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Research Article

Silibinin protects OTA-mediated TNF-α release from perfused rat livers and isolated rat Kupffer cells

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We studied the inhibitory effect of silibinin on ochratoxin A (OTA) and LPS-mediated tumor necrosis factor α (TNF-α) release and the leakage of cytotoxic markers glutamate dehydrogenase (GLDH) and lactate dehydrogenase (LDH), from isolated blood-free perfused rat livers, and from isolated pure rat Kupffer cells. In the recirculation perfusion model at the end point 90 min, 2.5 μmol/L OTA released 2600 pg/mL TNF-α without effects on liver vitality. LPS at 0.1 μg/mL induced 3000 pg TNF-α/mL with slight leakage of GLDH and LDH. Under similar experimental conditions, the addition of silibinin 10 min prior to OTA and LPS showed dose-dependent protection against OTA or LPS-induced hepatic TNF-α release. High-dose of silibinin (12.5 μg/mL) also completely restored GLDH and LDH levels in the perfusate. Pretreatment of isolated Kupffer cells with 0.02, 0.1, 0.5, 2.5, and 12.5 μg silibinin/mL 30 min prior to OTA reduced OTA-induced TNF-α levels to 90, 70, 25, 25, and 25% at 4 h, respectively, and abrogated any TNF-α release at 24 h. Similarly, in the presence of silibinin LPS-induced TNF-α levels decreased at 4 h to 71, 57, 18, 22, and 18%, respectively. However, after 24 h of LPS exposition the protection by silibinin vanished and TNF-α partially recurred into the incubation medium under LPS. In summary, silibinin had hepatoprotective effects against OTA- or LPS-mediated TNF-α release and also reduced the cytotoxicity of both toxins. Isolated Kupffer cells were even more sensitive to the protective effect than perfused livers and responded to very low concentrations of silibinin with a strong inhibition of toxins-mediated TNF-α release.

Keywords: Kupffer cells / Lipopolysaccharide / Ochratoxin A / Silibinin / TNF-α

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1 Introduction

Silibinin is the major pharmacologically active compound extracted from the seeds of the milk thistle *Silybum marianum*. It is a widely used traditional herbal/dietary supplement around the world for its strong hepatoprotective activity against several liver toxins (carbon tetrachloride, ethanol, acetaminophen, and phenylhydrazine) [1–4]. Protective effects of silibinin have been described in different models of experimental liver intoxication and were related to modulation of signaling cascades in hepatocytes and Kupffer cells causing inhibition of nitric oxide production, tumor necrosis factor-α (TNF-α) release and lipid peroxidation [5, 6]. Silibinin has been also shown to be an immune-response modifier *in vivo* [6] which restores impaired liver functions following partial hepatectomy [7].

Ochratoxin A (OTA) is a natural mycotoxin produced from aspergillus and penicillium species. Hepatotoxicity is one, albeit an important event among diverse toxicological responses for OTA [8–12]. Apart from kidney, the liver is a target organ of OTA because of its food-borne exposure *via* the portal vein after mycotoxin absorption from the gut and because of an enterohepatic circulation of OTA [13], which means repeated exposure of liver cells to internally circulating OTA.

The hepatotoxic effects of OTA are characterized by necrotic and apoptotic changes in the liver, by single-chain breakdown of DNA in hepatocytes, and liver tumor development in mice [11, 14–17]. A marked release of cytokines, in particular TNF-α and IL-6 has been observed during per-

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Abbreviations: GLDH, glutamate dehydrogenase; **iNOS,** inducible nitric oxide synthase; **LDH,** lactate dehydrogenase; **OTA,** ochratoxin A; **TNF-α,** tumor necrosis factor α

fusion of the isolated rat liver with OTA [18]. This rat liver-derived TNF-α originated from Kupffer cells [19]. Its release was affected by eicosanoids, whereby leucotrienes and CYP-450 metabolites stimulated cytokine release, while arachidonic acid and cyclooxygenase-derived metabolites suppressed it [20].

In this study, we investigated the effect of silibinin on TNF-α release from blood-free perfused rat livers in respond to OTA or LPS, and subsequently from isolated pure Kupffer cells which served as TNF-α source *in vitro* [19].

