[Frontiers in Bioscience E2, 1147-1153, June 1, 2010]

Role of autophagy in myocardial reperfusion injury

Yulan Jin [1,2], Huihua Wang [3], Xun Cui [1], Yuanzhe Jin [1], Zhelong Xu [3]

[1]Department of Physiology, Yanbian University, Yanji, China, [2]Heart Institute, North China Coal Medical University, Tangshan, China, [3]Department of Anesthesiology, the University of North Carolina at Chapel Hill, NC 27599

TABLE OF CONTENTS

1. ABSTRACT

While autophagy is induced by myocardial ischemia/reperfusion, it is unclear whether autophagy is detrimental or beneficial to myocardial survival during ischemia/reperfusion. Isolated rat hearts were subjected to 30 min regional ischemia followed by 2 h of reperfusion. Autophagy was determined by the ratio of LC3 -II to LC3-I with Western blotting. Autophagy was prominent upon reperfusion but not during ischemia in rat hearts, indicating that autophagy may play a role during reperfusion phase. Ischemia or reperfusion did not enhance Beclin 1 expression, suggesting that Beclin 1 may not be critical for the formation of autophagy in isolated rat hearts. 3-methyladenine (3-MA), a classical inhibitor of autophagy, suppressed reperfusion-induced autophagy and reduced the infarct size when introduced at reperfusion. NECA, an agonist of adenosine receptors, and morphine also reduced the formation of autophagy as well as the infarct size when introduced at reperfusion. These data suggest that autophagy may play a detrimental role during reperfusion and that modulation of autophagy may prevent reperfusion injury in rat hearts.

2. INTRODUCTION

Autophagy is the major cellular mechanism for the degradation and recycling of long-lived proteins and organelles (1). The initial step in autophagy is the formation of double-membrane autophagosomes which include proteins and organelles to lysosomes. Then, the autophagosomes fuse with lysosomes to deliver the contents into lysosomes, where their contents are degraded by lysosomal hydrolases and the resulting macromolecules are recycled (2). Autophagy occurs at basal levels but also can be induced by some stress conditions such as starvation and hypoxia (2). Autophagy not only serves as a cell survival mechanism by maintaining cellular homeostasis but may also play a role in cell death (type II programmed cell death) (3).

Recent studies have shown that autophagy may play a role in cardiac cell life/death (1). It has been reported that autophagy may serve as a homeostatic mechanism by inhibiting apoptosis in chronically ischemic pig myocardium (1). In support, autophagy was also found to play a protective role in HL-1 myocytes in the context of

ischemia/reperfusion (6, 7). However, it has also been documented that autophagy plays a detrimental role in myocardial ischemia/reperfusion injury and inhibition of autophagy leads to cardioprotection. In H9c2 cells, inhibition of autophagy by 3-methyladenine (3-MA) and LY294002 prevents cell death during glucose deprivation (1). It has also been reported that urocortin can inhibit beclin 1 mediated autophagic cell death in cardiac myocytes subjected to ischemia and reperfusion (9). Furthermore, recently Matsui *et al.* have proposed that autophagy is protective during ischemia but is detrimental during reperfusion and that AMP-activated protein kinase (10) and beclin 1 may mediate autophagy in the setting of ischemia/reperfusion (11). Thus, it is still unclear whether autophagy is protective or detrimental in the context of ischemia/reperfusion in the heart. Yet, little is known about the detailed time course for the induction and progression of autophagy during ischemia/reperfusion.

In the present study, we first determined whether autophagy is induced by ischemia/reperfusion in isolated rat heats. We then sought to define the time point at which autophagy is initiated during ischemia/reperfusion. Third, we examined if inhibition of autophagy could reduce infarct size. Lastly, we tested if cardioprotective interventions can inhibit autophagy.

3. MATERIALS AND METHODS

This study conforms to the NIH Guide for the Care and Use of Laboratory Animals (NIH publication NO. 85-23, revised 1996).

