

Synthesis of calcitonin gene-related peptide (CGRP) by rat arterial endothelial cells

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Summary. We investigated the protein and mRNA expression of calcitonin gene-related peptide (CGRP) in endothelial cells of the rat thoracic aorta and femoral artery. Light microscopic immunocytochemistry revealed that immunoreactivity for CGRP was preferentially located in the endothelium of both vessels. Immunoelectron microscopy showed that CGRP-immunoreactive gold particles were preferentially localized on cisterns of the rough endoplasmic reticulum and on the Weibel-Palade (WP) bodies in the endothelial cells. Prepro CGRP mRNA signals were also detected on the endothelium. Our results are the first to demonstrate that endothelial cells of both elastic and large muscular arteries synthesize CGRP and store it, in part, in WP bodies, implying that CGRP may act as an endothelium-derived relaxing factor in these vessels.

Key words: Calcitonin gene-related peptide, *In situ* hybridization, Immunocytochemistry, Immunoelectron microscopy, Weibel-Palade bodies

Introduction

Calcitonin gene-related peptide (CGRP) is a 37-amino-acid peptide encoded by tissue-specific alternative messenger RNA (mRNA) processing of the calcitonin gene (Amara et al., 1982). Initially isolated from the thyroid, CGRP was subsequently found to be ubiquitously distributed throughout the body and particularly abundant in the central nervous system and nerve fibers associated with blood vessels (Rosenfeld et al., 1983; Mulderry et al., 1985). Recently, Kawasaki et al. (1988) identified a vasodilatory action of CGRP as either neurotransmitter or neuromodulator, independent of noncholinergic nonadrenergic vasomotor nerve endings. By immunocytochemical analyses on endothelial cells of the human umbilical vein and artery, both *in situ* and in culture, Cai et al. (1993a,b)

demonstrated that immunoreactivity for CGRP was localized in regions of the rough endoplasmic reticulum (rER). Ozaka et al. (1997) showed that immunoreactivity for CGRP was observed in cisterns of the rER in endothelial cells of the rat carotid body artery and its branches. These data imply that such endothelial cells can synthesize CGRP. However, to our knowledge, mRNA expression of this peptide in endothelial cells of mammalian vessels has not been studied previously.

Following the proposal of endothelium-dependent vasodilation by Furchgott and Zawadzki (1980) and vasoconstriction by De May and Vanhoutte (1983), extensive pharmacological and biochemical analyses revealed vasomotor functions of endothelium-derived relaxing factors (EDRF) such as nitric oxide (NO) and prostacyclin (Rubanyi, 1991). In addition, endothelium-derived constricting factors (EDCF) such as endothelin (ET)-1, angiotensin-II and thromboxane-A₂ have been identified in a variety of vessels (Drexler and Hornig, 1999; Ruschitzka et al., 1999).

Endothelial cells possess Weibel-Palade (WP) bodies, Golgi apparatus-derived specific granules, in which ET-1 (Sakamoto et al., 1993; Doi et al., 1996; Nomiya et al., 1998; Kayashima et al., 1999) and NO synthase (Fukuda et al., 1995) are stored. Furthermore, Ozaka et al. (1997) demonstrated by the double immunolabeling that both ET-1 and CGRP coexist in the same WP bodies of the rat carotid body artery and its branches, implying that these peptides may be involved in regulating local blood flow through the carotid body. Although the significance of ET-1 as an EDCF is widely accepted (Yanagisawa et al., 1988), more detailed studies combined with *in situ* hybridization are necessary to elucidate whether WP bodies are involved in storage of CGRP in a wide range of vessels. The present study was designed to investigate expressions of CGRP protein and mRNA in rat thoracic aorta and femoral artery, in which endothelial cells possess many WP bodies.

