



Suberoylanilide Hydroxamic Acid (SAHA) Reduces Glutamate-Induced Oxidative Stress in Hippocampal Cells

Suberoilanilid Hidroksamik Asit (SAHA) Hipokampal Hücrelerde Glutamata Bağlı Oksidatif Stresi Azaltır

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Abstract

Introduction: Glutamate is an essential excitatory neurotransmitter in the brain, causing neuronal cell loss by overactivation in high concentrations. Epigenetic mechanisms, such as histone modifications, alter gene expression for maintaining cellular balance and are activated during sustained neuroinflammation and oxidative stress. Suberoylanilide hydroxamic acid (SAHA) is a well-known histone deacetylase inhibitor for its antitumor and anti-inflammatory properties. Therefore, in this study, we aimed to investigate the neuroprotective effect of SAHA against glutamate-induced oxidative stress in HT-22 hippocampal cells.

Materials and Methods: The HT-22 hippocampal neuronal cells were cultured in DMEM medium with 10% FBS and 1% penicillin/streptomycin and incubated at 37°C in a humidified atmosphere containing 5% CO₂. Cell viability was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay after administration of glutamate and SAHA to HT-22 cells, and then the absorbance levels were measured at 550 nm using a microplate reader. The neuroprotective effect of SAHA was evaluated by measuring the oxidative stress parameters like reduced glutathione (GSH) level, and antioxidant enzyme activities of glutathione reductase (GR) and glutathione peroxidase (GPx) by Enzyme-like immunosorbent assay (ELISA).

Results: SAHA has reduced glutamate-induced neuron death in HT-22 cells. Moreover, SAHA alleviated glutamate-induced oxidative stress by increasing GSH levels, and the activities of the antioxidant enzymes GR and GPx.

Conclusion: These results demonstrated that SAHA has antioxidant activity, reduces glutamate-induced oxidative stress, and confers protection against glutamate-induced neuronal death.

Keywords: Oxidative stress; glutamic acid; neurons; neuroprotection.

Özet

Giriş ve Amaç: Glutamat beyinde önemli bir uyarıcı nörotransmitterdir ve yüksek konsantrasyonlarda aşırı aktivasyonla nöronal hücre kaybına neden olur. Histon modifikasyonları gibi epigenetik mekanizmalar, hücresel dengeyi korumak için gen ekspresyonunu değiştirir ve kronik nöroinflamasyon ve oksidatif stres sırasında aktive edilir. Suberoilanilide hidroksamik asit (SAHA), antitümör ve antiinflamatuar özellikleriyle iyi bilinen bir histon deasetilaz inhibitörüdür. Bu nedenle, bu çalışmadaki amacımız, SAHA'nın HT-22 hipokampal hücrelerinde glutamata bağlı oksidatif strese karşı nöroprotektif etkisini araştırmaktır.

Gereç ve Yöntem: HT-22 hipokampal nöronal hücreler %10 FBS ve %1 penisilin/streptomisin içeren DMEM besiyeri içinde kültüre alındı ve %5 CO₂ içeren nemli atmosferde 37°C'de inkübe edildi. Hücre canlılığı, HT-22 hücrelerine glutamat ve SAHA uygulandıktan sonra MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) testi ile belirlendi ve sonra absorbans değerleri bir mikropilaka okuyucuda 550 nm'de ölçüldü. SAHA'nın nöroprotektif etkisi, ELISA (Enzyme-like immunosorbent assay) yöntemiyle, oksidatif stres parametreleri olan redukte glutatyon (GSH) seviyesi ve glutatyon redüktaz (GR) ve glutatyon peroksit (GPx) antioksidan enzim aktiviteleri ölçülerek değerlendirildi.

Bulgular: SAHA, HT-22 hücrelerinde glutamatın neden olduğu nöron ölümünü azaltmıştır. Ayrıca, SAHA, GSH seviyelerini ve antioksidan enzimler olan GR ve GPx'in aktivitelerini artırarak glutamatla indüklenen oksidatif stresi azalttı.

