

# GM3 ganglioside inhibits endothelin-1-mediated signal transduction in C6 glioma cells

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**Abstract** We found that sparse and confluent C6 glioma cells differ both in GM3 content, which increases with cell density, and in endothelin-1 (ET-1)-induced phosphoinositide hydrolysis, which was markedly higher in the sparse cells than in the confluent. Also after manipulation of the cellular GM3 content through treatment with exogenous GM3 or with drugs known to affect GM3 metabolism, the ET-1 effect was inversely related to GM3 cellular levels. Cell treatment with an anti-GM3 mAb resulted in the enhancement of ET-1-induced phospholipase C activation and restored the capacity of GM3-treated cells to respond to ET-1. These findings suggest that the GM3 ganglioside represents a physiological modulator of ET-1 signaling in glial cells. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

**Key words:** Endothelin; GM3 ganglioside; Inositol phosphate; Glial cell; C6 glioma cells

## 1. Introduction

Gangliosides are a family of sialic acid-containing glycosphingolipids located almost exclusively in the outer leaflet of the plasma membrane and particularly abundant in the nervous system [1]. The peculiar structure and the localization of gangliosides result in their involvement in several interaction mechanisms, such as those with external stimuli, receptors and transduction molecules [2–4]. In fact there is a significant body of literature that suggests that different gangliosides, particularly GM3, are able to affect the interaction of different ligands with their specific receptors and enzymes involved in protein phosphorylation/dephosphorylation, and second messenger generation [2,3]. These properties result in cellular gangliosides contributing to the regulation of crucial cell processes such as proliferation, differentiation and oncogenesis [4,5].

Within the plethora of extracellular signals that regulate cell function in the nervous system endothelin (ETs), and above all ET-1, represent a class of molecules that plays a crucial role in CNS functioning, acting as neurotransmitters or neu-

romodulators [6]. Besides neurons, also glial cells express ET genes and ET receptors, that are also involved in stimulating cell proliferation [6]. The role of ETs in glial cells is underscored by the evidence that these bioactive peptides are involved in patho-physiological processes such as traumatic injury, ischemia, and glial tumors [6]. In both astrocytes and glioma cells ET-1 exerts its effects through the signal transduction pathway that involves the stimulation of phosphatidylinositol (PI) turnover via phospholipase C (PLC) activation [7]. Notwithstanding this evidence, little is known on the regulatory molecules of ET-coupled signal transduction and, in particular, on the possible role played by gangliosides in this process, especially in glial cells.

In the present study we investigated the possible effect of gangliosides on ET-1 receptor-mediated signaling pathway and, for this purpose, C6 glioma cells were chosen as the experimental model. Like other glial cells, these cells exhibit both a simplified ganglioside pattern, characterized by the preponderance of GM3 species [8], and express ET receptors coupled with the activation of a PI-specific PLC [9–11]. The data obtained strongly support the action, in C6 cells, of cellular GM3 as a negative modulator of the ET-coupled signal transduction mechanism.

## 2. Materials and methods

### 2.1. Chemicals

Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), brefeldin A (BFA), bovine serum albumin (BSA), and Neu5Ac were from Sigma (St. Louis, MO, USA). *N*-Butyldeoxynojirimycin (NB-DNJ) was from Biomol (Plymouth Meeting, PA, USA). ET-1 was from Calbiochem (La Jolla, CA, USA). [*myo*-2-<sup>3</sup>H]inositol (22 Ci/mmol) was from New England Nuclear (Boston, MA, USA). The gangliosides GM3, GM2 and GM1 were prepared and purified as previously described [12]. The monoclonal antibody anti-GM3 DH2 (mouse IgG3) [13] was kindly provided by Prof. S.-I. Hakomori (Pacific Northwest Research Institute, Seattle, WA, USA). Total mouse IgG was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Dowex anion-exchange resin (AG1-X8, formate form, 200–400 mesh) was purchased from Bio-Rad (Richmond, CA, USA) and high performance thin layer chromatography (HPTLC) silica gel plates were from Merck (Darmstadt, Germany).

