This article was published in an Elsevier journal. The attached copy is furnished to the author for non-commercial research and education use, including for instruction at the author’s institution, sharing with colleagues and providing to institution administration.

Other uses, including reproduction and distribution, or selling or licensing copies, or posting to personal, institutional or third party websites are prohibited.

In most cases authors are permitted to post their version of the article (e.g. in Word or Tex form) to their personal website or institutional repository. Authors requiring further information regarding Elsevier’s archiving and manuscript policies are encouraged to visit:

http://www.elsevier.com/copyright
Dose-dependent neuroprotection of \textit{delta} opioid peptide [\textit{d}-Ala\textsubscript{2}, \textit{d}-Leu\textsubscript{5}] enkephalin in neuronal death and retarded behavior induced by forebrain ischemia in rats

Su Dian-san, Wang Zhen-hong, Zheng Yong-jun, Zhao Yan-hua, Wang Xiang-rui *

Department of Anesthesiology, RenJi Hospital, School of Medicine, Shanghai Jiaotong University, Shanghai, China

Received 22 May 2007; received in revised form 26 June 2007; accepted 27 June 2007

Abstract

Cerebral ischemic insult, mainly induced by cardiovascular disease, is one of the most severe neurological diseases in clinical. There’s mounting evidence showing that \textit{delta} opioid agonist [\textit{d}-Ala\textsubscript{2}, \textit{d}-Leu\textsubscript{5}] enkephalin (DADLE) has a tissue-protective effect. However, whether this property is effective to prevent neuronal death induced by forebrain ischemia is not clear. This study was aimed to investigate whether intracerebroventricular (ICV) administration of DADLE has a neuroprotective effect against forebrain ischemia in rats. We found in our study that administration of DADLE 45 min before forebrain ischemia had significant protective effect against CA1 neuronal lose. Further more, we found that DADLE had a dose-dependent protection for improving behavioral retardation revealed by Morris water maze and motor score test, while naltrindole, the antagonist of \textit{delta} opioid receptor, partially abolished neuroprotective effect of DADLE, which implicated that both opioid and non-opioid systems are involved in ischemic insults and neuroprotection.

© 2007 Elsevier Ireland Ltd. All rights reserved.

Keywords: DADLE; Intracerebroventricular administration; Neuroprotection; Forebrain ischemia; Morris water maze; Rats

It has been demonstrated that [\textit{d}-Ala\textsubscript{2}, \textit{d}-Leu\textsubscript{5}] enkephalin (DADLE), one of the \textit{delta} opioid agonists, possesses a tissue-protective property. The preconditioning or perfusion of DADLE remarkably prolonged the preservation time of isolated organs, such as the heart and lung [17,18,15]. DADLE also protects against psychostimulant-induced insults in the central nervous system [19,9] and against 6-hydroxydopamine-induced brain damage [4].

Early study has implicated that the involvement of \textit{delta} opioid receptor plays an important role in cerebral ischemia [22]. Zhang et al. reported that application of DADLE increases the tolerance of cultured cortical neurons against the hypoxia by employing an \textit{in vitro} model [21]. However, there is little direct evidence \textit{in vivo} proving the neuroprotective property of the DADLE against brain ischemia. Therefore, the purpose of the present study was to assess the effects of DADLE intracerebroventricular administration on the forebrain ischemia in the rat.

Male Sprague–Dawley weighing 350–400 g were anesthetized with 10% chloral hydrate (3 ml/kg, i.p.). Unilateral guide cannulae (22 gauge) were implanted 1 mm above the left lateral ventricle and then which was secured to the skull with dental acrylic and jeweller’s screws. The position of the cannula was confirmed by injections of methylene blue through the cannula at the time of sacrifice in a subset of rats. At least 6 days were allowed for recovery before testing. All experimental procedures were approved by the Animal Care and Use Committee of Shanghai Jiaotong University.

