

Differential Effects of Diabetes on Rat Choroid Plexus Ion Transporter Expression

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Though diabetes is a disease with vascular complications, little is known about its effects on the blood-brain barrier or the blood-cerebrospinal fluid barrier (BCSFB). The BCSFB is situated at choroid plexuses located in the lateral, third, and fourth ventricles. Choroid plexuses are the primary site of cerebrospinal fluid (CSF) production and express numerous ion transporters. Previous studies have shown a perturbation of ion transport in the periphery and brain during diabetes. In this study, we investigated the effect of diabetes on ion transporters in the choroid plexuses of streptozotocin (STZ)-induced diabetic rats. Diabetes was induced in male Sprague-Dawley rats by intraperitoneal injection of STZ (60 mg/kg in citrate buffer, confirmed by glucose analysis: 601 ± 22 mg/dl diabetic rats, 181 ± 46 mg/dl age-matched controls); and at 28 days, rats were killed, choroid plexuses harvested, and protein extracted. Western blot analyses were carried out using antibodies for ion transporters, including $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter and the $\text{Na}^+\text{-K}^+\text{-ATPase } \alpha\text{1-subunit}$. The efflux of the K^+ analog $^{86}\text{Rb}^+$ from choroid plexus was also studied. Diabetic rats showed an increase in expression of the $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter and the $\text{Na}^+\text{-K}^+\text{-ATPase } \alpha\text{1-subunit}$, as compared with age-matched controls, a decrease in $\text{Na}^+\text{-H}^+$ exchanger expression, and no change in $\text{Na}^+\text{-K}^+\text{-ATPase } \beta\text{1- or } \beta\text{2-subunit}$. The net effect of these changes was a 66% increase in $^{86}\text{Rb}^+$ efflux from diabetic choroid plexus compared with controls. These changes in expression may affect choroid plexus ion balance and thus significantly affect CSF production in diabetic rats. *Diabetes* 52:1496–1501, 2003

D diabetes is a disease with numerous vascular complications. Previous studies have shown perturbations in ion transport in both the periphery and the brain during diabetes (1–3). Though diabetes is a vascular disease, little is known regarding the effects of diabetes on the blood-brain barrier (BBB) or the blood-cerebrospinal fluid barrier (BCSFB). It has been reported that diabetes is a risk factor not only for stroke (4,5), but also for normal-pressure hydroceph-

alus (6,7). Hydrocephalus is caused by excessive retention or production of cerebrospinal fluid (CSF) within the central nervous system. CSF is produced by the choroidal epithelial cells of the choroid plexus (8). The choroid plexuses are also the site of the BCSFB and are located in the lateral, third, and fourth ventricles. Capillaries of the choroid plexus are fenestrated, allowing extracellular fluid formation (unlike other brain capillary beds). CSF production involves the combination of a number of transport systems in the choroid plexus (9) (Fig. 1).

Animal models of diabetes have shown that the $\text{Na}^+\text{-H}^+$ exchanger (10–12), $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter (13), and $\text{Na}^+\text{-K}^+\text{-ATPase}$ (14–17) have altered expression and activity in a number of vascular beds. These three transporters are critical for CSF production and pH maintenance (9). In this study, we have investigated the effects of the streptozotocin (STZ) model of type 1 diabetes on expression of the $\text{Na}^+\text{-H}^+$ exchanger, $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter, and $\text{Na}^+\text{-K}^+\text{-ATPase}$ in the rat choroid plexus.

RESEARCH DESIGN AND METHODS

Induction of diabetes. All protocols used in this study were approved by the University of Arizona Institutional Animal Care and Use Committee and abide by National Institutes of Health guidelines. Diabetes was induced in male 300- to 325-g Sprague-Dawley rats (Harlan, Indianapolis, IN) via an intraperitoneal injection of 60 mg/kg STZ (Sigma, St. Louis, MO) in sterile phosphate buffered saline. Control animals were injected intraperitoneally with phosphate buffered saline. The animals were housed under standard 12-h light-dark conditions and received food and water ad libitum for 28 days. Induction of diabetes was assessed by weight changes and blood glucose levels.

Blood and CSF chemistry. Heparinized blood samples were collected and analyzed using an ABL 505 blood gas analyzer (Radiometer Copenhagen). CSF samples were taken from the cisterna magna before decapitation and analyzed as for blood.

