Regulation of hypoxia-inducible factor-1α by cyclical mechanical stretch in rat vascular smooth muscle cells

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ABSTRACT

Vascular smooth muscle cells (VSMCs) are exposed to hormonal and mechanical stress in vivo. Hormonal factors have been shown to affect hypoxia-inducible factor-1α (HIF-1α). How mechanical stress affects the regulation of HIF-1α in VSMCs has not been reported previously, and therefore we sought to investigate the regulation of HIF-1α by cyclical mechanical stretch in cultured rat VSMCs. Rat VSMCs grown on a flexible membrane base were stretched by vacuum to 20% of the maximum elongation at 60 cycles/min. The levels of HIF-1α protein began to increase as early as 2 h after stretch was applied and reached a maximum of 2.8-fold over the control by 4 h. Real-time PCR showed that the levels of HIF-1α mRNA increased 2.1-fold after cyclical stretch for 4 h. Cyclical mechanical stretch also increased the immunohistochemical labelling of HIF-1α in VSMCs after cyclical stretch for 4 h. The phosphorylation of p42/p44 mitogen-activated protein kinase (MAP kinase) increased after stretch and this was inhibited by the MAP kinase kinase inhibitors PD98059 and U0126. PD98059 and U0126 also blocked HIF-1α gene expression induced by cyclical stretch. In conclusion, cyclical mechanical stretch activates the gene expression of HIF-1α in cultured VSMCs and this mechanical effect is possibly mediated by the p42/p44 MAP kinase pathway.

INTRODUCTION

Hypoxia-inducible factor-1 (HIF-1), a DNA-binding complex first identified as a factor critical for the inducible activity of the erythropoietin 3′ enhancer [1], is now recognized to be a key physiological regulator of gene expression that responds to changes in cellular oxygen tension [2]. HIF-1 is a heterodimeric DNA complex composed of two basic helix–loop–helix–Per-aryl hydrocarbon receptor nuclear translocator (ARNT)-Sim proteins (HIF-1α and HIF-1β). The HIF-1α subunit is an 826-amino-acid protein that is unique to HIF-1, whereas HIF-1β is identical with the ARNT protein and can dimerize with other basic helix–loop–helix proteins [3]. HIF-1α protein levels, which determine the level of HIF-1 DNA-binding and transcriptional activity, increase exponentially as cellular oxygen concentration is reduced [4]. Under hypoxic conditions, both HIF-1α protein levels and activity of the HIF-1α transactivation domains increase [4,5]. Pugh et al. [6] have demonstrated that sequences from HIF-1α, but not HIF-1β, convey hypoxia-inducible activity when fused to the...
DNA-binding domain of heterogeneous transcription factors. The HIF-1α gene has been shown to be involved in tumour angiogenesis and growth [7–9], and myocardial hypoxia is a potent inducer of HIF-1α protein expression [10]. Expression of HIF-1α protein is also induced by hypoxia in a variety of pulmonary cell types, including vascular endothelial cells and smooth muscle cells (SMCs), both in vivo and in cell culture [11].

In vascular SMCs (VSMCs), a range of different extracellular receptor agonists have been shown to induce HIF-1α mRNA and protein expression, including angiotensin II, platelet-derived growth factor and thrombin [12]. The involvement of reactive oxygen species in HIF-1α protein expression in VSMCs has only been demonstrated in response to these cytokines [12]. Cells in the cardiovascular system are continually subjected to mechanical forces due to changes in pressure and volume. SMCs 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 [13] and contribute to atherogenesis [14], the vascular hypertrophy associated with hypertension [15] and the acute rupture of atherosclerotic lesions [16,17]. The cyclical strain model system subjects cultured cells to repetitive stretch–relaxation at rates comparable with dynamic stretch overload in vivo. This model has been applied widely to study the molecular mechanisms of gene expression and signal transduction in many cell types [18–20].

How cyclical mechanical stretch affects the regulation of HIF-1α in SMCs has not been characterized previously. Using molecular approaches to study how haemodynamic overload affects neovascularization may provide the fundamental basis for the development of therapeutics. Mechanical induction of HIF-1α has been reported in heart tissue [21]; therefore, we hypothesized that HIF-1α gene may be mechanically responsive in VSMCs. In the present study, we sought to investigate the effect of cyclical stretch on HIF-1α gene expression in VSMCs and investigate the possible mechanism and signal pathways mediating the expression of HIF-1α by cyclical mechanical stretch.

