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Induction of *Escherichia coli* oh^{8}Gua Endonuclease by Some Chemicals in the Wild Type and mutM Mutant Strains

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The effects of nalidixic acid, mitomycin C, and cadmium chloride ($CdCl_2$) on the activity of 8-hydroxyguanine (oh^{8}Gua) endonuclease, a DNA repair enzyme for oxidatively modified guanine, oh^{8}Gua were studied. Nalidixic acid and mitomycin C, typical inducers of the SOS DNA repair response in *E. coli*, showed different effects. Nalidixic acid raised the activity of this enzyme, but mitomycin C did not show such an effect. Cadmium chloride also induced the enzyme activity. These results show that the expression of oh^{8}Gua endonuclease is regulated by multiple factors and can be induced under stressful conditions. In an attempt to demonstrate the importance of this enzyme in defense against DNA damage and mutagenesis, we also characterized *mutM* mutant for its oh^{8}Gua endonuclease activity. The *mutM* mutant showed no detectable oh^{8}Gua endonuclease activity, unlike its wild type showing high activity. In addition, paraquat, a superoxide producing compound, failed to elevate oh^{8}Gua endonuclease activity in this mutant. These results suggest that the *mutM* gene is identical to the oh^{8}Gua endonuclease gene of *E. coli*. Taken together with previous reports, these results suggest that oh^{8}Gua endonuclease plays a crucial role in the protection of aerobically growing organisms from threats of oxidative DNA damage and mutation.

DNA damages by reactive oxygen species generated in aerobically growing cells have been known to play an important role in spontaneous mutagenesis, carcinogenesis, and the aging process (Halliwell, 1991). The C-8 position of deoxyguanosine residues in DNA can be hydroxylated by reactive oxygen species to form 8-hydroxyguanine (oh^{8}Gua) residues (Kasai et al., 1994). Oh^{8}Gua in DNA can be miscoded to yield G-C to T-A transversion mutations (Kuchino et al., 1987), eventually leading to mutation and carcinogenesis (Cheng et al., 1992). Thus, aerobic organism need a defense mechanism(s) to prevent the impairment of genetic integrity induced by oh^{8}Gua residues in DNA.

The repair enzyme for oh^{8}Gua in DNA, oh^{8}Gua endonuclease, was purified from *E. coli* (Chung et al., 1991a). This enzyme cleaves DNA strand 3' and 5' to the oh^{8}Gua producing a single nucleotide gap in the modified nucleotide. Similar enzymatic activity was also detected in human leukocyte (Chung et al., 1991b). Meanwhile, this enzyme has been suggested to be identical to two other proteins encoded by previously known *E. coli* genes, *fpg* and *mutM*. The *fpg* gene encodes formamidopyrimidine (Fapy)-DNA glycosylase, which removes Fapy (imidazole ring open form of purines). Studies on the substrate specificity of this enzyme showed that oh^{8}Gua is an important substrate for this enzyme and strongly suggested that the two enzymes are identical (Tchou et al., 1991; Boiteux et al., 1992). It has also been shown that *mutM* gene, a mutator gene specifically leading to GC→TA transversion in *E. coli*, is identical to the *fpg* gene (Michaels et al., 1991) However, there still remains a possibility that other unidentified endonucleases might contribute to the overall oh^{8}Gua endonuclease activity.

The oxidative stress can be prevented by scavenger enzymes, such as superoxide dismutase (SOD) and catalase. In abnormally high levels of active oxygens, these enzymes counteract toxicity of active oxygens more efficiently. This adaptive response has been studied on oh^{8}Gua endonuclease (Kim et al., 1996). Expression of oh^{8}Gua endonuclease in *E. coli* was induced under various oxidative stress conditions, including anaerobic to aerobic shift, bubbling of O_2 into the growth medium, addition of O_2^--generating agents (Kim et al., 1996). These observations suggest that

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the expression of oh[8]Gua endonuclease might be regulated by several mechanisms. Thus, a question was raised on whether the SOS response is involved in the regulation of oh[8]Gua endonuclease. To answer this question, we tested the effects of nalidixic acid and mitomycin C, typical inducers of the SOS DNA repair response, on the activity of this enzyme. We also tested another chemical, cadmium chloride. In addition, we examined whether the *mutM* gene is responsible for the repair of oh[8]Gua in normally growing *E. coli.*

Materials and Methods

Bacterial strains and chemicals

The *E. coli* strains used in this study are listed in Table 1. AB1157 is the wild type without any mutation on the *recA* and *lexA* genes, which are essential for the SOS repair response (Walker, 1995). TT101 is a *mutM* deficient mutant derived from its wild type, CC104. Bacteria were grown in LB medium. Growth of bacteria was monitored by reading OD_{600nm}. Nalidixic acid, mitomycin C, cadmium chloride, and other reagents were all obtained from Sigma Chemical Co., unless otherwise indicated.

