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Short communication

Neutral sphingomyelinase: Localization in rat liver nuclei and involvement in regeneration/proliferation

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Abstract

We have studied the localization of neutral sphingomyelinase (N-SMase) in rat liver nuclei. The levels of neutral sphingomyelinase in regenerating liver nuclei were also assessed.

We found that rat liver nuclei contain a sphingomyelinase having a pH optima of 7.2 and a kDa of 92. In intact nuclei, neutral sphingomyelinase was associated predominantly with the nuclear envelope. In regenerating/proliferating rat liver (during DNA synthesis), neutral sphingomyelinase was translocated from the nuclear envelope to the nuclear matrix. The levels of sphingomyelin in whole nuclei decreased in reverse proportion to an increase in the levels of neutral sphingomyelinase. By contrast, there was a corresponding increase in the levels of ceramide and sphingosine during cell regeneration/proliferation. Thus, endogenous nuclear neutral sphingomyelinase may play a role in the regulation of sphingomyelin levels and in relevant signal transduction reactions involving cell regeneration/proliferation. The potential significance of ceramide generation may be aimed at programmed cell death to allow the regeneration of liver mediated via target proteins such as, ceramide activated protein kinases/phospholipases or other unknown mechanisms. (Mol Cell Biochem **143**: 169–174, 1995)

Key words: neutral sphingomyelinase (N-SMase), cell proliferation, nuclei, localization, signal transduction

Abbreviations: N-SMase – neutral sphingomyelinase; A-SMase – acid sphingomyelinase

Introduction

Sphingomyelinase(s)-type C (EC.3.1,4.12) are phospholipases that catalyze the hydrolysis of sphingomyelin to ceramide and phosphocholine [1]. Whereas, acid (A)-SMase is localized in the lysosomes in most tissues, neutral (N)-SMase has been primarily associated with the plasma membranes [1–3]. No information is available on the presence of sphingomyelinase in nuclei. Nor is much known about the role of sphingomyelin or its catabolic products in regenerating/proliferating liver cells. Consequently, we rationalized that endogenous nuclear sphingomyelinases may be involved in the regulation of sphingomyelin levels and in relevant signal transduction reaction that involve catabolic products of sphingomyelin, for example, ceramide, sphingosine and protein kinase-C [1, 4–6].

Materials and methods

Isotopes

[N-methyl-1-^{14}C] sphingomyelin (specific activity, 2.11GBq/mmol and [methyl-^{3}H]-thymidine (specific activity, 925GBq/

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mmol, 1.07 GBq/mmol) were purchased from Amersham Life Science, Arlington, IL. 'Liquiscint' was purchased from National Diagnostics, Atlanta, Georgia. Aquasol-II was purchased from New England, Nuclear, New York, N.Y. All other chemicals were purchased from Sigma Chemical Company, St. Louis, MO.

Animals

Experiments were carried out on male Wistar rats (100–120 g). Animals were housed for a week before starting the experimental procedures to adjust their day/night cycle and were fed rat chow and water. Partial hepatectomy was performed under light ether anaesthesia between 7 a.m. and 9 a.m. [7]. As a control we used liver from sham-operated animals. DNA synthesis in regenerating liver was measured by [^{3}H]-thymidine uptake in isolated nuclei [4].

Isolation of nuclei from liver cells

Liver from normal, sham-operated or partially hepatectomized rats were homogenized with buffer (50 mM Tris-HCl; pH 7.2, 0.25 M sucrose, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM PMSF, and 0.002% leupeptin) and the nuclear fraction was isolated [8]. The purity of nuclei preparations were assessed by means of light microscopy and marker enzyme assays [3].

Measurement of sphingomyelinase activity and product identification

The incubation mixture consisted of 50 mM Tris-HCl (pH 7.2–7.4), 10 uM Tris-acetate buffer, and 10 mM $MgCl_2$, 2 mg per ml Triton X-100, 2 mM N-methyl-[^{14}C]-sphingomyelin, and 3–4 mg per ml of nuclear protein [9]. Following incubation for 1 h at 37°C, the reaction was terminated with cold chloroform/methanol/water (100:42:6, v/v). The aqueous phase was withdrawn for radioactivity measurements. After termination of the N-SMase assay, a known amount of phosphocholine was added to the aqueous phase and the sample was separated by TLC with methanol, 0.5% NaCl, and NH_4OH (100:100:2 v/v) [10]. The gel area corresponding to phosphocholine was scraped, and the radioactivity was measured.

