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Shear Stress Down-Regulates Gene Transcription and Production of Adrenomedullin in Human Aortic Endothelial Cells

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Abstract Vascular endothelial cells are potent modulators of vascular tone in response to shear stress. Levels of vasoactive peptides such as adrenomedullin (AM), endothelin-1 (ET-1), C-type natriuretic peptide (CNP), and nitric oxide (NO) are affected by fluid shear stress. AM, a potent vasodilator and suppressor of smooth muscle cell proliferation, contains the shear stress responsive element (SSRE) ''GAGACC'' in its promoter region. To examine the role of AM in the shear stress response, cultured human aortic endothelial cells (HAoECs) were exposed to fluid shear stresses of 12 and 24 dynes/cm^2 in a cone-plate shear stress loading apparatus for various time periods, and the levels of AM gene expression and peptide secretion from HAoECs were measured by Northern blotting analysis and radioimmunoassay (RIA), respectively. Both AM gene transcription and AM peptide levels were down-regulated by fluid shear stress in a time- and magnitude-dependent manner. Our results demonstrate that the normal level of arterial shear stress down-regulates AM expression in HAoECs, suggesting that AM participates in the modulation of vascular tone by fluid shear stress. J. Cell. Biochem. 71:109–115, 1998.

Key words: fluid shear stress; adrenomedullin; endothelial cell; SSRE

Adrenomedullin (AM), a member of the calcitonin gene-related peptide superfamily, is a 52-amino acid peptide first isolated from human pheochromocytoma tissue [Kitamura et al., 1993a]. This peptide elicits a potent vasorelaxant response and suppresses proliferation of vascular smooth muscle cells [Ishiyama et al., 1993; Nuki et al. 1993; Kano et al., 1996]. AM is synthesized by endothelial cells and secreted in rat endothelial cells at a rate 60% that of ET-1 [Sugo et al., 1994]. It is known that chronic blood flow reduction causes a decrease in aortic diameter and alters the structure of the arterial wall. Langille and O'Donnell [1986] hypothesized that the endothelium plays a critical role as the vascular mechanotransducer, mediating the effect of shear stress on vascular remodeling. It has been suggested that wall shear stress in the vascular system is maintained at an optimal level by autoregulatory modulation of vascular structure [Kamiya et al., 1980; Zarins et al., 1987], and that this phenomenon is caused by the release of endothelium-derived mediators [Pohl et al., 1986; Rubanyi et al., 1993; Cooke et al., 1991]. Recently, several studies showed that endothelial cells express several vasoactive peptides, such as platelet-derived growth factor B chain (PDGF-B), transforming growth factor β (TGF-β), ET-1, thrombomodullin, tissue-type plasminogen activator (t-PA), and CNP that are regulated by shear stress [Ohno et al., 1995; Nishida et al., 1992; Tsuboi et al., 1995; Okahara et al., 1993]. Resnick et al. [1993] demonstrated that the SSRE core sequence, "GAGACC," is present in the promoter region of PDGF-B. This sequence is also found in the promoter regions of other endothelial cell-derived peptide genes, including AM. The aim of this study is to clarify the effect of fluid shear stress on AM gene transcription and peptide production in HAoEC.

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METHODS

Cell Culture

HAoECs were purchased from Clonetics Corp. (Walkersville, MD). The cells were maintained in BulletKit-endothelial cell growth medium (Clonetics Corp., Walkersville, MD) supplemented with 2% fetal bovine serum (FBS), 50 μg/ml gentamicin sulfate, 50 ng/ml amphotericin-B, 12 μg/ml bovine brain extract, 1 μg/ml hydrocortisone, and 10 ng/ml human recombinant epidermal growth factor. Cells were cultured in a CO_2 incubator in the presence of 95% air and 5% CO_2 at 37°C.

Shear Stress Loading Apparatus

We used a shear stress loading apparatus to expose cultured HAoECs to a well-defined fluid shear stress. We employed the cone-plate viscometer originally characterized by Bussolari and Dewey [1982] with some modifications. Briefly, the shear stress device consisted of a cone that rotated above a stationary base plate that contained the cultured HAoECs. The base plate was a 35-mm-diameter type I collagen-coated polystyrene dish (Iwaki Glass Company, Ltd., Tokyo, Japan). The cone, coupled to a variable speed motor, made an angle of 1° with the culture plate. The distance between the cone and the culture dish was adjusted to 50 μm. The shear stress device was operated in a CO_2 incubator at 37°C.

