Morphological and biochemical characterization of basal and starvation-induced autophagy in isolated adult rat cardiomyocytes


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Autophagy is a type of programmed cell death that occurs during tissue and organ development to eliminate unnecessary cells (4, 9, 24), but it also has survival-oriented functions, occurring under both basal conditions and conditions of stress, such as starvation (9, 12, 24). During autophagic degeneration, degraded membrane lipids and proteins within autophagosomes are recruited to maintain ATP production and protein synthesis, thereby promoting cell survival. Autophagic cell death is actually 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. But this definition tells us nothing about the pathophysiological significance of autophagy in disease processes. Consequently, although cardiomyocytes showing characteristics of autophagy have recently been reported in failing hearts, suggesting autophagic hyperfunction (6, 10, 11, 23, 27), it is still undetermined whether an abnormal increase in autophagic vacuoles is the primary cause of cardiomyocyte death in heart failure or even whether it reflects hyperfunction or hypofunction of the autophagic process. Danon’s disease may epitomize the complicated issue of autophagy in the heart. In this disease, digestion of the contents of autophagic vacuoles is impaired (hypofunction) due to a gene-related deficiency in the lysosomal enzyme lysosome-associated membrane protein-2 (LAMP-2), which results in accumulation of autophagolysosomes with undigested contents in the cytoplasm and cardiomyocyte death, ultimately leading to heart failure (18, 25). Thus, autophagic hypofunction is apparently detrimental in the case of Danon’s disease.

Our aim in the present study was to test whether basal and starvation-induced autophagy are protective or detrimental processes in cultured adult rat ventricular cardiomyocytes (ARVCs). For that purpose, we inhibited or enhanced autophagy by using several reagents and small interfering RNA (siRNA) and then assessed the survival rates among the cells and the ultrastructural changes they underwent.

MATERIALS AND METHODS

ARVC isolation and culture. This study was approved by our Institutional Animal Research Committee and conformed to the animal care guidelines of the American Physiological Society. We isolated ARVCs from male Sprague-Dawley rats (200 to 250 g) as previously described (14). The cells were plated on laminin-coated dishes or slide glass chambers at the concentration of 5.0 \times 10^4 cells/ml and incubated in DMEM containing 50 µg/ml gentamicin in a CO₂ incubator (95% room air-CO₂).

Cell treatments. One day after plating, the normal culture medium of some ARVCs was replaced with glucose-free DMEM (No. 11966, GIBCO), in which glucose was substituted with 11 mM mannitol to...
induce starvation-induced autophagy, and the cells were incubated for up to 4 days. Cells incubated in glucose (11 mM)-containing medium served as controls. In some experiments, an inhibitor of autophagy, either 3-methyladenine (3-MA; an inhibitor of phagophore formation; Sigma-Aldrich) or leupeptin (an inhibitor of lysosomal cysteine protease to suppress subsequent digestion of the contents within autophagolysosomes; Sigma-Aldrich) (27), was added when the medium was changed. Rapamycin (an inhibitor of mammalian target of rapamycin, which negatively controls autophagy by mediating class I phosphatidylinositol 3-kinases, Sigma-Aldrich) was alternatively used in an attempt to enhance autophagy (19). Because 3-MA and leupeptin are not specific inhibitors of autophagy, we attempted the knockdown of ATG5 and LAMP-2 using siRNA delivered by lentivirus. ARVCs were incubated in virus-containing medium for 24 h and then exposed to starvation. The efficiency of transfection (% positive) was calculated by counting green fluorescent protein (GFP)-positive cells in five random high-power fields (×400) of ARVCs transfected with a lentiviral GFP expression vector at 24 h after lentivirus mediated GFP transfection.

**Lentivirus-mediated delivery of siRNA.** The oligonucleotides used for siRNA were as follows: ATG5-1 sense, TCGGAGTGTCAACAGGAAAT; ATG5-2 antisense, ATTCCTGTTGACCTGAC; LAMP-2-1 sense, GCAGTTGTGCTTTA; ATG5-1 antisense, TAAAGCACGTTGGAATCCGC; LAMP-2 antisense, 5′-TTGCCATCAACGACCCCTTC-3′; and GAPDH sense, 5′-CTGGCTACCATGGGGCTGCA-3′; LAMP-2 sense, 5′-GACGCTGGTAACTGACCA-3′; ATG5-2 antisense, 5′-CTGGCTACCATGGGGCTGCA-3′; LAMP-2 antisense, 5′-ATCCAGTATGATGGCGCTTGAGA-3′; and GAPDH antisense, 5′-TGGTCATGGATGACCTTGGC-3′. A nonsilencing control sequence was designed according to the sequences of a negative-control siRNA purchased from B-Bridge International. Every siRNA construct was made using pSINsi-mU6 silencing control sequence designed according to the sequences of a negative-control siRNA purchased from B-Bridge International. Every siRNA construct was made using pSINsi-mU6 silencing control sequence designed according to the sequences of a negative-control siRNA purchased from B-Bridge International. We produced lentiviral stocks in 293FT cells introduced to lentivirus vector plasmid (pLenti6/V5-D-TOPO vector (Takara Bio), and the siRNA-producing constructs were transfection.

