C/EBP beta and C/EBP delta expression is elevated in the early phase of ethanol-induced hepatosteatosis in mice

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Aim: Alcohol, which is predominantly metabolized in the liver, is a major hepatic toxicant that readily induces hepatic steatosis. The expression of CCAAT enhancer binding protein (C/EBP), especially the C/EBP delta variety, is increased in the early phase of adipogenesis. However, the role of C/EBP delta in ethanol-induced hepatosteatosis is unclear.

Methods: Male C57BL/6J mice were randomized to one of four groups: a control group, a group receiving orally administered ethanol (4 g ethanol/kg body weight) (EtOH), a high-fat-diet (HF) group and an EtOH+HF group. Mice were sacrificed after 5 or 10 weeks for various measurements. The in vitro effect of ethanol on the expression of C/EBP alpha, beta and delta was studied in HepG2 cells.

Results: By week 5, ethanol treatment had significantly increased liver C/EBP delta and beta protein expression (by 2.3- and 1.4-fold, respectively), which then returned to the control level by week 10. In contrast, the expression of C/EBP alpha was evident only at week 10. The in vitro study shows that C/EBP delta expression was elevated significantly at 24 h but not at 48 or 72 h. C/EBP beta expression was highest at 48 h, whereas C/EBP alpha expression was highest at 72 h. We also found that a low concentration of ethanol plus oleic acid enhanced C/EBP delta expression in HepG2 cells.

Conclusion: C/EBP delta expression appears to play an important role in the early phase of ethanol-induced hepatosteatosis in mice and in ethanol-treated HepG2 cells. In addition, EtOH+HF enhances the expression of C/EBP delta in HepG2 cells. Thus, C/EBP delta might be a therapeutic target in alcoholic hepatosteatosis.

Keywords: C/EBP delta; ethanol; hepatosteatosis; adipogenesis

Original Article

Acta Pharmacologica Sinica (2009) 30: 1138–1143; doi: 10.1038/aps.2009.109; published online 20 July 2009

Introduction

Acute or chronic alcohol consumption can cause severe liver injury[1]. Alcohol is known to impair fat oxidation and to stimulate lipogenesis in the liver[2, 3]. Thus, alcohol consumption can lead to the development of hepatic steatosis. Most studies on alcoholic hepatic steatosis have focused on the ability of ethanol to shift the redox state in the liver and to inhibit fatty acid oxidation[3, 4]. Indeed, previous studies have shown the repression of some enzymes involved in fatty acid oxidation and induction of lipogenic enzymes in ethanol-fed animals[2, 3]. The de novo synthesis of fatty acids in the liver was shown to increase with ethanol feeding in conjunction with the induction of adipogenic enzymes, including peroxisome proliferator-activated receptor alpha (PPAR alpha) and/or sterol regulatory element binding protein 1 (SREBP-1)[2, 6]. However, the mechanisms by which ethanol causes steatosis are multiple and complex.

The CCAAT enhancer binding proteins (C/EBPs) are a family of basic leucine zipper transcription factors involved in the regulation of cellular differentiation and functions[7–9]. Six members of the C/EBP family have been described, including C/EBP alpha, beta, gamma, delta, epsilon and zeta[10]. C/EBP alpha, beta, and delta are important transcription factors in adipose differentiation. C/EBPs may affect adipogenesis by regulation and coordination of a cascade of transcription factors that together lead to the establishment of the differentiated state[11]. For instance, C/EBP beta and delta have been shown to be expressed early in adipogenesis, whereas C/EBP alpha is expressed much later[12, 13]. The vital role played by C/EBP beta and delta in adipogenesis was demonstrated by disruption of both genes, which prevents the normal development of adipose tissue[14]. Furthermore, silencing of C/EBP
delta impairs the expression of factor for adipocyte differentiation 49 (fad49), which is up-regulated in adipogenesis and appears to play a crucial role early in the process.[13]

McKnight et al.[15] have shown that C/EBP delta alone possesses minimal adipogenic activity and C/EBP beta and C/EBP delta play important roles in inducing expression of C/EBP alpha and PPAR gamma. Lai et al.[17] have demonstrated that induction of C/EBP delta expression transiently activates PPAR gamma 2 transcription, which is involved in adipocyte-like lipogenesis in HepG2 cells. The temporal pattern of expression of these three C/EBP isoforms during adipose differentiation may reflect the underpinnings of a regulatory cascade that controls the process of terminal cell differentiation.[18]

Previous studies have examined the effect of ethanol on C/EBPs in animals and in cell cultures, but the results are inconsistent[19, 20]. In addition, little or no information is available about the effect of ethanol on C/EBP delta. In this study, we conducted both in vivo and in vitro experiments to investigate the changes of C/EBP delta expression in ethanol-induced hepatosteatosis. We postulated that C/EBP delta was an important transcription factor in the early phase of ethanol-induced hepatosteatosis.

