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Alleviative effects of s-allyl cysteine and s-ethyl cysteine on MCD diet-induced hepatotoxicity in mice

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ABSTRACT

Alleviative effects of s-allyl cysteine (SAC) and s-ethyl cysteine (SEC) upon methionine and choline deficient (MCD) diet-induced hepatotoxicity in mice were examined. SAC or SEC at 1 g/L was added into drinking water for 7 weeks with MCD diet. MCD feeding significantly increased hepatic triglyceride and cholesterol levels, and elevated the activity of glucose-6-phosphate dehydrogenase (G6PDH), malic enzyme, fatty acid synthase (FAS) and 3-hydroxy-3-methylglutaryl coenzyme A reductase ($P < 0.05$). However, the intake of SAC or SEC significantly decreased hepatic triglyceride accumulation, and reduced G6PDH and FAS activities ($P < 0.05$). MCD feeding significantly lowered serum and hepatic glutathione (GSH) levels, increased malondialdehyde (MDA) and oxidized glutathione (GSSG) formation, and suppressed the activity and mRNA expression of glutathione peroxidase (GPX), superoxide dismutase (SOD) and catalase ($P < 0.05$). The intake of SAC or SEC significantly increased serum and hepatic GSH levels, decreased MDA and GSSG formation, restored the activity and mRNA expression of GPX, SOD and catalase ($P < 0.05$). MCD feeding significantly enhanced the mRNA expression of interleukin (IL)-1beta, IL-6, tumor necrosis factor (TNF)-alpha, transforming growth factor (TGF)-beta1, matrix metalloproteinases-9 (MMP-9) and collagen-alpha1 ($P < 0.05$). The intake of SAC and SEC significantly blunted the mRNA expression of IL-1beta, IL-6, TNF-alpha, TGF-beta1 and collagen-alpha1 ($P < 0.05$). SEC was greater than SAC in suppressing IL-6 and TNF-alpha expression ($P < 0.05$), but SAC was greater than SEC in suppressing collagen-alpha1 and TGF-beta1 expression ($P < 0.05$). These data suggest that SAC and SEC are potent agents against MCD-induced hepatotoxicity.

1. Introduction

Nonalcoholic steatohepatitis (NASH) is the most common cause of cryptogenic cirrhosis in the United States and other developed countries (Brunt, 2001; Angulo, 2002). The clinical features of NASH include hepatic lipid accumulation, oxidative, inflammatory and fibrotic injury. It has been documented that rodents via feeding a methionine and choline deficient (MCD) diet could develop a steatohepatitis, which is pathologically similar to NASH observed in human (Weltman et al., 1996; George et al., 2003; Ip et al., 2003; Koppe et al., 2004). Thus, this MCD dietary model has been widely used to investigate pathological development and/or possible therapeutic strategy for NASH.

MCD-induced steatosis is mainly due to the elimination of methionine and choline from this diet impair very low density lipoprotein production and hepatic triglyceride secretion, which consequently causes triglyceride accumulation in liver (Vance and Vance, 1985; Kulinski et al., 2004). It is known that fatty livers are more sensitive to oxidative stress, and MCD diet causes hepatic glutathione (GSH) depletion (George et al., 2003; Oz et al., 2006); thus, oxidative injury is one of the major characteristics occurred in MCD-fed rodents. However, the impact of MCD diet upon the hepatic mRNA expression of anti-oxidant enzymes such as glutathione peroxidase (GPX), superoxide dismutase (SOD) and catalase remains unknown. In addition, MCD feeding markedly upregulated hepatic mRNA expression of several inflammation and fibrosis associated cytokines or factors such as tumor necrosis factor (TNF)-alpha, and collagen-alpha1 (George et al., 2003; Koppe et al., 2004), which recruited or accelerated the progression of

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; FAS, fatty acid synthase; G6PDH, glucose-6-phosphate dehydrogenase; GPX, glutathione peroxidase; GSH, glutathione; GSSG, oxidized glutathione; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl coenzyme A reductase; IL-1beta, interleukin-1beta; IL-6, interleukin-6; MCD, methionine and choline deficient; MDA, malondialdehyde; MMP-9, matrix metalloproteinase; NASH, nonalcoholic steatohepatitis; PCR, polymerase chain reaction; SAC, s-allyl cysteine; SEC, s-ethyl cysteine; SOD, superoxide dismutase; TGF-beta1, transforming growth factor-beta1; TG, triglyceride; TNF-alpha, tumor necrosis factor-alpha.

