3-Morpholinopropyl isothiocyanate is a novel synthetic isothiocyanate that strongly induces the antioxidant response element-dependent Nrf2-mediated detoxifying/antioxidant enzymes in vitro and in vivo

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The induction of NF-E2-related factor-2 (Nrf2)-mediated detoxifying/antioxidant enzymes is recognized as an effective strategy for cancer chemoprevention. Here, we report that 3-morpholinopropyl isothiocyanate (3MP-ITC) is an exceptionally strong chemical inducer of these enzymes. Exposure of 3MP-ITC in HepG2C8 cells not only induced endogenous Nrf2 protein but also suppressed endogenous Kelch-like ECH-associated protein 1, resulting in an increased nuclear accumulation of Nrf2. Using chemical inhibitors of protein synthesis (cycloheximide) and 26S proteasomal degradation (MG-132), we observed that the induction of Nrf2 protein by 3MP-ITC appeared to be post-translational regulated. 3MP-ITC activated ERK1/2 and JNK1/2 and the activation of antioxidant response element (ARE) by 3MP-ITC was significantly attenuated by chemical inhibition of PKC and PI3K signaling pathways in HepG2C8 cells. Treatment with 3MP-ITC significantly depleted the intracellular level of glutathione (GSH) in HepG2C8 cells and oral administration of 3MP-ITC increased the protein expression of hepatic NAD[P]H:quinone oxidoreductase-1 and Nrf2 in Nrf2 (+/+ ) but not in Nrf2 (−/−) mice, whereas UDP-glucuronosyl transferase 1A1 was induced in both genotypes. Our results indicate that 3MP-ITC is a novel ITC that strongly induces Nrf2-dependent ARE-mediated detoxifying/antioxidant enzymes in vitro and in vivo via the Nrf2 signaling pathway coupled with GSH depletion and activation of multiple signaling kinase pathways, which could be potentially useful agent for cancer chemoprevention.

Introduction

Numerous epidemiological studies have demonstrated that consumption of cruciferous vegetables is effective against development of numerous types of cancer in human (1). Significant portion of chemopreventive effects by cruciferous vegetables is attributed to enhancing carcinogen detoxification and antioxidant system, which is mostly mediated by the cellular NF-E2-related factor-2 (Nrf2)-mediated detoxifying/antioxidant enzymes, such as glutathione-S-transferase, UDP-glucuronosyl transferase, NAD[P]H:quinone oxidoreductase-1 (NQO1) and heme oxygenase-1 (HO-1) (2,3). Accumulating evidence indicates that a coordinated induction of Nrf2-mediated detoxifying/antioxidant enzymes is regulated by a cis-acting element called the antioxidant response element (ARE), which is located in the 5’-upstream promoter region of these genes (4,5). Molecular mechanism studies have identified two key cellular sensors proteins, which constitute the principal components for ARE activation, Nrf2, a positive transcriptional factor of ARE and Kelch-like ECH-associated protein 1 (Keap1), a cytosolic inhibitor of Nrf2 (6). Under basal condition, Nrf2 is sequestered in the cytoplasm as an inactive complex with Keap1. Exposure of cells to ARE inducers will result in the dissociation of Nrf2 from Keap1 and facilitates Nrf2 translocation to the nucleus, where it binds to and transcriptionally activates ARE by heterodimerizing with small Maf proteins (7). The physiological importance of Nrf2 and Keap1 has been highlighted in knockout animal studies. Whereas mice lacking Nrf2 exhibited a higher sensitivity to tissue injury against acetaminophen, butylated-hydroxytoluene, benzo[a]pyrene and diesel exhausts (8), Keap1 knockout mice were post-natally lethal (9).

