Neuropharmacology 71 (2013) 10–18

Contents lists available at SciVerse ScienceDirect

Neuropharmacology

journal homepage: www.elsevier.com/locate/neuropharm

Effect of naloxone on ischemic acute kidney injury in the mouse

Junpei Mutoh, Masahiro Ohsawa*, Hiroaki Hisa

Second Department of Pharmacology, School of Pharmaceutical Sciences, Kyushu University of Health and Welfare, 1714-1 Yoshino-machi, Nobeoka-shi, Miyazaki 882-8508, Japan

ARTICLE INFO

Article history:
Received 17 March 2012
Received in revised form
22 February 2013
Accepted 3 March 2013

Keywords:
Acute kidney injury
Hypothalamus
Naloxone
Opioid

ABSTRACT

Renal ischemia produces sympathoexcitation, which is responsible for the development of ischemic acute kidney injury. Stimulation of central opioid receptors activates the renal sympathetic nerve. The present study examined the effect of an opioid receptor antagonist naloxone on the ischemia/reperfusion-induced renal dysfunction in mice. Blood urea nitrogen (BUN) and plasma creatinine increased 24 h after the renal ischemia/reperfusion. Intraperitoneal or intracerebroventricular, but not intrathecal, pretreatment with naloxone suppressed the renal ischemia/reperfusion-induced increases in BUN and plasma creatinine. This effect of naloxone was reversed by subcutaneous pretreatment with morphine. Selective MOP receptor antagonist β-funaltrexamine (FNA) also suppressed the renal ischemia/reperfusion-induced increases in BUN and plasma creatinine. Moreover, tyrosine hydroxylase expression in the renal tissue increased 24 h after renal ischemia/reperfusion, which was abolished by intraperitoneal or intracerebroventricular pretreatment with naloxone and FNA. Immunohistochemical experiments revealed a significant increase in the number of the Fos family proteins (c-Fos, FosB, Fra-1, and Fra-2) positive cells in the paraventricular nucleus of hypothalamus and supraoptic nucleus 24 h after the renal ischemia/reperfusion. Intracerebroventricular pretreatment with naloxone attenuated the renal ischemia/reperfusion-induced increase in the number of the Fos family proteins positive cells in these areas. Finally, we observed that i.c.v. pretreatment with antiserum against β-endorphin also suppressed the increased blood urea and plasma creatinine. These results suggest that the blockade of central opioid receptors can attenuate the ischemic acute kidney injury through the inhibition of renal sympathoexcitation. The central opioid receptors may thus be a new target for the treatment of ischemic organ failures.

1. Introduction

Postischemic failure of organs such as the heart, the brain, and the kidney represents a major problem in clinical medicine. Ischemic acute kidney injury (AKI) is a frequently observed clinical syndrome with high morbidity and mortality. Renal ischemia/reperfusion (I/R) is observed after transplantation and as a major complication in the cardiac and vascular surgery, the septic as well as hypovolemic shock, and the trauma (Bonventre and Weinberg, 2003). Detailed mechanisms underlying the I/R-induced renal injury, however, remain unclear because many intracellular events have been shown to contribute to this injury (Edelstein et al., 1997).

Sympathetic nerve activity is elevated during ischemia of several organs. Myocardial ischemia-induced sympathoexcitation has been well investigated (White, 1957; Zahner et al., 2003; Zheng et al., 2009; Infanger et al., 2010; Sharma et al., 2011). This sympathoexcitation is thought to be closely related to the progression and prognosis of heart failure that is subsequent to myocardial ischemia (see review Watson et al., 2006). In the case of the kidney, the sympathoexcitation is also shown to contribute in part to the I/R-induced AKI (Kobuchi et al., 2011). Indeed, patients with chronic renal failure show an increased sympathetic nerve activity as evaluated by the measurement of serum noradrenaline concentration (see review Koomans et al., 2004). Moreover, renal application of noradrenaline also produces AKI in rats (Conger et al., 1991). Although this phenomenon is important for the progression of I/R-induced renal dysfunction, the mechanisms underlying the sympathetic nerve activation after renal I/R remain poorly understood.

Opioid peptides and opiate alkaloids affect many physiological functions including the sensory system, the neuroendocrine function, the food consumption, and the autonomic regulation. Three

* Corresponding author. Present address. Laboratory of CNS Pharmacology, Graduate School of Pharmacy and Pharmaceutical Sciences, Nagoya City University, 3-1, Tanabe-dori, Mizuho-ku, Nagoya 467-8603, Japan. Tel./fax: +81 52 836 3410.
E-mail address: ohsawa@phar.nagoya-cu.ac.jp (M. Ohsawa).

