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Autophagy in the brains of young patients with poorly controlled T1DM and fatal diabetic ketoacidosis

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Abstract

Semi-quantitative neuroradiologic studies, quantitative neuron density studies and immunocytochemistry markers of oxidative stress and neuroinflammation indicate neuronal injury and deficits in young patients with chronic poorly controlled type 1 diabetes mellitus (T1DM). Present data suggest that pathogenesis of the neuronal deficits in young patients, who die as the result of diabetic ketoacidosis (DKA) and brain edema (BE), does not involve apoptosis, a prominent form of regulated cell death in many disease states. To further address this we studied mediators of macroautophagy, endoplasmic reticulum (ER) stress and apoptosis. In all areas studied we demonstrated increased levels of macroautophagy-associated proteins including light chain-3 (LC3) and autophagy related protein-4 (Atg4), as well as increased levels of the ER-associated glucose-regulated protein78/binding immunoglobulin protein (GRP78/BiP) in T1DM. In contrast, cleaved caspase-3 was rarely detected in any T1DM brain regions. These results suggest that chronic metabolic instability and oxidative stress may cause alterations in the autophagy-lysosomal pathway but not apoptosis, and macroautophagy-associated molecules may serve as useful candidates for further study in the pathogenesis of early neuronal deficits in T1DM.

Keywords

Autophagic gene-4; Cleaved caspase-3; Diabetic encephalopathy; Diabetic ketoacidosis; Glucose-regulated protein78; Light chain-3; Oxidative stress

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Introduction

The metabolic instability of suboptimally controlled T1DM includes extremes of hypoglycemia, hyperglycemia and ketonuria, events that can recur from the time of diagnosis. Each of these perturbations of stability can mediate systemic oxidative stress and inflammation, even at an early age (Ceriello, 2006; Esposito et al., 2002; Flores et al., 2004; Gogitidze et al., 2010; Jain et al., 2006; Singh et al., 2004). The treatment of severe diabetic ketoacidosis (DKA), a life-threatening form of metabolic instability, is associated with the paradoxical effects of increasing oxidative stress and systemic inflammation (Dalton et al., 2003; Hoffman et al., 2003a,b; Jerath et al., 2005; Lee et al., 2002; Turk et al., 2006) during the time that subclinical brain edema (BE) is progressing, clinical BE is most likely to occur (Edge et al., 2001; Glaser et al., 2001; Hoffman et al., 1988) and metabolic correction is occurring. These oxidative and immunologic stresses compromise neuronal homeostasis (Duarte et al., 2006), neuronal polarity (Sosa et al., 2006) and neurotrophic support (Hoffman et al., 2010, 2011; Sima and Li, 2005).

Neuronal deficits, markers of oxidative stress and neuroinflammation have been reported in the brains of diabetic rat models (Li et al., 2002), in neurodegenerative diseases (Sayre et al., 2008), and in young patients with chronic poorly controlled T1DM resulting in the fatal BE/DKA (Hoffman et al., 2006, 2007, 2008, 2009, 2010, 2011). Of importance, metabolic dysfunction and oxidative stress have been suggested to be major factors in the onset of Alzheimer disease (AD) (Casadesus et al., 2007). Oxidative stress and neuroinflammation are also involved in the pathogenesis of neuronal deficits of acute trauma (Clark et al., 2008; Sadasivan et al., 2008). However, despite the similarities of the markers there are apparent differences in the pathogenesis of the neuronal death in young patients with poorly controlled T1DM. These include normal neuronal morphology (Hoffman et al., 2011), the absence of TUNEL (Hoffman et al., 2006), and several other immunocytochemical markers of the mitochondrial apoptotic pathway (Hoffman et al., 2011), the most prominent form of regulated cell death in neurodegenerative diseases (Szegezdi et al., 2006). These dissimilarities suggest the involvement of alternative forms of cell death (Ferraro and Cecconi, 2007; Hoffman et al., 2011; Maiuri et al., 2007; Tan et al., 1998).

