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Xanthohumol suppresses inflammatory response to warm ischemia–reperfusion induced liver injury

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ABSTRACT

Liver ischemia/reperfusion (I/R) leads to formation of reactive oxygen species (ROS), which cause hepatic injury and initiate an inflammatory response, which is a critical problem after liver surgery and transplantation. Xanthohumol, the major prenylated chalcone found in hops, has been discussed for its anti-inflammatory and ROS-scavenging properties, and thus, we aimed to investigate the effect of xanthohumol in a model of warm I/R liver injury. Xanthohumol was applied to BALB/c mice orally at a dose of 1 mg/g body weight for 5 days before I/R-injury was induced by clamping the vascular blood supply to the median and left lateral liver lobe for 1 h followed by a 6 h period of reperfusion. At this time, HPLC analysis revealed hepatic xanthohumol levels of approximately 2 μM, a concentration which has been shown to inhibit inflammatory effects *in vitro*. Assessment of hepatic HMOX1 expression, hepatic glutathione content and immunohistochemical analysis for proteins conjugated with the reactive aldehyde 4-hydroxynonenal indicated that I/R-induced oxidative stress was significantly inhibited in xanthohumol-fed compared to control mice. Histological analysis, TUNEL staining and determination of transaminase serum levels revealed no significant effects of xanthohumol on acute hepatocellular injury. However, at the same time point, pretreatment with xanthohumol almost completely blunted the I/R-induced AKT and NFκB activation and the expression of the proinflammatory genes IL-1alpha, IL-6, MCP-1 and ICAM-1, which are known to play a crucial role in the subacute phase of I/R-induced liver damage. In conclusion, these data indicate the potential of xanthohumol application to prevent adverse inflammatory responses to I/R-induced liver damage such as after surgical liver resection or transplantation.

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Introduction

Hepatic ischemia/reperfusion (I/R) injury occurs in a variety of clinical scenarios, including transplantation, liver resection, trauma, and hypovolemic shock. The molecular mechanisms causing liver injury after I/R have been subject of several studies, which revealed a series of complex interactions of various participant inflammatory pathways (Walsh et al., 2009; Zwacka et al., 1998a). The process of hepatic I/R-injury can be divided into two phases; an acute phase (the first 6 h after reperfusion) and the following subacute phase (Fan et al., 1999). The acute phase is characterized by acute generation of reactive oxygen species (ROS) subsequent to reoxygenation of the liver leading to marked hepatocellular damage, measurable by an increase of serum alanine transaminase levels peaking 3 to 6 h after reperfusion (Parks and Granger, 1988; Rauen et al., 1994). The secondary subacute phase is associated with vigorous inflammatory responses and the progression of necrotic processes (Gujral et al., 2001; Zwacka et al., 1998a). This can lead to chronic liver inflammation, a decline in liver function, and eventually complete organ failure.

Whereas earlier studies suggested that hepatic I/R-damage was mainly inflicted in the acute phase by ROS directly, more recent data indicate that oxidative stress may injure the tissue more indirectly by initiating a cascade of adverse cellular responses leading to inflammation, *e.g.* by activating redox-sensitive transcription factors such as NFκB (Jaeschke, 1995; Shin et al., 2008; Zwacka et al., 1998b). Antioxidant as well as anti-inflammatory therapies depict two practicable and medically sensible treatment options for preventing or ameliorating I/R-induced liver injury. Antioxidant substances could reduce I/R-induced tissue damage directly caused by ROS and, more importantly, attenuate the ROS-triggered inflammatory response which could be further suppressed by additional anti-inflammatory therapies.

Xanthohumol (XN), the principal prenylated chalcone of the hop plant (*Humulus lupulus* L.), has been discussed for its antioxidant (Gerhauser et al., 2002; Miranda et al., 2000; Yamaguchi et al., 2009) as well as anti-inflammatory properties (Dorn et al., 2010b; Lupinacci

