Sphingosine inhibits nitric oxide synthase from cerebellar granule cells differentiated in vitro

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Abstract The effects of different bioactive sphingoid molecules on NOS activity of differentiated cerebellar granule cells were investigated by measuring the conversion of [^{3}H]arginine to [^{3}H]citrulline. Cytosolic Ca^{2+}-dependent NOS activity was strongly inhibited in a dose-dependent manner by sphingosine in concentrations of 1–40 μM. This inhibition seems to be peculiar to sphingosine in that ceramide, *N*-acetylsphingosine, sphingosine-1P, sphinganine and tetradecylamine have no effect on the cytosolic enzyme at the considered concentrations, suggesting that it is the bulk of the sphingosine hydrophilic portion that is critical for cytosolic NOS inhibition. This inhibition of cytosolic NOS is not reversed by increasing the arginine concentration, so a competitive mechanism can be excluded. Instead, increasing the concentrations of calmodulin led to loss of sphingosine inhibition, suggesting that sphingosine interferes with the calmodulin-dependent activation of the enzyme by a competitive mechanism. Sphingosine and related compounds had no effect on the particulate Ca^{2+}-independent NOS activity. The data obtained suggest that sphingosine could be involved in the regulation of NO production in neurons.

Key words: Nitric oxide synthase; Sphingosine; Ceramide; Cerebellar granule cell; Neuronal differentiation

1. Introduction

Studies on several cell types have brought to light evidence that specific sphingoid molecules, mainly ceramide, sphingosine and sphingosine-1-phosphate, are involved in the mechanism of cell response to different extracellular agents (for reviews, see [1–4]). This signalling mechanism is emerging as a complex one, which can operate both by affecting direct intracellular targets (including specific protein kinases and phosphatases) and, interestingly, by cross-talking with other signalling pathways. Also in nervous system cells it has been revealed [5–11] that sphingoid molecules act as intracellular mediators of the effects of specific stimuli, especially with regard to neuronal differentiation [5–7,10,11]. In fact, the administration of differentiating agents to neuroblastoma cells results in increased intracellular ceramide which in turn acts as a mediator of their neuronal-like differentiation [7]. Further evidence supporting sphingoid molecule involvement in neuronal differentiation lies in the fact that in primary cultures of cerebellar granule cells the cellular content of both ceramide and sphingosine increases throughout their differentiation in culture, and is maintained at high levels in differentiated cells [10].

From among the different signalling pathways operating in the nervous system, one that is peculiar is that mediated by nitric oxide (NO), a highly diffusible, short-lived messenger involved in the modulation of several neural functions, including neurotransmission and differentiation [12–16]. In a previous study [17] we demonstrated that cerebellar granule cells contain a cytosolic Ca^{2+}-dependent NO synthase (NOS) activity and a particulate one that is almost Ca^{2+}-independent, and that these enzymes progressively and markedly increase during differentiation; such an enzyme increase represents a specific expression of differentiated cells.

The involvement of sphingoid molecules and NO in differentiating cerebellar granule cells prompted us to investigate the possible occurrence of cross-talk between the sphingoid and the nitric oxide signalling pathways in these neurons. As a first approach we studied the possible effects of different bioactive sphingoid molecules on NOS activity in differentiated cerebellar granule cells, and we observed a specific inhibitory effect of sphingosine on cytosolic Ca^{2+}-dependent NOS activity, likely due to competition with calmodulin, the known physiological activator of the enzyme.

2. Materials and methods

2.1. Materials

Basal modified Eagle's medium (BME), fetal calf serum (FCS), NADPH tetrasodium salt, (*6R*)-5,6,7,8-tetrahydrobiopterin dihydrochloride (BH_4), arginine, HEPES, EGTA, ceramide from bovine brain sphingomyelin (Cer), D-*erythro*-sphingosine (Sph), sphingosine-1-phosphate (Sph-1P), D-*erythro*-sphinganine (Sa), *N*-acetyl sphingosine (C2-Cer), tetradecylamine (TdA), bovine brain calmodulin (CaM), were from Sigma (St. Louis, MO, USA); HPTLC silica gel plates from Merck (Darmstadt, Germany); L-[2,3,4,5-^{3}H]arginine monohydrochloride (64 Ci/mmol) from Amersham (Bucks, UK); Coomassie protein assay reagent and bovine serum albumin (BSA) from Pierce Chemical Co. (Rockford, IL, USA).