2 Materials and methods

2.1 Chemicals and reagents

OTA was purchased from CSIR, Food Science and Technology, Pretoria, South Africa; it was free of LPS contamination as tested using the Limulus Amebocyte Lysate Endosafe® KTA test kit from (Charles River Wiga Sulzfeld, Germany). Silibinin was provided kindly from Dr. K. Odenthal from Madaus AG, Cologne, Germany. LPS (*Escherichia coli* serotype 0111:B4), heat fetal calf serum, trypane blue, modified Hank's balanced salt solution Ca^{2+} free, as well as modified Hank's balanced salt solution with Ca^{2+} were purchased from Sigma–Aldrich (Steinheim, Germany). Collagenase type CIS 212 U/mg and RPMI 1640 medium were from Biochrom AG (Berlin, Germany). Penicillin and streptomycin, trypsin/EDTA; were from Gibco® (Paisley, Scotland, UK). DNAase I was obtained from Boehringer (Mannheim, Germany) and Nycodenz® 5-(*N*-2,3-dihydroxypropylactemido)-2,4,6,tri-iodo-*N*,*N*′-bis (2,3 dihydroxypropyl) isophthalamide was from Axis-Shield PoCAS (Oslo, Norway). Pronase E was from E. Merck (Darmstadt, Germany). Enzyme linked immunosorbant assay (ELISA) kit Cytoscreen® was purchased from BioSource International (Camarillo, Canada), with antibodies selective for detection of rat TNF-α. Other materials and chemicals were purchased from international research suppliers.

2.2 Animals

Male Wistar rats (200–280 g) were used to prepare blood-free perfused livers or also isolated Kupffer cells. The animals were fed *ad libitum* with Altromin® standard diet and received water *ad libitum*. They were kept under 12 h 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.

2.3 Preparations of isolated blood-free perfused rat liver

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 died by exsanguination. Then, the liver was excorporated and perfused with Krebs–Henseleit solution for 10–15 min to completely remove the blood. The liver was then forwarded to a temperature- and oxygen-controlled perfusion system or was used for Kupffer cell preparations.

2.4 Liver perfusion

Isolated rat livers were perfused as described previously by Al-Anati *et al*. 2005 [20]. Briefly, blood-free rat livers were placed in an experimental perfusion setup, installed in a temperature-controlled hood. Livers were recirculated with 100 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. The livers were equilibrated with the perfusion buffer (without test compounds) during a pre-experimental period of 10 min. After that zero-samples from the perfusate were taken. Then, silibinin at different tested doses was injected *via* a portal vein inlet to the perfusion medium and 10 min later OTA, 1 μg/mL (2.5 μmol/L), or 0.1 μg/mL LPS were added at time point 20 min after zero-time.

For controls, each tested concentration of silibinin was given alone and then tested for in combination with OTA or LPS under identical experimental conditions. Basal TNF-α release was measured on isolated perfused rat livers without treatment.

2.5 Isolation of Kupffer cells

Kupffer cells were isolated according to the collagenase/pronase digestion method of Eyhorn *et al*. 1988 [21]. Briefly, blood-free isolated rats livers were perfused *via* the portal vein with a modified Hank's balanced Ca^{2+} free salt solution at 7.5 mL/min for 5–10 min. Then, livers were re-perfused with 80 mL RPMI 1.640 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 perfused for 30 min at 20 mL/min under gassing with air containing 5% CO_2. After that preperiod, livers were dissected and tissues digested for 15–30 min in 120 mL RPMI 1.640 medium containing 20 mg 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 three tubes and centrifuged (1800 rpm, 10 min at 4°C). The pellets were re-suspended in 10 mL Hank's balanced salt solution with

Ca^{2+}, and then centrifuged (1800 rpm, 10 min at 4°C) 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 two tubes. Fresh Hank's balanced salt solution (1 mL) 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. Afterwards, the upper three of four 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 and rotor. The outlets starting from samples 8 to 14 (32–51 mL/min) contained pure Kupffer cells as estimated by adspection under a microscope. The cells were collected and centrifuged at 1800 rpm for 10 min at 4°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 in a Bürker-Türk glass chamber and adjusted to 2×10^6/mL which were seeded in tissue culture plates. Experiments were carried out under culture conditions.

