FULL PAPER *Physiology*

Indole-3-Carbinol Prevents H_2O_2-Induced Inhibition of Gap Junctional Intercellular Communication by Inactivation of PKB/Akt

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(Received 25 December 2007/Accepted 10 June 2008)

ABSTRACT. Indole-3-carbinol (I3C) is a phytochemical found in cruciferous vegetables and possesses a variety of biological and biochemical effects. Despite a wealth of data about the chemopreventive properties of I3C, its effects on gap junctional intercellular communication (GJIC), which is associated with the promotion and progression phases of the multi-stage process of carcinogenesis, has not been studied. In this study, we examined the ability of I3C to prevent H_2O_2-induced inhibition of GJIC in WB-F344 rat liver epithelial cells (WB cells). The cells were preincubated with I3C for 48 hr, and then treated with 1 mM H_2O_2 for 1 hr. We found that I3C could prevent the H_2O_2-induced inhibition of GJIC through prevention of the phosphorylated state of gap junction protein connexin 43 (Cx43) phosphorylation. Prevention of GJIC by I3C was dependent upon inactivation of Akt, but not MAPK, although inhibition of GJIC by H_2O_2 leads to activation of both. Similar to I3C, modulation of Akt activation through the phosphoinositide-3 kinase inhibitor, LY294002, could also prevent H_2O_2-induced inhibition of GJIC and phosphorylation of Cx43. Our results suggest that I3C might exert its dietary chemopreventive effects by interfering with the Akt signaling pathway, which appears to be linked to modulating GJIC, a cellular mechanisms regulating cell proliferation, differentiation and apoptosis.

KEY WORDS: gap junctional intercellular communication, indole-3-carbinol, oxidative stress, PKB/Akt.

J. Vet. Med. Sci. 70(10): 1057–1063, 2008

Gap junctions consist of 2 adjacent hemichannels comprised of hexamers of connexin proteins that have intracellular amino and carboxyl termini and four membrane spanning regions [18, 45]. Gap junction channels have been associated with homeostatic regulation of cell growth, differentiation and other physiological process via their ability to allow direct exchange of cytoplasmic components, such as ions, amino acids, short polypeptides, nucleotides, second messengers, high-energy phosphatates and other metabolites [18, 24, 36]. Gap junction channels are regulated in response to various stimuli, including endogenous growth factors, inflammatory mediators, cytokines, hormones, cell adhesion and extracellular matrices and exogenous agents, such as environmental toxicants, dietary agents, drugs and microbial toxins. Upon interaction with a cell, these agents trigger various intracellular signals, such as modulation of Ca^{2+}, pH, redox state, activation of transcription factors, and activation of kinases [40]. Gap junctional intercellular communication (GJIC) is generally reduced or inhibited in physiologically abnormal cells, such as cancer cells [27]. Chemical tumor promoters (phorbol esters, DDT, phenobarbital, saccharin) and various oncogenes (ras, raf, neu, src, mos) downregulate GJIC, reversibly or stably, respectively. This inhibition of GJIC is suspected to be involved in the mechanism of tumor promotion and progression [39]. The conformational changes of connexin 43 (Cx43), a major gap junction protein in astrocytes, cardiac myocytes and WB-F344 rat liver epithelial cells (WB cells), leading to GJIC inhibition are correlated with phosphorylation of the serine or tyrosine residue of its C-terminus [45].

A diet rich in fruit and vegetables provides phytochemicals, which are bioactive nonnutrient plant compounds. Recent dietary and epidemiological studies have shown that diet plays a crucial role in health promotion and disease prevention, and phytochemicals might be responsible for this disease prevention via their potent antioxidant activities [22, 37]. In addition, it has been proposed that prevention of the inhibition of GJIC by various tumor promoters or restoration of GJIC in cancer cells can be a strategy for cancer chemoprevention and chemotherapy [41]. Since Indole-3-carbinol (I3C), a presumptive cancer chemopreventive phytochemical found in cruciferous vegetables, such as cabbage, broccoli, cauliflower and brussel sprouts, possesses a variety of biological and biochemical effects, it has been hypothesized that it might work by preventing inhibition of GJIC [38], such as through consumption of green tea [33], resveratrol [30, 42], caffeic acid ethyl ester [28] and β-sitosterol [29]. It has also been reported that I3C might play an important role in prevention of human prostate [3], colon [8] and breast cancers [26].

Reactive oxygen species (ROS) are considered potentially toxic because of the oxidative damage they can cause to cellular components, such as lipids, nucleic acids and proteins. However, ROSs are not simply toxic byproducts of metabolism or used only in specialized microbicidal roles during phagocytosis, but they play crucial roles in many cellular processes, including various signal transduction networks [6, 32]. The principal mediator of ROS-dependent signaling is the two electron reduction product of oxygen

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and hydrogen peroxide (H_2O_2). Generation of ROSs in response to various external stimuli has been related to activation of transcription factors such as NF-κB [34] and AP-1 [23], mitogen-activated protein kinases (MAPK) [43] and protein kinase B (PKB)/Akt (Akt) [35]. In particular, it is well known that H_2O_2 regulates the phosphoinositide 3-kinase (PI3K) cellular signaling pathway [20] via inactivation of the phosphatase and tensin homologue deleted on chromosome 10 (PTEN) [21] and activation of Akt [35].

