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A SIRT3/AMPK/autophagy network orchestrates the protective effects of *trans*-resveratrol in stressed peritoneal macrophages and RAW 264.7 macrophages

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Abbreviations: AAPH, 2,2'-azobis (2-amidinopropane) dihydrochloride; AMPK, AMP-activated protein kinase; ComC, compound C; Edar, edaravone, 3-MA, 3-methyladenine; MMP, mitochondrial membrane potential; Rapa, rapamycin; Resv, *trans*-resveratrol; VitC, vitamin C.

Abstract:

Resveratrol gains a great interest for its strong antioxidant properties, while the molecular mechanisms underlie the beneficial effects on psychosocial stress remain controversial. In this study, we demonstrated that resveratrol protected peritoneal macrophages and RAW 264.7 cells from stress-induced decrease in the total cell count, phagocytic capability, reactive oxygen species generation, monodansylcadaverine and mitochondrial membrane potential in stressed mice. Resveratrol promoted stress-induced autophagy in both models. Modulation of autophagy by rapamycin or 3-methyladenine regulated the protective effect of resveratrol, suggesting a role of autophagy in the protective mechanisms of resveratrol. The comparison studies revealed that distinct mechanisms were implicated in the protective effect of resveratrol and other antioxidants (vitamin C and edaravone). Resveratrol promoted autophagy via upregulating SIRT3 expression and phosphorylation of AMP-activated protein kinase (AMPK). Knockdown of *SIRT3* resulted in decreased autophagy and abolished protective effect of resveratrol. SIRT1 was also involved in the protective mechanism of resveratrol, although its effect on autophagy was unnoticeable. Pharmacological manipulation of autophagy modulated the effects of resveratrol on SIRT3 and AMPK, revealing the engagement of a positive feedback loop. In sharp contrast, vitamin C and edaravone effectively protected macrophages from stress-induced cytotoxicity, accompanied by downregulated SIRT3 expression and AMPK phosphorylation, and decreased level of autophagy response. Taken together, we conclude that a SIRT3/AMPK/autophagy network orchestrates in the protective effect of resveratrol in macrophages.

Keywords:

autophagy; macrophage; mitochondria; resveratrol; oxidative stress; SIRT3

Introduction

Chronic psychosocial stress (e.g., unemployment, low socioeconomic status, or work stress) is known to provoke immunosuppression and diverse associated pathologies, including diabetes, cardiovascular disease and *etc*. [1, 2]. Numerous previous studies have provided a link between stress and increased immune cell apoptosis [3]. Of all the immune cells, macrophages are the most important as they play an essential role in innate immunity and serve as key effectors of the ensuing adaptive response. Thus, interventions protect immune cells, particularly macrophages, from stress-induced impairment may provide a reference for the design of therapeutic approaches to avoid the appearance of stress disorders [4]. It has been well documented that oxidative stress is implicated in the pathogenesis of a variety of diseases [5-8] with reactive oxygen species (ROS) as an important effectors of damage [9, 10]. Oxidative stress occurs when the production of ROS exceeds the antioxidant defense mechanisms present in the body, resulting in impairment of physiological function, cell death and immunocompromise [5]. Immune cells are particularly sensitive to oxidative stress because (i) their membranes contain high concentrations of polyunsaturated fatty acids that are very susceptible to peroxidation, and (ii) they produce large amounts of ROS when stimulated [11, 12]. Therefore, the antioxidant therapy could be a viable therapeutic option for patients with chronic psychosocial stress exposing.

Resveratrol (3,5,4'-trihydroxy-*trans*-stilbene, Resv), a dietary polyphenolic phytoalexin found naturally in a variety of food and medicinal plants[13], has recently gained a great interest for its strong antioxidant properties [14, 15]. The anti-apoptotic effect of Resv has been recently demonstrated in testis under chronic immobilized stress [16]. The exact mechanisms by which Resv exerts its protective effect, however, remain under debate. The notion that the effects of Resv to be ascribed solely to its antioxidant activity has been challenged by reports showing that Resv could potently induce ROS production under certain

experimental conditions [17-19]. The implication of Resv in the regulation of autophagy indicated a more complex mechanism of its effects. Morselli *et al.* [20] have shown that autophagy mediated the pharmacological lifespan extension by Resv in nematodes, and Resv ameliorated the fitness of human cells undergoing metabolic stress by autophagy induction. Furthermore, Miki *et al.* [21] reported that Resv induced apoptosis via ROS-triggered autophagy in human colon cancer cells, while Lin *et al*. [22] indicated Resv enhanced temozolomide-induced apoptotic cell death in malignant glioma by inhibiting autophagy. Finally, despite the extensive *in vivo* and *in vitro* studies of Resv-induced autophagy [23-25], the understanding and knowledge of the mechanisms of its actions are still limited, particularly in the immune system.

In this study, we employed the physical restraint of mouse, a widely used experimental model for psychosocial stress [26, 27], to investigate the effect of Resv on stress-induced impairment of peritoneal macrophages. To further explore the detailed molecular mechanisms of the pharmacological actions of Resv, we utilized murine macrophage RAW 264.7 cells challenged with 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH, a water-soluble azo compound that can generate free radicals in cells as a comparable *in vitro* model.

Materials and Methods

Reagents

Resv, identified as pure *trans*-resveratrol, was generously supplied by Tianjin Jianfeng Natural Product R&D (Tianjin, China). 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) and Sodium fluorescein were purchased from Wako Pure Chemical Industries (Osaka, Japan). 4’,6-diamidino-2-phenylindole (DAPI), 2’,7’-dichlorofluorescein diacetate (DCFH-DA), edaravone (Edar), JC-1, Hoechst 33258, 3-methyladenine (3-MA),

monodansylcadaverine (MDC), Mito Red, 3-(4,5-dimetrylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), rapamycin (Rapa), tetramethylrhodamine methyl ester (TMRM) and vitamin C (VitC) were purchased from Sigma Chemical (St. Louis, MO, USA). Lipid peroxidation MDA assay kit was purchased from Beyotime Institute of Biotechnology (Haimen, Jiangsu, China). Compound C (ComC) was purchased from Abcam Public Company (Cambridge, UK). EX 527 was purchased from Selleck Chemicals (Houston, TX, USA). Sodium fluorescein was purchased from Wako Pure Chemical Industries (Osaka, Japan). FluoSpheres® carboxylate-modified fluorescent microspheres and Alexa Fluor® 488 goat anti-rabbit IgG were purchased from Invitrogen (Carlsbad, CA, USA). Polyclonal antibodies against Beclin1, LC3β, SIRT3, AMPK, p-AMPK, β-Actin and horseradish peroxidase-conjugated secondary antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA).

