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Original Articles

Honokiol induces autophagy of neuroblastoma cells through activating the PI3K/Akt/mTOR and endoplasmic reticular stress/ERK1/2 signaling pathways and suppressing cell migration

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ABSTRACT

In children, neuroblastomas are the most common and deadly solid tumor. Our previous study showed that honokiol, a small-molecule polyphenol, can traverse the blood–brain barrier and kill neuroblastoma cells. In this study, we further investigated the mechanisms of honokiol-induced insults to neuroblastoma cells. Treatment of neuroblastoma neuro-2a cells with honokiol elevated the levels of microtubule-associated protein light chain 3 (LC3)-II and induced cell autophagy in time- and concentration-dependent manners. Interestingly, pretreatment with 3-methyladenine (3-MA), an inhibitor of autophagy, led to the simultaneous attenuation of honokiol-induced cell autophagy and apoptosis but did not influence cell necrosis. As to the mechanisms, exposure of neuro-2a cells to honokiol time-dependently decreased the amount of phosphatidylinositol 3-kinase (PI3K). Sequentially, honokiol downregulated phosphorylation of protein kinase B (Akt) and mammalian target of rapamycin (mTOR) in neuro-2a cells. Furthermore, honokiol elevated the levels of glucose-regulated protein (GpR)78, an endoplasmic reticular stress (ERS)-associated protein, and amounts of intracellular reactive oxygen species (ROS). In contrast, reducing production of intracellular ROS using N-acetylcysteine, a scavenger of ROS, concurrently suppressed honokiol-induced cellular autophagy. Consequently, honokiol stimulated phosphorylation of extracellular signal-regulated kinase (ERK)1/2. However, pretreatment of neuro-2a cells with PD98059, an inhibitor of ERK1/2, lowered honokiol-induced autophagy. The effects of honokiol on inducing autophagy and apoptosis of neuroblastoma cells were further confirmed using mouse neuroblastoma NB41A3 cells as our experimental model. Fascinatingly, treatment of neuroblastoma neuro-2a and NB41A3 cells with honokiol for 12 h did not affect cell autophagy or apoptosis but caused significant suppression of cell migration. Taken together, this study showed that honokiol can induce autophagy of neuroblastoma cells and consequent apoptosis through activating the PI3K/Akt/mTOR and ERS/ROS/ERK1/2 signaling pathways and suppressing cell migration. Thus, honokiol has potential for treating neuroblastomas.

Abbreviations: Akt, protein kinase B; ANOVA, analysis of variance; BBB, blood–brain barrier; BrdU, 5-bromo-2′-deoxyuridine; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; ERK1/2, extracellular signal-regulated kinase 1/2; ERS, endoplasmic reticular stress; FBS, fetal bovine serum; GpR78, glucose-regulated protein 78; LC3, light chain 3; 3-MA, 3-methyladenine; mAb, monoclonal antibody; mTOR, mammalian target of rapamycin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NAC, N-acetylcysteine; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline buffer; PI, propidium iodide; PI3K, phosphatidylinositol 3-kinase; RIPA, ice-cold radioimmunoprecipitation assay; ROS, reactive oxygen species; SDS, sodium dodecylsulfate.

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Introduction

In children, childhood cancer is the leading cause of disease-related deaths [1]. Among tumors diagnosed in childhood, neuroblastomas are the most common solid cancer. Neuroblastomas are neuroendocrine tumors that develop from neural crest elements of the sympathetic nervous system [2]. In the clinic, neuroblastomas can be found in several areas of the body, including the adrenal glands, abdomen, neck, chest, spine, and pelvis [3]. According to risk categories, neuroblastomas are classified into low, intermediate, and high risk. Unfortunately, children who suffer from a neuroblastoma are usually diagnosed as being high-risk patients [4]. Furthermore, more than 50% of neuroblastoma patients present with metastasis into the bone marrow, cytoskeleton, lymph nodes, liver, and intracranial orbital sites [5]. As a result, neuroblastomas are still a major problem in pediatric oncology. Patients who suffer from an adverse neuroblastoma are frequently treated with a coordinated sequence of chemotherapy, surgery, radiation, or combined therapy [6]. As to chemotherapy, strengthening conventional cancer drugs by combining them with 13-cis-retinoic acid remarkably improved the outcomes of neuroblastoma patients. Nevertheless, for children with a high-risk neuroblastoma, only one-third of cases are predicted to have long-term survival when treated with existing regimens [7]. In addition, the costs of conservative therapies are fairly high. More importantly, children with a high-risk neuroblastoma may achieve a long-term cure but usually develop complications of hearing loss, cardiac dysfunction, infertility, and secondary malignancies after therapy [8]. Clinically, high-dose chemotherapy to achieve minimal residual disease is a common cause leading to therapeutic failure with neuroblastomas [7]. Consequently, discovering more-effective and less-toxic drugs that can improve survival rates but decrease side effects is certainly needed to establish new therapeutic strategies for treating neuroblastomas.

Honokiol (2-(4-hydroxy-3-prop-2-enyl-phenyl)-4-prop-2-enyl-phenol) is a small-molecule polyphenol that was proven to be the most important active pharmaceutical ingredient of Houpo (*Magnolia officinalis* Rehd. et Wils.), a traditional Chinese medicine [9,10]. Previous studies showed that honokiol had advantageous effects on a variety of diseases, such as anti-anxiety, anti-depression, anti-oxidant, anti-inflammatory, antibacterial, anti-platelet, and anti-arrhythmic functions [10,11]. A previous clinical trial reported that 13-cis-retinoic acid is helpful for children with a neuroblastoma following administration of chemotherapy or transplantation [12]. Structurally, honokiol was identified as a new natural rexinoid and can activate the retinoid X receptor to upregulate the expression of the ATP-binding cassette transporter A1 gene [13]. The ATP-binding cassette transporter A1 protein plays therapeutic and teratogenic roles in therapy for neuroblastoma patients because it acts to maintain cholesterol homeostasis [14]. Honokiol can also stimulate neurite growth and protect neurons against oxidative stress-induced insults [15,16]. Recent oncological studies presented honokiol as a novel and multifunctional natural agent against tumors and angiogenesis [11,17]. Moreover, our previous study demonstrated that honokiol can pass through the blood–brain barrier (BBB) and kill neuroblastoma cells without affecting the viability of normal brain cells [18]. Therefore, honokiol can be used as an effective candidate drug for treating patients with high-risk neuroblastomas.

Autophagy is a preserved progression that uses double-membrane vesicles to deliver cytoplasmic contents to lysosomes for degradation [19]. Autophagic cells can consequently undergo cell survival or death [20]. In particular, autophagy has been shown to closely crosstalk with apoptosis, a programmed cell death. Hence, autophagy censoriously maintains homeostasis between survival and death in eukaryotic cells. Microtubule-associated protein 1A/1B-light chain 3 (LC3) is ubiquitously distributed in mammalian cells [21]. During autophagy, a cytosolic form of LC3 (LC3-I) is conjugated to phosphatidylethanolamine to form an LC3–phosphatidylethanolamine conjugate (LC3-II), which is recruited to autophagosomal membranes. Thus, LC3-I/II can be immunodetected to monitor autophagy and autophagy-related processes. As to the mechanisms, mammalian target of rapamycin (mTOR), a serine/threonine kinase, is a major negative regulator of cellular autophagy [22]. mTOR signaling is deregulated in many human diseases because the process participates in regulating cell growth and proliferation. Various protein kinases are involved in regulating mTOR activation [23]. The phosphoinositide-3 kinase (PI3K)-mediated activation of protein kinase B (Akt) can subsequently stimulate phosphorylation of mTOR [23,24]. In contrast, extracellular signal-regulated kinase 1/2 (ERK1/2) signaling negatively adjusts mTOR phosphorylation [25]. Furthermore, endoplasmic reticular stress (ERS) is reported to be a stimulus that can induce both autophagy and apoptosis [26]. Thus, ERS-induced autophagy critically implies the therapeutic potential in a variety of cancers. A previous study reported that ERS can elevate intracellular reactive oxygen species (ROS) levels and then simulate phosphorylation of ERK1/2 [27]. In addition to apoptosis, autophagy and autophagy-induced cell death are important factors when examining cancer drugs. Our previous study showed that honokiol can induce apoptotic insults to neuroblastoma cells [18]. In this study, we further attempted to evaluate the effects of honokiol on autophagy of neuroblastoma cells and possible molecular mechanisms.

