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Simulated diabetic ketoacidosis therapy in vitro elicits brain cell swelling via sodium-hydrogen exchange and anion transport

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1Department of Physiology and Pharmacology, Western University, London, Ontario, Canada; 2Children’s Health Research Institute, London, Ontario, Canada; 3Centre for Critical Illness Research, London, Ontario, Canada; 4Department of Paediatrics, Western University, London, Ontario, Canada; 5Department of Clinical Neurological Sciences, Western University, London, Ontario, Canada; and 6Translational Research Centre, London, Ontario, Canada

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Rose KL, Watson AJ, Drysdale TA, Cepinskas G, Chan M, Rupar CA, Fraser DD. Simulated diabetic ketoacidosis therapy in vitro elicits brain cell swelling via sodium-hydrogen exchange and anion transport. Am J Physiol Endocrinol Metab 309: E370–E379, 2015. First published June 16, 2015; doi:10.1152/ajpendo.00107.2015.—A common complication of type 1 diabetes mellitus is diabetic ketoacidosis (DKA), a state of severe insulin deficiency. A potentially harmful consequence of DKA therapy in children is cerebral edema (DKA-CE); however, the mechanisms of therapy-induced DKA-CE are largely unknown (37). Clinical studies link young age, longer duration of symptoms, and increased DKA severity to the development of DKA-CE (9, 37). Treatment factors implicated in the development of DKA-CE include bolused administration of either insulin or bicarbonate (20, 21, 27), suggesting that rapid correction of glucose and acidosis in DKA may aggravate DKA-CE via osmolar shifts and/or alkalinization.

Type 1 diabetes mellitus is often complicated by diabetic ketoacidosis (DKA), a state of hyperglycemia with accumulation of acidic ketone bodies in blood (54). Early recognition and treatment of DKA is critical to avoid hyperosmolar coma and death. DKA is treated with insulin and rehydration in a monitored hospital setting; however, a potential consequence and treatment of DKA in children is cerebral edema (DKA-CE). DKA-CE mortality is estimated at 21–25%, with significant neurological morbidity at 15–26% (37). Both vasogenic and cellular mechanisms are implicated in DKA-CE (29, 34, 49); however, the pathological mechanisms of therapy-induced DKA-CE are largely unknown (37).

Clinical studies link young age, longer duration of symptoms, and increased DKA severity to the development of DKA-CE (9, 37). Treatment factors implicated in the development of DKA-CE include bolused administration of either insulin or bicarbonate (20, 21, 27), suggesting that rapid correction of glucose and acidosis in DKA may aggravate DKA-CE via osmolar shifts and/or alkalinization.

A role for the sodium hydrogen exchanger (NHE) in DKA-CE had been proposed previously but not investigated (51). NHE1-9 are a family of transporters that regulate pH and cell volume by exchange of one intracellular H+ for an extracellular Na+ (15, 40). In the brain, the NHE1 isoform is widely distributed and expressed in both neurons and astrocytes (38, 46). In contrast, NHE2-5 expression is regional and significantly lower than NHE1 (6, 7, 11, 15, 38). NHE1 activity is essential for regulation of intracellular pH, and NHE1 is stimulated by insulin, acid loads, and hypo-osmotic stress (46, 50). Parallel activity of NHE1 and anion transport (AT) improves the efficiency of intracellular pH and cell volume regulation (2, 12, 30, 35).

In this study, we hypothesized that activation of membrane transporters with DKA therapies would induce brain cell swelling. Our aims were to investigate the DKA treatment factors that might contribute to cell swelling in a brain slice model and to investigate whether NHE and/or AT membrane transporters contribute to cell volume changes.

METHODS

This study was approved by the University Council on Animal Care at Western University (London, ON, Canada).

