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Regulation of resistin by cyclic mechanical stretch in cultured rat vascular smooth muscle cells

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ABSTRACT

Resistin has a potential role in atherosclerosis; however, the molecular mechanism underlying the increase in resistin expression in atherosclerosis remains unclear. As mechanical stretch plays an important role in atherosclerosis, in the present study we sought to investigate the cellular and molecular mechanisms underlying the regulation of resistin by cyclic mechanical stretch in VSMCs (vascular smooth muscle cells). VSMCs from thoracic aorta of adult Wistar rats were cultured and subjected to cyclic stretch. Cyclic mechanical stretch significantly increased resistin protein and mRNA expression as compared with control cells without stretch. The specific p38 MAPK (mitogen-activated protein kinase) inhibitor SB203580, the antioxidant *N*-acetylcysteine and p38 MAPK siRNA (small interfering RNA) attenuated the induction of resistin protein by cyclic stretch. Cyclic stretch significantly increased the phosphorylation of p38 MAPK, whereas pre-treatment with SB203580 and *N*-acetylcysteine significantly inhibited this effect. Cyclic stretch significantly increased ROS (reactive oxygen species) production, and pre-treatment with *N*-acetylcysteine significantly inhibited stretch-induced ROS production. Cyclic stretch also increased STAT3 (signal transducer and activator of transcription 3)-binding activity and resistin promoter activity, and resistin promoter activity was abolished when STAT3 in the promoter area was mutated. Pre-treatment with SB203580 and *N*-acetylcysteine significantly attenuated resistin promoter activity induced by cyclic stretch. Cyclic stretch increased the secretion of AngII (angiotensin II) and resistin from cultured VSMCs. Exogenous AngII increased resistin expression, and AngII receptor inhibition attenuated this effect. In conclusion, cyclic mechanical stretch increases resistin expression in cultured rat VSMCs. Stretch-induced resistin expression is mediated through ROS, and the p38 MAPK and STAT3 pathways. Therefore resistin induced by cyclic stretch may contribute to the pathogenesis of atherosclerosis under haemodynamic overload.

Key words: atherosclerosis, mitogen-activated protein kinase (MAPK), reactive oxygen species (ROS), resistin, smooth muscle cell, signal transducer and activator of transcription (STAT), stretch.

Abbreviations: AngII, angiotensin II; AP1, activator protein 1; AT_1 receptor, AngII type 1 receptor; Dp44mT, 2,2′-dipyridyl-*N*,*N*-dimethylsemicarbazone; EMSA, electrophoretic mobility-shift assay; ERK, extracellular-signal-regulated kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HIF1α, hypoxia-inducible factor 1α; MAPK, mitogen-activated protein kinase; NAC, *N*-acetylcysteine; NFATc, nuclear factor of activated T-cells family c; NF-κB, nuclear factor κB; ROS, reactive oxygen species; RT, reverse transcription; siRNA, small interfering RNA; STAT, signal transducer and activator of transcription protein; VSMC, vascular smooth muscle cell.

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INTRODUCTION

Resistin is an adipocytokine and has been shown to have potent pro-inflammatory properties [1]. In addition, the protein promotes endothelial cell activation [2], causes endothelial dysfunction of porcine coronary arteries [3], and has been shown suppress the expression of eNOS (endothelial NO synthase) and induce superoxide anion production in endothelial cells [4]. Resistin was found to have a potential role in atherosclerosis because the protein increased pro-inflammatory cytokine expression in vascular endothelial cells [5] and promoted VSMC (vascular smooth muscle cell) proliferation [6]. In the atheroma, resistin may contribute to atherogenesis by virtue of its effects on vascular endothelial cells and smooth muscle cells [7]. More recently, hyper-resistinaemia was found to be associated with hypertension in patients with Type 2 diabetes [8] and was associated with carotid atherosclerosis in hypertensive patients [9]. Therefore resistin may represent a novel link between metabolic signals, inflammation and atherosclerosis [10]. Although resistin plays a role in atherosclerosis, the molecular mechanism of its regulation remains unclear.

VSMCs are the major cellular components of the blood vessel wall and are subjected to a dynamic mechanical environment modulated by pulsatile pressure and oscillatory shear forces. The accompanying stress may regulate normal vascular tone and contribute to atherogenesis, vascular hypertrophy associated with hypertension and acute rupture of atherosclerotic lesions [11]. High blood pressure will increase vessel wall tension and stretch the vascular wall. How cyclic mechanical stretch affects the regulation of resistin in smooth muscle cells has not been characterized previously. Therefore the aim of the present study was to investigate the cellular and molecular mechanisms regulating resistin expression by cyclic mechanical stretch in VSMCs and to determine the possible signalling pathways underlying this effect.

MATERIALS AND METHODS

VSMC culture

Primary cultures of VSMCs were grown by the explant technique from the thoracic aorta of 200–250 g male Wistar rats, as described previously [12,13]. Cells were cultured in medium 199 containing 20 % (v/v) fetal bovine serum, 0.1 mmol/l non-essential amino acids, 1 mmol/l sodium pyruvate, 4 mmol/l L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin at 37 °C under 5 % CO_2/95 % air in a humidified incubator. When confluent, VSMC monolayers were passaged every 6–7 days after trypsinization, and were used for experiments from the passages 3–6. These cells were incubated for an additional 2 days to render them quiescent before the initiation of each experiment.

The study conforms to Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The study was reviewed and approved by the Institutional Animal Care and Use Committee of the Shin Kong Wu Ho-Su Memorial Hospital.