Materials and methods

Reagents

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco-BRL (Grand Island, NY, USA). Honokiol with purity >98%, 3-methyladenine (3-MA), acridine orange, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI), 5-bromo-2′-deoxyuridine (BrdU), PD98059, N-acetylcysteine (NAC), and dimethyl sulfoxide (DMSO) were obtained from Sigma (St. Louis, MO, USA). 2′,7′-Dichlorofluorescin diacetate was from Molecular Probes (Eugene, OR, USA).

Cell culture and drug treatments

Neuroblastoma neuro-2a and NB41A3 cells were purchased from American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM supplemented with 10% heat-inactivated FBS, L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin in 75-cm^2 flasks at 37 °C in a humidified atmosphere of 5% CO_2. Cells were grown to confluence prior to drug administration. Honokiol was purchased from Sigma and freshly dissolved in DMSO. Neuroblastoma cells were exposed to different concentrations of honokiol for various time intervals. For knockdown assays, cells were pretreated with 1 mM 3-MA (an inhibitor of autophagy), 30 μM PD98059 (an inhibitor of ERK1/2), or 1 mM NAC (a scavenger of ROS) for 1 h and then exposed to honokiol.

Assay of cell autophagy

Cell autophagy was assayed by quantifying cells with acidic vesicular organelles using flow cytometry as described previously [21]. After drug treatment, neuroblastoma neuro-2a and NB41A3 cells (10^5) were treated with 1 μg/ml acridine orange for 20 min. Then, cells were collected in phenol red-free DMEM. Green and red fluorescence levels of acridine orange in cells were measured with a flow cytometer (Becton Dickinson, San Jose, CA, USA). Fluorescent intensities were quantified with the aid of CellQuest software (Becton Dickinson). A fluorescent microscope (Nikon, Tokyo, Japan) was used to observe and photograph cells with fluorescent signals.

Analysis of cell viability

The toxicity of honokiol to neuro-2a and NB41A3 cells was determined using a colorimetric method as described previously [28]. Briefly, neuro-2a or NB41A3 cells (10^4 cells/well) were seeded in 96-well tissue culture plates overnight. After drug treatment, cells were cultured with a new medium containing 0.5 mg/ml MTT for a further 3 h. The blue formazan products in cells were dissolved in DMSO and spectrophotometrically measured at a wavelength of 550 nm.

Assay of DNA fragmentation

DNA fragmentation in neuroblastoma neuro-2a and NB41A3 cells was quantified using a cellular DNA fragmentation enzyme-linked immunosorbent assay kit (Boehringer Mannheim, Indianapolis, IN, USA) as described previously [29]. Briefly, neuroblastoma cells (2×10^5 cells) were sub-cultured in 24-well tissue culture plates and labeled with BrdU overnight. Cells were harvested and suspended in the culture medium. Then 100 μl of the cell suspension was added to each well of 96-well tissue culture plates. Neuroblastoma cells were co-cultured with honokiol for another 8 h at 37 °C in a humidified atmosphere of 5% CO_2. The amount of BrdU-labeled DNA in the cytoplasm was quantified using an Anthos 2010 microplate photometer (Anthos Labtec Instruments, Lagerhausstrasse, Wals/Salzburg, Austria) at a wavelength of 405 nm.

Quantification of apoptotic cells

Apoptosis of neuroblastoma cells was determined using PI to detect DNA injury in the nuclei according to a previously described method [30]. Briefly, after drug administration, neuroblastoma neuro-2a and NB41A3 cells were harvested and fixed in cold 80% ethanol. Following centrifugation and washing, fixed cells were stained with PI and analyzed using a FACScan flow cytometer (Becton Dickinson).

Determination of necrotic cells

Necrotic cells were determined using a photometric immunoassay according to a previously described method [18]. Briefly, neuroblastoma neuro-2a and NB41A3 (10^5) cells were seeded in 96-well tissue culture plates overnight. After drug treatment, cell lysates and culture medium were collected, and necrotic cells were immunodetected using a mouse monoclonal antibody (mAb) against histone. After the antibody reaction and washing, the colorimetric product was measured at 405 nm against a substrate solution as a blank.

Analysis of intracellular ROS

Levels of intracellular ROS in neuroblastoma cells were quantified as described previously [31]. Briefly, neuro-2a cells (5×10^5 cells/well) were cultured in 12-well tissue culture plates overnight. Cells were co-treated with honokiol and 2′,7′-dichlorofluorescin diacetate, an ROS-sensitive dye. After drug administration, neuro-2a cells were harvested and suspended in 1× phosphate-buffered saline (PBS) (0.14 M NaCl, 2.6 mM KCl, 8 mM Na_2HPO_4, and 1.5 mM KH_2PO_4). Relative fluorescent intensities in osteoblasts were quantified using a flow cytometer (Becton Dickinson).

Immunodetection of LC3-I, LC3-II, PI3K, p-Akt, Akt, p-mTOR, mTOR, glucose-regulated protein (Grp) 78, p-ERK1/2, ERK1, and β-actin

Immunoblotting assays were further carried out to determine cell autophagy and the possible molecular mechanisms as described previously [32]. After drug treatment, neuro-2a cells were washed with 1× PBS. Cell lysates were prepared in ice-cold radioimmunoprecipitation assay (RIPA) buffer (25 mM Tris–HCl (pH 7.2), 0.1% sodium dodecylsulfate (SDS), 1% Triton X-100, 1% sodium deoxycholate, 0.15 M NaCl, and 1 mM EDTA). To avoid protein degradation, a mixture of proteinase inhibitors, including 1 mM phenyl methyl sulfonyl fluoride, 1 mM sodium orthovanadate, and 5 μg/ml leupeptin, was added to the RIPA buffer. Protein concentrations were quantified by a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA). Cytosolic proteins (100 μg/well) were subjected to SDS–polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes. Membranes were blocked with 5% non-fat milk at 37 °C for 1 h. LC3-I, LC3-II, PI3K, p-Akt, p-mTOR, Grp78, and p-ERK1/2 were immunodetected using rabbit polyclonal antibodies or mouse mAbs against related proteins (Cell Signaling, Danvers, MA, USA). Cellular Akt, mTOR, ERK1, and β-actin proteins were immunodetected as internal standards (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Intensities of the immunoreactive bands were determined using an UVIDOCMW version 99.03 digital imaging system (UVtec, Cambridge, UK) as described previously [33].

Wound-healing assay

Effects of honokiol on cell migration were determined using a wound-healing assay as described previously [34]. Neuroblastoma neuro-2a and NB41A3 cells (5×10^4 cells/well) were seeded in 12-well plates. When the cells had grown to confluence, the monolayers were scratched with a sterile 1000-μl pipette tip. Cells were washed with PBS and then exposed to honokiol in DMEM. After drug treatment for 12 h, the wounded area was observed and photographed under a light microscope (Nikon). Migrated cells were counted and statistically analyzed.

Statistical analysis

Statistical differences between the control and drug-treated groups were considered significant when the p value of Duncan's multiple-range test was <0.05. Statistical analysis between drug-treated groups was carried out using a two-way analysis of variance (ANOVA).

Results

Honokiol induces autophagy and resulting apoptosis of neuroblastoma cells

Analyses of acidic vesicular organelles and LC3 proteins were carried out to determine the effects of honokiol on cellular autophagy (Fig. 1). Staining with acridine orange revealed that exposure of neuroblastoma neuro-2a cells to 25 μM honokiol for 72 h did not change the proportion of cells with acidic vesicular organelles (Fig. 1A). In comparison, at concentrations of 50 and 100 μM, proportions of neuro-2a cells stained with acridine orange were augmented. These fluorescent signals were quantified and statistically analyzed (Fig. 1B). Treatment of neuroblastoma neuro-2a cells with 50 and 100 μM for 72 h respectively induced cell autophagy by 29% and 59%. A time-dependent assay further showed that exposure of neuro-2a cells to 50 μM honokiol for 24, 48, and 72 h induced cellular autophagy in a time-dependent manner (Fig. 1C). Fluorescent signals were quantified and statistically analyzed (Fig. 1D). Treatment of neuro-2a cells with 50 μM honokiol for 24, 48, and 72 h respectively caused 14%, 23%, and 32% induction of cellular autophagy. In control neuro-2a cells, the LC3-II protein was not detected (Fig. 1E, top panel, lane 1). However, exposure of neuro-2a cells to 50 μM honokiol for 24 h slightly increased the levels of LC3-II (lane 2). In contrast, following exposure for 48 and 72 h, expressions of LC3-II in neuro-2a cells were obviously augmented by honokiol (lanes 3 and 4). The amount of β-actin was immunodetected as the internal control (bottom panel). These protein bands were quantified and statistically analyzed (Fig. 1F). Treatment of neuro-2a cells with honokiol for 48 and 72 h caused significant increases in levels of LC3-II.

