Delta opioid agonist [D-Ala2, D-Leu5] enkephalin (DADLE) reduced oxygen–glucose deprivation caused neuronal injury through the MAPK pathway

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ABSTRACT

It has been demonstrated that [D-Ala2, D-Leu5] enkephalin (DADLE), a delta opioid agonist, protected neuron from hypoxic neuronal injury by activating the delta opioid receptor (DOR). However, whether DADLE can prevent neuronal injury induced by severe hypoxia like oxygen–glucose deprivation (OGD) is not clear. Here, we investigated whether DADLE has a protective effect against neuronal injury induced by oxygen–glucose deprivation. Neuron viability was measured by MTT and neuron injury was assessed by lactate dehydrogenase (LDH) release. Protein expression was examined by Western blot. The results showed that DADLE protected the cortical neuron in a dose-dependent way from OGD injury. And this neuroprotective effect could be completely blocked by delta2 opioid antagonist Naltrindole. DADLE increased phosphorylation of ERK and prevented OGD-induced p38 phosphorylation. Neither DADLE nor Naltrindole had any appreciable effect on phosphorylation of JNK. One of the protective mechanisms of DADLE on OGD neurons may be due to the dynamic balance between the activation of ERK and the p38.

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1. Introduction

Neuronal death as a result of neuronal injuries such as stroke, is an irreversible process that leads to long term neurological deficit. The prevention of neuronal loss is therefore critical in rescuing the brain from neurological disaster. However, clinical strategies that mitigate hypoxic/ischemic injury induced neuronal loss are still very limited.

Recent data indicated that DADLE, can protect against ischemia–reperfusion-induced brain damage after transient middle cerebral artery occlusion (MCAO) in rats (Su, 2000). In our laboratory, Su Dian-san et al. (2007) also reported that intracerebroventricular treatment with DADLE attenuated the neuronal death and behavior retardation induced by forebrain ischemia in rats. However, the detailed mechanisms of its neuroprotection were not totally clarified.

Opioid receptors belong to the family of G protein-coupled receptors (GPCRs) (Lopez-Ilasaca et al., 1997; Polakiewicz et al., 1998) and their activation regulates multiple cellular processes, including activation of Mitogen-activated protein kinase (MAPK) (Burt et al., 1996; Fukuda et al., 1996; Wilson et al., 1997; Zhang et al., 1999). There are three members in MAP kinases, including extracellular signal-regulated kinases (ERK1/2), p38 kinase, and c-Jun N-terminal kinase (JNK). It has been reported that oxidative stress stimulates MAPK cascades through opioid receptors in neurons (Borlongan et al., 2000; Xia et al., 1995). However, the role of these MAPKs in neuronal oxidative injury is still unclear. In this study, we investigated...
the effects of delta opioid agonist DADLE on the expression of these three MAPK proteins as well as the cell viability and lactate dehydrogenase (LDH) release in the OGD-treated cortical neurons.

### 2. Results

#### 2.1. LDH level in culture medium was increased along with duration of OGD

We first investigated the effects of OGD on Medium LDH level by exposing the cortical neurons in OGD conditions at different times (0, 0.5, 1, 2, 4 and 6 h) at 37 °C. The results, as shown in Fig. 1, indicated that the cell injury was increased with an increase in the strength of OGD. A dramatic increment of the LDH activity was found at 2, 4 and 6 h of ischemia. The differences in the LDH activity between 4 and 6 h of OGD are greater than that between 2 and 4 h (Fig. 1). Therefore, 4 h of OGD was used in the following experiment.

#### 2.2. DADLE at 100 nM induced the best neuroprotective effect

We investigated the effects of different DADLE concentrations on the viabilities of ischemic cells. The cortical neurons were pre-incubated with different concentrations of DADLE for 1 h and then treated with ischemia (OGD) for 4 h. Cell viability was measured after the OGD treatment. It was found that cell viability increased progressively with the DADLE concentrations of added, it reached the highest value at 100 nM, and then decreased with the concentrations of DADLE. The value at 10 µM is lower than OGD (Fig. 2). Therefore, this dosage (100 nM) was used in the following experiments. DADLE exerts dual actions in the present study: the protection at low concentrations and the cytotoxicity at high concentrations. DADLE at low concentration (nM) shows high affinity to delta opioid receptor, while at high concentration (µM), it also shows considerable affinity to mu-opioid receptor. Although activation of delta opioid receptor provides neuroprotection against hypoxia induced injury, activation of mu-opioid receptor exacerbates the injury (Hayashi et al., 2002). Thus, the reduction of MTT level by 10 µM DADLE after OGD treatment may result from activation of mu-opioid receptor.

