Anthocyanins (ATCs) have been reported to induce apoptosis in various types of cancer cells, stimulating the development of ATCs as a cancer chemotherapeutic or chemopreventive agent. It was recently reported that ATCs can induce autophagy, however, the mechanism for this remains unclear. In the present report, we carried out mechanistic studies of the mechanism involved in ATC-induced autophagy using ATCs extracted from black soybeans (cv. Cheongja 3, Glycine max L.). ATCs clearly induced hallmark markers of autophagy, including LC3 puncta formation and the conversion of LC3-I to LC3-II in U2OS human osteosarcoma cells. The induction of autophagy was accompanied by the phosphorylation of multiple protein kinases including extracellular signal-regulated kinase (ERK)1/2, p38 mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK), protein kinase B (AKT) and adenosyl monophosphate-dependent protein kinase (AMPK). While chemical inhibitors against ERK1/2, p38 MAPK, JNK and AKT failed to inhibit ATC-induced autophagy, the suppression of AMPK by compound C (CC) as well as siRNA against AMPK reduced ATC-induced autophagy. The treatment of ATCs resulted in a decrease in intracellular ATP contents and the activation of AMPK by AICAR treatment also induced autophagy. It is noteworthy that the reduction of autophagy via the inhibition of AMPK resulted in enhanced apoptosis in ATC-treated cells. In addition, siRNA against forkhead box O3A (FOXO3a), a downstream target of AMPK, suppressed ATC-induced autophagy and p27KIP1 siRNA increased apoptosis in ATC-treated cells. Collectively, it can be concluded that ATCs induce autophagy in U2OS cells via activation of the AMPK-FOXO3a pathway and protect cells from ATC-induced apoptosis via the AMPK-p27KIP1 pathway. These results also suggest that autophagy-modulating agents could contribute to the efficient development of ATCs as anticancer therapy.

Introduction

Anthocyanins (ATCs) are natural phytochemicals which are produced by fruits and vegetables. ATCs have noteworthy biological functions, including anti-inflammatory and apoptosis-inducing effects. It has been reported that ATCs induce apoptosis in various cancer cell lines including hepatoma, colorectal carcinoma, gastric adenocarcinoma, leukemia and prostate cancer (1-5). The ATC-induced apoptosis occurs via both caspase-dependent and -independent mechanisms. It has been demonstrated that ATCs initially induced an alteration of protein kinase B (AKT) and mitogen-activated protein kinases (MAPKs) such as p38 MAPK, extracellular signal-regulated kinase (ERK)1/2 and c-Jun N-terminal kinase (JNK), which led to the upregulation of the BAX/BCL2 ratio and hence the activation of intrinsic apoptosis in cancer cells (1-4). However, in prostate cancer PC3 cells, ATCs induced caspase-independent apoptosis via the nuclear translocation of endonuclease G and AIF (5). Regarding their anti-inflammatory activity, ATCs prevent the LPS- and UVB-induced expression of inflammatory mediators such as cyclooxygenase (COX)-2 and prostaglandin E2 (PGE2), proinflammatory cytokines and oxidative stress. It has been reported that ATCs prevent the LPS- and UVB-induced expression of inflammatory mediators such as cyclooxygenase (COX)-2 and prostaglandin E2 (PGE2) by suppressing the nuclear factor of κ light polypeptide gene enhancer in activated B cells (NF-κB) and phosphoinositide 3-kinase (PI3K)/AKT pathways in...
human keratinocytes and macrophage cell lines (6,7). This anti-inflammatory activity of ATCs was shown to be effective in vivo to subside circulatory and multiple organ failures due to endotoxemia (8). Based on these apoptosis-inducing and anti-inflammatory as well as anti-invasive and anti-angiogenic effects (9,10), attempts have been made to develop ATCs as a chemopreventive agent, and promising results of pilot studies in rodent models of esophageal and colorectal cancers and human colorectal cancer patients have provided support for this hypothesis (11,12).

In addition to inducing apoptosis, it has been suggested that ATCs induce autophagy in cancer and glial cells (13,14), although the mechanism for this remains to be clarified. Autophagy is considered to be a cellular defense system that can protect cancer cells from anticancer therapeutics. Accordingly, autophagy inhibition augments ATC-induced apoptosis (13). A more detailed understanding of the mechanism underlying ATC-induced autophagy could contribute to the development of effective strategies for the use of ATCs as a chemopreventive or chemotherapeutic agent against cancer in the future.

