Impaired Autophagosome Clearance Contributes to Cardiomyocyte Death in Ischemia/Reperfusion Injury

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Background—In myocardial ischemia, induction of autophagy via the AMP-induced protein kinase pathway is protective, whereas reperfusion stimulates autophagy with BECLIN-1 upregulation and is implicated in causing cell death. We examined flux through the macroautophagy pathway as a determinant of the discrepant outcomes in cardiomyocyte cell death in this setting.

Methods and Results—Reversible left anterior descending coronary artery ligation was performed in mice with cardiomyocyte-restricted expression of green fluorescent protein–tagged microtubule–associated protein light chain-3 to induce ischemia (120 minutes) or ischemia/reperfusion (30–90 minutes) with saline or chloroquine pretreatment (n=4 per group). Autophagosome clearance, assessed as the ratio of punctate light chain-3 abundance in saline to chloroquine-treated samples, was markedly impaired with ischemia/reperfusion compared with sham controls. Reoxygenation increased cell death in neonatal rat cardiomyocytes compared with hypoxia alone, markedly increased autophagosomes but not autolysosomes (assessed as punctate dual fluorescent mCherry-green fluorescent protein tandem-tagged light chain-3 expression), and impaired clearance of polyglutamine aggregates, indicating impaired autophagic flux. The resultant autophagosome accumulation was associated with increased reactive oxygen species and mitochondrial permeabilization, leading to cell death, which was attenuated by cyclosporine A pretreatment. Hypoxia-reoxygenation injury was accompanied by reactive oxygen species–mediated BECLIN-1 upregulation and a reduction in lysosome-associated membrane protein-2, a critical determinant of autophagosome–lysosome fusion. Restoration of lysosome-associated membrane protein-2 levels synergizes with partial BECLIN-1 knockdown to restore autophagosome processing and to attenuate cell death after hypoxia-reoxygenation.

Conclusion—Ischemia/reperfusion injury impairs autophagosome clearance mediated in part by reactive oxygen species–induced decline in lysosome-associated membrane protein-2 and upregulation of BECLIN-1, contributing to increased cardiomyocyte death. (Circulation. 2012;125:3170-3181.)

Key Words: autophagy ■ cell death ■ ischemia ■ reperfusion injury

Autophagy is an intracellular lysosomal degradative process operative in homeostatic clearance of organelles and protein aggregates.1 Macropathagy involves segregation of cargo within double-membrane–bound autophagosomes that fuse with and are degraded within lysosomes, and efficient flux through the macroautophagy pathway is essential for cell survival.2 However, the frequent observation of autophagosomes in dying cells has stimulated interest in examining autophagy as a mechanism for cell death, called type II programmed cell death.3 Whether autophagy causes programmed cell death or is “guilty by association”4 remains a subject of active investigation.

Clinical Perspective on p 3181

Autophagy induction is critical for survival during the perinatal period of relative starvation,5 and suppression of constitutive cardiomyocyte autophagy with ablation of ATG56 or impairment of late stages of autophagy in the absence of lysosome-associated membrane protein-2 (LAMP2) in patients with Danon disease7 and in mice with LAMP2 ablation8 results in cardiomyopathy. Autophagy is also rapidly induced in the myocardium in response to stress such as fasting,9 pressure overload,6,9 and ischemia/reperfusion (IR) injury.10 In contrast to a clear prosurvival role for constitutive autophagy, stress-induced autophagy has been ascribed both salutary and deleterious roles in cardiomyocyte function and survival.9,10

Autophagosome prevalence, a commonly used readout for the state of autophagy activation, is determined by the rate of autophagosome formation (ie, induction of autophagy) and the rate of autophagosome destruction and is therefore a
function of “flux” through the autophagic pathway.\textsuperscript{11} It is not clear whether the increased abundance of autophagosomes in dying cells reflects upregulation of adaptive autophagy,\textsuperscript{2,4} an instance of dysregulated and excessive self-cannibalism,\textsuperscript{12} or an impairment in autophagic flux with reduced clearance of autophagosomes (and presumably cargo that would normally be degraded by autophagy) as postulated to occur in Danon disease,\textsuperscript{7} with secondary activation of programmed cell death. In this study, we have used an integrated approach to examine flux through the macroautophagy pathway in myocardial IR injury to test the hypothesis that impairment in late stages of autophagy with resultant autophagosome accumulation prevents its prosurvival role and triggers cardiomyocyte death.

Methods

IR Modeling

Adult male cardiomyocyte-specific green fluorescent protein–light chain-3 (GFP-LC3) transgenic mice\textsuperscript{9} and C57BL/6 mice (from Jackson Laboratories) were subjected to reversible left anterior descending artery coronary ligation in the presence of chloroquine 10 mg/kg or MnTMPyP 6 mg/kg IP, respectively, or saline control 1 mg/kg or MnTMPyP 6 mg/kg IP, respectively, or saline control 1 hour before surgery.\textsuperscript{11} All animal studies were approved by the Animal Studies Committee at the Washington University School of Medicine and the Institutional Animal Care and Use Committee at the John Cochran VA Medical Center.

Generation of Viral Constructs

Adenoviruses coding for rat LAMP2A and rat LAMP2B were generated with the Invitrogen Virapower system. Adenoviruses coding for Beclin-1 shRNA\textsuperscript{10} and cyan fluorescence protein–tagged polyglutamine Q19 and Q80 constructs\textsuperscript{14} and lentivirus coding for mCherry-GFP-LC3\textsuperscript{15} have been described.

Assessment of cell death, hypoxia-reoxygenation modeling, immunofluorescence imaging, flow cytometry, and quantitative polymerase chain reaction analysis was performed as described.\textsuperscript{15}

Statistical Analysis

Results are expressed as mean±SEM. Statistical differences were assessed with the unpaired Student \(t\) test for 2 independent groups, paired \(t\) test for dependent data, and 1-way ANOVA for multiple groups with SPSS software. Bonferroni post hoc testing was used after ANOVA for testing all pairwise comparisons between groups. A 2-tailed value of \(P<0.05\) was considered statistically significant.

Results

Autophagosome Clearance Is Impaired in Cardiomyocytes With IR Injury In Vivo

Ischemic insult activates cardiomyocyte autophagy, as evidenced by an increase in the ratio of LC3-II to LC3-I and increased numbers of punctate GFP-LC3–bearing autophagosomes after a brief episode of myocardial ischemia.\textsuperscript{10} Autophagosome abundance is further increased after reperfusion injury.\textsuperscript{10} Because the prevalence of autophagosomes at any point in time is determined by the rate of formation of autophagosomes (ie, induction of autophagy) and the rate of autophagosome processing (ie, flux through the macroautophagy pathway),\textsuperscript{11} we determined cumulative autophagic flux by determining the numbers of autophagosomes in the presence and absence of chloroquine, which inhibits lysosomal acidification and prevents autophagosome-lysosome fusion.\textsuperscript{11} We subjected mice with cardiomyocyte-specific expression of GFP-LC3\textsuperscript{9} to in vivo ischemia for 2 hours (confirmed by ST-segment elevation; Figure 1A, middle) or IR for an equivalent duration (ischemia for 30 minutes followed by reperfusion for 90 minutes with reversal of ST-segment elevation; Figure 1A, right) in the absence and presence of chloroquine and assessed LC3 distribution. Mice subjected to a sham procedure demonstrate a basal level of cardiomyocyte autophagy as evidenced by punctate localization of GFP-LC3 (on autophagosomes),\textsuperscript{9} with autophagosome accumulation (~6-fold increase) in the presence of chloroquine, suggesting intact autophagic flux (Figure 1B and 1C). Ischemia caused a 10-fold increase in autophagosome abundance compared with sham operation, implying the induction of autophagosome formation (Figure 1B and 1C). This was accompanied by partial impairment of autophagic flux (ratio of autophagosome abundance with and without chloroquine of ~2 with ischemia compared with ~6 in sham; Figure 1C).

Reperfusion after ischemia provoked a further increase in autophagosome abundance (23-fold), which was not altered in the presence of chloroquine, suggesting markedly impaired autophagic flux (ratio of autophagosome abundance with and without chloroquine of ~1; Figure 1C). Levels of GFP-tagged LC3-II and p62, a protein that links ubiquitinated aggregates for destruction within autophagosomes and is degraded on autophagosome processing,\textsuperscript{16} increased in IR-treated hearts to a degree comparable to that in hearts treated with chloroquine (Figure 1D–1F), further supporting the observation of impaired autophagic processing with IR injury.

Myocardial IR Injury Provokes a Decline in LAMP2 and an Increase in BECLIN-1 Abundance With Impaired Autophagosome Processing

Autophagosome processing can be impaired if lysosome numbers decline\textsuperscript{15} or if there is a selective impairment in autophagosome-lysosome fusion or lysosome function. Accordingly, we examined the expression of the lysosome membrane proteins LAMP1 and LAMP2. Interestingly, the level of LAMP2 declined by 41\% and of LAMP1 declined by 44\% in IR-treated GFPPL-LC3 transgenic hearts (Figure 1D, 1G, and 1H); only the decline in LAMP1, which closely tracks lysosome numbers,\textsuperscript{15} was prevented by chloroquine pretreatment, suggesting that the decline in LAMP2 was independent of lysosome pH and was not related to lysosome consumption in IR-induced autophagy. Interestingly, although LAMP1 deficiency in mice is of no serious consequence,\textsuperscript{17} LAMP2 is a critical determinant of autophagosome-lysosome fusion,\textsuperscript{18,19} and mutations causing LAMP2 deficiency result in Danon disease,\textsuperscript{7} which is characterized by autophagosome accumulation in multiple tissues, including the myocardium, and cardiomyopathy.\textsuperscript{8} As observed previously,\textsuperscript{10} BECLIN-1 levels were elevated with reperfusion injury and not during ischemia (Figure 1D and 1I).

To evaluate whether a reperfusion-induced surge in reactive oxygen species (ROS) generation mediated the observed changes, we treated adult male C57BL/6 mice with Mn(III)tetrakis(1-Methyl-4-pyridyl) porphyrin pentachloride (MnTMPyP), a cell-permeable superoxide dismutase mimetic that acts as an ROS scavenger, or diluent and subjected them
to reversible left anterior descending ligation for 30 minutes followed by reperfusion for 90 minutes or sham surgery. IR induced a marked increase in autophagosome-bound LC3-II abundance with p62 accumulation in the myocardium, which were both prevented by pretreatment with MnTMPyP (Figure 2A–2C). This suggests that IR-induced ROS generation may inhibit autophagosome processing in addition to damaging cellular structures that need removal through macroautophagy. Importantly, the observed IR-induced decline in LAMP2 and upregulation in BECLIN-1 were prevented with MnTMPyP pretreatment (Figure 2A, 2D, and 2E). Curiously, we did not observe an IR-induced decline in LAMP1 in this strain of mice, as observed in the GFL-LC3 reporter mice, and basal LAMP1 levels appeared to be negatively regulated by ROS (Figure 2A and 2F).

