Cytoprotective effect is one of common action pathways for antidepressants

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ABSTRACT

AIM: To explore the possible common action mechanism of antidepressants. **METHODS:** The cell viability was detected by MTT assay. The intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured by Fura 2-AM fluorescence labeling assay. Using RT-PCR, the mRNA level of nerve growth factor (NGF) was also detected. **RESULTS:** High concentration of corticosterone (0.2 mmol/L) was incubated with PC12 cells to simulate the lesion state of brain neurons in depressive illness. Three main kinds of antidepressants used in clinic [(1) tricyclic antidepressants (TCAs), such as desipramine (DIM) 0.625-10 μmol/L; (2) selective serotonin reuptake inhibitors (SSRIs), such as fluoxetine (FLU) 0.625-10 μmol/L; (3) monoamine oxidase inhibitors (MAOIs), such as moclobemide (MOC) 2.5-40 μmol/L] protected cells from the lesion induced by corticosterone. While antipsychotic drug chlorpromazine or anxiolytic agent diazepam 0.4-50 μmol/L had no such effect. Moreover, DIM 1, 5 μmol/L or FLU 1, 5 μmol/L attenuated the $[Ca^{2+}]_i$ overload induced by corticosterone 0.1 mmol/L for 48 h in PC12 cells. Furthermore, treatment with DIM or FLU 10 μmol/L for 48 h elevated the NGF mRNA expression in PC12 cells. **CONCLUSION:** Despite a remarkable structural diversity, the cytoprotective effect can be viewed as the common action pathway of the antidepressants. Moreover, attenuation of the intracellular Ca^{2+} overload and elevation of neurotrophic factor (such as NGF) expression is one of the mechanisms of cytoprotective effect of antidepressants.

INTRODUCTION

There are three main kinds of antidepressants [(1) tricyclic antidepressants (TCAs), such as desipramine (DIM); (2) selective serotonin reuptake inhibitors (SSRIs), such as fluoxetine (FLU); (3) monoamine oxidase inhibitors (MAOIs), such as moclobemide (MOC)] that have been used in clinic for several decades. Although these antidepressants with remarkable structural diversity all possess certain therapeutic effects, their mechanisms are still unclear, which limit the development of new drugs. Thereby, further studies on their mechanisms in detail, especially the common pathways, are very important.

It was reported that the volumes of the double-side hippocampus were reduced in the major depressive patients compared to the healthy control and there was a positive correlation between the hippocampus atrophy and the time course of the depression. This phenomenon is closely associated with the hyperaction of hypothalamic-pituitary-adrenal (HPA) axis and thereby the high concentration of glucocorticoids (GC) in blood[1,2]. Chronic psychosocial stress or corticosterone administration caused apical dendritic atrophy of hippocampal CA3 pyramidal neurons, which may be mediated by activation of HPA axis[1,2]. The HPA axis in major de-

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pressive patients is hyperactive and the effect of antiglucocorticoid therapy is very satisfactory, indicating that high concentration of GC in blood is the key point in the occurrence of depression[1,2]. The GC receptors in hippocampus are the richest in rat brain. These receptors are down-regulated in depressive patients because of the high blood level of GC and the negative feedback of hippocampus on the HPA axis is attenuated consequently, thereby, the high concentration of GC in blood is sustained permanently[3]. Since the hippocampus is a major site for emotional process, a role for excessive/long-lasting plasma GC levels has been suggested in conditions of mental impairment. The occurrence of depression may be closely related to the GC-induced lesion in hippocampus[1-3]. It is therefore important to look for pharmacological strategies that will avert or reduce these potential consequences on brain function. The rat adrenal pheochromocytoma cell line, designated PC12 cells, possesses typical features of brain neurons and is abundant in GC receptors[4]. The monoamine oxidase and monoamine transporters are also expressed in PC12 cells[5,6]. So, in this study, PC12 cells were incubated with high concentration of corticosterone to simulate the lesion state of brain neurons in depressive illness, and the neuroprotective action of three kinds of antidepressants and the possible mechanisms were observed.

MATERIALS AND METHODS

Drugs and reagents PC12 cells were kindly presented by Dr WAN You in Peking University. Corticosterone, DIM, and chlorpromazine were bought from Sigma (USA). FLU was from the No 4 Pharmaceutical Factory in Changzhou city (931201). MOC was provided by the Chemical Synthesis Lab of our institute; DMEM culture medium and Trizol reagent were from Gibco BRL (USA). Parenteral solution of diazepam was bought from the Huida Pharmaceutical Factory in Datong city (China). 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) was purchased from Merck Chemical Co (USA). Fura 2-AM was obtained from Fluka (Sweden). RT-PCR kit was the product of Promega (USA).

