IN VITRO INDUCTION OF TNF- BY OCHRATOXIN A

Lauy Mohammad Mahmood AL-Anati

لؤي العناتي

INAUGURAL-DISSERTATION
zur Erlangung des Grades eines
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DEDICATIONS

To my homeland;
Palestine

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List of abbreviations

Abbreviation	Description
Ca^{2+}	Calcium ion
cAMP	Cyclic adenosine monophoshate
CAPE	Caffeic acid phenylethyl ester
CO2	Carbon dioxide
COX	Cyclooxygenase
CYP-450	Cytochrome P-450
DMEM	Dulbeccos modified Eagles medium
FCS	Fatal calf serum
$GdCl_3$	Gadolinium chloride
GLDH	Glutamate dehydrogenase
HepG2	Human hepatoma cell line
hrs	Hours
IL-1,2,5,6,8,10	Interlukine-1,2,5,6,8,10
J774A.1	Mouse monocyte macrophages cell line
K^+	Potassium ion
L929	Connective tissue derived cell line
LDH	Lactate dehydrogenase

LIP-CLOD	Liposome-encapsulated clodronate
LIP-PBS	Liposome-encapsulated phosphate-buffered saline
LPS	Lipopolysaccharide
LPX	Lipoxygenase
LT-B,C	Leukotrienes-B,C
NDGA	Nordihydroguaiaretic acid
NF-kB	Nuclear transcription factor
OTA	Ochratoxin A
PGE	Prostaglandin
PMN	Polymorphonuclear cell
PLA_2	Phospholipase A_2
SEC	Sinusoidal endothelial cells
TACE	TNF-α-converting enzyme
TNFR1	Tumor necrosis factor receptor 1
TNF-α	Tumor necrosis factor-alpha

Chapter 1. Literature review

1.1 Ochratoxin A

1.1.1 Introduction

In 1920 an outbreak of porcine nephropathy occurred in Denmark and some years later in 1928 again a hitherto unknown renal porcine nephropathy was reported from there (Larsen, 1928). Today it is believed that the mycotoxin ochratoxin A (OTA) caused those nephropathies in swine. Ochratoxin A was isolated for the first time from *Aspergillus ochraceus* in 1965 by van der Merwe and his coworkers under an experimental survey to identify toxogenic fungus species. The name ochratoxin was derived from *Aspergillus ochraceus* and stands for a class of several mycotoxins designated by letters A, B and C (van der Merwe *et al.,* 1965a; b). At the same time this toxin was detected in field groups by Scott in 1965 (Scott*,* 1965). In 1957-1958 an unusual chronic kidney disease in humans called Balkan endemic nephropathy (BEN) occurred endemically in Yugoslavia, Rumania, and Bulgaria, mainly in rural areas where food is home grown. The suggestion was brought up that plant toxins or mycotoxins may be an environmental factor causing those diseases (Barnes, 1967; Hult *et al.,* 1982). In the 1970_s in Denmark, again nephritis was noted in pigs at slaughter. Analysis of pig feed showed that 50% of samples contained

ochratoxin A at levels up to 27 mg/kg. It further showed the presence of *Penicillium viridicatum* in the feed (Scott *et al.,* 1970; Krogh *et al.,* 1973). Since then, this toxin has occupied a major place in mycotoxin research and meanwhile it has been found to be ubiquitously present in all kind of food and feed. It is nowadays established that the toxin is continuously and unavoidably ingested with food, and consequently is present in blood in most, if not all tested people, in particular, in the USA and European populations (Jiménez *et al.,* 1999; Joint FAO/WHO, 2001).

1.1.2 Ochratoxin A-producing fungi

Ochratoxin A is a secondary metabolite of toxogenic species of Aspergillus and Penicillium fungi. It is produced redundant from *Aspergillus ochraceus* (van der Merwe *et al.,* 1965b), which grows in moderate temperatures and occurrs between 8-37 ^{0}C with optimum at 24-31 ^{0}C and 0.95-0.99 water activity (Pitt & Hocking, 1997; Joint FAO/WHO, 2001; Bakker & Pieters, 2002). Further studies showed the major mould responsible for ochratoxin A production is *Penicillium viridicatum* (Scott *et al.,* 1970; Krogh *et al.,* 1973), but more recently it has been shown to be *P. verrucosum* (Pitt, 1987). *P. verrucosum* was reported to grow in cool temperature regions below 30 ^{0}C with an optimum of 20 ^{0}C and with 0.8 water activity (Pitt & Hocking, 1997; Joint FAO/WHO,

2001; Bakker & Pieters, 2002). Recently, *A. carbonarius* was identified as a third major source of ochratoxin A in high temperature region. This fungus grows up to 40 ^{0}C with optimal temperatures of 32-35 ^{0}C (Téren *et al.,* 1996; Wicklow *et al.,* 1996). Some other fungal species (*Aspergillus alliaceus*, *Aspergillus auricomus*, *Aspergillus glaucus*, *Aspergillus melleus*, and *Aspergillus niger*) (Ciegler *et al.,* 1972; Abarca *et al.,* 1994; Bayman *et al.,* 2002) are less important producers of ochratoxins. In summary, ochratoxin A is a mycotoxin produced by several fungus species of the genera *Aspergillus* and *Penicillium* in different geographical areas under a wide variety of climate conditions. Thus the probability of its presence almost everywhere is very high. This adds OTA as a newly recognized risk factor for human an animal health.

1.1.3 Chemical properties of OTA

The systematical chemical name of OTA according to IUPAC is N-[[(3R)-5-chloro-8-hydroxy-3-methyl-1-oxo-7-isochromanyl]-carbonyl]-3-phenyl-L-alanine; other synonyms according to IARC are L-phenylalanine-N-[(5-chloro-3,4-dihydro-8-hydroxy-3-methyl-1-oxo-1H-2-benzopyran-7-yl)-carbonyl] or N-[(5-chloro-8-hydroxy-3-methyl-1-oxo-7-isochromanyl)-carbonyl]-3-phenyl-alanine. Its molecular weight is 403.8 g/mol. Ochratoxin A consists of a

chlorinated dihydroisocoumarin moiety linked through a 7-carboxyl group by an amide bond to one molecule of L-β-phenylalanine (Fig. 1).

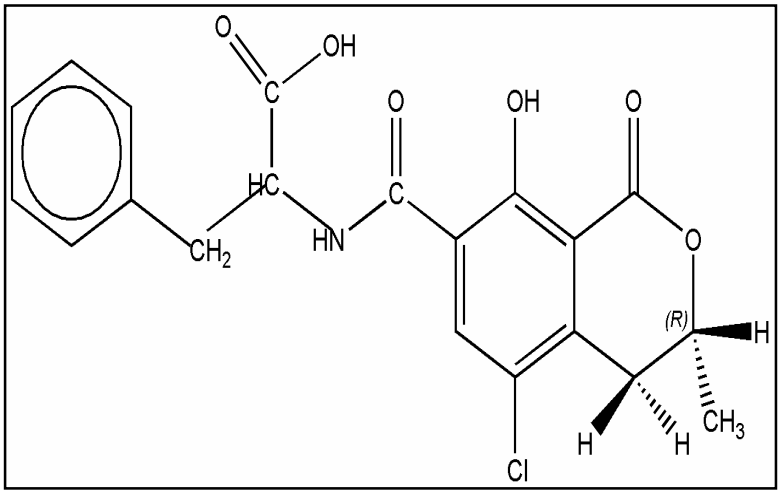

Fig. 1: Chemical structure of ochratoxin A

OTA forms a white crystalline powder when re-crystallized from xylene. The resoluted crystals emit green (in acid solution) and blue (in alkaline solution) fluorescence in ultraviolet light. The melting point of crystalline OTA is 169 ^{0}C. The free acid of ochratoxin A is soluble in organic solvents and the sodium salt form is soluble in water (IARC, 1983; 1993). Ethanol solution of OTA is stable for longer than a year if kept refrigerated and in the dark (USDHHS, 2002). It was reported that a methanol solution of OTA can be stably stored at –20 ^{0}C over a period of some years (Valenta, 1998), for at least 3 weeks at 4 ^{0}C and for at least 5 hrs at room temperature (Jiménez *et al.,* 1999).

1.1.4 Acceptable daily intake (ADI)

Based on risk assessments and the bulk data of toxicological adverse effects of ochratoxin A (Kuiper-Goodman & Scott, 1989; Walker, 2002), an acceptable daily intake (ADI) of OTA was suggested by several international committees in the last decade. In general, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) had evaluated ochratoxin A at its 37th meeting in 1991 (Joint FAO/WHO, 1991). In its assessments the carcinogenic effect of OTA was addressed, and the Lowest Observed Adverse Effect Level (LOAEL) of 8 μg/kg b.w. was set, based on renal dysfunctions in pigs. A Tolerable Daily Intake (TDI) of 16 ng/kg b.w. was established, which was converted to a Provisional Tolerable Weekly Intake (PTWI) of 112 ng/kg b.w. and a safety factor of 500 was applied. To follow the update, the Committee in 1995 at the 44th meeting reevaluated the toxicological profile of OTA. The evaluation was not changed and the (PTWI) value was rounded off to 100 ng/kg b.w. (Joint FAO/WHO, 1995).

In light of new information and development of analytical methods, the JECFA evaluated the data on OTA again in its 56th meeting in 2001. It was concluded that the new data raised further questions about the mechanisms by which OTA causes nephrotoxicity and renal carcinogenicity and the interdependence of these effects. The mechanisms by which OTA causes carcinogenicity are

unknown, although both genotoxic and non-genotoxic modes of action have been proposed. The Committee retained the previously established PTWI of 100 ng/kg body weight per week, pending the results of ongoing studies on the mechanisms of nephrotoxicity and carcinogenicity, and recommended a further review (Joint FAO/WHO, 2001; Bakker & Pieters, 2002).

According to the carcinogenic properties of OTA, the Canadian authorities had evaluated ochratoxin A in 1989, 1990, 1991 and 1996 (Kuiper-Goodman & Scott, 1989; Kuiper-Goodman, 1990; 1991, 1996), and calculated a Provisional Tolerable Daily Intake (PTDI) of 1.2-5.7 ng/kg b.w. Simultaneously in 1991 the Nordic expert group on food toxicology considered 5 ng/kg b.w. the highest Tolerable Daily Intake (NNT, 1991). Furthermore, the Europe Committee on Health and Consumer Protection in its opinion in 1994, stated that OTA is a potent nephrotoxic agent, a carcinogen, and has genotoxic properties. Therefore, they provisionally concluded that an Acceptable Daily Intake should fall in the range of a few ng/kg b.w./day and proposed to reconsider its opinion in the light of new information (Scientific Committee for Food 1996). However, the total daily intake of OTA from food in various European countries was between 0.9 ng/kg of b.w. in Germany and 4.6 ng/kg of b.w. in Italy (Wolff *et al.*, 2000)

1.1.5 Cytotoxicity and OTA-mediated cell death

Cytotoxic effects of OTA are based on the inhibition and/or activation of enzymes, of which several use phenylalanine as a substrate because this amino acid is part of the OTA molecule. It is believed that the penylalanine moiety in the OTA molecule interacts as a surrogate substrate. The main targeted enzyme is phenylalanine-tRNA synthetase which is inhibited in prokaryotes (Konrad & Röschenthaler, 1977), eukaryotic microorganisms (Creppy *et al.,* 1979), mammalian cells (Creppy *et al.,* 1983), and experimental animals *in vivo* (Creppy *et al.,* 1984). This inhibition causes a reduction in protein synthesis, which is an important effect of acute and subacute ochratoxin A toxicity. In addition to inhibition of protein synthesis RNA-synthesis inhibition is another end point of OTA toxicity (Dirheimer & Creppy, 1991), and probably DNA synthesis may be inhibited, too. An enzyme affected early by RNA inhibition is phosphoenolpyruvate carboxykinase, the key enzyme in the gluconeogenic pathway which depletes indirectly due to specific degradation of the mRNA coding for this enzyme (Meisner *et al.,* 1983). *In vitro* the addition of 1.0×10^{-4} M OTA to isolated rat liver mitochondria led to inhibition of succinate-cytochrome c reductase, succinate dehydrogenase, and succinate oxidase, due to effect on the mitochondrial respiration and oxidative phosphorylation through the impairment of the mitochondrial membrane and inhibition of the succinate-

supported electron transfer activities of the respiratory chain (Wei *et al.,* 1985). However, the concentrations required for these enzyme inhibitions are very high (in range of mmol range) and unlikely to occur *in vivo*.

With regard to cell death, the induction of apoptosis in several cell types of the urinary tract by OTA was reported. This organ system is a known OTA-target and clinically the most important one. Low doses of OTA activated apoptotic processes and oxidative damage in Wistar rat kidneys, particularly in both proximal and distal epithelial kidney cells (Petrik *et al.,* 2003). Apoptotic cell alterations were found when OTA at nanomolar concentrations was incubated with human proximal tubule-derived cells (IHKE) (Schwerdt *et al.,* 1999), and with dog renal collecting duct-derived cells (MDCK-C7) (Gekle *et al.,* 2000; Schwerdt *et al.,* 2004). Whatever, these changes were potentiated through inhibition and uncoupling of the mitochondrial respiratory chain (Schwerdt *et al.,* 2004). Interestingly OTA potentiated the apoptotic effect of TNF-α in MDCK-C7 cells (Gekle *et al.,* 2000). Furthermore apoptosis in hamster kidneys (HaK) and human HeLa cells was observed albeit at higher concentrations of OTA (Seegers *et al.,* 1994).

OTA-induced apoptosis is not limited to the urinary tract system but is found also in the immune system and the liver. Human peripheral blood lymphocytes and the human lymphoid T cell line, Kit 225 cells (Assaf *et al.,* 2004), bovine

lymphocytes (Lioi *et al.,* 2004), human hepatoma-derived cell line HepG2 (Renuzelli *et al.,* 2004) and liver of male mice (Atroshi *et al.,* 2000) proceed to cell death through the apoptotic pathway. In addition, the induction of DNA single strand breakdown and DNA adduct formation by OTA is considered as marker or evidence for OTA-induced apoptosis (Creppy *et al.,* 1985; Faucet *et al.,* 2004). Apart from apoptosis, necrosis also occurred under OTA burden. Necrotic changes were observed in rat liver (Aydin *et al.,* 2003), rat myocytes (Okutan *et al.,* 2004), and in germinal centres of the spleen and lymph nodes of Wistar rats (Kanisawa *et al.,* 1977), and dogs (Kitchen *et al.,* 1977). The parameters which determine the types of cell destruction are toxin dose and exposure time. E.g. one week after OTA administration to male mice only apoptotic without necrotic changes were observed in their livers, whereas centrilobular necrosis and apoptosis were seen after two weeks (Atroshi *et al.,* 2000). Gekle *et al.,* 2000, found that OTA at low dosage caused apoptosis and at higher dosage caused necrosis in MDCK-C11 cells (Gekle *et al.,* 2000). Others found only apoptotic but not necrotic changes in rat kidneys, which occurred in a dose- and time-dependent manner (Domijan *et al.,* 2004).

1.1.6 Hepatotoxicity of ochratoxin A

The liver is among the OTA-target organs because of its food-borne exposure via the portal vein to OTA after mycotoxin absorption from the gut and of an

enterohepatic circulation of OTA reported in rats (Fuchs *et al.,* 1988), indicating repeated exposure of liver cells to internally circulating OTA. Hepatotoxicity of OTA was observed previously in different species, especially in poultry. The older geese showed smaller liver weight at slaughter to be 400g compared with a normal weight of 600-900g. In a histopathological study the liver lesions in geese were caused by multifocal liver necrosis, containing inflammatory cells and sometimes bacterial colonies, while in other necrotic foci neither inflammatory reaction nor bacterial colonies were present. The organ weight reduction combined with gross changes was characterized by a fibrotic liver covered with fibrinous sheets. In the same study, the authors found the livers of broilers were enlarged and congested or, more often, shrunken. Fibrotic livers were covered by a sheet of fibrin (Schlosberg *et al.,* 1997). Similarly, chicks having received OTA before coccidiosis was induced experimentally, showed enlarged and congested livers (Stoev *et al.,* 2002). A short study was done in male Long-Evans and Sprague-Dawley rats given a single dose of benzene-free ochratoxin A by gavage. One of the earliest changes observed were multifocal hemorrhages and fibrin thrombi in the liver. The authors concluded that this occurred due to the activation of the extrinsic and intrinsic systems of coagulation (Albassam *et al.,* 1987). In accordance with this study Galtier *et al.,* 1979, found that ochratoxin A given via gavage to male Wister rats for 14 days

decreased blood coagulation factors II, VI, X, plasma fibrinogen, and thrombocyte counts (Galtier *et al.,* 1979).

In broilers, diffuse liver necrosis was common, usually without inflammatory cell infiltration. A subacute to chronic portal hepatitis was frequently observed. Some megalocytosis was often seen (Schlosberg *et al.,* 1997). Also in livers of fed chicks' cloudy swelling, granular degeneration, and rarely fatty changes of hepatic parenchymal cells were seen. Those changes usually were combined with activation of capillary endothelium and Kupffer cells, hyperemia and pericapillary edema as well as perivascular mononuclear cell infiltration (Steov *et al.,* 2002) However, at higher doses of OTA liver damage was presented in concert with nephrotoxicity in broiler chicks (Smith & Moss, 1985).

OTA effects on liver seem to be much less pronounced and specific than those of aflatoxins. Interestingly, OTA apparently prevented fatty degeneration of the liver caused by aflatoxin when the two toxins were given simultaneously to broiler chickens (Huff *et al.,* 1984). In rats treated with OTA, the histopathologic changes were found in the liver tissue, included granular or vacuolated degeneration and necrosis of the liver cells, sinusoidal and central vein dilatation, bile duct proliferation, enlargement of periportal areas with mononuclear cell inflammatory infiltration and mild degrees of fibrous tissue proliferation (Aydin *et al.,* 2003). Others found in young rats decreased

hepatocellular vacuolation, while in old rats hepatic erythropoiesis, inflammation, biliary/oval cell-proliferation and multinucleated giant cell formation (Dortant *et al.,* 2001).

Long-term studies of toxicity and carcinogenicity of ochratoxin A in mice, showed diets containing ochratoxin A in different doses and time exposures induced hepatic-cell tumours. It was not clearly indicated whether the liver tumours were benign or malignant (Kanisawa & Suzuki, 1978; Kanisawa, 1984). DNA single-strand breaks were observed *in vivo* in liver cells of mice after intraperitoneal injection of ochratoxin A. DNA repair, manifested as unscheduled DNA synthesis, was observed in most studies with primary cultures of rat and mouse hepatocytes (Joint FAO/WHO, 2001). The conclusion of these data was that OTA is direct genotoxic *in vitro*. It was also reported that *in vivo* OTA causes DNA adducts supporting a direct DNA reacting activity. However, it was never shown that OTA-derived radio activities occurred in DNA nor were such adducts ever documented by physiochemical analysis (Mally *et al.,* 2005). Therefore, the genotoxicity of OTA was recently questioned (Turesky, 2005). This means that it is likely that non-genotoxic, epigenetic disturbance would have caused cancer.

1.1.7 Cytokine modulation by OTA

The immune system is composed of very different cells, all capable of autonomous regulation. A very challenging understanding of this fine tuning emerged with the discovery of cytokines as new regulatory factors of the humoral immune system. The production of cytokines is influenced by several mycotoxins such as atranones B and E, trichodermin, 7-α-hydroxytrichodermol (Huttunen *et al.,* 2004) but most prominently by ochratoxin A. Ochratoxin A causes a significant release of pro-inflammatory cytokines TNF-α and IL-6 from blood-free perfused rat livers at micromoler concentration range and this release was comparable to that produced in rat livers by low concentration of LPS (Weidenbach *et al.,* 2000; Petzinger & Weidenbach, 2002). An antagonistic effect on OTA-mediated TNF-α release from rat livers was seen if other mycotoxins such as 3-acetoxydeoxynivalenol (3-Ac-DON), xanthomegnin (XAN), citrinin (CIT), and viomellein (VIO) were simultaneously co-applied (Petzinger & Weidenbach, 2002). The release was totally dependent upon extracellular calcium and was prevented by phosphodiesterase IV blocker rolipram (Weidenbach *et al.,* 2000).

Others reported TNF-α release from mouse RAW264.7 macrophage cell line upon OTA treatment (Huttunen *et al.,* 2004). Co-exposure of these cells to ochratoxin and *Streptococcus californicus*, the latter which causes TNF-α

release, had opposing effects (Huttunen *et al.,* 2004). OTA seems not to uniformly exert cytokine release. In the monocytic cell line THP-1 crude ochratoxin A from *Aspergillus ochraceus* as well as the pure toxin inhibited secretion of TNF-α (Heller *et al.,* 2002).

In contrast to hepatic IL-6 production, ochratoxin A failed to induce this interleukin from the RAW264.7 macrophage cell line, neither itself alone nor by concomitant exposure to ochratoxin A plus *Streptococcus californicus* (Huttunen *et al.,* 2004). In a thymoma cell line (EL4) which was stimulated by phorbol 12-myristate 12-acetate (PMA) exposure to ochratoxin A showed a marked increase of IL-2 production while IL-5 production was significantly decreased (Marin *et al.,* 1996). On the other hand, ochratoxin A inhibited IL-2 production from swine (Harvey *et al.,* 1992) lymphocytes which were stimulated by concanavalin A. Others found that OTA did not interfere with IL-2 levels released from stimulated murine lymphocytes (Thuvander *et al.,* 1995; 1996). In activated purified human T lymphocyte populations and subpopulations IL-2 production and IL-2 receptor expression were severely impaired by OTA, but pre-incubation of those cells with ochratoxin B (OTB) prior to OTA exposure reversed these inhibitory effects. The authors concluded that OTA abrogated the cells' ability to respond to activating stimuli *in vitro* through toxic interference with essential processes in cell metabolism (Lea *et*

al., 1989). Finally, IL-1 production from peritoneal mouse macrophages was inhibited when mice were pretreated with OTA for long time (Dhuley, 1997). Table 1 summarizes some of the mentioned effects of OTA on cytokine release.

Cytokine	Cell type/model	OTA concentration	Effects	Reference
TNF-α	Blood-free perfused rat livers	1 µg/ml	Stimulated	AL-Anati *et al.,* 2005
	Murine macrophages		Inhibited	Dhuley, 1997
	RAW264.7 macrophage cell line		Stimulated	Huttunen *et al.,* 2004
	Blood-free perfused rat livers	0.8 µg/ml	Stimulated	Petzinger & Weidenbach, 2002
	Blood-free perfused rat livers	0.8 µg/ml	Stimulated	Weidenbach *et al.,* 2000
	Monocytic cell line THP-1	400ng/ml pure OTA or 100ng crude OTA	Inhibited	Heller *et al.,* 2002
IL-6	Blood-free perfused rat livers	0.8 µg/ml	Stimulated	Petzinger & Weidenbach, 2002.
	Blood-free perfused rat livers	0.8 µg/ml	Stimulated	Weidenbach *et al.,* 2000
	RAW264.7 macrophage cell line		No effect	Huttunen *et al.,* 2004
IL-1	Murine macrophages		Inhibited	Dhuley, 1997
IL-5	Thymoma cell line (EL4)	5 or 10 µg/ml	Stimulated	Marin *et al.,* 1996
IL-2	Human T lymphocyte		Inhibited	Lea *et al.,* 1989
	Thymoma cell line (EL4)	5 or 10 µg/ml	Stimulated	Marin *et al.,* 1996
	Porcine lymphocytes		Inhibited	Harvey *et al.,* 1992
	Murine lymphocytes		No effects	Thuvander *et al.,* 1995
	Murine lymphocytes		No effects	Thuvander *et al.,* 1996

Table 1. Summary of cytokine modulation by OTA

1.2 TNF-alpha

1.2.1 Introduction

The proinflammatory cytokine tumor necrosis factor alpha (TNF-α) plays a fundamental role in immune defense. It was isolated in 1984, on the basis of its ability to kill tumor cells *in vitro* and to cause hemorrhagic necrosis of transplantable tumors in mice (Carswell *et al.,* 1975). It was described previously as hemorrhagic necrosis factor. Later, it was identified as the catabolic molecule cachectin in parasite-infested animals (Liz-Grana & Gomez-Reino Carnota, 2001). It continued to be the major topic of scientific investigation as indicated by several thousand citations in the last two decades (Aggarwal, 2000). Most of these studies demonstrated the powerful pro-inflammatory effects of TNF-α (Dayer *et al.,* 1985) and revealed its role as a central endogenous mediator of endotoxic shock (Beutler *et al.,* 1985; Tracey *et al.,* 1986). Furthermore, TNF-α known as pro-apoptotic cytokine was a double edged sword of activity: on one hand it mediated physiological processes and on the other hand promoted pathogenesis of several health disorders. Thus, TNF-α research is ongoing and has now reached mycotoxin research groups dealing apart from OTA with other toxins e.g. rubratoxin B (Nagashima *et al.,* 2001) and also fumonisins (He *et al.,* 2002).