Exposure of HAoECs to Fluid Shear Stress

Confluent HAoECs were washed with phosphate-buffered saline (PBS) and 1 ml of culture media containing 2% FBS. Plates were incubated in a CO_2 incubator for 30 min before being placed in the shear stress apparatus, and the cells were subjected to fluid shear stresses of 12 or 24 dynes/cm^2 for 3, 6, 12, and 24 h. The culture dishes were then removed from the device, and the media were collected. For RNA extraction, cells were washed with PBS, and then 0.8 ml of ISOGEN (Nippon Gene Co., Ltd., Toyama, Japan) was added [Chomczynski et al., 1993]. Control samples were obtained with the same procedures except for the absence of fluid shear stress exposure.

Northern Blot Analysis

Total RNAs from HAoECs, exposed and unexposed to fluid shear stress, were isolated using the method of Chomczynski and Sacchi [1987] with ISOGEN (Nippon Gene Co.). RNA concentrations were determined by measuring absorbance at 260 nm. RNA (5 μg/lane) was electrophoresed on 1% agarose gels and transferred to nylon membranes (Hybond N$^+$, Amersham International, Buckinghamshire, England). Full-length human AM cDNA (1449 bp), radiolabeled by random primer synthesis (Megaprime DNA labeling system, Amersham International) according to the manufacturer's instructions, was used to probe Northern blots [Kitamura et al., 1993b]. After hybridization [Church et al., 1984], filters were washed twice in 2× saline-sodium citrate (SSC) for 15 min, 1× SSC for 60 min, and 0.5× SSC for 30 min at 65°C, and exposed to Kodak XAR-5 film (Eastman Kodak, Rochester, NY) at −80°C for 48 h. Intensities of AM mRNA bands were determined by densitometric analysis using the MCID system (Imaging Research Inc., St. Catherines, Ontario, Canada), and band intensities were normalized according to β-actin control bands.

Radioimmunoassay for AM

Details of the preparation and characterization of antiserum #172-CI-7 against human AM[40–52] and its use in radioimmunoassay (RIA) have been described previously [Sakata et al., 1994]. The antiserum specifically recognizes the C-terminal amide structure of human AM. The N-Tyr derivative of human AM[40–52] was radioiodinated by the lactoperoxidase method, the monoiodinated form was isolated by reverse-phase high-performance liquid chromatography (RP-HPLC) and used as a tracer.

RESULTS

HAoECs subjected to fluid shear stress of 24 dynes/cm^2 for 0, 6, or 24 h were examined by phase-contrast microscopy (Fig. 1). There were no visible morphological changes in the cells after 3 h of exposure to fluid shear stress. After 6 h of exposure, cells changed from a polygonal, flat morphology to a spindle-like shape aligned with the direction of flow. This was also observed after 24 h of exposure. The cells began to detach from the culture dish after 24 h of exposure to fluid shear stress.

AM mRNA levels in HAoECs exposed to fluid shear stress were examined by Northern blot analysis. RNA was extracted from HAoECs after exposure to fluid shear stress of 24 dynes/cm^2 for 0, 3, 6, and 12 h. Northern blots demonstrated that fluid shear stress significantly

