Differential signal transductions of adrenoceptors in ischemia-reperfused heart and hypoxia-reoxygenated cardiomyocytes

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Directed by Professor Jun Hee Sul

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Ischemia/reperfusion has been associated with ventricular (LV) remodeling, including induction of interstitial fibrosis, cardiomyocyte apoptosis, and hypertrophy. Cardiac hypertrophy is a compensatory process which occurs in pathological conditions, such as hypertension, myocardial infarction, and some genetic heart diseases. α_1-Adrenoceptors (ARs) have been implicated in the pathogenesis of cardiac hypertrophy, ischemia-induced cardiac arrythmias and ischemic preconditioning. The goal of the present study is to test the hypothesis that α_1-AR mediated hypertrophy is selectively mediated via the oxidative modification of G_h during hypoxia/reoxygenation or

ischemia/reperfusion. To perform this work, we first examined whether α_1-AR mainly mediated hypertrophic response in norepinephrine (NE)-stimulated neonatal cardiomyocytes using the α_1 selective antagonist, prazocin. The increases in protein/DNA ratio and extracellular signal-regulated kinases (ERKs) phosphorylation as an index for the hypertrophic phenotype were observed in this experiment. Reactive oxygen species (ROS) production as a stimulator was also increased in hypoxia/reoxygenation (H/R). The mRNA levels of α_1-adrenoceptors in hypertrophic responses in both *in vivo* model of I/R myocardium and *in vitro* model of H/R cardiomyocytes were not increased but mRNA level of G_h protein was significantly increased. To further address the involvement of G_h in hypertrophic response of NE-stimulated cardiomyocytes, specific relation of G_h was confirmed by using G_h overexpression and inhibition into cardiomyocytes. Also mainly increased membrane G_h protein by ROS stimulation has a more sensitive effect on myocardial hypertrophy through MEK1,2/ERKs signal transduction pathway regardless of PLC δ_1 and induction of proto-oncogene, *c-fos* by α_1-AR in neonatal cardiomyocytes. These results provide that ROS production by I/R mediates G_h protein increase and increased G_h protein leads to more specific responsiveness to NE stimulated-hypertrophy in neonatal cardiomyocytes.

Key words: cardiomyocytes, ischemia/reperfusion, hypoxia/reoxygenation, hypertrophy, α–adrenergic receptor, G protein

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I. INTRODUCTION

Cardiac hypertrophy is a compensatory process which occurs in pathological conditions, such as hypertension, myocardial infarction, and some genetic heart diseases. Hypertrophy causes generally increase of cell size, protein synthesis and enhanced sarcomere organization. Although many previous studies involved in cardiac hypertrophy underlined the relation of Gαq and $G\alpha_{12/13}$[1,2], some groups have been recently reported that cardiac hypertrophy by α_1-AR stimulation was also related G_h pathway[3].

Adrenoceptors (ARs), members of the G protein-coupled receptor (PGCR)

superfamily, have an important role in interface between the sympathetic nervous system and the cardiovascular system, with integral roles in the rapid regulation of myocardial function[4,5,6]. Cardiac myocytes express at least six types of ARs, including three types of ß-ARs ($ß_1$, $ß_2$, and $ß_3$) and three types of α_1-ARs (α_{1a}, α_{1b}, and α_{1c})[7,8]. On the basis of receptor number, $ß_1$-AR is generally the predominant receptor subtype in cardiac myocytes, although the ratio between $ß_1$ and $ß_2$ receptors varies among species, and $ß_2$-AR signaling is species-specific and dependent on age and developmental stage[9]. Among α-ARs, the α_1-ARs but not the α_2-ARs exist in cardiomyocytes and α_1-ARs have been implicated in the pathogenesis of cardiac hypertrophy in ischemia-induced cardiac arrythmias, and ischemic preconditioning[10]. Chronic activation of α_1-ARs and ß-AR stimulate cardiomyocyte hypertrophy in animals[11,12]. Signaling pathways leading from α_1-ARs activation to hypertrophy are complex and may involve activation of extracellular signal-regulated protein kinase (ERK) and PI3K through G_q, PKC, Ras, and activation of calcineurin through Ca^{2+} and calmodulin. Both pathways lead to changes in gene expression through activation of transcription factors.

G_h, transglutaminase2 is ubiquitously expressed in mammalian tissues and acts as enzymatically-active transglutaminases and GTP-binding protein mediating intracellular signaling via the α_{1b}-AR[13]. Also, Szondy *et al* showed G_h participated in the AR signaling pathway in cardiac cells and was related to ischemia/reperfusion by

using knockout mice[14]. The mechanism of G_h activation induced by exact ischemia/reperfusion remains unclear.

Reactive oxygen species (ROS) such as superoxide generated endogenously or in response to environmental stress has been implicated in tissue injury including ischemia/reperfusion. Several researchers containing Amin *et al* have shown that ROS could link to the hypertrophy in neonatal rat ventricular myocytes (NRVM) as well as in adult rat ventricular myocytes (ARVM)[15,16,17]. Gabriela *et al* showed ROS may mediate α-AR stimulated hypertrophic signaling in ARVM via G protein modification[18].

Norepinephrine (NE), a known hypertrophic reagent like epinephrine activates α_1-ARs. Singal *et al* examined increases of cell size, atrial natriuretic factor (ANF) gene expression and protein synthesis in NE-induced cardiac hypertrophy[19]. Also it was reported that NE-induced hypertrophy through ROS was mediated Ras-Raf-MEK1,2-ERK1,2 signaling pathway via α-AR[16]. Cardiac hypertrophy normally observed with chronic administration of norepinephrine is absent in α_{1b}-AR knock out (KO) mice. Moreover, mice lacking both α_{1a} and α_{1b}-ARs have been reported to have abnormal postnatal cardiac development[20].

