Anesthesiology 2004; 100:562–8 © 2004 American Society of Anesthesiologists, Inc. Lippincott Williams & Wilkins, Inc.

Morphine Preconditions Purkinje Cells against Cell Death under In Vitro *Simulated Ischemia–Reperfusion Conditions*

Young Jin Lim, M.D., Ph.D., Shuqiu Zheng, M.D., Ph.D.,† Zhiyi Zuo, M.D., Ph.D.‡*

Background: **Morphine pretreatment *via* activation of δ_1-opioid receptors induces cardioprotection. In this study, the authors determined whether morphine preconditioning induces ischemic tolerance in neurons.**

Methods: **Cerebellar brain slices from adult Sprague-Dawley rats were incubated with morphine at 0.1–10 μM in the presence or absence of various antagonists for 30 min. They were then kept in morphine- and antagonist-free buffer for 30 min before they were subjected to simulated ischemia (oxygen–glucose deprivation) for 20 min. After being recovered in oxygenated artificial cerebrospinal fluid for 5 h, they were fixed for morphologic examination to determine the percentage of undamaged Purkinje cells.**

Results: **The survival rate of Purkinje cells was significantly higher in slices preconditioned with morphine ($\geq$ 0.3 μM) before the oxygen–glucose deprivation (57 ± 4% at 0.3 μM morphine) than that of the oxygen–glucose deprivation alone (39 ± 3%, $P < 0.05$). This morphine preconditioning–induced neuroprotection was abolished by naloxone, a non–type-selective opioid receptor antagonist, by naltrindole, a selective δ-opioid receptor antagonist, or by 7-benzylidenenaltrexone, a selective δ_1-opioid receptor antagonist. However, the effects were not blocked by the μ-, κ-, or δ_2-opioid receptor antagonists, β-funaltrexamine, nor-binaltorphimine, or naltriben, respectively. Morphine preconditioning–induced neuroprotection was partially blocked by the selective mitochondrial adenosine triphosphate–sensitive potassium channel antagonist, 5-hydroxydecanoate, or the mitochondrial electron transport inhibitor, myxothiazol. None of the inhibitors used in this study alone affected the simulated ischemia–induced neuronal death.**

Conclusions: **These data suggest that morphine preconditioning is neuroprotective. This neuroprotection may be δ_1-opioid receptor dependent and may involve mitochondrial adenosine triphosphate–sensitive potassium channel activation and free radical production. Because morphine is a commonly used analgesic, morphine preconditioning may be explored further for potential clinical use to reduce ischemic brain injury.**

ISCHEMIC brain injury contributes significantly to morbidity and mortality associated with common human diseases, such as stroke and brain trauma.[1,2] Although many interventions, such as hypothermia and glutamate receptor antagonists, have been explored for potential neuroprotection,[3,4] clinically effective and safe methods for reducing ischemic brain injury have not been established.

Opioids are an important class of therapeutic agents frequently used in the perioperative period for analgesia. Opioids are inhibitory neurotransmitters, and their receptors, classified mainly as δ-, μ-, and κ-opioid subtypes, are widely distributed throughout the central nervous system.[5] Recent studies, using cultured neocortical neurons from rats, suggest that opioids reduced glutamate- and hypoxia-induced neuronal death by activation of δ-opioid receptors.[6,7] Therefore, the presence of opioids during the application of these injuries provides neuroprotection.

Opioids have also been shown to induce cardioprotection when present only before ischemia.[8–11] This preconditioning-induced protection was δ_1-opioid receptor dependent.[8–11] Consistent with these results, it has been reported that ischemic preconditioning, a well-known phenomenon in which brief episodes of sublethal ischemia induce a robust protection against the deleterious effects of subsequent lethal ischemia in a variety of organ systems, including brain, heart, liver, intestine, and lung,[12,13] involves activation of δ_1-opioid receptors for its cardioprotection.[14] δ_1-Opioid receptors have also been implicated in the hypoxic preconditioning–induced increase in survival time of mice under subsequent lethal hypoxia.[15,16]

Therefore, we hypothesize that morphine, a commonly used opioid, induces ischemic tolerance in neurons and that this morphine preconditioning–induced neuroprotection is also δ-opioid receptor dependent. We used rat cerebellar slices and simulated ischemia *in vitro* by oxygen–glucose deprivation (OGD) to test these hypotheses. We chose to monitor the survival rate of Purkinje neurons in the study because these neurons are sensitive to ischemia and are easy to recognize in cerebellar sections, resulting in high accuracy of the data on cell injury and death.

Materials and Methods

The animal protocol was approved by the institutional Animal Care and Use Committee at the University of Virginia (Charlottesville, Virginia). All animal experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.[17] All efforts were made to minimize the number of animals used and their suffering.

* Visiting Associate Professor, Department of Anesthesiology, University of Virginia Health System, and Department of Anesthesiology and Pain Medicine, Seoul National University College of Medicine, Seoul, South Korea. † Research Assistant, ‡ Associate Professor, Department of Anesthesiology, University of Virginia Health System.

