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Mechanistic Insights into Chemopreventive Effects of Phenethyl Isothiocyanate in *N*-Nitrosobis(2-oxopropyl)amine-treated Hamsters

Akiyoshi Nishikawa,[1,3] In-Seon Lee,[1,4] Chikako Uneyama,[1] Fumio Furukawa,[1] Hyoung-Chin Kim,[1,5] Ken-ichiro Kasahara,[1] Namho Huh[2] and Michihito Takahashi[1]

[1]*Division of Pathology, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158 and* [2]*Department of Biochemistry, Faculty of Medicine, Toyama Medical and Pharmaceutical University, Sugitani, Toyama 930-01*

The influence of phenethyl isothiocyanate (PEITC) on cell kinetics in the target organs of *N*-nitrosobis(2-oxopropyl)amine (BOP) tumorigenicity and on xenobiotic-metabolizing enzymes was investigated in hamsters. Female 5-week-old Syrian hamsters were given a single s.c. dose of 0, 20 or 50 mg/kg of BOP 2 h after receiving PEITC by gavage at a dose of 0, 100 or 250 μmol/animal (0, 16.3 or 40.8 mg/animal). Six and 22 h after the BOP administration, hamsters were killed and tissues were sampled. Proliferating cell nuclear antigen immunohistochemistry demonstrated significant reduction ($P<0.05$–0.001) by PEITC of the labeling indices in the pancreatic acini and ducts, bronchioles, and renal tubules of the BOP-treated animals in a dose-dependent manner. In the lungs, the PEITC pretreatment significantly ($P<0.001$) reduced the O^6-methyldeoxyguanosine levels as compared to the BOP-alone value. Immunoblot analysis of liver cytochrome P450 isoenzymes showed CYP 2B1 to be mainly involved in the metabolic activation of BOP. PEITC significantly ($P<0.05$) inhibited the induction of several isoenzymes, including CYP 2B1, while lowering the hepatic glutathione *S*-transferase activity as well as glutathione levels, regardless of BOP administration. Our results thus suggest that PEITC exerts its chemopreventive activity against BOP initiation of carcinogenesis in hamsters by decreasing cell turnover and DNA methylation in the target organs, and by influencing hepatic xenobiotic-metabolizing phase I enzymes, although the relationship, if any, of the latter with the former events remains to be investigated.

Key words: Mechanism — PEITC — Chemoprevention — BOP — Hamster

PEITC, a natural constituent of cruciferous vegetables, has been extensively investigated as a chemopreventive agent in rats and mice.[1–8] It inhibits chemically induced lung, mammary gland, forestomach and esophagus tumorigenesis,[8] and we have recently shown dramatic decreases in lung and pancreatic carcinogenesis in hamsters receiving PEITC concurrently with BOP.[9] The analogue 3-phenylpropyl isothiocyanate also inhibits lung tumor development.[10] The principal underlying mechanism appears to be related to its ability to lower DNA alkylation by chemical carcinogens such as NNK in rats and mice.[1,2,11] It has been hypothesized that such a decrease in DNA adduct formation by PEITC is a consequence of modulation of xenobiotic metabolism by phase I and phase II enzymes.[12–15]

BOP has been shown to induce lung, pancreatic, liver and kidney tumors in Syrian hamsters,[16] and this animal model has been extensively used to assess the modifying effects of chemicals on pancreatic carcinogenicity,[17–19] based on the histological and biological similarities between human and BOP-induced hamster pancreatic tumors.[16] The hamster is also well documented as being suitable for examining the effects of carcinogenic substances which target the respiratory tract.[20] DNA alkylation is speculated to be an initiation-associated event in BOP tumorigenesis, because O^6- and 7-alkyl guanines are produced in the target organs.[21,22] Evidence has also been presented that division of cells during carcinogen exposure increases the likelihood of tumor initiation.[22]

The present short-term study was therefore conducted to investigate the effects of PEITC on cell kinetics and DNA methylation in Syrian hamsters, under similar conditions to those used for initiation in our previous long-term bioassay.[9,10] An investigation of metabolizing enzymes was included to assess their contribution to chemopreventive effects of PEITC during the initiation phase of

[3] To whom requests for reprints should be addressed.

[4] Present address: Department of Food Science and Technology, Keimyung University, 1000 Shindang-Dong, Dalseo-ku, Taegu 700-200, Korea.

[5] Present address: Toxicology Research Center, Korea Research Institute of Chemical Technology, 100 Jandong, Yusonku, Daejon 305-343, Korea.

