의학박사 학위논문

Role of AMPK in cardioprotection by leptin-preconditioning

렙틴 전처치에 의한 심장보호기전에서 AMPK 의 역할

2013 년 12 월 19 일

서울대학교 대학원

의학과 생리학 전공

김 기 석

A thesis of the Degree of Doctor of Philosophy

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The Department of Physiology,

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지도교수 호 원 경

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Role of AMPK in cardioprotection by leptin-preconditioning

by
Ki-Suk Kim

A thesis submitted to the Department of Physiology in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Physiology at Seoul National University College of Medicine

December 19, 2013

Approved by Thesis Committee:

Professor _____**Chairman**
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Professor _____
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Professor _____

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논문 제목: Role of AMPK in cardioprotection by leptin-preconditioning

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ABSTRACT

Introduction: Leptin, a product of the *ob* gene, is a 16-kDa peptide synthesized primarily by white adipose tissue. It is also produced in the heart, suggesting that it has a cardioprotective effect. AMP-activated protein kinase (AMPK) is a serine/threonine protein kinase that plays a key role in cardioprotection in the abnormal heart. However, it is not clear whether AMPK-mediated cardioprotection influences the effects of leptin in cardiac muscle. Here, I investigated the mechanism of cardioprotection in leptin preconditioning and the relationship between leptin preconditioning-induced cardioprotection and the regulation of AMPK.

Methods: A rat ischemia model was constructed using left anterior descending coronary artery occlusion (CAO). Infarct size was measured to evaluate the cardioprotective effect. A rat cardiac myoblast cell line (H9c2) was used to investigate the cardioprotective mechanism of leptin.

Results: Leptin decreased myocardial infarct size in rat cardiac ischemic reperfusion model. The effect of leptin was abolished in combination with compound C, an AMPK inhibitor or NAC, a ROS scavenger. Leptin phosphorylated AMPK dose- and time-dependently in H9c2 cells. Leptin also induced generation of reactive oxygen species (ROS) dose- and time-dependently in H9c2 cells. AMPK phosphorylation by leptin was abolished in combination with NAC, however, leptin-induced ROS generation was not affected by compound C. Therefore, Leptin

induced generation of ROS followed by activation of AMPK. Activated AMPK decreased cardiac ischemic damage.

Conclusions: AMPK activation plays a key role in leptin preconditioning-induced cardioprotection. Leptin-induced ROS generation results in AMPK activation.

Keywords: Leptin, Cardioprotection, AMPK, ROS, Ischemia, CAO

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LIST OF ABBREVIATIONS

AICAR : 5-aminoimidazole-4-carboxamide-1-b riboside

AMPK : AMP-activated protein kinase

CaMKKβ : Calmodulin-dependent protein kinase kinase-β

CAO : Coronary artery occlusion

ComC : Compound C

FBS : Fetal bovine serum

Gli : Glibenclamide

H/R : Hypoxia/reoxygenation

H_2DCFDA : Dichlorodihydrofluorescein

i.v. : Intravenous

I/R : Ischemia-reperfusion

IPC : Ischemic preconditioning

LepRb : the longest isoform of leptin receptor,

LKB : Liver kinase B

K_{ATP} : ATP-sensitive potassium

MAPK : Mitogen-activated protein kinase

MPTP : Mitochondrial permeability transition pore

NAC : N-acetyl-L-cysteine

PI3K : Phosphatidylinositol-3 kinase

RISK : Reperfusion injury salvation kinase

ROS : Reactive oxygen species

SD rat : Sprague-Dawley rats

SDS : Sodium dodecyl sulfate

TTC : Triphenyltetrazolium chloride

INTRODUCTION

Leptin, a product of the *ob* gene, is synthesized primarily by white adipose tissue (Zhang *et al*., 1994). Leptin is present in human serum at around 10-40 ng/ml (Considine and Caro, 1996) and the level of circulating leptin is proportional to the total amount of fat in the body. In addition to white adipose tissue (the major source of leptin), it can also be produced in various tissues, such as brown adipose tissue, placenta (syncytiotrophoblasts), ovaries, skeletal muscle, stomach (the lower part of the fundic glands), mammary epithelial cells, bone marrow, pituitary, and liver (Margetic *et al*., 2002). In spite that the primary function of leptin is known to be the regulation of food intake and energy expenditure, other functions mediated by direct actions on pheripheral organs are also recognized. In the pancreatic β-cells, leptin activates the ATP-sensitive potassium (K_{ATP}) channels trafficking to the surface membrane via AMP-activated protein kinase (AMPK) activation (Park *et al*., 2013). In the cardiovascular system, leptin is also produced by the heart, suggesting that it may act locally to mediate physiological effects (Purdham *et al*., 2004). Leptin modulates the immune response to atherosclerosis, of which obesity is a predisposing factor (Taleb *et al*., 2007). Leptin is also involved in the regulation of arterial pressure (Shek *et al*., 1998; Satoh *et al*., 1999; Shirasaka *et al*., 2003). For example, the leptin deficient *ob/ob* mice exhibit reduced arterial pressure as compared to their lean wild type controls despite being massively obese (Mark *et al*., 1999). On the other hand, chronic infusion of leptin or transgenic overexpression of

leptin elevated arterial pressure in spite of body weight reduction (Shek *et al*., 1998; Carlyle *et al*., 2002). Importantly, adrenergic blockade reverses leptin-induced arterial pressure increase highlighting the importance of the sympathetic nervous system in mediating the pressor effect of leptin (Carlyle *et al*., 2002). Effects of leptin on the heart was suggested in several studies strongly suggested in a recent study showing that cardioprotective effects of ischemic postconditioning is abolished in leptin-deficient *ob/ob* mice (Bouhidel *et al*., 2008). However, signaling mechanisms involved in leptin-induced cardiac protection remains unclear.

The metabolic actions of leptin are mediated via leptin receptor modulation (Zabeau *et al*., 2003). The leptin receptor, a product of *db/db* gene, belongs to the class I cytokine receptor family (Tartagila, 1997). Despite the existence of many splice variants of the leptin receptor, the longest isoform of leptin receptor, LepRb, has been shown to have full signaling capacity (Tartagila, 1997). A large number of signaling pathways have been associated with the LepRb. Leptin binding to LepRb leads to the activation of at least three major signaling pathways, such as the JAK/STAT pathways, the phosphatidylinositol-3 kinase (PI3K)-Akt (protein kinase B) and the mitogen-activated protein kinase (MAPK) pathways, and the AMP-activated protein kinase (AMPK) pathways. JAK/STAT pathways and PI3K/Akt/MAPK pathways are involved in the reperfusion injury salvation kinase (RISK) pathway (Hausenloy & Yellon, 2006; 2009), so that their activation by leptin may have potentials for protecting the myocardium against ischemia-reperfusion (I/R) injury. Indeed, there are reports that leptin induced

cardioprotection via JAK/STAT signaling (Smith *et al*., 2010) and PI3K/Akt/MAPK signaling (Smith *et al*., 2006). Involvement of AMPK in leptin effects was first shown in the stimulation of fatty acid oxidation in muscle by inhibiting acetyl coenzyme A carboxylase (Minokoshi *et al*., 2002), but in the heart the contribution of AMPK signaling in leptin effects is controversial. Leptin was shown to activate cardiac fatty acid oxidation, but it was independent of AMPK (Atkinson *et al*., 2002). Leptin exerted anti-apoptotic effects in H9c2 cells via MAPK and AMPK signaling (Shin *et al*., 2009). Considering that AMPK is involved in cardioprotective effect in ischemic preconditioning (Nishino *et al*., 2004; Sukhodub *et al*., 2007), it will be of particular interest to investigate whether leptin activates AMPK signaling in cardiaomyocytes and if so leptin-induced AMPK signaling contributes to leptin-induced cardioprotection.

AMPK is a serine/threonine protein kinase that is sensitive to a broad spectrum of stresses, especially those that cause changes in cellular energy status. AMPK is a heterotrimeric complex composed of catalytic α-subunits. Each subunit exists in multiple isoforms encoded by separate genes (α1, α2, β1, β2, γ1, γ2 and γ3) and their combinations give rise to a variety of AMPK holoenzymes. Phosphorylation of threonine 172 in the catalytic domain of the α-subunit of AMPK, which is required for its activation, is mediated by increase in AMP or by upstream kinase such as liver kinase B (LKB) or calmodulin-dependent protein kinase kinase-β (CaMKKβ) (Estañ *et al*., 2012; Hawley *et al*., 2005; Jaswal *et al*., 2010; Woods *et al*., 2005). In spite that AMPK activation is associated with myocardial ischemia (Calvert *et al*.,

2008; Kim *et al*., 2011; Lotz *et al*., 2011), it is not clear whether its activation has beneficial effects. A role of AMPK signaling in mediation of the effects of leptin has been demonstrated in skeletal muscle and the hypothalamus (Kahn *et al*., 2005), while in cardiomyocytes, the mechanism of leptin-induced AMPK activation is unclear and the effect of AMPK in cardioprotection controversial. Furthermore, the role of AMPK in leptin-induced cardioprotection has not been investigated. Therefore, this study investigated the cardioprotective mechanism of leptin, especially leptin preconditioning, and the relationship between leptin preconditioning-induced cardioprotection and the regulation of AMPK.

MATERIALS AND METHODS

Animals

The experiments were performed using Sprague-Dawley (SD) rats (male, 250-300 g, Orientbio Inc., Seoul, Korea). The animals were kept in a storage room under constant temperature (23±3°C), relative humidity (50±10%) and illumination (12-h light/dark cycles) until the initiation of the experiment. All animals were fed with standard animal chow daily and had access to drinking water *ad libitum*. Animals were randomized into groups. All animal handling procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of our institute (IACUC).

Drugs and reagents

Leptin, 5-aminoimidazole-4-carboxamide-1-b-riboside (AICAR), N-acetyl-L-cysteine (NAC), 2,3,5-triphenyltetrazoliumchloride (TTC), $CoCl_2$, hydrogen peroxide and glibenclamide were purchased from Sigma-Aldrich (St. Louis, USA). Compound C was purchased from Calbiochem (Darmstadt, Germany).

