

Oxidative Stress Stimulates Autophagic Flux During Ischemia/Reperfusion

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Abstract

Autophagy is a bulk degradation process in which cytosolic proteins and organelles are degraded through lysosomes. To evaluate autophagic flux in cardiac myocytes, we generated adenovirus and cardiac-specific transgenic mice harboring tandem fluorescent mRFP-GFP-LC3. Starvation significantly increased the number of mRFP-GFP-LC3 dots representing both autophagosomes and autolysosomes per cell, suggesting that autophagic flux is increased in cardiac myocytes. H_2O_2 significantly increased autophagic flux, which was attenuated in the presence of *N*-2-mercaptopropionyl glycine (MPG), an antioxidant, suggesting that oxidative stress stimulates autophagy in cardiac myocytes. Myocardial ischemia/reperfusion (I/R) increased both autophagosomes and autolysosomes, thereby increasing autophagic flux. Treatment with MPG attenuated I/R-induced increases in oxidative stress, autophagic flux, and Beclin-1 expression, accompanied by a decrease in the size of myocardial infarction (MI)/area at risk (AAR), suggesting that oxidative stress plays an important role in mediating autophagy and myocardial injury during I/R. MI/AAR after I/R was significantly reduced in *beclin1*^{+/-} mice, whereas *beclin1*^{+/-} mice treated with MPG exhibited no additional reduction in the size of MI/AAR after I/R. These results suggest that oxidative stress plays an important role in mediating autophagy during I/R, and that activation of autophagy through oxidative stress mediates myocardial injury in response to I/R in the mouse heart. *Antioxid. Redox Signal.* 14, 2179–2190.

Introduction

AUTOPHAGY is a bulk degradation process in which cytosolic proteins and organelles are sequestered by double-membrane structures of unknown origin called autophagosomes, transferred to lysosomes, and degraded by proteases therein (14). Autophagy is generally an adaptive mechanism by which amino acids and fatty acids are recycled for ATP production, and damaged organelles, protein aggregates, and exogenous pathogens, all of which could lead to cellular malfunction and cell death, are removed (15). However, excessive activation of autophagy can be toxic and may even induce cell death, termed type II programmed cell death (15).

Increasing lines of evidence suggest that autophagy is activated during various pathologic conditions in the heart (25). For example, both chronic and acute myocardial ischemia activate autophagy in the myocardium (18, 33), and autophagy also is activated during cardiac hypertrophy, atrophy, and heart failure (25). However, the function of autophagy in the heart appears to be stimulus dependent. For example, we showed previously that although autophagy can be protective during acute myocardial ischemia and chronic hibernation, it can be detrimental during myocardial reperfusion after a short period of ischemia [ischemia/reperfusion (I/R)] (18,

32). Because autophagy plays an important role in mediating survival and death of cardiac myocytes in a stimulus-dependent manner, it is important to determine whether autophagy is stimulated during various pathophysiologic conditions *in vivo* and to evaluate its functional significance.

Importantly, evaluating whether autophagy is stimulated is not an easy task, especially at the organ level *in vivo*. Autophagy is a dynamic process involving nucleation, autophagosome formation, fusion of autophagosomes to lysosomes, and degradation in lysosomes, each of which is governed by multiple signaling mechanisms (22). Although the assessment of autophagosome formation at a single time point may be used for evaluating the extent of autophagy, the results could be misleading because the extent of autophagosome formation often dissociates from the level of autophagic flux (14). Conversely, tandem fluorescent mRFP-GFP-LC3 (tf-LC3), recently developed by Kimura *et al.* (13), allows one to evaluate the extent of autophagosome and autolysosome formation simultaneously, because LC3 puncta labeled with both GFP and mRFP represent autophagosomes, whereas those labeled with mRFP alone represent autolysosomes. As GFP-LC3 mice developed by Mizushima and colleagues (23) have been a very useful tool for assessing the level of autophagosome formation in mice *in vivo*, we reasoned that

transgenic mice harboring tf-LC3 could also be a useful tool for conveniently evaluating the extent of autophagic flux in organs *in vivo*.

Here we generated transgenic mice with cardiac-specific expression of tf-LC3 (Tg-tf-LC3) and evaluated the effect of I/R on autophagic flux in the heart. Accumulating lines of evidence suggest that oxidative stress plays an important role in mediating autophagy in various cell types and organs (12). Because oxidative stress is an important cause of myocardial injury at the time of reperfusion (17), and because our previous results suggest that activation of autophagy during the reperfusion phase is detrimental (18), we hypothesized that oxidative stress activates autophagy during I/R and that activation of autophagy by oxidative stress plays an important role in mediating I/R injury in the heart. We tested (a) whether tf-LC3 can be used to evaluate autophagic flux in cardiac myocytes, (b) whether Tg-tf-LC3 can be used to evaluate the extent of autophagy during I/R in the mouse heart *in vivo*, and (c) whether oxidative stress plays an essential role in mediating autophagy and myocardial injury during I/R in the mouse heart *in vivo*.

Materials and Methods

Antibodies

Antibodies used in the study include those against LC3 (MBL Intl. Corp.; PD014), p62 (ARP Inc.; 03-GP62-C), Beclin1 (BD Biosciences; 612112), and α -Tubulin (Sigma-Aldrich; T6199).

Primary culture of cardiac myocytes

Primary cultures of left ventricular cardiac myocytes were prepared from 1-day-old Crl: (WI) BR-Wistar rats (Harlan Laboratories), as described previously (18). A cardiac myocytes-enriched fraction (>95%) was obtained by centrifugation through a discontinuous Percoll gradient (18). After 24 h of culture in gelatin-coated culture dishes, the media were changed to serum-free Dulbecco's modified Eagle's medium (DMEM)/F-12 (Mediatech Inc.). For amino acid deprivation (AAD), myocytes were washed 3 times with phosphate-buffered saline (PBS) and incubated with amino acid-free, serum-free Hanks Balanced Salt Solution (HBSS; Mediatech Inc.) for 2 h, as described previously (18).

Adenoviruses

The tf-LC3 plasmid construct (13) (courtesy of Dr Tamotsu Yoshimori, Osaka University, Osaka, Japan) was used to generate an adenovirus (Ad-tf-LC3) by using shuttle vector pDC316 and the Admax system (Microbix). Ad-LacZ was used as the control. Cardiac myocytes were transduced with 15 multiplicities of infection (MOIs) of adenovirus for 24 h.

Preparation of cell lysates and tissue homogenates

Protein lysates were prepared from myocytes cultured in 6-cm culture dishes by using boiled (95°C for 2 min) 2×SDS sample buffer containing 4% SDS, 20% glycerol, 120 mM Tris-HCl (pH 6.8), 0.01% bromophenol blue, and 5% β -mercaptoethanol. The protein samples were immediately boiled again at 95°C for 3 min.

Heart-tissue homogenates were prepared by using RIPA buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl,

0.1% SDS, 1% Igepal CA-630, and 0.5% sodium deoxycholate with protease inhibitors (Sigma; P8340) at a 1:400 dilution. After determining the protein concentration with BCA Assay (Thermo Scientific), equal amounts of proteins were loaded on SDS-PAGE gels with 6×sample buffer containing 0.35 M Tris-HCl (pH 6.8), 10.28% SDS, 36% glycerol, 0.01% bromophenol blue, and 5% β -mercaptoethanol.

Immunoblot analysis

In vivo samples containing 35 μ g of proteins were subjected to SDS-PAGE, as described previously (18). Densitometric analyses of the blots were carried out by using the public domain ImageJ program (NIH, Baltimore, MD).

Evaluation of fluorescent LC3 puncta

Cardiac myocytes cultured on coverslips were transduced with Ad-tf-LC3 at 15 MOI. Twenty-four hours after adenovirus transduction, the cells were washed with PBS, fixed with 4% paraformaldehyde, mounted with a reagent containing 4',6-diamidino-2-phenylindole (DAPI) (Vectashield; Vector Laboratories, Inc.), and viewed under a fluorescence microscope (Nikon Eclipse E800). The number of GFP and mRFP dots was determined by manual counting of fluorescent puncta in five fields from three different myocyte preparations with a 60×objective. The nuclear number was evaluated by counting the number of DAPI-stained nuclei in the same field. The number of dots per cell was obtained by dividing the total number of dots by the number of nuclei in each microscopic field.