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hepatic inflammation and fibrosis. Thus, any agent(s) with anti-oxidative, anti-inflammatory and/or anti-fibrotic activities may provide alleviative effects for liver against NASH development and/or deterioration.

s-Allyl cysteine (SAC) and s-ethyl cysteine (SEC) are two hydrophilic cysteine-containing compounds naturally formed in *Allium* plants such as garlic and onion (Fukushima et al., 1997; Jones et al., 2004). Our past studies have revealed that the intake of these compounds could attenuate hepatic lipogenesis and improve oxidative, inflammatory and fibrotic injury in diabetic and acetaminophen-tread mice (Hsu et al., 2006; Yin et al., 2007; Lin and Yin, 2008). Thus, we hypothesized that these compounds were potent agents against MCD-induced hepatotoxicity. The major purpose of this study was to investigate the alleviative effects of SAC and SEC upon MCD diet-induced hepatic lipid accumulation, oxidation, inflammation and fibrosis. The possible action modes of SAC and SEC were also examined.

2. Materials and methods

2.1. Materials, animals and diets

s-Allyl cysteine (SAC, 99%) was supplied by Wakunaga Pharmaceutical Co. Ltd. (Hiroshima, Japan), s-ethyl cysteine (SEC, 99.5%) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Male C57BL/6 mice, 3–4 weeks old, were obtained from National Laboratory Animal Center (National Science Council, Taipei City, Taiwan). Mice were housed on a 12-h light:dark schedule; water and mouse standard diet were consumed ad libitum. The use of mice was reviewed and approved by Chung Shan Medical University animal care committee. After one week acclimation, mice were divided into two groups, one continuously consumed normal diet and water (control group), and the other was switched to MCD diet (No. 960439; ICN Biomedicals, Irvine, CA, USA). This MCD formula comprised 17% protein, 65% carbohydrate (70:30 sucrose–starch), and 10% fat (as corn oil).

2.2. Experimental design

MCD diet groups were further divided into three sub-groups, in which water, SAC or SEC was supplied. Our previous study indicated that intake of SAC or SEC at 1 g/L effectively attenuated hepatic lipogenesis and improved oxidative stress (Lin and Yin, 2008). Thus, in our present study, each agent at 1 g/L was used and directly added into the drinking water. All mice had free access to food and water at all time. Body weight, consumed water volume and feed were recorded. After 7 weeks supplementation, mice were killed with carbon dioxide. Blood and liver were collected. Serum was separated from erythrocytes immediately. Liver at 0.2 g was homogenized on ice in 2 mL phosphate buffer saline (PBS, pH 7.2), and the filtrate was collected. The protein concentration of serum or liver filtrate was determined by the method of Lowry et al. (1951) using bovine serine albumin as a standard. In all experiments, sample was diluted to a final concentration of 1 g protein/L using PBS, pH 7.2.

2.3. Measurement of alanine aminotransferase (ALT), aspartate aminotransferase (AST), glucose and insulin

Serum activities of ALT and AST were determined by using commercial assay kits (Randox Laboratories Ltd., Crumlin, UK). Serum glucose level (mmol/L) was measured by a glucose HK kit (Sigma Chemical Co., St. Louis, MO, USA), and insulin (nmol/L) was measured by using a rat insulin RIA kit (SRI-13K; Linco Research, St. Charles, MO, USA).

2.4. Hepatic triglyceride (TG), and total cholesterol (TC) measurements

Hepatic TG (mg/g wet tissue) and TC (mg/g wet tissue) was extracted by the method of Folch et al. (1957) and Rudel and Morris (1973), respectively.

2.5. Assay for the activity of glucose-6-phosphate dehydrogenase (G6PDH), malic enzyme, fatty acid synthase (FAS) and 3-hydroxy-3-methylglutaryl coenzyme A (HMG–CoA) reductase

Hepatic G6PDH activity was assayed by a commercial kit (Sigma–Aldrich Co. Ltd., MO, USA), in which enzyme activity was determined by using a plate-reader spectrophotometer and measuring the absorbance increase at 340 nm due to the conversion of $NADP^+$ to NADPH. The activity of malic enzyme and FAS was measured according to the methods of Stelmanska et al. (2004), and Nepokroeff et al. (1975). Both were determined by spectrophotometric assays. FAS activity was determined from the rate of malonyl-CoA-dependent NADPH oxidation. HMG–CoA reductase activity in liver filtrate was measured by a radiochemical method as described in Kita et al. (1980), in which [3-^{14}C]HMG-CoA was used as a substrate, and [3-^{14}C]mevalonone synthesized during the assay was isolated by anion-exchange column chromatography.

2.6. Lipid oxidation, glutathione (GSH) and oxidized glutathione (GSSG) determination

Liver lipid oxidation was determined by measuring the level of malondialdehyde (MDA, nmol/mg protein) via an HPLC method described in Hsu et al. (2006). Briefly, 0.2 mL liver filtrate was suspended in 0.8 mL PBS. Then, 0.5 mL trichloroacetic acid (30%) was added. After vortexing and standing in ice for 2 h, sample was centrifuged at 2000*g* for 15 min. One milliliter supernatant was mixed with 0.25 mL thiobarbituric acid (1%) and the mixture was kept in boiling water bath for 15 min. The concentration of MDA–TBA complex was assayed using HPLC equipped with a reverse-phase Shodex KC-812 column with the UV–Vis detector at 532 nm. GSH and GSSG concentrations (nmol/mg protein) in liver were determined by commercial colorimetric GSH and GSSG assay kits (OxisResearch, Portland, OR, USA) according to the manufacture's instruction.

2.7. Catalase, glutathione peroxidase (GPX) and superoxide dismutase (SOD) assay

Catalase, GPX and SOD activities (U/mg protein) in liver were determined by commercial catalase, GPX and SOD assay kits (Calbiochem, EMD Biosciences, Inc. San Diego, CA, USA). For catalase activity assay, liver filtrate was mixed with 10 mM H_2O_2 and 50 mM PBS (pH 7.2). The absorbance change at 240 nm during the time interval of 30 s was measured. GPX activity was measured indirectly by monitoring the consumption of NADPH at 340 nm. SOD activity was measured by recording change in absorbance at 560 nm due to formation of Formosan, a reaction product of nitro blue tetrazolium salt.

2.8. Measurement of vascular H_2O_2 production

H_2O_2 level in tail artery was measured according to the method described in Sousa et al. (2008). After removing the connective tissue and blood, the arteries were placed in Krebs–HEPES medium at 37 °C. Vascular concentration of H_2O_2 was measured using an Amplex Red Hydrogen Peroxide Assay kit (Molecular Probes, Eugene, Oregon, USA) according to the manufacturer's protocol. Results are expressed as nmol/mg protein.

2.9. Hepatic cytokines and fibrotic factors determination

Hepatic levels of interleukin (IL)-1beta, IL-6, TNF-alpha and transforming growth factor (TGF)-beta1 were measured by commercial ELISA kits (R&D Systems, Minneapolis, MN, USA). Hepatic matrix metalloproteinases (MMP)-9 was quantified by a commercial kit (Amersham Biosciences, Piscataway, NJ, USA). Hepatic collagen level could be quantified by measuring hydroxyproline content. The chloramine T method described by Ellis et al. (1994) was used to determine hepatic hydroxyproline content. Briefly, 40 mg liver sample were hydrolyzed in 500 μL 6 N HCl at 100 °C for 24 h. After cooling, hydrolysate was neutralized with 500 μL 6 N NaOH, and centrifuged at 13,000*g* for 12 min. Forty microliter supernatant was added and incubated with 25 μL chloramine T solution containing 1 part 7% chloramine T and 4 parts citrate/acetate buffer (pH 6.0, 695 mM sodium acetate, 128 mM trisodium citrate, 29 mM citric acid, with 38.5% isopropanol) at room temperature for 10 min, and followed by adding 150 μL Ehrlich's solution, 1.4 M dimethylaminobenzaldehyde with 20% perchloric acid and 67% isopropanol. After incubation at 65 °C for 15 min, the absorbance was read at 561 nm. Hydroxyproline concentration was calculated from a standard curve and expressed as μg hydroxyproline/g liver.

2.10. Semi-quantitative polymerase chain reaction (PCR) for mRNA expression

The mRNA expression of GPX, SOD, catalase, IL-1beta, IL-6, TNF-alpha, collagen-alpha1, MMP-9 and TGF-beta1 were examined. Part of liver was homogenized in guanidine–thiocyanate, and total RNA was extracted. Two microgram of total RNA was used to generate cDNA, which was amplified using Taq DNA polymerase. PCR was carried out in 50 mL of reaction mixture containing Taq DNA polymerase buffer (20 mM Tris–HCl, pH 8.4, 50 mM KCl, 200 mM dNTP, 2.5 mM $MgCl_2$, 0.5 mM of each primer) and 2.5 U Taq DNA polymerase. The primers for PCR were synthesized based on previously published primer sequences (Oz et al., 2006; Song et al., 2006). GPX: forward, 5′-TTG GCT TGG TGA TTA CTG GC-3′, reverse, 5′-CAT TAG GTG GAA AGG CAT CG-3′; catalase: forward, 5′-AAC GCT GGA TGG ATT CTC CC-3′, reverse, 5′-GCC CTA ACC TTT CAT TTC CCT TCA G-3′; SOD: forward, 5′-CTA ACT CAA GCA TGG CGA TGA AA-3′; reverse, 5′-ACA CAG GGA ATG TTT ACT GCG C-3′; IL-1beta: forward, 5′-TGA TGT TCC CAT TAG ACA GCT GCA-3′, reverse, 5′-CCA TTG CTG TTT CCT AGG AAG ACA-3′; TNF-alpha: forward, 5′-GAA AGC ATG ATC CGA GAT GTG GAA-3′, reverse, 5′-GCT TGT CAC TCG AGT TTT GAG AAG-3′; IL-6: forward, 5′-GCC AGA GTC ATT CAG AGC AAT A-3′, reverse, 5′-GTG AGG AAT GTC CAC AAA CTG A-3′; collagen-alpha1: forward, 5′-CAG CGG TGA AGA AGG AAA GAG A-3′, reverse, 5′-GAA TCC GAT GTT GCC AGC TTC A-3′; MMP-9: forward, 5′-CTA GGC TAC