Assay for the transbilayer distribution of glycolipids: selective oxidation of glucosylceramide to glucuronylceramide by TEMPO nitroxyl radicals

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Abstract **In the present study, 2,2,6,6-tetramethylpiperidinooxy nitroxide (TEMPO) has been applied successfully to discriminate between glucosylceramide in the outer and inner leaflets of closed membrane bilayers. The nitroxyl radicals TEMPO and carboxy-TEMPO, once oxidized to nitrosonium ions, are capable of oxidizing residues that contain primary hydroxyl and amino groups. When applied to radiolabeled glucosylceramide in liposomes, oxidation with TEMPO led to an oxidized product that was easily separated from the original lipid by thin-layer chromatography, and that was identified by mass spectrometric analysis as the corresponding acid glucuronylceramide. To test whether oxidation was confined to the external leaflet, TEMPO was applied to large unilamellar vesicles (LUVs) consisting of egg phosphatidylcholine–egg phosphatidylethanolamine–cholesterol 55:5:40 (mol/mol). TEMPO oxidized most radiolabeled phosphatidylethanolamine, whereas carboxy-TEMPO oxidized only half. Hydrolysis by phospholipase A_2 confirmed that 50% of the phosphatidylethanolamine was accessible in the external bilayer leaflet, suggesting that TEMPO penetrated the lipid bilayer and carboxy-TEMPO did not. When applied to LUVs containing <1 mol% radiolabeled glucosylceramide or short-chain C_6-glucosylceramide, carboxy-TEMPO oxidized half the glucosylceramide. However, if surface C_6-glucosylceramide was first depleted by bovine serum albumin (BSA) (extracting 49 ± 1%), 94% of the remaining C_6-glucosylceramide was resistant to oxidation. Carboxy-TEMPO oxidized glucosylceramide on the surface of LUVs without affecting inner leaflet glucosylceramide. At pH 9.5 and at 0°C, the reaction reached completion by 20 min.**—Sillence, D. J., R. J. Raggers, D. C. A. Neville, D. J. Harvey, and G. van Meer. **Assay for the transbilayer distribution of glycolipids: selective oxidation of glucosylceramide to glucuronylceramide by TEMPO nitroxyl radicals. *J. Lipid Res.* 2000.** 41: **1252–1260.**

Supplementary key words glycosphingolipid • lipid asymmetry • ABC transporters

In studying the function of membrane lipids in cells, one major challenge is to elucidate the transmembrane distribution of lipids and how it is regulated. A particularly interesting case is the transmembrane distribution (or sidedness) of glycosphingolipids. Glycosphingolipids are thought to function on the mammalian cell surface (1) and, in line with this topology, the simple glycolipid galactosylceramide is synthesized on the noncytosolic surface of the endoplasmic reticulum (ER) and the complex glycolipids are synthesized on the noncytosolic surface of the Golgi. However, as an exception glucosylceramide (GlcCer), the precursor for the complex glycolipids, is synthesized on the cytosolic side of the Golgi. GlcCer must, therefore, cross the membrane to reach the site of higher glycolipid synthesis and the cell surface (2–6). Because the spontaneous transbilayer mobility of glycolipids has been found to be extremely slow (7), it is likely that a translocator is involved in the translocation of GlcCer across the Golgi membrane. Candidates for such a translocator include members of the ABC transporter family such as the multidrug resistance (MDR) glycoproteins. Both the MDR1 P-glycoprotein and the multidrug resistance protein MRP1 are capable of translocating short-chain analogs of GlcCer across the plasma membrane (8, 9). To study whether natural GlcCer is recognized by such transporters, it is necessary to develop a topology assay for natural GlcCer.

Abbreviations: amu, atomic mass units; BHT, butylated hydroxytoluene; BSA, bovine serum albumin; DHB, dihydroxybenzoic acid; EDTA, ethylenediamine tetraacetic acid; FAB, fast atom bombardment; GlcCer, glucosylceramide; LUVs, large unilamellar vesicles; MALDI-TOF, matrix-assisted laser desorption ionization/time-of-flight; C_{12}NBD-GlcCer, 12-[*N*-methyl-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)]aminododecanoyl sphingosyl-β-D-glucoside; Dulbecco's PBS, phosphate-buffered saline (without Ca^{2+} and Mg^{2+}); PC, phosphatidylcholine; PE, phosphatidylethanolamine; SUVs, small unilamellar vesicles; TEMPO, 2,2,6,6-tetramethylpiperidinooxy nitroxide; TEMPO·, free radical from TEMPO; $TEMPO^+$, nitrosonium ion from TEMPO; TEMPOH, TEMPO hydroxylamine; TLC, thin-layer chromatography.

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Several enzymes are available that, in principle, are capable of modifying GlcCer in membranes, but they are active at 37°C where interpretation of the data is complicated by the ongoing processes of exo- and endocytosis. Examples include glucocerebrosidase (2) and glycolipid *N*-deacylase (10). However, in practice, these enzymes display low reactivity on intact cells, probably because of insufficient penetration into the membrane. Therefore, we have searched for a suitable chemical procedure. Previously, chemical modification of glycolipids has been performed by a procedure employing sodium periodate (11, 12) as reviewed by Sillence, Raggers, and van Meer (13). Periodate is suitable for the oxidation of biomolecules that contain vicinal hydroxyl groups. Neuraminic acid and glycerol are, therefore, particularly sensitive to this oxidation procedure. The reaction runs at low concentrations of periodate and is rapid at low temperature (14, 15). Other naturally occurring polyol-containing compounds, such as ribose, galactose, and mannose, also contain vicinal ring hydroxyl groups that are *cis* to one another. However, in these cases the only available hydroxyl groups are part of a five- or six-membered ring and, therefore, are more difficult to oxidize. Thus, higher concentrations of periodate are required (16, 17). At these higher concentrations permeation of the bilayer can be a problem (18). Many carbohydrates and other polyols, such as the glucose group on GlcCer, contain vicinal ring hydroxyls that are *trans* to one another and are, therefore, even more resistant to oxidation by periodate.