Mouse model of DKA. We used our juvenile mouse model of DKA that develops DKA-CE with rehydration and insulin therapy (49). Briefly, 21-day-old mice were administered 200 mg/kg ip of both streptozocin (STZ) and alloxan (ALX). STZ-ALX was dissolved by cold citrate buffer (10 mM, pH 4.5) in glass immediately before injection. Control mice received the same volume of citrate buffer only. Tail vein blood analysis showed that mice developed DKA 72 h after STZ-ALX injection. DKA-CE was induced by ip injection with 3 ml of rehydration solution containing bicarbonate and insulin (1 U R and 1 U NPH; Eli Lilly, Toronto, ON, Canada). Brain water content (BWC) was measured as an indicator of DKA-CE and reflects the wet-to-dry brain weight ratio (49).

Brain slices. Brain slices were obtained from juvenile control and DKA mice (28–35 days old), as described previously (19, 20). Briefly, mice were sedated with midazolam (~20 mg/kg ip) and immediately decapitated. Coronal brain slices were cut in ice-cold
DKA artificial cerebrospinal fluid (DKA aCSF; Table 1) containing 0.5 mM kynurenic acid on a Vibratome Series 1000 (Warner Instruments, Hollister, MA). Slices were incubated in DKA aCSF at room temperature for 2 h before experiments were performed. Solution pH and oxygen levels were maintained by intermittent bubbling with 5% CO₂ (pH controller; Harvard Instruments) and with continuous bubbling with 100% O₂, respectively. For imaging, brain slices were individually submerged in a tissue bath on an inverted microscope (Zeiss, Toronto, ON, Canada) and rapidly perfused (4 ml/min) with DKA aCSF.

Experimental aCSF composition. For DKA imaging experiments, brain slices were perfused with DKA aCSF for 5 min before being switched over to an experimental aCSF for 25 min (Table 1). CSF composition in children with DKA is generally unknown, as a lumbar puncture might instigate transtentorial brain herniation secondary to elevated intracerebral pressure. One study on a mixed population of adults and adolescents demonstrated that DKA CSF had increased glucose, ketones, lactate, and osmolality, whereas the bicarbonate was reduced (44); during DKA treatment, all values approached normal values. Nominvasive NMR and MRS imaging have also demonstrated altered CSF composition during DKA, which included elevated glucose, lactate, and ketones, and reduced intracerebral pH (8, 13, 28, 55). Thus, experimental aCSF was approximated to these studies. The name of each aCSF indicates the components that change from DKA aCSF. For example, in Alk/Ins aCSF, aCSF pH was alkalinized using increased bicarbonate, and reduced lactate and β-OH-butyrate and insulin were added. NaCl concentrations were adjusted to maintain targeted osmolalities, a common method for maintaining osmolality in neurophysiology studies (42). Osmolalities were measured in triplicate on a calibrated osmometer prior to each experiment and varied by ≤5 mOsml. For control experiments, brain slices were perfused with only DKA aCSF throughout the experiments (Fig. 1).

Imaging light transmittance. Increased light transmittance (LT) imaged in brain slices provides an accurate measurement of cellular swelling; the technique has been described in detail previously (31, 39). Prior to all brain slice imaging experiments, low baseline LT was confirmed and verified tissue viability (5). Briefly, images of the hippocampus from 500 μM brain slices were acquired every 10 s with a digital charge-coupled device (CCD) camera controlled by a Biochrom 30 amino acid analyzer (Biochrom, Cambridge, UK) that measures the concentration of physiological amino acids, including taurine with postcolumn ninhydrin derivatization and using S-(2-aminoethyl)-l-cysteine as an internal standard. Data are expressed as nanomoles per milligram dry weight.

Physiological salts, reagents, and drugs. All reagents were purchased from Sigma-Aldrich (Oakville, ON, Canada) unless otherwise specified. Methyl-isobutyl-amiloride (MIA; LT, 50 μM) was added directly to DKA aCSF. Cariporide (1 μM; gift of Drs. H. Kleeman, W. Linz, and J. Pünter; Sanofi Aventis, Frankfurt, Germany) was serially diluted in distilled water and then into DKA aCSF. For DKA-corrected aCSF solutions throughout the experiment. NHE1 genotyping. Genotyping was performed using polymerase chain reaction (PCR), as described previously (22). Primers were synthesized by Sigma-Aldrich (wild type forward: 5'-CCT GAC CTG GTT CATCAA CA-3'; mutant forward: 5'-CCT GAC CTG GTT CATCA CT-3'; common reverse: 5'-TCA TGC CCT GCA CAAGA AGC CG-3'); PCR products were run on a 1.5% agarose gel containing ethidium bromide.

