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Oxidation of *N*-Alkyl-*N*-(3-carboxypropyl)nitrosamines by Iron Porphyrin and Oxidant Forms Alkylating Mutagens

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***N*-Alkyl-*N*-(3-carboxypropyl)nitrosamines are known to selectively induce urinary bladder tumor in rats and mice. To detect DNA damage by *N*-alkyl-*N*-(3-carboxypropyl)-nitrosamines, we evaluated their mutagenicity using the Ames assay in *S. typhimurium* and *E. coli* under oxidative conditions of chemical model for cytochrome P450. The activation system consisted of 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrinatoiron(III) pentachloride (4-MPy) plus an oxidant. The *N*-alkyl-*N*-(3-carboxypropyl)-nitrosamines; *N*-methyl-*N*-(3-carboxypropyl)nitrosamine (MCPN), *N*-ethyl-*N*-(3-carboxypropyl)nitrosamine (ECPN), *N*-propyl-*N*-(3-carboxypropyl)nitrosamine (PCPN), *N*-butyl-*N*-(3-carboxypropyl)nitrosamine (BCPN), were treated with 4-MPy and *t*-BuOOH in acetonitrile for 30 min at room temperature, the reaction mixture was extracted with dichloromethane, and the organic phase was assayed for their mutagenicity in *Salmonella typhimurium* TA1535 and *Escherichia coli* WP2 *uvrA*. The dichloromethane extract derived from the reaction mixture of MCPN, ECPN, PCPN or BCPN with 4-MPy plus *t*-BuOOH was mutagenic in both of the strains, indicating that *N*-alkyl-*N*-(3-carboxy-propyl)nitrosamines were oxidized to direct-acting mutagens by the 4-MPy plus *t*-BuOOH. The mutagenicity of oxidized BCPN extract in *S. typhimurium* YG7108 was higher than that in *S. typhimurium* TA1535, suggesting that the mutagenicity derived from BCPN was due to DNA alkylation. Furthermore, the DNA seemed to be butylated, not 3-carboxypropylated, exerting the mutagenicity of BCPN in the presence of 4-MPy and *t*-BuOOH.**

Key words: *N*-alkyl-*N*-(3-carboxypropyl)nitrosamine, chemical model, BCPN, metabolic activation, urinary bladder carcinogen

Introduction

N-Nitrosodibutylamine (NDB) is an environmental carcinogen which has been detected in foods, in the air of the work place, and in rubber articles (1–3). NDB is a bladder carcinogen that also induces tumors in liver and lung. Its 4-hydroxylated derivative, *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN) and its oxidation product *N*-butyl-*N*-(3-carboxypropyl)nitrosamine (BCPN) are also known to be selective urinary bladder carcinogens in rats and other animal species (4–6). The polar carboxylated analogue BCPN is excreted in high amounts in urine after oral application of NDB or BBN and also after direct injection of BCPN into the bladder. Intensive investigations on the structure-activity relationship revealed that this organotropic effect to the urinary bladder is related to oxidation of the terminal carbon atom of NDB. However, the mechanism by which BCPN exerts a tumor induction effect specifically in the urinary bladder is still unclear. To elucidate whether metabolic systems are capable of activating BCPN to genotoxic metabolites, we investigated the mutagenicity of BCPN in the *Salmonella* assay with a chemical model for cytochrome P450. The chemical model consists of water-soluble 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrinatoiron(III) chloride (4-MPy) and *tert*-butyl hydroperoxide (*t*-BuOOH) as an oxidant (7,8). *N*-Alkyl-(3-carboxypropyl)nitrosamines (alkyl = methyl, MCPN; ethyl, ECPN; propyl, PCPN) treated with the chemical model were assayed for their mutagenicity in *Salmonella typhimurium* TA1535 and *Escherichia coli* WP2 *uvrA* (Fig. 1). The mutagenicity of the extracts from the reaction of BCPN with the chemical model was compared between *S. typhimurium* TA1535 and *S. typhimuirum* YG7108. In order to investigate the correlation between the alkyl chain length and the mutagenicity, we compared the mutagenicity of *N*-alkyl-*N*-nitrosoureas (alkyl = methyl, MNU; ethyl,

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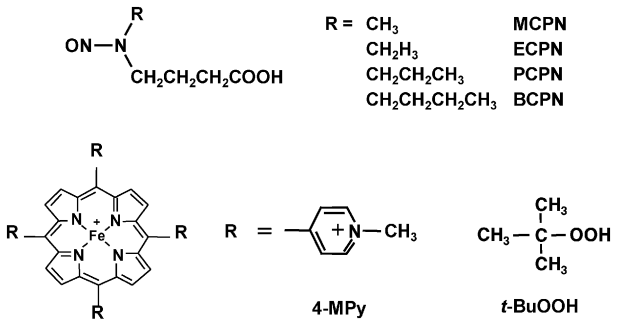

Fig. 1. Structures of chemicals used.