In vitro cyclic strain on cultured VSMCs

The strain unit Flexcell FX-2000 consists of a vacuum unit linked to a valve controlled by a computer program. VSMCs cultured on the flexible membrane base were subjected to cyclic stretch produced by this computer-controlled application of sinusoidal negative pressure at a frequency of 1 Hz (60 cycles/min) for various periods of time. VSMCs cultured on the flexible membrane base but not subjected to stretch were used as controls. The cells were placed in a humidified incubator with an atmosphere of 5 % CO_2 at 37 °C. Pre-treatment of cells with different inhibitors [SP600125 (20 μmol/l; Calbiochem), a potent cell-permeant selective and reversible inhibitor of JNK (c-Jun N-terminal kinase); SB203580 (3 μmol/l; Calbiochem), a highly specific cell-permeant inhibitor of p38 MAPK (mitogen-activated protein kinase); and PD98059 (50 μmol/l; Calbiochem), a specific and potent inhibitor of ERK (extracellular-signal-regulated kinase) kinase] was performed 30 min prior to cyclic stretch. NAC (*N*-acetylcysteine; 500 μmol/l; Calbiochem) is a free radical scavenger.

Western blot analysis

VSMCs exposed to cyclic stretch at 10 or 20 % elongation were harvested by scraping and were then centrifuged (300 ***g***) for 10 min at 4 °C. The pellet was resuspended and homogenized in reporter lysis buffer (Promega), followed by centrifugation at 10 600 ***g*** for 20 min at 4 °C. The protein content of the supernatant was determined using the Bio-Rad Laboratories protein assay using BSA as the standard. Western blot analysis was performed as described previously [13]. Polyclonal rabbit anti-(rat resistin) (Chemicon), polyclonal anti-(p38 MAPK) (Cell Signaling) and monoclonal anti-(phospho-p38 MAPK) antibodies were used.

Real-time RT (reverse transcription)–PCR

Total RNA from cultured VSMCs was extracted using the single-step acid guanidinium thiocyanate/phenol/chloroform extraction method. Real-time RT–PCR was performed as described previously [13]. The rat resistin primers were 5′-ACTTCAGCTCCCTACTG-3′ and 5′-GTCTATGCTTCCGCACT-3′.

RNA interference

Cultured VSMCs were transfected with 800 ng of p38 MAPK-annealed siRNA (small interfering RNA; Dharmacon) or resistin siRNA oligonucleotide

(Invitrogen). The p38 MAPK and resistin siRNAs are target-specific 20–25 nt siRNAs designed to knockdown gene expression of p38α MAPK and resistin. p38α MAPK sense and antisense of siRNA sequences were 5′-GUCAUCGGUAAGCUUCUGACUU-3′ and 5′-PUCAUCGGUAAGCUUCUGACUU-3′ respectively. Resistin sense and antisense of siRNA sequences were 5′-ACACAUUGUAUCCUCACGGACGUCCC-3′ and 5′-GGACGUCCGUGAGGATACAAUGUGU-3′ respectively. As a negative control, a non-targeting siRNA (scrambled siRNA) purchased from Dharmacon was used. VSMCs were transfected with siRNA oligonucleotides using Effectene transfection reagent (Qiagen) according to the manufacturer's recommendations.

Assay of ROS (reactive oxygen species)

ROS production was measured using the cell-permeant probe 2′-7′-dichlorodihydrofluorescin diacetate, which passively diffuses into cells where intracellular esterases cleave the acetate groups to form the impermeant $DCFH_2$ (2′,7′-dichlorofluorescein), which remains trapped within the cell [14]. After cyclic stretch, VSMCs were collected by trypsinization and resuspended in PBS. The ROS assay was performed according to the manufacturer's instruction (Invitrogen). Fluorescence microscopy was used to detect the green fluorescence.

EMSA (electrophoretic mobility-shift assay)

Nuclear protein concentrations from cultured VSMCs were determined using the Bio-Rad Laboratories protein assay. Consensus and control oligonucleotides (Research Biolabs) were labelled by polynucleotide kinase incorporation of [γ^{32}P]ATP. Oligonucleotides sequences of STAT3 (signal transducer and activator of transcription protein 3) were consensus 5′-GATCCTTCTGGGAATTCCTAGATC-3′. The mutant oligonucleotides sequences were 5′-GATCCTTCTGGGCCGTCCTAGATC-3′. The EMSA was performed as described previously [15]. Controls were performed in each case with mutant oligonucleotides or unlabelled oligonucleotides to compete with labelled sequences.

Promoter activity assay

A -741 to $+22$ bp rat resistin promoter construct was generated as follows. Rat genomic DNA was amplified with forward primer (5′-CTCAGCGGTAGAGCTCTTGCCTAGG-3′) and reverse primer (5′-GGAGAAATGAAAGGTTCTTCATCCTGTTA-3′). The amplified product was digested with MluI and BglII restriction enzymes and ligated into pGL3-basic luciferase plasmid vector (Promega) digested with the same enzymes. The resistin promoter contains STAT3-conserved sites (AAT) at -622 to -620 bp. For the mutant, the STAT3 sites were mutated using a Stratagene mutagenesis kit. Site-specific mutations were confirmed by DNA sequencing. Plasmids were transfected into VSMCs using a low-pressure-accelerated gene gun (Bioware Technologies), essentially following the protocol from the manufacturer. Briefly, 2 μg of plasmid DNA was suspended in 5 μl of PBS and was delivered to the cultured VSMCs at a helium pressure of 15 psi (where 1 psi = 6.9 kPa). The transfection efficiency using this method is 30 %. Following cyclic stretch for 2 h, cell extracts were prepared using the dual-luciferase reporter assay system (Promega) and were measured for dual luciferase activity by luminometer (Turner Designs).

Measurement of resistin and AngII (angiotensin II) concentrations

Conditioned medium from cultured VSMCs after cyclic stretch and those from control cells (without stretch) were collected for resistin and AngII measurements. The levels of resistin and AngII were measured by a quantitative sandwich enzyme immunoassay technique (Phoenix Pharmaceutical). The lower limit of detection of resistin and AngII was 0.07 ng/ml. Both the intra- and inter-observer coefficients of variance were < 10 %.

