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INCREASED SENSITIVITY TO ACUTE AND PERSISTENT PAIN IN NEURON-SPECIFIC ENDOTHELIN-1 KNOCKOUT MICE

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Abstract—Endothelin-1 (ET-1) exists in endothelial cells as well as a variety of other cell types. The presence of ET-1 and its receptors in neurons suggests its possible role as a neurotransmitter and/or neuromodulator. Studies utilizing exogenous ET-1 have suggested that ET-1 affects pain transmission. This study was designed to examine the possible role(s) of neuronal ET-1 in pain processing. We produced neuron-specific ET-1 knockout mice using the Cre/loxP system with a synapsin I promoter and examined the effects produced by the lack of neuronal ET-1 on pain behavior using common pain models and a model of stress-induced analgesia. In acute nociceptive pain models, paw withdrawal thresholds to radiant heat and mechanical stimuli applied with von Frey hairs were significantly lower in the knockout mice compared with control. This indicated that the absence of neuronal ET-1 leads to greater sensitivity to acute nociceptive stimuli. After inflammation was produced by intraplantar injection of carrageenan, there was a significantly greater degree of thermal hyperalgesia and mechanical allodynia in the knockout mice even after the difference in baseline values was compensated. Furthermore, in a neuropathic pain model produced by spinal nerve ligation, there was also a greater degree of mechanical allodynia in the knockout mice. Finally, in a swim-stress model, the magnitude of stress-induced analgesia was less in the knockout mice, indicating the involvement of neuronal ET-1 in stress-induced analgesia. The results suggest that there is a basal release of ET-1 from neurons that affect baseline pain thresholds as well as an additional release during persistent pain states that acts to suppress the pain. The involvement of neuronal ET-1 in stress-induced analgesia further suggests its role in endogenous pain inhibitory systems. To confirm that ET-1 is released in persistent pain states and to determine which part of the CNS is involved, we measured the concentrations of ET-1 before and after inducing peripheral inflammation in different parts of the CNS involved in endogenous pain inhibitory systems in normal mice. We found that ET-1 was increased in the hypothalamus while no significant increase was observed in the midbrain, medulla and spinal cord. The results of the present study suggest that neuronal ET-1 is involved in endogenous pain inhibitory control likely via pathways through the hypothalamus.

Key words: knockout mice, acute nociceptive pain, inflammatory pain, neuropathic pain, stress-induced analgesia.

The endothelins (ETs) are 21-amino acid peptides originally cloned from cultured porcine aortic endothelial cells (Yanagisawa et al., 1988). They are extremely potent vasoconstrictors and are expressed in vascular endothelium as well as in a variety of other tissues and cell types as three biologically active peptides, i.e. ET-1, ET-2 and ET-3 (Inoue et al., 1989). Sequent reports have shown that ET-1 is expressed in neurons of the brain and spinal cord (Lee et al., 1990; Giaid et al., 1991; Yamada and Kurokawa, 1998). ET-1 is also present in dorsal root ganglia (DRGs; Giaid et al., 1989; Kar et al., 1991). Likewise, receptors for ET-1 are present in neurons of the CNS (Kurokawa et al., 1997; Shibata et al., 1997; Yamada and Kurokawa, 1998) and those of the peripheral nervous system (Pomonis et al., 2001). Such presence of ET-1 and its receptors in neurons suggests its possible role as a neurotransmitter and/or neuromodulator.

Although some of the roles of ET-1 in the CNS have been suggested (Rubanyi and Polokoff, 1994; Kuwaki et al., 1997, 1999; van den Buuse and Webber, 2000), much of it remains elusive. Within the CNS, ET-1 has been detected in the cerebral cortex, the striatum, the hippocampus, the amygdaloid body, the hypothalamus, the Purkinje cell layer of the cerebellum, the raphe nuclei, and the dorsal horn and intermediate column of the spinal cord (Giaid et al., 1989, 1991; Lee et al., 1990). Such diverse distribution suggests that ET-1 is involved in a wide range of brain functions. ET receptors also have a widespread distribution. This includes the hypothalamus, the periaqueductal gray area (PAG), the locus coeruleus, the caudolateral area of the pontine tegmentum (A5) and the rostral pontine area of the lateral reticular formation (A7) (Kurokawa et al., 1997; Yamada and Kurokawa, 1998). These are areas involved in endogenous pain control systems (Lima and Almeida, 2002) and suggest the possibility that neuronal ET-1 may be involved in pain processing. A number of behavioral studies utilizing exogenous ET-1 administration to the CNS also suggest the involvement of ET-1 in pain processing. The administration of ET-1 to the lateral ventricle and to the PAG produced a significant increase in hot plate and tail flick latencies (TFLs; Nikolov

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Abbreviations: DRG, dorsal root ganglion; ET, endothelin; PAG, periaqueductal gray; PWL, paw withdrawal latency; TFL, tail flick latency.

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et al., 1993; D'Amico et al., 1996) and spinal administration produced a dose-dependent antinociceptive effect in the tail flick test and the formalin test (Kamei et al., 1993; Yamamoto et al., 1994).