2.2. Cell cultures

Primary cultures of granule cells were prepared from cerebella of 8 day old rats (Sprague-Dawley) and cultured in 60 mm dishes, in a humidified atmosphere of 5% CO_2 and 95% air at 37°C with 5 ml of BME medium supplemented with 10% FCS as previously described [18]. After 9 days in culture the cells, represented by ≥95% differentiated granule neurons, were rinsed twice with 50 mM HEPES (pH 7.4), 2.7 mM KCl and 100 mM NaCl, scraped off the plates in the same buffer solution (0.2 ml/dish) with a rubber policeman and homogenized by gentle pipetting up and down 50 times. The homogenates were centrifuged at 100 000 × *g* (30 min at 4°C) and the super-

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Abbreviations: BH_4, (*6R*)-5,6,7,8-tetrahydrobiopterin; BME, basal nodified Eagle's medium; BSA, bovine serum albumin; CaM, calmodulin; Cer, ceramide; C2-Cer, *N*-acetylsphingosine; FCS, fetal calf serum; NO, nitric oxide; NOS, NO synthase; iNOS, inducible NO synthase; Sa, sphinganine; Smase, sphingomyelinase; Sph, sphingosine; Sph-1P, sphingosine-1-phosphate; TdA, tetradecylamine

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natant and sedimented pellet collected and used respectively as the cytosolic and particulate fractions. Before use, the pellet was homogeneously suspended with 0.2 ml of the above buffer solution.

2.3. Determination of NOS activity

NOS activity was determined on fresh cytosolic and particulate fractions by measuring the conversion of [^{3}H]arginine to [^{3}H]citrulline. The assay was performed according to Bredt and Snyder [19] with some modifications [17] as follows: 10 μg protein of cytosolic fraction was incubated at 37°C for 60 min in 50 μl 0.45 mM $CaCl_2$, 2 mM NADPH, 10 μM BH_4, 50 mM HEPES buffer, pH 6.4, and in the presence of different concentrations (4–12 μM) of [^{3}H]arginine carrying 2 Ci/mmol. NOS activity on the particulate fraction was assayed both in the same reaction conditions used for cytosolic enzyme, or replacing $CaCl_2$ by 1 mM EGTA. Samples were then processed as previously described [17]. The enzyme activity, expressed as nmol [^{3}H]citrulline mg^{-1} protein h^{-1}, was calculated as previously described [17]. With both enzymes, and as established by ad hoc experiments, enzyme saturation was achieved with 10 μM arginine.

2.4. Effect of different sphingoid molecules and long chain alkylamines on NOS activity

The effect of different sphingoid molecules and tetradecylamine (TdA) was evaluated by measuring cytosolic and particulate NOS activity in the presence of different concentrations (1–40 μM) of each molecule. For this purpose stock solutions of Sph, Sph-1P, Sa, TdA in absolute ethanol were diluted in the enzyme suspension buffer (50 mM HEPES, 2.7 mM KCl, 100 mM NaCl, pH 6.4); 5 μl of the obtained solutions was added to 40 μl of the enzyme suspension and preincubated for 10 min at 4°C (ethanol final concentration in the enzyme assay ≤0.5%, v/v). The enzymatic reaction was then started by the addition of 5 μl of a mixture containing $CaCl_2$, NADPH, BH_4 and [^{3}H]arginine. When Cer and C2-Cer were used, stock solutions were prepared in ethanol/dodecane 98/2 (v/v) [20], then diluted and added to the enzyme suspensions as described above. Control experiments showed that the used concentrations of ethanol and ethanol/dodecane did not affect NOS activity.

2.5. Effect of sphingosine on cytosolic NOS activity in the presence of exogenously added calmodulin

The possible interactions between Sph and CaM, the known physiological activator of cytosolic NOS [21], were studied using the experimental conditions described in Section 2.4. CaM at different concentrations (0.06–6 μM) was added during the preincubation together with Sph, and the enzymatic reaction started as described above.

