Effect of Superoxide Anion Scavenger on Rat Hearts with Chronic Intermittent Hypoxia

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Running Head: O²⁻ scavenger on cardiac apoptosis

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ABSTRACT

Only very limited information regarding the protective effects of superoxide anion scavenger on chronic intermittent hypoxia-induced cardiac apoptosis is available. The purpose of this study is to evaluate the effects of superoxide anion scavenger on cardiac apoptotic and pro-survival pathways in rats with sleep apnea. Forty-two SD rats were divided into three groups, rats with normoxic exposure (CONTROL, 21% O₂, 1 month), rats with chronic intermittent hypoxia exposure (HYPOXIA, 3-7% O₂ versus 21% O₂ per 40 seconds cycle, 8 hrs per day, 1 month), and rats with pre-treatment of superoxide anion scavenger and chronic intermittent hypoxia exposure (HYPOXIA- O₂- SCAVENGER, MnTMPyP Pentachloride, 1 mg/kg, i.p. per day; 3-7% O₂ versus 21% O₂ per 40 seconds cycle, 8 hrs per day, 1 month) at 5-6 months of age. After 1 month, the protein levels and apoptotic cells of excised hearts from three groups were measured by Western blotting and TUNEL assay. Superoxide anion scavenger decreased HYPOXIA-induced myocardial architecture abnormalities, left ventricular hypertrophy and TUNEL-positive apoptosis. Superoxide anion scavenger decreased HYPOXIA-induced Fas ligand, Fas death receptors, Fas-associated death domain (FADD), activated caspase-8, and activated caspase-3 (Fas-dependent apoptotic pathway) as well as Bad, activated caspase-9 and activated caspase-3 (mitochondria-dependent apoptotic pathway), endonuclease G (EndoG), apoptosis-inducing factor (AIF) and TUNEL-positive apoptosis. Superoxide anion scavenger increased IGF-1, IGF-1R, p-PI3k, p-Akt, p-Bad, Bcl-2, Bcl-xL (survival pathway). Our findings imply that superoxide anion scavenger might prevent cardiac Fas-mediated and mitochondrial-mediated apoptosis and enhance IGF-1-related survival pathway in chronic intermittent hypoxia. Superoxide anion scavenger may prevent chronic sleep apnea-enhanced cardiac apoptotic pathways and enhances cardiac survival pathways.

Keywords: caspase, cell death, Fas receptor, hypoxia, heart, survival
INTRODUCTION

Obstructive sleep apnea (OSA) is a common sleep disorder (33) and is a risk factor for cardiovascular diseases, including hypertension, coronary artery disease, heart failure, heart attacks and stroke (1, 4-7, 9). Chronic intermittent hypoxia is used to mimic sleep apnea in animal models and has been associated with the development of myocardial dysfunction and heart failure (6, 7, 23). Our previous study showed that long-term not short-term intermittent hypoxia appeared to increase cardiac apoptosis (8, 12, 17, 19).

Apoptosis, a physiological program of cellular death, may contribute to many cardiac diseases (13, 16). The occurrence of apoptosis has been reported to contribute to the loss of cardiomyocytes, and is recognized as a predictor of adverse outcomes in patients with cardiac diseases or heart failure (21). The Fas receptor-dependent (type I) apoptotic pathway is initiated by binding the Fas ligand to the Fas receptor. Fas ligand binding followed by Fas-receptor oligomerization is known to lead to the formation of a death-inducing signal complex starting with recruitment of the Fas-associated death domain (FADD) (4). Fas receptor oligomerization recruits FADD and pro-caspase-8 to the complex and results in the activation of caspase-8. The activated caspase-8 cleaves pro-caspase-3, which then undergoes autocatalysis to form active caspase-3, a principle effector caspase of apoptosis (24, 25).

The mitochondrial-dependent (type II) apoptotic pathway is mediated by internal factors, especially in mitochondria. The mitochondria are the main sites of action for members of the apoptosis-regulating protein family exemplified by the Bcl-2 family, such as Bcl-2, and Bax (4). Anti-apoptotic proteins such as Bcl-2, Bcl-xL and p-Bad, prevent cytochrome c release whereas pro-apoptotic proteins such Bax, t-Bid, Bad, enhance cytochrome c release from mitochondria (4). Cytochrome c released from
mitochondria into the cytosol, is responsible for activating caspase-9, which activates caspase-3 and executes the apoptotic program (5).

Endonuclease G (EndoG), a caspase-independent and mitochondrion-specific nuclease, translocates to the nucleus during apoptosis. Once released from the mitochondria, EndoG cleaves chromatin DNA into nucleosomal fragments independently of caspases (18). The other caspase-independent apoptosis-inducing factor (AIF) induces chromatin condensation and large-scale DNA fragmentation (11, 30). During apoptosis, EndoG and AIF translocate to the nucleus where they causes oligonucleosomal DNA fragmentation (18) and represent caspase-independent apoptotic pathways initiated from the mitochondria. Insulin-like growth factor 1 (IGF-1) via the IGF-1 receptor triggers a signaling cascade and plays a key survival role in the heart (26, 29, 31). Phosphatidylinositol 3-kinase (PI3K) and protein kinase B (Akt) are key signaling factors in IGF-1 receptor (IGF-1R) mediated survival signaling (14, 26, 29, 31).

