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L-NAME prevents GM1 ganglioside-induced vasodilation in the rat brain

Ana Flávia Furian [a,b], Mauro Schneider Oliveira [a,b], Danieli Valnes Magni [a], Mauren Assis Souza [a], Vanessa Trindade Bortoluzzi [a], Lívia Maronesi Bueno [a], Luiz Fernando Freire Royes [a,c], Carlos Fernando Mello [a,*]

[a] Departamento de Fisiologia e Farmacologia, Universidade Federal de Santa Maria, 97105-900 Santa Maria, RS, Brazil
[b] Programa de Pós-Graduação em Ciências Biológicas: Bioquímica, Universidade Federal do Rio Grande do Sul, 90035-003 Porto Alegre, RS, Brazil
[c] Departamento de Educação Física e Desportos, Universidade Federal de Santa Maria, 97105-900 Santa Maria, RS, Brazil

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ABSTRACT

Monosialoganglioside (GM1) is a glycosphingolipid present in most cell membranes that displays antioxidant and neuroprotective properties. It has been recently described that GM1 induces vasodilation. However, the mechanisms underlying GM1-induced vasodilation were not evaluated to date. Therefore, in this study we investigated whether the nonspecific NOS inhibitor L-NAME prevents GM1-induced vasodilation in rats. The systemic injection of GM1 (50 mg/kg, i.p.) increased the outer diameter of pial vessels by 50% in anesthetized animals at 30 min, and this effect was fully prevented by the administration of the nitric oxide synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME, 60 mg/kg, i.p. 15 min before GM1 injection).

A 30 min exposure of cerebral cortex slices to GM1 (100 μM) increased the content of nitrite plus nitrate (NOx) by 50%. Addition of L-NAME (100 μM) to the incubation medium fully prevented GM1-induced NOx increase. Conversely, a 60 min exposure of slices to GM1 (100 μM) decreased NOx content, revealing a biphasic effect of GM1. Our results suggest that NO plays an important role in the vasodilation induced by GM1.

1. Introduction

Gangliosides, naturally occurring sialic acid containing glycosphingolipids, are components of most cell membranes. They are particularly abundant in the brain, where they represent the major lipid constituent of the neuronal surface (Ledeen and Yu, 1982; Tettamanti, 2004). Gangliosides are thought to play a modulatory role in different events associated with cell differentiation and oncogenic transformation (Schengrund, 1990; Zeller and Marchase, 1992), adaptive responses (Goettl et al., 2003; Perry et al., 2004), memory formation (Rahmann, 1995; Silva et al., 1996; Fong et al., 1997) and synaptic plasticity (Wieraszko and Seifert, 1985). Monosialoganglioside (GM1) protects the central nervous system against various neurotoxic agents or conditions, such as aspartic acid (Skaper et al., 1990), MPTP (Rothblat and Schneider, 1994), glutamic acid (Avrova et al., 2000), kainic acid (Wu et al., 2005), methylmalonic acid (Fighera et al., 2003) and glutaric acid exposure (Fighera et al., 2006), apoptosis (Gorria et al., 2006), anoxia (Carolei et al., 1991; Tan et al., 1993) and ischemia (Carolei et al., 1991; Kwak et al., 2005), Parkinson's (Schneider, 1998) and Alzheimer's diseases (Svennerholm, 1994; Yanagisawa, 2007), accompanied by an apparent absence of side effects.

Several neurochemical mechanisms have been proposed for GM1-induced neuroprotection. It has been proposed that GM1 interacts with neurotrophic factors and their receptors *ex vivo* and *in situ* (Duchemin et al., 1997, 1998, 2002; Rabin et al., 2002), activating MAPK (Mo et al., 2005) and PI3-kinase/Akt survival pathways (Duchemin et al., 2007). In addition, there are studies showing that GM1 displays antioxidant action, both *in situ* and *in vivo* (Avrova et al., 1994, 1998), and that systemic GM1 administration increases striatal ascorbic acid content (Fighera et al., 2003) and catalase activity in cerebral cortex homogenates *in vivo* (Fighera et al., 2004).

* Corresponding author at: Departamento de Fisiologia e Farmacologia, Universidade Federal de Santa Maria, Prédio 21, Sala 5219, 97105-900 Santa Maria, RS, Brazil. Tel.: +55 55 3220 9378; fax: +55 55 3220 8241.
E-mail addresses: cf.mello@smail.ufsm.br, furian.anaflavia@gmail.com (C.F. Mello).

Abbreviations: GM1, monosialoganglioside; aCSF, artificial cerebrospinal fluid; AUC, area under the curve; Hb, hemoglobin; i.p., intraperitoneal; NOx, nitrite plus nitrate; L-NAME, N^G-nitro-L-arginine methyl ester.

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It has been recently demonstrated that vasodilation underlies the GM1-induced increase of catalase content in brain homogenates, and suggested that this effect could be responsible for the neuroprotection induced by this ganglioside (Furian et al., 2007). In addition, it has been shown that GM1 administration significantly restores local cerebral blood flow and glucose metabolism in animals subjected to middle cerebral artery occlusion (Tanaka et al., 1986), and that GM1 improves neurological status and tends to increase cerebral blood flow in Alzheimer's diseased patients (Svennerholm et al., 2002), further suggesting that vasodilation and better perfusion may underlie the neuroprotection induced by GM1.