Animals

Male Kun-Ming (KM) mice (18-22 g) were purchased from Guangdong Medical Laboratory Animal Center (Guangzhou, China), with Permission No. SCXK 2008-0002. All mice were housed in a room at a mean constant temperature (23 ± 2°C) with a 12-h light-dark cycle, 50-60% relative humidity and free access to standard pellet chow and water. Mice were maintained in these facilities for at least 1 week before experiment. All animal care and experimental procedures were approved by the Animal Care and Use Committee of Sun Yat-sen University (Approval ID: SYXK 2007-0081), and were in accordance with the National Institute of Health's Guide for the Care and Use of Laboratory Animals.

Treatment to restrained mice

Mice were divided into groups, each consisting 6 mice. The groups were Cont (control),

restraint, three Resv groups (i.g. 7.5, 15 or 30 mg/kg Resv-L/M/H + restraint), Rapa (i.p. 1 mg/kg, Rapa + restraint), VitC (i.g. 30 mg/kg, VitC + restraint), Rapa + Resv (i.p. 1 mg/kg, Rapa + i.g. 15 mg/kg, Resv + restraint) and 3-MA + Resv (i.p. 30 mg/kg, 3-MA + i.g. 15 mg/kg, Resv + restraint). Mice were administered with samples mentioned above or 0.9% saline for 7 days and then fixed in restraint cages for 18 h, followed by a 12-h recovery stage before determinations of biochemical parameters.

Isolation of mouse peritoneal macrophages

Mice were sacrificed using diethyl ether anesthesia, cold PBS (7 mL) was injected intraperitoneally and peritoneal fluid was collected. Macrophages were obtained by centrifugation at 800 × *g* for 5 min.

Cell culture

The RAW 264.7 murine macrophage cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in DMEM medium (Gibco, Grand Island, NY) supplemented with 10% heat inactivated fetal bovine serum (Tianjin Haoyang Biological Manufacture, Tianjin, China), 2 mM $_{L}$-glutamine (Gibco), 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco) at 37°C with 5% CO_2. The cultures in exponential phase were used in the experiments.

MTT assay

The growth inhibitory effect of reagents on cells was measured by MTT assay. Cells were dispensed in 96-well plate at a density of 1×10^5 cells per well. After 24-h incubation, cells were treated with the tested agents for the indicated periods of time. A 20-μl aliquot of 0.5% MTT solution was added to each well followed by 4-h incubation. Optical density was

measured using an ELISA reader (Thermo Fisher Scientific, Franklin, MA, USA). The percentage of cell growth inhibition was calculated as follows:

Inhibitory ratio (%) = $(A_{570,\ control} - A_{570,\ sample}) / (A_{570,\ control} - A_{570,\ blank}) \times 100$

Flow Cytometry

Determination of phagocytic capability. Fluorescence labeled latex beads (2 μm) were opsonized by incubating with 1% BSA at 37°C for 40 min and subsequently ultrasounded for 10 min. Afterwards, beads were added to cells (1:15) for 1-h incubation at 37°C. At the end of incubation, cells were harvested for analysis by Beckman Coulter Epics XL flow cytometer equipped with Expo32 ADC (Brea, CA, USA).

TMRM assay for mitochondrial membrane potential (MMP). MMP ($\Delta\psi_m$) was measured with TMRM. After treatment, cells were incubated with 100 nM TMRM at 37°C for 30 min. Harvested cells were immediately analyzed for potential using Beckman Coulter Epics XL flow cytometer equipped with Expo32 ADC.

***Determination of ROS*.** Cells were incubated with 20 μM DCFH-DA at 37°C for 30 min. The intracellular ROS mediated oxidation of DCFH-DA to the fluorescent compound DCF. Harvested cells were immediately analyzed for potential using Beckman Coulter Epics XL flow cytometer equipped with Expo32 ADC.

MDC assay for autophagic vacuoles. To confirm that autophagy occurs, MDC, a fluorescent dye that is selectively incorporated into autophagosomes and autolysosomes was used [28, 29]. For quantification of MDC, cells were incubated with 10 μM MDC at 37°C for 1 h, intracellular MDC fluorescence was determined using Beckman Coulter Epics XL flow cytometer equipped with Expo32 ADC within 30 min after incubation.

Fluorescence imaging

Assessment of MMP using fluorescent microscopy. Following various treatments, cells were stained with 20 μM JC-1 at 37°C for 30 min. The images were recorded on a Leica CTR MIC fluorescent microscope (Leica Camera AG, Solms, Germany).

Assessment of autophagy. Peritoneal macrophages (or RAW 264.7) cells were stained with MitoRed at 37°C for 30 min, and then fixed in 4% paraformaldehyde. Samples were labeled with anti-LC3β at 4°C overnight and incubated with secondary antibody-Alexa flour 488® at 37°C for 4 h. Samples were counterstained with DAPI before being imaged using a Leica CTR MIC fluorescence microscope or Zeiss LSM510 Meta DuoScan laser scanning confocal microscope (Carl Zeiss AG, Oberkochen, Germany) as indicated.

Western blotting analysis

Cells were resuspended in lysis buffer (Beyotime Institute of Biotechnology) on ice for 5 min, and the supernatants were collected after centrifugation at 13,000 × *g* for 15 min. Protein lysates (30 μg) were separated in 10% or 15% SDS-PAGE and blotted onto nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ, USA). Proteins expression were detected using polyclonal antibody and visualized using anti-rabbit and anti-mouse IgG conjugated with horseradish peroxidase (HRP) and Pierce® ECL Western blotting Substrate (Thermo Fisher Scientific) as the substrate of HRP.

Transmission electron microscopy (TEM) for observation of autophagic ultrastructure

After incubation with AAPH for 24 h, RAW 264.7 cells were fixed, embedded, sliced and processed for imaging on a Philips Tecnai 10 transmission electron microscope (FEI, Hillsboro, OR, USA).