Protein isolation. Rats were anesthetized with sodium pentobarbital (64.8 mg/kg) and decapitated. The brains were rapidly removed, and the choroid plexuses of the lateral ventricles were removed. Protein was isolated from the choroid plexuses by incubating overnight in 6 mol/l urea buffer (6 mol/l urea, 10 mmol/l Tris, 1 mmol/l dithiothreitol, 5 mmol/l MgCl_2 , 5 mmol/l EGTA, 150 mmol/l NaCl, pH 8.0, complete mini EDTA free protease inhibitor [one tablet/10 ml of buffer; Roche, Mannheim, Germany]). Protein was quantified using the bicinchoninic acid method (Pierce, Indianapolis, IN).

Western blot analyses. Protein samples (20 μg) were separated on Novex 4–12% Tris-glycine gels using an electrophoretic field at 125 V for 75–90 min. Proteins were transferred to polyvinylidene fluoride membranes using 240 mA at 4°C for 30 min. The membranes were then blocked using 5% nonfat milk/Tris-buffered saline (20 mmol/l Tris, 137 mmol/l NaCl, pH 7.6) with 0.1% Tween-20. Membranes were incubated overnight at 4°C with primary antibodies (anti- $\text{Na}^+\text{-H}^+$ exchanger [1:250; Transduction Laboratories]; anti- $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter [1:2,000; The University of Iowa Hybridoma Bank]; anti-rat $\text{Na}^+\text{-K}^+\text{-ATPase } \alpha\text{1-}, \beta\text{1-}, \text{ and } \beta\text{2-subunits}$ [1:2,000; Research Diagnostics]). Primary antibodies were chosen based on their ability to bind to rat transporters. Following incubation with primary antibodies, the membranes were washed with 5% nonfat milk/TBS buffer before incubation with respective secondary antibodies (anti-mouse and anti-rabbit [Amersham, Springfield, IL] at 1:2,000 and 1:3,000 dilutions, respectively, in PBS/0.5% BSA) for 30 min at room temperature. Membranes were developed using the enzyme chemilumi-

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aCSF, artificial cerebrospinal fluid; BBB, blood-brain barrier; BCSFB, blood-cerebrospinal fluid barrier; CSF, cerebrospinal fluid; PKC, protein kinase C; STZ, streptozotocin.

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TABLE 2
Blood and CSF electrolytes in diabetic and control rats

	pH		Na ⁺ (meq/l)		K ⁺ (meq/l)		Ca ²⁺ (meq/l)	
	Plasma	CSF	Plasma	CSF	Plasma	CSF	Plasma	CSF
Control	7.40 ± 0.01	7.34 ± 0.02	139 ± 0.4	144 ± 2.0	2.91 ± 0.19	2.57 ± 0.06	1.19 ± 0.16	2.06 ± 0.01
Diabetic	7.41 ± 0.02	7.37 ± 0.03	137 ± 1.4	148 ± 3.5	3.67 ± 0.09*	2.65 ± 0.09	1.25 ± 0.10	2.13 ± 0.05

Each point represents the mean ± SEM of data even six rats. **P* < 0.01 indicates significant difference from control determined by Student's *t* test.

(22) and also, paradoxically, with increases in aqueductal CSF flow velocity (23, 24).

In this study, we investigated the effects of STZ-induced diabetes on the expression of choroid plexus ion transporters. We focused on three transport systems that have been shown to play important roles in both the formation of CSF and the maintenance of CSF composition: Na⁺-K⁺-ATPase, Na⁺-K⁺-2Cl⁻ cotransporter, and Na⁺-H⁺ exchanger. Na⁺-K⁺-ATPase is located on the apical membrane of the choroidal epithelial cells. Inhibition of this transporter with cardiac glycosides has been shown to inhibit CSF formation in the rabbit (25,26), the cat (27), and the dog (25). In the rat, the cardiac glycoside ouabain inhibits the uptake of ⁸⁶Rb⁺ into isolated choroid plexus (28,29) and increases its efflux (28). The Na⁺-K⁺-ATPase transporter has also been implicated in maintaining CSF K⁺ levels (30,31). Na⁺-K⁺-ATPase is composed of three subunits (α, β, and γ). The α-subunit is a multipass transmembrane protein that has binding sites for ATP, Na⁺, K⁺, and cardiac glycosides; it is often referred to as the catalytic subunit, while the β-subunit is the regulatory subunit (32). Both α- and β-subunits are required for activity (33,34). In the rat choroid plexus, only the α1-, β1-, and β2-subunits are present (29,35). Diabetic choroid plexus had a significant increase in the expression of the α1-subunit, but no significant change in the β1- or β2-subunit (Fig. 2A–C). A similar response in Na⁺-K⁺-ATPase transporter expression was observed by Klarr et al. (29) while investigating the effects of hyperkalemia on choroid

plexus Na⁺-K⁺-ATPase expression. It is interesting to note that in our study, plasma K⁺ levels were also elevated (Table 2).

