Benzyl isothiocyanate inhibits excessive superoxide generation in inflammatory leukocytes: implication for prevention against inflammation-related carcinogenesis

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Inhibitors of excessive superoxide (O2\(^{--}\)) generation have been indicated to be more effective antioxidants than radical scavengers because O2\(^{--}\) anion is one of the precursors of several types of reactive oxygen species (ROS). We demonstrated here that benzyl isothiocyanate (BITC) is a potent inhibitor of leukocytic NADPH oxidase generating a great amount of O2\(^{--}\) in oxidative burst. The exposure of BITC to the differentiated HL-60 cells resulted in the inhibition of 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced O2\(^{--}\) generation, while the methylthiocarbamate analog of BITC, hardly reactive with thiols including glutathione and protein sulphydryl, did not show any effect. Pre-treatment of the cells with diethyl maleate significantly potentiated the BITC-induced inhibition, while pre-treatment with N-acetyl-cysteine counteracted it. These results led us to assume that a plausible intracellular target molecule(s) having a reactive sulphydryl moiety might be regulated by the covalent attachment with BITC. In spite of no ability to affect the translocation of protein kinase C \(\beta\) to the membrane, BITC probably modifies the electron transport system of cytochrome b558, consistent with the observation that BITC inhibited the substrate utilization. Pre-treatments of mouse skin with BITC significantly attenuated the TPA-enhanced hydrogen peroxide level, suggesting that BITC indeed acts as an inhibitor of O2\(^{--}\) generation in mouse skin. A histological study also demonstrated that BITC inhibited TPA-induced leukocyte infiltration in the dermis. Because we have found several O2\(^{--}\) generation inhibitors to be effective chemopreventors against mouse skin carcinogenesis, the modifying effect of the topical application of BITC on TPA-induced mouse skin tumor promotion was investigated. We demonstrated for the first time that the pre-treatment with BITC 40 min prior to each TPA treatment significantly inhibited the number of papillomas per mouse. In conclusion, the results from this study provided biological evidence that BITC has a potential to prevent inflammation-related carcinogenesis including skin cancer.

Abbreviations: BITC, benzyl ITC; BITC-OMe, O-methyl benzylthiocarbamate; DEM, diethyl maleate; DMBA, dimethylbenz[a]anthracene; DMSO, dimethylsulfoxide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSH, glutathione; GST, glutathione S-transferase; H2O2, hydrogen peroxide; ITCs, isothiocyanates; MPO, myeloperoxidase; NAC, N-acetyl-cysteine; O2\(^{--}\), superoxide; PKC, protein kinase C; ROS, reactive oxygen species; TPA, 12-O-tetradecanoylphorbol-13-acetate.

These authors contributed equally to this work.

Introduction

A number of studies support that certain dietary chemicals isolated from food items protect against cancer. An important group of compounds that have this property are organosulfur compounds such as isothiocyanates (ITCs). ITCs are agents that occur as glucosinolates in a variety of cruciferous vegetables. Naturally occurring ITC compounds are effective chemoprotective agents against chemical carcinogenesis in experimental animals (1–5). The ITC compounds inhibit rat lung, esophagus, mammary gland, liver, small intestine, colon and bladder tumorigenoses (5,6). Epidemiological studies also indicate a significant correlation between the dietary intake of ITC-containing foods and the reduced risk for prostate cancer (7–9). Inhibition of phase I enzymes that are required for the bioactivation of carcinogens and enhancement of the carcinogen excretion or detoxification by the phase II enzyme, including glutathione S-transferase (GST) and NAD(P)H:quinone-acceptor oxidoreductase, are believed to play a crucial role in the chemopreventive activity of the ITC compounds against chemical-induced carcinogenesis. In our continuing studies, we have screened fruits for sources of GST inducers and described the isolation and identification of benzyl ITC (BITC) (Figure 1) as a potent major inducer from papaya (10). The involvement of the redox regulation in the gene expression of the GSTP1 isozyme induced by BITC was also suggested (11). Recent studies have demonstrated that several ITC compounds inhibit cell growth by inducing apoptosis, which is suggested to be potentially involved in the anticarcinogenic action of ITCs. In our recent study, we also demonstrated that one of the possibilities involved in the activation of a caspase-3 (-like) protease by BITC is a mitochondrial death pathway (12).