Preparation of the rat ischemic model

Male SD rats were anesthetized with sodium pentobarbital (50 mg/kg intravenous bolus) via the tail vein. After tracheotomy and tracheal cannulation, the animals were artificially ventilated (CWE; Colorado, USA) using positive pressure with air. After a left fourth thoracotomy, the left side of the heart was exposed and suspended in a pericardial cradle. A silk ligature (4-0) was placed halfway between the base and the apex of the heart around a prominent branch of the left anterior descending coronary artery to form a snare. By tightening the snare, a coronary artery occlusion (CAO) was produced and reperfusion was instituted by loosening the snare. CAO was verified by epicardial cyanosis and electrocardiographic changes. Adequate reperfusion was confirmed by epicardial hyperemia and reversion of the electrocardiographic changes. All rats were subjected to 30 min of CAO followed by 24 h of reperfusion. Leptin was administered at 1 h (1 mg/kg *i.v.*) or 24 h (1.5, 2.0 and 2.5 mg/kg *i.v.*) prior to CAO. AICAR (5 mg/kg *i.v.*), NAC (150 mg/kg *i.v.*), glibenclamide (0.1 mg/kg *i.v.*) or compound C (3.39 mg/kg *i.v.*) in combination with leptin was administered at 1 or 24 h prior to CAO (Fig. 1A). The rats then were euthanized with a lethal dose of pentobarbital injected into the tail vein and the heart was rapidly excised. The heart was cut into six slices from apex to base. Samples from ischemic and non-ischemic regions were incubated at 37°C for 5 min in 2% TTC in 0.2 mol/L of phosphate buffer adjusted to a pH of 7.4. Infarcted (pale) and non-infarcted (brick red) myocardium were distinguished. Infarct size was expressed as a percentage of the total heart using the Tina 2.0 software. Data are means ± standard error of the mean.

Cell culture

Rat cardiac myoblast cell line H9c2 was obtained from the Korean Cell Line Bank (Seoul, Korea). H9c2 cells were cultured in DMEM (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin: Pen-Strep, Gibco). The cells were incubated at 37°C in a 95% air-5% CO_2 gas mixture. The medium was replaced every 2 days.

Hypoxia/reoxygenation (H/R) and drug administration

For MTT assay, H9c2 cells were maintained in normal DMEM and replaced with serum-free DMEM 24 h prior to hypoxia and then pre-incubated in a hypoxic chamber containing 99% N_2 and 1% O_2 (monitored by an oxygen probe) for 12 h to imitate the *in vivo* ischemic state. After 12 h hypoxia, cells were rapidly transferred into a normoxic incubator with serum-free DMEM for reoxygenation (1 h). Cells were treated with leptin or without leptin 24 h prior to H/R (Fig. 1B).

(A)

(B)

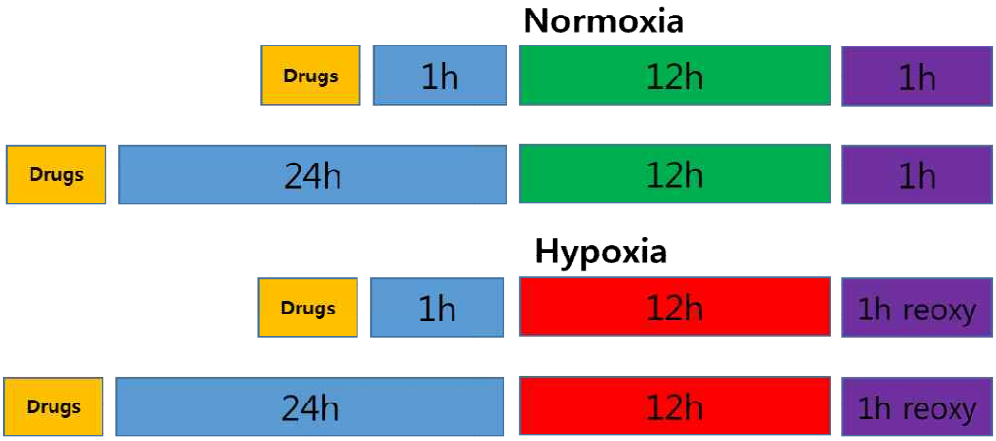


Figure 1. Study protocol *in vivo* (A) and *in vitro* (B).

Cell viability assay

After H/R treatment, MTT was added and the cells were incubated for 4 h at 37°C and solubilized with 150-μl DMSO. The absorbance at 492 nm was read using a multi-well scanning spectrophotometer reader. Cells in the control group were considered 100% viable. A live/dead assay was also performed to evaluate cell viability. The cells were incubated with the live/dead assay dye solution (abcam) containing 1 μl of live dye and 1 μl of propidium iodide in 1 ml of assay buffer. After 30-min incubation, cell were rinsed with PBS and cells were imaged by Fv10i confocal microscopy (Olympus, Tokyo, Japan). Cell viability was calculated as the ratio of green fluorescence (live cell) surface area to the combined surface area of green fluorescence (live cells) and red fluorescence (dead cells).

Measurement of intracellular reactive oxygen species (ROS) levels

Intracellular ROS generation was measured using dichlorodihydrofluorescein (H_2DCFDA, Calbiochem, San Diego, CA). H9c2 cells were plated in a 24-well plate and incubated with 5 μM H_2DCFDA at 37°C for 20 min in the dark. After incubation, the cells were washed three times with PBS and resuspended in PBS. The fluorescence signals of cells were immediately visualized using a LSM 510 Meta Confocal Microscope (ZEISS, Oberkochen, Germany). For the detection of green fluorescence (H_2DCFDA), cells were illuminated with 488- and 518-nm laser lines. Fluorescence images were analyzed using the LSM 510 Meta Confocal

Microscope (ZEISS, Oberkochen, Germany).

Western blot analysis

H9c2 cells (1×10^6/ 60-mm dish) were treated with the desired concentrations of leptin for the indicated time periods. The cells were vortexed in a protein extraction solution (PRO-PREP™, iNtRON Biotechnology Inc., Seongnam, Korea) containing 50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM dithiothreitol, 0.5% nonyl phenoxylpolyethoxylethanol, 1% Triton X-100, 1% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM EDTA and 1× protease inhibitor cocktail (Roshe Diagnostics, Indianapolis IN). The cell lysates were incubated for 30 min on ice with intermittent vortexing and were clarified by centrifugation at 13,000 rpm (Hanil, Incheon, Korea) at 4°C for 15 min. After centrifugation, the supernatant was separated and stored at -70°C until use. The protein concentrations in cell lysates were determined using the Bradford protein assay kit (Bio-Rad, CA). Equal amounts of proteins were mixed with 5× sample buffer, separated in an 8 or 10% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF, Millipore, Billerica, MA) membrane for 15 min (Bio-Rad, CA). Membranes were blocked with 5% (w/v) fat-free dry milk in TBST (20 mM Tris HCl (pH 8.0), 137 mM NaCl and 0.2% Tween-20) at RT for 60 min and then incubated with the desired primary antibodies; p-AMPK, AMPK or actin, at 4°C overnight. The primary antibody incubation was followed by incubation with a secondary horseradish peroxidase (HRP)-conjugated anti-rabbit antibody at 1:1,000 to

1:10,000. Immuno-positive bands were visualized by enhanced chemiluminescence (SuperSignal™ West Pico Chemiluminescent Substrate, Thermo Scientific) following the manufacturer's instructions. Relative protein levels were calculated using actin as the loading control.

Statistical analysis

All data are presented as means ± SE. All statistical analyses were performed by ANOVA. A value of $p < 0.05$ was taken to indicate statistical significance.

RESULTS

Leptin decreases ischemic myocardial infarct size in vivo

To investigate its effect on myocardial infarct size, leptin was administered 1 h (1.0 mg/kg *i.v.*) before CAO. The infarct was smaller in the leptin-treated group (16.0±2.0%, *n*=6) than in the ischemic controls (31.1±3.2%, *n*=4) (Fig. 2). Therefore, leptin had a cardioprotective effect when administered 1 h before CAO.

Leptin decreases ischemic myocardial infarct size via AMPK activation

To investigate the relationship between leptin-induced cardioprotection and leptin-induced AMPK activation in terms of myocardial infarct size, leptin was administered in combination with compound C, an AMPK inhibitor (3.39 mg/kg *i.v.*), 1 h before CAO. The infarct size was 31.0±4.7% (*n*=5), which was similar to that of the control group (31.1±3.2%, *n*=4) (Fig. 2). Therefore, compound C abolished the leptin-induced cardioprotective effect and decreased myocardial infarct size via AMPK activation.

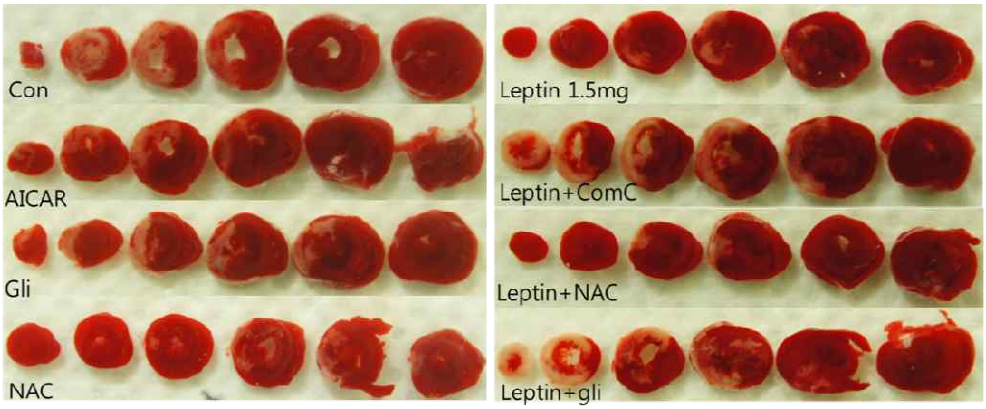
Leptin decreases ischemic myocardial infarct size via ROS induction

To investigate the relationship between leptin-induced cardioprotection and leptin-induced ROS induction in terms of myocardial infarct size, leptin was administered in combination with NAC, a ROS scavenger (150 mg/kg *i.v.*), 1 h before CAO. The infarct size was 14.1±2.0% (*n*=5) (Fig. 2). When NAC was administered alone 1 h before CAO, the infarct size was 10.2±2.1% (*n*=5), which was similar to that with NAC and leptin combined (Fig. 2). Therefore, NAC itself has a powerful cardioprotective effect independent of leptin.

Leptin decreases ischemic myocardial infarct size via the KATP channel

To investigate the relationship between leptin-induced cardioprotection and the K_{ATP} channel in terms of myocardial infarct size, leptin was administered in combination with glibenclamide, a K_{ATP} channel inhibitor (0.1 mg/kg *i.v.*), 1 h before CAO. The infarct size was 32.6±3.4% (*n*=4), which was similar to that of the control group (31.1±3.2%, *n*=4) (Fig. 2). Therefore, glibenclamide abolished the leptin-induced cardioprotective effect and decreased the myocardial infarct size via the K_{ATP} channel.

