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Original Research

# Effect of mechanical stretch on rat neonatal cardiomyocyte somatostatin receptor subtype 1 expression

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## Abstract

**Background:** Somatostatin receptors (SSTRs) have been proven to be involved in cardioprotection, but the effects of mechanical stress on their expression remain to be elucidated.

**Aim:** To investigate whether mechanical stretch is associated with increased expression of the SSTR in cardiomyocytes and if the phenomenon is mediated via an angiotensin II signaling pathway.

**Methods:** Using a well established cyclic mechanical stretch model exerting strain on primary cultured neonatal Wistar rat cardiomyocytes, we studied the expression of major SSTRs of cardiomyocyte under such stress. Western blot and immunoprecipitation were used to demonstrate the related changes. The role of endogenous angiotensin II and the effects of AT-1 receptor blockade were studied by pre-administration of losartan.

**Results:** Northern and western blotting revealed progressive increase of SSTR-1 mRNA and protein expression as the duration of stretch increased up to 48 hours. The maximal increases of SSTR-1 mRNA and protein were observed at 24 hours. The mechanical stretch significantly increased the angiotensin II in cardiomyocytes concomitantly. Losartan and PD98059 had a significant inhibitory effect on the expression of SSTR-1, indicating the angiotensin II and p42/p44 MAP kinases-related pathways were involved in the expression of SSTR-1 under mechanical stress. By contrast, tyrosine kinases, protein kinase A, protein kinase C, JAK-2, JNK and phosphoinositol-3-kinases had no effect.

**Conclusion:** This study demonstrated that accentuating the expression of SSTR-1mechanical stretch is angiotensin II and p42/p44 MAP kinases-dependent.

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**Keywords:** Mechanical stretch; Somatostatin; Receptor; Cardiomyocyte; Angiotensin

## 1. Introduction

Hypertensive cardiac disease is a common condition that emergency physicians have to deal with. Prolonged, uncontrolled hypertension always results in left ventricular hypertrophy, which is subsequently related to myocardial ischemia,

malignant arrhythmia and sudden cardiac death.<sup>1,2</sup> The underlying mechanism of myocardial hypertrophy and remodeling is multifactorial and involves multiple proto-oncogenes, growth factors, cytokines and extracellular matrices.<sup>3–8</sup> However, increased internal force to the myocardium might be the major contributing factor.

Although mechanical stress is responsive for myocardial hypertrophy, the possible candidate mediators or signaling pathways were investigated. Mechanical stress has been proven to be closely linked with the maintenance of myocardial structure and function. Progressive elevation of mechanical force affects

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signaling reactivity, alters the balance of forces among different components of the myocardium and transforms the physical properties of the tissues.<sup>9–11</sup> Increased physical stress might lead to maladaptive remodeling in many important clinical conditions, such as hypertension, ischemia or contractile abnormalities,<sup>12,13</sup> whereas cellular and extracellular remodeling tend to occur concomitantly and results in pathological processes.<sup>14</sup>

Among many mediators, somatostatin (SST) and its receptors (SSTR) have been proven to be expressed in myocardium.<sup>15</sup> It is well known that SST is produced by various cells, including cardiomyocytes in response to neuropeptides, neurotransmitters, thyroid and steroid hormones, growth factors and cytokines.<sup>15</sup> An earlier study demonstrated that SST affects contractile responses of atrial and ventricular cardiomyocytes.<sup>16</sup> Besides, our data revealed that an SST analog could mimic acute ischemic preconditioning in a rat myocardial infarction model.<sup>17</sup> Whether mechanical stress can affect expression of the SSTRs in myocardium deserves further investigation.

SSTR was considered to play an important role in cardiac hypertrophy in patients with acromegaly that is associated with excessive GH secretion.<sup>18</sup> However, there is no report concerning the relationship between SSTR and other pathological conditions associated with cardiac hypertrophy, such as hypertension. To elucidate the problem, we chose to use a well established cyclic mechanical stretch model to evaluate its effect on SSTR expression and to understand the possible cellular mechanisms involved.

