Jpn. J. Cancer Res. 83, 1204–1209, November 1992

Evidence of Direct Generation of Oxygen Free Radicals from Heterocyclic Amines by NADPH/Cytochrome P-450 Reductase *in vitro*

Keizo Sato,[1,2] Takaaki Akaike,[1] Yuichiro Kojima,[1] Masayuki Ando,[2] Minako Nagao[3] and Hiroshi Maeda[1,4]

Departments of [1]Microbiology and [2]Medicine, Kumamoto University School of Medicine, Honjo 2-2-1, Kumamoto 860 and [3]Carcinogenesis Division, National Cancer Center Research Institute, Tsukiji 5-1-1, Chuo-ku, Tokyo 104

Highly purified cytochrome P-450 reductase (also called cytochrome *c* reductase; EC 1.6.2.4.) and NADPH were used to generate superoxide radical ($O_2^{\cdot -}$) from 11 different heterocyclic amines (HCAs) as identified by electron spin resonance spectroscopy using the spin trapping method with 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO). The signal intensity of DMPO-OOH($\cdot O_2^-$) (i.e., the DMPO spin adduct of $O_2^{\cdot -}$) was strongest for 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ). The $O_2^{\cdot -}$ generation with HCAs decreased in the following order: 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) = 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) > 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline (diMeIQx) ≥ other HCAs; $O_2^{\cdot -}$ generation was lowest with 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole ·CH_3COOH (MeAαC). By using Lineweaver-Burk plots, K_m values of cytochrome P-450 reductase for mitomycin C, IQ, and MeIQ were determined to be 1.60×10^{-6} *M*, 1.97×10^{-5} *M*, and 2.83×10^{-6} *M*, respectively. The present findings have important implications for carcinogenesis because of the known effect of oxygen radicals on cell proliferation.

Key words: Heterocyclic amine — NADPH/cytochrome P-450 reductase — Oxygen radical — Carcinogenesis — ESR spin trapping

Generation of oxygen free radicals from various chemical carcinogens via cytochrome P-450 is a universal key step in carcinogenesis, cancer promotion and progression.[1-4] Oxygen free radicals interact with intracellular DNA, proteins and lipids; carbon-centered radicals also interact with nucleic acids and other vital molecules,[4-6] which alters genetic regulation in cells. It was recently reported that cytochrome P-450 reductase (also called cytochrome *c* reductase; EC 1.6.2.4) together with NADPH can generate oxygen radicals, specifically superoxide radical ($O_2^{\cdot -}$), from adriamycin and mitomycin C (MMC).[7,8] The oxyradicals generated by these anticancer agents may be important in mutagenicity and cytotoxicity.[9-11] We recently elucidated that alkylperoxyl radical (ROO·), which can be generated via lipid hydroperoxide (ROOH) and heme compounds, is the most potent cytotoxic oxyradical.[11] Superoxide ($O_2^{\cdot -}$) and hydroxyl radical (·OH) are known to undergo further reaction to generate alkylperoxyl radicals.[9,10] Alkylperoxide radicals appear to have a strong effect in tumor promotion.[12,13]

In the present study, we examined the possible generation of oxyradicals from various heterocyclic amines (HCAs[5]) (Fig. 1) by using purified cytochrome P-450 reductase. We identified this oxyradical generation ($O_2^{\cdot -}$) by using the electron spin resonance (ESR) spin trapping method as described recently.[11] The potency of $O_2^{\cdot -}$ generation from HCAs correlated well ($r = 0.88$) with mutagenicity in the Ames test system, although these two systems have no direct relation, because the former involves no hydroxylation of HCAs via cytochrome P-450 whereas the Ames test does require this step.

MATERIALS AND METHODS

Heterocyclic amines Names of HCAs tested in this experiment and their abbreviations are as follows: 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole ·CH_3COOH (Trp-P-1), 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole ·HCl·CH_3COOH (Trp-P-2), 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole ·HCl (Glu-P-1), 2-aminodipyrido[1,2-*a*:3′,2′-*d*]imidazole ·HCl (Glu-P-2), 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole ·CH_3COOH (MeAαC), 2-amino-9*H*-pyrido[2,3-*b*]indole ·CH_3COOH (AαC), 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), 2-amino-3,4,8-trimethylimidazo[4,5-*f*]-

[4] To whom correspondence and reprint requests should be addressed.