For *in vivo* determination of the number of fluorescent LC3 dots, fresh heart slices were embedded with Tissue-Tek OCT compound (Sakura Finetechnical Co., Ltd.) and frozen at −80°C. Sections 10 μ m thick were obtained from the frozen tissue samples by using a cryostat (CM3050 S; Leica), air-dried for 30 min, fixed by washing in 95% ethanol for 10 min, mounted by using a reagent containing DAPI, and viewed under a fluorescence microscope.

Dihydroethidium staining

The method used to identify the presence of oxidative stress with dihydroethidium (DHE) fluorescence was described previously (1).

Transgenic mice

Transgenic mice with cardiac-specific expression of tf-LC3 (Tg-tf-LC3) were generated on an FVB background with the murine α -Myosin Heavy Chain promoter, kindly provided by Dr. J. Robbins (Children's Hospital, Cincinnati, OH). Non-transgenic (NTg) mice were used as controls. Beclin-1-heterozygous knockout (*beclin1*^{+/-}) mice (courtesy of Dr. Beth Levine, University of Texas Southwestern, Dallas, TX) were described previously (18). For starvation studies, Tg-tf-LC3 mice were starved for 24 h, following which the mice were sacrificed. All protocols concerning the use of animals were approved by the Institutional Animal Care and Use Committee at the University of Medicine and Dentistry of New Jersey.

Oxidative stress and antioxidant treatment *in vitro*

Cardiac myocytes were treated with 200 μ M hydrogen peroxide (H₂O₂) for 1 h and 1 mM N-2-mercaptothionyl

glycine (MPG) for 4 h to induce oxidative stress and antioxidant treatment, respectively.

I/R surgery and antioxidant treatment in vivo

The method of inducing I/R in mice was described previously (18). Mice generated on an FVB background were subjected to ischemia for 45 min and to reperfusion for either 2 or 24 h. Mice generated on a C57BL/6 background were subjected to 30-min ischemia followed by reperfusion for 2 or 24 h. MPG, 100 mg/kg, was injected intraperitoneally into mice 24 h and 1 h before occlusion of the artery.

Assessment of area at risk and infarct size

The method used to determine the area at risk (AAR) and infarct size after I/R by using triphenyltetrazolium chloride (TTC) staining was described previously (18).

Statistics

Data are expressed as mean \pm SEM. Statistical analyses between groups of two were conducted with the unpaired Student *t* test. Groups of three or more were analyzed with one-way ANOVA, followed by the Newman-Keuls multiple comparison test. A value of $p < 0.05$ was considered statistically significant.

Results

tf-LC3 is a useful tool for evaluating autophagic flux in cardiac myocytes

Autophagic flux being a dynamic process, it is insufficient to evaluate only autophagosome formation to determine the extent of autophagy. It is imperative to distinguish between increased autophagosome formation and decreased autophagosome clearance (14). tf-LC3 staining is a very useful tool for evaluating the extent of autophagic flux, as it helps to differentiate between autophagosomes and autolysosomes (13). GFP fluorescence is quenched in the acidic pH of the lysosomal compartment, thereby limiting the use of GFP-LC3 to the identification of autophagosomes. However, mRFP continues to fluoresce, and mRFP-LC3 can be used to identify both autophagosomes and autolysosomes. By using tf-LC3 and determining the number of red dots that overlay green dots and appear yellow in merged images, the number of autophagosomes can be evaluated. The red dots that do not overlay green dots and appear red in merged images indicate autolysosome formation (13).

To evaluate autophagic flux in cultured cardiac myocytes, we generated an adenovirus harboring tf-LC3 (Ad-tf-LC3). Cardiac myocytes were transduced with Ad-tf-LC3 for 24 h. Expression of Ad-tf-LC3 does not affect the level of endogenous LC3 (Fig. 1A). We used amino acid deprivation (AAD) as a model to study the regulation of autophagic flux in cultured cardiac myocytes. Expression of LC3-II was significantly increased after 2 h of AAD (Fig. 1B). Expression of p62, a polyubiquitin-binding protein known to be sequestered and degraded during autophagy (2, 26), was significantly decreased after AAD, indicating enhanced autophagy (Fig. 1B). We then evaluated the extent of autophagosome and autolysosome formation by using fluorescent LC3 puncta. The numbers of GFP and mRFP dots per cell were both significantly

increased after AAD (Fig. 1C and D). In the merged images, more free red dots than yellow dots were seen, indicating significantly increased autolysosome formation compared with autophagosomes (Fig. 1E), and suggesting that AAD increases autophagic flux. Thus, the results of the analysis using Ad-tf-LC3 well corroborate those obtained with conventional methods.

Generation of transgenic mice with cardiac-specific expression of tf-LC3 (Tg-tf-LC3)

To evaluate the level of autophagic flux in the heart *in vivo* with tf-LC3, we generated transgenic mice with cardiac-specific expression of tf-LC3 (Tg-tf-LC3). Expression of tf-LC3 does not affect the level of endogenous LC3 at baseline or in response to starvation for 24 h *in vivo* (Fig. 2A). Starvation increased the level of endogenous LC3-II in both Tg-tf-LC3 and non-transgenic (NTg) control mice (Fig. 2A). Starvation also increased both green and red dots in Tg-tf-LC3 mice, and the merged images showed that starvation significantly increased both yellow dots representing autophagosome formation and red dots representing autolysosome formation in the mouse heart (Fig. 2B–D). These results suggest that Tg-tf-LC3 mice are useful for evaluating autophagic flux in the heart *in vivo*.

Antioxidant MPG inhibits oxidative stress-induced autophagy in cardiac myocytes in vitro

Oxidative stress plays an important role in mediating myocardial cell death in response to stress, such as I/R (6). Oxidative stress also stimulates autophagy in some cell types (6, 12). To evaluate the effect of oxidative stress on autophagy, we treated cardiac myocytes with 200 μ M H₂O₂ for 1 h. H₂O₂ treatment significantly increased LC3-II and decreased p62, suggesting that oxidative stress stimulates autophagy in cardiac myocytes (Fig. 3A, B). In cardiac myocytes transduced with Ad-tf-LC3, H₂O₂ increased the number of green and red dots, and the merged images showed that H₂O₂ increased both yellow dots representing autophagosomes and red dots representing autolysosomes (Fig. 3C, D). These results suggest that oxidative stress enhances autophagic flux in cultured myocytes.

To understand further the role of oxidative stress in mediating autophagy in cardiac myocytes, we used the antioxidant MPG. MPG is an amino acid derivative that contains a sulfhydryl or thiol group, and is known to act as a free radical scavenger (21). Treatment of cardiac myocytes with 1 mM MPG for 4 h significantly attenuated H₂O₂-induced increases in LC3-II and decreases in p62 accumulation (Fig. 3A, B), and significantly reduced both autophagosome and autolysosome formation, as evaluated by Ad-tf-LC3 (Fig. 3C, D). Taken together, the antioxidant, MPG, inhibits oxidative stress-induced autophagy in cardiac myocytes.

