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Molecules and Cells

Propofol Protects the Autophagic Cell Death Induced by the Ischemia/Reperfusion Injury in Rats

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Autophagy has been implicated in cardiac cell death during ischemia/reperfusion (I/R). In this study we investigated how propofol, an antioxidant widely used for anesthesia, affects the autophagic cell death induced by the myocardial I/R injury. The infarction size in the myocardium was dramatically reduced in rats treated with propofol during I/R compared with untreated rats. A large number of autophagic vacuoles were observed in the cardiomyocytes of I/R-injured rats but rarely in I/R-injured rats treated with propofol. While LC3-II formation, an autophagy marker, was up-regulated in the I/R-injured myocardium, it was significantly down-regulated in the myocardial tissues of I/R-injured and propofol-treated rats. Moreover, propofol inhibited the I/R-induced expression of Beclin-1, and it accelerated phosphorylation of mTOR during I/R and Beclin-1/Bcl-2 interaction in cells, which indicates that it facilitates the inhibitory pathway of autophagy. These data suggest that propofol protects the autophagic cell death induced by the myocardial I/R injury.

INTRODUCTION

Autophagy is an evolutionary conserved process involved in degradation of long-lived or damaged proteins and organelles (Levine and Klionsky, 2004). Autophagy has been implicated in myocardial ischemia/reperfusion (I/R). Nevertheless, the exact functional role of autophagy in the cell survival and death pathways associated with heart damage is still not clear. Some observations suggest that stimulation of autophagy can promote cell survival in response to stress conditions in cardiomyocytes (Hamacher-Brady et al., 2006a; Kuma et al., 2004). On the other hand, the excessive and long-term upregulation of autophagy under certain conditions inhibit cell survival, rather greatly accelerating the cell death in the heart (Hamacher-Brady et al., 2007; Matsui et al., 2007). However, these data clearly indicate that autophagy is specifically associated with cellular responses in cardiomyocytes.

Beclin-1 was initially identified as a Bcl-2-interacting protein (Liang et al., 1998). It directly interacts with class III PI3K (VPS 34) to induce the autophagic process (Sun et al., 2009). Beclin-1 also binds to Bcl-2 family proteins such as Bcl-2 (Pattingre et al., 2005). This Bcl-2 and Beclin-1 interaction blocks Beclin-1 binding to class III PI3K (Vps 34) and eventually inhibits autophagy (Pattingre et al., 2005). It has also been reported that Bcl-XL inhibits Beclin-1 activity by stabilizing Beclin-1 homodimeriztion (Feng et al., 2007). Therefore, disruption of Bcl-2 or Bcl-XL/Beclin-1 interaction is a critical mechanism to promote autophagy in cells.

Propofol is an intravenous anesthetic drug widely used for the induction and maintenance of anaesthesia. Propofol has a structural similarity to the endogenous antioxidant vitamin E and exhibits antioxidant activities (Gulcin et al., 2005). Therefore, it shows the protective effect on the hydrogen peroxide (H_2O_2)-induced apoptosis in cardiac cells (Wang et al., 2009) and myocardial ischemia and reperfusion (I/R) injury in rats (Jin et al., 2009). However, the mechanism of propofol on I/R injury has yet to be fully elucidated. Here we investigated how this antioxidant drug influences the autophagic cell death, a cellular damage occurring during the I/R process.

MATERIALS AND METHODS

Reagents

Propofol and intralipid were purchased from HanaPharm. Co. Ltd. (Korea). Antibodies against Beclin 1, p62 and Bcl-2 were purchased from Santa Cruz Biotechnology (USA), and PARP, mTOR and phospho-mTOR antibodies were purchased from Cell Signaling Technology (USA). Anti-LC3 antibody was from ABGENT (USA).

