Actinators of Spleen Glucocerebrosidase from Controls and Patients with Various Forms of Gaucher’s Disease*

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Glucocerebrosidase from normal human spleen, and spleen from cases of neurologic (types 2 and 3) and nonneurologic (type 1) Gaucher’s disease, was delipidated and inactivated by extraction from membranes with sodium cholate and ice-cold 1-butanol. Control glucocerebrosidase was stimulated markedly by large quantities (20–30 µg/assay) of phosphatidylserine (PS), or by a combination of smaller amounts (1–2 µg) of PS and 3 µg of a heat-stable factor (HSF) derived from the spleen of a patient with Gaucher’s disease. The residual glucocerebrosidase from a nonneurologic case, but not a neurologic case, was also responsive to PS and HSF. The combination of HSF and PS decreased the Kₘ of the normal enzyme for 4-methylumbelliferyl-β-D-glucopyranoside from 8.0 to 1.6 mM. These effectors also increased the reactivity of glucocerebrosidase to the inhibitor conduritol B epoxide; HSF alone had no effect (t₁/₂ = 19 ± 0.5 min) whereas the maximum rate of inactivation (t₁/₂ = 4.0 min) by conduritol B epoxide was achieved in the presence of a mixture of PS (1 µg) and HSF (3 µg).

Phosphatidylglycerol (PG) and phosphatidic acid, also acidic phospholipids, were effective activators of glucocerebrosidase. Varying the fatty acid composition of PG had little effect on its ability to stimulate glucocerebrosidase activity. However, in the case of phosphatidylycholine (PC), a weaker activator than PG or PS, fatty acid composition had a significant impact on the activity of this neutral lipid to activate glucocerebrosidase; dilinoleoyl-PC and dicaproyl-PC were almost totally inactive. The mono-, and di-, and trisialogangliosides (GM₁, GDI, and GT₁) were less than half as effective as PS as activators of glucocerebrosidase. These results indicate that acidic phospholipids and the heat-stable factor may both play a role in explaining the genetic heterogeneity of Gaucher’s disease.

Gaucher’s disease presents most often in a benign form (type 1) that involves visceral organs, but spares the central nervous system. Much less frequently, this autosomal recessive glycosphingolipidosis appears as a devastating neurologic disease with extensive central nervous system involvement resulting in death, usually before 2 years of age (type 2) or as a chronic disorder with progressive neurologic dysfunction, in which case death occurs during early adulthood (type 3) (1, 2).

The biochemical defect common to all forms of Gaucher’s disease is a profound, but rarely absolute, deficiency of the activity of lysosomal glucocerebrosidase (glucosylceramide gluchohydrolase, EC 3.2.1.45), the β-glucosidase responsible for catalyzing the hydrolysis of glucocerebroside to ceramide and glucose (3, 4).

A puzzling aspect of Gaucher’s disease is that estimations of residual glucocerebrosidase activity, measured in vitro in the presence of some bile salt, do not correlate with clinical severity or disease course (5, 6). Although it was shown nearly 10 years ago that glucocerebrosidase from control spleen or spleen of a patient with type 1 disease could be activated markedly by acidic phospholipids (e.g. phosphatidylserine), few comparative studies describing the activator lipid requirement of glucocerebrosidase from cases of neurologic and nonneurologic Gaucher’s disease have addressed this question (7–9). In fact, nearly all reports dealing with the enzymology of Gaucher’s disease published in the last decade have involved glucocerebrosidase assays in which, regardless of whether the glucocerebrosidase substrate was the natural one (glucocerebroside), or a nonphysiologic arylglucoside (4-methylumbelliferyl-β-D-glucopyranoside), the activator lipid was a bile salt such as sodium taurocholate (11–13) or sodium taurodeoxycholate (14).

The first evidence of a biochemical distinction between neurologic and nonneurologic cases of Gaucher’s disease was provided recently by Wenger and Roth (15). They demonstrated that β-glucosidase assays supplemented with a mixture of PS, Triton X-100, and partially purified HSF (16) activated β-glucosidase in crude extracts of leukocytes and fibroblasts from controls and a case of type 1 Gaucher’s disease, but not in an extract of the same kind of cells from a single case of neurologic Gaucher’s disease. In that report, the authors did not address the relative importance of the HSF and acidic phospholipid to the successful use of the β-glucosidase assay for the biochemical differentiation of neurologic and nonneurologic Gaucher’s disease cases.

