ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 1999 Nov; 20 (11): 991 - 994
http://www.simm.ac.cn; www.chinainfo.gov.cn/periodical

KN-62 provides neuroprotection against glutamate-induced excitotoxicity in neurons[1]

GAO Can, ZHANG Guang-Yi[2]
(*Research Center of Biochemistry and Molecular Biology, Xuzhou Medical College, Xuzhou 221002, China*)

KEY WORDS sodium glutamate; Ca^{2+}-calmodulin dependent protein kinase; KN-62; neurotoxins; cultured cells; neurons; phosphorylation; cell survival; piperazines

ABSTRACT

AIM: To study the effects of KN-62, an inhibitor of Ca^{2+}-calmodulin dependent protein kinase Ⅱ (CCDPK Ⅱ), on the damage of cortical neurons and mechanisms of the loss of CCDPK Ⅱ activity induced by sodium glutamate (Glu). **METHODS**: CCDPK Ⅱ activity was measured by ^{32}P incorporation and backphosphorylations of endogenous proteins were studied by autoradiography. **RESULTS**: 1) KN-62 provided partial protection against excitotoxical damage only before Glu (100 $\mu mol \cdot L^{-1}$, 10 min) treatment. 2) KN-62 markedly suppressed the loss of CCDPK Ⅱ activity induced by Glu from 48.0 % to 90.6 %. 3) Backphosphorylation of endogenous proteins (especially the 50 kDa protein) reduced to 78.2 % of control after treatment with Glu, and the reduction was protected with KN-62 added before Glu. **CONCLUSION**: KN-62 provided the protection against excitotoxicity and the loss of CCDPK Ⅱ activity as well as backphosphorylation of endogenous proteins induced by Glu. The neuroprotection provided by KN-62 was due to the inhibition of autophosphorylation of CCDPK Ⅱ.

INTRODUCTION

KN-62 (white solid, mp 130 ℃), 1-[*N*, *O*-bis (5-Isoquinolinesulfonyl)-*N*-methyl-*L*-tyrosyl]-4-phenylpiperazine, is a specific inhibitor of Ca^{2+}-calmodulin dependent protein kinase Ⅱ. Sodium glutamate (Glu), the major excitatory amino acid (EAA) in the CNS has been implicated in the injury associated with cerebral ischemia[1]. Ca^{2+}-calmodulin dependent protein kinase Ⅱ (CCDPK Ⅱ) which is enriched in CNS is very sensitive to ischemia. An early and persistent loss of CCDPK Ⅱ activity is a characteristic feature of cerebral ischemia[2]. CCDPK Ⅱ has characteristic properties in which autophosphorylation of the enzyme converts it from the Ca^{2+}-dependent form to the Ca^{2+}-independent species. It has been reported that elevation of intracellular Ca^{2+} levels in cultured neurons results in the autophosphorylation of CCDPK Ⅱ and produces the Ca^{2+}-independent form[3].

The mechanisms of the loss of CCDPK Ⅱ activity by stimulation of glutamate receptors (GluR) are not well understood. Although the NMDA receptor (NR) is physiologically important in a long-term potentiation (LTP) because of its high permeability to Ca^{2+}, the receptor may possibly be involved in pathological events such as neuronal cell death during brain ischemia and hypoxia[4]. Cerebral ischemia or excitotoxicity not only inhibits the enzyme activity but induces delayed neuronal death (DND)[5]. We investigated the effect of a toxic dose of Glu on cultured cortical neurons. The LDH release, CCDPK Ⅱ activity, and backphosphorylation of endogenous proteins were estimated to examine the mechanisms of the loss of CCDPK Ⅱ activity induced by Glu in attempts to establish an *in vitro* model of ischemia.

MATERIALS AND METHODS

Materials Sprague-Dawley rats (17- or 18-d fetus, ♀) were provided by the Animal Center of Xuzhou Medical College (purchased from Shanghai

[1] Project supported by the National Natural Science Foundation of China, No 39470168.
[2] Correspondence to Prof ZHANG Guang-Yi.
Phn 86-516-574-8423. Fax 86-516-574-8429.
E-mail xmcb@cumt.edu.cn
Received 1998-11-03 Accepted 1999-05-25

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 1999 Nov; 20 (11)
E-mail aps@server.shcnc.ac.cn Phn/Fax 86-21-6474-2629

Institute of Materia Medica, Grade Ⅱ, Certificate No 97017 and No 97032), China. Glu, KN-62, PNPP, histamine, NAD, poly-*l*-lysine were purchased from Sigma (USA). Neurobasal medium and B27 supplement were from Gibco (USA). Horse serum and fetal bovine serum were from Tianjing Biochemical Company. [γ-^{32}P] ATP was from Beijing Yahui Company. All the other chemicals were of AR.