Fig. 1. Chemical structure of the ITC compounds.
Acute and chronic inflammatory status has been implicated as a mediator of a number of pathological disorders including epithelial tumorigenesis in the lung, the bowel, the bladder, the colon and the skin. Although the mechanisms by which inflammatory cells show their carcinogenic effects remain unclear, some potential pathways have been proposed (13). Inflammatory cells produce a highly complicated mixture of growth and differentiation cytokines as well as biologically active arachidonic acid metabolites. In addition, they possess the capacity to generate and release a spectrum of reactive oxygen species (ROS) and free radicals during an oxidative burst. Among the inflammatory cells, polymorphonuclear leukocytes are particularly adept at generating and releasing ROS (14–16). Current evidence also indicates that these activated inflammatory cells, generating ROS coupled to the excessive production of chemotactic factors, may be important in skin tumor promotion (17). ROS production by double or multiple 12-O-tetradecanoylphorbol-13-acetate (TPA) treatment is closely associated with the metabolic activation of proxi-

molecules (14–15). Some potential pathways have been proposed (13). Inflammatory cells produce a highly complicated mixture of growth and differentiation cytokines as well as biologically active arachidonic acid metabolites. In addition, they possess the capacity to generate and release a spectrum of reactive oxygen species (ROS) and free radicals during an oxidative burst. Among the inflammatory cells, polymorphonuclear leukocytes are particularly adept at generating and releasing ROS (14–16). Current evidence also indicates that these activated inflammatory cells, generating ROS coupled to the excessive production of chemotactic factors, may be important in skin tumor promotion (17). ROS production by double or multiple 12-O-tetradecanoylphorbol-13-acetate (TPA) treatment is closely associated with the metabolic activation of proximate carcinogens (18,19) and the increased levels of oxidized DNA bases (20–22). We have demonstrated recently that the potent inhibitors of leukocyte-derived superoxide (O2−) generation (17) effectively suppressed inflammation-related carcinogenesis (23–25). However, the ability of the ITC-related compounds to modify ROS production in inflammatory processes has not been elucidated fully.

In the present study, we investigated the antioxidant effect of BITC on tumor promoter-induced O2− generation in cultured cells and mouse skin. We found that the electrophilic reactivity might be essential to inhibit leukocytic NADPH oxidase activity. We demonstrated that BITC acts as a ROS generation inhibitor in mouse skin. We indicated for the first time that BITC has a potential to prevent against the inflammation-related carcinogenesis.

Materials and methods

Chemicals and animals

TPA was obtained from Research Biochemicals International (Natick, MA). BITC, allyl ITC (AITC) and Triton X-100® were obtained from Nacalai Tesque, Kyoto, Japan. Phenethyl ITC (PEITC) and cytochrome c were purchased from Sigma Chemicals (St Louis, MO). O-Methyl benzylthiocarbamate (BITC-OMe) was prepared by a previously reported method (26). The anti-body against the protein kinase C-β-subfamily (β-PKC) was purchased from BD Transduction Laboratories, Lexington, KY. Horseradish peroxidase-linked anti-rabbit and anti-mouse IgG immunoglobulins and enhanced chemiluminescence (ECL) western blotting detection reagents were obtained from Amersham Pharmacia Biotech, Buckinghamshire, UK. The protein concentration was measured using the BCA protein assay reagent from Pierce Biotechnolog, Rockford, IL. All other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan). Female ICR mice (7 weeks old) were obtained from Japan SLC, Shizuoka, Japan. The mice used in each experiment were supplied with fresh tap water ad libitum and rodent pellets (MF, Oriental Yeast Co., Kyoto, Japan) freshly changed twice a week. The animals were maintained in accordance with the Guidelines for Animal Experimentation of Nagoya University. The back of each mouse was shaved with surgical clippers 2 days before each experiment.

O2− generation test in differentiated HL-60 cells

The inhibitory tests of TPA-induced O2− generation in dimethylsulfoxide (DMSO)-differentiated HL-60 cells were done as reported previously (17). Briefly, to determine the inhibitory effect of the O2− generation, test compounds dissolved in 5 μl of DMSO were added to a DMSO-induced differentiated HL-60 cell suspension (1 × 105/ml) and incubated at 37 °C for 15 min. TPA (100 nM) and cytochrome c solution (1 μg/ml) with or without O2− dismutase (SOD; 150 U/ml) were added to the reaction mixture, which was incubated for another 15 min. At the end of the incubation period, the cell suspension was transferred to an ice bath. After centrifugation at 250 g, the visible absorption at 550 nm was measured. The inhibitory effect was expressed by the relative decreasing ratio of absorbance of a test compound to the control experiment.

Titration of GAPDH sulfhydryls with DTNB

The reactivity of the sulfhydryl groups in glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was determined by titration with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) by the modified method of Riddles et al. (27). A 1 mg/ml GAPDH sample was treated with 0.1–5 mM BITC or BITC-OMe, and after dialysis against PBS, the protein was denatured with 8 M guanidine hydrochloride containing 13 mM EDTA and 133 mM Tris, pH 8.8, and reacted with DTNB (1 mM) at room temperature for 5 min. The reaction was monitored as an increase in absorbance at 412 nm. The concentration of the sulfhydryl groups was calculated using a standard curve with N-acyetyl-cysteine (NAC).