Using butanol-delipidated glucocerebrosidase preparations from the spleens of 11 cases of Gaucher’s disease, Glew et al. (6) recently found that a fluorometric β-glucosidase assay which relied solely upon PS as the exogenous activator was capable of distinguishing cases of type 1 Gaucher’s disease from type 3 cases.

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1 The abbreviations used are: PS, phosphatidylserine; HSF, heat-stable factor; lyoPS, lyso phosphatidylserine; NAPS, N-acetylphosphatidylserine; PG, phosphatidylglycerol; PA, phosphatidic acid; PC, phosphatidylycholine; GM₁, monosialoganglioside; GD₁, disialoganglioside; GT₁, trisialoganglioside; CBE, conduritol B epoxide; MUGlc, 4-methylumbelliferyl-β-D-glucopyranoside.
from type 2 and type 3 cases with extensive disease of the central nervous system. In that report, they established the validity of using the fluorometric $\beta$-glucosidase assay to specifically measure glucocerebrosidase activity which had been solubilized from membranes with sodium cholate and 1-butanol.

In an effort to learn more about the specificity of the lipid requirement for reconstitution of activity to delipidated glucocerebrosidase from control tissues, and tissues from neurologic and nonneurologic Gaucher's disease cases, we compared the glucocerebrosidase activator potency of various phospholipids, gangliosides, and the heat-stable factor.

**EXPERIMENTAL PROCEDURES AND RESULTS**

**DISCUSSION**

We have extended previous studies of the lipid requirement of glucocerebrosidase (6, 7). In the present study, we delipidated the enzyme by extracting spleen membranes with cholate and 1-butanol to render it sensitive to exogenous lipid activators. The fact that repeated extraction with 1-butanol does not render glucocerebrosidase activity absolutely dependent upon exogenous lipid could be due to the presence of endogenous lipids that resist butanol extraction.

The results of the present study of the residual glucocerebrosidase in neurologic and nonneurologic Gaucher's disease support our earlier findings (6); that is, residual enzyme in a case of nonneurologic Gaucher’s disease is stimulated markedly by PS, whereas residual glucocerebrosidase in neurologic cases is relatively unresponsive to this acidic phospholipid (Fig. 1). Thus, a major distinction between neurologic and nonneurologic cases of Gaucher’s disease may be the ability of the residual glucocerebrosidase of the latter to be stimulated by acidic phospholipids like phosphatidylserine.

Members of another class of acidic lipids, namely gangliosides, were only half as effective as PS in restoring activity to glucocerebrosidase from control spleen. This observation indicates that the presence of negative charge alone is not sufficient to confer upon a natural lipid the ability to activate glucocerebrosidase. Furthermore, gangliosides are much less efficient than PS in differentiating tissues from neurologic and nonneurologic cases of Gaucher’s disease.

Another way to distinguish subtypes of Gaucher’s disease has been suggested by Ginns et al. (30) who postulate that the mutations in nonneurologic and neurologic cases affect the processing of a precursor to the mature form of glucocerebrosidase differently and that the altered glucocerebrosidase in tissues of patients with different forms of Gaucher’s disease can be differentiated from each other by electrophoretic blotting. It remains to be seen whether the differential responsiveness of residual glucocerebrosidase activity in various subtypes of Gaucher’s disease to acidic phospholipids can also be explained by differences in enzyme processing.

With regard to specificity, the phospholipids that are most effective in activating the glucocerebrosidase from control tissue are those with an acidic character, namely PS > PG > PA. Blocking the amino acid group of PS with a long fatty acid, as in NAPS (Table I), results in nearly complete loss of its stimulatory potential. These results suggest that the polar head group influences the ability of a particular phospholipid to stimulate glucocerebrosidase activity. In the case of a neutral phospholipid, such as PC, fatty acid composition also has a large effect on the phospholipid's ability to activate glucocerebrosidase (Fig. 2). In sharp contrast, when the polar head group carries a net negative charge, as in the case for PG (Fig. 3), fatty acid composition has little effect on glucocerebrosidase activation potential. It may be that the polar head group determines primarily the degree to which the fatty acids in the sn-1 and sn-2 positions influence a phospholipid’s effectiveness as a glucocerebrosidase activator.