Isothiocyanates (ITCs) are among a class of chemopreventive compounds, which abundantly exist in cruciferous vegetables such as broccoli, watercress, Brussels sprouts, cabbage and cauliflower. ITCs are characterized by the chemical structure of R-N=C=S, where R designates an alkyl or aryl group (10). Multiple lines of evidence indicate that ITCs are not antioxidants themselves (11), but exert strong antioxidative effects in the cells through transcriptional activation of ARE by Nrf2 (12). At present, several mechanisms have been postulated to account for ARE-driven gene activation by ITCs. Talalay and colleagues have shown that ITCs could release Nrf2 from Keap1 by modulating the –SH groups of cysteines of Keap1, resulting in a conformational change of Keap1, which renders Keap1 unable to bind to Nrf2 protein (13,14). On the other hand, increasing the cellular level of Nrf2 appears yet to be another possible mechanism for ARE-dependent gene expression. This hypothesis agrees with many experimental data that overexpression of an expression vector, harboring Nrf2 complementary DNA, can activate ARE-driven reporter activity without the need of treatment with any chemical inducers (15). In addition, it appears that phosphorylation of intracellular kinases could play an important role in ARE-dependent gene activation, although the molecular mechanisms underlying how activation of these kinases contributes to the regulation of ARE-dependent gene expression still remain to be elucidated.

Sulforaphane and phenethyl ITC (Figure 1A and B) are effectively occurring chemopreventive ITCs. Their chemopreventive activities can be attributed to the induction of Nrf2-mediated detoxifying/antioxidant enzymes, apoptosis, cell cycle arrest and inhibition of histone deacetylases (16–18). However, chemopreventive activities of individual ITCs are quite specific, depending on the structure of ITCs, types of carcinogens and the experimental animal systems (19). Therefore, it can be assumed that understanding of the structure–activity relationship on Nrf2/ARE-dependent gene expression would be essential to optimize chemopreventive and anticarcinogenic activities of ITCs. Recently, we have identified 3-morpholinopropyl isothiocyanate (3MP-ITC, Figure 1C) as a novel strong ARE activator after evaluating the effects of many other synthetic ITCs on ARE-dependent gene expression in HepG2C8 cells (20) and report here that the molecular mechanisms underlying the induction of Nrf2-mediated detoxifying/antioxidant enzymes by 3MP-ITC could be attributed to cellular glutathione (GSH) status and Nrf2 activation coupled with ERK, JNK, PI3K and PKC signaling kinases in the HepG2C8 cells. Also, we have found that the in vivo induction of mouse hepatic NQO1 and Nrf2 protein by 3MP-ITC required Nrf2 genotype.

Materials and methods

Cell culture and reagents

HepG2C8 cells were maintained in modified F-12 medium supplemented with 10% fetal bovine serum, 1.17 mg/ml sodium bicarbonate, 100 units/ml...
Fig. 1. Chemical structure of (A) sulforaphane, (B) phenethyl ITC and (C) 3MP-ITC.

penicillin, 100 µg/ml streptomycin, 1% essential amino acids and 0.1% insulin in a humidified atmosphere of 5% CO₂ at 37°C. HepG2C8 cells were seeded in six-well plates, and allowed to grow up to 70% confluence. Before treatment with chemicals, HepG2C8 cells were starved overnight in F-12 medium, containing 0.5% fetal bovine serum.

Chemicals and antibodies

3MP-ITC was purchased from Lancaster Synthesis (Windham, NH). Sodium arsenite was purchased from Sigma (St Louis, MO). Sulforaphane and cycloheximide were purchased from LKT laboratories (St Paul, MN). Ro-32-0432, LY-294002 and MG-132 were obtained from Calbiochem (La Jolla, CA). Anti-bodies against phospho-ERK, phospho-JNK and phospho-p38 mitogen-activated protein kinase (MAPK) were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against ERK1, JNK/1,2, p38 MAPK, Nrf2, Keap1, HO-1, NQO1, UDG-glucuronyl transferase 1A1 (UGT1A1) and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Preparation of protein lysates, animal studies and western blotting

