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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 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 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

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