AMPK, which plays a central role in energy balance and metabolism, is one of the critical regulators of autophagy induction. AMPK becomes activated once it is phosphorylated by several kinases (15,16). Under metabolic stresses that restrain ATP synthesis, elevated AMP binds to the γ subunit of AMPK for both allosteric activation of AMPK and prevention of the dephosphorylation of AMPK, leading to a sustained activity of AMPK. Allosterically activated AMPK is phosphorylated by liver kinase B1 (LKB1) at Thr172 of the α subunit to become fully activated. In response to elevated intracellular calcium levels and cytokine signaling, calmodulin-dependent protein kinase kinase (CaMKK) and transforming growth factor-β-activated kinase 1 (TAK1), respectively, phosphorylate AMPK.

Activated AMPK phosphorylates mTORC1, an inhibitor of autophagy, and a serine/threonine kinase with homology to yeast atg1 (ULK1), an initiator of autophagy, thereby leading to autophagy induction (17,18). P27KIP1, a CDK inhibitor, is directly phosphorylated at Thr198 and stabilized by AMPK for the induction of autophagy and, notably, the knockdown of P27KIP1 in the presence of activated AMPK induced apoptosis instead of autophagy, an observation that explains the inhibitory effect of apoptosis by autophagy and the possibility that p27KIP1 may mediate the decision between apoptosis and autophagy (19). Forkhead box O3a (FOXO3a), an important regulator of glucose metabolism and tumor suppression, was reported to be phosphorylated by AMPK (20). Recently, FOXO3a was shown to upregulate P27KIP1 to induce cell cycle arrest in glioma cells (21,22). Therefore, it can be argued that under metabolic stress, autophagy formation can be induced through interactions among AMPK, FOXO3a, and P27KIP1.

ATC was recently reported to suppress IGF-1-induced mammalian target of rapamycin (mTOR) phosphorylation by AMPK activation in colon cancer cells, providing further support for its anticancer activity (23). However, the relationship between autophagy and AMPK in ATC-treated cells is not yet fully understood. To address this issue, we used ATCs extracted from black soybeans (cv Cheongja 3). These ATC extracts were shown to promote apoptosis in a model of prostate hyperplasia in vivo and to induce autophagy in glial cells, which proved to contain biologically active components of autophagy induction as well as apoptosis (14,24). In the present study, we observed the induction of autophagy and AMPK activation by ATCs and analyzed the role of AMPK in this autophagy.

**Materials and methods**

**Chemicals.** AICAR, compound C (CC), SP600125, SB203580, U0126, and triciribine were obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA) or Merck (Darmstadt, Germany). All chemicals used are of molecular biology or cell culture grade.

**Extraction of ATCs.** ATCs were extracted from black seed coated soybean, Cheongja 3, developed by the National Institute of Crop Science, Rural Development Administration at Miryang, Korea, as previously described, with minor modifications (25). Briefly, hand-peeled seed coats were extracted twice with 80% ethanol containing 0.1% acetic acid for 2 days at 4°C. These ethanol extracts were filtered through a 0.45-µm filter and concentrated in a rotary evaporator to obtain crude ATC extracts. The crude extracts were freeze-dried and kept below -70°C.

**Establishment of U2OS-GFP-LC3 cells.** U2OS cells were cultured in high glucose DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone Laboratories Inc., Logan, UT, USA), 100 U/ml penicillin/streptomycin (Hyclone). Plasmid EGFP-LC3 was generously donated by Professor S.H. Lee at the Department of Pathology, the Catholic University of Korea. To establish stable cell lines expressing GFP-LC3, pEGFP-empty vector or pEGFP-LC3 was transfected into U2OS cells. Following the selection of transfected cells using G418 for 14 days, viable cells were pooled, and GFP expression was observed under a fluorescence microscope. Total cellular extracts were prepared and subjected to immunoblot analysis against GFP or LC3.