Received from the Department of Anesthesiology, University of Virginia, Charlottesville, Virginia. Submitted for publication August 21, 2003. Accepted for publication November 24, 2003. Supported by Grant-in-Aid Award No. 0160326U (to Dr. Zuo) from the American Heart Association–Mid-Atlantic Affiliate, Baltimore, Maryland; grant No. RO1 GM065211 (to Dr. Zuo) from the National Institutes of Health, Bethesda, Maryland; and the Department of Anesthesiology, University of Virginia, Charlottesville, Virginia. Presented at the 16th Annual Meeting of the American Society of Anesthesiologists, San Francisco, California, October 11–16, 2003.

Address reprint requests to Dr. Zuo: Department of Anesthesiology, University of Virginia Health System, 1 Hospital Drive, P. O. Box 800710, Charlottesville, Virginia 22908-0710. Address electronic mail to: zz3c@virginia.edu. Individual article reprints may be purchased through the Journal Web site, www.anesthesiology.org.

Copyright © by the American Society of Anesthesiologists. Unauthorized reproduction of this article is prohibited.

Downloaded from http://asa2.silverchair.com/anesthesiology/article-pdf/100/3/562/353581/0000542-20040300-00015.pdf by guest on 20 March 2024

Materials

7-Benzylidenenaltrexone (BNTX) was purchased from Tocris Cookson (Ellisville, MO). All reagents, unless specified below, were obtained from Sigma Chemicals (St. Louis, MO).

Preparation of Brain Slices

As previously described,[18–20] cerebellar brain slices were prepared from 2- to 3-month-old, 230- to 270-g male Sprague-Dawley rats (Hilltop, Scottsdale, PA). Rats were anesthetized with 40 mg/kg pentobarbital intraperitoneally and then decapitated. The cerebellum was removed rapidly and placed in ice-cold artificial cerebrospinal fluid (aCSF) bubbled with 5% CO_2–95% O_2. The aCSF contained 116 mM NaCl, 26.2 mM $NaHCO_3$, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.9 mM $MgCl_2$, 0.9 mM NaH_2PO_4, and 5.6 mM glucose at a pH of 7.4. The cerebellum was then sectioned with a tissue slicer into 400-μm transverse slices. After sectioning, slices were placed into a tissue holder (made of plastic and with small holes in it to allow free diffusion of gases and water; this holder also helps to avoid direct gas bubbling on slices) and remained in the oxygenated aCSF at room temperature for approximately 1 h for recovery of synaptic function.[18]

Incubation

Ischemia was simulated *in vitro* by OGD. As previously described,[18–20] cerebellar slices were subjected to OGD by transferring them into a glass beaker containing 40 ml glucose-free aCSF (also containing 1 mM dithionite, an oxygen absorbent) bubbled with 95% N_2–5% CO_2 (30 min of bubbling before the addition of brain slices was performed to reduce the oxygen content in the solution). The partial pressure of oxygen in the aCSF as measured by means of a Clark oxygen electrode (Cameron Instrument Co., Port Aransas, TX) was less than 0.1 mmHg. The beaker containing the slices was immersed in a water bath to keep the temperature of aCSF at 37 ± 0.2°C. After 20 min under the OGD condition, slices were transferred to oxygenated aCSF and remained in this aCSF for 5 h at 37°C before they were fixed for morphologic examination. This period was used to simulate reperfusion and also to allow cell injury and death that may not be evident immediately after the OGD episode to become apparent.

Morphine preconditioning was performed by incubating cerebellar brain slices with various concentrations (0.1–10 μM) of morphine at 37°C for 30 min. After 30 min in morphine-free aCSF at 37°C, they were then exposed to OGD. Naloxone (a non–type-selective opioid antagonist, 50 μM), β-funaltrexamine (β-FNA; a selective μ-opioid receptor antagonist, 10 μM),[21] nor-binaltorphimine (nor-BNI; a selective κ-opioid receptor antagonist, 10 μM),[22] naltrindole (a selective δ-opioid receptor antagonist, 10 μM),[23] BNTX (a selective δ_1-opioid receptor antagonist, 0.5 μM),[23] naltriben (a selective δ_2-opioid receptor antagonist, 0.5 μM),[24] 5-hydroxydecanoate (a selective mitochondrial K_{ATP} channel blocker, 500 μM),[25] or myxothiazol (a mitochondrial electron transport inhibitor, 1 μM)[10] were present during the period from 30 min before the addition of morphine to the end of morphine pretreatment (total of 1 h). The concentration selection of these inhibitors was mainly based on previous studies.[6,8,10,11,24,25] As a control study, these inhibitors were present for the same duration of time in the incubations of some brain slices that were not preconditioned with morphine before OGD.

Morphologic Examination

After incubation, cerebellar slices were fixed in 4% paraformaldehyde in buffered saline overnight at 4°C. The slices were then paraffin embedded and sectioned into 4-μm-thick sections. The sections were cut from an interior region of the cerebellar slices (approximately 100 μm from the edge) to avoid areas subjected to slicing trauma during slice preparation. Morphologic examination was performed after staining the brain sections with hematoxylin and eosin. The sections were examined by an observer who was blinded to the group assignment to determine the percentage of undamaged Purkinje cells (survival rate). Damaged neurons were identified if the cells had one of the following characteristics: cell swelling; vacuolization; or presence of shrunken, darkened nuclei.[26] Purkinje cells were recognized based on the morphology and the location (between the molecular and granular layers). At least 50 Purkinje cells were counted from each of the sections and cells in total three sections were counted for each experimental condition (> 150 Purkinje cells) to compute the percentages.