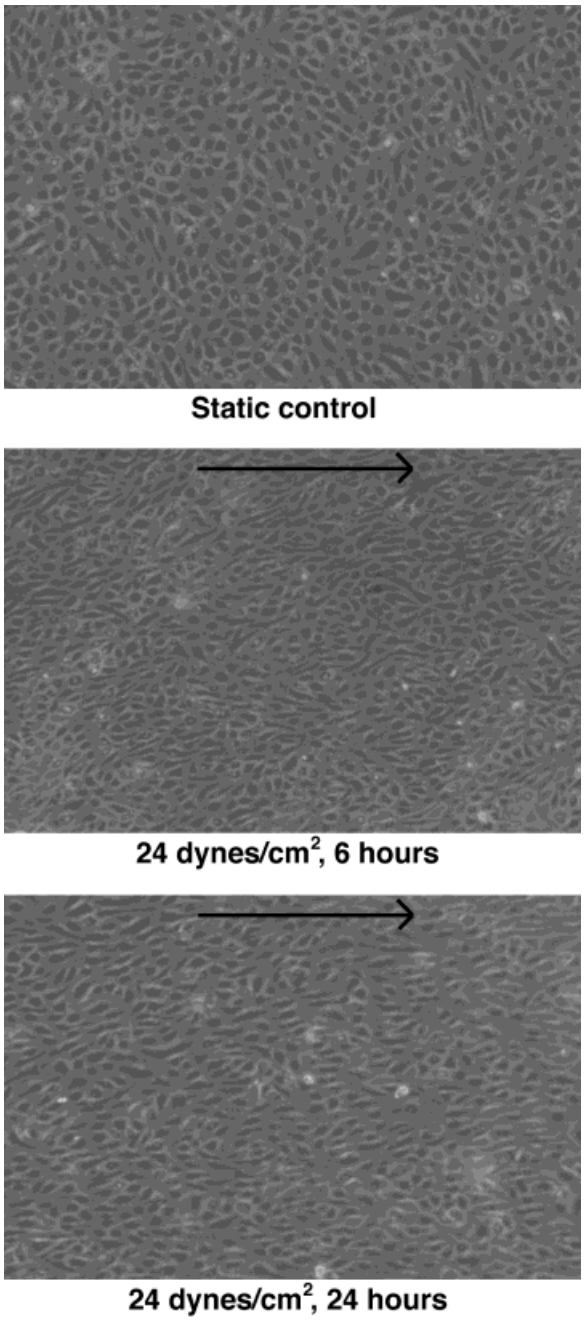

Fig. 1. Phase-contrast micrographs (×40): Changes in cell morphology and alignment in human aortic endothelial cells (HAoECs) exposed to fluid shear stress. Under static conditions, HAoECs exhibited no preferred orientation (top). HAoECs exposed to fluid shear stress for 6 h at 24 dynes/cm^2 showed little change in shape or alignment (middle). After 24 h, changes in morphology were observed in all cells (bottom). Direction of fluid shear stress is from left to right.

reduced AM mRNA levels after 3 h (Fig. 2). Northern blotting analysis of AM mRNA from untreated (control) HAoECs showed no significant change in mRNA levels (Fig. 2). Next, HAoECs were then exposed to several magnitudes of fluid shear stress (0, 12, 24 dynes/cm^2) for 6 h. The level of AM mRNA decreased in a magnitude-dependent manner (Fig. 3).

The amount of AM secreted from HAoECs after 6-h exposures to fluid shear stresses of 0, 12, and 24 dynes/cm^2 was assessed by RIA. Extracellular AM peptide levels decreased in a magnitude-dependent manner (Fig. 4). Thus, consistent with AM mRNA levels, AM peptide release from HAoECs was down-regulated by fluid shear stress.

DISCUSSION

Endothelial cells play a critical role in controlling vessel structure and function in response to acute and chronic alterations in blood flow [Pohl et al., 1986]. Alterations in blood flow are reflected by changes in the production of endothelium-derived factors, such as prostacyclin, NO, CNP, ET-1, thromboxane A_2, and angiotensin converting enzyme (ACE), which regulate the tone and proliferation of vascular smooth muscle cells. AM is also an endothelium-derived factor regulated by blood flow. Since atherosclerosis and formation of neointima are reported to have a close relation to blood flow, it is likely that fluid shear stress affects endothelial cell function in the pathophysiology of various diseases, including thrombosis, acute and chronic inflammation, hypertension, and atherosclerosis [Resnick et al., 1995].

The SSRE promoter motif "GAGACC," which is present in the promoter regions of genes such as the PDGF-B, TGF-β, t-PA, and intracellular adhesion molecule-1 (ICAM-1), is likely to be involved in shear stress-induced gene regulation [Resnick et al., 1993]. Expression of PDGF-B, TGF-β, t-PA, and ICAM-1 are all increased by fluid shear stress. Although AM has a potent vasodilating effect in vitro under static fluid conditions, the effect of fluid shear stress on AM expression has not yet been examined. Because the AM gene promoter contains the SSRE consensus sequence, we hypothesized that AM expression would be up-regulated by fluid shear stress. However, the present study demonstrates that AM expression levels in HAoECs are in fact lowered by normal arterial fluid shear stress.

