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Effects of 1-O-hexyl-2, 3, 5-trimethylhydroquinone in Carbon Tetrachloride-induced Hepatic Apoptosis with a Possible Relationship to Naofen

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Abstract: 1-O-hexyl-2, 3, 5-trimethylhydroquinone (HTHQ), a synthesized vitamin E derivative, is a superoxide scavenger which retains the original property of vitamin E as a natural lipophilic chain-breaking antioxidant. The present study was undertaken to evaluate the effects of HTHQ on hepatocyte apoptosis, using the carbon tetrachloride (CCl_4)-induced rat hepatic cirrhosis model. We also clarified the changes in expression of naofen, previously reported as an intracellular WD-repeat protein associated with apoptosis. CCl_4 was injected twice a week and HTHQ was mixed in the drinking water, with daily intake being allowed. Rats were divided into four groups: a CCl_4-treated group, a CCl_4 with daily intake of HTHQ group and solvent- or HTHQ-treated groups without CCl_4. Within eight weeks *in situ* hybridization studies showed that naofen-positive hepatocytes elicited positive reactions in the TUNEL assay. CCl_4 induced TUNEL-positive staining and also enhanced naofen mRNA expression in the livers. HTHQ inhibited both TUNEL staining and naofen expression caused by CCl_4. Furthermore, the levels of naofen mRNA expression in CCl_4-treated rat liver tissue were significantly reduced by treatment with HTHQ (1.90±0.18 vs. 1.29±0.08, $p<0.01$). HTHQ may inhibit both apoptosis and naofen expression in CCl_4-treated rats, thus reducing liver cirrhosis.

Key words: HTHQ, CCl_4, liver cirrhosis, WD-repeat protein

INTRODUCTION

1-O-hexyl-2, 3, 5-trimethylhydroquinone (HTHQ), a synthesized vitamin E derivative, is a superoxide scavenger (Nihro *et al*., 1994; Hino *et al*., 1998; Liu *et al*., 2002). It retains the original property of vitamin E as a natural lipophilic chain-breaking antioxidant (Tappel, 1980; Sharma, 2007). Carbon tetrachloride (CCl_4)-induced liver injury model is widely used to investigate the mechanisms of liver damage and regeneration (Patrick-Iwuanyanwu *et al*., 2007; Samudram *et al*., 2008; Dahiru *et al*., 2010; Sonkusale *et al*., 2011). Several lines of evidence indicate that vitamin E derivatives prevent both enhancement of lipid peroxidation and synthesis of type I collagen caused by CCl_4-induced chronic liver damage and cirrhosis (Parola *et al*., 1992a; Houglum *et al*., 1991; Chojkier *et al*., 1998; Parola *et al*., 1992b). Recently, we demonstrated that HTHQ can inhibit development of CCl_4-induced hepatic cirrhosis in rats more potently than does vitamin E (An *et al*., 2010). It is well known that hepatocytes are destructed and regenerated when continuously exposed to toxic substances such as CCl_4 (Weber *et al*., 2003). Although CCl_4 has been shown to induce apoptosis in hepatocytes (Yang *et al*., 2010; Lee *et al*., 2008; Tirkey *et al*., 2005; Khalaf *et al*., 2009; Obidah *et al*., 2011), it remains unknown whether HTHQ can suppress CCl_4-induced apoptotic processes in hepatocytes.

Recently, we characterized naofen, a novel WD40 repeat domain-containing protein (An *et al*., 2008; Feng *et al*., 2008, 2010; Sato *et al*., 2010). Functional analyses revealed that naofen is a new mediator of spontaneous apoptosis via activation of caspase-3 in the HEK293 cell line (Feng *et al*., 2010) and in renal tubular

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epithelial cells in the streptozotocin-induced rat diabetic model (Sato *et al*., 2010). Although naofen is found in hepatocytes it is still largely unknown whether treatment with CCl_4 induces expression of naofen in these cells. Therefore, we used the rat CCl_4-induced cirrhosis model to test the effects of HTHQ on hepatocyte apoptosis and the *in vivo* expression of naofen.

