Full-length article

Caspase-1 inhibitor Ac-YVAD-CHO attenuates quinolinic acid-induced increases in p53 and apoptosis in rat striatum[1]

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Key words

caspase 1; Ac-YVAD-CHO; Huntington disease; protein p53; NF-kappaB inhibitor alpha; apoptosis; NF-kappaB

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Abstract

Aim: To study the effects of the caspase-1 inhibitor Ac-YVAD-CHO on quinolinic acid (QA)-induced apoptosis. **Methods:** Rats were pre-treated with intrastriatal infusion of Ac-YVAD-CHO (2-8 μg) before intrastriatal injection of QA (60 nmol). Striatal total proteins, genomic DNA, and nuclear proteins were isolated. The effects of Ac-YVAD-CHO on QA-induced caspase-1 activity, internucleosomal DNA fragmentation, IκB-α degradation, NF-κB, and AP-1 activation, and increases in p53 protein levels were measured with enzyme assays, agarose gel electrophoresis, electrophoresis mobility shift assays, and Western blot analysis. **Results:** Pre-treatment with Ac-YVAD-CHO inhibited QA-induced internucleosomal DNA fragmentation. Ac-YVAD-CHO inhibited QA-induced increases in caspase-1 activity and p53 protein levels, but had no effect on QA-induced IκB-α degradation, NF-κB or AP-1 activation. **Conclusion:** Caspase-1 is involved in QA-induced p53 upregulation but not IκB-α degradation. Inhibition of caspase-1 attenuates QA-induced apoptosis in rat striatum.

Introduction

Excitotoxin-induced degeneration of striatal neurons in amimals has been used as an animal model of Huntington's disease (HD). Recent studies in transgenic animal models of HD demonstrated increased response of striatal neurons to excitotoxin, supporting a role of glutamate receptors in HD[1]. Excitotoxins acting on *N*-methyl-*D*-aspartate (NMDA) and kainic acid (KA) receptors will induce destruction of striatal GABAergic neurons by apoptotic mechanisms[2–4]. Caspases and tumor suppresser p53 play essential roles in apoptosis in a variety of cells including neurons[5–8]. Previous studies have reported that excitotoxin induces the activation of caspases and induces p53 expression[9–11]. Excitotoxin also reportedly activates nuclear factor-kappaB (NF-κB), but the mechanism by which this occurs remains unknown[12,13].

Recent studies have demonstrated that quinolinic acid (QA) induced significant increases in NF-κB binding activity in the nucleus[14]. NF-κB nuclear translocation mediates the upregulation of p53 and c-Myc in striatal neurons exposed to excitotoxic injury[15–17]. NMDA receptors activate NF-κB by selective degradation of an inhibitor protein, IκB-α. Excitotoxin-induced degradation of IκB-α involves a caspase-3-dependent mechanism in rat striatum and can be blunted by a caspase-3 inhibitor and a free radical scavenger[18,19]. In the present study we evaluated the role of caspase-1 inhibitor Ac-YVAD-CHO in QA-induced NF-κB activation, p53 induction, and apoptosis.

Materials and methods

Stereotaxic drug administration Sprague-Dawley rats (300–350 g) were obtained from the Experimental Animal Center of Soochow University (Certificate No 20020008, Grade II). Rats were anesthetized with pentobarbital sodium (50 mg/kg). Stereotaxic drug administration was performed using a Kopf stereotaxic apparatus as described by Qin *et al*[3]. To study the effects of a caspase-1 inhibitor on QA-induced internucleosomal DNA fragmentation, rats were either pre-treated with an intrastriatal infusion of Ac-YVAD-CHO (2–8 μg) or Me_2SO (2 μL) 10 min before instrastriatal injection of QA (60 nmol) and then killed 24 h after QA administration, or pre-treated with intrastriatal infusion of Ac-YVAD-CHO (4 μg) 10 min before instrastriatal injection of QA (60 nmol) and

