Resveratrol Reverses Remodeling in Hearts with Large, Old Myocardial Infarctions through Enhanced Autophagy-Activating AMP Kinase Pathway

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We investigated the effect of resveratrol, a popular natural polyphenolic compound with antioxidant and proautophagic actions, on postinfarction heart failure. Myocardial infarction was induced in mice by left coronary artery ligation. Four weeks postinfarction, when heart failure was established, the surviving mice were started on 2-week treatments with one of the following: vehicle, low- or high-dose resveratrol (5 or 50 mg/kg/day, respectively), chloroquine (an autophagy inhibitor), or high-dose resveratrol plus chloroquine. High-dose resveratrol partially reversed left ventricular dilation (reverse remodeling) and significantly improved cardiac function. Autophagy was augmented in those hearts, as indicated by up-regulation of myocardial microtubule-associated protein-1 light chain 3-II, ATP content, and autophagic vacuoles. The activities of AMP-activated protein kinase and silent information regulator-1 were enhanced in hearts treated with resveratrol, whereas Akt activity and manganese superoxide dismutase expression were unchanged, and the activities of mammalian target of rapamycin and p70 S6 kinase were suppressed. Chloroquine elicited opposite results, including exacerbation of cardiac remodeling associated with a reduction in autophagic activity. When resveratrol and chloroquine were administered together, the effects offset one another.

In vitro, compound C (AMP-activated protein kinase inhibitor) suppressed resveratrol-induced autophagy in cardiomyocytes, but did not affect the events evoked by chloroquine. In conclusion, resveratrol is a beneficial pharmacological tool that augments autophagy to bring about reverse remodeling in the postinfarction heart. (Am J Pathol 2013, 182: 701–713; http://dx.doi.org/10.1016/j.ajpath.2012.11.009)

Large myocardial infarctions are an important cause of heart failure.1,2 The postinfarction heart gradually dilates to maintain cardiac output in a process called remodeling.1 However, excessive remodeling late in the chronic stage leads to a loss of cardiac performance and heart failure.1 The process of remodeling is complex and involves a variety of factors, including myocyte hypertrophy, inflammation, oxidative stress, fibrosis, late cell death, angiogenesis, and the dynamics of the infarct scar.3,4 Autophagy is a physiological self-degradation process that proceeds via the lysosomal digestive pathway and functions to maintain the intracellular environment. Autophagic activation occurs in the normal heart under stable conditions, as well as in several heart diseases, including heart failure, hypertrophy, ischemic cardiomyopathy, and cardiac senescence.5–11 We previously reported that autophagy is activated following myocardial infarction and that it compensates for the lack of energy in affected cardiomyocytes through digestion and recycling of the constituents of the cells.12 In that study, augmenting autophagy mitigated adverse postinfarction cardiac remodeling and preserved cardiac performance, whereas inhibition

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of autophagy had the opposite effect. These findings suggest that enhancing autophagic activity could be a useful therapeutic strategy against the progression of postinfarction remodeling.

Resveratrol is a natural polyphenol present in many plant-based foods, including peanuts, cranberries, blueberries, and grapes. It is well known for its potential health benefits related to its reported anti-inflammatory, antioxidant, antiaging, cardioprotective, neuroprotective, and antitumorogenic properties. Early pharmacological studies showed that at therapeutic doses, this compound is nontoxic, easily absorbed, and well tolerated by humans. Importantly, resveratrol was recently reported to strongly accelerate autophagy in vivo. The signaling molecules stimulated by resveratrol in the cardiovascular system include AMP-activated protein kinase (AMPK), Akt, endothelial nitric oxide synthase (eNOS), manganese superoxide dismutase (MnSOD), peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α), silent information regulator-1 (Sirt1), IL-6, NF-kB, tumor necrosis factor-α (TNF-α), and adiponectin. However, the network formed by these molecules is complex, and the key autophagic factors associated with resveratrol have not yet been identified.

There have been a number of reports that resveratrol exerts various cardioprotective effects, including mitigation of left ventricular (LV) hypertrophy, reduction of myocardial infarct size, attenuation of ischemia-reperfusion injury, improvement of vascular function, and improvement of cardiac function in cardiomyopathy. On the other hand, there have been no reports on the effect of resveratrol on postinfarction cardiac remodeling and the role of autophagy. Our aim in the present study was to investigate the possible beneficial effect of resveratrol on postinfarction LV remodeling and to clarify the related molecular mechanisms. We addressed this issue by examining the effects of resveratrol in an established murine model of heart failure caused by an old myocardial infarction.

