Autophagy limits acute myocardial infarction induced by permanent coronary artery occlusion

Hiromitsu Kanamori,1,2 Genzou Takemura,1 Kazuko Goto,1 Rumi Maruyama,1 Koh Ono,3 Kazuya Nagao,3 Akiko Tsujimoto,1 Atsushi Ogino,1 Toshiaki Takeyama,1 Tomonori Kawaguchi,2 Takatomo Watanabe,1 Masanori Kawasaki,1 Takako Fujiwara,4 Hisayoshi Fujiwara,5 Mitsuru Seishima,2 and Shinya Minatoguchi1

Departments of 1Cardiology and 2Laboratory Medicine, Gifu University Graduate School of Medicine, Gifu; 3Department of Cardiovascular Medicine, Kyoto University; 4Department of Food Science, Kyoto Women’s University, Kyoto; and 5Division of Cardiovascular Medicine, Hyogo Prefectural Amagasaki Hospital, Hyogo, Japan

Submitted 20 October 2010; accepted in final form 11 March 2011

Kanamori H, Takemura G, Goto K, Maruyama R, Ono K, Nagao K, Tsujimoto A, Ogino A, Takeyama T, Kawaguchi T, Watanabe T, Kawasaki M, Fujiwara T, Fujiwara H, Seishima M, Minatoguchi S. Autophagy limits acute myocardial infarction induced by permanent coronary artery occlusion. Am J Physiol Heart Circ Physiol 300: H2261–H2271, 2011. First published March 18, 2011; doi:10.1152/ajpheart.01056.2010.—Ischemia is known to potentially stimulate autophagy in the heart, which may contribute to cardiomyocyte survival. In vitro, transfection with small interfering RNAs targeting Atg5 or Lamp-2 (an autophagy-related gene necessary, respectively, for the initiation and digestion step of autophagy), which specifically inhibited autophagy, diminished survival among cultured cardiomyocytes subjected to anoxia and significantly reduced their ATP content, confirming an autophagy-mediated protective effect against anoxia. We next examined the dynamics of cardiomyocyte autophagy and the effects of manipulating autophagy during acute myocardial infarction in vivo. Myocardial infarction was induced by permanent ligation of the left coronary artery in green fluorescent protein-microtubule-associated protein 1 light chain 3 (GFP-LC3) transgenic mice in which GFP-LC3 aggregates to be visible in the cytoplasm when autophagy is activated. Autophagy was rapidly (within 30 min after coronary ligation) activated in cardiomyocytes, and autophagic activity was particularly strong in salvaged cardiomyocytes bordering the infarcted area. Treatment with bafilomycin A1, an autophagy inhibitor, significantly increased infarct size (31% expansion) 24 h postinfarction. Interestingly, acute infarct size was significantly reduced (23% reduction) in starved mice showing prominent autophagy before infarction. Treatment with bafilomycin A1 reduced postinfarction myocardial ATP content, whereas starvation increased myocardial levels of amino acids and ATP, and the combined effects of bafilomycin A1 and starvation on acute infarct size offset one another. The present findings suggest that autophagy is an innate and potent process that protects cardiomyocytes from ischemic death during acute myocardial infarction.

ischemia; metabolism; infarct size; ultrastructure

AUTOPHAGY IS A PHYSIOLOGICAL process in which a cell digests its own constituents (e.g., subcellular organelles) via the lysosomal degradative pathway. This process occurs under both basal and stressful conditions and can be survival oriented, as degraded membrane lipids and proteins within autophagosomes are recruited to maintain needed levels of ATP production and protein synthesis, thereby promoting cell survival (21, 23, 35). In the heart, autophagy occurs constitutively in the normal myocardium but is substantially increased in several heart diseases including heart failure (18, 34, 38), hypertrophy (14, 30), and ischemic cardiomyopathy (41). Inefficient autophagy or its absence leads to poor myocardial performance, and inhibition of starvation-induced autophagy results in cardiac dysfunction and dilatation (17, 39).