**Impaired Autophagosome Clearance Is Sufficient to Cause Cell Death in Cardiomyocytes**

Autophagy has been implicated as a mechanism for programmed cell death. To test the hypothesis that impairment in autophagosome processing causes cardiomyocyte death, we subjected neonatal rat cardiomyocytes (NRCMs) to treatment with rapamycin, an irreversible inhibitor of mammalian target of rapamycin, to stimulate autophagy and blocked autophagosome processing with chloroquine pretreatment. The relative abundance of autophagosomes and autolysosomes was assessed with lentivirally transduced mCherry-GFP tandem-tagged LC3 (Figure 3A) as described previously. Induction of autophagy leads to punctate localization of LC3 on autophagosomes, which demonstrate both red and green fluorescence, with subsequent loss of green fluorescent signal on autophagosome-lysosome fusion and formation of autolysosomes resulting from the instability of GFP in the acidic intralysosomal environment. NRCMs in culture demonstrate constitutive (basal) autophagy with a preponderance of autolysosomes, which demonstrate both red and green fluorescence, with subsequent loss of green fluorescent signal on autophagosome-lysosome fusion and formation of autolysosomes resulting from the instability of GFP in the acidic intralysosomal environment. NRCMs in culture display a dynamic autophagy process, characterized by the periodic appearance of autophagosomes and autolysosomes. This process is influenced by various stimuli, including nutrient deprivation, growth factor withdrawal, and exposure to growth factors. To test the hypothesis that impaired autophagosome clearance is sufficient to cause cell death, we subjected NRCMs to treatment with rapamycin, an irreversible inhibitor of mammalian target of rapamycin, to stimulate autophagy and blocked autophagosome processing with chloroquine pretreatment. The relative abundance of autophagosomes and autolysosomes was assessed with lentivirally transduced mCherry-GFP tandem-tagged LC3 (Figure 3A) as described previously. Induction of autophagy leads to punctate localization of LC3 dots on autophagosomes, which demonstrate both red and green fluorescence, with subsequent loss of green fluorescent signal on autophagosome-lysosome fusion and formation of autolysosomes resulting from the instability of GFP in the acidic intralysosomal environment. NRCMs in culture display a dynamic autophagy process, characterized by the periodic appearance of autophagosomes and autolysosomes. This process is influenced by various stimuli, including nutrient deprivation, growth factor withdrawal, and exposure to growth factors.
Accumulation in Cardiomyocytes

Hypoxia-Reoxygenation Induces Autophagosome Accumulation

Autoimmune flux has been noted to be impaired in HL-1 cardiomyocytes, an immortalized cell line derived from atrial cardiomyocytes, with in vitro hypoxia-reoxygenation insult. Pretreatment with chloroquine increased the abundance of autophagosomes and autolysosomes, with in vitro hypoxia-reoxygenation insult. We subjected NRCMs to in vitro hypoxia (ambient O2 concentration <1%) followed by reoxygenation and determined the relative abundance of autophagosomes and autolysosomes. NRCMs cultured in a normoxic environment display basal autophagy with intact flux (Figure 4A; see also Figure 3A). Hypoxia for 24 hours markedly increased the abundance of autophagosomes (Figure 4A, middle), with an increase in both autophagosomes and autolysosomes (Figure 4B). Reoxygenation after 6 hours of hypoxia further increased the numbers of autophagic structures, which are predominantly autophagosomes (Figure 4A, bottom, and Figure 1A and IB in the online-only Data Supplement), without an increase in autolysosomes. Both prolonged hypoxia and hypoxia-reoxygenation increased LC3-II abundance, indicating increased autophagosome processing with reoxygenation after the onset of injury (Figure 4D and Figure IC in the online-only Data Supplement). Taken together, these data suggest impairment in autophagosome processing with reoxygenation after a hypoxic insult. To determine whether hypoxia-reoxygenation impairs proteosomal processing of proteins, which could result in an overload of the autophagic pathway for protein degradation and conceivably overwhelm the lysosomal machinery, we forcibly expressed polyglutamine repeats, which are known to be differentially processed via the ubiquitin-

**Figure 3B.** Treatment with rapamycin stimulates autophagy with enhanced autophagic flux, as evidenced by markedly increased autophagic structures, which are predominantly autophagosomes and autolysosomes. Rapamycin treatment provoked a decline in cumulative LC3 abundance, suggesting consumption during autophagy, and clearance of p62 (Figure 3C) compared with control, indicating intact flux. In contrast, impairment of autophagosome processing with chloroquine caused accumulation of autophagosome-bound LC3-II and p62 (Figure 3C), indicating impaired autophagosome clearance, which was sufficient to cause cell death in the basal state, and with rapamycin or nutrient deprivation–initiated autophagy, wherein autophagosome clearance was inhibited (Figure 3D). To investigate the mechanism of cell death with chloroquine, we posited that autophagosome accumulation prevents the clearance of damaged intracellular organelles and proteins, leading to increased ROS generation, which causes mitochondrial permeabilization and activation of programmed necrosis and/or necrosis. Indeed, chloroquine treatment increased ROS generation (Figure 3E and 3F) with loss of mitochondrial membrane potential (Figure 3E and 3G). Induction of ROS by chloroquine was not prevented by pretreatment with cyclosporine A, an inhibitor of mitochondrial permeability transition pore, indicating that ROS generation was upstream of mitochondrial permeabilization. Accordingly, pretreatment with cyclosporine A but not ZVAD-fmk (a pancaspase inhibitor) attenuated chloroquine-induced cell death under conditions of both basal and rapamycin-stimulated autophagy, suggesting activation of programmed necrosis pathway with autophagosome accumulation.

**Figure 2.** Scavenging reactive oxygen species restores autophagosome processing in myocardial ischemia/reperfusion injury in vivo. A through F, Representative immunoblots (A) and quantitative analysis of light chain-3 (LC3)-II (B), p62 (C), lysosome-associated membrane protein-2 (LAMP2; D), LAMP1 (E), and BECLIN-1 (F) expression in myocardial extracts from mice subjected to sham surgery or ischemia (30 minutes) followed by reperfusion (90 minutes) in the presence of MnTMPyP (6 mg/kg IP) or saline control injected 1 hour before surgery. n=5 to 6 per group. P values are by post hoc test after 1-way ANOVA. αSA indicates alpha-sarcomeric actin.

**Figure 3B.** Pretreatment with chloroquine resulted in accumulation of autophagosomes and the near absence of autolysosomes (Figure 3A, middle, and Figure 3B). Rapamycin treatment provoked a decline in cumulative LC3 abundance, suggesting consumption during autophagy, and clearance of p62 (Figure 3C) compared with control, indicating intact flux. In contrast, impairment of autophagosome processing with chloroquine caused accumulation of autophagosome-bound LC3-II and p62 (Figure 3C), indicating impaired autophagosome clearance, which was sufficient to cause cell death in the basal state, and with rapamycin or nutrient deprivation–initiated autophagy, wherein autophagosome clearance was inhibited (Figure 3D). To investigate the mechanism of cell death with chloroquine, we posited that autophagosome accumulation prevents the clearance of damaged intracellular organelles and proteins, leading to increased ROS generation, which causes mitochondrial permeabilization and activation of programmed necrosis and/or necrosis. Indeed, chloroquine treatment increased ROS generation (Figure 3E and 3F) with loss of mitochondrial membrane potential (Figure 3E and 3G). Induction of ROS by chloroquine was not prevented by pretreatment with cyclosporine A, an inhibitor of mitochondrial permeability transition pore, indicating that ROS generation was upstream of mitochondrial permeabilization. Accordingly, pretreatment with cyclosporine A but not ZVAD-fmk (a pancaspase inhibitor) attenuated chloroquine-induced cell death under conditions of both basal and rapamycin-stimulated autophagy, suggesting activation of programmed necrosis pathway with autophagosome accumulation.

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20. We subjected NRCMs to in vitro hypoxia (ambient O2 concentration <1%) followed by reoxygenation and determined the relative abundance of autophagosomes and autolysosomes. NRCMs cultured in a normoxic environment display basal autophagy with intact flux (Figure 4A; see also Figure 3A). Hypoxia for 24 hours markedly increased the abundance of autophagic structures (Figure 4A, middle), with an increase in both autophagosomes and autolysosomes (Figure 4B). Reoxygenation after 6 hours of hypoxia further increased the numbers of autophagic structures, which are predominantly autophagosomes (Figure 4A, bottom, and Figure 1A and IB in the online-only Data Supplement), without an increase in autolysosomes. Both prolonged hypoxia and hypoxia-reoxygenation increased LC3-II abundance, indicating increased numbers of autophagosomes (Figure 4C and Figure IC in the online-only Data Supplement) associated with a reduction in LAMP2 abundance (assessed with a monoclonal antibody directed to the matrix side of LAMP2 protein, which detects all isoforms; Figure 4C and 4D), without affecting LAMP1 levels except with longer duration (72 hours) of injury (Figure 4D). This was not due to a decline in the numbers or distribution of lysosomes (Figures ID, IIA, and IIB in the online-only Data Supplement), lysosomal permeabilization (Figure IIC in the online-only Data Supplement), or a change in lysosomal pH (Figure IID in the online-only Data Supplement). Hypoxia-reoxygenation–induced decline in LAMP2 was associated with BECLIN-1 upregulation compared with hypoxia alone and p62 accumulation up to 48 hours after the onset of injury (Figure 4D and Figure IC in the online-only Data Supplement). Taken together, these data suggest impairment in autophagosome processing with reoxygenation after a hypoxic insult. To determine whether hypoxia-reoxygenation impairs proteosomal processing of proteins, which could result in an overload of the autophagic pathway for protein degradation and conceivably overwhelm the lysosomal machinery, we forcibly expressed polyglutamine repeats, which are known to be differentially processed via the ubiquitin-

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proteasome pathway (Q19) and macroautophagy (Q80), in NRCMs and subjected them to hypoxia-reoxygenation injury. Hypoxia-reoxygenation impaired clearance of polyglutamine Q80 aggregates (Figure 5A and 5B) but not Q19 (Figure IIIA in the online-only Data Supplement), suggesting specific impairment in autophagy.

To determine whether hypoxia-reoxygenation–induced impairment in autophagosome processing contributes to cell death, we subjected NRCMs to matched durations of hypoxia and hypoxia-reoxygenation. Hypoxia increased cell death in NRCMs, with 45% of cells dead after 24 hours (Figure 5C and 5D and Figure IIIB in the online-only Data Supplement) compared with 6% in cells cultured in normoxic conditions. Interestingly, reoxygenation after a short hypoxic insult (6 hours) increased cell death even further (52%) compared with a comparable duration of hypoxia alone, suggesting an association between impaired autophagosome processing (see Figure 4) and reoxygenation-induced cell death in NRCMs. Experimental inhibition of autophagosome processing with chloroquine treatment increased hypoxia-induced cell death to levels comparable to hypoxia-reoxygenation but did not affect cell death with hypoxia-reoxygenation (Figure IIIC in the online-only Data Supplement), consistent with a severe impairment in autophagosome processing in cells subjected to hypoxia-reoxygenation injury. Autophagosome accumulation with hypoxia-reoxygenation was associated with ROS generation (Figure 5E and 5F) and mitochondrial permeabilization (Figure 5E and 5G), and
although mitochondrial permeabilization was prevented with cyclosporine A pretreatment (Figure 5E and 5G), ROS generation was not (Figure 5E and 5F), suggesting that it is upstream of mitochondrial permeabilization, mirroring the observations with chloroquine-induced autophagosome accumulation (Figure 3E–3G). Accordingly, cyclosporine A but not ZVAD-fmk attenuated hypoxia-reoxygenation–induced cell death, suggesting a mitochon-

Figure 4. Hypoxia-reoxygenation injury upregulates autophagy with impaired autophagosome processing in neonatal rat cardiomyocytes (NRCMs). A, Representative immunofluorescence images (×630) demonstrating mCherry–green fluorescent protein (GFP)–light chain-3 (LC3) localization in NRCMs cultured in a normoxic environment (as controls; top) subjected to hypoxia (24 hours; middle) or hypoxia (6 hours) followed by reoxygenation (18 hours; bottom). Nuclei are blue (DAPI). Representative of n=3 experiments. B, Quantitative analysis of autophagosomes (white bars), autolysosomes (AL; black bars), and both (gray bars) in NRCMs treated as in A. P values are by post hoc test between groups and paired t test within a group. n=30 to 50 nuclei per group. C and D, Representative immunoblots depicting LC3 processing and lysosome-associated membrane protein-2 (LAMP2) abundance in response to hypoxia and hypoxia-reoxygenation injury for 24 and 48 hours (C) and time course for change in LAMP2, LAMP1, BECLIN-1, and p62 abundance in response to hypoxia-reoxygenation injury (D). Representative of n=2 experiments. αSA indicates alpha-sarcomeric actin.

Figure 5. Impaired autophagosome processing is associated with cell death in neonatal rat cardiomyocytes (NRCMs) subjected to hypoxia-reoxygenation injury. A and B, Representative immunofluorescence images (×630) depicting cyan fluorescence protein–tagged polyglutamine Q80 (Poly-Q80) accumulation (green; A) and quantitative analysis of Q80 aggregates (B) in NRCMs subjected to hypoxia (Hyp)-reoxygenation (Reox) and hypoxia and normoxic controls (Nor). Nuclei are red (TOPRO-3). P value is by post hoc test. Representative of n=2 experiments. n=30 to 50 nuclei per group. C and D, Representative immunofluorescence images (×200; C) and quantitative analysis (D) of alive (green) and dead (red) NRCMs treated as in A. P<0.05 by post hoc test; n=24 to 40 per group. E, Representative immunofluorescence images (×630) of NRCMs loaded with the reactive oxygen species (ROS) indicator carboxy-H2DCFDA (top) or tetramethyl rhodamine ethyl ester (TMRE; middle) and merged (bottom) and subjected to hypoxia-reoxygenation as in A in the presence of cyclosporine A (CsA; 20 μmol/L) or dimethyl sulfoxide (DMSO) control. F and G, Quantification of mean fluorescence of flow cytometric analysis of NRCMs treated as in E for ROS (F) and TMRE (G). P<0.05 vs normoxic control by post hoc test (n=3–4 per group). H, Cell death in NRCMs subjected to hypoxia-reoxygenation as in A in the presence of ZVAD-fmk (20 μmol/L), CsA (20 μmol/L), or DMSO and normoxic control. P<0.05 vs normoxic control by post hoc test (n=10–20 per group).
The decline in LAMP2 and the increase in BECLIN-1 abundance are observed very early (2 hours) after the onset of reperfusion/reoxygenation (Figure 2A, 2D, and 2E and Figure 1 in the online-only Data Supplement), suggesting a posttranslational rather than a transcriptional mechanism. As observed in vivo IR injury, treatment with MnTMPyP to scavenge ROS prevented a reoxygenation-induced decline in LAMP2 abundance (Figure 6A) and attenuated the increase in BECLIN-1 abundance (Figure 6B), with restoration of autolysosome predominance after hypoxia-reoxygenation injury compared with a predominant increase in autolysosomes in NRCMs subjected to hypoxia-reoxygenation (Figure 6C and 6D). This suggests that the reoxygenation-induced increase in ROS provokes autophagosome formation but impairs their clearance.