The endoplasmic reticulum (ER) is a major metabolic organelle and a nutrient sensor (Mandl et al., 2009). In addition to misfolded proteins triggering the unfolded protein response (UPR) (Raghubir et al., 2011), compensatory ER mechanisms reduce the stress of protein misfolding (Zhang and Kaufman, 2006). Other triggers of UPR include the metabolic factors of hyperglycemia (Inagi, 2011; Sheikh-Ali et al., 2010), hypoglycemia (Ikesugi et al., 2006), lipids (Pineau et al., 2009) and C5b-9, the membrane attack complex (Cybulsky et al., 2002). If compensation is not adequate, continued ER stress can lead to apoptosis (Fribley et al., 2009; Szegezdi et al., 2006) and/or the autophagy-lysosomal pathway, an important lysosome mediated degradation pathway (Bernales et al., 2006). Autophagy plays a major role in cellular homeostasis, serving as 1) a degradation pathway of damaged proteins and organelles that result from oxidative/nitrosative stress (Scherz-Shouval et al., 2007); 2) an adaptive response to metabolic challenges (Kaushik et al., 2010); and 3) possibly a neurotoxic role in neurodegenerative diseases (Cherra and Chu, 2008; Hoozemans et al., 2005).

Two major types of autophagy are macroautophagy (Ravikumar et al., 2010) and chaperone-mediated autophagy (CMA) (Kaushik et al., 2008), both constitutively expressed with the lysosome being their common endpoint. However, they differ in the conditions of activation, their targeted substrate, and regulation. Macroautophagy is the major form of autophagy, and is involved in tissue homeostasis and innate and adaptive immunity (Schmid and Munz, 2007). It has a non-selective response to various forms of stress, is regulated by autophagy-related (Atg) genes and is involved in bulk sequestration of cytosolic components. CMA is dependent on the constitutively expressed heat-shock cognate70 (hsc70), shares 80% homology with the heat-shock protein70 (HSP70), and identifies peptide sequences of cytoplasmic substrates, thus being more selective in its degradation. CMA serves to balance dysregulated energy and is maximally activated by nutrient/metabolic (Cuervo, 2010) and oxidative/nitrosative stresses. Cross-talk has also been established between these two forms of autophagy (Kaushik et al., 2008; Massey et al., 2008; Mehrpour et al., 2010).

We investigated whether the milieu of chronic suboptimal metabolic control in T1DM and the resulting oxidative stress induced markers of macroautophagy in non-dividing differentiated cells (Cuervo, 2004), and is thus a candidate for early neuronal death and diabetic encephalopathy (Nijholt et al., 2011; Pivtoraiko et al., 2009). We addressed this by studying: 1) microtubule-associated protein 1 light chain-3 (LC3/ Atg8), a well-accepted marker of autophagic vacuoles (Tanida et al., 2008); 2) autophagic-related gene-4 (Atg4), a target of oxidative stress (Scherz-Shouval et al., 2007) and an essential protease required for cleavage of LC3; 3) glucose-regulated protein 78/binding immunoglobulin protein chaperone (GRP78/BiP) (Rao et al., 2002) an ER-associated protein that is induced by changes in cellular redox and by hyperglycemia (Liu et al., 2010), and is a regulator of stress-induced UPR and CMA (Li et al., 2008), with a primary function to bind misfolded proteins, and 4) cleaved caspase-3, a key enzyme activated by ER stress (Hitomi et al., 2004) and a critical late stage regulator of both extrinsic (death receptor-mediated) and intrinsic (mitochondrial) apoptosis (Stennicke and Salvesen, 2000).

Materials and methods

Case 1

An adolescent girl with a 4-year history of poorly controlled T1DM, which resulted in recurrent hospitalizations for DKA. There was no history of other medical conditions or microvascular disease. There was no history of recurrent fever or enteritis. The final admission was preceded by a 12-h history of abdominal pain and several episodes of emesis. On physical examination, she was oriented but drowsy. Her height was 163 cm; weight was 68.5 kg; blood pressure was 140/70 mm Hg; pulse was 140/min; respirations were 30/min; and temperature was 98.5 F. There were no signs of infection. The mucous membranes were dry, the abdomen was diffusely tender, and bowel pattern sounds were hypoactive. Tanner stage was the-larche stage 3 and pubarche stage 2 (Marshall and Tanner, 1969). Admission laboratory tests consisted of pH 7.10; pCO₂, 15 mm Hg; pO₂, 106 mm Hg; glucose, 810 mg/dl; Na, 132 meq/L; K, 5.7 meq/L; Cl, 93 meq/L; HCO₃, 5 meq/L; BUN, 30 mg/dl; and calculated effective plasma osmolality, 309 Osm. Treatment was in a pediatric intensive care unit, and correction of the hyperglycemia and metabolic acidosis was unremarkable. Twelve

