The treatment of C6 glioma cells with the nitric oxide donor, PAPANONOnate, resulted in a dose-dependent inhibition of cell proliferation. This was associated to a rapid and significant increase of ceramide levels and was mimicked by treatments that augment cellular ceramide. Metabolic experiments with radioactive sphingosine, serine, and choline showed that nitric oxide strongly reduced the utilization of ceramide for the biosynthesis of both sphingomyelin and glucosylceramide. Nevertheless, nitric oxide did not modify the activity of different enzymes of ceramide metabolism. The possibility that nitric oxide impairs the availability of ceramide for sphingolipid biosynthesis was then investigated. The metabolism of N-hexanoyl-[3H]sphingosine demonstrated that nitric oxide did not affect the biosynthesis of N-hexanoyl-[3H]sphingolipids but inhibited the metabolic utilization of long chain [3H]ceramide, synthesized in the endoplasmic reticulum (ER) from the recycled [3H]sphingosine. Moreover, results obtained with fluorescent ceramides, brefeldin A, ATP depletion, as well as in a ceramide transport assay indicate that nitric oxide impairs the traffic of ceramide from ER to Golgi apparatus. All this supports that, in glioma cells, the modulation of ceramide traffic can contribute to the regulation of its intracellular levels and participate in the nitric oxide-activated signaling pathway involved in the control of cell proliferation.

During the past 10 years, a great amount of evidence has demonstrated that ceramide (Cer), a key molecule in sphingo-
standing this evidence, the molecular mechanisms underlying these effects on glial cell growth are still largely unknown. Furthermore, in some extraneuronal cells, recent evidence shows that NO may exert an apoptotic effect by increasing Cer level through the regulation of the metabolic pathways involved in its generation or removal (26, 27).

In this study we investigate the possible involvement of Cer in the inhibitory role exerted by NO on C6 glioma cell proliferation. In particular, we focus on the effects of NO on the in vivo mechanisms involved in the control of cellular Cer levels. Here, we present evidence that in C6 glioma cells NO promotes a Cer increase by inhibiting its transport, possibly by a NO-mediated, from ER to Golgi apparatus. As a consequence, newly synthesized Cer accumulates and appears to act as a mediator to the antiproliferative effect of NO.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco’s modified Eagle’s medium (DMEM), fetal calf serum (FCS), brefeldin A (BFA), bovine serum albumin fraction V (BSA), fatty acid free-BSA, sphingomyelinase (SMAse) from Staphylococcus aureus, UDP-glucose (UDP-GlcF), fumonisin B1 (FB1), N-acetyl-D-erythro-sphingosine (C2-Cer), N-(hexoyl-N-erythro-sphingosine (C6-Cer), and N-acetyl-N-dihydrophosphingosine (C2-DHCer) were from Sigma Chemical Co. (St. Louis, MO). [methyl-3H]Thymidine (20 Ci/mmol), [3H]choline (19 Ci/mmol), [3H]sphingosine (23 Ci/mmol), DOG-3H-acl-lys (C2-DHCer) were from PANonoate, 10 M cGMP, 10 M PAPANOate. In chase experiments, after a 1-h pulse with the radiolabeled compound (2 μCi/ml), the cells were maintained in DMEM plus 10% FCS with or without 400 μM PAPANOate. At appropriate pulse or chase times, the cells were washed twice with PBS at 4 °C and harvested, and total lipids were extracted and processed as previously described (28).

[sph–H]Sphingomyelin, [3H]Sphingosine, and N-Hexanoyl-[3H]-sphingosine Metabolism in Intact Cells—C6 glioma cells plated at 1.5 × 10^5/cm² were grown on a 35-mm dish. The cultures were incubated in DMEM plus 10% FCS. Stock solutions of [Sph–H]Sph, [3H]Sph, and [Sph–H]C6-Cer in absolute ethanol were prepared and added to fresh medium. In all cases the final concentration of ethanol never exceeded 0.1% (v/v). The cells were pulsed for different times with [Sph–H]Sph (1 μCi/ml), [Sph–H]C6-Cer (0.6 μCi/ml) with or without 400 μM PAPANOate. To evaluate the effect of BFA and ATP depletion on [H]Sph metabolism, the cells were preincubated for 30 min at 37 °C with 1 μg/ml BFA or 20 μM 2-deoxy-d-glucose, 2 mM NaF (28), in all cases the same conditions were maintained during the [H]Sph pulse. At different pulse times the cells were washed twice with PBS at 4 °C and harvested, and total lipids were extracted and processed as previously described (28).