We recently demonstrated that activation of extracellular signal-related kinase (ERK) and p38 are involved in the H_2O_2-induced inhibition mechanism of GJIC [5], and sulforaphane could prevent downregulation of GJIC by inactivation of ERK and p38 [5, 14]. I3C and sulforaphane are constituents of cruciferous vegetables and have similar activities in relation to their antioxidant and anticancer properties. In this study, we therefore examined the ability of I3C to prevent H_2O_2-induced inhibition of GJIC in WB cells. There is little published data concerning MAPK regulation by I3C that indicates antioxidant or anticancer activities. Most of the studies that have shown antitumor activity for I3C have looked at the PI3K-Akt pathway [4, 12, 31]. We also investigated the possibility of a relationship between the Akt pathway and GJIC.

MATERIALS AND METHODS

Chemicals: Indole-3-carbinol, hydrogen peroxide (H_2O_2), Lucifer yellow CH and monoclonal β-actin antibodies were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Mouse anti-Cx43 monoclonal antibody was obtained from Chemicon Laboratory (Temecula, CA, U.S.A.). Anti-active MAPK pAb and anti-active p38 pAb were supplied by Promega (Madison, WI, U.S.A.). Anti-phospho JNK, anti JNK1 and p38 antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.). Akt and phosphor-Akt antibodies were purchased from Cell Signaling Technology, Inc. (Hitchin, United Kingdom). Rabbit anti-Map Kinase, horse radish peroxidase (HRP)-goat anti-mouse IgG conjugate and HRP-goat anti-rabbit conjugate antibody were all obtained from Zymed Laboratories (San Francisco, CA, U.S.A.). LY294002 and wortmannin were from Calbiochem.

Cell cultures and chemical treatment: WB-F344 rat liver epithelial cells (WB cells) were kindly provided by Dr. James E. Trosko at Michigan State University (U.S.A.). The cells were cultured in D-media (Formula No. 78–5470EB, Gibco BRL, Grand Island, NY, U.S.A.) supplemented with 5% fetal bovine serum (Gibco BRL) and a penicillin-streptomycin mixture (Gibco BRL). They were then incubated at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. The cells were grown in 75 mm tissue culture plates, and the culture medium was changed every other day. Passage 8–22 cells were used in all experiments. The cells were pretreated with I3C for 48 hr before being treated with 1mM H_2O_2 for 1 hr.

Cell viability assay: The cytotoxic effects of I3C on WB cells were measured by MTT assay based on the ability of live cells to convert tetrazolium salt into purple formazan. In brief, the cells were seeded in 24-well microplates and incubated overnight. The cells were then treated with different concentrations of I3C or its vehicle, dimethyl sulfoxide (DMSO; 0.1%), for 48 hr. At the end of these periods, 50 m*l* of MTT stock solution (5 mg/m*l*, Sigma) was added to each well, and the plates were further incubated for 4 hr at 37°C. The supernatant was removed, and 500 m*l* of DMSO was added to each well to solubilize the water insoluble purple formazan crystals; it was and then transferred into a 96-well microplate for reading. The absorbency at a wavelength of 570 nm was measured with an EL800 microplate reader (BIO-TEK Instruments, Winooski, VT, U.S.A.). All measurements were performed in triplicate. The results are expressed as the percentage of proliferation with respect to the vehicle control group.

Determination of GJIC: GJIC was determined by the scrape loading/dye transfer (SL/DT) technique as described previously [7]. The SL/DT assay is a manner in which a cell's ability to perform gap junctional intercellular communication can be determined. This assay was conducted at non-cytotoxic dose levels for the samples, as determined by the MTT assay. Cells were pretreated with I3C for 48 hr prior to the addition of H_2O_2 for 1 hr. Following incubation, the cells were gently scraped with a scalpel blade in the presence of Lucifer yellow. The dye enters cells through transient membranes disruptions created by the scalpel blade. If the cells contain functional gap junctions, the Lucifer yellow dye travels through the gap junctions away from the scrape line into neighboring cells. After the cells were fixed with 10% neutral formalin, the distance traveled by the dye in a direction perpendicular to the scrape was observed with an inverted fluorescence microscope (Olympus IX70, Okaya, Japan).

Western blot analysis: Cells were grown in a 100 mm tissue culture dish (Nunc, Rochester, NY, U.S.A.) to the same confluency as in the SL/DT assay. The cells were then treated with each test agent in the same manner as described in the SL/DT assay. Western blot analysis of Cx43 was performed as described previously [46]. Proteins were extracted with 20% SDS solution containing 1 mM phenylmethylsulfonyl fluoride, 10 mM iodoacetamide, 1 mM leupeptin, 1 mM antipain, 0.1 mM sodium orthovanadate and 5 mM sodium fluoride. The protein concentration was determined using a DC assay kit (Bio-Rad, Hercules, CA, U.S.A.) and separated on 12% SDS-PAGE. They were then transferred to nitrocellulose membranes at 100 V, 350 mA for 1 hr. All antibodies were used according to the manufacturer's instructions, and protein bands were detected using an ECL detection kit (Amersham, Piscataway, NJ, U.S.A.).

Statistical analysis: The data are represented as the mean ± SE (standard error) of three separate experiments. Statistical comparisons were performed using analysis of variance (ANOVA) and Dunnet's *t*-test. A P-value of less than 0.05 was considered significant.