During acute myocardial infarction, the myocardium is subjected to sudden ischemia due to interruption of its blood supply, a condition that might be comparable to extreme starvation. Moreover, earlier studies (11, 26, 33) showed a correlation between suppression of autophagy and exacerbation of the myocardial damage caused by transient acute myocardial ischemia, suggesting ischemia-induced autophagy may contribute to the survival of cardiomyocytes. Still, it has never been clearly demonstrated that autophagy does protect the cardiomyocytes from death caused by prolonged ischemia in vivo. For example, the studies cited above used an ischemia/reperfusion model with a short ischemic interval (20 or 45 min), making it possible to assume that autophagy only delays ischemic injury (26, 33). In addition, information is still lacking about the time course and pathophysiological significance of autophagic activity in the heart under conditions of permanent ischemia causing infarction. In the present study, therefore, we used an acute myocardial infarction model entailing permanent occlusion of the left coronary artery to investigate the dynamics of autophagy in the heart for up to 24 h after coronary occlusion. We then examined the effects of inhibiting or enhancing autophagy on acute myocardial infarction. Preceding the in vivo experiments, we assessed autophagy-specific effects on ischemia using small interfering (si)RNAs targeting autophagy-related genes in cardiomyocytes in vitro.

MATERIALS AND METHODS

Cell culture and treatments. Cardiomyocytes were isolated from the hearts of 1-day-old C57BL/6 mice as described previously (42). We knocked down Atg5 and Lamp-2 using siRNA delivered using a lentiviral vector that also encoded green fluorescent protein (GFP). The oligonucleotides for the siRNAs and a nonsilencing control sequence were designed as previously reported (25). The transfection efficiency (%positive) was found to be 75 ± 5.6% based on the rate of GFP positivity among cells in five random high-power fields of cardiomyocytes 24 h after transfection.

Cells on slides and in plates were subjected to simulated ischemia by changing the buffer to ischemia-mimetic solution (in mM: 125 NaCl, 8 KCl, 1.2 KH2PO4, 1.25 MgSO4, 1.2 CaCl2, 6.25 NaHCO3, 5 sodium lactate, and 20 HEPES pH 6.6) and placing them in hypoxic chambers equilibrated with 95% N2-5% CO2, as previously described (11). After
the cells were incubated for the indicated intervals, cell viability was assessed using 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8) assays (Cell Counting Kit-8; Dojindo Laboratories) and in situ deoxynucleotidyl transferase dUTP-mediated nick-end labeling (TUNEL) assays (Chemicon International), and total ATP content was measured using an ATP bioluminescent assay kit (Sigma).

siRNAs. The oligonucleotides used to construct the siRNAs were as follows: Atg5–1 sense, GCCGGTGAGGCTCCTATTTA; Atg5–1 antisense, TAAAGTGAGGCTCCTAAACGCC; Atg5–2 sense, GGAGCTGGAATTTTACCAAGGA; Atg5–2 antisense, ATCTTGCTATTCGACCTCC; Lamp-2–1 sense, GCCGGTGACCTCAATTGAT; Lamp-2–1 antisense, ATGCGTTAGGCTAACGAGG; Lamp-2–2 sense, GGGAGATGGAATTTCAACAAT; and Lamp-2–2 antisense, ATTGTTGAATACTCTCCC. A nonsilencing control sequence was designed on the sequences of a negative-control siRNA purchased from B-D International. Every siRNA construct was made using pSilNG-mi6 vector (Takara Bio), and the siRNA-producing constructs were introduced into a lentiviral vector plasmid (pLent6/V5-D-TOPO vector; Invitrogen). Knockdown efficiency was assessed through total RNA isolation, cDNA synthesis, and real-time PCR using murine mouse 3T3L1 cells as previously described (25). Primer sequences used for quantification of Atg5, Lamp-2, and GAPDH were as follows: Atg5 sense, 5'-ATATGAAGGCACACCCCTGA-3'; Atg5 antisense, 5'-CATCAAGATAATCAGGT-3'; Lamp-2 sense, 5'GGGCTGT-3'TTGCGAATTCT-3'; Lamp-2 antisense, 5'-TGAGATCATGATGCTGAC-3'; and GAPDH antisense, 5'-TTGTCAATGGATGACCTTGGC-3'. Transfection of cardiomyocytes was accomplished by replacing the culture medium with virus-containing medium for 24 h.

Animal models. This study conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85–23, revised 1996) and was approved by our Institutional Animal Research Committee. Pathogen-free heterozygous GFP-labeled microtubule-associated protein 1 light chain 3 (LC3) transgenic mice (strain GFP-LC3#53; RIKEN BioResource Center), which harbor a rat LC3-EGFP fusion construct under the control of the chicken β-actin promoter (29), were housed in a temperature-controlled environment with a 12-h light-dark cycle. The mice received food and water ad libitum. Genotyping of GFP-LC3 reporter mice was accomplished using PCR with GFP-LC3 primers (yielding a 500-bp PCR product) and mouse LC3 internal control primers (yielding a 400-bp PCR product).