NHE1 immunoblotting. Flash-frozen dissected mouse brain regions isolated 72 h after STZ-ALX or sham injection were homogenized in ice-cold RIPA buffer containing a broad-range protease inhibitor.

Table 1. Components of DKA aCSF and simulated DKA therapy aCSFs

<table>
<thead>
<tr>
<th>Component</th>
<th>pH</th>
<th>Bicarbonate, mM</th>
<th>Sodium, mM</th>
<th>Glucose, mM</th>
<th>Osmol, m</th>
<th>β-OH-butyrate, mM</th>
<th>Lactate, mM</th>
<th>Insulin, μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>DKA aCSF</td>
<td>7.1</td>
<td>10</td>
<td>134</td>
<td>30</td>
<td>344</td>
<td>4</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Alk</td>
<td>7.4</td>
<td>26</td>
<td>122</td>
<td>30</td>
<td>344</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ins</td>
<td>7.1</td>
<td>10</td>
<td>134</td>
<td>30</td>
<td>344</td>
<td>4</td>
<td>4</td>
<td>0.1</td>
</tr>
<tr>
<td>Alk/Ins</td>
<td>7.4</td>
<td>26</td>
<td>122</td>
<td>30</td>
<td>344</td>
<td>0</td>
<td>0</td>
<td>0.1</td>
</tr>
<tr>
<td>↓ Osmol</td>
<td>7.1</td>
<td>10</td>
<td>117</td>
<td>10</td>
<td>290</td>
<td>4</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>DKA corrected</td>
<td>7.4</td>
<td>26</td>
<td>105.5</td>
<td>10</td>
<td>291</td>
<td>0</td>
<td>0</td>
<td>0.1</td>
</tr>
</tbody>
</table>

DKA, diabetic ketoacidosis; aCSF, artificial cerebrospinal fluid; Alk, alkalinization; Ins, insulin; Osmol, osmolality. DKA-corrected aCSF included Alk/Ins and decreased Osmol. Components that were common to all aCSFs are as follows (in mM): 3.3 KCl, 1.1 MgSO₄, 2.0 CaCl₂, and 1.1 NaH₂PO₄.
cocktail (Calbiochem, La Jolla, CA) and then centrifuged at 1,000 g for 20 min to remove insoluble material. NHE1 expression was detected as described previously, with some modifications (47). Briefly, 20 μg/H9262 g of protein from each sample was mixed with Laemmli loading buffer and run on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis gel. The samples were transferred onto PVDF membrane at 30 V overnight at 4°C. The membrane was blocked with 5% nonfat milk in TBS-T for 1 h and then incubated overnight at 4°C with NHE1 antibody (1:1,000; Chemicon, Temecula, CA). The membrane was blocked with 5% nonfat milk in TBS-T with frequent changes and then incubated for 1 h with anti-mouse antibody conjugated to horseradish peroxidase (Cell Signaling Technology, Danvers, MA). Antibody binding was detected with chemiluminescent reagent (Lumiglo Reagent; Cell Signaling Technology).

Statistics. Data are presented as means ± SE. Data groups were prescreened for normality and then compared with either a Student’s t-test or Mann-Whitney U-test. Multiple groups were compared with analysis of variance and Holm-Sidak post hoc test. A P value of <0.05 was considered significant.

RESULTS

For LT imaging experiments, data traces were collected from brain slices that underwent simulated diabetic ketoacidosis (DKA) therapy (corrected DKA) and from brain slices that remained in DKA-like conditions as controls (DKA-only) (Fig. 1A). Time points from multiple experiments were averaged to create the raw DKA-corrected trace and the raw DKA trace, respectively (Fig. 1B).