[5] The abbreviations used are: HCAs, heterocyclic amines; ESR, electron spin resonance; SOD, superoxide dismutase; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; DTPA, diethylenetriaminepentaacetic acid; MMC, mitomycin C; Tempol, 2,2,6,6-tetramethyl-4-hydroxypiperidine.

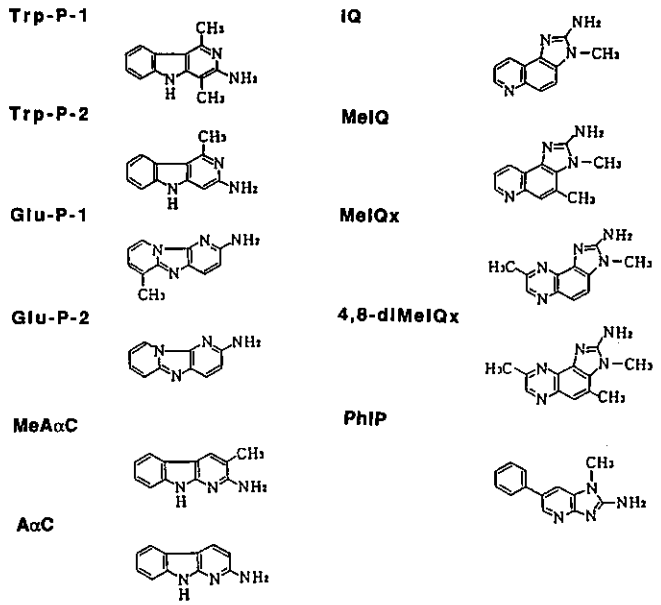

Fig. 1. Structures and abbreviations of heterocyclic amines.

quinoxaline (4.8-diMeIQx), 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP). Glu-P-1 and Glu-P-2 were provided from Katsura Chemical Co., Tokyo. Other HCAs were from Nard Institute, Osaka.

Chemicals We used 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) throughout the experiments as a spin trapping agent. DMPO of the highest purity was synthesized and purified by Dojindo Laboratories, Kumamoto. Nicotinamide adenine nucleotide diphosphate, reduced form (NADPH, coenzyme-II) was from Oriental Yeast Co., Ltd., Tokyo. Dimethyl sulfoxide was from Nacalai Tesque Inc., Kyoto. Diethylenetriaminepentaacetic acid (DTPA) was purchased from Dojindo Laboratories, Kumamoto. MMC was a kind gift from Kyowa Hakko Co., Ltd., Tokyo. Tempol was obtained from Sigma Chemical Co., St. Louis, Mo.

Enzymes and proteins NADPH/cytochrome P-450 reductase was purified according to the method of Ardies *et al.*[14] from rat liver after induction by injecting phenobarbital sodium. This protein preparation was more than 95% pure as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Catalase was from Boehringer Mannheim GmbH, Mannheim, Germany, and human recombinant Cu,Zn-superoxide dismutase (SOD) was a gift from Nippon Kayaku Co., Ltd., Tokyo.

Quantification of superoxide radical by using ESR spectroscopy ESR spin trapping with DMPO was used to identify the radical species generated in the reaction systems of NADPH/cytochrome P-450 reductase with various HCAs (ESR instrument: JEOL JES-RE1X spectrometer, with a quartz flat cell of 160 μl effective volume), as described recently.[11] $O_2^{\cdot -}$ was entrapped by DMPO, and DMPO-OOH($-O_2^-$) formed was quantified by normalization of the DMPO-OOH signal height against the standard signal intensity of manganese oxide (MnO). Conditions are given in detail in the figure legends. Absolute concentrations of the spin adduct of $O_2^{\cdot -}$ radical were determined by double integration of the ESR spectrum; a 1.0 μM Tempol solution was used as a primary standard of ESR absorption as described recently.[11] ESR spectra were recorded at room temperature under the following conditions: modulation frequency, 100 kHz; modulation amplitude, 0.079 mT; scanning field, 336.1$\pm$5 mT; receiver gain, 100 or 500; response time, 0.3 s; sweep time, 2 min; microwave power, 20 mW; and microwave frequency, 9.421 GHz.