(A)


Con
AICAR
Gli
NAC
Leptin 1.5mg
Leptin+ComC
Leptin+NAC
Leptin+gli

(B)

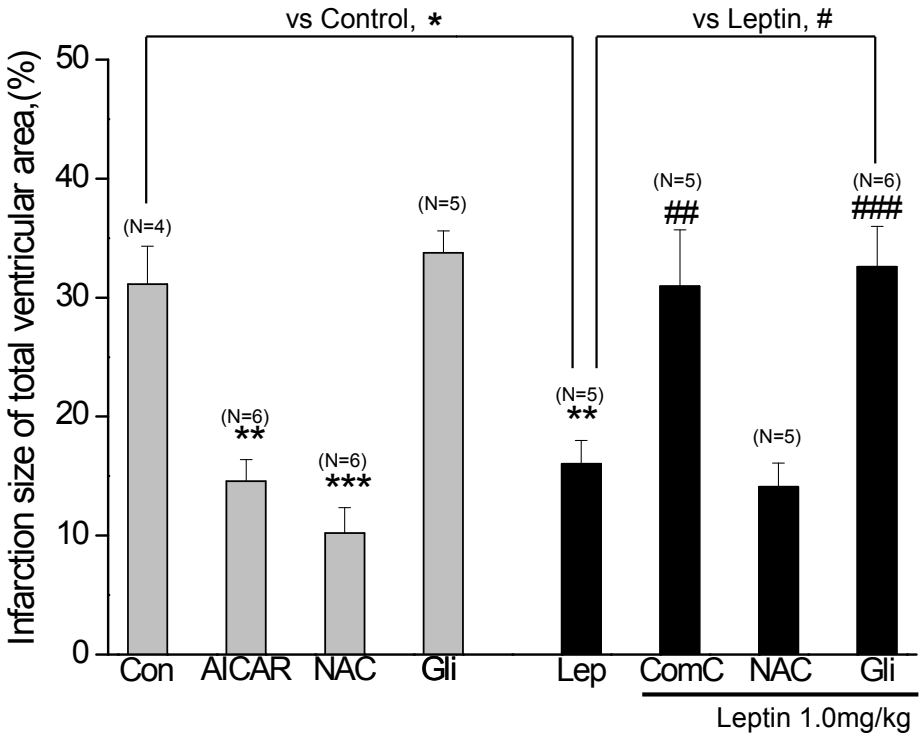

vs Control, *
vs Leptin, #
Infarction size of total ventricular area,(%)
50
40
30
20
10
0
(N=4)
(N=6)
**
(N=6)
***
(N=5)
(N=5)
**
(N=5)
##
(N=5)
(N=6)
###
Con
AICAR
NAC
Gli
Lep
ComC
NAC
Gli
Leptin 1.0mg/kg

Figure 2. Effect of leptin on ischemic myocardial infarcts *in vivo*.

(**A**) Representative heart sections from the control, AlCAR, NAC, glibenclamide, leptin, leptin+compound C, leptin+NAC and leptin+glibenclamide groups when the compounds were administered *i.v.* 1 h prior to CAO into SD rats. (**B**) Quantitative analysis of infarct size (infarct area/total ventricular area) in the control, AlCAR, NAC, glibenclamide, leptin, leptin+compound C, leptin+NAC and leptin+glibenclamide groups when the compounds were administered *i.v.* 1 h prior to CAO into SD rats. ** P <0.01, *** P <0.001 versus control; ## P <0.01, ### P <0.001 versus leptin.

Leptin preconditioning decreases ischemic myocardial infarct size dose-dependently in vivo

To investigate the effect of leptin-preconditioning on myocardial infarct size, leptin was administered 24 h (1.5, 2.0, and 2.5 mg/kg *i.v.*) before CAO. The leptin-treated infarct was decreased to 24.0±2.1% (*n*=6), 17.6±1.2% (*n*=5), and 14.6±2.5% (*n*=5), respectively, compared with the controls (32.7±1.6%, *n*=4) (Fig. 3). Therefore, leptin had a cardioprotective effect 24 h before CAO.

Leptin preconditioning decreases ischemic myocardial infarct size via AMPK activation

To investigate the relationship between leptin-induced cardioprotection and AMPK activation in terms of myocardial infarct size, leptin was administered in combination with compound C, an AMPK inhibitor (3.39 mg/kg *i.v.*), 24 h before CAO. The control infarct size was 33.8±2.4% (*n*=5) (Fig. 3). When leptin (2.5 mg/kg) was administered in combination with compound C, the infarct size was the same as when administered 1 h before CAO (31.0±4.7%, *n*=5) (Fig. 2). In addition, different groups of animals were given an AMPK activator, 5-aminoimidazole-4-carboxamide-1-b-riboside (AICAR), to investigate the effect of AMPK activation. AICAR alone was administered 24 h (5 mg/kg *i.v.*) before CAO. AICAR decreased the infarct size (14.6±2.3%, n=6), similar to the results with leptin (Fig. 3). Therefore, leptin decreases myocardial infarct size via AMPK activation.

Leptin preconditioning decreases ischemic myocardial infarct size via ROS induction

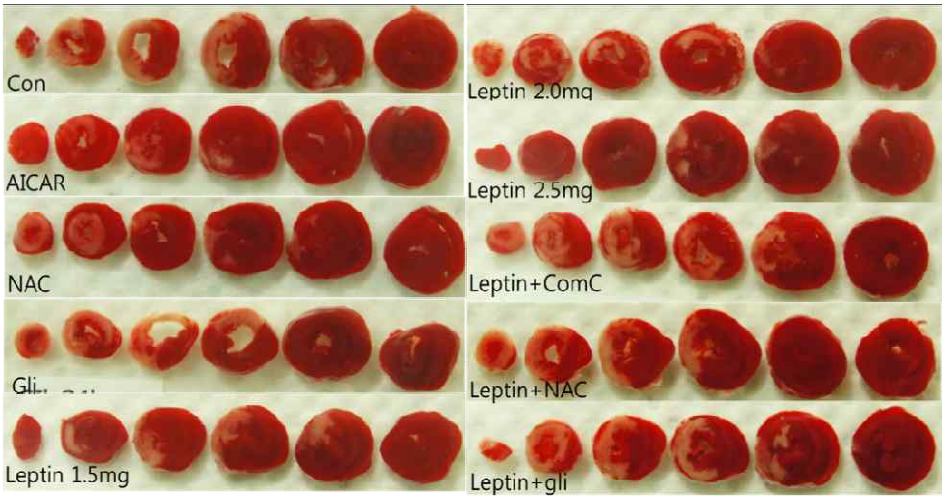
To investigate the relationship between leptin-induced cardioprotection and leptin-induced ROS induction in terms of myocardial infarct size, leptin was administered in combination with NAC, the ROS scavenger (150 mg/kg *i.v.*), 24 hours before CAO. In this case, NAC abolished the leptin-induced cardioprotection (28.6±5.3%, *n*=6), but less so than when given 1 hour before CAO (14.1±2.0%, *n*=5). When administered 24 hours before CAO, NAC alone had no effect on the infarct size (25.8±1.9%, *n*=6) (Fig. 3). Based on the results 1 hour before CAO, NAC itself has a powerful cardioprotective effect. However, the effect of NAC-induced cardioprotection disappeared 24 hours before CAO. Nevertheless, NAC abolished the leptin preconditioning-induced cardioprotective effect. Therefore, leptin-preconditioning decreases myocardial infarct size via ROS induction.

Leptin preconditioning decreases ischemic myocardial infarct size via the KATP channel

To investigate the relationship between leptin-induced cardioprotection and the K_{ATP} channel in terms of myocardial infarct size, leptin was administered in combination with glibenclamide, a K_{ATP} channel inhibitor (0.1 mg/kg *i.v.*), 24 h before CAO. When leptin (2.5 mg/kg) was administered in combination with glibenclamide, the infarct size was similar than that when administered 1 h before CAO (34.5±3.1%,

n=5) (Fig. 3). In addition, different animals were given glibenclamide alone to investigate the effect of K_{ATP} channel blockage. Glibenclamide was administered 24 h before CAO. Glibenclamide alone decreased the infarct size (26.7±1.7%, n=6). Therefore, leptin-preconditioning decreases infarct size via effects on the K_{ATP} channel.

(A)


Con
AICAR
NAC
Gli
Leptin 1.5mg
Leptin 2.0mg
Leptin 2.5mg
Leptin+ComC
Leptin+NAC
Leptin+gli

(B)

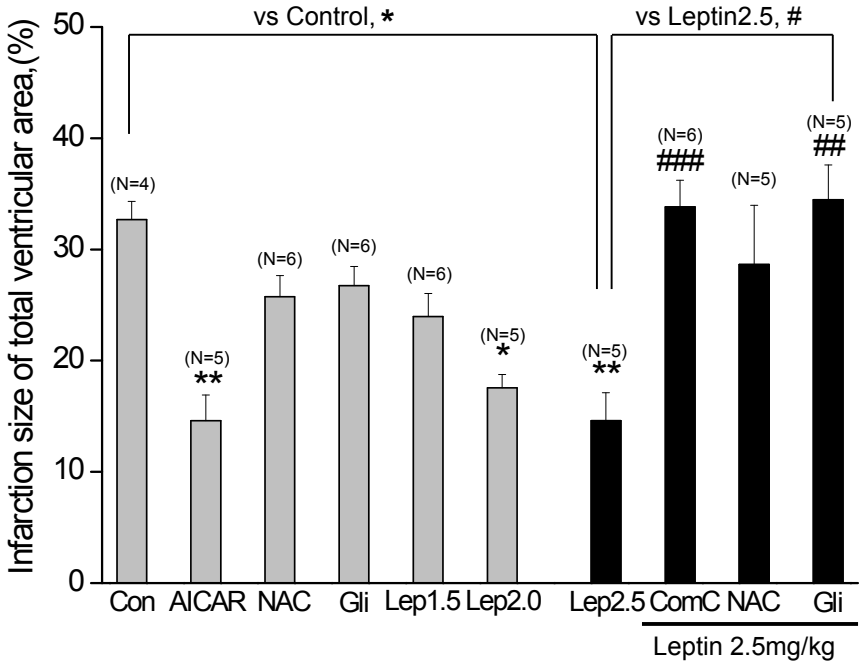


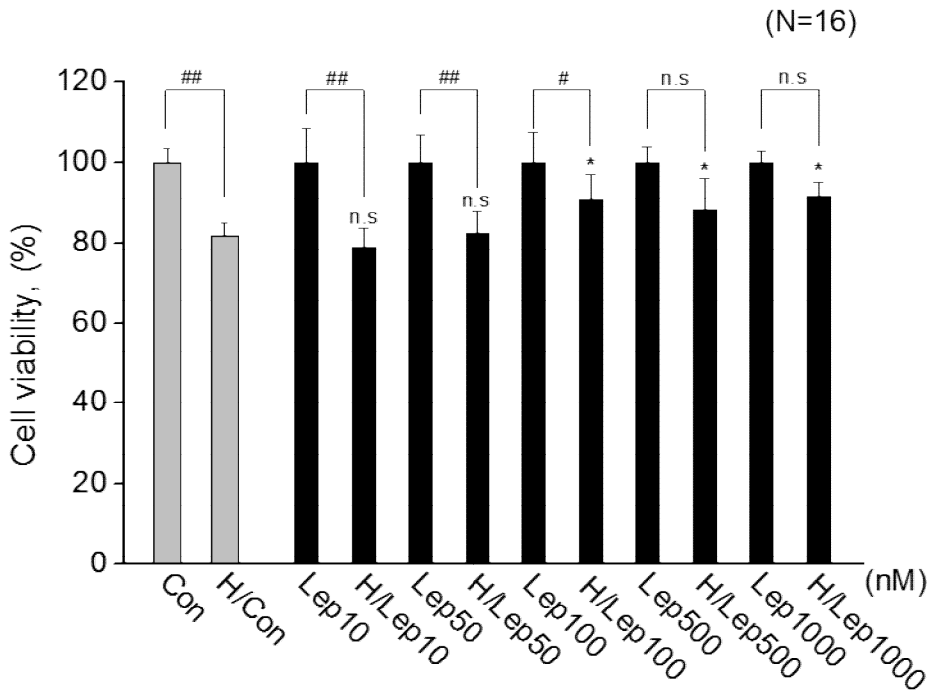
Figure 3. Effect of leptin-preconditioning on ischemic myocardial infarcts *in vivo*.