The raw DKA trace was subtracted from the DKA-corrected trace to obtain the subtracted DKA-corrected data, which represents the net effect of simulated DKA correction. DG, dentate gyrus.

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Fig. 1. Imaging light transmittance (LT; cell edema) in hippocampal slices during simulated diabetic ketoacidosis (DKA) therapy. A: representative LT Images. Shown are 1) an initial grayscale image of the brain slice with the standard region of interest (box) in the CA1 stratum radiatum, 2) a pseudocolor LT image from a brain slice perfused in DKA artificial cerebrospinal fluid (DKA aCSF) for 20 min, and 3) a pseudocolor LT image from a brain slice during DKA correction aCSF for 15 min. The pseudocolor scale represents progressive cell swelling and progresses as follows: blue < green < yellow < red. B: raw traces. LT data were collected from a single region of interest over the course of an experiment. For this figure, each averaged trace is from 3 independent experiments. The switchover to DKA-corrected solution is indicated by a single arrow, and the time at which images (A2, A3) were obtained is indicated by double arrows. To control for non-experimental sources of cell swelling, raw averaged traces of LT were obtained from a separate brain slice in DKA-aCSF only (no switchover). C: baseline subtraction. LT from the DKA trace (red line in B) was subtracted from the experimental trace (blue line in B) to obtain the averaged subtracted DKA-corrected trace (purple line), which represents the net effect of simulated DKA correction. DG, dentate gyrus.

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due to decreased osmolality measured above, we tested for evidence of increased intracellular taurine, a primary idiogenic osmole, in DKA brain tissue. Our data confirmed that juvenile DKA mice had significantly elevated brain taurine compared with control mice (brain taurine concentrations were 3.0 ± 0.1 and 2.5 ± 0.2 mmol/mg dry wt in DKA and control brains, respectively; \( P < 0.05, n = 9–10 \) /group; Fig. 2C), potentially adding to the osmotic gradient across the brain cell membrane during DKA correction.

The role of NHEs in cellular DKA-CE was assessed with MIA, an NHE inhibitor. MIA reduced cellular DKA-CE significantly in brain tissue treated with DKA-corrected aCSF (67% decrease in LT; Fig. 3, A and C). MIA failed to reduce cell swelling in brain tissue treated with Alk/Ins aCSF (29% decrease in LT; Fig. 3, B and C), indicating that MIA-sensitive NHEs likely mediate the osmotic component of cellular DKA-CE.

Since MIA crosses the blood-brain barrier (BBB) (22), in vitro data was confirmed in vivo using juvenile DKA mice that develop DKA-CE when treated with insulin and rehydration fluid (49). Mice injected with STZ-ALX developed DKA, as indicated by elevated blood glucose (DKA 29.3 ± 0.4 mmol/l, \( n = 66 \); control 10.5 ± 0.3 mmol/l, \( n = 35 \); \( P < 0.001 \)) and β-OH-butyrate (DKA 4.46 ± 0.08 mmol/l, \( n = 66 \); control 0.19 ± 0.02 mmol/l, \( n = 35 \); \( P < 0.001 \)), as well as decreased body weight (DKA 18.7 ± 0.2 mmol/l, \( n = 66 \); control 23.9 ± 0.4 mmol/l, \( n = 35 \); \( P < 0.001 \)). DKA therapy resulted in rapid correction of serum glucose (7.6 ± 0.8 mmol/l, \( n = 34 \); \( P < 0.001 \)) and was associated with increased brain water content, consistent with the development of DKA-CE. Administration of MIA to DKA mice significantly reduced the DKA-CE elicited with DKA therapy (Fig. 3D), indicating that activity of brain NHEs contribute to cellular DKA-CE.