hours into treatment, she developed a sudden onset of labored respirations and within 20 min, had a cardio-respiratory arrest. An emergency CT scan showed sulcal effacement and cerebral and pontine edema with evidence of herniation. Efforts at resuscitation were unsuccessful, and she was pronounced dead 1.5 h after the cardiorespiratory arrest.

Case 2

An adolescent girl had an 8-year history of poorly controlled T1DM, which had resulted in recurrent hospitalizations for DKA. There was no history of other medical conditions or microvascular disease. The final admission was preceded by an 18-h history of capillary blood glucoses of over 300 mg/dl, ketonuria, a 4-h history of headache, and several episodes of emesis. There was no history of fever or enteritis. On physical examination, she was slightly confused and lethargic. Her height was 154 cm; weight was 45 kg; blood pressure was 135/68 mm Hg; pulse was 132/min; respirations were 26/min; and temperature was 97 F. There were no signs of infection. Diffuse abdominal tenderness and decreased bowel sounds were present. The Tanner stage was thelarche stage 2 and pubarche stage 1 (Marshall and Tanner, 1969). Admission laboratory tests consisted of pH 7.16; pCO₂, 17 mm Hg; pO₂, 100 mm Hg; blood glucose, 581 mg/dl; Na, 130 meq/L; K, 4.8 meq/L; Cl, 89 meq/L; HCO₃, 6 meq/L; BUN, 28 mg/dl; and calculated effective plasma 292 Osm. Treatment was in a pediatric intensive care unit, and correction of the hyperglycemia and metabolic acidosis was unremarkable. Ten hours into treatment, she became unresponsive and was treated with mannitol and hyperventilation and placed on mechanical ventilation. A CT head scan showed diffuse cerebral edema and decreased intercaudate diameter. She was pronounced dead approximately 10 h after the cardiorespiratory arrest.

Control brain tissue consisted of two age matched, non-diabetic cases.

Immunostaining

Brain tissue samples were fixed in 10% buffered formalin and embedded in paraffin wax by conventional techniques. The paraffin blocks were then cut into 5 µm thick sections that were subsequently mounted. The slides were deparaffinized with xylene, rehydrated through a descending ethanol series (100%, 95%, 70%, 50%, 3 min each) and washed in Tris-buffered saline for 2–5 min. Antigen retrieval was achieved by immersing slides in 0.01 M citrate buffer (pH 6) and microwaving at 800 W for 3–5 min, followed by a 20 minute rest period. The slides were then incubated in blocking solution [5% normal goat serum (NGS), 5% bovine serum albumin (BSA) and 0.1% TritonX-100 in phosphate-buffered saline (PBS)] for 30 min at room temperature to prevent non-specific background staining. For double-labeling immunofluorescence experiments the samples were exposed to either mouse anti-human β-III-tubulin, mouse anti-Iba1 (Abcam, MA) or mouse anti-GFAP (Sigma Aldrich, MO) at 4 °C overnight, and visualized with either FITC- or Texas Red-conjugated secondary anti-mouse antibody (Vector Laboratory, CA). The sections were then incubated overnight with either rabbit anti-human GRP78, LC3A/B or sheep-anti human Atg4C antibody (all from Abcam, MO), followed by incubation with either FITC or Texas Red-conjugated anti-rabbit or anti-sheep antibody (Vector Laboratory, CA). The sections were mounted in Vectashield Mounting Medium (Vector Laboratory, Burlingame, CA) and viewed on a confocal laser scanning microscope (LSM 510 Zeiss, Germany, objective

40×1.3 NA). Negative controls included sections that were treated in the same manner with omission of the primary antibody as well as controls treated with mouse, sheep or rabbit IgG isotype control.

