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Kainic acid induces early and transient autophagic stress in mouse hippocampus

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Abstract

Kainic acid (KA) treatment is a well-established model of hippocampal neuron death mediated in large part by KA receptor-induced excitotoxicity. KA-induced, delayed neuron death has been shown previously to follow the induction of seizures and exhibit characteristics of both apoptosis and necrosis. Growing evidence supports a role of autophagic stress-induced death of neurons in several *in vitro* and *in vivo* models of neuron death and neurodegeneration. However, whether autophagic stress also plays a role in KA-induced excitotoxicity has not been previously investigated. To examine whether KA alters the levels of proteins associated with or known to regulate the formation of autophagic vacuoles, we isolated hippocampal extracts from control mice and in mice following 2–16 h KA injection. KA induced a significant increase in the amount of LC3-II, a specific marker of autophagic vacuoles, at 4–6 h following KA, which indicates a transient induction of autophagic stress. Levels of autophagy-associated proteins including ATG5 (conjugated to ATG12), ATG6 and ATG7 did not change significantly after treatment with KA. However, ratios of phospho-mTOR/mTOR were elevated from 6 to 16 h, and ratios of phospho-Akt/Akt were elevated at 16 h following KA treatment, suggesting a potential negative feedback loop to inhibit further stimulation of autophagic stress. Together these data indicate the transient induction of autophagic stress by KA which may serve to regulate excitotoxic death in mouse hippocampus.

Keywords: Kainic acid; Hippocampus; Autophagy; ATG5; ATG7; Akt; mTOR; LC3

The excitatory amino acid neurotransmitter glutamate is known to play an important role in a vast array of neuronal activities as well as in the induction of excitotoxic neurodegeneration through massive activation of its receptors [18,19]. Kainic acid (KA) is a potent glutamate receptor agonist with selectivity towards non-*N*-methyl-D-aspartate (NMDA)-type glutamate receptors [20,8]. KA is well known for its ability to induce seizures within minutes of its administration and is followed by a delayed excitotoxic neuron death in the hippocampus several hours later, in part through an increase in intracellular calcium and activation of calcium-dependent neuron death pathways [1,15,31]. Both apoptotic and necrotic death of neurons are associated with KA-induced excitotoxicity *in vivo* [30,11], suggesting the existence of multiple death pathways induced by glutamate receptor neurotoxicity.

Autophagic stress results from alterations in autophagy, a lysosomal degradation pathway that is responsible for the homeostatically regulated turnover of macronutrients and organelles [4]. Macroautophagy, the most prominent form of autophagy in cells, occurs via the formation of double-membraned autophagic vacuoles (AVs) that sequester and shuttle damaged organelles and macronutritents to lysosomes for degradation by acidic lysosomal hydrolases [32]. Our laboratory and others have shown that autophagic stress leads to an accumulation of AVs that occurs either from an induction of their nascent formation, or from an inhibition of their recycling due to a dysfunction of acidic organelles [16,3,21,24]. If left unchecked, autophagic stress can lead to autophagic cell death [16,3,21,24], which has been shown morphologically to possess elements of both apoptosis and necrosis [6]. Recent reports have implicated a role for the induction of autophagic stress in glutamate

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receptor-mediated excitotoxicity of motor neurons [17,29]. Although multiple types of cell death have been delineated previously in excitotoxic neuron death, the contribution of autophagic stress in models of glutamate receptor-induced excitotoxicity has not been previously investigated.

The stimulation, assembly and recycling of AVs in macroautophagy is exquisitely regulated by a large group of proteins (ATGs) isolated originally in yeast [32]. The importance of ATG proteins in macroautophagy is emphasized by studies of their genetic manipulation, such that over-expression of ATG5, ATG6 (Beclin) and ATG7 induce macroautophagy [13,9,25,27,26] and their targeted deletion (or haploinsufficiency in the case of Beclin) inhibits macroautophagy [33,10,14]. Deficiencies in ATG5 and ATG7 in particular have been shown to induce neurodegeneration in mice [10,14], which highlights the importance of autophagy-associated proteins in maintaining neuron survival. In addition, the induction of macroautophagy has been shown to be regulated by different classes of phosphatidylinositol 3-kinase (PI3-K). Class I PI3-K activates pro-survival, Akt-mediated signaling and has been shown to inhibit macroautophagy, whereas the activation of class III PI3-K has been shown to stimulate macroautophagy [23].