ENU; propyl, PNU; butyl, BNU) in *S. typhimurium* TA1535 and *E. coli* WP2 *uvrA* with that of *N*-alkyl-*N*-(3-carboxypropyl)nitrosamines in both bacterial strains.

Materials and Methods

Chemicals: Sodium ammonium hydrogenphosphate tetrahydrate and iron(II) chloride tetrahydrate were purchased from Merck (Darmstadt, Germany). Ammonium hexafluorophosphate was obtained from Kanto Chemical Co. Ltd (Tokyo, Japan). Tetrakis(1-methylpyridinium-4-yl)porphyrine-*p*-toluenesulfonate, sodium hydride, L-tryptophan and tetrabutylammonium chloride were purchased from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan). Bacto agar and bacto nutrient broth were obtained from Becton Dickinson Microbiology Systems (Sparks, USA). MNU, ENU, PNU and BNU were purchased from Toshin Gousei (Tokyo, Japan). *t*-BuOOH was obtained from Sigma-Aldrich, Inc. (St. Louis, MO) and used as received. Other reagents used were purchased from Wako Pure Chemical Industries (Osaka, Japan). 4-MPy was synthesized as described (9). The purity of 4-MPy was determined by ultraviolet spectroscopy [Yield; 94.0%, at λ_{max} = 400 nm, (ε in 3 M HCl = 0.96×10^4)], and *N*-alkyl-*N*-(3-carboxypropyl)nitrosamines were synthesized by alkylation of 2-pyrrolidone, followed by the nitrosation of the corresponding *N*-alkyl-2-pyrrolidone with $NaNO_2$ under acidic conditions (10). To decompose unknown direct-acting mutagen formed in trace amounts, the *N*-alkyl-*N*-(3-carboxypropyl)nitrosamine was dissolved in methanol with saturated sodium hydroxide and the entire solution was stirred overnight at room temperature (11). The reaction mixture was extracted three times with CH_2Cl_2, and the combined organic phases were dried over Na_2SO_4, filtered, and then evaporated *in vacuo* to give an oil. The structures and purities of compounds were confirmed by nuclear magnetic resonance spectroscopy (JEOL JNM-LA400). Mutagenicity of *N*-alkyl-*N*-(3-carboxypropyl)nitrosamines was assayed in *S. typhimurium* TA1535, *E. coli* WP2 *uvrA* and *S. typhimurium* YG7108 before use. The results in *S. typhimurium* TA1535 and *E. coli* WP2 *uvrA* was shown in Table 1, and the result of BCPN in *S. typhimurium* YG7108 were the following revertants; 20 ± 1 at 0 μmol/plate, 27 ± 1 at 5 μmol/plate, 25 ± 1 at 10 μmol/plate, 22 ± 3 at 20 μmol/plate, 20 ± 1 at 40 μmol/plate.

Reaction of *N*-alkyl-*N*-(3-carboxypropyl)nitrosamines with 4-MPy plus *t*-BuOOH for bacterial mutation assay: To a solution of *N*-alkyl-*N*-(3-carboxypropyl)nitrosamine (2.66 mmol) and 4-MPy (30 mg, 33 μmol) in acetonitrile (120 mL), *t*-BuOOH (23 μL, 165 μmol) was added, and then the mixture was incubated for 30 min at room temperature. Water (500 mL) was added to the reaction mixture and the mixture was extracted six times with CH_2Cl_2 (50 mL). The combined organic phases were dried over Na_2SO_4, filtered, and evaporated *in vacuo* to give a pale yellow oil. The crude product yielded MCPN (recovery yield 98%), ECPN (recovery yield 101%), PCPN (recovery yield 99%) and BCPN (recovery yield 97%). The residue was tested in the Ames assay as described below.