Surgical protocol

Male Sprague-Dawley rats (7-8 weeks of age) were surgically prepared for I/R injury as described previously (Jin et al., 2009). All procedures were performed in accordance with the Guide for Care and Use of Laboratory Animals published by the National Institutes of Health (Guide for the Care and Use of Laboratory Animals, 1996). The protocol was approved by the Animal Research Committee of the Gyeongsang National University, Korea. The animals were anesthetized with ketamine 50 mg/kg and xylazine 5 mg/kg into the gluteus maximus muscle, and were ventilated artificially with room air using rodent venti-

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lator (Type 7025, Ugo Basile, Italy). Respiratory rate was synchronized with the rat's spontaneous rate (60-80 strokes/min, 8 ml/kg body weight). Arterial blood pH and blood gases were maintained within normal physiological limits (pH 7.35-7.45; $_PCO_2$: 30-35 mmHg; $_PO_2$: 85-100 mmHg) by adjusting the respiratory rate and tidal volume. The chest was opened via a left thoracotomy, followed by a pericardiotomy. A 4-0 black silk suture (4/0 nonabsorbable, Catania, Italy) was passed around the left ascending (LAD) coronary artery, and the ends were pulled through a small vinyl tube to form a snare and then tightened. Coronary artery occlusion was verified by epicardial cyanosis. After 25 min of ischemia, the myocardium was reperfused for 24 h. The rats were randomized into four groups as follows. Group 1 rats (sham), surgically operated, but no tightening of the coronary sutures. Group 2 rats (I/R), received saline (1.2 ml/kg/h) and were subjected to 25 min of ischemia followed by reperfusion. Group 3 rats (I/R with propofol), received propofol (12 mg/kg/h) and were subjected to 25 min of ischemia followed by reperfusion. Group 4 rats (I/R with intralipid), received intralipid (1.2 ml/kg/h).

Western blotting and immunoprecipitation

For western blot analysis, animals were killed at 24 h after reperfusion (n = 6 per group). The hearts were homogenized and lysed in buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40 (NP-40) and protease inhibitors (1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml aprotinin, 1 μg/ml leupeptin). Lysates were centrifuged at 14,000 × *g* for 20 min to remove tissue debris. Total cellular proteins (30 μg) were separated in a 10% SDS-PAGE gel and transferred onto the nitrocellulose membrane. The membranes were blocked with 5% skimmed milk and sequentially incubated with the primary antibodies. Control for protein loading was performed by reprobing membranes with an antibody against β-actin (Santa Cruz Biotechnology). Membranes were then incubated with horseradish peroxide (HRP) conjugated secondary antibodies (Caltag, USA) followed by ECL detection (Pierce, USA). These experiments were repeated twice at least and showed reproducible results. Images were scanned and intensity analysis was carried out using Sigma Gel V1.0 (Jandel Scientific, USA). For immunoprecipitation, protein samples (500 μg) were mixed with protein A/G agarose beads (Santa Cruz Biotechnology) and incubated for 1 h at 4°C, and then the sample was centrifuged at 12,000 × *g* for 1 min. The supernatant was incubated with 2 μg of immunoprecipitaion (IP) antibodies overnight at 4°C and then incubated with protein A/G agarose beads for 2 h at 4°C. The negative control was prepared with protein A/G agarose beads without antibodies. The protein-bead complex was then washed and collected by centrifugation, and protein samples were boiled in loading buffer to remove agarose beads and separated in a 10% SDS-PAGE gel. Proteins were then transferred to the membrane and probed with antibodies against the interacting protein of interest as described above.

Immunohistochemistry

Hearts were removed, fixed overnight in 0.1 M phosphate buffered saline (PBS) containing 4% paraformaldehyde at 4°C. And then, tissues were paraffin-embedded. Serial sections were cut at 5 μm thickness using a microtome. The tissue sections were rinsed three times in PBS, incubated in 2% normal goat serum for 1 h at room temperature, then incubated overnight at 4°C with rabbit anti-LC3 antibody (Abgent, 1:1000) diluted in PBS containing 2% normal goat serum and 0.3% Triton X-100. On the following day, sections were washed with PBS three times, incubated with biotinylated anti-rabbit IgG (Vector Laboratories, USA) for 1 h at room temperature, incubated with avidin-biotin complex (ABC; Vector) for 1 h at room temperature, rinsed with PBS and then treated with DAB (Sigma) to reveal LC3-like immunoreactivity.