1.2.2 TNF-α production and release

The human TNF-α gene is located in human chromosome 6 within the major histocompatibility complex (MHC), in the 6p21.3 Class III HLA zone. On both sides of it, in the 3' and 5' position, are the genes coding for the α- and ß-lymphotoxins, respectively. Even though the expression of these genes is independently regulated, the gene organization is quite similar. The TNF-α gene has 3,634 base pairs distributed into four exons and three introns (intron 1, 606 bp; intron 2, 186 bp; intron 3, 300 bp) the fourth and last exon codes for over 80% of the protein. The TNF cDNA has 1,585 base pairs and translates into a 230-amino acid-protein precursor. The TNF-α gene promoter contains recognition sequences for transcription factors such as AP-1, AP-2, CREBPß, CRE, Egr1, Ets, NF-AT, NF-kB and SP-1 (reviewed by Liz-Grana & Gomez-Reino Carnota, 2001).

TNF-α is produced by a wide variety of cell types in response to various inflammatory stimuli, such as lipopolysaccharides (LPS), phorbol ester, zymosan, ultraviolet light, TNF-α itself, other cytokines such as interleukin (IL)-1, IL-2, interferone (IFN)-γ, IFN-α, Granulocyte-Macrophage Colony-Stimulating factor (GM-CSF), the Transforming Growth Factor (TGF)-ß (Liz-Grana & Gomez-Reino Carnota, 2001).

1.2.3 TNF-α receptors and signaling pathway

In 1989, several groups independently reported the isolation of a TNF-α binding protein or TNF-α inhibitor from human urine that turned out to be the soluble form of the TNF-α receptor (Seckinger *et al.,* 1989; Lantz *et al.,* 1990). From the amino acid sequence of this protein the cDNA was isolated and cloned (Loetscher *et al.,* 1990; Nophar *et al.,* 1990). Simultaneously, the cDNA for a second TNF-α receptor was isolated and cloned (Kohno *et al.,* 1990; Smith *et al.,* 1990). It is now clear that TNF-α binds with almost equal affinity to two distinct receptors referred to as p60 (also called p55 or type I or CD120a) and p80 (also called p75 or type II or CD120b), with an approximate molecular mass of 60 kDa and 80 kDa, respectively (Aggarwal, 2000; Liz-Grana & Gomez-Reino Carnota, 2001). The human p60 receptor has 426 amino acid residues consisting of an extracellular domain (ECD) of 182 amino acids, a transmembrane domain (TMD) of 21 amino acids and an intracellular domain (ICD) of 221 amino acids. From this the predicted molecular mass of this receptor was about 47.5 kDa. As the apparent molecular mass of the p60 receptor is between 55 and 60 kDa, the difference most probably is attributable to three potential N-linked glycosylation sites present in the ECD of the receptors. The ECD of the p60 receptor has a net charge opposite to that of the TNF-α molecule, suggesting strong electrostatic interaction. The human p80

receptor is a 46 kDa protein, and it consists of 439 amino acid residues with an ECD of 235 amino acids, a transmembrane domain (TMD) of 30 amino acid residues, and an intracellular domain (ICD) of 174 amino acids. This receptor is glycosylated as well (Bazzoni & Beutler, 1996; Aggarwal, 2000).

The two receptors bind TNF-α with almost equal affinity. The receptors exhibit in their ECD four cysteine-rich regions, each consisting of six cysteine residues. These cysteines are conserved within the two receptors. In contrast the structure of the ICD of the two receptors is quite distinct and lacks enzymatic activity. The ICD of the p60 receptor contains a homophilic interaction region of approximately 80 amino acid residues towards its carboxyl terminal, called the death domain (DD) (Tartaglia *et al.,* 1993) which is absent in the p80 receptor. This region was found to be required for TNF-α induced apoptosis, antiviral activity, and nitric oxide synthase induction. Within the past decade, major advances have been made in understanding how TNF-α receptors transduce their signals. A series of signaling molecules have been discovered that play a critical part in the TNF-α induced cellular responses. Some of the major TNFα induced cellular responses were reviewed by Bazzoni & Beutler, 1996 and Aggarwal, 2000.

1.2.4 Biological activity of TNF-α and interference with pathological conditions

TNF-α has a broad spectrum of biologic activities and acts as a double-edged sword. On the one hand, it plays a major part in many physiological processes such as growth regulation, differentiation, viral replication, and liver regeneration (Goeddel *et al.,* 1986; Aggarwal & Natarajan, 1996). Furthermore, chronic, low-levels stimulate monocyte-macrophages and contribute to bacterial, parasitic (Liz-Grana & Gomez-Reino Carnota, 2001), and viral elimination (Van Reeth *et al.,* 2002; Korten *et al.,* 2005), and lead to bone resorption (Bezerra *et al.*, 2005).

On the other hand TNF-α, if released systemically in large amounts all at once, may induce tissue damage, shock and death. Whatever, a TNF-α increase in blood plays an important role in the pathogenesis of bacterial, viral and fungal infections, protozoa infestation, in addition to non-infectious disorders as e.g. silicosis (Piguet *et al.,* 1990; Ding *et al.,* 2002). The development of granulomatous inflammation in patients with chronic beryllium disease is associated with the production of numerous inflammatory cytokines, in particularly TNF-α (Maier, 2002; Maier *et al.,* 2002). Thus, the overproduction or inappropriate production of TNF-α should promote the pathogenesis of

several health disorders in particular chronic parasitic diseases such as trypanosomiasis caused by *Trypanosoma brucei rhodesiense* (Naessens *et al.,* 2005), *Trypanosoma brucei brucei*, *Trypanosoma cruzi* and babesiosis caused by *Babesia bovis* (Shoda *et al.,* 2001), toxoplasmosis caused by *Toxoplasma gondii* and schistosomiasis caused by *Schistosoma mansoni* (Marshall *et al.,* 1999), malaria caused by *Plasmodium berghei* (Hirunpetcharat *et al.,* 1999) and *Plasmodium falciparum* (Ramasamy, 1998).

Nervous system disorders were also reported to be modulated by TNF-α such as neurotoxicity induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Leng *et al.,* 2005), experimental autoimmune encephalomyelitis (EAE) (Djerbi *et al.,* 2003) and cerebral injury especially in infants (Mohan *et al.,* 2004). Rheumatoid arthritis (Taberner *et al.,* 2005), ankylosing spondylitis and psoriatic arthritis (Braun & Sieper, 2003) are also influenced by TNF-α.

In cardiovascular disorders, such as systemic lupus erythematosus, multiple sclerosis and systemic vasculitis, chronic heart failure (Dibbs *et al.,* 1999). TNF-α was involved as well as in chronic otitis media (Maeda *et al.,* 2004) and juvenile dermatomyositis (Pachman *et al.,* 2001).

Gastrointestinal tract diseases are worsened by TNF-α such as Crohn's disease (Brown & Abreu, 2005), pancreatitis (Kim *et al.,* 2000), and gastritis induced

by *Helicobacter pylori* (von Herbay & Rudi, 2000). Among the gastrointestinal tract disorders, chronic liver disorders are particularly promoted via TNF-α as alcoholic liver disease (Hirano *et al.,* 2003; Song *et al.,* 2004), septicaemia accompanying liver cirrhosis (Byl *et al.,* 1993; Ceydeli *et al.,* 2003), chronic inflammatory liver disease (Adams & Afford, 2002; McClain *et al.,* 2004), and primary sclerosing cholangitis (Bernal *et al.,* 1999; Mitchell *et al.,* 2001).

The acute diseases as well as inflammatory bowl disease, septic shock syndrome (Bazzoni & Beutler, 1996; Liz-Grana & Gomez-Reino Carnota, 2001) and asthma (Mattoli *et al.,* 1991; Broide *et al.,* 1992) were influenced by TNF-α.

1.2.5 TNF-α causing apoptosis and necrosis

The major hot topic of TNF-α research is the pro-apoptotic effect of TNF-α in primary cells or culture cell lines. Apoptotic effects of TNF-α were demonstrated in HeLa cells (Cozzi *et al.,* 2003), U937 cells (Misasi *et al.,* 2004), endometrial cells (Okazaki *et al.,* 2005), endothelial cells of rat coronary artery (Csiszar *et al.,* 2004), and T-lymphocytes (Bonetti *et al.,* 2003), neuronal cells in the rat cerebral cortex but not in hippocampus (Montes-Rodriguez *et al.,* 2004).

Pretreatment with TNF-α was reported to sensitize several tumor cells to apoptosis, such as Hodgkin tumor HD-MyZ cells which were subjected to apoptotic cell death induced by antineoplastic agents and by ceramide (Schmelz *et al.,* 2004). Furthermore, TNF-α treatment sensitized human thyroid cells to apoptosis (Mezosi *et al.,* 2005), Also pretreatment with luteolin, a plant flavonoid, greatly sensitized TNF-α-induced apoptotic cell death in a number of human cancer cell lines, including colorectal cancer COLO205, HCT116 cells and cervical cancer HeLa cells (Shi *et al.,* 2004). Aspirin sensitizes HeLa cells to TNF-α-induced apoptosis as well (Kutuk & Basaga, 2004). These results suggest that aspirin could be used to potentiate the effectiveness of TNF-α-based therapeutic interventions in cancer treatment (Kutuk & Basaga, 2004). Tubuloside B has the neuroprotective capacity to antagonize TNF-α-induced apoptosis in SH-SY5Y cells and may be useful in treating some neurodegenerative diseases (Deng *et al.,* 2004).

TNF-α represents one of the first pulmonary responses to hyperoxia, subsequently induced apoptosis in type II pneumocytes (TII cells). Eliminating the TNF-α effect *in vivo* by anti-TNF-α antibodies prevents the pro-apoptotic sensitization of TII cells pneumocytes (Guthmann *et al.,* 2005).

Vascular endothelial and/or smooth muscle cells also express TNF-α and IL-17, which can be up-regulated in pro-atherogenic pathophysiological conditions in

the coronary arteries. TNF-α has been shown to exert pro-inflammatory vascular effects (e.g., induction of oxidative stress, endothelial apoptosis, up-regulation of adhesion molecules and chemokines) (Csiszar & Ungvari, 2004). TNF-α causes apoptosis in both rat and human vascular smooth muscle cells and is intimately involved in the atherosclerotic process. Thus, inhibition of TNF-α is a useful approach in novel atherosclerosis therapies (Tang *et al.,* 2005).

1.2.6 TNF-α regulation by immunesuppressants, phosphodiesterase inhibitors, adenosine receptor antagonist, and non steroidal anti-inflammatory compound

The studies related with biosynthesis and cellular responses to TNF-α suggested at least three intervention strategies to suppress TNF-α release or TNF-α action or both (Henderson & Black, 1992): these included receptor antagonism (Prabhakar *et al.,* 1995), blockade of target cell signalling pathways (Ramirez *et al.,* 1999; Eigler *et al.,* 2000), and biosynthesis inhibition (Lee *et al.,* 1995). Compounds either inhibiting activation or synthesis of TNF-α were classified in different categories according to their mechanism of action.

1.2.6.1 Glucocorticoids

Glucocorticoids are among the most potent and widely used anti-inflammatory agents which were the earliest class of compounds identified to inhibit TNF-α expression (Ochalski *at al.,* 1993). Dexamethasone and hydrocortisone inhibited LPS-stimulated TNF-α release from leukocytes (Wirtz *et al.,* 2004). Dexamethasone suppressed and delayed the expression of TNF-α via downstream nuclear factor kappa B (NF-kB), signal transduction and activator of transcription 3 (STAT3), and activation protein 1 (AP-1) activation (Debonera *et al.,* 2003), thus indicating that TNF-α release is regulated at the transcriptional level and/or mRNA stability. Moreover, this class of compounds inhibited TNF-α action e.g. dexamethasone inhibited the induction of IL-8 by TNF-α (Nyhlen *et al.,* 2004), also dehydroepiandrosterone (DHEA) and DHEA-sulfate (DHEAS) inhibited TNF-α-induced NF-kB activation at transcription level (Iwasaki *et al.,* 2004).

1.2.6.2 cAMP elevating agents

The second class of TNF-α inhibitors, that have been studied extensively, are the cAMP elevating agents (Tannenbaum & Hamilton, 1989; Irie *et al.,* 2001), such as dibutyryl cAMP (Endres *et al.,* 1991), isoproterenol (Severn *et al.,*

1992), and prostaglandin E (Renz *et al.,* 1988; Lee *et al.,* 1995; Liz-Grana & Gomez-Reino Carnota, 2001). Also first and second generations of selective and nonselective phosphodiesterase PDE IV inhibitors such as rolipram (Brideau *et al.,* 1999; Hartmann *et al.,* 2000) suppress TNF-α release, too. It has already been shown that rolipram completely abolished OTA mediated TNF-α release from rat liver, indicating that this release required cAMP (Weidenbach *et al.,* 2000). The cAMP-elevating PDE-inhibitors inhibited LPS-stimulated TNF-α release without effects neither on TNF-α mRNA expression nor on NF-kB activation (Shames *et al.,* 2001). Thus the mechanisms are different from the transcription-related mechanism provoked by glucocorticoids. However, the cAMP elevating agents suppress TNF-α release via different mechanisms such as inhibition of leukotrienes or through elevation of prostaglandins or IL-10 levels.

1.2.6.3 Adenosine agonist

The third group of compounds, which was reported to suppress TNF-α release were adenosine agonists. Adenosine is an endogenous nucleoside that regulates numerous cellular functions including anti-inflammation processes (Jacobson *et al.,* 1992). Adenosine acts via cell surface receptors sub-typed as A1, A2A,

A2B, and A3. The A2A receptor (A2AR), in particular, has been linked to anti-inflammatory effects of adenosine (Lappin & Whaley, 1984). Therefore, an A2AR agonist namely 2-p-(2-carboxyethyl)phenethylamino-5'-N-ethylcarboxamido adenosine (CGS 21680) decreased TNF-α production from stimulated monocytes by LPS, whereas A2AR antagonism significantly increased TNF-α and blocked the inhibitory effect of CGS 21680. This A2AR-dependent inhibitory pathway involves the formation of cyclic adenosine monophosphate (cAMP) to activate protein kinase A, resulting in phosphorylation of cAMP response element-binding protein (CREB). Phospho-CREB has been shown to inhibit NF-kB transcriptional activity (Bshesh *et al.,* 2002). Similarly, the non-selective adenosine receptor agonist 5'-N-ethylcarboxamidoadenosine (NECA) inhibited LPS-induced TNF-α release from XS-106 cells. Furthermore, treatment with the selective adenosine A3 receptor agonist 1-[2-chloro-6[[(3 iodophenyl) methyl] amino] - 9H – purine - 9 - yl]- 1- deoxy – N - methyl-beta-D-ribofuranuronamide (Cl-IB-MECA) or the selective adenosine A2A receptor agonist 4-[2-[[-6-amino-9-(N-ethyl-beta-D-ribofuranuronamidosyl)-9H-purin-2-yl]amino]ethyl]benzene-propanoic acid CGS 21680 alone partially inhibited lipopolysaccharide-induced TNF-α release (when compared to NECA), whereas a combination of both agonists resulted in the inhibition of TNF-α release comparable to that observed with NECA alone.

Thus, at least two adenosine receptors (A2A, A3), activated by different agonists, triggered inhibition of LPS-induced TNF-α release in various cell lines. On the other hand, treatment of cells with the adenosine A2A receptor selective antagonists 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5ylamino]ethyl)phenol (ZM 241385) and 5-amino-2-(2-furyl)-7-phenylethyl-pyrazolo[4,3-e]-1,2,4-triazolo[1,5c]pyrimidine (SCH 58261) and the adenosine A3 receptor selective antagonist N-[9-chloro-2-(2-furanyl)[1,2,4]-triazolo[1,5-c]quinazolin-5-benzeneacetamide (MRS 1220) partially blocked the inhibitory effects of NECA on lipopolysaccharide-induced TNF-α release. Combined addition of MRS 1220 and SCH 58261 completely blocked the inhibitory effects of NECA on lipopolysaccharide-induced TNF-α release (Dickenson *et al.,* 2003).

In order to clarify the mechanistic effect of adenosine agonist-mediated TNF-α inhibition and to determine the receptor subtype involved in this effect, a study on the human macrophage cell line U937 was carried out. In this study A1/A3 agonist N6-(4-amino-3-iodobenzyl) adenosine (I-ABA) decreased LPS-stimulated TNF-α protein production. The mechanism was pre-translational, as adenosine receptor stimulation caused a marked decrease in TNF-α mRNA. The rank order of agonists as TNF-α inhibitors suggested that preferentially the A3 receptor is involved because N6-(3-iodobenzyl)-9-[5-(methylcarbamoyl)-beta-

D-ribofuranosyl] adenosine > 2-chloroadenosine ≥ I-ABA > N6-benzyl 5'-N-ethylcarboxamidoadenosine N6-benzyl-NECA > NECA > CGS21680 > N6-cyclohexyladenosine. This was supported by the fact that a mixed A1/A3 antagonist (xanthine amine congener) reversed the effect, whereas A1-specific (1,3-dipropyl-8-cyclopentylxanthine) and A2-specific (3,7-dimethyl-1-propargylxanthine) antagonists did not (Sajjadi *et al.,* 1996).

Adenosine (ADO) and the synthetic ADO analogue MDL201112 inhibited TNF-α production from mouse peritoneal macrophages, J774 and RAW-264 cells. MDL201112 selectively inhibited the expression of steady-state TNF-α RNA in LPS plus IFN-gamma-activated J774 and RAW-264 cells. ADO had no effect on RNA levels for TNF-α suggesting that ADO acts at a post-transcriptional biosynthetic step. Furthermore, the authors found a single i.p. injection of MDL201112 in LPS-challenged animals inhibited the appearance of TNF-α in the serum compared with the control group (Parmely *et al.,* 1993).

1.2.6.4 Matrix metalloproteinases, TACE, and TNF-α

Matrix metalloproteinases (MMPs) are a family of structurally related proteins with the collective capability to degrade all components of the extracellular matrix. Although MMP-mediated degradation of the extracellular matrix occurs

physiologically and under numerous pathological conditions (reviewed by Nozell *et al.,* 2004), a number of reports identified and characterized a membrane-associated metalloproteinase believed to be responsible for enzymatic processing of a membrane-bound precursor of TNF-α (Mohler *et al.,* 1994; Solorzano *et al.,* 1997). *In vitro* experiments showed that the matrix metalloproteinase inhibitor [4-(N-hydroxyamino)-2R-isobutyl-3S-(phenylthiomethyl)-succinyl]-L- phenylalanine-N-methylamide (GI 129471) inhibited *in vitro* the elevation of TNF-α in LPS-stimulated human and mouse whole blood (Murakami *et al.,* 1998). GM-6001 also inhibited the release of TNF-α and soluble TNF receptor (p75) from peripheral blood mononuclear cells when stimulated with endotoxin and/or exogenous TNF-α and significantly attenuated plasma TNF-α response in endotoxin challenged C57BL/6 mice (Solorzano *et al.,* 1997). The other proteinase inhibitor of this group is KB-R7785, which inhibited soluble TNF-α production in spleen cell cultures when stimulated by heat-killed *Listeria monocytogenes.* It reduced serum TNF-α level in *Listeria monocytogenes* infected mice (Yamada *et al.,* 2000). In addition, BB-2275, a synthetic inhibitor of matrix metalloproteinase activity (MMP), significantly reduced the levels of soluble TNF-α, p55 sTNF-R, and p75 sTNF-R released from rheumatoid synovial membrane cell cultures (Williams *et al.,* 1996). The piperine compound inhibits the matrix

metalloproteinase production and nuclear translocation of p65, p50, c-Rel subunits of NF-kappa B and other transcription factors such as ATF-2, c-Fos and CREB and subsequently inhibited TNF-α production from B16F-10 cells (Pradeep & Kuttan, 2004).

On the other hand, TNF-α and TNF-β strongly stimulated the production of MMP-2 and MMP-9 from bovine endometrial monolayer containing both epithelial and stromal cells (Hashizume *et al.,* 2003). In addition, PDE4 inhibitors rolipram, cilomilast, and CI-1044 are effective inhibitors for pro-MMP-2 and pro-MMP-1 secretion induced by TNF-α (Martin-Chouly *et al.,* 2004).

It was found that the Chinese medicine Reduqing (RDQ) inhibited the transcription of TNF-α mRNA induced by LPS stimulation through inhibition of TNF-α-converting enzyme (TACE), and subsequently inhibited sTNF-α secretion (Wang *et al.,* 2003). In another study Reduqing was reported to have double inhibitory effects on sTNF-α production and on the gene expression of TACE stimulated by LPS (Wang *et al.,* 2001). Also GW3333, a dual inhibitor of (TACE) and matrix metalloproteinases (MMPs), completely blocked increases in plasma TNF-α in LPS challenged mice and TNFα in the pleural cavity after intrapleural zymosan injection (Conway *et al.,* 2001).

1.2.6.5 CSAID Cytokine inhibitors

Bicyclic imidazoles are a novel class of nonsteroidal anti-inflammatory compounds that display unique pharmacological profiles by reducing cytokine production and arachidonic acid metabolism. Pretreatment of pigs with SK&F 86002 significantly attenuated LPS-induced increases in plasma TNF-α (Triplett *et al.,* 1996), and also inhibited serum TNF-α in LPS-challenged mice (Badger *et al.,* 1989; Spinelle-Jaegle *et al.,* 2001) and in male Sprague-Dawley rats (Smith *et al.,* 1991). Similar effects of SK&F 86002 have been shown in LPS treated human monocytes (Prabhakar *et al.,* 1993; Prichett *et al.,* 1995). This inhibition didn't effect TNF mRNA accumulation, suggesting a post-transcriptional action (Prichett *et al.,* 1995). Furthermore, inhibitory effects of SK&F 86002 and related analogs of the pyridinyl imidazole class on TNF-α production were mediated via a cAMP-dependent mechanism, although the pyridinyl imidazole compounds were found to be generally weak phosphodiesterase inhibitors, which did not affect cAMP levels in human monocytes, alone or in the presence of LPS (Kassis *et al.,* 1993).

The test compounds SK&F 105809, SK&F 105561, SK&F 104351, SK&F 104493 have been shown to inhibit the production TNF-α from human monocytes *in vitro*, and *in vivo* they reduced the plasma level of TNF-α in LPS

challenged mice (Griswold *et al.,* 1993; Votta & Bertolini, 1994). Two lysophosphatidylcholine acyltransferase (LPCAT) inhibitors SK&F 98625 (diethyl 7-(3,4,5-triphenyl-2-oxo2,3-dihydro-imidazole-1-yl) heptane phosphonate) and YM 50201 (3-hydroxyethyl 5,3'-thiophenyl pyridine) strongly inhibited TNF-α production in response to LPS in both unprimed MonoMac-6 cells and in cells primed with IFN-γ. The inhibitory effects of SK&F 98625 resulted from reduction of TNF-α mRNA levels in MonoMac-6 cells (Schmid *et al.,* 2003). Furthermore, SK&F 98625-induced inhibition of TNF-α production from peritoneal macrophages incubated in medium containing thapsigargin (Yamada *et al.,* 1998a).

1.3 Arachidonic acid

Arachidonic acid is a polyunsaturated fatty acid is derived directly from linolenic acid or is ingested as a dietary constituent. Arachidonic acid is stored in the cell membrane of virtually all cells and is released in response to stimluli such as histamine and platelet-derived growth factor. Arachidonic acid can be released by three pathways: (1) conversion of phosphatidyl ethanolamine or phosphatidyl choline to phosphatidic acid in a reaction catalysed by phospholipase D (PLD), followed by formation of diglyceride and monoglyceride and the release of arachidonic acid; (2) degradation of phosphatidylinositol via a sequence of reactions beginning with PLC cleavage of the phosphodiester bond of membrane lipids to yield diacylglycerol, followed by the action of dilglyceride lipase and monoglyceride lipase to release arachidonic acid and glycerol; and (3) direct action of PLA_2 on a phospholipid.

