CHARLES E. SCHMIDT COLLEGE OF MEDICINE

Calpain Cleavage of GAD65 is Pathological and Impairs GABA Neurotransmission

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Low GABA is associated with a plethora of neurodegenerative diseases, a few of which are epilepsy, Parkinson's disease, Huntington's chorea etc. The GABA synthesizing enzyme, L-glutamic acid decarboxylase 65 (GAD65), is cleaved to form its truncated form (ΔGAD65). Previously, we showed by in vitro biochemical characterization that ΔGAD65 was 2-3 times more stable and stronger than the full length form (FLGAD65). The enzyme that caused cleavage was later identified as calpain. Calpain is known to cleave its substrates either under a physiological stimulus or upon a sustained pathological insult. However, the precise role of calpain cleavage of GAD65 is poorly understood. In this communication, we aimed to investigate the significance of GAD65 cleavage and understand its implications on GABA neurotransmission. Specifically, we addressed under what circumstances - physiological or pathological, is the formation of ΔGAD65 favored. We used diverse in vitro and in vivo methods employing techniques such as western blotting, immuno-precipitation, radioactive GAD activity assay, along with a rat model of epilepsy to study the implications of GAD65 cleavage. Our data indicate that ΔGAD65 progressively accumulates with increasing excitotoxic stimulus. After cleavage, the more active ΔGAD65 detaches from the synaptic vesicles, thereby resulting in reduced GABA synthesis. Efforts are underway for optimizing conditions to study calpain cleavage of GAD65 in a rat model of epilepsy. So far, our data suggest that calpain mediated cleavage of GAD65 is pathological and that it leads to decreased GABA synthesis at the synaptic vesicles that result in poor uptake causing local inhibitory circuit dysfunction.

CALPAIN CLEAVAGE OF GAD65 IS PATHOLOGICAL AND IMPAIRS GABA NEUROTRANSMISSION

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ABSTRACT

BACKGROUND: Low GABA levels are associated with a plethora of neurodegenerative diseases such as epilepsy, spasticity, Parkinson's disease, Huntington's disease etc. GAD65, the synthesizing enzyme of GABA is cleaved by calpain to release its truncated form. *In vitro* biochemical characterization revealed that truncated (GAD65 (ΔGAD65) was 2-3 times more stable and stronger than the full length form (FL GAD65).

OBJECTIVES: To investigate the significance of GAD65 cleavage and thereby understand its implications on GABA neurotransmission.

METHODS: Format: E17 rat embryo primary neuronal cultures, fresh adult rat brains, Ischemic rat model brains. Techniques: Western Blot, semi-quantitative RT-PCR, Cell based fluorescent assays, Co-immunoprecipitation, Radioactive GAD activity Assays and GABA Uptake Assays.

RESULTS: The cleaved GAD65 accumulates under increasing excitotoxic stimulus, delinked from SVs, results in reduced GABA production and uptake.

CONCLUSIONS: Our data suggest that GAD65 cleavage is pathological and that it leads to low levels of GABA synthesis that result in poor uptake and release at the synapse.

INTRODUCTION

The brain constantly strives to maintain a balance between the excitatory and inhibitory networks, whose key players are the neurotransmitters L-glutamic acid and γ-amino butyric acid (GABA), respectively. Too much excitation or two little inhibition could tip the balance and is linked to a plethora of neurodegenerative diseases such as epilepsy, spasticity, Parkinson's disease, Huntington's chorea etc (Wong et al. 2003). The decision in maintaining the state of equilibrium is controlled by the activity of the GABA synthesizing enzyme, L-glutamic acid decarboxylase (GAD) which utilizes L-glutamic acid as its substrate. Our studies focus on understanding the factors that govern the regulation of the GAD enzyme and thereby understand its implications on GABA neurotransmission.

