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·基础研究·

Activation of Muscarinic Cholinergic Receptors Protects Cerebellar Granule Neurons from Dopamine-Induced Apoptosis via ERK Pathway

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Abstract: 【Objective】 To investigate the molecular mechanism of dopamine (DA)-induced apoptosis in cultured cerebellar granule neurons (CGNs) and the effect of muscarinic cholinergic receptor (mAChR) agonist carbachol on it. 【Methods】 The apoptosis of neurons was measured by phase-contrast microscopy, Hoechst 33258 nucleus staining and DNA fragmentation agarose gel electrophoresis. The neuronal viability was measured by fluorescein diacetate (FDA) staining. The activation of extracellular signal-regulated protein kinase (ERK) was determined by Western blot. 【Results】 Dopamine increases the phosphorylation of ERK and induces apoptosis in CGNs, which is blocked by both carbachol and PD 98059. The protective effect and the inhibiting ERK phosphorylation of carbachol were blocked by atropine. 【Conclusion】 DA-induced apoptosis in CGNs may be mediated by activation of ERK. Carbachol protects CGNs from DA-induced apoptosis by activating mAChR and subsequent inhibition of activation of ERK.

Key words: dopamine; apoptosis; muscarinic cholinergic receptor; extracellular signal-regulated protein kinase

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氨甲酰胆碱对多巴胺诱导的小脑颗粒细胞凋亡的保护机制

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摘 要: 【目的】 研究多巴胺诱导小脑颗粒神经元凋亡的分子机制, 以及胆碱受体激动剂氨甲酰胆碱对多巴胺诱导凋亡的作用。【方法】 在培养的小脑颗粒神经元建立多巴胺凋亡模型。用相差显微镜观察形态学, DNA 凝胶电泳和 Hoechst 33258 核染色分析神经元凋亡, 细胞的存活率用二乙酸荧光素(FDA)染色法检测。采用 Western blot 分析细胞外信号调控的蛋白激酶(ERK)激活情况。【结果】 多巴胺可诱导小脑颗粒神经元凋亡, 并可持续激活 ERK, 二者均可被氨甲酰胆碱和 PD 98059 抑制。氨甲酰胆碱对神经元的保护作用及对 ERK 激活的抑制作用可被阿托品阻断。【结论】 多巴胺在小脑颗粒神经元诱导凋亡可能是通过持续激活 ERK 介导的。氨甲酰胆碱通过激活 M 胆碱受体, 继而抑制了 ERK 的激活, 从而起到对神经元的保护作用。

关键词: 多巴胺; 凋亡; M 胆碱受体; 细胞外信号调控的蛋白激酶

Dopamine (DA) is a neurotransmitter under physiological conditions in central nervous system. However, accumulative evidences indicate that in certain conditions DA may also serve as a neurotoxin and participate in neurodegenerative processes, such as Parkinson's disease (PD). DA induces typical neuronal apoptosis in cortical, striatal, forebrain, cerebellar granule neurons (CGNs)[1] and in several types of cell lines. Until now, the molecular events involved in the process of DA-induced apoptosis are still unknown. Recent evidence suggests that the ERK (extracellular signal-regulated protein kinase) pathway may play an important role in triggering apoptosis in response to oxidative toxicity[2], and DA

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neurotoxicity is highly linked to oxidative metabolism[3]. In this study, we examined ERK pathway in DA-induced apoptosis and the effects of carbachol on it. The data presented here demonstrated that carbachol could protect CGNs from DA-induced death by inhibiting the phosphorylation of ERK.

1 Materials and Methods

1.1 Reagents

Basal medium eagle (BME), fetal bovine serum, and gentamycin were purchased from GIBCO. Dopamine (DA), carbamylcholine (Carbachol), atropine, hexamethonium, fluorescein diacetate (FDA), Hoechst 33258, and PD98059 were purchased from Sigma Chemical Co.

1.2 Preparation of cultured CGNs

Rat CGNs were prepared from postnatal day 8 Sprague Dawley rat pups (15 ~ 19 g, SUMS' s animal farms) as described by Novelli *et al*[4].