The effect of superoxide anion scavenger on cardiac apoptosis in chronic intermittent hypoxia is not understood. Since superoxide anion scavenger may prevent damage by free radicals, we hypothesized that superoxide anion scavenger may prevent cardiac Fas-mediated, mitochondrial-mediated, or EndoG/AIF-mediated apoptosis and enhance IGF-1-related survival pathways during exposure to chronic intermittent hypoxia.

**Materials and Methods**

**Animal model**

The study was performed on forty-two Sprague-Dawley (SD) male rats. Ambient
temperature was maintained at 25°C and the animals were kept on an artificial 12-h light-dark cycle, beginning at 7:00 a.m. Rats were provided with standard laboratory chow (Lab Diet 5001; PMI Nutrition International Inc., Brentwood, MO, USA) and water *ad libitum*. All protocols were approved by the Institutional Animal Care and Use Committee of China Medical University, Taichung, Taiwan, and the principles of laboratory animal care (NIH publication) were followed.

**Chronic intermittent hypoxia and Superoxide anion scavenger**

Forty-two SD rats were divided into three groups, CONTROL group: rats with normoxic exposure (normoxia 21% O₂, 1 month); HYPOXIA group: rats with chronic intermittent hypoxia exposure (3-7% O₂ versus 21% O₂ per 40 seconds cycle, 8 hours per day, 1 month); HYPOXIA-O²⁻ SCAVENGER group: rats with pre-treatment of superoxide anion scavenger (MnTMPyP Pentachloride, 1 mg/kg, i.p. per day, 1 month) and during chronic intermittent hypoxia (3-7% O₂ versus 21% O₂ per 40 seconds cycle, 8 hours per day, 1 month) exposure at 5-6 months of age. All hypoxia experiments were performed between 9:00 a.m. and 5:00 p.m. Oxygen concentration was detected and confirmed by SYSTECH O₂ analyzer in each chamber.

**Cardiac characteristics**

The hearts of eight rats from each of the three groups (CONTROL, HYPOXIA, and HYPOXIA-O²⁻SCAVENGER) were excised and cleaned with phosphate-buffered saline (PBS). The eight left ventricles in each group were separated and weighed. The right tibias were also separated and tibial lengths were measured by an electronic digital vernier caliper to adjust the whole heart weight. The ratios of the total heart weight to body weight, the left ventricle weight to the whole heart weight, and the
whole heart weight to tibia length were calculated.

**Echocardiography**

All echocardiographic examinations were performed on the one day before the animal been sacrificed by an experienced cardiologist who was blinded to the three animal groups and interpreted echocardiographic data independently. Rats were secured in a supine position on a plastic board and the precordial chest wall was shaved. Rats were lightly anesthetized with inhaled isoflurane at a concentration of 2% in a 70% oxygen mixture. A commercially available echocardiographic machine (ultrasound system -Vivid i, GE Healthcare, Milwaukee, WS, USA) was used with a 10-MHz transducer (GE 10S-RS) for image acquisition. The heart was first imaged in the two-dimensional mode in the parasternal long-axis and a two-dimensional short-axis view of the left ventricle obtained at the level of the papillary muscles. These views were used to determine the optimal position of the M-mode cursor that was perpendicular to the ventricular septum and posterior wall of left ventricle. M-mode tracings were recorded through the anterior and posterior LV walls. Anterior and posterior wall thicknesses (end-diastolic and end-systolic) and LV internal dimensions were measured using a modification of the American Society for Echocardiography leading edge method from at least three consecutive cardiac cycles on the M-mode tracings.

**H&E staining and TUNEL Assay**

The hearts of six animals from each group were soaked in formalin, dehydrated through graded alcohols, and embedded in paraffin wax. Sections of 2 µm thickness were cut, deparaffinized by immersion in xylene, and rehydrated. The slices
were stained with hematoxylin-eosin (H&E staining) and then dehydrated in increasing grades (100%, 90%, 80% and 70%) of ethanol-water solutions. Finally, they were soaked in Xylene twice. Photomicrographs were obtained using Zeiss Axiophot microscopes. For the Terminal Deoxynucleotide Transferase-mediated dUTP Nick End Labeling (TUNEL) assay, the sections were incubated with proteinase K, washed in PBS, incubated with permeabilization solution, blocking buffer, and then washed twice with PBS. A terminal deoxynucleotidyl transferase and fluorescein isothiocyanate-dUTP assay proceeded for 60 min at 37 °C using an apoptosis detection kit (Roche Applied Science, Indianapolis, IN, USA) for detection. TUNEL-positive nuclei (fragmented DNA) fluoresced bright green at 450-500 nm. The mean number of TUNEL-positive cells were counted for at least 5-6 separate fields x 2 slices x 3 regions of the left ventricle (upper, middle, lower) excised from six rat hearts from each group. All counts were performed by at least two independent individuals in a blinded manner.