MATERIALS AND METHODS

Animals, treatment and specimen collection: All rats received humane care with the prior approval of the Animal Care Committee of Aichi Medical University. The rats were kept in a climate-controlled room under a 12 h light-dark cycle with free access to food and tap water throughout the studies. They were divided into four groups: a solvent-treated group, a CCl_4-treated group, a CCl_4 plus HTHQ-treated group and a HTHQ-treated group. The method of administration and preparation of paraffin block of liver sample were performed essentially as described in a previous report (An *et al*., 2010). The consumption of drinking water was checked and there were no clear differences in the amounts of drinking water between the solvent-treated group and the HTHQ-treated groups.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay: Apoptosis was determined by the TUNEL assay using the ApopTag® Plus *in situ* Apoptosis Detection Kit (Chemicon International, USA) according to the manufacturer's instructions. The slides were counterstained with 0.5% methyl green for microscopic examination. Five (100x magnification) fields were randomly selected per slide and total hepatocytes counted per field. The mean percentage of apoptotic hepatocytes was calculated and compared between the different experimental groups.

***In situ* hybridization:** Based upon the previous report (An *et al*., 2008), the sequence of the naofen probe was designed as follows: 5'-GCAGAAAGGATATATGCT CTGTGTACTTTA-3'. Hybridization was performed at 105°C for five min and 74°C for 45 min, with the probe diluted at 1:1500. Positive regions were visualized with DAB (Falma, Tokyo, Japan) and then counterstained with hematoxylin for microscopic examination. Five (100x magnification) fields were randomly selected per slide and total hepatocytes counted per field. The mean percentage of naofen-positive hepatocytes was calculated and compared between different experimental groups. Five independent experiments were performed.

Real-time polymerase chain reaction (PCR): Total RNA was extracted from frozen control and CCl_4-treated rat liver tissues using TRIzol® reagent (Invitrogen, USA). Equal amounts of DNA-free total RNA from each sample were converted to cDNA using the ReverTra Plus kit (Toyobo, Osaka, Japan). Reverse transcription was performed at 65°C for five min, 42°C for 60 min and 85°C for five min. Quantitative real-time PCR was performed using TaqMan primers and probes for naofen and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control. PCR reactions and analyses were carried out using the Step One real-time PCR system and software (Applied Biosystems). The relative abundance of the target genes was obtained by normalizing to GAPDH expression according to the $\Delta\Delta C_T$ method as previously reported by An *et al*. (2010). Five independent experiments were performed.

Statistics: The significance of differences between the means of the total numbers of hepatocytes was determined by analysis of variance (ANOVA), evaluated by Scheffe's method. The rates were analyzed with a non-parametric method, Kruskal-Wallis one-way ANOVA. Statistical significance was accepted at 0.05.

RESULTS

Eight weeks after starting injections of CCl_4, the liver architecture was completely distorted by fibrous septa and the formation of regenerative nodules (pseudo-lobules) (Fig. 1a). In order to evaluate the effects of agents on apoptosis, TUNEL staining was performed. The apoptosis of hepatocytes was observed around Glisson's area in the CCl_4-treated group (Fig. 1b). The percentage of apoptotic cells in the liver of the CCl_4-treated group was 45.34±1.71, significantly greater than that in the solvent-only group ($p<0.01$) (Fig. 1e). In contrast, in the CCl_4 plus HTHQ-treated group, the formation of interlobular fibrosis and pseudo-lobules was clearly diminished, although the vacuoles and formation of enlarged portal veins were little affected by HTHQ (Fig. 1c, d). The percentage of apoptotic hepatocytes obtained in the CCl_4 plus HTHQ-treated group was 26.64±0.89, significantly smaller than that in the CCl_4-treated group ($p<0.01$).