However, little is known about hypertrophic signaling initiated by ROS in ischemia-reperfused heart. In this study, it will be addressed that ischemia-reperfusion injury of heart leads to differential signals through ARs and tested the hypothesis that

NE-stimulated hypertrophy undergoes specific signaling pathway by the oxidative modification of G_h in hypoxia/reoxygenation(H/R) or ischemia/reperfusion(I/R) injury.

II. MATERIALS AND METHODS

1. Myocardial ischemia-reperfusion protocol

All experimental procedures for animal studies were approved by the Committee for the Care and Use of Laboratory Animals, Yonsei University College of Medicine and were performed in accordance with the Committee's Guidelines and Regulations for Animal Care. Myocardial infarction was produced in male Sprague–Dawley rats (200±50 g) by surgical occlusion of left anterior descending coronary artery, according to the method described previously with minor modifications[21]. Briefly, rats were anesthetized by intramuscular injection of zoletil (50 mg/kg) and xylazine (5 mg/kg) and the chest was opened by cutting the third and fourth ribs. The heart was exteriorized through the intercostal space and the left coronary artery was ligated 2–3 mm from its origin with a 6-0 prolene suture (ETHICON Inc., NJ, USA). Coronary occlusion was maintained for 1 hour, followed by removal of the suture and reperfusion for 3 hours. Throughout the operation, the animals were ventilated with 95% O_2 and 5% CO_2, using a Harvard ventilator (Harvard Apparatus, Millis, MA).

Sham-operated animals were treated similarly, except that the coronary suture was not tied.

2. Culture of cardiomyocytes and *in vitro* hypoxia-reoxygenation treatment

Neonatal rat cardiomyocytes were prepared by an enzymatic method. Briefly, hearts of 1- to 2-day-old Sprague–Dawley rat pups were dissected, minced, enzymatically dispersed with 10 ml of collagenase I (0.8 mg/ml, 262 U/mg, Gibco BRL, Paisley, UK) and centrifuged differentially to yield 5×10^5 cells/mL. After incubation for 4–6 hours, the cells were rinsed twice with cell culture medium and 0.1 μM BrdU (Sigma Chemical St., Louis, MO) was added. Cells were then cultured in a CO_2 incubator at 37 °C for 1-2 days. For the treatment of hypoxia/reoxygenation, the cells were anaerobically incubated for 1 hour using deoxygenated serum free α-MEM (Gibco BRL, Paisley, UK) in an anaerobic chamber (Thermo Forma Anaerobic System Model 1025, Marietta, USA), followed by aerobic culture for different times.

3. Quantification of total protein and DNA from cardiomycytes

Total protein content/DNA ratios were measured after solubilizing the cells in 1 N NaOH at 60 °C for 30 minutes. Total protein content was determined with BCA protein reagent (Pierce Chemicals, Ontario, USA) with a bovine albumin standard according to the manufacturer's direction. For the quantitative measurement of DNA,

cells were lysed by adding Sodium Dodecyl Sulfate (SDS, Sigma Chemical St., Louis, MO) and proteinase K (Bioneer, Daejon, Korea), and extraction of DNA was performed with phenol. The absorbance of the purified DNA was measured at 260 nm.

4. Measurement of intracellular reactive oxygen species generation

Neonatal rat cardiomyocytes were labeled with 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA; Molecular Probe, CA, USA). The probe H_2DCFDA (10 μM) enters the cell and the acetate group on H_2DCFDA is cleaved by cellular esterases, trapping the nonfluorescent 2′,7′-dichlorofluorescin (DCFH) inside. Subsequent oxidation by reactive oxygen species yields the fluorescent product DCF. The dye, when exposed to an excitation wavelength of 480 nm, emits light at 535 nm only when it has been oxidized. Labeled cells were examined using a luminescence spectrophotometer for oxidized dye.

5. Flow cytometry

Cardiomyocytes were retrieved with a standard trypsinization technique. Cells were washed in phosphate buffered saline (PBS) and fixed in 70 % ethanol at 4 ℃ for 30 min with agitation. Cells were washed twice in PBS and resuspended at 2×10^6 cells/ml in blocking buffer (1 % BSA, 0.1 % FBS) containing anti-G_h antibody (Santa Cruz Biotechnology, Inc., CA, USA) diluted at 1/200. The labeling reaction mixture

was agitated for 20 minutes at room temperature. Cells were washed twice and then labeled with anti-goat-FITC conjugated IgG (Jackson ImmunoResearch Laboratories, Inc., PA, USA) diluted to 1/400 for 20 min at room temperature in the dark. After two more washes, flow cytometric analysis was performed on a FACS Calibur system (Becton Dickinson, CA, USA) using CellQuest™software with 10,000 events recorded for each sample. Data was acquired in single parameter histogram with appropriate particle size and light scatter gating.

6. Immunoblot analysis

Proteins were separated by SDS-PAGE using 10–12 % polyacrylamide gels and then electrotransferred to methanol-treated polyvinylidene difluoride membranes. The blotted membranes were washed twice with water and blocked by incubation with 5% nonfat dried milk in PBS buffer (8.0 g NaCl, 0.2 g KCl, 1.5 g NaH_2PO_4, 0.2 g K_2HPO_4 per liter). For the phosphorylation analysis of mitogen-activated protein (MAP) kinases, the membranes were probed with anti-phospho-p38 kinase, JNK and ERK antibodies (Santa Cruz Biotechnology, Inc., CA, USA), followed by goat anti-rabbit and goat anti-mouse IgG-peroxidase. The blots were detected using enhanced chemiluminescence kits (ECL, Amersham Pharmacia Biotech, Piscataway, NJ)

