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The role of opioid receptors in hypoxic preconditioning against seizures in brain

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Abstract

Preconditioning is defined as an adaptive mechanism produced by short periods of hypoxia/ischemia, resulting in protection against subsequent ischemic insult and development of seizures. Results of the present study demonstrate that an episode of normobar hypoxia reduces the susceptibility to convulsions induced by pentylenetetrazol (PTZ) 30 min, 24 h, as well as 4 and 7 days later. Administration of morphine showed similar effects after 24 h. Naloxone, given before ischemic preconditioning, as well as morphine, blocked the development of the protection. Administration of D-Ala-Met-enkephalin-Gly-ol (DAMGO — a selective mu-opioid receptor agonist), as well as *trans*-3,4-dichloro-*N*-methyl-*N*-[7-(1-pyrrolidinyl) cycloexilbenzeneacetamide ethane sulfonate] (U-69,593 — a selective kappa-opioid receptor agonist), mimicked the effects of hypoxic preconditioning (HPC). (−)-*N*-(Cyclopropylmethyl)-4,14-dimethoxymorphinan-6-one (cyprodime — a selective mu-opioid receptor antagonist, as well as nor-binaltorphimine dihydrochloride (nor-BNI — selective kappa-opioid receptors antagonist), given before HPC as well as before respective opioid receptor agonists, blocked the development of the protection. This study provides evidence that mu- and kappa-opioid receptors are involved in HPC against seizures in the brain.

Keywords: Hypoxia; Seizures; Opioid receptors; Preconditioning

1. Introduction

A transient stop of the normal cerebral blood flow produces a number of changes in the CNS including cell damage and changes in transmitter release. However, how moderate reductions in cerebral blood flow influence neuronal channel function is not clear in all aspects. Hypoxic/ischemic preconditioning is a protective mechanism against subsequent hypoxic/ischemic injury. That kind of phenomenon occurs in a variety of organ systems, including the heart [17], brain [5,9–13,16,32], lung [19], and liver [14]. The experimental data suggest that two separate periods of protection can be distinguished following a single ischemic episode. An immediate and delayed protective effect can be observed and the importance of each phase varies between species and organ systems [14,16,20,27,28,33]. Hypoxic/ischemic preconditioning seems to be a multifactorial process requiring the interaction of numerous signals, second messengers, and effector mechanisms, and still is not clearly defined. It has been reported that the mechanism of ischemic tolerance in the brain involves a cascade of events including A1 receptor-mediated stimulation of K^+ ATP-dependent channels [7], induction of heat-shock protein [9], increase in nitric oxide synthase activity [6], involvement of NMDA receptors [18,22] and the GABA-ergic system [26], and activation of specific transcription factors (immediate early genes (IEGs), c-*jun*, junB, and c-*fos*) [14,28]. Protein kinase C (PKC), which facilitates opening of ATP-sensitive potassium (KATP), has been proposed as a primary cellular mediator of ischemic preconditioning in the heart [27,33]. Recent evidence indicates that, in contrast to cardiac tissue,

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ischemic preconditioning in neurons does not involve activation of PKC [29]. This suggests that there are differences between the preconditioning phenomena seen in neuronal and cardiac tissues.

There is a growing body of evidence that suggests that activation of the opioid system may be involved in the protective effects of ischemic preconditioning in the heart. It has been shown that morphine mimics the cardioprotective effect of ischemic preconditioning in the rat heart [23]. Involvement of the kappa-opioid system in the phenomenon of preconditioning protection has also been suggested. In his study, Wu et al. [34] provided evidence that kappa-opioid receptors mediate the cardioprotection, which may involve PKC. Moreover, it has been suggested that the cardioprotective effects of ischemic preconditioning may be related to a reduction in the affinity of kappa receptor binding [35]. On the other hand, it has also been shown that delta-opioid receptors play an important role in the cardioprotective effect of ischemic preconditioning in the rat heart [25].

The aim of this study was to test the hypothesis that endogenous opioids are involved in preconditioning phenomena against seizures in brain and, to determine whether administration of opioid receptor agonists can mimic the neuroprotective effect of hypoxic preconditioning (HPC).

2. Materials and methods

2.1. Animals

Female Albino Swiss mice (25 g) were used in the studies. The animals were allowed to settle under standard conditions: the temperature was maintained $20 \pm 1^{\circ}C$, a natural light–dark cycle was used, and animals had free access to food and water. Each experimental group consisted of 20 animals.

