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ORIGINAL ARTICLE

Angiotensin II mediates urotensin II expression by hypoxia in cultured cardiac fibroblast

Kou-Gi Shyu[*,†,1], Bao-Wei Wang[*,1], Wei-Jan Chen[‡], Peiliang Kuan[*] and Chiu-Mei Lin[§,¶]

[*]Division of Cardiology, Shin Kong Wu Ho-Su Memorial Hospital, Taipei, Taiwan, [†]Graduate Institute of Clinical Medicine, Taipei Medical University, Taipei, Taiwan, [‡]First Cardiovascular Division, Chang Gung Memorial Hospital, Chang Gung University College of Medicine, Kwei-Shan, Tao-Yuan, Taiwan, [§]Department of Emergency Medicine, Shin Kong Wu Ho-Su Memorial Hospital, Taipei, Taiwan, [¶]Institute of Injury Prevention and Control, College of Public Health, Taipei Medical University, Taipei, Taiwan

ABSTRACT

Background Urotensin II plays a role in myocardial remodelling. Cardiac fibroblasts play a critical role in the development of cardiac fibrosis. The effect of hypoxia on urotensin II expression in cardiac fibroblasts is poorly understood. We sought to investigate the regulation of urotensin II by hypoxia in cardiac fibroblasts and the effect of angiotensin II in the interaction with urotensin II.

Methods and results Rat cardiac fibroblasts were cultured in hypoxic chamber. Hypoxia significantly increased urotensin II expression and reactive oxygen species (ROS) production in cultured cardiac fibroblasts. Hypoxia-induced increase in urotensin II protein and ROS was significantly attenuated after the addition of SP600125, JNK siRNA or N-acetylcysteine before hypoxia treatment. The phosphorylated JNK protein was induced by hypoxia and was abolished by pretreatment with SP600125, losartan (an angiotensin II receptor antagonist) or N-acetylcysteine. The increased urotensin II expression by exogenous addition of angiotensin II was similar to that by hypoxia. Addition of losartan and angiotensin II antibody before hypoxia almost completely inhibited the increase in urotensin II induced by hypoxia. Hypoxia significantly increased the secretion of angiotensin II from cardiac fibroblasts and increased the collagen I protein expression. Hypoxia significantly increased the urotensin II promoter activity by 4·3-fold as compared to normoxic control. Urotensin II siRNA almost completely attenuated the collagen I protein expression induced by hypoxia.

Conclusions Hypoxia-induced urotensin II expression in cardiac fibroblast is mediated by angiotensin II and through ROS and JNK pathway. Urotensin II is a mediator of angiotensin II-induced cardiac fibrosis under hypoxia.

Keywords Cytokine, fibrosis, hypoxia, reactive oxygen species, urotensin II.

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Introduction

Cardiac fibrosis leads to the loss of normal cardiac function and is closely associated with heart failure [1]. Urotensin II (U II) is a somatostatin-like cyclic peptide synthesized by proteolytic cleavage from a precursor molecule, prepro-U II [2], and plays a role in the progressive remodelling process in heart failure [3,4]. U II has been shown to increase cardiac fibroblast proliferation and collagen synthesis in the in vitro and in vivo studies [3,4]. Reactive oxygen species (ROS) is involved in the U II-induced proliferation of cardiac fibroblasts [5].

Hypoxia is a stimulus to collagen synthesis in cardiac fibroblasts [6]. Chronic exposure to hypoxia in rats has been shown to enhance pulmonary artery response to U II [7]. Chronic hypoxia also has been shown to increase U II expression in the rat myocardium [8]. Hypoxia can stimulate cardiac fibroblast to secrete cytokines and growth factors [9]. The effect of hypoxia on U II expression in cardiac fibroblasts has not been previously reported. Angiotensin II (Ang II) has been shown to induce cardiac fibrosis by increasing the oxidative stress and pro-collagen I expression. [10] The effect of Ang II on U II has not been reported. We hypothesized that hypoxia may regulate the expression of U II in cardiac fibroblasts and Ang II may act

[1]Both authors contributed equally to this work.

as an upstream regulator of U II. In the study, we designed to investigate the U II expression in cardiac fibroblasts under hypoxia and under Ang II stimulation and tried to seek the possible molecular mechanisms and signal pathways mediating the expression of U II under hypoxic condition and Ang II stimulation. Transcriptional regulation of U II receptor by nuclear receptor-κB and hypoxia-inducible factor-1α has been reported [11,12]. The transcriptional regulation of U II remains poorly understood. C-Jun N-terminal kinase (JNK) pathway has been reported to mediate the oxidative stress induced by U II [13]. We therefore hypothesized that activating protein-1 (AP-1), a well-characterized downstream target of JNK, may play a role in the transcriptional regulation of U II.

Methods

Cardiac fibroblast culture

Cardiac fibroblasts were prepared from adult male Sprague-Dawley rat hearts weighing 250–300 g using a method described by Gustafsson and Brunton [14]. Briefly, after rapid excision of the hearts, the ventricles were isolated, minced, pooled and placed in a solution of 100 U mL^{-1} collagenase I and 0·1% trypsin. Sequential 10-min period of digestion was performed with constant stirring at 37 °C. After discarding the eluate from the first digestion period, digested fibroblasts from nine more digestion periods were pooled, pelleted and resuspended in the Dullbecco's modified Eagle's medium supplemented with penicillin, streptomycin, fungizone and 10% foetal bovine serum. After a 30-min period of attachment to uncoated culture plates, cells that were weakly attached or unattached were rinsed free and discarded. After 2–3 days, confluent cultures were amplified by trypsinization and seeding onto new dishes. The purity of these cultured cardiac fibroblasts were > 95% as determined by positive staining for vimentin and negative staining for smooth muscle α-actin, von Willebrand factor, and desmin. Cell cultures were incubated at 37 °C in a humidified atmosphere of 5% CO_2/95% air. Studies were conducted on cardiac fibroblasts (passages two through four) that were grown to subconfluence in serum-containing media and then growth arrested for 24 h in serum-free medium before treatment. All animals received humane care according to the guidelines stated in the Guide for the Care and Use of Laboratory Animals.

