methods

High-precision fluorescence assay for sphingomyelinase activity of isolated enzymes and cell lysates

A. Loidl,* R. Claus,† H. P. Deigner,§ and A. Hermetter[1,]*

Department of Biochemistry,* Technische Universität Graz, Petersgasse 12, A-8010 Graz, Austria; Clinic for Anaesthesiology and Intensive Care Therapy,† FSU Jena, Bachstrasse 18, D-07740 Jena, Germany; and Zentrallaboratorium Deutscher Apotheker,§ Carl-Mannich-Strasse 20, D-65760 Eschborn, Germany

Abstract Sphingomyelinases are important enzymes of signal transduction. They catalyze the hydrolysis of sphingomyelin, giving rise to the intracellular formation of biologically active ceramide and phosphatidylcholine. Here we report on a fluorescence method for the fast and accurate determination of this enzyme in biological samples. The assay is based on a fluorescent sphingomyelin analog carrying fluorescent 7-nitro-2-1,3-benzooxadiazolyl amino-dodecanoic acid instead of an aliphatic acyl chain at the nitrogen atom. The fluorescent substrate is hydrolysed by sphingomyelinases to form fluorescent ceramide, which can be separated from the remaining substrate using TLC on silica gel. The fluorescence intensity pattern obtained on the TLC plate can accurately be determined using a CCD camera. Typically, a large number of samples can be analyzed simultaneously. Examples for the quantitative analysis of sphingomyelinases from freshly prepared cellular homogenates as well as from commercial sources are given.—Loidl, A., R. Claus, H. P. Deigner, and A. Hermetter. **High-precision fluorescence assay for sphingomyelinase activity of isolated enzymes and cell lysates.** ***J. Lipid Res.*** **2002.** 43: **815–823.**

Supplementary key words intracellular signaling • ceramide • lipid second messenger • atherosclerosis • smooth muscle cells • low density lipoprotein • apoptosis • cell proliferation

Sphingomyelinases represent central elements of the so-called sphingomyelin/ceramide signaling pathway (1). They play an important role in induction of cell proliferation (2), apoptosis (3), and cell activation (4), which are considered key events in atherogenesis, cancer, and immunological diseases. Oxidatively modified LDL induces proliferation and apoptosis of macrophages (5, 6) and smooth muscle cells (SMCs) (7), thus contributing to the onset and development of atherosclerosis. Recent studies have defined the sphingomyelin transduction pathway as an upstream mechanism for mediating cellular responses after exposure of SMCs (8) as well as MΦ and fibroblasts to oxidized LDL (oxLDL), resulting in an autoloop activation of acid sphingomyelinase (aSMase) by its own product, ceramide (9). Considering the broad variety of different cellular events where sphingomyelinases are involved, there is an urgent need for a valuable, reliable, and rapid method for determination of sphingomyelinase (SMase)-activity in biological samples.

Presently, several assays for SMase activity are known, including an in situ assay for acid SMase (10), a robotic assay for sphingomyelinase activity (11), a high-throughput technique (12), and several others (13, 14) that all require radioactive substrates.

Here we describe a fluorescence method for the accurate and fast determination of SMase-activity in a large number of samples. It is based on a fluorescently labeled sphingomyelin substrate that is cleaved hydrolytically by SMase to give fluorescent ceramide. This product can be separated from the remaining substrate on TLC plates followed by determination of fluorescence intensity using a CCD-camera [detection-limit: 0.2 pmol of the *N*-7-nitrobenz-2-oxa-1,3-diazol (NBD)-fluorophore]. The linearity range is between 0.1 pmol and at least 2,000 pmol label. This assay is suitable as a routine method for determination of SMase-activity in pure enzymes and crude enzyme preparations as well as in complex biological samples (arterial SMCs).

MATERIALS AND METHODS

Materials

Chemicals were purchased from Merck (Darmstadt, Germany). Egg yolk sphingomyelin, neutral sphingomyelinase from *Bacillus cereus* (347 U/mg Protein), and acid sphingomyelinase (177 U/mg protein) from human placenta were from Sigma (Vienna, Austria). Radioactive sphingomyelin (SM) (egg) ([^{3}H]choline methyl) (81.5 Ci/mmol) was purchased from Perkin Elmer

Abbreviations: mmLDL, minimally modified LDL; natLDL, native LDL; oxLDL, oxidized LDL; SM, sphingomyelin; SMase, sphingomyelinase; aSMase, acid sphingomyelinase; SMC, smooth muscle cell; TNFα, tumor necrosis factor α; NBD, *N*-7-nitrobenz-2-oxa-1,3-diazol.

[1] To whom correspondence should be addressed.
e-mail: albin.hermetter@tugraz.at

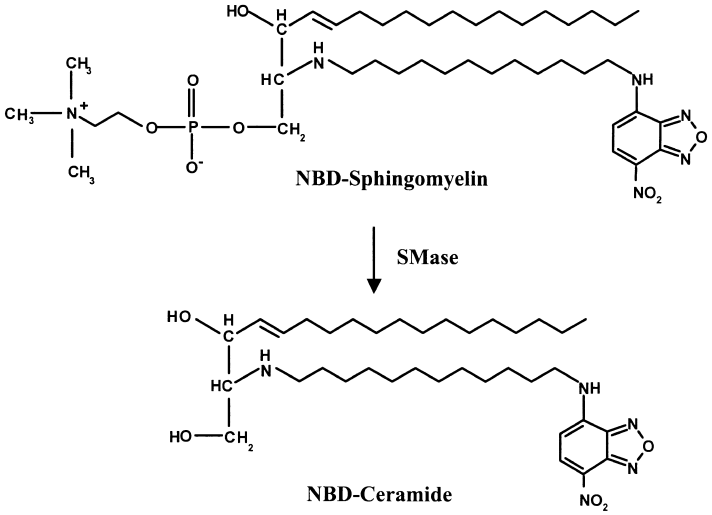

Fig. 1. Fluorescence spectroscopic assay for the determination of sphingomyelinase (SMase)-activity. Fluorescent *N*-7-nitrobenz-2-oxa-1,3-diazol (NBD)-C_{12}-sphingomyelin is hydrolyzed by sphingomyelinase, leading to the formation of corresponding fluorescent ceramide.

Life Sciences (Boston, MA). LCS Safety Cocktail for liquid scintillation counting was from Mallinckrodt Baker (Deventer, Holland). Plastic-ware for cell culture was purchased from Greiner, Frickenhausen, Germany. Media and supplements for cell culture were purchased from Paa Laboratories (Linz, Austria). Tumor necrosis factor α (TNFα) (human recombinant, over expressed in *Escherichia coli*) and [C_6]Ceramide (solution in EtOH) were from Calbiochem (Bad Soden, Germany).