Bacterial mutation assay: The bacterial mutation assay was carried out as described by Maron *et al.* and Brusick *et al.* (12,13). Professor B. N. Ames (University of California, Berkeley, USA) provided the *S. typhimurium* TA1535, and Dr T. Nohmi (National Institute of Health Sciences, Tokyo, Japan) kindly provided the *S. typhimurium* YG7108 and *E. coli* WP2 *uvrA*. The oil obtained above or *N*-nitroso-*N*-alkylurea was diluted to the desired concentrations per 50 μL acetonitrile or DMSO, respectively. Each solution with the concentration was put into a test tube, was added 0.5 mL of 0.1 M sodium phosphate buffer (pH 7.4), and then 0.1 mL of a culture of the tester strain, and finally 2 mL of top agar. The mixture was then poured onto a minimal-glucose agar plate. After incubation for 44 h at 37°C, the colonies were counted. Each sample was assayed using duplicate plates and the data are presented as the number of mean revertant colonies per plate ± standard error (SE) of three independent assay. The results were considered positive if the test produced a reproducible, dose-related increase in the number of revertant colonies, and if the number of the colonies was double the background number of colonies (14).

Results

The *N*-alkyl-*N*-(3-carboxypropyl)nitrosamines were not mutagenic in either *S. typhimurium* TA1535 or *E. coli* WP2 *uvrA* without the activation system (Table 1). Since the *N*-alkyl-*N*-(3-carboxypropyl)nitrosamines have a highly polar carboxyl group, they were easily conjugated with glycine in the mitochondrial fraction or glucuronic acid in the microsomal fraction for excretion from the body. The phase II enzyme in S-9mix could inhibit the activation of BCPN (4,15) and thus, we used a chemical model for cytochrome P450 in the Ames

assay (7,8). The dichloromethane extracts from the reaction mixture contained the chemical model and the *N*-alkyl-*N*-(3-carboxypropyl)nitrosamines MCPN, ECPN, PCPN, BCPN and the extracts were mutagenic in both *S. typhimurium* TA1535 and *E. coli* WP2 *uvrA* (Fig. 2).

The DNA-damaging mechanism of BCPN treated with 4-MPy plus *t*-BuOOH was investigated by comparison of BCPN mutagenicity in *S. typhimurium* TA1535 versus *S. typhimurium* YG7108. Since *S. typhimurium* YG7108 is deficient in O^6-methylguanine methyltransferase, it is highly sensitive to alkylating agents (16). The mutagenicity of BCPN in *S. typhimurium* YG7108 was higher than that in the parent strain *S. typhimurium* TA1535 (Fig. 3).

Since BCPN has two α-methylenes, it can be α-hydroxylated at either alkyl chain, with the consequent formation of both a butylation and a carboxypropylation species. To investigate the effect of the alkyl group with respect to mutagenicity of the *N*-alkyl-*N*-(3-carboxypropyl)nitrosamines, we compared the relative mutagenicity of direct-acting *N*-alkyl-*N*-nitrosoureas in *S. typhimurium* TA1535 and *E. coli* WP2 *uvrA* with that of *N*-alkyl-*N*-(3-carboxypropyl)nitrosamines in both bacterial strains. MNU mutagenicity in *S. typhimurium* TA1535 was higher than that in *E. coli* WP2 *uvrA*, ENU or PNU mutagenicities were similar in both strains, and BNU mutagenicity in *S. typhimurium* TA1535 was lower than that in *E. coli* WP2 *uvrA* (Fig. 4). The relative mutagenicity was calculated from the

Table 1. Direct mutagenic activity of *N*-alkyl-*N*-(3-carboxypropyl)-nitrosamines in *S. typhimurium* TA1535 and *E. coli* WP2 *uvrA*

Concentration (μmol/plate)	*S. typhimurium* TA1535	*E. coli* WP2 *uvrA*
MCPN		
0	10±3	18±1
20	21±2	21±4
40	27±1	17±4
ECPN		
0	15±5	23±2
6.3	14±4	21±1
12.5	17±2	22±1
25	16±6	20±1
50	17±4	17±3
PCPN		
0	13±1	14±1
5.6	13±3	17±8
11	10±1	19±1
23	12±0	15±2
45	10±1	11±1
BCPN		
0	26±5	20±1
5	25±0	27±1
10	19±6	25±1
20	22±8	22±3
40	24±6	20±1

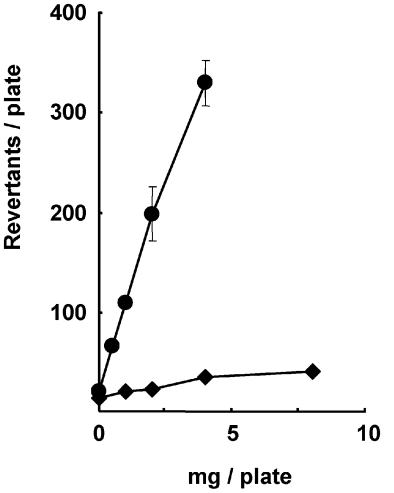

Fig. 3. Mutagenicity of BCPN by 4-MPy plus *t*-BuOOH in *S. typhimurium* YG7108 (●) and TA1535 (◆).

