Anti-inflammatory effect of resveratrol in rodent models and RAW 264.7 murine macrophages: Role of NF-κβ, SIRT1 and HO-1/Nrf2 pathways.

Authored by

Basel A. Abdel-Wahab Saad A. Al-Qahtani

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, Basel A. Abdel-Wahab[1,2], Saad A. Al-Qahtani[3]

[1] Department of Pharmacology, College of Medicine, Assiut University, Assiut, Egypt.

[2] Department of Pharmacology, College of Pharmacy, Najran University, Najran, Saudi Arabia.

[3]Department of Clinical Pharmacy, College of Pharmacy, Najran University, Najran, Saudi Arabia

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Possible molecular mechanisms of resveratrol's anti-inflammatory effect

Corresponding author:

Basel A. Abdel-Wahab

Department of Pharmacology, College of Medicine, Assiut University, Assiut, Egypt.

Current address: Department of Pharmacology, College of Pharmacy, Najran University, Najran, Saudi Arabia

E-mail: basel_post@msn.com

Tel: 00966553899185

Fax: 0020-75442419

Abstract

Resveratrol (RSV) is a polyphenolic compound with potent antioxidant, antitumor and neuroprotective effects. Here, we have investigated the anti-inflammatory and anti-osteoarthritic effects of RSV in four animal models of inflammation (xylene-induced ear edema, acetic acid-induced vascular permeability, monosodium iodoacetate (MIA) -induced osteoarthritis and carrageenan-induced paw edema). In order to elucidate the mechanisms of RSV anti-inflammatory effects and the possible role of proinflammatory mediators and SIRT1, HO-1/Nrf2 and NF-κβ pathways, the effects of RSV were studied on lipopolysaccharide (LPS, 1 µg/ml) -stimulated RAW 264.7 murine macrophages. RSV (25, 50 and 100 mg/kg, p.o) attenuated the clinical signs of inflammation in the four tested animal models in a dose-dependent effect with an effect similar or superior to the positive control, diclofenac (10 mg/kg, p.o) in the large RSV dose (100 mg/kg). LPS stimulation increased significantly the production of NO, PGE2 and LBT4 with a corresponding increase in iNOS, COX-2 and 5-LOX mRNA expression. Moreover, LPS significantly stimulated the activities and mRNA expression of NF-κβ p65 and inhibited the activities and mRNA expression of SIRT1, HO-1 and Nrf2. RSV (0.1, 1 and 10 µM) significantly antagonized all the LPS-mediated effects on the murine macrophages only in its high concentrations (1 and 10 µM). In this study, we report that RSV, in the doses under this study, has effective anti-inflammatory effect possibly mediated via inhibiting NF- κβ p65 and activating SIRT1 and HO-1/Nrf2 pathways. Hence, RSV could represent a good and safer therapeutic approach relative to NSAIDs in the management of inflammation and osteoarthritis.

Keywords: Resveratrol, anti-inflammatory effect, NF-κβ, SIRT1, HO-1/Nrf2, RAW 264.7 murine macrophages.

1. Introduction

Inflammation is a physiological protective response of the body to remove injurious factors of various intrinsic or extrinsic stimuli and it initiates the process of injury healing. However, chronic inflammation, can contribute to some disease states as chronic obstructive pulmonary diseases, type 2 diabetes, atherosclerosis, heart attack, neurodegenerative diseases, rheumatoid arthritis, and cancer.[1-6] The inflammatory response is accompanied by increased vascular permeability, increased neutrophil-derived reactive oxygen species (ROS) and excessive release of nitric oxide (NO) and other proinflammatory mediators.[7]

Lipopolysaccharide (LPS), a pathogenic endotoxin that is a component of the outer membrane of Gram-negative bacteria. It can evoke innate immune responses and following inflammatory reactions by stimulating the expression of many pro-inflammatory genes in macrophages. It is frequently used to induce pharmacological research models of inflammation.[8] Previous studies suggested that, LPS can activate nuclear factor-kappa B (NF-κβ) and three mitogen-activated protein kinase (MAPK) pathways. These pathways include, the c-Jun NH2-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), and p38 pathways. Activation of these pathways result in the overexpression of inflammatory mediators such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), NO, leukotrienes (LTs) and prostaglandin (PG) E2.[9] Cyclooxygenase-2 (COX-2), 5-lipooxygenase (5-LOX) and inducible nitric oxide synthase (iNOS) are responsible for the production of PGE2, LTs and NO, respectively.[10] NO produced by iNOS is considered a critical mediator of inflammatory responses, wherever, high levels of NO can cause many inflammatory diseases, such as atherosclerosis, inflammatory bowel diseases and rheumatoid.[11]

NF-κβ is an inducible transcription factor, promote the expression of NF-κβ-regulated genes, which thereby contributes to the further enhancement of proinflammatory cytokines

and defense against oxidative stress.[12] In addition, NF-κβ-regulates the expression of COX-2 and iNOS.[13]

Previous studies have indicated the importance of the antioxidant pathway involving Nuclear Factor Erythroid 2-Like 2 (Nrf2) in the inflammatory processes.[14] Under unstressed conditions, Nrf2 presents in the cytoplasm together with its repressor protein, Kelch-like ECH-associated protein 1 (Keap1). Under stress conditions, Nrf2 dissociates from Keap1, translocates into the nucleus and combines with the antioxidant response element (ARE). This interaction leads to the induction of phase II detoxifying enzymes and cytoprotective homeostatic gene regulatory network,[15] such as heme oxygenase 1 (HO-1).[16] HO-1 is the major anti-inflammatory and anti-oxidative enzyme among the genes that are mediated by Nrf2 activation.[17] Several reports have suggested that, the activation of HO-1 attenuates the inflammatory and oxidative damage that occurs in LPS-induced inflammation.[18]

Sirtuins (SIRTs) are a class of NAD+- dependent deacetylases, which regulate systems that control the redox environment, thereby counteracting oxidative damage.[19] It has been previously suggested that activation of the SIRT1 gene expression and/or proteins with several agents may have anti-inflammatory and other useful health effects.[20]

Several anti-inflammatory or anti-oxidative phytochemicals suppress NF-κβ signaling and activate the Nrf2-ARE pathway suggesting that the suppression of NF-κβ signaling and the activation of the Nrf2 pathway may crosstalk with each other.[21] Trans-resveratrol (RSV) (3,5,4-trihydroxystilbene), a polyphenolic compound that is isolated from grapes and traditional Chinese medicinal plants such as *Polygonum cuspidatum*.[22] It exhibits a wide range of biological properties, including its potent antioxidant in addition to neuroprotective, and promising anti-inflammatory properties.[23] Studies have revealed that RSV modulates genes related to redox pathways. It stimulated the expression of Nrf2 in an ischemia rat model.[24] Moreover; studies have attributed the anti-oxidative/anti-nitrosative effects of RSV

to its ability to inhibit the NF-κβ and to up-regulate the Nrf2 transcriptional pathways.[25] Furthermore, neuroprotection against oxidative stress by RSV is at least partially mediated through the activation of the SIRT1 pathway.[26] However, the mechanism(s) of RSV in inflammation and its ability to modulate these pathways remain elusive.

Hence, the aim of this study was to investigate the anti-inflammatory activity of RSV in different inflammation rodent models. In addition to investigate whether the mechanisms underlying the anti-inflammatory effects of RSV are associated with inhibition of proinflammatory mediators and iNOS, COX-2 and 5-LOX expression and possible modulation of SIRT1, HO-1/Nrf2 and NF-κβ p65 pathways in LPS-stimulated RAW 264.7 murine macrophage cell culture was studied.