Western blot analysis. Protein samples (100 µg) extracted from hearts (n = 3 to 6 from each group) were subjected to 10 or 15% PAGE (10% gel for cathepsin D and α-tubulin and 15% gel for LC3) and then transferred to PVDF membranes. The membranes were then probed using primary antibodies against LC3, p62 and cathepsin D after which the blots were visualized using enhanced chemiluminescence (Amersham). α-Tubulin (analyzed using an antibody from Santa Cruz) served as the loading control.

Measurement of amino acid concentrations. Hearts were weighed while frozen and homogenized in glass microhomogenizers in the presence of cold 5% sulphosalicylic acid. Free amino acids in the supernatants were then measured using an automated amino acid analyzer (Hitachi L8500). Amino acid concentrations were expressed as the sum of the concentrations of Asp, Thr, Ser, Asn, Glu, Gin, Pro, Gly, Ala, Val, Cys, Met, Ile, Leu, Tyr, Phe, Lys, His, and Arg.

Histogramal analyses. The hearts were removed, weighed, and cut into transverse slices through the middle of the ventricles between the atrioventricular groove and the apex. The basal slices were fixed in 10% buffered formalin, embedded in paraffin or cryomold, cut into 4-µm-thick sections, and stained with hematoxylin-eosin, Masson’s trichrome, or Oil Red O.

Immunochemistry was performed on deparaffinized sections using a primary polyclonal antibody against myoglobin (DAKO). A Vectastain Elite ABC system (Vector Laboratories) was then used to immunostain the sections; diaminobenzidine served as the chromogen, and the nucleoli were counterstained with hematoxycyan. The infarct/ischemia territory was demarcated by the staining defect of myoglobin (8). The extent of myocardial infarction was measured in the transverse sections of the ventricles cut at the midpapillary muscle level using a LUZEX F multipurpose color image processor (Nireco), and the values were expressed as the ratio of the myoglobin-absent area to the total area of the left ventricular wall.

For double immunofluorescent labeling, sections were labeled with primary antibodies against GFP (Molecular Probes), cathepsin D (Santa Cruz), and/or myoglobin, and then with Alexa Fluor 488- and 568-conjugated secondary antibodies (Molecular Probes). The sections were counterstained with Hoechst 33342 and then observed under a confocal microscope (LSM510; Zeiss).

For detection of apoptosis, we performed TUNEL assays (Chemicon International). Quantitative assessments, including the number of immunopositive dots or nuclei, were carried out in 20 randomly chosen high-power fields (×600).

Electron microscopy. Cardiac tissue was quickly cut into 1-mm cubes, immersion fixed overnight at 4°C in 2.5% glutaraldehyde in 0.1 mol/l phosphate buffer (pH 7.4), and postfixed in 1% buffered osmium tetroxide. The specimens were conventionally processed and examined under an electron microscope (H-800; Hitachi).

Immunoelectron microscopy. Immunoelectron microscopy was performed for detection of cathepsin D, one of the lysosomal enzymes, using the preembedding method as previously described (17).

Enzyme cytochemistry for acid phosphatase. To detect activity of acid phosphatase, one of the lysosomal enzymes, cytochemical analyses were carried out using the method of Robinson and Karnovsky (32) with some modification (17).

Measurement of blood pressure and heart rate. Blood pressure and heart rate were measured before and 4 h after surgery using the tail cuff method (BP-98A; Softron).

Measurement of serum troponin I level. Serum concentrations of cardiac troponin I were measured 24 h after coronary artery ligation using an enzyme immunoassay kit (Access AccuTnI; Beckman Coulter).
Myocardial ATP content. Myocardial ATP content was measured using an ATP bioluminescent assay kit (TOYO Ink).

Statistical analysis. Values were shown as means ± SE. The significance of the differences in variance was evaluated using Bartlett test. When the variance was significantly different, the significance of differences was tested by Kruskal-Wallis test. All data were tested for normal distribution with the Kolmogorov-Smirnov test. The significance of differences between groups was evaluated using one-way ANOVA with a post hoc Newman-Keul’s multiple comparison test or a repeated-measures ANOVA. Values of $P < 0.05$ were considered significant.

