Chronic ethanol treatment of human hepatocytes inhibits the activation of the insulin signaling pathway by increasing cytosolic free calcium levels

YI-MIN CHEN[1], JIN-FANG ZHAO[1], YONG-LIN LIU[1], JIE CHEN[2] and RONG-LIN JIANG[3]

[1]Department of Clinical Laboratory, The First Affiliated Hospital of Zhejiang Chinese Medical University, Hangzhou, Zhejiang 310006; [2]Department of Endocrinology, The First Clinical Medical College, Zhejiang Chinese Medical University, Hangzhou, Zhejiang 310053; [3]Department of Intensive Care Unit, The First Affiliated Hospital of Zhejiang Chinese Medical University, Hangzhou, Zhejiang 310006, P.R. China

Abstract. The present study aimed to investigate the effects of ethanol treatment on the induction of intracellular calcium ($[Ca^{2+}]_i$) levels and the inhibition of the activation of the insulin signaling pathway in human hepatocytes. L-02 cells were treated with various concentrations of ethanol for different periods of time. Cell viability and alanine aminotransferase (ALT)/aspartate aminotransferase (AST) leakage in the culture supernatant were evaluated. Changes in $[Ca^{2+}]_i$ levels were detected by flow cytometry and confocal microscopy. Total RNA and protein were extracted to examine the mRNA and protein levels of insulin receptor substrate (IRS)1, IRS2, phosphatidylinositol 3-kinase (PI3K) and glucose transporter 2 (GLUT2) by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analysis, respectively. Furthermore, insulin was added to the ethanol-treated L-02 cells, and the phosphorylation levels of PI3K and protein kinase B (PKB) were determined by western blot analysis before and after Ca^{2+} blockage. No significant changes were observed in cell viability, $[Ca^{2+}]_i$ levels and in the expression and phosphorylation levels of insulin signal transduction molecules when the L-02 cells were treated with 0.5 or 1% ethanol. However, treatment with 2 or 4% ethanol resulted in a significant decrease in cell viability and in the mRNA levels of *IRS1*, *IRS2*, *PI3K* (p85α) and *GLUT2*, as well as in an increase in ALT/AST leakage and in the $[Ca^{2+}]_i$ levels (P<0.05). The expression and phosphorylation levels of PI3K (p85α) and PKB were also inhibited by treatment with 2 or 4% ethanol. These cytological effects induced by ethanol treatment were partially reversed by Ca^{2+} blockage. These results suggest that ethanol treatment inhibits the activation of the insulin signal transduction pathway in a dose-, time- and Ca^{2+}-dependent manner. The inhibition of *IRS1/2*, *PI3K* (p85α), *PKB* and *GLUT2* expression and of PI3K (p85α) and PKB phosphorylation by the high concentrations of ethanol may be the core molecular mechanism of ethanol-induced insulin resistance, and may be related to the induction of $[Ca^{2+}]_i$ levels.

Introduction

Studies on the correlation between alcohol and type 2 diabetes mellitus (T2DM) have indicated that ethanolism is one of the predisposing factors for T2DM (1-3). As demonstrated by previous studies (1-4), there is a U- or J-shaped correlation between alcohol consumption and diabetes, namely the risk of diabetes is lowest for subjects with appropriate alcohol intake, while for patients with excessive ethanol consumption, the risk is relatively high. However, the relevant etiology of T2DM induced by excessive alcohol consumption has not yet been elucidated.

Insulin resistance is the pathological basis for T2DM, and is associated with an insulin-mediated decrease in the glucose uptake capability of the liver, skeletal muscle and adipocytes, as well as a reduction in glycogen synthesis in hepatocytes (5,6). Since the liver is the main organ responsible for glucose and lipid metabolism, it is able to regulate blood glucose through hepatocellular glycogen synthesis and gluconeogenesis (7). The dysfunction of the insulin signaling pathway has been reported to be one of the important factors for the development of insulin resistance. Normally, the insulin and insulin receptor (IR) complex are activated by phosphorylation, and in turn stimulate the downstream insulin receptor substrate (IRS), phosphatidylinositol 3-kinase (PI3K) and protein kinase B (PKB, also known as Akt), resulting in the transduction of insulin-mediated signaling and the regulation of hepatocellular glycogen synthesis and gluconeogenesis (8). It has been demonstrated that alcohol consumption induces an

Correspondence to: Professor Rong-Lin Jiang, Department of Intensive Care Unit, The First Affiliated Hospital of Zhejiang Chinese Medical University, 54 Youdian Road, Hangzhou, Zhejiang 310006, P.R. China
E-mail: jiangronglin@126.com

increase in intracellular calcium ([Ca^{2+}]$_i$) levels and hepatocellular damage. Hepatocellular calcium overload significantly contributes to ethanol-induced hepatic injury (9). In addition to causing direct damage to hepatocytes, ethanol intake may induce insulin resistance by interfering with glycogen synthesis, gluconeogenesis and relevant signal transduction. In the present study, human hepatocytes were treated with various concentrations of ethanol. Changes in the [Ca^{2+}]$_i$ levels and in the expression of key signaling molecules of the insulin signaling pathway, as well as changes in relevant phosphorylation profiles were determined. The effects of [Ca^{2+}]$_i$ on insulin signaling were examined by Ca^{2+} channel blockage assay in order to elucidate the mechanisms responsible for the ethanol-induced increase in hepatocellular calcium ions and its effects on the insulin signaling pathway.