Sonuç: Bu sonuçlar, SAHA'nın antioksidan aktiviteye sahip olduğunu, glutamatla indüklenen oksidatif stresi azalttığını ve glutamat kaynaklı nöronal ölüme karşı koruma sağladığını göstermiştir.

Anahtar Kelimeler: Oksidatif stress; glutamik asit; nöron; nöroproteksiyon.

Introduction

Neurodegenerative diseases are delineated by harm and loss of neuronal cells, leading to impaired motor and cognitive functions. The pathophysiology of neurodegenerative diseases has not yet been fully explained, but it has been suggested that oxidative stress may have a role in these diseases (1). In neurodegenerative diseases,

preclinical and clinical studies have shown that oxidative stress biomarkers are high, and antioxidant defense biomarkers are at low levels in the brain (2). Oxidative stress is triggered by free radicals produced in exogenous or endogenous processes. The production of reactive oxygen species (ROS) can also occur as a side effect of aerobic respiration in the organism. Due to its

high lipid content and oxygen using up, brain tissue is more prone to ROS damage (3, 4). Antioxidant systems consist of enzymes (such as catalase, glutathione reductase, peroxidase, and superoxide dismutase) and non-enzymatic components (like glutathione, vitamins A, E, and C) that are available to protect organisms from ROS's harmful effects. These systems are responsible for maintaining the redox balance in oxidative stress (5). It is crucial to maintain the balance between stimulating and inhibitory neuronal connections so that the central nervous system (CNS) can maintain normal functioning. Glutamate is one of the key neurotransmitters in the CNS of mammals, and the greater part of excitatory signals is mediated by glutamate (6). Glutamate receptors are distributed in almost all neuronal cell types. Glutamate-induced oxidative stress and toxicology have been demonstrated in primary neuronal cultures, neuronal cell lines, and oligodendrocytes. This oxidative stress is thought to contribute to many neurodegenerative diseases (7). Therefore, extracellular glutamate levels in the physiological range are crucial for the survival and function of neurons. Impairment of glutamate homeostasis causes neurological and neurodegenerative diseases such as Parkinson's and Alzheimer's disease (8). The development of histone deacetylase inhibitors (HDACi) has increased the interest in epigenetic pharmacology. Inhibition of HDAC can transform the cellular acetylation balance in favor of hyperacetylation, affecting gene expression profiles. HDACi have recently been used clinically in many neoplastic diseases (9). It has been confirmed that suberoylanilide hydroxamic acid (SAHA), a histone deacetylase pan-inhibitor, has notable anticancer activity on various tumors (10). Moreover, evidence from clinical studies has shown that HDACi can be a potential treatment option for CNS diseases (11). Evidence from experimental studies demonstrated that prevention of HDACs have a remarkably effect on immune cell activation, which may be the underlying cause of the neuroprotective effect of HDACi. However, the mechanism of immunosuppression caused by HDACi is not fully known (12). HT-22 cells are mouse hippocampal cells and often used for studies on the mechanism of neurotoxicity caused by oxidative stress as an *in vitro* model (13). In our study, we aimed to investigate the neuroprotective effect of SAHA in glutamate-treated HT-22 cells.

Materials and Methods

Reagents: Fetal bovine serum albumin (FBS), penicillin/streptomycin, Trypsin-EDTA (0.25% and 0.0025%), Dulbecco's Modified Eagle medium (DMEM), L-glutamine, and Dulbecco's Phosphate Buffered Saline (dPBS) were purchased from Gibco BRL (Grand Island, NY, USA). Suberoylanilide hydroxamic acid (SAHA) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture: The HT-22 hippocampal neuronal cell line was derived from the mouse hippocampus, and HT-22 cells were used to investigate glutamate-induced neurotoxicity. HT-22 cells were cultured in DMEM with 10% FBS and 1% penicillin/streptomycin and incubated at 37°C in a humidified atmosphere containing 5% CO₂.

