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Activity Change of Sphingomyelin Anabolic Enzymes during Dimethylnitrosamine-induced Hepatic Fibrosis in Rats

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Abstract – In the present study, we investigated activity change of sphingomyelin anabolic enzymes such as sphingomyelin synthase and ceramide synthase. Sprague-Dawley male rats treated with 10 mg/kg of DMN intraperitoneally were used as a hepatic fibrosis model. Sphingomyelin synthase and ceramide synthase activities were measured in 1-week, 2-week, 3-week and 4-week DMN-treated rats along with respective control group rats. We found the increased sphingomyelin synthase activity in 4-week DMN-treated liver but not in kidney. Ceramide synthase activity was significantly increased in DMN-treated kidney after 2-week treatment and in DMN-treated liver after 3-week treatment. Although further investigation is necessary to elucidate meanings of sphingolipid metabolites during the liver fibrosis, activity change of sphingolipid anabolic enzymes may imply that sphingolipid metabolism and sphingolipid metabolites could be involved in liver fibrosis especially under oxidative stress.

Keywords : Dimethylnitrosamine, Hepatic fibrosis, Sphingomyelin cycle

INTRODUCTION

Dimethylnitrosamine (DMN) is a potent hepatotoxin, carcinogen and mutagen (Haggerty and Holsapple, 1990). At doses as small as 20 mg/kg, DMN can cause massive liver necrosis and death in many species (Hashimoto *et al.*, 1989). In rats, DMN-induced liver fibrosis is a well-established, reproducible animal model with severe hepatic necrosis and formation of septa with micronodular cirrhosis after 3 weeks of treatment (Kim *et al.*, 1998; Jenkins *et al.*, 1985; Jezequel *et al.*, 1989). DMN causes excessive deposition of extracellular matrix proteins, especially collagen (Jezequel *et al.*, 1987; George and Chandrakasan, 1996; George *et al.*, 2001; Shiba *et al.*, 1998), and a rapid increase of the proportion of proliferative hepatic stellate cells in rat liver (Kim *et al.*, 1998; Paolucci *et al.*, 1990; Mancini *et al.*, 1992), which produces profound liver fibrosis over a short period. Therefore, this model is considered to be appropriate for investigation of the contribution of hepatic stellate cells activation to the early events in the development of fibrosis.

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Bioactive sphingolipids, especially ceramide and sphingosine 1-phosphate, have now been identified as strategic intermediates in cellular responses to inflammation, proliferation, differentiation, cell cycle arrest, senescence, and cell death (Cuvillier and Levade, 2003; Hannun *et al.*, 2001; Kolesnick, 2002; Mathias *et al.*, 1998; Obeid and Hannun, 2003). Hepatocellular injury activates signal transduction pathways that mediate cell repair, proliferation, or even cell death (Chalfant *et al.*, 2002; Chen *et al.*, 1995; Chun *et al.*, 2003; Cock *et al.*, 1998). Interestingly, bioactive sphingolipids are increasingly appreciated as important participants in liver responses to these perturbations. A number of extracellular stimuli are known to induce ceramide levels in cells, including tumor necrosis factor-α (TNF-α), Fas ligand, lipopolysaccharide (LPS or endotoxin), and chemotherapeutic agents (Hannun, 1996). Furthermore, many of these inducers of ceramide are also known to be involved in mediating liver injury. Since ceramide has been implicated in pathways regulating cell proliferation and the induction of apoptosis, it follows that ceramide signaling may be an important mediator in the liver homeostatic response. Activation of acidic sphingomyelinase and ceramide secretion were reported to induce hepatocyte apoptosis (Lang *et al.*, 2007). Acidic sphingomyelinase knockout mice showed defective TNF-α-mediated hepatocellular apoptosis and

liver damage (Garcia-Ruiz *et al.*, 2003)

Previously, we reported activity changes of sphingomyelin catabolic enzymes such as sphingomyelinase and ceramidases in DMN-treated liver fibrosis model (Sacket and Im, 2008). In the present study, sphingomyelin anabolic enzymes such as sphingomyelin synthase and ceramide synthase were measured in the liver and kidney from DMN-treated rats.

