科技部補助專題研究計畫成果報告
期末報告

探討心肌缺氧壓力下其心房顫動及心臟纖維化的機制及其訊息傳遞路徑(第3年)

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中 文 摘 要 ： 心臟缺血(Ischemia)、缺氧(Hypoxia)會造成許多心臟疾病，如心肌梗塞、充血性心臟衰竭(congestive heart failure)、動脈硬化(atherosclerosis)、冠狀阻塞(coronary occlusion)及手術。心臟缺血會導致心肌細胞減少氧氣的供給而造成心房顫動（atrial fibrillation, AF），使心肌細胞損傷，進而促使心臟重構(cardiac remodeling)、纖維化(fibrogenesis)最終導致心衰竭(heart failure)，而心房顫動是臨床最常見的心律不整（cardiac rhythm disorders）。研究上發現，心房顫動會增加低氧指標(markers)及血管新生指標因子的表現，並會向上調控低氧誘導因子（hypoxia-inducible factor, HIF）及纖維化路徑。心臟重構是心房顫動及心衰竭重要的特徵，具有心臟纖維化(cardiac fibrosis)及細胞外間質(exteracelluar matrix, ECM)，心肌細胞肥大(hypertrophy)的特徵。心臟纖維化的部位會有大量的細胞外間質的沉積，且會增加細胞外間質中第一型膠原蛋白的比例。研究指出心肌缺氧壓力下，會刺激心臟細胞活化低氧誘導因子來調控相關基因表現，以保護心肌細胞。以目前的研究，在心肌缺氧壓力下所導致的心房顫動進而促使心臟重構及纖維化的因果中，其作用的相關細胞分子機轉，目前尚不是很清楚。目前有許多研究指出在缺氧壓力下，細胞會經MAPK訊息傳導路徑進而誘導下游基因的表現以調控細胞。在我們實驗結果顯示，在心肌缺氧壓力下，會誘導JNK 訊息傳導路徑並且調控心肌纖維化因子(TGF-1, -SMA, COL1A, COL3A)並促進纖維化因子蛋白表現量上升，我們進一步利用JNK 抑制劑、Dominate-Negative JNK以及N-acetylcysteine (NAC)抗氧化因子進一步表現在缺氧壓力下的心肌細胞，藉由西方點墨法、細胞免疫螢光染色等實驗方法，證實在缺氧壓力下，心肌細胞是經由ROS/JNK/c-Jun/ATF2路徑來調控缺氧所刺激的纖維化。在基礎研究上，我們的研究結果指出一新機制：缺氧壓力會藉由調控ROS/JNK/c-Jun/ ATF2路徑來造成心房心肌纖維化；在臨床研究上，我們的研究結果提供一個新方向來解釋缺氧性心房心肌纖維化。

中 文 關 鍵 詞 ： 心房顫動、缺氧、纖維化、低氧誘導因子、細胞外間質

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fibrosis, and the ratio of collagen type I increases in fibrosis. Moreover, in respond to hypoxia, several HIF-1α-regulated cardioprotective genes have been reported. There is a need for a better understanding of the causes and consequences of hypoxia, and for the development of atrial fibrillation and cardiac remodeling (i.e., fibrosis). However, numerous paper indicate JNK signaling pathway involved in hypoxia mechanism1-5. In our study, the protein levels of TGF-1, -SMA, COL1A and COL3A increased in hypoxia craidomyocyte. The addition before hypoxia of the c-Jun N-terminal kinase (JNK) pathway inhibitor (SP600125) and JNK dominate-negative had the effect of inhibiting JNK phosphorylation and fibrosis markers expression by western blot and immunocytochemistry assay. The JNK signalling pathway, SEK/MKK4, c-Jun and ATF2 are also activated by hypoxia stimulation. Hypoxia also significantly increased reactive oxygen species (ROS) production in cultured cardiaomyocyte.In addiotion, the phosphorylated JNK and fibrosis amrkers were induced by hypoxia and were abolished by pretreatment N-acetylcysteine (NAC). ROS/JNK/ATF2/c-Junk signaling pathway play a crucial role in hypoxa induced fibrosis. Our study suggests that Hypoxia-induced COL1A and COL3A expression in HL-1 cardiomycyte is mediated by ROS and through JNK and c-Jun/ATF2 pathway.

英文關鍵詞：fibrogenesis, hypoxia, cardiac remodeling, atrial ischemia, atrial fibrillation, exteracelluar matrix, JNK, reactive oxygen species

十一、研究計畫中英文摘要：請就本計畫要點作一概述，並依本計畫性質自訂關鍵詞。

（一）計畫中文摘要。（五百字以內）

心臟缺血(Ischemia)、缺氧(Hypoxia)會造成許多心臟疾病，如心肌梗塞、充血性心臟衰竭(congestive heart failure)、動脈硬化(atherosclerosis)、冠狀阻塞(coronary occlusion)及手術。心臟缺血會導致心肌細胞減少氧氣的供給而造成心房顫動（atrial fibrillation, AF），使心肌細胞損傷，進而促使心臟重構(cardiac remodeling)、纖維化(fibrogenesis)最終導致心衰竭(heart failure)，而心房顫動是臨床最常見的心律不整(cardiac rhythm disorders)。研究上發現，心房顫動會增加低氧指標(markers)及血管新生指標因子的表現，並會向上調控低氧誘導因子(hypoxia-inducible factor, HIF)及纖維化路徑。心臟重構是心房顫動及心衰竭重要的特徵，具有心臟纖維化(cardiac fibrosis)及細胞外間質(exteracelluar matrix, ECM)，心肌細胞肥大(hypertrophy)的特徵。心臟纖維化的部位會有大量的細胞外間質的沉積，且會增加細胞外間質中第一型膠原蛋白的比例。研究指出心肌缺氧壓力下，會刺激心臟細胞活化低氧誘導因子來調控相關基因表現，以保護心肌細胞。以目前的研究，在心肌缺氧壓力下所導致的心房顫動進而促使心臟重構及纖維化的因果中，其作用的相關細胞分子機轉，目前尚不是很清楚。目前有許多研究指出在缺氧壓力下，細胞會經 MAPK 訊息傳導路徑進而誘導下游基因的表現以調控細胞。在我們實驗結果顯示，在心肌缺氧壓力下，會誘導 JNK 訊息傳導路徑並且調控心肌纖維化因子(TGF-β1, α-SMA, COL1A, COL3A)並促進纖維化因子蛋白表現量上升，我們進一步利用 JNK 抑制劑、Dominate-Negative JNK 以及 N-acetylcysteine (NAC)抗氧化因子進一步表現在缺氧壓力下的心肌細胞，藉由西方點墨法、細胞免疫螢光染色等實驗方法，證實在缺氧壓力下，心肌細胞是經由 ROS/JNK/c-Jun/ATF2 路徑來調控缺氧所刺激的纖維化。在基礎研究上，我們的研究結果指出一新機制：缺氧壓力會藉由調控 ROS/JNK/c-Jun/ ATF2 路徑來造成心房心肌纖維化；在臨床研究上，我們的研究結果提供一個新方向來解釋缺氧性心房心肌纖維化。

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Introduction

Ischemia (reduced blood flow) and hypoxia (reduced oxygen) are mainly subject in cardiology, occur in myocardial infarction, congestive heart failure, atherosclerosis, coronary occlusion or surgery. Ischemic heart diease is the leading cause of death worldwide[6]. Myocardial ischemia may cause the reduction in oxygen tension and nutrients and increase in toxic metabolites. Cardiac hypoxia can result in atrial fibrillation, cellular damage, and ultimately cell death[7]. The deficient oxygen supply may result in myocardial dysfunction, promote cardiac remodeling and fibrogenesis and leading to development of heart failure on the ventricular level and dysrhythmias like atrial fibrillation, which is raised by selective atrial ischemia [8,9]. The hypoxia-inducible factor (HIF) pathway has been implicated in atrial fibrillation development [10,11]. In atrial fibrillation, the increased expression of hypoxic and angiogenic markersis are found, and also proven the up-regulation of the HIF pathway and paralleled by fibrogenesis in atrial myocardium [9,12]. **There is a need for a better understanding of the causes and consequences of hypoxia, and for the development of atrial fibrillation and cardiac remodeling (i.e., fibrosis)**.