(**A**) Representative heart sections from the control, AlCAR, NAC, glibenclamide, leptin (1.5 mg/kg), leptin (2.0 mg/kg), leptin (2.5 mg/kg), leptin+compound C, leptin+NAC and leptin+glibenclamide groups when the compounds were administered *i.v.* 24 h prior to CAO into SD rats. (**B**) Quantitative analysis of infarct size (infarct area/total ventricular area) in the control, AlCAR, NAC, glibenclamide, leptin (1.5 mg/kg), leptin (2.0 mg/kg), leptin (2.5 mg/kg), leptin+compound C, leptin+NAC and leptin+glibenclamide groups when the compounds were administered *i.v.* 1 h prior to CAO into SD rats. * P <0.05, ** P <0.01 versus control; ## P <0.01, ### P <0.001 versus leptin.

Leptin-preconditioning rescues the hypoxia/reperfusion-induced cell death in H9c2 cells

To assess whether leptin-preconditioning increases cell viability in the presence of hypoxia/reperfusion (HR), H9c2 cells were treated with 0, 10, 50, 100, 500, or 1000 nM leptin 24 h prior to hypoxia. In the untreated controls, the H9c2 cell viability decreased significantly in the presence of HR. In the groups treated with low concentrations of leptin (10 and 50 nM), the viability of H9c2 cells was decreased similar to that of the control group in the presence of HR. However, in the groups treated with high concentrations of leptin (500 and 1000 nM), even though the viability of H9c2 cells decreased slightly in the presence of HR, this change was not significant. Therefore, in the groups treated with leptin, cell viability increased dose-dependently compared with the untreated controls (Fig. 4A). To confirm that leptin-preconditioning increases cell viability in the presence of HR, a live/dead assay was performed. H9c2 cells were treated with 0 and 500 nM leptin 24 h prior to hypoxia. In the presence of HR, leptin-preconditioning increased cell viability. When treated with $CoCl_2$, a hypoxia-mimicking agent, the effect of leptin preconditioning was the same as that of leptin in the presence of HR. Therefore, leptin preconditioning has a cardioprotective effect in the rat ischemic heart *in vivo* and in H9c2 cells *in vitro* (Fig. 4B).

(A)


(N=16)
Cell viability, (%)
120
100
80
60
40
20
0
##
##
##
#
n.s
n.s
n.s
n.s
n.s
*
*
*
(nM)
Con
H/Con
Lep10
H/Lep10
Lep50
H/Lep50
Lep100
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H/Lep500
Lep1000
H/Lep1000

(B)

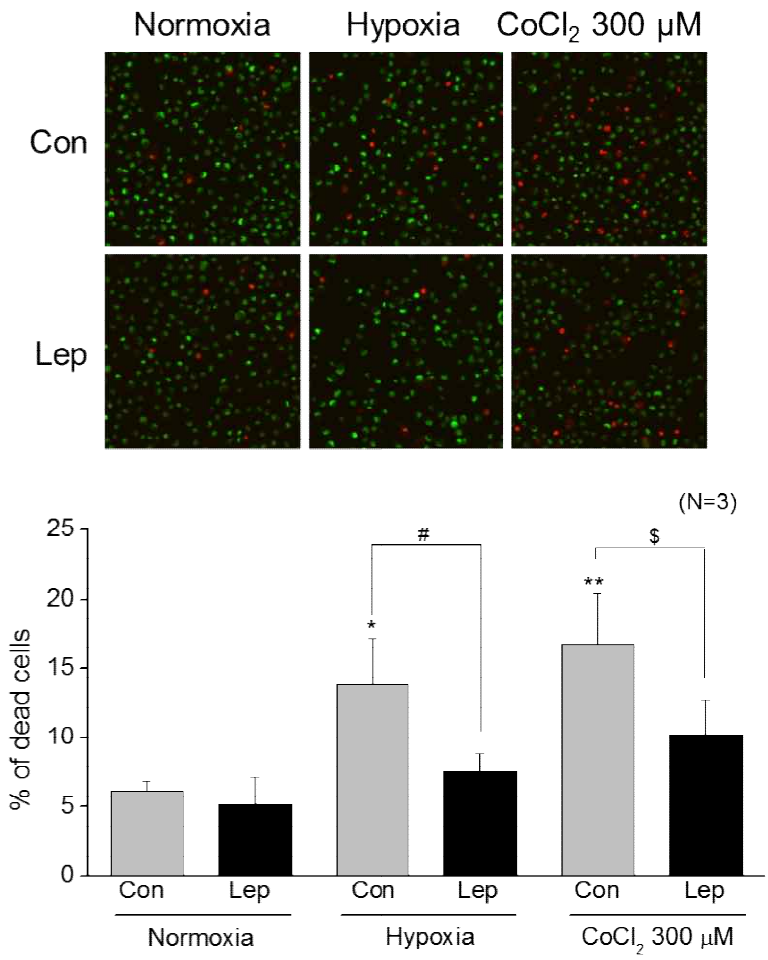


Figure 4. Effect of leptin-preconditioning in the presence of hypoxia/reperfusion in H9c2 cells.

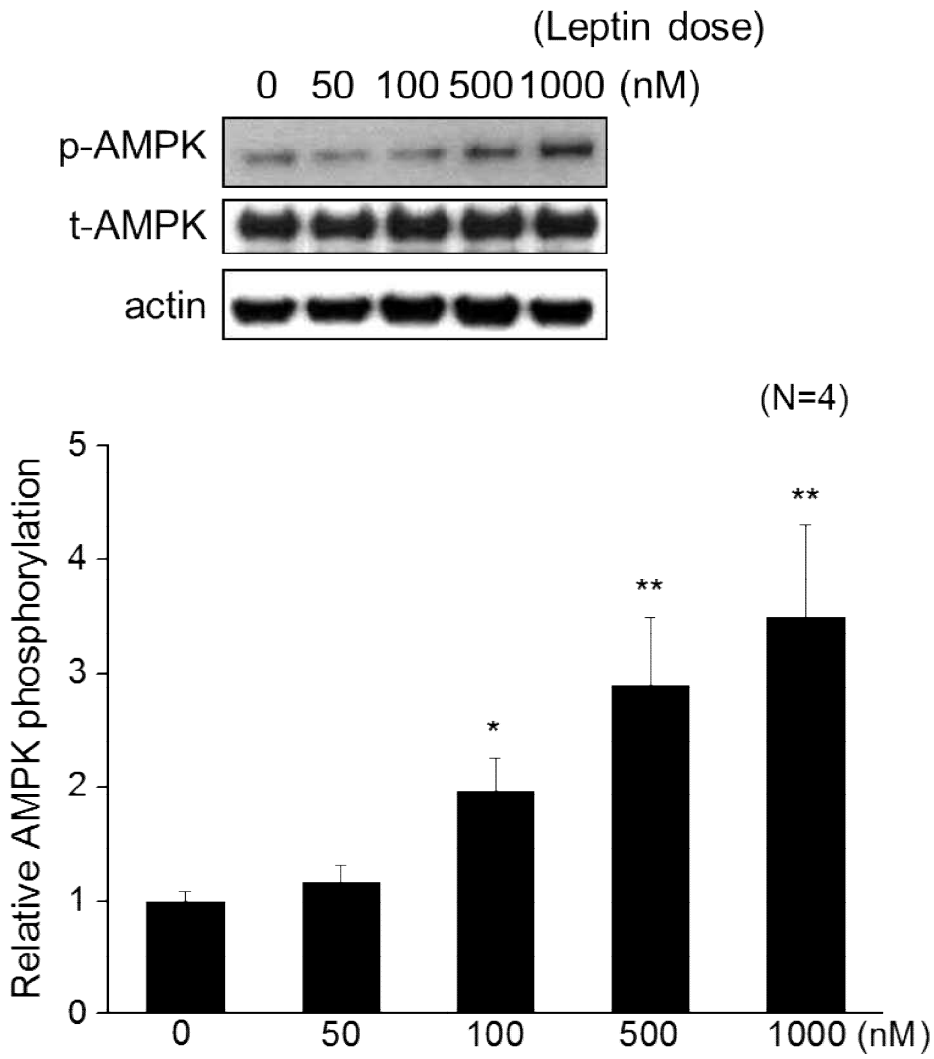
(**A**) Cell viability was measured by MTT assay. Hypoxia for 12 h and reoxygenation for 1 h. * P <0.05 versus control under hypoxic state, # P <0.05, ## P <0.01 under normoxic state versus under hypoxic state. 'n.s' means 'not

significant'. 'H' means group in the presence of hypoxia/reoxygenation. (B) Cell viability was measured by live/dead assay. Hypoxia for 12 h and reoxygenation for 1 h. * P <0.05, ** P <0.01 versus control under normoxic state, # P <0.05 versus control under hypoxia, $ P <0.05 versus control under $CoCl_2$ application 'H' means group in the presence of hypoxia/reoxygenation.