To better define the cellular origin of cellular DKA-CE, we imaged calcein fluorescence dye dilution in brain slices as an indicator of neuronal swelling (31) during simulated DKA therapy. Calcein dye dilution experiments suggest that simulated DKA therapy elicits cell swelling in the stratum radiatum that is at least in part neuronal in origin (81% decrease in fluorescence; \( n = 3 \); Fig. 4A). Neuronal swelling with simulated DKA therapy is supported by the observation that calcein dilution was similar in the stratum pyramidale, an anatomic region with neuronal cell bodies that are largely devoid of glia (\( n = 3 \); data not shown). MIA pretreatment of calcein-loaded brain slices failed to significantly reduce cell swelling elicited with simulated DKA therapy (10% decrease in fluorescence, \( n = 5 \); Fig. 4B), indicating that the MIA-sensitive osmotic component resides in nonneuronal cells such as astrocytes.

Chronic acidosis increases NHE1 expression in some tissues (25); however, NHE1 expression determined using immunoblotting was similar in both control and DKA cortex [optical density (OD) \( C = 2.4 ± 0.4 \) arbitrary units (AU); OD\(_{DKA} = 2.4 ± 0.5 \) AU], hippocampus (OD\(_ C = 2.3 ± 0.5 \) AU; OD\(_{DKA} = 2.4 ± 0.5 \) AU), brainstem-diencephalon (OD\(_ C = 2.4 ± 0.5 \) AU; OD\(_{DKA} = 2.2 ± 0.5 \) AU), and cerebellum (OD\(_ C = 2.5 ± 0.4 \) AU; OD\(_{DKA} = 2.7 ± 0.4 \) AU) (\( P = 0.948, n = 4 \)/group; Fig. 5, A and 5B). Similar NHE1 expression levels were also observed in both control and DKA brain following fractioning and immunoblotting of membrane vs. cytosolic components (data not shown; \( n = 3 \)/group). Finally, similar levels of cellular DKA-CE were elicited in brain slices obtained from either control (sham-injected) or DKA (STZ/ALX-injected) mice (134.8 and 78.4% increase in LT, respectively; Fig. 5C) preincubated for 2 h in DKA aCSF. Taken together, these results demonstrate that NHE1 expression is unchanged by DKA.
Slow-wave epilepsy (“swe”) mice, which endogenously express a mutated NHE1 (17), were used to determine whether NHE1 activity is important for cellular DKA-CE during DKA therapy. Genotyping and Western blotting confirmed that NHE1 expression was significantly reduced in swe/H11001/mouse brain, and NHE1 was altogether absent in swe/swe mice (n = 3/group, 1 forebrain/sample, OD/H11001 = 5.94 ± 0.38 AU, OD/swe/H11001 = 2.98 ± 0.62 AU, OD/swe/swe = 0.05 ± 0.04 AU, P < 0.05; Fig. 6, A and B). Swe/swe mice did not survive to juvenile age (35 days postnatal) for DKA induction in vivo, and brain slices from swe/swe mice did not survive incubation in DKA aCSF (n = 5; LT values were exceedingly high, and tissue failed to respond to osmotic stimuli). These data suggest that NHE1 expression is critical for brain tolerance and cell survival during DKA. In contrast, swe/+ mice survived DKA conditions despite a 50% reduction in NHE1 expression. Imaging LT showed that DKA correction caused cellular DKA-CE in live brain slices from swe/+ mice (91.8% increase in LT) that was similar to cellular DKA-CE in brain slices from wild-type littermates (116.1% increase in LT; Fig. 6, C and D). Moreover, like NHE1+/+ mice, we observed DKA-CE in vivo when DKA swe/+ were treated therapeutically with insulin and rehydration fluid (swe/+ DKA, BWC = 3.55 ± 0.2 l/kg dry brain wt; swe/+ DKA-CE, BWC = 3.71 ± 0.03 l/kg dry brain wt; Fig. 6E).