PC12 cell culture and detection of cell viability with MTT assay PC12 cells were seeded into 96-well plates (Costar, USA) at a density of 2×10^8/L and cultured in the medium consisting of 90 % DMEM, 5 % heat-inactivated horse serum, 5 % fetal calf serum, benzylpenicillin 200 kU/L, and streptomycin 100 mg/L in a humidified incubator (Napco, USA) with 5 % CO_2 for 3-4 d. The MTT assay was described in previous study [7]. The medium was replaced with DMEM consisting of indicating drugs respectively (including DIM 0.625-10 μmol/L, FLU 0.625-10 μmol/L, MOC 2.5-40 μmol/L, diazepam 0.4-50 μmol/L, or chlorpromazine 0.4-50 μmol/L) and corticosterone 0.2 mmol/L, and then cells were incubated for another 48 h. Having been washed with *D*-Hanks' solution twice, cells were incubated with DMEM containing MTT 0.5 g/L at 37ºC for another 4 h. After remove of the medium, 10 % SDS 100 μL per well was added. After the blue granules dissolved completely (about 12-16 h needed), the absorbance at 570 nm ($A_{570\ nm}$) was detected with MCC/340 spectrophotometer (Flow, USA).

Preparation of total RNA PC12 cells were planted in 50 mL plastic flask (Costar, USA) at a density of 2×10^8/L in the growth medium for 3-4 d, and then the cells were classified into following groups: control, DIM (10 μmol/L) or FLU (10 μmol/L) group. After incubation for another 48 h, cells were harvested and the total RNA was extracted by using Trizol reagent, following the instructions provided by the manufacture. The yield and purity of total RNA was determined with UV spectrophotometer (Shimadzu, Japan) at 260 and 280 nm.

RT-PCR RT-PCR was performed following the instruction provided by the manufacturer. Total RNA 1 μg was added into 50 μL of RT-PCR reaction system containing Mg^{2+} 0.5 mmol/L and a pair of primers 0.3 μg×2. Sequences of the NGF primers specific for rats were: TCATCCACCCACCCA GTCT (5'), CACGCAG-GCTGTATCTATC (3'). The expected size of NGF was 330 bp. The mixture was overlaid with mineral oil and amplified in Perkin-Elmer thermal cycle 480 (USA). The reaction mixtures were incubated for 45 min at 48 ºC followed by 2 min at 94 ºC to denature the template, and then thirty cycles (30 s at 94 ºC, 1 min at 55 ºC, and 2 min at 72 ºC) and a final cycle (7 min at 72 ºC) were performed. The RT-PCR products were stored at 4 ºC.

RT-PCR product 10 μL and λDNA (*EcoR* I/*Hind* III restriction digest) marker were electrophoresed in 2 % agarose gel, stained with 0.5 mg/L ethidium bromide, visualized under an ultraviolet light, and photographed with UVP gel imaging system (USA). The amount of RT-PCR product was semiquantitatively determined by measuring the density of the specific bands.

Measurements of intracellular Ca^{2+} concentration in PC12 cells The intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in PC12 cells was monitored by fluorometry, using the Ca^{2+}-sensitive dye Fura 2-AM. The cells were seeded in 24-well plates at the density of 2×10^8/L. After 3-4 d, the medium was replaced with serum-free DMEM containing DIM (1, 5 μmol/L) or FLU (1, 5 μmol/L) in the presence of 0.1 mmol/L corticosterone, and then cells were cultured for another 48 h. For Fura 2-AM loading, cells were collected and incubated with the complete medium containing 5 μmol/L Fura 2-AM at 37 ºC for 45 min, subsequently, cells were washed and resuspended again with cold BSS buffer (NaCl 130 mmol/L, KCl l5.4 mmol/L, $CaCl_2$ 1.8 mmol/L, glucose 5.5 mmol/L, Hepes 20 mmol/L, pH 7.4) containing 0.2 % BSA. The cells were incubated at 37 ºC for another 5 min just prior to measurement. $[Ca^{2+}]_i$ was determined by alternating excitation wavelengths of between 340 nm and 380 nm with emission at 510 nm on a fluorescence spectrophotometer (F-4500, HITACHI, Japan) and the data analyzed with customized software provided by F-4500. The ratio of fluorescence intensities excited by 340 or 380 nm was calculated after subtraction of the background fluore-scence.

Data analysis The results were expressed as mean±SD. The differences between groups were determined by the one-way ANOVA analysis.