Cell culture

Human transformed SMCs (ACBRI #443 Human Coronary Artery SMCs from Applied Cell Biology Research Institute, Kirk-

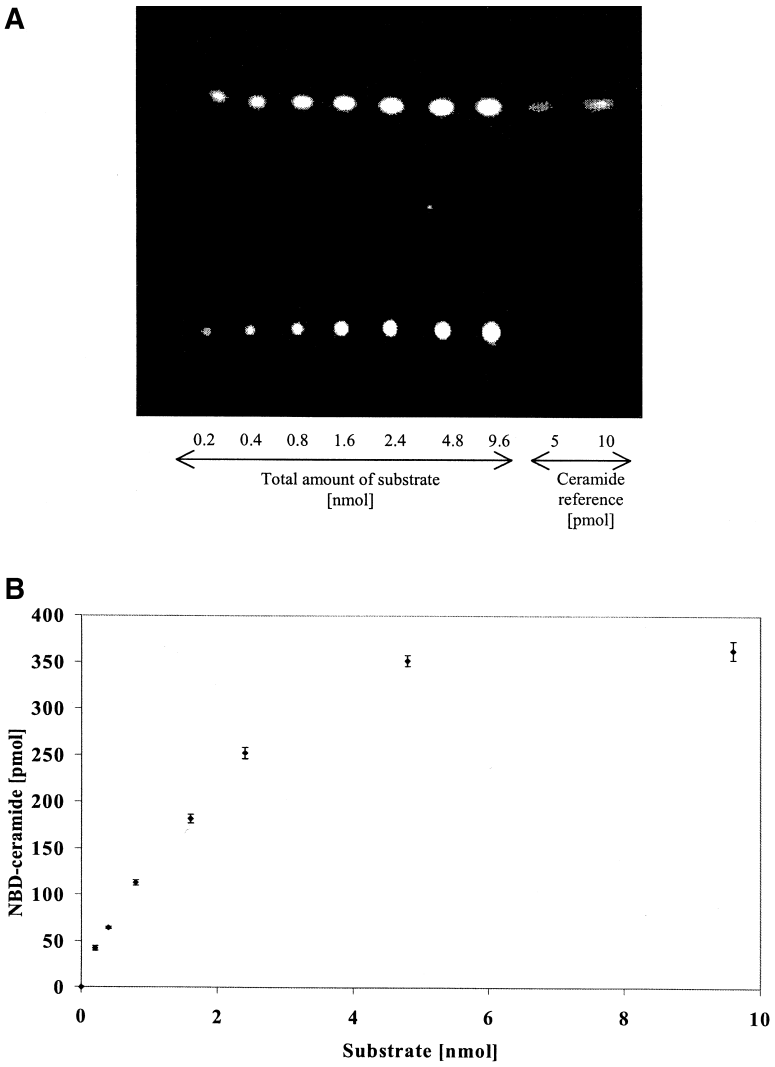

Fig. 2. Activity of acid SMase from human placenta. Effect of substrate concentration. A: Acid SMase from human placenta (1.6 units) was incubated with increasing amounts (0.2 to 9.6 nmol) of NBD-sphingomyelin in acid reaction buffer (250 mM Sodium-Acetate, 1 mM EDTA, pH 5.0) in a total volume of 200 μl. After incubation at 37°C for 1 h the reaction was stopped by addition of 600 μl $CHCl_3$-CH_3OH, 2:1 (v/v). Lipids were extracted and an aliquot of the organic lipid phase (100 μl) was evaporated under a N_2 stream. The residue was dissolved in 10 μl $CHCl_3$. NBD-ceramide was separated from the remaining substrate by TLC using $CHCl_3$-CH_3OH-H_2O, 65:25:4 (v/v/v) as a solvent. The remaining substrate [NBD-sphingomyelin (SM)] and the product (NBD-ceramide) appeared as single spots at R_F = 0.15 and R_F = 0.85, respectively. NBD-ceramide was used as a reference lipid. The fluorescence intensities of the NBD-ceramide spots formed were determined using a CCD camera followed by software-based data processing. The amounts of pmol ceramide were obtained from the respective fluorescence intensities using the calibration plot shown in Fig. 3. B: Plot of pmol NBD-ceramide generated from NBD-SM by acid SMase versus the amount of substrate in the reaction mixture. The indicated values are means ± SD (n ⩾ 3).

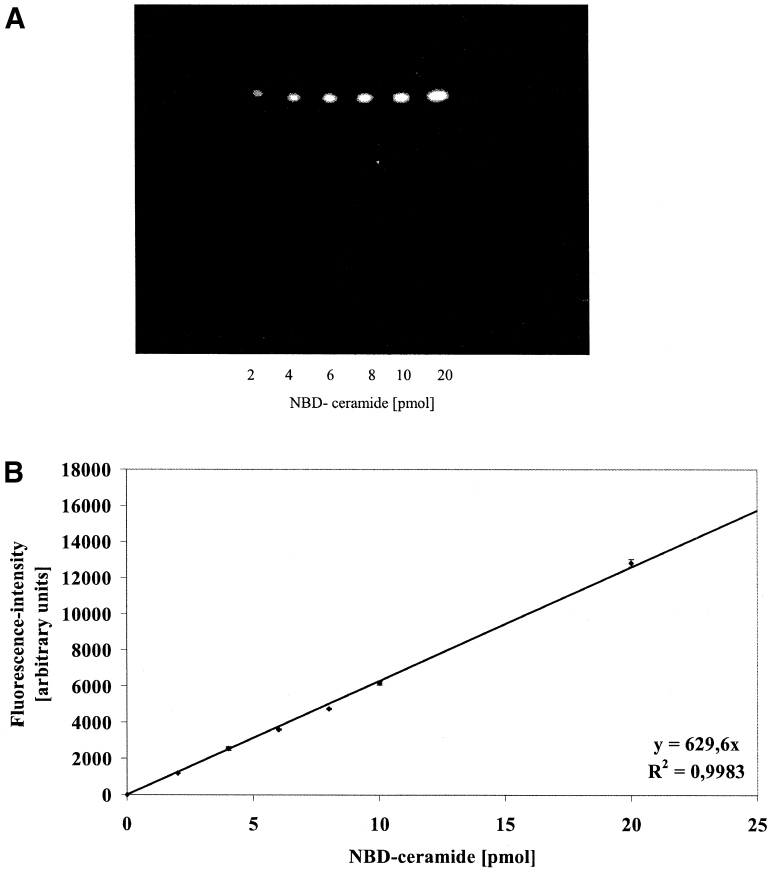

Fig. 3. Calibration of NBD-ceramide fluorescence on TLC plates. A: Increasing amounts of fluorescent ceramide were applied onto a TLC plate followed by chromatography using the same solvent as indicated in Fig. 2. Under these conditions NBD-ceramide appeared as a single spot at $R_F = 0.85$. The emission intensities of the fluorescent ceramide spots were measured as described in the legend to Fig. 2. A representative chromatogram is shown. B: Calibration plot of NBD-ceramide concentration versus fluorescence intensity as determined on silica gel plates. Indicated values are means ± SD ($n \geqslant 3$).

land WA) were routinely grown in DMEM supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin/streptomycin and 25 mM HEPES at 37°C in humidified CO_2 (8%) atmosphere. For SMase experiments cells were growth-arrested by incubation with DMEM + 0.1% FCS for 24 h.