Abbreviations: PEITC, phenethyl isothiocyanate; BOP, *N*-nitrosobis(2-oxopropyl)amine; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; PCNA, proliferating cell nuclear antigen; O^6-MedGuo, O^6-methyldeoxyguanosine; GST, glutathione *S*-transferase; GSH, glutathione.

BOP-induced carcinogenesis in hamsters, since BOP requires metabolic activation to yield alkylating agents.[23]

MATERIALS AND METHODS

Animals and chemicals Female Syrian hamsters (Japan SLC, Shizuoka), 5-weeks old with an initial body weight of approximately 80 g, were used in the experiment. They were housed, five per polycarbonate cage, in an air-conditioned room at 23±2°C and 60±5% humidity under a daily cycle of alternating 12 h periods of light and darkness. Oriental MF (Oriental Yeast, Tokyo) was freely available as the basal diet. BOP was obtained from Nacalai Tesque (Kyoto) and PEITC (purity >99%) was purchased from Aldrich Chem. (Milwaukee, WI).

Animal treatment Animal groups, each consisting of 5 hamsters, received BOP (20 or 50 mg/kg) s.c. 2 h after administration by gavage of PEITC at a concentration of 0, 100 or 250 μmol/animal (0, 16.3 or 40.8 mg/animal) dissolved in 0.1 ml corn oil. Vehicle controls for PEITC consisting of 5 animals were given corn oil alone without the BOP treatment. Six and 22 h after the BOP administration, animals were killed and tissue samples of liver, lung, pancreas and kidney were taken.

Assay of cell proliferation and DNA methylation Cell proliferative activity was examined in terms of the PCNA-labeling index. Lung, liver, pancreas and kidney tissues from 5 animals per group examined were immediately fixed in methanolic Carnoy's fixative (methanol : chloroform : acetic acid = 6 : 3 : 1) at autopsy and routinely processed for embedding in paraffin. Sections were cut for immunohistochemistry with anti-PCNA antibody PC10 (Dako, Kyoto) using the streptoavidin-biotin complex method. Only nuclei stained dark brown with diaminobenzidine were judged to be PCNA-labeled. More than 500 cells per tissue section were counted under the microscope and PCNA-labeling indices were expressed as percentages of the total numbers of cells. DNA was isolated from the lungs of each of 5 hamsters treated with 50 mg/kg BOP following 250 μmol of PEITC or corn oil alone, and the levels of O^6-MedGuo were measured by radioimmunoassay, as described previously.[24]

Assay for phase I and II enzymes, and GSH Phase I isoenzymes were analyzed by immunoblotting. Briefly, liver, lung and pancreas samples from 5 animals per group were homogenized in 3 volumes of 1.15% KCl, microsome fractions were prepared by differential centrifugation and resuspension in 1.15% KCl/20% glycerol, and proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis on 7.5% polyacrylamide gels according to the method of Laemmli.[25] After transfer to polyvinylidene fluoride membranes, they were probed with specific goat anti-rat CYP 1A, 2B, 2E, 3A and 4A sera (Daiichi Pure Chem., Tokyo). A rabbit peroxidase-labeled affinity-purified antibody to goat IgG (H+L) (BioMakor, Israel) was used as the secondary antibody. Livers from 5 animals per group were analyzed for GST activity and GSH level. GST activity was determined using GSH and 1-chloro-2,4-dinitrobenzene as the substrate.[26] GSH levels were measured by reaction with *O*-phthalaldehyde to form a fluorescent product with an emission peak at 420 nm on excitation at 350 nm.[27]

Statistics The results were statistically evaluated by analysis of variance.

RESULTS

Effects of PEITC on cell proliferation induced by BOP As shown in Fig. 1, the PCNA-labeling indices (%) in the pancreatic acini and ducts, bronchioles and renal tubules were significantly ($P<0.05$–0.001) increased as compared to the vehicle control values for PEITC, 6 h after the BOP treatment. This effect was significantly ($P<0.05$–0.001) decreased by the PEITC pretreatment

