Inhibition of Akt signaling and enhanced ERK1/2 activity are involved in induction of macroautophagy by triterpenoid B-group soyasaponins in colon cancer cells

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Triterpenoid B-group soyasaponins have been found to induce macroautophagy in human colon cancer cells at concentrations obtainable through consumption of legume foodstuffs. In the present studies the mechanism(s) for this autophagy-inducing action of soyasaponins was evaluated by measuring changes in signal transduction pathways associated with autophagy. Specifically, inhibition of the Akt signaling pathway and enhanced activity of ERK1/2 have previously been implicated in controlling induction of macroautophagy in mammalian cancer cells. Here we show that these pathways are also involved in B-group soyasaponin-induced macroautophagy, as changes in enzyme activities preceded significant increases in autophagic activity. The autophagic capacity of HCT-15 cells was significantly increased by 6 h post-saponin exposure, which led us to measure alterations in signaling events that preceded this time point. We determined that exposure to B-group soyasaponins suppressed Akt activity maximally by 50%, which was associated with a reduction in the activating phosphorylation of the Akt-serine[473]** residue. In addition, ERK1/2 activity was significantly increased by 60%, and was determined to be necessary for B-group soyasaponin-induced autophagy. The raf-1 kinase has been identified as a potential point of cross-talk between the Akt and ERK1/2 signaling cascades. Following B-group soyasaponin treatment activity of raf-1 was significantly increased by a maximal 200%, suggesting that this enzyme in part modulates the enhanced ERK1/2 activity. These results provide new insights into the signaling events that control induction of autophagy by B-group soyasaponins in human colon cancer cells and suggest that soyasaponins warrant further study as potential colon cancer chemopreventive agents.**

Introduction

Colon carcinoma is the second largest cause of cancer-related mortality in the United States, and both prevention and treatment of this disease are the focus of intensive research efforts (1). Various dietary patterns, including those that emphasize legumes, have been associated with reduced colon cancer risk (2–4). Epidemiological data indicate that dietary inclusion of at least two servings of legumes per week has been associated with a significant reduction in colon cancer incidence (5,6). In particular, soy legumes have received increased scrutiny as a food component with potential colon chemoprotective attributes. In populations in which soy intake is typically low, higher consumption of foods derived from the soy legume has been associated with reduced colon cancer incidence (7). In addition, a yearlong prospective double-blind intervention trial found that daily dietary inclusion of 39 g of soy protein reduced the recurrence of adenomatous polyps (8). Legumes contain a variety of phytochemicals that could contribute to prevention of colon carcinogenesis. There is increasing evidence that the triterpenoid soyasaponins may be constituents of legumes involved in suppressing colon cancer.

Saponins are amphiphilic compounds composed of a lipid soluble aglycone, designated the sapogenol, to which water-soluble sugar moieties are attached. Under in vitro conditions, numerous saponins have demonstrated antimutagenic, anticarcinogenic and antimetastatic effects against multiple cell lines (9). The triterpenoid soyasaponins are composed of a neutral, non-polar oleane-12-ene triterpene aglycone to which various sugar residues are linked at one or more glycosylation sites, with the B-group as the predominant class found in intact legumes and soy foods (10). Following ingestion of soy foods, soyasaponins appear to pass undigested through the small intestine, and are then partially metabolized to sapogenols by bacterial glycosidases in the colon (11–13). The ensuing mixture of saponins and sapogenols is presumably bioavailable to the colonic epithelium, suggesting that soyasaponins could potentially modulate colon carcinogenesis.

In rodent models, chemically induced colon carcinogenesis was diminished by dietary administration of foods that contain soyasaponins, including garbanzo bean flour, soy protein isolate, soy flour and soy flakes (14–16), while ingestion of soy foods devoid of soyasaponins conferred no protective effects (16). Specifically, administration of a 3% crude soyasaponin diet to CF-1 mice post-initiation significantly reduced both incidence and multiplicity of aberrant crypt foci (17).

In cell culture studies, crude soyasaponin mixtures are growth inhibitory against multiple human colon adenocarcinoma cell lines (18–21). In our laboratory, we have recently demonstrated that a fraction of purified B-group soyasaponins inhibits the proliferation of HCT-15 human colon adenocarcinoma cells at concentrations attainable from the diet (22). Of particular interest, we observed that B-group soyasaponin treatment was associated with ultrastructural alterations such as cytoplasmic vacuolization, increased intracellular incorporation of monodansylcadaverine (MDC) and elevated concentrations of microtubule associated protein light chain-3 protein, which collectively are phenomena indicative of sustained autophagy, the hallmark of Type II cell death (22).

Abbreviations: MDC, monodansylcadaverine; PI3K, phosphotidyl-inositol-3-kinase; PIP$_3$, phosphotidyl-inositol-3,4,5-phosphate; TSC, tuberous sclerosis complex.

Macroautoutophagy (hereafter called autophagy) is the physiological process of controlled self-digestion utilized for the turnover of cellular constituents (23). Following induction of autophagy, an isolation membrane of indeterminate origin sequesters cytoplasm, organelles and proteins to form the double-membrane autophagic vacuole (24). Subsequent to acidification, the autophagic vacuole fuses with the lysosome, releasing the enclosed cellular components for degradation by lysosomal enzymes (25).

Although down-regulated in malignant cancers (26), induction of autophagy can promote both tumor progression and cancer cell death (27). Tumor progression can be favored under stressful conditions including nutrient limitation, hypoxia, radiation and chemotherapy. Autophagy can serve as a mechanism of self-defense by recycling essential molecules thus enhancing survival of catabolic cells centrally located within tumors, and by contributing to therapy resistance by sequestering toxic agents and damaged mitochondria thereby diminishing apoptotic triggers (27–30).