## 2. Methods

### 2.1. Primary culture of neonatal rat cardiomyocytes

Neonatal cardiomyocytes were obtained from 2–3-day-old Wistar rats by trypsinization. Fetal hearts were excised and washed in phosphate-buffered saline (PBS). The tissue was minced and digested repeatedly in a pancreatin solution. The first two supernatants were discarded and cells released into the remaining supernatants were maintained at 37°C in a 5% CO<sub>2</sub>/95% O<sub>2</sub> atmosphere in heart medium (Hank's buffered salt solution, MEM vitamins, MEM essential amino acids, MEM nonessential amino acids, 2 mM L-glutamine, 0.05% (w/v) glycine, 0.0125% hypoxanthine, 50 IU/mL penicillin, 50 µg/mL streptomycin, 1.65 µg/L sodium bicarbonate and 10% (v/v) fetal calf serum) until digestion was complete. The cultures were then enriched for myocytes by plating for 1 hour, to deplete the culture of non-muscle cells, which adhere more rapidly. Cardiac myocytes in the medium were then plated into six-well culture plates (Flex I, Flexcell Co., MaKeesport, PA, USA) at a cell density of  $1.6 \times 10^3$  cells/well. After 2 days in culture, cells were transferred to serum-free medium (Ham's F-12/DMEM 1:1 (v/v)) and maintained for another 2 days. The cultured myocytes obtained were >95% pure as judged by observation of contractile characteristics with a light microscope and staining with anti-desmin antibody. The enriched myocytes were subjected to cyclical stretch.

The animals were cared for according to the Guide for the Care and Use of Laboratory Animals (NIH publication no.

85023, revised 1996), and the protocol was approved by our Institute Review Board.

### 2.2. In vitro cyclical mechanical stretch

The experiment was done with a cyclical strain unit (Flexcell I FX-2000, Flexcell International Co., PA, USA) that consisted of a vacuum unit linked to a valve controlled by a computer program. Neonatal cardiomyocytes cultured on plates with a flexible membrane base coated with type 1 rat collagen were subjected to cyclical strain. A pressure of ~15 kPa was applied repeatedly to the flexible baseplate membrane at 60 cycles/min. Application of the reduced pressure resulted in maximal elongation of 20% of cells at the periphery of the well, with strain declining toward the center of the well. The cardiomyocytes were stretched at 37°C in a humidified incubator with a 5% CO<sub>2</sub>/95% O<sub>2</sub> atmosphere.

### 2.3. RNA isolation and northern blot analysis

Total RNA was isolated from cardiomyocytes by solubilizing cardiomyocytes with the Ultraspec RNA kit (BIOTECHX Laboratory, Houston, TX, USA). The cells were washed three times with PBS at 4°C and whole-cell RNA was isolated. RNA was quantified as the ratio of the absorbance at 260 nm (A<sub>260</sub>) and at 280 nm (A<sub>280</sub>) in a spectrophotometer (U-2010, HITACHI Co., Japan), denatured and subjected to electrophoresis in 1.0% formaldehyde, 10× Denhardt's solution, 0.1% (w/v) SDS, 0.2 mg/mL denatured salmon sperm DNA, 10 mM EDTA, 25% 4 × RNA, 1 M NaCl, 0.6 M Tris-HCl pH 7.5, 0.18 M NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O, 0.24 M Na<sub>2</sub>HPO<sub>4</sub>, 0.01 M sodium pyrophosphate at 42°C for at least 2 hours. The hybridization solution was identical except that it contained 10% dextran sulfate and the labeled probe. Equal quantities (20 µg) of total RNA were loaded into each lane of a Hybond-N<sup>+</sup> nylon membrane. The rat cDNA clones for SSTR-1-5, angiotensinogen and the pTRI-GAPDH (Ambion Inc., TX, USA) were used, and purified insert DNA was labeled using Redivue [ $\alpha$ -<sup>32</sup>P]dCTP (Amersham Pharmacia Biotech Co., NJ, USA) for hybridization in Rapid-hyb buffer (Amersham, Buckinghamshire, UK) at 65°C for 4 hours. Blots were then washed in 0.1% SDS and 0.2 × standard saline citrate at 65°C. Quantitative analysis was done with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA) and assays were done for at least five times. The primers used are listed below.

