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# Resveratrol attenuates aging-induced mitochondrial dysfunction and mitochondria-mediated apoptosis in the rat heart

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## ABSTRACT

**BACKGROUD/OBJECTIVES:** Resveratrol, a natural polyphenolic compound, has potent antioxidant and anti-inflammatory properties, leading to beneficial effects against cardiovascular diseases. The purpose of this study was to determine whether resveratrol induces protective effects against aging-induced cardiac remodeling, mitochondrial dysfunction, and mitochondria-mediated apoptosis in the heart.
**MATERIALS/METHODS:** Thirty-two male Fischer 344 rats were divided into 4 groups: 2 groups that were orally treated with resveratrol (50 mg/kg/day) for 6 weeks (young and old resveratrol groups), and 2 control groups (young and old control groups). Mitochondrial function and mitochondria-mediated apoptotic pathway were analyzed in cardiac muscle fibers from the left ventricle.
**RESULTS:** Resveratrol significantly reduced cardiac hypertrophy and remodeling in aging hearts. In addition, resveratrol significantly ameliorated aging-induced mitochondrial dysfunction (e.g., decreased oxygen respiration and increased hydrogen peroxide emission) and mitochondria-dependent apoptotic signaling (the Bax/Bcl-2 ratio, mitochondrial permeability transition pore opening sensitivity, and cleaved caspase-3 protein levels). Resveratrol also significantly attenuated aging-induced apoptosis (determined via cleaved caspase-3 staining and TUNEL-positive myonuclei) in cardiac muscles.
**CONCLUSION:** This study demonstrates that resveratrol treatment has a beneficial effect on aging-induced cardiac remodeling by ameliorating mitochondrial dysfunction and inhibiting mitochondria-mediated apoptosis in the heart.

**Keywords:** Aging; resveratrol; heart; mitochondria; oxidative stress

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**Conflict of Interest**

The authors declare no potential conflicts of interest.

**Author Contributions**

Conceptualization: No MH, Kwak HB. Data curation: No MH, Choi Y, Park DH, Kang JH, Kim CJ, Seo DY, Han J, Kwak HB. Formal analysis: No MH, Choi Y, Cho EJ, Kwak HB. Funding acquisition: Kwak HB. Investigation: No MH, Heo JW. Writing - original draft: Choi Y, No MH. Writing - review & editing: Choi Y, Kwak HB.

## INTRODUCTION

Cardiovascular disease is the leading cause of both death and disability worldwide, with the number of fatalities projected to increase to over 22.2 million a year by 2030 [1,2]. Aging is characterized by progressive abnormalities in the structure and function of the heart. More specifically, aging induces an increased susceptibility to oxidative stress [3] and inflammation [4], a decline in contractile function, and cardiac remodeling [5], leading to an increased risk of heart disease and failure [6]. Apoptosis, a process of programmed cell death, is accelerated with advancing age, and excessive apoptosis in the heart results in diminished cardiomyocyte number and impaired myocardial contractile function, contributing to the development and progression of cardiac diseases [7].

Mitochondria, which account for approximately 35% of the cardiomyocyte volume [8], exhibit major functional and structural changes with aging that play a crucial role in regulating apoptosis in the heart. In particular, mitochondria-mediated apoptotic signaling via the Bcl-2 family of proteins is known play a key role in the regulation of apoptosis by controlling cytochrome *c* release [9]. We previously reported that excessive apoptosis with aging is related to the mitochondria-driven apoptotic pathway, particularly the Bax/Bcl-2 ratio and the cleaved caspase-3 levels [10-12], concomitant with mitochondrial dysfunction, which ultimately results in the decreased production of adenosine triphosphate (ATP, the main source of intracellular energy) and impaired myocardial contractile function. Moreover, since mitochondria are both the main sources and primary targets of reactive oxygen species (ROS), oxidative stress is considered a central contributor to the impairment of mitochondrial function and structure in the aging heart [13]. Thus, these findings suggest that the development of successful antioxidant therapeutics targeting the mitochondria could be a key strategy against cardiac aging.

Resveratrol is a natural polyphenolic compound obtained from various plants, such as grapes, berries, and peanuts. Resveratrol, which exerts antioxidant and anti-inflammatory properties [14], has been shown to have cardioprotective effects in several pathological conditions, including cardiac hypertrophy, myocardial ischemic/reperfusion, and heart failure [15,16]. In animal models of cardiac dysfunction caused by various cardiomyopathies, resveratrol has been shown to reduce pathological cardiac remodeling, potentially through the regulation of autophagy in the hearts under pressure overload. In addition to the regulation of autophagy, resveratrol also exerts both direct and indirect antioxidant effects by scavenging ROS—including superoxide radicals ($O_2^-$) and hydroxyl radicals ($OH^-$)—and by inhibiting oxidative damage (i.e., DNA damage due to $OH^-$) [17]. These antioxidant effects are critical in mitigating mitochondrial dysfunction, which plays a key role in aging. Given that mitochondrial dysfunction and oxidative stress are central contributors to the aging process, the mitochondrial protective effects of resveratrol may offer potential for the prevention or amelioration of age-related cardiac dysfunction. Despite the cardioprotective effects of resveratrol on pathological conditions, it is still unknown whether prolonged resveratrol intake can counteract age-dependent degeneration and decline in heart function by improving both mitochondrial function and the mitochondria-dependent apoptotic pathway.

Accordingly, this study aimed to determine whether resveratrol protects against cardiac aging, including cardiac hypertrophy and remodeling, apoptosis, and mitochondrial dysfunction in the heart. To address this, we investigated the effects of resveratrol on 1) mitochondrial function (e.g., mitochondrial oxygen ($O_2$) respiration, hydrogen peroxide ($H_2O_2$) emission,

and calcium ($Ca^{2+}$) retention capacity), 2) the mitochondria-dependent apoptotic pathway (Bax/Bcl-2 ratio, mitochondrial permeability transition pore [mPTP] opening sensitivity, and cleaved caspase-3 protein levels), 3) apoptosis (cleaved caspase-3 staining and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling [TUNEL]-positive myonuclei), and 4) cardiac remodeling (structural and morphological changes) in aged rat hearts.

