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Wogonin Induces Cross-Regulation Between Autophagy and Apoptosis via a Variety of Akt Pathway in Human Nasopharyngeal Carcinoma Cells

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ABSTRACT

Autophagy as well as apoptosis is an emerging target for cancer therapy. Wogonin, a flavonoid compound derived from the traditional Chinese medicine of Huang-Qin, has anticancer activity in many cancer cells including human nasopharyngeal carcinoma (NPC). However, the involvement of autophagy in the wogonin-induced apoptosis of NPC cells was still uninvestigated. In this study, we found wogonin-induced autophagy had interference on the process of apoptosis. Wogonin-induced autophagy formation evidenced by LC3 I/II cleavage, acridine orange (AO)-stained vacuoles and the autophagosome/autolysosome images of TEM analysis. Activation of autophagy with rapamycin resulted in increased wogonin-mediated autophagy via inhibition of mTOR/P70S6K pathway. The functional relevance of autophagy in the antitumor activity was investigated by annexin V-positive stained cells and PARP cleavage. Induction of autophagy by rapamycin ameliorated the wogonin-mediated apoptosis, whereas inhibition of autophagy by 3-methyladenine (3-MA) or bafilomycin A1 increased the apoptotic effect. Interestingly, this study also found, in addition the mTOR/P70S6K pathway, wogonin also inhibited Raf/ERK pathway, a variety of Akt pathways. Inactivation of PI_3K/Akt by their inhibitors significantly induced apoptosis and markedly sensitized the NPC cells to wogonin-induced apoptosis. This anticancer effect of Akt was further confirmed by SH6, a specific inhibitor of Akt. Importantly, inactivation of its downstream molecule ERK by PD98059, a MEK inhibitor, also induced apoptosis. This study indicated wogonin-induced both autophagy and apoptosis through a variety of Akt pathways and suggested modulation of autophagy might provide profoundly the potential therapeutic effect. J. Cell. Biochem. 113: 3476–3485, 2012.

KEY WORDS: AKT; AUTOPHAGY; APOPTOSIS; WOGONIN; NPC

Autophagy is implicated in various cellular metabolism and disease [White and DiPaola, 2009]. In particular, autophagy is extensively documented to regulate cancer development and progression and to determine the response of tumor cells to anticancer therapy [Chen and Karantza, 2011]. Recent reports indicate that autophagy and apoptosis are often induced by the anticancer agents, but the role of autophagy is still controversial [Eisenberg-Lerner et al., 2009; Wirawan et al., 2011]. Wogonin, a natural flavonoid compound derived from the traditional Chinese medicine of Huang-Qin, recognized as a new source of anticancer drug due to its broad toxicities to various types of tumor cell lines and the low toxicities to normal tissues [Baumann et al., 2008; Chow et al., 2011]. However, the modulation of autophagy in the cancer therapy of wogonin was still unclear.

Autophagy is often considered a survival pathway by eliminating unwanted organelles to maintain the cellular homeostasis during starvation and other stress condition [Meijer and Codogno, 2004]. In mammalian cells, microtubule-associated protein 1 light chain 3 (LC3) is conjugated to phosphatidyl ethanolamine for insertion into the autophagosome membrane. The most distinctive feature of

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autophagy is the formation of autophagosomes, which are double-membrane vesicles that fuse with lysosomes for hydrolytic cleavage of engulfed proteins and organelles [White and DiPaola, 2009]. The key mediators such as, Beclin-1, UVRAG, Bcl-2, Class III and I PI3K, mammalian target of rapamycin (mTOR), and p53, play pivotal roles in autophagic signaling networks in cancer [Liu et al., 2010]. Suppression of mTOR/P70S6K and constitutive activation of ERK1/2 pathways have both shown to involve in regulating autophagy formation [Shinojima et al., 2007]. The 4E-BP1/PHAS that inhibits the initiation of translation and the kinase p70S6K that phosphorylates the 40S ribosomal protein S6 and enhances the translation of mRNAs are two characterized targets of mTOR [Schmelzle and Hall, 2000]. Additionally, the Ras/Raf/ERK signaling pathway plays a crucial role in autophagy regulation as well [Chappell et al., 2011]. Upon receptor activation, membrane-bound Ras recruits one of the Raf kinases (A-raf, B-Raf or C-Raf) into a complex and phosphorylates MEK1/2 which in turn activates ERK1/2, subsequently leads to autophagy stimulation [Cagnol and Chambard, 2010].

Hence, the common upstream target of mTOR/p70s6k and Ras/Raf/ERK might be a good candidate for the control of autophagy. Evolutionarily conserved serine/threonine kinase Akt, also known as protein kinase B (PKB), is one of the most frequently activated protein kinase in human cancer [Hay, 2005]. Hyperactivation of Akt is associated with resistance to apoptosis, increased cell growth, cellular proliferation, and cellular energy metabolism [Nicholson and Anderson, 2002]. Akt also can potentially phosphorylate diverse downstream targets such as Bad, IκB kinase glycogen synthase kinase 3 (GSK-3β), or mTOR. In addition, Raf-1/MEK1/ERK pathway interrelated with Akt has shown to regulate cell apoptosis [McCubrey et al., 2006]. Thus, Akt is an interesting and promising target for pharamacological intervention in cancer therapy.

Nasopharyngeal carcinoma is one of the dominant head and neck cancer in southern China and South East Asia. In contrast to other head and neck squamous cell carcinomas, NPC is always associated with Epstein-Barr virus, which has high probability of advanced or metastatic disease at initial presentation [Bensouda et al., 2011]. NPC is highly chemosensitive, but the use of chemotherapy for treating patients has been found with recurrent or metastatic NPC [Chan et al., 2005]. Previous study has shown the capability of wogonin-induced NPC cell apoptosis is dependent on the GSK-3β/ΔNp63α pathway [Chow et al., 2011]. In this study, we aimed to investigate the potency of wogonin and the involved signal pathways modulating autophagy and apoptosis in NPC cells. We found the Akt response to wogonin might contribute to cell death and autophagy through suppression of a variety of Akt pathways.