Table 1. Comparison of myocardial infarction size in I/R-injured rats treated with saline (control), propofol, or intralipid

	I/R injury (n = 10)	I/R + propofol (n = 10)	I/R + intralipid (n = 10)
Body weight (gram)	245 ± 20	240 ± 15	247 ± 16
LV weight (gram)	0.60 ± 0.02	0.55 ± 0.03	0.63 ± 0.03
Area at risk (gram)	0.24 ± 0.01	0.25 ± 0.02	0.31 ± 0.02
Infarct area (gram)	0.14 ± 0.01	0.06 ± 0.02	0.14 ± 0.02
Infarct area/area at risk (%)	57.4 ± 4.5	24.3 ± 8.6	45.1 ± 7.7

LV, left ventricle

Transmission Electron Microscopy (TEM) analysis

Tissues were fixed in a 2.5% glutaraldehyde solution (1XPBS) for 6 h at 4°C and washed in PBS-sucrose (300 mOsm, pH 7.2). The tissues were post-fixed in 2% osmium tetroxide for 2 h, dehydrated in ethanol (50% to 95% for 20 min and 100% for 30 min) and finally substituted by propylene oxide solution. After being embedded in Poly/Bed mixture (19.3 ml Poly/Bed 812, 12.3 ml dodecenylsuccinic anhydrade, 9.4 ml nadic methyl anhydride, 0.6 ml DMP-30), the TEM analysis was carried out as described (Dadakhujaev et al., 2008).

RESULTS

Propofol prohibits the I/R-induced myocardial infarction

The myocardial I/R injury is a major cause for many cardiovascular diseases. To explore how propofol influences the I/R-injured rat myocardium, we carried out the surgical operation to generate the left coronary artery occlusion for 25 min and reperfuse the myocardium in the rats with/without propofol. After 24 h, we measured the weight of infarction area. The weight of infarction area of the left ventricle obtained from I/R-injured rats was 0.14 ± 0.01 (*g*), whereas the infarction weight of I/R-injured rats treated with propofol was significantly decreased to 0.06 ± 0.02 (Table 1, $P < 0.05$, Fig. 1A). The vehicle (intralipid) did not affect the reduction of infarction size by propofol (Fig. 1A). The portion of infarction area to the total risk area was similarly changed: 57.4 ± 4.5% (I/R-injured rats), 24.3 ± 8.6 [I/R-injured rats with propofol, $P < 0.05$ (Table 1, Fig. 1B)]. These data clearly suggest that propofol notably prohibits the myocardial damage induced by I/R.

Propofol prevents formation of autophagosomes during myocardial I/R

Autophagic vacuoles (AVs) including autophagosomes and autophagolysosomes are generally formed in cells undergoing the autophagic process. Therefore, observation of AVs inside cells can be used as an indicator to analyze the induction of autophagy. During the myocardial I/R injury, autophagy has been suggested that it contributes to some portions of cell death in the myocardium (Kostin et al., 2003; Uchiyama et al., 2008). To investigate the role of autophagy in the I/R-induced myocardial damage and the effect of propofol in the occurrence of autophagy, first we directly observed formation of AVs in cardiomyocytes of I/R-damaged rats using electron microscope

