International Scholars Journals

International Journal of Pharmacy and Pharmacology ISSN: 2326-7267 Vol. 7 (9), pp. 001-005, September, 2018. Available online at www.internationalscholarsjournals.org © International Scholars Journals

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Full Length Research Paper

Protective effects of calpeptin on rat hippocampal slices exposed to oxygen and glucose deprivation

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Accepted 14 February, 2018

The present study investigated the protective effects of calpeptin on rat hippocampal slices exposed to oxygen and glucose deprivation (OGD). Forty SD rat hippocampal slices were randomly assigned into control group and calpeptin group. According to the calpeptin concentration of artificial cerebrospinal fluid, the calpeptin group was subdivided into 1, 10, 100 and 200 mol/ l group (n=8 per group). Extracellular recording technique was employed to determine the effects of calpeptin on the orthodromic population spikes (OPS) and hypoxic injury potential (HIP) of hippocampal slices exposed to OGD. TUNEL staining was conducted to detect the apoptosis of pyramidal cells with or without calpeptin treatment. In the 10, 100 and 200 mol/l group, the presence of HIP and the number of apoptotic cells were markedly increased, and the rate and amplitude of OPS recovery were significantly elevated when compared with control group. However, there were no remarkable differences in these parameters between 10, 100 and 200 mol/l group. Our results showed calpeptin of 10 to 200 mol/l could dramatically improve the injury of OGD to rat hippocampal slices in which decreased apoptosis of neurons in the CA1 region by calpeptin played an important role.

Key words: Calpain, oxygen and glucose deprivation, hippocampal slice, orthodromic population spike, hypoxic injury potential.

INTRODUCTION

Calpain is a calcium dependent cysteine protease and widely expressed in the nervous system. After ischemia, the intracellular calcium overload activates calpain which then cause the apoptosis and necrosis of neurons playing an important role in the ischemic brain injury (Zhao et al., 1999; Ray et al., 2000) . It has been shown that specific calpain inhibitor could markedly decrease the neuron death after brain ischemia (Seyfried et al., 2001). Calpeptin is a synthetic, effective and cell-permeable calpain inhibitor and can effectively inhibit the activities of -calpain and m-calpain. Calpeptin has the advantage with high efficiency, specificity and good membrane permeability and it do not directly inhibit the activity of Caspase-3 (Tamura et al., 2003). Several studies have shown that Calpeptin could effectively reduce the kidney injury (Takaoka et al., 2000), cardiac ischemic injury (Feng et al., 2001) and excitatory amino acidinduced neuronal injury (Das et al., 2005). In the present study, we investigated the effects of calpeptin on the orthodromic population spike (OPS) and hypoxic injury potential (HIP) as well as the apoptosis of neurons in the CA1 region of hippocampal sclices exposed to oxygen and glucose deprivation (OGD) aiming to explore the protective effects of calpeptin on the ischemic brain injury and the potential mechanism.

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MATERIALS AND METHODS

Materials

Forty healthy male Sprague-Dawley rat (30 to 40 days) weighing 80 to 110 g were purchased from the Animal Center of Xuzhou Medical College. Calpeptin (Calbiochem, USA), TUNEL kit (Boster, China) and dimethyl sulfoxide (DMSO; Sigma, USA) were used in the present study. This study has been approved by the ethics committee of our hospital.

Preparation of artificial cerebral spinal fluid ($ACSF_n$)

The $ACSF_n$ (pH=7.35 to 7.45) contained 124 mmol/l NaCl, 3.3 mmol/l KCl, 1.24 mmol/l NaH_2PO_4, 2.4 mmol/l $MgSO_4$, 25.7 mmol/l $NaHCO_3$, 2.4 mmoll $CaCl_2$, and glucose 10.0 mmol/l. The $ACSF_n$ was pre-bubbled with gas mixtures containing 95% O_2 and 5% CO_2 for 15 min before experiment. In the OGD, the glucose was removed from the $ACSF_n$ which then bubbled with gas mixtures containing 95% N_2 and 5% CO_2. The slices were completely submerged and protected from the vigorous bubbling in the chamber by a semi-permeable nylon mesh.

Preparation of hippocampal slices

Rats were decapitated after anesthesia with ethyl ether and hippocampi were separated at 4°C. The hippocampi were transversely sliced (400 μ m thick) with a tissue slicer (Shanghai Haitian, China) and stored in the oxygen bubbled ACSFn at 34°C for 2 to 3 h.