Neuronal cultures Cortical cell cultures were prepared using method[6] with minor modifications. Cells were pipetted into poly-*l*-lysine-coated wells or flasks yielding a final cell counts of 2×10^5 cells/m^2 using Gibco DMEM (DMEM: containing 10 % horse and 10 % fetal bovine serum; glutamine 4 mmol·L^{-1}). Neurobasal medium (containing B27 supplement) was changed at second day and neurons were fed twice weekly. Experiments were performed on 11 – 15 d (postisolation) old neurons.

Cell viability assay Quantitative assessment of neuronal injury were made using lactate dehydrogenase (LDH) release as a marker for membrane breakage and cell death[7]. Cultured cells were washed 4 times with DMEM (serum free) and Glu (100 μmol·L^{-1}) was added directly to the culture media. After 10 min incubation at room temperature, cells were washed 4 times with serum-free DMEM. KN-62 was added according to the designs. Glucose concentration was kept at 10 mmol·L^{-1} throughout the experiment period. The cultures were incubated for 24 h to measure the release of LDH.

CCDPK Ⅱ activity assay The cultures was washed 3 or 4 times with cold Hanks' solution followed by supersonic treatment after being scraped in the presence of ice-cold homogenate buffer (containing Tris-HCl 20, edetic acid 2, NaF 5, PMSF 0.5 mmol·L^{-1} and Triton-100 0.1 %, pH 7.4). The cytosolic fraction was obtained by centrifugation at 10 000 × *g* for 5 min and stored at liquid N_2 for the assay of CCDPK Ⅱ activity. Cells were collected immediately to measure the activity of CCDPK Ⅱ. The reaction was performed at 30 ℃ for 5 min in a final volume of 50 μL according to the document[8].

Ca^{2+}/CaM-dependent backphosphorylation of endogenous proteins The backphosphorylation of endogenous proteins were carried out according to the methods of Nestler[9].

Protein assay Protein content was determined by the method of Lowry *et al*, using BSA as the standard.

Statistical evaluation Data were compared by *t* test.

RESULTS

Effects of KN-62 on the glutamate-induced LDH release in cultured cortical neurons A KN-62 concentration-dependent reduction in LDH release was observed, being the most effective at 1.0 – 10 μmol·L^{-1}. KN-62 was given 30 min before Glu (100 μmol·L^{-1}, 10 min) application. LDH release was measured after a 24-h incubation. The maximal release was 70.6 % ($P < 0.05$ *vs* control) at 1.0 μmol·L^{-1}, 61.5 %, and 56.2 % ($P < 0.01$ *vs* control) at 5.0, 10.0 μmol·L^{-1}, respectively (Fig 1).

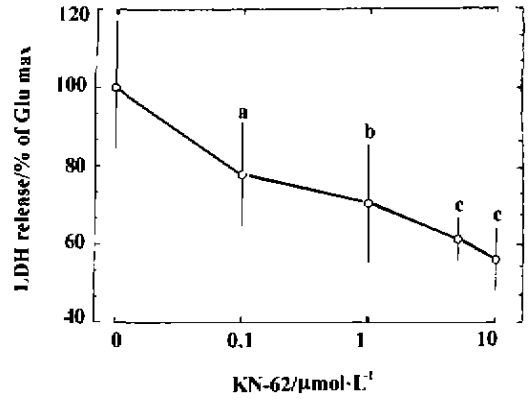

Fig 1. Concentration-response relation of KN-62 against Glu excitotoxicity in cultured rat cortical neurons. $n = 4$. $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs* control.

Neuroprotection achieved by KN-62 (5 μmol·L^{-1}) was strong only when it was added before Glu (100 μmol·L^{-1}, 10 min) application and included throughout the postincubation period. Neither simultaneous administration with Glu nor post-Glu application of KN-62 showed neuroprotection (Tab 1).

Effect of KN-62 on the Glu-induced loss of CCDPK Ⅱ activity in cultured cortical neurons Excessive Glu caused the loss of CCDPK Ⅱ activity. KN-62 protected the activity of the enzyme significantly from 48.0 % ($P < 0.01$, *vs* control) to 90.6 % ($P > 0.05$, *vs* control), whereas KN-62 alone did not influence the activity of CCDPK Ⅱ (Tab 2).

Tab 1. Effect of mode of administration of KN-62 on the degree of protection against Glu in cultured cortical neurons. $n=4$. $\bar{x} \pm s$. $^aP>0.05$, $^cP<0.01$ *vs* Glu.

Addition	LDH release/ %
Glu	100 ± 17
Glu + KN-62 *	92 ± 6[a]
(Glu + KN-62) * *	91 ± 11[a]
KN-62 + Glu	62 ± 6[c]
KN-62 + Glu + KN-62	49 ± 11[c]

* : post-Glu application; ** : simultaneous administration.