7. RT-PCR analysis

The expression levels of α_1-ARs mRNA were analyzed by the reverse transcription polymerase chain reaction (RT-PCR) technique. Total RNA was prepared by Ultraspect™-II RNA system (Biotecx Laboratories, USA) and single-stranded cDNA was then synthesized from isolated total RNA by Avian myeloblastosis virus (AMV) reverse transcriptase. A 20-μl reverse transcription reaction mixture containing 1 μg of total RNA, 1× reverse transcription buffer (10 mM Tris–HCl, pH 9.0, 50 mM KCl, 0.1 % Triton X-100), 1 mM deoxynucleoside triphosphates (dNTPs) 0.5 unit of RNase inhibitor, 0.5 μg of oligo(dT)15, and 15 units of AMV reverse transcriptase were incubated at 42 °C for 15 minutes, heated at 99 °C for 5 minutes, and then incubated at 0–5 °C for 5 minutes. PCRs were performed for 35 cycles with 3′ and 5′ primers based on the sequences of α_1-ARs gene primers (α_{1a}, 5'-GAGAATTCCGAG GCCTCAAGTCCGGCCT-3' and 5'-TTGAATTCTCGGGAAAACTTGGCAG-3'; α_{1b} , 5'-CTGGGGAGAGTTGAAAGATGCC-3' and 5'-CCGACAGGATGACCAAG ATGTT-3'; α_{1d}, 5'-TTGAATTCCTACAGA GACCCACGACCCAG-3' and 5'-AGGG CACGTAGAAGGAGAC-3'). The GAPDH primers (5'-CTCCCAACGTGTCTGTT GTG-3’ and 5’- TGAGCTTGACAAAGTGGTCG-3') were used as the internal standard. The signal intensity of the amplification product was normalized to its respective GADPH signal intensity.

8. Confocal microscopy and fluorescence measurements.

The measurement of cytosolic free Ca^{2+} concentration was estimated by the confocal microscopy analysis. Neonatal rat cardiomyocytes are plated on 4 well slide chamber coated with 1.5 % gelatin for 1 day in α-MEM containing 10% fetal bovine serum (Gibco BRL, Paisley, UK) and 0.1 μM BrdU (Sigma Chemical St., Louis, MO). After incubation, the cells were washed with modified Tyrode's solution with 0.265 g/L $CaCl_2$, 0.214 g/L $MgCl_2$, 0.2 g/L KCl, 8.0 g/L NaCl, 1 g/L glucose, 0.05 g/L NaH_2PO_4, and 1.0 g/L $NaHCO_3$. Cells were then loaded with 5 mM of the acetoxymethyl ester of fluo-4 (Fluo-4 AM, Molecular Probes, CA, USA) for 20 min, in the dark and at 37 °C. Fluorescence images were collected using a confocal microscope (Leica, Solms, Germany) excited by 488 nm line of argon laser and emitted light collected through a 510-560 nm band-pass filter. Relative data of intracellular Ca^{2+} was determined by measuring fluorescent intensity.

9. Statistical analysis

All data are presented as means ±S.E. Data were analyzed by 1-way ANOVA followed by Dunnett's test. *P* values <0.05 were considered significant.

III. Results

1. Selectivity of adrenoceptors in NE-stimulated cardiomyocytes

To confirm selectivity of adrenoceptors in NE-stimulated cardiomyocytes, cardiomyocyte protein synthesis was measured as an index for the hypertrophic phenotype by NE. After α_1 selective antagonist, prazosin (100 nM) and β antagonist, propanolol (2 μM) were treated for 30 minutes, norepinephrine (10 μM) was treated with cardiomycytes for 24 hours. Norepinephrine significantly increased the protein/DNA ratio up to 130 %, based on the control. Whereas α_1-antagonist, prazocin, decreased the protein/DNA ratio increased by NE treatment, β-antagonist, propranolol, did not affect NE-induced protein/DNA ratio (Figure 1A). Phosphorylation of ERKs also was significantly increased to 3 folds in cardiomyocytes induced by NE treatment and the phosphorylation of ERKs was specifically inhibited to that of control by prazocin (Figure 1B). These data indicates that α_1-AR mainly mediated hypertrophic response in NE-stimulated neonatal cardiomyocytes.

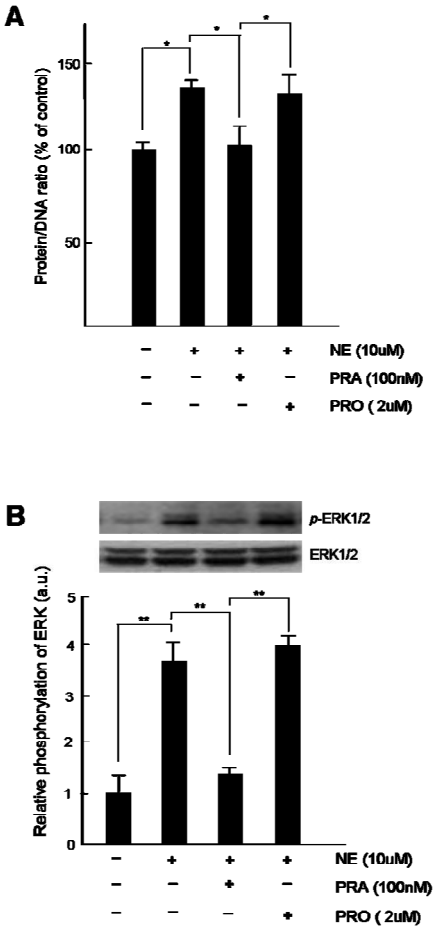

Figure 1. Selectivity of adrenoceptors in NE-stimulated cardiomyocytes.

