced expression of proinflammatory responses (Eilertsen *et al.*, 2003).

Furthermore, the optimal dosage of aspirin for complete inhibition of platelet aggregation is subject to great interindividual variability and we also observed that the aspirin-effect was absent in some individuals whereas it was very pronounced in others.

Aspirin irreversibly inhibits the constitutive platelet COX-1, thus the platelet production of thromboxane and PGE_2 is reduced. Østerud et al. further demonstrated that activity in monocytes without LPS-stimulated decreased after aspirin addition. This is an indication of a different activation mechanism when LPS is not the stimulating agent (Østerud *et al.*, 1992).

Selective COX-2 and COX-1 inhibitors. The present study demonstrates that CAY10404, a selective COX-2 inhibitor (coxib), does not significantly affect LPS-induced TF activity, or LPS-induced TF mRNA expression. However, at 5 μM CAY10404, a trend for reduction of mRNA expression for all the investigated genes, except IL-1 was observed. In fact, lower concentrations of the coxib caused enhanced expression of both IL-8 and IL-1β. The concentrations of the coxib used in our study are all above the IC_{50} for inhibition of COX-2, which is <1 nM in isolated cells.

Free arachidonic acid activated cyclooxygenase and lipooxygenase in platelets. When the COX-2 inhibitor was added, an inhibition of cyclooxygenase pathway occurred. Since lipoxyenase is not inhibited, the oxygenation of liberated arachidonate should continue until no arachidonate is longer available (Hamberg & Hamberg, 1980).
Furthermore, the treatment with COX-2 inhibitor enhanced the effect on TF activity generation, which indicate that the lipooxygenase, and not cyclooxygenase pathway, was involved in the generation of TF activity.

COX-2 is inducible by proinflammatory stimuli, including cytokines and endotoxin, and is hence considered to be responsible for proinflammatory prostaglandin formation. The addition of COX-2 inhibitor does not only reduce the synthesis of PGE_2, but may redirect free arachidonate into the lipooxygenase pathway, thus enhancing the leukotrienes generation (Brock *et al.*, 1999; Østerud *et al.*, 1992). LTB_4 is known to enhance LPS-induced IL-8 release from blood cells (Lund & Østerud, 2003). Similar explanation can be accounted for the increased IL-8 expression when samples were incubated with the selective COX-1 inhibitor.

After LPS stimulation, TNF-α is the first cytokine to be detected in the circulation, thereafter IL-1 is observed (DeForge *et al.*, 1992). The TNF-α expression decreased as the coxib was added to samples, while IL-1β expression increased as lower doses were used. In contrast to this observation, in whole blood culture, selective COX-2 inhibition was found to increase the

TNF-α production, detected by ELISA ((Härtel *et al.*, 2004). Moreover, at the highest concentration of 50 μM, TNF-α production increased to 153.9% as compared to stimulated cells incubated without COX-2 inhibitor (Härtel *et al.*, 2004). This discrepancy might be explained by that PGE_2 has cellular actions which can be considered both pro-inflammatory and anti-inflammatory. Via anti-inflammatory, it suppresses monocyte TNF-α and IL-1β production (Hart *et al.*, 1989).

When the effects of the selective COX-1 inhibitor SC-560 were investigated, very similar results were obtained. An enhancing effect of the LPS-induced gene expression was observed for the same genes except TNF-α as the COX-1 inhibitor had no effect on the gene expression of TNF-α.

A trend for increased expression of MCP-1 mRNA was observed after addition of the selective COX-1 inhibitor as it was for aspirin. When the COX-2 inhibitor was used it did not seem to consistently affect the MCP-1 expression. MCP-1 is mainly expressed by macrophages in response to cytokines, such as TNF-α and IL-1β, and our results indicate that inhibition of platelet COX-1 leads to an increase in MCP-1 expression, whereas this is not the case when the inducible COX-2 activity is inhibited. It is difficult to speculate about the underlying mechanism for this observation.

Inhibition of mPGES-1 expression and selective mPGES-1 inhibition. When the selective mPGES-1 inhibitor MF63 is added to freshly collected blood prior to stimulation with LPS, a selective inhibition of the production of PGE_2 (with an IC_{50} of 1.3 μM) and no concomitant TxB_2 inhibition were observed (Côté *et al.*, 2007). The IC_{50} concentration is within the concentration range used in our study. With the addition of selective mPGES-1 inhibitor prior to LPS-stimulation, TF activity was dose-dependently increased. The effects on mRNA expression of the investigated proinflammatory genes were less pronounced and not significant for any of the genes. The mPGES-1 is inducible by various inflammatory stimuli and is primarily coupled to COX-2 (Murakami *et al.*, 2003). Furthermore, it is up-regulated in response to various proinflammatory stimuli with a concomitant increase of COX-2 expression.

