Mutation Spectrum Induced by Dihydropyrazines in *Escherichia coli*

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Dihydropyrazine (DHP), which induces mutagenesis in *E. coli*, was investigated. From analyzing mutations in the chromosomal *rpoB* gene, the mutation spectrum in *uvrB* strain revealed the different behavior on exposure to two DHP derivatives 3-hydro-2,2,5,6-tetramethylpyrazine (HTMP), and 2,3-dihydro-5,6-dimethylpyrazine (DHDMP). A higher level of DHP-induced mutation was observed, with base substitutions at G:C pairs predominant. HTMP and DHDMP increased the frequency of G:C to T:A transversions. HTMP increased the frequency of G:C to A:T transitions, than did DHDMP. These findings suggest that DHPs prefer to attack the G:C pair and that different DHP derivatives may prefer distinct mutagenic base pairs; and further, that nucleotide excision repair may be involved in the repair of DHP-induced mutations.

Key words dihydropyrazine; DNA damage; *Escherichia coli*; mutation

Dihydropyrazine (DHP) is produced by the condensation of two molecules of D-glucosamine,1 and some have DNA strand-cleaving activity in *vivo*.2,3 DHPs generate hydroxyl and carbon-centered radicals, the reactive species responsible for DNA strand-cleaving reactions in *vivo*.4 In addition, highly reactive DHPs enter into a diamine-exchange reaction,5,6 and some of the products also cause DNA strand breakage in *vivo*.7 Since many pyrazine derivatives have been found in various foods8 and human urine,9 we speculated that DHPs, which are presumed precursors of pyrazines, would be potentially damaging in *vivo*. DHPs are universal in the human body,10 however, there is little reported concerning the biological and physiological roles of DHPs.

Recently, we have reported that DHPs inhibited growth and induced mutagenesis in *E. coli*.11 In the present study, to investigate the mutation spectrum of DHPs using *Escherichia coli*, we exposed wild-type and *uvrB* strains with two DHPs: 3-hydro-2,2,5,6-tetramethylpyrazine (HTMP) and 2,3-dihydro-5,6-dimethylpyrazine (DHDMP), which showed different radical signal pattern and revealed distinct DNA strand-cleaving activities, respectively. We analyzed that DHP induced mutations in *rpoB* gene, and then observed the variations in the mutational effects of the two DHPs.

MATERIALS AND METHODS

Synthesis of DHPs The DHPs (Fig. 1) were synthesized by condensation of diketones and diamines. HTMP and DHDMP were prepared by the method of Yamaguchi et al.21

Bacterial Strains *E. coli* K-12 strains, AB1157 [ara-14 argE3 galK2 his-4 lacY1 leuB6 metl-1 proA2 rpsL31 supE44 thi-1 thr-1 tss-33 ydl-5]12 and AB1885 [AB1157*uvrB5*]12 were obtained from the National Institute of Genetics, Stocks Research Center (Mishima, Japan).

Survival Assay Overnight cultures of *E. coli* were diluted 100 fold in fresh LB medium and grown at 37 °C until the OD600 reached about 0.1. Cells were treated with DHPs for 1 h with shaking at 37 °C, plated on LB agar, and then incubated overnight at 37 °C. Survival rates were calculated as the percentage of cells surviving after treated with HTMP or DHDMP. All experiments were conducted at least four times.

Mutagenesis Assay Overnight cultures of *E. coli* were diluted 100 fold in fresh LB medium and grown at 37 °C until the OD600 reached about 0.1. Cells were treated with DHPs for 1 h with shaking at 37 °C. Samples were washed twice in M9 salts, plated on LB agar containing rifampicin (100 μg/ml), and incubated overnight at 37 °C. The number of cells was measured by plating on LB agar and counting colonies. All experiments were conducted at least four times.

Analysis of Mutations The rifampicin-resistant (Rif+)

mutants were selected as described above. To obtain the independent mutants from each case, we randomly picked up 96 colonies from 100 μg/ml rifampicin containing plates. Each mutant was restreaked on fresh 100 μg/ml rifampicin containing plates, isolated, and resuspended into 20 μl of Ex Taq PCR mixture (TaKaRa, Shiga, Japan). The *rpoB* mutation cluster II fragment was amplified by PCR with 5′-tcgaggcttc-

cggatactctgagc-3′ and 5′-ggtacgtgcgttctgtaac-3′. Amplified fragments were purified using Wizard PCR Prep DNA purification system (Promega), sequenced by the sequencing primer 5′-ctccgatggtcggcag-3′ and the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) using an ABI PRISM 3100 automatic sequencer (Applied Biosystems).

Fig. 1. Chemical Structures of the DHPs Employed in This Study

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RESULTS

Recently, we had demonstrated that DHPs induced mutagenesis by using uvrB-deficient or uvrB-proficient E. coli strains with different backgrounds. To control for that in this work, we examined the DHP induced mutant frequency by using strains with an isogenic background.

Figure 2 shows the survival rate and the frequency of rifampicin-resistant mutation induced by DHPs in wild-type or uvrB. In AB1157 background, consistent with the previous study, HTMP and DHDMP increased mutation frequency in the uvrB strain, however, hardly increased mutation frequency in the wild-type strain.

To identify DHP-induced mutations, we chose 15 mM of DHPs as the dose for the selection of the Rif r mutants and amplified a 1 kb fragment including the rpoB mutation cluster II, which is the major mutation hot spot in the rpoB gene. As a result, above 90% of the Rif r mutants were identified in cluster II (data not shown).

