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Effects of dexamethasone pretreatment on NF-κB activation and neuronal apoptosis in the brain of neonatal rats following hypoxia-ischemia

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Abstract: Objective Previous studies suggest that dexamethasome (DEX) pretreatment can reduce hypoxic-ischemic brain damage (HIBD), but the mechanism for this effect has not been identified. This study examined the effects of DXM pretreatment on NF-κB activation and neuronal apoptosis in the brain of neonatal rats with HIBD, in order to identify a possible mechanism for the protective effect of DEX pretreatment in HIBD. **Methods** Forty-two 6-day-old Sprague-Dawley (SD) rats were randomly assigned to 5 groups: Normal controls (n=8), Sham-operated (n=8), HIBD (n=8), DEX pretreated (P-DEX, n=9) and DEX treated (DEX, n=9). HIBD was induced by hypoxia exposure combined with ligation of the left common carotid artery. DEX (0.1 mg/kg) was administered to rats in the P-DEX group 24 hrs before HI and to rats in the DEX group immediately after HI. After 72 hrs of HI, the rats were sacrificed and then the brain tissues were removed. Apoptosis was examined by means of terminal-deoxynucleotidyl transferase mediated d-UTP nick end labeling (TUNEL). The expression of p65 protein in tissue sections was detected by immunohistochemistry. The expression of IκBα protein was measured by Western blotting. Co-localization of p65 protein expression and apoptosis was determined with double-label immunofluorescence. **Results** The number of p65 positive cells and apoptotic cells was greater and the expression of the IκBα protein was less in the HIBD and DEX groups ($P<0.01$) when compared with the Normal control and Sham-operated groups ($P<0.01$). There were fewer p65 positive cells and apoptotic cells, and the level of IκBα protein expression was greater in the P-DEX group ($P<0.01$) compared with the HIBD group. There were no significant differences between the DEX and HIBD groups. In the HIBD group, the level of NF-κB activation and the extent of neuronal apoptosis were significantly correlated ($r=0.775$, $P<0.01$). **Conclusions** The activation of NF-κB may play an important role in the development of neuronal apoptosis in neonatal rats with HIBD. The protective effects of DEX pretreatment may work through the inhibition of NF-κB activation which may then inhibit neuronal apoptosis.

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Key words: NF-κB; Apoptosis; Hypoxia-ischemia, brain; Dexamethasone; Rat, newborn

地塞米松预处理对新生大鼠缺氧缺血后脑内 NF-κB 活性及神经细胞凋亡的影响

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[**摘 要**] **目的** 探讨地塞米松预处理对新生大鼠缺氧缺血后脑内 NF-κB 活性及神经细胞凋亡的影响。**方法** 42 只新生大鼠随机分为 5 组,即正常对照组(n=8),假手术组(n=8),缺氧缺血组(HIBD,n=8),地塞米松治疗组(DEX,n=9)及地塞米松预处理组(P-DEX,n=9)。于缺氧缺血(HI)后 72 h 取脑,以 Western 印迹法检测脑组织中 NF-κB 抑制蛋白 IκBα 表达,TUNEL 法检测细胞凋亡,免疫组织化学法检测 NF-κB 亚基 p65 核移位情况,免疫荧光双标法检测 P65 核移位与细胞凋亡共表达。**结果** 与正常对照组和假手术组比较,HIBD 组及 DEX 组 p65 阳性细胞及 TUNEL 阳性细胞数明显增加($P<0.01$),IκBα 蛋白表达明显减少($P<0.01$)。P-DEX 组也可见 p65 阳性细胞及 TUNEL 阳性细胞表达,但较 HIBD 组及 DEX 组明显减少($P<0.01$),而 IκBα 蛋白表达明显增