RESULTS

Effect of specific inhibition of autophagy using siRNAs in cardiomyocytes rendered anoxic in vitro. We used siRNAs to knock down the autophagy-related genes Atg5 and Lamp-2 in cultured neonatal cardiomyocytes (Fig. 1A) and then examined the effect of anoxia. Hypoxia/anoxia is a potent stimulator of autophagy in cultured cardiomyocytes (11), and siRNAs targeting Atg5 specifically inhibit the initiation of autophagy (phagophore formation), while those targeting Lamp-2 inhibit a later step (digestion of the contents of autophagosomes) (23, 35). We found that the survival rate among cardiomyocytes subjected to 24 h of anoxia was significantly reduced by treatment with either of these siRNAs (Fig. 1B). The anoxia also increased the numbers of cardiomyocytes positive for TUNEL, but the percent TUNEL was similar among the groups (Fig. 1C), suggesting the frequency of apoptotic death was unaffected by knocking down anoxia-induced autophagy. Six hours of anoxia markedly reduced the ATP content of cardiomyocytes, and functional inhibition of autophagy using siRNA targeting either Atg5 or Lamp-2 further reduced their ATP content, although at this time there was not yet an apparent difference in survival rate among the groups (Fig. 1D). These findings indicate the specificity of the autophagy-mediated protective effect against anoxia, of which mechanism might be preservation of cellular ATP level.

Development of autophagy following acute myocardial infarction. In the in vivo model of myocardial infarction, we noted a significant increase in the number of GFP-LC3 dots in cardiomyocytes.
the ischemic area as early as 30 min after coronary ligation (Fig. 2). The numbers of dots increased in both the ischemic and nonischemic regions of the myocardium, but the increase occurred sooner and was larger in ischemic and border regions (Fig. 2B). On the other hand, during the later stage of infarction (24 h after onset), the numbers of GFP-LC3 dots declined dramatically in the infarcted area but were preserved or somewhat increased in noninfarcted regions (Fig. 2B).

Electron microscopic observation revealed the presence of cytoplasmic vacuoles of homogenous electron density in cardiomyocytes as early as 30 min after coronary ligation (Fig. 3A). Such vacuoles were rarely seen in cardiomyocytes from sham-operated mice and resembled those that emerge in the cardiomyocytes of mice subjected to starvation, which we previously reported to be lysosomes (17). The term lysosome is used to describe a group of membrane-bound organelles that contain acid hydrolases (10). Immunoelectron microscopy for cathepsin D and enzyme cytochemistry for acid phosphatase activity further confirmed that the ischemia-associated vacuoles were lysosomes (Fig. 3B). Although Oil Red O staining was performed on the cryosections because of presumed lipid droplets, the reaction was negative in any groups (data not shown), suggesting that those structures were not lipid droplets. During the later stages of acute infarction, most of the vacuoles in the infarcted area resembled those seen in necrotic cells; however, many of the cytoplasmic vacuoles in the salvaged cardiomyocytes turned out to be typical autophagosomes containing intracellular organelles, such as degraded mitochondria and membrane-like structures (Fig. 3A).

Following coronary artery ligation, levels of LC3-I initially declined, reaching a minimum after 4 h, and then increased to levels somewhat higher than control (Fig. 4). By contrast, levels of LC3-II increased continuously. As a result, the LC3-II-to-LC3-I ratio, an established indicator of autophagic turnover (15), reached a maximum 4 h postinfarction. The ubiquitin- and LC3-binding protein p62 regulates the formation of protein aggregates and is removed by autophagy (19). In the infarcted hearts, p62 levels increased at 24 h postinfarction (Fig. 4), while myocardial expression of cathepsin D increased as early as at 30 min and peaked at 24 h (Fig. 5, A and B). Moreover, at early stages of infarction, cathepsin D dots did not always coincide with the GFP-LC3 dots, suggesting phagophores or autophagosomes had formed but had not yet fused with lysosomes. By the later stages of infarction, almost all of the autophagosomes and lysosomes had merged, suggesting the formation of autolysosomes (Fig. 5C).

Collectively, these findings suggest that, on the whole, autophagic activity is increased in the ischemic heart during the early stages of infarction (up to 4 h after the onset) but is impaired during later stages. This apparent late impairment may be partially attributable to massive necrosis, which interrupted all functions, including autophagy.