**METHODS**

**VSMC culture**

Primary cultures of VSMCs were grown by the explant technique from the thoracic aorta of 200–250 g male Sprague–Dawley rats, as described previously [22]. Cells were cultured in medium 199 containing 20 % (v/v) fetal calf 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% CO2/95% air in a humidified incubator.

When confluent, VSMC monolayers were passaged every 6–7 days after trypsinization and were used for experiments from the third to sixth passages. These third to sixth passage cells were then cultured in Flexcell I flexible membrane dishes in medium 199 containing 0.5 % fetal calf serum, and the cells were incubated for a further 2 days to render them quiescent before the initiation of each experiment. To determine the role of mitogen-activated protein kinase (MAP kinase) in stretch-induced HIF-1α expression, VSMCs were pretreated with PD98059 (50 µM) or U0126 (25 µM) for 30 min, followed by cyclical stretch. PD98059 is a selective and cell-permeant inhibitor of MAP kinase kinase (MEK), and U0126 is a potent and specific inhibitor of MEK. PD98059 and U0126 were purchased from Calbiochem.

**In vitro cyclical strain on cultured VSMCs**

The strain unit Flexcell FX-2000 (Flexcell International) consists of a vacuum unit linked to a valve controlled by a computer program. VSMCs cultured on the flexible membrane base were subjected to cyclical stretch produced by this computer-controlled application of sinusoidal negative pressure. VSMCs cultured on the flexible membrane base, but not subjected to stretch, were used as control. The flexible membranes supporting the cultured cells were deformed by a sinusoidal negative pressure with a peak level of ±15 kPa at a frequency of 1 Hz (60 cycles/min) for various periods of time. Strain profile analysis indicates that radial strain responds non-linearly to increasing vacuum pressure. At a peak level of ~15 kPa, this stretching model produces a strain on cells ranging from minimal strain at the centre of the membrane to a peak value of 20% at the periphery (with an average strain of 11% elongation) [23]. The cells were placed in a humidified incubator with an atmosphere of 5% CO2 at 37 °C. After the stretch, the total RNA and protein from the stretched cells were collected for real-time reverse transcriptase–PCR and Western-blot analysis. Pretreatment of cells with different kinds of inhibitor was performed 30 min prior to cyclical stretch.

**Cytotoxicity studies**

VSMCs were adjusted to 3×10⁴ cells/ml in medium 199. Aliquots (20 ml) of cell suspension were plated in 40-mm Petri dishes. After incubation for 24 h, the medium was replaced with fresh medium containing PD98059 (5, 50 and 100 µM) or U0126 (5, 25 and 50 µM). After incubation for 24 h, the medium was aspirated and 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide solution was added and the incubation continued for another 4 h. At the end of the incubation period, the medium was removed from the attached cells and the converted dye crystals were dissolved with DMSO. The absorbance was measured at a wavelength of 570 nm.
Western-blot analysis

VSMCs exposed to cyclical stretch (20 % elongation) were harvested by scraping and then centrifuged (300 g) for 10 min at 4 °C. The pellet was resuspended, homogenized in a Reporter Lysis Buffer (Promega) and centrifuged at 10 600 g for 20 min. Protein content of the supernatant was determined by the Bio-Rad Protein Assay using BSA as the standard. Equal amounts of protein (15 µg) were loaded on to a SDS/12.5 % (w/v) polyacrylamide minigel, followed by electrophoresis. Proteins were electroblotted on to nitrocellulose. The blots were incubated overnight in Tris-buffered saline containing 5 % (v/v) dried milk to block non-specific binding of the antibody. Proteins of interest were revealed with specific antibodies (mouse anti-(HIF-1α) monoclonal antibody from BD Biosciences; mouse anti-(vascular endothelial growth factor [VEGF]) monoclonal antibody and rabbit anti-VEGF polyclonal antibody from Santa Cruz Biotechnology; mouse anti-(phospho-p42/p44 MAP kinase) and anti-(total p42/p44 MAP kinase) monoclonal antibodies from Cell Signaling) as indicated (1:1000 dilution) for 1 h at room temperature, followed by incubation with a 1:5000 dilution of horseradish peroxidase-conjugated polyclonal anti-(rabbit IgG) or anti-(mouse IgG) antibodies for 1 h at room temperature. The membrane was then detected with an enhanced chemiluminescence detection system (ECL®, Amershams Biociences). Equal protein loading of the samples was verified further by staining with a mouse anti-(glyceraldehyde-3-phosphate dehydrogenase [GAPDH]) monoclonal antibody (Santa Cruz Biotechnology). All Western blots were quantified using densitometry.