Extracellular recording

The hippocampal slices were immersed in a tank 2 mm under the surface. Then, perfusion with gas mixtures (95% O_2 + 5% CO_2) bubbled $ACSF_n$ was performed at a rate of 3 ml/min. Bipolar stimulating electrodes were put on the Schaffer branch of CA1 region and stimulation was done with the intensity of 0.2 to 0.8 mA. The glass microelectrode filled with NaCl (impedance: 4 to 10 M) was put in the pyramidal cells of CA1 region. The OPS and HIP were recorded. When the amplitude of OPS was greater than 3 mV and maintained stable for 15 min, electrophysiological examination was conducted. Then temperature of ACSF and liquid in the tank was maintained at 34°C.

Grouping

A total of 40 rat hippocampal slices were randomly assigned into two groups: control group and calpeptin group. According to the calpeptin concentration of $ACSF_{OGD}$, the calpeptin group was subdivided into 1, 10, 100 and 200 mol/l group (n=8 per group). In the control group, the slices were perfused with $ACSF_{OGD}$ containing 0.1% DMSO and pre- bubbled with 95% N_2+5% CO_2 for 14 min when the OPS of slices was stable for 15 min (Liu et al., 2005) , and then with ACSFn for 60 min. In the calpeptin groups, slices were perfused with $ACSF_{OGD}$ containing calpeptin of different concentrations and 0.1% DMSO for 14 min and then with ACSFn for 60 min. The calpeptin was dissolved in the DMSO and then added to $ACSF_{OGD}$.

Observation indexes

1. Time to OPS disappearance: from the beginning of OGD to the OPS disappearance.
2. Amplitude of OPS recovery: the proportion of OPS amplitude after 60 min of perfusion with $ACSF_n$ to that before OGD.
3. Rate of OPS recovery: The proportion of slices with OPS recovery to the total number of slices in one group. OPS recovery was defined as the OPS amplitude after 60 min of perfusion with $ACSF_n$ reaches more than 60% of that before OGD.
4. Time to HIP appearance: From the beginning of OGD to the HIP appearance.
5. Rate of HIP presence: the proportion of slices with HIP to the total number of slice in one group.
6. Apoptosis of neurons in the CA1 region: Immediately after electrophysiological examination, the slices were fixed with 4% paraformaldehyde for 12 h and frozen sections (20 m thick) were obtained followed by TUNEL staining according to manufacturer's instructions. Three fields were randomly selected from the CA1 region at 400× and the number of positive cells (brown cells) was determined followed by averaging (Nisticò et al., 2008; Zheng et al., 2005).

Statistical analysis

Quantitative data were expressed as means ± standard deviation (mean±SD) and statistical analysis was performed with SPSS version 13.0 statistical software. Chi square test was employed to compare the rates and quantitative data were analyzed with one way analysis of variance. A value of $P<0.05$ was considered statistically significant.

RESULTS

Effects of calpeptin on the OPS and HIP of hippocampal slices exposed to OGD

In the control group, the OPS disappeared within several minutes after OGD exposure followed by the presence of HIP. 60 min after ACSFn treatment, OPS was only observed in one slice in the control group. After perfusion with $ACSF_{OGD}$ containing 1, 10, 100 mol/l or 200 mol/l calpeptin, the presence of HIP was decreased, but the rate and amplitude of OPS recovery were improved after perfusion with ACSFn. There were no marked differences in these parameters between control group and 1 mol/l group. When compared with the control group, after perfusion with $ACSF_{OGD}$ containing 10, 100 mol/l or 200 mol/l calpeptin, the presence of HIP was significantly decreased and the rate and amplitude of OPS recovery were dramatically improved after perfusion with ACSFn. Nevertheless, no marked differences in these parameters were observed between 100, 200 and 10 mol/l group (Table 1 and Figure 1).

Effects of calpeptin on OGD induced apoptosis of pyramidal cells in the CA1 region

Apoptosis was observed in all groups to different extents. There was no marked difference in the number of apoptotic cells between 1 mol/l group and control group. In the 10, 100 and 200 mol/l group, the number of apoptotic pyramidal cells was remarkably decreased when compared with control group. Moreover, no significant difference was noted between 10, 100 and 200 mol/l group (Table 2 and Figure 2).