To induce forebrain ischemia, animals were anesthetized again with 10% chloral hydrate (3 ml/kg, i.p.). Lungs were ventilated mechanically with a mixture of oxygen and air for FiO\textsubscript{2} 0.3 to maintain normocapnia. The pericranial temperature was maintained to 37.5 ± 0.5 °C by surface heating or cooling, and the EEG was monitored simultaneously and continuously. The tail artery was cannulated for mean arterial pressure (MAP) monitoring and blood sampling for arterial blood gases and hematocrit analysis 10 min before ischemia and 10 and 60 min after ischemia. The right jugular vein was cannulated with PE-60
tubing for withdrawing blood. Ischemia was induced by withdrawal of 6–10 ml blood from the jugular catheter, once the MAP fell to 25 mmHg, the bilateral common carotid arteries were occluded with aneurysm clips, and a timer was started. Ischemia period was lasted for 10 min and was confirmed by the presence of an isoelectric electroencephalogram. To terminate ischemia, the aneurysm clips were removed and shed blood was reinfused. After the trachea was extubated, the animals were transferred to recovery chamber. A rectal probe was used for intermittent temperature monitoring in the first 3 h post-operatively and controlled to 37.5 ± 1.0 °C by surface heating or cooling or managing the temperature of recovery chambers because delta opioid receptor stimulation may induce hypothermia [1].

The animals were randomly allocated to one of six groups (n = 25 per group). ICV administration dose was determined based on the previous study [21]. A total volume of 10 μl artificial cerebrospinal fluid (ACSF) containing 0, 0.2, 2, 20 mM DADLE or 2 mM DADLE in addition with 2 mM naltrindole was administered 45 min prior to forebrain ischemia. Followed by a 10 min of bilateral carotid artery occlusion to induce ischemia. Sham operations were also performed using the same procedures as in the DADLE-0 groups without ischemia (n = 25). All drugs were injected using a microinfusion pump and infusions were delivered over a 60 s period.

At post-ischemia day 5, motor functional tests were performed by an investigator blinded to group assignment according to a well-established paradigm, including assays of prehensile traction and balance beam performance as described previously [8]. The motor score was graded in a 0–9 scale (best score = 9). The Morris water maze (MWM, Shanghai Jiliang Software Technology Co. Ltd., China) trials were performed at the 6th, 7th, and 8th day post-ischemia as previously described [14,7]. Briefly, a hidden black plastic platform (12 cm diameter) was fixed 40 cm away from the wall of the water maze (160 cm diameter, 35 cm deep) and 2 cm below the water surface. Five trials in each session were conducted daily, with three sessions in total. At the beginning of each trial, the rat was placed into the water, facing the wall of the pool at one of four starting points (north, south, west, or east). The starting point was randomly selected, but protocol was fixed at the beginning of each trial and was maintained throughout Trials 1–5 (acquisition trials). Each rat was allowed to find the platform in 90 s and to mount the platform. Thirty seconds after mounting the platform, the animal was removed and placed in a holding cage and warmed with a heating lamp. Rats failing to locate and mount the platform within 90 s were placed on the platform for 30 s before they were transferred to the holding cage. The interval between each trial was 20 s. The amounts of time spent to find and mount the platform (escape latency) and swimming speed were recorded. Following the five acquisition trials, each rat underwent a single probe trial, in which the platform was removed from the water tank and the animal was allowed to swim freely for 60 s. The probe trial is used to assess the development of spatial bias. The time spent in each quadrant of the former platform position was recorded. Overall, each rat completed 15 acquisition trials and three probe trials over a 3-day period.

At the 9th day post-ischemia, the animals were anesthetized by 10% chloral hydrate and sacrificed for histological analysis. Briefly, the rats underwent transcardial perfusion with isotonic saline (150 ml) and 4% (400 ml) paraformaldehyde in a phosphate buffer solution (0.1 M, pH 7.4). Brains were removed and post-fixed in 4% paraformaldehyde in 0.1 M PB overnight. After dehydration in graded concentration of ethanol and butanol, the brains were embedded in paraffin. Four-micrometer-thick coronal sections was obtained and stained with hematoxylin and eosin.