Hypoxic stimulation

A humidified temperature-controlled incubator Proox model 110 (BioSpherix, Redfield, NY, USA) was used as hypoxic chamber. For hypoxia conditions, the concentration of oxygen was reduced to 2·5% by replacement with N_2, keeping CO_2 constant at 5%, and incubated at 37 °C for different times. Control was defined as 95% air and 5% CO_2. For the investigation of signal pathways, cells were pretreated with inhibitors for 30 min and then exposed to hypoxia without changing medium. SP600125 (20 μM Calbiochem®; EMD Biosciences Inc., San Diego, CA, USA) is a potent, cell-permeable, selective and reversible inhibitor of JNK. SB203580 (3 μM, Calbiochem®) is a highly specific, cell-permeable inhibitor of p38 kinase. PD98059 (50 μM Calbiochem®) is a specific and potent inhibitor of ERK kinase. N-acetylcysteine (NAC, 500 μM Calbiochem®) is a free radical scavenger. Losartan (100 nM, Merck, NJ, USA) is a specific antagonist of Ang II receptor. 2,2′-Dipyridyl–N, N-dimethylsemicarbazone (Dp44mT, 30 μM, Calbiochem®) is a ROS generator.

Western blot analysis

Western blot was performed as previously described [15]. Polyclonal anti-U II, anti-U II receptor, anti-collagen I (catalogue no. sc-8788), and anti-angiotensin antibodies (Santa Cruz Biotechnology, Santa-Cruz, CA, USA), monoclonal anti-α-tubulin antibody (Sigma, St Louis, MI, USA), polyclonal anti-JNK and monoclonal anti-phospho JNK antibodies (Cell Signaling, Beverly, MA, USA) were used.

Real-time reverse transcription-polymerase chain reaction

Total RNA from cultured cardiac fibroblasts was extracted using the single-step acid guanidinium thiocyanate/phenol/chloroform extraction method [16]. Real-time reverse transcription-polymerase chain reaction (PCR) was performed as previously described [17]. The rat U II primers were 5′-CGGACACTGGTGAAATG-3′ and 5′-CCTGGTTCTCGCCAAA-3′.

RNA interference

Cultured cardiac fibroblasts were transfected with 800 ng of JNK annealed siRNA, or U II siRNA oligonucleotide (Dharmacon Inc., Lafayette, CO, USA). JNK or U II siRNA is a target-specific 20-25 nt siRNA designed to knockdown gene expression. JNK sense and antisense of siRNA sequences were 5′-CGUGGAUUUAUGGUCUGUGdTdT and 5′-CACAGACCAUAAAUCCACGdTdT, respectively. U II sense and antisense of siRNA sequences were 5′-CACGGACACUGGUGAAAUGUU and 5′-PCAUUUCACCAGUGUCCGUGUU, respectively. As a negative control, a nontargeting siRNA (scrambled siRNA) purchased from Dharmacon was used. Cardiac fibroblasts were transfected with siRNA oligonucleotides using Effectene transfection reagent kit as suggested by the manufacture (Qiagen Inc, Valencia, CA, USA).

Reactive oxygen species assay

Reactive oxygen species production was measured using the cell-permeable probe 2′-7′-dichlorodihydrofluorescin diacetate,

which passively diffuses into cells where intracellular esterases cleave the acetate groups to form the impermeable $DCFH_2$ which remains trapped within the cell [18]. After hypoxia treatment, cells were collected by trypsinization and resuspended in phosphate-buffered saline medium. ROS assay was performed according to the manufacturer's instruction (Invitrogen, Eugene, OR, USA). Fluorescence microscopy was used to detect the green fluorescence.

Electrophoretic mobility shift assay (EMSA)

Nuclear protein concentrations from cultured cardiac fibroblasts were determined by Biorad protein assay. Consensus and control oligonucleotides (Research Biolabs, Singapore) were labelled by polynucleotides kinase incorporation of [γ^{32}P]ATP. Oligonucleotides sequences of AP-1 were consensus 5′-CGCTTGATGACTCAGCCGGAA-3′. The AP-1 mutant oligonucleotides sequences were 5′-CGCTTGATGACTTGG-CCGGAA-3′. EMSA was performed as previously described [16]. Controls were performed in each case with mutant oligonucleotides or cold oligonucleotides to compete with labelled sequences.

Promoter activity assay

A −664 to +44 bp rat U II promoter construct was generated as follows. Rat genomic DNA was amplified with forward primer (CACAGCACTTTGCCAGGTTGA) and reverse primer (AGGAGTCCTACGAAGAGCAGG). The amplified product was digested with MluI and BglII restriction enzymes and ligated into pGL3-basic luciferase plasmid vector (Promega Corp., Madison, WI, USA) digested with the same enzymes. The U II promoter contains AP-1 conserved sites (CA) at −621 to −620 bp. For the mutant, the AP-1 binding sites were mutated using the mutagenesis kit (Mission Biotech, Taipei, Taiwan). Site-specific mutations were confirmed by DNA sequencing. Plasmids were transfected into cardiac fibroblasts using a low-pressure-accelerated gene gun (Bioware Technologies, Taipei, Taiwan) essentially following the protocol from the manufacturer. In brief, 2 μg of plasmid DNA was suspended in 5 μL of PBS and was delivered to the cultured cardiac fibroblasts at a helium pressure of 15 psi. Following hypoxia treatment for 1·5 h, cell extracts were prepared using Dual-Luciferase Reporter Assay System (Promega) and measured for dual luciferase activity by luminometer (Turner Designs Inc., Sunnyvale, CA, USA).

Measurement of angiotensin II concentration

Conditioned media from cultured cardiac fibroblasts under hypoxia and those from control cells (normoxia) were collected for Ang II measurement. The level of Ang II was measured by a quantitative sandwich enzyme immunoassay technique (SPI-BIO, Massy Cedex, France). The lower limit of detection of Ang II was 0·5–1·5 pg mL^{-1}. Both the intra-observer and the interobserver coefficient of variance were < 10%.