MATERIALS AND METHODS

CELL CULTURE AND REAGENTS

Human nasopharyngeal carcinoma cell lines NPC-TW076 and NPC-TW039 were isolated from keratinized nasopharyngeal squamous cell carcinoma [Lin et al., 1990]. The cells were maintained in basal medium (DMEM/F-12 at 1:1 v/v; Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum in a humidified incubator at 37°C under 5% CO_2/95% air. Most chemicals were obtained from Sigma–Aldrich (St. Louis, MO) unless otherwise indicated. Antibodies to p-Akt, Akt, p-mTOR, p-P70S6K, LC3B I/II, PARP with cleaved PARP, cRaf and p-Erk1/2 were purchased from cell signaling (Beverly, MA). Rapamycin and SH-6 (Akt inhibitor III) were purchased from Cayman (Ann Arbor, MI) and Merck (Darmstadt, Germany), respectively. PD98059 was purchased from Sigma–Aldrich.

AUTOPHAGY DETECTION

Autophagy is a lysosomal degradation pathway for cytoplasmic material. The acidic intracellular compartments were visualized by supravital AO staining. After incubation, cells were washed with phosphate-buffered saline and stained with 1 μg/ml AO (Sigma–Aldrich, 318337) for 15 min at 37°C. Subsequently, cells were washed and analyzed under the inverted fluorescent microscope. Analysis was performed via fluorescence microscopy using 490-nm band-pass blue excitation filters and a 515-nm long-pass barrier filter. Depending on their acidity, autophagic lysosomes appeared as orange/red fluorescent cytoplasmic vesicles, while cytoplasm and nucleolus were green. Alternatively, AO-stained cells were trypsinized, washed, and quantified by measuring the ratio of red/green fluorescence (FL3/FL1) on a FACSCalibur flow cytometer (Becton, Dickinson and Company) and analyzed using Cell Quest Pro software.

The transmission electron microscopy (TEM) was utilized for analyzing the ultra-structural images of autophagosomes and autolysosmes. Trypsinized cells were fixed with 2.5% glutaraldehyde in phosphate-buffered saline, followed by 2% OsO_4. After dehydration, thin sections were stained with uranyl acetate and lead citrate for observation.

WESTERN BLOT ANALYSIS

The cells were seeded at a 3×10^5 amount per 60-mm culture dish. After treatment, cells were washed with ice-cold phosphate-buffered saline and lysed in Mammalian Protein Extraction Reagent (M-PER; Pierce Chemical Co., Rockford, IL). Protein samples (20 μg per lane) were separated on a 10% SDS–polyacrylamide gel electrophoresis (PAGE) and blotted onto polyvinylidene difluoride membranes (Immobilon(TM)-P, Millipore, Bedford, MA), blocked in 5% nonfat milk in PBS and probed with primary antibodies overnight at 4°C. The membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies. The immunoreactive protein bands were developed by Enhanced Chemiluminescence (ECL) (Amersham Pharmacia Biotech, Freiburg, Germany).

APOPTOSIS ASSAY

Cell apoptosis was assayed by annexin V-Cy5 and PI staining (BioVision, Mountain View, CA). The cells were treated with Ly294002, wortmannin, SH6, rapamycin, bafilomycin A1, 3-MA, or PD98059 at indicated concentrations in the presence or absence of wogonin (50 μM) for 24 h. The cells were pelleted and resuspended in annexin V-binding buffer (10 mM HEPES, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, pH 7.4) containing annexin V-Cy5 (1:1,000) and 1 mg/ml PI. After incubation at room temperature for 5 min, the cells were analyzed with a FACSCalibur flow cytometer

(Becton Dickinson). The percentage of total apoptotic events was defined as the sum of the cells in the early stage (annexin V-Cy5 positive/PI negative) or late stage (annexin V-Cy5 positive/PI positive) of apoptosis as previously described [Chow et al., 2011].

STATISTICAL ANALYSES

Data are presented as means ± SEM, n = 3. The statistical differences were determined using Student–Newman–Keuls Test and Dunn's Test (Sigma Stat Software Program, Jandel Scientific, San Rafael, CA). A *P*-value of 0.05 or less was considered as significant.

RESULTS

WOGONIN INDUCES AUTOPHAGY IN NPC CELLS

Wogonin has shown to induce NPC cell apoptosis [Chow et al., 2011]. To investigate whether wogonin-induced autophagy in NPC cells, the autophagic responses were assayed by LC3-I/II cleavage, the AO staining and the double-membrane autophagic vacuole development. NPC cells were incubated with 50 μM wogonin in different duration. As shown in Figure 1A, wogonin rapidly induced an increase of LC3-II level at 4 h and declined at 8–24 h. The process of autophagy starts with the autophagosome formation and then progresses to autolysosome through the fusion of acidic lysosomes with autophagosomes (Hippert et al., 2006; Wang et al., 2008). AO is a fluorescent weak base that accumulates in acidic compartments such as autolysosome and lysosome to fluoresce bright red, whereas cytoplasm and nucleolus fluoresce bright green (Palmgren, 1991). Therefore, AO staining of the live cells was also employed to visualize acidic autolysosomes in control and wogonin-treated NPC cells. As shown in Figure 1B, after 2 h wogonin treatment the yellow vesicles became visible and increased in number that compared with the control group. After 8 h incubation markedly induced larger bright-red vacuoles (bold arrows), suggesting wogonin-induced autolysosomes. Furthermore, TEM images showed a few basal autophagosomes with double-membrane appearance in the control group. After 24 h wogonin treatment abundant and enormous vacuoles of autophagosomes/autolysosomes with degraded organelles became visible (Fig. 1C). These data indicated that wogonin activated the autophagic process in a time-dependent manner in NPC cells.