**Immunoblot analysis.** U2OS cells treated with ATCs with or without other chemicals as indicated were lysed with RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.25% Na-deoxycholate, 1% NP-40, 1 mM EDTA) containing protease inhibitors (Roche Applied Sciences, Indianapolis, IN, USA). Total cellular proteins were separated by SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Millipore, Bedford, MA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone Laboratories Inc., Logan, UT, USA), which were hybridized with primary and then secondary antibodies. Finally, protein bands were detected by enhanced chemiluminescence (ECL; GE Healthcare, UK). Antibodies against phospho-AMPK-Thr172, phospho-p38 MAPK (T180/Y182), p38 MAPK, JNK, Erk1/2, phospho-Erk1/2 (T202/Y204), mTOR, and phospho-mTOR (S2448) were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA) and antibodies against phospho-JNK (Y185/Y223), AKT, phospho-AKT (S473), ACC, phospho-ACC (S79), and cleaved PARP were from Epitomics, Inc. (Burlingame, CA, USA). Antibodies against actin, α-tubulin, and GAPDH from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and LC3 from Sigma-Aldrich Corp. were used.
Transfection of small interfering RNA. Small interfering RNA against AMPKα1 (siAMPK) was purchased from Sigma-Aldrich Corp., and small interfering RNAs against p27KIP1 and FOXO3a were from Bioneer (Daejeon, Korea). Each siRNA was transfected into U2OS cells or U2OS-GFP-LC3 cells by the reverse transfection method using RNAiMAX reagent (Invitrogen) according to the manufacturer’s protocol. The sequences of duplex siRNAs are as follows: siAMPK, 5'-CCCAUAUUAUUUGCGUGUA(dTdT)::5'-UACACGCAAAUAAUAUGGG(dTdT); sip27KIP1, 5'-CGAGCGAUUCUUCUCAA(dTdT)::5'-UUGAGUAGAAGAUCGUCG(dTdT); siFOXO3a, 5'-GACGAUGAUGCGCCUCUCU(dTdT)::5'-AGAGAGGCGCAUCAUCGU(dTdT).

Assessment of cell death. Cell death was examined by measuring sub-G1 cells and phosphatidylserine translocation. For measurement of sub-G1 cells, cells were fixed in ice-cold 70% ethanol for 2 h at 4°C and stained with propidium iodide (PI; 0.2 mg/ml), followed by flow cytometric analysis (FACSCalibur, BD Bioscience, San Jose, CA, USA). To determine phosphatidylserine translocation, the cells stained with PI and annexin V using ApoScan Kit (BioBud, Gyunggi-do, Korea) were analyzed using a previously described method (26).

Measurement of intracellular ATP levels. Intracellular ATP levels were measured using a CellTiter Glo Kit (Promega, Madison, WI, USA) following the manufacturer’s instructions.

Statistical analysis. Statistical significances were evaluated using the one-way ANOVA test. The data represent three independent experiments performed in triplicate as the mean ± SD.

Results

ATCs induce autophagy in U2OS cells. When human osteosarcoma U2OS cells were treated with ATCs, their growth was not inhibited in the first 24 h. However, after 24 h, ATCs suppressed the growth of U2OS cells associated with apoptosis (Fig. 1A and B), suggesting that ATCs activate the cell death program after 24 h in this cell line and autophagy may be induced before 24 h. To investigate this further, we examined the autophagy by ATCs before 24 h. For this end, we generated U2OS cells stably expressing GFP-LC3 (U2OS-GFP-LC3) and first observed LC3 puncta formation in these cells. When U2OS-GFP-LC3 cells were treated with ATCs for 6 h, it appeared that GFP-LC3 puncta were formed (Fig. 1C). Consistent with LC3 foci formation, the conversion of LC3-I to LC3-II occurred in both U2OS (Fig. 1D) and U2OS-GFP-LC3 cells (data not shown) in a dose-dependent manner, reaching a plateau level at concentrations of ATCs above 200 µg/ml of ATCs. These findings demonstrated that ATCs induced autophagy in U2OS cells prior to the activation of apoptosis.

ATC-induced autophagy is not prevented by inhibitors of MAPks or AKT. To identify the signal transduction pathway leading to autophagy in ATC-treated cells, we investigated the phosphorylation of signal transduction proteins which have been reported to be involved in ATC-induced apoptosis. As shown in Fig. 2A, the phosphorylation of ERK1/2, p38 MAPK, JNK, and AKT was increased by ATC treatment, suggesting that they are involved in this autophagy. However, neither the formation of GFP-LC3 puncta nor LC3-I conversion to LC3-II was prevented by any of the chemical inhibitors of AKT,
ATC-induced autophagy is prevented by AMPK inhibition. We then explored the activation of AMPK in ATC-treated cells. While the phosphorylation of mTOR was decreased, the phosphorylation of both AMPKα1 and its substrate, acetyl CoA carboxylase (ACC) increased according to concentrations
of ATCs, indicating that AMPK is activated in this autophagy (Fig. 3A). Pretreatment with CC, an inhibitor of AMPK, reduced both the GFP-LC3 puncta formation (Fig. 3B) and the conversion of LC3-I to LC3-II (Fig. 3C). Consistent with the effect of CC, the knockdown of AMPKα1 using siRNA against AMPKα1 also suppressed the conversion of LC3-I to LC3-II (Fig. 3D). Furthermore, ATCs diminished intracellular ATP levels (Fig. 3E) and AICAR, an analog of AMP, also induced autophagy in U2OS cells (Fig. 3F). Collectively, these findings suggest that ATC-induced autophagy is dependent on AMPK activation which may be due to the depletion of ATP.