Materials and methods

Cell source and culture. Human hepatocytes (L-02), supplied by the Cell Bank of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China), were incubated in RPMI-1640 medium (Gibco, Carlsbad, CA, USA) containing 10% fetal calf serum (FCS) at 37˚C and 5% CO$_2$.

Ethanol treatment of hepatocytes. The L-02 cells were inoculated into 6-well plates (1x10^5 cells/well) and cultured overnight at 37˚C in 5% CO$_2$ to form a cellular monolayer. The medium was then replaced with serum-free RPMI-1640 medium containing ethanol at various concentrations (0, 0.5, 1, 2 or 4%, v/v), based on the reaction time and concentrations specified in the literature (9-11). After being sealed with parafilm, the culture was incubated at 37˚C in 5% CO$_2$ for 0, 2, 6, 12 or 24 h. Due to the concentration reduction caused by ethanol volatilization, the culture medium was replaced with fresh medium containing ethanol (concentration unchanged) every 6 h.

Examination of biochemical parameters. Following incubation, the supernatants were collected and the concentration of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) was determined for each culture using an Aeroset automatic biochemical analyzer (Abbott Laboratories, Abbott Park, IL, USA). The extent of ethanol-induced hepatocellular damage at different concentrations was analyzed according to the ALT and AST leakage.

Cell viability assays. The L-02 cells were inoculated into 96-well plates (1x10^4 cells/well) and cultured overnight at 37˚C in 5% CO$_2$ to form a cellular monolayer. The cultures were treated with various concentrations of ethanol for 24 h, followed by the addition of 20 μl of CCK-8 solution (Beyotime Institute of Technology, Haimen, China). The mixture was incubated for a further 2 h. The absorbance was then measured at 450 nm using an iMark Microplate Absorbance Reader (Bio-Rad, Hercules, CA, USA).

[Ca^{2+}]$_i$ assays. The cell cultures were treated with various concentrations of ethanol for various periods of time prior to trypsinization with 0.25% trypsin. The cell suspensions were collected by centrifugation at 500 x g for 5 min. After washing

with D-Hank's solution and centrifugation, the cell pellets were resuspended in 500 μl of RPMI-1640 medium containing 5 μM Fluo-4 AM (Ca^{2+} fluorescence indicator; Molecular Probes, Eugene, OR, USA), 0.1% Pluronic F-127 (Sigma-Aldrich, St. Louis, MO, USA) and 0.2% BSA (Amresco LLC, Solon, OH, USA). After staining for 30 min at 37˚C, the cells were centrifuged and washed twice with D-Hank's solution to remove the staining reagent, followed by a 30-min incubation with serum-free RPMI-1640 medium for AM de-esterification to release the indicator. The fluorescence intensity of intracellular Ca^{2+} was then determined by flow cytometry using a BD FACSCanto™ II flow cytometer (BD Diagnostic Systems, Sparks, MD, USA), and the mean fluorescence intensity of a total of 10,000 cells was evaluated using BD FACSDiva software version 8.0 (BD Diagnostic Systems). Furthermore, 5x10^4 cells were seeded in 12-well culture plates (Corning Inc., Union City, CA, USA) containing 20-mm diameter coverslips and incubated overnight at 37˚C to form a cell monolayer. Following treatment with 2 or 4% ethanol for 24 h, the cells were washed thoroughly with D-Hank's solution and stained with Fluo-4 AM as described above. After staining, the coverslips were removed and the cells were fixed with ice-cold methanol, and then mounted on a glass slide for the determination of the [Ca^{2+}]$_i$ fluorescence intensity under a laser confocal scanning microscope (LSM510; Zeiss, Jena, Germany) with an excitation wavelength of 494 nm and emission wavelength of 516 nm.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The PCR primer sequences for human insulin receptor substrate 1 (*IRS1*), *IRS2*, *PI3K* and *β-actin* (as the internal control) for RT-qPCR were obtained from the RTPrimerDB database (the real-time PCR primer and probe database). The primer sequences for human glucose transporter 2 (*GLUT2*) were as previously described (12). The above-mentioned primers were synthesized by Invitrogen Co., (Shanghai, China) (Table I). The cell cultures were treated with various concentrations of ethanol for 24 h and the cells were harvested using a scraper. Total RNA was extracted using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). The content and purity of the RNA were determined by spectrophotometry. Using a PrimeScript® RT reagent kit (Takara Bio, Inc., Shiga, Japan), cDNA was prepared by the reverse transcription of equal amounts of total RNA from each cell culture, according to the manufacturer's instructions. Each sample was subjected to PCR amplification using a SYBR® Premix Ex Taq™ II kit (Takara Bio, Inc.) on an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The reaction volume was 20 μl, containing 0.2 μM primers and 2 μl cDNA template. The PCR program used was: 95˚C for 30 sec, followed by 40 cycles of 95˚C for 5 sec and 60˚C for 20 sec. The RT-qPCR results were quantitatively analyzed using REST 2005 software and the ΔΔCT relative quantification method, as previously described (13).