Although it has been note for a decade that HIF transcription factors are modulated by environmental stress, the molecular mechanisms of HIF members in cardiac ischemic remain unclear. HIF1 is a highly conserved transcription factor that regulates ~100 genes and plays an important role in normal growth and development as well as in the pathophyiology of ischemia, inflammation and cancer [13,14]. HIF-1 is a basic helix-loop-helix transcription factor including of oxygen-regulated HIF-α subunits(HIF-1α, HIF-2αand HIF-3α) and constitutive expressed HIF-β [also known as aryl hydrocarbon receptor nuclear translocator (ARNT)] [15]. Among the three HIFα isoforms, HIF-1α and HIF-2α are similar in their transcriptional activities. HIF-1α is stable in hypoxia, but in the presence of oxygen it is hydroxylated by prolyl hydroxylase enzymes [16,17]. Under hypoxia conditions, prolyl hydroxylase activity is inhibited, which results in the stabilization and nuclear translocation of the HIF-1α, enabling it to bind to HIF-1β and recruit of transcriptional coactivators, and subsequent binding to hypoxia response elements (HRE) of target genes [18]. Recently report implicates the role of HIF-1 in the cardiorespiratory physiology response to hypoxia [19]. In addition, HIF-1 also play a role in cardioprotective, cardiac metabolism and and angiogenesis during ischemia [20]. In respond to hypoxia, several HIF-1α-regulated cardioprotective genes have been reported, such as adrenomedullin, heme oxgenase-1 (HO-1), cardiotrophin-1 (CT-1), connective tissue growth factor (CTGF) and G protein-coupled receptor 30 (GPR30/GPER) et al. [9,20,21,22].

Cardiac remodeling is characterized by cardiac fibrosis, exteracelluar matrix (ECM) deposition and hypertrophy; the cardiac fibrosis is characterized by abundant accumulation of matrix proteins in extracellular space. ECM is comprised of Collagens, smaller amounts of fibronectin, laminin and elastin. The collagenous matrix family has at least 19 genetically distinct types. In the myocardium, the collagen proteins is consist of types I, III, IV, V and VI, the fibrillar interstitial types I and III collagens are the major structural components of the cardiac ECM, with structural type I collagen being the most predominant (up to 85%) and promote myocardial stiffness [23,24]. Abundant deposition of ECM is found in the maturation of fibrosis, and the ratio of collagen type I increases in fibrosis [25]. However, cardiac remodeling, the important pathological feature of the failing heart make alterations in ECM, including its structure and properties[26]. Recent studies indicate that renin–angiotensin system (RAS) and TGF-β1 are the mediator of the cardiac remodeling; the angiotensin II (Ang II) and TGF-β1 act as part of a network that promotes cardiac remodeling [27-30]. However, Ang II have been report to active mineralocorticoid receptor (MR) mediated gene expression through the type 1 angiotensin reportor (AT1) [31,32]. Therefore, it is reasonable to hypothesize that MR is involved in the effect of myocardial fibrosis under low oxygen stress. **In the present study, we evaluated whether hypoxia could modulated cardiac remodelind, fibrosis molecular mechanism of HL-1 cardiomyocytes and RAS pathway.**

The myocardium is a highly organism structure that is composed of cardiomyocytes, fibroblasts, endothelial cells, vascular and neuronal networks. The complex ECM serves as a scaffold for cells and is synthesized by myofibroblasts. Recently report implicated that the cardiomyocytes are also an important source of the ECM [33-36]. Moreover, the ECM could directly modulate the calcium dynamics and electrophysiologic characteristics of cardiomyocytes [37]. Respond to the specific environments stress, the cardiomyocyte expressed the cardiac fibrotic regulator such as CTGF [38], TGF-β1 [36,39,40] and ET-1[41]. **Therefore, we evaluate the role of ECM protein in hypoxia treatment cardiomyocytes, HL-1 cell**.

HL-1 cells have been extensively characterize [42] and provided a model system to study cardiac physiological properties as well as toxicological research [43,44]. Mouse HL-1 cell are derived from the AT-1 mouse atrial cardiomyocytes tumor lineage that retain cardiac phenotypic characteristics such as morphologic, biochemical and electrophysiologic properties of differentiated cardiomyocytes [45-47]. HL-1 cardiomyocytes have been widely reported for hypoxia research that is consistent with previously described for adult cardiomyocytes [22,48-50], and strongly supported HL-1 cells for the study of hypoxia regulation. In addition, there are several report indicate that HL-1 serves as a general model for studying atrial fibrillation[51,52] . Overall, we used the HL-1 atrial cardiomyocyte system to investigate the effect of hypoxia on cardiac remodeling.

The present study is conducted to examine the two major hypotheses: (1) to clarify the fibrotic molecular mechanisms involved in the response of cardiomyocytes to hypoxic conditions, which may mediate cardiac remodeling in the cardiomyocyte; (2) to test the MR activation under hypoxia in cardiomyocyte.

Method

Culture of Atrial Myocytes and Fibroblasts

The HL1 atrial cell line was derived from adult mouse atria, which was obtained from National Taiwan University Hospital, Taipei (with courtesy of Dr. Chia-TIi Tsai), and was sub-cultured by following the protocol from Louisiana State University health sciences center in New Orleans, La. HL-1 cells were cultured in the Claycomb medium [42] (Sigma) supplemented with 10% foetal bovine serum (FBS), 2 mM L-glutamine, 0.1 mM norepinephrine, 0.1 mg/mL streptomycin, and 100 U/mL penicillin at 37°C in a humid atmosphere of 5% CO_2. Cells were plated at 25,000 cells/cm^2 density on precoated plate (5 ng/ml fibronectin in 0.02% gelatin solution). Prior to experiments, cells were arrested overnight in the culture medium in reduced serum media (2% FBS).

Hypoxia Treatments and Medical Preparation

For all the assay, HL-1 cells were incubated in reduced serum media (2% FBS). For hypoxia, HL-1 cells were placed in hypoxia chamber (NexBiOxy Inc.,Taiwan) and maintained at 37°C with a humidified hypoxic atmosphere of 1% O_2, 5% CO_2, and 93% N_2. Controls were maintained at 5% CO2 and 95% air at 37°C. Prolyl hydroxylase inhibitor, CoCl2 was prepared by diluting 100 mM stock solutions. For certain experiments, cells were treated with inhibitors for 30 min before the hypoxic stimulus. The inhibitors used were SB201980 (p38 inhibitor), U0126 (MAPKK/MEK inhibitor) and SP600125 (JNK inhibitor) were purchased from Calbiochem (La Jolla, CA, USA) and 10 mM stock solution made up in DMSO. N-acetyl cysteine, NAC, a general ROS scavenger was dissolved in the buffer (20 mM HEPES,pH 7.0) at 100 mM as a stock solution, cells were pre-treated with 2 mM of NAC for 2 hr to prevents formation of ROS.

