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ARAŞTIRMA / RESEARCH

The investigation of endoplasmic reticulum stress markers ATF5 and phosphorylated eIF2α after kainic acid treatment in neuroblastoma cells

Endoplazmik retikulum stres belirteçlerinden ATF5 ve fosforile IF2α düzeylerinin nöroblastoma hücrelerinde kainik asit muamelesi sonrası incelenmesi

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Abstract

Purpose: The aim of this study was to investigate the relationship between kainic acid induced excitotoxicity and endoplasmic resticulum (ER) stress by analyzing two major ER stress markers such as ATF5 and phosphorylated eIF2α in neuroblastoma cells.

Materials and Methods: Neuroblastoma cells were treated with 1 mM kainic acid for 24 hours. ATP measurement was performed in kainic acid-treated and vehicle-treated neuroblastoma cells via ATP bioluminescence assay. Total protein was isolated from kainic acid-treated and control cells. Via western blotting, the expression levels of ATF5 and phosphorylated eIF2α were analyzed.

Results: We showed for the first time that as a result of kainic acid treatment in neuroblastoma cells, the protein expression levels of ER stress markers ATF5 and phosphorylated eIF2α did not display any change when compared to control cells. We also showed that ATP levels were decreased in kainic acid-treated cells.

Conclusion: This study may show that the level of stress that kainic acid causes at 1 mM for 24 hours in neuroblastoma cells was not adequate to lead to ER stress which is measurable by ATF5 and phosphorylated eIF2α. Either an increased level of treatment of kainic acid via increased duration or concentration is necessary or different markers should be tried. The investigation of the ER stress pathways in the excitotoxicity-related brain diseases will pave the way for new therapies based on ER stress and combat more than one disease simultaneously.

Keywords:. endoplasmic reticulum, stress, ATF5, eIF2

Öz

Amaç: Bu çalışmada, amacımız, kainik asite bağlı eksitotoksisite ve endoplazmik retikulum (ER) stres arasındaki ilişkiyi iki majör endoplazmik retikulum stres markırı olan ATF5 ve fosforile olmuş eIF2a analiz ederek incelemektir.

Gereç ve Yöntem: Neuroblastoma hücrelerini 1 mM kainik asit ile 24 saat muamele ettik. ATP ölçümü, kainik asit muamelesi yapılan hücrelerde ya da kontrol hücrelerinde bioluminesans bir yöntem ile yapılmıştır. Total protein, kainik asit muamelesi yapılmış ya da kontrol hücrelerinden izole edilmiş ve ATF5 ve fosforile olmuş eIF2α markırları western blot ile incelenmiştir.

Bulgular: Kainik asit ile muamele edilmiş neuroblastoma hücrelerinde ATF5 ve fosforile olmuş eIF2a seviyelerinin kontrole göre değişmediğini ilk kez gösterdik. Kainik asit ile muamele edilmiş hücrelerde ATP seviyesinin düştüğünü gösterdik.

Sonuç: 1 mM ve 24 saat süren kainik asit muamelesi, ATF5 ve fosforile olmuş eIF2α ile gösterilebilecek endoplazmik retikulum stresi yaratmak için yeterli olmayabilir. Süre ve konsantrasyon olarak arttırılmış kainik asit muamelesi ya da farklı markırlar gerekmektedir. Eksitotoksisiteye bağlı beyin hastalıklarında ER stres yolaklarını araştırmak, yeni tedavi yolları bulmak ve birden fazla hastalığı aynı anda engellemek adına önemli olacaktır.

Anahtar kelimeler: endoplasmic reticulum, stress, ATF5, eIF2

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INTRODUCTION

The endoplasmic reticulum (ER) is the main compartment of secretory pathways in the cell[1]. The two types of ER are known as the smooth and rough ER where rough ER contains ribosomes. There fore ER is not only responsible from the folding and secretion of proteins but also from the production of proteins. The quality control of proteins, lipid synthesis and Ca^{+2} homeostasis are the other major functions of ER. As a result of ER stress, Unfolded Protein Response (UPR) is induced, which is one of the major signaling pathways[2]. UPR initially restores ER homeostasis and normalizes cellular functions. However, if this is not achieved, apoptosis is induced. The UPR is controlled by three major stress markers inositol requiring enzyme 1 (IRE1)[3], protein kinase RNA-activated (PKR)-like ER kinase (PERK) and activating transcription factor 6 (ATF6)[4].