3-MA, an inhibitor of autophagy, was applied to neuroblastoma cells to evaluate the effects of honokiol-induced autophagy on resulting cellular insults (Fig. 2). Exposure of neuro-2a cells to honokiol induced cell autophagy by 31% (Fig. 2A). 3-MA alone did not influence cell autophagy but lowered honokiol-induced autophagy by 69%. Treatment of neuro-2a cells with honokiol respectively caused significant 2.4-fold and 40% increases in DNA fragmentation and cell apoptosis (Fig. 2B, C). In comparison, pretreatment with 3-MA significantly attenuated honokiol-induced DNA fragmentation and cell apoptosis by 39% and 42%, respectively. Additionally, treatment with 3-MA, honokiol, and a combination of 3-MA and honokiol did not affect cell necrosis (Fig. 2C). Separately, exposure to honokiol led to a 51% decrease in the viability of neuro-2a cells (Fig. 2D). However, 3-MA lessened honokiol-induced cell death by 53%. Pretreatment with 3-MA alone did not change DNA fragmentation, cell apoptosis, or cell death (Fig. 2B–D).

Honokiol triggers autophagy via activation of the PI3K/Akt/mTOR signaling pathway

Immunoblotting analyses of the PI3K/Akt/mTOR signaling pathway were further performed to determine the mechanisms of honokiol-induced cellular autophagy (Fig. 3). PI3K was immunodetected in control neuroblastoma neuro-2a cells (Fig. 3A, top panel, lane 1). However, treatment of neuro-2a cells with honokiol for 24 h decreased the levels of PI3K (lane 2). After exposure to honokiol for 48 and 72 h, the amount of PI3K in neuro-2a cells was obviously diminished (lanes 3 and 4). β-Actin was analyzed as the internal control (bottom panel). These protein bands were quantified and statistically analyzed (Fig. 3B). Exposure of neuro-2a cells to honokiol for 24, 48, and 72 h respectively caused significant 15%, 54%, and 63% reductions in the amount of PI3K. Sequentially, treatment with honokiol for 24 h decreased the levels of p-AKT and p-mTOR in neuro-2a cells (Fig. 3C, E, top panels, lane 2). However, the amounts of p-AKT and p-mTOR were downregulated

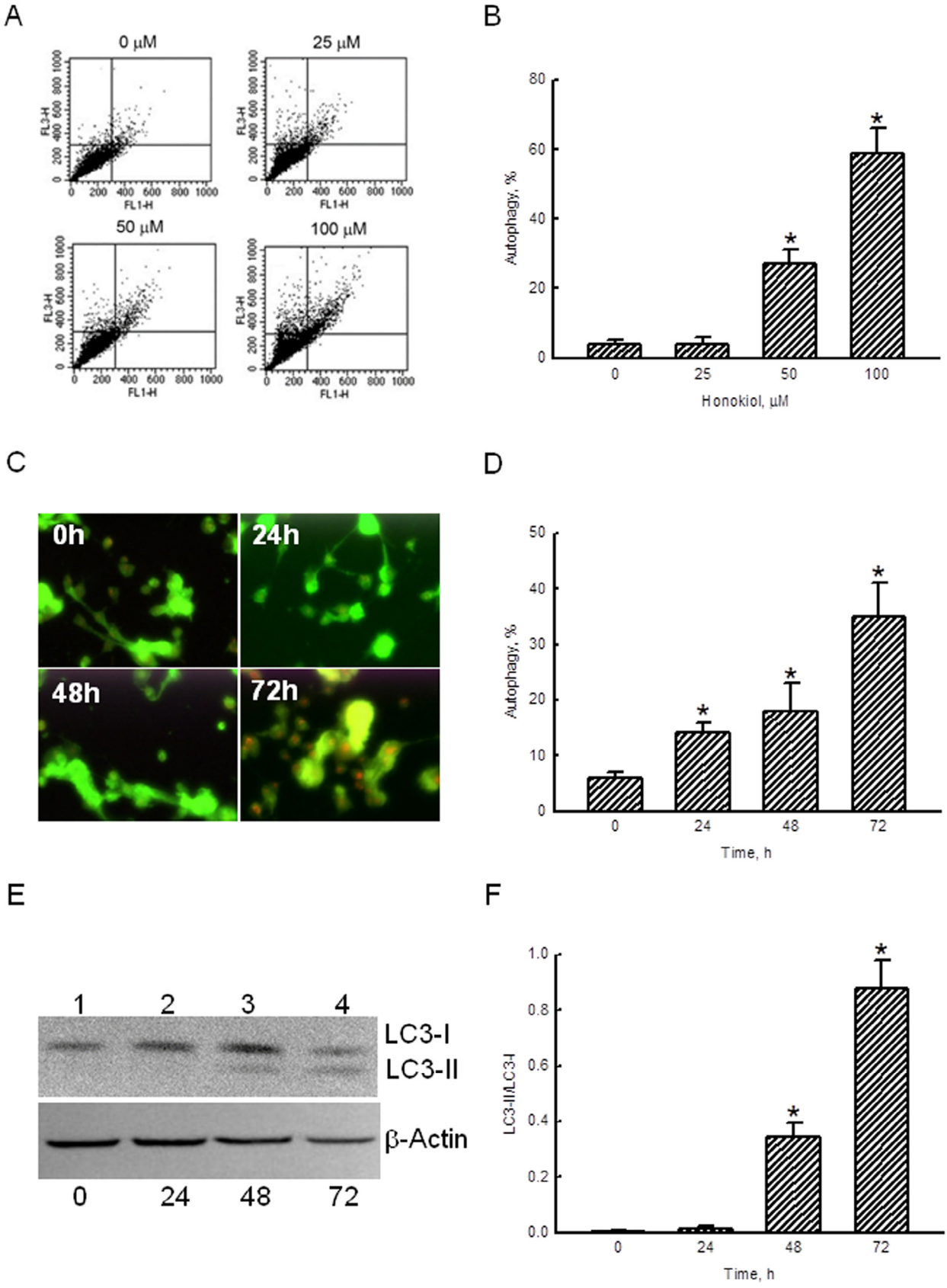

Fig. 1. Effects of honokiol on autophagy of neuroblastoma cells. Neuro-2a cells were treated with 25, 50, and 100 μM honokiol for 72 h (A, B) or with 50 μM honokiol for 24, 48, and 72 h (C, D). After staining with acridine orange, cellular autophagy was quantified using flow cytometry to detect the formation of acidic vesicular organelles (A, B, D). Cells with fluorescent signals were observed and photographed with a fluorescent microscope at 200× (C). Levels of LC3-I and LC3-II were immunodetected (E, top panel). β-Actin was analyzed as the internal control (bottom panel). These protein bands were quantified and statistically analyzed (F). Each value represents the mean ± SEM for $n = 6$. An asterisk (*) indicates that values significantly differed from the respective control, $p < 0.05$.