Materials and Methods

Animals and Experimental Protocols

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 the institutional animal research committee of Gifu University. Myocardial infarction was generated in male C57BL/6J mice (8 to 10 weeks of age; CLEA Japan, Tokyo, Japan) by ligating the left coronary artery as previously described. Sham-treated animals underwent the same surgical procedure without left coronary artery ligation.

We induced myocardial infarction in 107 mice, of which 80 survived 4 weeks after the procedure (survival rate: 75%). Each of the surviving mice was assigned to one of the following five treatment groups after echocardiographic examination: vehicle (control, n = 16); 5 mg/kg per day, or low-dose, resveratrol (Sigma-Aldrich, St. Louis, MO; n = 16); 50 mg/kg per day, or high-dose, resveratrol (n = 16); 10 mg/kg per day chloroquine (Sigma-Aldrich; n = 16); and high-dose resveratrol plus chloroquine (n = 16). The agents were administered for 14 days using subcutaneously embedded osmotic minipumps (ALZET; DURECT, Cupertino, CA). We used two doses of resveratrol (5 mg/kg and 50 mg/kg) to assess the dose-dependent effects. Both doses were previously reported not to cause apparent adverse effects in the rodent heart. Chloroquine is a membrane-permeant lysosomal inhibitor that acts by inhibiting vacuolar H⁺-ATPase. It also inhibits autophagosome–lysosome fusion, thereby preventing the final digestion step in autophagy. Chloroquine is frequently used to experimentally inhibit autophagy, and the dose of chloroquine used here was previously reported to inhibit autophagy in mice, without apparent side effects. To assess the effects of these treatments on mice without infarction, sham-operated mice were assigned to the same groups 4 weeks after surgery (n = 8 each). All mice were examined 2 weeks after starting treatment (6 weeks after surgery).

Physiological Studies

Echocardiography and cardiac catheterization were performed before sacrifice as described previously.

Histology

Once the physiological measurements were complete, mice were sacrificed, and the hearts were removed, weighed, and cut into halves transversely between the atrioventricular groove and the apex. The basal specimens were fixed in 10% buffered formalin, embedded in paraffin, cut into sections (4-μm thick), and then stained with H&E or Masson’s trichrome. Cardiomyocyte size (measured as the transverse diameter of myocytes cut at the level of the nucleus) was assessed in randomly chosen high-power fields (600×) in each section.

Immunohistochemistry

After deparaffinization, the sections (4-μm thick) were incubated with a primary antibody against microtubule-associated protein-1 light chain 3 (LC3; MBL International, Woburn, MA) or atrial natriuretic peptide (ANP; Santa Cruz Biotechnology, Santa Cruz, CA). To observe autophagic activity in cardiomyocytes, sections immunostained with anti-LC3 followed by Alexa 488 (green; Molecular Probes, Sunnyvale, CA) were also labeled with anti-myoglobin antibody (DAKO Japan, Kyoto, Japan) followed by Alexa 568 (red; Molecular Probes). These sections were then counterstained with Hoechst 33342 and observed under a confocal microscope (LSM510; Carl Zeiss, Oberkochen, Germany). A Vectastain Elite ABC system (Vector
Laboratories, Burlingame, CA) was used for immunostaining of ANP; diaminobenzidine served as the chromogen, and the nuclei were counterstained with hematoxylin. For in situ terminal dUTP nick end-labeling (TUNEL), tissue sections were first stained with Fluorescein-FragEL (Oncogene Research Products, Boston, MA) and then labeled with antimyoglobin antibody followed by Alexa 568.

Quantitative assessments, including numbers of immunopositive dots within cells, were performed in 20 randomly chosen high-power fields (600×) using a multipurpose color image processor (Nireco, Kyoto, Japan). Border areas were defined as areas containing both infarcted and salvaged myocardium within a high-power field, whereas remote areas were myocardial regions far remote from any infarction.

Electron Microscopy

Cardiac tissue was quickly cut into 1-mm cubes, immersion fixed in 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.4) overnight at 4°C, and postfixed in 1% buffered osmium tetroxide. The specimens were then dehydrated through a graded ethanol series and embedded in epoxy resin. Ultrathin sections (90 nm), double-stained with uranyl acetate and lead citrate, were examined in an electron microscope (H-800; Hitachi, Tokyo, Japan).

Western Blot Analysis

Proteins (50 μg) extracted from hearts were subjected to 10% or 15% polyacrylamide gel electrophoresis and then transferred onto polyvinylidene difluoride membranes. The membranes were then probed using primary antibodies against LC3 (MBL International), p62 (MBL International), ANP (Santa Cruz International), Sirt1 (Millipore, Billerica, MA), and AMPK, phosphorylated AMPK (p-AMPK), p70 S6 kinase (p70S6K), phosphorylated p70 S6 kinase (p-p70S6K), mTOR, phosphorylated mTOR (p-mTOR), Akt, phosphorylated Akt (p-Akt; all from Cell Signaling Technology, Danvers, MA), histone H3, acetylated-histone H3 (both from Calbiochem, Darmstadt, Germany), and manganese-superoxide dismutase (MnSOD; Millipore), after which the blots were visualized using enhanced chemiluminescence (Amersham/GE Healthcare, Little Chalfont, United Kingdom). α-Tubulin (analyzed using an antibody from Santa Cruz Biotechnology) served as the loading control.