2.6. Protein determination

Protein content in the cytosolic and particulate fractions was determined with the Coomassie protein assay reagent using BSA as the standard.

3. Results

As our aim was to evaluate the possible influence of sphingoid molecules on NOS activity in differentiated granule cells, we first considered ceramide and sphingosine, two bioactive sphingoids present at high levels in these cells differentiated in culture [10]. Cerebellar granule cells used on the 9th day in culture showed a cytosolic NOS activity which is Ca^{2+}-dependent and a particulate one which is Ca^{2+}-independent [17]; in particular NOS activity assayed in the cytosolic fraction was 6.7 ± 0.9 nmol mg^{-1} protein h^{-1}, and was abolished by the presence of 1 mM EGTA. On the other hand, NOS activity evaluated on the particulate fraction was 1.4 ± 0.2 nmol mg^{-1} protein h^{-1}, the same value being obtained in the presence of either 1 mM EGTA or 0.45 mM $CaCl_2$. Using 10 μg protein of the cytosolic or particulate fraction, we found that Sph exerted a strong dose-dependent inhibitory effect on Ca^{2+}-dependent cytosolic NOS, reaching 80% and 95% inhibition at 20 and 40 μM respectively (Fig. 1, left panel). In contrast, concentrations up to 40 μM of Sph had no effect on the activity of the Ca^{2+}-independent particulate NOS assayed in presence of 1 mM EGTA (Fig. 1, right panel). At all the used concentrations Sph did not influence particulate NOS even when the enzyme assay was performed in the presence of 0.45 mM $CaCl_2$. Under the same experimental conditions Cer (up to 40 μM) did not modify the activity of either enzyme.

The potent inhibitory effect of sphingosine on cytosolic NOS, prompted us to evaluate the effects of different Sph analogues, namely its phosphorylated metabolite Sph-1P, *N*-acetyl derivative (C2-Cer), the saturated stereoisomer D-*erythro*-sphinganine (Sa), and the long chain alkylamine tetradecylamine (TdA) on the same enzyme. As shown in Fig. 2, at both 10 and 20 μM concentration, none of these molecules had any significant effect on cytosolic NOS activity; nor was any effect observed at 40 μM effector. Also, the activity of the particle-bound NOS remained unaffected by these molecules, under the same experimental conditions (data not shown).

In order to explore the mechanism by which Sph interferes with cytosolic NOS, a kinetic analysis of the enzyme activity was carried out in the presence or absence of 10 μM Sph (Fig. 3). Sph was found to decrease the apparent V_{max} of the en-

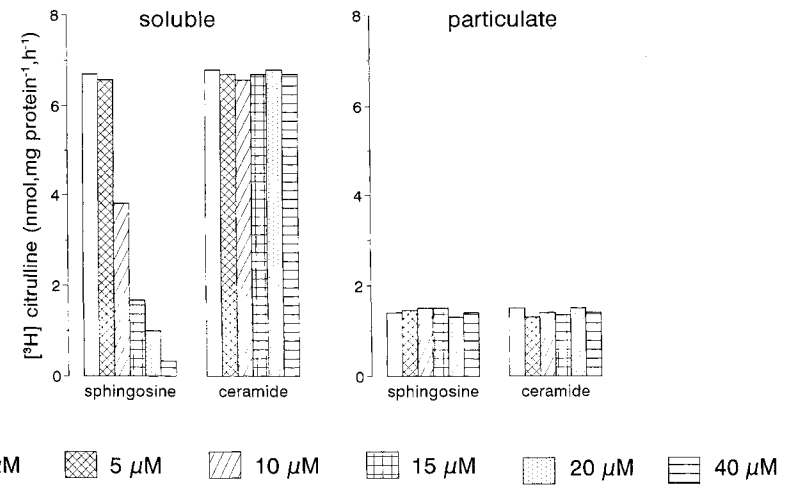

Fig. 1. Influence of sphingosine and ceramide on cytosolic and particulate NOS activity of cerebellar granule cells. NOS activity was determined in the presence of different concentrations of Sph or Cer (0–20 μM) as described in Section 2. Data are the mean values of at least three experiments, each in duplicate; S.D. never exceeded 15% of the mean.