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多($P<0.01$)。直线回归分析显示,在 HIBD 组中 NF-κB 的活化与缺氧缺血后神经细胞凋亡密切相关($r=0.775$, $P<0.01$)。**结论** NF-κB 的活化与缺氧缺血后神经细胞凋亡密切相关。DEX 预处理对缺氧缺血性脑损伤的保护作用可能与抑制 NF-κB 的活化,减少细胞凋亡有关。 **[中国当代儿科杂志,2004, 6(4): 251－255]**

[关 键 词] 核因子－κB;凋亡;缺氧缺血,脑;地塞米松;大鼠,新生
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Brain damage resulting from hypoxia-ischemia (HI) in the perinatal period remains a major cause of permanent neurological dysfunction, such as cerebral palsy, mental retardation and epilepsy. For the prevention of the HI sequels in the brain, proper treatment is indispensable during the perinatal period.

It has been shown that there are two patterns of cell death in hypoxic-ischemic brain damage (HIBD), that is apoptosis and necrosis. Further studies have shown that apoptosis is the cause of delayed neuronal death[1,2], but the mechanism of apoptosis following HI injury has not been indentified. NF-κB, one of the nuclear transcription factors, works as a transducer transporting information to the nucleus to initiate gene expression. Its regulation of programmed cell death is gradually becoming understood[3]. But the role of NF-κB is still controversial. It may exert either anti- or pro-apoptotic effects depending on different cell types and apoptotic stimuli[4]. Therefore, the role of NF-κB on HIBD in neonatal rats is to be elucidated.

Glucocorticoids are extensively used in the human fetus and newborns as promoters of fetal lung maturation and as an adjunct to therapy in bronchopulmonary dysplasia. Glucocorticoids used to be considered as potent inductors of apoptosis in thymocyte cells. But recently many studies have demonstrated a protective effect of dexamethasone (DEX) on the neonatal rats when it was adminstered before HI insults[5－7]. In addition, the protective effect was dose-and time-dependent[8]. However, the exact mechanism for this protective effect has not been elucidated. This paper examined the effects of DEX pretreatment on NF-κB activation and neuronal apoptosis in neonatal rat model of HIBD so as to identify the mechanism of this protective effect.

Materials and methods

Animal model and grouping

Forty-two 6-day-old Sprague-Dawley rats were randomly assigned into five groups: Normal control (n=8), Sham-operated (n=8), HIBD (n=8), DEX pretreated (P-DEX, n=9) and DEX treated (DEX, n=9). In the P-DEX group, 0.1 mg/kg of DEX dissolved in saline was injected intraperitoneally 24 hours before cerebral HI. In the DEX group, 0.1 mg/kg of DEX was injected intraperitoneally immediately after cerebral HI.

According to the Rice[9] method, 7-day-old Sprague Dawley rats were anesthetized with ether. After the left common carotid arteries had been ligated, the rats were sent back to their dams and allowed to suckle for 1 hour. Following the recovery period, these rats were exposed to hypoxia with mixed gas (8% oxygen with 92% nitrogen) for 2 hours in a 30 cm × 40 cm × 50 cm plastic chamber, maintaining its temperature at 37℃. After 72 hours of cerebral HI, the rats were sacrificed and then the brain tissues were removed.

Detection of p65 protein expression by immunohistochemistry

Enzyme digesting methods for paraffin section were used. Sections of 4 μm thick were de-waxed, rehydrated, inactivated by 3% H_2O_2, digested by complex digestion liquid and blocked by goat blood serum. After the sections were incubated with monoclonal antibody of p65 (1∶100 dilution in PBS) overnight at 4℃, biotinated goat anti-mouse IgG was added next day. They were stained with streptavidin-biotin complex (SABC) and diaminobenzidine (DAB). The first antibody was replaced by phosphated-buffer saline (PBS) as negative control. Under a light microscope, cells with brown particles in the nucleus were positive cells. Posi-

tive cells of cortex and hippocampus in five fields of vision in every section were counted at ×400 magnification. For double-labeling, sections were incubated with monoclonal antibody of p65 and TdT and Dig-dUTP buffer, followed by incubation with Cy3-conjugated goat anti-mouse secondary antibodies. Immunostaining was visualized by fluorescence microscope. Adjacent sections were stained with hematoxylin and eosin (H&E) for cellular morphology.

