Protective Effects of the Delta Opioid Peptide [D-Ala2, D-Leu5]enkephalin in an *Ex Vivo* Model of Ischemia/Reperfusion in Brain Slices

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Keywords

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SUMMARY

Introduction: The delta opioid peptide [D-Ala2, D-Leu5]enkephalin (DADLE) plays a key role in neuronal protection against both hypoxic and ischemic conditions. However, the cellular mechanisms of action of DADLE under these conditions remain unclear. **Methods:** Ischemia was simulated with perfusing the brain slices with glucose-free artificial cerebrospinal fluid. Apoptosis was examined using an *in situ* cell death detection kit and expressed as the percentage of positively labeled neurons relative to total number of neurons. PCR was performed by adding cDNA, 5 pm dNTP, 1 μ L Taqase, and primers. PCR products were separated with electrophoresis, stained with ethidium bromide, and visualized under ultraviolet light. **Aims:** To investigate the potential effects of DADLE in an *ex vivo* model of cerebral ischemia/reperfusion. **Results:** DADLE attenuated lactic dehydrogenase release and neuronal apoptosis in a concentration-dependent manner. The protective effects of DADLE were attenuated by representative selective delta2, but not delta1 opioid antagonists. Treatment with PD98059, a selective inhibitor of ERK kinase (MEK), also blocked the protective effect of DADLE as well as ERK phosphorylation induced by DADLE. **Conclusions:** Endogenous opioid peptides could promote cell survival via delta2 opioid receptors, possibly through the downstream MEK-ERK pathway.

Introduction

[D-Ala2,D-Leu5]enkephalin (DADLE), a delta opioid peptide, protects the brain against ischemia-induced insults [1,2]. Putative mechanisms underlying the protective properties of DADLE include antioxidation [3]. Other studies have indicated that the neuroprotective effects of DADLE against methamphetamine-induced dopaminergic terminal damage are mediated by opioid receptors [4,5]. Antiapoptotic effects apparently also contribute to the protective action of delta opioids both *in vitro* and *in vivo* [2,5]. Delta opioid receptors could stimulate extracellular signal-regulated protein kinases (ERK), which are members of the mitogen-activated protein kinases (MAPK) [6]. Because ERKs are implicated in cell survival [7,8], it is possible that DADLE might induce an antiapoptotic property by activating the MAPK pathway via opioid receptors [8]. Indeed, it is suggested that ERK activation may promote cell survival via the MEK-ERK pathway perhaps through delta2 opioid receptors [9].

In the current study, we examined the protective effects of DADLE against ischemia/reperfusion injury in a model system using rat brain slices. Ischemia/reperfusion was simulated by removing oxygen/glucose from the perfusate, followed by re-oxygenation [10,11]. Potential role of delta receptor subtypes and the MAPK pathway were also investigated.

Methods

Animals

Male Sprague-Dawley rats (Grade II, Certificate No. 152) weighing 90–120 g were purchased from Shanghai Experimental Animal Center, Chinese Academy of Sciences. All animal experiments were approved by the Institutional Animal Care and Use Committee.

Reagents and Drugs

Lactic dehydrogenase (LDH) detection kit was purchased from Jiancheng Bioengineering Institution (Jiancheng, Nanjing, China). [D-Ala, D-Leu]enkephalinamide (DADLE, a selective delta opioid receptor agonist), naltrindole (a selective delta opioid receptor antagonist), 7-benzylidene-naltrexone (BNTX; a selective delta1 antagonist), naltriben (a selective delta2 opioid antagonist), and naloxone (a nonselective opioid antagonist) were all purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cerebral Cortical Slice Preparation

Brain slices (400 μ m thick) were prepared as previously described [11] using a vibrating tissue slicer (ZQP-86; Zhixin Co., Ltd.,

Figure 1 Effects of DADLE on I/R injury in brain slices. **(A)** Effects of DADLE alone. * $P < 0.05$ versus blank control. **(B)** Interaction of DADLE (10^{-5} M) with representative opioid antagonists (all at $10 \mu\text{M}$). # $P < 0.05$ versus I/R plus DADLE. The values represent mean $\pm$ SD from five to six independent experiments.