In the next experiments, we examined whether treatment of CCl_4 induced expression of naofen in apoptotic hepatocytes. The expression of naofen mRNA in the HTHQ-treated group was almost identical to that in the solvent-treated group (Fig. 2a, d). In the CCl_4-treated group, expression of naofen mRNA increased robustly in the hepatocytes and its expression in the

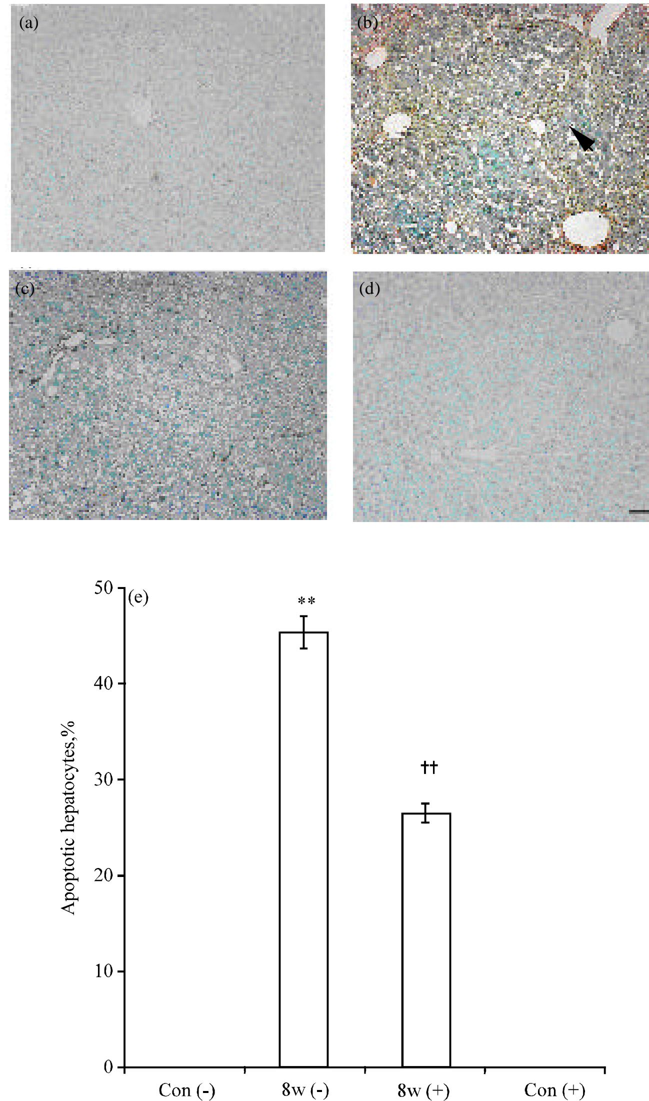

Fig. 1(a-e): TUNEL expression in rat livers following carbon tetrachloride (CCl_4)-induced cirrhosis, TUNEL staining. (a), Solvent-treated group, (b), CCl_4-treated group, (c), CCl_4 plus HTHQ-treated group, (d), HTHQ-treated group, Horizontal bar indicates 100 μm, (e) Percentage of apoptosis-positive hepatocytes in CCl_4-treated rats, Con (-): Solvent-treated group, 8w (-): CCl_4-treated group, 8w (+): CCl_4+HTHQ-treated group, Con (+): HTHQ-treated group, **$p<0.01$ compared to the control group, $^{††}p<0.01$ compared to the CCl_4-treated group (n = 5, each)

pseudo lobules were greater than that in other areas (Fig. 2b). The mean percentage of naofen-positive hepatocytes in the CCl_4-treated group was 78.40±4.16 (Fig. 2e), significantly greater than that obtained in the solvent-treated group ($p<0.01$). In contrast, in the CCl_4 plus HTHQ-treated group (Fig. 2c), the amount of naofen mRNA in the cytoplasm of hepatocytes around Glisson's area was clearly diminished compared to the CCl_4-treated group. The mean percentage of naofen-positive hepatocytes in the CCl_4 plus HTHQ-treated group was 7.47±1.25 (Fig. 2e), significantly smaller than that in the CCl_4-treated group ($p<0.01$). Arrows show the same hepatocyte, indicating that among the abundant naofen-positive hepatocytes, some underwent apoptosis (Fig. 1b, Fig. 2b).

