The mechanisms responsible for cerebral edema formation in diabetic ketoacidosis (DKA) are not well understood, although evidence suggests ischemia as a contributing factor. Previous studies have shown that the Na-K-Cl cotransporter of cerebral microvascular endothelial cells and astrocytes is a major participant in ischemia-induced cerebral edema in stroke. The present study was conducted to test the hypothesis that the Na-K-Cl cotransporter also contributes to cerebral edema in DKA. Sprague-Dawley rats were administered streptozotocin to induce DKA, and then cerebral edema was assessed by determination of apparent diffusion coefficients (ADC) with magnetic resonance diffusion-weighted imaging. Cerebral ADC values in DKA rats were significantly reduced in both cortex and striatum compared with non-DKA control rats, indicating the presence of cerebral edema. Intravenous administration of bumetanide to DKA rats abolished the drop in cortical ADC values, while having no significant effect in the striatum. Insulin and saline treatment had no effect when given after bumetanide but increased both cortical and striatal ADC values when given before bumetanide. Evidence is also presented here that acetate and β-hydroxybutyrate stimulate brain microvascular Na-K-Cl cotransporter activity. These findings suggest that the Na-K-Cl cotransporter contributes to brain edema in DKA.

**Bumetanide Reduces Cerebral Edema Formation in Rats With Diabetic Ketoacidosis**

Tina I. Lam, Steven E. Anderson, Nicole Glaser, and Martha E. O’Donnell

Cerebral edema is the most common serious complication of diabetic ketoacidosis (DKA) in children, yet little is known of the mechanisms responsible (1,2). Clinically apparent cerebral edema has a mortality rate of 21–90% (2–5). It is estimated to occur in 1% of DKA episodes (2,6) and accounts for 50–60% of diabetes-related deaths in children (7,8). In addition to symptomatic cerebral edema, it appears that asymptomatic cerebral swelling occurs and is, in fact, prevalent in DKA episodes in children (3,9). Swelling has also been reported to worsen with therapy (3,9). These and other findings have led some to hypothesize that cerebral edema associated with DKA in children is primarily the result of intravenous infusion fluid therapy and rapid changes in serum osmolality. However, case reports have demonstrated the presence of symptomatic cerebral edema in children with DKA before therapy, suggesting that cerebral edema is not simply the result of therapeutic interventions (2,8,10,11).

A number of findings have led to the hypothesis that cerebral ischemia may underlie DKA-associated cerebral edema. Previous studies have demonstrated that hypocapnia resulting from therapeutic hyperventilation in patients with brain injuries can cause cerebral vasoconstriction and diminish cerebral blood flow sufficiently enough to cause ischemia (12–14). More recently, Glaser et al. (2) demonstrated that the degree of hypocapnia and severity of dehydration in children with DKA are significantly associated with cerebral edema. These findings raise the question of whether cerebral hypoperfusion might result from the combination of severe hypocapnia and circulatory volume depletion.

In studies focusing on mechanisms of edema formation in ischemic stroke, we and others (15–17) have found evidence that the Na-K-Cl cotransporter of brain microvascular endothelial cells (blood-brain barrier [BBB] endothelial cells) and brain astrocytes play a prominent role in mediating brain edema formation. In those studies, the Na-K-Cl cotransport inhibitor bumetanide was found to reduce both edema formation and infarct occurring during ischemia (15,16) or ischemia plus reperfusion (17). Previous studies have also shown that hypoxia, vasopressin, and endothelin, factors present during cerebral ischemia (18–21), all stimulate activity of the BBB Na-K-Cl cotransporter (22–26). The Na-K-Cl cotransporter of astrocytes appears to be stimulated by conditions of cerebral ischemia as well (27,28). In the case of cerebral ischemia occurring in thrombotic stroke, edema formation is thought to occur in the presence of an intact BBB during the first 4–6 h, with barrier breakdown occurring thereafter (29–33). In this scenario, stimulated activity of the astrocyte Na-K-Cl cotransporter causes those cells to swell (cytotoxic edema) (27,34–36), while stimulation of the BBB Na-K-Cl cotransporter causes an elevated secretion of NaCl and water across the BBB from blood into brain, in essence facilitating cytotoxic edema formation (15,16). There is some evidence that stimulation of the BBB Na-K-Cl cotransporter can also cause the endothelial cells to swell as ischemia progresses, further decreasing cerebral perfusion. Another previous study has shown that the

From the 1Department of Physiology and Membrane Biology, University of California, Davis, California; and the 2Department of Pediatrics, University of California, Davis, California.