AUTOPHAGY MODULATES WOGONIN-INDUCED APOPTOSIS

To clarify the autophagy formation in NPC cells, rapamycin, a known inducer of autophagy was used to detect the effect on wogonin-induced autophagic vacuoles. As shown in Figure 2A, the smaller and orange AO-stained vacuoles presented in rapamycin-treated cells that different from the larger bright-red ones in wogonin-treated cells. Interestingly, rapamycin pretreatment markedly elevated the numbers of bright-red autophagic vacuoles (Fig. 2A) and showed a significant increase in red

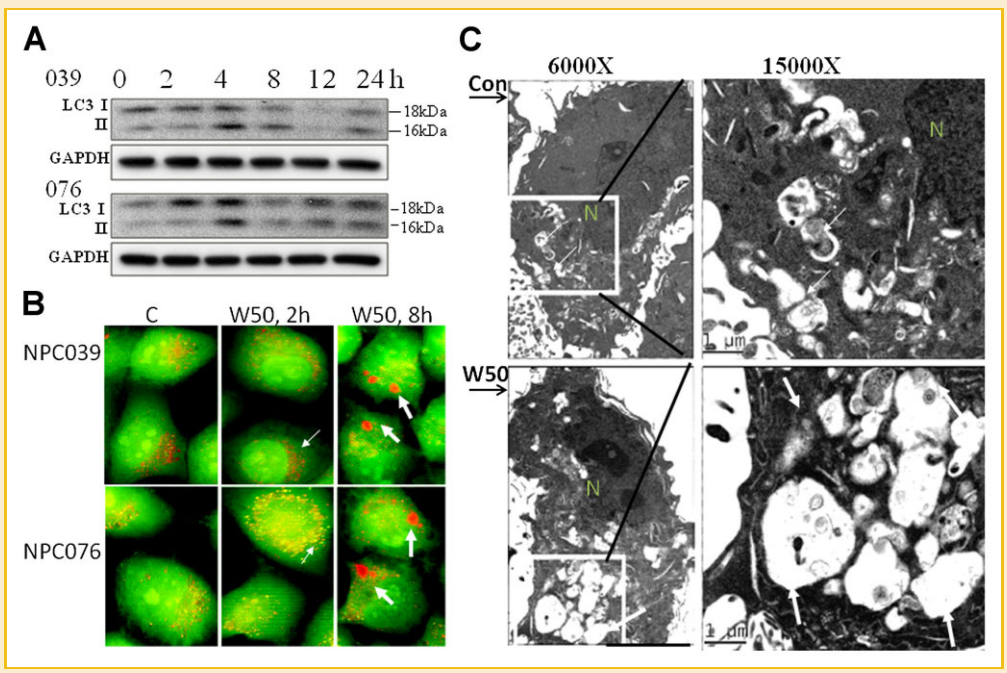

Fig. 1. Wogonin induces autophagy in NPC cells. A: Cells were treated with 50 μM wogonin for indicated time periods and followed by Western blot analysis for detection the levels of LC3 I/II. GAPDH was used as an equal loading control. The blots shown are representative of three independent experiments. B: NPC cells were treated with 50 μM wogonin for 2 and 8 h periods, the red fluorescence of autophagic vacuoles was observed under a fluorescence microscope by AO staining. C: NPC cells were treated with 50 μM wogonin for 24 h. The ultra-structure images were examined by transmission electron microscope. Autophagosomes contained organelles undergoing degenerative changes (arrow). Typical autolysosomes contained remnant of digested organelles or cytoplasm (bold arrow). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

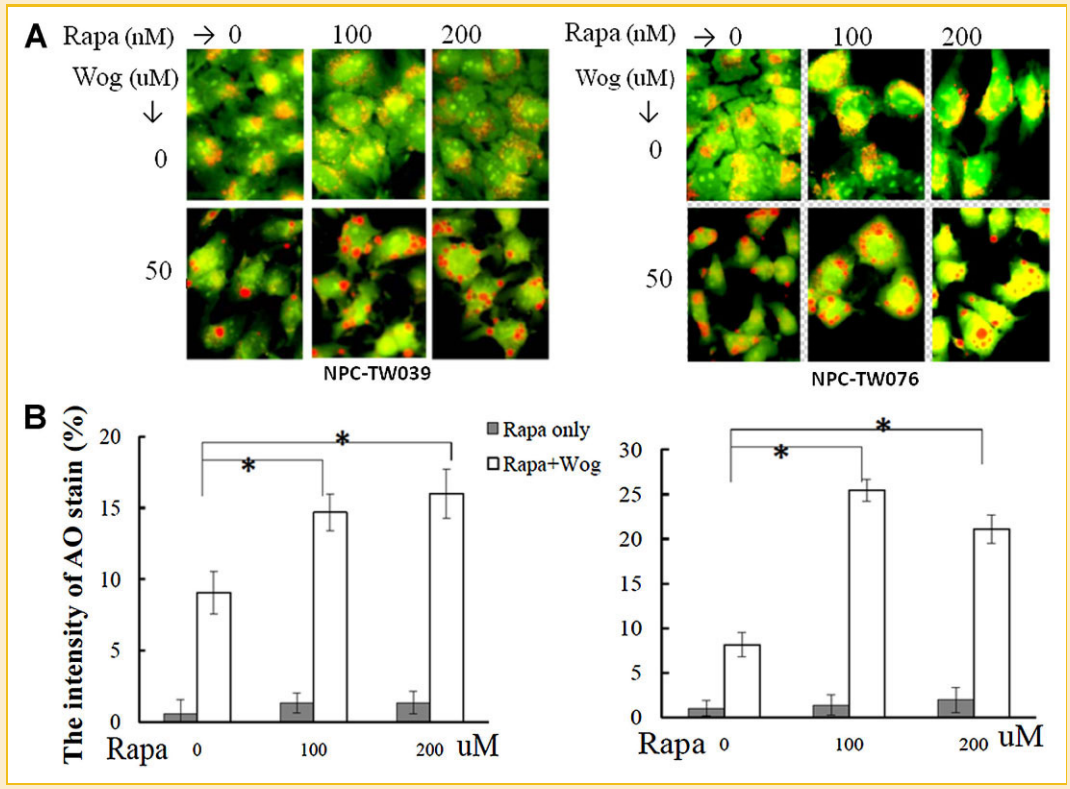

Fig. 2. Rapamycin induces autophagy and enhances wogonin-mediated autophagy. NPC cell were pretreated with rapamycin (100 and 200 nM) for 24 h prior to incubation with or without 50 μM wogonin for 24 h. A: The autophagosome vacuoles were observed under a fluorescence microscope by AO staining. B: The red fluorescent of AO-stained cell was analyzed by flow cytometry. Data were presented as means ± SD, n = 3. Data from each only rapamycin or only wogonin-treated cells were compared to untreated cells and rapamycin plus wogonin-treated cells were compared with only wogonin-treated cells by analysis of variance. $^{*}P < 0.001$. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