2.2. Drugs

The following substances were used in the experiments: morphine hydrochloride (POLFA, Warsaw, Poland); intraperitoneally (IP), naloxone hydrochloride (Tocris Cookson, Bristol, UK); IP, D-Ala-Met-enkephalin-Gly-ol (DAMGO, Tocris Cookson) — the selective mu-opioid receptor agonist, (−)-*N*-(cyclopropylmethyl)-4,14-dimethoxymorphinan-6-one (cyprodime, Tocris Cookson) — the selective mu-opioid receptor antagonist, *trans*-3,4-dichloro-*N*-methyl-*N*-[7-(1-pyrrolidinyl) cycloexilbenzeneacetamide ethane sulfonate] (U-69,593, Tocris Cookson) — the selective kappa-opioid receptor agonist, nor-binaltorphimine dihydrochloride (nor-BNI, Tocris Cookson) — the selective kappa-opioid receptor antagonist, pentylenetetrazol (PTZ, Sigma, St. Louis, MO); subcutaneously (SC).

All selective ligands were administrated intracerebroventricularly (ICV), in the constant volume of 2 μl. Exact doses are given in the description of each experimental procedure.

2.3. Hypoxic preconditioning

The preconditioned animals were placed in a plastic airtight chamber (volume: 10 l) and allowed to breathe spontaneously with normobaric gas mixture (5.5% oxygen, 94.5% nitrogen, flow: 10 l/min) for 5.5 min. After the hypoxic episode mice were allowed to recover in the home cages.

2.4. Opioid preconditioning

Opioid preconditioning was induced by either morphine, administered at the doses of 5, 10, 30, or 75 mg/kg, or selective kappa-opioid receptor agonist, U-69.593, given ICV at the doses of 70, 150, or 300 nmol, or selective mu-opioid receptor agonist, DAMGO, at the doses of 5 and 10 nmol, ICV.

2.5. Seizures

Clonic seizures were induced by PTZ, administered SC 30 min, 1 and 24 h, as well as 4 and 7 days after the HPC according to the procedures mentioned above. CD_{50}, the dose of the applied convulsant, which is necessary to produce clonic seizures in 50% of the tested animals, was estimated in milligrams per kilogram (mg/kg) of body weight according to the method described by Litchfield and Wilcoxon [15] using 12 animals for each dose of PTZ.

To test the hypothesis that opioid receptors are involved in the early and late phases of preconditioning, the clonic seizures were induced by PTZ, 30 min and 24 h after the mimicked opioid preconditioning. Thirty minutes and twenty-four hours were chosen to examine the early and late phase of preconditioning, respectively.

2.6. Experimental protocol

Experimental protocol was approved by the Bioethical Committee of Lublin Medical University for the use of animal subjects. The animals were divided into the following experimental groups: control group, which was injected with the same volume of vehicle, HPC group, HPC + naloxone (3 mg/kg), morphine (5 mg/kg) morphine (10 mg/kg), morphine (30 mg/kg), morphine (75 mg/kg), morphine (10 mg/kg) + naloxone (3 mg/kg) morphine (30 mg/kg) + naloxone (3 mg/kg), HPC + nor-BNI (1 nmol), U-69,593 (70 nmol), U-69,593 (150 nmol), U-69,593 (300 nmol), U-69,593 (300 nmol) + nor-BNI (1 nmol), HPC + cyprodime (5 μmol), DAMGO (1 nmol), DAMGO (5 nmol), DAMGO (10 nmol), DAMGO (10 nmol) + cyprodime (5 μmol). For each experimental group mentioned above, the CD_{50} for clonic seizures induced by PTZ was estimated. Each dose of PTZ was injected to 12 animals.

In the case of HPC groups, the clonic seizures were induced by injection of PTZ, 30 min, 1, and 24 h as well as 4 and 7 days after the hypoxic episode. The clonic seizures

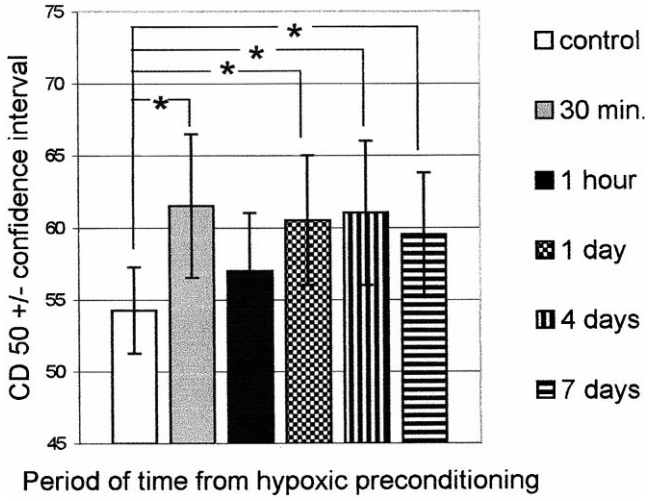

Fig. 1. The influence of HPC (5.5 min in 5.5% oxygen and 94.5% nitrogen) on CD_{50} (convulsive dose in mg/kg for 50% of tested animals) in PTZ-induced clonic seizures; * $p < 0.05$.

