Full Paper

**N-Stearoyltyrosine Protects Against Glutamate-Induced Oxidative Toxicity by an Apoptosis-Inducing Factor (AIF)-Mediated Caspase-Independent Cell Death Pathway**

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**Abstract.**  N-Stearoyltyrosine (NsTyr), a synthesized anandamide (AEA) analogue, could exert potent neuroprotective effects on cerebral ischemia models both in vivo and in vitro via intervening in multiple injuries. Glutamate, a major excitatory neurotransmitter, plays a critical role during stroke/cerebral ischemia. In this study, we explored the protective effects of NsTyr on glutamate neurotoxicity in PC12 cells and investigated its underlying mechanisms. NsTyr treatment attenuated glutamate-induced oxidative toxicity in a dose-dependent manner and the best performance was observed at 10 μM. NsTyr treatment suppressed glutamate-induced upregulation of lipoxygenase 12/15 (LOX 12/15) activity and reactive oxygen species (ROS) elevation, attenuated the increase of BH3-interacting domain death agonist (Bid) in the mitochondria, prevented the loss of mitochondria membrane potential and consequently inhibited apoptosis-inducing factor (AIF) translocation into the nucleus. The results demonstrated that NsTyr could protect cells against AIF-mediated caspase-independent cell death induced by glutamate, which may be due to the blockage of Bid-mediated mitochondrial damage via reducing LOX 12/15 activity and ROS accumulation.

**Keywords:**  N-stearoyltyrosine, glutamate, oxidative toxicity, apoptosis-inducing factor (AIF), caspase-independent

**Introduction**

Stroke/cerebral ischemia, the second leading cause of mortality in the world, involves complex pathophysiological processes (1). Disturbance of glutamate levels is the primary cause of neuronal death in stroke/cerebral ischemia through excitotoxicity triggered by the over-activation of glutamate ionotropic receptors and oxidative toxicity initiated by the blockade of the cysteine/glutamate antiporter (2). The oxidative toxicity is an important component of glutamate neurotoxicity during cerebral ischemia (3).

The pharmaceutical approaches intervening in multiple injuries instead of a single injury may function effectively for multifaceted neuronal death in stroke/cerebral ischemia patients (4, 5). Endocannabinoid anandamide (AEA) has been reported to protect neurons from ischemic injury via intervening in multiple injuries (6 – 8). However, AEA with a short metabolic half-life in vivo could not satisfy the requirements in clinical practice (9). Developing novel AEA analogues without the above-mentioned defect would be an alternation for neuroprotection.

We have synthesized diverse N-fatty-acyl amino acids as AEA analogues and evaluated their neuroprotective effects. One of them, N-steaoryltyrosine (NsTyr, Fig. 1), showed potent activities on stroke/cerebral ischemia models and is a promising neuroprotective candidate currently in preclinical studies in China. The underlying mechanisms of NsTyr were proved to intervene in

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multiple injuries, such as antagonizing against OGD-induced neuronal injury, inhibiting the inflammatory response, and regulating neuronal plasticity (10 – 12). Glutamate neurotoxicity plays an important role in neuronal death under stroke/cerebral ischemia conditions (2). In the present study, we verified that NsTyr exerts its neuroprotective effects by acting on glutamate toxicity in PC12 cells. Its neuroprotective effects on glutamate-induced oxidative toxicity are exerted at least partially through the blockage of the apoptosis-inducing factor (AIF)-mediated caspase-independent cell death pathway.

Materials and Methods

Reagents

DMEM medium, fetal bovine serum (FBS), glutamine, penicillin, streptomycin, and JC-1 assay kit were purchased from Gibco Invitrogen (Carlsbad, CA, USA). Annexin V-FITC apoptosis detection kit, TUNEL (dT-T-mediated dUTP nick-end labeling) assay kit, and ROS assay kit were purchased from Becton Dickinson Company (San Jose, CA, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), MK-801, CNQX, NAC, z-VAD-FMK, BI-6C9, baicalein, nuclear extraction kit, and mitochondria extraction kit were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) unless specified in the text. The other chemicals and reagents were analytical grade and were purchased from local commercial suppliers.