**Hypoxia-Reoxygenation–Induced LAMP2 Decline and BECLIN-1 Upregulation Are Mediated by ROS**

The decline in LAMP2 and the increase in BECLIN-1 abundance are observed very early (2 hours) after the onset of reperfusion/reoxygenation (Figure 2A, 2D, and 2E and Figure 1 in the online-only Data Supplement), suggesting a posttranslational rather than a transcriptional mechanism. As observed in vivo IR injury, treatment with MnTMPyP to scavenge ROS prevented a reoxygenation-induced decline in LAMP2 abundance (Figure 6A) and attenuated the increase in BECLIN-1 abundance (Figure 6B), with restoration of a relative excess of autolysosomes and a decrease in total autophagic structures in NRCMs subjected to hypoxia-reoxygenation (Figure 6C and 6D). This suggests that the reoxygenation-induced increase in ROS provokes autophagosome formation but impairs their clearance.

**Beclin-1 Knockdown Synergizes With Exogenous Expression of LAMP2 Isoforms to Restore Autophagosome Processing**

Multiple studies have demonstrated the salutary effects of BECN1 haploinsufficiency in pathological states, including reperfusion injury, in contrast to its essential role in autophagosome formation, which is critical for autophagy-mediated cell survival. Whether the observed reperfusion/reoxygenation-induced increase in BECLIN-1 abundance (to supranormal levels; Figures 1D, 1I, 2A, 2E, and 4D) or the decline in LAMP2 levels (Figures 1D, 1G, 2A, 2D, 4C, and 4D) contributes to ROS-induced impairment in autophagosome processing is not known. Accordingly, to test the hypothesis that partial knockdown of BECLIN-1 and restoration of LAMP2 by exogenous expression will synergistically restore autophagosome processing and attenuate hypoxia-reoxygenation–induced cell death, we transduced NRCMs with increasing doses of adenovirus coding for Beclin-1 shRNA expression and rat LAMP2A and rat LAMP2B, the 2 cardiac-expressed isoforms of LAMP2, either individually or in combination. BECLIN-1 knockdown was minimal with the low infective dose of virus (multiplicity of infection [MOI]=1) and substantial with a higher dose (MOI=10) compared with control (Figure 7A). Exogenous expression of LAMP2A and LAMP2B prevented the decline in LAMP2 levels after hypoxia-reoxygenation injury (Figure 7B and Figure IV in the online-only Data Supplement). Importantly, exogenous expression of LAMP2A and LAMP2B resulted in restoration of autophagosome processing, as indicated by reduced LC3-II and p62 accumulation (compared with respective normoxia control; Figure 7B), and restoration of autolysosome predominance after hypoxia-reoxygenation injury compared with a predominant increase in autophagosomes in cells treated with control viruses (Figure 7C and 7D). The effects of Beclin-1 knockdown were dose dependent. Although minimal Beclin-1 knockdown (Beclin-1 shRNA MOI=1; Figure 7A) restored autophagosome processing either singly, as indicated by the increased ratio of autolysosomes to autophagosomes and reduced LC3-II and p62 accumulation (compared with respective normoxia control; Figure 7A and Figure VA and VB in the online-only Data Supplement), or in combination with exogenous LAMP2A and LAMP2B expression (Figure 7C and 7D and Figure VC in the online-only Data Supplement), further knockdown of Beclin-1 (with a higher viral dose, MOI=10; Figure 7A) decreased the abundance of autophagic structures, indicating the suppression of autophagosome formation (Figures 7C and 7D).
These data suggest that the increased levels of BECLIN-1 seen with reperfusion/reoxygenation contribute to the impairment in autophagosome clearance, whereas the basal levels of BECLIN-1 are necessary for the induction of autophagosome formation in response to hypoxia-reoxygenation injury. The restoration of autophagosome processing with partial Beclin-1 knockdown (low dose) and LAMP2A/B overexpression was individually associated with the reduction in hypoxia-reoxygenation–induced cell death, with the largest benefit (32% reduction in cell death) observed with a combination of these strategies (Figure 7E). Further knockdown of Beclin-1 (MOI=10) with a resultant inhibition of autophagosome formation (Figure 7D and Figure VA in the online-only Data Supplement) markedly increased cell death in NRCMs cultured under normoxic conditions and in response to hypoxia-reoxygenation injury (Figure 7E), underscoring a critical role for autophagy in cardiomyocyte survival.10,20,26

BECLIN-1 Abundance Regulates Transcription of Autophagy Genes

In addition to the established role for BECLIN-1 in inducing autophagosome formation, our results indicate that partial
Beclin-1 knockdown enhances autophagosome processing. Indeed, whereas the increased total LC3 and/or p62 abundance with partial Beclin-1 knockdown (Figure 7A and Figure VC in the online-only Data Supplement) could indicate suppression of autophagosome formation with secondary accumulation of these proteins, the increase in the ratio of autolysosomes to autophagosomes with hypoxia-reoxygenation (Figure 7C and Figure VA and VB in the online-only Data Supplement) suggests otherwise. Therefore, we examined the serial changes in protein and transcript abundance of LC3 and p62, along with that of LAMP1, the levels of which track lysosome numbers and do not change with hypoxia-reoxygenation injury (Figure 4D), and RAB7, a small GTP binding protein that mediates autophagosome-lysosome fusion, with Beclin-1 knockdown (with a low viral dose of MOI = 1). Interestingly, partial Beclin-1 knockdown increased total LC3, p62, LAMP1, and RAB7 abundance at 48 and 72 hours (Figure 8A) compared with nontargeting LacZ shRNA-transduced cells, which was preceded or accompanied by an increase in respective gene transcripts, followed by a suppression in transcript levels at 72 hours for MAP1LC3B, LAMP1, and RAB7 but not SQSTM1 (Figure 8B–8E). These observations suggest that Beclin-1 knockdown activates the transcriptional machinery early (on or before 48 hours) to increase the synthesis of autophagy and lysosomal proteins, which is subsequently suppressed (at 72 hours) when levels of these proteins build up, suggesting a mechanism for restoring flux by priming the autophagy pathway. Indeed, exogenous expression of BECLIN-1 for 48 hours stimulated autophagosome formation but impaired autophagosome pro-
cessing (Figure 8F and 8G) and increased levels of autophagosome-bound LC3-II and p62 with a decline in LAMP1 and RAB7 levels (Figure 8H) in a dose-dependent fashion. Importantly, high levels of BECLIN-1 expression (MOI=100) suppressed gene transcription (predating the observed protein changes) for MAP1LC3B (Figure 8I), LAMP1 (Figure 8K), and RAB7 (Figure 8L) but not SQSTM1 (Figure 8J) and increased cell death (Figure 8M). BECLIN-1 protein levels thus appear to be a “sensor” for transcriptional priming of the cellular autophagic machinery and an important cellular control point for dictating relative abundance of relevant autophagy-lysosomal proteins. Taken together, these data suggest that the ROS-induced increase in BECLIN-1 abundance could suppress autophagosome maturation via transcriptional downregulation of components of the autophagy-lysosome machinery.

**Discussion**

In this study, we demonstrate that in IR injury, cardiomyocyte autophagy is upregulated as a stress-response mechanism but autophagosome clearance is impaired, contributing to cell death. Specifically, we found a rapid reperfusion-induced decline in LAMP2, a protein critical for autophagosome-lysosome fusion, and upregulation of BECLIN-1, which impairs autophagosome processing, with increased ROS generation and mitochondrial permeabilization, thereby provoking cardiomyocyte death. Facilitating autophagosome processing by restoring LAMP2 levels and partially knocking down BECLIN-1 restores autophagosome processing and attenuates reoxygenation-induced cell death.

Contemporary studies in cardiomyocytes subjected to hypoxia-reoxygenation have demonstrated the accumulation of autophagosomes,\textsuperscript{20} LC3-II, and p62\textsuperscript{10,28} and a decline in nicotinamide phosphoribosyltransferase, which impairs autophagosome processing,\textsuperscript{28} indicating impaired “autophagic flux” in vitro. Conversely, increases in both autophagosomes and autolysosomes (as indicated by mRFP-GFP-LC3)\textsuperscript{29} were interpreted as “intact” flux in vivo. These discrepant observations reflect the limitations of various imperfect tools to monitor flux through the autophagy pathway. Indeed, the lack of equivalent fluorescence intensity and photostability of mRFP and GFP use in the dual fluorescent construct\textsuperscript{20} and differential kinetics of GFP degradation, depending on the state of lysosome acidification,\textsuperscript{30} may account for the variable readouts. We have attempted to overcome these limitations by generating an LC3 construct tandem tagged with mCherry and GFP probes, which have comparable intensities and photostability,\textsuperscript{31} ruling out alterations in lysosomal acidification, and using complementary approaches permitting increased accuracy in the assessment of autophagic flux.

Our results explain why autophagy, which facilitates cardiomyocyte survival (with reduced infarct size) during ischemia,\textsuperscript{10,32} has been implicated in causing cell death (with increased infarct size) on reperfusion of the ischemic myocardium.\textsuperscript{10} In the study by Matsui et al\textsuperscript{10}, reperfusion provoked a 7-fold increase in autophagosome abundance in the absence of further AMP-induced protein kinase activation or mammalian target of rapamycin inhibition during the ischemic phase. Importantly, reperfusion was associated with a rapid upregulation of BECLIN-1 (within 30 minutes), and BECN1 haploinsufficient mice demonstrated a decrease in cardiomyocyte autophagosome abundance, reduced programmed cell death, and reduced infarct size with in vivo IR injury compared with wild-type controls. The assumption that BECN1 haploinsufficiency leads to a reduction in autophagosome abundance primarily by preventing autophagy initiation is challenged by our results that demonstrate that it increased autophagosome processing, ie, increased autophagic flux, likely by relieving the transcriptional down-regulation of the autophagy-lysosome machinery by supra-normal BECLIN-1 levels, while enough BECLIN-1 protein is available to facilitate autophagosome formation (Figure 8). A more complete knockdown of BECLIN-1, however, impairs autophagosome formation, thereby impairing autophagy, with increased cardiomyocyte death. Beclin-1 knockdown may also reduce autophagosome processing by disrupting the interaction with Rubicon, a GTPase-regulating protein that localizes to late endosome/autophagosomes and inhibits autophagic flux.\textsuperscript{33} The observation that Beclin-1 knockdown confers benefit above and beyond that with LAMP2 overexpression, despite restoration of autophagy to a similar extent (Figure 7C and 7D), suggests that other mechanisms such as a reduction in p53 levels\textsuperscript{34} or restoration of mitochondrial homeostasis\textsuperscript{22} could be operative. Interestingly, the possibility that Beclin-1 knockdown may upregulate transcription of LAMP2 may explain the very similar degree of benefit obtained with Beclin-1 knockdown alone versus in combination with LAMP2A +2B overexpression (Figure 7E). Thus, BECLIN-1 abundance may act as a switch to determine whether the end result of autophagy is efficient autophagic degradation of targeted cargo to ensure cellular homeostasis and survival or autophagosome accumulation, triggering programmed cell death. Such a role for BECLIN-1 is consistent with the observation that it acts as tumor suppressor protein.\textsuperscript{23,24}

A novel observation of this study is the rapid decline in LAMP2 abundance with reperfusion. LAMP2 is a critical determinant of autophagosome-lysosome fusion.\textsuperscript{18,19} LAMP2 knockdown impairs autophagy in nutrient-starved adult rat ventricular myocytes and causes cell death at levels comparable to autophagy inhibition with 3MA\textsuperscript{26} and ablation of LAMP2\textsuperscript{20} or loss of LAMP2 protein owing to mutations in individuals with Danon disease\textsuperscript{7} causes cardiomyocyte autophagosome accumulation and extensive myocardial fibrosis,\textsuperscript{35} suggesting cell death as the pathogenic mechanism. Interestingly, exogenous expression of both LAMP2A, a protein receptor for chaperone-mediated autophagy,\textsuperscript{56} and LAMP2B, which is postulated to be involved in autophagosome-lysosome fusion,\textsuperscript{7,18} accelerated autophagosome processing, suggesting a redundant role for these isoforms in the heart.

