Protective effects of curcumin against human immunodeficiency virus 1 gp120 V3 loop-induced neuronal injury in rats

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Abstract
Curcumin improves the learning and memory deficits in rats induced by the gp120 V3 loop. The present study cultured rat hippocampal neurons with 1 nM gp120 V3 loop and 1 \( \mu \)M curcumin for 24 hours. The results showed that curcumin inhibited the gp120 V3 loop-induced mitochondrial membrane potential decrease, reduced the mRNA expression of the pro-apoptotic gene caspase-3, and attenuated hippocampal neuronal injury.

Key Words: curcumin; gp120 V3 loop; hippocampal neurons; mitochondrial membrane potential; caspase-3; human immunodeficiency virus-associated neurocognitive function; neural regeneration

INTRODUCTION
Although 50–60% acquired immune deficiency syndrome (AIDS) patients develop human immunodeficiency virus (HIV)-associated neurocognitive disorders (HAND). Most of these are mild in neurocognitive impairment\textsuperscript{[1]}, which makes them problematic to treat. Normally, an effective intervention for mild HAND might prevent the progression or elicit remission\textsuperscript{[2]}

The V3 loop domain is the core structure of the HIV-1 envelope glycoprotein 120 (gp120). The gp120 V3 loop can induce serious inflammation of the central nervous system which results in neuronal insult by direct and indirect means\textsuperscript{[3-5]}. The long term effect is one of the primary mechanisms for complication for HIV infected patients. Treatments with anti-retroviral therapy had sharply reduced the incidences of HAND\textsuperscript{[6]}. Nevertheless, a markedly increasing prevalence of HAND has been observed by epidemiologists during combination (two or more) antiretroviral therapy over the past two decades\textsuperscript{[7]}. Curcumin derived from turmeric has a wide range of pharmacological properties, including anti-inflammatory, anti-oxidant and anti-cancer properties\textsuperscript{[8-10]}. Curcumin also has a long tradition of use as a dietary compound and as a molecular tracer biomarker, which can be administered orally to animals and humans with little or no toxicity. Our previous work has shown that curcumin can improve spatial memory impairment induced by the HIV type 1 glycoprotein 120 V3 loop peptide in rats\textsuperscript{[11]}. Cellular energy metabolism may be impaired with the mitochondrial dysfunction found in neurodegenerative diseases\textsuperscript{[12]}. Dysfunctional mitochondria are often associated with the start of apoptosis and a subsequent progressive loss of neurons. Changes leading to mitochondrial dysfunction may be involved in such pathologies as HAND\textsuperscript{[13]}. In the intrinsic pathway of apoptosis, mitochondrial membrane potential (\( \Psi_{m} \)) alteration is an early apoptosis sign\textsuperscript{[14]}. Caspase-3 is an essential downstream effector of the apoptotic cascade and is activated after exposure to pro-apoptotic elements\textsuperscript{[15]}. Thus, the prevention of mitochondrial dysfunction may prohibit downstream signal transmission including the caspase-3 activation of apoptosis.

The present study investigated the possible neuroprotective mechanisms of curcumin against gp120-induced apoptosis. These experiments detected \( \Psi_{m} \) and measured the expression of caspase-3 mRNA in vitro.

RESULTS

The morphological characteristics of hippocampal neurons
Under an inverted phase contrast
microscope, primary cultures of hippocampal neurons were largely attached to the plate at 4 hours. Most of the glial cells and fibroblasts were removed after being cultured in serum-free medium. On the first day of culture, most of the hippocampal neurons developed short neurites with special haloes as seen with hematoxylin and eosin staining. On the third day, neuronal bodies increased markedly in size, and many neurites extended around the cell body (Figures 1A and B). At 7 days of culture, we observed an intertwined network of axons and dendrites in the culture dish using a confocal laser scanning microscope (Figure 1C). Cy3 and microtubule-associated protein 2 (MAP-2) staining was additionally used to identify neurons, and the cellular nuclei were stained with Hoechst. Plates of hippocampal neurons that grew well with at least 90% of the cells labeled with the MAP-2 specific cell marker were used for further experiments.

