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WIN55, 212-2 increased the level of BDNF mediated PKAC-β and C-FOS in cerebral cortex after ICH in rats

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ABSTRACT

To observe whether cannabinoid receptor (CB1R) agonist improve neurological function accompanied by an increase in Brain-Derived Neurotrophic Factor (BDNF), mediated by C-FOS and a decrease in PKA C-β in cerebrum cortex after intracerebral hemorrhage (ICH) in rats. Intracerebral hemorrhage model of rat was made by VII Collagenase, which was killed for brain tissue as specimens with 24 hours. The mRNA levels of PKAC-β, C-FOS and BDNF expression were detected by semi-quantitative RT-PCR. The expression of PKAC-β, C-FOS and BDNF proteins were determined by Western blotting and immunohistochemistry. Intraperitoneal (i.p.) injection of WIN55-212-2 could obviously improve some nervous deficit symptoms and increased the mRNA and protein levels of BDNF. Increased BDNF synthesis was accompanied by increased activation of C-FOS and downregulation of PKA in the ipsilateral cerebral cortex prepared from WIN55-212-2 rats. PKAC-β, C-FOS, and BDNF proteins are expressed on membrane of neuron, nucleus of neuron or the cytoplasm of glial cells respectively. The expression of BDNF is induced not only by activation of C-FOS, but also by inhibiton of PKA through stimulation of MAP kinase in WIN55, 212-2 rats.

KEYWORDS

WIN55-212-2;
ICH;
BDNF;
PKAC-β;
C-FOS.

INTRODUCTION

ICH is bleeding in the brain caused by the rupture of a blood vessel in the head. Blood irritates the brain tissues, causing cerebral swelling. It can collect into a mass called a hematoma. Either swelling or a hematoma will increase pressure on nearby brain tissues and can quickly destroy them. So the clinical symptoms usually develop suddenly, without warning, often during activity. Intracerebral hemorrhagic stroke accounted for 10% -25% of stroke. In central nervous system (CNS), cannabinoids（CBs）can be broadly defined as compounds with actions on cannabinoid receptor‘I to protect the neurons. When CB1R is activated, it will trigger a cascade of intracellular changes. Signalling pathways have been the focus of research into the mechanisms of cannabinoids. Signal transduction mechanisms of CB1R-mediated include inhibition of adenylyl cyclase and N-type Ca^{2+}, but stimulation of MAP kinase and PI3K/PKB[1]. BDNF binding toTrk receptor is

important for synaptic plasticity and neuroprotection. Cannabinoids signalling pathways have an effect on the expression of BDNF through regulation of CREB. So the scientists make an effort to find the relation between CB1R and BDNF. CB1 receptor stimulation may regulate MAP kinase activity indirectly through its effects on cAMP accumulation, inducing the expression of BDNF[2-4]. Cannabinoids may induce the expression of C-FOS, containing both serum response element (SRE) and cAMP response element (CRE) in their promoter, which is important to increase the levels of BDNF[4]. The main purpose of our experiment is to explore the effect of WIN55, 212-2 on the expression of PKAC-β, C-FOS and BDNF in ipsilateral cortex, so as to provide a theoretical basis for CBs treating ICH.

ANIMALS AND GROUPS

The study was performed according to the guidelines of the Institutional Animal Care Committee. forty-eight (48) male SD rats, of clean grade, weighing from 180 to 220g, were provided by Animal Center of chongqing medical university. The rats were randomly divided into 6 groups. Group 1: WIN55, 212-2 high dose group(5mg/kg). Group 2: WIN55, 212-2 middle dose group(3mg/kg). Group 3: WIN55, 212-2 low dose group(1mg/kg). Group 4: Nimodipine injection control group(1mg/kg). Group 5: Model control group (saline and DMSO compounds). Group 6: Sham-operation control group(saline and DMSO compounds).

DRUGS AND ADMINISTRATION

(R)-(+)-[2, 3-Dihydro-5-methyl-3[(4-morpholinyl)methyl]pyrrolo[1, 2, 3-de]-1, 4-benzoxazinyl]-(1-naphthalenyl)methanone mesylate salt (WIN55, 212-2), batch number: 027k4604, offered by America Sigma company. Nimodipine, batch number: BXCC7K1, was from the first affiliated hospital of chong qing medical university. WIN55, 212-2 were dissolved in 60% dimethyl sulfoxide (DMSO) and saline. The control groups, except Nimodipine group, received injections of vehicle (60% DMSO in saline). The rats were injected by the intraperitoneal route, thirty minuts after the operation.

