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Cytotoxicity of Benzyl Isothiocyanate in Normal Renal Proximal Tubular Cells and Its Modulation by Glutathione

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ABSTRACT: In the present study, we examined the toxicity of benzyl ITC (BITC) and its urinary mercapturic acid metabolite (BITC-NAC), using a normal renal proximal tubular cell line, pig LLC-PK1. BITC increased cell death with an IC_{50} value of about 7 μM, whereas the cytotoxic effect of BITC-NAC was five times weaker than that of BITC. We observed a significant necrosis of the compounds on LLC-PK1 cells with oxidative stress. In the presence of 5 mM glutathione (GSH), comparable to physiological levels, the cytotoxicity of BITC-NAC as well as BITC was significantly reduced. Furthermore, the increase in intracellular GSH levels by pretreatment with NAC before the BITC treatment resulted in inhibition of the BITC-induced necrotic events as well as intracellular oxidative stress. These results suggest that GSH is a determinant of cellular resistance against the BITC-mediated and oxidative stress-dependent cytotoxicity in renal proximal tubular cells.

KEYWORDS: *benzyl isothiocyanate, necrosis, LLC-PK1 cell, ATP, reactive oxygen species*

■ INTRODUCTION

Isothiocyanates (ITCs) are compounds in a variety of cruciferous vegetables such as broccoli, watercress, and Japanese radish.[1] They are stored as glucosinolates (GSs) in plants and are released when the plant tissue is damaged or ground. The conversion from GSs into ITCs is catalyzed by myrosinase, a thioglucosidase that is physically separated from glucosinolates under normal conditions. GSs may also be hydrolyzed in the intestinal tract because the microflora possess a myrosinase-like activity.[2] Naturally occurring ITC compounds have been regarded as promising preventive agents against carcinogenesis in the lung, esophagus, mammary gland, liver, small intestine, colon, and bladder.[1,3] Epidemiological studies also indicate a significant correlation between the dietary intake of ITC-containing foods and the reduced risk of several cancers.[4] Various ITCs can inhibit both the formation and development of a cancer cell through multiple pathways, i.e., the inhibition of P450 monooxygenases, induction of drug metabolizing phase 2 enzymes, induction of apoptosis, and inhibition of the cell cycle progression.[5] Thiol conjugates of ITCs are formed by nonenzymatic and enzymatic conjugation with glutathione (GSH) as a major route of ITC metabolism.[5] In the kidney, stepwise enzymatic removal of glutamine and glycine from GSH yields L-cysteine-ITC conjugates, which are subsequently acetylated to yield *N*-acetyl-L-cysteine (NAC) conjugates of ITCs (mercapturic acids) excreted in the urine.

In recent years, there have been several reports on the toxicity of ITCs with cellular stress in several tissues.[6] With respect to in vitro effects, they include cell proliferation reduction and an increase in cell death in a variety of normal cell types as well as cancer cells.[7−10] The in vivo toxic effects of natural and artificial ITCs include urinary bladder carcinogenicity in rats,[11] tumor promotion effects in several tissues including the bladder,[12] cholestatic hepatocellular injury,[13] and maternal toxicity.[14] For instance, benzyl ITC (BITC) and phenethyl ITC (PEITC), both of which are derived from cruciferous vegetables, promote urinary bladder carcinogenesis in rats treated with diethylnitrosamine and *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine.[12] Since the employed dose was higher than that required for chemoprevention against carcinogenesis in other tissues, the promotional effect of ITCs could be dependent on their concentration. Another paper suggested that necrotic cell death induced by higher concentrations of these ITCs might be involved in its ability to promote carcinogenesis.[15] Although high levels of Brussels sprouts, an ITC-rich source among cruciferous vegetables, in the diet was shown to cause impaired kidney functions,[16] direct effect of ITCs on renal cells has not been clarified.