**Tissue Extraction**

Cardiac tissue extracts were obtained by homogenizing the left ventricle samples in a RIPA buffer (R0278, Sigma-Aldrich, MO, USA) at a ratio of 100 mg tissue/1ml buffer for 1 min. The homogenates were placed on ice for 10 minutes and then centrifuged at 12,000g for 40 min at 4°C. The supernatant of each sample was collected and stored at -70°C for further investigation.

**Electrophoresis and Western Blot**

Protein concentration of cardiac tissue extracts was determined by the Bradford method (Bio-Rad Protein Assay, Hercules, CA). Protein samples (40μg/lane) were
separated on a 10% SDS polyacrylamide gel by electrophoresis (SDS-PAGE) with a constant voltage of 75 V. Electrophoresed proteins were transferred to polyvinylidene difluoride (PVDF) membrane (0.45 μm pore size Millipore, Bedford, MA, USA) with a transfer apparatus (Bio-rad). PVDF membranes were incubated with 5% skim milk in Tris buffered saline (TBS) for 30 min. Primary antibodies including IGF-1, IGF-1R, p-PI3K, Bcl-2, Bcl-xL, p-Bad, Bad, Bax, cytochrome c, Fas ligand, Fas receptor, FADD, EndoG, AIF, caspase-8, caspase-9, caspase-3 and α-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and p-Akt (Cell Signaling Technology Inc., Beverly, MA, USA) were diluted to 1:500 in antibody binding buffer overnight at 4°C. The immunoblots were washed three times in TBS for 10 min and then immersed in the second antibody solution containing goat anti-mouse IgG-HRP, goat anti-rabbit IgG-HRP, or donkey anti goat IgG-HRP (Santa Cruz) for 1 hour and diluted 500-fold in TBS buffer. The immunoblots were then washed three times in TBS for 10 min each times. The immunoblotted proteins were visualized using an enhanced chemiluminescence ECL western blotting luminal reagent (Santa Cruz, CA, USA) and quantified using a Fujifilm LAS-3000 chemiluminescence detection system (Tokyo, Japan).

**Statistical Analysis**

The all data of weight index, protein levels, and the percentage of TUNEL positive cells were compared between the CONTROL, HYPOXIA, and HYPOXIA-O2-SCAVENGER groups using one-way analysis of variance with pre-planned contrast comparison for negative or positive control. In all cases, P<0.05 was considered significant.
Results

Body weight and cardiac characteristics

The whole heart weight (WHW), left ventricular weight (LVW), whole heart weight normalized by body weight (WHW/BW), left ventricular weight normalized by body weight (LVW/BW), whole heart weight normalized by tibia length (WHW/tibia) and left ventricular weight normalized by tibia length (LVW/tibia) in the HYPOXIA group were higher than those in the CONTROL group. The LVW, WHW/BW, LVW/BW, WHW/tibia and LVW/tibia in the HYPOXIA-O2-SCAVENGER group were lower than in the HYPOXIA group (Table 1), which imply daily pre-treatment with the superoxide anion scavenger attenuated chronic intermittent hypoxia-induced left ventricular hypertrophy.

Echocardiography revealed significantly reduced LV wall thickness in the HYPOXIA-O2-SCAVENGER groups, compared with HYPOXIA group (Table 1). Left ventricular fractional shortening (FS) was not significantly different between the CONTROL, HYPOXIA and HYPOXIA-O2-SCAVENGER groups (Table 1 and Figure 1A). To investigate the effect of superoxide anion scavenger on myocardial architecture under chronic intermittent hypoxia, histopathological analyses of ventricular tissues by H and E staining was done on the LV tissues of CONTROL, HYPOXIA, and HYPOXIA-O2-SCAVENGER groups. After viewing images at x400 magnification, it was found that the ventricular myocardium in the HYPOXIA group showed abnormal myocardial architecture and increased interstitial spacing, compared with the CONTROL group. The abnormalities of myocardial architecture in the HYPOXIA-O2-SCAVENGER group were less than those in the HYPOXIA group (Figure 1 B).
TUNEL-positive apoptotic cells from cardiac tissues

To evaluate the anti-apoptotic effect of superoxide anion after chronic intermittent hypoxia, DAPI staining and TUNEL assay were measured in all groups. It was observed that the left ventricle of the HYPOXIA group had a greater percentage of TUNEL-positive cardiac cells than the left ventricles in the CONTROL group. Decreases in the percentage of TUNEL-positive cardiac cells were observed in the HYPOXIA-O2 SCAVENGER group, compared with the HYPOXIA group (Figure 1C, D).

Cardiac IGF-1R/PI3K/AKT survival pathway

To investigate the pro-survival effects of superoxide anion scavenger treatment on the cardiac IGF-1R/PI3K/AKT survival pathway after chronic intermittent hypoxia, the pro-survival relative protein expression was measured in hearts excised from all the groups. The pro-survival proteins IGF-1 and p-PI3K appeared to have a compensatory pro-survival response in the HYPOXIA group. IGF-1, IGF-1R, p-PI3K, and p-Akt were increased in the HYPOXIA-O2 SCAVENGER group, compared with the CONTROL group. IGF-1, IGF-1R, and p-Akt were enhanced by treatment with superoxide anion scavenger, compared with the HYPOXIA group (Figure 2).