Oligodeoxynucleotide for substrate

The oligodeoxynucleotide used as a substrate for the endonuclease assay was chemically synthesized as described previously (Kuchino et al., 1987). It is a 46 mer oligodeoxynucleotide containing one oh[8]Gua residue at the defined position. Its sequence and the site of base modification is shown in Fig. 1. The oligodeoxynucleotide was labeled with ^{32}P at the 3' terminus as described by Chung et al., (1991a). To obtain a duplex DNA substrate, the 3'-labeled oligodeoxynucleotide was annealed with an excess amount of unlabeled complementary oligodeoxynucleotide for 10 min at 65℃ and slowly cooled to room temperature.

Treatment of bacterial culture with chemicals

Overnight cultures of *E. coli* were used to inoculate fresh media. The cultures were allowed to grow to OD_{600} of 0.3-0.4 with shaking at 200 rpm. The cells were further incubated in the absence and presence of nalidixic acid, mitomycin C, cadmium chloride, or paraquat (methyl viologen) for 1-3 h at 37℃ depending on the chemicals used. The growth of bacteria was monitored by measuring OD_{600} before and after addition of the chemicals. The cells were then harvested for enzyme assays.

Table 1. *E. coli* strains used in this study

Strain	Genotype and Characteristics	Reference
AB1157	F- *thr-1 leu-6 proA2 his4 thi-1 argE3 lacY1 galK2 ara-14 xyl-5 mtl-1 tsx-33 strA31 sup-37* (wild type, *nfo+ nth+ xth+ recA+ lexA+*)	Bachmann, 1972
CC104	*ara*, Δ(*gpt -lac*)$_5$, *rpsL* [F'*lacI*378, *lacZ*461, *proA*$^+$*B*$^+$] (wild type)	Cupples and Miller, 1989
TT101	Identical to CC104 except for *mutM*::mini-*tet* (*mutM* mutant)	Michaels *et al.*, 1991

mutM, mutator gene that specifically increases GC→TA transversions.

5'-CAGCCAATCAGT(G-OH)CACCATCCCGGGTCGTTTT
AGAACGTCGTGACT-3'

Fig. 1. Sequence of an oligonucleotide substrate used in the oh[8]Gua endonuclease assay. G-OH indicates the position of oh[8]Gua (8-hydroxyguanine)

Cell extracts

Crude extracts of *E. coli* cells were prepared as follows. The cultures were chilled and centrifuged at 10,000 g for 15 min and the pellets were washed once with 1 ml of 50 mM potassium phosphate buffer containing 1 mM EDTA and stored at -70℃ until use. The pellets were thawed and suspended in 50 mM potassium phosphate buffer containing 1 mM EDTA (pH 7.0) and disrupted by sonication for 1 min (12 sec × 5). Cell debris was removed by centrifugation at 27,000 g for 20 min, and the supernatant was used for enzyme assays. The protein concentration was determined by the bicinchoninic acid method (Smith et al., 1985) using bovine serum albumin as a standard.

Oh[8]Gua endonuclease assay

The assay for the oh[8]Gua endonuclease activity was performed as described previously (Chung et al., 1991a). The reaction mixture (20 μl) contained 1.0 pmol of ^{32}P-labeled duplex oligodeoxynucleotide, 50 mM Tris-HCl (pH 7.5), 50 mM KCl, 5 mM EDTA, and 5-10 μg of *E. coli* crude extract. After incubation at 37℃ for 15 min, reactions were terminated by extraction with phenol-chloroform (1:1, v/v). The amount of DNA fragments cleaved at the position of oh[8]Gua were analyzed by running on 20% (w/v) polyacrylamide gel containing 8 M urea. For determination of cleavage location, 1 M piperidine was treated at 90℃ for 30 min, under which condition the oh[8]Gua-containing oligonucleotide is cleaved at the same position as the oh[8]Gua endonuclease (Chung et al., 1992). After electrophoresis at 2000 V for 2 h, cleaved oligonucelotide bands were detected by autoradiography. For quantitation of the results, the transmittance of the bands were measured using a video densitometer (Bio-Rad, Model 620). One unit of the enzyme activity was defined as 1.0 pmol of the substrate oligonucleotide cleaved per 5 min.

