Role of endothelin-1 in stress response in the central nervous system

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Received 10 December 1999; accepted in final form 29 February 2000

Endothelin-1 (ET-1) is a 21-amino acid peptide that induces a variety of biological activities, including vasoconstriction and cell proliferation, and its likely involvement in cardiovascular and other diseases has recently led to broad clinical trials of ET receptor antagonists. ET-1 is widely distributed in the central nervous system (CNS), where it is thought to regulate hormone and neurotransmitter release. Here we show that CNS responses to emotional and physical stressors are differentially affected in heterozygous ET-1-knockout mice, which exhibited diminished aggressive and autonomic responses toward intruders (emotional stressors) but responded to restraint-induced (physical) stress more intensely than wild-type mice. This suggests differing roles of ET-1 in the central pathways mediating responses to different types of stress. Hypothalamic levels of ET-1 and the catecholamine metabolite 3-methoxy-4-hydroxyphenylglycol (MHPG) were both increased in wild-type mice subjected to intruder stress, whereas MHPG levels were not significantly affected in ET-1-knockout mice. Furthermore, immunohistochemical analysis showed that ET-1 and tyrosine hydroxylase, an enzyme in the catecholamine synthesis pathway, were colocalized within certain neurons of the hypothalamus and amygdala. Our findings suggest that ET-1 modulates central coordination of stress responses in close association with catecholamine metabolism.

knockout mice; catecholamine

RESPONSES TO ENVIRONMENTAL stressors involve activation of complex central nervous system (CNS) pathways leading from stress perception to behavioral, autonomic, and endocrine responses. As represented by Selye’s adaptation syndrome, general responses to stressors can lead to pathophysiological changes and may thus provide a pathogenic basis for such diseases as hypertension and ischemic heart disease (9, 35). The hypothalamus and limbic system, including the amygdala, are thought to integrate stressor-evoked signals into such physiological responses. In addition, accumulating evidence indicates that neuroendocrine factors, such as catecholamines, corticotrophin-releasing hormone (CRH), serotonin, and vasopressin function within the central circuits mediating stress responses (3, 4, 8, 17, 32, 34, 36, 39, 40); however, the precise allocation of these factors within neuroanatomical structures and their mutual interactions have not been fully explored.

Endothelin-1 (ET-1) is a 21-amino acid peptide that was first identified as a vasoconstrictor in the supernatant of cultured vascular endothelial cells (42). Three isopeptides (ET-1, -2, and -3) encoded by separate gene loci constitute a gene family whose products act via two distinct G protein-coupled receptors (ET-A and ET-B) with differing affinities (26, 28, 33, 41). In addition to its vasoconstrictor effects on vascular smooth muscle cells, in other cell types, ET-1 exhibits a diverse set of biological activities, including stimulation of proliferation and modulation of hormone release (26, 28, 33, 41). In the CNS, ET-1 expression is widely distributed in the cerebral cortex, striatum, hippocampus, amygdala, pituitary gland, suprarenal glands, and ventricular nuclei of the hypothalamus, cerebellar Purkinje cells, raphe nuclei, dorsal motor nucleus of the vagus nerve and the dorsal horn, and intermediolateral cell column in the spinal cord (6, 25, 38, 43). The expression pattern of ET-1 and its effects on the release of various neuropeptides (26, 28, 33, 41, 43) suggests that it may serve as a neurotransmitter and/or neuromodulator within the CNS. When we used gene targeting to disrupt the mouse Edn 1 locus encoding ET-1 (17), the resultant mice homozygous for the ET-1 null mutation exhibited morphological abnormalities in cranial neural crest-derived craniofacial tissues.
and organs (16, 17). Our further analysis of these ET-1-knockout mice revealed the central role of ET-1 in the autonomic nervous regulation of cardiopulmonary homeostasis (17, 18, 22, 23, 27). Moreover, the high levels of ET-1 expression in the hypothalamus and limbic system and the close association between ET-1 and central and peripheral sympathetic nerve activity (15, 18–23, 27, 29) led us to hypothesize that ET-1 plays a significant role in stress responses. In the present study, we tested this hypothesis by applying stress-inducing stimuli to ET-1-knockout mice and analyzing selected behavioral, autonomic, and neurochemical responses.