1.3 Exposure of CGNs to drugs

CGNs were maintained in standard medium (BME, 100 mL/L fetal bovine serum, 25 mmol/L KCl, 2 mmol/L glutamine, 50 mg/L gentamycin and 1 000 mg/L glucose) for 7 days and then the medium was replaced with serum-free standard medium. All experiments were carried out with neurons on the 8th day *in vitro* (DIV), and neuronal viability was determined 24 h later as indicated below. The following drugs were added to the granule neuron cultures at indicated time: DA (0.6 mmol/L), carbachol (1.25 mmol/L), atropine (0.01 mmol/L), hexamethonium (0.1 mmol/L), PD98059 (0.02 mmol/L, dissolved in 1 ml/L dimethyl sulfoxide).

1.4 Assessment of neuronal viability

Granule neuron viability was quantified after staining of the cells with fluorescein diacetate (FDA), which is deesterified only by living cells.

1.5 Detection of apoptosis

DNA fragmentation was detected by electrophoresis as described by Yan *et al*[5]. CGNs were visualized using Leica optics to detect morphological evidence of apoptosis and following stained with the fluorescent dye Hoechst 33258 to reveal nuclear condensation/ aggregation.

1.6 Western blotting

Protein level and activation (diphosphorylation of Thr202/Tyr204) level of ERK1/2 were determined by Western immunoblot using Cell Signal Phospho Plus p44/p42 MAP Kinase (Thr202/Tyr204) antibody Kit (#9100), and were expressed as fold *vs* control of optical density of certain band from Western immunoblot.

1.7 Stastical analysis

Data were expressed as mean ± SEM values, and statistical significance was assessed by using one-way ANOVA followed by Tukey-Kramer's multiple range tests. Differences were considered significant at $P < 0.05$.

2 Results

2.1 DA induces apoptosis in cultured CGNs

Cultured CGNs were exposed to 0.6 mmol/L DA in serum free BME for 4 h. DA induced a typical apoptotic DNA ladder with a 200-base pair range increase 24 h later (Fig. 1).

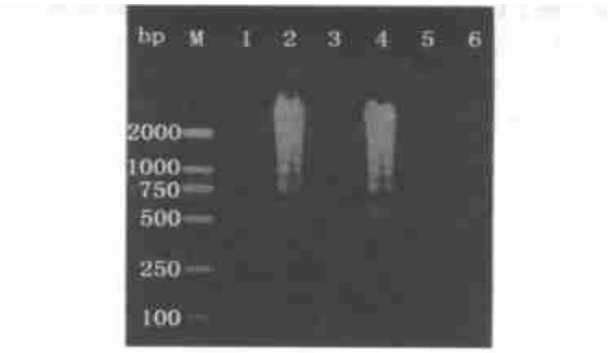

Fig. 1 DNA fragmentation of CGNs revealed by agarose gel electrophoresis

M, DNA size marker; Lane 1, control; Lane 2, DA; Lane 3, DA+carbachol; Lane 4, DA+carbachol+atropine; Lane 5, DA+carbachol+hexamethonium; Lane 6, DA+PD 98059

CGNs exposed to DA displayed morphological features of apoptosis, including cytoplasmic blebbing and heterochromatic clumping revealed by microscopy (Fig. 2b). DA induced nuclear changes were evaluated under a fluorescence microscope using

Hoechst 33258 staining. In control cells, the nuclei showed uniform staining, indicating that cells were healthy and nuclei were intact. With DA treatment, some nuclei exhibited typical apoptotic characteristics, such as condensation of nuclear chromatin and aggregation at the nuclear membrane (Fig. 3b). Neuronal viability was assessed after 24 h, more than 80% of the neurons died (Fig. 4).