Collagen synthesis assay

Cardiac fibroblasts were cultured with serum-free medium in ViewPlate for 60 min (Packard Instrument Co., Meriden, CT, USA). Recombinant U II (12 pM; Phoenix Pharmaceutical, Burlingame, CA, USA) was added to the medium in normoxia. The cells were then labelled with 100 μCi mL^{-1} ^{3}H-proline for 16 h. Various inhibitors or U II siRNA were added to the medium 30 min before the addition of induction of hypoxia. Cells were washed with PBS twice. Fifty microlitres of MicroScint-20 was added, and the plate was read with TopCount (Packard Instrument Co.).

Statistical analysis

The data were expressed as mean ± SD. Statistical significance was performed with analysis of variance (GraphPad Software Inc., San Diego, CA, USA). The Dunnett's test was used to compare multiple groups to a single control group. Tukey–Kramer comparison test was used for pairwise comparisons between multiple groups after the ANOVA. A value of $P < 0·05$ was considered to denote statistical significance.

Results

Hypoxia increases U II expression in cultured cardiac fibroblasts

To test the effect of hypoxia on U II expression, different degrees of hypoxia were used. As shown in Supplemental Fig. S1, hypoxia at 2·5% and 5% oxygen for 2 h significantly induced U II protein expression, while hypoxia at 10% oxygen had no effect on U II expression. We then used 2·5% oxygen as hypoxia to the following experiments. As shown in Fig. 1, hypoxia at 2·5% oxygen induced U II protein expression maximally at 2 h after hypoxia treatment and maintained elevated for 8 h (Fig. 1a,b). Hypoxia at 2·5% oxygen also induced U II receptor protein expression from 2 to 4 h. The levels of U II mRNA also significantly increased from 1·5 to 4 h after hypoxia treatment (Fig. 1c).

Hypoxia-induced U II protein expression in cardiac fibroblasts is mediated by reactive oxygen species and JNK

To investigate the possible signalling pathways mediating hypoxia-induced U II expression, different inhibitors were used. As shown in Fig. 2a,b, the Western blots demonstrated that hypoxia-induced increase in U II protein was significantly attenuated after the addition of SP600125 or NAC 30 min before hypoxia treatment. The U II protein induced by hypoxia

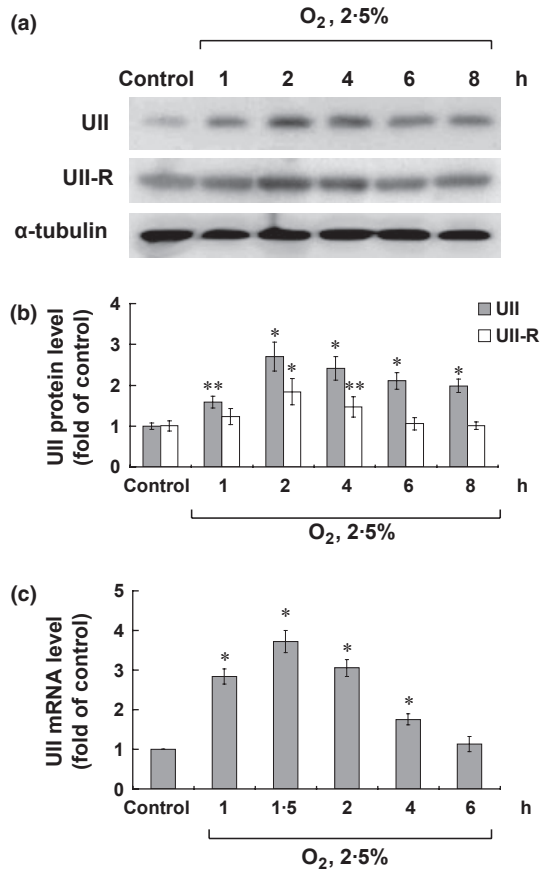


Figure 1 Hypoxia increases urotensin II (U-II) and U-II receptor (U-II-R) expression in cultured cardiac fibroblasts. (a) Representative Western blots for U-II in cardiac fibroblasts treated with 2·5% O_2 for periods of time. (b) Quantitative analysis of protein levels. The values from treated fibroblasts have been normalized to values in control cells (n = 4 per group). *P < 0·001 vs. control. **P < 0·05 vs. control. (c) Quantitative analysis of U-II mRNA levels. The mRNA levels were measured by real-time PCR. The values from hypoxic fibroblasts have been normalized to matched α-tubulin measurement and then expressed as a ratio of normalized values to mRNA in control cells (n = 3 per group). *P < 0·01 vs. control.

was partially affected by the addition of PD98059 and SB203580. DMSO, as the vehicle for SP600125, did not affect U II expression induced by hypoxia. Addition of Dp44mT alone without hypoxia treatment significantly increased U II expression. JNK siRNA also completely blocked the U II expression induced by hypoxia. The control siRNA did not affect U II expression induced by hypoxia. Addition of hydrogen peroxide at 100 μM also induced the expression of U II protein expression, effect similar to hypoxia or Dp44mT (Supplemental Fig. S2).

As shown in Fig. 2c,d, phosphorylated JNK protein was induced by hypoxia in a time-dependent manner. The phosphorylated JNK protein was maximally induced at 1·5 h of hypoxia treatment and remained elevated until 4 h. The pattern of increase in phosphorylated JNK protein after hypoxia was slightly earlier than that of U II protein after hypoxia as shown in Fig. 1. The phosphorylated JNK was abolished by pretreatment with SP600125, losartan (an Ang II receptor antagonist) or NAC.