The kidney is well-known to play a critical role in eliminating non-nutrient chemicals from foods as well as natural end products and drugs. Active drug secretion and reabsorption mainly take place in the proximal tubule cells, which are equipped with separate transport systems for organic anions and cations. Although the rate-limiting step for excretion of organic anions is the uptake step at the basolateral membrane (BLM), their concentration inside the proximal tubule cells is higher than that outside, either at the BLM or the brush border membrane (BBM) side.[17] In a contrast to the rapidly declining plasma concentrations of the ITC metabolites and possibly low concentrations in other tissues, urinary concentrations of the ITC metabolites may reach very high levels after ITC ingestion.[4] Furthermore, ITC-containing supplements are appearing on the market because a dietary supplement or fortified food can overcome the aversion of cruciferous vegetables. A series of information led us to the hypothesis that the renal proximal tubule cell is a potential target for the side effects by higher doses of ITCs.

In vitro experiments using cell culture systems are useful for toxicity prediction of target organs on drugs or chemical

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exposure.[18] Renal proximal tubular cells represent a primary target site for several documented nephrotoxins in vivo.[19,20] Cultured mammalian renal tubular cells can serve as useful tools for assessing the biochemical and physiological functions of the kidney. LLC-PK1 is an established cell line derived from normal porcine kidneys that has been widely used to study renal functions because LLC-PK1 cells retain many properties of native proximal tubular epithelial cells including transport of hexose, amino acids, phosphate, and organic cations.[21] In addition, the effects of the selective nephrotoxins and non-nephrotoxins on normal porcine LLC-PK1 cells are consistent with in vivo findings.[22]

Among ITCs, our group has recently focused on BITC isolated from the extract of papaya (*Carica papaya*) fruits and demonstrated its potent chemoprotective effects.[3] More recently, BITC as well as PEITC and sulforaphane were found as metabolites in serum from a human subject eating broccoli, garden cress, and watercress, suggesting that BITC could be daily consumed from a cruciferous vegetables-containing diet.[23] In this study, we have analyzed the cytotoxicity of BITC and its urinary mercapturic acid metabolite (BITC-NAC) by using a renal proximal tubular cell line, pig LLC-PK1. This study also adds an additional risk of ITCs to renal proximal tubular cells with the critical role of the cellular GSH level.

■ MATERIALS AND METHODS

Chemicals. BITC was purchased from LKT Laboratories, Inc. (MN, USA). NAC, propidium iodide (PI), GSH, GSSG, and Medium 199 were purchased from Sigma-Aldrich (MO, USA). Fetal bovine serum (FBS) was purchased from Nichirei Corporation (Tokyo, Japan). 2′,7′-Dichlorodihydrofluorescein diacetate (H_2DCF-DA) was purchased from Nakalai tesque (Kyoto, Japan). All other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Synthesis of BITC-NAC. BITC-NAC was synthesized by following the method of Vermeulen et al.[24] BITC (14.9 mmol) was gradually added to a solution of NAC (13.6 mmol) and sodium bicarbonate (13.6 mmol) in 33 mL of aqueous 82% (v/v) ethanol. All NAC had reacted within a few hours after BITC was added. Ethanol was evaporated, and the mixture was acidified with HCl. After crystallization, the product was washed on filter with cold water and dried on filter with gentle suction, yielding 11.5 mmol (84.6%) of BITC-NAC as yellowish-white crystals.

Cell Culture. Pig kidney epithelial cell line LLC-PK1 was purchased from Human Science Research Resources Bank (Osaka, Japan). Cells were cultured in Medium 199 supplemented with 5% fetal bovine serum and penicillin−streptomycin (100 U/mL) at 37 °C in a humidified chamber of 95% air and 5% CO_2.

Trypan Blue Exclusion Test. LLC-PK1 cells were suspended at a density of 5×10^5 cells per well in a 6-well plate. After overnight preculture, LLC-PK1 cells were incubated with BITC or BITC-NAC for 24 h. The treated cells were collected and centrifuged. The pellet was resuspended in a proper volume of PBS, and an aliquot of the suspension was mixed 1:1 with trypan blue. The total cells and viable cells (cell that excluded blue dye) were counted using a hemocytometer (Bürker-Türk) under a light microscope.

Measurement of Necrotic Cells. Both the attached and floating LLC-PK1 cells were collected and washed with PBS. The cells were suspended in 500 μL of PBS and stained with 20 μL of PI for 20 min on ice. Fluorescence intensity was measured by using a flow cytometer (EPICSXL, Beckman Coulter, CA, USA) and analyzed by Win MDI 2.9 Software Program.