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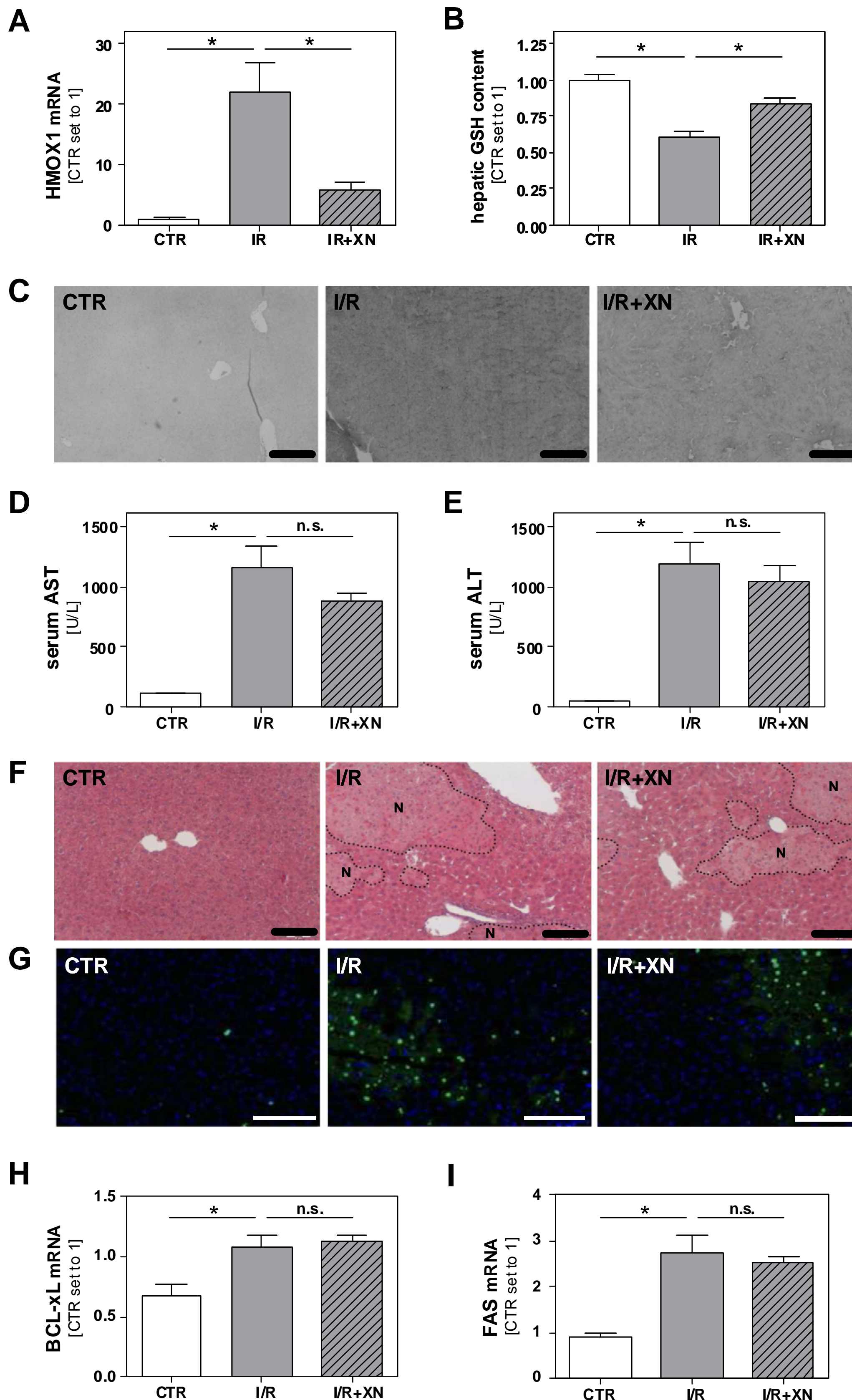
et al., 2009; Stevens and Page, 2004) suggesting this natural substance as a potential candidate for an I/R-damage-preventing treatment of the liver. In addition, XN is known as an inhibitor of the transcription factor NFκB (Albini et al., 2006; Colgate et al., 2007; Dorn et al., 2010c) whose activity increases upon hepatic I/R (Suetsugu et al., 2005; Tacke et al., 2009). Whereas it has been unclear for a long time whether NFκB-dependent signaling withholds a protective or damaging role in I/R, more recent data show that the NFκB pathway does not serve as a



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survival pathway in hepatic I/R, but instead can aggravate hepatocellular death and liver damage (Luedde et al., 2005; Suetsugu et al., 2005). NFκB-mediated expression of adhesion molecules like ICAM-1 (Inter-Cellular Adhesion Molecule-1) may be responsible for increased tissue damage inflicted by infiltrating neutrophils in the late phase of hepatic I/R-injury (Jaeschke, 2006; Jaeschke et al., 1996). However, as shown by Beraza et al. (2007), complete abolishment of NFκB activation in conditional NEMO-knockout mice resulted in massive hepatic inflammation and apoptosis after I/R indicating that suppression of NFκB induction is preferable to complete inhibition of basal NFκB activity for preventing I/R-induced liver injury.