The viability of primary hippocampal neurons exposed to curcumin and the gp120 V3 loop

The neurotoxicity of the gp120 V3 loop and curcumin was assessed with the methyl thiazolyl tetrazolium (MTT) assay. Curcumin resulted in a markedly concentration-dependent decrease in the viability of hippocampal neurons. The 1 µM curcumin group showed no significant differences at 24 and 48 hours compared with the control group (no treatment) for viability of the hippocampal neurons, whereas the 5 µM and the 10 µM curcumin groups at 6 hours showed a significant decrease in cell viability compared to the control. Therefore, a concentration of 1 µM curcumin exposure to neurons for 24 hours was selected for subsequent experiments (Figure 2A). The exposure of the gp120 V3 loop to hippocampal neurons decreased neuronal viability in a time-dependent manner. After incubation for 2, 4, 8 or 12 hours with a concentration of 0.5, 1 or 2 nM gp120 V3 loop, the viability of hippocampal neurons showed no significant differences compared with the control group. However, at 24 hours, the viability of hippocampal neurons in the 1 nM gp120 V3 group significantly decreased compared with the control group ($P < 0.05$; Figure 2B). Thus, to demonstrate the pathological features of apoptosis of HAND, the concentration of 1 nM gp120 V3 loop exposure to neurons for 24 hours was used in subsequent experiments.

Curcumin protected hippocampal neurons against the gp120 V3 loop-induced impairment of mitochondrial function

To investigate whether the gp120 V3 loop-induced cytotoxicity was associated with mitochondrial dysfunction, the integrity of the $\Psi_m$ was detected using the mitochondrial specific fluorochrome, JC-1. Compared with control neurons, the gp120 V3 loop group showed a significantly impaired mitochondrial function, but the mitochondrial function remained unchanged when using curcumin alone. However, the mitochondrial function of the curcumin+gp120 V3 loop group was significantly better than the gp120 V3 loop group (Figure 3).

Curcumin inhibited the caspase-3 mRNA expression induced by the gp120 V3 loop in hippocampal neurons

Real-time fluorescent quantitative RT-PCR was used to assess the level of caspase-3 mRNA. The level of caspase-3 mRNA expression was higher in the gp120 V3 loop group compared with control group ($P < 0.01$), but no significant difference was observed between curcumin and control groups. In addition, the level of caspase-3 mRNA expression was significantly lower in the curcumin-gp120 V3 loop group compared with the gp120 group ($P < 0.05$; Figure 4).
The present study assessed the effects of 0.5, 1, 2, 5 and 10 μM curcumin on cell viability at different time points using the MTT assay. The results showed that curcumin dose-dependently inhibited the activity of hippocampal neurons. However, there was not a time-dependent effect from the lower to higher concentrations. These data confirm that the cytotoxicities of curcumin are low and acceptable, consistent with its use in clinical studies[10]. Neurons are vulnerable to gp120[17]. The viability of hippocampal neurons was time-dependently decreased after exposure to the 120 V3 loop peptide in the present study. We used 1 nM gp120 V3 loop in these experiments which is a dose that simulates the pathological processes of HAND. Because increased caspase-3 expression and decreased ∆Ψm may influence neuronal apoptosis, the activation of caspase-3 appears to be necessary for nuclear fragmentation and apoptotic body formation, but is not necessary for chromatin condensation[18]. Furthermore, this process occurs just prior to the characteristic morphological changes associated with apoptosis[19]. The results from the present study revealed that in vitro exposure of gp120 to hippocampal neurons resulted in a decreased ∆Ψm, concordant with previous findings[20]. However, pretreatment of the cells with 1 μM curcumin greatly inhibited this decrease, which provided experimental evidence that curcumin treatment reversed the injury effects.