Proliferation and migration assays

The proliferation of VSMCs was determined using [^{3}H]thymidine incorporation. VSMCs were seeded on to ViewPlate (Packard Instruments) for 60 min at a density of 5×10^3 cells/well in serum-free medium and were incubated overnight. Recombinant resistin (20 μg/ml), resistin siRNA, NAC or Dp44mT [2,2′-dipyridyl-*N*,*N*-dimethylsemicarbozaone (30 μmol/l); Calbiochem] was added to the plate. Cells were pre-incubated with siRNA for 16 h before performing the migration and proliferation assays. Thymidine uptake was studied by the addition of 500 nCi/ml [^{3}H]thymidine (Perkin Elmer) for 6 and 18 h. Cells were washed twice with PBS, MicroScint-20 (50 μl) was added, and the plate was read with a TopCount (Packard Instrument).

The migration activity of VSMCs was determined using the growth-factor-reduced Matrigel invasion system (Becton Dickinson), following the protocol provided by the manufacturer. The migration assay was performed as described previously [16].

Statistical analysis

Values are expressed as means $\pm$ S.D. Statistical significance was performed with ANOVA (GraphPad). The Dunnett's test was used to compare multiple groups to a single control group. The Tukey–Kramer comparison test was used for pairwise comparisons between multiple groups after ANOVA. A value of $P < 0.05$ was considered to denote statistical significance.

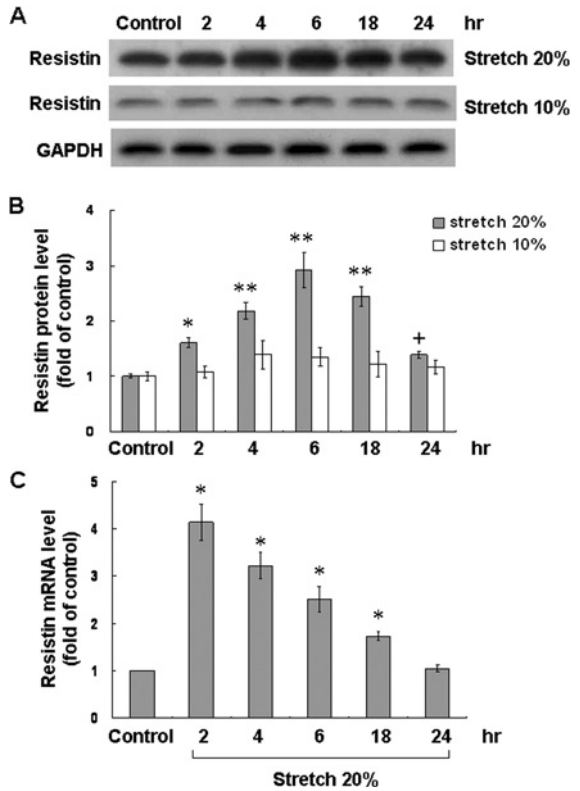

Figure 1 Cyclic stretch increases resistin protein and mRNA expression in VSMCs

(A) Representative Western blots of resistin in VSMCs subjected to cyclic stretch at 10 or 20% elongation for various periods of time. (B) Quantitative analysis of resistin protein levels. The values from stretched VSMCs have been normalized to values in control cells, and the results from four independent experiments are shown. $^{+}P < 0.05$, $^{*}P < 0.01$ and $^{**}P < 0.001$ compared with controls. (C) Fold increases in resistin mRNA induced by cyclic stretch at 20% elongation for various periods of time. The values from stretched VSMCs have been normalized to matched GAPDH (glyceraldehyde-3-phosphate dehydrogenase) measurements and are then expressed as a ratio of normalized values to mRNA in control cells ($n = 3$–4 per group). $^{*}P < 0.01$ compared with control.

RESULTS

Cyclic stretch enhances resistin protein and mRNA expression in cultured VSMCs

To test the effect of cyclic stretch on the resistin expression, stretch at 10 and 20% elongation were used. The levels of resistin protein began to increase as early as 2 h after stretch when 20% elongation was applied, reached a maximum of 2.9-fold over the control by 6 h and remained elevated up to 24 h (Figure 1). Stretch-induced resistin protein expression was load-dependent. When VSMCs were stretched at 10% elongation, the levels of resistin protein did not increase significantly compared with control cells without stretch (Figures 1A and 1B). The levels of resistin mRNA also significantly increased from 2 to 18 h after cyclic stretch (Figure 1C).

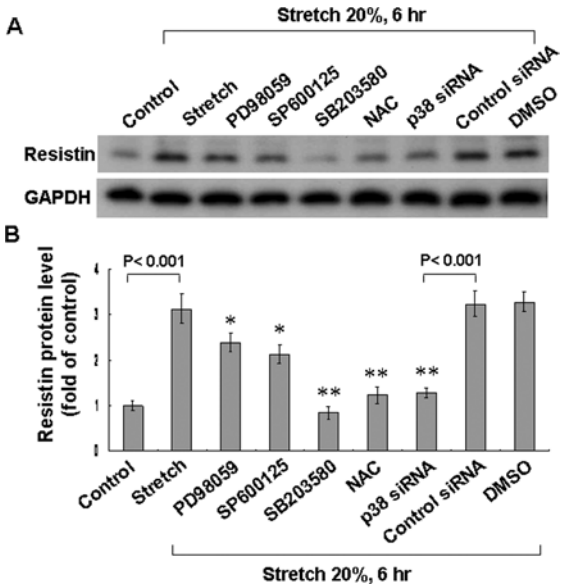

Figure 2 ROS and p38 MAPK are important regulators that mediate stretch-induced resistin expression in VSMCs

(A) Representative Western blots of resistin protein levels in VSMCs subjected to cyclic stretch for 6 h or control cells without stretch in the absence or presence of different inhibitors and siRNA. (B) Quantification of resistin protein levels. The values from stretched VSMCs have been normalized to values in control cells ($n = 4$ per group). $^{*}P < 0.01$ and $^{**}P < 0.001$ compared with stretch.

Cyclic stretch-induced resistin protein expression in VSMCs is mediated by ROS and p38 MAPK

To investigate the possible signalling pathways mediating stretch-induced resistin expression, different inhibitors were used. Western blot analysis demonstrated that the cyclic-stretch-induced increase in resistin protein was completely attenuated after the addition of SB203580 or NAC 30 min before stretch (Figure 2). Resistin protein induction by stretch was partially attenuated by the addition of PD98059 or SP600125. DMSO as the vehicle for PD98059 did not affect resistin expression induced by stretch. p38α MAPK siRNA also completely blocked resistin expression induced by stretch, whereas the control siRNA had no effect. These findings indicate that ROS and p38 MAPK pathways are important regulators in mediating resistin expression induced by cyclic stretch.