Western blot analysis. The cell cultures were treated with various concentrations of ethanol for 24 h, and then harvested using a scraper followed by centrifugation at 500 x g for 5 min. The cell pellets were lysed with 200 μl lysis buffer (Beyotime Institute of Technology) and the lysates were centrifuged at

Table I. Primers for RT-qPCR.

Gene	Forward primer (5'→3')	Reverse primer (5'→3')	Size (bp)
IRS1	CAGCTCACCTTCTGTCAGG	AGGTCCATCTTCATGTACTCC	90
IRS2	ATTGACTTCTTGTCCCACCA	TCCAAAAGAAAACTGCAAGC	157
PI3K (p85α)	GATTCTCAGCAGCCAGCTCTGAT	GCAGGCTGTCGTTCATTCCAT	91
GLUT2	TGGGCTGAGGAAGAGACTGT	CATAGGAACCAGGCCTGAAA	282
β-actin	CTGGAACGGTGAAGGTGACA	AAGGGACTTCCTGTAACAATGCA	140

IRS, insulin receptor substrate; PI3K, phosphatidylinositol 3-kinase; GLUT2, glucose transporter 2.

17,200 x g for 5 min to collect the supernatant specimens for western blot analysis. Following protein quantification, 50 μg of protein were resolved by SDS-PAGE and electrically transferred onto PVDF membranes. Using rabbit-IgG against human PI3K (Cat. no. 93-3959-100) or Akt/PKB (Cat. no. 93-3247-100; dilution 1:500; both from BioVision, Inc., Milpitas, CA, USA) as the primary antibody, and HRP-conjugated goat anti-rabbit-IgG (Cat. no. LK-ab5178-100; 1:3,000; LiankeBio, Hangzhou, China) as the secondary antibody, western blot analyses were performed to determine the effects of various concentrations of ethanol on the expression of PI3K and PKB, with β-actin as the internal reference. To examine the effects of ethanol on the phosphorylation profiles of critical signaling molecules in the insulin signaling pathway, the medium was changed to ethanol- and serum-free RPMI-1640 medium following the 24-h ethanol treatments. Insulin (100 nM) was then added to each well and the culture was incubated for a further 30 min. The cells were harvested as described above and were used for protein extraction. Western blot analyses for detecting the phosphorylation profiles of PI3K and PKB were performed as described above, with rabbit-IgG against human phospho-Tyr508-PI3K (p85α; Cat. no. sc-12929-R) and phospho-Thr308-Akt/PKB (Cat. no. sc-16646-R) or phospho-Ser473-Akt/PKB (Cat. no. sc-7985-R; all from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) as the primary antibodies.

Ca²⁺ channel blockage assays. The L-02 cells were inoculated into 6-well plates (1x10⁵ cells/well) and cultured overnight at 37°C in 5% CO_2 to form a cell monolayer. The culture medium was replaced, followed by the addition of 100 μl of a Ca^{2+} channel blocker and/or a chelating agent with the following final concentrations: 1 mM EGTA (extracellular Ca^{2+} chelator), 50 μM 2-APB (IP3 receptor antagonist), or 1 mM EGTA + 50 μM 2-APB. The cells were pre-treated for 30 min at 37°C prior to treatment with 2% ethanol for 24 h. The medium was replaced with ethanol- and serum-free RPMI-1640 containing 100 nM insulin and incubated for a further 30 min. The fluorescence intensity of $[Ca^{2+}]_i$ was assayed by flow cytometry. Simultaneously, cells were harvested for protein extraction. The effects of ethanol on the phosphorylation profiles of PI3K and PKB following Ca^{2+} blockage were examined by western blot analysis as described above.

Statistical analysis. The results are presented as the means ± SD. SPSS 16.0 software was used to perform the Student's t-test for
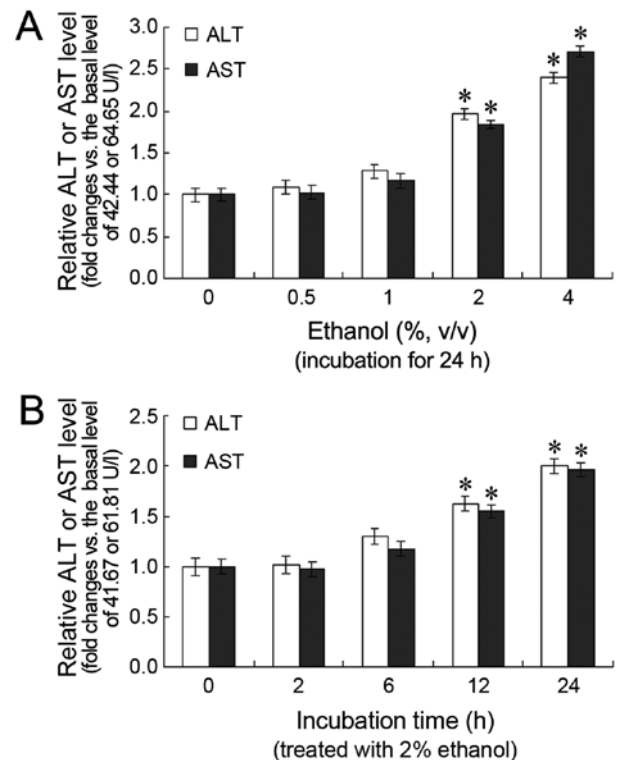