MPG protects against myocardial reperfusion injury by partial inhibition of autophagy

Reperfusion of the ischemic myocardium increases production of reactive oxygen species (ROS), which play an important role in mediating reperfusion injuries (19). In mouse hearts subjected to 45 min of ischemia followed by 2 h of reperfusion, DHE fluorescence, an indicator of superoxide, was significantly

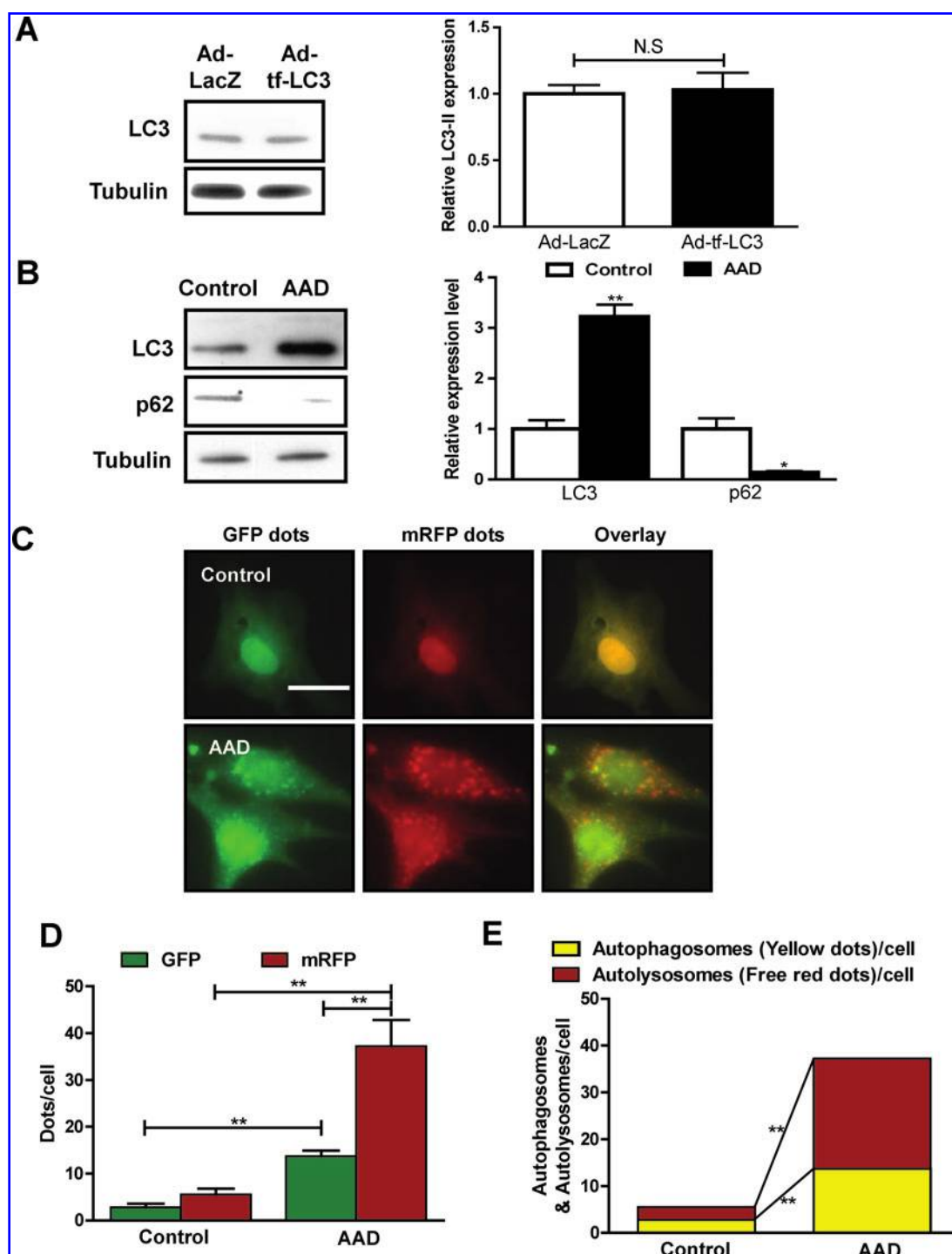


FIG. 1. tf-LC3 can be used to evaluate autophagic flux in cultured cardiac myocytes. (A) Neonatal rat ventricular myocytes were transduced with adenovirus harboring tf-LC3 (Ad-tf-LC3) or control Ad-LacZ for 24 h. Immunoblot and densitometric analyses indicating expression of LC3-II are shown. Tubulin was used as an internal control. (B) Cardiac myocytes were subjected to amino acid deprivation (AAD) by treatment with serum-free, amino acid-free Hanks balanced salt solution for 2 h. Immunoblot and densitometric analyses indicating expression of LC3-II, p62, and tubulin are shown. (C) Myocytes were transduced with Ad-tf-LC3 for 24 h and were subjected to AAD for 2 h. Representative images of fluorescent LC3 puncta are shown. (D) Mean number of GFP and mRFP dots per cell. (E) Mean number of autophagosomes (dots with both red and green color; *i.e.*, dots with yellow color in merged images) and autolysosomes (dots with only red but not green color; *i.e.*, dots with red color in merged images) per cell. Adenovirus was transduced at 15 MOI. Results represent the means from at least three independent experiments. * $p < 0.05$; ** $p < 0.01$; N.S., not significant. Scale bar represents 50 μm . (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

