

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

Santosh J. SACKET and Dong-Soon IM

Laboratory of Pharmacology, College of Pharmacy (BK21 Project) and Longevity Life Science and Technology Institutes, Pusan National University, Busan 609-735, Republic of Korea

(Received March 7, 2008; Accepted March 13, 2008)

**Abstract** – Oxidative stress may represent a common link between chronic liver damage and hepatic fibrosis. In the present study, we investigated activity changes of sphingomyelin catabolic enzymes, such as sphingomyelinases and ceramidases by using dimethylnitrosamine (DMN)-treated Sprague-Dawley (SD) male rats hepatic fibrosis model as a hepatic fibrosis model. Twenty rats divided into five groups received: (1) saline; (2) DMN for 1 week, (3) DMN for 2 weeks, (4) DMN for 3 weeks, and (5) DMN for 4 weeks by intraperitoneally 10 mg/kg of body weight for three consecutive days a week. Activities of acidic and neutral sphingomyelinases and acidic, neutral and alkaline ceramidases were measured in the liver and kidney from DMN-treated rats. We found increased ceramidase activities from 2-week and/or 3-week DMN treated rat livers compared to control rat liver. Acidic sphingomyelinase and alkaline ceramidase activities were significantly increased in 3-week DMN-treated rat kidneys compared to control rat kidney. Therefore, sphingolipid metabolizing enzymes and sphingolipid metabolites are supposed to be involved in liver fibrosis, although further investigation is necessary to elucidate meanings of sphingolipids during the liver fibrosis

**Keywords** □ Dimethylnitrosamine, Hepatic fibrosis, sphingomyelin cycle

### INTRODUCTION

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 in the liver, i.e. the hepatic stellate cell (HSC) (Friedman, 1993). Liver fibrosis is a chronic illness characterized by generalized damage and formation of regeneration nodules. Secondary to the anatomical alterations, there are functional and hemodynamic disturbances (Iredale, 2007). Hepatic fibrosis is wound healing response to chronic liver injury, which if persistent leads to cirrhosis and liver failure (Li and Friedman, 1999). It is characterized by the excess production and deposition of ECM components. Viral infection, alcoholic or drug toxicity, or any other factors that cause damage to hepatocytes elicit an inflammatory reaction in the liver. The damaged hepatocytes, their membrane components, metabolites of toxic agents, and

infiltrating inflammatory cells are the activators of Kupffer cells. The activated Kupffer cells release a number of soluble agents, including cytokines, reactive oxygen species (ROS), and other factors (Roberts *et al.*, 2007).

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 HSCs 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 HSC activation to the early events in the development of fibrosis.

Bioactive sphingolipids, especially ceramide and sphin-

\*Corresponding author

Tel: +82-51-510-2817, Fax: +82-51-513-6754

E-mail: imds@pusan.ac.kr

goline 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). By means of exquisite homeostatic mechanisms, the liver maintains the distinctive capacity to regulate its tissue mass by inductive cell division and apoptosis. 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). In addition, recent findings suggest that a complex series of signaling events drive the liver homeostatic responses under conditions where the liver tissue mass is perturbed. These perturbations can include drug-induced injury, viral infection, partial hepatectomy, ischemia/reperfusion, fulminant liver disease, and hepatocellular carcinoma. 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- $\alpha$  (TNF- $\alpha$ ), 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.

In the present study, therefore, we measured sphingomyelin-catabolizing enzyme activities such as acidic and neutral sphingomyelinases and acidic, neutral and alkaline ceramidases from the liver and kidney of DMN-treated rats.

## MATERIALS AND METHODS

### Materials

Dimethylnitrosamine (DMN) was from Sigma-Aldrich, Inc, St. Louis, [N-methyl-  $^{14}\text{C}$ ] -sphingomyelin, [N-palmitoyl- $^{14}\text{C}$ ]-sphingosine from Moravak Biochemicals. D-erythro-sphingosine (synthetic) was purchased from Avanti Polar Lipids Inc. (Alabaster, USA). While EN<sup>3</sup> HANCE spray was purchased from PerkinElmer Life & Analytical Sciences (Boston, USA). Silica gel HPTLC (60 F<sub>254</sub>, 20×20) 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 1 week, 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. 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. 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°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 above mentioned activi-

ties. 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 sphingomyelinase:

