

# Propofol Depresses Angiotensin II-induced Cell Proliferation in Rat Cardiac Fibroblasts

Tzu-Hung Cheng, Ph.D.,\* Yuk-Man Leung, Ph.D.,† Chi-Wai Cheung, M.B., B.S.,‡ Cheng-Hsien Chen, Ph.D.,§ Yen-Ling Chen, M.S.,|| Kar-Lok Wong, M.D., Ph.D.#

## ABSTRACT

**Background:** Propofol may have beneficial effects on the prevention of angiotensin II (Ang II)-induced cardiac fibroblast proliferation via its antioxidative properties. The authors hypothesized that propofol may alter Ang II-induced cell proliferation and aimed to identify the putative underlying signaling pathways in rat cardiac fibroblasts.

**Methods:** Cultured rat cardiac fibroblasts were pretreated with propofol then stimulated with Ang II; cell proliferation and endothelin-1 gene expression were examined. The effect of propofol on Ang II-induced nicotinamide adenine dinucleotide phosphate-oxidase activity, reactive oxygen species formation, extracellular signal-regulated kinase phosphorylation, and activator protein 1-mediated reporter activity were also examined. The effect of propofol on nitric oxide production and protein kinase B and endothelial nitric oxide synthase phosphorylations were also tested to elucidate the intracellular mechanism of propofol in proliferation.

**Results:** Ang II (100 nM) increased cell proliferation and endothelin-1 expression, which were partially inhibited by propofol (10 or 30  $\mu$ M). Propofol also inhibited Ang II-increased nicotinamide adenine dinucleotide phosphate-oxidase activity, reactive oxygen species formation, extracellular signal-regulated kinase phosphorylation, and activator protein 1-mediated reporter activity. Propofol was also found to increase nitric oxide generation and protein kinase B and nitric oxide synthase phosphorylations. Nitric oxide synthase inhibi-

tor (*N*-nitro-L-arginine methylester) and the short interfering RNA transfection for protein kinase B or endothelial nitric oxide synthase markedly attenuated the inhibitory effect of propofol on Ang II-induced cell proliferation.

**Conclusions:** The authors' results suggest that propofol prevents cardiac fibroblast proliferation by interfering with the generation of reactive oxygen species and involves the activation of the protein kinase B-endothelial nitric oxide synthase-nitric oxide pathway.

## What We Already Know about This Topic

- ❖ Diastolic dysfunction in heart failure reflects interstitial fibrosis in part due to angiotensin acting on cardiac fibroblasts
- ❖ Propofol is sometimes infused for long periods of time in patients with heart failure

## What This Article Tells Us That Is New

- ❖ In cultured rat fibroblasts, propofol prevented fibroblast activation and proliferation in response to angiotensin

**I**N up to 70% of cases, heart failure occurs exclusively on the basis of an impairment of diastolic function related to cardiac fibrosis as well as increased formation of endothelin 1 (ET-1).<sup>1–3</sup> The heart is composed of not only cardiac myocytes but also nonmyocytes, particularly fibroblasts. Unlike cardiac myocytes, cardiac fibroblasts can proliferate and increase the deposition of extracellular matrix proteins, which leads to interstitial fibrosis.<sup>3–5</sup> Therefore, fibroblasts play a crucial role in the development of cardiac fibrosis, which enhances intrinsic myocardial stiffness and results in diastolic dysfunction.<sup>1–4</sup>

Propofol is an intravenous sedative-hypnotic agent introduced in the United States in 1989. It is indicated for induction and maintenance of general anesthesia as well as for sedation of critical patients in the intensive care unit.<sup>6,7</sup> Propofol also is an antioxidant.<sup>8–13</sup> Recently, pharmacologic intervention with angiotensin-converting enzyme inhibitors and angiotensin type 1 receptor antagonists has demonstrated that angiotensin II (Ang II) plays an important role in the mediation of human hypertensive cardiac fibrosis or pressure overload-induced cardiac fibrosis as well as pulmonary fibrosis in different experimental models.<sup>3,4,14,15</sup>

Accumulated evidence suggests that reactive oxygen species (ROS) are centrally involved in the development of interstitial cardiac fibrosis.<sup>4,15,16</sup> We have previously reported

\* Associate Professor, Department of Biological Science and Technology, College of Life Sciences, † Associate Professor, Graduate Institute of Neural and Cognitive Sciences, China Medical University, ‡ Clinical Assistant Professor, Department of Anesthesiology, Li Ka Shing Faculty of Medicine, University of Hong Kong, Hong Kong, China. § Associate Professor, Department of Medicine, Taipei Medical University, Taipei, Taiwan. || Research Assistant, Graduate Institute of Medical Sciences, National Defense Medical Center, Taipei, Taiwan. # Associate Professor, Department of Anesthesiology, Institute of Clinical Medical Sciences, and Cardiovascular Biology Research Group, China Medical University and Hospital.

Received from the Department of Anesthesiology, Institute of Clinical Medical Sciences, and Cardiovascular Biology Research Group, China Medical University and Hospital, Taichung, Taiwan. Submitted for publication March 3, 2009. Accepted for publication July 21, 2009. Supported by grant No. NSC-96-2320-B-039-009-MY3 (to Dr. Wong) from the National Science Council, Taipei, Taiwan, and grant No. DMR-98-080 (to Dr. Wong) from the China Medical University, Taichung, Taiwan. Presented in part at the Annual Meeting of the European Society of Anaesthesiology, Milan, Italy, June 6–9, 2009.

Address correspondence to Dr. Wong: Department of Anesthesiology, China Medical University and Hospital, No. 2, Yuh-Der Road Taichung 404, Taiwan. klwong@mail.cmuh.org.tw. Information on purchasing reprints may be found at [www.anesthesiology.org](http://www.anesthesiology.org) or on the masthead page at the beginning of this issue. ANESTHESIOLOGY's articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

that ROS are essential for Ang II–induced cell proliferation and ET-1 gene expression in cardiac fibroblasts.<sup>14,16</sup> Prevention of excessive ROS production has been reported to have beneficial effects in the prevention of injury to the cardiovascular system.<sup>4</sup> Propofol is an intravenous anesthetic agent that is widely used for continuous sedation in critically ill patients.<sup>6,7</sup> It is therefore important to determine the effects of propofol on the cardiovascular system. Propofol is known to act as an antioxidant, reacting with free radicals to prevent oxidative cell damage in several types of preparations.<sup>8–13</sup> Application of propofol also stimulates the production of nitric oxide from cultured vascular endothelial cells.<sup>12</sup> Nitric oxide derived from endothelial nitric oxide synthase (eNOS) was implicated in modulating cytotoxic mechanisms,<sup>17–19</sup> presumably by influencing oxidative stress as well as to modulate many of the processes leading to ventricular remodeling; also, considerable evidence suggests that nitric oxide can ameliorate cardiac fibrosis.<sup>4</sup> It has been known that protein kinase B (Akt) contributes to enhanced phosphorylation of eNOS and the production of nitric oxide. There is increasing evidence which implicates that Akt, nitric oxide, or both, act as downstream effectors of survival signaling in cells.<sup>17–19</sup> However, no study has addressed the effects of propofol on cardiac fibroblast proliferation. The aims of this study were to investigate the antiproliferative effect of propofol on Ang II–induced cardiac fibroblast cell growth and to identify the possible mechanisms for the beneficial effects of propofol.

## Materials and Methods

### Materials

Dulbecco's modified Eagle's medium (DMEM), fetal calf serum, and tissue culture reagents were from Life Technologies, Inc. (Gaithersburg, MD). Rat ET-1 complementary DNA (cDNA) probe (accession No. M64711) was obtained as previously described.<sup>14</sup> 2',7'-Dichlorofluorescein diacetate was obtained from Molecular Probes (Eugene, OR). Propofol (B. Braun, Melsungen, Germany; diluted in 0.1% dimethylsulfoxide [DMSO]), *N*-nitro-*L*-arginine methyl ester (*L*-NAME), and all other reagent-grade chemicals were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). DMSO controls were included in the various assay systems. The plasmid activator protein 1 (AP-1)–Luc containing the firefly luciferase reporter gene driven by a basic promoter element (TATA box) joined to tandem repeats of AP-1 binding element was obtained from Stratagene (La Jolla, CA). Rabbit polyclonal anti-phospho-extracellular signal-regulated kinase (ERK) antibody and anti-ERK antibody were purchased from New England Biolabs (Beverly, MA) and Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-Ser1177 phospho-eNOS antibodies, anti-Ser473 phospho-Akt antibodies, and anti-Akt antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-eNOS antibodies were from BD Bioscience (San Jose, CA).