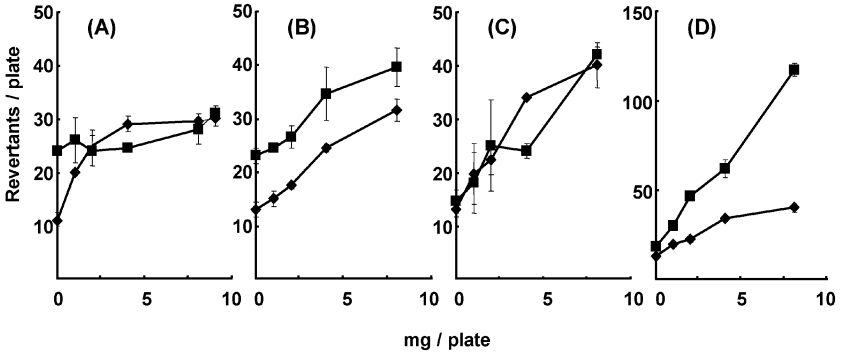

Fig. 2. Mutagenicity of *N*-alkyl-*N*-(3-carboxypropyl)nitrosamines by a chemical model in *S. typhimurium* TA1535 (◆) and *E. coli* WP2 *uvrA* (■). (A) MCPN, (B) ECPN, (C) PCPN, (D) BCPN. The *N*-alkyl-*N*-(3-carboxypropyl)nitrosamines were reacted with 4-MPy and *t*-BuOOH at room temperature for 30 min and the reaction solution was extracted six times with dichloromethane. The solvent layer was dried over Na_2SO_4, filtered, and evaporated *in vacuo* to obtain an oil.

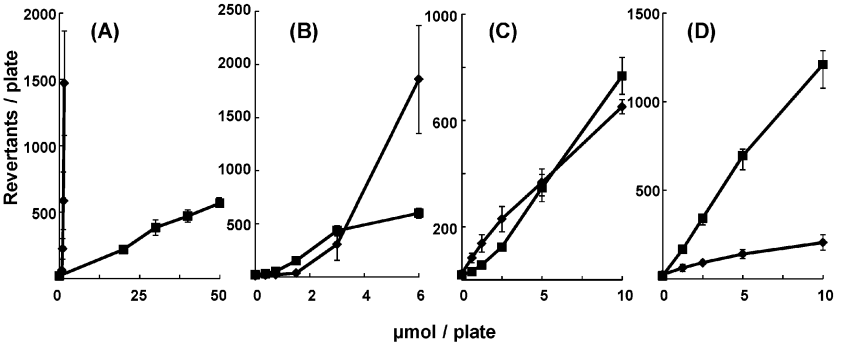

Fig. 4. Mutagenicity of *N*-alkyl-*N*-nitrosoureas by a chemical model in *S. typhimurium* TA1535 (◆) and *E. coli* WP2 *uvrA* (■). (A) MNU, (B) ENU, (C) PNU, (D) BNU.

Table 2. Relative mutagenicity of activated *N*-alkyl-*N*-(3-carboxypropyl)nitrosamines and *N*-alkyl-*N*-nitrosoureas

Mutagens	TA1535:WP2 *uvrA* (Revertants/mg)*	TA1535:WP2 *uvrA* (Percent ratio)**
MNU	7359:108	98:2
ENU	2148:885	71:29
PNU	497:542	48:52
BNU	135:847	14:86
Activated MCPN	7:1	93:7
Activated ECPN	3:2	61:39
Activated PCPN	6:5	56:44
Activated BCPN	4:12	22:78

*The relative mutagenicity was calculated from the slope in linear dose response relationship in the concentration range before the toxic effect appeared. **Percent ratio was calculated from dividing each revertants of *S. typhimurium* TA1535 or *E. coli* WP2 *uvrA* by the total revertants of *S. typhimurium* TA1535 and *E. coli* WP2 *uvrA*, the relative mutagenicity in *S. typhimurium* TA1535 and *E. coli* WP2 *uvrA* can be easily understandable by the percent ratio.

slope of the linear dose response relationship in the range of concentration before the appearance of the toxic effect (Table 2). For purposes of comparison, we also expressed the relative mutagenicity of *N*-alkyl-*N*-(3-carboxypropyl)nitrosamines or *N*-alkyl-*N*-nitrosoureas in the two strains in terms of percent ratio. The percent ratio of the mutagenicity of *N*-alkyl-*N*-(3-carboxypropyl)nitrosamines in both strains were similar to that observed with *N*-alkyl-*N*-nitrosoureas. Thus the data showed that DNA alkylation, not 3-carboxypropylation, was relevant to the mutagenic effect.