The activity of neutral and acidic sphingomyelinase was determined as reported by Liu and Hannun (Liu and Hannun, 2000). Briefly, the tissue homogenates were centrifuged at 1000 g for 10 min and the supernatant (~5 mg) was used for further analysis. The activity of both sphingomyelinases was measured using radiolabeled substrate, [N-methyl- $^{14}\text{C}$ ]-sphingomyelin. For neutral sphingomyelinase the reaction mixture contained 100 nmol of sphingomyelin (1154 dpm/nmol) in 100 mM Tris-HCl (pH 7.4), 5 mM  $\text{MgCl}_2$ , 0.1% Triton X-100 and 5 mM dithiothreitol in a final volume of 0.2 ml. In the case of acidic sphingomyelinase, the assay mixture contained 100 nmol of sphingomyelin (1154 dpm/nmol) in 100 mM sodium acetate (pH 5.0), 0.1% Triton X-100 and 0.1 mM EDTA. After incubation at 37°C for 1 h the reaction was stopped by adding 1.5 ml of chloroform: methanol (2:1), followed by addition of 0.2 ml of water. A portion of the aqueous phase was transferred to scintillation vials and counted in a liquid scintillation counter for the radioactivity of the reaction product,  $^{14}\text{C}$ -choline phosphate.

#### Activity of ceramidases:

The activity of alkaline, neutral and acidic ceramidases was determined by the method of Nikolova-Karakashian and Merrill (Nikolova-Karakashian and Merrill, 2000). The activity of the enzymes was measured using radiolabeled

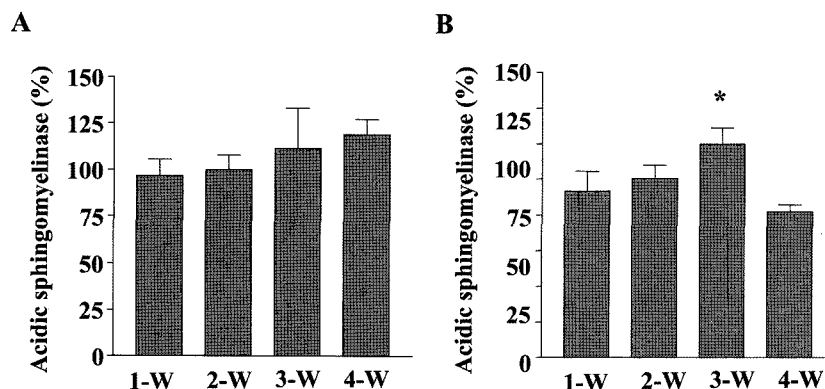
substrate [N-palmitoyl-1- $^{14}\text{C}$ ]-sphingosine. The tissue homogenates were centrifuged at 1000 g for 10 min and the supernatant (~5 mg) was used for the analysis. The reaction was started by the addition of supernatant to the tubes containing 20  $\mu\text{l}$  of substrate mixture (50 nmol of ceramide - 2353 dpm/nmol, 2.5 mg Triton X-100, 1 mg Tween 20, 0.4 mg sodium cholate) and 130  $\mu\text{l}$  of a reaction buffer. The reaction buffer contained 125 mM sucrose, 0.01 mM EDTA and 125 mM sodium acetate (pH 4.5) or 10 mM Tris-HCl (pH 7.2) or 125 mM HEPES (pH 8.0) for acidic, neutral and alkaline ceramidases activity assay, respectively. After incubation at 37°C for 1 h the reaction was stopped by adding 2 ml of basic Doyle's solution (isopropanol:heptane:1N NaOH, 40:10:1, v/v/v), 1.8 ml of heptane and 1.6 ml of water. Samples were then centrifuged and the upper phase was discarded. The lower phase was washed twice with 1.6 ml heptane and then 1 ml of 1 N  $\text{H}_2\text{SO}_4$  and 2.4 ml of heptane were added. After centrifugation, 1 ml aliquots from the upper phase were transferred to scintillation vials and analyzed for the radioactivity of the reaction product,  $^{14}\text{C}$ -palmitate.

#### Statistical analysis

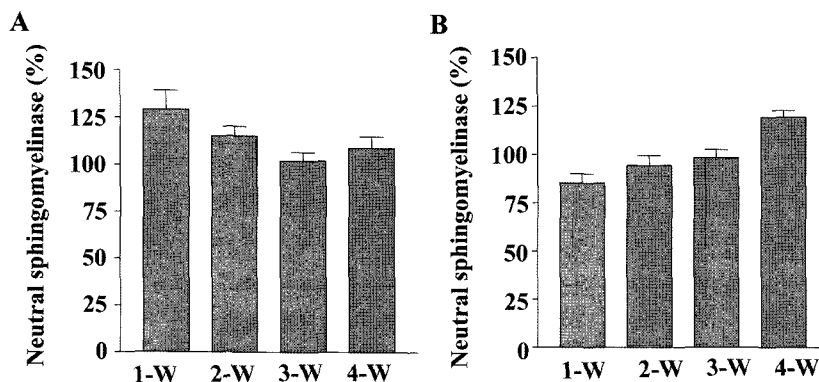
The results are expressed as mean $\pm$ SE of three determinations. Statistical significance of differences was determined by student-t test. Significance was accepted when  $P < 0.05$ .

