Indole-3-Carbinol Prevents H₂O₂-Induced Inhibition of Gap Junctional Intercellular Communication by Inactivation of PKB/Akt

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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 H₂O₂-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 H₂O₂ for 1 hr. We found that I3C could prevent the H₂O₂-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 H₂O₂ leads to activation of both. Similar to I3C, modulation of Akt activation through the phosphoinositide-3 kinase inhibitor, LY294002, could also prevent H₂O₂-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 mechanism regulating cell proliferation, differentiation and apoptosis.

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

FULL PAPER

In this study, we examined the ability of I3C to prevent H₂O₂-induced inhibition of GJIC in 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...
and hydrogen peroxide (H$_2$O$_2$). Generation of ROSs in response to various external stimuli has been related to activation of transcription factors such as NF-kB [34] and AP-1 [23], mitogen-activated protein kinases (MAPK) [43] and protein kinase B (PKB/Akt) [35]. In particular, it is well known that H$_2$O$_2$ 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$_2$O$_2$-induced inhibition mechanism of GJIC in WB cells. There is little published data concerning MAPK regulations by I3C that indicates antioxidant or anticancer activities. In this study, we therefore examined the ability of I3C to prevent H$_2$O$_2$-induced inhibition of GJIC in WB cells. We also investigated the possibility of a relationship between the Akt pathway and GJIC.

MATERIALS AND METHODS

Chemicals: Indole-3-carbinol, hydrogen peroxide (H$_2$O$_2$), 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 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 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 Chemicon Laboratory (Temecula, 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. Troso 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). The cells 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$_2$O$_2$ 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 ml of MTT stock solution (5 mg/ml, Sigma) was added to each well, and the plates were further incubated for 4 hr at 37°C. The supernatant was removed, and 500 ml of DMSO was added to each well to solubilize the water insoluble purple formazan crystals; it was 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$_2$O$_2$ 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 confluence 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 H2O2-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 H2O2 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 H2O2, 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 H2O2. 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 H2O2 (Fig. 2C). In the cells of the control group and I3C treatment groups not exposed to H2O2, 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]. H2O2 treatment caused the P0 and P1 bands to disappear and induced an increase in the P2 band. However, the treatment of I3C with H2O2 prevented phosphorylation of the P0 and P1 bands in a dose-dependent fashion.

Mechanism of prevention of H2O2-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 H2O2-induced inhibition of GJIC. As previously described [28], we could observe that H2O2 activated ERK1/2, p38 and JNK MAP kinases (Fig. 3A). Given that I3C could prevent inhibition of GJIC and prevent hyperphosphorylation 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 H2O2 inhibition of GJIC. As indicated by Western blot analysis, H2O2 could activate Akt. I3C treatment prior to H2O2 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 H2O2-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 H2O2, 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 H2O2 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 H2O2-induced inhibition of GJIC.

DISCUSSION

In these studies designed to test the hypothesis that the illaged 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 H2O2-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 H2O2 was shown to implicate activa-
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 H2O2, TPA and 18β-glycyrrhetinic acid inhibit GJIC via very different mechanisms.

We have previously reported that H2O2 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-
CHEMOPREVENTION BY INDOLE-3-CARBINOL

Foraphane, could prevent H2O2-induced inhibition of GJIC in WB cells [5, 14]. In this study, we found that the inhibition of GJIC by 1 mM H2O2 also occurred through activation of ERK1/2, p38 and JNK MAP kinases. However, pretreatment with 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 H2O2-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 H2O2 induces phosphorylation of many intracellular proteins, including the PI3K/Akt and MAPK pathway [5, 35]. Here, we showed that H2O2-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 H2O2 treatment. As shown in Fig. 4, LY294002 prevented H2O2-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 H2O2-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 H2O2-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$_2$O$_2$ could directly induce tyrosine phosphorylation of EGFR. Data presented by Wang et al. [44] indicates that H$_2$O$_2$ induces Akt phosphorylation through an EGFR-dependent PI3K/Akt pathway. It has also been 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.

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