fluorescence in the presence of wogonin for 24 h as compared with the control (Fig. 2B). To verify the possible autophagy pathway induced by wogonin, the key autophagic pathway mTOR/P70S6K was investigated by Western blotting (Fig. 3). The levels of p-mTOR and p-P70S6K expressions were decreased in the cells treated with wogonin or rapamycin for 24 h. Interestingly, rapamycin pretreatment decreased the level of wogonin-inhibited p-P70S6K expression. This data indicated the mTOR/P70S6K pathway was involved in wogonin-induced autophagy.

Further, the functional relevance of mTOR pathway in the antitumor activity was investigated by annexin V-positive stained cells and PARP cleavage conditions (Fig. 4). Rapamycin pretreatment significantly ameliorated wogonin-induced apoptosis investigated by decreased annexin V-positive stained cells and PARP cleavage (Fig. 4). Bafilomycin A1, an inhibitor of vacuolar H^{+}ATPase, disrupts the fusion of autophagosomes to lysosomes and prevents the maturation of autophagic vacuoles [Yoshimori et al., 1991]. The NPC cells were coincubated with bafilomycin at indicated concentration in the presence or absence of wogonin for 24 h and the autophagic and apoptotic effects were investigated (Fig. 5). As shown in Figure 5A (upper left panel), after AO staining the control cells displayed yellow fluoresce, indicated

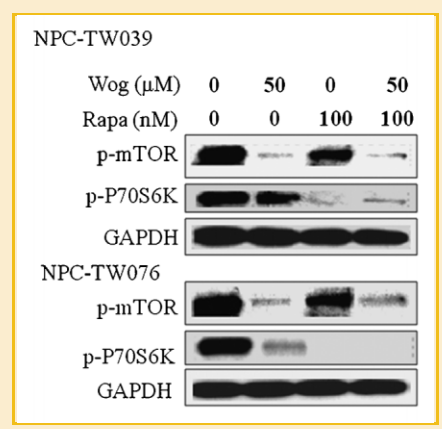

Fig. 3. Rapamycin or wognin induced autophagy via inhibition of mTOR/P70S6K pathway. NPC cells were pretreated with 100 nM rapamycin for 24 h prior to incubation with or without 50 μM wogonin for 24 h. Cell lysates were prepared and subjected to Western blot with p-mTOR, p-P70S6K and GAPDH antibodies. The blots shown are representative of three independent experiments.

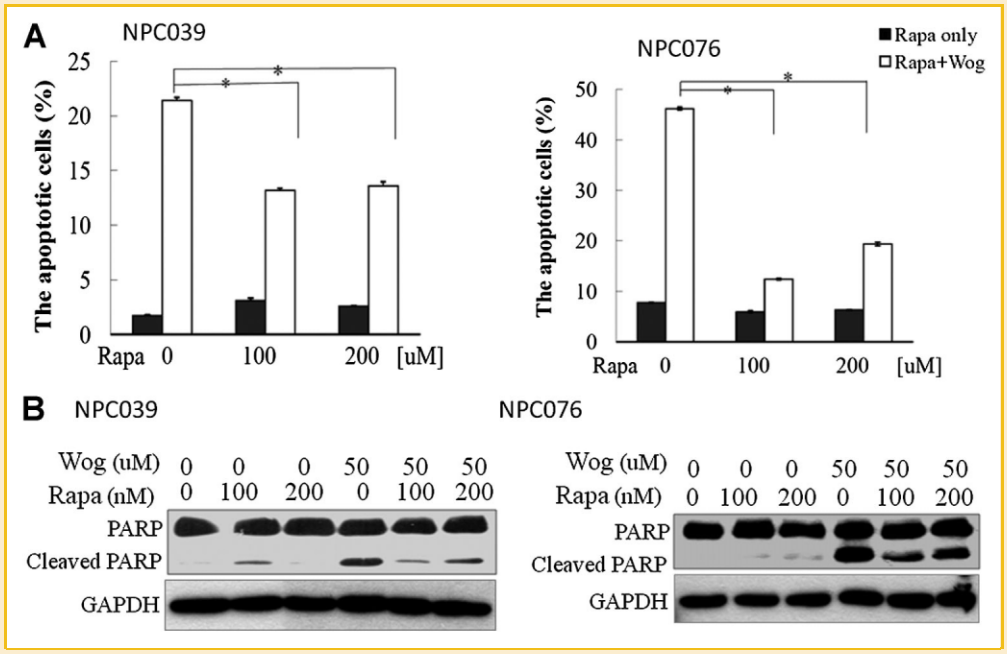

Fig. 4. Rapamycin-induced autophagy abrogates wogonin-induced cell apoptosis. NPC cells were pretreated with rapamycin (100 and 200 nM) for 24 h prior to incubation with 50 μM wogonin for 24 h. A: The apoptotic cells were stained with annexin V-cy5/PI analyzed by flow cytometry. Data were presented as means ± SD, n = 3. B: The apoptotic cell lysates were analyzed by Western blot for detection the level of PARP/cleaved PARP expression. GAPDH was used as an equal loading control. The blots shown are representative of three independent experiments. Data from each only rapamycin or only wogonin-treated cells were compared to untreated cells and rapamycin plus wogonin-treated cells were compared with only wogonin-treated cells by analysis of variance. n = 3, $^{*}P < 0.001$.

there was a basal autophagy formation. Bafilomycin A1 pretreatment apparently abrogated the bright-red fluoresce in the wogonin-treated cells (Fig. 5A), confirming the autophagosomes evoked by wogonin underwent the same maturation process of the fusion with lysosomes. Bafilomycin A1 blocked the maturation of autophagy induced dose-dependently an accumulation of wogonin-induced LC3-II (Fig. 5B) and resulted in enhancement of wogonin-mediated apoptosis (Fig. 5C). Finally, 3-MA, an inhibitor of autophagy that inactivated type-III PI_3K, also enhanced the wogonin-mediated apoptosis (Fig. 5D). Taken together, these data indicated autophagy formation might impede the anticancer activity of wogonin.