In Vitro Enzyme Assays—SMAse, SM synthase, and GlcCer synthase activities were assessed using as enzyme source cell homogenate (obtained by sonication in H2O three times, 10 s at 4 °C) of control and PAPANOate-treated cells. Mg2+-dependent neutral sphingomyelinase (N-SMase) and acidic sphingomyelinase (A-SMase) were assayed using [Sph–H]Sph as substrate as previously described (29). The N-SMase incubation mixture contained 20 mM Tris-Cl (pH 7.4), 10 mM MgCl2, 0.1% Triton X-100, 250 μM [Sph–H]Sph (0.1 μCi), and 5-20 μg of cell protein in a final volume of 25 μl. The A-SMase incubation mixture contained 200 mM acetic acid buffer (pH 5.0), 10 mM EDTA, 0.1% Triton X-100, 250 μM [Sph–H]Sph (0.1 μCi), and 2-10 μg of cell protein in a final volume of 25 μl. After 30-min incubation, the reactions were stopped by adding 100 μl of chloroform/methanol (2:1, by volume). That [3H]sphingosine kinase activity was assessed as previously described (31), with minor modifications. The incubation mixture contained 50 mM Tris-Cl (pH 7.4), 25 mM KC1, 0.5 mM EDTA, 2 mM (0.05 μCi) of [Sph–H]C6-Cer (as 1:1 complex with fatty acid-free BSA), and 15 μg of cell protein in a final volume of 50 μl. After 15-min incubation at 37 °C, the reaction was stopped by adding 150 μl of chloroform/methanol (1:2, by volume) at 4 °C. GlcCer synthase activity was assessed as previously described (30) with minor modifications. The incubation mixture contained 50 mM Tris-Cl (pH 7.4), 25 mM KC1, 0.5 mM EDTA, 2 mM (0.05 μCi) of [Sph–H]C6-Cer (as 1:1 complex with fatty acid-free BSA), and 15 μg of cell protein in a final volume of 50 μl. After 15-min incubation at 37 °C, the reaction was stopped by adding 150 μl of chloroform/methanol (1:2, by volume) at 4 °C. In all cases, after lipid extraction and phase separation, the [3H]lipids were resolved by HPTLC as previously described (16).

Sphingosine kinase activity was assessed as described (31). Briefly, control and PAPANOate-treated cells were washed with PBS and scraped in Sph kinase buffer (20 mM Tris-Cl (pH 7.4) containing 20% glycerol, 1 mM mercaptoethanol, 1 mM orthovanadate, and 1 mM EDTA, 5 mM NaF, 0.5 mM 4-deoxyyporidine, 0.4 mM phenylmethylsulfonyl fluoroacetate, 0.2 mM β-mercaptoethanol, and 10 μg/ml each of leupeptin, aprotinin, and trypsin inhibitor). Cells were disrupted by freeze and thawing and centrifuged at 105,000 × g for 90 min. An aliquot of the supernatant (20–50 μg of protein) was diluted in Sph kinase buffer, then Sph (50 μM final concentration as a 1:1 complex with fatty acid-free BSA), and 15 μg of cell protein in a final volume of 50 μl. After 15-min incubation at 37 °C, the reaction was stopped by adding 150 μl of chloroform/methanol (1:2, by volume) at 4 °C. In all cases, after lipid extraction and phase separation, the [3H]lipids were resolved by HPTLC as previously described (16).
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**RESULTS**

