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Retinoic acid-related orphan receptors regulate autophagy and cell survival in cardiac myocytes during hypoxic stress

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Abstract

Introduction: Autophagy is a highly conserved evolutionary process that regulates cell quality control through protein degradation, organelle turnover, and recycling of cellular components by fusing with lysosomes. Defects in autophagy can lead to increased reactive oxygen species (ROS) and oxidative stress from impaired mitochondrial clearance by mitophagy. These defects are commonly associated with chronic human diseases such as cancer, myocardial infarction, neurodegenerative diseases, and aging.

Aim: Herein, we show that the gene Retinoic Acid-Related Orphan Receptors α (*Rora*) is cardioprotective through modulation of autophagy and clearance of damaged ROS-producing mitochondria in cardiac myocytes.

Methods and results: We show that RORα is downregulated during hypoxia, leading to increased death of cardiac cells and enhanced mitochondrial perturbations. We demonstrate that the small molecule Nobiletin, a polymethoxy flavonoid, can induce *RORα* activation and downregulate the aging-associated marker p16, coincident with reduced ROS-producing mitochondria. We further show that Nobiletin binds directly to the *Rora* gene promoter, leading to activation of autophagic function and increased cell survival of cardiac myocytes during hypoxia. Interestingly, loss of RORα activity during hypoxia resulted in the failure of Nobiletin to rescue autophagy

and inhibits its capacity for cardiac protection. Furthermore, the inactivation of autophagy by ATG7 knockdown abrogated the cytoprotective effects of Nobiletin on autophagic activation.

Conclusion: Collectively, these results demonstrate that RORα regulates autophagic processes linked to aging upon activation with Nobiletin. Interventions that activate RORα may prove beneficial in reducing hypoxia-induced mitochondrial ROS associated with cardiac aging.

Keywords: RORα, nobiletin, autophagy, mitochondria, cell death

INTRODUCTION

Mammalian cells discard damaged organelles and macromolecular proteins through an evolutionary conserved process known as autophagy[1]. Autophagy provides a vital cellular quality control mechanism for preventing the accumulation of damaged organelles, such as mitochondria, which are a major source of reactive oxygen species (ROS), as well as oxidized proteins that would otherwise cause cell death[2]. Autophagy involves the sequestration and degradation of cellular material through a highly regulated multistep process that involves the fusion of autophagosomes with acid-rich lysosomes, which hydrolyzes the autophagic cargo[3]. Defects in autophagy have been associated with a number of human diseases, including tumorigenesis, myocardial infarction, neurodegenerative diseases, and aging[2]. In fact, the accumulation of ROS-producing mitochondrial and cellular debris is viewed as a harbinger of aging[3].

Indeed, previous work from our laboratory and others has shown that disruption of autophagy/mitophagy following hypoxia resulted in the accumulation of damaged mitochondria and senescence markers such as p53[4,5]. Conversely, activating autophagy through the gain of function of ATG7 or Tat-Beclin-1 restored mitochondrial quality control and suppressed cardiac injury following myocardial infarction[4,5]. Further, autophagy activation suppressed premature aging and senescence[6]. Hence, these studies highlight the important role of autophagy as a cellular quality control mechanism during cardiac stress.

For this reason, there has been considerable growing interest in identifying new compounds that can activate autophagy and avert the accumulation of damaged ROS-producing mitochondria during cardiac stress. In particular, Nobiletin (Nob), a polymethoxy compound, has gained heightened interest over the years for its medicinal properties and potential therapeutic application in a number of human diseases associated with impaired autophagy such as cancer, inflammation, and aging[7-10]. At the cellular level, the biological effects of Nobiletin have been attributed to its ability to activate the orphan retinoic acid receptor (ROR)[8]. ROR is comprised of a superfamily of nuclear transcription factors that include Rora, Rorb, and Rorc[11]. These factors activate target gene expression by binding to sequence specific RORE elements within their cognate target gene promoters[11].

Notably, our *in silico* analysis revealed that several genes required for autophagy were found to contain canonical AGGTCA DNA elements for Rora binding, raising the possibility that Rora may be an important regulator of autophagy. However, the connection between ROR signaling and autophagy-regulated cellular quality control during cellular stress in the heart remains cryptic. Therefore, given the critical connection between autophagy dysregulation and aging, we investigated the impact of Nobiletin-mediated ROR signaling on cardiac autophagy signaling in cardiac myocytes subjected to hypoxic stress.

MATERIALS AND METHODS

Cardiac cell culture and treatments

Cardiomyocyte cells were isolated from 1- to 2-day-old Sprague-Dawley rats. For cell isolation, hearts were removed and treated enzymatically to isolate primary neonatal cardiomyocytes, as previously reported[4]. 24 h after plating, cells were subjected to serum-free DMEM (DMEM/F12 [Thermo Fisher Scientific, MT10092 CV]- 1 pack, $NaHCO_3$- 0.25 g, HEPES (Sigma-Aldrich, H3375-1 Kg) - 3.57 g, Na selenite (Sigma-Aldrich, S5261-100 G) -1 mL, transferrin (Sigma-Aldrich, T1408-500 MG) -1 mL, LiCl-1 mL, ascorbic acid (Sigma-Aldrich, A4034-100 G) -1 mL, insulin (Sigma-Aldrich, I0516-5 mL)-0.25 mL, T3 [L-Thyroxine-1 mL, gentamicin-1 mL] (Thermo Fisher Scientific, 15710072), as previously described[4]. Isolated cultured cardiomyocyte cells were treated with 25 μM Nobiletin (Sigma-Aldrich, N1538-5MGXXX) for 6 h prior to hypoxia. For hypoxia studies, cardiomyocyte cells were moved into an air-tight chamber continually gassed with 95% N_2, and po2 ≤ 5 mm Hg.

