Daphnetin-mediated Nrf2 antioxidant signaling pathways ameliorate tert-butyl hydroperoxide (*t*-BHP)-induced mitochondrial dysfunction and cell death

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ARTICLE INFO

Keywords:

Daphnetin

Oxidative damage

Mitochondrial dysfunction

ROS

Nrf2

ABSTRACT

Daphnetin (Daph), a natural coumarin derivative isolated from plants of the *Genus Daphne*, possesses abundant biological activities, such as anti-inflammatory, antioxidant and anticancer properties. In the present study, we focused on investigating the protective effect of Daph against *tert*-butyl hydroperoxide (*t*-BHP)-induced oxidative damage, mitochondrial dysfunction and the involvement of underlying molecular mechanisms. Our findings indicated that Daph effectively inhibited *t*-BHP-stimulated cytotoxicity, cell apoptosis, and mitochondrial dysfunction, which are associated with suppressed reactive oxygen species (ROS) generation, decreased malondialdehyde (MDA) formation, increased superoxide dismutase (SOD) levels and glutathione (GSH)/GSSG (oxidized GSH) ratio. Further investigation indicated that Daph significantly suppressed cytochrome *c* release and NLRP3 inflammasome activation and modulated apoptosis-related protein Bcl-2, Bax, and caspase-3 expression. Moreover, Daph dramatically induced the expression of the glutamate-cysteine ligase modifier (GCLM) subunit and the glutamate-cysteine ligase catalytic (GCLC) subunit, heme oxygenase-1 (HO-1), and NAD (P) H: quinone oxidoreductase (NQO1), which is largely dependent on upregulating the nuclear factor-erythroid 2-related factor 2 (Nrf2) nuclear translocation, reducing the Keap1 protein expression, and strengthening the antioxidant response element (ARE) promoter activity. Additionally, Daph remarkably activated a c-Jun NH2-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) phosphorylation, but ERK and JNK inhibitor pretreatment exhibited an evident decrease of the level of Daph-enhanced Nrf2 nuclear translocation. Furthermore, Daph exposure suppressed *t*-BHP-induced cytotoxicity and ROS overproduction, which are mostly blocked in Nrf2 knockout RAW 264.7 cells and peritoneal macrophages. Accordingly, Daph exhibited protective roles against *t*-BHP-triggered oxidative damage and mitochondrial dysfunction by the upregulation of Nrf2 antioxidant signaling pathways, which may be involved in the activation of JNK and ERK.

1. Introduction

Nuclear factor-erythroid 2-related factor 2 (Nrf2), an essential transcription factor, is required to ameliorate the progression of various diseases, especially those resulting from oxidative stress [1]. Previous studies have indicated that Nrf2 activators effectively attenuate renal damage and preserve renal function in streptozotocin-induced diabetic Nrf2^{+/+} mice, whereas this protective effect is not observed in Nrf2^{-/-} mice. This indicates that targeting Nrf2 activation might be used therapeutically to ameliorate metabolic disorders [2]. Under physiological conditions, Nrf2 is sequestered in the cytoplasm by Keap1 (Kelch-like ECH-associated protein 1, also known as a repressor of Nrf2). Under stress conditions, Nrf2 detaches from Keap1, translocates into the

nucleus and binds to the antioxidant response element (ARE), which leads to the expression of several antioxidant and detoxification genes, such as heme oxygenase-1 (HO-1), superoxide dismutase (SOD), NAD (P) H: quinone oxidoreductase (NQO1), the glutamate-cysteine ligase modifier (GCLM) and the catalytic (GCLC) subunit, which are necessary for glutathione (GSH) biosynthesis [3,4]. Additionally, increasing evidence suggests that multiple signal transduction pathways, including phosphatidylinositol 3-kinase (PI3K) [5], protein kinase C (PKC) [6,7] and mitogen-activated protein kinase (MAPK) pathways [8], play crucial roles in the regulation of the Nrf2 pathway, which can similarly induce the expression of various antioxidant enzymes. These antioxidant enzymes are essential for maintaining cellular redox homeostasis against oxidative injury [9].

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<http://dx.doi.org/10.1016/j.freeradbiomed.2017.02.016>

Received 4 November 2016; Received in revised form 25 January 2017; Accepted 6 February 2017

Available online 07 February 2017

0891-5849/ © 2017 Published by Elsevier Inc.

Oxidative damage and mitochondrial dysfunction are strongly related to the pathogenesis of various diseases, such as age-related macular degeneration (AMD), Parkinson's disease, heart ischemia/reperfusion injury, alcoholic hepatitis, diabetes and cardiovascular diseases [10]. Excess reactive oxygen species (ROS) generation and release induces a variety of oxidative stress responses, which further exacerbate mitochondrial dysfunction and result in cell injury and death to accelerate disease progression [11,12]. Numerous reports have suggested that cytochrome *c* is released into the cytoplasm, which is recognized as a marker of severe mitochondrial injury [13]. In fact, cell apoptosis results in a change in a battery of protein expression levels, such as the upregulation of antiapoptotic protein Bcl-2, the down-regulation of the proapoptotic protein Bax and the reduction of caspase 3 activity [14]. Moreover, oxidative stress both directly activates inflammatory responses and induces mitochondrial dysfunction, which plays a key role in further modulating inflammation. In contrast, mitochondrial dysfunction also boosts oxidative stress and results in membrane permeability transition (MPT) and mtDNA translocation to the cytoplasm [15]. Cytosolic mtDNA in bone marrow-derived macrophages (BMDMs) and increased ROS initiate NACHT, LRR and PYD domain-containing protein 3 (NLRP3) inflammasome assemblies [16,17], which promote caspase-1 activation with subsequent pro-inflammatory IL-1 β and IL-18 release, resulting in inflammatory responses [18]. Accumulating evidence suggests that Nrf2, as a key target of ROS inhibition, is essential for suppressing ROS-induced inflammasome activation [19].