DISCUSSION

The conditions for hippocampal slice culture can be well controlled and the influences of blood pressure,

Table 1. Effects of calpeptin on the OPS and HIP of hippocampal slices exposed to OGD (mean±SD, n=8).

Group	OPS abolishing	OPS recovery rate	OPS recovery amplitude	HIP appearing	HIP appearance rate
Control	132.3±21.1	12.5	14.4±20.2	584.5±82.4	100
1 mol/L	138.2±23.4*	12.5*	22.4±26.5*	609.7±91.2*	87.5(7/8)*
10 mol/L	211.1±34.0 ##	75#	69.3±13.8 ##	674.3±87.3##	25(2/8) ##
100 mol/L	192.3±25.3 ##	87. 5#	68.6±17.3##	649.1±88.4#	12.5(1/8) ##
200 mol/L	228.3±28.9 ##	87.5#	74.3±18.3##	676.2±104.2#	12.5(1/8) ##

*P>0.05 vs Control, #P<0.05, ##P<0.01 vs Control.

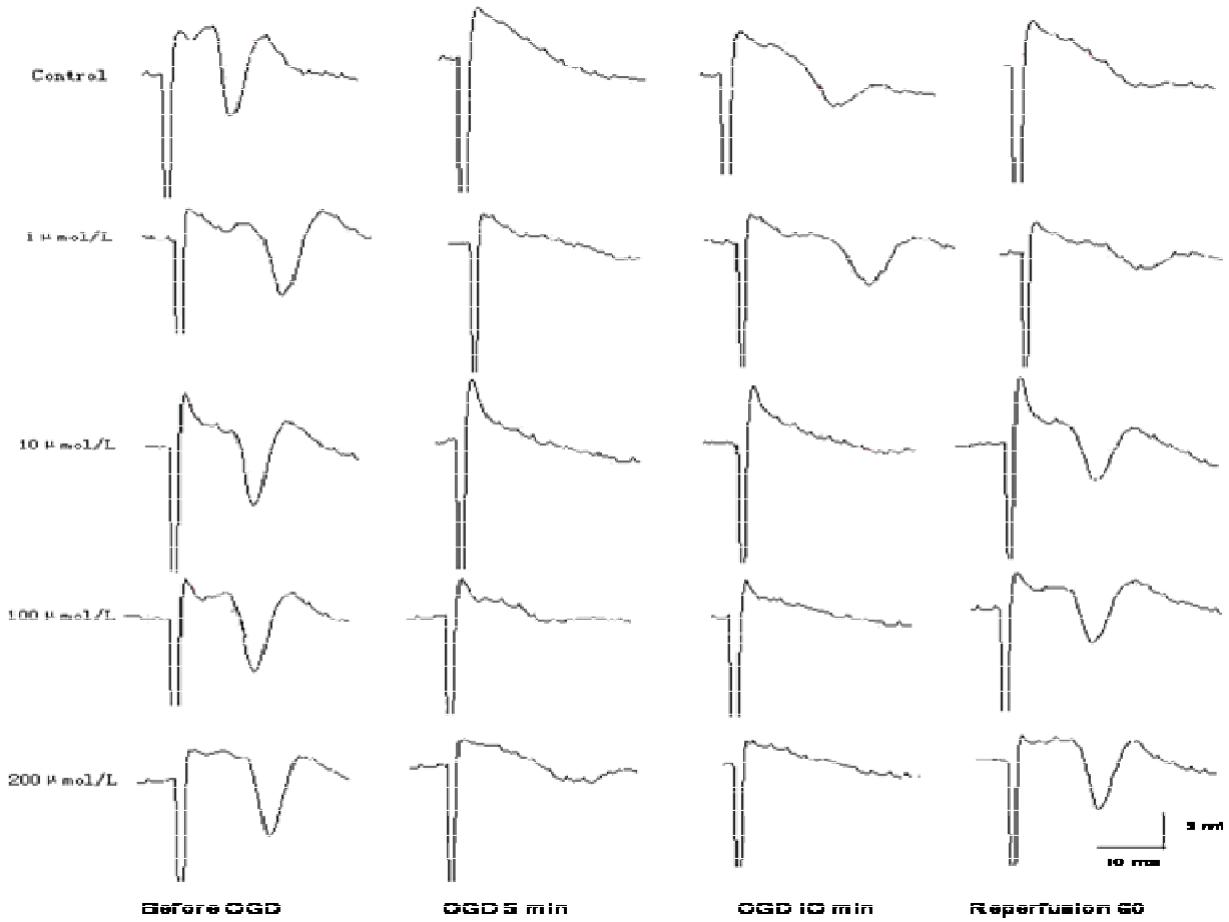

Figure 1. Effects of calpeptin on OPS and HIP of rat OGD injury hippocampal slices (mean±SD, n=8), *P>0.05 vs Control, #P<0.05, ##P<0.01 vs Control.

Table 2. Effects of calpeptin on the neuronal apoptosis in the CA1 region after OGD (mean ± SD, n=8).

Group	Apoptotic rate
Control	14.1±3.7
1 mol/L	12.9±1.9*
10 mol/L	7.9±2.5**
100 mol/L	8.3±1.8**
200 mol/L	8.8±2.4**

*P>0.05 vs Control,, **P<0.01 vs Control.

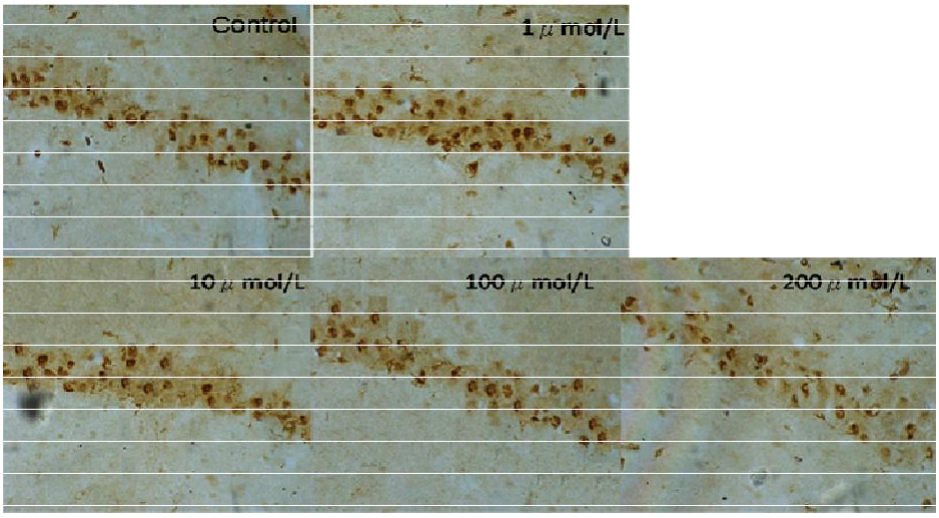

Figure 2. Effects of calpeptin on neuronal apoptosis in CA1 region after hypoxia/glucose deprivation on rat hippocampal slices (mean±SD, n=8), **P*>0.05 1 mol/L group vs control, ***P*<0.01 10 and 100 and 200 mol/L group vs control.