Given the exploratory nature of the study, the type I error rate was not controlled across multiple experimental outcomes, which would require additional studies focusing on specific associations. Nevertheless, our results, which require confirmation in adult cardiac myocytes and the in vivo heart, suggest that impaired autophagosome clearance results in
increased ROS levels, which trigger mitochondrial permeabilization, leading to a necrotic mechanism of cell death in cardiac IR injury, and underscore the need for careful evaluation of autophagic flux to determine its role in cardiac stress and physiology.

Acknowledgments

We thank Michel Jadot, PhD, Laboratoire de Chimie Physiologique, Namur, for his kind gift of the monoclonal antibody targeted to matrix side of LAMP2 and Junichi Sadoshima, PhD, UMDNJ, and Conrad Weihl, MD, PhD, Washington University, for their generous gifts of adenovirus expressing shRNA targeting Beclin-1 and cyan fluorescence protein–tagged polyglutamine constructs, respectively. We express our sincere gratitude to Paul Saftig, PhD, University of Kiel, and Douglas L. Mann, MD, Washington University, for their insightful comments and support, as well as Eric Novak, MS, Washington University, for statistical assistance.

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Disclosures

None.

References

Ma et al. Impaired Autophagy in Cardiomyocyte Death

In myocardial infarction resulting from coronary occlusion, contemporary therapy is focused on expedient reperfusion to prevent loss of myocardium. Despite reperfusion and often as a result of reperfusion injury, there is ongoing cardiomyocyte cell death, which can be targeted therapeutically to maximize myocardial salvage. Autophagy is an evolutionarily conserved lysosomal pathway to break down damaged cellular constituents, to provide nutrients, and to facilitate cell survival. Autophagy has also been implicated as a mechanism for programmed cell death. In cardiac ischemia/reperfusion injury, autophagy is upregulated, and previous studies have suggested that it plays a dichotomous role in facilitating cell survival during ischemia but provoking cell death during reperfusion. To examine the hypothesis that autophagy is upregulated in reperfusion injury but impaired, thus preventing its prosurvival function, we have carefully examined flux through the autophagy pathway in cardiomyocytes subjected to ischemia and reperfusion. Our results demonstrate that autophagosomes accumulate during reperfusion injury after myocardial ischemia, suggesting impaired autophagosome processing. This accumulation of autophagosomes, presumably with the deleterious cargo that is not degraded, is associated with an increased prevalence of reactive oxygen species and mitochondrial permeabilization, resulting in necrotic cardiomyocyte death. The underlying mechanism for the impairment in autophagosome processing appears to be a reperfusion-induced decline in lysosome-associated membrane protein-2 levels, a protein critical for autophagosome-lysosome fusion, and an increase in the abundance of BECLIN-1. Restoring lysosome-associated membrane protein-2 levels and knocking down BECLIN-1 restore autophagosome processing with the attenuation of cell death. Thus, facilitating autophagy with enhanced flux through the autophagy pathway is a promising strategy to achieve myocardial salvage in ischemia/reperfusion injury.
Impaired Autophagosome Clearance Contributes to Cardiomyocyte Death in Ischemia/Reperfusion Injury
Xiucui Ma, Haiyan Liu, Sarah R. Foyil, Rebecca J. Godar, Carla J. Weinheimer, Joseph A. Hill and Abhinav Diwan

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SUPPLEMENTARY MATERIAL

Expanded Methods

*In vivo ischemia-reperfusion modeling:* Adult transgenic mice (8-12 weeks of age) with cardiac myocyte specific expression of GFP-tagged LC3\(^1\) were subjected to reversible left anterior descending artery coronary ligation as described\(^2\). Briefly, mice were anesthetized with a mixture of ketamine (100mg/kg) and xylazine (10mg/kg); surgically prepped and ventilated. After thoracotomy, the left anterior descending artery (LAD) was identified and ligated with a 9-0 polypropolene suture that was passed under the LAD. A knot was tied over a 1-mm section of PE-10 tubing placed directly over the vessel to create the occlusion. Ischemia was confirmed by absence of blood flow verified visually under the microscope, and presence of ST elevations on EKG (see Figure 1A, *middle* panel). Reperfusion was induced by cutting the knot on top of the PE tubing, allowing release of the occlusion; and confirmed by resolution of ST segment elevation (see Figure 1A, *right* panel). A sham procedure constituted the surgical incision without LAD ligation. Mice were monitored closely for warmth, oxygenation saturation and depth of anesthesia until the end of the ischemia/reperfusion time, when the animal was euthanized.

The aorta was cannulated, and the heart was briefly perfused with cold cardioplegie solution (KCl 50mmol/L, NaCl 70mmol/L, NaHCO3 12mmol/L, Glucose 11mmol/L, Ethylene glycol-bis (β-aminoethyl ether)-N,N,N’,N’-Tetraacetic acid 10mmol/L; 2,3 butanedione monoxime 30mmol/L and Heparin 100IU) then excised, atria cut away and ventricular chamber sliced in 3 ring-shaped sections. The apical section was flash frozen in liquid nitrogen and processed as ischemic tissue for immunoblots, while the middle section was fixed with 4% paraformaldehyde for 1 hour followed by 30% sucrose overnight for cryoprotection at 4°C. The sections were embedded and frozen in OCT solution (Optimal Cutting Temperature, Sakura) prior to sectioning with cryostat (8µm thick sections, Leica CM3000 cryostat). Air-dried sections were kept frozen until fluorescence imaging. In separate studies, adult male C57/Bl6 mice (obtained from Jackson Laboratories) were pretreated with superoxide dismutase mimetic, MnTMPyP (6
mg/kg IP as ROS scavenger) or saline and subjected to in-vivo ischemia-reperfusion injury as described above. All animal studies were approved by the Animal Studies Committee at Washington University School of Medicine and the Institutional Animal Care and Use Committee at the John Cochran Veterans Affairs Medical Center.

**Isolation of neonatal rat cardiac myocytes (NRCMs):** Rat neonatal cardiomyocyte cultures were prepared as described\(^3\). Briefly, hearts were removed from 1-day-old Sprague-Dawley rats, the atria and great vessels were trimmed off, and tissue was finely minced followed by sequential digestion with 0.5 mg/ml collagenase (WAKO, Richmond, VA). Ventricular cardiomyocytes were separated from fibroblasts by differential plating and were cultured in gelatin-coated 12-well tissue culture plates (0.4 hearts/well) in media containing Dulbecco's modified Eagle's medium, 10% horse serum, 5% fetal calf serum, 100 \(\mu\)mol/L bromodeoxyuridine, penicillin, streptomycin, and L-glutamine. Nutrient deprivation was induced by culturing in serum-free, amino-acid free, low-glucose medium\(^3\).

**Hypoxia-reoxygenation modeling in vitro:** Cells were subjected to hypoxia in vitro in an oxygen control cabinet (Coy Laboratories, Grass Lake, MI) mounted within an incubator and equipped with oxygen controller and sensor for continuous oxygen level monitoring. A mixture of 95% nitrogen and 5% CO\(_2\) was utilized to create hypoxia and oxygen levels in the chamber were monitored and maintained at <1%, as described\(^3\).

**Generation of viral constructs:** The generation of mCherry-GFP-LC3 particles has been described\(^3\). Coding sequences for rat \(LAMP2A\) and rat \(LAMP2B\); and C-terminal HA-tagged mouse BECLIN-1 were cloned into pENTR-TOPO vector (Invitrogen) and recombinant adenoviral constructs generated by employing Clonase recombinase (ViraPower, Invitrogen). Adenoviruses were subsequently generated in
HEK293A cells and titered per manufacturer’s instructions. Adenoviral particles coding for Beclin-1-1 shRNA, and CFP-tagged polyglutamine Q19 and Q80 constructs have been previously described.

Assessment of cell death: Cell death assays were performed in 96 well plate and chamber slide (Nunc, Fisher) format with the Live-Dead Cytotoxicity Viability kit for Mammalian cells (Invitrogen) per manufacturer’s instructions as described3.

Flow cytometry: Flow cytometric analysis on a BD FACScan flow cytometer with analysis of 20,000 events per sample as described3,6. Briefly, NRCMs were incubated with tetra-methyl rhodamine ester (TMRE, an indicator of intact mitochondrial inner membrane potential) at a final concentration of 50nmol/L for 30 minutes, trypsinized and subjected to flow cytometric analysis with emission in the FL2 channel. For intracellular ROS measurement, NRCMs were washed and incubated with carboxy-H2DCFDA (a cell-permeant ROS indicator) at a final concentration of 10µmol/L for 30 minutes in HBSS at 37 degrees, trypsinized and subjected to flow cytometry with emission in the FL1 channel. Assessment for LysoTracker Red staining was performed as described3. Cyflogic software (freeware from cyflogic.com) was employed for analysis.

Immunoflourescence imaging: Imaging studies for GFP-LC3 in myocardial sections was performed as described1. All visible GFP puncta (small and large) were counted. Imaging for mCherry-GFP-LC3 localization was performed as described3. LysoTracker staining and assessment of polyglutamine (Q80 and Q19) distribution were performed on 4% paraformaldehyde fixed cells using Axioscap upright microscope; AxioCam HRC camera and Plan Neofluar objective (63X, NA1.25, oil) (Zeiss) fitted with appropriate filter cubes. Images were acquired and analyzed using Zeiss Axiovision software Punctate
fluorescent tagged LC3 and Q80 dots were counted and expressed as number per nucleus. Imaging for LAMP2 was performed on NRCMs fixed with 4% paraformaldehyde (20 minutes) followed by permeabilization with 0.1% Triton X-100 (5 minutes), using the monoclonal antibody (10D10) against matrix side of LAMP2 protein. Organelle imaging was performed using LysoTracker Red and Green, Mitotracker Red CMX-ROS, TOPRO-3 iodide (Invitrogen) per manufacturer’s protocols; and Acridine Orange (Biotium) as described. Assessment of lysosomal pH was performed with pH-sensitive dye-Lysosensor yellow/blue DND-160 (Invitrogen) following manufacturer’s instructions, as described. Imaging for mitochondrial membrane potential and intracellular reactive oxygen species were performed on cells treated with TMRE and carboxy-H2DCFDA, as described.

**Immunoblotting:** Immunoblotting was performed on cardiac and cellular extracts using previously described techniques. Antibodies employed were as follows: LAMP2-Mouse monoclonal ABL-93 (Developmental Studies Hybridoma Bank) for murine tissues and mouse monoclonal 10D10 for rat tissues (kind gift from Dr. Michel Jadot), LAMP1- sc-19992 (Santa Cruz Biotechnology) and ab24170 (Abcam); LC3- NB100-2220 (Novus Biologicals); p62- ab5416 (Abcam); Beclin-1–ab16998 (Abcam); and α-Sarcomeric actin-ab7799 (Abcam). Image J software was employed for quantitative analysis. Protein abundance was normalized to α-sarcomeric protein expression and reported as fold change versus control. Chemicals employed were obtained as follows (Vendor, Catalog number): Rapamycin (EMD4Biosciences, 553212), Chloroquine (Sigma, CC6628), MnTMPyP (Calbiochem, 475872), TMRE (Invitrogen, T-699), 5-(and-6)-carboxy-2’7’-dichlorodihydrofluorescein diacetate (H2DCFDA; Invitrogen, C-400), cyclosporine A (Calbiochem, 239835) and ZVAD-fmk (Calbiochem, 627610).
Quantitative PCR: Real time PCR was performed as described. Briefly, total RNA was prepared from NRCMs using a RNA-easy mini kit (Qiagen) and cDNA was synthesized with 1 microgram of total RNA using SuperScript III first-strand synthesis system (Invitrogen). 1ul of cDNA template was mixed with 12.5 ul of 2xSYBR Green PCR Master Mix (Invitrogen) and subjected to quantitative PCR in triplicate under the following conditions: 50 °C, 2 min: 95 °C, 10 min, followed by 40 cycles of 95 °C, 15s, 60 °C, 1 min in the ABI7500 Fast RealTime PCR system. The housekeeping gene GAPDH was also amplified in parallel as a reference for the quantification of transcripts coding for LC3, p62, RAB7 and LAMP1. Primers employed were as follows: 1) Rat MAP1LC3B (LC3): forward 5’- TTTGTAAGGCGGTTCTGAC-3’ and reverse 5’- CAGGTAGCAGGAAGCAGAGG-3’; 2) Rat SQSTM1 (p62): forward 5’-GCTGCCCTGTACCCACATCT-3’; and reverse 5’- CGCCTTCATCCGAGAAAC-3’ modified from; 3) Rat RAB7 forward 5’-TTACTTGCAGACCGTGCCAAGGA-3’ and reverse 5’-TGTCAGTTTGGATGGGTCGGGA-3’; 4) Rat LAMP1 forward 5’-TCTTCAGCAGCAGTCCAG-3’ and reverse 5’-ATGAGGACGATGAGGACCAG-3’ developed and validated internally; and 5) Rat GAPDH forward 5’-ACTCCCACTCTTCCACCTTC-3’ and reverse 5’-TCCTGCTCAGTGTCCTTGC-3’. Results were calculated using the ∆-∆Ct method and expressed as previously described.

