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Altered glycaemia differentially modulates efflux transporter expression and activity in hCMEC/D3 cell line

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Abstract

The unique phenotype of blood-brain barrier (BBB) endothelium is partly maintained by abundant expression of ATP-binding cassette superfamily of efflux transporters that strictly restrict the CNS access to toxic substances including xenobiotics in circulation. Previously, we have shown that diabetes-related altered glycemic conditions differentially affect and compromise BBB integrity. However, the impact of diabetes on BBB efflux transporters is less understood. In this study, we examined the effects of single or repeated episodes of hypo- and hyperglycemia on major BBB efflux transporters expression/function in human cerebromicrovascular endothelial cell line (hCMEC/D3). Cells were exposed to normal (5.5mM), hypo (2.2mM) or hyper (25 or 35mM)-glycemic media containing D-glucose for 12h (acute) or two 3h episodes/day of hypo- or hyperglycemia with an intercalated 2h normal glycemic exposure for 3 days ("glycemic variability", see Methods). Acute hypoglycemic exposure (12h) up-regulated BBB endothelial mRNA and protein expression of P-glycoprotein, BCRP and other multidrug resistance associated proteins (MRP1 and 4) paralleled by an increase in transporter-specific efflux activity (~2-fold vs. control). Although, 12h hyperglycemia did not affect the efflux transporter expression (except for MRP4), a significant increase in BCRP activity was observed. By contrast, DNA microarray data revealed that repeated hyperglycemic episodes (but not hypoglycemia) significantly up-regulate P-glycoprotein expression and activity. Thus, this study suggests a differential impact of altered glycemic conditions on major BBB drug efflux transporters expression/function, sensitive to the length of exposure (acute vs. repeated), with an implication for altered CNS drug disposition in diabetic population.

Keywords

Alternative; BBB; Diabetes; Drug resistance; Glycemic variability; hCMEC/D3; Hypoglycemia; Transporter

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Introduction

Blood-brain barrier (BBB), constituted by brain microvascular endothelial cells, is a dynamic physical interface that critically regulates CNS homeostasis for optimal neuronal function[1]. Brain microvascular endothelial phenotype is also enriched with ABC superfamily of drug efflux transporters that strictly restrict the brain penetration and accumulation of a broad spectrum of neurotoxic chemicals including xenobiotics[2;3]. Thus, ABC efflux transporter family constitutes an important component integral to the BBB function. Pathological alterations in the expression/functional profiles of these molecular “gate keepers” result in enhanced neurotoxicity and altered CNS drug disposition[4;5].

Impairment of BBB is a major cerebrovascular complication of diabetes mellitus resulting in an increased risk of various brain disorders [6]. Recently, we have shown that diabetes-related altered glycaemia potentially impairs expression/distribution of endothelial tight junction proteins and nutrient transporters, evoking a compromised BBB integrity [7]. However, the impact of diabetes on various human BBB drug efflux transporters is currently less understood and studies from rodent models of diabetes produced conflicting data demonstrating a marked down-regulation, induction or no change in transporter expression and activity[8;9]. Therefore, we investigated the effects (acute or chronic “glycemic variability”) of hypo- and hyperglycemia on major BBB efflux transporters such as P-glycoprotein and breast cancer resistance protein (BCRP) in human brain microvascular endothelial cell line (hCMEC/D3), a well-established *in vitro* human BBB model for mechanistic understanding of drug transport across BBB[10;11].

Materials and methods

Antibodies were obtained from the following sources: Mouse anti-P-glycoprotein (#C219) from EMD Millipore Corp (Billerica, MA); rabbit anti-BCRP (#4477) and anti-MRP4 (D1Z3W) from Cell Signaling (Danvers, MA); mouse anti- β -actin (#A5441) from Sigma-Aldrich (Saint Louis, MO). Rhodamine 123, D-luciferin and Ko143 were purchased from Sigma-Aldrich. Tariquidar was obtained from Apexbio Technology (Houston, TX), while cascade blue dextran (3kDa) was from Life Technologies (Carlsbad, CA). Sterile culture ware and molecular biology grade chemicals and reagents were obtained from standard commercial sources.