Transient transfection

For transient transfections, HL-1 cell were transfected with lipofectamine 2000 (invitrogene) following the manufacturer's instructions. Typically, 2 x 106 cells were plated in 6 cm cell culture dish. The next day cell were transfect with 5 ug/ dish of dominate-negative JNK construct and negative control pCDNA3.1. After 48 hours, the cell were treated with hypoxia for following assay. The dominate-negative JNK construct were a kind gift from Dr. Tang Chih-Hsin.

Western Blot Analysis

Total cellular protein was extracted from several conditioned cells using a Pro-prep protein extraction solution (iNtRON. Biotechnology, Republic of Korea). The homogenates were centrifuged at 15,000×g for 20 min at 4°C and stored at –20°C. Nuclear fractions were obtained by Kontes Dounce Homogenizer with hypotonic lysis buffer(10 mM HEPES, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT, 0.05% Igepal, pH 7.9). The lysate were centrifuge for 900g for 5 min then the nuclear pellet subject to lyse by Pro-prep protein extraction solution. Protein concentrations were determined using the DC Protein Assay (BioRad Laboratories, Inc., USA) and 25 µg of soluble protein per sample was analysed by 8% or 12% SDS poly-acrylamide gels SDS-PAGE. Samples were electrophoresed, transferred to polyvinylidene difluoridep (PVDF) membrane (Millipore).

The membranes were blocked with 3% bovine serum albumin (BSA) for 1 hr at RT and incubated with either of the antibody against HIF-1α (Santa Cruze), α-SMA (Santa Cruz Biotechnology), TGF-β1 (Geneway), COL1A (Abcam),p-c-Jun, p-ATF2, p-SEK/MKK4 (Cell Signaling), p38α, p38 MAPK(pT180/pY182), pan-JNK/SAPK, JNK/SAPK(pT183/pY185), ERK, ERK(pT201/pY204), Akt and pAkt (BD biosciences) at 4°C for overnight. A duplicate membrane was probed with anti-β-Actin (Geneway). The membranes were then incubated with a species-specific horseradish peroxidase labeled

secondary antibody (BD Biosciences: 1:5000) for 1 h at 37°C. Between each step, the membranes were thoroughly washed with TBST. Peroxidase activity was detected on LA4000 (Fuji) using the ECL detection reagents (Millipore). For quantification purposes, blots were quantified using a luminescent imager (LAS-1000 Image Analyzer, Fujifilm, Berlin, Germany) and FluorChem image software (Alpha Innotech, San Leandro.).

Measurements of reactive oxygen species (ROS)

Intracellular ROS production was estimated by using a fluorescent probe, 2',7'-dichlorofluorescin diacetate (DCFH-DA)(MERCK). Cells were seeded in 96-well plates and exposed to DCFH-DA for 30min followed by NAC pretreatment for 2 hr and then treatment with hypoxia. After incubation with 20uM DCFH-DA for 30min, the fluorescence was detected and read at 485nm for excitation and 530nm for emission via a Synergy™ HT Multi-Mode Microplate Reader for fluorescence and normalized by the cell viability measured using the Cell Counting Kit-8(CCK-8)(Sigma).

Result

Effects of Hypoxia on fibrosis maker and Mineralocorticoid Receptor in Cardiomyocytes

To characterize the hypoxia expose time-scale for induction of fibrosis marker: TGF-β1, COL1A, COL3A and αSMA protein expression, we cultured HL-1 cardiomyocytes to 3, 6, 9, and 24 h of hypoxia (1% O_2). Hypoxia up-regulated HIF-1α expression in a time-dependent manner. HIF-1α expression was significantly increased at 6 h in cells maintained under hypoxia than in normoxic cells and stabilize expressed following expose to hypoxia (Figure 1). As shown in Figure 1, fibrosis marker gene, TGF-β1, COL1A, COL3A, and αSMA markedly increased in hypoxia treatment cardiomyocytes. These results support that hypoxia treatment up-regulation fibrosis gene expression.

To further analyze the effects of hypoxia on the expression of fibrosis genes, prolyl hydroxylase (PHD) inhibitors cobalt chloride ($CoCl_2$) were used to treat-with cardiomyocyte on HIF-1α induction. As shown in Figure 2, increased HIF-1α stabilization was observed following exposure to hypoxia and to $CoCl_2$. A similar trend was observed in up-regulation on TGF-β1, COL1A, COL3A, and αSMA protein expression.

Previous [53-56] research have shown that GR and AT2 were be regulated under hypoxia condition. In our research, we exam the effect of hypoxia on renin–angiotensin system (RAS) in cardiomyyocyte. As shown in supFigure, hypoxia and $CoCl_2$ enhanced the MR, GR, AT-1 and AT2 protein expression. These findings indicate that oxygen stress could up-regulate the renin–angiotensin system (RAS).

Effects of Hypoxia on Intracellular Oxidant Signaling in Cardiomyocytes

To confirm that signaling pathway was responsible for hypoxia, under low oxygen condition and $CoCl_2$ treatment, we identified the hypoxia could up-regulate JNK signaling pathway. As shown in Figure 4D, the JNK phosphorylation increase under hypoxia condition. To further demonstrate MAPK pathway is involved in Hypoxia-stimulated fibrosis gene expression, we pre-treatment of hypoxia HL-1 cells with MAPK pathway inhibitor, SB201980 (p38 inhibitor), U0126 (ERK inhibitor), SP600125 (JNK inhibitor). As shown in Figure 3, the fibrosis marker COL1A, COL3A, αSMA and TGF-β1 were significantly abrogate on the SP600125 treatment HL-1 cell. The increase in JNK activation was accompanied by rise in p-SEK/MMK4 phosphorylation (Figure 4D), which suggests a possible association between these events. To confirm this, HL-1 cell were treated with JNK inhibitor SP600125, and analysis by Western blotting (Figure 4E). Taken together, these findings suggest that JNK acts as an upstream mediator of hypoxia-induced fibrosis signaling.

Role of JNK pathway in activation of fibrosis expression by hypoxia in HL-1 cell

It has been previously report that HIF-1α nuclear translocation in response to hypoxia [48,57], in our research the HIF-1α nuclear translocation also observed under low oxygen condition (Figure 7A). According these data, we confirm the fibrosis markers, COL1A and COL3A are up-regulate by hypoxia and mediate by JNK pathway. There are still lots of unknown down-stream signaling by hypoxia induction. Since the JNK inhibitor could modulate COL1A and COL3A expression, we focus on the JNK signal transduction pathway. To confirm that the inhibitors were functional in HL-1 cell, cells were treated with hypoxia, with or without the JNK inhibitor. The activation state of the pathways was then measured by Western blot analysis (Figure 4E). HL-1 cell displayed induction of COL1A and COL3A in response to hypoxia treatment that was significantly suppressed by SP600125 pre-treatment.

To confirm that JNK activity was involved in the hypoxia-induced fibrosis marker expression in HL-1 cell, we investigated whether administration of a JNK inhibitor or expression of dominant-negative JNK could affect hypoxia-induced activation of COL1A and COL3A gene. The activation state of the

pathways was then measured by western blot analysis (Figure 5A). COL1A and COL3A induction by hypoxia was significantly inhibited by pre-treatment with SP600125, and the inhibition was also found in JNK dominate-negative expression HL-1 cell.