RESULTS

Protective effects of antidepressants on PC12 cells from the lesion induced by corticosterone After the treatment of PC12 with corticosterone 0.2 mmol/L for 48 h, the $A_{570\ nm}$ decreased compared with control ($P<0.01$), indicating that the cells were impaired or some of them were dead. While in the presence of DIM (0.625-10 μmol/L), FLU (0.625-10 μmol/L) or MOC (2.5-40 μmol/L), the A_{570nm} values increased in a concentration-dependent manner, indicating that these drugs all could protect the cells from the lesion induced by corticosterone (Fig 1). However, anxiolytic diazepam or antipsychotic chlorpromazine had no such effect (Fig 2).

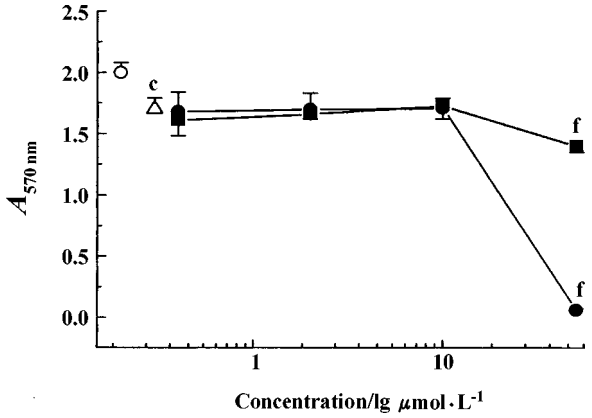

Fig 2. Effect of chlorpromazine or diazepam on the corticosterone-induced lesion in PC12 cells. (○) Control; (△) Corticosterone; (●) Corticosterone+chlorpromazine; (■) Corticosterone+diazepam. *n*=6. Mean±SD. $^cP<0.01$ *vs* corresponding control. $^fP<0.01$ *vs* corticosterone-treated group.

Effects of antidepressants on corticosterone-induced $[Ca^{2+}]_i$ overloading in PC12 cells After the treatment of PC12 cells with corticosterone 0.1 mmol/L for 48 h, $[Ca^{2+}]_i$ elevated significantly compared with

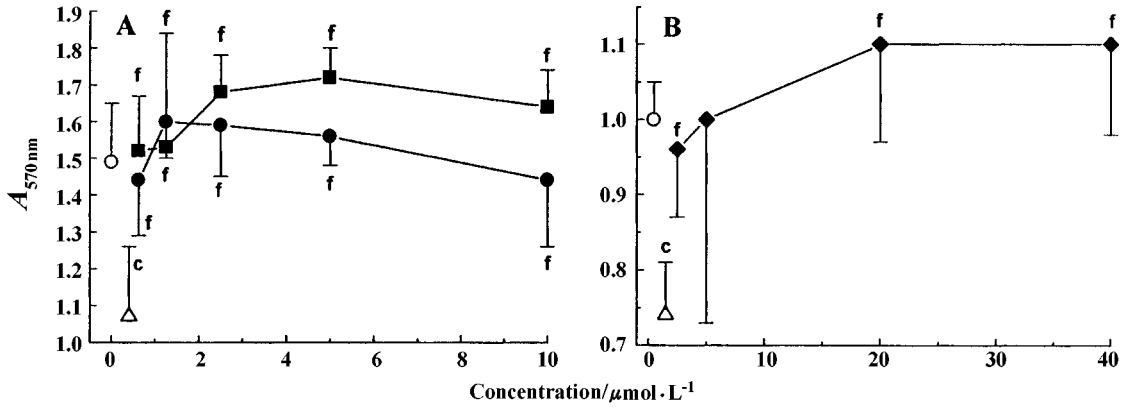

Fig 1. Inhibitory effect of DIM, FLU (A), or MOC (B) on the corticosterone-induced lesion in PC12 cells. (○) Control; (△) Corticosterone; (●) Corticosterone+FLU; (■) Corticosterone+DIM; (◆) Corticosterone+MOC. *n*=6. Mean±SD. $^cP<0.01$ *vs* corresponding control. $^fP<0.01$ *vs* corresponding corticosterone-treated group.

the control (P<0.01). While in the presence of DIM (1, 5 μmol/L) or FLU (1, 5 μmol/L), the corticosterone-induced $[Ca^{2+}]_i$ overloading was attenuated (Tab 1). These results indicated that the cytoprotective action of antidepressants might be associated with their reducing the $[Ca^{2+}]_i$ overloading.

Tab 1. Effect of DIM or FLU on the corticosterone (0.1 mmol/L)-induced $[Ca^{2+}]_i$ overloading in PC12 cells. n=6. Mean±SD. ^cP<0.01 *vs* corresponding control. ^eP<0.05, ^fP<0.01 *vs* corresponding corticosterone group.