Role of autophagy in acute infarct expansion. Bafilomycin A1 was administered to mice to inhibit autophagy. Four hours after coronary ligation in bafilomycin A1-treated mice, the myocardial LC3-II-to-LC3-I ratio had increased beyond the levels seen in the hearts of saline-treated mice (Fig. 6), suggesting accumulation of LC3-II due to inhibition of autophagosome degradation. Ultrastructurally, salvaged cardiomyocytes neighboring necrotic ones in bafilomycin A1-treated hearts showed numerous homogenously electron-dense, lyso-

Fig. 2. Time course of autophagy development in cardiomyocytes during acute myocardial infarction. A: distributions of green fluorescent protein-microtubule-associated protein 1 light chain 3 (GFP-LC3)-positive dots within cardiomyocytes in ventricular transverse sections at the indicated times after coronary artery ligation. Left: distribution of myoglobin in immunohistochemical preparations; myoglobin-positive areas (brown) are viable myocardium. Bars = 1 mm. Right: immunofluorescent labeling of myoglobin (red) and GFP (green) in serial sections corresponding to those at left. Bars = 20 μm. *Ischemic/infarcted region. B: numbers of LC3-GFP dots per high-power field (×600) of cardiomyocytes in the remote (open column), border (dotted column), and ischemic/infarct (closed column) areas. Numbers within or above the columns indicate the number of hearts analyzed in each group. Statistical significance was evaluated using a repeated-measures ANOVA.
some-like structures, which were also abundant in necrotic cardiomyocytes in saline-treated hearts (Fig. 7). Blood pressure was somewhat lower than control in bafilomycin A1-treated mice 30 min after induction of infarction and 4 and 24 h postinfarction (Fig. 8A). Twenty-four hours postinfarction, serum levels of troponin I, an enzyme released from infarcted myocardium, was significantly higher in bafilomycin A1-treated mice (94.1 ± 9.7 ng/ml) than in saline-treated control mice (85.1 ± 8.6 ng/ml; P = 0.04), which strongly suggests exacerbation of the acute infarction by bafilomycin A1 (Fig. 8B). Indeed, histological examination of hearts collected 24 h postinfarction confirmed that bafilomycin A1 administration significantly increased infarct size by 31% compared with saline (%infarct area in the left ventricle: 34 ± 1.1 vs. 26 ± 0.55%; P < 0.0001; Fig. 8B). Furthermore, the time frame of this result suggests treatment with bafilomycin A1 expands rather than simply accelerates acute ischemic death among cardiomyocytes.

We next tested whether starvation-induced autophagy would influence the progression of acute infarction. We confirmed the
presence of prominent autophagy in the hearts of mice starved for 24 h as previously reported (17). Myocardial infarction was induced by coronary artery ligation in the mice after starvation for 24 h. The animals were then given access to food. Four hours postinfarction, the LC3-II-to-LC3-I ratio was greatly increased in the hearts of the starved mice (Fig. 6), and greater numbers of autophagosomes were observed in viable cardiomyocytes from starved mice than from saline-treated mice (Fig. 7). Interestingly, starvation led to a 23% reduction in myocardial ATP content. Unexpectedly, in starved mice also treated with bafilomycin A1, the myocardial ATP content was similar to that in bafilomycin A1-treated mice.

**DISCUSSION**

The present study detailed development of cardiomyocyte autophagy in acute myocardial infarction and is the first report examining the acute effects of manipulating autophagy in permanently ischemic hearts in vivo.

**Autophagy and autophagic cell death in acute myocardial infarction.** We observed a time-dependent transition in the ultrastructure of autophagic vacuoles within cardiomyocytes during acute myocardial infarction. During the early stage, the content of the majority of vacuoles was electron-dense, homogenous lipid-like droplets; relatively few vacuoles contained intracellular organelles and showed the typical features of autophagosomes. Immunoelectron microscopy and enzyme cytochemistry suggested that such lipid-like homogenous vacuoles might be lysosomes containing cathepsin D antigenicity and acid phosphatase activity. In salvaged cardiomyocytes, however, the ratio of the types of vacuoles present was reversed during the later stage: most of the vacuoles were typical autophagosomes, with only a few probable lysosomes. This transition in autophagic morphology in cardiomyocytes is strikingly similar to that observed in starved mice (17) and suggests that lysosomal activation precedes the formation of morphologically typical autophagosomes, which is consistent with earlier observations (6). In necrotic cardiomyocytes, by contrast, many vacuoles remained homogenously electron-dense lysosomes. The autophagic process was probably interrupted in those cells by cell death, which may contribute to the apparent impairment of autophagy in hearts with later stage infarctions.

