

Original Article

Protective effect of xanthohumol on toxin-induced liver inflammation and fibrosis

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Received November 1, 2011; accepted December 6, 2011; Epub January 1, 2012; Published January 15, 2012

Abstract: Xanthohumol, the major prenylated chalcone found in hops, is known for its anti-inflammatory properties. We have recently shown that xanthohumol inhibits hepatic inflammation and fibrosis in a murine model of non-alcoholic steatohepatitis. The aim of this study was to investigate the effect of xanthohumol in an acute model of liver injury. Carbon tetrachloride (CCl₄), an industrial solvent, is a hepatotoxic agent and its administration is widely used as an animal model of toxin-induced liver injury. Xanthohumol was applied orally at a dose of 1 mg/g body weight 2 days prior as well as during and after exposure to CCl₄. 72 h after a single CCl₄ application histomorphology and serum levels of transaminases revealed considerable hepatocellular necrosis, which was accompanied by significantly enhanced hepatic expression of pro-inflammatory cytokines. Furthermore, elevated hepatic alpha-smooth muscle actin expression indicated activation of hepatic stellate cells, and in accordance, we detected enhanced hepatic expression levels of TGF- β and collagen type I reflecting a marked fibrogenic response to CCl₄ exposure. While the degree of hepatocellular damage in response to CCl₄ was similar in mice which received xanthohumol and the control group, pro-inflammatory and profibrogenic hepatic gene expression were almost completely blunted in xanthohumol fed mice. Furthermore, xanthohumol fed mice revealed decreased hepatic NF κ B activity. These results suggest that the protective effects of xanthohumol in this toxic liver injury model involves direct mechanisms related to its ability to block both hepatic inflammation and the activation of hepatic stellate cells, presumable at least in part *via* decreasing NF κ B activity. Thus, this study further indicates the potential of xanthohumol application to prevent or ameliorate the development and progression of liver fibrosis in response to hepatic injury.

Keywords: Xanthohumol, carbon tetrachloride, fibrosis, inflammation, acute liver injury

Introduction

Xanthohumol (XN) is the principal prenylated chalcone of the female inflorescences (hop cones, hops) of the hop plant *Humulus lupulus* L. and it has been shown to have several beneficial biological activities. Among them its chemopreventive and anti-inflammatory properties are the most extensively investigated [1, 2], and these are at least in part mediated *via* inhibition of the NF κ B signaling pathway [3-6]. We have shown previously that XN inhibits hepatic inflammation and fibrosis in a murine model of non-alcoholic fatty liver disease (NAFLD) [6]. NAFLD is considered as the most frequent liver disease in Western countries [7-9]. It is characterized by hepatocellular lipid accumulation, on the ground of which inflammation and fibrosis may develop. The histological picture closely

resembles alcoholic liver disease [10]. In non-alcoholic steatohepatitis (NASH) and alcoholic steatohepatitis (ASH) as well as in other chronic liver diseases like viral hepatitis, hepatic fibrosis is the peril that determines morbidity and mortality. Cirrhosis, as the end stage of hepatic fibrosis, is a major clinical issue for its high prevalence in the world and its tight relationship with hepatocellular carcinoma incidence [11-13]. Hepatic fibrosis is characterized by an excessive and aberrant deposition of extracellular matrix (ECM) proteins in the liver, the most abundant of which is collagen type I [14]. Activated hepatic stellate cells (HSC) are the cellular source of the excessive ECM deposition [15-17]. Normally, these cells are quiescent and produce only small amounts of ECM components, such as laminin and collagen type IV, during the formation of basement membrane

[18]. However, in response to hepatic injury HSC get activated and transform into a myofibroblast-like phenotype, expressing alpha-smooth muscle actin (α -SMA), and dramatically increase the production of collagens [19]. Importantly, the transcription factor NF κ B plays a crucial role in HSC activation [20, 21, 15, 22].