MATERIALS AND METHODS

Materials

DMN was from Sigma-Aldrich, Inc, St. Louis, [N-palmitoyl-1-^{14}C]-sphingosine from Moravek Biochemicals (Brea, CA, USA). D-*erythro*-[3-^{3}H]-sphingosine and EN^{3} HANCE spray was purchased from PerkinElmer Life & Analytical Sciences (Boston, MA, USA). Silica gel HPTLC (60 F_{254}, 20 x 20) were obtained from Merck (Germany) and Kodak Medical X-ray film from Eastman Kodak Company (NY, USA). All other materials were purchased from Sigma-Aldrich Korea (St. Louis, MO, USA).

Animal care and maintenance

Animal care and all experimental procedures were conducted in accordance with the Guide for Animal Experiments edited by the Korean Academy of Medical Sciences. Experiments were carried out in male Sprague–Dawley rats weighing 225 - 265 g. Following shipment all animals were acclimatized in the animal care facility for at least 1 week prior to any studies. Animals were kept on standard rat chow with free access to tap water, in a temperature- and humidity-controlled animal house under 12-h light–dark cycles.

Induction of liver fibrosis

The protocol used for inducing the liver injury by the chemical DMN is a modified version of that originally described by Lee *et al.* (Lee *et al.*, 2004). In brief, prior to the start of the study twenty rats were randomized on the basis of their body weight and assigned them to five groups of four each: control, DMN 1week, DMN 2 weeks, DMN 3 weeks and DMN 4 weeks such that each group had similar distribution of body weights. The animals were given intraperitoneal injections of either vehicle or DMN (diluted with saline) at a dose of 10 mg/kg of body weight for three consecutive days per week. Body weights were measured each day prior to the injections of DMN. Blood and tissue samples for biochemical and pathological examinations were collected from the animals at sacrifice at the end of each week, as described specifically below. At the end of the first, second, third and fourth week, all rats from respective groups were sacrificed under ether anesthesia and their livers and kidneys were excised. Blood samples for biochemical analyses were obtained from the inferior *vena cava*. The liver specimens were immediately fixed in 10% neutral buffered formalin for histochemical studies. The remaining liver tissue and kidney tissues were used for homogenate preparation as described below.

Preparation of tissue homogenates

Rat tissue homogenates were prepared as described by others (Igarashi and Hakomori, 1989) with some modifications. Tissues including kidney and liver were removed from rats and washed in cold phosphate-buffered saline (PBS) separately. The tissues were placed in 10 ml of cold 20 mM Tris-HCl (pH 7.5) and 2 mM EDTA solution, homogenized by Tekmar homogenizer (OH, USA) at 4^{o}C. The homogenate was centrifuged at 1000 *g* for 10 min to remove unbroken cell debris, and the supernatant was used as an enzyme source for all below mentioned activities. Protein concentrations were determined by the Micro BCA Protein Assay Reagent Kit (Pierce, Rockford, IL, USA) with bovine serum albumin (BSA) as a standard.

Activity of sphingomyelin synthase

The activity of sphingomyelin synthase was measured as described (Futerman *et al.*, 1990). In brief, the reaction mixture consisted of 50 mM Tris-HCl (pH 7.4), 25 mM KCl, and 0.5 mM EDTA and homogenate containing 5 mg of protein (Luberto and Hannun, 1998). The reaction was started by addition of [N-palmitoyl-1-^{14}C]-sphingosine (20 nmol) as an equimolar complex with fatty acid free BSA (dBSA) (complex specific activity: ~ 9 × 10^{3} cpm/nmol) and proceeded for up to 60 min. The reaction was stopped by addition of 3 ml of chloroform: methanol (1:2); the mixture was vortexed and kept on ice. Lipids were extracted as indicated by the Bligh and Dyer method (Bligh and Dyer, 1959) and resolved by TLC in chloroform:methanol:15 mM anhydrous $CaCl_2$ (60:35:8). The [N-palmitoyl-1-^{14}C]-SM produced was detected by autoradiography, scraped from the plates and quantitated by liquid scintillation counting. Values for blanks were subtracted from total values of [N-palmitoyl-1-^{14}C]-SM to yield the amount of [N-palmitoyl-1-^{14}C]-SM produced.