Figure 2 shows the three major pathways involved in arachidonic acid metabolism (a) The cyclooxygenase (COX) pathway results in the formation of prostaglandin G2 (PGG_2) from arachidonic acid by a cyclooxygenase reaction. In a subsequent peroxidase reaction, PGG_2 undergoes a two-electron reduction to PGH_2. Both of these reactions are catalysed by COX (prostaglandin synthase H). PGG_2 serves as a substrate for cell-specific isomerases and synthases,

producing other eicosanoids such as prostacyclin (PGI_2) and thromboxane A_2 (TXA_2). (b) The lipoxygenase pathway forms hydroperoxyeicosatetraenoic acids (HPETEs) and dihydroxyeicosatetraenoic acid (DEA) by lipoxygenase and subsequently converts these to (1) hydroxyeicosatetraenoic acids (HETEs) by peroxidases, (2) leukotrienes (e.g. LTC_4) by hydrase and glutathione S-transferase (GST), and (3) lipoxins by lipoxygenases. (c) The epoxygenase pathway forms epoxyeicosatrienoic acid (EET) and dihydroxyacids by cytochrome P-450 epoxygenase (Holtzmann, 1992; Brash, 2001; Zeldin, 2001). These products and the nonenzymatic transformations have well-substantiated bioactivities. Unchanged arachidonic acid itself has biological activity and is involved in cellular signaling as a second messenger (Brash, 2001).

However, TNF-α action (Vondracek *et al.,* 2001), or release is modulated by arachidonic acid (Stuhlmeier *et al.,* 1996) and some of its metabolites (Renz *et al.,* 1988). On the other hand, in the presence of TNF-α arachidonic acid and its products were released in several cell lines due to the activation of phospholipase A2, which mediated and potentiated TNF-α toxicity (Reid *et al.,* 1991; Hayakawa *et al.,* 1993; Liu and McHowat, 1998).

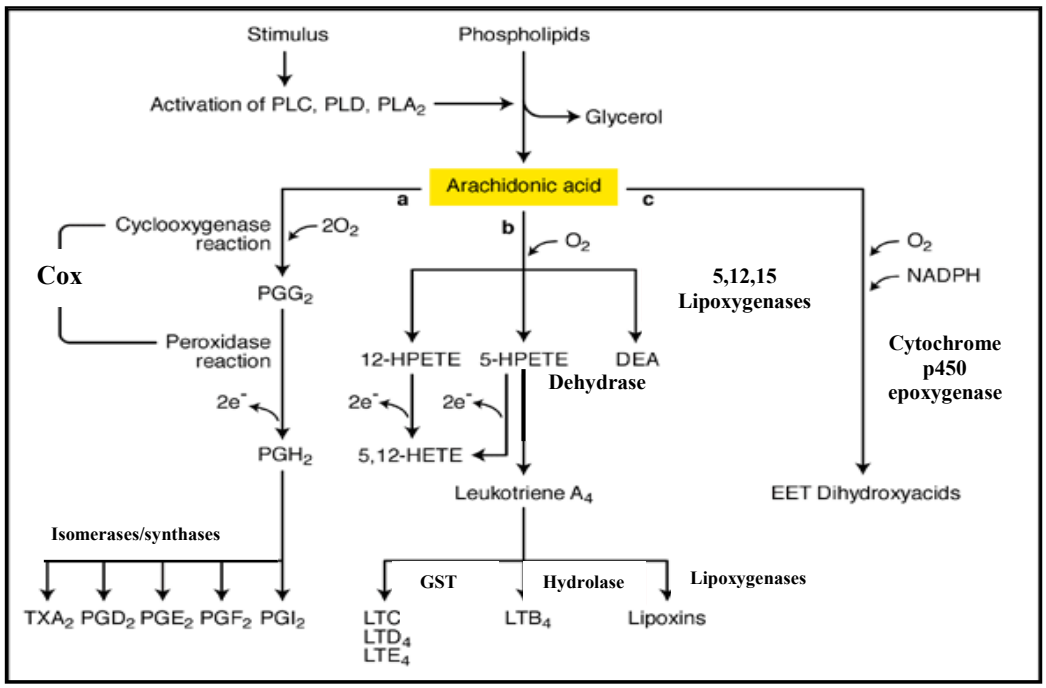

Fig. 2 Arachidonic acid metabolic signaling cascade

Chapter 2. Research objectives

The liver is among the target organs of OTA because of its food-borne exposure via the portal vein after mycotoxin absorption from the gut. Because an enterohepatic circulation of OTA was reported in rat repeated exposure of liver cells to internally circulating OTA occurs as well. Ochratoxin A is transported from blood into hepatocytes by carrier-mediated transport. The liver is also a major organ for systemic release of inflammatory cytokines, i.e. TNF-α and IL-6, upon exposure to gut-derived bacterial toxins i.e. lipopolysaccharides. TNF-α plays an important role in liver regeneration, and promotes the pathogenesis of chronic liver disorders. Therefore, the aim of this study is summarized in following questions.

1- Does experimental passage of OTA via the portal vein of blood-free perfused rat livers induce TNF-α?
2- Does this release have significant meaning in comparison with LPS?
3- Which liver cell types in blood-free perfused rat livers serve as TNF-α source in response to OTA?
4- Is the OTA effect restricted to the liver or do other organs contribute to TNF-α release?

5- Are the arachidonic acid and its metabolites influenced by OTA-mediated TNF-α release from blood-free perfused rat livers?

6- Which signaling pathway does OTA use to induce TNF-α release?

Chapter 3. Materials & Methods

3.1 Materials

3.1.1 Chemicals & Reagents

Chemical Name	Source
Anti-CD14 (mouse IgG1)	*Alexis Biochemicals, Grünberg, Germany*
Arachidonic acid (5, 8, 11, 14-eicosatetraenoic acid) sodium salt	*Sigma-Aldrich Co. Steinheim, Germany*
Aristolochic acid sodium salt (type I & II) ($C_{17}H_{10}NHaO_7$)	*Sigma-Aldrich Co. Steinheim, Germany*
Caffeic acid phenylethyl ester	*Alexis Biochemicals, Grünberg, Germany*
Calcium chloride dihydrate($CaCl_2 \bullet 2H_2O$)	*E. Merck Darmstadt, Germany*
Collagen type VII, from rat tail	*Sigma-Aldrich Co. Steinheim, Germany*
Collagenase type CIS, 212U/mg	*Biochrom AG, Berlin, Germany*
Collagenase NB 4 from Cl. histolyticum 0.161 PZU/mg	*SERVA Electrophoresis GmbH, Heidelberg, Germany*
Dextran (Leuconostoc mesenteroides, strain No. B-512)	*Sigma-Aldrich Co. Steinheim, Germany*
Digitonin p.A	*SERVA Electrophoresis GmbH, Heidelberg, Germany*
DMSO	*SERVA Electrophoresis GmbH, Heidelberg, Germany*
DNase I	*Boehringer, Germany*
Dulbeccos modified Egales medium (DMEM)	*GIBCO^{TM} , Paisley, Scotland, UK*
Ethanol	*E. Merck Darmstadt, Germany*

Gadolinium chloride hexahydrate ($GdCl_3$)	*Sigma-Aldrich Co. Steinheim, Germany*
Glucose anhydrous ($C_6H_{12}O_6$)	*E. Merck Darmstadt, Germany*
Heat inactivated fetal calf serum	*Sigma-Aldrich Co. Steinheim, Germany*
Heparin, Liquemin® 5000 IU/ml	*Sigma-Aldrich Co. Steinheim, Germany*
Indomethacin ($C_{19}H_{16}CINO_4$)	*Sigma-Aldrich Co. Steinheim, Germany*
Lipopolysaccharide (*E. Coli* serotype 0111:B4)	*Sigma-Aldrich Co. Steinheim, Germany*
Magnesium chloride hexahydrate ($MgCl_2•6H_2O$)	*E. Merck Darmstadt, Germany*
Magnesium sulphate heptahydrate ($MgSO_4•7H_2O$)	*E. Merck Darmstadt, Germany*
Metyrapone (2-methyl-1, 2-di-3-pyridyl-1-propane)	*Sigma-Aldrich Co. Steinheim, Germany*
Modified HANK's balanced salt solution Ca^{2+} free	*Sigma-Aldrich Co. Steinheim, Germany*
Modified HANK's balanced salt solution with Ca^{2+}	*Sigma-Aldrich Co. Steinheim, Germany*
Nordihydroguaiaretic acid (NDGA) (1, 4-bis[3,4-Dihydroxyphenyl]-2,3-dimethylbutane), from Larrea divaricata (creosotebush)	*Sigma-Aldrich Co. Steinheim, Germany*
Nycodenz® 5-(N-2,3-dihydroxypropylactemido)-2, 4, 6,tri-iodo-N,N'-bis (2,3 dihydroxypropyl) isophthalamide	*AXIS-SHIELD PoC AS, Oslo, Norway*
Ochratoxin A (MT-I-161A)	*CSIR, Food Science and Technology, Pretoria, South Africa*
Penicillin & Streptomycin	*GIBCO™ , Paisley, Scotland, UK*
Potassium chloride (KCl)	*E. Merck Darmstadt, Germany*
Potassium dihydrogen phosphate (KH_2PO4)	*E. Merck Darmstadt, Germany*
Pronase E	*E. Merck Darmstadt, Germany*
RPMI 1640 medium	*Biochrom AG, Berlin, Germany*
Sodium chloride (NaCl)	*E. Merck Darmstadt, Germany*
Sodium dihydrogen phosphate monohydrate ($NaH_2PO_4•H_2O$)	*E. Merck Darmstadt, Germany*
Sodium hydrogen carbonate ($NaHCO_3$)	*E. Merck Darmstadt, Germany*

Trypane blue	*Sigma-Aldrich Co. Steinheim, Germany*
Trypsin/EDTA	*GIBCO^{TM} , Paisley, Scotland, UK*
Urethane	*Fluka chemie AG Switzerland*

3.1.2 Kits

TNF-α Enzyme linked immunosorbant assay (ELISA) kit Cytoscreen®	*BioSource International, Camarillo, Canada, with antibodies selective for detection of rat TNF-α*
Lactate dehydrogenase (LDH)	*Roche Diagnostics Corporation, Indianapolis, USA*
Glutamate dehydrogenase (GLDH)	*Roche Diagnostics Corporation, Indianapolis, USA*
Lactate	*Roche Diagnostics Corporation, Indianapolis, USA*

3.1.3 Instruments

Autoclave	*SANOClav, Lam-201, Geislineen, Germany*
Balance	*CAHN Microbalance C-30. INC. Cerritos, California, USA*
Balance	*METTLER AE 260, DeltaRange®,Gießen, Germany*
Benchmark microplate reader	*BIO-RAD labrotories GmbH, Munch, Germany*
Biohazard Laminar flow cabinet	*danLAF® VFR 1806, Denmark*
Centrifuge	*Eppendorf 4515D, Eppendorf-Nether-Hinz GmbH, Hamburg,Germany*
Centrifuge	*BHG HERMLE Z2364, Gosheim, Germany*
Gradient centrifuge	*Sigma 4k15, sigma international*
JE-6B elutriation system and rotor	*Beckman Instruments, Inc. Palo Alto, USA*
Freezer (-20 ^{0}C)	*LIEBHERR Premium, -20*
Freezer (-80 ^{0}C)	*Nap Coil UF 400*
Heating magnetic stirrer	*Heidolph MR82*

Incubator	*New Brunswick Scientific, New Jersey USA*
Light microscope	*ORTHOMAT^{TM},Leitz Fluovert, Wetzlar, Germany*
Microliter pipettes (10 µl – 1000 µl)	*Eppendorf-Nether-Hinz GmbH, Hamburg,Germany*
Perfusion system	*House made, Gießen, Germany*
pH meter	*CG 841, Schott, Mainz Germany*
Refrigerator	*LIEBHERR Premium.*
Surgical and anatomical set	*Hebu GmbH Weilheim, Germany*
Vortex mixer	*Heidolph, REAX 1DR, Germany*
Water bath	*Julabo SW1, Julabo Labortechnik, seelbach, Germany*

3.1.4 Disposable materials:

All disposable materials were obtained from Sarstedt, Aktiengesellschaft & Co., Nümbrecht, Germany

1) Syringes (1ml, 5ml, 10ml and 20ml)
2) Non pyrogenic plane tubes (1.5, 15, 50 ml)
3) Tissue culture plates (different size)
4) Blue, yellow and white tips

3.1.5 Cell types

Cell name	Identification	Sources
L929	NCTC clone 929, clone strain L, connective tissue, mouse	Obtained from Dr. Ayub Darji, Institute of Medical Microbiology, Justus-Liebig-University Gieβen

J774 A.1	Mouse Monocyte macrophage cell line	Obtained from Dr. Ayub Darji, Institute of Medical Microbiology, Justus-Liebig-University Gieβen
HepG2	Human hepatoma cell line	Obtained from Dr. Ayub Darji, Institute of Medical Microbiology, Justus-Liebig-University Gieβen
Hepatocytes	Primary cells	Prepared in Pharmacology & Toxicology Institute, Justus-Liebig-University Gieβen
Kupffer cells	Primary cells	Prepared in Clinics of Gastroenterology, Hepatology and Infectiology. Heinrich-Heine University Düsseldorf
Peritoneal rat macrophages	Primary cells	Prepared in Pharmacology & Toxicology Institute, Justus-Liebig-University Gieβen
Sinusoidal endothelial cells	Primary cells	Prepared in Clinics of Gastroenterology, Hepatology and Infectiology. Heinrich-Heine University Düsseldorf

3.2 Animals

Male Wistar rats (200-280g) were used in all experiments. The animals were fed *ad libitum* with Altromin® standard diet and received water *ad libitum*. They were kept under 12-hr light-dark cycles at 22°C temperature and ventilation under standard conditions. The health of rats was routinely tested by sentinel animals and the animals were found to be free of chronic infections and parasites.

3.3 Solutions & buffers

3.3.1 Krebs-Henseleit buffer

Component	mM	g/L
Glucose anhydrous	5.56	1.0
$NaHCO_3$	25	2.1
NaCl	118	6.9
KCl	4.7	0.35
$MgSO_4 \cdot 7H_2O$	1.2	0.3
$KH_2PO_4 \cdot 2H_2O$	1.2	0.16
$CaCl_2 \cdot 2H_2O$	1.7	0.25

The volume was completed to 1000 ml by deionized water. The mixture was dissolved well and transferred to a shaker water bath at 37 ^{0}C with continuous gas (95% O_2-5% CO_2) supplement. Finally, pH was adjusted to 7.4 by 1 N HCl.

3.3.2 Krebs-Henseleit buffer Ca^{2+} free

Component	mM	g/L
Glucose anhydrous	5.56	1.0
$NaHCO_3$	25	2.1
NaCl	118	6.9

KCl	4.7	0.35
$MgSO_4 \cdot 7H_2O$	1.2	0.3
$KH_2PO_4 \cdot 2H_2O$	1.2	0.16

The volume was completed to 1000 ml by deionized water. The mixture was dissolved well and transferred to a shaker water bath at 37 ^{0}C with continuous gas (95% O_2-5% CO_2) supplement. Finally, pH was adjusted to 7.4 by 1 N HCl.

3.3.3 Tyrode buffer

Component	**mM**	**g/L**
Glucose anhydrous	5.56	1.0
$NaHCO_3$	25	2.1
NaCl	219	12.8
KCl	4.7	0.35
$MgSO_4 \cdot 7H_2O$	1.18	0.29
KH_2PO_4	1.18	0.21

The volume was completed to 1000 ml by deionized water. The mixture was dissolved well and transferred to a shaker water bath at 37 ^{0}C with continuous gas (95% O_2-5% CO_2) supplement. Finally, pH was adjusted to 7.4 by 1 N HCl.

3.3.4 Phosphate buffer

Component	mM	g/L
NaCl	137	8.0
KCl	2.68	0.28
KH_2PO_4	1.14	0.160
$Na_2PO_4 \cdot 7H_2O$	7.3	1.14

The volume was completed to 1000 ml by deionized water. The mixture was dissolved well. Finally, pH was adjusted to 7.4 and autoclaved.

3.3.5 Modifid HANK's balanced salt solution with Ca^{2+}

Component	mM	g/L
$CaCl_2 \cdot H_2O$	1.25	0.185
$MgSO_4$ (anhydrous)	0.831	0.09767
KCl	5.37	0.4
KH_2PO_4 (anhydrous)	0.34	0.06
$NaHCO_3$	4.17	0.35
NaCl	136.89	8.0
$NaHPO_4$ (anhydrous)	0.4	0.04788

D-Glucose	5.55	1.0

3.3.6 Modifid HANK's balanced salt solution Ca^{2+} free

Component	mM	g/L
KCl	0.831	0.4
KH_2PO_4 (anhydrous)	5.37	0.06
$NaHCO_3$	0.34	0.35
NaCl	4.17	8.0
$NaHPO_4$ (anhydrous)	136.89	0.04788
D-Glucose	0.4	1.0

3.4 Methods

3.4.1 Ochratoxin A and tested compound preparations

Compounds were weighted using sensitive balance (*CAHN Microbalance C-30. INC. Cerritos, California, USA)*, then dissolved according to their chemical properties using absolute ethanol or buffers and stored at 4 ^{0}C no longer than one week.

3.4.2 Liposome preparation and administration

The multilamellar liposomes were prepared as described earlier by Van Rooijen and coworker (Van Rooijen & Sanders, 1994). First, 11 mg cholesterol and 75 mg phosphatidylcholine were dissolved in chloroform in a 500-ml round-bottom flask. After low-vacuum rotary evaporation a thin film was formed on the interior of the flask. This film was dispersed by rotation for 10 min in 10ml phosphate-buffered saline (PBS) solution in which 0.7M clodronate (a kind gift of Roche Diagnostics, Mannheim, Germany) was dissolved. After removal of the lipid film, the suspension was kept for 2 hrs at room temperature and sonicated for 3 min in a water bath sonicator. This suspension remained for another 2 hrs at room temperature for liposome swelling. The liposomes were centrifuged for 30 min at 25,000 g to wash out the free clodronate. In order to get the proper concentration, 6 mg clodronate per ml suspension, the liposomes were resuspended in 4 ml PBS. During this period no perceptible leakage of clodronate out of the liposomes can be found. Livers were prepared from rats which received 48 hrs before liver preparation 2ml/rat liposome encapsulated clodronate by i.p. injection and, for control, 2ml/rat liposomes encapsulated buffer medium. Liposome-encapsulated clodronate (LIP-CLOD) and liposome-encapsulated phosphate-buffered saline (LIP-PBS) were sent by air mail in kind of cooperation, then stored at 4°C for no longer than 2 weeks.

3.4.3 Rat liver preparations

The rats were anesthetized by an intraperitoneal injection of 1-1.5 ml 20% urethane solution, and heparinized with 0.3 ml/kg of b.w. Liquemin® 5000 IU/ml i.v. into the femoral vein. After laparotomy a catheter was inserted into the portal vein and another catheter inserted in the cranial vena cava. By perfusion with Krebs-Henseleit solution the rats were killed by exsanguination. Then, the liver was excarporated and perfused with Krebs-Henseleit solution about 10-15 min to completely remove the blood (Fig. 3A).

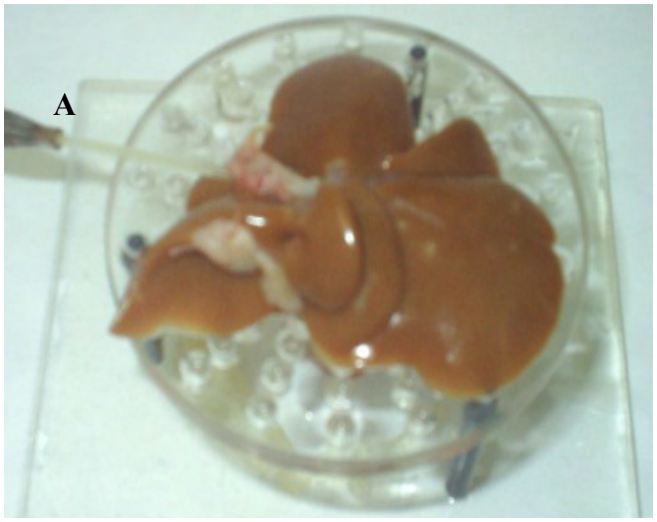

Fig. 3A: Blood-free rat liver

The liver of a male Wistar rat was placed on a support and was washed with Krebs-Henseleit solution for about 10-15 minutes to completely remove the blood. One catheter (A) was inserted into the cranial vena cava and the other catheter (not shown) was inserted into the portal vein.

3.4.4 Isolated blood-free liver perfusion

Isolated blood-free rat livers were perfused as described previously by AL-Anati *et al.,* 2005. Briefly, blood-free rat livers were placed in an experimental perfusion setup, installed in a temperature-controlled hood. Livers were re-circulated with 75 ml of 2% dextran Krebs-Henseleit solution via the portal vein catheter at 37°C. The system was supplemented by 95% O_2- 5% CO_2 gassing, the flow rate and the pH of perfusate were monitored and controlled during the experiments (Fig. 3B). The livers were equilibrated with the perfusion buffer (without tested compound) during a pre-experimental period of 10 min. After that zero-samples from the perfusate were taken. Then, the corresponding substances were injected via a portal vein inlet to the perfusion medium and 10 min later ochratoxin A, 1µg/ml (2.5µmol/L), or 0.1 µg/ml LPS were added at t=20min after zero-time.

For controls, each tested compound was given alone and then tested for TNF-α in combination with ochratoxin A under identical experimental conditions. Basal TNF-α release was measured on isolated perfused rat livers without treatment.

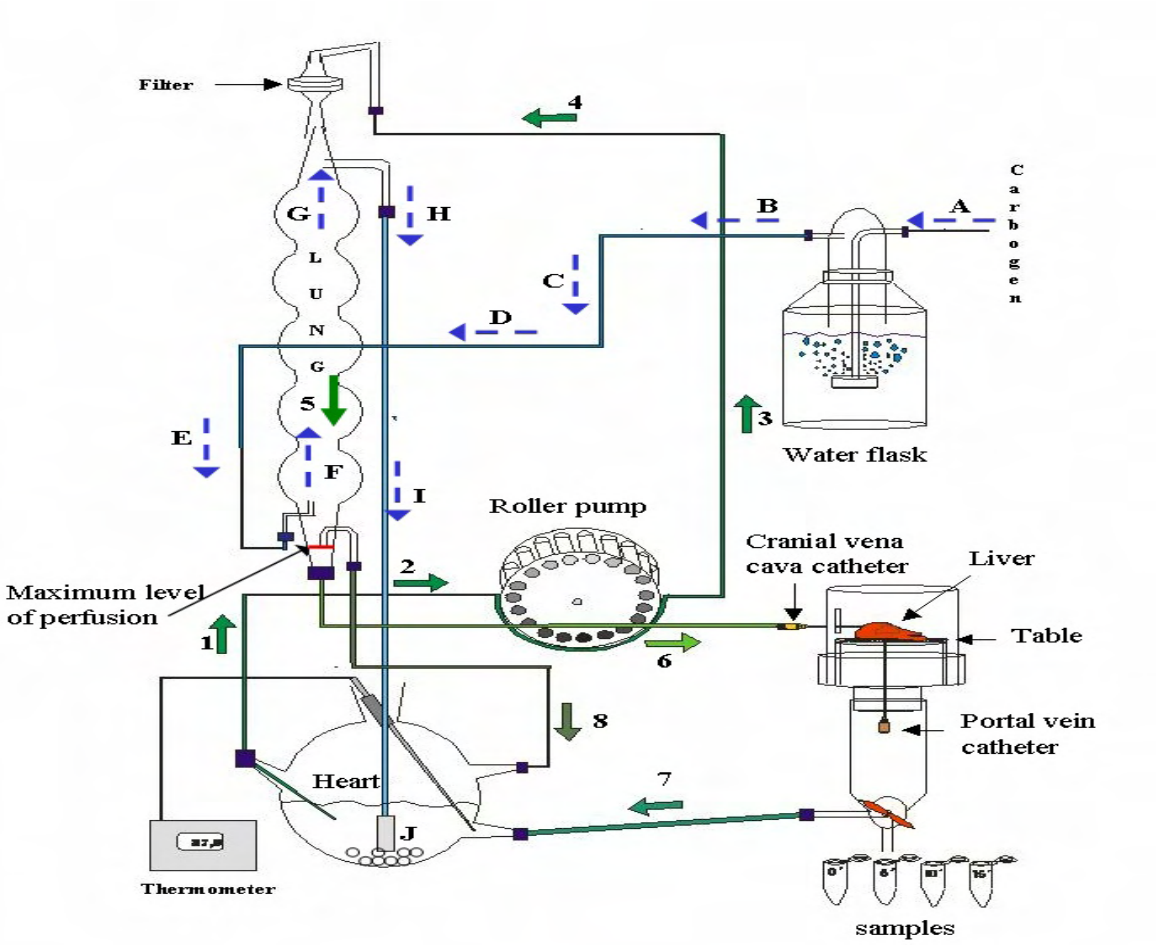

Fig. 3B: Schedule of perfusion

The figure shows the flow of gas and perfusion fluid in the experimental perfusion setup. The flow of $O_2\backslash Co_2$ gas comes from (A) a carbogen gas bottle through a water bottle, then moves through a tube (B, C, D, and E) to enter the artificial lung (F and G) and then leaves the lung through tube (H and I) to reach the perfusion fluid (J). The perfusion fluid starts from the artificial heart (1 and 2) by the action of a roller pump, then passes (3 and 4) a filter on the top of the lung. The fluid accumulates in the basis of the lung and passes (6) to the liver via the cranial vena cava catheter. The fluid leaves the liver through the portal vein catheter from where the samples are taken and circulates back to the heart (7). From the maximum fluid level in the basal part of the lung, the extra fluid returns back to the heart as shown by label (8).