Upstream of cardiac mitochondria dependent apoptotic pathway

In order to identify the anti-apoptotic effect of superoxide anion scavenger treatment upstream of the cardiac mitochondria dependent apoptotic pathway after chronic intermittent hypoxia, the Bcl-2 families were measured by Western blotting in the hearts excised from all groups. The anti-apoptotic protein levels of Bcl-2, Bcl-xL, p-Bad in the HYPOXIA- O2 SCAVENGER group were significantly higher than
those in the HYPOXIA group, while the pro-apoptotic protein levels of Bad, Bax and cytochrome c in the HYPOXIA-O$_2^-$ SCAVENGER group were significantly lower than those in the HYPOXIA group (Figure 3).

**Upstream components of cardiac Fas receptor dependent apoptotic pathways**

To investigate the effect of superoxide anion scavenger treatment on upstream cardiac Fas receptor dependent apoptotic pathways after chronic intermittent hypoxia, the protein levels of Fas Ligand, Fas and FADD were measured in hearts excised from the three groups. Compared with the CONTROL group, Fas Ligand, Fas receptor, and FADD were significantly increased in the HYPOXIA group. Fas Ligand, Fas receptor, and FADD in the HYPOXIA- O$_2^-$ SCAVENGER group were significantly lower than those in the HYPOXIA group (Figure 4).

**Downstream components of cardiac Fas receptor and mitochondria dependent apoptotic pathways**

In order to identify the changes of downstream of cardiac Fas receptor and mitochondrial dependent apoptotic pathways, the pro-form and active form of caspases-8, -9 and -3 were measured in the excised hearts of three groups by Western blotting. The protein levels of activated caspases-8, -9, and -3 were elevated in the HYPOXIA groups compared to the CONTROL group. The protein levels of activated caspases-8, -9, and -3 in the HYPOXIA-O$_2^-$ SCAVENGER group were significantly lower than those in the HYPOXIA group (Figure 5).

**EndoG-AIF dependent apoptotic pathways**

To investigate the effect of superoxide anion scavenger treatment on cardiac caspase-independent apoptotic pathways after chronic intermittent hypoxia, the
EndoG and AIF were measured in hearts excised from the three groups. The protein levels of EndoG and AIF were significantly increased in the HYPOXIA group, compared with the CONTROL group. The protein levels of EndoG and AIF in the HYPOXIA-O$_2^-$ SCAVENGER group were significantly lower than those in the HYPOXIA group (Figure 6).

**Discussion**

**Major findings**

Our main findings can be summarized as follows: (1) Daily pre-treatment of superoxide anion scavenger before intermittent hypoxic exposure decreased the myocardial architecture abnormalities, attenuated left ventricular hypertrophy and TUNEL-positive apoptosis. (2) Superoxide anion scavenger treatment enhanced the cardiac IGF-1-related survival pathway under chronic intermittent hypoxia. (3) Pre-treatment of superoxide anion scavenger attenuated the activated cardiac Fas-dependent apoptotic pathway and mitochondria-dependent apoptotic pathways under chronic intermittent hypoxia. (4) Superoxide anion scavenger attenuated cardiac EndoG and AIF caspase-independent apoptosis under chronic intermittent hypoxia. Therefore, superoxide anion might play a pathophysiologic role and superoxide anion scavenger might play a therapeutic role in cardiac apoptosis under chronic intermittent hypoxia which may occur in patients with long-term sleep apnea. Further, left ventricular chamber size and fractional shortening (FS) did not change after one-month intermittent hypoxia.

Insulin-like growth factor 1 (IGF-1), is a key survival factor in the heart. IGF-1 can be released into the bloodstream by the liver, or synthesized locally by muscles and neural cells. Acting in an autocrine or paracrine fashion, IGF-1 via the IGF-1
receptor triggers a signaling cascade that plays many essential regulatory roles in
multiple aspects of cardiac biology. Phosphatidylinositol 3-kinase (PI3K) and protein
kinase B (Akt) are key signaling factors in insulin and IGF-1 receptor (IGF-1R)(2, 10, 26). In the current study, superoxide anion scavenger treatment appeared to enhance
the cardiac IGF-1-related survival pathway under chronic intermittent hypoxia, which
might imply that superoxide anion scavenger treatment has pro-survival effects on
chronic intermittent hypoxia-induced heart diseases.