Western blot

Cardiomyocyte cells were lysed in RIPA buffer (1% triton x [Sigma-Aldrich, X100-1 L]-0.5 mL, 0.1% SDS-250 μL of 20% SDS, 140 mM NaCl-1.4 mL 5 M NaCl, 10 mM Tris-500 μL 1 M Tris pH 8, sodium deoxycholate-0.5 g, pH of RIPA 7.7). Protein was extracted and measured by bicinchoninic acid (BCA; Thermo Fisher Scientific, 23225). Cell lysate of 20 μg protein was resolved on denaturing SDS PAGE gels, followed by transfer to nitrocellulose membranes (Bio-Rad, 1620094). Membranes were probed with primary antibodies against LC3 (NEB, 2775S), BECN1/Beclin-1 (NEB, 3495S), RORα (Thermo Fisher Scientific, BS-20687R), GAPDH (NEB, 97166S) or ACTA1/ACTIN (Sigma-Aldrich, A2172) at a dilution of 1:1,000 in TBS-T (5X 1 L [36.55 g NaCl, 15.5 g Tris, pH 7.4, 5 mL Tween-20 {VWR, Ac23336-0010}], dilute to x1), overnight at 4 °C. On the next day, membranes were incubated with secondary HRP conjugated anti-murine or rabbit antibodies (Cederlane, 715-035-150, 715-035-152). Bands were detected using enhanced ECL (GE Healthcare, CA89168-782).

siRNAs and adenoviruses

Cardiomyocyte cells were transfected or infected for 18 h before hypoxia treatment. For transfection of siRNA against RORα (siRORα, 60 nM), cells were treated with Effectene reagent (Qiagen, Inc., 301427). siRORα was custom-designed and synthesized by Invitrogen. For infection with Adenoviruses harboring shRNA against Atg7 (shAtg7), cells were infected for 24 h before hypoxia at a multiplicity of infection of 10-20 (hairpin-forming oligonucleotides: 5′-CGGTCAAAGGACAAAGTTAACATTCAAGAGATGTTAACTTTGTCCTTTGACCTTTTTA-3′). Empty cytomegalovirus (CMV) adenovirus (provided by Lorrie Kirshenbaum, University of Manitoba, Winnipeg, Manitoba, Canada[4]) served as control.

Cell viability assay

Cardiomyocyte cells were treated with the vital dyes calcein acetoxymethyl ester (Calcein-AM, 2 μM; Life Technologies, C3100MP) which stains live cells in green and ethidium homodimer-1 (2 μM) which stains dead cells in red. Live and dead cells were visualized by epifluorescence microscopy at ×200 magnification, using Olympus AX-70 research fluorescence microscope.

Reactive oxygen species

Cardiomyocyte cells were treated with dihydroethidium (Invitrogen, D23107) (2.5 μM) for 30 min to visualize red fluorescence, which indicates an increase in superoxide species production by epifluorescence microscopy using Olympus AX-70 fluorescence microscope.

Mitochondrial membrane potential ($\Delta\Psi$M)

Cardiomyocyte cells were treated with tetra-methylrhodamine methyl ester perchlorate (TMRM; Thermo Fisher Scientific, T-668) (50 nM) to visualize normal mitochondrial membrane potential with bright red staining, and diffuse red staining when mitochondrial membrane potential disperses. Images were captured with epifluorescence microscopy and fluorescence intensity was analyzed by ImageJ software.

Mitochondrial permeability transition pore opening

Cardiomyocyte cells were treated with 5 μmol/L calceinAM (Molecular Probes) in the presence of 2 μmol/L cobalt chloride to assess mitochondrial permeability transition pore opening (mPTP). Reduced green staining is indicative of mPTP opening. Images were captured with epifluorescence microscopy and fluorescence intensity was analyzed by ImageJ software. The integrated optical density of green staining was used as an index of mPTP.

Autophagic flux

For autophagy flux assessment, cardiac myocyte cells were plated on coverslips at a density of 4×10^4, and co-infected with adenoviruses encoding green fluorescent protein (GFP)-fused LC3 (provided by Junichi Sadoshima, Rutgers University, New Jersey, USA[12]). Cells were treated with 10 mM Chloroquine (Sigma-Aldrich C6628-25 g) as a positive control for autophagic flux. After hypoxia, cardiac cells were fixed with 4% paraformaldehyde (Electron Microscopy Science, 15710), and mounted with FluorSave™ Reagent (Millipore, 345789). Confocal microscopy (Carl Zeiss Axio Observer SD) with 630× magnification and Zen Software (Carl Zeiss) were used for imaging[4]. For analysis, the number of GFP puncta was counted manually from three different myocyte isolations. The number of puncta per cell was calculated by dividing the total number of puncta by the number of cells per image.

Oxygen consumption rate

Cardiomyocyte cells were cultured in 96-well plates and assessed for mitochondrial oxygen consumption rate (OCR) using XFe96 Cell Mito Stress Analyzer (Agilent). Culture medium was changed 1 h before the assay to XF Base medium (Agilent, 102353-100), (pH 7.4) supplemented with 1 mM pyruvate (Thermo Fisher Scientific, 11360-070), and 10 mM D-glucose (Thermo Fisher Scientific, D16-500), followed by sequential injection of oligomycin (1 μM; Sigma-Aldrich, O4876-5 MG), FCCP (2-[2-{4-(trifluoromethoxy)phenyl}hydrazinylidene]-propanedinitrile) (1 μM; Sigma-Aldrich, C2920), and rotenone (1 μM; Sigma-Aldrich, R8875-1 G) with antimycin (1 μM; Sigma-Aldrich, A8674-25 MG).

Mitochondrial morphology assay

Cardiomyocyte cells were plated on collagen from rat tails coated coverslips at a density of 4×10^4 upon isolation. After hypoxia/normoxia, cells were fixed with 4% paraformaldehyde (Electron Microscopy Science, 15710), permeabilized with 0.5% Triton-x100 for 10 min, and blocked with 1% Triton X-100 + 2% bovine serum albumin (BSA; Roche, 10775835001) for 15 min. HSPD1/HSP60 antibody (Santa Cruz Biotechnology, sc-13115) was used for mitochondrial staining in blocking solution for 1 h at RT. Secondary antibody donkey anti-mouse IgG, Alexa Fluor® 546 conjugate (Thermo Fisher Scientific, LSA10036) was added for 1 h at RT. Hoechst (Thermo Fisher Scientific, 33342) was used for nuclear counterstaing, followed by mounting with FluorSave™ Reagent (Millipore, 345789). Images were captured by confocal microscopy (Carl Zeiss Axio Observer SD) using magnification 630× and Zen Software. For analysis, form factor (FF: perimeter2/4π·Area), as a measure of length and branching corresponding to a circular mitochondrion, was calculated using ImageJ software.