**Measurement of ATP content.** The total ATP levels within ARVCs were determined using an ATP Bioluminescent Assay Kit (Sigma), following the manufacturer’s instructions. After we harvested the cells by centrifugation at 4,500 g for 5 min at 4°C, ATP was extracted from the cell pellets by incubating with 1% trichloroacetic acid-4 mM EDTA solution for 10 min on ice. The extracts were then collected and centrifuged at 12,000 g for 10 min at 4°C, after which the supernatants were used for ATP determination. Experiments were done in triplicate.

**Western blot analysis.** Proteins extracted from ARVCs were subjected to 14% polyacrylamide gel electrophoresis and then transferred onto polyvinylidene difluoride membranes. The membranes were then probed using primary antibodies against LC3 (Santa Cruz Biotechnology) and the cleaved forms of caspase-3 (Cell Signaling), after which the blots were visualized using enhanced chemiluminescence (Amersham). α-Tubulin (analyzed using an antibody from Santa Cruz) served as the loading control. Experiments were done in triplicate.

**Statistical Analysis**

Values were expressed as means ± SE. Statistical comparisons were made using t-tests or ANOVA followed by Newman-Keuls multiple comparisons test. Values of P < 0.05 were considered significant.

![Image](http://ajpheart.physiology.org/)

**Fig. 1.** Adult rat ventricular cardiomyocyte (ARVC) viability. A: viability of ARVCs assessed by Trypan blue dye exclusion. A, left: representative photomicrographs of ARVCs from control and glucose-starved groups. Arrows indicate Trypan blue-stained dead ARVCs. Bars, 20 μm. A, right: survival curves for the ARVCs up to 4 days (n = 6 experiments for each group). B, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)-positivity rates in the control (G, glucose) and glucose-starved (M, mannitol) groups (n = 3 experiments each). There is no significant (NS) difference between the groups.

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RESULTS

ARVC viability and morphology. The survival rate among control ARVCs, as indicated by Trypan blue dye exclusion, gradually declined until it reached 55 ± 8.3% after 4 days of incubation (Fig. 1A). Survival declined more steeply among the glucose-starved ARVCs and reached 19 ± 5.2% (P < 0.005 vs. control) after 4 days of incubation (Fig. 1A). Nonetheless, the incidences of apoptosis, as indicated by terminal deoxy-nucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) positivity, were similar among the control and glucose-starved cells after 4 days of incubation (Fig. 1B).

Necrotic ARVCs were often observed in both groups. Under electron microscopic examination, these necrotic cells showed disrupted plasma membranes, mitochondria that were swollen and containing flocculent dense bodies, and sarcomeres that were supercontracted (Fig. 2). In living ARVCs in both groups, membrane-bound autophagic vacuoles were apparent, within which were intracellular organelles (mostly mitochondria) being digested. Notably, these vacuoles were much more numerous in the glucose-starved ARVCs than in the controls (Fig. 2).

Immunohistochemical analysis revealed that control ARVCs expressed some LC3 (Fig. 3), but comparatively small numbers of LC3-positive “dots” were seen (3.5 ± 0.7 dots/cell). By contrast, glucose-starved cells expressed an abundance of LC3-positive dots (21 ± 1.5 dots/cell, P < 0.05). The dots were also observed in necrotic ARVCs with damaged plasma membranes but were not seen in apoptotic ARVCs (Fig. 3). Western blot analysis revealed that LC3-II was significantly increased in the glucose-starved ARVCs (Fig. 4).

Intervention in ARVC autophagy. We used 3-MA and leupeptin to examine the effects of inhibiting autophagy in these cells. Both inhibitors dose-dependently reduced viability among control and glucose-starved ARVCs after 3 days of incubation (Fig. 5A), suggesting that the inhibition of either basal or starvation-induced autophagy is detrimental to the isolated ARVCs. In contrast, the treatment with rapamycin significantly attenuated the starvation-induced death of ARVCs in a dose-dependent manner (Fig. 5A).