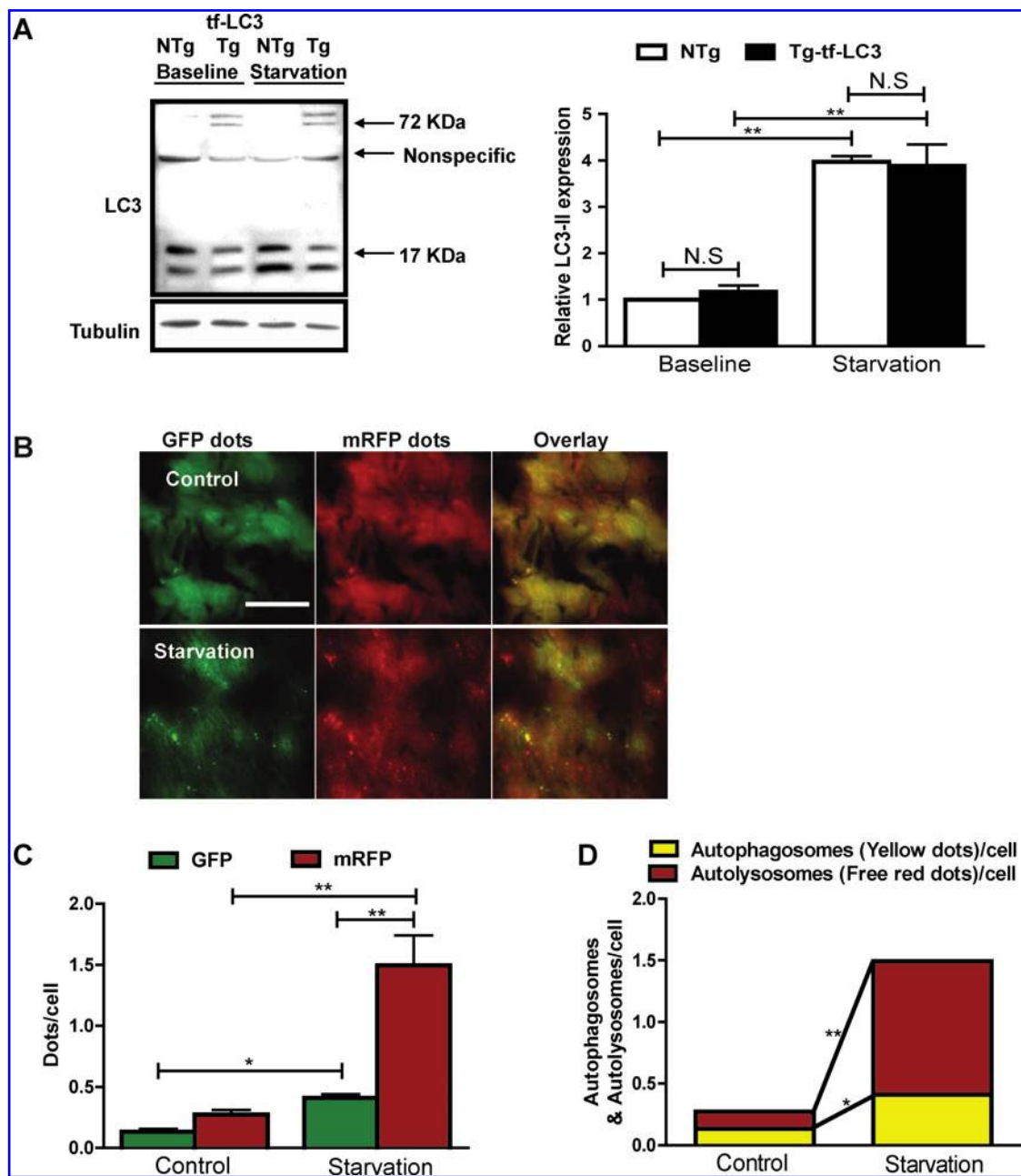


FIG. 2. Transgenic mice harboring tf-LC3 can be used to study autophagy in the heart. Transgenic mice harboring cardiac-specific expressed tf-LC3 (Tg-tf-LC3) were generated. Age-matched non-transgenic mice (NTg) were used as controls. The mice were starved for 24 h, after which they were sacrificed, and the hearts were removed for biochemical analyses. **(A)** Immunoblot analyses indicating expression of endogenous LC3 and mRFP-GFP-LC3, both at baseline and after 24-h starvation in the Tg-tf-LC3 and NTg mice. Bands of endogenous LC3-I and LC3-II were detected at ~17 kDa, and those of mRFP-GFP-LC3 were detected at ~72 kDa. A nonspecific band was observed at ~55 kDa. Quantitative analyses showed that starvation significantly increases the level of endogenous LC3-II in both NTg and Tg-tf-LC3. **(B)** Frozen sections were made from hearts of Tg-tf-LC3 mice at baseline and after starvation. Representative images of fluorescent LC3 puncta, GFP dots, mRFP dots, and their merged images are shown. **(C)** Mean numbers of green and red dots per cell. **(D)** Mean numbers of autophagosomes represented by yellow dots in merged images and autolysosomes represented by red dots in merged images per cell. Results represent the means from at least three mice. * $p < 0.05$; ** $p < 0.01$; N.S., not significant. Scale bar represents 50 μm . (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

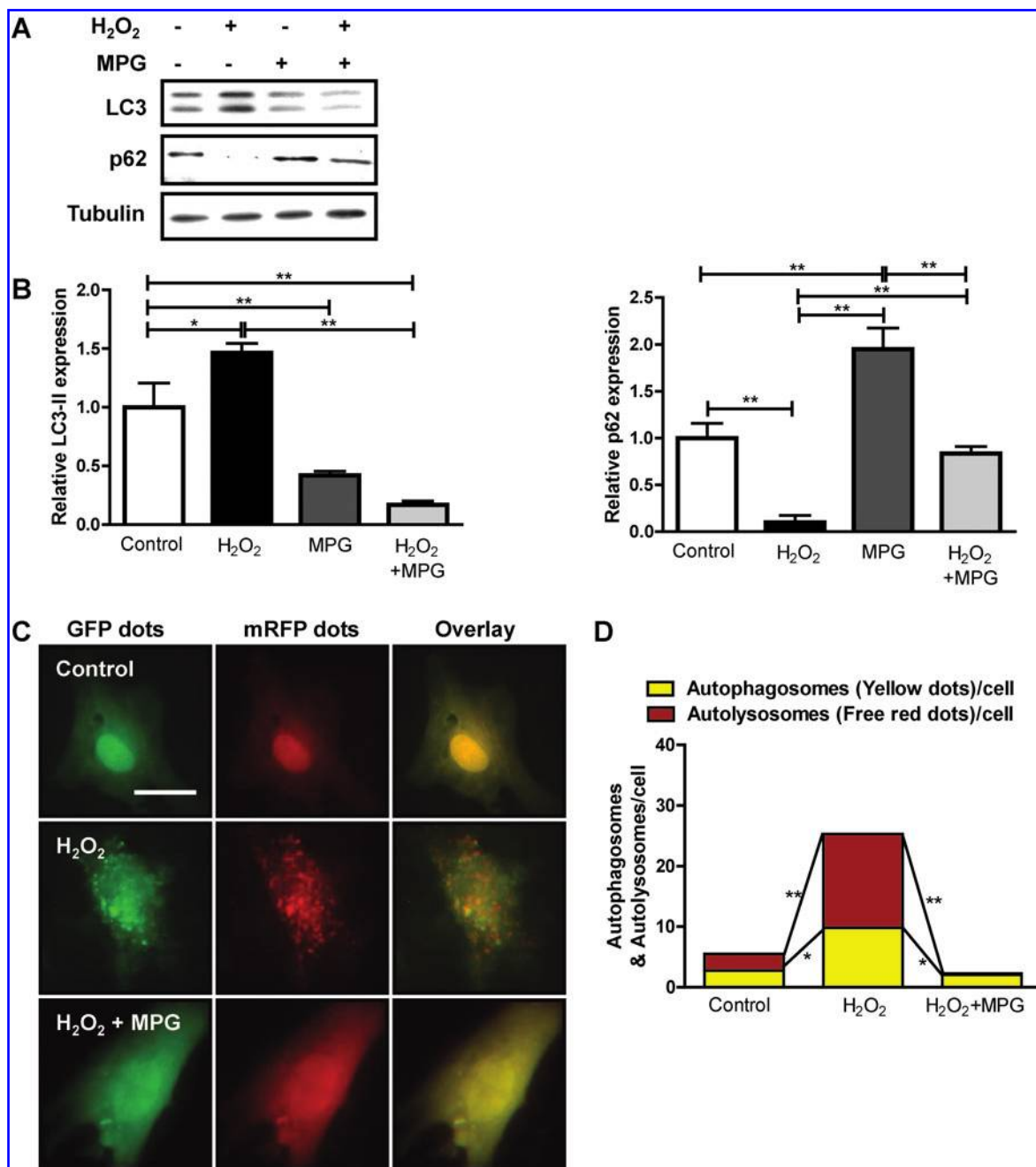


FIG. 3. N-2-Mercaptopropionyl glycine (MPG) inhibits hydrogen peroxide (H₂O₂)-induced autophagy *in vitro*. Cardiac myocytes were treated with 1 mM antioxidant, MPG, for 4 h, followed by 200 μ M H₂O₂ for 1 h. **(A)** Immunoblots indicating expression of LC3, p62, and tubulin. **(B)** Densitometric analyses. **(C)** Cardiac myocytes were transduced with 15 MOI of Ad-tf-LC3 for 24 h, after which they were treated with H₂O₂ in the presence or absence of MPG at the indicated doses and times. Representative images of fluorescent LC3 puncta are shown. Scale bar represents 50 μ m. **(D)** Mean number of autophagosomes represented by yellow dots in merged images and autolysosomes represented by red dots in merged images per cell. Results represent the means from at least three independent experiments. **p* < 0.05; ***p* < 0.01. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

increased (Fig. 4A). However, when mice were injected intraperitoneally with 100 mg/kg of the antioxidant, MPG, 24 h and 1 h before ischemia followed by reperfusion, the amount of oxidative stress was significantly decreased (Fig. 4A).

We showed previously that autophagy is robustly increased during I/R and has a detrimental effect (18). To evaluate the role of oxidative stress in mediating autophagy

during I/R, mice were subjected to I/R in the presence or absence of MPG. As previously reported, I/R significantly increased LC3-II and decreased p62 accumulation, indicative of increased autophagy (Fig. 4B, C). Although treatment with MPG increased the level of LC3-II, it also increased accumulation of p62, indicating that autophagic flux is inhibited (Fig. 4B, C). To evaluate further the formation of

autophagosomes and autolysosomes in cardiac myocytes during I/R, Tg-tf-LC3 mice were subjected to I/R in the presence and absence of MPG. I/R significantly increased red and green puncta, with more autolysosome formation, as evidenced by red puncta, than autophagosome formation, indicated by yellow puncta in merged images (Fig. 4D–F). Treatment with MPG partially decreased both green and red dots after I/R (Fig. 4D, E). Overlaid images showed that MPG significantly decreased I/R-induced increases in yellow dots and red dots, with a more prominent effect on red dots, indicating that MPG significantly decreased autophagic flux during I/R (Fig. 4F). These results suggest that an MPG-sensitive mechanism, most likely oxidative stress, plays an important role in mediating autophagy in response to I/R in the heart.