Superoxide dismutase assay

Total superoxide dismutase (SOD) activity of the crude extract was assayed by the ferricytochrome c reduction method (McCord and Fridovich, 1969). Xanthine and xanthine oxidase were added as the source of super-

oxide (O_2^-) radicals. Reduction of cytochrome c by O_2^- was monitored by spectrophotometric reading at 550 nm. Xanthine oxidase was added in an appropriate amount that results in an OD_{550} change of about 0.025/min. One unit of SOD was defined as the activity required for 50% inhibition of the rate of cytochrome c reduction under the assay conditions.

Results and Discussion

Effects of SOS inducers

The SOS DNA repair system of *E. coli* is a specialized DNA repair and cellular survival response to DNA damage and is regulated by the RecA and LexA proteins (Walker, 1995). It serves as the mechanism for both survival and mutagenesis after the treatment of lethal dose of DNA damaging agents. Many genes are known to be induced by this system, several of which are unknown for their functions (Walker, 1995). In this study, we examined the relation between regulation of oh^{8}Gua endonuclease and SOS DNA repair system. Therefore, we examined the effects of nalidixic acid and mitomycin C on the oh^{8}Gua endonuclease. In wild type *E. coli* AB1157, nalidixic acid and mitomycin C showed different effects on both the SOD and oh^{8}Gua endonuclease activities (Fig. 2). Nalidixic acid increased the activities of both enzymes in a dose-dependent fashion. On the other hand, mitomycin C showed little or no effect on either of the enzymes at any concentration treated. As shown in upper panels of Fig. 2, nalidixic acid and mitomycin C suppressed the growth of *E. coli* to a similar extent at the treated concentrations. These results suggest that the oh^{8}Gua endonuclease is not regulated by the SOS repair system. Instead, the induction of this enzyme by nalidixic acid might be due to other mechanisms, such as oxidative stress. On the other hand, mitomycin C might not have caused sufficient oxidative stress to induce oh^{8}Gua endonuclease. With the present results alone, it is not known whether the induction by nalidixic acid is indeed mediated by oxidative stress. However, some reports have suggested a possibility that oxidative stress is involved in the induction. For example, primary action mechanism of nalidixic acid and other quinone is independent of the SOS response and the induction of *recA* and SOS response is consequential (Piddock et al., 1990). Further studies using strains with mutations in the *recA* or *lexA* gene will help in clarification of the relationship between the regulation of oh^{8}Gua endonuclease and the SOS repair system.

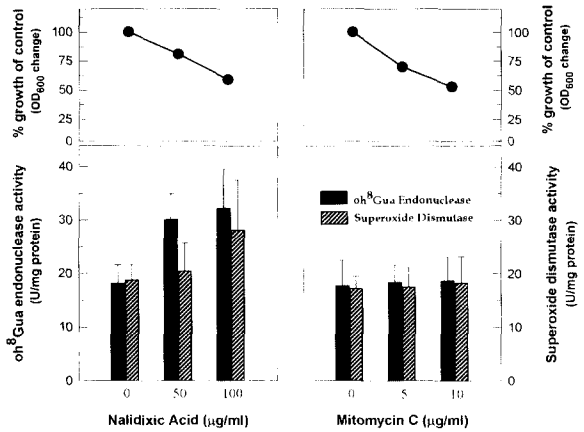

Fig. 2. Effects of nalidixic acid and mitomycin C on the superoxide dismutase and oh^{8}Gua endonuclease activities of *E. coli* AB1157. E. coli were grown at 37℃ in LB medium to reach 0.3-0.4 of OD_{600}. Nalidixic or mitomycin C was added to the culture and further incubated for 1 h. The cells were pelleted, and its extract was assayed for superoxide dismutase and oh^{8}Gua endonuclease. Upper panel shows the degree of growth inhibition by the chemical treatments. The OD_{600} observed without the treatment was expressed as 100%, and the others were as its relative values. Values are mean+S.D. from 3 separate experiments.

Effect of cadmium chloride

Cadmium (Cd) is a highly toxic heavy metal and a ubiquitous environmental contaminant (Moffatt et al., 1992). Cadmium chloride has been known to induce many cellular proteins both in prokaryotic and eukaryotic cells (Craig and Dekker, 1988; Rosenberg and Kappas, 1991; Davalli et al., 1992). In addition, involvement of reactive oxygen species in these inductions has also been suggested (VanBogelen et al., 1987; Abe et al., 1994; Siow et al., 1995).