The aim of this study was to investigate the effects of XN on oxidative stress, hepatocellular damage and inflammation in the acute phase of a murine model of warm I/R-injury.

Methods

Animal model of warm ischemia/reperfusion injury of the liver

Male BALB/c mice were purchased from Charles River Laboratories (Sulzfeld, Germany) at 8 weeks of age and housed in a 22 °C controlled room under a 12 h light–dark cycle with free access to food and water. After one week of acclimatization mice were fed either with standard diet (control) or standard diet supplemented with 0.5% (w/w) XN. XN was obtained from the Nookandeh Institute for Natural Chemicals (Homburg/Saar, Germany) with a purity ≥98% determined by HPLC. All chows were prepared by Ssniff (Soest, Germany). After 5 days of feeding mice were subjected to a model of warm ischemia–reperfusion injury as described in detail by Abe et al. (2009). Briefly, mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) by intraperitoneal (*i.p.*) injection. Median laparotomy was performed and portal vein, hepatic artery and bile duct were clamped with a non-traumatic microvascular clamp (Fine Science Tools, Heidelberg, Germany) just above the branching to the right lateral lobe, resulting in ischemia in approximately 70% of the liver. After 60 min clamps were removed leading to fast reperfusion of the ischemic liver lobes. Six hours after reperfusion mice were killed by heart puncture under ketamine/xylazine anesthesia, and blood samples and liver tissue were collected for further analyses. All animals received humane care in compliance with institutional guidelines. The study was approved by the local Institutional Review Board.

Histological and immunohistological analysis

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 μm) were deparaffinized with xylene and stained with haematoxylin/eosin (H&E) as described (Gabele et al., 2008). TUNEL and 4-hydroxynonenal staining were performed as described previously (Amann et al., 2010; Gabele et al., 2011).

Hepatic glutathione content

Hepatic glutathione (GSH) content was measured using the colorimetric microplate assay from Oxford Biomedical Research (Oxford, MI, USA) according to manufacturer's instructions using 40 mg of snap-frozen liver tissue.

Quantification of activated NFkB concentration in hepatic tissue

NFκB was quantified in liver extracts with the ELISA-based kit PathScan Phospho-NFκB from Cell Signaling Technology (Danvers, MA, USA) as described (Dorn et al., 2010b).

Western blotting

Protein extraction and Western blotting were performed as described (Dorn et al., 2010b) applying anti-rabbit anti-bodies against phospho-IκB-α (#2859), IκB-α (#4812), phospho-AKT (#4058) and AKT (#9272), all from Cell Signaling Technology according to manufacturer's recommendations (all diluted 1:1000).

Quantitative real time-PCR analysis

RNA isolation from liver tissue and reverse transcription were performed as described (Hellerbrand et al., 2006). Quantitative real time-PCR was performed applying LightCycler technology (Roche, Mannheim, Germany) as described (Muhlbauer et al., 2003) applying the following pairs of primers: 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).

Quantification of xanthohumol concentration in hepatic tissue

Liver tissue samples (200 mg) were thoroughly homogenized in 5 ml of methanol at 4 °C using a MICCRA D1 homogenizer from ART Prozess- & Labortechnik (Müllheim, Germany) and subsequently sonicated with a Sonopuls HD 70 from Bandelin electronics (Berlin, Germany). After centrifugation (20,000 *g*, 4 °C, 15 min) the supernatants were further purified using NanoSep centrifugal filter devices (#5168502 from VWR, Darmstadt, Germany). Subsequently, the purified fraction was applied to HPLC analysis. HPLC was performed using a 1200 SL Agilent system (Santa Clara, California, US) with Diode Array Detection at 370 nm and a water/acetonitrile gradient.

Statistical analysis

Values are presented as mean ± SEM. Comparison between groups was made using the Student's unpaired *t*-test. Welch's correction was performed if required. A p-value <0.05 was considered statistically significant. All calculations were performed using the statistical computer package GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA).