3.4.5 Isolation of sinusoidal endothelial and Kupffer cells

Kupffer cells and sinusoidal endothelial cells were isolated according to the collagenase/pronase digestion method of Eyhorn *et al*. 1988. Briefly, blood-free isolated rats livers were perfused via the portal vein with a modified HANK's balanced salt solution Ca^{2+} free at 7.5 ml/min for 5-10 min. Then livers were re-perfused with 80 ml RPMI 1640 medium containing 34.4 mg/ml pronase E for 10 min at a flow rate of 10 ml/min. The medium was replaced by 100 ml RPMI 1640 medium containing 40 mg collagenase type CIS and 22.6 mg pronase E and livers were re-circulated for 30 min at 20 ml/min with gassing with air containing 5% CO_2. After that livers were dissected and tissues digested for 15-30 min in 120 ml RPMI 1640 medium containing 20mg collagenase type CIS, 5.3 mg/ml pronase E and 5 mg/ml DNase. During the incubation, the pH was carefully controlled to 7.4-7.6 by gassing with air containing 5% CO_2 and adding small amounts of 0.1 M NaOH. Subsequently, the cell suspension was filtered through sterile mesh 300 μm into 3 tubes and centrifuged (1800 rpm, 10 min at 4^0C) (*Sigma 4k15*). The pellets were re-suspended in 10 ml HANK's balanced salt solution with Ca^{2+}, then centrifuged (1800 rpm, 10 min at 4^0C) (*Sigma 4k15*) again. The cell pellets were re-suspended by HANK's balanced salt solution with Ca^{2+} in 10 ml maximum when 14 ml of Nycodenz® was added to this suspension, then divided to 2 tube's. 1ml of fresh HANK's

balanced salt solution with Ca^{2+} was dropped carefully on the tubes wall to form a clear layer above the suspension surface. The tubes were subjected to a density centrifugation at 3500 rpm for 20 min (*Sigma 4k15*). Afterwards, the upper 3 of 4 formed layers from each tube were collected and the volume was completed to be 10 ml by cold RPMI 1640 medium, mixed and injected into the JE-6B elutriation system & rotor (*Beckman Instruments*). The speed was gradually raised from 4.4 (19ml/min) to 6.7 (27ml/min). The outlets were collected in tubes, which contained pure sinusoidal endothelial cells. The outlets starting from 8 to 14 (32-51ml/min) contained pure Kupffer cells. The cells were collected separately and centrifuged at 1800 rpm for 10 min at 4 ^{0}C. The pellets finally were re-suspended in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum and penicillin (100 IU/ml) and streptomycin (100 µg/ml). The cells were counted by Bürker-Türk counter and adjusted to be 2x 10^6 cells/ml which were seeded in tissue culture plats. Experiments were carried out under culture conditions.

3.4.6 Isolation of hepatocytes

Hepatocytes were isolated by a collagenase perfusion method as already described by Petzinger *et al.,* 1989. Briefly, isolated blood-free rat livers prepared as above were placed in an experimental perfusion setup, installed in a

temperature controlled hood. Livers were perfused with 75 ml of Ca^{2+} free Krebs-Henseleit solution containing 60mg collagenase type NB 4 for 15 min in temperature-controlled hood at 37°C under 95% O_2 -5% CO_2 gassing. After that livers were carefully dissected and tissue digested for 5 min in the perfusate solutions by bubbling gas. The cell suspensions were filtered through double layers sterile gauze into 4 tubes. The volume was adjusted by Krebs-Henseleit solution Ca^{2+} and centrifuged at 300-400 rpm for 10 min (*BHG HERMLE Z2364*). The pellets were re-suspended by Tyrode buffer 5.5 mM glucose without albumin then washed 3 times. The cell suspensions were filtered through 4-6 layers gauze into 4 tubes. The cells regenerated through incubation these suspensions in shaker water bath (*Julabo SW1*) adjusted at 37^{o}C and provided by 95% O_2 -5% CO_2 gas for 30 min, then centrifuged again (*BHG HERMLE Z2364*). Finally, cells were suspended in Dulbeccos Modified Eagles medium (DMEM) containing 10% heat-inactivated fetal calf serum, penicillin (100 IU/ml), and streptomycin (100 µg/ml). The cells were counted under microscope by Bürker-Türk counter and adjusted to be 2x 10^6 cells/ml, then were seeded in collagen pre-coated tissue culture plates. Experiments were carried out under culture conditions.

3.4.7 Peritoneal rat macrophages preparation

Peritoneal macrophages were prepared as described previously by Renz *et al.,* 1988 with some modification. Rats received 5µg concavalin A in 1ml buffer i.p. 5 days before being killed by carbon dioxide gas. The abdominal skin was removed and sterile phosphate buffer 50-60 ml containing 50 IU heparin and 5% glucose was injected into the abdominal cavity. After 5 min fluid was aspirated (about 50ml) and transfered to sterile tubes. Tubes were centrifuged at 1200 rpm about 10-15 min (*BHG HERMLE Z2364*), the supernatant aspirated and cells were washed with phosphate buffer 3 times, then resuspended by Dulbeccos modified Eagles medium (DMEM) supplemented with 10% fetal calf serum, 100 IU/ml penicillin and 100 mg/ml streptomycin. Cell suspensions were seeded in culture plates and incubated at 37 °C with humidified 5% CO_2 atmosphere for 2 hr to allow macrophages to adhere on flask bottom. Culture medium with flooded cells was aspirated and adhered cells harvested by trypsin, then re-suspended by culture medium and 5 ml of $2x10^6$ cells/ml again seeded in culture plates and incubated at 37 °C with humidified 5% CO_2 atmosphere for 2 hr to allow macrophages to adhere on flask bottom again. Experiments were carried out under culture conditions.

3.4.8 Cell lines L929, HepG2, and J774A.1

Connective tissue derived cell line (L929), human hepatoma cell line (HepG2) and mouse monocyte macrophages (J774A.1) cell line were cultured under routine techniques. Briefly, the cells were taken out from liquid nitrogen and were thawed at 37 °C in water bath, then were suspended in Dulbeccos modified Eagles medium (DMEM) supplemented with 10% fetal calf serum, 100 IU/ml penicillin and 100 mg/ml streptomycin. Cells were seeded in tissue culture plates, and incubated at 37 °C with humidified 5% CO_2 atmosphere. The cells were monitored during the growth with replacement of culture medium each 72 hrs until the plates became overcrowded with cells. Then the cells were harvested by trypsin and the cells resuspended by culture medium and finally transferred to several culture flasks. When these became full of cells (about 10x 10^6 cells), then 5 ml of new medium were added and experiments were carried out under culture conditions.

3.4.9 Cell counting

1) 400 µl Tyrode buffer
2) 50 µl cell suspension
3) 50 µl trypan blue stain

From this mixture 10 µl were counted in a Bürker-Türk counter chamber. The mean of alive cells in four large counted squares was taken and multiplied by 0.1625 to get the number of cells/ml of original suspension. Then the cell number was adjusted to $2x10^6$ cells/ml into the particular cell medium.

3.4.10 Culture conditions

5 ml of each single cell suspension, each at 2 x 10^6 cells/ml, were incubated in media supplemented with 10% heat-inactivated fetal calf serum and penicillin/streptomycin (100IU & 100µg/ml respectively), at 37°C in a humidified atmosphere of 5% CO_2 air. Freshly prepared hepatocytes and sinusoidal endothelial cells were transferred to tissue culture plates pre-coated with collagen, while Kupffer cells, peritoneal macrophages, and cell lines L929, J774A.1, and HepG2 were seeded in uncoated tissue culture plates. Primary cells and cell lines were incubated without OTA or LPS during a pre-experimental period of 2 hrs. After that zero-culture medium samples were taken. The tested substances were added to Kupffer cells culture only after 30 min of zero time before 1µg/ml (2.5µmol/L) OTA or 0.1 µg/ml of LPS were added to culture media after 1 hr after zero sample.

For controls each compound was given alone and then tested for TNF-α in combination with ochratoxin A under similar experimental conditions. Basal TNF-α release was measured in culture media without treatments.

3.4.11 Sampling schedule

Perfusate samples were collected directly from perfused liver outlets according to the following time schedule at 0, 20, 30, 50, 70, and 90 min (end point). Culture samples were collected under sterile laminar flow at 0, 0.5, 1, 1.5, 2, 4 and 24 hrs (the end point). Samples were stored at -70 °C until analysis by a rat TNF-α ELISA test system according to the company's instruction (see TNF-α assay section). Samples for cytotoxicity studies were analyzed in the Institute of Clinical Chemistry and Pathochemistry, Justus-Liebig-University Gieβen, in kind of cooperation.

3.4.12 Cytotoxicity markers

According to Guillouzo, 1998, the vitality of the liver was determined by assaying leakages to perfusate of lactate dehydrogenase (LDH), glutamate dehydrogenase (GLDH), lactate and potassium ion (K^+). In the same way direct cytotoxicity of 1µg/ml OTA (2.5µmol/L) or 0.1µg/ml LPS were investigated

and compared with cytotoxicity exerted by 0.01% digitonin in the perfusion system. OTA and digitonin were added 20 min after zero time.

3.4.13 TNF-α assay

1- The number of 8-well strips needed for the assay were determined and inserted in the frame for current use (the extra strips and frame re-bagged and stored in refrigerator for another use).

2- 50 µl of *Incubation Buffer* was added to all wells. Wells reserved for chromogen blank were left empty.

3- 100 µl of *Standard Diluent Buffer* was added to zero wells. Wells reserved for chromogen blank were left empty.

4- 100 µl of *Standards* were added to the appropriate microtiter wells. (Standard was reconstituted according to the labeled vial, to be 1000 pg/ml, then two fold dilution was done to reach 15.6 pg/ml). 50 µl of *Standard Diluent Buffer* was added to each well followed by 50 µl of diluted samples. Samples were diluted to be within the range of determination, then the plate was taped gently on the side and thoroughly mixed.

5- 50 µl of biotinylated anti-TNF-α (*Biotin Conjugate*) solution was pipetted into each well except the chromogen blank, then the plate was taped gently on the side and thoroughly mixed.

6- The plate was covered with *plastic plate cover* (provided in kit) and incubated for 1 hour and 30 minutes at room temperature.

7- The solution was thoroughly aspirated from each well and discarded. Wells were washed 4 times with 250 µl, each time using *washing buffer* (25ml of the provided washing buffer was diluted in 1.25 L of deionized water).

8- 100 µl of *Streptavidin-HRP Working Solution* was added to each well except the chromogen blank. (*Streptavidin-HRP Working Solution* was prepared by mixing of 120 µl 100x concentrated solution with 12 ml of *Streptavidin-HRP Diluent*).

9- The plate was covered with *plastic plate cover* (provided in kit) and incubated for 45 minutes at room temperature.

10- The solution was thoroughly aspirated from each well and the liquid was discarded. Wells were washed 4 times with 250 µl each time using *washing buffer* (25 ml of the provided washing buffer was diluted in 1.25L of deionized water).

11- 100 µl of *Stabilized chromogen* was added to each well. The liquid in the wells turned to colorize blue.

12- The plate was covered with *plastic plate cover* (provided in kit) and incubated for 30 minutes at room temperature in dark place.

13- 100 µl of *Stop Solution* was added to each well, then the plate was taped gently on the side and thoroughly mixed. The solutions in each well were changed from blue to yellow.

14- The absorbance of each well was registered at 450 nm having blanked the plate reader against a chromogen blank composed of 100 µl each of *Stabilized Chromogen* and *Stop Solution*.

15- Absorbances of the standards were plotted against the standard concentrations to obtain the best fitted curve.

16- The concentrations of unknown samples were calculated from the standard curve plotted in step 15. The concentration was multiplied by a dilution factor used during samples preparation.

3.5 Statistical analysis

Data are presented as mean ± SEM for at least three separated trials for each experiment. An unpaired student's *t*-test was used for comparison of TNF-α release under the tested compounds with basal TNF-α release, while the

experiments of concurrently administered compounds with ochratoxin A were compared with ochratoxin A alone. For multiple group comparisons two way ANOVA used for analysis of variance followed by Bonferroni *t* test. A *P* value *<0.05, **< 0.01, ***< 0.001 compared with control values was considered statistically significant. Data were analyzed by Graphpad Prism software version 4.0 (San Diego, California, USA).

Chapter 4. Results

4.1 Induction of TNF-α from blood free perfused rat livers

The isolated perfused liver represents an *in vitro* model copying the *in vivo* liver situation. Major advantages are that the three-dimensional architecture and bile flow are preserved and that bile can be collected and analyzed separately. It is also the only *in vitro* model that allows consideration of issues related to hemodynamics (Conway *et al,* 1983). Therefore, in our study we used blood-free perfused rat livers to investigate the effects of OTA on cytokine release, in particular of TNF-α. Blood-free rat livers were perfused with 75 ml Krebs-Henseleit buffer containing 2% dextran 90 min and buffer samples were collected and tested for TNF-α at indicated time points. Figure 4 shows TNF-α concentrations in perfusates increasing gradually from 35 pg/ml at zero time to 265 pg/ml at 90 min. These basal TNF-α concentrations were detected in perfusate samples, which were obtained from untreated livers.

Figure 4 also shows experiments after adding OTA in different concentrations at the time point 20 min. The addition of 0.5 µmol OTA/l to the perfusion system almost doubled TNF-α concentrations and an almost 2-fold TNF-α ($P<0.001$) elevation was seen at 90 min to be about 500 pg TNF-α/ml. When OTA was added into the perfusion system at 2.5 µmol/l, TNF-α concentration

sharply increased 30 min after its application and reached 2600 pg/ml at 90 min. The increase was ten times of the basal level at this time end point. The differences in TNF-α concentrations are statistically significant at $P<0.001$ from 50 min until the end of perfusion (Fig. 4). Higher OTA concentration at 12.5 µmol OTA/l caused amplification of TNF-α concentration in the perfusate to be 3000 pg/ml at 90 min. Livers responded ten min after this dosage and elevation in TNF-α levels was significant at $P<0.001$ starting from 30 min to the end of perfusion (Fig. 4).

In order to have a positive control, the livers were perfused with 0.1µg/ml LPS, the known TNF-α inducer. Re-circulation of LPS in the perfusate caused significant ($P<0.001$) release of TNF-α from 50 min to 3017 pg/ml at 90 min (Fig. 4).

TNF-α released by 2.5 µmol OTA/l was significantly ($P<0.001$) higher than that released by 0.5 µmol OTA/l at all tested time points. However, it was less than that released by 12.5 µmol OTA/l in particular at 50 and 70 min ($P<0.001$). At 90 min TNF-α released by two doses (2.5 & 12.5 µmol OTA/l) showed similar perfusate levels.

TNF-α released by 0.1 µg/ml LPS was significantly ($P<0.01$) higher at 50 min and ($P<0.001$) at 70 min than that released by 2.5 µmol OTA/l. At 90 min no significant difference was observed between 0.1 µg/ml LPS and 2.5 µmol

OTA/l. OTA at 12.5 µmol/l had stronger effects than 0.1 µg/ml LPS at 30 min ($P<0.05$) and at 50 min ($P<0.01$), then became similar at the end of perfusion.

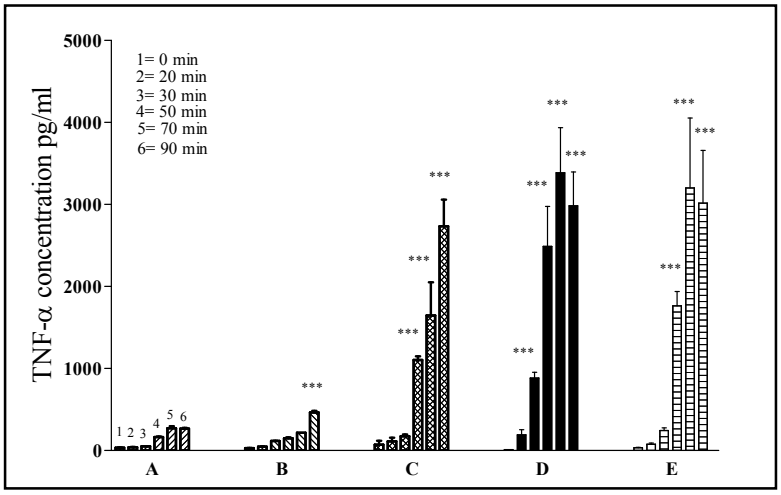

Fig. 4: Induction of TNF-α by OTA

TNF-α concentrations were measured in perfusate samples at 1) 0, 2) 20, 3) 30, 4) 50, 5) 70 and 6) 90 min. Samples obtained from blood-free rat livers perfused with: **A)** 2% dextran Krebs-Henseleit buffer medium alone (untreated); **B)** 0.5µmol/L OTA; **C)** 2.5 µmol/L OTA; **D)** 12.5 µmol/L OTA; **E)** 0.1µg/ml LPS. OTA and LPS were applied at 20 min into the perfusate. Values represent the mean ± SEM of 3 livers for each group (* $P<0.05$, ** $P<0.01$, *** $P<0.001$).

4.2 Markers of cytotoxicity

In order to exclude direct cytotoxicity of ochratoxin A as the cause of TNF-α release, lactate dehydrogenase (LDH) (Fig. 5), glutamate dehydrogenase (GLDH) (Fig. 6), lactate (Fig. 7) and potassium ions (Fig. 8) were measured at parallel times. Whereas TNF-α release was stimulated 10 fold during 90 min of

perfusion in the presence of 2.5µmol/L OTA, the cytotoxic markers in the perfusate changed only slightly but not significantly at the indicated time points (Fig. 5B, 6B, 7B, 8B).

A five-fold higher OTA concentration (12.5µmol/L OTA) didn't cause significant alteration in the potassium ion concentrations in perfusate (Fig. 8C), but caused slight release of GLDH (Fig. 6C), which became significant ($P<0.05$) at 90 min. At 30 and 50 min LDH increased significantly at $P<0.001$, but at 70 and 90 min the difference were significant at $P<0.01$ (Fig. 5C). Lactate increased starting from 30 to the end of perfusion. It was significant at 30, 50, 70, and 90 min at ($P<0.001$, $P<0.001$, $P<0.05$, $P<0.01$, respectively) (Fig. 7C).

The effects of 12.5µmol/L OTA compared with 2.5µmol/L OTA on potassium ion concentrations in perfusate were comparable (Fig. 8B, 8C). The GLDH concentration in perfusate after adding of 12.5µmol/L OTA was higher at 90 min than that released by 2.5µmol/L OTA, but statistically not significant (Fig. 6B, 6C). 12.5µmol/L OTA released significant amounts of LDH (Fig. 5B, 5C), and lactate (Fig. 7B, 7C) comparing with 2.5µmol/L OTA induced level ($P<0.001$). These results indicate that 2.5µmol OTA /L in contrast to 12.5µmol OTA /L didn't alter the liver vitality criteria LDH and lactate release.

Under the same conditions, these markers were measured when 0.1 µg/ml LPS was present in perfusate. We found significant release of LDH at 70 and 90 min

(P<0.05 and P<0.001, respectively) (Fig. 5D), and even more obvious of 0.1 µg/ml LPS effects were seen on GLDH levels, which increased from 30 min (P<0.05) to reach the highest level at 50 min (P<0.001) (Fig. 6D). These levels returned back at 90 min to be still higher than control group (untreated livers), but this was statistically not significant (Fig. 6A, 6D). The potassium ion (Fig. 8D) and lactate (Fig. 7D) concentrations were still comparable at indicated time points with control group (untreated livers).

GLDH released into perfusate at 50 min in presence of 0.1 µg/ml LPS was more than twic of that released by OTA at 2.5µmol/L OTA or 12.5µmol/L OTA. LDH released by 0.1 µg/ml LPS at 70 and 90 min were higher than that released by 2.5µmol/L OTA but equaled 12.5µmol/L OTA. With respect to lactate release LPS had similar effects to 2.5 µmol/L OTA but was less toxic than 12.5 µmol/L OTA.

The direct cell destruction was measured by 0.01% digitonin in perfusate. 0.01% digitonin in comparison to the control liver group caused significant (P<0.001) increase in LDH beginning from 30 min until the end of perfusion (Fig. 5E). Lactate released by 0.01% digitonin was significantly (P<0.01) higher than untreated livers at 30 and 50 min, then the difference became significant (P<0.05) at 70 and 90 min (Fig. 7E). While potassium ion was still unaltered (Fig. 8E), the GLDH release was significant at 50 min (P<0.05), and even more

significant (P<0.001) at 70 and 90 min (Fig. 6E). At the end point of perfusion 2.5 µmol/L OTA release 35% LDH, 50% GLDH and 45% Lactate of 0.01% digitonin end point. 12.5µmol/L OTA release similar level of LDH and lactate compared to 0.01% digitonin, but the GLDH release by 12.5µmol/L OTA was 60% of 0.01% digitonin at the end point.

The liver cells vitality was less effected by 0.1 µg/ml LPS compared to 0.01% digitonin. This means, lactate, GLDH, LDH, and potassium ion released by 0.1 µg/ml LPS were significantly less than that released by 0.01% digitonin.

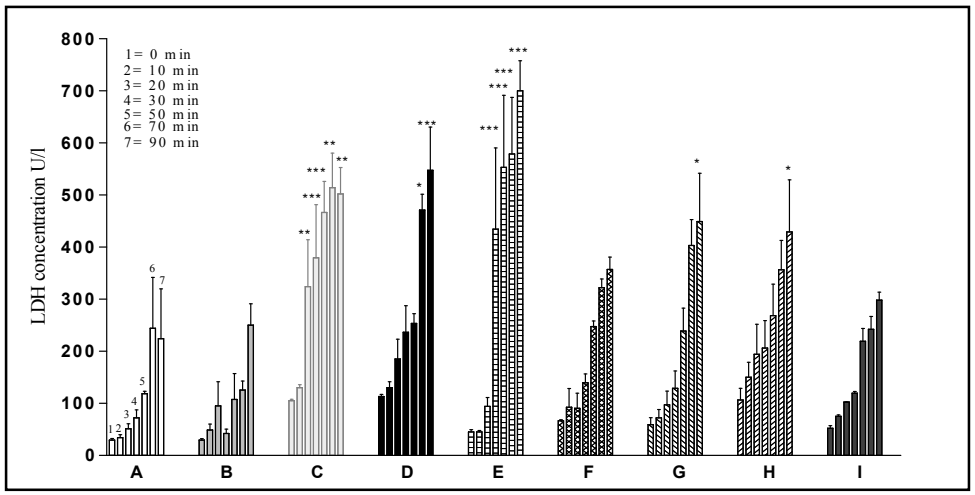

Fig. 5: LDH released into perfusate

LDH concentrations were measured in perfusate samples at 1) 0, 2) 10, 3) 20, 4) 30, 5) 50, 6) 70 and 7) 90 min. Samples were obtained from blood-free rat livers perfused with: **A)** 2% dextran Krebs-Henseleit buffer medium alone (untreated); **B)** 2.5µmol/L OTA; **C)** 12.5µmol/L OTA; **D)** 0.1 µg/ml LPS; **E)** 0.01% Digitonin; **F)** 15µmol/L gadolinium chloride alone; **G)** 15µmol/L gadolinium chloride followed by OTA; **H)** 2.5µmol/L OTA (livers pretreated 48 hrs with 2ml LIP-PBS); **I)** 2.5µmol/L OTA (livers pretreated 48 hrs with 2ml LIP-CLOD). The tested compounds were applied at 10 min after zero time then followed by 2.5µmol/L OTA or LPS at 20 min (see Materials and Methods). Values represent the mean ± SEM of 3 livers for each group, (* P<0.05, ** P<0.01, *** P<0.001).