Many carbohydrates contain a primary hydroxyl group that can, in principle, be oxidized to an aldehyde, or further to a carboxylic acid. Oxidation of this group in glycosphingolipids has been performed with $KMnO_4$ in the presence of an excess of $NaIO_4$ (19). However, $KMnO_4$ also mediates the oxidative cleavage of double bonds and ring hydroxyl groups and there is still a search for milder, more selective reagents. A relatively specific procedure has been developed that permits the oxidation of primary alcohol groups even in the presence of secondary alcohol groups (20–24). Such a selective approach is promising both in organic synthesis and in biochemical applications. This reaction utilizes 2,2,6,6-tetramethylpiperidinooxy nitroxide (TEMPO) analogs that are nitroxyl radicals, containing the N,N-disubstituted NO group with one unpaired electron. Oxidation of nitroxyl radicals by hypochlorite–hypobromite yields an active nitrosonium ion (**Fig. 1A**). TEMPO nitrosonium ions are soluble in aqueous solution and are capable of the complete oxidation of primary alcohol groups on a wide variety of carbohydrates, thus producing the corresponding uronic acids (22, 24, 25). We have successfully modified this method for use in specifically oxidizing surface GlcCer to glucuronylceramide in large unilamellar vesicles. The method is simple and the reaction quickly goes to completion at 0°C.

MATERIALS AND METHODS

Preparation of [^{14}C]GlcCer

[^{14}C]C_6GlcCer was synthesized by a method similar to that used by Kishimoto (26). Briefly, 17 μmol of the appropriate glucosphingosine, 17 μmol of [^{14}C]hexanoic acid, 34 μmol of triphenylphosphine, and 34 μmol of 2,2-dithiodipyridine were dissolved in 325 μL of dichloromethane and left at room temperature overnight. The solvent was then removed under a stream of nitrogen and the lipids were resuspended in 60 μL of chloroform–methanol 2:1 (v/v). After application to a thin-layer chromatography (TLC) plate, the [^{14}C]C_6GlcCer was separated from the unreacted hexanoic acid by chloroform–methanol–acetic acid 90:2:8 (v/v/v). Radiolabeled natural GlcCer was prepared by labeling a confluent 75-cm^2 flask of fibroblasts with 50 μCi of [^{14}C]acetate. GlcCer was then purified by lipid extraction in 3.2 mL of chloroform–methanol 1:2.2 (v/v) and two-dimensional TLC as described below.

Preparation of large unilamellar vesicles by ether injection

Large unilamellar vesicles (LUVs) were made by ether injection, similar to the procedure described by Deamer and Bangham (27). Briefly, 1100 nmol of egg phosphatidylcholine (PC), 800 nmol of cholesterol (Chol), and 100 nmol of egg phosphatidylethanolamine (PE) 55:40:5 (mol/mol) and either 10 nmol of [^{14}C]C_6GlcCer, an estimated 2 nmol of natural [^{14}C]GlcCer, or 3 nmol of 1-palmitoyl-2-[1-^{14}C]linoleoyl L-3-PE were taken up in 700 μL of diethyl ether. This mixture was then slowly injected with a 250-μL syringe into 2 mL of phosphate-buffered saline (PBS) at 55–65°C. At this temperature the ether evaporates and the dissolved lipids form liposomes. After cooling, the liposomes were sized by extrusion through a polycarbonate filter (pore size, 200 nm; Nuclepore, Pleasanton, CA) at pressures up to 6 bar (28). The final concentration of lipid was 0.9 μmol/mL. Small unilamellar vesicles were made by sonicating the preceding lipids, but without GlcCer, for 10 min on ice at 60 W, using a Vibra Cell (Sonics & Materials, Danbury, CT). [^{14}C]C_6GlcCer or C_{12}NBD-GlcCer was then inserted into the outer leaflet of the SUVs by incubating them in the presence of 0.03% bovine serum albumin (BSA), which had been complexed to the GlcCer in a prior incubation for 30 min at 0°C (25 nmol of C_6GlcCer or 10 nmol of C_{12}NBD-GlcCer into 50 μL of 0.03% BSA; plus 1 mL of SUVs).

BSA depletion of LUVs

[^{14}C]C_6GlcCer LUVs (20 μl, 18 nmol of total lipid containing 92 pmol of [^{14}C]C_6GlcCer, 1,100 dpm) were mixed with 600 μL of 0.5% BSA in PBS and then incubated at 20°C for 30 min. The molar excess of the BSA over the C_6GlcCer (500-fold) suffices to deplete fully the C_6GlcCer from the outer bilayer leaflet. The vesicles were then pelleted (SW55, 30 min, 28,900 rpm, 4°C) and analyzed, or resuspended in 20 μL of PBS.

Phospholipase A_2 assay for lipid localization in LUVs

Liposomes were diluted with 3 volumes of PBS (with Ca^{2+} and Mg^{2+}) containing bee venom phospholipase A_2 (33 IU/mL) and incubated at 10°C. At varying time intervals the reaction was stopped by the addition of 320-μL samples to 300 μL of an ice-cold solution of 10 mM EDTA in water, pH 5 (29).

Oxidation by carboxy-TEMPO nitrosonium ions

This oxidation reaction relies on the generation of an active nitrosonium ion by in situ oxidation of the carboxy-TEMPO free radical by OCl^-/Br^-. For this, we prepared a reaction mixture. Carboxy-TEMPO can be purchased from several commercial sources as a dry powder in the free acid form. Its solubility is greatly enhanced by conversion to the sodium salt. As a first step, 10 mg (final concentration, 15 mM) of carboxy-TEMPO was sonicated in 830 μL of 80 mM $NaHCO_3$ (pH 13) with 53 μL of 1 M NaOH. After cooling on ice the nitrosonium ion was gen-