2. Materials and Methods

2.1. Animals

Swiss albino male mice weighing between 25 to 30 g and Wistar male Albino rats weighing between 150–200 g obtained from the animal Care Unit of King Saud University, Riyadh, Saudi Arabia were utilized in this study. The animals were accommodated at room temperature (28 ± 5) ℃ with a relative humidity of 55±5% in a standard wire meshed plastic cages for 4 to 5 days prior to commencement of the experiment. During the entire period of the study, the animals were kept on a 12/ 12 h light/dark cycle with free access to standard pellet diet and water *ad libitum*. Animals were kept on a 12 h light-dark cycle (6:00 am–6:00 pm), where all experimental testing were conducted during the light phase, from 9:00 am–2:00 pm. Animals either rats or mice were randomly divided into five groups (n= 8). Group 1 (control): received the vehicle (0.5% carboxymethyl cellulose (CMC)). Group 2: was treated with Diclofenac (10 mg/kg, p.o., suspended in 0.5% CMC) (Srivastava et al., 2013). Groups (3-5): were treated with RSV in doses 25, 50 and 100 mg/kg (from preliminary studies in our lab) suspended in 0.5% CMC respectively. The experimental protocol was approved by the Institutional Animal Use and Care Committee, College of Medicine, King Khaled University, Saudi Arabia and is in close agreement with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 8023, revised 1978).

2. Chemicals

All chemicals and reagents used were of analytical grade. *Trans*-resveratrol (3,5,4-trihydroxy-*trans*-stilbene), carrageenan, xylene, monosodium iodoacetate, Griess reagent (1:1 mixture of 0.1% N-(1-naphthyl) ethylene-diamine dihydrochloride in water and 1% sulfanilamide in 5% phosphoric acid), diclofenac and Escherichia coli LPS (O55:B5) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Due to the limited water solubility of

RSV, it was suspended in 0.5 % w/v solution of carboxymethyl cellulose (CMC), as a suspending agent, in distilled water and given to the animals orally at a dose level 25, 50 and 100 mg/kg body weight through a gavage tube. LPS was dissolved in phosphate-buffered saline (PBS) at 1 µg/ml. The stock solutions were stored at -20℃. The solution was diluted with medium to the desired concentration just before use. RAW264.7 cells were purchased from the American Type Culture Collection (Rockville, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and TRIzol reagent were purchased from Gibco/BRL (Grand Island, USA).

2.3. Animal Testing

2.3.1. Xylene-induced ear edema test.

The xylene-induced ear edema test was performed as previously described with minor modifications.[27] RSV was administered orally, at doses of 25, 50, 100 mg/kg 1 h before application of xylene. A total of 20 µl of xylene was applied to the inner and outer surfaces of the right ear of each mouse. The left ear remained untreated. Control animals received the vehicle (0.5% CMC). Diclofenac (10 mg/kg, p.o., suspended in 0.5% CMC) was used as reference drug. The animals were euthanized by cervical dislocation 1 h later, and two earplugs (7 mm in diameter) were removed from both the treated ear and the untreated ear. Weights of treated and untreated earplugs were measured with an electronic balance. The difference in weight of the two earplugs was taken as a measure of edematous response.

2.3.2. Acetic acid-induced vascular permeability

The acetic acid-induced vascular permeability test was carried out using a modification of the previously described method.[28] Thirty minutes after oral administration of vehicle (0.5% CMC), RSV (25, 50 and 100 mg/kg) or diclofenac (10 mg/kg), 10 ml/kg body weight of 2% Evans blue was injected intravenously into each mouse tail. Twenty minutes later, 10 ml/kg body weight of 1% acetic acid in saline was injected intraperitoneally. Twenty minutes

after the injection of acetic acid, the mice were sacrificed by cervical dislocation. After 5 ml of saline was injected into the abdominal cavity, the washings were collected into test tubes, and then centrifuged at 2000 rpm for10 min. The absorbance of the supernatant was read at 650 NM using an ELISA reader (Bio-Rad, Hercules, USA) and the amounts of Evans blue leakage in abdominal cavity were measured from the absorbance measurement in the supernatant. Vascular permeability was represented as the amounts of Evans blue leakage in the abdominal cavities.

2.3.3. MIA-induced osteoarthritis

The test was carried out according the method described previously.[29] Rats were anesthetized with a thiopental sodium (40 mg/kg, i.p), and were given a single intra-articular injection of monosodium iodoacetate (MIA) through the infrapatella ligament of the right knee. MIA was dissolved in 0.9% sterile saline and administered in a volume of 50 µl (60 mg/ml) using a 26 gauge, 0.5-inch needle. The effect of joint damage on weight distribution through the right (arthritic) and left (untreated) knee was assessed using an in capacitance tester (LintonInstru- mentation, Norfolk, UK). Briefly, an in capacitance tester measures weight distribution on the two hind paws. The force exerted by each hind limb is measured in grams. Each experimental group was administered orally with vehicle (0.5% CMC in distilled water), RSV (25, 50 or 100 mg/kg), or diclofenac (10 mg/kg) for 14 days. The effect of joint damage on weight distribution through the right (arthritic) and left (untreated) knee was measured at 1,7,14 days after drug treatment.

2.3.4. Carrageenan-induced paw edema

The procedures conducted according to the previously described method.[30] One hundred microliters of 1 % suspension of carrageenan in saline was injected into the plantar side of the left hind paws of the rats. RSV (25, 50 and 100 mg/kg) or diclofenac (10 mg/kg) and the vehicle (0.5 % CMC in distilled water) were administered orally (p.o.) 1 h before the

carrageenan treatment. The volume of paw edema was measured by a plethysmometer (Ugo Basile, Comerio, Varese, Italy) prior to carrageenan injection and every hour for 6 h. Edema was expressed as the increase in paw volume (ml) after carrageenan injection relative to the pre-injection value for each animal. Results are expressed as paw volume change (ml).

2.4. Cell culture

2.4.1. Cell viability assays

RAW264.7, a murine macrophage cell line, was cultured in DMEM supplemented with 10% heat-inactivated FBS and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) at 37℃ in an atmosphere containing 5% CO2. For cell viability assays, cells (1 × 104 cells/well) were seeded in 96-well plates, pretreated with 0.1, 1, 10 μM RSV for 1 h, and stimulated with or without 1 μg/ml LPS for 24 h. After incubation, 20 μl of CCK-8 reagent was added to each well, and the plates were incubated for 2 h at 37℃. The absorbance of the resulting solution was then measured using a microplate reader (Bio-Rad, Hercules, USA) at the test wavelength of 450 nm and the reference wavelength of 570 nm. The results were expressed as fold changes by normalizing the data to the control. Four replicates were performed for each treatment.

2.4.2. Determination of NO, PGE2, LTB4 levels

To investigate the anti-inflammatory mechanisms of RSV, the change in NO, PGE2, LTB4 levels in LPS-stimulated RAW264.7 murine macrophage cells were studied. For this purpose, RAW 246.7 cells were seeded in 96-well plates at a density of 2×10^5 cells per well and grown for adherence. The cells were then pretreated with various concentrations of RES (0.1, 1, 10 μM) for 4 h before stimulation with LPS (1 μg/ml) and incubation for 12 h.

The nitrite concentration in the culture medium was measured as an indicator of NO production according to the Griess reaction. Briefly, 100 μl of cell culture supernatant was reacted with 100 μl of Griess reagent (1:1 mixture of 0.1% N-(1-naphthyl) ethylene-diamine

dihydrochloride in water and 1% sulfanilamide in 5% phosphoric acid) in a 96-well plate, and absorbance at 540 nm was recorded using the ELISA reader (Bio-rad, Hercules, USA).

PGE2 and LTB4 levels were measured using an enzyme-linked immune Sorbent assay (ELISA) commercial kit (Abcam Chemical Co., London, UK) according to the manufacturer's instructions. The absorbance of the resulting solution was then measured using a microplate reader (Bio-Rad, Hercules, USA) at the test wavelength of 450 nm.