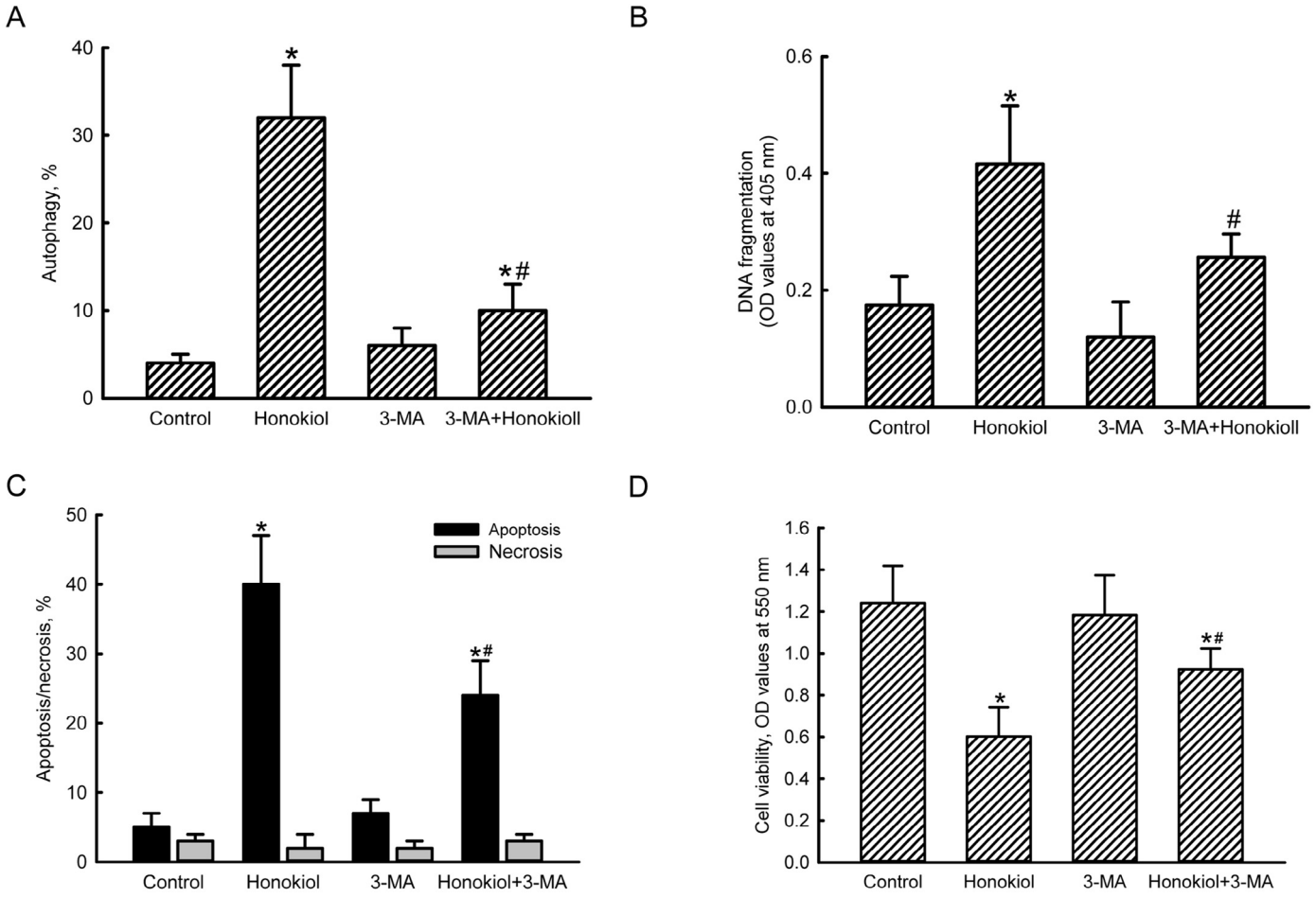

Fig. 2. Effects of 3-methyladenine (3-MA) on honokiol-induced autophagy, DNA fragmentation, apoptosis, and cell viability. Neuro-2a cells were pretreated with 1 mM 3-MA for 1 h and then exposed to 50 μM honokiol for another 72 h. Cellular autophagy (A) and apoptosis (C) were analyzed using flow cytometry. DNA fragmentation (B) and cell necrosis (C) were quantified with a photometric immunoassay. Cell viability was assayed using a colorimetric method (D). Each value represents the mean ± SEM, $n = 6$. The symbols * and # indicate that a value significantly ($p < 0.05$) differed from the respective control and honokiol-treated groups, respectively.

following exposure to honokiol for 48 and 72 h (lanes 3 and 4). Akt and mTOR were analyzed as respective internal controls for p-Akt and p-mTOR (Fig. 3C, E, bottom panels). These protein bands were quantified and statistically analyzed (Fig. 3D, F). Treatment with honokiol for 24, 48, and 72 h respectively led to significant decreases of 26%, 49%, and 64% for p-Akt and 24%, 54%, and 61% for p-mTOR.

ERS and ROS participates in honokiol-induced autophagy of neuroblastoma cells

To determine the effects of honokiol on ERS, levels of Grp78 and intracellular ROS were assayed (Fig. 4A). Exposure of neuroblastoma neuro-2a cells to honokiol for up to 24 h did not change the amount of Grp78 (Fig. 4A, top panel, lane 2). Following treatment with honokiol for 48 and 72 h, the amount of Grp78 in neuro-2a cells was obviously augmented (lanes 3 and 4). β-Actin was analyzed as the internal control (bottom panel). These protein bands were quantified and statistically analyzed (Fig. 4B). Exposure of neuro-2a cells to honokiol for 48 and 72 h respectively elevated the amount of Gpr78 by 2.1- and 2.3-fold. Treatment of neuro-2a cells with honokiol for 24, 48, and 72 h respectively caused significant 58%, 138%, 179% increases in the levels of intracellular ROS (Fig. 4C). In comparison, pretreatment with NAC significantly suppressed honokiol-induced intracellular ROS by 80% (Fig. 4D). Interestingly, honokiol-induced autophagy of neuro-2a cells was significantly suppressed by 43% following pretreatment with NAC (Fig. 4E).

Honokiol stimulates ERK1/2 phosphorylation and consequent autophagy of neuroblastoma cells

Phosphorylation of ERK1/2 (p-ERK1/2) was analyzed to determine the roles of ERS and intracellular ROS in honokiol-induced cellular autophagy (Fig. 5). Exposure of neuro-2a cells to honokiol for 24 h did not change the p-ERK1/2 level (Fig. 5A, top panel, lane 2). In contrast, levels of p-ERK1/2 in neuro-2a cells were obviously elevated following honokiol administration for 48 and 72 h (lanes 3 and 4). The amount of ERK1 was analyzed as the internal control (bottom panel). These protein bands were quantified and statistically analyzed (Fig. 5B). Our results showed that treatment of neuro-2a cells with honokiol for 48 and 72 h respectively led to significant 3.5- and 3.3-fold increases in the amount of p-ERK1. In parallel, after exposure for 48 and 72 h, honokiol respectively stimulated phosphorylation of ERK2 by 41% and 39% (Fig. 5B). To further determine the roles of ERK1/2 in honokiol-induced autophagy, neuro-2a cells were pretreated with PD98059, an inhibitor of ERK1/2 (Fig. 5C). Treatment with honokiol caused 31% of neuro-2a cells to undergo autophagy. Pretreatment with PD98059 alone did not influence cell autophagy (Fig. 5C). In comparison, PD98059 downregulated honokiol-induced autophagy by 42%.