The Na⁺-K⁺-2Cl⁻ cotransporter is expressed on both the basolateral and apical membranes of the choroid plexus (Fig. 1) (36). This transporter has also been implicated in CSF production. CSF production is decreased by bumetanide, a Na⁺-K⁺-2Cl⁻ cotransporter inhibitor (37). Uptake of Na⁺, K⁺, and Cl⁻ by isolated choroid plexus can be inhibited by bumetanide (18,28). Efflux of ⁸⁶Rb⁺ is also inhibited by bumetanide (18,28,29). In this study, we saw a significant increase in expression of Na⁺-K⁺-2Cl⁻ cotransporter (Fig. 2D).

The Na⁺-H⁺ exchanger is located on the basal membrane of the choroid plexus, and it is involved in transport of Na⁺ from the blood in exchange for H⁺ and important for choroid plexus pH regulation. Inhibition of this transporter reduces both CSF production in rabbit (38) and Na⁺ uptake from blood to choroid plexus in the rat (39). In contrast to both Na⁺-K⁺-ATPase and Na⁺-K⁺-2Cl⁻ cotransporter, the expression of Na⁺-H⁺ exchanger was reduced during diabetes (Fig. 2E). Expression of these three ion transporters during diabetes has been shown to vary in a tissue-specific manner. Na⁺-K⁺-ATPase α1-subunit expression is reduced in the aorta of STZ-induced diabetic rats without changes in Na⁺-K⁺-ATPase α1-subunit in soleus muscle (13). In general, this transporter has reduced activity in diabetic models (13,16,17). In diabetes,

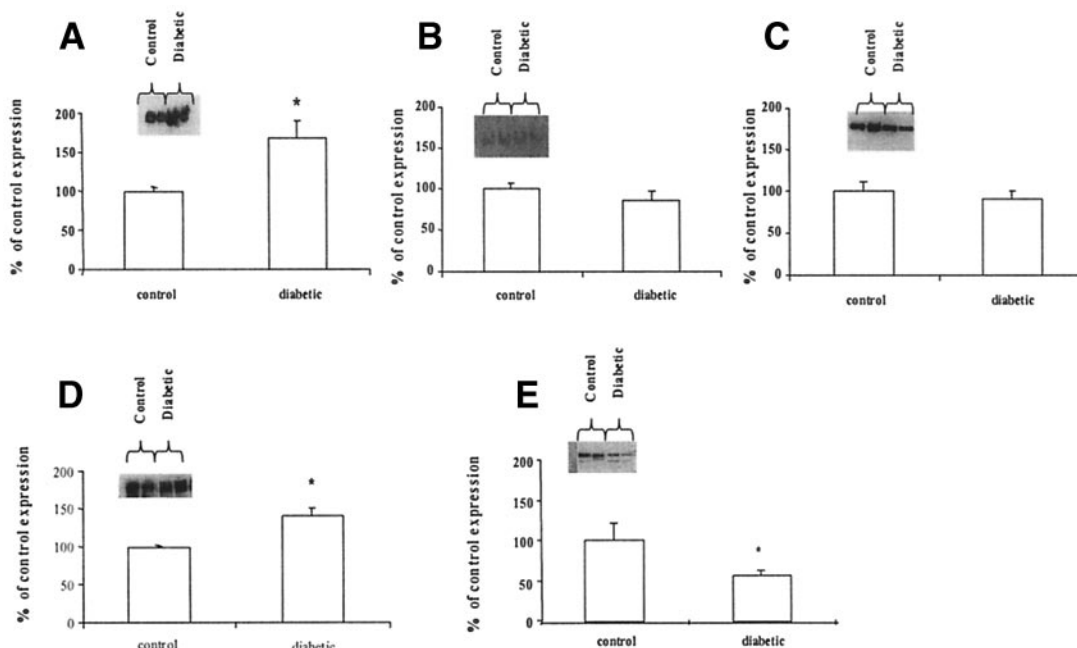


FIG. 2. Western blot analyses of ion transporters in rat control and diabetic choroid plexuses. Each bar represents the mean and SE percentage difference between diabetic and control for four separate animals. **P* < 0.05 using Student's *t* test. Expression of Na⁺-K⁺-ATPase α1-subunit (A) increases with diabetes. Expressions of Na⁺-K⁺-ATPase β1- (B) and β2-subunits (C) are not altered by diabetes. Na⁺-K⁺-2Cl⁻ cotransporter (D) expression is significantly increased in diabetic choroid plexuses. Na⁺-H⁺ exchanger (E) expression is significantly reduced in diabetic choroid plexuses compared with controls.