2.4.3. Determination of and SIRT1, HO-1, Nrf2 and NF-KB p65 activities

For further investigation of the anti-inflammatory mechanisms of RSV, the activities of the nuclear factors SIRT1, HO-1, Nrf2 and NF-KB p65 in LPS-stimulated RAW264.7 cells were examined. For this purpose, RAW 264.7 cells were treated in a similar fashion to that described above for determination of NO, PGE2 and LTB4, using the same selected concentrations of RSV. The activities of HO-1, SIRT1, Nrf2 and NF-KB p65 in the supernatant of cell culture were determined using a new sensitive enzyme immunoassay kit for HO-1, SIRT1 and NF-KB p65 (Abcam Chemical Co., London, UK) and for Nrf2 (Cayman Chemical Company, Ann, Arbor, MI, USA) which measure the nuclear binding activity of the tested transcription factors with DNA. All procedures were conducted according to the manufacturer's instructions. The absorbance of the resulting solution was then measured using a microplate reader (Bio-Rad, Hercules, USA) at the test wavelength of 450 nm and the reference wavelength of 570 nm. The results were expressed as fold changes by normalizing the data to the control. Four replicates were performed for each treatment.

2.4.3. Quantitative real-time polymerase chain reaction (qRT-PCR)

For further investigation of the anti-inflammatory mechanism of RSV, the expression of mRNA of iNOS, COX-2 and 5-LOX enzymes and the nuclear factors SIRT1, Nrf2 and NF-KB p65 in the supernatant of cell culture were determined using qRT-PCR. Cells were seeded in 6-well culture plates at a density of 1×10^6 cells/ well and serum-starved for 18 h. Cells

were then pretreated with RSV for 1 h and stimulated with 1 μg/ml LPS for 4 h. Total RNA was isolated from cells using TRIzol reagent following the manufacturer's instructions, and complementary DNA was synthesized using 3 μg of RNA in a reverse transcription reaction from real-time polymerase chain reaction (RT-PCR) kit (Invitrogen, Carlsbad, USA). Quantitative RT-PCR (qRT-PCR) analyses were performed using C1000 thermal cycler (Bio-Rad) with SYBR-Green (Invitrogen). The relative levels of gene expression were determined using the cycle threshold method of relative quantification. Each assay was normalized to the level of β-actin mRNA. The primer pairs used are shown in table 2.

2.5. Statistical analysis

All values are expressed as mean ± standard error of the mean (SEM) of (n) observations. For the in vivo studies, n represents the number of animals. For cell culture studies, proinflammatory mediators, and transcriptional factors analyses, the figures shown are representative of at least four experiments performed on different experimental days on the samples collected from RAW 264.7 cells in each group. Results were analyzed by one-way ANOVA followed by a Tuky's post hoc test for multiple comparisons using SigmaState (version 11.0) software. For the arthritis studies, Mann-Whitney U test (two-tailed, independent) was used to compare medians of the arthritic indices. A P value of less than 0.05 was considered significant.

3. Results

2.1. Anti-inflammatory activity of RSV in vivo

2.1.1. Effect of RSV on xylene-induced ear edema in mice

The anti-inflammatory effect of the RSV (25, 50 and 100 mg/kg, p.o) on acute inflammation was evaluated on xylene-induced ear edema in mice. Topical application of xylene resulted in cutaneous inflammation in the ears of mice, which was reflected in significant [F (4,39) = 5.335, p = 0.0018] increase in ear plug weight of the right ear when compared to the vehicle-treated left ear. Treating the mice with diclofenac (10 mg/kg), as a positive control, significantly ($p < 0.01$) reduced the ear plug weight by about 50.8%. RSV significantly ($p< 0.05$ and $p< 0.01$) reduced the ear plug weight in xylene-induced ear edema formation respectively in a dose-dependent manner with a maximal suppression > 62% with the dose 100 mg/kg (Fig. 1). These findings indicate that RSV particularly in dose 100 mg/kg shows anti-inflammatory activity, which is more potent than diclofenac.

2.1.2. Effect of RSV on acetic acid-induced vascular permeability

The effect of RSV on the vascular permeability was tested via measurement of the amount of Evans blue permeated after its intravenous injection in the peritoneal cavity stimulated by intraperitoneal (i.p.) injection of acetic acid in mice. Intraperitoneal injection of acetic acid resulted in significant [F (4,39) = 491.7, p =.9...0] increase in amount of Evans blue dye infiltered in mice peritoneal cavity. Animals treated with RSV exhibited significant inhibition ($p< 0.05$, $p< 0.01$ and $p< 0.001$) at the doses 25, 50 and 100 mg/kg of RSV respectively in vascular permeability. The percent of inhibition with RSV dose 100 mg/kg, p.o. showed higher potency than that of diclofenac (10 mg/kg) (40.97 and 32.89% respectively) (Fig 2).

2.1.3. Effect of RSV on MIA-induced osteoarthritis

This experiment was designed to study the effectiveness of RSV in reducing the osteoarthritis pain by determining of the effect of RSV on MIA-induced weight-bearing differential that were conducted after MIA-induced osteoarthritis. RSV significantly reduced the change in hind paw weight distribution in a dose-dependent manner ($p< 0.05$, $p< 0.01$ and $p< 0.001$) with the doses 25, 50 and 100 mg/kg, respectively, 7 [$F (4,39) = 7.162$, $p = 0.0003$] and 14 [$F (4,39) = 6.309$, $p = .9...0$] days after treatment. Throughout these times, diclofenac (10 mg/kg) produced a similar or inferior effect to RSV at a dose 100 mg/kg (Table 1). This result suggests that RSV 100 mg/kg could be a superior anti-osteoarthritic agent, compared to diclofenac.

2.1.4. Effect of RSV on carrageenan-induced paw edema

Carrageenan-induced paw edema is a common tool for testing the antiinflamatory activity of drugs in rats. Intraplantar injection of carragenan caused a time-dependent rise in paw volume that was highest after 5 h in rats (Fig. 3). RSV in doses 50 and 100 mg/kg, p.o. significantly inhibited the development of carrageenan-induced paw edema, the effect that was more obvious after 3 h of treatment in a dose-dependent manner (Fig. 3). This inhibitory effect of carrageenan-induced inflammation by RSV (50 mg/kg) was similar to that produced by the positive control diclofenac (10 mg/kg) ($p < 0.05$). However, RSV at 100 mg/kg showed higher significant level ($p < 0.01$) of inhibition to paw edema than diclofenac.

2.2. Anti-inflammatory activity of RSV in RAW264.7 murine macrophages

2.3.1. Cell culture and viability of RAW264.7 cells

To assess the cytotoxic effects of RSV on RAW264.7 cells, cells were pretreated with different concentrations of RSV for 1 h and then were stimulated with 1 μg/ml LPS for 24 h. Cell viability was evaluated using the CCK-8 reagent. Data revealed that 0–20 μM RSV did

not affect cell viability over the 24-h period tested (Fig 4A). These non-toxic concentrations of RSV (0–10 μM) were used in the subsequent experiments.

2.3.2. Effect of RSV on LPS-induced NO, PGE2 and LTB4 secretion

To examine the potential anti-inflammatory properties of RSV on LPS-induced NO, PGE2 and LTB4 production in RAW 264.7 cells, cells were treated with or without RSV (0.1, 1, 10 μM) and then stimulated with LPS (1 μg/ml) for 24 h. NO, PGE2 and LTB4 concentrations were measured in the culture supernatants by the Griess reaction and ELISA assays, respectively. There was no any significant measurable changes in NO, PGE2 and LTB4 concentrations in the normal non-stimulated culture media. LPS stimulation of macrophages significantly increased the concentrations of NO [$F(3,15) = 20.81$, $P<0.0001$], PGE2 [$F(3,15) = 9.301$, $p= 0.0019$] and LTB4 [$F(3,15) = 7.805$, $p= 0.0037$]. After stimulation with LPS, the accumulation of NO, PGE2 and LTB4 in RAW264.7 cells has 4.5, 3.1, and 2.8-fold increases respectively. Pretreatment with RSV significantly inhibited LPS-induced NO, PGE2 and LTB4 production in a concentration-dependent manner (Figure 4A, 5A, 6A). These findings suggested that RSV inhibits both of NO, LTs and PGs production stimulated by LPS.