Materials and methods

Animals

Male adult Wistar rats weighing 280±20 g were used

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for the present study. We followed the Guiding Principles for the Care and Use of Animals approved by the University of Occupational and Environmental Health in accordance with the principles of the declaration of Helsinki (1983) for the care and use of animals. Animals were deeply anesthetized with an intraperitoneal injection of pentobarbital (50 mg/kg). The thoracic aorta and femoral artery were isolated after perfusion with a physiological saline followed by a solution of 2% paraformaldehyde (PFA) in 0.1M phosphate buffer via the left ventricle for 5 min each.

Tissue preparation

For light microscopy, specimens were fixed in a solution of 4% PFA in 0.1M phosphate buffer for two days at 4 °C. For conventional electron microscopy, specimens were fixed in a mixture of 2% PFA and 2.5% glutaraldehyde in 0.1M phosphate buffer for 2 hr at 4 °C, postfixed in 1% osmium tetroxide in the same buffer for 2 hr at 4 °C, dehydrated in ethanol, and embedded in epoxy resin. Ultrathin sections were prepared using an ultramicrotome (LKB 2128), were stained with saturated uranyl acetate and lead citrate, and examined using a JEM 1200 EX electron microscope.

Light microscopic immunocytochemistry

Paraffin-embedded sections (approximately 5- μ m thick) were deparaffinized, rinsed briefly in 0.1M phosphate-buffered saline (PBS), digested with 0.4% pepsin in distilled water containing 0.01N HCl, treated with 0.3% H₂O₂ in absolute methanol to block endogenous peroxidase activity, and thoroughly washed in 0.1M PBS. Sections were incubated in a humid chamber with rabbit anti-rat CGRP polyclonal (whole serum) antibody (Sigma-Genosys, The Woodlands, TX) diluted to 1:200 with 0.1% bovine serum albumin (BSA) in 0.1M PBS for 18 hr at 4 °C. After rinsing in 0.1M PBS, sections were reacted using the indirect immunoperoxidase method (Histfine Simple Stain PO Kit, Nichirei, Tokyo, Japan). The peroxidase complex was visualized by treatment with a freshly prepared diaminobenzidine tetrahydrochloride (DAB; 0.1 mg/ml) solution with 0.01% H₂O₂ for 5 min. The specificity of the immunostaining was confirmed by substituting the primary antibodies for normal rabbit serum or PBS.

Immunoelectron microscopy

For the post-embedding method, specimens were immersed in a periodate-lysine-PFA solution (McLean and Nakane, 1974) for 6 hr at 4 °C, dehydrated in ethanol, and embedded in epoxy resin. After non-specific binding was blocked with 1% BSA in 0.1M PBS, ultrathin sections of gold interference colors were immunolabeled with rabbit anti-rat CGRP antibody at a dilution of 1:800 to 1:1600 in 1% BSA in 0.1M PBS for 4 hr at room temperature. Sections were washed in 0.1M

PBS, blocked in 1% BSA in 0.1M PBS for 20 min, incubated with goat anti-rabbit IgG-coated 15 nm colloidal gold (Ultra Biosols, Liverpool, UK) with a dilution of 1:100 in 0.1% BSA in 0.1M PBS for 1 hr at room temperature, and then rinsed in 0.1M PBS. The specificity of the immunostaining was confirmed by substituting the primary antibodies for normal rabbit serum or PBS.