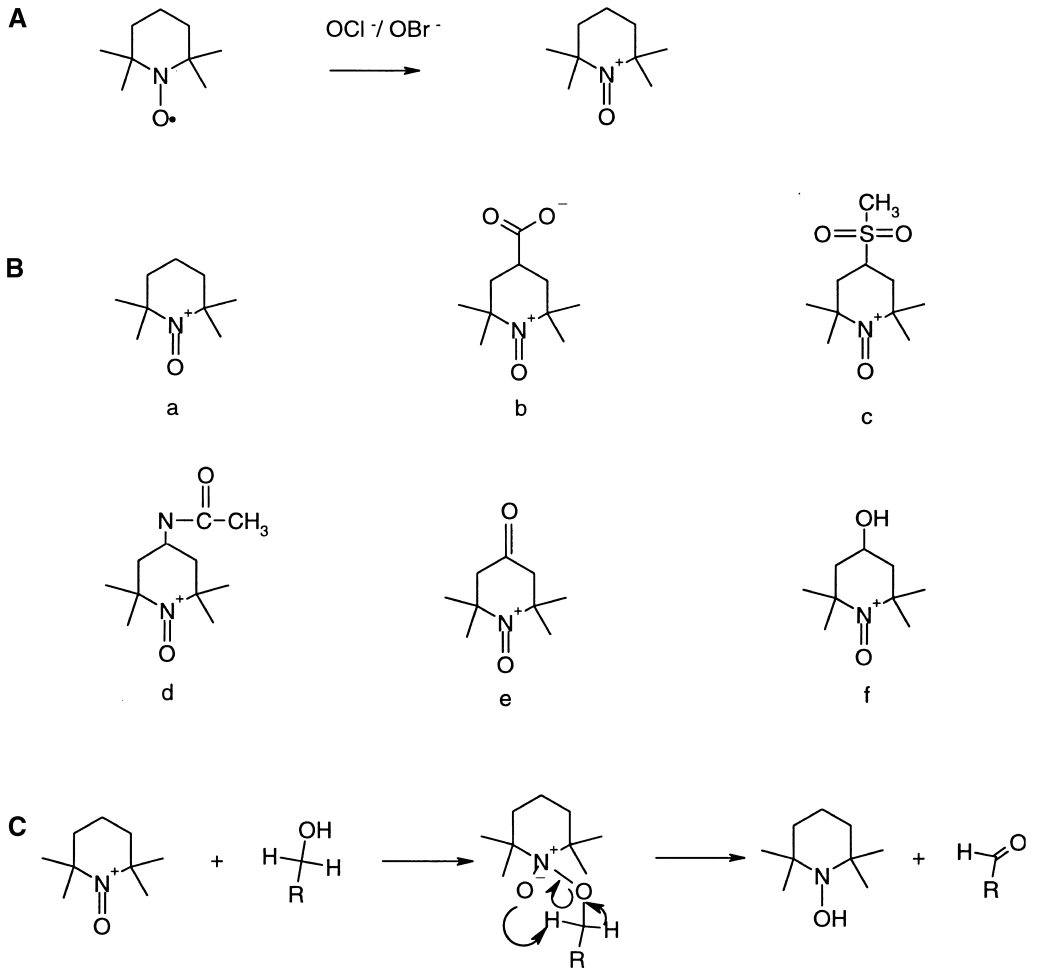

Fig. 1. TEMPO oxidation reaction (25). (A) The oxidation of the TEMPO nitroxyl radical to the corresponding nitrosonium ion. (B) The various analogs of TEMPO (as their nitrosonium ions): (a) TEMPO, (b) carboxy-TEMPO, (c) 4-methylsulfoxy-TEMPO, (d) 4-acetylamino-TEMPO, (e) 4-oxo-TEMPO, and (f) 4-hydroxy-TEMPO. (C) Proposed reaction scheme.

erated by the addition of NaBr (6 μL at 1 M; final concentration, 1.9 mM) followed by concentrated NaOCl (25 μL, pH 13, 15% [w/v]; final concentration, 16 mM). This mixture was then diluted to 290 mOsm by the addition of 300 μL of water and 2 mL of buffer A (200 mL of PBS plus 1.5 g of $NaHCO_3$, in a final volume of 300 mL; pH 9.5), and adjusted to pH 9.5 at 0°C with 1 M NaOH. At this point butylated hydroxytoluene (BHT) was added (13 μL at 25 mM; final concentration, 100 μM). Liposomes (100 μL) were then incubated with 800 μL of this reaction mixture at 0°C. After various times up to 1 h, the reaction mixture was quenched with 100 μL of ethanol. Lipids were then extracted by the addition of 3.2 mL of chloroform–methanol 1:2.2 (v/v) and the phases were separated by the addition of 1 mL of chloroform and 1 mL of buffer A. An alkaline extraction was necessary in the presence of high concentrations of carboxy-TEMPO to minimize the solubility of this compound in the lower phase and to eliminate competing reactions after the phase split. Alkaline phase splits increase the possibility of losing acidic short-chain lipids to the upper phase. To recover the remaining lipid from the upper phase, this phase was neutralized with 10 μL of concentrated HCl and passed through a C_{18} Sep-Pak column (Waters, Milford, MA). The column was then washed with 10 mL of water and the lipids were eluted from the column with chloroform–methanol–10 mM acetic acid 1:2.2:0.2 (v/v/v). Subsequently, the lipids were dried and separated by TLC as described below.

Analysis of oxidation products by TLC and mass spectrometry

Lipids were separated on boric acid-impregnated TLC plates, in two dimensions: *i*) chloroform–methanol–25% aqueous ammonia–water 65:35:4:4 (v/v/v) and *ii*) chloroform–acetone–methanol–acetic acid–water 50:20:10:10:5 (v/v/v/v/v) (30). In the case of radioactive samples, the TLC plates were dried and exposed to a ^{14}C-sensitive screen. Radioactive spots were quantitated with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and ImageQuant software by comparison with internal standards. For mass spectrometry, a fluorescent C_{12}NBD-GlcCer was used to allow easy localization on the TLC plate. C_{12}NBD-GlcCer and its reaction product were then eluted from the silica with chloroform–methanol–10 mM acetic acid 1:2.2:1 (v/v/v) followed by a phase split as described above. They were examined by electrospray tandem mass spectrometry (MS/MS), using a Micromass (Beverly, MA) Q-TOF mass spectrometer. Each compound was dissolved in methanol at a concentration of 10 μM and infused, using the nanoflow probe at 200 nl/min. The needle voltage was 3,000 V and the ion source was maintained at 80°C. Argon was used as the collision gas, the voltage on the col-

lision cell was 80 V, and the cone voltage was 100 V. The resolution was adjusted so that isotope peaks could just be resolved.

