Atrial Natriuretic Peptide Reduces Ischemia/Reperfusion-Induced Spinal Cord Injury in Rats by Enhancing Sensory Neuron Activation

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ABSTRACT

We recently demonstrated that calcitonin gene-related peptide (CGRP) released from sensory neurons reduces spinal cord injury (SCI) by inhibiting neutrophil activation through an increase in the endothelial production of prostacyclin (PGI2). Carperitide, a synthetic α-human atrial natriuretic peptide (ANP), reduces ischemia/reperfusion (I/R)-induced tissue injury. However, its precise therapeutic mechanism(s) remains to be elucidated. In the present study, we examined whether ANP reduces I/R-induced spinal cord injury by enhancing sensory neuron activation using rats. ANP increased CGRP release and cellular cAMP levels in dorsal root ganglion neurons isolated from rats in vitro. The increase in CGRP release induced by ANP was reversed by pretreatment with capsazepine, an inhibitor of vanilloid receptor-1 activation, or with (9S,10S,12R)-2,3,9,10,11,12-hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1H-diindolo[1,2,3-fg;3′,2′,1′-kl]pyrrolo[3,4-ij][1,6]-benzodiazone-10-carboxylic acid hexyl ester (KT5720), an inhibitor of protein kinase A (PKA), suggesting that ANP might increase CGRP release from sensory neurons by activating PKA through an increase in the cellular cAMP level. Spinal cord ischemia was induced in rats using a balloon catheter placed in the aorta. ANP reduced mortality and motor disturbances by inhibiting reduction of the number of motor neurons in animals subjected to SCI. ANP significantly enhanced I/R-induced increases in spinal cord tissue levels of CGRP and 6-keto-prostaglandin F1α, a stable metabolite of PGI2. ANP inhibited I/R-induced increases in spinal cord tissue levels of tumor necrosis factor and myeloperoxidase. Pretreatment with 4′-chloro-3-methoxy-cinnamnamide (SB366791), a specific vanilloid receptor-1 antagonist, and indomethacin reversed the effects of ANP. These results strongly suggest that ANP might reduce I/R-induced SCI in rats by inhibiting neutrophil activation through enhancement of sensory neuron activation.

Paraplegia is a serious complication that occurs after surgical repair of thoracoabdominal aortic aneurysms (Gilling-Smith et al., 1995). This complication has been attributed to temporary or permanent ischemia of the spinal cord caused by interruption of the blood supply during aortic cross-clamping (Gilling-Smith et al., 1995). Dissection, rupture, and prolonged clamp times are closely related to an increased incidence of paraplegia (Lintott et al., 1998). Recently, it has been shown that neutrophils play a central role in development of the pathologic condition of ischemia/reperfusion (I/R)-induced tissue injury (Bednar et al., 1997). We previously demonstrated that physiologic anticoagulants, such as activated protein C (Hirose et al., 2000) and antithrombin (Hirose et al., 2004), reduce I/R-induced SCI in rats by inhibiting neutrophil activation.

Carperitide, a synthetic α-human atrial natriuretic peptide (ANP), was isolated and identified in 1984 by Kangawa and Matsuo (1984). Because ANP decreases preload and afterload by diuresis (Maack et al., 1984; Ishihara et al., 1985) and induces vasodilation (Wedel and Garbers, 2001), it has been used mainly for improvement of cardiac function in treatment of heart failure (Kikuchi et al., 2001) and in postoperative management of cardiac surgery (Tsuneyoshi et al., 2004). Although recent studies have demonstrated that ANP...
reduces I/R-induced cardiac injury by increasing nitric oxide production (Okawa et al., 2003), its therapeutic mechanism(s) is not fully understood.

Capsaicin-sensitive sensory neurons are nociceptive neurons that are activated by a wide variety of noxious physical and chemical stimuli (Dray, 1995). On activation, sensory neurons release calcitonin gene-related peptide (CGRP), a neuropeptide that has potent vasodilatory activity (Bull et al., 1996). Because ablation of the sensory fibers can result in a marked increase in severity of inflammation (Szallasi and Blumberg, 1996) and inflammatory responses in CGRP knockout mice subjected to hepatic I/R are markedly enhanced (Harada et al., 2006), sensory neurons might play a role in the maintenance of tissue integrity by attenuating inflammatory responses (Okajima and Harada, 2006). We demonstrated that CGRP increases endothelial production of PGI2 via activation of both endothelial nitric oxide synthase and cyclooxygenase-1 in rats subjected to hepatic I/R, thereby reducing I/R-induced liver injury through inhibition of neutrophil activation (Harada et al., 2002).

