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The Involvement of Nitric Oxide in Synergistic Neuronal Damage Induced by β-amyloid Peptide and Glutamate in Primary Rat Cortical Neurons

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Abstract

Abnormal extracellular accumulation of β-amyloid (Aβ), the major component of senile plaques in the Alzheimer's brain and the excitatory amino acid glutamate are both considered to be associated with neurodegeneration. We studied whether nitric oxide (NO) was involved in neuronal damage induced by Aβ and glutamate in primary rat cortical neurons. Our results demonstrated that (1) Both neuronal damage and NO production were synergistically induced by Aβ-(25-35) and glutamate; (2) This synergistic neuronal damage induced by Aβ-(25-35) and glutamate was attenuated by selective inhibitors of NO synthase. We propose that cytotoxic characteristics of NO, at least in part, are involved in the synergistic neuronal damage induced by Aβ and glutamate, presumably seen in Alzheimer's brains.

Key Words: Alzheimer's disease, β-amyloid peptide, glutamate, neurodegeneration, nitric oxide,

Introduction

In Alzheimer's disease, abnormal extracellular accumulations of β-amyloid (Aβ), the major component of senile plaques in the Alzheimer's brain, are considered to be associated with neurodegeneration (18). Using primary cortical neurons or a variety of neuronal cell lines, Aβ or its active residue (25-35 fragment) can make neurons more susceptible to surrounding insults, such as excitotoxicity, hypoglycemia, or ischemia (5, 10, 13). Recently, this lowing of threshold for neuronal vulnerability is considered to be the consequence of the loss of neuronal Ca^{2+} homeostasis (10, 13).

Within the central nervous system, nitric oxide (NO) plays an important role in the pathogenesis involved in neuronal death (3, 4). The generation of NO by the calcium-dependent stimulation of NO synthase (NOS) was reported to be associated with neurotoxic effects (3, 4, 8). Since Aβ can destabilize intracellular calcium homeostasis leading to an increase of intracellular calcium, it raises a possibility that Aβ-induced neurotoxicity may involve abnormal generation of NO via the over-activation of Ca^{2+}-dependent NOS. In addition, several studies also indicated that Aβ-(25-35) can synergistically act with cytokines (e.g. interferon) to damage neurons by excessive production of NO in astrocytes or neuronal cell lines (14, 17).

Previous investigators had demonstrated that Aβ-(25-35) can interact with glutamate, an important pathogenesis underlying excitotoxicity in the central nervous system (2), to synergistically damage cortical neurons (5, 10, 13). However, there is no clear evidence that demonstrates whether nitric oxide is involved in this synergistic neuronal damage induced by the interactions of Aβ-(25-35) and glutamate. Thus, the major purpose of the present study was to investigate the above issue using primary rat cortical neurons. In addition, selective inhibitors of NOS were used to determine whether these pharmacological agents had therapeutic potentials.

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Materials and Methods

The culture of primary mixed cortical neurons was performed as before (12). Basically, primary mixed cortical cultures containing both neuronal and glial elements were prepared from post-natal SD rats (< day 1). Following sacrifice of the newborn rats, the brains were removed and placed in an isotonic salt solution containing penicillin G (100 unit/ml), streptomycin (100 μg/ml), and amphotericin B (0.25 μg/ml). The dissected cerebral cortices were suspended in 15 ml of 0.25% (weight/volume) trypsin and isotonic salt solution (pH = 7.4) and placed in a shaking water bath for 6 min at 37ÑaC to dissociate the cells. The dissociated cells were then treated with 160 μg of DNAase I (5 min, 37°C) and diluted with serum supplemented medium (SSM: Dulbecco's modified Eagles medium with Ham's nutrient mixture F-12 containing 15 mM HEPES, 0.365 g/L of L-glutamine, 1.2 g/L of $NaHCO_3$, 100 units each of penicillin and streptomycin, and 10% fetal bovine serum). The cell-containing solution was centrifuged at 1,000g for 10 min. The supernatant was discarded and the remaining pellet was resuspended in 10-15 ml of SSM and further dissociated by trituration. These cells were again diluted in SSM, and then plated at a density of 1 million in 35 mm diameter round culture dishes precoated with poly-L-lysine (10 μg/ml). Cells were then incubated at 37°C in a humidified incubator with 5% CO_2 plus 95% air. Forty-eight hours later, the SSM medium was removed and replaced with fresh SSM containing 10 μM cytosine arabinoside. Following another 48-hour interval, the cultures were rinsed once with fresh SSM medium and were then incubated for an additional 4 days prior to experimentation. The purity of enriched neuronal cultures was around 90%.