### 2.2. Cell culture

Rat C6 glioma cells were cultured in DMEM supplemented with 10% FCS, 100 U/ml of penicillin and 100 µg/ml streptomycin, in an atmosphere of 5% CO<sub>2</sub>/95% humidified air. The cells were plated in 35- and 100-mm diameter dishes at 4000 and 16000 cells/cm<sup>2</sup> in order to obtain sparse and confluent cell populations respectively. The culture medium was changed 48 h after plating and the cells were grown for another 24 h at 37°C. BFA was added to the growth medium at 1 µg/ml final concentration, NB-DNJ and gangliosides GM3, GM2

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**Abbreviations:** BFA, brefeldin A; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; ET, endothelin; FCS, fetal calf serum; HPTLC, high performance thin layer chromatography; IP, inositol phosphate; NB-DNJ, *N*-butyldeoxynojirimycin; PI, phosphatidylinositol; PLC, phospholipase C

and GM1 at 50  $\mu$ M unless otherwise stated. All treatments were done for 24 h in the presence or absence of [*myo*- $^3$ H]inositol for IP determination and ganglioside analysis, respectively.

### 2.3. Determination of inositol phosphate (IP) formation

The phosphoinositide hydrolysis was measured as previously described [11,14] with minor modifications. Cells, grown in 35-mm culture dishes for 72 h, were labeled to equilibrium in the last 24 h with [*myo*- $^3$ H]inositol (1  $\mu$ Ci/ml) in DMEM supplemented with 10% FCS. After labeling, the medium was removed and the cells were washed three times with physiological saline solution (118 mM NaCl, 4.7 mM KCl, 1.8 mM  $\text{CaCl}_2$ , 1.2 mM  $\text{MgCl}_2$ , 1.2 mM  $\text{KH}_2\text{PO}_4$ , 11 mM glucose and 20 mM HEPES, pH 7.4). After a 15-min preincubation at 37°C in the same buffer containing 20 mM LiCl, the cells were stimulated with 100 nM ET-1. In most experiments the incubation time was 15 min. At the end, after rapid aspiration of the solution from the cells, 0.5 ml ice-cold methanol/concentrated HCl (100:1, v/v) was added and the cells were scraped off the plates and transferred to test tubes. Chloroform (0.5 ml) and 10 mM EDTA (0.25 ml) were added and, after vortexing, the aqueous and organic phases were separated by centrifugation (5000  $\times$  g, 5 min, 4°C). The aqueous phase, containing water-soluble [ $^3$ H]inositol-labeled compounds, was then applied to a Dowex AG 1  $\times$  8 (200–400 mesh, formate form) column. After the elution of free [ $^3$ H]inositol and [ $^3$ H]glycerophosphoinositols, the total [ $^3$ H]IPs ( $\text{IP}_1$ ,  $\text{IP}_2$  and  $\text{IP}_3$ ) were eluted in a single step with 5 ml of 1 M ammonium formate/0.1 M formic acid and then counted for radioactivity. [ $^3$ H]IP levels are expressed as the percentage of the total radioactivity associated with [ $^3$ H]inositol-labeled phospholipids, recovered in the organic phase.

When anti-GM3 mAb DH2 (IgG3) and total mouse IgG were used, the cells were incubated with these agents at 1  $\mu$ g/ml during the preincubation in the presence of lithium and before ET-1 stimulation.

### 2.4. Ganglioside analysis

The cells (grown on 100-mm dishes) were scraped off the plates, lyophilized and then subjected to lipid extraction [15]. The total lipid extract was subjected to a two-phase partitioning [16], with an additional washing of the organic phase. After this washing the recovery of cellular GM3 in the aqueous phase was found to be higher than 93%. The obtained aqueous phase, containing gangliosides, was separated by HPTLC using chloroform/methanol/0.2%  $\text{CaCl}_2$  (55:45:10, by volume). After spraying the plates with a *p*-dimethylaminobenzaldehyde reagent [17], GM3 was quantified by densitometric analysis (Molecular Analyst program, Bio-Rad Laboratories).

### 2.5. Other methods

Ganglioside-bound sialic acid was determined by the resorcinol-HCl method [18] using Neu5Ac as the standard; cell protein were assayed [19] using BSA as the standard. Statistical significance of differences was determined by Student's *t*-test.

## 3. Results and discussion

Preliminary experiments were carried out to determine the kinetic profile of PI hydrolysis after ET-1 stimulation of the C6 glioma cells. The administration of 100 nM ET-1 to C6 cells induced a significant and time-dependent increase in [ $^3$ H]IPs (Fig. 1). This increase in the PI hydrolysis products was already evident 5 min after stimulation, increasing thereafter and reaching the highest levels (365% of unstimulated