Sources of reagents

Triphenylphosphine, 2,2-dithiodipyridine (aldrithiol-2), glucosylpsychosine, egg PE, egg PC, cholesterol, bee venom phospholipase A_2, [1-^{14}C]acetic acid (50 Ci/mol), C_{12}NBD-GlcCer, and [^{14}C]hexanoic acid (5.2 Ci/mol) were obtained from Sigma (Zwijndrecht, The Netherlands). Carboxy-TEMPO, TEMPO, 4-methylsulfoxy-TEMPO, 4-acetylamino-TEMPO, and sodium bromide were obtained from Aldrich (Zwijndrecht, The Netherlands). Sodium hypochlorite was a kind gift from Brenntag Falleyway Chemicals (West Midlands, UK). 1-Palmitoyl-2-[1-^{14}C]linoleoyl L-3-PE (56 Ci/mol) was obtained from Amersham (Bucks, UK).

RESULTS

Direct translation of the chemical method with catalytic amounts of TEMPO in the presence of excess hypochlorite (24, 25) showed that this method was not directly applicable to biomembranes because it caused nonspecific peroxidation of unsaturated phospholipids. Nonspecific peroxidation was then minimized in four ways: *i*) The hypochlorite concentration was minimized by using excess TEMPO over hypochlorite; *ii*) the reaction was performed in the presence of 100 μM butylated hydroxytoluene, which is widely used to inhibit nonspecific peroxidation of lipids; *iii*) the concentration of NaBr that, in the presence of NaOCl, yields OBr^- (which is a stronger oxidant than OCl^-), was minimized; and *iv*) the reaction was carried out at pH 9.5 instead of pH 10. The reaction is pH dependent, with a pH optimum at 10. However, competing β-elimination of hydroxy or alkoxy groups is reduced by performing the reaction at pH 9.5. Using these conditions, GlcCer gave an oxidation product that was separable by two-dimensional thin-layer chromatography (**Fig. 2**). Under similar conditions PE also gave an oxidation product, whereas PC did not.

Analysis of the products of the reaction of TEMPO with natural GlcCer and NBD-GlcCer by matrix-assisted laser desorption ionization (MALDI) and electrospray mass spectrometry showed that oxidation had occurred only in the sugar ring. The reaction did not affect the heterocyclic group of NBD-GlcCer as shown in the electrospray MS/MS spectrum (**Fig. 3**). The top spectrum (Fig. 3A) is that of the starting glycolipid and the product is shown underneath (Fig. 3B). The starting glycolipid and product were detected as sodium adducts $[M+Na]^+$ at mass-to-charge (m/z) ratios of 858.6 and 872.5, respectively. The mass difference of 14 mass units between the two compounds is consistent with oxidation of a primary alcohol to a carboxylic acid. The spectrum of the unoxidized NBD-GlcCer was dominated by fragments from the NBD moiety, that is, loss of HNO_3 (63 mass units) to give the ion at m/z 795.6. The presence of an ion at m/z 633.5 is indicative of the loss of glucose (residue mass 162) from the m/z 795.6 ion, as shown in Fig. 3A, and elimination of a further water molecule (18 mass units) gave the ion at m/z 615.5. Corresponding losses from the molecular ion gave

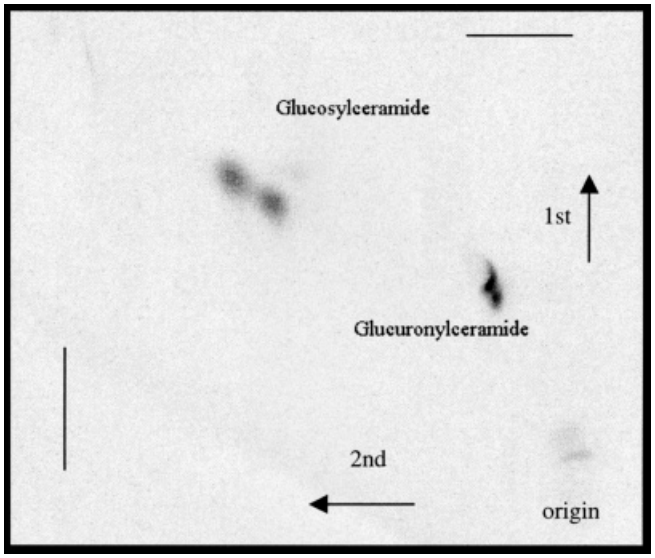

Fig. 2. Two-dimensional thin-layer chromatogram of glucosylceramide and its oxidized product glucuronylceramide. Natural [^{14}C]GlcCer-containing large unilamellar vesicles (80 nmol of total lipid; ~80 pmol of [^{14}C]GlcCer) prepared by ether injection and sized through a 0.2-μm pore size filter, were incubated with 15 mM carboxy-TEMPO at 0°C for 60 min. The oxidation reaction was stopped with cold ethanol. The lipids were extracted and separated by 2D-TLC in the first (alkaline) and second (acid) solvents as described in Materials and Methods. Natural GlcCer appears as two spots due to fatty acyl chain heterogeneity. The glucuronylceramide migrated slower in the acid than in the alkaline solvent (even as a protonated species), which may be due to an overriding effect of the increased polarity of the carboxyl group because the acidic solvent is less polar.

the ions at m/z 696.5 and 678.5. The spectrum of the compound after oxidation (Fig. 3B) showed the same general features but, because of the more labile hydrogen atom of the carboxy group, loss of the glucuronic acid moiety (residue mass 176) became the dominant fragmentation to give the ion at m/z 696.5. Loss of HNO_3 then occurred from this ion to give m/z 633.5.