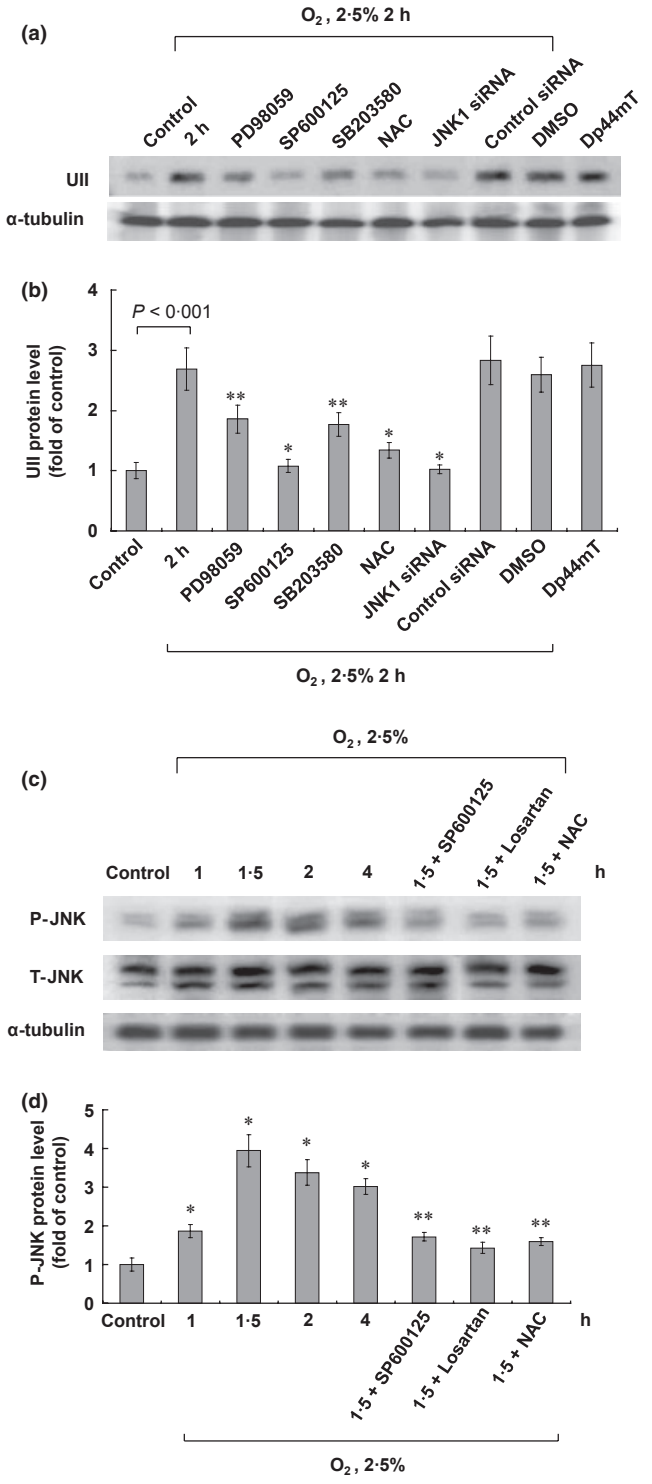
As shown in Fig. 3, hypoxia for 1·5 h significantly increased the ROS production and maintained elevated for 6 h by using ROS assay with fluorescent microscope. Pretreatment with NAC 30 min before hypoxia treatment significantly blocked the induction of ROS by hypoxia. In the control group with normoxic treatment, very few cardiac fibroblasts expressed green fluorescence. Exogenous addition of Dp44mT to the normoxic cell significantly increased ROS production.

Angiotensin II mediates the induction of urotensin II by hypoxia

Exogenous addition of Ang II at 10 nM significantly increased U II protein expression. The increased U II expression by Ang II was similar to that by hypoxia (Fig. 4). Addition of losartan (100 nM, an antagonist of Ang II receptor) and Ang II antibody 30 min before hypoxia almost completely inhibited the increase in U II induced by hypoxia. Hypoxia for 1–4 h significantly increased the secretion of Ang II from cardiac fibroblasts (Fig. 5). This finding indicates that Ang II mediates increase in U II by hypoxia through Ang II receptor. Addition of JNK siRNA but not losartan 30 min before Dp44mT (30 μM) stimulation under hypoxia completely attenuated U II expression induced by hypoxia (Fig. 4c,d). Addition of NAC 30 min before anisomycin (JNK stimulator) treatment did not affect U II expression induced by hypoxia, indicating that Ang II receptor is activated before ROS production and ROS production activates JNK. Hypoxia at 2·5% and 5% oxygen also significantly increased Ang II receptor expression in the cultured fibroblasts as shown in Supplemental Fig. S3.

Hypoxia increases U II promoter activity

The rat U II promoter construct contains CBF, AP-1, NF-κB, Stat1 and Ets binding sites. Hypoxia for 1·5 h significantly increased U II promoter activity by 4·3-fold as compared to normoxic control (Fig. 6a,b). Addition of SP600125, NAC and losartan 30 min before hypoxia abolished the increased U II promoter activity. Addition of Ang II alone without hypoxia significantly increased U II promoter activity similar to hypoxia. When the AP-1 binding sites were mutated, the increased promoter activity induced by hypoxia and Ang II was abolished. This finding indicates that hypoxia regulates U



II in cardiac fibroblasts at transcriptional level and that AP-1 binding site in the U II promoter is essential for the transcriptional regulation.

Figure 2 Reactive oxygen species and JNK MAP kinase are important regulators that mediate hypoxia-induced U-II expression in cardiac fibroblasts. (a) Representative Western blots for U-II protein levels in fibroblasts subjected to hypoxia stimulation for 2 h or control cells without hypoxia in the absence or presence of inhibitors, and siRNA. Dp44mT was added to fibroblasts without hypoxia. (b) Quantitative analysis of U-II protein levels. The values from hypoxic fibroblasts have been normalized to values in control cells (n = 3 per group). *P < 0·001 vs. 2 h. **P < 0·05 vs. 2 h. (c) Representative Western blots for phosphorylated and total JNK in fibroblasts subjected to 2·5% oxygen stimulation for various periods of time in the presence or absence of inhibitors. (d) Quantitative analysis of phosphorylated JNK protein levels. The values from hypoxic fibroblasts have been normalized to values in control cells (n = 3 per group). *P < 0·01 vs. control. **P < 0·01 vs. 1·5 h.

Hypoxia increases AP-1-binding activity

Hypoxia treatment of cardiac fibroblasts for 1·5–4 h significantly increased the DNA-protein binding activity of AP-1 (Fig. 6c). An excess of unlabelled AP-1 oligonucleotide competed with the probe for binding AP-1 protein, whereas an oligonucleotide containing a 2-bp substitution in the AP-1 binding site did not compete for binding. Addition of SP600125, losartan and NAC 30 min before hypoxia abolished the DNA-protein binding activity induced by hypoxia. DNA-binding complexes induced by hypoxia could be supershifted by a monoclonal c-Jun antibody, indicating the presence of this protein in these complexes. The c-Jun protein was also significantly induced by hypoxia as shown in Supplemental Fig. S4.