It is possible that other promoter elements play a role in transcriptional control of AM, and that various mechanisms may activate SSREs in response to fluid shear stress. ACE is also down-regulated by fluid shear stress despite

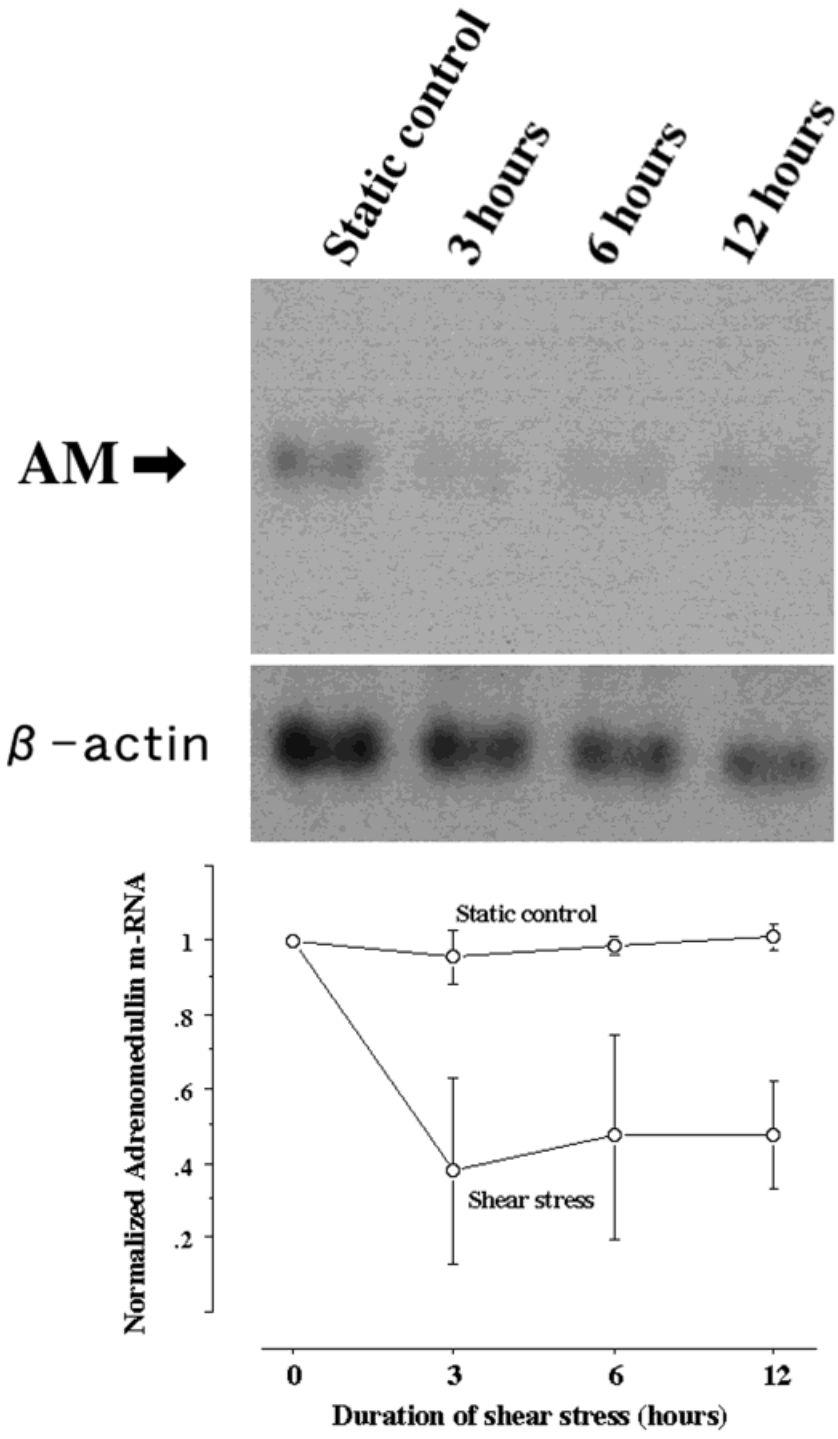

Fig. 2. Northern blotting analysis of adrenomedullin (AM) mRNA from human aortic endothelial cells (HAoECs) exposed to fluid shear stress of 24 dynes/cm^2 for increasing periods of time (0, 3, 6, and 12 h). Total RNA was extracted as described under Methods. Each lane was loaded with 5 μg of total RNA. Northern blots were probed with the full-length cDNA for AM (top), and β-actin cDNA (middle). Fluid shear stress caused down-regulation of AM mRNA levels that was evident after 3 h of fluid shear. Density of the AM bands were normalized with respect to β-actin band density in the linear range of x-ray film sensitivity (n = 2) (bottom).

the presence of an SSRE [Eisenberg et al., 1994], and PDGF-A expression is up-regulated by fluid shear stress in the absence of SSRE [Halnon et al., 1994]. While the SSRE sequence is necessary for fluid shear stress induced up-regulation of PDGF-B [Resnick et al., 1993], it is not required for the up-regulation of TGF-β [Ohno et al., 1995]. Therefore, it is possible that other types of SSREs may alter gene expression by different mechanisms or modify the regulatory functions of the factors that bind to the GAGACC motif.

Recently, Chun et al. [1997] reported that AM mRNA expression in human umbilical vein endothelial cells increased under fluid shear stress. Although the reason for these discor-