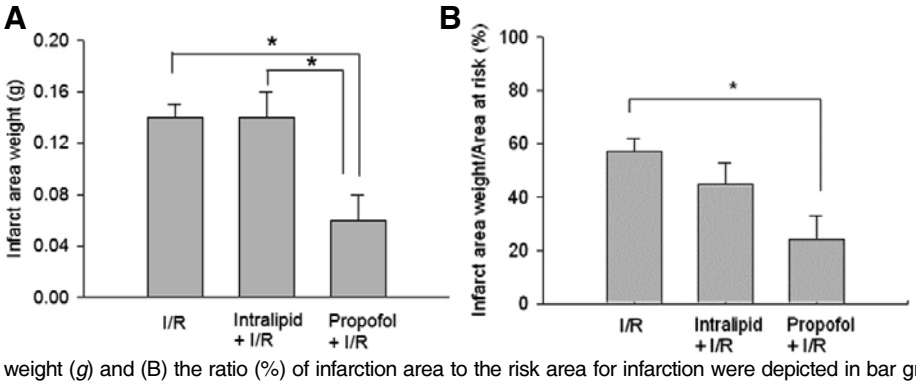

Fig. 1. Comparison of myocardial infarction size in I/R-induced rats. Myocardial I/R injuries were induced by occluding the left anterior descending coronary artery for 25 min followed by 24 h reperfusion. Saline (1.2 ml/kg/h), propofol (12 mg/kg/h) or intralipid (1.2 ml/kg/h) was administrated via femoral vein beginning 15 min before reperfusion and continued for 30 min into reperfusion. The infarction size was determined by Evans blue staining. (A) Infarction area weight (*g*) and (B) the ratio (%) of infarction area to the risk area for infarction were depicted in bar graphs. Data are means ± SE. (n = 10 in each group). *Represents $P < 0.05$ compared to control group.

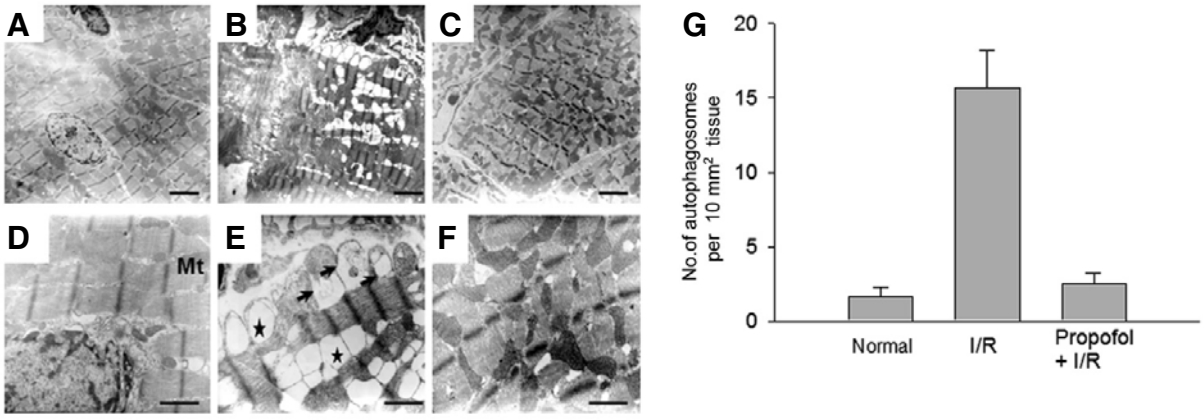

Fig. 2. Electron microscopic analysis in the I/R-injured cardiomyocytes. Control hearts treated with intralipid (A and D), I/R-injured hearts (B and E), and I/R-injured hearts treated with propofol (C and F) were represented, irrespectively. Autophagic vacuoles containing mitochondria were indicated by arrows. Myofibrillar lyses (asterisks) were observed in cardiomyocytes undergoing autophagic cell death. Bars, 5 μm (A-C) and 1 μm (D-F). Numbers of autophagic vacuoles per a specific area were determined from EM images and represented in the graph (G).

analysis (TEM). I/R caused accumulation of abundant AVs containing partially digested cytoplasmic contents and myofibrillar lysis, which was associated with degenerated mitochondria and myelin figures, in cardiomyocytes (Figs. 2B and 2E) compared to the control group (Figs. 2A and 2D). However, propofol treatment during I/R dramatically decreased formation of cytosolic vacuoles and myofibrillar lysis in cardiomyocytes of I/R-damaged rats (Figs. 2C and 2F), indicating that autophagy is associated with I/R-induced cell death, and propofol protects this autophagic cell death (Fig. 2G).