We recently reported that CGRP reduces the severity of compression-induced SCI in rats by inhibiting neutrophil activation through the promotion of endothelial PGI2 production (Kitamura et al., 2007). Thus, it is possible that I/R-induced activation of sensory neurons in the spinal cord reduces I/R-induced SCI by inhibiting neutrophil activation. Because neutrophils are critically involved in the development of I/R-induced cardiac injury (Vinten-Johansen, 2004), it is possible that ANP reduces I/R-induced SCI by inhibiting neutrophil activation. We hypothesized that ANP might enhance CGRP release from sensory neurons in the spinal cord, thereby increasing endothelial production of nitric oxide and PGI2, both of which are capable of inhibiting neutrophil activation directly or indirectly (Okajima et al., 2004). In the present study, we examined this possibility using a rat model of I/R-induced SCI.

Materials and Methods

Materials. Carperitide (α-human ANP) was the generous gift of Daiichi Pharmaceutical Co. (Tokyo, Japan). KT5720, an inhibitor of PKA, was purchased from Alexis (Basel, Switzerland). Indomethacin (IM), a nonselective inhibitor of cyclooxygenase, SB366791, a specific vanilloid receptor-1 antagonist (Varga et al., 2005), and capsazepine (CPZ), an inhibitor of vanilloid receptor-1 activation (Perkins and Campbell, 1992), were purchased from Sigma-Aldrich (St. Louis, MO). All other reagents were of analytical grade.

Preparation of Various Agents. ANP was dissolved in sterile distilled water. KT5720 was dissolved in 10% Tween 20/10% methanol (10%) with normal saline. IM was suspended in bicarbonate-buffered saline. SB366791 was dissolved in dimethyl sulfoxide (1%) with normal saline. CPZ was dissolved in 10% Tween 20/10% ethanol (10%) with normal saline. Solutions were prepared immediately before the experiments. Each saline-treated SCI animal received the vehicle in these experiments. However, results in control experiments using the vehicle of each solution were not significantly different from those obtained by using saline (data not shown). Therefore, we used as a representative control the data obtained by using saline in the present study.

Animals. The study protocol was approved by the Nagoya City University Animal Care and Use Committee. The care and handling of the animals were conducted in accordance with the guidelines of the National Institutes of Health. Adult pathogen-free male SD rats (Nihon SLC, Hamamatsu, Japan) weighting 300 to 350 g were used in the experiments.

Isolation and Culture of Dorsal Root Ganglion Neurons and Measurement of CGRP in Culture and cAMP Measurement. DRGs isolated from the lumbar, cervical, and thoracic region were dissected from rats as described previously (Harada et al., 2006). In brief, DRGs were placed in ice-cold sterile calcium- and magnesium-free Dulbecco’s phosphate-buffered saline (PBS) (Invitrogen, Grand Island, NY). Ganglia were chopped and incubated at 37°C for 15 min in Dulbecco’s PBS containing 20 U/ml papain ( Worthington Biochemicals, Lakewood, NJ). The tissue was then incubated at 37°C for 15 min in Dulbecco’s PBS containing 4 mg/ml collagenase type II ( Worthington Biochemicals). The tissue was incubated for a further 30 min in Dulbecco’s PBS containing 2000 U/ml dispase 1 (Godo Shusei, Tokyo, Japan) at 37°C. Individual cells were then dissociated by trituration through a fire-polished Pasteur pipette. After centrifugation at 2500g for 5 min, the resultant pellet was washed twice in serum-free Ham’s F-12 medium (Hyclone, Logan, UT). Cells were plated on 60-mm polystyrene dish precoated with Vitrogen (Cohesion Technologies, Palo Alto, CA) in Ham’s F-12 medium containing 10% supplemented calf serum, 2 mM glutamine, and 50 ng/ml mouse 2.5S nerve growth factor (Upstate Biotechnology, Lake Placid, NY). After 24 h, the culture medium was removed and replaced every 2 days.