RNA isolation and reverse transcription

Total RNA was isolated from VSMCs using the single-step acid guanidinium thiocyanate/phenol/chloroform extraction method. Total RNA (1 µg) was incubated with 200 units of Moloney murine leukaemia virus reverse transcriptase in a buffer containing a final concentration of 50 mmol/l Tris/HCl (pH 8.3), 75 mmol/l KCl, 3 mmol/l MgCl2, 20 units of RNase inhibitor, 1 µmol/l poly(dT) oligomer and 0.5 mmol/l of each dNTP in a final volume of 20 µl. The reaction mixture was incubated at 42 °C for 1 h and then at 94 °C for 5 min to inactivate the enzyme. A total of 80 µl of diethyl pyrocarbonate-treated water was added to the reaction mixture before storage at −70 °C.

Real-time PCR

A Lightcycler (Roche Diagnostics) was used for real-time PCR. cDNA was diluted 1:10 with nuclease-free water. A portion (2 µl) of the solution was used for the Lightcycler SYBR-Green mastermix (Roche Diagnostics) with 0.5 µmol/l primer, 5 mmol/l MgCl2 and 2 µl Master SYBR-Green in nuclease-free water in a final volume of 20 µl. The primers used were as follows: HIF-1α, 5'-d(AGTCGGGACACGCTCAC)-3' (forward) and 5'-d(TGCTGCTTTGTATGGA)-3' (reverse); GAPDH, 5'-d(CATCACCATCTCCAGAGG)-3' (forward) and 5'-d(GGATGAGTTCTGGGCTGCC)-3' (reverse); and VEGF, 5'-d(GAGGGCAATCCAGAGGAGT)-3' (forward) and 5'-d(TGAGAGGATCTGGTTCCCCGAAA)-3' (reverse). The initial denaturation phase for rat HIF-1α and VEGF was 5 min at 95 °C, followed by an amplification phase as detailed below: denaturation at 95 °C for 10 s, annealing at 57 °C for 10 s, elongation at 72 °C for 15 s, detection at 80 °C and for 45 cycles. Amplification, fluorescence detection and post-processing calculation were performed using the Lightcycler apparatus. Individual PCR products were analysed for DNA sequence to confirm the purity of the product.

Immunohistochemical labelling of cultured VSMCs

After cyclical stretch, VSMCs were rinsed with PBS, fixed for 20 min in 4 % paraformaldehyde in PBS at room temperature, rinsed with PBS, and then incubated with 0.3 % H2O2 in methanol for 30 min at room temperature. The cells were rinsed with PBS and incubated in permeabilization solution (0.1 % Triton X-100 in 0.1 % sodium citrate) for 2 min on ice. Cells were incubated in primary antibody [anti-(HIF-1α)] at 1:150 dilution overnight at 4 °C, washed extensively, incubated with secondary antibodies [biotinylated anti-(mouse IgG) antibody] for 1 h, then incubated with avidin–peroxidase for an additional 1 h. The cells were finally examined with a light microscope.

Analysis of VEGF protein treated with antisense oligonucleotide for HIF-1α

We have demonstrated previously [24] that VEGF is induced by cyclical stretch. As HIF-1 is the transcriptional factor for VEGF, we have tested whether HIF-1α mediates an increase in VEGF after cyclical stretch by preparing an antisense oligonucleotide for HIF-1α (CCTCCATGGGCAATCCGTTGCT). As a control, scrambled oligonucleotide (ACTCGTACCCGCGCA- GTTCG) was also prepared. The antisense or scrambled oligonucleotide (7.5 µmol/l of each) was added to VSMCs by use of Effectene transfection reagent (Qiagen) and incubated for 6 h, followed by 48 h of incubation in medium containing 0.5 % (v/v) fetal calf 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 6 h before protein isolation, antisense oligonucleotide, scrambled oligonucleotide or non-Effectene-treated VSMCs were cyclically stretched. Total protein was isolated for Western blotting.
Electrophoretic mobility-shift assay (EMSA)