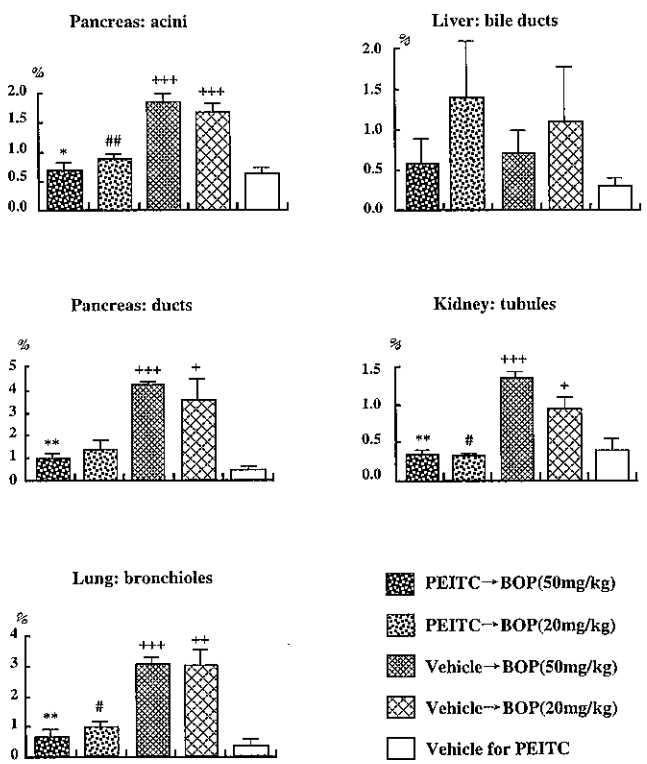

Fig. 1. PCNA-labeling indices (%) 6 h after BOP treatment. Data represent mean±SE. * $P<0.01$, ** $P<0.001$ vs. the Vehicle→BOP (50 mg/kg) group. # $P<0.05$, ## $P<0.01$ vs. the Vehicle→BOP (20 mg/kg) group. + $P<0.05$, ++ $P<0.01$, +++ $P<0.001$ vs. the Vehicle control group.

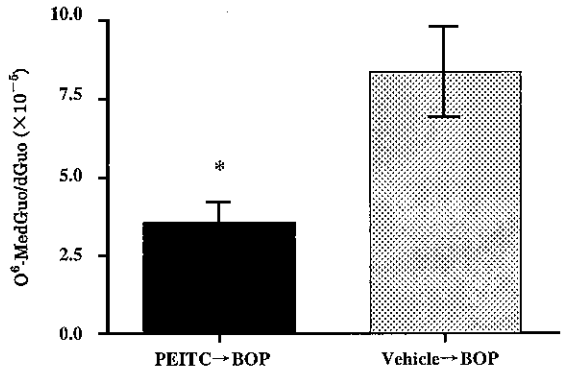

Fig. 2. Effects of PEITC (100 μmol) on O^6-MedGuo formation in hamster lungs treated with BOP (50 mg/kg) at 6 h after the BOP treatment. Data represent mean±SD. * $P<$ 0.001 vs. the Vehicle→BOP group.

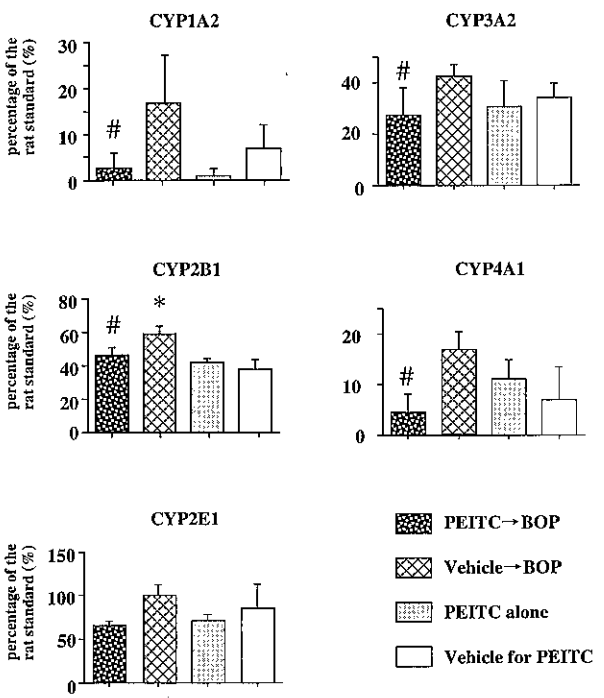

Fig. 3. Levels of liver microsomal P450 isoenzymes in hamsters treated with BOP (50 mg/kg) and/or PEITC (250 μmol) at 6 h after the BOP treatment. Data represent mean ±SD as a percentage of the corresponding rat CYP standard. The designations CYP 1A2, CYP 2B1, CYP 2E1, CYP 3A2 and CYP 4A1 are based on the rat antibodies used. * $P<$ 0.05 vs. the Vehicle control group; # $P<0.05$ vs. the Vehicle →BOP group.

in a dose-dependent manner. Similar tendencies were noted 22 h after the BOP treatment, but with lower statistical significance (data not shown).