**AMPK inhibition enhances ATC-induced apoptosis.** Based on the report that autophagy inhibition augmented the ATC-induced apoptosis in hepatocellular carcinoma cells (13), we analyzed the effect of AMPK on apoptosis in ATC-treated U2OS cells. As shown in Fig. 4, while ATCs induced a subtle apoptosis at 6 h in U2OS cells that had been transfected with control siRNA, ATCs dramatically induced apoptotic features such as accumulation of hypo-diploidic cells (Fig. 4A) and poly(ADP-ribose) polymerase-1 (PARP) cleavage (Fig. 4B) in AMPKα1 siRNA-transfected U2OS cells. Thus, these data suggest that ATC-activated AMPK protects U2OS cells from apoptosis.

**Differential effects of p27KIP1 and FOXO3a on ATC-induced autophagy.** To investigate the downstream targets of AMPK that play a role in the induction of autophagy, we attempted the transfection of siRNAs against ULK1, p27KIP1, and FOXO3a.
which have been demonstrated to be autophagy modulators under AMPK. The knockdown of ULK1 by ULK1 siRNA did not exert an inhibitory effect on either ATC-induced autophagy or apoptosis, ruling out the involvement of ULK1 in this autophagy (data not shown). As shown in Fig. 5, both ATC-induced LC3 puncta formation (Fig. 5A) and the conversion of LC3-I to LC3-II (Fig. 5B) were reduced, but ATC-induced PARP cleavage was not altered by FOXO3a knockdown, suggesting that FOXO3a may be responsible for the induction of autophagy induction under active AMPK. In contrast to the effect of FOXO3a siRNA, p70S6K siRNA potentiated ATC-induced PARP cleavage but had no effect on LC3-II levels (Fig. 5C), suggesting that p70S6K may inhibit ATC-induced apoptosis, but has no effect on autophagy. Thus, it can be suggested that AMPK activated by ATCs may induce autophagy via FOXO3a and inhibit apoptosis via p70S6K.

Discussion

The findings reported herein indicate that ATCs induce autophagy in human osteosarcoma U2OS cells, accompanied by the activation of MAPKs and AMPK. By analyzing the effects of chemical inhibitors and siRNAs against MAPKs and AMPK on ATC-induced autophagy, the findings suggest that AMPK plays a critical role in this autophagy. However, how ATCs specifically activate AMPK, remains unknown. It has been reported that phytochemicals such as galegine, berberine, as well as resveratrol activate AMPK in an AMP-dependent manner (27). ATCs also reduced ATP levels in this cell line and AICAR, an analog of AMP, induced autophagy (Fig. 3E and F), indicating that AMP/LKB1-dependent AMPK activation results from ATC treatment. However, LKB1 was not phosphorylated by ATCs and AMPK was still phosphorylated in U2OS cells that had been transfectioned with LKB1 siRNA (data not shown). In addition, ATC-induced AMPK phosphorylation was not altered by BAPTA-AM, a calcium chelator, CaMKK siRNA, TAK1 siRNA or ROS scavengers (data not shown). Collectively, it can be assumed that ATCs may enrich intracellular AMP levels which induce the allosteric activation of AMPK without the action of LKB1 or inhibit the dephosphorylation of AMPK that was phosphorylated by unidentified enzymes other than LKB1. However, as it is known that the allosteric activation of AMPK by AMP is induced in AMPK phosphorylated by basal LKB1 activity, this assumption leaves many questions to be resolved. Considering the numerous studies reporting that AMPK-activating compounds including phytochemicals inhibit mitochondrial respiratory complexes to reduce synthesis of ATP, we speculate that ATCs might contain two major features for activating AMPK: one is to reduce ATP synthesis possibly via the inhibition of mitochondrial respiratory complexes and the other is to induce the basal phosphorylation of AMPK or the allosteric activation of AMPK in conjunction with AMP even in the absence of LKB1 activity.