Conversely, autophagy can inhibit tumor progression by slowing cell proliferation and inhibiting angiogenesis (28). Extensive autophagy can also result in destruction of vital cell constituents, committing the cell to a caspase-independent death characterized by the formation of numerous autophagic vacuoles followed by cytoplasmic destruction (31). Of interest, tamoxifen and arsenic trioxide, compounds that induce autophagy, have demonstrated the potential to be effective chemotherapeutic agents in clinical trials (32,33). *In vitro* exposure to phytochemicals including resveratrol and the B-group soyasaponins can reduce the viability of cancer cells through sustained autophagy (22,34).

Little is known about the molecular pathways that regulate autophagy in cancer cells, especially how dietary factors may initiate this process. Notably, disruption of the PI3K/Akt signaling pathway, culminating in inhibition of Akt, a central regulator of cell survival, has been consistently associated with indicators of autophagy in cancer cells (35–38). Regulation of Akt activity by phosphotidyl-inositol-3-kinase (PI3K) through upstream second messengers and secondary enzymes has been recently reviewed (39). Following activation by receptor tyrosine kinases, PI3K can generate large quantities of membrane-bound phosphotidyl-inositol-3,4,5-phosphate (PIP_3), a second messenger that recruits both PI3K-dependent kinase-1 (PDK-1) and Akt to the plasma membrane, an event antagonized by phosphatase activity (40). The constitutively active PDK-1 can then phosphorylate the threonine[308] residue located in the catalytic region of Akt, revealing both ATP and substrate binding sites (39). Of importance, maximal activation of Akt requires phosphorylation of the serine[473] residue located in the regulatory carboxyterminal hydrophobic motif by an unidentified enzyme, PI3K-dependent kinase-2 (PDK-2) (39). Activated Akt has been linked to signaling pathways involved in aberrant cell growth and differentiation regulation characteristic of carcinogenesis as well as of cancer chemotherapy resistance making it an attractive target in drug discovery strategies (41). Thus, understanding the role of dietary factors such as soyasaponins in modulating this key survival pathway is important.

Although targets of Akt involved in the regulation of autophagic capacity are uncertain, one proposed downstream effector is the mTor kinase (42). Akt contributes to the positive regulation of mTor activity through an inhibitory phosphorylation on tuberous sclerosis complex-2-thr[1462] (TSC-2),

a GTPase activating protein that reduces the activity of Rheb, an activator of mTor (43,44). Thus, inhibition of Akt signaling may reduce the activity of mTor. In regard to autophagy, it has been demonstrated that insulin/PI3K/Akt signaling regulated autophagy during nutrient deprivation through an mTor-dependent pathway in rat hepatocytes (45). However, it has not been established whether mTor plays a role in mediating autophagic response following exposure to chemotherapeutic agents.

Another signaling pathway recently associated with regulation of autophagy is the *ras*/raf-1/MEK/ERK cascade. Dysregulation of *ras* function is believed to play a key role in a variety of human carcinomas (46). Specifically, up-regulation of ERK1/2 activity via enhanced activity of the *ras*/raf-1/MEK/ERK pathway is imperative for induction of autophagy in HT-29 colon cancer cells, potentially through controlling isolation membrane flux by initiating events that ultimately inhibit a G-protein coupled receptor associated with the endoplasmic reticulum and golgi apparatus (47–49).

In light of the colonic exposure to dietary B-group soyasaponins and the capacity of these plant compounds to sustain autophagy in colon cancer cells *in vitro*, examination of soyasaponin modulation of key signaling pathways involved in colon carcinogenesis is warranted. Therefore, in the current studies we investigated involvement of Akt, mTor and ERK1/2 signaling pathways in B-group soyasaponin-induced autophagy.

Materials and methods

Reagents
The purified B-group soyasaponin sample was prepared by Mark Berhow (USDA, Agricultural Research Service, National Center for Agricultural Utilization Research, Peoria, IL) from material provided by Organic Technologies (Coshockton, OH) and was dissolved in DMSO (Sigma Chemical, St Louis, MO). Monodansylcadaverine (MDC) was purchased from Sigma Chemical. The primary antibodies against Akt, phospho-glycogen synthase kinase-α/β[ser21/9], phospho-Akt-thr[308], phospho-Akt-ser[473], TSC-2, phospho-TSC-2-thr[1462], mTor, ERK1/2, phospho-Elk-1[ser383], phospho-ERK1/2-thr[202/tyr204], phospho-MEK1/2-ser[217/221] and phospho-raf-1-ser[259] were purchased from Cell Signaling Technology (Beverly, MA). The primary antibodies against raf-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture
Human colon adenocarcinoma HCT-15 cells were purchased from the American Type Culture Collection (Rockville, MD). Cells were cultured in DMEM (GIBCO BRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO BRL), 50 U/ml penicillin and 50 µg/ml streptomycin. Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2, and were used in subsequent experiments between subculture passages six and ten. Cells were routinely plated at a density of 12 000/cm^2, and allowed to grow for 48 h. At this time, media was replaced and either soyasaponin treatment or DMSO vehicle was added. In select experiments cells were exposed to enzyme inhibitors for 1 h prior to saponin treatment: 100 nM rapamycin to inhibit mTor, 50 µM LY294002 to inhibit PI3K and 10 µM U0126 to inhibit MEK1/2, all purchased from Cell Signaling Technology.

MDC incorporation assay
MDC has been shown to selectively accumulate in acidic autophagic vacuoles (50). Measuring MDC incorporation is a rapid, convenient approach to assay autophagy in cultured cells (51), and therefore intracellular concentrations of MDC were quantified using procedures previously described (52). In brief, cells were incubated with 50 µM MDC for 60 min at 37°C, and then collected in 10 mM Tris–HCl, pH 8, and containing 0.1% Triton-X 100. MDC incorporation was measured using fluorescence photometry in a Packard Fluorocount microplate reader (excitation wavelength 380 nm and emission filter 525 nm). MDC values were normalized to cell number by adding ethidium bromide solution to a final concentration of 0.2 µM and measuring DNA fluorescence

(excitation wavelength 530 nm and emission filter 590 nm), and were expressed as arbitrary units of specific activity.

