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Ethanol metabolism activates cell cycle checkpoint kinase, Chk2

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Abstract

Chronic ethanol abuse results in hepatocyte injury and impairs hepatocyte replication. We have previously shown that ethanol metabolism results in cell cycle arrest at the G2/M transition, which is partially mediated by inhibitory phosphorylation of the cyclin-dependent kinase, Cdc2. To further delineate the mechanisms by which ethanol metabolism mediates this G2/M arrest, we investigated the involvement of upstream regulators of Cdc2 activity. Cdc2 is activated by the phosphatase Cdc25C. The activity of Cdc25C can, in turn, be regulated by the checkpoint kinase, Chk2, which is regulated by the kinase ataxia telangiectasia mutated (ATM). To investigate the involvement of the regulators of Cdc2 activity, VA-13 cells, which are Hep G2 cells modified to efficiently express alcohol dehydrogenase, were cultured in the presence or absence of 25 mM ethanol. Immunoblots were performed to determine the effects of ethanol metabolism on the activation of Cdc25C, Chk2, and ATM. Ethanol metabolism increased the active forms of ATM and Chk2, as well as the phosphorylated form of Cdc25C. Additionally, inhibition of ATM resulted in approximately 50% of the cells being rescued from the G2/M cell cycle arrest and ameliorated the inhibitory phosphorylation of Cdc2. Our findings demonstrated that ethanol metabolism activates ATM. ATM can activate the checkpoint kinase Chk2, resulting in phosphorylation of Cdc25C and ultimately in the accumulation of inactive Cdc2. This may, in part, explain the ethanol metabolism–mediated impairment in hepatocyte replication, which may be important in the initiation and progression of alcoholic liver injury. Published by Elsevier Inc.

Keywords: Alcoholic liver disease; Ethanol metabolism; Liver regeneration; Checkpoint kinase; Ataxia telangiectasia mutated (ATM); Cdc2

Introduction

The liver is the primary site of ethanol metabolism and can result in hepatotoxicity. There is extensive evidence that ethanol metabolism delays or impairs the replication of hepatocytes and cultured hepatic cells (Clemens et al., 2002, 2003; Koteish et al., 2002; Yang et al., 1998). Because of these facts, it appears that chronic ethanol consumption not only results in cell damage and death but also alters the ability of the liver to respond appropriately to injury and replace damaged cells.

There are two mechanisms by which lost hepatocytes can be replaced (Diehl, 2005; Santoni-Rugiu et al., 2005). Replacement of damaged hepatocytes normally occurs by the replication of existing mature hepatocytes (Sell, 2001). If the replication of existing hepatocytes is inhibited, as is the case in many chronic liver diseases, replacement of damaged cells occurs by activation of a population of bipotential hepatic cells known as liver progenitor cells or oval cells (Kuwahara et al., 2008; Ohlson et al., 1998; Roskams et al., 2003; Yang et al., 2004). Because liver progenitor cells are bipotential and can differentiate into hepatocytes or bile ductular epithelium, it has been proposed that replacement of hepatocytes by liver progenitor cells not only leads to the replacement of hepatocytes but also results in increased proliferation of ductals (Ray et al., 1993; Roskams et al., 2003). In patients suffering from alcoholic liver disease, it has been shown that increased expression of ductular epithelia correlates highly with portal inflammation, fibrosis, and cirrhosis (Ray et al., 1993). Thus, increased expression of ductular epithelia may be a precursor to the accumulation of fibrotic tissue in the liver. Furthermore, impairment of normal hepatocyte replication by ethanol metabolism may have a role in the fibrotic scarring associated with alcoholic liver disease.

We have previously shown that ethanol metabolism by recombinant Hep G2 cells, which have been engineered to efficiently metabolize ethanol, results in impaired cellular replication (Clemens et al., 2002; Donohue et al., 2006). This ethanol metabolism–meditated impaired cellular replication is, at least partially, the result of a cell

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cycle arrest at the G2/M transition of the cell cycle. Furthermore, this impairment is caused, at least in part, by an accumulation of the cyclin-dependent kinase, Cdc2, in the inactive phosphorylated form (Clemens et al., 2003).

The activity of Cdc2 is required for the transition from the G2 phase to M phase of the cell cycle (Nurse, 1990) and is regulated both positively and negatively by phosphorylation (Hunter, 1995). Cdc2 is maintained in an inactive state throughout most of the cell cycle. This is accomplished by phosphorylation at threonine 14 (Thr 14) and tyrosine 15 (Tyr 15), which renders Cdc2 incapable of binding ATP and thus inactive (Atherton-Fessler et al., 1993). The phosphorylation state of these two amino acid residues is regulated by a balance between the activities of the kinases Myt1 and Wee1, as well as the Cdc25 family of phosphatases (Liu et al., 1997, 1999; McGowan and Russell, 1993; Wells et al., 1999). In late G2 phase, Cdc2 is activated by Cdc25C, and mitosis progresses (Peng et al., 1997). Thus, the activity of Cdc25C is critical for the activation of Cdc2 and the occurrence of mitosis.