With regard to the HSF, our results indicate that this low molecular weight glycoprotein activator of glucocerebrosidase acts primarily by increasing the sensitivity of the enzyme to exogenous lipid activators, particularly PS, and HSF alone is only a weak activator of glucocerebrosidase and, in the absence of PS, relatively large amounts of the factor are required to produce even the slightest degree of stimulation of enzyme activity (Fig. 5). Berent and Radin (31) came to the same conclusions about a similar HSF they isolated from bovine spleen. This conclusion is supported by the results of the experiment (Fig. 8 and Table III) in which we observed that the HSF alone was incapable of increasing the reactivity of the active site of glucocerebrosidase to CBE. It seems that the HSF acts as a co-activator; thus, Berent and Radin’s use of the term “coglucosidase” (31, 32) may be appropriate. These observations lead us to conclude that the acidic phospholipid is the dominant component of the PS:HSF activator pair used by Wenger and Roth (15) to optimize assay conditions that distinguish the residual $\beta$-glucosidase from cases of neurologic and nonneurologic Gaucher’s disease.

The physiologic significance of the HSF was questioned by us (33) and others (34, 35). One reason for considering it to be physiologically irrelevant was that large amounts of HSF were needed to cause moderate stimulation of the glucocerebrosidase preparations in use at that time (33). Using butanol-delipidated preparations of glucocerebrosidase, we now show that in the presence of small amount of PS only submicrogram quantities of HSF are needed to stimulate glucocerebrosidase activity. Hirabayashi et al. (36) have recently reported that a similar type of heat-stable activator protein is indeed essential for the metabolism of the ganglioside GM2. In light of our revised optimized assay conditions, and our use of cholate- and butanol-extracted glucocerebrosidase as a source of enzyme, the physiologic significance of HSF warrants reconsideration. Also, because a nonphysiologic $\beta$-glucosidase substrate was used in the present study, the physiologic relevance of our glucocerebrosidase activator studies remains a question to be addressed when someone identifies a detergent that disperses glucocerebroside substrate without interfering with the ability of phospholipids and HSF to activate glucocerebrosidase.

The results of the present study should stimulate further studies of the lipid requirement of glucocerebrosidase as well as inquiry into the mode of action and structure of complexes formed between glucocerebrosidase, acidic lipids, and HSF. The results of such investigations could provide insight into the structural and kinetic differences between the mutant glucocerebrosidases that occur in cases of neurologic and nonneurologic Gaucher’s disease.

**REFERENCES**


Supplementary Material to Activators of Spleen Glucocerebrosidase

ACTIVATORS OF SPLEEN GLUCOCEREBROSIDASE FROM CONTROLS AND PATIENTS WITH VARIOUS FORMS OF GAUCHER'S DISEASE

ALANKANANDA BASU, ROBERT F. GLEM, LIDIA R. DANIELS, AND LINDA S. CLARK

EXPERIMENTAL PROCEDURE

Materials - Acetyl-ethylphosphoryl-choline, lyso-PC (18:0, 18:1, 18:2), lyso-PE (18:0, 18:1, 18:2), lyso-PS (18:0, 18:1, 18:2), and lyso-PS (18:0, 18:1, 18:2) were purchased from Sigma Chemical Co., St. Louis, MO. Boston Collaborative Co., Lexington, MA, and Generi Chemicals, Inc., Burlington, MA. 

Enzyme preparations - Glucocerebrosidase from control spleen and type I Gaucher's disease was generously provided by Dr. Elizabeth Morrison of the Department of Medicine, University of Michigan, Ann Arbor, MI. Type II and type III Gaucher's disease were provided by Dr. Robert H. Uditsky of the Department of Medicine, University of Illinois, Chicago, IL. 

Tissue samples - Tissue samples were obtained (with the consent of the patients) by surgical biopsy of 4 patients with type I Gaucher's disease and of 12 patients with type II or type III Gaucher's disease for the preparation of enzyme. The glucocerebrosidase was stored at -70°C until assay. 

Gangliosides - Gangliosides from type II and type III Gaucher's disease were isolated from fresh and frozen spleen of the cases by the method of Brown and associates (13). These gangliosides confirmed the results of the previous study (13) and were not stimulated by PS.

RESULTS

Comparative assay of activators from type I Gaucher's disease and controls - The results of these studies are summarized in Table 1. The results clearly show that activators are effective in the enzyme from type II and type III Gaucher's disease but not in the enzyme from type I Gaucher's disease, and that the activators are not present in the enzyme from type I Gaucher's disease.

Comparison of the effects of purified activators from type I and II Gaucher's disease and controls - The results of these studies are summarized in Table 2. The results show that the activators in the enzyme from type I Gaucher's disease are not as effective as the activators in the enzyme from type II Gaucher's disease and controls.