Fas receptor-dependent and mitochondrial-dependent apoptotic pathways
appeared to induced by various types of chronic intermittent hypoxia such as the
model of nocturnal sleep apnea in the current study (3-7% O₂ versus 21% O₂ per 40
seconds cycle, 8 hours per day, 1 month) and our previous studies (7% O₂ 60 seconds,
20% O₂ alternating 60 seconds, 8 hours per day, 2 months) (15) were also activated by
nocturnal eight hours sustained hypoxia (17, 19). A study found that cardiac ischemia
induced the release of cytochrome c, EndoG and AIF from mitochondria into the
cytosol in rat postnatal differentiated cardiomyocytes (3). In the study reported here,
superoxide anion scavenger was found to significantly prevent more cardiac activated
pro-apoptotic protein AIF and EndoG after chronic intermittent hypoxia. Our findings
imply that superoxide anion scavenger has both caspase-dependent and
caspase-independent anti-apoptotic effects on the chronic intermittent hypoxic heart.
To our knowledge, no previous studies have reported on the cardiac Fas-dependent
and mitochondrial-dependent apoptotic pathway as well as EndoG and AIF under
chronic intermittent hypoxia treated with superoxide anion scavenger.

This preventive effect of superoxide anion scavenger is consistent with other
reports in the literature that have demonstrated that activation of antioxidant enzymes
or treatment with antioxidant agents ameliorates ischemia/reperfusion injury. In
previous studies, the neuroprotective effect of MnTMPyP, which is a cell-permeable superoxide dismutase (28) mimetic and a peroxynitrite scavenger as well as an oxidative stress inhibitor, reduced the quantity of ROS produced ischemia/reperfusion injury (27, 32). A vitro study showed that MnII-containing superoxide anion scavenger has a direct protective action on H9c2 rat cardiac muscle cells subjected to hypoxia and reoxygenation (22). Our data indirectly supports chronic intermittent hypoxia as a possible cause of cardiac apoptosis via free radicals (superoxide anion). Of course, further studies are required to elucidate underlying mechanisms responsible for the therapeutic effects of cleaning free radicals in sleep apnea and further clinical studies are required to clarify the possible human therapeutic applications.

**Limitation.** Some limitations can be found in the current study. Since apnea duration might range from 10 sec to over 1 minute as well as intrathoracic negative pressure swings occur in obstructive sleep apnea, chronic intermittent hypoxia alternating 40 seconds can be partially mimic to obstructive sleep apnea in human. In our experimental design, hypoxia was applied systemically, which consequently produced a widespread systematic action. Thus any effect noted in intermittent hypoxia-induced cardiac events cannot be isolated to any specific effects such as free radicals, oxidative stress, re-oxygenation or only hypoxia. The goal of the present study, however, was to determine the protective effects of superoxide anion scavenger on chronic intermittent hypoxia-induced cardiac apoptosis. Another notable account is, while the treatment groups received 1 mg/kg MnTMPyP Pentachloride, the control groups were free of any administration not even a vehicle.

**Hypothesized clinical application**
Since chronic intermittent hypoxia (e.g. in obstructive sleep apnea) might enhance cardiac apoptosis, chronic intermittent hypoxia or severe obstructive sleep apnea should be highly aware of the possibility of progressive development in cardiac abnormality and be considered as a major risk factor for the development of heart failure. Because cardiac tissues are difficult to extract from human hearts, the current animal model provides an important therapeutic possibility of superoxide anion scavenger to prevent sleep apnea induced cardiac apoptosis. Based on the current evidence from animal study, we might further hypothesize severe OSA patients may cause cardiac apoptosis via hypoxia and free radicals (superoxide anion). Future clinical study is needed to prove the apoptotic effect of heart in patients with severe OSA and clarify the possible therapeutic application in human.

Acknowledgements

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Conflict of interest

The authors declare no conflicts of interest.
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Figure Legends

Figure 1

(A) Representative echocardiography (ECHO) from three groups. Two-dimensional short-axis view at the level of the papillary muscles and M-mode tracings were recorded through the anterior and posterior LV walls. (B) Representative hematoxylin and eosin stain (H&E). (C) Representative TUNEL assay (low panels, green spots) and DAPI staining (upper panels, blue spots) of cardiac sections from left ventricles in rats with normoxic exposure (CONTROL), rats with nocturnal intermittent hypoxia (HYPOXIA) and rats with treated superoxide anion scavenger under nocturnal intermittent hypoxia (HYPOXIA-O\(^2\)-SCAVENGER). The images were magnified x400. (D) Bars represent the percentage of TUNEL-positive cells and indicate mean values ± SD, (n=6). ***P<0.001 indicates significant differences from the CONTROL group. ###P<0.001 indicates significant differences between the HYPOXIA group and the HYPOXIA-O\(^2\)-SCAVENGER group.

Figure 2

(A) The representative protein products of insulin-like growth factor 1 (IGF-1) and IGF-1 receptor, phosphates phosphoinositide-3 kinase (p-PI3K) and p-Akt extracted from the left ventricles of excised hearts in three rats with normoxic exposure (CONTROL), from three rats with nocturnal intermittent hypoxia (HYPOXIA), and from three rats with treated superoxide anion scavenger under nocturnal intermittent hypoxia (HYPOXIA-O\(^2\)-SCAVENGER), as measured by Western blotting analysis. \(\alpha\)-tubulin was used as an internal control. (B) Bars represent the relative fold changes of protein quantification relative to the CONTROL group in IGF-1, IGF-1 receptor,
p-PI3K and p-Akt based on α-tubulin and indicate the mean values ±SD (n=6 in each group). *P <0.05, **P <0.01, indicate significant differences from the CONTROL group. #P<0.05, ##P<0.01 indicate significant differences between the HYPOXIA group and the HYPOXIA-O²⁻ SCAVENGER group.