Groups		$[Ca^{2+}]_i$/nmol·L^{-1}
Control		232±28
Corticosterone		313±17^c
Corticosterone+DIM	1 μmol/L	285±22
	5 μmol/L	248±18^f
Control		201±19
Corticosterone		341±24^c
Corticosterone+FLU	1 μmol/L	301±9^{ec}
	5 μmol/L	264±14^{fc}

Effects of antidepressants on mRNA level of NGF in PC12 cells NGF mRNA level in PC12 cells was determined by RT-PCR and the corresponding bands in electrophoresis were semiquantitatively calculated. DIM 10 μmol/L or FLU 10 μmol/L increased the NGF mRNA level compared with the normal control (P<0.01), indicating that the neuroprotection of antidepressants might be associated with the increase in expression of neurotrophic factors (Tab 2).

Tab 2. Effect of DIM or FLU on the mRNA level of NGF in PC12 cells. n=6. Mean±SD. ^cP<0.01 *vs* control.

Groups	Concentration/ μmol·L^{-1}	mRNA level/ % of control
Control	–	100
DIM	10	168± 9^c
FLU	10	182±56^c

DISCUSSION

Our previous studies found that classical antidepressants also protected the primarily cultured rat hippocampal neurons from the lesion induced by corticosterone, which was consistent with the results in PC12 cells. In addition, buspirone (a 5-HT receptor agonist, possessed antidepressant and anxiolytic effects) had the same cytoprotective effect on PC12 cells[7]. Furthermore, the new compounds under development in our lab (such as oligosaccharides extracted from *Morinda officinalis*, extract of cotton seeds, indole derivatives etc) all antagonized the lesion induced by corticosterone in PC12 cells and their antidepressant effects were well demonstrated in animal models subsequently[8,9]. On the other hand, *N*-methyl-*D*-aspartate (NMDA) receptor antagonists are regarded as one of the most representative developing direction for antidepressant[10]. MK801, amantadine, Zn^{2+} or ketamine, for example, all had notable antidepressant effects in animal models or depressive patients[10]. Interestingly, we also found that they protected the PC12 cells from the corticosterone- or NMDA- induced lesion, suggesting that NMDA may be involved in or even mediated the corticosterone-induced lesion in PC12 cells. All of these evidences supported that the cytoprotective action might be the common pathway of the antidepressants.

There are now several independent studies reporting that the volume of hippocampus is decreased in patients suffering from depression or post traumatic stress disorder, which is closely related to the down-regulation of the rate of neurogenesis in adult hippocampus[1,11]. Exposure to inescapable footshock in the learned helplessness model of depression results in a long lasting down-regulation of neurogenesis. Up-regulation of neurogenesis is dependent on long-term antidepressant administration (2-4 weeks), consistent with the time course for the therapeutic action of antidepressant treatment. Other non-antidepressant psychotropic drugs tested, including haloperidol or morphine, do not up-regulate adult neurogenesis[11,12]. The neurogenesis action of antidepressants is closely associated with the up-regulation of cAMP-CREB (cAMP-response element binding protein) cascade and expression of brain-derived neurotrophic factor (BDNF) in hippocampus[11,12]. Combined with the results of our studies, the hypothesis that the cytoprotection is a common action pathway of antidepressants is further supported. Certainly, the mechanisms in detail need more studies.

The mechanism of corticosterone-induced lesion

of neurons is still unclear, which maybe closely related to the energy metabolism disorder or $[Ca^{2+}]_i$ overloading in neurons[13]. In this study, corticosterone did induce the $[Ca^{2+}]_i$ overloading in PC12 cells, while DIM or FLU reversed these changes which might be one of the mechanisms of their cytoprotection. Our previous studies also found that corticosterone decreased the NGF mRNA level in PC12 cells, while antidepressants elevated it in this study, in which the mechanism may be that antidepressants activated the adenylate cyclase (AC) in cell membrane and increased the cAMP level, thereby up-regulated cAMP-CREB (cAMP-response element binding protein) cascade and expression of neurotrophic factors (such as NGF)[7,11,14,15]. Elevation of NGF expression also may be one of the mechanisms of cytoprotection of antidepressants, while the relationship between $[Ca^{2+}]_i$ overloading and NGF expression should be further studied.

The relatively swift and specific models for new antidepressant agent screening *in vitro* are very few. We have much evidence to speculate that the cytoprotective effect is one of the common action pathway for antidepressants, which will contribute to the new drug screening and development.

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