**Effect of NO on C6 Glioma Cell Proliferation**—To investigate the effect of exogenously delivered NO on C6 glioma cell proliferation, cells were treated with different concentrations of the NO-releasing molecule PAPANONOate. In the 100–400 μM range, PAPANONOate induced a dose-dependent inhibition of cell proliferation (Fig. 1, left panel). The maximal inhibitory effect on [3H]thymidine incorporation of about 80% was reached at 400 μM PAPANONOate. In these experimental conditions, at both 24 and 48 h after treatment with the NO donor, cell viability, assessed by trypan blue exclusion, was not affected. This indicates that the effect of the NO-releasing compound on [3H]thymidine incorporation results from the inhibition of cell growth and not from toxicity. Moreover, when PAPANONOate was used after being decayed for 24 h, no effect on [3H]thymidine incorporation was observed, indicating that the effect of PAPANONOate on C6 glioma cell proliferation was due to NO generation. As shown in Fig. 1 (right panel), dibutyl cGMP (2 mM) was also effective in inhibiting [3H]thymidine incorporation, suggesting a role for the cGMP/PKA pathway in NO-mediated effects on cell proliferation.

**Fig. 1. Effect of NO and cGMP on C6 glioma cell proliferation.** Quiescent C6 glioma cells were incubated 24 h in DMEM plus 10% FCS with different concentrations of PAPANONOate (left panel) or dibutyryl-cGMP (right panel). In the last 4 h of incubation, cells were pulsed with 1 μCi of [3H]thymidine, and the radioactivity associated with trichloroacetic-insoluble material was determined. Results are shown as a percentage of control, untreated cells. All values are the mean ± S.D. of at least three individual experiments. *, p < 0.001 versus control.
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Involvement of Cer in the NO-induced Cell Growth Inhibition of C6 Glioma Cells—We next evaluated the possible involvement of Cer as a mediator for the antiproliferative activity of NO in C6 glioma cells. To this purpose, we first measured intracellular Cer levels at various intervals after the administration of PAPANONOate to cells labeled to the equilibrium with \(^{3}H\)Sph. In preliminary experiments, we set up the labeling conditions so as to obtain a \(^{3}H\)GM3/\(^{3}H\)SM ratio corresponding to that of the endogenous compounds as an index for a steady-state labeling of cell sphingolipids. As shown in Fig. 2, in these conditions, exogenously delivered NO caused a rapid and significant increase of the Cer cellular level. The maximum increase, corresponding to 167% of the control levels, was reached 4 h after NO treatment. Cer levels in NO-treated cells remained significantly higher than in controls, even after 24 h.

In addition, we obtained evidence that those treatments able to increase Cer cellular levels mimicked the NO-induced cell growth inhibition (Fig. 3). In particular, the incubation of C6 cells with cell-permeable analogs C2- or C6-Cer as well as the treatment with bacterial SMase resulted in a significant reduction of \(^{3}H\)thymidine incorporation into DNA. Under the same conditions, the C2-Cer dihydro-derivative had no effect.

Effect of NO on \(^{1}H\)SM and \(^{1}H\)Choline Metabolism and on SMase and SM Synthase Activities—To investigate the metabolic pathways involved in NO-induced modification of Cer levels, we first examined the effect of NO on SM degradation and biosynthesis. The possible contribution of SM degradation to the increase of Cer levels observed in NO-treated cells was evaluated in pulse experiments with [Sph-\(^{3}H\)]Sph, performed with or without PAPANONOate. After a 1-h pulse, similar values of [\(^{3}H\)]SM and \(^{3}H\)Cer were found in control and treated cells (Fig. 4, upper panel). In these conditions, \(^{3}H\)Sph formed from \(^{3}H\)Cer hydrolysis was 3.3% total incorporated radioactivity in control and 3.1% in treated cells, indicating that NO did not modify Cer cleavage by ceramidases. We also evaluated the effect of NO on \textit{in vitro} activity of N-SMase and A-SMase. Using homogenates obtained from control or NO-treated cells as the enzyme source, and adding PAPANONOate to the homogenate of control cells, no difference was observed in either N-SMase or A-SMase activities (Fig. 4, lower panel).