### Culture of Cardiac Fibroblasts

The investigation conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National

Institutes of Health (NIH Publication No. 85-23, revised 1996) and was also approved by the Institutional Animal Care and Use Committee of China Medical University (Taichung, Taiwan). Primary cultures of neonatal rat cardiac fibroblasts were prepared as previously described.<sup>16</sup> Briefly, ventricles from 1- to 2-day-old neonatal Sprague-Dawley rats were cut into chunks of approximately 1 mm<sup>3</sup> by using scissors and were subjected to trypsin (0.125%; Invitrogen, Carlsbad, CA) digestion in phosphate-buffered saline. Dispersed cells were incubated on 100-mm culture dishes for 30 min in a 5% CO<sub>2</sub> incubator. Nonmyocytes attached to the bottom of the dishes were subsequently incubated with DMEM supplemented with 10% fetal calf serum for an additional 2–4 days. Confluent nonmyocytes were treated with trypsin and subcultured. Subconfluent (approximately 70% confluency) cardiac fibroblasts grown in culture dishes from the second to fourth passage were used in the experiments and were greater than 99% positive for vimentin antibodies (Sigma-Aldrich). Serum-containing medium from the cultured cells was replaced with serum-free medium, and the cells were then exposed to the agents as indicated.

### Evaluation of Cytotoxicity

Equal numbers of cardiac fibroblasts were plated on a 96-well microplate (1 × 10<sup>4</sup> cells/well). Increasing concentrations of propofol (1, 3, 10, or 30 μM) were added. After 24 h of incubation, cytotoxicity was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The assay is based on the transformation of the tetrazolium salt MTT by active mitochondria to an insoluble formazan salt. MTT was added to each well under sterile conditions (with a final concentration of 0.5 mg/ml), and the plates were incubated for 4 h at 37°C. Untransformed MTT was removed by aspiration, and formazan crystals were dissolved in DMSO (150 μl/well). Formazan was quantified photometrically at 540-nm excitation wavelength with a Bio-Rad automated Enzyme Immunoassay Analyzer (Bio-Rad, Hercules, CA).

### Cell Proliferation

Proliferation was assessed by quantifying 5-bromo-2'-deoxyuridine (BrdU) incorporation in the presence or absence of reagents as indicated. The rate of cellular proliferation was determined by cell counting. Cells were removed from the culture dish by addition of trypsin and then centrifuged. The pellet was resuspended in 1 ml DMEM, and cells were counted in an automatic cell counter (S.ST.II/ZM; Coulter Electronics Ltd., Miami, FL). Cell proliferation was assessed by the incorporation of BrdU. Cells (1 × 10<sup>4</sup> cells/well) were incubated in 96-well plastic plates. Then, BrdU (10 μM) was added to the medium and the cells were incubated for another 18 h. Subsequently, the cells were fixed and BrdU incorporation was determined with a Cell Proliferation ELISA Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

### **RNA Isolation and Northern Blot Analysis**

Preparation of total RNA and Northern blot analyses of ET-1 and 18S RNA were performed as described previously.<sup>20</sup> Total RNA was isolated from cells by the guanidine isothiocyanate–phenol chloroform method. Blots of specific messenger RNA (mRNA) bands were detected by autoradiography and analyzed with a densitometer (Computing Densitometer 300S; Molecular Dynamics, Sunnyvale, CA). Blots were stripped and reprobed for 18S cDNA probe (American Type Culture Collection, Manassas, VA) to control for loading. Expression of ET-1 mRNA was quantitated and was normalized to the 18S signal.

### **NADPH Oxidase Activity Assay**

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase was measured as previously described.<sup>21</sup> In brief, NADPH oxidase activity was measured using the lucigenin-enhanced chemiluminescence method in microsomal membrane fractions. For the isolation of microsomal membranes, cell homogenates were prepared in 250 mM sucrose, 5 mM HEPES (pH 7.4), 1 mM phenylmethylsulfonyl fluoride, 10  $\mu$ g/ml aprotinin, and 5  $\mu$ g/ml leupeptin, followed by centrifugation at 1,000g (10 min, 4°C). The pellet was discarded, and the supernatant was spun at 8,000g (10 min, 4°C). The microsomal fraction was separated from cytosol by centrifugation of the supernatant at 29,100g (20 min, 4°C). The protein concentration was measured using the bicinchoninic acid protein assay (Pierce, Rockford, IL) and was adjusted to 2 mg/ml. Total cell suspension with a volume of 250  $\mu$ l was mixed with 250  $\mu$ l Hank's Balanced Salt Solution containing 500  $\mu$ M lucigenin and was kept at 37°C for 10 min. NADPH oxidase activity assay was initiated by adding 10  $\mu$ l NADPH (100  $\mu$ M) as substrate. The photon emission was measured, and the respective background counts were subtracted. Neither the cellular fraction alone nor NADPH alone evoked any lucigenin chemiluminescence signal.

### **Detection of Superoxide Production**

Superoxide production was measured by lucigenin-amplified chemiluminescence as previously described.<sup>22</sup> Briefly, cardiac fibroblasts after treatment were lysed immediately with a lysis buffer containing lucigenin (500  $\mu$ M). Readings were begun immediately after addition of lysis buffer. Each reading was recorded as single photon counts using a microplate scintillation counter (Topcount; Packard Instrument Co., Meriden, CT).

### **Flow Cytometric Assay of 2',7'-Dichlorodihydrofluorescein Oxidation**

The determination of intracellular reactive oxygen species production was based on the oxidation of 2',7'-dichlorodihydrofluorescein to a fluorescent 2',7'-dichlorofluorescein. 2',7'-Dichlorodihydrofluorescein was added at a final concentration of 10  $\mu$ M and incubated for 30 min at 37°C. The cells were then washed once with phosphate-buffered saline and maintained in a 1-ml culture medium. After drug treatment, the medium was aspirated, and cells were washed twice with phosphate-buffered saline and then dissociated with

trypsin. Cellular fluorescence was determined by flow cytometry (FACS-SCAN; Becton-Dickinson, Franklin Lakes, NJ). Cells were excited with an argon laser at 488 nm, and measurements were taken at 510–540 nm.

### **Western Blot Analysis**

Western blot analysis was performed as previously described.<sup>14</sup> Whole-cell extracts were obtained in a radioimmunoprecipitation assay buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 0.1% SDS, 1.0% Triton X-100, 1% deoxycholate, 5 mM EDTA, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail; Complete; Roche Diagnostics GmbH, Mannheim, Germany). Extracts or proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by electrotransfer to polyvinylidene difluoride membranes and probed with antisera, followed by horseradish peroxidase–conjugated secondary antibodies. The proteins were visualized by chemiluminescence, according to the manufacturer's instructions (Pierce).

### **Transfection and Luciferase Assay**

For the transient transfections, cells were transfected with different expression vectors by the calcium phosphate<sup>23</sup> Cardiac fibroblasts plated on six-well (35-mm) dishes were transfected using the luciferase reporter construct possessing consensus AP-1 binding sites (AP-1–Luc; Stratagene). After incubation for 5 h, cells were then washed three times with phosphate-buffered saline and incubated with 10% serum DMEM. After 24 h, cells were washed with serum-free medium and incubated in the same medium for an additional 24 h. Cells were then treated with different agents and assayed for luciferase activity with a luciferase reporter assay kit. As was the case for AP-1 transcriptional activity, the specific firefly luciferase activity was normalized for transfection efficiency to its respective  $\beta$ -galactosidase activity and expressed relative to the control.

### **Measurement of Nitrate/Nitrite Levels**

The culture medium was stored at –70°C until use. After the medium had been thawed, the sample was deproteinized with two volumes of 4°C 99% ethanol and centrifuged (3,000g for 10 min). These medium samples (100  $\mu$ l) were injected into a collection chamber containing 5% VCl<sub>3</sub>. This strong reducing environment converts both nitrate and nitrite to nitric oxide. A constant stream of helium gas carried nitric oxide into a nitric oxide analyzer (Seivers 270B NOA; Seivers Instruments Inc., Boulder, CO), where nitric oxide reacted with ozone, resulting in the emission of light. Light emission is proportional to the quantity of nitric oxide formed; standard amounts of nitrate were used for calibration.