Intriguingly, numerous drugs, including abundant phytochemicals, can induce activation of the Nrf2 signaling pathway, which increases the capacity of antioxidation and decreases apoptotic cell death by reversing the protein levels of Bcl-2, Bax and caspase 3 in apoptotic cells [20]. To date, abundant natural product-derived compounds, such as polyphenols, quinones, flavonoids and coumarins, have been widely accepted as alternative and adjuvant medicines for various pathologies, including inflammation, oxidative stress, cancer and apoptosis, via upregulation of the Nrf2/ARE signaling pathway [1,21]. Daphnetin (7, 8-dihydroxycoumarin, Daph; Fig. 1A), a natural coumarin derivative, is isolated from plants of the genus *Daphne* and has been used clinically in the treatment of various diseases, such as rheumatoid arthritis and coagulation disorders [22]. Furthermore, recent reports have suggested that Daph effectively attenuates oxidative stress-related hepatotoxicity and inhibits DMBA-induced mammary carcinogenesis by activating the Nrf2 signaling pathway [23,24]. However, the cytoprotective effect of Daph against oxidative damage and mitochondrial dysfunction and the underlying molecular mechanisms have yet to be elucidated. Consequently, we investigated the cytoprotective effect of Daph and the mechanism related to oxidative stress and mitochondrial dysfunction in *t*-BHP-induced RAW 264.7 cells.

2. Material and methods

2.1. Reagents and chemical

Daphnetin (Daph) with a purity > 98% was purchased from the National Institute for Food and Drug Control. *tert*-butyl hydroperoxide (*t*-BHP), hydrogen peroxide (H₂O₂), and Pyocaine (Pyo, a ROS inducer), or N-acetyl-L-cysteine (NAC, a ROS inhibitor), GF109203X, LY294002, SP600125, U0126, and SB203580 (specific inhibitors of the PKC δ , PI3K/AKT, JNK1/2, ERK1/2, and p38, respectively) were provided by Sigma-Aldrich (St. Louis, MO). Biotin-PEAC5-maleimide (BPM) or an HRP-linked anti-biotin antibody were offered by DOJINDO (Kumamoto, Japan) or (Cell Signaling Technology, Beverly, MA), respectively. Penicillin and streptomycin, Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were acquired from Invitrogen-Gibco (Grand Island, NY). Antibodies against Nrf2, GCLC, GCLM, HO-1, Keap1, NQO1, p-PI3K, PI3K, p-AKT, AKT, p-JNK, JNK, p-PKC δ , p-ERK, ERK, p-p38, p38, NLRP3, caspase-1, ASC, IL-1 β ,

casapase-3, Bax, Bcl-2, cytochrome *c*, Lamin B and β -actin were supplied by Cell Signaling (Boston, MA, USA) or Abcam (Cambridge, MA, USA). Prime-Script RT-PCR kit and Faststart Universal SYBR Green Master were offered by Takara (Dalian, China) and Roche (Basel, Switzerland), respectively. In addition, SOD and MDA test kit were supplied by Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The GSH and GSSG test kits were purchased from Beyotime Biotechnology (Shanghai, China). The Annexin V fluorescein isothiocyanate (FITC)/Propidium Iodide (PI) apoptosis kit and the lipophilic cationic probe 5, 5', 6, 6' -tetrachloro-1, 1', 3, 3' -tetraethyl-benzimidazolyl -carbocyanine iodide (JC-1) were offered from Beyotime Institute of Biotechnology (Jinlin, China). All other chemicals were offered by Sigma-Aldrich (St. Louis, MO, USA), if not otherwise indicated.

2.2. Animals

Wild-type (WT) and Nrf2^{-/-} (knockout) C57BL/6 mice were purchased from Liaoning Changsheng Technology Industrial Co., LTD (Certificate SCXK2010-0001; Liaoning, China) or The Jackson Laboratory (Bar Harbor, ME, USA), respectively. All animals were raised under SPF-condition after feeding for several days. All studies were in accordance with the International Guiding Principles for Biomedical Research Involving Animals, which was published by the Council for the International Organizations of Medical Sciences.

2.3. Cell culture

The RAW 264.7 mouse macrophage cell line, supplied by the China Cell Line Bank (Beijing China), was maintained in DMEM medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL of penicillin, 100 U/mL of streptomycin and 3 mM glutamine at 37 °C in a humidified atmosphere containing 5% CO₂. In all experiments, cells were allowed to acclimate for 24 h before any treatments.

2.4. Generation of peritoneal macrophage

WT and Nrf2^{-/-} mice were injected intraperitoneally with 700 μ g of alum diluted in sterile PBS for 5 days of stimulation. Then, PEMs were isolated by flushing the peritoneal cavity with 10 mL ice cold PBS. Cells were centrifuged and resuspended in serum free RPMI-1640 with 1% L-glutamine and 1% Pen/Strep, pooled and plated with a density of 2.5 $\times$ 10⁴ cells/cm² and incubated at 37 °C and 5% CO₂ 2 h for PEMs to adhere. Subsequently, took 1 $\times$ 10⁴ adherent cells and stained with the cell surface markers CD11b, and more than 95% of cells were CD11b⁺ by flow cytometry analysis (LSR II Flow Cytometer; BD Biosciences, San Jose, CA, USA).

2.5. MTT assay

RAW 264.7 cells were grown in 96-well plates (2 $\times$ 10⁴ cells/well) for 24 h and then treated with Daph (2.5, 5 or 10 μ g/mL) for 18 h and subsequently stimulated with *t*-BHP (5 mM) for an additional 3 h. Additionally, Nrf2^{-/-} RAW 264.7 cells were grown in 96-well plates (2 $\times$ 10⁴ cells/well) for 24 h and then subjected to Daph (10 μ g/mL) for 18 h and exposed to *t*-BHP (5 mM) for an additional 3 h. Moreover, WT and Nrf2^{-/-} peritoneal macrophages were grown in 96-well plates at a concentration of 2 $\times$ 10⁴ cells/well for 24 h, exposed to Daph (10 μ g/mL) for 18 h and stimulated by *t*-BHP (5 mM) for an additional 3 h. Cell viability was measured by MTT assay in accordance with the manufacturer's instructions. MTT (5 mg/mL) was added to the cells and incubated for another 4 h; the supernatant was removed, and DMSO was added to each well to lyse the cells. The absorbance of MTT was measured at 570 nm.

Fig. 1. Effects of Daph on *t*-BHP-induced RAW 264.7 cell cytotoxicity, apoptosis and apoptosis-related proteins expression. (A) The chemical structure of daphnetin (Daph). RAW 264.7 cells were subjected to Daph (2.5, 5 or 10 µg/mL) for 18 h, subsequently stimulated with *t*-BHP (5 mM) for 3 h. (B) Cell viability was determined by MTT assay, and (C–D) the percentage of cell apoptosis and necrosis were measured using flow cytometry. In addition, Cells were exposed to Daph (2.5, 5 or 10 µg/mL) for 1 h, and then treated with or without *t*-BHP (12.5 µM) for an additional 18 h. (E and F) The expression of apoptosis-related proteins (Bax, Bcl-2 and caspase-3) were determined by Western blot analysis. (G and H) Quantification of HO-1 protein expression was performed by densitometric analysis and β-actin was acted as an internal control. All results were expressed as means ± SEM of three independent experiments. **p* < 0.05, ***p* < 0.01 vs the control (Con) group; ^{##}*p* < 0.05, ^{###}*p* < 0.01 vs the *t*-BHP group.