2.3.3. Effect of RSV on LPS-induced changes in iNOS, COX-2 and 5-LOX mRNAs expression

Next, the effect of RSV on COX-2, iNOS and 5-LOX mRNA expression was investigated using quantitative real-time PCR. It was found that the expression levels of COX-2, iNOS and 5-LOX in RAW 264.7 cells were very low in the absence of LPS. When LPS was added to the culture media, all the levels of iNOS [$F(4 ,19) = 8.258$, $p= 0.0010$], COX-2 [$F(4 ,19) = 10.19$, $p =0.0003$] and 5-LOX [$F(4 ,19) = 7.822$, $p=0.0013$] mRNA significantly increased after 4 h of treatment with LPS. Pretreatment with RSV followed by LPS stimulation for 4 h attenuated the expression of COX-2, iNOS and 5-LOX mRNA levels

in a concentration-dependent manner, with the highest inhibitory effect ($p< 0.01$) with the dose 10 μM of RSV (Fig. 4B, 5B, 6B).

2.3.4. Effect of RSV on LPS-induced changes in SIRT1, HO-1 and Nrf2 activities and mRNA expression

For further investigation of the possible mechanisms of the anti-inflammatory effect of RSV on both the activities and mRNA expression of the nuclear factors SIRT1, Nrf2 and NF-κβ p65. Although, the activities of these factors usually measured by immunoblotting of the phosphorelated factors or cytosolic activating factors such as IKβα and IKK for NF- κβ which unfortunately was not available in our lab. We utilized a new sensitive ELISA assay kits, which measure the binding activities of these factors with the DNA. Stimulation of RAW 264.7 cells with LPS resulted in a significant decrease in both the activities measured by ELISA assay and mRNA expression, measured by real-time PCR, of SIRT1 [$F(4 ,19) = 24.61$, $P<0.0001$] and [$F(4 ,19) =14.88$, $P<0.0001$] respectively. Pretreatment with RSV (0.1, 1, 10 μM) followed by LPS stimulation for 4 h significantly increased both the activities and mRNA expression of SIRT1 in a concentration-dependent manner (Fig. 7). In addition, LPS stimulation to RAW 264.7 cells resulted in a significant increase in both the activity and mRNA expression of Nrf2 [$F(4 ,19) = 7.822$, $p= 0.0013$] and [$F(4 ,19) = 8.908$, $p= 0.0007$] respectively. Pretreatment with RSV (0.1, 1, 10 μM) caused further increase in both the activity and mRNA expression of Nrf2 in a concentration-dependent manner relative to the LPS levels. Moreover, stimulation of RAW 264.7 cells with LPS resulted in a significant increase in both the activity and mRNA expression of HO-1 [[$F(4 ,19) =17.34$, $P<0.0001$] and [$F(4 ,19) =14.71$, $p = 0.0001$]. Pretreatment with RSV (0.1, 1, 10 μM) followed by LPS stimulation for 4 h significantly increased both the activities and mRNA expression of HO-1 in a concentration-dependent manner relative to the LPS levels (Fig 8).

2.3.5. Effect of RSV on LPS-induced changes in NF-κβ activity and mRNA expression

Stimulation of RAW 264.7 cells with LPS resulted in a significant increase in both the activities and mRNA expression, measured by real-time PCR, of NF-KB p65 [[$F(4, 19) = 16.02$, $P<0.0001$] and [$F(4, 19) = 7.822$, $p = 0.0013$]. Pretreatment with RSV (0.1, 1, 10 μM) followed by LPS stimulation for 4 h significantly decreased both the activities and mRNA expression of NF-KB p65 in a concentration-dependent manner, with maximal inhibitory activity at the dose 10 μM ($p< 0.001$ and $p< 0.01$ for inhibition of NF-κβ p65 activity and expression respectively) (Fig 9).

4. Discussion

Many plant-derived compounds, including RSV have promising anti-inflammatory effects.[31] For this reason, they are candidate molecules for the treatment and/or control of chronic inflammatory states such as osteoarthritis, inflammatory bowel diseases, atherosclerosis, etc. RSV has been identified as a dual inhibitor of COX and 5-LOX with anti-inflammatory activity and low side effects, especially improved gastrointestinal side effects.[32] The present study describes the anti-inflammatory activity of RSV and investigates its possible modulation of pro-inflammatory mediators as well as the associated changes in the intracellular signaling pathways.

To elucidate the anti-inflammatory activity of RSV, four in vivo models were used: xylene-induced ear edema, vascular permeability, MIT- induced arthritis and carrageenan-induced paw edema. In the acute model, i.e. the xylene-induced ear edema in mice, topical application of xylene induces significant inflammatory responses characterized by edema, neutrophil infiltration, PGS production and increases in vascular permeability.[33] RSV at 25, 50 and 100 mg/kg, significantly suppressed xylene-induced ear edema formation in a dose-dependent fashion which was more pronounced than the positive control diclofenac in the large dose of RSV (100 mg/kg). The majority of the activities of xylene appear to be involved or to be dependent on arachidonic acid release and metabolism.[34] Therefore, the results of the present study suggest that RSV may have pharmacological properties similar to both COX and LOX inhibitors.

In the early stage of inflammatory responses, the anti-inflammatory activity was assessed by inhibition of vascular permeability, which is a prominent feature of the inflammatory pathological process. Vascular permeability induced by acetic acid was measured by the amount of Evans blue dye from the abdominal cavity.[35] The increase in vascular permeability is related to leakage of plasma from the blood vessels in the

inflammation site leading to dilation of arterioles and venules and increased vascular permeability.[36] As demonstrated in Fig. 2, RSV at 25, 50 and 100 mg/kg showed significant decrease in the amount of Evans blue measured in vascular permeability assay. These results indicate that RSV significantly reduced vascular permeability, which may in turn, contributes in its anti-inflammatory effect.

The monosodium iodoacetate (MIA) model of OA has been widely used and well described in rats, especially in terms of pathological progression of the disease, and peripheral nerve sensitization. Injection of MIA in the joints of animals inhibits glyceraldehye-3-phosphate dehydrogenase activity in chondrocytes, leading to disruption of glycolysis and eventual cell death.[37] The progressive loss of chondrocytes leads to morphological and histological alterations in the articular cartilage, closely similar to those seen in human OA.[38] RSV significantly decreased change in hind paw weight distribution in a dose-dependent manner at 0, 7, and 14 days after treatment in the MIA-induced osteoarthritis animal model. In addition, diclofenac has a similar or inferior effect to RSV at 100 mg/kg at 0, 7, and 14 days after treatment (Table 1). Those results suggest that RSV might be a superior anti-osteoarthritis agent, compared to diclofenac through acute and chronic inflammatory pain relief and inflammation inhibition. It has been reported that RSV has dual inhibitory effect of both COX-2 and 5-LOX enzymes.[32] Therefore, it is thought that RSV may work as an anti-osteoarthritic agent by decreasing inflammation and pain through inhibition of both PGs and LTs production.

Carrageenan-induced paw edema is considered one of the frequently used models for the study of inflammation, inflammatory pain, and the anti-inflammatory activity of different compounds. Previous studies have shown that, proinflammatory cytokines such as TNF-α and IL-1 are released after carrageenan injections in the hind paw.[39] In addition, it is known that the third phase of the carrageenan-induced edema, in which the edema reaches its largest

volume, is characterized by the excessive release of PGs and other compounds of inflammatory reaction.[40] In this study, the inhibitory effect of carrageenan-induced inflammation by RSV (50 and 100 mg/kg) was similar to that produced by the positive control, diclofanac (10 mg/kg) (Fig. 3,). Hence, RSV showed a potent anti-inflammatory effect against carrageenan-induced inflammation and ability to inhibit the production of proinflammatory mediators, including PGs and LTs induced by carrageenan.