Measurement of lipid levels in regenerating rat liver nuclei during DNA synthesis

Lipids were extracted [11] and sphingomyelin, ceramides and sphingosine were separated by TLC. Ceramides were separated successively in two systems: (1) ethyl ether; (2) chloroform-hexane-ethyl ether-acetic acid (85:15:1 by volume). The chromatograms were developed by spraying with 10% $CuSO_4$ in 8% phosphoric acid, followed by heating for 10 min at 160°C and were subjected to densitometry on a scanning densitometer from Joyce Loebl (England) with a 300–400 nm filter, with aperture 0.5 mm as described [4].

Western immunoblot assay of N-SMase

2–3 µg of purified urinary N-SMase, nuclear proteins, and standard proteins of known molecular weight were subjected to western immunoblot assay employing monospecific polycolonal antibody against N-SMase as described previously [10].

Results

Effect of pH on N-SMase activity in nuclei and product characterization

Rat liver nuclei exhibited activity in a wide pH-range [5–8]. However, optimal sphingomyelinase activity was observed at pH 7.2 (Fig. 1). When the products of N-SMase activity were analyzed following TLC and radioactivity measurements, most (>95%, 950 cpm out of 1000 cpm in aqueous phase) of the radiolabeled product co-migrated with authentic phosphocholine.

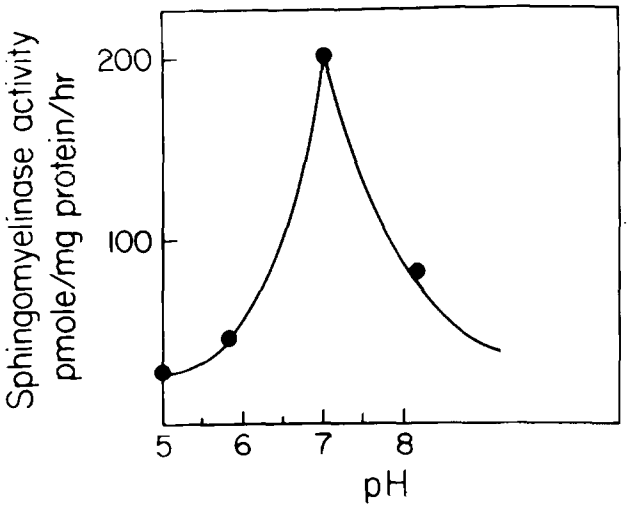

Fig. 1. Effects of pH on sphingomyelinase activity in rat liver cell nuclei. Sphingomyelinase activity (SMase) was measured in nuclear homogenates as described in the text.

Table 1. Distribution of marker enzymes in homogenates and nuclei from rat liver. The purity of the isolated nuclei was assessed by their marker enzyme activity as described in 'Material and methods'

Fraction	Acid phosphatase*	Alkaline phosphatase*	NADPH-cytochrome-C reductase**	Succinate dehydrogenase**	Galacosyl-transferase***
Homogenate	96.8 ± 1.6	76.1 ± 2.1	61.3 ± 1.3	36.2 ± 1.7	29.5 ± 2.1
Nuclei	–	–	3.8 ± 0.5	1.4 ± 0.3	1.8 ± 0.6

* umol P/mg protein/h
** umol P/mg protein/min
*** umol galactose/mg protein/h

Distribution of sphingomyelinase activity in rat liver nuclei and the effects of Triton X-100

We found that the specific activity of the N-SMase in the nuclei (76 nmole/h/mg protein) was one third that in the whole cell homogenate (222 nmole/h/protein) on a per mg protein basis. Most of the nuclear N-SMase was solubilized (71 nmole/mg protein) with 1% Triton X-100.