Measurement of Myocardial ATP Content

The ATP content in the heart was measured using an ATP bioluminescent assay kit (TOYO Ink, Tokyo, Japan).

In Vitro Study

Cardiomyocytes were isolated from 1-day-old neonatal C57BL6 mice by the previously reported method. The cardiomyocytes were plated on laminin-coated culture dishes or slide glass chambers and incubated in Dulbecco’s modified Eagle's medium (Sigma-Aldrich) containing 5% fetal bovine serum (HyClone; Thermo Scientific, Waltham, MA) at 37°C. Two days after plating, the cells were treated with saline, 100 μmol/L resveratrol, or 3 μmol/L chloroquine, with or without simultaneous treatment with 20 μmol/L AMPK inhibitor compound C (Calbiochem). Four hours later, the cells were used for Western blot analysis for AMPK and p-AMPK, immunofluorescence for LC3 using anti-LC3 antibody as the primary antibody and Alexa 488 as the secondary antibody, electron microscopy, and ATP measurement (Sigma-Aldrich).

Table 1 Cardiac Function during Physiological Examinations Carried Out Before and After Treatment (4 and 6 Weeks after Surgery, Respectively)

<table>
<thead>
<tr>
<th></th>
<th>Sham Vehicle n = 8</th>
<th>Myocardial infarction Vehicle n = 16</th>
<th>RSV 5 mg n = 16</th>
<th>RSV 50 mg n = 16</th>
<th>Chloroquine n = 16</th>
<th>RSV + Cq n = 16</th>
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<tbody>
<tr>
<td><strong>Before treatment</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>LVEDd, mm</td>
<td>3.21 ± 0.04</td>
<td>4.77 ± 0.08*</td>
<td>4.77 ± 0.08*</td>
<td>4.68 ± 0.06*</td>
<td>4.742 ± 0.03*</td>
<td>4.69 ± 0.05*</td>
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<tr>
<td>LVEF, %</td>
<td>73.9 ± 1.3</td>
<td>38.8 ± 1.3*</td>
<td>36.4 ± 1.5*</td>
<td>38.5 ± 0.8*</td>
<td>38.5 ± 1.0*</td>
<td>38.6 ± 0.43*</td>
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<tr>
<td>Heart rate, b.p.m.</td>
<td>499 ± 9</td>
<td>491 ± 10</td>
<td>489 ± 11</td>
<td>488 ± 7</td>
<td>488 ± 4</td>
<td>486 ± 5</td>
</tr>
<tr>
<td><strong>After treatment</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LVEDd, mm</td>
<td>3.17 ± 0.05</td>
<td>4.93 ± 0.09*</td>
<td>4.98 ± 0.06*</td>
<td>4.14 ± 0.04*</td>
<td>5.20 ± 0.06*</td>
<td>4.92 ± 0.04*</td>
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<tr>
<td>LVEF, %</td>
<td>73.2 ± 0.78</td>
<td>35.7 ± 1.2*</td>
<td>34.8 ± 1.1*</td>
<td>46.9 ± 0.86*</td>
<td>32.0 ± 1.4*</td>
<td>35.7 ± 0.58*</td>
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<tr>
<td>Heart rate, b.p.m.</td>
<td>497 ± 17</td>
<td>490 ± 7</td>
<td>484 ± 8</td>
<td>487 ± 7</td>
<td>494 ± 7</td>
<td>493 ± 6</td>
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<td>SBP, mm Hg</td>
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<td>88.0 ± 1.5*</td>
<td>91.5 ± 0.7*</td>
<td>95.2 ± 0.9*</td>
<td>89.6 ± 1.7*</td>
<td>90.3 ± 1.4*</td>
</tr>
<tr>
<td>LVEDP, mm Hg</td>
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<td>5.7 ± 0.7*</td>
<td>1.6 ± 0.1*</td>
<td>7.9 ± 0.6*</td>
<td>5.5 ± 0.4*</td>
</tr>
<tr>
<td>+dp/dt, mm Hg/s</td>
<td>10.246 ± 113</td>
<td>4435 ± 79*</td>
<td>4661 ± 153*</td>
<td>6675 ± 251*</td>
<td>3627 ± 105*</td>
<td>4540 ± 121*</td>
</tr>
<tr>
<td>-dp/dt, mm Hg/s</td>
<td>-9017 ± 359</td>
<td>-3743 ± 137*</td>
<td>-3548 ± 158*</td>
<td>-5005 ± 282*</td>
<td>-3324 ± 117*</td>
<td>-4112 ± 127*</td>
</tr>
</tbody>
</table>