Knockdown of *SIRT3/SIRT1* using siRNA procedure

Small interfering siRNA targeting to *SIRT3/SIRT1* or non-targeting negative control

siRNA were purchased from Ribobio (Guangzhou, China). RAW 264.7 cells at 50% confluency were transfected for 48 h with siRNA of *SIRT3/SIRT1* or control siRNA using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. Transfection efficiency was optimized by trying a range of siRNA and Lipofectamine 2000 concentrations.

Statistics

All data were expressed as means ± SEM of at least three independent experiments. The data were analysed by ANOVA using Statistics Package for Social Science (SPSS) software (version 19.0; SPSS, Chicago, IL, USA) and LSD-post-hoc test was employed to assess the statistical significance of difference between control and treated groups. In case $p < 0.05$ was considered statistically significant.

Results

Resv protected mouse peritoneal macrophages from restraint stress-induced impairment

Consistent with previous reports [29], restraint stress in mice led to a significant reduction of total peritoneal macrophages as compared to the control group (Fig. 1A). Mice administrated with Resv showed increased macrophage number in a dose-dependent manner. Furthermore, peritoneal macrophages in restrained mice showed suppressed phagocytic capability for opsonized latex beads. Ingestion of beads rose to significantly higher levels in restrained mice administrated with Resv. Administration of 30 mg/kg Resv completely restored the restraint-related reduction in phagocytic capability of macrophages (Fig. 1B).

Mitochondria, known as "power plants", also function as the complex integrators of cell metabolism and signaling, and play a pivotal role in the life and death decisions [30].

Mitochondrial membrane potential (MMP) has been proposed as an ideal biomarker for environmental stress [31]. Our data demonstrated that restraint stress resulted in a dramatic drop of MMP in peritoneal macrophages, as reflected by the lower level of TMRM fluorescence (Fig. 1C). Alterations of MMP *in situ* was further observed using JC-1, a lipid dye emits differential fluorescence that is depended on different polymerization forms. In control group, mitochondria were healthy with stable MMP, as shown by sustained aggregation of the red JC-1 fluorescence. In restrained mice, the fluorescence changed to a green signal, indicated decreased MMP and damaged mitochondria (Fig. 1D).

Resv enhanced autophagy in peritoneal macrophages in restraint stressed mice

We next investigated whether autophagy was involved in the protective effect of Resv on peritoneal macrophages in restraint stressed mice. The occurrence of autophagy was first evaluated by conversion of LC3-I to autophagosome associated phosphatidylethanolamine (PE)-conjugated LC3-II form [32]. A bright punctate pattern in stressed macrophages indicated the formation of isolation membranes and autophagosomes (Fig. 2A). Treatment of mice with 15 mg/kg Resv enhanced LC3β aggregation as reflected by the increased LC3β punctate. We further confirmed the occurrence of autophagic flux in macrophages using Western blotting. Restraint stress resulted in a slight accumulation of LC3-II in peritoneal macrophages (Fig. 2B). Administration of Resv significantly enhanced LC3-II accumulation with concomitant decrease of LC3-I. Consistent with increased autophagic flux, Resv also induced an elevated expression on Beclin1 (Fig. 2B), one of the first mammalian proteins discovered to mediate autophagy [33]. Rapa, a canonical mTOR (mammalian target of rapamycin) inhibitor, enhanced Resv-induced LC3-I/II conversion and Beclin1 expression (Fig. 2B), which were suppressed by 3-MA, a pan phosphoinositide 3-kinase (PI3K) inhibitor.

Resv prevented AAPH-induced proliferation inhibition and impairment of phagocytic activity in RAW 264.7 macrophages

To better understand the molecular mechanisms of the beneficial effects of Resv, we utilized RAW 264.7 macrophages challenged with AAPH, a known trigger of oxidative stress in cell culture system [34]. MTT assay showed that AAPH inhibited cell proliferation in concentration- and time-dependent manners (Fig. 3A). Nuclear morphological study showed that AAPH induced typical apoptotic DNA fragmentation in cells after 24-h incubation (Fig. 3B). The growth inhibition induced by AAPH were significantly attenuated by Resv (Fig. 3C). AAPH led to increasing generation of ROS and MDA and collapse of MMP, which were all reversed by Resv treatment (Fig. 3D-F). In addition, treatment of RAW 264.7 cells with Resv significantly reversed the reduction of phagocytic capabilities as shown by utilizing BSA-incubated fluorescence latex beads (Fig. 3E and F).

Autophagy partially mediated the beneficial effects of Resv in RAW 264.7 cells and peritoneal macrophages

To test whether autophagy was implicated in the protective effects Resv in RAW 264.7 macrophages challenged with AAPH, three different methods were employed to evaluate autophagy stimulation: (i) formation of acidic vacuole by MDC staining, (ii) TEM, (iii) modulation of autophagy proteins by Western blotting. In agreement with others [35], trigger of oxidative stress in macrophages by AAPH stimulated autophagy as evidenced by the increased MDC positive ratio (Fig. 4A). Presence of Resv alone or in combination with rapamycin increased the ratio of MDC staining in AAPH stressed cells. No surprisingly, autophagy inhibitor 3-MA blocked the increase of MDC staining induced by AAPH and Resv. Using TEM, we showed that control cells exhibited a normal tubular mitochondrial network.

In contrast, mitochondria were rounded, swollen with loss of cristae (yellow arrows) in AAPH-treated cells (Fig. 4B). Autophagosome containing mitochondrion was observed in Resv/AAPH-treated macrophages (red arrow, Fig. 4B). Finally, we demonstrated that AAPH slightly upregulated the expression of Beclin1 and LC3-I/II conversion (Fig. 4C). Consistently, expression of Beclin1 and LC3-I/II conversion were both enhanced in Resv/AAPH-treated cells compared to AAPH-treated cells. The effects of Resv on AAPH-induced Beclin1 expression and LC3-I/II conversion was positively modulated by rapamycin, while was negatively regulated by 3-MA.

Combined, the presented data suggested that upregulated autophagy mediated, at least partially, the protective effects of Resv in AAPH-treated RAW 264.7 macrophages. The protective action of autophagy on macrophages was also demonstrated in restraint stressed mice by administration of Rapa alone (Fig. 1).