Nitric oxide (NO) is involved in several cellular functions, including regulation of blood-vessel tonus, immune and inflammatory response and neurotransmission (Calabrese et al., 2007). NO is a short-lived gas, which is synthesized from L-arginine by the members of the NO synthase (NOS) family of proteins (EC 1.14.13.39) (Calabrese et al., 2007). The NOS family consists of three isoforms: neuronal NOS (nNOS or NOS-1), inducible or macrophage NOS (iNOS or NOS-2) and endothelial NOS (eNOS or NOS-3). NO synthesized in endothelial cells diffuses to smooth muscle cells where it activates soluble guanylate cyclase, resulting in cyclic guanylyl monophosphate-dependent vasodilation (Katsuki et al., 1977; Furchgott and Zawadzki, 1980; Palmer et al., 1987), an important process in the homeostasis of blood pressure and flow. Since NO is a key mediator in vasodilation, and the mechanisms by which GM1 induce vasodilation were not evaluated to date, this study aimed to investigate the involvement of NO-related mechanisms in the vasodilatory action of GM1 (Furian et al., 2007).

2. Experimental procedures

2.1. Animals and reagents

Adult male Wistar rats (250–280 g), maintained on a 12-h light/dark cycle (lights on at 7:00), with free access to tap water and standard laboratory chow (Guabi, Santa Maria, RS, Brazil) were used. All experimental protocols (including statistical evaluation) were designed aiming to keep the number of animals used to a minimum, as well as their suffering. The Ethics Committee for animal research of the Federal University of Santa Maria approved the experimental design. All reagents were purchased from Sigma (St. Louis, MO, USA) and respective solutions were prepared in type I ultra pure water. GM1 ganglioside was kindly donated by TRB Pharma Laboratories, São Paulo, Brazil.

2.2. Outer diameter of cerebral vessels measurement

Animals were deeply anesthetized with Equithesin (1% phenobarbital, 2% magnesium sulfate, 4% chloral hydrate, 42% propylene glycol, 11% ethanol; 3 ml/kg, i.p.) and had their right brain hemisphere exposed by removal of the right parietal bone. Care was taken to leave the dura mater intact, and the cranial window was continuously poured with sterile physiological saline. The animals were then injected with 0.9% NaCl (1 ml/kg, i.p.) or L-NAME (60 mg/kg, i.p.) (Adachi et al., 2000) and 15 min thereafter they were injected with 0.9% NaCl or GM1 (50 mg/kg, i.p.) (Furian et al., 2007). Changes in outer diameter of pial vessels were monitored using a digital video camera coupled to a PZO stereomicroscope (40×), up to 60 min after drug administration, and pial vessels were selected as follows: the optical field was divided in 4 quadrants and 2–3 vessels that had a baseline outer diameter between 5 and 100 μm were randomly selected in each quadrant to follow up along time. This resulted in the observation of 8–12 pial vessels/animal along time. The resulting mean of these measurements at each time point (0 and 30 min) was used for posterior statistical analysis. The state of circulation in cerebral vessels was estimated by the change in the outer diameter of pial vessels (Sobey and Quan, 1999) along time, according Podoprigora et al. (2005).

2.3. Colorimetric determination of NOx content

For the *ex vivo* experiments, the animals received GM1 (50 mg/kg, i.p.) or saline (0.9% NaCl, 1 ml/kg, i.p.), and 15, 30 or 60 min after the injection, they were sacrificed by decapitation and their brains were rapidly removed. The hippocampi and cerebral cortices were dissected on an inverted ice-cold Petri dish and homogenized with $ZnSO_4$ (200 mM) and acetonitrile (96%), centrifuged at 16,000 × *g* for 30 min at 4 °C, and the supernatant was collected for assay of the nitrite plus nitrate (NOx) content (Miranda et al., 2001). The resulting pellet was suspended in NaOH (6 M) for protein determination. NOx content was estimated in a medium containing 300 μl of 2% VCl_3 (in 5% HCl), 200 μl of 0.1% *N*-(1-naphthyl) ethylene-diamine dihydrochloride, 200 μl of 2% sulfanilamide (in 5% HCl). After incubating at 37 °C for 60 min, nitrite levels were determined spectrophotometrically at 540 nm, based on the reduction of nitrate to nitrite by VCl_3. Tissue nitrite and nitrate levels were expressed as nmol of NOx/mg of protein.

2.4. In situ experiments

Animals were sacrificed by decapitation and the cerebral cortex was immediately dissected and used for the preparation of slices (400 μm thick) with a McIlwain tissue chopper. Slices were suspended in a pregassed (carbogen) artificial cerebrospinal fluid (aCSF) containing (in mM): 1.25 NaH_2PO_4; 22 NaH_2CO_3; 1.8 $MgSO_4$; 129.0 NaCl; 1.8 $CaCl_2$; 3.5 KCl; 10 D-glucose, and pH was adjusted to 7.4 with carbogen, and incubated for 30 min at 37 °C with or without GM1 (10, 30 or 100 μM). The effect of GM1 (100 μM) on NOx content along time was determined at 10, 30 or 60 min. In those experiments designed to evaluate the effect of L-NAME on GM1-induced NOx increase, slices were incubated with aCSF, GM1 (100 μM), L-NAME (100 μM) or GM1 plus L-NAME for 30 min at 37 °C. The concentration of L-NAME was chosen based on a pilot dose-response curve. After incubation, slices were homogenized with $ZnSO_4$ (200 mM) and acetonitrile (96%). The homogenates were centrifuged at 16,000 × *g* for 30 min at 4 °C, and the supernatant was collected for determination of NOx content as described above.

