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Protective effect of gamma-aminobutyric acid (GABA) against cytotoxicity of ethanol in isolated rat hepatocytes involves modulations in cellular polyamine levels

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Summary. Gamma-aminobutyric acid (GABA) is considered to be a multifunctional molecule with various physiological effects throughout the body. It is also evident that the liver contains GABA and its transporter. However, the functions of GABA in liver have not been well documented. In this study, the cytoprotective effect of GABA against ethanol-induced hepatotoxicity was evaluated in primary cultured rat hepatocytes. Addition of ethanol induced decrease of cell viability in a dose-dependent manner. However, treatment with GABA resulted in a dose-dependent recovery from ethanol (150 mM)-induced cytotoxicity.

GABA reversed the ethanol-induced decrease in intracellular polyamine levels. Furthermore, the addition of polyamines also reversed the ethanol-induced decrease of cell viability. These results suggest that GABA is protective against the cytotoxicity of ethanol in isolated rat hepatocytes and this effect may be modulated by the maintenance of intracellular polyamine levels.

Keywords: GABA – Ethanol – Hepatocytes – Polyamines

Introduction

Gamma-aminobutyric acid (GABA) is well known as a potent amino acid neurotransmitter. It is reported to have various physiological functions in the body and also used for hypertension treatment (Vemulapalli and Barletta, 1984). Rat and human liver contain high concentration of GABA and its metabolic enzyme systems (Minuk, 1993). The uptake system of GABA has been extensively studied and GABA transporters identified and cloned in human liver (Gong et al., 2001). Furthermore, it was reported that the serum concentrations of GABA were increased in patients with acute and chronic liver failure in a clinical study (Levy et al., 1987). However, the relationship between GABA and liver failure has not been well documented.

Alcohol-induced liver disease is the most common hepatic disease in western countries (McCullough, 1999). Hepatocyte apoptosis is recognized in the liver of both clinical (Natori et al., 2001) and experimental (Goldin et al., 1993) alcohol-related injuries, and currently identified as a common feature of alcoholic liver disease. The evaluation of ethanol-induced hepatic disease in *in vitro* models has thus become important and been studied by methods using hepatocytes extensively. However, the concentration of ethanol to induce hepatotoxicity in available literature range from 1 (Castilla et al., 2004) to 200 mM (Lee et al., 2002). Furthermore, mechanisms and mediators responsible for ethanol-induced liver failure are not clearly understood.

It has been suggested that ethanol induced a decrease of intracellular polyamines (putrescine, spermidine and spermine), which are ubiquitous polycationic metabolites and essential for a normal growth rate in mammalian cells (Sessa and Perin, 1997). We previously reported that the maintenance of intracellular polyamine levels was an important mechanisms in the reduction of cytotoxicity induced by carbon tetrachloride (Chen et al., 2000).

The objective of the present study was to examine the cytoprotective effect of GABA against ethanol-induced hepatic injury and its related mechanism. To address this, we investigated the role of GABA in the regulation of cell injury in isolated primary cultured rat hepatocytes, as well as the mediation of intracellular polyamine levels in this role.

Materials and methods

Materials

GABA was purchased from Wako (Osaka, Japan). Fetal bovine serum (FBS) was purchased from Equitech-Bio Inc. (Kerrville, Texas, USA). Putrescine, spermidine and spermine were purchased from Sigma-Aldrich Fine Chemicals (Tokyo, Japan). Other chemicals used in this study were special grade commercial products.

Method

Hepatocyte preparation and culture

The Animal Research Committee of the Osaka City University approved the protocol for this experiment, and care of the animals was in accordance with the standard of this institution (Guide for Animal Experimentation, Osaka City University). Hepatocyte were isolated from 10-week-old male Wister rats anesthetized with diethyl ester by collagenase perfusion (Moldeus et al., 1978). The viability of the isolated hepatocytes was over 90% as determined by 0.2% trypan blue exclusion. The cells were plated in 35 mm plastic dishes at a density of 2.5×10^5 cells/ml in 2 ml Williams' Medium E supplemented with 10% FBS, and were cultured in humidified atmosphere of 5% CO_2 and 95% air at 37 °C overnight. This medium was treated with or without GABA the next day. Two hours after this treatment, the medium was changed for 10% horse serum containing fresh Williams' medium E with or without ethanol and polyamines. It has been reported that exogenous addition of polyamine can be irreversibly degraded by oxidative deamination catalysed by amine oxidase and the products of this degradation are unstable and toxic (Schipper et al., 2000). To lower the influence of this oxidation, we used horse serum which is known to contain less amine oxidase.