The integrated stress response (ISR) is a detailed signaling pathway in eukaryotic cells activated in response to different physiological or pathological conditions such as hypoxia, amino acid deprivation, glucose deprivation, and viral infection[5]. The main sensor for ISR is eIF2α phosphorylation, which leads to the attenuation of translation.

Activating transcription factor-5 (ATF5) is a transcription factor induced upon extracellular stressors such as fasting, amino-acid limitation, cadmium or arsenite[6]. ATF5 is also a neuroprotective stress-response transcription factor promoting transcription of anti-apoptotic target genes like MCL[1].

Excitotoxicity is induced when the excess glutamate remains in the synaptic cleft of neurons[7]. It is one of the major molecular pathways underlying almost all brain diseases including neurodegeneration, epilepsy and traumas[7]. There are several reasons for excessive glutamate accumulation. Firstly, secreted glutamate might not be taken up by glutamate transporters expressed on astrocytes[8]. Secondly, the amount of glutamate released from the glutamatergic neurons might be more than normal levels. The majority of the excess glutamate is absorbed by GLT-1 (Glutamate Transporter 1) expressed on astrocytes[9]. Absorbed glutamate either enters TCA cycle via glutamate dehydrogenase (GDH) or converted to glutamine by Glutamine Synthetase (GS)[10]. After being converted to glutamine, it is metabolized by glutaminase to glutamate in glutamatergic neurons[11,12].

Kainic acid is a potent toxin leading to excitotoxicity in cellular and animal models[13] and leads to glutamate accumulation in the synaptic cleft. Kainic acid-induced excitotoxicity definitely causes a stress in the cells. Exogenous stress leads to ER stress and the induction of UPR pathways[14].

In this study, we wanted to investigate the relationship between kainic acid induced excitotoxicity and ER stress by analyzing two major ER stress markers such as ATF5 and phosphorylated eIF2α in neuroblastoma cells. We for the first time showed that as a result of kainic acid treatment in neuroblastoma cells, the protein expression levels of ER stress markers ATF5 and phosphorylated eIF2α did not display any change when compared to control cells. This study may show that the level of stress that kainic acid causes at 1 mM for 24 hours in neuroblastoma cells was not adequate to lead to ER stress which is measurable by ATF5 and phosphorylated eIF2α levels. Either an increased level of treatment by kainic acid is necessary or different markers should be tried. However, we showed that ATP levels were decreased in kainic acid treated cells. The investigation of the ER stress pathways in an excitotoxic cellular model will lead to a better understanding of the relation between ER and excitotoxicity and enlighten our knowledge about excitotoxicity related brain diseases.

MATERIALS AND METHODS

Cell culture

Neuroblastoma (N2A) cells were obtained from ECACC. The N2A cells were maintained in MEM with %10 FBS, 100 µg/ml penisilin/streptomisin, 2 mM L-glutamin at 37 °C incubators with %5 CO_2.

Kainic acid treatment

Kainic acid was dissolved in H_2O. Control represents vehicle-treated cells. N2A cells were treated with 1 mM kainic acid for 24 hours.

ATP measurement

ATP levels of control cells and kainic acid treated (1 mM for 24 hours) N2A cells were measured by using an ATP bioluminescence assay kit according to the manufacturer's guidelines (Roche Diagnostics). ATP levels are represented as Relative Luminescence Units

(RLU) according to the assay kit. 4 measurements were averaged.

Western blotting

Protein was made from N2A whole cell extracts using RIPA Lysis buffer according to the manufacturer's guidelines (VWR). Protease and phosphatase inhibitors (MedChem Express-100X) were added. Cell extracts were loaded onto 4-15 % gradient SDS-PAGE gels (BioRad) after incubating with loading buffer (126 mM Tris HCl pH6.5, %25 glycerol, %5 SDS, ¾ beta-mercaptoetanol, %0.25 BPB (bromophenol blue) at 95˚C for 5 min. Proteins were immunoblotted with anti-ATF5 antibody (Elabscience) and anti-phosphorylated eIF2α antibody at 1:1000 concentration and with anti-tubulin antibody (Elabscience) at 1:5000 concentration. The membranes were then probed with horseradish peroxidase-conjugated secondary antibodies (Elabscience) and developed with enhanced chemiluminescence western blotting substrate (Thermo Scientific). Blots were exposed to HyBlot autoradiography film or visualized with detection system. The protein bands were quantified using the densitometry analysis in ImageJ software (NIH, Washington, DC, USA).

