Effect of saposins on acid sphingomyelinase

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The effect of saposins (A, B, C and D) on acid sphingomyelinase activity was determined using a crude human kidney sphingomyelinase preparation and a purified sphingomyelinase preparation from human placenta. Saposin D stimulated the activity of the crude enzyme by increasing its apparent K_m and $V_{max.}$ values for sphingomyelin hydrolysis. Unlike the crude enzyme, the activity of the purified enzyme was strongly inhibited by saposin D as well as other saposins. Saposin D decreased the apparent K_m and $V_{max.}$ values of purified sphingomyelinase activity. The effects of saposin D on the activity of different sphingomyelinase preparations appear to depend on Triton X-100, which is present in the crude enzyme but not in the purified enzyme. When the detergent was removed from the crude preparation, the effect of saposin D changed from being stimulatory to inhibitory. Conversely, when the detergent is added to the purified enzyme, the effect of saposin D on sphingomyelinase activity changed from being inhibitory to stimulatory. While other saposins were inhibitory or had no effect on sphingomyelinase activity in the above assay system, not only saposin D but also saposins A and C exhibited a stimulatory effect upon purified sphingomyelinase activity when the substrate, sphingomyelin, was added in the form of liposomes without detergent. Saposin B was not only inhibitory in the liposome system, but also reduced the stimulatory effect of saposins A, C and D. These observations indicate that the stimulatory effect of saposins A, C and D on acid sphingomyelinase activity is greatly influenced by the physical environment of the enzyme and suggest that similar effects by saposins may be exerted in lysosomal membranes.

INTRODUCTION

Sphingomyelin is a major lipid constituent of animal cell membranes, including plasma membranes, myelin sheath and erythrocyte stroma. It is degraded in lysosomes by acid sphingomyelinase (sphingomyelin phosphodiesterase; EC 3.1.4.12) yielding phosphorylcholine and ceramide. Abnormalities in sphingomyelin metabolism have been found in atherosclerosis, cancer and a genetically transmitted disease, namely Niemann–Pick disease [1]. With the exception of Niemann–Pick disease, the underlying causes of these abnormalities are not known. Niemann–Pick disease is a group of inherited metabolic diseases, which are characterized by abnormal accumulation of sphingomyelin in patients' tissues. Several types of this disease, classified according to clinical, pathological, and biochemical criteria, are known. In the most recent classification, Spence and Callahan [2] separated this group of diseases into two types. Type I includes three subgroups all of which are caused by sphingomyelinase deficiency. Type II also includes three subgroups in which the primary defect is not known.

All saposins (A, B, C and D) are produced by partial proteolysis of a single precursor protein, prosaposin, and are involved in lysosomal hydrolysis of many sphingolipids [3]. We have previously shown that saposin D stimulates acid sphingomyelinase activity of a human placenta preparation [4] and that abnormal accumulation of saposin D, and other saposins to a lesser extent, occurs in tissues of patients with Niemann–Pick disease [5]. Stimulation of sphingomyelinase by a saposin C preparation has been reported previously [6–8], but possible contamination of the preparations involved by saposin D was not excluded. These findings suggest that saposin D may be involved in the mechanism of this genetic disease, especially in the Type II phenotype, in which patients have apparently normal *in vitro* sphingomyelinase activity.

In addition to its clinical importance, saposin D may also be involved in controlling sphingomyelin turnover in animal tissues. A significant link between sphingomyelin turnover and protein kinase C has been suggested [1,9]. Since saposin D may be involved in controlling sphingomyelinase activity in tissues, it may be playing a significant role in producing important secondary messengers, namely ceramide, sphingosine, and choline, the compounds produced by the hydrolysis of sphingomyelin. The present investigation was undertaken to further characterize the stimulatory effect of saposin D and the other saposins on sphingomyelinase activity in order to understand its role in sphingomyelin metabolism in tissues.

MATERIALS AND METHODS

Sphingomyelinase assay with sphingomyelin in micelles

The assay was performed as described previously using [*choline-methyl*-^{14}C]sphingomyelin (830 c.p.m./nmol) as the substrate [4]. Additional Triton X-100 (Calbiochem) was added when needed, as described in specific experiments. A Triton X-100 extract of a particulate fraction of human kidney, prepared as described previously [4], was used as the crude enzyme preparation. A purified acid sphingomyelinase preparation was prepared from human placenta as described previously [10]. As estimated from SDS/PAGE, this preparation was approx. 50% pure. The enzyme solution (0.12 mg of protein/ml) was diluted before use with 20 vol. of 0.1 M sodium acetate buffer, pH 5.0, containing 10 mg/ml BSA (Sigma Chemical Co.).

