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Decreased blood–brain barrier permeability to fluorescein in streptozotocin-treated rats

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Abstract

Investigations of the blood–brain barrier (BBB) in diabetes have yielded contradictory results. It is possible that diabetes differentially affects paracellular and transcellular permeabilities via modulation of tight junction and transport proteins, respectively. Fluorescein (FL), a marker for paracellular permeability, is a substrate for the transport proteins organic anion transporter (OAT)-3 and multidrug resistance protein (MRP)-2 at the BBB. Furthermore, MRP-2-mediated efflux of FL can be upregulated by glucose. In this study, streptozotocin-induced diabetes led to decreased brain distribution of FL measured by in situ brain perfusion, consistent with activation of an efflux transport system for FL at the BBB. This change was paralleled by increased protein expression of MRP-2, but not OAT-3, in cerebral microvessels. These data indicate that diabetes may lead to changes in efflux transporters at the BBB and have implications for delivery of therapeutics to the central nervous system.
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Keywords: Blood–brain barrier; Diabetes; Multidrug resistance protein; Organic anion transporter; Fluorescein; In situ brain perfusion

Diabetes is characterized by multifocal microvascular pathologies. However, the specific effects of diabetes on the blood–brain barrier (BBB) remain controversial. Studies utilizing the streptozotocin (STZ)-induced model of diabetes to assess its effect on the BBB have indicated that the BBB remains intact [4], whereas recent work [29] indicates that BBB permeability is increased in STZ diabetes. Clinical studies of BBB function in diabetes have been similarly disparate [7,38].

One explanation for these conflicting results is that paracellular and transcellular permeabilities of the BBB are differentially regulated in diabetes. Whereas permeability to macromolecules and most small, water-soluble molecules is limited through the paracellular pathway by epithelial-like tight junctions (TJ) [12], transcellular movement of lipid-soluble molecules and organic ions is highly regulated by numerous transport and efflux proteins [27].

Fluorescein (FL) has been used as a marker of paracellular BBB disruption by osmotic shock [13]. However, FL is also a substrate for at least two groups of transporters expressed at the BBB: multidrug resistance proteins (MRP) and organic ion transporters (OAT) [39]. Therefore, the distribution of FL into the brain can be affected by both the integrity of TJ and the expression/activity of these transporters. Interestingly, MRP-2-mediated efflux of FL in the gut can be stimulated by hyperglycemia [25], indicating that efflux transport may be upregulated in diabetes.

We previously demonstrated that TJ are compromised in STZ diabetes, and that BBB permeability to sucrose is concurrently increased [Hawkins et al., submitted]. In the present study, we have examined the effects of STZ treatment on distribution of FL into the brain using in situ brain perfusion and on the expression of the FL-transporting proteins MRP-2 and OAT-3 in cerebral microvessels.

Animal protocols used in this study were approved by the University of Arizona Institutional Animal Care and Use Committee and conform to NIH guidelines. Male Sprague–Dawley rats (328 ± 4 g, 2–4 months old) were purchased from Harlan (Indianapolis, IN) and fasted for 6 h prior to i.p. injection of either 60 mg/kg streptozotocin (STZ) in 0.9% saline or saline alone (SAL). Following injection, animals were returned to their cages, maintained under standard 12-h light:12-h dark conditions, and given food and water ad libitum for the remainder of the study (14 days for all endpoints). Development of diabetes was confirmed by blood glucose analysis; STZ-treated animals

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with non-fasting blood glucose < 300 mg/dl at sacrifice (2 out of 56, 4%) were excluded from the study.

Rabbit polyclonal affinity-purified antibody to rat OAT-3 was obtained from Alpha Diagnostic (San Antonio, TX). Mouse monoclonal antibody to MRP-2 was obtained from Alexis (San Diego, CA). All other reagents were purchased from Sigma (St. Louis, MO), unless otherwise stated.