It has been proposed that the inhibition of one or both COX enzymes, while reducing the levels of gastroprotective prostaglandins, may result in alternative processing of AA via the 5-LOX pathway. This increases the production of cysteinyl leukotrienes and LT B4, which contribute to gastrointestinal toxicity by promoting the migration of leukocytes, breaking down the mucosal barrier and stimulating gastric acid secretion.[41] For this reason, compounds that inhibit COX and 5-LOX simultaneously, like RSV, may have enhanced anti-inflammatory effect and reduced undesirable side effects with improved gastrointestinal safety profile.

To explore the mechanism underlying potentially beneficial effects of RSV, its effects on RAW 264.7 murine macrophage functions related to inflammation were investigated. Activated macrophages by LPS secrete large amounts of proinflammatory mediators, such as PGE2 and LTB4, via the inducible isoforms of COX-2 and 5-LOX respectively.[41] This fact is in agreement with the results obtained in this study that showed increased expression of COX-2 and 5-LOX mRNA and levels of PGE2 and LTB4 in LPS-stimulated macrophages. Considering the fact that NO/iNOS also play an important role in the progress of inflammation, we also evaluated the effect of RSV in this work on the NO/iNOS system in LPS-induced RAW 264.7 murine macrophage cells. Results showed that both the level of NO and expression of iNOS mRNA were inhibited by various concentrations of RSV (0.1–10 μM) in a concentration dependent manner. Hence, RSV not only has dual inhibition of COX-

2/ 5-LOX but more interestingly, it also exerted inhibitory effect on NO/iNOS system. During the inflammatory response, iNOS is expressed in response to endotoxin, such as LPS and various proinflammatory cytokines, including INF-γ and TNF-α, where, NO produced by iNOS is regarded as a critical mediator of inflammatory responses.[42]

Transmission of the LPS signal within the macrophages is initiated by binding and activation of LPS receptor complex, which is, comprised of Toll-like receptor4 (TLR4) and myeloid differentiation protein-2 (MD-2). Activated TLR4 subsequently transfers the signal via two main signaling pathways: myeloid differentiation factor 88 (MyD88) -dependent and Toll/IL-1 receptor domain that contains the adapter inducing interferon-β (TRIF) -dependent pathways. Through the MyD88-dependent pathway, phosphorylation of mitogen activated protein kinases (MAPKs) and ubiquitin-degradation of inhibitor κβα (Iκβα) result in the activation of several transcription factors such as NF-κβ, activating protein-1 (AP-1), cAMP-responsive element binding protein (CREB), and CCAAT-enhancer box binding protein (C/EBP) which, in turn, promote expression of COX-2, 5-LOX and iNOS (Palsson-McDermott and O'Neill, 2004). Since iNOS and COX-2/5-LOX are governed by the MyD88- and TRIF-dependent pathways and in the MyD88-dependent pathway; signal transduction is dependent on NF-κβ.[41] These facts are in agreement with other study showed that, the expressions of unease and COX-2 in murine macrophages have been shown to be dependent on NF-κβ activity.[43,44] Thus the possibility that RSV to inhibit NF-κβ activation was examined. Our results indicate that inhibition of iNOS and COX-2 expression of mRNA by RSV were probably due to the suppression of both NF-κβ activity and mRNA expression. Supporting to this concept, the findings of previous studies, showed that NF-κβ response elements are present on the promoters of the iNOS, COX-2 and TNF-α genes.[45,46] NF-κβ is a critical key transcription factor that expresses the genes involved in inflammation and has attracted attention as a target for treating inflammatory diseases.

NF-κβ is composed of a range of homo- or heterodimeric combinations of NF-κβ/Rel proteins, such as Rel (cRel), RelA (p65), RelB, NF-κβ1 (p50), and NF-κβ2 (p52) in mammals. The main inducible form is a heterodimeric consisting of the p50/p65 subunit. NF-κβ is present in the cytoplasm as an inactive complex associated with an inhibitory protein called Iκβ. Various external or internal stimuli cause the dissociation of the NF-κβ/Iκβ complex through the phosphorylation and degradation of Iκβ. The degradation of Iκβ results from the phosphorylation of particular serine sites where IKK catalyzes this step. The complex of IKK is composed of three subunits; catalytic (IKK-α and IKK-β) and regulatory subunits (IKKγ).[47] After activation, the NF-κβ heterodimer (p65/p50) is translocated rapidly from the cytoplasm to the nucleus. In the nucleus, p65/p50 of the NF-κβ subunit binds to a specific DNA binding site and modulates the transcription of target genes. These genes include proinflammatory mediators, such as iNOS, COX-2, 5-LOX, various cytokines (TNF-α and IL-1, etc.), chemokines, and adhesion molecules.[48] The present study showed that RSV not only can enhance the expression of mRNA of NF-κβ p65 but also it enhances its activity. This effect may be mediated through inhibition LPS-induced degradation and phosphorylation of IKK-α and IKK-β, the fact that needs further study.

For further exploration of the basis of the anti-inflammatory effect of RSV, upstream signal transduction molecules such as HO-1, Nrf2 and SIRT-1were examined. Results showed that, while LPS increased HO-1 and Nrf2 activities and expression and decreased SIRT1 activity and expression, RSV significantly increased both the activities and mRNA expression of the three transcriptional factors, in LPS-stimulated RAW 264.7 macrophages in a concentration-dependent effect relative to the levels measured with LPS stimulation alone. Under normal oxidative conditions, Nrf2 is present in the cytoplasm but unable to operate. When the redox status altered as in the case of inflammation, this transcription factor can move into the nucleus of the cell and affects on the ARE found in the promoter regions of

genes encoding phase II detoxification enzymes and antioxidant proteins.[15] A group of Nrf2 target genes cooperatively function to repress proinflammatory signals, such as those of TNF-α or interleukin-1β. Human peroxiredoxin I (PrxI or PAG) is one of the Nrf2 target genes that are likely to influence the inflammatory process.[49] PrxI was identified as a negative regulator of macrophage migration inhibitory factor (MIF), a crucial factor in the regulation of inflammation and sepsis.[50] Many Nrf2-dependent genes encode antioxidant proteins, which enable cells to counteract nitrosative/oxidative stress, which occurs during inflammation and restore redox homeostasis such as HO-1.[51] HO-1 is the major anti- inflammatory and anti-oxidative enzyme among the genes that are mediated by Nrf2 activation.[17] Several studies have suggested that the activation of HO-1 counteracts the inflammatory and oxidative damage that occurs in LPS-induced inflammation.[18] HO-1 also plays a protective role in LPS-induced acute lung injury in rats.[52] Our findings demonstrated that RSV enhanced Nrf2 and HO-1 expression in LPS-induced RAW 264.7 macrophages. These observations indicated that RSV can exert its anti-inflammatory activity by up regulating the Nrf2/HO-1 signaling pathway.