2.6. Apoptosis quantification

RAW 264.7 cells were grown in a 12-well plate (4×10^5 cells/well) for 24 h and were then pretreated with different dosages of Daph (2.5, 5 or 10 $\mu\text{g/mL}$) for 18 h followed by exposure to *t*-BHP (5 mM) for an additional 1 h. Cells were washed twice with ice-cold PBS, collected and centrifuged at 1500 rpm for 5 min at 4 °C. Next, cells were examined with FITC-labeled annexin V and propidium iodide staining, and the percentages of apoptosis and necrosis were detected using flow cytometry.

2.7. Intracellular ROS measurement

To detect intracellular ROS production, RAW 264.7 cells were seeded into 96-well plates (2×10^4 cells/well) for 24 h and then in serum-free DMEM for 6 h; the cells were then pretreated with different dosages of Daph or NAC (a ROS inhibitor) for 18 h. Next, the cells were incubated with 50 μM of DCFH-DA for 30 min and subsequently exposed to *t*-BHP (5 mM), H_2O_2 , or Pyocine (a ROS inducer) for 5 min to trigger ROS generation. DCF fluorescence intensities were detected by flow cytometry or a multi-detection reader at excitation and emission wavelengths of 485 and 535 nm, respectively.

2.8. Measurement of intracellular GSH/GSSG ratio, SOD and MDA levels

RAW 264.7 cells were plated into a 60 mm² dish (3×10^6 cells/well) for 24 h and then pretreated with Daph (2.5, 5 or 10 $\mu\text{g/mL}$) for 18 h; the cells were then exposed to *t*-BHP (5 mM) for 1 h. Next, levels of intracellular hydrogen peroxide (H_2O_2) and superoxide (O_2^-), GSH, GSSG, SOD and MDA were quantified using commercially available kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) in accordance with the manufacturer's instructions.

2.9. JC-1 assay for mitochondrial membrane potential (MMP)

Mitochondrial membrane potential ($\Delta\Psi$) was assessed by JC-1 staining. Briefly, RAW 264.7 cells were grown in 12-well plates at a density of 4×10^5 cells/well for 24 h. Subsequently, the cells were pretreated with different dosages of Daph (2.5, 5 or 10 $\mu\text{g/mL}$) for 18 h, followed by exposure to *t*-BHP (5 mM) for an additional 1 h. Next, the cells were washed with PBS and incubated with JC-1 (10 $\mu\text{g/mL}$) at 37 °C in the dark for 20 min. The $\Delta\Psi\text{m}$ was analyzed for intracellular fluorescence by flow cytometry.

2.10. Western blot analysis

The cells were lysed in a RIPA buffer with protease and phosphatase inhibitors for 15 min. The protein concentrations were measured using a BCA protein assay kit (Beyotime, China). An equal amount of protein (20 μg) for each sample was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% gel and then electrophoretically transferred onto a polyvinylidene difluoride membranes (PVDF), which was purchased from Bio-Rad (Hercules, CA). The membrane was blocked with blocking solution (5% (w/v) nonfat dry milk) for 2 h, and followed by an overnight incubation at 4 °C with specific primary antibody, including cytochrome c, caspase-3, Bax, Bcl-2, Nrf2, GCLC, GCLM, HO-1, Keap1, NQO1, p-PI3K, PI3K, p-AKT, AKT, p-JNK, JNK, p-PKC δ , p-ERK, ERK, p-p38, p38, NLRP3, caspase-1, ASC, IL-1 β , Lamin B and β -actin. Next day, after thoroughly washing with PBST for three times, the membrane was incubated for an additional 2 h with a peroxidase conjugated secondary antibody at room temperature, and followed by ECL detection (Millipore corporation, Billerica, MA, USA). Band intensities were quantified by using Image J gel analysis software. The experiments were repeated three times for each experimental condition.

2.11. Preparation of nuclear and cytosolic fractions

The nuclear and cytoplasmic extracts were prepared using an NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Pierce Biotechnology, Rockford, IL, USA) in accordance with the manufacturer's instructions. All steps were carried out on ice or at 4 °C unless stated otherwise.

2.12. Total RNA extraction and qPCR

Total RNA was isolated from cells using the Trizol reagent, and then reverses transcription into cDNA by employing the Prime-Script RT-PCR kit (Takara) in accordance with the procedure described by the manufacturer. The following PCR primer sequences (forward and reverse, respectively) were used: Mouse GCLC: 5'-CAC TGC CAG AAC ACA GAC CC, 5'-ATG GTC TGG CTG AGA AGC CT-3'; Mouse GCLM: 5'-GGG CAC AGG TAA AAC CCA AT-3', 5'-GCT TCC TGG AAA CTT GCC TC-3'; NQO-1: 5'-GCC TGA GCC CAG ATA TTG TG-3', 5'-GGA AAG GAC CGT TGT CGT-3'; HO-1: 5'-GAG ATA GAG CGC AAC AAG CAG-3', 5'-CTT GAC CTC AGG TGT CAT CTC-3'; β -actin: 5'-TCC TTC GTT GCC GGT CCA CA-3', CGT CTC CGG AGT CCA TCA CA-3'. Gene expression changes were calculated by the comparative Ct method and the values were analyzed by normalizing with β -actin mRNA expression.

2.13. Measurement of free -SH groups and Keap1 modification by Daph

As previously described [25], Keap1 was treated with Daph for 30 min at 25 °C in Tris-HCl. Aliquots of the reaction mixture were mixed with a solution containing of reaction buffer. After 2 min, the concentration of thionitrobenzoic acid released was measured by a multi-detection reader at 412 nm. In addition, a BPM-labeling assay was performed to analyze the availability of protein thiol groups after exposure to Daph. In this procedure, the target protein (s) treat with Daph, and the reaction mixture is then subjected to BPM. In the cell-free system, recombinant murine Keap1 protein was reacted with Daph for 30 min at Tris-HCl and then subjected to 50 μM BPM for 30 min at 37 °C. The resulting protein was analyzed by Western blot analysis with an HRP-linked anti-biotin antibody.

