Diallyl Trisulfide Enhances Benzo[a]pyrene-induced *CYP1A1* Expression and Metabolic Activation in Hepatic HepG2 Cells

SHIGEYUKI UNO¹, MIZUKI SAKAI^{1,2}, YUI FUJINARI^{1,2}, TAKASHI HOSONO²,
TAIICHIRO SEKI² and MAKOTO MAKISHIMA¹

¹Division of Biochemistry, Department of Biomedical Sciences, Nihon University School of Medicine, Tokyo, Japan;

²Department of Chemistry and Life Science, Nihon University College of Bioresource Sciences, Kanagawa, Japan

Abstract. *Background/Aim:* Benzo[a]pyrene (BaP), an environmental pollutant produced by combustion processes, induces expression of cytochrome P450 (CYP) 1A1 via the activation of aryl hydrocarbon receptor (AHR). Induced CYP1A1 is involved in BaP metabolism, resulting in either detoxification or metabolic activation in a context-dependent manner. The effect of diallyl trisulfide (DATS), a garlic-derived organosulfur compound, on BaP metabolism has not been investigated. *Materials and Methods:* The combined effect of DATS and BaP on BaP metabolism in hepatocyte-derived HepG2 cells was examined. *Results:* DATS enhanced BaP-induced CYP1A1 and CYP1B1 mRNA expression, BaP hydroxylation and BaP–DNA adduct formation. Combined treatment of BaP and DATS also increased reactive oxygen species levels. DATS enhanced BaP-induced AHR recruitment and histone H3 acetylation on the CYP1A1 promoter. *Conclusion:* DATS combined treatment enhances BaP metabolic activation through an AHR-modulating mechanism.

Benzo[a]pyrene (BaP) is a polycyclic aromatic hydrocarbon pollutant found in cigarette smoke, charcoal-grilled food and coke ovens, that has been reported to increase the risk of several human diseases, such as lung and colon cancer and atherosclerosis (1-3). The aryl hydrocarbon receptor (AHR) is activated by BaP and induces the expression of genes involved in xenobiotic metabolism, such as cytochrome P450 (CYP) 1A1, CYP1A2, CYP1B, NAD(P)H:quinone oxidoreductase-1 (NQO1) and glutathione S-transferase A1 (4, 5). Sequential coordinated reactions by xenobiotic enzymes induce effective BaP detoxification (3, 6).

However, the induced xenobiotic enzymes, such as CYP1A1 and CYP1B1, can also mediate BaP metabolic activation, a mechanism that enhances BaP toxicity leading to DNA adduct formation and reactive oxygen species (ROS) production (1, 7, 8). *Cyp1a1*(–/–) mice have higher BaP–DNA adduct levels and lower BaP clearance compared to wild-type mice (9), and CYP1A1 overexpression suppresses BaP–DNA adduct formation in hepatocytes (10), indicating that CYP1A1 plays a role in BaP detoxification. By contrast, CYP1A1-null hepatocytes are resistant to BaP toxicity (11) and CYP1A1 is necessary for generation of the potent carcinogen BaP-7,8-diol-9,10-epoxide (6, 7, 12). Compensatory induction of CYP1B1 in *Cyp1a1*(–/–) mice enhances BaP metabolic activation (13, 14). Thus, CYP1 enzymes metabolize BaP, resulting in either detoxication or metabolic activation in a context-dependent manner.

Garlic (*Allium sativum* L.) has been used as a spice and as an effective phytochemical medicine in ancient China, Egypt, Greece, India and Roma (15). Garlic has anticancer, antioxidant, antidiabetic, and cardio-protective activities (15-18). Alliin, a sulfoxide contained in garlic, is decomposed into allicin, which is an odorous component that is converted into several sulfides, such as diallyl sulfide, diallyl disulfide (DADS) and diallyl trisulfide (DATS), in crushed garlic (19). DATS is included in 39% of garlic oil and exerts the therapeutic effects of garlic (15-18, 20). DATS is the most potent garlic-derived sulfide for exhibiting anticancer and hepatoprotective activities (15, 21). However, the underlying mechanisms of DATS effect remain largely unknown.

Diallyl polysulfide induces expression of *CYP2B* and *CYP3A* genes through a mechanism mediated by constitutive androstane receptor, a xenobiotic-sensing nuclear receptor, in rat liver (22), indicating that garlic constituents influence xenobiotic metabolism. BaP is found not only in cigarette smoke but also in foods, such as hamburger and smoked cheese (3, 13). The combined effect of the dietary factors BaP and DATS has been poorly investigated. In this study, we examined the effect of DATS on BaP metabolism in hepatocytes.