The inhibition of autophagy by 3-MA or leupeptin increased the numbers of necrotic ARVCs but had no effect on apoptosis, i.e., no effect on the incidence of TUNEL-positive cells or caspase-3 activation (Fig. 5, B and C). Rapamycin did not affect apoptosis either. Moreover, the two inhibitors induced ultrastructural changes that were specific to each, particularly in living ARVCs (Fig. 6). Treatment with 3-MA markedly reduced the numbers of autophagic vacuoles in glucose-starved ARVCs, irrespective of whether they were living or dead (Fig. 6, A and B). By contrast, the numbers of autophagic vacuoles and their size were greatly increased in the leupeptin-treated ARVCs (Fig. 6, C–F). Within the enlarged autophagolysosomes was an abundance of incompletely digested mitochondria and other membranous structures. Rapamycin increased the vacuoles containing digested organelles and also empty...
Fig. 3. Confocal micrographs of ARVCs double stained for α-sarcomeric actin (α-Sr actin) and light chain 3 (LC3). The LC3-positive dots are more intense and numerous in glucose-starved ARVCs than in control cells (A and B). LC3-positivity was seen in necrotic ARVCs but not in apoptotic ones (C and D). Treatment of the glucose-starved ARVCs with 3-methyladenine (3-MA) decreased the LC3 dots (E), whereas leupeptin (Leu) and rapamycin (Rap) augmented them (F and G). Bars, 10 μm. A–G, rightmost: higher magnification of the boxed area of respective image at its left.
vacuoles; the latter might be phagophore or autophagolysosome that finished digestion of the contents (Fig. 6G).

The LC3-immunopositive dots were significantly decreased in the glucose-starved ARVCs by the treatment with 3-MA, whereas they were increased by leupeptin and rapamycin. Such immunofluorescent findings were supported by the Western blot analysis for LC3-II (Fig. 4). The increase of LC3-II in the leupeptin-treated ARVCs may be explained by the accumulation of undigested LC3-II.

We next performed experiments using siRNAs for ATG5 to specifically inhibit the initiation step of autophagy (formation of phagophore) and also siRNAs for LAMP-2 to inhibit the digestion step of autophagy. We generated two siRNA constructs directed against different regions of ATG5 (ATG5-1 and ATG5-2) and two siRNA against LAMP-2 (LAMP-2-1 and LAMP-2-2). ATG5-1 and ATG5-2 decreased the expression of ATG5 at mRNA level, whereas LAMP-2-1 and LAMP-2-2 decreased the expression of LAMP-2 at mRNA level (Fig. 7A). However, the transfection efficiency was relatively low: only 29% of ARVCs transfected with a lentiviral GFP expression vector. Despite such poor transfection efficiency, all siRNA significantly, though not markedly, decreased the viability of the ARVCs exposed to glucose depletion for 3 days (Fig. 7B), although no

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Fig. 4. Western blots for LC3 in ARVCs (top) and the densitometry showing relative intensity of LC3-II expression (bottom, n = 3 experiments each). *P < 0.05 compared with the control (G) group; #P < 0.05 compared with the glucose-starved (Mannitol) group.

Fig. 5. Intervention of basal and starvation-induced autophagy. A: both 3-MA and Leu dose-dependently reduced the viability of both control and glucose-starved ARVCs, whereas Rap improved the viability of glucose-starved ARVCs. B and C: apoptosis evaluated by TUNEL assay was not affected by any treatments in the glucose-starved ARVCs (B) and Western blot analysis failed to detect cleaved (active forms of) caspase-3 in all groups (C) (n = 3 experiments each).
The effect of siRNAs was seen upon cell survival in nonstarved cells (basal autophagy), probably because basal autophagy is originally low level and because transfection efficiency was substantially low. We evaluated the effect of siRNAs upon autophagy by counting LC3-positive dots in the glucose-starved cardiomyocytes under confocal microscopy. When compared with the control siRNA (23 ± 1.5 dots/cell, n = 6), both ATG5 siRNA significantly reduced the numbers of dots (18 ± 0.62 in ATG5-1, and 19 ± 0.71 in ATG5-2, n = 6 each), whereas both LAMP-2 siRNA significantly increased them (36 ± 0.60 in LAMP-2-1, and 34 ± 0.54 in LAMP-2-2, n = 6 each) (photographs not shown).
siRNA. 

Experiment. *P < 0.05 compared with control siRNA. 

The cardiomyocytes-transfected control siRNA was set at 100% in each starved ARVCs after 3 days of incubation (1.59). The ATP content to be significantly diminished in the glucose-starved ARVCs. We found that this necrosis was accelerated by inhibition of autophagy, necrosis was increased among glucose-starved ARVCs and cardiomyocytes (21). Since we observed that the incidence in ATP content is known to be tightly related to necrosis in ARVCs (29). The reduction in the intracellular ATP content in ARVCs. 