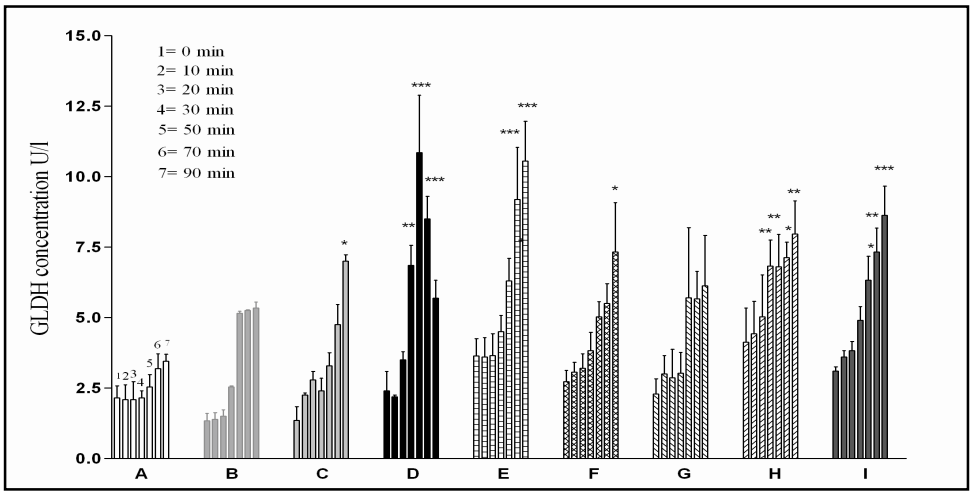

Fig. 6: GLDH released into perfusate

GLDH concentrations were measured in perfusate samples at 1) 0, 2) 10, 3) 20, 4) 30, 5) 50, 6) 70 and 7) 90 min. Samples were obtained from blood-free rat livers perfused with: **A)** 2% dextran Krebs-Henseleit buffer medium alone (untreated); **B)** 2.5µmol/L OTA; **C)** 12.5µmol/L OTA; **D)** 0.1 µg/ml LPS; **E)** 0.01% Digitonin; **F)** 15µmol/L gadolinium chloride alone; **G)** 15µmol/L gadolinium chloride followed by OTA; **H)** 2.5µmol/L OTA (livers pretreated 48 hrs with 2ml LIP-PBS); **I)** 2.5µmol/L OTA (livers pretreated 48 hrs with 2ml LIP-CLOD). The tested compounds were applied at 10 min after zero time then followed by 2.5µmol/L OTA or LPS at 20 min (see Materials and Methods). Values represent the mean ± SEM of 3 livers for each group, (* $P<0.05$, ** $P<0.01$, *** $P<0.001$).

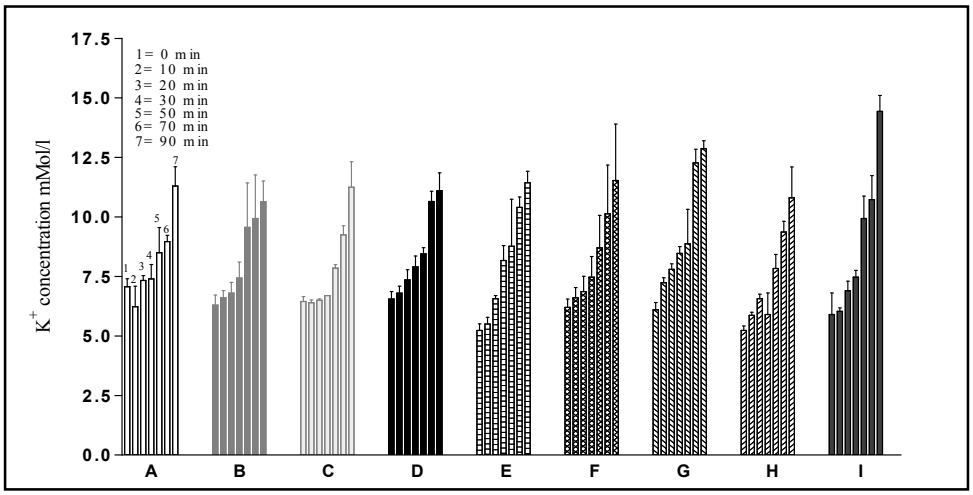

Fig. 7: Lactate released into perfusate

Lactate concentrations were measured in perfusate samples at 1) 0, 2) 20, 3) 30, 4) 50, 5) 70 and 6) 90 min. Samples were obtained from blood-free rat livers perfused with: **A)** 2%

dextran Krebs-Henseleit buffer medium alone (untreated); **B)** 2.5µmol/L OTA; **C)** 12.5µmol/L OTA; **D)** 0.1 µg/ml LPS; **E)** 0.01% Digitonin; The tested compounds were applied at 10 min after zero time then followed by 2.5µmol/L OTA or LPS at 20 min (see Materials and Methods). Values represent the mean ± SEM of 3 livers for each group, (* P<0.05, ** P<0.01, *** P<0.001).

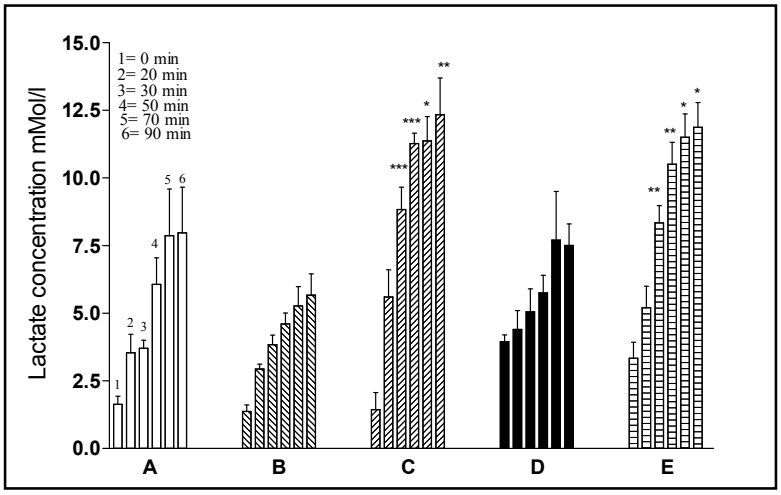

Fig. 8: Potassium ion released into perfusate

K^+ concentrations were measured in perfusate samples at 1) 0, 2) 10, 3) 20, 4) 30, 5) 50, 6) 70 and 7) 90 min. Samples were obtained from blood-free rat livers perfused with: **A)** 2% dextran Krebs-Henseleit buffer medium alone (untreated); **B)** 2.5µmol/L OTA; **C)** 12.5µmol/L OTA; **D)** 0.1 µg/ml LPS; **E)** 0.01% Digitonin; **F)** 15µmol/L gadolinium chloride alone; **G)** 15µmol/L gadolinium chloride followed by OTA; **H)** 2.5µmol/L OTA (livers pretreated 48 hrs with 2ml LIP-PBS); **I)** 2.5µmol/L OTA (livers pretreated 48 hrs with 2ml LIP-CLOD). The tested compounds were applied at 10 min after zero time then followed by 2.5µmol/L OTA or LPS at 20 min (see Materials and Methods). Values represent the mean ± SEM of 3 livers for each group, (* P<0.05, ** P<0.01, *** P<0.001).

According to these findings OTA at 2.5µmol/L and LPS at 0.1µg/ml caused significant release of TNF-α with minor changes of liver cell vitality. Therefore, these doses were selected in the further experiments.

4.3 The role of Kupffer cells in OTA-mediated TNF-α release

In the perfused liver different cell types may serve as a source of TNF-α. Most likely are Kupffer cells, since TNF-α is typically released by macrophages and TNF-α release by OTA was reported from a mouse macrophage cell line (Huttunen *et al.,* 2004). Therefore, the aim of this part of the study was to elucidate which liver cell type respond to OTA by releasing TNF-α into the perfusate of blood-free perfused rat livers. For this reason, Kupffer cells were blocked *in vitro* by addition of the heavy metal gadolinium chloride to the perfusate, and *in vivo* by the Kupffer-cell poisoning toxin clodronate. Isolated livers taken from these animals were taken as livers depleted in this responding cell type. In addition Kupffer cells, sinusoidal endothelial cells, and hepatocytes were isolated from rat livers and were exposed in single cell culture to 2.5 µmol/ml OTA for 24 hours. The positive control LPS at 0.1 µg/ml was used in this cell culture model in addition.

4.3.1 Blockade of Kupffer cells by gadolinium chloride ($GdCl_3$)

The rare earth metal salt gadolinium chloride ($GdCl_3$) was reported to depress reticuluendothelial (RES) activity (Lazar, 1973), and selectively depresses phagocytic activity (Brown *et al.,* 1997, Yang *et al.,* 1999). It abolished the hepatic expression of certain Kupffer cell specific antigens (Klein *et al.,* 1994;

Kim & Choi, 1997), without effecting the number of Kupffer cells (Rai *et al.,* 1996).

For this study we blocked Kupffer cells *in vitro* by adding 15µmol/l gadolinium chloride into the perfusion system at 10 min after the starting point. $GdCl_3$ alone or when it was applied 10 min prior to 2.5µmol/L OTA didn't cause significant changes in LDH (Fig. 5G), GLDH (Fig. 6G) and potassium ion (Fig. 8G) concentrations in perfusate compared to the control group (untreated livers). These findings indicate the tested dose of $GdCl_3$ was not toxic for liver cells. Our findings correlated with Lee *et al.,* 2004, who found that gadolinium chloride didn't alter the Kupffer cells vitality *in vitro* up to 27 µmol/l (Lee *et al.,* 2004).

On control livers 15 µmol/l $GdCl_3$ caused slight but not significant reduction in TNF-α basal levels, i.e. TNF-α was 176 pg/ml in comparison to 265 pg/ml in untreated livers at 90 min (Fig. 9). However, when 15 µmol/l $GdCl_3$ was co-applied 10 min prior to 2.5 µmol/l of OTA to the perfusion system, it completely abolished OTA-mediated TNF-α release over 90 min (Fig. 9). TNF-α levels were significantly ($P<0.001$) lower than OTA induced levels from 50 min to the end of perfusion. These results correlate with findings that $GdCl_3$ reduced TNF-α concentration in the livers (Lazar *et al.,* 1995), and in plasma (Yee *et al.,* 2003) of LPS challenged mice.

4.3.2 Depletion of Kupffer cells by liposomes-encapsulated clodronate

Another physical method to eliminate Kupffer cells is by using dichlorometheline biphosphonate. The compound was particularly effective against macrophages when it was encapsulated into liposomes, enabling its cell selective uptake (Van Rooijen & Sanders; 1994, Bautista *et al.,* 1994). The compound selectively induced cell death in macrophages of the liver and the spleen (Meijer *et al.,* 2000; Alves-Rosa *et al.,* 2003).

Accordingly, we depleted Kupffer cells by *in vivo* i.p. administration of 2ml/rat liposome-encapsulated clodronate (LIP-CLOD) 48 hrs prior to liver preparations. Livers pre-treated with LIP-CLOD and perfused with 2.5 μmol/l OTA didn't release significant effects on LDH (Fig. 5I), or potassium ion (Fig. 8I), comparing with OTA induced levels. The main effect of LIP-CLOD was seen on GLDH levels, which increased significantly at 50, 70 and 90 min ($P<0.05$, $P<0.001$ and $P<0.001$, respectively) (Fig. 6I). These results indicate LIP-CLOD added harmful effects to livers perfused with 2.5 μmol/l OTA. For control, clodronate vehicle (liposomes suspended in phosphate buffer) was used in the same fashion. LIP-PBS had no effects on potassium ion concentrations in comparison with control (untreated livers) (Fig. 8H) or with OTA released potassium levels, but increased slightly LDH concentration at 90 min ($P<0.05$) (Fig. 5H). The significant changes took place in GLDH from 30 to 90 min

(P<0.01) compared to untreated livers (Fig. 6H), without interference with 2.5 µmol/l OTA released levels.

The pre-treatment of rats by LIP-CLOD abrogated any OTA-mediated TNF-α release into the perfusate. TNF-α levels were significantly lower than OTA induced levels at P<0.001 from 50 min to the end of perfusion. It was 100 pg/ml, which was lower than the basal TNF-α level released by untreated livers (Fig. 9). For control, the clodronate vehicle (LIP-PBS) was used in the same experimental fashion. LIP-PBS showed no influence on 2.5 µmol/l OTA induced TNF-α levels (Fig. 9).

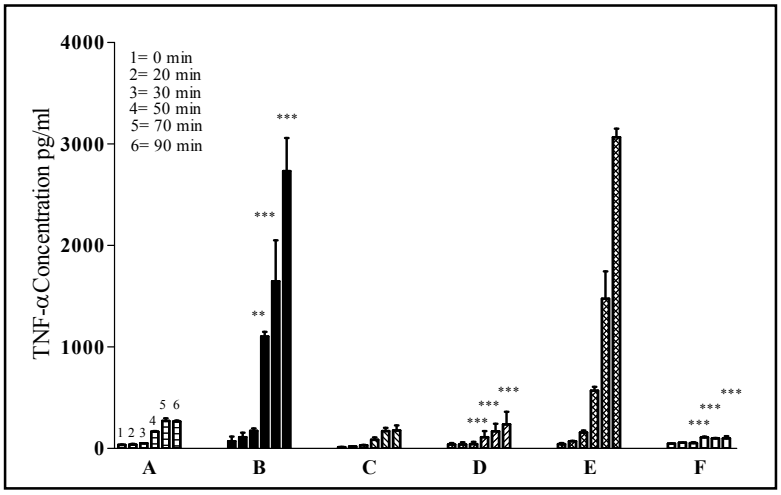

Fig. 9: OTA-mediated release of TNF-α from blood-free perfused rat livers under Kupffer cell blockage

TNF-α concentrations were measured in perfusate samples at 1) 0, 2) 20, 3) 30, 4) 50, 5) 70 and 6) 90 min. Samples obtained from blood-free rat livers perfused with: **A)** 2% dextran Krebs-Henseleit buffer medium alone (untreated); **B)** 2.5µmol/L of OTA; **C)** 15µmol/L gadolinium chloride alone; **D)** 15 µmol/L gadolinium chloride followed by 2.5 µmol/L OTA; **E)** 2.5 µmol/L OTA (livers pretreated by in vivo i.p injection of liposome encapsulated phosphate buffer); **F)** 2.5 µmol/L OTA (livers pretreated with in vivo i.p injection of liposome encapsulated clodronate) (see Materials & Methods section).

Values represent the mean ± SEM of 3 livers for each group, (* P<0.05, ** P<0.01, *** P<0.001).

4.3.3 OTA-mediated TNF-α release from isolated Kupffer cells

In order to analyze in more details the cellular target of OTA in the perfused rat liver model, the direct contact of OTA with Kupffer cells in cell culture was carried out. The change in TNF-α level was monitored in OTA-free Kupffer cell culture, which slightly increased from 50 pg/ml at zero time to 110 pg/ml after 24 hrs (Fig. 10). A significant amount of TNF-α was released into incubation medium of isolated Kupffer cells when 2.5µmol/l OTA was added 1hr after starting the experiments up to 24 hrs. OTA caused slight release of TNF-α after 4 hrs, but this release was statistically significant (P<0.001) at the end of incubation. Here, TNF-α reached 1000 pg/ml in the incubation medium. This increase was 10 times the basal TNF-α release from control culture (Fig. 10 & Table 2).

For a positive control, Kupffer cells culture was exposed to 0.1 µg of LPS/ml. LPS caused significant (P<0.001) release of TNF-α into the incubation medium 1 hr after its addition. It was 3000 pg/ml after 24hrs (Fig. 10 & Table 2). In comparison OTA required more than 3 hrs to release TNF-α from Kupffer cells, while LPS needed about 1 hr to yield a comparable effect. The LPS effects were stronger than the OTA effects, i.e. TNF-α released by LPS was significantly

(P<0.001) higher than that released by OTA from the time point 2 hrs up to the end of incubation at 24 hrs.

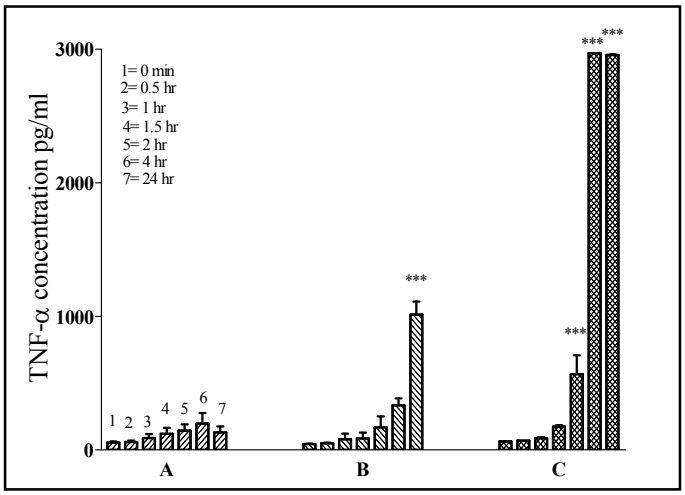

Fig. 10: TNF-α release from Kupffer cell culture
TNF-α concentrations were measured in incubated media of isolated cell in single cell culture at 1) 0, 2) 0.5, 3) 1, 4) 1.5, 5) 2, 6) 4 and 7) 24 hrs indicated by single columns. **A)** Kupffer cells without treatment; **B)** Kupffer cells exposed to 2.5 µmol/L OTA. **C)** Kupffer cells exposed to 0.1 µg/ml LPS. OTA and LPS were added 1hr after zero samples, (see Materials & Methods section). Values represent the mean ± SEM of 3 cell preparations for each group, (* P<0.05, ** P<0.01, *** P<0.001).

4.3.4 TNF-α release from isolated sinusoidal endothelial cells

To study the role of sinusoidal endothelial cells in OTA-mediated TNF-α release from blood-free perfused rat livers, isolated sinusoidal endothelial cells were freshly prepared from rat livers and subjected in single cell cultures to OTA exposure.

In the incubation medium TNF-α basal level was increased from 45 pg/ml at zero time to 220 pg/ml after 24 hrs (Fig. 11). When the culture was exposed to

2.5 µmol/l OTA, the TNF-α level was increased to the same extent from 49 pg/ml to 230 pg/ml up to 24 hrs (Fig. 11). This means that sinusoidal endothelial cells didn't respond to OTA. However, after adding 0.1 µg of LPS/ml to the incubation medium at 1 hr a significant (P<0.001) release of TNF-α to the incubation medium starting from 4 hrs to be 2000 pg/ml after 24 hrs was seen (Fig. 11 & Table 2). These data show that whereas sinusoidal endothelial cells respond to LPS they were insensitive to OTA.

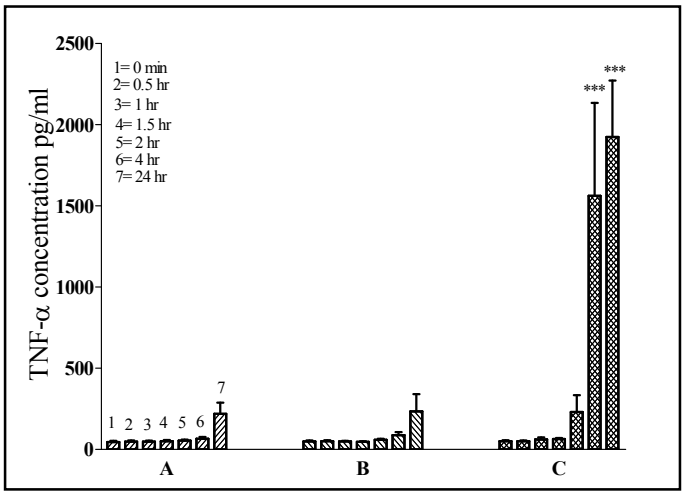

Fig. 11: TNF-α release from sinusoidal endothelial cell culture
TNF-α concentrations were measured in incubated media of isolated cell in single cell culture at 1) 0, 2) 0.5, 3) 1, 4) 1.5, 5) 2, 6) 4 and 7) 24 hrs indicated by single columns. **A)** Sinusoidal endothelial cells without treatment; **B)** Sinusoidal endothelial cells exposed to 2.5 µmol/L OTA. **C)** Sinusoidal endothelial cells exposed to 0.1 µg/ml LPS. OTA and LPS were added 1hr after zero samples, (see Materials & Methods section). Values represent the mean ± SEM of 3 cell preparations for each group, (* P<0.05, ** P<0.01, *** P<0.001).

4.3.5 TNF-α release from isolated hepatocytes

To elicit the possibility of hepatocyte participation in OTA-mediated TNF-α release from blood-free perfused rat liver, primary rat hepatocytes were prepared in single cell cultures. The basal levels of TNF-α release from the hepatocyte cell culture were higher than those released from sinusoidal endothelial cells and Kupffer cells. This level decreased with time from 295pg/ml to 47 pg/ml at 24 hrs (Fig. 12). The presence of 2.5µmol/l OTA at 1 hr didn't cause significant changes in TNF-α levels at indicated time points (Fig. 12). This means that hepatocytes didn't participate in OTA-mediated TNF-α release from blood-free perfused rat livers.

On the other hand, a positive result was obtained if 0.1 µg/ml LPS was added to the culture medium under similar experimental conditions. This caused a slight release of TNF-α, which was significant at $p<0.5$ at 1.5 and 2 hrs, then became significant at $P<0.01$ at 4 hrs, which later at the end of the incubation period became significant at $P<0.001$ in comparison with treated or with untreated cultures (Fig. 12 & Table 2).

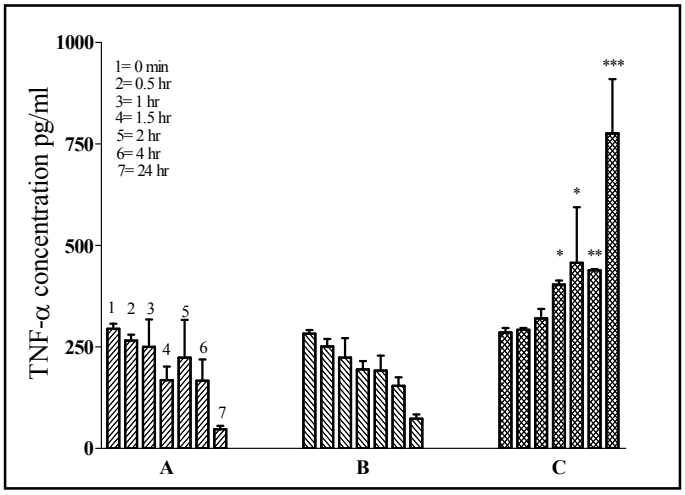

Fig. 12: TNF-α release from hepatocytes cell culture
TNF-α concentrations were measured in incubated media of isolated cell in single cell culture at 1) 0, 2) 0.5, 3) 1, 4) 1.5, 5) 2, 6) 4 and 7) 24 hrs indicated by single columns. **A)** Hepatocyte cells without treatment; **B)** Hepatocyte cells exposed to 2.5 µmol/L OTA. **C)** Hepatocyte cells exposed to 0.1 µg/ml LPS. OTA and LPS were added 1hr after zero samples, (see Materials & Methods section). Values represent the mean ± SEM of 3 cell preparations for each group, (* $P<0.05$, ** $P<0.01$, *** $P<0.001$).

4.4 TNF-α release from macrophages

The aim of this part of the study was to extent and generalize the OTA effect on the liver cells to various other cell types. For this reason, primary rat peritoneal macrophages and the mouse macrophages cell line J774A.1 were used and also the HepG2 and L929 cell line. All cell types were adjusted to $2x10^6$ cells/ml and exposed to 2.5µmol/l OTA 1hr after zero time up to 24 hrs. Furthermore, under similar experimental conditions these cells were incubated with 0.1 µg/ml LPS, the known inducer of TNF-α, as the positive control.

4.4.1 OTA-mediated TNF-α release from isolated peritoneal macrophages

Macrophages were collected from rats by peritoneal lavage and brought in culture. TNF-α levels increased in the incubation medium of control cells to be 160 pg/ml after 24 hrs (Fig. 13). 2.5μmol/l OTA stimulated these cells to release significant amounts of TNF-α during 24 hrs of exposure time. About 1560 pg TNF-α/ml of culture medium was detected at the end of incubation. The response of these cells started after 1 hr of OTA contact. The elevation had statistical meaning ($P<0.01$) at 4 hrs, then became significant ($P<0.001$) at the end of incubation, when it was 10 times the basal TNF-α release from control culture (Fig. 13 & Table 2).