As shown in Figure 3, cyclic stretch for 2–6 h significantly increased ROS production as determined using a ROS assay with a fluorescent microscope. The increase in ROS by cyclic stretch was transient because ROS production declined after stretch for 18 h. Pre-treatment with NAC significantly blocked the induction of ROS by cyclic stretch. In the control group without stretch, very few VSMCs expressed green fluorescence. Exogenous addition of resistin at 20 μg/ml significantly increased ROS

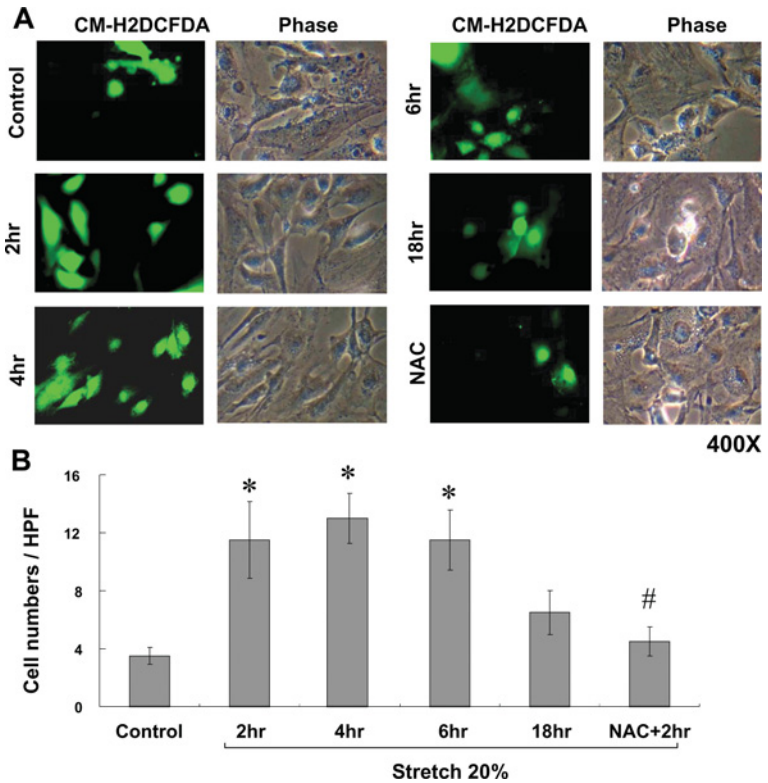

Figure 3 Effect of cyclic stretch on ROS formation in VSMCs

(A) Representative microscopic images from the ROS assay with {left-hand panels; CM-H2DCFDA [5-(and-6)-chloromethyl-2′7′-dichlorodihydrofluorescein diacetate acetyl ester]} or without (right-hand panels; Phase) green fluorescence in VSMCs subjected to cyclic stretch for 2–18 h, or control cells without stretch, in the absence or presence of NAC. (B) Quantification of the positive fluorescent cells ($n = 4$ per group). $^{*}P < 0.001$ compared with control; $^{\#}P < 0.001$ compared with 2 h. HPF, high-power field.

production (see Supplementary Figure S1 available at http://www.ClinSci.org/cs/118/cs1180221add.htm). To block the effect of AngII, an antibody against AngII was added to the conditioned medium generated from VSMCs following stretch. ROS was produced after the inhibition of AngII, as shown in Supplementary Figure S1. This finding implicates the autocrine effect of resistin on ROS production in rat VSMCs.

As shown in Figures 4(A) and 4(B), phosphorylated p38 MAPK was maximally induced at 2 h after cyclic stretch and remained elevated until 24 h. Phosphorylation of p38 MAPK was abolished by pre-treatment with SB203580 or NAC. p38α MAPK siRNA knocked-down p38 protein expression 72% (from 2.8-fold to 0.78-fold).

Exogenous addition of AngII at 10 nmol/l significantly increased resistin protein expression. Increased resistin expression induced by AngII was similar to that by cyclic stretch (Figure 5). Addition of losartan (100 nmol/l), an antagonist of the AT_1 receptor (AngII type 1 receptor), and an antibody against AngII for 30 min before cyclic stretch completely inhibited the cyclic-stretch-induced increase in resistin. This finding indicates that AngII mediated the increase in resistin by cyclic stretch through the AT_1 receptor. Addition of Dp44mT alone without stretch also significantly increased resistin expression. Addition of p38α MAPK siRNA 30 min before Dp44mT treatment completely attenuated the increase in resistin induced by Dp44mT, indicating that ROS was activated before p38 MAPK stimulation.

Cyclic stretch increases STAT3-binding activity

Cyclic stretch of cultured VSMCs for 2–24 h significantly increased the DNA–protein-binding activity of STAT3 (Figure 6). An excess of unlabelled STAT3 oligonucleotide competed with the probe for binding STAT3 protein, whereas an oligonucleotide containing a 3-bp substitution in the STAT3-binding site did not compete for binding. Addition of SB203580 and NAC 30 min before stretch abolished the DNA–protein-binding activity induced by stretch.