## MATERIALS AND METHODS

### Animals and resveratrol dosage regimen

Forty male Fischer 344 rats were categorized according to age and divided into control and resveratrol-treated groups: 1) young control group (YC, 4 mon, n = 8), 2) young resveratrol group (YR, 4 mon, n = 8), 3) old control group (OC, 20 mon, n = 8), and 4) old resveratrol group (OR, 20 mon, n = 8). The sample size was determined using the resource equation method for analysis of variance (ANOVA) [18]. A sample size of more than 6 animals per group ensured adequate sample size to detect significant results in the study. Finally, a total of 32 Fischer 344 male rats were used in this study to consider expected attrition (e.g., death of animals). All animals were housed two per cage in a temperature-controlled room (20 ± 2°C) with a 12-h light-dark diurnal cycle (light from 8 a.m. to 8 p.m.). Rats were allowed to adapt to the environment for approximately 7 days in the animal cage prior to beginning the experiment. During the experiment, the rats were provided standard laboratory chow (LabDiet 5L79; Orient Bio Inc., Seongnam, Korea) and tap water *ad libitum*. All experimental procedures were performed in accordance with the animal care guidelines of the Animal Care and Use Committee of Kyung Hee University (Seoul, Korea).

The young and old rats were acclimatized for 4 or 20 mon, respectively. The resveratrol group received resveratrol (trans-form, CAS 501-36-0; Tokyo Chemical Industries Co., Ltd., Tokyo, Japan) dissolved in 0.5% carboxymethyl cellulose (CMC) sodium solution via oral gavage, starting at 16 weeks of age for the young rats and 80 weeks for the old rats, and continuing for 6 weeks. The dosage (50 mg/kg/day) and administration route for resveratrol were confirmed the bioavailability of resveratrol and its conversion into dihydroresveratrol by measuring resveratrol levels in plasma [19]. The control groups were administrated equal volumes of 0.5% CMC sodium solution.

### Tissue preparation and homogenization

Rats in the treatment groups were sacrificed with pentobarbital sodium (5 mg/100 g) 24 h after their last treatment. The left ventricle (LV) samples were snap-frozen in liquid nitrogen for protein expression analysis. Samples were stored at −80°C until further analyses. Tissues for morphological experiments were fixed in 10% natural formalin buffer (Sigma-Aldrich, St. Louis, MO, USA) for 7 days at room temperature (RT) as around 24–26°C.

Frozen LV tissues were minced into fine pieces with scissors and homogenized with lysis buffer (pH 7.6) containing non-ionic detergent, protease inhibitors (4 mM 4-[2-Aminoethyl] benzenesulfonyl fluoride hydrochloride [AEBSF], 1 ug/mL benzamidine, 1 ug/mL leupeptin, 1 ug/mL pepstatin, 1 mM ethylenediaminetetraacetic acid [EDTA], 1 mM ethylene glycol-bis [β-aminoethyl ether]-N,N,N′,N′-tetraacetic acid), and phosphatase inhibitors (1 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM beta-glycerophosphate, and 2.5 mM sodium pyrophosphate) using an OMNI TH homogenizer (OMNI International, Kennesaw, GA, USA) for 20 s. The homogenized tissue was centrifuged twice at 10,000 × g for 20 min and


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10 min at 4°C. Total protein concentrations were determined using a BCA Protein Assay Kit (Beyotime Inc., Shanghai, China).

## Mitochondrial function

*Preparation of permeabilized cardiac myofiber bundles*

Immediately after sacrifice, 2–3 mg of an LV muscle biopsy sample was transferred into a Petri dish containing ice-cold buffer X, consisting of 7.23 mM $K_2$EGTA, 2.77 mM Ca$K_2$EGTA, 20 mM imidazole, 0.5 mM 1,4-dithiothreitol, 20 mM taurine, 5.7 mM ATP, 14.3 mM phosphocreatine, 6.56 $MgCl_2$-6 $H_2O$ (pH 7.1, 290 mOsm) and trimmed of adipose tissue, connective tissue, and blood vessels using scissors and forceps as previously described [10,11]. The small bundle was separated along its longitudinal axis using forceps and then treated with 50 μg/mL of saponin for 30 min. Saponin selectively permeabilizes the sarcolemmal membrane while keeping the mitochondrial membrane intact. The permeabilized LV fiber bundles were washed in ice-cold buffer Z, containing 50 μM EGTA (washing buffer), 105 mM K-MES, 30 mM KCl, 10 mM $KH_2PO_4$, 5 mM $MgCl_2$-6$H_2O$, and 0.5 mM bovine serum albumin (BSA; pH 7.1), for a minimum of 15 min in a shaker, after which they remained in buffer Z on a rotator at 4°C until analysis. The permeabilized LV fibers were used to measure mitochondrial $O_2$ respiration, $H_2O_2$ emission, and $Ca^{2+}$ retention capacity to determine the effects of aging and resveratrol on mitochondrial function.

*Mitochondrial $O_2$ respiration*

The mitochondrial $O_2$ consumption rate was determined from a polarographic high-respirometry measurement using an Oroboros $O_2$K Oxygraph (Oroboros Instruments, Innsbruck, Austria) as previously described [10,20]. This method caps two chambers and monitors the $O_2$ concentration with a polarographic $O_2$ electrode using two parallel measurement methods. The $O_2$ flow rate was calculated as the negative derivative of $O_2$ concentration, measured continuously while sequentially adding multiple substrates at 30°C under the following conditions: 1) 5 mM glutamate (G; complex I substrate) + 2 mM malate (M; complex I substrate, state 2 conditions), 2) 4 mM adenosine diphosphate (ADP; state 3 conditions), and 3) 10 mM succinate (SUCC; complex II substrate). Changes in $O_2$ respiration were recorded and analyzed using Datlab software (Datlab Version 6.0; Oroboros Instruments). After the experiment, the raw data for mitochondrial $O_2$ respiration were normalized by wet tissue weight.

*Mitochondrial $H_2O_2$ emission*

Mitochondrial $H_2O_2$ emission was determined as previously described for cardiac muscle [10,20], which was continuously monitored for the oxidation of Amplex Red in buffer Z at 37°C (ΔF/min) during state 4 respiration with 10 μg/mL oligomycin. The oxidation of Amplex Red (excitation/emission wavelengths λ = 567/587 nm) was measured using a SPEX Fluormax 4 spectrofluorometer (Horiba, Edison, NJ, USA) with the following steps: 2 mg of LV muscle fiber, 10 μM Amplex Red, 1.5 U/mL horseradish peroxidase (HRP), 10 μg/mL oligomycin in the cuvette and turn on the stirbar, and 5 mM G (complex I substrate) + 2 mM M (complex I substrate), 10 mM SUCC (complex II substrate), and 10 mM glycerol-3 phosphate (G3P; lipid substrate) into the cuvette in the order. The mitochondrial $H_2O_2$ emission rate after removing the background value from each standard value was calculated from the slope of the ΔF/min gradient values. After the experiment, the raw data from mitochondrial $H_2O_2$ emissions were normalized by wet tissue weight.