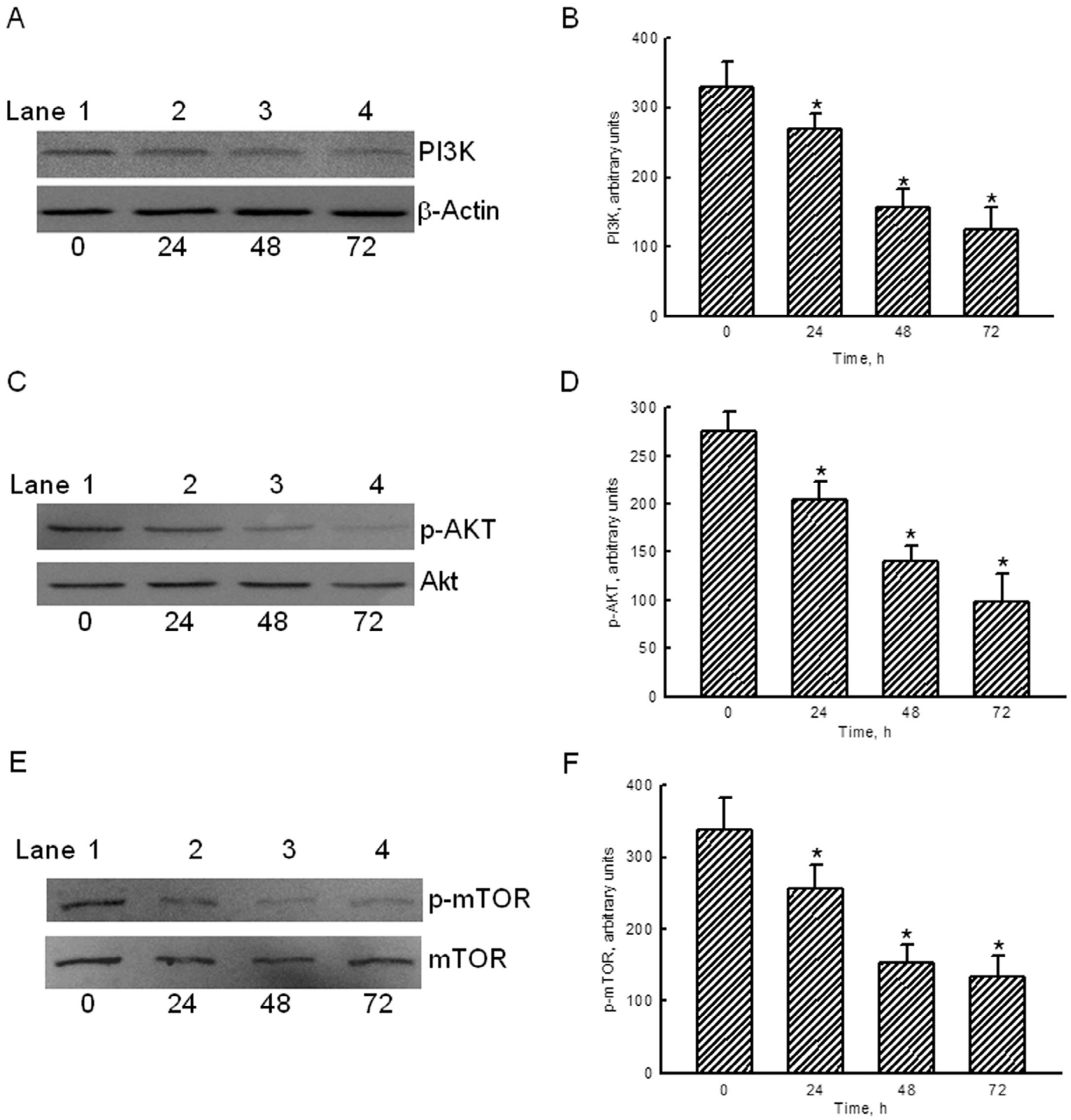

Fig. 3. Effects of honokiol on downregulation of PI3K/Akt/mTOR signaling. Neuro-2a cells were treated with 50 μM honokiol for 24, 48, and 72 h. Amounts of PI3K, p-Akt, and p-mTOR were immunodetected (A, C, E, top panels). β-Actin, Akt, and mTOR were respectively analyzed as the internal controls for PI3K, p-Akt, and p-mTOR (A, C, E, bottom panels). These protein bands were quantified and statistically analyzed (B, D, F). Each value represents the mean ± SEM, $n = 6$. An asterisk (*) indicates that a value significantly ($p < 0.05$) differed from the respective control.

Honokiol-induced autophagy of neuroblastoma cells is confirmed using NB413 cells as another experimental model

To confirm the effects of honokiol on inducing autophagy in neuroblastoma cells, NB41A3 cells were further used as our experimental model (Fig. 6). Exposure of neuroblastoma NB41A3 cells to honokiol induced cellular autophagy by 36% (Fig. 6A). However, pretreatment with 3-MA led to a 64% reduction in honokiol-induced autophagy. In parallel, treatment of NB41A3 cells with honokiol induced DNA fragmentation by 2.3-fold (Fig. 6B). The

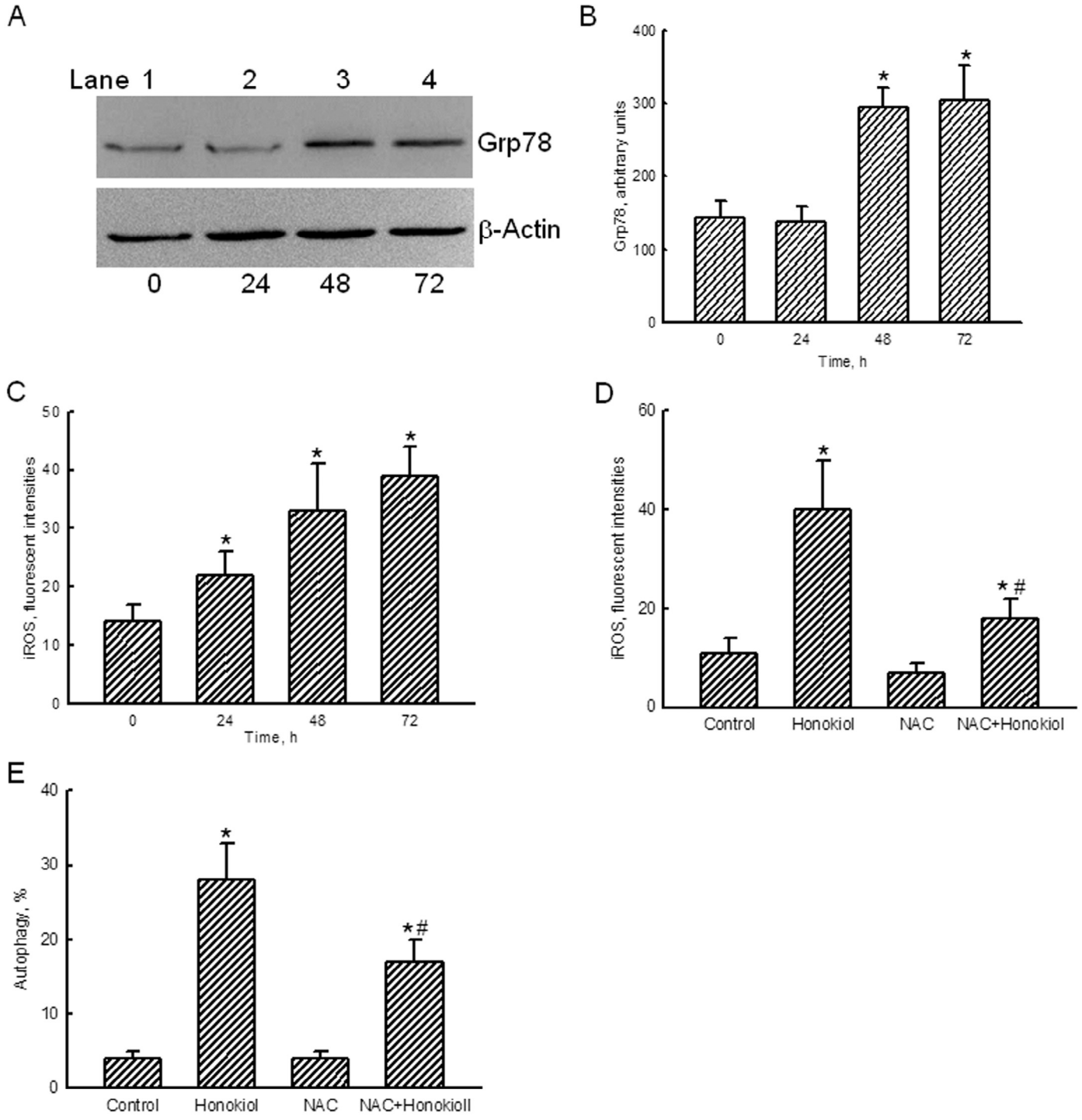

Fig. 4. Effect of honokiol on endoplasmic reticular stress (ERS)-induced production of Grp78 and intracellular reactive oxygen species (iROS). Neuro-2a cells were treated with 50 μM honokiol for 24, 48, and 72 h. The amount of Grp78 was immunodetected (A, top panel). β-Actin was analyzed as the internal control (bottom panel). These protein bands were quantified and statistically analyzed (B). Levels of iROS were assayed using flow cytometry (C). To determine the roles of ROS in honokiol-induced autophagy, neuro-2a cells were pretreated with 1 mM N-acetylcysteine (NAC), a scavenger of ROS, for 1 h and then exposed to 50 μM honokiol for 72 h. iROS (D) and cellular autophagy (E) were quantified using flow cytometry. Each value represents the mean ± SEM, $n = 6$. The symbols * and # respectively indicate that a value significantly ($p < 0.05$) differed from the control and honokiol-treated groups.

honokiol-triggered DNA damage was significantly alleviated by 3-MA. Sequentially, honokiol triggered 41% of NB41A3 cells to undergo apoptosis (Fig. 6C). 3-MA decreased honokiol-induced apoptosis of NB41A3 cells by 49%. Consequently, treatment of NB41A3 cells with honokiol led to a 52% decrease in cell viability (Fig. 6D).