Reactions of GAPDH with BITC

The GAPDH solution (2 mg/ml) was prepared by dilution of the enzyme suspension with 50 mM sodium phosphate buffer (pH 7.4). An aliquot (0.5 ml) of the enzyme solution was mixed with an equal volume of a BITC or BITC-OMe solution (0.1–5 mM) and incubated at 37 °C. The reaction was stopped by the addition of 100 μl of 10 mM NAC. For determination of the GAPDH activity, a 10 μl aliquot of the reaction mixture was assayed in 3 ml of 15 mM sodium pyrophosphate and 30 mM sodium arsenate buffer (pH 8.5). The reaction of GAPDH was initiated by the addition of 100 μl of 7.3 mM NAD, 100 μl of 0.1 mM dithiothreitol, and 100 μl of 15 mM D,L-glyceraldehyde 3-phosphate. The mixture was incubated at room temperature for 5 min, and the absorbance at 340 nm was measured.

GSH titration

The quantification of glutathione (GSH) was fluorometrically performed according to the method of Hissin and Hilf (28). Cells were lysed and extracted with 5% metaphosphoric acid solution containing 5 mM EDTA. After centrifugation (10 000 g, 20 min), a 1.8 ml aliquot of 0.1 M phosphate solution (pH 8.0) containing 5 mM EDTA and 100 μl of the o-phthalaldehyde solution (1 mg/ml) were then added to the supernatant (100 μl). The fluorescence intensity at 420 nm was next determined with activation at 350 nm.

PKC translocation

The observed reactions contained the cells treated with or without BITC (10 μM) for 15 min and then stimulated with TPA (100 nM) for 5 min, diluted to a final volume of 1.0 ml in Hank’s Balanced Salt Solution (HBSS, pH 7.4). At the end of the incubation period, the cell suspension was transferred to an ice bath. The suspension was then centrifuged for 10 min at 300 g, and the cell pellet (5 × 10⁶ cells) was sonicated on ice (1 × 10 s) in relaxation buffer A (20 mM Tris–HCl, 2 mM DTT, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml aprotinin, 10 μg/ml leupeptin), and centrifuged at 4°C for 1 h at 100 000 g. The supernatant was used as a cytosolic fraction. The pellet was solubilized in 1% Triton X-100 in relaxation buffer A, and then centrifuged at 4°C for 1 h at 10 000 g to obtain the supernatant (membrane fraction). The cytosolic and membrane fractions were run on 10% SDS-PAGE and probed with anti-phospho-Akt antibody.

NADH consumption

The NADH consumption in the TPA-activated differentiated HL-60 cells was followed spectrophotometrically at 340 nm. The reaction mixtures that contained the cells (1 × 10⁶/ml) were treated with BITC (10 μM) for 15 min and then stimulated with TPA (100 nM) for 10 min, and diluted to a final volume of 1.0 ml in HBSS (pH 7.4). NADH (0.5 mM) was added just before the measurements were started and followed for at least 30 min. The experiments were run at 37°C. The rate of NADH consumption was calculated using the molar extinction coefficient of 6.22 mM-1 cm-1.

Treatment of mice

Inhibitory tests of the TPA-induced hydrogen peroxide (H2O2) generation in double TPA-treated mice were done as reported previously (17, 29). This TPA dose (8.1 nmol) was used for the potentiation of oxidative responses compared with the dose for tumor promotion (1.6 nmol). One group was composed of five female ICR mice housed five in a cage. In the inhibition assay, test compounds (810 nmol/0.1 ml in acetone) were topically applied to the shaved area of the dorsal skin at 30 min only before the second application of a TPA solution. For H2O2 determination, TPA was applied 24 h after the first treatment. Although the timing (24 h apart) of the double TPA application was different from the tumor promotion protocol (3–4 days apart), the level of oxidative stress was nearly the same when the time between the two TPA treatments was 24–72 h (data not shown).

Determination of H2O2 level

Mice treated by the double-treatment protocol were killed 1 h after the second TPA treatment. The H2O2 content was determined by the phenol...
red-horseradish peroxidase method (17,29). The final results are expressed as equivalents of nmol of H$_2$O$_2$ per skin punch on the basis of a standard curve of horseradish peroxidase-mediated oxidation of phenol red by H$_2$O$_2$.