Similar stimulation took place in response to 0.1 μg of LPS/ml after 1 hr of LPS addition. LPS caused significant ($P<0.001$) release of TNF-α to the culture medium starting from 4 hrs up to the end of incubation. TNF-α concentrations in the incubated medium reached 2600 pg/ml after 24 hrs of LPS exposure (Fig. 13 & Table 2). TNF-α release by OTA was significantly lower than by LPS indicating that LPS is more powerful than OTA for TNF-α release.

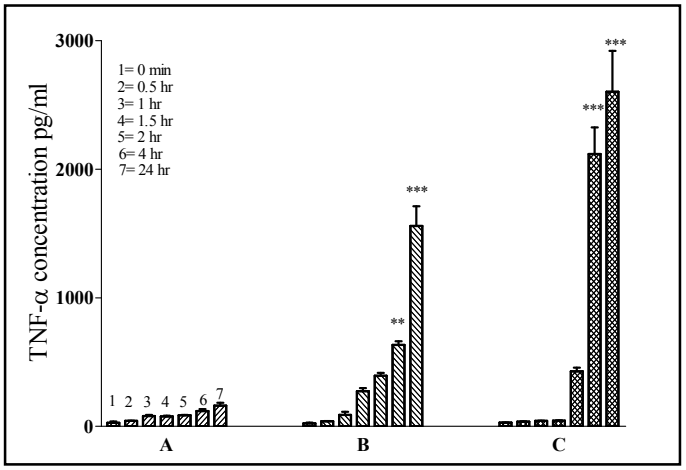

Fig. 13: TNF-α release from rat peritoneal macrophages cell culture
TNF-α concentrations were measured in incubated media of isolated cell in single cell culture at 1) 0, 2) 0.5, 3) 1, 4) 1.5, 5) 2, 6) 4 and 7) 24 hrs indicated by single columns. **A)** Peritoneal macrophages without treatment; **B)** Peritoneal macrophages exposed to 2.5 µmol/L OTA. **C)** Peritoneal macrophages exposed to 0.1 µg/ml LPS. OTA and LPS were added 1hr after zero samples, (see Materials & Methods section). Values represent the mean ± SEM of 3 cell preparations for each group, (* $P<0.05$, ** $P<0.01$, *** $P<0.001$).

4.4.2 OTA-mediated TNF-α release from J774A.1 cells

The mouse macrophages cell line J774.A1 was used to confirm the effects of OTA on rat peritoneal macrophages. We observed an increase in TNF-α levels in the culture medium of J774.A1 cells from 32 pg/ml at zero time to 111 pg/ml after 24 hrs in the absence of OTA (Fig. 14). In it's presence (2.5µmol/l OTA) release of TNF-α was 635 pg/ml after 24 hrs (Fig. 14 & Table 2). This increase was significant ($P<0.05$) at 4 hrs, and even more ($P<0.001$) at 24 hrs. At the end point this increase was 6 times the basal TNF-α release of the control culture. Similarly the cells exposed to 0.1 µg of LPS/ml released significant ($P<0.05$) amounts of TNF-α into the incubation medium at 2 hrs. From then TNF-α

concentrations were significant at P<0.001 from 4 hrs to the end of incubation, when it reached about 2115 pg/ml (Fig. 14 & Table 2).

The amount of TNF-α released by OTA was significantly lower than by LPS at P<0.001 from the time point of 4 hrs up to the end of incubation. Our data suggest that the effect of LPS on J774A.1 is faster and greater than that promoted by OTA.

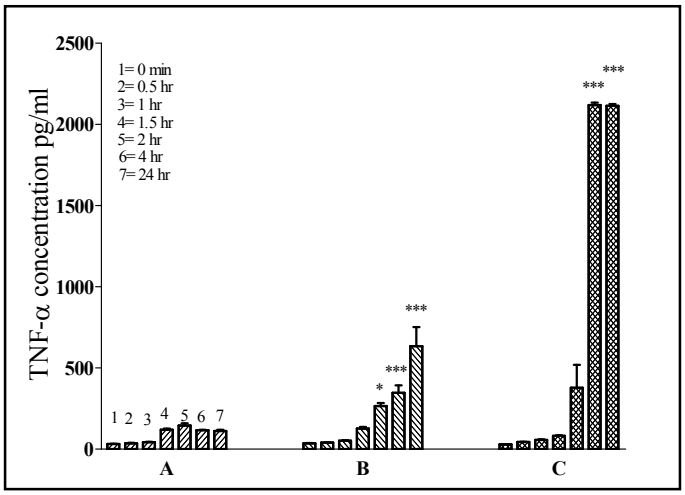

Fig. 14: TNF-α release from J774A.1 cell line
TNF-α concentrations were measured in incubated media of isolated cell in single cell culture at 1) 0, 2) 0.5, 3) 1, 4) 1.5, 5) 2, 6) 4 and 7) 24 hrs indicated by single columns. **A)** J774A.1 cell line without treatment; **B)** J774A.1 cell line exposed to 2.5 µmol/L OTA. **C)** J774A.1 cell line exposed to 0.1 µg/ml LPS. OTA and LPS were added 1hr after zero samples, (see Materials & Methods section). Values represent the mean ± SEM of 3 cell preparations for each group, (* P<0.05, ** P<0.01, *** P<0.001).

4.4.3 TNF-α release from $HepG_2$ cells

The human HepG2 cell is a suitable model for *in vitro* studies of hepatocyte cytotoxicity in particular the ethanol-induced hepatocyte toxicity and/or apoptosis (Cameron *et al.,* 1998; Nakayama *et al.,* 2001). Human HepG2

responded to ethanol at 80mmol/l and released TNF-α, which subsequently mediated apoptotic processes (Neuman *et al.,* 1998). Ethanol at 50 mmol/l stimulated expression of TNFR1 in HepG2 which promoted the effects of TNF-α (Rodriguez *et al.,* 2004).

In this study TNF-α basal levels increased in the culture medium from 30 pg/ml to only 36 pg/ml after 24 hrs. Neither 2.5µmol/l OTA nor 0.1 µg/ml LPS changed TNF-α concentrations in comparison with control (untreated cells) at any indicated time points (Fig. 15 & Table 2).

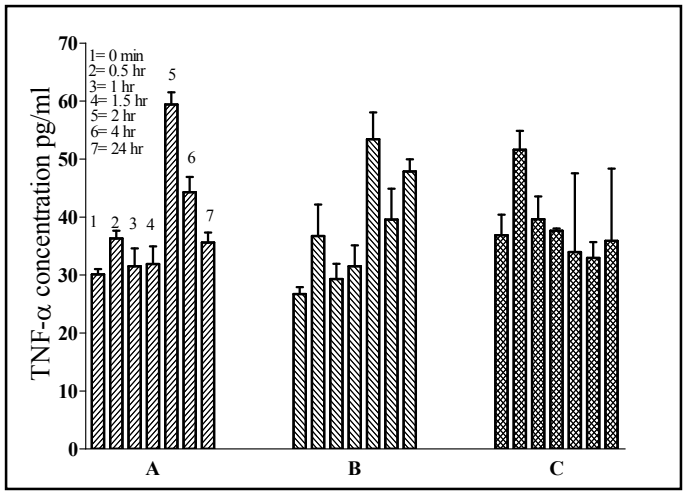

Fig. 15: TNF-α release from HepG2 cell line
TNF-α concentrations were measured in incubated media of isolated cell in single cell culture at 1) 0, 2) 0.5, 3) 1, 4) 1.5, 5) 2, 6) 4 and 7) 24 hrs indicated by single columns. **A)** HepG2 cell line without treatment; **B)** HepG2 cell line exposed to 2.5 µmol/L OTA. **C)** HepG2 cell line exposed to 0.1 µg/ml LPS. OTA and LPS were added 1hr after zero samples, (see Materials & Methods section). Values represent the mean ± SEM of 3 cell preparations for each group, (* $P<0.05$, ** $P<0.01$, *** $P<0.001$).

4.4.4 TNF-α release from L929 cells

The L929 cell line is a sensitive cell line for TNF-α apoptotic action (Hayakawa *et al.,* 1991; 1993). The cells may be used as a marker to measure TNF-α mediated cytotoxic effects of TNF-α inducers. In our hands, however the cells showed no response to OTA. Again the TNF-α basal level was measured in the incubation medium during culture in the absence of OTA. This level increased from 30 pg/ml to 36 pg/ml after 24 hrs (Fig. 16). 2.5µmol/l OTA added to the incubation medium at 1 hr didn't change TNF-α concentrations in incubation medium at indicated time points compared with control group (Fig. 16).

0.1 µg/ml LPS, however, caused a slight release at 24 hrs, when TNF-α concentrations were 91 pg/ml. This was significant in comparison to the control group or to the OTA treated group at $P<0.001$ (Fig. 16 & Table 2). Apparently L929 cells responded although slightly only to LPS.

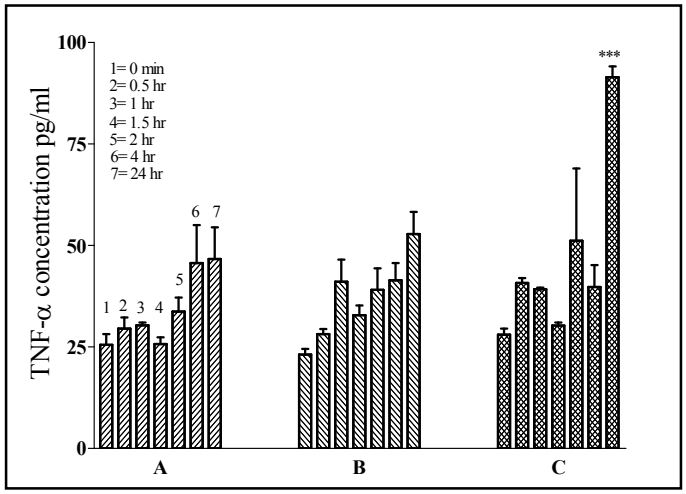

Fig. 16: TNF-α release from L929 cell line

TNF-α concentrations were measured in incubated media of isolated cell in single cell culture at 1) 0, 2) 0.5, 3) 1, 4) 1.5, 5) 2, 6) 4 and 7) 24 hrs indicated by single columns. **A)** L929 cell line without treatment; **B)** L929 cell line exposed to 2.5 µmol/L OTA. **C)** L929 cell line exposed to 0.1 µg/ml LPS. OTA and LPS were added 1hr after zero samples, (see Materials & Methods section). Values represent the mean ± SEM of 3 cell preparations for each group, (* P<0.05, ** P<0.01, *** P<0.001).

Cell type	TNF-α concentrations in incubated medium at 24 hrs		
	Without treatment	2.5 µmol/l OTA	0.1 µg/ml LPS
Kupffer cells	131	1000***	3000***
Peritoneal macrophages	161	1560***	2600***
J774A.1 cell line	111	635***	2115***
Hepatocytes	47	73	776 ***
HepG2 cell line	36	48	36
Sinusoidal endothelial cells	219	235	1925***
L929 cell line	47	53	92***

Table 2: TNF-α concentrations in incubated medium of different cells at 24 hrs in the absence or presence of 2.5 µmol/l OTA or 0.1 µg/ml. OTA and LPS were applied at 1 hr from zero time (see Materials and Methods). Values represent the mean ± SEM of 3 cultures for each group, (* P<0.05, ** P<0.01, *** P<0.001).

4.5 The role of arachidonic acid and its metabolites on OTA-mediated TNF-α release from rat livers

4.5.1 Effect of PLA_2 inhibitor aristolochic acid

Most inflammatory diseases associated with increased $sPLA_2$ levels are characterized by overproduction of cytokines such as TNF-α, IL-1, and IL-6 (Remick, 1997). Two $sPLA_2$ isoforms, human group IB (hGIB) and human

group X (hGX) $sPLA_2$ were reported to induce TNF-α and IL-6 from human lung macrophages (Granata *et al.,* 2005).

In order to inhibit enzymatic activity of phospholipase A_2 in liver tissue, 50µmol/L of aristolochic acid, a potent PLA_2 enzyme inhibitor (Rosenthal *et al.,* 1992) was applied 10 min prior to 2.5µmol/l OTA in the perfusion system. Under these conditions aristolochic acid markedly reduced TNF-α concentrations beginning from the time point 50 min to be 188 pg/ml at 90 min (Fig. 17). This is below basal secretions in untreated controls. This reduction was significant at $P<0.001$ compared to OTA induced levels but not for basal levels. Aristolochic acid alone also reduced the basal TNF-α concentration to 139 pg/ml at 90 min but this reduction was not significant versus controls (Fig. 18 & Table 3). Our results indicate that the induction of TNF-α by 2.5µmol/l OTA from blood-free perfused rats livers required phospholipase A_2 or, subsequently, one of its metabolic products, in particular arachidonic acid derivatives.

4.5.2 Inhibition of pathways of arachidonic acid metabolism

In order to clarify which metabolic pathway of arachidonic acid could interfere with TNF-α release by 2.5µmol/l OTA, we blocked each individual pathway. Then, concurrent inhibitions were carried out. However, blocking the

cyclooxygenases pathways by 10µmol/L of indomethacin slightly elevated TNF-α concentrations compared with untreated controls, i.e. 467 pg/ml versus 256 pg/ml at 90 min. This elevation was not significant in comparison with basal release and/or with OTA induced levels (Fig. 17). Indomethacin, applied 10 min prior to OTA, however, provoked additional TNF-α release in the perfusate starting to be significant ($P<0.001$) at 70 min, then ending at two-fold higher concentrations over the OTA induced level at 90 min. I.e. TNF-α concentration was elevated by indomethcin from 2600 pg/ml (OTA alone) to 5500 pg/ml at the end of perfusion (Fig. 18 & Table 3).

In order to block lipoxygenases pathways, nordihydroguaiaretic acid (NDGA) was used at 30µmol/L to inhibit the production of leukotrienes. In the presence of (NDGA) alone the TNF-α level increased to be 330 pg/ml at 90 min (Fig. 17), which was not significant compared with TNF-α basal release. Administration of nordihydroguaiaretic acid 10 min before OTA diminished the TNF-α level in the perfusate at 90 min to be 6% of OTA-induced level, i.e. TNF-α concentration was 175 pg/ml versus 2600 pg/ml at 90 min (Fig. 18 & Table 3). Our findings suggest that the induction of TNF-α by OTA from blood-free rat livers requires some of the lipoxygenases metabolites.

Finally, inhibition of the third major pathway of arachidonic acid metabolites, the CYP-450 pathway by 100µmol/L metyrapone, was performed. In the

presence of metyrapone alone no significant interference with TNF-α basal release occurred. However, TNF-α concentrations in perfusate were significantly ($P<0.001$) lower than the levels released by OTA in particular at 50, 70 and 90 min, namely 233 pg/ml at 90 min (Fig. 17). Administration of metyrapone 10 min before OTA reduced TNF-α level in the perfusate to 7% of the OTA induced level at 90 min, i.e. TNF-α concentration was 200 pg/ml versus 2600 pg/ml at 90 min (Fig. 18 & Table 3). Our finding indicate that the induction of TNF-α by OTA from blood-free rat livers requires some of the CYP-450 metabolites.

Blocking of cyclooxygenases by indomethacin in combination with blockage of lipoxygenases by NDGA caused significant ($P<0.001$) reduction in TNF-α levels beginning from 50 min to 90 min. At this end point only 5% of OTA induced levels at 90 min were reached, i.e. TNF-α concentration at the end of perfusion was 135 pg/ml (Fig. 18 & Table 3). Blockage of cyclooxygenases plus CYP-450 pathways also significantly ($P<0.001$) reduced TNF-α concentrations from 50 min to 90 min, to 8% of the OTA induced levels at 90 min, i.e. TNF-α concentration was 220 pg/ml (Fig. 18 & Table 3). Finally, blockage of lipoxygenases and CYP-450 pathways, significantly reduced TNF-α concentrations from 50 min to 90 min, i.e. TNF-α concentration was 151 pg/ml at 90 min (Fig. 18 & Table 3). The reduction in TNF-α concentrations in

each combination was lower than in OTA-free perfused control livers (statistically not significant). The results indicated so far that in the absence of arachidonic acid metabolites, generated in the lipoxygenase and CYP-450 pathway, OTA-mediated TNF-α released from blood-free perfused rat livers got lost.

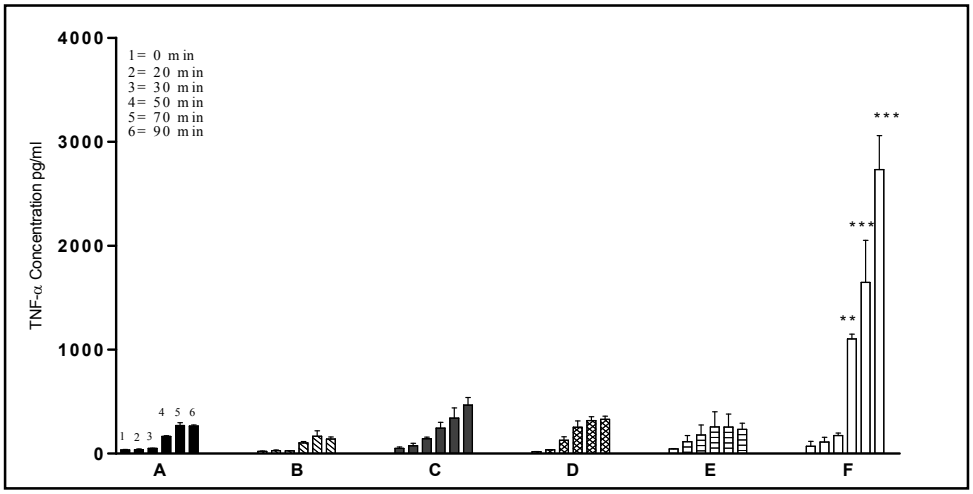

Fig. 17: Basal TNF-α release from blood-free rat livers
TNF-α concentrations were measured in perfusate samples at 1) 0, 2) 20, 3) 30, 4) 50, 5) 70 and 6) 90 min, indicated by single columns. Samples obtained from blood-free rat livers perfused with: **A)** 2% dextran Krebs-Henseleit buffer medium alone (untreated); **B)** 50µmol/L aristolochic acid; **C)** 10µmol/L indomethacin; **D)** 30µmol/L of nordihydroguaiaretic acid; **E)** 100µmol/L of metyrapone; **F)** 2.5µmol/L of OTA. The tested compounds were applied at 10 min while OTA was applied at 20 min from zero time (see Materials and Methods). Values represent the mean ± SEM of 3 livers for each group, (* $P<0.05$, ** $P<0.01$, *** $P<0.001$).

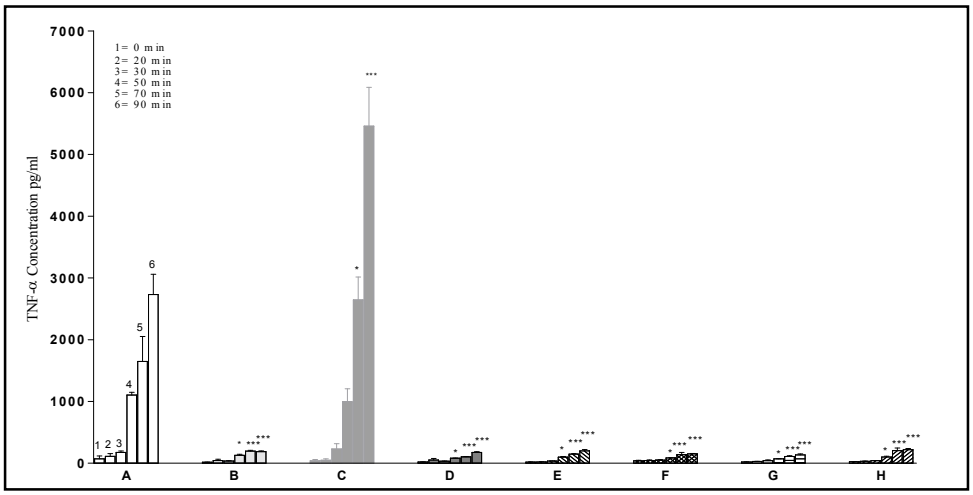

Fig. 18: Effects of blocking arachidonic acid release and its major pathways on TNF-α release in the presence of OTA

TNF-α concentrations were measured in perfusate samples at 1) 0, 2) 20, 3) 30, 4) 50, 5) 70 and 6) 90 min. Samples were obtained from blood-free rat livers perfused with: **A)** 2.5µmol/L OTA; **B)** 50µmol/L aristolochic acid followed by OTA; **C)** 10µmol/L indomethacin followed by OTA; **D)** 30µmol/L nordihydroguaiaretic acid followed by OTA; **E)** 100µmol/L metyrapone followed by OTA; **F)** 100µmol/L metyrapone and 30µmol/L nordihydroguaiaretic acid followed by OTA; **G)** 10µmol/L indomethacin and 30µmol/L nordihydroguaiaretic acid followed by OTA; **H)** 10µmol/L indomethacin and 10µmol/L metyrapone followed by OTA. The tested compounds were applied at 10 min after zero time then followed by 2.5µmol/L OTA at 20 min (see Materials and Methods). Values represent the mean ± SEM of 3 livers for each group, (* $P<0.05$, ** $P<0.01$, *** $P<0.001$).

4.5.3 Effect of exogenous arachidonic acid supplementation

The results so far let the question open how arachidonic acid itself inferes with OTA-mediated TNF-α release. To test this, 10µmol/L of exogenous arachidonic acid sodium salt was added to the perfusate at 10 min after zero time. The exogenous arachidonic acid alone didn't show significant interference with basal TNF-α release, which was 300 pg/ml at 90 min (Fig. 19). When it was applied 10 min prior to OTA, it caused a complete blockage of TNF-α release

into perfusate starting from 50 min to 90 min (P<0.001). At the end of perfusion TNF-α concentration was 196 pg/ml (Fig. 19 & Table 3). This was even lower than TNF-α release exerted by arachidonic acid alone and also lower than basal release from untreated controls. Our data suggest an important controller function for arachidonic acid and its metabolites on this mycotoxin provoked TNF-α release from rat livers, namely a stimulation by LPX and CYP-450 metabolites and inhibition by COX metabolites and arachidonic acid itself.

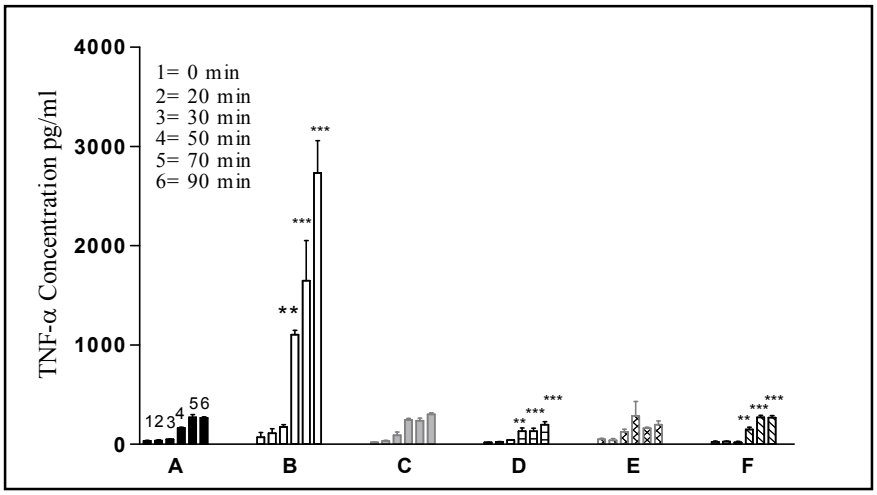

Fig. 19: Effects of inhibition of NF-kB and addition of arachidonic acid on TNF-α release

TNF-α concentrations were measured in perfusate samples at 1) 0, 2) 20, 3) 30, 4) 50, 5) 70 and 6) 90 min. Samples obtained from blood-free rat livers perfused with: **A)** ■ 2% dextran Krebs-Henseleit buffer medium alone (untreated); **B)** ▭ 2.5µmol/L OTA; **C)** ▩ 10µmol/L exogenous arachidonic acid; **D)** ▤ 10µmol/L exogenous arachidonic acid followed by OTA; **E)** ▩ 10µmol/L caffeic acid phenylethyl ester (CAPE); **F)** ▨ 10µmol/L caffeic acid phenylethyl ester (CAPE) followed by OTA. The tested compounds were applied at 10 min while 2.5µmol/L OTA was applied at 20 min into the perfusate after zero time (see Materials and Methods). Values represent the mean ± SEM of 3 livers for each group, (* P<0.05, ** P<0.01, *** P<0.001).