2.6 Culture conditions

Five milliliter of Kupffer cells suspension was incubated in media supplemented with 10% heat-inactivated fetal calf serum and penicillin/streptomycin at 37°C in a humidified atmosphere of 5% CO_2 air. The cells were incubated without OTA or LPS during a pre-experimental period of 2 h. After that pre-period, culture medium samples were taken. Then, 30 min later, silibinin at different doses was added into the incubation medium followed by 1 μg/mL (2.5 μmol/L) OTA or 0.1 μg/mL of LPS 1 h after zero-time sample.

2.7 Sampling schedule

Perfusate samples were collected at 0, 20, 30, 50, 70, and 90 min (the end point). Culture medium samples were collected at 0, 0.5, 1, 1.5, 2, 4 and 24 h (the end point). Samples were stored at –80°C until analysis of TNF-α by an ELISA test system detecting rat TNF-α according to the company's instruction.

2.8 Cytotoxicity markers

Vitality of the liver was determined by assaying lactate dehydrogenase (LDH) and glutamate dehydrogenase (GLDH) from the perfusate at 0, 20, 30, 50, 70, and 90 min in the buffer using diagnostic kits from Roche (Switzerland).

2.9 Statistical analysis

Data are presented as mean ± SEM of at least three separate trials *per* preparation obtained form different animals. Two way ANOVA statistical test was used for the analysis of variance followed by Bonferroni *t*-test. A *p*-value * < 0.05, ** < 0.01, and *** < 0.001 compared with control values was considered statistically significant. Data were analyzed by Graphpad Prism software, version 4.0 (San Diego, California, USA).

3 Results

3.1 OTA- and LPS-mediated TNF-α release from blood-free perfused rat livers

In previous studies, we had documented the effect of OTA on TNF-α release from blood-free perfused rat livers [19, 20] and compared it with well known LPS effects [10, 22]. In this study, we again found in the recirculating perfusion medium significant release of TNF-α following the addition of OTA into the perfusate. At 2.5 μmol/L OTA, 2600 pg/mL TNF-α occurred in the perfusion medium after 90 min without a significant increase in the cytotoxicity markers LDH and GLDH. LPS at 0.1 μg/mL released a similar amount of TNF-α, *i.e.*, 3000 pg/mL, at the end point, but this release occurred in parallel with a slight leakage of GLDH and LDH into the perfusate. Other cytotoxicity markers (lactate and potassium ion) were still low and their levels were much lower than those obtained after perfusing 0.01% digitonin, which caused destruction of liver cells and was used as a 100% positive control value [23]. Accordingly, these toxin doses which induced significant levels of TNF-α without effecting liver vitality, were selected for the further protection studies.

3.2 Inhibitory effects of silibinin on OTA- and LPS-mediated TNF-α release from blood-free perfused rat livers

Control perfusions in the presence of different doses of silibinin (0.5, 2.5 and 12.5 μg/mL) did not cause significant change of the level of basal TNF-α at any perfusion time point (Figs. 1B, 2B, and 3B, respectively). Furthermore, the basal levels of cytotoxic markers LDH and GLDH were not altered.

At its lowest concentration silibinin, 0.5 μg/mL, only slightly reduced OTA or LPS effects in the perfused liver model (Figs. 1C and F and Table 1). Addition of 2.5 μg/mL silibinin into the perfusion system inhibited 50% of OTA-induced TNF-α level at the end point 90 min (Fig. 2C). Also strong reductions on LPS-mediated TNF-α release were observed at 50, 70 and 90 min when 2.5 μg/mL silibinin was added prior to LPS, which amounted to 20, 40, and 50% of LPS-induced levels, respectively (Fig. 2F). The strongest effects of 2.5 μg/mL silibinin on LDH and GLDH levels took place 30 min after addition of the toxins (50 min after starting point). LDH and GLDH levels in the perfusate amounted to 100 and 63.4% of OTA-induced levels and 27.6 and 13.8% of LPS-induced levels, respectively (Table 1).