Therefore, it is of interest to test the effect of cad-

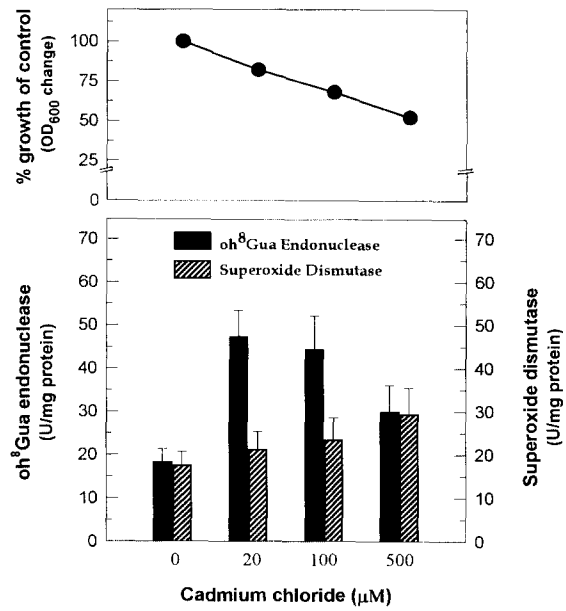

Fig. 3. Effect of cadmium chloride on the superoxide dismutase and oh^{8}Gua endonuclease activities of *E. coli* AB1157. *E. coli* were grown at 37℃ in LB medium until OD_{600} of 0.3-0.4. The culture was then added with cadminum chloride and further incubated for 1 h. The cells were pelleted, and its extract was assayed for superoxide dismutase and oh^{8}Gua endonuclease. Upper panel shows the degree of growth inhibition by cadmium chloride treatment. The OD_{600} observed without the treatment was expressed as 100%, and the others were as its relative values. Enzyme activity data represent mean+S.D. from 3 separate experiments.

mium chloride on the induction of oh[8]Gua endonuclease. Treatment with 20 μM of cadmium chloride, which suppressed bacterial growth to 84% of untreated control (Fig. 3, upper panel), markedly induced the oh[8]Gua endonuclease activity in the AB1157 wild type *E. coli* (Fig. 3, lower panel). At this concentration, SOD showed an slight increase in its activity. Induction of oh[8]Gua endonuclease was attenuated at high concentrations (100 uM and 500 uM), probably as a result of greater inhibition of bacterial growth by this toxic heavy metal (Fig. 3).

The precise mechanisms of cadmium-mediated toxicity and protein induction are unknown. However, some toxic effects of cadmium have been suggested to have resulted from its reaction with essential sulfhydryl groups or zinc (Zn) in protein, causing denaturation of proteins. In addition, production of reactive oxygen species by cadmium is in part due to the inhibition of Zn-containing superoxide dismutase (Jungmann et al., 1993; Abe et al., 1994). The present results, a marked induction of *E. coli* oh[8]Gua endonuclease by cadmium chloride, therefore, would suggest that the oh[8]Gua endonuclease may play an important defensive role against the oxidative stress.

Absence of oh[8]Gua endonuclease activity in mutM strain

Recent studies have suggested that the *mutM* gene of *E. coli* is identical to the *fpg* gene which encodes Fapy-DNA glycosylase (Michaels et al., 1991). There also exist evidences that oh[8]Gua endonuclease of *E. coli* is identical to Fapy-DNA glycosylase (Tchou et al., 1991; Boiteux et al., 1992). It has also been shown that accumulation of oh[8]Gua residues in DNA in the *mutM* mutant strain, suggesting the involvement of the *mut M* gene in repair of oh[8]Gua. There still remains a possibility, however, that other unidentified endonucleases might contribute to the overall oh[8]Gua endonuclease activity. To verify this possibility and to determine to what extent the *mutM* gene is involved in the repair of oh[8]Gua residues in normally growing *E. coli*, we compared the endonuclease activity for oh[8]Gua in the *mutM*-deficient to that in the wild type *E. coli* strains. In addition, we tested for induction of the oh[8]Gua endonuclease activity in both strains upon treatment of paraquat.