Apoptosis detection

Cell apoptosis was detected by using an in situ cell death detection kit (Roche, USA) according to the manufacturer's protocol. Briefly, sections were incubated with TdT and Dig-dUTP buffer for 1 hour at 37℃, followed by incubation with POD thans form buffer, then sections were stained with diaminobenzidine (DAB). Under a light microscope, cells with brown particles in the nucleus were positive ones. Positive cells of cortex and hippocampus were counted.

Western blot

The left brain tissues were finely minced and homogenized in 5 volumes of ice-colded protein-extracting buffer. The homogenate was centrifuged at 10 000 r/m at 4℃ for 15 minutes, then the supernatant was collected. The protein concentration was determined by the improved Lowry method. Proteins were loaded and separated on 8% SDS-polyacrylamide gel electrophoresis gel under denatured conditions. Proteins were then transferred to nitrocellulose membranes. After incubation in blocking solution [5% milk in Tris buffered saline with Tween 20 (TBST)] at room temperature for 1 hour, the monoclonal antibody of $I\kappa B\alpha$ (Santa Cruze, CA) was added and they were reacted at 4℃ overnight. The second antibody alkaline phosphatase-conjugated goat anti-mouse IgG was added next day. The results of Western blot was analysed by an automated gel-imaging system (GIS-700 D).

Statistical analysis

All data were expressed as the mean±standard deviation ($\bar{x} \pm s$). The Dunner t test was used to analyse the differences of multi-means. The relationship between activiation of NF-κB and neuronal apoptosis was analysed by linear correlation.

Results

Pathological changes of cortex and hippocampus

In the Normal control and Sham-operated groups, the number and structure of neurons in cortex and hippocampus were normal. In the HIBD and DEX groups, cells in cortex and hippolampus were disordered and degenerated. Karyopyknosis was seen and the cytoplasm was stained by eosin. There were few changes in the P-DEX group. See Figure 1.

Expression of p65 protein and apoptotic cells in cortex and hippocampus

In the Normal control and Sham-operated groups, there were few apoptotic cells and p65 positive cells in the cortex and hippocampus. In the HIBD group, the apoptotic cells and p65 positive cells in the cortex and hippocampus were significantly more than those in the Normal control and Sham-operated groups (all $P<0.01$). In the P-DEX group, the number of apoptotic cells and p65 positive cells in the cortex and hippocampus were significantly less when compared with those of the HIBD group (all $P<0.01$), while in the DEX group, they still remained very high. There were no significant differences compared with those of the HIBD group ($P>0.05$). See Table 1 and Figures 2 and 3. Double-label immunofluorescence experiments revealed p65 immunoreactivity in TUNEL-positive cells in the HIBD group, which confirmed the close relationship between the apoptotic cells and p65 positive cells by a linear correlation analysis ($r=0.775$, $P<0.01$). See Figure 4 and Figure 5.

Table 1 Results of p65 immunihistochemistry and TUNEL ($\bar{x} \pm s$)

Group	n	Number of p65 positive cells	Number of TUNEL positive cells

Normal control	8	2.5±0.93	6.00±0.76
Sham-operated	8	2.625±1.061	6.50±1.20
HIBD	8	352.63±5.90[a,b]	384.05±11.42[a,b]
DEX	9	355±10.2	372.75±12.31
P-DEX	9	132.67±4.24[c]	142.25±6.76[c]

Note: a vs Normal control group $P<0.01$; b vs Sham-operated group $P<0.01$; c vs HIBD group $P<0.01$

Figure 5 Result of the linear correlation analysis

IκBα protein expression in the brain

Western blot showed that the expression of IκBα protein was obvious in the brain tissue of the Normal control and Sham-operated groups. In the HIBD and DEX groups, the expression of IκBα protein was significantly lower than those in the Normal control and Sham-operated groups (both $P<0.01$). In the P-DEX group, the expression of IκBα protein was significantly higher than those in the HIBD and DEX groups (both $P<0.01$). See Figure 6 and Table 2.

