The role of endogenous reactive oxygen species in cardiac myocyte autophagy

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Summary

Autophagy is implicated in the maintenance of cardiac homeostasis. Autophagy is activated in heart failure, in which reactive oxygen species (ROS) are increased. Exogenous ROS have been shown to induce cardiomyocyte autophagy alterations. However, little is known about the influences of physiological levels of endogenous ROS on cardiomyocyte autophagy. In the present study, we tested the hypothesis that endogenous ROS in cardiomyocytes play an important role in inducing autophagy. Cultured H9C2 cardiomyocytes or Sprague-Dawley rats were treated with the antioxidant N-acetyl-cysteine (NAC) or the superoxide dismutase mimic tempol under the basal or nutrient deprivation conditions. The autophagic flux was assessed by the lysosomal inhibitor chloroquine. In H9C2 cardiomyocytes, under a basal condition, NAC or tempol increased the ratio of LC3 II/I proteins and reduced LC3 II autophagic flux. Under nutrient deprivation, NAC increased the LC3 II/I ratio and reduced LC3 II autophagic flux. In vivo studies in rats, NAC treatment increased the LC3 II/I ratio and p-Akt protein expression in myocardium. We concluded that the antioxidants reduced autophagic flux in cardiomyocytes under the basal or nutrient deprivation conditions, suggesting that endogenous ROS promote autophagy flux under physiological conditions, and this effect is mediated, at least in part, through Akt inhibition.

Key words: Reactive oxygen species; endogenous; autophagy; cardiomyocytes; rats

Introduction

Autophagy is a degradation process in which cytosolic proteins and organelles are degraded and recycled through lysosomes(Lavandero *et al.*, 2013). It occurs in the normal myocardium and plays an important role in maintaining cardiac structure and function. Inefficient autophagy or its absence causes cardiac dysfunction and dilation(Nakai *et al.*, 2007). Increasing evidence suggests that autophagy is activated during various pathologic conditions in the heart. For example, myocyte autophagy is activated during acute myocardial ischemia, myocardial ischemia and reperfusion(Matsui *et al.*, 2007), myocardial infarction(Kanamori *et al.*, 2011), cardiac hypertrophy and heart failure(Zhu *et al.*, 2007). The function of autophagy in the heart appears to be stimulus dependent.

Several mechanisms mediate myocyte autophagy. One of these mechanisms is the production of reactive oxygen species (ROS). Antioxidant N-2-mercaptopropionyl glycine attenuates myocyte autophagy during myocardial ischemia and reperfusion, suggesting oxidative stress mediates myocyte autophagy(Hariharan *et al.*, 2011). In cardiac myocyte in vitro, glucose deprivation-stimulated ROS induce autophagy(Marambio *et al.*, 2010). However, little is known about the influences of normal physiological levels of endogenous ROS on cardiac myocyte autophagy. We hypothesize that physiological levels of ROS production in cardiac myocytes play an important role in inducing autophagy. In the present study, we first evaluate the functional roles of endogenous ROS in the regulation of basal and moderate stress-triggered autophagy in cardiomyocytes.

The sources of ROS include mitochondrial respiration, NADPH oxidase and xanthine Oxidase(Santos *et al.*, 2016). NADPH oxidase is the major source of ROS in cardiomyocytes. However, the contribution of the NADPH oxidase-derived ROS to the regulation of basal

autophagy in cardiac myocytes remains to be fully elucidated. Therefore, the second objective of this study is to evaluate the functional roles of NADPH oxidase-derived ROS in the regulation of basal autophagy in cardiomyocytes.

The mechanisms through which ROS regulate autophagy have not been fully understood. High levels of ROS during myocardial ischemia and reperfusion induce myocyte autophagy mediated by Beclin1 expression(Hariharan *et al.*, 2011). A high dose of hydrogen peroxide (H_2O_2) induces myocyte autophagy in association with decreased phosphorylated mTOR protein expression and increased phosphorylated AMPK expression(Essick *et al.*, 2013). However, it is unknown about the mechanisms of endogenous ROS-regulated myocyte autophagy. Accordingly, the third objective of this study is to determine the signaling transduction pathways of physiological levels of ROS-regulated myocyte autophagy.

Material and methods

H9C2 cardiomyocyte culture

The H9C2 cardiomyocytes were cultured in 75 cm^2 flask in Dulbecco's modified Earle's medium (DMEM) (Gibco, Life Technologies, USA) containing 4.5 g/L D-glucose, 3.7 g/L sodium bicarbonate and 110 mg/L sodium pyruvate, supplemented with 10% fetal bovine serum (Australia) and penicillin (100 units/ml) and streptomycin (100 μg/ml) in a humidified incubator with 95% air and 5% CO_2 at 37 °C, as described in our previous study(Li *et al.*, 2015). After 4 to 5 days, cells were passaged at a 1:5 ratio and seeded at the density of 0.15×10^6 cells per 35-mm well of six-well plates. These cells were cultured for 4 days and then performed treatments.