After 5 days in culture, the medium was aspirated gently and washed with serum-free Ham’s F-12 medium. Cells were incubated with ANP (1, 10, and 100 nM) for 30 min in Ham’s F-12 medium containing 1% supplemented calf serum without nerve growth factor. After incubation, supernatants were sampled and stored at −20°C for CGRP measurement. To determine whether ANP increased CGRP release from DRGs via vanilloid receptor-1 activation, we examined the effect of CPZ, an inhibitor of vanilloid receptor-1 activation (Perkins and Campbell, 1992), on ANP-induced CGRP release from DRGs. CGRP levels were determined using a commercial rat CGRP enzyme immunoassay kit (SPI-BIO, Massy, France). Recent studies demonstrated that cAMP plays a critical role in CGRP production from sensory neurons by phosphorylating vanilloid receptor-1 through activation of PKA (Mohapatra and Nau, 2003), and cAMP-dependent PKA activation is critically involved in CGRP production in DRGs (Hou and Wang, 2001). Therefore, we measured the intracellular cAMP levels in DRGs. We examined the effect of KT5720 on CGRP release from DRGs at a concentration of 10 μM, as described previously (Harada et al., 2006). After collection of supernatants, plates were placed on ice, media were removed, and cells were washed by ice-cold PBS. Thereafter, ice-cold 65% ethanol were added to each well and placed on ice. Ethanol were collected and dried under nitrogen gas. Intracellular levels of cAMP were determined with an enzyme immunoassay kit (GE Healthcare, Buckinghamshire, UK) according to the manufacturer’s instructions.

Animal Model of Transient Ischemia of the Spinal Cord. During surgical preparation, body temperature was monitored using a rectal probe and maintained at 36.5–37.5°C with a thermal pad and a heat lamp. Under pentobarbital anesthesia (45 mg/kg i.p.; Abbott Laboratories, North Chicago, IL), a 2-F Fogarty catheter ( Edwards Lifesciences, Irvine, CA) was inserted into the left femoral artery, and the balloon was placed at the end of the aortic arch (Hirose et al., 2000, 2004). To induce spinal cord ischemia, the balloon was inflated with 0.05 ml of distilled water for 20 min. Complete occlusion of the descending aorta was evidenced by sustained loss of any detectable pulse measured by Doppler sonography in the right femoral artery. At the end of the ischemic period, the catheter was deflated and removed. After the wound was closed, animals were returned to the cages and allowed to recover. This technique creates spinal cord injury in the lumbar-sacral segment, which causes paraplegia in a reproducible manner (Hirose et al., 2000, 2004).

In the pretreatment group, ANP was infused continuously via the internal jugular vein from 30 min before ischemia to 60 min after reperfusion. In the post-treatment group, ANP was infused continuously via the internal jugular vein from 15 min after reperfusion to
105 min after reperfusion. IM (20 mg/kg) was injected s.c. at 60 min before ischemia. SB366791 (500 μg/kg) was injected i.p. at 60 min before ischemia. In sham-operated animals, the balloon catheter was placed in the aorta but not inflated.

**Evaluation of Survival and Neurologic Status.** To evaluate the effect of ANP on survival, animals were allowed to survive for 21 days. Animals that never recovered completely from the surgery and then died within 24 h after reperfusion were excluded from the analyses. The motor function of rats was assessed in a masked manner using the Tarlov's scale and the inclined-plane test as described previously (Hirose et al., 2000, 2004). The Tarlov's motor scale is as follows: 0, no voluntary movement (complete paraplegia); 1, perceptible movement at the joint; 2, good joint mobility but inability to stand; 3, ability to stand and walk; and 4, complete recovery. For the inclined-plane test, we used a smooth-surface inclined plane, and the maximum inclination of the plane at which rats could maintain themselves for 5 s without falling from the plane was recorded.

**Histopathologic Examination of the Spinal Cord.** The lumbar enlargement of the spinal cord under the L1 vertebra was harvested and postfixed in the fixative overnight. The specimens were embedded into paraffin, and transverse sections (5-μm thick) were stained with hematoxylin and eosin. When significant injury was present, motor neuron cells in the ventral gray matter showed eosinophilic, structureless cytoplasm and loss of nuclear hematoxylin stainability (Hirose et al., 2000). The cells were considered viable if they demonstrated basophilic stippling. The total number of intact motor neurons in the ventral gray matter region was counted using five serial sections of each animal.

**Measurement of Spinal Cord Tumor Necrosis Factor Levels.** Spinal cord levels of tumor necrosis factor (TNF) were determined by a modification of the methods described previously (Hirose et al., 2000). In brief, after the indicated time period of reperfusion, spinal cords were removed, immediately plunged into liquid nitrogen for rapid freezing, and stored at −80°C for later biochemical analysis. TNF concentrations were measured at five segmental levels (cervical, upper thoracic, lower thoracic, lumbar, and sacral segments). The frozen sample was weighed and homogenized (1:10, w/v) in 0.1 M phosphate buffer (pH 7.4) containing 0.05% sodium azide in an ice bath. The homogenate was sonicated for 30 s and centrifuged (4500g at 4°C for 20 min). The concentration of TNF in the supernatant was determined using an enzyme-linked immunosorbent assay kit for rat TNF (BioSource International, Camarillo, CA). Results are expressed as picograms of TNF per gram of tissue. Because we have previously demonstrated that levels of TNF in spinal cord tissue are increased after I/R, peaking at 3 h of reperfusion (Hirose et al., 2000), we measured the spinal cord TNF levels 3 h after reperfusion in this study.