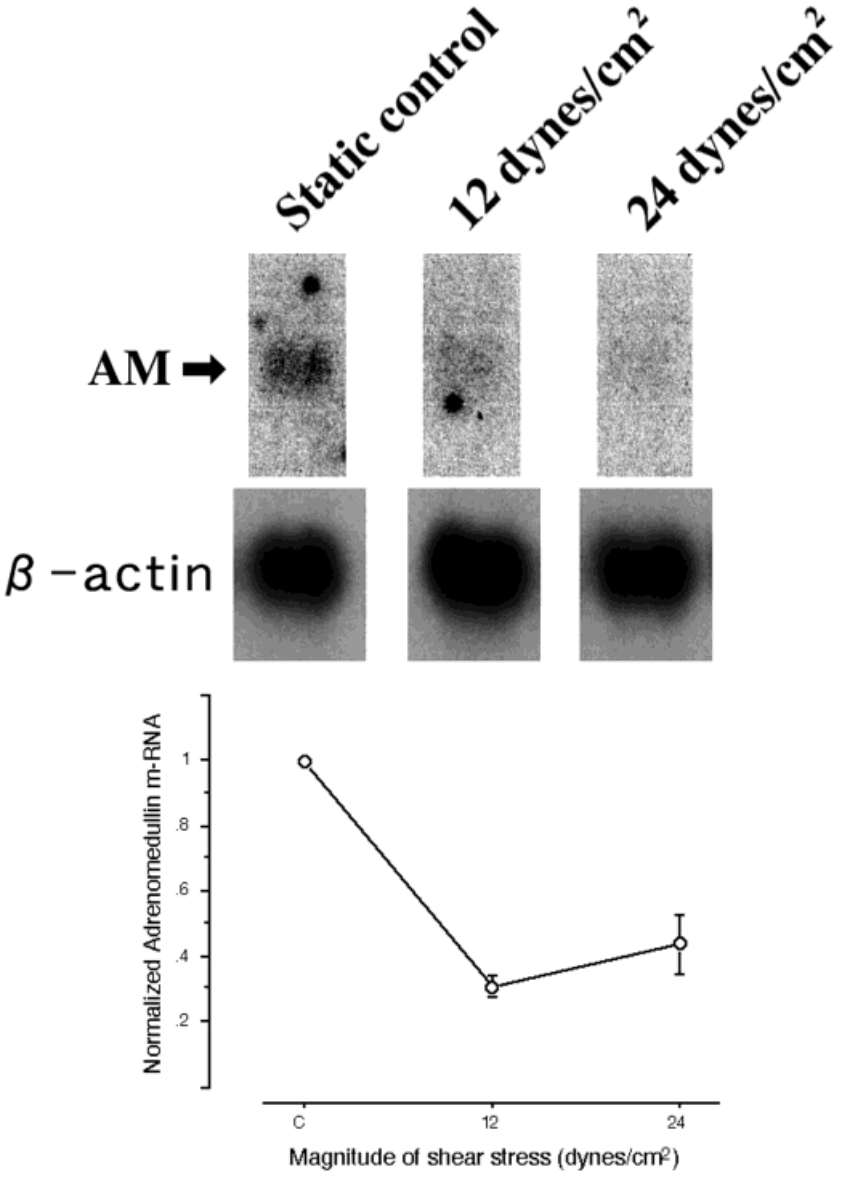

Fig. 3. Northern blotting analysis of adrenomedulin (AM) mRNA obtained from human aortic endothelial cells (HAoECs) exposed to various magnitudes of fluid shear stress for 6 h (static control, 12 and 24 dynes/cm^2). Each lane was loaded with 5 μg of total RNA. Northern blots were probed with the full-length cDNA for AM (top), and β-actin cDNA (middle). Density of the AM bands was normalized with respect to β-actin band density in the linear range of x-ray film sensitivity (bottom). A reduction in AM mRNA levels is observed in response to fluid shear stress in a magnitude-dependent manner.

dant results is not clear, it may be due to differences in the shear stress loading apparatus (cone-plate type vs parallel-plate type flow chamber) or endothelial cells used (human aortic endothelial cells vs human umbilical vein endothelial cells). The cone-plate apparatus has the advantage of not adding hydrostatic pressure to the fluid shear stress stimulus. Thus, we suggest our results are free of such possible artifacts and due to fluid shear stress alone.

AM has potent vasorelaxant activity [Ishiyama et al., 1993; Nuki et al., 1993] and suppresses proliferation in vascular smooth muscle cells [Kano et al., 1996]. We observed a significant decrease in AM mRNA levels after 3 h of a fluid shear