2.14. CRISPR/Cas9 knockout of Nrf2 gene

RAW 264.7 cells were cultured in 12-well plates at the density of 6×10^5 cells/well for 24 h. The plasmids of expressing Cas9 with Nrf2-sgRNA and puromycin resistant gene were co-transfected into RAW 264.7 cells using Viafect transfection reagent (Promega). At 48 h after transfection, cells were added puromycin at a concentration of 2 $\mu\text{g/mL}$ and harvested for immunoblotting analysis with Nrf2 antibody. After 7 days, cells were cultured in 96-well plates (1 cell/well). The efficiency of gene editing was evaluated by the Western blot and DNA sequencing was performed to confirm the edited gene of Nrf2.

2.15. ARE promoter activity

RAW 264.7 cells were grown in 96-well plates (2×10^4 cells/well) until reaching approximately 75% confluence. According to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA), pGL4.37 and pGL4.74 plasmids were transfected into cells using Viafect transfection reagent. After Daph (10 $\mu\text{g/mL}$) treatment for different time periods, we used a dual-luciferase reporter assay system (Dual-Glo[®] Luciferase Assay System) for detecting and analyzing ARE-driven promoter activity.

2.16. Statistical analysis

All data referenced above were expressed as the means $\pm$ SEM and analyzed using SPSS19.0 (IBM). Comparisons between experimental

groups were conducted using one-way ANOVA, whereas multiple comparisons were made using the LSD method. Statistical significance was defined as $p < 0.05$ or $p < 0.01$.

3. Results

3.1. Daph inhibited *t*-BHP-induced cytotoxicity and reduced cell apoptosis and apoptosis-related protein expression in RAW 264.7 cells

t-BHP, a chemical toxin, can result in various cellular injuries by inducing oxidative stress in biological systems. Thus, to investigate the potential cytoprotective effect of Daph, we employed *t*-BHP-induced toxicity in RAW 264.7 cells, and cell viability was determined by an MTT assay. Our results indicated that Daph remarkably protected the cells from *t*-BHP-triggered oxidative cytotoxicity (Fig. 1B). Moreover, *t*-BHP also significantly led to cell death by promoting apoptosis and necrosis in total cells, whereas Daph effectively reduced *t*-BHP-induced cell apoptosis at the most effective concentration of 10 $\mu\text{g/mL}$ (Fig. 1C and D). To further demonstrate the possible mechanisms of the role of Daph in anti-apoptosis, we tested its effect on *t*-BHP-induced apoptosis-related proteins. In this study, we found that 10 $\mu\text{g/mL}$ of Daph dramatically increased Bcl-2 protein expression and the form of procaspase-3. In contrast, 10 $\mu\text{g/mL}$ of Daph dramatically decreased Bax protein expression and the form of cleaved-caspase-3 (Fig. 1E–H).

3.2. Daph decreased *t*-BHP-induced ROS generation and MDA formation and increased the GSH/GSSG ratio and SOD content in RAW 264.7 cells

Because *t*-BHP promotes ROS overproduction and MDA formation, which induce cellular oxidative injury, we investigated whether Daph-mediated protection against *t*-BHP-stimulated oxidative damage is associated with reducing intracellular ROS production and MDA formation in RAW 264.7 cells. In this study, we found that *t*-BHP treatment enhanced ROS, H_2O_2 and O_2^- production and MDA formation, which were inhibited by Daph exposure (Fig. 2A–E). In addition, the formation of ROS was measured by the GSH-to-GSSG ratio, which was significantly higher in the Daph-treated group than in the *t*-BHP-induced group (Fig. 2F). SOD is thought to be an essential antioxidant that protects against *t*-BHP-induced oxidative damage. Fig. 2G shows that *t*-BHP exposure effectively enhanced SOD depletion, whereas Daph treatment considerably reduced *t*-BHP-induced SOD depletion.

3.3. Daph ameliorated *t*-BHP-induced mitochondrial dysfunction in RAW 264.7 cells

Mitochondria, which play a vital role in the process of apoptosis in different cells, are strongly associated with the loss of mitochondrial membrane potential (MMP, $\Delta\Psi\text{m}$). To investigate whether Daph inhibited *t*-BHP-induced apoptosis related to the mitochondria pathway, RAW 264.7 cells were pretreated with Daph and exposed to *t*-BHP (5 mM) to induce mitochondrial dysfunction, and JC-1 staining was then used to detect the change of $\Delta\Psi\text{m}$. Numerous reports have indicated that normal cells exhibit JC-1 red fluorescence, but a reduction of MMP resulted in the replacement of red fluorescence by green JC-1 monomers. Panels A and B in Fig. 3 showed that the $\Delta\Psi\text{m}$ of cells was reduced with *t*-BHP alone but was enhanced after pretreatment with Daph in a dose-dependent manner. These results suggested that Daph inhibited *t*-BHP-induced mitochondrial dysfunction in RAW 264.7 cells. To further determine the protective effect of Daph on mitochondria, we investigated whether Daph could regulate *t*-BHP-induced cytochrome *c* expression in RAW 264.7 cell cytoplasm and mitochondria. Our results showed that various concentrations of Daph inhibited the lower dose (12.5 μM) or higher dose (5 mM) *t*-BHP-induced concentrations in the release of cytochrome *c* into cytoplasm in RAW 264.7 cells (Fig. 3C–F).

3.4. Daph suppressed *t*-BHP-activated NLRP3 inflammasomes in RAW 264.7 cells

Both ROS accumulation and mitochondrial dysfunction are closely associated with activation of the NLRP3 inflammasome. Thus, we detected the *t*-BHP-induced activation of the NLRP3 inflammasome and the inhibitory effect of Daph on the *t*-BHP-activated NLRP3 inflammasome. Given that *t*-BHP exhibits cytotoxicity, we chose different lower dosages of *t*-BHP to stimulate RAW 264.7 cells. Our results indicated that exposing cells to *t*-BHP (12.5 μM) for 18 h did not exhibit cytotoxicity and activated NLRP3, ASC, caspase-1 and IL-1 β protein expression (Fig. 4A and B). Therefore, this dose was chosen as the treatment dose to induce NLRP3 inflammasome activation and investigate the effect of Daph. As shown in Fig. 4C and D, 10 $\mu\text{g/mL}$ of Daph effectively inhibited the *t*-BHP-induced NLRP3, ASC, caspase-1 and IL-1 β protein expression.