(A) To assess hypertrophic response, total protein and genomic DNA were measured as $2x10^6$ cells after 24 hour-culture in response to different condition ($^{*}P < 0.05$). (B) Immunoblots for p-ERK1,2 and ERK1,2 in neonatal cardiomyocytes was performed by prazocin (PRA) or propanolol (PRO) pre-treatment followed by NE. The monoclonal antibody was used for detection specifically recognized the 42-kDa and 44-kDa. Data are presented as means ±SE and summarized from 3 individual experiments ($^{**}P < 0.01$).

2. Reactive oxygen species (ROS) production in hypoxia/reoxygenated cardiomyocyte.

To show the ROS production in hypoxia/reoxygenated(H/R) cardiomyocytes as compared with ischemia/reperfused (I/R) myocardium, ROS production was monitored at different times of reoxygenation in cardiomyocytes. And exogenously added H_2O_2 was chosen as positive control to show an increase in intracellular ROS.

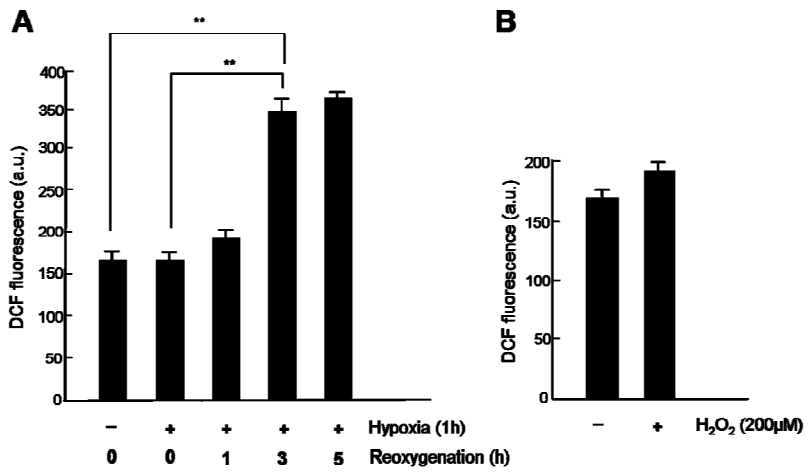

Figure 2. Reactive oxygen species (ROS) production in hypoxia/reoxygenated cardiomyocytes. (A) Neonatal cardiomyoctes subjected to hypoxia at reoxygenation time-dependent manner. A significant rise was shown in cardiomyocytes treated to reoxygenation 3 hours and 5 hours after hypoxia. (B) H_2O_2 (200 μM) was added in cardiomyocytes. Data are presented as means ±SE and summarized from 3 individual experiments ($^{**}P < 0.01$).

ROS production was increased on 10% by H_2O_2 (200 μM) treatment for 30 minutes in cardiomyocytes. H (1 h)/R (3 h) caused a marginal increase in DCF fluorescence over the labeled normoxic control and hypoxic control cells (Figure 2). These data indicates that H (1 h)/R (3 h) leads to meaningful ROS production.

3. Expression of α_1-adrenoceptors in H/R cardiomyocytes and I/R myocardium.

To determine the involvement of α_1-adrenoceptors in hypertrophic responses in both *in vivo* model of I/R myocardium and *in vitro* model of H/R cardiomyocyte, mRNA levels of α_1-adrenoceptors were primarily examined. After H (1 h) / R (3 h) treatment of cardiomyocytes, cells were stimulated with NE (10 μM) for 30 minutes. Expressions of α_{1a}, α_{1b} and α_{1d} were not changed in H/R cardiomyocytes. To address these phenomena *in vivo*, mRNA levels of α_1-adrenoceptors were also measured in I (1 h) / R (3 h) myocardium. Like H/R cardiomyocytes, expressions of α_{1a}, α_{1b} and α_{1d} were not changed in I/R myocardium (Figure 3). These data indicate that hypertrophic response by ROS production was not related to the expression of α_1-adrenoceptors in both H/R cardiomyocytes and I/R myocardium.

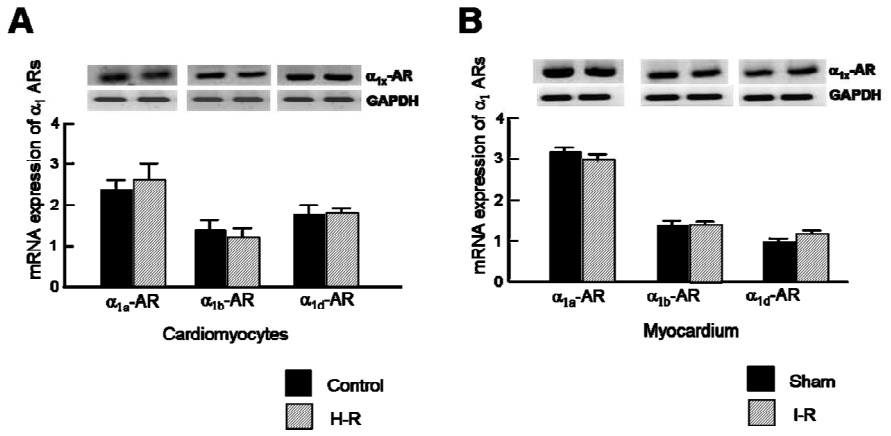

Figure 3. Expression of α $_1$-adrenergic receptors in H/R cardiomyocytes and I/R myocardium. Cardiomyocytes and heart tissues were each obtained from H(1h)/ R(3 h) and I(1 h)/ R(3 h). The α_1-adrenergic receptor subtypes were not shown differences in expression (P > 0.05). Data are presented as means ±SE and summarized from 3 individual experiments for all groups.

