Postganglionic sympathetic neurons express endothelin

DEBORAH H. DAMON
Department of Pharmacology, University of Vermont, Burlington, Vermont 05405

Damon, Deborah H. Postganglionic sympathetic neurons express endothelin. Am. J. Physiol. 274 (Regulatory Integrative Comp. Physiol. 43): R873–R878, 1998.—Endothelin (ET) is a peptide originally identified as an endothelial-derived vasoconstrictor. It is now recognized that ET is produced by and acts on many other tissues including the brain and spinal cord, where it is believed to modulate neurotransmission. The present studies demonstrate that ET is synthesized by and secreted from postganglionic sympathetic neurons. With the use of Northern analysis, ET-1 mRNA was detected in cultures of sympathetic superior cervical ganglion (SCG) neurons isolated from 3- to 5-day old rat pups. ET-1 and ET-3 peptides were also detected in cultured SCG neurons using immunohistochemistry. ET-1 (50 pg/106 cells) and ET-3 (173 pg/106 cells) were detected by radioimmunoassay of media conditioned by cultured SCG. ET-1 (77 pg/mg protein) and ET-3 (30 pg/mg protein) were also detected by radioimmunoassay of extracts of adult SCG.

blood pressure; neuropeptide; neuromodulator

ENDOTHELINS (ETs) are a family of peptides that are important modulators of vascular structure and function. ET-1 is a potent vasoconstrictor (20) and a mitogen for vascular smooth muscle cells (2) that has implicated in the vascular response to injury (5). ETs also modulate the vasoreactivity of the sympathetic nervous system. ET inhibits sympathetic neurotransmission in the guinea pig femoral artery (19). Intracerebroventricular injection of ET-1 has been reported to increase blood pressure and plasma catecholamines, and the increase in blood pressure was inhibited by intravenous administration of an α-adrenergic receptor antagonist (16). Intrathecal injection of ET-1 and ET-3 decreased arterial pressure (7), but the mechanism of these actions was not determined. ET-1 has also been shown to modulate the release of catecholamines from the adrenal gland (1).

Vascular cells (20) produce ET-1 that can modulate vascular cell function and postganglionic sympathetic neurotransmission. ET-1 and ET-3 have been detected in neurons of the brain and spinal cord (6, 10, 22), but the precise source of ET that could modulate central sympathetic or preganglionic neurotransmission has not been identified. The present study tests the hypothesis that postganglionic sympathetic neurons synthesize and secrete ET.

MATERIALS AND METHODS

Materials. Bovine serum albumin (fraction V), normal goat serum cytosine arabinoside (Ara C), and mitomycin C were purchased from Sigma Chemical (St. Louis, MO). Collagenase (type 2), hyaluronidase, and trypsin (3X) were purchased from Worthington Biochemical (Freehold, NJ). Collagen and nerve growth factor (NGF) were purchased from Collaborative Biomedical Products (Bedford, CA). Dulbecco’s modified Eagle’s medium (DMEM), penicillin-streptomycin, and glutamine were purchased from Gibco-BRL (Grand Island, NY). Fetal calf serum (FCS) was purchased from Summit Biotechnologies (Fort Collins, CO). Primary antibody to tyrosine hydroxylase (TH) (rabbit anti-TH) and corresponding rhodamine-labeled secondary antibody [donkey anti-rabbit rhodamine-labeled immunoglobulin (lg)] were purchased from Chemicon International (Temecula, CA). Primary and secondary antibodies for detecting ET-1 were obtained from Peninsula Laboratories (Belmont, CA) and Biogenesis (Sanvonn, NH). ET-3 antibodies were obtained from Peninsula Laboratores.

SCG cultures. Rat pups (3–4 days of age) were anesthetized with metofane and euthanized by removing their hearts. SCG were collected and enzymatically dissociated for 20 min at 37°C in a collagenase-hyaluronidase solution (10 mg/ml bovine serum albumin, 4 mg/ml collagenase, 1 mg/ml hyaluronidase) and then for 3 min in trypsin (3 mg/ml). Dissociated neurons were applied to collagen-coated dishes and grown in DMEM supplemented with 10% FCS, 50 ng/ml NGF, penicillin-streptomycin, and glutamine. One and three days after plating, the cultures were treated with an antimitotic agent [Ara C or mitomycin C (10 mg/ml)] to suppress the growth of nonneuronal cells. Experiments were performed on cells that had been in culture for 4–8 days.

Northern analysis. RNA was isolated as described by Chirgwin et al. (4). Briefly, cells were lysed with 4 M guanidium isothiocyanate centrifuged through a gradient of cesium chloride. Pelleted RNA was purified by chloroform: butanol extraction and ethanol precipitation, separated by electrophoresis through an agarose (1.2%)-formaldehyde gel, and transferred to nitrocellulose. The RNA was immobilized on the nitrocellulose by baking for 2 h at 80°C. After prehybridization, RNA was labeled by hybridization to radioactively labeled cDNAs encoding for rat ET-1 (17). After hybridization, nitrocellulose membranes were washed under stringent conditions and exposed to X-ray film.