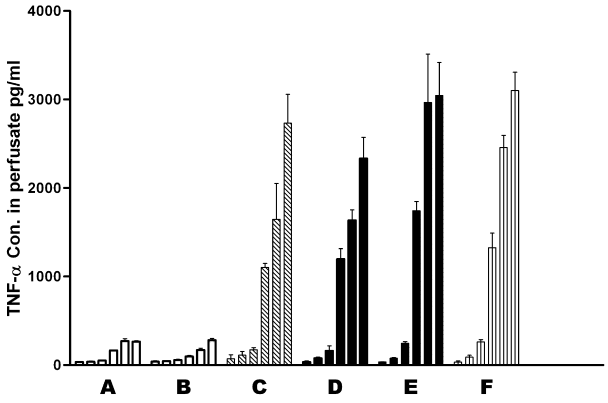

Figure 1. Effects of 0.5 μg/mL silibinin on OTA or LPS-mediated TNF-α release from blood-free perfused rat livers: TNF-α concentrations were measured in perfusate samples at 0, 20, 30, 50, 70, and 90 min. Samples were obtained from blood-free rat livers perfused with: (A) 2% dextran Krebs–Henseleit buffer medium alone (untreated), (B) 0.5 μg/mL silibinin, (C) 2.5 μmol/L OTA, (D) 0.5 μg/mL silibinin followed by 2.5 μmol/L OTA, (E) 0.1 μg/mL LPS, and (F) 0.5 μg/mL silibinin followed by 0.1 μg/mL LPS. The silibinin was applied at 10 min after zero time, while 2.5 μmol/L OTA or 0.1 μg/mL LPS at 20 min. Values represent the mean ± SEM of three livers for each group (*$p < 0.05$, ** $p < 0.01$, and ***$p < 0.001$).

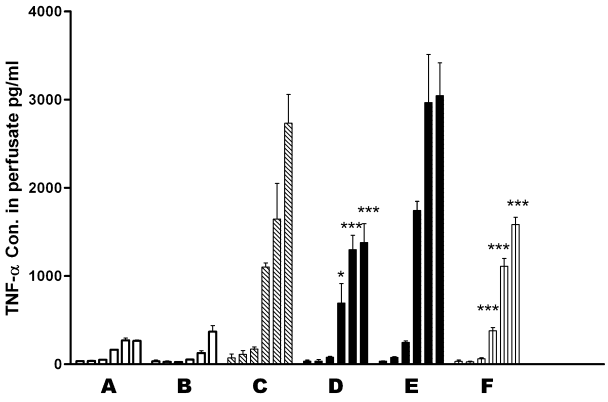

Figure 2. Effects of 2.5 μg/mL silibinin on OTA or LPS-mediated TNF-α release from blood-free perfused rat livers: TNF-α concentrations were measured in perfusate samples at 0, 20, 30, 50, 70, and 90 min. Samples were obtained from blood-free rat livers perfused with: (A) 2% dextran Krebs–Henseleit buffer medium alone (untreated), (B) 2.5 μg/mL silibinin, (C) 2.5 μmol/L OTA, (D) 2.5 μg/mL silibinin followed by 2.5 μmol/L OTA, (E) 0.1 μg/mL LPS, and (F) 2.5 μg/mL silibinin followed by 0.1 μg/mL LPS. The silibinin was applied at 10 min after zero time, while 2.5 μmol/L OTA or 0.1 μg/mL LPS at 20 min. Values represent the mean ± SEM of three livers for each group (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

At the highest concentration of silibinin (12.5 μg/mL) TNF-α levels amounted at the end point to only 20 and 30% of OTA- and LPS-mediated release (Figs. 3C and F), respectively, but were still higher than the basal control levels (without toxin treatment). At high-dose silibinin (12.5 μg/mL) LDH and GLDH levels in the perfusate