Address correspondence and reprint requests to Dr. Martha E. O’Donnell, Department of Physiology and Membrane Biology, School of Medicine, University of California, One Shields Avenue, Davis, CA 95616. E-mail: medonnell@ucdavis.edu.

Received for publication 21 May 2004 and accepted in revised form 10 November 2004.

ADC, apparent diffusion coefficient; BBB, blood-brain barrier; CMEC, cerebral microvascular endothelial cell; DKA, diabetic ketoacidosis; DMEM, Dulbecco’s modified Eagle’s medium; DWI, diffusion-weighted spin echo images; STZ, streptozotocin.

© 2005 by the American Diabetes Association.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
ketoadic acetoacetate increases the release of endothelin-1 by BBB endothelial cells (37). Thus, it is possible that an ischemia-induced and/or ketoadic-induced stimulation of Na-K-Cl cotransporter activity in BBB endothelial cells and/or astrocytes contributes to cerebral edema formation in DKA. The present study was conducted to evaluate the effect of the Na-K-Cl cotransport inhibitor bumetanide on cerebral edema in DKA using the streptozotocin (STZ) rat model of DKA and magnetic resonance imaging.

**RESEARCH DESIGN AND METHODS**

**Induction of diabetes and DKA.** This study was conducted in accordance with the Animal Use and Care Guidelines issued by the National Institutes of Health using a protocol approved by the Animal Use and Care Committee of the University of California, Davis, California. Four-week-old (150-g) normo-
tensive Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were given an intraperitoneal injection of 150 mg/kg STZ in 0.05 mol/l citric acid (Sigma, St. Louis, MO), pH 4.3. Control rats received an injection of 0.05 mol/l citric acid only (vehicle for STZ). To prevent hypoglycemia, rats were given unlimited access to D10W (water with 10% dextrose; Fisher Scientific, Santa Clara, CA) in the first 24-h period after STZ injection. Subsequently, rats were allowed unlimited access to tap water and food (standard rat diet). Each day rats were weighed and urine glucose, ketoadics (assessed as acetoadacete, and), and pH were evaluated using Multistix urinalysis strips (Bayer; Fisher Scientific). Significantly elevated urine acetoadacetate was seen within 2 weeks after STZ injection. DKA was considered to be present when urine glucose concentration was >2000 mg/dl and urine acetoadectate concentrations were ≥160 mg/dl. Twenty-four hours before imaging, rats were deprived of drinking water to induce dehydration and ensure acidosis.

**Magnetic resonance diffusion-weighted imaging analysis of brain edema.** For imaging, rats were anesthetized using Na pentobarbital (65 mg/kg i.p.). The left femoral vein was then cannulated with PE-50 polyethylene tubing for additional anesthesia (as needed), for administration of compounds to be tested, and for blood sampling. In experiments using nephrectomized rats, a dorsoventral incision was made into the abdominal cavity, and renal blood vessels and ureter were ligated. Kidneys were then excised and the incision closed using a 9-mm Autoclip Applier (Vibratome, St. Louis, MO). Body temperature was monitored with a rectal probe (Cole-Parmer Instruments, Vernon Hills, IL) and maintained at 36.8–37.0°C using a heating pad with circulating water throughout surgery and the subsequent brain imaging (Gaymar, Orchard Park, NY).