BACKGROUND AND SIGNIFICANCE

GAD exists in two isoforms - GAD65 and GAD67, where 65 and 67 denote their respective molecular weights in KDa. GAD67, a cytosolic protein is constitutively active and is responsible for 90% of GABA in the brain which is used for non-neurotransmission purposes such as a trophic factor during synaptogenesis, differentiation, cell plasticity etc. On the other hand, GAD65 is concentrated in the nerve terminals, is transiently activated only when there is a demand to generate GABA solely for neurotransmission (Pinal & Tobin 1998). This notion is supported by our recent data that there is a functional coupling between synaptic vesicles (SV) associated GAD65 and vesicular GABA transporter (VGAT), and that, such an alignment in close proximity promotes and is necessary for efficient GABA synthesis and packaging into the SVs to be released subsequently on the onset of an imminent neuronal stimulus (Jin et al. 2003). This is also corroborated by an independent finding that GAD65$^{-/-}$ mice are more susceptible to seizures and exhibit increased anxiety (Kash et al. 1999).

Over the past few decades, dramatic progress has been made in understanding the regulation of the GAD enzyme at the transcriptional, translational and post-translational levels (Wei & Wu 2008). So far, with respect to post-translational modifications, the role of palmitoylation, phosphorylation and proteolytic cleavage of GADs have been addressed. Since GAD65 is directly involved in GABA neurotransmission, the work presented here revolves around it. GAD65 cleavage was particularly intriguing because it was reported that GAD65 was readily cleaved to release a stable truncated form in the presence of mild trypsin treatment during the course of protein purification both *in vitro* and *in vivo* (Christgau et al. 1992) (Wei et al. 2003). Interestingly, biochemical analysis of the truncated GAD65 (ΔGAD65) versus full length (FLGAD65) counterpart revealed that ΔGAD65 was 2-3 times stronger and more stable than FLGAD65 (Wei et al. 2003). The enzyme responsible for cleavage was later identified to be calpain (Wei et al. 2006). Calpain is a very important modulator of signaling pathways in the brain and is known to cleave its substrates, both under physiological as well as pathological conditions. However, the precise role of calpain cleavage of GAD65 was unclear.

HYPOTHESIS

The calpain cleavage of FLGAD65 to ΔGAD65 is pathological.

OBJECTIVES

- What is the purpose of cleavage of GAD65?
- Since ΔGAD65 is more active than FLGAD65, does GAD65 undergo cleavage to meet the sudden extra demand for GABA neurotransmission or is ΔGAD65 released from its site of action, the synaptic vesicles and hence results in the impairment of GABA neurotransmission?
- If the formation of ΔGAD65 is pathological, is it observed in a disease brain tissue such as a rat model of focal cerebral ischemia accomplished through Middle Cerebral Artery Occlusion (MCAO)?

RESULTS

1. Accumulation of ΔGAD65 with increase in excitotoxic stimulus.

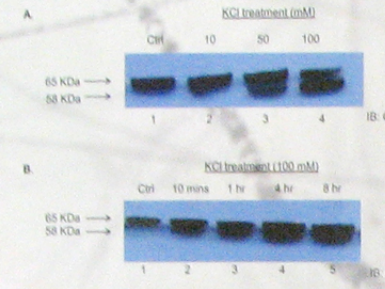


Fig.1.A. High K^+ stimulation of rat brain synaptosomes induces proteolytic cleavage of FL GAD65 in a dose dependent manner. Fig.1.B. E17 primary rat neuronal cell cultures at 11 DIV were exposed to 100 mM KCl for different time intervals as shown (lanes 2-5). Lane 1 represents the untreated control. Similar results were obtained when neuronal cultures were exposed to increasing concentrations of either glutamate or H_2O_2.

2. Assessment of total GAD activity in rat brain synaptosomes and cell viability in neuronal cells under high K^+ stimulation.

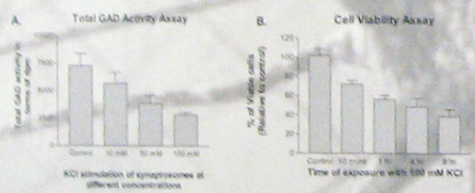


Fig.2.A. Total GAD activity in rat brain synaptosomes was measured under high K^+ stimulation. Fig.2.B. Cell viability assay as a measure of total ATP in metabolically active cells was measured in rat embryo primary neuronal cultures that were stimulated with high K^+.