Preparation of fluorescent sphingomyelin and ceramide

Sphingosylphosphocholine was prepared from egg sphingomyelin according to the method of Kaller (15). It was acylated by NBD-amino-dodecanoylhydroxysuccinimide ester in 5% aqueous $NaHCO_3$-Ethanol, 9:1 (v/v) to give crude *N*-(NBD-amino-dodecanoyl)-sphingomyelin. The pure product was obtained after purification by preparative TLC on silica gel using $CHCl_3$-CH_3OH-H_2O, 65:25:4 (v/v/v) as a solvent. It showed a single spot on TLC (R_F: 0.15; solvent as above).

N-(NBD-amino-dodecanoyl)-ceramide was obtained by hydrolytic cleavage of the corresponding sphingomyelin with sphingomyelinase from *Bacillus cereus* in 20 mM HEPES buffer, 1 mM $MgCl_2$, pH 7.4. The crude product was purified by column chromatography as described above. The pure compound showed a single spot on TLC (R_F: 0.85; conditions as above).

Isolation and oxidation of low-density lipoprotein

Human LDL (d = 1.019–1.063) was isolated from pooled fresh plasma using a Beckman NVT-rotor (16). LDL was oxidatively modified (oxLDL) as described by Watson (17). According to the latter procedure, mildly oxidized LDL was obtained. It is characterized by a moderate content of lipid peroxidation products [30–60 nmol of peroxide equivalents/mg of apolipoprotein B (apoB)] (18), and a slightly enhanced electrophoretic mobility (REM) (0.34) as compared with native LDL (0.30) in 0.8% agarose-gels.The protein content of isolated and modified LDL was determined by the method of Lowry (19).

Preparation of an aqueous dispersion of the fluorescent substrate

A solution of NBD-sphingomyelin in tetrahydrofuran (20 nmol in 30 μl) was injected with a hamilton syringe into 2 ml acid reaction buffer (250 mM sodium-acetate, 1 mM EDTA, pH 5.0) for determination of acid SMase-activity, into 2 ml of neutral Mg^{2+}-containing reaction buffer (20 mM HEPES, 1 mM $MgCl_2$, pH 7.4) for determination of neutral Mg^{2+}-dependent SMase-activity or into 2 ml of neutral reaction buffer (20 mM HEPES, pH 7.4) for determination of neutral Mg^{2+}-independent SMase-activity, under stirring at room temperature. The final substrate concentration was 10 μM.

Fluorescence sphingomyelinase assay

For determination of SMase-activity as a function of substrate concentration, 1.6 units of acid SMase from human placenta were incubated with NBD-sphingomyelin (absolute amounts between 0.2 and 9.6 nmol) in a total volume of 200 μl for 1 h at 37°C. The reaction was stopped by adding 600 μl $CHCl_3$-CH_3OH, 2:1 (v/v) for lipid extraction. The solvent of a 100 μl aliquot of the organic phase was removed under N_2, the residue redissolved in 10 μl $CHCl_3$, and the resultant solution was applied onto a TLC plate.

The labeled lipids were separated on silica TLC plates using $CHCl_3$-CH_3OH-H_2O, 65:25:4 (v/v/v) as solvent (20). Under these conditions, NBD-SM and NBD-ceramide appear as single spots at $R_F = 0.15$ and $R_F = 0.85$, respectively. The fluorescence intensities were determined with a Herolab CCD camera (excitation at 365 nm) using EasyWin®-software for data acquisition and

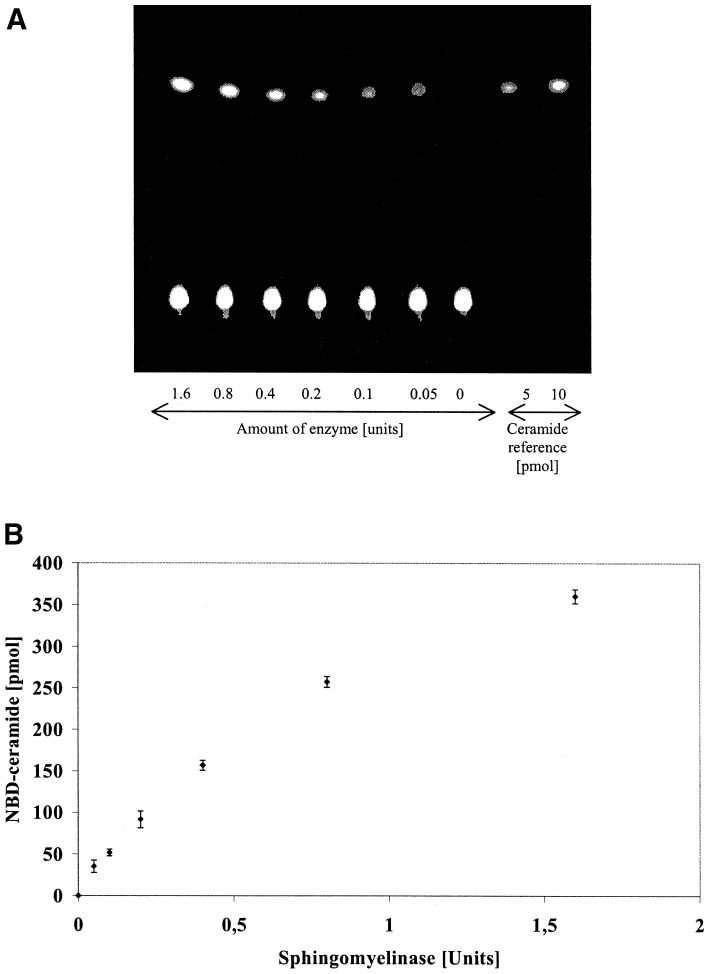

Fig. 4. Activity of acid sphingomyelinase (aSMase). Effect of enzyme conzentration. A: Increasing amounts of acid SMase from human placenta (0.05 units to 1.6 units) were incubated with 9.6 nmol NBD-SM in acid reaction buffer (250 mM sodium-acetate, 1 mM EDTA, pH 5.0) in a total volume of 200 μl. Enzyme activities were determined as indicated in the legend to Fig. 2A. B: Plot of pmol NBD-ceramide generated from NBD-SM by acid SMase vs. the amount of enzyme in the reaction mixture. The indicated values are means ± SD. ($n \geqslant 3$).

processing. Absolute amounts of ceramide/pmol were obtained from a calibration plot of the amount of NBD-ceramide (0.2 to 20 pmols) versus fluorescence intensity.