**Assay of Myeloperoxidase Levels in Spinal Cord Tissue.** Spinal cord levels of myeloperoxidase (MPO) were determined by a modification of the methods described previously (Hirose et al., 2000). In brief, the frozen samples were weighed, homogenized (1:10, w/v) in 0.1 M phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethyl ammonium bromide (Sigma-Aldrich) in an ice bath, and sonicated for 30 s. After centrifugation (4500g at 4°C for 20 min), the supernatant was assayed for MPO activity. The assay started by adding 0.1 ml of sample to 0.6 ml of 0.1 M phosphate buffer (pH 6.0) containing 1.25 mg/ml o-dianisidine and 0.05% hydrogen peroxide. The change in absorbance at 460 nm after 20 min was measured using a spectrophotometer (DU-54; Beckman Coulter, Irvine, CA), and the MPO activity in each sample was calculated using a standard curve prepared using purified MPO. We measured the spinal cord MPO levels at 24 h after reperfusion as described previously (Hirose et al., 2000).

**Measurement of Spinal Cord Tissue Levels of CGRP.** Spinal cord tissue levels of CGRP were determined by a modification of the methods described previously (Kitamura et al., 2007). In brief, the frozen sample was weighed and homogenized (1:10, w/v) in 2 N acetic acid. The homogenates were bathed in 90°C water for 20 min and then centrifuged (4500g at 4°C for 20 min). CGRP was extracted from the supernatant using reverse-phase C18 columns (GE Healthcare). Columns were prepared by washing with 5 ml of methanol, followed by 10 ml of water, before use. The supernatant was applied onto the column, followed by washing with 20 ml of 0.1% trifluoroacetic acid. CGRP was eluted with 3 ml of 60% acetonitrile in 0.1% trifluoroacetic acid, and the solvent was evaporated under a stream of nitrogen gas. The concentration of CGRP was assayed using a specific enzyme immunoassay kit (SPI-BIO). The sensitivity of the CGRP assay was 10 pg/ml. The cross-reactivity of the antiserum of rat α- and β-CGRP was 100% as specified in the manufacturer’s data sheet. Results are expressed as picograms of CGRP per gram of tissue.

**Assay of Levels of 6-Keto-PGF1α in Spinal Cord Tissue.** Spinal cord tissue levels of 6-keto-PGF1α, a stable metabolite of PGI2, were determined by a modification of the methods described previously (Hirose et al., 2004). In brief, the frozen sample was weighed and homogenized (1:10, w/v) in 0.1 M phosphate buffer (pH 7.4) containing 2 mM indomethacin at 4°C. The homogenates were centrifuged at 4500g for 20 min at 4°C. The supernatant was then acidified with 1 M HCl. 6-Keto-PGF1α was extracted from the supernatant using columns packed with ethyl-bonded silica gel (ethyl C25; GE Healthcare). Columns were prepared by washing with 2 ml of methanol followed by 2 ml of water before use. The acidified supernatant was applied onto the column, followed by washing sequentially with 5 ml of 10% ethanol and 5 ml of hexane. 6-Keto-PGF1α was eluted with 5 ml of methyl formate, and the solvent was evaporated under a stream of nitrogen gas. The concentration of 6-keto-PGF1α was assayed using a specific enzyme immunoassay kit (GE Healthcare). The cross-reactivities of this assay with PGE2, PGE2-fom bromoxane B2, and arachidonic acid were 2.8, 1.4, 0.03, and 0.01, respectively, according to the manufacturer’s data sheet. Results are expressed as nanograms of 6-keto-PGF1α per gram of tissue. We measured the spinal cord 6-keto-PGF1α levels 3 h after reperfusion as described previously (Hirose et al., 2004).

**Statistical Analysis.** All values are expressed as mean ± S.D. Survival rate was analyzed with Fisher’s exact probability test. Differences in motor scores were assessed using the nonparametric Mann-Whitney U test or the Wilcoxon signed-rank test. The results between two groups were analyzed with Student’s t test. Multiple group comparisons were performed using analysis of variance followed by Scheffé’s post hoc test. p < 0.05 was considered statistically significant.