RESULTS

Cell viability and I3C: To test the effects of I3C on cell viability, we treated WB cells with 0.1% DMSO as a control or with 50 to 250 μM I3C dissolved in DMSO (final concentration: 0.1%) for 48 hr. As shown in Fig. 1, I3C decreased the viability of WB cells in a dose-dependent fashion. Treatment with more than 200 μM of I3C significantly decreased cell viability. Although I3C may have potential for cytotoxicity at a high concentration, further experiments were performed with a concentration of I3C that had fewer cytotoxic effects on WB cells.

Effects of I3C on H_2O_2-induced inhibition of GJIC: The GJIC of WB cells was assessed using the SL/DT assay. WB cells were treated with 50, 100 or 150 μM I3C or its vehicle, 0.1% DMSO, for 48 hr prior to addition of 1 mM H_2O_2 for 1 hr. When we treated WB cells with only I3C, we did not find any change in GJIC compared with the control (Fig. 2A). After exposing the cells to H_2O_2, about 40% inhibition of GJIC was detected compared to control cells (Figs. 2A and 2B). However, the cells pretreated with I3C showed a dose-dependent reduction in inhibition of GJIC induced by H_2O_2. I3C alone did not enhance GJIC in the WB cells of the control group. It has been suggested that opening or closure of GJIC might be regulated by phosphorylation of connexin proteins, including Cx43, the prominent connexin in WB cells [16]. Western blot analysis was performed to detect the state of Cx43 phosphorylation after treatment with I3C and H_2O_2 (Fig. 2C). In the cells of the control group and I3C treatment groups not exposed to H_2O_2, three major isoforms of Cx43, which correspond to different phosphorylated forms of Cx43, were detected: an unphosphorylated form (P0) and two phosphorylated forms (P1 and P2) [25]. H_2O_2 treatment caused the P0 and P1 bands to disappear and induced an increase in the P2 band. However, the treatment of I3C with H_2O_2 prevented phosphorylation of the P0 and P1 bands in a dose-dependent fashion.

Mechanism of prevention of H_2O_2-induced GJIC inhibition by I3C: Previous investigations from our and other laboratories have suggested involvement of activated MAPK in phosphorylation of Cx43 and eventual inhibition of induction of GJIC by various agents [5, 10, 11, 15]. We therefore examined the activity of ERK1/2, p38 and JNK MAP kinases, using Western blot analysis to identify the protective mechanism of I3C in relation to H_2O_2-induced inhibition of GJIC. As previously described [28], we could observe that H_2O_2 activated ERK1/2, p38 and JNK MAP kinases (Fig.3A). Given that I3C could prevent inhibition of GJIC and prevent hyperphorsphorylation of Cx43, continuous activation of MAP kinases in the I3C treatment groups suggested an alternative mechanism is present that might control phosphorylation of Cx43 in WB cells.

Howells *et al.* showed that I3C inhibits Akt and induces apoptosis in human breast cancer cells [12]. Based on these findings, we examined whether Akt singling might contribute to I3C prevention of H_2O_2 inhibition of GJIC. As indicated by Western blot analysis, H_2O_2 could activate Akt.

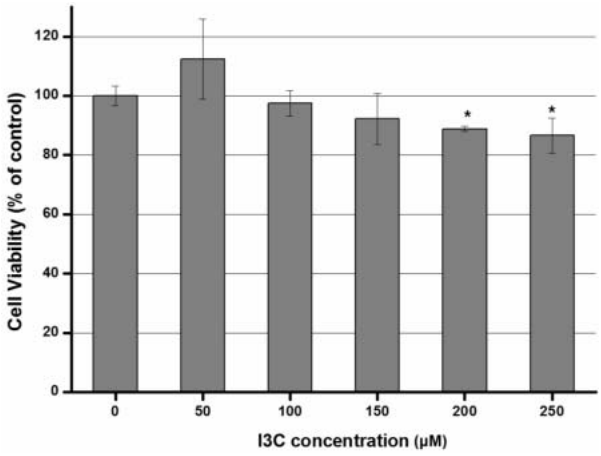

Fig. 1. Cell viability and I3C. Cells were treated with I3C for 48 hr. The cell viability rate was determined by MTT assay. Three independent experiments were performed in triplicate. Asterisks(*) indicate significant differences from the non-treated control group.

I3C treatment prior to H_2O_2 induced a dose-dependent decrease in activated Akt (Fig. 3B).

Effect of Akt on GJIC using inhibitors: To determine whether Akt was indeed involved in the preventive effect of I3C on H_2O_2-inhibited GJIC, we treated cells with two chemically-unrelated PI3K inhibitors, LY294002 and wortmannin. Akt is a downstream target of PI3K. LY294002 and wortmannin are known as PI3K inhibitors usually used to inhibit Akt activation [4, 31]. In the SL/DT assay, the results showed that LY294002 could also prevent inhibition of GJIC induced by H_2O_2, as was shown for I3C (Figs. 4A and 4B). Likewise, treatment of cells with wortmannin interfered with inhibition of GJIC and activation of Cx43 (data not shown). In the western blot analysis, we observed that PI3K inhibitors reduced the P2 band of Cx43 protein in a dose-dependent manner (Fig. 4C). There was no change in MAPK activation or phosphorylation by treatment with PI3K inhibitor (Fig.5A). Phosphorylation of Akt by H_2O_2 treatment could also be reduced by LY294002 (Fig. 5B). Therefore, these results suggested that the Akt pathway might be closely related to regulation of GJIC in WB cells as well as to the mechanism underlying the preventive effects of I3C in relation to H_2O_2-induced inhibition of GJIC.

