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In vitro studies of inflammation and cytotoxicity

Ylva Edling

Karolinska Institutet

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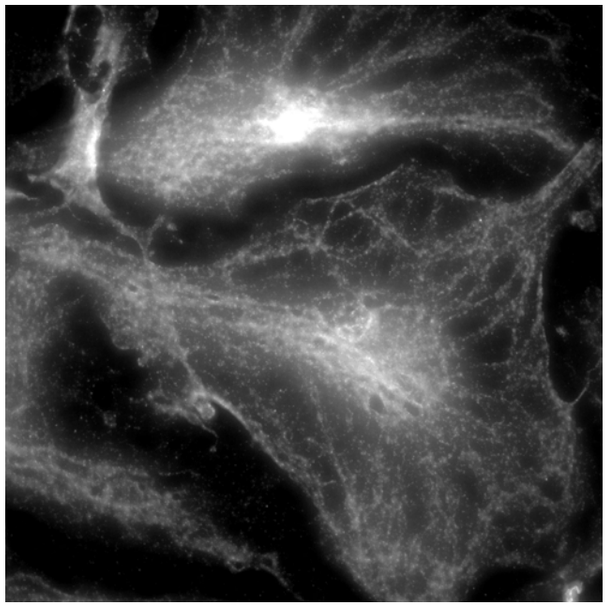
Endotoxin-, glutamate- and drug-induced inflammation and cytotoxicity with emphasis on signal transduction mechanisms



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ABSTRACT

Inflammatory cells such as monocytes and glial cells play an important role in the pathogenesis of ischemic- or drug-induced brain or liver injury. Excessive concentrations of glutamate is toxic to neural cells, and triggers a series of transcriptional events beginning with the expression of the immediate early genes like *c-fos* and *c-jun*, which in turn affect the expression of other genes necessary for the development of brain injury. Glial cells are important mediators in such response as they are responsible for the secretion of pro-inflammatory cytokines and chemokines which in turn will cause recruitment of immune cells into the brain. Using primary rat glial cell cultures we found that lipopolysaccharide (LPS)-induced *c-fos* expression was p38 MAPK-dependent and occurred via the activation of the SRE or the CRE elements in the promoter. In contrast to what has been shown in neurons, we found that glutamate-induced *c-fos* expression in glial cells involves a novel calcium-dependent pathway. This mechanism requires the participation of mGluR5, mobilization of ER-calcium and de-repression of DREAM at the DRE element in the *c-fos* promoter.

Similar mechanisms of inflammation as seen in the brain also occur in the liver, where Kupffer cells play a similar role functioning as the hepatic macrophages in their ability to release pro-inflammatory cytokines. Drug-induced hepatotoxicity is a major problem in drug development since preclinical *in vitro* as well as *in vivo* animal models usually are of little value for prediction of hepatotoxicity in humans. Pure hepatocyte cultures are generally not a sensitive enough model system to predict drug-induced cytotoxicity. We therefore developed a novel *in vitro* system containing both monocytes and hepatocytes. Ximelagatran (thrombin inhibitor) as well as the PPAR-γ agonists' troglitazone (hepatotoxic) and rosiglitazone (not hepatotoxic) were used as model compounds.

Studies in single cultures of monocytes (THP-1) showed a ximelagatran dependent release of pro-inflammatory chemokines and decreased cell viability, which was shown by inhibitors to involve the JNK- and ERK-signal transduction pathways.

A novel human *in vitro* co-culture model system containing THP-1 and hepatocytes (Huh-7) was established where the cells were separated by a permeable membrane. In such co-cultures troglitazone-induced cytotoxicity was more apparent and observed earlier than using single cultures of either Huh-7 or THP-1 cells, whereas rosiglitazone showed no cytotoxicity in either system. The troglitazone effect was accompanied by a much greater expression of genes encoding pro-inflammatory cytokines, chemokines and several other stress-related genes using the co-culture system as compared to single cell type cultures. Conditioned medium from troglitazone-treated THP-1 cells decreased the viability of Huh-7 cells indicate the release of monocyte-derived mediators. It is concluded that such co-culture system might constitute a valuable tool for predictions of drug-induced hepatotoxicity.

LIST OF PUBLICATIONS

I. Simi A, **Edling Y**, Ingelman-Sundberg M, Tindberg N. *c-fos* activation by lipopolysaccharide in glial cells via p38 MAPK-dependent activation of SRE or CRE elements. *J Neurochem*. 2005, **92**:915-924.

II. **Edling Y**, Ingelman-Sundberg M, Simi A. Glutamate activates *c-fos* in glial cells via a novel mechanism involving the glutamate receptor subtype mGlu5 and the transcriptional repressor DREAM. *Glia*. 2007, **55**:328-340.

III. **Edling Y**, Andersson T. B., Porsmyr-Palmertz M, Ingelman-Sundberg M. Pro-inflammatory response and adverse drug reactions: mechanisms of action of ximelagatran on chemokine and cytokine activation in a monocyte *in vitro* model. 2007, submitted *Toxicology In Vitro*

IV. **Edling Y**, Sivertsson L, Butura A, Ingelman-Sundberg M, Ek M. Increased sensitivity for drug-induced hepatotoxicity using a novel human in vitro co-culture model. 2008, submitted *Toxicology and Applied Pharmacology*

CONTENTS

LIST OF ABBREVIATIONS

AC	Adenylyl cyclase
ALD	Alcoholic liver disease
ALT	Alanine aminotransferase
AMPA	(*S*)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AP-1	Activator protein-1
ATF	Activating transcription factor
CaMK	Calcium-calmodulin dependent protein kinases
CMZ	Chlomethiazole
CNS	Central nervous system
COX	Cyclooxygenase
CRE	cAMP-response element
CYP	Cytochorome P450
DAG	1,2-diacylglycerol
DILI	Drug-induced liver injury
DRE	Downstream regulatory element
DREAM	DRE-antagonistic modulator
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
GFAP	Glial fibrillary acidic protein
GFP	Green Fluorescent protein
IEG	Immediate early gene
IFN-γ	Interferon-γ
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IL-8	Interleukin-8
iNOS	Inducible nitric oxide synthase
IP_3	Inositol 1,4,5-triphospate
JAK	Janus activated kinase
JNK	c-jun N-terminal kinase
KA	Kainic acid
LPS	Lipopolysaccharide
LTP	Long term potentiation
MAPK	Mitogen-activated protein kinases
MAPKK	MAPK kinase
MAPKKK	MAPK kinase kinase
MCAO	Middle cerebral artery occlusion
MCP-1	Monocyte chemoattractant protein-1
MEK	MAPK/ERK kinase
mGluR	Metabotropic glutamate receptors
MPT	Membrane permeability transition
NAC	N-acetyl-L-cysteine
NF-κB	Nuclear factor kappa B
NGF	Nerve growth factor
NK	Natural killer

NMDA	*N*-methyl-D-aspartic acid
NO	Nitric oxide
PGE_2	Prostaglandin E_2
PIP_2	Phosphatidylinositol-4,5-bisphosphate
PKA	Protein kinase A
PLA2	Phospholipase A2
PLC	Phospholipase C
PPAR-γ	Peroxisome proliferator-activated receptor-γ
PPRE	PPARγ response element
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RXR	Retinoid X receptor
SIE	*Sis*-inducible enhancer element
SRE	Serum response element
TGF-β	Transforming growth factor β
TNFα	Tumor necrosis factor α
ULN	Upper limit of normal
VEGF	Vascular epidermal growth factor

1 INTRODUCTION

Inflammatory reactions are important in damage caused by different insults e.g. ischemia or drug-induced injury, both in the liver as well as in the brain.

In the central nervous system (CNS) inflammation is important in the physiological processes occurring after the onset of an ischemic stroke or head injury. Stroke is a very common cause of death in the industrial part of the world and every year 30,000 people suffer from stroke in Sweden. At present no efficient pharmacological treatment is available to treat the damages which occur after the insult. In the liver, inflammation is also a key component of virus and drug-induced injury and toxicity. Approximately 4 % of newly developed drugs are withdrawn from the market due to adverse drug reactions and adverse drug reactions cause 100,000 deaths per year in the US (cf. (Eichelbaum et al. 2006)). Moreover, during 1995-2005 at least 34 drugs were withdrawn from the market, mainly due to hepatotoxic or cardiotoxic effects (Need et al. 2005). At present there are no good *in vitro* or animal models to study and detect the potential toxic effects of new drugs during development.

1.1 INFLAMMATION

Inflammation is a common response of living tissue to injury. Nowadays inflammation is not only viewed in the classical clinical way (edema, erythema and pain), but also in terms of activation of cells and production of mediators that participate in host defense and have the potential to cause further tissue injury. This response may be initiated by physical injury, chemical substances, micro-organisms or other agents. The main function of the inflammatory response is to alert the immune system; destroy and eliminate the foreign agent, and remove the damaged tissues and cells. Although inflammation is necessary for the elimination of pathogen(s), inappropriate or unregulated inflammation can cause tissue or organ injury. During inflammation the balance between pro-inflammatory and anti-inflammatory mediators is a key point, i.e. the release of specific cytokines and chemokines (see 1.1.2.1). The magnitude of inflammatory response is fundamental since insufficient response result in immunodeficiency and eventually to infection; excessive immune responses on the

other hand can cause disease where inflammation is a constituent such as rheumatoid arthritis, diabetes, multiple sclerosis to give a few examples (Tracey 2002).

1.1.1 Immune cells of brain and liver

All organs in the human body have immune cells such as macrophages which are derived from circulating immune cells. Four organs in the body have resident specialized immune cells; lung alveolar macrophages, osteoclasts in bone, microglia in the brain and Kupffer cells in the liver. The cells function in instances of injury, entrance of a foreign substance and also by alerting circulating immune cells. This work focuses on immune responses and inflammatory signaling in both the brain and in the liver.

The human brain consists mainly of two cell types; neurons and glial cells. Brain function is completely dependent on a constant supply of glucose and oxygen via the blood. The most vulnerable cells to any interruption in the continuous flow are the neurons; whereas the glial cells, endothelial cells and oligodendrocytes are more resistant (Siesjo 1988). The glial cell population can be subdivided into several categories; astrocytes, oligodendrocytes and microglia. Glial cells were for a long time considered only as packing material for the neurons, "gluing" them together, hence their name glia which comes from the Greek word for glue. However, the glial cell population has numerous functions, some of them being supportive to neuronal function. For example, the reuptake of the excitatory amino acid glutamate is of key importance. Another crucial function is the regulation of homeostasis of ions such as Ca^{2+} and K^{+} (reviewed in (Ogawa et al. 2007). Glial cells, mainly the astrocytes and microglia, also have immunological functions; cytokine secretion, phagocytosis of damaged cells and recruitment of monocytes and neutrophils to areas of cellular injury.

The liver harbors mainly hepatocytes, Kupffer cells, endothelial cells and stellate cells. Hepatocytes form the majority of the liver tissue, and are responsible for lipogenesis, glucogenesis, and production of proteins as well as the metabolism of endogenous and exogenous substances. Similar to interactions between neurons and the glial cell population in the brain, Kupffer cells affect the environment of the hepatocytes via the production of various inflammatory mediators in response to both endogenous and exogenous substances. Under normal conditions Kupffer cells, the resident immune cells, produce low levels of cytokines. However, if the liver has been damaged – for

example, by trauma, drugs or excessive alcohol consumption - inflammatory responses are initiated. The inflammatory response is generally initiated by activation of monocytes or Kupffer cells, thereby causing the release of cytokines. If the inflammation does not decrease after a short period, this increase in cytokine production can cause activation of stellate cells resulting in fibrosis (scaring of the liver). Alcoholic liver disease (ALD) is an example where persistent high levels of cytokines cause chronic inflammation of the liver (Neuman 2003) (see 1.3.1.1).

1.1.2 Inflammatory mediators

There are several types of mediators involved in inflammation e.g. cytokines, chemokines, prostaglandins, leukotrienes and nitric oxide (NO). The following text will emphasize the role of cytokines and chemokines.

1.1.2.1 Cytokines and chemokines

Cytokines are polypeptides that are associated with inflammation, immune responses as well as cell death. They are produced by macrophages, monocytes, lymphocytes as well as endothelial cells and many other cell types. In the central nervous system (CNS) the microglia and astrocytes are the major producers of cytokines, whereas in the liver Kupffer cells, the resident liver macrophages, are responsible. There are both pro-inflammatory, exemplified by tumor necrosis factor α (TNFα), interleukin (IL)-1β, IL-6 as well as anti-inflammatory cytokines such as IL-4, IL-10, IL-13 and transforming growth factor-β (TGF-β). Macrophage-produced TNFα, IL-1β and IL-6 are normally the first cytokines to be released in an inflammatory cascade. Moreover, TNFα can stimulate the synthesis of nitric oxide and other inflammatory mediators that drive chronic inflammatory responses. Anti-inflammatory cytokines such as IL-10 and TGF-β can in turn attenuate TNFα activity. Balance in the activation of immune cells is crucial. For example, increased levels of TNFα can lead to chronically activated cells, increased levels of oxidative stress causing DNA and tissue damages which in turn can lead to promotion of tumor development. In contrast to their cytotoxic effects, macrophages are also involved in tissue regeneration. For example, TNFα participates in hepatocyte regeneration.