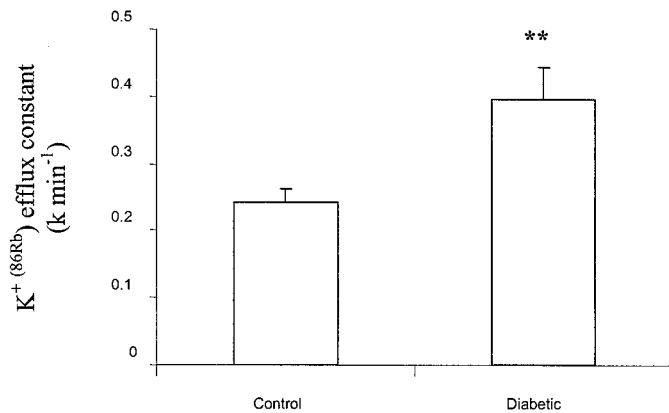


FIG. 3. $^{86}\text{Rb}^+$ efflux constants from preloaded diabetic and nondiabetic rat choroid plexuses. Each bar represents the mean and SE from six rats. $*P < 0.05$ using Student's *t* test. Efflux of $^{86}\text{Rb}^+$ from diabetic rats is significantly higher than that seen from controls.

the $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter was upregulated in the aorta with no change in expression in the heart (13).

We also investigated the effect of these transporter protein expression changes on the efflux of $^{86}\text{Rb}^+$ from preloaded choroid plexuses. $^{86}\text{Rb}^+$ is a marker for K^+ transport and thus exits the cell via the $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter along the gradient and reenters the cell via ATPase transporters against the gradient. From Fig. 3, it is apparent that diabetic rats have an $^{86}\text{Rb}^+$ efflux rate approximately double that of the nondiabetic rats. This indicates that the transport of K^+ into the CSF is higher in diabetic rat choroid plexuses than in controls. In our study, the control efflux constant of $0.24 \pm 0.02 \text{ min}^{-1}$ is similar to the K^+ efflux constants obtained previously (18). The efflux constant in diabetic rats was significantly greater ($P < 0.05$), at $0.40 \pm 0.05 \text{ min}^{-1}$. Keep and colleagues observed a similar increase in K^+ ($^{86}\text{Rb}^+$) efflux either by inhibiting $\text{Na}^+\text{-K}^+\text{-ATPase}$ (28) or by increasing aCSF osmolality (40). The increase of $^{86}\text{Rb}^+$ efflux in this study may be due to an inhibition of $\text{Na}^+\text{-K}^+\text{-ATPase}$, as shown by Keep (28), to an increase in cotransporter activity, or to a combination.

All three of the transporters investigated in this study have previously been shown to be modulated not only in numerous organs during diabetes, but also under various conditions in the choroid plexus. In this study, we saw an increased expression of the $\text{Na}^+\text{-K}^+\text{-ATPase}$ α -1 subunit and the $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter and a decreased expression of the $\text{Na}^+\text{-H}^+$ exchanger. During diabetes, a number of changes occur within the plasma. Perhaps most importantly for ion transport, plasma osmolality and K^+ levels are elevated (41). Though we did not measure osmolality, we did observe a significant increase in the levels of plasma K^+ . Further, Arieff and Kleeman (41) measured a plasma glucose that is similar to our diabetic value of 600 mg/dl, this group concluded that the increase in osmolality was due to the increased glucose. Thus, we can assume that the osmolality of the plasma in our studies was also elevated. Despite the probable change in osmolality and the observed change in plasma K^+ , we saw no significant difference in CSF K^+ levels, again similar to the study of Arieff and Kleeman (41). During hyperkalemia, CSF K^+ levels were maintained despite increased plasma K^+ , and a decreased $^{86}\text{Rb}^+$ transport across both the BBB

and the BCSFB (42). Hyperkalemia also led to an increase in choroidal $\text{Na}^+\text{-K}^+\text{-ATPase}$ α 1-subunit expression, but no effect was seen in efflux of $^{86}\text{Rb}^+$ (29). Osmolality can also modulate choroid plexus efflux of $^{86}\text{Rb}^+$. In isolated choroid plexus studies, Keep et al. (40) raised the osmolality of aCSF to 420 mOsm/kg and observed an increase in $^{86}\text{Rb}^+$ efflux. It is unlikely that the change in osmolality in our study would reach this level based on the study of Arieff and Kleeman (41). Furthermore, in the current study, the aCSF was of the same osmolality for both control and diabetic rat choroid plexuses. However, it is possible that the increased efflux may be a response of the choroid plexus to being transferred from an environment of high osmolality to one of normal osmolality.