All mice were cared for in accordance with the guidelines of the NIH Guide for the Care and Use of Laboratory Animals. All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham. Mice with 129SvJ × C57BL/6J background were used in all experiments. The experiments were repeated with multiple litters ($n = 3$ for each time point). Mice were injected with KA (20 mg/kg i.p.) as previously reported [34] and were euthanized from 2 to 16 h following KA administration with subsequent removal of their hippocampi and freezing on dry ice. Sham (0 h) mice not receiving KA served as controls for each litter tested. Hippocampi were homogenized in lysis buffer containing 25 mM HEPES, 5 mM EDTA, 5 mM $MgCl_2$, 1% SDS, 1% Triton X-100, 1 mM PMSF, 1% protease inhibitor cocktail and 1% phosphatase inhibitor cocktail (Sigma). Twenty-five micrograms of protein per lane were resolved via SDS-PAGE and transferred to PVDF. Blots were blocked for 1 h, RT (5% milk), followed by overnight incubation at 4 °C with primary antibody (goat anti-ATG-5 (Santa Cruz); goat anti-ATG-7 (Santa Cruz); rabbit anti-beclin (Santa Cruz); rabbit anti-phospho-mTOR (Ser 2448) and rabbit anti-mTOR (Cell Signaling); rabbit anti-phospho-Akt (Ser 473) and rabbit anti-total Akt (Cell Signaling); and rabbit anti-LC3 (Uchiyama laboratory). All blots were also probed for rabbit anti-β-tubulin (Santa Cruz), which served as a loading control. Blots were washed with 1× TBS containing 0.1% Tween 20, then incubated with secondary antibody (goat anti-rabbit IgG, 1 h, RT) and washed. Signal was detected using Supersignal chemiluminescence (Pierce). Blots were scanned for densitometric analysis using UN-Scan-IT software (Orem, Utah). Blots for phospho-specific proteins (Akt and mTOR) were stripped subsequently using ReStore® Western Blot stripping buffer (Pierce) then re-probed for total Akt and mTOR. Protein levels from hippocampi of KA-treated mice (2–16 h) were normalized to control levels (0 h) in each western blot experiment, with three different western blot experiments

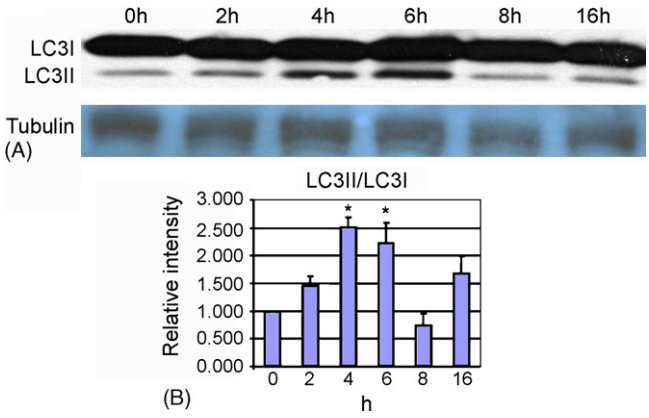

Fig. 1. LC3II/I ratio in the hippocampus is significantly increased by greater than two-fold at 4–6 h post-KA treatment, indicating an increased accumulation of AVs. Equal amounts of total hippocampal protein was loaded and detected by western blot (A) followed by scanning and densitometric analysis of the bands. Graphical representation (B) of the ratio of LC3-II relative to LC3-I (mean ± S.E.M.). $^{*}p < 0.05$ compared to 0 h sham control by one factor ANOVA with Tukey's multiple comparison post hoc test.

representing three separate time courses. Levels of protein were analyzed for significance over time via one factor ANOVA and Tukey's multiple comparison post hoc test. In all cases, a level of $p < 0.05$ was considered significant.

We began our study by assessing the effects of KA on LC3-II, the mammalian homologue of ATG8 and a selective biochemical marker for AVs [12]. LC3-II is the cleaved and lipidated form of the cytosolic LC3-I, and these post-translational modifications allow it to insert into the outer membrane of AVs [28]. Significant increases in the ratio of LC3-II/LC3-I were specific for 4 h (2.5-fold increase) and 6 h (2.2-fold increase) following KA administration (Fig. 1). These data indicate the transient induction of autophagic stress resulting from acute KA administration.

Levels of ATG5, ATG7 and Beclin were also measured following acute KA administration, as these proteins are known to be essential for the induction of autophagy [33,10,14]. While slight increases in ATG5, ATG6 and ATG7 were observed following KA administration, they were not found to be statistically significant (Fig. 2). These data suggest that a change in steady-state protein levels of ATG proteins is not necessarily required for the transient induction of autophagic stress. In addition, the Beclin-independent induction of autophagic stress has been documented recently in SH-SY5Y cells treated with the dopaminergic neurotoxin MPP^{+} [5], which suggests that Beclin may not be always required for the induction of autophagic stress through the formation of new vacuoles. Conversely, the transient induction of autophagic stress by KA may result from impairment of lysosomal degradation pathways, which by definition does not involve the ATG5, ATG6 or ATG7-dependent formation of new vacuoles.

Activation of class I PI3-K is known to negatively regulate the induction of autophagy [23]. To determine whether the transient induction of autophagic stress by KA was associated with changes in PI3-K-related signaling molecules, the phosphorylation of mTOR and Akt was measured (Fig. 3).