in mimicked preconditioning groups were induced by injection of PTZ, 30 min and 24 h after the injection of morphine. Because morphine failed to induce the preconditioning after 30 min, its effect after 24 h was studied according to the protocol mentioned above.

Naloxone, a nonselective opioid receptor antagonist, was given 30 min before the HPC as well as 30 min before morphine.

Mice were also pretreated with the selective antagonists nor-BNI or cyprodime, 30 min before the administration of respective selective agonists or preconditioning.

2.7. Histological analysis

Three experimental and three control animals were randomly selected. Animals were killed 3 days after the HPC. Brains were postfixed with Baker solution (1% $CaCl_2$ in 10% buffered formalin) for 2 weeks. The brains then were dehydrated, embedded in paraffin, and sectioned at 6-μm thickness for staining with Cresyl violet for light microscopic analysis. The following brain structures were taken into consideration: hypocampus and cerebral cortex.

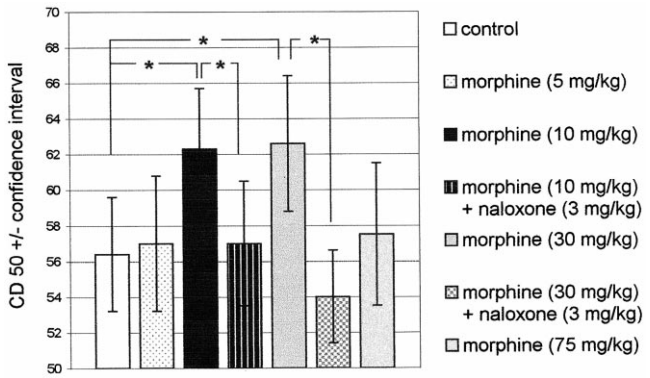

Fig. 2. The influence of morphine administration on CD_{50} in clonic seizures induced by PTZ 24 h later; * $p < 0.05$.

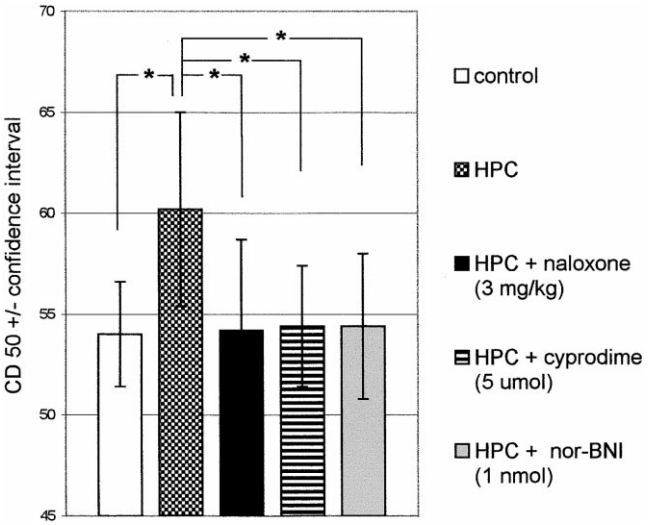

Fig. 3. The influence of naloxone (3 mg/kg), cyprodime (5 μmol), and nor-BNI (1 nmol) on CD_{50} in clonic seizures induced by PTZ 24 h after HPC; * $p < 0.05$.

2.8. Statistical analysis

The CD_{50} values for each group were calculated, fitting the data by linear regression analysis according to Litchfield and Wilcoxon [15]. CD_{50} values were determined on the basis of the equation of the determined regression line, $P = a \log(CD) + b$, where CD is a dose for which the expected effect is P probits, and b is initial ordinate confidence interval (f). The dose was calculated for the expected effects being equal to 50%.