Synthesis of NsTyr

NsTyr used in the study was synthesized in our laboratory as described (10). Briefly, a mixture of 9 g N,N'-dicyclohexylcarbodiimide (43.6 mmol), 10 g stearic acid (35.2 mmol), 6 g N-hydroxysuccinimide (52.1 mmol), and 80 ml tetrahydrofuran (THF) were placed in an ice-bath with stirring for 16 h to provide stearic acid 2,5-dioxo-pyrrolidin-1-yl ester. After filtration, evaporation, and recrystallization in isopropanol, the N-hydroxysuccinimide ester of stearic acid was obtained with a yield 90%. Then 5 mmol N-hydroxysuccinimide ester of stearic acid in 20 ml THF was added dropwise to a 20-ml aqueous solution of 5.5 mmol l-tyrosine and 15 mmol NaHCO3. The mixture was stirred for 6 h at room temperature. The solvent was removed under reduced pressure. The residue was dissolved in water and adjusted with 1 M HCl to pH 3, and the solution was extracted with ethyl acetate (2 times × 50 ml). The combined organic extracts were successively washed with water and saturated NaCl solution and then dried over anhydrous sodium sulfate and evaporated to dryness. The structure of NsTyr was confirmed by the physical constants and spectral data (1H NMR and 13C NMR) (10). Its DMSO stock solution (10 mM) was stored at −80°C.

Cell culture and drug treatment

PC12 cells were obtained from the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in DMEM supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, and 10 μg/ml streptomycin at 37°C in a humidified atmosphere of 95% air and 5% CO2. The cells were plated at an appropriate density according to each experimental scale and NsTyr, MK-801 (5 μM), CNQX (20 μM), NAC (5 mM), and z-VAD-FMK were added 1 h before addition of glutamate. All inhibitors were freshly constituted in DMSO.

Cell viability assay

Briefly, PC12 cells were seeded into 96-well plates at 2 × 104 cells per well and maintained in a CO2 incubator at 37°C overnight. After 24 h glutamate treatment, MTT was added to each well at final concentration of 1 mg/ml. After additional incubation of 4 h at 37°C, the supernatants were removed, and 100 μl of DMSO was added to dissolve the insoluble dark blue formazan crystals. The absorbance was measured at 570 nm with a reference wavelength of 630 nm using a microtiter plate reader (Quant; Bio-Tek Instruments Inc., Winooski, VT, USA).

Flow cytometry

After glutamate treatment with or without NsTyr, PC12 cells were double-labeled with Annexin V-FITC and propidium iodide (PI) using an Annexin V-FITC Apoptosis Detection Kit for flow cytometry (FCM) analysis. Briefly, after the indicated treatment, the cells were washed with PBS, dissociated with 0.25% trypsin-EDTA, and resuspended in 1 ml Annexin V binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl2, pH 7.4). Cells (2 × 105) were stained with 5 μl Annexin V-FITC and 0.5 μg/ml PI in 100 μl binding buffer at 4°C, according to manufacturer’s instructions. Samples
Photographs were taken using a Philip CM-120 transmission electron microscope CM120 (Eindhoven, Netherlands).

**Immunocytochemistry**

After glutamate treatment with or without NsTyr for 15 h, PC12 cells were fixed with 4% PFA for 20 min at room temperature and then blocked and permeabilized with PBS containing 10% goat serum and 0.1% Triton X-100 for 1 h at room temperature. Following incubation with monoclonal anti-AIF antibody (1:100 in blocking solution) at 4°C overnight, the cells were exposed to the FITC-coupled goat anti-mouse secondary antibody (1:400) at room temperature for 1 h. The specificity of AIF immunoreactivity was captured by the Zeiss 510 laser scanning confocal microscope with the appropriate fluorescence filters (excitation wavelength of 488 nm and emission wavelength of 525 nm).

**Cytoplasmic and nuclear protein extraction**

PC12 cells were incubated with or without 10 mM glutamate and/or 10 μM NsTyr for 15 h. The cytoplasmic fraction and the nuclei were isolated with a nuclear extraction kit according to the manufacturer’s instructions. Cytoplasmic and nuclear protein concentration was determined by the Thermo BCA protein assay (Pierce, Rockford, IL, USA). Samples of 10 μg cytoplasmic or nuclear proteins were used for western blot analysis.

**Mitochondria isolation**

After treatment with or without 10 mM glutamate and/or 10 μM NsTyr for 12 h, PC12 cells were harvested from 10-cm dishes and centrifuged at 700 × g for 2 min. Then, the supernatants were removed and the mitochondria were prepared. Mitochondrial proteins were extracted by a mitochondria extraction kit according to the manufacturer’s instructions. Samples of 5 μg mitochondrial proteins were used for western blot analysis.