Materials and methods

Animals and diet

Experiments were performed on 6-week-old male C57BL/6J mice obtained from the Animal Center of National Cheng Kung University Medical College, Tainan, Taiwan. C57BL/6J mice were used because it has been shown that they consume alcohol more readily than most strains[21]. The mice were used because of the results are inconsistent[19, 20], and in addition, little or no information is available about the effect of ethanol on C/EBP delta. In this study, we conducted both in vivo and in vitro experiments to investigate the changes of C/EBP delta expression in ethanol-induced hepatosteatosis. We postulated that C/EBP delta was an important transcription factor in the early phase of ethanol-induced hepatosteatosis.

Assay of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT)

The mice were sacrificed following 5 or 10 weeks of treatment. Blood samples were obtained from the orbital vascular plexus of each mouse and centrifuged at 1000×g at 4 °C for 5 min to separate serum. Serum levels of AST and ALT were determined using clinical test kits (AppliedBio assay kits; Hercules, CA, USA).

Liver morphology

Immediately after the mice had been killed, the liver tissue was fixed in 10% neutral buffered formalin. The tissue was then processed with graded alcohol and xylene and embedded in paraffin. Liver sections (3–5 μm thick) were stained with hematoxylin and eosin (HE). Pathological changes were observed by light microscopy.

Cell culture conditions

The human hepatoma HepG2 cell line was purchased from Biosource Resource and Research Center (Food Industry Research and Development Institute, Hsinchu, Taiwan). These cells were maintained in Dulbecco’s Modified Essential Media (DMEM) containing 10% FBS at 37 °C with 5% CO2 and 95% humidity. HepG2 cells were treated with ethanol at different concentrations (10, 100, 200, or 400 mmol/L) as described previously.[22] Then, the cells were gently sealed with parafilm to avoid the evaporation of ethanol. Control cells not treated with ethanol were sealed in parafilm as well. The cells were then incubated for 24–72 h at 37 °C with 5% CO2. The cells did not show any change in viability following sealing with parafilm.

Induction of steatosis in HepG2 cells was obtained using oleic acid (Sigma-Aldrich Inc, Saint Louis, MO, USA) as described previously.[23] Oleic acid was added to the culture medium to obtain a final concentration of 25, 50, 100, or 200 μmol/L. The duration of this treatment was 72 h.

Western blot analysis

Expression levels of C/EBP alpha, beta, and delta in HepG2 cells and mouse liver tissues were determined by Western blot techniques. Proteins were extracted using RIPA buffer and separated by SDS-PAGE and then transferred and immobilized on a nitrocellulose membrane. The membrane was blocked with 5% non-fat dry milk in phosphate-buffered saline containing 0.1% Tween 20 (PBS-T) for a 2-h incubation at room temperature. The membrane was then washed in PBS-T and hybridized with primary antibodies diluted in PBS-T for 16 h. Proper dilutions of specific antibodies for C/EBP alpha, beta, delta (1:200) and actin (1:1000) purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA) were used. Incubation with secondary antibodies and detection of the antigen-antibody complex were performed using the ECL kit (Amersham Biosciences, UK). Densities of the obtained immunoblots were quantified using Image J (NIH).

Statistical analysis

Data are expressed as the means±SD. Statistical significance of the differences between groups means was analyzed by one-way ANOVA followed by Duncan’s multiple comparisons, with P<0.05 considered significant.

Results

Ethanol administration and high-fat diet increase liver function abnormality

After 5 weeks of treatment, serum levels of AST and ALT were markedly higher in mice in the EtOH group, the HF group and the EtOH+HF group than in those in the control group (P<0.05). The levels of AST and ALT in these three groups...
of mice were further increased after 10 weeks of treatment (Table 1).