The possible effect of NO on SM biosynthesis was then evaluated by pulsing cells with \(^{3}H\)choline with or without PAPANONOate (Fig. 5, upper panel). At 2-h pulse, the incorporation of \(^{3}H\)choline into SM in NO-treated cells was about 40% of that measured in control cells, whereas the incorporation of the same precursor into phosphatidylcholine, the phosphorylcholine donor for the conversion of Cer to SM, was not affected by NO. On the basis of this evidence we assessed the effect of NO on \textit{in vitro} activity of SM synthase. Here too, the homogenates obtained from control or NO-treated cells were used as the enzyme source, and, in the case of control cells, the enzymatic activity was also measured in the presence of PAPANONOate. As shown in Fig. 5 (lower panel), neither NO cell treatment nor NO addition in the enzymatic assay affected SM synthase activity in C6 glioma cells.

Effect of NO on \(^{3}H\)Sph Metabolism—For further information on the effect of NO on SM biosynthesis, taking into account that in glial cells exogenous Sph is rapidly incorporated first in Cer and then in SM and glycosphingolipids (37, 38), we performed an additional metabolic study using \(^{3}H\)Sph. When C6 glioma cells were pulsed for 1 h with \(^{3}H\)Sph with or without PAPANONOate, the radioactive precursor was rapidly and efficiently incorporated in both control and treated cells. In both cases, \(^{3}H\)Sph was mainly metabolized to N-acylated compounds, most represented by Cer, SM, and, in lower amounts, GlcCer and GM3 (Fig. 6, upper panel). After 1-h pulse, the uptake of Sph and the incorporation of radioactivity into N-acylated compounds (as the sum of tritiated Cer, SM, GlcCer, and GM3) were very similar in control and NO-treated cells. However, treatment with NO strongly modified the distribution of radioactivity between the different Sph metabolites; in fact, \(^{3}H\)Cer was about 2-fold higher in NO-treated than in control cells (Fig. 6, upper panel). At the same time, in NO-treated cells, the radioactivity incorporated into SM,
GlcCer, and GM3 was 40, 35, and 34% less, respectively, than in controls. In these conditions, treatment with NO donor did not modify the amount of radioactivity incorporated into sphingosine 1-phosphate (Fig. 6, upper panel). As in the case of SM synthase, the in vitro activity of GlcCer synthase was similar in control (0.27 ± 0.04 nmol/mg of protein/min) and NO-treated cells (0.26 ± 0.039 nmol/mg of protein/min), and this was also found after addition of PAPANONOate to the incubation mixture of control cell assay (0.28 ± 0.045 nmol/mg of protein/min).

In addition, the activity of Sph kinase assayed in vitro was very similar in control (0.12 ± 0.013 nmol/min/mg of protein) and NO-treated (0.13 ± 0.015 nmol/min/mg of protein) cells.

**Effect of NO on Sphingolipid Metabolism from [3H]Serine—**

The possible effect of NO on the de novo biosynthesis and metabolic processing of Cer was investigated after administration of [3H]serine. After a 1-h pulse, the incorporation of radioactivity into total lipids and the amount of radioactivity associated to sphingolipids were very similar in control and NO-treated cells indicating that NO did not affect the de novo biosynthesis of Cer. At this pulse time, NO treatment promoted an increase of the radioactivity associated to Cer with a concomitant decrease of that associated to SM (Fig. 6, lower left panel). This effect was found to be more marked when NO was administered during chase. In particular, as shown in Fig. 6 (lower right panel), NO induced a 40% increase in [3H]Cer levels, paralleled by a significant decrease of the radioactivity incorporated into SM.