A previous study showed that caspase-3 proteolytic activity as a molecular apoptosis mechanism in neurons increased in cerebrocortical cultures exposed to gp120[21]. Similarly, the expression of caspase-3 markedly increased in injured hippocampal neurons in the present study which supports previous results[22]. Caspase inhibition also prevented in vivo dendrite degeneration in HIV/gp120 transgenic mice, and alleviated neuronal loss in the central nervous system[23]. Together, these findings suggest a causal link between caspase-3 activation and apoptotic molecular mechanisms in the pathogenesis of HAND. To verify the protective mechanisms of curcumin for gp120-induced caspase-3 activation in neurons, we measured the caspase-3 mRNA expression in hippocampal neurons cultures which were pretreated with the gp120V3 loop peptide and then exposed to curcumin. The results indicated that the increased caspase-3 expression caused by the gp120V3 loop was significantly reduced in the presence of curcumin. These experiments reveal the protective effects of curcumin on hippocampal neurons are dependent on antagonizing caspase-3 expression. This suggests that curcumin intervention targets the caspase enzyme.

**DISCUSSION**

The mitochondrial function of rat hippocampal neurons. (A) Fluorescence images of JC-1-stained rat hippocampal neurons in four groups. In this image, more highly polarized mitochondria are distinctly red, and less polarized mitochondria are yellow. (A1- A4) control, curcumin, gp120V3 loop, curcumin+ gp120 V3 loop groups, respectively. Arrows indicate mitochondria of neurons from four groups. (B) Fluorescence ratio of JC-1-stained hippocampal neurons for the four groups (n = 6), *P < 0.01, vs. the control group; **P < 0.01, vs. the gp120V3 loop group. The data were expressed as mean ± SD and analyzed using one-way analysis of variance, followed by multiple comparisons using Fisher’s protected least significant difference test.

Caspase-3 expression in hippocampal neurons of the four groups as detected by quantitative real time RT-PCR. *P < 0.01, vs. the control group; **P < 0.05, vs. the gp120 V3 loop group (n = 6). The data were expressed as mean ± SD and analyzed using one-way analysis of variance, followed by multiple comparisons using Fisher’s protected least significant difference test. The dissociation curves were unique for each amplicon and confirmed gene target specificity.
pathway which may be beneficial for the prevention of HAND.
In conclusion, curcumin protected hippocampal neurons from insult induced by the gp120 V3 loop in vitro through stabilizing ΔΨm and preventing caspase-3 activation. Identifying the pathological and pharmacological mechanisms of curcumin may yield important insights and alternative drugs to alleviate the neuronal damage in HAND.

MATERIALS AND METHODS

Design
A controlled study of cytobiology.

Time and setting
The experiments were performed at the Key Laboratory of State Administration of Traditional Chinese Medicine, the Medical College of Jinan University, China, from December 2008 to June 2011.

Materials

Animals
A total of 90 one-day neonatal Sprague-Dawley rats were purchased from the Animal Experimental Center of Southern Medical University, China for hippocampal neuron culture (license: No. SCXK (Yue) 2006A051, 2006B023).

Drugs and reagents
The HIV-1 gp120 V3 loop fragment has an amino acid sequence of: NNTRKSIRIQRGPGRAFVTIGKIG, a chemical formula of: C_{114}H_{199}N_{44}O_{31}, and a molecular weight of 2640.06. Curcumin (chemical formula and molecular weight: C_{16}H_{12}O_{6}, 368.37) was purchased from Fluka, Buchs, Grisons, Switzerland; Figure 5).

Methods

Primary culture of hippocampal neurons
Hippocampal neurons were isolated and cultured according to previously described methods with some modifications. A total of 10^5 or 10^6 cells/well in 96- or 6-well culture plates were cultured with 90% Dulbecco’s modified Eagle medium/F12 and 10% fetal bovine serum (Sijiqing, Hangzhou, China) in a sterile environment. After 4 hours, serum free medium containing 98% neurobasal medium (Gibco, Carlsbad, California, USA) and 2% B27 (Gibco) replaced the culture medium. Finally, 60% stearle medium was replaced twice a week.