In addition to metabolic overload, alcohol or viral infection the liver is frequently exposed to various insults, including toxic chemicals [23, 24]. Liver damage caused by hepatotoxic chemicals induces compensatory hepatic hyperplasia after severe liver necrosis due to direct damage of hepatocytes and subsequent inflammation [25]. Carbon tetrachloride (CCl₄), an industrial solvent, is a hepatotoxic agent and its administration is widely used as an animal model of toxin-induced liver injury that allows the evaluation of both necrosis and subsequent inflammation [26] as well as fibrosis [27]. In contrast to our previously used NASH-model which led to only mild hepatocellular damage and inflammation [6], CCl₄ application results in excessive necrotic and apoptotic death of hepatocytes, which induces the activation of HSC.

To investigate the effect of XN on acute liver injury and to further study its role in liver fibrosis, we subjected mice, which were treated with and without XN at a dose of approximately 1 mg/g body weight, to acute CCl₄-induced liver damage.

Methods

Chemicals and animal feeds

Carbon tetrachloride (CCl₄) and olive oil were obtained from Sigma Pharmaceuticals (Hamburg, Germany). Xanthohumol (XN) was obtained from Alexis Biochemicals (Lausen, Switzerland) with a purity \geq 98% determined by HPLC. All chows were prepared by Ssniff (Soest, Germany).

CCl₄-induced acute liver injury

A single dose of CCl₄ (1 μ l/g body weight in olive oil) was intraperitoneally injected to 10 weeks old female BALB/c mice. 72 h after CCl₄ injection, mice were killed by heart puncture under deep ketamine/xylazine (2:1) anesthesia, and liver tissue and blood samples were collected for further analysis. Livers and blood from olive oil treated animals served as controls.

Histology

For histological analysis murine liver tissue specimens were fixed for 24 h in 4% formalin at room temperature, dehydrated by graded ethanol and embedded in paraffin. Tissue sections (thickness 5 μ m) were deparaffinized with xylene and stained with eosin/haematoxylin (H&E) as described [28].

Quantitative real time-PCR analysis

RNA isolation from liver tissue and reverse transcription were performed as described [29]. Quantitative real time-PCR was performed applying LightCycler technology (Roche, Mannheim, Germany) as described [30] applying the following pairs of primers: murine collagen-I (for: 5'-CGG GCA GGA CTT GGG TA; rev: 5'-CGG AAT CTG AAT GGT CTG ACT) and murine MCP-1 (for: 5'-TGG GCC TGC TGT TCA CA; rev: 5'-TCC GAT CCA GGT TTT TAA TGT A). All other mRNA expression analyses were performed using QuantiTect Primer Assays according to the manufacturer's instructions (Qiagen, Hilden, Germany). Amplification of cDNA derived from 18s rRNA (for: 5'-AAA CGG CTA CCA CAT CCA AG; rev: 5'-CCT CCA ATG GAT CCT CGT TA) was used for normalization.

Quantification of NF κ B activity

Liver tissue extracts were obtained by homogenization of snap-frozen liver tissue in Cell Lysis Buffer from Cell Signaling (Danvers, MA, USA) supplemented with 1 mM PMSF and a protease inhibitor cocktail (cOmplete Mini Protease Inhibitor Cocktail Tablets from Roche Diagnostics, Mannheim, Germany) using a MICCRA D1 homogenizer (ART Prozess- & Labortechnik, Mülheim, Germany), and subsequent sonication (with a Sonopuls HD 70 from Bandelin electronics, Berlin, Germany) and centrifugation. Activated NF κ B was quantified in liver tissue extracts via ELISA-technique using the PathScan Phospho-NF κ B p65 (Ser536) Sandwich ELISA Antibody Pair from Cell Signaling following the manufacturer's protocols as described [6].

Statistical analysis

Values are presented as mean \pm SEM. Comparison between groups was made using the Mann Whitney test. A p value $<$ 0.05 was considered statistically significant. All calculations were performed using the statistical computer pack-

age GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, Ca, USA).