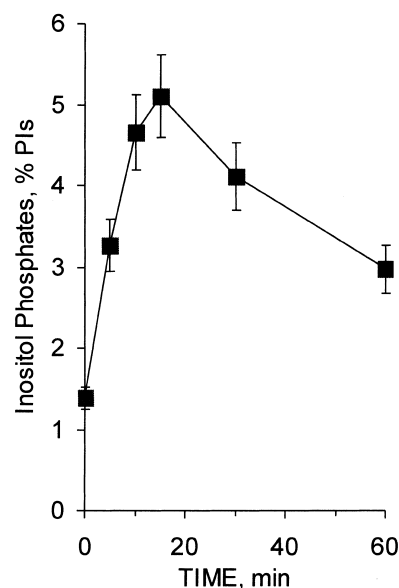


Fig. 1. Time course of ET-1-induced IP formation in C6 cells. Cells were labeled to equilibrium with [*myo*- $^3$ H]inositol and then incubated with 100 nM ET-1 for different times, up to 60 min. Total [ $^3$ H]IPs were extracted and separated as described in Section 2. Data are expressed as the percentage of total [ $^3$ H]PIs, and are the mean values  $\pm$  S.D. of three independent experiments. Basal [ $^3$ H]IPs levels were similar at all the incubation times.

cells) at 15 min. With continued incubation, for up to 1 h, the [ $^3$ H]IPs level declined gradually. Thus 15 min cell stimulation with 100 nM ET-1 was used throughout the study.

In agreement with previous studies [8], the analysis of the ganglioside composition demonstrated that the ganglioside pattern of C6 cells is simplified and characterized by the prevalence (more than 95%) of GM3. The preponderance of GM3 was found in the C6 cells at both the sparse and the confluent stage. However, at these two stages of growth in culture, the amount of cellular GM3 was significantly different (Table 1). In particular the increase in cell density was paralleled by the elevation of the cellular GM3 content, the confluent cells containing about 1.5-fold the amount of GM3 of sparse C6 cells. These data are in agreement with previous, early findings [20–22] demonstrating that in some cultured cells the content of GM3 (and other glycosphingolipids) is dependent on the cell density. Interestingly, we found that sparse and confluent C6 cells also displayed a different response to ET-1. In fact, as shown in Table 1, the radiolabeled IP amount accumulated in the sparse cells was about 150% that of the confluent cells.

The evidence that C6 cells at different stages of confluence differ both in their GM3 content and response to ET-1, prompted us to focus our study on the possible role of GM3 as a cellular modulator of ET-1 receptor-coupled PLC activation. Our initial approach was to evaluate whether C6 glioma cells, at the same growth stage but with different GM3 content, respond differently to ET-1. We first administered GM3 ganglioside (in the range 2–50  $\mu$ M) to sparse C6 cells for 24 h. After removal of the exogenous ganglioside the GM3-treated cells were analyzed for both GM3 content and ET-1 response. In the experimental conditions used the administration of GM3 resulted in a dose-dependent increase in cellular GM3 content (Fig. 2, upper panel). This is in agreement with previous evidence demonstrating that exogenous

Table 1  
GM3 content and ET-1 response in C6 glioma cells at different density

Cell density	GM3 (nmol/mg protein)	IP (% of basal level)
Sparse cells	2.90 $\pm$ 0.37	365 $\pm$ 11
Confluent cells	4.25 $\pm$ 0.53**	245 $\pm$ 15**

Sparse and confluent cells were obtained as described in Section 2. IP accumulation was measured after 15 min stimulation with 100 nM ET-1. Data are the mean  $\pm$  S.D. of three independent experiments in duplicate. \*\**P* < 0.001 vs. sparse cells.