**Effect of NO on [Sph-3H]C6-Cer Metabolism—**

On the basis of these results we investigated the possibility that NO impairs the availability of Cer for the biosynthesis of complex sphingolipids. To this purpose we evaluated the effect of PAPANONOate on [Sph-3H]C6-Cer metabolism. As shown in Fig. 7, after 2-h pulse, the biosynthesis of [Sph-3H]C6-SM and [Sph-3H]C6-GlcCer (both derived from the direct metabolic use of the short chain, free diffusing Cer) was not affected by NO. On the contrary the utilization of long-chain [3H]Cer, produced in the...
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ER from \(^{3}\)H[Sph derived from [Sph-\(^{3}\)H]C6-Cer catabolism (39), was strongly inhibited by NO. In fact, in NO-treated cells \(^{3}\)H[Cer was about 2-fold higher, whereas \(^{3}\)H[Sph and \(^{3}\)H[GlcCer were 40 and 30\% less than those measured in control cells. Noteworthy, the amount of \(^{3}\)H[Sph produced from the degradation of [Sph-\(^{3}\)H]C6-Cer by ceramidase and represented by the sum of Sph and its sphingolipid metabolites was very similar in control and NO-treated cells.

Effect of NO on the Intracellular Distribution of BODIPY-C5-Cer and NBD-C6-Cer—The transport of natural Cer from ER to the Golgi apparatus can be qualitatively evaluated from the analysis of BODIPY-C5-Cer redistribution into cells (32, 40). We thus investigated the effect of NO on the behavior of this fluorescent Cer into intact C6 glioma cells. After labeling ER and other intracellular membranes, cells were chased, in the presence or absence of 400 \(\mu\)M PAPANOate to allow the redistribution of BODIPY-C5-Cer. In control cells most of fluorescence accumulated in the perinuclear region (Fig. 8A), representative of the Golgi apparatus (32), whereas in NO-treated cells the accumulation of fluorescence in the Golgi region was strongly reduced (Fig. 8B). In contrast, when cells were labeled with NBD-C6-Cer, which spontaneously moves between intracellular membranes, NO did not appreciably modify the accumulation of NBD fluorescence in the perinuclear Golgi region (Fig. 8, C and D).

Effect of NO on SM and GlcCer Formation from \(^{3}\)H[Sph in BFA-treated Cells—To obtain further evidence for the effect of NO on Cer traffic, we then investigated the effect of NO on \(^{3}\)H[Sph metabolism in C6 glioma cells treated with BFA, which leads to the fusion of the cis-Golgi membranes with the ER (41). Our working hypothesis was that, if NO determines a defect in ER to Golgi apparatus trafficking of Cer, treatment with BFA should make Cer metabolism insensitive to NO. After pretreatment with BFA for 30 min at 37 °C, cells were pulsed for 30 min with \(^{3}\)H[Sph in the presence of BFA alone or with PAPANOate. Then, the conversion of \(^{3}\)H[Cer to \(^{3}\)H[SM and \(^{3}\)H[GlcCer was compared in control and BFA-treated cells (Fig. 9). In BFA-treated cells, the amount of \(^{3}\)H[SM and \(^{3}\)H[GlcCer synthesized from \(^{3}\)H[Cer was found to be about 2-fold higher than in control ones. In the presence of BFA, NO was no more able to impair \(^{3}\)H[Cer metabolic utilization, thus implying that NO affects the translocation of Cer from the ER to the Golgi apparatus.

Effect of NO on SM Formation from \(^{3}\)H[Sph after ATP Depletion—To gain insight into the possible mechanisms of Cer transport affected by NO, we next examined the effect of NO on \(^{3}\)H[Cer metabolism, after promoting ATP depletion of cells. After pulse with \(^{3}\)H[Sph in an ATP-depleting medium, the incorporation of radioactivity into SM and GlcCer was found to be about 70 and 30\% less than controls (Fig. 10). This was paralleled by a 2-fold increase of \(^{3}\)H[Cer levels. Moreover, ATP depletion did not reduce either \(^{3}\)H[Sph uptake or its incorporation into N-acylated compounds (as the sum of tritiated Cer SM and GlcCer, Fig. 10). This ruled out the possibility that the decrease in \(^{3}\)H[SM formation in ATP-depleted cells was due to a decrease in the cellular uptake of \(^{3}\)H[Sph or its utilization for \(^{3}\)H[Cer synthesis. In ATP-depleted cells, NO treatment did not further modify the conversion of \(^{3}\)H[Cer to either \(^{3}\)H[SM or \(^{3}\)H[GlcCer. Thus, these results show that in C6 glioma cells the trafficking of Cer to the site of SM and, in a lesser extent, GlcCer biosynthesis is mainly ATP-dependent and that ATP depletion mimicked the NO effect on Cer metabolism.