## RESULTS

### Sphingomyelinase activity in kidney and liver



**Fig. 1.** Acidic sphingomyelinase activity in DMN-treated rat liver and kidney. Acidic sphingomyelinase 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 $\pm$ S.E. of three independent measurements. Statistical significance: \* $P < 0.05$  vs. control.



**Fig. 2.** Neutral sphingomyelinase activity in DMN-treated rat liver and kidney Neutral sphingomyelinase 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  $\pm$ S.E. of three independent measurements.

Acidic and neutral sphingomyelinase activities were measured in DMN-treated rat liver and kidney tissue homogenates. We found that acidic sphingomyelinase activity was not changed in the liver by DMN treatment (Fig 1-A). In the 3-week DMN-treated kidney homogenates, acidic sphingomyelinase was significantly increased (Fig 1-B). While neutral sphingomyelinase activity in liver was 2.5 times higher than kidney homogenates, DMN-treated rat kidney and liver did not show significant increase or decrease of the activity than control (Fig. 2).

### Ceramidase activity in kidney and liver

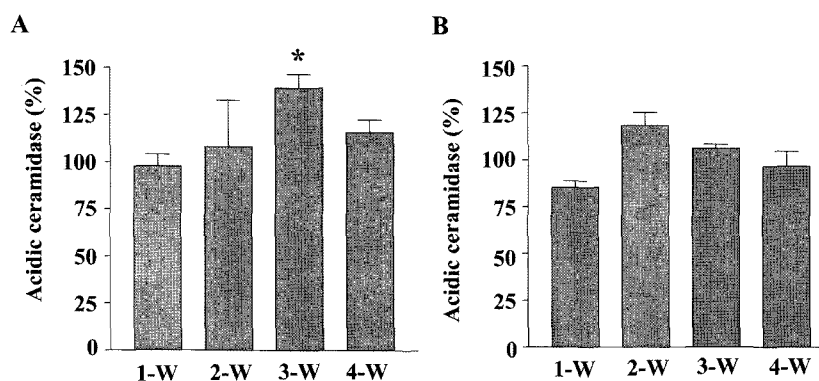
Similarly, acidic, neutral and alkaline ceramidase activities were measured in rat liver and kidney tissue homogenates and we found that activity of all isoforms of ceramidases are higher in rat kidney than in liver. Acidic ceramidase activity was increased in 3-week-DMN-treated

liver than control (Fig. 3). Neutral ceramidase activity was increased in 2-week-DMN-treated liver (Fig. 4). Alkaline ceramidase activity was significantly higher in 2-week and 3-week-DMN-treated liver (Fig. 5). Ceramidase activities were not changed in DMN-treated rat kidney compared to the control except alkaline ceramidase from 3-week-DMN-treated kidney (Fig. 3-5).

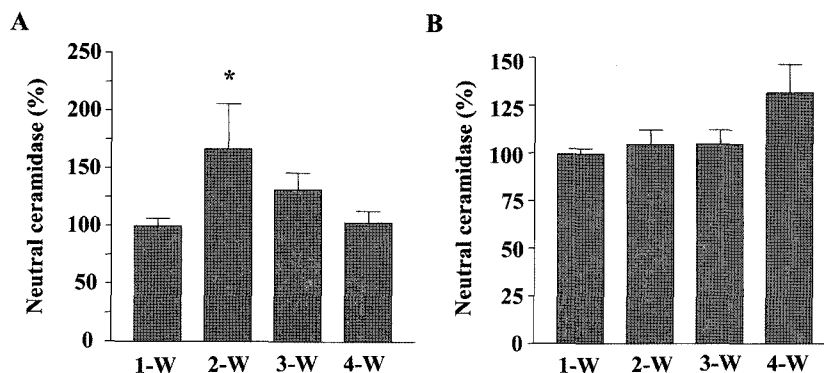
## DISCUSSION

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 extracellular matrix proteins. Hepatic fibrogenesis is accompanied by hepatocellular necrosis and inflammation.