Statistical Analysis

Results are presented as mean ± SEM. Each experiment included a control group and an OGD group. Each experimental condition was repeated with cerebellar slices from 9–14 rats. Statistical analysis was performed by means of Kruskal-Wallis test followed by Mann–Whitney rank sum test when appropriate. A $P < 0.05$ was considered significant.

Results

Morphine Preconditioning Dose-dependently Reduces OGD-induced Purkinje Cell Death

Oxygen–glucose deprivation and reoxygenation induced Purkinje cell death (fig. 1). A 30-min preconditioning of the cerebellar brain slices with morphine increased the survival rate of Purkinje cells after OGD and reoxygenation (fig. 2). The protective effects of morphine preconditioning were concentration dependent and were maximal at concentrations higher than 0.3 μM

Copyright © by the American Society of Anesthesiologists. Unauthorized reproduction of this article is prohibited.

Downloaded from http://asa2.silverchair.com/anesthesiology/article-pdf/100/3/562/353581/0000542-20040300-00015.pdf by guest on 20 March 2024

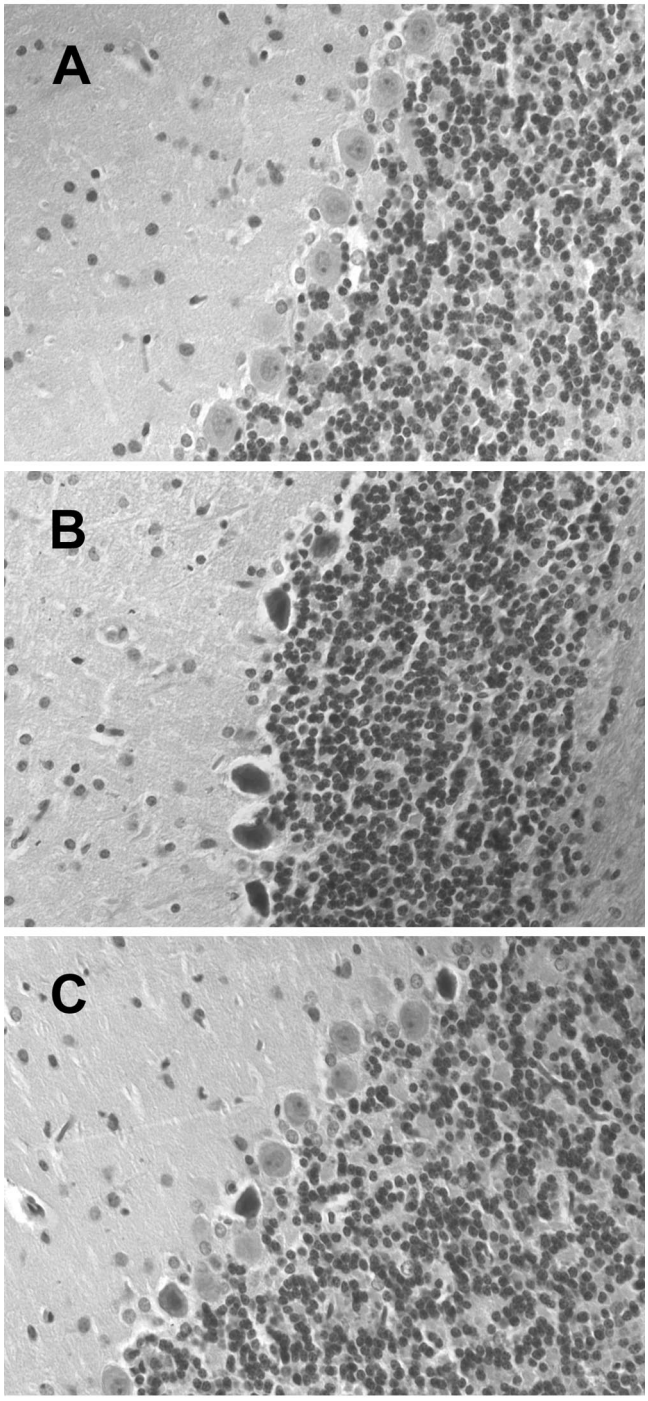

Fig. 1. Representative sections of cerebellar slices stained with hematoxylin and eosin. Cerebellar brain slices were incubated with 3 μM morphine for 30 min and then maintained in morphine-free artificial cerebrospinal fluid for 30 min before being exposed to a 20-min oxygen–glucose deprivation at 37°C. Brain slices were kept in oxygenated artificial cerebrospinal fluid for 5 h before they were fixed for staining. Brain slices under control (*A*), oxygen–glucose deprivation (*B*), or morphine preconditioning plus oxygen–glucose deprivation (*C*) conditions are shown.

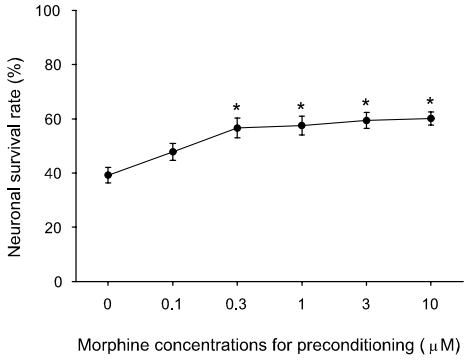

Fig. 2. Dose–responses of morphine preconditioning–induced neuroprotection in Purkinje cells. Cerebellar brain slices were incubated with various concentrations of morphine for 30 min and then maintained in morphine-free artificial cerebrospinal fluid for 30 min before being exposed to a 20-min oxygen–glucose deprivation at 37°C. Brain slices were kept in oxygenated artificial cerebrospinal fluid for 5 h before they were fixed for morphologic examination. Results are presented as mean ± SEM (n = 9). * $P < 0.05$ compared with oxygen–glucose deprivation alone.