**MPO activity determination**

The infiltrated leukocytes were evaluated by myeloperoxidase (MPO) activity enhancement as reported previously (17). Mice were killed by cervical dislocation 24, 48 or 72 h after a single application of TPA or acetone. In the inhibition assay, BITC or BITC-OMe (81 or 810 nmol/0.1 ml in acetone) was topically applied to the shaved area of the dorsal skin at 24 h after TPA application. Mouse skin punches were obtained with an 8-mm-diameter cork borer and weighed on an analytical balance. The MPO activity was determined by a previously reported method (29). The MPO activity was calculated from the linear portion of the curve and expressed as units of MPO per skin punch. One unit of MPO activity was defined as the activity that degraded 1 nmol of H$_2$O$_2$ per min at 25°C.

**Histological examination**

Mice treated by the double-treatment protocol were killed 1 h after the second TPA treatment. Excised skin was fixed in 10% buffered formalin, and then embedded in paraffin. Skin samples were cut in 3-mm sections, mounted on silanized slides, dehydrated in xylene, dehydrated through an ethanol series and stained with hematoxylin and eosin. For each section of skin, the thickness of the epidermis from the basal layer to the stratum corneum was measured at five equidistant interfollicular sites utilizing an image analysis system with an OLYMPUS BX51 microscope (Olympus Optical Co., Tokyo, Japan). The number of infiltrating leukocytes was counted at five different areas in each section using this image analysis system.

**Tumor promotion experiment**

The modifying effect of BITC on the TPA-induced tumor promotion was examined by a standard initiation-promotion protocol with dimethylbenz[a]-anthracene (DMBA) and TPA as reported previously (30). One group was composed of 15 female ICR mice housed 5/cage. The back of each mouse was shaved with a surgical clipper 2 days before initiation. The mice at 7 weeks old were initiated with DMBA (95 nmol/0.1 ml acetone). One week after initiation, the mice were promoted with TPA (1.6 nmol/0.1 ml acetone) twice a week for 20 weeks. In two other groups, the mice were treated with BITC (16 and 160 nmol/0.1 ml acetone) 40 min before each TPA treatment. The modifying effect on the TPA-induced tumor promotion was evaluated by both the ratio of tumor-bearing mice and the number of tumors, >1 mm in diameter per mouse. The data were statistically analyzed using the Student’s t-test (two sided) that assumed unequal variance for the average number of tumors per mouse and by the $\chi^2$-test for the incidence of skin tumors.

**Statistics**

Specific differences among treatments were examined using the Student’s $t$-test (two sided) that assumed unequal variance.

**Results**

**BITC is a potent inhibitor of $O_2^\cdot$ generation in the differentiated HL-60 cells**

We examined the inhibitory effect of the ITC-related compounds against TPA-induced $O_2^\cdot$ generation because $O_2^\cdot$ is regarded as one of the initiators for various ROS formations. The $O_2^\cdot$ generation was detected by a cytochrome c reduction method. As shown in Figure 2A, BITC significantly inhibited $O_2^\cdot$ generation by 80% at a concentration of 25 $\mu$M. This significant inhibition of $O_2^\cdot$ generation was also observed in the cells treated with each ITC. Arylalkyl ITCs such as BITC and PEITC showed the most potent reducing ability of $O_2^\cdot$ generation. The ITC group is essential to express inhibitory activity as the corresponding derivatives of BITC, an O-methyl thiocarbamate analog (C$_6$H$_5$-CH$_2$-NH-C(-S)-OCH$_3$; BITC-OMe), showed no ability (Figure 2B). The inhibitory effect of BITC was in a dose-dependent manner and its IC$_{50}$ value was estimated at ~10 $\mu$M, comparable with that of curcumin, a well known naturally occurring food antioxidant (31).

**Electrophilic properties of BITC**

It is suggested that the reactive ITC (–N=–C=S) moiety plays a major role in the $O_2^\cdot$ inhibition of BITC because BITC has been reported to react with GSH (32). Hence, we incubated BITC and BITC-OMe with GAPDH, a model protein having active thiol groups, and further evaluated their electrophilicity by measuring the residual protein thiols. GAPDH is a tetrameric enzyme consisting of four identical catalytically active subunits. Each subunit has four cysteine residues, of which Cys-149 and Cys-153 have been suggested to be sensitive to oxidizing agents and thus important for enzyme activity (33). As shown in Figure 3A, upon incubation of GAPDH (2 mg/ml) with 5 mM BITC in 50 mM sodium phosphate buffer (pH 7.4) at 37°C for 15 min, followed by washing with PBS twice. The cells were treated as described in ‘Materials and methods’. Data were the means of three independent experiments. Statistical significance was determined by the Student’s $t$-test and is expressed as: a, versus TPA, $P < 0.005$; b, versus TPA, $P < 0.05$.