Comparative assay of activators from controls and patients with various forms of Gaucher's disease - The results of these studies are summarized in Table 3. The results show that activators are effective in the enzyme from all patients with Gaucher's disease, and that the activators are not present in the enzyme from type I Gaucher's disease.

Comparison of the effects of activators from controls and patients with various forms of Gaucher's disease - The results of these studies are summarized in Table 4. The results show that activators are effective in the enzyme from all patients with Gaucher's disease, and that the activators are not present in the enzyme from type I Gaucher's disease.

The activators in the enzyme from type II and type III Gaucher's disease are not as effective as the activators in the enzyme from type I Gaucher's disease and controls.

The activators in the enzyme from type I Gaucher's disease are not as effective as the activators in the enzyme from type II and type III Gaucher's disease and controls.

The activators in the enzyme from type I Gaucher's disease are not as effective as the activators in the enzyme from type II and type III Gaucher's disease and controls.

The activators in the enzyme from type I Gaucher's disease are not as effective as the activators in the enzyme from type II and type III Gaucher's disease and controls.

The activators in the enzyme from type I Gaucher's disease are not as effective as the activators in the enzyme from type II and type III Gaucher's disease and controls.

The activators in the enzyme from type I Gaucher's disease are not as effective as the activators in the enzyme from type II and type III Gaucher's disease and controls.

The activators in the enzyme from type I Gaucher's disease are not as effective as the activators in the enzyme from type II and type III Gaucher's disease and controls.

The activators in the enzyme from type I Gaucher's disease are not as effective as the activators in the enzyme from type II and type III Gaucher's disease and controls.

The activators in the enzyme from type I Gaucher's disease are not as effective as the activators in the enzyme from type II and type III Gaucher's disease and controls.

The activators in the enzyme from type I Gaucher's disease are not as effective as the activators in the enzyme from type II and type III Gaucher's disease and controls.

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The activators in the enzyme from type I Gaucher's disease are not as effective as the activators in the enzyme from type II and type III Gaucher's disease and controls.
Addition 

<table>
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<th>Specific activity (units per mg protein)</th>
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<th>Type 2</th>
<th>Type 3</th>
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Table I

The effect of sodium taurocholate, phosphatidylserine, lysophosphatidylserine and N-acylphosphatidylserine on the activity of spleen glucocerebrosidase from a control and patients with type 1, type 2 and type 3 Gaucher's disease.

Table II

The effect of phosphatidylserine and heat-stable factor, separately and in combination, on bacterial-extracted spleen membrane glucocerebrosidase from a control and patients with nonneurologic (type 1) and neurologic (type 2) Gaucher's disease.

Addition 

<table>
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<tr>
<th>Specific activity (units per mg protein)</th>
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<th>Type 2</th>
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<tr>
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<td>10.7</td>
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HSF, heat-stable factor; PS, phosphatidylserine

The effect of phosphatidylserine and heat-stable factor on $k_{m}$, Vmax and the rate of inactivation of control spleen glucocerebrosidase by conduritol B epoxide.

Table III

<table>
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<th>Effector</th>
<th>$k_{m}$ (mM)</th>
<th>Vmax (units/mg)</th>
<th>Rate of inactivation</th>
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<tr>
<td>None</td>
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<td></td>
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<tr>
<td>PS (10 μg)</td>
<td>4.9</td>
<td>750</td>
<td>0.3</td>
<td>10.5</td>
</tr>
<tr>
<td>PS (10 μg) + HSF (10 μg)</td>
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<td>750</td>
<td>0.3</td>
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<tr>
<td>PS (25 μg)</td>
<td>1.9</td>
<td>870</td>
<td>0.3</td>
<td>10.5</td>
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</table>

HSF, conduritol B epoxide; PS, phosphatidylserine; HSF, heat-stable factor.

1 The number in this column indicates the quantity of effector per 100 μl of preincubation medium.

2 The number in parenthesis indicates the quantity of effector per 100 μl of preincubation medium.

Fig. 1. The effect of phosphatidylserine and various gangliosides on spleen glucocerebrosidase. Spleen glucocerebrosidase from a control (a) and patients with nonneurologic (b) or neurologic (c) Gaucher's disease was partially purified and extracted twice with 1-butanol as described under "Experimental Procedures." The butanol-insoluble fractions were resuspended in 50 mM sodium acetate buffer, pH 5.5, and sodium taurocholate, 1.2 percent (w/v). In the absence of CBE, activity was completely stable for 45 min at 37°C.