Immunoblotting and immunoprecipitation

Cells were harvested in non-denaturing lysis buffer [20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton-X, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 2 mM DTT, 1 mM sodium orthovanadate, 0.02 mg/ml aprotinin, 0.001 mg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride (PMSF) in distilled water]; mTor lysis buffer [50 mM Tris, pH 7.4, 100 mM NaCl, 50 mM β-glycerophosphate, 10% glycerol, 1% Tween-20, 1 mM EDTA, 1 mM EGTA, 0.01 mg/ml aprotinin, 0.01 mg/ml leupeptin, 0.001 mg/ml pepstatin A, 2 mM PMSF, and 25 mM NaFl in phosphate-buffered saline (PBS)]; or raf-1 lysis buffer (20 mM Tris, pH 7.9, 137 mM NaCl, 5 mM EDTA, 1 mM EGTA, 10 mM NaF, 1 mM sodium pyrophosphate, 100 μM β-glycerophosphate, 0.01 mg/ml aprotinin, 1 mM PMSF, 10% glycerol and 1% Triton-X in distilled water). For immunoblotting, equivalent amounts of protein were separated using SDS–PAGE, and then transferred to a nitrocellulose membrane. Membranes were blocked with blocking buffer for 1 h, probed with primary antibodies overnight and then incubated with the HRP-conjugated secondary antibody for 1 h. Antibody binding was detected by enhanced chemiluminescence (Amersham, Buckinghamshire, UK). Density for each band was analyzed using Image J (NIH, Bethesda, MD). Equal protein loading was confirmed by probing β-actin. Values obtained for phosphorylated enzymes were normalized to total enzyme concentration, and expressed as percentage of control value. For immunoprecipitation, 300 μg of lysate proteins were immunoprecipitated overnight with the primary antibody of interest along with protein A agarose beads in lysis buffer, and then used for kinase activity assays.

Kinase activity assays

mTor activity was measured using the methods of Chiang *et al.* (53). mTor immunoprecipitates were washed three times with mTor lysis buffer, once with high salt wash buffer (100 mM Tris–HCl, pH 7.4 and 500 mM LiCl in PBS), and once with kinase assay buffer (10 mM HEPES, pH 7.4, 50 mM NaCl, 50 mM β-glycerophosphate and 10% glycerol in PBS), and then resuspended in kinase assay buffer supplemented with 50 μM ATP, 1 mM DTT, and 10 mM MnCl$_2$. 4 μg PHIP-1 substrate (Stratagene, La Jolla, CA) and 5 μCi of [γ-^{32}P]ATP (7000 Ci/mmol) were added to each sample to initiate the kinase activity reaction. Mixtures were incubated for 20 min at 37°C, and the reaction was stopped with the addition of equal amounts of 2× SDS–PAGE sample buffer. Samples were boiled for 3 min, and then separated using SDS–PAGE prior to transfer to a nitrocellulose membrane. Reaction products were detected by autoradiography and densitometry was performed as described above. Kinase activity of both Akt and ERK1/2 was determined using non-radioactive kinase assay kits according to manufacturer's instructions (Cell Signaling Technology). In brief, immunoprecipitates were washed twice with non-denaturing lysis buffer and then twice with kinase buffer (25 mM Tris pH 7.5, 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM sodium orthovanadate, and 10 mM MgCl$_2$ in distilled water). Pellets were then suspended in 30 μl kinase buffer supplemented with 0.2 mM ATP and 2 μg substrate protein, and incubated for 30 min at 37°C. The reaction was stopped with the addition of 25 μl 3× SDS–PAGE sample buffer. Samples were boiled for 5 min, and then separated using SDS–PAGE prior to transfer to a nitrocellulose membrane. Reaction products were detected using immunoblotting with phospho-specific antibodies as described above. Raf-1 activity was measured using the methods of Bondzai *et al.* (54). Raf-1 immunoprecipitates were washed three times with Raf-1 lysis buffer, once with washing buffer (10 mM HEPES, pH 7.4, 100 mM NaCl, 0.02 mg/ml aprotinin and 0.5% NP-40 in distilled water), and once with reaction buffer (20 mM Tris, pH 7.4, 20 mM NaCl, 1 mM DTT, 10 mM MgCl and 1 mM MnCl in distilled water), and then resuspended in reaction buffer supplemented with 20 μM ATP and 500 ng MEK-1 substrate (Santa Cruz Biotechnology). Mixtures were incubated for 30 min at 37°C, and the reaction was stopped with the addition of equal amounts of 2× SDS–PAGE sample buffer. Samples were boiled for 10 min, and then separated using SDS–PAGE prior to transfer to a nitrocellulose membrane. Reaction products were detected using immunoblotting with phospho-specific antibodies as described above. Values obtained for enzyme activity were normalized to total enzyme concentration, and expressed as percentage of control value.

Statistical analysis

Experiments were performed in triplicate, and were repeated at least twice. Statistical differences between control and treated samples were determined by Student's *t*-test using SAS software. *P* values of <0.05 were considered significant.

Results

Autophagy is induced by B-group soyasaponins in HCT-15 cells

MDC has been shown to selectively accumulate in acidic autophagic vacuoles (51), and therefore measurement of MDC incorporation is a rapid, convenient means to assay autophagy in cultured cells (51). We have previously observed that MDC incorporation parallels microtubule associated protein light chain-3 protein upregulation in response to B-group soyasaponins (22). Exposure of HCT-15 cells to B-group soyasaponins led to a time- and dose-dependent increase in intracellular MDC incorporation (Figure 1). Specific activity rose above controls by 3 h post-treatment, and reached significance by 6 h post-treatment, at which time it was 26 and 79% higher, for cells treated with doses of 25 and 100 p.p.m., respectively, compared to control cells. These results indicate that autophagic capacity was significantly increased in B-group soyasaponin-treated cells by 6 h post-treatment. Therefore we evaluated alterations in signaling pathways that preceded this time-point.