H_2O_2 treatment

H9C2 cardiomyocytes were exposed to 10, 100, and 1000 μM of H_2O_2 (Sigma-Aldrich, St. Louis, MO) for 60 min or phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin used as a control.

Antioxidant treatment

H9C2 cardiomyocytes were incubated with N-acetyl-cysteine (NAC) (Sigma-Aldrich, St. Louis, MO) at 2 mM or tempol (Sigma-Aldrich, St. Louis, MO) at 10 mM for 6 h. PBS containing 0.1% bovine serum albumin was used as a control in all protocols. The dosage for NAC and tempol used in this study was based on the previous studies(Peng *et al.*, 2011;Rahman *et al.*, 2014). NAC is a precursor of the cellular antioxidant glutathione. Tempol is a membrane-permeable piperidine nitroxide that mimics SODs by dismuting O_2^- anions. Tempol also detoxifies redox-reactive transition metal ions and directly reacts with many ROS forming adducts.

NADPH oxidase inhibitor apocynin treatment

H9C2 cardiomyocytes were treated with apocynin (100 μmol/L) (Sigma-Aldrich, St. Louis, MO) for 6 h. DMSO (0.02%) was used as control. The dose used in this study was based on the previous report(Qin *et al.*, 2007).

Nutrient deprivation for induction of autophagy

H9C2 cardiomyocytes were cultured in glucose-and serum-free DMEM for 6 h (a nutrient deprivation condition) for induction of autophagy.

Autophagic flux measurements in vitro

H9C2 cardiomyocytes were incubated in DMEM containing vehicle (PBS), NAC, tempol, PBS plus a lysosomal inhibitor chloroquine, NAC plus chloroquine or tempol plus chloroquine for

6 h (a basal condition) or glucose-and serum-free DMEM containing vehicle (PBS), NAC, tempol, PBS plus chloroquine, NAC plus chloroquine or tempol plus chloroquine for 6 h (a nutrient deprivation condition). LC3B-II protein detection was performed using Western blot. The autophagic flux was assessed by the lysosomal inhibitor chloroquine(Rahman *et al.*, 2014), and determined by calculating the difference between absolute LC3B-II protein levels measured in the presence and absence of chloroquine in H9C2 cardiomyocytes preincubated with vehicle, NAC or tempol.

Effects of antioxidants on myocyte autophagy in vivo

The protocol for the care and use of all animals in this study was approved by the Shanxi Medical University Committee on Animal Resources and conformed to "the guiding principles in the Care and Use of Animals" of the American Physiological Society and the Guide for the Care and Use of Laboratory Animals as outlined by the National Research Council and the US National Institutes of Health Publication No. 85-23, revised 1996. Male Sprague-Dawley rats weighing 180-200g were obtained from the Academy of Military Medical Sciences. All animals had access to the standard laboratory diet and drinking water. The animals were randomized to receive an antioxidant NAC (Sigma-Aldrich, St Louis, MO) (500mg/kg/day) or normal saline treatment for 2 weeks (n=6). The dose used in this study was based on the previous reports(Grieve *et al.*, 2006).

Western blot

H9C2 cardiomyocytes or left ventricular myocardial tissue were homogenized in a lysis buffer containing 1 M Tris-HCl (pH 7.4), 0.25 M sucrose, 0.5 M EDTA (pH 8.0), 100 mM dithiothreitol, 100 mM phenylmethysulfoyl fluoride, apotinin (2.2 mg/ml), leupeptin (5 mg/ml). The homogenate was centrifuged at 12000 g for 30 min at 4 °C and the supernatant was collected

as protein extracts. Protein extracts (40-60 μg) were resolved by 12% SDS-polyacrylamide gel and transferred to polyvinylidene fluoride membranes. The blots were incubated overnight with rabbit anti-LC3 (dilution 1:500) (Novus, Biologicals, Littleton, CO), autophagy-related gene 5 (Atg5) (dilution 1:100), sequestosome 1 (SQSTM1/P62) (dilution 1:1000), adenosine 5'-monophosphate (AMP)-activated protein kinase (AMPK) (dilution 1:1000) and protein kinase B (Akt, a serine/threonine-specific protein kinase) (dilution 1:200) (Santa Cruz Biotechnology, Dallas, TX) polyclonal antibodies. Mouse anti-GAPDH monoclonal antibody (dilution 1:10000) (Abcam, Cambridge, MA) was used to confirm equal loading conditions. The blots were then incubated with the secondary antibodies goat anti-rabbit IgG-HRP or goat anti-mouse IgG-HRP (dilution 1:2000). The Western blot bands were analyzed using the Image Lab™ acquisition and analysis software (Bio-Lab, Hercules, CA). The optical density of tissue samples was normalized to a control sample in an arbitrary densitometry unit.

Statistical analysis

Data are presented as mean ± SEM. Student's t test for unpaired data was used to determine the statistical significance of differences between the two means. The statistical significance of differences among groups was determined using analysis of variance and the Bonferroni correction for multiple comparisons. A value of $P < 0.05$ was considered statistically significant.

