Simulated Diabetic Ketoacidosis Therapy *In Vitro* Elicits Brain Cell Swelling

Via Sodium-Hydrogen Exchange and Anion Transport

Running Head: Cell swelling caused by DKA therapy

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Abstract

A common complication of type-1 diabetes mellitus is diabetic ketoacidosis (DKA), a state of severe insulin deficiency. A potential harmful consequence of DKA therapy in children is cerebral edema (DKA-CE); however, the mechanisms of therapy-induced DKA-CE are unknown. Our aims were to identify the DKA treatment factors and membrane mechanisms that might contribute specifically to brain cell swelling. To this end, DKA was induced in juvenile mice with the administration of the pancreatic toxins streptozocin and alloxan. Brain slices were prepared and exposed to DKA-like conditions in vitro. Cell volume changes were imaged in response to simulated DKA-therapy. Our experiments showed that cell swelling was elicited with isolated DKA treatment components, including alkalinization, insulin/alkalinization or rapid reductions in osmolality. Methyl-isobutyl-amiloride, a non-selective inhibitor of sodium-hydrogen exchangers (NHEs), reduced cell swelling in brain slices elicited with simulated DKA-therapy (in vitro) and decreased brain water content in juvenile DKA mice administered insulin and rehydration therapy (in vivo). Specific pharmacological inhibition of the NHE1 isoform with Cariporide also inhibited cell swelling, but only in the presence of the anion transport (AT) inhibitor 4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid. DKA did not alter brain NHE1 isoform expression, suggesting that the cell swelling attributed to the NHE1 was activity-dependent. In conclusion, our data raises the possibility that brain cell swelling can be elicited by DKA treatment factors and that it is mediated by NHEs and/or co-activation of NHE1 and AT.
Introduction

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 re-hydration in a monitored hospital setting, however, a potential consequence of DKA and its therapy 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 alkanization.

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 brain, the NHE1 isoform is widely distributed and expressed in both neurons and astrocytes (38, 46). In contrast, NHE2-5 expression is region-specific 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 the 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 (intraperitoneal; 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 (1U 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 previously described (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) 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 minutes before switching 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, while the bicarbonate was reduced (44); during DKA treatment all values approached normal values. Noninvasive NMR and MRS imaging have also demonstrated altered CSF composition during DKA that 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 was 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 mOsmol. For control experiments, brain slices were perfused with only DKA aCSF throughout the experiments (Figure 1).

Imaging Light Transmittance (LT). Increased 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 10s with a digital CCD controlled by Northern Eclipse Imaging Software (Empix Imaging Inc., Mississauga, ON). The first image served as control (T_cont), and subsequent experimental images
were subtracted in a pixel-by-pixel fashion from the control image \((T_{\text{exp}}-T_{\text{cont}})\). This value was divided by the \(T_{\text{cont}}\) and the gain of intrinsic signal, then expressed as a percentage of the digital intensity of the control image: \(LT = \frac{(T_{\text{exp}}-T_{\text{cont}})}{(\text{gain} \times T_{\text{cont}})} \times 100 = \Delta T/T\%\). The control image was displayed in bright field, and each subtracted image was displayed with the change in LT for each pixel indicated using pseudo-colour intensity scale. Regions of interest (ROI) were identified in the control image, and size and positioning were consistent in all experiments (CA1 stratum radiatum). LT time points were averaged over several experiments and then graphed over time. For graphical comparison, mean LT for each experimental time point was subtracted from the mean LT for its respective timepoint on the baseline recording. For statistical analysis, experimental LT were averaged at single timepoint (22 min) without subtraction (raw data).

**Imaging Calcein Fluorescence.** Neurons preferentially load with calcein-am in brain slices (31). Briefly, 150-300 μm brain slices were incubated for 1 h in DKA aCSF containing calcein (Invitrogen, Carlsbad, CA, 20 μM), and then rinsed for 20 min in DKA aCSF. Brain slices were individually transferred to an imaging chamber, perfused with DKA aCSF and the hippocampus viewed with an inverted microscope. Calcein was excited with light from a xenon lamp passed through a 485 nm excitation filter cube every 10 s controlled by Northern Eclipse Software. After passing through a 440 nm emission filter, images were acquired every 10 s by a CCD. ROI were identified in the control image, and size and positioning were consistent in all experiments (CA1 stratum radiatum). To control for possible variation in calcein loading and auto-fluorescence between slices, mean fluorescence of the ROI from the initial image was subtracted from all subsequent images to calculate the net change in fluorescence at each time point. Time points were averaged from several brain slices then graphed against time. For statistical analysis, change in fluorescence was averaged at a single time point (20 min).
Brain Taurine Measurements. Brains from DKA and control juvenile mice were isolated, weighed and homogenized by sonication in 1ml of a solution containing 5-sulfosalicylic acid (0.2M), S-(2-aminoethyl)-L-cysteine (0.17mM) in a lithium citrate (133mM) buffer pH 2.6. A dilution of the brain homogenate was analyzed on a Biochrom 30 aminoacid analyzer (Biochrom LTD., Cambridge, UK) that measures the concentration of physiological amino acids including taurine with post-column ninydrin derivatization and using S-(2-aminoethyl)-L-cysteine as an internal standard. Data is expressed as nmol/mg dry weight.