Reduced Akt activity is associated with B-group soyasaponin-induced autophagy

Inhibition of the Akt signaling pathway has previously been associated with induction of autophagy (35–38). Therefore, we measured Akt activity by western blot analysis of phosphorylated GSKα/β substrate following an *in vitro* kinase assay. Akt activity was significantly suppressed at both soyasaponin concentrations studied by 1 h post-treatment, an effect that was sustained through 6 h of treatment (Figure 2A). Maximal inhibition was observed at 6 h post-treatment, when activity was suppressed by 35 and 49% for doses of 25 and 100 p.p.m., respectively, compared to controls. This indicates that B-group soyasaponins are stimulating autophagy in part by inhibiting cell survival-associated Akt activity.

Although Akt activity is regulated by a variety of factors, a major route of regulation is through bisphosphorylation of thr^{308} and ser^{473} residues (55). Akt monophosphorylated at the thr^{308} residue may be only 10% as active as the bisphosphorylated protein (56). As total protein concentrations of the Akt enzyme were unaffected by B-group soyasaponin treatment, we measured the degree of phosphorylation at these regulatory sites on the Akt protein by western blot analysis. In the presence of B-group soyasaponins phosphorylation of
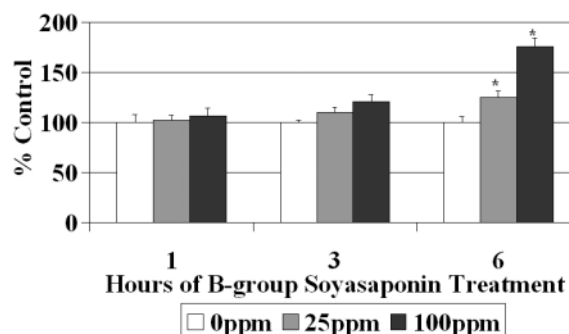

Fig. 1. MDC accumulates in HCT-15 cells in a dose- and time-dependent manner following B-group soyasaponin treatment. Intracellular MDC was measured using fluorescence photometry, normalized to total cell number, and expressed as specific activity with control values regarded as 100%. Results shown are means + SD and are representative of three independent experiments. Asterisk indicates *P*-value less than 0.05.
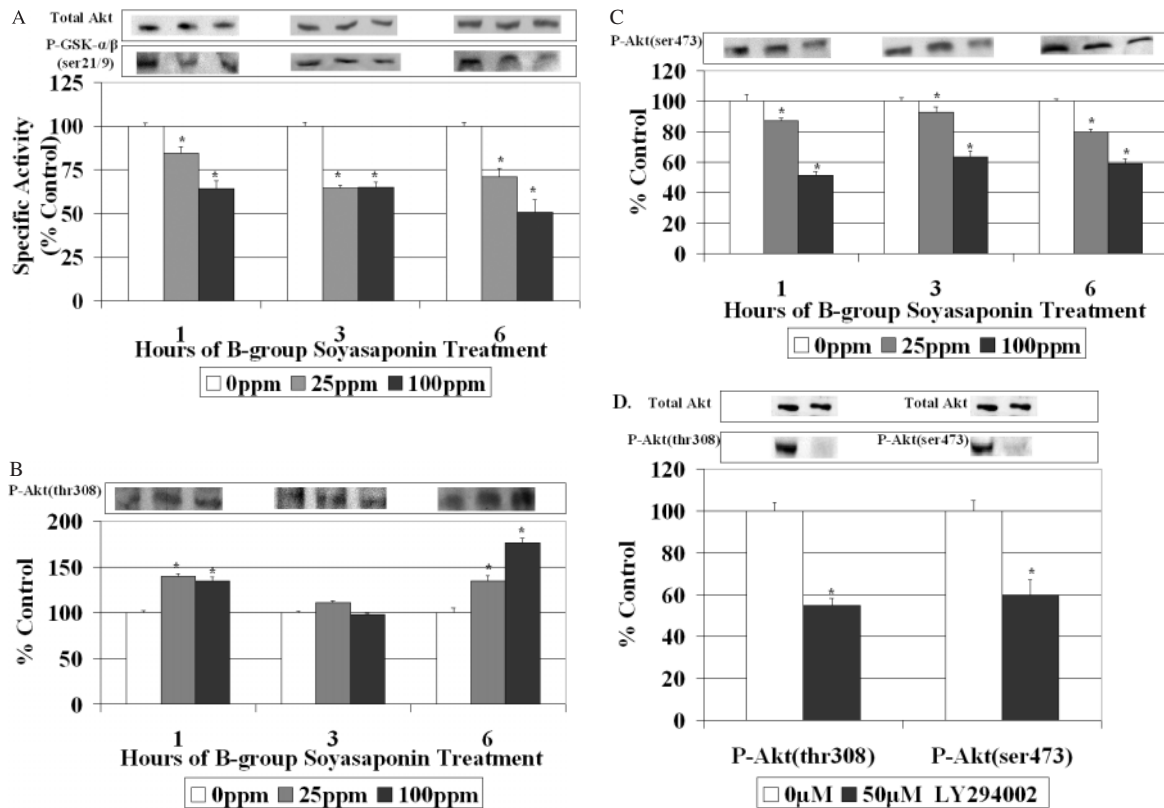
Fig. 2. Suppression of Akt activity is associated with B-group soyasaponin-induced autophagy in HCT-15 cells. Results shown are means + SD normalized to control values, and are representative of three independent experiments. Representative western blots are shown above graph. Asterisk indicates *P*-value less than 0.05. (**A**) Suppression of Akt activity is associated with B-group soyasaponin treatment in HCT-15 cells. Akt activity was measured with an *in vitro* kinase assay, using the GSKα/β substrate. Phosphorylated GSKα/β-ser[21/9] was detected by immunoblotting with phosphospecific GSKα/β-ser[21/9] antibodies. (**B**) The activating phosphorylation on threonine[308] residue is increased, as detected by immunoblotting with phosphospecific antibodies. (**C**) The activating phosphorylation on serine[473] residue is reduced, as detected by immunoblotting with phosphospecific antibodies. (**D**) Treatment with the PI3K inhibitor LY294002 (50 μM) for 1 h suppresses activating phosphorylations of both Akt-threonine[308] and Akt-serine[473], as detected by immunoblotting with phosphospecific antibodies.

thr[308] increased (Figure 2B). In contrast, phosphorylation of ser[473] was significantly suppressed at all time points examined, averaging a decline of 17% (25 p.p.m.) and 43% (100 p.p.m.) below control levels (Figure 2C).