#### 2.3. DOR antagonist Naltrindole abolished the neuroprotective effect of DADLE

To test whether the neuroprotective effect of DADLE was through activation of delta opioid receptor, we use MTT and LDH assays to examine whether DOR antagonist Naltrindole could block DADLE-induced neuroprotection. Naltrindole and DADLE were co-administered to neuronal cultures for 1 h before treated with OGD. MTT and LDH assays revealed that 1 µM Naltrindole could completely abolish DADLE-induced neuroprotection of cortical neurons on OGD model (Fig. 3). Therefore, this dosage of Naltrindole (1 µM) was used in the following experiments.

#### 2.4. DADLE had a reciprocal effect on phosphorylation of ERK and p38

To understand the potential mechanisms involved in the protective effects of DADLE against OGD injury, we investigated the effects of DADLE on the level of total and phosphorylated p38, ERK and JNK. As shown in Fig. 4A, OGD significantly increased the levels of phosphorylated p38 MAPK (pp38) (156±21% versus 100% in normoxia; \(p<0.01\)). DADLE blocked OGD-induced upregulation of phospho-P38, while Naltrindole treatment reversed the effect of DADLE on phospho-p38 level. DADLE induced a significant increase in phospho-ERK levels in neurons during OGD (311±20% in DA+OGD and OGD alone; \(p<0.05\)).
191±10% in OGD as compared with the normoxia, *p* < 0.01). And this effect was abolished by Naltrindole treatment (Fig. 5B). These results indicated that DADLE, acting via DOR activation, reciprocally regulated phosphorylation of ERK and p38. Neither DADLE nor Naltrindole had any appreciable effect on phosphorylated c-Jun N-terminal protein kinase (pJNK) (Fig. 4C).

2.5. DADLE modulated cell viability through regulating phosphorylation of ERK and p38

Because phosphorylation of MAPKs can be pharmacologically inhibited (Davies et al., 2000; Torocsik and Szeberenyi, 2000), we used several specific MAPK inhibitors to block MAPK phosphorylation. As shown in Fig. 5A, OGD alone had no effect on pJNK levels but significantly increased phospho-p38 levels. Treatment with U0126, an ERK upstream kinase inhibitor, blocked the DADLE-induced increase in phospho-ERK; in addition, phospho-p38 levels were increased significantly in the same time, suggesting an interaction between activation of the two MAPKs. Treatment with the p38 MAPK inhibitor, SB203580, and the JNK blocker, SP600125, reversed the OGD-induced phospho-p38 increase and phospho-ERK decrease with no significant effect on pJNK. The above results suggested that OGD and DADLE triggered the phosphorylation of different MAPKs as a signaling mechanism to regulate levels of different protective or harmful proteins, thereby rescuing the cell or resulting in cell death. To strengthen these conclusions, we measured LDH release to evaluate changes in cell viability associated with changes in MAPK activity. LDH release was not significantly affected in normoxia neurons treated with MAPK inhibitors at the concentrations used, suggesting that these concentrations were not harmful to neurons within the experimental period (Fig. 5B). OGD increased LDH release, and this was partially reversed by treatment with SB203580 plus SP600125. These data indicated an important role of p38 MAPK phosphorylation in neuronal injury during OGD. DADLE reduced OGD-induced LDH leakage, and this effect was attenuated by U0126, suggesting that ERK activation is essential for DADLE-induced neuroprotection.