Radioimmunoassay. Radioimmunoassays were performed using commercially available kits from Peninsula Laboratories. ET-1 and ET-3 were assayed in serum-free media that had been conditioned by SCG cultures for 72 h and in extracts of homogenized adult SCG. ET-1 was also assayed in freeze/thaw cell lysates of cultured SCG and extracts of homogenized adult celiac ganglia. Assays were performed in duplicate.

Immunohistochemistry. Cells were rinsed with 0.1 M phosphate-buffered saline (PBS; 19 mM sodium phosphate monobasic, 81 mM sodium phosphate dibasic, 0.05 M sodium chloride, pH 7.4) and then fixed for 2 h in 4% paraformaldehyde. The cells were then permeabilized (1 h in 0.1 M PBS, 0.2% Triton X-100, 0.9% hydrogen peroxide), blocked (30 min
in 5% normal goat serum), and incubated with primary antibody for 18–24 h at 4°C (ET-1 and ET-3; 1:200) or room temperature (TH; 1:4,000). The primary antibodies would bind specifically to their corresponding antigens. Cells were then washed with 0.1 M PBS and incubated with secondary antibodies for 60 min (ET-1 and ET-3; goat anti-rabbit IgG fluorescein, 1:100) or overnight (TH; donkey anti-rabbit IgG tetramethylrhodamine isothiocyanate, 1:200). The secondary antibodies would only bind to cells that had primary antibody bound to them, and thus only cells expressing ET or TH antigens would exhibit fluorescence associated with the secondary antibodies.

RESULTS

Preproendothelin 1 (prepro-ET-1) mRNA [2.3 kilobase (kb)] is expressed in cultures of rat postganglionic sympathetic neurons (Fig. 1). These neurons were characterized as sympathetic because they survived in NGF and expressed TH, the rate-limiting enzyme in catecholamine synthesis. prepro-ET-1 mRNA was expressed in four independent isolates of SCG. This mRNA has previously been detected in cultures of rat aortic vascular smooth muscle, and thus these cells served as a positive control (Fig. 1). For both vascular smooth muscle and SCG cultures, only a single band, and thus a single size, of prepro-ET-1 mRNA was detected (data not shown). Although mRNA expression was not quantitated, prepro-ET-1 mRNA expression in sympathetic neuronal cultures was approximately equal to that in the vascular smooth muscle cultures. SCG expression of prepro-ET-1 mRNA remained constant for 4–16 days in culture (data not shown).

Immunoreactive ET-1 was detected in cell extracts and in media conditioned by cultured postganglionic sympathetic neurons (Table 1). Immunoreactive ET-1 was also detected in extracts of adult SCG (Table 1) and in an extract of adult celiac ganglia (6.3 pg/mg protein). Immunoreactive ET-3 was detected in media conditioned by cultured postganglionic sympathetic neurons and in extracts of adult SCG (Table 1). Immunoreactive ET-1 was detected in all neurons.

Immunohistochemistry was performed on SCG cultures. Phase contrast microscopy of the SCG cultures indicated the presence of phase-bright neurons as well as other unidentified cells (Fig. 1). Immunoreactive ET-1 was localized in postganglionic sympathetic neuronal cell bodies and in neuronal processes (Fig. 2B). This was also the case for ET-3 (Fig. 3 and data not shown). ET immunoreactivity was detected in all neurons.

DISCUSSION

The present study indicates that ET-1 and ET-3 are synthesized in and secreted by postganglionic sympathetic neurons. This study extends previous reports that ET-1 and ET-3 are expressed by neurons in the brain and spinal cord (6, 10, 22). ET secreted from postganglionic sympathetic neurons may represent a novel mechanism or mechanisms for controlling vascular function. Sympathetic ET could modulate vascular function by acting directly on vascular smooth muscle or by acting indirectly on preganglionic or postganglionic sympathetic neurons.

ET expression was studied in cultures of sympathetic neurons that were generated from enzymatically dissociated SCG of 3- to 4-day-old rat pups. This model is a well-characterized in vitro neuronal system that is routinely used to study many aspects of neuronal function, including neuronal survival and neurotransmitter and neuropeptide expression. Addition of an antimitotic agent produced cultures relatively free of nonneuronal cells. Immunohistochemical labeling with antibodies to ET (Fig. 2) confirmed that the neurons in the cultures were catecholamine synthesizing and thus

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<td>Cultured neurons media, pg/10^6 cells</td>
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Data for the media of cultured neurons are the means ± SE from 3 independently isolated cultures. Cell lysate data are the mean of 2 isolates. Extract data are the means from 2 independent extractions.
sympathetic. Application of acetylcholine to these neurons caused marked increases in intracellular calcium (unpublished observation), indicating that these cells were responsive to an appropriate physiological agonist.