Cer Transport from the ER to the Site of SM and GlcCer Biosynthesis in Control and NO-treated Semi-intact Cells—\(^{3}\)H[Cer enriched semi-intact cells obtained from control and NO-treated cells were used to obtain further insights on the effect of NO on Cer transport from ER to the site of SM and GlcCer synthesis. When control semi-intact cells were incubated in the buffer, \(^{3}\)H[SM represented about 4\% and \(^{3}\)H[GlcCer 1\% of N-acylated lipids (Fig. 11, upper left panel). In the transport mixture containing the cytosol obtained from control cells, the formation of both \(^{3}\)H[SM and \(^{3}\)H[GlcCer was increased by about 4-fold; the same increase in \(^{3}\)H[GlcCer was observed after adding UDP-Glc to the buffer (Fig. 11, upper left panel). The removal of the ATP-regenerating system from the transport mixture led to a strong reduction of SM but not GlcCer formation (Fig. 11, upper left panel).

As shown in Fig. 11 (upper right panel), incubation of NO-treated semi-intact cells with buffer alone led to \(^{3}\)H[SM and \(^{3}\)H[GlcCer biosynthesis similar to that observed in control semi-intact cells. The addition of NO-treated cytosol and the transport mixture promoted a nearly 3-fold increase in \(^{3}\)H[GlcCer formation without any significant change in \(^{3}\)H[SM; in the absence of the ATP-regenerating system, SM formation was not modified. Here too, the presence of UDP-Glc in the buffer was sufficient to promote \(^{3}\)H[GlcCer biosynthesis. Thus, these results confirm that, in C6 glioma cells, NO impairs the ATP, cytosol-dependent, inter-membrane Cer traffic needed mainly for SM biosynthesis and, to a lesser extent, for GlcCer.
To see if any specific cellular component is involved in the NO-induced defective Cer translocation, we carried out fraction exchange experiments in the transport assay (Fig. 11, lower panel). The use of NO-treated cytosol with control membranes did not significantly modify the synthesis of [3H]SM or [3H]GlcCer. Moreover, the use of cytosol obtained from control cells in combination with NO-treated cells did not restore the capacity to synthesize [3H]SM and [3H]GlcCer (Fig. 11, lower panel), thus suggesting that NO exerts its effect on Cer transport at the level of the membranous compartment involved in Cer traffic.

**DISCUSSION**

The first evidence provided by this study is that NO exerts a dose-dependent antiproliferative effect on C6 glioma cells. As observed in other cell types (42, 43), this effect was not mimicked by a membrane-permeant non-hydrolyzable analog of cGMP, supporting the evidence that also in these cells the antimitogenic effect of NO is independent from the activation of guanylate cyclase, and thus from cGMP. Instead, we found that NO induces an early and significant increase in Cer levels and that treatments resulting in the increase of cell Cer are able to mimic the antiproliferative effect of NO in C6 glioma cells. Altogether, these data strongly suggest that Cer may be a mediator of the antiproliferative effect of NO. In this context, it is noteworthy that Cer has been recently indicated as a mediator of the apoptotic response to NO in glomeruloendothelial and mesangial and HL60 cells (26, 27). Furthermore, these results strengthen the general role of Cer in the control of cell proliferation (5) and, in particular, as a negative regulator of glial growth (13, 29, 44).