2.5. Hemoglobin content

The content of hemoglobin in brain and slice homogenates was spectrophotometrically estimated by the absorbance of the Soret band (405–435 nm, multiple wavelength scan), according to Henry (1991) in a Hitachi U-2001 double beam spectrophotometer (Hitachi Instruments Incorporation, Schaumburg, IL, USA). The area under the curve (AUC) of the Soret band was quantified by Autodesk (AutoCAD® 2005), corrected for protein content and expressed as AUC/mg of protein as an index of hemoglobin content.

2.6. Protein determination

Protein content was measured colorimetrically by the method of Bradford (1976), and bovine serum albumin (1 mg/ml) was used as standard.

2.7. Statistical analysis

Variations in the outer diameter of pial vessels induced by GM1 were analyzed by a 2 (NaCl or L-NAME) × 2 (NaCl or GM1) × 2 (measures) factorial ANOVA considering the "measures" factor as a within-subject factor. Brain NOx and Hb content *ex vivo* were analyzed by a 2 (NaCl or GM1) × 3 (times) × 2 (hippocampus or cortex) factorial ANOVA, with the cerebral structure treated as a within-subject factor. Data from the experiments that evaluated the effect of increasing concentrations of GM1 on NOx and Hb content *in situ* were analyzed by one-way ANOVA. Data from the experiment that assessed the effect of GM1 on NOx content *in situ* were analyzed by a 2 (aCSF or GM1) × 3 (10, 30 or 60 min) factorial ANOVA. In those experiments involving L-NAME, NOx and hemoglobin content were analyzed by a two-way ANOVA. Post hoc analysis was carried out by the Student–Newman–Keuls test. A $P < 0.05$ was considered significant. All data are reported as mean + S.E.M.

3. Results

In order to investigate whether nitric oxide is involved in the vasodilation induced by GM1, we measured the outer diameter of pial vessels after the systemic injection of GM1 (50 mg/kg, i.p.) or its vehicle, in the presence or absence of L-NAME (60 mg/kg, i.p.) in anesthetized rats (Fig. 1). Baseline diameter of pial vessels was 41.7 ± 0.44 μm (range 7.5–95 μm, median = 44.6 μm). Statistical analysis showed that GM1 increased in 50% the outer diameter of pial vessels compared with baseline at 30 min [two-way ANOVA: $F(1,16) = 26.87$, $P = 0.001$, Fig. 2A and B], confirming our previous work (Furian et al., 2007). The administration of NaCl or L-NAME alone did not change the outer diameter of the pial vessels [$F(1,16) = 0.40$, $P = 0.535$, data not shown]. However, the administration of L-NAME fully prevented GM1-induced vasodilation [$F(1,16) = 24.49$, $P = 0.001$, Fig. 2C and D].

The fact that L-NAME blunted GM1-induced vasodilation *in vivo* suggested the involvement of nitric oxide in such an effect. Since

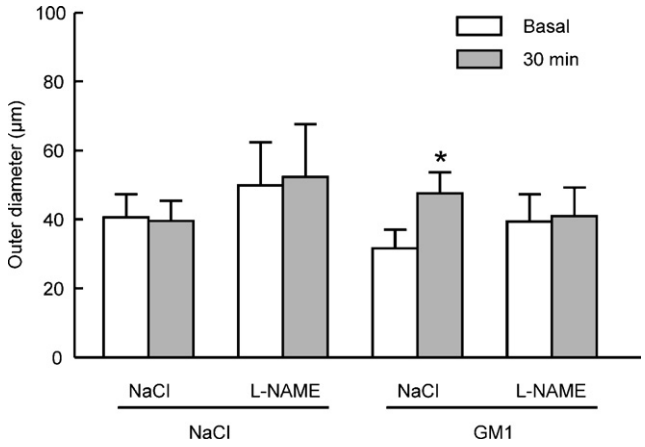

Fig. 1. Effect of NaCl (0.9%), GM1 (50 mg/kg, i.p.), L-NAME (60 mg/kg, i.p.) and L-NAME plus GM1 on the outer diameter of pial vessels (in μm) at 30 min. Data are mean + S.E.M. for $n = 5$ animals in each group, from five different experiments. *Indicates a significant difference ($P = 0.001$) compared with basal values.

determination of NOx levels has been used to infer variations in NO production (Bryan and Grisham, 2007), we investigated whether GM1 administration increased NOx content *ex vivo* and *in situ*. Fig. 3 shows the effect of systemic injection of GM1 (50 mg/kg, i.p.) 15, 30 or 60 min before sacrifice on the NOx content of the hippocampus (3A) and cerebral cortex (3B). Statistical analysis revealed a significant effect of treatment [$F(1,42) = 7.28$, $P = 0.010$] and a significant effect of time [$F(2,42) = 4.44$, $P = 0.018$] indicating that GM1 decreased NOx content 60 min after administration in hippocampus and in cerebral cortex *ex vivo*. The effect of GM1 on hemoglobin content was also determined. Statistical analyses revealed a significant effect of treatment [$F(1,42) = 4.74$, $P < 0.05$] and a significant effect of brain structure [$F(1,42) = 47.26$, $P < 0.001$, data not shown] on hemoglobin content. Post hoc analysis revealed that GM1 increased hemoglobin content in all cerebral structures, and that cerebral cortex presents the highest hemoglobin content among the studied structures, replicating our previous findings (Furian et al., 2007).