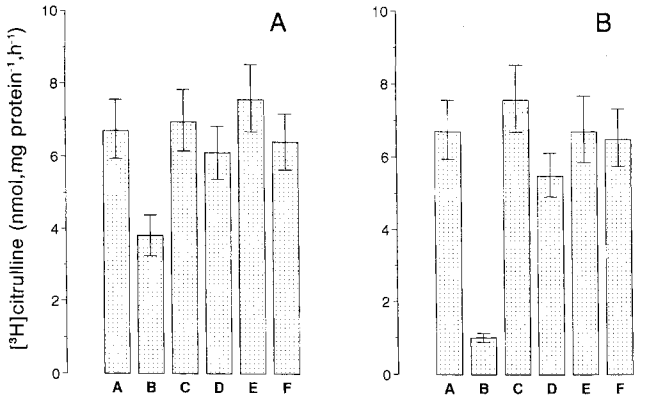

Fig. 2. Influence of different sphingoid molecules and long chain alkyl amines on cytosolic NOS activity of cerebellar granule cells. The cytosolic NOS activity was determined in the presence of 10 μM (panel A) and 20 μM (panel B) of none (A), Sph (B), Sph-1P (C), Sa (D), TdA (E), or C2-Cer (F). Data are the mean values ± S.D. of at least three experiments in duplicate.

zyme with no alteration of K_m values, indicating a non-competitive inhibition.

It is well known that Ca^{2+}-promoted CaM binding is required for the activity of cytosolic neuronal NOS, including cerebellar granule cell NOS activity [21]; thus we measured the effect of Sph on this enzyme in the presence of different concentrations of CaM. In the absence of Sph NOS activity was not appreciably affected by CaM (Fig. 4), whereas in the presence of Sph increasing the concentration of CaM (from 0.06 to 6 μM) led to the loss of the Sph inhibitory effect in a dose-dependent manner. Sph concentrations determining a 50% inhibition of NOS were 10, 15, 20 μM in presence of no, 0.06 and 0.6 μM exogenously added CaM, respectively.

4. Discussion

Previous studies on the effect of sphingoid molecules on the NO signalling pathway mainly regarded the cytokine-mediated iNOS expression in non-neuronal tissues or cells [22–25]. In addition, it has been reported that ceramide-induced vasodilation in rat thoracic aortic rings is partially dependent on endothelium-derived NO [26], and that sphingosine and SMase treatment significantly attenuates endothelium-dependent relaxation in response to different stimuli in coronary arteries [27].

In this study we provide evidence that sphingosine exerts a

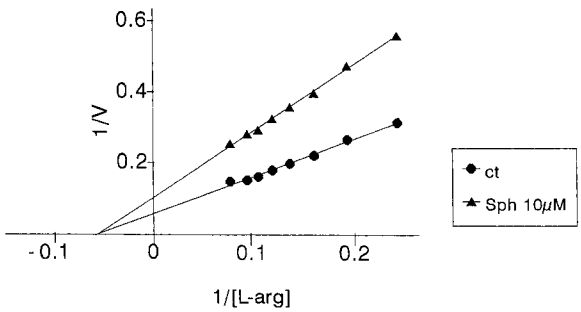

Fig. 3. Kinetic analysis of sphingosine inhibition of cytosolic NOS activity. The enzyme was assayed alone (●) or with 10 μM sphingosine (▲) in the presence of different concentrations of arginine (4–12 μM). Data are the mean values of at least three experiments in duplicate; S.D. never exceeded 15% of the mean.