Results

The effects of exogenous H_2O_2 on autophagy in H9C2 cardiomyocytes

Evidence has accumulated that autophagy is activated under pathological conditions in the heart (Hariharan *et al.*, 2011). For example, autophagy is activated during myocardial ischemia and reperfusion and heart failure (Nakai *et al.*, 2007;Zhu *et al.*, 2007), which are associated with

increased oxidative stress(Burgoyne *et al.*, 2012). To determine whether increased oxidative stress regulates myocyte autophagy, H9C2 cardiomyocytes were treated with exogenous H_2O_2 (10, 100 or 1000 μM) for 60 min. Figure 1A and B show that H_2O_2 at 10 μM had no effect on the ratio of LC3 II to LC3 I proteins, a marker of autophagy, in H9C2 cardiomyocytes, but H_2O_2 at 100 or 1000 μM caused a marked increase in autophagy in H9C2 cardiomyocytes. Figure 1C and D show that the autophagic protein Atg5 expression had no change in H9C2 cardiomyocytes after exposure to 10 μM of H_2O_2, but Atg5 protein expression was significantly increased in H9C2 cardiomyocytes after exposure to 100 or 1000 μM of H_2O_2 These findings suggest that high concentrations of exogenous H_2O_2 significantly induce myocyte autophagy.

The role of endogenous ROS in autophagy in H9C2 cardiomyocytes

To determine the functional roles of endogenous ROS in the regulation of myocyte autophagy, two general antioxidants, NAC and tempol, were used to treat H9C2 cardiomyocytes. Under a basal condition, H9C2 cardiomyocytes were treated with NAC, tempol or placebo for 6 h. Figure 2A and B show that NAC induced a significant increase in the LC3 II/I ratio. Similarly, tempol also caused an increase in the LC3 II/I ratio (Figure 2C and D). These findings suggest that NAC or tempol triggers the accumulation of autophagosomes, which is the result of either increased synthesis or decreased degradation.

To determine whether NADPH oxidase is a major source of endogenous ROS which regulate autophagy, H9C2 cardiomyocytes were treated with apocynin, an inhibitor of NADPH oxidase or placebo for 6h. Figure 2E and F show that apocynin markly increased the LC3 II/I ratio. These results suggest that NADPH oxidase may be a major source of endogenous ROS which regulate myocyte autophagy.

To assess which of these two possibilities is more likely, we measured P62 protein expression and autophagic flux using the lysosomal inhibitor chloroquine (50 μM). Figure 2G and H show that tempol increased P62 protein expression in H9C2 cardiomyocytes under a basal condition. The autophagic flux was further determined by calculating the difference between absolute LC3B-II protein levels measured in the presence and absence of chloroquine in H9C2 cardiomyocytes preincubated with vehicle, NAC or tempol. Under a basal condition, in NAC preincubated H9C2 cardiomyocytes, autophagic flux was significantly lower than those measured in vehicle preincubated H9C2 cardiomyocytes (Figure 2I and J). Likewise, in tempol preincubated cardiomyocytes, autophagic flux under a basal condition was lower than those measured in vehicle preincubated H9C2 cardiomyocytes (Figure 2K and L). These results indicate that endogenous ROS promote myocyte autophagic flux under a basal condition.

Nutrient deprivation-induced ROS mediate autophagy in H9C2 cardiomyocytes

The nutrient deprivation has been shown to induce ROS(Sciarretta *et al.*, 2013). To further determine the role of endogenous ROS in the regulation of autophagy under moderate stress conditions, H9C2 cardiomyocytes were exposed to the glucose- and serum-free medium with or without NAC or tempol treatment. Figure 3A and B show that the nutrient deprivation induces an increase in the LC3 II/I ratio, and the increase was preserved by NAC. Tempol treatment caused a further increase in the LC3 II/I ratio in H9C2 cardiomyocytes subjected to the nutrient deprivation (Figure 3C and D).

To examine the effects of nutrient deprivation-induced ROS on autophagic flux, H9C2 cardiomyocytes were exposed to the glucose- and serum-free medium, and treated with vehicle, chloroquine (50 μM), NAC or chloroquine plus NAC for 6 h. Figure 3E and F show autophagic flux was significantly lower in the NAC treated group compared with those measured in the vehicle

treated group. These findings suggest that moderate stress-induced ROS promote autophagic flux in H9C2 cardiomyocytes.

The role of endogenous ROS in myocyte autophagy in rats in vivo

The role of endogenous ROS in the regulation of basal myocyte autophagy in vivo were evaluated by administering NAC to rats and by measuring changes in the levels of LC3 I and LC3 II proteins and the ratio of LC3 II to LC I proteins in myocardium. NAC treatment significantly increased the level of LC3 II protein and the ratio of LC3 II to LC3 I proteins (Figure 4 A and B).