We showed previously that Beclin-1, a key molecule mediating autophagy, is significantly upregulated during I/R, and that autophagy is induced in a Beclin-1-dependent manner (18). Although MPG did not affect expression of Beclin-1 at baseline (data not shown), MPG significantly attenuated I/R-induced upregulation of Beclin-1 (Fig. 4G, H). Beclin-1 forms a complex with class III phosphatidylinositol 3-kinase and other autophagy-related proteins, and is localized to the pre-autophagosomal structure mediating vesicle nucleation (18, 28). However, Beclin-1 does not affect the conjugation of LC3-I with phosphatidylethanolamine, leading to LC3-II formation (18, 29). This may explain why treatment with MPG did not affect LC3-II accumulation, while significantly inhibiting autophagosome formation.

MPG plays a cytoprotective role during I/R in dog and mouse hearts (11, 20, 21). In mouse hearts subjected to 45-min ischemia and 24-h reperfusion, although no significant difference was found in the area at risk (AAR) (Fig. 5A, C), the size of myocardial infarction (MI)/AAR, as evaluated by triphenyltetrazolium chloride staining, was significantly decreased in the presence of MPG (Fig. 5A, B), suggesting that oxidative stress plays an important role in mediating I/R injury in our model, consistent with previous results (18).

Because MPG treatment inhibited both autophagic flux and myocardial injury, and because stimulation of autophagy during I/R could be detrimental (18), the MPG-induced reduction in infarct size may be mediated through its inhibition of autophagy. To test this hypothesis, we used *beclin1* heterozygous knockout mice (*beclin1*^{+/-}), in which autophagy during I/R is significantly attenuated (18). As shown previously, *beclin1*^{+/-} mice exhibited a significantly smaller size of MI/AAR after I/R (Fig. 5A, B), suggesting that Beclin-1-induced autophagy after I/R is detrimental (18). Although MPG significantly reduced the size of MI/AAR in control mice, it did not further reduce the size of MI/AAR in *beclin1*^{+/-} mice (Fig. 5A, B). Neither MPG nor downregulation of Beclin-1 significantly affected the size of AAR (Fig. 5C). These results are consistent with the notion that MPG inhibits I/R injury through suppression of autophagy. We confirmed that I/R-induced upregulation of Beclin-1 is suppressed both by MPG and in *beclin1*^{+/-} mice, either alone or in combination (Fig. 5D).

Discussion

In this study, we showed that tf-LC3 can be used to evaluate the level of autophagic flux in cardiac myocytes both *in vitro* and *in vivo*. By using Tg-tf-LC3, we demonstrated that

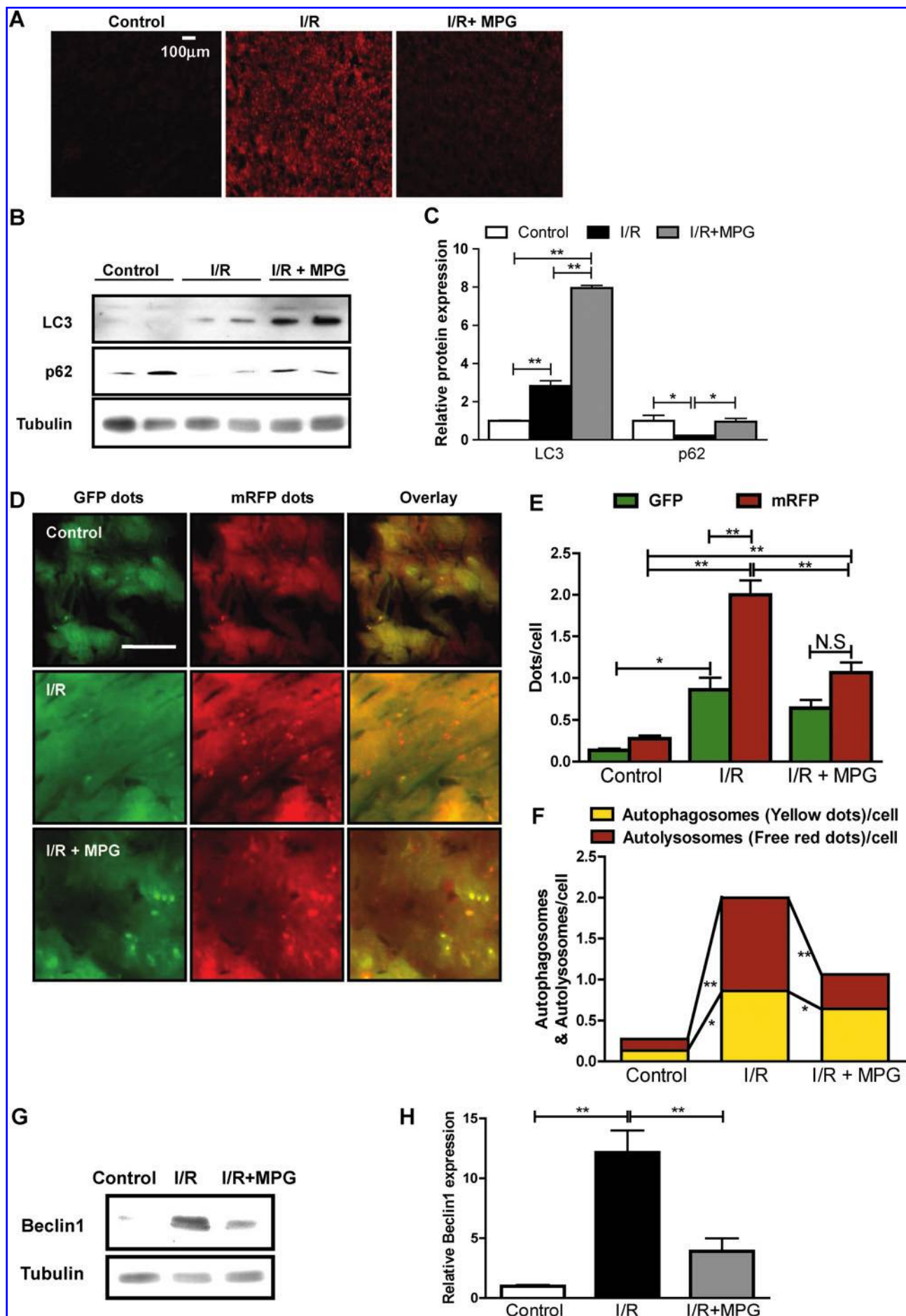
I/R increases autophagic flux in cardiac myocytes through an oxidative stress-dependent mechanism *in vivo*.

tf-LC3 is a useful tool for evaluating the formation of both autophagosomes and autolysosomes simultaneously in cardiac myocytes. The results of our experiments with tf-LC3 showed that AAR increases both autophagosomes and autolysosomes, an observation also supported by conventional indexes of autophagy, including increases in LC3-II and decreases in p62 accumulation in cardiac myocytes. Although autophagosomes accumulate when fusion between autophagosomes and lysosomes is inhibited, a concurrent increase in both autophagosomes and autolysosomes indicates that autophagic flux is stimulated (14). Although autophagic flux can be evaluated by comparing the extent of autophagosome accumulation in the presence or absence of inhibitors of lysosomal fusion or degradation or both, such interventions are usually toxic, which would confound analyses of the role of autophagy in mediating biologic functions *in vivo* (14, 24). Tg-tf-LC3 mice are useful not only in that the extent of autophagic flux can be monitored *in vivo*, but also in that one can investigate the effect of various interventions on formation of autophagosomes and autolysosomes independently.

Terada and colleagues (23, 31) previously reported double-transgenic mice generated by cross-breeding cardiac-specific mCherry-LC3 mice with systemic GFP-LC3 mice. Compared with those mice, our transgenic mice with cardiac-specific expression of a single transgene, tf-LC3, have several advantages. First, because either green or red color can be emitted from the same LC3 molecule in Tg-tf-LC3, more-accurate quantification of autophagosomes and autolysosomes can be made than in the cross between cardiac-specific mCherry-LC3 and systemic GFP-LC3 mice, in which the number of mCherry-LC3 and GFP-LC3 molecules are not identical and GFP-LC3 is not cardiac myocyte specific. Second, the mouse model reported by Terada requires cross-breeding between mCherry-LC3 mice and GFP-LC3 mice, whereas our model does not, because both GFP and mRFP are expressed in a single transgene. Finally, because tf-LC3 is expressed in a cardiac myocyte-specific manner in Tg-tf-LC3, our model allows one to quantitate the extent of autophagic flux specifically in cardiac myocytes.