4. Expression of G proteins in H/R cardiomyocytes and I/R myocardium.

To find whether the changes of G protein expression levels were involved with hypertrophy in H/R and I/R, expression level of the G proteins was examined. In H/R cardiomyocytes, G_h protein expression was mainly increased but the other G proteins expression were not significantly changed, compared to normal cardiomyocytes (Figure 4A). And to address these phenomena *in vivo*, expression levels of G proteins were also measured in I (1 h) / R (3 h) myocardium. Like H/R cardiomyocytes, expression of G_h was increased but the other G proteins was not changed in I/R

myocardium. (Figure 4B). Furthermore G_h protein translocated in cellular membrane was more increased than cytosolic G protein after H (1 h) / R (3 h) (Figure 4C). Also increase of G_h protein by reoxygenation time was confirmed (Figure 5).

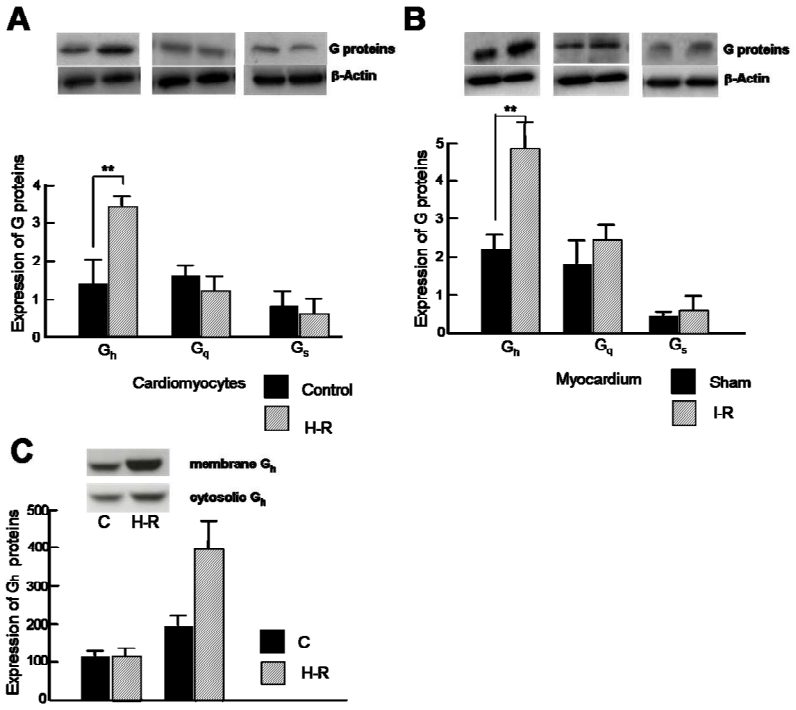

Figure 4. Expression of G proteins in H/R cardiomyocytes and I/R myocardium.

(A) Cardiomycoytes obtained from H/R were lysed, and 20 ug of protein of protein were fractionated on 10 % SDS-PAGE. (B) Heart tissues obtained from I/R and sham rats were homogenized, and 20 ug of protein were fractionated on 10 % SDS-PAGE. G_h protein subtypes were shown differences in expression. (C) expression levels of membrane and cytosolic G_h were estimated ($^{**}P < 0.01$).

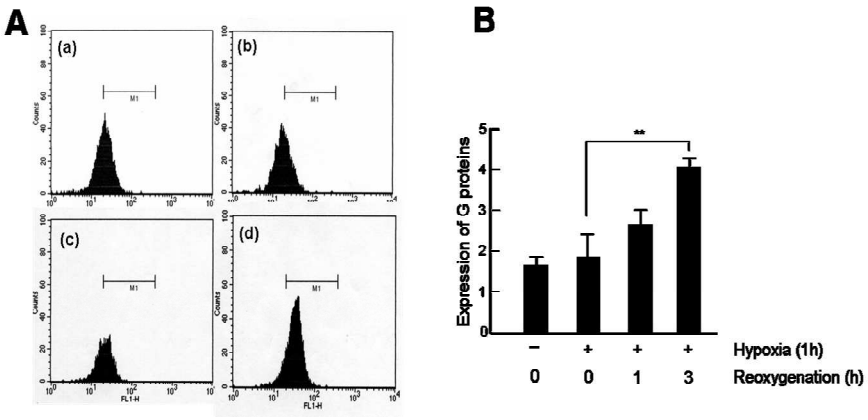

Figure 5. Expression level of G_h in H/R cardiomyocytes.

Cells were cultured in serum-free medium in anaerobic chamber for 1 hour, and then reoxygenation with 10 % FBS in time-dependent manner. (A) The expression level of G_h of H/R cardiomyocytes was detected by flow cytometry. (B) Results are presented as means ±SE and summarized from 3 individual experiments ($^{**}P < 0.01$).

To further address the involvement of G_h in hypertrophic responses of NE-treated cardiomyocytes, G_h gene was transfected into cardiomyocytes for overexpression. In G_h-transfected cells, G_h protein was highly expressed both in membrane and in cytosolic fraction (Figure 6A). NE further increases ERKs phosphorylation compared to the control. To test if the increased phosphorylation of ERKs was tightly related to G_h overexpression in NE-stimulated cardiomyocytes, siRNA for G_h was transfected into cells for transient knock down for G_h. In cells treated with siRNA for G_h, NE-stimulated cardiomyocytes abolished ERKs phosphorylation induced by α_1 adrenoceptor (Figure 6B). These data indicate that H/R stimulation and I/R injury affect the expression of G_h, leading to ERKs phosphorylation regardless of expression

level of α_1 adrenoceptors.