Chemokines are also polypeptides, but generally smaller than cytokines, involved in cellular communications and recruitment of inflammatory cells such as monocytes and neutrophils to the area of insult. Chemokines can be subdivided into four groups

depending on the spacing of their first two cysteine (C) residues; where IL-8 and monocyte chemoattractant protein-1 (MCP-1) are examples of CC and CXC chemokines and the most widely expressed. IL-8 has been implicated in the recruitment of neutrophils to the CNS in cerebral ischemia. Increased levels of MCP-1, and IL-1β have been observed in patients with acute ischemic stroke (Emsley and Tyrrell 2002; Rothwell 1999). Moreover, MCP-1 is believed to be involved in the infiltration of monocytes and macrophages into the CNS (reviewed in (Emsley and Tyrrell 2002). Thus, chemokines are also major contributors to inflammation.

1.1.3 Inflammatory signaling

Released inflammatory mediators such as TNF, IL-1 and IL-6 function via binding to receptors on the cellular surface. TNF and IL-1 family members exert their biological effects though binding to TNF-receptor (TNFR) and IL-1 receptor (IL-1R), respectively. Receptor binding triggers a series of intracellular events, which eventually leads to activation of mitogen-activated protein kinases (MAPKs) as well as nuclear factor kappa B (NF-κB) and subsequent expression of inflammatory proteins (Akira and Takeda 2004; O'Neill 2000; Shen and Pervaiz 2006). For example, IL-6 binds to the IL-6 receptor complex which consists of the receptor and a signal transducer. This complex causes the downstream cascade which eventually leads to activation of Janus activated kinase (JAK) and transduction of signal transducer and activator of transcription (STAT) family proteins to the nucleus (Kishimoto 2006).

1.1.3.1 The mitogen activated kinase family

MAPK cascades are collectively composed of three protein kinases that act sequentially of each other; a MAPK is activated by a MAPK kinase (MAPKK) which in turn is activated by a MAPK kinase kinase (MAPKKK) (see figure 1). The signal is transferred form the cell surface to the nucleus either via interaction with small GTP-binding proteins of the Ras superfamily or through interaction with membrane receptors. Activated MAPK translocates from the cytosol to the nucleus where they phosphorylates a variety of transcription factors and thereby activate transcription.

MAPKs are generally divided into three groups; i) extracellular signal-regulated kinase (ERK), *ii*) c-jun N-terminal kinase (JNK) and *iii*) the p38 MAPK group kinases (figure 1). They all are activated by dual phosphorylation of both threonine and tyrosine residues (Raingeaud et al. 1995). The mammalian ERK cascade is involved in the

control of cell proliferation and differentiation by mitogenic stimuli and growth factors, while JNK and p38 are regulated by environmental stress such as UV radiation, osmotic shock, ischemic injury, and by inflammatory cytokines such as TNFα, IL-1β. The MAPK pathways coordinate activation of gene transcription, protein synthesis, cell death and differentiation (reviewed in (Kyriakis and Avruch 2001)).

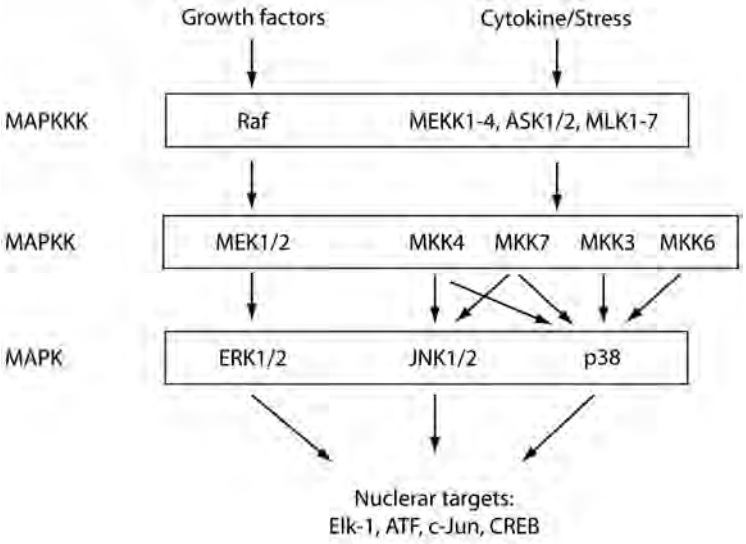

Figure 1. MAPK signaling pathway. Note that arrows do not indicate direct phosphorylation events, but rather pathways.

1.1.3.1.1 Biological functions of ERK, JNK and p38

There are numerous reports demonstrating the involvement of the p38 MAPK pathway in inflammation. The p38 pathway plays a significant role in production of pro-inflammatory cytokines such as IL-1β, TNFα, IL-6 and IL-8; induction of cyclooxygenase (COX) enzyme COX-2; as well as expression of inducible nitric oxide synthase (iNOS) (reviewed in (Ono and Han 2000)). Inhibition of p38 MAPK with the pyridinylimidazole compound SB203580 reduces pro-inflammatory cytokine production in monocytes, macrophages, neutrophils and T-lymphocytes (Kaminska 2005). SB203580 was originally developed as an inhibitor of inflammatory cytokine synthesis (Badger et al. 1996), which was later found to inhibit p38 MAPK by binding to the ATP-pocket of the enzyme (Tong et al. 1997).

It has been shown that there is sustained activation of p38 MAPK in glial cells following cerebral ischemia (Barone et al. 2001). Treatment with SB203580 significantly reduced the size of the infarct in the gerbil ischemia model (Sugino et al.

2000). Even more intriguing is the fact that the infarct volume was significantly reduced when SB203580 was administrated even up to 12 hours after middle cerebral artery occlusion (MCAO). This also reduced the induction of inflammatory components such as iNOS, TNFα, IL-1β and cyclooxygenase (COX)-2 (Piao et al. 2003). A second generation p38 MAPK inhibitor SB239063 has been shown to reduce infarct size, as well as reduce expression of TNFα and IL-1β mRNA (Barone et al. 2001). SB239063 potently inhibits the p38 enzymatic activity but not the phosphorylation of the enzyme (Barone et al. 2001).

Experiments in primary cultures of neurons have shown that withdrawal of nerve growth factor (NGF) or nutrients cause apoptosis, and that this apoptotic program require JNKs and p38s (Kyriakis and Avruch 2001). Studies in PC-12 neuronal cells revealed that NGF withdrawal leads to activation of JNK and p38 along with a decrease in ERK (Xia et al. 1995). NGF normally activates Ras, which leads to activation of Raf then MAPK/ERK kinases (MEK 1/2) followed by the ERK pathway with the net outcome of cell survival. So NGF withdrawal may not only trigger JNK and p38 activation but also concurrently suppress the activation of ERKs. The inactivation of ERK-cell survival pathway together with the activation of JNK- and p38-pathways appears to be critical for NGF-induced apoptosis in PC-12 cells (Xia et al. 1995). Activation of ERK pathway is also required for oxidative stress-induced cell death (Guyton et al. 1996).

Both JNKs and p38 have been shown to regulate levels of functional protein both on a translational as well as a posttranscriptional level. High turnover proteins such as inflammatory cytokines and chemokines such as IL-1β, IL-6 and IL-8, contain AU-rich elements (ARE) in their 3'-untranslated region (repeats of AUUUA sequences). Activation of p38 has been demonstrated to increase the stability of IL-8 mRNA, and this increased stability is a result of the activity of the p38 substrate MAPKAP-K2 (Kyriakis and Avruch 2001).

1.1.3.2 Regulation of AP-1 gene expression by MAPK

Apart from affecting mRNA stability, the MAPKs are also involved in transcriptional control of gene expression. The major transcription factors involved in mediating inflammatory responses apart from activator protein-1 (AP-1) are NF-κB and the novel member DREAM (see 1.1.3.3). AP-1 is a transcription factor complex, composed of

members of the Jun and Fos family as homodimers (of Jun-family members) or heterodimers. The *c-fos* component of the AP-1 complex, will serve as an example of MAPK regulation of gene expression.

The AP-1 transcription factor complex binds to AP-1 recognition sequences within target gene promoter regions (Su and Karin 1996). As mentioned, both *c-jun* and *c-fos* are immediate early genes, i.e. genes whose transcription is rapidly induced by a variety of stimuli. Induction of *c-fos* transcription is mediated by different *cis*-acting elements in the proximal promoter region (see figure 2). These *cis*-elements include a *Sis*-inducible enhancer element (SIE), a serum response element (SRE), a cAMP-response element (CRE), and the downstream regulatory element (DRE) (see figure 2). SIE is the target for Janus protein tyrosine kinase (JAK)-mediated cytokine signaling; SRE receives signals generated by growth factors, cytokines and other MAPK activating factors; CRE typically respond to neurotransmitter and hormone signaling; and DRE is responsive to intracellular calcium changes (Carrion et al. 1999; Su and Karin 1996). Induction of *c-fos* transcription via these transcriptional response elements requires phosphorylation of the respective transcription factors binding to the elements (see figure 2). These phosphorylations occur via several different upstream kinases which show some degree of cross-activity (see figure 2). p38 regulates *c-fos* transcription via phosphorylation of either Elk-1 or activating transcription factor (ATF)-2 which bind to the SRE and CRE element, respectively (see figure 2).

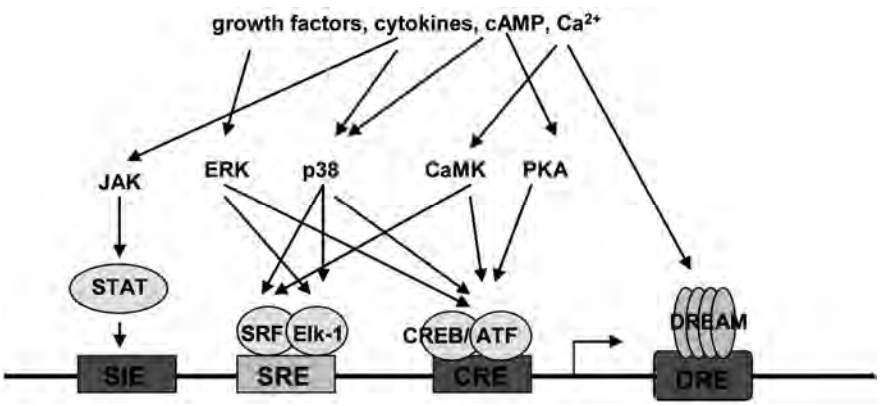

Figure 2. Signaling pathways involved in *c-fos* transcription by different extracellular stimulus. Arrows are not necessarily direct phosphorylation events, rather refer to pathways.

1.1.3.3 Involvement of the transcriptional repressor DREAM in gene regulation and inflammation

The importance of the transcriptional repressor element, downstream response element (DRE), was first described in the regulation of the prodynorphin gene which is involved in memory acquisition and pain (Carrion et al. 1998). The DRE recognition sequence, GAGTCAAGG, is typically located downstream of the gene's transcription start (Carrion et al. 1998). Carrión and co-workers identified a 110-kDa nuclear complex binding to the element, which upon activation of protein kinase A (PKA) the binding of the complex was reduced and the prodynorphin gene was transcribed (Carrion et al. 1998). Carrión and collaborators identified the 110-kDa repressor complex that binds specifically to the DRE; the complex was named DRE-antagonistic modulator (DREAM) (Carrion et al. 1999). Structure and sequence analysis of the DREAM complex revealed that it contained four calcium binding domains (EF-hands). Carrión and co-workers also showed that upon stimulation with calcium, DREAM lost its ability to bind to the DRE as well as its repressor function (Carrion et al. 1999). Furthermore, mutation of any of the four EF-hands prevented DREAM to respond to calcium (Carrion et al. 1999). Aside form the prodynorphin gene, Carrión and co-worker also showed that DREAM binds to a DRE site in the *c-fos* promoter (Carrion et al. 1999). Thus, DREAM was the first known calcium-binding protein to function as a DNA-binding transcriptional regulator, again stressing the importance of calcium as a second messenger in the cell.

In a knock-out mouse model DREAM-deficient mice showed a greatly reduced pain behavior compared to wild type mice using a model of both tissue injury and inflammation (Cheng et al. 2002). Another *in vivo* study showed that peripheral inflammation caused up-regulation of DREAM in the spinal cord, mainly in IB4-positive cells which are typically inflammatory cells of monocytic lineage such as macrophages, monocytes and microglia (Zhang et al. 2007). Furthermore, DREAM is also involved in regulation of cytokines, e.g. IL-2, IL-4 and interferon (IFN)-γ, thus indicating it may have a central role in the immune response (Savignac et al. 2005).