Chromogenic in situ detection of cleaved caspase-3

Sections from paraffin-embedded brains were deparaffinized and subjected to antigen retrieval in citrate buffer with heat, then incubated for 5 min with 3% H₂O₂ in PBS to reduce endogenous peroxidase activity. Sections were incubated in PBS blocking buffer (PBSBB; 1% BSA, 0.2% evaporated milk, 0.3% Triton X-100 in PBS) for 30 min, followed by overnight (4°C) incubation in PBSBB with rabbit anti-cleaved caspase-3 (Cell Signaling Technologies). After PBS washes, slides were incubated with HRP-conjugated goat anti-rabbit secondary antibody (SuperPicture, Zymed) followed by PBS wash. Antibody localization was detected via Tyramide Signal Amplification (Perkin Elmer Life Sciences) using biotin-conjugated tyramide (30 min, RT followed by PBS wash), Vectastain ABC (Vector Labs; 30 min, RT followed by PBS wash) and DAB chromogen (Pierce, Rockford, IL; 10–15 min, RT followed by distilled H₂O wash). Slides were counter-stained with hematoxylin, de-colored with acid-ethanol and incubated with bluing reagents (1% ammoniumhydroxide), dehydrated in increasing percentages of ethanol, cleared in xylene and then coverslipped with Cytoseal 60TM.

Evaluation of immunostaining

For quantification of immunostaining intensity brain images were taken at 20× magnification using identical camera settings so that the number and intensity of pixels would reflect the differences in their protein expression. The protein level of GRP78, LC3 and Atg4 was determined by measuring the area (number of pixels) and fluorescent intensity (average intensity of pixels) of the staining from twenty four images captured randomly in the brain parenchyma from DKA and control sections. The unit area of each image was 0.172 mm². Analysis was performed using the Image J program (NIH software program). The relative expression was calculated by multiplying the number of pixels (area) by the average intensity of pixels.

The number of neurons, astrocytes and microglial cells that expressed GRP78, LC3 and Atg4, were evaluated by counting the number of β III-tubulin+, GFAP+, or Iba1+ which were GRP78+, LC3+ or Atg4+, in five randomly chosen microscopic fields (at 10× magnification per section). A mean of the percentage β III-tubulin+, GFAP+, or Iba1+/-SD was computed by dividing the number of these cells in the DKA cases by the number in the controls multiplied by 100.

Statistics

All values are expressed as mean±standard deviation (SD). Unpaired t test was performed to identify the differences between the two groups. A probability value <0.05 was regarded as statistically significant. Prism software (Graph Pad Prism 3.0, San Diego, CA) was used for statistics.

Results

GRP78 levels in neurons

Immunocytochemistry of brain sections showed that GRP78, localized primarily to the cytoplasm of cells in basal ganglia, cerebellum, frontal cortex, hippocampus, parieto-occipital cortex, pons and medulla. Diffuse cytoplasmic staining was predominantly present in neurons (β III tubulin+cells), (Fig. 1A) while sporadic expression of GRP78 was seen in microglia and astrocytes. Quantitative immunohistochemical analysis showed a specific distribution pattern for GRP78+ neurons. The majority of neurons in the hippocampus ($59\pm4.3\%$) of the DKA cases were GRP78+ while the basal ganglia, cerebellum, frontal cortex, parieto-occipital cortex and medulla had GRP78+neurons in the range of $24\pm 3.2\%$, $18\pm3.5\%$, 22 ± 5 , $15\pm4\%$, $15\pm3\%$, respectively (Fig. 1B). Sporadic expression of GRP78 was present in the neurons of the pons and spinal cord. In contrast the correspondent areas of the control brain samples showed very weak or absent GRP78+ staining, in neurons and glial cells and quantitative analysis indicated significantly less GRP78+ staining compared with DKA brains ($p<0.001$). The analysis of the GRP78 immunostaining in gray (cerebral cortex, subcortical gray matter, cerebellar cortex, brain stem nuclei, and spinal central gray matter) and white matter did not show any differences in the intensity for GRP78 staining in the DKA brains (Fig. 1C).