Ion transporter expression can be modulated by a number of post-translational modifications. It is thus possible that the change in expression observed in this study may not be an increase in protein but an elongation of the blots by modifications such as phosphorylation and or glycosylation. All three of the transport mechanisms in this study have previously been shown to be modulated by post-translational mechanisms. The $\text{Na}^+\text{-K}^+\text{-ATPase}$ α 1-subunit contains a number of serine and threonine amino acids, which can be phosphorylated by protein kinase C (PKC). This phosphorylation can be stimulated by glucose (43), parathyroid hormone (44), and serotonin (45). In diabetes, high levels of glucose are present in the plasma (Table 1). Serine and threonine phosphorylation of $\text{Na}^+\text{-K}^+\text{-ATPase}$ α -subunits has been linked with a downregulation of activity either by stimulating $\text{Na}^+\text{-K}^+\text{-ATPase}$ endocytosis (46) or by inhibiting enzyme activity itself. Insulin increases the membrane content of $\text{Na}^+\text{-K}^+\text{-ATPase}$ (47) via both a PKC-mediated and a tyrosine kinase-mediated mechanism (43), leading to an increase in $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity. From Fig. 2A, it is apparent that the increased expression is predominately due to a smearing/elongation of the blot, potentially indicative of post-translational modifications such as phosphorylation. In vitro studies of $\text{Na}^+\text{-K}^+\text{-ATPase}$ have shown that in the presence of high glucose, α -subunits can be glycosylated, resulting in reduced activity of ATPase (48,49). Nonenzymatic glycosylation of cell membrane and plasma protein amide groups via the Amadori reaction is a common feature in diabetes (50,51) and has been reported in the CSF of subjects with neurological disorders (52). Either of these mechanisms or a combination of both could explain the increased expression of $\text{Na}^+\text{-K}^+\text{-ATPase}$ α 1-subunit in our study.

$\text{Na}^+\text{-K}^+\text{-2Cl}^-$ activity can also be affected by phosphorylation. The nonspecific PKC activator phorbol 12-myristate 13-acetate inhibits the $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter (53), though osmotic shock stimulates $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransport via a PKC- δ -mediated phosphorylation event, thus indicating that various members of the PKC family have different effects on ion transport. Finally, $\text{Na}^+\text{-H}^+$ exchanger activity is stimulated by insulin via a PKC- ξ -mediated mechanism. Therefore, lack of insulin in diabetic rats could lead to a decrease in the levels of $\text{Na}^+\text{-H}^+$ exchanger on the basolateral membrane of the choroidal epithelium.

A change in choroid plexus transporter expression may effect either the composition or the production of CSF.

From our study, it is apparent that the ionic composition of the CSF is not altered during diabetes. All three transporters are essential for maintaining Cl^- , K^+ , and Na^+ concentrations and pH of the CSF, as well as being the major driving forces of CSF production. Previous studies have shown that altering the activity of these transporters can alter the production of CSF. It is thus likely that diabetic animals have a different rate of CSF turnover than do normal animals. A change in CSF flow rate and/or turnover could reduce the ability of the CSF to compensate for alterations in brain extracellular fluid composition and thus contribute to tissue damage during pathological insults such as stroke or hydrocephalus.

The alteration of levels and/or activity of ion transporters at the BCSFB could have an effect on CSF production. There were, however, no major changes in the levels of the major CSF cations (Table 2), indicating that even though changes occurred in transporter expression levels, net CSF ion concentrations were not adversely affected during the early stages (28 days) of untreated type 1 diabetes in the rat. Rb^+ efflux is increased in isolated choroid plexuses (Fig. 3); we would thus expect some change in ion concentrations in the CSF. This indicates that there may be alterations in other transporters or ion channels that compensate for the change in Na^+/K^+ -ATPase. This study has investigated the effects of a short-term diabetic insult. In human type 1 diabetes, the disease manifests over a considerably longer time period. It would thus be interesting to investigate the long-term effects of ion transport changes on CSF dynamics. Inhibition of the three main transporters investigated in this study has previously been reported to inhibit CSF production (25–27,37–38). We have shown a decrease in expression of the Na^+/H^+ exchanger and a probable increase in phosphorylation of the Na^+/K^+ -ATPase $\alpha 1$ -subunit. This would indicate that both transporters have a decreased activity, potentially resulting in reduced production of balanced CSF by the choroid plexuses. These changes in transporters may have an effect on the long-term health of people with type 1 diabetes and may contribute to the increased probability of stroke and normal-pressure hydrocephalus in diabetes.

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