Enhanced Adrenomedullin Production by Mechanical Stretching in Cultured Rat Cardiomyocytes

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Abstract—Adrenomedullin (AM) is secreted from cultured cardiac myocytes. In this study, we examined whether mechanical stretching stimulates AM production in cardiac myocytes, and if so, whether angiotensin II (Ang II) is involved in that mechanism. Neonatal rat cardiac myocytes cultured in serum-free medium were stretched 10% or 20% on flexible silicone rubber culture dishes, and AM mRNA expression was examined by quantitative polymerase chain reaction. The AM mRNA levels in the myocytes stretched 10% and 20% for 24 hours significantly increased by 56% (P<0.05) and 88% (P<0.01), respectively, when compared with the levels in nonstretched cells. AM secretion into the medium after the myocytes were stretched 10% and 20% increased by 22% (P<0.05) and 45% (P<0.01), respectively. In nonstretched myocytes incubated with 10⁻⁶ mol/L Ang II for 24 hours, AM mRNA and secretion increased by 86% (P<0.05) and 36% (P<0.01), respectively. These effects of Ang II were abolished by 10⁻⁶ mol/L CV-11974, an Ang II type I (AT₁) receptor antagonist, but not by 10⁻⁶ mol/L PD-123319, an Ang II type II antagonist. Stretch-induced increases of AM gene expression and secretion were significantly inhibited (P<0.05) in the presence of 10⁻⁶ mol/L CV-11974 by 46% and 52%, respectively; however, they were not affected by 10⁻⁶ mol/L PD-123319. These findings indicate that AM production from cardiac myocytes is augmented by mechanical stretching, partially through the AT₁ receptors, which suggests a local interaction between AM and the renin-angiotensin system in stretched cardiac myocytes. *(Hypertension. 2000;35:1210-1214.)*

Key Words: adrenomedullin ■ hypertrophy ■ mechanical stretch ■ angiotensin II ■ receptors, angiotensin

Left ventricular hypertrophy has been shown to be an important risk factor for cardiovascular disease.¹ ² Cardiac hypertrophy is basically an adaptation mechanism that develops in response to an increased cardiac workload, but it can ultimately progress to heart failure. Substantial evidence³ ⁵ ⁷ suggests that mechanical load is an important factor in the process of cardiac hypertrophy, and recent studies⁸ ¹⁰ ¹¹ indicate that humoral factors such as angiotensin II (Ang II) or endothelin-1 (ET-1) also play an important role in hypertrophy in cardiomyocytes and hyperplasia of the interstitial space in the heart. Adrenomedullin (AM) is a potent vasorelaxant peptide discovered in human pheochromocytomas.⁹ AM mRNA is expressed in various organs of rats and humans, including the normal adrenal medulla and cardiac ventricle.¹⁰ ¹¹ Recent reports¹² ¹⁵ have shown that AM content and mRNA expression in the hypertrophic left ventricle induced by hemodynamic overload are increased when compared with levels in normal rats. We have shown that AM is synthesized and secreted from cultured neonatal rat cardiac myocytes and fibroblasts and that secreted AM acts on the myocytes to inhibit hypertrophy and on the fibroblasts to inhibit growth. This suggests the possible role of AM in modulating cardiac hypertrophy and interstitial fibrosis.¹⁶ ¹⁷ However, little is known about the mechanisms that regulate AM synthesis and secretion in the cardiac ventricle. To determine whether mechanical stretching stimulates AM production we stretched neonatal rat cardiac myocytes cultured on silicone rubber dishes and then measured AM gene expression and secretion. We also used Ang II receptor antagonists to evaluate the role of the local renin-angiotensin system in stretch-induced AM production.

**Methods**

**Chemicals**

Ang II and rat AM were purchased from Peptide Institute Inc. CV-11974 was a gift from Takeda Chemical Industries Ltd, Osaka, Japan, and PD-123319 was purchased from Research Biochemicals International. Holo-transferrin (human), collagenase (type IV), and trypsin (bovine pancreas) were purchased from Sigma Chemical Co, and fibronectin was obtained from Collaborative Biomedical.

**Cell Culture**

Primary cultures of cardiac myocytes were prepared from cardiac ventricles of 1- to 2-day-old Wistar rats as described previously.¹⁶
Digestion of the minced ventricles was accomplished with 0.12% trypsin and 0.03% collagenase, after which cells were placed in culture dishes for 30 minutes at 37°C to allow selective attachment of nonmyocytes (primarily cardiac fibroblasts). Cardiomyocyte-enriched suspensions were removed from the culture dishes and were plated at a density of 1×10^6 cells/cm^2 onto collagen type I–coated 24-well culture plates (Sumitomo Bakelite Co Ltd) or silicone rubber dishes that had been coated with fibronectin. Cells were cultured for 48 hours with DMEM containing 15 mmol/L HEPES, 10% fetal bovine serum, 10 μg/mL insulin, 10 μg/mL transferrin, and 0.1 mmol/L bromodeoxyuridine (BrDU). The culture medium was then changed for serum-free DMEM containing the same additives with the exception of BrDu. After having been incubated for 24 hours, the cardiomyocytes were exposed to Ang II or were stretched 10% or 20% on the silicone dishes with or without the Ang II receptor antagonists.