After treatments, HepG2C8 cells were washed with ice-cold phosphate-buffered saline (PBS) (pH 7.4) and harvested with 200 µl of a whole-cell lysis buffer (pH 7.4), containing 10 mM Tris–HCl, 50 mM sodium chloride, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 100 µM sodium orthovanadate, 2 mM iodoacetic acid, 5 mM ZnCl₂, 1 mM phenylmethylsulfonyl fluoride and 0.5% Triton-X 100. Cell lysates were vigorously vortexed, homogenized in an ultrasonicator for 10 s and left on ice for 30 min. The homogenates were centrifuged at 13 000 rpm for 15 min at 4°C and the supernatants were collected. Nuclear and cytoplasmic extracts of HepG2C8 cells were prepared, using NE-PER nuclear and cytoplasmic extraction reagent (Pierce Biotechnology, Rockford, IL) as recommended by the manufacturer. We have originally obtained the Nrf2 knockout mice from Drs Jefferson Y. Chan and Yuet Wai Kan (from the University of California at San Francisco) and backcrossed with Nrf2 (from the University of California at San Francisco) and backcrossed with C57BL/6 wild-type mice in our laboratory. The C57BL/6 Nrf2 (+/+), and Nrf2 (−/−) mice were housed in sterile filter-capped cages and provided with diet and water ad libitum. The protocol for the animal study was approved by the Rutgers University Institutional Animal Care and Use Committee. For preparation of mouse hepatic protein lysates, mice were fasted overnight and orally administered with 40 mg/kg of 3MP-ITC, dissolved in 50% polyethylene glycol 400 and 50% H₂O. After killing by CO₂ inhalation, the liver was excised, frozen down in liquid nitrogen and stored at −80°C until analyses. Frozen liver samples were thawed and ground by homogenizer in 400 µl ice-cold cell lysis buffer, centrifuged at 13 000 rpm for 15 min at 4°C and the supernatants were collected for western blotting. The protein concentration of lysates was determined by BCA protein assay kit (Pierce Biotechnology, Rockford, IL). Equal sample amount of proteins was mixed with protein loading buffer and heated at 95°C for 5 min. The samples were then resolved in a 10% sodium dodeyl phosphate–polyacrylamide gel electrophoresis at 200 V and transferred onto polyvinylidene difluoride membrane (Immobilon-P, Millipore, Bedford, MA) for 2 h at 200 mA, using a semi-dry transfer system (Fisher Scientific, Pittsburgh, PA). The membrane was blocked with 5% non-fat dry milk in 1× PBST buffer (0.1% Tween 20 in PBS) for 1 h at room temperature and incubated with primary antibodies in 3% non-fat dry milk of 1× PBS (1:1000 dilution) overnight at 4°C. After hybridization with primary antibody, the membrane was washed three times with 1× PBST and incubated with secondary antibody conjugated with horseradish peroxidase for 1 h at room temperature, followed by washing with PBST three times. Final detection was performed with enhanced chemiluminescence western blotting reagents (Amersham Pharmacia, Piscataway, NJ) and the bands were visualized with Bio-Rad ChemiDoc XR5 system (Hercules, CA).

Determination of total intracellular GSH

Intracellular GSH level of HepG2C8 cells was measured with a total GSH quantification kit (Dojindo Molecular Technologies, Gaithersburg, MD) as recommended by the manufacturer.

Luciferase assay

ARE–luciferase activity in HepG2C8 cells was determined using a luciferase kit from Promega (Madison, WI). Briefly, the cells were washed twice with ice-cold PBS (pH 7.4) after treatments and harvested in 1× reporter lysis buffer (Promega, Madison, WI). After centrifugation at 13 000 rpm for 10 min, aliquot (10 µl) of the supernatant was assayed for luciferase activity with a SIRIUS luminometer (Berthold Detection System GmbH, Pforzheim, Germany). The luciferase activity was normalized against protein concentration and expressed as a fold induction over the luciferase activity in control vehicle-treated cells. Data were statistically analyzed by analysis of variance, followed by the Student’s t-test.