H_2DCF-DA Assay. Intracellular reactive oxygen species (ROS) products were detected by H_2DCF-DA as an intracellular fluorescence probe.[9] Briefly, after treatment, LLC-PK1 cells were incubated with H_2DCF-DA (10 μM) for 15 min at 37 °C. The cells were harvested and suspended in 500 μL of PBS. Then, flow cytometric analysis was used to detect dichlorofluorescein (DCF) formed by the reaction of H_2DCF with the intracellular ROS products. Data were collected and analyzed by Win MDI 2.9 Software Program.

Measurement of Intracellular ATP Level. Intracellular ATP was measured using an ATP Detection Kit (Promega, WI, USA) according to the manufacturer's protocol. Briefly, the attached cells were harvested with 100 μL of 100 mM Tris−HCl and 4 mM EDTA, boiled for 2 min, and centrifuged at 1,5006g for 2 min. After mixing 450 μL from each of the supernatants or the 0−20 μM ATP standard solution with rL/L Reagent, the emitted light was measured. Aliquots from each sample were used for measuring protein concentration by the Biorad protein assay (Bio-Rad Laboratories, CA, USA). The ATP concentrations were calculated from the linear part of a standard curve and expressed as the relative ATP content per mg protein.

Assay of Intracellular GSH Contents. LLC-PK1 cells were collected and homogenized in 50 mM KH_2PO_4 buffer (pH 8.0) with 5 mM EDTA with mixing for 30 min. After centrifuging at 10,000*g* for 5 min, the supernatant was used for the determination of total GSH and oxidized form of GSH (GSSG) contents. GSH contents were determined following the method of Baker et al.[25] For the determination of GSH+GSSG contents, 50 μL of sample solution was placed in the wells and 100 μL of reaction solution added. GSSG was extracted with 97% 2-vinylpyridine and 20% triethanolamine from the sample solution, and the extract was centrifuged at 8,000*g* for 1 min. Fifty microliters of the lower phase of extract or each GSSG standard solution was placed in the wells and 100 μL of reaction solution added. Reaction solution contained 5 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB, 140 μL), 50 mM NADPH (18.75 μL), 50 mM KH_2PO_4 buffer with 5 mM EDTA (pH 8.0, 2.94 mL), and glutathione reductase (1 U). The changes in absorbance for both GSH+GSSG and GSSG were recorded at 415 nm using a microplate reader (model 680, Nippon Bio-Rad Laboratories, Tokyo, Japan). Aliquots from each sample were used for measuring protein concentration by the Biorad protein assay. The GSH+GSSG or GSSG concentrations were calculated from the linear part of a standard curve and expressed as the relative GSH+GSSG or GSSG content per mg protein. The GSH content was calculated by subtracting the GSSG from GSH+GSSG.

Statistical Analysis. All values were expressed as means ± SD. Statistical significance was assessed by Student's paired two-tailed *t*-test or analysis of variance on untransformed data, followed by comparison of group averages by contrast analysis, using the Super ANOVA statistical program (Abacus Concepts, Berkeley, CA). A *P* value of 0.05 was considered to be statistically significant.

■ RESULTS AND DISCUSSION

Cytotoxic Effect of BITC and BITC-NAC on LLC-PK1 Cells. We assessed the cytotoxic effect of BITC and BITC-NAC on renal proximal tubular cells, the general pig cell line LLC-PK1. We synthesized BITC-NAC, the main metabolite of BITC in the urine, as previously reported.[24] As shown in Figure 1A, not only BITC but also BITC-NAC inhibited the cell proliferation in a dose-dependent manner. The value of IC_{50} for BITC-NAC was approximately five times greater than that of BITC (38 μM versus 7 μM). The effective concentration of BITC for LLC-PK1 cells were comparable with that for several cancer cell models, human pancreatic cancer BxPC-3 cells (∼10 μM[26]), human breast cancer MDA-MB-231 cells (<2.5 μM[27]), and human T lymphocytic leukemia Jurkat cells (6 μM[28]), but considerably lower than normal tissue-originated cell lines including immortalized human pancreatic HPDE-6 cells (>40 μM[26]), rat liver RL34 cells (∼20 μM[9]), and quiescent human colon fibroblastoid CCD-18Co cells (>20 μM[29]). It should be noted that LLC-PK1 cells have very low levels of endogenous multidrug resistance protein 2 (MRP2)-mediated efflux activities,[30] which might play a pivotal role in the transport of