The signal pathways of endogenous ROS in the regulation of myocyte autophagy

Autophagy is initiated under control of the energy sensor AMP activated protein kinase (AMPK) and the amino-acid sensor mammalian target of rapamycin complex I (mTOR)(Matsui *et al.*, 2007;Xiao *et al.*, 2015). Autophagy is inhibited by protein kinase B (Akt) activation(Hua *et al.*, 2011). AMPK, mTOR, and Akt are well known to be under the influence of ROS(Essick *et al.*, 2013;Horie *et al.*, 2008). To examine ROS regulation of p-AMPK and p-Akt activity, healthy adult male rats were treated with antioxidant NAC under a basal condition. Compared with the vehicle treated group, in the NAC treated group, p-AMPK protein had no change under the basal condition (Data not shown), but p-Akt protein expression was significantly increased under the basal condition (Figure 4C and D).

Discussion

In the present study, we have demonstrated that (1) in cultured H9C2 cardiomyocytes, under a basal condition, antioxidant NAC or tempol and NADPH oxidase inhibitor apocynin induced an increase in the ratio of LC3 II/I proteins; tempol increased P62 protein expression; NAC or tempol reduced LC3 II autophagic flux; (2) under a nutrient deprivation condition, the LC3 II/I ratio was

increased and the increase was preserved by NAC and promoted by tempol; under this condition, NAC also reduced LC3 II autophagic flux; (3) In rats in vivo, NAC treatment caused an increase in the LC3 II/I ratio in myocardium; (4) NAC treatment markedly increased p-Akt protein expression. These results suggest that endogenous ROS promote autophagy flux under physiological conditions, and this effect was mediated, at least in part, through Akt inhibition.

Autophagy has been shown to increase during myocardial ischemia and reperfusion and heart failure (Nakai *et al.*, 2007;Zhu *et al.*, 2007), which are associated with increased oxidative stress(Burgoyne *et al.*, 2012). In adult rat ventricular cardiomyocytes in culture, high concentrations of H_2O_2 increase autophagy(Hariharan *et al.*, 2011;Essick *et al.*, 2013). In H9C2 cardiomyocytes, high concentrations of H_2O_2 also induce autophagy(Hsu *et al.*, 2014). Our present study further confirmed these results showing that H_2O_2 induces autophagy as evidenced by increases in the ratio of LC3 II/I proteins and Atg5 protein expression in H9C2 cardiomyocytes at a dose-dependent manner. These findings suggest that exogenous ROS mediate myocyte autophagy.

Autophagy is either positively or negatively regulated by oxidative stress(Kubli & Gustafsson, 2012). However, there is no report about the role of endogenous ROS in myocyte autophagy. In the present study, we have found that antioxidant NAC or tempol increased the LC3 II/I ratio in cardiomyocyte in vitro and in myocardium in rats in vivo under a basal physiological condition. These results indicate that antioxidants trigger the accumulation of autophagosomes, which results from either increased synthesis or decreased degradation. P62, an adaptor protein that links aggregated proteins in autophagosomes and is degraded in autolysosomes, has been used to indicate autophagic flux(Matsui *et al.*, 2007). We have found that tempo increased P62 protein expression in cardiomyocytes under a basal condition, indicating impaired autophagic flux. We

have further found that NAC or tempol inhibited autophagic flux under the basal condition evaluated by the lysosomal inhibitor chloroquine. These findings suggest that endogenous ROS promote autophagic flux in cardiac myocytes under the basal physiological condition. These findings are supported by the previous studies showing that overexpression of catalase that detoxifies H_2O_2 tends to increase myocardial LC3 II/I although this study did not reach statistical significance and did not show the effect of catalase on autophagic flux(Turdi *et al.*, 2012). In agreement with the present study, endogenous ROS has been shown to promote autophagic flux in skeletal muscle cells(Rahman *et al.*, 2014). In addition, we have found that NADPH oxidase inhibitor apocynin increased the LC3 II/I ratio under a basal physiological condition, suggesting that NADPH oxidase may be a major source of endogenous ROS which regulate myocyte autophagy.

The levels of intracellular ROS increase in response to nutrient deprivation(Scherz-Shouval *et al.*, 2007). In the present study, we have found that nutrient deprivation increased the ratio of LC3 II/I proteins and the increase was preserved by NAC or enhanced by tempol treatment. We have further demonstrated that NAC or tempol treatment reduced autophagic flux. These results indicate that endogenous ROS induce autophagic flux under the nutrient deprivation condition. These findings are in consistent with the previous reports showing that starvation induces autophagic flux(Hariharan *et al.*, 2011). The notion is also supported by other report demonstrating that nutrient deprivation-derived ROS induce autophagic flux in skeletal muscle(Rahman *et al.*, 2014). Under glucose deprivation conditions, a study from Sadoshima's group has shown that NADPH oxidase subunit Nox4 promotes myocyte autophagy, suggesting that Nox4 mediates autophagy in response to energy stress in myocytes(Sciarretta *et al.*, 2013).

Studies have shown that AMPK/mTOR, Akt and Beclin1 are involved in the regulation of myocyte autophagy(Matsui *et al.*, 2007). AMPKα activation has been shown to inhibit mTOR activation and the inhibition of mTOR induces myocyte autophagy(Matsui *et al.*, 2007;Xiao *et al.*, 2015). In the present study, we have demonstrated that under the basal condition, NAC markedly increased Akt protein expression. These results indicate that endogenous ROS may inhibit Akt activity. Since Akt is an inhibitor of autophagy, these findings suggest that endogenous ROS promote myocyte autophagy probably through Akt inhibition.