Because TEMPO is hydrophobic and soluble in organic solvents we suspected that it might be able to penetrate membranes. We therefore also used carboxy-TEMPO, which at alkaline pH as its nitrosonium ion, would be expected to be zwitterionic and, therefore, membrane impermeable. To optimize further the carboxy-TEMPO-mediated oxidation of GlcCer, a concentration curve was created with SUVs onto which C_6GlcCer had been complexed to the outer surface. The results are shown in **Fig. 4**. At concentrations of carboxy-TEMPO above 10 mM, after 30 min of incubation at 0°C, 86 ± 0.8% of the 2.5 nmol of C_6GlcCer was oxidized. The small percentage of C_6GlcCer that was resistant to oxidation may have been caused by spontaneous translocation of a small fraction of the C_6GlcCer from the outer to the inner leaflet of the SUV preparation. In addition, a small amount of the C_6GlcCer might still be located on any remaining BSA, where it may be shielded from oxidation by the protein. Therefore, carboxy-TEMPO was used at a concentration of 15 mM throughout the study.

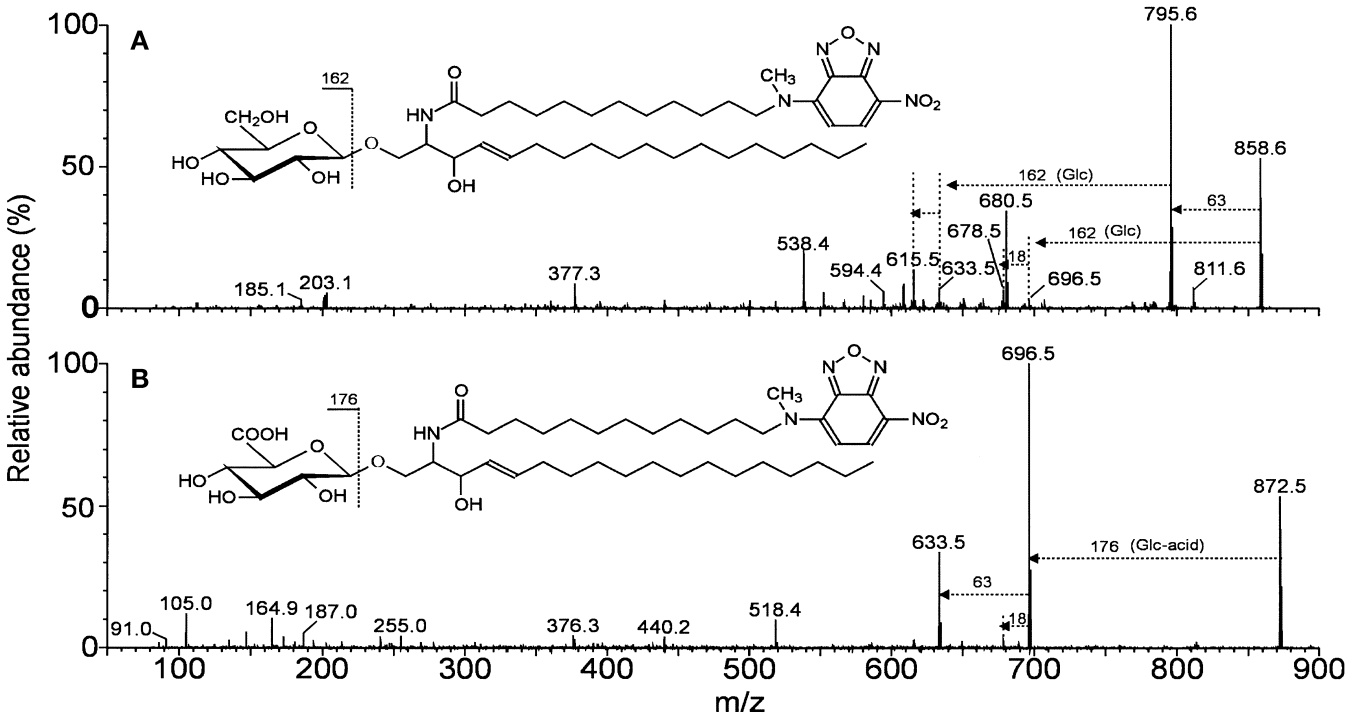

Fig. 3. (A) Electrospray MS/MS spectrum of the C_{12}NBD-GlcCer $[M+Na]^+$ ion at m/z 858.6. Fragments showing the presence of the glucose moiety are indicated by arrows. (B) Corresponding spectrum of the oxidized molecule at m/z 872.5. C_{12}NBD-GlcCer was incorporated into the outer leaflet of SUVs and oxidized as described in Materials and Methods. Oxidation of glucose to glucuronic acid is shown by the prominent loss of 176 mass units from the molecular ion.

To address whether it is possible to use carboxy-TEMPO nitrosonium ions as a probe specifically to recognize lipid on the surface of membrane bilayers, large unilamellar vesicles (LUVs) of a defined size and composition were made as described in Materials and Methods. LUVs are a useful model system because it is well established that natural phospholipid is symmetrically distributed in these model bilayers (29–31). TEMPO nitrosonium ions are also capable of oxidizing primary amines and hence are able to oxidize PE. To investigate whether TEMPO and carboxy-TEMPO specifically oxidize lipid in the outer leaflet, experiments were devised to check that indeed PE was evenly distributed over the two bilayer leaflets, and then to see to what extent PE in the LUVs was accessible for oxidation. To do this, PC–PE–cholesterol LUVs containing radiolabeled PE were first treated with phospholipase A_2 at low temperature (29). The results of such an experiment are shown in **Fig. 5A** and show a rapid hydrolysis of the PE in the first 20 min followed by a plateau, indicating no further hydrolysis beyond 50%. Roughly 90% of the PE was degraded when the liposomes were treated for 60 min with phospholipase A_2 in the presence of 0.1% Triton X-100. These results confirm that PE is symmetrically distributed in LUVs.