In situ hybridization

Deparaffinized and hydrated sections (approximately 5- μ m thick) were blocked with 0.1% H₂O₂ in methanol for 20 min to destroy endogenous peroxidase, digested with 1 μ g/ml of proteinase K in 10 mM Tris-HCl (pH 8.0), 1 mM ethylenediamine- N,N,N',N'-tetraacetic acid (EDTA) for 5 min, fixed in 4% PFA at room temperature for 10 min, immersed in 0.2M HCl for 10 min to remove basic substances and then acetylated with 0.25% acetic anhydride, 0.1M triethanolamine-HCl (pH 8.0) for 10 min. Subsequently, the sections were dehydrated and air-dried at room temperature for 30 min. Sections were preincubated with a hybridization buffer containing 20 mM Tris-HCl (pH 7.5), 0.9M NaCl, 6 mM EDTA, 1X Denhardt's reagent (0.02% Ficoll, 0.02% polyvinyl pyrrolidone, and 0.02% BSA), at room temperature for 1 hr in a humid chamber. The oligonucleotides for prepro CGRP mRNA used in this *in situ* hybridization study were labeled at the 3' tail with digoxigenin (DIG)-11-dUTP using a DIG Oligonucleotide Tailing Kit (Boehringer Mannheim, Mannheim, Germany). The sequence of the antisense oligonucleotide probe (5'-AAGCCTGCCAGCCGATGGGTCACGCAGGTGGCAGTGTTCAGGATCTCTT-3'; 50 mer) was determined from the sequence of the cDNA encoding prepro CGRP and synthesized by Takara Custom DNA Services (Takara Shuzo, Kyoto, Japan). The probe was diluted in the hybridization buffer to which 10% dextran sulfate had been added, and 10 ng probe/100 μ l was applied to each glass slide. Parafilm M (American National Can, Greenwich, CT) was placed over the sections, and the sections were incubated overnight at 45 °C in a humid chamber. After removing the coverslips in 2x saline-sodium citrate (SCC; 1x SCC; 150 mM NaCl, 15 mM sodium citrate; pH 7.0) at room temperature, the sections were washed in 2x SCC at room temperature for 20 min, twice in 1x SCC at 45 °C for 30 min each, and in 2x SCC at room temperature for 20 min. They were then immunoreacted with peroxidase-conjugated anti-DIG antibody (Boehringer Mannheim, Mannheim, Germany). After rinsing with 0.1M Tris-HCl (pH 7.5), 0.15 M NaCl, and 0.05% Tween 20, the peroxidase complex was amplified by Tyramide Signal Amplification Kit (DuPont NEN, Boston, MA) and visualized by treatment with a freshly prepared DAB (0.1 mg/ml) solution with 0.01% H₂O₂ for 5 min at room temperature. After dehydration, sections were counterstained with methylgreen, and then mounted with cover slips by Entellan neu (Merck, Darmstadt, Germany).