Since the 50% reduction in NHE1 expression in swe/+ mice may not be sufficient to inhibit cellular DKA-CE, we also used two potent NHE1 isoform-specific antagonists to inhibit cellular DKA-CE [KR-33028 (32) and cariporide (41)]. Cellular DKA-CE was not reduced by pharmacological inhibition of NHE1 with either cariporide (3.15% increase in LT; Fig. 7A) or KR-33028 (1.4% increase in LT; Fig. 7B). An inhibitor of AT also failed to reduce cellular DKA-CE (DIDS, 13.4% increase in LT; Fig. 7C). In contrast, coinhibition of NHE1 and AT with application of cariporide and DIDS, respectively, partially reduced cellular DKA-CE (15.1% decrease in LT; Fig. 7D). Thus, both NHE1 and AT have compensatory actions and require coinhibition to reduce cellular DKA-CE elicited by DKA therapy.

**DISCUSSION**

In this study, we used an in vitro paradigm (33) to investigate the potential DKA therapies and membrane mechanisms contributing to cellular DKA-CE. DKA-CE occurs almost exclusively in children, typically within the first 24 h of DKA therapy (20, 29). Clinical studies suggest that bolused insulin, bicarbonate administration, and/or rapid rehydration may precipitate DKA-CE (20, 21, 27). Previously, we reported that DKA-CE was elicited with combined insulin/bicarbonate administration in juvenile DKA mice (49) and now extend our investigations to live brain slices. The DKA correction with simulated DKA therapy in vitro mimics an insulin bolus in combination with rapid alkalization and reduction in osmo-
lality. Mechanistic studies on the origins of cellular DKA-CE were accomplished by altering aCSF composition during simulated DKA therapy. To our knowledge, this is one of the few studies to use in vitro techniques to directly study DKA-CE (33) and the first to use intact brain slices that allow for preservation of both intact neuron-astrocyte relationships and synaptic functions and permits accurate drug dosing for transporter inhibition. Brain slice experiments also allow for the exclusive study of cell edema independent of changes in cerebral blood flow and vasogenic edema (1, 5). Finally, cell edema is accurately quantified in brain slices by imaging either LT or intracellular calcein dye dilution (3, 31, 39).

The CSF composition is altered by DKA (44), possibly because of increased BBB permeability. BBB dysfunction may be secondary to DKA-associated inflammation and leukocyte adherence to the brain microvascular endothelium (16, 45). DKA-induced BBB permeability may exacerbate increases in brain glucose, lactate, and ketone levels during DKA (13, 28, 55) and underlie the vasogenic edema observed on brain MRI with apparent diffusion coefficients (19, 23, 29). In this study, we examined the potential for cellular DKA-CE to be mediated by changes in CSF composition during DKA therapy. Our data suggest that rapid alkalization (with or without insulin) and/or a rapid decrease in osmolality in CSF could lead to cellular DKA-CE in children during DKA therapy. These data are in agreement with previous in vivo data (49), where many factors exist that are not present under in vitro conditions (i.e., circulating counterregulatory hormones, inflammatory factors, etc.). Our data may also explain why clinical studies have had...
difficulty identifying a single therapeutic component that causes DKA-CE.

NHEs are hypothesized to underlie cell swelling in DKA-CE (51). In this study, DKA mice that were pretreated with MIA exhibited significantly decreased BWC after DKA therapy, supporting the hypothesis that NHE activity might in part underlie DKA-CE. To determine whether the inhibitory action of MIA on DKA-CE was due to reduced cellular DKA-CE, as opposed to vasogenic accumulation of extracellular fluid, we performed in vitro experiments specifically examining cellular edema. Indeed, a reduction in cellular DKA-CE after pretreatment with MIA was observed in brain slices following simulated DKA therapy. MIA failed to inhibit cell swelling due to simulated therapy with only bicarbonate-insulin in the absence of an osmolar decrease. Taken together, our data suggest that the NHE antagonist MIA inhibits only the osmotic-mediated component of cell swelling during simulated DKA therapy.