Mitophagy assessment

Cardiomyocyte cells were infected with MitoKeima adenovirus (provided by Junichi Sadoshima, Rutgers University, New Jersey, USA, as previously described[4]. Following hypoxia, cells were fixed with 4% paraformaldehyde (Electron Microscopy Science, 15710) and mounted with FluorSave™ Reagent (Millipore, 345789). Images were captured by confocal microscopy (Carl Zeiss Axio Observer SD) using the magnification 63× and Zen Software (Carl Zeiss). Mitophagy was assessed by measuring the fluorescence signal ratio 560/440 nm and the size of MitoKeima puncta using Zeiss ZEN Blue software, as previously described[4]. The size of MitoKeima puncta was calculated from the five largest puncta in each image and the fold-change in area was calculated by averaging 3-5 images from each condition. All imaging parameters remained the same among all conditions and images.

Statistical analysis

Quantitative data is presented as Average ± SEM. One-way ANOVA was used for multiple comparisons between groups. Tukey's post hoc test was used to determine differences among groups. Unpaired two-tailed Student's *t*-test was used to compare mean differences between two groups. Differences were considered to be statistically significant at a level of $^{*}P < 0.05$ or $^{**}P < 0.01$. In all studies, data were obtained from at least $n = 3$-5 independent myocyte isolations for each condition tested.

RESULTS

Nobiletin rescues hypoxia-induced mitochondrial perturbations and cardiac cell death

Since earlier work demonstrated that mitochondria are the major source of ROS and cellular damage associated with aging phenotype[13], we first explored the impact of Nobiletin on markers of aging in cardiac myocytes. As shown in Figure 1, increased levels of p16 were observed in cardiac myocytes subjected to hypoxia; however, treatment with Nobiletin downregulated p16 expression to levels comparable to normoxic controls. Furthermore, aging markers improved, coincided with improved mitochondrial function and reduced ROS production in cardiac myocytes treated with Nobiletin during hypoxia. As shown in Figure 2A, in contrast to normoxic control cells that exhibited a highly organized reticular mitochondrial network, cardiac myocytes subjected to hypoxia exhibited severe morphological defects consistent with mitochondrial fragmentation and disruption of the mitochondrial reticular network that was abrogated by Nobiletin treatment. Concordant with these findings, the mitochondrial network disruption was accompanied by increased mitochondrial permeability transition pore opening [Figure 2B], decline in mitochondrial membrane potential [Figure 2C], increased ROS production [Figure 2D], impaired mitochondrial respiration [Figure 3A] and increased cell death [Figure 3B]. Interestingly, hypoxia- induced mitochondrial damage was suppressed by Nobiletin treatment. These findings demonstrate that Nobiletin protects cardiac myocytes from mitochondrial perturbations, leading to increased ROS production and cell death subjected to hypoxic stress.

Nobiletin upregulates autophagy and mitophagy during hypoxia

Given that cellular quality control processes, such as autophagy or mitophagy, play a critical role in removing damaged mitochondria and cellular debris during cell stress, we reasoned that the accumulation of damaged mitochondria and cellular debris from impaired autophagic processes may result in cell death associated with hypoxia. To explore this possibility, we monitored autophagy in cardiac myocytes under normoxic conditions and during hypoxia. As shown by Western Blot analysis in Figure 4A, we observed a marked reduction in autophagy proteins, LC3II, and BECLIN1 in cardiac myocytes subjected to hypoxia compared to normoxic control cells. Further, the reduction in autophagy protein abundance was accompanied by a reduction in autophagic flux as indicated by the reduced number of green GFP-LC3 staining puncta as shown by epifluorescence microscopy Figure 4B. However, cardiac myocytes treated with Nobiletin exhibited increased autophagic protein abundance and GFP-LC3 positive puncta indicative of

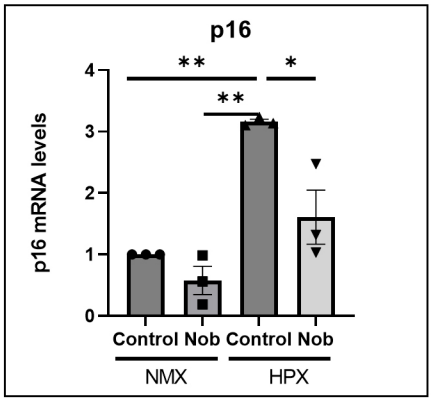

Figure 1. Nobiletin rescues aging during hypoxia. mRNA levels of p16 under normoxia (NMX) and hypoxia (HPX) in the presence and absence of Nob. Data are expressed as mean ± SEM. *$P < 0.05$; **$P < 0.01$, $n = 3$ independent myocyte isolations.