*P < 0.05 versus the vehicle-treated sham group.
†P < 0.05 versus the vehicle-treated infarction group.
Data are expressed as means ± SEM. The significance of differences between groups was evaluated using one-way analysis of variance with a post hoc Newman-Keuls multiple comparisons test or a repeated measures analysis of variance (Table 1). Values of $P < 0.05$ were considered significant.

Results

Resveratrol Reverses Remodeling and Improves Cardiac Function in Established Heart Failure

All of the mice in each group survived through the 2 weeks of treatment (6 weeks after surgery). However, echocardiography and cardiac catheterization revealed that in the vehicle-treated group, there was a progressive worsening of LV remodeling during this period, with enlargement of the LV cavity and diminishing cardiac performance, ie, reduced LV ejection fraction, reduced maximal and minimal change in pressure over time ($\pm$LV dP/dt), reduced LV systolic pressure, and increased LV end-diastolic pressure (Table 1 and Figure 1A). By contrast, the LV dilation and dysfunction were attenuated in the high-dose (but not the low-dose) resveratrol group; LV systolic pressure was preserved, and LV end-diastolic pressure was significantly lower than in the other treatment groups. Treatment with chloroquine had the opposite effect; it increased LV end-diastolic pressure, dilated the LV cavity, and exacerbated the reduction in cardiac performance. Moreover, the group treated with a combination of high-dose resveratrol plus chloroquine showed cardiac remodeling and dysfunction similar to that in the vehicle group. In other words, chloroquine appeared to block the beneficial effects of high-dose resveratrol. Neither high-dose resveratrol nor chloroquine...
affected left ventricular geometry or cardiac function in the sham-operated heart (data not shown).

Treatment with high-dose resveratrol also significantly suppressed elongation of the infarct wall segment and reduced heart-to-body weight ratios, lung-to-body weight ratios, and cardiomyocyte size (Figure 1B and Table 2). Thus, high-dose resveratrol attenuated cardiac hypertrophy, adverse remodeling, and pulmonary congestion. Conversely, treatment with chloroquine exacerbated LV dilation and the accompanying elongation of the infarct wall segment (Figure 1B and Table 2).

Our physiological and morphological studies revealed that only the high-dose, not low-dose, resveratrol was effective, suggesting a dose-dependent effect of this compound. Thus, we performed the subsequent mechanistic studies (ie, biochemical analyses) using only the group treated with the high-dose, rather than the low-dose, resveratrol. We evaluated myocardial ANP expression as an index of heart failure severity. Western blot and immunohistochemical analyses showed that ANP expression in ventricular cardiomyocytes neighboring the infarct area was suppressed in hearts treated with resveratrol, whereas it was greatly up-regulated in hearts treated with chloroquine, as compared to vehicle-treated hearts (Figure 1C). When resveratrol and chloroquine were administered together, their respective effects on ANP expression were offsetting. Thus, myocardial ANP expression levels mostly reflected the degree of cardiac dysfunction, but the relationship was not completely parallel, and the reasons remain unknown.

Resveratrol Augments Cardiomyocyte Autophagy in Postinfarction Hearts

Double immunofluorescent labeling revealed that the numbers of LC3-positive autophagic vacuoles were significantly increased within surviving cardiomyocytes following infarction (Figure 2). The immunopositive dots were distributed in cardiomyocytes situated in the area bordering the infarction as well as in more remote areas. Within the infarct area, however, the dot number was extremely low, which is consistent with an earlier report.12 In hearts treated with resveratrol, dot numbers were significantly increased in both the border and the remote areas, whereas chloroquine significantly reduced dot numbers in those areas (Figure 2).

Electron microscopic examination revealed that both autophagic vacuoles and lysosomes were abundant within cardiomyocytes in the postinfarction heart, and their numbers appeared to be further increased in cardiomyocytes from resveratrol-treated hearts (Figure 3). Conversely, their numbers were markedly diminished in chloroquine-treated hearts.