It was confirmed that bisphosphorylation of Akt is dependent upon PI3K activity in HCT-15 cells (Figure 2D). Addition of the PI3K inhibitor LY294002 (50 μM) abolished phosphorylation of Akt at both thr[308] and ser[473].

mTor, a downstream target of Akt, does not play a central role in B-group soyasaponin-induced autophagy

The mTor kinase is a downstream target of Akt that has been suggested to modulate autophagic response (42). Akt contributes to the regulation of mTor activity through an inhibitory phosphorylation at thr[1462] on TSC-2, a GTPase activating protein directed toward Rheb, which is an activator of mTor (43,44). Hence it could be expected that a reduction in Akt activity would result in inhibition of mTor.

Despite an initial increase, phosphorylation of TSC-2-thr[1462] declined significantly over time, with a maximal decrease of 44% (25 p.p.m.) and 64% (100 p.p.m.) observed at 6 h post-treatment (Figure 3A). mTor activity also underwent a modest, yet significant, increase of 17% (25 p.p.m.) and 32% (100 p.p.m.), compared to controls, at 1 h post-treatment, although no significant alterations in mTor activity were observed at subsequent exposure times (Figure 3B). To ensure

the efficacy of the kinase activity assay, cells were treated with the mTor inhibitor rapamycin (100 nM), which significantly depressed mTor activity by 48% (Figure 3C). MDC incorporation was also measured in order to evaluate whether mTor inhibition could induce autophagy in HCT-15 cells (Figure 3D). Although rapamycin treatment resulted in a significant 40% increase in MDC accumulation compared to control, incorporation was almost doubled in B-group soyasaponin treated cells, as it increased by 78% at the 100 p.p.m. dose. In addition, when cells were treated with rapamycin prior to soyasaponin exposure, MDC incorporation was limited to that observed with rapamycin alone.

Increased ERK 1/2 activity is necessary for B-group soyasaponin-induced autophagy

Independent of Akt signaling, increased activity of ERK1/2 has been reported to be required for induction of autophagy in HT-29 colon cancer cells, potentially through controlling isolation membrane flux (51). Therefore, we measured ERK1/2 activity by western blot analysis of phosphorylated Elk-1 substrate following an *in vitro* kinase assay. ERK1/2 activity was significantly elevated at all time points evaluated (Figure 4A). In cells treated with 25 p.p.m. B-group soyasaponins, activity increased 49% by 1 h post-treatment, and maximal activity increased by 61% above that in control cells at 6 h. In cells treated with 100 p.p.m. B-group
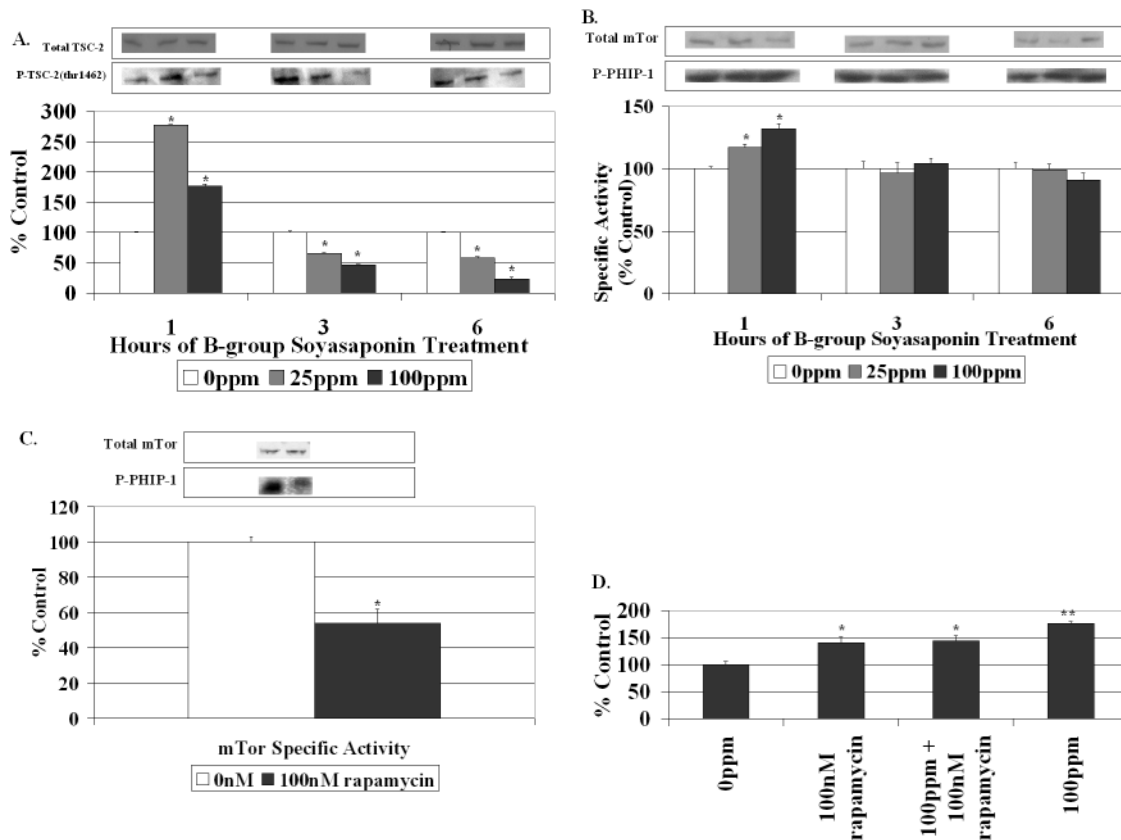
Fig. 3. Inhibition of mTor activity is not associated with B-group soyasaponin induced autophagy in HCT-15 cells. Results shown are means + SD normalized to control values, and are representative of two independent experiments. Representative western blots are shown above graph. Asterisk indicates P-value < 0.05; double asterisk indicates P-value < 0.01. (**A**) Inhibitory phosphorylation of TSC2-thr[1462], a negative regulator of mTor, is reduced following B-group soyasaponin treatment for 3 and 6 h, as detected by immunoblotting with phosphospecific antibodies. (**B**) mTor activity is initially increased by treatment with B-group soyasaponins. mTor activity was measured with an *in vitro* kinase assay, using the PHIP-1 substrate. Incorporation of radiolabeled phosphate was detected by autoradiography. (**C**) HCT-15 cells are sensitive to mTor inhibition. Cells were treated with 100 nM rapamycin for 1 h, and mTor activity was measured as described above. (**D**) Inhibition of mTor limits B-group soyasaponin-induced autophagy, as assessed by MDC incorporation.