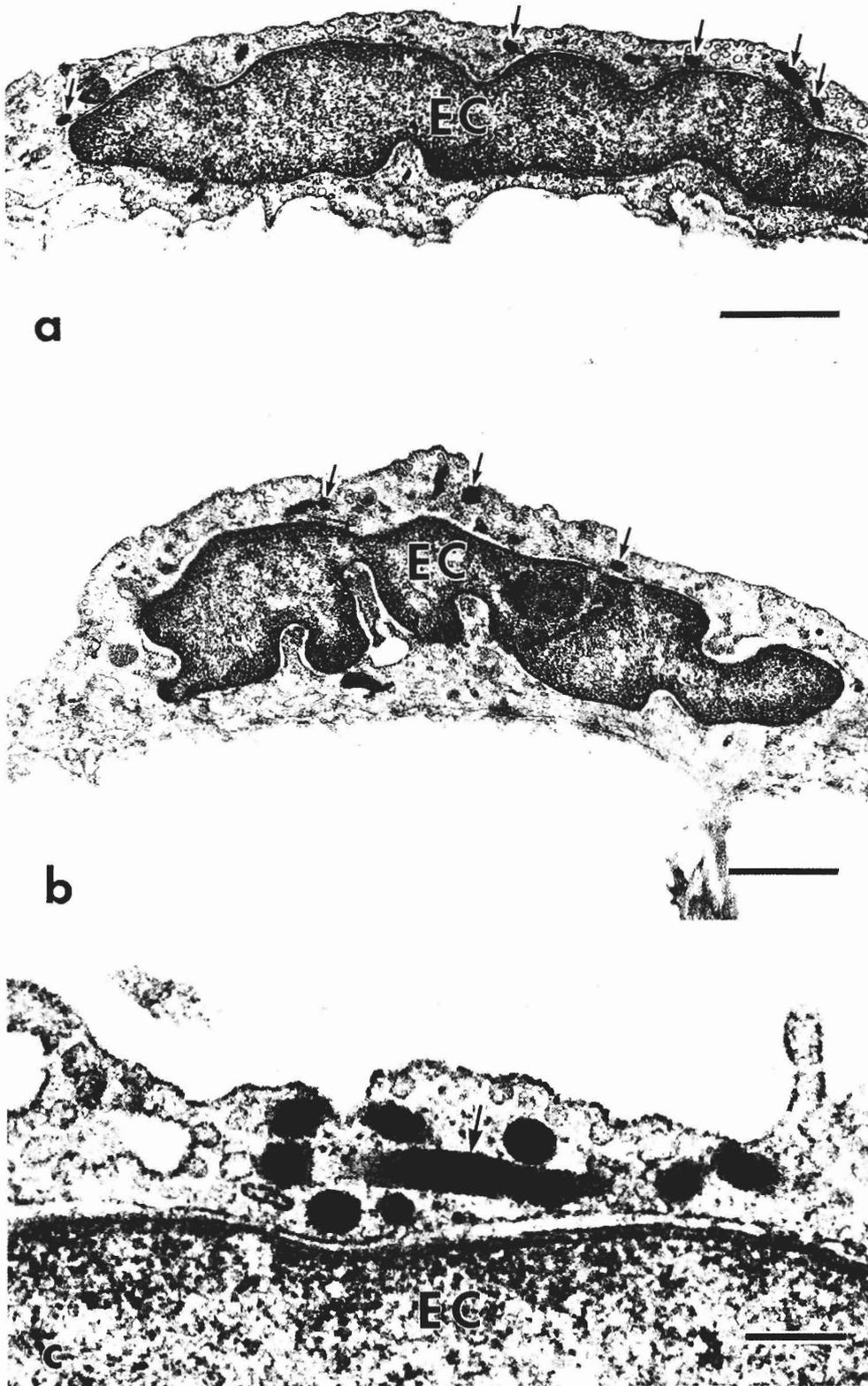


Fig. 1. Endothelial cells (EC) of the thoracic aorta (**a, c**) and femoral artery (**b**) contain a considerable number of WP bodies (arrows). WP bodies are round or oval in shape but occasionally elongated (arrow in **c**) containing crystalloid structures in their interior. Bars: 1 μm (in **a** and **b**), 0.2 μm (in **c**).

As a control, sense probe (5'-AAGAGATCCTGCA ACACTGCCACCTGCGTGACCCATCGGCTGGCAG GCTT-3'; 50 mer) was substituted for the antisense probe, and sections adjacent to those for *in situ* hybridization were immunostained for CGRP.

Results

Electron microscopy

Figure 1 shows WP bodies in the nuclear region of endothelial cells of the thoracic aorta (Fig. 1a,c) and femoral artery (Fig. 1b). Elongated WP bodies occasionally included crystalloid structures in their interior (Fig. 1c).

Immunocytochemistry of CGRP

Light microscopic immunoreactivity for CGRP was

preferentially observed in the endothelium of the thoracic aorta (Fig. 2a) and femoral artery (Fig. 2c). Negative controls, in which the primary antibody was substituted by normal rabbit serum, exhibited little or no immunoreactivity in either vessels (Fig. 2b,d).

Immunoelectron micrographs showed preferential localization of CGRP-immunoreactive gold particles on cisterns of the rER and WP bodies in endothelial cells of the thoracic aorta (Fig. 3a) and femoral artery (Fig. 3b). Negative controls exhibited few or no gold particles on the above sites.