Since it has been demonstrated that NO end products like nitrites and nitrates can be removed from brain *in vivo* by blood flow (Kumura et al., 1994), a possible effect of GM1 on NOx levels *ex vivo* could be masked. Therefore, we decided to investigate the effect of GM1 (0, 10, 30 or 100 μM) on NOx content in slices of cerebral cortex (Fig. 4A). The slice preparation is devoid of a functional vascular system and, therefore, would be expected to reveal a possible effect of GM1 on NOx levels. Statistical analysis revealed that incubation of slices with GM1 (100 μM) for 30 min significantly increased NOx levels [$F(3,24) = 3.74$, $P = 0.024$]. As expected, hemoglobin content in cortical slices was not altered by incubation with GM1 [$F(3,24) = 0.291$, $P = 0.832$, Fig. 4B]. In addition, we investigated whether GM1 (100 μM) altered NOx content along time. Statistical analysis revealed a highly significant treatment by time interaction [$F(2,16) = 12.72$, $P < 0.001$]. Post hoc analysis revealed that GM1 increased NOx content in cortical slices at 30 min, but decreased at 60 min (Fig. 5A). As expected, no significant effect of GM1 on hemoglobin content of slices was found along time [$F(2,16) = 1.90$, $P > 0.05$, Fig. 5B].

The effect of L-NAME (30, 100 and 300 μM, for 30 min) on NOx and hemoglobin content of cortical slices was also evaluated. Statistical analysis revealed no significant effect of L-NAME *per se* on NOx [$F(3,12) = 0.295$, $P = 0.829$, data not shown] or hemoglobin [$F(3,12) = 0.057$, $P = 0.981$, data not shown] content.

In order to obtain pharmacological evidence for the role of nitric oxide synthase (NOS) in GM1-induced increase of NOx content *in situ*, cortical slices were incubated with aCSF or L-NAME (100 μM)

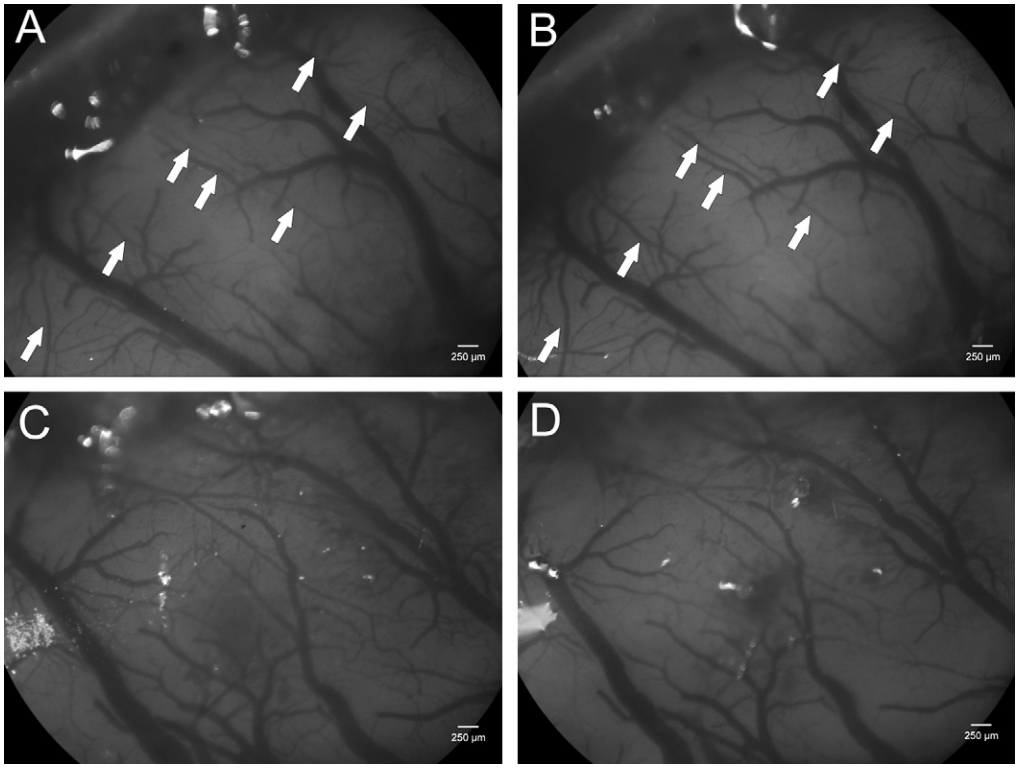

Fig. 2. GM1 increases the outer diameter of pial vessels. (A) and (B) shows representative micrographs of pial vessels before (baseline) and 30 min after the administration of GM1 (50 mg/kg, i.p.), respectively. Arrows indicate vasodilated pial vessels, compared to baseline (40×). (C) and (D) shows representative micrographs of pial vessels before (baseline) and 30 min after the administration of GM1 (50 mg/kg, i.p.) to an animal previously injected with L-NAME (60 mg/kg, i.p., 15 min before GM1), respectively.