Autophagy is also a mode of cell death that occurs during tissue and organ development to eliminate unnecessary cells (4, 21, 23, 35). Autophagic cell death is a morphological term derived from electron microscopic observations and denotes a form of cell death in which abundant autophagic vacuoles are present in the cytoplasm (20). However, how can one be certain that such a cell is actually dead? Indeed, following...
coronary artery ligation, we observed cardiomyocytes containing numerous autophagic vacuoles, but we were not certain the cells were dead, as their plasma membrane, organelles, and cytoplasm appeared intact (salvaged cardiomyocytes at the border zone). We only deemed autophagic cardiomyocytes to be dead when necrotic features were also present (infarcted cardiomyocytes). Based on our observations, necrotic cardiomyocytes within the infarcted area contained both lysosomes and autophagosomes, but lysosomes were most prevalent during the early stage of infarction (Figs. 3A and 7). This suggests autophagy is a compensatory, prosurvival response to ischemic stress and that cardiomyocyte death is, at least in part, an unsuccessful outcome brought on by necrosis. Thus previous studies (1, 9) and our present observations collectively suggest that autophagic cell death can occur along with autophagy and that autophagy likely represents a prosurvival mechanism activated during acute myocardial infarction in a struggle against stress and cell death, i.e., necrosis with autophagy may reflect failure of a survival effort due to insufficient compensation by autophagy.

TUNEL assays revealed significantly increased numbers of apoptotic cells in infarcted hearts (in vivo) and anoxic cardiomyocytes (in vitro). However, we found that inhibiting autophagy with bafilomycin A1 does not cause a further increase in apoptotic cells. This observation differs from earlier ones

Fig. 5. Time course of cathepsin D expression in cardiomyocytes during acute myocardial infarction. A: distribution of cathepsin D-positive dots at the indicated times within cardiomyocytes in transverse ventricular sections assessed by immunofluorescent labeling of myoglobin (red) and cathepsin D (green); nuclei are counterstained with Hoechst 33342 (blue). Bars = 20 μm. *Ischemic/infarcted region. B: Western blotting for cathepsin D and densitometry in sham-operated hearts and hearts with acute myocardial infarction. Numbers within or above the columns indicate the number of hearts analyzed in each group. Statistical significance was evaluated using one-way ANOVA. C: colocalization of LC3-GFP (green) and cathepsin D (red) assessed by double immunofluorescent labeling of the ischemic border zone in hearts 4 and 24 h postinfarction. Yellow color displays the merge of green and red color.
made using cell lines (1). Cardiomyocytes are reportedly very insensitive to apoptotic inducers such as Fas stimulation (12, 43), and our findings suggest they are also not susceptible to apoptosis induced by inhibition of autophagy.

Role of autophagy in acute myocardial infarction. We have demonstrated the critical role played by autophagy in limiting the extent of acute myocardial infarction in vivo. Upon coronary artery occlusion, cardiomyocytes within the risk area experience a sudden disruption of their blood supply, which causes a rapid depletion of myocardial ATP. For cardiomyocytes, in particular, disruption of active ion transport among the cytosol, sarcoplasmic reticulum, and extracellular space caused by ATP depletion poses a serious problem, as these cells are highly sensitive to changes in the cytosolic Ca\(^{2+}\) concentration. For example, an uncontrolled increase in cytosolic Ca\(^{2+}\) causes supercontraction of the sarcomeres, which destroys the plasma membrane and cytoplasmic architecture, leading to necrotic cell death. This necrosis spreads through the ischemic myocardium at the periphery in the risk area in a time-dependent manner, creating a wavefront phenomenon (31), and it is generally accepted that expansion of the infarction is complete within several hours, i.e., 1–2 h in rats (2), 3 h in dogs (31), and 3–6 h in humans (13). In humans, the optimal time window for coronary reperfusion therapy is within 6 h after the onset of acute infarction; beyond that time window, therapy cannot effectively limit infarct size (3). Our results showed that induction of autophagy by starvation reduces infarct size 24 h after coronary artery occlusion in mice, while, conversely, inhibition of autophagy by bafilomycin A1 increases infarct size. This time frame suggests autophagy prevents ischemic cardiomyocyte death rather than just delaying it.