DISCUSSION

Although autophagy is a form of cell death, it is generally accepted that autophagy is also essential for cell survival in cases of nutrient or growth-factor removal (3, 13). Indeed, Kuma et al. (12) reported that autophagy is necessary for the survival of neonatal mice during periods of starvation after birth. The present study confirmed these findings in ARVCs by showing that two autophagy inhibitors, 3-MA and leupeptin, reduced survival among ARVCs subjected to glucose depletion. Experiments using siRNA for ATG5 and LAMP-2 strengthened the findings in terms of specific inhibition of autophagy. Notably, these inhibitors also reduced survival among control ARVCs without apparent nutrient depletion, suggesting that autophagy plays an essential physiological role necessary for ARVC survival, even under basal culture conditions. That said, the in vitro conditions that the cells experienced in the present study almost certainly differ significantly from the in vivo conditions. 

Although both 3-MA and leupeptin inhibit autophagy, their mechanisms of action differ: 3-MA inhibits formation of phagophores, whereas leupeptin suppresses digestion of the contents of autophagolysosomes. The use of leupeptin may mimic the pathophysiological conditions of Danon’s disease (18), and we actually observed the accumulation of gigantic autophagolysosomes containing undigested organelles in ARVCs treated with leupeptin. In contrast, ARVCs treated with 3-MA lacked autophagic features. Despite their differences, both inhibitors caused autophagic hypofunction and markedly accelerated ARVC necrosis, accompanied by a reduction in intracellular ATP. ATP depletion upsets the transmembrane balance of electrolytes by disrupting the activities ATP-dependent ion pumps, thereby causing the cells to become edematous (2, 21). In addition, ATP depletion disturbs the homeostasis of ARVCs and the increase in the incidence of necrosis under both basal conditions and after glucose depletion. 

ATP content in ARVCs. The reduction in the intracellular ATP content is known to be tightly related to necrosis in cardiomyocytes (21). Since we observed that the incidence in necrosis was increased among glucose-starved ARVCs and that this necrosis was accelerated by inhibition of autophagy, we next measured the ATP contents in the ARVCs. We found the ATP content to be significantly diminished in the glucose-starved ARVCs after 3 days of incubation (1.59 ± 0.14 × 10^-10 mol/μg protein) compared with controls (2.41 ± 0.06 × 10^-10 mol/μg protein, P < 0.05). Interestingly, the ATP content was further reduced in both control and glucose-starved cells by treatment with an autophagy inhibitor: in controls, 1.94 ± 0.47 × 10^-10 mol/μg protein by 3-MA and 2.09 ± 0.21 × 10^-10 mol/μg protein by leupeptin; and in glucose-starved cells, 1.08 ± 0.11 × 10^-10 mol/μg protein by 3-MA and 1.18 ± 0.03 × 10^-10 mol/μg protein by leupeptin (Fig. 8). Conversely, the ATP content was significantly increased by the treatment with rapamycin in the glucose-starved cells (2.11 ± 0.25 × 10^-10 mol/μg protein). There was thus a significant correlation between reduction in the ATP content of ARVCs and the increase in the incidence of necrosis under both basal conditions and after glucose depletion.
maintained by mitochondrial ATP energy production and the synthesis of proteins essential for cell survival. Together, these effects could have caused the cells to eventually become necrotic. Thus the necrosis among ARVCs observed in the present study might be the result of energy depletion (primary necrosis) caused by the prevention of recycled energy production via autophagy. This idea might be supported by the fact that the upregulation of autophagy by rapamycin preserved the ATP content and increased cell survival. However, another possibility is that the ATP levels dropped as the result of cell rupture. It is difficult to strictly determine the cause-and-effect relationship between necrosis and ATP loss in the present study protocol, and to resolve this issue, it would be necessary to monitor the morphology and ATP content in a single cell level.

Earlier studies have shown that expression of autophagic genes is needed for autophagic cell death (20, 22, 29). Furthermore, the true function of autophagy, whether protective or detrimental, is still controversial in the context of cardiovascular disease. For instance, Akazawa et al. (1) developed a mouse model of diphtheria toxin-induced heart failure in which the cardiomyocytes showed abundant autophagic vacuoles, and in a hamster cardiomyopathy model induced by δ-sarcoglycan deficiency, we observed severe autophagic degeneration in cardiomyocytes and found that treatment with granulocyte colony-stimulating factor mitigated both the heart failure and the associated autophagic findings (16). Autophagy may also be an adaptive or maladaptive feature of hearts subjected to pressure overload (17, 30) and was recently highlighted in ischemic heart disease, where it appears to protect ischemic cardiomyocytes (5, 15, 27). It thus appears that the determination of whether autophagy is protective or detrimental in diseased hearts will require further investigation. However, the findings of the present study suggest that the autophagic degeneration of cultured ARVCs reflects a prosurvival, compensatory response to glucose depletion and that ARVC death may represent the unsuccessful outcome due to necrosis.

GRANTS

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