Morphologic and biochemical characterization of rat liver nuclei

Light microscopic observations revealed that the nuclei preparations were 100% homogeneous and devoid of other cell organelles and whole cells (data not shown). The distribution of marker enzymes in rat liver homogenates and nuclei (Table 1) showed significant activity of enzymes known to serve as markers for various organelles. For example, nuclei preparations were devoid of acid phosphatase and alkaline phosphatase, indicating the absence of lysosomes and plasma membrane. However, the nuclei preparations did contain some NADPH cytochrome C-reductase, succinate dehydrogenase, and galactosyltransferase activity.

Western immunoblot analysis of N-SMase

We found that the monospecific polyclonal antibody raised against N-SMase recognized polypeptides, similar to urinary N-SMase in whole nuclei and nuclei extracted with 1% Triton X-100 (Fig. 2). The apparent molecular weight of N-SMase in rat liver nuclei was on the order of 92 kDa.

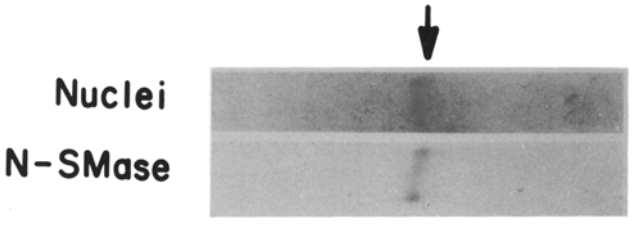

Fig. 2. Western immunoblot analysis of N-SMase in whole rat liver nuclei and urinary N-SMase. For further details see the text and reference 10.

Incorporation of [^{3}H]-thymidine into DNA in regenerating rat liver nuclei

There was a lag in the incorporation of [^{3}H]-thymidine into DNA for up to 10 h after hepatectomy (Fig. 3). Thereafter, there was a marked increase in the incorporation of [^{3}H]-thymidine into DNA that reached a peak level at approxi-

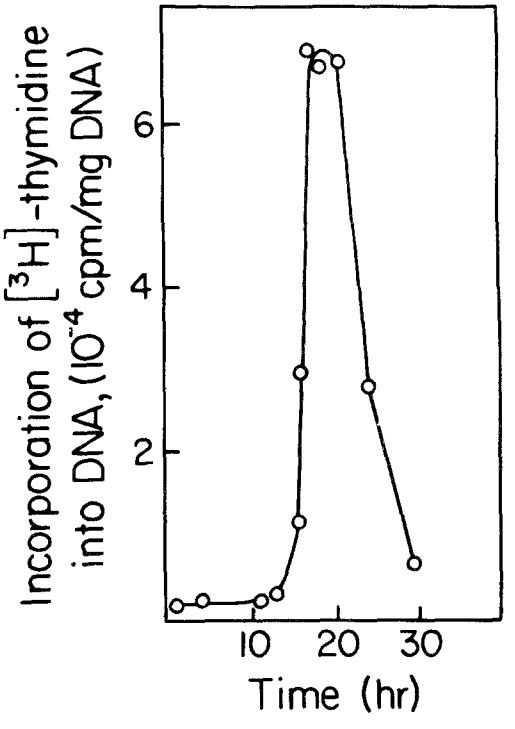

Fig. 3. Incorporation of [^{3}H]-thymidine in regenerating rat liver nuclei. The labeling of newly synthesized DNA in regenerating rat liver was carried out by incorporation of [^{3}H]-thymidine into DNA after injection of 200 uCi of methyl-[^{3}H]-thymidine (specific activity 15 Ci/mmol) per rat for 30 min before decapitation of animals. Specific radioactivity of the incorporated thymidine into DNA was expressed as cpm/mg DNA.

mately 20 h. At this time, about 35-fold increase in the incorporation of [^{3}H]-thymidine into DNA occurred compared to control. This stimulation of [^{3}H]-thymidine into DNA in rat liver nuclei was sustained for an additional 2 h. Then, we observed a decrease in the incorporation of [^{3}H]-thymidine into DNA but, the level was still 15-fold higher than the 10 h time point. Finally, the incorporation of [^{3}H]-thymidine into DNA declined to almost basal levels at 30 h.

