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Research report

Glycerophosphoinositol and dexamethasone improve transendothelial electrical resistance in an in vitro study of the blood–brain barrier

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Abstract

The blood–brain barrier (BBB) maintains the homeostasis of the brain microenvironment, which is crucial for neuronal activity and function. Under pathological conditions, the BBB may fail due to yet unknown mechanisms. BBB failure is accompanied by an increase in the transendothelial permeability to substances such as sucrose that are normally extruded. Furthermore, altered BBB function may also lead to development of abnormal drug extrusion mechanisms including expression of multiple drug resistance proteins. Therefore, it is not surprising that strategies have been developed to "repair" the BBB in order to restore normal brain homeostasis and penetration/extrusion of pharmacologically active (noxious) substances. To this end, steroidal hormones and synthetic analogues such as dexamethasone (DEX) have been used to counteract BBB failure. However, several side effects limit the usefulness of steroid treatment in humans leading to the quest for developing novel strategies for BBB repair. We here show that, in an in vitro model of the BBB based on a co-culture of endothelial cells (EC) and glia, the natural compound glycerophosphoinositol (GPI) may replicate the effects of DEX. Thus, GPI in concentrations ranging from 3 to 100 μM promoted both BBB formation and repair in a dose dependent fashion. Similar effects were obtained with an elevated dose of DEX (10 μM); at higher concentrations (100 μM), DEX was cytotoxic. We conclude that the endogenous anti-inflammatory agent GPI may ameliorate BBB function with efficacy comparable to that of steroids, but with significantly fewer side effects. Further experiments will confirm the efficacy of this treatment in vivo and elucidate the pathways that lead to BBB repair after exposure to GPI.

Keywords: Blood–brain barrier; Dexamethasone; GPI anti-inflammatory agent; Glucocorticoid; Arachidonic acid; In vitro model

1. Introduction

The blood–brain barrier (BBB) is composed of a continuous layer of cerebrovascular endothelial cells (EC) that lie on a basal lamina. EC are joined by tight intercellular junctions that provide a biological barrier to maintain the homeostasis of the brain microenvironment. This specialized barrier serves as an interface between the circulating blood, brain interstitium and parenchyma, isolating brain tissue from blood constituents [1]. Given the crucial importance of BBB functions in maintaining the central nervous system (CNS) homeostasis by preventing entry of substances that might alter neuronal function in the CNS, it becomes evident how failure or partial deterioration of the BBB could play a role in the pathogenesis of brain disease. Inflammatory processes at the BBB level, occurring for whatever reason, are one of the main causes of BBB disruption [11,20,28]. Matrix metalloproteinases such as gelatinase A (MMP_2) and gelatinase B (MMP_9) are directly involved in this process due their degrading action on the extracellular matrix that exist between EC and their astrocytic counterpart [15,23,27].

Glucocorticoids, a subclass of anti-inflammatory steroid hormones called corticosteroids (CS), have been extensively used to treat a broad variety of autoimmune disorders and to reduce brain edema [10]. CS exerts their complex effects on cells involved in immune and inflammatory responses primarily by modulating gene transcription. However, they also influence the translational and post-translational mechanisms by which proteins are synthesized, processed and exported from cells [14,17,26]. The