Results

Effect of xanthohumol on hepatocellular damage in toxin induced liver injury

To assess the effect of xanthohumol (XN) in the model of carbon tetrachloride (CCl₄) induced toxic liver injury mice were fed with control chow or a diet containing 0.5% (w/w) XN resulting in a daily dose of approximately 1 mg/g body weight (BW). After 2 days of XN-feeding, mice received a single dose of CCl₄ (1 µl/g BW) or the same volume of the pure solvent (olive oil). Feeding of XN or control chow was continued, and 3 days after CCl₄ application mice were sacrificed. At this time body weight did not differ significantly between control mice (18.2 ± 1.3 g) and XN-fed (18.3 ± 0.2 g) animals, and mice receiving CCl₄ alone (16.4 ± 1.3 g) or CCl₄ together with XN (16.8 ± 0.4 g). However, liver weight and liver to body weight ratio, respectively, were significantly elevated in CCl₄-treated mice but did not differ between XN- and control chow-fed mice (**Figure 1A**). Macroscopically, livers of CCl₄-treated mice were larger and revealed a pale and irregular surface indicative of severe hepatocellular damage (**Figure 1B**). Histopathological analysis confirmed large areas of necrotic tissue in the central zones of the livers of CCl₄-treated mice with no significant differences between the XN-fed and control chow-fed group (**Figure 1C**). Hepatocellular damage in the CCl₄ groups was also reflected by a marked increase of serum transaminases compared to both control groups, which was similar in XN-fed and control chow-fed mice (**Figure 1D and 1E**).

Effect of xanthohumol on hepatic inflammation and fibrosis in toxin induced liver injury

To determine whether XN affects the inflammatory response after acute CCl₄-mediated liver injury we analyzed the expression of pro-inflammatory cytokines in injured livers using semi-quantitative real-time PCR. 72 h after CCl₄ injection, hepatic mRNA expression levels of tumor necrosis factor (TNF) and interleukin-1 alpha (IL-1α) were significantly elevated in mice without XN-feeding. In contrast, inflammatory gene expression was almost completely blunted in CCl₄-treated mice by XN-feeding (**Figure 2A and 2B**). Similar results were obtained when

analyzing hepatic mRNA expression of monocyte chemoattractant protein-1 (MCP-1) and intercellular adhesion molecule-1 (ICAM-1) (**Figure 2C and 2D**), both genes which are strongly regulated by the transcription factor NFκB. In line with these and previous *in vitro* findings by our group [6, 31] and others [3-6] we found 72 h after CCl₄-injection significantly lower hepatic NFκB activity in XN-fed mice compared to control chow-fed animals (**Figure 2D**).

Next, we determined if the reduced inflammation seen in CCl₄-treated and XN-fed mice is reflected by down-regulation of genes that mediate the fibrotic response. Levels of TGF-β mRNA were markedly induced in control chow-fed CCl₄-treated mice while in XN-fed CCl₄-treated mice expression levels of this cytokine, which plays a crucial pathophysiological role in liver fibrosis [32, 33], were not elevated compared to control mice (**Figure 3A**). In addition, expression of genes encoding collagen type I (Coll-I) and tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) were induced after CCl₄-treatment, but this increase was inhibited by XN application (**Figure 3B and 3C**). The expression of these genes reflects the onset of tissue remodeling processes, which can lead to fibrosis [34]. In line with this, a significant increase of alpha-smooth muscle actin (α-SMA) expression was observable in control chow-fed but not in XN-fed mice after CCl₄-treatment, indicating a XN-mediated inhibition of injury-triggered HSC activation (**Figure 3D**). These data indicate that livers of XN-fed animals display a diminished pro-inflammatory and pro-fibrotic response after CCl₄-induced injury.