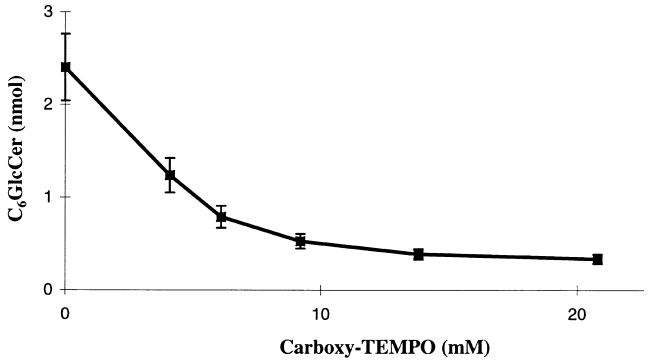

Fig. 4. Concentration curve for the oxidation of surface C_6GlcCer with carboxy-TEMPO. Small unilamellar vesicles (100 nmol of total lipid) containing 2.5 nmol of [^{14}C]C_6GlcCer (27,500 dpm) in the outer bilayer leaflet were prepared as described in Materials and Methods. Surface C_6GlcCer was then oxidized with the indicated concentrations of carboxy-TEMPO in a total volume of 900 μL as described in Materials and Methods. The amount of C_6GlcCer (nmol) was calculated from the ^{14}C signal as measured by a PhosphorImager that was calibrated by a known amount of [^{14}C]lipid. The curve is the mean of one experiment carried out in duplicate (values ± range) and is representative of two separate experiments.

Experiments with TEMPO and carboxy-TEMPO were, in this case, complicated by competing side reactions during the lipid extraction. We were unable to avoid these reactions completely, but they were minimized *i*) by quenching the reaction with 100 μL of cold ethanol and *ii*) by keeping the pH at 9.5 during the phase split. The effect of these adaptations was assessed by adding the reaction mixture to a lipid extraction mixture that contained the LUV lipids. Only 10 ± 1% of the PE reacted during the subsequent standard lipid extraction. In contrast, GlcCer did not react under these circumstances, abolishing the need for such controls in GlcCer experiments. Carboxy-TEMPO oxidized

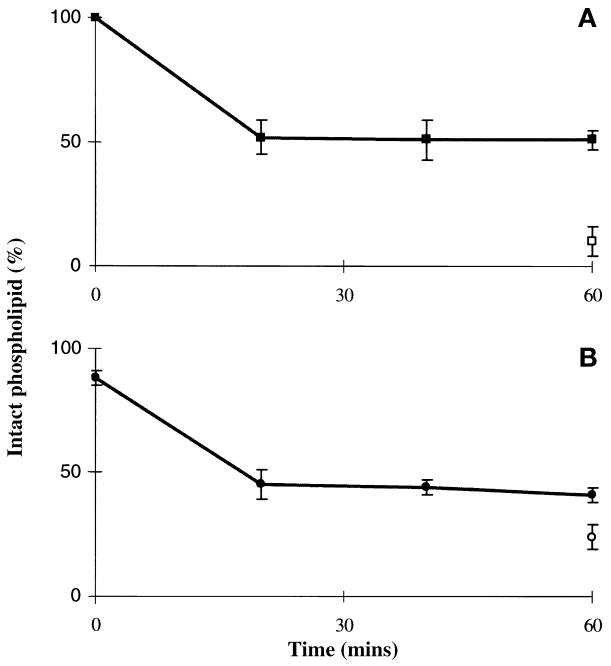

Fig. 5. Phosphatidylethanolamine (PE) is symmetrically distributed in large unilamellar vesicles as measured by phospholipase A_2 hydrolysis and carboxy-TEMPO oxidation. (A) For each time point, LUVs (80 nmol of total lipid, 4 nmol of PE, 0.12 nmol of [^{14}C]PE, 15,000 dpm), prepared by ether injection as described in the text, were incubated with 8 IU of bee venom phospholipase A_2 at 10°C (solid squares) plus 0.1% Triton (open square). Hydrolysis was stopped by EDTA and the lipids were extracted and separated, after which radioactivity in the PE spot was quantitated as described in Material and Methods. The values are expressed as a percentage of the radioactivity in [^{14}C]PE before phospholipase treatment, and represent means ± SD of two experiments performed in duplicate (n = 4). (B) Time course for the oxidation of [^{14}C]PE-containing LUVs with carboxy-TEMPO. LUVs (see above) were incubated with 15 mM carboxy-TEMPO at 0°C for the indicated times (solid circles). In the same incubation, TEMPO produced 75 ± 10% oxidation (open circle). The oxidation reaction was stopped with cold ethanol. The lipids were extracted and separated, and radioactivity in the PE spot quantitated as described in Material and Methods. The values are expressed as a percentage of the radioactivity in [^{14}C]PE before oxidation, and represent the means ± SD of two experiments performed in duplicate (n = 4).

53 ± 4% of the total PE during the 1-h incubation. This contrasts with TEMPO, which oxidized 75 ± 10% of the PE in 1 h (the mean ± SD of 3 to 5 experiments carried out in duplicate). It is notable that the reaction of TEMPO with lipid in the lumenal leaflet of liposomes did not go to completion. This is probably because the buffer on the inside of the liposomes (in which the liposomes had been prepared) was not optimized for the TEMPO reaction. To investigate this more fully a time course was performed, similar to the time course performed with phospholipase A_2. The results of such an experiment are illustrated in Fig. 5B, which shows a rapid oxidation phase followed by a plateau. This result resembles the hydrolysis with phospholipase A_2 (Fig. 5A), and is consistent with the proposition that carboxy-TEMPO is capable of selectively oxidizing outer leaflet PE without oxidizing inner leaflet PE.

In our main quest for a suitable selective assay for GlcCer, we first tested the reactivity of various commercially available TEMPO derivatives (Fig. 1B) in LUVs containing natural [^{14}C]GlcCer, as in Fig. 2. TEMPO derivatives such as the 4-hydroxy and 4-oxo forms are inexpensive and commercially available but were not used because their nitrosonium ions are labile at high pH. When applied at a concentration of 15 mM for 1 h at 0°C, there were large differences in the level of [^{14}C]GlcCer oxidation by the other TEMPO derivatives depending on the substituent at the 4-position. 4-Acetylamino-TEMPO and 4-methylsulfoxy-TEMPO showed little reactivity, oxidizing 3 ± 3 and 8 ± 3% (mean ± SD, n = 4), respectively. However, both TEMPO and carboxy-TEMPO showed high reactivity with natural [^{14}C]GlcCer, oxidizing 60 ± 3 and 44 ± 3% (mean ± SD, n = 18), respectively. Consequently, subsequent experiments focused on the latter compounds.