### **Measurement of NOS Activity**

To measure NOS activity, L-arginine to L-citrulline conversion was assayed in cells with the NOS detection assay kit (Calbiochem, EMD Chemicals Inc., Gibbstown, NJ) according to the manufacturer's instructions. Briefly, cells were

lysed with buffer containing 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS. After incubation on ice for 30 min, cell extracts were centrifuged to remove cell debris. Protein extracts were then incubated for 60 min at 37°C in a solution of 10  $\mu\text{M}$  L-[ $^3\text{H}$ ]arginine, 1 mM NADPH, 1  $\mu\text{M}$  Flavin adenine dinucleotide, 1  $\mu\text{M}$  Flavin mononucleotide, 100 nM calmodulin, 600  $\mu\text{M}$   $\text{CaCl}_2$ , and 3  $\mu\text{M}$  tetrahydrobiopterin in a final volume of 40  $\mu\text{l}$ . The reaction was stopped by the addition of 400  $\mu\text{l}$  stop buffer (10 mM EDTA, 50 mM HEPES buffer, pH 5.5) to the reaction mixture. Then, 100  $\mu\text{l}$  equilibrated resin was added to each mixture. Reaction samples were transferred to spin cups and centrifuged at 10,000g for 30 s. The radioactivity of the flow through was measured by liquid scintillation counting. Enzyme activity was expressed as citrulline production in  $\text{pmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$ .

### Short Interfering RNA Transfection

We purchased Akt short interfering RNA (siRNA) and eNOS siRNA from Santa Cruz Biotechnology. Akt siRNA, eNOS siRNA, and mock control oligonucleotides were transfected using the lipofectamine reagent according to the manufacturer's instructions. The final concentration of siRNAs for transfection was 10 or 100 nM. We washed transfected cells and incubated them in new culture media for an additional treatment as indicated and Western blot assays.

### Experimental Design

The cultured cardiac fibroblasts were randomly assigned to one of the following groups:

**Group 1 for Cell Viability Analysis.** Cardiac fibroblasts were cultured in microplate and were incubated with increasing concentrations of propofol (1, 3, 10, 30, or 100  $\mu\text{M}$ ). After 24 h, cell viability was determined with the MTT assay.

**Group 2 for Cell Proliferation Analysis.** Cells were preincubated with propofol (1–30  $\mu\text{M}$ ) and treated with Ang II (100 nM) for 24 h as indicated. Cell proliferation was assessed by the incorporation of BrdU. Cells and BrdU incorporation were determined with a Cell Proliferation ELISA.

**Group 3 for ET-1 mRNA Analysis.** Cardiac fibroblasts were either controls or treated with different concentrations of propofol (1–30  $\mu\text{M}$ ) in the absence or the presence of Ang II (100 nM) for 30 min.

**Group 4 for ROS Detection.** Cardiac fibroblasts were treated with propofol (1–10  $\mu\text{M}$ ) for 30 min and then stimulated with Ang II (100 nM) for 30 min or not.

**Group 5 for ERK Phosphorylation Analysis.** Cardiac fibroblasts were preincubated with propofol (10  $\mu\text{M}$ ) or apocynin (1  $\mu\text{M}$ ) and then stimulated with Ang II (100 nM) for 30 min. The phosphorylation of ERK was detected by Western blotting.

**Group 6 for AP-1-mediated Reporter Activity Analysis.** Cardiac fibroblasts, transfected with AP-1-Luc, were treated as indicated. Cells were preincubated with propofol (10  $\mu\text{M}$ )

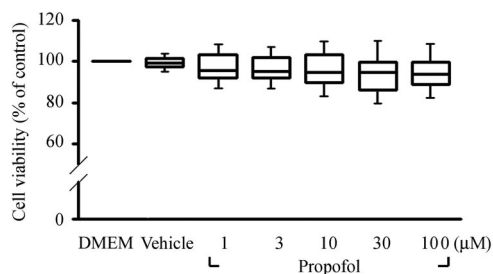


Fig. 1. Box plots of effects of propofol on the viability of cardiac fibroblasts. Cardiac fibroblasts were cultured in microplates and were incubated with increasing concentration of propofol. After 24 h, cell viability was determined with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. Data are reported as mean  $\pm$  SD ( $n = 6$ ). The line within the box plots indicates the mean value, the lower and upper sides of the box indicate the 25th and 75th percentiles, and the error bars indicate the 10th and 90th percentiles. DMEM = Dulbecco's modified Eagle's medium.

or apocynin (1  $\mu\text{M}$ ) and then stimulated with Ang II (100 nM) for 24 h or not.

**Group 7 for eNOS and Akt Phosphorylation Analysis.** Cardiac fibroblasts were in control condition, treated with propofol (10  $\mu\text{M}$ ) for 10, 30, 60, or 120 min.

**Group 8 for Akt-eNOS-NO Pathway Analysis.** Transfected cells (transfected with Akt siRNA [10 or 100 nM] to get Akt knockdown cells and transfected with or eNOS siRNA [10 or 100 nM] to get eNOS knockdown) were pretreated with or without propofol (10  $\mu\text{M}$ ) for 30 min and then treated with Ang II (100 nM) for 30 min.

### Statistical Analysis

Results are expressed as mean  $\pm$  SD for at least six experiments. Statistical analysis was performed using the Student *t* test or analysis of variance followed by Tukey multiple comparisons using GraphPad Prism (GraphPad Software, San Diego, CA). A value of  $P < 0.05$  was considered to be statistically significant.

## Results

### Effects of Propofol on Cardiac Fibroblast Viability

Exposure of cultured cardiac fibroblasts to propofol (1, 3, 10, or 30  $\mu\text{M}$ ) for 24 h did not induce any significant effect on cardiac fibroblast viability (fig. 1).

### Effects of Propofol on Ang II-induced Cell Proliferation of Cardiac Fibroblasts

The effects of propofol on Ang II-stimulated rat cardiac fibroblast proliferation were assessed by analyzing DNA synthesis with BrdU incorporation and cell counting. Pretreatment of cardiac fibroblasts with propofol (10 or 30  $\mu\text{M}$ ) for 30 min followed by exposure to Ang II (100 nM) for 24 h resulted in a significant decrease in Ang II-increased cell number and BrdU incorporation (figs. 2A and B). These data clearly suggest that propofol inhibited Ang II-induced proliferation of cardiac fibroblasts.

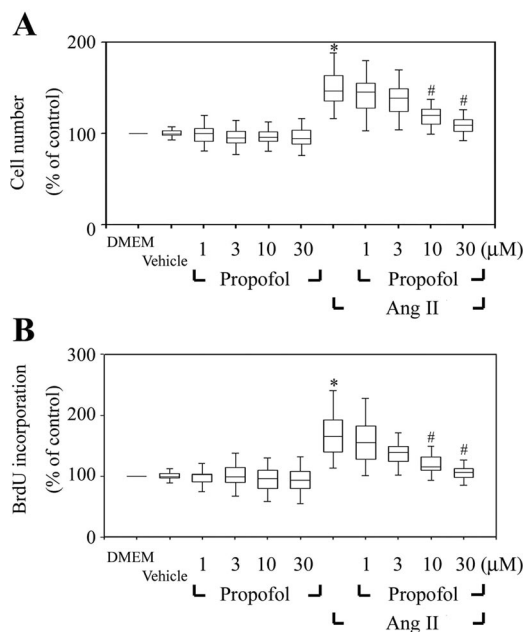


Fig. 2. Box plots of effects of propofol on the angiotensin II (Ang II)-induced cell proliferation in cardiac fibroblasts. Cells were preincubated with the indicated doses of propofol and treated with Ang II (100 nM) for 24 h as indicated. Cell number and 5-bromo-2'-deoxyuridine (BrdU) incorporation were expressed as percentage of Dulbecco's modified Eagle's medium (DMEM) control. Results are shown as mean  $\pm$  SD (n = 6). The line within the box plots indicates the mean value, the lower and upper sides of the box indicate the 25th and 75th percentiles, and the error bars indicate the 10th and 90th percentiles. \*  $P < 0.05$  versus dimethylsulfoxide control (vehicle). #  $P < 0.05$  versus Ang II alone. (A) Propofol inhibits Ang II-induced cell proliferation. Cells were counted for cell number and calculated as a percentage of the DMEM control value. (B) Propofol inhibits Ang II-induced DNA synthesis. Cell proliferation was estimated from the incorporation of BrdU and calculated as a percentage of the DMEM control value.