These studies suggest that ET-1 in the CNS suppressively modulates pain transmission and plays a role in endogenous pain inhibitory control. In contrast to these effects in the CNS, peripheral administrations of ET-1 induced hyperalgesia and nociception. The acute administration onto the sciatic nerve induced pain behavior (Davar et al., 1998; Fareed et al., 2000) and intra-articular or s.c. injection caused acute nociception and/or potentiated formalin-induced nociception (De-Melo et al., 1998; Gokin et al., 2001; Piovezan et al., 2000). Peripherally released ET-1 acts as an algogen and is implicated in the pathogenesis of a variety of pain states such as inflammatory pain, neuropathic pain and cancer pain (Ferreira et al., 1989; Jarvis et al., 2000; Wacnik et al., 2001). Thus ET-1 in the CNS and that in the periphery appear to play distinctly different roles in pain generation and transmission. Peripheral ET-1 involved in pathological states is released from non-neuronal cells, e.g. keratinocytes, cardiomyocytes, and cancer cells (Ahn et al., 1998; Pernow and Wang, 1997; Wacnik et al., 2001). On the other hand, the pain inhibitory actions of exogenous ET-1 in the CNS may reflect ET-1 of neuronal origin (neuronal ET-1), but may also reflect ET-1 released from non-neuronal elements within the CNS. Endothelial cells of cerebral microvessels produce ET-1 and release it through the adventitial side of the cell to the extracellular space of the CNS (Yoshimoto et al., 1990). ETs are also present in glial cells (MacCumber et al., 1990). Such non-neuronal ET-1 in the CNS may reach ET receptors on neurons in a paracrine manner or by way of CSF and affect pain transmission.

In the present study, to tease out the possible actions of neuronal ET-1 on pain transmission, we produced neuron-specific ET-1 knockout mice using the Cre/loxP system with a synapsin I promoter. We investigated the effects produced by the lack of neuronal ET-1 on acute nociceptive pain, inflammatory pain and neuropathic pain by behavioral testing of these mice using common pain models. In addition, we assessed the difference in stress-induced analgesia between these mice and control mice. Furthermore, we measured the concentrations of ET-1 in normal mice before and after the induction of peripheral inflammation in parts of the CNS that have been implicated in endogenous pain inhibitory control.

EXPERIMENTAL PROCEDURES

Experiments were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Use Committee of Chiba University Graduate School of Medicine.

Animals

Neuron-specific ET-1 knockout mice were generated by the Cre/loxP system (Gu et al., 1994). Conditional ET-1 allele (ET-1 flox) mice were generated as described previously (Huang et al., 2002). Cre expression was controlled by the synapsin I promoter, which had been shown to drive transgenic expression specifically in neuronal cells (Hoesche et al., 1993; Zhu et al., 2001). By mating ET-1 (flox/flox) mice with synapsin I-Cre (±) transgenic mice (a generous gift from Dr. J. D. Marth), we obtained synapsin I-Cre (+); ET-1 flox mice (neuron-specific ET-1 knockout mice) and synapsin I-Cre (−); ET-1 flox mice (control mice). The genotype of knockout mice was identified by polymerase chain reaction assay and/or Southern blot analysis on DNA extracted from tail biopsy as described (Huang et al., 2002). C57BL/6 wild type mice were used in the study measuring ET-1 levels in the brain. Male mice were used in all experiments. All mice were housed in plastic cages in a room maintained at 23–25 °C with lights on at 7:00 A.M. and off at 7:00 P.M. Mice had food and water available *ad libitum*. All efforts were made to minimize the number of animals used and their suffering.

Immunohistochemistry

Immunohistochemical double staining of the spinal cord and the amygdala for ET-1 and Neu-N, a neuronal marker, was performed to confirm the knockout of neuronal ET-1. ET-1 staining of DRG cells was also performed. Spinal cord, brain and DRG tissues were harvested from control and knockout mice and were prepared for staining as follows. All animals were deeply anesthetized with sodium pentobarbital and transcardially perfused with 30 ml of 4% paraformaldehyde following 8 ml of heparinized 0.9% saline. Both solutions had been cooled to 4 °C. After perfusion, brain tissues, spinal cords and DRGs were removed and postfixed in 4% paraformaldehyde overnight at 4 °C and then immersed in 20% sucrose solution for 16 h at 4 °C. The lumbar segment of the spinal cord and the amygdaloidal area of the brain were sectioned at 40-μm thickness on a freezing microtome and were collected in 0.01 M phosphate buffer saline. The DRGs were sectioned at 20 μm. Endogenous tissue peroxidase activity was quenched by soaking the sections for 30 min in 0.3% hydrogen peroxide solution, then the sections were treated for 60 min at room temperature in a blocking solution, which consisted of 0.3% Triton X-100 and 0.5% normal goat serum. They were then processed for double staining by a free-floating avidin–biotin-complex technique. Brain and spinal cord sections were incubated for 20 h at 4 °C in rabbit antibody against ET-1 (Peninsula Laboratories, San Carlos, CA, USA) and mouse antibody against Neu-N (Chemicon International, Temecula, CA, USA) diluted with the blocking solution. Next, sections were washed and incubated with biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA) followed by Alexa Fluor 488 streptavidin conjugate (Molecular Probes, Eugene, OR, USA) and Alexa Fluor 594 goat anti-mouse IgG (Molecular Probes). Both incubations were performed for 1 h at room temperature. DRG sections were incubated with rabbit antibody against ET-1 then with biotinylated goat anti-rabbit IgG followed by Alexa Fluor 488 streptavidin conjugate. Processed sections were observed with a fluorescence microscope (Eclipse E-800; Nikon, Tokyo, Japan) and their images were captured by cooled CCD digital camera systems (Penguin 600CL; Pixera, Los Gatos, CA, USA).

Behavioral testing

Testing was performed during the day portion of the circadian cycle (07:00–19:00 h). In studies 1 through 3, animals were allowed several 1-h habituation periods inside the testing chamber (see below) on the day before testing until they were well acclimated to the chamber. On the day of testing, animals were placed in the chamber and behavioral testings were conducted after exploration behavior terminated. The experimenter was blinded to the animals' genotype. No difference in appearance, weight, behavior, grooming or feeding was observed between knockout and control mice. No other difference could be observed that might confound the blinded analysis.