Evidence obtained from non-neural cells indicates that the modulation of Cer levels following NO treatment can depend on both the activation of N- and A-SMase and the inhibition of ceramidases (26, 27). The studies here performed to single out the metabolic pathway responsible for NO-dependent Cer accumulation in C6 glioma cells indicate that the degradation of...
and then labeled with 0.3 μCi/ml [3H]Sph for 30 min in BFA-containing medium with or without 400 μM PAPANONate. At the end, cell lipids were extracted and analyzed as described under “Experimental Procedures.” Closed bars, control cells; open bars, BFA-treated cells; striped bars, cells treated with BFA and NO. All values are the mean ± S.D. of at least three individual experiments.

![Graph showing effect of NO on [3H]Sph metabolism in BFA-treated C6 glioma cells.](image)

**Fig. 9.** Effect of NO on [3H]Sph metabolism in BFA-treated C6 glioma cells. Cells were preincubated in DMEM plus 10% FCS containing BFA (1 μg/ml) for 30 min at 37 °C, and then labeled with 0.3 μCi/ml [3H]Sph for 1 h in ATP-depleting medium with or without 400 μM PAPANONate. After 1 h, lipids were extracted and analyzed as described under “Experimental Procedures.” Closed bars, control cells; open bars, ATP-depleted cells; striped bars, ATP-depleted and NO-treated cells. All values are the mean ± S.D. of at least three individual experiments.

both SM and Cer is not involved in the NO-induced regulation of Cer levels. By using different experimental approaches, the major pathway responsible for the NO-dependent Cer increase was found to involve a reduced utilization of Cer for the biosynthesis of complex sphingolipids, mainly SM. Thus, unlike what was observed in other cell types (26, 27), in C6 glioma cells SM or Cer cleavage does not appear to be involved in the modulation of Cer levels by NO. Instead, metabolic experiments performed with [3H]choline, [3H]serine, and [3H]Sph, all indicate that NO strongly reduces the conversion of Cer to SM without affecting the biosynthesis of [3H]Cer and [3H]phosphatidylcholine, the two immediate precursors for SM formation. These results strongly support that Cer utilization for SM biosynthesis represents a key element/module in the Cer signaling involved in the control of glial cell growth (16, 44) and more in general in cell proliferation (for a review see Ref. 5). Notwithstanding the evidence of a NO-dependent inhibition of both SM and GlcCer biosynthesis, no direct effect of NO on the enzymatic activity of either SM or GlcCer synthase was detected. Thus, differently from what occurs in cerebellar astrocytes and hippocampal neurons after stimulation with basic fibroblast growth factor (16, 45), the control of Cer levels might be achieved by a mechanism other than the direct regulation of SM- and/or GlcCer-synthase. A reasonable possibility is that NO acts by reducing the availability of Cer as substrate for both enzymes, possibly inducing a defect in the translocation of Cer, synthesized in the ER, to the sites where SM-synthase and GlcCer-synthase are localized. Very convincing support to this hypothesis was obtained by treating C6 glioma cells with [Sph-3H]C6-Cer, with or without the NO-releasing agent. In fact, in the presence of NO, the direct utilization of radiolabeled C6-Cer for the biosynthesis of C6-SM and C6-GlcCer, which does not require Cer exit from the ER, was unmodified. Moreover, treatment of C6 glioma cells with C6-Cer also resulted in the generation of endogenous long-chain Cer, as in A549 cells (39). This process most reasonably involves the recycling of Sph for the biosynthesis of long-chain Cer at the ER level. Opposite to what was observed for short-chain Cer, the utilization of long-chain Cer for the biosynthesis of complex sphingolipids was strongly inhibited by NO. This finding further confirms that newly synthesized Cer in the ER is no longer available for complex sphingolipid biosynthesis in the presence of NO.