SMase-activity as a function of enzyme-concentration (0.05 to 1.6 U) was determined at nonlimiting amount substrate concentration (9.6 nmol lipid in the incubation mix).

Radioactive sphingomyelinase assay

Basically, the same procedure was used as described previously (21). A mixture of [^{3}H]sphingomyelin (^{3}H-labeled in the choline moiety, 81.5 Ci /mmol) and unlabeled egg yolk sphingomyelin served as a substrate. Experimental conditions (buffers, total lipid concentration) for assaying SMase activity were the same as indicated above for the fluorescence method. Radioactivity in a typical assay mixture was 1×10^6 cpm. SMase activity was determined from the amount of radioactive choline released into the aqueous phase using LCS safety cocktail for liquid scintillation counting.

Determination of acid and neutral SMase-activity in SMC cell extracts

SMCs were incubated with 0.1 ng/ml TNFα, native LDL, minimally modified LDL (60 μg Protein/ml each), or 10 μM [C_6]ceramide in six-well plates (9.6 cm^2/well) for 15 min to 8 h. Cells were washed with ice-cold PBS, scraped from the multi-well plates, and isolated by centrifugation. Acid lysis buffer (250 mM sodium-acetate, 0.2% Triton X-100, pH 5.0) for analyzing acid SMase-activity, neutral Mg^{2+}-containing lysis buffer (20 mM HEPES, 10 mM $MgCl_2$, 2 mM EDTA, 5 mM DTT, 1 mM NaF, 0.1 mM Na_2MoO_4, 1 mM PMSF, 10 μg/ml Leupeptine, 10 μg/ml Benzamidine, 10 μg/ml Soja-Trypsine inhibitor, 0.2% Triton X-100, pH 7.4) for analyzing neutral Mg^{2+}-dependent SMase or neutral lysis buffer (20 mM HEPES, 2 mM EDTA, 5 mM DTT, 1 mM NaF, 0.1 mM Na_2MoO_4, 1 mM PMSF, 10 μg/ml Leupeptine, 10 μg/ml benzamidine, 10 μg/ml Soja-Trypsine inhibitor, 0.2% Triton X-100, pH 7.4) for analyzing neutral Mg^{2+}-independent Smase were added (60 μl/9.6 cm^2 well), and samples were incubated on ice for 1 h (vortexing every 15 minutes). The samples were then subjected to centrifugation at 14,000 *g* at 4°C for 5 min. The supernatant was isolated and an aliquot containing 20 μg protein (determined by the method of Bradford) (22) was analyzed for acid and neutral SMase-activity, respectively, under the conditions described above. The reaction was stopped by addition of organic solvent. For the determination of acid SMase-activity, an 100 μl aliquot of the organic phase was evaporated under a nitrogen stream. The residue was dissolved in 10 μl $CHCl_3$ and applied to a TLC silica-gel plate. For TLC analysis of neutral SMase-activity a 300 μl aliquot of the organic phase was exposed to a N_2 stream and the residue was dissolved in 10 μl $CHCl_3$ for TLC analysis.

RESULTS

Determination of SMase-activity

Sphingomyelinases in intact cells hydrolyze sphingomyelin leading to the formation of ceramide and phosphatidylcholine (23). For determination of SMase-activity in vitro,

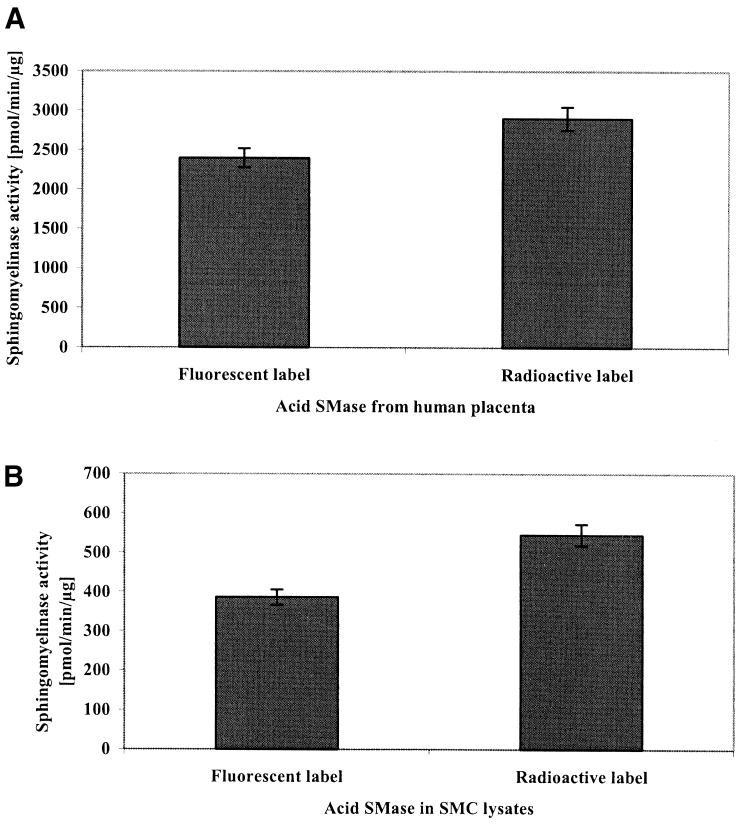

Fig. 5. Activity of acid SMase. Comparison of fluorescent and radiolabeled substrate. B: Determination of aSMase activity in smooth muscle cell (SMC) lysates. Fluorescent NBD-sphingomyelin (2 nmol) or a mixture of unlabeled sphingomyelin with radiolabeled [^{3}H]SM (total lipid 2 nmol) in acid reaction buffer (250 mM sodium-acetate, 1 mM EDTA, pH 5.0) were added to lysates of SMC containing 30 µg protein. Lysates were prepared and enzyme activities were determined as described in Materials and Methods. A: Determination of activity of acid SMase from human placenta. Fluorescent NBD-SM or radiolabeled [^{3}H]choline methyl SM in acid reaction buffer were incubated with 1.6 U aSMase. SMase activities were measured as described above. Indicated SMase activities are means ± SD ($n \geq 4$).

we have developed a high-throughput method based on a fluorescent substrate analog (NBD-sphingomyelin) (**Fig.1**) that is hydrolyzed to form the corresponding ceramide. After the reaction the remaining substrate and the product are extracted by organic solvent and separated by TLC followed by fluorescence analysis using a CCD camera.