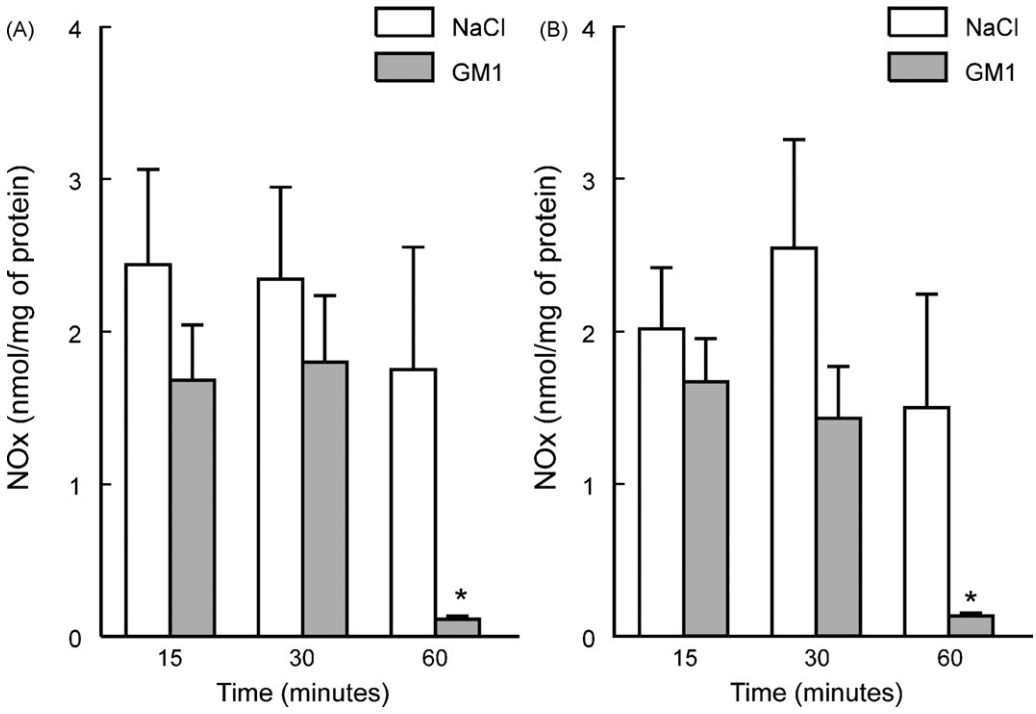

Fig. 3. Effect of GM1 (50 mg/kg, i.p.) injected 15, 30 and 60 min before sacrifice, on NOx content in hippocampus (3A) and cortex (3B) ex vivo. Data are mean + S.E.M. for $n = 8$ in each group, from five different experiments. *Indicates a significant difference ($P = 0.010$) compared with the respective NaCl group.

in the presence or absence of GM1 (100 μM) for 30 min, and the NOx content was measured (Fig. 6A). Statistical analysis revealed a significant NOS inhibitor (aCSF or L-NAME) by glycolipid (GM1 or aCSF) interaction: $F(1,16) = 18.24$, $P = 0.001$, because incubation with L-NAME blunted GM1-induced increase of NOx content. As expected, neither the glycolipid nor the NOS inhibitor altered the content of hemoglobin in the slices [$F(1,16) = 0.43$, $P = 0.524$, Fig. 6B].

4. Discussion

In the current study we showed that GM1-induced increase of pial vessels outer diameter (50% increase) is fully prevented by NOS inhibitor L-NAME, suggesting that NO underlies the vasodilation induced by GM1 (Figs. 1 and 2). We also showed that GM1 increases NOx content by 50% in cortical slices after a 30 min exposure and decreases NOx content after 60 min (Figs. 4A and