**METHODS**

**Mice.** ET-1-knockout mice were established by gene targeting as previously described (17). After extensive backcross breeding, the genetic background had been altered to ICR or to C57B6. Most experiments were performed using mice with the ICR background; for the restraint stress test, however, mice with the genetic background of the 129Sv/J × ICR hybrid were used. The mice were housed in a room maintained at 25°C on a light-dark cycle (lights were on from 0900 to 2100) and were fed a normal diet and water ad libitum. Only males were used in this study.

**Resident-intruder test.** Heterozygous ET-1-knockout mice and their wild-type litter mates (10–14 wk old) were housed as isolated individuals for 4 wk. On the day of experimentation, each mouse (resident) in its home cage was placed on top of a radio receiver (RLA1020; Data Sciences). Telemetric monitoring of electrocardiogram (ECG) and body temperature was then accomplished using a radio transmitter (TA10ETA-F20; Data Sciences) implanted in advance in the abdominal cavities of the animals under halothane anesthesia. After baseline parameters were measured for 10 min, a

![Fig. 1. Behavioral and autonomic responses during the resident-intruder test. Tests were repeated with an interval of 1 wk.](http://ajpregu.physiology.org/)

**A and B:** aggressive response. Resident mice were wild-type (Wild; n = 12 for the first test, n = 7 for the 2nd test) and endothelin (ET)-1-knockout heterozygotes (Hetero; n = 19 for the 1st test, n = 11 for the second test). **A:** latency to the first attack. Cumulative percentages of mice attacking an intruder were plotted and compared between the genotypes using Gehan-Wilcoxon’s nonparametric test. **B:** numbers of attacks within 5 min of intrusion during the same experiment shown in A. C and D: changes in heart rate (C) and body temperature (D) of resident mice (Wild, n = 7; Hetero, n = 9) are plotted as a function of time; the presence of an intruder is indicated by the bar.
group-housed, wild-type mouse (intruder) was placed in the cage for 5 min. Behavioral responses of the resident, namely latency before attack of the intruder and the number of attacks, were observed through a transparent wall of the cage. The ECG and body temperature of the resident was continuously monitored throughout the experimental period and processed using a computer-assisted data acquisition system (Dataquest IV; Data Sciences) that yielded values for heart rate and body temperature every 1 min. Tests were repeated after an interval of 1 wk, in the same individuals when possible.

Restraint test. A femoral artery in each animal was cannulated with a polyethylene tube under halothane anesthesia as previously described (17). Beginning on the day after the surgery, arterial blood pressure and heart rate were monitored continuously through the cannula using a pressure transducer (AP601G; Nihon Koden) and a tachometer (AT600G; Nihon Koden), respectively. Initially, baseline parameters were measured for 10 min in the awake, unrestrained animals, after which the mice were placed in a narrow stainless steel mesh tube (2.5 cm in diameter) for 10 min. Mean arterial blood pressure was obtained by passing the instantaneous blood pressure signal through a low-pass filter (corner frequency 0.2 Hz) while heart rate signals were sampled at the rate of 1 Hz using an analog-to-digital converter (MacLab/16s; ADInstruments). The mean values for these variables over periods of successive 2-, 5-, or 10-min intervals were then calculated by computer (Power Macintosh 9500/120; Apple Computer).

