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Shear stress induces expression of CNP gene in human endothelial cells

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Abstract To elucidate the effect of blood flow on gene transcription of C-type natriuretic peptide (CNP), human umbilical vein endothelial cells were exposed to shear stress in a cone-plate viscometer. Expression of CNP mRNA, evaluated by reverse transcription-polymerase chain reaction, was markedly increased by exposure to shear stress of 24 dyne/cm^2 at 3 h. The CNP mRNA level was maintained until 12 h. Thus, the present study demonstrated for the first time that shear stress induces expression of CNP gene in human endothelial cells.

Key words: Shear stress; C-type natriuretic peptide; Human endothelial cell; mRNA

1. Introduction

C-type natriuretic peptide (CNP), a new member of the natriuretic peptide family, was identified in the porcine brain [1]. In contrast to other natriuretic peptides; ANP and BNP, CNP has not been detected in the heart and blood, and was believed to be a neuropeptide [2]. Recently, CNP has been identified in endothelial cells and recognized to be a vascular local mediator [3,4]. Natriuretic peptide family has at least three receptors, namely ANP-A receptor, ANP-B receptor and clearance receptor. ANP-A receptor and ANP-B receptor possess an intracellular guanylate cyclase domain [5]. Accordingly, natriuretic peptides increase intracellular cGMP via ANP-A receptor and ANP-B receptor. ANP and BNP activate selectively ANP-A receptor, whereas CNP potently and selectively activates the ANP-B receptor [1,6]. ANP-A receptor and ANP-B receptor are both expressed in intact aortic media. However, in cultured smooth muscle cells that are known to be 'synthetic phenotype', ANP-A receptor becomes undetectable and ANP-B receptor becomes to be expressed dominantly [7]. Synthetic smooth muscle cells are major pathological contents of atherosclerosis and neointimal lesion. Thus, CNP potentially plays an important role in vascular remodeling. Actually, Furuya et al. reported that CNP inhibits proliferation of cultured rat vascular smooth muscle cells and that administration of CNP inhibits the development of intimal lesions induced by air-drying injury in rat carotid arteries [8,9]. The endothelial production of CNP is reported to be augmented by cytokines such as TGF-β [3]. Recently, several studies indicated that mRNA expression of several peptides, that are synthesized in endothelial cell, are regulated by shear stress [10–12]. In the present study, to examine the effect of shear stress on CNP gene expression, we have evaluated the presence of mRNA of CNP by reverse transcription-polymerase chain reaction (RT-PCR), in human endothelial cells exposed to a various degree of shear stress.

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Abbreviations: CNP, C-type natriuretic peptide; RT-PCR, reverse transcription-polymerase chain reaction; ANP, atrium natriuretic peptide; BNP, brain natriuretic peptide; cGMP, guanosine 3′:5′-cyclic monophosphate; TGFβ, transforming growth factor-beta; HUVEC, human umbilical vein endothelial cell; FBS, fetal bovine serum; FGF, fibroblast growth factor; PBS, phosphate buffered saline; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; SDS, sodium dodecyl sulfate; ICAM, intracellular adhesion molecule; cNOS, constitutive nitric oxide synthase; SSRE, shear stress responsive element.

2. Materials and methods

2.1. Human umbilical vein endothelial cell culture

Human umbilical vein endothelial cells (HUVEC) were isolated from the umbilical vein as described previously [13]. The cells were maintained in MCDB131 (Chlorella Inc., Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin and 10 μg/ml human recombinant basic FGF (Dainippon Pharmaceutical Co., LTD., Osaka, Japan). Cells were subcultured, and the identification was performed by immunocytochemistry using anti-factor VIII antibody. The purity of these cells was >97%.

2.2. Shear stress loading apparatus

We used a shear stress loading apparatus (Toray Industries, Inc., Tokyo, Japan) that allowed us to expose cultured HUVEC to a well-defined laminar fluid flow. Our system is modified from the cone-plate viscometer device first described and characterized by Bussolari and Dewey [14]. The shear stress device consists of a cone that rotates above a stationary base plate containing the cultured HUVEC. The base plate is made by the 35-mm-diameter collagen coated polystyrene dish (Iwaki Glass Company, LTD., Tokyo, Japan). The cone makes an angle of 0.5° with the culture plate and is coupled to a variable motor. The distance between the cone and the culture dish was adjusted to be 50 μm. This shear stress device was operated in CO_2 incubator.