Cyclic stretch increases resistin promoter activity

The rat resistin promoter construct contains STAT3-, AP1 (activator protein 1)-, NFATc (nuclear factor of

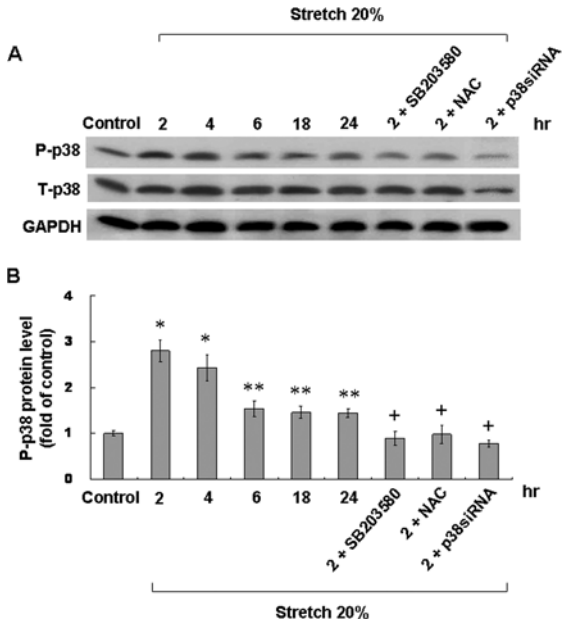

Figure 4 Effect of cyclic stretch on phosphorylation of p38 MAPK in VSMCs

(A) Representative Western blots of phosphorylated (P-p38) and total p38 (T-p38) MAPK in VSMCs after cyclic stretch at 20 % elongation for various periods of time and in the presence of SB203580, NAC and p38α MAPK siRNA. (B) Quantification of phosphorylated p38 MAPK. The values from stretched VSMCs have been normalized to matched GAPDH and the corresponding total protein measurement, and are then expressed as a ratio of normalized values to each phosphorylated protein in control cells. Values are from four independent experiments. $^{*}P < 0.001$ and $^{**}P < 0.01$ compared with controls; $^{+}P < 0.001$ compared with 2 h.

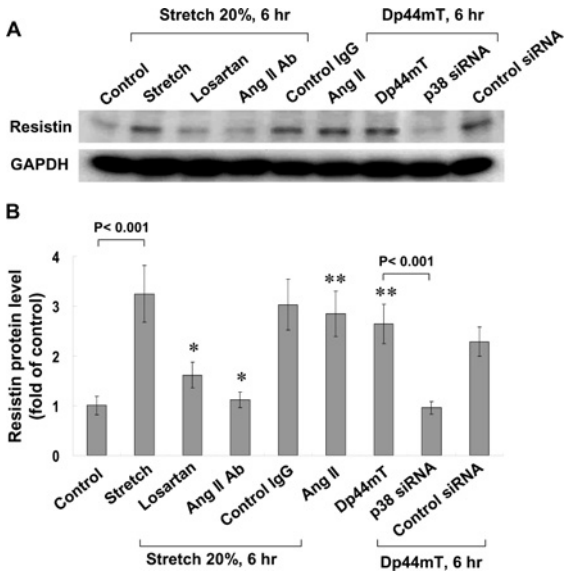

Figure 5 AngII mediates the increase in resistin by cyclic stretch through the AT_1 receptor

(A) Representative Western blots of resistin in VSMCs subjected to cyclic stretch at 20 % elongation for 6 h or without stretch in the absence or presence of inhibitors. (B) Quantification of resistin protein levels. The values from stretched VSMCs have been normalized to values in control cells, and the values are from four independent experiments. $^{*}P < 0.01$ and $^{**}P < 0.001$ compared with controls.

activated T-cells family c)-, NF-κB (nuclear factor κB)- and HIF1α (hypoxia-inducible factor 1α)-binding sites. Cyclic stretch for 2 h significantly increased the resistin promoter activity 5.3-fold compared with control cells without stretch (Figure 6C). Addition of SB203580, NAC and losartan 30 min before stretch abolished the increase in resistin promoter activity. Addition of AngII alone without stretch significantly increased resistin promoter activity similar to cyclic stretch. When the STAT3-binding sites were mutated, the increased promoter activity induced by cyclic stretch and AngII was abolished. This finding indicates that cyclic stretch regulates resistin in VSMCs at the transcriptional level and that STAT3-binding sites in the resistin promoter are essential for the transcriptional regulation.

Cyclic stretch stimulates the secretion of resistin and AngII from VSMCs

As shown in Figure 7, cyclic stretch significantly increased resistin secretion from VSMCs beginning at 2 h after cyclic stretch and remaining elevated for 24 h. The mean concentration of resistin rose from 92 ± 7 pg/ml before stretch to 547 ± 32 pg/ml after stretch for 6 h ($P < 0.001$). AngII was also secreted from stretched VSMCs from 62 ± 13 ng/ml before stretch to 138 ± 29 ng/ml after stretch for 4 h ($P < 0.001$).

Resistin increases proliferation and migration of VSMCs

Rat VSMCs cultured in the conditioned medium generated from stretched cells migrated significantly through the filter membrane compared with those cultured in non-conditioned medium (Figures 8A and 8B). Both inhibition of resistin activity by siRNA and free radical activity by NAC decreased VSMC migration activity. Resistin siRNA significantly knocked-down resistin protein expression in VSMCs (see Supplementary Figure S2 available at http://www.ClinSci.org/cs/118/cs1180221add.htm). Rat VSMCs also migrated significantly through the filter membrane after exogenous addition of recombinant resistin (results not shown) and Dp44mT compared with the control group without treatment. The migration activity of VSMCs was similar in control and scrambled siRNA-treated groups. Conditioned medium generated from stretched cells and exogenous addition of Dp44mT significantly increased thymidine incorporation compared with cells cultured in non-conditioned medium (Figure 8C). Both inhibition of resistin activity by siRNA and free radical activity by NAC decreased thymidine incorporation. These findings suggest that resistin mediates the proliferation and migration of VSMCs induced by cyclic stretch.