An EMSA was carried out to detect the formation of HIF-1α–DNA complexes. Nuclear protein concentrations from VSMCs were determined by the Bio-Rad protein assay. Consensus and control oligonucleotides (Santa Cruz Biotechnology) were labelled by polynucleotide kinase incorporation of $[^{32}P]dATP$. Oligonucleotides sequences for HIF-1α consensus were 5′-TCTGTACGTGACCACACTCACC-3′. The HIF-1α mutant oligonucleotides sequences was 5′-TCTGTAAAAGACCACACTCACC-3′. After the oligonucleotide was radiolabelled, the nuclear extract (4 µg of protein in 2 µl of nuclear extract) was mixed with 20 pmol of the appropriate $[^{32}P]$dATP-labelled consensus or mutant oligonucleotide in a total volume of 20 µl for 30 min at room temperature. The samples were then resolved on a 4 % (w/v) polyacrylamide gel. The gel was transferred on to Whatman paper, dried and visualized by autoradiography. Controls were performed in each case with mutant oligonucleotides or unlabelled oligonucleotides to compete with labelled sequences. For supershift analysis, 1 µl of anti-(HIF-1α) antibody was added to the EMSA reaction.

Statistical analysis

Data are expressed as means ± S.E.M. Statistical significance was evaluated using ANOVA, followed by Dunnett’s and Bonferroni tests (GraphPad Software). A value of $P < 0.05$ was considered to denote statistical significance.

RESULTS

Effect of cyclical stretch on HIF-1α protein expression

To test the effect of cyclical mechanical stretch on HIF-1α protein expression, VSMCs were cyclically stretched for various periods of time and HIF-1α protein levels subsequently measured. The level of HIF-1α protein began to increase as early as 2 h after stretch to 20 % elongation was applied and reached a maximum of 2.8-fold over the control by 4 h (Figure 1). The HIF-1α protein returned to the pre-stretch level after 24 h after stretch. The band position of HIF-1α protein after stretch is the same as HIF-1α protein induced by hypoxia on Western blot. Stretch-induced HIF-1α protein expression was load-dependent. When VSMCs were stretched to 10 % elongation for 4 h, HIF-1α protein expression increased 1.6-fold compared with control cells without stretch. VEGF protein also increased 2.6 ± 0.5-, 2.2 ± 0.4-, 2.3 ± 0.3- and 1.6 ± 0.2-fold at 2, 4, 6 and 24 h respectively, after stretch to 20 % elongation compared with control cells without stretch ($P < 0.01$).

Figure 1 Time course of the effect of cyclical stretch on HIF-1α protein expression in VSMCs

(A) Representative Western blot for HIF-1α in VSMCs subjected to cyclical stretch to 20 % elongation for various periods of time. Equal protein loading of the samples was verified by staining with a GAPDH-specific monoclonal antibody. Hypoxic cells were cultured in 5 % $O_2$ for 2 h. (B) Quantification of HIF-1α protein levels in VSMCs as a result of cyclical stretch to 20 % elongation. The values from stretched cells have been normalized to values in control cells of four independent experiments.

Figure 2 Effect of MEK inhibitors on cyclical stretch-induced HIF-1α protein expression

(A) Representative Western blot for HIF-1α in VSMCs subjected to cyclical stretch to 20 % elongation for 4 h in the absence or presence of PD98059 and U0126. (B) Quantification of HIF-1α protein expression in the presence or absence of inhibitors after cyclical stretch for 4 h. The HIF-1α protein levels have been normalized to levels in control cells in each of four independent experiments.
Hypoxia-inducible factor-1α induced by mechanical stretch