1.2 NEUROINFLAMMATION AND THE ROLE OF GLUTAMATE

As mentioned, CNS inflammation is important factor in many diseases, as an example I'll use ischemic stroke of the brain. One of the events occurring after the onset of

cerebral ischemia is inflammatory response and inflammatory mediated events. The insult itself leads to the immediate cell death by necrosis. Around the core area is a zone with compromised oxygenation and energy supply, where the cells are still metabolically active even though ATP levels have dropped to 50-70% of normal levels, and the cells can thus undergo programmed cell death (Mehta et al. 2007; Rossi et al. 2007). As the insult progresses and with reperfusion of the affected area, various processes arise such as inflammation, excitotoxicity, production of reactive species as well as apoptosis (Emsley and Tyrrell 2002).

There are overwhelming data suggesting that inflammation contributes to cerebral ischemic injury (Price et al. 2003), however the mechanisms leading to the inflammatory response are not fully understood. Thus, the identification of the cell populations that are responsible for inflammation and their affect on the environment is of great importance. One characteristic feature of the inflammatory response is the activation of microglia and astrocytes, and subsequently their expression and release of inflammatory cytokines, for instance IL-1β and TNFα and chemokines, for example IL-8 and MCP-1 (Zhang et al. 2000), as well as release of glutamate (reviewed in (Panickar and Norenberg 2005). IL-1 or TNFα administrated by intracerebral injection exacerbates the ischemic injury *in vivo*, whereas administration of anti-inflammatory mediators such as IL-10 or IL-1 receptor antagonist reduces the injury (Allan and Rothwell 2001; Barone and Feuerstein 1999) and references therein). Recently more data are emerging stating the effect that glial derived mediators have on the cellular environment of the brain. Taylor et al. (2005) show how activation of specific glutamate receptors in microglial cells induced expression and release of TNFα, which in turn contributed to neurotoxicity. Furthermore, Patel and collaborators (2006) show that IL-1 enhances the brain damage induced by glutamate receptor activation, and that the enhancement is via prolongation of the seizure. Additionally, prostaglandin E_2 (PGE_2) which is known to contribute to inflammation and has been shown to modulate neuronal activity in the spinal cord, by affecting the frequency of what the neurons are firing (Lu et al. 2007).

1.2.1 Glutamate in relation to brain injury

Glutamate is one of the major excitatory neurotransmitters in the CNS, and is necessary for normal brain function. However during CNS injury, excessive exposure of glutamate to neural cells is lethal. Under normal conditions the concentration of

glutamate in the extracellular space is in the low micromolar range; however following cerebral ischemia there is an excessive release and increase in glutamate concentration (100-300 μM) (Benveniste et al. 1984; Budd 1998; Takagi et al. 1993). This effect is potentiated by the breakdown of astrocytic functions with respect to re-uptake of glutamate from the synaptic cleft (Mori et al. 2004). There are numerous studies on how glutamate elicits its toxic effects on neurons (reviewed in (Ogawa et al. 2007)). However, there are few studies that focus on how the excitotoxicity affect glial cells. Although far more resistant, glial cells are also exposed to the increased glutamate concentrations during ischemia. Furthermore, lately it has also been known that astrocytes play a role in glutamate signaling; they express transporters which regulate the extracellular concentration, they express glutamate receptors as well as having different mechanisms of releasing glutamate (Rossi et al. 2007).

The increased concentration of glutamate from presynaptic neurons into the synaptic cleft cause overstimulation of both ionotropic and metabotropic glutamate receptors (see below 1.3.1.1) in postsynaptic cells. Activation of metabotropic receptors causes further increase of calcium levels by release from intracellular stores via the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) to generate inositol 1,4,5-triphospate (IP_3) (Choi 1990). The high extracellular concentrations of glutamate can induce excitotoxic cell death involving overstimulation of glutamate receptors, and as mentioned this activation causes intracellular accumulation of calcium, which in turn leads to massive calcium-dependent activation of enzymes such as phospholipase A2 (PLA2) and nitric oxide synthase, generation of ROS and mitochondria dysfunction. Under normal conditions glutamate is taken up by surrounding astrocytes, thereby keeping the concentration of glutamate low in the synaptic cleft and minimizing the exposure. However, *in vivo* studies have shown astrocytic cell death even though they were thought to resist glutamate excitotoxicity (reviewed in (Had-Aissouni et al. 2002)).

Cornell-Bell et al. showed that cultured astrocytes express glutamate receptors, which are used to receive and respond to signals from neurons (Cornell-Bell et al. 1990). The authors showed that the binding of glutamate to the receptor leads to extracellular calcium flow into the cell, as well as release of calcium from intracellular stores. This increase triggers a rise in calcium levels in neighboring cells; consequently the signal is passed from one astrocyte to the next one (Cornell-Bell et al. 1990).

1.2.1.1 Glutamate and its' receptors in CNS

Glutamate can act on two different types of receptors; ligand gated ion channels (ionotropic receptors) and G-protein coupled (metabotropic) receptors. The ionotropic receptors are subdivided into three classes based on their binding of exogenous substrates; *N*-methyl-D-aspartic acid (NMDA), (*S*)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainic acid (KA). Upon binding of glutamate these receptors let charged ions such as Na^+ and Ca^{2+} pass into the cell.

The G-protein coupled metabotropic glutamate receptors (mGluR) are widely expressed not only in neurons but also in glia cells (Petralia et al. 1996; Prezeau et al. 1994). Metabotropic glutamate receptors trigger the signaling cascade by activating phospholipase C (PLC) or adenylyl cyclase (AC). There are at least eight different subtypes of metabotropic receptors known, which are arranged into three different groups; group 1 (mGluR 1/5), group 2 (mGluR 2/3) and group 3 (mGluR 4/6/7/8). Group 1 is positively coupled to PLC, thus stimulating hydrolysis of PIP_2 into IP_3 and 1,2-diacylglycerol (DAG), whereas group 2 and 3 are negatively coupled to AC leading to an inhibitory cAMP cascade.

1.2.2 Calcium in response to glutamate

The calcium ion is a very versatile second messenger which can enter the cell via numerous sources; via charged ion channel pores, such as NMDA, AMPA and KA receptor channels (see above 1.2.1.1), as well as being released from intra-cellular calcium stores. Under normal conditions the concentration of calcium in the cytosol is kept low, around 100 nM. This level is tightly regulated by Ca^{2+} influx, Ca^{2+} efflux, Ca^{2+} -binding proteins and intracellular Ca^{2+} storage. So even a small local increase in calcium concentration, for example around an ion channel or at intracellular store release sites, can activate calcium-dependent enzymes as well as neighboring channels or receptors (reviewed in (Arundine and Tymianski 2003)). However, due to the low levels of ATP during and after an ischemic insult, sequestering Ca^{2+} into the mitochondria as well as the endoplasmic reticulum (ER) is compromised (Siesjo 1988).

As mentioned above, activation of metabotropic glutamate receptors cause increased calcium levels. Group 1 of metabotropic receptors coupled to the hydrolysis of PIP_2 into IP_3 and DAG. IP_3 binds to the IP_3-receptors on the ER membrane, which then in turn releases calcium from the ER. Although cellular Ca^{2+} excess is not solely

responsible for mediating neuronal cell death, the importance of calcium in glutamate-induced excitotoxicity has been accentuated (Choi 1987; Choi et al. 1987).

1.2.3 The immediate early gene *c-fos* in CNS

As mentioned before excitotoxicity, oxidative stress and inflammation contribute to neuronal damage. Among the early events that occur are the release of excitatory amino acids, like glutamate, and accumulation of intracellular calcium. These signals are believed to trigger a series of transcriptional events advancing from the expression of immediate early genes (IEG) to the synthesis of proteins, which will in turn regulate the expression of other genes with potential to increase the brain injury. Glutamate, but also inflammatory stimuli like lipopolysaccharide (LPS) and cytokines, induce the expression of the immediate early response genes like *c-fos* and *c-jun* in the brain. In neurons, *c-fos* expression is important for coupling of neuronal activity to gene expression, thus mediating physiological functions such as activity-dependent survival, plasticity and long-term potentiation (LTP) (Abraham et al. 1991; Morgan and Curran 1991). Moreover, glial *c-fos* expression has been documented in pathological conditions such as ischemia (Yu et al. 1995) and CNS or systemic infections (Matsunaga et al. 2001; Rubio and Martin-Clemente 1999), where it seems to contribute to glial activation and inflammatory gene expression. Additionally, neuronal expression of *c-fos* has also been documented in pathological conditions such as cerebral ischemia (Dragunow et al. 1994; Nowak et al. 1990) and studies in animal models of cerebral ischemia using *c-fos* anti-sense oligonucleotides revealed a great neuroprotective potential (Chiasson et al. 1997; Lu et al. 1997).

1.3 LIVER INJURY AND THE ROLE OF KUPFFER CELLS

Drug-induced hepatotoxicity can cause hepatitis (inflammation in the liver), hepatic failure and can even be lethal. Preclinical *in vivo* animal toxicity studies are usually of little value for prediction of hepatotoxicity in humans, due to fundamental species differences in drug metabolism and transport. Cultured hepatocytes have been widely used as a tool to study drug metabolism and toxicity. Though primary cultures of human hepatocytes are available, one of the drawbacks is the rapid deterioration of liver specific enzymes involved in the metabolism after days in culture (LeCluyse 2001). Regarding human *in vitro* models, primary hepatocytes or hepatoma cell lines, such as HepG2, have been widely used but show a low extent of sensitivity and

generally a relatively low degree of prediction for drug toxicity (reviewed in (Dambach et al. 2005; Groneberg et al. 2002)). Drug-induced hepatotoxicity requires the involvement of Kupffer cells, which are responsible for mediating the inflammatory processes through the release of several pro-inflammatory cytokines such as IL-1β, TGF-β, TNFα and IL-6. Indeed, inactivation of Kupffer cells by *in vivo* treatment of rats with gadolinium chloride prevents the toxicity exerted by e.g. paracetamol (Michael et al. 1999) and carbon tetrachloride (Muriel and Escobar 2003).

The primary functions of Kupffer cells are phagocytosis, processing of ingested material, antigen presentation and secretion of biologically active products. The activation process of the Kupffer cells is a two-step process which requires a priming factor, e.g. interferon-γ (IFN-γ). Primed macrophages are characterized by increased secretion of reactive oxygen species (ROS), whereas fully activated macrophages secrete high levels of above mentioned cytokines. This activation of macrophages implies that certain genes, like immediate early genes, are induced to produce proteins to carry out the fully activated macrophage-functions. Cytokine expression in connection to liver damage is not only unfavorable, since cytokines are involved in regeneration of the liver. In spite of this, if the inflammation does not subside after a short time the persistent production of the same cytokines may cause liver damage and cirrhosis.

1.3.1.1 Alcoholic liver disease and the importance of Kupffer cells

Animal models of alcoholic liver disease (ALD) have shown amelioration of hepatic injury with pre-treatment with gadolinium chloride, which depletes the liver resident macrophages (Kupffer cells), and thereby lessens the production of TNFα produced. Additionally, administration of TNFα antibodies attenuated the ethanol-induced liver injury, as well as the importance of macrophage-produced TNFα was shown in knock-out mice which lack TNF receptor 1 (Tsukamoto and Lu 2001; Yin et al. 1999). Thus, Kupffer cells have a direct regulatory role in ethanol-induced hepatocyte injury, and the activation of monocytes has been shown to play an important role in ALD (McClain et al. 2002). One important aspect to keep in mind is the fact that TNFα is involved in the initiation of liver regeneration, so elucidating a way to inhibit the mechanisms behind the priming mechanisms behind TNFα expression without interfering with the pro-regenerative effects is of great importance when developing an anti-inflammatory therapy for ALD (reviewed in (Tsukamoto and Lu 2001)). Additionally, inactivation of

Kupffer cells with gadolinium chloride alleviated symptoms of ALD mainly by reducing ethanol-induced steatosis and cytochrome P450 (CYP) 2E1-meidated ROS production (Jarvelainen et al. 2000). Furthermore, *in vitro* studies have shown that co-cultures of macrophages and hepatocytes render the hepatocytes more sensitive to ethanol-induced oxidative stress (Griffon et al. 2000).

1.3.1.2 Drug-induced liver injury

There are many different mechanisms contributing to drug-induced liver injury (Lee 2003; Navarro and Senior 2006; Park et al. 2005). One cause of hepatotoxicity is dependent on formation of metabolites from drug metabolizing enzymes, e.g. by cytochrome P450 (CYP). The CYP-dependent formation of reactive metabolites are either directly hepatotoxic or form adducts with hepatic proteins, potentially triggering an immune response, which might cause hepatitis, hepatic failure and even death. Drug-induced liver injury is a frequent reason for the withdrawal of an approved drug form the market, or why drugs are stopped during development (for review see (Kaplowitz 2005)).