Assay of cell viability

Cell viability was measured by Neutral Red assay as described previously (Zhang et al., 1990). Neutral Red stock solution (0.4% Neutral Red in water) was diluted 1:80 in phosphate-buffer saline. Hepatocytes were incubated with the Neutral Red solution for 2 h at 37 °C to allow the lysosomes of viable cells to take up the dye. The Neutral Red solution was then removed and the cultures were washed rapidly (in less than 2.5 min) with a mixture of 1% formaldehyde-1% calcium chloride. A mixture of 1% acetic acid-50% ethanol was added to the cells to extract the Neutral Red from hepatocytes at room temperature for 30 min. Each sample was then measured at 540 nm with a spectrophotometer.

LDH assay

Measuring the LDH activity in the suspension buffer as previously described (Bergmeyer et al., 1965), monitored the degree of cell injury. Assay conditions included 0.6 mM sodium pyruvate, 0.18 mM NADH, and a suitable volume of enzyme solution at 25 °C in a total volume of 3.15 ml.

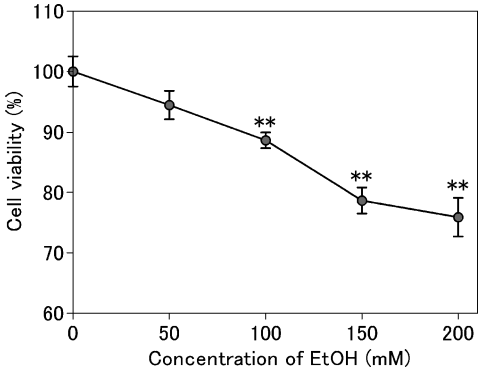

Fig. 1. Dose-dependent cytotoxicity of ethanol on cell viability. Cells (2.5×10^5 cells/ml) were treated with varying concentrations of ethanol for 24 h. Cell viability was determined as described in Materials and methods. Cell viability was expressed as the percentage of absorbance at 540 nm to the average of non-EtOH-treated cultures. Results show means ± S.D. of three different determinations. Data were analyzed by one-way ANOVA followed by Tukey multiple comparison test. The differences between means were significant at $^{**}p < 0.01$, compared with non-EtOH-treated cultures

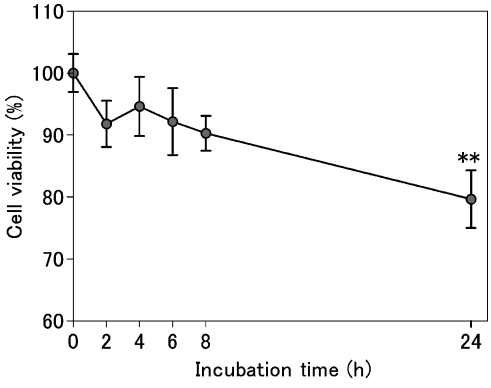

Fig. 2. Time course effect of ethanol on cell viability. Cells (2.5×10^5 cells/ml) were incubated for the indicated period with or without ethanol (150 mM). Cell viability was determined as described in Materials and methods, and the level relative to that of each time of control (100%). Results show means ± S.D. of three different determinations. Data were analyzed by one-way ANOVA followed by Tukey multiple comparison test. The differences between means were significant at $^{**}p < 0.01$, compared with 0 h incubation cultures