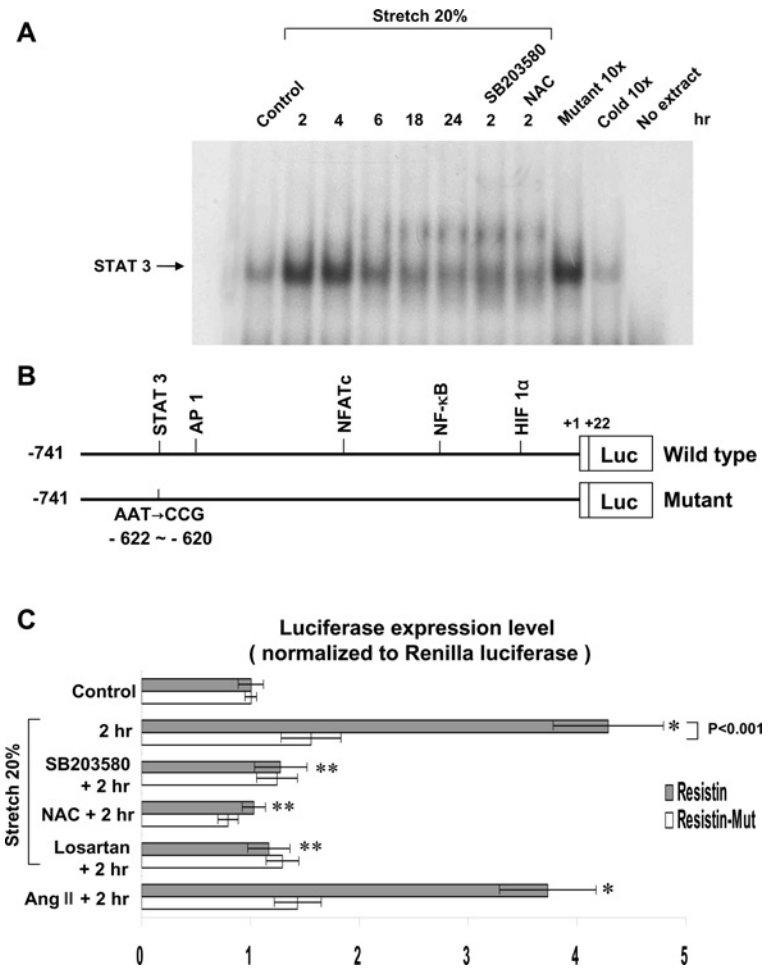

Figure 6 Cyclic stretch increases STAT3-binding activity and resistin promoter activity

(A) A representative EMSA showing protein binding to the STAT3 oligonucleotide in nuclear extracts of VSMCs after cyclic stretch for various periods of time and in the presence of inhibitors. Similar results were found in another two independent experiments. Cold oligo, unlablelled STAT3 oligonucleotides. (B) Constructs of the resistin promoter gene. +1, initiation site for resistin transcription; Luc, luciferase. (C) Quantification of resistin promoter activity. VSMCs were transiently transfected with pResistin-Luc using a gene gun. The luciferase activity in cell lysates was measured and normalized to *Renilla* activity ($n = 3$ per group). $^{*}P < 0.001$ compared with control; $^{**}P < 0.001$ compared with 2 h. Mut, mutant.

DISCUSSION

In the present study, we have demonstrated several significant findings. First, cyclic stretch up-regulates resistin expression and increases the secretion of resistin in rat VSMCs; secondly, AngII acts as an autocrine factor in mediating the increase in resistin expression induced by cyclic stretch; thirdly, oxidative stress, p38 MAPK and the STAT3 transcription factor are involved in the signalling pathway resulting in resistin induction; and fourthly, resistin increases the migration and proliferation of rat VSMCs. Resistin in VSMCs was up-regulated in both a time- and load-dependent manner by cyclic stretch. As rhythmic distension of the vessel wall is a component of pulsatile flow, our present findings clearly indicate that haemodynamic forces play a crucial role in the modulation of resistin expression in VSMCs.

The induction of resistin protein by cyclic stretch was largely mediated by ROS and p38 MAPK pathways because the potent antioxidant NAC and the specific and potent inhibitor of an upstream p38 MAPK SB203580 completely inhibited the induction of resistin protein. Cyclic stretch at 20% elongation increased ROS formation in VSMCs and NAC reduced the ROS formation induced by cyclic stretch. A ROS-generating agent, Dp44mT, increased resistin protein expression similar to cyclic stretch. This signalling pathway of p38 MAPK was confirmed further by the finding that p38α MAPK siRNA inhibited the induction of resistin protein by cyclic stretch. Cyclic stretch increased p38 MAPK phosphorylation, which was inhibited by NAC. p38α MAPK siRNA completely attenuated the increase in resistin by the ROS-generating agent, indicating that cyclic stretch activates ROS upstream of p38 MAPK.

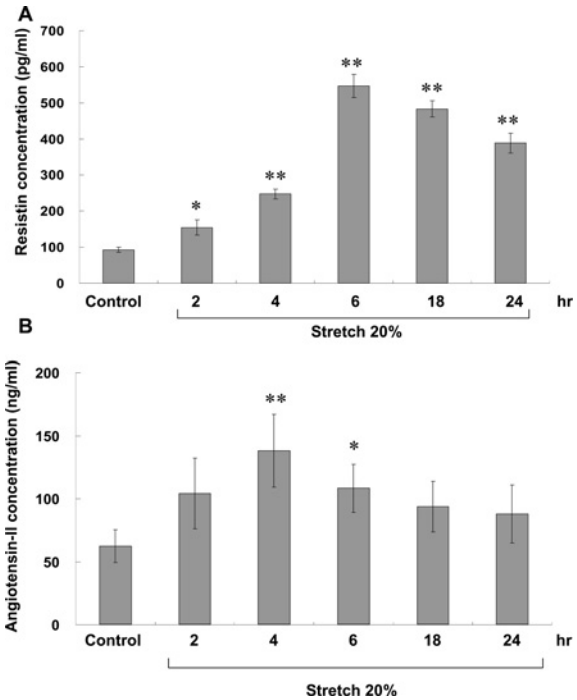

Figure 7 Cyclic stretch increases the release of resistin and AngII from VSMCs subjected to cyclic stretch at 20% elongation for various periods of time

Cultured VSMCs were stretched for various periods of time and the cultured medium was collected for measurement of resistin (A) and AngII (B) via immunoassay ($n = 5$ per group). $^{*}P < 0.05$ and $^{**}P < 0.001$ compared with control.