### Effects of Propofol on Ang II-induced ET-1 Expression in Cardiac Fibroblasts

Cardiac fibroblasts were preincubated with propofol (1, 3, or 10  $\mu$ M, 30 min) before exposure to Ang II (100 nM) for 30 min and then assayed for propofol-inhibited Ang II-induced ET-1 mRNA expression. Propofol (10  $\mu$ M) caused a down-regulation of Ang II-induced ET-1 mRNA (fig. 3A). Exposure of cardiac fibroblasts to Ang II (100 nM) for 24 h significantly increased ET-1 peptide secretion (fig. 3B). Pretreatment of cardiac fibroblasts with propofol (10  $\mu$ M) inhibited Ang II-induced ET-1 secretion (fig. 3B). These data indicate that propofol inhibited Ang II-induced ET-1 expression in cardiac fibroblasts.

### Effects of Propofol on Ang II-increased NADPH Oxidase Activity and ROS Formation

We have demonstrated that Ang II stimulated ROS production in cardiac fibroblasts.<sup>16</sup> In this study, we further examined whether propofol prevented Ang II-increased NADPH oxidase activity and ROS formation in cardiac fibroblasts. Cardiac fibroblasts were pretreated with propofol (1, 3, or 10

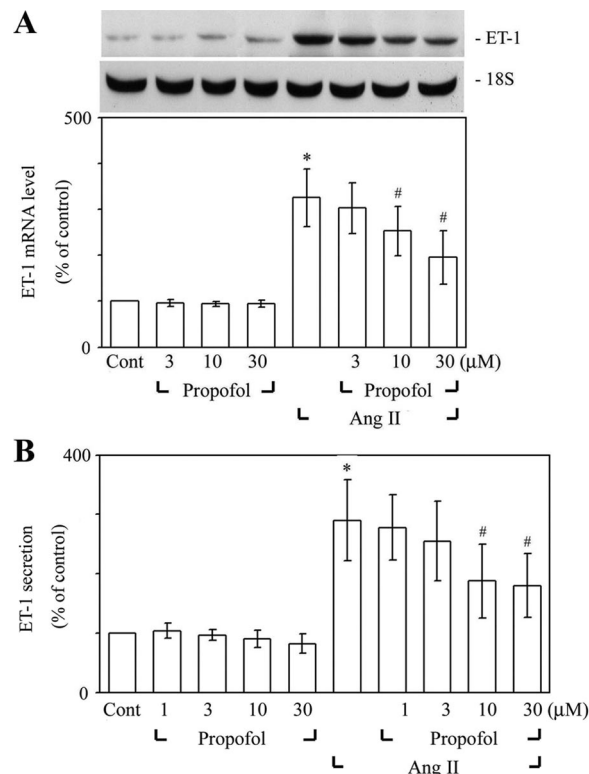


Fig. 3. Propofol down-regulates angiotensin II (Ang II)-induced endothelin-1 (ET-1) expression in cardiac fibroblasts. Results are shown as mean  $\pm$  SD (n = 6). \*  $P < 0.05$  versus control (Cont). #  $P < 0.05$  versus Ang II alone. (A) Down-regulation of Ang II-induced ET-1 messenger RNA (mRNA) by propofol. Cells were preincubated with propofol (1, 3, and 10  $\mu$ M) and then stimulated with Ang II (100 nM) for 30 min or not. (B) Propofol inhibits Ang II-induced ET-1 secretion. Cells were preincubated with propofol (1, 3, and 10  $\mu$ M) and then stimulated with Ang II (100 nM) for 24 h or not.

$\mu$ M, 30 min) and then treated with Ang II (100 nM) for 30 min. Pretreatment of cultured cardiac fibroblasts with propofol (10  $\mu$ M) significantly inhibited Ang II-induced NADPH oxidase activity, superoxide formation, and ROS formation as measured after Ang II treatment (fig. 4A–D). These findings support that propofol inhibited Ang II-increased NADPH oxidase activity and intracellular ROS levels in cardiac fibroblasts.

### Effects of Propofol on Ang II-activated ERK Phosphorylation and AP-1-mediated Reporter Activity in Cardiac Fibroblasts

We previously reported that ROS were involved in the activation of the ERK pathway, which culminated in ET-1 gene expression.<sup>14,16</sup> As shown in figure 5A, exposure of cardiac fibroblasts to Ang II (100 nM) for 30 min rapidly activated phosphorylation of ERK. However, cardiac fibroblasts pretreated with propofol (10  $\mu$ M, 30 min) had significantly decreased levels of Ang II-induced ERK phosphorylation. Moreover, Ang II-increased AP-1 activation is involved in ET-1 gene induction.<sup>14,17</sup> We further evaluated the effects of

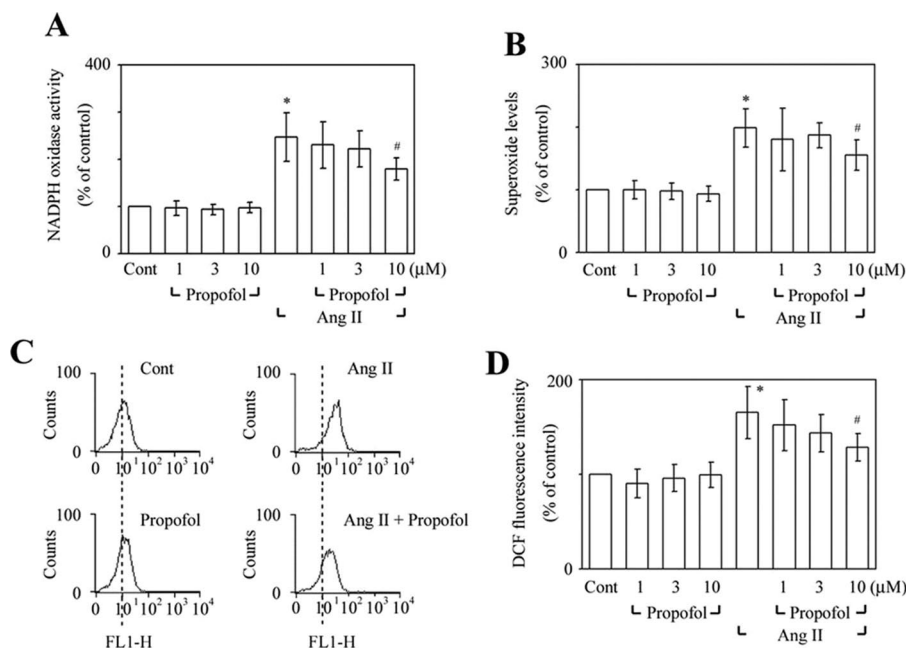


Fig. 4. Effects of propofol on angiotensin II (Ang II)-increased nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity and reactive oxygen species formation. Cells were preincubated with propofol (1, 3, or 10  $\mu\text{M}$ ) for 30 min and then stimulated with Ang II (100 nM) for 30 min or not. Results are shown as mean  $\pm$  SD ( $n = 6$ ). \*  $P < 0.05$  versus control (Cont). #  $P < 0.05$  versus Ang II alone. (A) Effect of propofol on Ang II-increased NADPH oxidase activity. Cardiac fibroblasts after treatment were lysed and immediately followed with NADPH oxidase activity assay. (B) Effect of propofol on Ang II-induced superoxide formation. Cardiac fibroblasts after treatment were lysed and immediately followed with superoxide assay by lucigenin method. (C) Effect of propofol on Ang II-induced reactive oxygen species generation. Flow cytometric histogram of dichlorofluorescein (DCF) in cardiac fibroblasts. Cardiac fibroblasts were treated with vehicle control, propofol (10  $\mu\text{M}$ ) for 60 min, Ang II (100 nM) for 30 min, or preincubation with propofol (10  $\mu\text{M}$ ) for 30 min and then stimulation with Ang II. Counts = cell number; FL1-H = relative DCF fluorescence intensity. (D) Column bar graph of mean cell fluorescence for DCF. The fluorescence intensities in untreated control cells are expressed as 100%.

propofol on Ang II-induced AP-1 functional activity using a reporter gene assay. Propofol (10  $\mu\text{M}$ ) also significantly attenuated Ang II-induced AP-1-mediated reporter activation (fig. 5B). These findings demonstrate that propofol inhibited Ang II-activated ERK signaling pathway and AP-1 activation in cardiac fibroblasts.