soyasaponins, maximal activity was observed 1 h post-treatment, (66% above control levels), and activity was maintained at 37% above control at both 3 and 6 h post-treatment.

To further characterize soyasaponin-induced changes in signaling upstream of ERK1/2, we evaluated involvement of both MEK1/2 and raf-1 in the activation of ERK1/2 using phospho-specific antibodies, and obtained mixed results (Figure 4B and C). MEK1/2 can activate ERK1/2 by phosphorylating ERK1/2-thr[202/tyr204]. Although we observed significant increases in phosphorylation of this site at both 1 and 3 h post-treatment, reaching a maximal 100 and 75% above control levels at B-group soyasaponin concentrations of 25 and 100 p.p.m., respectively, phosphorylation returned to the control level by 6 h post-treatment. Similar results were observed when we evaluated the raf-1-mediated activating phosphorylations on MEK1/2-ser[217/221]. Phosphorylation was increased significantly following both 25 and 100 p.p.m. B-group soyasaponin treatment at 1 and 3 h post-treatment. Maximal phosphorylation occurred by 3 h post-treatment, a 50% increase at 25 p.p.m. and a 75% increase at 100 p.p.m. above control values, yet returned to the control levels by 6 h post-treatment. Taken together these data suggest that the elevations in ERK1/2 activity are in part due to increased signaling through the raf/MEK/ERK signaling cascade occurring during the initial 3 h following soyasaponin treatment.

In order to evaluate the necessity of enhanced ERK1/2 activity in B-group soyasaponin induced autophagy, we treated cells with the MEK1/2 inhibitor U0126 (0.01 mM) prior to exposure to either DMSO vehicle or soyasaponins (Figure 4D). Compared to controls, these conditions resulted in a 27 and 23% suppression in MDC incorporation, respectively, indicating that ERK1/2 activity is necessary to maintain basal levels of autophagic capacity in HCT-15 cells, and for induction of autophagy by B-group soyasaponins.

Cross-talk between Akt and ras/raf/MEK/ERK signaling pathways
The activity of the *ras*/raf-1/MEK/ERK signaling pathway may be constitutively increased as the HCT-15 cell line expresses oncogenic *ras* protein. However, the activity of raf-1 is also regulated by an Akt-mediated inhibitory phosphorylation at ser[259], and may represent a point of cross-talk between the two signaling pathways (57). Therefore, it could be expected that the suppression of Akt activity would result in enhanced raf-1 activity.