3. Discussion

The major findings of our study were as follows: (1) delta opioid receptor agonist, DADLE, protected against OGD neuronal injury. (2) A maximum of protective effect was seen at a concentration of 100 nM for DADLE. (3) This protective effect could be completely abolished by the delta opioid receptor antagonist, Naltrindole; (4) DADLE induced a significant increase in phospho-ERK and decrease in phospho-p38 levels in cultured neuron after OGD treatment, and this effect was abolished partially by Naltrindole. (5) There is a dynamic balance between phospho-ERK and phospho-p38 in DADLA-induced neuronal protection in OGD injury.

It has long been known that the brain is sensitive to hypoxia and ischemia (Coyle and Puttfarcken, 1993). In our in vitro study, neuron viabilities decreased to 25–35% of control after 4 h incubation under OGD condition. In the present study we showed that DADLE protected cortical neuron against OGD-induced neuronal injury and this protective effect could be blocked by DOR antagonist Naltrindole.

DADLE is widely used for DOR activation in cultured cells (Hayashi et al., 1999; Ma et al., 2005; Zhang et al., 2000, 2002). In animal experiments, we and others demonstrated dose-dependently neuroprotection effect of DADLE in neuronal death and retarded behavior induced by brain ischemia in rats (Hayashi et al., 2002; Horiuchi et al., 2004; Su et al., 2007). Yet controversy over the effects of different doses of DADLE on neuronal protection still remains. Previous studies showed that DADLE had potential antiapoptotic effect at pilo-molar to nalo-molar by activating DOR but was cytotoxic at micro-molar by activating mu-opioid receptor in serum deprived pheochromocytoma cell (PC12) (Hayashi et al., 2002). However,
current studies have observed that 10 μM of DADLE induced protective effect on hypoxic cortical neuronal cultures by activating DOR but not mu-opioid receptor (Zhang et al., 2000, 2002). We also found in our study that DADLE exerted dual actions: the protection at low concentrations against cell death (below 1 μM) and the cytotoxicity at high concentrations (at 10 μM). These different results may be a result of the differences in experimental designs. The pathophysiologic responses to different kinds of insults are inherently different. Furthermore, the distribution and densities of delta- and mu-opioid receptors may be quite different among all these cells mentioned above.

An interesting finding in this work was the reciprocal change in the phosphorylation of ERK and p38 MAPK upon OGD treatment which was regulated by DADLE. First, increased phospho-p38 and decreased phospho-ERK levels were observed in our DADLE+Naltrindole+OGD group. Furthermore, the ERK inhibitor, U0126, not only reduced ERK activity but also increased p38MAPK activity, whereas the opposite was true for p38 MAPK inhibition. Taken together, these results suggested that there may be cross-talk between the phosphorylation of ERK and p38 MAPK. The phosphorylation of p38 plays a critical role during OGD treatment, leading to neuronal injury, whereas DADLE increases the phosphorylation of the ERK and decreases the phosphorylation of p38, thus protecting neurons from injury.

Activation of intracellular ERK has been suggested to induce neuroprotection by preventing neuronal death in stroke (Park et al., 2004; Tataroglu et al., 2002). For example, blockade of ERK function induces cell death in various models of neuronal injury (Hayashi et al., 2002, Hetman et al., 1999). However, there is also evidence that ERK blockade protects neurons from stress (Tagawa et al., 2000). Our data strongly support the neuroprotective role of ERK in cortical neurons in OGD injury. This is based on the findings that 1) DADLE increased p-ERK levels and reduced neuronal injury and 2) ERK blockade reversed DADLE protection. Since there is evidence for signal coupling of DOR to ERK activation (Burt et al., 1996; Yenari et al., 2003), ERK activity may be a downstream signal of DOR activation in DADLE treated OGD neurons. Indeed, our data showed that antagonizing of DOR activation abolished both the ERK upregulation and neuroprotection induced by DADLE. Since blocking of DOR by Naltrindole in DADLE-treated neurons eliminated both the activation of the p-ERK and the neuroprotective effect, we believe that DADLE-induced neuroprotection is dependent on the activation of p-ERK.