Treatment with honokiol at a low concentration for a short-term interval can suppress migration of neuroblastoma cells

Effects of honokiol administration at a low concentration and for a short-term interval on migration of neuroblastoma cells were

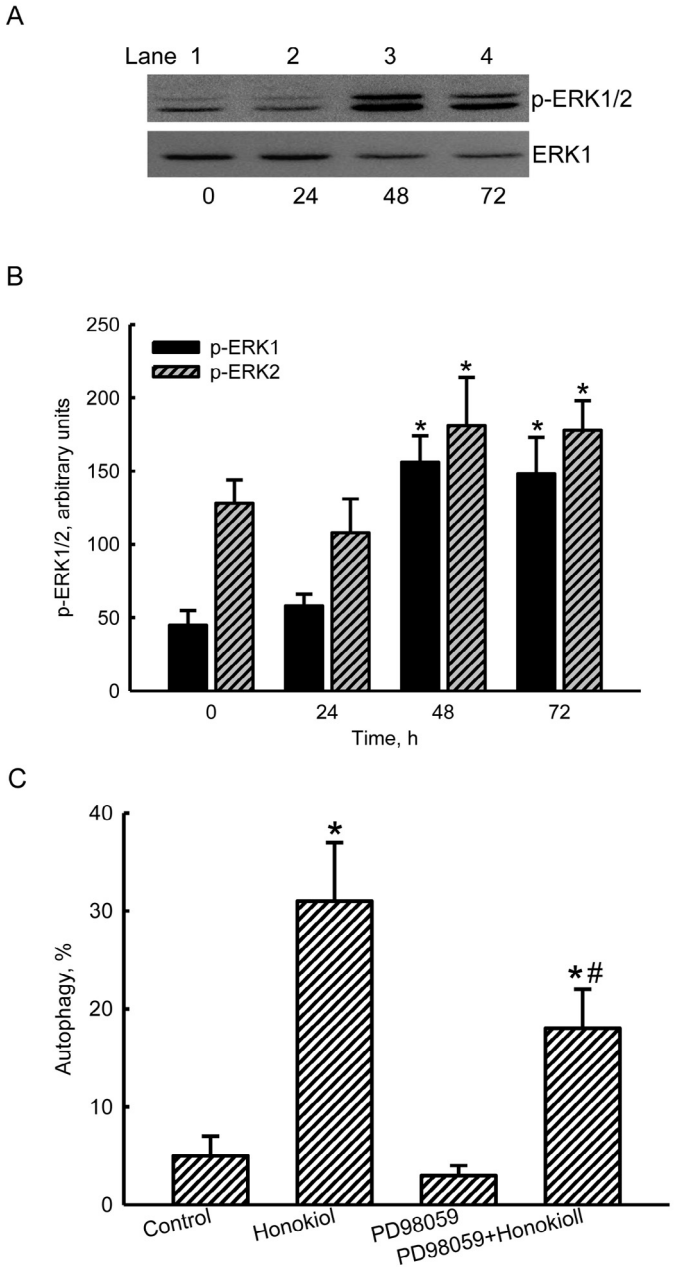

Fig. 5. Roles of extracellular signal-regulated kinase (ERK)1/2 in honokiol-induced autophagy. Neuro-2a cells were treated with 50 μM honokiol for 24, 48, and 72 h. Phosphorylation of ERK1/2 was immunodetected (A, top panel). The amount of ERK1 was analyzed using the internal control (bottom panel). These protein bands were quantified and statistically analyzed (B). Neuro-2a cells were pretreated with 30 μM PD98059, an inhibitor of ERK1/2, for 1 h and then exposed to honokiol for 72 h. Cellular autophagy was quantified using flow cytometry (C). Each value represents the mean ± SEM, n = 12. The symbols * and # respectively indicate that a value significantly ($p < 0.05$) differed from the control and honokiol-treated groups.

assessed (Fig. 7). Exposure of neuroblastoma neuro-2a and NB41A3 cells to 25 μM honokiol for 12 h did not affect cell viability (Fig. 7A). After treatment for 12 h, honokiol at 25 μM also did not induce autophagy or apoptosis of neuro-2a or NB41A3 cells (Fig. 7B). Remarkably, exposure to honokiol at such a low concentration for 12 h repressed migration of neuro-2a cells (Fig. 7C, right panel). Migration of NB41A3 cells was also blocked by honokiol (left panel). These migrated cells were counted and statistically analyzed (Fig. 7D). Treatment of neuro-2a and NB41A3 cells with honokiol respectively caused 46% and 41% decreases in migration of neuroblastoma neuro-2a and NB41A3 cells.

Discussion

Honokiol can induce autophagy and consequent death of neuroblastoma cells. Autophagy is a self-degradative process that can be detected by staining with acridine orange [19]. The present results by flow cytometry indicate an increase in the proportions of neuroblastoma neuro-2a cells with acidic vesicular organelles. In addition, the ratio of LC3-II to LC3-I is usually applied to monitor cellular autophagy [35]. Our data reveal that after honokiol administration, the amount of LC3-II was raised. NB41A3 cells are another neuroblastoma cell line that is derived from mouse brains [36]. Our present results showed that exposure to honokiol also increased proportions of NB41A3 cells with acidic vesicular organelles. Thus, honokiol can induce autophagic insults to neuroblastoma cells. When cells suffer autophagy, they may reverse to survival or proceed to death [20]. 3-MA is an inhibitor of cellular autophagy because this chemical can block the formation of autophagosomes [37]. This study showed that pretreatment with 3-MA caused significant alleviation of honokiol-induced autophagy of neuroblastoma neuro-2a and NB41A3 cells. More interestingly, this study demonstrated that pretreatment with 3-MA lowered honokiol-triggered apoptosis and concurrently defended against the death of neuroblastoma cells. As a result, we can conclude that honokiol can induce autophagy and autophagic cell death in neuroblastoma cells. Previous studies presented that honokiol induced autophagy of various types of tumor cells via diverse mechanisms [38–40]. Lv et al. stated the beneficial results of a combined treatment with honokiol and chloroquine, an autophagy inhibitor, on killing human non-small cell lung cancer cells by inhibiting autophagy and inducing apoptosis due to a caspase-dependent mechanism [38]. To melanoma stem cells, honokiol decreased cell viability by suppressing Notch-2 signaling [39]. In comparison, Hahm et al. reported the honokiol-induced cytoprotective autophagy in prostate cancer LNCaP, C4-2, or TRAMP-C1 cells [40]. However, our present results show that honokiol induces autophagy and autophagic cell death in neuroblastoma cells. Thus, the action mechanisms and final outcomes of honokiol-induced autophagy to different types of tumor cells can be tissue specific. The major pathways of honokiol-induced insults to tumor cells can be p53-dependent or independent [11]. In human neuroglioma H4 cells, honokiol is reported to activate p53 and then induces cell cycle arrest and apoptosis [41]. In contrast, honokiol-induced cell cycle arrest in human prostate cancer PC-3 and LNCaP cells is p53-independent [42]. Sahu et al. showed that 8-methoxypyrimido[4′,5′:4,5]thieno(2,3-b)quinoline-4(3H)-one, a novel anticancer agent, induces apoptosis of neuro-2a cells through a p53-dependent pathway [43]. Nevertheless, the roles of p53 in honokiol-induced autophagy and autophagic apoptosis in neuroblastoma neuro-2a and NB41A3 cells are still unknown and should be evaluated. Traditionally, current strategies for treating patients with a high-risk neuroblastoma are usually complicated by serious side effects [8]. Previous studies done in our and other labs showed the safety of honokiol to normal brain cells [11,18]. Therefore, the present study provides *in vitro* data to demonstrate the potential of honokiol to be clinically applied for neuroblastoma therapy.