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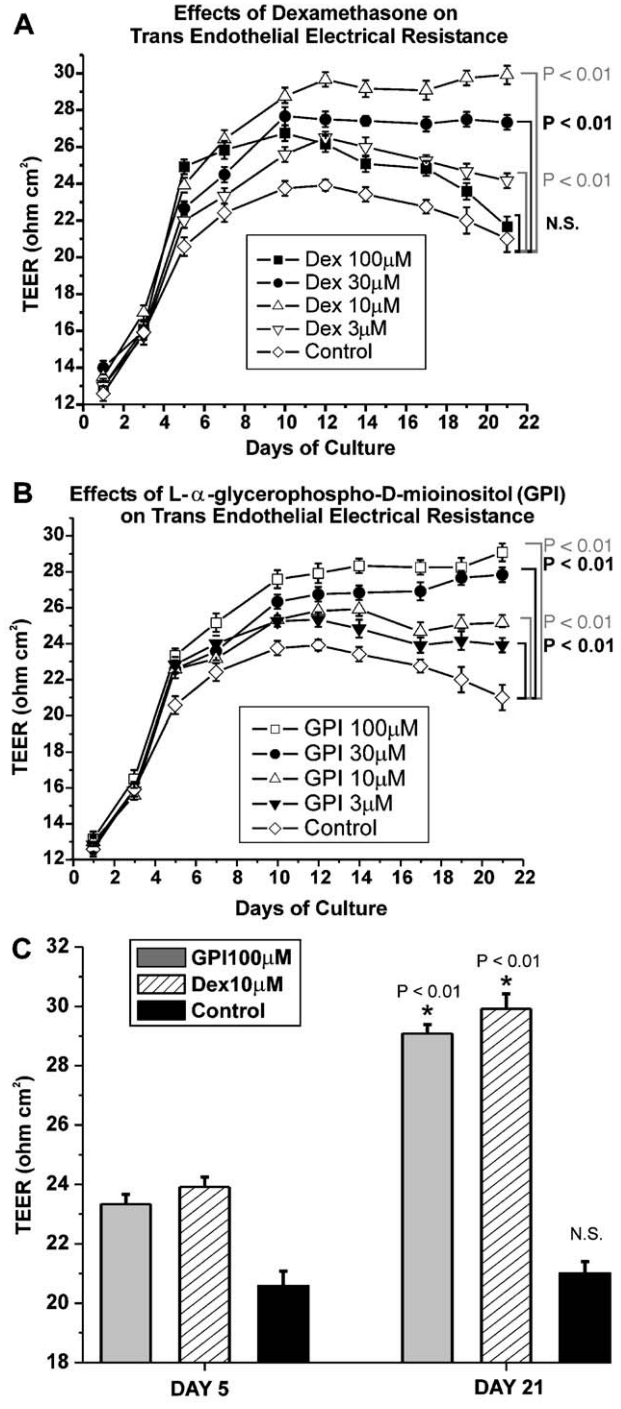

Fig. 1. (A) Dose-dependent effects of dexamethasone on TEER in a co-culture model. (B) Dose-dependent effects of GPI on TEER in a co-culture model. The experimental design was the same used for dexamethasone. (C) Summary of the experimental results after 5 and 21 days of treatment.

usefulness of CS is however limited by their toxicity [31,32].

To test the hypothesis that naturally occurring, non-steroidal compounds may have overlapping therapeutic effects but limited toxicity, we tested the efficacy of a putative ‘‘BBB drug’’, glycerophosphoinositol (GPI) [8,16]. GPI inhibits arachidonic acid (AA) release, impeding the production of pro-inflammatory molecules that generally lead to BBB failure. We compared these results with the analog effects of dexamethasone (DEX) in an in vitro model of the mammalian blood–brain barrier.

2. Methods

2.1. Transwell apparatus

Cells were co-cultured using sets of a 12-well Transwell-Clear Polyester Membrane apparatus (Costar cat. #3460) that feature a vertical side by side diffusion system through a thin, microscopically transparent polyester membrane of 12 mm diameter and 0.4 μm pore size mounted on a double chamber. The top one represents the intracapillary space (Lumen) and the bottom one the extracapillary space (ECS). The membrane allows nutrient exchange between the two compartments, but at the same time provides a barrier to cell movement. Each well-membrane is pre-treated with adhesion factors for optimal cell attachment and growth. The apparatus itself provides cell visibility under phase contrast microscopy, allows assessment of cell viability and monolayer formation under the experimental conditions and the study of bidirectional solute’s transport across the BBB making this model a suitable system to determine their kinetic parameters [5,18,33].

2.2. Cell lines used and experimental set up

Rat glioma cells (C6) and bovine aortic endothelial cells (BAEC) were grown to confluence in 75-cm^2 flasks, removed with trypsin and resuspended in DMEM containing 10% FBS, 1% PSF and 1% anti-mycotic [29,30]. The $7.5*10^4$ of C6 and $8.5*10^4$ of BAEC were used for each

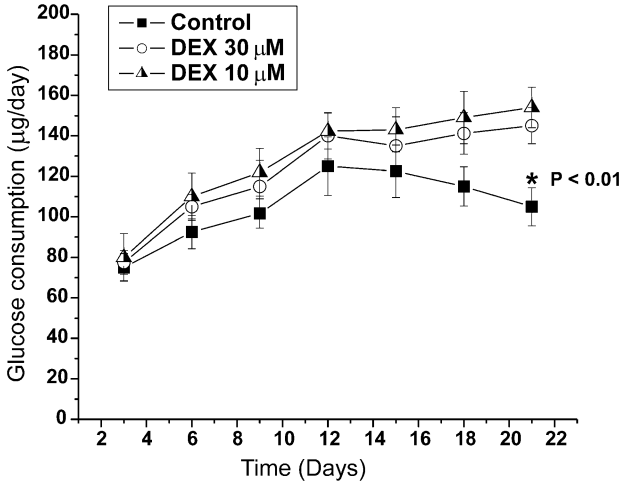

Fig. 2. Cellular growth was monitored by determination of the glucose consumption. Note that, unlike in treated co-cultures, glucose consumption in the control declined at the end of the experiment.