DISCUSSION

In these studies designed to test the hypothesis that the illedged chemopreventive effects of I3C might occur via prevention of inhibition of GJIC by oxidative stress-related mechanisms during the tumor promotion phase of carcinogenesis, we showed for the first time that I3C can prevent inhibition of GJIC by H_2O_2-mediated oxidative stress. We presented evidence that prevention of GJIC by I3C is dependent upon inactivation of Akt, but not MAPK, although inhibition of GJIC by H_2O_2 was shown to implicate activa-

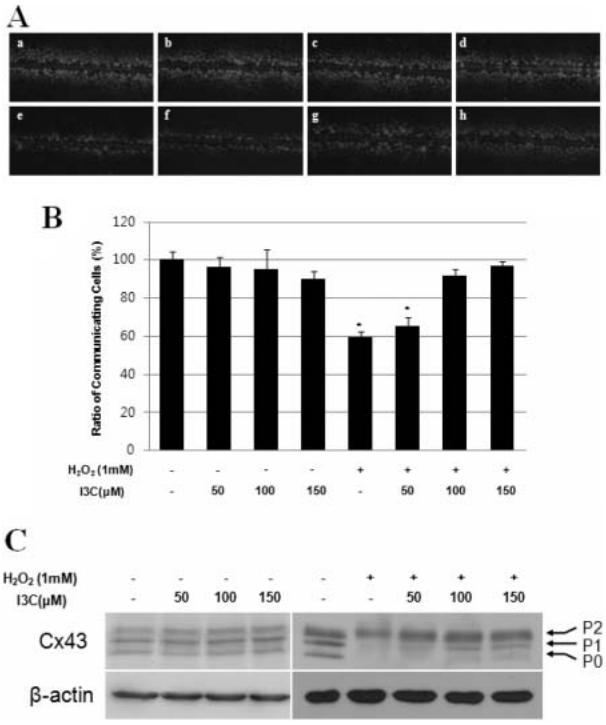

Fig. 2. Recovery effect of I3C on inhibition of GJIC by H_2O_2 as determined by the SL/DT assay and western blotting. (A) Cells were treated with (a, e) 0.1% DMSO, (b, f) 50 μM, (c, g) 100 μM or (d, h) 150 μM for 48 hr. The cells were then cultured without (a-d) or with (e-f) 1 mM H_2O_2 for 1 hr and scrape loaded. Each figure is representative of three independent experiments. (B) The recovery rate was counted under an inverted fluorescent microscope. Each value represents the average ± standard deviation determined from six measurements of scrape loads of two culture dishes. * Significantly different from the control ($P < 0.05$ by Dunnet's *t*-test). (C) The effect of I3C on the phosphorylation pattern of Cx43 was measured. Total cellular protein extracts were prepared and western blot analysis was performed with 20 μg protein using antibody specific for Cx43, indicating a connexin of a molecular weight close to 43 kDa. β-actin was the control for protein loading. This is a representative blot from three independent experiments.

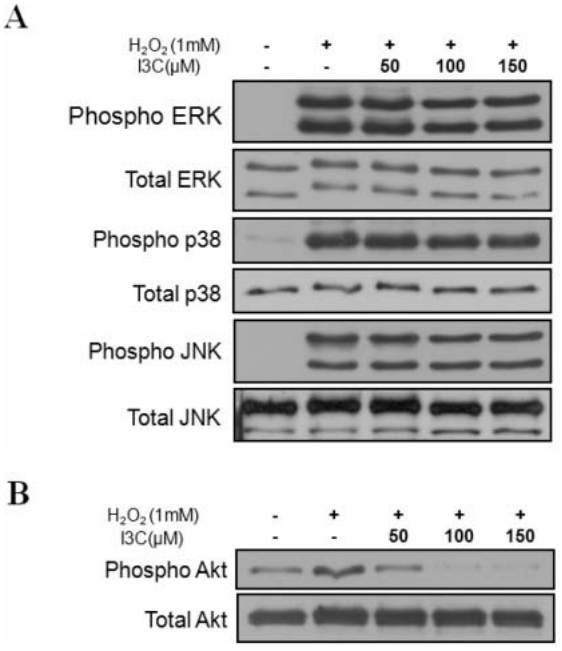

Fig. 3. Effect of I3C on H_2O_2-induced phosphorylation of MAPKs and Akt. WB cells were treated with 50–150 μM I3C for 48 hr prior to addition of 1 mM H_2O_2 for 1 hr. Western blots were developed with the antibodies indicated on the left. (A) Total cellular protein extracts were prepared and western blot analysis was performed with 20 μg protein using antibody specific for phospho-ERK or total ERK, phopho-p38 or total p38 and phospho-JNK or total JNK. (B) Proteins were detected with antibodies against phospho-Akt and total Akt. These are representative blots from three independent experiments.

tion of both signaling pathways. We also confirmed that activity of Akt might be indispensable for GJIC modulation in WB cells using two chemically-unrelated PI3K inhibitors, LY294002 and wortmannin.

Despite a wealth of data about the chemopreventive properties of I3C, their effects on GJIC, a cellular process related to the promotion/progression phases of carcinogenesis, have not been extensively studied. Herrmann *et al.* [9] observed the inhibition of GJIC in rat hepatocytes by indolo[3,2-*b*]carbazole (ICZ), which is an acid-catalyzed product of I3C. I3C is converted to numerous products with distinctive biological activities. ICZ, a condensation product of I3C formed in the acidic environment of the stomach, has both structural and functional similarity with a well-known tumor promoter, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) [17]. Several studies have revealed that I3C exerts distinct responses to ICZ *in vitro* and *in vivo* [1, 2]. Therefore, the beneficial activity of dietary I3C should not be attributed completely to the production of ICZ, although a partial response due to ICZ conversion might be feasible.