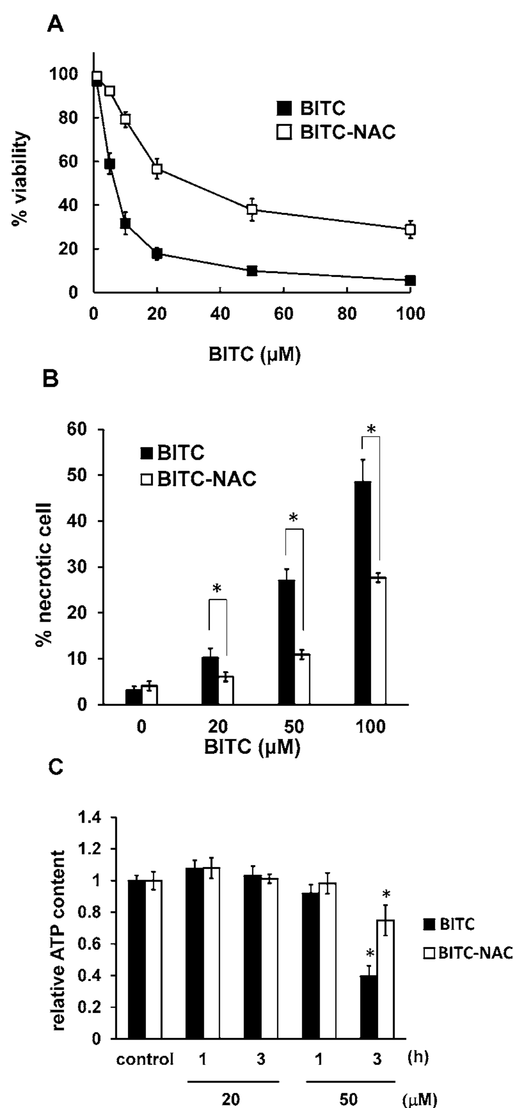

Figure 1. BITC and BITC-NAC induced cytotoxicity in renal proximal tubular LLC-PK1 cells. (A) Effect of BITC or BITC-NAC on cell viability. LLC-PK1 cells were treated with different concentrations of BITC or BITC-NAC for 24 h. The cell viability was determined by a trypan blue exclusion method. (B) Necrosis induction by BITC or BITC-NAC. LLC-PK1 cells were treated with different concentrations of BITC or BITC-NAC for 2 h. Necrotic cells with PI uptake were measured by a flow cytometer. The values represent means ± SD of three separate experiments (*$P < 0.05$). (C) Effect of BITC or BITC-NAC on intracellular ATP level. LLC-PK1 cells were treated with BITC or BITC-NAC at the indicated concentrations for 1 or 3 h. The intracellular ATP level was measured using an ATP Detection Kit. The values represent means ± SD of three separate experiments (*$P < 0.05$ vs control).

aromatic isothiocyanate.[31] Anyway, LLC-PK1 cells are more sensitive to BITC than other normal cell lines.

In the previous study, when subjects were fed 200 μmol ITCs contained in broccoli sprout extracts, the cumulative urinary excretion of the ITC metabolites at 8 h was about 60% of the ingested dose.[32] This study also assumed that if 1 L of urine was produced in 8 h, the total urinary ITC metabolites on average could be estimated to be 117 μM, about 100-fold higher than that in the plasma. This information strongly supported the idea that a toxic concentration of ITC mercapturic acid metabolites could be locally achieved in the urinary tract.

BITC Induced Necrotic Biochemical Changes with Oxidative Stress in LLC-PK1 Cells. We next examined whether BITC or BITC-NAC induced necrotic cell death by measuring the PI incorporation level and intracellular ATP concentration. In order to see early response to necrotic effect of BITC clearly, higher concentrations of BITC (20 or 50 μM) were treated for shorter time periods (1−3 h), even though the required concentration to inhibit cell viability after a 24 h-incubation was relatively lower (Figure 1). As shown in Figure 1B, BITC treatment for 2 h increased the number of PI-incorporated cells in a dose-dependent manner, whereas the percent of permeable cells induced by BITC-NAC was significantly lower than that of BITC at each concentration. After a 3 h-treatment, 50 μM BITC significantly decreased the intracellular ATP concentration. Under the same condition, the effect of BITC-NAC treatment was not remarkable as compared to BITC (Figure 1C). These results suggested that BITC-NAC at high concentrations could induce necrotic cell death even though the effect was weaker than BITC. At the lower BITC concentration of 20 μM, ATP depletion did not totally account for early response of the cytotoxic effect.