Fig. 2. The effect of chain length and degree of unsaturation of bacterial-extracted glucocerebrosidase from a control spleen on the activity of control spleen glucocerebrosidase. Bacterial-extracted glucocerebrosidase from control spleen was prepared and assayed as described under "Experimental Procedures." 18:1, dilauroyl phosphatidylcholine; 18:2, dilinoleoyl phosphatidylcholine; 18:3, trilinoleoyl phosphatidylcholine; 18:3, dilinolenoyl phosphatidylcholine; 18:4, arachidonyl phosphatidylcholine; 18:4, arachidonyl phosphatidylcholine; 18:0, dipalmitoyl phosphatidylcholine; 18:0, dipalmitoyl phosphatidylcholine; 18.0, dipalmitoyl phosphatidylcholine; 18.0, dipalmitoyl phosphatidylcholine; 18.0, dipalmitoyl phosphatidylcholine.
Fig. 3. The effect of chain length and degree of unsaturation of the fatty acids of phosphatidylglycerol on the activity of spleen glucocerebrosidase. Neutral-extracted glucocerebrosidase from control spleen was prepared and assayed as described under "Experimental Procedures." 12:0, disacetyl phosphatidylglycerol; 14:0, dimeristic phosphatidylglycerol; 15:0, dimethylammonium phosphatidylglycerol; 16:0, distearoyl phosphatidylglycerol; 18:1, dilauroyl phosphatidylglycerol; 18:2, dioleoyl phosphatidylglycerol.

Fig. 4. The effect of mixtures of phosphatidylserine and Triton X-100 on control spleen glucocerebrosidase. Spleen glucocerebrosidase from control spleen was assayed as described under "Experimental Procedures" with increasing amounts of phosphatidylserine to which 0.05%, 0.1%, 0.5%, and 1% (w/w) Triton X-100. The insert shows the effect of Triton X-100 concentration on specific activity in the absence (0) or presence (•) of 75 μg phosphatidylserine.

Fig. 5. Comparison of the activation of control spleen glucocerebrosidase by heat-stable factor in the absence or presence of phosphatidylserine (2 μg). Glucocerebroidase activity was measured as described in the legend to Table III in the absence (0) or presence (•) of 2 μg phosphatidylserine in the indicated range of heat-stable factor concentrations.

Fig. 6. Comparison of the activation of control spleen glucocerebrosidase by phosphatidylserine in the absence and presence of heat-stable factor. Fig. 6A, Glucocerebrosidase was assayed as described under "Experimental Procedures" over the indicated range of phosphatidylserine concentration in the absence or presence of 3 μg heat-stable factor. Fig. 6B shows the increase in specific activity as a function of the amount of phosphatidylserine in the assay; the increase in specific activity was obtained by subtracting the activity in the absence of HSF from the activity obtained in the presence of the HSF.

Fig. 7. The effect of heat-stable factor in presence of phosphatidylserine or ganglioside (GPI) on the activity of glucocerebrosidase from control spleen, Gaucher's disease, and adrenal medullary and neuropathic cases of Gaucher's disease. Partially purified spleen glucocerebrosidase preparations isolated from membranes from control (A) and cases of type 1 (B) and type 3 (C) Gaucher's disease were assayed using HUGlcpe as substrate as described under "Experimental Procedures" in the presence of the indicated amount of heat-stable factor together with 2 μg phosphatidylserine (0), 50 μg phosphatidylserine (0), or none (0). The increase in specific activity was plotted versus the amount of heat-stable factor in the assay.

Fig. 8. The effect of heat-stable factor and phosphatidylserine on the rate of inactivation of control spleen glucocerebrosidase by 5.25 mM conductant. This experiment was performed as described in the legend to Table III in the absence (0) or presence (•) of phosphatidylserine (2 μg), (A) phosphatidylserine (3 μg), (B) phosphatidylserine (1 μg), (C) phosphatidylserine (2 μg), and (D) phosphatidylserine (1 μg) plus heat-stable factor (5 μg), where the number in parenthesis indicates the quantity of effector per 100 μg of preincubation medium.

Fig. 9. Comparison of the activation of control spleen glucocerebrosidase by heat-stable factor in the absence or presence of phosphatidylserine (2 μg). Glucocerebrosidase activity was measured as described in the legend to Table III in the absence (0) or presence (•) of 2 μg phosphatidylserine in the indicated range of heat-stable factor concentrations.
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