#### Effects of Propofol on Nitric Oxide Synthesis, NOS Activity, Phospho-eNOS, and Phospho-Akt in Cardiac Fibroblasts

Exposure of cardiac fibroblasts to propofol time- and dose-dependently enhanced nitric oxide generation (figs. 6A and B). All three NOS isoforms characterized to date depend on calmodulin activation but are distinguished from each other by their calcium sensitivity and enzymatic.<sup>19</sup> A constitutive nitric oxide synthase activity was detected and quantified in whole extracts of cells, and this activity was dependent on the presence of calcium (fig. 6C). Exposure of cardiac fibroblasts to propofol (10  $\mu\text{M}$ ) also enhanced the strain-increased NOS activity at 30 min after stimulation (fig. 6C). In addition, propofol treatment in cardiac fibroblasts significantly enhanced phospho-Akt (fig. 6D) and phospho-eNOS (fig. 6E). These findings reveal that propofol increased nitric oxide production and Akt/eNOS phosphorylation in cardiac fibroblasts.

#### Role of Akt-eNOS-Nitric Oxide Pathway in the Inhibitory Effect of Propofol on Ang II-induced Cardiac Fibroblast Proliferation

To identify the signaling pathways involved in the effect of propofol, L-NAME (a NOS inhibitor), and siRNA for Akt and eNOS were applied in cardiac fibroblasts. The Akt and eNOS protein levels were markedly reduced by Akt and eNOS siRNA transfection, respectively, as shown in figure 7A and B. The inhibitory effect of propofol on the Ang II-induced cell proliferation was partially reversed by L-NAME and Akt and eNOS siRNA transfection (fig. 7C). Similarly, the inhibitory effect of propofol on Ang II-increased BrdU incorporation was also reduced by L-NAME and Akt and eNOS siRNA transfection (fig. 7D). These results reveal the involvement of the Akt-eNOS-nitric oxide signaling pathway in the effect of propofol on Ang II-induced cardiac fibroblast proliferation.

#### Discussion

The major finding of this study is that propofol prevents cardiac fibroblast proliferation by interfering with the generation of ROS and involves the activation of the Akt-eNOS-NO pathway. Cardiovascular morbidity and mortality resulting from congestive heart failure are major concerns for critical care.<sup>1</sup> In up to two thirds of cases, heart failure

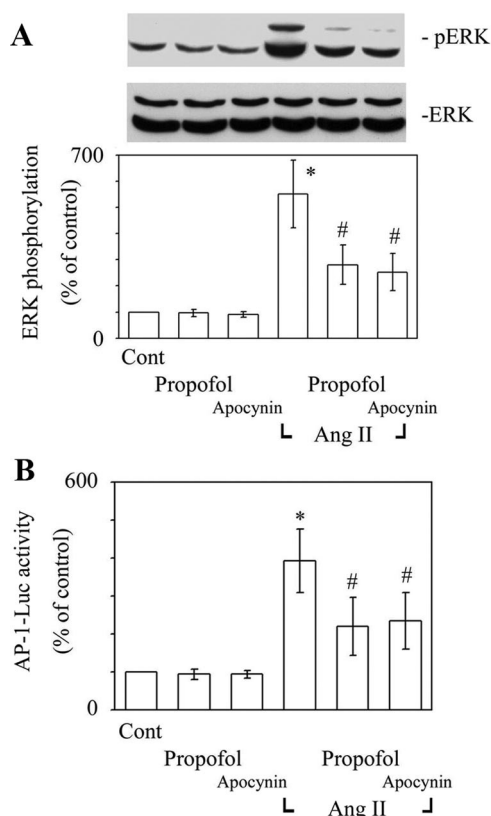


Fig. 5. Inhibitory effect of propofol on angiotensin II (Ang II)-increased extracellular signal-regulated kinase (ERK) phosphorylation and activator protein 1 (AP-1)-mediated reporter activity in cardiac fibroblasts. \*  $P < 0.05$  versus control. #  $P < 0.05$  versus Ang II alone. (A) Effects of propofol on Ang II-increased ERK phosphorylation. Cells were preincubated with propofol (10  $\mu\text{M}$ ) or apocynin (1  $\mu\text{M}$ ) and then stimulated with Ang II (100 nM) for 30 min. Phosphorylation of ERK was detected by Western blotting using anti-phospho-ERK antibody. Densitometric analyses were performed with a densitometer. Data are shown as fold increase relative to control groups. Results are shown as mean  $\pm$  SD ( $n = 6$ ). (B) Effects of propofol on Ang II-increased AP-1-mediated reporter activity. Cardiac fibroblasts, transfected with AP-1-Luc, were treated as indicated. Cells were preincubated with propofol (10  $\mu\text{M}$ ) or apocynin (1  $\mu\text{M}$ ) and then stimulated with Ang II (100 nM) for 24 h or not. Luciferase activity was expressed as activity relative to untreated control (Cont). Results are shown as mean  $\pm$  SD ( $n = 6$ ).

occurs exclusively on the basis of an impairment of diastolic function related to cardiac fibrosis as well as increased formation of ET-1.<sup>1-4</sup> Cardiac fibrosis is a characterization of heart disease and is the result of a variety of structural changes that occur after pathologic stimuli to the cardiovascular system.<sup>2-5</sup> The fibroblasts play a pivotal role in the development of cardiac fibrosis and progression of left ventricular remodeling and results in diastolic dysfunction, accounting for 50–70% of congestive heart failure in clinical practice.<sup>1</sup> Some of the antioxidants that are derived from plants<sup>24,25</sup> may have potential therapeutic benefits on clinical use. Animal studies also revealed that the antioxidant dimethylthiourea<sup>26</sup> and probucol<sup>27</sup> markedly improve left ventricular remodeling in chronic heart failure, predominantly by reducing cardiac fibrosis. Plant-derived propofol is a potent intravenous hypnotic agent. It is

indicated for induction and maintenance of general anesthesia as well as for sedation of intubated, mechanically ventilated patients in the intensive care unit.<sup>6,7</sup> Experimental studies including our recent report also have shown some degree of protective effects on cardiomyocytes and endothelial cells subjected to oxidant stress<sup>11,28</sup>; however, the direct effect of propofol on cardiac cell growth remains unclear.

The increased levels of different humoral factors such as insulin-like growth factor 1, Ang II, and ET-1 may cause the development of cardiac hypertrophy and cardiac fibrosis, leading to an enhancement of cardiac remodeling during the development of heart failure.<sup>1-5</sup> Cardiac fibrosis is characterized by a disproportionate proliferation of fibrillar collagen in response to different pathologic insult, such as after myocyte death, stretch, cytokines, and hormones, and has been shown to stimulate differentiation, collagen deposition, migration, and proliferation.<sup>1-5,14,16</sup> It has also been shown that ET-1 stimulates membrane-bound NADPH oxidase, which generates ROS in many types of cells, including cardiac fibroblasts.<sup>4</sup> Ample evidence demonstrated that Ang II may act as a potent stimulator of fibroblast proliferation.<sup>4,14,16</sup> Despite the pathophysiologic significance of fibrosis, no effective clinical therapy strategies currently exist.<sup>1,4</sup> However, there is also no existing study addressing the interference of propofol on ET-1 expression, ROS, and nitric oxide production in cardiac fibroblasts. The current study is aimed to investigate the effect of propofol on nitric oxide production and Ang II-induced ET-1 expression and to identify the signaling protein kinase cascades that may be responsible for the putative effect of propofol.

In our experiments, propofol was diluted in 0.1% DMSO. To exclude the potential impact of emulsion, the solvent DMSO alone (< 0.2%) did not affect the cardiac fibroblast proliferation induced by Ang II (data not shown). Studies on the antioxidative effect of propofol in inhibiting radical production revealed that it possesses a certain degree of protective effects *in vitro* and *in vivo*, including neuroprotective properties,<sup>10</sup> improvement of endothelial dysfunction,<sup>28</sup> and protection against injuries caused by ischemia/reoxygenation.<sup>11,12</sup> These protective characteristics may be associated with their antiinflammatory capacity and antioxidant activity.