SIRT1 is an enzyme of SIRTs that can transfer from the cytoplasm to the nucleus, there it starts to deacetylate nucleosomal histones and contribute to telomere maintenance.[53]In addition, SIRT1 deacetylates other nuclear transcription factors such as Peroxisome proliferator-activated receptor-α (PPAR-α),[54] p53, [55] and can repress NF-κβ activity.[56] Hence, the observed inhibition of NF-κβ activity by RSV may be mediated via its activation of SIRT1 activity and expression, the fact that needs further study. Activation of SIRT1 by RSV may also increase the expression of several transcription factors, which increases the target genes of antioxidant enzyme defenses, such as catalase and glutathione peroxidase, to reduce ROS levels in inflamed tissues, as another mechanism for RSV anti-inflammatory activity and accounts for its potent antioxidant properties.[15]

Conclusions

In conclusion, RSV exhibits potent anti-inflammatory activities, particularly in relatively large doses (100 mg/kg) in different inflammation and osteoarthritis animal models. Moreover, RSV showed a potent inhibitory effect on LPS-induced NO, PGE2 and LTB4 production in RAW 264.7 murine macrophage cells, the effect that was accompanied by inhibition of the LPS-induced mRNA expression of iNOS, COX-2 and 5-LOX. Furthermore, these effects were associated with modulatory effects of RSV on SIRT1, HO-1/Nrf2 and NF-κβ activities and mRNA expression in LPS-stimulated RAW 264.7 murine macrophage cell culture. Results of this study suggest that RSV has remarkable anti-inflammatory activity with inhibitory effect on COX/5-LOX and NF-Kβ and activation of SIRT1 and HO-1/Nrf2 pathway. The present study directs the attention toward the potential mechanisms of the anti-inflammatory and antiosteoarthretic actions of RSV as promising alternative for the traditional NSAIDs and selective COX-2 inhibitors for the treatment of inflammatory diseases.

Conflict of interest

The authors do not have any conflict of interest.

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Tables

Table 1. Effect of resveratrol, RSV (25, 50 and 100 mg/kg, p.o.) on MIA-induced osteoarthritis in rats. The control group was treated with vehicle contains 0.5% carboxymethyl cellulose (CMS). Diclofenac (Dic., 10 mg/kg, p.o) was used as reference standard.

Treatment (mg/kg)	Change in hind paw weight distribution (%)		
	0 day	7 day	14 day
Control (0.5% CMS)	32.8 ± 1.3	24.6 ± 0.7	24.4 ± 1.1
Diclofenac (10)	32.8 ± 1.5	28.6 ± 0.6 [b]	30.8 ± 1.4 [b]
RSV (25)	32.7 ± 1.7	25.4 ± 0.6	24.5 ± 1.2
RSV (50)	32.7 ± 1.4	26.3 ± 0.8 [a]	25.9 ± 0.8
RSV (100)	32.7 ± 1.9	28.4 ± 0.7[b]	29.3 ± 0.9 [a]

The values represent the mean ± S.E.M (n = 8).

[a] $p < 0.05$ vs. vehicle control group.

[b] $p < 0.01$ vs. vehicle control group.

Table 2. Primer pairs used for the determination of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), 5-lipooxygenase (5-LOX), Sirtuin-1 (SIRT1), heme oxygenase 1 (HO-1), Nuclear Factor Erythroid 2-Like 2 (Nrf2) and nuclear factor kappa-beta (NF-κβ) gene expression with respect to B-actin in RAW264.7 murine macrophage cell culture.

	Forward primer (5`→ 3`)	Reverse primer (5`→ 3`)
iNOS	CCCTTCCGAAGTTTCTGGCAGC	GGCTGTCAGAGCCTCGTGGCTT
COX-2	TCTCCAACCTCTCCTACTAC	GCACGTAGTCTTCGATCACT
5-LOX	GGCACCGACGACTACATCTAC	CAATTTTGCACGTCCATCCC
SIRT-1	CGGTCTGTCAGCATCATCTTCC	CGCCTTATCCTCTAGTTCCTGTG
HO-1	CGTGCAGAGAATTCTGAGTTC	AGACGCTTTACGTAGTGCTG
Nrf2	GATTCGTGCACAGCAGCA	GCCAGCTGAACTCCTTAGAC
NF-KB	GGGTCAGAGGCCAATAGAGA	CCTAGCTTTCTCTGAACTGCAAA
β-actin	TCATGAAGTGTGACGTTGACATCCGT	CCTAGAAGCATTTGCGGTGCACGATG

Figures

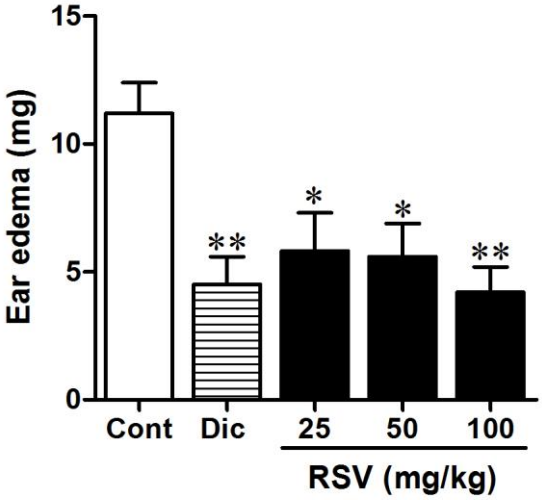

Figure 1. Effects of resveratrol, (RSV, 25, 50 and 100 mg/kg, p.o) on xylene-induced ear edema in mice. Diclofenac (Dic., 10 mg/kg, p.o) was used as reference standard. Increase in ear thickness (%) was attained as it follows; {(Right ear thickness−Left ear thickness)/Left ear thickness}×100. The values are expressed as the mean ± S.E.M. (n = 8).

*$p < 0.05$ vs. the vehicle control group.

**$p < 0.01$ vs. the vehicle control group.

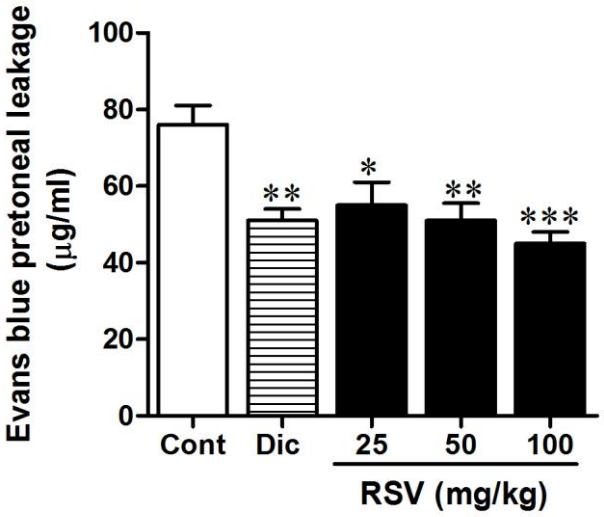

Figure 2. Effects of resveratrol (RSV, 25, 50 and 100 mg/kg, p.o) on acetic acid-induced vascular permeability in mice. Diclofenac (Dic., 10 mg/kg, p.o) was used as reference standard. Evans blue dye leakage was determined 20 min after acetic acid injection. The values are expressed as the mean ± S.E.M. (n = 8).

*$p < 0.05$ vs. the vehicle control group.

**$p < 0.01$ vs. the vehicle control group.

***$p < 0.001$ vs. the vehicle control group.

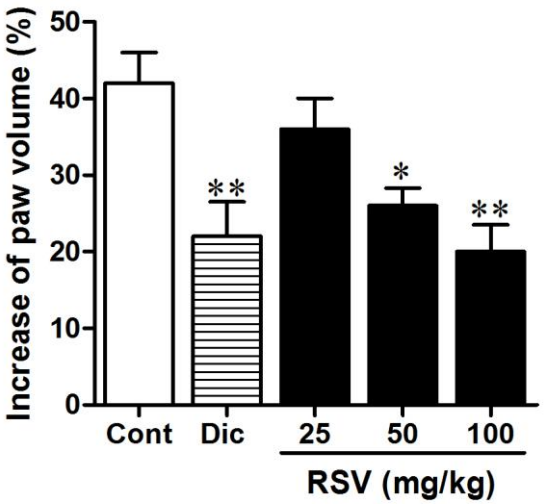

Figure 3. Effects of resveratrol (RSV, 25, 50 and 100 mg/kg, p.o) on carrageenan-induced paw edema in rats. Diclofenac (Dic., 10 mg/kg, p.o) was used as reference standard. Increase of paw volume (%) was attained by following equation; (a−b)/b100.