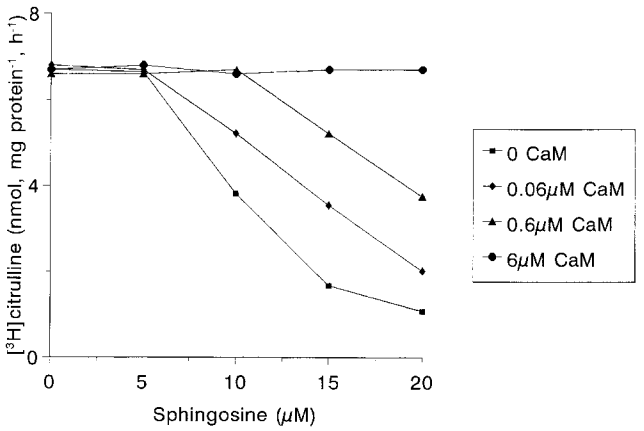

Fig. 4. Effect of sphingosine on cytosolic NOS activity of cerebellar granule cells: influence of calmodulin. 10 μg of cytosolic enzyme was incubated in the presence of both sphingosine (5–20 μM) and calmodulin 0 (■), 0.06 (◆), 0.6 (▲) and 6 (●) μM. Data are the mean values of at least three experiments in duplicate; S.D. never exceeded 15% of the mean.

significant dose-dependent inhibitory effect on the in vitro NOS activity associated with the cytosolic fraction of differentiated cerebellar granule cells, but does not influence the in vitro activity of the particulate Ca^{2+}-independent enzyme. Sph concentrations effective on NOS are similar to or lower than those used in previous studies that demonstrated an in vitro modulatory effect of Sph on different enzymes ([28,29] and references in [4]). The inhibition exerted by sphingosine appears to be specific and to require the bulk of the hydrophilic portion of the molecule. In fact, *N*-acylated sphingosine, Cer, the metabolic precursor of sphingosine, and the water-soluble analog C2-Cer are not effective in modulating the activity of cytosolic NOS, thus indicating that the amino group of Sph is necessary for its inhibitory activity on NOS. The amino group is not the only requirement for the Sph-mediated inhibition of NOS since the long chain alkylamine, TdA, is also inefficient as inhibitor of cytosolic NOS. Furthermore, sphingosine phosphorylated at C-1 (Sph-1P), a bioactive molecule [30] intermediate in Sph catabolism [31], does not modify cytosolic NOS activity, indicating that the primary hydroxyl function is also required for Sph inhibitory activity on NOS. Also the sphingolipid metabolic precursor sphinganine, which differs from sphingosine in that it lacks the double bond between C4 and C5, is without effect, suggesting that NOS inhibition by sphingoid bases is also sensitive to the presence of the sphingosine double bond. Kinetic experiments demonstrate that Sph decreases the apparent maximal velocity but does not alter the apparent K_m value, thus acting as a non-competitive inhibitor of NOS.

Finally the possibility that cytosolic NOS inhibition by sphingosine is linked to NOS activity dependence on CaM was investigated. The results we obtained demonstrate that increasing concentrations of CaM promote a rightward shift of the Sph-dependent inhibition curve of cytosolic NOS, indicating that Sph interferes with the CaM-dependent activation of the enzyme, presumably by a competitive mechanism. This result is in agreement with previous studies that demonstrate that Sph is able to inhibit other CaM-dependent enzymes [32]. Since in the enzyme assay Sph is always present at much higher concentrations than CaM, it is unlikely that calmodulin simply lowers the effective concentration of Sph

by acting as a Sph binding protein. The Sph inhibitory activity could be explained either by a binding of Sph to the Ca-calmodulin complex or by its interaction with the calmodulin binding domain of cytosolic NOS. The evidence that Sph interferes with the CaM activation of cytosolic NOS could also explain the Sph failure to inhibit the Ca^{2+}-independent NOS activity associated to the particulate fraction of cerebellar granule cells.

In conclusion, the data obtained demonstrate that sphingosine, but not other structurally related bioactive sphingoid molecules like ceramide, sphingosine-1P and sphinganine, is able to modulate the Ca^{2+}-dependent NOS of cerebellar neurons, possibly through a CaM-dependent mechanism. The physiological relevance of these results derives from the evidence that sphingosine cellular concentrations have been found in the micromolar range in different cell types (reviewed in [33]) and sphingosine intracellular levels can rapidly and transiently increase in response to extracellular stimuli (reviewed in [34]). To the best of our knowledge, this is the first evidence suggesting the involvement of Sph in the regulation of NO production in neurons, implying the occurrence of cross-talk between sphingolipid- and NO-mediated pathways of signal transduction operating in these cells. The occurrence of NOS inhibition by sphingoid bioregulators in in vivo systems is under current investigation in our laboratory.

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