Leptin induces AMPK phosphorylation in H9c2 cells in a dose- and time-dependent manner

To assess whether leptin treatment increases AMPK activity dose-dependently, H9c2 cells were treated with 0, 50, 100, 500, or 1000 nM leptin. Considerable AMPK phosphorylation was observed after 2 h of treatment, as indicated by immunoblotting of cell lysates with an AMPK Thr-172 phospho-specific antibody (Fig. 5A). Treatment with leptin increased AMPK phosphorylation in a dose-dependent manner. To determine whether treatment with leptin increases AMPK activity time-dependently, H9c2 cells were treated with 500 nM leptin for 0, 0.5, 1, 2, 3, 5, 6, 12, or 24 h. Treatment with 500 nM leptin increased the AMPK phosphorylation until 6 h in a time-dependent manner, but the level of phosphorylation decreased after 6 h (Fig. 5B).

(A)


(Leptin dose)
0 50 100 500 1000 (nM)
p-AMPK
t-AMPK
actin
(N=4)
Relative AMPK phosphorylation
0
1
2
3
4
5
*
**
**
0
50
100
500
1000 (nM)

(B)

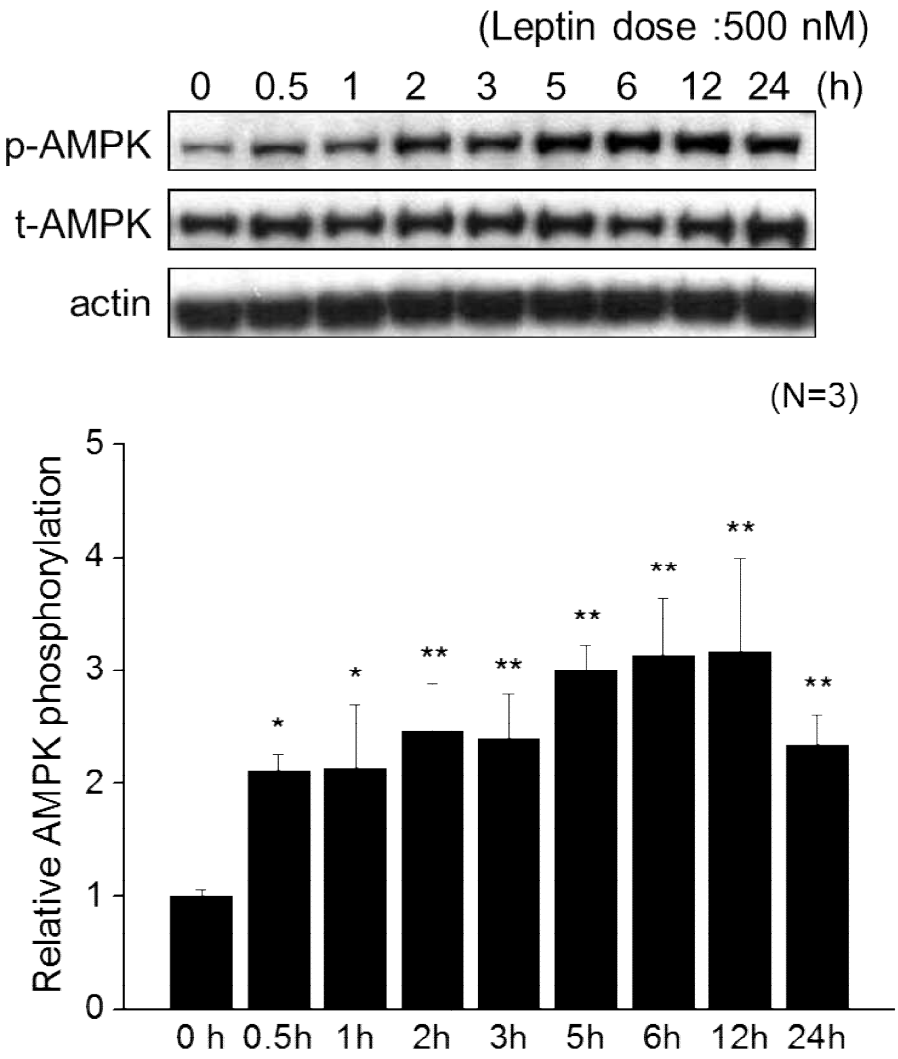


Figure 5. Leptin induced AMPK activation in C2H9 cells in a both dose-(A) and time-(B) dependent manner.

Western blot analysis of phosphorylated and total AMPK. * $P < 0.05$, **$P < 0.01$ versus 0 nM leptin group (A), * $P < 0.05$, **$P < 0.01$ versus group before

administration of leptin (B).

Leptin induces AMPK phosphorylation under both normoxia and hypoxia in H9c2 cells

Treatment with 500 nM leptin increased AMPK activation under both normoxia and hypoxia, similar to treatment with AICAR, a chemical activator of AMPK. Leptin-induced AMPK activation was decreased by the administration of compound C, a chemical inhibitor of AMPK, under both normoxia and hypoxia (Fig. 6).

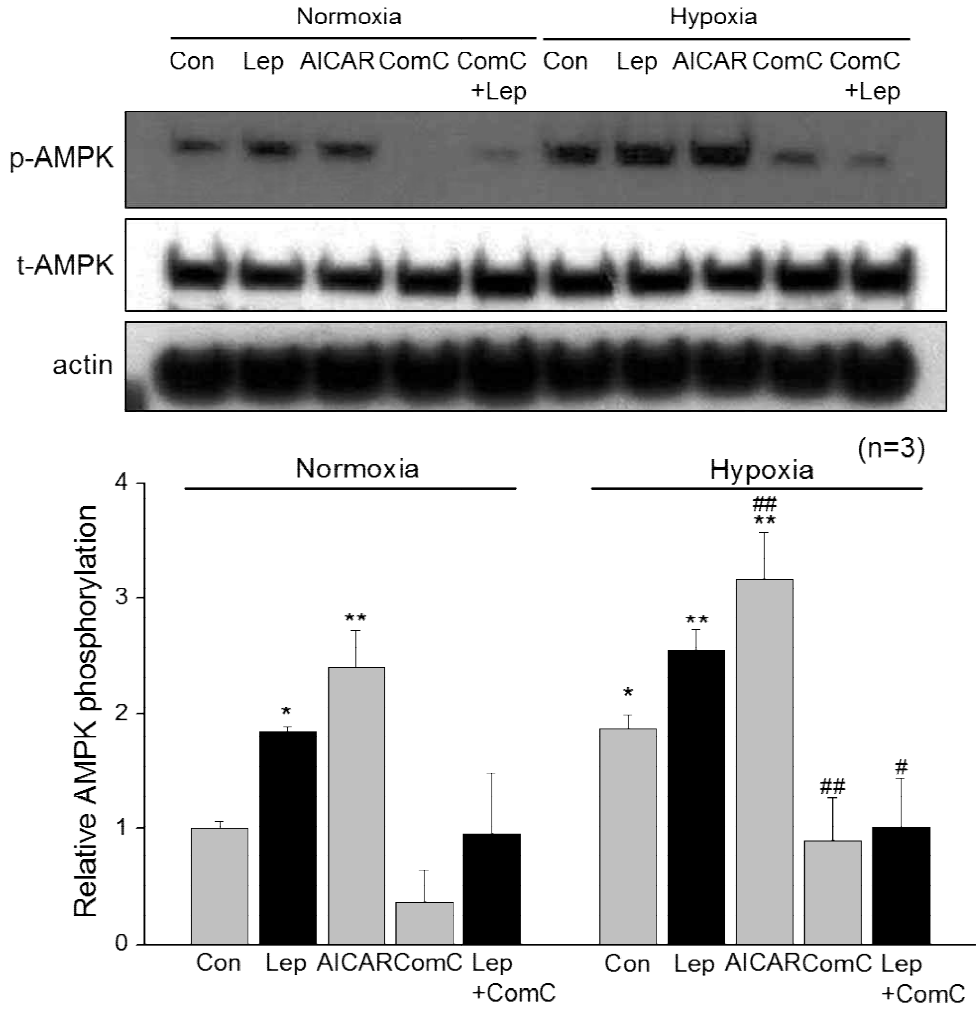


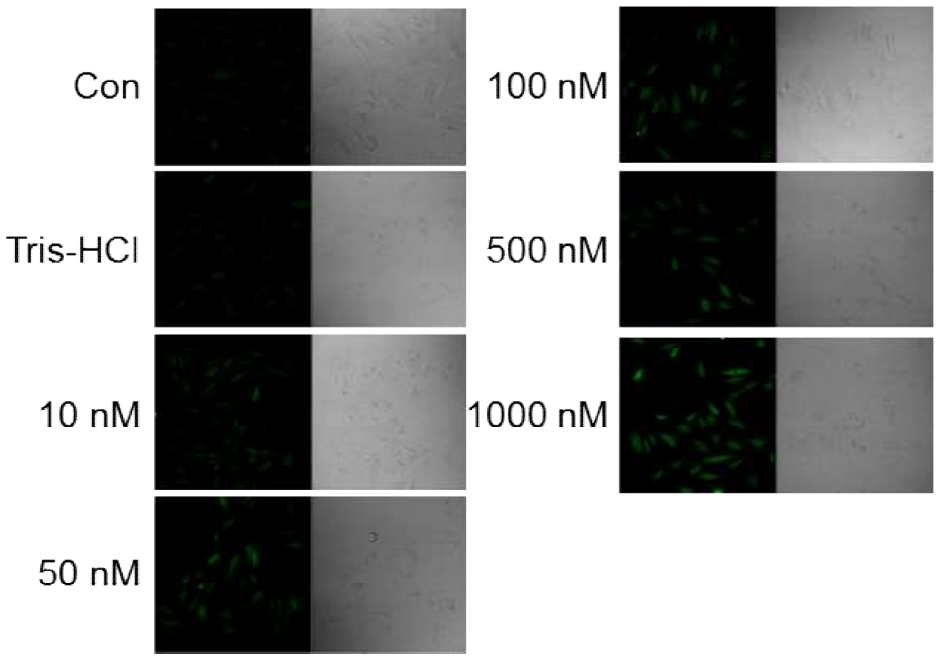
Figure 6. Leptin-induced AMPK activation is blocked by compound C under normoxia and hypoxia.

Western blotting analysis of phosphorylated and total AMPK. * P<0.05, **P<0.01 versus control group under normoxia, #P<0.05, ##P<0.01 versus control group under hypoxia.