2.3. Experimental protocol

Confluent HUVEC on 35-mm-diameter collagen coated polystyrene dish was washed with PBS for three times and added 1 ml of culture media containing 10% FBS. The plate was placed in CO_2 incubator for 30 min before exposure to shear stress. The culture dish was then placed in the cone-plate viscometer, and the cells were subjected to shear stress from 3 to 24 dyne/cm^2 for 1, 3, 6 or 12 h. The culture dish was then detached from the device, and the medium was discarded. For RNA isolation, 1 ml of Isogen (Nippon Gene Co., Ltd. Toyama, Japan) was added to the cells immediately, and the lysate was prepared. Stationary samples were obtained with the same procedure except shear stress exposure.

2.4. RNA isolation

Total RNA was isolated according to the manufacture's protocol of Isogen [15]. After isolation, total RNA samples were checked by gel electrophoresis in a 0.8% agarose gel (Molecular Grade Agarose, Bio-Rad, Richmond USA) stained by ethidium bromide. The concentrations of total RNA were then calculated by spectrophotometric measurements at 260 nm wavelength.

2.5. Reverse transcription-polymerase chain reaction

Semiquantative assessment of CNP transcript was performed as fol-

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lows. First strand cDNA was synthesized in 20 μl of 0.5 mM of dATP, dGTP, dTTP and dCTP, 2.5 ng/μl random hexamers, 50 mM KCl, 20mM Tris-HCl, pH 8.4, 2.5 mM $MgCl_2$, and 10 unit of SuperScript II RT (SUPERSCRIPT Preamplification System, Gibco, Gaithersburg, USA) at 42°C for 50 min using as template 1 μg aliquots of total RNA. One μg of cDNA synthesis product was amplified in a thermal cycler with Taq polymerase 2.5 U (Gibco) with 1 μM of forward/reverse primer for CNP (5'-ATGCATCTCTCCCAGCTGCT/3'- TACTCGCCGGACCCTACAAT) and for GAPDH. Primers for GAPDH is synthesized as previously described [16]. The expected amplified products were 380 and 379 bp, respectively. After a 3 min 94°C denudation step, samples were subjected to 40 cycles of denudation (94°C for 1 min), annealing (CNP, 55°C; GAPDH, 48°C for 1 min) and extension (72°C for 1 min). Then 2.5 μl of PCR products was electrophoresed in 2% agarose gels containing ethidium bromide and examined under UV light. And then PCR products of CNP were transfered from agarose gels to Nylon membranes (Hybond-N+, Amersham, Buckinghamshire, UK) and hybridized γ^{32}P-labelled mid-region oligonucleotide (5'-GCCGAAGGTCCCGC) corresponding to CNP nucleotide 392–398 and 843–849, in 7% polyethylene glycol and 10% SDS at 37°C overnight. The blots were washed in 2× SSC at room temperature for 10 min 3 times. The blots were exposed to X-ray film (X- OMAT AR, Kodak, Rochester, USA) for 3 h at −80°C.

3. Results

3.1. Induction of CNP mRNA expression in HUVEC by shear stress

Expression of CNP mRNA was not detected in the stationary HUVEC. However, an exposure of HUVEC to a steady laminar fluid shear stress (magnitude, 20 dyne/cm^2) caused a marked increase in expression of CNP mRNA. Expected 380 bp products were detected from 3 h to 12 h after onset of flow (Fig. 1). The degree of CNP mRNA expression per GAPDH measured by densitometry increased in a time dependent manner (57.8%/3 h, 84.8%/6 h, 100%/12 h).

The expression of CNP mRNA was not detected in HUVEC which were subjected to a low grade of shear stress (0 to 3 dyne/cm^2) for the period of 12 h. However, a higher shear stress exposure (12 and 24 dyne/cm^2) to HUVEC markedly increased the expression of CNP mRNA (Fig. 2). GAPDH expression was not altered by any of the shear stress examined in this study, as shown in the figures.