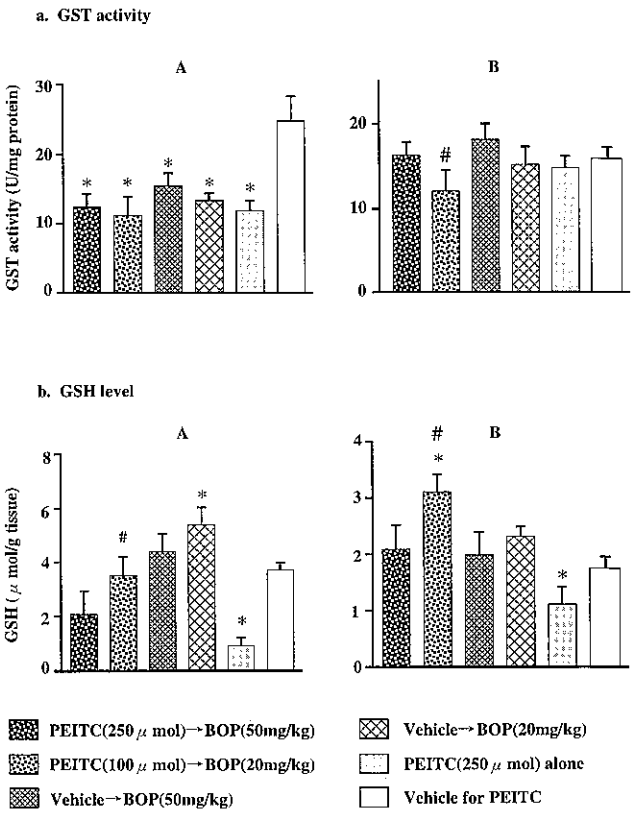

Fig. 4. Effects of PEITC on hepatic GST activity (a) and GSH levels (b) in hamsters at 6 (A) and 22 h (B) after the BOP treatment. Data represent mean±SD. * $P<0.05$ vs. the Vehicle control group; # $P<0.05$ vs. the Vehicle→BOP (20 mg/kg) group.

Effects of PEITC on O^6-MedGuo formation As shown in Fig. 2, 6 h after the BOP treatment, the average level (3.56×10^{-5}) of O^6-MedGuo per deoxyguanosine in the lung DNA of hamsters given 50 mg/kg BOP and 250 μmol of PEITC previously was significantly ($P<0.001$) lower than that (8.36×10^{-5}) in hamsters given 50 mg/kg BOP and corn oil.

Effects of BOP and/or PEITC on cytochrome P450 isoenzymes As demonstrated in Fig. 3, the 50 mg/kg BOP treatment significantly ($P<0.05$) increased the level of hepatic CYP 2B1, as compared to the vehicle control value, 6 h after the BOP treatment, and also showed a tendency to increase the levels of CYP 1A2, 3A2 and 4A1. However, the combined administration of 250 μmol of PEITC with 50 mg/kg BOP significantly ($P<0.05$) reduced the levels of CYP 1A2, CYP 2B1, CYP 3A2 and CYP 4A1 compared with those in the BOP-treated group. The PEITC treatment also showed a tendency to depress the CYP 2E1 level. Similar tenden-

cies were seen 22 h after the BOP treatment but the statistical significance was less than at 6 h after (data not shown). PEITC did not affect the P450 isoenzyme levels in the pancreas and lung (data not shown).

Effects of BOP and/or PEITC on hepatic GST and GSH

As shown in Fig. 4a, hepatic GST activity was significantly ($P<0.05$) decreased in all groups treated with PEITC and/or BOP, as compared to the vehicle control value, 6 h after the BOP treatment. Hepatic GSH levels were also significantly ($P<0.05$) decreased by the PEITC administration as compared to the vehicle→BOP or vehicle alone group value after 6 h, although recovery was advanced by the 22 h time point (Fig. 4b).

