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Brain microvessel endothelin type A receptors are coupled to ceramide production

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Abstract

Treatment of brain microvessels with endothelin-1 evoked an early decrease in the sphingomyelin levels concomitantly with an increase in those of ceramides. These responses were time- and concentration-dependent. Evidence also shows that endothelin type A receptors are involved. This is the first report on the involvement of an agonist in the regulation of the ceramide signal transduction system on blood–brain barrier and shows a new pathway likely involved in the regulation of the cerebral microvascular functioning.

Keywords: Endothelin 1; Ceramide; Blood–brain barrier

The disruption of the blood–brain barrier (BBB) leads to the unrestricted passage of cells and molecules into the brain leading to pathophysiologic processes. This event plays a pivotal role in the etiology and/or the development of a variety of central nervous system pathologies [1,2]. Brain microvessels form the anatomical and functional basis of the blood–brain barrier. Although all BBB experimental models have limitations [2,3], a great deal of available evidence has demonstrated that several BBB properties are acquired when intact microvessels are studied due to the important role of paracrine interactions between the cells (endothelial cells, pericytes, and astrocytes) comprising the brain microvessels [3]. Although the existence of many conditions and agonists that alter the BBB permeability are well known, the second messenger systems involved are far to be fully understood [4].

It is well known that endothelin-1 (ET-1) is a vasoactive peptide involved in the control of BBB permeability phenomena [5]. Our interest has been focused on the study of modulation by agonists of brain microvessel signal transduction systems [6–10] and, more specifically, on the mechanism(s) by which ET-1 exerts its actions on nervous system, including brain microvessels [11–13] as well as neuronal systems [14,15]. Thus, in studies with isolated brain microvessels, we have reported the involvement of the phospholipase C-evoked phosphoinositide hydrolysis [11], the protein kinase C-evoked phosphorylation of a 80 kDa protein [12], the platelet-activating factor (PAF) synthesis [11], and the cAMP signal transduction system [13] in the ET-1 action. To our knowledge, little or no information is available on the sphingolipid metabolism in the BBB. There is evidence that tumor necrosis factor-α triggers expression of adhesion molecules and ceramide production in rat endothelial cells from large brain microvessels [16]. Taking into account the important role of ceramide in permeability phenomena, at least in epidermal tissues [17], it is very surprising that to date the BBB ceramide signal transduction system is so poorly understood. Since we have reported that sphingolipid hydrolysis and ceramide production are involved in the ET-1 signal transduction mechanism in neuronal tissue [14,15], we hypothesized that ET-1 may evoke ceramide production in BBB leading to a downstream activation

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of a signal pathway still unexplored in the BBB. The present work is an attempt for the first time to investigate this possibility.

Materials and methods

Chemicals. [γ-^{32}P]ATP (specific activity 3000 Ci/mmol) was purchased from Nuclear Ibérica, Spain. *Escherichia coli* diacylglycerol kinase was from Calbiochem, San Diego, CA. ET-1, BQ-123, and BQ-788, ceramides type III from bovine brain sphingomyelin (SM), containing primarily stearic and nervonic acids and ceramides type IV from bovine brain galactocerebrosides, containing α-hydroxy fatty acids were from Sigma Chemical (St. Louis, MO). High-performance thin layer chromatography plates were purchased from Merck (Darmstad, Germany). All other reagents were of the highest analytical grade available.

Isolation of brain microvessels. Cerebral microvessels were isolated from bovine brains basically as described previously [7], in a buffer containing in millimolar: 103 NaCl, 4.7 KCl, 1.2 KPO_4H_2, 1.2 $MgSO_4$, 25 $NaCO_3H$, 1.4 $CaCl_2$, 10 glucose, and 15 Hepes, pH 7.4. Prior to the isolation of microvessels, the meninges were carefully removed and discarded, and cerebral cortex grey matter was homogenized using a hand-driven pestle in 5 volume of the buffer described above. After centrifugation at 1000*g* for 10 min at 4 °C, the pellet was resuspended on 4 volume of the buffer containing 15% dextran and centrifugated at 4000*g* for 15 min for removing the myelin. The resulting pellet, consisting mainly of microvessels, was resuspended in the same buffer without dextran. Samples were poured onto successive 600, 200, and 50 μm pore-size nylon sieves. The purity of the preparation was quite satisfactory from both morphological and biochemical criteria, and neuronal contaminants were negligible [7].