Shanghai, China), with minor modifications. Briefly, rats were decapitated, and the brain was quickly removed and immersed in ice-cold oxygenated (95% O_2 /5% CO_2) artificial cerebrospinal fluid (ACSF) with the following composition (in mM): NaCl 119, KCl 2.5, CaCl_2 2, MgSO_4 1, NaH_2PO_4 1.25, NaHCO_3 26.2, glucose 10, with pH 7.4. Slices were equilibrated at room temperature (24°C) for 90 min and then at 37°C for 30 min prior to experiments.

Ischemia/Reperfusion Experiments

Ischemia was simulated with perfusing the brain slices with glucose-free ACSF (glucose was substituted by sucrose of equimolar concentration) and saturated with 95% N_2 + 5% CO_2 for 10 min. Reperfusion was simulated with perfusion in oxygenated normal ACSF (containing glucose) and saturated with 95% O_2 /5% CO_2 for 2 h.

DADLE (10^{-9} – 10^{-5} M), in the presence or absence of one of the following compounds, was included in the ACSF in some experiments: naltrindole ($10 \mu\text{M}$), BNTX ($10 \mu\text{M}$), NTB ($10 \mu\text{M}$), and naloxone ($10 \mu\text{M}$).

Lactate Dehydrogenase Assay

Lactic dehydrogenase in the perfusate was examined as previously described [12] using a commercial kit from Jiancheng

Figure 2 Effects of DADLE on neuronal apoptosis. **(A)** Representative slides of TUNEL staining; $\times 400$. **(B)** Summary of the results. The values represent mean $\pm$ SD of four independent experiments. * $P < 0.05$ versus I/R injury alone.

Bioengineering Institution and used to reflect neuronal damage. LDH concentration in brain slices was subjected to ischemia/reperfusion, but not any other drug treatment was set at 100%.

In Situ Labeling of DNA Fragmentation

At the end of the experiments, brain slices were fixed at 4°C in 4% paraformaldehyde for 2 h. Sections (6- μ m thick) were prepared with routine procedures. Apoptosis was examined using an *in situ* cell death detection kit from Roche Applied Science (Mannheim, Germany). Briefly, tissue sections were rehydrated by heating at 60°C, followed by washing in xylene and rehydration through a graded series of ethanol. Sections were then incubated with protease for 15 min at 37°C, followed by 1% Triton X-100 for 8 min. Slides were then rinsed twice with PBS. Fifty microliter of TUNEL reaction mixture was added to the tissue, and incubation lasted for 60 min at 37°C in a humidified atmosphere in the absence of light. The slides were rinsed three times in PBS prior to addition of 50 μ L converter-POD. Tissue sections were incubated in a humidified chamber for 30 additional min at 37°C and rinsed three times with PBS prior to DAB color reaction and microscopic examination using an Axioplan 2 imaging system (ZEISS, Jena, Germany). Apoptosis is expressed as the percentage of positively labeled neurons relative to total number of neurons.

RT-PCR Procedures

Total RNA was extracted from brain slices using a Trizol reagent (Invitrogen, CA, USA). cDNA was synthesized from 1 μ g of pooled mRNA using Oligo dT Primer (50 pmol), 20 pmol dNTPs, 20 U RNase Inhibitor (Promega, San Luis Obispo, CA, USA), and 200 μ M-MLV Rtase (Promega). PCR was performed by adding cDNA, 5 pmol dNTP, 1 μ L Taqase, and one of the following primers:

Bcl-2 sense, 5'-CTGGTGGACAACATCGCTCTG-3' and antisense, 5'-GGTCTGCTGACCTCACTTGTG-3'; *Bax* sense, 5'-TCCAGGATC-GAGCAGA-3' and antisense, 5'-AAGTAGAAGAGGGCAACC-3'; β -actin sense, 5'-ATTGTAACCAACTGGGACG-3' and antisense, 5'-TTGCCGATAGTGATGACCT-3'. All primers were designed using the DNASTAR PrimerSelect program (Lasergene, Madison, WI, USA) and synthesized by CASarray (Shanghai, China). PCR products were separated with electrophoresis, stained with ethidium bromide, and visualized under ultraviolet light. Optical density was semiquantitatively analyzed with a Tanon GIS gel imaging system (Bio-Tanon, Shanghai, China). mRNA expression was normalized using β -actin as an internal control.