LC3-II level is decreased by propofol during I/R

To further confirm whether propofol can modulate autophagic cell death in the I/R-injured myocardium, we examined the level of LC3 expression. The production of phosphatidylethanolamine-conjugated LC3 (LC3-II) has been suggested as another marker for autophagy induction. LC3-II formation was dramatically increased in the myocardium exposed to I/R injury compared to the control group (Figs. 3A and 3B). However, in the case of propofol treatment during I/R, LC3-II production was significantly decreased compared to the I/R-only group (Figs. 3A and 3B). Furthermore, expression of p62, an autophagy-associated protein was alleviated in the I/R-induced myocardium, but it was recovered by treatment of propofol to the I/R-injured rats to the similar level shown in the control group (Fig. 3A). We also examined total expression of LC3 in the myocardium using immunohistochemical staining. LC3 was weakly expressed in all area of myocardium in normal and propofol-treated rats although some expression was detected in endocardium. On the contrary, the strong expression was observed in the papillary muscle of the I/R injured rats (Fig. 3C). This data indicates again that the myocardial I/R injury induces autophagy leading to cell death and propofol inhibits this autophagic cell death.

Effect of propofol on expression of autophagy-related proteins during I/R

Autophagy is majorly regulated by two central pathways: Beclin-1 and mTOR-dependent mechanism. To explore how propofol regulates the I/R-induced autophagy, we analyzed the expression of several autophagy-related proteins involved in these pathways in the I/R-injured myocardium. The myocardial I/R injury resulted in a significant increase of Beclin-1 and Bcl-2 expression compared to the control group (Figs. 4A and 4B). On the other hand, the phosphorylation of mTOR at the 2418th serine residue, which negatively mediates autophagy, was greatly downregulated in the I/R-injured myocardium although total mTOR levels were not changed by I/R. However, treatment with propofol to I/R-injured animals significantly prevented I/R-dependent Beclin-1 expression and also increased phos-