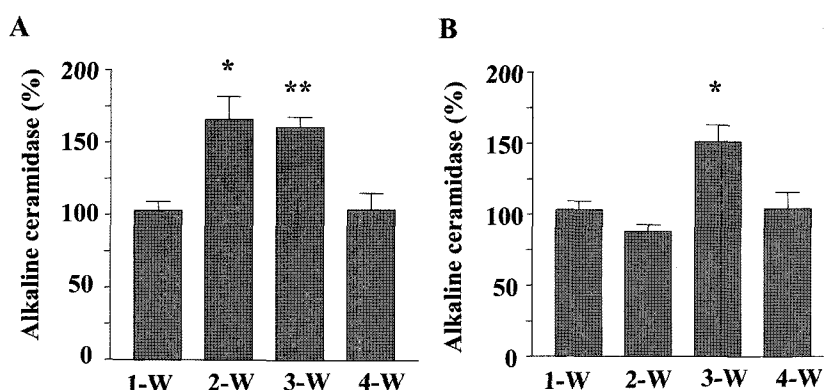
In the present study we investigated sphingomyelinase



**Fig. 3.** Acidic ceramidase activity in DMN-treated rat liver and kidney Acidic ceramidase 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  $\pm$ S.E. of three independent measurements. Statistical significance: \* $P$ <0.05 vs. control.



**Fig. 4.** Neutral ceramidase activity in DMN-treated rat liver and kidney Neutral ceramidase 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. Statistical significance: \* $P < 0.05$  vs. control.



**Fig. 5.** Alkaline ceramidase activity in DMN-treated rat liver and kidney Alkaline ceramidase 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. Statistical significance: \* $P < 0.05$  or \*\* $P < 0.01$  vs. control.

(acidic and neutral) and ceramidase (acidic, neutral and alkaline) activities. DMN treated rat liver showed no change in acidic and neutral sphingomyelinase activities during liver fibrosis. On the other hand, ceramidase (acidic, neutral and alkaline) activities were increased during 2-week and/or 3-week-DMN-treated liver. Rat kidney showed increased activities of acidic sphingomyelinase and alkaline ceramidase in 3-week-DMN-treated kidney. Further investigation is necessary to elucidate meanings of sphingolipids during the liver fibrosis by investigating sphingolipid anabolic enzymes and quantitative and qualitative analysis of sphingolipids.

#### ACKNOWLEDGMENT

This work was supported by the Grand-in-aid for 2007

Korea-Japan Joint Research Project from Korea Science and Engineering Foundation (F01-2007-000-10005-0).

#### REFERENCES

- Chalfant, C. E., Rathman, K., Pinkerman, R. L., Wood, R. E., Obeid, L. M., Ogretmen, B. and Hannun, Y. A. (2002). De novo ceramide regulates the alternative splicing of caspase 9 and Bcl-x in A549 lung adenocarcinoma cells. Dependence on protein phosphatase-1. *J Biol Chem* **277**, 12587-12595.
- Chen, J., Nikolova-Karakashian, M., Merrill, A. H., Jr. and Morgan, E. T. (1995). Regulation of cytochrome P450 2C11 (CYP2C11) gene expression by interleukin-1, sphingomyelin hydrolysis, and ceramides in rat hepatocytes. *J Biol Chem* **270**, 25233-25238.
- Chun, Y. J., Park, S. and Yang, S. A. (2003). Activation of Fas receptor modulates cytochrome P450 3A4 expression in human colon carcinoma cells. *Toxicol Lett* **146**, 75-81.