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killed 12, 24, or 48 h after QA administration. Striatal genomic DNA was isolated and electrophoresed on a 2% agarose gel. To study the effect of a caspase-1 inhibitor on QA-induced increases in caspase-1 activity, rats were pre-treated with an intrastriatal infusion of Ac-YVAD-CHO (4 μg) or Me_2SO (2 μL) 10 min before intrastriatal injection of QA (60 nmol) and then killed 12 h after QA treatment. Striatal homogenates were used for assay of caspase-1 activity. To study the effect of a caspase-1 inhibitor on QA-induced increases in p53 proteins and NF-κB and AP-1 binding activities, rats were pre-treated with intrastriatal infusion of Ac-YVAD-CHO (4 μg) or Me_2SO (2 μL) 10 min before intrastriatal injection of QA (60 nmol) and then killed 24 h after QA treatment. Total striatal proteins were extracted for Western blot analysis. Other animals were killed 12 h after QA treatment and nuclear proteins were isolated from the striatum for an electrophoresis mobility shift assay.

Isolation of genomic DNA and electrophoresis Striatal genomic DNA was prepared as described by Qin *et al*[3]. Briefly, striatal tissues were homogenized in a buffer containing NaCl 100 mmol/L, edetic acid 25 mmol/L, Tris-HCl 10 mmol/L (pH 8.0), 0.5% SDS, and RNase A 0.5 mg/L. Homogenates were incubated at 55 ºC for 2 h. Incubation was continued overnight after 0.6 mg protease K was added to the hoogenates. DNA was extracted with phenol: chlorform:isoamyl alcohol (24:25:1). DNA fragments were separated on 2% agarose gel and detected with a UV trasmilluminator after being stained with ethidium bromide.

Caspase-1 activity assay The caspase-1 activity assay was performed with an enzyme activity assay kit (Caspase-I/ ICE Colorimetric Assay Kit, BioVision) according to the manufacturer's instructions. Each striatal tissue was homogenized in 500 μL cell lysis buffer and centrifuged for 10 min at 10 000×*g*. The supernatant was transferred to a fresh tube and kept on ice. Protein concentration was determined using BCA kit (Pierce, Rockford, IL). For each 100 mg protein was diluted to 50 μL with cell lysis buffer and 50 μL 2×reaction buffer (containing DTT 10 mmol/L) was added, then 5 μL of the 4 μmol/L YVAD-pNA substrate (200 μmol/L) and the mixture was inculated at 37 ºC for 1 h. Read samples at 405 nm in a spectrophotometer (Bio-Rad Smart Spec 3000).

Western blot analysis Western blot analysis was performed as described previously[16]. Striatal tissues were homogenized in a buffer containing Tris-HCl 10 mmol/L (pH 7.4), NaCl 150 mmol/L, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, edetic acid 5 mmol/L, PMSF 1 mmol/L, aprotinin 0.28 kU/L, leupeptin 50 mg/L, benzamidine 1 mmol/L, pepstain A 7 mg/L. Protein concentration was determined using the BCA kit. Thirty micrograms of protein from each sample was subjected to electrophoresis on a 12% SDS-PAGE gel using a constant current. Proteins were transferred to nitrocellulose membranes and incubated with anti-p53 antibody (p53, p240, Santa Cruz, CA) in Tris buffered saline containing 0.2% Tween-20 (TBST) and 3% nonfat dry milk for 3 h. Membranes were washed and incubated with horseradish peroxidase-conjugated second antibody in TBST containing 3% nonfat dry milk for 1 h. Immunoreactivity was measured with enhanced chemoluminescent autoradiography (ECL kit, Amersham, Arlington Heights, IL) according to the manufacturer's instructions.

Electrophoresis mobility shift assay Striatal nuclear proteins were prepared as described by Qin *et al*[14]. Protein concentration was determined with the BCA kit (Pierce, Rockford, IL). Synthetic double-stranded NF-κB and AP-1 oligonucleotidyl probes were purchased from Promega (Madison, WI) and labeled with [^{32}P]ATP using T4 polynucleotide kinase (Promega). Nuclear proteins (6–14 μg) were incubated with [^{32}P]labeled probes (40 000 c/min) for 15 min at room temperature in the binding buffer (Promega). Reaction mixtures were electrophoresed on a 4.5% non-denaturing polyacrymide gel, then dried and exposed to x-ray film at –80 ºC with intensifying screens for 24 to 48 h. The results were quantitatively analyzed using an image analyzer (SigmaPlot Pro 4).