**Results**

**Effect of ANP on CGRP Release and Cellular cAMP Levels in DRGs Isolated from Rats in Vitro.** To determine whether ANP increases CGRP release from sensory neurons, we examined the effect of ANP on CGRP release from DRGs isolated from rats. ANP, at concentrations higher than 1 nM, enhanced CGRP release from DRGs (Fig. 1A). Cyclic AMP has been shown to play an important role in CGRP release from sensory neurons on activation (Mohapatra and Nau, 2003). ANP at concentrations higher than 1 nM increased cellular cAMP levels in DRGs isolated from rats (Fig. 1B).

**Effects of Pretreatment with Capsazepine, an Inhibitor of Vanilloid Receptor-1 Activation, and KT5720, an Inhibitor of PKA, on CGRP Release in DRGs Isolated from Rats.** Pretreatment with CPZ reversed the ANP-increased CGRP release from DRGs (Fig. 2). Because vanilloid receptor-1 is activated by phosphorylation through cAMP-dependent PKA (Hou and Wang, 2001) and ANP increased cellular cAMP levels in DRGs, it is possible that ANP increases CGRP release from DRGs by activating PKA. To examine this possibility, we analyzed the effect of pretreat-
ment with KT5720, an inhibitor of PKA (Harada et al., 2006), on ANP-induced CGRP release from DRGs isolated from rats. As shown in Fig. 2, the ANP-induced increase in CGRP release from DRGs was completely reversed by pretreatment with KT5720.

Effect of ANP on Survival and Motor Disturbances in Rats Subjected to Spinal Cord I/R. To examine whether ANP reduces I/R-induced SCI in rats, we analyzed the effect of ANP on motor disturbances observed in rats subjected to spinal cord I/R. Although i.v. administration of ANP at a dose of 1 μg/kg/min significantly reduced motor disturbances, ANP at doses of 0.1 and 0.5 μg/kg/min did not (data not shown). Thus, we examined the therapeutic mechanism of ANP at a dose of 1 μg/kg/min on I/R-induced SCI in rats in the present study. The overall survival rate at 21 days after aortic occlusion was significantly higher in ANP-treated SCI animals (80%, n = 10) than in saline-treated SCI animals (20%, n = 10) (p < 0.03) (Fig. 3). Although saline-treated SCI animals showed remarkable neurologic dysfunction and did not show recovery from paralysis, the neurologic scores (Tarlov's score) of ANP-treated SCI animals (n = 10) did not decrease and were significantly higher each day for 7 days than those of saline-treated SCI animals (n = 10) (p < 0.01) (Fig. 4). The angle of the inclined plane was higher in ANP-treated SCI animals (n = 10) than that in saline-treated SCI animals (n = 10) at 24 h after reperfusion (Fig. 5). In contrast with results in animals administered ANP before spinal cord I/R, motor disturbances were not reduced in animals administered ANP (1 μg/kg/min for 90 min) at 15 min after the spinal cord I/R (data not shown).

Effect of ANP on I/R-Induced Histopathologic Changes in the Spinal Cord Tissue. There were no histopathologic changes in spinal cord tissue sections from sham-operated animals (Fig. 6, inset A). Nissl's substance was observed in the cytoplasm of normal neurons in these animals (Fig. 6, inset A). In contrast, spinal cord tissue sections from saline-treated SCI animals showed neuronal injury in the ventral gray matter; i.e., many neurons were

![Fig. 1. Effect of ANP on CGRP release from DRGs (A) and intracellular cAMP levels in DRGs (B). DRGs were incubated with ANP (1, 10, and 100 nM) for 30 min. Supernatants were collected, and CGRP levels were measured by enzyme immunoassay. Intracellular cAMP levels were measured by enzyme immunoassay. Each value represents the mean ± S.D. from five experiments. * p < 0.01 versus media. † p < 0.01 versus ANP.](https://jpet.aspetjournals.org/)

![Fig. 2. Effect of CPZ and KT5720 on ANP-induced increase in CGRP release from DRGs. DRGs were incubated with ANP (10 nM) for 30 min in the presence or absence of CPZ (10 μM), an inhibitor of vanilloid receptor-1 activation, or KT5720 (10 μM), a PKA inhibitor. Each value represents the mean ± S.D. from five experiments. * p < 0.01 versus media. † p < 0.01 versus ANP.](https://jpet.aspetjournals.org/)