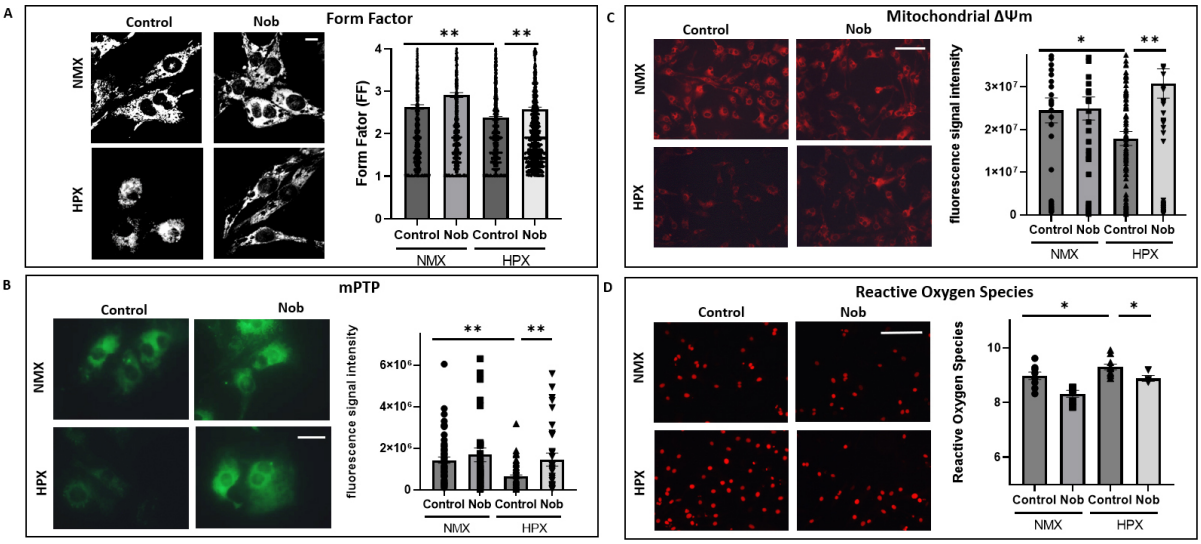

Figure 2. Nobiletin rescues mitochondrial perturbations during hypoxia. (A) epifluorescence stinging of mitochondrial morphology aspect ratio with mitochondrial HSPD1 in cardiac myocytes under NMX and HPX conditions in the presence and absence of Nob, bar: 10 μm, histogram shows mitochondrial form factor (FF) which quantifies mitochondrial morphology as an index of mitochondrial fusion and fission. (B) green fluorescence staining of mitochondrial permeability transition pore opening (mPTP) of cardiac myocytes under normoxia (NMX) and hypoxia (HPX) in the presence and absence of Nob, bar: 20 μm; (C) red fluorescence staining of mitochondrial membrane potential (ΔΨm) by TMRM in cardiac myocytes under NMX and HPX conditions in the presence and absence of Nob, bar: 20 μm; (D) red fluorescence staining of mitochondrial reactive oxygen species (ROS) by dihydroethidine in cardiac myocytes under NMX and HPX conditions in the presence and absence of Nob, bar: 100 μm; Data are expressed as mean ± SEM. *$P < 0.05$; **$P < 0.01$, n = 3-4 independent myocyte isolations, counting > 200 cells for each condition tested.

increased autophagosome formation during hypoxia. These findings indicate that general autophagy, which is impaired in cardiac myocytes during hypoxia, can be restored by Nobiletin treatment. Since damaged mitochondria are cleared by a specialized process known as mitophagy, we next explored whether mitophagy was impaired during hypoxia. For these studies, we utilized the dual emission mitophagy reporter MitoKeima that fluoresces green following lysosomal clearance of mitophagosomes (neutral pH) or fluoresces yellow/red when mitophagy is impaired (acidic pH). The size of puncta is another parameter for the status of mitophagy, as indicated by large puncta when mitophagosomes accumulate due to disrupted mitochondrial - lysosomal clearance, or smaller puncta when mitochondrial clearance is normal[4,14]. As shown in Figure 5, we observed a marked reduction in mitophagy, as indicated by the presence of large red

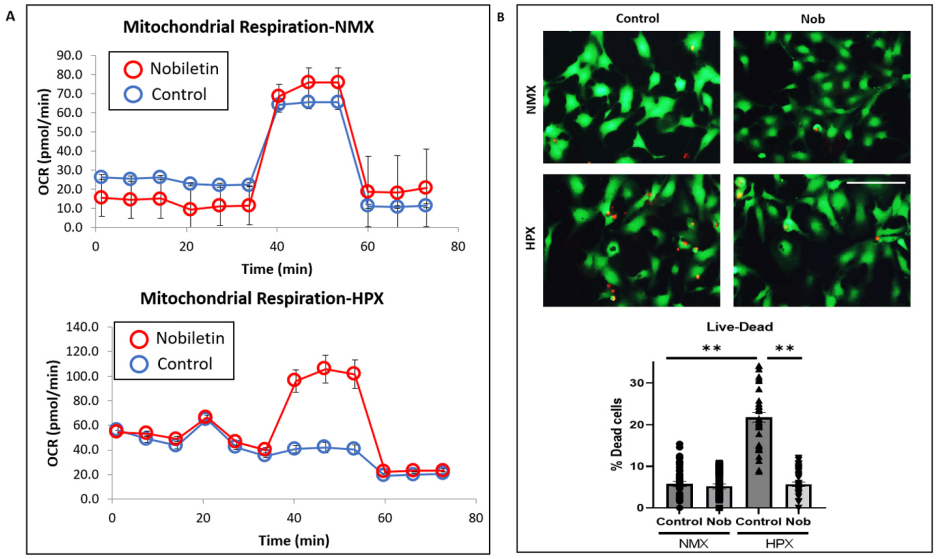

Figure 3. Nobiletin rescues mitochondrial oxygen consumption rate (OCR) and cardiac cell death during hypoxia. (A) mitochondrial OCR in cardiac myocytes under normoxia (NMX) and hypoxia (HPX) in the presence and absence of Nob. (B) Cell viability assessed with vital dyes calcein-AM (green; live cells) and ethidium-homodimer-1 (red; dead cells), bar: 100 µm. Data are expressed as mean ± SEM. $^{**}P < 0.01$, n = 3-4 independent myocyte isolations, counting > 200 cells for each condition tested.

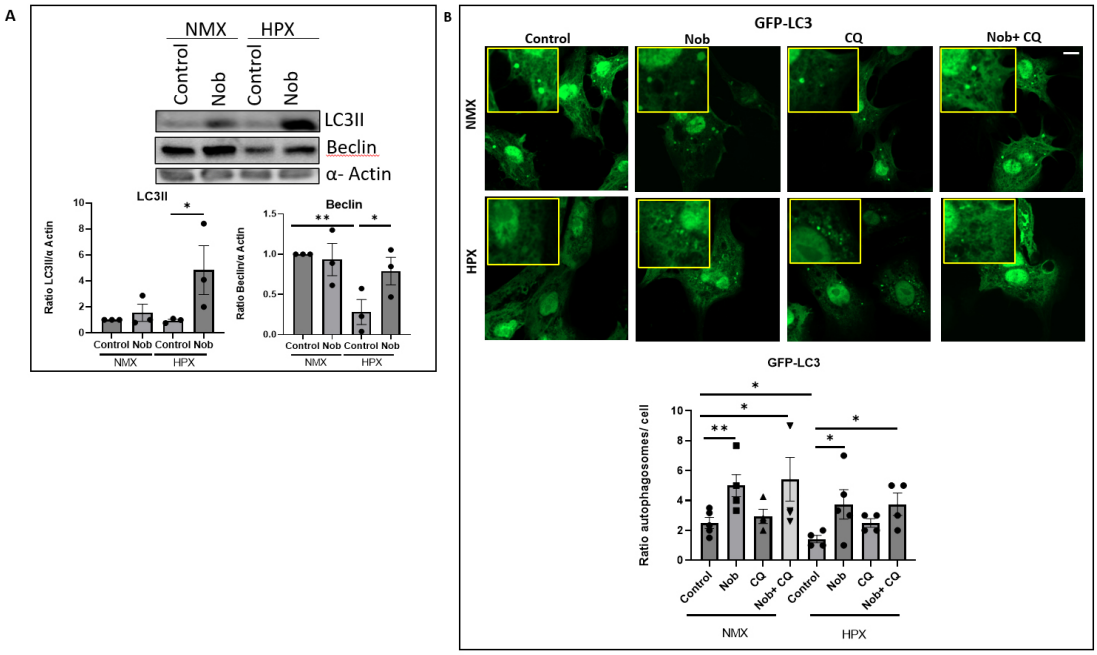

Figure 4. Nobiletin upregulates autophagic function of cardiac cells during hypoxia. (A) western blot analysis of LC3 and Beclin protein expression in cardiac myocytes under normoxia (NMX) and hypoxia (HPX) in the presence and absence of Nob. Histogram represents quantitative data for the western blot, relative to α sarcomeric Actin as a loading control. (B) Upper panel, epifluorescence microscopy of cardiac myocytes expressing autophagy reporter GFP-LC3 (green puncta) with or without Chloroquine (CQ) to assess autophagic flux under normoxia (NMX) and hypoxia (HPX) in the presence and absence of Nob, bar: 10 µm, magnified regions are depicted by the yellow boxes. Bottom panel, analysis of conditions tested on upper panel. Data are expressed as mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$, n = 3-4 independent myocyte isolations.

