PRIMARY HUMAN HEPATOCYTES ARE PROTECTED AGAINST PROLONGED AND REPEATED EXPOSURE TO ETHANOL BY SILIBININ-DIHEMISUCCINATE

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Abstract — Aims and methods: We investigated the effect of silibinin-C-2',3'-dihydrogensuccinate (SDH) on primary human hepatocytes when exposed to ethanol for 14 days. At regular intervals, the medium was refreshed and liver enzymes and secreted protein in the medium were determined. Results: The ethanol-induced release of lactate dehydrogenase (at 34 mM ethanol) was completely blocked by 20 μM SDH. SDH itself stimulated fibrinogen release and had no toxic effect. Conclusions: We can conclude that SDH has a beneficial effect on human hepatocytes when exposed to ethanol in vitro.

INTRODUCTION

Silymarin is a flavonoid extracted from the milk thistle Silybum marianum. The biologically active isomer has been identified as silibinin-C-2',3'-dihydrogensuccinate (SDH) and is commercially available under the name Legalon®. It has been used in the treatment of acute poisoning by the mushroom Amanita phalloides (Trost and Halbach, 1978; Hruby et al., 1987), and a variety of liver diseases, mainly in Central and Southern Europe (Ferenci et al., 1989). Although the hepatoprotective properties of SDH and its derivatives have been reported both from in vitro and in vivo studies (Salmi and Sarna, 1982; Varga et al., 1991; Carini et al., 1992; Pares et al., 1998), its mechanism of action still has not been established. Different mechanisms have been proposed. Silymarin (or its derivatives) might function as an antioxidative-scavenger of free radicals (mainly active towards HO and HOCl and less so for H2O2 or O2–); it might function as a regulator of membrane permeability and stability or of transport across the membrane; or it might function as a stimulator of polymerase I and rRNA transcription, leading to increased rRNA synthesis (Valenzuela and Garrido, 1994; Wellington and Jarvis, 2001).

It has been suggested that SDH might also be beneficial in alcohol intoxication (Varga et al., 1991). Ethanol is predominantly metabolized in the liver by alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) (Castaneda and Kinne, 2000). At low doses of ethanol, the cytochrome P450 2E1 isoenzyme, previously referred to as microsomal ethanol-oxidizing system or MEOS, is in general not important (Lieber, 1994), but is induced by ethanol at high concentrations (Roberts et al., 1995). Via the cytochrome P450 2E1-pathway, ethanol is involved in the generation of reactive oxygen species. In addition, ethanol accounts for increased acetaldehyde production. Both factors are thought to be responsible for most of the increased hepatotoxicity of ethanol at higher doses (Lieber, 1993; Kurose et al., 1997). Beneficial effects of SDH towards alcohol toxicity have been investigated, in vivo, in a rat model (Valenzuela et al., 1989; Comoglio et al., 1995) and in vitro by using isolated rat hepatocytes (Corrazi et al., 1982; Carini et al., 1992). However, data from in vitro studies using human hepatocytes are sparse. In the present study we examined, for the first time, cell injury induced by repeated exposure to ethanol (over a period of 14 days) of primary human hepatocytes. We monitored cell viability and the release into the medium of cellular enzymes, and we investigated whether SDH could protect liver cells against the cytotoxic effects of ethanol.

MATERIALS AND METHODS

Isolation and culture of primary human hepatocytes

Hepatocytes were isolated from liver obtained from post-mortem organ donors when the tissue could not be used for transplantation. For these experiments, permission from the Medical Ethical Committee for experimentation using human tissues with informed consent of the family had been obtained. A two-step collagenase (Roche; Mannheim, Germany) perfusion method was used as described previously (Rijnjtes et al., 1986). Donor 1 was an 18-year-old male, cells after isolation had a viability of 80% as determined by the trypan blue exclusion test. Donor 2 was a 30-year-old male, the cells after isolation had a viability of 79%. Both donors died as a result of trauma and had no history of ethanol misuse. The cells were seeded at a density of 175 × 103 viable cells per cm2 in plastic culture flasks coated with human liver bio-matrix (Rijnjtes et al., 1986). Cells were cultured in Williams’ E medium supplemented with: 10% fetal calf serum, 2 mM L-glutamine, 50 nM dexamethasone, 0.02 U/ml insulin, 2.5 mg/ml fungizone, 100 mg/ml vancomycin, 50 mg/ml gentamycin, 100 mg/ml streptomycin, and 100 U/ml penicillin (WEM-C). Two days after cell seeding, cells were incubated as described in ‘Experimental procedure’.