With our unique mouse model, we here provide evidence that oxidative stress plays an important role in mediating autophagy during reperfusion (Fig. 6). Reperfusion causes various cellular responses relevant to autophagy, including oxidative stress, mitochondrial permeability transition pore (mPTP) opening, endoplasmic reticulum stress, Ca²⁺ overloading, and mitochondrial damage (7, 17, 30). However, to our knowledge, the role of oxidative stress in mediating increases in autophagic flux during I/R has not been clearly demonstrated. Our results suggest that increases in oxidative stress are both necessary and sufficient for inducing autophagy during I/R in cardiac myocytes.

Oxidative stress affects autophagy through multiple mechanisms. During starvation, H₂O₂ directly oxidizes HsAtg4 and inhibits its cysteine protease activity, thereby causing increased LC3 lipidation and autophagy in cancer cell lines (27). Oxidative stress affects lysosomal membrane permeability through disulfide bond formation in the lysosomal membrane proteins (6, 12). Autophagy is also activated as a compensatory response to damages in intracellular organelles (12). For example, increases in oxidative stress in mitochondria induce opening of the mPTP, leading to depolarization of



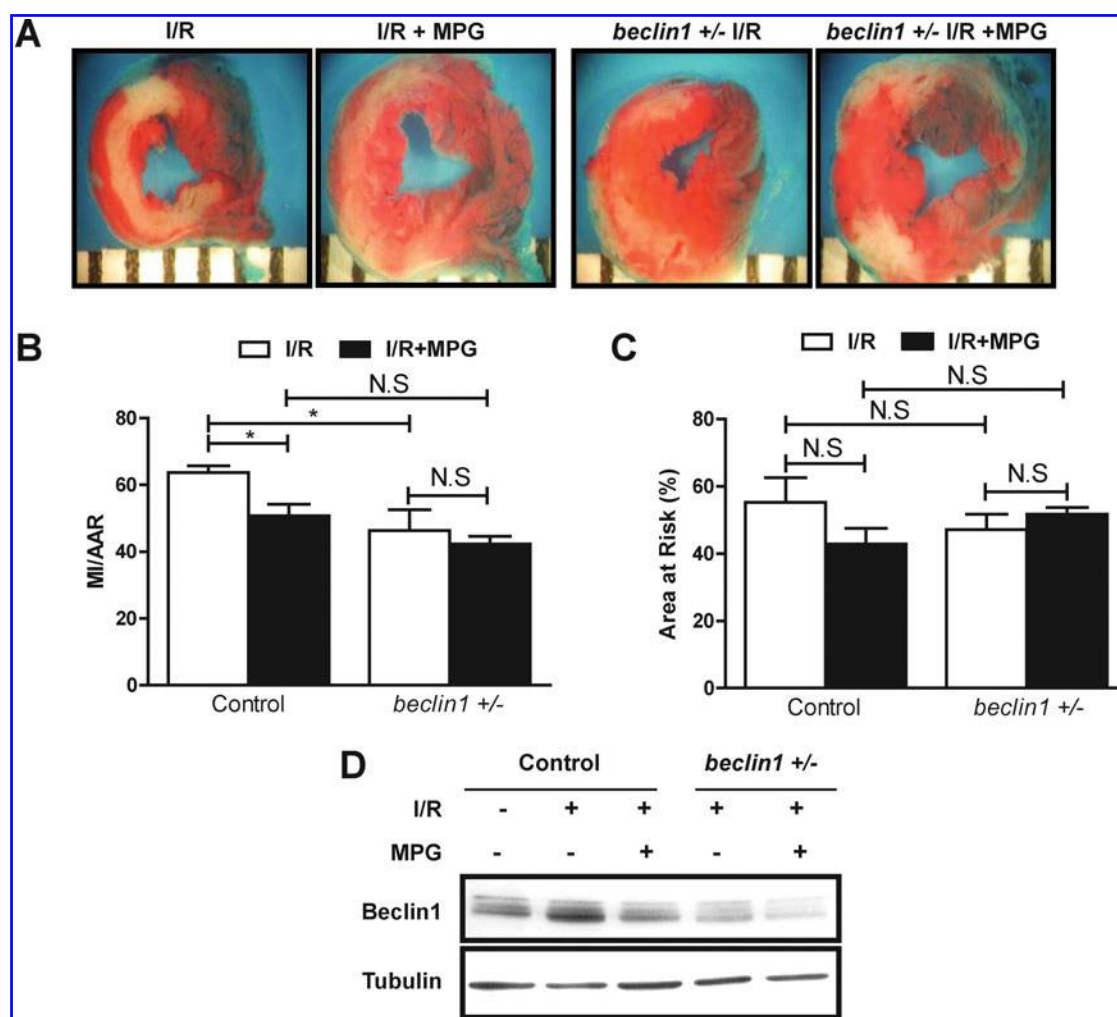


FIG. 5. MPG decreases infarction size after I/R through inhibition of autophagy. (A–C) The 3- to 4-month-old wild-type mice and *beclin1* heterozygous knockout mice (*beclin1*^{+/-}) bred on a C57 background were each subjected to 30 min of myocardial ischemia followed by 24 h of reperfusion. Antioxidant MPG (100 mg/kg) was injected intraperitoneally 24 h and 1 h before ischemia. Mice were sacrificed, and the hearts were removed. Myocardial infarction size was determined after staining with triphenyltetrazolium chloride (TTC). (A) Representative images of LV tissue sections from wild-type and *beclin1*^{+/-} mice subjected to IR in the presence or absence of MPG. (B) The myocardial infarction (MI) size/area at risk (AAR) expressed as a percentage. (C) The percentage of area at risk. (D) Mice were subjected to 30 min of ischemia followed by 2 h of reperfusion. Immunoblots indicating the expression of Beclin-1 and tubulin are shown. Results represent the means from at least three mice each. **p* < 0.05; ***p* < 0.01; N.S., not significant. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

FIG. 4. MPG partially inhibits autophagy after ischemia/reperfusion (I/R). The 3- to 4-month-old wild-type FVB strain mice were subjected to 45-min myocardial ischemia followed by 2-h reperfusion. Antioxidant MPG (100 mg/kg) was injected intraperitoneally 24 h and 1 h before ischemia. (A) Frozen sections were prepared from the mouse hearts and stained with DHE to evaluate the presence of oxidative stress. Scale bar indicates 100 μ m. (B) Immunoblots indicating expression of LC3, p62, and tubulin in mouse hearts subjected to I/R in the presence or absence of MPG. (C) Densitometric analyses. (D) Tg-tf-LC3 mice were subjected to I/R in the presence or absence of MPG. Representative images of fluorescent LC3 puncta are shown. Scale bar indicates 50 μ m. (E) Mean numbers of green and red dots per cell. (F) Mean numbers of autophagosomes represented by yellow dots in merged images and autolysosomes represented by red dots in merged images per cell. Results represent the means from at least three independent experiments. (G) Immunoblots indicating expression of Beclin-1 and tubulin after I/R in the presence or absence of MPG. (H) Densitometric analyses. Results represent the means from at least three mice each. **p* < 0.05; ***p* < 0.01; N.S., not significant. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