Mitochondrial injury in drug-induced liver injury has become more valued recently as a possible mechanism of action of hepatotoxicity. Among the suggested mechanisms are; uncoupling of oxidative phosphorylation, inhibition of electron transport chain, opening of membrane permeability transition (MPT) pores. Drug-induced hepatoxicity can often have a delayed onset, up to weeks, after initiation of drug treatment. Why there is this delayed onset is not clear, however one hypothesis is that genetic or acquired abnormalities of electron transport chain or factors of the antioxidant system might augment the mitochondrial oxidative stress (Boelsterli 2003; Boelsterli and Lim 2007; Ong et al. 2007).

Troglitazone and ximelagatran are two examples of drugs that have been withdrawn from the market due to hepatotoxicity where the mechanisms of toxicity are unknown.

1.3.1.2.1 Troglitazone

Troglitazone, (±)-5-[4-(6-hydroxy-2,5,7,8-tetramethylchroman-2-ylmethoxy)benzyl]-thiazolidine-2,4-dione, (Rezulin®, Resulin® and Romozin®) (figure 3) was the first thiazolidinedione, or glitazone, developed on the market for the treatment of type II diabetes. However, troglitazone was withdrawn from the market in 2000 after clinical

signs of hepatotoxicity, which were increased levels of alanine aminotransferases (ALT) in serum (Masubuchi 2006). Later rosiglitazone (figure 3), also belonging to the glitazone family, was developed and is still used in the clinic. Troglitazone is an agonist to the peroxisome proliferator-activated receptor-γ (PPARγ) and ameliorates insulin resistance thus enhancing the sensitivity of the liver, muscle and adipose tissue to circulating insulin (reviewed in (Van Gaal and Scheen 2002)). Endogenous agonists to PPARγ are polyunsaturated fatty acids such as linoleic acid, arachidonic acid, as well as prostaglandin-related compounds such as 15-deoxy-delta12-14-PGJ2, and examples of synthetic compounds are the glitazones; troglitazone, rosiglitazone, ciglitazone and pioglitaozone (Kota et al. 2005). Upon binding of the ligand PPARγ forms a heterodimer with retinoid X receptor (RXR), which subsequently binds to PPARγ response element (PPRE) in target genes. PPREs are found in genes involved in lipid metabolism and energy balance.

In the patients treated with troglitazone an increase of ALT levels was observed, which is almost always a consequence of already dead or dying hepatocytes. More than 1.9% of patients treated with troglitazone in the clinical trials showed the increased levels of ALT that was more than three times the upper limit of normal (3x ULN) value as well as other signs of liver injury, and in a number of cases hepatic failure was evident (Watkins and Whitcomb 1998). Additionally, a case study showed that the troglitazone-induced hepatic liver injury had a delayed onset (months) after start of treatment, which could quite abruptly develop into hepatic failure (reviewed in (Graham et al. 2003)). Extensive *in vitro* studies have been carried out in an attempt to elucidate the mechanisms behind the troglitazone-induced hepatotoxicity. One hypothesis is that reactive quinone-metabolites of troglitazone, produced by CYP3A4, are involved in the hepatotoxicity and that these reactive metabolites can form adducts to proteins like glutathione (He et al. 2004). Another proposed mechanism of troglitazone-induced toxicity is via mitochondrial dysfunction, with decreased ATP levels and the loss of mitochondrial potential, as well as increased levels of ROS (reviewed in (Masubuchi 2006; Smith 2003)). The loss of mitochondrial potential often precedes the detection of metabolic products and therefore contradicts the involvement of reactive troglitazone-intermediates.

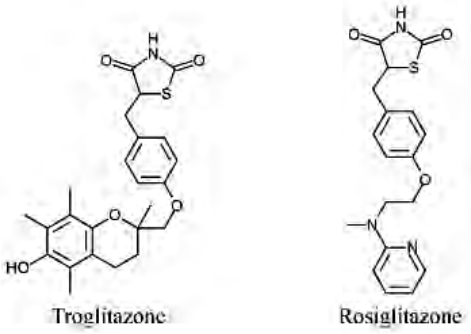

Figure 3. Structure of the glitazones troglitazone and rosiglitazone.

1.3.1.2.2 Ximelagatran

Ximelagatran (figure 4), Exanta® or Exarta®, is a prodrug converted into the active form melagatran which constitutes a potent thrombin inhibitor (Gustafsson et al. 1998), and was developed as a replacement for warfarin. Warfarin, also known as waran; is commonly used in the clinic as an anti-coagulant inhibitor. However, obstacles with adjusting the warfarin dosage are the influence of genetic and environmental factors. The dose-response relationship is influenced by genetic variations of CYP2C9 and VKORC1, together with various disease states, other drugs and dietary factors. Therefore in order to optimize the therapeutic effect of warfarin without risking dangerous side effects, such as bleeding, routine monitoring of the level of anti-coagulation is required (reviewed in (Hirsh et al. 2001)). Thus, other drugs are warranted.

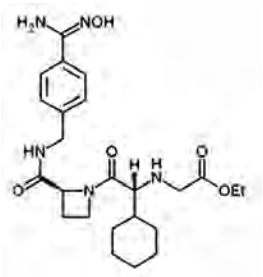

Figure 4. Structure of the specific thrombin inhibitor ximelagtran

With this background melagatran was developed as a selective thrombin inhibitor. Because of low membrane permeability melagatran is not suited for oral

administration; therefore to improve the absorption ximelagatran was developed which is rapidly biotransformed after oral intake (Gustafsson et al. 2001). Because of the consistent pharmacokinetic properties of ximelagatran there is a lesser need for routine coagulation monitoring than for anti-coagulant drugs currently on the market (Petersen et al. 2003).

The pro-drug ximelagatran is as mentioned rapidly bioconverted after oral intake. The metabolites of ximelagatran are ethylmelagatran, hydroxymelagatran and melagatran, which are formed through ester cleavage and reduction of the amidoxime group (figure 5) (Eriksson et al. 2003b). Both *in vitro* and *in vivo* studies showed that none of the major hepatic drug metabolizing enzymes appears to be involved in either of these steps (Bredberg et al. 2003; Clement and Lopian 2003). The reduction step is carried out by a NADH-dependent enzyme system present in the endoplasmic reticulum and the outer mitochondrial membrane (Andersson et al. 2005).

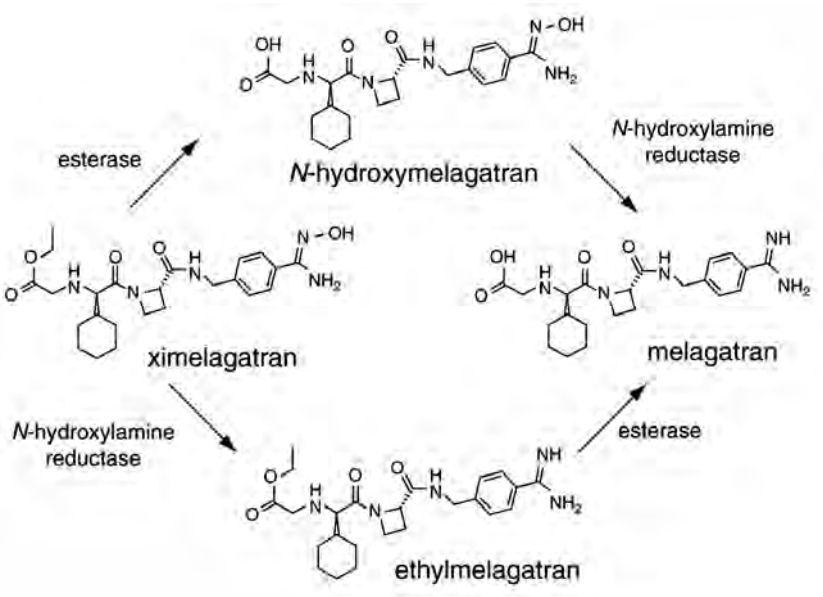

Figure 5. Metabolism of the specific thrombin inhibitor ximelagatran.

Pre-clinical animal studies and toxicological studies did not indicate any potential liver effects by ximelagatran, nor did short-term trails following orthopedic surgery (Eriksson et al. 2003a; Francis et al. 2002). However, long-term (>35 days exposure)

trials of ximelagatran revealed elevations in liver enzymes >3x ULN in 7.9% of the patients treated with ximelagatran versus 1.2% in comparator group who received a warfarin based regime (Lee et al. 2005). Though, the increased aminotransferases were transient in their appearance (Petersen et al. 2003), the mechanisms behind the increase of liver enzymes and the potential liver effects are not yet understood. As a result of the observed drug-induced liver injury ximelagatran was withdrawn from the market in 2006 (AstraZeneca 2006). Retrospective genetic studies have indicated an association between the elevated transferases and presence of the MHC class II alleles *DRB1*07* and *DQA1*02*, suggesting a contribution of the immune system to the observed liver effect (Kindmark et al. 2007).

2 OUTLINE

The present thesis studies were undertaken in order to investigate signal transduction mechanisms and systems connected with the inflammatory processes involved in brain and liver damage. Specifically, the following projects were the focus:

- In order to understand pathways involved in glial activation during brain inflammation and injury, intracellular signaling transduction pathways leading to *c-fos* activation after stimulation with inflammatory stimulus LPS were identified.
- In order to understand glial responses to excessive concentrations of glutamate, signal transduction pathways from the particular plasma membrane receptor to the responsible *cis*-acting element(s) in the proximal promoter region of *c-fos* and the involvement of transcriptional repressor DREAM in glial cells were identified.
- An *in vitro* co-culture model with higher sensitivity and better predictability was established, in order to evaluate the inflammatory involvement of monocytes in drug-induced liver damage.
- Signal transduction pathways of relevance for drug-induced toxicity were investigated.

3 COMMENTS ON METHODOLOGY

3.1 PRIMARY CULTURES OF CORTICAL GLIAL CELLS

Primary cortical glial cell cultures were established from cortices of newborn rats (6 - 24 hrs) as originally described by Saneto et al (1990). By immunocytochemistry primary cortical glial cell cultures were found to contain ~85% glial fibrillary acidic protein (GFAP) positive cells (astrocytes) and ~3-5% $CD11b^+$ cells (microglia), remaining cells were fibroblasts and ependymal cells (described previously by (Tindberg et al. 1996)). All experimental procedures involving animals were approved by the ethical committee for animal experiments at Northern Stockholm area.

3.2 CO-CULTURE MODEL

In order to study the influence of monocytes in drug-induced hepatotoxicity we aimed to set up a human *in vitro* co-culture system. In the model we used a human monocytic (THP-1) and a human hepatoma (Huh-7) cell line. Much work was put on the establishment of a suitable system. We evaluated different co-culture model set ups, such as a well-insert model, a sandwich-culture with collagen layer between the two different cell types, as well as a culture where the cells were in direct contact with each other (mixed culture). The difficulty with these two latter models was the issue of separating the two cell types in order to assess their respective mRNA expression levels and viability. In the insert-model the two cell types are separated with a porous membrane which allows molecules to passively diffuse in the medium (figure 6). On the other hand, one drawback with the insert-model is the relatively long distance between the compartments with the two cell types, 1 mm.

We continued with the insert-model as this model permitted us to evaluate the different cells separately in relation to viability and gene expression, and it also showed to be more sensitive in relation to viability. In the co-cultures, the cells were grown in a mixed medium of THP-1 and Huh-7 medium (1:1) in a trans-well system where the cells were separated by a porous membrane (pore size 3 μM). In co-cultures, the Huh-7 cells were first seeded at 75 x 10^4 cells/well, cultured over night and thereafter the inserts and the THP-1 cells were added (30 x 10^4 cells/well). We also performed cultures where we used conditioned medium from treated monocytes, which was later

transferred to hepatocytes for evaluation of THP-1-released mediators. For cultures with conditioned medium, THP-1 and Huh-7 cells were cultured in 1:1 mixed medium for one day at densities as above. The THP-1 cells were thereafter treated with 50 µM troglitazone for 24 hours, centrifuged and the medium was subsequently transferred to Huh-7 cells, which were cultured for another 24 hours before cell viability was evaluated. In some experiments Huh-7 cells were also co-treated with troglitazone when the conditioned medium was added.

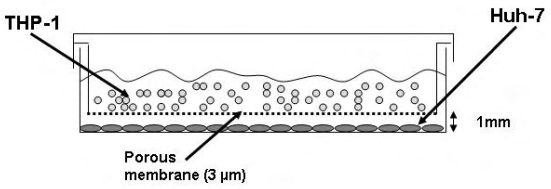

Figure 6. Schematic figure of the insert model of the co-culture system. The monocytes (THP-1) are kept in the upper compartment, and the hepatoma cells (Huh-7) are kept in the lower compartment, separated by a porous membrane.

3.3 CELL TRANSFECTIONS

There are many different methods to introduce foreign DNA into a cell, e.g. in order to express a protein of interest. In paper I and II Lipofectamine 2000 was used, which is based on a liposome method. Liposomes are formed around the negatively charged DNA in order to deliver the DNA into the cell via fusion with the plasma membrane. Other means of transfection are e.g. DEAE-dextran, calcium-phosphate or electroporation. These methods cause the cells to be transiently transfected since the foreign DNA introduced is usually not inserted into the nuclear genome, and thereby lost during cell division.