Somatostatin receptor subtype 1 (318 bp PCR product)

sense, 5'-ATGGTGGCCCTCAAGGCCGG-3'  
antisense, 5'-CGCGGTGGCGTAATAGTCAA-3'

Somatostatin receptor subtype 2 (318 bp PCR product)

sense, 5'-TCCTCTGGAATCCGAGTGGG-3'  
antisense, 5'-TTGTCCTGCTTACTGTCACT-3'

Somatostatin receptor subtype 3 (332 bp PCR product)

sense, 5'-TGCCACCCTGGGCAACGTGT-3'  
antisense, 5'-CAGGCAGAATATGCTGGTGA-3'

Somatostatin receptor subtype 4 (323 bp PCR product)

sense, 5'-GCGCGCGGCGACCTACCGGC-3'  
antisense, 5'-GCCTGGTGATTTTCTTCTCC-3'

Somatostatin receptor subtype 5 (259 bp PCR product)

sense, 5'-CTGGTGGGGCCGGCGCCCTC-3'  
antisense, 5'-CCAGGCGGCACAGGACGGGG-3'

#### 2.4. Western blot analysis

Protein extracts were obtained from cultures by rinsing the cells with ice-cold PBS once and with Tris-buffered saline (pH 7.4). Cells were resuspended in cold lysis buffer (150 mmol/L NaCl, 20 mmol/L Tris-HCl pH 7.5, 1 mmol/L EDTA pH 8.0, 0.5% (w/v) sodium deoxycholate, 1% (v/v) Nonidet P-40, 10 µg/mL aprotinin, 10 µg/mL leupeptin and 1 µmol/L phenylmethylsulfonyl fluoride), and cellular debris was removed by centrifugation at 14,000g for 10 min at 4°C. The protein content of the supernatant was determined by the Bio-Rad Protein assay kit and an ELISA reader ( $A_{595}$ ) using bovine serum albumin as the standard. Equal amounts of protein extracts (30 µg) were subjected to electrophoresis in SDS 10% (w/v) polyacrylamide gels and then electroblotted onto nitrocellulose membranes, which were incubated overnight in PBS containing 5% (v/v) skim milk to block nonspecific binding of the antibody. Proteins of interest were revealed using specific antibodies (mouse monoclonal anti-SSTR-1 and anti-SSTR-2 antibodies from Chemicon International, Inc., CA, USA) as indicated (1:1000 dilution) for 1 hour at room temperature, followed by incubation with a dilution of 1:5000 of horseradish peroxidase-conjugated polyclonal anti-rabbit antibody for 1 hour at room temperature. The membrane was examined with an enhanced chemiluminescence system (ECL, Amersham Corp., Buckinghamshire, UK) and the signal intensity was quantified by densitometry. Equal protein loading of the samples was further verified by staining with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) specific monoclonal antibody.

#### 2.5. Treatment protocol

To investigate the possible signaling pathways, different groups of cardiomyocytes were treated with losartan (200 nM, an angiotensin AT1 receptor inhibitor), octreotide (1 µg/mL), H89 (10 µM, a protein kinase A inhibitor), forskolin (10 µM, an adenylyl cyclase activator), genistein (40 µM, a tyrosine kinase inhibitor), AG-490 (100 µM, a specific inhibitor of Janus kinase or JAK-2), PD 98059 (50 µM, an inhibitor of p42/p44 MAP kinase), Gö6976 (16 nM, a selective protein kinase C inhibitor), SP600125 (25 µM, a c-Jun N-terminal kinase or JNK inhibitor)

wortmannin (50 nM, a phosphoinositol-3-kinase inhibitor) at 30 minutes before the stretch.

#### 2.6. Measurement of the concentration of angiotensin II

Culture media from cardiac myocytes subjected to cyclical mechanical stretch were collected for the measurement of angiotensin II. After adding EDTA (to 1 mg/mL) and captopril (to 10 µM) and subsequent centrifugation at 1000g at 4°C for 5 minutes, the supernatant was collected and frozen at -20°C. The level of angiotensin II was measured by a quantitative sandwich enzyme immunoassay technique using a commercial kit (Peninsula Laboratories, Inc., Belmont, CA, USA). The lower limit of detection of angiotensin II was 0.05 ng/mL and the interassay variation was <5%.