Immunohistochemistry. Mice were anesthetized with ether and perfused with Zamboni’s solution. The brains were dissected, prefixed, embedded in OCT compound, and serially cut into 10-μm frozen sections using a cryostat. Slide-mounted tissue sections were washed with PBS, treated with 0.3% H2O2 in methanol, preincubated with goat nonimmune serum, and incubated with rabbit anti-ET-1 polyclonal antibody for 2 days at 4°C (43). The sections were then incubated with biotinylated goat anti-rabbit IgG (Immu-Biological Laboratories) for 1 h at 37°C. After washing, the sections were treated with avidin-biotinylated horseradish peroxidase complex ( Vectastain ABC kit, Vector Labs) and developed with 0.004% H2O2 and 0.02% diaminobenzidine tetrahydrochloride. Double-label immunostaining was accomplished by first treating sections with rabbit anti-ET-1 polyclonal antibody followed by incubation with rhodamine-conjugated swine anti-rabbit IgG (Dako) for 1 h at 37°C. The sections were then treated with anti-tyrosine hydroxylase (Chemicon) or anti-gial fibrillary acidic protein (GFAP) monoclonal antibody for 1 h at 37°C and stained with fluorescein-labeled horse anti-mouse IgG (Dako) for an additional 1 h. Samples were examined under a MRC1024 confocal laser scanning microscope (BioRad).

Measurement of ET-1 and MHPG levels. The brains of mice just subjected to the resident-intruder test (see Resident-intruder test) and of untreated controls were fixed by applying microwave radiation (5 kW; model TMW6402C; Toshiba) to their heads for 1.2 s. The brain tissue was then removed and dissected (7), isolating the hypothalamus and medulla oblongata, which were weighed and stored at −80°C until use. ET-1 levels in tissue homogenates were measured by enzyme-linked immunosorbent assay. Tissue contents of a noradrenaline metabolite, 3-methoxy-4-hydroxyphenylglycol (MHPG), were measured with high-performance liquid chromatography.

Statistical analysis. Gehan-Wilcoxon’s nonparametric test was used to compare attack latency during the resident-intruder test; attack numbers were compared using Student’s t-test. Heart rates, arterial blood pressures, and body temperatures were analyzed using repeated-measures ANOVA. ET-1 and MHPG levels were compared using Student’s t-test and two-factor (genotype × stress) ANOVA, respectively; the latter was carried out using the Statistica statistics package (StatSoft; detailed explanation of the method and references are supplied in the manufacturer’s manual). Quantitative data were expressed as means ± SE. Values of P < 0.05 were considered significant.

RESULTS

Impaired stress responses in ET-1-knockout mice. To investigate the role of ET-1 in the central circuits mediating behavioral responses to emotional stress, we performed resident-intruder tests in which the latency before attacking an intruder and the frequency of the attacks was assessed. When confronted with an intruder, resident heterozygous ET-1-knockout mice (ICR background) were slower to attack the intruder (Fig. 1A) and attacked less frequently (Fig. 1B) than their wild-type litter mates. Increased aggressiveness and intensity of attack, presumably due to fighting experience, was noted in both groups during a second test carried out 1 wk later (Fig. 1, A
and B). Similar results were obtained using mice with the C57B6 background, although they were generally less aggressive than mice with the ICR background (data not shown).

Changes in heart rate and body temperature were also analyzed using telemetric monitoring (Fig. 1, C and D). In parallel with the aggressive behavior, heart rate and body temperature were both significantly increased on intrusion by a stranger. As with behavior, these responses were significantly diminished in ET-1-knockout mice (Fig. 1, C and D), although the difference in body temperature between the two groups was not significant.

To examine whether stress responses are globally diminished by mutation of the ET-1 gene, we subjected ET-1-knockout and wild-type mice to the physical stress of being restrained within a narrow tube and monitored their arterial blood pressures and heart rates. As observed previously (20), ET-1-knockout mice had higher arterial blood pressures than wild-type mice under baseline conditions. The physical stress of restraint evoked sustained increases in mean arterial blood pressure that had similar time courses in both wild-type and ET-1-knockout mice (Fig. 2A). Heart rates in wild-type mice increased transiently and then returned to the baseline within 5 min (Fig. 2B). On the other hand, heart rates in ET-1-knockout mice were significantly elevated above baseline throughout the entire restraint period (Fig. 2B), reflecting a significant interaction between genotype and the time course of the change of heart rate. The lack of acclimatization of heart rate in response to a sustained stress, which is thought to be mediated by the hypothalamus and by cardiac vagal afferents (31), indicates that at least some central pathways involved in processing