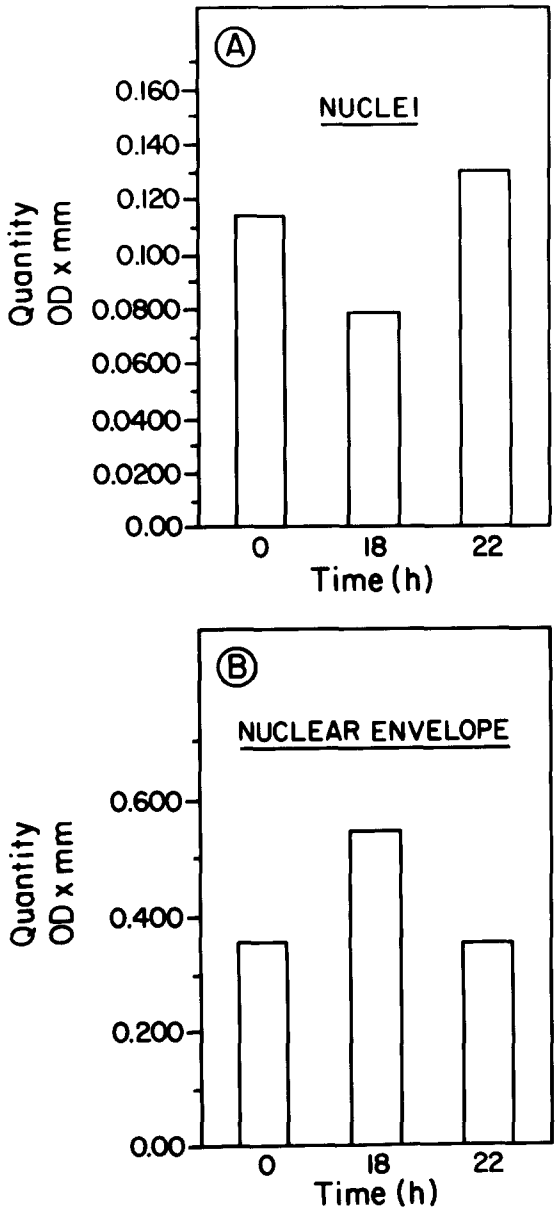

Fig. 4. Level of neutral sphingomyelinase in regenerating rat liver nuclei and nuclear envelope. At the indicated time following sham hepatectomy, liver was excised from the rats and the levels of N-SMase in nuclei and nuclear membrane was quantified employing Western immunoblot assays followed by densitometric scanning. See text for further details.

Distribution of N-SMase in whole nuclei and nuclear envelope in regenerating rat liver

The basal level (zero time) of N-SMase in nuclear envelope was about triple the level in nuclei from control rat liver (Fig. 4A, B). Eighteen hours after hepactectomy, we observed a 50% decrease in the level of N-SMase in whole nuclei (Fig. 4A). In contrast, eighteen hours after surgery the level of N-SMase in nuclear envelope increased about 50% compared to zero time (Fig. 4B). Twenty-two hours after surgery, the level of N-SMase in whole nuclei and nuclear envelope returned to basal levels (Fig. 4A, B).

Determination of the levels of sphingomyelin, ceramides and sphingosine in regenerating liver nuclei during DNA synthesis

Sixteen hours after surgery, the level of sphingomyelin in rat liver nuclei in control (sham operated) animals increased approximately 50%. Thereafter (up to 22 h), the level of sphingomyelin did not change appreciably (Table 2). In contrast, at 16 h the level of sphingomyelin in the experimental group of rats was about 43% lower than the level in the control group, and it remained lower up to 18 h. After 18 h, the levels of sphingomyelin in experimental animals were higher than corresponding control values by 30–56% and more than 200% higher than the animals in the experimental group (16 h time point). Increases in the level of ceramide in regenerating rat liver nuclei followed the same pattern as increases in sphingomyelin levels, but on a smaller scale. During the 20–24 h period, ceramide levels increased 24–55% compared to corresponding control. A rapid increase (150%) in the level of sphingosine was observed in the control group sixteen hours after sham hepactectomy. Thereafter (20–24 h after sham hepactectomy), the level of sphingosine was similar to the zero hour time point. However, in regenerating rat liver, the sphingosine content in nuclei was increased at least 200% compared to the corresponding control value. Twenty-four hours after surgery, the experimental group of rat liver nuclei contained about 360% higher than the corresponding control value.