Figure 2A shows a typical fluorescence image of a thin layer chromatogram of organic lipid extracts that were obtained from reaction mixtures containing different amounts of substrates after treatment with acid SMase. The amount of ceramide formed (pmol/min) was determined from the fluorescence intensity of the upper spot and plotted against the substrate concentration used (Fig. 2B).

For the determination of absolute amounts of the reaction product, a calibration plot was generated with defined amounts of NBD-ceramide. The respective lipid samples were subjected to TLC and fluorescence intensities of the fluorescent lipid spots were determined. If adequately low amounts of fluorescent NBD-lipids (pmoles) are applied onto the silica gel plate, a linear relationship between fluorescence intensity and fluorophore concentration was found (**Fig. 3**) with NBD-ceramide as well as NBD-SM (results not shown). The correlation coefficient r^2 was $\geq$0.99 in both cases. The linearity range is between 0.1 pmol and 2,000 pmol fluorophore (detection limit: 0.2 pmol of the fluorophore).

Figure 4 shows the results of a typical fluorescence analysis when NBD-SM substrate was subjected to various amounts of acid SMase. The amount of the fluorescent reaction product (ceramide, R_F = 0.85) was determined as a measure for enzyme activity, and showed a linear relationship with enzyme concentration. Activities based on the fluorescent substrate were 85% and 82.5% of the activities that were determined using the radiolabeled substrate for aSMase in cell lysates and isolated aSMase from human placenta, respectively (**Fig. 5**).

SMase-activity in human SMCs

The described fluorescence assay was applied to the determination of neutral and acid SMase activities in human SMC after incubation with various agents, namely native LDL (natLDL), minimally modified LDL (mmLDL) (each 60 µg Protein/ml), ceramide (10 µM), or TNFα (0.1 ng/ml) for 1 h. The respective systems promote (intracellular) signaling at different levels and as a consequence influence cell growth or death (24).

SMase-activity in SMC was determined as pmol ceramide formed/µg protein (**Figs. 6–8**). Absolute levels of SMases in untreated cells were 386 pmol/min/µg protein for acid, 39.2 pmol/min/µg protein for neutral Mg^{2+}-dependent Smase, and 53 pmol/min/µg for Mg^{2+}-independent neutral SMase (Fig. 6).

TNFα (0.1 ng/ml), which is known to activate both acid and neutral SMase (25, 26), led to a strong increase (926 pmol/min/µg, 240% of control) in acid SMase-activity of SMCs after 1 h (Fig. 7). TNFα also stimulated neutral SMase (Fig. 8) in SMCs (76,6 pmol/min/µg, 195% of control), although to a lower extent as compared with acid SMase (Fig. 7).

Modified LDL at a concentration that is mitogenic for SMC (60 µg/ml of apoB) induced an increase in acid (Fig. 7) and neutral (Fig. 8) SMase-activities, whereas natLDL produced similar but much lower effects. MmLDL (60 µg protein/ml) activated acid SMase (741 pmol/min/µg, 192% of control) nearly as effectively as a stimulation by 0.1

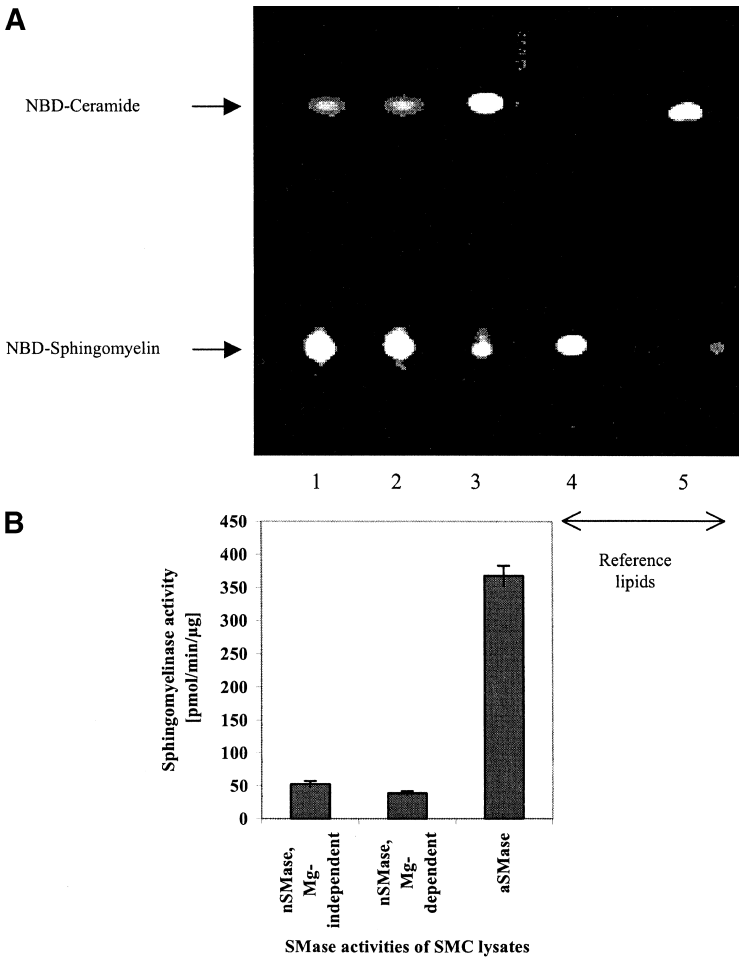

Fig. 6. Activities of acid, neutral Mg^{2+}-dependent, and neutral Mg^{2+}-independent SMase in SMC lysates. SMCs were lyzed in lysis buffer for assaying neutral Mg^{2+}-independent (1), neutral Mg^{2+}-dependent (2), and acid (3) SMases. Enzyme activities were determined as described under Materials and Methods. For TLC, NBD-SM (4) and NBD-ceramide (5) were used as reference lipids. Indicated enzyme activities are means ± SD (n = 3).

ng/ml TNFα (Fig. 7). Neutral SMase in SMC was also activated by mmLDL (Fig. 8)

Stimulation of SMC with 10 μM ceramide for 1 h resulted in an increase of acid SMase-activity by 170% of the control (656 pmol/min/μg, Fig. 8A). On the other hand, neutral SMase remained unaffected in the presence of 10 μM ceramide after 1 h (Fig. 8B). Activation of neutral SMase by 10 μM ceramide was only observed after incubation of SMC for 7–8 h (data not shown).