To investigate the effect of I3C on GJIC, we treated cells with a well-known tumor promoter, TPA, and a specific blocker of gap junction, 18β-glycyrrhetinic acid (GA), after incubation with I3C for 48 hr in a preliminary study. We were able to detect significant inhibition of GJIC by both TPA and GA in time- and dose-dependent manners. Interestingly, I3C could not prevent or restore the inhibition of GJIC induced by those chemicals. This indicates that H_2O_2, TPA and 18β-glycyrrhetinic acid inhibit GJIC via very different mechanisms.

We have previously reported that H_2O_2 inhibits GJIC in WB cells and that this is accompanied by phosphorylation of Cx43 through activation of ERK1/2 and p38 MAP kinases [5]. We have also shown that inactivation of ERK1/2 and p38 MAP kinases, using pharmaceutical inhibitors of each signal pathway or a chemopreventive agent, sul-

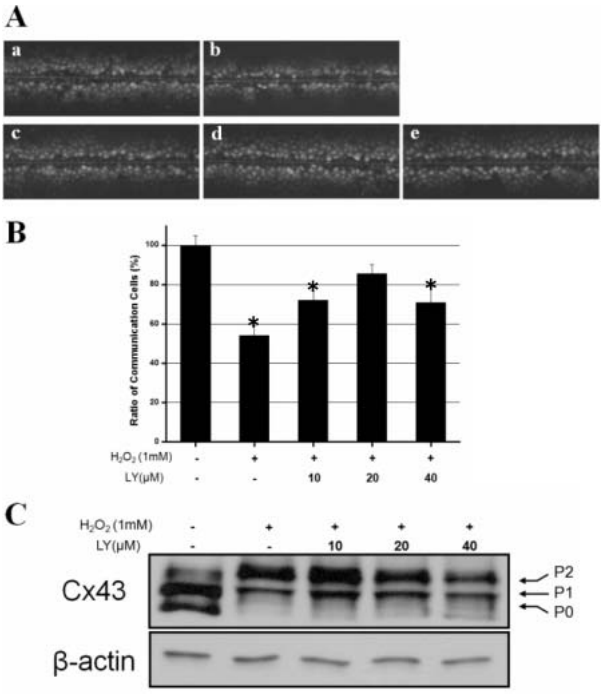

Fig. 4. Recovery effect of PI3K inhibitor LY294002 on inhibition of GJIC by H_2O_2 as determined by the SL/DT assay and western blotting. (A) The cells were treated without (a and b) or with (c) 10 μM, (d) 20 μM and (e) 40 μM for 6 hr. The cells were then cultured without (a) or with (b-e) 1 mM H_2O_2 for 1 hr and scrape loaded. Each figure is representative of three independent experiments. (B) The recovery rate was counted under an inverted fluorescent microscope. Each value represents the average ± standard deviation determined from six measurements of scrape loads of two culture dishes. * Significantly different from the control (P<0.05 by Dunnet's t-test). (C) The effect of I3C on the phosphorylation pattern of Cx43 was measured. Total cellular protein extracts were prepared and western blot analysis was performed with 20 μg protein using antibody specific for Cx43. β-actin was the control for protein loading. This is a representative blot from three independent experiments.

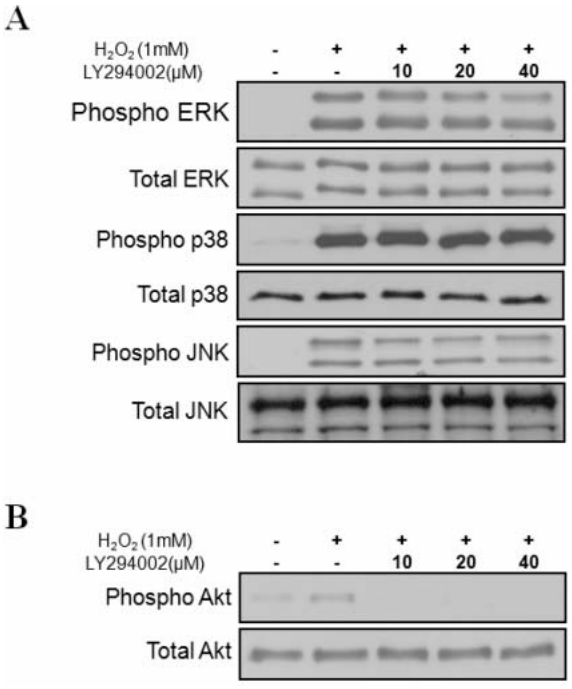

Fig. 5. Effect of the PI3K inhibitor LY294002 on H_2O_2-induced phosphorylation of MAPKs and Akt. WB cells were treated without or with 10–40 μM LY294002 for 6 hr prior to the addition of 1 mM H_2O_2 for 1 hr. Western blots were developed with the antibodies indicated on the left. (A) Total cellular protein extracts were prepared and western blot analysis was performed with 20 μg protein using antibody specific for phospho-ERK or total ERK, phopho-p38 or total p38 and phospho-JNK or total JNK. (B) Proteins were detected with antibodies against phospho-Akt and total Akt. These are representative blots from three independent experiments.

foraphane, could prevent H_2O_2-induced inhibition of GJIC in WB cells [5, 14]. In this study, we found that the inhibition of GJIC by 1 mM H_2O_2 also occurred through activation of ERK1/2, p38 and JNK MAP kinases. However, pretreatment of I3C could prevent inhibition of GJIC and phosphorylation of Cx43 without affecting the activity of MAP kinases (Figs. 2, 3A). These results suggested that at least one alternative pathway is present that mediates Cx43 phosphorylation and by which I3C might exert a protective effect on H_2O_2-induced GJIC inhibition of WB cells.