Western blot analysis showed that expression of LC3-II and p62 was up-regulated in the postinfarction heart (Figure 4). As a result, the LC3-II/LC3-I ratio was significantly increased and was increased still further by resveratrol treatment (Figure 4), which is indicative of increased autophagic turnover.21 In the heart treated with the combination of resveratrol plus chloroquine, the LC3-II/LC3-I ratios were similar to those seen in vehicle-treated hearts. The LC3-binding protein p62 regulates the formation of protein aggregates and is removed in the final digestion step during autophagy.22 Thus, an increased level of p62 indicates an increase in protein aggregation or impairment of their digestion. Levels of p62 were also significantly increased in the postinfarction heart, were reduced by high-dose resveratrol, but were increased by chloroquine (Figure 4). P62 levels of the combination group showed an intermediate value between those of the resveratrol alone and the chloroquine alone groups (Figure 4).

Resveratrol Improves the Energy Status of Postinfarction Hearts

We observed a significant increase in the level of activated AMPK (p-AMPK) in the postinfarction heart, and the level was increased still further by both resveratrol and chloroquine (Figure 5A). Conversely, direct measurements

Table 2 Cardiac Morphometry and Pathology

<table>
<thead>
<tr>
<th></th>
<th>Sham Vehicle n = 8</th>
<th>Myocardial infarction</th>
<th>RSV 5 mg n = 16</th>
<th>RSV 50 mg n = 16</th>
<th>Chloroquine n = 16</th>
<th>RSV + Cq n = 16</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>30.2 ± 0.46</td>
<td>26.7 ± 0.44*</td>
<td>26.9 ± 0.42*</td>
<td>27.1 ± 0.35*</td>
<td>26.3 ± 0.40*</td>
<td>27.1 ± 0.41*</td>
</tr>
<tr>
<td>Heart weight (g)</td>
<td>0.12 ± 0.004</td>
<td>0.14 ± 0.007*</td>
<td>0.13 ± 0.004*</td>
<td>0.12 ± 0.004*</td>
<td>0.13 ± 0.006*</td>
<td>0.13 ± 0.005*</td>
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<td>Heart/body ratio (mg/g)</td>
<td>3.83 ± 0.10</td>
<td>5.18 ± 0.21*</td>
<td>4.78 ± 0.15*</td>
<td>4.24 ± 0.12*</td>
<td>4.96 ± 0.17*</td>
<td>4.72 ± 0.17*</td>
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<tr>
<td>Lung/Body ratio (mg/g)</td>
<td>4.92 ± 0.10</td>
<td>5.49 ± 0.13*</td>
<td>5.56 ± 0.12*</td>
<td>4.83 ± 0.12*</td>
<td>5.67 ± 0.19*</td>
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<tr>
<td>%Infarct segment</td>
<td>49.9 ± 1.49</td>
<td>50.9 ± 1.53</td>
<td>40.6 ± 2.06*</td>
<td>56.4 ± 1.68*</td>
<td>47.2 ± 1.55</td>
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<tr>
<td>Infarct length (mm)</td>
<td>16.0 ± 0.47</td>
<td>15.5 ± 0.39</td>
<td>14.4 ± 0.25*</td>
<td>16.7 ± 0.44*</td>
<td>15.1 ± 0.58</td>
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<tr>
<td>Infarct thickness (mm)</td>
<td>2.4 ± 0.06</td>
<td>2.4 ± 0.08</td>
<td>2.4 ± 0.05</td>
<td>2.4 ± 0.03</td>
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<tr>
<td>Myocyte size (μm)</td>
<td>12.7 ± 0.3</td>
<td>16.5 ± 0.3*</td>
<td>16.4 ± 0.2*</td>
<td>13.5 ± 0.1*</td>
<td>16.5 ± 0.2*</td>
<td>16.5 ± 0.12*</td>
</tr>
</tbody>
</table>

*P < 0.05 versus the vehicle-treated sham group.

†P < 0.05 versus the vehicle-treated infarction group.

Cq, chloroquine; RSV, resveratrol.
revealed that although resveratrol significantly increased myocardial ATP content, chloroquine reduced it (Figure 5B), and the combined treatment resulted in an intermediate value.

Effect of Resveratrol on mTOR and p70 S6 Kinase Activity

We found that p-mTOR levels were elevated in the post-infarction heart, but were significantly reduced to the level seen in sham-operated hearts by treatment with resveratrol (Figure 6A). In addition, levels of p-p70S6K, a direct downstream target of mTOR, paralleled those of p-mTOR (Figure 6B). Both kinases were significantly activated in hearts treated with chloroquine. In hearts treated with resveratrol plus chloroquine, the activities of these kinases reached an intermediate level, similar to that seen in vehicle-treated hearts (Figure 6).