*Mitochondrial $Ca^{2+}$ retention capacity and mPTP opening sensitivity*

Mitochondrial $Ca^{2+}$ retention capacity was measured to assess the susceptibility of mPTP


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opening as previously described with minor modifications [10]. Briefly, after LV tissue was separated, permeabilized with saponin, and washed with washing buffer (buffer Z supplemented with 50 μM EGTA), overlaid traces of changes in $Ca^{2+}$-induced fluorescence by calcium green-5 N were measured continuously (ΔF/min) at 37°C during state 4 conditions (10 μg/mL oligomycin) using a Spex Fluormax 4 spectrofluorometer (Horiba). After establishing the background ΔF/min, the reaction was initiated by the addition of $Ca^{2+}$ pulses (30 nM) with excitation and emission wavelengths set at 506 nm (excitation wavelength) and 532 nm (emission wavelength), respectively. After completing the experiment, the level of mitochondrial $Ca^{2+}$ retention capacity was normalized by wet tissue weight. Total mitochondrial $Ca^{2+}$ retention capacity prior to PTP opening (i.e., the release of $Ca^{2+}$) was expressed as picomoles/mg wet tissue weight. The opening sensitivity of the mPTP was calculated using the $Ca^{2+}$ retention capacity graph. mPTP was indirectly calculated by dividing the YC value of the $Ca^{2+}$ retention capacity by the respective groups, and comparisons were indicated with percentage values.

## Western blotting

Protein levels for Bax, Bcl-2, and cleaved caspase-3 were determined by Western blot analysis, following the procedure previously described by our laboratory with minor modification [11]. LV tissues were homogenized in RIPA buffer (Beyotime Inc.) supplemented with a protease inhibitor cocktail (Roche, Mannheim, Germany) and phosphatase inhibitor cocktail (Roche). Equal amounts of protein (20 μg) from different samples were loaded onto 10–12% sodium dodecyl sulfate-polyacrylamide gels and separated by electrophoresis at 110 V for 2 h. The proteins were transferred onto PVDF membranes (Pall Life Science, Ann Arbor, MI, USA) at 100 V for 1 h in ice-cold water. After staining with Ponceau S (Sigma-Aldrich) to verify equal loading and transfer of proteins to the membranes. The membranes were blocked using 5% non-fat milk in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) for 2 h at RT. The membranes were incubated with different primary antibodies against β-actin (sc-47778, 1:5,000; Santa Cruz, Dallas, TX, USA), Bax (sc-7480, 1:1,000; Santa Cruz), Bcl-2 (sc-7382, 1:1,000; Santa Cruz), and cleaved caspase-3 (Asp175, 1:1,000; Cell Signaling Technology, Beverly, MA, USA) for 16–20 h at 4°C. The next day, the membranes were washed three times with TBS-T and incubated with HRP-conjugated anti-mouse (1:3,000; Santa Cruz) or anti-rabbit (1:3,000; Santa Cruz) secondary antibodies for 1 h at RT. The membranes were subsequently washed three times with TBS-T, and the protein bands were detected using an enhanced chemiluminescence detection kit (Thermo Scientific, Rockford, IL, USA) using Chemidoc (Bio-Rad, Hercules, CA, USA). The band intensities were quantified using Image-Pro Plus software (Media Cybernetics, Bethesda, MD, USA).

## Immunohistochemistry

LV tissue was paraffin-embedded and cut into 5-μm thick cross-sections. The sections were deparaffinized in xylene, hydrated in decreasing ethanol concentrations, and treated with 3% $H_2O_2$ in 0.05 M Tris-HCl (0.25% Triton X-100) for 15 min at RT. They were then washed three times with phosphate-buffered saline (PBS) for 3 min each. After blocking with 5% diluent solution (Invitrogen, Carlsbad, CA, USA) in 0.05 M Tris-HCl containing 1 mg/mL BSA, 1 mM NaF, and 0.05% Triton X-100 for 1 h at RT, sections were incubated with anti-cleaved caspase-3 primary antibody (Cell Signaling Technology) overnight at 4°C. The sections were then rinsed thrice with PBS. Positive staining was detected using HRP-conjugated secondary antibodies by incubating the sections in 3,3'-diaminobenzidine (DAB) peroxidase substrates at RT (Dako Cytomation, Glostrup, Denmark). The sections were then visualized with 0.05% DAB solution in 0.003% $H_2O_2$, washed, dehydrated, cleared, and coverslipped with synthetic


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mounting medium. Digital images were captured via Axioplan 2 imaging (Carl Zeiss, Jena, Germany). The cleaved caspase-3-positive cells were quantified from multiple LV sections using the Image J analysis program (National Institutes of Health, Bethesda, MD, USA).

### Hematoxylin and eosin (H&E) staining

Hematoxylin was used as a general stain for cardiomyocytes, nuclei, extracellular area, and geometry. First, the sections were de-waxed and then stained with hematoxylin for 3 min. Next, the sections were washed twice in distilled water and then soaked in 85% alcohol for 1 min. Sections were stained with eosin for 5 min and immersed in 70%, 80%, and 90% alcohol. The sections were then soaked in 100% alcohol for 2 min and twice in xylene for 30 s. The sections were then sorted and photographed using Axioplan 2 imaging (Carl Zeiss).

### TUNEL-positive myonuclei determination

TUNEL staining for strand breaks in paraffinized sections was performed using an ApopTag™ peroxidase *in situ* apoptosis detection kit (Chemicon International Inc., Billerica, MA, USA) according to the manufacturer's instructions. Images (40× magnification) were captured via Axioplan 2 imaging and software (Carl Zeiss). TUNEL-positive myonuclei were counted in multiple LV sections and are indicated as a percentage of all myonuclei.

### Statistical analysis

All data are expressed as the mean ± standard error of the mean (SEM). Statistical analysis was performed using 2-way ANOVA followed by Tukey's *post hoc* test to assess mean differences among groups for aging and resveratrol effects. The level of statistical significance was set at $P < 0.05$. All graphs were generated using Prism software (GraphPad, La Jolla, CA, USA).