We evaluated activity of raf-1 by western blot analysis of phosphorylated MEK-1 substrate following an *in vitro* kinase assay (Figure 5A). In accordance with the enhanced phosphorylation of MEK1/2 reported above, we observed a significant increase in raf-1 activity at both 1 and 3 h post-treatment with B-group soyasaponins, although activity had declined to
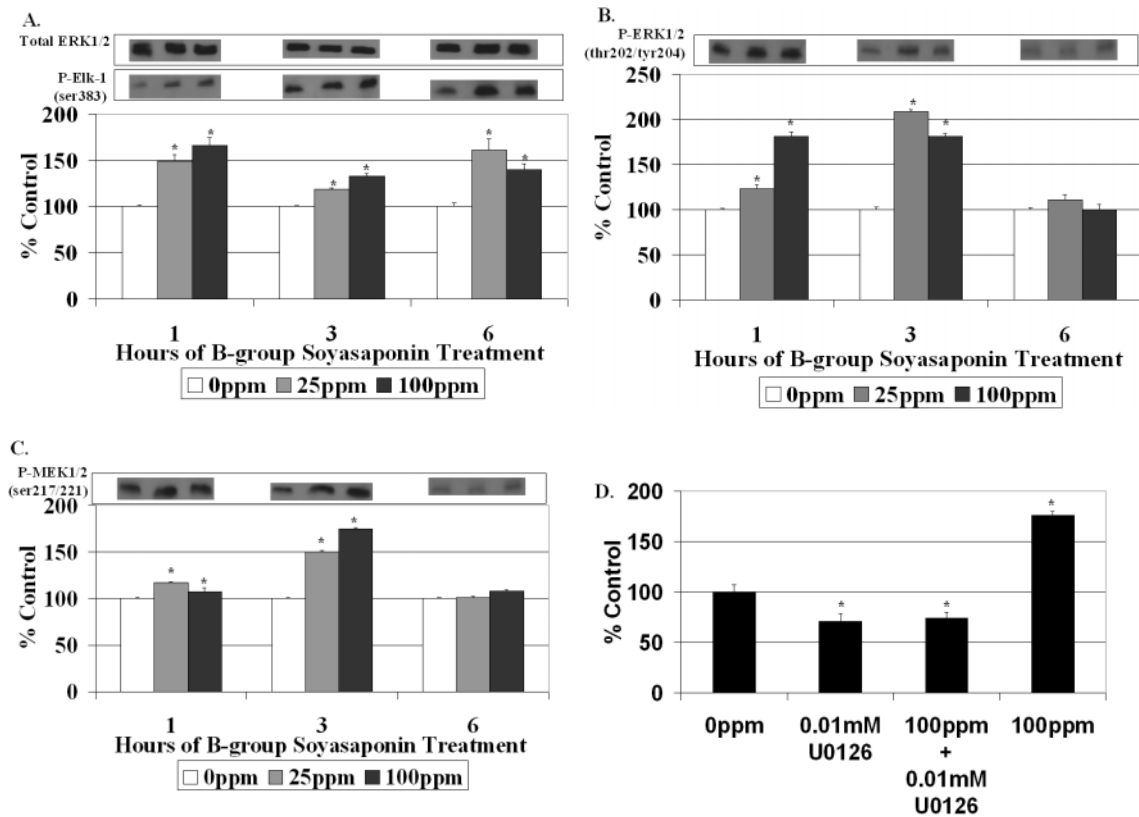
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Fig. 4. Enhanced ERK1/2 activity is necessary for B-group soyasaponin-induced autophagy in HCT-15 cells. Results shown are means + SD normalized to control values, and are representative of three independent experiments. Representative western blots are shown above graph. Asterisk indicates P-value <0.05. (**A**) ERK1/2 activity is increased following treatment with B-group soyasaponins. ERK1/2 activity was measured with an *in vitro* kinase assay, using the Elk-1 substrate. Phosphorylated Elk1-serine[383] was detected by immunoblotting with phosphospecific antibodies. (**B**) The activating phosphorylations on ERK1/2- thr[202]/tyr[204] are increased following B-group soyasaponin treatment for 1 and 3 h, as detected by immunoblotting with phosphospecific antibodies. (**C**) The activating phosphorylations on MEK1/2-ser[217/221] are increased following B-group soyasaponin treatment for 1 and 3 h, as detected by immunoblotting with phosphospecific antibodies. (**D**) Inhibition of ERK1/2 prevents B-group soyasaponin induced-autophagy, as assessed by MDC incorporation.
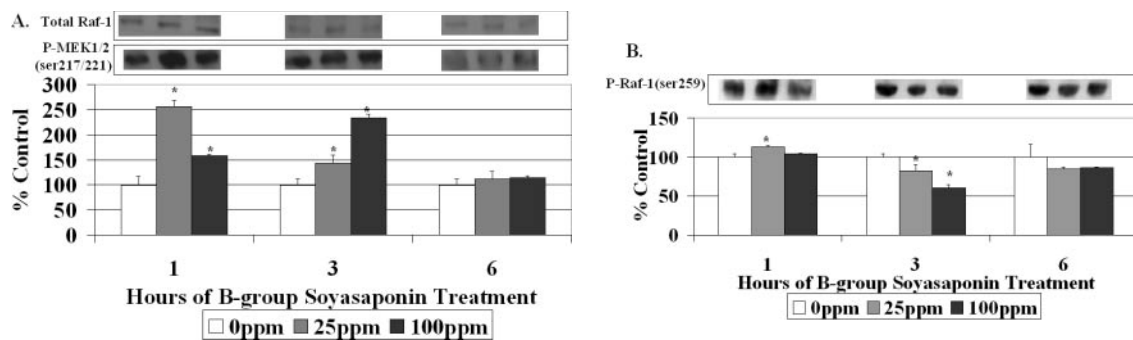

Fig. 5. Enhanced Raf-1 activity contributes to upregulation of the RAF/MEK/ERK signaling cascade. Results shown are means + SD normalized to control values, and are representative of three independent experiments. Representative western blots are shown above graph. Asterisk indicates P-value <0.05. (**A**) Raf-1 activity is significantly increased at 1 and 3 h following B-group soyasaponin treatment. Raf-1 activity was measured with an *in vitro* kinase assay, using the MEK substrate. Phosphorylated MEK-ser[217/221] was detected by immunoblotting with phosphospecific antibodies. (**B**) The inhibitory phosphorylation mediated by Akt on raf-1-ser[259] is decreased following B-group soyasaponin treatment for 3 and 6 h, as detected by immunoblotting with phosphospecific antibodies.

control levels by 6 h post-treatment. Thus, we evaluated the phosphorylation status of raf-1-ser[259], expecting to observe reduced phosphorylation of this residue. However, we observed an initial increase in the inhibitory phosphorylation at 1 h post-treatment (Figure 5B). At both 3 and 6 h post-treatment, a decline in phosphorylation of raf-1-ser[259] was observed. This inhibition of phosphorylation was sustained at 14% below control levels for cells treated at the 25 p.p.m. dose at both time points, while it declined to 39% (3 h) and then rebounded to 13% (6 h) below control levels following treatment with 100 p.p.m. B-group soyas-aponins. Enhanced activity of the raf-1 enzyme appears to explain, in part, increased signaling through the raf/MEK/ERK pathway.

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Discussion

The present studies demonstrate that B-group soyasaponin-induced autophagy is associated with suppression of Akt activity and enhancement of ERK1/2 signaling. This is the first report characterizing the involvement of these signaling pathways in B-group soyasaponin induced autophagy in HCT-15 cells.

