·Original Article in English·

Insulin2like growth factor21 reduces β2amyloid precursor protein expression after ischemic white matter damage in near2term fetal sheep

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Abstract: Objective β2amyloid precursor protein (β2APP) is thought to be a sensitive marker for brain white matter damage (WMD) and participates in the mechanisms of hypoxic2ischemic brain damage. This paper aims to study the influence of ischemia and IGF21 treatment on the expression of β2APP in white matter of near2term fetal sheep. **Methods** Romney2Suffolk fetal sheep were instrumented at 117 to 124 days of gestation (term = 147 days). Reversible cerebral ischemia was induced by occlusion of bilateral carotid arteries for 30 mins. After damage the sheep were randomly divided into two groups: the Ischemic group (n = 8) and the IGF21 treatment group (Treatment group, n = 9). The sham2operation group (n = 5) was used as Control group. In the Treatment group, 3 μg (1 ml) recombinant human IGF21 (rhIGF21) was infused into the left lateral ventricle, 90 mins after reperfusion. The Control group received infusion of 1 ml artificial cerebrospinal fluid into the left ventricle. Ninety2six hrs after ischemia, the sheep were sacrificed and the brains were fixed. Immunohistochemical staining was performed to assess glial fibrillary acidic protein (GFAP), β2APP positive cells and the myelin basic protein (MBP) density in the white matter. Fluorescent staining was performed for double labeling. **Results** The MBP density of the Ischemic group (4.7 ±7.1) was significantly reduced as compared with the Control group (27.8 ±4.8, P < 0.001). β2APP positive cells were not observed in the Control group. β2APP positive cells of the Ischemic group increased significantly after ischemia (49.6 ±23.7, P < 0.001). IGF treatment significantly reduced the β2APP positive cells (17.9 ±16.5, P < 0.01). Fluorescent double labeling showed that the β2APP positive cells were co2localized with GFAP positive cells. **Conclusions** Ischemia increases the expression of β2APP by astrocytes in near2term fetal sheep white matter, which may underlie the mechanisms of ischemic WMD. IGF21can reduce the expression of β2APP, which may be mechanism of its protective effect against WMD.

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Key words: Insulin2like growth factor21; White matter damage; β2amyloid precursor protein; Fetal sheep

胰岛素样生长因子21 减少胎羊缺血性脑白质损伤后淀粉样前体蛋白表达

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[**摘 要**] **目的** 淀粉样前体蛋白(β2APP)是脑白质损伤早期敏感的指标,并参与缺氧缺血性脑损伤机制。本研究观察胎羊缺血性脑白质损伤及胰岛素样生长因子21(IGF21)治疗对淀粉样前体蛋白(β2APP)表达的影响。**方法** 胎羊于胎龄 1172124 天(足月为 147 天)时通过双侧颈动脉阻塞 30 min 造成双侧脑缺血损伤,损伤后胎羊随机分为损伤组(n = 8)和重组人 IGF21(rhIGF21)治疗组(n = 9);另设正常对照组(n = 5),为假手术动物。治疗组缺血后 90 min 经侧脑室注射 3 μg rhIGF21;损伤组经侧脑室注射等量人工脑脊液。缺血损伤后 96 h 结束实验,处死动物,取出胎羊,固定脑组织。免疫组化法检测脑白质胶质原纤维酸性蛋白(GFAP)、β2APP 阳性细胞及白质

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内髓鞘碱性蛋白(MBP)密度。应用免疫荧光双标记观察 APP 表达阳性细胞。结果　与正常对照组(27.8 ±4.8)比较,缺血损伤组 MBP 密度(4.7 ±7.1, $P < 0.001$)明显减少。正常对照组未见β2APP 阳性细胞,损伤后阳性细胞数明显增加(49.6 ±23.7, $P < 0.001$),rhIGF21 治疗可减少β2APP 阳性细胞数(17.9 ±16.5, $P < 0.01$)。免疫荧光双标记显示部分细胞为β2APP2GFAP 双标阳性细胞。结论　胎羊缺血性脑白质损伤可导致星形胶质细胞表达β2APP,β2APP 表达增加可能与脑损伤有关。IGF21 可减少β2APP 表达,可能是减轻脑白质损伤的机制之一。

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[**关　键　词**]　胰岛素样生长因子21;白质损伤;β2淀粉样前体蛋白;胎羊

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Hypoxic2ischemic encephalopathy (HIE) during the perinatal period remains a major disease that caus2es neurological system sequelae in childhood[1]. There is increasing evidence suggesting that white matter of premature brain is highly vulnerable to ischemia[2,3]. Perinatal brain white matter damage (WMD) can cause neurological sequelae such as epilepsy, cerebral palsy and neurobehavioral abnormalities[4]. β2amyloid precursor protein (β2APP) is a transmembrane pro2tein widely distributed in the central nervous system. Normally, axonal transport of β2APP is rapid. WMD may disturb axonal transport, which in turn lead to β2APP accumulation in axone. Thus β2APP is thought to be a sensitive marker for axonal lesions in brain white matter. Increased β2APP expression after injury suggests that it is an acute response protein after injury and may participate in hypoxic ischemic brain injury[5].