![Fig. 3. Comparison of survival curves after transient ischemia of the spinal cord in saline-treated and ANP-treated SCI animals. Solid line, saline-treated SCI animals (n = 10); dashed line, ANP-treated SCI animals (n = 10). * p < 0.05 versus ANP-treated SCI group.](https://jpet.aspetjournals.org/)
characterized by eosinophilic, structureless cytoplasm and loss of nuclear hematoxylin stainability (Fig. 6, inset B). The tissue sections from ANP-treated SCI animals showed a marked reduction of these histopathologic changes (Fig. 6, inset C). Although approximately 70% of motor neurons in the ventral gray matter were lost in saline-treated SCI animals, only approximately 25% of motor neurons were lost in ANP-treated SCI animals (Fig. 6).

Effect of ANP on Spinal Cord Tissue Levels of CGRP in Rats Subjected to Spinal Cord I/R. To examine whether ANP reduces I/R-induced SCI by sensory neuron activation in rats, we analyzed the effect of ANP on spinal cord tissue levels of CGRP in rats subjected to spinal cord I/R. Spinal cord tissue levels of CGRP were significantly increased after spinal cord I/R, peaking at 1 h after reperfusion, compared with those in sham-operated animals (Fig. 7A). ANP markedly enhanced I/R-induced increases in spinal cord tissue levels of CGRP at 1 h after reperfusion (Fig. 7B).

Effect of ANP on Spinal Cord Tissue Levels of 6-Keto-PGF₁α, TNF, and MPO in Rats Subjected to Spinal Cord I/R. Spinal cord tissue levels of 6-keto-PGF₁α, a stable metabolite of PGI₂, were increased after spinal cord I/R, peaking at 1 h after reperfusion (Hirose et al., 2004). Administration of ANP significantly enhanced I/R-induced increases in spinal cord tissue levels of 6-keto-PGF₁α at 1 h after reperfusion (Fig. 8A). Spinal cord tissue levels of TNF were increased after spinal cord I/R, peaking at 3 h after reperfusion (Hirose et al., 2000). Administration of ANP inhibited I/R-induced increases in spinal cord tissue levels of TNF at 3 h after reperfusion (Fig. 8B). Accumulation of neutrophils in the postischemic spinal cord tissue was evaluated by measuring MPO levels. Spinal cord tissue levels of MPO were increased after spinal cord I/R, peaking at 24 h after reperfusion (Hirose et al., 2000). ANP significantly inhibited increases in MPO levels at 24 h after reperfusion (Fig. 8C).

Effects of ANP, SB366791, and/or IM on Motor Disturbances in Rats Subjected to Spinal Cord I/R. Because ANP enhanced I/R-induced increases in spinal cord tissue levels of CGRP, it is possible that sensory neuron activation is implicated in therapeutic effects of ANP in rats subjected to spinal cord I/R. To examine this possibility, we analyzed the effect of SB366791, a specific antagonist of vanilloid receptor-1 (Kitamura et al., 2007), on ANP-induced reduction of motor disturbances in rats subjected to spinal
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Effects of ANP and/or SB366791 on Increases in Spinal Cord Tissue Levels of CGRP in Rats Subjected to Spinal Cord I/R. Pretreatment with SB366791 inhibited I/R-induced increases in spinal cord tissue levels of CGRP, and it also completely inhibited ANP-induced increases in spinal cord tissue levels of CGRP at 1 h after reperfusion (Fig. 7B). Thus, sensory neuron activation might play a critical role in ANP-induced enhancement of spinal cord tissue levels of CGRP in rats subjected to spinal cord I/R.

Effects of ANP, SB366791 and/or IM on Spinal Cord Tissue Levels of 6-Keto-PGF₁α, TNF, and MPO in Animals Subjected to Spinal Cord I/R. Pretreatment with SB366791 and IM inhibited I/R-induced increases in spinal cord I/R. Although pretreatment with SB366791 did not exacerbate motor disturbances, it completely reversed ANP-induced reduction of motor disturbances in animals subjected to spinal cord I/R (Fig. 5).
cable tissue levels of 6-keto-PGF$_{1α}$, and it also completely reversed ANP-induced enhancement of increases in spinal cord tissue levels of 6-keto-PGF$_{1α}$, in rats subjected to spinal cord I/R (Fig. 8A).

Pretreatment with SB366791 and IM had no effects on I/R-induced increases in spinal cord tissue levels of TNF and MPO (Fig. 8, B and C). However, ANP-induced effects on spinal cord tissue levels of TNF and MPO in rats subjected to spinal cord I/R were completely abrogated by pretreatment with SB366791 and IM (Fig. 8, B and C).