Discussion

The aim of this study was to investigate the effect of xanthohumol (XN) in an acute model of liver injury and to further study its role in liver fibrosis. For this purpose we subjected mice fed with and without XN (at a dose of approximately 1 mg/g body weight) to acute CCl₄-induced liver damage. Our results reveal a profound inhibitory effect of XN on pro-inflammatory and pro-fibrogenic hepatic gene expression in this model. Noteworthy, these effects occurred despite the fact that hepatocellular injury as reflected by serum levels of transaminases or histomorphological analysis was comparable between control mice and XN-fed mice 72 h after CCl₄-injection. These findings suggest that

Xanthohumol effects in toxic liver injury

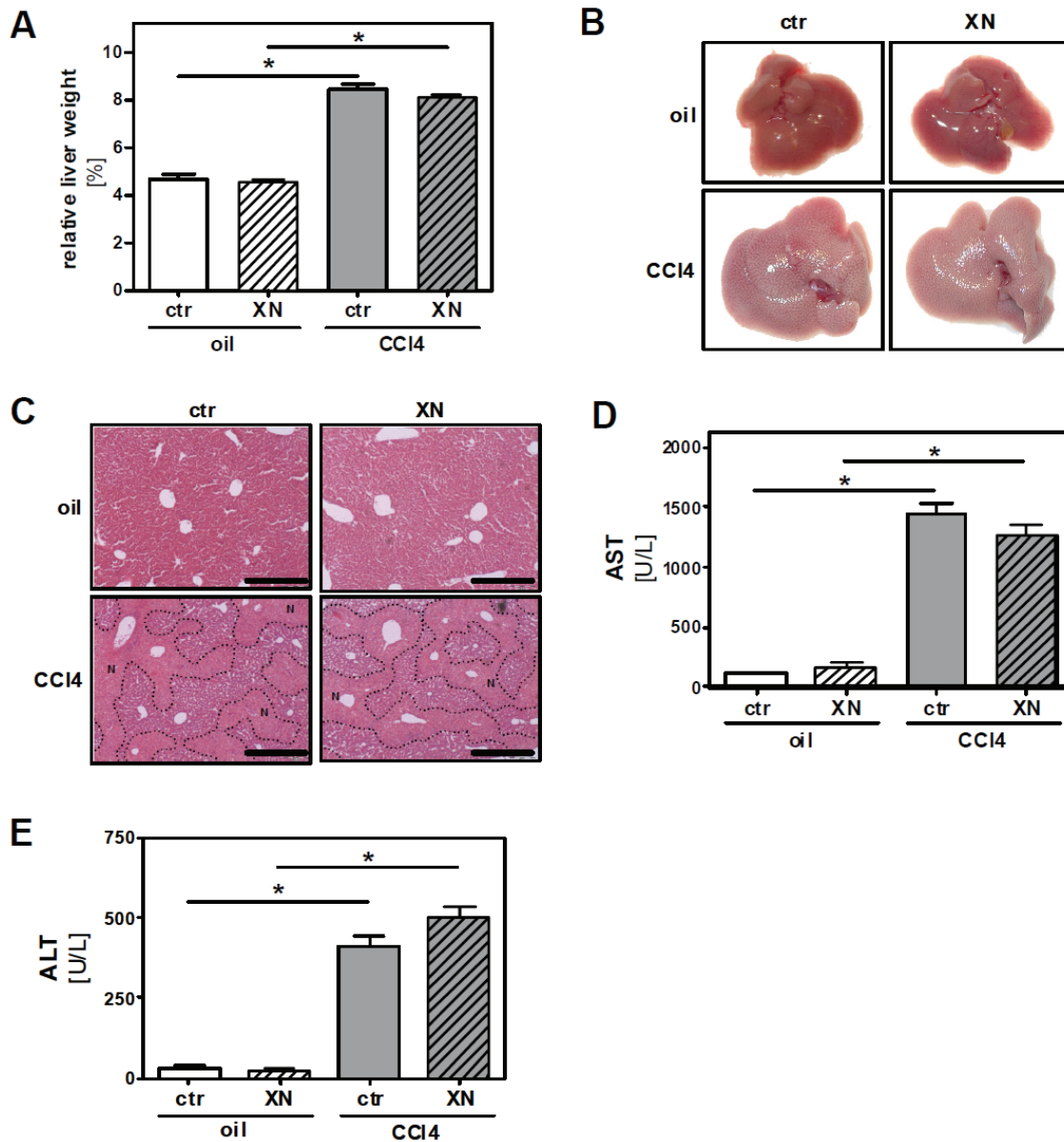


Figure 1. Effect of xanthohumol on hepatocellular damage in toxin induced liver injury. Mice were fed either with control chow (ctr.) or with the same chow supplemented with 0.5% (w/w) xanthohumol (XN). After 2 days of feeding a single dose of CCl₄ (1 μl/g body weight in olive oil) or olive oil alone (oil) was injected intraperitoneally to mice from both the control chow-fed and XN-fed group. 72 h after CCl₄ or olive oil injection mice were sacrificed. (A) Liver-to-body-weight-ratio (*: p<0.05). (B) Representative macroscopic images of livers from the four treatment groups. (C) HE-staining of liver tissue. Necrotic areas are marked out by dotted lines. Black bars represent 0.5 mm. (D) AST and (E) ALT serum levels (*: p<0.05).

the suppressive effect of XN against the progress of acute CCl₄-induced hepatic fibrosis involves direct mechanisms related to its ability to block both hepatic inflammation and the activation of hepatic stellate cells (HSC). Actually, we have previously shown that XN exhibits direct

anti-inflammatory effects on HSC and inhibits the activation of these cells, respectively, by inhibiting IκBα degradation and subsequent NFκB activation [6]. Notably, these anti-fibrogenic effects have been observed at concentrations as low as 5 μM. Previous studies

