Reductive Metabolism of Stilbene Oxide and Styrene Oxide to the Olefins in Rats

Shigeyuki KITAMURA,* Yoshiki SHIRAISHI, Masashi MITA, Hiromi SUEKI, Chiharu MAEDA, and Kiyoshi TATSUMI

Institute of Pharmaceutical Science, Hiroshima University School of Medicine, Kasumi 1–2–3 Minami-ku, Hiroshima 734–8551, Japan. Received July 21, 1999; accepted October 13, 1999

The present study provides the first evidence that stilbene oxide and styrene oxide are reductively metabolized to the corresponding olefins in rats. When *cis*- or *trans*-stilbene oxide was given orally to rats, both *cis*- and *trans*-stilbene were isolated from the urine and feces. Styrene was also isolated from the urine and feces of rats given styrene oxide. These metabolites were identified unequivocally by UV and mass spectral comparison with authentic samples, and on the basis of their TLC and HPLC behavior. However, these olefins were not detected in the urine or feces of antibiotics-treated rats dosed with *cis*- or *trans*-stilbene oxide. Cecal contents of the untreated rats exhibited olefin oxide reductase activities toward *cis*- and *trans*-stilbene oxides under anaerobic conditions. The results suggest that intestinal bacteria play an important role in the reduction of olefin oxides to the corresponding olefins in the animal body.

Key words epoxide reduction; intestinal bacteria; olefin oxide; *trans*-stilbene oxide; *cis*-stilbene oxide; styrene oxide

Aromatic hydrocarbons or olefin compounds undergo metabolic oxidation to their epoxides. Some epoxides are known to be active intermediates in carcinogenesis and mutagenesis.^{1–3} Olefin oxides of propylene, butadiene, styrene, ethylene, tamoxifen and allylbenzene analogs are toxic metabolites of the parent olefins.^{4–7} Styrene oxide, the primary metabolite of the major industrial chemical styrene, reacts to form adduct at O⁶-position of guanine moiety of DNA, and is considered to induce carcinogenicity and mutagenicity of styrene.⁸ Some olefin oxides also exist as a naturally occurring toxin like nivalenol, a trichothecene mycotoxin, and are used as pesticides such as dieldrin and endrin.⁹ Furthermore, *trans*-stilbene oxide is known as a potent inducer for cytochrome P450 2B subfamily, epoxide hydrolase and DT-diaphorase.^{10,11} Stilbene oxide also induces the metallothionein synthesis in rats *in vivo*.¹² In this case, *trans*-stilbene oxide is more effective than *cis*-isomer.

The epoxides are metabolized to the corresponding dihydrodiols and to glutathione conjugates.^{13–16} Of the epoxide compounds, the arene oxides are also known to be reduced to the parent arenes.^{17–22} The participation of cytochrome P450 and aldehyde oxidase in the arene oxide reduction was demonstrated.^{18–22} However, the reduction of olefin oxides to the parent olefins has not yet been reported. Sugiura *et al.*²⁰ found that benzo[*a*]pyrene 7,8-dihydrodiol 9,10-oxide was not reduced by the cytochrome P450 system of rat liver microsomes. Hirao *et al.*²² also reported that the reduction of the olefin oxides such as styrene oxide and *trans*-stilbene oxide did not occur in the cytosol fraction of rat liver even in the presence of an electron donor for aldehyde oxidase.

In the present study, we thoroughly examined the *in vivo* metabolism of *trans*-stilbene oxide, *cis*-stilbene oxide and styrene oxide in rats, focusing on their reduction to the olefin products.

MATERIALS AND METHODS

Chemicals Chemicals used were obtained from the following sources: *trans*-stilbene oxide, *trans*-stilbene and sty-

rene oxide from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), *cis*-stilbene and styrene from Nacalai Tesque, Inc. (Kyoto, Japan), and *cis*-stilbene oxide from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.).

Animals and Antibiotic-Treatment Male Wistar rats (210–240 g) were used. Treatment of rats with antibiotics was performed according to the method of Kinouchi *et al.*²³ A mixture of neomycin sulfate, streptomycin sulfate and bacitracin dissolved in sterilized water was given orally to rats twice daily for 6 d at a dose of 200 mg/kg each time. The antibiotic treatment was continued for 3 more d after administration of the olefin oxides.