3.4 IMMUNOCYTOCHEMISTRY

For the expression studies of wild type and mutant variants of DREAM (EFwt and EFmut; where the calcium-binding domains, EF, has been mutated) and c-Fos in paper II primary rat glial cells were transiently transfected, stimulated with glutamate and subsequently immunocytochemistry was performed. Control experiments using a plasmid with Green Fluorescent protein (pGFP) expression vector showed that with both the DMRIE-C and Fugene 6 transfection protocols the protein of interest were

only expressed in astrocytes. Cells were seeded on coverslips in 6-well plates and grown to approximately 90% confluence before transfection with Fugene 6 and DNA (ratio 3:1) with pCMV-FLAG (mock), pCMV-EFwt-FLAG or pCMV-EFmut-FLAG. For the expression studies of EFwt- and EFmut-variants of DREAM, coverslips blocked with a mixture of horse, goat and fetal bovine serum in order to reduce background as much as possible. The immunostaining was analyzed using a Delta vision deconvolution microscope (DeltaVision® RT Restoration Imaging System from Applied Precision) using a 100x or a 60x objective. Image stacks (typically of 14-16 widefield images within a ~4 μm thickness cell layer) were deconvolved and projected to a flattened image. The flattened images were saved as TIFF files and formatted using the Image J software. Comparing confocal to deconvolution microscope, traditional confocal microscope uses pinholes to exclude all surrounding light except the light that originates from the current plane of focus of the sample. This increases the signal-to-noise ratio. Drawbacks are though that the focus and realignment of pinholes needs to be reset for each image in a Z-series, as well as decreased signal. On the contrary, with deconvolution microscope the focus needs to be set near the center of ones interest, thereafter settings of the microscope can be adjusted to how many images should be taken within a decided range of upper and lower Z axis. The picture is then formed by flattening of the stack of images. Another user-advantage with deconvolution microscope is that it uses ordinary white light which is filtered with different filters to get the right wavelength, while confocal microscope uses laser to excite the specimen.

3.5 MAP KINASE INHIBITORS

The kinase inhibitors used in this thesis work are SB203580, PD98059, U0126 and KN-62, which are all commercially available, and chlomethiazole (CMZ) (structure figure 7).

The pyridinyl imidazole compound SB203580 (4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole) is a competitive inhibitor of p38 MAPK, and binds to the ATP-binding site of the kinase (Young et al. 1997).

KN-62 (1-[N,O-*bis*-(5-Isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine) is an inhibitor of calcium/calmodulin-dependent kinase II (Ca^{2+}/CaM kinase II), but not

via competitive binding to ATP pocket but rather to calmodulin since KN-62 does not affect calmodulin-independent activity of Ca^{2+}/CaM kinase II (Tokumitsu et al. 1990).

U0126 (1,4-Diamino-2,3-dicyano-1,4-*bis*(2-aminophenylthio)butadiene) was found to be an inhibitor of AP-1 transactivation in a cell-based reporter system, as well as inhibiting endogenous promoters containing AP-1 elements without affecting the genes lacking AP-1 element. This inhibition was found to be mediated by inhibition of the MEK 1 and MEK 2 kinases, in a noncompetitive fashion with the ATP binding site (Favata et al. 1998).

PD98059 [2-(2'-amino-3'-methoxyphenyl)-oxanaphtalen-4-one] is a cell permeable inhibitor and was found to block the activation of ERK1/2 kinases via an indirect way by inhibiting the MEK1/2 kinases upstream of ERK. MEK activates its substrate ERK by phosphorylation of its tyrosine and threonine residues. This activity is effectively inhibited by PD98059 (Dudley et al. 1995).

CMZ [5-(2-chloroethyl)-4-methyl-1,3-thiazole] is a cell permeable p38 MAPK inhibitor, that inhibits the downstream activity of p38 and not direct by binding to the ATP-pocket and obstruct the phosphorylation (Simi et al. 2000).

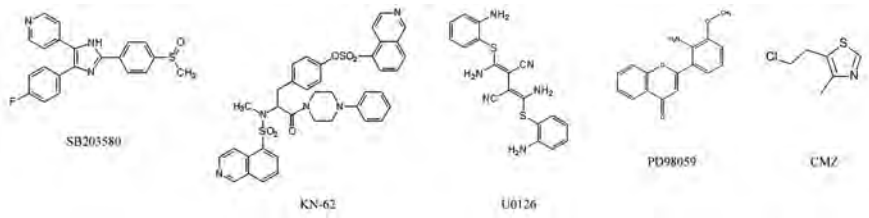

Figure 7. Schematic figures of the MAPK inhibitors used: SB203580, KN-62, U0126, PD98059 and CMZ.

4 RESULTS

4.1 PAPER I – *c-FOS* ACTIVATION BY LIPOPOLYSACCHARIDE IN GLIAL CELLS VIA P38 MAPK-DEPENDENT ACTIVATION OF SRE OR CRE ELEMENTS.

Given that glial cells are important in CNS injury and that *c-fos* is important component of their activation, the present study was undertaken in order to characterize the involvement of the different *cis*-acting elements in the *c-fos* proximal promoter region in response to the inflammatory stimuli LPS in cortical glial cells. In the study we used luciferase constructs containing the SIE, SRE, CRE and DRE elements present in the proximal promoter of *c-fos* (presented in figure 2A of paper I).

We observed that LPS causes an increase of c-fos mRNA expression, and the magnitude of induction was on average up to ~5 fold after 30 min (see figure 1 paper I). The induction of c-fos mRNA levels following 30 min of LPS treatment was inhibited by the p38 MAPK inhibitor SB203580 as well as by chlomethiazole (CMZ) (see figure 1 paper I), a neuroprotective compound that was previously shown to inhibit p38 MAPK activity (Simi et al. 2000). No significant involvement of calcium-calmodulin dependent protein kinases (CaMKs) could be seen based on the usage of the inhibitor KN-62 in the LPS-induced c-fos mRNA expression (see figure 1 paper I). A slight decrease in the *c-fos* induction was observed after treatment with the ERK MAP kinase pathway inhibitor U0126 at 1 μM, but it was not significant (see figure 1 paper I), nor dose-dependent.

Furthermore, LPS induced both the basic *c-fos* promoter (containing SIE, SRE and CRE elements)-luciferase reporter as well as reporter constructs with isolated SRE or CRE element. Mutation of either the SRE or the CRE in the basic construct alone did not affect the LPS-inducibility, but mutations in both elements abolished the affect of LPS (see figure 3 in paper I). Additionally, LPS increased the phosphorylation of the Elk-1 and CREB/ATF-1 transcription factors, which mediate the transcriptional activation of *c-fos* at the SRE and CRE elements, respectively (Sassone-Corsi et al. 1988) (see figure 5 in paper I). Moreover, LPS also activated GAL4-Elk-1 and GAL4-CREB chimerical proteins that subsequently increased the expression of GAL4-driven

luciferase reporters and where LPS-induced GAL4-Elk-1 was sensitive to addition of the MAPK inhibitors CMZ and SB203580 (see figure 4 in paper I).

To conclude, we have found that LPS activates *c-fos* by p38-dependent phosphorylation of the transcription factors binding to either the SRE or the CRE element in the promoter of *c-fos* in rat cortical glial cells.

4.2 PAPER II – GLUTAMATE ACTIVATES *c-FOS* IN GLIAL CELLS VIA A NOVEL MECHANISM INVOLVING THE GLUTAMATE RECEPTOR SUBTYPE MGLU5 AND THE TRANSCRIPTIONAL REPRESSOR DREAM

In this study we investigated the effect of glutamate on the activation of *c-fos* in primary mixed cortical glial cell culture, since so little is known in how glial cells respond to glutamate. We showed that glutamate rapidly increases c-fos mRNA expression with a peak after 30 min, where after the mRNA declines back again to control levels after 1 hour (see figure 1 in paper II). Chelating intracellular calcium release by BAPTA-AM significantly attenuated the expression of c-fos mRNA, suggesting the importance of calcium in glutamate signaling (see figure 1 in paper II). The induction of c-fos mRNA expression by glutamate is not sensitive to the inhibitors of ERK-, p38 MAPK- or CaMK-pathways (see figure 2 in paper II); even though a moderate increase in phosphorylation of ERK1/2 could be observed (data not shown). In human hepatoma cells the level of c-fos mRNA expression was additively induced in the presence of thapsigargin and teleocidin, which empties the ER-calcium stores and a tumor promoter activating PKC, respectively (Tsukamoto et al. 1993). On the contrary, in our study thapsigargin did not further enhance the glutamate effect in the primary glial cells (see figure 4 in paper II), suggesting that glutamate and thapsigargin uses the same mechanism of activating *c-fos*.

Furthermore, blocking the NMDA receptor with the antagonist MK-801 failed to reduce the expression in c-fos mRNA levels after glutamate stimulation. On the contrary, the metabotropic glutamate receptor 5 antagonist MPEP significantly diminished the mRNA levels (see figure 3 in paper II). This points to the direction to the involvement of intracellular calcium and not extracellular influx, since mGluR5 is a

G-protein coupled receptor positively coupled to PLC which upon activation subsequently leads to release of calcium from intracellular stores.

Activation of cytosolic kinase pathways such as ERK and CaMK causes activation of *c-fos* by phosphorylation of CREB and/or Elk1 transcription factors at the CRE and/or SRE elements of the *c-fos* promoter, respectively, and these signaling mechanisms seem to be valid in neuronal cells. To determine how ER-mediated calcium increase downstream mGluR5 activates *c-fos* transcription in cortical glial cells, we used a panel of luciferase reporter constructs in transient transfections. One of these variants, called c-fos+DRE, included the whole rat *c-fos* proximal promoter up to the translation start (- 421 to + 180 bp), containing all SIE, SRE, CRE and DRE elements, of which the three latter ones can respond to calcium signals. Glutamate induced expression of the c-fos+DRE construct, but not the expression of the c-fos*Δ*DRE variant, which carries a 3' deletion that eliminated the DRE element, at any time point examined (3 to 7 hrs) (see figure 5 in paper II).

To confirm that indeed the *DRE* element alone could confer glutamate induction without the involvement of the other calcium-responsive elements of the *c-fos* promoter (SRE and CRE), we used luciferase reporter constructs containing the isolated elements. In agreement with experiments using the whole rat *c-fos* promoter, glutamate induced expression of the DRE-driven, but not the SRE- or CRE-driven luciferase reporter constructs at any time point examined (1 to 8 hrs) (see figure 5 in paper II). To the DRE element four factors are binding, constituting the repressor, DREAM (DRE antagonistic modulator). We examined the subcellular localization of DREAM in cortical glial cells by immunofluorescence microscopy. When glial cells were serum-starved for 16 hrs, DREAM was almost exclusively localized in the cell nucleus. Administering glutamate (30 min to 1 hr) decreased the nuclear and restored the whole-cell distribution of DREAM (see figure 6 in paper II). The timing of the re-distribution of DREAM upon glutamate treatment in GFAP+ astrocytes was consistent with a role in mediating *c-fos* transcription, as DREAM was detected outside the nucleus already at 30 min, which is the peak of c-fos mRNA induction by glutamate, and assumed a whole cell-distribution after 1hr of glutamate treatment, when c-Fos protein has accumulated in the nucleus.

To further evaluate the involvement of DREAM in c-Fos expression we transiently expressed two variants of DREAM in cortical glial cells, the wild type protein (EFwt) and a variant where all four calcium binding EF-hands had been mutated (EFmut) (described by (Carrion et al. 1999)). The EFmut DREAM acts as a constitutive repressor as it can no longer be released from the DNA upon binding of calcium. Both EFwt and EFmut DREAM variants were FLAG-tagged in order to identify their expression in transfected cells. c-Fos immunoreactivity in EFwt DREAM expressing cells was on average the same as in non-transfected cells in the same culture. However, in cultures transfected with the EFmut vector, non-transfected cells still responded to glutamate by up-regulating c-Fos whereas c-Fos immunoreactivity was substantially lesser in cells expressing the EFmut DREAM. When counting only the transfected cells, the number of cells with nuclear c-Fos immunostaining was substantially lower in cells expressing the EFmut DREAM as compared to cells expressing EFwt DREAM (see figure 7 in paper II).

In conclusion, we have found a novel calcium- and DRE/DREAM-dependent pathway leading to *c-fos* activation by glutamate in glial cells (see figure 8).