**Statistical analysis.** Data are means ± SE of 13 control and 13 DKA rats. Plasma values of the parameters shown were determined for control (non-DKA) rats and DKA rats without mannitol and saline infusion. Ketoadic values shown are for β-hydroxybutyrate. *Values are significantly different than control values.

**Assay of Na-K-Cl cotransporter activity**

**Cell culture.** Bovine cerebral microvascular endothelial cells (CMECs) were grown on collagen type I and fibronectin-coated multwell plates in Dulbecco's modified Eagle's medium (DMEM), 5 mMol/l glucose, 1% (vol/vol) l-glutamine, 0.1% (vol/vol) gentamicin, 0.05% (vol/vol) basic fibroblast growth factor, 0% horse serum, and 0% calf serum. Growth medium was changed every other day. Two days before each experiment, cells were refed with a 50:50 (vol/vol) mixture of fresh DMEM containing 10% fetal bovine serum and astrocyte-conditioned medium. Astrocyte-conditioned medium was prepared by exposing primary rat astrocytes to DME containing 10% fetal bovine serum for 72 h and then filtering through a 0.2-μm membrane.

**K influx assays.** Na-K-Cl cotransport activity was measured as ouabain-sensitive, bumetanide-sensitive K influx, using 86Rb as a tracer for K as previously described (23). CMECs cultured on multwell plates (24-well cluster plates) were pretreated for 7 min with acetoadacetate (Li salt) or β-hydroxybutyrate (Na salt) (each 0 or 10 μM/l), as well as bumetanide (0 or 10 μM/l) and ouabain (0 or 100 μM/l) in HEPES-buffered minimal essential medium (pH 7.4 for all media). CMECs were then exposed to assay medium for 7 min (identical to pretreatment medium but containing 86Rb). After rapidly rinsing away extracellular radioactivity with ice-cold 0.1-M NaOH/MTG cells were extracted 0.2% sodium dodecyl sulfate for protein determination (bicinchonic acid [BCA] method) or 24Rb quantitation (liquid scintillation analysis, Tri-Carb 2500 TR liquid scintillation counter). By these methods, addition of the ketoadics as their Li and Na salts (acetoadacetate and β-hydroxybutyrate, respectively) did not result in any changes in extracellular pH or intracellular pH (pHi), the latter determined using the pH-sensitive dye BCECF, respectively.

**Statistical analysis.** All values are presented as means ± SE. Data shown were analyzed for significance using ANOVA for repeated measures with a Bonferroni-Dunn post hoc test, unless otherwise noted. P values <0.05 were considered significant.

**RESULTS**

Rats were evaluated for evidence of DKA before experiments by daily monitoring of urine glucose and ketoadics. However, at the end of every imaging experiment, rats were additionally evaluated for blood chemistry parameters of ketoacidosis. Table 1 illustrates that glucose, blood urea nitrogen, anion gap, and β-hydroxybutyrate were all elevated, while total CO2 and pH were reduced in DKA rats compared with control rats.

**Evidence of cerebral edema in rats exhibiting DKA.** Figure 1 is a magnetic resonance imaging proton DWI of a DKA rat brain, illustrating the brain regions evaluated for the presence of edema in these studies. From the DWI data, we calculated ADC values for the indicated brain regions in both hemispheres (regions L1–L4 and R1–R4, respectively). Figure 2 shows the results of cerebral
edema analyses conducted in healthy (control) rats and in rats administered STZ and then subsequently allowed to develop DKA (DKA rats). In these studies, we found significantly reduced ADC values in both cortex and striatum of DKA rats compared with control rats, indicating the presence of cerebral edema in the DKA rats. Cortical ADC values shown in this and subsequent figures are the average of cortical regions 1, 2, and 3 depicted in Fig. 1.