RNA extraction and semi-quantitative reverse transcription–polymerase chain reaction

Total RNA from HepG2C8 cells was isolated with an RNAeasy Mini Kit (Qiagen, Valencia, CA). RNA samples were converted to single-stranded cDNA by the Superscript First-Strand Synthesis System III (Invitrogen, Carlsbad, CA) and the resulting cDNA was amplified by the PCR supermix kit (Invitrogen). Polymerase chain reaction conditions are as follows: 94°C for 5 min followed by cycles of denaturation at 94°C for 30 s, annealing at 55°C for 45 s, extension at 72°C for 45 s and a final extension at 72°C for 10 min. The 5′ and 3′ primers used for amplifying HO-1 were 5′-CTTCTTCACCTTCACCCCAACA-3′ and 5′-ATGCCTGGTATGTCCTTCCC-3′. The 5′ and 3′ primers used for amplifying NQO1 were 5′-GCGCGACCTCTGTGATATT-3′ and 5′-AAATGATGGCCACCAAGAC-3′. The 5′ and 3′ primers used for amplifying UGT1A1 were 5′-TAAAGGTGCAACCCAAAAAGC-3′ and 5′-TCTGTGATTGTGCGCCTTTC-3′. Glyceraldehyde-3-phosphate dehydrogenase was used as a control and was amplified with the 5′ and 3′ primers 5′-CCGGACAGCCCTTGTGATATT-3′ and 5′-AAATGATGGCCACCAAGAC-3′. Polymerase chain reaction products were resolved on 1.5% agarose gels and visualized under ultraviolet lamps.

Results

3MP-ITC strongly activates ARE-dependent gene expression and induces HO-1, NQO1, UGT1A1 and Nrf2 proteins in HepG2C8 cells

HepG2C8 cells were established in our laboratory after a transfection of HepG2 cells with ARE–luciferase reporter plasmid (21) and G418 selection. Using HepG2C8 cells, we have found that treatment of 3MP-ITC strongly increases ARE-dependent luciferase activity in HepG2C8 cells (20). To examine whether ARE activation by 3MP-ITC is translated to the induction of ARE-dependent protein expression, HepG2C8 cells were treated with 3MP-ITC and the protein expression of Nrf2-mediated detoxifying/antioxidant enzymes, such as HO-1, NQO1 and UGT1A1 was measured by western blotting. Sodium arsenite (NaAsO₂) and sulforaphane were used as positive controls. As seen in Figure 2A, expression of 3MP-ITC to HepG2C8 cells strongly induced the protein expression of HO-1, NQO1 and UGT1A1 in a time-dependent manner. In addition, semi-quantitative reverse transcription–polymerase chain reaction demonstrated that treatment of 3MP-ITC resulted in an increase in HO-1, NQO1 and UGT1A1 mRNAs that were apparent at 3 h, increased up to 12 h and declined after 24 h (Figure 2B). This fact suggests that a strong induction of HO-1, NQO1 and UGT1A1 enzymes by 3MP-ITC is regulated at the transcriptional level.

Induction of Nrf2 protein and suppression of Keap1 protein contributes to the nuclear translocation of Nrf2 protein by 3MP-ITC, in which Nrf2 protein induction is post-translationally regulated

Next, we examined the effect of 3MP-ITC on two key regulatory proteins of ARE, e.g. Nrf2 and Keap1. As seen in Figure 3A, treatment of 3MP-ITC not only increased the protein expression of Nrf2 but also strongly suppressed that of Keap1 in HepG2C8 cells. Using the nuclear and cytosolic extracts, we found that Nrf2, induced by 3MP-ITC, was mostly located in the nucleus (Figure 3B), suggesting that suppression of Keap1 by 3MP-ITC might result in a decrease of...
Nrf2 protein retention in the cytosol, which in turn rendered Nrf2 to translocate into the nucleus and activated ARE-dependent gene expression.

Although it is evident that Nrf2 protein induction is an important mechanism for ARE activation, how cellular Nrf2 expression is induced is still controversial. Kwak et al. (22) have found that an ARE-like sequence exists in the Nrf2 promoter region and that treatment of 3H-1,2-dithiole-3-thione could lead to an increase of Nrf2 mRNA in murine keratinocytes by providing the binding site of Nrf2 itself. In contrast, we and other investigators have reported that increasing the level of Nrf2 protein was regulated in response to ARE inducers by attenuating the ubiquitin-dependent 26S proteosomal degradation with Nrf2 mRNA steady and unaffected (23–26). This discrepancy prompted us to ask whether an increased Nrf2 protein expression by 3MP-ITC is post-translationally regulated.