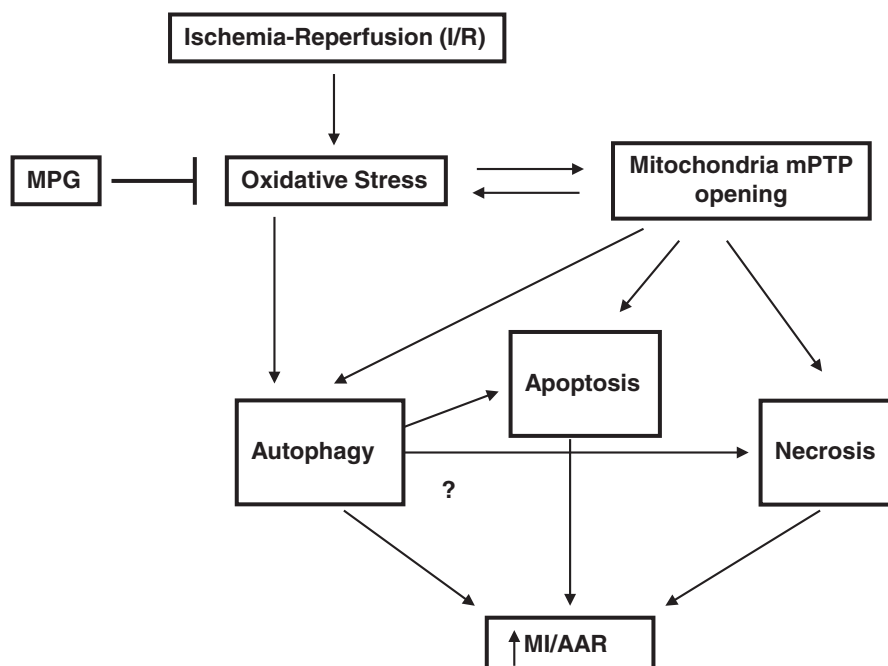


FIG. 6. Schematic representation of the role of oxidative stress in mediating autophagy and myocardial injury in response to I/R. Myocardial I/R stimulates autophagy through increases in oxidative stress. Stimulation of autophagy plays an important role in mediating myocardial injury.

the mitochondrial membrane potential, swelling, and damage to mitochondrial proteins (3, 5, 9). Cyclophilin D, a component of the mPTP, is involved in starvation-induced mitophagy in cardiac myocytes (4). BH3-only proteins, such as Bnip3, are upregulated during mPTP opening and mediate autophagy during I/R (10, 30). Oxidative stress also triggers ER stress, which, in turn, induces autophagy (17, 30).

Although the molecular mechanism by which oxidative stress induces autophagy during myocardial reperfusion remains to be elucidated, we showed previously that Beclin-1 is dramatically upregulated during the reperfusion phase in the mouse heart, which, in turn, plays an essential role in mediating increases in autophagy (18). Because MPG treatment significantly inhibited upregulation of Beclin-1 during the reperfusion phase, it is possible that oxidative stress induces autophagy through upregulation of Beclin-1. Whether the dramatic upregulation of Beclin-1 during the reperfusion phase is mediated through a transcriptional mechanism, and, if so, which transcription factor is involved in this process, remains to be elucidated.

Our results showed that MPG treatment inhibited formation of both autophagosomes and autolysosomes during reperfusion. Interestingly, we noted that the formation of the latter is more strongly affected. Although the precise mechanism by which this regulation takes place is yet to be identified, it is possible that mechanisms mediating autolysosome formation, such as the fusion of autophagosomes and lysosomes, may be sensitive to oxidative stress. Interestingly, a study by Li *et al.* (16) showed that MPG neutralizes 3-aminopropanol, a neurotoxin formed during cerebral ischemia that damages the lysosome.

Although oxidative stress activates various mechanisms leading to an increase in death of cardiac myocytes (19), we propose that oxidative stress induces reperfusion injury in part through stimulation of autophagy. Several lines of evidence support our hypothesis. First, suppression of oxidative stress inhibits autophagy during the reperfusion phase, and a

similar extent of autophagy suppression by Beclin-1 down-regulation was sufficient to reduce the level of reperfusion injury. Second, although MPG significantly reduced the size of MI/AAR in control mice, it did not further reduce the size of MI/AAR when autophagy is suppressed in *beclin1*^{+/-} mice, consistent with the notion that oxidative stress regulates cell survival/death through regulation of autophagy. Whether stimulation of autophagy can be detrimental during reperfusion is controversial (8, 18). Although our results indicating that MPG suppresses both autophagy and myocardial injury support the notion that autophagy during reperfusion is detrimental, further studies are needed to elucidate the functional significance of autophagy during I/R.

At present, the contribution of autophagic cell death to overall myocardial injury is unknown. Because I/R-induced increases in apoptosis are attenuated in *beclin1*^{+/-} mice, it is possible that autophagy and apoptosis may be linked. Although mPTP opening may initially induce both autophagy and apoptosis, severe depletion of ATP caused by mPTP opening may lead to necrosis as well. Whether strong activation of lysosomal degradation by autophagy contributes to necrotic cell death during I/R remains to be elucidated.

In summary, our results suggest that tf-LC3 is a useful tool for investigating the level of autophagic flux in the heart and the cardiac myocytes therein. With Tg-tf-LC3 mice, we showed that oxidative stress plays an important role in stimulating autophagic flux, which contributes to myocardial injury during I/R *in vivo*. We propose that suppression of oxidative stress during the reperfusion phase prevents myocardial injury in part through suppression of excessive autophagy.