Correspondence to: Makoto Makishima, Division of Biochemistry, Department of Biomedical Sciences, Nihon University School of Medicine, Itabashi-ku, Tokyo 173-8610, Japan. Tel/Fax: +81 339728199, e-mail: makishima.makoto@nihon-u.ac.jp

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Materials and Methods

Cell culture. Human hepatocyte-derived HepG2 cells (RIKEN Cell Bank, Tsukuba, Japan) were cultured in DMEM containing 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were treated with vehicle control, 1 µM BaP (Tokyo Chemical Industry, Tokyo, Japan), and/or 50-200 µM DATS (21) for 24 h. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) (1 nM; Wako Pure Chemical, Osaka, Japan) was also used as a potent AHR activator.

Reverse transcription and real-time quantitative polymerase chain reaction. Total RNA was extracted using the acid guanidium thiocyanate/phenol/chloroform method, and cDNA was synthesized using the ImProm-II Reverse Transcription system (Promega Corporation, Madison, WI, USA) (23). Real-time polymerase chain reaction was performed with Chromo4 Real Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) using Power SYBR Green PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA). Primer sequences for human *CYP1A1*, *CYP1A2*, *CYP1B1* and *GAPDH* (the gene encoding glyceraldehyde-3-phosphate dehydrogenase) were reported previously (24, 25). Other primer sequences used were as follows: *HMOX1*, 5'-CTC TTT GAG GAG TTG CAG GA-3' and 5'-CCA GAA AGC TGA GTG TAA GG-3'; *NQO1*, 5'-CAC GGG GAC ATG AAT GTC AT-3' and 5'-CAG GCG TTT CTT CCA TCC TT-3'. The mRNA levels were quantified with standard curves using the corresponding cloned cDNAs inserted into expression plasmids and adjusted to those of *GAPDH* as reported previously (14).

BaP hydroxylation. Extracts from cell microsomal fractions were subjected to BaP hydroxylation assay (26, 27). Briefly, 50 µg of microsomal protein was incubated in 100 mM potassium phosphate buffer (pH 7.4) containing 1 mg/ml bovine serum albumin, 80 µM BaP and 0.3 mM NADPH at 37°C for 30 min. The reaction was stopped by the addition of acetone/n-hexane (1:3). After the organic phase was extracted with NaOH, the concentration of 3-hydroxy-BaP was measured spectrofluorometrically with activation at 396 nm and fluorescence at 522 nm.

Measurement of DNA adducts. BaP-induced DNA adduct levels in HepG2 cells were determined by a ³²P-postlabeling method (9). After DNA extraction, hydrolysis to 3'-phosphodeoxynucleotides with micrococcal endonuclease and spleen phosphodiesterase and treatment with nuclease P1 (Yamasa Corporation, Chiba, Japan) were carried out to enrich the adducts. The 3'-phosphodeoxynucleotides were labeled at the 5' positions with [³²P]ATP and T4 polynucleotide kinase. The ³²P-labeled DNA adducts were resolved with two-dimensional thin-layer chromatography on polyethyleneimine cellulose sheets using the following solvents: D1=1 M sodium phosphate, pH 6.0; D3=2.7 M lithium formate/7.6 M urea; D4=0.8 M lithium chloride/0.4 M Tris-HCl/8.2 M urea, pH 8.0; and D5=1.5 M sodium phosphate, pH 6.0, visualized and quantified by scintillation counting. DNA adduct levels were evaluated as relative adduct labeling values ([cpm in adducts]/[cpm in total nucleotides]) normalized to the amount of DNA in each sample (23).

Measurement of ROS levels. Total ROS and reactive nitrogen species in conditioned media of cell culture were determined as dichlorofluorescein signal levels using the OxiSelect In Vitro ROS/RNS assay kit (Cell Biolabs, San Diego, CA, USA).

Chromatin immunoprecipitation. Chromatin immunoprecipitation (ChIP) was performed as reported previously (24). After nuclear proteins were cross-linked to DNA in 1% formaldehyde for 15 min, cells were washed and lysed in lysis buffer (50 mM Tris-HCl, pH 8.1, 1% SDS, and 10 mM EDTA). After sonication and removal of cellular debris, the lysates were diluted in ChIP dilution buffer (16.7 mM Tris-HCl, pH 8.1, 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, and 167 mM NaCl). ChIP was performed with control IgG antibody, anti-AHR antibody, or anti-Acetyl-Histone H3 (Lys9/Lys14) antibody (Cell Signaling Technology, Boston, MA, USA). DNA was purified with MonoFas DNA Purification Kit (GL Sciences, Torrance, CA, USA). For quantitative polymerase reaction, the Chromo4 Real Time PCR Detection System (Bio-Rad Laboratories Inc.) was used with the Power SYBR Green PCR Master Mix (Thermo Fisher Scientific). The primers 5'-GAA CGC TGG GCG TGC AGA TGC CTC-3' and 5'-CAC TAA GGC GAT CCT AGA GGC TG-3' were used to detect the -379 to -693 region in the *CYP1A1* promoter as reported previously (25).