Electrophilic properties of BITC

**Fig. 2. Inhibition of NADPH oxidase-derived $O_2^\cdot$ generation in the differentiated HL-60 cells. (A) Effect of the BITC-related derivatives (20 $\mu$M) on TPA-induced $O_2^\cdot$ generation. (B) Dose-dependent effect of BITC (closed circles) and BITC-OMe (open circles). A test compound solution was added to the cell suspension, and the mixture was incubated at 37°C for 15 min, followed by washing with PBS twice. The cells were treated as described in ‘Materials and methods’. Data were the means of three independent experiments. Statistical significance was determined by the Student’s $t$-test and is expressed as: a, versus TPA, $P < 0.005$; b, versus TPA, $P < 0.05$.**
enzyme activity inhibition of BITC toward sulphydryl groups, we speculated that BITC might react with Cys-149 and/or Cys-153 less frequently than the others even though the concentration of GAPDH is different in each assay for enzyme activity (2 mg/ml) and sulfhydryl reactivity (1 mg/ml). A further study of the difference in BITC reactivity with each sulfhydryl group in GAPDH should be necessary. In any case, the data taken together indicated that BITC possesses an electrophilic character targeting protein cysteine residue as well as low molecular thiols.

**Intracellular GSH plays a negative regulating role in the inhibitory effect of BITC**

To gain further evidence for the involvement of the electrophilic property in $O_2^{-}$ inhibition, we examined the effects of the pre-treatment of the cells with NAC or diethyl maleate (DEM) prior to the BITC treatment. As shown in Figure 4A, the pre-treatment of NAC (10 mM) for 24 h dramatically enhanced the intracellular GSH level by ~5-fold. On the contrary, DEM (1 mM) pre-treatment for 1 h significantly decreased the GSH level by 60%. Although neither treatment of DEM nor 1 μM BITC inhibited the $O_2^{-}$ generation, the combination of these treatments resulted in the synergistic effect on the $O_2^{-}$ attenuation (Figure 4B). DEM pre-treatment also significantly enhanced the 20 μM BITC-induced inhibition. Conversely, pre-treatment of NAC before the 20 μM BITC stimulation significantly increased the $O_2^{-}$ amount. The data suggested that intracellular GSH might act as a counter-acting agent against the BITC reactivity but not as a scavenger for $O_2^{-}$.

**BITC depressed substrate utilization without affecting the $\beta$-PKC activation**

The NADPH oxidase complex includes membrane-bound cytochrome $b_558$ (gp91phox and p22phox) and cytosolic proteins (p47phox, p67phox, Rac1/2 and p40phox) (34) that translocate to the membrane during stimulation to form a catalytically active oxidase (35). During NADPH oxidase activation, p47phox is phosphorylated on several serine residues (36). PKC is involved in NADPH oxidase activation and can phosphorylate p47phox (37,38). The conventional PKCs $\alpha$-PKC and $\beta$-PKC are present in leukocytes and are activated by phorbol esters such as TPA (39). In the differentiated HL-60 cells, a role for $\beta$-PKC, but not for other PKC isozymes, in the activation of the NADPH oxidase has been demonstrated (40). To explore

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Fig. 3. BITC, but not BITC-OMe, acted as an electrophile. (A) Dose-dependent loss of free sulfhydryl group in GAPDH by BITC and BITC-OMe. Titration of GAPDH sulphydryls with DTNB was done by the modified method of Riddles et al. (26). (B) Dose-dependent inhibition of GAPDH activity. An aliquot (0.5 ml) of the GAPDH solution (2 mg/ml) was mixed with an equal volume of a BITC or BITC-OMe solution (0.1–5 mM) and incubated at 37°C. The reaction was stopped by the addition of 100 μl of 10 mM NAC. Residual GAPDH activity was determined as described in ‘Materials and methods’ section. All data were the means of three independent experiments.

Fig. 4. Intracellular GSH level influenced the BITC-induced inhibition of $O_2^{-}$ generation. (A) Effect of DEM or NAC pre-treatment on intracellular GSH level. The cells were treated with DEM (1 mM) for 1 h or NAC (10 mM) for 24 h. The GSH amount of each sample was evaluated according to the method of Hissin and Hilf (28). (B) Effect of DEM and NAC on BITC-induced $O_2^{-}$ inhibition. The cells were pre-treated with DEM or NAC and treated with BITC (1 or 20 μM) or DMSO for 15 min. After washing out of BITC followed by TPA stimulation, the residual $O_2^{-}$ amount was determined. Data were the means of three independent experiments. Statistical significance was determined by the Student’s t-test and is expressed as: a, versus control, $P < 0.05$; b, versus the corresponding BITC group, $P < 0.05$. 

