Experimental and Molecular Pathology 92 (2012) 222–228

Contents lists available at SciVerse ScienceDirect

Experimental and Molecular Pathology

journal homepage: www.elsevier.com/locate/yexmp

Hop bitter acids exhibit anti-fibrogenic effects on hepatic stellate cells in vitro

Michael Saugspier [a], Christoph Dorn [a], Wolfgang E. Thasler [b], Manfred Gehrig [c], Jörg Heilmann [d], Claus Hellerbrand [a,*]

[a] Department of Internal Medicine I, University Hospital Regensburg, Germany
[b] Grosshadern Tissue Bank and Center for Liver Cell Research, Department of Surgery, Ludwig-Maximilians-University Munich, Germany
[c] Nateco$_2$, Wolnzach, Germany
[d] Department of Pharmaceutical Biology, Institute of Pharmacy, University of Regensburg, Germany

ARTICLE INFO

Article history:
Received 5 September 2011
Available online 25 November 2011

Keywords:
Hop
Bitter acids
Hepatic fibrosis
Hepatic stellate cells
Nuclear factor kappa B

ABSTRACT

Female inflorescences of the hop plant *Humulus lupulus* L. contain a variety of secondary metabolites with bitter acids (BA) as quantitatively dominating secondary metabolites. The use of hops in beer brewing has a long history due to the antibacterial effects of the BA and their typical bitter taste. Furthermore, hop cones are used in traditional medicine and for pharmaceutical purposes. Recent studies indicate that BA may affect activity of the transcription factor NFκB. NFκB plays a key role in the activation process of hepatic stellate cells (HSC), which is the key event of hepatic fibrosis. The aim of this study was to investigate the effect of BA on HSC (activation) and their potential to inhibit molecular processes involved in the pathogenesis of hepatic fibrosis. HSC were isolated from murine and human liver tissue and incubated with a characterized fraction of bitter acids purified from a CO_2 hop extract. At a concentration of 25 μg/ml BA started to induce LDH leakage. Already at lower concentrations BA lead to a dose dependent inhibition of HSC proliferation and inhibited IκB-α-phosphorylation, nuclear p65 translocation and binding activity in a dose dependent way (up to 10 μg/ml). Accordingly, the same BA-doses inhibited the expression of pro-inflammatory and NFκB regulated genes as MCP-1 and RANTES, but did not affect expression of genes not related to NFκB signaling. In addition to the effect on activated HSC, BA inhibited the in vitro activation process of freshly isolated HSC as evidenced by delayed expression of collagen I and α-SMA mRNA and protein. Together, these findings indicate that BA inhibit NFκB activation, and herewith the activation and development of profibrogenic phenotype of HSC. Thus, bitter acids appear as potential functional nutrients for the prevention or treatment hepatic fibrosis in chronic liver disease.

Introduction

Hop cones obtained from the plant *Humulus lupulus* L. are traditionally used as natural preserving agent and to add bitterness and flavor to beer. Particularly the quantitatively dominating secondary metabolites in hops, the bitter acids (BA), are responsible for both effects. About 95% of worldwide cultivated hop is used for brewing purposes, the remainder utilized for the production of phytomedicines and dietary supplements (Van et al., 2009a). BA consist of two related series, the α-acids (or humulones) and β-acids (or lupulones), which are both characterized as prenylated acylphloroglucinols. Recent studies have shown that BA exhibit several biological properties with promising effects in cancer therapy and prevention. Thus, BA inhibit cell growth of invasive cancer cells and angiogenesis and induce apoptosis in fast-growing tumor cells (Tobe et al., 1997; Shimamura et al., 2001; Lamy et al., 2007). Moreover, it has been shown that BA may play a role in the prevention of lifestyle-related disorders since they reveal positive effects on lipid metabolism, glucose tolerance, and body weight in vivo (Yajima et al., 2004; Miura et al., 2005; Yajima et al., 2005). The prevalence of metabolic diseases, a cluster of abnormalities like obesity, glucose tolerance and insulin resistance, has reached epidemic proportions worldwide. Accordingly, the non-alcoholic fatty liver disease (NAFLD) has emerged as a considerable health concern because a significant number of patients progress to more severe stages of liver disease including non-alcoholic steatohepatitis (NASH) (Powell et al., 1990; Caldwell et al., 1999).

Hepatic stellate cells (HSC) represent the central mediators of hepatic fibrosis in chronic liver disease including NASH. They are resident retinoid storing cells located in the perisinusoidal space of Disse, in between fenestrated endothelium and hepatocytes (Atzori et al., 2009). As response to various stimuli during hepatic injury, quiescent HSC get activated leading to highly proliferative myofibroblast-like cells, which exhibit pro-fibrogenic and pro-inflammatory gene expression (Mann et al., 2007). We and others have shown that activation of the transcription factor NFκB plays a critical role in HSC activation

* Corresponding author at: Dept. of Internal Medicine I, University Hospital Regensburg, D-93042 Regensburg, Germany. Fax: +49 941 944 7154.
E-mail address: claus.hellerbrand@klinik.uni-regensburg.de (C. Hellerbrand).

0014-4800/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.yexmp.2011.11.005

(Hellerbrand et al., 1998a; Hellerbrand et al., 1998b; Elsharkawy et al., 2005). Moreover, increased hepatic NFκB activity and responsiveness during activation of HSC are known to promote inflammation and fibrosis (Lee et al., 1995; Hellerbrand et al., 1998a; Lang et al., 2000). Notably, BA have been shown to exhibit their anti-inflammatory and chemopreventive effects in part via decreasing NFκB activity (Lee et al., 2007; Van et al., 2009b), however, the biological activity related to hepatic fibrosis has not yet been examined.