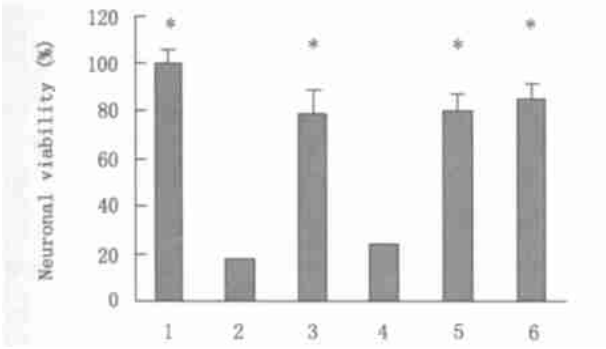

Fig. 4 Effect on the survival of CGNs

1, control; 2, DA; 3, DA+carbachol; 4, DA+carbachol+atropine; 5, DA+carbachol+hexamethonium; 6, DA+PD 98059; * $P<0.05$, compared with DA

2.2 DA activates the ERK1/2 pathway

To assess ERK phosphorylation in the presence or absence of DA, we used an antibody that recognizes only the activated, dually phosphorylated forms of ERKs. As shown in Fig. 5b, treatment of CGNs with 0.6 mmol/L DA for 5 min led to a 3.5-fold phosphorylation of ERKs relative to control, untreated cells. Persistent phosphorylation of ERKs in CGNs exposed to DA for 5 min ~ 2 h was observed. However, total ERK levels were not significantly affected by DA treatment (Fig. 5a).

2.3 The MEK-1/2 inhibitor, PD98059 protects CGNs from DA-induced apoptosis

Treatment of CGNs with PD98059, a selective inhibitor of the dual specific ERK-activating kinases MEK-1/2, markedly blocked DA-induced phosphorylation of ERKs, as shown in Fig. 6b. Moreover, PD 98059 also protected CGNs from DA-induced apoptosis at the same concentration (Fig. 1, Fig. 4).

2.4 Carbachol blocks DA-induced apoptosis of CGNs through activating mAChR

CGNs were pretreated with 1.25 mmol/L carbachol for 30 min before exposed to DA. Pretreatment of

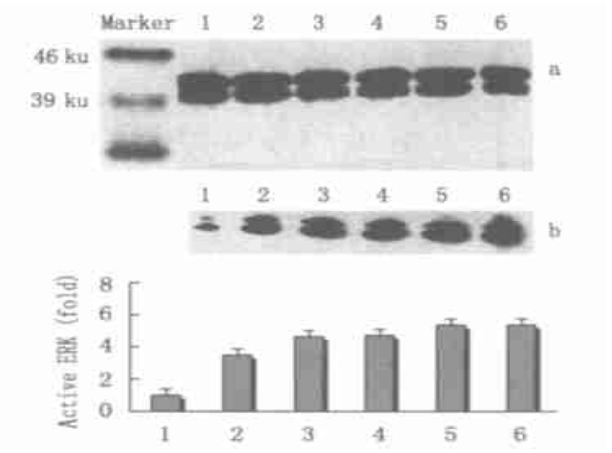

Fig. 5 Total ERKs(a) and phosphorylatin of ERKs(b) induced by dopamine examined by Western blot relative active ERK levels in control cells was given a value of 1

Lane 1, control; Lane 2, 5 min; Lane 3, 15 min; Lane 4, 30 min; Lane 5, 1 h; Lane 6, 2 h

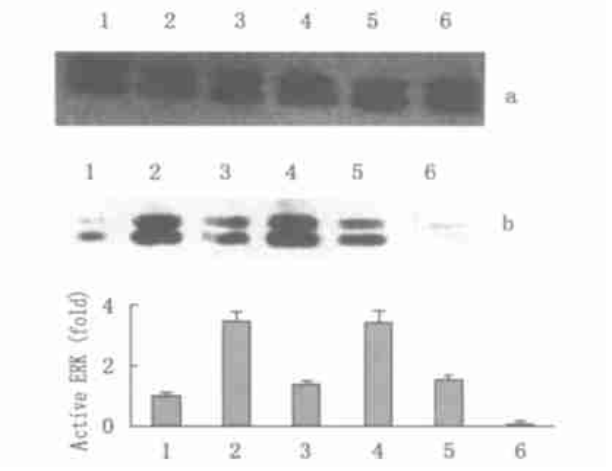

Fig. 6 Inhibition of DA-induced ERK phosphorylation in cerebellar granule neurons by carbachol and PD 98059

Lane 1, control; Lane 2, DA; Lane 3, DA+carbachol; Lane 4, DA+carbachol+atropine; Lane 5, DA+carbachol+hexamethonium; Lane 6, DA+PD98059

neurons with carbachol resulted in a significant increase in survival (Fig. 4) and prevented the DNA fragmentation (Fig. 1).