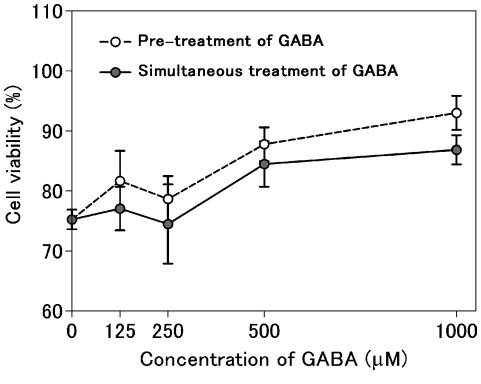

Fig. 3. The protective effect of GABA on cell viability in ethanol-treated hepatocyte. Cells (2.5×10^5 cells/ml) were incubated for 24 h with ethanol (150 mM) and GABA (125–1000 μM). Treatment with GABA was carried out by the simultaneous or pre-addition (2 h.) of GABA before ethanol-treatment. Cell viability was expressed as the percentage of absorbance at 540 nm to the average of non-EtOH-treated cultures. Results show means ± S.D. of three different determinations. The data were analyzed by two-way ANOVA to establish the dose-dependent effect of GABA and to compare the effect between simultaneous and pre-treatment with GABA. $p < 0.05$ was used to indicate a statistically significant difference

The initial rate of NADH loss, measured as a reduction in absorbance at 340 nm, was used as an indication of LDH activity. Under these assay conditions the loss of NADH was linear with respect to time and enzyme concentration over the range of enzyme activity monitored.

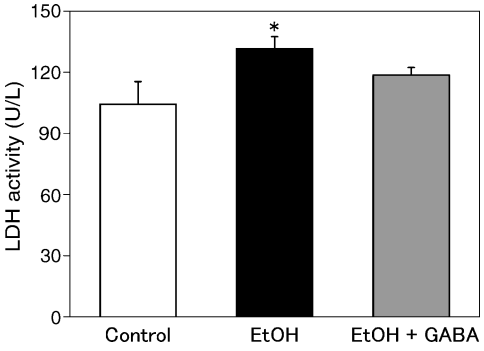

Fig. 4. The protective effects of GABA on ethanol-induced cytotoxicity measured as leakage of LDH, a biochemical marker of cell injury. Cells (2.5×10^5 cells/ml) were incubated for 24 h with or without ethanol (150 mM) and pre-addition with GABA (1000 μM). Leakage of LDH was determined as described in Materials and methods. Results show means ± S.D. of three different determinations. Data were analyzed by one-way ANOVA followed by Tukey multiple comparison test. The differences between means were significant at $^*p < 0.05$, compared with control cultures

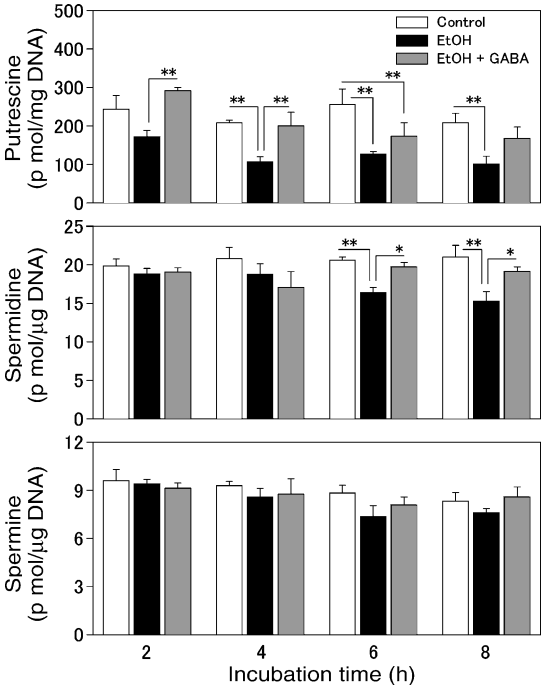

Fig. 5. The effects of GABA on cellular polyamine levels in ethanol-treated rat hepatocyte. Cells (2.5×10^5 cells/ml) were incubated for the indicated period with or without ethanol (150 mM) and pre-addition of GABA (1000 μM). Cellular polyamine levels were determined as described in Materials and methods. Results show means ± S.D. of three different determinations. Data were analyzed by one-way ANOVA followed by Tukey multiple comparison test. The differences between means were significant at $^*p < 0.05$ and $^{**}p < 0.01$