Statistical analysis: Statistical differences were assessed with the unpaired Student’s t-test for assessing differences between two independent groups; and with a paired t-test for assessing differences in autophagosomes and autolysosomes measured from the same cells within a group (i.e for dependent data). One-way ANOVA was employed for multiple groups and assumptions of normality and equality of variances were verified. Bonferroni’s post-hoc testing (with adjustment factor value of (K choose 2) =
K(K-1)/2) was employed after ANOVA for testing all pairwise comparisons. A two-tailed P value of less than 0.05 was considered statistically significant. All analyses were conducted with SPSS software (SPSS Inc., Chicago, IL).

Additional Acknowledgements:

The product ABL-93 (Mouse monoclonal against mouse LAMP2) developed by J. Thomas August, was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242. Confocal imaging studies employed in this work were supported by the Hope Center Alafi Neuroimaging Lab and a P30 Neuroscience Blueprint Interdisciplinary Center Core award to Washington University (P30 NS057105).”

Reference List


Supplemental figure legends:

**Figure S1: Autophagosome processing is impaired by reoxygenation following hypoxic insult. A)** Representative immunofluorescence images (630X) demonstrating mCherry-GFP-LC3 localization in NRCMs subjected to hypoxia or normoxia as control (both 6 hours); and hypoxia (6 hours) followed reoxygenation (2 hours) or normoxia (8 hours) as control. Nuclei are blue (DAPI). **B)** Quantitative analysis of autophagosomes (white bars), autolysosomes (black bars) and both (gray bars) in NRCMs subjected to hypoxia (Hyp) followed by reoxygenation (Reox) as in A; n=20-30 nuclei/group. * and # indicate P< 0.05 vs respective Normoxic control for autophagosomes and both (autophagosomes and autolysosomes), respectively, by post-hoc test. P values indicated are by paired t-test within each treatment. **C)** Immunoblot depicting LC3, p62, LAMP2 and BECLIN-1 expression in NRCMs subjected to hypoxia for 6 hours without reoxygenation or after 2 hours of reoxygenation; and normoxic controls cultured for the same duration in parallel. Representative of n=2 experiments. **D)** Representative flow cytometric tracing of LysoTracker Red staining (left) and quantitation (right) of mean LysoTracker Red expression (as fold change) by flow cytometry in NRCMs subjected to hypoxia (6 hours) followed by reoxygenation (2 hours; Red); and normoxia (8 hours; Black) as control. N=3-6/group. P value is by t-test.

**Figure S2: Hypoxia-reoxygenation does not alter lysosome numbers, cause lysosomal permeabilization, or alter lysosome pH in NRCMs. A)** Distribution of lysosomes by LysoTracker red (top) and LysoTracker green (bottom, with mitotracker red costaining) staining in NRCMs subjected to hypoxia, hypoxia-reoxygenation and normoxia (control). **B)** Representative flow cytometric analysis of tracing of LysoTracker red staining (left) and quantitation (right) of mean LysoTracker Red expression
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Figure S3: Clearance of short polyglutamine (Q19) repeat peptide and cell death in NRCMs subjected to hypoxia-reoxygenation injury. A) Representative epifluorescence images for NRCMs infected with adenoviruses expressing CFP tagged polyglutamine Q19 repeats (at 100 MOI) and subjected to hypoxia, hypoxia-reoxygenation and normoxia (control). B) NRCMs were subjected to hypoxia or normoxia (control) for the indicated time periods; or hypoxia followed by 18 hours of reoxygenation for the same time period and cell death assessed at the indicated time points. * indicated P<0.05 vs normoxic control in the same treatment group at 24 hours by post-hoc test after one-way ANOVA (n=14-40/group). Data at 24 hours are from Figure 5D. C) Cell death in NRCMs subjected to hypoxia (for 24 hours) or hypoxia followed by reoxygenation (6-18 hours) in presence of chloroquine (CQ, 10µmol/L) or diluent control. P values indicated are by post-hoc test after one-way ANOVA (n=8-12/group).
Figure S4: Adenoviral transduction of LAMP2 isoforms in NRCMs subjected to hypoxia-reoxygenation injury. Representative epifluorescence images (630X) of NRCMs adenovirally transduced with rat LAMP2A, rat LAMP2B, both together or LacZ as control (each at MOI=10) and subjected to hypoxia (6 hours) followed by reoxygenation (18 hours); or normoxia (24 hours) as control, and immunostained for LAMP2 (with antibody against the matrix side of LAMP2 protein). Nuclei are blue (DAPI).

Figure S5: Effects of Beclin-1 knockdown on autophagy. A) Representative immunofluorescence images (630X) depicting mCherry-GFP-LC3 expression in NRCMs transduced with adenoviruses expressing Beclin-1 shRNA (MOI=1 and 10), or LacZ shRNA control (MOI=1) and subjected to hypoxia-reoxygenation (6-18 hours) and normoxic growth conditions as control. B) Quantitative analysis of autophagosomes (white bars), autolysosomes (black bars) and both (gray bars) in NRCMs subjected to hypoxia followed by reoxygenation (Hyp/Reox) or normoxia (Nor) as in A; n=10-15 nuclei/group. * and # indicate P< 0.05 vs respective Normoxic control for autophagosomes and both (autophagosomes and autolysosomes), respectively, by post-hoc test. P values depicted are by paired t-test within each treatment. See Figure 7D for quantitative analysis of Beclin shRNA MOI-10 samples. C) Immunoblot depicting BECLIN-1, LAMP2, LC3 and p62 expression in NRCMs adenovirally transduced with Beclin-1 shRNA (MOI=1 for 48 hours) and LAMP2A+LAMP2B (each at MOI=10 for 24 hours); and LacZ control (MOI=21) and thereafter subjected to hypoxia (6 hours) followed by reoxygenation (18 hours). Representative of n=2 experiments.
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자가포식소체 기능조절로 허혈/재관류 시 심근보호가 가능한 것으로 보인다

백 상 흥 교수 가톨릭대학교 서울성모병원 순환기내과

Summary

배경
심근허혈에서 AMP-유발 단백질 kinase 기전에 의한 자가포식은 심근보호 작용이 있지만, 재관류는 BECLIN-1의 증가와 세포사멸을 일으킨다. 자가포식은 세포소기관의 항상성 유지를 위한 세포 내 분해 과정이며, 허혈/재관류 손상(ischemia/reperfusion injury)과 같은 스트레스 반응으로 유발된다. 거대자가포식은 이중막과 결합한 자가포식소체(autophagosome) 내에서 분해될 물질과의 리소좀을 통한 분해에 관여하며, 거대자가포식을 통한 효과적인 유동성은 세포 생존에 필수적이다. 본 연구에서는 심근세포의 허혈/재관류 손상에서 거대자가포식 경로를 통해 유동성을 관찰하여 자가포식소체의 축적 결과, 세포의 초기 생존과 심근세포 사멸을 예방할 수 있는지를 검증하였다.

방법 및 결과
1. In vivo에서 허혈/재관류 손상 시, 심근세포에서 자가포식소체의 제거 기전이 손상

마우스의 심근세포에 허혈 자극(2시간) 또는 허혈 30분 후 재관류를 90분 동안 시행한 두 가지 경우에서 심근세포에 특이적으로 GFP-LC3가 발현하는 실험 대상을 정립하고, chloroquine의 존재하에서 LC3의 분포를 측정하였다. 수술 대조군과 비교 시, 허혈 자극의 경우 자가포식소체가 10배 이상 증가함을 확인하여 자가포식소체 형성의 유발을 확인하였다.

2. 손상된 자가포식소체의 활동 과정 중 심근세포의 허혈/재관류 손상은 LAMP2의 감소와 BECLIN-1의 증가를 유도

자가포식소체의 활동 과정은 리소좀 수의 감소, 자가포식소체-리소좀 결합, 또는 리소좀의 기능이상에 의해 억제된다. 리소좀 막단백질인 LAMP1(lysosome-associated membrane protein-1)의 결핍은 자가포식소체의 변화에는 영향을 주지 않았으나, LAMP2의 결핍은 자가포식소체-리소좀 결합에서 중요한 요소임을 확인
인하였다. BECLIN-1의 수치는 재관류 동안 증가하지만, 허혈 동안에는 증가하지 않았다. 허혈/재관류 손상 후 유지되는 활성 산소종(reactive oxygen species)의 생성은 자가포식소체의 활동 과정을 억제하며, 세포 구조에 영향을 주어 거대자가포식을 통한 제거가 필요하다. 허혈/재관류 손상에 의한 LAMP2의 감소와 BECLIN-1의 증가는 활성 산소종 억제제인 MnTMPyP\([\text{Mn(III) tetrakis(1-Methyl-4-pyridyl) porphyrin pentachloride}]\)의 처리로 억제된다.

3. 심근세포에서 손상된 자가포식소체의 제거 기전은 세포사멸을 일으킴
자가포식소체 제거 기전의 손상이 심근세포의 사멸을 일으킨다고 가정하고, 실험 진행 시 neonatal rat cardiomyocytes(NRCMs)에 rapamycin으로 처리한 후, 영양재를 통해 자가포식의 발현을 촉진하고, chloroquine 처리를 통해 자가포식소체 기전을 차단하였다. Chloroquine의 처리를 통한 자가포식소체 기전의 손상은 자가포식소체와 결합하는 LC3-Ⅱ, p62의 축적을 야기하고, 이는 자가포식소체의 제거 기전을 손상시켜 세포사멸에서 세포사멸의 충분한 원인이 되었다.

4. 심근세포에서 Hypoxia-Reoxygenation이 자가포식소체의 축적을 유도
24시간 동안 NRCMs의 hypoxia 상태는 자가포식소체와 autolysosome를 증가시키고, hypoxia 6시간 후 reoxygenation은 자가포식소체를 증가시키지만, autolysosome는 증가시키지 않는다. 지속되는 hypoxia 또는 hypoxia-reoxygenation은 줄 다 LC3-Ⅱ를 증가시키며, 자가포식소체가 증가된다. Hypoxia-reoxygenation은 hypoxia 상태만 유지한 경우에 비해 세포사멸이 증가한다. Hypoxia-reoxygenation에서 자가포식소체의 축적은 활성 산소종 발생 및 미토콘드리아의 투과성과 연관되고, hypoxia-reoxygenation에 의한 세포사멸을 cyclosporine A가 약화시킬 것을 확인하였다.

5. Hypoxia-Reoxygenation 시 활성 산소종이 LAMP2의 감소와 BECLIN-1의 증가를 유도
Reoxygenation 2시간 후 LAMP2가 감소하고 BECLIN-1이 증가되며, 이를 억제하기 위해 in vivo에서 MnTMPyP의 처리와 함께 허혈/재관류 손상을 시행한 결과, MnTMPyP는 LAMP2의 감소와 BECLIN-1의 증가를 억제시킨다. Reoxygenation이 유발하는 활성 산소종의 증가는 자가포식소체의 형성을 유도하지만, 그들의 제거 기전의 손상을 가져온다.

6. BECLIN-1의 발현 억제가 LAMP2 isoform의 외인성 발현과 함께 작용하여 자가포식소체의 작용을 회복
BECLIN-1은 자가포식소체의 형성에 필수적인 역할을 하므로 자가포식을 통한 세포 생존에 중요한데, BECLIN-1의 부분적인 제거와 외인성 발현(adenovirus를 이용한 과발현)에 의한 LAMP2의 발현은 자가포식소체 기전의 미니아 효과를 가져올 것이며, hypoxia-reoxygenation이 유도한 세포사멸이 약화될 것이라고 가정한다. 부분적인 BECLIN-1의 제거와 LAMP2A/B의 과발현에 의한 자가포식소체 기전의 회복은 hypoxia-reoxygenation에 의해 유도된 세포사멸을 약화시킨다. 일반적인 산소 농도 조건에서 배양된 NRCMs에서 hypoxia-reoxygenation injury에 대한 반응으로 자가포식소체 형성의 억제와 함께 BECLIN-1의 제거는 세포사멸을 증가시킨다.

7. BECLIN-1이 충분히 존재하면 자가포식 유전자의 전사를 조절함
hypoxia-reoxygenation injury 조건의 변화 없이 LAMP1과 함께 LC3, p62의 단백질의 연속적인 변화와 전사를 관찰한 결과, 부분적으로 BECLIN-1을 제거하면 LC3, p62, LAMP1이 증가된다. BECLIN-1의 발현은 자가
포식소체 관련 유전자 전사를 억제하며, 세포사멸이 증가된다. 이러한 연구 결과는 활성 산소종이 BECLIN-1의 증가를 유도해 자가포식소체-리소좀 기전의 구성요소들의 전사 억제를 통해 자가포식소체의 성숙을 억제함을 보여준다.