RESULTS AND DISCUSSION

HCAs were reacted with 0.5 μM cytochrome P-450 reductase in the presence of 150 μM NADPH and 500 μM DTPA, and 200 μl of the reaction mixture was subjected to ESR spectroscopy with the spin trap DMPO (45 mM) in 10 mM sodium phosphate buffer (pH 6.5). The ESR spectrum obtained for MeIQ, shown in Fig. 2, indicates $O_2^{\cdot -}$ generation since appearance of the DMPO-OOH signals having a hyperfine coupling constant (hfc) of $a_N=1.43$ mT, $a_H^\beta=1.15$ mT, $a_H^\gamma=0.13$ mT was completely inhibited by Cu,Zn-SOD. In addition, $O_2^{\cdot -}$ generation increased in a dose-dependent manner with MeIQ in this system (Fig. 3). All other HCAs tested showed similar signals, which varied in their magnitude. Namely, MeIQ, MeIQx, IQ, and diMeIQx are better $O_2^{\cdot -}$ generators than Glu-P-1, Glu-P-2, Trp-P-1, Trp-P-2, AαC, MeAαC, and PhIP (Fig. 3.).

The $O_2^{\cdot -}$ generation by MeIQ or MeIQx was also tested in rat liver microsomes by ESR spin trapping as described above. The results showed the an appreciable amount of $O_2^{\cdot -}$ was indeed generated in this system, as observed in the purified cytochrome P-450 reductase system (data not shown).

Kinetic analyses of $O_2^{\cdot -}$ generation were performed by drawing Lineweaver-Burk plots of DMPO-OOH generated during the first 1 min of incubation as a measure of the reaction (velocity) versus concentration of HCA or MMC (substrate). The K_m values calculated were 1.60×10^{-6} M for MMC, $1.97\times10^{-5}M$ for IQ and $2.83\times10^{-6}M$ for MeIQ (Fig. 4). The K_m value for MeIQ was comparable to that of MMC. This indicates that HCAs can indeed be substrates for cytochrome P-450 reductase and can be relatively potent generators of oxygen radicals in biological systems, like MMC. Furthermore the trend of mutagenic activation of HCAs by

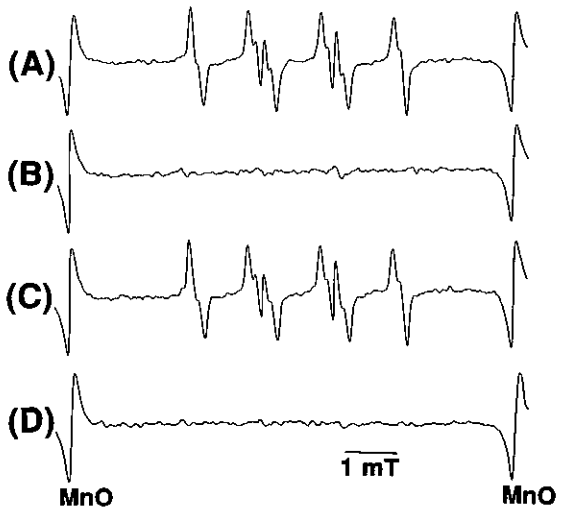

Fig. 2. ESR spectra of DMPO spin adducts of $O_2^{\cdot -}$ generated in the NADPH/cytochrome P-450 reductase/MeIQ system. (A) The spectrum obtained in the reaction mixture of 150 μM NADPH, 0.5 μM cytochrome P-450 reductase, 6 mM MeIQ, 500 μM DTPA and 45 mM DMPO in 10 mM phosphate buffer (pH 6.5). (B) The spectrum obtained in the same reaction mixture as (A) with 500 U/ml Cu,Zn-SOD. (C) The spectrum obtained in the same reaction mixture as (A) with 100 U/ml catalase. (D) The spectrum obtained in the same reaction mixture as (A) with 500 U/ml Cu,Zn-SOD and 100 U/ml catalase. All spectra were recorded at 1 min after addition of DMPO. In addition, no DMPO spin adduct signal was observed in the reaction mixture that contained only 150 μM NADPH, 0.5 μM cytochrome P-450 reductase, and 500 μM DTPA. Conditions of ESR measurement are noted in the text.