(survival rates were 57 ± 4 and 39 ± 3% in slices preconditioned with 0.3 μM morphine before OGD and in slices subjected to OGD alone, respectively, n = 9, $P < 0.05$; fig. 2).

δ_1-Opioid Receptors Mediate Morphine Preconditioning–induced Neuroprotection

Morphine (3 μM) preconditioning–induced neuroprotection was abolished by the non–type-selective opioid receptor antagonist naloxone (naloxone reduced survival rates from 56 ± 2% in slices with morphine preconditioning plus OGD to 39 ± 3%, n = 11, $P < 0.001$, which was not different from that in slices subjected to OGD alone, 36 ± 3%; fig. 3). To identify the subtypes of opioid receptors that mediate morphine preconditioning–induced neuroprotection, selective antagonists for the μ-, κ-, or δ-opioid receptors were used in the study. When they were present with morphine during the preconditioning period, only naltrindole, a δ-opioid receptor antagonist, inhibited morphine preconditioning–induced neuroprotection (naltrindole reduced survival rates from 49 ± 2% in slices with morphine preconditioning plus OGD to 28 ± 2%, n = 11, $P < 0.001$, which was not different from that in slices subjected to OGD alone, 27 ± 2%; fig. 3). Either β-FNA or nor-BNI, antagonists for μ- or κ-opioid receptors, respectively, did not affect morphine preconditioning–induced neuroprotection (fig. 3). Additional data using selective δ_1- or δ_2-opioid receptor antagonists showed that the neuroprotection induced by morphine preconditioning was abolished by the δ_1-opioid receptor antagonist BNTX (BNTX reduced survival rates from 56 ± 2% in slices with morphine preconditioning plus OGD to 38 ± 3%, n = 11, $P < 0.001$, which was not different from that in slices subjected to OGD alone, 36 ± 3%) and was not

Copyright © by the American Society of Anesthesiologists. Unauthorized reproduction of this article is prohibited.

Downloaded from http://asa2.silverchair.com/anesthesiology/article-pdf/100/3/562/353581/0000542-20040300-00015.pdf by guest on 20 March 2024

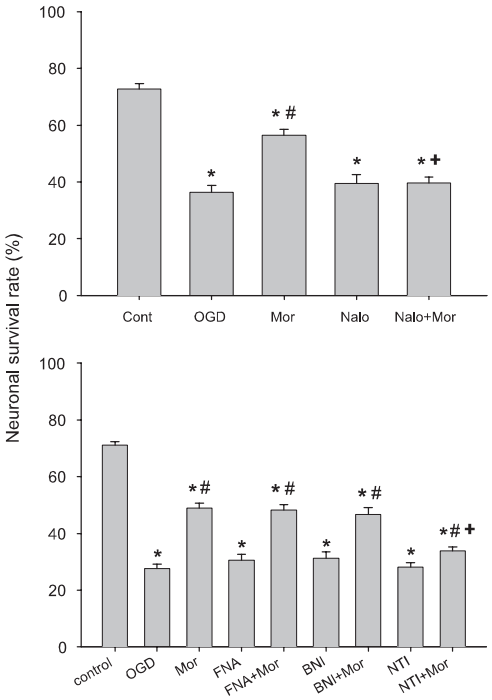

Fig. 3. The effects of opioid receptor antagonists on neuroprotection induced by morphine preconditioning. Cerebellar brain slices were incubated with 3 μM morphine for 30 min and then maintained in morphine-free artificial cerebrospinal fluid for 30 min before being exposed to a 20-min oxygen–glucose deprivation (OGD) at 37°C. Brain slices were kept in oxygenated artificial cerebrospinal fluid for 5 h before they were fixed for morphologic examination. Each antagonist was present during the period from 30 min before the addition of morphine to the end of morphine pretreatment (total of 1 h). Results are presented as mean ± SEM (n = 11). * $P < 0.05$ compared with control. # $P < 0.05$ compared with OGD alone. + $P < 0.05$ compared with morphine preconditioning plus OGD. BNI = 10 μM nor-binaltorphimine plus OGD; BNI + Mor = 10 μM nor-binaltorphimine plus morphine preconditioning plus OGD; Cont = control; FNA = 10 μM β-funaltrexamine plus OGD; FNA + Mor = 10 μM β-funaltrexamine plus morphine preconditioning plus OGD; Mor = morphine preconditioning plus OGD; Nalo = 50 μM naloxone plus OGD; Nalo + Mor = 50 μM naloxone plus morphine preconditioning plus OGD; NTI = 10 μM naltrindole plus OGD; NTI + Mor = 10 μM naltrindole plus morphine preconditioning plus OGD.