Oxidative stress is well known to be profibrotic in many organs, particularly in the heart.<sup>4</sup> Experimental evidence supports an important contribution of increased oxidative stress to contractile dysfunction in congestive heart failure.<sup>1,4</sup> The complex enzymes called NADPH oxidases are especially important with regard to redox signaling in congestive heart failure and its antecedent conditions.<sup>4</sup> Importantly, the effects of cell proliferation are increasingly being attributed to ROS produced by NADPH oxidases, including endothelial cells and fibroblasts.<sup>14,25,29</sup> Substantial evidence suggests that fibroblast NADPH oxidase is a harbinger and initiator of vascular disease and remodeling.<sup>4</sup> The results from our study demonstrate that propofol inhibited Ang II-induced ET-1 expression and cell proliferation of cardiac fibroblasts.

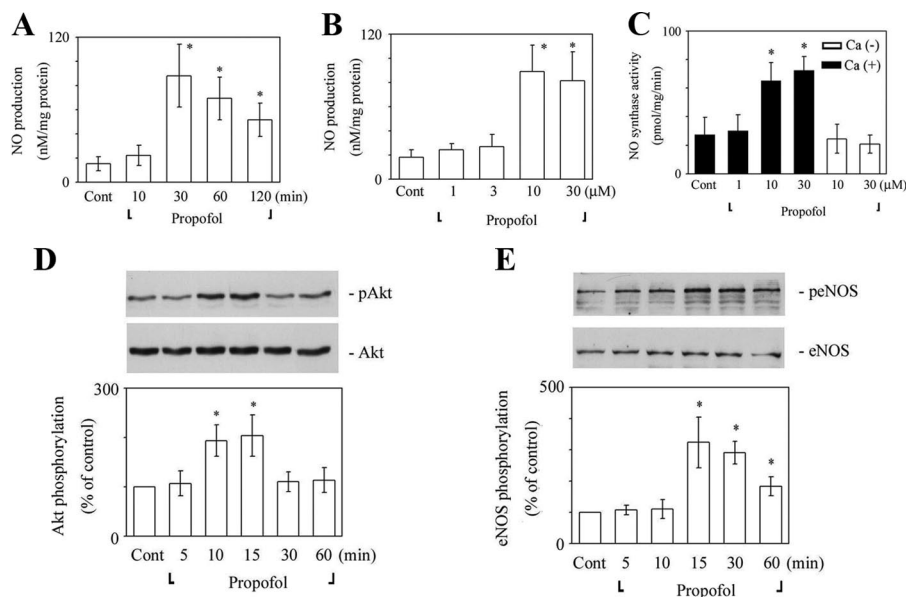


Fig. 6. Effect of propofol on nitric oxide (NO) production, nitric oxide synthase (NOS) activity, and Akt and endothelial NOS (eNOS) phosphorylation. \*  $P < 0.05$  versus control. (A) Time course of propofol-induced NO production in cardiac fibroblasts. Cells were in control condition (Cont), treated with propofol (10  $\mu\text{M}$ ) for 10, 30, 60, or 120 min. Results are shown as mean  $\pm$  SD ( $n = 6$ ). (B) Cells from either control or treated with propofol (1, 3, 10, or 30  $\mu\text{M}$ ) for 30 min. Results are shown as mean  $\pm$  SD ( $n = 6$ ). (C) Cells were treated with propofol (1, 10, or 30  $\mu\text{M}$ ) for 30 min. NOS activities were measured by citrulline formation from cell lysate incubated with (black bars) or without  $\text{Ca}^{2+}$  (white bars). NOS activity in the absence of  $\text{Ca}^{2+}$  indicates inducible NOS activity, whereas its activity in the presence of  $\text{Ca}^{2+}$  represents constitutive NOS activity. Results are shown as mean  $\pm$  SD ( $n = 6$ ). (D) Effects of propofol on phosphorylation of Akt in cardiac fibroblasts. Cells were treated with the vehicle control or propofol (10  $\mu\text{M}$ ) for indicated times. Western analysis was performed to detect phospho-Akt (Ser473) and total Akt. Data were represented as fold increase relative to control groups. Results are shown as mean  $\pm$  SD ( $n = 6$ ). (E) Effects of propofol on phosphorylation of eNOS in cardiac fibroblasts. Western analysis was performed to detect phospho-eNOS (Ser1177) and total eNOS. Results are shown as mean  $\pm$  SD ( $n = 6$ ).

Pretreatment of cultured cardiac fibroblasts with propofol (10  $\mu\text{M}$ ) significantly inhibited Ang II–induced superoxide and ROS formation, and also inhibited Ang II–increased NADPH oxidase activity as measured after Ang II treatment. These findings support that propofol inhibited Ang II–increased NADPH oxidase activity and intracellular ROS levels in cardiac fibroblasts.

It has been reported that nitric oxide can trigger apoptosis or protect cells from apoptotic stimuli, depending on the concentration of nitric oxide.<sup>17,18</sup> The relatively low physiologic concentration of nitric oxide generated by eNOS or neuronal NOS can act as a beneficial protection effector in several cell systems, including cardiac fibroblasts.<sup>17–19</sup> The serine–threonine kinase Akt activation has been reported to activate eNOS, which leads to nitric oxide production and enhancement of cell survival. The Akt–eNOS–nitric oxide pathway has been identified as an important survival pathway in many cell types, including cardiomyocytes, cardiac fibroblasts, vascular smooth muscle cells, and endothelial cells.<sup>18,19,30</sup> Nitric oxide has been suggested to inhibit fibroblasts proliferation.<sup>31</sup> Some other results also revealed that the benefit effects of angiotensin-converting enzyme inhibitor or plant-derived antioxidant are associated with Akt–eNOS–nitric oxide pathway within human cardiac fibroblasts.<sup>31,32</sup> Propofol can stimulate nitric oxide release from cultured human umbilical vein endothelial cells.<sup>12</sup> Our recent study also implies that propofol-induced nitric oxide

production and suppression of cyclic strain-induced ET-1 expression *via* its antioxidative effects could be considered as one of the mechanisms responsible for the protective effect of propofol in endothelial cells.<sup>28</sup> In this study, we clearly demonstrated that propofol modulated Ang II–induced cell proliferation and ET-1 gene expression in cardiac fibroblasts. Propofol treatment in cardiac fibroblasts significantly enhanced phospho-eNOS (fig. 6E) and phospho-Akt (fig. 6D). These findings reveal that propofol increased NO production and the Akt–eNOS–nitric oxide signaling pathway was involved in the effect of propofol on Ang II–induced cardiac fibroblast proliferation.

It is considered that ROS are mediators for intracellular signaling, which may involve the induction and/or the development of various physiologic and pathophysiologic events such as hypertrophy of cardiomyocytes and proliferation of cardiac fibroblasts.<sup>4</sup> In addition, increased ROS levels are involved in cell proliferation and ET-1 induction, which can be attenuated by antioxidant pretreatment of cells.<sup>20,24,25</sup> More specifically, propofol also prevented Ang II–mediated NADPH oxidase activity (fig. 4A). Furthermore, our previous report demonstrated that activation of ERK is redox-sensitive<sup>14,17,33</sup> and that suppression of ROS generation inhibits ET-1 gene expression and may have a beneficial effect of cell protection.<sup>20,23,24</sup> We previously reported that ROS were involved in the activation of the ERK pathway, which culminated in ET-1 gene expression.<sup>14,16,20</sup> To gain insight