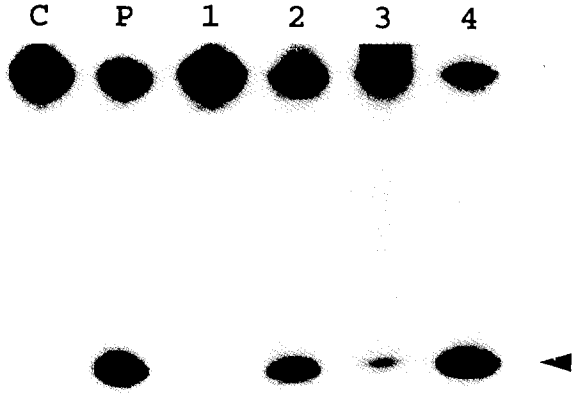

Fig. 4. Absence of oh[8]Gua endonuclease activity in the *mutM* strain. Both wild type (lane 1 and 3) and *mutM* (lane 2 and 4) *E. coli* strains were grown in LB medium with vigorous shaking to saturation. The cells were pelleted, and its extracts in 2 different doses (5 μg, lane 1 and 2; 10 μg, lane 3 and 4) were assayed for oh[8]Gua endonuclease. The assay products were analyzed on a 20% denaturing polyacrylamide gel and autoradiographed as descibed in Materials and Methods. In lane C, no extract was added to the incubation mixture. Lane P shows the piperidine-treated, radiolabeled substrate used as a position marker (see Materials and Methods). The arrowhead indicates the fragment cleaved at the position of oh[8]Gua.

The wild type (CC104) and *mutM* mutant (TT101) strains were grown to saturation at 37℃ in LB medium. Crude extracts prepared from these cultures were assayed for the oh[8]Gua endonuclease activity. As shown in Fig. 4, 5 μg (lane 2) and 10 μg (lane 4) of the CC104 extract generated distinct lower bands which are the cleavage products at the position of oh[8]Gua. On the other hand, TT101 extract did not hydrolyze the p^{32}-labeled oligonucleotide at the same doses (lanes 1 and 3). These results clearly show that the *mutM* mutant strain has negligible repair activity for oh[8]Gua.

Effect of paraquat on oh[8]Gua endonuclease activity

We have previously reported that oh[8]Gua endonuclease could be induced upon treatment of superoxide (O_2^-) producing agents such as paraquat (Kim et al., 1996). Therefore we tested whether the *mutM* or its wild type strain can induce oh[8]Gua endonuclease activity by the paraquat treatment. We also measured SOD activity in the cells, which is known to be considerably induced by the same treatment. (Nunoshiba et al., 1992).

Basal SOD activities without the paraquat treatment were similar in both strains. And paraquat raised the total SOD activity in both strains as shown in Table 2. In contrast to the result with SOD, the paraquat treatment (0.5 mM, 3 h) did not produce any detectable oh[8]Gua endonuclease activity in the *mutM* strain, while the same treatment elevated this enzyme activity in the wild type strain (Table 3). These results suggest that the *mutM* gene is identical to the oh[8]Gua endonuclease gene of *E. coli*. In addition, similar basal and paraquat-induced levels of SOD activity in both

Table 2. Effect of paraquat on the activity of superoxide dismutase of *mutM* and wild type *E. coli* strains

Paraquat Treatment	*E. coli* strains	
	CC104 (wild)	TT101 (*mutM*)
0 mM	17.7±2.3	16.6±2.2
0.1 mM	20.8±3.5	19.1±3.4
0.5 mM	21.9±2.9	18.6±3.2

E. coli strains were grown at 37℃ in LB medium to reach 0.3-0.4 of OD_{600}. Paraquat was added to the culture and further incubated for 3 h. The cells were pelleted, and its extract was assayed for superoxide dismutase. One unit of SOD was defined as the activity to inhibit the rate of cytochrome *c* reduction by xanthine/xanthine oxidase to 50%. Values are mean±S.D. from 3 separate experiments.

Table 3. Effect of paraquat on the activity of oh^{8}Gua endonuclease of *mutM* and wild type *E. coli* strains

Paraquat Treatment	*E. coli* strains	
	CC104 (wild)	TT101 (*mutM*)
0 mM	7.9+2.8	U.D.
0.1 mM	12.4+2.4	U.D.
0.5 mM	11.1+2.2	U.D.

E. coli strains were grown at 37℃ in LB medium to reach 0.3-0.4 of OD_{600}. Paraquat was added to the culture and further incubated for 3 h. The cells were pelleted, and its extract was assayed for oh^{8}Gua endonuclease. One unit of endonuclease was defined as the activity to cleave 1.0 pmol of the substrste DNA for 5 min. Values are mean+S.D. from 3 separate experiments. U.D., undetectable.

the *mutM* and the wild type strain support the idea that mutation of the *mutM* gene shows little or no influence on the primary defense system against superoxide radical.

From the results obtained in this study, it can be concluded that the *E. coli mutM* (*fpg*) gene product is identical to the oh^{8}Gua endonuclease and that this enzyme plays the predominant role in the endonucleolytic repair of oh^{8}Gua residues of damaged DNA.

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