Physiological salts, reagents and drugs. All reagents were purchased from Sigma Aldrich (Oakville, ON) unless otherwise specified. Methyl-isobutyl-amiloride (MIA; LT, 50 μM; in vivo, 50 mg/kg) was solubilized in dimethylsulfoxide (DMSO), and then diluted 1:1000 into DKA aCSF. Cariporide (1 μM; gift of Drs. H-Kleeman, W. Linz, J. Pünter; Sanofi Aventis, Frankfurt, Germany) was serially diluted in distilled water and then into DKA aCSF. KR-33028 (30 μM; gift of Dr. K. H. Kim; Yonsei University College of Medicine, Korea) was solubilized in DMSO and then diluted 1:1000 into DKA aCSF. DIDS (4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid; 500 μM) was added directly to DKA aCSF. For LT imaging experiments, antagonists were added to DKA aCSF and experimental DKA aCSF solutions throughout the experiment.

NHE1 Genotyping. Genotyping was performed using polymerase chain reaction (PCR) as previously described (22). Primers were synthesized by Sigma Aldrich (wildtype forward: 5’-CCT GAC CTG GTT CAT CAA CA-3’; mutant forward: 5’-CCT GAC CTG GTT CAT CAA CT-3’; common reverse: 5’-TCA TGC CCT GCA CAA AGA 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 cocktail (Calbiochem, La Jolla, CA), then centrifuged at 1000 x g for 20 min to remove insoluble material. NHE1 expression was detected as previously described, with some modifications (47). Briefly, 20 μg of protein from each sample was mixed with Laemmli loading buffer and run on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS/PAGE) gel. The samples were transferred onto PVDF membrane at 30V overnight at 4°C. The membrane was blocked with 5% non-fat milk in TBS-T for 1 h, and then incubated overnight at 4°C with NHE1 antibody (1:1000; Chemicon, Temicula, CA). The membrane was blocked with 5% non-fat milk in TBS-T with frequent changes, then incubated for 1 h with antimouse antibody conjugated to horse radish peroxidase (Cell Signaling Technology, Danvers, MA). Antibody binding was detected with chemiluminescent reagent (Lumiglo Reagent, Cell Signaling Technology).

Statistics. Data is presented as mean±standard error. 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 < 0.05 was considered significant.
Results

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

The first aim of this study was to determine which components of DKA therapy could potentially lead to cellular DKA-CE, including aggressive insulin administration, or rapid correction of either acidosis or hyperosmolality (Table 1). Simulated DKA therapy resulted in a significant increase in LT, or cell edema, at 22 min in brain tissue treated with either alkalinization alone (38.1% increase in LT) or with combined insulin and alkalinization (70% increase in LT) (Figure 2A, 2B). The greatest increase in LT resulted from complete DKA correction with insulin, alkalinization and decreased osmolality (99.4% increase in LT), as well as decreased osmolality alone (98.9% increase in LT). No significant difference in LT was observed with therapy consisting of insulin only (6.4% increase in LT), consistent with the low bioavailability of insulin in acidic media (24). Overall, these data suggest that multiple DKA treatment factors potentially cause cellular DKA-CE.