Resv enhanced stress-induced autophagy through a SIRT3/AMPK positive feedback pathway

To further understand the molecular mechanisms underlying the protective effects of Resv, a comparison study was performed using two different antioxidants (i.e., VitC and Edar). VitC and Edar protected RAW 264.7 macrophages from AAPH-induced growth inhibition, loss of MMP and decreased phagocytosis activity (Fig. 3C-F). However, VitC and Edar markedly inhibited AAPH-induced formation of acidic vacuole (Fig. 4A), and upregulation of Beclin1 and LC3-I/II conversion (Fig. 4C). Similarly, VitC protected peritoneal macrophages from the detrimental effects (decrease of macrophage count, phagocytic capability and MMP, Fig. 1A, B and C) of restraint stress, accompanied by decreased levels of Beclin1 and LC3-II. These data are consistent with notion that antioxidant can inhibit basal autophagy [36]. Thus, the presented data indicated that two distinct

mechanisms were involved in the protective effects of Resv and antioxidants (VitC and Edar).

We next examined the levels of AMPK and SIRT3, two important metabolic sensors [37, 38], which have been implicated in autophagy induction and the protective effects of Resv. Results of Western blotting analysis showed that AAPH treatment increased expression of SIRT3 and phosphorylation of AMPK, possibly due to the adaptive response to oxidative stress. Treatment of cells with Resv further elevated SIRT3 expression and AMPK activity (Fig. 5A). Knockdown of *SIRT3* using RNAi procedure (Fig. 5B) almost completely inhibited AAPH- or AAPH/Resv-upregulated SIRT3 expression, AMPK phosphorylation, Beclin1 expression, and LC3-I/II conversion (Fig. 5C). Data also revealed accumulation of LC3-II foci and increased level of co-localization of LC3β puncta and mitochondria in cells treated with AAPH, suggesting the initiation of mitochondrial autophagy. The co-localization of mitochondria and LC3-II puncta was promoted by Resv treatment. In contrast, knockdown of *SIRT3* resulted in decrease of LC3-II foci (Fig. 5D). These data indicated that SIRT3/AMPK axis play an important role in the modulation of autophagy in the Resv-treated macrophages. Interestingly, we found that Resv-induced SIRT3 expression and AMPK phosphorylation were enhanced by mTOR inhibitor Rapa, while inhibited by autophagy inhibitor 3-MA, suggesting a cross-talk between autophagy and SIRT3/AMPK pathway in the protective action of Resv.

Inhibition of SIRT1 and AMPK were conducted to evaluated the effects of Resv on activation of AMPK and induction of autophagy

Resv is a well-known activator of SIRT1, thus, siRNA of *SIRT1* (Fig. 6A) was utilized to assess the effects of Resv. Western blotting results showed that the activation of AMPK was not affected by siRNA *SIRT1*, probably due to the compensatory effect of SIRT3. Compared to SIRT3, the effect of SIRT1 on autophagy-related proteins was much less obvious (Fig. 6B).

In addition, inhibitor of SIRT1, EX 527, played a similar role to siRNA SIRT1 (Fig. 6C). At the same time, inhibitor of AMPK, ComC, showed remarkable inhibitive effects on the activation of AMPK and the expressions of autophagy-related proteins (Fig. 6C).

SIRT3 and SIRT1 participated in the effect of Resv on phagocytic capability

Knockdown of both *SIRT3 and SIRT1* significantly blocked the protective effect of Resv on phagocytic capability of RAW 264.7 cells (Fig. 7A and B). In addition, data showed that both inhibitors weakened the effect of Resv on phagocytic capability (Fig. 7C). This effect of Resv was partly blocked by both siRNA of *SIRT3 and SIRT1*, while fully inhibited by ComC, indicating a crucial role of AMPK.

Discussion

Restraint has been demonstrated in our previous studies to be able to induce splenic immunocompromise [5] and energy metabolism disorder [7] through evoking oxidative stress and mitochondrial dysfunction [39]. In agreement with the previous findings, we showed that mitochondrial oxidative damage was involved in restraint stress-induced deleterious effects on peritoneal macrophages via a mechanism of apoptotic cell death. The dysfunctional mitochondria have been indicated as the major source of ROS generation. Ironically, mitochondria are also the primary target of the ROS due to the vicinity to the site of ROS generation and their vulnerability due to lack of protection mechanism [40]. Therefore, protection for mitochondria from oxidative damage using antioxidants or by removing of dysfunctional mitochondria via mitophagy has been considered as ideal strategies for

psychological stress.

Resv, itself, has antioxidant properties to remove mitochondrial ROS [41]. It has also been reported that Resv upregulates cellular mechanisms of oxidative resistance by inducing antioxidant enzymes such as MnSOD [42]. In consistent with many other studies which demonstrated multiple roles of Resv on reducing oxidative stress, we demonstrated that administration of Resv effectively blocked restraint stress-induced drop of MMP. Most importantly, the decline of total count and phagocytic capability of macrophages induced by stress was ameliorated, indicating beneficial effects on macrophage function. As a comparison, an antioxidant agent, VitC, exhibited similar protective effects on peritoneal macrophages in restraint stressed mice.

Although it is well accepted that antioxidant properties of Resv are critical for its protective effects, other mechanisms could not be excluded. Studies from several groups have shown that the beneficial effects of Resv result from the activation of SIRT1, SIRT3, LKB1, and AMPK [43-46]. For instance, Lagouge *et al* [46] have shown that Resv improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1α. Additionally, Resv protected cardiomyocytes from oxidative stress-induced cell death by modulating NF-κB [47]. It is clear that protective effect of Resv is complex and multifaceted. In this study, we employed azo compound AAPH challenged RAW 264.7 macrophage cells as a comparable *in vitro* model [48] to study the protective mechanism of Resv. We showed that Resv was effective in recovering oxidative stress altered mitochondrial dysfunction and apoptotic death in macrophages. These protective effects were tightly associated with enhanced induction of autophagy, which is one of the most important oxidative stress responses to prevent further damage by the removal of already damaged components. Our TEM and confocal fluorescence imaging data clearly indicated that Resv promoted the mitophagy, leading to maintenance of healthy mitochondria network.