On comparing Fig 1.A. with Fig.2.A and Fig.1B. with Fig.2.B, it is evident that conditions favoring accumulation of ΔGAD65 also caused either loss of total GAD activity or neuronal cell death, which are both pathological conditions.

3. Status of mRNA of both GAD65 and GAD67 under high K^+ stimulation.

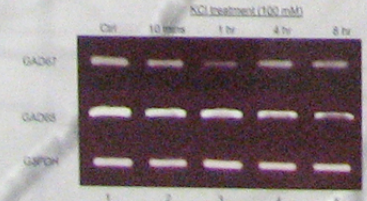


Fig.3. Status of mRNA, under conditions that caused the formation of ΔGAD65 were investigated. With increase in exposure to high K^+, the level of expression of GAD65 deteriorated unlike GAD67, whose levels were either constant or slightly up-regulated. Since GAD65 is directly involved in neurotransmission, any down-regulation of GAD65 mRNA could not compensate for the loss of GAD65 at the synapse.

4. Synaptic vesicle (SV) membrane bound GAD65 was released under high K^+ stimulation. The cleavage site of GAD65 on the SVs was not masked by protein-protein interactions but was amenable to attack by calpain.

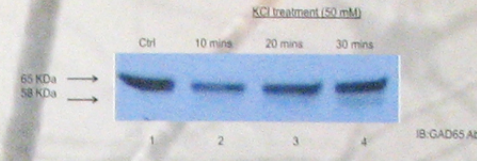


Fig.4. GAD65 is predominantly a synaptic vesicle (SV) membrane protein. Upon high K^+ stimulation *in vivo*, GAD65 on the SVs, which is the site of action for the synthesizing neurotransmitter GABA, was not masked by protein-protein interactions through interacting partners such as HSC70, CSP etc., but was amenable to attack by calpain.

5. Truncated GAD65 was no longer attached to synaptic vesicles but was released into the surrounding buffer. This indicates a loss of GAD65 at the synapse, since truncated GAD65 bears the active co-factor PLP binding site.

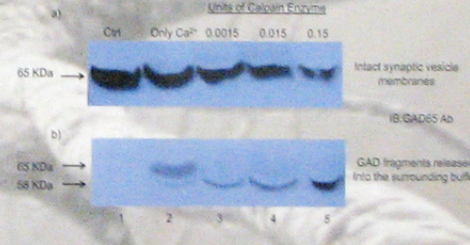


Fig. 5: Status of truncated GAD65 on intact synaptic vesicles that were subjected to *in vitro* calpain treatment. a) Washed intact synaptic vesicles b) Released GAD fragments collected in the surrounding buffer. As indicated, both lanes 1 and 2 in a) and b) served as controls. Lanes 3-5 in a) represent synaptic vesicles that were washed after *in vitro* calpain treatment. Lanes 3-5 in b) represent the GAD fragments released into the surrounding buffer.

6. GAD67 could not compensate for the loss of GAD65 on the SVs.

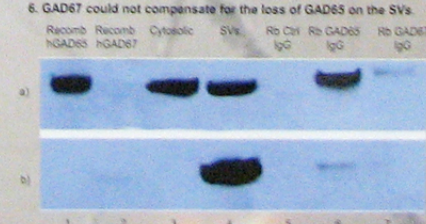
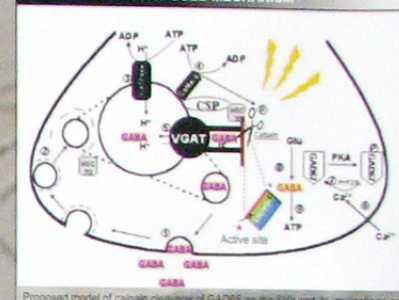