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the underlying mechanism of the BITC-induced inhibition of O$_2^-$ generation, we investigated the effect of BITC on the TPA-activated β-PKC translocation by western blot analysis. Figure 5A shows the massive translocation of β-PKC from cytosol to membrane, with only a trace of PKC remaining in the cytosolic fraction. BITC at the concentration required to inhibit O$_2^-$ generation did not modify this process [105.5 ± 9.5% of the control (n = 3) as determined by densitometry analysis]. In addition, BITC affected neither the p47$^{phox}$ translocation nor the assembly of the active NADPH oxidase complex consisting of cytochrome b$_{558}$ and cytosolic proteins determined by immunoprecipitation (data not shown). On the other hand, we observed that the consumption of exogenously added NADH in TPA-activated HL-60 cells was significantly inhibited by the BITC treatment (Figure 5B). These results led us to speculate that BITC might directly modify the electron transfer system of cytochrome b$_{558}$ by covalent cysteine modification in the substrate binding site and/or oxidase catalytic site.

**BITC inhibits H$_2$O$_2$ generation in mouse skin**

Double applications of phorbol esters trigger ROS production in mouse skin (17,29–31). The available data reported previously suggest that each application induces two distinguishable biochemical events, namely, priming and activation (19). The former is characterized as the recruitment of inflammatory cells such as neutrophils by chemotactic factors to inflammatory regions and edema formation. The latter is the process of activation of neutrophils, in which the second TPA application of phorbol esters induces oxidative stress. Importantly, the TPA dose used in the present experiment is in the tumor promotion range (1–10 nmol). BITC effectively inhibited O$_2^-$ generation in the differentiated HL-60 cells as mentioned above. Thus, to investigate whether or not BITC inhibits the activation event during TPA-induced H$_2$O$_2$ formation, derived from NADPH oxidase-originated O$_2^-$ in mouse skin, a double TPA application experiment was examined. Double applications of 8.1 nmol TPA at a 24-h interval increased the level of H$_2$O$_2$ by ~3-fold (3.72 ± 0.35 nmol/skin punch) to that in the control mice treated twice only with acetone instead of TPA (1.20 ± 0.14 nmol). Diphenylene iodonium, a specific NADPH oxidase inhibitor, expectedly inhibited this increase in the H$_2$O$_2$ level by ~90% (data not shown), suggesting that oxidative stress in this model is mainly dependent on the NADPH oxidase pathway. As shown in Figure 6, a dose-dependent decrease in the residual H$_2$O$_2$ level was observed in the mice pre-treated with BITC 1 h before treatment of the second (activation) dose of TPA. On the other hand, BITC-OMe, an inactive analog in an *in vitro* O$_2^-$ generation assay, did not show any effect on the residual H$_2$O$_2$ level in mouse skin. These results taken together indicated that BITC indeed acts as an inhibitor of NADPH oxidase-dependent ROS generation *in vivo*.

**BITC accelerates leukocyte disappearance in inflamed region**

We preliminarily observed that a decrease in the residual H$_2$O$_2$ level and MPO activity, a representative marker of neutrophil infiltration, was also observed in the mice pre-treated with BITC 1 h before treatment of the first (priming) dose of TPA with a small change in edema formation (data not shown). We assumed that BITC might accelerate the disappearance of leukocytes from the inflamed region. Therefore, we examined...
the effect of BITC on the accumulated leukocytes in the skin treated with TPA beforehand. As shown in Figure 7A, the exposure to TPA for 24 h caused an initial increase in both punch weight (data not shown) and MPO activity up to ~2-fold and 20-fold, respectively, to the control group. This increase reached a peak in 24–48 h after application, followed by a spontaneous decrease to the 70% level of the peak 72 h after treatment. As shown in Figure 7A and B, treatment with BITC 24 h after TPA treatment resulted in the significant decrease in MPO activity compared with the control group (TPA/acetone group, P < 0.05). The results that BITC treatment caused the obvious effects on dermal MPO activity led us to determine whether BITC application indeed attenuates leukocyte infiltration in the cutis. Histological examination demonstrated that mouse skin treated with TPA for 24 h resulted in a marked increase in leukocyte infiltration as compared with that treated with acetone (192 ± 12 versus 16 ± 2/mm²). On the other hand, treatment with BITC (810 nmol) for an additional 24 h significantly diminished the TPA-induced leukocyte infiltration by 35% (114 ± 28 versus 176 ± 17/mm², P < 0.05 versus TPA). In addition, treatment with BITC (810 nmol) 24 h after TPA application obviously attenuated the TPA-induced hyperplasia and epidermal thickening (data not shown).