#### 2.7. Statistical analysis

All values are expressed as mean ± SEM. Data from densitometry of northern and western blotting were analyzed by nonlinear regression curve fitting using SSPS 10.0 software. Statistical analysis was done with the Student 2-tailed paired *t* test or 2-way analysis of variance (ANOVA) followed by the Bonferroni test when appropriate. The level of statistically significant difference was set at  $p \leq 0.05$ .

### 3. Results

#### 3.1. Effect of mechanical stretch on SSTR-1 mRNA expression

A pressure of ~15 kPa was applied repeatedly to the flexible baseplate membrane at 60 cycles/min. Application of the reduced pressure resulted in maximal elongation of 20% of cells at the periphery of the well, with strain declining toward the center. Northern blotting revealed a progressive increase of SSTR-1 mRNA expression as the duration of stretch increased up to 24 hours. The maximal increase ( $p < 0.01$ ) of SSTR-1 mRNA at 24 hours was  $88 \pm 12\%$  (Fig. 1).

Other subtypes of SSTR mRNA that could be detected were SSTR-2 and SSTR-4. However, there no significant increase of these SSTR mRNAs was induced by cyclical mechanical

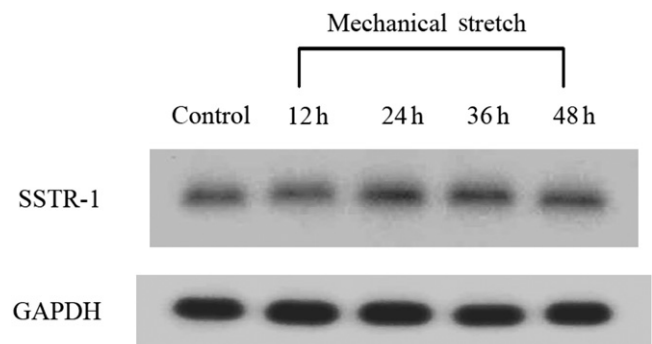


Fig. 1. Representative northern blots for SSTR-1 mRNA levels in neonatal rat cardiomyocytes subjected to 20% stretch for various lengths of time.

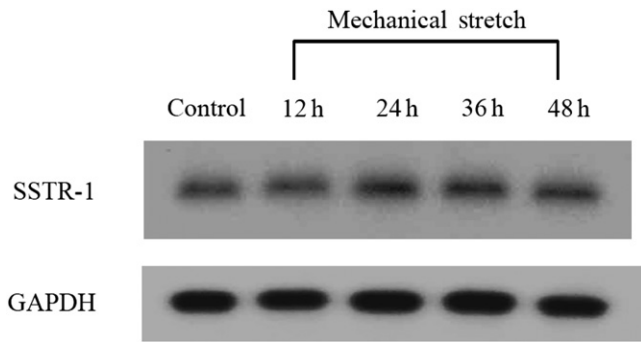


Fig. 2. Representative western blots for SSTR-1 protein levels in cardiomyocytes subjected to 20% stretch for various lengths of time.

stretch; the increase was  $10 \pm 12\%$  for SSTR-2 and  $6 \pm 7\%$  for SSTR-4. No SSTR-3 or SSTR-5 mRNA was detected before or after mechanical stretch for various lengths of time.

### 3.2. Effect of mechanical stretch on SSTR-1 protein expression

Western blotting revealed a progressive increase of SSTR-1 protein expression as the duration of stretch increased up to 48 hours. The maximal increase ( $p < 0.001$ ) of SSTR-1 mRNA was  $110 \pm 32\%$  at 24 hours (Fig. 2).

SSTR-2 and SSTR-4 mRNAs were detected; however, there was no significant increase of these SSTR mRNAs induced by cyclical mechanical stretch. No SSTR-3 or SSTR-5 mRNA was detectable either before or after mechanical stretch for various lengths of time.