3.5. Daph enhanced the expression of GCLC, GCLM, HO-1 and NQO1 protein in RAW 264.7 cells

Recent reports have suggested that various antioxidative enzymes, including GCLC, GCLM, HO-1 and NQO1, play essential roles in the amelioration of oxidative injury. Consequently, we measured whether Daph could induce GCLC, GCLM, HO-1 and NQO1 expression to enhance the resistance of cells to oxidative damage. In our studies, RAW 264.7 cells were exposed to different dosages of Daph for 18 h to detect the most effective dosage for inducing the expression of these proteins (Fig. 5A and B). Additionally, cells were cultured with Daph (10 $\mu\text{g/mL}$) for several time points to test the optimal exposure period for increasing the expression of these genes and proteins (Fig. 5C–F). These observations indicated that cells exposed to 10 $\mu\text{g/mL}$ of Daph for 18 h significantly upregulated the expression of these antioxidative enzymes in RAW 264.7 cells.

3.6. Daph upregulated the Keap1-Nrf2/ARE signaling pathway in RAW 264.7 cells

Due to the Keap1-Nrf2/ARE signaling pathway is essential for regulating antioxidant responses and modulating the expression of numerous antioxidant enzymes, such as GCLC, GCLM, HO-1 and NQO1, we examined whether Daph could upregulate the Keap1-Nrf2/ARE signaling pathway. Cells were treated with Daph (1.25, 2.5, 5 or 10 $\mu\text{g/mL}$) for 6 h, and total protein was then extracted from the cells for Western blot analysis. The results showed that Daph dramatically enhanced the total protein expression of Nrf2 and Keap1 degradation in a dose-dependent manner (Fig. 6A and C). Additionally, our results further indicated that 10 $\mu\text{g/mL}$ of Daph effectively induced an increase in the nuclear expression and a concomitant decrease in the cytoplasmic expression of Nrf2 in a time-dependent manner (Fig. 6B and D). Furthermore, because ARE activation plays a crucial role in increasing Nrf2 nuclear transcription, cells were transiently transfected with an ARE-luciferase plasmid and were maintained with Daph, and changes in luciferase activity then acted as a measure of ARE activation. This result indicated that Daph also remarkably enhanced ARE-driven luciferase activity in a dose- and time-dependent manner (Fig. 6E and F). Due to Keap1 modification by an electrophile could be responsible for Nrf2 activation, our further work measured whether Daph was covalently attached to Keap1. Incubation of recombinant murine Keap1 with Daph did not exhibit an inhibition of effect on thiol groups in the protein (Fig. 6G). Under these conditions, Daph did not inhibit the level of Keap1 modified by BPM (Fig. 6H–I), suggesting that Daph covalently binding to Keap1 was difficult.

Fig. 2. Effects of Daph on t-BHP-induced ROS generation, GSH/GSSG ratio, SOD and MDA levels in RAW 264.7 cells. Cells were treated with or without Daph for 18 h, and were stained with 50 µM of DCFH-DA for 30 min, and subsequently exposed to t-BHP (5 mM) for 5 min to induce the ROS generation. (A and B) DCF fluorescence intensities were detected by flow cytometry or a fluorescent microscope. Cells were treated with or without Daph for 18 h, and subsequently exposed to t-BHP (5 mM) for 1 h. (C–G) Effect of Daph on t-BHP-induced production of H₂O₂ and O₂⁻, formation of MDA and depletion of SOD, as well as reduction of GSH/GSSG ratio, were determined using their commercial test kit, respectively. All results were expressed as means ± SEM of three independent experiments. **p* < 0.05, ***p* < 0.01 vs the control group; #*p* < 0.05, ##*p* < 0.01 vs the t-BHP group.

3.7. Daph modulated Nrf2 protein expression via ERK and JNK activation in RAW 264.7 cells

Various signal transduction pathways, including PI3K/Akt, PKCδ and MAPK pathways, are strongly related to the regulation of Nrf2 nuclear translocation. To further investigate whether Daph-induced Nrf2 activation is regulated by these signaling pathways, we determined the effect of Daph on the activation of PI3K/Akt, PKCδ and

MAPK pathways. RAW 264.7 cells were treated with Daph (10 µg/mL) at four time points (1, 3, 6 or 18 h), and total protein was then extracted from these cells for Western blot analysis using specific antibodies. We found that Daph clearly increased ERK and JNK phosphorylation, but this effect was not exhibited in the other pathways (Fig. 7A–L). Simultaneously, cells were pretreated for 1 h with or without LY294002, SP600125, U0126, SB203580 and GF109203X, which are the specific inhibitors of the PI3K/Akt, JNK, ERK, P38 and PKCδ

pathways, respectively, and exposed to Daph (10 $\mu\text{g/mL}$) for 18 h. Subsequently, we investigated the effects of these inhibitors on Nrf2 nuclear translocation and found that Daph-mediated Nrf2 activation

was dramatically inhibited by JNK and ERK kinase inhibitors (Fig. 7M and N). In addition, as shown in Fig. 7O–R, SP600125 and U0126 could effectively inhibit Daph-induced JNK and ERK phosphorylation, re-

Fig. 3. Effects of Daph on *t*-BHP-induced mitochondrial dysfunction in RAW 264.7 cells. Cells were treated with various concentrations of Daph (2.5, 5 or 10 $\mu\text{g/mL}$) for 18 h, and subsequently exposed to *t*-BHP (5 mM) for 1 h. (A and B) The effects of Daph on mitochondrial membrane potential were tested using the JC-1 method and were determined by flow cytometry and a fluorescent microscope. (C) Cytochrome *c* was measured in cytosolic and mitochondrial cells using Western blot analysis. (D) Cells were treated with various concentrations of Daph (2.5, 5 or 10 $\mu\text{g/mL}$) for 1 h, and subsequently exposed to *t*-BHP (12.5 μM) for an additional 24 h. The expression of cytochrome *c* protein in the cytosolic and mitochondrial fractions was measured by Western blot analysis. (E and F) Quantification of cytochrome *c* protein expression was performed by densitometric analysis and β -actin was acted as an internal control. All results were expressed as means $\pm$ SEM of three independent experiments. * $p < 0.05$, ** $p < 0.01$ vs the control group; # $p < 0.05$, ## $p < 0.01$ vs the *t*-BHP group.

spectively, which is consistent with previous reports. These investigations indicated that Daph regulates Nrf2 nuclear translocation via the activation of JNK and ERK signaling in RAW 264.7 cells.