**Figure 3**

(A) The representative protein products of B-cell lymphoma 2 gene (Bcl-2), B-cell lymphoma-extra large (Bcl-xL), phosphates Bcl-2-associated death promoter (p-Bad), Bcl-2-associated death promoter (Bad), Bcl-2–associated X protein (20) and cytosolic cytochrome c extracted from the left ventricles of excised hearts in three rats with normoxic exposure (CONTROL), three rats with nocturnal intermittent hypoxia (HYPOXIA), and three rats with treated superoxide anion scavenger under nocturnal intermittent hypoxia (HYPOXIA-O²⁻SCAVENGER), as measured by Western blotting analysis. α-tubulin was use as an internal control. (B) Bars represent the relative fold changes of protein quantification relative to the CONTROL group in Bcl-2, Bcl-xL, p-Bad, Bad, Bax and cytochrome c on α-tubulin and indicate the mean values ±SD (n=6 in each group). **P <0.01, ***P <0.001, significant differences from the Control group. ##P<0.01, ###P<0.001, indicate significant differences between the Hypoxia group and the HYPOXIA-O²⁻SCAVENGER group.

**Figure 4**

(A) The representative protein products of Fas ligand, Fas receptor (Fas) and Fas-associated death domain (FADD) extracted from the left ventricles of excised hearts in three rats with normoxic exposure (CONTROL), three rats with nocturnal intermittent hypoxia (HYPOXIA), and three rats with treated superoxide anion scavenger...
scavenger under nocturnal intermittent hypoxia (HYPOXIA-O^2-SCAVENGER) as measured by Western blotting analysis. α-tubulin was use as an internal control. (B) Bars represent the relative fold changes of protein quantification relative to the CONTROL group in FasL, Fas and FADD on α-tubulin and mean values ±SD (n=6 in each group). *P<0.05, **P<0.01, ***P<0.001 indicate significant differences from the CONTROL group. #P<0.05, ##P<0.01, indicate significant differences between the HYPOXIA group and the HYPOXIA-O^2-SCAVENGER group.

Figure 5
(A) The representative protein products of caspase-8, caspase-9 and caspase-3 extracted from the left ventricles of excised hearts in three rats with normoxic exposure (CONTROL), three rats with nocturnal intermittent hypoxia (HYPOXIA), and three rats with treated superoxide anion scavenger under nocturnal intermittent hypoxia (HYPOXIA-O^2-SCAVENGER), as measured by Western blotting analysis. α-tubulin was used as an internal control. (B) Bars represent the relative fold changes of protein quantification relative to the CONTROL group in active caspase-8, caspase-9, caspase-3 on α-tubulin and mean values ±SD (n=6 in each group). *P<0.05, indicates significant differences from the CONTROL group. #P<0.05, indicates significant differences between the HYPOXIA group and the HYPOXIA-O^2-SCAVENGER group.

Figure 6
(A) The representative protein products of Endonuclease G (EndoG) and apoptosis-inducing factor (AIF) extracted from the left ventricles of excised hearts in three rats with normoxic exposure (CONTROL), three rats with nocturnal intermittent
hypoxia (HYPOXIA), and three rats with treated superoxide anion scavenger under nocturnal intermittent hypoxia (HYPOXIA-O2-SCAVenger), as measured by Western blotting analysis. α-tubulin was used as an internal control. (B) Bars represent the relative fold changes of protein quantification relative to the CONTROL group in EndoG and AIF on α-tubulin and mean values ±SD (n=6 in each group). *P<0.05, **P<0.01, ***P<0.001 indicate significant differences from CONTROL group. ##P<0.01, ###P<0.001, indicate significant differences between HYPOXIA group and the HYPOXIA-O2-SCAVenger group.

Figure 7. Proposed schematic diagram from the current study showing that the cardiac Fas-dependent and the mitochondria-dependent apoptotic pathway will be activated after chronic intermittent hypoxia, and these two major apoptotic pathways appear to be suppressed, or not activated, by pre-treated superoxide anion scavenger. The caspase-independent EndoG and AIF will be activated after chronic intermittent hypoxia, and those appear to be suppressed, or not activated, by pre-treated superoxide anion scavenger. In contrast, the cardiac pro-survival IGF-1-PI3K-Akt pathway becomes more activated under chronic intermittent hypoxia after pre-treated superoxide anion scavenger.
### (A)