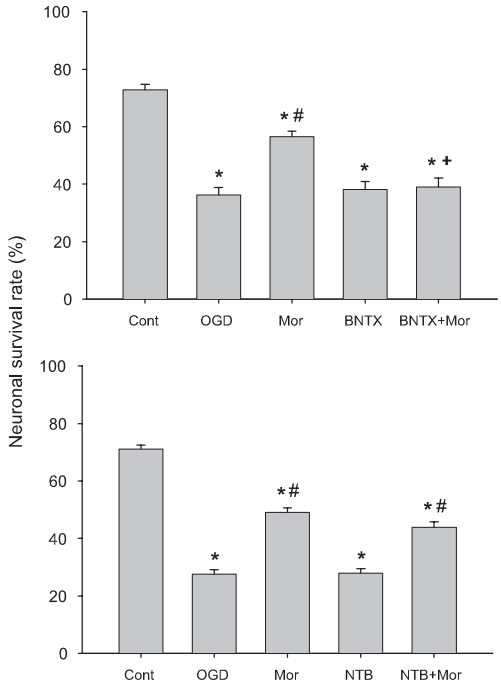

Fig. 4. The effects of δ-opioid receptor antagonists on neuroprotection induced by morphine preconditioning. Cerebellar brain slices were incubated with 3 μM morphine for 30 min and then maintained in morphine-free artificial cerebrospinal fluid for 30 min before being exposed to a 20-min oxygen–glucose deprivation (OGD) at 37°C. Brain slices were kept in oxygenated artificial cerebrospinal fluid for 5 h before they were fixed for morphologic examination. Each antagonist was present during the period from 30 min before the addition of morphine to the end of morphine pretreatment (total of 1 h). Results are presented as mean ± SEM (n = 11). * $P < 0.05$ compared with control. # $P < 0.05$ compared with OGD alone. + $P < 0.05$ compared with morphine preconditioning plus OGD. BNTX = 0.5 μM 7-benzylidenenaltrexone plus OGD; BNTX + Mor = 0.5 μM 7-benzylidenenaltrexone plus morphine preconditioning plus OGD; Cont = control; Mor = morphine preconditioning plus OGD; NTB = 0.5 μM naltriben plus OGD; NTB + Mor = 0.5 μM naltriben plus morphine preconditioning plus OGD.

affected by the δ_2-opioid receptor antagonist naltriben (fig. 4). The presence of naloxone, β-FNA, nor-BNI, naltrindole, BNTX, or naltriben alone did not change the survival rates of Purkinje neurons under the OGD and reoxygenation conditions (figs. 3 and 4).

Mitochondrial K_{ATP} Channels and Mitochondrial Electron Transport System May Be Involved in Morphine Preconditioning–induced Neuroprotection

Concomitant presence of 5-hydroxydecanoate or myxothiazol, inhibitors for mitochondrial K_{ATP} channels and mitochondrial electron transport, respectively, during the 30-min exposure of cerebellar slices to morphine (3 μM) at least partially blocked morphine preconditioning–induced neuroprotection (5-hydroxydecanoate and myxothiazol reduced survival rates from 55 ± 2% in slices with morphine preconditioning plus OGD to 44 ± 3% and 44 ± 3%, respectively, n = 12, $P < 0.05$, which were also different from that in slices subjected to OGD alone, 34 ± 2%, n = 12, $P < 0.05$; fig. 5). 5-Hydroxydecanoate or myxothiazol alone had no effects on OGD-/reoxygenation-induced Purkinje cell death.

Discussion

Our study demonstrated the following major findings: (1) morphine preconditioning induced neuroprotection; (2) this neuroprotection was mediated by δ_1-opioid receptors and may not depend on the activation of μ-, κ-, or δ_2-opioid receptors; and (3) additional intracellular components, such as mitochondrial K_{ATP} channels and electron transport system, may also be involved in morphine preconditioning–induced neuroprotection.

An earlier study showed that ischemic precondition-

Copyright © by the American Society of Anesthesiologists. Unauthorized reproduction of this article is prohibited.

Downloaded from http://asa2.silverchair.com/anesthesiology/article-pdf/100/3/562/353581/0000542-20040300-00015.pdf by guest on 20 March 2024

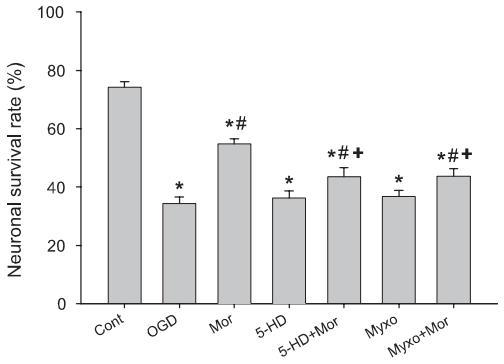

Fig. 5. The effects of mitochondrial adenosine triphosphate–sensitive potassium channels and mitochondrial electron transport inhibitors on neuroprotection induced by morphine preconditioning. Cerebellar brain slices were incubated with 3 μM morphine for 30 min and then maintained in morphine-free artificial cerebrospinal fluid for 30 min before being exposed to a 20-min oxygen–glucose deprivation (OGD) at 37°C. Brain slices were kept in oxygenated artificial cerebrospinal fluid for 5 h before they were fixed for morphologic examination. Each antagonist was present during the period from 30 min before the addition of morphine to the end of morphine pretreatment (total of 1 h). Results are presented as mean ± SEM (n = 12). * $P < 0.05$ compared with control. # $P < 0.05$ compared with OGD alone. + $P < 0.05$ compared with morphine preconditioning plus OGD. Cont = control; 5-HD = 500 μM 5-hydroxydecanoate plus OGD; 5-HD + Mor = 500 μM 5-hydroxydecanoate plus morphine preconditioning plus OGD; Mor = morphine preconditioning plus OGD; Myxo = 1 μM myxothiazol plus OGD; Myxo + Mor = 1 μM myxothiazol plus morphine preconditioning plus OGD.