Fig. 3. Levels of ET-1 and 3-methoxy-4-hydroxyphenylglycol (MHPG) in the hypothalamus during the resident-intruder test. A: hypothalamic ET-1 levels in wild-type mice and ET-1-knockout heterozygotes under baseline (Wild, n = 11; Hetero, n = 5) and stressed (Wild, n = 7; Hetero, n = 6) conditions. B and C: hypothalamic (B) and medullary (C) MHPG levels in wild-type mice and ET-1-knockout heterozygotes under baseline (Wild, n = 7; Hetero, n = 9) and stressed (Wild, n = 6; Hetero, n = 8) conditions. #P < 0.05; ##P < 0.01 vs. baseline; *P < 0.05; **P < 0.01 vs. wild-type mice.
stress-induced responses are impaired in ET-1-knockout mice.

Stress-induced changes in brain ET-1 levels associated with catecholamine metabolism. Catecholamines are among the factors known to play major roles in central stress responses. To investigate whether ET-1 production is affected by stress and to compare it with catecholamine metabolism, we measured hypothalamic ET-1 and MHPG and found that both were significantly elevated in wild-type mice during the resident-intruder test (Fig. 3, A and B). The increase in MHPG is consistent with earlier findings demonstrating the role of hypothalamic catecholamine metabolism in the central processing of stress responses (11, 30). The increase in hypothalamic ET-1 levels in ET-1-knockout heterozygotes, whose basal ET-1 levels in the hypothalamus were ~70% of those of wild-type litter mates, was not significant (Fig. 3A). Furthermore, the stress-induced increase in hypothalamic MHPG was significantly diminished in ET-1-knockout mice (Fig. 3B).

We also measured MHPG in the medulla oblongata. Wild-type mice again exhibited increased MHPG levels in response to intruder stress, although the increase was small compared with that seen in the hypothalamus (Fig. 3C). In contrast, no stress-induced changes in medullary MHPG were seen in ET-1-knockout mice (Fig. 3C). It is noteworthy that baseline medullary MHPG levels were higher in ET-1-knockout mice than in wild-type mice (Fig. 3C). We previously reported that as a consequence of augmented sympathetic nerve activity, blood pressure was ~10 mmHg higher in ET-1-knockout mice than in wild-type mice (17, 18, 22, 23, 27). The elevation of baseline MHPG levels in the medullas of ET-1-knockout mice is consistent with that earlier finding, because sympathoexcitatory neurons in the rostroventrolateral medulla can be activated by catecholamines via β-receptors (37).

Expression of ET-1 in the brain. Expression of ET-1 in the brain was examined immunohistochemically. Intense immunoreactive (ir) ET-1 signaling was detected in the hypothalamic paraventricular nucleus and in the amygdala (Fig. 4), both of which are involved in coordinating stress responses (24). Many but not all irET-1-positive cells were small in size and were identified as glial cells by colocalization with GFAP, a glial cell marker (data not shown). Double labeling revealed that many irET-1-positive neurons in the hypothalamus and amygdala were also positive for tyrosine hydroxylase, an enzyme in the catecholamine synthesis pathway (Fig. 4). Thus ET-1 and catecholamines are coexpressed within certain neurons situated in centers coordinating stress responses.
DISCUSSION

Confrontation with an intruder evokes aggressive responses accompanied by sympathetic neural and humoral activation in some animals, including mice housed in isolation. This response has served as a useful experimental model of emotional stress and has been applied to various genetically engineered animals to clarify the molecular mechanisms responsible for central stress responses (34, 39). In the present study, we demonstrate that the responses to an intruder were significantly diminished in ET-1-knockout mice. Because stress-induced behavioral and autonomic responses as well as increases in hypothalamic catecholamine metabolism were all impaired in ET-1-knockout mice, we suggest that ET-1 plays a significant role in the coordination of central stress responses in the hypothalamus and limbic system and that ET-1 may even directly regulate catecholamine-mediated signaling. In contrast, the response to the physical stress imposed by restraint within a narrow tube was augmented in ET-1-knockout mice, suggesting that vagal inhibition of the stress-mediating neural circuit may also be impaired in ET-1-knockout mice.