Preparation of Cecal Contents Cecal contents of animals were suspended in four volumes of deoxygenated GAM broth (Nissui, Tokyo, Japan) and gently centrifuged at 500×*g* for 5 min to exclude food materials. The supernatant was used as the suspension of cecal contents.

Thin-Layer Chromatography (TLC) and High-Performance Liquid Chromatography (HPLC) Silica gel plates (Kieselgel 60 GF₂₅₄, 0.25 mm thick; Merck, Darmstadt, Germany) were developed in (a) *n*-hexane–benzene (7:3, v/v) or (b) *n*-hexane–ethyl ether (95:5, v/v). Spots were visualized under UV light (254 nm). The *R_f* values of authentic olefins and olefin oxides were as follows: *trans*- and *cis*-stilbene oxide 0.22, *trans*-stilbene 0.42 and *cis*-stilbene 0.46 in solvent (a); styrene oxide 0.27 and styrene 0.60 in solvent (b).

HPLC was performed in a Hitachi L-6000 chromatograph (Tokyo, Japan) fitted with a 250×4.6 mm Capcell Pak C₁₈ UG 120 Å (5 μm) column (Shiseido, Tokyo, Japan). The mobile phase was acetonitrile–H₂O (7:3, v/v). The chromatograph was operated at a flow rate of 1.0 or 0.5 ml/min in the case of stilbene oxide or styrene oxide, respectively, at a wavelength of 254 nm. The elution times of *cis*-stilbene oxide, *trans*-stilbene oxide, *trans*-stilbene, *cis*-stilbene, diphenyl (an internal standard in the case of stilbene oxide), styrene oxide, styrene and diphenyl (an internal standard in the case of styrene oxide) were 6.0, 7.5, 10.2, 10.8, 9.0, 7.8, 12.6 and 17.8 min, respectively.

Isolation of Olefin Oxide Metabolites from Urine and

* To whom correspondence should be addressed.

Fig. 1. HPLC of Urinary and Fecal Metabolites of Rats Dosed with *trans*-Stilbene Oxide

Extracts of the urine and feces of rats dosed with *trans*-stilbene oxide for 3 d as described in Materials and Methods were injected to HPLC. A dotted line shows the chromatogram of authentic *trans*-stilbene oxide, *cis*-stilbene and *trans*-stilbene.

Feces *trans*-Stilbene oxide, *cis*-stilbene oxide and styrene oxide were each given orally to five rats at a dose of 200 mg/kg once daily for 3 d. The urine and feces were collected separately in a light-intercepting vessel for 2 d following administration of the olefin oxides. The urine and feces from the rats before administration were used as controls. The following experiments were conducted in the dark. The urine (530–550 ml) was adjusted to pH 10 with 1 N NaOH and extracted twice with three volumes of benzene in the case of *cis*- and *trans*-stilbene oxides, or with diethyl ether in the case of styrene oxide. The combined benzene or diethyl ether extract was evaporated to dryness *in vacuo*. In the case of *cis*- and *trans*-stilbene oxides, the extracts were evaporated and the residues were dissolved with 50 ml of distilled water adjusted to pH 10 with 1 N NaOH, and extracted again twice with 100 ml of benzene. The combined extracts were evaporated to dryness *in vacuo*. TLC showed that either extract contained two kinds of metabolites with *R_f* values corresponding to *cis*- and *trans*-stilbene. In the case of styrene oxide, TLC showed that the residue contained one metabolite with an *R_f* value corresponding to that of styrene. These metabolites were purified by preparative TLC using solvent a or b.

The feces (80–110 g) were dried, pulverized in a mortar and extracted twice with 10 volumes of benzene or diethyl ether by sonication and shaking for 20 min each in the case of *cis*- and *trans*-stilbene oxides or styrene oxide, respectively. The combined benzene or diethyl ether extract was evaporated and dissolved in 50 ml of distilled water adjusted to pH 10, and again extracted twice with 100 ml of benzene and diethyl ether, respectively. The combined extracts were passed through a Florisil (Wako Pure Chemical Industries Ltd. Osaka, Japan, 5×40 mm) column, and the metabolites were purified by preparative TLC as described above.