### 3.3. Mechanical stretch causes secretion of angiotensin II from cardiomyocytes

The mechanical stretch significantly increased ( $p < 0.001$ ) angiotensin II in cardiomyocytes; the concentration of angiotensin II increased from  $29.8 \pm 1.2$  to  $123.4 \pm 4.8$  pg/mL after a stretch for 6 hours, which was comparable with our earlier study.<sup>17</sup> No angiotensin II was detected in the conditioned medium of fibroblasts either under mechanical stress or not.

### 3.4. Signaling mechanisms of stretch-induced SSTR-1 expression

In the process of investigating the possible signaling pathways, cardiomyocytes were treated with losartan (200 nM, an angiotensin AT1 receptor inhibitor), octreotide (1  $\mu$ g/mL), H89 (10  $\mu$ M, a protein kinase A inhibitor), forskolin (10  $\mu$ M, an adenylyl cyclase activator), genistein (40  $\mu$ M, a tyrosine kinase inhibitor), AG-490 (100  $\mu$ M, a specific inhibitor of Janus kinase or JAK-2), PD 98059 (50  $\mu$ M, an inhibitor of p42/p44 MAP kinase), Gö6976 (16 nM, a selective protein kinase C inhibitor), SP600125 (25  $\mu$ M, a JNK inhibitor) wortmannin (50 nM, a phosphoinositol-3-kinase inhibitor) at 30 minutes before the stretch.

Losartan prevented SSTR-1 expression almost completely (Fig. 3A), and PD98059 had an inhibitory effect on stretch-induced expression of SSTR-1 (Fig. 3B). By contrast, treatment with octreotide, H89, forskolin, genistein, AG-490, Gö6976, SP600125 or wortmannin had no significant effect on stretch-induced SSTR-1 expression (Fig. 3A and B). In summary, the angiotensin II and p42/p44 MAP kinase-related

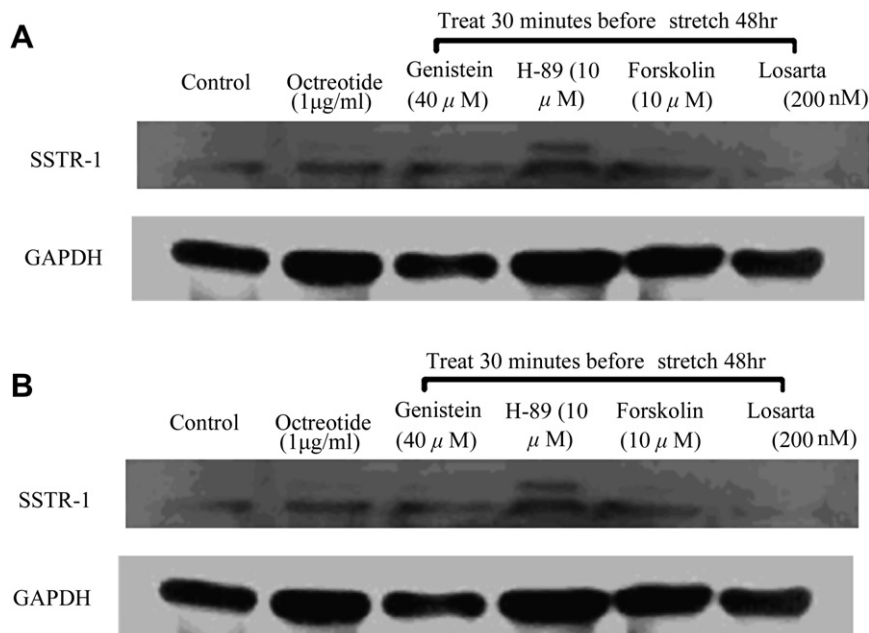


Fig. 3. (A) Western blots for SSTR-1 protein levels in cardiomyocytes subjected to 20% stretch for 18 hours in the absence or in the presence of octreotide, losartan, H89, forskolin and genistein; only losartan had a significant inhibitory effect on the expression of SSTR-1. (B) Western blots for SSTR-1 protein levels in cardiomyocytes subjected to 20% stretch for 18 hours in the absence or in the presence of AG-490, Gö6976, PD98059, SP600125 and wortmannin; only PD98059 had a significant inhibitory effect on the expression of SSTR-1.

pathways were involved in expression of SSTR-1 under mechanical stress whereas other signaling pathways, such as tyrosine kinases, protein kinase A, protein kinase C, JAK-2, JNK and phosphoinositol-3-kinases, were not the contributing mechanisms.