The test of the significance of the differences consists in comparing the following values: PR = CD1/CD2, $f_{PR} = 10_{\uparrow}$

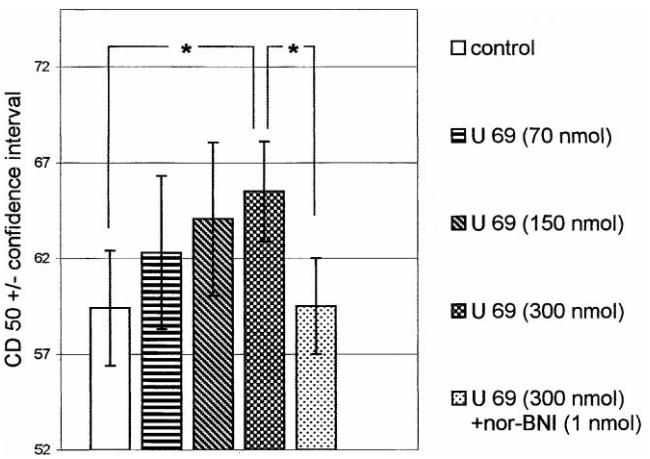

Fig. 4. The influence of administration of kappa-selective agonist (U-69,593) and antagonist (nor-BNI) on CD_{50} in clonic seizures induced by PTZ, 24 h later; * $p < 0.05$.

[k sqrt($\log^2(f1) + \log^2(f2)$)]; $k = 1$ for $p < 0.05$, $k = 1.31$ for $p < 0.01$, $k = 1.68$ for $p < 0.001$. The difference between compounds was taken to be significant when PR $< f_{PR}$.

3. Results

The present investigations demonstrate that an episode of normobar hypoxia (5.5 min) reduces the susceptibility to convulsions induced by PTZ 30 min, 24 h as well as 4 and 7 days later (Fig. 1). Our results confirm the previous studies showing the two, early and late, phases of HPC. The protective effect observed after 30 min was not visible after 1 h. The same protective effect of HPC was observed after 24 h and was observable even 7 days later (Fig. 1). No significant histological damage occurs in this model of experimental hypoxia, suggesting that functional alterations take place in neurons when exposed to HPC.

To test the hypothesis that opioid receptors are involved in HPC in the brain, and to determine whether administration of opioid receptor agonists can mimic the early or late neuroprotective effect of moderate hypoxia, morphine was administrated 30 min and 24 h before PTZ seizures.

As shown at Fig. 2, administration of morphine at the doses of 10 and 30 mg/kg induced protection observed after 24 h. At all applied doses, morphine failed to induce the early phase of preconditioning. That means that morphine mimics only the late effect of preconditioning.

Naloxone (3 mg/kg), a nonselective opioid receptor antagonist, given 30 min before morphine (10 or 30 mg/kg) abolished its protection (Fig. 2).

Pretreatment with naloxone as well as both selective mu- and kappa-opioid receptor antagonists, prevented the development of the protection by HPC (Fig. 3).

Furthermore, U-69,593 (selective kappa-opioid receptor agonist), only at the highest of applied doses (300 nmol ICV) mimicked the preconditioning, and this effect was antagonised by the pretreatment with nor-BNI (Fig. 4).

Also, DAMGO (selective mu-opioid receptor agonist) produced a protective effect at the highest dose used (10 nmol, ICV) observed 24 h after administration. Cyprodime, highly selective mu-opioid receptor antagonist, at the dose of 5 μmol before mu-opioid receptor agonist, blocked development of the protection (Fig. 5).

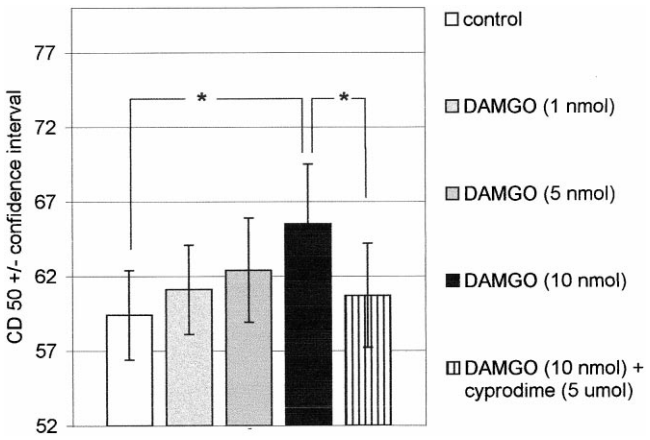

Fig. 5. The influence of administration of mu-selective agonist DAMGO and antagonist cyprodime on CD_{50} in clonic seizures induced by PTZ 24 h later; * $p < 0.05$.

4. Discussion

The study provides evidence that opioid receptor activation plays an important role in ischemic preconditioning against seizures in the brain. HPC increases CD_{50} in PTZ-induced seizures. Naloxone and selective kappa- or mu-antagonist pretreatment attenuates this protective effect. Both morphine and a selective kappa-agonist were seen to mimic the effect of preconditioning on PTZ-evoked seizures.