AKT IS A TARGET OF WOGONIN IN KILLING OF NPC CELLS

The Akt signaling pathway is implicated in the regulation of a wide variety of cellular processes including survival, proliferation, growth, metabolism, autophagy and apoptosis [Nicholson and Anderson, 2002]. The role of Akt was further investigated after co-incubation of a PI_3K inhibitor (wortmannin or LY-294002) in the presence or absence of wogonin for 24 h. As shown in Figure 6A, treatment with wogonin or the two PI3K inhibitors decreased the phosphorylation level of Akt (p-Akt) in NPC cells. Notably, the two PI3K inhibitors significantly induced cell apoptosis and markedly enhanced the wogonin-induced apoptosis (Fig. 6A,B). This apoptotic effect was further confirmed by SH6, a specific inhibitor of Akt. As shown in Figure 7, specific suppression of Akt by 10 μM SH6 induced cell apoptosis, and also markedly enhanced the wogonin-induced apoptosis in a dose-dependent manner. The data suggested Akt was a target of wogonin in killing of NPC cells.

AKT INACTIVAION INDUCES DUAL-SUPPRESSION OF mTOR/P70S6K AND cRaf/ERK PATHWAYS

Furthermore, a variety of Akt signal pathways related to apoptosis and autophagy were investigated. As shown in Figure 8A, SH6 simultaneously decreased the levels of p-mTOR/p-P70S6K expression and the levels of c-Raf and phosphorylated ERK1/2 (p-ERK) expression, liked the effect of wogonin. Notably, co-incubation of wogonin with SH6 did not show any synergistic inhibition of c-Raf/p-ERK expression (Fig. 8B). The data indicated inactivation of Akt induced dual-suppression of mTOR/P70S6K and c-Raf/ERK pathways in NPC cells.

INACTIVATION OF ERK INDUCES NPC CELL APOPTOSIS

To further investigate the role of ERK in NPC cells, PD98059, a MEK inhibitor, was coincubated in the presence or absence of wogonin for 24 h and the apoptotic effect was investigated. As shown in Figure 8C, PD98059 (25–50 μM) significantly induced cell apoptosis in a dose-dependent manner (Fig. 8C). Co-incubation of wogonin with PD98059 did not show any synergistic effect. Thus, this data suggested Akt/Raf/ERK was associated with wogonin-induced apoptosis.