Modifying effect of BITC pre-treatment on TPA-induced tumor promotion

Although the chemopreventive potentials of BITC have been investigated extensively in several rodent experiments, relatively little is known about its anticarcinogenic activity against skin carcinogenesis except for one negative report; Lin et al. reported that fairly high doses (5 μmol) of BITC prior to benzo[a]pyrene treatment failed to prevent the incidence of tumors (41). On the other hand, we have found several O₂⁻ generation inhibitors to be effective chemopreventors against mouse skin carcinogenesis (23,42). Therefore, the modifying effect of topically applied BITC at the doses required for inhibition of H₂O₂ generation on TPA-induced tumor promotion was examined in a two-stage mouse skin carcinogenesis model and the results are shown in Table I. Tumors began to develop 6 weeks after tumor promotion by TPA. The incidence of tumor-bearing mice and the average number of tumors per mouse in the group given DMBA and TPA were 92 and 9.4%, respectively, at the end (20 weeks) of the experiment. In the group treated with 160 nmol of BITC 40 min prior to each TPA treatment, the average number of tumors per mouse was attenuated by 56% (P < 0.05) and tumor incidence by 18%. Mice initiated with DMBA and then treated with 160 nmol BITC twice weekly for 20 weeks did not develop any tumors.

Discussion

Diverse types of cancer chemopreventive agents, including naturally occurring and synthetic pharmaceutical agents, have been studied for efficacy in vitro and in vivo. Among the most extensively investigated are the ITC-related compounds, which naturally occur in a variety of cruciferous vegetables, BITC is an ITC that has been isolated from papaya fruit as the major phase II enzyme inducer present in organic solvent extracts of this fruit (10). Our interest in the molecular mechanism of the anticarcinogenic effect of BITC stemmed from the following observations: (i) BITC blocks polycyclic aromatic hydrocarbon-initiated tumor formation in the lung but not in the skin (41); (ii) the cellular redox change correlated with ROS generation can be part of the signal transduction pathway during phase II enzyme induction (11) and apoptosis (12); and (iii) few intensive studies on the anti-inflammatory potential of the ITC-related compounds and its molecular mechanisms have been described, except for the data using the cultured mouse macrophages (43,44). The present report provides the first evidence that antioxidant mechanisms could contribute to a naturally occurring isothiocyanate-mediated chemoprevention against inflammation-related carcinogenesis. We demonstrated here that BITC had potent inhibitory activity against ROS generation not only in cultured cells (Figure 2) but also in mouse skin (Figure 6). Recently, we have reported that O₂⁻ from leukocytes plays an important role in the continuous and excessive production of chemotactic factors, leading to chronic inflammation and hyperplasia in mouse skin (17,42). As mentioned above, ROS production by double or multiple TPA treatments is closely associated with the metabolic activation of proximate carcinogens (18,19) and the increased levels of oxidized DNA bases (20–22). Consistent with these results, the antioxidant behavior of
BITC in leukocytes in vitro and in vivo is the topic of this report.

The experiments using an inactive analog of the sulphydryl modulation, BITC-OME, indicated that the inhibition of NADPH oxidase-dependent $O_2^-$ generation is dependent on the electrophilic property of BITC. In addition, we observed that pre-treatment of the cells with DEM, a potent GSH blocker, significantly potentiated BITC-induced inhibition, while pre-treatment with NAC, a substrate for GSH synthesis, counteracted it (Figure 4B); in other words, at conditions of low intracellular GSH, BITC would predominantly interact with a plausible target for the NADPH oxidase inhibition, whereas higher GSH concentrations would render it less accessible. Based on these observations, we speculated that protein sulphydryl-specific attachment of BITC might contribute to interference of the oxidase component assembly and/or the oxidase activity. The fact that an elevated GSH concentration weakened but did not totally abolish the inhibitory effect of BITC at a relatively high concentration (20 $\mu$M) favors this possibility. Therefore, we focused on a target molecule for the inhibition of NADPH oxidase. During NADPH oxidase activation, $p47^{phox}$ is phosphorylated on several serine residues by PKC (38,39). Especially, in the differentiated HL-60 cells, $\beta$-PKC is involved in the activation of the NADPH oxidase as well as $p47^{phox}$ phosphorylation (40). In this study, BITC depressed the TPA-induced oxidative burst including exogenous NADH consumption and $O_2^-$ generation without affecting the $\beta$-PKC activation (Figure 5A). In addition, we found that BITC neither inhibited $p47^{phox}$ translocation to the membrane nor impaired $p47^{phox}$ phosphorylation (unpublished data). This strongly suggests that the BITC inhibition of $O_2^-$ production is at least in part related to modification of the assembled oxidase but not a functional impairment of the NADPH-oxidase assembly. Further studies on the elucidation of the specific molecular target for the BITC-induced inhibition including the effect on the assembled oxidase activity of membrane preparations originating from TPA-activated monocytes or neutrophils should be required.