Since we did not measure the direct effects on the production of the specific prostanoids (e.g. TxA_2 and PGE_2) in this study, we are not able to tell whether the addition of mPGES-1/ COX-2 inhibitor might have inhibited the generation of both thromboxanes and PGE_2.

Dual COX-2/ 5-LOX inhibitor. To compare the results of the COX and prostaglandin E synthase inhibitors with the effects of inhibition of leukotrienes biosynthesis, we included a dual COX-2/ 5-LOX inhibitor in the study. However, as it did not significantly affect the LPS-induced TF activity, we did not include this inhibitor in the mRNA expression analysis. Yet, it was evident from the TF activity measurements that this inhibitor followed a different pattern compared to the other inhibitors since there was a trend for reduced TF-activity after treatment with the dual inhibitor. In LPS stimulated blood, the COX-2/ 5-LOX inhibitor has previously been reported to inhibit the release of TNF-α, IL-1β and IL-8, measured by ELISA. The IC_{50s} for the inhibition of those cytokines were 33 μM, 11 μM and 6.7 μM, respectively (Hartman *et al.*, 1995). Due to the solubility of the inhibitor, the highest dose used in our studies was 10 μM. This should be sufficient to significantly inhibit the production of TxA_2.

5.3. Leukocyte-platelet interactions measured by flow cytometry

Platelet activation can be measured by the quantification of platelet-monocyte aggregates or heteroconjugates. It has recently been suggested that platelet-monocyte aggregates is a more robust indicator of *in vivo* platelet activation than platelet-leukocyte aggregates (Michelson *et al.*, 2001). To compare the binding of platelets to monocytes and granulocytes, the effect of LPS and LPS+PAF in combination to stimulate platelets and leukocytes, measured as heteroconjugate formation, were examined. Most of the platelets were conjugated to the monocytes at all induction states of the blood, and especially after LPS+PAF stimulation. PAF is a relatively potent platelet inducer but also stimulate certain activation signals in leukocytes. After LPS stimulation alone, we hardly detected any CD42a, platelet marker, in the granulocyte window (Figure 22). The amount of platelets detected in the granulocyte window was much lower as compared to the situation in the monocyte window. It is further observed a great interindividual response to stimuli (monocyte population), which can also be explained by high responders and low responders (very low response to a stimulating agent). However, the relatively low amount of platelet binding to granulocytes compared to monocytes, suggest that platelets binding to granulocytes may require stronger stimulation or stimulation of a platelet-specific or granulocyte-specific activator.

As expected, LPS stimulation markedly increased specific surface markers of the granulocyte- and monocyte population (Figure 21). However, when whole blood stimulation with LPS and PAF in combination, a slightly reduction was seen in monocyte population compared to LPS alone. This effect was earlier observed in various cytokines expression when whole blood was stimulated with both LPS and different doses of PAF. This indicates that PAF only has a limited effect when used as a pre-stimulus of LPS-induced expression of proinflammatory mediators and molecules in whole blood.

Granulocytes generate PAF, which interact with- and activate platelets, when blood is exposed to LPS (O'Flaherty & Wykle, 1983). Thus the small amount of detected platelets population may also caused by the activated granulocytes. For the granulocyte population, a small increase was observed when samples were stimulated with LPS and PAF. This might indicates that granulocytes release PAF (and granular enzymes), and PAF acts both as an autocrine activator, thus amplifying the granulocytes activation, but also as a paracrine which stimulate platelets and monocytes.

The clear increase in the number of CD42a positive events in the monocyte gate is observed for samples stimulated with both LPS and PAF (Figure 22), which is indicative of platelet-monocyte conjugates, thereby confirms the ability of PAF to enhance the LPS-inducing effect in stimulating platelet. Furthermore, our results confirm that PAF activates platelets and monocytes and stimulates platelet-monocyte interactions, without affecting the granulocytes to the same degree. This was demonstrated by Groscurth et al. using PAF as a single stimulus to whole citrated blood (Groscurth *et al.*, 1988). They observed morphological changes in both monocytes and granulocytes, yet only monocytes came into close contact with platelets and platelet aggregates (Groscurth *et al.*, 1988). As for the TF activity and mRNA expression studies, cell-cell interactions displayed a great interindividual response to the stimuli, which can also be explained by high responders and low responders phenomenon reported by Groscurth et al.