ROS play essential roles in signal transduction and physiological regulation of vascular function. We have demonstrated previously that ROS and ERK kinase mediate resistin expression by hypoxia in VSMCs [18]. In cardiomyocytes, cyclic-stretch-induced resistin is mediated by the ERK kinase and NF-κB pathways [19]. The signalling pathways mediating resistin expression may be different in different cell types and experimental models; however, the functional consequence of resistin up-regulation in VSMCs and cardiomyocytes is the same. Resistin up-regulation decreases glucose uptake in both cells types and experimental models. These results confirm that resistin functions as a mediator of insulin resistance.

Mechanical stretch may induce secretion or synthesis of bioactive molecules from VSMCs [11]. In the present study, we have demonstrated that stretched VSMCs secreted AngII. Although losartan has many potential effects besides AT_1 receptor antagonism [20,21], both an antibody against AngII and losartan blocked the increases in resistin protein induced by cyclic stretch. This finding indicates that AngII through the AT_1 receptor mediates the increase in resistin induced by stretch. The increase in resistin protein expression by AngII was similar to that by stretch. These results provide the first evidence for AngII mediating cyclic-stretch-induced expression of resistin in rat VSMCs. These results have established autocrine or paracrine production in rat VSMCs in response to cyclic stretch. Actually, VSMCs have been shown to secrete AngII in response to high glucose and mechanical stretch [13,23]. The autocrine role of AngII in VSMCs has also been reported previously [11,23]. In the present study, we confirmed further the autocrine role of AngII in VSMCs. Zou et al. [24] have demonstrated that mechanical stress activates the AT_1 receptor without the involvement of AngII in cardiomyocytes during a short period of stretch (5–8 min). In the present study, we have used longer periods (more than 2 h) of mechanical stress in VSMCs. Thus different periods of stress and cell types may have activated different mechanisms in response to mechanical stretch.

Biomarkers that integrate metabolic and inflammatory signals are powerful candidates for defining the risk of atherosclerotic cardiovascular disease [25]. Hyperresistinaemia impairs glucose tolerance and induces hepatic insulin resistance in rodents [26], whereas mice deficient in resistin are protected from obesity-associated insulin resistance [27]. Our present study using a VSMC culture system has demonstrated an impairment of glucose transport by resistin. Glucose uptake in VSMCs was reduced by resistin up-regulation (results not shown). Impairment of glucose transport may explain the potential mechanism of resistin induction in insulin resistance. In the present study, we have demonstrated that cyclic stretch increased resistin secretion from VSMCs, indicating that this protein plays an autocrine or paracrine function in VSMCs. Resistin secreted from stretched VSMCs increased migration and proliferation of VSMCs. Antioxidant and resistin siRNA attenuated the migration and proliferation induced by stretch and resistin. These findings suggest that resistin may play an important role in the remodelling of vascular disease.

Oxidative stress has been demonstrated to modulate STATs [28,29]. Many of the effects of STATs involve the direct binding to DNA and transcriptional activation of target genes. STAT3 plays an important role in vascular wall remodelling after arterial injury [30]. We have demonstrated previously that hypoxia generates ROS in VSMCs [18]. In the present study, we have demonstrated that the stimulation of STAT3 DNA-binding activity by cyclic stretch required at least p38 MAPK phosphorylation and ROS, as p38 MAPK inhibition and NAC abolished STAT3-binding activity. We have demonstrated further that cyclic stretch increased resistin promoter activity, and the binding site of STAT3 in the resistin promoter is essential for transcriptional regulation. Taken together, our present results indicate that cyclic stretch may increase STAT3 transcriptional activity in VSMCs. As STAT3 has an apoptotic effect and promotes cellular proliferation [31], the essential role of STAT3