Human patients with DKA are generally administered insulin and saline infusion treatment immediately upon DKA diagnosis, before any evaluation of cerebral edema. We therefore evaluated any interventions for cerebral edema not only in untreated DKA, but also in the context of concomitant treatment with insulin and saline infusion. To gather additional baseline data, we evaluated our DKA rats for cerebral edema after treating them for a brief period (approximately 1 h) with a regimen of subcutaneous insulin injection followed by intravenous saline infusion as described in RESEARCH DESIGN AND METHODS. We found that DKA rats exposed to insulin/saline infusion exhibited reduced ADC values in both cortex and striatum compared with infused control rats in a manner similar to the noninfused rats. Specifically, cortex ADC values for infused control compared with infused DKA rats were 8.14 ± 0.12 and 6.13 ± 0.10, respectively. Similarly, striatum ADC values for infused control compared with DKA rats were 7.33 ± 0.13 and 5.83 ± 0.06, respectively. All values are means ± SE and represent four rats each. Both cortex and striatum ADC values for DKA rats are significantly different from their control ADC values (P < 0.0001). In these experiments, we also found small but significant increases in ADC values of cortex and striatum in infused control rats compared with noninfused control rats (Fig. 2). The reason for this is not clear but may be related to increased cerebral perfusion. However, DKA rats treated with this brief infusion of saline and insulin exhibited no significant differences in ADC values for either cortex or striatum compared with noninfused DKA rats.

**Effect of bumetanide on cerebral edema in rats with DKA.** To determine whether the Na-K-Cl cotransporter inhibitor bumetanide might reduce edema associated with DKA as it reduces edema in ischemic stroke, we administered bumetanide (30 mg/kg) intravenously to both control and DKA rats and determined cerebral ADC values. Figure 3 shows that DKA rats treated with bumetanide exhibited significantly less cerebral edema (i.e., significantly greater ADC values) than DKA rats given vehicle only. Bumetanide did not produce a significant change in ADC values in control rats. We also did not find an effect of bumetanide on ADC values in the striatum of DKA rats.

Bumetanide is well known to have renal diuretic actions. Thus, we tested whether bumetanide effects on
brain edema in DKA occur even in the absence of diuretic effects. To do this, rats were nephrectomized as described in RESEARCH DESIGN AND METHODS, then immediately subjected to DWI analysis of cortical and striatal ADC values. We observed a similar effect of bumetanide in nephrectomized rats as in intact non-nephrectomized rats in that intravenous administration of bumetanide to DKA rats significantly elevated ADC values. Specifically, cortex ADC values for nephrectomized DKA rats were 8.21 ± 0.07 and 5.78 ± 0.08 with and without intravenous bumetanide treatment, respectively (n = 4 rats each, P < 0.0001). As with the intact non-nephrectomized rats (Fig. 3), bumetanide had no significant effect on striatum ADC values in the nephrectomized rats (5.61 ± 0.16 and 5.45 ± 0.34 with and without bumetanide, respectively, n = 4 rats each).

**Effect of bumetanide on cerebral edema in DKA following insulin/saline infusion.** Because treatment with insulin and saline infusion is standard practice for both children and adults diagnosed with DKA, in the present study we also evaluated the effect of bumetanide on cerebral edema in combination with insulin/saline infusion. As shown in Fig. 4A, we found that bumetanide increased cortical ADC values in rats with DKA and that insulin/saline infusion during the subsequent 50 min did not further alter the ADC values. Bumetanide was without significant effect on striatal ADC values. In another set of experiments, we evaluated cerebral ADC values in DKA rats given a 68-min insulin/saline infusion followed by bumetanide (Fig. 4B). In this case, we found that the insulin/saline infusion alone had no effect on either cortical or striatal ADC values but that bumetanide significantly elevated ADC values and that this effect was observed for both cortex and striatum.

Mannitol is frequently used to treat symptomatic cerebral edema during DKA. Thus, we conducted experiments to compare mannitol effects on ADC with those of bumetanide. In these experiments, rats were given an intravenous injection of mannitol (1 g/kg) before an insulin/saline infusion and ADC values were determined. The data shown in Fig. 5 reveal that like bumetanide, mannitol increased ADC values in cortex with no significant effect in the striatum. Thus, bumetanide and mannitol were equally effective in reducing cerebral edema in DKA.
Effective in reversing edema in DKA. Other aspects of these findings will be considered in Discussion.