Hexamethonium, a specific neuronal nAChR antagonist, did not alter the protective effects of carbachol at concentration of 0.1 mmol/L (Fig. 4). In contrast, relatively low concentration of atropine (0.01 mmol/L), a specific mAChR antagonist, completely blocked the protective effects of carbachol

(Fig.4). Hexamethonium or atropine alone failed to induce apoptosis in neurons (data not shown).

Pretreatment of neurons with carbachol (1.25 mmol/L) before exposure to DA rescued most of the neurons and maintained normal nuclear morphology (Fig.3c), which was reversed by atropine (10 μM) (Fig.3d), but not by hexamethonium (data not shown).

2.5 Carbachol decreases phosphorylation of ERK1/2 increased by DA in CGNs

Pretreatment with carbachol reduced the DA-stimulated phosphorylation levels of ERK (Fig.6b). Total ERK levels in the CGNs were not affected by either DA or carbachol (Fig.6a). The inhibition of phosphorylation of ERK by carbachol was blocked by atropine but not by hexamethonium (Fig.6b).

3 Discussion

DA, the endogenous neurotransmitter of the nigro-striatal pathway, is a powerful oxidant that exerts its toxic potential through its oxidative metabolites. In this paper, we demonstrated that DA induced apoptosis in cerebral granule neurons.

We also provided evidences that the DA-induced apoptosis was mediated by the ERK activation pathway. Firstly, exposure of the cells to DA persistently activates the ERK pathway, i.e. phosphorylation of ERK1/2. DA strongly stimulates ERK activity after 5 min exposure of cells and lasts for at least 2 hours. Secondary, PD98059, a MEK1/2 inhibitor, can block the phosphorylation of ERK as well as apoptosis. Our results indicate that DA-induced persistent phosphorylation of the ERK may play a critical role in initiating the apoptotic suicide program.

ERK activation is typically associated with cell survival, proliferation and differentiation given their activation by mitogens and some cell survival factors[6]. However, ERK does not appear to act universally to promote cell survival in all models of neurodegeneration. For example, sustained activation of ERKs brought about by protein phosphatase inhibition induced neuronal cell death in hippocampal slice cultures[7]. Furthermore, the specific MEK1/2 inhibitor PD98059 reduced neuronal injury in focal cerebral ischemia[8]. It is possible that the precise parameters of ERKs, or other MAPKs, activation ultimately dictate whether the activated kinases participate in a cell death-promoting or cell survival pathway.

In the present study we have demonstrated that the AChR agonist carbachol prevented apoptosis of cultured CGNs induced by DA. The protective effect of carbachol is supported by morphological and biochemical data (Fig. 1, 2, 3). The survival-promoting effects of carbachol correlate with its activity in activating mAChR, and was completely blocked by atropine but not by hexamethonium. These data suggest that mAChR may mediate the antiapoptotic actions of carbachol. Carbachol inhibited the activation of ERK1/2 by DA, which was blocked by mAChR antagonist atropine but not by nAChR antagonist hexamethonium. It indicates that the protective effect of carbachol is mediated by activation of mAChR and subsequent inhibition of ERK phosphorylation.

We have previously reported that activation of the mAChR (possibly of the m3 subtype) blocked apoptosis of cultured CGNs exposed to physiological concentration of K^+ (nondepolarizing conditions)[5]. Carbachol also blocks beta-amyloid induced apoptosis in cultured cortical neurons through reversing or ameliorating the beta-amyloid induced downregulation of bcl-2 and upregulation of bax, p53, and c-fos genes[9]. In addition, carbachol protects against beta-adrenergic receptors-stimulated apoptosis via the activation of muscarinic receptor coupled G(i) protein and subsequent activation of p38 kinase[10]. These data indicate that mAChR may play an important role in neuronal survival.

The mAChR belongs to a large family of structurally related G protein-coupled receptors linked to several effector systems. Molecular cloning and pharmacological studies have demonstrated the existence of at least five different mAChR subtypes,

(contined on page 169)

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designated m1-m5. Which mAChR subtype is responsible for the protective effect and its molecular mechanism should be further studied.