Fig. 6. Analysis of DKA-cerebral edema (DKA-CE) in NHE1 mutant [slow-wave epilepsy (swe)] and wild-type mice. A: genotypes of swe mouse line pups were determined by PCR analysis. B: representative immunoblot showing reduced functional NHE1 expression in swe/+ mice (P < 0.05, n = 3). C and D: in vitro experiments. Imaging reveals significant LT increases at 22 min in brain slices from swe/+ and +/- mice during treatment with DKA-corrected aCSF (ANOVA with Holm-Sidak post hoc test; *P = 0.05, n = 5–9). E: in vivo experiments. Elevated BWC (DKA-CE) was observed 2 h after DKA therapy in swe/+ mice despite a 50% reduction in NHE1 expression (*P < 0.001, n = 11–16).

Fig. 7. Coinhibition of NHE1 and anion transport inhibit cellular DKA-CE. Statistical analysis shows no differences in nonsubtracted LT values at 22 min in brain slices pretreated with the specific NHE1 inhibitors cariporide (P = 0.732, n = 5; A) or KR-33028 (P = 0.880; n = 4–6; B). Pharmacological inhibition of anion transport with DIDS (4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid) also failed to reduce cellular DKA-CE (P = 0.249, n = 5; C). In contrast, coinhibition of both NHE1 and anion transport decreased significantly LT at 22 min (P = 0.034, n = 6).
Both neurons and astrocytes are important sources of LT in brain slice experiments (3). For this study, LT was imaged from within the CA1 stratum radiatum, an easily identifiable region enriched with neuronal apical dendrites and with a relatively small number of astrocytes (52). Neuronal swelling in response to simulated DKA therapy was further suggested by calcein fluorescence dilution imaging (31). Concurrent neuronal and astrocytic swelling has been observed previously in a model of ischemic brain injury (48), but only astrocytic swelling is observed in brain slices exposed to hypo-osmotic stimuli (4, 48). Thus, our data suggests that the neuronal swelling due to simulated DKA therapy is not mediated by a hypo-osmotic shift but perhaps by rapid alkalization and/or the addition of insulin.

Pretreatment with MIA had no effect on neuronal swelling, as observed in calcein dye dilution experiments, suggesting that MIA inhibited only NHEs on astrocytes that are exquisitely sensitive to osmotic swelling. Indeed, astrocytes rapidly alter cell volume in response to osmotic stress via aquaporin channels, whereas neuronal swelling occurs slowly, due to other uncharacterized transport mechanisms (4, 48). Based on our MIA data, we conclude that neuronal and astrocytic swelling are mediated by different mechanisms during DKA therapy. Ultimately, precise determination of the relative contributions of neuronal and astrocytic swelling to DKA-CE will require two photon microscopy combined with cell-specific expression of fluorescent indicators (18, 43).

NHE1 is a key regulator of cell volume and pH in brain (46), and NHE1 expression is upregulated in several tissues by chronic acidosis (25) and/or hyperglycemia (53). Surprisingly, NHE1 expression was not elevated in DKA brain tissue, which is when aggressive DKA therapy may instigate cellular DKA-CE. In addition, cellular DKA-CE with simulated DKA therapy was similar in brain slices obtained from control and DKA mice. Together, these data indicate that NHE activity, but not expression, is increased by DKA therapy.

To define the role of NHE1 activity in DKA-CE, NHE1 mutant mice were employed in both brain slice (in vitro) and BWG (in vivo) experiments. The NHE1 mutant mice did not survive for the induction of DKA, and brain tissue from NHE1 mutant mice did not survive incubation in DKA aCSF. Thus, NHE1 is critical for brain tolerance and cell survival during DKA. Swe/+ mice, which express about half the functional NHE1 of wild-type mice, exhibited elevated BWG after DKA therapy, and in vitro experiments showed cell swelling in swe/+ mouse brain slices during simulated DKA therapy. These data indicate that partial expression of NHE1 is sufficient for cell swelling in DKA-CE, although it is also possible that other NHE isoforms or transporters functionally compensated for NHE1 in swe/+ mice (56).