Autophagy-induced apoptosis is involved in honokiol-induced insults to neuroblastoma cells. In parallel with induction of cellular autophagy, exposure of neuroblastoma neuro-2a and NB41A3 cells to honokiol prompted DNA fragmentation and sequential cell cycle arrest at the sub-G_1 phase. DNA fragmentation and cell cycle arrest at the sub-G_1 phase are two typical characteristics that indicate that cells are undergoing apoptosis [29,44]. Our previous study also showed that honokiol can induce apoptotic insults to neuroblastoma cells [18]. In contrast, pretreatment of neuroblastoma

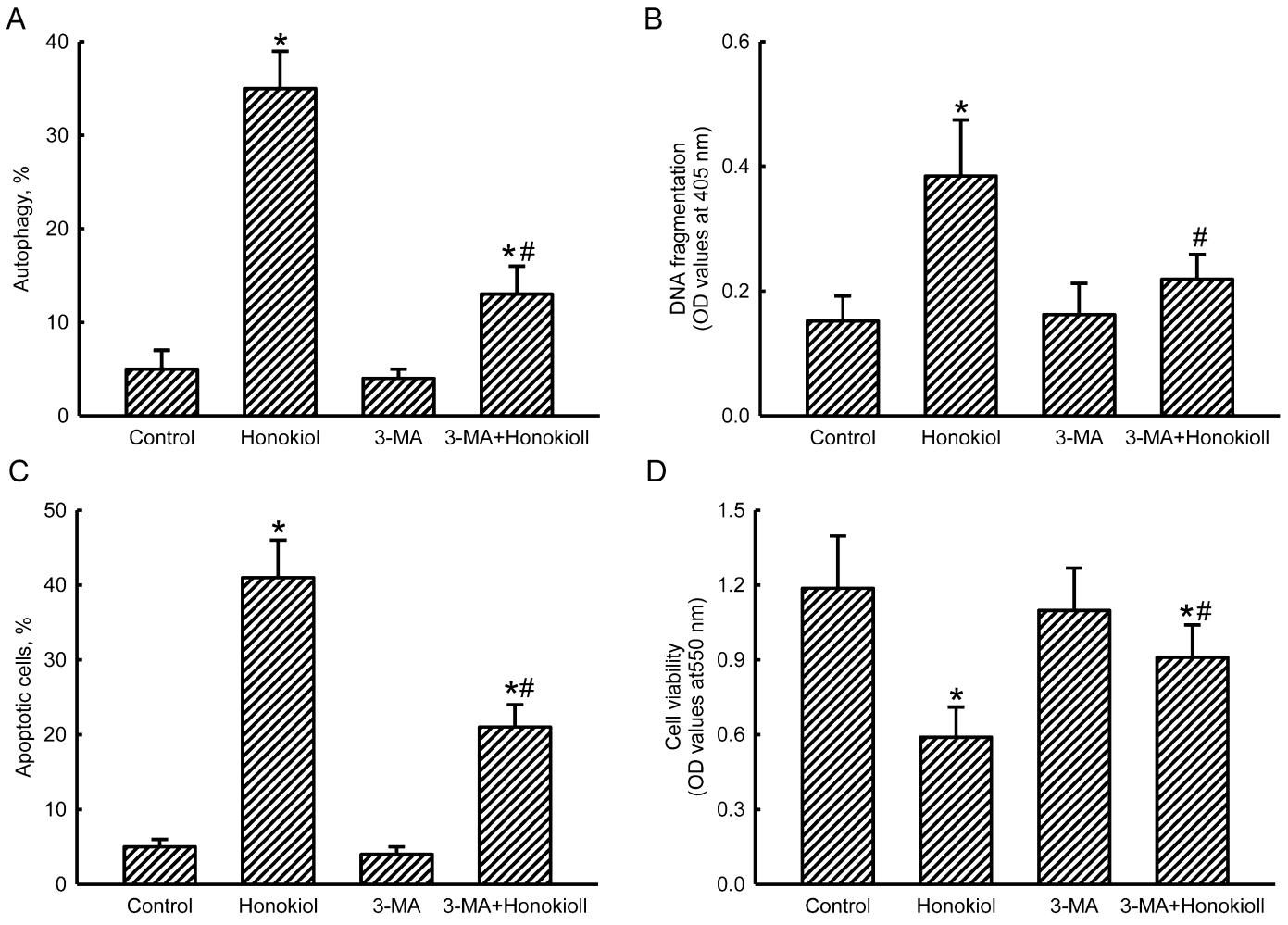

Fig. 6. Effects of honokiol on autophagy, DNA fragmentation, apoptosis, and cell viability of neuroblastoma NB41A3 cells. NB41A3 cells were pretreated with 1 mM 3-methyladenine (3-MA) for 1 h and then exposed to 50 μM honokiol for another 72 h. Cellular autophagy (A) and DNA fragmentation (B) were analyzed using flow cytometry. Apoptosis of NB41A3 cells was quantified with a photometric immunoassay (C). Cell viability was assayed using a colorimetric method (D). Each value represents the mean ± SEM, $n = 6$. The symbols * and # respectively indicate that a value significantly ($p < 0.05$) differed from the respective control and honokiol-treated groups.

neuro-2a and NB41A3 cells with 3-MA alone did not influence cell apoptosis but caused significant attenuation of honokiol-induced apoptotic injury. Autophagy is reported to closely cross-talk with apoptosis [20,45]. In general, autophagy can block apoptosis, and certain apoptosis-associated proteins disrupt the autophagic process. Nevertheless, in some cases, autophagy may induce cell apoptosis or necrosis, also known as autophagic cell death [45]. The chief principles of cancer therapy focus on inducing cell death and inhibiting cell survival [46]. A previous study showed similar effects of honokiol of inducing autophagic and apoptotic insults to glioblastoma multiforme cells [47]. The present study further supported that honokiol can be used to kill neuroblastoma cells through inducing autophagy and successive autophagic cell death, especially apoptosis.

The PI3K/Akt/mTOR signaling pathway participates in honokiol-induced cellular autophagy. mTOR is a master negative regulator of cellular autophagy [22]. Following phosphorylation, mTOR can inhibit activation of downstream protein kinases, including ULK1 and ATG13, and subsequently suppress cellular autophagy [48]. Our data reveal that exposure of neuroblastoma cells to honokiol caused a significant downregulation of mTOR phosphorylation. Accordingly, the honokiol-induced autophagy of neuroblastoma cells is due to suppression of mTOR activation. Chronologically, Akt is an upstream protein that can stimulate phosphorylation of mTOR [24]. In addition, PI3K sequentially contributes to activation of Akt [23]. Our present results showed that levels of PI3K and phosphorylated Akt significantly dropped following honokiol administration. Thus, the PI3k/Akt signaling pathway is involved in honokiol-induced downregulation of mTOR phosphorylation and consequent autophagic insults to neuroblastoma cells. Recently, targeting the PI3K/Akt/mTOR pathway was thought to have practical potential for discovery of anticancer drugs, design of clinical trials, and even personalized medicine [49]. A previous study reported that honokiol can decrease the PI3K/mTOR pathway-mediated immunoresistance of glioma, breast, and prostate cancer cell lines, but did not affect T cell functions [50]. So, honokiol may be clinically available to augment T cell-mediated cancer immunotherapy. Different studies also presented the beneficial antitumor activities of honokiol.