**Effect of acetoacetate and β-hydroxybutyrate on Na-K-Cl cotransporter activity of CMECs.** The finding that bumetanide effectively reduces cerebral edema in DKA provides strong evidence that the Na-K-Cl cotransporter of brain microvascular endothelial cells and/or astrocytes is involved in formation of edema in DKA. Consistent with this, previous studies have shown that acetoacetate increases release of endothelin-1 from brain microvascular endothelial cells and that both acetoacetate and β-hydroxybutyrate increase intracellular [Ca] (\([\text{Ca}]_i\)) of these cells (37). Since both elevated [Ca] and endothelin are known stimulators of the brain microvascular Na-K-Cl cotransporters (22–26), it is possible that ketoadcids present in DKA may stimulate the cotransporter, leading to edema formation. Thus, we have evaluated this possibility in our present studies. As shown in Table 2, in these initial studies we found that both acetoacetate and β-hydroxybutyrate (10 nmol/l each) did indeed significantly stimulate activity of the Na-K-Cl cotransporter of brain microvascular endothelial cells.

**DISCUSSION**

The results of the present study demonstrate that rats with DKA exhibit reduced cerebral ADC values, as determined by magnetic resonance DWI, indicating the presence of significant edema. Intravenous administration of the Na-K-Cl cotransport inhibitor bumetanide reverses this reduction in cortex ADC values. In addition, we have found that the ketoacids acetoacetate and β-hydroxybutyrate stimulate activity of the brain microvascular endothelial Na-K-Cl cotransporter. Our findings suggest that Na-K-Cl cotransporter activity contributes to cerebral edema in DKA.

The mechanism of DKA-related cerebral edema has been a subject of great controversy. Many previous investigators have hypothesized that rapid osmotic changes coupled with intravenous fluid infusion lead to cell swelling during DKA treatment (4,39,40). This hypothesis has been challenged by data demonstrating that cerebral edema in DKA patients may be present before initiation of treatment for DKA (8,10,11). In addition, studies have not detected an association between cerebral edema and rates of fluid infusion or rates of change of glucose or sodium concentrations during treatment for DKA (2,5,41–43). These data suggest that mechanisms other than osmotic shifts might be involved. In other studies, Glaser et al. (2)

**TABLE 2**

<table>
<thead>
<tr>
<th>Control</th>
<th>Acetoacetate</th>
<th>β-Hydroxybutyrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>19.20 ± 2.22</td>
<td>26.30 ± 2.14*</td>
<td>25.26 ± 1.51*</td>
</tr>
</tbody>
</table>

Data are means ± SE of three experiments (each with quadruplicate replicates). Cultured brain microvascular endothelial cell monolayers were exposed to either acetoacetate (0 or 10 nmol/l) or β-hydroxybutyrate (0 or 10 nmol/l) for 7 min, then Na-K-Cl cotransporter activity assessed as bumetanide-sensitive K influx, as described in Research Design and Methods. *Value significantly different from control (P < 0.02 and P < 0.05 for acetoacetate and β-hydroxybutyrate, respectively).

Our current DWI studies reveal that rats with untreated DKA have significantly reduced ADC values in both cortex and striatum compared with control non-DKA rats. Previous studies in both rats and humans have demonstrated that a drop in cerebral ADC correlates well with cytotoxic edema during ischemia (44–46). Thus, the reduced ADC is consistent with the presence of cytotoxic edema in the untreated DKA rats. These results lend further support to the hypothesis that untreated DKA may cause cerebral ischemia. It is possible, however, that factors other than ischemia associated with DKA may also underlie cerebral edema formation and further studies are needed to address this issue.