## RESULTS

### Resveratrol attenuates aging-associated cardiac hypertrophy and remodeling

Two-way ANOVA reveals a significant age-by-resveratrol interaction in the body weight ($F = 4.39$, $P < 0.05$), a main effect of age in the heart mass ($F = 25.43$, $P < 0.001$), and a main effect of resveratrol in the heart mass/body weight mass ($F = 7.15$, $P < 0.05$). The body weight and absolute heart mass were significantly increased with aging (all $P < 0.05$; $P < 0.05$; **Table 1**). After resveratrol administration, we found that body weight significantly decreased, and the heart mass/body weight ratio significantly increased in the OR group compared to the OC group.

**Table 1.** Effects of resveratrol on body weight, heart mass, and the heart mass/body weight ratio

| Variable | YC | YR | OC | OR |
|---|---|---|---|---|
| Body weight (g) | 308.62 ± 5.61 | 306.88 ± 13.23 | 361.13 ± 12.15* | 309.50 ± 14.58† |
| Heart mass (mg) | 810.88 ± 12.50 | 833.99 ± 14.38 | 904.38 ± 27.05* | 915.38 ± 10.30* |
| Heart mass/body weight (mg/g) | 2.63 ± 0.46 | 2.74 ± 0.09 | 2.53 ± 0.13 | 3.00 ± 0.15† |

Two-way ANOVA reveals a significant age-by-resveratrol interaction in the body weight ($P < 0.05$), a main effect of age in the heart mass ($P < 0.001$), and a main effect of resveratrol in the heart mass/body weight mass ($P < 0.05$). Subjects were categorized according to age and divided into control and resveratrol-treated groups: young control (YC), young resveratrol (YR), old control (OC), and old resveratrol (OR) groups.
All data are presented as the mean ± standard error of the mean.
*$P < 0.05$ vs. YC; †$P < 0.05$ vs. OC.


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Histological analysis showed dramatic remodeling of cardiac muscle during aging (**Fig. 1A**). In the 2-way ANOVA, significant age-by-resveratrol interactions were observed in the percentage of extramyocyte space (F = 4.87, $P < 0.05$) and myocyte cross-sectional area (CSA) (F = 5.47, $P < 0.05$). Additionally, a significant main effect of age was observed in the number of myocytes (F = 45.66, $P < 0.001$). The percentage of extramyocyte space and the mean myocyte CSA in the LV were significantly increased in the OC group compared to the YC and YR groups (all $P < 0.05$; **Fig. 1B and D**). Additionally, a significant reduction in the number of myocytes per 100,000 μm² area in the LV was observed in the OC group compared to the YC and YR groups ($P < 0.05$; **Fig. 1C**). Resveratrol administration prevented aging-induced changes in the myocyte CSA in the LV compared to the OC group ($P < 0.05$; **Fig. 1D**). There were no significant differences in the percentage of extramyocyte space ($P = 0.059$; **Fig. 1B**) and the number of myocytes per 100,000 μm² area (**Fig. 1C**) between the OC and OR groups. Together, these results suggest that resveratrol treatment effectively attenuates aging-associated cardiac hypertrophy and remodeling in aged rats.

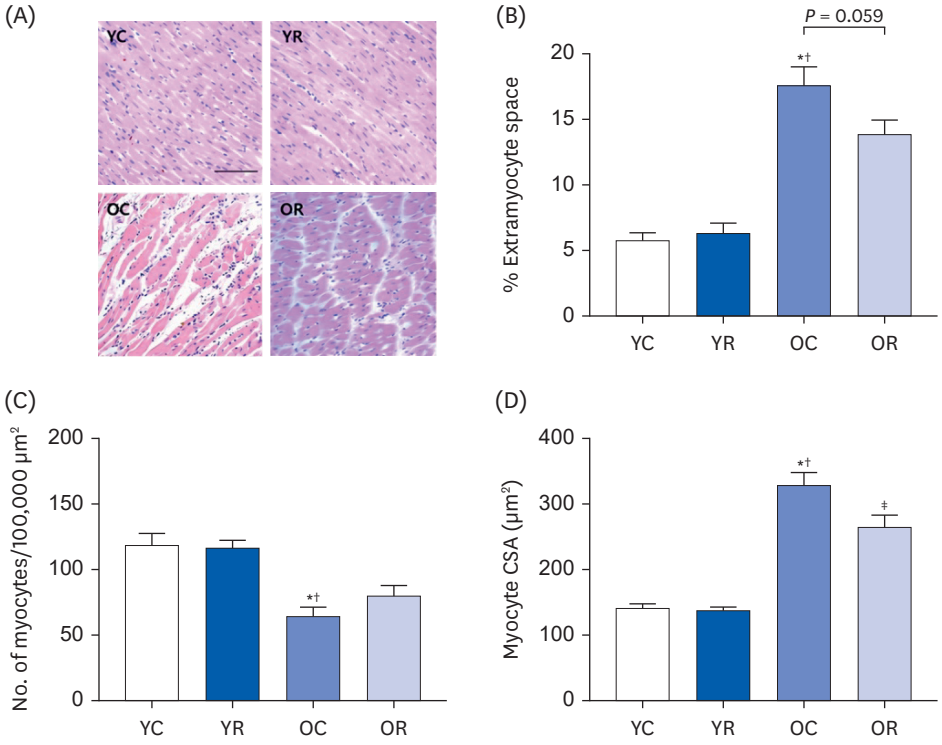


**Fig. 1.** Effects of aging and resveratrol on morphology. (A) Representative histological cross-sections of the LV tissues of rats from the YC, YR, OC, and OR groups stained with hematoxylin and eosin (magnification: 40× and scale bar: 100 μm). Unstained areas indicate the extramyocyte space. (B) Quantification of the percentage of extramyocyte space. (C) The number of myocytes per 100,000 μm² in the LV tissues. (D) The myocyte CSA of LV tissue histological sections, in square micrometers. The scale bar indicates 100 m. In the 2-way ANOVA, significant age-by-resveratrol interactions were observed in the percentage of extramyocyte space ($P < 0.05$) and myocyte CSA ($P < 0.05$). Additionally, a significant main effect of age was observed in the number of myocytes ($P < 0.001$).
Subjects were categorized according to age and divided into control and resveratrol-treated groups: young control (YC), young resveratrol (YR), old control (OC), and old resveratrol (OR) groups.
Data are presented as the mean ± standard error of the mean.
LV, left ventricle; CSA, cross-sectional area; ANOVA, analysis of variance.
*$P < 0.05$ vs. YC; †$P < 0.05$ vs. YR; and ‡$P < 0.05$ vs. OC.