연구 결과, 허혈/재관류 손상 시 자가포식소체의 제거 기전이 수술 대조군보다 손상되었음을 확인하였다. 자가포식소체의 축적은 활성 산소종의 증가와 미토콘드리아 투과성과 연관되어 세포사멸을 유도하나, 이를 cyclosporine A에 의해 억제된다. LAMP2 수치의 회복은 부분적인 BECLIN-1의 발현 억제와의 시너지 효과로 자가포식소체의 기전을 회복시키며, hypoxia-reoxygenation 후 세포사멸을 약화시킨다.

결론
본 연구는 자가포식이 왜 허혈 동안 경색부위를 줄이고, 심근세포의 생존을 가능하게 하는지에 대한 기전이 세포의 사멸과 관련되어 있음을 확인하였다. 또한, 재관류와 함께 LAMP2의 급격한 감소를 관찰하였고, LAMP2의 발현 억제는 영양결핍 상태에서 자가포식을 손상시키며, 손상된 자가포식소체는 활성 산소종의 증가와 연관이 있다. 이는 미토콘드리아의 두과정을 유발하고, 심장의 허혈/재관류 손상에서 세포사멸의 메커니즘을 따르며, 심장의 스트레스와 다른 여러 조건에서 이들의 메커니즘 파악을 위해서는 자가포식의 유동성을 평가가 필요하다.
결론적으로, 허혈/재관류 손상은 자가포식소체의 제거 기전을 손상시키며, LAMP2의 감소와 BECLIN-1의 증가를 유도하여 심근세포의 사멸을 증가시킨다.

Commentary
현재 심근경색의 치료는 심근 손상 방지를 위한 재관류에 초점을 맞추고 있다. 하지만 성공적인 재관류에도 불구하고, 재관류 후 손상에 의한 심근세포의 사멸이 진행되는데, 이러한 세포사멸에서 심근세포를 보호하는 것이 귀중적인 치료 목표이다. 심근경색 후 재관류 시 손상에 의한 자가포식소체와 기타 유해한 물질의 축적에 의한 심근세포의 활성 산소종 증가와 미토콘드리아 투과성 증가로 인한 심근세포의 세포사멸을 유발한다. 하지만 LAMP2 발현 회복과 BECLIN-1 억제는 자가포식소체 기전을 회복시키며 세포사멸을 억제시킨다. 따라서 자가포식소체 기전을 이용한 강한 유동성을 지닌 자가포식은 허혈 후 재관류 시 심근세포 손상을 줄이는 긍정적인 치료 전략이 될 것으로 사료된다.
Impaired Autophagosome Clearance Contributes to Cardiomyocyte Death in Ischemia/Reperfusion Injury

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Background—In myocardial ischemia, induction of autophagy via the AMP-induced protein kinase pathway is protective, whereas reperfusion stimulates autophagy with BECLIN-1 upregulation and is implicated in causing cell death. We examined flux through the macroautophagy pathway as a determinant of the discrepant outcomes in cardiomyocyte cell death in this setting.

Methods and Results—Reversible left anterior descending coronary artery ligation was performed in mice with cardiomyocyte-restricted expression of green fluorescent protein–tagged microtubule–associated protein light chain-3 to induce ischemia (120 minutes) or ischemia/reperfusion (30–90 minutes) with saline or chloroquine pretreatment (n = 4 per group). Autophagosome clearance, assessed as the ratio of punctate light chain-3 abundance in saline to chloroquine-treated samples, was markedly impaired with ischemia/reperfusion compared with sham controls. Reoxygenation increased cell death in neonatal rat cardiomyocytes compared with hypoxia alone, markedly increased autophagosomes but not autolysosomes (assessed as punctate dual fluorescent mCherry-green fluorescent protein tandem-tagged light chain-3 expression), and impaired clearance of polyglutamine aggregates, indicating impaired autophagic flux. The resultant autophagosome accumulation was associated with increased reactive oxygen species and mitochondrial permeabilization, leading to cell death, which was attenuated by cyclosporine A pretreatment. Hypoxia-reoxygenation injury was accompanied by reactive oxygen species–mediated BECLIN-1 upregulation and a reduction in lysosome-associated membrane protein-2, a critical determinant of autophagosome-lysosome fusion. Restoration of lysosome-associated membrane protein-2 levels synergizes with partial BECLIN-1 knockdown to restore autophagosome processing and to attenuate cell death after hypoxia-reoxygenation.

Conclusion—Ischemia/reperfusion injury impairs autophagosome clearance mediated in part by reactive oxygen species–induced decline in lysosome-associated membrane protein-2 and upregulation of BECLIN-1, contributing to increased cardiomyocyte death. (Circulation. 2012;125:3170-3181.)

Key Words: autophagy • cell death • ischemia • reperfusion injury

Autophagy is an intracellular lysosomal degradative process operative in homeostatic clearance of organelles and protein aggregates.1 Macroautophagy involves segregation of cargo within double-membrane–bound autophagosomes that fuse with and are degraded within lysosomes, and efficient flux through the macroautophagy pathway is essential for cell survival.2 However, the frequent observation of autophagosomes in dying cells has stimulated interest in examining autophagy as a mechanism for cell death, called type II programmed cell death.3 Whether autophagy causes programmed cell death or is “guilty by association”4 remains a subject of active investigation.

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Autophagy induction is critical for survival during the perinatal period of relative starvation,5 and suppression of constitutive cardiomyocyte autophagy with ablation of ATG56 or impairment of late stages of autophagy in the absence of lysosome-associated membrane protein-2 (LAMP2) in patients with Danon disease7 and in mice with LAMP2 ablation8 results in cardiomyopathy. Autophagy is also rapidly induced in the myocardium in response to stress such as fasting,9 pressure overload,6,9 and ischemia/reperfusion (IR) injury.10 In contrast to a clear prosurvival role for constitutive autophagy, stress-induced autophagy has been ascribed both salutary and deleterious roles in cardiomyocyte function and survival.9,10

Autophagosome prevalence, a commonly used readout for the state of autophagy activation, is determined by the rate of autophagosome formation (ie, induction of autophagy) and the rate of autophagosome destruction and is therefore a
function of “flux” through the autophagic pathway. It is not clear whether the increased abundance of autophagosomes in dying cells reflects upregulation of adaptive autophagy, an instance of dysregulated and excessive self-cannibalism, or an impairment in autophagic flux with reduced clearance of autophagosomes (and presumably cargo) that would normally be degraded by autophagy as postulated to occur in Danon disease, with secondary activation of programmed cell death.

In this study, we have used an integrated approach to examine flux through the macroautophagy pathway in myocardial IR injury to test the hypothesis that impairment in late stages of autophagy with resultant autophagosome accumulation prevents its prosurvival role and triggers cardiomyocyte death.

**Methods**

**IR Modeling**

Adult male cardiomyocyte-specific green fluorescent protein–light chain-3 (GFP-LC3) transgenic mice and C57BL/6 mice (from Jackson Laboratories) were subjected to reversible left anterior descending artery coronary ligation in the presence of chloroquine 10 mg/kg or MnTMPyP 6 mg/kg IP, respectively, or saline control 1 hour before surgery. All animal studies were approved by the Animal Studies Committee at the Washington University School of Medicine and the Institutional Animal Care and Use Committee at the John Cochran VA Medical Center.

**Generation of Viral Constructs**

Adenoviruses coding for rat LAMP2A and rat LAMP2B were generated with the Invitrogen Virapower system. Adenoviruses coding for Beclin1 shRNA and cyan fluorescence protein–tagged polyglutamine Q19 and Q80 constructs and lentivirus coding for mCherry-GFP-LC3 have been described.

Assessment of cell death, hypoxia-reoxygenation modeling, immunofluorescence imaging, flow cytometry, and quantitative polymerase chain reaction analysis was performed as described.

**Statistical Analysis**

Results are expressed as mean ± SEM. Statistical differences were assessed with the unpaired Student’s t test for 2 independent groups, paired t test for dependent data, and 1-way ANOVA for multiple groups with SPSS software. Bonferroni post hoc testing was used after ANOVA for testing all pairwise comparisons between groups. A 2-tailed value of P<0.05 was considered statistically significant.

**Results**

**Autophagosome Clearance Is Impaired in Cardiomyocytes With IR Injury In Vivo**

Ischemic insult activates cardiomyocyte autophagy, as evidenced by an increase in the ratio of LC3-II to LC3-I and increased numbers of punctate GFP-LC3–bearing autophagosomes after a brief episode of myocardial ischemia. Autophagosome abundance is further increased after reperfusion injury. Because the prevalence of autophagosomes at any point in time is determined by the rate of formation of autophagosomes (ie, induction of autophagy) and the rate of autophagosome processing (ie, flux through the macroautophagy pathway), we determined cumulative autophagic flux by determining the numbers of autophagosomes in the presence and absence of chloroquine, which inhibits lysosomal acidification and prevents autophagosome-lysosome fusion. We subjected mice with cardiomyocyte-specific expression of GFP-LC3 to in vivo ischemia for 2 hours (confirmed by ST-segment elevation; Figure 1A, middle) or IR for an equivalent duration (ischemia for 30 minutes followed by reperfusion for 90 minutes with reversal of ST-segment elevation; Figure 1A, right) in the absence and presence of chloroquine and assessed LC3 distribution. Mice subjected to a sham procedure demonstrate a basal level of cardiomyocyte autophagy as evidenced by punctate localization of GFP-LC3 (on autophagosomes), with autophagosome accumulation (~6-fold increase) in the presence of chloroquine, suggesting intact autophagic flux (Figure 1B and 1C). Ischemia caused a 10-fold increase in autophagosome abundance compared with sham operation, implying the induction of autophagosome formation (Figure 1B and 1C). This was accompanied by partial impairment of autophagic flux (ratio of autophagosome abundance with and without chloroquine of ~2 with ischemia compared with ~6 in sham; Figure 1C). Reperfusion after ischemia provoked a further increase in autophagosome abundance (23-fold), which was not altered in the presence of chloroquine, suggesting markedly impaired autophagic flux (ratio of autophagosome abundance with and without chloroquine of ~1; Figure 1C). Levels of GFP-tagged LC3-II and p62, a protein that links ubiquitinated aggregates for destruction within autophagosomes and is degraded on autophagosome processing, increased in IR-treated hearts to a degree comparable to that in hearts treated with chloroquine (Figure 1D–1F), further supporting the observation of impaired autophagosome processing with IR injury.

**Myocardial IR Injury Provokes a Decline in LAMP2 and an Increase in BECLIN-1 Abundance With Impaired Autophagosome Processing**

Autophagosome processing can be impaired if lysosome numbers decline or if there is a selective impairment in autophagosome-lysosome fusion or lysosome function. Accordingly, we examined the expression of the lysosome membrane proteins LAMP1 and LAMP2. Interestingly, the level of LAMP2 declined by 41% and of LAMP1 declined by 44% in IR-treated GFP-LC3 transgenic hearts (Figure 1D, 1G, and 1H); only the decline in LAMP1, which closely tracks lysosome numbers, was prevented by chloroquine pretreatment, suggesting that the decline in LAMP2 was independent of lysosome pH and was not related to lysosome consumption in IR-induced autophagy. Interestingly, although LAMP1 deficiency in mice is of no serious consequence, LAMP2 is a critical determinant of autophagosome-lysosome fusion, and mutations causing LAMP2 deficiency result in Danon disease, which is characterized by autophagosome accumulation in multiple tissues, including the myocardium, and cardiomyopathy. As observed previously, BECLIN-1 levels were elevated with reperfusion injury and not during ischemia (Figure 1D and 1I).

To evaluate whether a reperfusion-induced surge in reactive oxygen species (ROS) generation mediated the observed changes, we treated adult male C57BL/6 mice with Mn(III)tetrakis(1-Methyl-4-pyridyl) porphyrin pentachloride (MnTMPyP), a cell-permeable superoxide dismutase mimetic that acts as a ROS scavenger, or diluent and subjected them...
to reversible left anterior descending ligation for 30 minutes followed by reperfusion for 90 minutes or sham surgery. IR induced a marked increase in autophagosome-bound LC3-II abundance with p62 accumulation in the myocardium, which were both prevented by pretreatment with MnTMPyP (Figure 2A–2C). This suggests that IR-induced ROS generation may inhibit autophagosome processing in addition to damaging cellular structures that need removal through macroautophagy. Importantly, the observed IR-induced decline in LAMP2 and upregulation in BECLIN-1 were prevented with MnTMPyP pretreatment (Figure 2A, 2D, and 2E). Curiously, we did not observe an IR-induced decline in LAMP1 in this strain of mice, as observed in the GFL-LC3 reporter mice, and basal LAMP1 levels appeared to be negatively regulated by ROS (Figure 2A and 2F).