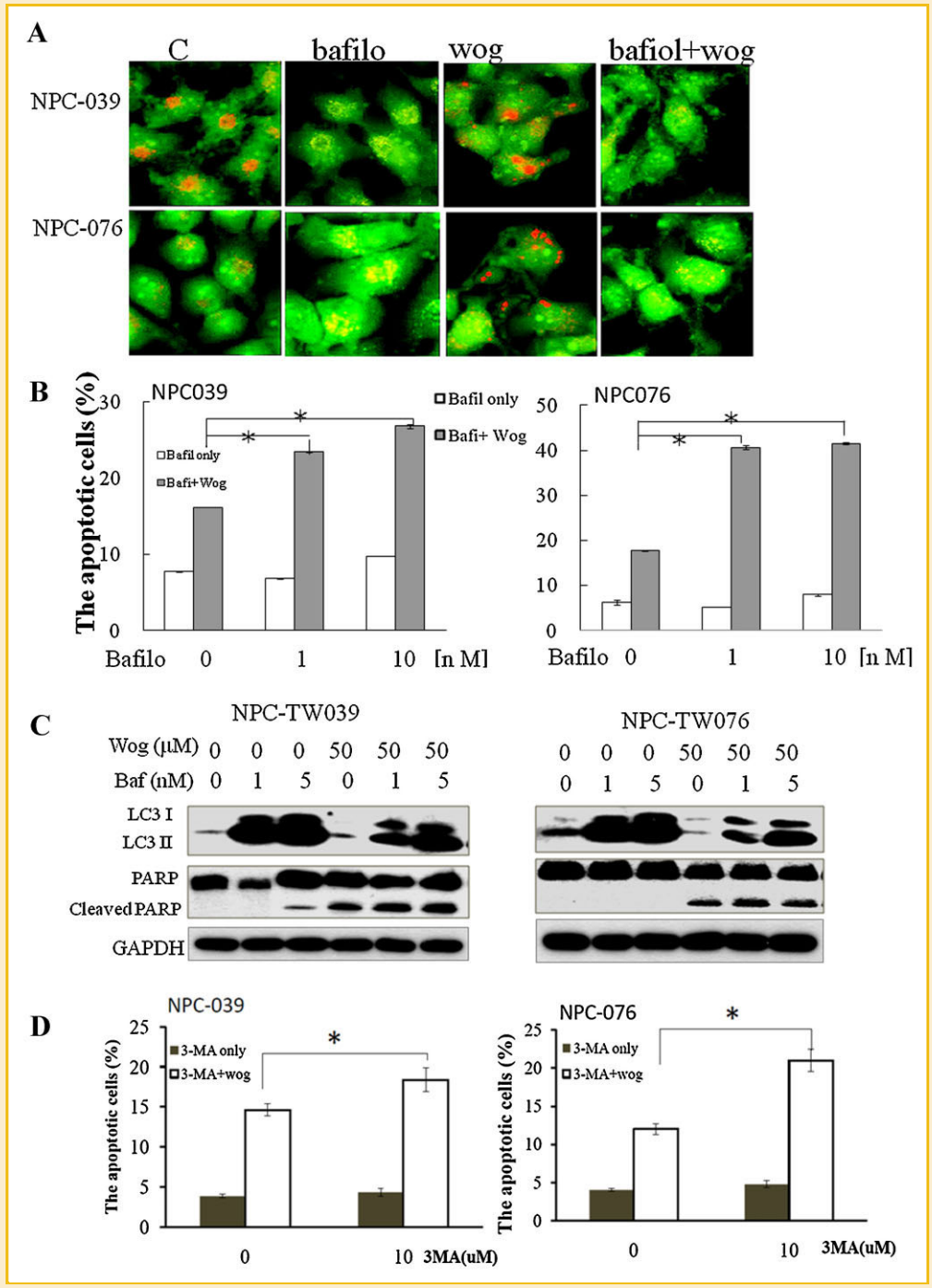

Fig. 5. A–C: Suppression of autolysosome formation enhances wogonin-mediated apoptosis. NPC cells were pretreated with bafilomycin as indicated concentration for 2 h prior to treatment with or without 50 μM wogonin for 24 h. A: The red fluorescence of autophagic morphology stained with AO was observed in fluorescence microscope. B: The apoptotic cell lysates were analyzed the levels of LC3I/II and PARP/cleaved PARP by Western blot. GAPDH was used as an equal loading control. The blots shown are representative of three independent experiments. C: The apoptotic cells were stained with annexin V-cy5/PI and assessed by flow cytometry. D: Suppression of autophagy by 3-MA enhanced the wogonin-mediated apoptosis. NPC cells were pretreated with 10 μM 3-MA for 2 h prior to treatment with or without 50 μM wogonin for 24 h. The apoptotic cells were stained with annexin V-cy5/PI and assessed by flow cytometry. Data were presented as means ± SD, n = 3. Data from bafilomycin, 3-MA or only wogonin-treated cells were compared to untreated cells and bafilomycin plus wogonin-treated cells were compared with only wogonin-treated cells by analysis of variance. $^{*}P < 0.001$. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

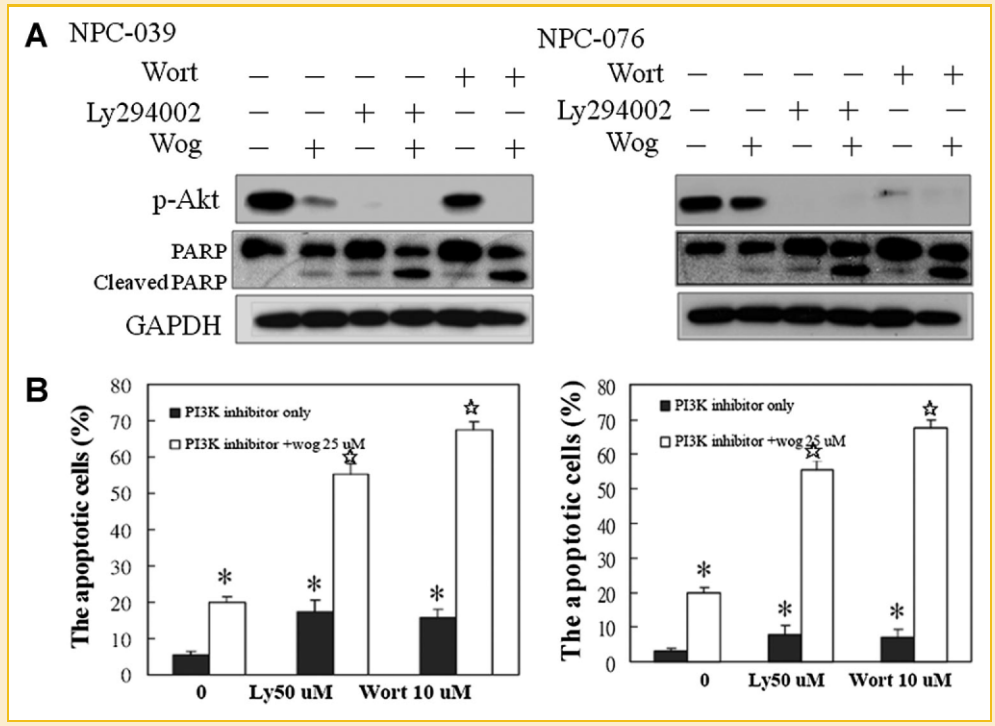

Fig. 6. Wogonin-inactivated Akt leads to cell apoptosis. NPC cells were treated with PI3K inhibitors Ly294002 or wortmannin for 24 h in the presence or absence of 50 μM wogonin. A: The cell lysates were analyzed the levels of p-Akt and PARP/cleaved PARP by Western blot. GAPDH was used as an equal loading control. The blots shown are representative of three independent experiments. B: The apoptotic cells were assessed by annexin V-cy5/PI staining and flow cytometry. Data were presented as means ± SD, n = 3. $^{*}P < 0.001$ versus untreated (control) group; ☆ $P < 0.001$ versus wogonin-treated group.

DISCUSSION

Wogonin is a new source of anti-cancer agent and much effort has been devoted to the apoptotic effect [Baumann et al., 2008]. This study found wogonin-induced autophagy and apoptosis accompanied with downregulation of Akt pathway. Modulation of autophagy in NPC therapy was shown to play a protective role through downregulation of mTOR/p70S6K pathway. However, inactivation of Akt by PI3K or Akt inhibitors significantly induced cell apoptosis and dramatically enhanced the wogonin-induced apoptosis. Downregulation of Akt/c-Raf/ERK pathway was shown to enhance the apoptotic effect of wogonin, indicating wogonin was proved useful in cancer treatment. This study revealed that wogonin-inhibited a variety of Akt pathways might induce a cross-talk between apoptosis and autophagy. Thus, it was suggested that blockade of autophagy or combination of PI3K/Akt inhibitors effectively enhanced the anticancer activity of wogonin.