The analysis of intracellular distribution of BODIPY-C5-Cer, which mimics the intracellular movements of natural Cer, provides additional evidence that NO can influence the intracellular traffic of Cer from ER to the Golgi apparatus. Moreover, results obtained with BFA demonstrate that, in C6 glioma cells, the cis-Golgi represents the major subcellular site for both GlcCer and SM biosynthesis and supports that NO induces a defect in Cer translocation from ER to the Golgi apparatus. In fact, when cells were treated with BFA, which causes Golgi disassembly and redistribution to the ER (41), the conversion of Cer to both GlcCer and SM was strongly increased. The increase in GlcCer is in agreement with the generally observed cis-medial Golgi location of the glucosyltransferase involved in its biosynthesis (46, 47). A different consideration must be made for the BFA-dependent SM increase in C6 glioma cells. As occurs in many cell types (48–50), the results here obtained indicate that even in C6 glioma cells SM biosynthesis occurs mainly in the cis-medial Golgi. Nevertheless, in different cell types, SM synthesis can also occur at different subcellular sites (51–53), and, in rat cerebellar astrocytes, the activation of an SM synthase located in a compartment other than cis-medial Golgi is involved in the basic fibroblast growth factor signaling pathway involved in cell proliferation (16). The direct availability of Cer for SM and GlcCer synthases determined by BFA makes C6 glioma cells no more sensitive to NO-dependent inhibition of Cer utilization. This evidence further confirms that SM- and GlcCer-synthases are not the NO target and strengthens the notion that the NO-dependent Cer increase is mainly due to a defect in its translocation from ER to cis-Golgi.

The way Cer moves from the ER to Golgi is still unclear but, on the basis of many experimental results, two main mechanisms appear to be involved and include an ATP-dependent vesicle-mediated Cer transport, and a non-vesicular Cer trans-
location that can involve the participation of transfer proteins as well as ER-Golgi membrane contacts (54–56). Using a cell line with a specific defect in SM biosynthesis, evidence was recently presented that Cer may preferentially use an ATP-independent non-vesicular pathway for glycosphingolipid production and an ATP-dependent vesicle-mediated mechanism for the biosynthesis of SM (40). These same researchers also demonstrated that the transport of Cer for SM biosynthesis strongly depends on some unidentified cytosolic factors, whereas the biosynthesis of GlcCer is mainly cytosol-independent (33). The results obtained in our present study on ATP-depleted cells and on in vitro Cer transport assays are in agreement with this hypothesis. In fact, in C6 glioma cells, ATP depletion strongly affects SM biosynthesis and, to a lesser extent, that of GlcCer. On the other hand, the conversion of Cer into SM in semi-intact cells was strongly dependent on the presence of ATP and cytosol, whereas that of GlcCer appears to be mainly dependent on the availability of UDP-Glc. We also obtained evidence that ATP depletion mimics the inhibitory effect of NO on the biosynthesis of complex sphingolipids, in particular SM, and that NO is no longer able to exert any effect in ATP-depleted cells. Thus the ATP-dependent vesicle-mediated transport of Cer, primarily for SM biosynthesis, is involved in the NO-dependent Cer increase. Moreover, results obtained by using semi-intact cells and cytosol derived from NO-treated cells, led us to exclude the involvement of a cytosolic factor as a NO target and strongly suggest that the effect of NO on Cer traffic resides in the membrane component involved in this process. To explain Cer accumulation consequent to a NO-mediated impairment in its traffic, two major mechanisms can be hypothesized. First, in the presence of NO, newly synthesized Cer cannot move from the ER, thus accumulating in this compartment where it may exert its biological effects (57). Second, Cer-transferring structures, possibly vesicles, actually leave the ER but, in the presence of NO, their Golgi-specific targeting is impaired, thus determining an accumulation of Cer-containing structures available for the interaction with Cer targets. Studies are in progress in our laboratory to investigate these possibilities.

In conclusion, to the best of our knowledge this represents the first evidence for an active role for Cer traffic in Cer signaling. The results provided here demonstrate that NO is able to modulate the intracellular traffic of Cer between the ER and the Golgi apparatus. As a consequence, newly synthesized Cer
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acumulates and appears to act as a mediator of the antiproliferative effect of NO in these cells. These results point out the relevance of Cer intracellular movements and of the role of specific signaling pools, in defining the biological effects of Cer (7, 8). Altogether, this strengthens the paradigm that the understanding of the complex signaling role of Cer cannot leave out of consideration the metabolic origin, the topology of production, and the intracellular traffic of this bioactive lipid and its interplay with other signaling pathways.

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