Cell culture and treatment

HCMEC/D3 cells (p 27-29) were cultured in buffered EBM-2 medium with growth supplements and antibiotics at 37°C with 5% CO₂ under humidified conditions[7;11]. After reaching confluence, cells were exposed to normal (5.5mM), hypo (2.2mM) or hyper (25mM) glycemic concentrations of D-glucose for 12h (acute). As mentioned in our previous studies [7], these conditions did not impair the endothelial cell viability. To determine the effects of glycemic variability, a previously described protocol [12] was followed with minor modifications. As shown in **Fig. 3A**, hCMEC/D3 cells in groups I-III were exposed to two 3h-episodes of normal, hypo or hyperglycemia (5.5, 2.2 or 35mM D-glucose, respectively), with an intermittent 5.5mM episode (2h) for 3 days. In group IV, cells were exposed to two 3h episodes of 35mM alternating with 2.2mM (abrupt glucose

fluctuation;[13]). Following last treatment on each day, treatment media were replaced overnight with fresh EBM-2 media (with 1% human serum and antibiotics only).

Quantitative RT-PCR

Transporter mRNA expression was analyzed by real time quantitative RT-PCR as described earlier [14;15], with primer sequences shown in **table 1**. Total RNA was extracted from harvested cells using RNeasy plus mini kit with an indigenous DNA decontamination step, as per the manufacturer's instructions (QuiagenINc, Santa Clarita, CA). RNA purity and quantity were analyzed by Nanodrop ND-1000 spectrophotometer and reverse transcription to cDNA was performed with 1µg total RNA using superscript III first strand synthesis system (Life technologies, Carlsabad, CA). A SYBR green-based florescence method was used for gene amplification by qPCR on Bio-Rad CFX96 Touch™ system [7]. Relative expression of target genes was determined by Ct method, with β-actin as an internal control.

Western blotting

A previously described procedure was followed to determine the protein expression in hCMEC/D3 cells [7]. Briefly, cells were lysed and protein content was estimated by bicinchoninic acid assay. Equal amount of denatured protein (30µg) was subjected to SDS-PAGE (4-20% graded gels). After electrotransferring, the PVDF membranes were blocked and blots were incubated overnight with primary antibodies in cold blocking buffer (1:250-500). Following repeated washes with Tris buffered saline containing 0.1% tween20 blots were incubated with HRP-linked secondary antibodies(1:8000). After successive washes, blots were imaged by enhanced chemiluminescence and protein band optical density was analyzed by Image Studio™ with β-actin used as a loading control.

DNA microarray analysis

To determine the effects of glycemic variability, total RNA was extracted as mentioned above following the last treatment on day 3. RNA quality was determined and global gene expression profiling was carried out on Illumina platform (Human HT12) by Microarray Core Facility at Children's Hospital Boston. Data were analyzed by GenomeStudio and normalized with housekeeping genes for each sample.

Efflux activity

P-glycoprotein and BCRP functional activities were probed by measuring active efflux of their respective substrates, rhodamine123 [16] and D-luciferin [17], respectively. Briefly, cells were washed with calcium and magnesium free cold Hank's balanced salt solution (HBSS) and incubated on ice with rhodamine123 (10µg/ml) or D-luciferin (100µM) in efflux buffer with or without tariquidar (200nM, a potential inhibitor of P-glycoprotein; [16]) and Ko143 (2µM, a BCRP inhibitor; [11]). After 1h, cells were washed with pre-warmed HBSS and incubated in efflux buffer at 37°C for 1h with tariquidar (0 or 200nM) or 30 min with Ko143 (0 or 2µM) under gentle agitation. Finally, cells were rinsed with ice-cold PBS and lysed with 0.3% triton x-100. Fluorescence of cell lysates was measured and normalized to protein content of the sample.

Bi-directional transport assay

A previously established procedure [10] was adapted with some modifications. HCMEC/D3 cells in the apical chambers of the transwell inserts were exposed to normal or hypoglycemic concentrations for 12h. Following treatment, cells were washed with warm HBSS and fresh efflux buffer was added to apical (150µL) and basal (500µL) chambers. After 15 min of equilibration, apical or basal side of inserts was added a mixture of rhodamine123 (10µg/ml) and cascade blue dextran (3kDa; 50µg/ml) and incubated at 37°C for 1h. Cascade blue dextran was used as an internal marker for BBB integrity. Media from apical or basal side were sampled for fluorescence readings and permeability coefficients (P_e) were determined according to published protocols [10;15]. Permeability of rhodamine123 was normalized to dextran P_e in each direction for all samples to determine the efflux ratio of rhodamine123 (basal to apical, B-A/apical to basal, A-B apparent) [10].