Activity of ceramide synthase:

Ceramide synthase (*Sphingosine N-Acyltransferase*) activity was measured (Wang *et al.*, 1991). The assay mixture for ceramide synthase contained 1 μM [^{3}H]D-

erythro-sphingosine, 25 mM potassium phosphate buffer (pH 7.4), 0.5 mM dithiothreitol, 200 μM palmitoyl-CoA, and homogenate containing approximately 0.2 mg of protein in a total volume of 0.1 ml, which were found to be optimal assay conditions as reported previously (Morell and Radin, 1970). The reaction was initiated by adding palmitoyl-CoA, and after incubation at 37°C for 15 min, the products were extracted, resolved by TLC, and detected by autoradiography, scraped from the plates and quantitated by liquid scintillation counting.

Statistical analysis

The results are expressed as mean ± SE of three determinations. Statistical significance of differences was determined by student-t test. Significance was accepted when $P<0.05$.

RESULTS

Sphingomyelin synthase activity in kidney and liver

Previously, we reported increased ceramidase activities from 2-week and/or 3-week DMN-treated rat livers compared to control rat liver (Sacket and Im, 2008). And also acidic sphingomyelinase and alkaline ceramidase activities were observed to be significantly increased in 3-week DMN-treated rat kidneys compared to control rat kidney. In the present study, sphingomyelin anabolic enzyme, sphingomyelin synthase was measured in DMN-treated rat liver and kidney tissue homogenates. DMN-treated rat kidney and liver showed slightly increased activity than control but this change was not significant than control. And among the all DMN-treated groups, sphingomyelin synthase activity was significantly increased only in 4-week DMN-treated rat liver but not in kidney homogenate (Fig. 1).

Ceramide synthase activity in kidney and liver

Similarly, ceramide synthase activity was measured in rat liver and kidney tissue homogenates. Ceramide synthase activity was increased in 3-week and 4-week-DMN-treated liver than control (Fig. 2-A). Ceramide synthase activity was significantly increased in DMN-treated kidney after 2-week treatment also (Fig. 2-B).

Discussion

Liver fibrosis occurs as a result of an imbalance between fibrogenesis and fibrolysis in the liver and many works have been published concerning extracellular matrix (ECM) synthesis and the main ECM-producing cell

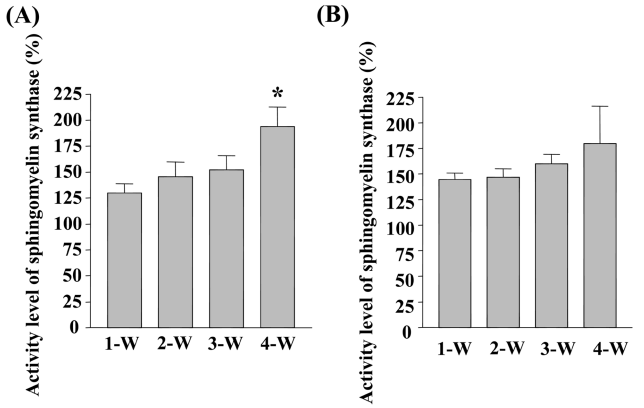

Fig. 1. Sphingomyelin synthase activity in DMN-treated rat liver and kidney.
Sphingomyelin synthase activities were measured from 1-week, 2-week, 3-week, 4-week-DMN treated rat liver (A) or kidney (B). Activities were shown as percentage of control (saline-treated rat liver or kidney). Histogram shows mean ±S.E. of three independent measurements. *$P<0.05$ is significant level compared to the respective control group activity.

in the liver, i.e. the hepatic stellate cell (Friedman, 1993). Hepatic fibrosis represents a common response to chronic liver injuries of variable origin, *e.g.* viral, metabolic and toxic. Regardless of the type of insults, liver fibrosis is characterized by the increased production of ECM proteins. Hepatic fibrogenesis is accompanied by hepatocellular necrosis and inflammation. Hepatic stellate cells are regarded as the primary target cells for inflammatory stimuli in the injured liver (George *et al.*, 1999; Li and Friedman, 1999). However, changes of sph-