We further demonstrated that SIRT3, a mitochondrial nicotinamide adenine dinucleotide $(NAD)^+$-dependent protein deacetylase, was upregulated in peritoneal macrophages in response to restraint stress, indicating an adaptive response. The expression of SIRT3 was significantly higher in stressed mice supplemented with Resv compared to mice without Resv treatment. SIRT3 has been recently highlighted as a regulator of autophagy in its mechanisms of action, in addition to its pivotal role in the regulation of ROS production and detoxification [49]. Mukherjee *et al.* [50] reported that a modified Resv enhanced the expression of autophagy related proteins, LC3-II and Beclin1 in rat. This was accompanied with significantly increase of SIRT1 and SIRT3, indicating that SIRTs correlated with induction of autophagy. Indeed, Resv-induced SIRT3 upregulation in macrophages is accompanied with increased AMPK activation, as well as elevated LC3-II/I ratio and Beclin1 level. SIRT3 deacetylates and activates LKB1, a serine-threonine kinase that directly phosphorylates and activates AMPK [51]. AMPK then promotes autophagy by directly activating Ulk1 through phosphorylation of Ser 317 and Ser 777 [52]. Knockdown of *SIRT3* in the RAW 264.7 macrophages blocked the effects of Resv on AMPK phosphorylation and autophagy related proteins upregulation, supporting the notion that these events are likely dependent on SIRT3.

Interestingly, application of mTOR inhibitor Rapa with Resv not only enhanced autophagy induction, but also increased SIRT3 expression and AMPK activation. Conversely, autophagy inhibitor 3-MA blocked SIRT3 expression and AMPK phosphorylation in Resv-challenged RAW 264.7 macrophages. Rapa has been shown to be able to increase the phosphorylation at Thr172 of AMPK [53]. AMPK leads to increased peroxisome PGC-1α expression directly [54] or by modulating CREB family of transcription factors [55, 56]. PGC-1α is important for mitochondrial function, as well as for the expression of multiple key mitochondrial proteins, including SIRT3 [57]. The mechanism by which 3-MA regulates SIRT3/AMPK axis pathway is less clear. In consistent with our findings, Yun *et al* [58] has

demonstrated that treatment of KBM-5 leukemia cells with 3-MA inhibited tanshinone IIA-induced AMPK phosphorylation.

Recent studies have revealed various antioxidant functions of SIRT3 in mitochondria. SIRT3 controls mitochondrial oxidative courses and ROS generation, and activates enzymes responsible for ROS clearance [37]. On the other hand, many studies demonstrate multiple roles of resveratrol on reducing oxidative stress. For instance, resveratrol preserves activities of superoxide dismutase, glutathione peroxidase and catalase in rodents [42]. *In vitro* systems, it is illustrated that resveratrol scavenges superoxide and peroxynitrite [41], and blocks the oxidation of low-density lipoproteins [59]. However, the research and development of antioxidant is still facing problems and obstacles, such as the difficulty to address specifically to mitochondrial interior, unfavorable side effect due to the quenching of ROS involved in certain metabolic regulations, and the inevitable clearance by cellular enzymes [60]. Autophagic mechanism may contribute to solving some of these issues. Damaged mitochondria can be recognized specifically by selective autophagy, named mitophagy, through illustrated cargo-receptor proteins. A recently identified Atg protein Atg32 in yeast, anchored on mitochondrial outer-membrane, acts as a receptor to bind Atg11 during mitophagy [61]. Besides, Nix that has been defined as a receptor on mitochondria is elucidated to mediate mammalian mitophagy through interacting with LC3β [62].

The multifaceted mechanisms of resveratrol, thus, make it a unique and potential more useful addition to the strategies to prevent/remove mitochondrial ROS. Here we demonstrated that resveratrol rescued macrophages and its function of phagocytic via activating SIRT3/AMPK/autophagy positive feedback loop, which removed and recycled dysfunctional mitochondria and mitigated oxidative stress. This study may provide a new insight for the investigation in antioxidant mechanism as well as in mitophagy signaling pathway.

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Conflicts of interest

The authors declare no competing financial or commercial conflict of interest.

Figure legends

Fig. 1. Effects of Resv on peritoneal macrophages in restraint stressed mice. Mice were administered with indicated chemicals or 0.9% saline (vehicle control) for 7 days and then fixed in restraint cages for 18 h, followed by a 12-h recovery stage before determinations of biochemical parameters. Cold PBS was injected intraperitoneally and peritoneal fluid was collected, acrophages were obtained by centrifugation. (**A**) Number of peritoneal macrophages measured by cell counter. (**B**) Phagocytic capability of peritoneal macrophages evaluated by engulfment of BSA-incubated fluorescence latex beads using flow cytometer. (**C**) Mitochondrial membrane potential (MMP) of peritoneal macrophages determined by TMRM staining using flow cytometry. (**D**) Fluorescence microscopy of peritoneal macrophages stained with JC-1. Cont, control; Resv-L/M/H: 7.5, 15 or 30 mg/kg; Rapa: 1

mg/kg; VitC: 30 mg/kg. Results presented are the means ± SEM (n = 6). $^{**}p < 0.01$ *vs* Cont group, $^{\#}p < 0.05$ and $^{\#}p < 0.01$ *vs* restraint group.

Fig. 2. Resv enhanced stress-induced autophagy in mouse peritoneal macrophages. (A) Fluorescence microscopy in macrophages showing LC3β distribution and MitoRed-labeled mitochondria. Cells were couterstained with DAPI. Apoptotic body was indicated with arrow. (**B**) Western blotting analysis (left panel) and quantitative densitometry (right panel) of the LC3-I/II conversion and expression of Beclin1. (**C**) Western blotting analysis (left panel) and quantitative densitometry (right panel) of the protein expressions of SIRT3, AMPK and p-AMPK in peritoneal macrophages. Cont, control; Resv, 15 mg/kg; Rapa, 1 mg/kg; 3-MA, 30 mg/kg; VitC, 30 mg/kg. Data are expressed as the means ± SEM (n = 6). $^{**}p < 0.01$ *vs* Cont group, $^{\#\#}p < 0.01$ *vs* restraint group, $^{\Delta}p < 0.05$ and $^{\Delta\Delta}p < 0.01$ *vs* Resv + restraint group.

