Toxicology 280 (2011) 152–163

Contents lists available at ScienceDirect

Toxicology

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

Betulin and betulinic acid attenuate ethanol-induced liver stellate cell activation by inhibiting reactive oxygen species (ROS), cytokine (TNF-α, TGF-β) production and by influencing intracellular signaling

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ARTICLE INFO

Article history:
Received 21 October 2010
Received in revised form
24 November 2010
Accepted 13 December 2010
Available online 21 December 2010

Keywords:
Liver stellate cells
Ethanol
Betulin
Betulinic acid
Cytokines
MMPs
TIMPs
MAPK
NFκB

ABSTRACT

Background/aims: Liver fibrosis has been reported to be inhibited in vivo by oleanolic and ursolic acids. However, the mechanisms of the action of those triterpenoids are poorly understood. In this study, we aimed to determine the antifibrotic potential of other triterpenes, betulin and betulinic acid, and to characterize their influence on the signal transduction pathways involved in ethanol-activated hepatic stellate cells (HSCs).

Methods: Investigated was the influence of preincubation of rat HSCs with betulin and betulinic acid, at non-toxic concentrations, on ethanol-induced toxicity, migration, and several markers of HSC activation such as smooth muscle α-actin (α-SMA) and procollagen I expression, release of reactive oxygen species (ROS) and cytokines: tumor necrosis factor-α (TNF-α) and tumor growth factor-β1 (TGF-β1), and production of metalloproteinase-2 (MMP-2) and tissue inhibitors of metalloproteinases (TIMP-1 and TIMP-2). To assess the mechanism of the action of those triterpenes, intracellular signals such as nuclear factor-κB (NFκB), c-Jun N-terminal kinase (JNK), and p38 mitogen-activated protein kinase (p38 MAPK) induced by ethanol were examined.

Results: In vitro, betulin, but not betulinic acid, protected HSCs against ethanol toxicity. However, both betulin and betulinic acid inhibited the production of ROS by HSCs treated with ethanol and inhibited their migration as well as ethanol-induced TNF-α, and TGF-β1, production. Betulin and betulinic acid down-regulated ethanol-induced production of TIMP-1 and TIMP-2. Betulin and betulinic acid, also decreased ethanol-induced activity of MMP-2. In ethanol-induced HSCs, betulin inhibited the activation of the p38 MAPK and the JNK transduction pathways, while betulinic acid inhibited the JNK transduction pathway only. They also significantly inhibited phosphorylation of IκB and Smad 3 and attenuated the activation of TGF-β1 and NFκB/IκB transduction signaling.

Conclusion: The results indicated that betulin and betulinic acid inhibited ethanol-induced activation of HSCs on different levels, acting as antioxidants, inhibitors of cytokine production, and inhibitors of TGF-β, and NFκB/IκB transduction signaling. Betulin was also inhibitor of both JNK and p38 MAPK signal transduction, while betulinic acid inhibited only JNK. The remarkable inhibition of several markers of HCS activation makes triterpenes, especially betulin, promising agents for anti-fibrotic combination therapies.

1. Introduction

Alcohol abuse is the major cause of liver fibrosis and cirrhosis in developed countries. Before alcoholic liver fibrosis becomes evident, the liver undergoes several stages of alcoholic liver disease (ALD) including steatosis and steatohepatitis. These stages, including early liver fibrosis, can be treated if the cause of liver injury is eliminated. Hepatic fibrosis is a consequence of the wound-healing response to repeated injury (Bataller and Brenner, 2005). Fibrosis is characterized by an extensive deposition of extracellular matrix (ECM) and loss of parenchyma tissue. Hepatic stellate cells (HSCs) exist in the normal liver as quiescent retinoid-storing cells, and, following a fibrogenic stimulus, they undergo a complex activation process in which the cells change from quiescent to an activated myofibroblast-like phenotype, proliferate, and migrate to the site of liver injury producing extensive amounts of ECM

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doi:10.1016/j.tox.2010.12.006

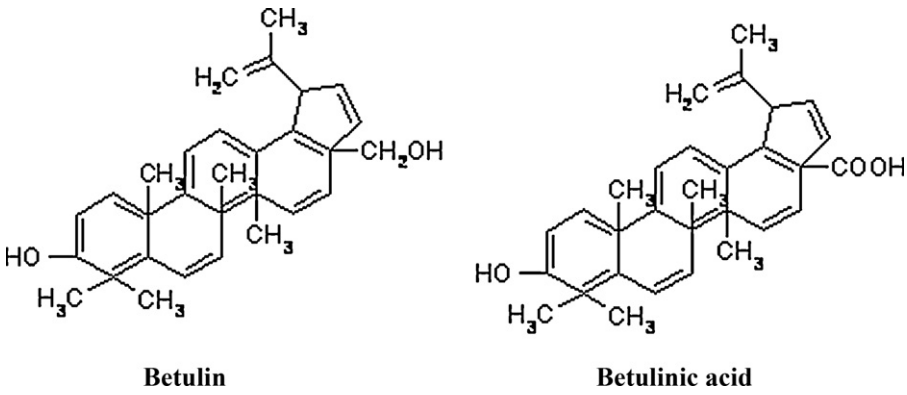

Fig. 1. Structures of betulin and betulinic acid.