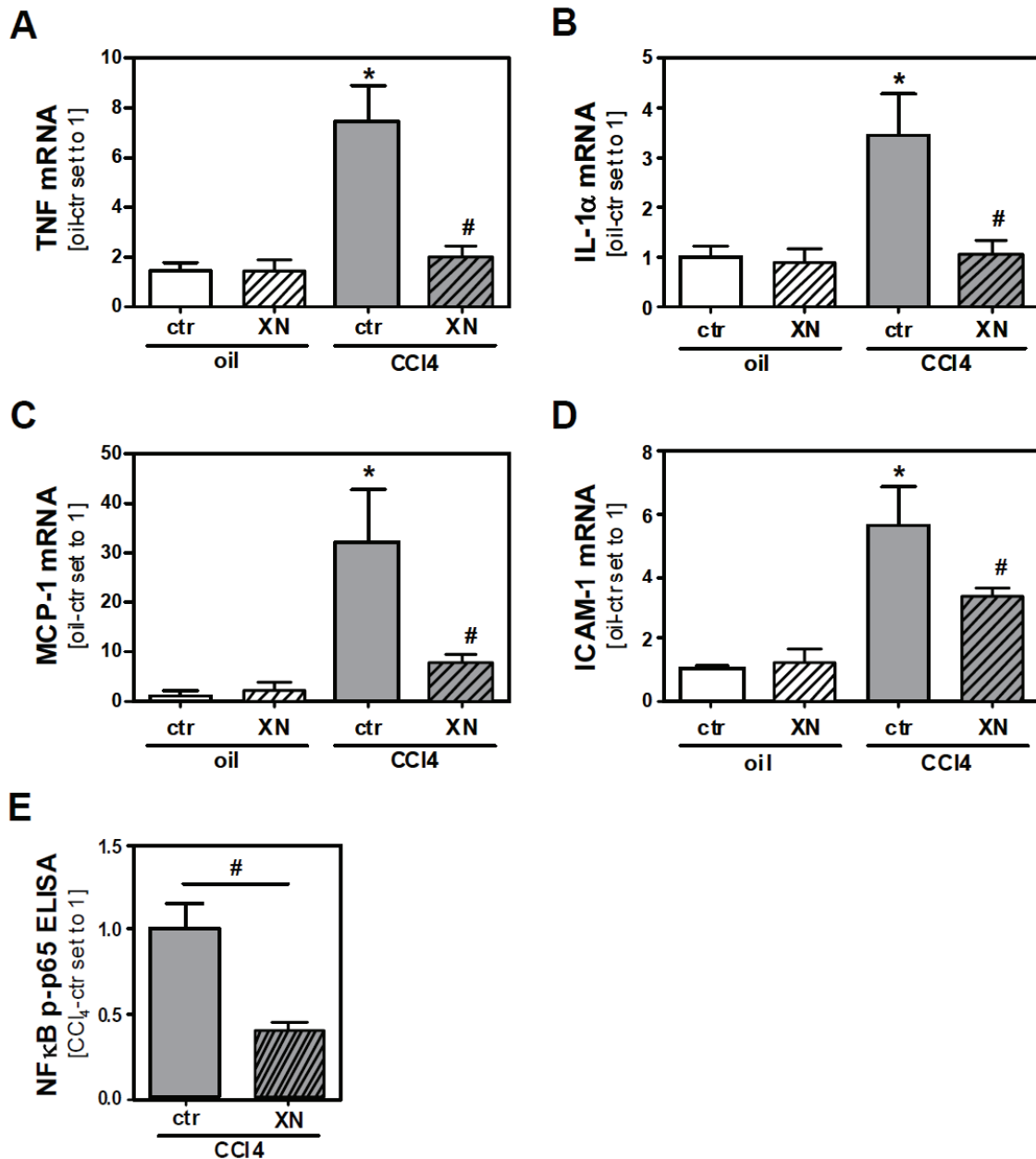


Figure 2. Effect of xanthohumol on hepatic inflammation in toxin induced liver injury. Mice were fed either with control chow (ctr) or with the same chow supplemented with 0.5% (w/w) xanthohumol (XN). After 2 days of feeding a single dose of CCl₄ (1 μl/g body weight in olive oil) or olive oil alone (oil) was injected intraperitoneally to mice from both the control chow-fed and XN-fed group. 72 h after CCl₄ or olive oil injection mice were sacrificed. Analysis of hepatic mRNA levels of (A) TNF, (B) IL-1α, (C) MCP-1 and (D) ICAM-1 by quantitative RT-PCR (*: p<0.05 compared to oil-ctr; #: p<0.05 compared to CCl₄-ctr). (E) Comparison of NFκB activity in livers of control chow-fed and XN-fed mice 72 h after CCl₄-injection via ELISA-based quantification of phospho-p65 (Ser536) (*: p<0.05).

could not detect (unmetabolized and unconjugated) XN in the systemic circulation upon oral application [35, 36]. However, the anatomical situation of the liver has to be considered. It can

be expected that after oral intake the XN concentration in the portal vein is higher than in the systemic circulation. Further, HSC are located in the liver in the space of Disse (or perisinusoidal