Previous research has suggested that osmotic disequilibrium in brain cells during DKA therapy is due, in part, to intracellular idiogenic osmoles that are elevated to compensate for the DKA-associated hyperosmolality (33). Given the cell swelling 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 as compared to control mice (brain taurine concentrations were 3.0±0.1 and 2.5±0.2
[nmol/mg dry weight] in DKA and control brains, respectively; p<0.05, n=9-10 per group;
Figure 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
significantly reduced cellular DKA-CE in brain tissue treated with DKA-corrected aCSF (67%
decrease in LT; Figure 3A, 3C). MIA failed to reduce cell swelling in brain tissue treated with
Alk/Ins aCSF (29% decrease in LT; Figure 3B, 3C), 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 (Figure 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 the 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; Figure 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 is largely devoid of glia (data not shown; n=3). MIA pre-treatment of calcein-loaded brain slices failed to significantly reduce cell swelling elicited with simulated DKA therapy (10% decrease in fluorescence; n=5; Figure 4B), indicating that the MIA-sensitive osmotic component resides in non-neuronal 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= 2.2 ± 0.5 AU; OD_DKA= 2.3 ± 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; Figure 5A, 5B). Similar NHE1 expression levels were also observed in both control and DKA brain following fractioning and immunoblotting of membrane versus 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; Figure 5C), pre-incubated for 2 hours 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 if NHE1 activity is important for cellular DKA-CE during DKA therapy.
Genotyping and western blotting confirmed that NHE1 expression was significantly reduced in swe/+ mouse brain and NHE1 was altogether absent in swe/swe mice (n=3/group, 1 forebrain per sample, OD_{swe/+} = 5.94 ± 0.38 AU, OD_{swe/swe} = 2.98 ± 0.62 AU, OD_{swe/swe} = 0.05 ± 0.04 AU; P<0.05) (Figure 6A, 6B). Swe/swe mice did not survive to juvenile age (35 days post-natal) 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; Figure 6C, D). Moreover, like NHE1+/+ mice, we observed DKA-CE in vivo when DKA swe/+ were therapeutically treated with insulin and rehydration fluid (swe/+ DKA, BWC= 3.55 ± 0.2 l/kg dry brain weight; swe/+ DKA-CE, BWC= 3.71 ± 0.03 l/kg dry brain weight, Figure 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, Figure 7A) or KR-33028 (1.4% increase in LT, Figure 7B). An inhibitor of anion transport (AT) also failed to reduce cellular DKA-CE (DIDS, 13.4% increase in LT; Figure 7C). In contrast, co-inhibition of NHE1 and AT with application of Cariporide and DIDS, respectively, partially reduced cellular DKA-CE (15.1% decrease in LT; Figure 7D). Thus, both NHE1 and AT have compensatory actions and require co-inhibition to reduce cellular DKA-CE elicited with 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 hours of DKA therapy (20, 29). Clinical studies suggest that bolused insulin, bicarbonate administration, and/or rapid re-hydration, may precipitate DKA-CE (20, 21, 27). We previously 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 alkalinization and reduction in osmolality. 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 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 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 due to 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).
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 alkalinization (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 counter-regulatory 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 pre-treated with MIA exhibited significantly decreased BWC after DKA therapy, supporting the hypothesis that NHE activity might, in part, underlie DKA-CE. To determine if 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 on cellular edema. Indeed, a reduction in cellular DKA-CE after pre-treatment 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 suggests that the NHE antagonist MIA inhibits only the osmotic-mediated component of cell swelling during simulated DKA therapy.

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 previously been observed 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 alkalinization 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, while neuronal swelling occurs slowly, due to other uncharacterized transport mechanisms (4, 48). Based on our MIA data, we conclude that neuronal and astrocytic swelling is 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 endogenous 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 BWC (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 wildtype mice, exhibited elevated BWC 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 were employed, KR-33028 (32) and Cariporide (41). 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 mortality 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 co-transport (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, while brain slices offer excellent experimental control, physical damage may occur to the superficial surface 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, while 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 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 if insulin resistance occurs in brain cells during DKA. Thirdly, 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 while the 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 demonstrates 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 alkalinization, 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 in order to reduce the chances of instigating cellular DKA-CE by causing rapid and/or drastic changes in CSF 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:

None.

Author Contributions:

KLR and DDF designed the experiments; KLR, MC and CAR performed experiments; KLR, AJW, TAD, MC, CAR and DDF analyzed data; KLR, AJW, TAD, GC, MC, CAR and DDF interpreted results of experiments; KLR and DDF prepared figures; KLR drafted the manuscript; AJW, TAD, GC, MC, CAR and DDF edited and revised manuscript; all authors approved final version of manuscript.


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Figure Legends

Figure 1. Imaging Light Transmittance (LT; cell edema) in hippocampal slices during simulated DKA therapy. A. Representative LT Images. Shown is: 1) an initial grey scale 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-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 was 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; B) was subtracted from the experimental trace (blue; B) to obtain the averaged subtracted DKA-corrected trace (purple), which represents the net effect of simulated DKA-correction.