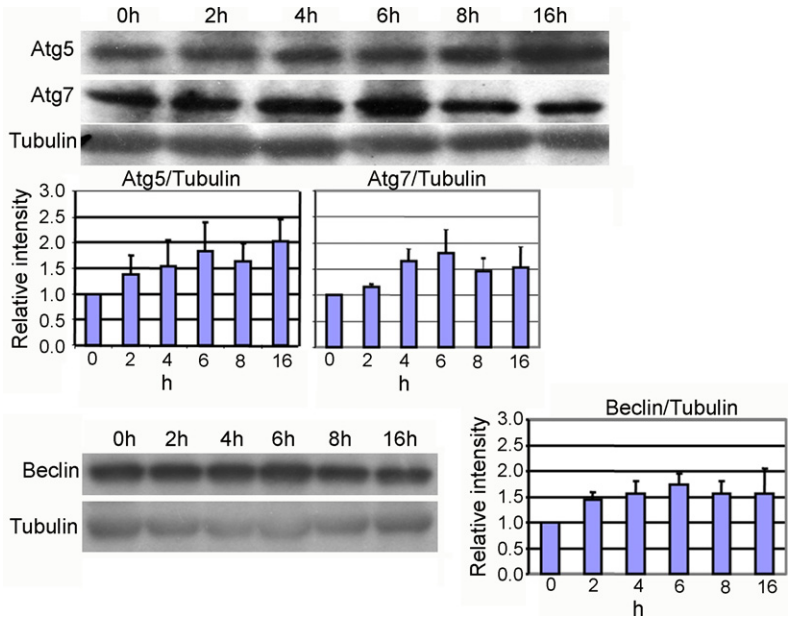

Fig. 2. Levels of ATG5 (conjugated to ATG12), ATG7 and Beclin were not significantly altered post-KA treatment. Equal amounts of total hippocampal protein was loaded and detected by western blot (A) followed by scanning and densitometric analysis of the bands. Graphical representation (B) of each band, expressed relative to levels of β-tubulin (mean ± S.E.M.).

The ratio of phospho-mTOR/mTOR increased significantly by 2–3-fold from 6 to 16 h following KA treatment, and a significant, 2.8-fold increase in the ratio of phospho-Akt/Akt was observed at 16 h following KA treatment. The KA-induced activation of Akt and mTOR may result from a stress response in the population of neurons surviving KA treatment. In addition, the KA induced increase in activation of Akt and mTOR may serve to negatively regulate the induction of autophagy, as evidenced by the decrease in the ratio of LC3-II/I by 8–16 h after KA treatment. Previous studies in our laboratory have found a KA-induced, c-fos-regulated increase in BDNF [7], which may induce the activation of Akt [2]. A previous study of intrastriatal KA administration in the rat showed a transient decrease in Akt phosphorylation measured 24–48 h following KA [22], but differences in species, route of administration and time course may explain this apparent discrepancy with the results of our study.

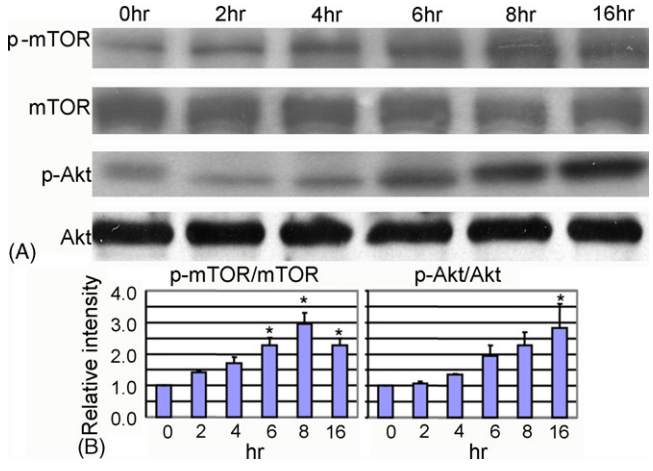

Fig. 3. Significant increases in the ratios of phospho-mTOR (2–3-fold, 6–16 h) and phospho-Akt/Akt (2.8-fold, 16 h) were observed post-KA treatment. Equal amounts of total hippocampal protein was loaded and detected by western blot (A) followed by scanning and densitometric analysis of the bands. Graphical representation (B) of the ratio of phospho-mTOR/mTOR or phospho-Akt/Akt (mean ± S.E.M.). $^{*}p < 0.05$ compared to 0 h sham control by one factor ANOVA with Tukey's multiple comparison post hoc test.

The present study indicates the early and transient induction of autophagic stress in the mouse hippocampus following KA administration. The induction of autophagic stress by KA may be a stress response of neurons to increase the turnover of proteins and damaged mitochondria under conditions of low trophic support. Furthermore, the transient nature of autophagic stress observed in the present study may be due to the compensatory increase in activation of Akt and mTOR, molecules known to negatively regulate macroautophagy. The compensatory increase in pro-survival Akt signaling may thus serve an important role in regulating the induction of autophagic stress, which if left unchecked has been shown to cause cell death [16]. The transient increase in autophagic stress observed in the present study may result from a stress response of increased seizure-induced activity in the mouse and closely follows the temporal induction in seizures by KA, but occurs well before the advent of noticeable neuron death as reported previously by our laboratory [34]. Future studies are warranted to determine the mechanisms and consequences of autophagic stress induction in models of excitotoxic neurodegeneration, and whether its time course or extent can be altered with pharmacological manipulation or additional trophic support.

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