In situ hybridization

Prepro CGRP mRNA signals were detected using the antisense probe on the endothelium of the thoracic aorta (Fig. 4a) and femoral artery (Fig. 4c). *In situ* hybridization using the sense probe elicited no

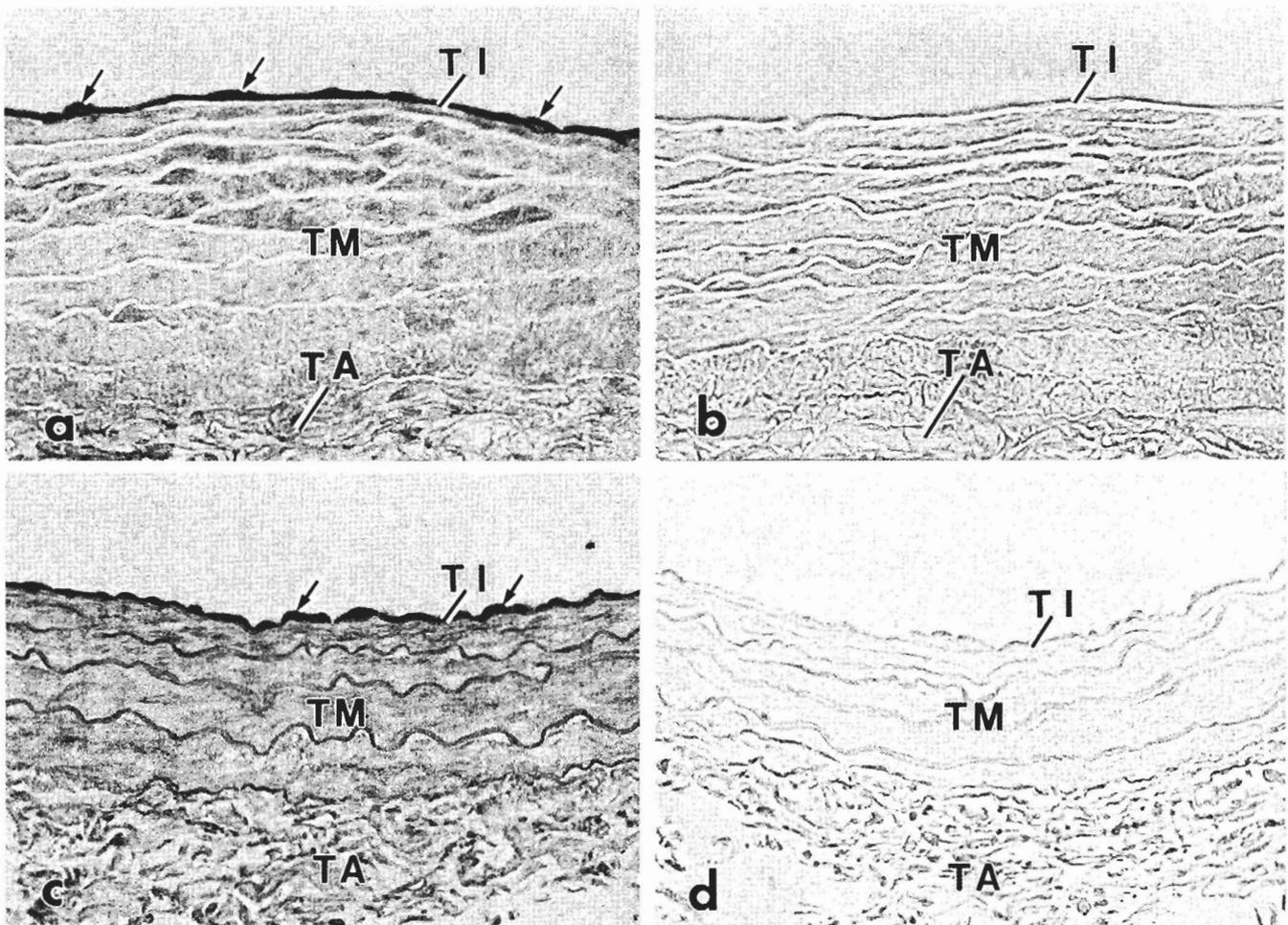


Fig. 2. Immunoreactivity for CGRP (arrows) is preferentially seen along the endothelium of the thoracic aorta (a) and femoral artery (c). Negative controls exhibit little or no immunoreactivity in the thoracic aorta (b) and femoral artery (d). TI: tunica intima; TM: tunica media; TA: tunica adventitia. x 1,200

detectable signals in either vessels (Fig. 4b,d).