Aβ-(25-35), L-glutamate, N^G-Nitro-L-arginine methyl ester hydrochloride (L-NAME, a selective inhibitor of NOS) (1), and 7-Nitroindazole (7-NI, a selective inhibitor of neuronal NOS) (15) were purchased from Research Biochemicals International (Natick, MA, USA). Aminoguanidine (AG, a selective inhibitor of iNOS) (18) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). One day prior to any experimentation, AÉ"-(25-35) and other drugs were solubilized in sterile, distilled water as stock concentrations and were stored as aliquots at -20°C (7-NI was solubilized in glycerol). All cultured neurons (the 9 days *in vitro*) were routinely exposed to experimental drugs for 48 hours prior to the assessment of NO production and neuronal damage.

The levels of NO, lactate dehydrogenase (LDH), and neuronal morphology were evaluated from a same culture. In brief, NO was reflected by nitrite, a stable oxidation breakdown product of NO (6). Nitrite levels were analyzed by mixing 100 μl of culture medium with 100 μl of Greiss reagent (1 part 1% sulfanilamide in 60% acetic acid plus 1 part 0.1% naphthylenediamine dihydrochloride in distilled water) for 10 min, and read at 546 nm. Neuronal damage was first evaluated by the examination of neuronal morphology under phase-contrast microscopy followed by quantification of LDH released from damaged or destroyed cells into the culture medium, as previously described (5, 8, 13). The peak absorbance at 450 nm of the spectrophotometer was used to determine LDH units using LDH colorimetric assay kit (Cat. No. 500, Sigma). Unless otherwise stated, data are expressed as mean±SEM error of mean. Statistical evaluation was performed using one-way ANOVA followed by Dunnett's *t*-test for *post hoc* comparisons.

Results

To determine whether Aβ-(25-35)-induced neuronal damage is associated with an elevation in NO production, experiments were conducted using primary cortical neurons from rats. As demonstrated in Fig. 1Aa, Aβ-(25-35) induced both neuronal damage and NO production in a concentration-dependent manner. However, the significant increases in NO production were generated by relatively higher concentrations (25-50 μM) of Aβ-(25-35). In addition, the increased LDH release in response to Aβ-(25-35) correlates with the elevated NO production (Fig. 1Ab, $r = 0.79$, $p < 0.05$).

To determine whether glutamate can potentiate both Aβ-(25-35)-induced neuronal damage and NO production observed in Fig. 1A*a*, glutamate was applied to cortical cultures in the presence of Aβ-(25-35). As the concentrations of glutamate were given from 0.1 to 0.5 mM, the magnitudes of Aβ-(25-35)-induced LDH release and NO production were concentration-dependently potentiated by glutamate (Fig. 1Ba). The increased LDH release in response to Aβ-(25-35) and glutamate correlates with the elevated NO production (Fig. 1Bb, $r = 0.89$, $p < 0.05$). Similarly, while the concentrations of Aβ-(25-35) were at 1, 10, 25, and 50 μM, the magnitudes of glutamate-induced neuronal damage and NO production were potentiated by Aβ-(25-35) in a concentration-dependent fashion (Fig. 1Ca), accompanying a significant correlation between LDH release and NO production (Fig. 1Cb, $r = 0.92$, $p < 0.05$). In addition to LDH release, synergistic neuronal damage induced by Aβ-(25-35) and glutamate was also exhibited at the morphological level. Damaged neurons were identified by a morphological change, i.e., irregular shrunken cell bodies with dystrophic or fragmented neurites (Fig. 2), as compared to a healthy neuron having smooth