Incubation of microvessels. Before assays, microvessels were resuspended in a balanced salt solution (BSS), in millimolar: 135 NaCl, 4.5 KCl, 1.5 $CaCl_2$, 0.5 $MgCl_2$, 5.6 glucose, and 10 Hepes, pH 7.4. Aliquots of 300 μl of microvessels were transferred into glass tubes containing BSS and then, either ET-1 or diluent (water) alone was added and incubated for 30 min at 37 °C [11]. To know the receptor subtypes (ET_A or ET_B) involved in ET-1 actions, experiments with specific antagonist receptors were done. The microvessels were preincubated with or without BQ-123 and BQ-788 for 10 min before adding ET-1 for 30 min. The antagonists were dissolved in DMSO or ethanol, respectively, and the diluent final concentration was never higher than 1%. The incubation mixtures were continuously gassed with 95% O_2/5% CO_2. The incubations were stopped by addition of 0.38 ml of BSS containing 10 mM EDTA and 1 ml of chloroform/methanol/13 M HCl (100:100:1, by volume). The samples were homogenized twice for 5 s with Polytron PT 1200 at the maximum speed and then they were sonicated.

Extraction of total lipids—separation of sphingolipids. Lipids were extracted by the method described previously [18]. The organic phases were dried under N_2 atmosphere and the total lipids were weighed and dissolved in chloroform/methanol (2:1, by volume). One aliquot of total lipid was used for determination of total phospholipid (see later) and the other one was subjected to alkaline hydrolysis in 0.1 M methanolic KOH at 37 °C for 1 h to remove glycerolipids, as previously described [19]. SM and ceramide were resolved by sequential one-dimensional TLC using chloroform/methanol/acetic acid/water (25:15:4:1.5) and chloroform/methanol/acetic acid (65:2.5:4). The former solvent system reached 3 cm from the top plate, whereas the latter was developed through the plate, as described previously [19]. Lipid standards were co-chromatographed with samples. Lipids were visualized by iodine vapors and the bands corresponding to ceramides were scraped from the plates and extracted with chloroform/methanol (4:5, by volume) and dried under N_2 atmosphere for posterior quantitation. SM bands and the aliquot of total lipid were quantitated by measurement of inorganic phosphorous content [20].

Radioenzymatic determination of ceramide levels. Extracted ceramide was phosphorylated in the presence of diacylglycerol kinase, basically as described previously [19]. Ceramides were solubilized in 40 μl of octyl-β-D-glucoside/cardiolipin solution: 3.75% octylglucoside, 12.5 mM cardiolipin in 1 mM diethylenetriaminepentaacetic acid, and sonicated in a bath sonicator (50/60 Hz) for 1 min to achieve consistent solubilization of the ceramides. The reaction mixture contained (in millimolar): 120 Hepes buffer, pH 7.4, 100 LiCl, 25 $MgCl_2$, 2 EGTA, 2 dithiothreitol, and 5 μg of enzyme and the emulsified ceramide substrate. After preincubation at room temperature for 10 min, the reaction was started by adding 10 μl of 10 mM [γ-^{32}P]ATP in 20 mM imidazol buffer, pH 6.6. After 10 min, phosphorylated derivative of ceramide was extracted, fractionated by TLC using chloroform/pyridine/formic acid (60:30:7), visualized by autoradiography using Kodak X-Omat film, and quantitated by liquid scintillation counting. Calibration curves were performed using known amounts of ceramide.

Statistical analysis. Statistical analysis was made using Student's *t* test for paired observations. *P* values smaller than 0.05 were considered as significant.

Results and discussion

Bovine cerebral microvessels were treated with ET-1 and SM and ceramide levels were measured. It was noteworthy that, at least, two different molecular species of phosphorylated ceramide were found in the radioenzymatic assay, as we have previously described in intact rat aorta rings [15]. The phosphorylated derivative of the major band had a Rf value of 0.2, whereas a value of 0.4 was observed for the minor band. The level of the ceramide specie(s) present in the major band (1.5 nmol/mg total lipids) was about 40 times that of the minor one (34.4 pmol/mg total lipids). These bands run in parallel with the band corresponding to the phosphorylated ceramide type IV standard, which is rich in hydroxy fatty acids. The phosphorylated derivative of the ceramide type III standard (without hydroxy acids) was found to overlap to some degree with that of ceramide type IV. Therefore, it may only be concluded that cerebral microvessel ceramides exist both with or without hydroxy acids. In addition, differences in the length of the fatty acid chain cannot be discarded.