To investigate this alternative mechanism, we examined the phosphorylation level of Akt protein, a downstream signaling pathway of PI3K. It has been reported that acute treatment of cells with H_2O_2 induces phosphorylation of many intracellular proteins, including the PI3K/Akt and MAPK pathway [5, 35]. Here, we showed that H_2O_2 induced activation of Akt that was prevented by I3C (Fig. 3B). Therefore, we hypothesized that blockage of the Akt pathway might be one of the primary mechanisms of I3C in prevention of inhibition of GJIC. To confirm this hypothesis, we treated cells with two chemically-unrelated PI3K inhibitors, LY294002 and wortmannin, prior to H_2O_2 treatment. As shown in Fig. 4, LY294002 prevented H_2O_2-induced inhibition of GJIC and phosphorylation of Cx43. Likewise, treatment of cells with wortmannin interfered with inhibition of GJIC and activation of Cx43 (data not shown). In agreement with the results of I3C treatment, prevention of GJIC in LY294002-treated cells had no relationship with the phosphorylation level of MAPK. These results suggested that inhibition of Akt phosphorylation in WB cells by I3C could prevent H_2O_2-induced inhibition of GJIC. Because we could not clarify the mechanism of the relationship between the phosphorylation of Cx43 and phosphorylation of Akt, further studies are required to elucidate it.

One important question remains to be answered. How did I3C prevent H_2O_2-induced phosphorylation of Akt? A possible candidate for mediating the effects of I3C-induced

inhibition of Akt signaling under oxidative stress might be the epidermal growth factor receptor (EGFR), which is the prototypical member of the Erb-B family of receptor tyrosine kinases. The EGFR signal transduction pathways have been correlated with various processes that contribute to development of malignancies, such as effects on cell cycle progression, inhibition of apoptosis, angiogenesis, tumor cell motility and metastasis [19]. Huang *et al.* [13] reported that H_2O_2 could directly induce tyrosine phosphorylation of EGFR. Data presented by Wang *et al.* [44] indicates that H_2O_2 induces Akt phosphorylation through an EGFR-dependent PI3K/Akt pathway. It has also beem reported that Akt phosphorylation and its corresponding biological activity through EGFR signaling is mediated by PI3K and that these signaling pathways are downregulated by I3C [4]. According to these reports, it is evident that EGFR might be implicated in the response to I3C under oxidative stress.

The preventive effect of I3C on inhibition of GJIC by oxidative stress was apparent in the data presented here, thus suggesting that the chemopreventive effect of I3C might be beneficial for preservation of differentiated functions in the liver under oxidative stress.

ACKNOWLEDGEMENTS. This work was supported by a grant from the BK21 program for veterinary science. We appreciate the great contributions of Dr. James E. Trosko (National Food Safety Toxicology Center, Department of Pediatrics and Human Development, Michigan State University, East Lansing, MI 48824, U.S.A.), who helped to revise and fully discussed this manuscript.