Statistical analyses

Data were expressed as mean $\pm$ SEM and analyzed by one-way ANOVA followed by multiple comparison tests. Student's t-test (two-tailed, unpaired) was used when appropriate. For statistical significance, P value was set to less than 0.05.

Results

Hypoglycemia (12h) up-regulates BBB efflux transporter expression/activity

As shown in **Fig. 1**, hypoglycemia significantly increased the steady state mRNA levels of P-glycoprotein and BCRP ($P < 0.01$ and $P < 0.05$ vs. control, respectively). In addition, MRP1 and 4 transcript levels were also found to be up-regulated ($P < 0.05$ vs. control). However, hyperglycemic exposure (12h) did not significantly affect gene expression of P-glycoprotein and BCRP, although a moderate increase in MRP4 or down-regulation of MRP1 mRNA was observed following 12h hyperglycemia. In congruence with mRNA expression, western blots revealed a significant increase in cellular P-glycoprotein, BCRP and MRP4 protein levels by hypoglycemia in hCMEC/D3 cultures (**Fig. 1**). By contrast, hyperglycemic exposure differentially affected ABC transporter protein expression, with an increase (MRP4), decrease (P-glycoprotein) or no change (BCRP), when compared to normoglycaemic controls.

We next determined the effects of acute hypo and hyperglycemia on P-glycoprotein and BCRP activity. As shown in **Fig. 2A**, one-way ANOVA indicated a significant increase in P-glycoprotein activity, as determined by rhodamine123 efflux, following 12h exposure to hypoglycemic challenge ($P < 0.01$ vs. control) that strongly correlates with increased P-glycoprotein expression. Hydrocortisone (100nM) exposure for 24h was used as a positive control [18]. Moreover, hypoglycemia-induced rhodamine123 efflux was significantly attenuated by the presence of tariquidar, a non-competitive P-glycoprotein inhibitor, during cold and warm incubation procedures (**Fig. 2B**, $P < 0.05$). Additionally, acute hypoglycemic exposure evoked a significant increase in BCRP activity (**Fig. 2C**, $P < 0.01$), as revealed by a potential decrease in intracellular accumulation of D-luciferin. As shown here (**Fig. 2C**) and mentioned earlier [17], Ko143, a known BCRP inhibitor, blocked D-luciferin efflux in hCMEC/D3 cells (by ~45%), thus indicating D-luciferin as BCRP substrate [17]. By

contrast, acute hyperglycemic challenge (12h) did not affect P-glycoprotein functionality (**Fig. 2A**). However, a marked increase in efflux activity of BCRP was observed following 12h hyperglycemia (**Fig. 2C**), although the BCRP expression was found to be unaltered (**Fig. 1**).

We also assessed the effects of hypoglycemia on the bi-directional flux of rhodamine123 across the hCMEC/D3 monolayers in transwell inserts using a paracellular tracer as a measure of BBB integrity. The bi-directional P_e values for rhodamine123 and cascade blue were shown in **Fig. 2D**. As illustrated in **Fig. 2E**, our data indicate a significant increase in efflux ratio of rhodamine123 following hypoglycemic exposure (12h), possibly through a reduced A-B P_e , ($P<0.05$ vs. control), thus suggesting an increased P-glycoprotein activity at the apical membranes. Interestingly, there were no discernable changes in TEER values ($\Omega\cdot\text{cm}^2$) across the endothelial barriers in culture inserts following normal or hypoglycemic exposures (**Fig. 2F**), which is in agreement with our previous reports [7].