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<td>p-Akt</td>
<td><img src="p-Akt-Control.png" alt="Image" /></td>
<td><img src="p-Akt-Hypoxia.png" alt="Image" /></td>
<td><img src="p-Akt-Hypoxia-SCAVENGER.png" alt="Image" /></td>
</tr>
<tr>
<td>α-Tubulin</td>
<td><img src="%CE%B1-Tubulin-Control.png" alt="Image" /></td>
<td><img src="%CE%B1-Tubulin-Hypoxia.png" alt="Image" /></td>
<td><img src="%CE%B1-Tubulin-Hypoxia-SCAVENGER.png" alt="Image" /></td>
</tr>
</tbody>
</table>

### (B)

#### IGF-1/α-Tubulin

- Control: ![Image](IGF-1-Control.png)
- Hypoxia: ![Image](IGF-1-Hypoxia.png)
- Hypoxia + O²-SCAVENGER: ![Image](IGF-1-Hypoxia-SCAVENGER.png)

#### IGF-1R/α-Tubulin

- Control: ![Image](IGF-1R-Control.png)
- Hypoxia: ![Image](IGF-1R-Hypoxia.png)
- Hypoxia + O²-SCAVENGER: ![Image](IGF-1R-Hypoxia-SCAVENGER.png)

#### p-PI3K/α-Tubulin

- Control: ![Image](p-PI3K-Control.png)
- Hypoxia: ![Image](p-PI3K-Hypoxia.png)
- Hypoxia + O²-SCAVENGER: ![Image](p-PI3K-Hypoxia-SCAVENGER.png)

#### p-Akt/α-Tubulin

- Control: ![Image](p-Akt-Control.png)
- Hypoxia: ![Image](p-Akt-Hypoxia.png)
- Hypoxia + O²-SCAVENGER: ![Image](p-Akt-Hypoxia-SCAVENGER.png)
Fig 3.

(A) | CONTROL | HYPOXIA | HYPOXIA + O2-SCAVENER
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Bcl-2</td>
<td></td>
<td>28 kDa</td>
</tr>
<tr>
<td>Bcl-xL</td>
<td></td>
<td>30 kDa</td>
</tr>
<tr>
<td>p-Bad</td>
<td></td>
<td>25 kDa</td>
</tr>
<tr>
<td>Bad</td>
<td></td>
<td>25 kDa</td>
</tr>
<tr>
<td>Bax</td>
<td></td>
<td>23 kDa</td>
</tr>
<tr>
<td>Cytochrome c</td>
<td></td>
<td>15 kDa</td>
</tr>
<tr>
<td>α-Tubulin</td>
<td></td>
<td>55 kDa</td>
</tr>
</tbody>
</table>

(B)

<table>
<thead>
<tr>
<th>Control</th>
<th>Hypoxia</th>
<th>Hypoxia-O2-SCAVENER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bcl-2/α-Tubulin</td>
<td></td>
<td>***</td>
</tr>
<tr>
<td>Bcl-xL/α-Tubulin</td>
<td></td>
<td>#</td>
</tr>
<tr>
<td>p-Bad/α-Tubulin</td>
<td></td>
<td>**</td>
</tr>
<tr>
<td>Bad/α-Tubulin</td>
<td></td>
<td>*</td>
</tr>
<tr>
<td>Bax/α-Tubulin</td>
<td></td>
<td>** **</td>
</tr>
<tr>
<td>Cytochrome c/α-Tubulin</td>
<td></td>
<td>** #</td>
</tr>
</tbody>
</table>
Fig 4.

(A) 

<table>
<thead>
<tr>
<th></th>
<th>CONTROL</th>
<th>HYPOXIA</th>
<th>HYPOXIA + O2-SCAVENGER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fas Ligand</td>
<td></td>
<td></td>
<td>48 kDa</td>
</tr>
<tr>
<td>Fas</td>
<td></td>
<td></td>
<td>48 kDa</td>
</tr>
<tr>
<td>FADD</td>
<td></td>
<td></td>
<td>30 kDa</td>
</tr>
<tr>
<td>α-Tubulin</td>
<td></td>
<td></td>
<td>55 kDa</td>
</tr>
</tbody>
</table>

(B) 

- Fas Ligand/α-Tubulin: 
  - Control vs Hypoxia: **
  - Hypoxia vs Hypoxia-O2-SCAVENGER: ##

- Fas/α-Tubulin: 
  - Control vs Hypoxia: **
  - Hypoxia vs Hypoxia-O2-SCAVENGER: ##

- FADD/α-Tubulin: 
  - Control vs Hypoxia: *
  - Hypoxia vs Hypoxia-O2-SCAVENGER: #
Fig 5.

(A) Western Blot Analysis

<table>
<thead>
<tr>
<th></th>
<th>CONTROL</th>
<th>HYPOXIA</th>
<th>HYPOXIA + O2-SCAVENGER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caspase-8</td>
<td>Pro</td>
<td>Active</td>
<td>54 kDa</td>
</tr>
<tr>
<td>Caspase-9</td>
<td>Pro</td>
<td></td>
<td>43 kDa</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>Pro</td>
<td>Active</td>
<td>35 kDa</td>
</tr>
<tr>
<td>α-Tubulin</td>
<td></td>
<td></td>
<td>35 kDa</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>55 kDa</td>
</tr>
</tbody>
</table>

(B) Bar Graph Analysis

- Active caspase-8/α-Tubulin
- Active caspase-9/α-Tubulin
- Active caspase-3/α-Tubulin
Fig 6.