0028-3908/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.neuropharm.2013.03.001

classes of opioid receptor subtypes, MOP, DOP, and KOP, have been identified, each of which displays unique distribution in the central nervous systems (Mansour et al., 1987). All classes of opioid receptors expressed in the forebrain region regulate the sympathetic nerve activity. Stimulation of central MOP receptors causes the initial suppression and following sustained elevation of the renal sympathetic nerve activity (Cao and Morrison, 2004). Central endogenous KOP systems also modulate the renal sympathetic tone (Gottlieb and Kapusta, 2005). Taken together, the activation of central opioid systems increases the renal sympathetic nerve activity.

To the best of our knowledge, there is no evidence on the possible contribution of opioid systems in I/R-induced dysfunction of several organs including the heart and the kidney. The present study was undertaken to define the role of central opioid systems on the I/R-induced renal dysfunction. In the first step of this study, we examined whether the peripheral or central application of naloxone reverses the I/R-induced renal dysfunction in mice. In the next step, changes in the expression of renal tyrosine hydroxylase (TH), a rate-limiting enzyme for noradrenaline biosynthesis, and the expression of hypothalamic Fos family proteins (c-Fos, FosB, Fra-1, and Fra-2) were examined in ischemic AKI mice. If these parameters could be influenced by the renal I/R, effects of naloxone on these changes were also examined. The findings in this study can provide new evidence that the inhibition of opioid systems improves the progression and prognosis of the I/R-induced dysfunction of organs based upon the increase in sympathetic nerve activity.

2. Methods

The present study was conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, Kyushu University of Health and Welfare, as adopted by Committee on Animal Research of Kyushu University of Health and Welfare, which are accredited by the Ministry of Education, Culture, Sports, Science and Technology of Japan.

2.1. Animals

Male ICR mice (SLC Japan Inc., Saga, Japan) weighing 20 to 30 g were used in this study. Animals were housed five per cage in a room maintained at 23 ± 0.5 °C with an alternating 12 h light–dark cycle. Food and water were available *ad libitum*. Animals were used only once in all experiments.

Acute kidney injury (AKI) was induced by clamping both renal pedicles for 30 min with a sterile surgical procedure under pentobarbital anesthesia (60 mg/kg, i.p.). At the end of the ischemic period, the clamps were released for reperfusion. In sham-operated animals, both kidneys were treated identically without clamping the renal pedicles. Twenty-four after the ischemia/reperfusion (I/R), blood samples were drawn from the abdominal aorta to evaluate renal function. EDTA (15 μg) was used as an anticoagulant for blood collection. The blood samples were then centrifuged at 1000×*g* for 10 min at room temperature, and resulting supernatant was collected as the plasma samples. Blood urea nitrogen (BUN) and plasma creatinine levels were measured using a commercial assay kit, the BUN-test-Wako and Creatinine-test-Wako (Wako Pure Chemicals, Osaka, Japan), respectively. The treatment and intervention regimens are listed in the Table 1.

Table 1
Summary of animal groups with intervention and treatment in this study.

Group	Procedure	Drug	Administration route
Sham	Sham operation	None	None
Control	I/R	None	None
Nx IP 1.0	I/R	Naloxone 1.0 mg/kg	Intraperitoneal
Nx IP 3.0	I/R	Naloxone 3.0 mg/kg	Intraperitoneal
Nx ICV	I/R	Naloxone 10 μg	Intracerebroventricular
Nx IT	I/R	Naloxone 10 μg	Intrathecal
FNA	I/R	β-funaltrexamine (20 μg)	Intracerebroventricular
AS	I/R	Antiserum against β-endorphin	Intracerebroventricular