Statistical analysis

All the statistical analyses were performed using statistical software (GraphPad Software, Inc., San Diego, USA). Namely, "unpaired, one-tail, equal variance and two-sample t-test" was performed using Prism 5 software. Significant differences are shown by asterisks indicating *p<0.05. Error bars in figures represent standard error of the mean (SEM).

RESULTS

Kainic acid treatment leads to a decrease in ATP levels in N2A cells

To determine whether kainic acid induced excitototoxicity leads to a change in ATP levels, we measured the ATP levels in neuroblastoma cells after 1 mM kainic acid treatment for 24 hours and compared with vehicle-treated control cells. We observed that ATP levels were decreased after kainic acid treatment when compared to control cells (Figure 1). Therefore, we concluded that excitotoxicity may lead a dysfunction in mitochondria resulting in reduced ATP production.

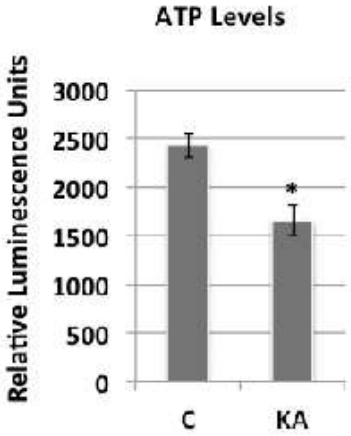

Figure 1. ATP levels in kainic-acid treated and vehicle-treated control neuroblastoma cells.

ATP levels were measured with ATP bioluminescence assay kit. The graph shows the average of four different measurements. Student's t-test was performed. The values significantly different from the relative controls are indicated with symbols. p*: 0.00486< 0.05. C: vehicle-treated cells KA: kainic acid-treated cells.

ATF5 and phosphorylated eIF2a levels do not change after kainic acid treatment in N2A cells

Since kainic acid causes a stress condition in the cell, we wondered whether endoplasmic reticulum stress is induced. Since ATP levels were decreased after 1 mM kainic acid treatment for 24 hours, we speculated that ER stress might be turned on. Therefore, we analyzed the protein levels of two ER stress markers via western blotting. Total protein was isolated from control N2A cells and also from cells treated with 1 mM kainic acid for 24 hours. There was no difference between the protein levels of ATF5 (Figure 2) or phosphorylated eIF2α (Figure 3) when control N2A cells and kainic acid treated N2A cells were compared. This result shows that ER stress is not induced. This study may show that the level of stress that kainic acid causes at 1 mM for 24 hours in neuroblastoma cells was not adequate to lead to ER stress which is measurable by ATF5 and phosphorylated eIF2α. Either an increased level of treatment by kainic acid is necessary or different markers should be tried.

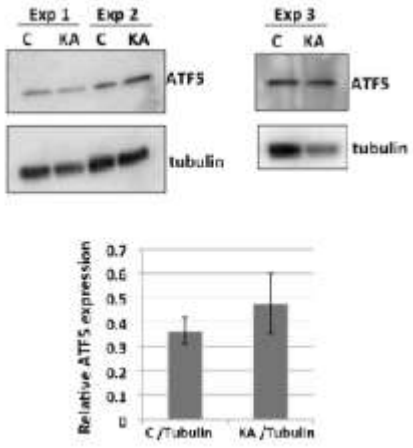

Figure 2. ATF5 protein levels do not show any difference between kainic acid-treated and control cells.

A western blot of vehicle-treated or kainic acid-treated N2A cell extracts show expression levels of ATF5, with tubulin as a loading control. Relative protein levels from the blot (the average of three independent experiments shown as Exp 1, Exp 2, Exp 3) are quantified below. Student's t-test was performed. p: 0.22433>0.05. No statistical significance was found. C: non-treated cells KA: kainic acid-treated cells.

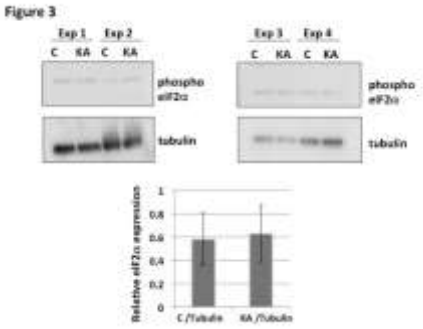

Figure 3. Phosphorylated eIF2 protein levels do not show any difference between kainic acid treated and control cells.