Inhibitor	Inhibitee	TNF-α change Δt=90 min (100%=265pg/ml)
Without OTA		
Ø medium	**Ø**	
50µmol/L aristolochic acid	Phospholipase A2	52%
10µmol/L indomethacin	Cyclooxygenase	182%
30µmol/L nordihydroguaiaretic acid	Lipoxygenase	91%
100µmol/L metyrapone	CYP-450	128%
10µmol/L caffeic acid phenylethyl ester (CAPE)	NF-kB	76%
10µmol/L arachidonic acid	Exogenous arachidonic acid	117%
With 2.5µmol OTA/l		**(100%=2600 pg/ml)**
Ø medium	**Ø**	
50µmol/L aristolochic acid	Phospholipase A2	7%
10µmol/L indomethacin	Cyclooxygenase	211%
30µmol/L nordihydroguaiaretic acid	Lipoxygenase	6%
100µmol/L metyrapone	CYP-450	7%
10µmol/L caffeic acid phenylethyl ester (CAPE)	NF-kB	7%
10µmol/L arachidonic acid	Exogenous arachidonic acid	10%
10µmol/L indomethacin and 30µmol/L nordihydroguaiaretic acid	Cyclooxygenase and Lipoxygenase	5%
10µmol/L indomethacin and 100µmol/L metyrapone	Cyclooxygenase and CYP-450	8%
30µmol/L nordihydroguaiaretic acid and 100µmol/L metyrapone	Lipoxygenase and CYP-450	5%

Table 3: Percentages of TNF-α release in the absence or presence of 2.5 µmol/l OTA in the blood-free perfused rat liver model.

4.6 Inhibition of OTA-induced TNF-α signaling cascade

4.6.1 Effect of calcium on OTA-mediated TNF-α release

To study the effects of calcium on TNF-α release by OTA, blood-free rat livers were perfused with 75 ml Krebs-Henseleit calcium-free buffer containing 2% dextran under the same experimental conditions. In control livers TNF-α levels were less than basal release. Under these conditions livers didn't respond to OTA effects indicating that calcium is required in the perfusion medium (Fig. 20).

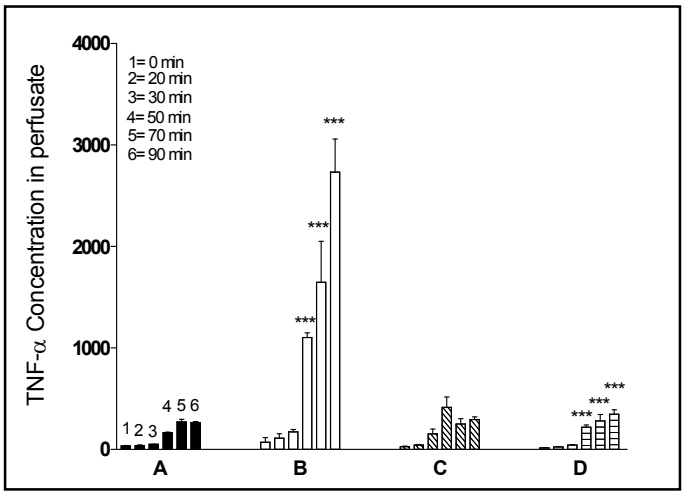

Fig. 20: Effect of calcium on OTA-mediated TNF-α release from blood-free perfused rat liver

TNF-α concentrations were measured in perfusate samples at 1) 0, 2) 20, 3) 30, 4) 50, 5) 70 and 6) 90 min. Samples obtained from blood-free rat livers perfused with: **A)** ■ 2% dextran Krebs-Henseleit buffer medium alone (untreated); **B)** ▭ 2.5µmol/L OTA; **C)** ▨ 2% dextran Krebs-Henseleit Ca^{+2} buffer medium alone (untreated); **D)** ▤ 2.5µmol/L OTA in 2% dextran Krebs-Henseleit buffer medium. OTA was applied at 20 min into the perfusate after zero time (see Materials and Methods). Values represent the mean ± SEM of 3 livers for each group, (* P<0.05, ** P<0.01, *** P<0.001).

4.6.2 Effect of NF-kB inhibitor CAPE

TNF-α synthesis is under strong control of the transcription factor NF-kB (Aggarwal, 2000; Denk *et al.,* 2001; Liz-Grana and Gomez-Reino Carnota, 2001; Ye *et al.,* 2003). To estimate the influence of NF-kB on OTA-mediated TNF-α release, 10μmol/L caffeic acid phenylethyl ester (CAPE) which potentially inhibits NF-kB activation (Natarajan *et al.,* 1996) was added to Kupffer cell cultures or to isolated perfused rat livers.

CAPE alone had no effects on basal TNF-α levels released by Kupffer cells in the incubation medium, but when it was added 30 min prior to OTA, a significant reduction in TNF-α concentration ($P<0.001$) was observed to be 200 pg/ml at 24 hrs (Fig. 21). Similarly, in the presence of CAPE the basal concentration of TNF-α in the perfusate of perfused rat livers didn't change. When it was added 10 min prior to OTA to the perfusion medium a significant reduction in TNF-α concentration ($P<0.001$) was observed in the perfusate to be 277 pg/ml at 90 min (Fig. 19 & Table 3). This indicates that NF-kB is required for OTA-mediated release of TNF-α from perfused rat livers and from isolated Kupffer cells in culture.

4.6.3 Involvement of CD14 in OTA-mediated TNF-α release from Kupffer cells

Induction of TNF-α in response to LPS required the CD14 molecule to be a binding bridge between LPS and the Toll-like receptor 4. To test the influence of the CD14 molecule in OTA-mediated TNF-α release, 3ng/ml of Anti-CD14 was added to the incubation medium of Kupffer cells 30 min prior to OTA, which decreased the TNF-α level in the incubation medium from over 1000 to 375 pg/ml after 24 hrs (Fig. 21). These results indicated the signal cascade leading to TNF-α release requires CD14 molecules, which probably acts as a connecting signal between OTA and TNFR.

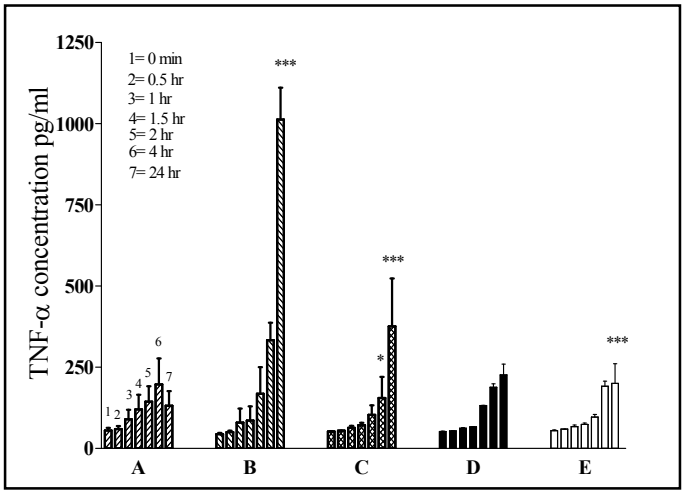

Fig. 21: Involvement of CD14 and NF-kB in TNF-α release from Kupffer cells
TNF-α concentrations were measured in incubated media of isolated cell in single culture at 1) 0, 2) 0.5, 3) 1, 4) 1.5, 5) 2, 6) 4 and 7) 24 hrs indicated by single columns. **A)** medium alone (untreated); **B)** 2.5µmol/L of OTA; **C)** 3ng/ml AntiCD14 antibody followed by 2.5 µmol/L OTA; **D)** 10µmol/L caffeic acid phenylethyl ester (CAPE); **E)** 10µmol/L caffeic acid phenylethyl ester (CAPE) followed by OTA. The tested compounds were applied at 30 min while 2.5µmol/L OTA was applied at 1 hr into the incubation medium

after zero time (see Materials & Methods section). Values represent the mean ± SEM of 3 culturs for each group, (* $P<0.05$, ** $P<0.01$, *** $P<0.001$).

Chapter 5. Discussion

5.1 Meaning of OTA-mediated release of TNF-α in the liver

Because the liver is placed between the digestive tract and the systemic circulation, the liver occupies a central role in metabolism of nutrients and drugs. It receives via the portal vein large amounts of nutrients and noxious compounds from the digestive tract. The liver is the principal organ involved in the biotransformation of exogenous substances and converts hydrophobic substances into water-soluble products. However, potentially hepatotoxic chemicals may produce liver damage *in vivo* due to an interaction with complex cellular processes that follow their uptake, biotransformation, and elimination. Such a hepatotoxin may react with fundamental cellular constituents; i.e. proteins, lipids, RNA, and DNA and may induce lesions of the liver which often cause steatosis (Guillouzo, 1998).

The liver is also a major organ for systemic release of inflammatory cytokines, e.g. TNF-α and IL-6, upon exposure to gut-derived bacterial toxins, i.e. lipopolysaccharides (LPS). The release of cytokines from livers, in particular TNF-α, had two sword edges. On the one hand, it mediates the physiological processes of liver regeneration, and also plays an important role in the defense mechanisms against bacteria, viruses, fungi and parasitic infections. On the other hand it promotes the pathogenesis of several liver disorders, in particular

chronic disorders, such as alcoholic liver disease (Hirano *et al.,* 2003; Song *et al.,* 2004), liver-cirrhosis accompanying septicaemia (Byl *et al.,* 1993, Ceydeli *et al.,* 2003), chronic inflammatory liver disease (Adams & Afford 2002; McClain *et al.,* 2004) and primary sclerosing cholangitis (Bernal *et al.,* 1999; Mitchell *et al.,* 2001).

The liver functions are under the influence of various endogenous and exogenous factors. Therefore, *in vivo* it is difficult to distinguish between the primary effects of a compound on liver functions from those induced secondarily, that result in complex interactions with other organs. *In vitro* liver models represent an experimental approach to screen potential hepatotoxic compounds and to investigate mechanism(s) by which chemicals induce liver lesions. For this reason the isolated perfused liver preparation, liver tissue slices, isolated and cultured hepatocytes, and subcellular fractions have been used (Conway *et al.,* 1983; Guillouzo, 1998).

In vitro, the isolated perfused liver appear to be the nearest to the *in vivo* situation. This model has been used for investigating drug- and chemical-induced hepatotoxicity (Plaa, 1993) and represents a useful tool to study toxicokinetics. In our experiments we used isolated blood-free perfused rat livers to investigate the effect of OTA on TNF-α release in comparison to LPS, the known inducer of TNF-α.

Because of the ubiquitous presence of ochratoxin A in almost every kind of food (Gareis & Scheuer, 2000), it is reasonable that following absorption from the gut, this toxin may exert effects first in the liver. OTA is clearly a potent nephrotoxin and is the most important agent causing Balkan Endemic Nephropathy (BEN) (Pfohl-Leszkowicz *et al.,* 2002). However, the liver is also among the OTA-target organs because of its food-borne exposure via the portal vein after absorption from the gut. Furthermore, an enterohepatic circulation of OTA was reported (Fuchs *et al.,* 1988), providing repeated exposure of liver cells to internally circulating OTA. OTA is transported from blood into hepatocytes by carrier-mediated transport (Kontaxi *et al.,* 1996). These reasons may explain why the liver is exposed to the highest peak concentration of OTA following the oral route and may cause damage to the liver as indicated by the detoriation of liver DNA by OTA (Pfohl-Leszkowicz *et al.,* 1993) and liver tumors (Boorman, 1989). In contrast to subchronic and chronic effects, acute toxic OTA effects to the liver are not clear. In previous studies with isolated liver cell cultures direct cytotoxicity occured on hepatocytes at concentrations as low as 1 µM OTA (Dörrenhaus & Föllmann, 1997).

A new effect of OTA is the release of TNF-α from blood-free perfused rat livers (Weidenbach *et al.,* 2000). This release was not observed with several other mycotoxins such as 3-acetoxydeoxynivalenol (3-Ac-DON), xanthomegnin

(XAN), citrinin (CIT), and viomellein (VIO) and was regarded specific for OTA (Petzinger & Weidenbach, 2002).

In experiments shown in this thesis, the release of the proinflammatory cytokine TNF-α was observed during an ongoing toxin passage through an isolated perfused rat liver at concentrations as low as 0.5 µM. The release of TNF-α increased ten fold at 2.5 µmol/L OTA without observing signs of general cytotoxicity as assayed by LDH, GLDH, lactate and K^+ ion release. The highest tested dose of OTA (12.5 µmol/L) caused somewhat higher elevations of TNF-α concentration in the perfusate compared with 2.5 µmol/L but also caused alterations in the liver cells' vitality, indicated by the release of high amounts of LDH, GLDH and lactate. The increase of cytotoxic markers by the highest tested dose of OTA correlated with released TNF-α. This could mean that TNF-α probably promotes the cytotoxic effects of OTA at this dose. The known inducer of TNF-α, LPS, was used in our experiments as a positive control to estimate the efficacy of the perfused liver model. If used at 0.1 µg/ml LPS under similar experimental conditions, LPS in a previous study induced a similar TNF-α release from blood-free perfused rat livers as with 2.5 µmol/l OTA (Weidenbach *et al.,* 2000).

The liver lobule is formed by parenchymal cells, i.e. hepatocytes and non-parenchymal cells. In contrast to hepatocytes that occupy almost 80% of the

total liver volume and cell mass and perform the majority of numerous liver functions, non-parenchymal liver cells contribute ca. 6.5% to the liver volume, but 40% to the total number of liver cells. These cells are localized in the sinusoidal cell fraction of the liver tissue. The walls of hepatic sinusoids are lined by three different cell types: sinusoidal endothelial cells (SEC), Kupffer cells (KC), and hepatic stellate cells (HSC, formerly known as fat-storing cells, Ito cells, lipocytes, perisinusoidal cells, or vitamin A-rich cells). Additionally, intrahepatic lymphocytes (IHL), including pit cells, i.e. liver-specific natural killer cells, are often present in the sinusoidal lumen. It has been increasingly recognized that both under normal and pathological conditions, many hepatocyte functions are regulated by substances released from neighbouring nonparenchymal cells (Milosevic, *et al.,* 1999; Khetani *et al.,* 2004). That means mediators released from non-parenchymal liver cells may evidently regulate functions of neighbouring hepatocytes and non-hepatocytes, respectively. Key mediators involved in the intercellular communication in the liver are prostanoids, nitric oxide, endothelin-1, TNF-α, interleukins, and chemokines, growth factors (TGF-beta, PDGF, IGF-I, HGF), and reactive oxygen species (ROS). Paradoxically, the cooperation among liver cells is better understood under some pathological conditions (i.e. in experimental models of liver injury) than under normal conditions (Kmiec, 2001).

The intact liver has a complex structure and function. This means, it is difficult (in the isolated organ) to distinguish the primary site of OTA effects from those resulting from cross-talk interactions between liver cells. Accordingly, many cells may serve as a TNF-α source in response to OTA in the isolated organ. To elucidate which liver cell type responds to OTA by releasing TNF-α into the perfusate of blood-free perfused rat livers, Kupffer cells were blocked *in vitro* by the heavy metal gadolinium chloride and *in vivo* by i.p. injection of the Kupffer cells toxin clodronate. Furthermore, the major liver cell populations were isolated in single cell cultures and exposed to OTA or to LPS separately for 24 hrs.

Kupffer cells, the resident macrophages of the liver, are localized in the lumen of the sinusoids and anchored to the endothelium by cytoplasmic protrusions (Laskin, 1990). They are in constant contact with gut-derived particulate materials and soluble bacterial products so that a subthreshold level of their activation in the normal liver may be anticipated. Hepatic macrophages secrete potent mediators of the inflammatory response (reactive oxygen species, eicosanoids, nitric oxide, carbon monoxide, TNF-α and other cytokines), and thus control the early phase of liver inflammation, playing an important part in the innate immune defense system. Exposure of Kupffer cells to bacterial products, especially endotoxins (lipopolysaccharides, LPS), can lead to the

intensive production of inflammatory mediators, and ultimately to liver injury. Besides typical macrophage activities, Kupffer cells play an important role in the clearance of senescent and damaged erythrocytes. Liver macrophages modulate immune responses via antigen presentation (Knolle & Gerken, 2000; Kmiec, 2001), and suppression of T-cell activation by antigen-presenting sinusoidal endothelial cells via paracrine actions (Knolle & Gerken, 2000). They also participate in the development of oral tolerance to bacterial superantigens (Kmiec, 2001). Moreover, during liver injury and inflammation, Kupffer cells secrete enzymes and cytokines that may damage hepatocytes, and are active in the remodelling of extracellular matrix. However, much evidence has accumulated in support of a role for Kupffer cells in various models of liver diseases (Iimuro *et al.,* 1994; Sarphie *et al.,* 1996).

We blocked Kupffer cells *in vitro* by the rare earth metal salt gadolinium chloride ($GdCl_3$), as this compound was reported to depress the reticuloendothelial (RES) activity (Lazar, 1973), and phagocytic activity (Brown *et al.,* 1997, Yang *et al.,* 1999). It abolishes the hepatic expression of some Kupffer cells' specific antigens (Klein *et al.,* 1994; Kim & Choi, 1997), without affecting the number of Kupffer cells (Rai *et al.,* 1996). $GdCl_3$ reduces superoxide production and TNF-α mRNA expression by Kupffer cells in response to injury (Iimuro *et al.,* 1994; Lazar *et al.,* 1994). Alternatively,

Kupffer cells isolated from $GdCl_3$-treated animals produced more superoxide and TNF-α compared with control cells (Ahmad *et al.,* 1999). Furthermore, total hepatic TNF-α mRNA levels were reduced to approximately 60% up to 5 days in rats pre-treated with gadolinium chloride and challenged with LPS 4 hrs before being scarified. In that study the authors found the LPS responses reversed to normal on day 8 (Lee *et al.,* 2004). This contradiction in the action of $GdCl_3$ *in vivo* was explained by the survival of a subpopulation of Kupffer cells which remain viable after systemic $GdCl_3$ administration. In our experiments, $GdCl_3$ at 15μmol/l added to the perfusion medium didn't produce specific toxicity of the liver, i.e. no leakage of cytotoxicity markers occurred into the perfusate. However, it achieved complete blockage of OTA-mediated TNF-α release from blood-free perfused rat livers.

Apart from *in vitro* blockage, we also blocked Kupffer cells *in vivo* by clodronate. Clodronate was encapsulated into liposomes and was injected intaperitoneally. This was reported to enhance its efficacy against macrophages. Clodronate requires rather a long time to block Kupffer cells via induction of apoptosis, too long for an application to perfused rat livers *in vitro.* Therefore, liposome encapsulated clodronate, LIP-CLOD, was injected i.p. into rats 48 hrs before liver preparation. The livers isolated from LIP-CLOD pre-treated rats showed slight release of cytotoxicity markers (in the absence of OTA). This

indicated LIP-CLOD at the recommended dose may produce harmful effects on the liver. However, LIP-CLOD successfully blocked the effect of OTA by completely inhibiting TNF-α release. The inhibitory effect of LIP-CLOD on OTA-mediated TNF-α release could result from the preceeding death of Kupffer cells. Consequently, OTA would not be able to find the target cell for TNF-α release. Kupffer cells depletion by LIP-CLOD caused delayed liver regeneration after partial hepatectomy in rats due to the reduction of TNF-α mRNA (Meijer *et al.,* 2000). The vehicle of clodronate in our experiments was used under similar experimental conditions and didn't significantly modify OTA actions. This means, the effects produced by liposome-encapsulated LIP-CLOD resulted from clodronate action.

The data obtained from the above experiments suggest that Kupffer cells are the target for OTA mediated TNF-α release. This was confirmed with cell preparations from rat liver containing isolated hepatocytes, or sinusoidal endothelial cells, respectively. A non-toxic dose of OTA or LPS was used which already had produced significant release of TNF-α from the blood-free perfused liver. From the single cells preparations neither sinusoidal endothelial cell nor hepatocytes showed any response during OTA exposure. However, in contrast to OTA, all cells responded to LPS, albeit not with equal efficacy. This

discrepancy in TNF-α release could indicate separate signalling mechanisms of OTA versus LPS on the different cell types.

Liver parenchymal cells were reported already as a minor source for TNF-α release (Hunt *et al.,* 1992). E.g. Hasmall *et al.,* 2000, found that basal level of TNF-α in pure hepatocytes cultures was higher than that detected from non-parenchymal liver cells and only non-parenchymal liver cells responded to nafenopin by releasing TNF-α. On the other hand, the parenchymal cell was reported to produce TNF-α four times more upon LPS stimulation than without LPS. Furthermore, rat hepatocytes released TNF-α after 1 hr of exposure to *Listeria monocytogenes*. This response was time-dose- and density-dependent (Santos *et al.,* 2005).

TNF-α was also released from a human liver sinusoidal endothelial cell line during hypoxia re-oxygenation injury. It increased significantly in a time-dependent manner, while sinusoidal cell function decreased (Wang *et al.,* 2002). Sinusoidal cells expressed TNF-α mRNA when rat liver was chronically cannulated and subjected to haemorrhage (Yamashita, *et al.,* 2002), or when rats were stimulated i.v. by plasmid DNA-cationic liposome complex (lipoplex) (Sakurai *et al.,* 2002). From this data we conclude that in principle the release of TNF-α from hepatocytes or sinusoidal endothelial cells is feasible, but that hepatocytes and sinusoidal endothelial cells lack any response to release TNF-α

upon OTA binding. It seems that OTA could trigger this specific signalling cascade only in Kupffer cells.

The primary targets for the OTA mediated cytokine release in rat livers were Kupffer cells, whereas TNF-α release in response to LPS is a summation of the outcome of liver parenchymal and non-parenchymal cells.

5.2 Non-Liver cells and OTA: Differential sensitivity between OTA and LPS on releasing TNF-α

Kupffer cells represent the liver macrophage, which produces a number of mediators, including cytokines and chemokines (Lewis & McGee, 1992). Apart from liver tissue macrophages are widely present in the lung, bone marrow, and synovia (Lewis & McGee, 1992), possibly indicating that *in vivo* this cytokine response to OTA is likely not restricted to the liver and may include many other organs as well. Accordingly, the observed phenomenon of TNF-α release indicates a general, previously unconsidered, toxicological property of this mycotoxin. For this reason, the ability of other cell types to release TNF-α by ochratoxin A or by LPS was investigated. A preparation of primary rat peritoneal macrophages and also the mouse monocyte macrophage cell line (J774A.1) were used as were the human hepatoma cell line (HepG2) and the connective tissue cell line (L929) culture. All cell types were exposed to

2.5µmol/l OTA for 1-24 hrs. Furthermore, under similar experimental conditions these cells were incubated with 0.1 µg/ml LPS as the positive control.

We found rat peritoneal macrophages, and mouse macrophages J774A.1 released TNF-α in respond to OTA, but the HepG2 and the L929 cell line didn't respond to OTA at any indicated time points. OTA-mediated TNF-α release was 10 times that of basal levels in peritoneal macrophages, whereas the elevation was 6 times in the case of the J774A.1 cell line. On the other hand, all cell types exposed in our experiments to LPS showed significant release of TNF-α except HepG2 which even didn't respond to LPS. Hong *et al.,* 2004 found 10µg/ml LPS expressed TNF-α protein from HepG2. The contradiction to our findings could be explained by the Gutierrez-Ruiz *et al.,* 1999 finding. When they found TNF-α released from HepG2 by 1µg/ml LPS as well as other stimulators. The authors concluded HepG2 cells participated in a differential cytokine release, which differs according to the toxic agent and the time of exposure (Gutierrez-Ruiz *et al.,* 1999).

Our data suggest different kind of macrophages (Kupffer cells, peritoneal macrophages, J774A.1 cell line) as a general source for TNF-α release upon OTA exposure. In contrast, LPS induces TNF-α from a wide variety of cell types. In addition, the LPS reaction was more pronounced than that by OTA.

5.3 Endogenous protection of OTA mediated TNF-α release by prostanoids

TNF-α also potently stimulates apoptosis in many cell types (Aggarwal, 2000; Gupta, 2002). This mechanism may convey another type of cell toxicity of OTA. However, in liver parenchymal cells promotion of liver cell regeneration has also been reported (Yamada *et al.,* 1998b; Diehl, 1999). The regenerating effects on hepatocytes require a primary stimulus such as tissue destruction which was performed experimentally by a partial hepatectomy (Webber *et al.,* 1994). Also TNF-α can promote liver injury in a number of ways. For example, *in vitro* TNF-α renders hepatocytes more susceptible to toxicity (Admson & Billings, 1992; Hoek & Pastorino, 2002), TNF-α also can prime PMNs to release toxic products (i.e. ROS and proteases) that can damage nearby cells (Kushimoto *et al.,* 1996). In non-injured, "normal" livers OTA mediated strong release of TNF-α. This could, however, cause a disaster to the liver if other effects did not provide protection. The protective effect provided by other liver cells could be the release of other inflammatory mediators i.e. PLA_2, or PLA_2 derived metabolic prostanoid products (AL-Anati *et al.,* 2005), or the release of other cytokines, which have antagonistic effects with TNF-α such as IL-10. However, this mechanistic concept requires further studies.