Effects of glycemic variability on efflux transporter gene expression in hCMEC/D3 cells

The effects of recurrent episodes of hyper and/or hypoglycemia on BBB endothelial efflux transporter expression was assessed by global transcriptome profiling of hCMEC/D3 cells. As shown in **Fig. 3B**, one-way ANOVA indicated that recurrent episodes of hyperglycemia for 3 days significantly up-regulated gene expression of P-glycoprotein (*ABCB1*, by 35% vs. control, $P<0.05$); while the BCRP (*ABCG2*) expression was markedly down-regulated (~50%, $P<0.05$ vs. control). Further, repeated hyperglycemic insults (25 or 35mM D-glucose) significantly enhanced P-glycoprotein function (**Fig. 3C**, $P<0.01$, vs. control). No significant effects of hyperglycemia on MRP4 (*ABCC4*) or MRP10 (*ABCC10*) expression were observed, although *ABCB10* (mitochondrial ABC transporter) was dramatically increased ($P<0.05$).

On the other hand, recurrent hypoglycemic insults did not significantly affect P-glycoprotein gene expression (**Fig. 3B**) or its activity (**Fig. 3C**) although a moderate decrease in BCRP expression was observed (**Fig. 3B**). Importantly, expression levels of *ABCB1*, *ABCB10* and *ABCG2* in hCMEC/D3 cells were significantly down-regulated by repeated exposures to abrupt glucose fluctuations (40-75% of control; $P<0.05$ and $P<0.01$; **Fig. 3B**); while the expression of *ABCC4* and *ABCC10* was found to be up-regulated ($P<0.05$ and $P<0.01$, vs. control, respectively).

Discussion

Diabetic conditions are shown to differentially modulate the expression/function of various drug efflux transporters in hepatic and renal tissues [19]. Additionally, long-standing diabetes in rodents attenuated cerebral P-glycoprotein and BCRP expression resulting in enhanced brain uptake and pharmacological/toxicological activities of their substrates [8;20;21]. However, subsequent studies demonstrated a dramatic increase [9] or lack of effect [22] on P-glycoprotein, BCRP or MRP2 expression/function in diabetic rats. A major limitation of these studies was the use of whole brain samples that does not directly reflect BBB-specific localization. Also, they have less translational value due to significant differences in BBB efflux transporter expression between human and rodent species [23].

The hCMEC/D3 cell line exhibits polarized expression of functional efflux transporters in rank order comparable to primary cultures of BBB endothelium [10;11;16] and freshly isolated human brain microvessels[24], albeit to a reduced level. Given its broad utility as a surrogate alternative for understanding the molecular regulation of *in vivo* BBB efflux transporters in health and disease, the findings of our study may hold clinical significance. Importantly, our data indicate differential and time-dependent effects of hypo- and hyperglycemia on BBB efflux transporters (P-glycoprotein and BCRP) expression/function. Further, this study corroborates with the polarized distribution of P-glycoprotein to apical membranes of hCMEC/D3 cells (**Fig. 2C**;[16]). Also, we have determined the effects of glycemic variability, a more deleterious factor than sustained hyperglycemia leading to enhanced oxidative stress[12;25]. Interestingly, global analysis of ABC transporter family revealed a potential and opposite regulation of P-glycoprotein and BCRP expression/function by hyperglycemic excursions, in contrast to acute conditions. On the other hand, cellular adaptive responses could possibly mediate the reduced impact of hypoglycemia in glycemic variability paradigm.

Analogous to human brain microvessels, hCMEC/D3 cells abundantly express aromatic hydrocarbon receptor (AhR), compared to other nuclear receptors[14]. Therefore, it is possible that AhR activation could likely mediate hypo- or hyperglycemia evoked responses in BBB efflux transporter expression. In fact, preliminary results from our group suggested an unaltered total protein expression of pregnane-X-receptor under acute hypo- or hyperglycemic conditions (data not shown). In addition, increased oxidative/inflammatory stress [7], could have a strong role in induction of BBB efflux transporter expression/function by altered glycaemia[4;9]. However, further investigation will be necessary to unveil the molecular mechanisms involved.

In summary, our study suggests that diabetes-related altered glycemic conditions could have potential impact on expression and functional profiles of major BBB drug efflux transporters. This would have potential clinical implication as most of the diabetic patients have co-morbid disorders and are poly-medicine users. For example, diabetes-related alterations in BBB drug efflux transporter profiles lead to increased brain uptake of peripherally acting drugs such as cimetidine [21] or reduced efficacy of CNS therapeutics [8]. Further, ABC efflux transporter expression/functional changes can impact the brain distribution of oral antidiabetic drugs [26;27]. Moreover, increased BBB transport and brain accumulation of endogenous substrates such as amyloid beta could represent another possible pathological manifestations[28]. Taken together, this study underlies the emphatic need for assessment of clinical drug-drug interactions and altered CNS pharmacokinetics in diabetic subjects.