The insulin2like growth factor21 (IGF21) pro2tects against WMD caused by hypoxia2ischemia[6]. However, whether IGF21 has an influence on β2APP expression remains unclear. The purpose of this study is to examine β2APP expression after hypoxic2is2chemic WMD in fetal lambs and the effect of IGF21 on β2APP expression.

Materials and methods

Grouping

The experiments were approved by the Animal Ethics Committee of the University of Auckland, New Zealand. Romney2Suffolk fetal lambs were in2strumented at 117 to 124 days of gestation (term = 147 days), and were randomly assigned to the Control group ($n = 5$), the Ischemic group ($n = 8$) and the IGF21 treatment group (Treatment group, $n = 9$).

Establishment of animal model

Animals in the Ischemic and Treatment groups were operated on under anesthesia. Bilateral subaxil2lary arteries were catheterized and occipital2vertebral artery anastomoses were ligated so as to block the blood supply from the vertebral artery to the carotid arteries. A pair of ballooned inflatable occluder cuff was placed around each carotid artery. Electroen2cephalogram (EEG) electrodes (AS63325SSF, Coon2erf Wire, USA) were placed on the dura overlying the parietal lobe in the parasagittal region. The left lateral ventricle was cannulated. Reversible cerebral ischemia was induced by occlusion of bilateral carotid arteries through saline infusion into the cuffs for 30 minutes via float catheters. Successful occlusion (is2chemic injury) was indicated when isoelectric EEG lasted for 30 seconds. Ninety minutes after reperfu2sion, the Treatment group received a 12hour infusion of 3 μg recombinant human IGF21 (rhIGF21, Chi2ron, USA) diluted in 1 ml artificial cerebrospinal flu2id (CSF) into the lateral ventricle. The Ischemic group only received an infusion of 1 ml artificial CSF. The animals in the Control group were sham2operated and catheterized but did not experience ischemia and intracerebroventricular injection. Fetal lambs were returned to the ewes' uteri and gentamycin was ad2ministrated into the amnionic amniotic cavities. The ewes were kept in a temperature2controlled room (16 ℃, humidity 50 %) for 3 days, and received gen2tamycin injections to prevent infection. Fetal arterial blood gas analyses were monitored and catheters were flushed by heparinized saline daily. Perfusion of arti2ficial CSF was performed daily to keep lateral ventri2cle catheter patent. Ninety2six hours after ischemia, the ewes were sacrificed and the fetuses were immedi2ately removed through an abdominal incision and then perfused with normal saline followed by 10 % forma2lin. Fetal brain tissues were then fixed, embedded and sectioned.

Immunohistochemistry

Mouse monoclonal anti2GFAP antibody (1∶500, Sigma, USA) and anti2MBP (1∶100, Roche, Ger2many) were used as primary antibody respectively. Antibody 22C11 was used to detect β2APP, since it can recognize β2APP isoforms with or without the KPI domain. Slides were incubated with the primary antibodies for 12 - 28 hours at 4 ℃. Biotinylated horse anti2mouse or sheep anti2rabbit secondary anti2body (1∶200, Sigma, USA) were added for 12 hours at 4 ℃. Sections were stained by DAB. For im2munofluorescence, FITC2conjugated horse anti2mouse antibody (Molecular Probes, Eugene, USA) was used to detect GFAP, Texas red2conjugated horse an2ti2mouse or sheep anti2rabbit antibody was used to de2tect β2APP, and FITC2labeled Griffonia simplicifolia (Sigma, USA) were used to detect IB4.

The GFAP, IB4 and APP positive cells in the white matter were quantified under light microscope. The number of positive cells was counted in the white matter of transverse sectional with a vertical length of 0.54 mm (Figure 1 on the front back cover). MBP densities in the white matter of the same region as well as in the adjacent grey matter were measured by an image analysis instrument (Sigmascan) and the differences were calculated. The average difference of areas 1 - 3 was used in the statistical analysis. Results of immunofluorescence were analyzed by the Leica TCS4D system and confocal microscope.

Statistical analysis

Data were analyzed by the Prism 3.0 software. All numbers were represented as $x \pm s$. Comparison between two groups was done by the Student's t test and comparison among multiple groups was done by ANOVA, followed by a comparison between groups using Dunnett2 t test if statistical differences were ob2served. A P of < 0.05 was considered statistically significant.