These experiments were performed according to the regulations of the Animal Research Committee of Miyazaki Medical College (1998-037-2). This investigation conformed with the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No.85-23, revised 1996).

**Assay of AM in Conditioned Medium**

Conditioned medium collected from 24-well culture plates or from silicone dishes was acidified with acetic acid to a final concentration of 1.0 mol/L. The medium was heated at 100°C for 10 minutes to inactivate proteases and was applied to a Sep-Pak C18 cartridge (Millipore-Waters). After the cartridge was washed with 10% CH,CN in 0.1% trifluoroacetic acid, the adsorbed materials were eluted with 60% CH,CN in 0.1% trifluoroacetic acid, lyophilized, and stored at −30°C. The lyophilized samples were dissolved in radioimmunoassay (RIA) buffer and were subjected to RIA for rat AM as described previously.

The recovery of AM in this assay procedure was 82%.

**AM mRNA Measurement by Real-Time Quantitative Polymerase Chain Reaction**

Total RNA Isolation Reagent (GIBCO BRL) was used to extract 2 μg of total RNA, which then underwent reverse-transcription by means of SuperScript reverse transcriptase (Life Technologies Inc) into cDNA. To measure rat AM mRNA levels, we used a novel quantitative polymerase chain reaction (PCR) method, Real-Time Quantitative PCR (Prism 7700 Sequence Detector, Applied Biosystems) as previously reported, with the following oligonucleotide-18 amplified with the following pairs of oligonucleotides: CGCCCAAGGCAAGCTCAGACAC (nucleotides 387 to 410) for AM and ATCCACATTCTCCAGGAGGC (nucleotides 244 to 267) for GAPDH. cDNA of rat AM and GAPDH was amplified with the following pairs of oligonucleotides: CGCACTTCCGAAAGAATGG (nucleotides 236 to 255, forward primer) and CGTGTGACTCGAATGTGGGC (nucleotides 412 to 431, reverse primer) for AM cDNA and CGCAAGTCTCAAGTGCACA (nucleotides 183 to 201, forward primer) and AAGAGGCTAGTACACAGTCC (nucleotides 308 to 329, reverse primer) for GAPDH. cDNA from rat lungs was used as a standard and levels of AM mRNA were compared after they had been normalized relative to those of GAPDH.

**Statistical Analysis**

Student’s *t* test was used for comparison of the 2 variables. Multiple comparison was assessed first with 1-way ANOVA and then with the Scheffé test. All data were expressed as the mean±SEM of the samples examined; *P*<0.05 was considered significant. Cells isolated separately from different groups of neonatal rats were used to repeat the experiments, and identical results were obtained.

**Results**

**Mechanical Stretching Augments AM Production from Cardiac Myocytes**

To examine whether mechanical stretching affects AM synthesis and secretion, cultured cardiac myocytes were stretched on silicone dishes for 24 hours. AM mRNA expression in cells stretched 10% and 20% increased by 56% (P<0.05) and 88% (P<0.01), respectively, when compared with the levels of AM mRNA expression in nonstretched myocytes (Figure 1A). AM secretion from cells stretched 10% and 20% also increased by 22% (P<0.05) and 45% (P<0.01), respectively, when compared with the AM secretion in controls (Figure 1B). To examine the sequential changes of the rate of AM secretion, we collected conditioned medium of stretched or nonstretched myocytes every 4 hours and measured the AM concentration. As shown in Figure 2,
Mechanical Stretching Stimulates AM Production in Part via the AT\textsubscript{1} Receptors

To clarify the role of the endogenous renin-angiotensin system in stretch-induced AM production, the myocytes were stretched 20% for 24 hours in the presence or absence of the Ang II receptor antagonists. As shown in Figures 4A and 4B, when myocytes were incubated with CV-11974, the stretch-induced increases of AM mRNA expression and AM secretion were significantly attenuated ($P<0.05$) by 46% and 52% respectively; however, PD-123319 produced little effect on the stretch-stimulated AM gene expression and secretion. To evaluate Ang II secretion from the myocytes, we measured Ang II concentrations in the conditioned media of the control cells and of the cells stretched for 30 minutes or 24 hours by a specific radioimmunoassay.\cite{21} The Ang II levels in most of the samples from those myocytes were $<3.0 \times 10^{-12}$ mol/L, a level undetectable by our assay.