Cell viability measurement: MTT test was conducted to measure cellular viability. HT-22 cells were seeded at the 96-well plates at a density of 10⁴ cells per well and then incubated overnight at 37°C humidified atmosphere containing 5% CO₂. Subsequently, cells were treated with glutamate (5mM), SAHA (0.25, 0.50, 0.75, and 1 μM), and α-tocopherol (50 μM) as a positive control. Following 24-hour SAHA and glutamate treatment, mediums were aspirated, and 100 μl of MTT solution (5 mg/mL) was added to the wells and incubated at 37°C for 4-hour in the dark. After incubation, mediums were discarded, and 100 μl DMSO was used for dissolving formazan crystals. The absorbance levels were measured at 550 nm using a microplate reader (TECAN, Switzerland).

Measurement of glutathione levels and glutathione reductase and glutathione peroxidase enzyme activities: Hippocampal neuronal cells were seeded into 12-well plates at 10⁵ cells per well, and then incubated for 24 hours. Following the incubation period, SAHA and glutamate were administered to HT-22 cells for a 24-hour. After the treatment period, cells were detached with Trypsin-EDTA solution and neutralized with medium containing FBS, then the lysate was centrifuged at 12,000 rpm for 30 min at 4°C. The supernatants (100 μl of each) were collected for an Enzyme-like immunosorbent assay (ELISA). Glutathione levels, glutathione peroxidase and glutathione reductase activities were determined with ELISA kits (GSH and GPx assay kit, #703002, #703102 Cayman Chemicals, Michigan, USA), according to instructions of the manufacturer.

Statistical analysis: Descriptive statistics for the continuous variables were presented as Mean and Standard deviation. One-way ANOVA was used to compare group means. Following the ANOVA, Duncan multiple comparison test were used to identify different groups. Statistical significance

level was considered as 5% and SPSS (ver: 21) statistical program was used for all statistical computations.

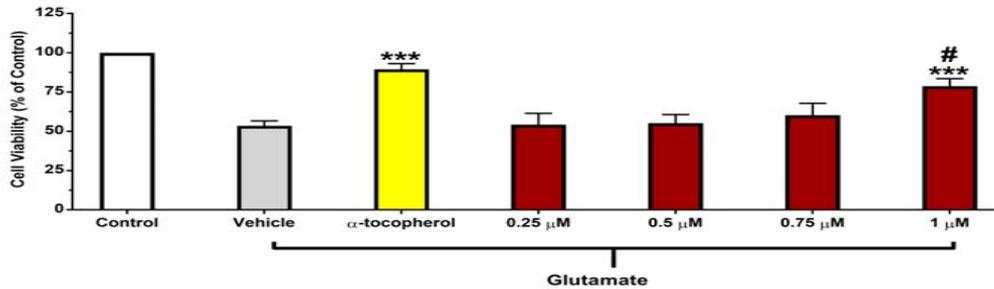


Figure 1. The neuroprotective effect of SAHA on glutamate-mediated cytotoxicity in HT-22 cells. Data are represented as the mean cell viability \pm standard deviation (SD) of at least six replicates. *** $p < 0.001$ versus vehicle, # $p < 0.05$ versus glutamate+ SAHA. SAHA: suberoylanilide hydroxamic acid.