ing–induced cardioprotection is mediated by δ_1-opioid receptors.[14] In addition, pretreatment with morphine or δ_1-opioid receptor agonists also induced cardioprotection against ischemia.[8–11] In isolated rabbit heart, morphine pretreatment and ischemic preconditioning were equally cardioprotective.[27] In chick myocyte cultures, morphine or the selective δ-opioid receptor agonist BW373U86 attenuated ischemia–reperfusion injury.[8,10] The protection induced by morphine was abolished by naloxone or BNTX.[9] In isolated rat heart, activation of δ-opioid receptors also improved cardiac function.[28] These findings led us to hypothesize that morphine preconditioning might also induce neuroprotection against ischemia. To avoid the presence of morphine in brain slices during OGD, we placed brain slices in morphine-free aCSF for 30 min before OGD. Our results indicate that morphine preconditioning induced a dose-dependent neuroprotective effect and the maximal protection was achieved at morphine concentrations higher than 0.3 μM, similar to the results of a previous study using chick myocyte cultures.[8] Because the minimum effective concentrations in the blood for morphine to have analgesic effects are 0.06–0.6 μM,[29] the concentrations for morphine to induce neuroprotection in our study are clinically relevant.

We then investigated which opioid receptors were involved in morphine preconditioning–induced neuroprotection. Three major opioid receptors, μ-, κ-, and δ-receptors, and two δ subtypes of opioid receptors, δ_1 and δ_2, have been characterized.[5,30] Previous studies have indicated that δ_1-opioid receptors are important for morphine preconditioning– and ischemic preconditioning–induced cardioprotection.[8–11,14] In addition, it has been shown that activation of neuronal δ-opioid receptors during the application of injuries (not qualified as preconditioning) protected neurons from hypoxic injury[6] or glutamate excitotoxicity[7] in rat cortical neuron cultures. Inhibition of δ-opioid receptors but not μ- or κ-opioid receptors during hypoxia caused more severe neuronal injury than hypoxia alone.[6] In whole animal experiments using [D-Pen,^{2} D-Pen^5]-enkephalin, an δ_1-opioid receptor agonist, and BNTX, it has been shown that the δ_1-opioid receptors mediated the increased survival time of mice to hypoxic environments.[16] Consistent with these previous results, our data suggest that δ_1-opioid receptors play an important role in morphine preconditioning–induced neuroprotection because this neuroprotection was inhibited by naloxone, a non–type-selective opioid receptor antagonist, by naltrindole, a selective δ-opioid receptor antagonist, and by BNTX, a selective δ_1-opioid receptor antagonist. Our results also suggest that δ_2-, μ-, or κ-receptors may not be important for morphine preconditioning–induced neuroprotection because naltriben, β-FNA, or nor-BNI, antagonists for these receptors, respectively, did not inhibit the neuroprotection. β-FNA, nor-BNI, and naltrindole have been shown to have extremely high binding affinities for their respective subtypes of opioid receptors (IC_{50} is in the nanomolar to picomolar range).[21,31–33] In our study, we used 10 μM β-FNA, nor-BNI, and naltrindole. We assumed that such high concentrations of these antagonists would effectively block their respective receptors. In addition, 10 μM was the highest concentration used to indicate no involvement of μ or κ receptors in opioid-induced cardioprotection and neuroprotection in the previous studies.[6,11] BNTX was reported to be approximately 10-fold less potent than naltriben to inhibit [^{3}H]naltriben binding.[24] In this study, although the same concentrations (0.5 μM) of naltriben and BNTX were used, naltriben did not inhibit morphine preconditioning–induced neuroprotection.

After activation of δ_1-opioid receptors in the cell membrane, what are the intracellular mediators for morphine preconditioning–induced neuroprotection? K_{ATP} channels, especially mitochondrial K_{ATP} channels, have been shown to mediate morphine preconditioning–induced cardioprotection.[8,9] In addition, oxygen free radicals have also been found to be important in this cardioprotection.[9,10,34] In fact, a positive feedback circle has been proposed for mitochondrial K_{ATP} channels and free radicals: free radicals activate K_{ATP} channels, and activation of K_{ATP} channels can induce free radical production.[9,10] We tested whether K_{ATP} channels and free radicals played a role in morphine preconditioning–induced neuroprotection by using 5-hydroxydecanoate, a selective

Copyright © by the American Society of Anesthesiologists. Unauthorized reproduction of this article is prohibited.

Downloaded from http://asa2.silverchair.com/anesthesiology/article-pdf/100/3/562/353581/0000542-20040300-00015.pdf by guest on 20 March 2024

mitochondrial K_{ATP} channel inhibitor with no effects on sarcolemmal K_{ATP} channels,[25] and myxothiazol, a mitochondrial site III electron transport inhibitor that can inhibit production of oxygen free radicals in mitochondria.[10,35] Our results suggest that both K_{ATP} channels and free radical production in mitochondria may be involved in morphine preconditioning–induced neuroprotection.