Table 2 Expression of IκBα protein

($\bar{x}\pm s$)

Group	Gray scale
Normal control	1
Sham-operated	0.9840±0.008
HIBD	0.1834±0.033[a,b]
DEX	0.1835±0.028
P-DEX	0.9185±0.012[c]

Note: a vs Normal control group $P<0.01$; b vs Sham-operated group, $P<0.01$; c vs HIBD group $P<0.01$

Discussion

Since the protective effect of DEX in HIBD was discovered in 1991, several mechanisms have been proposed to explain the protective effect of DEX, including activiation of antioxidant enzymes, increased cerebral blood flow or systemic circulation and hyperglycemia. But some researchers consider that none of the above mechanisms contributes to this protective effect[10,11]. Recently, several reports have shown that apoptosis increased dramatically after cerebral HI[1,2]. In this study, we also found the number of apoptotic cells increased significantly 72 hours after cerebral HI, which further confirmed that apoptosis may play an important role in HIBD.

NF-κB is one of the transcription factors. In most cells, NF-κB is sequestered in the cytoplasm by interaction with IκB family, including IκBα. Once stimulated, IκBα is phosphorylated and degraded, then free NF-κB dimmers (p65, p50 and c-Rel etc.) translocate into nucleus and bind with the κB site of the corresponding target genes[3]. Therefore, the activation of NF-κB is closely related with phosphorylation and degradation of IκB. In the present study, we found the expression of IκBα protein was weakened, p65 protein translocated into nucleus and apoptotic cells increased in the HIBD group. Double-label immunofluorescence revealed p65 immunoreactivity in TUNEL-positive cells, which indicated the activation of NF-κB was associated with neuronal apoptosis.

Glucocorticoids were proved to inhibit NF-κB activiation through the following three pathways. Mechanically, glucocorticoids diffuse through the cell membrane and bind to their inactive glucocorticoids receptors (GRs). Activated GRs can then modulate transcriptional events by directly associating glucocorticoids GCs response elements (GRE) or by antagonizing the activity of transcription factors (including NF-κB) by the following methods: (1) Inducing gene transcripton and protein synthe-

sis of the NF-κB inhibitor, IκB. (2) Inhibiting of NF-κB binding to DNA, or association with NF-κB bounding to the κB DNA site. (3) Competing with NF-κB for nuclear coactiviators[12]. In the present study, we found the level of IκBα protein expression in P-DEX group was higher than that of HIBD group, which indicated DEX may antagonize NF-κB activiation by inducing IκBα protein synthesis. Further studies are needed to investigate whether DEX may interfere through the other two pathways.

Why was there no protective effect in the DEX group? Two reasons may contribute to the result: (1) Because of the incomplete development of brain collateral circulation in neonatal rats, DEX could not effectively get to brain tissues through the ligated left common carotid artery. Therefore, it could not influence the expression of IκBα protein. (2) Once triggered, NF-κB is activiated immediately, and its free NF-κB dimmers translocate into nucleus and bind to corresponding DNA sites to modulate transcription events. At this time, even if DEX could induce and increase the expression of IκBα protein, the IκBα proteins located in the cytoplasm can not bind with p65/p50 protein located in the nucleus. But some research has reported that newly-synthesised IκBα protein can dissociate NF-κB-DNA complex out from nucleus[13]. However, this phenomenon has not been detected in this study. (All figures are on the back cover)

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· 消息 ·

中国当代儿科杂志被美国《化学文摘》收录

从 2004 年第 6 卷第 1 期起，中国当代儿科杂志被国际著名检索系统美国《化学文摘》(CA)收录。这标