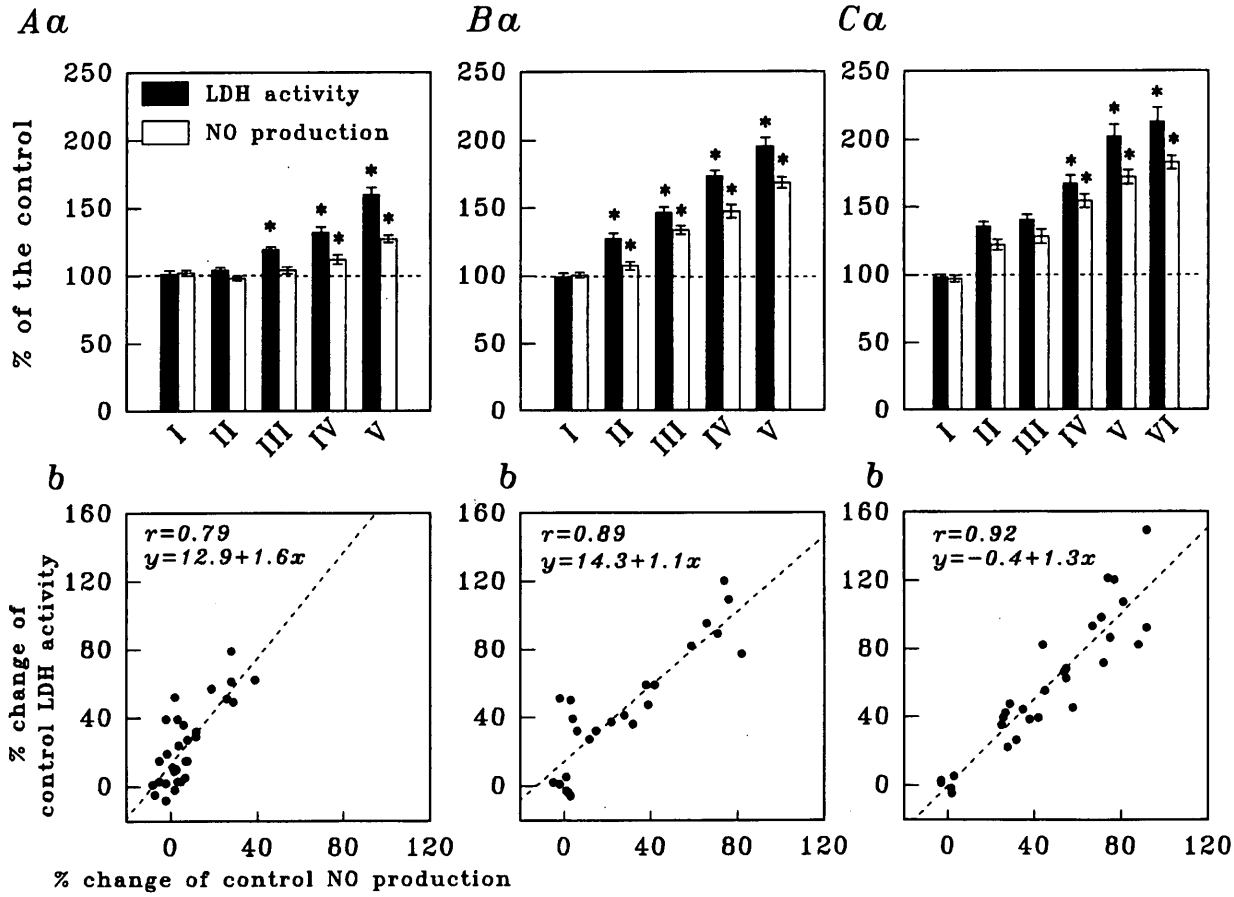

Fig. 1. Synergistic neuronal damage and NO production induced by Aβ-(25-35) and glutamate. (A). A summary of five separate experiments. Abbreviations: *I*, vehicle; *II*, 1 μM Aβ-(25-35); *III*, 10 μM Aβ-(25-35); *IV*, 25 μM Aβ-(25-35); V, 50 μM Aβ-(25-35). *p<0.05 (vs. the cultures only subjected to the vehicle). The average of the control nitrite per well (10^6 cells) was 2.97±0.36 nmole. (B). A summary of five separate experiments. Abbreviations: *I*, vehicle; *II*, 25 μM Aβ-(25-35); *III*, 0.1 mM glutamate + *II*; *IV*, 0.3 mM glutamate + *II*; V, 0.5 mM glutamate + *II*. *p<0.05 [vs. the cultures only subjected to 25 μM Aβ-(25-35)]. The average of the control nitrite per well (106 cells) was 3.17±0.41 nmole. (C). Abbreviations: *I*, vehicle; *II*, 0.5 mM glutamate; *III*, 1 μM Aβ-(25-35) + *II*; *IV*, 10 μM Aβ-(25-35) + *II*; *V*, 25 μM Aβ-(25-35) + *II*; *VI*, 50 μM Aβ-(25-35) + *II*. *p<0.05 (vs. the cultures only subjected to 0.5 mM glutamate). The average of the control nitrite per well (10^6 cells) was 3.25±0.41 nmole.

and round soma with clear neurites and axons.