To elucidate the cytotoxic mechanism of BITC on LLC-PK1 cells, we evaluated the intracellular levels of ROS and GSH/GSSG. BITC treatment increased the intracellular ROS level in a dose-dependent manner after 1 h (Figure 2A) and 3 h (data not shown). As shown in Figure 2B, BITC dose-dependently decreased the GSH level along with an increase in the GSSG level after 3 h. These results suggest that BITC significantly induces oxidative stress in renal proximal tubular LLC-PK1 cells. We initially observed that BITC induced DNA fragmentation at 20 μM but not at higher concentrations (data not shown). Thus, BITC-NAC as well as BITC at the higher concentration (∼50 μM) inhibited cell proliferation through biochemical events related to necrosis such as PI incorporation and ATP depletion as early response.

Inhibitory Effect of Extracellular GSH on Cytotoxic Effects of BITC and BITC-NAC. To examine the effect of the extracellular GSH on the cytotoxic effects of BITC and BITC-NAC, LLC-PK1 cells were pretreated with a physiological concentration of GSH (5 mM) for 30 min followed by cotreatment of BITC and BITC-NAC with GSH. As shown in Figure 3, GSH significantly counteracted the cytotoxicity induced by not only BITC but also BITC-NAC. It is well known that the thiol conjugates of ITCs in an aqueous solution exist in equilibrium with the free form having an electrophilic reactivity.[5] In other words, the ITC-thiol conjugates are deconjugated over time to yield ITCs and thiols until a state of equilibrium is reached. Exogenously added GSH significantly counteracted cell death induced by BITC-NAC, suggesting that modulation of the equilibrium by increasing the free thiol might inhibit the production of free ITCs. We initially observed that the amount of BITC-NAC in the culture medium was spontaneously decomposed to 40% 2 h after incubation (data not shown), which is consistent with the previous report showing that the half-life of the decomposition for BITC-NAC was 157 min in a buffer at pH 7.4 and 37 °C.[33] If all decomposed BITC-NAC is converted into BITC, BITC-NAC-induced toxicity should be more significant compared with the present data. This discrepancy can be explained by a previous paper showing that free ITC itself is decomposed into amine and thiourea in aqueous solution.[34] Zhang reported that

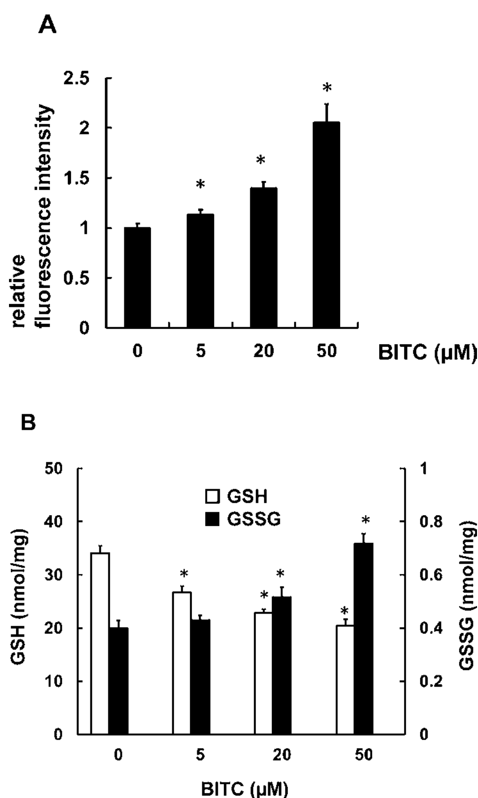

Figure 2. BITC-induced intracellular oxidative stress in LLC-PK1 cells. (A) Effect of BITC on intracellular DCF level. LLC-PK1 cells were treated with BITC at the indicated concentrations for 1 h and then incubated with H_2DCF-DA (10 μM) for 15 min. The intracellular DCF level was measured by a flow cytometer. The values represent means ± SD of three separate experiments (*$P < 0.05$ vs control). (B) Effect of BITC on intracellular GSH/GSSG level. LLC-PK1 cells were treated with BITC at the indicated concentrations for 3 h. The values represent means ± SD of three separate experiments (*$P < 0.05$ vs control).