Furthermore, the immunocytochemistry staining further validated the JNK pathway modulated in hypoxia-induced fibrotic markers expression (Figure 6). As shown in Figure 6, increased fibrosis was detected in the hypoxia HL-1 cell; while fibrosis elements were down-regulated by JNK inhibitor, where the COL1A and COL3A performed in HL-1 cell. Herein we shown that an upstream activator of the JNK signaling pathway, SEK/MKK4, were modulate fibrotic marker expression(Figure 4D, 4E). However, DN-JNK and SP600125-treated cell demostrated significantly decreased hypoxia-indced fibrosis(Figure 5, 6).

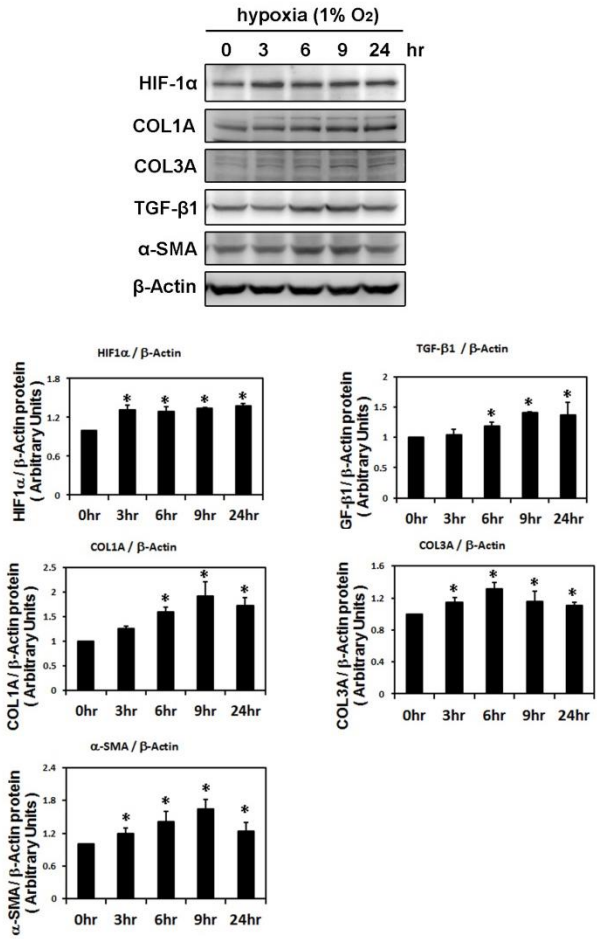
The c-Jun and ATF-2(activating transcription factor 2), were been identified as substrates for JNK[58]. SAPK/JNK, when active as a dimer, could translocate to the nucleus and regulate transcription through its effects on c-Jun, ATF-2, and other transcription factors[59]. As shown in Figure 7A, hypoxia and CoCl2 treatment activated nuclear c-Jun and ATF-2 phosphorylation in HL-1 cell. The addition of SP600125 reduced the phosphorylation of c-Jun and ATF2 in hypoxia HL-1 cell(Figure 7B). Ultimately, the low oxigen induced SAPK/JNK down-stream signaling that active and phosphorylated of c-Jun and ATF2.

ROS modulate Hypoxia-induced fibrosis marker expression

Reactive oxygen species (ROS) have been implicated in pathangenesis of a variety of diease such as Atrial Fibrillation[60] and play a role as second messengers to regulate mitogenic signal transduction in rat cardiac myocyte [61]. Since hypoxia is known to increase intracellular reactive oxygen species (ROS) [62], we evaluated the role of ROS on the hypoxia induce cardiomyocyte cardiac fibrosis. For investigated the role of ROS, we used antioxidant N-acetyl cysteine (NAC) effectiviely inhibits HIF1a sythesis and fibrosis markers (COL1A and COL3A) expression in hypoxia HL-1 cell (Figure 8A), and abrogate phosphorylation of JNK, c-Jun and ATF2, that hypoxia induce JNK pathway(Figure 8B, C). The ROS levels were assessed with dichlorofluorescin diacetate (DCFH-DA), there was a significant difference in ROS production between hypoxia and normoxia. The level of ROS was great in hypoxic cells compared to cells grown under normoxia and the NAC treatment cells resulted in abrogation of ROS (Figure 8D). These results indicate the ROS productions were associated with COL1A and COL3A expression which stimulate by hypoxia. This was interesting to found that ROS inhibitor could abrogate the fibrotic marker induced by hypoxia.

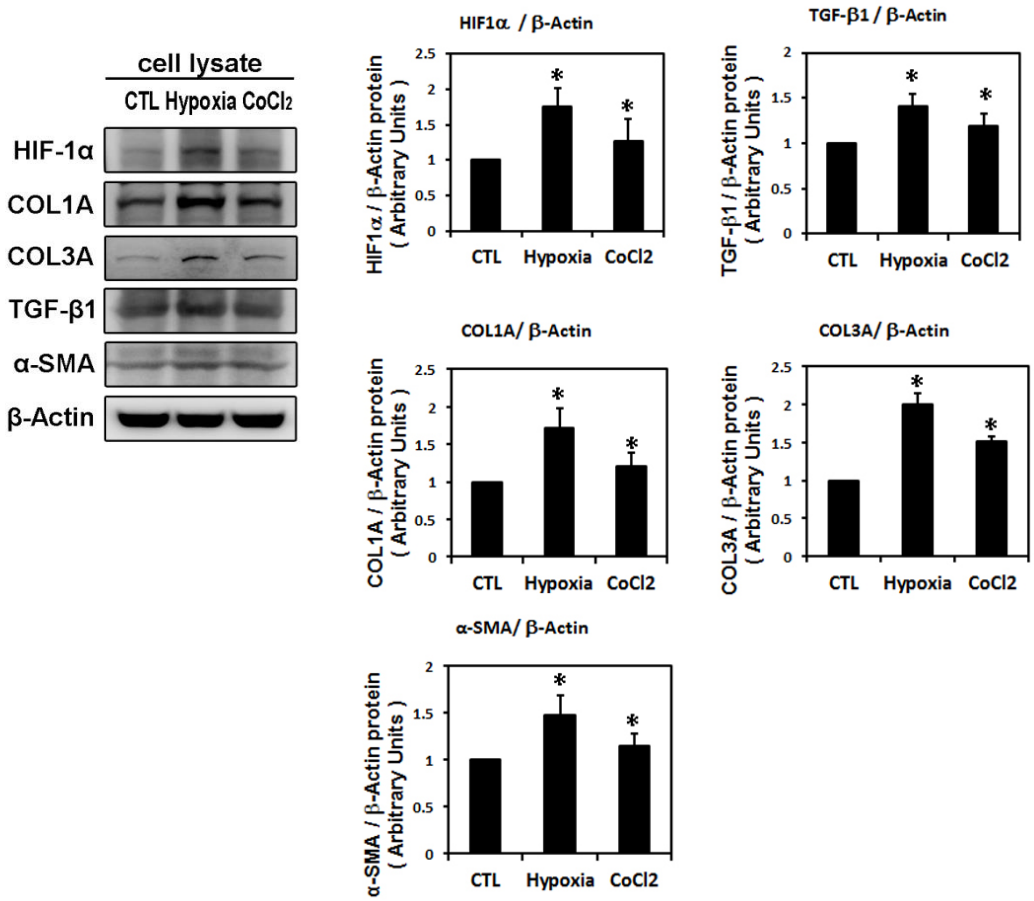
Our results suggestion hypoxia triggers cardiomyocyte fibrotic marker COL1A and COL3A expression via a ROS/JNK/ATF2 pathway. These results indicate that JNK pathway plays an important role in the hypoxia induced fibrosis mechanism, ROS/JNK/ATF2/c-Jun may be a rational target for fibrosis cardiomyocyte to low oxigen stress.