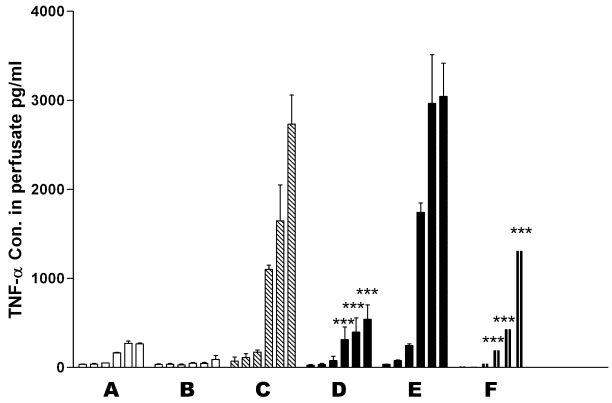

Figure 3. Effects of 12.5 μg/mL silibinin on OTA or LPS-mediated TNF-α release from blood-free perfused rat livers: TNF-α concentrations were measured in perfusate samples at 0, 20, 30, 50, 70, and 90 min. Samples were obtained from blood-free rat livers perfused with: (A) 2% dextran Krebs–Henseleit buffer medium alone (untreated), (B) 12.5 μg/mL silibinin, (C) 2.5 μmol/L OTA, (D) 12.5 μg/mL silibinin followed by 2.5 μmol/L OTA, (E) 0.1 μg/mL LPS, and (F) 12.5 μg/mL silibinin followed by 0.1 μg/mL LPS. The silibinin was applied at 10 min after zero time, while OTA or LPS were added at 20 min. Values represent the mean ± SEM of three livers for each group (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

30 min after the addition of each toxin were 90 and 55% of the OTA induced levels and 24 and 8% of the LPS induced levels (Table 1).

3.3 Inhibitory effect of silibinin on OTA- and LPS-mediated TNF-α release from Kupffer cells

Our previous findings showed that Kupffer cells strongly responded to 2.5 μmol/L OTA and released about 1000 pg TNF-α/mL culture medium within 24 h. This increase was ten times the basal TNF-α release from control cells. LPS at 0.1 μg/mL induced an even stronger and more rapid onset of TNF-α release on Kupffer cells. Silibinin at increasing concentrations of 0.02, 0.1, 0.5, 2.5, and 12.5 μg/mL diminished TNF-α release in a dose dependent-manner when added 30 min before OTA or LPS into the culture medium (Figs. 4 and 5).

The lowest silibinin doses 0.02 and 0.1 μg/mL reduced OTA-induced TNF-α level to 90 and 70% at 4 and 24 h, respectively. Higher doses of 0.5, 2.5, and 12.5 μg/mL abrogated any OTA-induced TNF-α release within 24 h (Fig. 4B).

Regarding pretreatment of Kupffer cells with silibinin at 0.02 or 0.1 μg/mL, a reduction of LPS-induced TNF-α levels occurred at 4 h to 71 and 57%, respectively. In the presence of higher silibinin concentrations (0.5, 2.5, and 12.5 μg/mL) TNF-α levels at 4 h were even more reduced to 20% of LPS-induced level (Fig. 5A). However, this protective effect of silibinin vanished at the end of the incubation time (24 h) when TNF-α recurred into the incubation

Table 1. Percentages of LDH and GLDH released from blood-free perfused rat livers during 30 min after addition of toxins (that is 50 min after starting perfusion), livers pretreated with 0.5 or 2.5 or 12.5 μg/mL silibinin 10 min before adding OTA or LPS

0.5 μg/mL Silibinin		2.5 μg/mL Silibinin		12.5 μg/mL Silibinin	
% of LDH release after 30 min of OTA or LPS addition					
2.5 μmol/L OTA 100% = 250.3 U/L	0.1 μg/mL LPS 100% = 547.5 U/L	2.5 μmol/L OTA 100% = 250.3 U/L	0.1 μg/mL LPS 100% = 547.5 U/L	2.5 μmol/L OTA 100% = 250.3 U/L	0.1 μg/mL LPS 100% = 547.5 U/L
100%	90%	100%	27.6%	88.9%	24.0%
% of GLDH release after 30 min of OTA or LPS addition					
2.5 μmol/L OTA 100% = 5.35 U/L	0.1 μg/mL LPS 100% = 5.7 U/L	2.5 μmol/L OTA 100% = 5.35 U/L	0.1 μg/mL LPS 100% = 5.7 U/L	2.5 μmol/L OTA 100% = 5.35 U/L	0.1 μg/mL LPS 100% = 5.7 U/L
100%	85%	63.4%	13.8%	55.3%	8.3%