Results

Effect of xanthohumol on oxidative stress induced during the acute phase of hepatic ischemia–reperfusion

To assess the effect of XN on ischemia/reperfusion (I/R) injury, XN was applied orally to mice by supplementation of the standard diet with XN at a concentration of 5 mg/g chow starting 5 days prior to

Fig. 1. Effect of xanthohumol on oxidative stress and hepatocellular death in the acute phase of hepatic I/R-injury. Mice were fed either with standard chow or the same chow supplemented with 0.5% (w/w) xanthohumol (XN) for 5 days before subjecting them to a model of warm ischemia–reperfusion (I/R) liver injury (I/R and I/R+XN, respectively). Control group (CTR) received standard chow and underwent sham-surgery. (A) Analysis of hepatic heme oxigenase 1 (HMOX1) mRNA expression and (B) relative quantification of hepatic glutathione (GSH) content. (C) Representative microscopic images of liver tissue sections stained for 4-hydroxynonenal (4-HNE). Black bars represent 0.2 mm. (D) Aspartate aminotransferase (AST) and (E) alanine aminotransferase (ALT) serum levels. (F) Representative microscopic images of haematoxylin/eosin- or (G) TUNEL-stained liver tissue sections, respectively. Necrotic areas (N) are marked out by dotted lines. Black and white bars represent 0.2 mm. (H) Hepatic mRNA expression of the anti-apoptotic BCL-xL and (I) the pro-apoptotic FAS (CD95) gene. Bars and whiskers represent mean ± SEM, respectively (*: p<0.05; n.s.: not significant).

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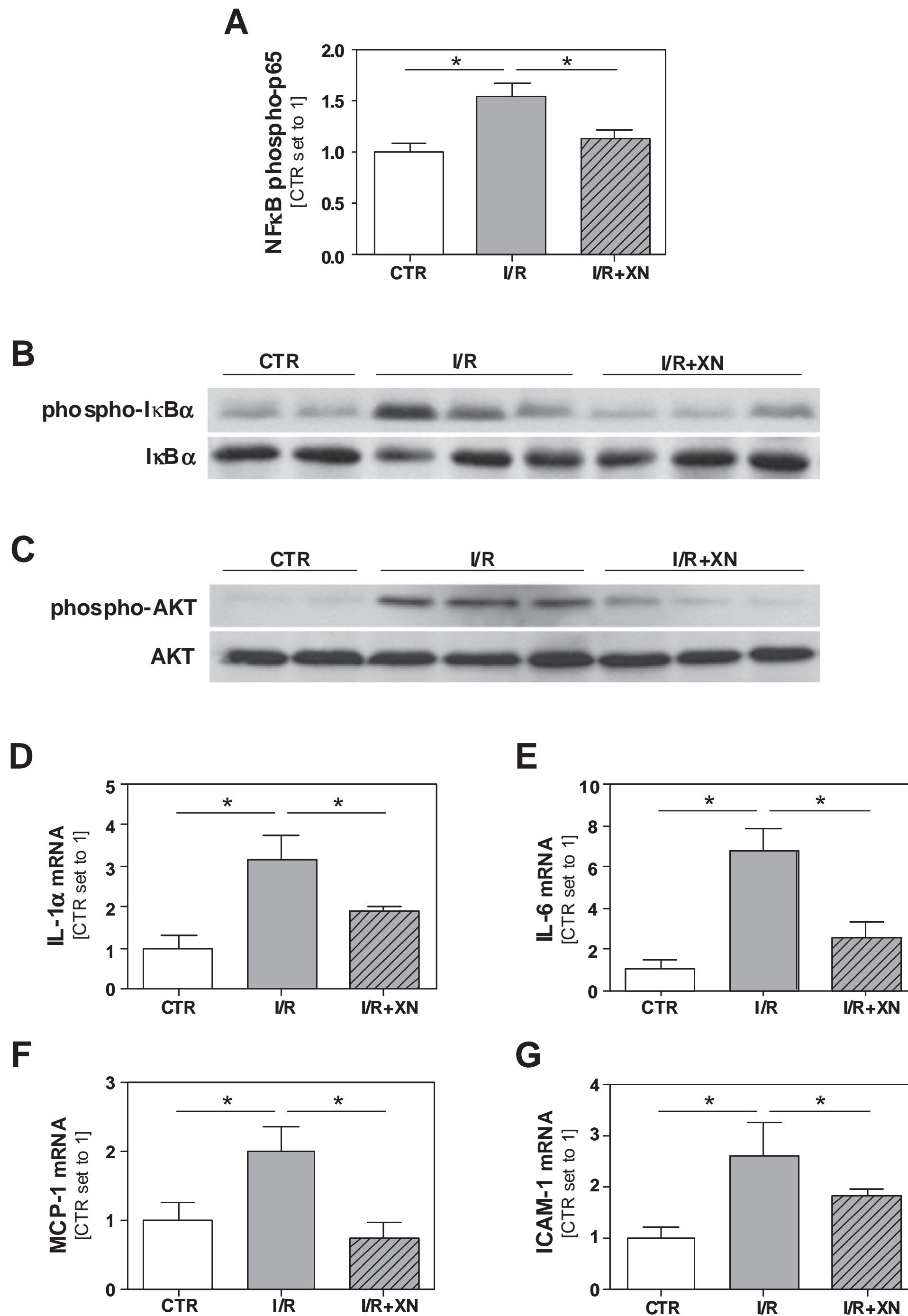