Western Blot Analysis

Brain slices were homogenized in 20 mM HEPES (pH 7.5) buffer containing: 250 mM sucrose, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 2 μ g/mL aprotinin, 10 μ g/mL leupeptin, 5 μ g/mL pepstatin, and 12.5 μ g/mL Nacetyl-leu-leu-norleucinal, and 1:100 dilution phosphatase inhibitor cocktail I and II (Sigma-Aldrich, St. Louis, MO, USA). The homogenate was centrifuged at 8000 $\times g$ for 20 min at 4°C. Protein concentration of the supernatant was determined using a Bradford method (Bio-Rad, Hercules, CA, USA). Twenty micrograms of protein from each sample was

separated by 12% SDS-PAGE and transferred to a polyvinylidene difluoride membrane, prior to incubation with a primary antibody against one of the following proteins: phospho- versus total ERK1, phospho- versus total ERK2 (all at 1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA). After incubation with an appropriate secondary antibody and color reaction, the bands of interest were quantified using an image analysis system (Image; NIH, Bethesda, MD, USA).

Statistical Analysis

Morphologic assessments and biochemical assays were performed in a minimum of eight slices for each treatment condition. All data are presented as mean $\pm$ SEM and analyzed with one-way ANOVA followed by Fisher's PLSD *post hoc* test. Statistical significance was set at $P < 0.05$.

Results

DADLE attenuated LDH release from the brain slices in a concentration-dependent manner (Figure 1A). The inhibitory effects of DADLE at a representative concentration (10 mM) were attenuated by nonselective opioid antagonists naltrindole and naloxone, the selective delta2 opioid antagonist naltriben, but not the selective delta1 antagonist BNTX (Figure 1B). Consistent with the LDH results, DADLE (10^{-6} and 10^{-5} M) significantly attenuated neuronal apoptosis (Figure 2).

Ischemia/reperfusion increased the mRNAs (Figure 3A) and protein (Figure 4A) level for both *Bcl-2* and *Bax*. Treatment with DADLE (10^{-6} and 10^{-5} M) significantly enhanced *Bcl-2* mRNA expression but suppressed *Bax* mRNA expression (Figure 3B). As

Figure 3 Effects of DADLE on Bax and Bcl-2 mRNA expression. (A) Representative mRNA blots. (B) Summary of results. All values represent mean $\pm$ SD of three independent experiments. * $P < 0.05$ versus I/R injury alone.

Figure 4 Effects of DADLE on Bax and Bcl-2 protein expression. (A) Representative immunoblots. (B) Effects on Bax/Bcl-2 ratio; a summary of results. * $P < 0.05$ versus I/R injury alone. All values represent mean $\pm$ SD of three independent experiments.

Figure 5 Effects of DADLE on ERK phosphorylation. (A) Representative immunoblots. (B) Summary of results. All values represent mean $\pm$ SD of four independent experiments. * $P < 0.05$ versus I/R injury alone.

a result, the Bax/Bcl-2 ratio was reduced with DADLE (Figure 4B).

DADLE increased ERK phosphorylation (Figure 5). Increased ERK phosphorylation by DADLE was abolished by naltrindole (Figure 6A,B). Treatment of the brain slices with PD98059, a selective MEK inhibitor upstream of ERK, also abolished the DADLE-induced phosphorylation of ERK (Figure 6C,D).