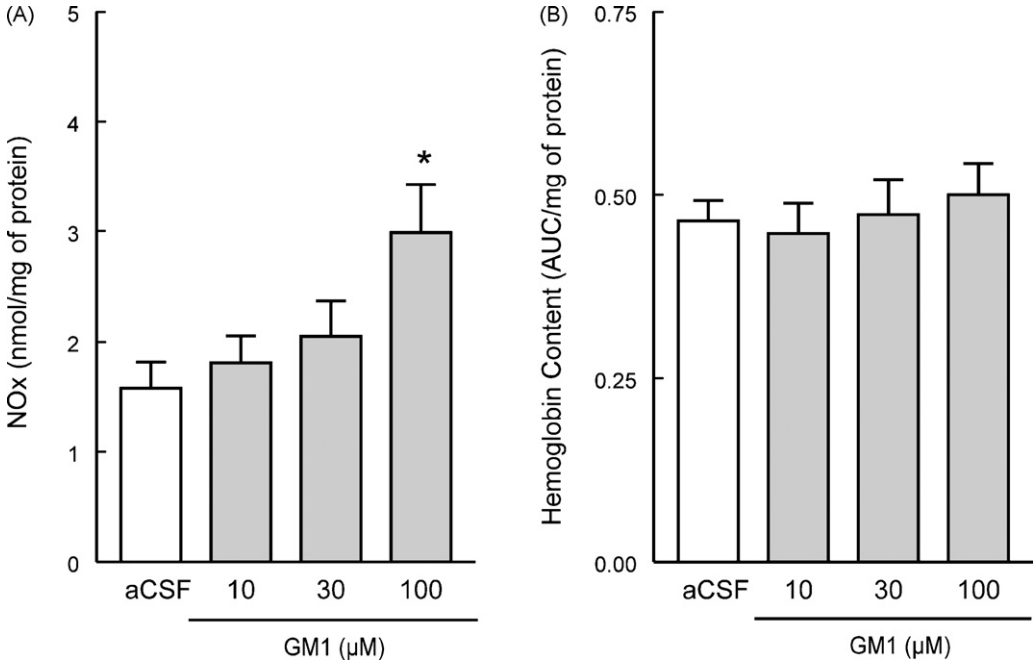

Fig. 4. Effect of GM1 (0, 10, 30 or 100 μM) on NOx (4A) and hemoglobin content (4B) *in situ*. Data are mean + S.E.M. for $n = 7$ in each group, from three different experiments. *Indicates a significant difference ($P = 0.024$) compared with the aCSF group.

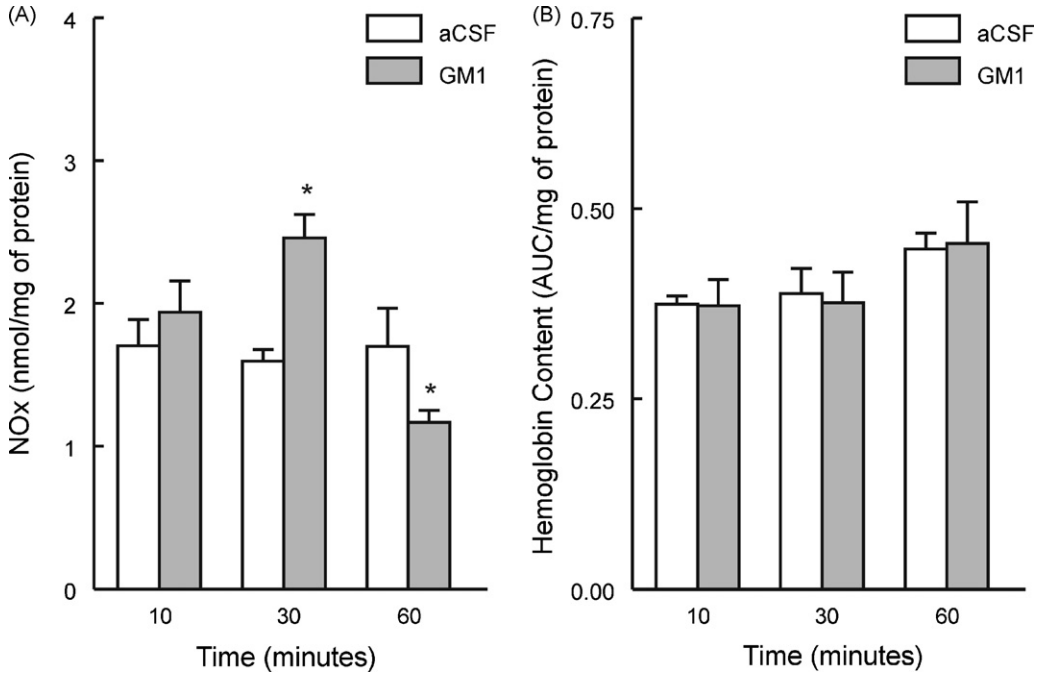

Fig. 5. Effect of GM1 (100 μM) exposure for 10, 30 or 60 min on NOx (5A) and hemoglobin content (5B) *in situ*. Data are mean + S.E.M. for $n = 9$ in each group, from five different experiments. *Indicates a significant difference ($P = 0.001$) compared with the respective aCSF group.

5A), and that the stimulatory effect of GM1 is fully prevented by L-NAME (Fig. 6A). Interestingly, the systemic administration of GM1 did not modified NOx content *ex vivo* within 30 min (Fig. 3), but caused vasodilation measured by the increase in pial vessels outer diameter (Figs. 1 and 2) and by the increase in hemoglobin content in hippocampal and cortical samples (data not shown).