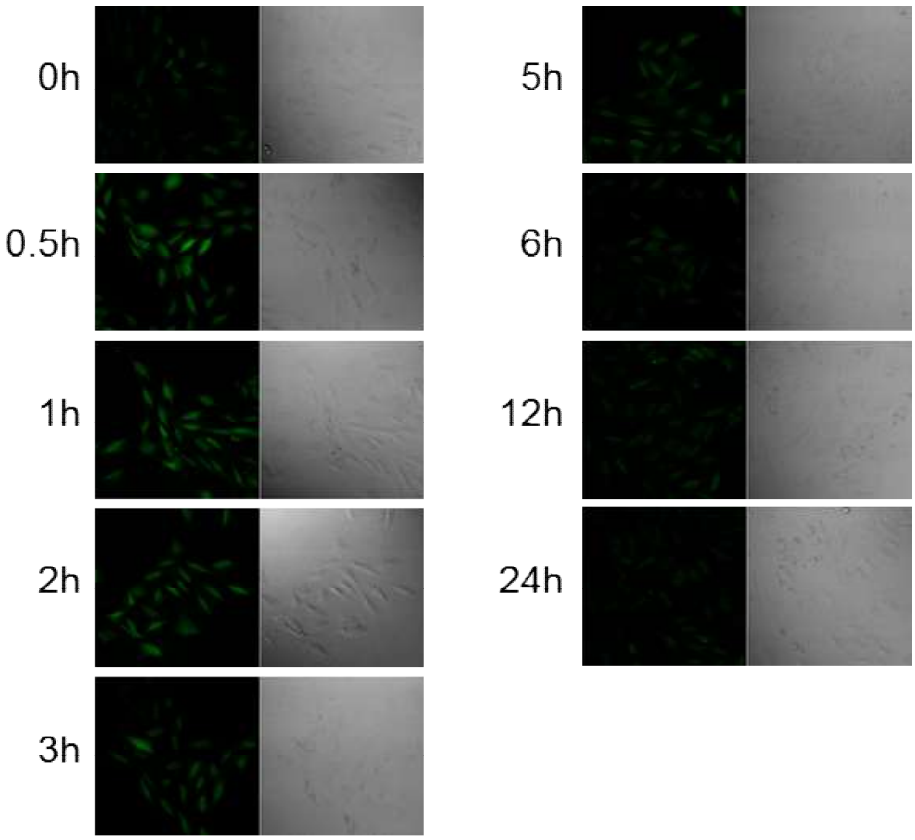
Leptin induces ROS generation in H9c2 cells in a dose- and time- dependent manner

To determine whether leptin treatment increases ROS generation in a dose-dependent manner, H9c2 cells were treated with 0, 1, 10, 30, 100, 300, or 1000 nM leptin. Leptin treatment increased ROS generation in a dose-dependent manner, as indicated by the relative dichlorofluorescein diacetate intensity (Fig. 7A,C). To determine whether treatment with leptin increases ROS generation time-dependently, H9c2 cells were treated with 500 nM leptin for 0, 0.5, 1, 2, 3, 5, 6, 12, or 24 h. Treatment with 500 nM leptin increased ROS generation for 2 h, but decreased it thereafter (Fig. 7B,D). None of the leptin concentrations tested affected cell viability (Fig. 7C). These findings confirm that leptin induces ROS.

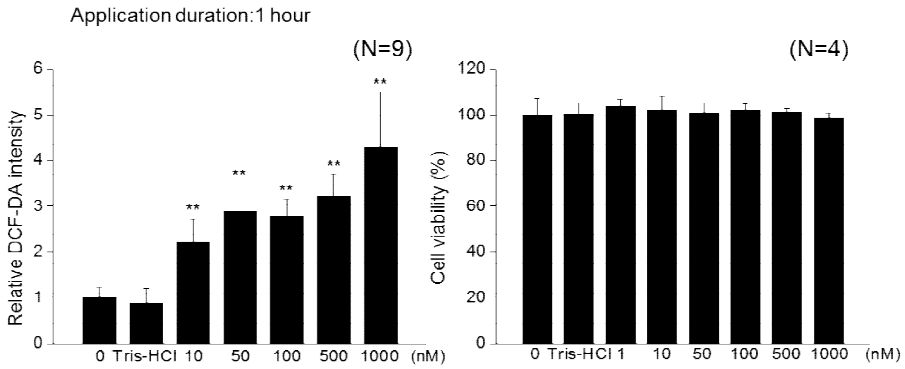
(A)



(B)



(C)



(D)

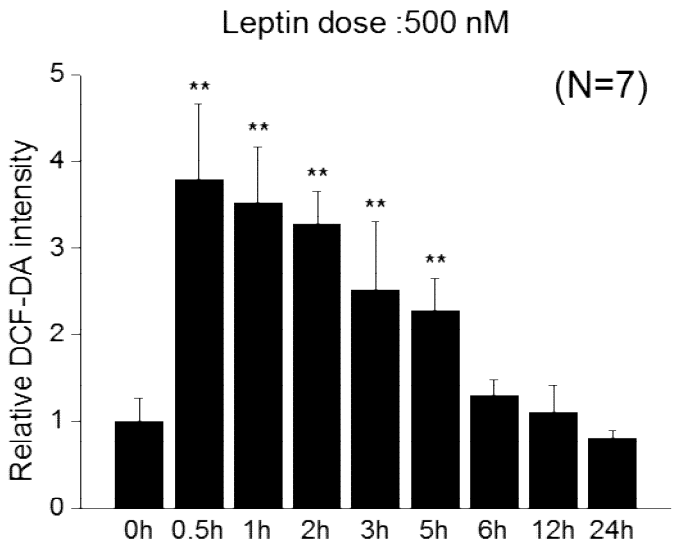


Figure 7. Leptin induces ROS generation in H9c2 cells in a both dose-(A) and time-(B) dependent manner.

(A) Fluorescence microscopy images showing the DCF-DA signal intensity in H9c2 cells. Leptin significantly increased ROS generation in a dose-dependent manner.

(B) Fluorescence microscopy images showing the DCF-DA signal intensity in H9c2 cells. Leptin also significantly increased ROS generation in a time-dependent manner. (C) Relative DCF-DA intensities were measured dose-dependently. Cell viability after treatment with 0 to 1000 nM leptin was evaluated by MTT assay (lower). **P < 0.01 versus control. (D) Relative DCF-DA intensities were measured time-dependently. **P < 0.01 versus control.

AMPK activation by leptin-preconditioning occurs downstream of ROS generation

To investigate the relationship between cardioprotection by AMPK activation and leptin-induced ROS induction, AICAR, an AMPK activator was administered alone or in combination with NAC, a ROS scavenger (150 mg/kg *i.v.*), 24 h prior to CAO. AICAR alone decreased the ischemic myocardial infarct size (18.7±3.4%, n=6), and AICAR in combination with NAC decreased the ischemic myocardial infarct size to the same degree as AICAR alone (Fig. 8). NAC had no effect on AICAR-induced cardioprotection.

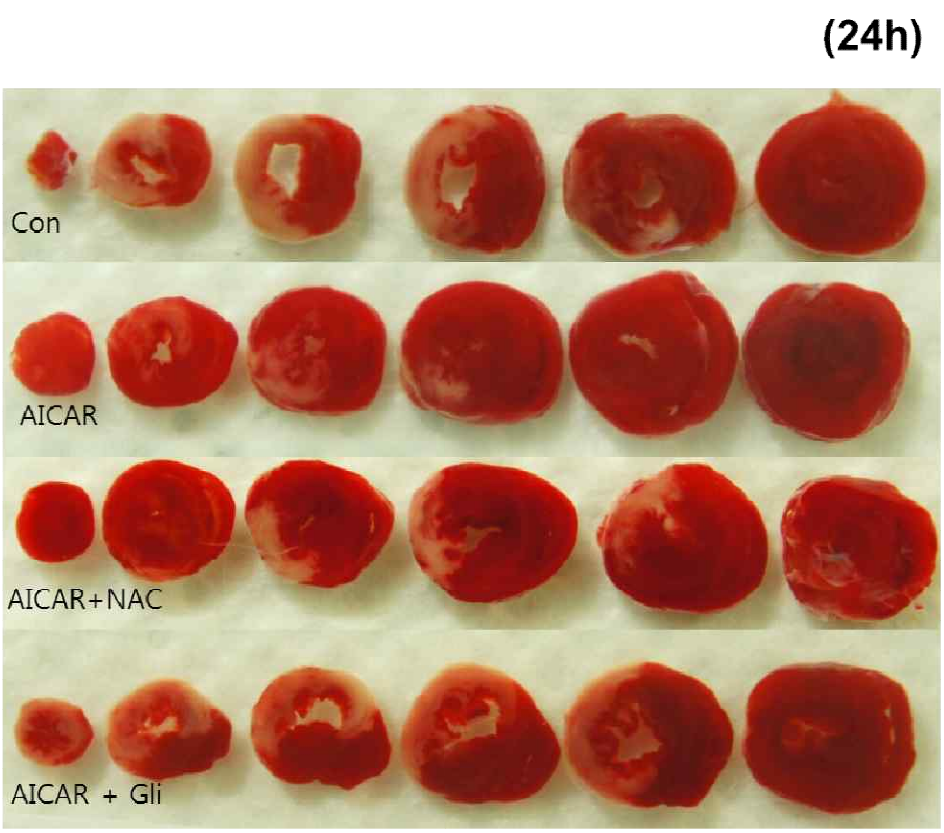
In H9c2 cells, leptin-induced ROS generation was not affected by administration of leptin in combination with compound C. AICAR alone did not induce ROS generation, but leptin-induced ROS generation was decreased by administration of NAC, a ROS scavenger (Fig. 9A,B). But, in H9c2 cells, leptin-induced AMPK phosphorylation was affected by administration of leptin in combination with NAC, ROS scavenger. Leptin-preconditioning alone phosphorylate AMPK. But, leptin-induced AMPK phosphorylation was decreased by administration of NAC, a ROS scavenger (Fig. 9C,D). NAC alone had no effect on AMPK phosphorylation (Fig. 9C,D).

These findings confirm that leptin-induced ROS generation is not downstream of AMPK activation, but that AMPK activation by leptin-preconditioning occurs downstream of ROS generation.

Cardioprotection mediated by leptin-induced AMPK activation is dependent on the K_{ATP} channel

To investigate the relationship between cardioprotection by AMPK activation and the K_{ATP} channel, AICAR, an AMPK activator, was administered alone or in combination with glibenclamide, a K_{ATP} channel blocker (150 mg/kg *i.v.*), 24 h before CAO. Administration of AICAR alone decreased the ischemic myocardial infarct size (14.6±2.3%, *n*=6); this effect was enhanced by administration of AICAR in combination with glibenclamide (Fig. 8). Glibenclamide decreased the AICAR-induced cardioprotection (32.7±3.8%, *n*=5). These findings confirmed that cardioprotection mediated by AMPK activation was dependent on the K_{ATP} channel channel, and therefore AMPK activation induced by leptin-preconditioning may occur upstream of the K_{ATP} channel.