Several pieces of evidence support the notion that ET-1 is involved in CNS functions related to stress responses. For example, ET-1 is widely expressed throughout the CNS, including in the limbic system and the hypothalamus (6, 25, 38, 43). Moreover, ET receptors are distributed in those regions of the forebrain, cerebellum, and brain stem where ET-1 is expressed (5, 10, 13, 14). Intracerebroventricular administration of ET-1 or ET-3 evokes changes in sympathetic nerve activity and in behavioral responses such as barrel rotation (13, 29) and evokes c-fos expression in the ventrolateral septum, stria terminalis, paraventricular hypothalamic nucleus, and central amygdaloid nucleus (44). This pattern is similar to that induced by intracerebroventricular infusion of vasopressin or CRH (1), neuropeptides also implicated in stress responses (3, 8, 32, 36, 39, 40). In the context of these earlier studies, the present results suggest that participation by ET-1 in the central responses to emotional and physical stresses involves modulation of separate CNS pathways.

When mice were subjected to intruder stress, hypothalamic levels of ET-1 and MHPG were both increased in wild-type mice, but no change in MHPG levels was observed in ET-1-knockout mice, suggesting that ET-1- and catecholamine-mediated signaling are sequentially activated in response to stress. In fact, ET-1 and ET-3 have been shown to induce catecholamine release from striatal slice (12) and adrenomedullary chromaffin cells (2). Furthermore, ET-1 colocalized with tyrosine hydroxylase in some neurons of the hypothalamus and amygdala. Although there is presently no direct evidence for the involvement of cells coexpressing ET-1 and catecholamines in central stress responses, our findings make it reasonable to hypothesize that activation of ET-1-catecholamine pathways in the hypothalamus and amygdala contributes significantly to that process.

We previously showed that heterozygous ET-1-knockout mice have high blood pressure that is primarily attributable to excessive sympathetic activity, whereas baroreflex control of heart rate was not affected (17, 18, 22, 23, 27). This may seem to contradict the present finding of the relationship between ET-1 and catecholamines in stress responses, but it is likely that the relationship between ET-1 and catecholamines varies among different regions in the CNS. For instance, within the medulla oblongata, topical administration of ET-1 elicits differing effects on sympathetic nerve activity depending on the specific sites of administration (19), indicating that ET-1 acts on both the stimulatory and inhibitory pathways involved in central control of sympathetic activity.

The present findings are also important in practical terms, because ET antagonism is now likely to emerge as an important therapeutic strategy for the treatment of several cardiovascular and other diseases. Indeed, despite the fact that the chronic effects of these antagonists on ET metabolism and function in the CNS have not been fully investigated, a number of ET receptor antagonists are currently undergoing clinical trials. Our findings indicate that a chronic ~50% reduction in systemic ET-1 production, including in the brain, can result in significant changes in the pathways mediating central stress processing. Evaluation of the effects of chronic administration of ET receptor antagonists on CNS function would thus seem warranted.

In conclusion, ET-1 has emerged as a potentially important modulator of stress responses in the CNS. Nonetheless, further analysis of the relationship between the ET and catecholamine systems, as well as between ET-1 and other factors, such as CRH, vasopressin, and serotonin, will be necessary before a complete understanding of the CNS pathways mediating stress is achieved.

We thank Dr. Takeshi Iwatsubo (University of Tokyo) for helpful advice and discussion and Dr. Takamura Muraki (Tokyo Women’s Medical University) for technical support. Y. Kurihara is a Research Fellow in the Program for Promotion of Fundamental Studies in Health Sciences of the Organization for Drug ADR Relief, Research and Development Promotion and Product Review of Japan.

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan; the Japan Cardiovascular Research Foundation; the Kanae Foundation of Research for New Medicine; Tanabe Medical Frontier Conference; Suzuken Memorial Foundation; the Ryoichi Naito Foundation for Medical Research; Tokyo Biochemical Research Foundation; the Naito Foundation; the Takeda Science Foundation and the Mochida Memorial Foundation for Medical and Pharmaceutical Research (to H. Kurihara and T. Kuwaki).

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