Hypoxia modulates collagen I protein expression

As shown in Fig. 7a,b, hypoxia for 4–8 h significantly increased collagen I protein expression in cardiac fibroblasts. To investigate the effect of U II on collagen I protein expression under hypoxia, U II siRNA was transfected to knock down the U II gene in cardiac fibroblast. As shown in Fig. 7c,d, U II siRNA almost completely attenuated collagen I protein expression induced by hypoxia. The scrambled siRNA did not change collagen I expression induced by hypoxia. Addition of SP600125 and NAC 30 min before hypoxia completely inhibited collagen I protein expression induced by hypoxia. Exogenous addition of recombinant U II protein at 10 pM increased collagen I protein expression similar to that of hypoxia. The U II siRNA significantly reduced U II protein expression while scramble siRNA (control siRNA) did not affect U II protein expression (Supplemental Fig. S5). Confocal microscope confirmed the Western blot findings. As shown in Supplemental Fig. S6, labelling of collagen I protein stain increased after hypoxia and U II recombinant protein treatment. SP600125, NAC and U II siRNA reduced collagen I protein labelling induced by hypoxia.

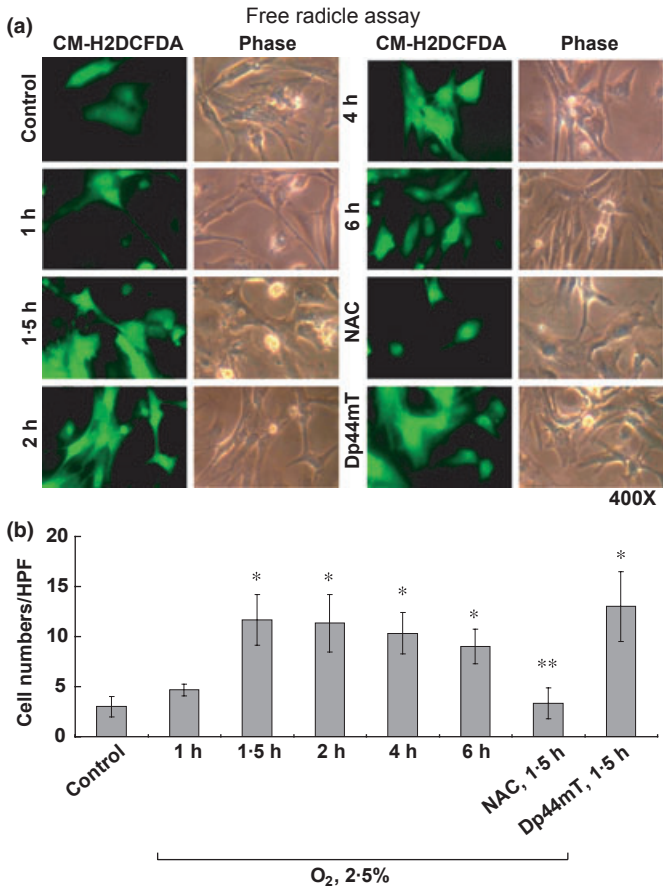


Figure 3 Hypoxia increases reactive oxygen species (ROS) formation in cardiac fibroblasts. (a) Representative microscopic image for ROS assay with (left panel) or without green fluorescence (right panel) in fibroblasts subjected to hypoxia stimulation for various periods of time or control cells without hypoxia in the absence or presence of NAC. NAC (500 μM) was added 30 min before hypoxia stimulation. (b) Quantitative analysis of the positive fluorescent cells. Control group indicates normoxia group. (n = 4 per group). *P < 0·001 vs. control. **P < 0·001 vs. 1·5 h.

U II modulates cardiac fibroblast protein synthesis

As shown in Supplemental Fig. S7, hypoxia, U II recombinant protein, Ang II and Dp44mT significantly increased ^{3}H-proline incorporation into the cultured fibroblasts as compared to control cells. Pretreatment with U II siRNA, losartan and NAC significantly attenuated protein synthesis induced by hypoxia. Scramble U II siRNA did not have inhibitory effect on proline incorporation induced by hypoxia. Addition of Ang II significantly increased collagen I protein expression. Pretreatment with U II siRNA before exogenous Ang II stimulation significantly attenuated fibroblast protein synthesis induced by