Figure 1.



Time course of fibrosis marker gene, TGF-β1, COL1A, COL3A, and α-SMA expression markedly increased in hypoxia treatment cardiomyocytes. HL-1 cell were incubated with the hypoxia condition (1%O_2) for the indicated times. Fibrosis markers were detected in cellular homogenates by Western blot analysis. Western blots contained 25μg total protein / lane and were probed with the indicated antibody. As a control for equal loading and blotting, the β-Actin is shown. **Quantification of WB** The bar graph shows the value of each sample relative to that of control that received 0hr, the none treatment. Representative blot from at least three independent experiments is shown. The data are means±SD (n=3, *p<0.05 vs. control).

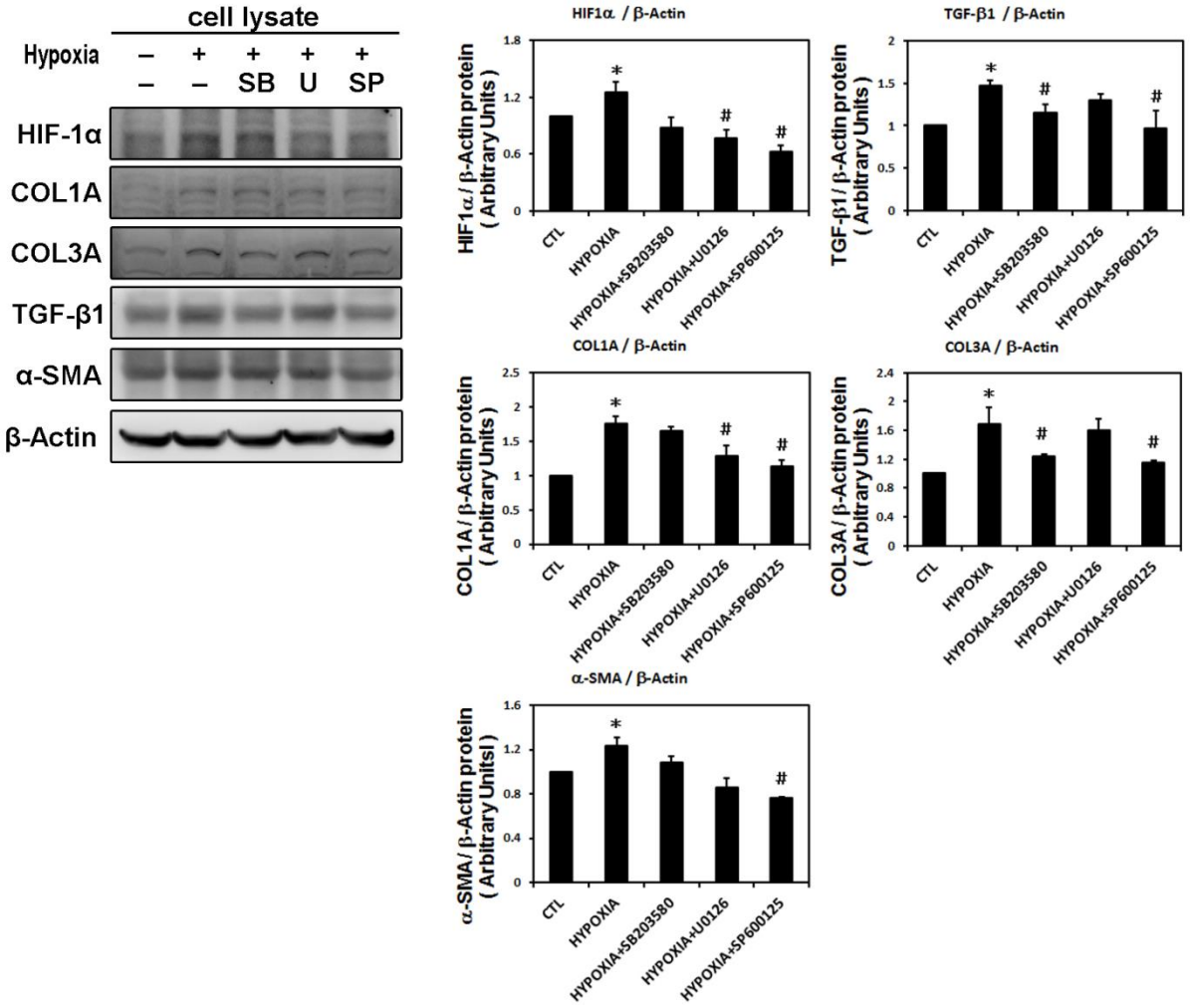
Figure 2.



Effect of fibrosis marker COL1A, COL3A, α-SMA and TGF-β1 on oxidative stress and cobalt chloride (CoCl2) treatment by hypoxia.

Hif1a, COL1A, COL3A, TGF-β1 and α-SMA protein expression levels were examined in hypoxia (1%O_2) or treat-with CoCl2(10^{-4}M) for 6hr using Western blot analysis. Western blots contained 25μg total protein / lane and were probed with the indicated antibody. As a control for equal loading and blotting, the β-Actin is shown. **Quantification of WB** The bar graph shows the value of each sample relative to that of control, the none treatment. Representative blot from at least three independent experiments is shown. The data are means±SD (n=3, *p<0.05 vs. control).

Figure 3.