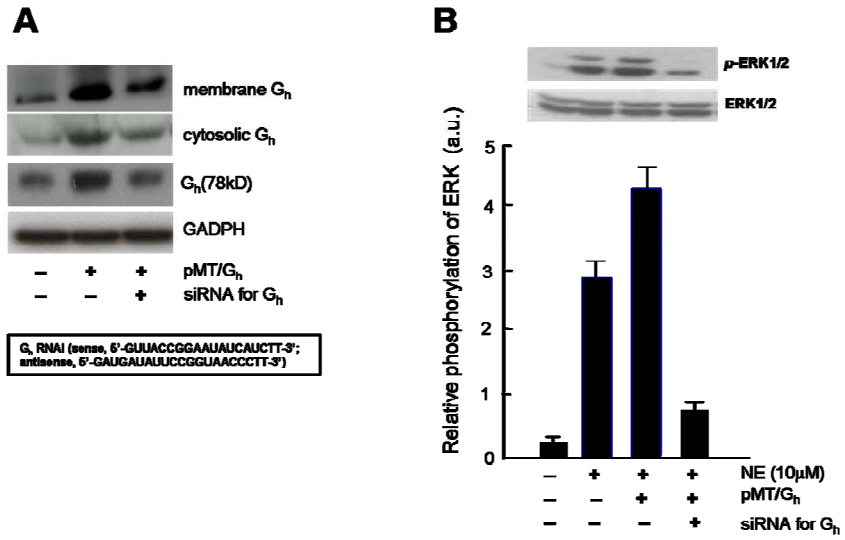

Figure 6. Coupling of G_h and ERK1/2 activation in NE-stimulated cardiomyocytes. Cardiomyocytes were treated siRNA for 12 hours and/or transfected pMT/G_h for additional 12 hours and then NE stimulation was added for 10 minutes. G_h expression (A) and ERK activation (B) were detected by RT-PCR and immunoblot analysis. All data was summarized from 3 individual experiments.

5. Effect of upstream regulators of ERKs in H/R cardiomyocytes followed by norepinephrine stimulation

To test if overexpression of G_h induced by H/R affects upstream regulators of ERKs, MAPKs and MEK1,2 was examined in H (1h) /R (3h). As shown in previous data, increased G_h protein inducing H/R affected more highly on the hypertrophic marker MAPKs by NE stimulation in neonatal cardiomyocytes. The phosphorylation

of ERKs, not P38 and JNK, was up-regulated by the treatment with H/R followed by additional NE (10minutes) treatment compared with NE treatment in control (Figure 7). The phosphorylation of MEK1,2 upstream regulator of ERKs was significantly increased at reoxygenation 3 hours (Figure 8). These results showed that intracellular signaling pathway induced by norepinephrine is primarily processed by MEK1,2/ERKs cascade through α_1-AR in cardiomyocytes and MEK1,2/ERKs cascade was more increased by G_h protein increase throughout H/R.

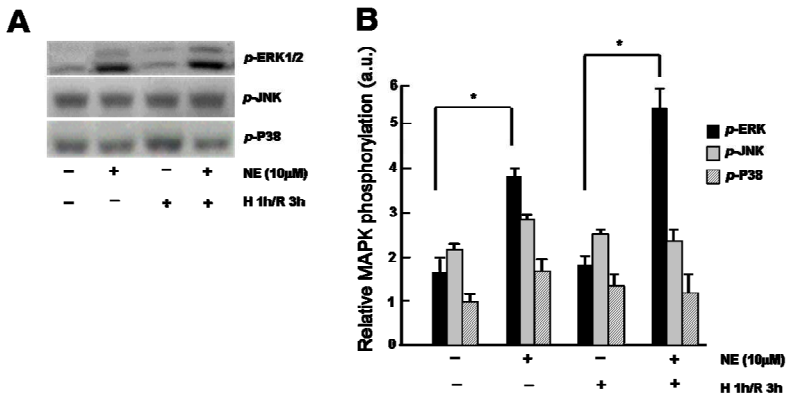

Figure 7. Phosphorylation of MAPKs in H/R cardiomyocytes.

(A) Phosphorylation of ERK, JNK and P38 was assessed under H/R with or without NE stimulation. This was performed by immunoblot. (B) Relative phosphorylation was represented the ratio to the lowest activity and results are presented from 3 individual experiments ($^{*}p < 0.05$).

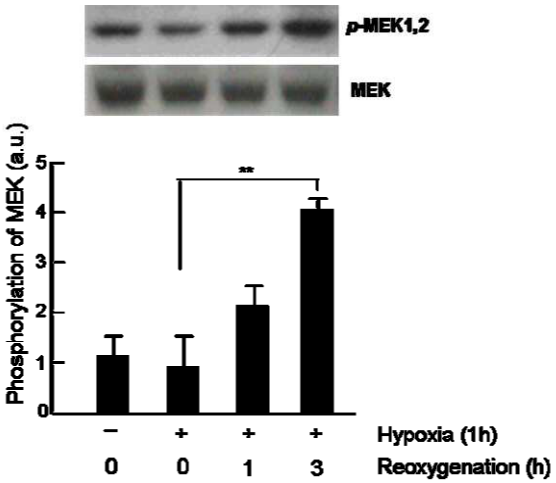

Figure 8. Effects of G_h on upstream regulators of ERK1/2 in H/R cardiomyocytes.
Cardiomyocytes were exposed to 1 hour-hypoxia followed by 1 hour- and 3 hours-reoxygenation. 100 ug of cell lysate was fractionated on 12 % SDS-PAGE, blotted on PMSF membrane, and immunostained with rabbit polyclonal anti-MEK1,2 antibody (1:1000). Phosphorylation of MEK1,2 was detected finally by ECL detection system. Results are presented as means ±SE and summarized from 3 individual experiments ($^{**}P < 0.01$).