Expression of ET was detected using multiple independent assays. Hybridization of mRNA isolated from SCG cultures with a cDNA specific for rat prepro-ET-1 detected one 2.3-kb band, strongly suggesting that these SCG cultures contained cells that synthesized prepro-ET-1 mRNA. Cell lysates of and media conditioned by SCG cultures contained immunoreactive ET-1 and ET-3. Immunoreactive ET-1 and ET-3 were also detected in homogenates of adult SCG and celiac ganglia, indicating that these peptides are expressed by sympathetic neurons in vivo as well as in vitro. The antibodies used to detect ET-1 and ET-3 in radioimmunoassay exhibited no crossreactivity with each other or with many other neuropeptides [brain natriuretic peptide 26 (porcine), angiotensin II, calcitonin gene-related peptide (human), arginine8-vasopressin, vasoactive intestinal peptide, substance P; Peninsula Laboratories]. It is possible that the ET immunoreactivity we detected in media conditioned by SCG cultures was secreted by nonneuronal cells or was released from neuronal or nonneuronal cells that had died during the period of

Fig. 2. ET-1 is expressed by postganglionic sympathetic neurons. A culture of SCG (phase contrast shown in A) was labeled with an ET-1 primary antibody and fluorescein-labeled secondary antibody (B) and then labeled with a tyrosine hydroxylase primary antibody and a rhodamine-labeled secondary antibody (C). A companion culture (phase contrast shown in D) was also labeled with only secondary antibody (E).
culture. We think this is unlikely. Less than 10% of cells in the SCG cultures in which the secretion of ET was measured were nonneuronal cells, and, thus, if these cells were secreting ET, they would need to be secreting unusually large amounts per cell. Preliminary experiments with these cells indicate that this is not the case. We do not think that dead cells released ET, because there was no evidence of cell death in our cultures. ET-1 and ET-3 were also detected in neurons in SCG culture by immunohistochemical analysis with antibodies from Peninsula Laboratories that were distinct from those used in the radioimmunoassay. In addition, immunohistochemical analysis with an ET-1 antibody from an alternate source (Biogenesis) also detected ET-1 immunoreactivity in sympathetic neurons in SCG culture.

Our data suggest that ET is expressed by neonatal and adult postganglionic sympathetic neurons. Our data do not allow us to quantitatively compare neonatal versus adult neuronal expression of ET. It is possible that ET expression is developmentally regulated and may vary as a function of the age of the animal. This possibility is currently under investigation.

Are ETs neurotransmitters for postganglionic sympathetic neurons? Our data do not allow us to unequivocally answer this question. Figures 2 and 3 indicate that immunoreactive ET-1 and ET-3 are localized in cell bodies and processes of SCG neurons. Without further experimentation, the significance of this localization is unclear, but the data suggest that ETs could be released from cell bodies and/or from nerve terminals. The data
in Table 1 clearly indicate that ET-1 and ET-3 are constitutively secreted from SCG sympathetic neurons, but the site and mechanism of this secretion remain to be elucidated. Preliminary experiments indicate that stimulation of SCG cultures with a nicotinic agonist did not increase release of ET-1 into the media (data not shown), suggesting that ET-1 secretion is not dependent on neuronal activity. Further experiments are in progress to verify this preliminary observation. The work of Shinkai-Goromaru et al. (18), however, suggests that in the rat iris sphincter ET-3 release is regulated by neuronal activity. In many nonneuronal cells there does not appear to be a significant amount of ET-1 stored intracellularly, and alterations in ET secretion are dependent on alterations in ET mRNA. We are currently investigating this potential mechanism of ET regulation.

ETs are potent modulators of cellular function. Minimally effective concentrations of ET-1 and ET-3 range from 10^{-11} to 10^{-9} M (8, 11, 15). The amounts of ET-1 and ET-3 secreted by sympathetic neurons in culture (Table 1) are within this range, assuming a volume of distribution of ≈1 ml. These amounts of ET are also within the range that has been detected in media conditioned by other cell types in culture (3, 14, 21, 23). ET expression is positively and negatively regulated in nonneuronal cells and tissues by many physiological, pharmacological, and pathological mechanisms (3, 13, 20). Regulation of ET expression in sympathetic neurons is currently under investigation.

The present study identifies a novel source of ET. The physiological or pathological significance of this source remains to be determined. However, several lines of evidence suggest multiple roles for sympathetically derived ET in regulating vascular function. If ET is released from postganglionic sympathetic nerve terminals, it could act directly on vascular smooth muscle. ET is a mitogen (2), and thus sympathetic-derived ET could modulate physiological and/or pathological vascular smooth muscle growth. ET is also a vasoconstrictor (20), and thus sympathetic-derived ET could mediate or modulate sympathetic control of vascular diameter. It has been reported that ET modulates the release of neurotransmitter from sympathetic neurons (19). This observation suggests the possibility that ET released from sympathetic neurons could act autocrinely to modulate neurotransmitter release. Intrathecal injection of ET modulates blood pressure (7), indicating that ET modulates the activity of preganglionic sympathetic neurons. ET released from postganglionic sympathetic cell bodies could act at preganglionic sympathetic nerve terminals to modulate the release of neurotransmitter.

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**REFERENCES**