LC3 and Atg4 levels in neurons

Emerging studies indicate that a variety of cellular stresses, such as endoplasmic reticulum stress or mitochondrial dysfunction can induce autophagy, which could be selectively involved in removal of the oxidative modified macromolecules and organelles. Due to the fact that we found increased levels of markers for oxidative stress and ER stress in DKA we analyzed relative levels of autophagy associated proteins in DKA neurons.

Immunofluorescence staining indicated DKA neurons exhibited increased levels of microtubule-associated protein light-chain 3 (LC3), an autophagosome marker. In the observed areas of the DKA brains neurons exhibited punctate perinuclear LC3+ immunoreactivity while glial cells (predominantly astrocytes and microglia) exhibited faint or absent immunoreactivity. The distribution of LC3+ neurons was similar to GRP78+ neurons. The LC3 levels were slightly greater in hippocampal neurons although there were similar numbers of LC3+ neurons in the basal ganglia and frontal cortex (24 ± 3.2 ; 22 ± 3.1 ; 21 ± 4.1 , retrospectively) (Figs. 2A, B). Approximately 10% of β III neurons in the cerebellum, pons and medulla showed LC3 expression. Similar to GRP78 there was no difference in LC3 immunostaining intensity in structures of gray and white matter (Fig. 2C).

The Atg4 family of cysteine proteases plays an important role in regulation of autophagosome formation and degradation. Oxidative stress is considered a potent regulator of Atg4 expression. Thus we analyzed expression of Atg4. Atg4 had patterns of distribution and levels similar to LC3 in the neurons of the observed areas (Figs. 3A, B). There were slightly higher numbers of Atg4+ microglial cells than for LC3+ in the DKA cases (data not shown). In the DKA cases Atg4 was only sporadically present in astrocytes. Analysis of gray and white matter also showed similar levels of expression of Atg4 (Fig. 3C).

Cleaved caspase-3 in neurons

To determine if apoptosis played a role in the regulation of neuronal cell death in poorly controlled T1DM and was relevant to the observed alterations in markers of ER stress and autophagy, cleaved caspase-3 immunoreactivity was assessed. Cleaved caspase-3 positive neuronal cells were very rare in brains of DKA cases numbers of which were not different from controls (data not shown). Thus apoptosis, at least that driven by caspase-dependent mechanisms, does not appear to play an important role in regulating neuron death in DKA nor associated with the alterations in autophagy observed in the DKA brains.

Discussion

Increased levels of LC3 and Atg4 in the brains of young patients with T1DM and chronic poor metabolic control are in agreement with the potential oxidative stress-induced alteration in macroautophagy and enhanced levels of GRP78 that implicate changes in both macroautophagy and CMA (Kiffin et al., 2006). The disrupted metabolic control in DKA results in numerous aberrant nutrient, hormone and metabolic signals that could induce autophagy. Specific candidates for induction and stimulation of autophagy include: insulin deficiency/resistance (Barrett et al., 1982; Evans et al., 2005; Hoffman et al., 2010; Yu et al., 2008); deficiency of insulin growth factor-1 (IGF-1) and insulin growth factor-1 receptor (IGF-1R) (Bains et al., 2011; Cinaz et al., 1996; Hoffman et al., 2010); hyperglucagonemia (Schworer and Mortimore, 1979); and hyperglycemia (Liu et al., 2010). Based on the expression of 8OHG and NT (Greenacre and Ischiropoulos, 2001; Hoffman et al., 2011; Nunomura et al., 2006) other candidates for perturbation of autophagy include: alteration of protein synthesis and degradation (Ding et al., 2007) due to the oxidative stress of RNA (Castellani et al., 2008; Hoffman et al., 2011), protein damage, altered lipid metabolism (Decsi et al., 2005; Kim et al., 2007; Singh et al., 2009); increased production of ketones and aldehydes (Finn and Dice, 2005; Hill et al., 2008; Wood et al., 2006) and lipid peroxidation (Hoffman et al., 2011; Kostolanska et al., 2009; Lee et al., 2002; Muller et al., 2011).