Discussion

Besides reports on immunoreactivity for CGRP in endothelial cells of the human umbilical vein and artery (Cai et al., 1993) and of the rat carotid body artery (Ozaka et al., 1997), the present study revealed that endothelial cells of both elastic and large muscular arteries synthesize CGRP and store it, in part, in WP bodies. Since the first description of WP bodies in the rat

small artery by Weibel and Palade (1964), their nature and function have been analyzed by biochemical, physiological and immunocytochemical approaches using various vessels. Among their roles, these inclusions are a storage site for von Willebrand factor (Ewenstein et al., 1987; Kagawa and Fujimoto, 1987), tissue-type plasminogen activator (Rosnoblet et al., 1999), histamine (Fujimoto et al., 1982; Doi et al., 1995), ET-1 (Sakamoto et al., 1993; Doi et al., 1996; Nomiya et al., 1998; Kayashima et al., 1999), ET

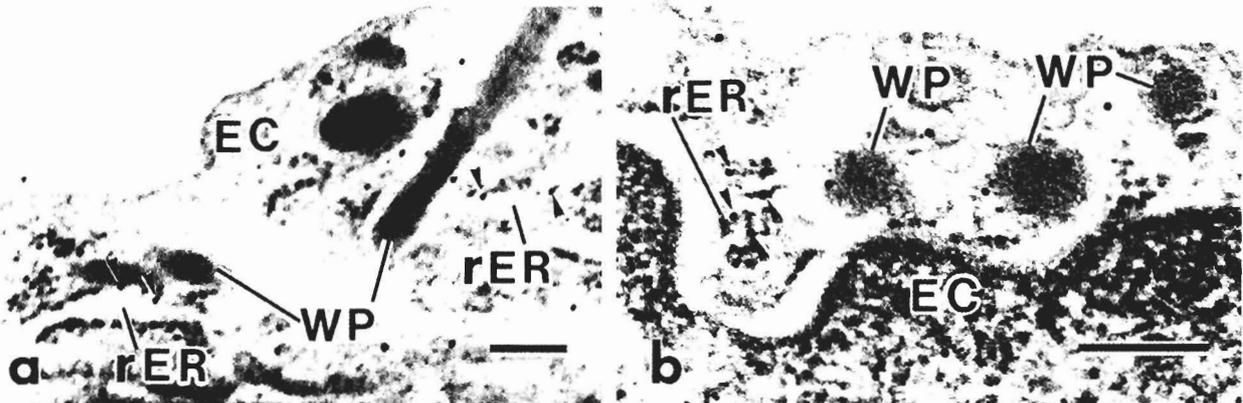


Fig. 3. By immunoelectron microscopy, CGRP-immunoreactive gold particles are preferentially localized on cisterns of the rER (arrowheads) and on WP bodies (WP) in endothelial cells (EC) of the thoracic aorta (a) and femoral artery (b). Bars: 0.2 μ m.