Wogonin is a natural compound with anticancer activity and low toxicity against macrophage and normal cells [Baumann et al., 2008]. It can inhibit the growth of human NPC cells by inducing cell apoptosis via GSK-3β/ΔNp63α pathway [Chow et al., 2011]. In this study, we demonstrated that wogonin-induced autophagy formation might impede the process of apoptotic death. Autophagy begins with the cytosolic components sequestration, including intracellular organelles degradation and double-membrane structure formation [Meijer and Codogno, 2004]. As compared with the effect of rapamycin treatment, wogonin-induced autophagy generation by rapidly induction of the LC3 cleavage and larger bright-red AO-stained vacuoles (Fig. 1A,B). The bright-red vacuoles implied wogonin provoked higher intensity of autophagic response associated with activation of lysosome and was shown in the TEM images called autolysosome with degraded organelles (Fig. 1C). During the formation of autophagosomes, LC3 is lipidated and conjugated LC3-II to localize on autophagosomes and autolysosomes [Tanida et al., 2005]. However, the endogenous LC3-II might be rapidly degraded in the autolysosomes after wogonin treatment for 8–24 h. This effect was evidenced by an accumulation of wogonin-mediated LC3-II through inhibition of the fusion of autophagosome with lysosome (Fig. 1A).

Under normal conditions, basal autophagy is a mechanism for the turnover of proteins and elimination of damaged or aged organelles and cytoplasmic components to maintain cell homeostasis. Rapamycin-mediated autophagy displayed relatively smaller and yellow to orange AO-stained vacuoles as compared with that of wogonin treatment (Fig. 2A). Interestingly, rapamycin pretreatment increased the quantity (the bright red vacuoles) and the quality (the intensity of red fluorescence) of AO positive vacuoles induced by wogonin (Fig. 2A,B). This rapamycin-induced protection might be mediated by recycling degraded metabolites from energy production and protein synthesis, and allowing adaptation against cellular

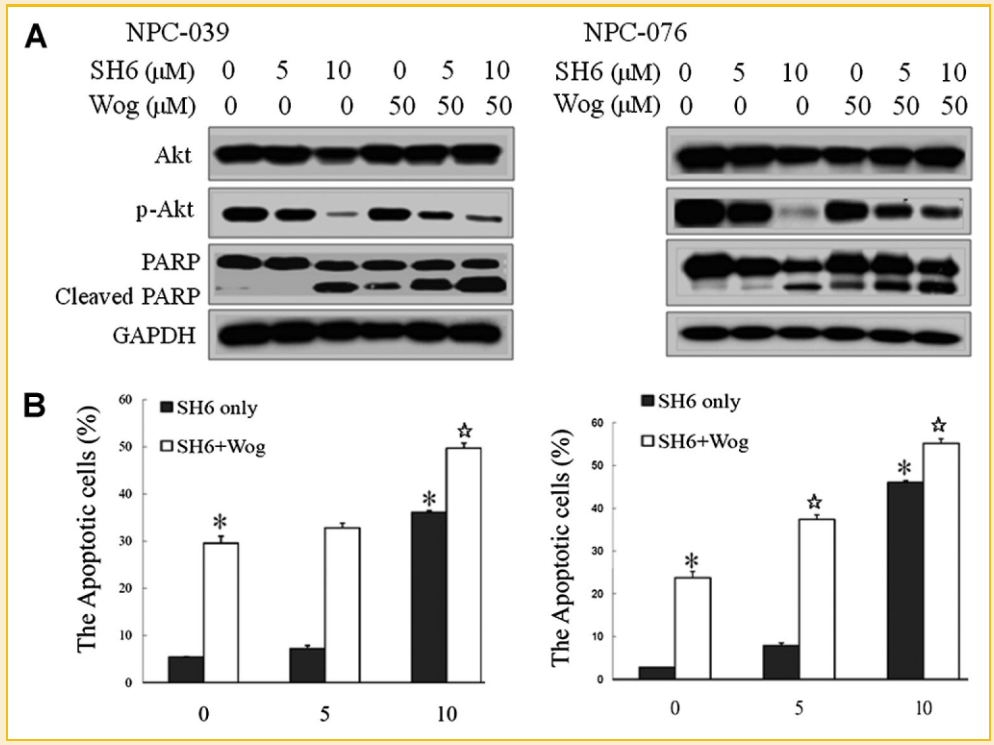

Fig. 7. SH6 induces cell apoptosis and enhances wogonin-mediated cell death. NPC cells were treated SH6 at concentration indicated in the presence or absence of wogonin (50 μM). A: Cell lysates were prepared by Western blotting analysis with Akt, p-Akt and PARP/cleaved PARP antibodies. GAPDH was used as an equal loading control. The blots shown are representative of three independent experiments. B: The apoptotic event was assayed by annexin V-cy5/PI staining with flow cytometry. Data were presented as means ± SD, n = 3. *$P < 0.001$ versus untreated (control) group; ☆ $P < 0.001$ versus wogonin-treated group.

environmental changes. The possible molecular cytoprotection of autophagy may be involved in interference with both the proapoptotic shift in the expression of Bcl2 family protein and the prevention of oxidative stress [Vucicevic et al., 2011]. The exact mechanisms underlying the antiapoptotic action of autophagy in wogonin remained to be explored. However, inhibition of autophagy by bafilomycin A1 or 3-MA enhanced wogonin-mediated cell apoptosis (Fig. 5C,D). In addition, rapamycin treatment induced downregulation of mTOR/P70S6K pathway similar to the effect of wogonin. Notably, rapamycin markedly enhanced the wogonin-mediated dephosphorylation of P70S6K, suggesting autophagy induction through inactivation of mTOR pathway.

Oncogenic activation of Akt is believed to promote proliferation and increase cell survival and thereby contribute to cancer progression [Nicholson and Anderson, 2002; Hay, 2005]. PI_3K is the main modulator in the upstream of Akt pathway. Blockage of Akt signaling results in apoptosis and growth inhibition of tumor cells [Cheng et al., 2005]. However, inhibiting components of PI3K/Akt pathway often does not induce substantial apoptosis without additional insults [Fan et al., 2006; Degtyarev et al., 2008]. It has been suggested that Akt isoforms may be differentially regulated depending on the external stimuli and may rectify distinct aspects of cellular processes in a cell-specific manner [Degtyarev et al., 2008]. In this study, we presented inactivation of PI_3K/Akt pathway induced apoptosis in NPC cells and dramatically sensitized tumor cells to wogonin-induced cell apoptosis, suggesting target of Akt was proved useful in cancer treatment. In particular, SH6 also inhibited the mTOR/P70S6K pathway (Fig. 8A), indicating mTOR inactivation was mediated by inhibition of Akt in NPC cells.