Statistical analysis. Data are presented as means±S.D. We performed one-way ANOVA followed by Tukey's multiple comparisons or Student's *t*-test to assess significant differences.

Results

DATS enhances BaP-induced CYP1A1 expression in hepatocytes. To examine the combined effect of BaP and DATS on CYP1A1 expression, hepatocyte-derived HepG2 cells were treated with several concentrations of DATS in the presence or absence of BaP. In the absence of BaP, DATS at 50-200 µM did not increase *CYP1A1* mRNA levels in HepG2 cells (Figure 1A). Interestingly, DATS combined treatment further increased *CYP1A1* mRNA levels in cells treated with 1 µM BaP in a concentration-dependent manner. We next examined the combined effect of DATS and another AHR ligand, TCDD, on *CYP1A1* expression. TCDD is a high-affinity AHR ligand that is resistant to metabolism in cells (28). TCDD at 1 nM effectively induced *CYP1A1* mRNA expression in HepG2 cells as reported previously (29), and DATS at 100 µM further elevated its expression in cells treated with TCDD (Figure 1B).

We examined the combined effect of DATS and BaP on the expression of other AHR target genes, *CYP1A2*, *CYP1B1* and *NQO1*. DATS alone had no effect on gene expression (Figure 2A), although it has been previously reported to induce NQO1 expression through activation of nuclear factor E2-related factor 2 (NRF2) (30). Combined treatment with BaP and DATS effectively induced *CYP1B1* expression but not *CYP1A2* or *NQO1* expression (Figure 2A). These findings indicate that the enhancing effect of DATS on AHR target gene expression is gene selective. We also examined the combined effect of BaP and DATS on another NRF2 target gene, *HMOX1*, which encodes heme oxygenase 1 (30, 31). Combined treatment with BaP and DATS had no effect on *HMOX1* expression (Figure 2B).

Figure 1. Combined treatment with aryl hydrocarbon receptor (AHR) ligand and diallyl trisulfide (DATS) increases cytochrome P450 1A1 (*CYP1A1*) expression. (A) Effect of the combination of benzo[a]pyrene (BaP) and DATS. HepG2 cells were treated with 0, 50, 100 or 200 μ M DATS with or without 1 μ M BaP for 24 h ($n=3$ for each group). (B) Effect of the combination of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and DATS. HepG2 cells were treated with vehicle control (-), 100 μ M DATS and/or 1 nM TCDD for 24 h. mRNA values are relative to those of cells treated with BaP alone ($n=4$ for each group). * $p<0.05$; ** $p<0.01$; *** $p<0.001$ (one-way ANOVA followed by Tukey's comparisons).

DATS enhances BaP metabolism. Since CYP1 enzymes are involved in both BaP detoxification and metabolic activation, BaP metabolism in HepG2 cells treated with BaP and DATS was examined. BaP hydroxylation into 3-hydroxy BaP is catalyzed by CYP1A1 and not by CYP1A2 or CYP1B1 (10). Treatment of cells with BaP, but not DATS, increased BaP hydroxylation activity and the combination of BaP and DATS further increased BaP hydroxylation (Figure 3A). The combined treatment with DATS also enhanced BaP-DNA adduct formation (Figure 3B). While treatment with BaP or DATS alone did not increase ROS levels, the combined treatment with these compounds increased ROS levels in culture media (Figure 3C). These findings indicate that DATS enhances BaP metabolism, likely favoring metabolic activation.

DATS enhances AHR transcriptional activity on the *CYP1A1* gene in cells treated with BaP. To examine the effect of DATS on AHR recruitment on the *CYP1A1* gene, chromatin immunoprecipitation (ChIP) assay was performed using the anti-AHR antibody. BaP, but not DATS, increased AHR binding to the *CYP1A1* promoter sequence, although the extent of binding was not statistically significant, and the combination of BaP and DATS effectively increased AHR recruitment (Figure 4). AHR recruitment was correlated with increased acetylated histone H3 at the *CYP1A1* promoter in cells treated with BaP or BaP plus DATS (Figure 4). Therefore, DATS enhanced BaP-induced AHR recruitment and epigenomic modification of histone at the *CYP1A1* gene.