Experimental procedure

Legalon®Sil was obtained from MADAUS AG (Cologne, Germany). One vial Legalon®Sil contained 528.5 mg of silibinin-C-2',3'-dihydrogensuccinate disodium salt (SDH) (corresponding to 350 mg silibinin). SDH was dissolved in sterile phosphate-buffered saline (PBS) at a concentration of 2 mM.
On day –2, cells were isolated and seeded in 75-cm² culture flasks. After 18–24 h the cells were washed with PBS to remove dead and non-attached cells and the medium was refreshed. On day 0, the medium was collected, used for control analysis and replaced with fresh medium containing ethanol (8.5, 17 or 34 mM) or SDH (5, 10 or 20 µM) or a combination of 34 mM ethanol with SDH. In parallel, cells were incubated in WEM-C as controls. On days 3, 5, 7, 10, 12 and 14, the medium was collected and replaced by fresh medium with the same concentration of ethanol and/or SDH. All incubations were carried out in triplicate.

In order to investigate whether pre-incubation of human hepatocytes had any additional effect on their behaviour towards exposure to ethanol, 5, 10 or 20 µM SDH was added to the cells 6 h before ethanol (34 mM) was introduced in the culture. Medium was collected and refreshed as indicated above.

Sample collection and analysis

At the given time-points the medium was collected, centrifuged for 10 min at 3000 r.p.m. (1100 g) at 4°C. Aliquots of the medium were stored at –20°C until further analysis. The release of lactate dehydrogenase (LDH), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and γ-glutamyl transferase (γ-GT) into the culture medium was determined on a Roche/Hitachi 747 analyser using Roche reagents. LDH is expressed as U/l released into the medium in 24 h in a 75-cm² culture flask (equal to 13 × 10⁶ human hepatocytes). Human albumin and fibrinogen, secreted into the culture medium, were determined by ELISA using specific monoclonal antibodies, as described previously (Fourneau et al., 1997).

Statistical analysis of results

This was performed using Student’s t-test.

RESULTS

When cells were incubated with ethanol at different concentrations (0–34 mM), no change was observed in the medium for the commonly used parameters of liver cell injury: AST, ALT or γ-GT. Only LDH in the medium was significantly increased after incubation of the hepatocytes with 34 mM of ethanol after 7–14 days incubation (Fig. 1A). Incubation with 34 mM ethanol in combination with 5 or 10 µM SDH protected the cells from LDH loss starting from day 7 (Fig. 1B). 20 µM SDH reduced the LDH release starting on day 3. Pre-incubation of the cells with SDH for 6 h before addition of 34 mM ethanol protected the cells against LDH release starting on day 3 (Fig. 2). No changes were observed when primary human hepatocytes were incubated with SDH alone at a concentration of 20 µM for a period of up to 14 days (data not shown). At the end of the experiments, the amounts of albumin and fibrinogen released by the hepatocytes into the medium were determined (Fig. 3). Ethanol (34 mM) led to a decrease, whereas SDH (20 µM) led to an increase in the proteins in the medium; however, these changes reached statistically significant differences only for fibrinogen (Student’s t-test). This effect was sustained in the combination experiments, where SDH decreased the inhibitory effect of ethanol.