Discussion

N-Nitrosodialkylamines require enzymatic activation through oxidation by cytochrome P450 in the S–9 mix to exert their mutagenic effects (17). The mutagenicity can be detected by the *Salmonella* assay, which is a short term screening assay for DNA damage (12). Metabolism of NDB and BBN leads to excretion of a large amount of BCPN in urine, and these compounds induce urinary bladder cancer (4–6). Although the BCPN analogues MCPN, ECPN and PCPN also induce urinary bladder cancer, the corresponding carboxymethyl- or carboxyethyl-homologues did not induce the cancer (5). Thus, the presence of a carboxypropyl group in *N*-nitrosodialkylamine is a key factor in the induction of urinary bladder cancer. Our results showed that the *N*-alkyl-*N*-(3-carboxypropyl)nitrosamines were not mutagenic in *S. typhimurium* TA1535 or *E. coli* WP2 *uvrA*. Nagao *et al.* suggested that the BCPN acted as a direct-acting mutagen and was the ultimate carcinogen after absorption into the bladder epithelium (15). In contrast, Pool *et al.* showed that BCPN was not mutagenic in *Salmonella* strains in the absence of an activation system (18). This negative result agreed with our data. Nagao *et al.* used the BCPN which was synthesized by oxidation of the corresponding alcohols with permanganese (15,19). In the present study, *N*-alkyl-*N*-(3-carboxypropyl)nitrosamine was prepared from 2-pyrrolidone and alkyl halide, followed by nitrosation by $NaNO_2$ under acidic conditions (10). Thus, the BCPN used in those studies were synthesized by different routes.

In the present study, we applied a chemical model for cytochrome P450 as an alternative for metabolic activation to the Ames assay (7,8). Previous reports have shown that the chemical models consisting of iron porphyrin or ruthenium porphyrin plus an oxidant have induced mutagenicity in aromatic amines, heterocyclic amines, polyaromatic hydrocarbons, and *N*-nitrosodialkylamines in *Salmonella* strains (20–23). The 4-MPy plus *t*-BuOOH system activated MCPN, ECPN, PCPN and BCPN into direct-acting mutagens. Since the *N*-alkyl-*N*-(3-carboxypropyl)nitrosamines have a highly polar carboxyl group, these compounds were easily conjugated with glycine or glucuronic acid before excretion from the body (4). The glycine conjugate of BCPN has been reported to be no mutagenic with or without the S-9 mix. The chemical model lacks phase II enzymes and thus the activation using the chemical model was

not influenced by any phase II enzyme.

The mutagenicity of BCPN in the presence of the 4-MPy plus *t*-BuOOH was compared in *S. typhimurium* YG7108 versus *S. typhimurium* TA1535 to investigate the DNA-damaging pathway of *N*-nitrosodialkylamines by the chemical model. The oxidized BCPN mutagenicity in *S. typhimurium* YG7108 was higher than that in *S. typhimurium* TA1535, indicating that the mutagenicity by the chemical model was due to DNA alkylation.

We have already reported that the effect of alkyl group on the pattern of mutagenicity is similar in α-substituted *N*-nitrosamines; α-acetoxy nitrosamines, α-hydroperoxy nitrosamines, α-phosphonooxy nitrosamines and alkanediazohydroxide (24), the mutagenicity of *N*-alkyl-*N*-(3-carboxypropyl)nitrosamines was also compared in both bacterial strains. The DNA alkylation seemed to be a butylation resulting from α-hydroxylation of the 3-carboxypropyl group and the lesion was concerned with the mutagenic effect. In studies using rat liver and pig urinary bladder microsomes, Janzowski *et al.* showed that BCPN was preferentially oxidized at the α-position of the alkyl chain bearing the carboxyl group rather than the butyl chain (25). Airoldi *et al.* also demonstrated that O^6-butylguanine from BBN in rat urothelial and hepatic cells was detected (26,27). These data are in agreement with our observation that *N*-alkyl-*N*-(3-carboxypropyl)nitrosamines mutagenicity in the presence of activation system is due to the butyrated DNA.

This chemical model can be also used under various reaction conditions (pH, temperature, ionic strength etc.), thus simplifying the isolation and identification of the unstable mutagenic compound from the reaction mixture for elucidation of the activation mechanism (28). We are continuing to investigate the identification of the direct-acting mutagen and the mechanism of formation of the mutagen.

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Conflicts of interest: None of the authors have any conflicts of interest associated with this study.

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