(A)



(B)

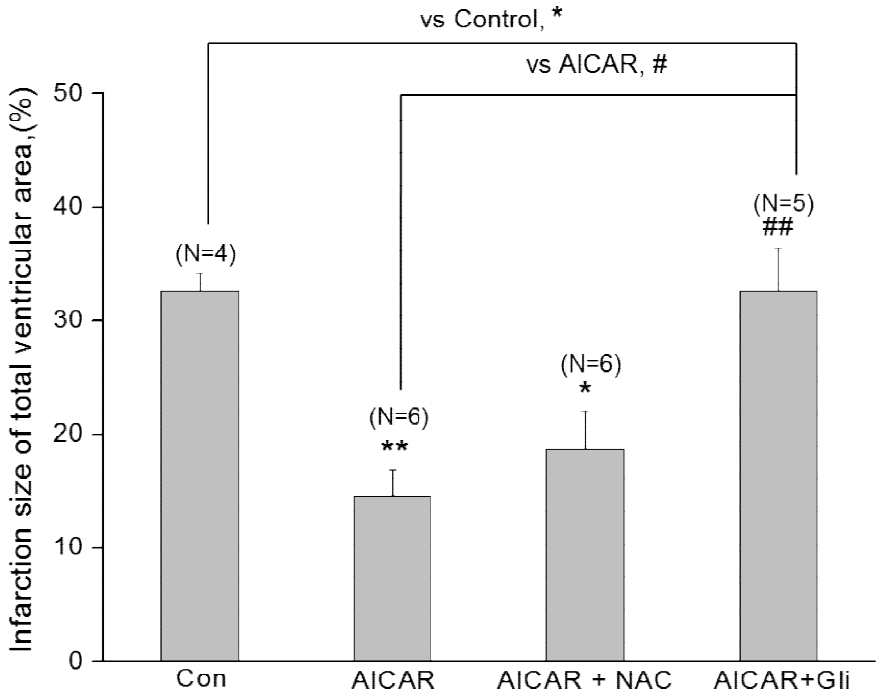
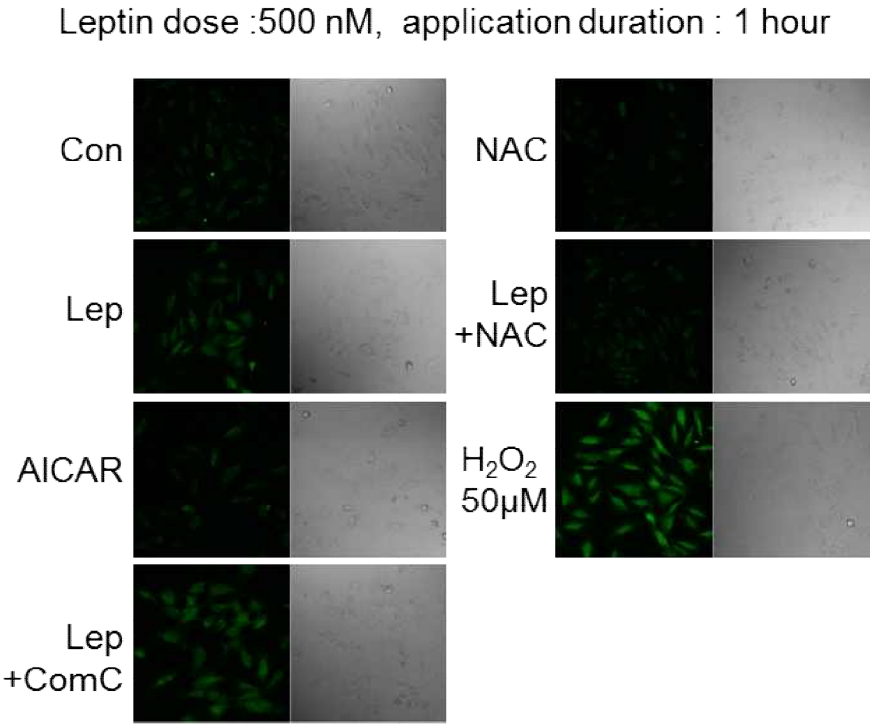


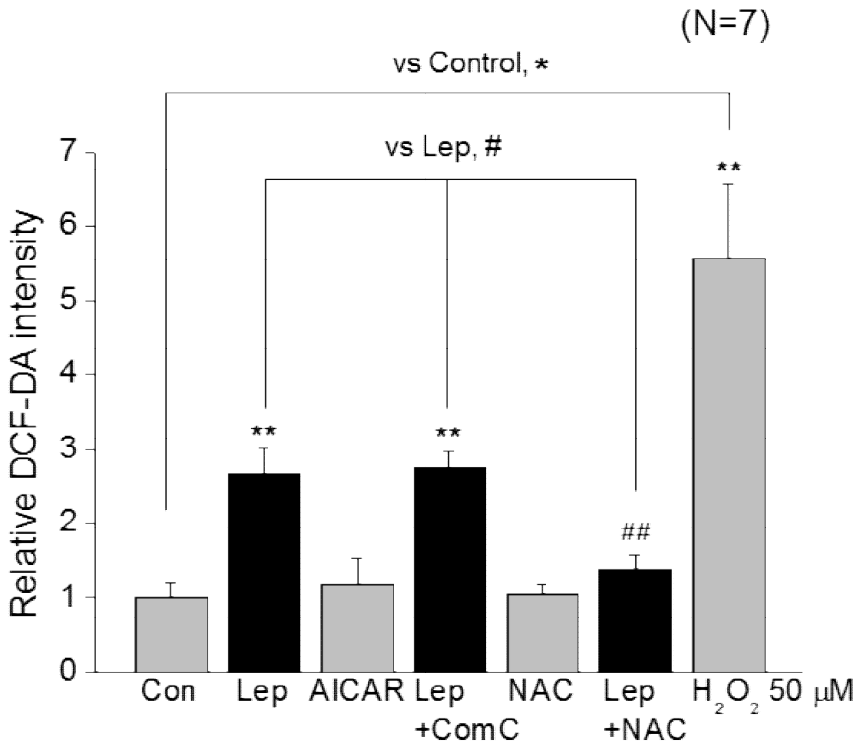
Figure 8. AMPK activation by leptin-preconditioning occurs downstream of ROS generation.

(**A**) Representative heart sections from the control, AlCAR, AICAR+NAC and AICAR+glibenclamide groups when compounds were administered *i.v.* 24 h prior to CAO into SD rats. (**B**) Quantitative analysis of infarct size (infarct area/total ventricular area) in the control, AlCAR, AICAR+NAC and AICAR+glibenclamide groups. * P <0.05, ** P <0.01 versus control, ## P <0.01 versus AICAR . **(Data of both control and AICAR were used as a duplicate with figure 3)**

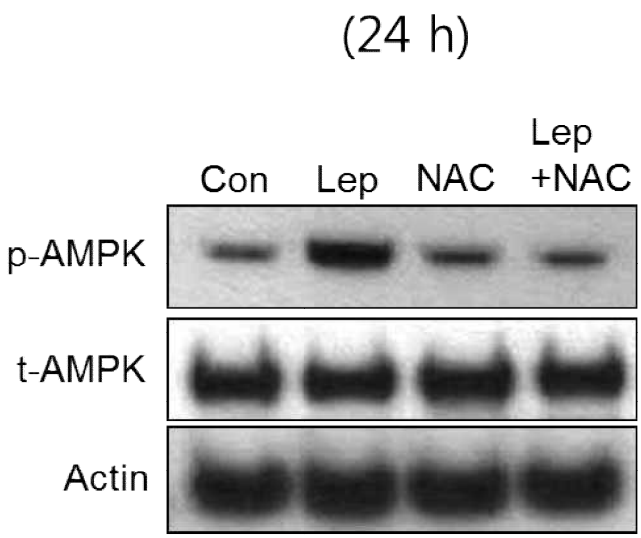
(A)


Leptin dose :500 nM, application duration : 1 hour
Con
Lep
AICAR
Lep
+ComC
NAC
Lep
+NAC
H2O2
50μM

(B)


(N=7)
vs Control, *
vs Lep, #
Relative DCF-DA intensity
0
1
2
3
4
5
6
7
**
**
**
##
Con
Lep
AICAR
Lep +ComC
NAC
Lep +NAC
H2O2 50 μM

(C)


(24 h)
Con
Lep
NAC
Lep +NAC
p-AMPK
t-AMPK
Actin

(D)

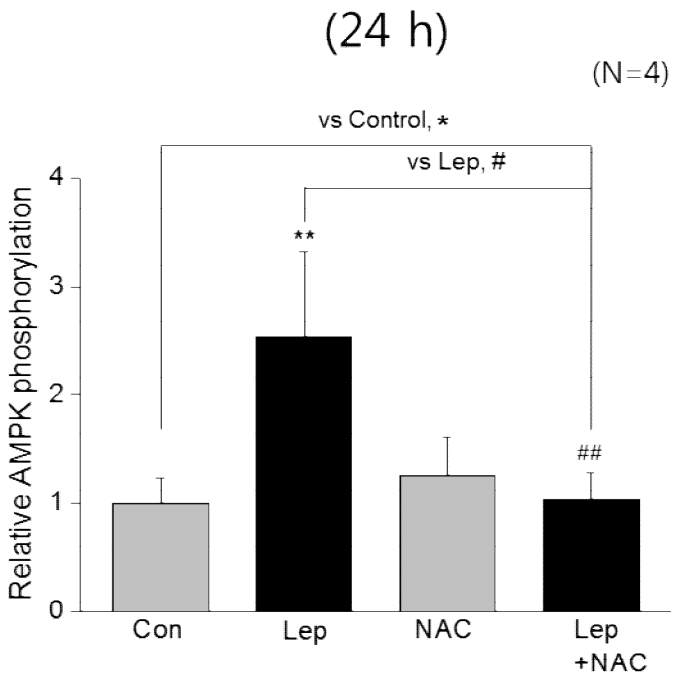


Figure 9. AMPK activation by leptin-preconditioning occurs downstream of ROS generation.

(A) Fluorescence microscopy images showing the DCF-DA signal intensity in H9c2 cells in the control, leptin, AICAR, NAC, leptin+NAC, leptin+compound C and H_2O_2 (positive control) groups. (B) Relative DCF-DA intensities were measured in the control, leptin, AICAR, NAC, leptin+NAC, leptin+compound C and H_2O_2 (positive control) groups. ** P <0.01 versus control, ## P <0.01 versus leptin. (C) Western blotting photograph of phosphorylated and total AMPK of H9c2 cells in the control, leptin, NAC and leptin+NAC groups. (D) Western blotting analysis of phosphorylated and total AMPK in the control, leptin, NAC and leptin+NAC groups. ** P <0.01 versus control, ## P <0.01 versus leptin.