Results

Ischemic WMD

MBP staining was compact in normal white mat2ter and its arrangement was consistent with axons un2der high magnification. Ischemia led to a marked loosening of MBP staining (Figure 2 on the front back cover). MBP density in the Ischemic group (4.7 ± 7.1) was significantly lower than that of the Control group (27.8 ± 4.8, $t = 7.013$, $P < 0.001$). Increasing GFAP positive cells were found in the white matter of the Ischemic group compared with the Control group (35.1 ± 15.4 vs 24.9 ± 13.4). But the difference failed to reach statistical signifi2cance. Astrocytes appeared hypertrophy after is2chemia (Figure 2 on the front back cover).

β2APP expressions in white matter

β2APP positive cells could not be detected in the white matter of the Control group, while the cells markedly increased in the Ischemic group (49.6 ± 23.7, $P < 0.001$). The β2APP positive cell de2creased after rhIGF21 treatment (17.92 ± 16.50, $P < 0.01$) as compared with the Ischemic group. Nine2ty2six hours after ischemia, β2APP was mainly local2ized in the cytoplasm, but not in the axons. Under high magnification, the morphology of β2APP positive cells appeared to be similar to that of astrocyte and microglia, however double immunofluorescence showed β2APP and GFAP co2localization (Figure 3 on the front back cover) and no β2APP/ IB4 double posi2tive cells were observed.

Discussion

The animal model in this research was fetal lamb brain damage due to intrauterine ischemia. Fetal lambs at 117 - 124 days of gestation are near2term fe2tuses. According to comparative anatomy, the brain development at this stage is similar to that of full2term human fetuses. The pathological changes of the brain damage seen in this model are similar to those in full2term neonates with HIE. The main injury sites are the parasagittal region (both white and grey mat2ters) and the hippocampus. Since the sulcus and gyral structures of the sheep brain are similar to those of hu2man, and there is abundant subcortical white matter in the sheep brain, sheep are useful for studying WMD.

The results of this research indicate that in2trauterine hypoxia2ischemia induced WMD and in2crease β2APP expression by astrocytes in the white matter. A single dose of IGF21 injection to the lateral

ventricle could decrease β2APP expression.

Physiologically, β2APP nourishes neurons, promotes neurite growth and synaptogenesis. However, under pathological conditions, β2APP is associated with pathogenesis of brain damage. Over2expression of β2APP can activate caspase23 and induce microglial pro2inflammatory response, which are associated with neuronal apoptosis[7]. Cleavage products of β2APP directly participate in apoptosis. High levels of β2APP is neurotoxic and is associated with ischemic brain injury[8]. Clinical research has also observed that in cadaveric specimens of premature infants with periventricular leukomalacia (PVL), increased β2APP expression was found around the infarct tissues, which was correlated with the extent of injury[9]. However in this study, β2APP was localized in the cytoplasm not in the axons after ischemia. The reason that axonal β2APP aggregation was not observed at 96 hours post2ischemia may be that β2APP has a short half2life and its axonal aggregation only occurs at an early time after injury.

This research found β2APP positive cells were morphologically similar to astrocytes and microglia, while double immunofluorescence results suggested that β2APP was expressed by astrocytes in this study. The finding is similar to Pluta's study[10], indicating that astrocytic expression of β2APP after ischemic injury is associated with neurodegenerative changes after injury.

Previous research has shown that the same model can lead to an increased expression of caspase23 in brain white matter[11] and IGF21 treatment could decrease the caspase23 expression and brain WMD. β2APP can activate caspase23 and also serve as a substrate for caspases, which in turn participate in the pathogenesis of ischemic brain injury by promoting apoptosis[12]. The increased expression of β2APP may have participated in the pathogenesis of ischemic WMD in fetal lambs in this research. IGF21 can inhibit β2APP2induced neuronal apoptosis by binding to its homologous receptor[13]. The result that IGF21 treatment decreased β2APP expression after ischemia may be related to the protective effect of IGF21 against ischemic WMD in fetal lambs. Further studies are required to examine the time course of the changes of β2APP expression after ischemia and their correlation, as well as the dose2effect of IGF21. These studies will be helpful in clarifying the role of IGF21 in protecting immature brain from ischemic damage.

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Insulin-like growth factor-1 reduces β-amyloid precursor protein expression after ischemic white matter damage in nearterm fetal sheep

(These figures refer to the paper on page 449)

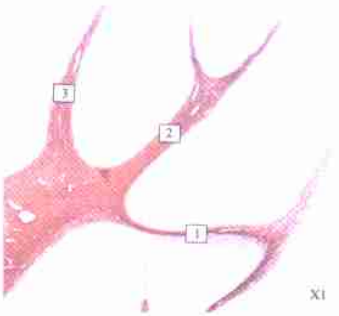

Figure 1 Brain white matter (× 1)

Note: The GFAP, IB4 and APP positive cells and MBP densities were measured in the areas 1, 2 and 3.