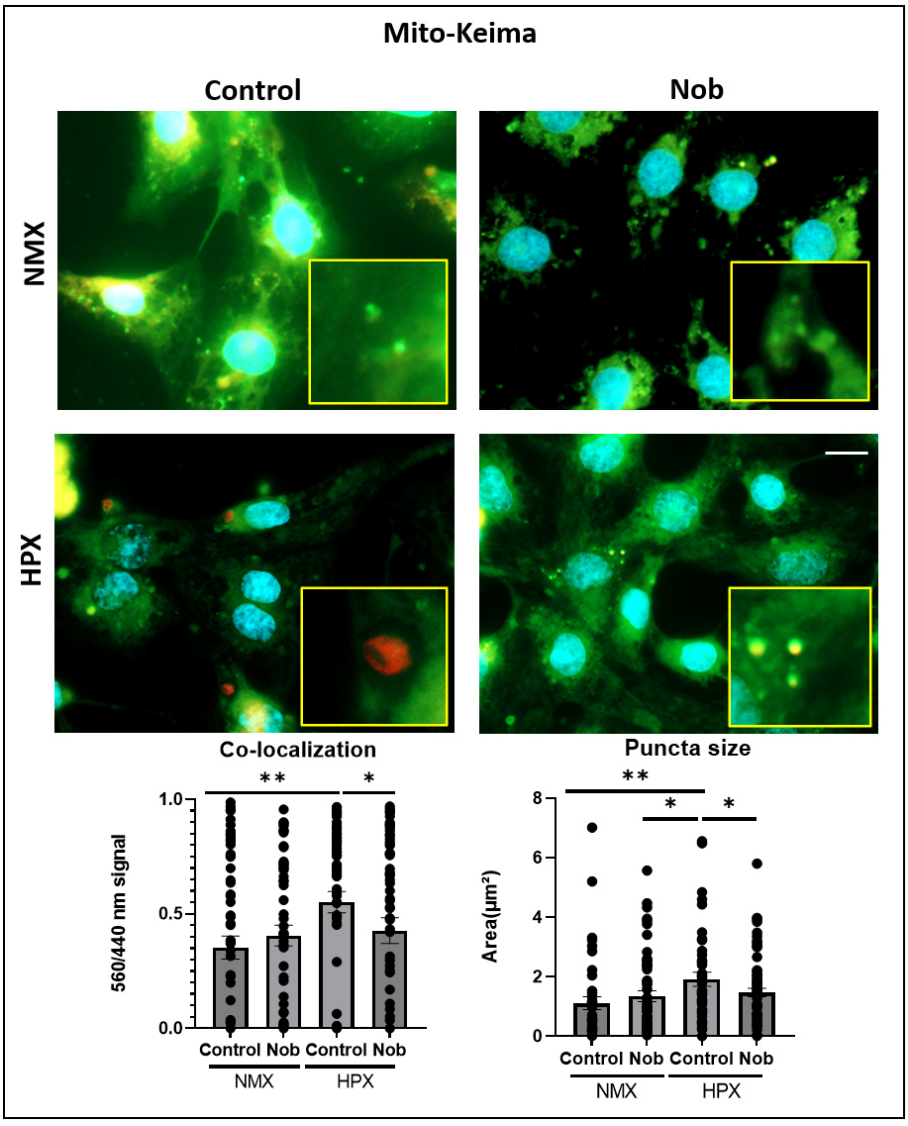

Figure 5. Nobiletin upregulates mitophagy of cardiac cells during hypoxia. Upper panel, representative images for MitoKeima staining, as an index of mitophagy in cardiac myocytes under normoxia (NMX) and hypoxia (HPX) in the presence and absence of Nob, bars: 10 μm. Magnified regions are depicted by the yellow boxes, mitochondria that are unfused with lysosomes (neutral pH) demarked as green fluorescent puncta, mitochondria that have fused with lysosomes (acidic pH) are demarked as red/yellow fluorescent puncta. Bottom panel, quantitative analysis for conditions shown above. Data are expressed as mean ± SEM. *$P < 0.05$; **$P < 0.01$, $n = 3$-4 independent myocyte isolations.

fluorescent puncta in cardiac myocytes subjected to hypoxia compared to normoxic control cells. Importantly, hypoxia-induced impairment of mitophagy was restored in cardiac myocytes treated with Nobiletin. Taken together, these findings demonstrate that impaired autophagy/mitophagy in cardiac myocytes subjected to hypoxia can be upregulated with Nobiletin treatment. Furthermore, these findings support our view that Nobiletin activates critical cellular quality control mechanisms that prevent the accumulation of damaged ROS-generating mitochondria that would otherwise cause cell death.