Table 1 shows the types of spontaneous or DHP-induced mutations in cluster II within wild-type or uvrB strains, in which the majority of the identified mutations were single base substitutions. In wild-type, most of the spontaneous mutations are G:C to A:T transitions (71.9%), followed by G:C to T:A transversions (18.8%) and A:T to T:A transversions (9.4%). In uvrB, most of the spontaneous mutations are G:C to A:T transitions (41.9%), followed by G:C to T:A transversions (32.6%), G:C to C:G transversions (9.3%), A:T to T:A transversions (4.7%), and A:T to G:C transitions (4.7%); most of the HTMP induced mutations are G:C to A:T transitions (52.2%), followed by G:C to T:A transversions (44.6%), A:T to T:A transversions (2.2%), and A:T to G:C transitions (1.1%); most of the DHDMP induced mutations are G:C to T:A transversions (54.1%), followed by G:C to A:T transitions (36.5%), G:C to C:G transversions (2.4%), A:T to T:A transversions (1.2%), and A:T to G:C transitions (1.2%).

Figure 3 shows the sites of the single base substitutions detected in the uvrB strain. There were four apparent hot spots, 1535C, 1546G, 1592C, and 1691C. HTMP increased the G:C to T:A mutation at 1535C and the G:C to A:T mutation at 1592C and 1691C, respectively; and DHDMP increased the G:C to T:A mutation at 1535C and the G:C to A:T mutation at 1691C.

DISCUSSION

To investigate the mutation spectrum caused by DHPs, we used two DHP derivatives HTMP and DHDMP, and employed Rif r mutation assay. The target of rifampicin is the β subunit of RNA polymerase, which is a product of the rpoB gene. Rif r mutants predominantly have a base substitutional missense mutation in the rpoB gene. The two regions of the β subunit of RNA polymerase are known to accumulate Rif r mutants: cluster I (amino acids 140—148) and cluster II (amino acids 508—574), with the latter being the major mutational hot spot. Consistent with this, spontaneous or DHP-induced mutations within wild-type or uvrB strain in our study were mainly single base substitutions located in the cluster II.
HTMP and DHDMP increased mutation frequency more in the uvrB strain, however, not in the wild-type (Fig. 2). In addition, the effects of HTMP and DHDMP on the overall mutation spectra are different in the uvrB strain (Table 1): HTMP increased G:C to T:A transversions and G:C to A:T transitions, while DHDMP increased the frequency of G:C to T:A transversions. Furthermore, the mutation hot spots, which were induced in the uvrB strain by HTMP and DHDMP, were also distinct (Fig. 3). These findings suggest that DHPs prefer to attack the G:C pair, which is consistent with our previous finding that DHPs preferentially cleave at guanine sites in vitro.\(^1\) Moreover, it is suggested that different DHP species cause different mutagenic base pairs.

A possible explanation for the increase of G:C to T:A transversions might arise from the oxidation of G to 8-hydroxydeoxyguanine in DNA.\(^1\)\(^5\)\(^6\)\(^7\) Other damaged DNA bases, \(O^6\)-alkylguanine or 5-hydroxydeoxycytidine, might increase G:C to A:T transitions.\(^1\)\(^7\)\(^8\)\(^9\) Previously, we reported that DHPs generate hydroxyl and carbon-centered radicals; and moreover, we have recently observed the formation of 8-hydroxydeoxyguanosine by DHPs;\(^4\)\(^9\)\(^10\) and participation of \(\cdot O_2\) in the effects of DHPs.\(^1\)\(^0\) Therefore, it is suggested that DHPs might instigate oxidative DNA damage and/or DNA alkylation, which resulted in mutation. In addition, HTMP has considerably stronger DNA strand-cleaving activity than DHDMP;\(^1\)\(^1\) and the ESR spectra of HTMP and DHDMP show remarkable differences.\(^1\)\(^2\)\(^1\)\(^3\) Thus, it is speculated that radicals generated from HTMP could cause DNA alkylation more effectively than that of DHDMP;\(^1\)\(^9\) we will examine that in the future.

The uvrB gene codes UvrB protein, a subunit of the UvrABC endonuclease that plays a role in the repair of various types of DNA lesions: UvrA dimer and UvrB monomer form a complex and initially recognize DNA damage, including oxidative DNA damage, abasic sites, and bulky adducts.\(^1\)\(^1\)\(^2\)\(^1\)\(^3\) Considering the results together, it is suggested that nucleotide excision repair may be involved in the repair of DHP-induced mutations.

DHP species are included in the Maillard reaction intermediates. The Maillard reaction is thought to play a role in the pathophysiology of aging and diabetes.\(^2\)\(^3\) Thus, DHP may be involved in aging and diabetes related diseases. Moreover, studies of porphyria suggest that the DNA damaging ability of DHPs are potentially carcinogenic. In acute intermittent porphyria (AIP), the heme precursor 5-aminolevulinic acid (ALA) accumulates. Since the DHP, 2,5-dicarboxyethyl-3,6-dihydropyrazine, generated by chemical dimerization of two ALA molecules, promotes DNA strand-cleavage in vitro, it is likely that DNA damage induced by ALA dimerization would lead to an increased risk of hepatocellular carcinoma in AIP patients.\(^2\)\(^4\)\(^2\)\(^5\)

In conclusion, DHPs appear to cause DNA mutations with different preferences in vivo, and nucleotide excision repair may be involved in the repair and the fixation of DHP-induced mutations. Further studies will be necessary to determine the DNA repair systems involved in the removal of the DHP-induced DNA lesions.

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