We conclude that the CNS endogenous opioid pathways may mediate the neuroprotective mechanisms of hypoxic/ischemic preconditioning.

It is known that severe hypoxia/ischemia may elicit seizures. It has previously been found that transient hypoxia/ischemia, for example, transient occlusion of rat carotid arteries, decreases susceptibility to seizures evoked with bicuculline [26], kainic acid [20], and PTZ [22]. Our results demonstrate that an episode of hypoxia increases the convulsive threshold for clonic seizures in the PTZ model. It confirms the anticonvulsive/neuroprotective properties of a short period of hypoxia against subsequent seizures. We observed two, early and late, phases of HPC. The early phase was noted after 30 min and disappeared after 1 h. HPC induced the late phase of protection after 24 h and the effect lasted for at least 7 days. Similarly, in the heart, ischemic preconditioning has been associated with both an early or acute phase of protection, lasting approximately 1–2 h, as well as a delayed phase seen at least 24 h following the initial sublethal ischemic insult, and lasting up to 72 h. It is believed that both responses are triggered by similar receptor mediated events and also involve similar signalling pathways involving kinase cascades. However, it is thought that the ultimate target or end-effector through which the protection is manifested may be different for the early vs. late effects [2].

In our experimental protocol, early and late protective effects were studied 30 min and 24 h after the preconditioning episode, respectively.

To test the hypothesis whether opioid receptors are involved in HPC in the brain and whether administration of opioid receptor agonists can mimic the early and late neuroprotective effect of hypoxia, morphine was administrated 30 min and 24 h before PTZ.

We found that administration of morphine can result in a protective phenomenon 24 h later. No early phase protection was observed following morphine administration. Because of the fact that pretreatment with naloxone abolished both hypoxic- and morphine-induced preconditioning, we suggest that opioid systems are involved in the phenomenon.

Cyprodime, a selective mu-opioid receptor antagonist, as well as nor-BNI, a selective kappa-opioid receptor antagonist, given before the HPC abolished the development of the protection. Furthermore, administration of the selective kappa-opioid receptor agonist U69 as well as DAMGO, a selective mu-opioid receptor agonist, induced a protective effect (observed after 24 h after administration). Administration of antagonists before respective opioid receptor agonists, blocked the development of the mimicked opioid protection. These results indicate the involvement of both mu- and kappa-opioid receptors in the mechanism of the preconditioning protection.

Previous studies of other research teams have indicated that reduction in affinity of kappa receptor binding might be responsible for cardioprotective effects of ischemic preconditioning [35]. On the other hand, Schulz et al. [25] reported that delta-1 but not mu- or kappa-opioid receptor activation mediates development of this phenomenon. The discrepancies in conclusions between these two studies may arise from different parameters analyzed as an evaluation of preconditioning. Besides, binding studies have shown that massive cerebral ischemia produces a significant increase in the number of kappa-opioid receptors, without changing affinity values [24]. Moreover, in different studies changes in opioid receptor (mu, delta, kappa) concentrations during temporary middle cerebral artery occlusion have been found [1,30]. This further supports the involvement of opioid pathways during ischemic/hypoxic episodes in the brain.

There is a growing body of evidence that suggests the involvement of opioid receptors in the convulsion activity. For example, according to the report of Comer et al. [3], a delta receptor-selective agonists produce a brief, nonlethal convulsion in mice. Moreover, intrahippocampal injection of a delta-opioid receptor antagonist suppressed the epileptogenic effects induced by the delta-opioid agonist [4].

On the other hand, kappa-opioid receptor agonists suppress absence seizures [21]. Moreover, blockade of mu-1 receptors has a proconvulsive effect, and activation of mu-1 receptors has a protective role against electrically induced convulsions [36]. The other findings suggest that the anticonvulsant effects of phenytoin may be mediated, at least in part, by the release of endogenous opioids and subsequent activation of opioid mu receptor [8]. Intracerebroventricular injections of the mu-selective enkephalin DAGO resulted in a dose-related protection in the flurothyl threshold test and the maximal electroshock test [31]. Anticonvulsive properties of mu- and kappa-opioid receptor agonists, as the neuroprotective effect, give us a clue to test their hypothetical involvement in preconditioning phenomenon.

Further studies are needed to specify the exact mechanisms of the hypoxic/ischemic preconditioning in the brain. For example, binding affinity of opioid receptors as well as involvement of second messenger systems should be studied.

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