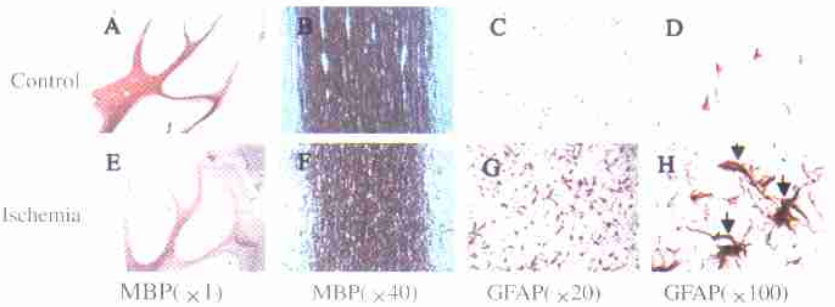

Figure 2 MBP and GFAP immunohistochemistry

Note: MBP staining was compact in normal white matter (A) and its arrangement was consistent whih axons under high magnification (B). Ischemia led to a marked loosening of MBP staining (E, F). GFAP positive cells could be found in the white matter of the Normal group (C, D), while the cells markedly increased and became hypertrophy after ischemia (G, H).

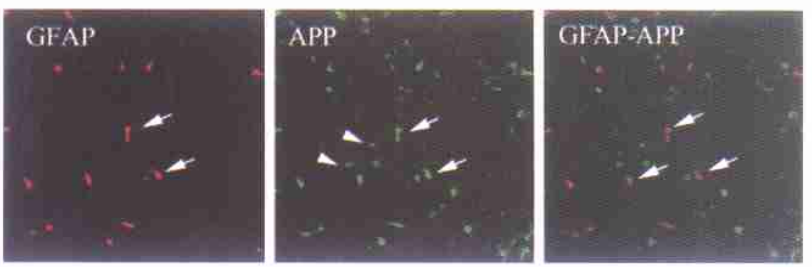

Figure 3 Double immunofluorescence showed the β-APP and GFAP double positive cells.

胰岛素样生长因子-1（IGF-1）对缺氧缺血脑损伤新生鼠内源性IGF-1和IGF-1受体基因表达的影响

（正文见第470页）

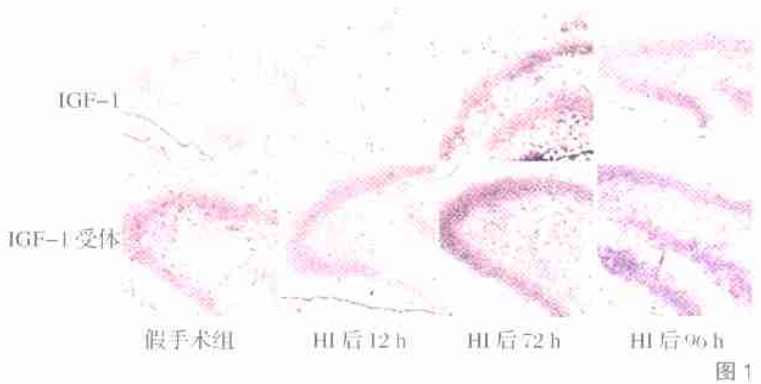

图1

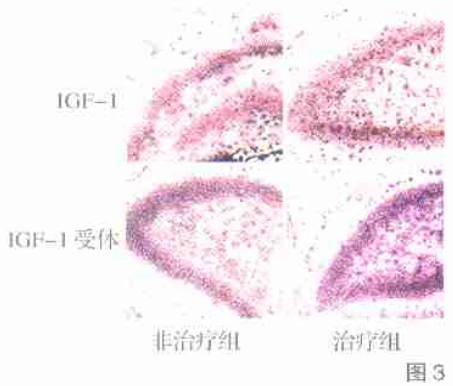

图3

图1 HIBD后海马IGF-1和IGF-1受体mRNA表达的变化（原位杂交，× 100）

Figure 1 Expressions of IGF-1 and IGF-1 receptor mRNA in the hippocampus after HIBD (in situ hybridization, × 100)

Note: Expressions of IGF-1 receptor and IGF-1 receptor mRNA in the hippocampus increased after HI, and reached a peak at 72 hrs post-damage.

图3 缺氧缺血后72小时，IGF-1治疗组与非治疗组IGF-1和IGF-1受体mRNA表达（原位杂交，× 100）

Figure 3 Expressions of IGF-1 and IGF-1 receptor mRNA in the IGF-1-treated and untreated groups at 72 hrs after hypoxia and ischemia (in situ hybridization, × 100)

Note: After rhIGF-1 treatment, the expression of IGF-1 mRNA in the hippocampus slightly decreased, while IGF-1 receptor mRNA expression significantly increased at 72 hrs post-damage as compared with the untreated group.