**Impaired Autophagosome Clearance Is Sufficient to Cause Cell Death in Cardiomyocytes**

Autophagy has been implicated as a mechanism for programmed cell death. To test the hypothesis that impairment in autophagosome processing causes cardiomyocyte death, we subjected neonatal rat cardiomyocytes (NRCMs) to treatment with rapamycin, an irreversible inhibitor of mammalian target of rapamycin, and nutrient deprivation to stimulate autophagy and blocked autophagosome processing with chloroquine pretreatment. The relative abundance of autophagosomes and autolysosomes was assessed with lentivirally transduced mCherry-GFP tandem-tagged LC3 (Figure 3A) as described previously. Induction of autophagy leads to punctate localization of LC3 on autophagosomes, which demonstrate both red and green fluorescence, with subsequent loss of green fluorescent signal on autophagosome-lysosome fusion and formation of autolysosomes resulting from the instability of GFP in the acidic intralysosomal environment. The relative abundance of autophagosomes and autolysosomes was assessed with lentivirally transduced mCherry-GFP tandem-tagged LC3 (Figure 3A) as described previously. Induction of autophagy leads to punctate localization of LC3 on autophagosomes, which demonstrate both red and green fluorescence, with subsequent loss of green fluorescent signal on autophagosome-lysosome fusion and formation of autolysosomes resulting from the instability of GFP in the acidic intralysosomal environment.
Figure 3B). Treatment with rapamycin stimulates autophagy with enhanced autophagic flux,15 as evidenced by markedly increased abundance of autolysosomes without a discernible accumulation of autophagosomes (Figure 3A, middle, and Figure 3B). Pretreatment with chloroquine resulted in accumulation of autophagosomes and the near absence of autolysosomes (Figure 3A, bottom, and Figure 3B). Rapamycin treatment provoked a decline in cumulative LC3 abundance, suggesting consumption during autophagy, and clearance of p62 (Figure 3C) compared with control, indicating intact flux. In contrast, impairment of autophagosome processing with chloroquine caused accumulation of autophagosome-bound LC3-II and p62 (Figure 3C), indicating impaired autophagosome clearance, which was sufficient to cause cell death in the basal state, and with rapamycin or nutrient deprivation–initiated autophagy, wherein autophagosome clearance was inhibited (Figure 3D). To investigate the mechanism of cell death with chloroquine, we posited that autophagosome accumulation prevents the clearance of damaged intracellular organelles and proteins, leading to increased ROS generation, which causes mitochondrial permeabilization and activation of programmed apoptosis and/or necrosis.3 Indeed, chloroquine treatment increased ROS generation (Figure 3E and 3F) with loss of mitochondrial membrane potential (Figure 3E and 3G). Induction of ROS by chloroquine was not prevented by pretreatment with cyclosporine A, an inhibitor of mitochondrial permeability transition pore, indicating that ROS generation was upstream of mitochondrial permeabilization. Accordingly, pretreatment with cyclosporine A but not ZVAD-fmk (a caspase inhibitor) attenuated chloroquine-induced cell death under conditions of both basal and rapamycin-stimulated autophagy, suggesting activation of programmed necrosis pathway with autophagosome accumulation.

**Hypoxia-Reoxygenation Induces Autophagosome Accumulation in Cardiomyocytes**

Autophagic flux has been noted to be impaired in HL-1 cardiomyocytes, an immortalized cell line derived from atrial myocytes, with in vitro hypoxia-reoxygenation insult.20 We subjected NRCMs to in vitro hypoxia (ambient O2 concentration <1%) followed by reoxygenation and determined the relative abundance of autophagosomes and autolysosomes.15 NRCMs cultured in a normoxic environment display basal autophagy with intact flux (Figure 4A; see also Figure 3A). Hypoxia for 24 hours markedly increased the abundance of autophagic structures (Figure 4A, middle), with an increase in both autophagosomes and autolysosomes (Figure 4B). Reoxygenation after 6 hours of hypoxia further increased the numbers of autophagic structures, which are predominantly autophagosomes (Figure 4A, bottom, and Figure 1A and IB in the online-only Data Supplement), without an increase in autolysosomes. Both prolonged hypoxia and hypoxia-reoxygenation increased LC3-II abundance, indicating increased numbers of autophagosomes (Figure 4C and Figure 4D)21 without affecting LAMP1 levels except with longer duration (72 hours) of injury (Figure 4D). This was not due to a decline in the numbers or distribution of lysosomes (Figures ID, IIA, and IIB in the online-only Data Supplement), lysosomal permeabilization (Figure IIC in the online-only Data Supplement), or a change in lysosomal pH (Figure IID in the online-only Data Supplement). Hypoxia-reoxygenation–induced decline in LAMP2 was associated with BECLIN-1 upregulation compared with hypoxia alone and p62 accumulation up to 48 hours after the onset of injury (Figure 4D and Figure IC in the online-only Data Supplement). Taken together, these data suggest impairment in autophagosome processing with reoxygenation after a hypoxic insult. To determine whether hypoxia-reoxygenation impairs proteasomal processing of proteins, which could result in an overload of the autophagic pathway for protein degradation and conceivably overwhelm the lysosomal machinery, we forcibly expressed polyglutamine repeats, which are known to be differentially processed via the ubiquitin-
proteasome pathway (Q19) and macroautophagy (Q80), in NRCMs and subjected them to hypoxia-reoxygenation injury. Hypoxia-reoxygenation impaired clearance of polyglutamine Q80 aggregates (Figure 5A and 5B) but not Q19 (Figure IIIA in the online-only Data Supplement), suggesting specific impairment in autophagy.

To determine whether hypoxia-reoxygenation–induced impairment in autophagosome processing contributes to cell death, we subjected NRCMs to matched durations of hypoxia and hypoxia-reoxygenation. Hypoxia increased cell death in NRCMs, with ≈45% of cells dead after 24 hours (Figure 5C and 5D and Figure IIIB in the online-only Data Supplement) compared with 6% in cells cultured in normoxic conditions. Interestingly, reoxygenation after a short hypoxic insult (6 hours) increased cell death even further (52%) compared with a comparable duration of hypoxia alone, suggesting an association between impaired autophagosome processing (see Figure 4) and reoxygenation-induced cell death in NRCMs. Experimental inhibition of autophagosome processing with chloroquine treatment increased hypoxia-induced cell death to levels comparable to hypoxia-reoxygenation but did not affect cell death with hypoxia-reoxygenation (Figure IIIC in the online-only Data Supplement), consistent with a severe impairment in autophagosome processing in cells subjected to hypoxia-reoxygenation injury. Autophagosome accumulation with hypoxia-reoxygenation was associated with ROS generation (Figure 5E and 5F) and mitochondrial permeabilization (Figure 5E and 5G), and...
although mitochondrial permeabilization was prevented with cyclosporine A pretreatment (Figure 5E and 5G), ROS generation was not (Figure 5E and 5F), suggesting that it is upstream of mitochondrial permeabilization, mirroring the observations with chloroquine-induced autophagosome accumulation (Figure 3E–3G). Accordingly, cyclosporine A but not ZVAD-fmk attenuated hypoxia-reoxygenation–induced cell death, suggesting a mitochon-

![Image](image_url)

**Figure 4.** Hypoxia-reoxygenation injury upregulates autophagy with impaired autophagosome processing in neonatal rat cardiomyocytes (NRCMs). A, Representative immunofluorescence images (×630) demonstrating mCherry–green fluorescent protein (GFP)-light chain-3 (LC3) localization in NRCMs cultured in a normoxic environment (as controls; top) subjected to hypoxia (24 hours; middle) or hypoxia (6 hours) followed by reoxygenation (18 hours; bottom). Nuclei are blue (DAPI). Representative of n = 3 experiments. B, Quantitative analysis of autophagosomes (white bars), autolysosomes (AL; black bars), and both (gray bars) in NRCMs treated as in A. P values are by post hoc test between groups and paired t test within a group. n = 30 to 50 nuclei per group. C and D, Representative immunoblots depicting LC3 processing and lysosome-associated membrane protein-2 (LAMP2) abundance in response to hypoxia and hypoxia-reoxygenation injury for 24 and 48 hours (C) and time course for change in LAMP2, LAMP1, BECLIN-1, and p62 abundance in response to hypoxia-reoxygenation injury (D). Representative of n = 2 experiments. αSA indicates alpha-sarcomeric actin.

![Image](image_url)

**Figure 5.** Impaired autophagosome processing is associated with cell death in neonatal rat cardiomyocytes (NRCMs) subjected to hypoxia-reoxygenation injury. A, Representative immunofluorescence images (×630) depicting cyan fluorescence protein–tagged polyglutamine Q80 (Poly-Q80) accumulation (green; A) and quantitative analysis of Q80 aggregates (B) in NRCMs subjected to hypoxia (Hyp)-reoxygenation (Reox) and hypoxia and normoxic controls (Nor). Nuclei are red (TOPO-3). P value is by post hoc test. Representative of n = 2 experiments. n = 30 to 50 nuclei per group. C and D, Representative immunofluorescence images (×200; C) and quantitative analysis (D) of alive (green) and dead (red) NRCMs treated as in A. *P<0.05 by post hoc test; n = 24 to 40 per group. E, Representative immunofluorescence images (×830) of NRCMs loaded with the reactive oxygen species (ROS) indicator carboxy-H2DCFDA (top) or tetramethyl rhodamine ethyl ester (TMRE; middle) and merged (bottom) and subjected to hypoxia-reoxygenation as in A in the presence of cyclosporine A (CsA; 20 μmol/L) or dimethyl sulfoxide (DMSO) control. F and G, Quantification of mean fluorescence of flow cytometric analysis of NRCMs treated as in E for ROS (F) and TMRE (G). *P<0.05 vs normoxic control by post hoc test (n=3–4 per group). H, Cell death in NRCMs subjected to hypoxia-reoxygenation as in A in the presence of ZVAD-fmk (20 μmol/L), CsA (20 μmol/L), or DMSO and normoxic control. *P<0.05 vs normoxic control by post hoc test (n=10–20 per group).


...drial permeability transition pore–driven necrotic mechanism of cell death

**Hypoxia-Reoxygenation–Induced LAMP2 Decline and BECLIN-1 Upregulation Are Mediated by ROS**

The decline in LAMP2 and the increase in BECLIN-1 abundance are observed very early (2 hours) after the onset of reperfusion/reoxygenation (Figure 2A, 2D, and 2E and Figure I in the online-only Data Supplement), suggesting a posttranslational rather than a transcriptional mechanism. As observed with in vivo IR injury, treatment with MnTMPyP to scavenge ROS prevented a reoxygenation-induced decline in LAMP2 abundance (Figure 6A) and attenuated the increase in BECLIN-1 abundance (Figure 6B), with restoration of a relative excess of autolysosomes and a decrease in total autophagic structures in NRCMs subjected to hypoxia-reoxygenation (Figure 6C and 6D). This suggests that the reoxygenation-induced increase in ROS provokes autophagosome formation but impairs their clearance.

**Beclin-1 Knockdown Synergizes With Exogenous Expression of LAMP2 Isoforms to Restore Autophagosome Processing**

Multiple studies have demonstrated the salutary effects of **BECN1** haploinsufficiency in pathological states, including reperfusion injury, in contrast to its essential role in autophagosome formation, which is critical for autophagy-mediated cell survival. Whether the observed reperfusion/reoxygenation-induced increase in **BECLIN-1** abundance (to supranormal levels; Figures 1D, 2A, 2E, and 4D) or the decline in LAMP2 levels (Figures 1D, 1G, 2A, 2D, 4C, and 4D) contributes to ROS-induced impairment in autophagosome processing is not known. Accordingly, to test the hypothesis that partial knockdown of **BECLIN-1** and restoration of LAMP2 by exogenous expression will synergistically restore autophagosome processing and attenuate hypoxia-reoxygenation–induced cell death, we transduced NRCMs with increasing doses of adenovirus coding for **Beclin-1** shRNA expression and rat LAMP2A and rat LAMP2B, the 2 cardiac-expressed isoforms of LAMP2, either individually or in combination. **Beclin-1** knockdown was minimal with the low infective dose of virus (multiplicity of infection [MOI]=1) and substantial with a higher dose (MOI=10) compared with control (Figure 7A). Exogenous expression of LAMP2A and LAMP2B prevented the decline in LAMP2 levels after hypoxia-reoxygenation injury (Figure 7B and Figure IV in the online-only Data Supplement). Importantly, exogenous expression of LAMP2A and LAMP2B resulted in restoration of autophagosome processing, as indicated by reduced LC3-II and p62 accumulation (compared with respective normoxia control; Figure 7B), and restoration of autolysosome predominance after hypoxia-reoxygenation injury compared with a predominant increase in autophagosomes in cells treated with control viruses (Figure 7C and 7D). The effects of **Beclin-1** knockdown were dose dependent. Although minimal **Beclin-1** knockdown (**Beclin-1** shRNA MOI=1; Figure 7A) restored autophagosome processing either singly, as indicated by the increased ratio of autolysosomes to autophagosomes and reduced LC3-II and p62 accumulation (compared with respective normoxia control; Figure 7B), further knockdown of **Beclin-1** (with a higher viral dose, MOI=10; Figure 7A) decreased the abundance of autophagic structures, indicating the suppression of autophagosome formation (Fig-
These data suggest that the increased levels of BECLIN-1 seen with reperfusion/reoxygenation contribute to the impairment in autophagosome clearance, whereas the basal levels of BECLIN-1 are necessary for the induction of autophagosome formation in response to hypoxia-reoxygenation injury. The restoration of autophagosome processing with partial Beclin-1 knockdown (low dose) and LAMP2A/B overexpression was individually associated with the reduction in hypoxia-reoxygenation–induced cell death, with the largest benefit (32% reduction in cell death) observed with a combination of these strategies (Figure 7E). Further knockdown of Beclin-1 (MOI=10) with a resultant inhibition of autophagosome formation (Figure 7D and Figure VA in the online-only Data Supplement) markedly increased cell death in NRCMs cultured under normoxic conditions and in response to hypoxia-reoxygenation injury (Figure 7E), underscoring a critical role for autophagy in cardiomyocyte survival.10,20,26