As known, platelet-leukocyte aggregates or conjugates are mainly formed via platelet-expressed P-selectin and its receptors PSGL-1 and CD15, and via fibrinogen bridging between glycoprotein IIb/ IIIa and CD11b/ CD18 (Lehr *et al.*, 1994). P-selectin is expressed only on the surface of activated platelets, while PSGL-1 and CD15 are constitutively expressed on leukocytes. In our experiments, we observed a totally different situation for the

platelet granule protein P-selectin. Upon platelet stimulation, the platelets release their granule content. As demonstrated, P-selectin associates with platelets, granulocytes and monocytes, and almost equally distributed (Figure 23 and 25). CD42a which is a constitutively expressed platelet surface receptor known to be shedded from the platelet surface under certain conditions. In our study, the vast majority of the CD42a remained in the platelet window and not associated with granulocytes, whereas a certain part of the CD42a was detected in the monocyte and platelet windows but probably still attached to the platelet surface (Figure 24).

Note, that the fluorescence signals detected for CD62P in our studies were quite low (Figure 23), which may indicate that the monoclonal antibody did not work optimally. Due to the limited amount of time, we did not have the possibility to confirm the results using a different batch of the antibody.

6. CONCLUSION

- Optimal incubation time for LPS stimulation was considered to be between 1.5 h and 2 h.
- LPS-stimulation of whole blood, as compared to saline controls, significantly increased the monocyte TF activity and cytokine mRNA expressions levels.
- Aspirin was observed to enhance the LPS-induced TF activity in monocytes and the proinflammatory cytokine gene expressions.
- As for aspirin, the COX-1 inhibitor SC-560, seemed to enhance the LPS-induced monocyte TF activity. The gene expression of proinflammatory cytokines was also increased.
- The selective COX-2 inhibitor CAY10404 enhanced the LPS-induced gene expression of IL-8, IL-1β and MCP-1 as wells as the monocyte TF activity. The TF and TNF-α mRNA expression were not affected by the inhibitor. At the highest concentration of CAY10404, it did not enhance any of the genes, on the contrary a non-significantly reduction was observed for most genes.
- The prostaglandin synthesis inhibitor CAY10526 was observed to have a biphasic effect in enhancing the monocyte TF activity, paralleled by an enhancing effect of the gene expression for all the proinflammatory cytokines.
- The selective mPGES-1 inhibitor, MF63, showed a dose-related enhancing effect of monocyte TF activity. The effects of MF63 on the mRNA expression of the cytokines were general low and not significant; however, there was a trend for an enhancing effect on the LPS-induced gene expression for all the genes, except TF.
- LPS-stimulation of whole blood significantly increased the respective markers of both the granulocyte- and monocyte populations.
- Platelet was observed to interact and bind easily to monocyte, whereas platelet binding to granulocytes seemed to require an additional/ stronger stimulus.

7. FUTURE PERSPECTIVES

This thesis was performed under a very limited amount of time; hence a lot of the links and necessary experiments to complete the project are still missing. First of all, we were unable to tell whether the addition of the various inhibitors of the prostanoid biosynthesis actually inhibited the production of PGE_2 and TxB_2, therefore a quantification of these eicosanoids should be carried out, for example using ELISA. Furthermore, we probably should address the expression levels of the enzymes under investigation, COX-1, COX-2, mPGES and possibly also the cytosolic PGE synthase, in whole blood.

The current research could be improved by using more parallels. By using more test persons, trends probably would become statistically significant, especially for MCP-1 gene expression. As it is great interindividual variations, the deletion of one subject could change the output of the result. Blood cells are observed to behave differently, depending on the type of anticoagulant used. It would be interesting to perform these experiments with different anticoagulants.

Optimally, we might investigate the high responder phenomenon and perform the experiments with a larger group of individuals and group them according to their response towards LPS. However, further studies on longer term expression (also analyze at 4 and 8 h) should be performed in order to gain a better understanding of the effect on responses to a stimulating agent. This is important as it has been reported that the prostanoid biosynthesis/ release is a very time dependent process, in addition to the fact that the cytokines MCP-1 and IL-8 are optimally expressed later than 2 h.

Moreover, in the flow cytometric analysis we need to address the link between the prostanoids and cell-cell interactions by performing experiments using flow cytometry on samples treated with the COX-1, COX-2 and mPGES inhibitors. Also in flow cytometric analysis anticoagulant test could be performed, as it has been shown that anticoagulant type has a marked effect on platelet-monocyte aggregate levels. Fragmin is known as a very effective inhibitor of platelet activation, and the use of citrate may result in quite different results. Finally, in future work a different batch of the CD62P antibody should be used to be sure that the fluorescence was OK and to confirm the obtained results.

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APPENDICES

APPENDIX 1 – Primers and probes concentrations for real-time PCR analysis

Calculation of primers and probes concentrations used in real-time PCR analysis.