3.8. Daph-mediated Nrf2 activation was independent upon ROS generation

To exclude whether Daph could trigger ROS generation, RAW264.7 cells were pretreated with Daph at final concentrations ranging from 0 to 40 $\mu\text{g/mL}$. This result indicated that Daph did not induce ROS production in a dose-dependent manner (Fig. 8A). Moreover, to explain whether Daph itself can react with *t*-BHP and that at least partially the

protective effect is due to such a reaction, cells were exposed to different ROS inducer, including *t*-BHP, H_2O_2 , and Pyocaine (Pyo, a ROS inducer), or N-acetyl-L-cysteine (NAC, a ROS inhibitor) serving as a negative control. Our finding indicated that Daph treatment could significantly reduce different ROS inducer-triggered ROS generation, indicating that the protective effect of Daph-exhibited may be not directly associated with reaction with *t*-BHP (Fig. 8B–D). Pretreatment with NAC markedly blocked intracellular production of ROS and did not exhibit a significant increase of Nrf2 expression, whereas Daph-induced Nrf2 activation was unaffected by NAC, suggesting that Daph-upregulated Nrf2 expression, ROS production is not the predominant

Fig. 4. Effects of Daph on *t*-BHP-activated NLRP3 inflammasome in RAW 264.7 cells. (A) Cells were exposed to *t*-BHP (6.25 or 12.5 μM) for 24 h. Protein expression of NLRP3, ASC, caspase-1 and IL-1 β were measured by Western blot analysis. Additionally, (C) cells were exposed to Daph (2.5, 5 or 10 $\mu\text{g/mL}$) for 1 h, and then treated with or without *t*-BHP (12.5 μM) for 24 h. Protein expressions of NLRP3, ASC, caspase-1 and IL-1 β were measured by Western blot analysis. (B and D) Quantification of expressions of NLRP3 inflammasome protein was performed by densitometric analysis and β -actin was acted as an internal control. All results were expressed as means $\pm$ SEM of three independent experiments. * $p < 0.05$, ** $p < 0.01$ vs the control group; # $p < 0.05$, ## $p < 0.01$ vs the *t*-BHP group.

Fig. 5. Effects of Daph on the activation of GCLC, GCLM, HO-1 and NQO1 in RAW 264.7 cells. (A) RAW 264.7 cells were treated with different concentrations of Daph (1.25, 2.5, 5 or 10 μg/mL) for 18 h, and (B) cells were exposed to Daph (10 μg/mL) for four time points (1, 3, 6 or 18 h). Protein expressions of GCLC, GCLM, HO-1 and NQO1 were measured by Western blot analysis. (C and D) Quantification of GCLC, GCLM, HO-1 and NQO1 protein expressions were performed by densitometric analysis and β-actin was acted as an internal control. (E) Cells were exposed to Daph (10 μg/mL) for three time points (3, 6 or 18 h). Effects of Daph on GCLC, GCLM, HO-1 and NQO1 genes expression. Total RNA was extracted from RAW 264.7 cells and genes expression was quantified using real-time PCR. All results were expressed as means ± SEM of three independent experiments. #*p* < 0.05, ##*p* < 0.01 vs the control group.

contributor (Fig. 8E–H).

3.9. Daph-mediated Nrf2 alleviated *t*-BHP-stimulated cytotoxicity and ROS generation in RAW 264.7 cells and peritoneal macrophages

Based on the outcomes described above, we hypothesized that the protective effects of Daph against *t*-BHP-induced oxidative damage result from the upregulation of the Nrf2 pathway. To verify the hypothesis, Nrf2 was knocked out in RAW 264.7 cells using the CRISPR/Cas9 gene editing system, and peritoneal macrophages from WT and Nrf2^{-/-} mice were used in the experiment. In this study, we measured several antioxidative enzyme expressions in WT and Nrf2^{-/-} RAW 264.7 cells, as well as peritoneal macrophages from WT and Nrf2^{-/-} mice. Our results suggested that Daph-mediated expression of antioxidative enzymes was almost abolished in Nrf2^{-/-} RAW 264.7

cells and Nrf2^{-/-} peritoneal macrophages (Fig. 9A–L). Additionally, we determined the effect of Daph on *t*-BHP-induced cell viability and ROS generation in Nrf2^{-/-} RAW 264.7 cells and Nrf2^{-/-} peritoneal macrophages. This result indicated that the inhibitory effect of Daph on cell viability and ROS generation was mostly blocked in Nrf2^{-/-} cells (Fig. 9M–P). These investigations demonstrated that Daph-exhibited cytoprotection may be associated with targeting Nrf2 pathway activation.

4. Discussion

Oxidative stress, a biochemical imbalance between endogenous antioxidant and oxidant formation, has been thought to play an essential role in the pathogenesis of various diseases [26]. Furthermore, mitochondria, key organelles in eukaryotic cells, are essential for cellular energy

Fig. 6. Effects of Daph on the Keap1-Nrf2/ARE signaling pathway. (A) Cells were exposed to various concentration of Daph (1.25, 2.5, 5 or 10 μg/mL) for 6 h, and the total protein were determined by Western blot analysis. (B) Cells were exposed to Daph (10 μg/mL) for three time points (1, 3 or 6 h), and the nuclear and cytoplasmic levels of Nrf2 were examined by Western blot analysis. (C and D) The relative density of protein was performed by densitometric analysis; β-actin and Lamin B were acted as an internal control, respectively. (E and F) The luciferase plasmids pGL-ARE and pRL-TK was transiently transfected into cells for 24 h and subsequently exposed to 10 μg/mL Daph for three time points (1, 3 or 6 h) or exposed to Daph (2.5, 5 or 10 μg/mL) for 6 h. ARE luciferase activity was detected by a dual-luciferase reporter assay system. In addition, recombinant murine Keap1 protein was treated with Daph (2.5, 5, or 10 μg/mL) for 30 min, and then exposed to BPM (50 μM) for 30 min (G) The number of free thiol groups was then determined. (H-I) The resulting protein was measured by Western blot analysis with an anti-biotin antibody, and the density of each band was normalized to that of Keap1. All results were expressed as means ± SEM of three independent experiments. #*p* < 0.05, ##*p* < 0.01 vs the control group.

production. Oxidative stress-induced damage of mitochondria mediates and accelerates the process of cell apoptosis and death [11]. Accordingly, attempts to inhibit oxidative stress may play significant roles in inhibiting mitochondrial dysfunction and cell damage. Increasing evidence shows that Nrf2-mediated antioxidant signaling pathways are crucial for the prevention of cell injury resulting from oxidative stress [27]. Daph, a

phytochemical that belongs to the coumarin compound, exists ubiquitously in the human diet and could upregulate Nrf2 and HO-1 protein expression to enhance antioxidant potentiation during carcinogenesis in rats [24]. In the present study, we focused on investigating the potential cytoprotective effects of Daph against *t*-BHP-induced oxidative damage, mitochondrial dysfunction, cell apoptosis and its underlying molecular

mechanisms. Moreover, we determined whether these effects were associated with inducing Nrf2-mediated antioxidant signaling pathways in RAW 264.7 cells.