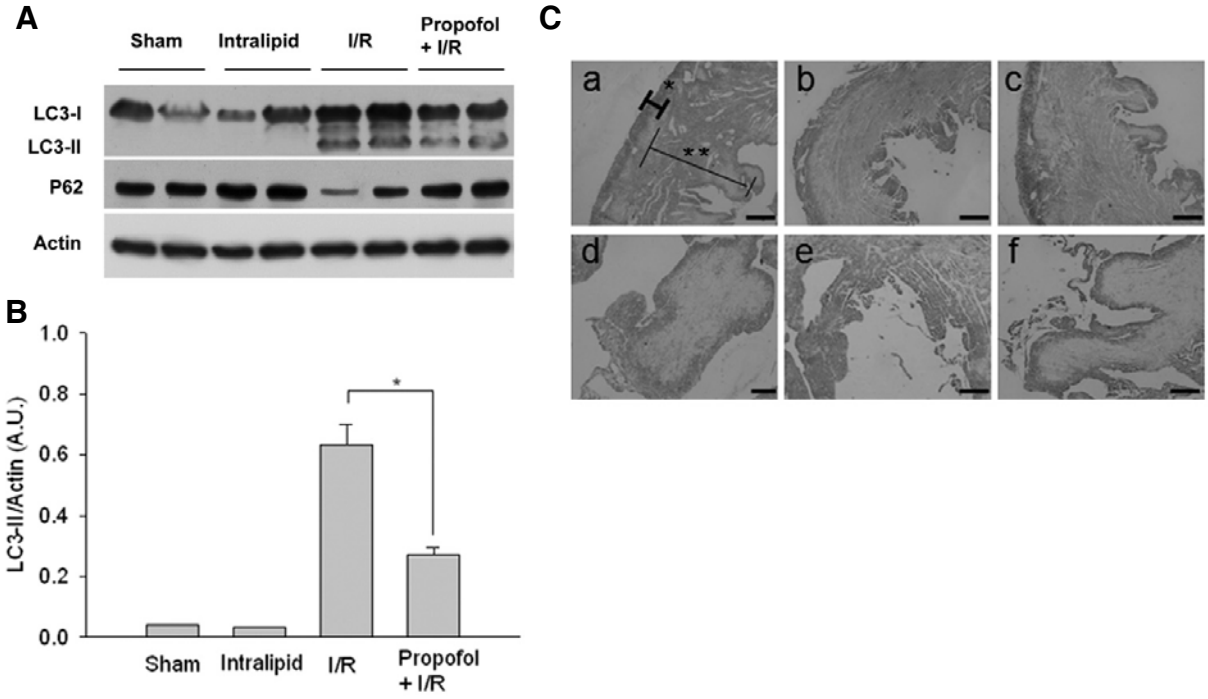

Fig. 3. Expression of LC3 in I/R-injured myocardium treated with propofol. (A) Western blot analysis. LC3 expression (LC3-I and LC3-II) in myocardial tissues was determined by blotting using anti-LC3 antibodies. β-actin was used as an internal control, and no difference was observed between the samples. (B) Quantification of LC3-II. Each LC3-II band shown above was quantified by the densitometric scan and normalized to β-actin values. Data (mean ± S.D.) are expressed as arbitrary unit (A.U.) (each n = 10). * Represents $P < 0.005$ compared to I/R-induced hearts; one-way ANOVA, followed by a Tukey's multiple-comparison test. (C) Immunohistochemistry of LC3 in I/R-injured myocardial tissues. Total LC3 expression in the endocardium (*) and myocardium (**) of normal (a and d), IR-induced (b and e), and IR-induced rat treated with propofol (c and f) was stained as described (Dadakhujaev et al., 2008). Scale bars, 100 μm (a-c), 25 μm (d-f).

phorylation of mTOR protein (Figs. 4A and 4B), suggesting that I/R-induced autophagic cell death is dependent of both Beclin-1 and mTOR pathways. According to previous data, propofol can also diminish I/R-induced apoptosis (Jin et al., 2009). Similarly, we observed that propofol prevented the PARP cleavage induced by the I/R injury (Fig. 4A).

To further confirm how Beclin-1/Bcl-2 interaction responds to I/R-induced autophagy in the presence of propofol, we performed immunoprecipitation analysis using total proteins obtained from the myocardial tissues of each experimental rat. The myocardial I/R significantly decreased interaction between Beclin-1 and Bcl-2, leading to the stimulation of Beclin-1-dependent autophagic cell death, while the administration of propofol to I/R-injured rats promoted Beclin-1/Bcl-2 interaction induced by I/R to a similar level as shown in the control group (Figs. 4C and 4D). These observations suggest that stimulation of Beclin-1/Bcl-2 binding by propofol in the I/R-injured myocardium eventually inhibits induction of autophagic cell death.

DISCUSSION

In this study, we showed that propofol significantly reduced the size of myocardial infarction induced by the myocardial I/R injury in rats. Additionally, we suggested that the cardioprotective effect of propofol during I/R was given by inhibiting the Bcl-2 dissociation from Beclin-1, resulting in a great impediment of autophagic cell death in the I/R injury. Autophagy plays a pivotal role in not only cell survival but also cell death. In particular, the specifically regulated autophagic process is involved in maintaining the normal heart function during perinatal starvation (Kuma et al., 2004). Although autophagy activation is essential for survival of cardiac myocytes in response to nutrient deprivation, prevention of glucose starvation-induced autophagy significantly reduced cell death in the H9C2-transformed cardiac myocyte cell line (Aki et al., 2003), suggesting that autophagy can be detrimental for cardiac cells. According to recent works, moreover, it has been suggested that autophagy plays two distinct roles during ischemia and reperfusion. In the ischemic phase, autophagy can be protective via AMPK activation and sequentially inhibition of mTOR signaling, but reperfusion after ischemia stimulates autophagic cell death through the different pathway. This autophagy process is beclin-1-dependent but AMPK-independent (Matsui et al., 2007). However, the role of autophagy in cardiac damage is still controversial. Autophagy protects cells from the I/R injury (Hamacher-Brady et al., 2006), but many I/R damage are associated with autophagy-induced cell death in the heart and other organs (Akazawa et al., 2004; Uchiyama et al., 2008). Furthermore, myocytes in human hearts die through the autophagic cell death (Kostin et al., 2003).