(A) Western blot analysis showing the expression levels of EndoG, AIF, and α-Tubulin in different conditions: CONTROL, HYPOXIA, HYPOXIA + O²-SCAVENGER. 

(B) Bar graph showing the relative expression levels of EndoG and AIF normalized to α-Tubulin in different conditions: Control, Hypoxia, and Hypoxia + O²-SCAVENGER. 

Means ± SEM, n = 3-5. 

### Statistical Analysis

- **EndoG**
  - Control: 0.95 ± 0.02
  - Hypoxia: 1.20 ± 0.03
  - Hypoxia + O²-SCAVENGER: 0.60 ± 0.01
  - Significance: *** p < 0.001

- **AIF**
  - Control: 0.90 ± 0.01
  - Hypoxia: 1.10 ± 0.02
  - Hypoxia + O²-SCAVENGER: 0.75 ± 0.03
  - Significance: ### p < 0.001

- **α-Tubulin**
  - Control: 0.98 ± 0.02
  - Hypoxia: 1.02 ± 0.01
  - Hypoxia + O²-SCAVENGER: 0.90 ± 0.01
  - Significance: * p < 0.05
Fig 7.
Table 1. Cardiac characteristics with CONTROL, HYPOXIA and HYPOXIA+O$^2$-SCAVENGER

<table>
<thead>
<tr>
<th></th>
<th>CONTROL</th>
<th>HYPOXIA</th>
<th>HYPOXIA+O$^2$-SCAVENGER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of animals</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>564±26</td>
<td>542±36</td>
<td>534±59</td>
</tr>
<tr>
<td>Whole heart weight, g</td>
<td>1.32±0.07</td>
<td>1.55±0.18**</td>
<td>1.25±0.25</td>
</tr>
<tr>
<td>Left ventricular weight, g</td>
<td>0.93±0.08</td>
<td>1.1±0.09**</td>
<td>0.89±0.17</td>
</tr>
<tr>
<td>Whole heart weight /Body weight ($\times 10^4$)</td>
<td>23.44±1.79</td>
<td>28.69±1.54**</td>
<td>25.32±1.63#</td>
</tr>
<tr>
<td>Left ventricular weight /Body weight ($\times 10^4$)</td>
<td>16.4±1.27</td>
<td>20.49±1.47**</td>
<td>17.99±1.24##</td>
</tr>
<tr>
<td>Left ventricular weight /Whole heart weight</td>
<td>0.701±0.051</td>
<td>0.714±0.033</td>
<td>0.710±0.025</td>
</tr>
<tr>
<td>Whole heart weight /Tibia length, g/mm</td>
<td>0.023±0.001</td>
<td>0.029±0.004**</td>
<td>0.022±0.003#</td>
</tr>
<tr>
<td>Left ventricular weight /Tibia length, g/mm</td>
<td>0.016±0.001</td>
<td>0.020±0.002**</td>
<td>0.016±0.002##</td>
</tr>
<tr>
<td>Heart rate</td>
<td>243±14</td>
<td>237±20</td>
<td>235±11</td>
</tr>
<tr>
<td>(IVSd),mm</td>
<td>2.04±0.23</td>
<td>2.10±0.10</td>
<td>1.75±0.23#</td>
</tr>
<tr>
<td>(LVPWd),mm</td>
<td>1.74±0.46</td>
<td>1.98±0.22</td>
<td>1.58±0.09##</td>
</tr>
<tr>
<td>(LVIDd),mm</td>
<td>8.10±0.84</td>
<td>8.75±0.65</td>
<td>8.59±0.25</td>
</tr>
<tr>
<td>(LVIDs),mm</td>
<td>5.04±0.70</td>
<td>5.72±0.69</td>
<td>5.60±0.70</td>
</tr>
<tr>
<td>Fractional Shortening (FS),%</td>
<td>38.3±6.5</td>
<td>35.4±7.9</td>
<td>37.2±6.5</td>
</tr>
</tbody>
</table>

Values are mean ± SD for the rats with normoxic exposure (CONTROL) and rats with nocturnal intermittent hypoxia exposure (HYPOXIA), and rats with pre-treatment of superoxide anion scavenger and nocturnal intermittent hypoxia exposure (HYPOXIA-O$^2$-SCAVENGER). IVSd: interventricular septum at diastole; LVPWd: left ventricular posterior wall thickness at diastole; LVIDd: internal dimension at diastole of left ventricle; LVIDs: internal dimension at systole of left ventricle; FS: (LVIDd-LVIDs)/LVIDd×100. *P<0.05, **P<0.01 indicate significant differences between CONTROL and HYPOXIA or HYPOXIA-O$^2$-SCAVENGER group. #P<0.05, ##P<0.01 indicates significant differences between HYPOXIA group and HYPOXIA-O$^2$-SCAVENGER group.