Therefore, this study was performed to identify the effect of hop bitter acids on primary murine and human HSC in vitro.

Methods

Cell isolation and cell culture

Isolation of primary hepatic stellate cells (HSC) from 8 week old female BALB/c mice (Charles River Laboratories, Sulzfeld, Germany) or primary human HSC was performed as described previously (Bosserhoff et al., 2003). Remnant liver samples were obtained from patients with informed consent through the Grosshadern Tissue Bank after partial hepatectomy. This tissue bank is regulated according to the guidelines of the non-profit state-controlled HTCR (Human Tissue and Cell Research) foundation following study approval Thasler et al., 2003 (Thasler et al., 2003). HSC were cultured in high glucose DMEM supplemented with 10% FCS, 100 units of penicillin and 100 μg/ml streptomycin (PAA, Pasching, Austria) at 37 °C in a humidified atmosphere of 5% CO_2 in air. In vitro activation of HSC was achieved by cell culture on uncoated tissue culture dishes as described (Arthur et al., 1999).

Chemicals

A supercritical carbon dioxide hop extract (HE-BA) was provided by NATECO$_2$ (Wolnzach, Germany). This hop extract contains 13.0% (w/w) α-acids (humulone, cohumulone and adhumulone) and 51.9% (w/w) β-acids (lupulone, colupulone and adlupulone) as revealed by HPLC analysis (data not shown). Besides the BA, this extract also contains components belonging to the hop essential oil (detailed chemical composition and quantity are given as supplementary data) (Supplementary Table 1) and further mentioned as remnant lipophilic compounds. A stock solution (100 mg/ml) was prepared by dissolving HE-BA in DMSO. After a centrifugation step, unsolved compounds were removed and the stock solution was aliquoted and stored at −20 °C prior to use. Samples indicated as controls received DMSO at the same concentration as HE-BA treated samples. The final concentration of DMSO did not exceed 0.1% in all experiments. Tumor necrosis factor (TNF) was obtained from R&D (Wiesbaden-Nordenstadt, Germany), and all other chemicals were from Sigma Pharmaceuticals (Hamburg, Germany).

For further purification of bitter acids and separation from other compounds HE-BA was first solved in hexane. The obtained solution was transferred to a separating funnel and a liquid–liquid extraction was done with sodium hydroxide (pH 12). The sodium hydroxide solution (containing the deprotonated BA) was acidified with hydrochloric acid (37%) followed by a liquid–liquid extraction with petroleum ether as second phase. Subsequently, purified BA as well as the fraction containing remnant lipophilic compounds (not soluble at pH 12) were dried using a rotary evaporator.

Separation success of BA and remnant lipophilic compounds was checked by thin layer chromatography using silica gel plates (Merck, Darmstadt, Germany) and a solvent mixture of cyclohexane, ethyl acetate and glacial acetic acid (ratio 70:29:1). For visualization anisaldehyde spray reagent was used as described (Kritchevsky et al., 1963).

Proliferation assay

For quantification of cell proliferation the colorimetric XTT assay was used (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

Expression analysis

Total cellular RNA isolation and reverse transcription were performed as described (Dorn et al., 2010). Quantitative real time-PCR for mRNA expression analysis was done with specific set of primers (Table 1) as described (Muhlbauer et al., 2003). Expression of α-smooth muscle actin (α-SMA), CCL-5 (RANTES) and keratinocyte growth factor (KGF) was analyzed applying the QuantiTect Primer Assay according to the manufacturer's instructions (Qiagen, Hilden, Germany).

Protein analysis

Protein extraction and Western blotting were performed applying anti-bodies against phospho-IκB-α and phospho-JNK as well as their not phosphorylated types (Cell Signaling Technology, Boston, USA) as described (Hellerbrand et al., 1998a).

Immunofluorescence

Immunofluorescence staining was done by applying an antibody against p65 (Santa Cruz Biotechnology, Santa Cruz, USA) followed by an incubation with a secondary antibody (Invitrogen Life Technologies, Carlsbad, USA) as described (Muhlbauer et al., 2004).

Statistical analysis

Values are presented as mean ± SEM. Comparison between groups was made using the Student's unpaired *t*-test. A p value <0.05 was considered statistically significant. All calculations were performed using the statistical computer package GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, USA).

Results

BA enriched extract inhibits the in vitro activation of HSC

The activation of HSC is one of the central pathophysiological mechanisms of liver fibrogenesis (Bataller and Brenner, 2005; Friedman, 2008). First, we therefore aimed to analyze the effect of BA enriched extract (HE-BA) on the in vitro activation process of murine HSC. Two days after isolation HSC were exposed to HE-BA at two different doses (2.5 and 5 μg/ml) for 3 days. Here, and in subsequent experiments control cells were treated with DMSO at the same concentration as used as solvent for HE-BA. Subsequently, mRNA expression of two established markers of HSC activation, namely collagen type I (col I) and alpha-smooth muscle actin (α-SMA), was determined by quantitative RT-PCR analysis. Treatment with HE-BA did not affect cell morphology (Fig. 1A) but significantly reduced the expression of collagen type I (Fig. 1B) and

Table 1
Set of primers used for quantitative RT-PCR analysis.

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
18S	AAA CGG CTA CCA CAT CCA AG	CCT CCA ATG GAT CCT CGT TA
Collagen type I	CGG GCA GGA CTT GGG TA	CGG AAT CTG AAT GGT CTG ACT
MCP-1	TGG GCC TGC TGT TCA CA	TCC GAT CCA GGT TTT TAA TGT A