6. Regulation of proto-oncogene expression in H/R cardiomyocytes.

To determine the immediate early genes were involved in hypertrophy via H/R, we examined the mRNAs for *c-jun, c-fos, c-myc* in H/R cardiomyocytes. Significant increases in *c-jun, c-fos* and *c-myc* were observed by NE stimulation or H/R, but only *c-fos* was significantly elevated by H/R with additional NE treatment (Figure 9). The induction of *c-jun* and c-*myc* was the independent consequence of H/R or NE. It seems that these genes do not have involvement in hypertrophic

response by oxidative modification of G protein. That is, these data show that increased expression level of G_h in H/R mainly affects *c-fos* and activation of *c-fos* maybe provoke myocardial hypertrophy.

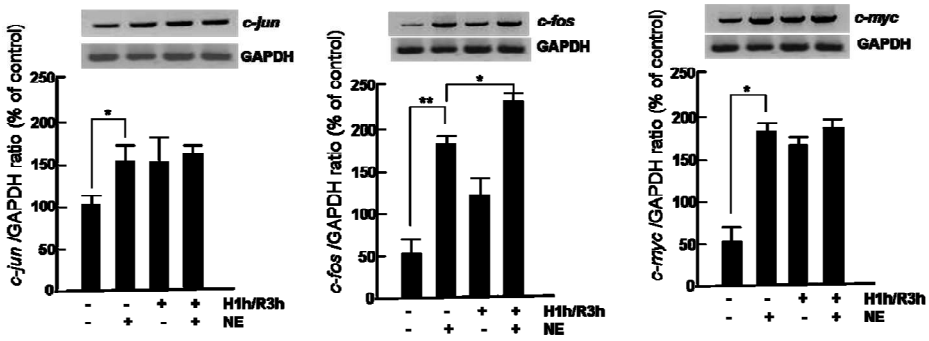

Figure 9. Regulation of proto-oncogenes expression in H/R cardiomyocytes.
The mRNA expression in cardiomyocytes was evaluated by differential RT-PCR analysis. Cardiomyocytes were 10 minutes stimulation with NE after H(1h)/R(3h). Results are presented as means ±SE and summarized from 3 individual experiments ($^{*}P < 0.05$, $^{**}P < 0.01$).

7. Expression of G proteins on PLC δ_1 in H/R cardiomyocytes and I/R myocardium.

We previously examined that PLC δ_1 is the effector of G_h-mediated signaling. As the enzymatic activity of all PLC isozymes is dependent on Ca^{2+} concentration, the increase in intracellular Ca^{2+} may contribute to the increase in PLC δ_1 expression. But PLC δ_1 was decreased in H (1 h)/R (3 h) and I (1 h)/ R(3h) and did

not coincide with elevated G_h protein (Figure 10).

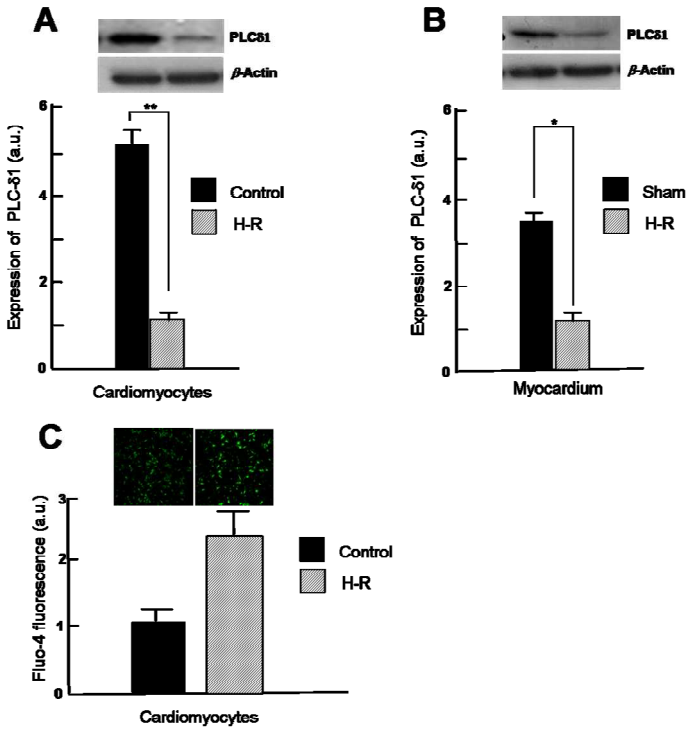

Figure 10. PLC δ_1 expression in H/R cardiomyocytes and I/R myocardium.

(A) Cardiomyocytes and (B) heart tissues were each obtained from H(1 h)/ R(3 h) and I(1 h)/ R(3 h). 100ug of cell lysate was fractionated on 10 % SDS-PAGE, blotted on PMSF membrane, and immunostained with mouse monoclonal anti-PLC δ_1 antibody (1:1000). Phosphorylation of PLCδ_1 was detected finally by ECL detection system. (C) cardiomyocytes were exposed to H (1 h)/ R (3 h) and representative $[Ca^{2+}]_i$ images were presented with fluo-4 fluorescence. Results are presented as means ±SE and summarized from 3 individual experiments ($^{*}P < 0.05$, $^{**}P < 0.01$).

Enhanced expression level of G_h by H/R or I/R was influenced by ROS

production and not by increased intracellular Ca^{2+} concentration. These results mean that this hypertrophic mechanism involved in H/R and I/R was independent from pathway related to PLC δ_1.

IV. Discussion

Myocardial infarction, acute renal failure, adult respiratory distress syndrome, and organ transplantation are all characterized by a period of ischemia followed by reperfusion that results in increased regional vascular resistance and decreased organ perfusion[22,23,24,25]. Increase in vascular tone after ischemia-reperfusion (I/R) was shown to be related to damaged vascular smooth muscle and endothelial cells, resulting in a shift in balance between vasoconstrictor and vasodilator tone. I/R has also been associated with a variety of events involved in vascular remodeling, including accumulation of growth factors, protein tyrosine phosphorylation, and cell proliferation.