temperature, electrolytes and brain blood barrier are excluded. Therefore, the hippocampal slice culture has been extensively applied in the neurophysiological and neu-ropharmacological researches (Wang et al., 1999). To mimic hypoxia *in vitro* , the glucose was removed and the oxygen was replaced with nitrogen. In the electrophysiological examination, Schaffer branch in the CA1 region was stimulated with electrodes, and the induced peak potential of pyramidal cells was recorded. The temporal changes in the electrophysiological features can be observed after OGD treatment (Fairchild et al., 1988). Before the presence of HIP, the damaged synaptic function is reversible, and the presence of HIP represents the irreversible hypoxic injury of synaptic function. In the present study, HIP was observed in all slices after OGD and last for 1 to 3 min. After perfusion with ACSFn, the rate and amplitude of OPS were increased but at a relatively low level. These findings suggested the hippocampal neurons were markedly injured.

During the mid-1960s, the calcium-dependent proteolytic activities caused by a “calcium-activated neutral protease” (CANP) were detected in brain, lens of the eye and other tissues. In the late 1960s, the enzymes were isolated and characterized independently in both rat brain and skeletal muscle. These activities were caused by an intracellular cysteine protease not associated with the lysosome and having an optimum activity at neutral pH, which clearly distinguished it from the cathepsin family of proteases. The calcium-dependent activity, intracellular localization, along with the limited, specific proteolysis on its substrates, highlighted calpain’s role as a regulatory, rather than a digestive protease.