BECLIN-1 Abundance Regulates Transcription of Autophagy Genes

In addition to the established role for BECLIN-1 in inducing autophagosome formation, our results indicate that partial...
Beclin-1 knockdown enhances autophagosome processing. Indeed, whereas the increased total LC3 and/or p62 abundance with partial Beclin-1 knockdown (Figure 7A and Figure VC in the online-only Data Supplement) could indicate suppression of autophagosome formation with secondary accumulation of these proteins, the increase in the ratio of autolysosomes to autophagosomes with hypoxia-reoxygenation (Figure 7C and Figure VA and VB in the online-only Data Supplement) suggests otherwise. Therefore, we examined the serial changes in protein and transcript abundance of LC3 and p62, along with that of LAMP1, the levels of which track lysosome numbers15 and do not change with hypoxia-reoxygenation injury (Figure 4D), and RAB7, a small GTP binding protein that mediates autophagosome-lysosome fusion,27 with Beclin-1 knockdown (with a low viral dose of MOI=1). Interestingly, partial Beclin-1 knockdown increased total LC3, p62, LAMP1, and RAB7 abundance at 48 and 72 hours (Figure 8A) compared with nontargeting LacZ shRNA-transduced cells, which was preceded or accompanied by an increase in respective gene transcripts, followed by a suppression in transcript levels at 72 hours for MAP1LC3B, LAMP1, and RAB7 but not SQSTM1 (Figure 8B–8E). These observations suggest that Beclin-1 knockdown activates the transcriptional machinery early (on or before 48 hours) to increase the synthesis of autophagy and lysosomal proteins, which is subsequently suppressed (at 72 hours) when levels of these proteins build up, suggesting a mechanism for restoring flux by priming the autophagy pathway.15 Indeed, exogenous expression of BECLIN-1 for 48 hours stimulated autophagosome formation but impaired autophagosome pro-

Figure 8. BECLIN-1 levels regulate transcription of autophagy genes to affect autophagosome processing. A, Representative immunoblot depicting the time course of changes in protein levels of lysosome-associated membrane protein-1 (LAMP1), light chain-3 (LC3), p62, and RAB7 in neonatal rat cardiomyocytes (NRCMs) adenovirally transduced with shRNA targeting Beclin-1 or a LacZ as nontargeting (NT) shRNA control (both at multiplicity of infection [MOI]=1) at 24, 48, and 72 hours after treatment with viral particles. Representative of n=2 experiments. B through E, Real-time polymerase chain reaction (PCR)–based quantification of MAP1LC3B (coding for LC3), SQSTM1 (for p62), LAMP1, and RAB7 transcripts in samples treated as in A. n=4 per group at each time point. P values are by t test vs control at each time point. F, Representative immunofluorescence images (×630) demonstrating mCherry–green fluorescent protein (GFP)–light chain-3 (LC3) localization in NRCMs infected with increasing doses of adenoviruses (MOI=1, 10, 50, and 100) coding for HA-tagged BECLIN-1 or LacZ as control for 48 hours. G, Quantitative analysis of autophagosomes (white bars), autolysosomes (black bars), and both (gray bars) in NRCMs treated as in A; n=10 to 20 nuclei per group. *P<0.05 vs respective control for autophagosomes, #P<0.05 vs respective control for autolysosomes by posthoc test. P value within each group is by paired t test. H, Immunoblots (representative of n=2 experiments) depicting Beclin-1 (HA), LC3, p62, RAB7, and LAMP1 expression in NRCMs treated as in F. I through L, Real-time PCR-based quantification of MAP1LC3B (I), SQSTM1 (J), LAMP1 (K), and RAB7 (L) transcripts in NRCMs adenovirally transduced with Beclin-1-HA or LacZ (100 MOI) as control for 24 hours. n=4 per group at each time point. P values are by t test vs control. M, Cell death in NRCMs treated as in F; n=8 to 16 per group. P values noted are by post hoc test. aSA indicates alpha-sarcomeric actin.
cessing (Figure 8F and 8G) and increased levels of autophagosome-bound LC3-II and p62 with a decline in LAMP1 and Rab7 levels (Figure 8H) in a dose-dependent fashion. Importantly, high levels of BECLIN-1 expression (MOI = 100) suppressed gene transcription (predating the observed protein changes) for MAP1LC3B (Figure 8I), LAMP1 (Figure 8K), and Rab7 (Figure 8L) but not SQSTM1 (Figure 8J) and increased cell death (Figure 8M). BECLIN-1 protein levels thus appear to be a “sensor” for transcriptional priming of the cellular autophagic machinery and an important cellular control point for dictating relative abundance of relevant autophagy-lysosomal proteins. Taken together, these data suggest that the ROS-induced increase in BECLIN-1 abundance could suppress autophagosome maturation via transcriptional downregulation of components of the autophagy-lysosome machinery.

Discussion

In this study, we demonstrate that in IR injury, cardiomyocyte autophagy is upregulated as a stress-response mechanism but autophagosome clearance is impaired, contributing to cell death. Specifically, we found a rapid reperfusion-induced decline in LAMP2, a protein critical for autophagosome-lysosome fusion, and upregulation of BECLIN-1, which impairs autophagosome processing, with increased ROS generation and mitochondrial permeabilization, thereby provoking cardiomyocyte death. Facilitating autophagosome processing by restoring LAMP2 levels and partially knocking down BECLIN-1 restores autophagosome processing and attenuates reoxygenation-induced cell death.

Contemporary studies in cardiomyocytes subjected to hypoxia-reoxygenation have demonstrated the accumulation of autophagosomes, LC3-II, and p62 indicating impaired “autophagic flux” in vitro. Conversely, increases in both autophagosomes and autolysosomes (as indicated by mRFP-GFP LC3) were interpreted as “intact” flux in vivo. These discrepant observations reflect the limitations of various imperfect tools to interpret autophagic flux. Conversely, increases in both autophagosomes and autolysosomes (as indicated by mRFP-GFP LC3) were interpreted as “intact” flux in vivo. These discrepant observations reflect the limitations of various imperfect tools to interpret autophagic flux. Indeed, the lack of equivalent fluorescence intensity and photostability of mRFP and GFP use in the dual fluorescent construct and differential kinetics of GFP degradation, depending on the state of lysosome acidification, may account for the variable readouts. We have attempted to overcome these limitations by generating an LC3 construct tandem tagged with mCherry and GFP probes, which have comparable intensities and photostability, ruling out alterations in lysosomal acidification, and using complementary approaches permitting increased accuracy in the assessment of autophagic flux.

Our results explain why autophagy, which facilitates cardiomyocyte survival (with reduced infarct size) during ischemia, has been implicated in causing cell death (with increased infarct size) on reperfusion of the ischemic myocardium. In this study by Matsui et al., reperfusion provoked a 7-fold increase in autophagosome abundance in the absence of further AMP-induced protein kinase activation or mammalian target of rapamycin inhibition during the ischemic phase. Importantly, reperfusion was associated with a rapid upregulation of BECLIN-1 (within 30 minutes), and BECN1 haploinsufficient mice demonstrated a decrease in cardiomyocyte autophagosome abundance, reduced programmed cell death, and reduced infarct size with in vivo IR injury compared with wild-type controls. The assumption that BECN1 haploinsufficiency leads to a reduction in autophagosome abundance primarily by preventing autophagy initiation is challenged by our results that demonstrate that it increased autophagosome processing, i.e., increased autophagic flux, likely by relieving the transcriptional downregulation of the autophagy-lysosome machinery by supranormal BECLIN-1 levels, while enough BECLIN-1 protein is available to facilitate autophagosome formation (Figure 8). A more complete knockdown of BECLIN-1, however, impairs autophagosome formation, thereby impairing autophagy, with increased cardiomyocyte death. Beclin-1 knockdown may also restore autophagosome processing by interrupting the interaction with Rubicon, a GTPase-regulating protein that localizes to late endosome/autophagosomes and inhibits autophagic flux. The observation that Beclin-1 knockdown confers benefit above and beyond that with LAMP2 overexpression, despite restoration of autophagy to a similar extent (Figure 7C and 7D), suggests that other mechanisms such as a reduction in p53 levels or restoration of mitochondrial homeostasis could be operative. Interestingly, the possibility that Beclin-1 knockdown may upregulate transcription of LAMP2 may explain the very similar degree of benefit obtained with Beclin-1 knockdown alone versus in combination with LAMP2A +2B overexpression (Figure 7E). Thus, BECLIN-1 abundance may act as a switch to determine whether the end result of autophagy is efficient autophagic degradation of targeted cargo to ensure cellular homeostasis and survival or autophagosome accumulation, triggering programmed cell death. Such a role for BECLIN-1 is consistent with the observation that it acts as tumor suppressor protein.

A novel observation of this study is the rapid decline in LAMP2 abundance with reperfusion. LAMP2 is a critical determinant of autophagosome-lysosome fusion. LAMP2 knockdown impairs autophagy in nutrient-starved adult rat ventricular myocytes and causes cell death at levels comparable to autophagy inhibition with 3MA and ablation of LAMP2 or loss of LAMP2 protein owing to mutations in individuals with Danon disease causes cardiomyocyte autophagosome accumulation and extensive myocardial fibrosis, suggesting cell death as the pathogenic mechanism. Interestingly, exogenous expression of both LAMP2A, a protein receptor for chaperone-mediated autophagy, and LAMP2B, which is postulated to be involved in autophagosome-lysosome fusion, accelerated autophagosome processing, suggesting a redundant role for these isoforms in the heart.

Given the exploratory nature of the study, the type I error rate was not controlled across multiple experimental outcomes, which would require additional studies focusing on specific associations. Nevertheless, our results, which require confirmation in adult cardiac myocytes and in vivo heart, suggest that impaired autophagosome clearance results in
increased ROS levels, which trigger mitochondrial permeabilization, leading to a necrotic mechanism of cell death in cardiac IR injury, and underscore the need for careful evaluation of autophagic flux to determine its role in cardiac stress and physiology.

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None.

References

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References

CLINICAL PERSPECTIVE

In myocardial infarction resulting from coronary occlusion, contemporary therapy is focused on expedient reperfusion to prevent loss of myocardium. Despite reperfusion and often as a result of reperfusion injury, there is ongoing cardiomyocyte cell death, which can be targeted therapeutically to maximize myocardial salvage. Autophagy is an evolutionarily conserved lysosomal pathway to break down damaged cellular constituents, to provide nutrients, and to facilitate cell survival. Autophagy has also been implicated as a mechanism for programmed cell death. In cardiac ischemia/reperfusion injury, autophagy is upregulated, and previous studies have suggested that it plays a dichotomous role in facilitating cell survival during ischemia but provoking cell death during reperfusion. To examine the hypothesis that autophagy is upregulated in reperfusion injury but impaired, thus preventing its prosurvival function, we have carefully examined flux through the autophagy pathway in cardiomyocytes subjected to ischemia and reperfusion. Our results demonstrate that autophagosomes accumulate during reperfusion injury after myocardial ischemia, suggesting impaired autophagosome processing. This accumulation of autophagosomes, presumably with the deleterious cargo that is not degraded, is associated with an increased prevalence of reactive oxygen species and mitochondrial permeabilization, resulting in necrotic cardiomyocyte death. The underlying mechanism for the impairment in autophagosome processing appears to be a reperfusion-induced decline in lysosome-associated membrane protein-2 levels, a protein critical for autophagosome-lysosome fusion, and an increase in the abundance of BECLIN-1. Restoring lysosome-associated membrane protein-2 levels and knocking down BECLIN-1 restore autophagosome processing with the attenuation of cell death. Thus, facilitating autophagy with enhanced flux through the autophagy pathway is a promising strategy to achieve myocardial salvage in ischemia/reperfusion injury.