REFERENCES

1. Anderton, M. J., Manson, M. M., Verschoyle, R. D., Gescher, A., Lamb, J. H., Farmer, P. B., Steward, W. P. and Williams, M. L. 2004. Pharmacokinetics and tissue disposition of indole-3-carbinol and its acid condensation products after oral administration to mice. *Clin. Cancer Res.* **10**: 5233–5241.
2. Chen, Y. H. and Yang, D. 2002. Differential effects of vegetable-derived indoles on the induction of quinone reductase in hepatoma cells. *J. Nutr. Sci. Vitaminol. (Tokyo)* **48**: 477–482.
3. Chinni, S. R., Li, Y., Upadhyay, S., Koppolu, P. K. and Sarkar, F. H. 2001. Indole-3-carbinol (I3C) induced cell growth inhibition, G1 cell cycle arrest and apoptosis in prostate cancer cells. *Oncogene* **20**: 2927–2936.
4. Chinni, S. R. and Sarkar, F. H. 2002. Akt inactivation is a key event in indole-3-carbinol-induced apoptosis in PC-3 cells. *Clin. Cancer Res.* **8**: 1228–1236.
5. Cho, J. H., Cho, S. D., Hu, H., Kim, S. H., Lee, S. K., Lee, Y. S. and Kang, K. S. 2002. The roles of ERK1/2 and p38 MAP kinases in the preventive mechanisms of mushroom Phellinus linteus against the inhibition of gap junctional intercellular communication by hydrogen peroxide. *Carcinogenesis* **23**: 1163–1169.
6. Droge, W. 2002. Free radicals in the physiological control of cell function. *Physiol. Rev.* **82**: 47–95.
7. el-Fouly, M. H., Trosko, J. E. and Chang, C. C. 1987. Scrape-loading and dye transfer. A rapid and simple technique to study gap junctional intercellular communication. *Exp. Cell. Res.* **168**: 422–430.
8. Frydoonfar, H. R., McGrath, D. R. and Spigelman, A. D. 2002. Inhibition of proliferation of a colon cancer cell line by indole-3-carbinol. *Colorectal. Dis.* **4**: 205–207.
9. Herrmann, S., Seidelin, M., Bisgaard, H. C. and Vang, O. 2002. Indolo[3,2-b]carbazole inhibits gap junctional intercellular communication in rat primary hepatocytes and acts as a potential tumor promoter. *Carcinogenesis* 23: 1861–1868.
10. Hill, C. S., Oh, S. Y., Schmidt, S. A., Clark, K. J. and Murray, A. W. 1994. Lysophosphatidic acid inhibits gap-junctional communication and stimulates phosphorylation of connexin-43 in WB cells: possible involvement of the mitogen-activated protein kinase cascade. *Biochem. J.* **303** (**Pt 2**): 475–479.
11. Hossain, M. Z., Ao, P. and Boynton, A. L. 1998. Platelet-derived growth factor-induced disruption of gap junctional communication and phosphorylation of connexin43 involves protein kinase C and mitogen-activated protein kinase. *J. Cell. Physiol.* 176: 332–341.
12. Howells, L. M., Gallacher-Horley, B., Houghton, C. E., Manson, M. M. and Hudson, E. A. 2002. Indole-3-carbinol inhibits protein kinase B/Akt and induces apoptosis in the human breast tumor cell line MDA MB468 but not in the nontumorigenic HBL100 line. *Mol. Cancer Ther.* **1**: 1161–1172.
13. Huang, R. P., Peng, A., Golard, A., Hossain, M. Z., Huang, R., Liu, Y. G. and Boynton, A. L. 2001. Hydrogen peroxide promotes transformation of rat liver non-neoplastic epithelial cells through activation of epidermal growth factor receptor. *Mol. Carcinog.* **30**: 209–217.
14. Hwang, J. W., Park, J. S., Jo, E. H., Kim, S. J., Yoon, B. S., Kim, S. H., Lee, Y. S. and Kang, K. S. 2005. Chinese cabbage extracts and sulforaphane can protect H_2O_2-induced inhibition of gap junctional intercellular communication through the inactivation of ERK1/2 and p38 MAP kinases. *J. Agric. Food. Chem.* **53**: 8205–8210.
15. Kanemitsu, M. Y. and Lau, A. F. 1993. Epidermal growth factor stimulates the disruption of gap junctional communication and connexin43 phosphorylation independent of 12-0-tetradecanoylphorbol 13-acetate-sensitive protein kinase C: the possible involvement of mitogen-activated protein kinase. *Mol. Biol. Cell* **4**: 837–848.
16. Klotz, L. O., Patak, P., Ale-Agha, N., Buchczyk, D. P., Abdelmohsen, K., Gerber, P. A., von Montfort, C. and Sies, H. 2002. 2-Methyl-1,4-naphthoquinone, vitamin K(3), decreases gap-junctional intercellular communication via activation of the epidermal growth factor receptor/extracellular signal-regulated kinase cascade. *Cancer Res.* **62**: 4922–4928.
17. Kociba, R. J., Keyes, D. G., Beyer, J. E., Carreon, R. M., Wade, C. E., Dittenber, D. A., Kalnins, R. P., Frauson, L. E., Park, C. N., Barnard, S. D., Hummel, R. A. and Humiston, C. G. 1978. Results of a two-year chronic toxicity and oncogenicity study of 2,3,7,8-tetrachlorodibenzo-p-dioxin in rats. *Toxicol. Appl. Pharmacol.* **46**: 279–303
18. Kumar, N. M. and Gilula, N. B. 1996. The gap junction communication channel. *Cell* **84**: 381–388.
19. Leithe, E. and Rivedal, E. 2004. Epidermal growth factor regulates ubiquitination, internalization and proteasome-dependent degradation of connexin43. *J. Cell Sci.* **117**: 1211–1220.
20. Leslie, N. R. 2006. The redox regulation of PI 3-kinase-dependent signaling. *Antioxid. Redox Signal.* **8**: 1765–1774
21. Leslie, N. R., Bennett, D., Lindsay, Y. E., Stewart, H., Gray, A. and Downes, C. P. 2003. Redox regulation of PI 3-kinase signalling via inactivation of PTEN. *Embo J.* **22**: 5501–5510
22. Liu, R. H. 2003. Health benefits of fruit and vegetables are