Animals were anesthetized with 1 ml/kg ketamine cocktail (acepromazine 0.6 mg/ml, ketamine 78.3 mg/ml, and xylazine 3.1 mg/ml) and given heparin (10,000 units/kg, i.p.), then perfused with an oxygenated Ringer's solution containing FL (sodium fluorescein salt, Sigma) via bilateral common carotid artery cannulation as previously described [13]. Cerebral hemispheres were mechanically homogenized in 7.5% (w/v) trichloroacetic acid (TCA) and neutralized with 5N NaOH for fluorimetric determination of FL (excitation 485 nm, emission 535 nm). Samples of perfusate were collected from the perfusion circuit, diluted in 7.5% TCA, and measured by the same methods. The ratio of FL in brain to that in the perfusate, R_{br}, was calculated by the following:

$$R_{br}\,(\mu l/g) = \frac{[FL]_{susp} V_{susp}}{[FL]_p m_{sam}} \quad (1)$$

where $[FL]_{susp}$ is the measured concentration of FL in the tissue suspension, V_{susp} the total volume of the tissue suspension, $[FL]_p$ the measured concentration of FL in the perfusate, and m_{sam} is the mass of the brain tissue sampled.

Unidirectional transfer coefficients (K_{in}) were calculated from the slope of the least-squares fit to linear regressions of R_{br} versus time (T):

$$R_{br} = K_{in}(T) + A \quad (2)$$

Alternatively, non-linear uptake data was fit to the equation:

$$R_{br} = \frac{K_{in}}{k_{out}}(1 - e^{-k_{out}T}) \quad (3)$$

where k_{out} is an efflux constant [26] using Sigma Plot 2001 v. 7.0.

Cerebral microvessel protein was isolated from freshly harvested brains as previously described [14] and quantified using the bicinchoninic acid method (Pierce, Indianapolis, IL) with BSA as a standard. Samples were frozen at −20 °C for use within a week or at −80 °C for use at a later time. Forty micrograms of protein was loaded onto Tris–HCl gels (Criterion, Biorad, Hercules, CA), separated (200 V, 60 min) and transferred to PVDF membranes (240 mA, 45 min). Membranes were sealed in plastic and frozen at −20 °C until use, blocked 4 h in TBS with 0.5% Tween-20 and 5% non-fat dry milk, and incubated in primary antibody 1 h at RT. Membranes were washed several times with TBS/Tween-20/milk followed by TBS/Tween-20 without milk prior to incubation with HRP-conjugated secondary antibody (Amersham) for 30 min at RT, washed again, developed using enzyme chemiluminescence (ECLplus, Amersham), and visualized on X-ray film. Semiquantitation of scanned films was performed using a Kodak Image Station, with gel staining (Gelcode; Pierce, Rockford, IL) as a loading control. Results are reported as percent expression of control. Primary and secondary antibodies were diluted in 0.5% BSA in PBS (anti-OAT-3, 1:500, anti-MRP-2, 1:50, anti-Rb and anti-Ms secondary, 1:4000).

For immunofluorescence studies, animals were anesthetized as described above. Following transcardiac perfusion with 0.9% saline, brains were removed and snap-frozen in 2-methyl butane with dry ice. Brains were stored at −80 °C until cutting into 20-μm coronal sections and mounting onto gelatin-coated slides (minimum of 48 h). All slides were stored at −80 °C until use. Slides were brought to room temperature, air dried, fixed in 100% ethanol (acetone was used for MRP-2 per manufacturer's indications) for 10 min, then washed in PBS followed by wash buffer (1% BSA/0.2% Tween-20 in PBS). After blocking 90 min in normal goat serum (Vector Labs, Burlingame, CA) diluted 1:50 in wash buffer, slides were incubated with primary antibody diluted in wash buffer (anti-MRP-2 diluted 1:50, anti OAT-3 diluted 1:500) in humidified chambers overnight at 4 °C. After rinsing with wash buffer, slides were incubated with appropriate fluorescent-tagged secondary antibody (Alexafluor488 conjugated anti-Rb or anti-Ms IgG, Molecular Probes, Eugene, OR) diluted 1:500 in wash buffer for 1 h at room temperature, then rinsed with wash buffer and PBS and coverslips were mounted with Vectashield (Vector Labs). Microvessels were visualized on a Zeiss 510 Metaseries laser scanning confocal microscope.

Student's t-test was used for the comparison of two means (SAL versus STZ). Linear regression coefficients were compared by methods described in [14].