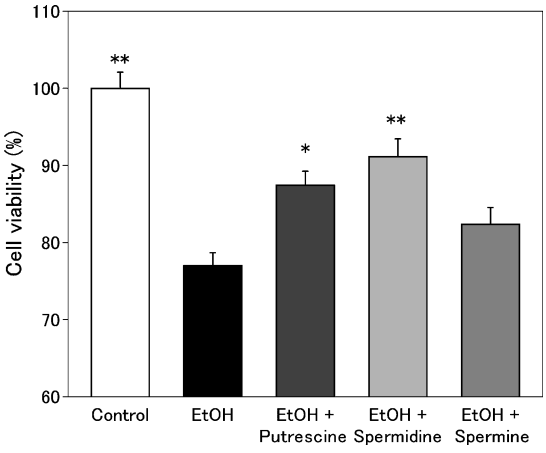

Fig. 6. The effects of polyamines in ethanol-treated isolated rat hepatocyte. Cells (2.5×10^5 cells/ml) were incubated for 24 h with or without ethanol (150 mM) and 400 μM polyamines (putrescine, spermidine, spermine). Cell viability was determined as described in Materials and methods, and the level relative to that of control (100%) was determined. Results show means ± S.D. of three different determinations. Data were analyzed by one-way ANOVA followed by Tukey multiple comparison test. The differences between means were significant at $^*p < 0.05$ and $^{**}p < 0.01$, compared with ethanol-treated cultures

Determination of intracellular polyamines

Cells (5×10^5) were collected and an extract was obtained by treating with 0.35 ml of 0.4 N perchloric acid, and centrifuged at 14,000 × *g* for 20 min. The supernatant obtained was used for injection into the HPLC system. The polyamines were separated on an ODS column (Mightysil RP-18 GP, 4.6 × 150 mm, particle size 5 μm, Kanto chemical, Tokyo, Japan), with a solvent composed of 10 mM 1-hexanesulfonic acid sodium salt/100 mM sodium perchloric acid (pH = 4.0) as solvent A and solvent A/methanol (1:3) as solvent B (pH = 3.0). The sample was eluted with a programmed solvent gradient using a linear gradient curve. The gradient changed from 4 to 55% of solvent B from 0 to 30 min at flow rate of 0.7 ml/min. Eluted fractions were mixed with 6 mM *O*-phthalaldehyde (0.7 ml/min), and the fluorescence was measured at excitation and emission wavelength of 345 and 440 nm, respectively, for assay of the polyamines with an FP1520 fluorescence detector (JASCO, Tokyo, Japan). The DNA content of the perchloric acid-precipitable materials was determined as previously described (Burton, 1968).

Statistical analysis

The data in Figs. 1–6 were analyzed by one-way ANOVA, and the differences between means were analyzed using Tukey multiple comparison tests. The data of Fig. 3 were analyzed by two-way ANOVA to establish the dose-dependent effect of GABA and to compare the effect between simultaneous and pre-treatment with GABA. $p < 0.05$ was used to indicate a statistically significant difference.

Results

Initially, hepatocytes were treated with various concentrations of ethanol for 24 h. The effect of ethanol on cell viability was examined by the neutral red assay as described above. Figure 1 shows that treatment with ethanol significantly decreased cell viability in a dose-depen-

dent manner. It has been reported that the concentration of ethanol in a heavy drinker was from 100 to 200 mM (Henzel et al., 2004). Therefore, we regarded 150 mM ethanol as a suitable concentration to examine ethanol-induce liver injury. Hence, this concentration was used in subsequent experiments. As shown in Fig. 2, a significant decrease of cell viability induced by ethanol (150 mM) was observed at 24 h significantly. However, addition of GABA reversed this decrease in a dose-dependent manner (125–1000 μM). Furthermore, a two-hour pre-treatment with GABA before addition of ethanol showed significantly stronger cytoprotective effect than simultaneous treatment (Fig. 3). To confirm the cytoprotective effect of GABA, the degree of cell injury was examined by LDH assay, which is applied to clinical diagnosis of organic necrosis. It has been shown in cell tissue culture studies that the majority of apoptotic cells are not phagocytosed but rather enter a process termed secondary necrosis (Vahrmeijer et al., 1999). Therefore, we examined this enzymatic activity as a biomarker of apoptotic and/or necrotic type of cell death induced by ethanol in this study. Figure 4 shows that ethanol (150 mM) induced significant leakage of LDH, but pre-treatment with GABA (1000 μM) reduced this leakage.

In Fig. 5, intracellular polyamine (putrescine, spermidine, spermine) levels were assayed to examine the cytoprotective mechanism of GABA. In ethanol-treated cells, depletion of intracellular putrescine and spermidine were observed before 8 h. There was, however, no change in intracellular spermine levels in ethanol-treated cells. However, these decreases were significantly reversed by pre-treatment of GABA.