4. Discussion

Shear stress is a tractive force that acts in the direction of blood flow on the surface of the blood vessel and endothelial cells are always exposed to shear stress. Endothelial cells were once thought to be a passive, non-thrombogenic barrier. However, endothelial cells are known to have mechanoreceptors by

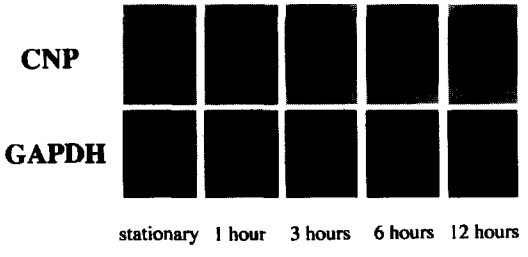

Fig. 1. CNP and GAPDH gene expression in HUVEC exposure to shear stress (24 dyne/cm^2) for 1, 3, 6, 12 h. PCR products of CNP were hybridized to γ^{32}P-labelled mid-region oligonucleotide.

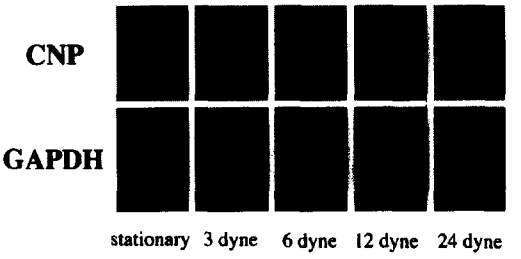

Fig. 2. Southern RT-PCR analysis of CNP mRNA in HUVEC subjected to shear stresses of 3 to 24 dyne/cm^2 for 12 h.

which changes in blood flow or shear stress are recognized by the endothelial cell and the signal is transmitted to intracellular organelles [17]. Recently, several studies clarified that shear stress exerts an influence on the expression of mRNA such as tissue plasminogen activator, ICAM-1, TGF-β1 and constitutive nitric oxide synthase (cNOS) mRNA [10–12,18]. Promoter analysis of these substance revealed a specific base arrangement; 'GAGACC' or 'GGTCTC', shear stress responsive element (SSRE), exists in the promoter region of these gene [19]. Several in vivo investigations have revealed that high shear stress decreases wall thickening in diseased arteries and vascular grafts [20,21]. The elevated expressions of TGF-β and cNOS may have been contributed to the suppression of neointima in response to hemodynamic force.

CNP has a less potent of vasodilatation, but has a magnitude more potent of growth suppression of vascular smooth muscle cells than ANP [1]. We hypothesized that the expression of CNP mRNA is regulated by shear stress and evaluated the expression of CNP mRNA by RT-PCR. It has been reported that CNP mRNA was detectable in cultured bovine aortic endothelial cell [3]. However, CNP or CNP mRNA has not been identified in human endothelial cells. In our experiments, CNP mRNA was not detected in the stationary HUVEC. However, exposure of 24 dyne/cm^2 shear stress to HUVEC clearly increased CNP mRNA within 3 h. Shear stress of 12 dyne/cm^2 for 12 h also induced the marked expression of CNP mRNA in HUVEC. Thus, the present study demonstrated for the first time that shear stress induces expression of CNP gene in human endothelial cells. As physiological arterial shear stress is reported to be approximately 20 dyne/cm^2 [17], the present findings implicate that shear stress might be a major regulatory factor of in vivo CNP production in endothelial cells. Considering the previous reports on TGF-β [3], there was a possibility that the expression of CNP in the present study may be augumented by TGF-β, possibly up-regulated by shear stress. However, the expression of CNP mRNA was not detected by stimulating the same HUVEC by TGF-β (100 pM,1 nM; 6 h) (data not shown), indicating that shear stress directly induced the expression of CNP mRNA. Regarding SSRE considered to be essential for shear-induced transcription, Ogawa et al. has recently identified the element in the second coding region and the 3'-untranslated region of CNP gene in mice [22]. The element has been also identified in the 3'-untranslated region of human CNP gene (personal communication with Dr. Shouji Tanaka of Suntory Research Institute, Kyoto, Japan). Changes in shear stress have been implicated in various clinical settings including atherosclerosis. Therefore, CNP which suppresses smooth muscle cell proliferation, especially of synthetic pheno-

types, might be one of the mechanisms of shear stress-regulating vascular remodeling.

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