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List of abbreviations

ABC	ATP-binding cassette
BBB	Blood-brain barrier
BCRP	Breast cancer resistant protein
hCMEC/D3	human cerebral microvascular endothelial cell line
MRP	Multi-drug resistance associated protein
TQ	tariquidar
AhR	aromatic hydrocarbon receptor
P_e	apparent permeability
TEER	trans-endothelial electrical resistance

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Highlights

- Hypoglycaemia upregulates Pgp and BCRP in BBB endothelial cell line
- Acute hyperglycemia increased only MRP4 expression
- Chronic hyperglycemic fluctuation upregulate Pgp expression and activity

Figure 1. Effects of altered glycaemia (12h) on mRNA and protein expression of major BBB efflux transporters

HCMC/D3 cells were exposed to 12h normal, hypo or hyperglycemic media containing 5.5, 2.2 or 25mM D-glucose, respectively. Efflux transporters mRNA and protein expression were determined by RT-PCR and western blotting. ** $P < 0.01$, * $P < 0.05$, vs. control (5.5mM). $N = 3-4$ /conditions ($n = 2-3$ biological replicates each).

Figure 2. Effects of hypo- and hyperglycemic challenge (12h) on P-glycoprotein and BCRP efflux activity
Following treatment, intracellular accumulation of transporter substrates rhodamine123 (P-glycoprotein) and D-luciferin (BCRP) was determined by relative fluorescence units normalized to protein content (RFU/mg protein) and expressed as % control. (A) Cellular accumulation of rhodamine123, with 100nM hydrocortisone (HC) as a positive control (24h); (B) Hypoglycemia (HG)-induced rhodamine123 efflux was determined in presence or absence of tariquidar (TQ, 200nM); (C) Both hypo and hyperglycemic conditions (12h)

significantly increase BCRP activity as determined by D-luciferin efflux that was sensitive to BCRP inhibitor, Ko143 (2 μ M); **(D)** Bi-directional permeability coefficients (P_e , $\times 10^{-3}$ cm/s) for rhodamine123 and cascade blue (3kDa) under normal and hypoglycemic conditions; **(E)** Efflux ratio of rhodamine123 across hCMEC/D3 monolayers following 12h hypoglycemic challenge, with insets showing apical to basal (A-B) and basal to apical (B-A) P_e ; **(F)** Transendothelial electric resistance across the hCMEC/D3 monolayers on transwell membrane filters; ** $P < 0.01$, * $P < 0.05$, # $P < 0.05$, vs. control (5.5mM); \$ $P < 0.05$, vs. HG. N=5-6/ conditions (n = 2-3 biological replicates each).

Figure 3. Effects of glycemic variability on efflux transporter gene expression and activity (A) Experimental design followed for recurrent episodes of normal (5.5mM), hyper (35mM) and/or hypoglycemic (2.2mM) insults (see Methods); (B) Gene expression of major efflux transporters in hCMEC/D3 cells (fold change vs. control) assessed by DNA microarray. (C) Rhodamine123 (Rho) accumulation in hCMEC/D3 cells (expressed as % control) as a measure of P-glycoprotein activity. **P<0.01, *P<0.05 vs. control; N=3-6/conditions(n = 3 biological replicates each).

Table 1

Forward and reverse primer sequences used for quantitative RT-PCR

Target gene	Forward	Reverse
MDR1	GCTCCTGACTATGCCAAAGC	TCTTCACCTCCAGGCTCAGT
MRP1	AGTGGAACCCCTCTCTGTTAAG	CCTGATACGTCTTGGTCTTCATC
MRP4	TGATGAGCCGTATGTTTTC	CTTCGGAACGACTTGACAT
BCRP	CACCTTATTGGCCTCAGGAA	CCTGCTTGAAGGCTCTATG
PXR	CGCTTCCTGAGTCTTTCA	CGCCTGCCGATGAGTACA
β -actin	CTGGCCCGGACCTGACAGA	GCCGCAGTGGCCATCTCTT