**Western blot analysis**

After treatment, PC12 cells were rinsed with PBS and lysed with an ice-cold lysis buffer and then centrifuged at 15,000 × g for 15 min at 4°C. The supernatants were collected, and the protein concentration was evaluated with the Thermo BCA protein assay. Proteins from cell lysates were denatured at 99°C for 10 min and 35 μg proteins were separated on a 10% SDS–polyacrylamide gel and electrotransferred to a nitrocellulose membrane. Membranes were blocked with 5% non-fat milk in Tris-buffered saline–0.1% Tween 20 (TBS-T) for 1 h. After incubation overnight at 4°C with monoclonal mouse anti-AIF antibody, polyclonal rabbit anti-Bid antibody (1:200), monoclonal rabbit anti-GAPDH antibody
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(1:1000), and polyclonal rabbit anti-LOX 12/15 (1:1000 dilution; Cayman Chemical, Ann Arbor, MI, USA), the membranes were washed and then incubated with secondary antibody, anti-rabbit or anti-mouse IgG HRP-linked antibody (1:2000), for 1 h. Proteins were visualized by an enhanced chemiluminescence detection kit (Pierce). The optical density of each band was quantified using image analysis software (Quantity One v4.4.0, Bio-Rad).

**15-HETE measurement**

The lipooxygenase 12/15 (LOX 12/15) activity was measured by determining the levels of its metabolite 15-hydroxyeicosatetraenoic acid (15-HETE). Cells ($2 \times 10^6$) seeded on a 6-cm² dish. After exposure to glutamate for 8 h with or without NsTyr, 15-HETE concentrations were measured with the 15(S)-HETE Enzyme Immunoassay kit (Assay Designs, Ann Arbor, MI, USA), in accordance with the manufacturer’s instructions.

**Results**

Glutamate-induced PC12 cell death was due to non-receptor–mediated oxidative toxicity through a caspase-independent pathway

After the treatment with 10 mM glutamate for 24 h, the viability of PC12 cells evaluated by MTT assay was reduced to about 25% of that in the control group ($P < 0.001$) (Fig. 2A). To understand the mechanisms of the glutamate-induced PC12 cell death, the antioxidant (5 mM NAC), caspase inhibitor (10 – 30 μM z-VAD-FMK), and glutamate receptor antagonists (5 μM MK-801 and 20 μM CNQX) were applied, respectively, 1 h prior to the treatment of glutamate. Only NAC resulted in a significant enhancement of cell viability ($P < 0.001$) in the presence of glutamate, while z-VAD-FMK, MK-801, and CNQX (Fig. 2B) each showed no obvious effect ($P > 0.05$), suggesting that glutamate-induced PC12 cell death was mainly due to non-receptor–mediated oxidative toxicity through a caspase-independent pathway.

**NsTyr treatment resulted in the protective effects on glutamate-induced cell death**

The protective effects of NsTyr on glutamate neurotoxicity were observed in PC12 cells. NsTyr at 1 – 10 μM did not affect the viability of PC12 cells for the control group, but reduced glutamate-induced cell death in a concentration- and time-dependent manner (Fig. 3: A, B). Pretreatment with 10 μM NsTyr for 1 h almost completely protected the PC12 cells against glutamate-induced cell death ($P < 0.001$), which was consistent
NsTyr’s Effect on Glutamate Toxicity

with the observations by microscopy and flow cytometry (Fig. 3: C, D).

**NsTyr inhibited glutamate-induced AIF nuclear translocation**

In glutamate-induced caspase-independent cell death, translocation of AIF into the nucleus led to large-scale DNA fragmentation (> 50 kbp) and subsequent cell death (13). Blockage of the AIF nuclear translocation could resist glutamate-induced cell death (14). Therefore, we were interested in the relationship of neuroprotective effects of NsTyr and AIF nuclear translocation. NsTyr alone had no obvious effects on AIF levels in the nucleus, cytoplasm, and mitochondria of PC12 cells. As illustrated in Fig. 4A, glutamate treatment up-regulated the AIF levels in the nucleus and cytoplasm and down-regulated the AIF levels in mitochondria (P < 0.01), while NsTyr treatment significantly reversed the glutamate effects. Taken together, NsTyr treatment significantly prevented the glutamate-induced AIF release and nuclear translocation, which was also confirmed by observation with confocal microscopy (Fig. 4B).
NsTyr blocked the Bid-mediated mitochondrial damage induced by glutamate

Mitochondrial damage is an essential earlier event for AIF nuclear translocation and cell death (13, 15), and the depletion of MMP is an important early determinant for the mitochondrial damage (16). As shown in Fig. 5A, glutamate treatment for 12 h caused a significant loss of JC-1 red fluorescence, indicating the loss of MMP, whereas NsTyr treatment almost completely abrogated the MMP reduction. In addition, observation under the transmission electron microscopy (Fig. 5B) showed most mitochondria of cells after glutamate treatment for 15 h were swollen and some of them even lost cristae, whereas NsTyr treatment reversed the morphological alterations. These data suggested that NsTyr prevented glutamate-induced mitochondrial damage.