A more detailed analysis was carried out to study the time-dependence of the activation of acid and neutral SMase by mmLDL and native LDL in SMCs (**Figs. 9A**, **B**).

Freshly prepared LDL showed only marginal effects on both enzymes after short incubation times. In contrast, activation maxima were observed already after 60 min exposure of SMC to modified lipoproteins. Both native and mmLDL led to similar activation effects on acid and neutral SMases after prolongated (6 h) exposure times.

DISCUSSION

Sphingomyelinase is an important enzyme of signal transduction (27). It generates ceramide, which is an important second messenger involved in cell proliferation (28) as well as apoptosis (29, 30, 31). Whether ceramide induces proliferation or apoptosis does not only depend on the cell type but also on the localisation and pH-optimum of the respective SMase isoform (32). Whereas lysosomal acid SMase is likely to play an important role in induction of cell death (22, 33), activation of Mg^{2+}-dependent neutral SMase may lead to proliferation (33, 34).

Our assay, which is based on a fluorescent substrate, is reliable since it measures very similar activities as compared with established assays based on radioactive substrates. It is suitable for determination of all isoforms of SMase. It replaces the common radioactive methods (10–14) and avoids obvious expenditures for waste and contamination.

A linear correlation was found between enzyme mass and enzyme-activity between 0.05 and 1 unit, determined under conditions of non-limiting substrate concentrations.

We examined the activities of neutral and acid SMase in stimulated SMC using the new assay and found an increase in SMase activities depending on the isoform of the enzyme and the stimulus. TNFα is a cytokine known to be a signal for cells to undergo apoptosis (27). NatLDL represents a growth signal, whereas oxLDL is a pathophysiologic stimulus of proliferation and/or death of vascular cells. All these agents exerted stimulating effects on the activity of aSMase and neutral SMase in SMC, leading to the formation of ceramide, a lipid second messenger. Cer-

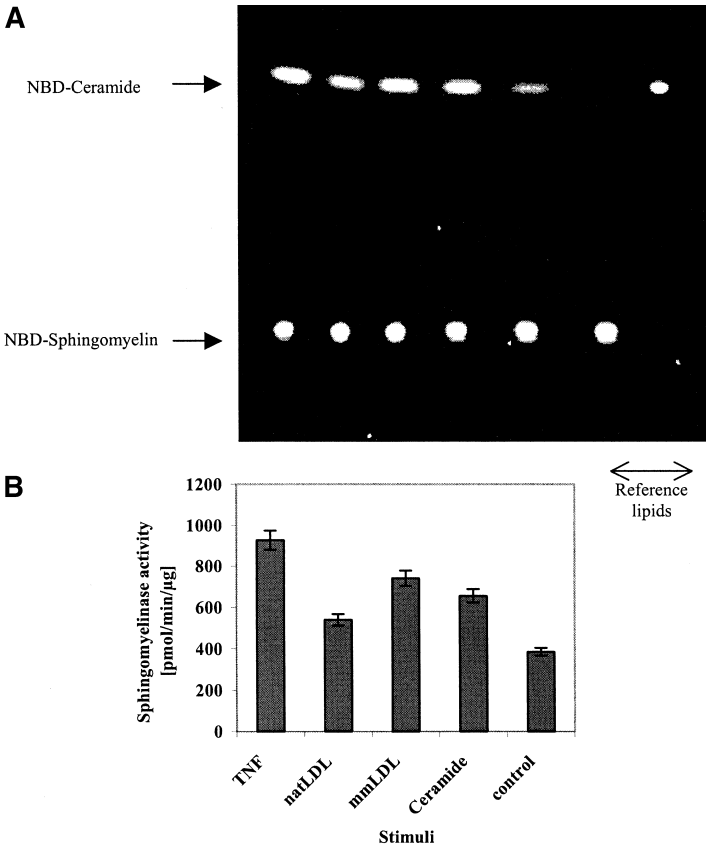

Fig. 7. Acid SMase activity in SMC lysates. Effects of LDL, tumor necrosis factor α (TNFα), and ceramide. A: TLC analysis of lipids. Cells were stimulated by TNFα (0.1 ng/ml), native (nat)LDL (60 μg Protein/ml), minimally modified (mm)LDL (60 μg Protein/ml) or 10 μM ceramide in DMEM + 0.1% FCS or DMEM + 0.1% FCS only (control) for 1 h followed by cell lysis in acid lysis buffer. Enzyme activities were determined as described under Materials and Methods. For TLC, NBD-SM and ceramide (5 pmol each) were used as reference lipids. B: Acid SMase activities (pmol/min/μg). SMase activities of stimulated SMCs were determined from the formation of fluorescent ceramide (upper panel). Indicated values are means ± SD (n ⩾ 3).

amide interferes with several down-stream targets, which are part of the pathways leading to apoptosis and proliferation, respectively (35).

In contrast to results obtained with other cell types (36), we found an increase of enzyme activities of both SMase isoforms in SMCs with a maximum after 1 h for both isoforms.

MmLDL but not natLDL led to a pronounced activation of acid and neutral SMase already between 30 and 60 min incubation time. Freshly prepared LDL showed much lower effects under these conditions. In addition, a second increase of acid and neutral SMase-activities was reached after several hours of incubation with mmLDL. However, the same long-term effect was obtained with natLDL.

It is striking that acid and neutral SMase are similarly affected by mmLDL after very short time. Thus, it is likely that immediate physical interactions between the lipoproteins (lipids) and the cell surface membrane or forthcoming oxidative modifications are involved in these phenomena. This would be in agreement with the hypothesis that both isoforms are associated with the plasma membrane and thus are subject to modulations by membrane-active components (6, 37). Research is in progress to define the molecular basis for SMase activation by mmLDL lipids.

In this paper we have described the principle and useful applications of the fluorescence SMase-assay for the analysis of SMase activities in vitro and in cells (SMC). The described new method may be useful for determination of SMase activity in various fields of pathobiochemistry, where these enzymes may play a role, e.g., atherosclerosis, cancer, and immunology (4, 35, 38). For this purpose, the described assay for the determination of SMase-activity provides a sensitive, reproducible, and very simple method in the research laboratory and for routine laboratory practice.

The authors are grateful for financial support by Österreichische Nationalbank (project No. 6939), Österreichischer Herzfonds, and Österreichischer Forschungsfonds (FWF project No. P14714-PHA).