Effect of Resveratrol on Akt and Sirt1 Activity and MnSOD Expression

We also assessed the postinfarct activity of Akt, a key prosurvival molecule that negatively regulates myocardial AMPK activity and is reportedly inhibited by resveratrol.23,24 Western blot analysis showed that p-Akt levels were significantly higher in postinfarction hearts than in sham-operated hearts, but were unaffected by any of the

Figure 2  Postinfarction accumulation of autophagic vacuoles. A: Immunofluorescent labeling of LC3 (green) and myoglobin (red) in ventricular tissues in an area remote from the infarct (top row), in tissue bordering the infarct (middle row), and in the infarct area (bottom row). Scale bars: 10 μm. B: Number of LC3 dots per high-power fields (HPF) (600×) in the remote, border, and infarct areas. *P < 0.05 versus the sham-operated group; †P < 0.05 versus the vehicle-treated control group. Cq/C, chloroquine; R+C, resveratrol 50 mg/kg + chloroquine; RSV50/R, resveratrol 50 mg/kg; S, sham; Veh/V, vehicle.
treatments in this study (Figure 7A). This suggests resveratrol acts directly or indirectly on AMPK without affecting the Akt pathway in the present model.

Sirt1, an NAD$^+$-dependent protein/histone deacetylase, is a mammalian orthologue of yeast Sir2, which deacetylates histone polypeptides with a preference for histone H3 lysine 9. We found that Sirt1 levels were similar in the five treatment groups (Figure 7B). On the other hand, the level of acetyl-histone, which is inversely proportional to the Sirt1 activity, was significantly increased in the postinfarction heart. The increase in acetyl-histone, ie, inactivity of Sirt1, was attenuated by resveratrol treatment, but was unaffected by chloroquine (Figure 7B). Myocardial Sirt1 thus appears to be activated by resveratrol in the postinfarction heart, but the lack of effect of chloroquine suggests Sirt1 is not involved in the autophagic response. Myocardial MnSOD levels were similar between the groups with myocardial infarction (Figure 7C).

Effect of Resveratrol on Apoptosis

Because earlier studies reported that autophagic cell death could switch to apoptotic cell death when autophagy was inhibited, we next assessed the apoptosis incidence. Double immunofluorescent labeling using TUNEL and an anti-myoglobin antibody revealed that TUNEL positivity was rare in the postinfarction heart, though the incidence among cardiomyocytes and nonmyocytes was significantly higher in the postinfarction hearts than in sham-operated hearts, and it did not differ among the treatment groups (Figure 8A). Activation of caspase-3, the majority of which was probably of nonmyocyte origin, was confirmed in the postinfarction heart (Figure 8B), but was apparently unaffected by the treatments.

Effect of an AMPK Inhibitor on Cultured Cardiomyocytes

Finally, we performed in vitro experiments to investigate the mechanistic role of AMPK activation for resveratrol-induced autophagy in cardiomyocytes. Isolated neonatal mouse cardiomyocytes were treated with resveratrol or chloroquine with or without simultaneous treatment with compound C (an AMPK inhibitor). Resveratrol brought about an increase in p-AMPK expression, LC3 dot number, autophagosomes and autolysosomes, and ATP content in the cardiomyocytes, but simultaneous treatment with compound C completely canceled such effects of resveratrol (Figure 9). Conversely, treatment with chloroquine resulted in an increase in p-AMPK, an increase in LC3 dots and lysosomes, and a decrease in ATP content in the cardiomyocytes, none of which, except for AMPK inactivation, was affected by simultaneous treatment with the AMPK inhibitor. These results indicate that resveratrol enhances autophagy through the AMPK pathway and that resveratrol and chloroquine regulate AMPK activity and subsequent events in a distinct manner, despite the fact that both indeed
activate AMPK. It is believed that resveratrol directly activates AMPK to promote autophagy, because autophagy evoked by resveratrol is inhibited by the AMPK inhibitor. On the other hand, chloroquine may inhibit autophagy, as previously reported, by interfering with fusion between autophagosomes and lysosomes to result in a reduction in ATP content that in turn stimulates AMPK activity (Figure 9E).

Discussion

Mechanisms for the Beneficial Effects of Resveratrol in the Postinfarction Heart

The main finding of this study is that systemic treatment with resveratrol partially reversed the adverse LV remodeling and mitigated the heart failure seen following a large myocardial infarction, with no major side effects. Resveratrol was administered to established heart failure, closely resembling the frequently met clinical situation because patients often do not seek treatment until after developing the symptoms. We suggest that these beneficial effects of resveratrol mainly reflect its ability to increase autophagic activity, as the autophagic inhibitor chloroquine produced opposite outcomes when administered alone and offset the effects of resveratrol when the two agents were administered together. In addition, autophagic activity (Figures 2–4), myocardial ATP content (Figure 5B), and the degree of improvement in LV remodeling and function (Figure 1) varied in parallel in the postinfarction hearts, supporting the notion that the increased autophagy accelerated energy recycling to the benefit of postinfarction processes in the heart.