Results

Effects of Ac-YVAD-CHO on QA-induced inter-nucleosomal DNA fragmentation To evaluate the consequences of caspase-1 inhibition on apoptosis, we studied the effect of Ac-YVAD-CHO on QA-induced internucleosomal DNA fragmentation. The results showed that QA (60 nmol) induced intense internucleo-somal DNA fragmentation 24 h after drug administration. The DNA fragments were generally multimers of 180–200 base pairs, indicating internucleosomal DNA digestion by an endonuclease. QA-induced DNA fragmentation was strongly attenuated by Ac-YVAD-CHO in a dose-dependent manner (Figure 1A). The results also showed that Ac-YVAD-CHO (4 μg) inhibited QA-induced DNA fragmentation at all time points examined (12, 24, and 48 h after QA administration; Figure 1B).

Effects of Ac-YVAD-CHO on a QA-induced increase in caspase-1 activity QA injection significantly activated caspase-1 activity in comparison with saline injected animals ($P<0.05$, $n=6$). The increase in caspase-1 activity induced by QA was significantly inhibited by Ac-YVAD-CHO ($P<0.05$, $n=6$); however, pre-treatment with Me_2SO had no effect on the QA-induced activation of caspase-1 (Figure 2).

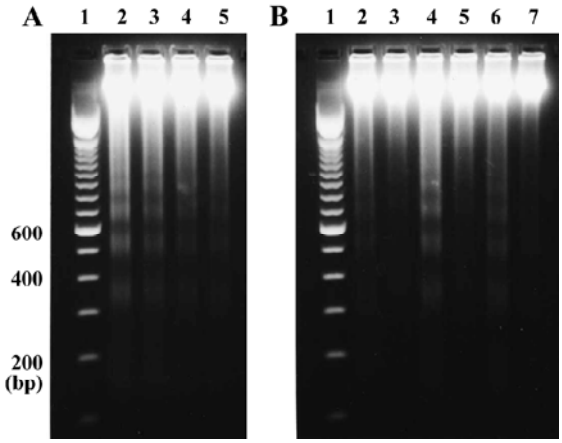

Figure 1. Effects of Ac-YVAD-CHO on QA-induced internucleosomal DNA fragmentation. (A): Rats were pre-treated with intrastriatal injection of Ac-YVAD-CHO (2–8 μg) or Me_2SO (2 μL) 10 min before intrastriatal injection of QA (60 nmol) and then killed 24 h after QA treatment. Genomic DNA from the injected striatum was isolated and electrophoresed on a 2% agarose gel. Lane 1:100-base pair DNA ladder; 2: QA+Vehicle; 3: QA+Ac-YVAD-CHO (2 μg); 4: QA+Ac-YVAD-CHO (4 μg); 5: QA+Ac-YVAD-CHO (8 μg). (B): Rats were pre-treated with Ac-YVAD-CHO (4 μg) and QA (60 nmol) as described above. Lane 1: 100-base pair DNA ladder; 2: QA+Vehicle, 12 h; 3: QA+Ac-YVAD-CHO, 12 h; 4: QA+Vehicle, 24 h; 5: QA+Ac-YVAD-CHO, 24 h; 6: QA+Vehicle, 48 h; 7: QA+Ac-YVAD-CHO, 48 h.

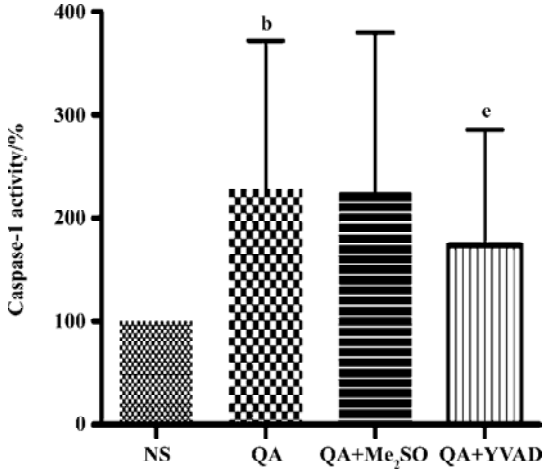

Figure 2. Effects of Ac-YVAD-CHO on the QA-induced increase in caspase-1 activity. The results were expressed as a percentage of control (saline injection) after using statistical analysis. Statistical analysis was carried out with Student's *t*-test. n=6. Mean±SD. $^bP<0.05$ *vs* NS; $^eP<0.05$ *vs* QA-treated group.