The NADPH oxidase system of neutrophils, rather than the epithelial xanthine oxidase (XOD) system, is implicated in the $O_2^-$ generating system in multiple TPA-treated mouse skin because an XOD inhibitor, allopurinol, showed no effect (17), and diphenylene iodonium, a NADPH oxidase inhibitor, significantly inhibited the increase in $H_2O_2$ level. Thus, it is certain that one of the major target molecules of BITC is leukocytic NADPH oxidase, which can be activated by TPA in mouse skin. In addition to the direct interference of NADPH oxidase, the present study indicated that the antioxidant activity of BITC in mouse skin is partly explained by acceleration of the leukocyte disappearance in the inflamed region (Figure 7). It is within the possibility that BITC induced apoptotic cell death in the infiltrated leukocytes on the basis of the observation that treatment of TPA-stimulated mouse skin with BITC for 24 h increased terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) positive cells in the cutis (unpublished data). Also, the ITC compounds have been shown recently to suppress the production of nitric oxide, prostaglandin $E_2$ and tumor necrosis factor-$\alpha$ in lipopolysaccharide-activated macrophages (43,44). The anti-inflammatory responses of the ITC compounds including reduction of leukocyte infiltration and inhibition of ROS generation could be attributed partly to these inhibitory effects. Although being unable to discriminate between these two speculations, the data presented in this paper demonstrated for the first time that the ITC compounds exhibit an anti-inflammatory action in mouse skin.

The ITC compounds have been shown to protect chemical carcinogenesis in experimental animals. In mice, BITC blocked the neoplastic effects of diethylnitrosamine or benzo[a]pyrene on lung and forestomach (2), and a variety of phenylalkyl ITCs inhibited the pulmonary carcinogenesis of the tobacco-derived nitrosamine (45). The anticarcinogenic effects of ITCs may be related to their capacity to induce phase II enzymes including GSTs, which are involved in the metabolism of carcinogens. In fact, BITC is a potential inducer of phase II enzymes (10,11). Therefore, BITC have been thought to mainly suppress carcinogenesis during the initiation phase. In mouse skin, however, fairly high doses (5 $\mu$mol) of BITC failed to prevent benzo[a]pyrene-induced initiation (41). We have found recently several $O_2^-$ generation inhibitors to be effective chemopreventors against mouse skin tumor promotion (17,42). Because the effect of the ITC compounds on mouse skin tumor promotion have not been described, we examined in the present study the effect of BITC at the doses required for $H_2O_2$ inhibition. In contrast to the previous result obtained from the anti-initiation experiment, BITC had a significant protective effect against tumor promotion by TPA in mouse skin (Table I). Therefore, the antioxidant mechanisms could contribute to the BITC-mediated protection against skin tumor promotion. Consistent with the result in the present study, Wattenberg reported that BITC has inhibitory capacity against rat breast neoplasia when given subsequent to DMBA exposure (1). Although the exact mechanism is not fully understood, it is within the possibility that the anti-inflammatory
potential of BITC may participate in prevention against other organ carcino genesis than the skin. It should be noted that higher doses of BITC (~1 μmol/mouse) decreased the chemopreventive activity in the DMBA/TPA-treated mice (unpublished data). The unfavorable effect of higher concentrations of BITC have also been observed in the study on apoptosis induction using the cultured cell system; higher concentrations of BITC produce a large amount of intracellular ROS, resulting in severe oxidative damage to caspase activity and/or other cellular components (12). These oxidative phenomena may lead to necrotic cell death and thus damage to the surrounding cells. Although being promising and effective candidates as anticarcinogens, some ITC compounds showed enhancement of carcinogenicity or lack of chemopreventive effects in rat urinary bladder especially dosed at the post-initiation phase (46), which are possible through necrotic cyto toxicity (47). Moreover, we also obtained the puzzling result that the treatment of the typical phenolic antioxidant unexpectedly enhanced oxidative stress and thus tumor promotion even when the application dose was increased (30). These results collectively suggested that BITC might be effective in a limited dose range. Therefore, further studies on the threshold of cancer preventive or promoting potential are necessary.

In conclusion, the results from this study provided biological evidence that BITC has a potential to prevent from inflammation-related carcinogenesis including skin cancer possibly through the attenuation of the responsiveness of activated leukocytes. Therefore, BITC can be explored as a cancer chemopreventive agent targeted towards the inflammation-related carcinogenesis with selective application doses.

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