The values are expressed as the mean ±S.E.M. (n = 8).

*$p < 0.05$ vs. the vehicle control group.

**$p < 0.01$ vs. the vehicle control group.

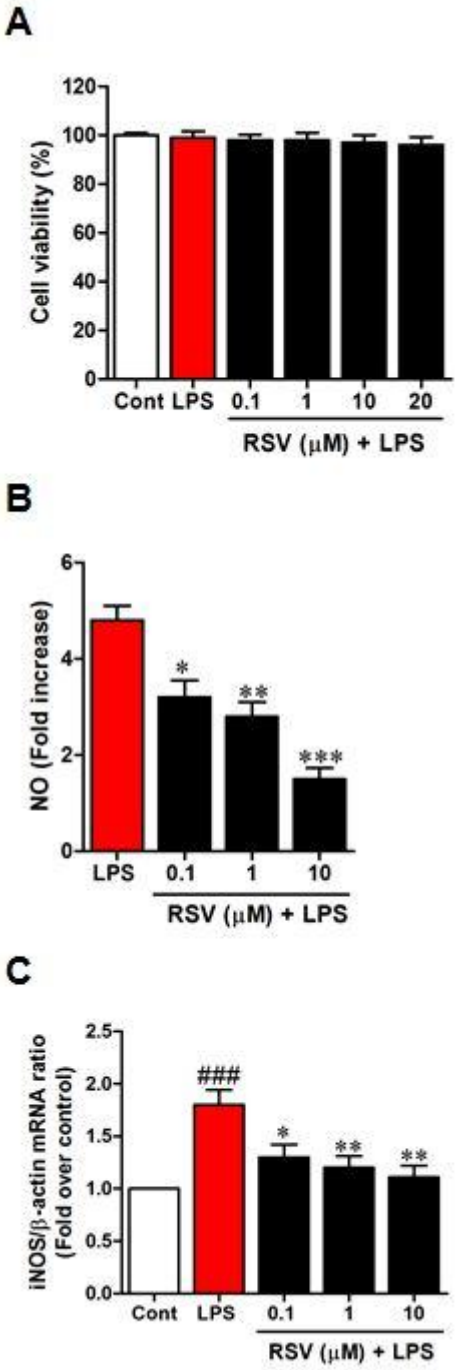

Figure 4: The effects of resveratrol (RSV, 0.1, 1 and 10 μM) on LPS (1 μg/ml) -induced changes in (A) RAW 264.7 murine macrophages cell viability (B) NO level (C) iNOS mRNA expression in RAW264.7 cells. Values represent means ± SEM of Four independent experiments. The values are expressed as the mean ± SEM. (n = 4).

###p< 0.001 vs. the vehicle control group.

*p< 0.05 vs. LPS-treated group.
**p< 0.01 vs. LPS-treated group.
***p< 0.001 vs. LPS-treated group.

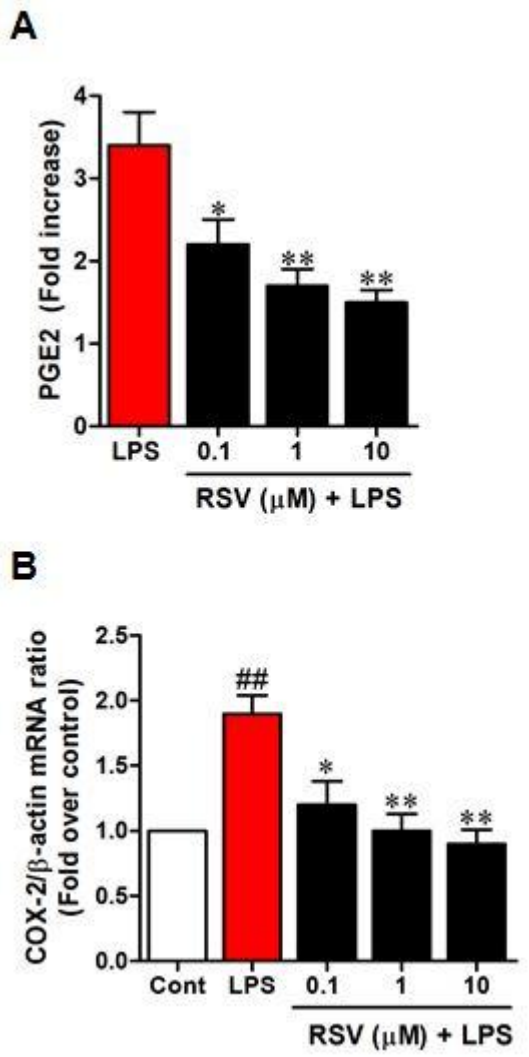

Figure 5: The effects of resveratrol (RSV, 0.1, 1 and 10 μM) on LPS (1 μg/ml) -induced changes in (A) PGE2 level (B) COX-2 mRNA expression in RAW264.7 cells. Values represent means ± SEM of four independent experiments.

##p< 0.01 vs. the vehicle control group.

*p< 0.05 vs. LPS-treated group.

**p< 0.01 vs. LPS-treated group.

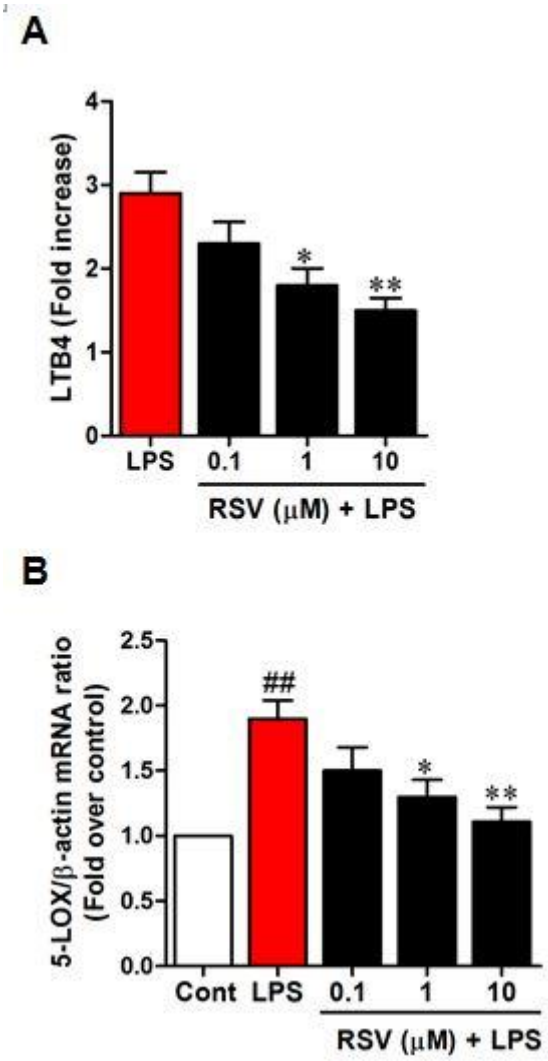

Figure 6: The effects of resveratrol (RSV, 0.1, 1 and 10 μM) on LPS (1 μg/ml) -induced changes in (A) LTB4 level (B) 5-LOX mRNA expression in RAW264.7 cells. Values represent means ± SEM of four independent experiments.

##$p < 0.01$ vs. the vehicle control group.

*$p < 0.05$ vs. LPS-treated group.