Bid, a pro-apoptosis Bcl-2 family protein, can be activated in response to glutamate toxicity and insert into the outer mitochondrial membrane, resulting in mitochondrial damage and cell death (17). Bid inhibitor BI-6C9 (20 μM) reversed glutamate-induced PC12 cell death (Fig. 5C) and the glutamate treatment did not alter the total Bid expression, but elevated Bid levels in the mitochondria almost doubly, and NsTyr reduced...
the mitochondrial Bid levels to normal levels (Fig. 5D). Taken together, those results suggested that NsTyr prevented glutamate-induced Bid-mediated mitochondrial damage.

**NsTyr could reduce glutamate-induced ROS accumulation via inhibiting LOX 12/15 activity**

Excessive glutamate could result in the accumulation of ROS, which further elicited Bid-mediated mitochondrial damage (15). Using H2DCF-DA, a fluorescent dye, we observed that the intracellular ROS increased significantly in the PC12 cells treated with glutamate for 8 h compared with untreated cells ($P < 0.001$, Fig. 6A), while NsTyr reduced the increase of ROS levels ($P < 0.001$).

The inhibition of LOX 12/15, one of the key mediators
in glutamate-induced oxidative toxicity, could alleviate ROS accumulation (18). At 10 μM, baicalein, an LOX 12/15 inhibitor, reduced ROS accumulation and attenuated glutamate-induced PC12 cell death significantly (Fig. 6: A, B). Treatment of glutamate for 8 h resulted in no significant effects on LOX 12/15 expression (Fig. 6C). In contrast, the activity of 12/15-LOX in the glutamate group, measured by levels of its metabolite 15-HETE, increased to about 2.3-fold that of the control group, while 10 μM NsTyr treatment produced 48.1% reduction in LOX 12/15 activity (Fig. 6D).

Discussion

Stroke/cerebral ischemia is a multifactorial pathophysiologic process such as oxygen and glucose deprivation (OGD), glutamate disturbance, and inflammation (1). Agents with the potential to intervene in multiple injuries would offer a novel therapeutic strategy for stroke/cerebral ischemia (4, 5). Endocannabinoid AEA can protect neurons from damages through intervening in multiple injuries, such as inhibiting glutamate release (19–21), scavengering free radicals (6, 22, 23), reducing Ca$^{2+}$ influx (24, 25), and decreasing inflammatory response (8). However, it is difficult to use AEA in clinical practice because it is rapidly degraded (26). Hence, developing novel AEA analogues with predominant pharmaceutical activity may offer an alternative candidate for stroke/cerebral ischemia therapy.

NsTyr, an AEA analogue developed in our laboratory, could remarkably decrease hippocampal neuron apoptosis and ameliorate learning and memory in the rats followed by acute or chronic cerebral ischemic injury (11, 12). The mechanism study revealed that NsTyr protected neurons against OGD or LPS injury through reducing Ca$^{2+}$ influx, regulating expression of apoptosis-related genes (8).
associated genes, and decreasing the inflammation response (10), implicating NsTyr’s neuroprotective effect are exerted by intervening in multiple injuries. The good pharmacokinetics of oral administration of NsTyr was also summarized by its metabolic half-life of 3 h and the presence in the brain at 8 h after NsTyr administration (data not shown). Although glutamate is one of vital pathophysiological factors in cerebral ischemic injury, the effects of NsTyr on glutamate neurotoxicity are unclear. To clarify NsTyr’s neuroprotective activity through intervening in multiple injuries, the present work investigates the effects of NsTyr on glutamate-elicited cell injury and its underlying mechanisms.

Excessive glutamate could decrease cystine uptake through interfering with the glutamate/cystine antiporter, resulting in intracellular GSH depletion, and finally in oxidative toxicity, which is a critical component of glutamate neurotoxicity during cerebral ischemia (27–29). In the present study, the protective effects of antioxidant rather than the glutamate-receptor antagonists against glutamate-induced PC12 cell death were observed, indicating that glutamate induces cellular injury mainly through oxidative toxicity. Treatment of PC12 cells with NsTyr significantly abated cell damage after glutamate exposure, showing NsTyr’s ability to prominently protect cells from glutamate-induced oxidative toxicity.