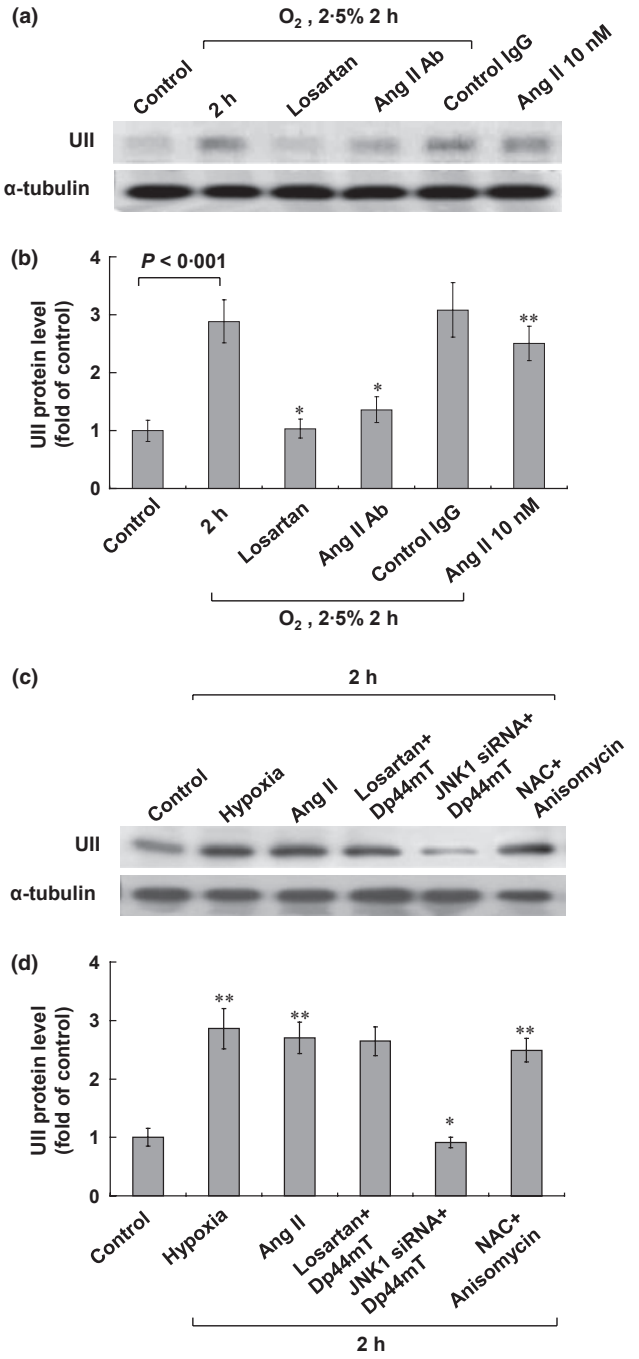


Figure 4 Angiotensin II (Ang II) mediates the induction of urotensin II (U-II) by hypoxia. (a and c) Representative Western blots for U-II protein levels in fibroblasts subjected to hypoxia stimulation for 2 h or control cells without hypoxia in the absence or presence of inhibitor, antibody or siRNA. (b and d) Quantitative analysis of U-II protein levels. The values from hypoxic fibroblasts have been normalized to values in control cells (n = 4 per group). *P < 0·001 vs. hypoxia 2 h. **P < 0·001 vs. control.

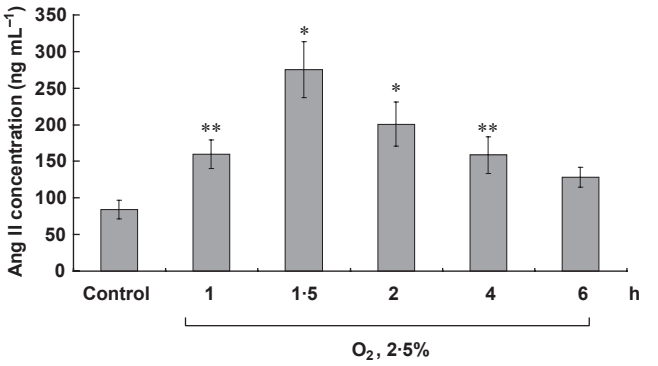


Figure 5 Hypoxia increases the secretion of angiotensin II (Ang II) from cardiac fibroblasts. Ang II was measured by ELISA method from the conditioned medium (n = 4 per group). *P < 0·001 vs. control.

Ang II (Fig. 7c,d). Knockdown of Ang II by Ang II antibody significantly attenuated the collagen I protein expression induced by hypoxia as shown in Supplemental Fig. S8.

Discussion

U II, as a hypertrophic and fibrotic modulator, plays an important role in myocardial remodelling [4,19]. Elevated levels of myocardial U II expression and plasma U II were found in patients with congestive heart failure [20,21] and acute myocardial infarction [22], and in rats with diabetic cardiomyopathy [23]. Bousette *et al.* [24] have reported that blockade of U II attenuates cardiac dysfunction in a rat model of coronary ligation model. More recently, Tran *et al.* [25] have demonstrated that chronic U II infusion induces diastolic dysfunction and enhances collagen production in rats, indicating U II as a major contributor for the diastolic dysfunction. Most of the functions of U II involved in the cardiac remodelling are investigated on cardiac myocytes. A few studies investigated the role of U II in cardiac fibroblast. Cardiac fibroblasts play a critical role in the development of cardiac fibrosis, one of the processes in cardiac remodelling, because of their ability to increase collagen protein synthesis and secretion in the extracellular matrix [9]. Tissue hypoxia is an essential feature of chronic inflammatory disease, and hypoxia might contribute to the development of myocardial fibrosis, which finally leads to arrhythmia. In this study, we have demonstrated that hypoxia increased U II protein and messenger RNA expression. U II receptor protein expression was also induced by hypoxia. The U II-induced by hypoxia contributes to collagen protein expression and synthesis in cardiac fibroblasts. Our study reports for the first time that U II may play a crucial role in cardiac fibrosis under hypoxia. Actually, U II expression was increased in atrial tissue from patients with