2.2. Western blot

The kidney was removed quickly after decapitation of mice and homogenized in ice-cold buffered sucrose solution containing 20 mM Tris-HCl (pH 7.4), 2 mM EDTA, 0.5 mM EGTA, and 1 mM phenylmethylsulfonyl fluoride plus 250 μg/ml leupeptin, 250 μg/ml aprotinin, and 0.32 M sucrose. The homogenate was then centrifuged at 1000×*g* for 10 min at 4 °C, and resulting supernatant was used as protein samples for Western blot analysis. The protein concentration was measured using Bradford assay kit (Bio-Rad Laboratories, Hercules, CA). A same amount of protein samples was diluted with an equal volume of 2× electrophoresis sample buffer containing 2% SDS and 10% glycerol with 0.2 M dithiothreitol. Proteins (25 μg) were separated by SDS-PAGE (12%) using buffer system of Laemmli (1970). After electrophoresis, proteins were transferred to nitrocellulose membrane (Bio-Rad Laboratories) in Tris/glycine buffer containing 25 mM Tris and 192 mM glycine and 20% methanol. The membrane was soaked in a blocking buffer [3% bovine serum albumin (Sigma-Aldrich, St. Louise, MO) in Tris-buffered saline (pH 7.6) containing 0.05% Tween-20 (TBS/T)] for 1 h at room temperature. The membrane was washed with TBS/T for three 10-min intervals. For detection of tyrosine hydroxylase (TH), the membrane was incubated with the mouse monoclonal anti TH (1:2000; Chemicon, Temecula, CA) for overnight at 4 °C with gentle agitation. The membrane was then washed in TBS/T three 10-min intervals before being incubated in horseradish peroxidase-conjugated goat anti-rabbit IgG (1:200,000; Cell Signaling Technology Inc., Beverly, MA) for 1.5 h at room temperature. After three 5-min washes in TBS/T and two 5-min washes in TBS, the antigen–antibody peroxidase complex was detected by enhanced chemiluminescence (Pierce, Rockford, IL) and visualized using VersaDoc 5000 (Bio-Rad Laboratories). The intensity of the band was analyzed and semiquantified by computer-assisted densitometry using Quality One software (Bio-Rad Laboratories). Each value was normalized by the respective value for β-actin (1:10,000; Sigma-Aldrich) as an internal control.

2.3. Immunohistochemistry

Twenty four hours after the renal ischemia reperfusion, mice were anesthetized with pentobarbital (60 mg/kg, i.p) and were intracardially perfused with phosphate buffered saline (PBS) followed by 4% paraformaldehyde in PBS. Brain was removed, postfixed in the same fixative for 2 h, and immersed in 30% sucrose/PBS solution overnight. Coronal brain sections of 50 μm were prepared with the use of a vibratome (VIB-1500; The Vibratome Co., St. Louis, MO).

The brain sections were blocked in 10% normal goat serum in 0.1 M PBS for 2 h at room temperature. Each primary antibody was diluted in 0.1 M PBS containing 0.5% bovine serum albumin and 0.4% Triton-X 100 [1:5000 rabbit anti Fos (Santa Cruz Biotechnology, Santa Cruz, CA)] and was incubated for 2 days overnight at 4 °C. This antibody recognizes c-Fos, FosB, Fra-1, and Fra-2. The slices were then rinsed and incubated with the secondary antibody conjugated with Alexa 488 (Invitrogen, Carlsbad, CA) for 2 h at room temperature. The slices were mounted on the slide and coverslipped with fluorescence mounting medium (Dako, Carpentaria, CA).

Sections were examined on a laser scanning confocal microscope (LMS 510 META, Carl Zeiss) with excitation/emission wave length 488/520 nm.

Quantifications of the Fos-positive cells were done by counting the number of immunoreactivity on either side of the midline within the hypothalamus.

2.4. Drugs and drug injection

Drugs used in the present study were naloxone hydrochloride (Sigma-Aldrich), β-funaltrexamine (FNA; Sigma-Aldrich), and morphine (Sankyo Co., Tokyo, Japan). Antiserum to β-endorphin was purchased from Phoenix Pharmaceuticals Inc. (Belmont, CA). Other reagents used in the present study were molecular biology grade. Naloxone and morphine were dissolved in physiological saline. Intraperitoneal (I.p.) administration of naloxone was performed 30 min prior to renal ischemia. Intracerebroventricular (I.c.v.) or intrathecal (i.t.) injection of naloxone was performed 10 min prior to renal ischemia. Morphine was injected subcutaneously (s.c.) 30 min prior to i.c.v. injection of naloxone. I.c.v injection of FNA was performed 24 h prior to renal ischemia. Antiserum to β-endorphin was injected 1 h prior to renal ischemia. Naloxone was injected at doses of 1.0 and 3.0 mg/kg in a volume of 0.1 ml/10 g when given i.p., at dose of 10 μg in a volume of 4 μl when given i.c.v., and at dose of 10 μg in a volume of 5 μl when given i.t.. Morphine was injected s.c. at 30 mg/kg in a volume of 0.1 ml/10 g. Antiserum to β-endorphin was injected i.c.v. at 1:500 and 1:100 dilution in a volume of 4 μl.

I.c.v. injection in a volume of 4 μl was performed according to the methods of Haley and McCormick (1957). The mouse was manually restrained and 31-gauge needle mated to 10 μl Hamilton syringe was inserted 1.5 mm from mid line, 0 mm from the bregma and 3.0 mm from the surface of the skull.

I.t. injection in a volume of 5 μl was performed according to the methods of Hylden and Wilcox (1980). The mouse was manually restrained and 30-gauge needle mated to 25 ml Hamilton syringe was inserted between L5 and L6 of the mouse spinal column. Both i.c.v. and i.t. injections were carried out under sodium pentobarbital anesthesia (60 mg/kg, i.p.).