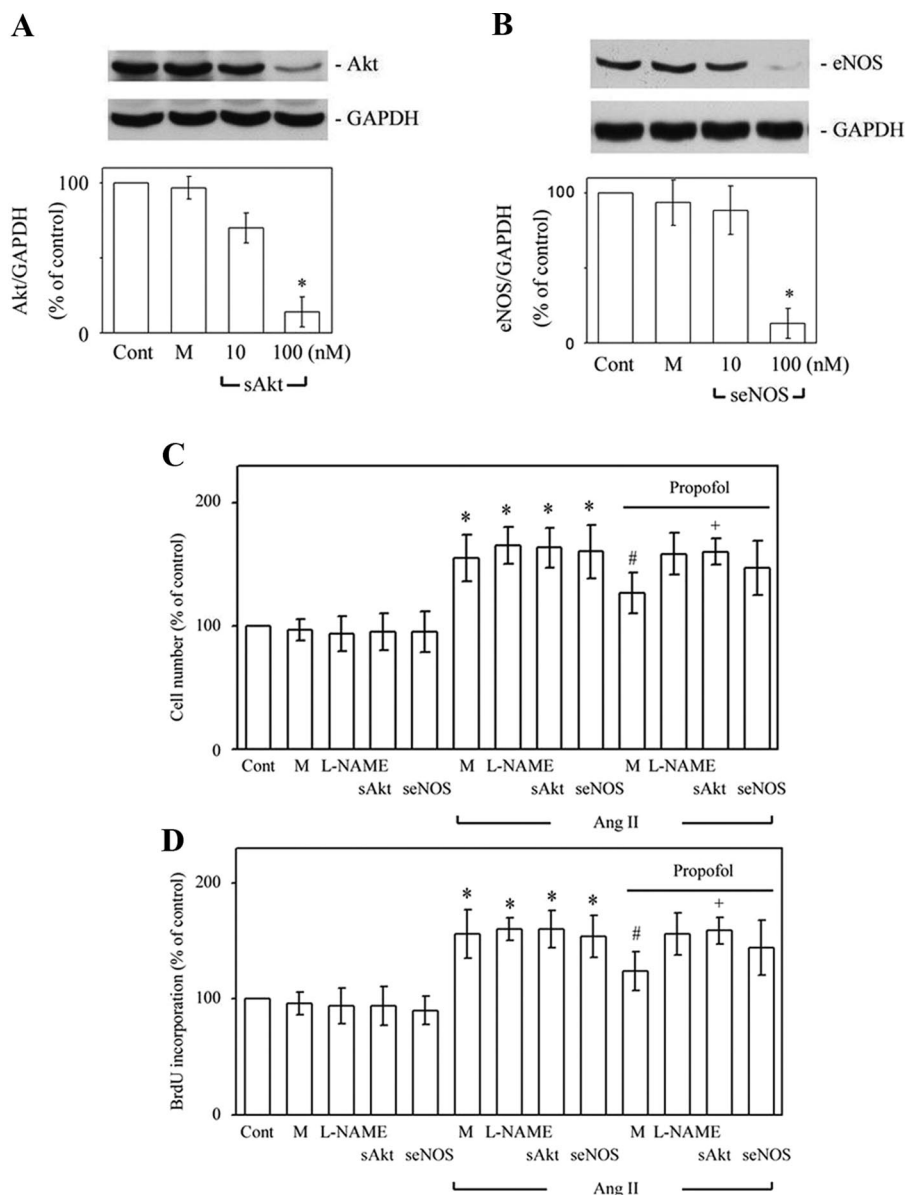


Fig. 7. Blockage of Akt–endothelial nitric oxide synthase (eNOS)–nitric oxide pathway attenuated the inhibitory effect of propofol on angiotensin II (Ang II)–induced cell proliferation in cardiac fibroblasts. \*  $P < 0.05$  versus mock control. #  $P < 0.05$  versus Ang II treatment. +  $P < 0.05$  versus propofol and Ang II treatment. (A) The effect of Akt short interfering RNA (siRNA) transfection on Akt protein levels in cardiac fibroblasts. The cells were transfected with Akt siRNA (sAkt; 10 or 100 nM) to get Akt knockdown cells. Control siRNA was also applied as mock controls (M). Western blotting was performed with the specific antibody against Akt. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. Results are shown as mean  $\pm$  SD ( $n = 6$ ). (B) The effect of eNOS siRNA transfection on eNOS protein levels in cardiac fibroblasts. The cells were transfected with or eNOS siRNA (seNOS; 10 or 100 nM) to get eNOS knockdown cells. Western blotting was performed with the specific antibody against eNOS. Results are shown as mean  $\pm$  SD ( $n = 6$ ). (C) The effect of *N*-nitro-*L*-arginine methylester (L-NAME), Akt siRNA, and eNOS siRNA on propofol-decreased Ang II–induced cell proliferation in cardiac fibroblasts. Transfected cells were pretreated with or without propofol (10  $\mu$ M) for 30 min and then treated with Ang II (100 nM) for 30 min. For inhibiting the activity of eNOS, the cells were pretreated with L-NAME (100  $\mu$ M) for 30 min. Cont = untransfected control. Results are shown as mean  $\pm$  SD ( $n = 6$ ). (D) The effect of L-NAME, Akt siRNA, and eNOS siRNA on propofol-decreased Ang II–induced DNA synthesis in cardiac fibroblasts. Transfected cells were pretreated with or without propofol (10  $\mu$ M) for 30 min and then treated with Ang II (100 nM) for 24 h. Results are shown as mean  $\pm$  SD ( $n = 6$ ).

into the mechanism of action of propofol, we examined whether propofol affected the Ang II–activated ERK pathway of cardiac fibroblasts. As shown in figure 5A, exposure of cardiac fibroblasts to Ang II (100 nM) for 30 min rapidly activated phosphorylation of ERK. However, cardiac fibroblasts pretreated with propofol (10  $\mu$ M, 30 min)

had significantly decreased levels of Ang II–induced ERK phosphorylation.

It has also been shown that activation of AP-1 stimulates fibronectin expression in various cell types.<sup>14,16,20</sup> Our previous report indicated that AP-1 binding element was mainly responsible for the induction of ET-1 gene expression by

ET-1 in cardiac fibroblasts.<sup>14,16</sup> While intracellular ROS generation plays an important role in the ET-1–induced proliferation of cardiac fibroblasts, antioxidants attenuated ET-1–stimulated AP-1 binding activity.<sup>14</sup> One possible explanation for the inhibitory effect of propofol on Ang II–induced cell proliferation and ET-1 gene expression may thus be its ability to attenuate ROS formation and then inhibit ERK phosphorylation and AP-1–mediated reporter activity in cardiac fibroblasts.

Reactive oxygen species are believed to contribute the tissue injuries associated with many pathologic processes such as tissue anoxia, inflammatory process, and cardiovascular diseases.<sup>4</sup> In such diseases, antioxidants can protect tissues by inhibiting lipid peroxide formation or increasing the activity of the glutathione antioxidant system or through other mechanisms.<sup>4,8-13</sup> Alternatively, our results showed that propofol may inhibit Ang II–induced cell proliferation and ET-1 gene expression by increasing radical scavenging enzyme activity and expression. Other major effects of Ang II stimulation in cardiac fibroblasts are the liberation of calcium from intracellular stores and the production of diacylglycerol, which induces protein kinase C activation.<sup>34</sup> Moreover, myocardial protection by propofol is known to involve activation of protein kinase C.<sup>35</sup> Therefore, further experiments will be necessary to identify the detailed mechanisms of the inhibitory effects of propofol.

A better understanding of the distinct roles of oxidative stress and redox signaling pathways in different components of the process of heart failure may therefore provide the basis for contriving new therapeutic strategies for this disease. The current study delivers important new insight into the molecular mechanisms of action of propofol in cardiac fibroblasts. Although the precise mechanism by which propofol inhibits the proliferation of cardiac fibroblasts remains to be further clarified. These findings may provide an explanation for some of the factors responsible for the antiproliferative effects of propofol on cardiac fibroblasts. In summary, this study has shown that propofol inhibited Ang II–induced ROS formation, ERK phosphorylation, AP-1–mediated reporter activity, and ET-1 gene expression. Moreover, propofol also increased Akt and eNOS phosphorylation, and thereafter nitric oxide production in cardiac fibroblasts. It seems plausible that the Ang II–activated signaling pathway consists of a number of redox-sensitive steps and that propofol treatment could modulate the redox state of the cell.

The authors thank David Chiu-Yin Kwan, Ph.D. (Professor, Department of Medicine, Faculty of Health Sciences, McMaster University, Hamilton, Ontario, Canada), for his helpful comments and critical review during the preparation of this manuscript. The authors also thank the Graduate Institute of Biostatistics, China Medical University, Taichung, Taiwan, for their statistical assistance.