Immunocytochemical identification in hippocampal neurons
Neurons cultured for 4 hours or for 3 days were stained with hematoxylin and eosin as previously described and photographed under an inverted phase contrast microscope (Olympus, Tokyo, Japan). At 7 days, Hoechst 33342 (Beyotime, Haimen, Jiangsu, China) and MAP-2 (Sigma, St. Louis, MO, USA) staining was used to identify neurons. Briefly, cells were fixed in ice-cold 4% paraformaldehyde and incubated with the neuron specific MAP-2 antibody in blocking serum at 4°C overnight. After incubation in a species-specific IgG conjugated with Cy3, cells were washed with PBS, followed by Hoechst 33342 staining at 37°C for 5 minutes and photographed with a confocal laser scanning microscope (Zeiss, LSM 510, Oberkochen, Baden-Württemberg, Germany).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for neuronal viability
Cells were seeded in 96-well plates (1 x 10^4 cells/mL in 200 µL per well aliquots) until the cells adhered. At 6 days, hippocampal neurons were treated with culture medium containing curcumin, the gp120 V3 loop, or nothing (control), respectively. Hippocampal neurons were pretreated with 0.5, 1, 2, 5, or 10 µM of curcumin for 2, 4, 8, 12, 24 or 48 hours, and treated with 0.5, 1, or 2 nM of the gp120 V3 loop for 2, 4, 6, 8, 12, 24 or 48 hours. Curcumin and gp120 V3 loop peptide concentration was determined using MTT assay (Sigma) as previously described. In subsequent experiments, hippocampal neurons were randomly assigned to four groups: control, curcumin, gp120 V3 loop, or the curcumin+gp120 V3 loop group. Curcumin or the gp120 V3 loop was placed in the culture medium and homogenized. Curcumin and gp120 were mixed and placed in the culture medium for the curcumin+gp120 V3 loop group.

JC-1 measurement of mitochondrial membrane potential
Mitochondrial membrane potential was observed with the mitochondrial indicator JC-1 (fluorescent dye bisbenzimide, Beyotime, China). Red and green fluorescence of JC-1 reflected changes of ΔΨm. At 24 hours post-drug treatment, hippocampal neurons were incubated with the filtered culture medium containing JC-1 (10 µg/mL) at 37°C for 20 minutes in the dark. The cells were rinsed twice with buffer and images were obtained using a fluorescence microscope (Olympus IX-71). The JC-1 red/green fluorescence ratio was normalized to the control ratio with Image-Pro Plus 6.0 software (Bethesda, Maryland, USA).

Real-time fluorescent quantitative RT-PCR for caspase-3 expression
Total cellular RNA was extracted from hippocampal neurons cultured in 6-well culture plates using the TRIzol Reagent (TaKaRa, Kyoto, Japan) according to the manufacturer’s protocol. The synthesis of the cDNA was performed with the Reverse Transcription System (TaKaRa) using 500 ng of RNA. All samples were run in duplicate on 96-well optical PCR plates in a final reaction volume of 20 µL. The PCR parameters were 1 cycle at 95°C for 30 minutes, and 40 cycles at 95°C for 5 seconds and 60°C for 20 seconds. The caspase-3...
specific gene primers and the internal control gene primers (β-actin) are as follows:

| Gene       | Primer sequence          | Product length (bp) |
|------------|--------------------------|---------------------|
| Caspase-3  | Sense: 5'-GGG AGC GGT CAT GGT | 162                 |
|            | TCA-3'                   |                     |
|            | Antisense: 5'-AGG GGT TCT GTT TCT |                     |
|            | TTG CA-3'                |                     |
| β-actin    | Sense: 5'-GTA AAG ACC TCT ATG CCA | 227                 |
|            | ACA-3'                   |                     |
|            | Antisense: 5'-GGA CTC ATC GTA CTC |                     |
|            | CTG CT-3'                |                     |

The products of PCR reactions were analyzed using a Light cycler 480 PCR Instruments System (Roche, Basel, Switzerland). The relative gene expression was calculated using $2^{-\Delta \Delta CT}$ method.[28]

**Statistical analysis**

Data were expressed as mean ± SD and analyzed using one-way analysis of variance, followed by multiple comparisons using Fisher’s protected least significant difference test with SPSS 18.0. A value of $P < 0.05$ was considered statistically significant.

**Author contributions:** Zheng Gong, Lijuan Yang, Hongmei Tang, and Jun Dong designed the study. Zheng Gong, Lijuan Yang, Sai Xie, Luyan Guo, Junbin Wang, Qinyin Deng, and Guoyin Xiong, and Yanyan Xing conducted the experiments. Rui Pan provided analytic tools and analyzed the data.

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