**Discussion**

In the present study, ANP increased CGRP release from DRGs isolated from rats in vitro. To examine whether ANP increases CGRP release by activating vanilloid receptor-1 in sensory neurons in vitro, we analyzed the effect of SB366791, a vanilloid receptor-1 inhibitor more specific than CPZ (Varga et al., 2005), on the ANP-induced increase in CGRP release from isolated DRGs. However, preliminary experiments showed that SB366791 increased CGRP release from DRGs isolated from rats, and this effect might be induced by dimethyl sulfoxide present in SB366791 solution (data not shown). Thus, we used CPZ instead of SB366791 for in vitro experiments in the present study. Pretreatment with CPZ reversed the ANP-induced increase in CGRP release from DRGs, suggesting that vanilloid receptor-1 activation might be critically involved in the ANP-induced increase in CGRP release from sensory neurons. However, the mechanism(s) by which ANP activates vanilloid receptor-1 is still not fully understood. ANP activates PKA in the ischemic liver of rats, thereby preventing apoptosis (Kulhanek-Heinze et al., 2004). Activation of PKA has been shown to induce phosphorylation of vanilloid receptor-1, thereby sensitizing sensory neuron activation by inflammatory mediators such as bradykinin (Tominaga et al., 2001). Consistent with this hypothesis are observations in the present study demonstrating that ANP increased cellular cAMP levels in DRGs and that the ANP-induced increase in CGRP release from DRGs was completely inhibited by KT5720. These observations suggested that ANP might increase CGRP release by activating PKA through an increase in the cellular cAMP level in sensory neurons.

ANP reduced motor disturbances in rats subjected to transient spinal cord ischemia as shown in the present study. ANP inhibited I/R-induced increases in spinal cord tissue levels of TNF. Because TNF has been shown to be an important causal substance in the development of I/R-induced spinal cord damage (Cassada et al., 2002), ANP might reduce spinal cord damage by inhibiting TNF production. TNF activates endothelial cells and neutrophils, thereby inducing endothelial cell injury (Kitamura et al., 2007). Vascular permeability was markedly increased as a consequence of activated neutrophil-induced endothelial cell damage, leading to spinal cord ischemia (Kitamura et al., 2007). Such microcirculatory disturbances might lead to microinfarction of the spinal cord and reduction of the number of motor neurons, thereby inducing a motor disturbance (Hirose et al., 2000). Thus, it is possible that ANP inhibits the decrease in the number of motor neurons by inhibiting activated neutrophil-induced endothelial cell damage through inhibition of TNF production, which contributes to the reduction of mortality and motor disturbances in this rat model of spinal cord damage. Consistent with this notion is our present observation demonstrating that ANP inhibited I/R-induced increases in spinal cord tissue levels of MPO.

Because complete occlusion of the descending aorta might induce damage to the lower limbs or peripheral nerves in rats, motor disturbances observed in the present study might be derived not only from SCI but also from damage to the peripheral nerves and limbs. However, this possibility seems unlikely, because motor disturbances observed in the present study were almost the same as those in rats subjected to direct spinal cord damage induced by compression trauma (Kitamura et al., 2007).

Spinal cord tissue levels of CGRP were significantly increased after spinal cord I/R as shown in the present study. We recently demonstrated that immunohistochemical expression of CGRP is clearly observed in the superficial layer of the dorsal horns of the spinal cord in rats subjected to compression trauma of the spinal cord (Kitamura et al., 2007). CGRP mRNA-positive neurons are also observed in the mouse dorsal horn (Tie-Jun et al., 2001). These observations suggested that I/R-induced increases in spinal cord tissue levels of CGRP might be explained mainly by increases in the release of CGRP from spinal cord sensory neurons in rats. Sensory neurons are activated by endogenous inflammatory mediators such as bradykinin in vitro (Tominaga et al., 2001). Spinal cord tissue levels of bradykinin are increased during development of compression-induced SCI (Pan et al., 2001). Because local I/R plays a critical role in development of compression-induced SCI (Taoka and Oka-jima, 2003), these substances might increase spinal cord tissue levels of CGRP by activating sensory neurons in rats subjected to spinal cord I/R. Consistent with this hypothesis are observations in the present study demonstrating that SB366791 inhibited increases in spinal cord tissue levels of CGRP in rats subjected to spinal cord I/R. ANP significantly enhanced I/R-induced increases in spinal cord tissue levels of CGRP, and these increases were inhibited by pretreatment with SB366791 in the present study, suggesting that ANP might enhance sensory neuron activation in the spinal cord after I/R.