Values represent the mean ± SEM of three cell preparations for each group (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

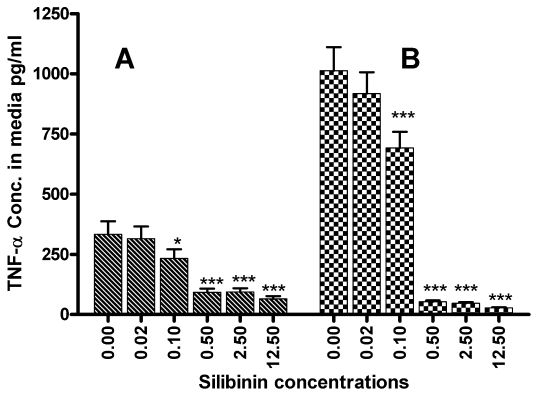

Figure 4. Effects of silibinin on OTA-mediated TNF-α release from Kupffer cells: TNF-α concentrations were measured in culture media samples, group A at 4 h and group B at 24 h. Kupffer cells were exposed to 0, 0.02, 0.1, 0.5, 2.5, and 12.5 μg/mL silibinin which was applied at 30 min after zero time, then 2.5 μmol/L OTA were added 30 min later. Values represent the mean ± SEM of three cell preparations for each group (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

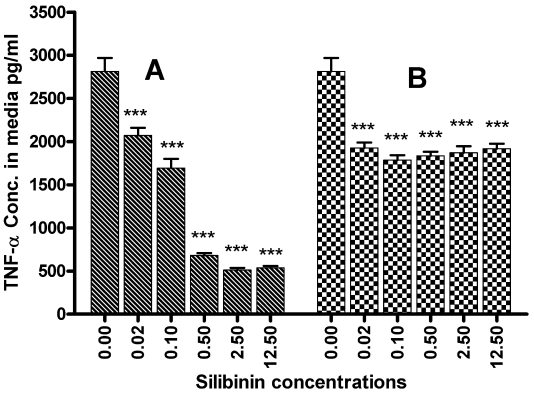

Figure 5. Effects of silibinin on LPS-mediated TNF-α release from Kupffer cells: TNF-α concentrations were measured in culture media samples, group A at 4 h and group B at 24 h. Kupffer cells were exposed to 0, 0.02, 0.1, 0.5, 2.5 and 12.5 μg/ml silibinin which was applied at 30 min after zero time, then 0.1 μg/ml LPS were added 30 min later. Values represent the mean ± SEM of three cell preparation for each group, (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

medium and reached 60–70% of LPS control levels (Fig. 5B).

4 Discussion

Silibinin is a flavanolignane isolated from the silymarin complex which derives from the seeds of the milk thistle (*S. marianum*). Silibinin is a hepatoprotective phytodrug approved for the treatment of chronic liver disease such as hepatitis and cirrhosis [24, 25] and for acute amanita phalloides mushroom poisoning [26, 27]. Silibinin manifests a variety of biological activities at different cellular levels in hepatocytes, including inhibition of the uptake of amatoxins [28] and phallotoxins [29]. It exerts strong antioxidative and membrane stabilizing activities as do other flavonoids [3, 30, 31]. The latter effects are due to the scavenging of free radicals and the inhibition of lipid peroxidation [5, 32]. Recently described effects include anti-inflammatory and immunomodulatory activity *via* the inhibition of NFκB activation [33, 34], which evoked benefits in a multiple sclerosis animal model [35]. Silibinin exerts an inhibitory effect on the expression of TNF-α and other proinflammatory cytokines [36] as well as on the intrahepatic synthesis of liver disease relevant mRNAs, *i.e.*, for IFN-γ, IL-4, and IL-2, and an intrahepatic reduction of inducible NO synthase (iNOS) and NFκB [34].

In previous papers we have described the release of TNF-α by the mould toxin OTA [19, 20] and described OTA-mediated hepatoxicity [23]. In line with this data we report in this paper on the protective effect of silibinin on TNF-α

release from perfused rat livers mediated by OTA and *E. coli* LPS.