Tab 2. Effect of KN-62 on the Glu-induced loss of CCDPK Ⅱ activity in cultured cortical neurons. $n=4$. $\bar{x} \pm s$. $^aP>0.05$, $^cP<0.01$ *vs* control.

Addition	CCDPK Ⅱ activity/ %
Control	100 ± 5
Glu	48 ± 4[c]
KN-62 + Glu	91 ± 6[a]
KN-62	102 ± 11[a]

Effect of KN-62 on the backphosphorylation of endogenous proteins Backphosphorylation of endogenous proteins, especially the 50 kDa, decreased when cortical neurons were exposed to Glu (100 μmol · L^{-1}) for 10 min. Pretreatment with 5 μmol · L^{-1} of KN-62 for 30 min prevented the decrease of backphosphorylation of endogenous proteins caused by Glu. Whereas KN-62 itself did not influence the level of backphosphorylation of the 50 kDa protein. When the 50 kDa subunit level was examined using a Ultra Violet Products Analyzer, lane B is 78.2 % of lane A, whereas lane C is 95.3 %, lane D is 100.0 % (Fig 2).

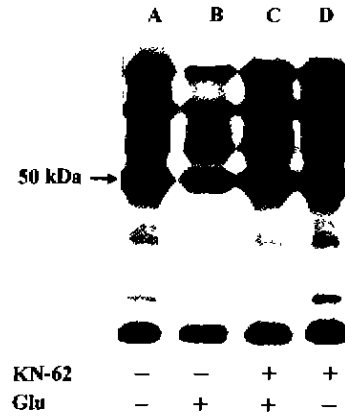

Fig 2. Effect of KN-62 on the Glu-induced backphosphorylation of endogenous proteins.

DISCUSSION

CCDPK Ⅱ is highly enriched in neurons both in the pre- and post-synaptic compartments where it is essential to neurotransmitter release and induction of LTP[10]. CCDPK Ⅱ is a serine-threonine kinase which can be activated by an increase in $[Ca^{2+}]_i$ (bound to calmodulin), following an appropriate agonist stimulation such as Glu. Once activated by the Ca^{2+}/CaM, 20 % – 80 % of the enzyme activity is maintained by its conversion to a Ca^{2+}-independent form via autophosphorylation. The catalytic activity of CCDPK Ⅱ is regulated by the autophosphorylation of Thr286/Thr287 (in the α/β subunit) which are responsible for generation of the Ca^{2+}-independant activity[11]. Therefore, to examine the role of CCDPK Ⅱ in EAA-mediated excitotoxicity in fetal rat cerebrocortical cultures, we used the specific CCDPK Ⅱ inhibitor KN-62 with proven cell permeability. KN-62 inhibited CCDPK Ⅱ competitively with respect to Ca^{2+}/CaM and was effective at inhibiting the activity of CCDPK Ⅱ only before its autophosphorylation[12]. It has proved that KN-62 can reduce Ca^{2+} accumulation in NMDA toxicity and in oxygen/glucose deprivation[13]. The observation that pretreatment with KN-62 is necessary in reducing Glu-induced excitotoxicity indicates that the neuroprotective effects of this compound are mediated by an intracellular mechanism. KN-62 does not provide a complete blockade of Glu-mediated excitotoxicity implicating that such an insult is multifactorial.

Since KN-62 can provide partial protection against excitotoxicity, we further investigated if it can protect the activity of CCDPK Ⅱ. Our results showed that pretreatment with KN-62 almost completely antagonized the loss of activity of the enzyme induced by Glu, whereas KN-62 itself had no effect on the activity. The changes of backphosphorylation of endogenous proteins were consistent with the changes of the activity of CCDPK Ⅱ, which means that Glu caused the

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 1999 Nov; 20 (11)
E-mail aps@server.shcnc.ac.cn Phn/Fax 86-21-6474-2629

decrease of levels of backphosphorylation of endogenous proteins (especially the 50 kDa protein), whereas pretreatment with KN-62 may protect it.

Other evidence have verified that the molecular weight of the α subunit of CCDPK Ⅱ, which can be autophosphorylated by increasing of $[Ca^{2+}]_i$, is 50 kDa[2]. Our results indicated that Glu caused the decrease of backphosphorylation of the 50 kDa protein *in vitro*, which represented the increase of autophosphorylation of α subunit *in vivo*, thus the Ca^{2+}-dependent CCDPK Ⅱ activity decreased. The activated CCDPK Ⅱ (Ca^{2+}-independent) then phosphorated substrates including NR, which can induce a serious of physiological/pathological reaction caused by the enhancement of Ca^{2+}-influx[14].