SURGERY

Rats were anesthetized with intraperitoneal pentobarbital (40mg/kg) and then were placed in a stereotactic frame. The scalp was incised longitudinally in the midline and a 1mm burr hole was made in the skull, using a dental drill at 1mm posterior and 3.2mm lateral to bregma. Asterile needle was then lowered vertically into the right caudate nucleus at 5.6mm. Collagenase VII (Sigma) 0.5μ/2μl were injected into right caudate nucleus of rats in ICH groups respectively. The same operation was taken in sham-operation control rats, but infused with saline. The needle was removed and the skull sealed. Following surgery, neurological function was graded using the longa scores. Zero point represented normal scores, One point indicated that the rats could not extend their left anterior limbs, Two points indicated that the rats circled to the left, Three points described tumbling towards the left, Four points represented conscious disturbance, with no walking. Rat scoring 1-3 points indicated successful model establishment. The rats, choosed for the experiment, were killed 24 hours after the operation.

IMMUNOHISTOCHEMISTRY

The rats were sacrificed 24 h after the operation. Rats were deeply anesthetized with pentobarbital (40mg/kg, i.p.) and transcardially perfused with phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde in PBS. Their brains were removed, postûxed in the same ûxative for 24 h. Serial tissue sections were cut in the vertical meridian. The thickness of sections is 5um. Immunohistochemical Staining of PKAC-β, C-FOS and BDNF Protein was conducted according to the instruction of sABC kit (Boster, China). Sections were incubated with mouse anti- rat polyclonal antibody PKAC-β (1:120, Bioworld, US), C-FOS (1:120, Bioworld, US) or BDNF (1:50, Santa Cruiz, US) followed by incubation with biotinilated anti-rabbit IgG and the streptavidiperoxidase complex. The positive cells were observed and its images were obtained under light microscope (Leica, Germany). All sections were analyses under the same magnification times and the same light intensity.

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SEMI-QUANTITATIVE RT-PCR

Rats were sacrifced at 24 h after reperfusion with 0.9% saline solution. Ipsilateral cortex of Cerebrum was rapidly harvested, frozen and pooled under 80°C for future use. Total RNA was isolated from cortex according to the manufacturer's (Bio-flux, China) instructions. 1ug of total RNA was transcribed into cDNA using AMV reverse transcriptase (Bio-flux, Chinese) with 0.5ul oligo-(dT)18 (Bio-flux, Chinese) as primers. The Primers (Sbsbio-Genosys) are listed in TABLE 1. The annealing temperature is 54°C for PKAC-β, 60 I for C-FOS, 62 I for BDNF or 57I for ®-actin. The PCR specific optimum cycle numbers of ampliûcation were operated as follows: 30 cycles for PKAC-β or C-FOS, 35 cycles for BDNF, and 28 cycles for β-actin. Amplified cDNA was then separated by electrophoresis in 2% agarose (Biowest, Bio Sun, China) gels stained with ethidium bromide (Promega, US). Band size was recognized using DNA ladder and its optical density was measured using Bio-Rad video imaging system. Quantification was performed by expressing the optical density (OD) of the respective bands as percent of β-actin.

Western blotting Cerebral cortex was homogenized in lysis buffer, Cell lysates were centrifuged at 12, 000g for 15 min, and protein content was measured using the BCA assay. Samples were diluted 1:1 in PBS and denatured at 95°C for 5 min. Proteins (50ug/lane) were separated by SDS-PAGE (10% or 12% resolving gel) and transferred onto PVDF (polyvinyldifluoridine) membranes by semidry transfer. Blots were blocked for 1h at room temperature with blocking buffer and incubated with rabbit polyclonal anti-PKAC-β (1:120;Bioworld, US), anti-BDNF (1:100;Santa Cruiz, US) or anti-β-Actin (1:500; ABZOOM, US) antibodies in blocking buffer overnight at 4°C. After incubation with a horseradish peroxidase-coupled secondary antibody (1:3000, Beyotime, China) for 2h at room temperature, bands were visualized using the ECL detection system. The membrane was subsequently exposed to photographic film. The optical densities (ODs) of bands were calculated using Bio-Rad video imaging system. Quantification was also performed by expressing the optical density of the respective bands as percent of β-actin.