Cell cycle arrest can result from the activation of pathways known as cell cycle checkpoints. Induction of these pathways can be initiated by the activation of signal transduction pathways mediated by the kinase, ataxia telangiectasia mutated (ATM). ATM is activated by a number of stimuli, including genotoxic assault, delayed or incomplete DNA replication, viral infection, and oxidative stress (Abraham, 2001; Marshall et al., 2005). Many of the downstream actions of this kinase are mediated through the actions of the serine/threonine kinase, checkpoint kinase 2 (Chk2) (Zhou and Elledge, 2000). Chk2 is activated by phosphorylation at Thr 68 by ATM (Ahn et al., 2000) and is able to phosphorylate Cdc25C at serine 216 (Ser 216), a site known to be involved in the negative regulation of this important cell cycle regulator (Abraham, 2001; Chaturvedi et al., 1999; Zhou et al., 2000). Phosphorylation of Cdc25C at Ser 216 creates a binding site for 14-3-3 proteins. Binding of Cdc25C to 14-3-3 proteins sequesters Cdc25C in the cytoplasm, effectively nullifying its ability to activate the nuclear protein Cdc2 (Peng et al., 1997). Thus, the checkpoint kinase Chk2 can impair progression through the G2/M transition of the cell cycle by phosphorylating Cdc25C.

Using the recombinant Hep G2 cell line VA-13, which efficiently metabolizes ethanol, we have investigated the involvement of the cell cycle checkpoints in the ethanol metabolism–mediated cell cycle arrest (Clemens et al., 2002). The results of these studies demonstrate that ethanol metabolism is associated with the activation of ATM and the checkpoint kinase Chk2. Additionally, we show that Ser 216-phosphorylated Cdc25C is increased. These findings provide an explanation for the increase in the phosphorylated form of Cdc2, which is observed in VA-13 cells actively metabolizing ethanol. Activation of the kinases involved in the cell cycle checkpoints may have a role in the impairment of normal hepatocyte replication that has been observed in the liver of individuals suffering from alcoholic liver disease.

Materials and methods

Cell culture

VA-13 cells are recombinant Hep G2 cells that have been stably transfected to express alcohol dehydrogenase. The construction and characterization of these cell lines have been previously described (Clemens et al., 2002). Cells were maintained in Dulbecco's modified Eagle's medium containing high glucose and supplemented with 10% fetal bovine serum, 2 mM glutamine, 400 μg/mL zeocin, 50 μg/mL gentamicin, 1,000 units/mL penicillin, and 100 μg/mL streptomycin (complete Dulbecco's modified Eagle's medium). For ethanol experiments, cells were cultured as above, but 25 mM N-(2-hydroxyethyl) piperazine-N-(2-ethanesulfonic acid) was included in the growth media of all flasks, and 25 mM ethanol was included in the growth media of cells exposed to ethanol. During ethanol experiments, all flasks were tightly sealed to minimize evaporation of ethanol and acetaldehyde. ATM was inhibited by inclusion of caffeine at a concentration of 1 mM (Zhou et al., 2000). Alcohol dehydrogenase activity was inhibited by inclusion of 2 mM 4-methyl pyrazole.

Immunoblotting and antibodies

Cells were removed from the flasks by trypsinization and collected by centrifugation. The cell pellets were lysed in radioimmunoprecipitation assay buffer (50 mM Tris, pH 7.4; 1% nonidet P-40; 0.25% sodium deoxycholate; 150 mM NaCl; 1 mM EDTA; 1 mM phenylmethylsulfonyl fluoride; 1 mM Na_3VO_2; 1 mM NaF). Complete miniprotease cocktail was added to the buffer as recommended by the manufacturer (Roche, Indianapolis, IN). The protein concentrations of the lysates were determined using the Bio-Rad protein reagent (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin as a standard. Thirty micrograms of protein from each sample were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Proteins were electroblotted onto polyvinyl difluoride membranes in 25 mM Tris and 192 mM glycine at 100 V for 60 min at 4°C. The membranes were blocked in Tris-buffered saline with Tween 20 (TBST) (20 mM Tris, pH 7.6; 136 mM NaCl; 0.1% Tween 20) containing 5% nonfat dry milk for 1 h at room temperature and incubated at 4°C overnight with the primary antibody diluted in TBST/milk solution. The membranes were washed in TBST and incubated for 1 h with a peroxidase-conjugated secondary antibody diluted in TBST/milk, washed, and the proteins visualized by chemiluminescence using the SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL). The resulting bands were quantified by densitometry using Quantity One software (Bio-Rad). Antibodies used were anti-Cdc2 (Upstate Biotechnology, Lake Placid, NY), anti-phospho-Cdc2 (Tyr 15), anti-phospho-Cdc25C (Ser 216), anti-Chk2, anti-phospho-Chk2 (Thr 68) (Cell Signaling, Beverly, MA), anti-Cdc25C (Santa