An adverse impact of the metabolic milieu on susceptible long-lived, post-mitotic neurons is strengthened considering that with a normal metabolic state, mild oxidative stress gradually results in increased lysosomal burden with the accumulation of toxic protein aggregates, damaged organelles and further oxidative stress (Brunk and Terman, 2002). This stress results in the gradual impairment of neuronal function, decreased production of ATP, and possible cell death (Terman et al., 2007). It is important to recognize that HSP70, a stabilizer of protein metabolism, is diffusely expressed in DKA/BE (Hoffman et al., 2007) and is increased systemically prior to treatment of severe uncomplicated (without BE) DKA (Oglesbee et al., 2005). Thus HSP70 could provide protection against the oxidative stress during normal metabolism and lysosomal membrane permeabilization (Nylandsted et al., 2004) such as occurs in retinal pigment epithelial cells in macular degeneration (Kaamiranta et al., 2009).

Results of IHC indicate increased LC3 immunoreactivity, suggesting an alteration in the autophagy-lysosome pathway in poorly controlled T1DM. These findings are complemented by a correlative increase in Atg4 immunoreactivity, which indicates an enhancement of autophagy induction. While at present we are limited in the manner in which our specimens

are processed to further assess the more biochemical nature of autophagy dysfunction, our data clearly indicate an alteration in the autophagy-lysosome pathway that suggests induction of autophagy. We appreciate that LC3 IHC cannot definitely delineate the accumulation of autophagic vacuoles, as antibodies used to detect LC3 recognize both cytosolic LC3-I as well as the autophagic vacuole-associated LC3-II (Kabeya et al., 2000). Furthermore, studies of autophagic flux would be required to assess whether accumulation of autophagic vacuoles occurs as a result of nascent autophagic vacuole formation, or as a result of lysosome dysfunction (Klionsky et al., 2008). Regardless, the metabolic instability and negative nitrogen balance in T1DM, even without DKA (Mordier et al., 2000; Umpleby et al., 1986), suggest that alterations in macroautophagy induction are occurring and further studies are warranted to validate the manner in which macroautophagy is altered. Examples of the significant catabolism in DKA and potential impact on autophagy include: a transient decrease in numerous amino acids during severe uncomplicated DKA (Carl et al., 2002) with arginine deprivation inhibiting the mammalian target rapamycin (mTOR), a regulator of autophagy and possible stimulation (Savaraj et al., 2010). In contrast, glutamine which is also decreased during DKA and its treatment (Carl et al., 2002) has a direct relationship with autophagy (Nicklin et al., 2009) that could transiently impair autophagy. In addition to the decrease of the antioxidant vitamins A and C, during DKA (Lee et al., 2002), the depletion of cysteine and glycine (Carl et al., 2002) two of the three amino acids that make up glutathione, a primary antioxidant that is depleted in poorly controlled T1DM (Darmaun et al., 2005) results in further increases of oxidative stress that may contribute to autophagic induction.

A protective role for autophagy cannot be ruled out (Vanhorebeek et al., 2011; Zhang et al., 2008). However, dysfunctional autophagy due to both chronic poor control and the acute metabolic instability of DKA is supported by magnetic resonance spectroscopy (MRS) reports of neuronal injury/deficits in young patients with poorly controlled T1DM (Makimattila et al., 2004; Sarac et al., 2005) unrelated to DKA. MRS has also identified transient neuronal injury during the acute insult of DKA and its treatment (Wootton-Gorges et al., 2007). The regions of neuronal insult demonstrated by MRS correlate well with the regions of immunocytochemical (ICC) markers of oxidative/nitrosative stress and the decreased neuronal density in the fatal DKA/BE (Hoffman et al., 2008, 2010, 2011). LC3, Atg-4 and GRP78 immunoreactivity is greatest in the hippocampus, the region that exhibits significant elevations in NT (Hoffman et al., 2010) and 8OHG (Hoffman et al., 2011) and the greatest neuronal deficit (Hoffman et al., 2008), as well as the region exhibiting maximal down-regulation of IGF-1R and insulin receptors (IR) (Hoffman et al., 2010). In contrast, immunoreactivity of autophagy markers was comparatively low in the cerebellum. This is in keeping with no decrease in Purkinje cell density and only minimal decreases of insulin growth factor-1 (IGF-1) and insulin receptors in DKA/BE (Hoffman et al., 2008). This preserved histology agrees with the report by Bains et al. (2009) that IGF-1 protects Purkinje neurons from autophagic death.