t-BHP, an organic hydroperoxide, is commonly employed to explore the mechanism of cell damage stimulated by oxidative stress [28]. Indeed, previous studies suggested that *t*-BHP exposure to ARPE-19

Fig. 7. Effects of Daph on the activation of JNK and ERK pathways in RAW 264.7 cells. (A–F) Cells were treated with Daph (10 $\mu\text{g/mL}$) for four time points (1, 3, 6 or 18 h), and whole cell lysates were prepared and detected by Western blot analysis for phosphorylated and total PI3K, AKT, ERK, JNK, p38, and PKC δ protein expression. (M) Cells were pretreated with or without LY294002 (20 μM), SP600125 (20 μM), U0126 (10 μM), SB203580 (10 μM), and GF109203X (5 μM), respectively for 1 h and exposed to Daph (10 $\mu\text{g/mL}$) for 18 h. The total protein of Nrf2 was measured by Western blot analysis. (O and P) Cells were pretreated with or without SP600125 (20 μM) and U0126 (10 μM) for 1 h, and then exposed to Daph (10 $\mu\text{g/mL}$) for an additional 18 h. (G, H, I, J, K, L, N, Q and R) Quantification of induction of PI3K/AKT, MAPKs and PKC δ phosphorylation and total Nrf2 were performed by densitometric analysis and its unphosphorylated forms or β -actin was acted as an internal control, respectively. All results were expressed as means $\pm$ SEM of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ vs the control group; $^{+}p < 0.05$, $^{++}p < 0.01$ vs the Daph group.

cells not only resulted in cell death by promoting apoptosis but also led to oxidative stress by increasing ROS accumulation [29]. Similarly, the present results showed that *t*-BHP-induced RAW 264.7 cells reduced cell viability and promoted cell apoptosis and necrosis compared with the control group, whereas Daph pretreatment remarkably enhanced cell viability and reduced *t*-BHP-induced cell apoptosis. The overproduction of ROS triggers severe damage in various cells related to increased MDA levels and decreased SOD and GSH content [28,30]. MDA is an important marker of lipid peroxidation and indicates the presence of damage and destruction on cell membranes [31]. SOD is an essential antioxidant enzyme that inhibits the overproduction of ROS, largely including superoxide anion radicals [32]. Moreover, the glutathione pool, which is composed of reduced glutathione (GSH) and oxidized glutathione (GSSG) forms, is a key redox buffer in various cells, and the GSH/GSSG ratio is recognized as an indicator of intracellular redox status [33]. Our results indicated that Daph not only dramatically inhibited the overproduction of ROS induced by various inducers, including *t*-BHP, H_2O_2 , and Pyocaine, explaining that the protective effect of Daph-exhibited may be not directly associated with reaction with *t*-BHP, but also enhanced SOD levels and the GSH-to-GSSG ratio. However, the accumulation of ROS results in mitochondrial dysfunction, which promotes the release of cytochrome *c* and cell apoptosis [34,35]. Mitochondria are crucial for the maintenance of physiological function with normal MMP, whereas MMP dissipation is deemed to be a common early event of apoptosis [36]. Our JC-1

staining data suggested that *t*-BHP remarkably aggravated MMP collapse and increased the release of cytochrome *c* from mitochondria, whereas treatment with Daph effectively attenuated *t*-BHP-induced MMP collapse and the release of cytochrome *c*, indicating that Daph contributed to the amelioration of mitochondrial dysfunction. These investigations revealed that Daph is able to ameliorate oxidative stress-induced damage via improvement of mitochondrial dysfunction and reduction of cell apoptosis.

Based on the above outcome, we further investigated whether Daph-inhibited cell apoptosis is linked to its protection of mitochondrial dysfunction. Thus, mitochondria apoptosis-related markers, including caspase-3, Bax and Bcl-2 [37], were measured in the present study. Bcl-2 family proteins, which consist of the pro-apoptotic protein Bax and the anti-apoptotic protein Bcl-2, are essential regulators of mitochondrion-mediated apoptosis [38]. Moreover, caspase-3 activation plays an imperative role in the release of cytochrome *c* from the mitochondrial membrane to the cytosol [39]. Our findings indicated that Daph significantly enhanced the expression of the Bcl-2 protein, reduced the expression of the Bax protein, and displayed a decrease of cleavage-caspase-3 production and a concomitant increase of pro-caspase-3 in *t*-BHP-treated RAW 264.7 cells. More interestingly, many recent studies have suggested that the NLRP3 inflammasome can be activated by oxidative stress and induced by mitochondrial dysfunction, which promotes tissue inflammation [40,41]. We further investigated NLRP3 inflammasome activation by assessing the expression of NLRP3 inflam-

Fig. 8. Effects of ROS generation on Daph-mediated Nrf2 activation. Cells were treated with Daph (0, 2.5, 5, 10, 20 or 40) for 18 h, and were stained with 50 μM of DCFH-DA for 30 min (A) ROS generation was detected by a fluorescent microscope. Cells were treated with and without Daph or NAC (a ROS inhibitor) for 18 h, and were stained with DCFH-DA for 30 min, and subsequently exposed to *t*-BHP (5 mM), or H_2O_2 , or Pyocaine for 5 min (B–D) ROS generation was detected by a fluorescent microscope. In addition, (E–F) cells were exposed to various concentrations NAC (0, 2.5, 5, 10 or 20 mM) for 6 h, the expression of Nrf2 protein was measured by Western blot analysis. (G–H) Cells were exposed to various concentrations NAC (5 mM) for 1 h, and were treated with and without Daph (10 $\mu\text{g/mL}$) for 6 h, and then the expression of Nrf2 protein was measured by Western blot analysis. All results were expressed as means $\pm$ SEM of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ vs the control group; $^{+}p < 0.05$, $^{++}p < 0.01$ vs the *t*-BHP group; $^{*}p < 0.01$ vs the H_2O_2 group; $^{*}p < 0.01$ vs the Pyocaine group.