(Fig. 2, 3 see in inside front cover)

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氨甲酰胆碱对多巴胺诱导的小脑颗粒细胞凋亡的保护机制 （正文见第 161 页）

Activation of Muscarinic Cholinergic Receptors Protects Cerebellar Granule Neurons from Dopamine – Induced Apoptosis via ERK Pathway (Text in page 161)

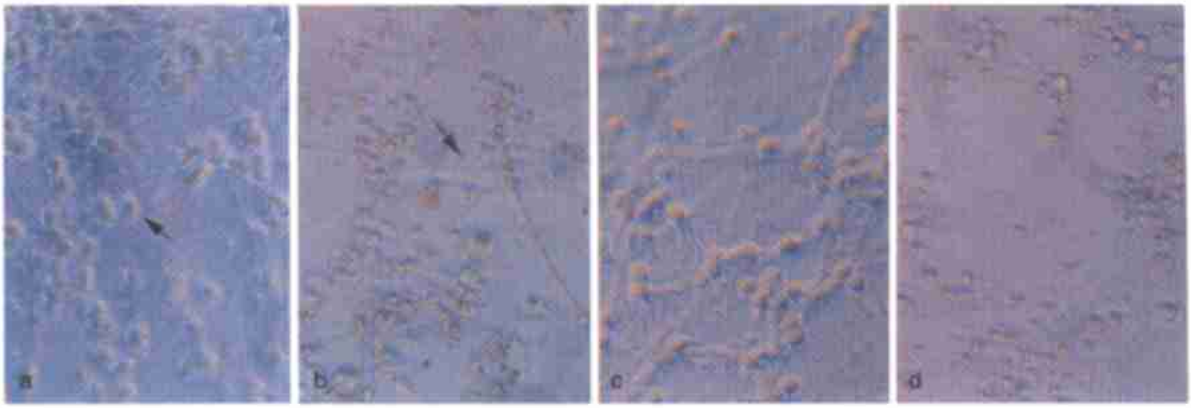

图 2　相差显微镜观察药物对小脑颗粒神经元形态的影响

Fig. 2　Morphological features of cerebellar granule neurons exposed to drugs revealed by phase contrast microscopy

a, control; b, DA alone; c, DA plus carbachol; d, DA plus carbachol plus atropine; Black arrows indicate neurons with normal morphology whereas white arrows indicate apoptotic neurons (×200)

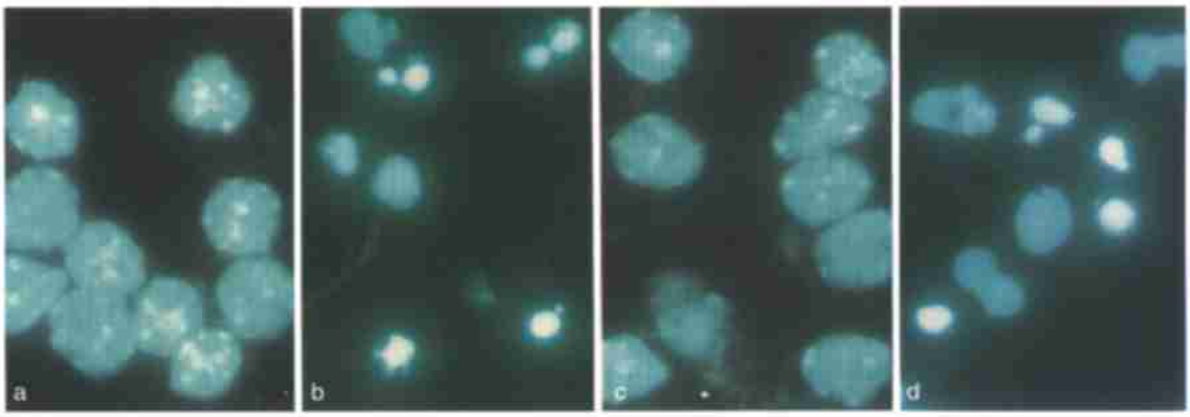

图 3　Hoechst 33258 核染色观察药物对小脑颗粒神经元核形态的影响

Fig. 3　Nuclear analysis of apoptotic cells stained with Hoechst 33258

a, control; b, DA alone; c, DA plus carbachol; d, DA plus carbachol plus atropine. (×1000)