The limitation of the present study is that we examined the role of endogenous ROS in myocyte autophagy using antioxidants. Further study is warranted to investigate the role of specific ROS such as superoxide and H_2O_2 in myocyte autophagy in cultured myocyte and transgenic animals. It is important to further elucidate the role of endogenous ROS-mediated autophagy on cell viability, metabolism and function.

In summary, antioxidant NAC or tempol decreased autophagic flux in cultured cardiomyocytes under the basal or nutrient deprivation conditions. NAC increased p-Akt protein expression. These findings suggest that endogenous ROS promote autophagy flux under physiological conditions, and this effect is mediated, at least in part, through Akt inhibition.

Conflicts of interest

There is no conflict of interest.

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Figure Legends

Figure 1. Effects of exogenous hydrogen peroxide (H_2O_2) on autophagy in H9C2 cardiomyocytes. **Panel A:** Representative Western blots of LC3 II and I proteins in H9C2 cardiomyocytes exposed to 10, 100 and 1000 μM of H_2O_2 or phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) as a control for 1 h. Equal loading of proteins is illustrated by GAPDH bands. **Panel B:** Respective group densitometry analysis. Values are means ± SEM; n=6. *P<0.01 vs. controls. **Panel C:** Representative Western blots of Atg5 protein in H9C2 cardiomyocytes exposed to 10, 100 and 1000 μM of H_2O_2 or PBS containing 0.1% BSA as a control for 1 h. Equal loading of proteins is illustrated by GAPDH bands. **Panel D:** Respective group densitometry analysis. Values are means ± SEM; n=6. *P<0.01 vs. controls.

Figure 2. Effects of antioxidants on autophagy in H9C2 cardiomyocytes under a basal condition. **Panel A:** Representative Western blot of LC3 II and I proteins in H9C2 cardiomyocytes treated with N-acetyl-cysteine (NAC) or saline as a control for 6 h. Equal loading of proteins is illustrated by GAPDH bands. **Panel B:** The ratio of LC3 II to LC3 I proteins. Values are means ± SEM; n=6. *P<0.001 vs. control. **Panel C:** Representative Western blot of LC3 II and I proteins in H9C2 cardiomyocytes treated with tempol or saline as a control for 6 h. Equal loading of proteins is illustrated by GAPDH bands. **Panel D:** The ratio of LC3 II to LC3 I proteins. Values are means ± SEM; n=6. *P<0.001 vs. control. **Panel E:** Representative Western blot of LC3 II and I proteins in H9C2 cardiomyocytes treated with NADPH oxidase inhibitor apocynin or saline as a control for 6 h. Equal loading of proteins is illustrated by GAPDH bands. **Panel F:** The ratio of LC3 II to

LC3 I proteins. Values are means ± SEM; n=6. *P<0.001 vs. control. **Panel G:** Representative Western blot of P62 protein in H9C2 cardiomyocytes treated with tempol or saline as a control for 6 h. Equal loading of proteins is illustrated by GAPDH bands. **Panel H:** Respective group densitometry analysis. Values are means ± SEM; n=6. *P<0.05 vs. controls. **Panel I:** Representative Western blot of LC3 II and I proteins in H9C2 cardiomyocytes treated with vehicle, NAC, chloroquine (CQ) and NAC plus CQ for 6 h. Equal loading of proteins is illustrated by GAPDH bands. **Panel J:** LC3 II autophagic flux in H9C2 cardiomyocytes treated with vehicle or NAC. Values are means ± SEM; n=6. *P<0.001 vs. vehicle. **Panel K:** Representative Western blot of LC3 II and I proteins in H9C2 cardiomyocytes treated with vehicle, tempol, chloroquine (CQ) and tempol plus CQ for 6 h. Equal loading of proteins is illustrated by GAPDH bands. **Panel L:** LC3 II autophagic flux in H9C2 cardiomyocytes treated with vehicle or tempol. Values are means ± SEM; n=6. *P<0.001 vs. vehicle.

Figure 3. Effects of antioxidants on autophagy in H9C2 cardiomyocytes under a nutrient deprivation (ND) condition. **Panel A:** Representative Western blot of LC3 II and I proteins in H9C2 cardiomyocytes in DMEM containing saline as a control or N-acetyl-cysteine (NAC) and in ND containing saline or NAC for 6 h. Equal loading of proteins is illustrated by GAPDH bands. **Panel B:** The ratio of LC3 II to LC3 I proteins. Values are means ± SEM; n=6. *P<0.01 vs. control. **Panel C:** Representative Western blot of LC3 II and I proteins in H9C2 cardiomyocytes in DMEM containing saline as a control or tempol and in ND containing saline or tempol for 6 h. Equal loading of proteins is illustrated by GAPDH bands. **Panel D:** The ratio of LC3 II to LC3 I proteins. Values are means ± SEM; n=6. *P<0.01 vs. control. **Panel E:** Representative Western blot of LC3 II and I proteins in H9C2 cardiomyocytes in ND treated with vehicle, NAC, chloroquine (CQ) and

NAC plus CQ for 6 h. Equal loading of proteins is illustrated by GAPDH bands. **Panel F:** LC3 II autophagic flux in H9C2 cardiomyocytes treated with vehicle or NAC. Values are means ± SEM; n=6. *P<0.01 vs. vehicle.