Gene: **cyclophilin A**

Both primers had a stock concentrations of 200 µM. This concentration was diluted twenty times, by adding 380 µL dH_2O to 20 µL of stock solution. The new concentration was then 10 µM. For 1 reaction, 0.4 µL of primer solution was used (from earlier knowledge). Thus the concentration was found out by following equation:

10000 nM * 0.4 µL = X nM * 20 µL
200 nM = X

Probe stock concentration was 100 µM. This was further diluted twenty times, which gave a new concentration of 5 µM. From earlier experience, 0.8 µL was used for each reaction. The probe concentration was found out:

5000 nM * 0.8 µL = X nM * 20 µL
200 nM = X

Concentrations for the rest of the genes used in this work were found out in similar way. Table A-1 below shows the amount of primers and probes used in this work.

Table A-1. Primers and probes concentrations.

Reagents	**Cyclophilin A**		**TF**		**TNF-α**	
	á	**x80**	**á**	**x80**	**á**	**x80**
2X mastermix	10	800	10	800	10	800
Forward primer	0.4	32 (200 nM)	0.4	32 (200 nM)	0.8	64 (400 nM)
Reverse primer	0.4	32 (200 nM)	0.4	32 (200 nM)	0.8	64 (400 nM)
Probe	0.8	64 (200 nM)	0.4	32 (100 nM)	0.8	64 (200 nM)
dH_2O	4.4	352	4.8	384	3.6	288
	16	1280	16	1280	16	1280

Reagents	**Interleukin 1β**		**Interleukin 8**		**MCP – 1**	
	á	**x80**	**á**	**x80**	**á**	**x80**
2X mastermix	10	800	10	800	10	800
Forward primer	0.8	64 (400 nM)	0.8	64 (400 nM)	0.8	64 (400 nM)
Reverse primer	0.8	64 (400 nM)	0.8	64 (400 nM)	0.8	64 (400 nM)
Probe	0.2	16 (100 nM)[2]	0.8	64 (200 nM)	0.2	16 (100 nM)[3]
dH_2O	4.2	336	3.6	288	4.2	336
	16	1280	16	1280	16	1280

[2] Probe #78, Roche Applied Science Universal Probe
[3] Probe #40, Roche Applied Science Universal Probe

APPENDIX 2 – Real-time PCR efficiency

In order to quantify the obtained results, standard curves were made for each of the genes. Relative gene expression was calculated based on the primer efficiency, C_t values of unknown sample and the respective sample of the reference gene.

Table A-2. Shows the slope, r^2 and efficiency values for each of the genes, calculated from the standard curves.

Subject 1.

Genes	*Slope*	*Efficiency*	*R^2*	*Y-intercept C_t*
Cyclo A	-3.466	0.9432	0.9975	35
TF	-3.3513	0.9879	0.9954	38
TNF-α	-3.4068	0.9658	0.9972	33
IL-1β	-3.3470	0.9896	0.9956	32
IL-8	-3.2820	1.017	0.9985	32

Subject 2.

Genes	*Slope*	*Efficiency*	*R^2*	*Y-intercept C_t*
Cyclo A	-3.3098	1.0051	0.9942	36
TF	-3.4170	0.9618	0.9922	38
TNF-α	-3.2830	1.0165	0.9982	33
IL-1β	-3.2877	1.0145	0.9972	32
IL-8	-3.2956	1.011	0.9989	31

Subject 3.

Genes	*Slope*	*Efficiency*	*R^2*	*Y-intercept C_t*
Cyclo A	-3.4119	0.9634	0.9991	34
TF	-3.3163	1.0023	0.9981	38
TNF-α	-3.4303	0.9567	0.9995	33
IL-1β	-3.3591	0.9847	0.9972	30
IL-8	-3.4218	0.9599	0.9988	30
MCP-1	-3.3649	0.9824	0.9972	38

Subject 4.

Genes	*Slope*	*Efficiency*	*R^2*	*Y-intercept C_t*
Cyclo A	-3.4792	0.9383	0.9990	36
TF	-3.4244	0.9589	0.9972	36
TNF-α	-3.4030	0.9672	0.9990	33
IL-1β	-3.2642	1.0247	0.9969	30
IL-8	-3.3495	0.9886	0.9994	29

Subject 5.

Genes	*Slope*	*Efficiency*	R^2	*Y-intercept* C_t
Cyclo A	-3.4587	0.9459	0.9972	36
TF	-3.4679	0.9425	0.9950	36
TNF-α	-3.3038	1.0076	0.9987	32
IL-1β	-3.3023	1.0083	0.9982	31
IL-8	-3.4134	0.9632	0.9992	30

Subject 6.

Genes	*Slope*	*Efficiency*	R^2	*Y-intercept* C_t
Cyclo A	-3.4521	0.9484	0.9980	35
TF	-3.3327	0.9955	0.9934	40
TNF-α	-3.3313	0.9961	0.9980	33
IL-1β	-3.4484	0.9498	0.9996	30
IL-8	-3.4524	0.9483	0.9991	30
MCP-1	-3.3520	0.9876	0.9969	40