Quantitative Determination of Olefin Oxide Metabolites Rats were given a single oral dose of 200 mg/kg of *cis*- or *trans*-stilbene oxide. The urine and feces were collected in a light-intercepting vessel for 3 d following the administration of the olefin oxide. The following experiments were conducted in the dark. An aliquot (5 ml) of the urine was adjusted to pH 10 and extracted three times with 30 ml of benzene. The dried and pulverized feces (2 g) were extracted three times with 30 ml of benzene. The benzene ex-

tracts from the urine and feces were applied to a silica gel (Kieselgel 60, Merck, 5×40 mm) column equilibrated with hexane. The columns washed with *n*-hexane were eluted with *n*-hexane–benzene (7:3, v/v). To the eluate was added 100 µg of biphenyl as an internal standard and evaporated to dryness. The residue was dissolved in 0.1 ml of methanol and an aliquot was subjected to HPLC to determine the olefins.

Incubation of Olefins or Olefin Oxides with Cecal Contents An incubation mixture consisted of 0.5 µmol of an olefin or an olefin oxide and 1 ml (equivalent to 20 mg) of cecal contents in a final volume of 2 ml of 0.1 M K₂Na-phosphate buffer (pH 7.4). The incubation was performed at 37 °C for 1–6 h in the dark under anaerobic conditions using a Thunberg tube. The side arm contained the substrate and the body contained all other components. The tube was gassed for 5 min with nitrogen, evacuated for 5 min, and again gassed with nitrogen. The reaction was started by mixing the components of the side arm and the body. After incubation, the whole added with 100 µg of biphenyl as an internal standard was extracted with 5 ml of benzene, and the extract was evaporated to dryness in the dark. The residue was dissolved in 0.1 ml of methanol and an aliquot was analyzed by HPLC.

RESULTS AND DISCUSSION

When *trans*-stilbene oxide was orally given to rats once per day for 3 d at 200 mg/kg, two metabolites were detected from the pooled urine and feces in HPLC (Fig. 1). The metabolites detected at 10.2 and 10.8 min were designated stilbene oxide-M1 and stilbene oxide-M2, respectively. These peaks were not detected in the chromatograms of control urine and feces. These metabolites were also detected in TLC, and isolated from the urine as described in the experimental section. The mass spectra of the two metabolites gave the same molecular ion at *m/z* 180, suggesting that these metabolites are the corresponding olefin compound. Furthermore, the spectra showed the same fragment ions at *m/z* 165, 102, 89 and 77 (Fig. 2). However, the UV spectra of these metabolites in ethanol revealed an absorption maximum at 296 nm with shoulders at 320, 309, and 242 nm for stilbene oxide-M1 and at 274 nm with shoulders at 332 and 242 nm for stilbene oxide-M2. The mass and UV spectra, and the TLC

Fig. 2. Mass Spectra of the *in Vivo* Metabolites of *trans*-Stilbene Oxide and Styrene Oxide in Rats

and HPLC behavior of these metabolites were identical to those of authentic samples of *trans*- and *cis*-stilbene. The two metabolites were also isolated from the urine of rats dosed with *cis*-stilbene oxide, and these were identical to those obtained from the urine of animals dosed with *trans*-stilbene oxide in terms of mass and UV spectra, and TLC and HPLC behavior (data not shown).

When styrene oxide was given orally to rats in a similar manner, one metabolite (styrene oxide-M1) was isolated from the urine. As shown in Fig. 2, the mass spectrum of the metabolite showed the molecular ion at m/z 104, suggesting that the metabolite is the corresponding olefin compound. Furthermore, the spectrum showed a fragment ion at m/z 78 ($M^+ - 26$) attributable to the loss of C_2H_2 . The UV spectrum of the metabolite in ethanol revealed an absorption maximum at 247 nm with shoulders at 220 and 269 nm. The mass and UV spectra, and the TLC and HPLC behavior of the metabolite were identical with those of an authentic sample of styrene. *trans*-Stilbene and *cis*-stilbene were also isolated from the feces of rats dosed with *cis*- or *trans*-stilbene oxide. Styrene was also isolated as a metabolite of styrene oxide from the feces of rats dosed with the olefin oxide (data not shown).