Multiple signaling kinase pathways are responsible for ARE activation by 3MP-ITC

MAPK cascades, consisting of ERK, JNK and p38 MAPK, are evolutionary conserved in all eukaryotes and play a central role in the cellular responses to various extracellular stimuli (27). Previous works from our group have demonstrated that ERK and JNK are positive regulators of ARE-dependent gene expression, but p38 MAPK is negatively involved (28). We found that treatment of 3MP-ITC resulted in activation of ERK1/2 and JNK, but not p38 MAPK, in HepG2C8 cells (Figure 4A) where phosphorylation level of ERK1/2 by 3MP-ITC was stronger than that by sodium arsenite or by sulforaphane, but that of JNK was much weaker compared with that by sulforaphane. In addition to MAPKs, activation of other kinases, including PI3K, PKC and PERK, has been reported to be responsible for ARE-dependent gene expression (28). Supporting this idea, we found that treatment of LY-294002, a chemical-specific inhibitor of PI3K, or Ro-32-0432, a chemical-specific inhibitor of PKC, strongly suppressed ARE-dependent gene expression, induced by 3MP-ITC in HepG2C8 cells, although these inhibitors failed to affect the basal level of ARE–luciferase activity (Figure 4B). These facts suggest that multiple signaling kinase pathways are involved in ARE-mediated gene activation by 3MP-ITC.

ARE-dependent gene activation by 3MP-ITC is closely correlated with depletion of intracellular GSH level

Kolm et al. (29) reported previously that ITCs could directly react with and deplete the level of intracellular GSH. In agreement with this observation, we found that exposure of 3MP-ITC to HepG2C8 cells resulted in a maximal suppression of intracellular GSH level after 6 h (45.2 ± 1.6%), followed by a gradual increase up to 12 h (75.5 ± 2.8%), possibly due to adaptive cellular responses (Figure 5), suggesting that depletion of intracellular GSH might be responsible for ARE-dependent gene activation by 3MP-ITC.

3MP-ITC is a chemical inducer of hepatic NQO1, UGT1A1 and Nrf2 proteins in vivo

In order to address whether hepatic HO-1, NQO1 and UGT1A1 proteins are inducible in vivo in response to 3MP-ITC, we orally
Discussion

We have found that 3MP-ITC, a novel synthetic ITC, strongly induced ARE-dependent gene activation and increased the protein expression of ARE-dependent Nrf2-mediated detoxifying/antioxidant enzymes, such as HO-1, NQO1 and UGT1A1 in HepG2C8 cells (Figure 2A). It appears that the cellular mechanisms responsible for ARE activation and the induction of Nrf2-mediated detoxifying/antioxidant enzymes by 3MP-ITC were similar to other naturally occurring chemopreventive agents in that the induction of Nrf2-mediated detoxifying/antioxidant enzymes by 3MP-ITC occurred through the induction of Nrf2 protein (Figure 3A) and its subsequent translocation (Figure 3B) into the nucleus. In addition, as is the case damages in the liver and other organs. Western blotting results show that oral administration of 3MP-ITC significantly induced the expression of hepatic NQO1 and UGT1A1 enzymes in vivo. Nrf2 (+/+) and Nrf2 (−/−) mice were orally administered with 3MP-ITC (40 mg/kg). After 6 h, the mice were killed and the expression of hepatic HO-1, NQO1, UGT1A1 and Nrf2 proteins was measured by western blotting (upper panel). Also, Nrf2 (+/+) and Nrf2 (−/−) mice were administered with the same dosage of 3MP-ITC and the protein expression of hepatic HO-1 and Nrf2 was measured by western blotting after a period of time indicated (lower panel).