## References

1. Hoit BD: Left ventricular diastolic function. *Crit Care Med* 2007; 35:S340-7
2. De Mello WC: Heart failure: How important is cellular sequestration? The role of the rennin-angiotensin-aldosterone system. *J Mol Cell Cardiol* 2004; 37:431-8
3. Mehta PK, Griendling KK: Angiotensin II cell signaling: Physiological and pathological effects in the cardiovascular system. *Am J Physiol Cell Physiol* 2007; 292:C82-97
4. Seddon M, Looi YH, Shah AM: Oxidative stress and redox signalling in cardiac hypertrophy and heart failure. *Heart* 2007; 93:903-7
5. Leask A: TGFbeta, cardiac fibroblasts, and the fibrotic response. *Cardiovasc Res* 2007; 74:207-12
6. Marik PE: Propofol: Therapeutic indications and side-effects. *Curr Pharm Des* 2004; 10:3639-49
7. Rigby-Jones AE, Nolan JA, Priston MJ, Wright PM, Sneyd JR, Wolf AR: Pharmacokinetics of propofol infusions in critically ill neonates, infants, and children in an intensive care unit. *ANESTHESIOLOGY* 2002; 97:1393-400
8. Marik PE: Propofol: An immunomodulating agent. *Pharmacotherapy* 2005; 25:288-335
9. Cavalca V, Colli S, Veglia F, Eligini S, Zingaro L, Squellerio I, Rondello N, Cighetti G, Tremoli E, Sisillo E: Anesthetic propofol enhances plasma gamma-tocopherol levels in patients undergoing cardiac surgery. *ANESTHESIOLOGY* 2008; 108:988-97
10. Acquaviva R, Campisi A, Murabito P, Raciti G, Avola R, Mangiameli S, Musumeci I, Barcellona ML, Vanella A, Li Volti G: Propofol attenuates peroxynitrite-mediated DNA damage and apoptosis in cultured astrocytes: An alternative protective mechanism. *ANESTHESIOLOGY* 2004; 101:1363-71
11. McDermott BJ, McWilliams S, Smyth K, Kelso EJ, Spiers JP, Zhao Y, Bell D, Mirakhor RK: Protection of cardiomyocyte function by propofol during simulated ischemia is associated with a direct action to reduce pro-oxidant activity. *J Mol Cell Cardiol* 2007; 42:600-8
12. Corcoran TB, O'Shea A, Engel A, Shorten GD: The influence of propofol on P-selectin expression and nitric oxide production in re-oxygenated human umbilical vein endothelial cells. *Acta Anaesthesiol Scand* 2006; 50:348-54
13. Xia Z, Godin DV, Ansley DM: Propofol enhances ischemic tolerance of middle-aged rat hearts: Effects on 15-F(2t)-isoprostane formation and tissue antioxidant capacity. *Cardiovasc Res* 2003; 59:113-21
14. Cheng CM, Hong HJ, Liu JC, Shih NL, Juan SH, Loh SH, Chan P, Chen JJ, Cheng TH: Crucial role of extracellular signal-regulated kinase pathway in reactive oxygen species-mediated endothelin-1 gene expression induced by endothelin-1 in rat cardiac fibroblasts. *Mol Pharmacol* 2003; 63:1002-11
15. Yamamoto K, Masuyama T, Sakata Y, Mano T, Nishikawa N, Kondo H, Akehi N, Kuzuya T, Miwa T, Hori M: Roles of renin-angiotensin and endothelin systems in development of diastolic heart failure in hypertensive hearts. *Cardiovasc Res* 2000; 47:274-83
16. Cheng TH, Cheng PY, Shih NL, Chen IB, Wang DL, Chen JJ: Involvement of reactive oxygen species in angiotensin II-induced endothelin-1 gene expression in rat cardiac fibroblasts. *J Am Coll Cardiol* 2003; 42:1845-54
17. Jones SP, Bolli R: The ubiquitous role of nitric oxide in cardioprotection. *J Mol Cell Cardiol* 2006; 40:16-23
18. Dimmeler S, Zeiher AM: Nitric oxide: An endothelial cell survival factor. *Cell Death Differ* 1999; 6:964-8
19. Tian B, Liu J, Bitterman P, Bache RJ: Angiotensin II modulates nitric oxide-induced cardiac fibroblast apoptosis by activation of AKT/PKB. *Am J Physiol Heart Circ Physiol* 2003; 285:H1105-12
20. Cheng TH, Shih NL, Chen SY, Loh SH, Cheng PY, Tsai CS, Liu SH, Wang DL, Chen JJ: Reactive oxygen species mediate cyclic strain-induced endothelin-1 gene expression via Ras/Raf/extracellular signal-regulated kinase pathway in endothelial cells. *J Mol Cell Cardiol* 2001; 33:1805-14
21. Griendling KK, Minieri CA, Ollerenshaw JD, Alexander RW: Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells. *Circ Res* 1994; 74:1141-8
22. Gyllenhammer H: Lucigenin chemiluminescence in the

- assessment on neutrophil superoxide production. *J Immunol Methods* 1987; 97:209-13
23. Cheng TH, Shih NL, Chen SY, Wang DL, Chen JJ: Reactive oxygen species modulate endothelin-I-induced c-fos gene expression in cardiomyocytes. *Cardiovasc Res* 1999; 41: 654-62
  24. Wong KL, Lin JW, Liu JH, Yang HY, Kao PF, Chen CH, Loh SH, Chiu WT, Cheng TH, Lin JG, Hong HJ: Antiproliferative effect of isosteviol on angiotensin II-treated rat aortic smooth muscle cells. *Pharmacology* 2006; 76:163-9
  25. Wong KL, Wu KC, Chou YH, Cheng TH, Hong HJ: Tetramethylpyrazine inhibits angiotensin II-increased NAD(P)H oxidase activity and subsequent proliferation in rat aortic smooth muscle cells. *Am J Chin Med* 2007; 35:1021-35
  26. Kinugawa S, Tsutsui H, Hayashidani S, Ide T, Suematsu N, Satoh S, Utsumi H, Takeshita A: Treatment with dimethylthiourea prevents left ventricular remodeling and failure after experimental myocardial infarction in mice: Role of oxidative stress. *Circ Res* 2000; 87:392-8
  27. Nakamura R, Egashira K, Machida Y, Hayashidani S, Takeya M, Utsumi H, Tsutsui H, Takeshita A: Probucol attenuates left ventricular dysfunction and remodeling in tachycardia-induced heart failure: Role of oxidative stress and inflammation. *Circulation* 2002; 106:362-7
  28. Cheng TH, Chen JJ, Chen CH, Wong KL: Effects of propofol on cyclic strain-induced endothelin-1 expression in human umbilical vein endothelial cells. *ANESTHESIOLOGY* 2009; 110:74-80
  29. Juan SH, Chen JJ, Chen CH, Lin H, Cheng CF, Liu JC, Hsieh MH, Chen YL, Chao HH, Chen TH, Chan P, Cheng TH: 17Beta-estradiol inhibits cyclic strain-induced endothelin-1 gene expression within vascular endothelial cells. *Am J Physiol Heart Circ Physiol* 2004; 287:H1254-61
  30. Oudit GY, Sun H, Kerfant BG, Crackower MA, Penninger JM, Backx PH: The role of phosphoinositide-3 kinase and PTEN in cardiovascular physiology and disease. *J Mol Cell Cardiol* 2004; 37:449-71
  31. Guo XG, Uzui H, Mizuguchi T, Ueda T, Chen JZ, Lee JD: Imidaprilat inhibits matrix metalloproteinase-2 activity in human cardiac fibroblasts induced by interleukin-1beta *via* NO-dependent pathway. *Int J Cardiol* 2008; 6:414-20
  32. Venkatachalam K, Mummidi S, Cortez DM, Prabhu SD, Valente AJ, Chandrasekar B: Resveratrol inhibits high glucose-induced PI3K/Akt/ERK-dependent interleukin-17 expression in primary mouse cardiac fibroblasts. *Am J Physiol Heart Circ Physiol* 2008; 294:H2078-87
  33. Hong HJ, Chan P, Liu JC, Juan SH, Huang MT, Lin JG, Cheng TH: Angiotensin II induces endothelin-1 gene expression *via* extracellular signal-regulated kinase pathway in rat aortic smooth muscle cells. *Cardiovasc Res* 2004; 61:159-68
  34. Olson ER, Shanhart PE, Naugle JE, Meszaros JG: Angiotensin II-induced extracellular signal-regulated kinase 1/2 activation is mediated by protein kinase C $\delta$  and intracellular calcium in adult rat cardiac fibroblasts. *Hypertension* 2008; 51:704-11
  35. Wickley PJ, Ding X, Murray PA, Damron DS: Propofol-induced activation of protein kinase C isoforms in adult rat ventricular myocytes. *ANESTHESIOLOGY* 2006; 104:970-7