Cell death independent of the caspases is well recognized (Kroemer and Martin, 2005), and therefore the lack of expression of cleaved caspase-3 and other markers of the energy-dependent apoptotic pathway including mitochondrial DNA deletion (mtDNA) (Hoffman et al., 2006, 2011), does not rule out the contribution of caspase-independent apoptosis or other

forms of cell death. Distinct temporal (Newcomb et al., 1999) and regional (Conti et al., 1998) apoptotic patterns have been reported in acute experimental traumatic brain injury. However, only the rare expression of cleaved caspase-3 in these DKA cases is in agreement with previous studies suggesting a lesser role for apoptosis (Hoffman et al., 2006, 2011) as a mechanism of chronic or acute neuronal loss, and also against apoptosis mediating ensuing cell death (Newcomb et al., 1999). Support for a limited role of apoptosis includes: 1) inhibition by polyunsaturated fatty acids (Kim et al., 2001); 2) a negative regulator effect by the receptor for advanced glycation end products (RAGE) (Kang et al., 2011), which is robustly expressed in the BE/DKA (Hoffman et al., 2008); 3) blockage of ER stress-induced apoptosis by complexes formed between GRP78 with caspase 7 and 12 (Rao et al., 2002); 4) an anti-apoptotic effect by HSP70 (Beere, 2005); 5) crosstalk between autophagy and apoptosis that could result in down regulation of apoptosis (Boya et al., 2005); and 6) preferential UPR activation of autophagy similar to Alzheimer disease (AD) (Scheper et al., 2011). Also, Zhu et al. (2006) have reported apoptosis to be a mathematical improbability in AD.

It is important to note that although not statistically significant, when areas of white matter were analyzed together there were increased levels of Atg-4 and LC3 immunoreactivity and equal levels of GRP78 in comparison to that observed in gray matter. The finding of significant expression of autophagic markers in both white and gray matter is in keeping with structural deficits in young patients with T1DM (Wessels et al., 2007; Aye et al., 2011) and the white matter atrophy in the frontal and temporal regions in these DKA cases (Hoffman et al., 2010). The increase of autophagy in white matter is consistent with the leukoencephalopathy, and activation of RAGE in a long-term diabetic mouse model and the neuroradiologic white matter injury in humans with T1DM (Toth et al., 2006).

This study demonstrates that autophagy is increased in the brains of young T1DM patients with chronic poor metabolic control and increased oxidative stress. Further study is needed on the relationship between autophagy and the pathogenesis of early onset diabetic encephalopathy in T1DM.

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Fig. 1.

(A) Representative images of GRP78+ neurons in the hippocampus of DKA brains and control cases. Scale bar=20 μ m. (B) Percentage of GRP78+ immunostaining neurons in the indicated areas of DKA and control cases. Error bars indicate $\pm$ SD. (**) $P < 0.001$ (C) Quantification of GRP78 fluorescence intensity in the gray and white matter of DKA brains. Data represent average of $\pm$ SD of five chosen microscopic fields per section at 10 $\times$ magnification.

Fig. 2.

(A) Representative images of LC3+ neurons in the hippocampus of DKA brains and control cases. Scale bar=20 μ m. (B) Percentage of LC3+ immunostaining neurons in the indicated areas of DKA and control cases. Error bars indicate $\pm$ SD. (**) $P < 0.001$ (C) Quantification of LC3+ fluorescence intensity in the gray and white matter of DKA brains. Data represent average $\pm$ SD of five chosen microscopic fields per section at 10 $\times$ magnification.

Fig. 3.

(A) Representative images of Atg4+ neurons in the hippocampus of DKA brains and control cases. Scale bar=20 μ m. (B) Percentage of Atg4+ immunostaining neurons in the indicated areas of DKA and control cases. Error bars indicate $\pm$ SD. (**) $P < 0.001$ (C) Quantification of Atg4+ fluorescence intensity in the gray and white matter of DKA brains. Data represent average $\pm$ SD of five chosen microscopic fields per section at 10 $\times$ magnification.