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## Resveratrol ameliorated aging-induced mitochondrial dysfunction in permeabilized cardiac muscle

Mitochondrial $O_2$ respiration was measured based on basal, glutamate-malate (GM)-supported respiration (electron inflow through complex I, state 2 conditions), ADP (state 3 conditions), and SUCC (complex II, state 3 conditions) in permeabilized cardiac fibers. In mitochondrial $O_2$ respiration, 2-way ANOVA reveals significant age-by-resveratrol interactions at ADP stage ($F = 7.40$, $P < 0.05$) and SUCC stage ($F = 6.20$, $P < 0.05$). Additionally, a significant main effect of aging was observed at GM stage ($F = 8.57$, $P < 0.05$). We found that aging resulted in decreased mitochondrial $O_2$ respiration at all stages, including those of GM, ADP, and SUCC (**Fig. 2A**). The administration of resveratrol to aged rats ameliorated the age-induced reductions in mitochondrial $O_2$ respiration, especially in the stages (state 3 conditions) of ADP and SUCC (**Fig. 2A**).

Mitochondrial $H_2O_2$ emission was measured during the following sequences 3 steps: basal, GM (substrates of complex I, GM) stage, SUCC (substrate of complex II, GM+S) stage, and G3P (lipid substrate, GMS+G3P) stage. As shown in **Fig. 2B**, there were no significant changes in mitochondrial $H_2O_2$ emissions among the groups with respect to complex I substrates. However, 2-way ANOVA reveals significant main effects of age and resveratrol at GM+S stage ($F = 41.64$, $P < 0.05$; $F = 9.94$, $P < 0.05$, respectively) and GMS+G3P stage ($F = 28.73$, $P < 0.05$; $F = 4.39$, $P < 0.05$, respectively). Mitochondrial $H_2O_2$ emission was dramatically elevated in the OC group compared with that in the YC group at the GM+S and GMS+G3P stages (both $P < 0.05$; **Fig. 2B**), indicating aging-induced excessive emission of mitochondrial oxidative stress. Furthermore, resveratrol administration to aged rats markedly attenuated mitochondrial $H_2O_2$ emission in the permeabilized cardiac muscle at the GM+S and GMS+G3P stages (both $P < 0.05$; **Fig. 2B**). These results suggest a protective effect of resveratrol against excessive oxidative stress in the cardiac muscles of aged rats.

In mitochondrial $Ca^{2+}$ retention capacity, 2-way ANOVA reveals significant main effects of age and resveratrol ($F = 5.95$, $P < 0.05$; $F = 12.45$, $P < 0.05$, respectively). The mitochondrial $Ca^{2+}$

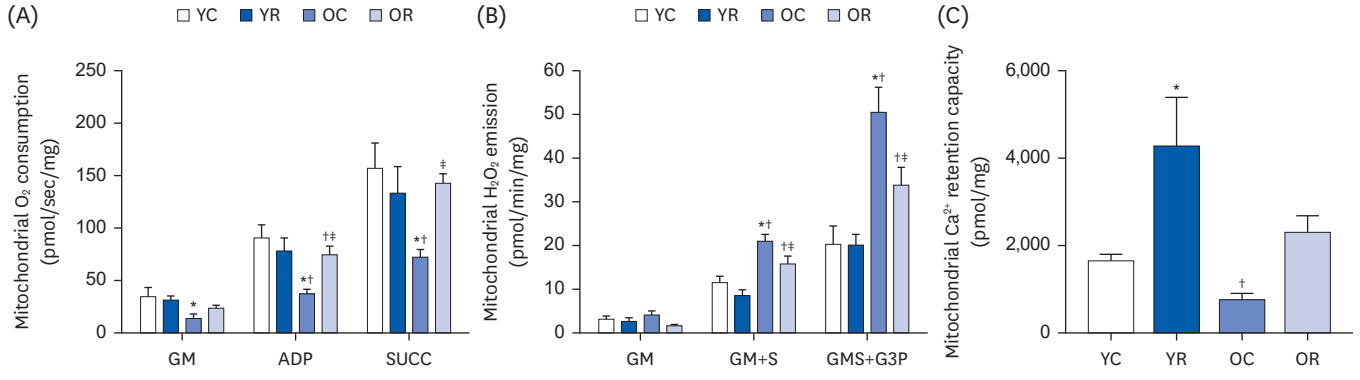


**Fig. 2.** Effects of aging and resveratrol on mitochondrial function in cardiac muscle. (A) In mitochondrial $O_2$ respiration, 2-way ANOVA reveals significant age-by-resveratrol interactions at ADP stage ($P < 0.05$) and SUCC stage ($P < 0.05$), and a significant main effect of aging was observed at GM stage ($P < 0.05$). (B) In mitochondrial $H_2O_2$ emission, two-way ANOVA reveals significant main effects of age and resveratrol at GM+S stage (all $P < 0.05$) and GMS+G3P stage (all $P < 0.05$). (C) In mitochondrial $Ca^{2+}$ retention capacity, 2-way ANOVA reveals significant main effects of age and resveratrol (all $P < 0.05$).
Subjects were categorized according to age and divided into control and resveratrol-treated groups: young control (YC), young resveratrol (YR) treatment, old control (OC), and old resveratrol (OR) treatment groups. Mitochondrial $H_2O_2$ emission was measured during the following sequences 3 steps: basal, GM (substrates of complex I, GM) stage, SUCC (substrate of complex II, GM+S) stage, and G3P (lipid substrate, GMS+G3P) stage.
Data are presented as the mean ± standard error of the mean.
$O_2$, oxygen; ANOVA, analysis of variance; ADP, adenosine diphosphate; SUCC, succinate; GM, glutamate-malate; G3P, glycerol-3 phosphate; $Ca^{2+}$, calcium; $H_2O_2$, hydrogen peroxide.
$^{*}P < 0.05$ vs. YC; $^{\dagger}P < 0.05$ vs. YR; and $^{\ddagger}P < 0.05$ vs. OC.


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retention capacity was significantly lower in the OC group than in the YC group ($P < 0.05$; **Fig. 2C**). Resveratrol administration significantly increased mitochondrial $Ca^{2+}$ retention capacity in the YE group compared with YC group ($P < 0.05$; **Fig. 2C**).