Fig. 9. Effects of Daph on Nrf2-mediated defense against *t*-BHP-stimulated cytotoxicity and ROS generation in RAW 264.7 cells. (A and B) WT and Nrf2^{-/-} RAW 264.7 cells or peritoneal macrophages were treated with or without exposed to Daph (10 μg/mL) for 6 h or 18 h. The total cell protein was determined by Western blot analysis. (C–L) The relative density of protein was performed by densitometric analysis; β-actin acted as the internal control. (M and O) WT and Nrf2^{-/-} RAW 264.7 cells or peritoneal macrophages were subjected to Daph (10 μg/mL) for 18 h and exposed to *t*-BHP (5 mM) for an additional 3 h; subsequently, cell viability was determined by MTT assay. (N and P) WT and Nrf2^{-/-} RAW 264.7 cells or peritoneal macrophages were treated with or without Daph for 18 h, stained with 50 μM of DCFH-DA for 40 min and subsequently exposed to *t*-BHP (5 mM) for 5 min to induce ROS generation, which was detected by a fluorescent microscope. #*p* < 0.05, ##*p* < 0.01 vs the control group; **p* < 0.05, ***p* < 0.01 vs the *t*-BHP group; +*p* < 0.05, ++*p* < 0.01 vs the Daph group.

masome member proteins. The present results discovered that *t*-BHP could evidently activate NLRP3 inflammasome through an increased expression of NLRP3, ASC, caspase-1 and IL-1β protein, whereas this increase was greatly decreased by pretreatment with Daph.

The Nrf2 signaling pathway, a primary host defense, plays an imperative role in maintaining the structural and functional integrity of mitochondria, which is of extraordinary importance under oxidative stress conditions [42]. In fact, recent studies have shown that numerous bioactive molecules, including natural compounds, were dependent on Nrf2 activation to induce various antioxidant enzymes expression, such as HO-1, GCLM, GCLC and NQO1, which are essential for inhibiting redox impairment and mitochondrial dysfunction [43–45]. It has been reported that Daph could protect from carcinogenesis via upregulation

of Nrf2 and HO-1 protein expression and enhance the ability of potential antioxidants in rats [24]. To further investigate the protective effect of Daph against oxidative damage and mitochondrial dysfunction associated with Nrf2 activation, we observed the effect of Daph on the Keap1/Nrf2/ARE antioxidant pathway and the regulation of antioxidant enzymes. In the present study, we demonstrated that various dosages and periods of Daph exposure dramatically increased antioxidant genes and the expression of HO-1, GCLM, GCLC and NQO1 in RAW 264.7 cells. Previous reports showed that activated-Nrf2 is released from Keap1 and translocated into the nucleus, whereupon it sequentially binds to ARE in the promoter region of its target genes, resulting in the induction of many cytoprotective genes and antioxidant enzymes [46]. Moreover, Keap1 modification by an electrophile

Fig. 10. Scheme summarizing the protection of *t*-BHP-induced RAW 264.7 cell mitochondrial dysfunction by Daph via activation of the Keap1-Nrf2/ARE signaling pathway. Daphnetin can induce c-Jun N-terminal kinase (JNK) and extracellular signal-regulated protein kinase (ERK) phosphorylation, which is assumed to detach Nrf2 from Keap1 and subsequently translocate and bind to the antioxidant response element (ARE), resulting in the expression of various antioxidant genes, such as GCLC, GCLM, HO-1 and NQO1. On the other hand, it is known that *t*-BHP can lead to ROS accumulation that triggers oxidative stress in RAW 264.7 cells to further induce mitochondrial dysfunction and release cytochrome c, which is closely associated with inflammatory responses and cell apoptosis. Importantly, Daphnetin enhances the induction of GCLC, GCLM, HO-1 and NQO1 to reduce ROS production and even alleviate mitochondrial dysfunction and cell damage.

has been reported to account for Nrf2 activation [25]. In this study, Daph significantly caused a decrease of Keap1 and an increase of Nrf2 in total cell lysates, which was associated with promoting the nuclear translocation of Nrf2 and strengthening ARE luciferase activity in a time- and dose-dependent manner. Moreover, our experiments explored that Daph is covalently combined with purified murine Keap1 using the BPM-labeling assay, indicating that the activation of Nrf2 by Daph may be independent upon the covalent modification of Keap1. On the other hand, while ROS production is regarded as playing an essential role in activation of Nrf2 [47], our results implicated that a higher dose of Daph did not induce ROS generation, and exposure to NAC failed to hinder Nrf2 activation resulted from Daph treatment. These findings indicated that Daph can ameliorate oxidative stress-induced mitochondrial dysfunction and cell injury through an underlying mechanism, which likely involves in Nrf2 activation. Therefore, we further observed the effect of Daph on *t*-BHP-induced ROS generation and cytotoxicity in Nrf2^{-/-} RAW 264.7 cells and peritoneal macrophages. These observations displayed that the inhibitory effects of Daph on ROS generation and cytotoxicity are mostly blocked in *t*-BHP-induced Nrf2^{-/-} RAW 264.7 cells and peritoneal macrophages. Given that Nrf2 activation is involved in the regulation of multiple signaling pathways, such as PCK, PI3K or MAPK [48], we explored whether Daph could regulate these signaling pathways to modulate the activation of Nrf2. In our studies, Daph strongly enhanced the phosphorylation of JNK and ERK, and the addition of their specific inhibitors (SP600125 and U0126, respectively) effectively abolished Daph-induced Nrf2 nuclear translocation, suggesting that Daph-mediated Nrf2 antioxidant signaling pathways may be regulated by the activation of ERK and JNK in RAW 264.7 cells.

5. Conclusion

In conclusion, as shown in Fig. 10, our findings revealed that Daphnetin (Daph) induced the expression of GCLC, GCLM, HO-1 and NQO1, which was largely dependent on the upregulation of the Nrf2 signaling pathway, via activating JNK and ERK phosphorylation, thereby protecting RAW 264.7 cells against *t*-BHP-induced oxidative damage, mitochondrial dysfunction, and apoptosis. These events were not only associated with reduced ROS generation and MDA formation but also increased the SOD level and the GSH/GSSG ratio. Further investigation suggested that Daph significantly suppressed cytochrome c release and NLRP3 inflammasome activation and regulated apoptosis-related protein Bcl-2, Bax, and caspase-3 expression. This study provides beneficial evidence for the application of Daph in the prevention of oxidative stress-induced mitochondrial dysfunction, indicating that Daph might contribute to the treatment of mitochondrial damage-related diseases.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content of this manuscript.

Acknowledgments

This work was in part supported by the National Science Foundation of China (Grant no. 81603174) and the General Financial Grant from the China Postdoctoral Science Foundation (Grant no. 168847).

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