Nobiletin fails to rescue cell death and autophagy in the presence of siRORα

Notably, *in silico* analysis revealed that several genes important for autophagy regulation contain canonical AGGTCA DNA elements for Rora [Figure 6A]. Based on this observation, we reasoned that the loss of ROR

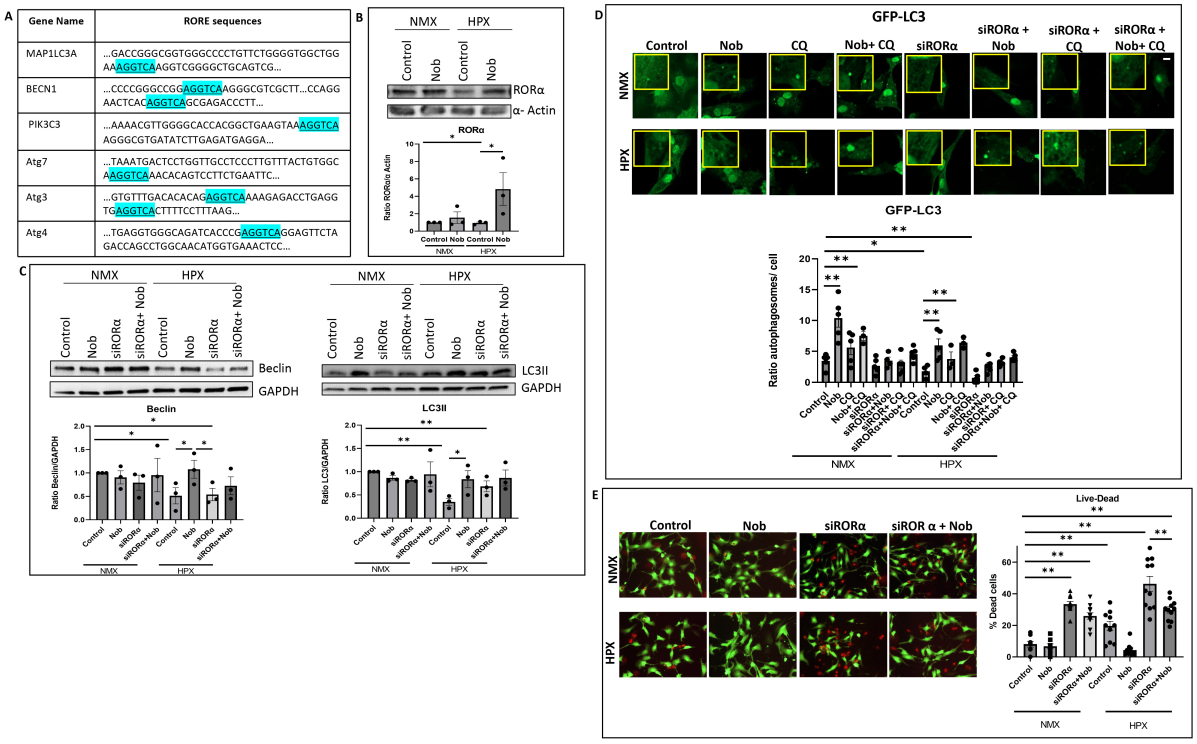

Figure 6. The ability of Nobiletin to rescue autophagy and cell death is lost in the presence of siROR. (A) ROR elements in the promoters of autophagy target genes are underlined and highlighted in blue. (B) Western blot analysis of RORα protein expression in cardiac myocytes under normoxia (NMX) and hypoxia (HPX) in the presence and absence of Nob. Histogram represents quantitative data for the western blot, relative to α sarcomeric Actin as a loading control. (C) Western blot analysis of Beclin, and LC3 protein expression in cardiac myocytes under normoxia (NMX) and hypoxia (HPX) in the presence and absence of Nob and siRORα. Histogram represents quantitative data for the western blot, relative to α sarcomeric Actin as a loading control. (D) Upper panel, epifluorescence microscopy of cardiac myocytes expressing autophagy reporter GFP-LC3 (green puncta) with or without Chloroquine (CQ) to assess autophagic flux under normoxia (NMX) and hypoxia (HPX) in the presence and absence of Nob and siRORα, bar: 10 μm, magnified regions are depicted by the yellow boxes. Bottom panel, analysis of conditions tested on upper panel. (E) Cell viability assessed with vital dyes calcein-AM (green; live cells) and ethidium-homodimer-1 (red; dead cells), bar: 100 μm. Data are expressed as mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$, n = 3-4 independent myocyte isolations, counting > 200 cells for each condition tested.

may underlie the impaired autophagy in cardiac cells subjected to hypoxia. Indeed, concordant with this view, as shown by Western Blot analysis, ROR protein abundance was markedly reduced in cardiac myocytes subjected to hypoxia compared to normoxic control cells; however, Nobiletin treatment increased expression levels of RORα in cardiac myocytes subjected to hypoxia [Figure 6B]. Interestingly, we observed no apparent changes in the expression levels of ROR or RORγ (data not shown), suggesting that loss of ROR activity is critical for regulating autophagy. Therefore, to verify that the cytoprotective effects conferred by Nobiletin on cardiac cell survival were dependent upon ROR, we next tested whether ROR inhibition would abrogate the ability of Nobiletin to suppress hypoxia-induced cell death of cardiac myocytes. For these studies, we tested the effects of Nobiletin in the absence and presence of siRNA directed against ROR. As shown in Figure 6C, in contrast to cardiac myocytes treated with vehicle and subjected to hypoxia, the ability of Nobiletin to rescue autophagy proteins BECLIN1 and LC3II in cardiac myocytes subjected to hypoxia was abrogated by ROR knockdown. Further, the reduction in autophagy proteins BECLIN1 and LC3II was accompanied by a reduction in autophagic flux as indicated by the reduced number of green GFP-LC3 staining puncta, Figure 6D. However, cardiac myocytes treated with Nobiletin exhibited increased GFP-LC3 positive puncta, which was abrogated in the presence of siRORα, indicating that autophagy cannot be restored by Nobiletin treatment when RORα is inhibited. This observation coincided with the

inability of Nobiletin to rescue cardiac cell death during hypoxia in the presence of siRORα [Figure 6E]. Together, these data indicate that ROR activation is critical for the cytoprotective effects conferred by Nobiletin to rescue autophagy and cell death.

The ability of Nob to rescue cell death is abrogated by knocking down autophagy

To verify that autophagic processes were important for the cell survival conferred by Nobiletin, we next examined whether autophagy inhibition by ATG7 knockdown would interfere with the ability of Nobiletin to suppress cell death of cardiac myocytes subjected to hypoxia. As shown by Western Blot analysis, Figure 7A, in contrast to vector control cells, Nobiletin failed to activate proteins such as LC3II required for autophagy. Furthermore, Nobiletin failed to suppress cell death following ATG7 knocked down, Figure 7B. Taken together, these findings strongly suggest that Nobiletin suppresses mitochondrial injury and cell death of cardiac myocytes through a mechanism that involves ROR-mediated activation of autophagy.