Table 1. Serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in C57BL/6J mice orally treated with ethanol (4 g/kg BW) with or without a high-fat diet for 5 or 10 weeks. Data are means±SD, n=6. Values not sharing a common alphabet letter are significantly different.  *P<0.05 vs control.  **P<0.05 vs corresponding group at 5 weeks.

| Group          | AST(U/L) | ALT(U/L) |
|----------------|----------|----------|
| 5 weeks        |          |          |
| Control        | 53.3±3   | 24.0±3   |
| EtOH           | 125±3*   | 65.0±9*  |
| HF             | 78.8±9*  | 39.3±5*  |
| EtOH+HF        | 103±20*  | 55.0±3*  |
| 10 weeks       |          |          |
| Control        | 54.0±15  | 26.3±2   |
| EtOH           | 215±41*  | 140±3*   |
| HF             | 121±6†   | 53.8±3*  |
| EtOH+HF        | 175±56‡  | 69.5±9*  |

Control mice (Control), ethanol administered mice (EtOH), high-fat diet mice (HF), or ethanol administered combined with high-fat diet (EtOH+HF).

Liver histology of experimental animals
Morphological changes in the liver induced by ethanol with or without the high-fat diet were examined. The HE stain showed a mild fatty degeneration in mice in the EtOH, HF and EtOH+HF groups after 5 weeks, but no such changes in the control mice (Figure 1A). After 10 weeks of treatment, hepatic steatosis was evident in the three intervention groups, but not in the control mice (Figure 1B). Notably, the EtOH+HF group exhibited the most severe fatty change of the intervention groups.

Protein expressions of C/EBP alpha, beta and delta in C57BL/6J (B6) mice with/without ethanol and with/without a high-fat diet
To investigate whether C/EBPs are involved in the progression of ethanol-induced hepatic steatosis, we determined the expression of C/EBP alpha, beta and delta in the mouse liver after treatment with ethanol, with or without HF diet, for 5 weeks and 10 weeks. After 5 weeks of treatment, the hepatic C/EBP delta expression increased significantly (2.3-fold) in ethanol-treated mice, as compared with that in the control mice. Moreover, the hepatic C/EBP delta expression in mice treated with EtOH+HF was higher than that in mice treated with either EtOH or HF alone. However, after 10 weeks of treatment, the hepatic expression of C/EBP delta in mice treated with EtOH+HF was restored to that in the control mice (Figure 2A).

As shown in Figure 2B, the hepatic C/EBP beta expression increased significantly in mice treated with EtOH or with EtOH+HF for 5 weeks (1.4-fold in each case), as compared with that in control mice. However, the hepatic expression of C/EBP beta in mice treated with EtOH and EtOH+HF for 10 weeks was restored to that in the control mice.

After 5 weeks of treatment, the hepatic C/EBP alpha expression in the four groups did not differ significantly. By contrast, after 10 weeks of treatment, the hepatic C/EBP alpha expression increased significantly in mice treated with EtOH and EtOH+HF, as compared with that in control mice (Figure 2C).

Protein expression of C/EBP alpha, beta and delta in HepG2 cells with/without ethanol
We incubated HepG2 cells with ethanol at different concentrations and determined the protein expression of C/EBP alpha, beta and delta. The expression of C/EBP delta in HepG2 cells incubated with 10 mmol/L ethanol for 24 h was higher than that of C/EBP alpha and beta. Treatment of cells with higher...
concentrations of ethanol (100–400 mmol/L) decreased the expression of C/EBPs and restored the expression to the control level (Figure 3A, 3B, and 3C).

We also determined the effect of ethanol on the expression of C/EBP delta mRNA in HepG2 cells by RT-PCR. We found that ethanol (10–400 mmol/L) did not significantly affect the mRNA expression of C/EBP delta (data not shown). The results suggest that the change in C/EBP delta protein expression may occur at the post-transcriptional level.

To determine the time course of C/EBP alpha, beta and delta protein expression, cells were exposed to 10 mmol/L ethanol for 24, 48, and 72 h. We found that C/EBP delta expression was significantly elevated at 24 h but not at 48 and or 72 h. By contrast, C/EBP beta expression was highest at 48 h, and the C/EBP alpha expression was highest at 72 h (Figure 4).

Protein expression of C/EBP delta in HepG2 cells with/without oleic acid

We treated HepG2 cells with different concentrations of oleic acid to mimic adipogenesis in hepatocytes. Oleic acid treatment increased C/EBP delta expression in HepG2 cells in a concentration-dependent manner (Figure 5).