Abbreviation used: ConA, concanavalin A.

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Sphingomyelinase assay with sphingomyelin in liposomes

The assay was done essentially as described by Poulos et al. [11]. Briefly, 0.5 mg of sphingomyelin containing 1×10^7 c.p.m. was sonicated with 2 ml of water in a bath-type sonicator and the nearly clear solution was used as substrate. The assay mixture contained 20 μl of the above substrate solution, 50 μl of 1 M sodium acetate buffer, pH 4.5, saposins, and the purified sphingomyelinase in a total volume of 100 μl. After shaking at 37 °C for 2 h, 0.3 ml of water and 1.5 ml of chloroform/methanol (2:1, v/v) were added, mixed, and centrifuged. This assay mixture did not contain Triton X-100. An aliquot (0.5 ml) of the upper (aqueous) layer was counted for radioactivity.

Saposins

Saposins A, C, and D were isolated from Gaucher spleen as described previously [4,5]. Saposin B was also purified from the same tissue by a slightly modified procedure (M. Hiraiwa, S. Soeda, Y. Kishimoto and J. S. O'Brien, unpublished work). Each purified saposin gave a single band after SDS/PAGE and showed no cross-contamination upon immunochemical examinations.

Other analyses

Determination of protein concentration and SDS/PAGE as well as Western-blot analysis were performed as described previously [4].

RESULTS AND DISCUSSION

In the presence of Triton X-100 sphingomyelinase activity of the human kidney preparation at pH 5.0 increased linearly as a function of added enzyme up to 60 μg of protein. With 15 μg of crude-extract protein, the activity increased linearly during incubation over a 3-h period. Under identical conditions, activity of the purified human placental sphingomyelinase preparation increased linearly with the amount of enzyme added up to 120 ng of protein. With 60 ng of purified enzyme protein, the activity increased linearly during a 3-h incubation period. In accordance with these observations, subsequent experiments were carried out with either the crude enzyme preparation containing 15 μg of protein and 36 μg of Triton X-100, or the purified enzyme preparation containing 60 ng of protein and 100 μg of BSA without Triton X-100, unless otherwise specified.

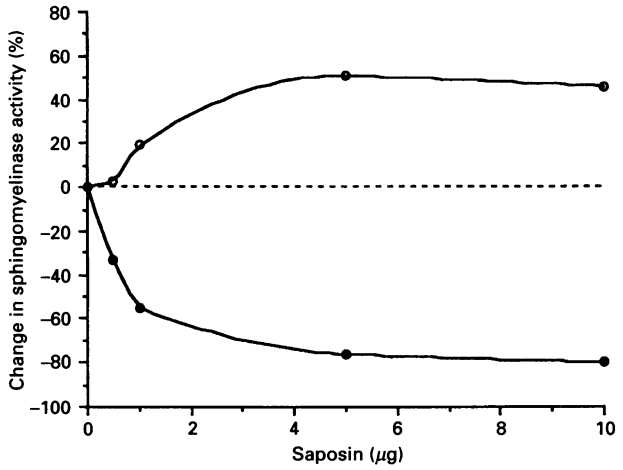

Figure 1 Effect of saposin D on sphingomyelinase activity of a crude preparation from human kidney (○) or purified sphingomyelinase (●)

Assay mixture contained 200 μM [*methyl*-^{14}C]sphingomyelin, containing 16200 c.p.m., 10 μg of Triton X-100, 50 μmol of sodium acetate buffer, pH 5.0, and a human kidney preparation containing 15 μg of protein and 36 μg of Triton X-100 or purified enzyme containing 60 ng of protein and 100 μg of BSA but no Triton X-100, with various amounts of saposin D in a total volume of 100 μl. Incubation was carried out at 37 °C for 2 h. The broken line denotes sphingomyelinase activity in the absence of added saposins.

Consistent with the previous observations using the crude human placental preparation [4], addition of saposin D stimulated acid sphingomyelinase activity in the crude preparation from human kidney (Figure 1). The results of kinetic studies indicate that saposin D increases both the apparent K_m value for sphingomyelin, from 0.05 mM to 0.5 mM, and the $V_{max.}$ value, from 0.1 nmol/h to 1.0 nmol/h.