Fig. 4. Multiple signaling kinases are involved in ARE-dependent gene activation by 3MP-ITC. (A) The same cell lysates in Figure 2A were used and phosphorylation of ERK1/2, JNK and p38 MAPK was analyzed by western blotting, using phospho-specific ERK1/2, JNK and p38 MAPK antibodies. Effect of 3MP-ITC on the endogenous levels of ERK2, JNK1/2 and total p38 MAPK was also measured, using polyclonal antibodies against ERK2, JNK1/2 and p38 MAPK. (B) Effects of pharmacological inhibitors of PKC and PI3K on ARE-dependent gene activation, induced by 3MP-ITC. HepG2C8 cells were incubated with 3MP-ITC in the absence or presence of LY-294002 (20 μM) or Ro-32-0432 (1 μM) for 24 h and the resulting ARE-luciferase activities were measured. The data shown are means ± standard deviation of luciferase activities in triplicate plates. Significant difference between the groups: ** P < 0.01.

Fig. 5. Depletion of intracellular total GSH by 3MP-ITC is responsible for ARE activation. After exposure of 3MP-ITC (20 μM) to HepG2C8 cells, the level of intracellular total GSH was measured in triplicate plates and expressed as percentage ratio over the control group. Significantly different from the control: ** P < 0.01.

administered 6- to 8-week-old male Nrf2 (+/+) and Nrf2 (−/−) mice (three mice per group) with 3MP-ITC for 6 h and measured the expression of hepatic HO-1, NQO1, UGT1A1 and Nrf2 proteins by western blotting. At autopsy, we observed no general organ
with sulforaphane (25), we found that 3MP-ITC induced the expression of Nrf2 protein by attenuating its proteasomal degradation (Figure 3D). Because, Keap1 is an adaptor protein for Cul3-based E3 ligase, which facilitates the polyubiquitinylation of Nrf2 (31,32), it is tempting to speculate that suppression of Keap1 protein (Figure 3A) by 3MP-ITC might result in the attenuation of Nrf2 proteosomal degradation.

We have observed that treatments of many natural and synthetic ITCs significantly stimulated ARE–luciferase activities in HepG2C8 cells with different dose–response and different potency, and most, if not all, of the ITCs had inducing effects (20). However, we could not find any ITC that would inhibit ARE-dependent gene expression in HepG2C8 cells, suggesting that the ITC class of compounds are probably universal ARE activators. In addition, we observed that the maximal ARE activation by many ITCs did not follow the same dose-dependent kinetics. Therefore, it is possible that, in addition to depletion of intracellular GSH as seen in Figure 5 or reported by others (33), other unknown factors or signaling pathways could be involved in ARE-dependent gene activation by ITCs since the degree of intracellular GSH depletion by ITCs would be stoichiometrically proportional to the amount of added ITCs.

Based on the principle that induction of Nrf2-mediated detoxifying/antioxidant enzymes in animals would reduce their susceptibility to the neoplastic and carcinogenic effects of carcinogens, many naturally occurring chemopreventive ITCs have been identified. For example, Talalay and colleagues have identified sulforaphane from broccoli extracts, using a simple bioassay to measure the activities of NQO1 in animal cells grown in microtiter plates (34). Morimitsu et al. (35) have also found that 6-(methylsulfonyl)hexyl ITC from Japanese horseradish wasabi (Wasabia japonica) was a key glutathione-S-transferase inducer in cultured rat liver epithelial RL-34 cells. Using murine hepatoma Hepa1c1c7 cells, Rose et al. (36) have identified 7-methylsulfinylheptyl and 8-methylsulfinyloctyl ITCs as potent inducers of phase-II enzymes from watercress. Although these ITCs were identified from dietary sources, there is every reason to believe that synthetic ITCs can be potential candidates for chemopreventive agents, if other important pharmaceutical or toxicological parameters, such as bioavailability and safety, are further evaluated. In line with this idea, we have identified that 3MP-ITC is a strong inducer of Nrf2-mediated detoxifying/antioxidant enzymes in vitro and in vivo and further studies aimed at testing the efficacy of 3MP-ITC as a potential chemopreventive compound using different carcinogenesis animal models are warranted in the future.

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