Statistical analysisComparison of two groups was made using one-way analysis of variance (ANOVA), and P values of <0.05 were considered to have statistical signiûcance.

EXPERIMENTAL DATA AND ANALYSIS

The position of PKAC-β, C-FOS and BDNF expression in the cell detected by immunohistochemistry

PKAC-β, C-FOS, and BDNF proteins are expressed on membrane of neuron, nucleus of neuron or the cytoplasm of glial cells respectively (Figure 1).

TABLE 1: The primer sequences of PKAC-β, C-FOS,BDNF and β-Actin

Target gene		Sequence	Target size(bp)
PKAC-β	Forward primer	GGTTCGCCAAGAGAGTCAAG	110bp
	Reverse primer	CCACCAGTCCACTGCCTTAT	
C-FOS	Forward primer	AGGCTGACTCCTTCCCTAGC	150bp
	Reverse primer	ATGCACCAGCTCAGTCAGTG	
BDNF	Forward primer	CATCTTAGGAGTGGAAAGGGTG	355bp
	Reverse primer	TATGAAGCCACCTAATCGACCT	
β-actin	Forward primer	GATGACCCAGATCATGTTTGA	535bp
	Reverse primer	TTGGCATAGAGGTCTTTACGG	

The effect of CB1R agonists WIN55, 212-2 on the mRNA expressions of PKAC-β, C-FOS and BDNF in Ipsilateral cortex of cerebrum (Figure 2)

C-FOS and BDNF mRNA expression in nimodipine was obviously upregulated(P<0.05vs model). Compared to nimodipine, WIN55, 212-2 significantly increased the levels of C-FOS and BDNF

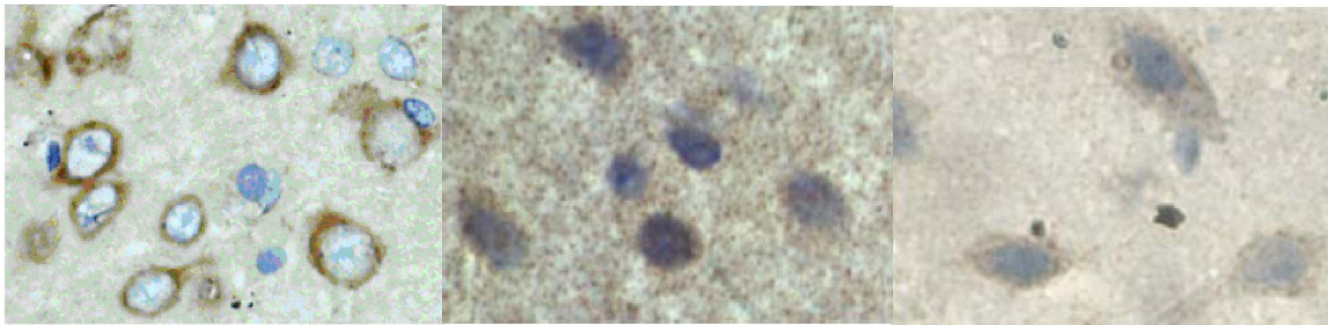

Figure 1 : Protein expression of BDNF, PKAC-β and C-FOS in cortex by immunohistochemistry (×400)