Effect of Ac-YVAD-CHO on QA-induced p53 induction QA induced significant increases in p53 protein levels by approximately 100%, which was significantly inhibited by Ac-YVAD-CHO ($P<0.05$, n=6, Figure 3).

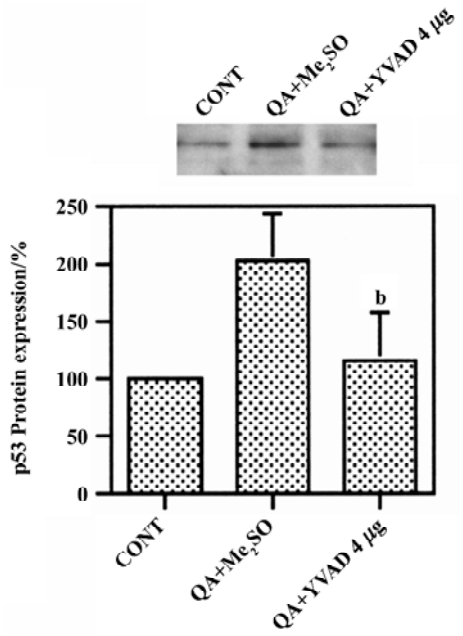

Figure 3. Effects of Ac-YVAD-CHO on QA-induced increase in p53 proteins expression. The results from 4 animals in each group were analyzed with an image analyzer and were expressed as percent of control (untreated animals). Statistical comparisons were carried out with ANOVA followed by Dunnett *t*-test. n=4. Mean±SD. $^bP<0.05$ *vs* QA+Me_2SO group.

Effects of Ac-YVAD-CHO on QA-induced degradation of IκB-α QA treatment significantly reduced protein levels of IκB-α, indicating that IκB-α was degraded after QA treatment. Pre-treatment with various doses of Ac-YVAD-CHO (2–8 mg) had no significant effect on QA-induced degradation of IκB-α (Figure 4).

Effect of Ac-YVAD-CHO on the QA-induced activation of NF-κB and AP-1 Changes in NF-κB and AP-1 binding in nuclear extracts were measured by using an electrophoresis mobility shift assay. QA induced dramatic increases in NF-κB binding activity in nuclei. However, Ac-YVAD-CHO (4 mg) had no significant effect on QA-induced NF-κB activation (Figure 5A). Similarly, AP-1 binding activity was increased markedly after QA administration and Ac-YVAD-CHO failed to inhibit the QA-induced activation of AP-1 (Figure 5B).

Discussion

Apoptotic mechanisms are involved in the degeneration of strial neurons induced by the glutamate receptor agonist QA. In the present study, the role of caspase-1 in QA-induced p53 upregulation and NF-κB activation was investigated using the selective cell-permeable caspase-1 inhibitor

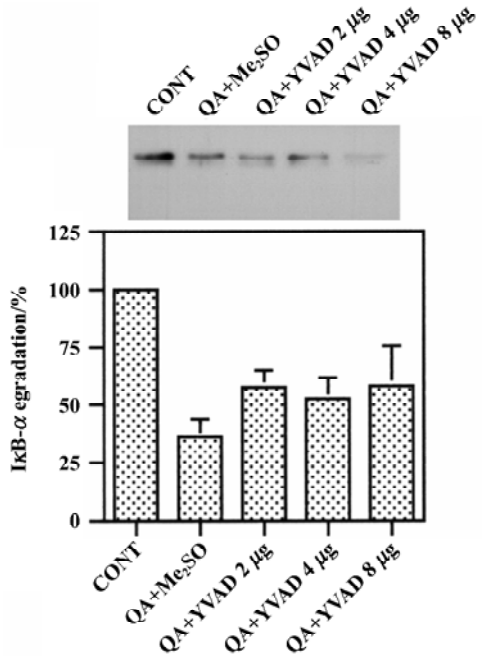

Figure 4. Effect of Ac-YVAD-CHO on the QA-induced degradation of IκB-α. The results from 5-6 animals in each group were analyzed with an image analyzer and expressed as percentage of control (untreated animals). Statistical comparisons were carried out using ANOVA analysis. Mean±SD.