Figure 4. Effects of antioxidant N-acetyl-cysteine (NAC) on autophagy and Akt protein in myocardium in rats. **Panel A:** Representative Western blot of LC3 II and I proteins in rats treated with NAC or normal saline (NS) for 2 weeks. Equal loading of proteins is illustrated by GAPDH bands. **Panel B:** The ratio of LC3 II to LC3 I proteins. Values are means ± SEM; n=6. *P<0.001 vs. NS. **Panel C:** Representative Western blot of Akt protein in rats treated with NAC or NS for 2 weeks. **Panel D:** Respective group densitometry analysis. Values are means ± SEM; n=4. *P<0.05 vs. NS.

Figure 1

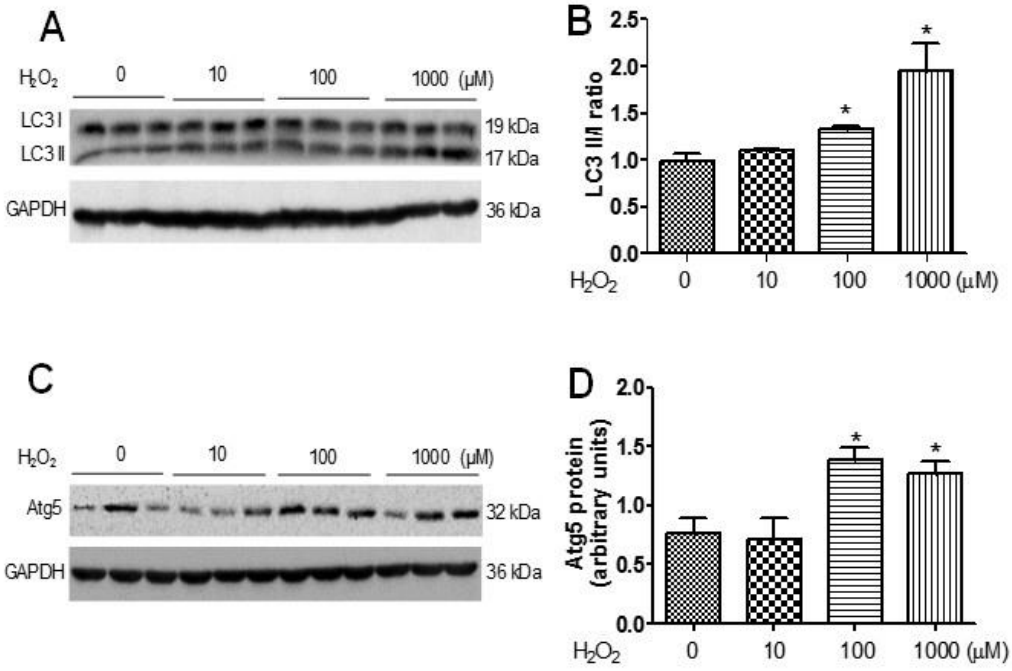

A
H2O2
0
10
100
1000 (μM)
LC3 I
LC3 II
19 kDa
17 kDa
GAPDH
36 kDa
B
LC3 II/I ratio
2.5
2.0
1.5
1.0
0.5
0.0
H2O2
0
10
100
1000 (μM)
C
H2O2
0
10
100
1000 (μM)
Atg5
32 kDa
GAPDH
36 kDa
D
Atg5 protein (arbitrary units)
2.0
1.5
1.0
0.5
0.0
H2O2
0
10
100
1000 (μM)