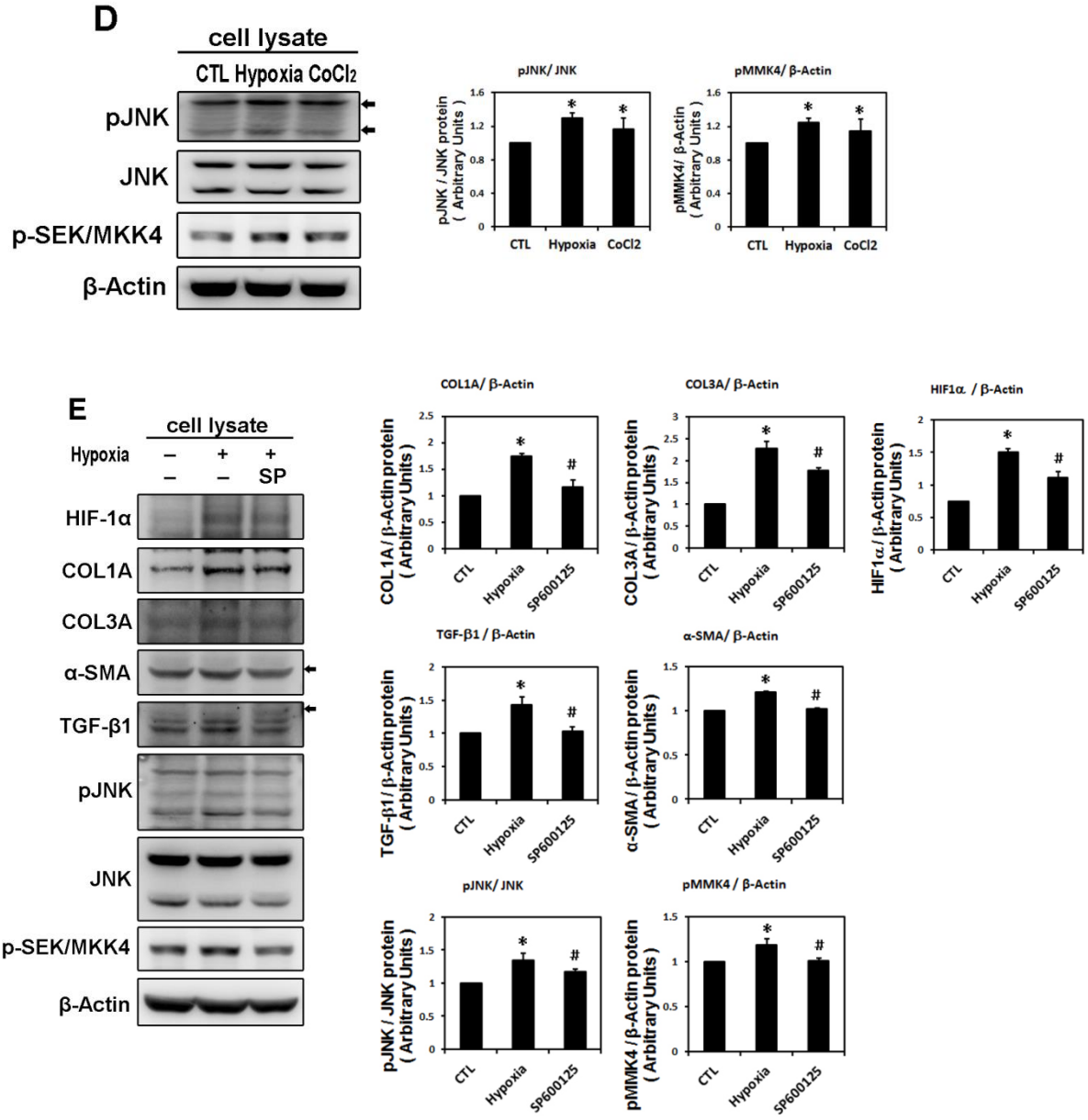


Effect of MAPK kinase inhibitor on the oxidative stress HL-1 cardiomyocyte cell.

HL-1 cells were pre-incubated with 10^{-5} M U0126, SB203580, SP600125, or DMSO as a vehicle for 30min and then treated with hypoxia stress for 6hr. Whole cell lysates were detected for COL1A, COL3A, TGF-β1 and α-SMA antibody by Western blot analysis. The blots contained 25mg total protein pre lane and were probed with the indicated antibody. As a control for equal loading and blotting, the β-Actin is shown.

Quantification of WB The bar graph shows the value of each sample relative to that of control that received vehicle, the none treatment. Representative blot from at least three independent experiments is shown. The data are means±SD (n=3, *p<0.05 vs. control; #p<0.05 vs. hypoxia).

Figure 4.

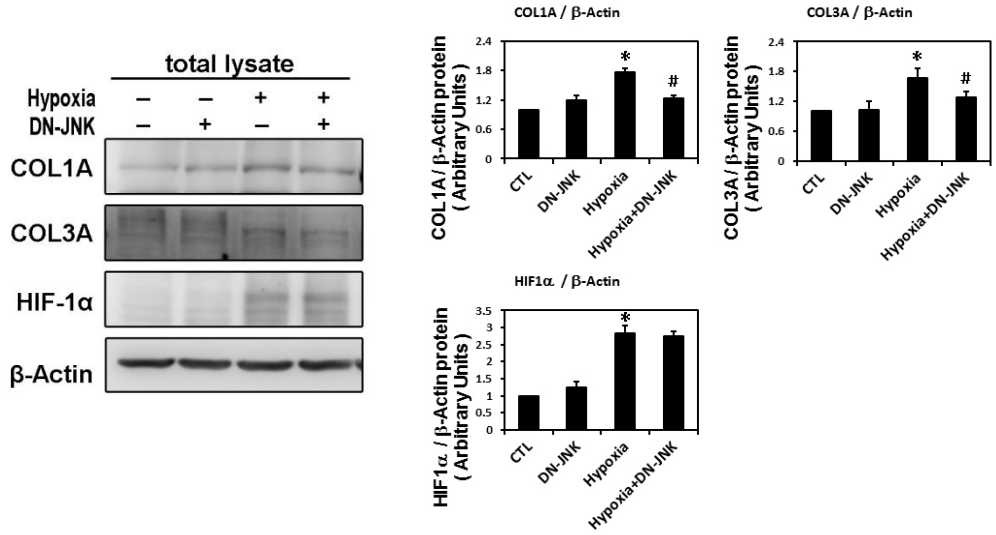


Hypoxia avtivates JNK pathway to modulate fibrosis marker expression.

(D) The HL-1 cell were exposed to hypoxia or 10^{-4}M CoCl2 for 6hr. Whole cell lysates were detected for JNK, pJNK and p-SEK/MKK4 antibody by Western blot analysis. (E) The HL-1 cell were exposed to 10^{-5}M SP600125 or DMSO as a vehicle for 30min and then treated with hypoxia stress for 6hr. Whole cell lysates were detected for JNK, pJNK, p-SEK/MKK4 COL1A, COL3A, TGF-β1 and α-SMA antibody by Western blot analysis. The blots contained 25mg total protein pre lane and were probed with the indicated antibody. As a control for equal loading and blotting, the β-Actin is shown.

Quantification of WB The bar graph shows the value of each sample relative to that of control that received vehicle, the none treatment. Representative blot from at least three independent experiments is shown. The data are means±SD (n=3, *p<0.05 vs. control; #p<0.05 vs. hypoxia).

Figure 5.