We think that the reversible phosphorylation of Glu receptors (GluR) is very important during ischemia. Taken together, the present data suggest that KN-62 inhibits autophosphorylation of CCDPK Ⅱ induced by Glu.

REFERENCES

1 Rajdev S, Reynolds IJ. Glutamate-induced intracellular calcium changes and neurotoxicity in cortical neurons *in vitro*: effect of chemical ischemia. Neuroscience 1994; 62: 667 – 79.

2 Yamamoto H, Fukunaga K, Lee K, Soderling TR. Ischemia-induced loss of brain calcium/calmodulin-dependent protein kinase Ⅱ. J Neurochem 1992; 58: 1110 – 7.

3 Fukunaga K, Soderling TR, Miyamoto E. Activation of Ca^{2+}/calmodulin-dependent protein kinase Ⅱ and protein kinase C by glutamate in cultured rat hippocampal neurons. J Biol Chem 1992; 267: 22527 – 33.

4 Black MA, Tremblay R, Mealing G, Ray R, Durkin JP, Whitfield JF, *et al*. *N*-methyl-aspartate- or glutamate-mediated toxicity in cultured rat cortical neurons is antagonized by FPL 15896AR. J Neurochem 1995; 65: 2170 – 7.

5 Churn SB, Limbrick D, Sombati S, Delorenzo RJ. Excitotoxic activation of the NMDA receptor results in inhibition of calcium/calmodulin kinase Ⅱ activity in cultured hippocampal neurons. J Neurosci 1995; 15: 3200 – 14.

6 Gao C, Zong YY, Zhang GY. Effects of glutamate on the activity of calcium/calmodulin-dependent protein kinase Ⅱ in cultured rat cortical neurons. Acta Acad Med Xuzhou 1997; 17: 551 – 4.

7 Koh JY, Choi DW. Quantitative deter mination of glutamate mediated cortical neuronal injury in cell culture by lactate dehydrogenase efflux assay. J Neurosci Meth 1987; 20: 83 – 90.

8 Tang FM, Zhang GY, Sao WJ, Sao SH. Changes of Ca^{2+}/CaM PK Ⅱ activity in cerebral ischemia and reperfusion and effects of bepridil on the enzyme activity. Chin J Pathophysiol 1994; 10: 139 – 42.

9 Nestler EJ, Greengard P. Protein phosphorylation in the brain. Nature 1983; 305: 583 – 8.

10 Larria A, Muller D, Derkach V, Griffith LC, Soderling TR. Regulatory phosphorylation of AMPA type glutamate receptors by CaM-K Ⅱ during long-term potentiation. Science 1997; 276: 2042 – 5.

11 Soderling TR. Structure and regulation of calcium/calmodulin-dependent protein kinases Ⅱ and Ⅳ. Biochem Biophysiol Acta 1996; 1297: 131 – 8.

12 Hidaka H, Kobayashi R. Pharmacology of protein kinase inhibitors. Annu Rev Pharmacol Toxicol 1992; 32: 377 – 97.

13 Hajimohammadreza I, Probert AW, Coughenour LL, Borosky SA, Marcoux FW, Wang K. A specific inhibitor of calcium/calmodulin-dependent protein kinase-Ⅱ provides neuroprotection against NMDA- and hypoxia/hypoglycemia-induced cell death. J Neurosci 1995; 15: 1093 – 101.

14 Soderling TR. Modulation of glutamate receptors by calcium/calmodulin-dependent protein kinase Ⅱ. Neurochem Int 1996; 28: 359 – 61.

KN-62 拮抗谷氨酸对神经细胞的兴奋毒作用[1]

高 灿, 张光毅[2] (徐州医学院生物化学与分子生物学研究中心, 徐州 221002, 中国)

关键词 谷氨酸钠; Ca^{2+}-钙调素依赖性蛋白激酶; KN-62; 神经毒素; 培养的细胞; 神经元; 磷酸化; 细胞存活; 哌嗪类

目的: 研究钙/钙调素依赖性蛋白激酶Ⅱ抑制剂 KN-62 对谷氨酸介导皮质神经元损伤及 CCDPK Ⅱ活性下降的影响. **方法**: LDH 释放代表神经元损伤, ^{32}P掺入法测 CCDPK Ⅱ活性, 放射自显影显示后磷酸化水平变化. **结果**: 只有在谷氨酸(100 μmol·L^{-1}, 10 min)作用前加 KN-62 可部分保护神经元损伤, 明显拮抗 CCDPK Ⅱ活性下降, 活性由 48.0 %恢复到 90.6 %, 显著抑制内源蛋白后磷酸化水平下降. **结论**: KN-62 对 CCDPK Ⅱ活性的保护作用是通过抑制 CCDPKⅡ的自身磷酸化而实现的.

(责任编辑 李 颖)