Figure 1. Changes in alanine aminotransferase (ALT) and aspartate amino-transferase (AST) levels in the supernatant of L-02 cells following ethanol treatment. (A) L-02 cells were treated with various concentrations of ethanol for 24 h. (B) L-02 cells were treated with 2% ethanol for different periods of time. *P<0.05 vs. the untreated group or at the 0 h time point.

comparisons between groups. A P-value <0.05 was considered to indicate a statistically significant difference.

Results

Effects of ethanol on ALT and AST levels in L-02 cells. Following treatment with various concentrations of ethanol for 24 h, the ALT and AST levels in the cell cultures increased in a dose-dependent manner, with a statistically significant difference observed between the 2 and 4% ethanol-treated groups (P<0.05) and the untreated group (Fig. 1A). Furthermore, the ALT and AST levels increased in a time-dependent manner. The ALT and AST levels were significantly higher after 12 and 24 h of treatment, compared with the 0 h time point (P<0.05; Fig. 1B).
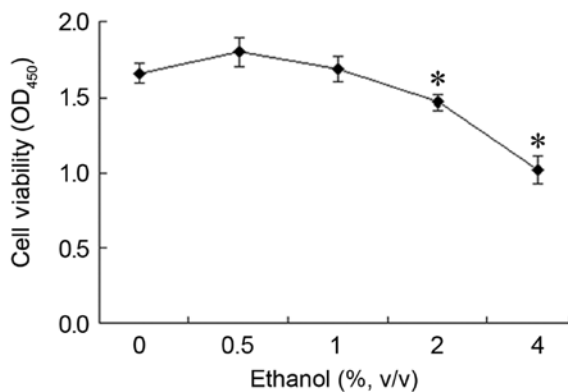
Figure 2. Viability of L-02 cells following treatment with various concentrations of ethanol for 24 h. *P<0.05 vs. untreated cells.

Effect of ethanol on L-02 cell viability. Following treatment with 0.5% ethanol for 24 h, an increase in cell viability was observed. However, this increase was not statistically significant compared with the control group (Fig. 2). By contrast, when the cells were treated with higher concentrations of ethanol, cell viability decreased in a concentration-dependent manner. The OD_{450} value which reflects the cell viability decreased from 1.66 to 1.46 or 1.02 following treatment with 2% or 4% ethanol for 24 h, representing a cell viability of 88.32% (1.46/1.66) and 61.41% (1.02/1.66) of the control group, respectively.

Changes in $[Ca^{2+}]_i$ levels. Following treatment with various concentrations of ethanol for different periods of time, the increase in the fluorescence intensity of $[Ca^{2+}]_i$ correlated with the increase in the ethanol concentration and the treatment duration, indicating that ethanol administration induces an increase in the $[Ca^{2+}]_i$ levels (Fig. 3A). A significant increase in the $[Ca^{2+}]_i$ levels was observed following treatment with 2 or 4% ethanol for 6 h (P<0.05). Moreover, following treatment with 2 or 4% ethanol for 24 h, the $[Ca^{2+}]_i$ levels were 319.19±13.50% and 356.65±25.75% of those of the control level, respectively. These

results were further confirmed by confocal microscopy; a significant increase in the Ca^{2+} fluorescence intensity was observed following treatment with 2 or 4% ethanol for 24 h (Fig. 3B).

Changes in the mRNA expression of IRS1, IRS2, PI3K and GLUT2. Following treatment of the L-02 cells with various concentrations of ethanol for 24 h, the mRNA expression levels of *PI3K* (*p85α*) and *GLUT2* decreased, although a slight increase in the mRNA expression levels of *IRS1* and *IRS2* was observed following treatment with 0.5% ethanol. However, these differences were not statistically significant. As the ethanol concentration increased, the mRNA expression levels of these genes decreased in a dose-dependent manner. The most significant decrease observed was in the mRNA expression of *IRS2* (Fig. 4). Following treatment with 1% ethanol for 24 h, a significant decrease was observed in the mRNA expression levels of *IRS2*, *PI3K* (*p85α*) and *GLUT2* (P<0.05). Following treatment with 2 or 4% ethanol for 24 h, the mRNA expression level of *IRS2* in the L-02 cells decreased to 0.41 and 0.34 fold of the control, respectively (Fig. 4).

Changes in the protein expression and the phosphorylation profiles of PI3K (p85α) and PKB. Following treatment with 0.5 or 1% ethanol for 24 h, no significant changes were observed in the protein expression of PI3K (p85α) and PKB in the L-02 cells. However, following treatment with 2 or 4% ethanol for 24 h, a significant decrease was observed in the protein expression of PI3K (p85α) and PKB compared with the untreated group (P<0.05; Fig. 5A). When the ethanol-treated cells were further stimulated with insulin, the insulin-induced phosphorylation of PKB and PI3K (p85α) decreased in an ethanol dose-dependent manner (Fig. 5B). The administration of 2 or 4% ethanol significantly suppressed the insulin-induced phosphorylation of PKB and PI3K (p85α) compared with the group not treated with ethanol.