Fig. 1 depicts the time course of ET-1 action on sphingolipid brain microvessels. ET-1 at 10^{-7} M decreased SM levels maximally at 30 min to 60% under control levels, concomitantly with an increase (nearly 60%) in the ceramide levels. Fig. 2 shows the dose-dependent alteration of ceramide induced by ET-1. ET-1 (10^{-7} M) was the lowest dose capable of evoking an accumulation of the two ceramide molecular species. By a still unknown reason, the major specie responds to a lower ET concentration. Taking into account that the minor specie only represents the 2.1% of total ceramide, in the following experiments the major specie was only studied.

To our knowledge, this is the first time that the bovine brain microvessels ceramide pattern has been described. A level of total ceramide of 1.7 pmol of total ceramide per nanomole lipid phosphate has been

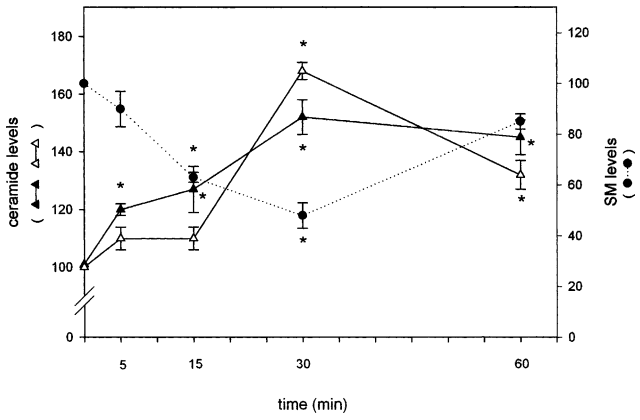

Fig. 1. Time course of the ET-1-induced alterations in the SM and ceramide levels. Cerebral cortex microvessels were stimulated with 10^{-7} M ET-1 for different times. Extraction and quantitation of sphingolipids were done as described in the text. Data are expressed as percentages of variation with respect to control value (100). Basal values were 1.50 ± 0.09 nmol/mg total lipids and 34.4 ± 2.3 pmol/mg total lipids for the major (filled triangle) and the minor (open triangle) specie of ceramide(s), respectively, and 5.03 ± 0.11 nmol/mg total lipids for SM (filled circles). Data represent the means $\pm$ SD ($n = 5$). *$P < 0.05$.

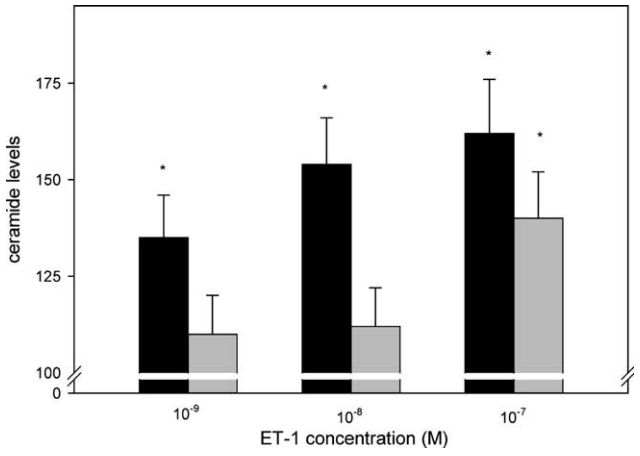

Fig. 2. Dose–response relationship for ET-1-induced ceramide production in cerebral cortex microvessels. Microvessels were stimulated with ET-1 for 30 min. Extraction and quantitation of ceramide were done as described in the text. Data are expressed as percentages of variation over control value (100). Basal values were 1.50 ± 0.09 nmol/mg total lipids and 36.58 ± 2.9 pmol/mg total lipids for the major (filled bars) and the minor (dotted bars) specie of ceramide(s), respectively. Data represent the means $\pm$ SD ($n = 3$). *$P < 0.05$.

previously reported in cultured rat brain capillary endothelial cells [16]. For comparative purposes, we have also normalized per lipid phosphate, and a value of 11.01 pmol of ceramide per nanomole lipid phosphate was found. The differences in the biological source and in the experimental procedure used to measure the levels of ceramide, are probably the cause of the differences found.