Here we found that I/R-induced cell death is associated with autophagy activated by expression of LC3-II, Beclin-1, deactivation of mTOR signaling and accumulation of autophagic vacuoles (Fig. 4). This autophagic cell death was protected by propofol through the reversing mechanism showed during I/R. Although necrosis and apoptosis have been suggested as the major cell death pathway following I/R, autophagy has also been implicated in the pathogenesis of several cardiovascular diseases (Kostin et al., 2003; Shaw and Kirshenbaum, 2008). In particular, two cell death mechanisms (apoptosis and autophagy) specifically interplay each other during the myocardial

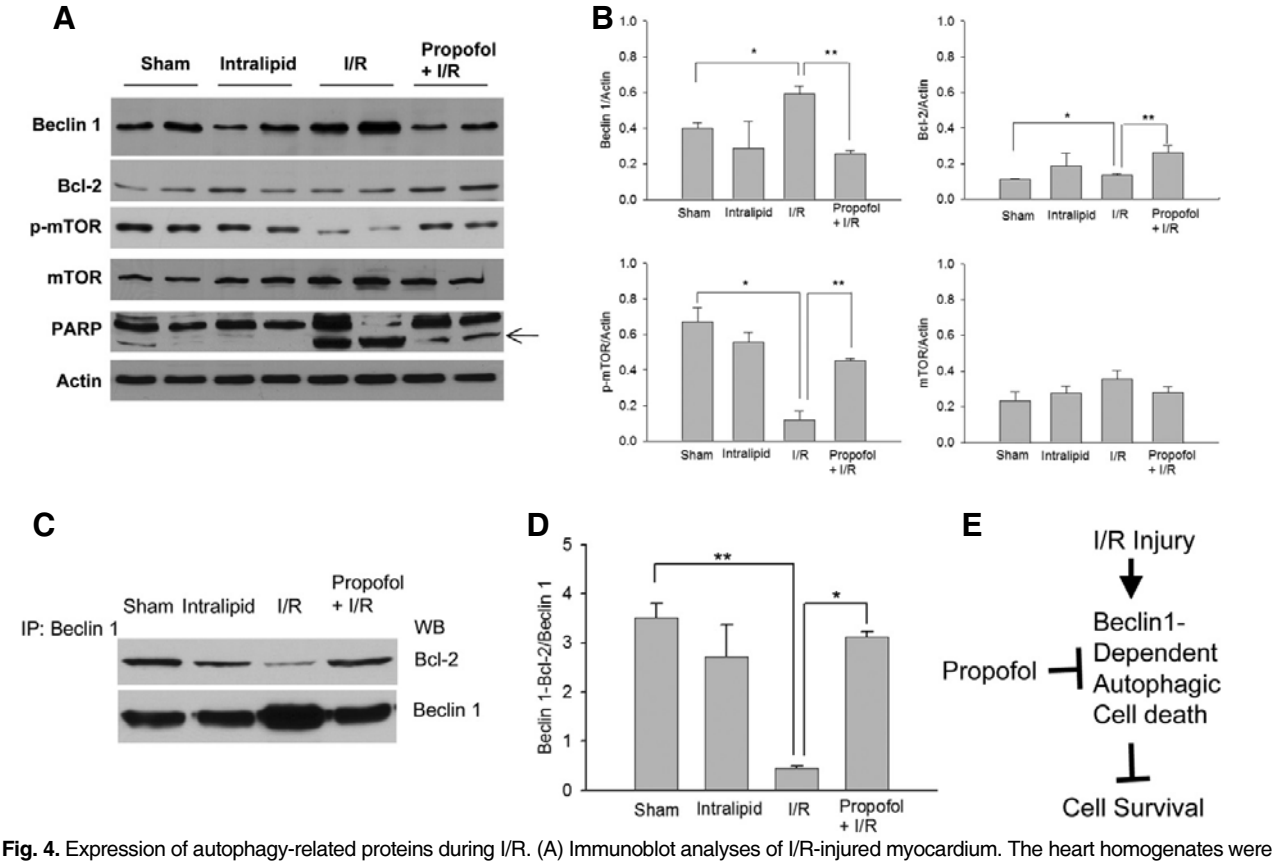

Fig. 4. Expression of autophagy-related proteins during I/R. (A) Immunoblot analyses of I/R-injured myocardium. The heart homogenates were analyzed by Western blot using specific antibody against each autophagy-related protein. Arrow indicates the cleaved PARP protein. Expression of β-actin was also examined for the protein loading control. (B) Quantification of Beclin-1, Bcl-2, p-mTOR, and mTOR expression. Each protein (Beclin-1, Bcl-2, p-mTOR, and mTOR) shown in Fig. 4C was quantified after densitometirc scan and normalized to β-actin. Data (mean ± S.D.) are expressed as arbitrary unit (A.U.) (each n = 10), and analyzed by one-way ANOVA followed by a Tukey's multiple-comparison test. $P < 0.05$ (*) and $P < 0.01$ (**). (C) Immunoprecipitation to determine Bcl-2/Beclin-1 interaction. Total tissue lysates were incubated with anti-Beclin-1 antibodies. Bcl-2 binding to Beclin-1 was determined by immunobblot using anti-Bcl-2 antibody. Total Beclin-1 in samples (input) was also determined by Western blot. (D) Quantification of Bcl-2/Beclin-1 interaction. Amount of Bcl-2 (Fig. 4C) was determined by densitometry and normalized to Beclin-1 band. Data (mean ± S.D.) represents three independent experiments. *$P < 0.05$ compared to I/R control, **$P < 0.01$ compared to sham. (E) Summary of propofol action during the myocardial I/R injury.