In the current study, administration of mannitol to DKA rats resulted in a rapid increase in ADC. In contrast, control rats exhibited a decline in ADC in response to mannitol, consistent with the osmotic effects of this agent (data not shown). In addition to its osmotic effects, mannitol is thought to have an additional, more rapid effect on cerebral blood flow via improving blood viscosity and erythrocyte deformability, leading to rapid improvements in oxygenation of ischemic tissues (47–49). In a rat model of cerebral ischemia followed by reperfusion, investigators found that mannitol administration improved cerebral blood flow and prevented obstruction of the microcirculation during the initial few minutes of reperfusion (49). In addition, studies in adults (47) and children (48) have found that mannitol administration may induce autoregulatory vasoconstriction, implying increased tissue oxygen delivery. Although further investigation of mannitol’s mechanism of action is necessary, it is possible that the rapid increase in ADC in rats with DKA treated with mannitol is a reflection of improvements in oxygen delivery. These findings thus lend further support to the hypothesis that DKA causes cerebral ischemia.

Our studies revealed no significant changes in cerebral ADC values of DKA rats treated with insulin and saline infusion over a 50- to 60-min period. In contrast, a recent study using DWI in children undergoing treatment for DKA demonstrated that ADC values were elevated above control values after several hours of treatment (41). In animal models of cerebral ischemia and in humans with ischemic stroke, cytotoxic edema is initially present and the BBB remains intact for the first 4–6 h of ischemia. With reperfusion of ischemic tissues, rapid breakdown of the BBB occurs with consequent vasogenic edema (29–33). We hypothesize that similar mechanisms may be responsible for the low ADC values observed in rats early in the course of treatment for DKA and the high ADC values observed in children after many hours of DKA treatment.

In animal models of cerebral ischemia, stimulation of the Na-K-Cl cotransporter of brain microvascular endothelial cells and brain astrocytes by conditions present during ischemia appears to play a prominent role in mediating brain edema formation (16,27,28,36). In the current study,
we found that intravenous administration of bumetanide resulted in rapid normalization of ADC values in both intact and nephrectomized rats with DKA. This suggests that inhibition of the Na-K-Cl cotransporter can reduce edema associated with DKA. The apparent edema-reducing effect of bumetanide on DKA rats is restricted to cortex unless an insulin/saline infusion precedes bumetanide treatment, in which case ADC values of both cortex and striatum are elevated and, in fact, slightly exceed those of non-DKA control rats. One possible explanation for this phenomenon is that the insulin/saline infusion may increase cerebral perfusion sufficiently enough to enhance delivery of bumetanide to cerebral microvessels. However, further experiments are needed to explore this phenomenon. The 30- mg/kg dose of bumetanide used in the present study is higher than the normal pharmacologic dose for use as a diuretic. In a previous study, we found that this dose of bumetanide effectively reduces ischemia-induced cerebral edema in rats subjected to middle cerebral artery occlusion (16). However, our studies have also shown that bumetanide is effective in reducing middle cerebral artery occlusion–induced edema at a dose of 7.5 mg/kg. Future experiments will need to test lower doses of bumetanide for effectiveness in reducing edema of DKA rats.

Clarification of the mechanisms underlying cerebral edema in DKA and the apparent edema-reducing effects of bumetanide will require further investigation. Previous studies have provided evidence that conditions present during cerebral ischemia, such as hypoxia, and increased vasopressin and endothelin-1 can stimulate the Na-K-Cl cotransporter of BBB endothelial cells (22,23,25,26). We have now shown in this study that the ketoacids acetocacetate and β-hydroxybutyrate can also stimulate the Na-K-Cl cotransporter of brain microvascular endothelial cells. A recent study has shown that acetocacetate increases the release of endothelin-1 from brain microvascular endothelial cells and that both acetocacetate and β-hydroxybutyrate elevate [Ca], in those cells (37). Thus, it is possible that in DKA either ketoacids and/or ischemia-related factors cause cerebral edema via a mechanism involving increased activity of the BBB Na-K-Cl cotransporter. While future studies are needed to further examine mechanisms of cerebral edema formation in DKA, our current studies indicate that the cotransporter does indeed contribute significantly to this phenomenon.

ACKNOWLEDGMENTS

This study was supported by the American Diabetes Association (to N.G.).

REFERENCES