Fig. 3. Effect of Resv on AAPH-induced cytotoxicity and impairment of phagocytic activity in RAW 264.7 cells. (**A**) Concentration and time dependent growth inhibitory effects of AAPH in RAW 264.7 cells. Cells were incubated with different concentrations (0.3~80 mM) of AAPH for 12, 24 or 48 h. The inhibitory ratio was determined by MTT assay. (**B**) AAPH (5 mM, 24 h)-induced apoptosis determined using Hoechst 33258 staining with fluorescence microscopy. Apoptotic bodies were indicated with white arrows. (**C**) Effect of Resv (2.5 μM), autophagy modulators (Rapa, 1.25 μM; 3-MA, 2 mM) and antioxidants (VitC, 5 μM; Edar, 2.5 μM) on AAPH-induced (5 mM/24 h) growth inhibition. (**D**) ROS was labeled with DCFH-DA and detected by flow cytometry. (**E**) Cellular malondialdehyde (MDA) was evaluated by glucosinolates barbituric acid chromogenic kit. (**F**) Evaluation of MMP with TMRM staining using flow cytometry. (**G, H**) Phagocytic capability of RAW 264.7 macrophages evaluated by engulfment of BSA-incubated fluorescence latex beads

using flow cytometer. Cont, control. Data are expressed as means ± SEM (n = 3). ** $p < 0.01$ *vs* Cont, $^{\#\#}$ $p < 0.01$ *vs* AAPH treated cells and $^{\Delta\Delta}p < 0.01$ *vs* Resv + AAPH treated group.

Fig. 4. Resv enhanced AAPH-induced autophagy in RAW 264.7 cells. Cells were incubated with indicated reagents for 24 h. **(A**) Formation of autophagic vacuoles was evaluated with MDC staining using flow cytometry. (**B**) Transmission electron microscopy of RAW 264.7 cells. Autolysosomes was indicated with green arrow; swelling mitochondria were indicated with yellow arrows; autophagy that swallowed mitochondrion was indicated with red arrow. (**C**) Western blotting analysis (upper panel) and quantitative densitometry (lower panel) of LC3-I/II conversion and Beclin1 expression. Cont, control; AAPH, 5 mM; Resv, 2.5 μM; Rapa, 1.25 μM; 3-MA, 2 mM; VitC, 5 μM; Edar, 2.5 μM. Data are expressed as the means ± SEM (n = 3). ** $p < 0.01$ *vs* Cont, $^{\#\#}$ $p < 0.01$ *vs* AAPH-treated cells, and $^{\Delta\Delta}p < 0.01$ *vs* Resv + AAPH treated cells.

Fig. 5. Effect of *SIRT3* knockdown on Resv regulated signaling pathway in RAW 264.7 macrophages. (A) Evaluation of protein expression levels of SIRT3, AMPK, p-AMPK by Western blotting. (**B**) Knockdown of *SIRT3* in RAW 264.7 cells using RNAi procedure. (**C**) Effect of *SIRT3* knockdown on protein expression levels of SIRT3, AMPK, p-AMPK, LC3-I/II, and Beclin1 determined by Western blotting analysis. (**D**) Confocal fluorescence microscopy of RAW 264.7 macrophages dually stained with LC3β (probed with primary anti-LC3β antibody, and a secondary antibody using Alexa Fluor, Cell Signaling Technology) and Mito Red. Cells were counter-stained with DAPI for DNA. Arrows indicate cells with poor shape. Cont, control; AAPH, 5 mM; Resv, 2.5 μM; Rapa, 1.25 μM; 3-MA, 2 mM; VitC, 5 μM; Edar, 2.5 μM. Data are expressed as the means ± SEM (n = 3). ** $p < 0.01$ *vs* Cont, $^{\#\#}$ $p < 0.01$ *vs* AAPH-treated cells, and $^{\Delta\Delta}p < 0.01$ *vs* Resv + AAPH treated cells.

Fig. 6. Effects of inhibition of SIRT1 and AMPK on activation of AMPK and induction of autophagy. (**A**) Knockdown of *SIRT1* in RAW 264.7 cells using RNAi procedure. Effect of *SIRT1* knockdown (**B**) and inhibitors (**C**) on protein expression levels of SIRT1, AMPK, p-AMPK, LC3-I/II, and Beclin1 determined by Western blotting analysis. Cont, control; AAPH, 5 mM; Resv, 2.5 μM; EX 527, 100 nM; ComC, 1 μM.

Fig. 7. Effect of *SIRT3/SIRT1* knockdown on phagocytic capability of RAW 264.7 macrophages. *SIRT3/SIRT1* siRNA transfected cells were incubated with indicated reagents for 24 h in **A** and **B**, and EX 527/ComC were utilized in **C**. Phagocytic capability of cells was evaluated by engulfment of BSA-incubated fluorescence latex beads using flow cytometer. Cont: control; AAPH, 5 mM; Resv, 2.5 μM; EX 527, 100 nM; ComC, 1 μM; NC, negative control. Data are expressed as the means ± SEM (n = 3). * $p < 0.05$ and ** $p < 0.01$. NS: not significant.

Fig. 8 Summary and hypothesis of the mechanism of Resv in RAW 264.7 cells and mouse peritoneal macrophages in restraint model. In both cells, AAPH or restraint induced loss of MMP, which could be scavenged by VitC or Edar. Mitochondrial oxidative stress induced compensatory upregulation of SIRT3, which might in turn activated phosphorylation of AMPK and subsequently triggered autophagy by upregulating Beclin1 expression and LC3 II/I conversion. Resv enhanced autophagy by activating SIRT3/AMPK and SIRT1/AMPK pathways and thus protected cells against apoptosis, which led to decline of phagocytosis capability. Autophagy not only preserved MMP but also activate SIRT3/AMPK pathway by a positive feedback.