DISCUSSION

In the present study, I demonstrated that leptin preconditioning in rat cardiomyocytes induced the production of ROS, which then activate AMPK. It resulted in protecting heart against cardiac ischemia-reperfusion. Relation of leptin-induced cardioprotection and K_{ATP} channels was suggested by the result that cardioprotective effect of leptin was abolished by glibenclamide, K_{ATP} channels blocker, but further investigation is required to clarify underlying mechanisms and causal relationships. Schematic diagram for signaling mechanisms for leptin-preconditioning is illustrated in Fig 10.

Mechanisms underlying cardioprotection by leptin-preconditioning

Ischemic heart disease is the main cause of heart failure and is the most common cause of death in most countries (He *et al*., 2001). It occurs when the heart muscle, or myocardium, receives insufficient blood flow. People with a family history of ischemic heart disease are usually commenced on several long-term medications with the aim of preventing myocardial infarctions. Such medications include antiplatelet drug, beta blocker and so on (Peters *et al*., 2003). In an experimental method, ischemic preconditioning (IPC) has been known to produce resistance to the loss of blood supply, and thus loss of oxygen. In the heart, IPC is an intrinsic process whereby repeated short episodes of ischaemia protect the myocardium against a subsequent ischemic

insult. It was first identified in 1986 by Murry *et al*. This group exposed anesthetised open-chest dogs to four periods of 5 minute coronary artery occlusions followed by a 5 minute period of reperfusion before the onset of a 40 minute sustained occlusion of the coronary artery. The control animals had no such period of IPC and had much larger infarct sizes compared with the dogs that did. Since its discovery in 1986, extensive studies have been undertaken to explain its mechanisms and induce protection pharmacologically. There is convincing evidence for the involvement of AMPK (Nishino *et al*., 2004), K_{ATP} channels (Sukhodub *et al*., 2007), MPTP (Smith *et al*., 2010), ROS (Garlid *et al*., 2013), chloride channels (Bozeat *et al*., 2011), the inward rectifier potassium ion channel (Diaz *et al*., 2004) and connexon 43 related channels (Jain *et al*., 2003). Especially, AMPK was shown to play a key role in cardioprotection in an abnormal heart. Activation of AMPK is crucial for ischemic-preconditioning-induced cardioprotection (Nishino *et al*., 2004; Sukhodub *et al*., 2007). AMPK direct-activator (A-769662) protects the heart against ischemia-reperfusion injury (Kim *et al*., 2011).

My study demonstrates that most of these factors, such as activation of AMPK, induction of ROS and activation of K_{ATP} channels, are downstream of leptin signaling. Activation of AMPK by leptin was reported in H9c2 cells (Shin *et al*., 2009). I confirmed it (Fig. 5), and further showed that the effect of leptin-preconditioning was abolished by compound C, AMPK inhibitor, in the ischemic heart (Fig. 2, 3). The molecular mechanism for leptin-induced AMPK activation in pancreatic β-cells is known to be mediated by CaMKKβ

(Park *et al*., 2013). This possibility was not examined in this study, but I showed that leptin-induced ROS generation mediates AMPK activation (Fig. 9). As for K_{ATP} channels, leptin has been reported to activate K_{ATP} channels trafficking by AMPK signaling in pancreatic β cells (Park *et al*., 2013, Chen *et al*., 2013). It needs to be investigated in future studies whether the same mechanism is involved in leptin-preconditioning in cardioprotection. Taken together, my study suggests that leptin preconditioning is the effective method to activate various targets that are potentially have cardioprotective effects. Furthermore, I demonstrated that leptin has prolonged effects, in that the cardioprotective effect of leptin administered 24 h before CAO is similar to that administered 1 h before CAO. Therefore, leptin might be a promising pharmacologic approach to limit cardiac injury and a target for preconditioning in translation medicine of ischemic heart. Similarly, adiponectin, a kind of adipocytokines, was reported to be effective for protection against myocardial ischemia-reperfusion injury, in that AMPK mediates antiapoptotic action while cyclooxygenase 2 mediates anti-inflammatory actions on cardiac cells (Shibata *et al*., 2005).

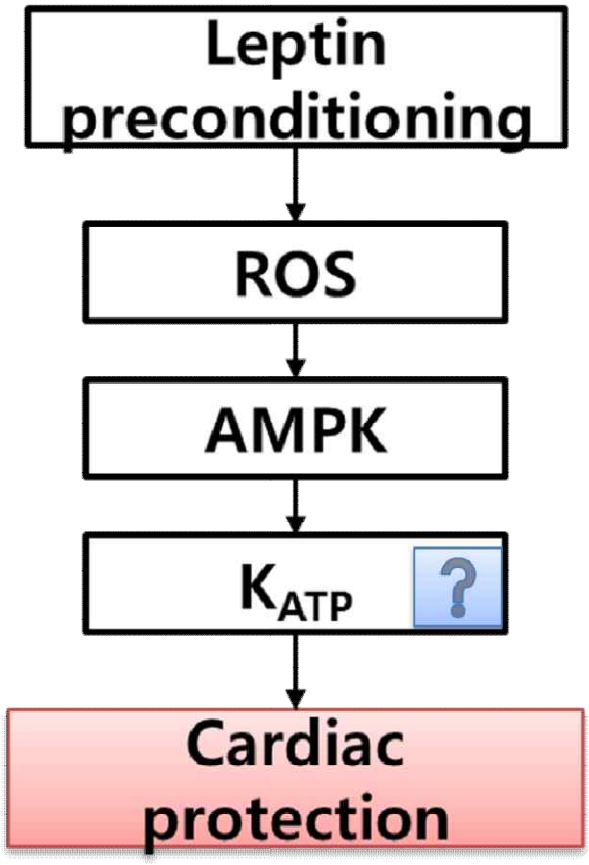


Figure 10. Signaling pathway of cardiac protection by leptin-preconditioning

Pros and Cons of antioxidants in relation to cardioprotection

In the present study, it was shown that infarct size after ischemic reperfusion is reduced by the antioxidant (Fig. 2). In contrast, cardioprotective effect by leptin-preconditioning is abolished by the antioxidant (Fig. 3), demonstrating that antioxidant treatment is not always beneficial for cardioprotection. I further elucidated that leptin induces ROS generation, which in turn activates AMPK, resulting in cardioprotection. Normally, under ischemia/reperfusion situation, induction of ROS is associated with heart failure and several conditions that predispose individuals to heart failure (Hafstad *et al*., 2013). Oxidative stress has been identified as a critical factor in most of the key steps in the pathophysiology of atherosclerosis and acute thrombotic events, including dyslipidemia leading to atheroma formation, LDL oxidation, endothelial dysfunction, plaque rupture, myocardial ischemic injury, and recurrent thrombosis (i.e., the secondary, or subsequent, clot that often occurs after initial thrombolysis; Dzau *et al*., 2006). ROS-induced pro-inflammatory cytokines are also involved in cardiac muscle dysfunction and in the complex syndrome of heart failure (Mann, 2002). Oxidative stress has also been implicated in diabetic cardiomyopathy (Bugger & Abel, 2010; Khullar *et al*., 2010), congestive cardiomyopathy (Pankuweit *et al*., 2010), and hypertensive heart disease (Shahbaz *et al*., 2010). Therefore, antioxidants such as vitamin E have been suggested as being potentially useful in the treatment of vascular disease (Ratcliffe, 1949). The use of vitamin E in the treatment of cardiovascular disease has been studied in animal models of arteriosclerosis

(Eisen & Gross, 1949; Ravin & Katz, 1949), and over the years, the oxidative stress hypothesis has encouraged the use of antioxidants (Rimm *et al*., 1993; Stanner *et al*., 2004). Studies on fruit and vegetable consumption, particularly those rich in vitamin C and other antioxidants, have shown correlations with a reduction in mortality due to cardiovascular disease (Verlangieri *et al*., 1985). Furthermore, the plasma level of vitamin E was shown to be inversely related to mortality from ischemic heart disease (Gey & Puska, 1989; Emmert & Kirchner, 1999). However, I showed that ROS generation mediated by leptin is crucial for inducing downstream events that lead to cardioprotection. In fact, signaling role of ROS is recently appreciated in various systems (Bae *et al*., 2011). ROS are not just byproducts of aerobic respiration to have damaging effects on the cell, but they are involved in various cellular signaling pathways. In this respect, taking vitamin E and other antioxidants daily to prevent oxidative stress may need to be reconsidered. In the case of leptin-induced cardioprotection and ischemic preconditioning, slight ROS generation may actually help protect the heart.

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국문 초록

서론: 렙틴은 *ob* 유전자에서 생산되는 물질로 주로 백색지방세포에서 합성되나 심장에서도 생산된다. 렙틴은 심장보호기능이 보고된 바 있다. AMP 의존성 인산화효소(AMPK)는 써린과 쓰레오닌의 단백질 인산화 효소로 비정상 심장상태에서 심근보호기능을 가지는 것으로 알려져 있다. 렙틴은 AMPK 를 활성화시킨다. 하지만 렙틴의 심장보호기능과 AMPK 의 심장보호기능과의 연관성은 아직 밝혀져 있지 않다. 본 연구에서는 렙틴 전처치에 의한 심장보호기전과 AMPK 의 조절에 의한 그것과의 관계를 밝히고자 하였다.

방법: 랫드의 허혈성 심장질환 모델은 좌측 관상동맥의 전방하강분지를 결찰함으로써 만들었고 심장보호효과를 판단하는 기준으로는 경색의 크기를 측정했다. 렙틴의 심장보호기전을 조사하기 위해서 랫드의 심장 근원세포주인 H9c2 세포를 사용했다.

결과: 렙틴은 허혈성 심장질환 모델에서 심근경색 크기를 감소시켰다. 이 효과는 AMPK 의 인산화 억제제인 compound C 와 활성산소 제거제인 NAC 의 전처치에 의해서 없어졌다. 렙틴은 H9c2 세포에서 AMPK 를 농도, 시간에 비례해서 인산화시켰고, 활성산소도 증가시켰다. 렙틴의 AMPK 인산화효과는 활성산소 제거제인 NAC 에 의해서 사라졌지만, 렙틴의 활성산소의 증가는 AMPK 의 인산화 억제제인 compound C 에 영향을 받지 않았다. 따라서, 렙틴에 의한 활성산

소생성의 증가는 AMPK 를 활성화시키고 허혈성 심장질환에 의한 심근의 손상을 감소시켰다.

결론: AMPK 는 렙틴 전처치에 의한 심장보호에 중요한 역할을 하고 있으며 AMPK 의 활성은 렙틴 전처치에 의한 활성산소의 발생에 의해 유발된다.

주요어 : 렙틴, 심장보호기능, AMP 의존성 인산화효소, 활성산소, 허혈성질환, 관상동맥결찰

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