STZ treatment led to significant changes in blood chemistry by day 14 (Table 1) and in weight (weight changes were +41 ± 5 and −16 ± 5 g for SAL and STZ, respectively).

Permeability of the BBB to FL was determined by in situ brain perfusion (Fig. 1). In the SAL group, brain entry of FL was limited ($K_{in} = 0.81 \pm 0.12\ \mu l\ g^{-1}\ min^{-1}$) and linear with respect to perfusion time ($R^2 = 0.98$) as previously reported [13]. STZ treatment significantly ($p < 0.01$) decreased brain entry of FL ($K_{in} = 0.35 \pm 0.07\ \mu l\ g^{-1}\ min^{-1}$, $R^2 = 0.90$), determined by comparison of linear regression coefficients [14]. Least-squares fitting of the STZ data to Eq. (3) improved the fit ($R^2 = 0.96$) and indicated an efflux component of FL transport not apparent in the SAL group ($k_{out} = 0.43 \pm 0.13\ min^{-1}$), while K_{in} ($0.93 \pm 0.33\ \mu l\ g^{-1}\ min^{-1}$) was not significantly different than SAL. Data from the SAL group could not be fit to the non-linear model, suggesting that efflux of FL at the BBB is negligible in the control condition.

Table 1
Blood chemistry data

	SAL	STZ
Glucose	242 ± 9	546 ± 12***
Ketones	6.1 ± 0.5	10.1 ± 0.8***
Triglycerides	54 ± 6	180 ± 24***
Cholesterol	85 ± 1	113 ± 3***
HDL	32 ± 2	52 ± 2*
LDL	38 ± 2	31 ± 3

Data are mean blood concentration (mg/dl) ± S.E.M.
* $p < 0.05$ vs. SAL.
*** $p < 0.001$ vs. SAL.

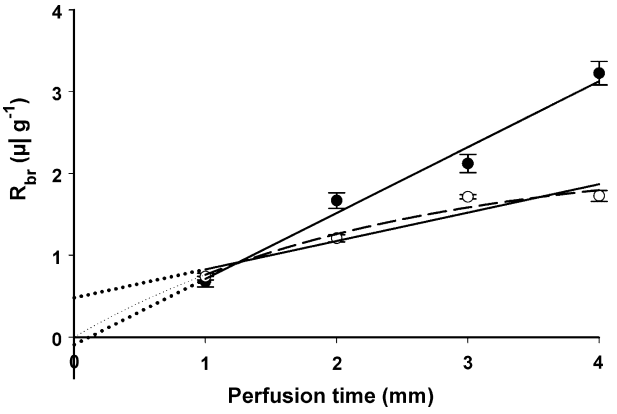


Fig. 1. In situ brain perfusion with sodium fluorescein. Data are mean $R_{br} \pm$ S.E.M., $n = 6$–11 per time point. Closed circles: SAL; open circles: STZ; solid lines: linear regressions fit to both data sets; dotted line: non-linear regression of STZ data fit to Eq. (3).

Expression of the FL-transporting proteins MRP-2 and OAT-3 was examined by western blotting of cerebral microvessel extracts [11,18]. The MRP-2 antibody M_2III-6 recognized a single band at approximately 170 kDa in liver extract (LE) [20], and faint band at approximately 150 kDa in cerebral microvessel extracts (Fig. 2A). STZ treatment was associated with a 49% increase in MRP-2 expression in cerebral microvessels (Fig. 2A). The OAT-3 antibody recognized a single band at 70 kDa in protein extracts from choroid plexus (CP) [40] and cerebral microvessels (Fig. 2B). No significant change was observed in protein expression of OAT-3 (Fig. 2B). Expression of both MRP-2 and OAT-3 was confirmed in cerebral microvessels by immunofluorescence microscopy of brain slices (Fig. 3).

FL has been used as a marker for studying BBB permeability for decades [1,15,41]. Though FL is a substrate for efflux transporters at the BBB [17,39], osmotic BBB disruption increases permeability to FL [13], indicating brain distribution of FL can be increased via opening of the paracellular route across the BBB. STZ-induced diabetes is associated with increased paracellular permeability of the BBB and decreased expression and/or degradation of BBB tight junction proteins [6,29]. In this study, brain uptake of FL was decreased in STZ-treated rats compared to controls (Fig. 1). Taking previously reported increases paracellular permeability into account, it is unlikely that this observation reflects a tightening of the diffusion barrier to FL.