Figure 2. Simulated DKA therapies (Table 1) cause cell swelling in hippocampal slices. A. Simulated DKA therapies elevate LT (cell edema) after baseline subtraction. B. Statistical analysis confirms that DKA corrected aCSF, decreased osmolality aCSF, Ins/Alk aCSF, and Alk alone aCSF lead to significant cell swelling. Statistical analysis was performed at 22 min with raw (non-subtracted) LT values. (p<0.05; n=5-29). A bar diagram illustrating the increased
taurine in brains of DKA juvenile mice as compared to control juvenile mice (p<0.05, n=9-10 animals per group).

**Figure 3. Non-selective NHE antagonism with methyl-isobutyl-amiloride (MIA) reduces both cellular DKA-CE in vitro and DKA-CE in vivo.** A-C. *In vitro* NHE antagonism. Non-selective NHE antagonism with MIA inhibits the osmotic component of cellular DKA-CE induced by DKA-corrected aCSF, as MIA failed to inhibit the cellular swelling elicited by Alk/Ins aCSF (p<0.05, n=10-20). D. *In vivo* NHE antagonism. Pre-treatment with MIA (50 μM) significantly reduced brain water content (DKA-CE) in DKA-treated mice compared to those pre-treated with vehicle only (p<0.05; n=26-28).

**Figure 4. Neuronal swelling elicited by simulated DKA therapy as determined by calcein fluorescence dilution imaging.** A. Averaged normalized fluorescence (F) traces show that neuronal swelling occurs in brain slices treated with DKA-corrected aCSF. Black arrow indicates time of switchover from DKA-aCSF to DKA-corrected aCSF. Inset: Statistical analysis at 20 min shows that the fluorescence signal decreased in brain slices treated with DKA-corrected aCSF (pink) compared to DKA aCSF (blue) (P=0.001; n=3). B. Calcein-loaded brain slices were pre-treated with MIA and treated with DKA-corrected aCSF. Statistical analysis at 20 min shows that NHE antagonism does not inhibit neuronal swelling (P<0.05; n=5).

**Figure 5. DKA does not change brain NHE1 expression.** A. Representative immunoblot showing that NHE1 expression is not elevated in DKA cortex (CO), hippocampus (HC), brainstem-diencephalon (BD) or cerebellum (CB). B. Optical densitometry confirms that NHE1
expression does not differ between control and DKA mouse brain (n=3); each sample contained
tissue from 3 [CO], 4 [CB, BD] or 5 [HC] mice). C. Statistical analysis reveals no significant
differences in cellular edema between control (sham-injected) and DKA (STZ/ALX-injected)
mouse brain slices when treated with DKA-corrected aCSF (P<0.05; n=4-10).

Figure 6. Analysis of DKA-CE in NHE1 mutant (swe) and wild-type mice. A. Genotypes of
swe mouse line pups were determined by PCR analysis. B. A 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-hoctest;
P=0.05; n=5-9). E. In vivo experiments. Elevated BWC (DKA-CE) was observed 2h after
DKA therapy in swe/+ mice, despite a 50% reduction in NHE1 expression (P<0.001; n=11-16).

Figure 7. Co-inhibition of NHE1 and AT inhibit cellular DKA-CE. Statistical analysis
shows no differences in non-subtracted LT values at 22 min in brain slices pre-treated with the
specific NHE1 inhibitors cariporide (A, P=0.732; n=5) or KR-33028 (B, P=0.880; n=4-6).
Pharmacological inhibition of anion transport with DIDS also failed to reduce cellular DKA-CE
(C, P=0.249; n=5). In contrast, co-inhibition of both NHE1 and AT significantly decreased LT
at 22 min (P=0.034; n=6).
Table 1. Components of DKA aCSF and simulated DKA therapy aCSFs.

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<th>pH</th>
<th>Bicarbonate (mM)</th>
<th>Sodium (mM)</th>
<th>Glucose (mM)</th>
<th>Osmolality (mOsmol)</th>
<th>β-OH-butyrate (mM)</th>
<th>Lactate (mM)</th>
<th>Insulin (μM)</th>
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Abbreviations: Alk, alkalinization; Ins, insulin; Osmol, osmolality. DKA corrected aCSF included Alk/Ins and decreased osmolality. Components that were common to all aCSF are as follows (in mM): 3.3 KCl, 1.1 MgSO₄, 2.0 CaCl₂, and 1.1 NaH₂PO₄.
A

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NHE1

GAPDH

B

C

Control mice

DKA

DKA corrected

Cell Swelling (ΔT/Δt%)

DKA

DKA corrected

* *