Induction of HIF-1α protein by stretch in VSMCs is p42/p44 MAP kinase-dependent

To test whether the p42/p44 MAP kinase pathway mediates the stretch-induced HIF-1α protein in VSMCs, cells were stretched by 20% for 4 h in the absence or presence of PD98059 or U0126, specific and potent inhibitors of MEK, an upstream p42/p44 MAP kinase kinase. As shown in Figure 2, the stretch-induced increase in HIF-1α protein was completely blocked after the addition of PD98059 (50 μM) or U0126 (25 μM) 30 min before stretch. PD98059 and U0126 also completely inhibited the increase in HIF-1α protein after stretch to 10% elongation for 4 h (results not shown). As shown in Figure 3, p42/p44 MAP kinase protein phosphorylation was induced by cyclical stretch to 20% elongation, and the increase in phosphorylated p42/p44 MAP kinase protein was blocked after the addition of PD98059 (50 μM) or U0126 (25 μM) 30 min before stretch. The total p42/p44 MAP kinase protein induced by stretch was not affected by the addition of PD98095 and U0126. The total p42/p44 MAP kinase protein increased 2.49 ± 0.28-fold after stretch for 4 h compared with control cells without stretch and also increased 2.20 ± 0.14- and 2.16 ± 0.1-fold after addition of PD98059 and U0126 respectively, before stretch (Figure 3B). These data suggest that the phosphorylation of p42/p44 MAP kinase is essential for induction of HIF-1α protein by stretch in VSMCs. To exclude the possibility that PD98095 and U0126 were cytotoxic, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide assay was performed. No difference in A570 was observed between control cells and cells treated with PD98095 or U0126 at different concentrations for up to 24 h. The data demonstrated that there was no cytotoxicity of PD98095 and U0126 on VSMCs.

Figure 3  Effect of cyclical stretch on the expression and phosphorylation of p42/p44 MAP kinase in VSMCs
Representative Western blot for phosphorylated (A) and total (B) p42/p44 MAP kinase in VSMC after stretch for 4 h in the absence or presence of inhibitors. Total cell extracts (20 μg) were resolved by SDS/PAGE and immunoblotted using anti-(phospho-p42/p44 MAP kinase) or anti-(total p42/p44 MAP kinase) monoclonal antibodies. Phosphorylated p42/p44 MAP kinase (phospho-P42/P44 MAPK) decreased after the addition of PD98059 and U0126, whereas total p42/p44 MAP kinase expression was not affected by the addition of the inhibitors. Western blots are representative of three independent experiments.

Figure 4  Time course of induction by cyclical stretch of HIF-1α mRNA expression in VSMCs by real-time PCR
(A) Representative real-time PCR observation for HIF-1α mRNA in VSMCs subjected to cyclical stretch to 20% elongation for various periods of time. (B) Fold increases in HIF-1α mRNA as a result of cyclical stretch for various periods of time. The values from stretched cells have been normalized to GAPDH levels and then expressed as a ratio of normalized values to mRNA in control cells. *P < 0.05 and †P < 0.01 compared with control. NC, negative control.

Figure 5  Quantitative analysis of HIF-1α mRNA after cyclical stretch for 4 h in the absence or presence of MEK inhibitors
The values from stretched cells have been normalized to matched GAPDH measurements and then expressed as a ratio of normalized values to mRNA in control cells. Results were obtained from three independent experiments.

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Cyclical stretch enhances mRNA expression of HIF-1α in VSMCs

The real-time PCR showed that HIF-1α mRNA increased significantly after 2 h of stretch to 20% elongation (Figure 4). The mRNA reached a maximal level after 4 h of stretch and then declined to the pre-stretch level. The stretch-induced increase in HIF-1α mRNA in VSMCs was also abolished by pretreatment with PD98059 (50 µM) or U0126 (25 µM) (Figure 5). After addition of PD98059 and U0126, HIF-1α mRNA levels in VSMCs stretched for 4 h decreased from 2.04 ± 0.18-fold in stretched cells to 1.12 ± 0.13- and 1.04 ± 0.12-fold respectively (Figure 5). These data suggest that MAP kinase may be involved in the induction of HIF-1α gene expression by cyclical stretch in VSMCs. As shown in Figure 6, inhibitors of an upstream p42/p44 MAP kinase kinase also blocked the induction of VEGF mRNA. VEGF mRNA also increased approx. 3-fold from 2 h to at least 24 h after stretch to 20% elongation compared with that in control cells without stretch.

Effect of cyclical stretch on HIF-1α immunohistochemical labelling in VSMCs

Cyclical stretch increased immunoreactive signals of HIF-1α in both the cytosol and nucleus of stretched VSMCs. The signals began to increase as early as 2 h after stretch to 20% elongation was applied and maximal staining was observed at 4 h after stretch (Figure 7). The immunoreactive signals also declined to the pre-stretch level at 24 h after stretch. These immunoreactive signals decreased after pretreatment with PD98059 (50 µM) or U0126 (25 µM) 30 min before stretch.