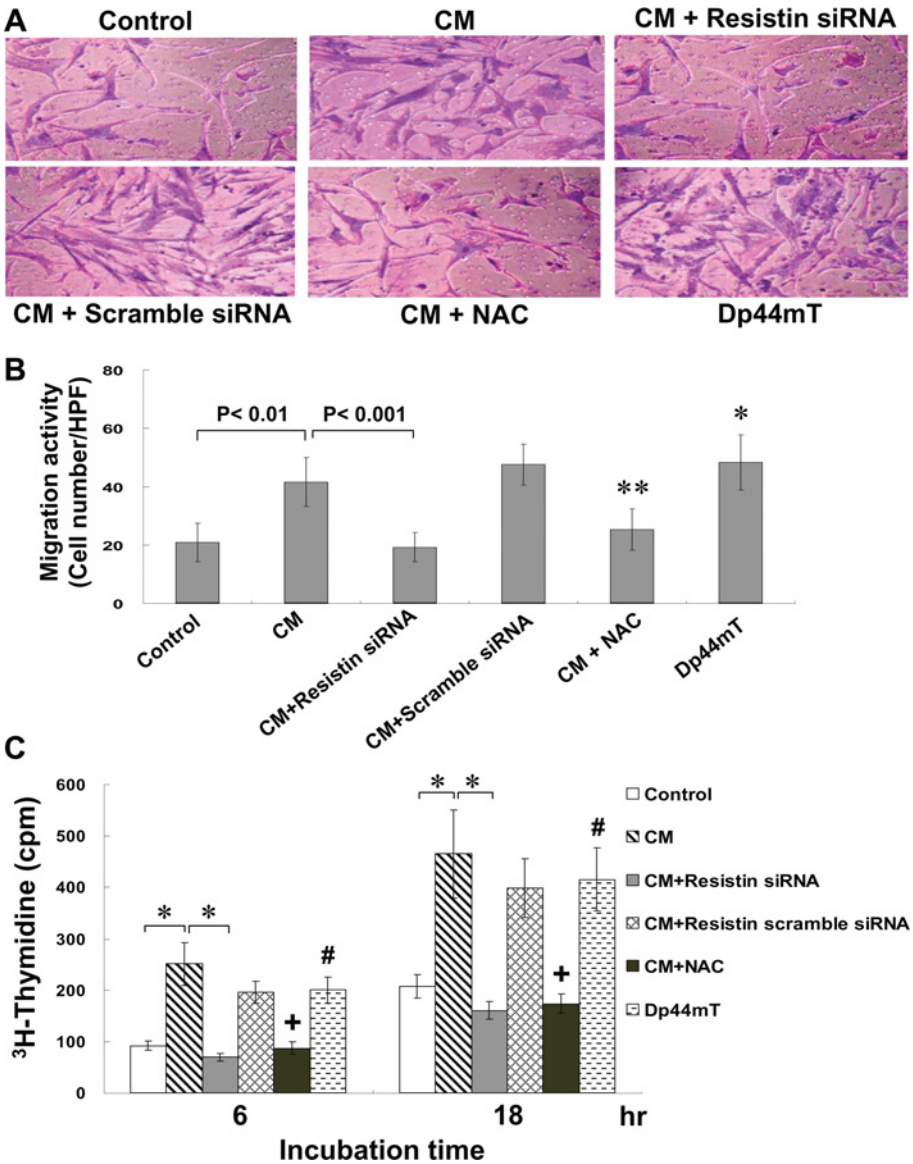

Figure 8 Effect of conditioned medium on the migration and proliferation of VSMCs

(A) Conditioned medium (CM) was obtained from VSMCs after cyclic stretch for 18 h. VSMCs migrating through the filter were stained. (B) The stained VSMCs were counted in four fields under a 400 × high-power field (HPF) ($n = 3$ per group). $^{*}P < 0.001$ compared with control; $^{**}P < 0.01$ compared with conditioned medium. (C) Proliferation of VSMCs was measured by [^{3}H]thymidine incorporation. $^{+}P < 0.001$ compared with conditioned medium; $^{\#}P < 0.01$ compared with control ($n = 3$ per group).

in the stretch model of VSMCs may contribute to the proliferation of VSMCs induced by cyclic stretch [11].

In summary, our present study reports for the first time that cyclic stretch enhances resistin expression in cultured rat VSMCs. Stretch-induced resistin expression is mediated through the ROS, p38 MAPK and STAT3 pathways. Resistin also increases migration and proliferation of VSMCs. Therefore resistin induced by cyclic stretch may contribute to the pathogenesis of atherosclerosis under haemodynamic overload.

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online data

SUPPLEMENTARY ONLINE DATA

Regulation of resistin by cyclic mechanical stretch in cultured rat vascular smooth muscle cells

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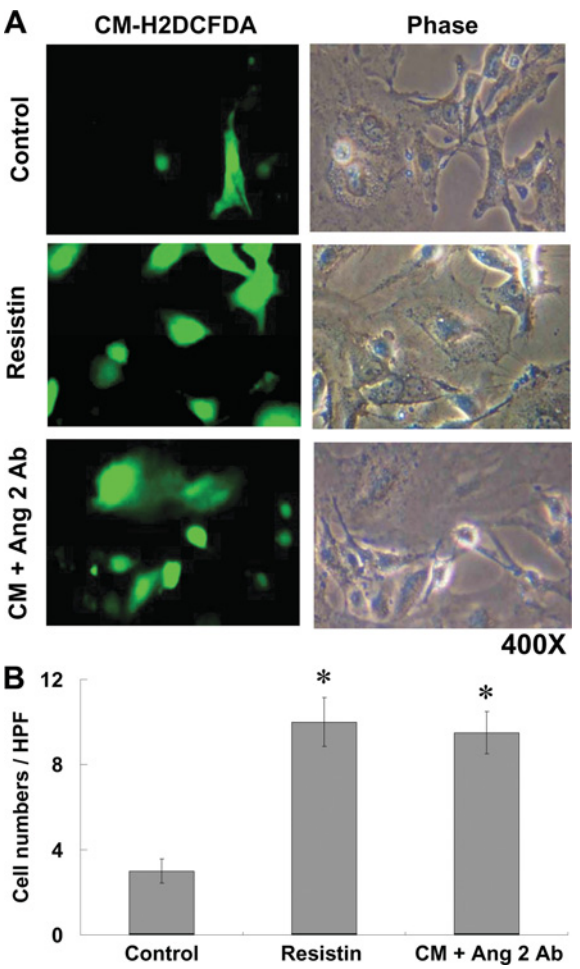

Figure S1 Effect of resistin on ROS formation in VSMCs

(A) Representative microscopic image from the ROS assay with (left-hand panels) or without (right-hand panels) green fluorescence in VSMCs subjected to resistin, conditioned medium (CM) with an antibody against AngII (AngII Ab) or control cells. (B) Quantification of fluorescent-positive cells ($n = 4$ per group). $^*P < 0.001$ compared with control.

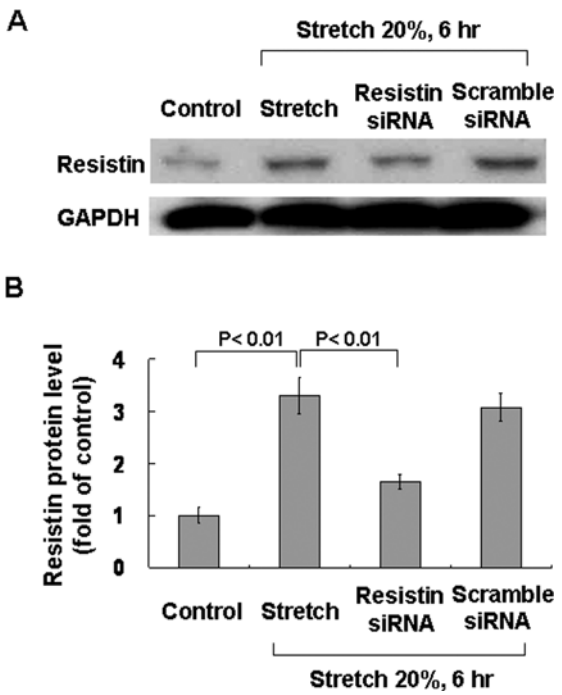

Figure S2 Effect of resistin siRNA on resistin protein expression

(A) Representative Western blots for resistin in VSMCs subjected to cyclic stretch at 20 % elongation for 6 h in the absence or presence of siRNA. (B) Quantitative analysis of resistin protein levels. The values from stretched VSMCs have been normalized to values in control cells ($n = 3$ per group).

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