Discussion

AM mRNA is expressed in various tissues and organs of rats and humans, including the normal adrenal medulla, cardiac atrium, and ventricle.\cite{10,11} AM is also present in human blood,\cite{11} and Sumimoto et al\cite{25} showed an elevated plasma AM level in patients with hypertension, particularly in those with left ventricular hypertrophy. Previous studies\cite{12-15} showed that AM gene expression and AM content are increased in rats with hypertrophy of the cardiac ventricles induced by hemodynamic overload when compared with control rats. In this study, we found that mechanical stretching stimulates AM mRNA expression and AM secretion in
cultured cardiac myocytes. When compared with the levels of gene expression and immunoreactive AM in the conditioned media, the increments in immunoreactive AM were smaller than those of AM mRNA in the myocytes that had been stretched or treated with Ang II. According to our unpublished observation, 47% of 10^{-7} mol/L of unlabelled AM added to the media disappeared after 24 hours of incubation with the cardiac myocytes, which suggests nonspecific or specific receptor binding or clearance of AM by the cells. The myocytes may be producing more AM than that detected in the conditioned media. It has been suggested that AM is secreted in a constitutive manner with a little intracellular storage from various types of cultured cells, including cardiac myocytes.\(^{16,17,23}\) In this study, the nonstretched myocytes constantly produced AM, and a significant increase in AM secretion occurred in the myocytes that had been stretched for ≥8 hours. This finding confirms those of previous observations\(^{16}\) about the constitutive secretion of AM.

In cultured vascular smooth muscle cells, AM production is increased in the presence of Ang II, ET-1, or cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β).\(^{12,24}\) AM production is stimulated by TNF-α and IL-1β in cultured cardiac myocytes and fibroblasts.\(^{25}\) In this study, synthetic Ang II stimulated AM mRNA expression and AM secretion in cultured cardiac myocytes. Those effects were completely abolished by CV-11974 (an AT₁ receptor antagonist) but were only slightly diminished by PD-123319, an AT₂ receptor antagonist. Because Ang II produced by cardiac myocytes is an important factor in hypertrophy stimulated by mechanical stretching,\(^{26,27}\) we examined the role of endogenous Ang II in stretch-induced AM production. As shown in Figure 4, CV-11974 significantly reduced the AM production in the stretched myocytes, whereas PD-123319 had little effect, which suggests that stretch-induced AM production is mediated in part by endogenous Ang II acting through the AT₁ receptor. We also evaluated Ang II production from the cells by measuring Ang II concentrations in the control cells and in stretched myocytes, but the concentrations were <3.0×10^{-12} mol/L, a level too low to be detected. This concentration is much lower than that of synthetic Ang II, which significantly elevated AM production in this study. Ang II levels in the cell surface or in extracellular cell-to-cell spaces may be higher than those in conditioned media, although this discrepancy is unexplained and should be the topic of future research.

CV-11974 did not reduce stretch-induced AM gene expression and secretion to the levels observed in controls, in spite of the complete inhibition of exogenous Ang II–induced AM production by that AT₁ antagonist in nonstretched myocytes. The AM mRNA expression and secretion were slightly reduced by PD-123319 in the control cells and in cells treated with Ang II. Horio et al.\(^{25}\) reported that Ang II had no significant effect on AM production in cultured cardiac myocytes isolated by a Percoll gradient that is apparently different from ours. The discrepancy between their results and ours suggests that a type of cell isolated by our method but not by theirs may be necessary for the Ang II– or stretch-induced AM production. Recently, nonmyocytes that secrete ET-1 were shown to have an important role in the Ang II–induced hypertrophy of cardiomyocytes.\(^{28}\) We observed that AM secretion from myocytes is augmented not only by Ang II but also by ET-1 or by fetal bovine serum that contains various growth-promoting factors (data not shown). Although endogenous Ang II acting through the AT₁ receptors seems important, other mechanisms including the AT₂ receptors, ET-1, or other growth-promoting factors may also be involved in the Ang II–induced or stretch-induced AM production. We have shown that AM attenuates Ang II–stimulated hypertrophy of cardiomyocytes and growth of cardiac fibroblasts,\(^{16,17}\) which suggests the possible role of AM in modulating cardiac hypertrophy and in remodeling as an autocrine or a paracrine factor. Augmentation of AM secretion from the myocytes by mechanical stretching supports our hypothesis that AM participates in the mechanism acting against cardiomyocyte hypertrophy, which is induced by hemodynamic overload of the heart.

In summary, this study revealed that mechanical stretching augments AM production from cultured cardiac myocytes, partially through the AT₁ receptors, which suggests interaction of the local renin-angiotensin system and AM in stretched myocytes.

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References


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