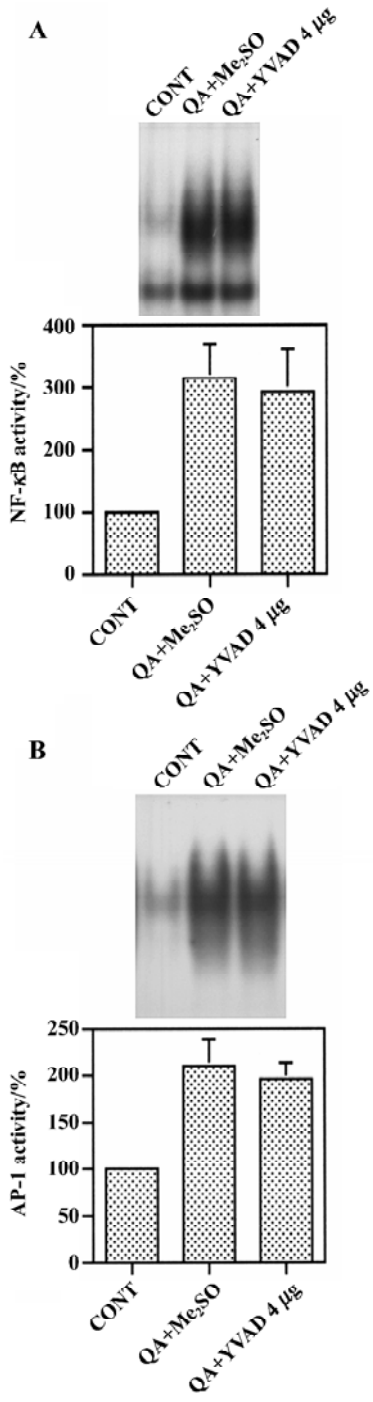

Figure 5. Effect of Ac-YVAD-CHO on QA-induced NF-κB activation. Vertical bars represent mean±SD. n=6. Statistical comparisons were carried out with ANOVA analysis. A: Effect of Ac-YVAD-CHO on the QA-induced NF-κB activation. B: Effect of Ac-YVAD-CHO on AP-1 activation.

Ac-YVAD-CHO. The results of the study showed that pretreatment with Ac-YVAD-CHO dose-dependently inhibited QA-induced internucleosomal DNA fragmentation. Ac-YVAD-CHO had no significant effect on the QA-induced IκB-α degradation and NF-κB activation. However, Ac-YVAD-CHO partially inhibited QA-induced increase in p53 protein levels. These results suggest that caspase-1 plays a role in QA-induced p53 upregulation but not QA-induced IκB-α degradation.

It has been reported that glutamate receptor agonists activate NF-κB via the degradation of IκB-α[15,16]. Previous studies have further suggested that caspase-3 , like protease, is involved in NMDA receptor-stimulated degradation of IκB-α[18,19]. Activation of NF-κB upregulates c-Myc and p53, indicating that NF-κB contributes to excitotoxin-induced apoptosis via the induction of cell cycle regulators[16,17,20]. p53 plays a critical role in cell death and survival. Its level is usually regulated by the rate of its degradation[21]. Now studies show that the levels of p53 can be regulated by NF-κB[16,17,22]. In the present study, caspase-1 inhibitor Ac-YVAD-CHO had no effect on either QA-induced IkB-α degradation or NF-κB activation. In contrast, Ac-YVAD-CHO significantly attenuated QA-induced increases in p53 protein levels and apoptosis. This study indicated that both NF-κB dependent and independent mechanisms are involved

in the QA-induced upregulation of p53 and apoptosis.

In conclusion, we found that the caspase-1 inhibitor Ac-YVAD-CHO inhibited the QA-induced increase in p53 protein levels and internucleosomal DNA fragmentation, but had no effect on QA-induced IκB-α degradation and NF-κB activation. These results suggest that caspase-1 plays an important role in QA-induced p53 induction and apoptosis, but caspase-1 does not contribute to the QA-induced degradation of IκB-α or NF-κB activation.

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