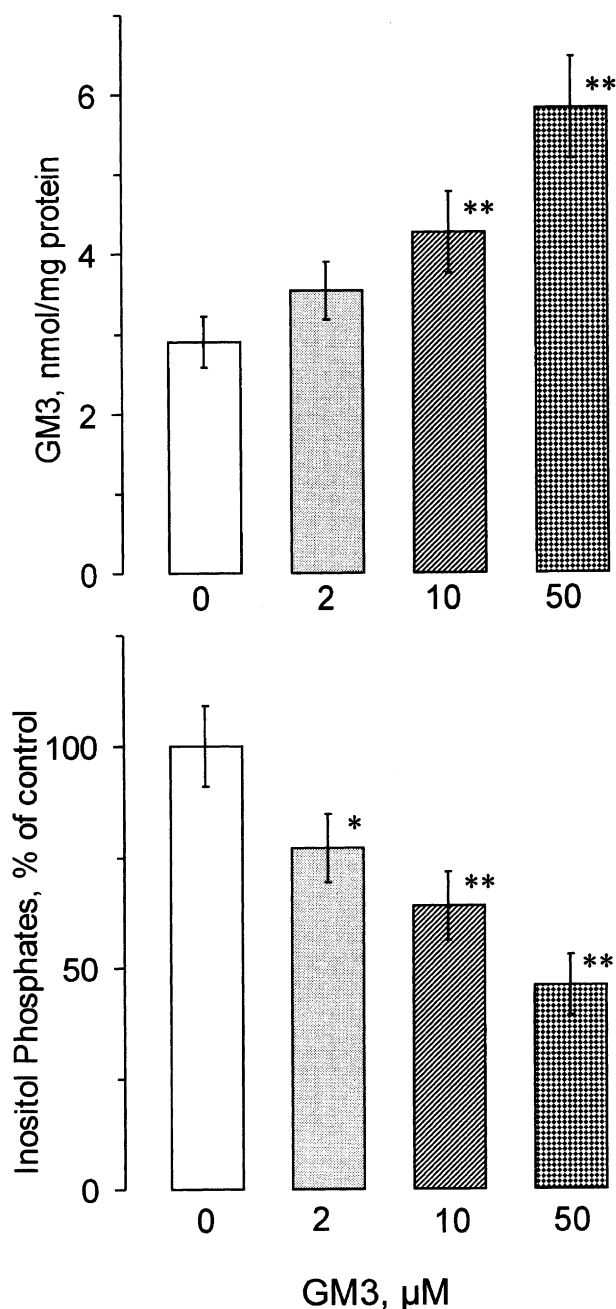


Fig. 2. Effect of exogenous GM3 on cellular GM3 content and ET-1-induced IP formation. Upper panel: Cells were incubated in the absence or presence of GM3 ganglioside at different concentrations. After 24 h, cells were subjected to ganglioside extraction and GM3 quantification as described in Section 2. Lower panel: Cells were incubated with [*myo*- $^3\text{H}$ ]inositol in the absence or presence of GM3 ganglioside at different concentrations. After 24 h, ET-1 (100 nM) was added for 15 min and accumulation of total [ $^3\text{H}$ ]IPs determined. Data are expressed as the percentage of control (cells incubated in the absence of GM3) and are the mean values  $\pm$  S.D. of at least three separate experiments. \* $P < 0.05$ ; \*\* $P < 0.01$ .

gangliosides can be taken up by cells and inserted into the plasma membrane, like endogenous compounds [23]. In GM3-treated cells ET-1-induced PLC activation, evaluated as PI hydrolysis, was lower than in untreated cells (lower panel, Fig. 2). The inhibitory effect was directly related to the amount of cellular GM3 and, at the highest dose, the ET-1-

induced IP formation was decreased by about 50%. This effect appeared to be specific, since in the same experimental conditions two other monosialogangliosides, GM2 and GM1 (up to 50  $\mu\text{M}$ ), did not produce any relevant effect on ET-1-stimulated IP formation ( $93 \pm 8.6$  and  $97 \pm 9.0\%$  of control, for GM2 and GM1, respectively).

In further experiments, ET-1 was incubated with the GM3 ganglioside (for 15 min at 37°C) prior to its administration to cells. The analysis of IP formation indicated that the preincubation of ET-1 with GM3 (up to 50  $\mu\text{M}$ ) had no effect on the PLC activation by the stimulus ( $96 \pm 10.5\%$  of control). Thus it appears that the effect of GM3 on ET-1 response does not occur through the direct interaction of the ganglioside with this peptide. On the basis of the present data and previous evidence supporting the implication of gangliosides in receptor function [2–4], it can be speculated that GM3 may act through the modulation of ET receptors.

To further support that cellular levels of GM3 affect ET-1-induced PLC activation, the endogenous content of GM3 was also modified by incubating the cells with drugs known to affect ganglioside metabolism. We administered either BFA, an inhibitor of the anterograde vesicular transport between Golgi compartments [24], or NB-DNJ, an inhibitor of glucosylceramide synthase [25]. As shown in Fig. 3 (left panel), both agents affected the cellular content of GM3 markedly, although in opposite directions: BFA caused a significant increase and NB-DNJ a marked decrease in cellular GM3. These data support previous studies [26–28] and demonstrate that in C6 cells GM3 metabolism, is perturbed by these drugs. Moreover, also in these experimental conditions the ET-1 effect on PI hydrolysis is inversely related to the GM3 cellular levels (Fig. 3, right panel). In fact, compared to untreated cells, the GM3 content in the BFA-treated cells increased by