Figure 2

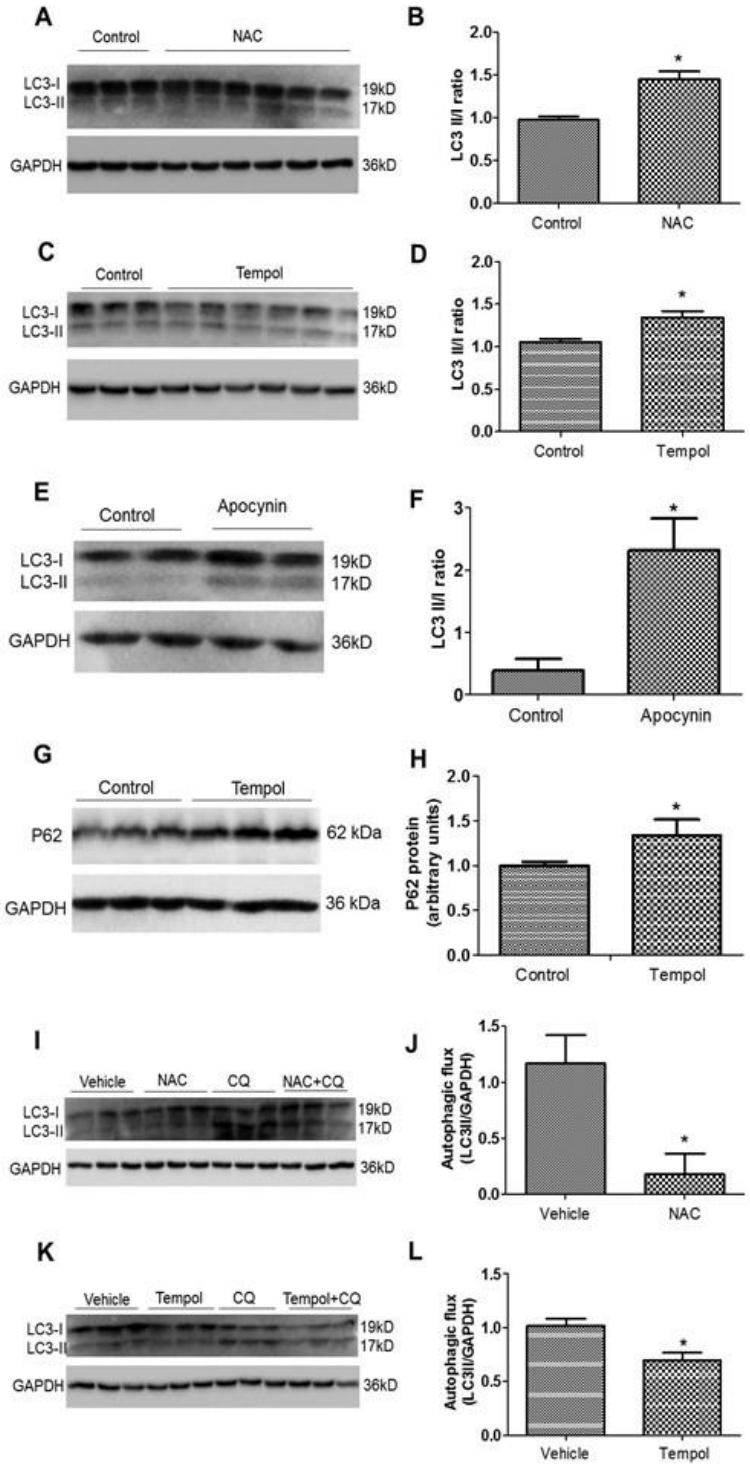

A
Control
NAC
LC3-I
LC3-II
19kD
17kD
GAPDH
36kD
B
LC3 II/I ratio
Control
NAC
C
Control
Tempol
D
E
Control
Apocynin
F
G
Control
Tempol
P62
62 kDa
GAPDH
36 kDa
H
P62 protein (arbitrary units)
I
Vehicle
NAC
CQ
NAC+CQ
J
Autophagic flux (LC3II/GAPDH)
K
Vehicle
Tempol
CQ
Tempol+CQ
L