## Resveratrol ameliorated aging-induced mitochondria-mediated apoptotic signaling and apoptosis in permeabilized cardiac muscle

We evaluated the protein expression of key regulators involved in mitochondria-driven apoptotic signaling and apoptosis, which members of the Bcl-2 family mainly regulate. Two-way ANOVA reveals significant main effects of age and resveratrol in the protein levels of Bax (F = 7.15, $P < 0.05$; F = 4.85, $P < 0.05$, respectively), Bcl-2 (F = 34.72, $P < 0.05$; F = 7.54, $P < 0.05$, respectively), and cleaved caspase-3 (F = 25.03, $P < 0.05$; F = 6.64, $P < 0.05$, respectively), and Bax/Bcl-2 ratio (F = 49.83, $P < 0.05$; F = 17.58, $P < 0.05$, respectively). As shown in **Fig. 3A and B**, there was an increase in Bax protein levels and a decrease in Bcl-2 expression in OC compared with YC (both $P < 0.05$). The Bax/Bcl-2 ratio, which is crucial in regulating mitochondria-mediated apoptosis, was markedly increased in OC compared with

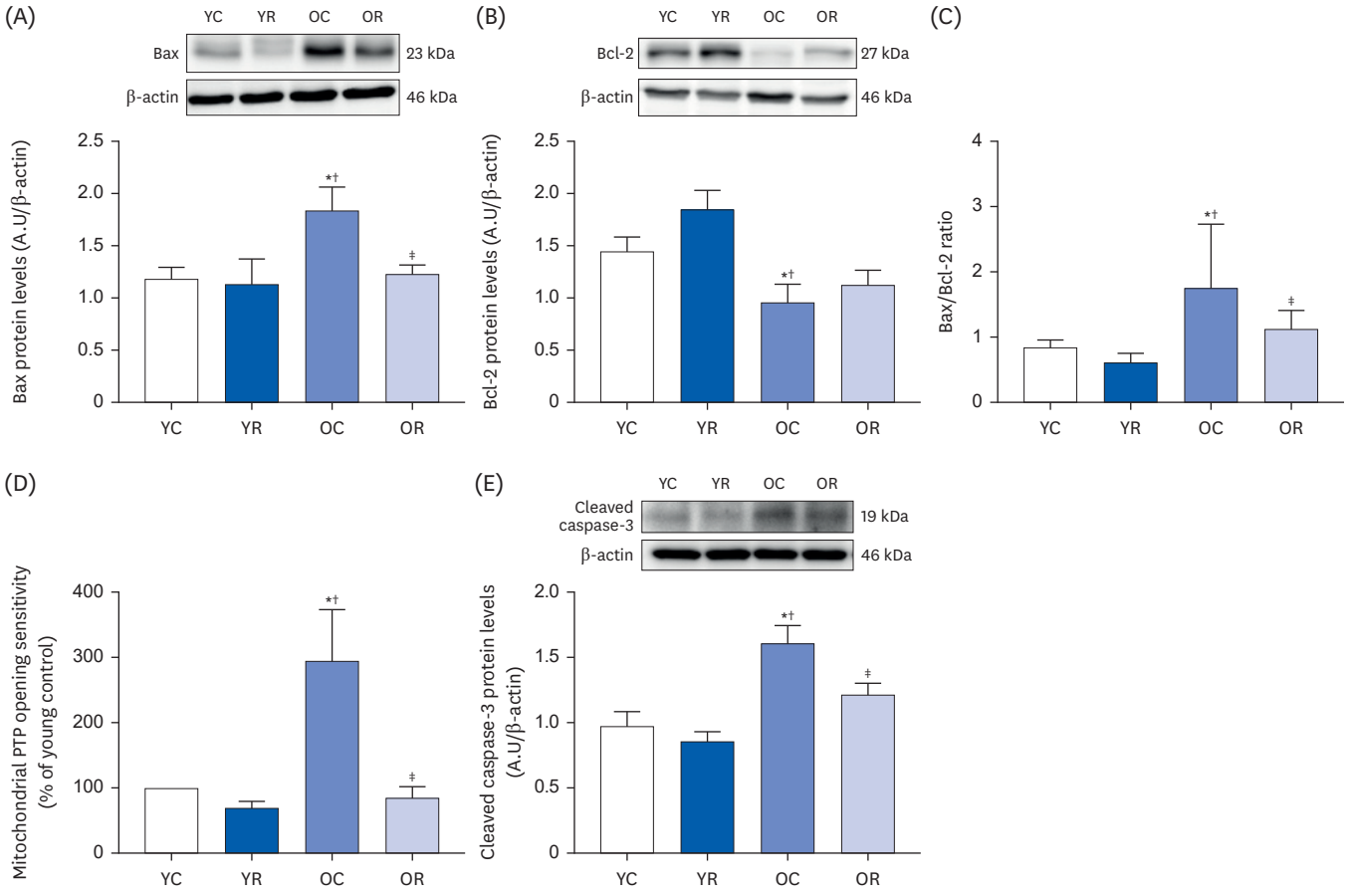


**Fig. 3.** Effects of aging and resveratrol treatment on mitochondria-mediated apoptotic signaling in cardiac muscle. Immunoblot bands of protein levels of (A) Bax, (B) Bcl-2, and (E) cleaved caspase-3. (C) Bax to Bcl-2 ratio. (D) mPTP opening. Two-way ANOVA reveals significant main effects of age and resveratrol in the protein levels of Bax, Bcl-2, and cleaved caspase-3, and Bax/Bcl-2 ratio (all $P < 0.05$). Additionally, a significant age-by-resveratrol interaction was observed in mPTP opening ($P < 0.05$). Quantification of relative western blot bands after normalization with β-actin.
Subjects were categorized according to age and divided into control and resveratrol-treated groups: young control (YC), young resveratrol (YR), old control (OC), and old resveratrol (OR) groups.
Data are presented as the mean ± standard error of the mean.
mPTP, mitochondrial permeability transition pore; ANOVA, analysis of variance.
*$P < 0.05$ vs. YC; †$P < 0.05$ vs. YR; ‡$P < 0.05$ vs. OC.