The accumulation of autophagic vacuoles can represent either their increased formation or impairment of their digestion. In the present study, we confirmed an increase in the LC3-II/LC3-I ratio, which is an established indicator of autophagic turnover. Moreover, to examine autophagic flux in vivo, we used chloroquine alone and in combination with resveratrol. Chloroquine inhibits autophagosome—lysosome fusion, thereby preventing the final digestion step in autophagy, and is frequently used as an autophagic inhibitor. In postinfarction hearts, chloroquine suppressed autophagic activity and reduced the numbers of autophagic vacuoles and LC3-II/LC3-I ratios, all of which were augmented by resveratrol. These findings indicate that resveratrol increases autophagic turnover or flux in failing hearts with old myocardial infarctions.

We recently observed the time course of autophagy in surviving cardiomyocytes in the postinfarction heart at 1, 2, and 3 weeks after infarction, and reported that autophagy...
was actively induced at the remote area as well as at the border, and the activity at the remote area was stronger during more chronic stages (2 and 3 weeks after infarction). The present study confirmed strong activity of autophagy in remote area even during 6 weeks post-infarction, a more chronic stage. These suggest that cardiac dysfunction and/or remodeling itself induces autophagy. Along with the progression of cardiac remodeling, wall stress increases in parallel with the ventricular dilation (Laplace’s law), causing tissue hypoxia, even in the remote myocardium, which may have contributed to the autophagy induction in the failing heart with a large, old infarction.

Molecular Signals Evoked by Resveratrol or Chloroquine

Energy recycling is an important function of autophagy, and we previously reported that autophagy increases myocardial ATP levels, leading to improved cardiac performance and cardiomyocyte survival. AMPK belongs to a conserved family of protein kinases and serves as a general integrator of the metabolic response to changes in energy availability; it is activated by ATP depletion and the concomitant AMP accumulation (increases in the AMP/ATP ratio). The observed up-regulation of p-AMPK in chloroquine-treated hearts is consistent with our earlier finding that inhibition of autophagy by bafilomycin A1 interferes with the supply of ATP to the ischemic myocardium, leading to AMPK activation. However, treatment with resveratrol also increased levels of p-AMPK, which goes against the initial assumption that augmenting autophagy with resveratrol would suppress levels of p-AMPK by recycling ATP. This suggests resveratrol activates AMPK directly or indirectly via other pathways, eg, upstream liver kinase B1 (LKB1), which regulates the intracellular energy status.
Our in vitro study suggested that despite the fact that both resveratrol and chloroquine activate AMPK, resveratrol directly activates AMPK to promote autophagy, whereas chloroquine may inhibit autophagy by interfering with fusion between autophagosomes and lysosomes as previously reported, to result in a reduction in ATP content, which in turn stimulates AMPK activity. Although we did not trace the resveratrol signaling to AMPK activation in the present study, we did confirm that resveratrol had no effect on the activity of Akt, which is known to associate with AMPK. 

Figure 8  Apoptosis among cardiomyocytes and nonmyocytes in the postinfarction heart. A: TUNEL (green) and immunostaining for myoglobin (red). Note the TUNEL+ cardiomyocyte (upper panels) and nonmyocyte (lower panels). Scale bars: 10 μm. Graphs show the incidences of TUNEL positivity among cardiomyocytes and nonmyocytes. B: Western blotting of procaspase-3 and cleaved caspase-3. *P < 0.05 versus the sham-operated group. Cq/C, chloroquine; R+C, resveratrol 50 mg/kg + chloroquine; RSV/R, resveratrol 50 mg/kg; S, sham; Veh/V, vehicle.

The beneficial effects of mTOR inhibition on LV remodeling after myocardial infarction. They demonstrated that mTOR inhibition using everolimus increased autophagy and concomitantly decreased proteasome activity in the border area. These reports are consistent with our present findings that resveratrol increased autophagy, increased levels of AMPK and suppressed levels of mTOR and p70S6K.

Resveratrol exerts multiple effects to regulate a variety of key molecules. Originally identified as a type of polyphenol, it reportedly shows strong antioxidant activity. Sirt1, an NAD+-dependent protein/histone deacetylase, is a mammalian orthologue of yeast Sir2, and deacetylates histone polypeptides with a preference for histone H3 lysine 9. Although we confirmed that resveratrol activates Sirt1, it remains unclear whether Sirt1 has a direct effect on autophagy. Tanno et al reported that resveratrol exerts antioxidant effects by inducing MnSOD through activation of Sirt1. Our study showed that Sirt1 activity in the postinfarction heart was maintained despite treatment with chloroquine (Figure 7B). That chloroquine exacerbated the LV remodeling and dysfunction without affecting Sirt1 activity implies a dissociation between Sirt1 activity and cardioprotection. Nadtocchiy et al reported that overexpression of Sirt1 protected against ischemia-reperfusion injury through stimulation of autophagy, whereas Kawasaki et al reported constitutive Sirt1 overexpression impairs mitochondrial function and reduces autophagy, resulting in cardiac dysfunction. Further study will be required to define the role of Sirt1 in postinfarction cardiac remodeling. The present study showed that myocardial MnSOD expression levels were similar between the groups. We would not deny the antioxidant property of resveratrol and its role in cardioprotection, as well as that of Sirt1, but we speculate this property might not be so strong in the present heart failure model.