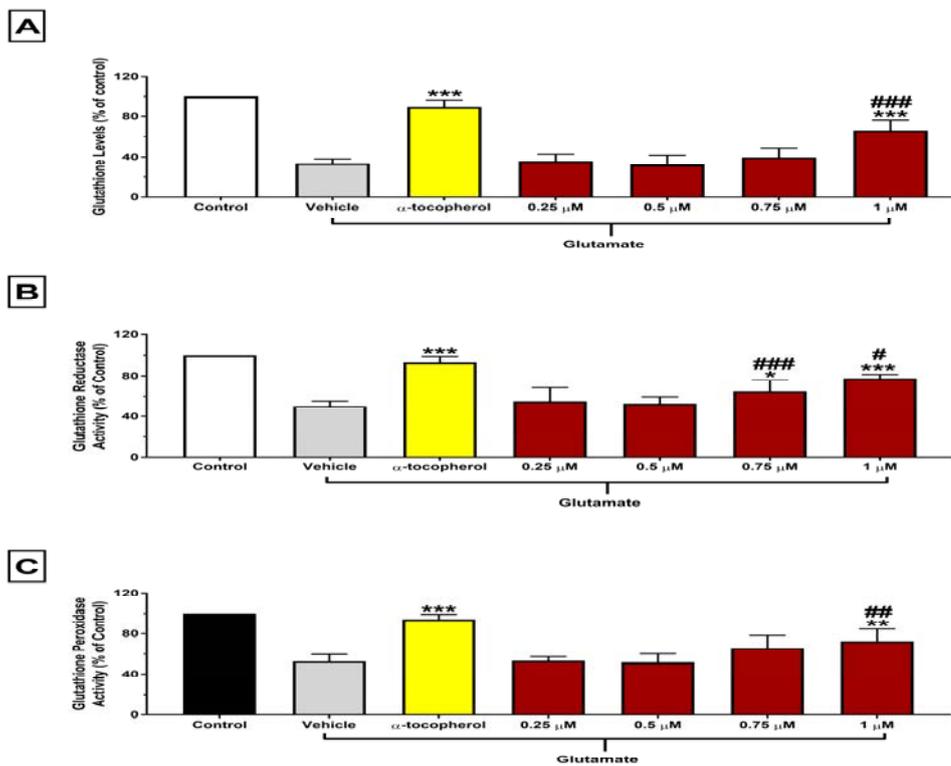


Figure 2. The effect of SAHA on (A) glutathione (GSH) levels, (B) glutathione reductase (GR), and (C) glutathione peroxidase (GPx) activities in HT-22 cells. Results are represented as mean (%) \pm SD of at least six replicates. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ versus vehicle, ### $p < 0.001$, ## $p < 0.01$, # $p < 0.05$ versus glutamate+SAHA in HT-22 cells. SAHA: suberoylanilide hydroxamic acid.

Results

SAHA significantly alleviated glutamate-induced cell death in hippocampal cells: To investigate the effect of the SAHA, HDACi, in the glutamate-induced microglial cells in vitro, we

added SAHA (0.25, 0.50, 0.75, and 1 μ M) to the cells exposed to glutamate (5mM). According to the MTT test results, glutamate (5mM) considerably lessened the amount of viable cells in microglial cells. SAHA (1 μ M) recovered

glutamate-caused cell death, and exhibited significant neuroprotective effects; however, the other doses of SAHA (0.25, 0.50, and 0.75 μM) had no impact on cellular viability (Figure 1). Besides, as a positive control, α -tocopherol (50 μM) also demonstrated a significant neuroprotective effect and protected hippocampal cells against glutamate-induced cell loss.

SAHA restored GSH, GR, and GPx activities:

The effect of SAHA on cellular GSH levels, GR, and GPx activities were investigated in hippocampal cells by ELISA. Glutathione is an essential antioxidant in the CNS. Also, the endogenous antioxidant enzymes GR/GPx are crucial for the production of GSH. Glutamate at the high concentrations results in a decrease in GSH. As shown in Figure 2, glutamate considerably diminished GSH levels, and GR/GPx activities in hippocampal cells. SAHA restored the reduced GSH levels, and GR/GPx activities in the cells after 24h exposure to glutamate. SAHA (1 μM) impeded the glutamate-caused decline of GSH levels in the cells. Moreover, SAHA ameliorated the glutamate-induced decline of GR (at the doses of 0.75 and 1 μM) and GPx (at the dose of 1 μM) activities in HT-22 cells (Figure 2A-C). In glutamate-induced cells, cellular GSH, GR, and GPx levels decreased to $33.53\pm 4.54\%$, $50\pm 5.33\%$, and $52.65\pm 7.14\%$, respectively. However, SAHA significantly alleviated the glutamate-induced decrease of GSH ($66.33\pm 9.9\%$ at 1 μM), GR ($64.57\pm 11.13\%$ at 0.75 μM and 77.42 ± 4.03 at 1 μM) and GPx ($72.6\pm 12.12\%$ at 1 μM).