In STZ-treated animals, FL uptake departs from linear with a flattening of the curve between 3 and 4 min (Fig. 1). Normally, due to the high concentration gradient between the perfusate and brain for FL (i.e., $[FL]_p \gg [FL]_{brain}$) we would expect unidirectional transfer (blood to brain) and the plot of uptake versus time to be linear; therefore, this departure from linearity indicates an efflux mechanism [3]. Thus, linear regression analysis would underestimate K_{in}. Previous studies have used fits to Eq. (3) to calculate K_{in} and k_{out} [26,35]. Fitting the STZ data to Eq. (3) improved the fit of the STZ data ($R^2 = 0.96$ versus 0.90 in the linear regression) and indicated no significant change in K_{in}. Rather, an efflux component to FL transport is indicated that is not observable in the control condition, as SAL data could not be fitted to the non-linear model. Furthermore, extrapolation of

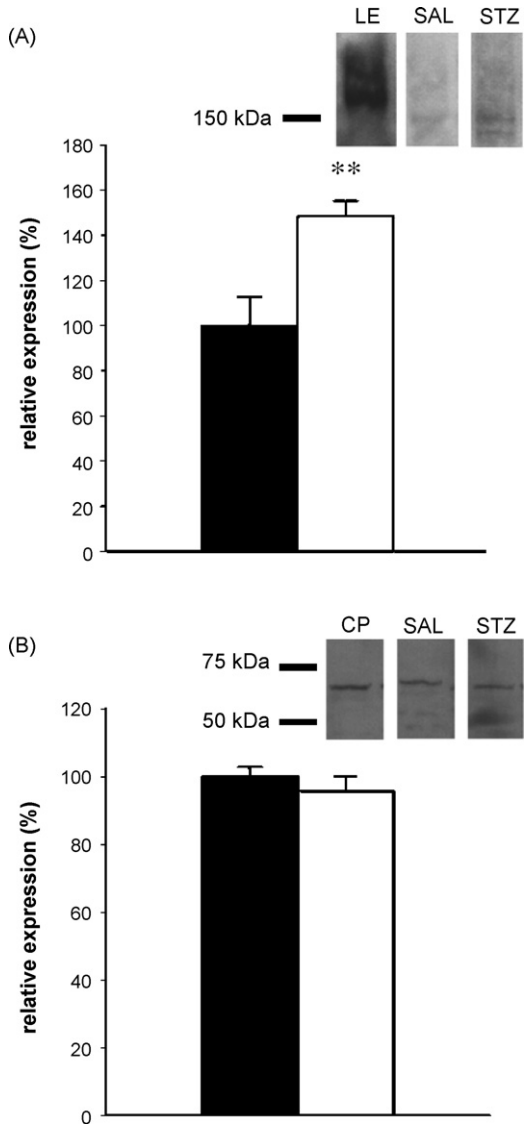


Fig. 2. Protein expression of MRP-2 and OAT-3. Shown are summary data and representative Western blots for MRP-2 (A) and OAT-3 (B) in cerebral microvessels isolated from SAL (solid bar) and STZ (open bar) animals. Data are mean percent relative expression normalized to SAL ± S.E.M., $n = 6$ per treatment group. Consistency of protein loading was confirmed by gel staining. LE: liver extract (positive control for MRP-2); CP: choroid plexus (positive control for OAT-3). $^{**}p < 0.01$.

the linear regression to $t = 0$ suggests an initial volume of distribution > 0 in the STZ group (Fig. 1). As the vascular volume has been corrected for by washout of the tracer at the end of the perfusion [13], this curve should intersect very close to the origin, further supporting fitting this data to the non-linear model.