**$p < 0.01$ vs. LPS-treated group.

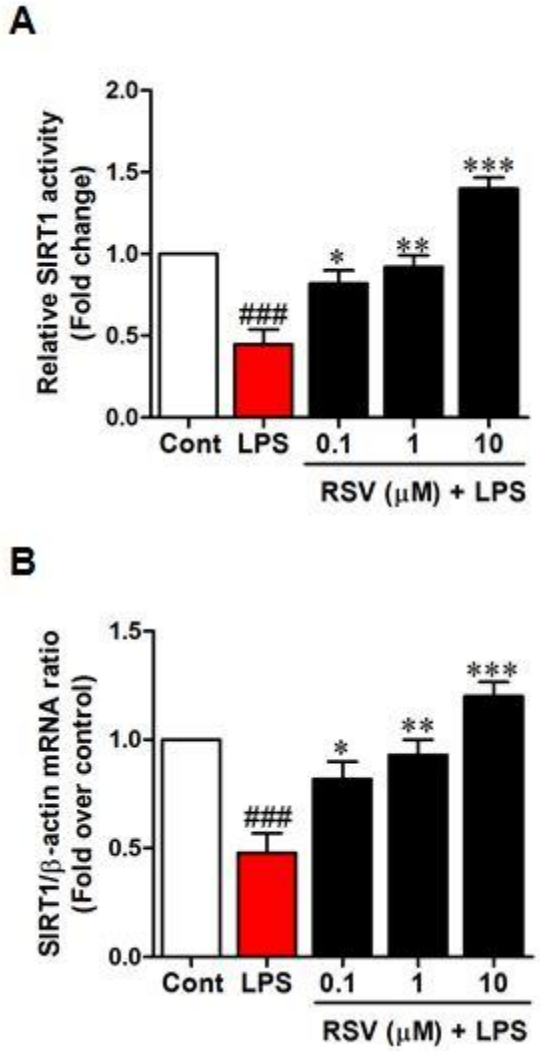

Figure 7: The effects of resveratrol (RSV, 0.1, 1 and 10 μM) on LPS (1 μg/ml) -induced changes in (A) SIRT1 activity (B) SIRT1 mRNA expression in RAW264.7 cells.

Values represent means ± SEM of four independent experiments.

###p< 0.001 vs. the vehicle control group.

*p< 0.05 vs. LPS-treated group.

**p< 0.01 vs. LPS-treated group.

***p< 0.001 vs. LPS-treated group.

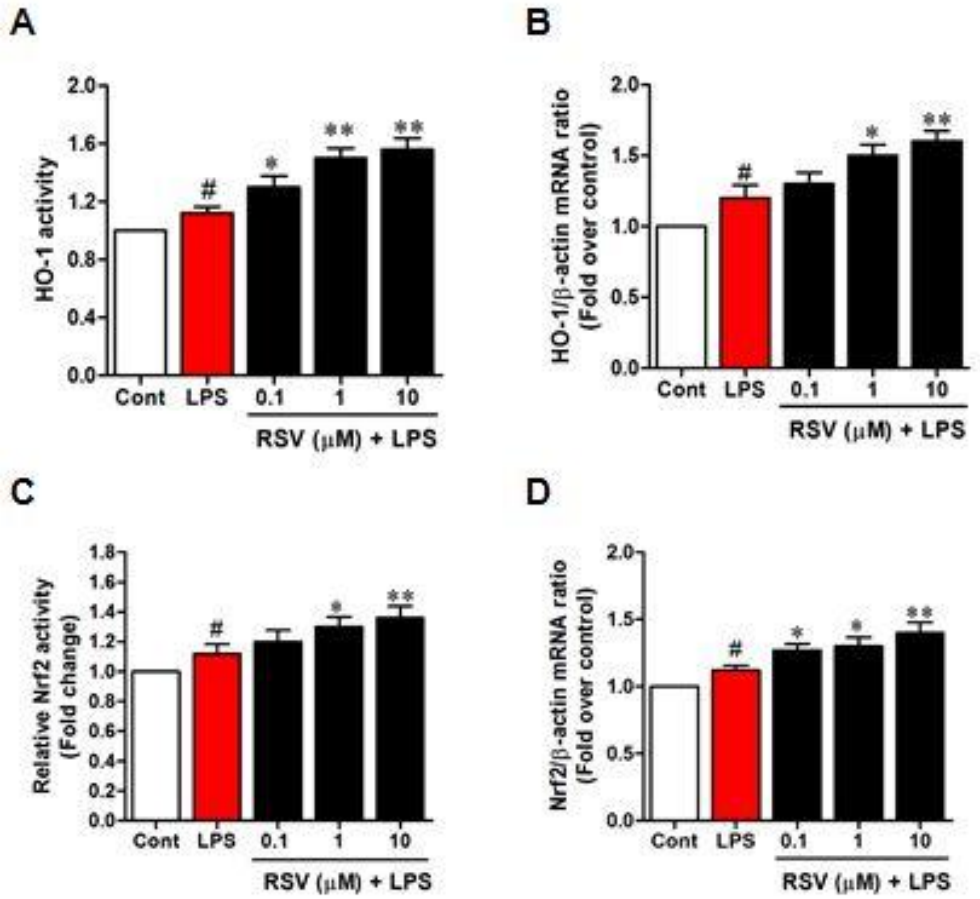

Figure 8: The effects of resveratrol (RSV, 0.1, 1 and 10 μM) on LPS (1 μg/ml) -induced changes in (A) HO-1 activity (B) HO-1 mRNA expression (C) Nrf2 activity (D) Nrf2 mRNA expression in RAW264.7 cells. Values represent means ± SEM of four independent experiments.

#p< 0.05 vs. the vehicle control group.

##p< 0.01 vs. the vehicle control group.

*p< 0.05 vs. LPS-treated group.

**p< 0.01 vs. LPS-treated group.

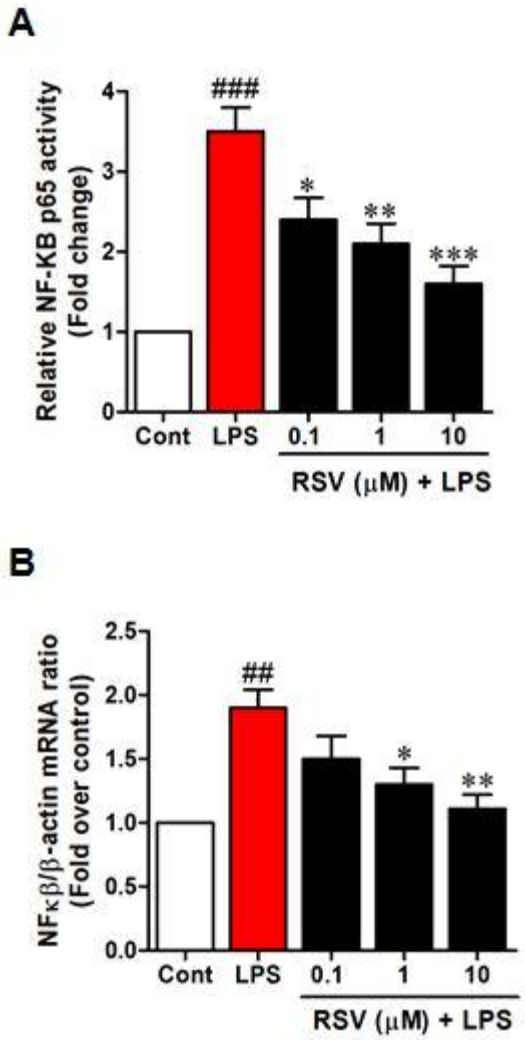

Figure 9: The effects of resveratrol (RSV, 0.1, 1, 10 μM) on LPS (1 μg/ml) -induced changes in (A) NF-κβ activity and (B) NF-κβ mRNA expression in RAW264.7 cells.

Values represent means ± SEM of four independent experiments.

##p< 0.01 vs. the vehicle control group.

###p< 0.001 vs. the vehicle control group.

*p< 0.05 vs. LPS-treated group.

**p< 0.01 vs. LPS-treated group.

***p< 0.001 vs. LPS-treated group.