PGI$_2$ inhibits monocyctic production of TNF by inhibiting nuclear factor-κB activation through the activation of protein kinase A (Eigler et al., 1998). We demonstrated that CGRP(8–37), a CGRP antagonist, inhibited compression trauma-induced increases in spinal cord tissue levels of 6-ke-to-PGF$_{1α}$, thereby exacerbating motor disturbances through enhancement of local inflammatory responses in rats (Kitamura et al., 2007). Thus, it is possible that ANP enhances CGRP release from the sensory neuron at the damaged segment of the spinal cord, thereby increasing endothelial production of PGI$_2$, which contributes to reduction of SCI in rats subjected to spinal cord I/R by inhibiting inflammatory responses. This notion is supported by the following observations in the present study: 1) ANP-induced increases in spinal cord tissue levels of 6-keto-PGF$_{1α}$ were inhibited by pretreatment with SB366791 and IM; 2) ANP-induced inhibition of increases in spinal cord tissue levels of TNF and MPO after spinal cord I/R was reversed by pretreatment with SB366791 and IM; and 3) reduction of motor disturbances by ANP was abrogated by pretreatment with SB366791 and IM.

Although pretreatment with SB366791 and IM reversed...
increases in spinal cord tissue levels of 6-keto-PGF1α, in rats subjected to spinal cord I/R, it did not exacerbate motor disturbances or enhance increases in spinal cord tissue levels of TNF and MPO. We reported previously that pretreatment with SB366791 and IM did not exacerbate motor disturbances or local inflammatory responses in rats subjected to compression trauma-induced spinal cord injury only when the motor disturbances and local inflammatory responses were maximum (Kitamura et al., 2007). Thus, it is likely that motor disturbances and local inflammatory responses observed in rats subjected to milder spinal cord ischemia are exacerbated by pretreatment with SB366791 and IM.

Because PGE2 is synthesized in endothelial cells from PGH2, a common precursor of PGI2 (Gerritsen and Cheli, 1983), it is likely that sensory neuron activation increases endothelial production of PGE2 as well as PGI2. Consistent with this hypothesis is our previous report demonstrating that sensory neuron activation increases gastric tissue levels of PGE2 as well as 6-keto-PGF1α, thereby attenuating local inflammatory responses in rats subjected to stress (Harada et al., 2003). Thus, it is likely that ANP increases the endothelial production of both PGE2 and PGI2 by increasing CGRP release from sensory neurons, thereby reducing SCI through attenuation of inflammatory responses in rats subjected to spinal cord I/R.

PGL2 and PGE2 have been shown to increase CGRP release from sensory neurons by increasing cAMP levels in sensory neurons (Rowlands et al., 2001). Thus, both PGL2 and PGE2 might further enhance ANP-induced sensory neuron activation, thereby contributing to attenuation of inflammatory responses in the spinal cord in rats subjected to spinal cord I/R.

ANP inhibits TNF production in monocytes stimulated by endotoxin in vitro (Kiemer et al., 2000), suggesting that ANP might directly inhibit TNF production, thereby reducing SCI in the present study. However, this possibility seems less likely, because pretreatment with SB366791 or IM completely abrogated therapeutic effects of ANP.

Although ANP reduced mortality as well as motor disturbances in this animal I/R-induced SCI animal model when administered before spinal cord ischemia, it did not do so when administered after ischemia. These observations raise the possibility that ANP may be useful in prevention of SCI when administered before surgical treatment of thoracoabdominal aortic aneurysms in the clinical setting.

Causes of death in this SCI animal model are not clear at present. However, because visceral I/R injury, an obligatory component of aortic occlusion, is an important cause of death in the postoperative phase of thoracoabdominal aortic aneurysm repair (Elbers et al., 2006), intestinal ischemia/reperfusion might at least partly contribute to the early deaths in the present study. We reported previously that sensory neuron activation reduces I/R-induced liver injury and stress-induced gastric injury by inhibiting neutrophil activation in rats (Harada et al., 2002, 2003). Thus, it is possible that ANP improves the outcome of animals subjected to I/R-induced SCI by attenuating inflammatory responses in both the visceral and spinal cord. This possibility should be investigated by further experiments in the near future.

Taken together, observations in the present study raised the possibility that ANP might reduce I/R-induced SCI in rats mainly by attenuating local inflammatory responses of the spinal cord through enhancement of sensory neuron activation.

References


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