We also quantified the level of naofen mRNA expression using GAPDH mRNA as an internal standard. In the CCl_4-treated group, the normalized expression of naofen was 1.90±0.18 which was significantly greater than that in the solvent-treated group ($p<0.01$). By contrast, in

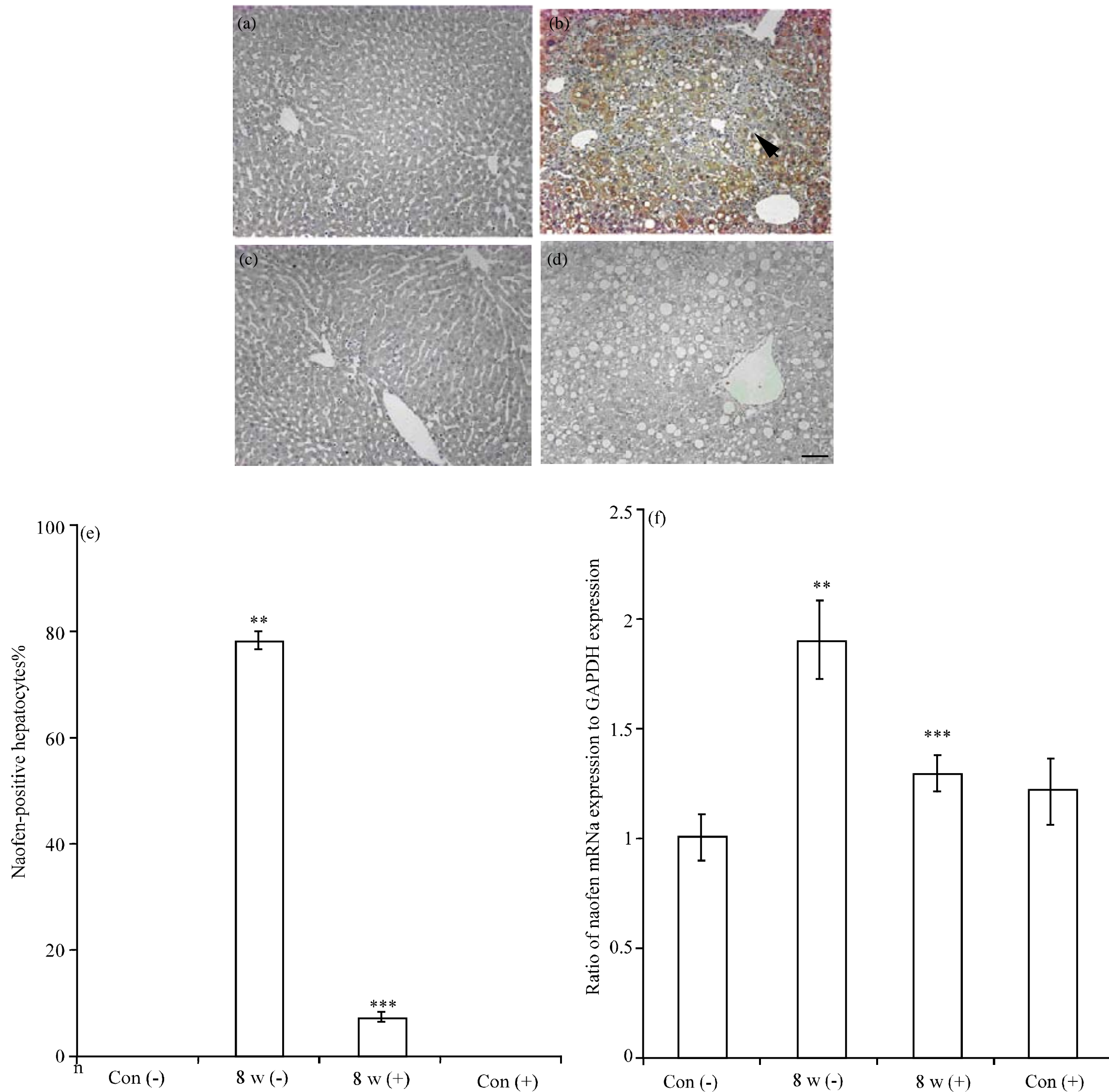

Fig. 2 (a-f): Naofen mRNA expression in rat livers following CCl_4-induced cirrhosis, (a) *In situ* hybridization assessment of naofen expression, (a) Solvent-treated group, (b) CCl_4-treated group, (c) CCl_4 plus HTHQ-treated group, (d) HTHQ-treated group, Horizontal bar indicates 100 μm, (e) Percentage of naofen-positive hepatocytes in CCl_4-treated rats, **p<0.01 compared to the control group, ***p<0.01 compared to the CCl_4-treated group (n = 5, each), (f) Ratios of naofen mRNA to GAPDH mRNA in liver tissues of CCl_4-treated rats, ** p<0.01 compared to the control group, ***p<0.01 compared to the CCl_4-treated group (n = 5)

the CCl_4 plus HTHQ-treated group, the normalized expression of naofen was 1.29±0.08 which was significantly smaller than that in the CCl_4-treated group (p<0.01) (Fig. 2f).

DISCUSSION

We have shown in the present study of rats that HTHQ effectively inhibits hepatocyte apoptosis caused by repeated hypodermic injections of CCl_4. The calculated doses of HTHQ from the consumed volumes of drinking water were in the range of 45.5-46.5 mg kg^{-1} body weight/day, comparable with a previous report (An *et al*., 2010).

Shi *et al*. (1998) reported that apoptosis in rat livers could be observed histochemically after CCl_4 administration and Sun *et al*. (2003) reported that the activity of caspase-3 was increased in the rat liver and