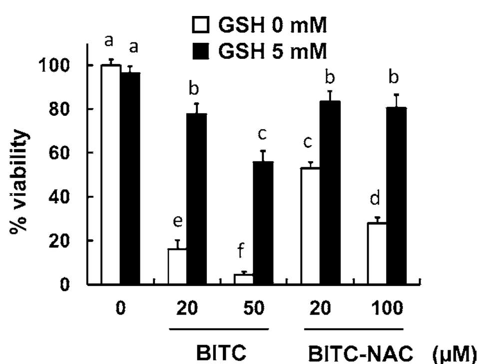

Figure 3. Exogenously added GSH attenuated the cytotoxicity induced by BITC and BITC-NAC. LLC-PK1 cells were treated with or without GSH (5 mM) for 30 min, followed by incubation with BITC or BITC-NAC for 24 h. The cell viability was determined by a trypan blue exclusion method. The values represent means ± SD of three separate experiments. Bars with the same letters are not significantly different at $P < 0.05$.

hydrolysis of the ITC-thiol conjugates was required for their cellular uptake and that only the free ITCs were transported through the cell membrane into the cytoplasm of murine hepatoma cells.[35] Furthermore, the addition of GSH and other thiols to the culture medium strongly inhibited the cellular uptake of the conjugates.[35] It is reported that incubation of an ITC-thiol conjugate with GSH results in an exchange of the isothiocyanate moiety between the two thiols and thus lowered free ITC level.[36] Therefore, the uptake of the released BITC might be involved in the cytotoxicity in renal proximal tubular cells.

Inhibitory Effect of NAC Pretreatment on the Cytotoxicity of BITC. To examine the role of the intracellular GSH in the cytotoxicity of BITC and BITC-NAC, LCC-PK1 cells were pretreated with NAC (1 mM) for 24 h. The NAC pretreatment significantly reduced every cytotoxic event induced by BITC such as decrease in cell numbers (Figure 4A), PI incorporation (Figure 4B), decline of intracellular ATP level (Figure 4C), and ROS production (Figure 4D), even though effects of NAC on ATP level and PI incorporation are only evident at a higher concentration of BITC (50 μM). We initially confirmed that the NAC pretreatment increased the intracellular GSH level by 40% in LCC-PK1 cells (data not shown). Taken together, the enhancement of intracellular GSH level could afford a resistance to BITC in LCC-PK1 cells.