Autophagy appears to effectively protect cardiomyocytes from acute ischemic insult and to rescue cardiomyocytes within permanently ischemic myocardium. Thus the present finding that enhanced autophagy can protect cardiomyocytes without an apparent blood supply from death suggests to us that there is an unexpectedly large gray (incompletely ischemic) myocardium (probably due to diffusion of oxygen from the endocardium and the nonischemic border area) or an unexpectedly rapid compensation by blood flow through
Fig. 8. Manipulation of autophagy during acute myocardial infarction. A: systolic blood pressure and heart rate before and 4 and 24 h postinfarction. B: size of the acute infarct assessed 24 h postinfarction based on serum levels of troponin I and on immunohistochemical detection of myoglobin. Photographs show representative immunohistochemical preparations from each group. Brown areas are myoglobin-positive viable myocardium. Bars = 1 mm. C: prevalence of TUNEL-positive cells 24 h postinfarction. D: myocardial amino acid content 4 h postinfarction. E: myocardial ATP content 4 h postinfarction. Numbers within or above the columns indicate the number of hearts analyzed in each group. Statistical significance was evaluated using one-way ANOVA.
collateralization or neovascularization of the ischemic myocardium. To inhibit the spread of ischemic cardiomyocyte death, glucose reserves within cardiomyocytes are utilized for energy production (5). In addition, autophagy is activated to maintain an adequate amino acid pool, which can be directly used as an energy source and can also be used for synthesis of proteins required for the proper response to the ischemic insult. Our results imply that augmenting autophagy may be a useful therapeutic strategy for treating acute myocardial infarction.

**Limitations of the study.** In the present study, we made measurement of the infarct size on the ventricular slices at the midpapillary muscle level. This approach is a common technique used in many literature including contemporary studies (16, 22, 36). However, at least seven slices from throughout the heart may be required to accurately determine infarct size and mice commonly develop apical aneurysms that contribute significantly to infarct size but cannot be reflected in an estimate based on midpapillary muscle sections (24, 37). According to the study by Michael et al. (27) that measured infarct size in percent weight using 4–5 slices of the ventricles in mice, the percent infarct size in left ventricle was 28.0 ± 2.8% at 24 h after the left coronary artery occlusion. This value is strikingly similar to the infarct size of the present study (26 ± 0.55% in the control group), which was calculated by percent area using the slice of midpapillary muscle level, supporting a notion that the ventricular slices at the midpapillary muscle level are assumed to be representative of the entire ventricle. In addition, a good correlation was noted between the calculated infarct size and serum troponin I levels. These facts may support the methodological validity of our histological evaluation of infarct size. However, it is needless to say that more accurate estimation of the infarct size has to be considered. Moreover, the risk region was not assessed either by coronary anatomy or regional blood flow and the infarct size was not thus assessed relative to the region at risk in the present study.

In the present study, we examined acute myocardial infarction up to 24 h after the onset. Even though the acute ischemic death of cardiomyocytes is presumed completed at that time, an infarcted myocardium shows further dramatic alterations thereafter. Thus it would be very interesting to investigate the dynamics and role of autophagy in the postmyocardial infarction remodeling process. Further investigation is warranted.

**Conclusions.** The present study demonstrated the development of cardiomyocyte autophagy in the heart during acute myocardial infarction; it was rapidly activated after the onset with specific distribution in the heart. Our findings suggest that autophagy is an innate protective mechanism against ischemic death of cardiomyocytes during acute myocardial infarction, to limit the size of acute infarcts, and imply that augmenting autophagy may be a useful therapeutic strategy for treating acute myocardial infarction.

**ACKNOWLEDGMENTS**

We thank the staff of Kyoto Women’s University (Chikako Koda, Aya Sakagami, Makiko Takeuchi, Megumi Tanigaki, Yuko Nakajima, and Ayako Nozu) for technical assistance.

**GRANTS**

This study was supported in part by Grants-in-Aid for Scientific Research from The Ministry of Education, Science, Grant-in-Aid of The Japanese Medical Association, and Culture of Japan and Research Grant from Gifu University.

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**REFERENCES**