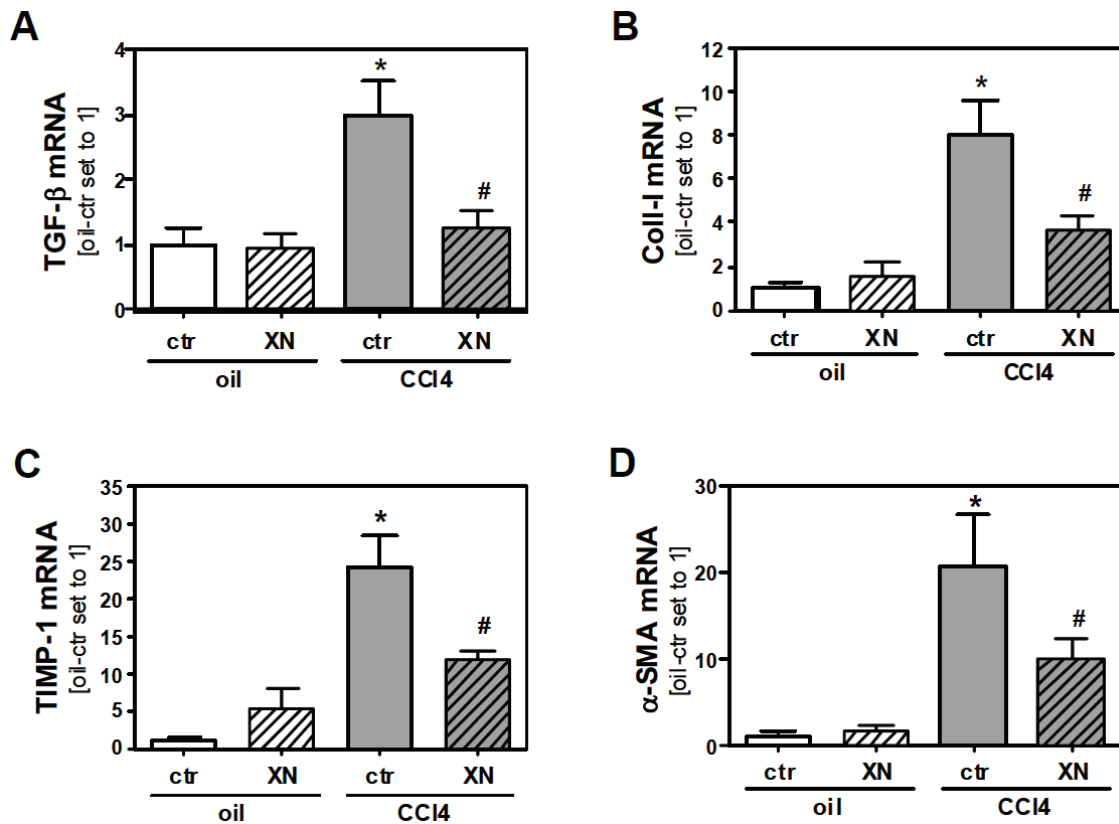


Figure 3. Effect of xanthohumol on toxin induced fibrogenic response of the liver. Mice were fed either with control chow (ctr) or with the same chow supplemented with 0.5% (w/w) xanthohumol (XN). After 2 days of feeding a single dose of CCl₄ (1 μl/g body weight in olive oil) or olive oil alone (oil) was injected intraperitoneally to mice from both the control chow-fed and XN-fed group. 72 h after CCl₄ or olive oil injection mice were sacrificed. Analysis of hepatic mRNA levels of (A) TGF-β, (B) Coll-I, (C) TIMP-1 and (D) α-SMA by quantitative RT-PCR (*: p<0.05 compared to oil-ctr; #: p<0.05 compared to CCl₄-ctr).

space), i.e. between the sinusoid and the hepatocytes. Herewith, HSC are directly exposed to XN concentration reaching the liver *via* the portal vein irrespective of (subsequent) metabolism in hepatocytes. Thus, XN concentrations reaching HSC in the space of Disse may be significantly higher than the levels in whole liver tissue.

In our previous study we have shown that XN inhibits hepatic inflammation and fibrosis in mice in a NASH model [6]. In this model hepatocellular lipid accumulation led to a mild inflammation and fibrogenic response after 3 weeks of feeding a NASH inducing diet. In contrast, the CCl₄-model is used to induce extended hepatocellular death and inflammation, which in turn promotes fibrosis. The marked anti-fibrogenic effect of XN under these acute and harsh ex-

perimental conditions further advances the concept that XN is a promising natural substance with the potential to inhibit the development and progression of hepatic fibrosis in patients with (chronic) liver disease.

Acknowledgements

We want to thank Ruth Schewior and Marina Fink for excellent technical assistance. This work was supported by grants from the German Research Association (He 2458/14-1 to C.H.) and the Medical Faculty of the University of Regensburg (ReForM) to C.H. Further, this project was supported in part by an unrestricted research grant from the Joh. Barth & Sohn GmbH (Nuremberg, Germany). Financial relationships of the authors with Joh. Barth & Sohn GmbH are as follows: C.H. is a consultant, and C.D. is work-

ing in the laboratory of C.H. All authors had complete and independent control over the study design, analysis and interpretation of data, report writing, and publication, regardless of results.

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