To characterize further the action of carboxy-TEMPO, a time course was performed to monitor the oxidation of LUVs containing natural GlcCer. The results in **Fig. 6** show a rapid oxidation of 50% of the GlcCer in the first 20 min, followed by a plateau. Because TEMPO nitrosonium ions are slowly inactivated in aqueous solution because of their (low) reactivity with hydroxide ions, it was necessary to exclude the possibility that the reaction was stopped at ~50% by the absence of reactive carboxy-TEMPO. For this, C_6GlcCer LUVs were added at the 60-min time point. This control experiment showed that even after 1 h in aqueous solution carboxy-TEMPO was still capable of oxidizing 70 pmol of C_6GlcCer (and LUV PE) between 60 and 80 min of the time course. Because, in Fig. 6, 100% corresponds to ~80 pmol of natural GlcCer it appears

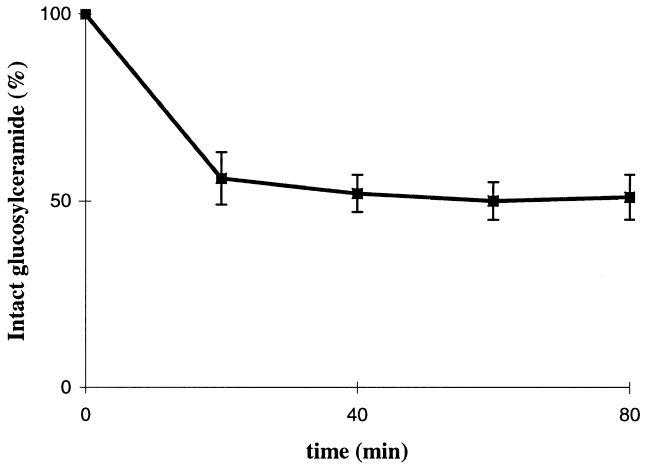

Fig. 6. Time course for the oxidation of natural GlcCer in large unilamellar vesicles with carboxy-TEMPO. [^{14}C]GlcCer-containing LUVs (see legend to Fig. 2) were incubated with 15 mM carboxy-TEMPO at 0°C for the times indicated. The oxidation reaction was stopped with cold ethanol. The lipids were extracted and separated as described in Materials and Methods. Each incubation contained ~80 pmol of natural GlcCer (80 nmol of total lipid, 4 nmol of PE). The values are expressed as a percentage of the radioactivity in [^{14}C]GlcCer before oxidation, and represent the means ± SD of two experiments performed in duplicate (n = 4).

that the plateau at 50% was not due to inactivation of the nitrosonium ions.

To perform a more definitive test for the asymmetry of the oxidations by TEMPO and carboxy-TEMPO a further set of experiments was performed with C_6GlcCer LUVs. These were chosen because an independent asymmetry assay for membrane C_6GlcCer has already been described, using depletion with BSA (6). Using the independent BSA depletion assay, roughly half of the C_6GlcCer was found to be accessible to BSA in C_6GlcCer LUVs (**Table 1**). It was then possible to compare the TEMPO and carboxy-TEMPO-mediated oxidation of C_6GlcCer with this independent result. Carboxy-TEMPO oxidized 51% of the C_6GlcCer in the LUV preparation, which agrees closely with the value obtained in the independent BSA assay. In contrast, TEMPO oxidized 81% of the C_6GlcCer in the symmetric LUVs, suggesting that, in addition to oxidizing surface GlcCer, it was also capable of oxidizing the inner leaflet GlcCer.

As a second control, C_6GlcCer was depleted from the outer leaflet of the LUVs with BSA, after which the LUVs were oxidized with either TEMPO derivative (Table 1). The ability of carboxy-TEMPO to oxidize C_6GlcCer was drastically reduced from 51% in the original LUVs to 6% in the surface-depleted LUVs. The C_6GlcCer that was still sensitive to oxidation by carboxy-TEMPO after BSA extraction was most likely incompletely extracted from the outer membrane leaflet. Table 1 shows that 52% of the C_6GlcCer remained after BSA extraction, and probably 2% of this was still outside; this implies that 4% of the remaining C_6GlcCer was accessible to subsequent carboxy-TEMPO oxidation. In addition, carboxy-TEMPO may also slowly penetrate the liposomal membrane. In contrast, TEMPO still oxidized half the C_6GlcCer left after removal of the outer leaflet C_6GlcCer. TEMPO was able to penetrate the bilayer rapidly and also oxidize inner leaflet C_6GlcCer.

DISCUSSION

In the search for a cell surface assay for glucosylceramide we have modified a specific oxidation method that has previously been used in organic synthesis. The spin-label TEMPO and its derivative carboxy-TEMPO are converted from their radical form into the corresponding nitrosonium ions. These, in turn, are used to specifically oxidize the primary hydroxyl on the glucose residue even in the presence of the ring hydroxyls and the allylic secondary hydroxyl on the sphingosine backbone. The oxidized product was easily separable by TLC and mass spectrometric analysis led to its identification as glucuronylceramide. Also, the primary amine on phospholipid PE was readily oxidized. In principle, this oxidation reaction should be applicable to any sugar-containing lipid and also to other lipids with primary hydroxyl groups, such as diacylglycerol, ceramide and sphingoid bases, or amines, such as phosphatidylserine (PS), sphingoid bases or sphingoid base-1-phosphates. In the present article, we demonstrate that oxidation of the aminophospholipid PE and of the glycolipid GlcCer by carboxy-TEMPO, but not by TEMPO itself, is limited to the accessible membrane surface in pure lipid membranes. This makes the carboxy-TEMPO reaction particularly useful for the determination of lipid asymmetry and, potentially, for the study of transmembrane translocation.