Injury to the hippocampal CA1 sector was evaluated under light microscopy by an investigator blinded to group assignment. The number of surviving neurons in CA1 was counted. Preserved neurons were considered to be those with a blue and visible nucleus along with an intact cytoplasm. The surviving neurons in three visual fields (94,879.82 μm²) in CA1 sector were counted and the average number was taken as the surviving neurons number of this section. The average number of the five successive sections represented the surviving neurons number of each rat. Then significant differences were determined by SPSS 10.0.

The motor scores were presented as the median and 95% confidence interval range, comparing among groups by the Kruskal–Wallis test. If the analysis results identified significant differences, Mann–Whitney U-test was used for intergroup comparisons. Mortality rate comparison was done using the Chi-square test or Fisher’s exact test. Morris water maze results were compared by repeated-measures analysis of variance followed by LSD post hoc test for intergroup comparisons. One-way ANOVA followed by LSD post hoc test was used to determine the CA1 neurons and the physiologic values. All data except for mortality rate and motor score are presented as the mean ± S.D., and p < 0.05 was considered statistically significant.

There were no significant differences for the physiologic values during the experiment among groups in three time points including the pH, partial arterial pressure of carbon dioxide, partial arterial pressure of oxygen, oxygen saturation as well as hematocrit (data not shown).

To study the consequence of surgery, we recorded the animal fatal percentage during recovery period. No animal died during the recovery period in the sham group. However, out of total 150 animals in experimental groups, 29 rats treated with DADLE or naltrindole died after ischemia, with some of them died in the early recovery period with apparent upper airway obstruction or seizure. However, the cause of other deaths was not identified as those deaths were not witnessed. There is no significant difference of the mortality percentage among experimental groups according to statistical analysis (data not shown).

To investigate whether administration of DADLE are able to improve animal behaviors, we employed the motor score test in our study. Motor scores are reported in Fig. 1. All the animals in sham group had a score of 8–9 (median 9, 95% confidence interval, 8.48–8.88). As expected, the motor score in the ACSF group decreased significantly, compared with that from the sham group after ischemic (median 3, 95% confidence interval, 3.17–3.73, p < 0.05). However, ICV injection of DADLE significantly improved the motor score after...
ischemia in a dose-dependent manner, compared with ACSF group (DAD0.2, median 5, 95% confidence interval, 4.48–5.52; DAD2, median 6, 95% confidence interval, 5.61–6.89; DAD20, median 8, 95% confidence interval, 7.34–7.80, \( p < 0.05 \)). Furthermore, in order to test the specificity of DADLE on motor score after ischemia, we used naltrindole, a specific antagonist of delta opioid receptor, to block its downstream signaling pathway. We co-administered naltrindole with 2 mM DADLE (DAD2 group), and found that the motor score significantly decreased in the DAD2 + NAT group (median 4, 95% confidence interval, 3.85–4.65, \( p < 0.05 \)), compared with the DAD2 which showed that DADLE executed its effect through delta opioid receptor.

It has been reported that after ischemic insults, patients or animals display memory retardation in different extent [16,12]. In our study, we used Morris water maze task to access the learning and memory.

Swimming speeds were not significantly different among the six groups as shown in Fig. 2. However, the escape latencies, denoting the cumulative time taken by animals to find the platform based on five trials of each day, were shorter in the DADLE group compared with the ACSF group (\( p < 0.05 \)). The escape latencies were dose-dependent shorter in the three DADLE groups, namely the higher administration dose of DADLE, the shorter latency, as demonstrated in DAD20 < DAD2 < DAD0.2 (\( p < 0.05 \)). Compared with the DAD2 group, the escape latency in DAD2 + NAT group was significantly longer (\( p < 0.05 \)), but still shorter than the ACSF group (\( p < 0.05 \)), which implicated the effective antagonism of NAT by blocking delta opioid receptor (Fig. 3).