loading. C6 were seeded on the bottom surface membrane of each well (the intraluminal space) and allowed 48 h in the incubator to attach before BAEC were introduced into the upper chamber. DEX was added on day 0 after BAEC were loaded in the apparatus. According to the experimental requirements, the final concentrations of DEX and GPI (the chemical name is L-α-glycero-phospho-D-myo-inositol) into the culture media were 3, 10, 30 and 100 μM. For each experimental condition, we used a set of 12 wells. Trans-endothelial electrical resistance (TEER) was monitored every 2 days since "day 1" of co-culture [12,34]. The equipment used consisted of a tissue resistance measurement chamber (Endohm chamber, WPI) that provide reproducible electrical resistance measurement of endothelial tissue culture cups. In conjunction with TEER monitoring, BAEC cells monolayer integrity check was assessed by inspection with phase contrast microscopy (data not shown), and cellular growth was monitored by measurement of glucose consumption [29] with the use of a biochemistry analyzer (YSI 2700 SELECT).

2.3. Permeability measurements

A bolus of ^{14}C sucrose was directly added to the media contained in the lumen. Samples were taken from both lumen and ECS over 40 min; ^{14}C sucrose diffusion was monitored by detection of the radioactive tracer. The permeability was calculated by graphical integration of the concentration of the tracer in the lumen and in the ECS [30].

3. Results

For the experiments presented here, a total of 10 sets of 12 wells each were set up. Data points represent a mean of 12 observations (± S.E.M.). Fig. 1 illustrates the effects of DEX and GPI applied at various concentrations to the co-cultures. The drug was applied 1 day after establishment of the co-culture. Fig. 1A shows the dose dependent effect of dexamethasone on TEER. Note that, after an initial increase in TEER, a time-dependent decline of BBB integrity is observed in drug-free co-cultures (◇). At low concentrations (3 μM, ∇; 10 μM, △; 30 μM, •), DEX promoted enhancement of barrier function and prevented the late decline in TEER with a maximal effect at 10 μM. At high concentrations (100 μM, ■), DEX failed to exert a positive effect on BBB integrity.

Fig. 1B summarizes the effects of GPI applied in cells grown in parallel experiments. Unlike in the case of DEX, the effects of GPI were consistently favorable to BBB integrity, even at concentrations as high as 100 μm. Fig. 1C shows a direct comparison between DEX 10 μM, GPI 100 μM and untreated cultures. ^{14}C sucrose permeability at day 21 was on average $4.95*10^{-5}$ and $3.34*10^{-5}$ cm/s for control and the treatment with DEX (10 μM), respectively.

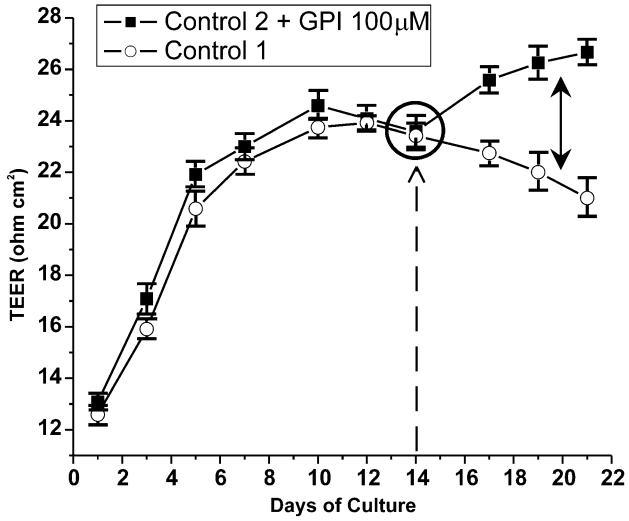

Fig. 3. Effects of GPI on time-induced BBB. GPI (at a final concentration of 100 μM) was added to control 2 after TEER started to decline. Note that GPI had a positive effect on declining TEER.