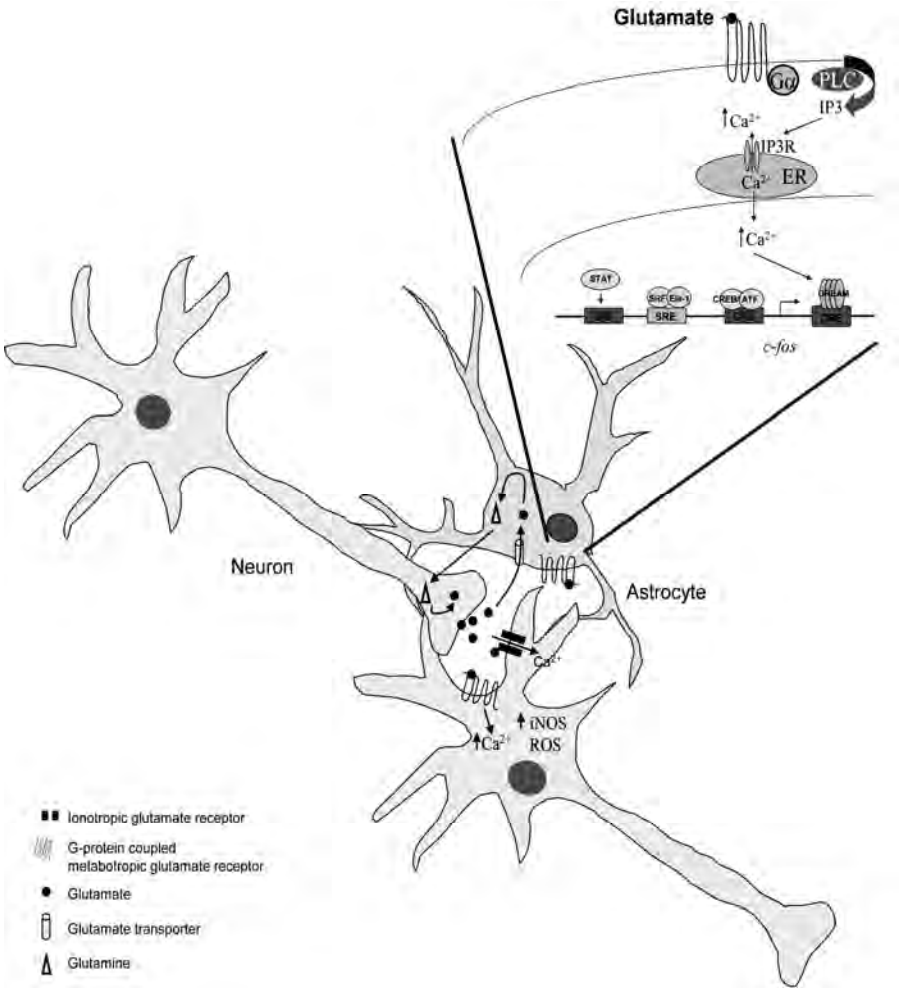

Figure 8. Increased glutamate concentrations in the synaptic cleft can cause overstimulation of both ionotropic and metabotropic glutamate receptors, which causes increase of calcium levels by influx and release from intracellular stores. The high extracellular concentrations of glutamate can induce excitotoxic cell death involving overstimulation of glutamate receptors, and as mentioned this activation causes intracellular accumulation of calcium, which in turn leads to massive calcium-dependent activation of enzymes, generation of ROS and mitochondrial dysfunction. In glial cells activation of metabotropic glutamate receptor by glutamate cause activation of *c-fos* via DRE/DREAM.

4.3 PAPER III – PRO-INFLAMMATORY RESPONSE AND ADVERSE DRUG REACTIONS: MECHANISMS OF ACTION OF XIMELAGATRAN ON CHEMOKINE AND CYTOKINE ACTIVATION IN A MONOCYTE IN VITRO MODEL

During recent years lots of drugs have been withdrawn, predominantly due to hepatotoxicity but also cardiotoxic effects (Need et al. 2005). To be able to avoid such problems late in development there is an urgent need to develop better *in vitro* and *in vivo* models that can predict drug-induced toxicity, and in particular hepatotoxicity. In the present study we elucidated pro-inflammatory cytokine and chemokine responses in a human *in vitro* system based on monocytes, in order to examine *in vitro* systems for possible prediction of drugs that may cause hepatotoxicity.

We observed that during THP-1 cell treatment with ximelagatran; an unexpectedly high intracellular accumulation of the esterhydrolysed ximelagatran metabolite hydroxymelagatran was obtained, reaching 3 mM concentration, with slow accumulation in the medium. The concentration of ximelagatran diminished in the medium over time (see figure 1 in paper III). Furthermore, ximelagatran caused activation of the JNK and ERK pathways thereby stimulating the release of IL-8 (see figure 3 and 4 in paper III). In addition, elevated expression of vascular epidermal growth factor (VEGF) and MCP-1 was observed as a result of ximelagatran treatment (see figure 3 in paper III).

Our results indicate that ximelagatran and/or the metabolites activate all three MAPK pathways; the ERK-, JNK- and the p38-signaling pathways (see figure 4 in paper III). In support of the participation of these cellular signaling systems were the findings that the addition of specific inhibitors of the JNK- and ERK-pathways indeed attenuated the release of IL-8, whereas SB203580 did not inhibit IL-8 release. Neither of the JNK-, ERK-, or p38-pathway inhibitors had any inhibitory effect on VEGF or MCP-1 release. By contrast, SB203580 itself stimulated the release and in combination with ximelagatran a further stimulatory effect was registered on IL-8 (see figure 4 in paper III).

In conclusion, we have used an *in vitro* cell model based on a monocytic cell line, to show the increased expression of IL-8, MCP-1 and VEGF by ximelagatran and metabolites, which might have a role in an immunomediated hepatotoxic action of the

drug. It is suggested that a monocytic *in vitro* cell system might add important information to the cytotoxic action of potential hepatotoxins that can be of value for predicting adverse effects of drugs *in vivo*.

4.4 PAPER IV – INCREASED SENSITIVITY FOR DRUG-INDUCED CYTOTOXICITY USING A NOVEL HUMAN *IN VITRO* CO-CULTURE MODEL

In continuation of evaluating the importance of immune responsive cells to drug-induced hepatotoxicity, we did set up a co-culture model with monocytes and hepatocytes, aiming to get a more sensitive system and better prediction of drug-induced hepatotoxicity. The importance of macrophages was demonstrated in a rat co-culture system of primary rat hepatocytes and RAW 264.7 macrophages, whereby the hepatocytes showed an increased sensitivity to ethanol-induced oxidative stress in co-cultures compared to single cultures (Griffon et al. 2000). Today human *in vitro* test systems for drug-induced hepatotoxicity is usually based on fractionated human tissue or the use of single cultures of human primary hepatocytes or hepatoma cell lines (Dambach et al. 2005; Groneberg et al. 2002). However, these *in vitro* methods do not take into account the participation of other cell types in drug-induced liver injury.

In the present investigation we established a human *in vitro* co-culture system using a monocytic (THP-1) and a hepatoma (Huh-7) cell line. The two cell types are separated by a porous membrane (see figure 1 paper IV), a model that has been used to evaluate the effect of hepatocyte-derived ROS on rat stellate cells (Nieto et al. 2002). As model drugs we chose two PPAR-γ-agonists aimed for treatment of type II diabetes, the hepatotoxic troglitazone and the non-hepatotoxic rosiglitazone

We show that co-cultures with monocytes and hepatocytes drastically increase the sensitivity (30% and 40%; in THP-1 and Huh-7 cells, respectively) to treatment with troglitazone, whereas the effect of rosiglitazone was insignificant in both singles and co-cultures except after 48 hours where a 30% reduction of hepatocytes could be observed (see figure 2 in paper IV). To further evaluate the role of monocytes we used conditioned medium from troglitazone treated THP-1 cells on Huh-7 cells, where we observed a synergistic effect of the medium together with troglitazone treatment (see figure 3 in paper IV). This suggests the involvement of monocyte-derived mediator in

the increased sensitivity of Huh-7 cells to troglitazone treatment. We found that troglitazone induces expression of several stress-related genes, such as DDIT3/CHOP, MT2A, CXCL2, CXCL10 and HspA6, in Huh-7 cells (see figure 5 in paper IV). In THP-1 cells we observed increased expression of CXCL2, CXCL10, MT2A and DDIT3 in co-cultures compared to single cultures (see figure 6 in paper IV). Overall the expression was significantly higher in co-cultures than in single cultures, and in general troglitazone treated cells had much higher expression levels compared to rosiglitazone treated cells.

To conclude, we observed an increased sensitivity of Huh-7 cells to troglitazone treatment in co-cultures with THP-1 cells, whereas rosiglitazone did not significantly affect the viability. Moreover, an increased expression of several stress-related genes was observed in Huh-7 cells of co-cultures treated with troglitazone.

4.5 SIGNALING MECHANISMS OF TROGLITAZONE AND ROSIGLITAZONE

In an attempt to elucidate the signaling pathways in the monocytic cell line THP-1 affected by troglitazone, a pilot study was carried out. We performed a Kinex™ Antibody microarray, which tracks over 600 proteins with both phosphospecific and protein-specific antibodies which track both the phosphorylation status and the expression level of proteins (Kinexus, Vancouver, Canada; www.kinexus.ca).

In this initial screen cell extracts from control (untreated) and troglitazone, rosiglitazone or vehicle treated THP-1 cells were prepared. The cells were stimulated for 10 minutes and subsequently harvested in a lysis buffer (supplied by Kinexus) according to manufactures protocol. The Kinex™ antibody microarray slides were run in pairs. The results were analyzed by Kinexus, and subsequently filtered with Excel according to percentage change (%CFC; the percentage change in normalized signal intensity from control); and flag ("flag" is an indication of the quality of the spot, based on its morphology and background; a flag of "0" means that the spot is acceptable, while a flag of "1" means the spot may not be reliable due to high local background and/or irregular spot morphology); signal/noise ratio and % error range control/treated. The results showed that 67 and 108 proteins had an increased phosphorylation (increase in %CFC; >20%) in troglitazone and rosiglitazone treated samples, respectively,

compared to control (DMSO treated cells), and 219 and 299 proteins showed decreased phosphorylation in response to troglitazone and rosiglitazone, respectively. Further analysis comparing the specific effects by the drugs revealed that troglitazone caused specifically increased phosphorylation of six proteins (table 1) whereas rosiglitazone treated cells increased phosphorylations in three specific proteins/pathways (table 1).

As evaluation of the proteins that had a decrease in phosphorylation status compared to control cells, revealed a fewer number of proteins that were specific for troglitazone compared to rosiglitazone (table 1). Noteworthy is the fact that the enzymes DGKZ and PKM2 that have increased phosphorylation status in rosiglitazone treated cells are the same proteins that have a lesser phosphorylation status in troglitazone treated cells (table 1). Furthermore, PCK2 and PKR1 that have increased phosphorylation in troglitazone treated cells whereas have less phosphorylation by rosiglitazone (table 1).

Table 1. Protein phosphorylation specifically affected by troglitazone and rosiglitazone.

Protein	Troglitazone	Rosiglitazone
AcCoA carboxylase	—	↓
Casp1	—	↓
Casp3	—	↓
Casp6	↑	—
CK1d	—	↓
CK1e	↑	—
DGKZ	↓	↑
Fas	—	↓
Hsp60	↑	—
Kit	—	↑
NME7	—	↓
PCK2	↑	↓
PKR1	↑	↓
PKM2	↓	↑
S6	—	↓
TTK	↑	—

Abbreviations. AcCoA carboxylase, Acetyl coenzyme A carboxylase; Casp1, Caspase 1; Casp3, Caspase 3; Casp6, Caspase 6; CK1d, Casein protein-serine kinase-1 delta; CK1e, Casein protein-serine kinase-1 epsilon; DGKZ, Diacylglycerol kinase zeta; Fas, Tumor necrosis factor superfamily member 6 (CD95); Hsp60, Heat shock 60 kDa protein; Kit, Kit/Steel factor receptor-tyrosine kinase; NME7, Nucleotide diphosphate kinase 7; PCK2, Phosphoenolpyruvate carboxykinase; PKR1, Double stranded RNA dependent protein-serine kinase 1; PKM2, Pyruvate kinase, isoenzyme M2; S6, 40S ribosomal protein S6; TTK, Dual specificity protein kinase.

Further analysis with Western blot of extracts prepared from troglitazone and rosiglitazone treated cells compared to vehicle treated cells (DMSO) showed that troglitazone induced increased levels of Hsp60 compared to vehicle treated samples or rosiglitazone treated THP-1 cells (figure 9).

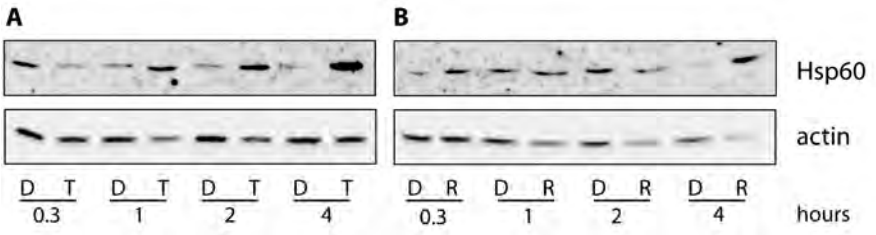

Figure 9. Western blot analysis of cell extracts from THP-1 cells, A) cell extract from cells treated with 50μM troglitazone (T) or vehicle (DMSO, D); and B) cell extracts from cells treated with 50 μM rosiglitazone (R) or vehicle (DMSO, D); for indicated times. Blots detected with anti-Hsp60 (upper blots) and anti-actin (lower blots) antibodies.