Figure 6 Quantitative analysis of VEGF mRNA after cyclical stretch for 4 h in the absence or presence of MEK inhibitors

The values from stretched cells have been normalized to matched GAPDH measurements and then expressed as a ratio of normalized values to mRNA in control cells. Results were obtained from three independent experiments.

Figure 7 Immunohistochemical localization of HIF-1α after cyclical stretch for 4 h in the absence or presence of MEK inhibitors

Representative microscopic images of the HIF-1α immunoreactive signals in control cells (A), and cyclically stretched VSMCs at 4 h in the absence (B) or presence of PD98059 (C) or U0126 (D) pretreatment. Arrows indicate strong nuclear localization of HIF-1α. Similar results were found in another three independent experiments.
Figure 8  Effect of HIF-1α antisense oligonucleotides on stretch-induced increase in VEGF protein expression in VSMCs

(A) Representative Western blot for VEGF and HIF-1α in VSMCs subjected to cyclical stretch to 20% elongation. (B) Change in VEGF protein expression in the presence of an HIF-1α antisense oligonucleotide after cyclical stretch for 4 h.

VEGF protein levels have been normalized to levels in control cells of three independent experiments. *P < 0.01 compared with control group (no stretch and no oligonucleotide); †P < 0.01 compared with HIF-1α antisense oligonucleotide plus stretch.

Antisense oligonucleotide for HIF-1α largely prevents the stretch-induced increase in VEGF protein expression

In VSMCs stretched for 4 h, the antisense oligonucleotide for HIF-1α prevented an increase in VEGF protein expression compared with controls (without stretch) or scrambled oligonucleotide-treated VSMCs (Figure 8). HIF-1α protein was also suppressed to pre-stretch levels after the addition of antisense oligonucleotide for HIF-1α. These data provide conclusive evidence that HIF-1α plays an obligatory role for VEGF induction in stretched VSMCs.

Cyclical stretch increases HIF-1α binding activity

At least two DNA–protein complexes with different migration properties were observed when nuclear extracts from VSMCs were incubated with the HIF-1α oligonucleotide (Figure 9). An excess of unlabelled HIF-1α oligonucleotide competed with the probe for binding to the HIF-1α protein, whereas an oligonucleotide containing a 3 bp substitution in the HIF-1α binding site did not compete for binding. Cyclical stretch of VSMCs for 2–6 h significantly increased the DNA–protein-binding activity of HIF-1α. Addition of PD98059 or U0126 30 min before stretch abolished the DNA–protein-binding activity induced by cyclical stretch. DNA–protein-binding complexes induced by cyclical stretch were supershifted by a specific anti-(HIF-1α) antibody, indicating the presence of this protein in these complexes.

DISCUSSION

In the present study, we found that cyclical mechanical stretch induced HIF-1α expression in VSMCs as determined by Western blotting, real-time PCR, immunohistochemical staining and EMSA. HIF-1α was up-regulated in both a time- and load-dependent manner by cyclical stretch. Cyclical stretching of VSMCs increased both HIF-1α protein and mRNA expression. The activation of HIF by hypoxia is complex and involves changes in protein stability, nuclear translocation, DNA-binding capability and transcriptional activation function [25]. The induction of HIF-1α protein by cyclical stretch may be caused by increased protein stability and decreased degradation [26]. Cyclical stretch also increased the HIF-1α transcriptional activity. Since rhythmic distension of the vessel wall is a component of pulsatile flow, our present study indicates that cyclical mechanical stretch is an important factor regulating HIF-1α in vascular wall cells. The induction of HIF-1α protein by cyclical stretch was mediated by the p42/p44 MAP kinase pathway, because the specific and potent inhibitors of an upstream p42/p44 MAP kinase kinase, PD98059 and U0126, inhibited the induction of HIF-1α protein.

To our knowledge, this is the first report suggesting the possible involvement of this pathway in cultured vessel wall cells undergoing mechanical stretch. Pages et al. [27] have demonstrated that p42/p44 MAP kinases stoichiometrically phosphorylate HIF-1α protein in vitro and that HIF-1α-dependent VEGF gene expression is strongly enhanced by the exclusive activation of p42/p44 MAP kinases. Therefore p42/p44 MAP kinases may play an important role in angiogenesis by mechanical stress.