In the probe trial, the time spent in the quadrant of the former platform was significantly longer in three DADLE groups compared with the ACSF group (\( p < 0.05 \)). Furthermore, the repeated-measures analysis of variance revealed that the higher dose of DADLE, the longer time spent in the quadrant of the former platform, shown as DAD20 > DAD2 > DAD0.2 (\( p < 0.05 \)). Compared with the DAD2 group, the time spent in the quadrant of the former platform in DAD2 + NAT group was significantly shorter (\( p < 0.05 \)), but still longer than the ACSF group (\( p < 0.05 \)) as shown in Fig. 4.

To further elucidate whether DADLE has a neuroprotective effect against cell death induced by forebrain ischemia, we counted surviving cells in CA1 of hippocampus, a brain region which is sensitive and susceptible to ischemia insult. The number of surviving hippocampal CA1 neurons was determined by hematoxylin and eosin staining. Compared with the ACSF group

---

**Fig. 1.** Motor scores. The motor scores were worst in the ACSF group (\( p < 0.05 \)); ICV administration of DADLE significantly improved the motor score after ischemia in a dose-dependently manner, namely DAD20 > DAD2 > DAD0.2. Comparing with the DAD2 group, the motor score was worse in the DAD2 + NAT group (\( p < 0.05 \)). *Compared with the ACSF group, \( p < 0.05 \). †Compared with the DAD2 group, \( p < 0.05 \).

**Fig. 2.** Swimming speeds in the Morris water maze. There were no significant differences among the groups.

**Fig. 3.** The escape latencies were dose-dependent shorter in the three DADLE group, namely the higher administration dose of DADLE, the shorter latency, DAD20 < DAD2 < DAD0.2 (\( p < 0.05 \)). The escape latencies in DAD2 + NAT group were significantly longer than the DAD2 group (\( p < 0.05 \)), but still shorter than the ACSF group (\( p < 0.05 \)). *Compared with the ACSF group, \( p < 0.05 \). †Compared with the DAD2 group, \( p < 0.05 \).

**Fig. 4.** The time spent in the quadrant of the former platform. The time spent in the quadrant of the former platform dose-depended increased, namely DAD20 > DAD2 > DAD0.2 (\( p < 0.05 \)). Compared with the DAD2 group, the time spent in the quadrant of the former platform in DAD2 + NAT group was significantly shorter (\( p < 0.05 \)), but still longer than the ACSF group (\( p < 0.05 \)). *Compared with the ACSF group, \( p < 0.05 \). †Compared with the DAD2 group, \( p < 0.05 \).
Inhibition of DADLE reduced neuronal injury after hypoxic exposure, while very similar to the present study [21]. They demonstrated that age in the striatum of rats [19].

Designs (intraperitoneal injection versus intracerebroventricular administration) appear to contribute to the different results, which might be a consequence of the intake efficacy of the compound or different final concentration in brain tissue. There is some evidence showing that DADLE is hard to across the blood–brain barrier (BBB). Using an in situ perfused rat brain, Chen and his colleagues studied the permeation characteristics of DADLE and its cyclic produgs across the BBB. They found that the apparent permeability coefficient (Papp) of DADLE across the BBB was very low (<10⁻⁷ cm/s), probably due to its unfavorable physicochemical properties (e.g., charge, hydrophilicity, and high hydrogen-bonding potential) [6].

DADLE is an agonist of delta opioid receptor. Upon the activation of delta opioid receptor, multiple intracellular signaling pathways are initiated and many signaling molecules are involved in, including protein kinase C, adenylate cyclase, and mitogen-activated protein kinase, which have been implicated to participate in the brain ischemic insult and may be involved in executing the neuroprotection of DADLE [2,13].

Zhang et al.’s study also indicated that the equal concentration naltrindole, an antagonist of delta opioid receptor, can entirely inhibit the effects of DADLE in vitro [21], however in our study, we found that naltrindole’s antagonistic effect was partial as the blocking could not be able to reverse the effect of DADLE entirely. Comparing with the DAD2 group, all parameter aggravated significantly in the DAD2 + NAT group, while still outstripped the ACSF group, which suggested that DADLE’s neuroprotective effects may also execute via a non-opioid mechanism.