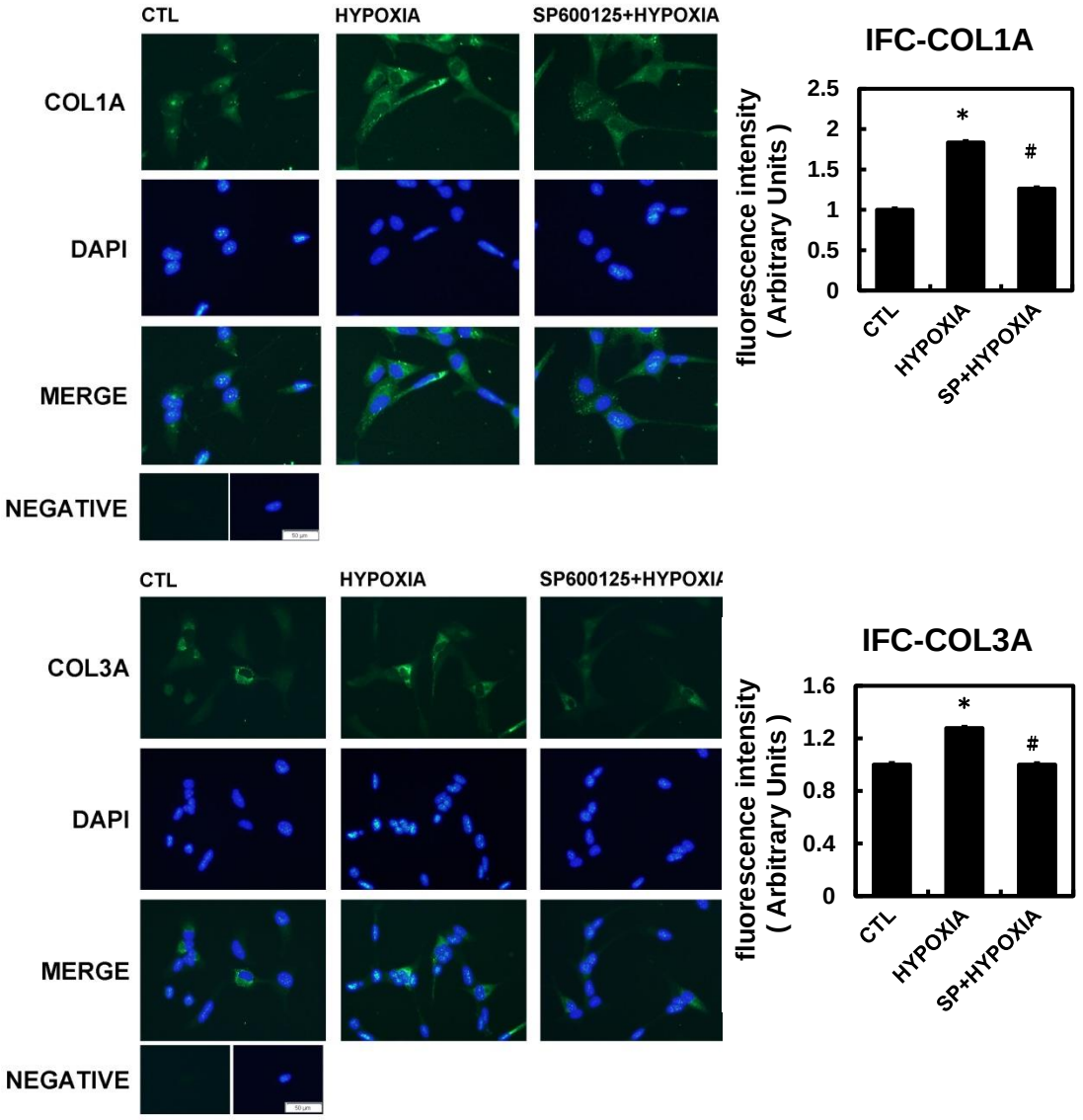


Effect of DN-JNK gene transfection decrease the hypoxia-induced COL1A and COL3A expression in HL-1 cell.

Western blot analysis with HIF-1α, COL1A and COL3A of HL-1 cell transfected with vehicle (pCDN3)(CTL) or DN-JNK for 24hr before 6hr hypoxia treatment. The blots contained 25mg total protein pre lane and were probed with the indicated antibody. As a control for equal loading and blotting, the β-Actin is shown.

Quantification of WB The bar graph shows the value of each sample relative to that of control that received 0hr, the none treatment. Representative blot from at least three independent experiments is shown. The data are means±SD (n=3, *p<0.05 vs. control; #p<0.05 vs. hypoxia).

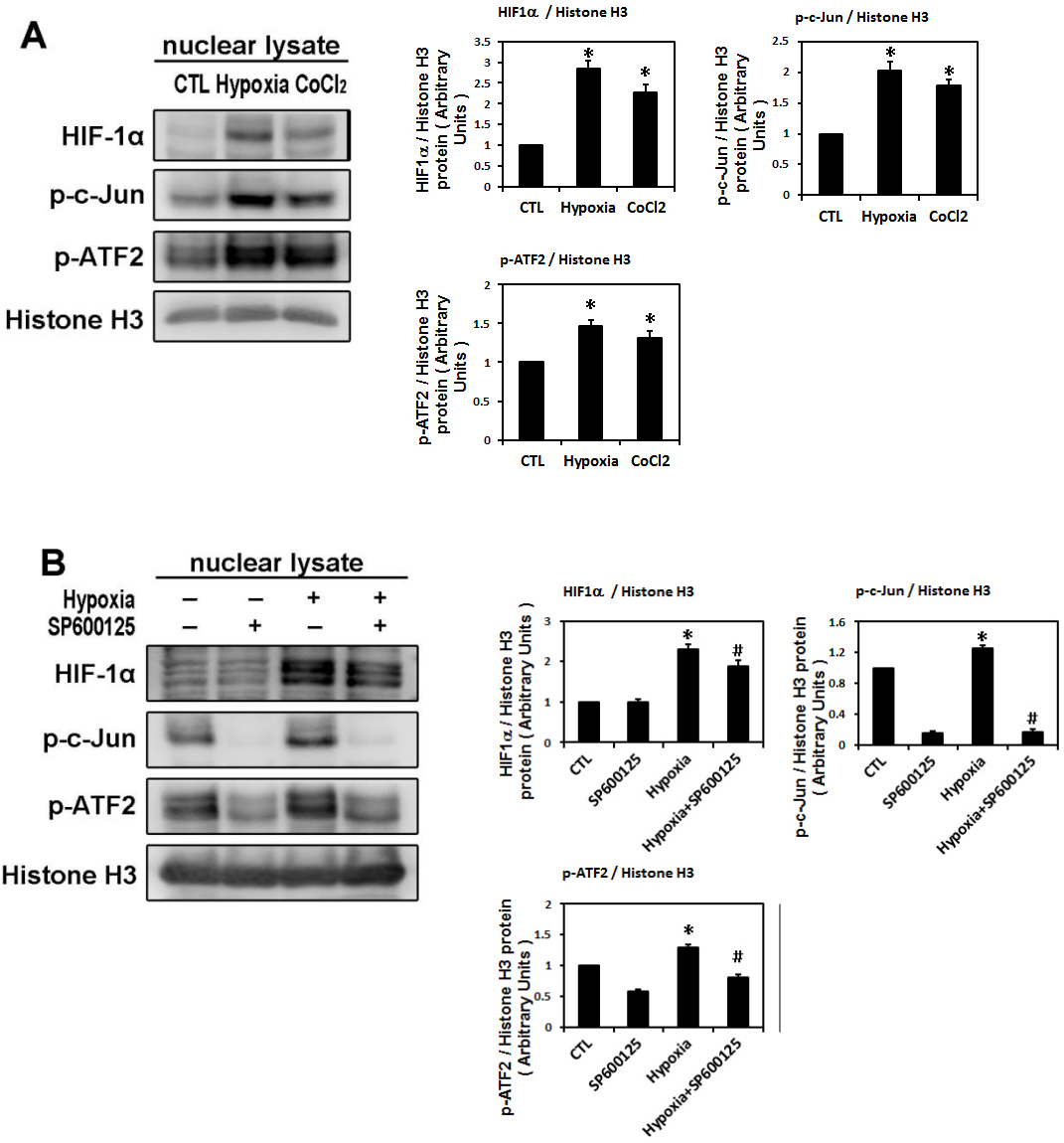
Figure 6.



Immunocytochemistry assay of fibrosis marker COL1A and COL3A in hypoxia cardiomyocyte HL-1 cell that modulate through by JNK kinase pathway

Fluorescent microscope analysis of HL-1 cell lines. The HL-1 cell were pre-treat with 10^{-5}M SP600125 or DMSO as a vehicle for 30min and then treated with hypoxia stress for 6hr. HL-1 cells were fixed and subjected to immunostaining with anti-COL1A or anti-COL3A Ab. The antibody dilution ratios were 1: 100, goat anti-mouse IgG conjugate or goat anti-rabbit IgG conjugate with fluoresce in isothiocyanate (FITC) 1: 200 (magnificationX20 on HL-1 cells). **Quantification of IHC** Pixel intensity of the cells displaying fluoresces accumulation was measured. All indicated fibrotic marker protein densities were significantly reduced in all the cells pre-treated by the blocker compared to treated by hypoxia only. Values are the arithmetic means of at least three independent experiments. The percentage of fluoresces cells was calculated per condition as described in Methods. The data are means±SEM (n >100, *P<0.01 vs. control; #p<0.05 vs. hypoxia).