Increased activity of p38 MAPK plays a critical role in cell death in neurons under stressful conditions (Takeda and

Fig. 4 - Representative immunoblots of total as well as phosphorylated p38, ERK and JNK in neurons of the cerebral cortex of normoxia (Nx), OGD and DADLE (DA)+OGD, or DA+Naltrindole (NTI)+OGD. Data is presented as mean ± S.D. (n=3). *p<0.05 versus OGD, $p<0.05 versus DA+OGD, †p<0.05 versus DA+Naltrindole (NTI)+OGD.
Ichijo, 2002). Inhibition of p38 MAPK confers neuroprotection in vitro against excitotoxic exposure (Legos et al., 2002) and reduces acute ischemic injury in vivo (Legos et al., 2001). In the present study, OGD caused a significant increase in p38 MAPK activity and serious injury to the neurons, whereas a p38 MAPK inhibitor reduced the increase in p-p38 level and reduced OGD-induced cell injury. Since DADLE suppressed the increase in p-p38 MAPK activity and reduced neuronal injury, we concluded that DOR activation suppressed p38 phosphorylation in OGD-treated neurons.

In summary, the possible protective mechanism of DADLE is dependent on the reciprocal change of the phosphorylation level of ERK and p38 MAPK in the OGD-treated neurons.

4. Experimental procedures
4.1. Animals

The experimental protocol used in this study was approved by the Ethics Committee for Animal Experimentation and was conducted according to the Guidelines for Animal Experimentation of Shanghai Jiao Tong University School of Medicine (Shanghai, China). The animals were studied at Shanghai Jiao Tong University School of Medicine (Shanghai, China). Sprague–Dawley pregnant (embryonic days 16–17) rats purchased from the Laboratory Animal Center, Shanghai Jiao Tong University School of Medicine. All reagents were obtained from Sigma Chemical (St. Louis, MO) unless specified in the text.

4.2. Neuronal cell culture

4.2.1. Preparation of neuronal cultures

Primary culture of cortical neuron from embryonic days 16–17 rats was prepared as previously described (Xia et al., 1995). In brief, fetuses were decapitated and cortical tissue was collected under sterile condition. The tissue was dispersed with a 1-ml pipette and then passed through an 80-µm nylon mesh with a Teflon pestle. Cells were resuspended in neuron-defined, serum-free Neurobasal medium (GIBCO-BRL, Grand Island, NY), supplemented with B-27, glutamine (0.5 mM), glutamate and a combination of penicillin (100 IU/ml) and streptomycin (100 µg/ml). The culture dishes were then kept in a humidified atmosphere of 95% air and 5% CO2 at 37 °C.

Fig. 5 – The phosphorylation of ERK and p38 were regulated by MAPKs inhibitors. After the addition of MAPK inhibitors (SB203580, SP600125 and U0126 for p38 MAPK, JNK, and ERK inhibition, respectively) to the medium (+ or * indicates with or without inhibitor), the cells underwent the indicated treatments, and then MAPK phosphorylation (A, n=4) and LDH leakage (B, n=8) were measured. Note that the DADLE-induced increases in phospho-ERK could be inhibited by U0126. Also, ERK inhibition induced an increase in phospho-p38 (A) and simultaneously abolished the ability of DADLE to protect cells from OGD, as indicated by LDH leakage (B). SB202190 plus SP600125 blocked the OGD-induced increase in phospho-p38 and led to an increase in phospho-ERK (A) and a decrease in OGD-induced neuronal injury (B). *p<0.05 versus OGD, †p<0.05 versus DA+OGD.
4.3. OGD treatment

To mimic cerebral ischemia in vitro, OGD was performed with the pretreated cells. For this purpose, we used a custom-made temperature-controlled anaerobic glove-box (Chavez et al., 2006), the glove-box system was set up at 37 °C with an atmosphere of 5% CO₂ and 95% N₂ (oxygen deprivation). All solutions were equilibrated for at least 12 h. Cells were transferred into the chamber, washed with PBS, and incubated with a preequilibrated glucose-free balanced salt solution for up to 2 h. For experimental controls, we used cultures that were subjected to the same procedures but maintained with glucose-containing media at normoxia in a standard cell culture incubator. The glove-box system used in this study was equipped with an inverted microscope that allowed us to visually inspect cell viability before terminating each experiment.