3.1. Perfusion of isolated rat heart

Male Wistar rats (250-350 g) were anesthetized with thiobutabarbital sodium (100 mg/kg i.p.). The hearts were removed rapidly and mounted on a Langendorff apparatus. The hearts were perfused with Krebs-Henseleit buffer containing (in mM) 118.5 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.8 $CaCl_2$, 24.8 $NaHCO_3$, 1.2 KH_2PO_4, and 10 glucose, which was heated to 37°C and gassed with 95 % O_2/5 % CO_2. A latex balloon connected to a pressure transducer was inserted into the left ventricle through the left atrium. The left ventricular pressure and heart rate were continuously recorded with a PowerLab system (ADInstruments, Mountain View, CA). A 5-0 silk suture was placed around the left coronary artery, and the ends of the suture were passed through a small piece of soft vinyl tubing to form a snare. All hearts were allowed to stabilize for at least 20 min. Ischemia was induced by pulling the snare and then fixing it by clamping the tubing with a small hemostat. Total coronary artery flow was measured by timed collection of the perfusate dripping from the heart into a graduated cylinder.

3.2. Measurement of infarct size

At the end of each experiment, the coronary artery was reoccluded, and fluorescent polymer microspheres (2-9 μM diameter, Duke Scientific Corp) were infused to demarcate the risk zone as the tissue without fluorescence. The hearts were weighed, frozen and cut into 1 mm slices. The slices were incubated in 1 % triphenyltetrazolium chloride (TTC) in sodium phosphate buffer at 37ºC for 20 min. The slices were immersed in 10 % formalin to enhance the contrast between stained (viable) and unstained (necrotic) tissue and then squeezed between glass plates spaced exactly 1 mm apart. The myocardium at risk was identified by illuminating the slices with U.V. light. The infarcted and risk zone regions were traced on a clear acetate sheet and quantified with ImageTool. The areas were converted into volumes by multiplying the areas by slice thickness. Infarct size is expressed as a percentage of the risk zone.

3.3. Autophagy assay

Autophagy was monitored by measuring the LC3 (microtubule-associated protein 1 light chain 3)-II to LC3-I ratio (LC3-II/LC3-I) with Western blotting analysis (See below).

3.4. Western blot analysis

Myocardial samples taken from risk zones were homogenized in ice-cold lysis buffer. Equal amounts of protein were loaded and eletrophoresed on SDS-polyacrylamide gel and transferred to a PVDF membrane. Membranes were blocked with nonfat milk, and then incubated with primary antibodies (1:1000) at 4 ºC overnight. The primary antibody bindings were detected with a secondary anti-rabbit antibody (1:2000) and visualized by the ECL method.

3.5. Experimental protocols

All hearts were subjected to a 30 min regional ischemia followed by 2 h of reperfusion. Biopsies were collected from the risk zones during ischemia and reperfusion. Infusion of NECA, morphine or 3-MA was started 5 min before the onset of reperfusion and continued for 35 min. Infarct size was measured 2 h after start of reperfusion.

3.6. Statistical Analysis

Data are expressed as mean ± SEM and were obtained from 5 to 10 separate experiments. Statistical significance was determined using Student t-test or one-way ANOVA followed by Tukey's test. A value of $P < 0.05$ was considered as statistically significant.

4. RESULTS

To determine if ischemia or/and reperfusion induces autophagy in the heart, isolated rat hearts were subjected 30 min regional ischemia followed by 2 h of reperfusion . Myocardial biopsies were taken from the risk zone and autophagy was assessed by determining LC3-II to LC3-I ratio (LC3-II/LC3-I), an established indicator of autophagy (1). As shown in Figure 1, 30 min ischemia did not significantly increase LC3-II/LC3-I, implying that autophagy was not induced during ischemia. In contrast, LC3-II/LC3-I was significantly increased upon reperfusion (Figure 2), suggesting that reperfusion induces autophagy in perfused rat hearts. To confirm the finding that the increase in LC3-II/LC3-I upon reperfusion was of autophagic origin, we perfused hearts with 1 mM 3-methyladenine (3-MA), an inhibitor of autophagosome formation (1), at reperfusion. As shown in Figure 3, LC3