plasma after CCl_4 administration. Furthermore it is well known that the mechanism of hepatic injury by CCl_4 involves hepatocyte membrane lipid peroxidation, causing destruction of the cells and their intracellular organelles (Khalaf *et al*., 2009). In addition, we showed the apoptosis of hepatocytes by using TUNEL assay (Moorthy *et al*., 2009; Amin, 2009; Ali *et al*., 2011; Khorshid *et al*., 2011). Based on these observations it would be reasonable to assume that orally administered HTHQ mixed into the rats' drinking water might reduce CCl_4-induced apoptotic changes by preventing enhancement of lipid peroxidation.

Naofen is a new intracellular component that may cause apoptosis by activating caspase-3 which mediates TNF-α-stimulated apoptosis (Feng *et al*., 2010). The present study revealed that the CCl_4-induced increase in expression of naofen mRNA correlated well with positive TUNEL staining in hepatocytes. In addition, HTHQ strongly suppressed the CCl_4-induced increase in naofen mRNA expression. It appears that HTHQ prevents fibrogenesis by inhibiting the naofen expression that mediates hepatocyte apoptosis.

The mechanism of induction of naofen mRNA in hepatocytes is currently obscure. When hepatocytes or cell lines were exposed to CCl_4, or to possible inflammatory mediators in *in vitro* studies, none of the examined mediators affected naofen expression (unpublished observation), implying that naofen expression may be induced by activation of non-parenchymal cells such as Kupffer cells. Further studies will determine whether non-parenchymal cells are involved.

In conclusion, we have demonstrated that HTHQ suppresses apoptosis during the development of CCl_4-induced liver cirrhosis in rats. In addition, the mode of action of HTHQ in preventing CCl_4-induced hepatic cirrhosis might include naofen-mediated apoptotic processes in rats.

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