Unlike the crude enzyme preparations, saposin D strongly inhibited the sphingomyelinase activity of the highly purified enzyme preparation from human placenta (Figure 1). A level of 50% inhibition was achieved with less than 1 μg of saposin D (0.001% saposin D in the incubation mixture). Kinetic studies showed that saposin D decreases the apparent K_m value for the substrate from 0.4 mM to 0.06 mM, while the $V_{max.}$ value decreases from 50 nmol/h to 0.8 nmol/h.

To understand better the observed difference in the effect of saposin D on sphingomyelinase activity in the crude versus the purified enzyme preparation, the crude enzyme was purified one step further by concanavalin A (ConA)–Sepharose-column chromatography as described previously [12]. Saposin D (10 μg) inhibited 90% of the activity retained by the ConA column (results not shown). In a separate experiment, the crude enzyme preparation was heated in boiling water for 5 min and then centrifuged. When the clear supernatant was added, together with 5 μg of saposin D, to either the crude enzyme preparation or to the purified enzyme, the sphingomyelinase activity increased by 56% and 80% respectively. These observations suggest that there is a heat-stable component in the crude preparation that changes the effect of saposin D on sphingomyelinase activity from inhibitory to stimulatory. This heat-stable component may be the Triton X-100 included in the crude sphingomyelinase preparation, as demonstrated by the following experiments.

One of the significant differences between the crude and purified enzyme preparations is the presence of Triton X-100. While 10 μl of the crude preparation (the amount routinely used for each assay) contained about 36 μg of Triton X-100, the purified preparation did not contain detergent. Therefore, we tested the effect of various concentrations of this detergent on the sphingomyelinase assay. The activity of both crude and purified sphingomyelinase in the absence of saposin D was highly dependent upon the amount of Triton X-100 added (Figure 2). When 5 μg of saposin D/assay was added to the crude enzyme preparation, the degree of stimulation (the ratio of activity in the presence and absence of saposin D) decreased gradually with increasing Triton X-100 concentration (Figure 3). Conversely, as described above, 5 μg of saposin D inhibited sphingomyelinase activity in the presence of low concentrations of Triton X-100. The inhibition was maximal at 10 μg of Triton X-100/assay. The degree of inhibition rapidly decreased with further increases of the detergent concentration. The effect of saposin D changed to slightly stimulatory at levels of 40 μg of Triton X-100/assay and higher.

Since the effect of 5 μg of saposin D/assay on purified enzyme was changed from inhibitory to stimulatory by the addition of 40 μg of Triton X-100/assay, i.e. approx. the same concentration that is present in the crude enzyme assay system, the effect of

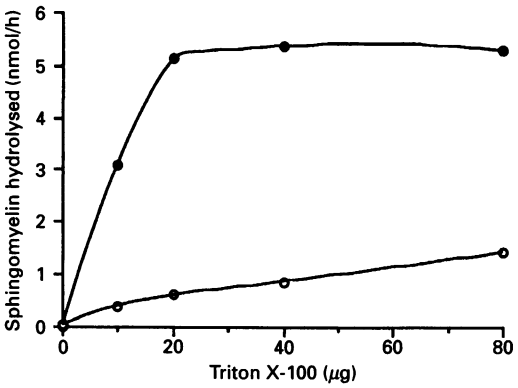

Figure 2 Effect of Triton X-100 concentration on sphingomyelinase activity of crude enzyme preparation (○) or purified enzyme preparation (●)

Assay was conducted as described in the legend to Figure 1. No saposins were added. The amount of Triton X-100 indicated is in addition to that already present in the crude enzyme preparation (36 μg).

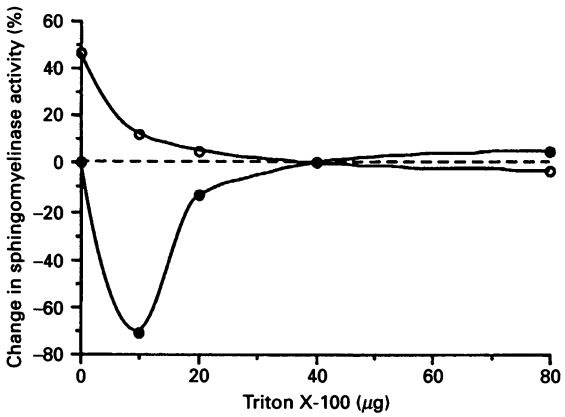

Figure 3 Effect of saposin D on assay mixture containing various amounts of Triton X-100

Five μg of saposin D was added to the assay mixture described for Figure 4. Results are plotted as the percentage change in sphingomyelinase activity. The broken line denotes sphingomyelinase activity without addition of saposins at the given Triton X-100 concentration. Key to symbols: (○), with crude sphingomyelinase; (●), with purified sphingomyelinase.