Figure 3

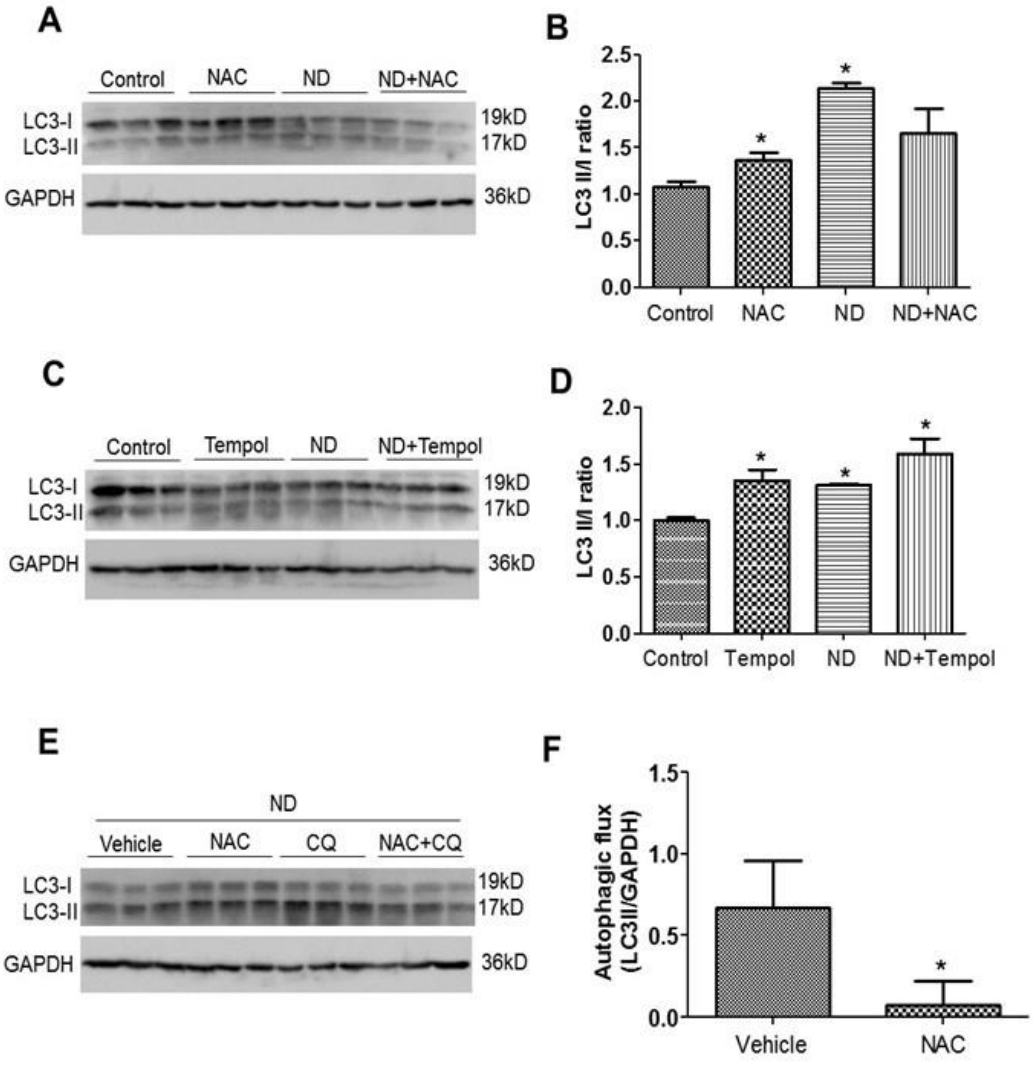
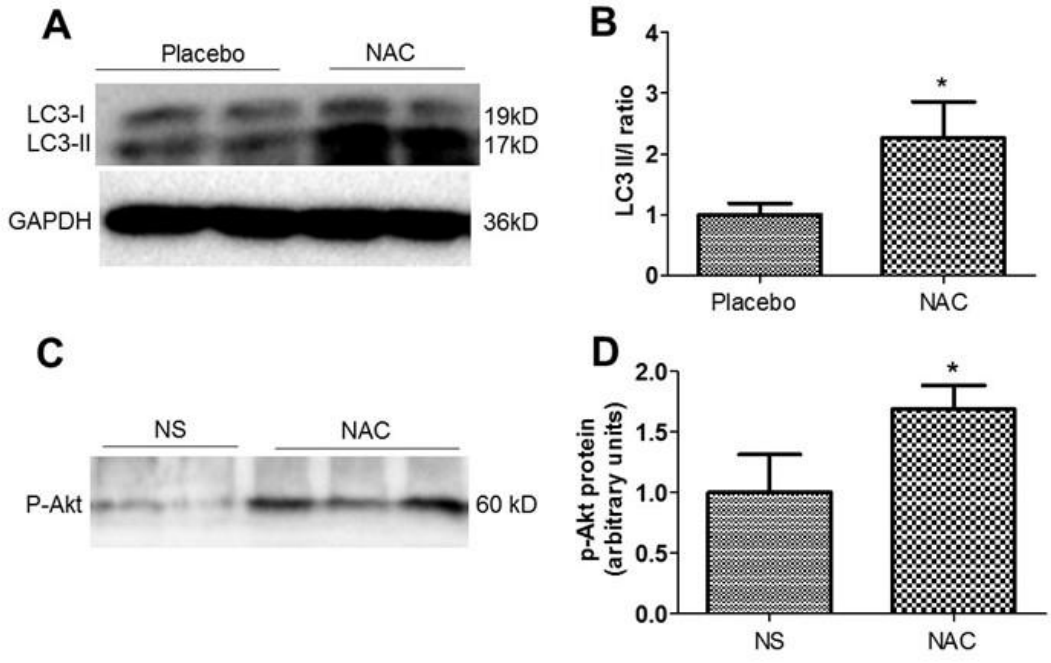

A
Control
NAC
ND
ND+NAC
LC3-I
LC3-II
19kD
17kD
GAPDH
36kD
B
LC3 II/I ratio
2.5
2.0
1.5
1.0
0.5
0.0
Control
NAC
ND
ND+NAC
C
Control
Tempol
ND
ND+Tempol
LC3-I
LC3-II
19kD
17kD
GAPDH
36kD
D
LC3 II/I ratio
2.0
1.5
1.0
0.5
0.0
Control
Tempol
ND
ND+Tempol
E
ND
Vehicle
NAC
CQ
NAC+CQ
LC3-I
LC3-II
19kD
17kD
GAPDH
36kD
F
Autophagic flux
(LC3II/GAPDH)
1.5
1.0
0.5
0.0
Vehicle
NAC

Figure 4


A
Placebo
NAC
LC3-I
LC3-II
19kD
17kD
GAPDH
36kD
B
LC3 II/I ratio
4
3
2
1
0
*
Placebo
NAC
C
NS
NAC
P-Akt
60 kD
D
p-Akt protein (arbitrary units)
2.0
1.5
1.0
0.5
0.0
*
NS
NAC