A recent study showed that AMPK phosphorylates ULK1 to initiate autophagy (18). In the present model, ATC-induced AMPK phosphorylation was accompanied by the dephosphorylation of mTOR (Fig. 3) and rapamycin, an inhibitor of mTOR, induced autophagy (data not shown). Moreover, ATCs induced autophagy in ULK1-knocked down cells to a comparable extent as in control siRNA-transfected U2OS cells (data not shown), suggesting that AMPK-induced mTORC1 inhibition may play a more important role in ATC-induced autophagy than does ULK1 activation. In addition to the mTOR and ULK1 pathways, AMPK modulates downstream target proteins such as p70S6K and FOXO3a to induce autophagy. P70S6K, an inhibitor of CDK1, which can be phosphorylated by AMPK and can be induced transcriptionally by FOXO3a as well, was reported to induce autophagy but inhibit apoptosis (19), suggesting that it is a strong molecular target of ATC-activated AMPK. Different from these reports, in our model, it appeared that p70S6K inhibited apoptosis but was not involved in autophagy, whereas FOXO3a contributed to the induction of autophagy but not apoptosis. Therefore, it can be concluded that AMPK activated by ATC-inhibited ATP synthesis induces autophagy by activating FOXO3a while suppressing mTORC1, and inhibits ATC-induced apoptosis via the upregulation of p70S6K, as summarized in Fig. 6. To verify this, however, the mechanism by which p70S6K and FOXO3a are regulated by ATCs remains to be clarified. As shown in Fig. 5C-E, p70S6K protein levels were upregulated by ATC treatment, which returned to basal levels in U2OS cells that had been pretreated with compound C (CC) or transfected with AMPliv siRNA before ATC treatment, suggesting that p70S6K may be stabilized by active AMPK. In contrast to p70S6K, FOXO3a protein levels were not consistently altered by ATCs and the phosphorylation of FOXO3a-Ser413, a site known to be phosphorylated by AMPK, was not detected either (data not shown), suggesting that the phosphorylation of Ser or Thr residues other than Ser413 by AMPK or an indirect effect of AMPK on FOXO3a induces autophagy.

The role of AMPK in cancer cells is known to be varied. AMPK can activate p53 by phosphorylating p53-Ser15 to induce cell cycle arrest and apoptosis (28,29). For example, a combined treatment with metformin and 2-deoxyguanosine, as well as vincristine, an anticancer agent, induced apoptosis in prostate and melanoma cells, respectively, by activating AMPK (30,31). Contrary to these reports, AMPK inhibition resulted in the sensitization of cancer cells to apoptosis by anticancer drugs such as cisplatin as well as metabolic stress such as hypoxia (32,33). This variable effect of AMPK on cell death may be due to both types of stresses and cellular
context. It was recently reported that autophagy inhibition can enhance the cytotoxicity of ATCs in hepatoma cells (13), which may be consistent with the data in our study showing that AMPK and p27\(^{kip1}\) siRNAs enhanced ATC-induced apoptosis (Figs. 4 and 5C). Therefore, AMPK activation by ATCs may contribute to the cancer cell survival via the induction of autophagy.

In non-transformed cells, autophagy serves to preserve genomic integrity, thereby preventing malignant transformations (34). This autophagy-inducing activity of ATCs could explain the chemopreventive effect of ATCs reported in the esophagus and the colon (11,12), although the autophagy-inducing activity of ATCs in non-transformed cells remains to be determined. A metabolic shift to aerobic glycolysis has generally been regarded as a driving force for carcinogenesis (35,36), and dysregulated metabolic homeostasis and obesity have been reported to increase the risk of certain types of cancer, such as breast cancer (37). Since AMPK is a central regulator of metabolism and preserves metabolic homeostasis, AMPK could prevent cancer formation under conditions of metabolic distress. Consistent with this assumption, metformin was reported to inhibit the formation of pre-neoplastic lesions in the colon and showed chemopreventive activity against breast cancer associated with obesity (38). Therefore, even if ATCs do not induce autophagy in non-transformed cells, only the AMPK-activating effect of ATCs could contribute to chemopreventive effects against cancer.

Autophagy can prevent the induction of apoptosis and, instead, initiate necrotic cell death in cancer cells in vivo, accompanied by an inflammatory response, which can contribute to the invasion and metastasis of cancer cells (39,40). This autophagy-inducing activity of ATCs could dampen the anticancer effect of ATCs, thereby constituting a negative feedback loop of ATC-induced apoptosis.

Collectively, it could be concluded that while ATCs may activate AMPK and, thus, prevent carcinogenesis in non-transformed cells by activating autophagy and preserving metabolic homeostasis, autophagy in cancer cells may diminish the apoptosis-inducing activity of ATCs. Therefore, to develop ATCs for use as efficient anticancer therapy or as a chemopreventive agent against cancer, the mechanism underlying the autophagy-inducing activity of ATCs as well as ATC-induced apoptosis needs to be thoroughly examined to determine whether it is possible to modulate genes to enhance the anticancer effects of ATCs.

Acknowledgements

This study was supported by the Biogreen 21 Program (code no. PJ007186), Rural Development Administration Korea. Part of this study was also supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2011-0027115).

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