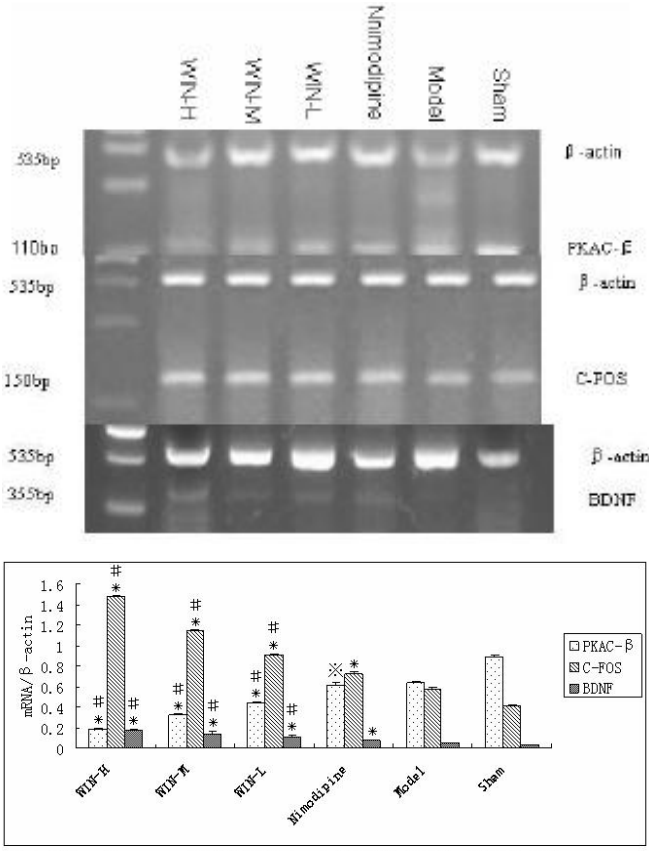

Fig. 2. Effect of treatment with WIN55,212-2 on the PAKCβ ,C-FOS and BDNF mRNA expression in ipsilateral cortex at 24 h after ICH. All drugs were injected i.p. 30 minuts after the operation. Data were shown as the mean± SD from three independent repeats. *P<0.05vs Model, ※P>0.05vs Model, #P<0.05vs nimodipine

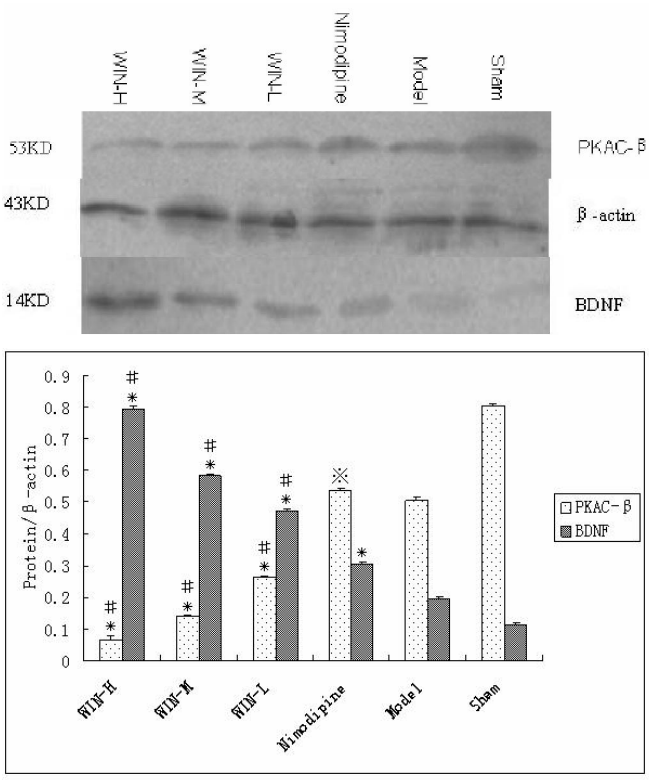

Fig.3. Effect of treatment with WIN55,212-2 on PKAC- β and BDNF proteins expression in ipsilateral cortex at 24 h after ICH. All drugs were injected i.p. 30 minuts after the operation. Data were shown as the mean±SD from three independent repeats. *P<0.05vs Model, ※ P>0.05vs Model, # P<0.05vs nimodipine

mRNA. Nimodipine had no significant effect on the expression of PKAC-βmRNA(P>0.05vs model). However, WIN55, 212-2 significantly reduced levels of PKAC-βmRNA expression as compared with nimodipine.

The effect of CB1R Agonists WIN55, 212-2 on the proteins expression of PKAC-β and BDNF in Ipsilateral Cortex of Cerebrum (Figure 3)

The expression of BDNF in nimodipine was obviously upregulated (P<0.05vs model). Compared to nimodipine, WIN55, 212-2 significantly increased the expression of BDNF protein. Nimodipine had no significant effect on the expression of PKAC-βprotein (P>0.05vs model). However, WIN55, 212-2 significantly reduced the expression of PKAC-βprotein as compared with nimodipine.