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Author Disclosure Statement

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References

1. Ago T, Kuroda J, Pain J, Fu C, Li H, and Sadoshima J. Up-regulation of Nox4 by hypertrophic stimuli promotes apoptosis and mitochondrial dysfunction in cardiac myocytes. *Circ Res* 106: 1253–1264, 2004.
2. Bjorkoy G, Lamark T, Brech A, Outzen H, Perander M, Overvatn A, Stenmark H, and Johansen T. p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death. *J Cell Biol* 171: 603–614, 2005.
3. Bopassa JC, Michel P, Gateau-Roesch O, Ovize M, and Ferrera R. Low-pressure reperfusion alters mitochondrial permeability transition. *Am J Physiol Heart Circ Physiol* 288: H2750–H2755, 2005.
4. Carreira RS, Lee Y, Ghochani M, Gustafsson AB, and Gottlieb RA. Cyclophilin D is required for mitochondrial removal by autophagy in cardiac cells. *Autophagy* 6, 2010 [epub ahead of print].
5. Garcia-Dorado D, Agullo L, Sartorio CL, and Ruiz-Meana M. Myocardial protection against reperfusion injury: the cGMP pathway. *Thromb Haemost* 101: 635–642, 2009.
6. Gurusamy N and Das DK. Autophagy, redox signaling, and ventricular remodeling. *Antioxid Redox Signal* 11: 1975–1988, 2009.
7. Gustafsson AB and Gottlieb RA. Autophagy in ischemic heart disease. *Circ Res* 104: 150–158, 2009.
8. Gustafsson AB and Gottlieb RA. Eat your heart out: role of autophagy in myocardial ischemia/reperfusion. *Autophagy* 4: 416–421, 2008.
9. Halestrap AP. Calcium, mitochondria and reperfusion injury: a pore way to die. *Biochem Soc Trans* 34: 232–237, 2006.
10. Hamacher-Brady A, Brady NR, Logue SE, Sayen MR, Jinno M, Kirshenbaum LA, Gottlieb RA, and Gustafsson AB. Response to myocardial ischemia/reperfusion injury involves Bnip3 and autophagy. *Cell Death Differ* 14: 146–157, 2007.
11. Horwitz LD, Fennessey PV, Shikes RH, and Kong Y. Marked reduction in myocardial infarct size due to prolonged infusion of an antioxidant during reperfusion. *Circulation* 89: 1792–1801, 1994.
12. Kiffin R, Bandyopadhyay U, and Cuervo AM. Oxidative stress and autophagy. *Antioxid Redox Signal* 8: 152–162, 2006.
13. Kimura S, Noda T, and Yoshimori T. Dissection of the autophagosome maturation process by a novel reporter protein, tandem fluorescent-tagged LC3. *Autophagy* 3: 452–460, 2007.
14. Klionsky DJ, Abeliovich H, Agostinis P, Agrawal DK, Aliev G, Askew DS, Baba M, Baehrecke EH, Bahr BA, Ballabio A, Bamber BA, Bascham DC, Bergamini E, Bi X, Biard-Piechaczyk M, Blum JS, Bredesen DE, Brodsky JL, Brumell JH, Brunk UT, Bursch W, Camougrand N, Cebollero E, Cecconi F, Chen Y, Chin LS, Choi A, Chu CT, Chung J, et al. Guidelines for the use and interpretation of assays for monitoring autophagy in higher eukaryotes. *Autophagy* 4: 151–175, 2008.
15. Levine B and Kroemer G. Autophagy in the pathogenesis of disease. *Cell* 132: 27–42, 2008.
16. Li W, Yuan XM, Ivanova S, Tracey KJ, Eaton JW, and Brunk UT. 3-Aminopropanol, formed during cerebral ischaemia, is a potent lysosomotropic neurotoxin. *Biochem J* 371: 429–436, 2003.
17. Matsui Y, Kyo S, Takagi H, Hsu CP, Hariharan N, Ago T, Vatner SF, and Sadoshima J. Molecular mechanisms and physiological significance of autophagy during myocardial ischemia and reperfusion. *Autophagy* 4: 409–415, 2008.
18. Matsui Y, Takagi H, Qu X, Abdellatif M, Sakoda H, Asano T, Levine B, and Sadoshima J. Distinct roles of autophagy in the heart during ischemia and reperfusion: roles of AMP-activated protein kinase and Beclin 1 in mediating autophagy. *Circ Res* 100: 914–922, 2007.
19. Misra MK, Sarwat M, Bhakuni P, Tuteja R, and Tuteja N. Oxidative stress and ischemic myocardial syndromes. *Med Sci Monit* 15: RA209–RA219, 2009.
20. Mitsos SE, Askew TE, Fantone JC, Kunkel SL, Abrams GD, Schork A, and Lucchesi BR. Protective effects of N-2-mercaptopyrionyl glycine against myocardial reperfusion injury after neutrophil depletion in the dog: evidence for the role of intracellular-derived free radicals. *Circulation* 73: 1077–1086, 1986.
21. Mitsos SE, Fantone JC, Gallagher KP, Walden KM, Simpson PJ, Abrams GD, Schork MA, and Lucchesi BR. Canine myocardial reperfusion injury: protection by a free radical scavenger, N-2-mercaptopyrionyl glycine. *J Cardiovasc Pharmacol* 8: 978–988, 1986.
22. Mizushima N. Autophagy: process and function. *Genes Dev* 21: 2861–2873, 2007.
23. Mizushima N, Yamamoto A, Matsui M, Yoshimori T, and Ohsumi Y. In vivo analysis of autophagy in response to nutrient starvation using transgenic mice expressing a fluorescent autophagosome marker. *Mol Biol Cell* 15: 1101–1111, 2004.
24. Mizushima N, Yoshimori T, and Levine B. Methods in mammalian autophagy research. *Cell* 140: 313–326, 2006.
25. Nishida K, Kyo S, Yamaguchi O, Sadoshima J, and Otsu K. The role of autophagy in the heart. *Cell Death Differ* 16: 31–38, 2009.
26. Pankiv S, Clausen TH, Lamark T, Brech A, Bruun JA, Outzen H, Overvatn A, Bjorkoy G, and Johansen T. p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. *J Biol Chem* 282: 24131–24145, 2007.
27. Scherz-Shouval R, Shvets E, Fass E, Shorer H, Gil L, and Elazar Z. Reactive oxygen species are essential for autophagy and specifically regulate the activity of Atg4. *EMBO J* 26: 1749–1760, 2007.
28. Sinha S and Levine B. The autophagy effector Beclin 1: a novel BH3-only protein. *Oncogene* 27 (suppl 1): S137–S148, 2008.
29. Suzuki K, Kirisako T, Kamada Y, Mizushima N, Noda T, and Ohsumi Y. The pre-autophagosomal structure organized by concerted functions of APG genes is essential for autophagosome formation. *EMBO J* 20: 5971–5981, 2001.
30. Takagi H, Matsui Y, and Sadoshima J. The role of autophagy in mediating cell survival and death during ischemia and reperfusion in the heart. *Antioxid Redox Signal* 9: 1373–1381, 2007.
31. Terada M, Nobori K, Munehisa Y, Kakizaki M, Ohba T, Takahashi Y, Koyama T, Terata Y, Ishida M, Iino K, Kosaka T, Watanabe H, Hasegawa H, and Ito H. Double transgenic mice crossed GFP-LC3 transgenic mice with alphaMyHC-mCherry-LC3 transgenic mice are a new and useful tool to

- examine the role of autophagy in the heart. *Circ J* 74: 203–206, 2008.
32. Yan L, Sadoshima J, Vatner DE, and Vatner SF. Autophagy in ischemic preconditioning and hibernating myocardium. *Autophagy* 5: 709–712, 2009.
 33. Yan L, Vatner DE, Kim SJ, Ge H, Masurekar M, Massover WH, Yang G, Matsui Y, Sadoshima J, and Vatner SF. Autophagy in chronically ischemic myocardium. *Proc Natl Acad Sci U S A* 102: 13807–13812, 2005.

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Abbreviations Used

AAD = amino acid deprivation
AAR = area at risk
Ad = adenovirus
DAPI = 4',6-diamidino-2-phenylindole
DHE = dihydroethidium
GFP = green fluorescent protein
H₂O₂ = hydrogen peroxide
I/R = ischemia/reperfusion
MI = myocardial infarction size
MOI = multiplicities of infection
MPG = N-2-mercaptopyrionyl glycine
mPTP = mitochondrial permeability transition pore
mRFP = monomeric red fluorescent protein
NTg = non-transgenic
PBS = phosphate-buffered saline
ROS = reactive oxygen species
tf-LC3 = tandem fluorescent mRFP-GFP-LC3
Tg = transgenic
TTC = triphenyltetrazolium chloride

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1. Ramón Rodrigo, Juan C. Prieto, Rodrigo Castillo. 2013. Cardioprotection against ischaemia/reperfusion by vitamins C and E plus n -3 fatty acids: molecular mechanisms and potential clinical applications. *Clinical Science* **124**:1, 1-15. [[CrossRef](#)]
2. Xuejun Wang, Erin J.M. Terpstra. 2012. Ubiquitin Receptors and Protein Quality Control. *Journal of Molecular and Cellular Cardiology* . [[CrossRef](#)]
3. Wajihah Mughal, Rimpdy Dhingra, Lorrie A. Kirshenbaum. 2012. Striking a Balance: Autophagy, Apoptosis, and Necrosis in a Normal and Failing Heart. *Current Hypertension Reports* . [[CrossRef](#)]
4. Sebastiano Sciarretta, Peiyong Zhai, Massimo Volpe, Junichi Sadoshima. 2012. Pharmacological Modulation of Autophagy During Cardiac Stress. *Journal of Cardiovascular Pharmacology* **60**:3, 235-241. [[CrossRef](#)]
5. Youngil Lee, Hwa-Youn Lee, Åsa B. Gustafsson. 2012. Regulation of Autophagy by Metabolic and Stress Signaling Pathways in the Heart. *Journal of Cardiovascular Pharmacology* **60**:2, 118-124. [[CrossRef](#)]
6. Daniela Zablocki , Junichi Sadoshima . Angiotensin II and Oxidative Stress in the Failing Heart. *Antioxidants & Redox Signaling*, ahead of print. [[Abstract](#)] [[Full Text HTML](#)] [[Full Text PDF](#)] [[Full Text PDF with Links](#)]
7. Jisun Lee, Samantha Giordano, Jianhua Zhang. 2012. Autophagy, mitochondria and oxidative stress: cross-talk and redox signalling. *Biochemical Journal* **441**:2, 523-540. [[CrossRef](#)]
8. K. Przyklenk, Y. Dong, V. V. Undyala, P. Whittaker. 2012. Autophagy as a therapeutic target for ischaemia/reperfusion injury? Concepts, controversies, and challenges. *Cardiovascular Research* . [[CrossRef](#)]
9. Joseph A. Hill, Sergio Lavandero, Beverly A. Rothermel Autophagy in Cardiac Physiology and Disease 405-422. [[CrossRef](#)]
10. Dipak K. Das . 2011. Autophagy: A Story of Live or Let Die. *Antioxidants & Redox Signaling* **14**:11, 2163-2164. [[Citation](#)] [[Full Text HTML](#)] [[Full Text PDF](#)] [[Full Text PDF with Links](#)]