Brain microvessels exhibited special characteristics, such as the presence of G_o protein [10], that is considered to be unique to neuronal tissues. Therefore, we have considered it interesting to compare the brain microvessel ceramide pattern and its response to ET-1 action with that previously described by us in neuronal and vascular tissue [14,15]. Thus, the ratio between the level of the two molecular species observed in brain microvessels is more similar to that found in the aorta than that observed in neuronal tissue, in particular in the cerebellum, where the difference was scarcely evident. In addition, the overall ceramide level in the aorta and neuronal tissue was found to be much higher than that found in cerebral microvessels. On the other hand, ET-1 was found to elicit enhanced ceramide levels in neuronal tissue [14,15] as in cerebral microvessels, but not in aortic tissue, at least in adult rats [15]. Therefore, it is again evident that brain microvessels exhibited specific characteristics, some of them are shared with the neuronal tissue and others with the vascular one.

To know the receptor subtype involved in the ceramide increasing effect of ET-1, we performed experiments with BQ-123 and BQ-788 as specific ET_A and ET_B receptor antagonists, respectively. In Table 1 it is shown that both antagonists did not evoke response by themselves. However, BQ-123 is able to fully prevent the ET-1 effect, unlike BQ-788 did, thus proving the involvement of ET_A receptor type in the modulation of the ceramide signal transduction system from BBB.

It has been reported that the responses of brain capillary endothelial cells to endothelins are specie specific. The fact that endothelial cells from human and rat brain capillaries are responsive to ET-1 and express ET_A receptors but not those from bovine, suggests that the peptide has both autocrine and paracrine actions in human and rat capillaries, but only paracrine actions in bovine capillaries [21]. As in bovine brain capillary, the pericytes express functional BQ-123-sensitive ET-1 receptors [21], the decrease in the SM levels concomitantly with the enhanced ceramide level could suggest that pericyte ET_A receptors are coupled to the SM cycle. Nevertheless, at present we cannot discard that an increased ceramide synthesis takes place also by ET-1

Table 1
Involvement of ET_A receptors in ceramide production elicited by ET-1

Treatment	Ceramide levels (nmol/mg total lipids)
Control	1.35 ± 0.1
ET-1	2.43 ± 0.1
BQ-123	1.51 ± 0.2
BQ-788	1.11 ± 0.2
ET-1 + BQ-123	1.27 ± 0.2
ET-1 + BQ-788	2.35 ± 0.2

Brain microvessels were preincubated with or without BQ-123 and BQ-788 for 10 min before adding ET-1 or vehicle (water) for 30 min. Ceramide was isolated and measured by radioenzymatic method, as indicated under Materials and methods. Results are expressed as means $\pm$ SD ($n = 5$).

action. In addition, the phosphoinositide hydrolysis and changes in the Ca^{2+} dynamics have been described to be coupled to ET_A receptors in the brain microvessels [22,11]. The study of cross-talk phenomena between these second messenger systems in the BBB is an interesting open question.

It is tempting to speculate on the physiological meaning of the present data. The regulation of BBB permeability by ET-1 has been reported to be mediated by ET_A-receptor [5]. Therefore, the present evidence suggests that ceramides are involved in the regulation of BBB permeability, like it has been reported in the epidermal permeability [17]. In contrast, in the microvasculature from other sources, such as that of rat mesentery, the ET_B receptors are involved [23]. It is noteworthy that ET-1 has been reported to induce Ca^{2+} mobilization and rearrangements of cytoskeleton F-actin filaments in human cerebromicrovascular endothelial cells [24]. The fact that ceramide has been reported to induce genes involved in cytoskeleton organization in endothelial cells [25], and that the vesicular transport and/or cell contraction are involved in permeability of the BBB [4], leads again to think that the ceramide production is, at least, one transduction system by which ET-1 evokes the alterations in the permeability of the BBB.

In summary, our data showing for the first time the involvement of ceramide signal transduction system in the ET-1 action of BBB open a new field in the study of the functionality of the BBB.

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