Discussion

In this study, DATS was found to enhance BaP-induced *CYP1A1* and *CYP1B1* expression and BaP metabolism in hepatocyte-derived HepG2 cells. The induction of *CYP1A1* mRNA expression was associated with increased AHR recruitment and histone H3 acetylation at the *CYP1A1* gene in cells treated with BaP plus DATS. Although DATS can activate another transcription factor, NRF2 (30, 32), the effect of DATS on *CYP1A1* expression is unlikely mediated by NRF2 activation, because we did not observe a combined effect of BaP and DATS in inducing NRF2 target genes. Allyl mercaptan, which is a metabolite of DADS and DATS (33), inhibits histone deacetylase (34). DADS increases histone H3 and H4 acetylation in rat intestine (35). These findings suggest that DATS enhances AHR transcriptional activity on *CYP1A1* through an epigenetic mechanism, such as histone modification. The combined treatment of BaP and DATS was not effective in inducing *CYP1A2* expression. Similar to DATS, 1 α ,25-dihydroxyvitamin D₃ enhanced BaP-induced transcription of *CYP1A1* but not *CYP1A2* in human monocyte/macrophage-derived cells (25). Although the human *CYP1A1* and *CYP1A2* genes are located in a head-to-head orientation on chromosome 15 and share a common regulatory region (36), transcription of these genes is thought to be regulated by AHR and additional gene-selective factor(s). Further studies are needed to elucidate a detailed molecular mechanism of DATS action.

Figure 2. Effect of combined treatment with benzo[a]pyrene (BaP) and diallyl trisulfide (DATS) on CYP1A2, CYP1B1, NQO1 and HMOX1 mRNA expression. (A) Expression of aryl hydrocarbon receptor (AHR) target genes, CYP1A2, CYP1B1 and NQO1. (B) Expression of a nuclear factor E2-related factor 2 target gene, HMOX1. HepG2 cells were treated with 0, 50, 100 or 200 μM DATS with or without 1 μM BaP for 24 h (n=3 for each group). mRNA values are relative to those of cells treated with BaP alone. ***p<0.001 (one-way ANOVA followed by Tukey's comparisons).

Combined treatment with BaP and DATS enhanced BaP–DNA adduct formation and increased ROS production. We previously reported that overexpression of CYP1A1, but not CYP1B1, decreases BaP–DNA adduct levels in HepG2 cells (10). CYP1B1 is responsible for the

increased BaP–DNA adduct formation in *Cyp1a1*(–/–) mice (14). Although CYP1A1 induces BaP detoxification rather than metabolic activation, the coincident induction of CYP1A1 and CYP1B1, not CYP1A2, in BaP plus DATS-treated cells may cause BaP metabolic activation

Figure 3. Effect of combined treatment with benzo[a]pyrene (BaP) and diallyl trisulfide (DATS) on BaP metabolism. (A) BaP hydroxylation activity. (B) DNA adduct formation. R.A.L.: Relative adduct labeling values. (C) Reactive oxygen species (ROS) production. ROS levels in conditioned media were determined as dichlorofluorescein (DCF) signal levels. HepG2 cells were treated with vehicle control (–), 100 µM DATS and/or 1 µM BaP for 24 h (*n*=3 for each group in (A) and (C); *n*=4 for each group in (B)). For BaP hydroxylation, extracts from cell microsomal fractions were subjected to BaP hydroxylation assay. **p*<0.05; ***p*<0.01 (one-way ANOVA followed by Tukey's comparisons) (A and C). **p*<0.05 (Student's *t* test) (B). n.d.: Not detected.

Figure 4. Diallyl trisulfide (DATS) enhances aryl hydrocarbon receptor (AHR) recruitment induced by benzo[a]pyrene (BaP) on the CYP1A1 gene. Chromatin immunoprecipitation (ChIP) analyses for AHR recruitment and histone H3 acetylation on the CYP1A1 promoter. Cells were treated with vehicle control (–), 100 µM DATS and/or 1 µM BaP for 6 h, and subjected for ChIP using anti-AHR antibody or anti-acetylated histone H3 antibody (*n*=4 for each group). **p*<0.05; ***p*<0.01 (one-way ANOVA followed by Tukey's comparisons).

(8), leading to increased BaP–DNA adduct formation and ROS production.

Dietary BaP enhances the pathogenesis of colon cancer, fatty liver change and atherosclerosis (13, 23, 37). On the other hand, garlic has been shown to protect against these diseases (15, 38, 39). Our results show that DATS, a garlic constituent, can disturb the balance of BaP metabolism and enhance BaP metabolic activation. Caution may be needed in limiting garlic intake in combination with a diet rich in grilled food due to increased BaP toxicity. Recently, AHR signaling has been implicated in intestinal and immune homeostasis by responding to tryptophan metabolites (40).

Further studies are needed to elucidate whether DATS enhances the beneficial effect of AHR.

Conflicts of Interest

The Authors declare no conflicts of interest in regard to this study.

Authors' Contributions

S.U. performed experiments, analyzed the data and wrote the manuscript. M.S., Y.F., and T.H. performed experiments and analyzed the data. T.S. supervised the study and edited the manuscript. M.M. supervised experiments, wrote the manuscript and edited the manuscript.

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