Manuscript received 19 November 2001 and in revised form 8 February 2002.

REFERENCES

1. Hannun, Y. A. 1994. The sphingomyelin cycle and the second messenger function of ceramide. *J. Biol. Chem.* **269:** 3125–3128.
2. Chatterjee, S. 1993. Neutral sphingomyelinases. *Adv. Lipid Res.* **26:** 25–48.
3. Hofmann, K., and V. M. Dixit. 1998. Ceramide in apoptosis—does it really matter? *Trends Biochem. Sci.* **23:** 374–377.
4. Prieschl, E. E., and T. Baumruker. 2000. Sphingolipids: second messengers, mediators and raft constituents in signalling. *Immunol. Today.* **21:** 555–560.
5. Kinscherf, R., R. Claus, H. P. Deigner, O. Nauen, C. Gehrke, A. Hermetter, S. Ruβwurm, V. Daniel, V. Hack, and J. Metz. 1997. Modified low density lipoprotein delivers substrate for ceramide formation and stimulates the sphingomyelin-ceramide pathway in human macrophages. *FEBS Lett.* **405:** 55–59.
6. Kinscherf, R., R. Claus, M. Wagner, C. Gehrke, H. Kamencic, D. Hou, O. Nauen, W. Schmiedt, G. Kovacs, J. Pill, J. Metz, and H. P. Deigner. 1998. Apoptosis caused by oxidized LDL is manganese superoxide dismutase and p53 dependent. *FASEB J.* **12:** 461–467.

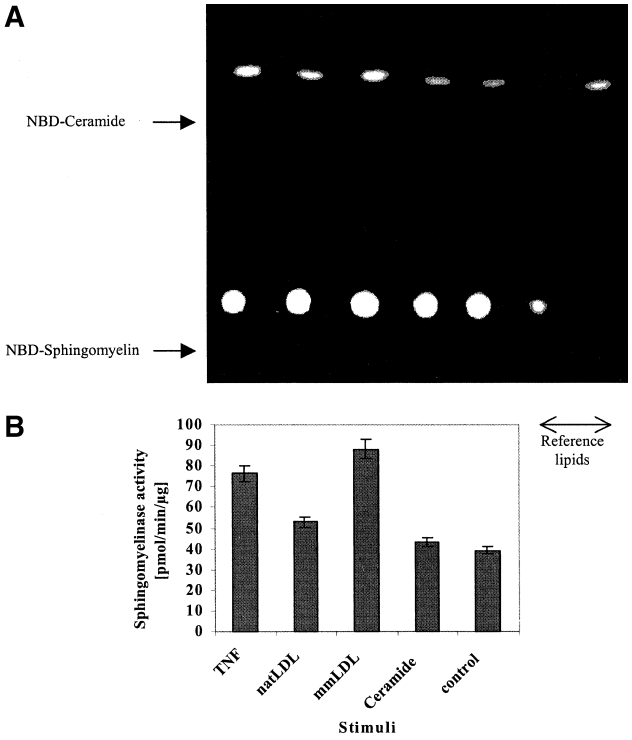

Fig. 8. Neutral SMase activity in SMC lysates. Effects of LDL, TNFα, and ceramide. A: TLC analysis of lipids. Cells were stimulated by TNFα (0.1 ng/ml), natLDL (60 μg Protein/ml), mmLDL (60 μg Protein/ml), 10 μM ceramide in DMEM + 0.1% FCS or DMEM + 0.1% FCS only (control) for 1 h followed by cell lysis in neutral Mg^{2+}-containing lysis buffer. Activites were determined as described in Materials and Methods. For TLC, NBD-SM and NBD-ceramide (5 pmol each) were used as reference lipids. B: Neutral SMase activities were determined from the formation of fluorescent ceramide. All indicated values are means ± SD (n ⩾ 3).

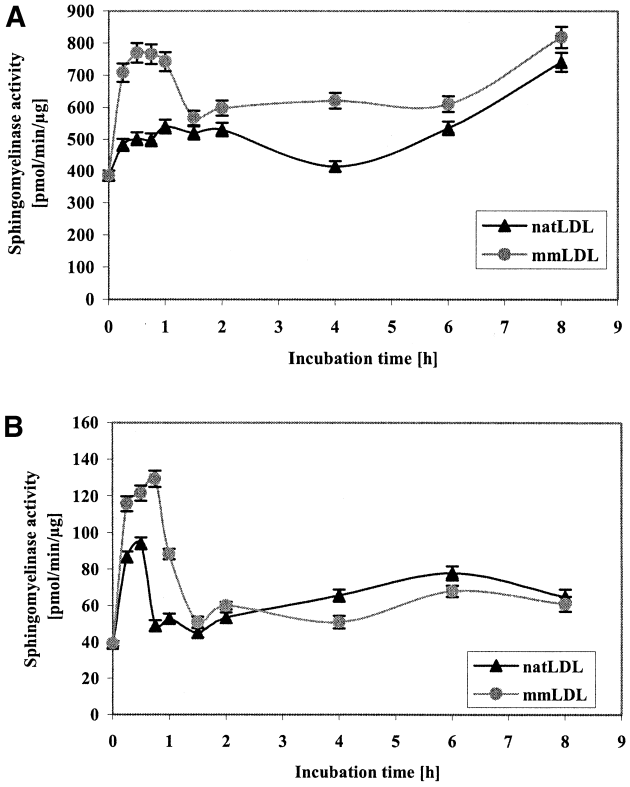

Fig. 9. Time-dependent activation of SMase-activity in SMCs by mmLDL and natLDL. A: Acid SMase. B: Neutral SMase. SMCs were incubated with LDL or mmLDL (60 μg Protein/ml each) or DMEM + 0.1% FCS (control) for different periods of times followed by analysis of enzyme activity as described in Figs. 7 and 8. Indicated activities are means ± SD (n ⩾ 4).