We found in this liver model that the addition of 0.5 μg/mL silibinin 10 min prior to OTA and LPS did not influence OTA or LPS-induced TNF-α levels, but 2.5 μg/mL silibinin lowered TNF-α by 50% and restored the basal levels of LDH and GLDH. At 12.5 μg/mL, silibinin had a stronger effect and levels amounted to only 20 and 30% of OTA and LPS induced levels, respectively. At that high silibinin dose GLDH and LDH levels in the perfusate were completely restored. The two findings reported here are thus: (i) suppression by silibinin of TNF-α release from perfused rat livers and (ii) restoration of hepatoxicity marker enzymes.

Suppression of TNF-α release is an effect obviously related to Kupffer cells, as Kupffer cells are the only source for OTA-mediated TNF-α release in our rat liver model [19, 20]. Silibinin must have targeted Kupffer cells by affecting TNF-α release *via* the NFκB pathway. This assumption conveys with our previous data on the dependence of OTA effects on activated NFκB [19, 20] and on inhibition of NFκB activation by silibinin reported by Schümann *et al*. 2003 [6]. They observed hepatoprotective activity of silibinin in a mouse model of T cell- and TNF-α dependent liver injury, resembling autoimmune hepatitis, *via* inhibition of intrahepatic NFκB activation. This prevented the subsequent synthesis of TNF-α, IFN-γ, IL-2, and iNOS. Furthermore, they found that silibinin significantly enhanced production of IL-10, which vice versa prevented release of further cytokines particularly from Kupffer cells. Since, Kupffer cells are predominantly involved in this autoimmune disease, silibinin had a direct hepatoprotective activity likely due to cytoprotection of Kupffer cells.

TNF-α is directly toxic on hepatocytes *via* induction of apoptosis [37, 38] and also mediates intrahepatic induction of iNOS [39], which *via* NO plays a significant role in liver injury [40]. Thus, the prevention of TNF-α release from Kupffer cells in our study in turn may have prevented the release of vitality marker enzymes from hepatocytes. The potential importance of Kupffer cells as target cells of silibinin was also supported by a previously shown direct influence of silibinin on isolated rat Kupffer cells [5]. Whereas in isolated activated rat Kupffer cells generation of superoxide anion radical and nitric oxide were markedly blocked at high doses, low doses of silibinin blocked the formation of leukotriene B4. In this cell model no effect on TNF-α was reported. Thus, release blockade of TNF-α and/or LTB4 in Kupffer cells could account for hepatoprotective effects of silibinin.

To address this question further we incubated isolated rat Kupffer cells at different doses of silibinin 10 min before applying OTA or LPS. TNF-α release from Kupffer cells occurred under both stimuli, and this release was strongly inhibited by silibinin in a dose-dependent manner. At its highest concentration of 12.5 μg silibinin/mL the cytokine release was 25% of OTA controls after 4 h and zero after 24 h. In case of LPS the cytokine release was 18% of LPS controls after 4 h, but vanished after 24 h, when TNF-α levels in the incubation medium began to increase. The reason for this unexpected effect is not understood.

Whether silibinin could have beneficial effects against mycotoxin induced liver damage in general has not been widely documented. Apart from OTA, silibinin protection of the liver against the mycotoxins aflatoxin B1 (AFB1) and fumonisin B1 (FB1) was reported [41, 42]. The protective effect against FB1 was shown to be related to inhibition of TNF-α release, whereas prevention of aflatoxicosis was due to an antioxidative mechanism. Silibinin also increased the microsomal content of xenobiotic detoxifying glutathione-S transferase in human and mice liver cells [43]. Thus, prevention of mycotoxin induced liver damage by silibinin may occur by different mechanisms.

In conclusion, we show that hepatoprotective effects of silibinin against OTA- and LPS-mediated hepatotoxicity were related to the inhibition of TNF-α release from Kupffer cells. We also documented a significant reduction of cellular cytoxicity markers into the perfusate. Whereas prevention by silibinin was long lasting (for 24 h) in the case of OTA, it was less strong regarding the LPS effects and finally ceased before 24 h had passed.

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The authors have declared no conflict of interest.

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