CONCLUSIONS

In recent years, the scientists are interesting in the relation between CB1R and BDNF. Khaspekov et al. report that genetic suppression or pharmacological antagonism of CB1R blocks the production of BDNF

following toxic administration of kainic acid, suggesting that BDNF may be another important mediator of the neuroprotective effects of CBs[5, 6]. Derkinderen et al. also found that Cannabinoids induced the expression of BDNF mRNA through activation of MAPK/ERK pathway[4]. In 2008, March et al. further proved that such BDNF up-regulation coincides with a higher binding activity and an increased protein expression of CB1R in rat model of striatal excitotoxicity[7]. In contrast, some scholars found that WIN 55, 212-2 significantly reduced BDNF levels in hippocampus, cortex or cerebellum. As for this phenomenon, differences in the model systems used should be taken into account when discussing such discrepancies[8, 9]. The result of our experiment is consistent with former. WIN55, 212-2 significantly increase BDNF in ipsilateral cortex.

Various signalling pathways, through CREB –mediated, can regulate a number of genes known as late response genes, which controled the expression of neurotrophic factors, such as BDNF. This mechanism may be particularly important for long-term changes in gene expression, and necessary for neuron protection. cAMP/PKA is one of the best characterized cannabinoid signaling pathways.

Childers and Deadwyler first reported that cannabinoids bind to receptors that couple to Gi/o-proteins and inhibit adenylyl cyclase in 1996[10]. After nearly 10 years, Huang CC and Kim SH also demonstrate that the neuroprotective effect of cannabinoids, acting through CB1R and Gi/o proteins, depend on suppression of cyclic AMP signaling through protein kinase A[11-13]. Our results is consistent with this conclusion. WIN55, 212-2 reduced the expression of PKAC-βin ipsilateral cortex. PKA contains catalytic subunits of α, β and γ, and theβsubunit is the highest concentration in neurons, which is necessary for synaptic plasticity. So we choose βsubunit in our experiment. In mouse hippocampus, D9-THC induced the expression of immediate-early gene (IGEs) products including BDNF and C-FOS proteins, through CB1R-mediated activation MAP kinase. Interestingly, CB1 receptor stimulation may also regulate MAP kinase activity indirectly through its effects on cAMP accumulation. A decrease in cAMP levels, and consequently in PKA activity, may participate in the stimulatory effects of CB1 activation on the MAP kinase signal pathway. This mechanisms suggest that WIN55, 212-2 may increase the expression of the BDNF through inhibition of PKA activity in ICH model[2 3 4].

IGE C-FOS regulates many cellular functions such as cell growth, transformation and apoptosis. C-FOS undergo rapid induction after extracellular stimulus, which is not only implicated in neuronal apoptosis but also important for recovery[14]. C-FOS contains both SRE and CRE, which is important for regulating the expression of BDNF[4]. Therefore, we focus our attention on C-FOS, in addition to PKA.

As early as 10 years ago, Mailleux P etal. discover that intraperitoneal injection of cannabinoid agonists significantly increases in concentration of the mRNAs for C-FOS, C-JUN and ZIF-268 in the cingulate cortex[15]. In consistent with Mailleux P, Derkinderen etal also showed that acute injection of THC not only increase the protein levlels of C-FOS, but also induce BDNF and Zif268 mRNA transcription through the ERK signaling pathway in hippocampus[4]. The result of our experiment also supported this conclusion.

In short, our results show that WIN55, 212-2 evoked a concentration-dependent increase in C-FOS and BDNF in rat cortex of ICH, but a decrease in PKA. WIN55, 212-2 are better in protecting the neurons compared with nimodipine. In the 24 hours after intraperitoneally injection, BDNF was increased accompany with C-FOS, suggesting that C-FOS positively regulate the expression of BDNF. In contrast, PKA was reduced by WIN55, 212-2, but it may induce the expression of the BDNF through activation of MAP kinase. This mechanism needs further demonstrated.

The functional crosstalk between CB1 receptor and BDNF signalling is so complicated that still not clear. However, evidence is emerging to suggest that cannabinoids increase the level of BDNF. This conclusion will be helpful in guiding future studies on mechanisms of cannabinoids neuroprotection and might represent a promising target for treatment of cerebrovascular diseases.

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