5 DISCUSSION

5.1 PAPER I AND II – *c-fos* REGULATION IN GLIA

We have studied the involvement of different *cis*-acting elements in *c-fos* expression in relation to LPS and glutamate stimulation. The inflammatory stimulus LPS activated *c-fos* in our glial cells via the p38 MAPK pathway which is consistent with the role of this pathway in stress and inflammatory responses, via the SRE or the CRE element (Lee and Young 1996). Since both elements need to be mutated in order to abolish the LPS-induced induction of *c-fos* the data indicate that LPS activates each element independently.

As mentioned, *c-fos* is induced by many different stimuli and despite the fact that it has been thoroughly studied there are conflicting reports regarding the involvement of the different elements. Several studies in the literature support a role of the ERK pathway in LPS signaling in monocytic cells and astrocytic cell lines (Schumann et al. 1998; Sweet and Hume 1996; Willis and Nisen 1996) and moreover, Guha and Mackman reported that LPS activation in THP-1 cells utilize the ERK pathway to activate the SRE element (Guha and Mackman 2001). In our study in primary cultures of cortical glial cells, LPS induced *c-fos* expression and the activation of GAL4-Elk1 via the p38 MAPK pathway (figures 1 and 4B, paper I), a difference that may be attributed to cell-specific factors or the differential regulation of *c-fos* expression in transformed vs. tumor cells. The SIE element does not apparently contribute to the activation of *c-fos* transcription by LPS in cortical glial cells, since a double mutation of the SRE and CRE elements completely abolished the increase in luciferase activity. Furthermore, it was shown that the transcriptional repressor DREAM is capable of repressing transcription at CRE sites by direct binding to the CREB family of proteins (Ledo et al. 2002). Thus, it may be plausible that activation of the *c-fos* CRE is also regulated by DREAM without the involvement of the adjacent DRE site, an issue that requires further studies to be resolved. Consequently, there still remains question marks to what extent the different elements can cooperate to affect the magnitude and/or the specificity of the induction.

The mechanism behind glutamate regulation of *c-fos* in neurons is well described; the induction is mediated via the calcium-dependent activation of CREB at the CRE and/or

Elk1 at the SRE by CaMKs and/or ERK pathway (Ahn et al. 1998; Hardingham et al. 1997; Vanhoutte et al. 1999; Xia et al. 1996). In our glial cellular model, we observed no involvement of the CRE and SRE elements or the transcription factors binding to them, which is in sharp contrast to the situation described in neurons. Since no involvement of neither of the MAPK pathways, nor the proposed neuronal CaMK-pathway is evident in glial cells, it thus seems likely that glutamate employs a novel mechanism, however calcium-dependent, for the activation of *c-fos* expression. Emptying the ER of calcium by thapsigargin and use of the calcium-chelator BAPTA showed the involvement of calcium in glutamate-induced *c-fos* expression in glial cells (figure 1 and 4 in paper II). Furthermore, it seems that glutamate and thapsigargin employ the same mechanisms of activation i.e. release of calcium from intracellular stores.

The DRE element of the *c-fos* promoter binds the calcium-binding transcriptional repressor DREAM (Carrion et al. 1999). However, the precise role of DREAM/*DRE* in activating *c-fos* transcription has not been investigated in response to glutamate. DREAM is bound to DNA and represses transcription in the absence of calcium, whereas at increased nuclear [Ca^{2+}], DREAM bind calcium and is subsequently released from the DNA. In H4 cells stably expressing DREAM, Zaidi and co-workers (Zaidi et al. 2004), showed that DREAM assumes a nuclear localization after serum starvation, which is lost when serum is replenished. From our expression studies with EFwt-DREAM and EFmut-DREAM constructs in glial cells we conclude that expression of the EFmut-DREAM inhibits glutamate-induced c-Fos protein expression in astrocytes (see figure 7 in paper II). This is in agreement with our results describing that glutamate induced changes in both c-Fos expression and the localization of endogenous DREAM in astrocytes (see figure 6 in paper II). This further supports the conclusion that DREAM is an important regulator of c-Fos expression in astrocytes. Transient expression studies with luciferase constructs carrying the DRE element present showed the necessity of the element in order to confer glutamate-induced c-fos mRNA expression. Together these data suggest that glutamate induces *c-fos* transcription via the DRE element alone through the de-repression of DREAM. This is to our knowledge the first demonstration of functionality of the *c-fos* DRE element in glial cells. Thus, in line with the research that show the involvement of DREAM in relation to pain, in expression of cytokines and our results with *c-fos* regulation, it

would be interesting to further elucidate the role of DREAM in inflammatory response in astrocytes.

Contradictory to what Eun and co-workers found in mouse microglial cells, we observed no involvement of the NMDA receptor in the activation of *c-fos* (see figure 3 in paper II) (Eun et al. 2004). Rather the glutamate-induced *c-fos* expression was transmitted via the involvement of the metabotropic glutamate receptor subtype 5, mGluR5 (see figure 3 in paper II). Although Eun and co-workers did not address the mechanism of *c-fos* activation by glutamate, their study suggests that there might be differences in glutamate responses among macroglia and microglia. In our mixed cortical glial cultures, c-Fos protein expression was restricted to astrocytes (see figure 6C and 7C in paper II), and it may be argued that microglia may behave differently if they are cultured alone or in the presence of astrocytes. It also seems that the pathway we describe in the present study for the activation of *c-fos* downstream mGluR5 is relevant mostly to cortical astrocytes. In line with all the above, Mukherjee and co-workers reported that although glutamate caused a marked activation of all three MAPK pathways in hippocampal neurons, a very small activation of MAPKs by glutamate was observed in hippocampal astrocytes, whereas NMDA did not activate astrocytic MAPKs at all (Mukherjee et al. 1999). The overall conclusion is that neurons and different types of glia cells do respond to glutamate via distinct pathways, which have been activated downstream of different glutamate receptor subtypes.

This shed some light over how *c-fos*, a member of the AP-1 transcription factor which mediates expression of inflammatory response genes such as iNOS, IL-1 and IL-6, gets transcriptionally activated by an inflammatory stimuli such as LPS as well as by the endogenous neurotransmitter glutamate in glial cells. Since glial cells are central to the inflammatory response elicited in the CNS following an insult or exposure to a pathogen, and since *c-fos* is an essential part of the activation of glial cells, the present result implicate glial p38 MAPK, Elk1, CREB/ATF-1 and DRE/DREAM as important mediators of inflammatory processes in the CNS.

5.2 PAPER III AND IV – DRUG-INDUCED HEPATOTOXICITY IN *IN VITRO* STUDIES

Drug-induced hepatotoxicity is the leading cause of failures in drug development at the clinical phase of the development. In most instances routine animal toxicology fails to identify the risk of subsequent problems in clinical stages (Kaplowitz 2005) and *in vitro* models using hepatocytes are not sensitive. We hypothesized that co-cultures with human hepatocytes and monocytes would constitute a more sensitive human *in vitro* model for hepatotoxicity.

In an initial attempt to investigate the role of immune cells in drug-induced cytotoxicity we used a monocytic cell line. In the case of ximelagatran we evaluated the inflammatory response from monocytes treated with ximelagatran. The effects seen on the THP-1 cells include cytokine/chemokine alterations and decreased cell viability, and could be caused by the high intracellular levels of the hydroxymelagatran metabolite as well as by the active metabolite melagatran, rather than by the parent compound. A requirement for intracellular metabolism would explain the delayed effect on the phosphorylation of ERK, JNK and p38 MAP kinases which occurred after 3-6 hours of stimulation, as well as the delayed release of the chemokines and cytokines. Both melagatran and hydroxymelagatran have poor membrane permeability (Gustafsson et al. 2001). The relative small rate of formation of melagatran or ethylmelagatran indicates that the THP-1 cells are relatively devoid of the amidoxime reductase system present in the outer mitochondrial membranes that are responsible for the production of the metabolites ethylmelagatran and melagatran (Andersson et al. 2005). The extrusion pump for ximelagatran, and its' metabolites, has not yet been identified, and it might be likely that the required transporter capacity is absent in the THP-1 cells.

MCP-1, IL-8 and VEGF all have implications in inflammatory processes and the infiltration of neutrophils and leukocytes to infected or inflamed sites (Clauss et al. 1990; Croll et al. 2004; Matsushima et al. 1988). Furthermore, MCP-1 is a potent chemotactic factor for monocytes. MCP-1 induces intracellular calcium influx, respiratory burst, expression of adhesion molecules such as β_2 integrins, and release of lysosomal enzymes in monocytes (see references within (Mukaida et al. 1998)). The expression of adhesion molecules has been correlated with the later stage of cell death of parenchymal cells (Jaeschke and Hasegawa 2006). Normally, the IL-8 protein is

barely secreted from non-induced cells, but its production is rapidly induced by a very wide range of stimuli encompassing pro-inflammatory cytokines such as TNF or IL-1 or cellular stress (Hoffmann et al. 2002; Mukaida et al. 1994). Intradermal injection of IL-8 in rats and rabbits caused a dose-dependent accumulation of neutrophils 2-3 h after injection, followed by lymphocyte infiltration (Mukaida et al. 1994). Newly recruited neutrophils generate and release reactive oxygen species and proteases which can cause death of parenchymal cells (Jaeschke et al. 1992). Inhibition of IL-8 by addition of anti-IL-8 antibodies has been shown to decrease tissue injury in various animal models. A reduced infiltration of neutrophils into the tissue, by IL-8 antibodies, is observed e.g. after LPS-induced dermatitis or cerebral ischemia-reperfusion injury (Mukaida et al. 1994) and see references within).

Similar to our results that SB203580 did not inhibit IL-8 release, studies done in fibroblasts and vascular endothelial cells have shown no inhibition of IL-1-induced IL-8 release by SB203580 (Ridley et al. 1997), whereas the IL-8 production in human peripheral blood mononuclear cells (PBMC) after osmotic stress was inhibited by SB203580 (Hoffmann et al. 2002). Furthermore, it has been shown using tetracycline-regulated reporter gene constructs, that activation of p38 MAPK leads to increased stability of IL-8 mRNA, while in non-stimulated cells the stability of IL-8 is low. Furthermore, IL-8 mRNA induction involves the JNK and ERK kinase pathways (Hoffmann et al. 2002) and see references within). Taken together, such opposing effects on IL-8 expression in different cell types, suggests that the regulation of IL-8 expression is both cell type as well as stimuli specific.

To conclude, in our human monocytic cell system, the ERK- and JNK-signaling pathways appear to play a role in IL-8 release and p38 MAPK appears to play a role in the production of IL-8, in a way that remains to be elucidated. On the other hand neither ERK-, nor JNK- or p38-signaling pathways were involved in release of VEGF or MCP-1 in response to ximelagatran exposure. Additionally, a recent pharmacogenetic study suggests a possible immune pathogenesis behind long-term exposure to ximelagatran. A genome-wide pharmacogenetic screening of 74 cases and 130 control subjects revealed a strong association between elevated ALAT levels and the genotype for the major histocompatibility complex (MHC) class II alleles, in particular *DRB*07* and *DQA1*02* (Kindmark et al. 2007). This thus indicates the involvement of the immune active cells in the potential hepatotoxic action of

ximelagatran and is in line with our current finding that the ximelagatran-dependent elevated expression of chemokines and cytokines, potentially attracting neutrophils and lymphocytes to the liver, might play a role in the development of liver injury.

To further develop our system in order to evaluate the contribution and importance of immune cells we established a human *in vitro* co-culture system using a monocytic (THP-1) and a hepatoma (Huh-7) cell line. Previously Tukov and co-workers have used a rat co-culture system to investigate the importance of LPS-induced responses of macrophages as a consequence to exposure to known hepatotoxic substances such as acetaminophen, chlorpromazine where the purpose of the macrophages was to generate endotoxin-induced pro-inflammatory cytokines (Tukov et al. 2006). In our system we have used the co-culture approach to evaluate the effect of the presence of monocytes *per se* for the cytotoxic action of troglitazone. Indeed, we found that co-cultures of human hepatocytes and monocytes treated with troglitazone were more sensitive than single cultures. Moreover, the effect was not observed when the co-cultures were treated with the non-hepatotoxic drug rosiglitazone (see figure 2 in paper IV). We also showed that conditioned medium from troglitazone-stimulated THP-1 cells increased the sensitivity of Huh-7 cells to troglitazone (see figure 3 in paper IV). It thus appears that soluble mediators released by THP-1 cells are involved in the increased sensitivity of co-cultures. Their identities are unknown, but several studies have previously shown that pro-inflammatory cytokines, such as TNF and IL-1β, might play an important role in drug-induced hepatotoxicity (Blazka et al. 1996; Blazka et al. 1995; Schwabe and Brenner 2006). Contradictory to what we and Desmet et al. have observed (2005), other groups have not detected increased levels of pro-inflammatory cytokines by troglitazone (Jiang et al. 1998; Ricote et al. 1998). The role pro-inflammatory cytokines play in our co-culture model is probably low, since increased levels of TNF and IL-6 mRNA are only seen in single cultures treated with troglitazone. It is likely that other (novel) mediators are involved in the increased sensitivity of Huh-7 cells to troglitazone treatment.