In the case of the GPI (100 μM), $P_{sucrose}$ was $3.45*10^{-5}$ cm/s ($n=3$ observations).

Glucose consumption was monitored over time every 3 days and the results are shown in Fig. 2. No significant differences were found between DEX or control cultures up to day 12 after plating. At this time point, DEX caused a modest yet significant increase in glucose consumption. This effect paralleled the positive effect of DEX on TEER suggesting that perhaps DEX promoted survival of ECs. Similar consideration may apply to GPI.

We further analyzed the effects of GPI and tested its capacity to revert the time-dependent TEER decline by adding the drug 14 days after the co-culture was established (Fig. 3). Note that GPI reversed BBB failure and preserved its integrity.

4. Discussion

Given the increasing evidence linking BBB failure to neurological disorders, it is not surprising that "BBB therapeutics" are becoming promising tools to treat brain edema and associated morbidity. In addition, it has been shown that BBB impairment may, in addition to having an etiologic role, cause enhanced toxicity to acute treatment of stroke [4,19]. Thus, if safe and effective drugs aimed at BBB repair were available, the use of drastic therapies (e.g., tissue plasminogen activator or tPA) may become more widespread. Furthermore, many chronic conditions such as multiple sclerosis and Alzheimer's dementia are characterized by a leaky BBB, suggesting that treatment with "BBB enhancers" may have therapeutic value. Finally, iatrogenic disruption of the blood–brain barrier is becoming a clinical reality, prompting the development of adequate measures to limit the extent and time window of potentially harmful side effects.

Corticosteroids such as dexamethasone have shown promise as putative agents capable of improving BBB function. While most of the mechanistic evidence linking their clinical efficacy to endothelium repair comes from in vitro studies, it appears that at least in the treatment of neurological diseases these drugs target the BBB [13]. Unfortunately, several factors limit their use, in particular for the treatment of chronic conditions [3,21]. Thus, alternative approaches have been sought, so far with little success. These strategies have included investigation of non-steroidal anti-inflammatory drugs, complement inhibitor, etc.

A new class of putative therapeutics has emerged with the discovery of specific inhibitors of cytosolic phospholipase A2 [2]. GPI was first detected as a biochemical intermediate of phosphatidylinositol (PI) metabolism initiated by $cPLA_2$ activity [2]. The main initial products of this pathway are AA and lysophosphatidylinositol; the latter is also a substrate for lysophospholipase A_1 ($LysoPLA_1$) activity, resulting in the release of the *sn*-1-linked fatty acid (i.e. stearic acid) and the formation of the GPI. The intracellular levels of GPI are thus increased when cPLA2 is activated by either external or internal stimuli and are widely modified in a variety of conditions.

Increased intracellular levels of both LysoPI and GPI have been observed in Ras transformed cells, thus indicating a correlation of the cPLA2 activity with Ras tumour induction. This evidence suggests that GPIs may be considered markers of Ras induced cell transformation [6].

An increase of GPI has also been observed in such diverse situations as physiological cell differentiation [22] or in tissue damage in acute brain injure [24] as well as in heart tissue following α1 adrenergic receptor stimulation [9].

Other experimental observations have substantiated for GPI a putative role in intracellular cell signalling. To date, the best documented GPI effects regard the selective inhibitory action of GPI on Gi-mediated cPLA2 activation [7,25]. Interestingly, exogenously added GPIs are reported to enter the cell by a plasma membrane transporter and accumulate within the cell cytoplasm, where they enter in cell homeostatic mechanisms and become part of endogenous signalling and metabolic pathways.

Our results have shown a dramatic similarity between the actions of GPI and those of DEX. However, these effects overlapped only in the therapeutic range of DEX actions. DEX exerted little or no effects at elevated concentrations, possibly due to a balance between its toxic and therapeutic effects. In contrast, GPI's effects were beneficial at all the concentrations tested. Thus, at least in vitro, GPI was able to improve BBB function by preventing culture-induced BBB decline after several days, as well by promoting recovery of function in compromised monolayers.

There is also plenty of scientific literature demonstrating an inhibitory role of corticosteroids on the PLA2 activity through increased gene expression of lipocortins. Hence, it may be possible that control of the cPLA2 activity by either GPI or DEX and consequent reduction of eicosanoids metabolism and active derivatives may be a possible common mechanism by which GPI and DEX modulate BBB permeability.

These pilot experiments warrant further investigations of possible BBB-protective effects of GPI in vivo.

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