To further define the role of NHE1 activity in cell swelling during DKA therapy, two highly selective NHE1 inhibitors, KR-33028 (32) and cariporide (41), were employed. NHE1 antagonists failed to inhibit the development of cellular DKA-CE during simulated DKA therapy, indicating that NHE1 inhibition alone cannot reduce cell swelling with DKA therapy. The brain tissue in these NHE1 antagonist experiments survived, and cell edema occurred in response to simulated DKA therapy, yet NHE1 mutant mouse brain tissue did not survive similar circumstances. The lack of tissue death observed during pharmacological inhibition of NHE1 suggests that NHE1 inhibition may be incomplete, or it may indicate that brain cells are capable of surviving in DKA conditions during transient but not permanent NHE1 inhibition.

MIA crosses the BBB and is a potent and specific NHE antagonist at the concentration used in this study (22, 26). MIA reduced DKA-CE in vivo and cellular DKA-CE in vitro during simulated DKA therapy, yet specific NHE1 antagonism did not reduce cell edema during simulated DKA therapy, suggesting an alternate mechanism for MIA. MIA may reduce cell swelling through the inhibition of NHE2-5 or perhaps by interacting with a currently unknown membrane transporter.

Neurons and astrocytes regulate intracellular pH using a number of ion transporters (15), and thus regulation of intracellular pH may occur even if one type of transporter is inhibited (36). Our results show that combined inhibition of NHE1 and AT are required to reduce cell swelling elicited in brain tissue with simulated DKA therapy. Studies show that both neuronal pH regulation (10) and regulatory volume increases in many cell types rely on tandem activation of NHE and AT (2, 30, 35). In vivo antagonism of Na-K-2Cl cotransport (NKCC) resulted in reduced cortical edema in DKA-CE mice, which suggests that NKCC may also contribute DKA-CE (34). Taken together, these studies suggest that inhibition of multiple NHE isoforms and/or multiple types of transporters should be more effective at reducing cellular DKA-CE than inhibition of a single isoform of NHE.

There are limitations to this study. First, although brain slices offer excellent experimental control, physical damage to the superficial surface may occur during preparation, and ischemia may occur at the tissue core. To reduce brain slice variability and potential tissue hypoxia, all brain slices were examined for viability before experiments, and oxygenated aCSF flow rates were maximized to reduce tissue hypoxia. Second, although we have attempted to mimic changes in the CSF composition during DKA therapy, CSF from children with DKA is not well defined. Thus, we investigated putative changes in CSF composition that are thought to occur in children with DKA as a result of increased BBB permeability (13, 19, 23, 29). It is also well established that systemic insulin crosses the BBB (8); however, studies have not yet addressed whether insulin resistance occurs in brain cells during DKA. Third, our work focuses on mechanisms of cell edema in hippocampal tissue during DKA, and we concede that cell swelling mechanisms may vary in other brain regions. Fourth, since this work was done with a mouse DKA model administered pancreatic toxins, caution must be used when extrapolating to human DKA. Unfortunately, we are not aware of other suitable juvenile DKA mouse models, and although the nonobese diabetic (NOD) mouse is similar in many respects to human autoimmune diabetes, NOD mice do not fully develop diabetes until adulthood, and they are resistant to ketoacidosis (14). Despite these study limitations, our data demonstrate the potential for aggressive DKA therapy to cause cellular DKA-CE.

In summary, we demonstrate that multiple DKA treatment factors can potentially elicit cell edema, including alkalization, insulin/alkalinization, and reduced osmolality (exacerbated by increased brain taurine), and that both neurons and astrocytes may swell in response to simulated DKA therapy. These results support clinical recommendations for slow and cautious treatment of children with DKA to reduce the chances
of instigating cellular DKA-CE by causing rapid and/or drastic changes in CSP pH, insulin levels, and/or osmolality during DKA therapy. Interestingly, general inhibition of NHE with MIA may reduce osmotic cell edema in astrocytes but not neurons. Moreover, our data provides the first experimental evidence that NHE activity and/or combined NHE1/AT might contribute to cellular DKA-CE.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES