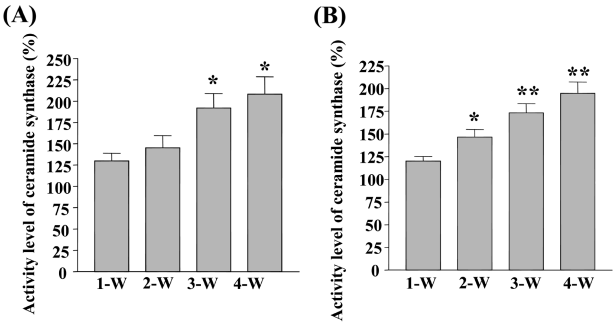

Fig. 2. Ceramide synthase activity in DMN-treated rat liver and kidney
Ceramide synthase activities were measured from 1-week, 2-week, 3-week, 4-week-DMN treated liver (A) or kidney (B). Activities were shown as percentage of control (saline-treated rat liver or kidney). Histogram shows mean ±S.E. of three independent measurements. *$P<0.05$ or **$P<0.01$ is significant level compared to the respective control group activity.

ingolipid metabolites and metabolism during liver fibrosis have not been explored. Changes of sphingolipid metabolism could result in concentration changes of sphingolipid metabolites in the tissue and in the cell.

Sphingomyelin is one of the most abundant sphingolipid species found in cell membranes. The enzyme responsible for the biosynthesis of sphingomyelin, sphingomyelin synthase, transfers the phosphocholine headgroup from phosphatidylcholine to ceramide. Sphingomyelin synthase activity has been attributed to the plasma membrane (Futerman *et al.*, 1990; Voelker and Kennedy, 1982), Golgi apparatus (Futerman *et al.*, 1990; Schweizer *et al.*, 1994) and endosomes (Allan and Kallen, 1994; Kallen *et al.*, 1994) and, in earlier studies, to the endoplasmic reticulum (van Golde *et al.*, 1974) and mitochondria. In our study, only 4-week DMN-treated liver showed significant increase of the enzyme activity, implying that this enzyme activity may not affect content changes of ceramide and sphingomyelin during liver fibrosis.

Ceramide is synthesized mainly from the reaction of a fatty acyl-CoA with a sphingoid base by an acyl-CoA-dependent ceramide synthase (Morell and Radin, 1970). Acylation of sphinganine to dihydroceramide (or to ceramide if sphingosine is available) is catalyzed by ceramide synthase (dihydroceramide synthase, sphinganine N-acyltransferase, sphingosine N-acyltransferase; EC 2.3.1.24). Dihydroceramide, a physiologically inactive intermediate, is converted to bioactive ceramide by dihydroceramide desaturase. In the *de novo* pathway, the molecular regulation of ceramide synthase may be of particular interest, as recent studies indicate that this enzyme is a metabolic target for TNF-α (Liu *et al.*, 2000; Xu *et al.*, 1998). Furthermore, many of these inducers of ceramide are also known to be involved in mediating liver injury. Since ceramide has been implicated in pathways regulating cell proliferation and the induction of apoptosis, it follows that ceramide signaling may be an important mediator in the liver homeostatic response. In the present study, increased activity of ceramide synthase was found from 3-week and 4-week DMN-treated liver compared to control liver. Furthermore, the activity was increased in 2-week, 3-week, and 4-week DMN-treated kidney. These results increased ceramide synthase activities from liver and kidney may result in accumulation of ceramide, which might induce apoptosis of hepatocytes.

In the previous study, we investigated sphingomyelinase (acidic and neutral) and ceramidase (acidic, neutral and alkaline) activities. But we did not find any significant change in the activities as much as we expected. DMN-treated rat kidney and liver showed slight increase in the all isoforms of ceramidase and sphingomyelinase activities as compared to control kidney and liver. But the activity of all the isoforms of sphingomyelinases and ceramidases were higher in rat kidney than liver. Now we investigated sphingomyelin synthase and ceramide synthase activities in the rat kidney and liver from DMN-treated rats. Sphingomyelin synthase activity was significantly increased in DMN-treated rat liver as compared to control liver. Ceramide synthase activity was significantly increased in DMN-treated kidney after 2-week treatment and in DMN-treated liver after 3-week treatment. Although DMN was used as a tool to induce liver fibrosis, oxidative stress by DMN could affect on other organs. In our experiment, kidney showed higher activities of sphingolipid metabolic enzymes and DMN affected enzyme activities in kidney, meaning that DMN-induced oxidative stress affects kidney function also. In summary, sphingolipid metabolizing enzymes and sphingolipid metabolites might be involved in oxidative stress-induced changes such as liver fibrosis, although further investigation is necessary to elucidate meanings of sphingolipid metabolites during the liver fibrosis

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