DISCUSSION

Exposure of primary hepatocytes (rat and human) or HepG2 cells to ethanol for 24 h results in a time- and dose-dependent increase of apoptosis and necrosis and in a reduction of cell proliferation (Neuman et al., 1993; Roberts et al., 1995;
Fig. 2. Effect of pre-incubation of cells with silibinin-dihemisuccinate (SDH) on the ethanol-induced release of lactate dehydrogenase (LDH) from primary human hepatocytes. Cells were isolated from human liver and cultured in monolayer. Two days after cell seeding the medium was changed to one containing different concentrations of SDH (5, 10 or 20 μM). After 6 h the medium was changed to one containing 34 mM ethanol in combination with 5, 10 or 20 μM SDH. Cells incubated with 34 mM ethanol, or cells cultured without the addition of SDH or ethanol, were included as controls. At the indicated time-points the medium was changed and samples taken for LDH determination. The experiment was performed in triplicate. LDH released into the medium per 24 h was calculated.

Neuman et al., 1999). A concentration of 40 mM ethanol was reported to result in 6% apoptosis in primary human hepatocytes (Neuman et al., 1999). Interestingly, the viability of HepG2 cells declined proportionally until 24 h of exposure to ethanol, but longer incubation (36 or 48 h) did not further reduce cell viability (Neuman et al., 1993). Both LDH release and apoptosis assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (activity of mitochondrial respiratory chain) are considered equally accurate for the quantification of the ethanol-induced toxicity on HepG2 cells (Neuman et al., 1993).

Little is known of how primary (human) hepatocytes respond in a more chronic situation of repeated exposure to ethanol. We observed a significant release of LDH into the medium from day 7 onwards (Fig. 1A), when primary human hepatocytes were cultured in a medium containing 34 mM (0.2% vol/vol.) ethanol but not at 8.5 or 17 mM. The LDH in the medium remained elevated up to day 14, although the medium was refreshed regularly. Under these conditions, a slight decrease of albumin and fibrinogen secretion was observed (Fig. 3). With the diminished availability of human liver tissue for experimental studies, we also tested the human hepatoma variant cell line HepG2-BD5 (van Pelt et al., 2003). We found that these cells were tolerant to at least 12.75-fold higher doses of ethanol and showed decreased LDH-release upon incubation with ethanol. This makes these cells unsuitable as an alternative to primary cells for this type of research.

In the present study we observed that incubation of primary human hepatocytes with 20 μM SDH completely blocked the release of LDH induced by 34 mM ethanol (Fig. 1B). This could involve the protection by SDH of cell membranes from radical-induced damage, because the LDH release was not related to mortality of the hepatocytes. One argument against ethanol-induced cell death in this experimental setting is that, during the entire experiment, no increase was observed of AST, ALT or γ-GT in the medium of cultures with or without ethanol (data not shown). The second argument is that protein synthesis by the hepatocytes, at the end of the experiment (as a measure for the number of cells), was not significantly different for the various medium combinations with ethanol and SDH when compared with cells in the control medium (Fig. 3). Therefore, the LDH measured in the medium must have leaked out or been transported from hepatocytes that were still viable. Interestingly, when primary hepatocytes were incubated with 20 μM SDH alone, total protein production was increased by around 30%. This increase was significant for fibrinogen but not for albumin (Fig. 3). Preincubation of the cells for 6 h with SDH prior to ethanol addition, moved forward the protective effect on the LDH release from day 7 to day 3 for a dose of either 5 or 10 μM SDH. At a dose of 20 μM SDH, preincubation of the cells did not have an additional protective effect on LDH release (Figs 1B and 2).

We can conclude from this study that SDH may have a beneficial effect on human hepatocytes when they are exposed to ethanol. In addition, the effect of SDH on protein synthesis in the absence of ethanol suggests a stimulating effect of this compound on cells. Whether these in vitro observations also have clinical significance remains to be clarified.
mechanism by which SDH might have a protective effect on the liver could involve multiple biochemical events (Valenzuela and Garrido, 1994; Wellington and Jarvis, 2001). This may explain the conflicting results obtained from in vitro and in vivo (patient) studies. Carefully designed in vitro studies using primary (human) hepatocytes are required to evaluate further the role of SDH at different levels, including ethanol transport, metabolism, toxicity and oxidative stress.

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