Fig 6: GAD65 and GAD67 interact at the synaptic vesicles. Since it is widely known that GABAergic neurotransmission is mainly carried out by the membrane associated GAD65, we were interested to observe whether GAD67 on the SVs had any role to play in GABAergic neurotransmission. It was first important to verify whether GAD67 was present on the SVs and whether it was interacting with GAD65 on the SVs. As indicated in the data, GAD65 and GAD67 interact at the synaptic vesicles. Using recombinant constructs, it was shown that GAD65 and GAD67 interact at the middle and the C terminus. However, this is the first line of evidence showing direct interaction of GAD65 and GAD67 using rat brain SVs. So, if cleavage occurs at the N terminus of GAD65, and is released into the cytosolic fraction as indicated in Fig. 5, then the interacting partner of GAD65, which is GAD67, is also released along with it and hence there is no compensation by GAD67 on the SVs.

PROPOSED MECHANISM



Proposed model of calpain cleavage of GAD65 on the SVs and its implications on GABA neurotransmission

REFERENCES

1.Christgau, S., Aanstoot, H. J., Schierbeck, H., Begley, K., Tullin, S., Hejnaes, K. and Baekkeskov, S. (1992) Membrane anchoring of the autoantigen GAD65 to microvesicles in pancreatic beta-cells by palmitoylation in the NH2-terminal domain. *J Cell Biol*, 118, 309-320.

2. Jin, H., Wu, H., Osterhaus, G., Wei, J., Davis, K., Sha, D., Floor, E., Hsu, C. C., Kopke, R. D. and Wu, J. Y. (2003) Demonstration of functional coupling between gamma -aminobutyric acid (GABA) synthesis and vesicular GABA transport into synaptic vesicles. *Proc Natl Acad Sci U S A*, 100, 4293-4298.

3. Kash, S. F., Tecott, L. H., Hodge, C. and Baekkeskov, S. (1999) Increased anxiety and altered responses to anxiolytics in mice deficient in the 65-kDa isoform of glutamic acid decarboxylase. *Proc Natl Acad Sci U S A*, 96, 1698-1703.

4.Pinal, C. S. and Tobin, A. J. (1998) Uniqueness and redundancy in GABA production. *Perspect Dev Neurobiol*, 5, 109-118.

5.Wei, J., Jin, Y., Wu, H., Sha, D. and Wu, J. Y. (2003) Identification and functional analysis of truncated human glutamic acid decarboxylase 65. *J Biomed Sci*, 10, 617-624.

6.Wei, J., Lin, C. H., Wu, H., Jin, Y., Lee, Y. H. and Wu, J. Y. (2006) Activity-dependent cleavage of brain glutamic acid decarboxylase 65 by calpain. *J Neurochem*, 98, 1669-1685.

7.Wei, J. and Wu, J. Y. (2008) Post-translational regulation of L-glutamic acid decarboxylase in the brain. *Neurochem Res*, 33, 1459-1466.

8.Wong, C. G., Bottiglieri, T. and Snead, O. C., 3rd (2003) GABA, gamma-hydroxybutyric acid, and neurological disease. *Ann Neurol*, 54 Suppl 6, S3-12.

CONCLUSIONS

- ΔGAD65 accumulates with increase in excitotoxic insult.
- The calpain cleavage site of GAD65 on the SV membrane is not masked by interacting partners, but is amenable to cleavage.
- The more active ΔGAD65 was not attached to SVs, but is released from its site of action, the SVs. The released part bears the active site of the enzyme.
- GAD65 and GAD67 interact on the SVs and the partnership falls off after cleavage. It is known that the partners interact at the middle and C terminus, and since the cleavage occurs at the N terminus, it leads to no compensation by GAD67 on SVs.

FUTURE DIRECTIONS

A rat model of focal cerebral ischemia is being accomplished by middle cerebral artery occlusion in the brain. We expect to observe ΔGAD65 in the brains of these models when compared to normal adult rat brains, upon synaptosomal lysis in the presence of protease inhibitors.

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