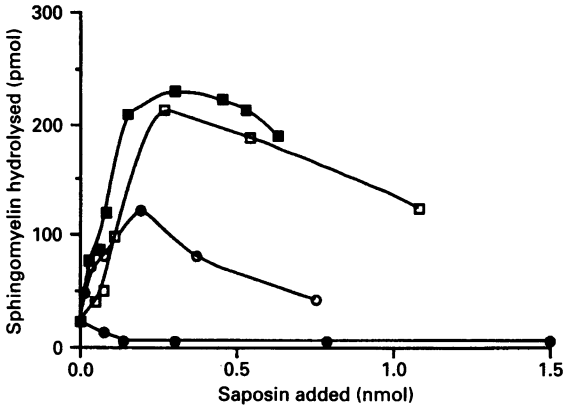

Figure 4 Effect of various amounts each of saposin A (○), B (●), C (□), and D (■) on sphingomyelinase activity in the liposome system

Various amounts of each saposin were added to the assay mixture containing a purified enzyme preparation and [^{14}C]sphingomyelin in liposomes. See the Materials and methods section for assay conditions.

Table 1 Effect of saposin B on stimulation of sphingomyelinase activity by other saposins

Assays were conducted by using purified sphingomyelinase and liposomal [^{14}C]sphingomyelin as described in the text. Amounts of saposins added were: saposins A and C, 0.25 nmol each; saposin B, 0.5 nmol; and saposin D, 0.2 nmol.

Addition	Sphingomyelinase activity (pmol/h)
None	22
Saposin B	7
Saposin A	34
Saposin A + saposin B	11
Saposin C	70
Saposin C + saposin B	15
Saposin D	53
Saposin D + saposin B	35

varying the amount of saposin D on the purified enzyme activity in the presence of this concentration of Triton X-100 was tested. Under these conditions, the addition of saposin D increased the enzyme activity at levels up to 1 μg/assay and then the degree of stimulation decreased slowly with increasing saposin D concentrations.

Since Triton X-100 variably affected the saposin D effect, the activity of sphingomyelinase was investigated by using the substrate in liposomes which contained no detergent as described by Poulos et al. [11]. The activity was increased by the addition of saposin D reaching a maximum (10-fold higher than that of the control) at a concentration of 3 μM and then declined, as shown in Figure 4. Unexpectedly, saposin C was as effective as saposin D for sphingomyelinase stimulation and saposin A was about half as effective. Interestingly, saposin B showed strong inhibition (> 70%) of enzyme activity. Kinetic studies (results not shown) demonstrated that the apparent K_m value for sphingomyelin was decreased by 3 μg of saposin D from 200 μM (the control value) to 100 μM, while the $V_{max.}$ value was increased from 50 pmol/h to 286 pmol/h. Similarly, the same amounts of saposin A and saposin C decrease the apparent K_m values to 100 μM and 45 μM, and $V_{max.}$ values to 72 and 182 pmol/h respectively. The stimulative effect of saposin A, C and D was counteracted by saposin B as shown in Table 1.

Acid sphingomyelinase is a glycoprotein strongly bound to the lysosomal membrane [2]. Its activity *in vitro* is highly dependent on the addition of a detergent or phospholipids, indicating that the membrane form is essential to exert its enzymic activity. The present investigation demonstrates that the sphingomyelinase activity *in vitro* is activated or inhibited by not only saposin D, as shown in our previous publication [4], but also by the other saposins A, B and C depending upon the composition of the micelle or liposome. We found that saposin D was inhibitory at low concentrations of Triton X-100 but became stimulatory at 0.04% or higher Triton X-100 concentrations. Under these conditions, other saposins (A, B, and C) were either inhibitory or had no effect on sphingomyelinase activity. On the other hand, when the substrate was presented in the form of liposomes without Triton X-100, all saposins, except saposin B, stimulated the sphingomyelinase activity to a significant degree. These observations suggest that similar modulation of sphingo-

myelinase activity may be occurring in tissues. Whether these saposins stimulate the spingomyelinase activity by interacting with the enzyme or substrate, or by simply changing micellar or liposomal nature, is to be investigated.

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