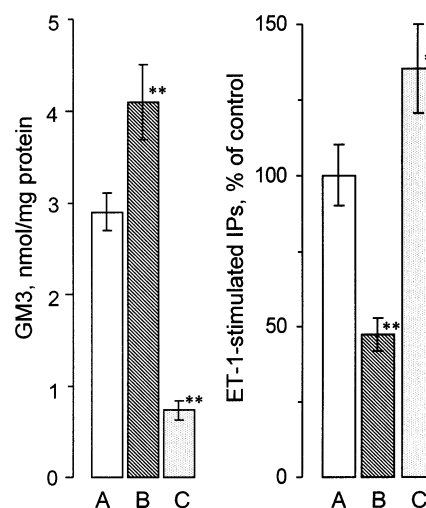


Fig. 3. Effect of different treatments on GM3 content and ET-1-induced IP formation. Cells were incubated for 24 h with [*myo*- $^3\text{H}$ ]inositol in the absence (A) or presence of 1  $\mu\text{g/ml}$  BFA (B) or 50  $\mu\text{M}$  NB-DNJ (C). After stimulation with 100 nM ET-1 for 15 min, GM3 content (left panel) and total [ $^3\text{H}$ ]IP levels (right panel) were measured. For each treatment the production of [ $^3\text{H}$ ]IPs observed in the presence of ET-1 was calculated by subtraction of the corresponding basal level without ET-1 treatment ( $1.39 \pm 0.21$ ,  $0.88 \pm 0.18$  and  $1.47 \pm 0.25$  for A, B and C, respectively) and expressed as the percentage of the response observed in ET-1-stimulated control cells (A). Each point is the mean  $\pm$  S.D. of three independent experiments. \*\* $P < 0.01$  vs. A.

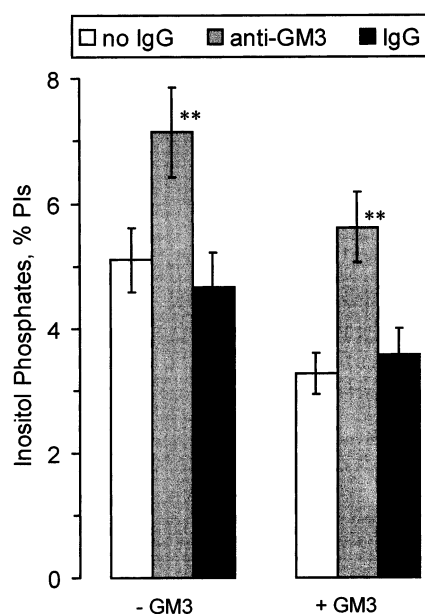


Fig. 4. Effect of anti-GM3 mAb on ET-1-induced PI turnover. C6 cells were incubated with [*myo*-<sup>3</sup>H]inositol in the absence (–GM3) or presence (+GM3) of 10  $\mu$ M GM3. After 24 h, cells were stimulated with ET-1 prior to (control) or after treatment (1  $\mu$ g/ml, 15 min) with anti-GM3 mAb DH2 (mouse IgG3) or total mouse IgG. Data are the mean  $\pm$  S.D. of three independent experiments. \*\* $P < 0.01$ .

40%, and IP formation induced by ET-1 was reduced to 50%. Conversely, in the NB-DNJ-treated cells there was a reduction in the GM3 levels (by about 60%) and a higher response to ET-1-stimulated IP formation (40% increase).

To confirm that cellular GM3 represents a modulator of ET-1 response in C6 glioma cells, cells carrying different amounts of GM3 were exposed to a specific anti-GM3 mAb before ET-1 stimulation. The treatment of sparse C6 cells with an anti-GM3 mAb (IgG3), but not with IgG, resulted in the enhancement of ET-1-induced PI hydrolysis (–GM3, Fig. 4). Moreover, mAb treatment of ‘GM3-loaded’ cells resulted in an increase in ET-1 response and appeared to restore the capacity of these cells, impaired after GM3 treatment, to respond to ET-1 (+GM3, Fig. 4).

The data presented here provide evidence that the response of C6 cells to ET-1 is inversely related to the cellular amount of GM3, and suggest that the ET-induced signaling activity in glial cells may depend on the amount of this ‘modulatory’ ganglioside present in the cells. Thus, the regulation of GM3 content may represent an effective mechanism to control ET-1 signaling and functional effects in glial cells. To the best of our knowledge our observations document the first evidence that the GM3 ganglioside is able to modulate the ET-1-mediated signaling pathway in cells, and provide a prompt starting point for future studies on the mechanism underlying this effect.

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