(Senoo, 2004; Bataller and Brenner, 2005). This event can be recapitulated in a culture model in which isolated HSCs are cultured on plastic in serum-containing media. The activated HSCs are a rich source of fibrillar types I and III collagen and also secrete high levels of the tissue inhibitor of metalloproteinase 1 (TIMP-1) (Siegmund et al., 2005). Many of the morphological and metabolic changes associated with HSC activation during fibrogenesis in vivo are also observed in HSCs grown in culture on plastic. Several soluble factors, including growth factors, cytokines, chemokines, and oxidative stress products, play a role in the activation of HSCs. Activation of HSCs is associated with sequential expression of several key cytokines and their surface receptors, including transforming growth factor β (TGF-β) and its receptors (Friedman et al., 1994). Exogenous expression of TGF-β in the liver induces liver fibrosis, and blockade of TGF-β signaling by multiple methods prevents progression of liver fibrosis in experimental animals (Yata et al., 2002). TGF-β downstream signaling is mediated by Smad 2 and Smad 3, structurally similar but functionally distinct. They are differentially activated by TGF-β in quiescent and activated HSCs and play a different role in HSC induction (Liu et al., 2003; Uemura et al., 2005).

The development of liver fibrosis in alcoholics has been linked to the oxidation of ethanol to the highly active compound acetaldehyde. At concentrations that were detected in hepatic venous blood during alcohol consumption, acetaldehyde stimulated type I collagen synthesis and gene transcription in cultured rat and human HSCs through activation of protein kinase C (PKC) (Svegliati-Baroni et al., 2001). Acetaldehyde was also shown to increase NFκB (p65) and its binding to the α_2(I) collagen promotor (Novitskiy et al., 2004) and enhance it by a mechanism dependent on the accumulation of H_2O_2 (Greenwel et al., 2000; Novitskiy et al., 2005; Svegliati-Baroni et al., 2005). CYP2E1 is an important source of reactive oxygen species (ROS) in alcohol-induced injury and fibrosis, generating superoxide (O_2^-) and hydrogen peroxide (H_2O_2). It has been detected that inhibition of CYP2E1 activity by diallyl sulfide (DAS) prevented induction of collagen I gene expression in rat stellate cells overexpressing CYP2E1 (Nieto et al., 2000). Oxidative stress also activates the c-Jun NH_2-terminal kinase (JNK), a protein which regulates secretion of proinflammatory cytokines by cultured HSCs (McCarroll et al., 2003; Nishitani and Matsumoto, 2006).

Matrix metalloproteinases (MMPs), a family of zinc metalloendopeptidases, are promptly expressed by HSCs in response to diverse hepatic toxins. In vitro experiments have demonstrated the role of MMPs in the activation of HSCs. Also proliferation of HSCs was promoted by pericellular collagen I proteolysis acting via αvβ3 integrins (Zhou et al., 2006). Conversely, MMPs may contribute to regression of liver fibrosis through cleavage of the fibrillar ECM and promotion of apoptosis among the activated HSCs. Thus MMPs play a dual role in liver fibrosis, depending on the timing (Han, 2006).

Natural triterpenoids such as ursolic and oleanolic acids have been investigated for their hepatoprotective effects. The mechanism of this effect is complex. It includes suppression of enzymes which play a role in liver damage such as cytochrome P450, cytochrome b5, CYP1A and CYP2A and an increase in antioxidant substances such as glutathione, metallothioneins, and glutathione-S-transferase with simultaneous protective effects on liver mitochondria. Oleanolic acid protects the mouse liver from hepatotoxic tetrachloromethane, acetaminophen, phalloidin, and

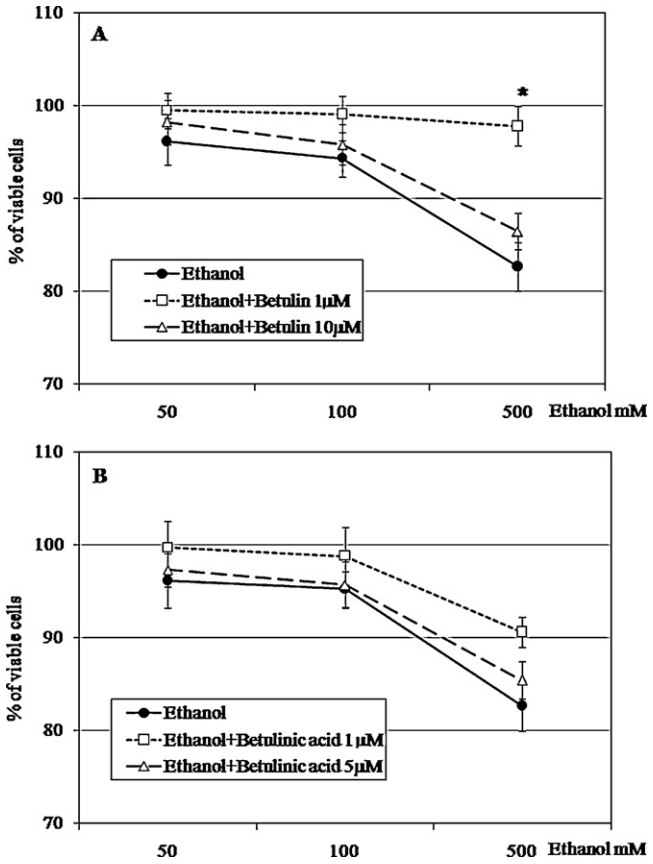

Fig. 2. The influence of betulin (A) and betulinic acid (B) on ethanol-induced toxicity in CFSC-2G cells. CFSC-2G cells were preincubated with the triterpenes for 24 h. Thereafter, ethanol at the indicated concentrations was added. After 24 h of incubation, the toxicity was determined by the MTT method. Values are means ± SD of results from five experiments. *Statistically significant at $p \leq 0.05$ in comparison to cells incubated with ethanol alone (Wilcoxon test).