DISCUSSION

Cellular quality control mechanisms such as autophagy/mitophagy are critical for maintaining cellular homeostasis and cell viability[4,15,16]. Defects in autophagy and mitochondrial function have been associated with advanced aging[17]. In the context of the heart, which is abundantly rich in mitochondria that are vital for supporting energy production through oxidative mechanisms, they are also a major source of ROS. Hence, the accumulation of damaged ROS-producing mitochondria from impaired cellular quality control mechanism is a harbinger of oxidation of proteins and lipids that ultimately results in cell death and cardiac dysfunction[4,15,16]. Indeed, excessive cellular ROS has been associated with increased aging and expression of p16[17]. Furthermore, previous studies have shown that Nobiletin could delay skeletal muscle aging by improving autophagy, decreasing ROS production, and reversing mitochondrial damage[18]. Interestingly, our study showed that Nobiletin suppressed mitochondrial ROS production coincident with a reduction in p16 levels. In fact, this observation is further substantiated by recent studies that have shown that clearance of damaged mitochondria through autophagy/mitophagy is essential for maintaining mitochondrial bioenergetics and cardiac function during cardiac stress[4,19]. Notably, our *in-silico* analysis revealed that several genes required for autophagy contain Rora cis-acting DNA elements in their gene promoters, suggesting that ROR may be an important regulator of autophagy. The decline in ROR protein abundance and mitophagy in cardiac myocytes subjected to hypoxic stress is consistent with the increased mitochondrial injury and ROS production during hypoxia. Further, the finding that ROR but not the other ROR proteins were altered in response to hypoxia would argue that ROR may be critically involved in autophagy regulation.

Since previous studies have demonstrated that Nobiletin is a specific activator of ROR, we explored the possibility of whether Nobiletin would activate autophagy during hypoxic stress. Interestingly, while hypoxia triggered severe cellular defects resulting in the accumulation of ROS-producing mitochondria and cell death from impaired autophagy/mitophagy, Nobiletin restored autophagy and clearance of damaged mitochondria during hypoxic stress. This also coincided with improved mitochondrial bioenergetics and cell viability. Although Nobiletin has been previously tested in the context of inflammation and cancer, the underlying mechanism was not determined. The fact that genetic interventions, including knockdown of ROR, abrogated the cytoprotective effects of Nobiletin on mitochondrial function, identifies ROR as a critical regulator of autophagy. This view is further substantiated by the fact that Nobiletin failed to suppress mitochondrial injury and cell death in cardiac myocytes rendered defective for ATG7. These findings support the notion that cytoprotective effects conferred by Nobiletin on autophagy and cell viability are dependent upon ROR. Although we did not examine the impact of Nobiletin on temporal effects of advanced age on cardiac function per se, which will require more detailed longitudinal studies, our studies

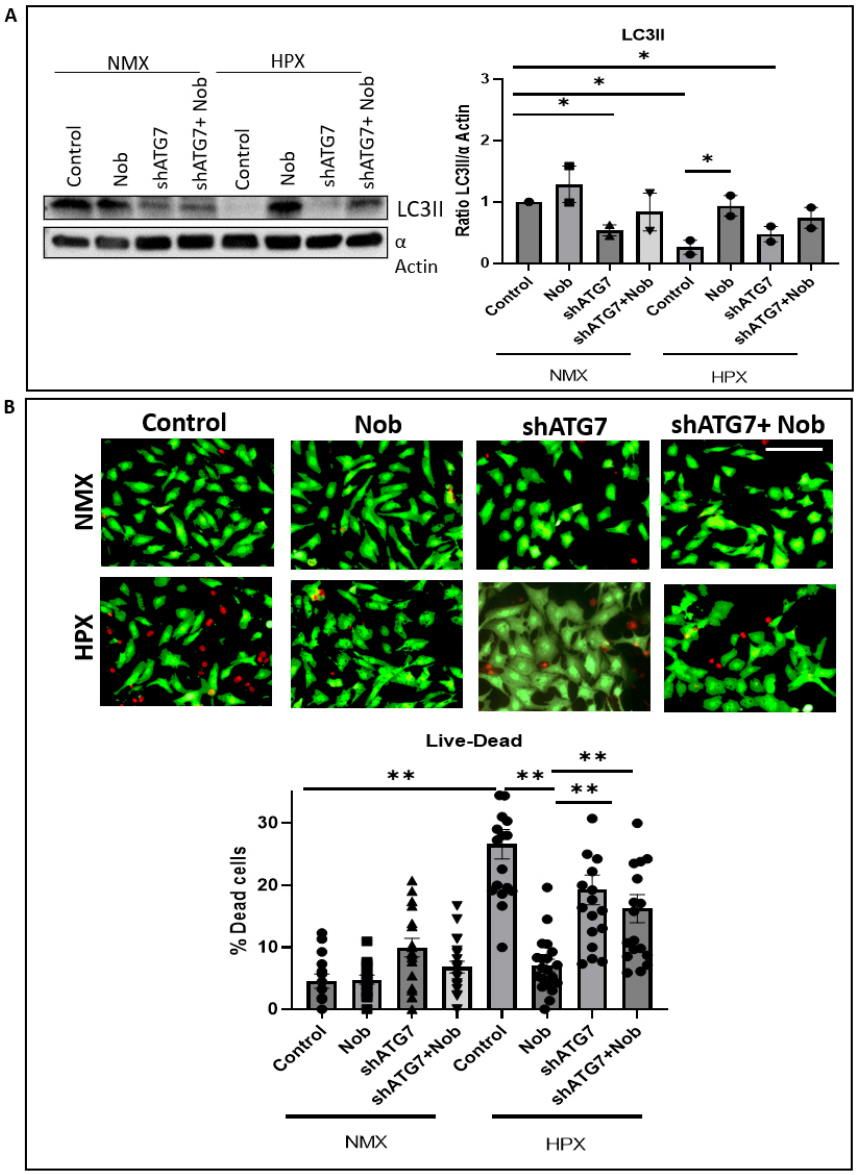

Figure 7. Nobiletin fails to rescue autophagy gene expression and cell death in the presence of shATG7. (A) Western blot analysis of LC3 protein expression in cardiac myocytes under normoxia (NMX) and hypoxia (HPX) in the presence and absence of Nob and shATG7. Histogram represents quantitative data for the western blot, relative to α sarcomeric Actin as a loading control. (B) Cell viability assessed with vital dyes calcein-AM (green; live cells) and ethidium-homodimer-1 (red; dead cells), bar: 100 μm. Data are expressed as mean ± SEM. *$P < 0.05$; **$P < 0.01$, n = 3-4 independent myocyte isolations, counting > 200 cells for each condition tested.

nevertheless support the notion that damaged ROS-producing mitochondria can be cleared by autophagic processes mediated by Nobiletin treatment. Nevertheless, whether the protective effect of Nobiletin is due to reduced oxidative stress which consequently signals the activation of autophagy and mitochondrial clearance, or whether Nobiletin-induced activation of autophagy leads to reduced oxidative stress has not been identified in our study. Therefore, it will be interesting to explore in future studies whether Nobiletin impinges on these or other cellular targets that confer protection during hypoxic stress.