- Cock, J. G., Tepper, A. D., de Vries, E., van Blitterswijk, W. J. and Borst, J. (1998). CD95 (Fas/APO-1) induces ceramide formation and apoptosis in the absence of a functional acid sphingomyelinase. *J Biol Chem* **273**, 7560-7565.
- Cuvillier, O. and Levade, T. (2003). Enzymes of sphingosine metabolism as potential pharmacological targets for therapeutic intervention in cancer. *Pharmacol Res* **47**, 439-445.
- Friedman, S. L. (1993). Seminars in medicine of the Beth Israel Hospital, Boston. The cellular basis of hepatic fibrosis. Mechanisms and treatment strategies. *N Engl J Med* **328**, 1828-1835.
- George, J. and Chandrakasan, G. (1996). Molecular characteristics of dimethylnitrosamine induced fibrotic liver collagen. *Biochim Biophys Acta* **1292**, 215-222.
- George, J., Rao, K. R., Stern, R. and Chandrakasan, G. (2001). Dimethylnitrosamine-induced liver injury in rats: the early deposition of collagen. *Toxicology* **156**, 129-138.
- Haggerty, H. G. and Holsapple, M. P. (1990). Role of metabolism in dimethylnitrosamine-induced immunosuppression: a review. *Toxicology* **63**, 1-23.
- Hannun, Y. A. (1996). Functions of ceramide in coordinating cellular responses to stress. *Science* **274**, 1855-1859.
- Hannun, Y. A., Luberto, C. and Argraves, K. M. (2001). Enzymes of sphingolipid metabolism: from modular to integrative signaling. *Biochemistry* **40**, 4893-4903.
- Hashimoto, N., Ishikawa, Y. and Utsunomiya, J. (1989). Effects of portacaval shunt, transposition, and dimethylnitrosamine-induced chronic liver injury on pancreatic hormones and amino acids in dog. *J Surg Res* **46**, 35-40.
- Igarashi, Y. and Hakomori, S. (1989). Enzymatic synthesis of N,N-dimethyl-sphingosine: demonstration of the sphingosine: N-methyltransferase in mouse brain. *Biochem Biophys Res Commun* **164**, 1411-1416.
- Iredale, J. P. (2007). Models of liver fibrosis: exploring the dynamic nature of inflammation and repair in a solid organ. *J Clin Invest* **117**, 539-548.
- Jenkins, S. A., Grandison, A., Baxter, J. N., Day, D. W., Taylor, I. and Shields, R. (1985). A dimethylnitrosamine-induced model of cirrhosis and portal hypertension in the rat. *J Hepatol* **1**, 489-499.
- Jezequel, A. M., Mancini, R., Rinaldesi, M. L., Macarri, G., Venturini, C. and Orlandi, F. (1987). A morphological study of the early stages of hepatic fibrosis induced by low doses of dimethylnitrosamine in the rat. *J Hepatol* **5**, 174-181.
- Jezequel, A. M., Mancini, R., Rinaldesi, M. L., Ballardini, G., Fallani, M., Bianchi, F. and Orlandi, F. (1989). Dimethylnitrosamine-induced cirrhosis. Evidence for an immunological mechanism. *J Hepatol* **8**, 42-52.
- Kim, Y., Ratziu, V., Choi, S. G., Lalazar, A., Theiss, G., Dang, Q., Kim, S. J. and Friedman, S. L. (1998). Transcriptional activation of transforming growth factor beta1 and its receptors by the Kruppel-like factor Zf9/core promoter-binding protein and Sp1. Potential mechanisms for autocrine fibrogenesis in response to injury. *J Biol Chem* **273**, 33750-33758.
- Kolesnick, R. (2002). The therapeutic potential of modulating the ceramide/sphingomyelin pathway. *J Clin Invest* **110**, 3-8.
- Lee, M. H., Yoon, S. and Moon, J. O. (2004). The flavonoid naringenin inhibits dimethylnitrosamine-induced liver damage in rats. *Biol Pharm Bull* **27**, 72-76.
- Li, D. and Friedman, S. L. (1999). Liver fibrogenesis and the role of hepatic stellate cells: new insights and prospects for therapy. *J Gastroenterol Hepatol* **14**, 618-633.
- Liu, B. and Hannun, Y. A. (2000). Sphingomyelinase assay using radiolabeled substrate. *Methods Enzymol* **311**, 164-167.
- Mancini, R., Jezequel, A. M., Benedetti, A., Paolucci, F., Trozzi, L. and Orlandi, F. (1992). Quantitative analysis of proliferating sinusoidal cells in dimethylnitrosamine-induced cirrhosis. An immunohistochemical study. *J Hepatol* **15**, 361-366.
- Mathias, S., Pena, L. A. and Kolesnick, R. N. (1998). Signal transduction of stress via ceramide. *Biochem J* **335** (Pt 3), 465-480.
- Nikolova-Karakashian, M. and Merrill, A. H., Jr. (2000). Ceramidases. *Methods Enzymol* **311**, 194-201.
- Obeid, L. M. and Hannun, Y. A. (2003). Ceramide, stress, and a "LAG" in aging. *Sci Aging Knowledge Environ* **2003**, PE27.
- Paolucci, F., Mancini, R., Marucci, L., Benedetti, A., Jezequel, A. M. and Orlandi, F. (1990). Immunohistochemical identification of proliferating cells following dimethylnitrosamine-induced liver injury. *Liver* **10**, 278-281.
- Roberts, R. A., Ganey, P. E., Ju, C., Kamendulis, L. M., Rusyn, I. and Klaunig, J. E. (2007). Role of the Kupffer cell in mediating hepatic toxicity and carcinogenesis. *Toxicol Sci* **96**, 2-15.
- Shiba, M., Shimizu, I., Yasuda, M., Ii, K. and Ito, S. (1998). Expression of type I and type III collagens during the course of dimethylnitrosamine-induced hepatic fibrosis in rats. *Liver* **18**, 196-204.