Expression of MRP-2 at the BBB is controversial, due to differential expression patterns among species, strains, and individuals [8]. Studies in postmortem human brain sections have variously indicated no brain immunoreactivity for MRP-2 [31] and heterogeneous expression in brain endothelium and parenchymal cells among individuals [23]. Mice have strain-specific expression patterns of MRP-2, with FVB mice in particular lacking MRP-2 in brain but not liver [37]. MRP-2 has been immunolocalized in rat brain microvessels [28], a finding

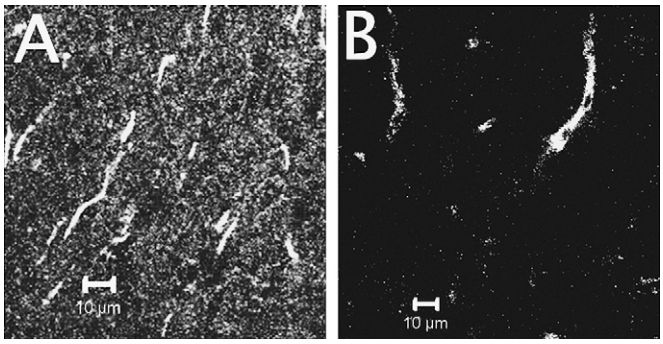


Fig. 3. Immunofluoresence of MRP-2 and OAT-3 in brain slices. Shown are representative images at 40× of cerebral sections stained for MRP-2 (A) and OAT-3 (B). Images were taken at 30% laser power, gain 500–700, pinhole = 66 μm. Localization to the vascular/perivascular region was confirmed by costaining with vascular markers ZO-1 and occludin (data not shown).

supported by increased brain distribution of MRP-2 substrates in TR- (MRP-2 deficient) rats [33]. Based on transport studies in isolated cerebral microvessels [28], MRP-2 is thought to be expressed at the luminal membrane of the capillary endothelium, where it is ideally situated to mediate efflux of substrates in a manner analogous to its role at the apical membranes of polarized epithelia in the kidney, liver, and gut [8].

It has been suggested that MRP-2 expression at the rat BBB is induced by status epilepticus but is below detection limits under normal conditions [16]. In this study, immunoreactivity for MRP-2 was observed in our enriched microvessel preparations from control animals but was very faint compared with the positive control (Fig. 2A). Fourteen-day STZ treatment increased expression of MRP-2, indicating that diabetes may lead to an upregulation of MRP-2 at the BBB.

Upregulation MRP-2-mediated efflux of FL in response to glucose was observed in preparations of rat jejunum [25]. Though that study examined activity of MRP-2 rather than expression, reactive oxygen species produced by hyperglycemia [5] can upregulate expression of *p*-glycoprotein and MRP isoforms [2]. In the liver, expression of MRP-2 can be regulated by several nuclear receptors [19,21], which can in turn be regulated by glucose [9] and hyperlipidemia [10]. Both hyperglycemia and hyperlipidemia are hallmarks of the STZ model, and were seen in this cohort (Table 1). Further study is warranted to see if these transporters are regulated at the BBB by similar mechanisms.

OAT-3, a member of the solute carrier (SLC) family of transporters, acts in concert with other transporters to mediate vectoral transport of xenobiotics and metabolites from blood to brain [24]. OAT-3 immunoreactivity has been shown at the abluminal membrane of cerebral microvessels [30,32], though there is evidence of luminal expression as well [22]. Western blots for OAT-3 have indicated molecular weights ranging from 40 to 130 kDa, depending on tissue, species, and assay conditions [34]. In rat brain capillaries, OAT-3 has been reported at approximately 75 kDa [22], similar to the band at approximately 70 kDa we observed (Fig. 2B). Though no changes in OAT-3 expression were observed in this study (Fig. 2B), this does not eliminate the possibility that OAT-3 activity is upregulated in STZ-treated animals [36]. Alternatively, the increase in MRP-2 activity alone may be sufficient to account for the changes in FL transport observed.

In summary, STZ diabetes leads to upregulation of efflux transport of FL at the BBB paralleled by increased expression of MRP-2. Substrates for MRP-2 include antiepileptic drugs [33] and HIV protease inhibitors [8], suggesting that the brain distribution of these therapeutics to the CNS may be altered in diabetic patients. As transcriptional mechanisms regulating the expression of multiple drug transporters may overlap and interact [2], it is possible that other efflux transporters such as *p*-glycoprotein or MRP-1 may be altered in diabetes as well. Additional study may prove critical for informed management of CNS therapeutic regimens in diabetic patients.

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