The excretion of the metabolites by untreated and antibiotics-treated rats after oral administration of the olefin oxides was examined quantitatively. Both *cis*- and *trans*-stilbenes were excreted in the urine and feces of untreated rats dosed with *cis*- or *trans*-stilbene oxide. After the dose of *trans*-stilbene oxide, 0.1 and 0.6% of the dose of *cis*- and *trans*-stilbenes were excreted in the urine, and 0.3 and 2.4% of the dose in the feces. After the dose of *cis*-stilbene oxide, 0.3 and 0.2% of the dose of *cis*- and *trans*-stilbenes were excreted in the urine, and 0.7 and 0.6% of the dose in the feces. When

Table 1. Reduction of Stilbene Oxide and *cis*-*trans* Isomerization of Stilbene by Rat Cecum Contents

Substrate	<i>cis</i> -Stilbene formed	<i>trans</i> -Stilbene formed
	(nmol/6 h/g cecum contents)	
<i>cis</i> -Stilbene oxide	62 ± 1	35 ± 2
<i>trans</i> -Stilbene oxide	71 ± 10	208 ± 17
	(nmol/h/g cecum contents)	
<i>cis</i> -Stilbene	—	115 ± 24
<i>trans</i> -Stilbene	35 ± 11	—

Each value represents the mean ± S.D. of three experiments. An olefin or an olefin oxide was incubated with rat cecum contents under anaerobic conditions for 1 or 6 h, respectively. Olefins formed were determined as described in Materials and Methods.

Fig. 3. Proposed Pathway of Styrene Oxide and Formation of *cis*- and *trans*-Stilbenes from *cis*- or *trans*-Stilbene Oxide in Rats

cis- or *trans*-stilbene oxide was administered to antibiotics-treated rats, *cis*- and *trans*-stilbenes were not detected in the urine or feces.

The reduction of stilbene oxide and the isomerization of stilbene by intestinal bacteria were examined. The cecal contents of untreated rats had the ability to catalyze the reduction of the olefin oxides to the olefins under anaerobic conditions. In this case, *cis*- and *trans*-stilbenes were formed simultaneously from *cis*- and *trans*-stilbene oxides. When *cis*- or *trans*-stilbene was incubated with rat cecal contents under anaerobic conditions, *cis*- and *trans*-isomers were formed from each isomer. The isomerization ratio from *cis*- to *trans*-isomer was higher than that from *trans*- to *cis*-isomer (Table 1). The activity was not influenced by oxygen. *cis*-Stilbene and *trans*-stilbene were also isomerized each other by light to some extent. This experiment of *cis*-*trans* isomerization of stilbene by intestinal bacteria was carried out in the dark. Perhaps the isomerization proceeded enzymatically, because the activity was not observed by the boiled cecum contents. These results suggest that styrene oxide, *cis*- and *trans*-stilbene oxides were reduced to the corresponding olefins by intestinal bacteria. In the case of stilbene oxides, *cis*-stilbene and *trans*-stilbene formed were also isomerized to each other by intestinal bacteria (Fig. 3).

Previous researchers have suggested that olefin oxides cannot be reduced in the animal body on the basis of findings that these oxides are not reduced by liver microsomes or cytosol *in vitro*.^{11,13} The present study provides evidence that *cis*- and *trans*-stilbene oxides, and styrene oxide administered to rats are reduced to the parent olefin compounds in the body, and that intestinal bacteria have the ability to re-

duce these oxides to olefins. The importance of intestinal bacteria in the metabolism of xenobiotics has been increasingly recognized. Involvement of intestinal bacteria in the reductions of nitro, azo, sulfoxide, hydroxamic acid and double bond compounds, and in the reductive cleavage of the benzisoxazole ring has been reported.^{24–32)} However, little is known about the reduction of olefin oxides by intestinal bacteria. Fukushima *et al.*³³⁾ have reported that an epoxysuccinic acid derivative was reduced by rat cecal contents. Recently, we presented evidence that an insecticide, dieldrin, was transformed to aldrin by epoxide reduction of intestinal bacteria.³⁴⁾ Further examination of the reductive metabolism of olefin oxides by intestinal bacteria is in progress.