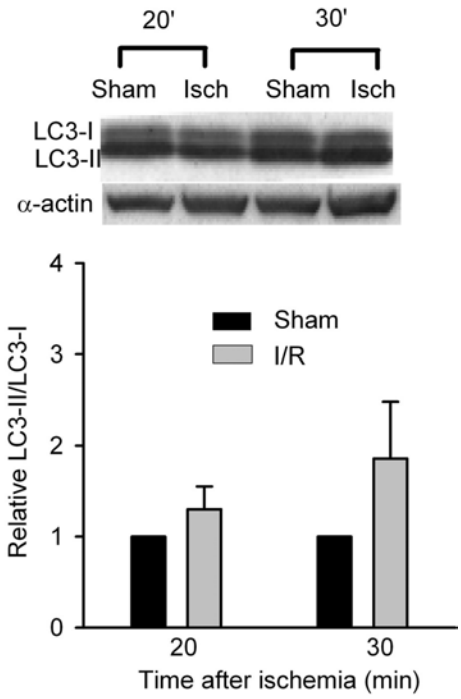

Figure 1. Western blotting analysis of LC3-I and LC3-II in isolated rat hearts. Ischemia (30 min) did not significantly increase LC3-II/LC3-I compared to the sham, indicating that autophagy is not prominent during ischemia. Data were obtained from 4 independent experiments.

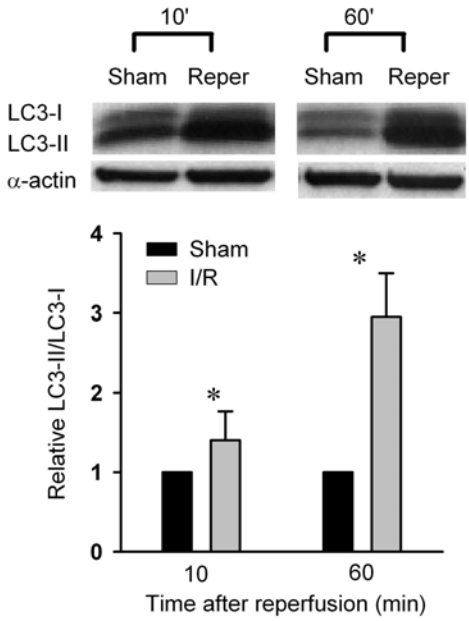

Figure 2. Western blotting analysis of LC3-I and LC3-II in isolated rat hearts. Reperfusion significantly increased LC3-II/LC3-I compared to the sham, indicating that reperfusion initiates autophagy in rat hearts. Data were obtained from 5 independent experiments. * $p<0.05$ vs. sham.

II/LC3-I was decreased by 3-MA, indicating the increase was due to the induction of autophagy.

Because Beclin 1 is a mammalian autophagy gene and has been reported to be involved in the mechanism of autophagy induced by myocardial ischemia/reperfusion (9), we then examined if ischemia/reperfusion could alter the expression of Beclin 1 protein in rat hearts. Unexpectedly, Beclin 1 protein expression was not up-regulated but was shown a tendency to decrease during both ischemia (Figure 4) and reperfusion (Figure 5), indicating that autophagy does not necessarily accompany upregulation of Beclin 1, at least in the isolated perfused rat heart model.

NECA (5'-N-ethylcarboxamidoadenosine) has been demonstrated to protect the heart from reperfusion injury by activating adenosine A_2 receptors. Interestingly, NECA (100 nM) prevented LC3-II/LC3-I increase during reperfusion (Figure 6), implying that prevention of autophagy may result in cardioprotection during reperfusion. Similarly, morphine (1 μM) given at reperfusion also reduced the LC3-II/LC3-I ratio (Figure 6).

To determine if autophagy is correlated with reperfusion injury, we tested the effects of 3-MA (1 mM), NECA (100 nM), and morphine (1 μM) which were shown to prevent autophagy formation, on myocardial infarction. As shown in Figure 7, 3-MA, NECA, and morphine significantly reduced infarct size compared to control. This result strongly suggests that autophagy contributes to reperfusion injury and may serve as a critical target of cardioprotective interventions during reperfusion.