Glutamate-induced oxidative injury can be mediated via the caspase-dependent or caspase-independent cell death pathway (14, 30). The caspase-independent AIF signaling is the main pathway (31, 32) through which glutamate induced cell death in immortalized HT-22 hippocampal neurons (13). In this study, inhibition of caspases did not block the PC12 cell damage triggered by glutamate (Fig. 2B) and a significant increase of AIF in nucleus was observed after glutamate exposure (Fig. 4), which might implicate that glutamate-induced damage in PC12 cells is mainly through the AIF-related caspase-independent pathway. Inhibiting AIF nuclear translocation or downregulating AIF levels would effectively antagonize OGD or glutamate-induced caspase-independent apoptosis (13, 33). We observed that the treatment with NsTyr could significantly reduce glutamate-induced AIF nuclear translocation in PC12 cells (Fig. 4) and inhibit the dramatic increase of the nuclear AIF in ischemic cerebral cortex and hippocampus of rats after middle cerebral artery occlusion (MCAO) (34). The results suggested that NsTyr could exert neuroprotective effects by blocking the AIF-related caspase-independent cell death pathway.

Mitochondria damage results in the release of pro-apoptotic proteins such as cytochrome c and AIF, which trigger caspase-dependent or caspase-independent cell death (35). Bid has been proved to play a pivotal role for mitochondrial damage in neuronal cell death (36, 37). During glutamate-induced oxidative toxicity, ROS accumulation can activate full-length Bid, which migrates to the mitochondrial membrane (38), leading to the depletion of MMP and mitochondrial damage (15). In this study, the Bid inhibitor BI-6C9 completely abolished glutamate injury (Fig. 5C), suggesting that Bid-mediated mitochondria damage is involved in glutamate-induced PC12 cell death (39). NsTyr treatment reduced ROS formation (Fig. 6A), reduced the full-length Bid levels in the mitochondria (Fig. 5D), and attenuated the mitochondrial damage (Fig. 5: A, B), implicating that NsTyr might block Bid-mediated mitochondrial damage through suppressing ROS elevation.

LOX 12/15, a principal lipoxygenase isoform in the CNS, plays a critical role in neuron death during glutamate injury (18, 40). The inhibition of LOX 12/15 could reduce the formation of ROS and lipid peroxidation and exert neuroprotective effects in vitro and in vivo (40–42). In this study, baicalein significantly suppressed ROS accumulation and protected PC12 cells from glutamate-induced oxidative toxicity (Fig. 6: A, B) and NsTyr inhibited LOX 12/15 activity but did not influence the levels of LOX 12/15 (Fig. 6: C, D), suggesting that LOX 12/15 is involved in the ROS accumulation induced by glutamate and that NsTyr’s effect on ROS was due to suppressing the activity of LOX 12/15. Moreover, our previous study showed that NsTyr could significantly increase the activities of antioxidative enzymes (43), which may also contribute to the suppression of NsTyr’s effects on ROS accumulation. Therefore, the suppression of NsTyr on ROS may be partially dependent on its inhibitory effect on LOX 12/15 activity.

Although NsTyr is an analog of AEA, no affinity of NsTyr with CB1 or CB2 receptor was observed (data not shown). Therefore, NsTyr should not be an agonist for CB receptors and NsTyr is an inhibitor of fatty acid amide hydrolase (FAAH) (IC50 < 1 μM), which is primarily responsible for the degradation of AEA. FAAH inhibitors have neuroprotective effects on several injuries (44–47), including the glutamatergic-related ones (48). So we suggest that NsTyr may exert neuroprotection through inhibiting FAAH activity and then regulating endocannabinoid levels following glutamate stimuli.

Above all, this study showed for the first time that NsTyr could protect against glutamate-induced AIF-mediated caspase-independent cell death, which may be at least due to the inhibition of Bid-mediated mitochondrial damage via reducing LOX 12/15 activity and ROS accumulation. In addition, our results strongly suggest that NsTyr exerts its neuroprotective effects by interven-
ing in multiple injuries and therefore would be a promising neuroprotective candidate for stroke/cerebral ischemia therapy.

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Conflicts of Interest

The authors declare no conflict of interest.

References

14. Zhang Y, Bhavnani BR. Glutamate-induced apoptosis in neuronal cells is mediated via caspase-dependent and independent mechanisms involving calpain and caspase-3 proteases as well as apoptosis inducing factor (AIF) and this process is inhibited by equine estrogens. BMC Neurosci. 2006;7:49.
28. Murphy TH, Miyamoto M, Sastre A, Schnaar RL, Coyle JT. Glutamate toxicity in a neuronal cell line involves inhibition of cystine transport leading to oxidative stress. Neuron. 1989;2:1547–1558.
36 Yin XM. Bid, a BH3-only multi-functional molecule, is at the cross road of life and death. Gene. 2006;369:7–19.