4.4. Drug treatments

All drugs were added directly to the culture medium at the appropriate concentration 1 h before OGD. [D-Ala², D-Leu⁵]-enkephalinamide (DADLE) and the specific DOR blocker, Naltrindole (RBI, St. Louis, MO), were used to block DOR function during OGD. The phosphorylated MAPks, p38 MAPK, JNK, and ERK, were blocked, respectively, using the specific inhibitors, SB203580 (10 mM), SP600125 (10 mM), and U0126 (10 mM; Cell Signaling, Beverly, MA).

4.5. Cell viability assessment

Functional mitochondria of living cells can deoxidize MTT to produce purple formazan, the measurement of which is used to determine the number of living cells (Lobner, 2000). After the cells were washed with PBS, 150 µl MTT (0.5 mg/ml) was added to the 96-well plate and the incubation continued for 4 h at 37 °C. The formazan accumulated in living cells was dissolved in 100 µl DMSO, and then quantified by optical density (OD) measured at 570 nm with a microplate reader (Tecan Sunrise, Switzerland). Survival of control group was defined as 100% and that of treated groups expressed as percentage of control levels.

4.6. Neuronal injury assay

Neuronal injury was quantitatively assessed by the measurement of LDH activity in the medium after the injury (Koh and Choi, 1987). LDH activity in the culture medium was measured with an LDH kit (Sigma Diagnostics Procedure No. 228-UV) and a Beckman DU-640 spectrophotometer system (Beckman Instruments, Fullerton, CA). Culture medium was sampled and centrifuged to remove cellular debris from the supernatant. Subsequently, 100 µl of the sample was added to a polystyrene cuvette containing 1 ml of LDH reagent (50 mM lactate and 7 mM NAD in 0.05% sodium azide buffer, pH 8.9). The cuvette was placed immediately into the spectrophotometer, maintained at 30 °C. After stabilization for 30 s, absorbance at 340 nm was recorded at 30-s intervals for 2 min. The change in absorbance was then expressed in concentration units (U) per liter and converted to percentage of control levels.

4.7. Western blot analysis

4.7.1. Immobolots

After removing the culture medium, the neuronal cells were washed twice with PBS and then suspended in PBS containing a protease inhibitor mixture (1 µM pepstatin, 200 µM phenylmethylsulfonyl fluoride, 1 µM leupeptin, and 1 mM EDTA; Roche Applied Science) followed by homogenization with a pipet pestle. All procedures were performed on ice as described previously (Li et al., 2006). The mixture was lysed by freeze-thawing three times and then centrifuged at 650 × g for 5 min to remove cellular debris, and samples of the supernatant were subjected to electrophoresis. Protein samples were separated on a 10–15% polyacrylamide gradient gel under denaturing conditions, and electrophoretically transferred to either a nitrocellulose (Bio-Rad) or polyvinylidene difluoride (Amersham Biosciences) membrane. The nonspecific binding was blocked by incubation with 5% skim milk or 1% bovine serum albumin (American Bioanalytical, Natick, MA) in 50 mM Tris-buffered saline (pH 7.4) and then incubated overnight at 4 °C with different primary antibodies diluted in PBST containing 1% bovine serum albumin; the antibodies and dilutions were p-p38, p-ERK, pJNK p38 MAPK, JNK, or ERK (1:500) (all from Cell Signaling). After washing, the membranes were incubated for 1 h at room temperature with goat anti-mouse IgG (Santa Cruz Biotechnology), or goat anti-rabbit IgG (Bio-Rad) diluted 1:400 in 3% skim milk and washed, and the bound antibody was visualized using a chemiluminescence kit (Amersham Biosciences). The densities of the bands were measured by densitometry using an image analysis system (Amersham Biosciences).

4.8. Statistical analysis

All values are presented as means ± S.D.. Comparisons among multiple groups involved one-way ANOVA followed by the Student–Newman–Keuls test and Dunnett’s test (for comparison of multiple experimental treatments to a common control value). All analyses were performed by using software SPSS 11.0. A value of p < 0.05 was considered significant.

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