Calpain is a soluble and calcium dependent protease and a papain family member of cysteine protease. The activity of calpain is found to be attributable to two main isoforms, dubbed ("mu")-calpain and m-calpain, that differed primarily in their calcium requirements *in vitro*. Their names reflect the fact that they are activated by micro- and nearly millimolar concentrations of Ca^{2+} within the cell, respectively (Glass et al., 2000). In the brain, the - calpain is mainly located in the cell body and dendrites of neurons and to a lesser extent in axons and glial cells, and the m-calpain is found in glia and a small amount in axons. Calpain is widely expressed in the cytoplasm, cell membrane and other organelle membrane in the mammalian nervous system.

Although the physiological role of calpains are still poorly understood, they have been shown to be involved in some processes including cell mobility and cell cycle progression, as well as cell-type specific functions. Under these physiological conditions, a transient and localized influx of calcium into the cell can activate a small local population of calpains, which then advance the signal transduction pathway by catalyzing the controlled proteolysis of its target proteins. Other reported roles of calpains are in cell function, helping to regulate clotting and the diameter of blood vessels, and playing a role in memory. Furthermore, calpains have been implicated in apoptotic cell death, and appear to be an essential component of necrosis (Liu et al., 2008).

After cerebrovascular accident (during the ischemic cascade) or some types of traumatic brain injury such as diffuse axonal injury), excessive amounts of calpain can be activated due to Ca^{2+} influx. Calpain activation leads to unregulated proteolysis of both target and non-target proteins and consequent irreversible tissue damage such as degradation of cytoskeletal proteins, voltage and ligand-gated ion channels, enzymes and lysosomal membrane resulting in cell death (Yamashima, 2000). Some studies have showed calpain inhibitor could exert

protective effects (Seyfried et al., 2001; Koumura et al., 2008). Calpeptin is a synthetic, effective and cell permeable calpain inhibitor and can significantly inhibit the activities of both isoforms of calpain. In the present study, after perfusion with $ACSF_{OGD}$ containing 10, 100 mol or 200 mol/l calpain, the times to OPS disappearance and the presence of HIP were markedly delayed and the rate and amplitude of OPS recovery were remarkably increased accompanied by decreased presence of HIP. These results indicated calpeptin could confer protective effects on the OGD induced injury of hippocampal slices.

It was found that calpain was only involved in the neuronal apoptosis post injury. Recently, calpain is also an important factor of neuronal apoptosis (Rami et al., 2000), but the exact mechanism is still poorly understood. Some researchers speculated that calpain could activate caspase-3 (Blomgren et al., 2001) and Bax, a pro-apoptotic protein and was related to the DNA cleavage and chromosome aggregation (Gao and Dou, 2000). Study showed there is a cross-talk between Calpain and Caspase-3 in penumbra and core during focal cerebral ischemia-reperfusion (Sun et al., 2008) and Calpeptin could reduce the neuronal apoptosis in hippocampal CA1 sector when the rats was subjected to the focal cerebral ischemia–reperfusion, the potential mechanism might be related to the inhibition of the expression of Caspase-3 by Calpeptin.

Our results showed the neuronal apoptosis was markedly improved after treatment with 10, 100 mol/l or 200 mol/l calpeptin, which also confirmed that calpain was involved in the neuronal apoptosis after ischemia. However, the specific mechanism underlying the protective effects of calpain on the post-ischemic apoptosis is unclear and further studies are required.

In the present study, 1 mol/l calpeptin did not exert obviously protective effects on the OGD induced injury of hippocampal slices which may be contributed to ineffective inhibition of OGD induced calpain activation. Moreover, calpeptin of 10 mol/l or higher concentrations had evident protective effects but the protective effects did not increase with the increase in concentrations. There were no significant differences between 100, 200 and 10 mol/l group. Maybe the inhibitory effects were maximized after 10 mol/l calpeptin and the calpeptin with increased concentration did not further enhance the protective effects.

Our study confirmed the calpeptin of appropriate concentrations could exert protective effects on the hippocampal slices exposed to OGD. Our results will provide basis for the development of new drugs and new strategies for the treatment of brain ischemia.

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