Moreover, the signal pathways of Akt downregulation involved with induction of apoptosis were investigated. Activation of Akt causes a variety of biological effects, including suppression of apoptosis by phosphorylation and inactivation of several targets along pro-apoptotic pathways, such as the Bcl-2 family member BAD or caspase-9. Repression of cRaf/MEK/ERK pathway has profound effects on anti-proliferative and apoptotic pathway in response to inactivation of Akt [Cagnol and Chambard, 2010; Chappell et al., 2011]. Wogonin-inhibited the Akt/mTOR/P70S6K pathway and the c-Raf/ERK pathway simultaneously (Fig. 8A,B), raising the possibility of linkage between Akt and Raf pathways [McCubrey et al., 2006; Chappell et al., 2011]. This effect was improved by SH6 treatment caused both inhibition of Raf/ERK and mTOR/P70S6K pathways [McCubrey et al., 2006]. Importantly, inactivation of ERK1/2 by PD98059, a MEK1 inhibitor, significantly led on apoptosis without enhancing cytotoxicity of wogonin. Wogonin-inactivated Akt conducted cell apoptosis might closely associate with downregulation of cRaf and inactivation of ERK

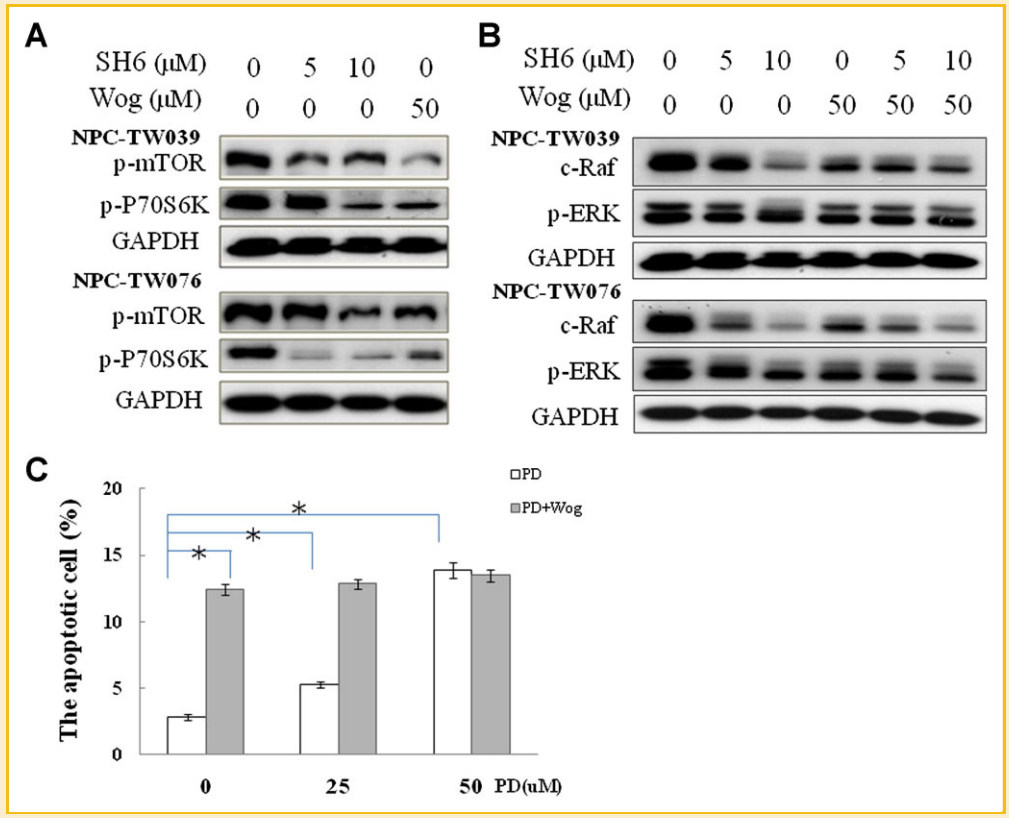

Fig. 8. Suppression of Akt induces dual suppression of mTOR and Raf/ERK pathways and inactivation of ERK induces cell apoptosis. A: NPC cells were treated 50 μM wogonin or SH6 at concentration indicated. Cell lysates were prepared and the levels of p-mTOR and p-P70S6K were detected by Western blot. B: NPC cells were treated SH6 at concentration indicated in the presence or absence of 50 μM wogonin. Cell lysates were prepared and the levels of c-Raf and p-ERK were detected by Western blot. GAPDH was used as an equal loading control. The blots shown are representative of three independent experiments. C: NPC cells were coincubated PD98059 (25–50 μM) with or without 50 μM wogonin for 24 h. The apoptotic event was assayed by annexin V-cy5/PI staining with flow cytometry. Data were presented as means ± SD, n = 3. Data from each experimental group were compared to the control (PD98059-untreated) group by analysis of variance. $^{*}P < 0.001$.

[Chappell et al., 2011], implying Akt/Raf/ERK pathway was a critical target pathway for wogonin in NPC cells.

This study found Akt was a major anti-apoptotic pathway mediator that conferred survival advantage and resistance of NPC cells against anticancer therapies. Wogonin might direct apoptosis and autophagy through inactivation of Akt pathway. It induced autophagy ameliorated wogonin-induced apoptosis through inhibition of mTOR/P70S6K pathway, but induced cell apoptosis and enhanced wogonin-induced cytotoxicity through inhibition of Akt/cRaf/ERK pathway. Thus, modulation of autophagy and combination with PI_3K/Akt inhibitors might profoundly enhance the apoptotic effect of wogonin and provide therapeutic implications to overcome drug resistance related to Akt pathway.

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