The present chemical approach offers a substantial advantage over previous oxidation methods because it is selective for primary hydroxyls. Therefore, it does not necessitate prior reactions, such as methylation or acetylation, to protect other hydroxyls. More importantly, this oxidation reaction can occur in aqueous solution and, therefore, has potential for applications in intact biological systems. TEMPO-mediated oxidation reactions are also rapid at 0°C, which is a temperature at which many biological processes (e.g., vesicular transport) are inhibited. It is not yet clear whether biological membranes stay intact when the PE, PS, and glycolipids on the outer surface are oxidized, but these may constitute only a small percentage of lipids in the outer leaflet of the plasma membrane of most cells, as the outer leaflet is thought to contain essentially none of the plasma membrane PS, about one-fifth of the PE, and only a minor amount of glycolipids (discussed in ref. 29).

One of the main complicating factors in using the original method for TEMPO oxidation (20, 24) in biological systems was that it used stoichiometric amounts of hypohalite in the presence of catalytic amounts of TEMPO. To adapt this method for use in a biological system it was first necessary to reduce the hypohalite concentration (31, 32). Ideally, it would have been possible to perform the oxidation in the absence of hypohalite, using stoichiometric amounts of synthetically prepared nitrosonium salts. However, the nitrosonium ion is likely to be unstable. Here we have attempted to mimic this approach by generating the nitrosonium ion in situ and using an excess of TEMPO over the primary oxidants NaOCl and NaOBr. This is the reverse of the previously published method. Theoretically, this approach generates a stoichiometric amount of nitrosonium ion and minimizes the hypohalite concentration through the following reactions.

TABLE 1. Oxidation of C_6GlcCer LUVs before and after depletion of C_6GlcCer from the surface

	% [^{14}C]C_6GlcCer Remaining	
	Control	BSA Extracted
BSA	52 ± 1	
Carboxy-TEMPO	49 ± 4	94 ± 2
TEMPO	19 ± 4	51 ± 5

Large unilamellar vesicles (80 nmol of total lipid and 400 pmol of [^{14}C]C_6GlcCer, prepared as described in Materials and Methods) were oxidized directly by 15 mM TEMPO and carboxy-TEMPO at 0°C as in Fig. 6. Alternatively, surface C_6GlcCer was first depleted by BSA as described in Materials and Methods. The LUVs were then spun down, resuspended, and oxidized. After 1 h the oxidation reaction was stopped with cold ethanol. The lipids were extracted and separated, and the radioactivity in the C_6GlcCer spot was quantitated as described in Materials and Methods. The values are expressed as a percentage of the radioactivity in [^{14}C]GlcCer before oxidation, and represent the mean ± SD of two experiments performed in duplicate.

$$OCl^- + Br^- \rightarrow OBr^- + Cl^- \quad \textit{Eq. 1)}$$

$$OBr^- + 2TEMPO^{\cdot} + H_2O \rightarrow 2TEMPO^+ + 2OH^- + Br^- \quad \textit{Eq. 2)}$$

The oxidation reaction can then proceed:

$$TEMPO^+ + OH^- + RCH_2OH \rightarrow TEMPOH + RCHO + H_2O \quad \textit{Eq. 3)}$$

$$TEMPO^+ + OH^- + TEMPOH \rightarrow 2TEMPO^{\cdot} + H_2O \quad \textit{Eq. 4)}$$

The regenerated TEMPO free radical can then go through reaction (2) again:

$$TEMPO^+ + OH^- + RCHO \rightarrow TEMPOH + RCOOH \quad \textit{Eq. 5)}$$

This gives the overall reaction:

$$RCH_2OH + 2NaOCl \rightarrow RCOOH + 2NaCl + H_2O$$

Apart from reducing hypohalite concentrations to a minimum, nonspecific oxidation was further reduced by the addition of butylated hydroxytoluene, which is a hydrophobic free radical scavenger. This compound may also inhibit unwanted halogenation by reacting with residual hypohalite. Nonspecific oxidation can also be reduced by the addition of chelating agents such as EDTA. These work by inhibiting nonspecific oxidation reactions catalyzed by transition metals. However, at high concentrations (0.1 mM) EDTA can inhibit the oxidation (D. J. Sillence, unpublished observations).

TEMPO radicals are stable only in the absence of α-hydrogens; hence the methyl substituents are necessary for stability but may also contribute to specificity. The reaction mechanism is still unclear, although a radical-based mechanism has been excluded (25). Under alkaline conditions the reaction has a greater specificity for primary than for secondary hydroxyl groups because, at alkaline pH, TEMPO-mediated oxidation takes place by a sterically hindered intermediate (Fig. 1C). In organic solvent nitrosonium ions can mediate the oxidation of hydroxyl groups to aldehydes and ketones (21–23). However, in aqueous solution nitrosonium ions catalyze complete and selective oxidation of primary alcohols to carboxylic acids (20, 24).

The capacity of the system to oxidize primary hydroxyl groups is dependent on the following factors: *i*) the buffering capacity of the system because, as the reaction progresses, the pH drops and inhibits the reaction; and *ii*) the concentration of OCl^- ions, because 2 mol of these is required for each mole of acid formed. It should be noted that the reaction is likely to proceed faster if all the reactants are in the same phase. This is not the case with the oxidation of a glycolipid because it is embedded in the membrane in a liquid-crystalline phase. It is interesting to note that this reaction can also be performed in a two-phase system (20, 22). Under these circumstances quaternary ammonium chloride is added as a phase-transfer catalyst. This catalyst is a hydrophobic compound that reacts with alcohol groups in the organic phase and forms a salt that is hydrophilic and therefore more likely to solvate in the aqueous phase, where it can be oxidized.

Egg PC–cholesterol–egg PE 55:40:5 (mol/mol) was chosen as the lipid mixture for the vesicles because it mimicks the composition of the plasma membrane outer leaflet with an intermediate level of fatty acid unsaturation and a high cholesterol content, although in the outer leaflet of plasma membranes half the PC would typically be replaced by sphingomyelin. Carboxy-TEMPO selectively oxidized GlcCer and PE in the outer leaflet of these model membranes. We are currently developing the assay for use on intact cells.

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