Figure 6 Interaction of DADLE with naltrindole and PD98059 on ERK phosphorylation. (A) Effects of DADLE in the absence versus presence of naltrindole (10 μ M), representative immunoblots. (B) Summary of results. * $P < 0.05$ versus I/R injury alone (DADLE -; naltrindole -); # $P < 0.05$ versus I/R plus DADLE. (C) Effects of DADLE in the absence versus presence of PD98059 (100 mM), representative immunoblots. (D) Summary of results. * $P < 0.05$ versus I/R injury alone (DADLE -; naltrindole -); # $P < 0.05$ versus I/R plus DADLE. All values represent mean $\pm$ SD of four independent experiments.

Conclusion

Results from this study suggested that the delta opioid peptide DADLE could protect the brain from ischemia/reperfusion injury in a concentration-dependent manner, most likely by attenuating apoptosis via the MEK/ERK antiapoptotic pathway. Consistent with a previous study [13], the protective effects of DADLE

seemed to be mediated by delta2 opioid receptor. It is noteworthy that DADLE has been reported to exhibit dual pharmacological actions in neocortical neurons [14,15]. It was recently reported that the delta opioid peptide 4-((2,5-dimethyl-4-(2-propenyl)-1-piperazinyl)(3-methoxyphenyl)methyl)-N, N-diethylbenzamide (2S-(1(S*),2alpha,5beta))-(9Cl) (SNC-80) could inhibit HIV-1 expression in DOR-transfected lymphocytes, whereas the delta2 antagonist naltrindole could block this effect [16].

The fact that the effects of DADLE could be attenuated by naltrindole supports the notion that the antiapoptotic action of DADLE is mediated by the activation of delta2 opioid receptors [17,18]. Although two delta opioid receptor subtypes have been defined pharmacologically [12], only one gene (DOR-1) was cloned [19]. A recent study on DOR-1 knockout mice demonstrated that both delta1 and delta2 subtypes of opioid receptors were abolished in these mice, suggesting that the pharmacologically distinct delta1 and delta2 subtypes of opioid receptors are encoded by the DOR-1 gene [20]. The different pharmacological profiles observed for the two delta subtypes might arise from post-translational protein modification and/or alternative splicing [21].

In our study, the protective effects of DADLE were observed at the micromolar range; such results were consistent with the previous findings in primary culture of neocortical neurons [22] but not in PC12 cells [13], suggesting that the results from immortalized cell line should be extrapolated with caution.

The results from this study demonstrated a role of MEK/ERK in the antiapoptotic action of DADLE. Potential role of Akt, also known as protein kinase B, a serine/threonine protein kinase that plays an important role in multiple cellular processes [23], in the action of DADLE cannot be ruled out [23]. Activation of the phos-

phoinositide 3-kinase (PI3K), and its downstream signaling molecules, for example, the MEK/ERK and the Akt/p70S6 pathways, produces potent antiapoptotic action [24]. It will be important that further studies clarify the potential involvement of Akt with DADLE action.

One recent study suggested that the cardioprotective effect of γ -opioid receptor agonists is mediated by Gi/Go proteins and mitochondrial K channels [25,26]. More specifically, functional inhibition or blocking of Gi/Go proteins and K channels could attenuate γ -opioid receptor-induced cardioprotection [25,26]. Opening of plasma membrane K channels has been shown to be protective in neuronal adaptation to hypoxic stress [26], but whether the G protein-K channel pathway is involved in the protective effects of γ -opioid receptors remains unclear. It is also possible that delta opioid receptor could produce neuroprotection by modulating protein kinase C (PKC) activity [25]. In fact, there is evidence showing that the cardioprotective effect of opioid receptors is mediated through a PKC pathway [27]. In addition, modulation of voltage-dependent Ca^{2+} channels may be involved in delta opioid receptor-mediated neuronal protection [28].

In summary, results from the current study indicated that endogenous opioid peptides may play important roles in regulating both cell survival and cell death through distinct subtypes of opioid receptors. Our findings also encourage development of delta opioids in the treatment of ischemic brain diseases.

Conflict of Interest

The authors declare no conflict of interest.

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