Fig. 2. Effects of xanthohumol on the inflammatory response to hepatic I/R-injury. Mice were fed either with standard chow or the same chow supplemented with 0.5% (w/w) xanthohumol (XN) for 5 days before subjecting them to a model of warm ischemia–reperfusion (I/R) liver injury (I/R and I/R+XN, respectively). Control group (CTR) received standard chow and underwent sham-surgery. (A) Comparison of NFκB activity in livers from the three experimental groups *via* ELISA-based quantification of phospho-p65 (Ser536). (B) Quantification of phosphorylated and unphosphorylated IκBα and (C) AKT, respectively, in hepatic tissue assessed by western blot analysis. (D) Measurement of hepatic mRNA expression of IL-1α, (E) IL-6, (F) MCP-1 and (G) ICAM-1 by quantitative RT-PCR in livers from the three experimental groups. Bars and whiskers represent mean ± SEM, respectively (*: $p<0.05$; n.s.: not significant).

warm I/R. Previously we have shown that this XN-concentration in the chow did not affect food consumption of mice and led to a daily uptake of approximately 1 mg/g body weight (Dorn et al., 2010a). Since we aimed to assess the XN effect on the acute phase of hepatic I/R-injury mice were sacrificed 6 h after reperfusion. HPLC analysis of hepatic tissue revealed a XN-concentration of 714 ± 65 ng XN per g of liver tissue (equivalent to approximately 2014 ± 182 nM) 6 h after I/R-injury. In this dose-range XN has been shown to exhibit antioxidant effects *in vitro* (Gerhauser et al., 2002; Yamaguchi et al., 2009), and it is known that reoxygenation of the ischemic hepatic tissue leads to acute ROS-formation (Abe et al., 2009; Klune and Tsung, 2010). Heme oxygenase 1 gene (HMOX1) is known as one of the most sensitive oxidative stress-inducible genes (Teoh, 2011; Tsuchihashi et al., 2004), and hepatic HMOX1 mRNA expression was significantly increased after I/R (Fig. 1A). This induction was significantly reduced in mice, which received XN-supplemented diet prior to I/R. Antioxidants like glutathione (GSH) are physiological countermeasures to free radicals such as ROS (Schauer et al., 2004), and after I/R the hepatic GSH content has

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been shown to be decreased (Liu et al., 1993; Stein et al., 1991). Also in the present study I/R livers had diminished GSH-levels, and this I/R-induced GSH-depletion was almost completely blunted in XN-fed mice (Fig. 1B). Moreover, I/R-induced oxidative stress leads to the formation of reactive aldehydes as 4-hydroxynonenal (4-HNE) (Fukai et al., 2005), and immunohistochemical analysis for 4-HNE-conjugated proteins revealed that XN markedly reduced immunosignal in I/R livers of XN-fed mice compared to control mice (Fig. 1C).

These data indicated that XN, directly or indirectly, ameliorates oxidative stress in the acute phase of I/R-induced liver damage.