A western blot of vehicle-treated or kainic acid-treated N2A cell extracts show expression levels of phosphorylated eIF2, with tubulin as a loading control. Relative protein levels from the blot (the average of four independent experiments shown as Exp 1, Exp 2, Exp 3, Exp 4) are quantified below. Student's t-test was performed. p: 0.4452>0.05. No statistical significance was found. C: non-treated cells KA: kainic acid-treated cells.

DISCUSSION

In this study, we formed a kainic acid induced excitotoxicity model in neuroblastoma cells. Kainic acid is used as a model for status epilepticus in cells and animals since it leads to excessive glutamate accumulation in the synaptic cleft[15]. Status epilepticus (SE) is defined as more than two consecutive seizures without a return to baseline mental status or continuous seizure activity for more than 30 minutes[16].

We treated the cells for 24 hours with 1 mM kainic acid. We then measured the ATP levels and found a decrease in kainic acid treated neuroblastoma cells when compared to vehicle treated control cells. Excitotoxic cells might have dysfunctional mitochondria leading to reduced ATP levels. Under normal conditions, the glutamate absorbed by the glutamate transporters on astrocytes is converted to α-ketoglutarate by glutamate dehydrogenase and enters into TCA cycle resulting in ATP synthesis. Therefore, an increase in ATP levels might occur. However, excitotoxicity might have also caused dysfunction in the cell metabolism and mitochondria causing a reduction in ATP synthesis.

We then wanted to investigate the endoplasmic reticulum stress induced by kainic acid in neuroblastoma cells. After treating the cells with kainic acid, we isolated total proteins and checked the protein expression levels of ATF5 and phosphorylated eIF2α, which are two endoplasmic reticulum stress markers. We did not find any difference between the expression levels of these two ER stress markers when kainic acid treated cells were compared with control cells. This result shows that the level of stress that kainic acid causes at 1 mM for 24 hours in neuroblastoma cells was not adequate to lead to ER stress which is measurable by ATF5 and phosphorylated eIF2α levels. A higher concentration or a longer duration of treatment may be necessary. Alternatively, different ER stress markers may be tried such as IRE1, PERK and ATF6. The expression levels of these markers may change after kainic acid treatment as an indication of ER stress. Although not significant, ATF5 protein levels have a tendency to increase after kainic acid treatment. Therefore, as mentioned above, a longer treatment or a higher concentration of kainic acid treatment might lead to an increase in ATF5 levels.

In the literature, the link between ER stress and excitotoxicity has been shown[17]. The inhibition of ER stress was shown to protect against excitotoxic neuronal injury in the rat brain[17]. ER stress was demonstrated to be induced by kainic acid treatment. ER stress inhibitor 4-phenylbutyric acid (PBA) decreases ER stress-mediated apoptosis and mitochondrial dysfunction[18]. A study demonstrated that inhibition of ER stress attenuates mitochondrial apoptosis and controls parkin-mediated neuronal death after kainic acid treatment and seizures[19]. The death of neurons after kainic acid treatment is mostly caused by apoptosis induced by mitochondria[20]. ER stress also leads to mitochondrial dysfunction. For this reason, in this study we wanted to investigate the ATP levels in the cells, which is the best indicator for mitochondrial function.

In future studies, kainic acid model of excitotoxicity might be applied to animals such as mice and then molecular pathways regarding ER stress might be studied in animal models. Kainic acid has been used successfully in mice and rats for some time now with some limitations and potentials[21]. The findings of the cell culture models can be further studied and confirmed in animals, which are administered with kainic acid, to explore ER stress and excitotoxicity.

The dysfunction of the ER stress response leads to complex diseases such as cancer, metabolic syndrome and neurodegeneration[22]. Therefore, any molecular pathway investigated, which is related to ER stress will pave the way for the development of therapeutics regarding these diseases. Since excitotoxicity is one of the major molecular pathways underlying the mechanisms of neurodegeneration, exploring ER stress related pathways in the context of excitotoxicity, will open new avenues for the understanding of brain diseases and the drug development to combat these diseases.