5. DISCUSSION

Autophagy is active at basal conditions in most cells to regulate the turnover of long-lived proteins and remove damaged structure. Autophagy can also be induced by stresses, such as nutrient depletion (14). Autophagy has been proposed to be involved in myocardial homeostasis, aging, and pathology (1). Recent studies have indicated that autophagy is induced by acute myocardial ischemia/reperfusion, although its exact role in ischemia/reperfusion injury remains unclear. In isolated rat hearts subjected to 30 min of global ischemia followed by 2 h of reperfusion, autophagy was detected after reperfusion (1). A recent study by Sadoshima's group documented that autophagy was induced by ischemia and further enhanced by reperfusion in *in vivo* mouse hearts (11). They further found that autophagy during ischemia was accompanied by activation of AMP-activated protein kinase (10), whereas up-regulation of Beclin 1 accounted for reperfusion-induced autophagy. These data suggest that both ischemia and reperfusion can induce autophagy in mouse hearts. However, autophagy was evident during reperfusion but not during ischemia in rat cardiomyocytes exposed to simulated ischemia and reperfusion (9). Similarly, Hamacher-Brady *et al.* reported that autophagic flux was null during ischemic period but increased at reperfusion in cardiac HL-1 cells subjected to simulated ischemia/reperfusion, although the flux did not reach the

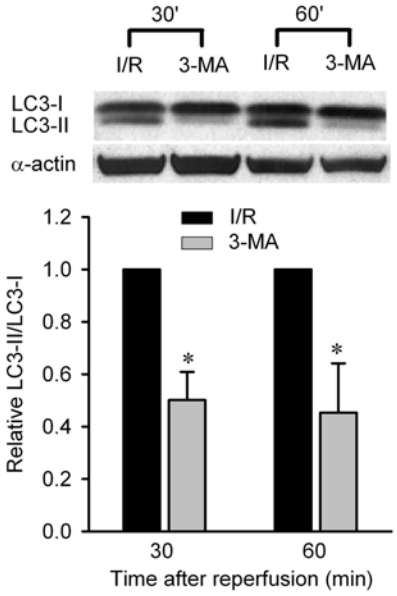

Figure 3. Western blotting analysis of LC3-I and LC3-II in isolated rat hearts. 3-MA (1 mM) given at reperfusion reduced LC3-II/LC3-I compared to I/R (ischemia/reperfusion) alone, indicating that reperfusion indeed induced autophagy in rat hearts. Data were obtained from 4 independent experiments. * $p<0.05$ vs. I/R.

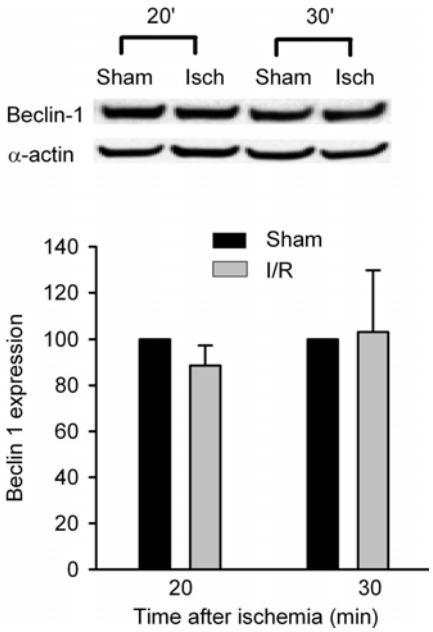

Figure 4. Western blotting analysis of Beclin 1 expression during ischemia in isolated rat hearts. Ischemia did not enhance Beclin 1 expression. Data were obtained from 4 independent experiments.

same degree as under normoxic conditions (1). These data indicate that reperfusion may accelerate the induction of autophagy in cardiomyocytes *in vitro*. In the present study, we found that autophagy was prominent upon reperfusion but not during the ischemic period, suggesting that autophagy may play a role in the reperfusion phase. Obviously, the current finding is distinguished from the above observation by Sadoshima's group. Although the reason for the difference is not clear, different experimental protocols and animal species may account for it.

While the above observations indicate that autophagy is induced by ischemia/reperfusion, it remains unclear whether autophagy is detrimental or protective during ischemia/reperfusion in the heart. In a recent study, Gottlieb's group found that simulated ischemia/reperfusion