A number of studies have showed that GM1 exerts vasomotor effects. In fact, it has been shown that GM1 administration (30 mg/kg, i.v.) significantly restores local cerebral blood flow and glucose metabolism in animals subjected to arterial occlusion (Tanaka et al., 1986). Moreover, GM1 reduces brain edema and increases cerebral metabolism after traumatic brain injury (Chen et al., 2003), and improves neurological status and tends to increase cerebral blood flow in Alzheimer's diseased patients (Svennerholm et al., 2002), further suggesting that vasodilation and better perfusion may underlie some pharmacological effects of GM1. In addition, we have recently demonstrated that GM1 induces vasodilation and increases catalase activity in brain samples (Furian et al., 2007), providing a possible convergent mechanism for the neuroprotective actions of GM1 against various neurodegenerative conditions, such as anoxia (Carolei et al., 1991; Tan et al., 1993), ischemia (Carolei et al., 1991; Kwak et al., 2005),

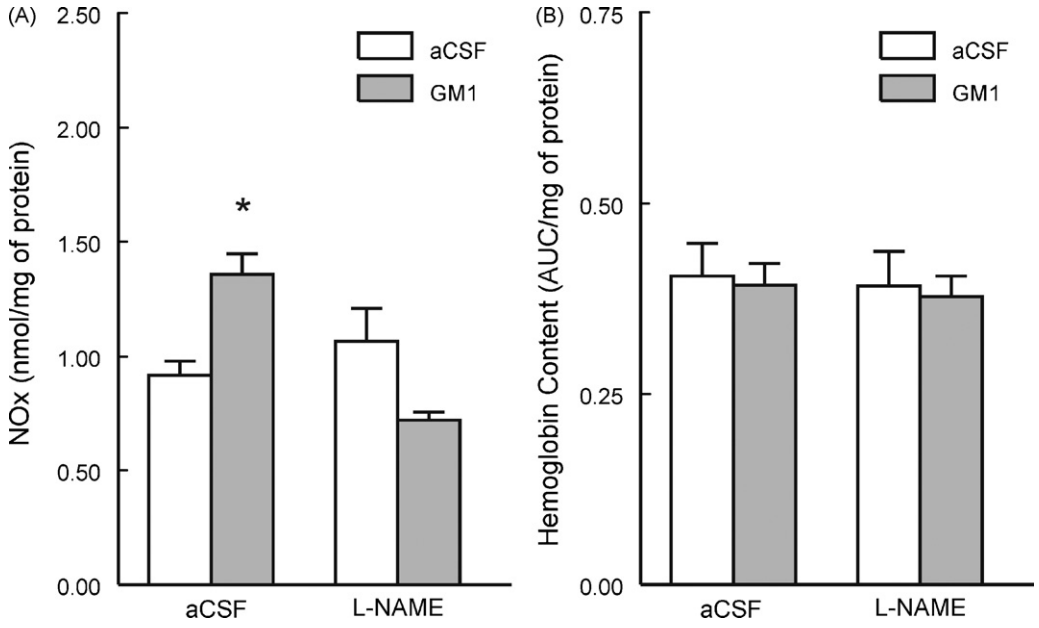

Fig. 6. Effect of L-NAME (100 μM) and GM1 (100 μM) on NOx (6A) and hemoglobin content (6B) *in situ*. Data are mean + S.E.M. for n = 5 in each group, from three different experiments. *Indicates a significant difference ($P = 0.001$) compared with the aCSF group.

Parkinson's (Schneider, 1998) and Alzheimer's diseases (Yanagisawa, 2007), in which there is a failure in brain antioxidant system and oxidative stress. However, this is the first study addressing the mechanisms underlying the vasomotor effects of GM1.

In the present study we show that GM1 causes vasodilation and consequently increases brain hemoglobin content through NOS, a key enzyme for vasodilatation control (Palmer et al., 1987), since the effects of GM1 were prevented by the NOS inhibitor L-NAME. Interestingly, it has been shown that GM1 activates Trk C receptors (Duchemin et al., 2002; Rabin et al., 2002), whose activation causes a fourfold increase in nitric oxide production by rat cerebral endothelial cells (Takeo et al., 2003). These findings are in agreement with the currently reported vasodilation induced by GM1, and its reversal by L-NAME. However, one must be aware that the high concentrations of GM1 used in the present study and the time required for the appearing of vasoactive effects of this ganglioside make it unlikely that endogenous GM1 is involved in the physiological regulation of the cerebral vessels tone.

NO is involved in several cellular functions, particularly in the brain, and has been recognized as a critical physiological mediator in the regulation of vascular tone (Vallance et al., 1989; Moncada and Higgs, 1991). NO is synthesized by NOS isoforms, nNOS and eNOS that are constitutively expressed and require the formation of Ca^{2+}-calmodulin complexes, and by iNOS, which does not require Ca^{2+}-calmodulin complexes formation for its activation (Calabrese et al., 2007). nNOS is abundantly expressed in selected brain areas, such as the cerebral cortex, striatum, amygdala, hippocampus, hypothalamus and thalamus (Vincent and Kimura, 1992; Rodrigo et al., 1994). Interestingly, nNOS has also been found in astrocytes and in the adventitia of rat brain blood vessels (Nozaki et al., 1993), while eNOS is mainly expressed in the endothelium (Marsden et al., 1993). Endothelial NOS has also been found in other cell types, such as human neuronal cells (Oka et al., 2004), human and rat astrocytes (Colasanti et al., 1998) and both have important role in regulating cerebral blood flow (Furchgott and Vanhoutte, 1989; Lee, 2000).