To study whether NO was involved in the synergistic neuronal damage induced by Aβ-(25-35) and glutamate, a selective NOS inhibitor was co-applied with the submaximal levels of Aβ-(25-35) and glutamate. Figure 3 illustrates that both neuronal damage and NO production induced by Aβ-(25-35) and glutamate were significantly attenuated by each addition of L-NAME, 7-NI, or AG.

Discussion

In consistence with previous reports (5, 10, 13), synergistic neuronal damage induced by Aβ and glutamate was observed in the present study. Furthermore, we demonstrated a possibility that NO was responsible for this synergistic neuronal damage in primary cortical neurons.

The cytotoxic characteristics of NO resulted from the interactions between Aβ and glutamate in this experiment make NO a candidate which leads neurons more susceptible to surrounding glutamate. The synergistic generation of NO can damage or render neurons more vulnerable to surrounding insults by several mechanisms, including enhancement of spontaneous glutamate release from cultured neurons (16), inhibition of DNA replication and mitochondria respiration (9), activation of ADP-ribosyltransferases (20), or interaction with oxygen free radicals (7). Therefore, in our results the production of NO by coapplication of Aβ and glutamate is in significant correlation with the secretion of LDH from the primary neuronal culture.

The synergistic generation of NO could presumably be synthesized by over-activation of NOS. Several previous studies have demonstrated that