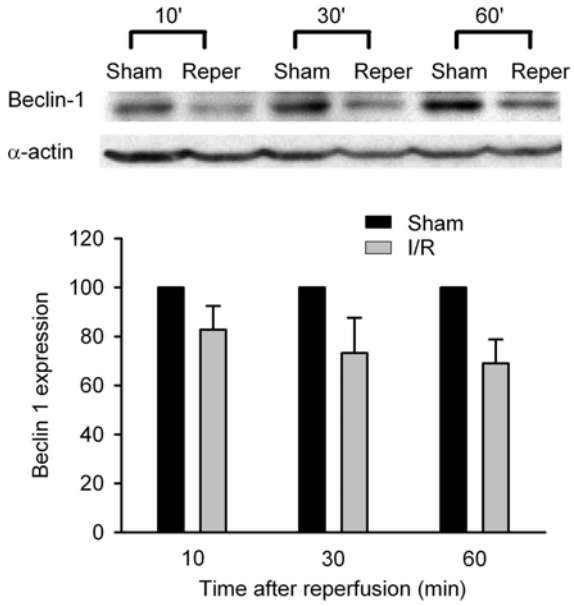

Figure 5. Western blotting analysis of Beclin 1 expression during reperfusion in isolated rat hearts. Reperfusion did not enhance Beclin 1 expression. Data were obtained from 8 independent experiments.

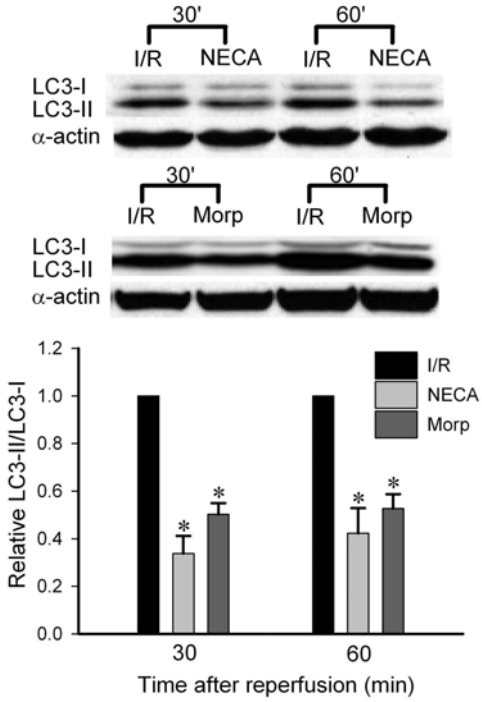

Figure 6. Western blotting analysis of LC3-I and LC3-II in isolated rat hearts. Both NECA (100 nM) and morphine (1 μM) given at reperfusion reduced LC3-II/LC3-I compared to I/R (ischemia/reperfusion) alone, indicating that NECA and morphine (Morp) prevent autophagy during reperfusion in rat hearts. Data were obtained from 5 independent experiments. * $p<0.05$ vs. I/R.

impaired autophagic flux in HL-1 cardiac cell line, and that overexpression of Beclin 1 enhanced autophagic flux following ischemia/reperfusion and significantly reduced activation of pro-apoptotic Bax, whereas knockdown of beclin-1 increased Bax activation (1). They also found that expression of dominant negative mutant of ATG5 increased cellular injury. The same group further reported that enhancement of autophagy by overexpression of ATG5 protected against Bnip3-mediated cell death in HL-1 cardiac myocytes exposed to 2 h of simulated ischemia followed by 12h of reperfusion, whereas inhibition of autophagy by ATG5K130R enhanced cell death, thus

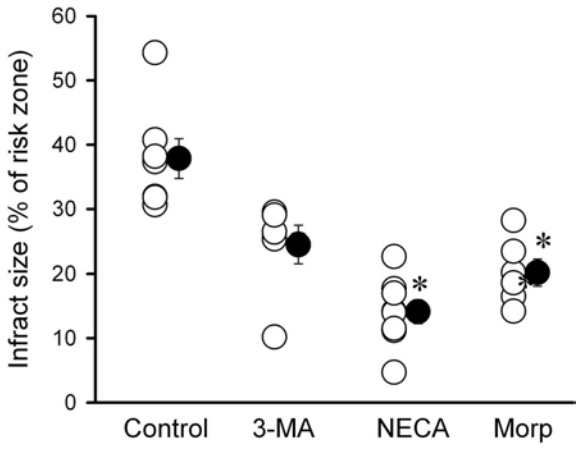

Figure 7. Effect of 3-MA, NECA, and morphine (Morp) on myocardial infarct size in isolated rat hearts. 3-MA (1 mM), NECA (100 nM), and morphine (1 μM) significantly reduced infarct size compared with control. Open circles represent individual experiments whereas closed circles depict group means with S.E.M. Data are from 6-8 independent experiments. * $p < 0.05$ vs. control.