Yazar Katkıları: Çalışma konsepti/Tasarımı: GDY; Veri toplama: GDY, AS; Veri analizi ve yorumlama: GDY; Yazı taslağı:GDY; İçeriğin eleştirel incelenmesi: GDY, AS; Son onay ve sorumluluk: GDY, AS; Teknik ve malzeme desteği: GDY, AS; Süpervizyon: GDY; Fon sağlama (mevcut ise): yok.
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REFERENCES

1. Oakes SA, Papa FR. the role of endoplasmic reticulum stress in human pathology. Annu Rev Pathology. 2015;10:173-94.
2. Hetz C. The unfolded protein response: controlling cell fate decisions under ER stress and beyond. Nature Rev Mol Cell Biol. 2012;13:89-102.
3. Adams CJ, Kopp MC, Larburu N, Nowak PR, Ali MMU. Structure and molecular mechanism of ER stress signaling by the unfolded protein response signal activator IRE1. Front Mol Biosci. 2019;6:11.
4. Liu CY, Kaufman RJ. The unfolded protein response. J Cell Sci. 2003;116:1861-2.
5. Pakos-Zebrucka K, Koryga I, Mnich K, Ljujic M, Samali A, Gorman AM. The integrated stress response. EMBO Rep. 2016;17:1374–95.
6. Torres-Peraza JF, Engel T, Martin-Ibanez R, Sanz-Rodriguez A, Fernandez-Fernandez MR, Esgleas M, et al. Protective neuronal induction of ATF5 in endoplasmic reticulum stress induced by status epilepticus. Brain. 2013:136;1161-76.
7. Dong X, Wang Y, Qin Z. Molecular mechanisms of excitotoxicity and their relevance to pathogenesis of neurodegenerative diseases. Acta Pharmacol Sin. 2009;30:379-87.
8. Zhou Y, Danbolt NC. Glutamate as a neurotransmitter in the healthy brain. J Neural Transm. 2014;121:799-817.
9. Olivares-Bañuelos TN, Chí-Castañeda D, Ortega A. Glutamate transporters: Gene expression regulation and signaling properties. Neuropharmacology. 2019;161:107550.
10. Karaca M, Frigerio F, Maechler P. From pancreatic islets to central nervous system, the importance of glutamate dehydrogenase for the control of energy homeostasis. Neurochem Int. 2011;59:510-7.
11. Danbolt NC. Glutamate uptake. Prog Neurobiol. 2001;65:1-105.
12. Lin CLG, Kong Q, Cuny GD, and Glicksman MA. Glutamate transporter EAAT2: a new target for the treatment of neurodegenerative diseases. Future Med Chem. 2012;4:1689-700.
13. Mohd Sairazi NS, Sirajudeen KNS, Asari MA, Muzaimi M Mummedy S, Sulaiman SA. Kainic acid-induced excitotoxicity experimental model: protective merits of natural products and plant extracts. Evid Based Complement Alternat Med. 2015;2015:972623.
14. Verdaguer E, Garcia-Jorda E, Jimenez A, Stranges A, Sureda FX, Canudas AM et al. Kainic acid-induced neuronal cell death in cerebellar granule cells is not prevented by caspase inhibitors. Br J Pharmacol. 2002;135:1297-307.

15. Vermoesen K, Smolders I, Massie A, Michotte Y, Clinckers R. The control of kainic acid-induced status epilepticus. Epilepsy Res. 2010;1-2:164-6.
16. Cherian A, Thomas SV. Status epilepticus. Ann Indian Acad Neurol. 2009;12:140–53.
17. Sokka AL, Putkonen N, Mudo G, Pryazhnikov E, Reijonen S, Khiroug L et al Endoplasmic reticulum stress inhibition protects against excitotoxic neuronal injury in the rat brain. J Neurosci. 2007;27:901-8.
18. Xue F, Shi C, Chen Q, Hang W, Xia L, Wu Y et al. Melatonin mediates protective effects against kainic acid-induced neuronal death through safeguarding er stress and mitochondrial disturbance. Front Mol Neurosci. 2017;10:49.
19. Kim JS, Heo RW, Kim H, Yi CO, Shin HJ, Han JW et al. Salubrinal, ER stress inhibitor, attenuates kainic acid-induced hippocampal cell death. J Neural Transm. 2014;121:1233.
20. Zhang XM, Zhu J. Kainic acid-induced neurotoxicity: targeting glial responses and glia-derived cytokines. Curr Neuropharmacol. 2011;9:388–98.
21. Zheng X, Zhang HL, Luo Q, Zhu J. Kainic acid-induced neurodegenerative model: potentials and limitations. J Biomed and Biotech. 2010;2011:457079.
22. Yoshida H. ER stress and diseases. FEBS J. 2007;274:630-58.