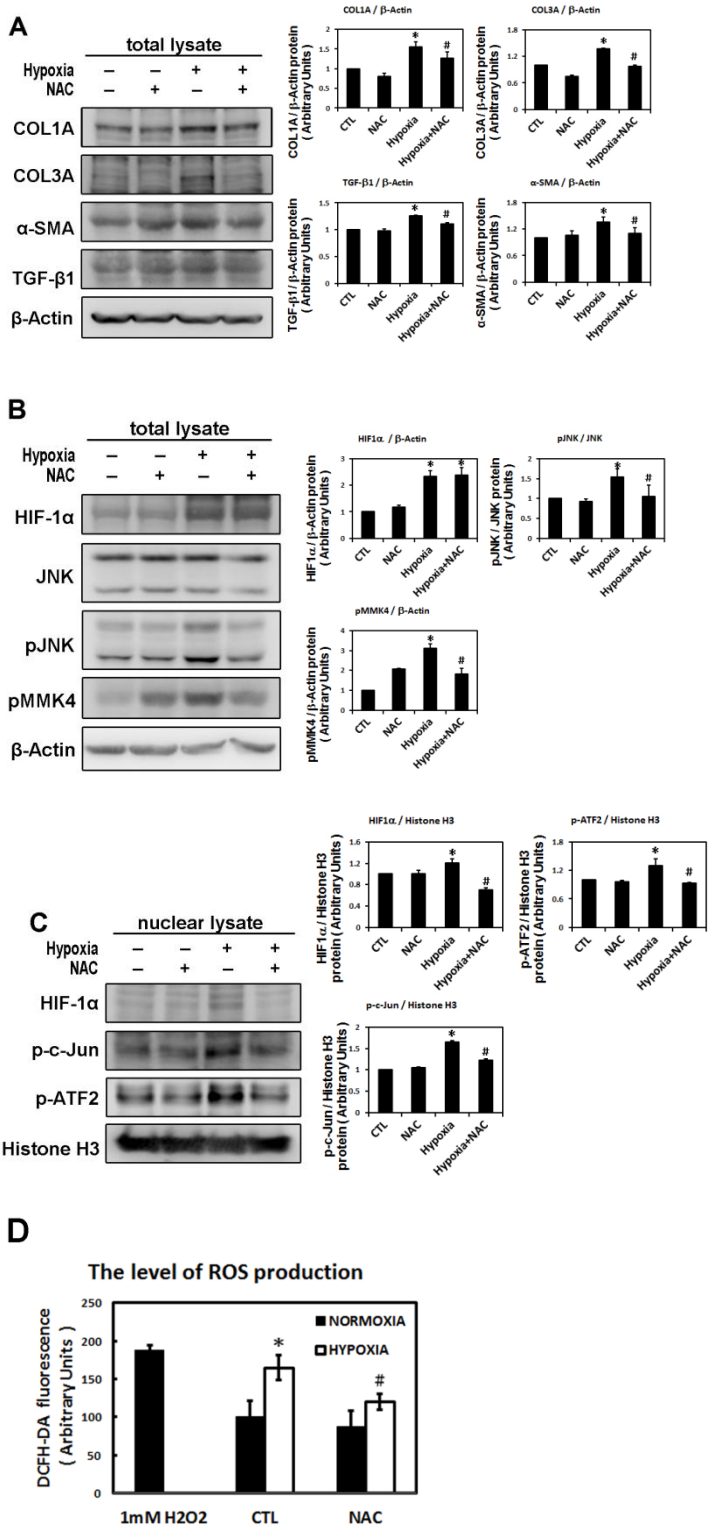
Figure 7.



Analysis of nuclear fraction from hypoxia cardiomyocyte HL-1 cell that modulated by JNK pathway.

(A) Western blot of p-c-Jun, p-ATF2 and HIF-1α nuclear lysate in cardiomyocyte under control condition, 10^{-4}M $CoCl_2$ or subjected to hypoxia (1%O_2, 6hr). (B) Western blot analysis with p-c-Jun, p-ATF2 and HIF-1a nuclear lysate of HL-1 cell pre-treat with 10^{-5}M SP600125 or DMSO as a vehicle for 30min and then treated with hypoxia stress for 6hr. The blots contained 25mg nuclear protein pre lane and were probed with the indicated antibody. As a control for equal loading and blotting, the β-Actin is shown. **Quantification of WB** The bar graph shows the value of each sample relative to that of control that received vehicle, the none treatment or relative to the hypoxia treatment. Representative blot from at least three independent experiments is shown. The data are means±SD (n=3, *p<0.05 vs. control; #p<0.05 vs. hypoxia).

Figure 8.



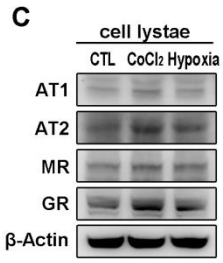
Effect of NAC on the hypoxia-induced fibrosis markers expression, phosphorylation of JNK kinase and ROS production.

HL-1 cell were pre-treated with 2 mM of NAC for 2 hr the treat with hypoxia or normoxia condition for 6hr. Western blot analysis (A, B) with Hif1a, COL1A, COL3A, TGF-β1, α-SMA, p-JNK and p-MMK4 of whole

cell lysate and (C) Hif1a, p-c-Jun, p-ATF2 of nuclear lysate. HL-1 cell with or without 2mMNAC were expose to hypoxia or normoxia and the 2',7'-dichlorofluorescin (DCF) fluorescence was measured by fluorescence Reader, as described in Materials and Methods

Quantification of WB and ROS prodution The bar graph shows the value of each sample relative to that of control, that received the none treatment or relative to the hypoxia treatment. Representative blot from at least three independent experiments is shown. The data are means±SD (n=3, *$p<0.05$ vs. control; #$p<0.05$ vs. hypoxia).

Sup figure



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科技部補助計畫衍生研發成果推廣資料表

日期:2015/10/19

科技部補助計畫	計畫名稱: 探討心肌缺氧壓力下其心房顫動及心臟纖維化的機制及其訊息傳遞路徑
	計畫主持人: 翁國昌
	計畫編號: 101-2314-B-040-018-MY3　　學門領域: 心胸內科

無研發成果推廣資料

101年度專題研究計畫研究成果彙整表

計畫主持人：翁國昌	**計畫編號：**101-2314-B-040-018-MY3
計畫名稱：探討心肌缺氧壓力下其心房顫動及心臟纖維化的機制及其訊息傳遞路徑	

成果項目			量化			單位	備註（質化說明：如數個計畫共同成果、成果列為該期刊之封面故事...等）
			實際已達成數（被接受或已發表）	預期總達成數（含實際已達成數）	本計畫實際貢獻百分比		
國內	論文著作	期刊論文	0	0	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	0	0	100%		
		專書	0	0	100%	章/本	
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（本國籍）	碩士生	0	0	100%	人次	
		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		
國外	論文著作	期刊論文	0	0	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	0	0	100%		
		專書	0	0	100%	章/本	
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（外國籍）	碩士生	0	0	100%	人次	
		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		

其他成果（無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。）	無

	成果項目	量化	名稱或內容性質簡述
科教處計畫加填項目	測驗工具(含質性與量性)	0	
	課程/模組	0	
	電腦及網路系統或工具	0	
	教材	0	
	舉辦之活動/競賽	0	
	研討會/工作坊	0	
	電子報、網站	0	
	計畫成果推廣之參與（閱聽）人數	0	

科技部補助專題研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估
   ■達成目標
   □未達成目標（請說明，以100字為限）
      □實驗失敗
      □因故實驗中斷
      □其他原因
   說明：

2. 研究成果在學術期刊發表或申請專利等情形：
   論文：□已發表 □未發表之文稿 ■撰寫中 □無
   專利：□已獲得 □申請中 ■無
   技轉：□已技轉 □洽談中 ■無
   其他：（以100字為限）

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）（以500字為限）