DISCUSSION

The present study demonstrated that PEITC effectively blocks the increase in cell proliferation induced by BOP treatment in its target organs, the pancreas, lung and kidney. This result is in line with an *in vitro* observation that PEITC and related isothiocyanates delay cell cycle progression in cultured Hela cells.[28] Also in the present study, the level of O^6-MedGuo in lung DNA was significantly decreased by the PEITC pretreatment. BOP has been shown primarily to cause DNA methylation, although low levels of hydroxypropylation may also occur.[21, 29] However, it remains to be investigated whether PEITC reduced the cell proliferation by inhibiting the activation of BOP or by directly decreasing cell turnover.

As suggested previously,[22] the balance among DNA damage, repair processes and cell proliferation presumably determines the potency and selectivity of carcinogenic nitrosamines like BOP. Cell division (i) fixes adducts as mutations, (ii) increases susceptibility to damage by providing single-stranded DNA without the protection of complementary DNA or histones, (iii) results in dedifferentiation and loss of 5-methylcytosine, and (iv) is associated with elevated expression of an array of oncogenes.[22] Recently, it was shown that PEITC activates c-*Jun* *N*-terminal kinase 1 in a dose- and time-dependent manner[30] so that it may have some ability to regulate such genes, although it is not known how this is related to the anti-proliferative effect. Further studies in this area are clearly warranted.

Previous studies using rats and mice have shown preventive effects of PEITC against NNK-induced lung carcinogenesis due to its inhibition of P450-mediated metabolic α-hydroxylation of the alkylating agent.[1, 2, 4, 12–14] Therefore, regarding the influence of PEITC on xenobiotic phase I enzymes, the results of the present study suggest that it exerts anti-initiating effects in hamsters by mechanisms similar to those observed in rats and mice.[3, 13] It has been shown that BOP is activated by CYP 2B1 and other related forms in mice, rats and hamsters.[23] In the latter species,[31] a particularly broad spectrum of phase I enzymes may be involved. In fact, in the present study, in addition to significant induction of P450 isoenzyme CYP 2B1 in the hamster liver, other isoenzymes such as CYP 1A2, CYP 2E1, CYP 3A2 and CYP 4A1 all showed tendencies for increase. As expected from the data for rats and humans,[15, 32, 33] in the present study PEITC consistently exerted inhibitory effects on phase I enzymes, including CYP 1A2 and CYP 2B1, in hamsters. CYP 2B1 and CYP 2E1 have been shown to activate NNK in rat or mouse lung microsomes[14, 34] and their involvement in NNK metabolism in hamsters has also been suggested on the basis of immunoblot analyses.[35] It is therefore possible that BOP and NNK share metabolic pathways leading to active carcinogenic forms. Since anti-rat CYP antibodies were used in the present study because of the lack of reliable anti-hamster CYP antibodies, it should be borne in mind that the results for immunoblotting of P450 isoenzymes may have some limitations. However, very recently we have confirmed in a comparative study using rats and hamsters that the influence of modulating factors such as cigarette smoke exposure on hepatic P450 isoenzymes was quite similar in the two species (unpublished data).

In contrast to the phase I enzyme case, the influence of PEITC on GST activity and GSH levels was different from the previous finding, in rats, of induction of phase II enzymes such as GST.[36, 37] This might be due to the species difference or time-point differences in the measurement. However, our results are in line with an *in vitro* study reporting no effect of PEITC on these parameters.[14, 38] In the present study PEITC was administered by a single gastric intubation, whereas it was given by feeding[36] or repeated dosing[32] in the previous experiments, so differences in experimental conditions may have been responsible for the observed anomaly. In rodents and humans, thiol conjugation with consumption of GSH is a major metabolic pathway of isothiocyanates,[39] which are known to induce a wide spectrum of phase II enzymes such as NAD(P)H:quinone reductase, epoxide hydrolase and UDP-glucuronosyltransferases due to their electrophilic potential.[12, 13, 30, 32, 40] In common with many chemically unrelated chemopreventive agents, isothiocyanates evoke a generalized electrophilic counterattack response in rodents, characterized by the induction of phase II enzymes, and thus further studies of this area are necessary.

In conclusion, our results clearly indicate that PEITC exerts chemopreventive effects against BOP-induced carcinogenesis in hamsters by inhibiting the associated increase in cell turnover and by reducing DNA alkylation, such as O^6-MedGuo formation, in target organs. This appears primarily to involve depression of phase I xenobiotic metabolizing enzymes in the liver, although

PEITC merely prevented the hepatic, but not the pancreatic or pulmonary isoenzyme induction. Further studies are required to investigate how hepatic phase I enzyme reduction influences cell proliferation and DNA methylation in the target organs other than the liver.

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