Effects of ethanol on $[Ca^{2+}]_i$ levels and the phosphorylation profiles of PI3K (p85α) and PKB following Ca^{2+} blockage. The extracellular Ca^{2+} chelator, EGTA, and the IP3 receptor
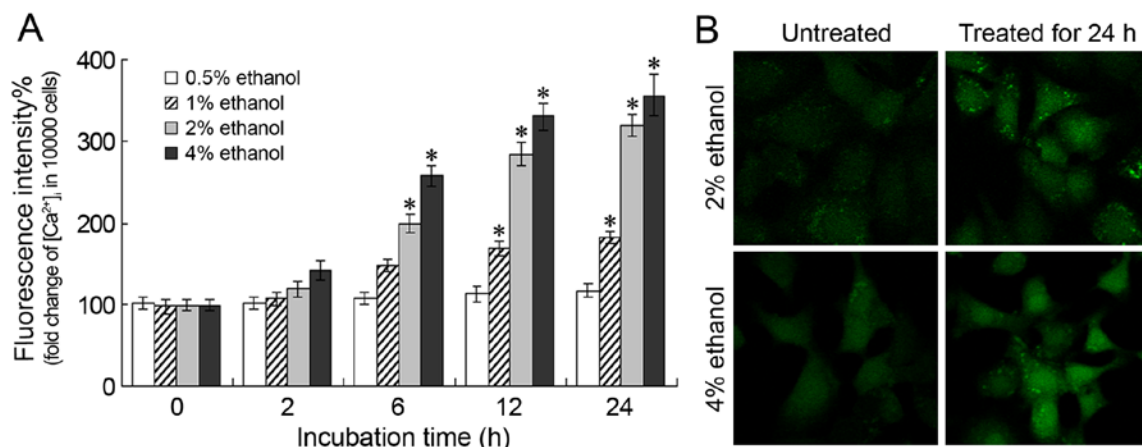

Figure 3. Changes in Ca^{2+} fluorescence intensity in L-02 cells following ethanol treatment. (A) The mean fluorescence intensity reflecting the $[Ca^{2+}]_i$ in L-02 cells was analyzed by flow cytometry following treatment with various concentrations of ethanol for different periods of time. A total of 10,000 cells were analyzed for each of the samples. Bars represent the means ± SD of 3 independent experiments. *P<0.05 vs. the untreated group. (B) Elevation in $[Ca^{2+}]_i$ levels in L-02 cells following treatment with 2% or 4% ethanol for 24 h, determined by laser confocal microscopy. The intensity of green fluorescence reflects the $[Ca^{2+}]_i$ levels in the L-02 cells.
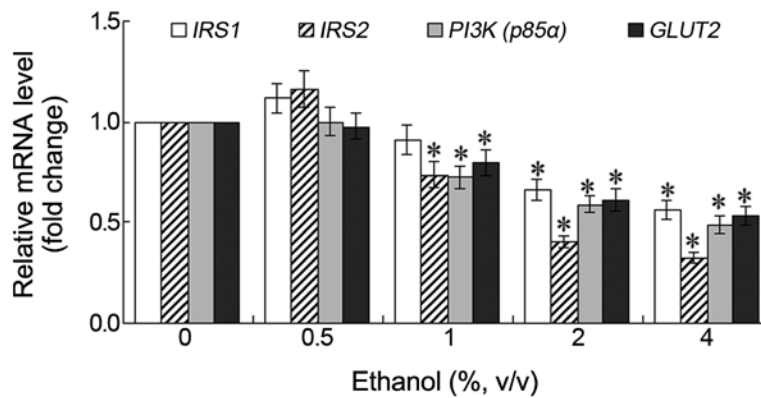
Figure 4. Relative mRNA levels of insulin receptor substrate (*IRS)1*, *IRS2*, phosphatidylinositol 3-kinase (*PI3K*) and glucose transporter 2 (*GLUT2*) in L-02 cells following treatment with various concentrations of ethanol for 24 h. *P<0.05 vs. the untreated group.
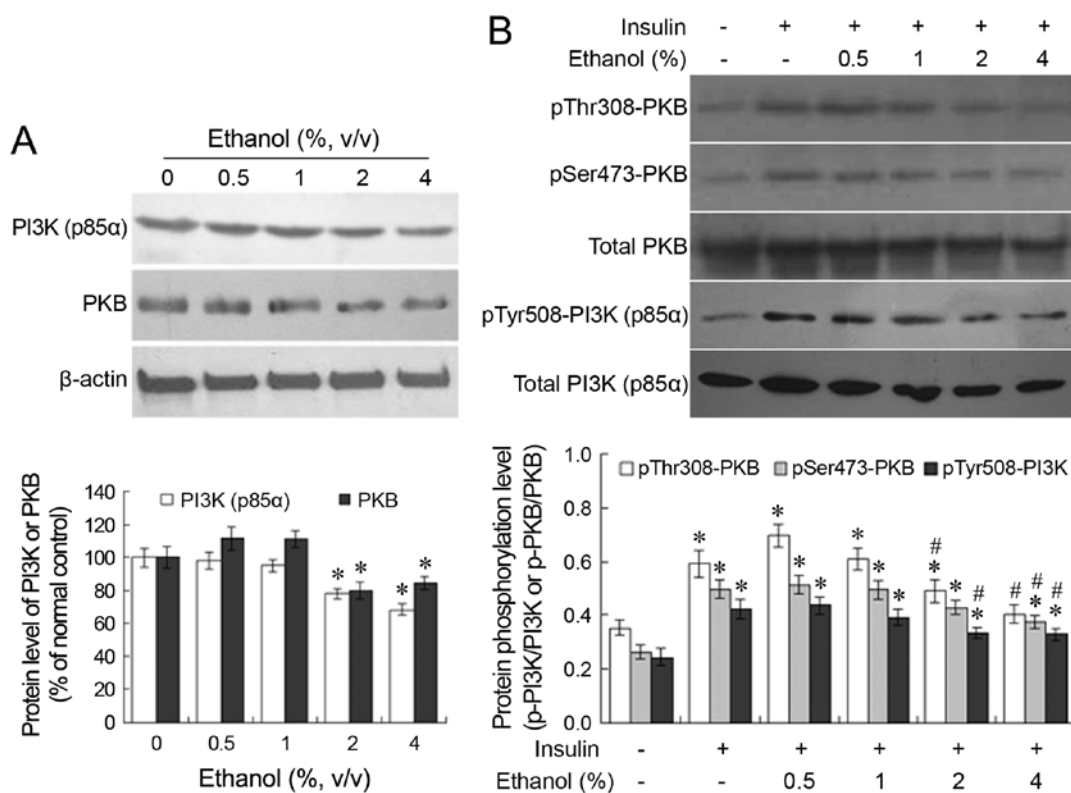