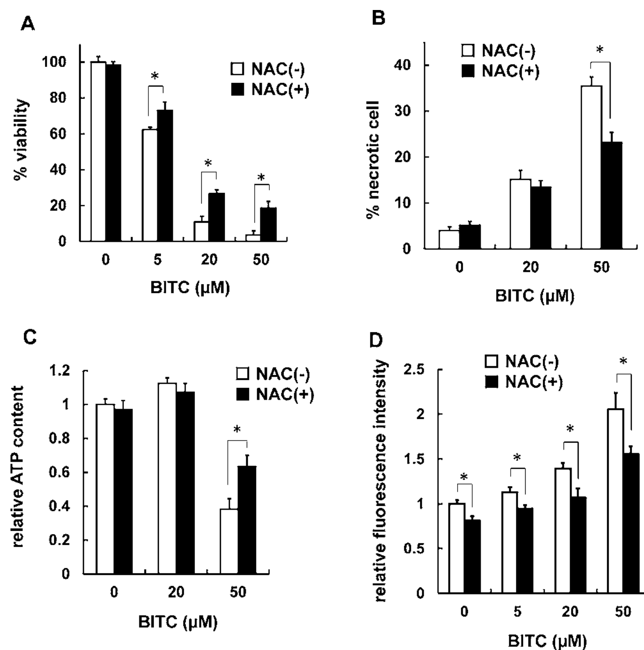

Figure 4. NAC pretreatment inhibited the BITC-induced cytotoxicity in LLC-PK1 cells. (A) Effect of NAC pretreatment on BITC-induced cell death. LLC-PK1 cells were pretreated with or without NAC (1 mM) for 24 h and then incubated with BITC for 24 h. Cell viability was determined by a trypan blue exclusion method. (B) Effect of NAC pretreatment on BITC-induced necrosis. After pretreatment with NAC, LLC-PK1 cells were incubated with BITC for 2 h. Necrotic cells with PI uptake were measured by a flow cytometer. (C) Effect of NAC pretreatment on BITC-induced depletion of ATP level. After pretreatment with NAC, LLC-PK1 cells were incubated with BITC for 3 h. The intracellular ATP level was measured using an ATP Detection Kit. (D) Effect of NAC pretreatment on the intracellular ROS level. After pretreatment with NAC, LLC-PK1 cells were incubated with BITC for 1 h. The values represent means ± SD of three separate experiments (*$P < 0.05$).

We showed that BITC treatment increased the intracellular ROS and GSSG levels with decrease in GSH (Figure 2), suggesting that BITC indeed induced oxidative stress in renal proximal tubular cells. We previously reported that the BITC-

induced intracellular oxidation of H_2DCF-DA is mainly due to the production of hydrogen peroxide derived from superoxide radical dismutation in rat hepatocytes.[9] It was also found that BITC directly modifies the respiratory chain of the isolated mitochondria[9] and activates ataxia telangiectasia-mutated (ATM) possibly in response to DNA oxidative damage and ATM-dependent p53 accumulation in normal colon fibroblasts.[29] Further study using the mitochondrial DNA-deficient (ρ^0) HeLa cells confirmed that BITC-induced ROS originated from the mitochondrial electron transfer chain.[8] Although the precise role of ROS in the BITC-induced cytotoxicity is unknown, the decline of GSH by the ROS-dependent GSSG formation might increase the frequency of electrophilic attack by BITC on thiol molecules including intracellular proteins. This idea was also supported by the finding that the NAC pretreatment significantly lessened every cytotoxic event with an increase in the intracellular GSH level (Figure 4). A positive correlation between the amount of ITCs binding to intracellular protein and their inhibitory effect on cell proliferation has been demonstrated, and several candidate proteins have been postulated as ITC targets.[3] Among them, a glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), is inhibited by BITC in the cells[8] through the loss of free sulfhydryl groups.[37] Therefore, inhibition of GAPDH may represent the BITC-mediated metabolic perturbation in maintenance of the ATP level. In addition to its role as a glycolytic enzyme, the GAPDH modification by BITC may seriously affect other cellular functions because GAPDH is known to take part in a broad array of biological activities including transcriptional and posttranscriptional gene regulation, receptor mediated cell signaling, chromatin structure, maintenance of DNA integrity, and cell death.[38]

The intracellular concentrations of GSH range from 0.5 to 10 mM.[39] Thus, it would be expected that the tissue concentrations of GSH could retard the deconjugation of the ITC-thiol conjugates; hence, free ITCs would not be readily available from thiol conjugates. The ITC-NAC conjugates in the kidney as well as the bladder are stored for a considerable time, which certainly facilitates the release of free ITCs and prolongs tissue exposure to ITC. Therefore, it is likely that the urinary tract epithelium that is transient and directly exposed to urine may be by far the most exposed tissue to the ingested ITCs. The decomposition of the thiol conjugates of ITCs was clearly pH dependent. A mechanism for the influence of hydroxyl ions on the decomposition of thiol conjugates of ITCs was proposed by others, in which a hydroxyl ion deprotonates the conjugate nitrogen, leading to elimination of the thiol leaving group and regeneration of the ITC.[40] Therefore, a change in the urinary pH or decrease in the GSH level in proximal tubule cells can manifest the risk of renal toxicity by ITCs.

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Author Contributions

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

ITCs, isothiocyanates; GSs, glucosinolates; GSH, glutathione (reduced form); NAC, *N*-acetyl-L-cysteine; BITC, benzyl ITC; BLM, basolateral membrane; BBM, brush border membrane; PI, propidium iodide; FBS, fetal bovine serum; H_2DCF-DA, 2′,7′-dichlorodihydrofluorescein diacetate; ROS, reactive oxygen species; DCF, dichlorofluorescein; GSSG, oxidized form of glutathione; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); ATM, ataxia telangiectasia-mutated; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

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