To examine the relationship between ethanol-induced cytotoxicity and depletion of intracellular polyamine, we evaluated the effect of exogenous addition of polyamine (putrescine, spermidine, spermine) on cell viability (Fig. 6). It has been reported that cells are equipped with an efficient transport system for uptake of polyamines from extracellular sources (Seiler et al., 1996) and exogenous addition of polyamine prevented polyamine depletion-related cell injury (Arimura et al., 2005; Wu et al., 1997). Treatment with putrescine and spermidine significantly reversed ethanol-induced cytotoxicity.

Discussion

The results obtained in the present study demonstrate for the first time, to the best of our knowledge, that GABA exhibits cytoprotective effect against ethanol-induced hepatic injury (Figs. 3, 4). Furthermore, we investigated this mechanism of GABA on ethanol-induced hepatocyte injury, and found that ethanol decreased cellular putrescine levels and treatment with GABA prevented this decrease (Fig. 5). These new findings suggest that the maintenance of cellular polyamine levels is one of the important roles in the protection of GABA against ethanol-induced hepatocyte injury.

Previous work in a number of model systems have shown that oxidative stress and oxygen-derived free radicals are well known to play an important role in the pathogenesis of ethanol-associated liver injury (Adachi and Ishii, 2002; Nagy, 2004). It was also reported that antioxidants such as green tea catechins (Ostrowska et al., 2004) and resveratrol (Kasdallah-Grissa et al., 2006) reduced ethanol-induced lipid peroxidation to nearly control levels in *in vivo* rat studies. In this study, pre-treatment of GABA reversed ethanol-induced polyamines depletions (Fig. 5), however, the high concentration more than 1000 μM of GABA did not reverse ethanol-induced cytotoxicity to control levels (data not shown). Furthermore, treatment with GABA and exogenous polyamines (putrescine and spermidine) reversed ethanol-induced cell injury to nearly the same extend, but not completely (Figs. 3, 6). These results suggest that depletion of cellular polyamine levels is one of the important pathways to cell death, besides other possible pathways which may exist in ethanol-induced hepatic cytotoxicity.

GABA is the predominant inhibitory neurotransmitter in the mammalian brain. Although initially thought to be confined to the central nervous system, GABAergic activity has also been described in other tissues throughout the body. Previous work in a number of model systems have shown that the liver is also equipped with transport, metabolic and generation systems of GABA (Minuk, 1993). In this study, pre-treatment with GABA before addition of ethanol showed significantly stronger cytoprotective effect than with simultaneous treatment (Fig. 3). This result suggests that the cytoprotective effect of GABA is not due to the direct reaction with ethanol in the medium, but the transport and/or metabolic of GABA may plays an important role in its activity.

On the relationship between cellular polyamine levels and liver injury, putrescine exhibited a protective effect against acute liver injury caused by hepatotoxins such as carbon tetrachloride (Nagoshi et al., 1994), D-galactosamine (Nishiguchi et al., 1990) and cadmium (Tzirogiannis et al., 2004) in *in vivo* and *in vitro* experimental models. Furthermore, many reports have shown that both acute and chronic exposure of ethanol induced suppression of intracellular putrescine levels, which were resulted from loss of ornithine decarboxylase activity (Klein et al., 1996; Sessa and Perin, 1997; Shibley et al., 1995; Tanaka et al., 1991). In this study, putrescine levels decreased

earlier than spermidine after the addition of ethanol (Fig. 5). Spermidine is synthesized from putrescine, hence this result suggest that the maintenance of putrescine level is an important mechanism of GABA to protect hepatocytes against ethanol-induced cytotoxicity. On the other hand, previous studies reported that ethanol potentiates GABAergic activities in liver and results in reduction of hepatic regenerative activity following partial hepatectomy, as well as following acute and chronic form of liver disease (Minuk et al., 1995).

Despite the fact that we have been able to show the involvement of intracellular polyamine levels in this study, the precise roles of GABA on the liver have yet to be clarified. We seek to further clarify this role and the associated mechanism(s) of the protective effect of GABA against ethanol-induced hepatic injury. This subject is currently under intense scrutiny in our laboratory.

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