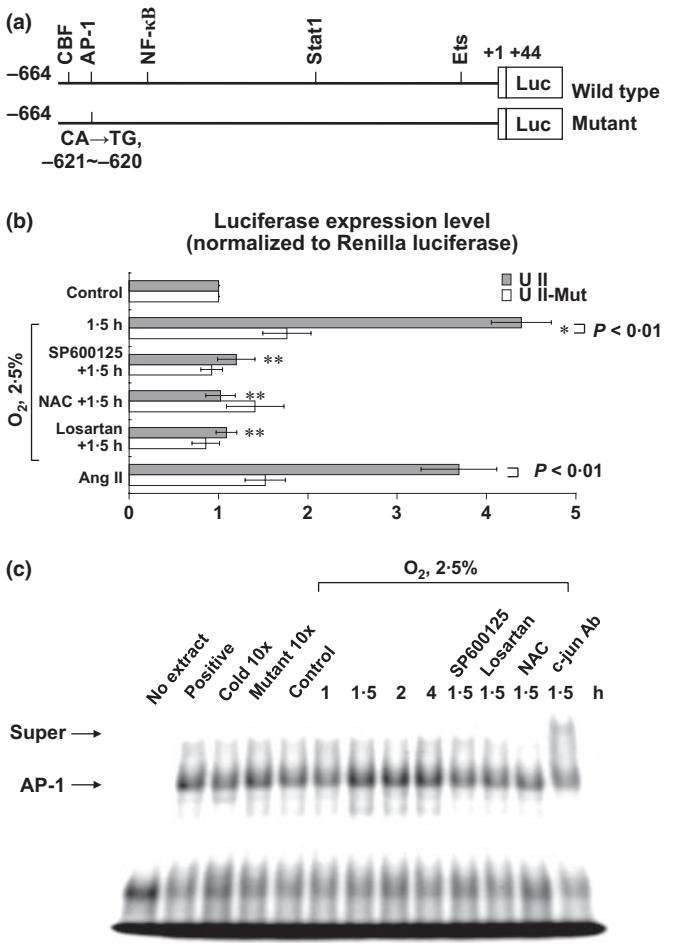


Figure 6 Hypoxia increases the urotensin II.(U-II) promoter activity and AP-1-binding activity. (a) Constructs of U-II promoter gene. Positive +1 demonstrates the initiation site for the U-II transcription. (b) Quantitative analysis of U-II promoter activity. Cardiac fibroblasts were transiently transfected with pUrotensin-II-Luc by gene gun. The luciferase activity in cell lysates was measured and was normalized with renilla activity (n = 3 per group). *P < 0·001 vs. control. **P < 0·001 vs. 1·5 h. (c) Representative Electrophoretic mobility shift assay showing protein binding to the AP-1 oligonucleotide in nuclear extracts of cardiac fibroblasts after hypoxia stimulation in the presence or absence of inhibitors. Arrow indicates the mobility of the complex. Similar results were found in another two independent experiments. Cold oligo means unlabelled AP-1 oligonucleotides. A significant supershifted complex (S) after incubation with c-Jun antibody was observed.

atrial fibrillation and rare U II was found in normal atrial tissue (Supplemental Fig. S9).

Reactive oxygen species is involved in U II-induced proliferation of cardiac fibroblasts [6]. Hypoxia increased ROS formation

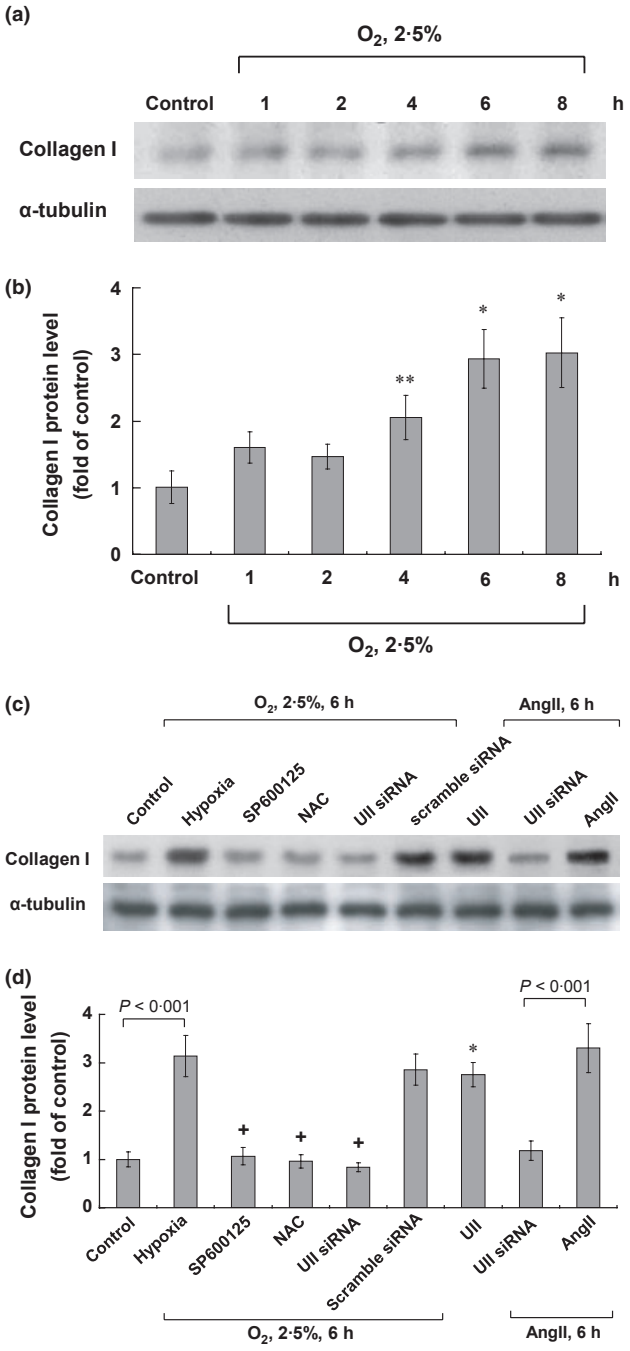


Figure 7 Effect of hypoxia on collagen I protein expression. (a and c) Representative Western blots for collagen I in fibroblast treated with 2·5% oxygen in the presence or absence of inhibitors and siRNA. (b and d) Quantitative analysis of collagen I protein levels. The values from treated fibroblasts have been normalized to values in control cells. (n = 4 per group). $^{*}P < 0{\cdot}01$ vs. control. $^{**}P < 0{\cdot}05$ vs. control. $^{+}P < 0{\cdot}001$ vs. hypoxia.

in cultured cardiac fibroblasts. Exogenous administration of ROS stimulant by hydrogen peroxide or ROS generator increased U II expression and collagen synthesis and antioxidant with NAC attenuated U II expression and collagen synthesis induced by hypoxia. Our data were consistent with previous studies that ROS is involved in cardiac fibroblast proliferation induced by U II. The increased ROS under hypoxia activated JNK pathway to increase U II expression because the addition of JNK siRNA before Dp44mT (30 μM) stimulation under hypoxia completely attenuated U II expression induced by hypoxia and the addition of NAC 30 min before anisomycin (JNK stimulator) treatment did not affect U II expression induced by hypoxia. Hypoxia has been shown to stimulate oxidative stress and increase ROS formation with subsequent cardiomyocyte hypertrophy [26,27]. U II has been shown to induce cardiomyocyte hypertrophy [28]. U II promotes cardiomyocyte hypertrophy via p38 and ERK pathways and not via JNK pathway. ERK is more common as a modulator of Ang II effects on various cell types including cardiac fibroblasts. However, in our study, JNK pathway was the main pathway to activate U II expression in cardiac fibroblast. Although ERK and p38 also seem to be involved in hypoxia-induced U II expression, they are not the major pathways because U II only partially activated p38 and ERK pathways. Different mitogen-activated protein (MAP) kinase pathways may be involved in different cell types in the regulation of U II. MAP kinases have been shown to regulate AP-1 activity [29]. SP600125, a potent inhibitor of JNK pathway, and c-Jun antibody inhibited the binding activity of AP-1 induced by hypoxia, indicating JNK pathway mediates the increased transcriptional activity of AP-1 in cardiac fibroblasts under hypoxia. The promoter activity assay confirmed the regulation of U II expression by the transcriptional regulation of AP-1 because the promoter activity in the mutant type of U II promoter without normal AP-1 binding site was not affected by hypoxia and Ang II stimulation. As fibroblasts are the major cell type found in an infarct zone following a myocardial infarction, modulating cardiac fibrosis or remodelling may target on cardiac fibroblast. If hypoxia can increase U II expression in both cardiomyocyte and cardiac fibroblast, therapeutic therapy targeted on U II may be potentially beneficial for the treatment of ischaemic heart disease.