Discussion

It is known that glutamate is a vital neurotransmitter in the CNS. In the last decades, knowledge about glutamate-mediated signaling in the brain has increased. Increased extracellular glutamate causes disruption of synaptic signal transduction, and neuronal death. Furthermore, diffusion of extrasynaptic glutamate is considerably associated with neuronal death. That disrupted glutamate homeostasis is a hallmark of several neurodegenerative diseases like Parkinson's disease (14). High levels of extracellular glutamate inhibit cysteine uptake, resulting in impaired cellular cysteine homeostasis, decreased cellular glutathione levels, and consequently accumulation of reactive oxygen species (7). In this study, it has been demonstrated that glutamate decreased the GSH levels, and GR/GPx activities, which are endogenous antioxidants, but the decreased antioxidant levels are restored by SAHA in glutamate-induced microglial cells in vitro. These

results suggest that SAHA has an antioxidant activity-mediated neuroprotective effect. Various investigations have been conducted to examine the association of oxidative stress and antioxidants with neurodegenerative diseases. Although several studies have been performed to explore whether antioxidants affect reducing neurodegenerative symptoms, there is no strong evidence showing the neuroprotective effect. Therefore, these mechanisms are needed to be investigated. Recently, it has been suggested that the imbalance among the ROS and antioxidant systems are associated with the etiology of Parkinson's disease (15). It is known that, compared to healthy controls, there is a 40% decrease in glutathione levels in the substantia nigra of patients with PD (16). This decrease is one of the initial biochemical alteration seen in PD. Also, GPx reduction in substantia nigra was detected in PD patients (17). In another pilot study in PD patients, it was shown that when the dopamine receptor agonist drug uptake was stopped for 12 hours, the GSH/GSSH ratio increased in the blood, compared to the period they were treated (18). These results indicate that the drugs used in Parkinson's disease may have adverse effects that can cause peripheral oxidative stress. Therefore, the use of oxidative stress-inhibiting therapeutic agents may be a better treatment alternative. Disturbances in acetylation homeostasis emerge as the leading cause leading to neuronal death. In some studies, it has been suggested that different inhibitors of HDAC have a protective effect in Huntington's disease models (19). The effect of HDACi on neuroimmunological response has not been fully elucidated yet. It has been suggested that pharmacological suppression of HDACs can be useful for treating neuroinflammatory diseases (20). A recent study demonstrated that impaired histone acetylation balance is also an indicator of PD (21). In animal models of Parkinson's disease, histone deacetylase inhibitors exhibited a beneficial effect neuroprotectively (22). Various histone deacetylase inhibitors have demonstrated antioxidant activities; nevertheless, these actions are mostly specific to cell type, and thus the comprehensive effect of histone deacetylases on oxidative stress is not apparent (23). It is known that SAHA used for cancer treatment is well tolerated in cancer patients; therefore, it is suitable for clinical use (24). A current study demonstrated that SAHA-treatment alleviated oxidative stress caused by rotenone, by rising GSH levels and sustaining the balance between GR and GPx (25). Our data showed that SAHA as an HDACi restored the decreased GSH, GR, and GPx

activities in glutamate-treated hippocampal cells in agreement with these studies.

Conclusion

The increase of oxidative stress in the many neurodegenerative diseases is well known. As a result of numerous clinical and preclinical studies, it was demonstrated that oxidative stress has a crucial effect on neurodegeneration. Correcting the imbalance between the antioxidant systems and oxidative stress, and reducing oxidative stress are important treatment goals in neurodegenerative diseases. As a result of our study, we concluded that SAHA, an HDACi, has antioxidant activity and neuroprotective effect, reduces oxidative stress mediated by glutamate, and might play a crucial role in the regulation of this balance, and promise as a treatment option. According to our knowledge, in this study, it was exhibited for the first time that SAHA showed neuroprotection against the oxidative stress mediated by glutamate in hippocampal cells; however, further studies are required to figure out mechanism of histone acetylation on the oxidative stress response.

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