from additive and synergistic combinations of phytochemicals. *Am. J. Clin. Nutr.* **78**: 517S–520S.
23. Lo, Y. Y. and Cruz, T. F. 1995. Involvement of reactive oxygen species in cytokine and growth factor induction of c-fos expression in chondrocytes. *J. Biol. Chem.* **270**: 11727–11730.
24. Loewenstein, W. R. 1981. Junctional intercellular communication: the cell-to-cell membrane channel. *Physiol. Rev.* **61**: 829–913
25. Matesic, D. F., Rupp, H. L., Bonney, W. J., Ruch, R. J. and Trosko, J. E. 1994. Changes in gap-junction permeability, phosphorylation, and number mediated by phorbol ester and non-phorbol-ester tumor promoters in rat liver epithelial cells. *Mol. Carcinog.* **10**: 226–236.
26. Meng, Q., Goldberg, I. D., Rosen, E. M. and Fan, S. 2000. Inhibitory effects of Indole-3-carbinol on invasion and migration in human breast cancer cells. *Breast Cancer Res. Treat.* **63**: 147–152.
27. Mesnil, M., Crespin, S., Avanzo, J. L. and Zaidan-Dagli, M. L. 2005. Defective gap junctional intercellular communication in the carcinogenic process. *Biochim. Biophys. Acta* **1719**: 125–145.
28. Na, H. K., Wilson, M. R., Kang, K. S., Chang, C. C., Grunberger, D. and Trosko, J. E. 2000. Restoration of gap junctional intercellular communication by caffeic acid phenethyl ester (CAPE) in a ras-transformed rat liver epithelial cell line. *Cancer Lett.* 157: 31–38.
29. Nakamura, Y., Yoshikawa, N., Hiroki, I., Sato, K., Ohtsuki, K., Chang, C. C., Upham, B. L. and Trosko, J. E. 2005. Beta-sitosterol from psyllium seed husk (Plantago ovata Forsk) restores gap junctional intercellular communication in Ha-ras transfected rat liver cells. *Nutr. Cancer* **51**: 218–225.
30. Nielsen, M., Ruch, R. J. and Vang, O. 2000. Resveratrol reverses tumor-promoter-induced inhibition of gap-junctional intercellular communication. *Biochem. Biophys. Res. Commun.* **275**: 804–809.
31. Rahman, K. M., Li, Y. and Sarkar, F. H. 2004. Inactivation of akt and NF-kappaB play important roles during indole-3-carbinol-induced apoptosis in breast cancer cells. *Nutr. Cancer* **48**: 84–94.
32. Rhee, S. G., Kang, S. W., Jeong, W., Chang, T. S., Yang, K. S. and Woo, H. A. 2005. Intracellular messenger function of hydrogen peroxide and its regulation by peroxiredoxins. *Curr. Opin. Cell Biol.* **17**: 183–189.
33. Sai, K., Kanno, J., Hasegawa, R., Trosko, J. E. and Inoue, T. 2000. Prevention of the down-regulation of gap junctional intercellular communication by green tea in the liver of mice fed pentachlorophenol. *Carcinogenesis* **21**: 1671–1676.
34. Schreck, R., Rieber, P. and Baeuerle, P. A. 1991. Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-kappa B transcription factor and HIV-1. *Embo. J.* **10**: 2247–2258.
35. Shaw, M., Cohen, P. and Alessi, D. R. 1998. The activation of protein kinase B by H_2O_2 or heat shock is mediated by phosphoinositide 3-kinase and not by mitogen-activated protein kinase-activated protein kinase-2. *Biochem. J.* **336** (**Pt 1**): 241–246.
36. Simon, A. M. and Goodenough, D. A. 1998. Diverse functions of vertebrate gap junctions. *Trends Cell Biol.* **8**: 477–483.
37. Steinmetz, K. A. and Potter, J. D. 1996. Vegetables, fruit, and cancer prevention: a review. *J. Am. Diet Assoc.* **96**: 1027–1039.
38. Trosko, J. E. 2006. Dietary modulation of the multistage, multimechanisms of human carcinogenesis: effects on initiated stem cells and cell-cell communication. *Nutr. Cancer* **54**: 102–110.
39. Trosko, J. E. and Chang, C. C. 2001. Mechanism of up-regulated gap junctional intercellular communication during chemoprevention and chemotherapy of cancer. *Mutat. Res.* **480–481**: 219–229.
40. Trosko, J. E., Chang, C. C., Upham, B. and Wilson, M. 1998. Epigenetic toxicology as toxicant-induced changes in intracellular signalling leading to altered gap junctional intercellular communication. *Toxicol. Lett.* **102–103**: 71–78.
41. Trosko, J. E. and Ruch, R. J. 2002. Gap junctions as targets for cancer chemoprevention and chemotherapy. *Curr. Drug Targets* **3**: 465–482.
42. Upham, B. L., Guzvic, M., Scott, J., Carbone, J. M., Blaha, L., Coe, C., Li, L. L., Rummel, A. M. and Trosko, J. E. 2007. Inhibition of gap junctional intercellular communication and activation of mitogen-activated protein kinase by tumor-promoting organic peroxides and protection by resveratrol. *Nutr. Cancer* **57**: 38–47.
43. Wang, X., Martindale, J. L., Liu, Y. and Holbrook, N. J. 1998. The cellular response to oxidative stress: influences of mitogen-activated protein kinase signalling pathways on cell survival. *Biochem. J.* **333** (**Pt 2**): 291–300.
44. Wang, X., McCullough, K. D., Franke, T. F. and Holbrook, N. J. 2000. Epidermal growth factor receptor-dependent Akt activation by oxidative stress enhances cell survival. *J. Biol. Chem.* **275**: 14624–14631.
45. Warn-Cramer, B. J., Cottrell, G. T., Burt, J. M. and Lau, A. F. 1998. Regulation of connexin-43 gap junctional intercellular communication by mitogen-activated protein kinase. *J. Biol. Chem.* **273**: 9188–9196.
46. Yang, S. R., Cho, S. D., Ahn, N. S., Jung, J. W., Park, J. S., Jo, E. H., Hwang, J. W., Jung, J. Y., Kim, T. Y., Yoon, B. S., Lee, B. H., Kang, K. S. and Lee, Y. S. 2005. Role of gap junctional intercellular communication (GJIC) through p38 and ERK1/2 pathway in the differentiation of rat neuronal stem cells. *J. Vet. Med. Sci.* **67**: 291–294.