I/R injury (Hamacher-Brady et al., 2006b), and Bnip3, a Bcl-2 family, controls both processes in the I/R damage (Lee and Paik, 2006; Shaw and Kirshenbaum, 2008). Bcl-2 inhibits the apoptotic process via the direct interaction with pro-apoptotic proteins (BAX/BAK). In addition, Bcl-2 can regulate autophagy by sequestrating Beclin-1. That is, the ability of Beclin-1 to promote autophagy is negatively regulated by interaction with Bcl-2 (Liang et al., 1998; Pattingre et al., 2005; Sun et al., 2009). The dissociation of Bcl-2 from Beclin-1/Bcl-2 complexes during I/R could enhance the autophagic activity of Beclin-1. Indeed, we showed that I/R injury itself causes decrease of interaction between Beclin-1 and Bcl-2 (Fig. 4), suggesting that myocardial I/R leads to heart damage via Beclin-1-dependent autophagy. However, we also showed that PARP cleavage, an indication of caspase activation during apoptosis, was greatly elevated in the I/R injury (Fig. 4A).

Propofol, an anesthetic drug, shows its anti-oxidant activity in some oxidative reactions. It actually blocks the apoptotic cell death induced in the myocardial I/R injury (Jin et al., 2009) and significantly reduces the H_2O_2-induced cellular toxicity in cardiac cells through activation of Akt protein and Bcl-2 upgulation (Wang et al., 2009). Additionally, propofol protects cell death of hepatic L02 cells under H_2O_2-induced oxidative stress via ERK activation (Wang et al., 2008). Those data indicate that propofol can act as an antioxidant by modulating the signaling proteins involved in stress responses. Indeed, treatment of propofol significantly prevented the myocardial damage induced by ischemia/reperfusion. It caused a great decrease of Beclin-1 expression and increase of mTOR activation (Figs. 4A and 4C), indicating that propofol prevents I/R-induced autophagic cell death. Furthermore, interaction of Beclin-1 with Bcl-2 was diminished by I/R injury, and recovered by propofol to the level shown in control. These results suggest that propofol can modulate autophagy via both Beclin-1-dependent and mTOR-dependent pathways. However, It is still unclear how propofol directly modulates expression of autophagy-related genes when the heart tissue is exposed to the I/R injury. Finally, we postulated here how propofol controls the autophagic process induced by myocardial I/R injury (Fig. 4E).

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