Effect of xanthohumol on hepatocellular necrosis and apoptosis in the acute phase of hepatic I/R-injury

Next, we assessed whether the inhibitory effect of XN on I/R-induced oxidative stress may be able to reduce hepatocellular damage directly inflicted by ROS in the acute phase of I/R-injury. Six hours after I/R serum levels of transaminases were markedly increased compared to sham-operated control mice, but the elevation was similar in XN-fed and chow-fed control mice (Fig. 1D and E). Histological analysis showed distinct areas of necrosis in both I/R-treated groups, which did not significantly differ in size and frequency between XN-fed and control mice (Fig. 1F). TUNEL staining of liver sections was predominantly positive in necrotic areas and revealed a similar staining pattern in XN-fed and control mice (Fig. 1G). To further characterize the effects of XN on hepatic tissue regarding a possible predisposition to apoptosis, we analyzed three apoptosis-related genes known to play important roles in hepatocellular homeostasis of cell death and survival. Whereas the expression of the anti-apoptotic *BCL-xL* gene was upregulated after I/R (Fig. 1H), expression of the pro-apoptotic *BAX* gene was not affected (data not shown). In line with a previous study (Nakajima et al., 2008), the expression of the pro-apoptotic FAS receptor (CD95) was significantly upregulated (Fig. 1I) in I/R compared to control livers. XN-feeding did not significantly affect the expression of any of the three analyzed genes in this setting. Together, these findings indicate that despite its oxidative stress-lowering effect, XN did not affect hepatocellular damage in the acute phase of I/R-injury.

Effects of xanthohumol on the inflammatory response in the acute phase of hepatic I/R

Oxidative stress is a known inducer of the transcription factor NFκB, which plays a pivotal role in the further course of (subacute) I/R-injury (Shin et al., 2008). In accordance to other studies (Shin et al., 2008; Zwacka et al., 1998b) we found an I/R-mediated induction of hepatic NFκB activity, which was almost completely blunted in XN-treated mice (Fig. 2A). In line with this, I/R-induced IκB-α phosphorylation was significantly reduced in XN-fed mice (Fig. 2B). One factor which lies upstream of IκB-α phosphorylation is the protein kinase B, also known as AKT, and we revealed that AKT gets phosphorylated after I/R (Fig. 2C). Notably, XN-pretreatment markedly abrogated AKT-phosphorylation in I/R-livers (Fig. 2C), which indicates that this effect also contributes to the XN-mediated inhibition of I/R-induced NFκB activation. Analysis of hepatic mRNA expression levels of four NFκB target genes (Pahl, 1999), which all play an important role in hepatic inflammation, underscored these findings. Thus, the significant upregulation of the expression of interleukin-1 alpha (IL-1α) and IL-6 as well as the chemokine monocyte chemoattractant protein-1 (MCP-1) and the intercellular adhesion molecule-1 (ICAM-1) in I/R-livers of control-fed mice was significantly suppressed in XN-fed animals (Fig. 2D–G). These cytokines play a crucial pathophysiological role in I/R-induced liver injury by recruitment and activation of T-lymphocytes and macrophages, and subsequently, by advancement of neutrophil infiltration in the subacute hepatic I/R-injury (Jaeschke, 2006; Jaeschke et al., 1996).