stress of 24 dynes/cm^2. We observed that the cells had morphological changes after a 6-h exposure to fluid shear stress but not at 3 h. Frank et al. [1984] demonstrated that an actin stress fiber found in endothelial cells changes as early as 3 h after fluid shear stress and before cell shape changes were observed. In the present study, the time course of AM mRNA level changes were similar to that of the actin stress fiber changes.

Both gene transcription and protein synthesis were down-regulated by fluid shear stress in a time- and magnitude-dependent manner. This indicates that AM is expressed most actively and contributes to controlling vascular tone in

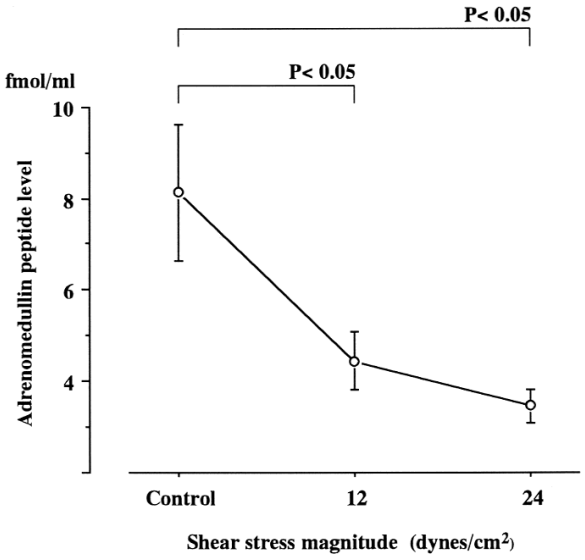

Fig. 4. Adrenomedullin (AM) peptide levels in the human aortic endothelial cells (HAoEC) culture medium were measured by radioimmunoassay. The levels of AM peptide decreased in response to fluid shear stress in a magnitude-dependent manner (control, 12 and 24 dynes/cm^2). Each point represents the mean ± SEM of six separate dishes. No immunoreactivity was detected in fresh culture medium containing 2% FBS.

low shear stress conditions. These findings imply that AM optimizes shear stress force against vasoconstrictors (example: ET-1) especially under low shear stress conditions.

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