REFERENCES

- Marquardt H., Kuroki T., Huberman E., Selkirk J. K., Heidelberg C., Grover P. L., Sims P., *Cancer Res.*, **32**, 716–720 (1992).
- Jerina D. M., Daly J. W., *Science*, **185**, 573–582 (1974).
- Hemminki A., Väyrynen T., Hemminki K., *Chem.-Biol. Interact.*, **93**, 51–58 (1994).
- Gadberry M. G., DeNicola D. B., Carlson G. P., *J. Toxicol. Environ. Health*, **48**, 273–294 (1996).
- Phillips D. H., Hewer A., White I. N. H., Farmer P. B., *Carcinogenesis*, **15**, 793–795 (1994).
- Luo G., Guenther T. M., *Drug Metab. Dispos.*, **24**, 1020–1027 (1996).
- Rios-Blanco M. N., Plna K., Faller T., Kessler W., Hakansson K., Kreuzer P. E., Ranasinghe A., Filser J. G., Segeräck D., Swenberg J. A., *Mutat. Res.*, **380**, 179–197 (1997).
- Vodicka P., Vodickova L., Trejbalova K., Scram R. J., Hemminki K., *Carcinogenesis*, **15**, 1949–1953 (1994).
- Tatsuno T., *Cancer Res.*, **28**, 2393–2396 (1968).
- Williams J. B., Wang R., Lu A. Y. H., Pickett C. B., *Arch. Biochem. Biophys.*, **232**, 408–413 (1984).
- Meijer J., DePierre J. W., Wang P. P., Guengerich F. P., *Biochim. Biophys. Acta*, **789**, 1–9 (1984).
- Sato M., Sasaki M., Oguro T., Kuroiwa Y., Yoshida T., *Chem.-Biol. Interact.*, **98**, 15–25 (1995).
- Watabe T., Akamatsu K., *Biochim. Biophys. Acta*, **279**, 297–305 (1972).
- Jakoby W. B., Habig W. H., “Enzymatic Basis of Detoxication,” Vol. II, ed. by Jakoby W. B., Academic Press, New York, 1980, pp. 63–94.
- Oesch F., “Enzymatic Basis of Detoxication,” Vol. II, ed. by Jakoby W. B., Academic Press, New York, 1980, pp. 277–290.
- Luo G., Guenther T. M., *Drug Metab. Dispos.*, **22**, 731–737 (1994).
- Booth J., Hewer A., Keysell G. R., Sims P., *Xenobiotica*, **5**, 197–203 (1975).
- Kato R., Iwasaki K., Shiraga T., Noguchi H., *Biochem. Biophys. Res. Commun.*, **70**, 681–687 (1976).
- Yamazoe Y., Sugiura M., Kamataki T., Kato R., *FEBS Lett.*, **88**, 337–340 (1978).
- Sugiura M., Yamazoe Y., Kamataki T., Kato R., *Cancer Res.*, **40**, 2910–2914 (1980).
- Wrighton S. A., Fahl W. E., Shinnick F. L. Jr., Jefcoate C. R., *Chem.-Biol. Interact.*, **40**, 345–356 (1982).
- Hirao Y., Kitamura S., Tatsumi K., *Carcinogenesis*, **15**, 739–743 (1994).
- Kinouchi T., Kataoka K., Miyanishi K., Akimoto S., Ohnishi Y., *Carcinogenesis*, **14**, 869–874 (1993).
- Scheline R. R., *Pharmacol. Rev.*, **25**, 451–523 (1973).
- Williams J. R., Grantham P. H., Marsh H. H., Weisburger J. H., *Biochem. Pharmacol.*, **19**, 173–188 (1970).
- Gingell R., Bridges J. W., Williams R. T., *Xenobiotica*, **1**, 143–156 (1971).
- Levin A. A., Dent J. G., *Drug Metab. Dispos.*, **10**, 450–454 (1982).
- Renwick A. G., Evans S. P., Sweatman T. W., Cumberland J., George C. F., *Biochem. Pharmacol.*, **31**, 2649–2656 (1982).
- Ishida M., Kitamura S., Tatsumi K., *Biochem. Mol. Biol. Int.*, **39**, 1157–1165 (1996).
- Kitamura S., Sugihara K., Kuwasako M., Tatsumi K., *J. Pharm. Pharmacol.*, **49**, 253–256 (1997).
- Kitamura S., Kuwasako M., Ohta S., Tatsumi K., *J. Pharm. Pharmacol.*, **51**, 79–84 (1998).
- Narai N., Kitamura S., Tatsumi K., *J. Pharmacobio-Dyn.*, **7**, 407–413 (1984).
- Fukushima K., Arai M., Suwa T., Satoh T., *Drug Metab. Dispos.*, **18**, 264–266 (1990).
- Kitamura S., Mita M., Shimizu Y., Sugihara K., Ohta S., *Biol. Pharm. Bull.*, **22**, 880–882 (1999).