#### 4. Discussion

This study demonstrated that mechanical stretch can accentuate SSTR-1 expression in an angiotensin II and p42/p44 MAP kinase-dependent manner. Tyrosine kinases, protein kinase A, protein kinase C, JAK-2, JNK and phosphoinositol-3-kinases are not involved in stretch-induced SSTR-1 expression.

It is well known that mechanical stress is closely linked to the maintenance of myocardial structure and function.<sup>19–21</sup> Chronic elevation of external force influences myocardial signaling reactivity, alters the balance of forces between different components of the myocardium and transforms the physical properties of these tissues.<sup>19–21</sup> Increased physical stress might lead to maladaptive remodeling in pathological conditions such as hypertension and ischemia, or contractile abnormalities that produce abnormally excessive stress.<sup>22,23</sup> Cellular and extracellular remodeling tends to occur concomitantly and these pathological processes affect each other because there are close interactions between the cardiomyocytes and the extracellular matrix.<sup>24</sup> Our colleagues have demonstrated that some matrix metalloproteinases (MMPs) were induced by tumor necrosis factor- $\alpha$  in a mechanical stretch model of endothelial cells,<sup>25</sup> whereas production of tumor necrosis factor- $\alpha$  could be induced in cardiac fibroblasts.<sup>26</sup> Earlier, we demonstrated that the cyclical mechanical stretch-induced increase of the expression of cardiac MMP-14 and MMP-2 is angiotensin II-dependent. The signaling pathways are mediated mainly by JAK-2 and STAT-1.<sup>27</sup> By contrast, the present study demonstrated that mechanical stretch can accentuate myocardial SSTR-1 expression with an angiotensin II and p42/p44 MAP kinase-dependent signaling pathway. This implies that the same mediator (angiotensin II) can initiate different signaling pathways to provoke different physiological effects, including protein expression.

The actions of SST are mediated by a family of G protein-coupled receptors of five distinct subtypes, SSTR1–5, that are encoded by separate genes on different chromosomes. The five receptors share common signaling pathways, such as the inhibition of adenylyl cyclase, activation of phosphotyrosine phosphatase and modulation of mitogen-activated protein kinase through G protein-dependent mechanisms. Some of the subtypes are also coupled to signaling effectors, such as inward rectifying K1 channels (SSTR-2–5), voltage-dependent calcium channels (SSTR-1 and -2), a Na<sup>+</sup>/H<sup>+</sup> exchanger (SSTR-1), AMPA/kainate glutamate channels (SSTR-1 and 2), phospholipase C (SSTR-2 and 5), and phospholipaseA2 (SSTR-4).<sup>15</sup> As mentioned above, mechanical stretch induces expression of SSTR-1 in neonatal cardiomyocytes. SSTR-1 is reported to be coupled with Na<sup>+</sup>/H<sup>+</sup> exchangers so it might play an important role in mechanical strain-related ischemic reperfusion injury, electrophysiological changes (arrhythmias) and even cardiac

hypertrophy.<sup>28–33</sup> Furthermore, the involvement of voltage-dependent calcium channels might contribute to subsequent arrhythmias.<sup>34</sup> SSTR is considered to play an important role in cardiac hypertrophy in those with acromegaly associated with excessive GH secretion.<sup>35</sup> However, there is still no report concerning the relationship between SSTR and other pathological conditions associated with cardiac hypertrophy, such as hypertension. The results of this study suggest that SSTR-1 is essential in mechanical strain-induced hypertrophy of cardiomyocytes.

In conclusion, this study demonstrated that the mechanical stretch-induced increase of SSTR-1 expression is angiotensin II and p42/p44 MAP kinase-dependent. This observation elucidates part of the pathophysiological mechanisms of cardiac hypertrophy, arrhythmias and even heart failure due to long-term hypertension.

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