Figure 5. Changes in (A) expression and (B) phosphorylation levels in phosphatidylinositol 3-kinase (PI3K) (p85α) and protein kinase B (PKB) in L-02 cells following treatment with various concentrations of ethanol for 24 h. *P<0.05 vs. the group not treated with ethanol or insulin; #P<0.05 vs. the group treated with insulin and not treated with ethanol.

antagonist, 2-APB, were employed to chelate the influx of extracellular calcium and to block the release of the intracellular calcium pool, respectively. Following treatment with EGTA or 2-APB, the ethanol-induced elevation in the hepatocellular Ca²⁺ concentration was significantly suppressed (P<0.05). This was particularly evident when intracellular and extracellular Ca²⁺ were blocked by the administration of both agents and the ethanol-induced [Ca²⁺]ᵢ decreased to 147.13% of the control (Fig. 6A). With the suppression of the increase in the [Ca²⁺]ᵢ levels, the ethanol-induced decrease in the phosphorylation of PI3K (p85α) and PKB was partially reversed. Following the concomitant administration of EGTA and 2-APB, the inhibitory effects of ethanol on the phosphorylation

of PI3K (p85α) and PKB were attenuated; the phosphorylation levels of PI3K (p85α) and PKB following treatment with both EGTA and 2-APB were similar to those of the control (Fig. 6B).

Discussion

It has been established that long-term excessive alcohol intake is associated with an increased risk of heart and hepatic diseases, hyperglycemia, diabetes and other related diseases (14). With the development of the Chinese economy, the proportion of the population with alcoholism has increased; alcohol abuse is a serious risk factor which is harmful to health in the long-term in China (15). Several epidemiological studies
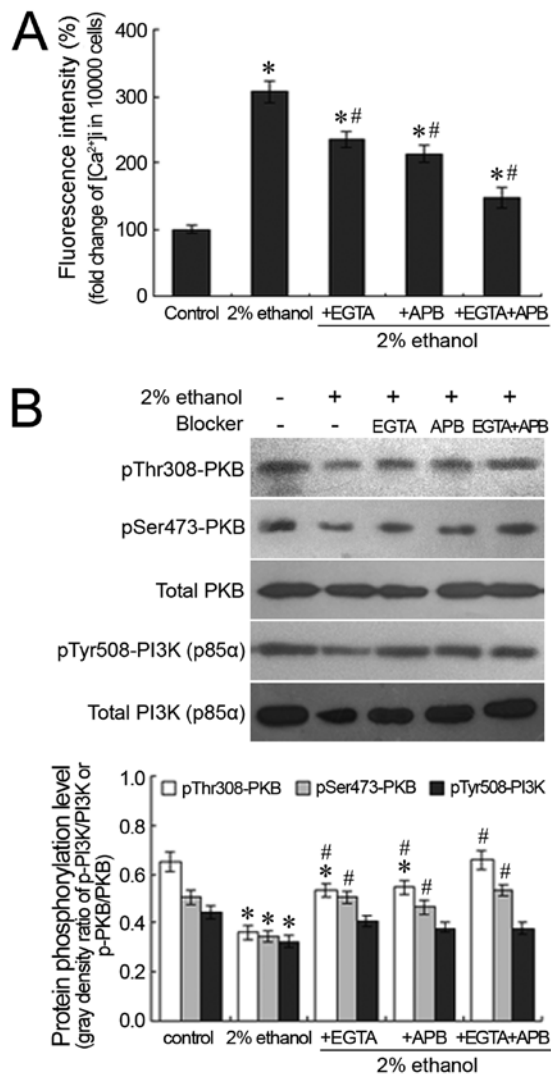
Figure 6. Changes (A) in the intracellular Ca^{2+} level and (B) in the phosphorylation level of phosphatidylinositol 3-kinase (PI3K) (p85α) and protein kinase B (PKB) in control or inhibitor-treated L-02 cells following treatment with 2% ethanol for 24 h. *P<0.05 vs. the untreated group; #P<0.05 vs. the 2% ethanol-treated group.