Therefore, our study suggests that the signaling pathway for morphine preconditioning–induced neuroprotection includes activation of δ_1-opioid receptors and mitochondrial K_{ATP} channels and production of free radicals. Because it has been shown that inhibition of mitochondrial K_{ATP} channels abolished free radical production induced by morphine and the selective δ-opioid receptor agonist BW373U86 in cultured myocytes,[9,10] it is possible that mitochondrial K_{ATP} channels may be an upstream event of free radical production in opioid preconditioning–induced neuroprotection. The positive feedback between K_{ATP} channels and production of free radicals in mitochondria may then amplify the signals to induce neuroprotection.

Our experiments were performed using brain slices. Brain slices have an advantage over cultured neurons because neurons in brain slices are similarly sensitive to hypoxia and simulated ischemia as are neurons in the intact brain.[6,18–20] The brain slice model also has an advantage over the intact animal model because it is easier to directly apply various drugs and to control variables such as oxygen tension, temperature, and chemical environments in brain slices. However, *in vitro* models differ in many ways from intact brains. For example, the extracellular partial pressure of carbon dioxide, H^+, glutamate, and lactic acid, which are important factors to determine cell death or survival after ischemia, might not change to the same degree as they would *in vivo* because of diffusion away from the slice, even in the interior region of the slice that was used for our cell count. Therefore, it is not appropriate to extrapolate our results directly to *in vivo* conditions. In addition, we evaluated cell death at 5 h after OGD because neuronal death in brain slices is manifest within 5 h after OGD, hypoxia, or overstimulation of glutamate receptors.[18–20] However, delayed cell death can take a few days to develop[1,36–38] and cannot be assessed by our slice model because neurons in the acutely prepared slices can be well preserved only for 5–10 h *in vitro* in oxygenated buffer containing glucose.[26,39] Therefore, our current study may have overestimated the morphine preconditioning–induced neuroprotection.

In summary, preconditioning brain slices with morphine at clinically relevant concentrations induced acute neuroprotection. This protection depends on activation of δ_1-opioid receptors. Activation of K_{ATP} channels and free radical production in mitochondria may be important downstream events for morphine preconditioning–induced neuroprotection.