Protein expression of C/EBP delta in HepG2 cells incubated with ethanol and with/without oleic acid

We then treated HepG2 cells with oleic acid (25 μmol/L) and with or without ethanol at a low concentration (10 mmol/L) to avoid ethanol cytotoxicity (Figure 6). We found that the combination of ethanol and oleic acid significantly increased C/EBP delta expression, and that this effect was additive or synergistic, as compared with oleic acid or ethanol alone, accord-
According to the following calculation: \[ \frac{(\text{combination} - \text{control})}{(\text{oleic acid} - \text{control}) + (\text{ethanol} - \text{control})} \].

**Discussion**

Steatosis is a critical stage of alcoholic liver disease (ALD), and it has been shown that prevention of steatosis may actually protect against more severe stages of ALD\(^2\). C/EBP alpha, beta, and delta are important transcription factors in adipose differentiation. Early expression of C/EBP delta is required for efficient induction of C/EBP alpha and PPAR gamma\(^2\). However, little is known whether C/EBPs are involved in the progression of alcoholic hepatosteatosis. In this study, we treated mice with ethanol to induce alcoholic hepatosteatosis, and we found that C/EBP delta expression was elevated in the initial phase of hepatosteatosis, suggesting that C/EBP delta plays a major role in the progression of alcoholic hepatosteatosis.

In the present study, we showed that ethanol administration induced liver injury, as evidenced by increased serum AST and ALT and by pathologic changes, which demonstrated that lipids started to accumulate in hepatocytes of mice treated with ethanol for 5 weeks. Mice treated with ethanol for 10 weeks developed more severe hepatosteatosis than those treated for 5 weeks. ALT was much lower in mice treated with EtOH+HF than in those treated with ethanol alone. We speculate that the high-fat diet may protect against ethanol-induced liver damage, possible by decreasing gastric absorption of ethanol. The more severe hepatosteatotic changes in the livers of mice treated with EtOH+HF was likely due to the combined effect of EtOH and HF, as indicated in the present study by the enhanced expression of C/EBP delta and perhaps other C/EBPs.
We showed that C/EBP delta expression was involved in the progression of ethanol-induced hepatic steatosis and that its expression was delta greater than that of C/EBP beta at week 5 (2.3- and 1.4-fold increases, respectively), whereas the expression of C/EBP alpha was not evident until week 10. Interestingly, hepatic expression of C/EBP beta and delta returned to the control level by week 10. These results suggest that C/EBP delta may play an important role in the early phase but not in the late phase of hepatosteatosis in this mouse model. By contrast, C/EBP alpha may participate in the late phase of hepatic steatosis in mice.

In HepG2 cells incubated with oleic acid, we observed that C/EBP delta expression was significantly increased. The data support the notion that C/EBP delta is involved in adipogenic differentiation[25]. The present in vitro study revealed that a low concentration (10 mmol/L) of ethanol not only increased C/EBP delta expression but also was synergistic with oleic acid in the promotion of C/EBP delta expression in HepG2 cells. Surprisingly, C/EBP alpha, beta, and delta expression in HepG2 cells were restored to a level similar to that of the control at higher concentrations of ethanol (200 and 400 mmol/L). It is possible that low and high ethanol concentrations may affect C/EBP expression via different mechanisms. The detailed mechanisms through which C/EBP delta coordinates with other C/EBPs to mediate additional adipogenic factors such as PPARs, adiponectin and SREBP remain to be investigated.

In conclusion, we have demonstrated an increase in C/EBP delta expression at a relatively early phase of ethanol-induced hepatosteatosis in mice. In addition, our in vitro study shows that ethanol plus oleic acid increases the expression of C/EBP delta in HepG2 cells. These results suggest that the inhibition of C/EBP delta is a potential therapeutic target in alcoholic hepatosteatosis.

Acknowledgements
This work was supported by grants from the Central Taiwan University of Science and Technology (CTU-96-23 to Yu-hsuan CHEN and Shih-pei CHANG).

Author contribution
Miao-lin HU and Yu-hsuan CHEN designed the study; Yu-hsuan CHEN, Chih-min YANG and Shih-pei CHANG performed the experiment and analyzed the data; Miao-lin HU and Yu-hsuan CHEN wrote the paper.

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