have demonstrated that excessive alcohol intake is associated with an increased risk of developing T2DM and other metabolic disorders (3,16,17); however, the molecular mechanisms responsible for alcohol-induced insulin resistance have not yet been elucidated.

Insulin resistance is the key pathological feature of T2DM, with the decreased sensitivity of peripheral tissues to insulin as the major presentation. The main manifestations include the decreased insulin-mediated glucose uptake capacity of the liver, skeletal muscle and adipocytes, and the dysfunction of hepatocellular glycogen synthesis (5,6). It has been demonstrated that excessive alcohol intake is associated with insulin resistance (18,19); however, the mechanisms involved remain unknown. As the main organ responsible for glucose and lipid metabolism, the liver regulates blood glucose through hepatocellular glycogen synthesis and gluconeogenesis. Xu *et al* observed that, in rats, alcohol administration correlated with a decrease in glucose synthesis in the liver, the activity of glycogen synthase and glucose uptake by skeletal muscle, as

well as in glycogen synthesis (20). One of the main physiological functions of insulin is to regulate glucose metabolism and to maintain blood glucose levels. The normal function of insulin depends on the effective transduction of the insulin signal, and its dysfunction is considered to be one of the important contributors to the development of insulin resistance. Thus, the present study, we aimed to elucidate the effects of ethanol on the insulin signaling pathway and the related mechanisms. We examined the expression levels of critical signaling molecules in the insulin signaling pathway and the changes in the phosphorylation profiles in L-02 human hepatic cells, which were selected as target cells and were treated with various concentrations of ethanol for various periods of time.

Our results demonstrated that low concentrations (0.5 or 1%) of ethanol had no suppressive effect on cell viability. However, with an increase in the ethanol concentration, cell viability decreased. Following treatment with 2 or 4% ethanol for 24 h, cell viability decreased to 88.32 and 61.41% of the control, respectively. Furthermore, ALT/AST leakage from of the cells increased in a time- and dose-dependent manner, suggesting that ethanol administration *in vitro* is associated with the exacerbation of cell damage.

It is well known that, during the insulin signaling process, the combination of insulin and its receptors results in receptor dimerization and autophosphorylation. IR tyrosine kinase activation subsequently recruits and phosphorylates several substrates, including IRS1-4, thereby providing specific docking sites for the recruitment of other downstream signaling proteins, leading to the activation of the PI3K→PKB signaling cascades (21,22). In the present study, following treatment with various concentrations of ethanol for 24 h, a slight increase in the mRNA expression levels of *IRS1* and *IRS2* in the L-02 cells was observed with a low ethanol concentration (0.5%); however, these expression levels decreased with higher ethanol concentrations in a dose-dependent manner, particularly those of *IRS2*. It has been shown that IRS1 or IRS2 deletion may induce glucose metabolism abnormalities and insulin resistance (23,24), although these effects are tissue-specific (25,26). IRS1 mainly affects skeletal muscle, while IRS2 has extensive effects on the liver, skeletal muscle and adipose tissues. IRS2 dominates the promotion of liver glycogen synthesis and the suppression of hepatic glucose output (25). Our results demonstrated that high concentrations of ethanol significantly decreased the mRNA expression levels of *IRS1* and *IRS2*, particularly those of *IRS2*, which suggests that both IRS1 and IRS2 are involved in the development of ethanol-mediated hepatic insulin resistance, with IRS2 being more dominant. The PI3K pathway is an important pathway for insulin signaling. PI3K is composed of a catalytic subunit of 110 kDa and a regulatory subunit (p85α) of 85 kDa. Changes in PI3K expression and activity have been demonstrated to closely correlate with insulin resistance (27). Glucose transport is based on the glucose transporter in the cell membrane. Although GLUT4 is of particular interest in the context of diabetes and insulin resistance, the liver is generally considered to lack significant expression of GLUT4 (28). In hepatocytes, GLUT2 is the main glucose transporter (29). In the present study, 24-h treatment with 2 or 4% ethanol significantly decreased the mRNA expression levels of *PI3K (p85α)* and *GLUT2* in the L-02 cells (P<0.05), indicating that ethanol administration suppressed the expression of these genes in a

dose-dependent manner. In summary, the administration of low concentrations of ethanol did not have a significant effect on the insulin signaling pathway in hepatocytes (L-02). Moderate and high concentrations of ethanol did have suppressive effects, of varying degrees, on the expression of key molecules in this pathway, which results in the interference with glycogen synthesis and gluconeogenesis, and relevant signal transduction, which in turn triggers insulin resistance in hepatocytes.