Discussion

The aim of this study was to investigate the effect of the prenylated chalcone xanthohumol (XN) on hepatic ischemia–reperfusion (I/R) injury, which is an important problem after liver transplantation and surgical resection (Bahde and Spiegel, 2010; Klune and Tsung, 2010). Based on the known pharmacological properties of XN we focused our analysis on the early phase of I/R-induced liver damage, which is characterized by ROS-formation following reoxygenation (Parks and Granger, 1988; Rauen et al., 1994). This leads to stimulation of inflammatory cells such liver-resident Kupffer cells, which are able to produce even more ROS (Jaeschke et al., 1992), and to activation of inflammatory pathways such as the redox-sensitive NFκB pathway (Li et al., 2010; Shin et al., 2008; Suetsugu et al., 2005). These processes are being further propelled by mediators which are released by necrotic hepatocytes (Abu-Amara et al., 2010). Noteworthy, pretreatment of mice with XN significantly ameliorated I/R-induced oxidative stress 6 h after reperfusion. However, at this early phase of I/R-injury hepatocellular damage was not modulated by XN. This finding is in line with previous studies (Galaris et al., 2006; Glantzounis et al., 2005), in which antioxidant treatment, although effective on the oxidative stress level, did not result in reduced acute phase hepatocellular injury. Still and remarkably, at the same time point NFκB activation and upregulation of the proinflammatory NFκB-dependent genes IL-1α, IL-6, MCP-1 and ICAM-1 were almost completely blunted in I/R livers of mice which have been pretreated with XN. These proinflammatory mediators play a crucial role in the later course of hepatic I/R-injury *via* recruitment and activation of proinflammtory cells, which cause hepatocellular damage directly as well as *via* further ROS-production (Jaeschke et al., 1992, 1996). Therefore, many attempts have been made to alleviate I/R-induced liver damage by applying either antioxidants like tocopherole, ascorbate, allopurinol, N-acetylcystein and α-lipoic acid or anti-inflammatory compounds as glucocorticoids or proteasome inhibitors with varying outcomes (Bahde and Spiegel, 2010). XN most probably combines both antioxidant (Gerhauser et al., 2002; Miranda et al., 2000; Yamaguchi et al., 2009) as well as anti-inflammatory properties (Dorn et al., 2010b; Lupinacci et al., 2009; Stevens and Page, 2004), and in our experimental setting we detected hepatic XN concentrations in a dose-range in which anti-inflammatory as well as antioxidant effects have been described *in vitro* (Gerhauser et al., 2002; Yamaguchi et al., 2009). Interestingly, expression levels of inducible NO synthase (iNOS), another ROS induced gene which has been shown to be crucial in I/R-induced inflammatory response (Chen et al., 2003), were similar in I/R-livers of XN-fed and control mice (data not shown). Since a previous study revealed an inhibitory effect of XN on iNOS expression in a model of ischemic stroke in rats (Yen et al., 2012) these data indicate that XN effects on specific target gene expression in response to a similar pathophysiological insult may vary in different organs.

In an *ex vivo*-model of cold hepatic I/R Hartkorn et al. (2009) injected XN into the portal vein of a rat liver at a very high dose (2.26 mM). Also in this *ex vivo*-model XN had an antioxidant effect and inhibitory effect on NFκB activity but led to a slight yet not significant increase of ALT and AST release. The processes of the inflammatory response and of cell injury in the reperfusion phase significantly vary depending on pre-existent warm versus cold ischemia (de and Rauen, 2007). Moreover, the XN-concentration used in the study by Hartkorn et al. (2.26 mM) appears quite high and may have caused concomitant cytotoxic effects. Furthermore, it appears unlikely that such high concentrations can be achieved *in vivo*, but the experimental conditions used by Hartkorn et al. may rather be translated into the application of XN as an additive to a transplant preservation solution.

In addition to I/R-injury, we previously reported that XN is able to inhibit hepatic inflammation and fibrosis in a murine model of non-alcoholic steatohepatitis (NASH) (Dorn et al., 2010b). Considering the fact, that steatotic livers pose an increasing problem in the field of liver transplantation (McCormack et al., 2011) and that steatotic

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livers are more susceptible to hepatic I/R-injury (Ezaki et al., 1992), a therapeutic agent which is known to exhibit its beneficial effects also in fatty livers, such as XN, seems to be especially suitably for prevention of I/R-induced inflammatory responses.

One prerequisite for the therapeutic application is a good safety profile of the used agent. Especially hepatotoxic properties have to be excluded, if applying a substance to patients with chronic liver disease. We and others have previously shown that XN meets these requirements (Dorn et al., 2010a; Hussong et al., 2005; Vanhoecke et al., 2005).

In conclusion, the present study revealed that XN is able to inhibit oxidative stress, and more importantly, almost completely block the inflammatory response in the acute phase of warm hepatic I/R-injury in mice. Noteworthy, XN did not lead to reduced hepatocellular damage in the acute phase of I/R-injury. However, suppression of the inflammatory response during the acute phase often translates to protection against subsequent inflammatory cell infiltration and later liver damage in response to I/R (Bahde and Spiegel, 2010). Still, further studies focusing on later phases of I/R-induced hepatic injury are required to conclusively demonstrate that XN inhibits liver damage in this clinically highly relevant condition. The present study indicates the potential of XN-application to ameliorate adverse inflammatory responses to I/R-induced liver damage such as after surgical liver resection or transplantation.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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S.M. and A.W. are employed by a Joh. Barth & Sohn subsidiary.

J.H. does not have a financial relationship with Joh. Barth & Sohn GmbH or another company related to this project.

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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