It has been reported that apoptotic loss of surviving cardiomyocytes may contribute to the progression of postinfarction cardiac remodeling and dysfunction. In addition, inactivation of autophagy reportedly triggers apoptosis in cell lines. In the present study, chloroquine inhibited autophagy in cardiomyocytes, but did not affect the rate of TUNEL positivity, and cardiac nonmyocytes showed relatively little autophagic activity after infarction, as indicated by the absence of LC3-II immunopositivity. The low rate of apoptosis, specifically among cardiomyocytes, may be due to the relatively low sensitivity of cardiomyocytes to apoptotic stimuli such as Fas stimulation.

Study Limitations

Chloroquine is thought to raise lysosomal pH, thereby inhibiting lysosomal activity, and is used in assays of short-term (several hours) autophagic flux. LC3-II protein and undigested autophagosomes would be expected to accumulate in hearts treated with chloroquine. Curiously, however, we did not observe such accumulation; instead, we
found that the numbers of LC3-immunopositive dots and the levels of both LC3-II and LC3-I were significantly reduced, suggesting that the initial step in the formation of autophagosomes (phagophore formation) was inhibited by chloroquine. Moreover, electron microscopic observation confirmed the impaired formation of autophagosomes in chloroquine-treated hearts. We previously observed the same phenomenon (reduced LC3-I and II) on administration of baflomycin A1 to mice that had been starved or subjected to myocardial infarction. Like chloroquine, baflomycin A1 reportedly interferes with the fusion of autophagosomes and lysosomes to inhibit formation of autophagolysosomes, suppressing the final digestion step in autophagy. Thus, both baflomycin A1 and chloroquine effectively inhibit autophagy, though the mechanisms are not yet fully understood.

We have found the beneficial effect of resveratrol on postinfarction remodeling and next elucidated the signal transduction evoked by resveratrol in in vitro cardiomyocytes using pharmacological intervention such as chloroquine and compound C. Chloroquine functionally inhibits autophagy as discussed above, and such an effect has been confirmed by many reports. Also, compound C is commonly used as an AMPK inhibitor and was actually shown to effectively inactivate AMPK in the present study. However, pharmacological approaches have limitations in specificity; an effective means of resolving this issue may be through the genetic approach. Such approaches may also lack specificity, as it was recently reported that there is an alternative autophagic pathway that is independent of Atg5 and Atg7, which had been believed to be essential for mammalian autophagy. It goes without saying that further investigation using more specific approaches is warranted in the future to confirm our findings.

Clinical Implications

Resveratrol was found to be therapeutically effective against established postinfarction heart failure. Although many reagents are reported to effectively prevent adverse postinfarction remodeling, patients often do not seek treatment until after developing the symptoms of established heart failure. In this context, a therapeutic agent, such as resveratrol, that could reverse the progression of heart failure and improve cardiac function would be highly desirable.

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A1 Resveratrol to Postinfarction Heart

![Figure A](image1)

**Figure 9** In vitro experiments using mouse neonatal cardiomyocytes. A: Western blots with densitometric analysis of AMPK and p-AMPK. Graphs show the intensity of each band in arbitrary units. B: Immunohistochemical labeling of LC3 (green dots) in cardiomyocytes. Scale bar = 20 μm. Graph shows the number of LC3 dots per cell. C: Electron microphotographs showing autophagic vacuoles. Resveratrol increased autophagic vacuoles (arrows). Chloroquine increased lysosomes (electron-dense spherical bodies) uncombined with autophagosomes, whereas compound C decreased resveratrol-induced autophagic vacuoles. Scale bar = 1 μm. Mf, myofibril; N, nucleus. D: ATP contents in the cardiomyocytes. E: Hypothesized signal transduction by resveratrol and chloroquine. *P < 0.05. CC = compound C; Cq, chloroquine; Cq + CC, chloroquine + compound C; N.S., not significant; Rsv, resveratrol 50 mg/kg; Rsv + CC, resveratrol 50 mg/kg + compound C; S, sham; Veh/V, vehicle.
References