In addition to the relevance to the expression profiles of key signaling molecules, insulin signal transduction closely correlates with the phosphorylation of signaling molecules. The activation of PI3K generates phosphatidylinositol (3,4,5)-trisphosphate (PIP3), a second messenger activating 3-phosphoinositide-dependent protein kinase-1 and -2 (PDK1 and PDK2), which mediate the effects of insulin on metabolism and pro-survival. PDK1 and PDK2 in turn activate the PKB, by inducing phosphorylation at T^{308} and S^{473}, respectively (21). In the present study, the ethanol-induced changes in the protein expression and phosphorylation levels of PI3K (p85α) and PKB were examined by western blot analysis. The results suggested that the administration of 0.5 or 1% ethanol had no significant suppressive effect on the expression and insulin-induced phosphorylation levels of PI3K (p85α) and PKB. By contrast, treatment with 2 or 4% ethanol significantly suppressed the expression and phosphorylation levels of these proteins (Fig. 5). Following treatment with 2 or 4% ethanol for 24 h, the expression of PI3K (p85α) and PKB significantly decreased to below the basal state in the absence of insulin stimulation. After the removal of ethanol and the addition of insulin, there was a significant increase in the phosphorylation levels of PI3K (p85α) and PKB compared with the cells not treated with ethanol, and the absolute content of the phosphorylated protein and its ratio relative to the total protein increased to varying extents. However, the phosphorylation levels in the 2 or 4% ethanol-treated groups were significantly lower than those of the group treated with insulin but not ethanol. One explanation is that the decrease in the insulin-induced phosphorylation levels of PI3K (p85α) and PKB was associated with the ethanol-induced decrease in the expression of these proteins. Conversely, the decrease in the phosphorylated protein ratio relative to the total protein suggests that ethanol administration suppressed the process of protein phosphorylation in addition to the inhibition of protein expression. These results demonstrate that ethanol interferes with insulin signal transduction by influencing both the protein expression and the phosphorylation levels of key molecules in the PI3K signaling pathway.

Calcium is an essential second messenger in hepatocytes, and homeostatic imbalance has been reported to be involved in the process of ethanol-induced hepatocyte damage (30,31). In the present study, changes in the $[Ca^{2+}]_i$ levels induced by ethanol treatment were examined by flow cytometry and confocal microscopy, and the results revealed that the increase in the $[Ca^{2+}]_i$ levels occurred in a concentration- and time-dependent manner. To confirm the roles of $[Ca^{2+}]_i$ in ethanol-induced hepatocyte damage and the suppression of the insulin signaling pathway, 2% was selected to be the ethanol concentration for the Ca^{2+} blockage assays in this study, as only a slight inhibition of cell viability was observed at this concentration, which is consistent with the results of other studies (9-11). Our results demonstrated that the ethanol-induced increase in $[Ca^{2+}]_i$ levels

was partially suppressed by the administration of the extracellular Ca^{2+} chelator, EGTA, and/or the IP3 receptor antagonist, 2-APB, although no complete blockage was observed (Fig. 6A). This suggests that other calcium channels may contribute to the increase in $[Ca^{2+}]_i$ levels induced by ethanol administration in addition to the extracellular Ca^{2+} influx and the IP3 receptor-mediated Ca^{2+} release from the calcium pool in the endoplasmic reticulum. An increased hepatocyte survival rate was observed as a result of the EGTA or 2-APB suppression of the ethanol-induced increase in $[Ca^{2+}]_i$ levels (data not shown), which suggests that the ethanol-induced increase in $[Ca^{2+}]_i$ levels is involved in the process of hepatocyte damage. Furthermore, the suppressive effects of EGTA or 2-APB on the ethanol-induced increase in the $[Ca^{2+}]_i$ levels correlated with the restoration of the phosphorylation levels of PI3K (p85α) and PKB in the presence of insulin stimulation (Fig. 6B), indicating that the inhibitory effects of ethanol on the key molecules of the insulin signaling pathway was $[Ca^{2+}]_i$-dependent.

In conclusion, the findings of our study demonstrated that ethanol treatment inhibited insulin signal transduction in a dose-, time- and Ca^{2+}-dependent manner. The inhibition of *IRS1/2*, *PI3K* (p85α), *PKB* and *GLUT2* expression and of PI3K (p85α) and PKB phosphorylation by high concentrations of ethanol may be the core molecular mechanism of ethanol-induced insulin resistance. Furthermore, although not statistically significant, treatment with 0.5% ethanol slightly increased the protein expression and phosphorylation levels of key components of the insulin signaling pathway. It is unknown whether ethanol at low concentrations indeed promotes the process of insulin signal transduction, and whether it correlates with the $[Ca^{2+}]_i$ levels. To clarify this issue, further investigation is required.

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