Discussion

The major findings reported in this paper are: (1) neutral sphingomyelinase was found predominantly associated with the nuclear envelope; and (2) whole nuclei from regenerating rat liver contained significant amounts of sphingomyelin, ceramide and sphingosine rose in tandem with increases in N-SMase.

Since N-SMase is predominantly associated with the

Table 2. Levels of sphingomyelin, ceramide and sphingosine in regenerating rat liver nuclei. Homogenate and nuclei preparations were subjected to extraction with organic solvents and processed for the measurement of lipid levels as described in 'Materials and methods'

Time after operation	Sphingomyelin (ug/mg protein of nuclei)		Ceramide (ug/mg nuclear protein)		Sphingosine (ng/mg nuclear protein)	
Hours	Control	Experimental	Control	Experimental	Control	Experimental
0	1.7 ± 0.4	–	0.77 ± 0.04	–	4.6 ± 0.7	–
16	2.6 ± 0.3	1.5 ± 0.36	0.95 ± 0.11	0.89 ± 0.05	7.1 ± 0.7	13.0 ± 1.4
18	N.D.	2.2 ± 0.32	N.D.	0.90 ± 0.1	4.6 ± 0.6	N.D.
20	2.7 ± 0.5	3.5 ± 0.5	0.93 ± 0.1	1.16 ± 0.11	N.D.	N.D.
22	2.5 ± 0.4	3.9 ± 0.4	0.77 ± 0.06	1.2 ± 0.11	N.D.	N.D.
24	N.D.	3.0 ± 0.5	N.D.	1.18 ± 0.10	5.3 ± 0.8	19.5 ± 3.6

plasma membrane [1, 2], we established employing morphological and biochemical criteria that our nuclear preparations were free of such organelles. In that, the nuclei preparations were free from other cellular organelles and contained marginal levels of 5′nucleotidase and galactosyltransferase that have been shown to be enzyme markers for plasma membrane and Golgi apparatus, respectively.

The nuclear N-SMase had physical-chemical and immunochemical properties similar to N-SMase from various sources [2, 10]. For example, studies determining the pH optima is in agreement with similar studies in a variety of cellular systems [1, 2]. Second, Western immunoblot assays revealed that antibody against N-SMase recognized a protein band having an apparent Mr on the order of 92 kDa. This finding agrees with our previous work showing that the human kidney, urinary N-SMase, and rabbit skeletal muscle[2] also have an apparent Mr on the order of 92 kDa [1, 10].

We found a possible relationship between the distribution of N-SMase in the nuclear envelope and the whole nuclei. Namely, we found that in regenerating rat liver (proliferating hepatocytes) 16 h after hepatectomy, the N-SMase levels in the whole nuclear envelope increased 50% compared to the control group. This increase was accompanied by a decrease in the whole nuclear levels of sphingomyelin. Ceramide, newly released due to N-SMase action, may have been rapidly catabolized to sphingosine via a ceramidase, and therefore, the level of ceramide in nuclei would not have changed in the experimental group of rat liver nuclei during 16–18 h post hepatectomy compared to control. Subsequently, however, the levels of ceramide in the experimental group were higher than the control group.

A correlation between increased masses of N-SMase with increased DNA synthesis was found as evidenced by an increase in the incorporation of [^{3}H]-thymidine into DNA in nuclei. The relevance of this finding is not clear from our study; however, we may speculate that increased activity of N-SMase may be required to produce ceramide and sphingosine. These compounds, in turn, may carry out a myriad of reactions involved in the signal transduction pathways, e.g., involving tumor necrosis factor-α [12–15]. Such ceramides may also be targeted towards regulating proteins such as, ceramide activated protein kinase [13] and/or ceramide dependent phosphatases [6]. Modulation of such regulating proteins may trigger a cascade of reactions that ultimately determine the regeneration of the liver. On the other hand, sphingosine, a potent inhibitor of protein kinase C may in turn, initiate programmed cell death or apoptosis, a cytotoxic signal that may cause liver degeneration. The role of N-SMase in the signal transduction pathway in regenerating liver merits further investigation.

Acknowledgements

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