Tsao et al.’s study [5] indicated that DADLE can act as a free radical scavenger and because it is well known that ischemia-induced neurotoxicity involves ROS, it is tempting to speculate that DADLE, at least in part, exert its brain protective effect through the sequestration of free radicals. However, further experiment to test the hypothesis that DADLE may sequester ischemia-induced free radical formation in vivo need to be carried out.

In summary, our work showed that intracerebroventricular administration of delta opioid peptide [d-Ala², d-Leu⁵] enkephalin (DADLE) in neuronal death and retarded behavior induced by forebrain ischemia in rats. We demonstrated that ICV administration of DADLE improved the motor functions and learning and memory after ischemic insult, as well as increasing the preserved neurons in hippocampal CA1 after forebrain ischemia.

There’s mounting evidence showing that DADLE has a protective property in the central nervous system. Zhang et al. demonstrated that DADLE reduced the glutamate-induced excitotoxic injury of neocortical neurons [21,20]. The results of Hayashi’s research indicated that DADLE at a low concentration had anti-apoptotic effect in serum-deprived pheochromocytoma cell (PC12) [10]. In animal experiments, Borlongan et al. reported that DADLE enhanced both in vitro and in vivo survival of dopaminergic neurons in mice [3]. Tsao et al. demonstrated that DADLE reduced methamphetamine-induced neuronal damage in the striatum of rats [19].

In regard to hypoxia or ischemia, Zhang et al.’s results are very similar to the present study [21]. They demonstrated that DADLE reduced neuronal injury after hypoxic exposure, while inhibition of mu and kappa opioid receptor had little effect on neuronal survival in hypoxic condition. However, Iwata’s [11] study revealed that intraperitoneal administration of DADLE had no neuroprotective effect on hippocampal injury induced by forebrain ischemia. The differences between experimental designs (intraperitoneal injection versus intracerebroventricular administration) appear to contribute to the different results, which might be a consequence of the intake efficacy of the compound or different final concentration in brain tissue. There is some evidence showing that DADLE is hard to across the blood–brain barrier (BBB). Using an in situ perfused rat brain, Chen and his colleagues studied the permeation characteristics of DADLE and its cyclic produgs across the BBB. They found that the apparent permeability coefficient (Papp) of DADLE across the BBB was very low (<10⁻⁷ cm/s), probably due to its unfavorable physicochemical properties (e.g., charge, hydrophilicity, and high hydrogen-bonding potential) [6].

DADLE is an agonist of delta opioid receptor. Upon the activation of delta opioid receptor, multiple intracellular signaling pathways are initiated and many signaling molecules are involved in, including protein kinase C, adenylate cyclase, and mitogen-activated protein kinase, which have been implicated to participate in the brain ischemic insult and may be involved in executing the neuroprotection of DADLE [2,13].

Zhang et al.’s study also indicated that the equal concentration naltrindole, an antagonist of delta opioid receptor, can entirely inhibit the effects of DADLE in vitro [21], however in our study, we found that naltrindole’s antagonistic effect was partial as the blocking could not be able to reverse the effect of DADLE entirely. Comparing with the DAD2 group, all parameter aggravated significantly in the DAD2 + NAT group, while still outstripped the ACSF group, which suggested that DADLE’s neuroprotective effects may also execute via a non-opioid mechanism.

Tsao et al.’s study [5] indicated that DADLE can act as a free radical scavenger and because it is well known that ischemia-induced neurotoxicity involves ROS, it is tempting to speculate that DADLE, at least in part, exert its brain protective effect through the sequestration of free radicals. However, further experiment to test the hypothesis that DADLE may sequester ischemia-induced free radical formation in vivo need to be carried out.

In summary, our work showed that intracerebroventricular administration of delta opioid peptide [d-Ala², d-Leu⁵] enkephalin induces dose-dependent neuroprotection against forebrain ischemia in rats, which might function through both opioid and non-opioid system, which implicated a potential drug target for clinical treatment of stroke as well as the prevention of ischemia insults.

Acknowledgements

These studies were supported by health bureau of Shang-hai (no. 01460), China. We thank Lu Ting-jia for technical and language assistance.

References