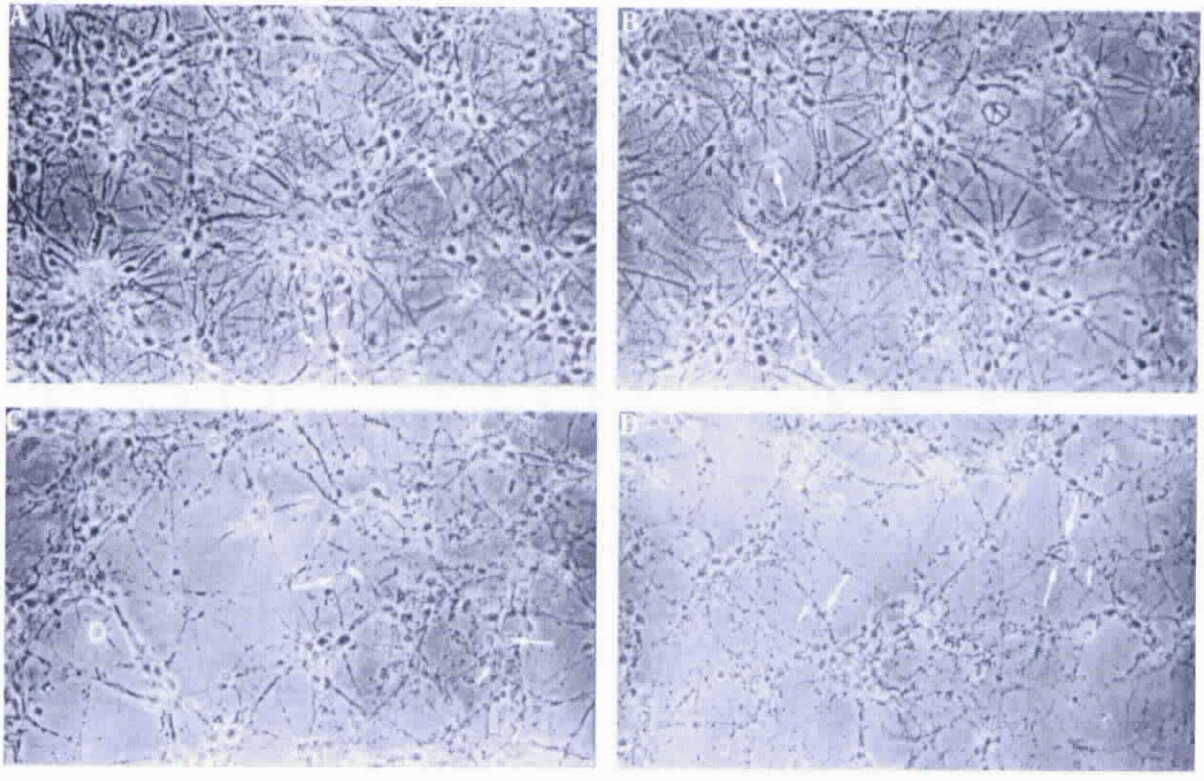

Fig. 2. Phase-contrast photomicrographs of primary cortical neurons taken 48 hours after the following treatments: (A). Control neurons (large arrow) showing extensive neurites with a relatively uniform diameter and a smooth appearance (small arrow); (B). Neurons treated with 0.5 mM glutamate exhibiting neurites (small arrow) and cell bodies (large arrow); (C). Neurons treated with 25 μM Aβ-(25-35) plus 0.5 mM glutamate demonstrating shrunken cell bodies (large arrow) with dystrophic or fragmented neurites (small arrow); (D). Neurons treated with 50 μM Aβ-(25-35) plus 0.5 mM glutamate indicating extensive neurite fragmentation (small arrow), beading, and cell lysis (large arrow). Scale bar, 50 μm.

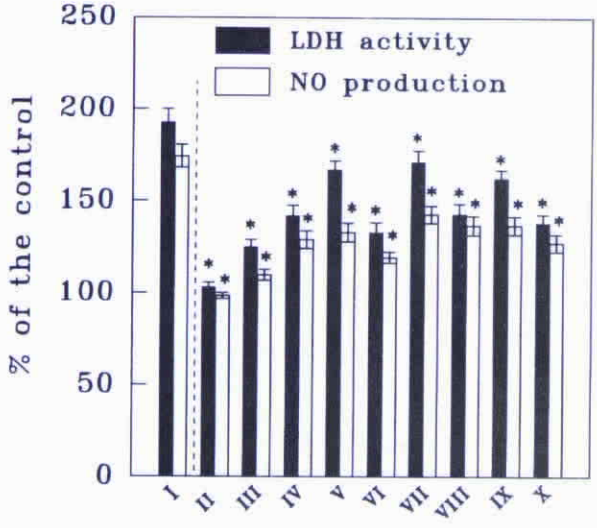

Fig. 3. Effects of selective inhibitors of NOS on both synergistic neuronal damage and NO production induced by the submaximal levels of Aβ-(25-35) and glutamate. A summary of five separate experiments. Abbreviations: *I*, 25 μM Aβ-(25-35) + 0.5 mM glutamate; *II*, vehicle; *III*, 25 μM Aβ-(25-35); *IV*, 0.5 mM glutamate; *V*, 10 μM L-NAME + *I*; *VI*, 100 μM L-NAME + *I*; *VII*, 10 μM 7-NI + *I*; *VIII*, 100 μM 7-NI + *I*; *IX*, 10 μM AG + *I*; *X*, 100 μM AG + *I*. The average of the control nitrite per well was 3.07±0.16 nmole. *$p<0.05$ (vs. the cultures only subjected to 25 μM Aβ-(25-35) and 0.5 mM glutamate).

interactions between Aβ and cytokines can synergistically increase the expression of inducible NOS in various types of astrocytes or cell lines (11, 14). The present study has also used selective inhibitors of neuronal NOS (7-NI) or inducible NOS (AG) to address the possible mechanism for the synergistic generation of NO resulted from the interactions between Aβ and glutamate. In the presence of Aβ-(25-35) and glutamate, selective NOS inhibitors attenuated NO production accompanying a significant lowering in LDH release (Fig. 3). Nevertheless, it remains unclear whether neuronal NOS or inducible NOS plays a dominant role in this type of neurodegeneration. We found that the application of 7-NI or AG can significantly decrease the production of NO as well as the secretion of LDH.

In conclusion, on the basis of these findings, we propose that NO is involved in synergistic neuronal damage induced by Aβ and glutamate. The involvement of NO in this synergistic neuronal damage is presumably seen in Alzheimer's brains.

Acknowledgments

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