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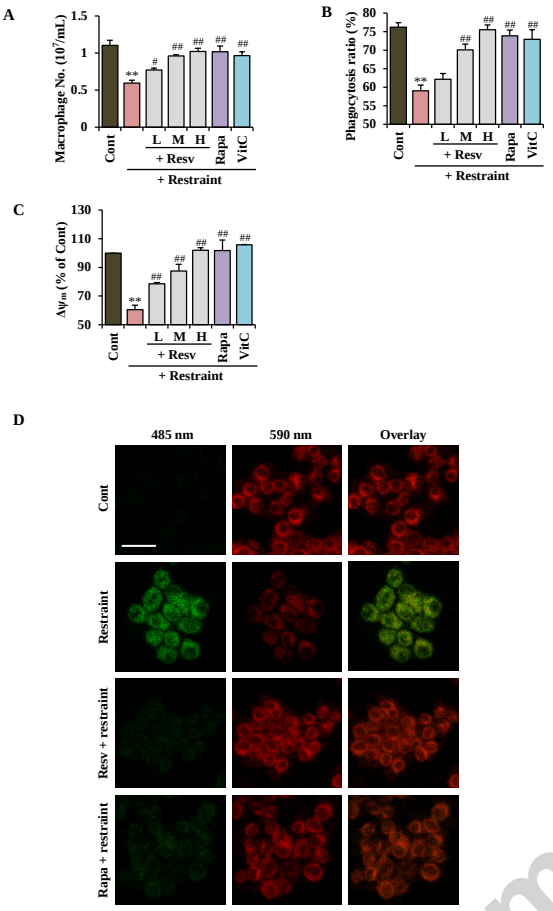
List of figures:

Fig. 1



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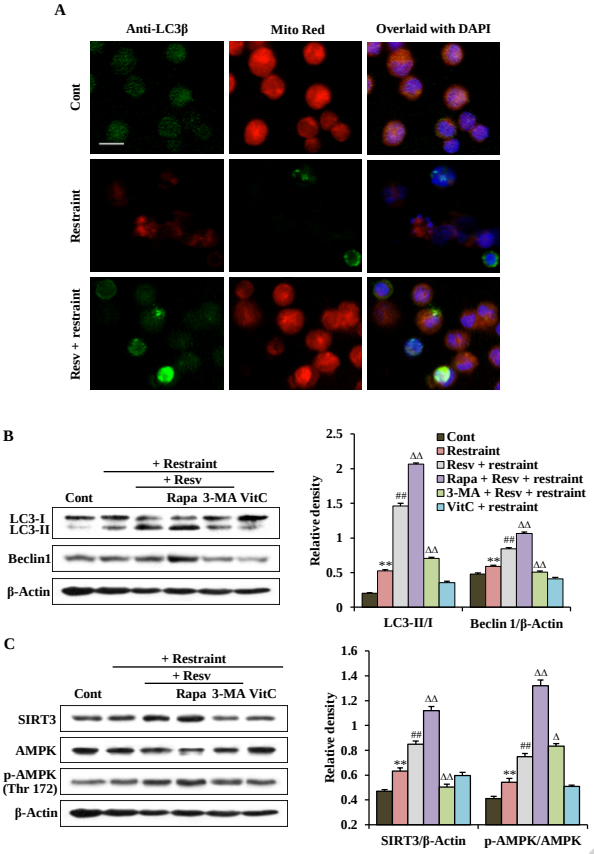

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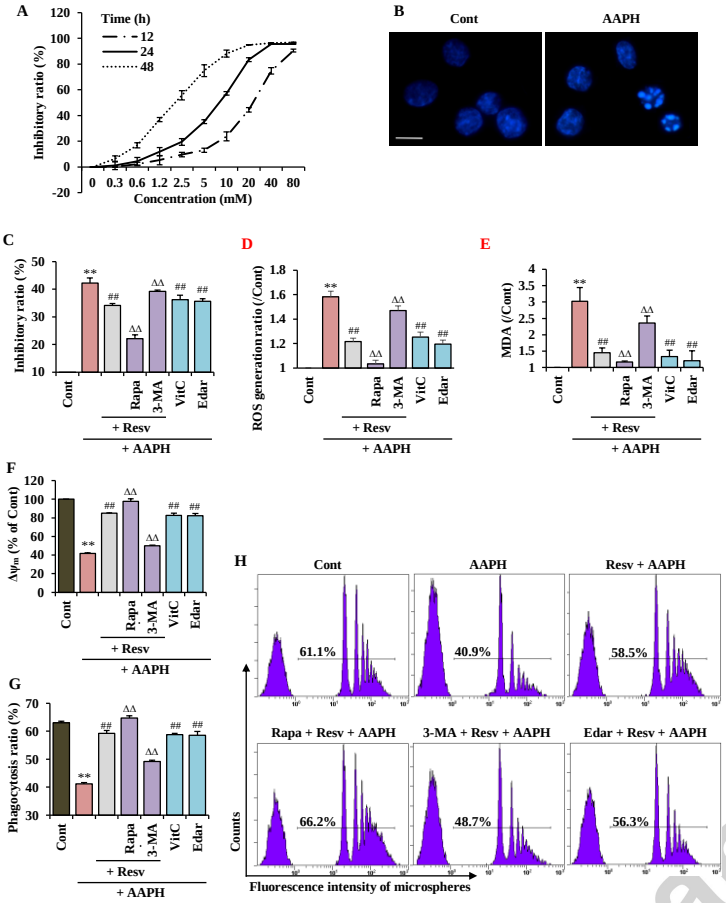

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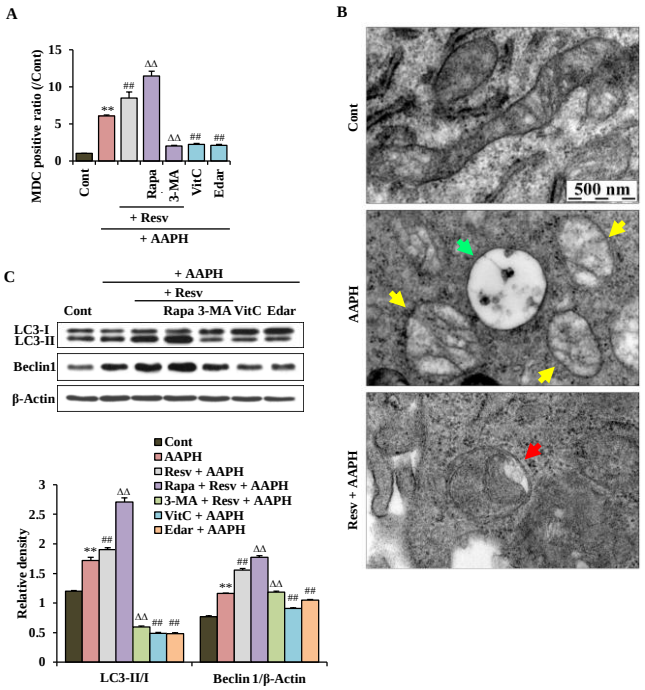