Given its high biological reactivity and diffusibility, NO biosynthesis is controlled by several mechanisms. These encompass modulation of the catalytic reaction, transcription, and post-transcriptional regulation of NOS. Short-term activation, as seen in our study, seems to be mediated by substrate and cofactor availability and phosphorylation (Kavya et al., 2006). Regarding this point, several consensus sequence sites for phosphorylation by protein kinases are found in all three NOS isoforms. For instance, phosphorylation of eNOS by phosphatidylinositol 3-kinase/Akt activates enzyme activity, resulting in increased synthesis of NO (Dimmeler et al., 1998; Fulton et al., 1999). Moreover, the phosphatidylinositol 3-kinase inhibitors wortmannin and LY-294002 prevent the nNOS-mediated increase in NO content (Canabal et al., 2007), further suggesting that phosphatidylinositol 3-kinase activates NOS. Since it has been demonstrated that GM1 activates phosphatidylinositol 3-kinase at concentrations comparable to that used in our study (Duchemin et al., 2007), it is tempting to propose that GM1 activates NOS through the phosphatidylinositol 3-kinase pathway. However, this discussion is speculative in nature, and further studies are necessary to determine how GM1 might activate NOS.

It has been shown that L-arginine infusion, a substrate for NO synthesis, increases pial vessels diameter by nitric oxide-dependent mechanism and improves regional cerebral blood flow (rCBF) distal to middle cerebral artery (MCA) occlusion. As a consequence, L-arginine reduces infarction volume in spontaneously hypertensive rats when administered before and after MCA occlusion, further supporting a role for nitric oxide in regulating rCBF (Morikawa et al., 1992, 1994). Interestingly, eNOS knockout mice present increased neuronal death after a stroke (Huang et al., 1996), which is attenuated by L-arginine. Since L-arginine-induced neuroprotection occurs with concomitant vasodilation, it has been argued that NO derived from other sources than eNOS increases blood flow and underlies such neuroprotection. Based on data from the present study we cannot conclude which NOS isoform is targeted by GM1, and more studies are necessary to determine if the actions of GM1 are due to an effect on a specific NOS isoform.

Since it has been demonstrated that NO end products like nitrites and nitrates can be removed from brain *in vivo* by blood flow (Kumura et al., 1994), a possible effect of GM1 on NOx levels *ex vivo* could be masked. Accordlingly, we have not found a GM1-induced increase in NOx content *ex vivo*. However, the experiments in slices of cerebral cortex revealed that GM1 increased NOx production *in situ* at 30 min, in the absence of blood circulation.

It is remarkable that GM1 reduced NOx content in the hippocampus and cerebral cortex (*ex vivo*) and in cortical slices (*in situ*) at 60 min, an effect that temporally coincided with the end of the vasodilatory effect of GM1 *in vivo*. An explanation for this fact come from the fact that nitric oxide accumulation by NOS activity is able to inhibit this enzyme by negative feedback (Assreuy et al., 1993), and thus it is plausible to propose that a significant increase in nitric oxide content 30 min after GM1 administration may result in subsequent inhibition of NOS acitivity, leading to the currently reported decrease in NOx content 60 min after the administration of GM1. Accordingly, Baumgartner et al. (1997) and Tseng et al. (1998) have shown that GM1 (30 mg/kg, i.v. for 3 days) prevents the increase in cerebral citrullin levels (a coproduct of nitric oxide formed from arginine as a result of nitric oxide synthase activity) in brain of dogs subjected to prolonged circulatory arrest. These results, which indicate a bimodal effect of GM1 on NOS activity determined by the duration of the exposition to the ganglioside, are in agreement with the results from Dawson et al. (1995), who have shown that a 2-h exposure of cells transfected with nNOS to gangliosides (including GM1) inhibits NOS activity (Dawson et al., 1995) and the study of Higashi and Yamagata (1992), who have shown that while low concentrations of gangliosides activate, high concentrations of gangliosides inhibit calmodulin-dependent enzymes. Considering that longer incubations would facilitate GM1 ganglioside incorporation to membranes (Riboni and Tettamanti, 1991) and increase its cellular availability, one might suppose a consequent dual effect on calmodulin binding that could activate or inhibit calmodulin-dependent NOS activity. However, more studies are needed to determine the underlying mechanisms of the currently reported dual effect of GM1 on NOx production *ex vivo* and *in situ*.

In addition, it is interesting to investigate whether GM1 is neuroprotective in other experimental models, such as hyperglycemic cerebral ischemia, since vascular production of superoxide, nitric oxide and peroxynitrite increases in this condition and could contribute for cerebral damage (Bemeur et al., 2007). This is in agreement with the view that NOS inhibitors with antioxidant properties, such as 7NI (Thomas et al., 2008) and GM1, may have a wide spectrum of clinical applications which were not fully considered. Despite the broad neuroprotective properties of exogenous and endogenous gangliosides, particularly GM1, one must be aware that genetic defects that induce ganglioside accumulation are deleterious. Interestingly Baek et al. (2008) have suggested the use of an imino sugar (NB-DGJ) to reduce total brain gangliosides and GM2 content without altering GM1 concentration, in β-hexosaminidase deficient mice.

In summary, in this study we demonstrated that NO plays a role in GM1-induced vasodilation in the brain and that longer exposure to GM1 decreases NO production. These findings further support the use of GM1 ganglioside, or related drugs, to treat clinical

conditions in which vasodilation is associated to a better prognosis, such as obstructive vascular, cardiac insufficiency and neurodegenerative diseases, combining the beneficial effects of early NO-mediated reperfusion with late inhibition of deleterious nitrosative species production.

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