The interaction of Ang II with U II has been shown to increase myocardial distensibility [30] and vasoconstriction [31]. The effect of Ang II and U II has not been reported in cardiac fibroblasts. The autocrine or paracrine effect of cardiac fibroblast has been confirmed because hypoxia can stimulate cardiac fibroblast to secrete cytokines and growth factors [9]. In this study, we have demonstrated that hypoxia increased the secretion of Ang II from cardiac fibroblasts and the secreted Ang II increased the U II expression, which resulted in increased

collagen expression and synthesis. Ang II has been shown to induce the secretion of syndecan, transforming growth factor-β1 and connective tissue growth factor in cardiac fibroblasts, which are all profibrotic factors [32–34]. In our study, exogenous Ang II stimulation increased the U II expression and U II promoter activity similar to the effect of hypoxia, and losartan and Ang II antibody inhibited the U II expression and promoter activity and cardiac fibroblast protein synthesis induced by U II stimulation. These findings indicate that U II is a mediator of Ang II-induced cardiac fibrosis under hypoxia. To the best of our knowledge, we report for the first time that hypoxia stimulates the cardiac fibroblasts to secrete Ang II, indicating the paracrine or autocrine function of cardiac fibroblast.

In summary, hypoxia-induced U II expression in cardiac fibroblast is mediated by Ang II and through ROS and JNK pathway. U II is a mediator of Ang II-induced cardiac fibrosis under hypoxia. U II may be a future potentially therapeutic target for the treatment of ischaemic heart disease to prevent cardiac fibrosis.

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Conflict of interest

None declared.

Address

Division of Cardiology, Shin Kong Wu Ho-Su Memorial Hospital, Taipei, Taiwan (K.-G. Shyu, B.-W. Wang, P. Kuan); Graduate Institute of Clinical Medicine, Taipei Medical University, Taipei, Taiwan (K.-G. Shyu); First Cardiovascular Division, Chang Gung Memorial Hospital, Chang Gung University College of Medicine, Kwei-Shan, Tao-Yuan, Taiwan (W.-J. Chen); Department of Emergency Medicine, Shin Kong Wu Ho-Su Memorial Hospital, Taipei, Taiwan (C.-M. Lin); Institute of Injury Prevention and Control, College of Public Health, Taipei Medical University, Taipei, Taiwan (C.-M. Lin).
Correspondence to: Dr Chiu-Mei Lin, Department of Emergency Medicine, Shin Kong Wu Ho-Su Memorial Hospital, 95 Wen-Chang Rd, Taipei 111, Taiwan. Tel.: 886 2 28332211; fax: 886 2 28365775; e-mail: mei882153@gmail.com

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Supporting information

Additional Supporting information may be found in the online version of the article:

Figure S1. Effect of hypoxia on urotensin II (U II) in cardiac fibroblasts. (a) Representative Western blots for U II in fibroblast treated with different degree of hypoxia. (b) Quantitative analysis of U II protein levels. The values from treated fibroblasts have been normalized to values in control cells. (n = 4 per group). $^{**}P < 0{\cdot}05$ vs. control. $^{*}P < 0{\cdot}001$ vs. control.

Figure S2. Hydrogen peroxide (H2O2) induces U II protein expression in cardiac fibroblasts. (a) Representative Western blots for U II in fibroblast treated with ROS stimulant. (b) Quantitative analysis of U II protein levels. The values from treated fibroblasts have been normalized to values in control cells. (n = 4 per group). $^{*}P < 0{\cdot}001$ vs. control.

Figure S3. Effect of hypoxia on angiotensin II receptor (Ang II R) in cardiac fibroblasts. (a) Representative Western blots for Ang II receptor in fibroblast treated with different degree of hypoxia. (b) Quantitative analysis of Ang II receptor protein levels. The values from treated fibroblasts have been normalized to values in control cells. (n = 4 per group). $^{**}P < 0{\cdot}001$ vs. control.

Figure S4. Effect of hypoxia on c-jun in cardiac fibroblasts. (a) Representative Western blots for c-jun in fibroblast treated with different degree of hypoxia. (b) Quantitative analysis of c-jun protein levels. The values from treated fibroblasts have been normalized to values in control cells. (n = 4 per group). $^{**}P < 0{\cdot}001$ vs. control. $^{*}P < 0{\cdot}05$ vs. control.

Figure S5. U II siRNA attenuates U II protein expression induced by hypoxia. (a) Representative Western blots for U II in fibroblast treated with 2·5% oxygen with or without siRNA. (b) Quantitative analysis of U II protein levels. The values from treated fibroblasts have been normalized to values in control cells. (n = 4 per group).

Figure S6. Hypoxia increases collagen I labeling in cardiac fibroblasts. Immunohistochemical stain of cultured cardiac fibroblasts was performed after hypoxia with or without inhibitor or siRNA treatment. Labeling of collagen I (arrow) decreased after treatment with N-acetylcysteine (NAC), JNK pathway inhibitor (SP), and U II siRNA induced by hypoxia.

Figure S7. U II modulates cardiac fibroblast protein synthesis. $^{*}P < 0{\cdot}01$ vs. control. $^{+}P < 0{\cdot}01$ vs. hypoxia. (n = 4 per group).

Figure S8. Knockdown of Ang II by Ang II antibody significantly attenuated the collagen I protein expression induced by hypoxia.

Figure S9. Urotensin II expression is increased in atrial tissue with atrial fibrillation. Confocal microscopic image for U II (arrow) labeling in atrial tissue from control patients (a) (normal atrial function with sinus rhythm) and from patients with atrial fibrillation (b). Left panel, vimentin staining. Mid-Panel, U II staining. Right panel, merged staining for vimentin and U II.

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