Inhibition of Akt has been consistently associated with enhanced autophagic capacity in cancer cells (35–38). Reduced Akt activity is also associated with B-group soyasaponin-induced autophagy. The data presented here suggest that the suppression of Akt activity is, in part, due to reduced activating phosphorylation of ser[473], and is likely independent of PI3K. Neither the reason for the dissimilar changes in phosphorylation of ser[473] and thr[308] of Akt nor the time-dependent increases in thr[308] phosphorylation are known. Phosphorylation of Akt-thr[308] is dependent upon membrane recruitment of both Akt and PDK-1, events that occur following PIP_3 production by PI3K. Phosphorylation of the thr[308] residue was significantly increased following B-group soyasaponin treatment, suggesting that this permissive phosphorylation was not a rate-limiting factor in regard to Akt activation. In addition, this observation suggests ample generation of PIP_3, thereby precluding involvement of PI3K in the inhibition of Akt activity. In support of this proposal, it was confirmed that thr[308] phosphorylation is dependent upon PI3K activity in HCT-15 cells. Addition of the PI3K inhibitor LY294002 abolished phosphorylation of both thr[308] and ser[473], a result consistent with deficient PIP_3 production. The decreased ser[473] phosphorylation of Akt could be due to inhibition of the unidentified PDK-2 enzyme, or to accelerated dephosphorylation as has been observed, for example, following exposure of MCF-7 breast cancer cells to ceramide (36). Additional experiments are required to clarify the mechanisms through which B-group soyasaponins contribute to a reduction of Akt phosphorylation at ser[473].

Although mTor has been proposed as a downstream effector of Akt to modulate autophagic capacity under conditions of nutrient deprivation, mTor does not appear to play a central role in autophagic induction in HCT-15 cells. We observed a modest elevation in mTor activity following 1 h of treatment with B-group soyasaponins, in accordance with the increased inhibitory phosphorylation of TSC-2-thr[1462] at this time point. However, at later times a lack of change in mTor activity occurred despite the B-group soyasaponin associated decrease in TSC-2-thr[1462] phosphorylation. The reasons for this disconnect between mTor activity and the inactivating phosphorylation of TSC-2 are not known. mTor is subject to various regulatory controls, in addition to that imposed by TSC-2, as recently reviewed (58). A significant increase in ERK1/2 activity, which we observed in response to B-group soyasaponin exposure, may have altered stability of the TSC complex (59), leading to mTor activity remaining unchanged. This warrants further study, as TSC-2-mediated modulation of mTor activity may be an additional means through which ERK1/2 contributes to regulation of the autophagic process. Of interest, we observed that rapamycin was able to increase MDC incorporation in HCT-15 cells, despite its suppression of mTor activity. Taken together our findings suggest that in HCT-15 cells the mTor kinase does not play a central role in induction of autophagy, although it may control the magnitude of autophagic response. This finding is in agreement with the observations that mTor-independent pathways regulate autophagy in other mammalian cell models, including C2C12 myotubes (60) and in HL-60 myeloblastic cells (61). Specifically, in the latter study autophagic regulation was dependant upon suppression of Akt signaling, while inhibition of mTor alone did not alter autophagic response. However, our findings are in contrast to that of Kanazawa *et al.* (45), who demonstrated that changes in insulin/PI3K/Akt signaling following nutrient deprivation regulated autophagy through an mTor dependent pathway in rat hepatocytes. This disparity may be attributed to differences in cell lines and experimental protocols particularly relating to adequacy of amino acid supply.

Another signaling pathway by which B-group soyasaponins may be stimulating autophagy in HCT-15 colon cancer cells is by activation of ERK1/2. This is consistent with findings of Ogier-Denis *et al.* (47) who observed that induction of autophagy was dependent upon stimulation of ERK1/2 and subsequent activation of GAIP through phosphorylation of ser[151]. Changes in GAIP activity and other GTP associated proteins have been linked to membrane associated events that could control autophagy at the initial sequestration step (47,51). It is not known whether B-group soyasaponin enhancement of ERK1/2 activity regulates autophagy in the same manner. Regardless, activation of ERK1/2 appears to be necessary for induction of autophagy in HCT-15 cells, as inhibition of the enzyme by U0126 both suppressed basal levels of autophagic capacity and completely eliminated the increases in autophagic capacity normally observed following exposure to 100 p.p.m. B-group soyasaponins.

We hypothesized that raf-1 might represent a point of cross-talk between the Akt and *ras*/raf/MEK/ERK pathways, as has previously been described in HT-29 colon cancer cells (62). We did observe that raf-1 activity was significantly increased by exposure to both concentrations of B-group soyasaponins. Therefore, we determined whether the reduced Akt activity in response to B-group soyasaponins would be accompanied by a reduction in the inhibitory phosphorylation at raf-1-ser[259], which could, in part, contribute to an increase in raf-1 activity, and thus in ERK1/2 activity. However, phosphorylation of this residue was increased at 1 h post-saponin treatment, despite reduced Akt activity and increased raf-1 activity at this time. The reason for this discrepancy is unclear. However, it would appear that the enhanced raf-1 activity contributes to the increased ERK1/2 activity that we observed following B-group soyasaponin treatment. Cross-talk between Akt and raf-1 cannot be proven from our studies. Therefore additional experimentation is required to determine whether enhanced raf-1 activity is directly attributable to inhibition of the Akt enzyme, and whether this is a definitive point of cross-talk.

In summary, in the present studies we have demonstrated for the first time that B-group soyasaponins induce autophagy in HCT-15 colon cancer cells through modulating the activity of two signaling pathways that have previously been implicated in the control of the induction of autophagy. B-group soyasaponin treatment diminishes Akt activity through reduction of the activating ser[473] phosphorylation. Treatment of cells also enhances activity of ERK1/2, which is necessary for induction of autophagy by this legume constituent. These findings represent a potential mechanism through which a diet rich in legumes that contain B-group soyasaponins may confer colon chemoprotective effects and suggest that further characterization of these compounds as cancer preventive agents is warranted.

Acknowledgements

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