The goal of the present study is to test hypothesis that α-AR stimulated hypertrophy is more selectively mediated via the oxidative modification of G_h during hypoxia/reoxygenation or ischemia/reperfusion injury. We and others have shown that α-AR mediated hypertrophic signaling and hypertrophy is mediated by reactive oxygen species (ROS). Nevertheless, little is known about the mechanism by which ROS initiates hypertrophic signaling.

Some studies showed that the functional consequences of G protein-coupled receptor activation depend not only on the levels of the signaling proteins involved but also on the efficiency of interaction between receptor and G protein. Using the coimmunoprecipitation technique to directly determine the association of specific α_1-receptor subtypes with Gα subunits, α_{1a}- and α_{1d}- adrenergic receptors are coupled to G$\alpha_{q/11}$ protein. Moreover, receptor stimulation enhances the coupling of all three α_1-adrenergic receptors to their G proteins. In the tail arteries obtained from rats subjected to I/R, receptor-stimulated coupling of the α_{1a}-adrenergic receptor to G$\alpha_{q/11}$ protein was increased[26]. We also demonstrated in neonatal cardiomycotes that α-AR stimulated hypertrophy is associated with ROS-mediated activiation of G_h. Although expression levels of α-ARs were not changed by ROS production, expression level of G_h was increased through ROS production. Moreover increased G_h affects the development of myocardial hypertrophy in hypoxia/reoxygenation. Interestingly, I/R did not affect the expression level of the other G protein associated α-adrenergic receptors.

And then we examined the signaling pathway on hypertrophy by G_h modification. After the exposure of the neonatal cardiomyocytes to I/R, NE stimulation leads to enhanced α_1-adrenergic-receptor-stimulated hypertrophy through enhanced G_h-MEK1,2-ERKs signaling and expression of *c-fos*.

The *c-fos*, a protooncogene, mediates both proliferative and cellular growth in

many cell types. Recently, Shuguang showed that the expression of *c-fos* in brain tissue was increased after I/R[27] and Xiao reported that expression of *c-fos* was also increased by hepatic ischemia/reperfusion in rats[28]. Komuro also showed c-fos was stimulated by activation of PKC and this was related to increase of skeletal α-actin genes, β-MHC and MLC-2a[29] which increase in hypertrophic response. These results indicated that myocardial hypertrophy may involve in the early phase of myocardial H/R.

Also we examined that this hypertrophic mechanism related with G_h was independent from pathway related to PLC δ_1. The hypertrophic signaling pathway via PLC was examined by many researchers[30,31]. But we revealed that PLC δ_1 has no relation in hypertrophy via ROS modification of G_h.

Taken together, these findings indicate that α-AR stimulation by ROS cause the oxidative posttranslational modification of G_h in neonatal cardiomyocytes. Increased G_h enhanced hypertrophic response through the MEK1,2-ERKs signaling and c-fos expression. Thus, modification of G_h by ROS may play a role in the phathophysiology and/or therapy of myocardial remodeling.

V. References

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Abstract (in Korean)

허혈-재관류 심장과 저산소-재산소화 심근 세포에서 아드레날린성 수용체의 특이적 신호전달

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김미진

허혈 후 재관류는 간질성 섬유화, 심근세포의 사멸과 심근비대의 유발을 내포하는 심실개조와 관련되어 있다. 심근의 비대는 고혈압, 심근경색, 유전성 심장 질환과 같은 병리학적인 병태에서 발생하는 보상과정이며, α_1-아드레날린성 수용체(adernoreceptor, AR)가 연관되어 있다. 본 연구의 목적은 심장에서 허혈/재관류나 저산소/재산소화에 의한 G_h의 산화적 조절이 α-AR에 의한 심근비대 반응을 선택적으로 매개하는지 확인하는데 있다. 먼저 신생백서의 심근세포를 배양한 뒤, α_1 길항체인 prazocin을 이용하여 노르에페네프린 (NE) 자극에 의한 심근비대반응이 α_1-AR에 의해 매개됨을 총단백질 생성량과 ERK의

인산화 정도를 이용하여 확인하였고, 저산소/재산소화 처리를 통하여 재산소화 시간증가에 따른 활성산소종 (ROS) 생성을 관찰하였다. 또한 허혈/재관류된 심장과 저산소/재산소화된 심근세포에서 발생하는 비대반응에 따른 α_1-AR의 mRNA 수준과 G 단백의 발현수준을 조사한 결과, α_1-AR에서는 아형들의 발현수준 변화가 없었으나, G 단백질은 G_h의 발현수준이 유의하게 증가하였다. 그리고, 비대 반응에서 G_h 의 특이적 연관성을 검증하기 위하여 G_h 유전자의 도입과 억제 실험을 한 결과, G_h 의 관련성이 확인되었다. 저산소/재산소화된 심근세포에서 ROS 자극에 의해 증가된 G_h는 주로 세포막 단백질이었으며 PLC δ_1와는 관계없이 MEK1,2/ERKs 신호전달 기작을 통해 심근비대를 매개하였다. 이상의 연구 결과를 토대로 저산소/재산소화에 노출된 심근세포는 α_1-AR와 증가된 G_h를 통하여 MEK1,2-ERKs의 활성과 원종양유전자인 *c-fos*의 발현 증가를 유도하여 심근비대를 유발시킴을 알 수 있었다. 또한 저산소/재산소화에서 G_h와 연관된 심근비대의 기작은 PLC δ_1와 무관함을 확인하였다.

핵심되는말: 심근세포, 허혈/재관류, 저산소/재산소화, 심근비대, α-아드레
날린 수용체, G 단백질