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YC ($P < 0.05$; **Fig. 3C**). Interestingly, resveratrol significantly attenuated Bax protein levels and the Bax/Bcl-2 ratio in OR compared with those in OC (both $P < 0.05$; **Fig. 3A and C**). In the mPTP opening sensitivity, 2-way ANOVA reveals a significant age-by-resveratrol interaction ($F = 4.65$, $P < 0.05$). The mPTP opening sensitivity was notably increased in OC compared with that in YC ($P < 0.05$; **Fig. 3D**), whereas OR attenuated the level of mPTP opening sensitivity compared with that of OC ($P < 0.05$; **Fig. 3D**). Similar results were found for cleaved caspase-3 protein levels in OC compared with YC ($P < 0.05$; **Fig. 3E**). The administration of resveratrol to aged rats significantly reduced the level of cleaved caspase-3 ($P < 0.05$; **Fig. 3E**). Together, these results suggest that resveratrol administration ameliorate aging-induced mitochondria-mediated apoptotic signaling.

Two-way ANOVA reveals significant age-by-resveratrol interactions in the cleaved caspase-3-positive cells ($F = 60.73$, $P < 0.001$) and TUNEL-positive myonuclei ($F = 11.47$, $P < 0.01$). The percentage of cleaved caspase-3-positive cells in the immunohistochemistry analysis was significantly increased in OC compared with that in YC ($P < 0.05$; **Fig. 4A and C**). Additionally, TUNEL-positive myonuclei were markedly increased in the OC cardiac muscle

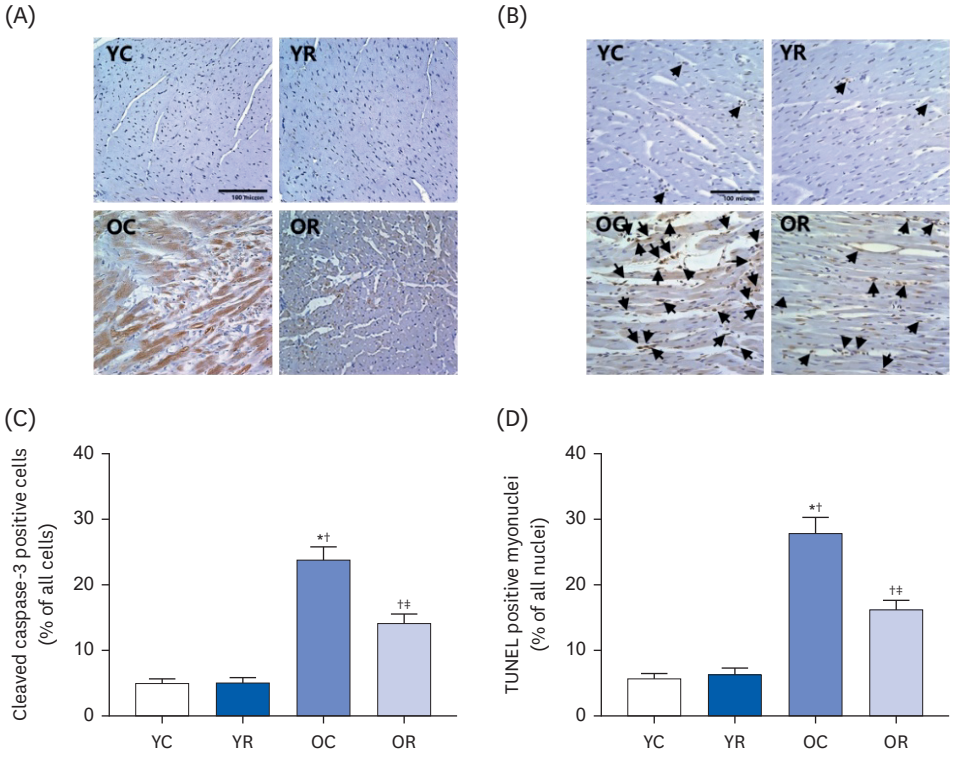


**Fig. 4.** Immunohistochemical assessment of apoptosis (cleaved caspase-3-positive cells and TUNEL-positive myonuclei) in the LV sections obtained from YC, YR, OC, and OR groups. Two-way ANOVA reveals significant age-by-resveratrol interactions in the cleaved caspase-3-positive cells ($P < 0.001$) and TUNEL-positive myonuclei ($P < 0.01$). (A) Representative photographs of LV sections immunohistochemically stained with anti-cleaved caspase-3 antibody (scale bar, 100 μm). (B) TUNEL staining photographs, in which brown-staining cells were TUNEL-positive myonuclei (magnification, 40×). Moreover, (C) quantification of cleaved caspase-3-positive cells, as assessed by immunohistochemical staining. (D) Quantification of TUNEL-positive myonuclei.
Subjects were categorized according to age and divided into control and resveratrol-treated groups: young control (YC), young resveratrol (YR), old control (OC), and old resveratrol (OR) groups.
Data are presented as the mean ± standard error of the mean.
TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; LV, left ventricle; ANOVA, analysis of variance.
$^{*}P < 0.05$ vs. YC; $^{\dagger}P < 0.05$ vs. YR; $^{\ddagger}P < 0.05$ vs. OC.


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sample compared the YC sample ($P < 0.05$; **Fig. 4B and D**). In contrast, resveratrol treatment in aged rats attenuated the aging-induced increase in the percentage of cleaved caspase-3-positive cells and TUNEL-positive myonuclei compared with that in OC (both $P < 0.05$; **Fig. 4**), suggesting that resveratrol protected against aging-induced apoptosis in cardiac muscle.

## DISCUSSION

The major finding of this study is that cardiac remodeling in aged rats was associated with impaired mitochondrial function and apoptotic cell death, which were rescued by treatment with resveratrol. More specifically, resveratrol effectively protected against age-induced cardiac hypertrophy and remodeling (e.g., increased extramyocyte space and myocyte CSA). In addition, resveratrol ameliorated age-induced mitochondrial dysfunction (e.g., decreased $O_2$ respiration and increased $H_2O_2$ emission) and mitochondria-dependent apoptotic signaling (e.g., increased Bax/Bcl-2 ratio, mPTP opening sensitivity, and cleaved caspase-3 protein levels). Finally, resveratrol protected against age-induced apoptosis (e.g., increased cleaved caspase-3-positive cells and TUNEL-positive myonuclei) in the cardiac muscle. To our knowledge, this study is the first to indicate that resveratrol treatment ameliorates mitochondrial dysfunction and mitochondria-mediated apoptotic pathways to suppress cardiac remodeling in the aging heart.