Honokiol induces autophagy of neuroblastoma cells through activating the ERS/ROS/ERK1/2 mechanism. After treatment with honokiol, the amount of Grp78 was significantly augmented in neuroblastoma cells. Grp78 is an ERS-associated protein and its expression is typically used to monitor the occurrence and process of ER injury [51]. Liu et al. showed that honokiol can trigger ERS and then suppress gastric tumor growth [52]. The present study further demonstrated the effects of honokiol on stimulating ERS in neuroblastoma cells. Exposure of neuroblastoma cells to honokiol augmented the amount of intracellular ROS and enhanced phosphorylation of ERK1/2. After initiation of ERS, cells can then produce

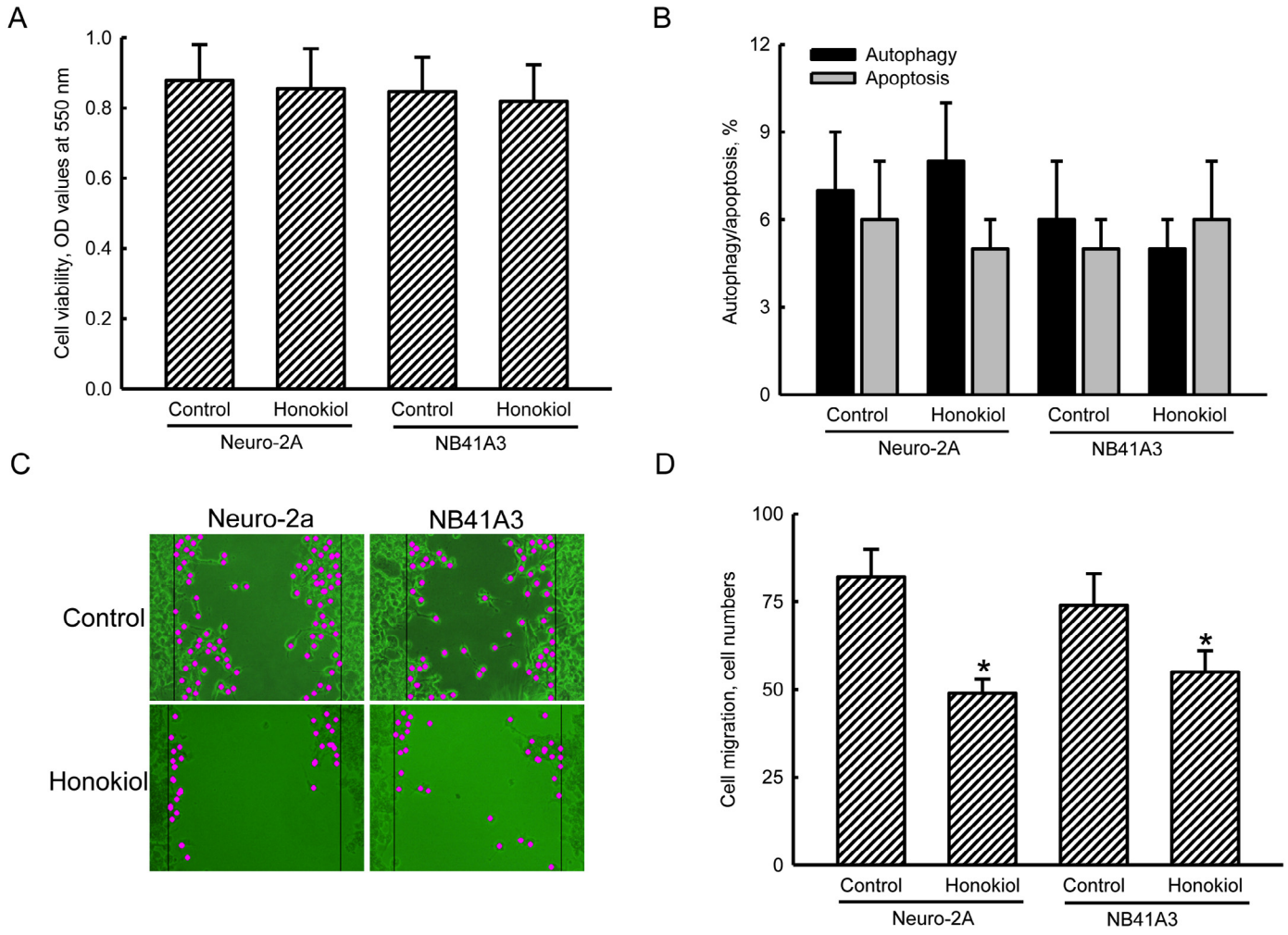

Fig. 7. Effects of honokiol administration at a low concentration for a short-term interval on cell migration and insults. Neuroblastoma neuro-2a and NB41A3 cells were exposed to 25 μM honokiol for 12 h. Cell viability was assayed using a colorimetric method (A). Autophagy and apoptosis were assayed using flow cytometry (B). A wound-healing assay was carried out to determine the migration of neuroblastoma cells (C). Migrated cells were counted and statistically analyzed (D). Each value represents the mean ± SEM for $n = 6$. An asterisk (*) indicates that values significantly differed from the respective control, $p < 0.05$.

large amount of ROS [27]. Hence, one of the possible sources of honokiol-induced augmentation in intracellular ROS is because of induction of ERS by this polyphenol. When suppressing ROS using NAC, honokiol-induced autophagy of neuroblastoma cells was simultaneously reduced. Sequentially, elevation of oxidative stress can excite ERK1/2 phosphorylation [27]. Our current results demonstrated that knocking down ERK1/2 activation by PD98059 concurrently lessened honokiol-induced autophagy of neuroblastoma cells. Therefore, besides the PI3K/Akt/mTOR mechanism, the ERS/ROS/ERK1/2 signaling pathway contributes to honokiol-induced autophagic insults to neuroblastoma cells.

Exposure to honokiol at a low concentration for short-term intervals did not induce insults to neuroblastoma-suppressed migration of neuroblastoma cells. Treatment of neuroblastoma neuro-2a and NB41A3 cells with 25 μM honokiol for 12 h did not affect cell apoptosis, autophagy, or death. Nevertheless, results of a wound-healing assay indicated that honokiol at such a low concentration and a short-term interval could inhibit the migration of neuroblastoma cells. In the clinic, more than 50% of neuroblastoma patients present with metastasis into the bone marrow, cytoskeleton, lymph nodes, liver, and intracranial orbital sites [5]. In other words, neuroblastoma cells derived from high-risk neuroblastomas are highly invasive. Zhu et al. showed the suppressive effects of 5-formylhonokiol, a derivative of honokiol, on the migration of human umbilical vein endothelial cells [53]. Recently, honokiol was shown to significantly block leptin-induced growth, invasion, and migration of breast cancer cells [54]. The present study further showed that although honokiol at a low concentration did not induce insults to neuroblastoma cells, this small polyphenol molecule suppressed the migration of neuroblastoma cells. Honokiol presents its multiple helpful effects on suppression of neuroblastoma development, including induction of autophagy and autophagic apoptosis and suppression of cell migration.

In summary, this study showed that treatment with honokiol increased proportions of neuroblastoma neuro-2a and NB41A3 cells with acidic vesicular organelles in concentration- and time-dependent manners. In parallel, levels of LC3-II in neuroblastoma cells increased following honokiol administration. Pretreatment with 3-MA attenuated honokiol-induced cellular autophagy, DNA fragmentation, apoptosis, and cell death. As to the mechanisms, exposure to honokiol sequentially reduced the levels of PI3K and phosphorylation of Akt and mTOR. In addition, honokiol augmented the levels of Grp78 and intracellular ROS in neuro-2a cells. Interestingly, reducing the amount of intracellular ROS instantaneously lowered honokiol-induced cellular autophagy. Honokiol enhanced phosphorylation of ERK1/2. However, knocking down ERK1/2 activity

caused significant alleviation of honokiol-triggered autophagic insults. More remarkably, treatment of neuroblastoma cells with honokiol at a low concentration for a short-term interval did not affect cellular autophagy, apoptosis, or death. Instead, honokiol under such a condition inhibited the migration of neuroblastoma cells. Taken together, this study shows the beneficial effects of honokiol of inducing autophagy of neuroblastoma cells through activating the PI3K/Akt/mTOR- and ERS/ROS/ERK1/2-dependent signaling pathways. Our results suggest that honokiol possesses potential for clinical application for treating neuroblastomas. A translational study using a xenograft animal model was conducted in our lab to confirm our *in vitro* results.

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Authors' contributions

PSY participated in the study design, conduction of most experiments, and partial preparation of the manuscript; WW performed the ERS assay and discussed the results; YAC conducted the apoptosis test and statistical analyses; CJL collected the data and discussed the results; JJW discussed the results and edited the manuscript; RMC obtained the funds, designed the research project, supervised all experiments, and drafted the manuscript. All authors reviewed the manuscript.

Conflict of interest

All of the authors of this paper declare that they have no conflict of interest.

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