The exact mechanisms for how troglitazone and other toxic glitazones, e.g. ciglitazone, cause hepatotoxicity is not known. In HepG2 cells it has been shown that troglitazone induces apoptosis while rosiglitazone does not (Guo et al. 2006; Smith 2003). Moreover, Gou et al showed using a global microarray system in primary rat hepatocytes that troglitazone differentially expressed genes involved in necrosis,

apoptosis and cell proliferation pathways compared to rosiglitazone (Guo et al. 2006). Furthermore, in HepG2 an increased induction of chaperone proteins were seen after treatment with troglitazone compared to rosiglitazone (Maniratanachote et al. 2005). It has been suggested that excessive ROS production might be part of the toxic effect of troglitazone (reviewed in (Lim et al. 2008; Masubuchi 2006; Smith 2003)). For example, it was recently shown that primary astrocytes and C6 glioma cells treated with ciglitazone have a decreased cell viability, increased apoptosis and increased ROS production compared to cells treated with rosiglitazone (Perez-Ortiz et al. 2004). Additionally, troglitazone has also been shown to affect the mitochondria, that is by inducing mitochondria swelling, decrease mitochondrial membrane potential, as well as induce mitochondrial permeability transition (Masubuchi et al. 2006; Perez-Ortiz et al. 2004; Tirmenstein et al. 2002). In order to investigate the involvement of ROS production in the troglitazone-induced cell death we treated co-cultures with different antioxidants. Pre-treatment of co-cultures with the antioxidant catalase partially protects the THP-1 cells, but not Huh-7 cells, from cell death at 48 hours. This might suggest that H_2O_2 released into the medium plays a more important role in troglitazone-induced cytotoxicity in THP-1 cells than in Huh-7 cells. In monocytes and macrophages H_2O_2 produced by NADPH-oxidase located in the plasma membrane is released during the respiratory burst, which is part of the defense mechanism against microorganisms and various exogenous compounds (reviewed in (Forman and Torres 2001)). ROS, including H_2O_2, and reactive nitrogen species (RNS) released during the respiratory burst may damage the adjacent cells as well as the monocyte/macrophage itself (Ferret et al. 2002). It is thus possible that the addition of exogenous catalase protects the THP-1 cells since it inhibits the autocrine action of THP-1 released H_2O_2. However, pretreatment with other antioxidants such as N-acetyl-L-cysteine (NAC) or trolox, a vitamin E derivate, did not protect either of the two cell types in our model, while they have been shown *in vitro* to protect against H_2O_2- and ROS-induced cell death (Dey and Cederbaum 2006; Forrest et al. 1994; Shishido et al. 2003).

As mentioned, exposure of human and rat hepatocytes to troglitazone leads to a decrease in mitochondrial transmembrane potential ($\Delta\Psi m$), and subsequently depletion of ATP levels (Haskins et al. 2001). The mitochondrial effect preceded the detection of metabolic products of troglitazone as well as was observed in peripheral blood mononuclear cells, which do not contain cytochrome P450 activity. Thus, the decrease in $\Delta\Psi m$ is unlikely due to covalent adduct formation of troglitazone metabolites, rather

by troglitazone itself (Haskins et al. 2001). Bova and co-workers (2005) have showed that the HepG2 $\Delta\Psi m$ is affected as early as after 7 hours of troglitazone-treatment, before any other effects could be observed (alterations in cell permeability and cell count). Similar results were observed in isolated mitochondria from mice, where Masubuchi and co-workers (2006) observed decrease of mitochondrial membrane potential and formation of mitochondrial permeability transition (MPT), which may contribute to the hepatotoxicity of troglitazone since this was not observed with rosiglitazone. Additionally to the mitochondrial alterations, troglitazone has been shown to cause oxidative stress *in vitro* (Haskins et al. 2001; Masubuchi et al. 2006; Ong et al. 2007; Shishido et al. 2003). Nadanaciva and co-workers (2007) showed that troglitazone inhibited complex II + III, IV and V while rosiglitazone did not have a potent effect on the mitochondrial respiratory complexes. Bova et al (2005) also observed troglitazone-induced activation of caspase-3, indicating that troglitazone treated cells undergo apoptosis. Furthermore, DNA fragmentation and typical morphological changes associated with apoptosis have been reported in HepG2 liver cells treated with troglitazone (Yamamoto et al. 2001).

It still remains to be resolved how our and others findings (describing a potential toxic risk using higher concentrations of the drug *in vitro*) can come in accordance with the rare but still significant cases of liver injury seen in patients who were treated with troglitazone. Patients who receive 400 and 600 mg/day reach concentrations of 3.6 and 6.3 μM in the plasma, respectively (Loi et al. 1999); although it has been shown that troglitazone accumulate in the liver reaching 15-20 higher concentrations than the plasma concentrations (Kawai et al. 1997; Sahi et al. 2000). Furthermore, it also remains to be shown how this correlates with the long lag-time of onset of troglitazone-induced liver injury observed in patients, which can be up to months (Graham et al. 2003). The long lag-time could be compatible with accumulated mitochondrial changes, since it appears that the effect on the mitochondrion plays an important role particularly in susceptible patients. Whether these deficiencies in the anti-oxidant system are genetic or acquired, remains to be elucidated (Boelsterli and Lim 2007). Evidence that supports the theory with defective mitochondrion has been shown in an *in vivo* model with heterozygous $Sod^{+/-}$ mice, where therapeutic doses of troglitazone induced mitochondrial injury and hepatocellular necrosis after four weeks of daily administration, but not in normal healthy mice (Ong et al. 2007).

To conclude, we have observed an increased sensitivity of Huh-7 cells to troglitazone treatment when co-cultured with THP-1 cells, whereas rosiglitazone did not significantly affect the viability. Moreover, an increased expression of several inflammatory and stress-related genes was observed in both THP-1 and Huh-7 cells of co-cultures treated with troglitazone. Our results show the importance of immune cells in *in vitro* systems in order to get a system with higher sensitivity and predictability. However, more research about the involvement of inflammatory cells in drug-induced liver injury is needed. Therefore further investigations of the effects of other known hepatotoxins in co-cultures of monocytes/macrophages and hepatocytes are important. In continuation the co-culture system can be further developed with other hepatotoxins as well as the introduction of other cell types such as endothelial cells which are important in the secretion of adhesion molecules and recruitment of immune cells, or stellate cells which when activated produce collagen that results in scarring of the tissue.

5.3 SIGNALING MECHANISMS OF TROGLITAZONE

Our preliminary data of elucidating different signaling pathways involved in troglitazone versus rosiglitazone signaling shows some differences. From our pilot experiment some of the proteins with increased phosphorylation status in rosiglitazone treated cells were decreased in troglitazone treated cells; DGKZ and PKM2 (see table 1). The same observation is seen when looking at increases in troglitazone treated cells, these enzymes were less phosphorylated in rosiglitazone treated samples; PCK and PKR (see table 1). The implications of this in our system is at this stage to early to say, or what kind of role (if any) it plays in our co-culture model together with Huh-7 cells. This remains to be investigated.

In human hepatocytes Lim and co-workers (2008) showed that troglitazone causes increased production of superoxide in the mitochondria which induced activation of the ASK1/JNK signaling pathway, which subsequently lead to apoptosis via mitochondrial permeability transition. This is in accordance with what we can observe in the THP-1 cells treated with troglitazone where we see an effect on activation of mitochondrial related proteins (PCK2), as well as increased levels of Hsp60 with troglitazone after 1 hour of stimulation, which is less apparent with rosiglitazone treatment (see preliminary results; figure 8). Hsp60 have both anti- as well as pro-apoptotic properties; cytosolic

Hsp60 can prevent translocation of pro-apoptotic Bax into the mitochondria. Whereas mitochondrial Hsp60 promotes maturation of pro-caspase 3 and thus has a pro-apoptotic effect (Arya et al. 2007). Whether we observe the mitochondrial or the cytosolic form remains to be investigated.

As revealed from the real-time PCR analysis from cells in the co-culture system, we observed an increased expression of DDIT3/CHOP in both cell types (figure 5 and 6, in paper IV). From the preliminary results with western blot analysis we observed an increase of Hsp60, whose promoter has been shown to contain a CHOP element, as a mitochondrial stress response element (Zhao et al. 2002). Additionally, DDIT3/CHOP has been shown to be up-regulated by oxidative stress (Guyton et al. 1996) as well as by mitochondrial stress (Zhao et al. 2002).

The data give some hints and ideas about early signal transduction mechanisms specific for troglitazone in immune responsive cells. Additionally, if these differences in signaling pathways have anything to do with the observed increased troglitazone-induced cytotoxicity in the co-cultures of THP-1 and Huh-7 remains to be elucidated.

6 CONCLUSIONS

The results of the current thesis can be summarized as follows.

6.1 *c-fos* REGULATION IN GLIAL CELLS

We found in our studies on the mechanisms of *c-fos* activation, conducted in primary cultures of rat glial cells, that:

- LPS exerted its induction via the SRE or the CRE element in the *c-fos* promoter.
- The activation of *c-fos* by LPS was p38 MAPK dependent.
- Glutamate induced *c-fos* expression involving a novel calcium-dependent pathway.
- The activation by glutamate involved the participation of mGluR5 at the plasma membrane and mobilization of calcium from the ER.
- The *c-fos* expression is dependent the de-repression of DREAM at the DRE element in the *c-fos* promoter, distinct from the pathway known in neurons.

In summary, our studies reveal details of how inflammatory stimulus such as LPS and endogenous stimulus such as glutamate can differently regulate the *c-fos* expression in glial cells. Our results shed some light over how different stimuli converge at the level of the *c-fos* promoter, activating the same *cis*-acting elements by phosphorylating the same transcription factors that occupy these elements, and transcriptionally activate *c-fos*. Since glial cells play a central to the inflammatory response elicited in the CNS following infection or an insult resulting in excessive concentrations of glutamate, i.e. ischemic injury, and *c-fos* is an essential part of the activation of glial cells, the present results implicate glial p38 MAPK, Elk1, CREB/ATF-1 and DRE/DREAM as crucial mediators of inflammatory and endogenous processes in the CNS. This can lay as a base in the understanding and development of novel therapeutics for example against pain, in relation to DRE/DREAM.

6.2 *IN VITRO* DRUG-INDUCED HEPATOXICITY

In our endeavor to establish a human *in vitro* model, with higher sensitivity and better prediction of drug-induced hepatotoxicity, we observed:

- Ximelagatran treatment of monocytes (THP-1) results in pro-inflammatory cytokine/chemokine alterations and decreased cell viability.
- A requirement for intracellular metabolism of ximelagatran would explain the delayed effect on the phosphorylation of ERK, JNK and p38 MAP in THP-1 cells.
- Co-cultures of transformed monocytes and hepatocytes treated with troglitazone were more sensitive compared to single cultures. This effect was not observed with the non-hepatotoxic drug rosiglitazone.
- The troglitazone-induced cytotoxicity in THP-1 cells could partly be blocked when co-cultures were pre-treated with the antioxidant catalase.
- Troglitazone apparently induced release of mediators from THP-1 cells affecting the Huh-7 cells sensitivity for cytotoxicity.
- Troglitazone treatment increased gene expression of the stress-related genes DDIT3/CHOP, MT2A, CXCL2 and CXCL10 in THP-1 cells. In Huh-7 cells troglitazone treatment caused increased mRNA levels of DDIT3/CHOP, MT2A, CXCL2, CXCL10 and HspA6.

In summary, the novel *in vitro* system could be used as a screening model in drug development as it appears that introduction of monocytes increases the sensitivity of the hepatocytes to troglitazone treatment. Further development of the system would include the introduction of other important cell types such as endothelial cells, involved in recruitment of immune cells such as monocytes and neutrophils, or stellate cells which produce and secrete collagen which in turn are important in the pathogenesis of liver damage, as well as use other hepatotoxins. Understanding the different cell-cell interactions, cell-cell signaling effects could serve as a base for better prediction of potential drug-induced liver damages.

To conclude, inflammation plays a central role in both brain and liver injuries, induced by different insults such as excitotoxicity or drugs. In my thesis work I have been studying and elucidated mechanisms involved signaling and inflammatory responses in glial cells and monocytes. Knowledge about signaling pathways involved in immune cells and the expression inflammatory mediators will help elucidating the importance of immune cells in the development of new drugs.

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