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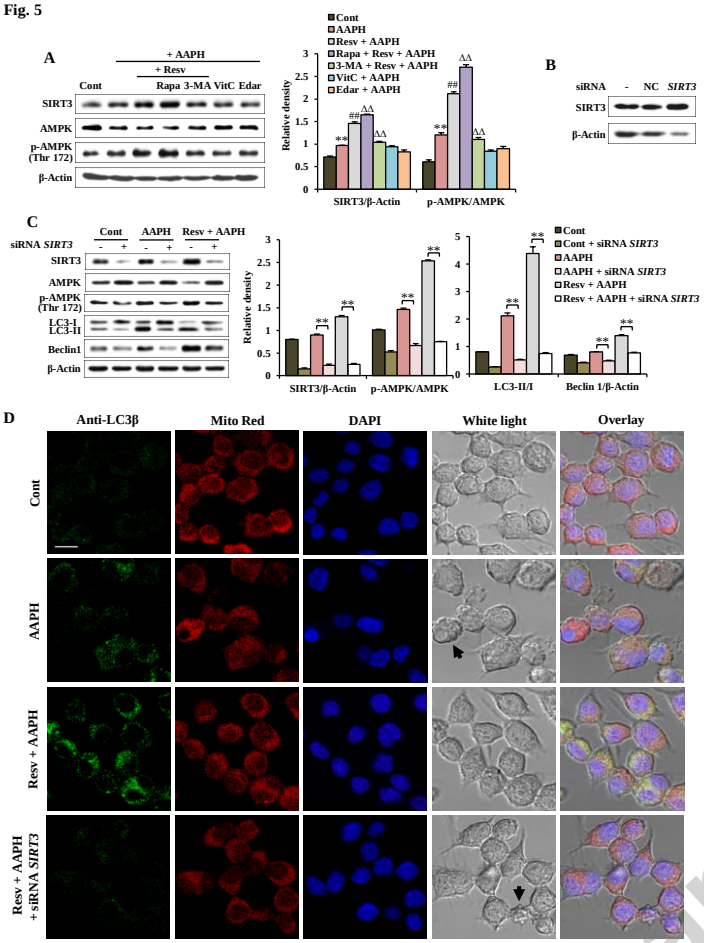

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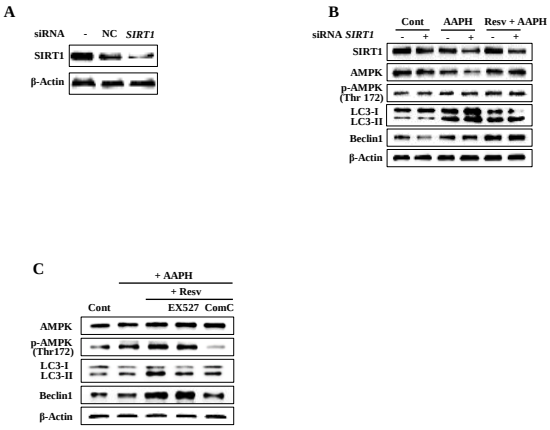


Fig. 7

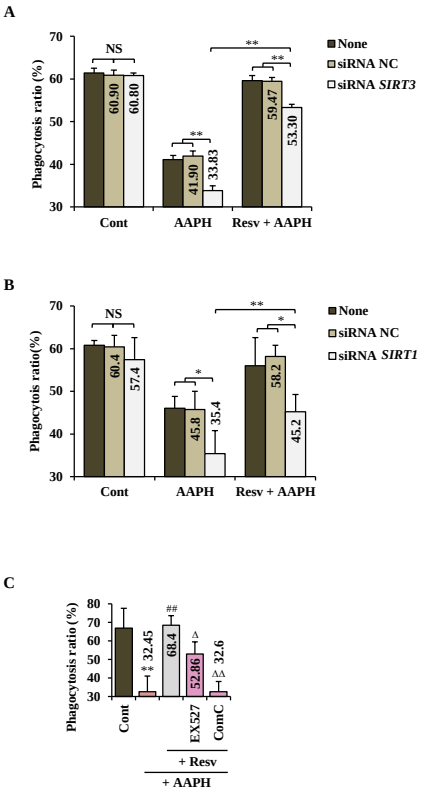
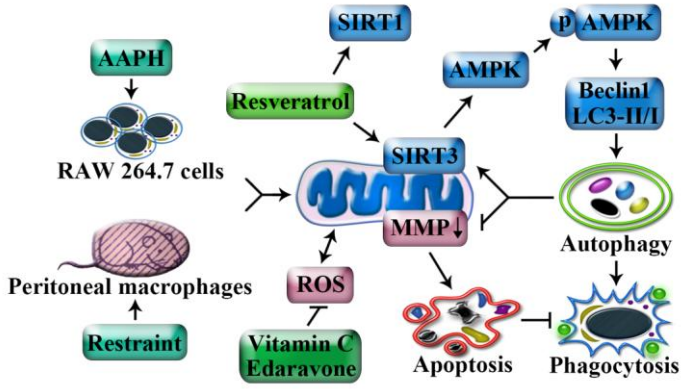


Fig. 8



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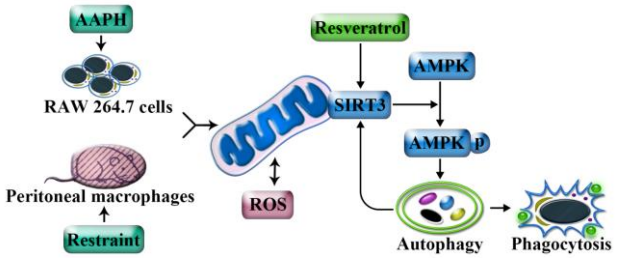
Graphical Abstract



Highlights

1. Resveratrol protects stress-induced impairment of macrophages in mice.
2. Resveratrol protects RAW 264.7 macrophages against AAPH-induced oxidative damage.
3. Resveratrol promotes mitochondrial autophagy via SIRT3 and AMPK dependent pathway.
4. SIRT3/AMPK/autophagy orchestrates in the action of resveratrol.