7. Augé, N., I. Escargueil-Blanc, I. Lajoie-Mazenc, I. Suc, N. Andrieu-Abadie, M. T. Pieraggi, M. Chatelut, J. C. Thiers, J. P. Jaffrézou, G. Laurent, T. Levade, A. Négre-Salvayre, and R. Salvayre. 1998. Potential role for ceramide in mitogen-activated protein kinase activation and proliferation of vascular smooth muscle cells induced by oxidized low density lipoprotein. *J. Biol. Chem.* **273:** 12893–12900.
8. Augé, N., N. Andrieu, A. Négre-Salvayre, J. C. Thiers, T. Levade, and R. Salvayre. 1996. The sphingomyelin-ceramide signaling pathway is involved in oxidized low density lipoprotein-induced cell proliferation. *J. Biol. Chem.* **271:** 19251–19255.
9. Deigner, H. P., R. Claus, G. A. Bonaterra, C. Gehrke, N. Bibak, M. Blaess, M. Cantz, J. Metz, and R. Kinscherf. 2001. Ceramide induces aSMase expression: implications for oxLDL-induced apoptosis. *FASEB J.* **15:** 807–814.
10. Levade, T., M. Leruth, D. Graber, A. Moisand, S. Vermeersch, R. Salvayre, and P. J. Courtoy. 1996. In situ assay of acid sphingomyelinase and ceramidase based on LDL-mediated lysosomal targeting of ceramide-labeled sphingomyelin. *J. Lipid Res.* **37:** 2525–2538.
11. Barbone, G. A., A. C. Jackson, D. M. Ritchie, and D. C. Argentieri. 1999. Robotic assay for sphingomyelinase activity for high throughput screening. *Methods Enzymol.* **311:** 168–176.
12. Hassler, D. F., R. M. Laethem, and G. K. Smith. 1999. A high throughput sphingomyelinase assay. *Methods Enzymol.* **311:** 176–184.
13. Liu, B., and Y. A. Hannun. 1999. Sphingomyelinase assay using a radiolabeled substrate. *Methods Enzymol.* **311:** 168–184.
14. Gulbins, E., and R. Kolesnick. 1999. Measurement of sphingomyelinase activity. *Methods Enzymol.* **322:** 382–388.
15. Kaller, H. 1961. Präparative Gewinnung von Sphingosin-phosphorylcholin. *Biochem. Z.* **334:** 451–456.
16. Chung, B. H., T. Wilkinson, J. C. Geer, and J. P. Segres. 1980. Preparative and quantitative isolation of plasma lipoproteins: rapid, simple discontinuous density gradient ultracentrifugation in a vertical rotor. *J. Lipid Res.* **21:** 284–291.
17. Watson, A. D., N. Leitinger, M. Navab, K. F. Faull, S. Horkko, J. L. Witztum, W. Palinski, D. Schwenke, R. G. Salomon, W. Sha, G. Subbanagounder, A. M. Fogelman, and J. A. Berliner. 1997. Structural identification by mass spectrometry of oxidized phospholipids in minimally oxidized low density lipoprotein that induce monocyte/endothelial interactions and evidence for their presence in vivo. *J. Biol. Chem.* **272:** 13597–13607.
18. el-Saadani, M., H. Esterbauer, M. el-Sayed, M. Goher, A. Y. Nassar, and G. Jurgen. 1989. A spectrophotometric assay for lipid peroxides in serum lipoproteins using a commercially available reagent. *J. Lipid Res.* **30:** 627–630.
19. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
20. Lipsky, N., and E. Pagano. 1983. Sphingolipid metabolism in cultured fibroblasts: microscopic and biochemical studies employing a fluorescent ceramide analogue. *Proc. Natl. Acad. Sci.* **80:** 2608–2612.
21. Lansmann, S., O. Bartelsen, and K. Sandhoff. 1999. Purification and characterization of recombinant human acid sphingomyelinase expressed in insect Sf21 cells. *Methods Enzymol.* **311:** 149–156.
22. Bradford, M. M. 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248–254.
23. Spence, M. W. 1993. Sphingomyelinases. *Adv. Lipid Res.* **26:** 3–21.
24. Mathias, S., L. A. Pena, and R. Kolesnick. 1998. Signal transduction of stress via ceramide. *Biochem. J.* **335:** 465–480.
25. Zhang, P., B. Liu, G. M. Jenkins, Y. A. Hannun, and L. M. Obeid. 1997. Expression of neutral sphingomyelinase identifies a distinct pool of sphingomyelin involved in apoptosis. *J. Biol. Chem.* **272:** 9609–9612.

26. Wiegmann, K., S. Schütze, T. Machleidt, D. Witte, and M. Krönke. 1994. Functional dichotomy of neutral and acidic sphingomyelinases in tumor necrosis factor signaling. *Cell.* **78:** 1005–1015.
27. Levade, T., and J. P. Jaffrézou. 1999. Signalling sphingomyelinases: which, where, how and why? *Biochim. Biophys. Act.* **1438:** 1–17.
28. Bielawska, A., M. Corinne, and Y. A. Hannun. 1992. Modulation of cell growth and differentation by ceramide. *FEBS.* **307:** 211–214.
29. Obeid, L. M., and Y. A. Hannun. 1995. Ceramide: a stress signal and mediator of growth suppression and apoptosis. *J. Cell. Biochem.* **58:** 191–198.
30. Hannun, Y. A., and L. M. Obeid. 1995. Ceramide: an intracellular signal for apoptosis. *Trends Biochem. Sci.* **20:** 73–77.
31. Kirschnek, S., F. Paris, M. Weller, H. Grassmé, K. Ferlinz, A. Riehle, Z. Fuks, R. Kolesnick, and E. Gulbins. 2000. CD95-mediated apoptosis *in vivo* involves acid sphingomyelinase. *J. Biol. Chem.* **275:** 27316–27323.
32. Testi, R. 1996. Sphingomyelin breakdown and cell fate. *Trends Biochem. Sci.* **21:** 468–471.
33. Kolesnick, R. N., and M. Krönke. 1998. Regulation of ceramide production and apoptosis. *Ann. Rev. Physiol.* **60:** 643–645.
34. Olivera, A., N. E. Buckley, and S. Spiegel. 1992. Sphingomyelinase and cell-permeable ceramide analogs stimulate cellular proliferation in quiescent swiss 3T3 fibroblasts. *J. Biol. Chem.* **267:** 26121–26127.
35. Rudin, C. M., and C. B. Thompson. 1997. Apoptosis and disease: regulation and clinical relevance of programmed cell death. *Annu. Rev. Med.* **48:** 267–281.
36. Jaffrézou, J. P., N. Maestre, V. De Mas-Mansat, C. Bezombez, T. Levade, and G. Laurent. 1998. Positive feedback control of neutral sphingomyelinase activity by ceramide. *FASEB J.* **12:** 999–1006.
37. Leitinger, N., and J. A. Berliner. 1999. MM-LDL and atherogenesis—a major role for phospholipid oxidation products. *In* Oxidative Stress and Vascular Disease, Developments in Cardiovascular Disease, Vol. 224. J. F. Keaney Jr., editor. Kluwer Academic publishers, Boston/Dordrecht/London. 117–134.
38. Chatterjee, S. 1998. Sphingolipids in atherosclerosis and vascular biology. *Arterioscler. Thromb. Vasc. Biol.* **18:** 1523–1533.