suggesting a beneficial (protective) role of autophagy during ischemia/reperfusion in cardiomyocytes (1). Thus, these findings may indicate that autophagy is protective during ischemia/reperfusion. However, this was challenged by a recent report in which urocortin, a peptide that was shown to protect cardiomyocytes, prevented Beclin 1 mediated cell death in rat cardiomyocytes exposed to simulated ischemia/reperfusion (9), suggesting that autophagy may be an additional cell death mechanism during ischemia/reperfusion in cardiomyocytes. Although these studies provide fresh and useful information regarding the role of autophagy in ischemia/reperfusion injury, it should be noted that these observations were made in cardiomyocytes *in vitro* and thus may not reflect the actual role of autophagy in the whole heart subjected to ischemia/reperfusion. Recently, Sadoshima's group has proposed that autophagy may be protective during ischemia but is detrimental during reperfusion in *in vivo* mouse hearts (11). In the present study, autophagy was prominent after the onset of reperfusion in rat hearts, and both 3-MA and NECA applied at reperfusion reduced autophagy as well as infarct size. Since 3-MA is a classic inhibitor of autophagy (1), and NECA and morphine have been demonstrated to be cardioprotective at reperfusion (17-21), our finding strongly suggests that autophagy contributes to reperfusion injury in the heart and that prevention of autophagy might be an important strategy to protect the heart from reperfusion injury.

Beclin 1, the first mammalian protein described to mediate autophagy (22), is the homologue of the yeast Atg6 (1). Beclin 1 plays an important role in autophagy by forming a protein complex with class III PI3-kinase within the autophagosome. Studies have shown that Beclin 1 mediates ischemia/reperfusion induced autophagy. In neonatal or adult rat cardiomyocytes, simulated ischemia/reperfusion up-regulated Beclin 1 expression, and urocortin inhibited autophagy partially by suppressing Beclin 1 expression, suggesting that Beclin 1 may mediate ischemia/reperfusion induced autophagy in cardiomyocytes (9). In addition, Beclin 1 is up-regulated during the reperfusion phase in mouse hearts, and induction of autophagy and cardiac death during reperfusion was attenuated in Beclin $1^{+/-}$ mice (11), suggesting a critical role of Beclin 1 in the formation of autophagy during reperfusion. Hence, we determined if Beclin 1 plays a role in autophagy formation in rat hearts. The result showed that Beclin 1 expression was not up-regulated by ischemia/reperfusion but to the contrary was slightly decreased, suggesting that Beclin 1 may not be critical for the formation of autophagy during ischemia/reperfusion in isolated perfused rat hearts.

In summary, autophagy becomes prominent upon reperfusion but not during ischemia in isolated rat hearts. Autophagy may contribute to the pathogenesis of myocardial reperfusion injury and modulation of autophagy formation might serve as a potential therapeutic intervention that reduces reperfusion injury in the clinical setting of acute myocardial infarction.

6. ACKNOWLEDGMENT

Drs Yulan Jin and Huihua Wang contributed equally to this work. This study was supported by Grant 2007136 from Bureau of Education, Hebei Provence, China and the National Institute of Health Grant 1R01HL083362.

7. REFERENCES

1. Levine B, Klionsky DJ: Development by Self-Digestion: Molecular Mechanisms and Biological Functions of Autophagy. *Dev Cell* 6, 463-477 (2004)

2. Shintani T, Klionsky DJ: Autophagy in Health and Disease: A Double-Edged Sword. *Science* 306, 990-995 (2004)

3. Baehrecke EH: Autophagy: dual roles in life and death? *Nat Rev Mol Cell Biol* 6, 505-510 (2005)

4. Hamacher-Brady A, Brady N, Gottlieb R: The Interplay between Pro-Death and Pro-Survival Signaling Pathways in Myocardial Ischemia/Reperfusion Injury: Apoptosis Meets Autophagy. *Cardiovasc Drugs Ther* 20, 445-462 (2006)

5. Yan L, Vatner DE, Kim S-J, Ge H, Masurekar M, Massover WH, Yang G, Matsui Y, Sadoshima J, Vatner SF: Autophagy in chronically ischemic myocardium. *Proc Natl Acad Sci U S A* 102, 13807-13812 (2005)

6. Hamacher-Brady A, Brady NR, Gottlieb RA: Enhancing Macroautophagy Protects against Ischemia/Reperfusion Injury in Cardiac Myocytes. *J. Biol. Chem.* 281, 29776-29787 (2006)