The extracellular phospholipase A_2 ($sPLA_2$) levels are increased in various

systemic inflammatory diseases such as adult respiratory distress syndrome, septic shock, and acute pancreatitis (Vadas, 1984; Kim *et al.,* 1995), autoimmune diseases like rheumatoid arthritis, Crohn's disease, and ulcerative colitis (Haapamaki *et al.,* 1998; Haapamaki *et al.,* 1999), and allergic disorders such as bronchial asthma and allergic rhinitis (Stadel *et al.,* 1994; Chilton *et al.,* 1996). In these diseases, $sPLA_2$ levels increase in plasma and inflammatory fluids like synovial fluid, bronchoalveolar lavage, and nasal lavage, indicating that $sPLA_2$s are released systemically as well as at sites of tissue inflammation. These observations implicate $sPLA_2$ in inflammation. Interestingly, most inflammatory diseases associated with increased $sPLA_2$ levels are characterized by overproduction of cytokines such as TNF-α, IL-1, and IL-6 (Remick, 1997). Others found two $sPLA_2$ isoforms, human group IB (hGIB) and human group X (hGX) induced TNF-α and IL-6 from human lung macrophage (Granata *et al.,* 2005). This data could explain why the phospholipase A_2 inhibitor aristolochic acid prevented TNF-α release from blood-free perfused liver in our experiments.

$sPLA_2$ has different biological activity and is implicated in the occurrence of diseases by different mechanisms. One important mechanism is the release of arachidonic acid (AA), the precursor of eicosanoids, from cell membranes or extracellular phospholipids (Murakami & Kudo, 2002). In the presence of TNF-

α arachidonic acid and its metabolic products were released in several cell lines due to the activation of phospholipase A_2, they mediated and potentiated TNF-α toxicity (Reid *et al.,* 1991; Hayakawa *et al.,* 1993). we expected that arachidonic acid may contribute to toxic effects observed with OTA. In this study the interference with TNF-α release by arachidonic acid and also by major metabolites derived from arachidonic acid was observed.

Our results clearly showed that arachidonic acid itself markedly suppressed OTA-mediated TNF-α cytokine release. The effect is probably not mediated by arachidonic acid alone but in addition by its cyclooxygenase metabolites, i.e. prostaglandins. These metabolites prevail over an opposing effect seen by arachidonic acid metabolites formed by lipoxygenase or P450 enzymes. If the cyclooxygenase/arachidonic acid shelter is blocked by indomethacin an excessive 2-fold higher release of TNF-α under OTA was observed, likely because arachidonic acid was shifted in the opposing lipoxygenase and/or P450 pathway. This situation may have clinical relevance as it sheds some doubts on the usefulness of clinical treatment with non-steroidal anti-inflammatory drugs under circumstances of ongoing mycotoxin burden.

In endothelial cells, arachidonic acid suppresses TNF-α release at the transcription level (Stuhlmeier *et al.,* 1996). This occurs by stabilization of IkBα/NF-kB complex, which lead to the inhibition of the transcription factor

NF-kB (Stuhlmeier *et al.,* 1997). The stabilization of the transcription factor complex forced NF-kB to reside in the cytosol, and this is why the activation of TNF-α and other genes ceases (Denk *et al.,* 2001; Ye *et al.,* 2003). Although the effect of OTA on the level of NF-kB is unknown, our results obtained from CAPE, an NF-kB blocker, would support this concept.

The effect of prostaglandin on TNF-α released from LPS-stimulated peritoneal rat macrophage was studied by Renz *et al.,* 1988. They found low concentrations of PGE_2 (0.1 to 10 ng/ml) stimulated, whereas higher concentrations (greater than 10 ng/ml) suppressed TNF-α release. The authors found PGE_2-stimulated TNF-α production was dependent on *de novo* protein synthesis and was associated with an intracellular rise of cGMP, because low PGE_2 concentrations preferentially increased cGMP but not cAMP. In tumor cytotoxicity assays, PGE_2-activated macrophages were active only against TNF-α-sensitive target cells. These findings demonstrate that TNF-α synthesis in macrophages is up-regulated by cGMP and down-regulated by cAMP, which indicates that cyclic nucleotides act as intracellular messengers for extracellular signals of macrophage activation (Renz *et al.,* 1988). Others found that the inhibitory effect of PGE_2 on TNF-α and IL-6 production by lipopolysaccharide (LPS)-stimulated murine peritoneal macrophages resulted from induction of IL-10 (Strassmann *et al.,* 1994). *In vivo*, the administration of PGE_2 before LPS

challenge significantly reduces circulating TNF-α and IL-6 levels. Anti-IL-10 antibody substantially enhanced the LPS-induced TNF-α and IL-6 levels in mice that received either LPS alone or LPS plus PGE_2 (Strassmann *et al.,* 1994). Similar findings were observed by Dooper *et al.,* 2002, when they found with LPS-stimulated freshly isolated human peripheral blood mononuclear cells (PBMC) that PGE_1, PGE_2, and PGE_3 caused inhibition of TNF-α production whereas IL-6 was unaffected but IL-10 increased (Dooper *et al.,* 2002). We conclude that PGs inhibited TNF-α release by different mechanisms and through an autocrine feedback mechanism. The elevation of TNF-α levels in our experiments in the presence of indomethacin is in accordance with this conclusion.

Cytochrome P4502E1 (CYP2E1) is up-regulated in Kupffer cells after ethanol treatment, this effect primes Kupffer cells, sensitizing them to increase TNF-α production in response to LPS. LPS caused overproduction of TNF-α from RAW 264.7 macrophages which were transfected with CYP2E1 (E2) (Cao *et al.,* 2005). The inhibition of Cytochrome P450 (CYP2J2) protected carcinoma cells from apoptosis induced by TNF-α (Jiang *et al.,* 2005). Fantuzzi *et al.,* 1993 found intraperitoneal administration of 100mg/kg of metyrapone suppressed serum TNF-α in LPS-challenged mice. Also *in vitro* production of TNF-α by endotoxin-stimulated human monocytes was also inhibited by metyrapone. The

author suggested the inhibition of TNF-α by metytrapone was resulted from cytochrome P450 inhibition (Fantuzzi *et al.,* 1993). However, these data could explain the effect of CYP-450 inhibitor metyrapone in our experiments, which caused blocking of OTA effects and the absence of TNF-α from perfusate.

Leukotrienes (LT), in particular LTB_4, are potent inflammatory mediators and immunomodulators (Gagnon *et al.,* 1989). In their interactions with leukocytes, LTB_4 can activate numerous functions of neutrophils and modulate the activities of various lymphocyte subsets. LTB_4 can also augment macrophage and monocyte cytotoxic activities and enhance their production of hydrogen peroxide and the monokines interleukin 1 and TNF-α (Gagnon *et al.,* 1989). Stimulated macrophages metabolize some protein of their membrane-derived arachidonic acid via the 5-lipooxygenase pathway, resulting primarily in the production of leukotrienes B_4 and C_4. It has been reported that the inhibition of 5-lipooxygenase by nordihydroguaiaretic acid (NDGA) and AA861 reduced asbestos- or silica-stimulated TNF-α release from rat alveolar macrophages. This inhibitory effect was partially restored by added exogenous LTB_4 (Dubois *et al.,* 1989). 15-HPETE regulates the production of the proinflammatory cytokine TNF-α posttranscriptionally by promoting degradation of LPS-induced TNFmRNA in a human monocytic cell line (Ferrante & Ferrante 2005). Blocking production of LTC_4 also caused reduction of LPS-induced synthesis of

TNF-α from murine peritoneal macrophages (Schade *et al.,* 1989). These results are similar to our observation in the perfused liver model. Others found that the 5-lipoxygenase inhibitors VZ 65 and AA-861 as well as methylxanthine pentoxyfilline (PTX) inhibited TNF-α production from LPS-stimulated murine macrophage line RAW264, independent of the simultaneously administration of LPS or 30 min after LPS treatment (Lin *et al.,* 2004). All these results indicate a substantial stimulation of TNF-α release by leukotrienes, irrespective which initiating trigger (OTA or LPS) is used.

Chapter 6. Conclusions

1-The experimental passages of OTA via portal vein induce TNF-α release in dose- and time-dependent fashion.

2-OTA at 2.5µmol/L releases significant amounts of TNF-α without influencing the liver cell's vitality.

3-Similar amounts of TNF-α are released by OTA at 2.5µmol/L and by LPS 0.1µg/ml.

4-Among liver cell populations only Kupffer cells are the source of OTA-mediated TNF-α release from blood-free perfused rat livers. In contrast, TNF-α release by LPS is a summation of release by Kupffer cells, hepatocytes, and sinusoidal endothelial cells.

5-Macrophages are the only source for TNF-α release upon OTA exposure, whereas LPS induces TNF-α from a wide variety of cell types.

6-LPS reaction was more pronounced than that by OTA in isolated cells.

7-Arachidonic acid and its cyclooxygenase metabolites are suppressors of OTA-mediated TNF-α release from blood-free perfused rat livers, whereas lipooxygenase -and CYP-450- metabolites have the opposite effect. (Fig. 22)

8-OTA-induced TNF-α release is likely to occur via the NF-kB transcription factor pathway. In addition, OTA apparently requires the expression of CD14 antigen in Kupffer cells probably for OTA binding to TNF receptors. (Fig. 22)

9-The *in vivo* cytokine response to OTA is likely not restricted to the liver and may include many other organs as well. Therefore, the observed phenomenon of TNF-α release indicates a general, previously unconsidered, toxicological property of this mycotoxin.

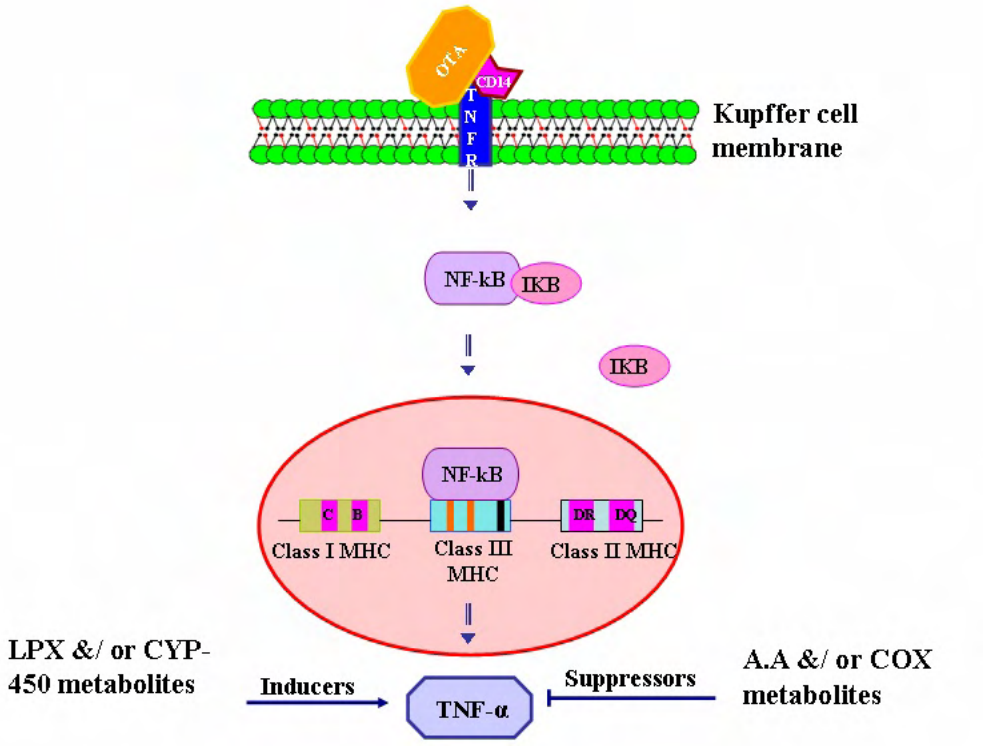

Fig. 22: Proposed mechanism of OTA-mediated TNF-α release from Kupffer cells: The binding of OTA with TNFR via CD14 on Kupffer cell membrane leads to activation of NF-kB followed by subsequent expression of major histocompatibility complex (MHC) Class III gene, which is modulated by lipoxygenase (LPX) and or Cytochrome P-450 (CYP-450) metabolites (inducer) and by arachidonic acid (A.A.) and or cyclooxygenase metaboiltes (COX) (suppressors).

Abstract

Introduction: Tumor necrosis factor alpha TNF-α is a pro-apoptotic cytokine which is produced by a wide variety of cell types in response to various inflammatory stimuli. It promotes the pathogenesis of several health disorders, in particular those related with chronic liver diseases. TNF-α is produced mainly by macrophages. That is why liver is considered a major source of whole body TNF-α, particularly if exposed to gut derived bacterial endotoxins (LPS). Also the liver is continuously exposed to the highest concentration of OTA via portal vein, and considered one of the important OTA-target organs.

Aim: We studied the mechanism of induction of TNF-α release from rat liver by OTA in comparison with 0.1 µg/ml lipopolysaccharide (LPS). For this purpose we used isolated blood free-perfused rat livers, isolated pure rat hepatocytes, sinusoidal endothelial and Kupffer cells. Furthermore, peritoneal rat macrophages, mouse macrophages J774A.1, HepG2, and L929 cell line were exposed to 2.5 µmol/L OTA or 0.1 µg/ml LPS. The primary cell preparations or cell lines were incubated for 24 hrs at 37^0C with 5% CO_2 in a culture medium containing 10% of FCS and penicillin/ streptomycin (100 IU & 100µg/ml respectively).

Results: In the perfusion model OTA induced TNF-α release in a time- and dose dependent fashion. OTA at 2.5µmol/L released 2600 pg/ml and similar amount of TNF-α was released by 0.1 µg/ml LPS (3017 pg/ml) after 90 min

without concomitant significant increase of cytotoxicity markers (LDH, GLDH, lactate and K^+ ion). Under similar experimental conditions blockade of Kupffer cells *in vitro* by 15µmol/L gadolinium chloride or *in vivo* cell depletion by 2ml of liposome encapsulated clodronate per rat abrogated all OTA-mediated TNF-α release from perfused rat livers. Further experiments were done to determine the target cells of OTA in the liver. Only isolated Kupffer cells released TNF-α when exposed separately to the same concentration of OTA, whereas isolated hepatocytes and sinusoidal endothelial cells remained unaltered. However, all these cells respond to LPS by releasing significant amounts of TNF-α into the culture media for up to 24 hrs. In further experiments peritoneal rat macrophages and mouse macrophages J774A.1 released TNF-α upon OTA or LPS exposure, whereas the L929 cell line responded to LPS only. In contrast, HepG2 was not affected by OTA or LPS.

We further investigated the role of arachidonic acid and its metabolites on OTA-mediated TNF-α release from blood free-perfused rat liver. Aristolochic acid, 50µmol/L, a phospholipase A_2 inhibitor, and 10µmol/L of exogenous arachidonic acid blocked OTA-induced TNF-α release even below basal levels. Indomethacin, 10µmol/L, a potent inhibitor of the cyclooxygenase (COX) pathway, almost doubled TNF-α concentrations in the perfusate yielding 5500 pg/ml after 90 min. On the other hand, inhibition of lipoxgenase (LPX) by

30µmol/L nordihydroguaiaretic acid (NDGA) and of the cytochrome P-450 (CYP) pathway by 100µmol/L metyrapone also decreased TNF-α below basal levels. Finally 10µmol/L caffeic acid phenylethyl ester, a NF-kB inhibitor, blocked OTA-mediated TNF-α release from perfused livers and also when applied to Kupffer cell suspensions. The addition of a monoclonal anti-CD14 antibody to Kupffer cells in suspension 30 min prior to OTA-treatment reduced TNF-α in the culture medium from 1000 pg/ml to 375pg/ml. OTA-mediated TNF-α release from blood free-perfused rat liver required Ca^{+2} ions.

Conclusion: OTA induces TNF-α from Kupffer cells. This release is suppressed by arachidonic acid and its cyclooxygenase metabolites, whereas LPX and CYP-450- metabolites have the opposite effect. OTA-induced TNF-α release is likely to occur via the NF-kB transcription factor pathway. In addition, OTA probably requires the expression of CD14 antigen in Kupffer cells probably for OTA-binding to TNF-α receptors. Our data suggest macrophages as a main source for TNF-α release upon OTA exposure. In contrast, LPS induced TNF-α from a wide variety of cell types. In addition, the LPS reaction was more pronounced than that by OTA. OTA-mediated TNF-α release from macrophages could explain general unconsidered immuno toxicological phenomena of OTA.

Zusammenfassung

Einleitung: Tumornekrosefaktor alpha (TNF-α) ist ein pro-apoptotisches Cytokin, das von einer großen Anzahl von Zelltypen als Antwort auf Entzündungsreize gebildet wird. Es fördert die Pathogenese mehrerer Krankheiten, vor allem solcher, die mit chronischen Lebererkrankungen einhergehen. TNF-α wird überwiegend von Makrophagen gebildet. Die Leber ist dann eine Hauptquelle, wenn aus dem Darm resorbierte bakterielle Endotoxine (LPS) auf sie einwirken. Allerdings ist die Leber auch einer ständigen Belastung mit hohen Konzentrationen von Ochratoxin A (OTA) über die Pfortader ausgesetzt und stellt daher ein wichtiges Zielorgan dieses Mycotoxins dar.

Ziel: Wir untersuchten den Mechanismus der Freisetzung von TNF- α aus der Leber durch OTA im Vergleich zu einer Freisetzung durch 0,1 µg/ml Lipopolysaccharid (LPS). Aus diesem Grund verwendeten wir die isolierte, Blutfrei-perfundierte Rattenleber sowie daraus isolierte Hepatozyten, sinusoidale Endothelzellen und Kupffer´sche Sternzellen. Weiterhin wurden peritoneale Rattenmakrophagen, Mausmakrophagenzellen J774A.1, HepG2-Zellen (humane Hepatozytentumorzellen) und die L929 Zelllinie mit 2,5µmol/L OTA oder 0,1µmol/L LPS inkubiert. Die Primärzellpäparationen bzw. Zelllinien wurden über 24 Stunden bei 37 ^{o}C unter 5% CO_2 in einem

Kulturmedium mit 10% fetalem Kälberserum (FKS) sowie Penicillin/Streptomycin (100 IU & 100µg/ml) kultiviert.

Ergebnisse: Im Perfusionsmodell induzierte OTA eine Zeit- und Dosis-abhängige Freisetzung von TNF-α. Unter 2,5µmol/L OTA wurden nach 90 Minuten 2600 pg/ml TNF-α gemessen, fast gleich viel wie unter 0,1µg/ml LPS (3017 pg/ml). Dabei stiegen die Zytotoxizitätsmarker LDH, GLDH, Laktat und Kalium-Ionen nicht nennenswert an.

Unter den gleichen experimentellen Bedingungen führte eine Blockade der Kupffer-Zellen *in vitro* durch 15µmol/L Gadoliniumchlorid oder eine *in vivo* Kupfferzellabnahme unter Einfluss von 2 ml/Ratte liposomalem Clodronat zu einem völligen Verschwinden der OTA-induzierten TNF-α Freisetzung aus der perfundierten Rattenleber. Weitere Experimente wurden unternommen, um die Zielzellen der OTA-Wirkung in der Leber zu ermitteln. Nur isolierte Kupffer-Zellen setzten TNF-α nach OTA-Exposition frei, während isolierte Hepatozyten und Sinusendothelzellen unverändert blieben. Allerdings reagierten alle Zellarten auf LPS, indem sie signifikante Mengen von TNF-α in die Kulturmedien innerhalb von 24 Stunden abgaben. In weiteren Experimenten setzten Ratten-Makrophagen sowie Maus-Makrophagen J774A.1 TNF-α unter OTA und LPS Einwirkung frei, während die L929-Zellen nur auf LPS reagierten. Im Unterschied dazu reagierten HepG2-Zellen weder auf OTA noch auf LPS.

Wir untersuchten weiterhin die Bedeutung von Arachidonsäure und ihren Metaboliten auf die OTA-vermittelte TNF-α Abgabe aus der blutfrei-perfundierten Rattenleber. Aristolochiasäure, 50µmol/L, ein Phospholipase A_2-Inhibitor, und 10µmol/L von exogen zugesetzter Arachidonsäure verminderten die OTA-induzierte TNF- α Freisetzung noch unter die basalen Spiegel. Indomethacin, 10µmol/L, eine potenter Hemmstoff des Cyclooxygenase-(COX)-Weges, verdoppelte beinahe die TNF-α Konzentration im Perfusat auf 5500 pg/ml nach 90 Minuten. Auf der anderen Seite bewirkte eine Hemmung der Lipoxigenase (LPX) durch 30µmol/L Nordihydroguairetinsäure (NDGA) und des Cytochrom P450- (CYP)- Weges durch 100µmol/L Metyrapone einen Abfall von TNF-α unter basale Konzentrationsspiegel. Schließlich blockierte 10µmol/L Coffeinsäurephenylester, ein NF-kB Hemmstoff, die OTA-vermittelte Freisetzung von TNF-α aus isolierten Kupffer-Zellen und perfundierten Lebern. Ein Zusatz des monoklonalen CD14-Antikörpers zu Kupffer-Zellen in Suspension 30 Minuten vor Zugabe von OTA verminderte den TNF-α Gehalt im Kulturmedium von 1000 pg/ml auf 375 pg/ml. Die OTA vermittelte TNF-α Abgabe aus der blutfrei-perfundierten Rattenleber benötigte Ca^{++}-Ionen.

Schlussfolgerungen: OTA induziert eine TNF-α Abgabe aus Kupffer´schen Sternzellen. Diese Freisetzung wird durch Arachidonsäure und ihre

Cyclooxygenase-Metabolite unterdrückt, während die LPX- und CYP450-gebildeten Metabolite den gegenteiligen Effekt haben. Die OTA-vermittelte TNF-α Freisetzung erfolgt wahrscheinlich mittels des NF-kB Transkriptionsweges. Zusätzlich benötigt OTA die Expression von CD14 Antigen auf Kupffer-Zellen, vermutlich für eine Bindung von OTA an TNF-α Rezeptoren. Unsere Ergebnisse legen dar, dass Makrophagen eine Hauptquelle des TNF-α unter OTA sind. Im Unterschied dazu induziert LPS TNF-α aus einer Vielzahl von Zelltypen. Zusätzlich ist aber die LPS Antwort stärker als unter OTA. Die OTA-vermittelte TNF-α Freisetzung aus Makrophagen könnte bisher nicht beachtete immuntoxikologische Eigenschaften des OTA erklären.

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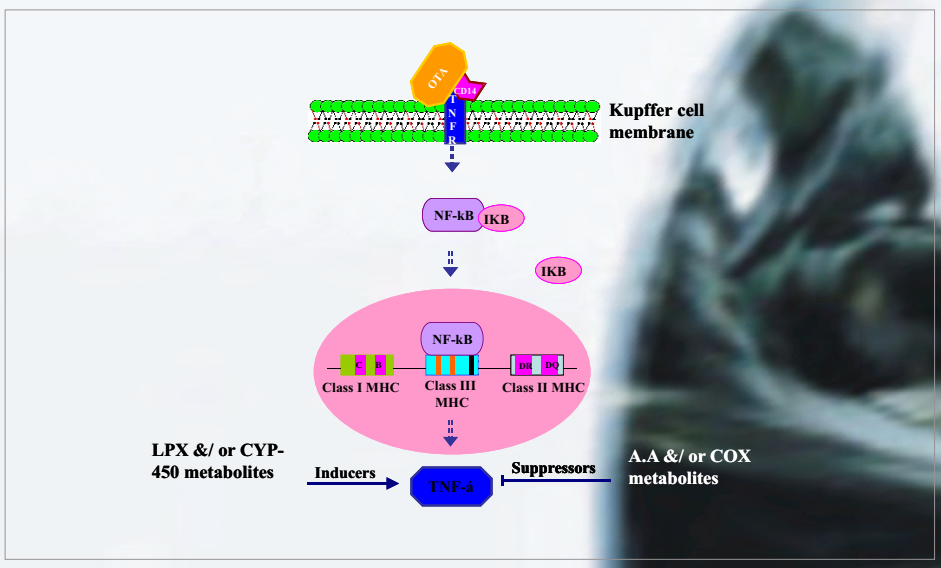

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