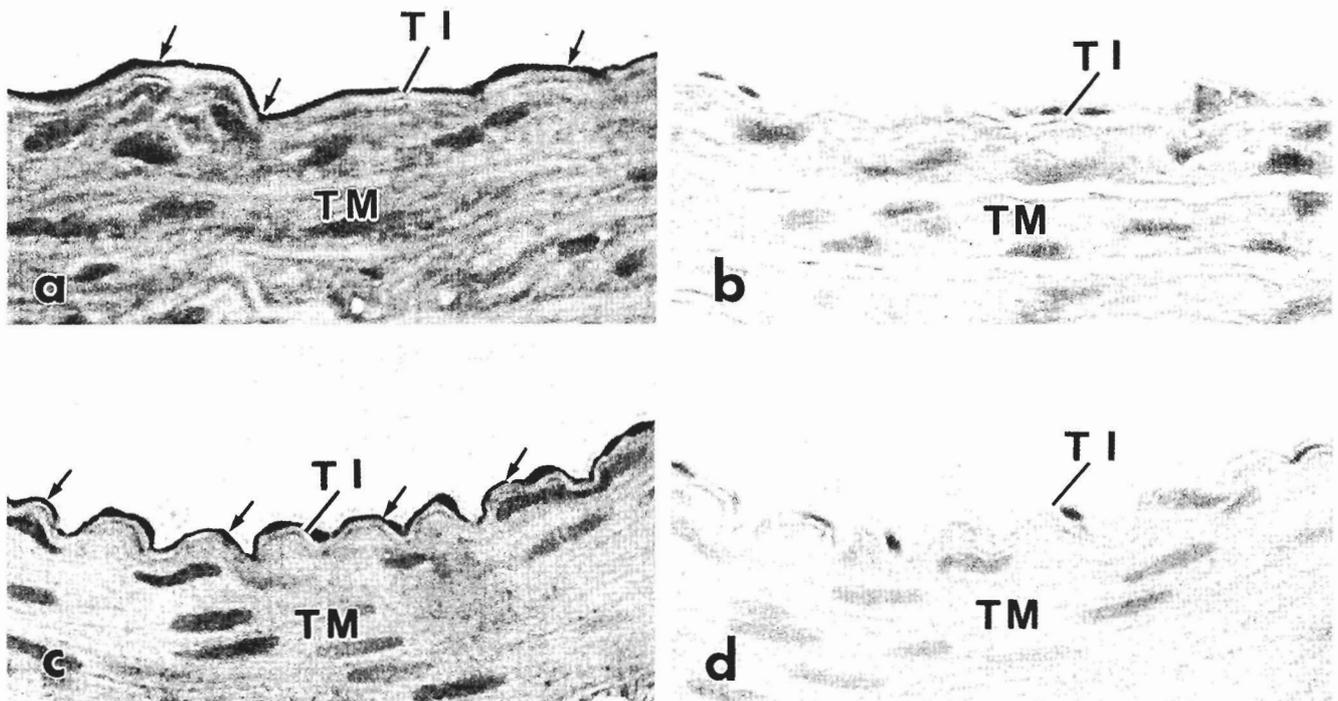


Fig. 4. Prepro CGRP mRNA signals (arrows) are detected along the endothelium of the thoracic aorta (a) and femoral artery (c). Hybridization using sense probe shows no detectable signals in the thoracic aorta (b) or femoral artery (d). TI: tunica intima; TM: tunica media. x 1,500

converting enzyme (Russell et al., 1998), and NO synthase (Fukuda et al., 1995). In addition, co-existence of histamine and von Willebrand factor (Ueda et al., 1992) and that of ET-1 and CGRP (Ozaka et al., 1997) in the same WP bodies were reported using double immunolabeling techniques.

Since the contents of WP bodies are extracellularly released by diacrine and/or exocytosis into both vascular lumen and subendothelial layer under normal and pathological conditions (Fujimoto, 1982; Doi et al., 1996), it is reasonable to consider that CGRP, which is stored in WP bodies and extracellularly released from these inclusions, may play a crucial role in endothelium-dependent vasodilatation. Saiag et al. (1999) provided pharmacological data which suggest that hypoxic-induced vasodilatation of the rat thoracic aorta is partially caused by certain EDRFs other than NO. Thus, CGRP may be one of the candidates. Further studies are necessary to confirm the vasodilative role of CGRP derived from WP bodies under hypoxic conditions.

Acknowledgements. The authors thank Dr. Takatoshi Ozaka for his participation in the early phase of this project. They also thank Ms. Toyono Nobukuni for typing the manuscript and Mr. Mitsuru Yokoyama for his photographic work. This work was supported in part by a Grant-in-Aid (number 11670029) for Scientific Research from the Ministry of Education, Science and Culture, Japan.

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Accepted June 18, 2001