References

1. Lipton P: Ischemic cell death in brain neurons. Physiol Res 1999; 79:1431–568
2. Martin JA, Smith BL, Mathews TJ, Ventura SJ: Births and deaths: Preliminary data for 1998. Natl Vit Stat Rep 1999; 47:1–45
3. Kataoka K, Yanase H: Mild hypothermia: A revived countermeasure against ischemic neuronal damages. Neurosci Res 1998; 32:103–17
4. Muir KW, Lees KR: Clinical experience with excitatory amino acid antagonist drugs. Stroke 1995; 26:503–13
5. Pasternak GW: Pharmacological mechanisms of opioid analgesics. Clin Neuropharmacol 1993; 16:1–18
6. Zhang J, Gibney GT, Zhao P, Xia Y: Neuroprotective role of delta-opioid receptors in cortical neurons. Am J Physiol Cell Physiol 2002; 282:C1225–34
7. Zhang J, Haddad GG, Xia Y: Delta-, but not mu- and kappa-, opioid receptor activation protects neocortical neurons from glutamate-induced excitotoxic injury. Brain Res 2000; 885:143–53
8. Liang BT, Gross GJ: Direct preconditioning of cardiac myocytes via opioid receptors and KATP channels. Circ Res 1999; 84:1396–400
9. McPherson BC, Yao Z: Morphine mimics preconditioning via free radical signals and mitochondrial K(ATP) channels in myocytes. Circulation 2001; 103: 290–5
10. McPherson BC, Yao Z: Signal transduction of opioid-induced cardioprotection in ischemia–reperfusion. ANESTHESIOLOGY 2001; 94:1082–8
11. Huh J, Gross GJ, Nagase H, Liang BT: Protection of cardiac myocytes via delta(1)-opioid receptors, protein kinase C, and mitochondrial K(ATP) channels. Am J Physiol Heart Circ Physiol 2001; 280:H377–83
12. Ferrari R, Ceconi C, Curello S, Percoco G, Toselli T, Antonioli G: Ischemic preconditioning, myocardial stunning, and hibernation: Basic aspects. Am Heart J 1999; 138:S61–8
13. Tomai F, Crea F, Chiariello L, Gioffre PA: Ischemic preconditioning in humans: Models, mediators, and clinical relevance. Circulation 1999; 100:559–63
14. Schultz JE, Hsu AK, Gross GJ: Ischemic preconditioning in the intact rat heart is mediated by delta1- but not mu- or kappa-opioid receptors. Circulation 1998; 97:1282–9
15. Mayfield KP, D'Alecy LG: Delta-1 opioid receptor dependence of acute hypoxic adaptation. J Pharmacol Exp Ther 1994; 268:74–7
16. Mayfield KP, D'Alecy LG: Delta-1 opioid agonist acutely increases hypoxic tolerance. J Pharmacol Exp Ther 1994; 268:683–8
17. National Institutes of Health: Guide for the Care and Use of Laboratory Animals. Bethesda, National Institutes of Health, revised 1996. NIH publication No. 80-23
18. Popovic R, Liniger R, Bickler PE: Anesthetics and mild hypothermia similarly prevent hippocampal neuron death in an *in vitro* model of cerebral ischemia. ANESTHESIOLOGY 2000; 92:1343–9
19. Zheng S, Zuo Z: Isoflurane preconditioning reduces Purkinje cell death in an *in vitro* model of rat cerebellar ischemia. Neuroscience 2003; 118:99–106
20. Li J, Zheng S, Zuo Z: Isoflurane decreases AMPA-induced dark cell degeneration and edematous damage of Purkinje neurons in the rat cerebellar slices. Brain Res 2002; 958:399–404
21. Takemori AE, Larson DL, Portoghese PS: The irreversible narcotic antagonistic and reversible agonistic properties of the fumaramate methyl ester derivative of naltrexone. Eur J Pharmacol 1981; 70:445–51
22. Portoghese PS, Lipkowski AW, Takemori AE: Binaltorphimine and norbinaltorphimine, potent and selective kappa-opioid receptor antagonists. Life Sci 1987; 40:1287–92
23. Portoghese PS, Sultana M, Takemori AE: Naltrindole, a highly selective and potent non-peptide delta opioid receptor antagonist. Eur J Pharmacol 1988; 146:185–6
24. Lever JR, Scheffel U: Selective *in vivo* binding of [^{3}H] naltriben to delta-opioid receptors in mouse brain. Eur J Pharmacol 1998; 350:335–44
25. Sato T, O'Rourke B, Marban E: Modulation of mitochondrial ATP-dependent K^+ channels by protein kinase C. Circ Res 1998; 83:110–4
26. Bak IJ, Misgeld U, Weiler M, Morgan E: The preservation of nerve cells in rat neostriatal slices maintained in vitro: A morphological study. Brain Res 1980; 197:341–53
27. Miki T, Cohen MV, Downey JM: Opioid receptor contributes to ischemic preconditioning through protein kinase C activation in rabbits. Mol Cell Biochem 1998; 186:3–12
28. Karck M, Tanaka S, Bolling SF, Simon A, Su TP, Oeltgen PR, Haverich A: Myocardial protection by ischemic preconditioning and delta-opioid receptor activation in the isolated working rat heart. J Thorac Cardiovasc Surg 2001; 122:986–92
29. Koska J: Intravenous agents, Clinical Anesthesia Practice. Edited by Kirby R, Gravenstein N. Philadelphia, Saunders, 1994, pp 597–605
30. Dhawan BN, Cesselin F, Raghubir R, Reisine T, Bradley PB, Portoghese PS, Hamon M: International Union of Pharmacology: XII. Classification of opioid receptors. Pharmacol Rev 1996; 48:567–92
31. Chang AC, Takemori AE, Ojala WH, Gleason WB, Portoghese PS: Kappa opioid receptor selective affinity labels: Electrophilic benzeneacetamides as kappa-selective opioid antagonists. J Med Chem 1994; 37:4490–8
32. Fang L, Knapp RJ, Horvath R, Matsunaga TO, Haaseth RC, Hruby VJ,

Copyright © by the American Society of Anesthesiologists. Unauthorized reproduction of this article is prohibited.

Downloaded from http://asa2.silverchair.com/anesthesiology/article-pdf/100/3/562/353581/0000542-20040300-00015.pdf by guest on 20 March 2024

Porreca F, Yamamura HI: Characterization of [^{3}H]naltrindole binding to delta opioid receptors in mouse brain and mouse vas deferens: Evidence for delta opioid receptor heterogeneity. J Pharmacol Exp Ther 1994; 268:836–46

33. Ward SJ, Fries DS, Larson DL, Portoghese PS, Takemori AE: Opioid receptor binding characteristics of the non-equilibrium mu antagonist, beta-funaltrexamine (beta-FNA). Eur J Pharmacol 1985; 107:323–30

34. Kukreja RC: Essential role of oxygen radicals in delayed pharmacological preconditioning. J Mol Cell Cardiol 2001; 33:1395–8

35. Freeman BA, Crapo JD: Biology of disease: Free radicals and tissue injury. Lab Invest 1982; 47:412–26

36. Kawakami M, Sekiguchi M, Sato K, Kozaki S, Takahashi M: Erythropoietin receptor-mediated inhibition of exocytotic glutamate release confers neuroprotection during chemical ischemia. J Biol Chem 2001; 276:39469–75

37. Du C, Hu R, Csernansky CA, Hsu CY, Choi D: Very delayed infarction after mild focal cerebral ischemia: A role for apoptosis? J Cereb Blood Flow Metab 1996; 16:195–201

38. Li Y, Chopp M, Jiang N, Yao F, Zaloga C: Temporal profile of in situ DNA fragmentation after transient middle cerebral artery occlusion in the rat. J Cereb Blood Flow Metab 1995; 15:389–97

39. Dingledine R: Brain Slices. New York, Plenum Press, 1984

Downloaded from http://asa2.silverchair.com/anesthesiology/article-pdf/100/3/562/353581/0000542-20040300-00015.pdf by guest on 20 March 2024

Copyright © by the American Society of Anesthesiologists. Unauthorized reproduction of this article is prohibited.