In summary, Nobiletin was previously shown to have multifunctional effects by interacting with several targets/pathways, such as N-methyl-D-aspartate (NMDA) receptor, NF-B, Pparg coactivator 1 alpha (PGC1α) and Phosphoinositide 3-kinase (PI3K)[20]. Furthermore, ROR is a positive regulator of the gene Basic Helix-Loop-Helix ARNT Like 1 (BMAL1), which is one of the core circadian genes[1], suggesting a role for Nobiletin in regluing circadian rhythms. Our findings reveal a novel signaling pathway that functionally links Nobiletin-mediated ROR signaling to cellular quality control mechanisms involving autophagy activation and cardiac cell survival. Given that impaired mitochondrial function and cellular quality control mechanisms are commonly associated with myocardial infarction and advanced aging, our data highlight a critical connection between ROR and autophagy regulation of mitochondrial quality control. Hence, the findings of the present study suggest that Nobiletin may be an effective therapeutic intervention for restoring cellular quality control mechanisms and cardiac function during cardiac stress conditions in the aged myocardium.

DECLARATIONS

Authors' contributions

Performed the studies, analyzed the data, and wrote the manuscript: Kirshenbaum E
performed the studies and analyzed the data: Nguyen H
Assisted with the microscopy studies: Margulets V
Assisted with the western blots: Crandall M
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Conceptualized and designed the study, and wrote the manuscript: Rabinovich-Nikitin I

Availability of data and materials

Data and materials are available upon request.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

All animal experiments conducted in this study were approved by the animal care committee of the University of Manitoba (22-048) and are in accordance with the guidelines laid for the protection of animals used for scientific purposes by the Canadian Council for Animal Care, directive 2010/63/EU, and National Institutes of Health (NIH).

Consent for publication

Not applicable.

REFERENCES

1. Rabinovich-Nikitin I, Lieberman B, Martino TA, Kirshenbaum LA. Circadian-regulated cell death in cardiovascular diseases. *Circulation* 2019;139:965-80. DOI PubMed

2. Rabinovich-Nikitin I, Love M, Kirshenbaum LA. Intersection of autophagy regulation and circadian rhythms in the heart. *Biochim Biophys Acta Mol Basis Dis* 2022;1868:166354. DOI PubMed
3. Sciarretta S, Maejima Y, Zablocki D, Sadoshima J. The role of autophagy in the heart. *Annu Rev Physiol* 2018;80:1-26. DOI PubMed
4. Rabinovich-Nikitin I, Rasouli M, Reitz CJ, et al. Mitochondrial autophagy and cell survival is regulated by the circadian clock gene in cardiac myocytes during ischemic stress. *Autophagy* 2021;17:3794-812. DOI PubMed PMC
5. Wang EY, Gang H, Aviv Y, Dhingra R, Margulets V, Kirshenbaum LA. p53 mediates autophagy and cell death by a mechanism contingent on Bnip3. *Hypertension* 2013;62:70-7. DOI PubMed
6. Cassidy LD, Narita M. Autophagy at the intersection of aging, senescence, and cancer. *Mol Oncol* 2022;16:3259-75. DOI PubMed PMC
7. Ito H, Negoro H, Kono J, et al. Effectiveness and safety of a mixture of nobiletin and tangeretin in nocturia patients: a randomized, placebo-controlled, double-blind, crossover study. *J Clin Med* 2023;12:2757. DOI PubMed PMC
8. Kim E, Kim YJ, Ji Z, et al. ROR activation by nobiletin enhances antitumor efficacy via suppression of IκB/NF-κB signaling in triple-negative breast cancer. *Cell Death Dis* 2022;13:374. DOI PubMed PMC
9. Dusabimana T, Kim SR, Kim HJ, Park SW, Kim H. Nobiletin ameliorates hepatic ischemia and reperfusion injury through the activation of SIRT-1/FOXO3a-mediated autophagy and mitochondrial biogenesis. *Exp Mol Med* 2019;51:1-16. DOI PubMed PMC
10. Moon JY, Cho SK. Nobiletin induces protective autophagy accompanied by ER-stress mediated apoptosis in human gastric cancer SNU-16 cells. *Molecules* 2016;21:914. DOI PubMed PMC
11. Lee JM, Kim H, Baek SH. Unraveling the physiological roles of retinoic acid receptor-related orphan receptor α. *Exp Mol Med* 2021;53:1278-86. DOI PubMed PMC
12. Hariharan N, Zhai P, Sadoshima J. Oxidative stress stimulates autophagic flux during ischemia/reperfusion. *Antioxid Redox Signal* 2011;14:2179-90. DOI PubMed PMC
13. Cui H, Kong Y, Zhang H. Oxidative stress, mitochondrial dysfunction, and aging. *J Signal Transduct* 2012;2012:646354. DOI PubMed PMC
14. Katayama H, Kogure T, Mizushima N, Yoshimori T, Miyawaki A. A sensitive and quantitative technique for detecting autophagic events based on lysosomal delivery. *Chem Biol* 2011;18:1042-52. DOI PubMed
15. Biala AK, Kirshenbaum LA. The interplay between cell death signaling pathways in the heart. *Trends Cardiovasc Med* 2014;24:325-31. DOI PubMed
16. Kirshenbaum LA. Regulation of autophagy in the heart in health and disease. *J Cardiovasc Pharmacol* 2012;60:109. DOI
17. Rubinsztein DC, Mariño G, Kroemer G. Autophagy and aging. *Cell* 2011;146:682-95. DOI PubMed
18. Wang HH, Sun YN, Qu TQ, et al. Nobiletin prevents D-galactose-induced C2C12 cell aging by improving mitochondrial function. *Int J Mol Sci* 2022;23:11963. DOI PubMed PMC
19. Dhingra R, Kirshenbaum LA. Regulation of mitochondrial dynamics and cell fate. *Circ J* 2014;78:803-10. DOI PubMed
20. Huang H, Li L, Shi W, et al. The multifunctional effects of nobiletin and its metabolites in vivo and in vitro. *Evid Based Complement Alternat Med* 2016;2016:2918796. DOI PubMed PMC