7. Hamacher-Brady A, Brady NR, Logue SE, Sayen MR, Jinno M, Kirshenbaum LA, Gottlieb RA, Gustafsson AB: Response to myocardial ischemia//reperfusion injury

involves Bnip3 and autophagy. *Cell Death Differ* 14, 146-157 (2006)

8. Aki T, Yamaguchi K, Fujimiya T, Mizukami Y: Phosphoinositide 3-kinase accelerates autophagic cell death during glucose deprivation in the rat cardiomyocyte-derived cell line H9c2. *Oncogene* 22, 8529-8535 (2003)

9. Valentim L, Laurence KM, Townsend PA, Carroll CJ, Soond S, Scarabelli TM, Knight RA, Latchman DS, Stephanou A: Urocortin inhibits Beclin1-mediated autophagic cell death in cardiac myocytes exposed to ischaemia/reperfusion injury. *J Mol Cell Cardiol* 40, 846-852 (2006)

10. Walter PL, Kampkotter A, Eckers A, Barthel A, Schmoll D, Sies H, Klotz LO: Modulation of FoxO signaling in human hepatoma cells by exposure to copper or zinc ions. *Arch Biochem Biophys* 454, 107-113 (2006)

11. Matsui Y, Takagi H, Qu X, Abdellatif M, Sakoda H, Asano T, Levine B, 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)

12. Kabeya Y, Mizushima N, Ueno T, Yamamoto A, Kirisako T, Noda T, Kominami E, Ohsumi Y, Yoshimori T: LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *EMBO J* 19, 5720-5728 (2000)

13. Schwarze PE, Seglen PO: Reduced autophagic activity, improved protein balance and enhanced *in vitro* survival of hepatocytes isolated from carcinogen-treated rats. *Exp Cell Res* 157, 15-28 (1985)

14. Klionsky DJ, Emr SD: Autophagy as a Regulated Pathway of Cellular Degradation. *Science* 290, 1717-1721 (2000)

15. Terman A, Brunk UT: Autophagy in cardiac myocyte homeostasis, aging, and pathology. *Cardiovasc Res* 68, 355-365 (2005)

16. Seglen PO, Gordon PB: 3-Methyladenine: Specific Inhibitor of Autophagic/Lysosomal Protein Degradation in Isolated Rat Hepatocytes. *PNAS* 79, 1889-1892 (1982)

17. Förster K, Paul I, Solenkova N, Staudt A, Cohen M, Downey J, Felix S, Krieg T: NECA at reperfusion limits infarction and inhibits formation of the mitochondrial permeability transition pore by activating p70S6 kinase. *Basic Res Cardiol* 101, 319-326 (2006)

18. Philipp S, Yang X-M, Cui L, Davis AM, Downey JM, Cohen MV: Postconditioning protects rabbit hearts through a protein kinase C-adenosine A_{2b} receptor cascade. *Cardiovasc Res* 70, 308-314 (2006)

19. Xu Z, Downey JM, Cohen MV: AMP 579 reduces contracture and limits infarction in rabbit heart by activating adenosine A_2 receptors. *J Cardiovasc Pharmacol* 38, 474-481 (2001)

20. Gross ER, Hsu AK, Gross GJ: Opioid-induced cardioprotection occurs via glycogen synthase kinase β inhibition during reperfusion in intact rat hearts. *Circ Res* 94, 960-966 (2004)

21. Jang Y, Xi J, Wang H, Mueller RA, Norfleet EA, Xu Z: Postconditioning prevents reperfusion injury by activating delta-opioid receptors. *Anesthesiology* 108, 243-250 (2008)

22. Liang XH, Jackson S, Seaman M, Brown K, Kempkes B, Hibshoosh H, Levine B: Induction of autophagy and inhibition of tumorigenesis by beclin 1. *Nature* 402, 672-676 (1999)

Key Words: Autophagy, Reperfusion Injury, LC3, Beclin 1, NECA, Morphine

Send corresponding to: Zhelong Xu, Department of Anesthesiology, CB# 7010, The University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, Tel: 919-843-4174, Fax: 919-843-3805, E-mail: zxu@aims.unc.edu

http://www.bioscience.org/current/vol2E.htm