This model of cyclical stretching of SMC cultures grown on a flexible membrane is an artificial environment for cells. At a peak level of ∼15 kPa of stretch, VEGF protein and mRNA expression were induced in cardiac myocytes and VSMCs [23,28,29]. The transient expression of HIF-1α protein may play an important role, because VEGF is a target gene of HIF-1. When blood pressure is fluctuating, high blood pressure will increase the vessel wall tension and stretch the vascular wall cells. To our knowledge, this is the first report suggesting the possible involvement of this pathway in cultured vessel wall cells undergoing mechanical stretch. Pages et al. [27] have demonstrated that p42/p44 MAP kinases stoichiometrically phosphorylate HIF-1α protein in vitro and that HIF-1α-dependent VEGF gene expression is strongly enhanced by the exclusive activation of p42/p44 MAP kinases. Therefore p42/p44 MAP kinases may play an important role in angiogenesis by mechanical stress.

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wall. Therefore the transient increase in HIF-1α gene expression after cyclical stretch may be important in patients with fluctuating high blood pressure. The increase in HIF-1α protein will increase expression of its target gene, VEGF, and may therefore accelerate the process of atherosclerosis in the vessel wall. Based on this viewpoint, our present study implies that the control of hypertension to prevent fluctuation of blood pressure and morning surge in hypertensive patients is very important.

Because HIF-1α induction was first described for conditions of decreased oxygen pressure, most mechanistic studies on HIF-1α regulation have concerned hypoxic conditions. However, recent studies suggest that HIF-1α mRNA and protein can be induced under normoxic conditions by growth factors and hormones in VSMCs [12] and by mechanical stress in rat myocardium [21]. Myocardial expression of HIF-1α in response to stretch is mediated by the phosphoinositide 3-kinase pathway [21]. In the present study, because we did not investigate this pathway, we cannot exclude involvement of this pathway in VSMCs. Induction of HIF-1α gene by growth factors and hormones in VSMCs is not mediated through activation of p42/p44 MAP kinase. Two reports [30,31] have shown a role of the p42/p44 MAP kinase pathway in enhancing the transcriptional activity of HIF-1α. In the present study, we found that p42/p44 MAP kinase-dependent HIF-1α protein induction occurred in VSMCs under mechanical stretch. Taken together, the mechanism of HIF-1α induction in VSMCs varies when they respond to different stimuli.

The transcriptional up-regulation of VEGF has been shown to play a major role in the induction of VEGF, which is mediated by the binding of HIF-1 to the hypoxia-response elements in the 5′-flanking region [32]. Increased vascular permeability and neovascularization are prominent features of the microvascular complications of diabetes mellitus. Cyclical stretching-induced increases HIF-1α mRNA and protein expression could play a role in the vascular injury that complicates disease states such as diabetes mellitus, because of the capacity of VEGF to function simultaneously as a potent vascular permeabilizing agent and mitogen [33–35]. VEGF is likely to be an important protein released from

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**Figure 9** Effect of cyclical stretch on HIF-1α binding activity

Representative EMSA showing protein binding to the HIF-1α oligonucleotide in nuclear extracts of VSMCs after cyclical stretch in the absence or presence of MEK inhibitors is shown. Arrows indicate the mobility of the complex. Similar results were found in another independent experiment. Cold oligo, unlabelled HIF-1α oligonucleotide. A significant supershifted complex (S) after incubation with anti-(HIF-1α) antibody (HIF-1α Ab) was observed.
SMCs under mechanical stress such as balloon angioplasty [36] and cyclical stretch [30]. We [24] and others [37] have demonstrated that VEGF is induced by mechanical stress. The present study, demonstrating that an antisense oligonucleotide for HIF-1α prevents VEGF protein expression, provides conclusive evidence that HIF-1α plays an obligatory role in VEGF expression in VSMCs after cyclical stretch. Therefore increased transient HIF-1α gene expression induced by cyclical stretching may be relevant to pathological states of the cardiovascular system, including atherosclerotic plaque stability and hypertension. This novel stretch-induction of HIF-1α gene in VSMCs may play a role in vascular remodelling.

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