The exponential increase in total death due to cardiovascular disease in the elderly population indicates that intrinsic cardiac aging is also a major risk factor for cardiovascular disease [21-23]. Emerging evidence suggests that age-associated changes in cardiac structure and function in rodent models closely recapitulate older human hearts, including cardiac hypertrophy, increased fibrosis, reduced functional reserve, and adaptive capacity to stress [24-26]. The present findings of dramatic cardiac remodeling and hypertrophy, such as a loss in cardiomyocytes and increased extramyocyte space in aged rats compared with young rats, support our previous findings [10,11], which also indicated cardiac aging phenotypes in humans.

Several studies have reported that resveratrol reverses cardiomyopathic changes in the remodeling associated with cardiac diseases such as cardiac hypertrophy [27] and heart failure [28]. Most of these protective effects are usually attributed to the antioxidant effects of resveratrol [29] and ROS scavenging [17], which can protect against apoptotic cell death [30]. However, little information is available regarding the effects of resveratrol on cardiac aging, which is considered a major determinant of the development of cardiac diseases. Notably, we found that resveratrol treatment repressed the age-induced changes in extramyocyte space and myocyte CSA, suggesting that resveratrol exerts beneficial effects on age-induced cardiac hypertrophy and remodeling. Moreover, in accordance with previous findings [29], we observed that resveratrol treatment of young rats with healthy hearts had no effect on cardiac hypertrophy and remodeling, indicating that it would seem difficult to expect the cardioprotective effects of resveratrol on healthy cardiomyocytes. Altogether, these findings provide new evidence regarding the protective effects of resveratrol treatment on aging-induced cardiac remodeling.

Mitochondria have emerged as central regulators of the aging heart [9,13,31]. Age-dependent impairment of mitochondrial function is mainly associated with diminished ADP-stimulated maximal mitochondrial respiration and subsequent mitochondrial ROS production, leading to mitochondrial oxidative damage [24]. In the present study, we determined the effects of


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resveratrol on aging-induced mitochondrial dysfunction. We found that resveratrol alleviated decreased mitochondrial $O_2$ respiration and excessive mitochondrial $H_2O_2$ emission and decreased mitochondrial $Ca^{2+}$ retention capacity, even though resveratrol did not significantly affect mitochondrial $Ca^{2+}$ retention capacity in aged cardiac muscle. Using permeabilized cardiac muscle fibers, we showed that basal respiration (GM) and maximal respiration with ADP- and SUCC-stimulated state 3 conditions were decreased with advancing age. Resveratrol treatment greatly rescued age-associated decreases in mitochondrial ADP- and SUCC-stimulated maximal $O_2$ respiration in aged rats, but not in young rats.

Mitochondrial ROS production and mitochondrial respiratory dysfunction are important factors in cardiac aging [24]. Thus, we sought to determine the effect of resveratrol on mitochondrial $H_2O_2$ emission, which depends on the balance between the rate of $H_2O_2$ generation and that of its scavenging in aged cardiac muscle. It is well known that mitochondria contribute predominantly to ROS production in cells, including the generation of $O_2^{\cdot-}$, $H_2O_2$, and $OH^-$ [32]. Excessive ROS levels overwhelm both antioxidant defense mechanisms and the ROS scavenger system. Among the antioxidant enzymes, manganese superoxide dismutase (Mn-SOD) plays a pivotal role in $O_2^-$ detoxification. For example, mitochondrial Mn-SOD mutant mice showed enlarged hearts with endocardial fibrosis [33]. In this respect, the present finding regarding the prevention of the age-associated increases in mitochondrial $H_2O_2$ emission following resveratrol treatment may explain its antioxidant effect against age-related ROS production. Indeed, Leonard *et al.* [17] showed that resveratrol is an effective scavenger of ROS, including $O_2^-$ and $OH^-$. Furthermore, resveratrol treatment induced the elevation of cardiac Mn-SOD in animals with chronic heart failure [29], suggesting that resveratrol may play an important role in cardiomyopathy protection via increased Mn-SOD levels. Alterations in mitochondrial $Ca^{2+}$ retention capacity are associated with mitochondrial dysfunction and mPTP opening, triggering the mitochondrial apoptotic pathway [34]. The increased opening sensitivity of the mPTP causes mitochondrial cytochrome *c* release in the cytosol, ultimately leading to apoptosis [35,36]. Increased apoptosis of cardiac muscle with aging reduces the number of myocytes and contractile units, closely related to decreased contractility, cardiac remodeling, and reactive hypertrophy [12]. Indeed, we observed increased mitochondria-mediated apoptosis and altered cardiac morphology in aged rats. As expected, the observed activation of mitochondria-dependent apoptotic signaling and the concomitant increase in apoptosis in aged hearts are consistent with our earlier findings of an increased Bax/Bcl-2 ratio, mPTP opening sensitivity, and cleaved caspase-3, leading to DNA fragmentation (i.e., apoptosis) [10-12]. Moreover, resveratrol administration markedly downregulated the pro-apoptotic protein Bax, the Bax/Bcl-2 ratio, mPTP opening sensitivity, and apoptosis (TUNEL-positive myonuclei) in the aging heart. Taken together, these results indicate that resveratrol treatment in aging hearts attenuates apoptosis by regulating the mitochondrial apoptotic signaling pathway, suggesting that resveratrol may play a crucial role as a defensive anti-apoptotic mechanism in the aging heart.

The present study has some limitations. During the experimental period, all animals had unrestricted access to food and water, although we did not monitor daily food intake or activity levels. In addition to its cardioprotective effects, resveratrol has been reported to exhibit weight-lowing effects [37]. Our results also showed that resveratrol decreased body weight in the aged rat. Further study is needed to clarify the mechanisms underlying the weight-lowing effects of resveratrol during the aging process.


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In summary, six weeks of resveratrol treatment protected against aging-induced cardiac remodeling, including a decreased extramyocyte space and increased myocyte CSA in the heart, and attenuated the mitochondrial impairment in the aging rat heart. Resveratrol treatment protected against decreased mitochondrial $O_2$ consumption, increased mitochondrial $H_2O_2$ emission in the aging heart. Moreover, resveratrol treatment in the old group led to a decrease in the Bax protein levels, the Bax/Bcl-2 ratio, the mPTP opening sensitivity, the cleaved caspase-3 protein levels, and the number of TUNEL-positive myonuclei. These data demonstrate that resveratrol treatment prevents mitochondrial dysfunction and apoptotic signaling induced by aging in the rat heart and supports a therapeutic role for resveratrol in managing cardiac aging.

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