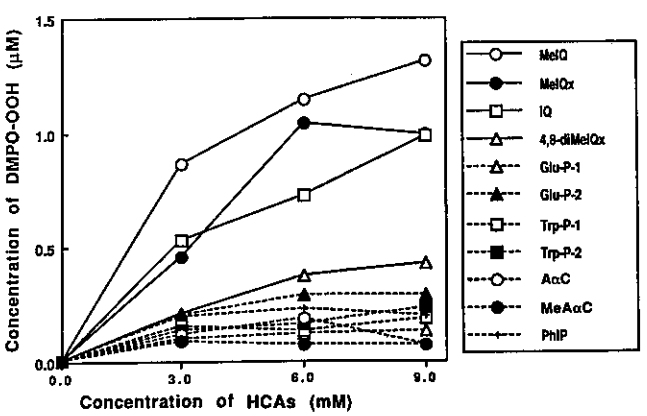

Fig. 3. Generation of $O_2^{\cdot -}$ spin adduct (DMPO-OOH) in NADPH/cytochrome P-450 reductase and various HCAs. All reaction mixtures contained 150 μM NADPH, 0.5 μM cytochrome P-450 reductase, 500 μM DTPA and 45 mM DMPO, with various concentrations of HCAs in 10 mM sodium phosphate buffer (pH 6.5). The spectra were recorded at 1 min after addition of DMPO, and the absolute concentration of spin adduct was determined. See the text for other details.

cytochrome P-450 was found to be similar to the present results[15]; MeIQ was almost 4.5 times more potent than IQ in two different assay systems, and V_{max}/K_m of MeIQ is about 30% of that of MMC (Table I).

HCAs interact with DNA after activation with cytochrome P-450 system, generating *N*-hydroxyl derivatives of HCAs which are directly responsible for the chemical modification of DNA[16] or mutagenesis. In this report, we have demonstrated that the enzyme cytochrome P-450 reductase itself, which is a component of the microsomal cytochrome P-450 system, can also participate in the generation of superoxide radicals when HCAs are present. Thus, HCAs can presumably damage DNA, proteins, lipids and also the cell membrane via generation of oxyradicals in view of their known reactivity,[9] as can lipid peroxide radicals.[12]

As shown in Fig. 5, we examined the correlation between the signal intensity of DMPO-OOH ($-O_2^-$) (relative amount of generated superoxide) and mutagenic potential in the Ames test using *Salmonella typhimurium*, as reviewed by Sugimura,[17] among nine water-soluble compounds tested. A significant correlation (r=0.88)

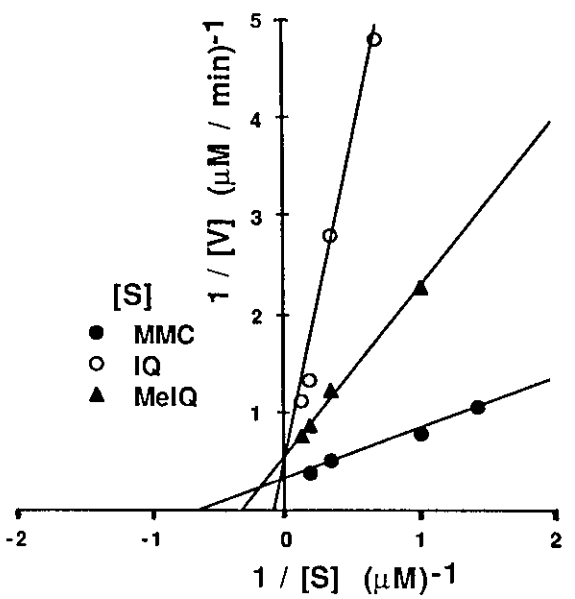

Fig. 4. Lineweaver-Burk plots of the rate of DMPO-OOH generation against concentration of HCAs or MMC. All reaction mixtures contained 150 μM NADPH, 0.5 μM cytochrome P-450 reductase, 500 μM DTPA and 45 mM DMPO, with various concentrations of HCAs or MMC in 10 mM sodium phosphate buffer (pH 6.5). The spectra were recorded at 1 min after addition of DMPO, and the amount of DMPO-OOH, the adduct with $O_2^{\cdot -}$, generated during the first 1-min of incubation was determined as described in the text.

Table I. Kinetic Parameters of Purified Cytochrome P-450 Reductase from Rat Liver in $O_2^{\cdot -}$ Generation from Mitomycin C and Heterocyclic Amines[a]

Substrate	K_m (μM)	V_{max} (min^{-1})	V_{max}/K_m ($\mu M^{-1}\ min^{-1}$)
MMC	1.60	6.34	3.96
IQ	19.6	5.76	0.29
MeIQ	2.83	3.35	1.19

a) The amount of superoxide anion radical ($O_2^{\cdot -}$) was quantitated by ESR spin trapping (see text). The reaction was performed in the presence of 150 μM NADPH in 50 mM phosphate buffer (pH 6.5) containing 45 mM DMPO. Values in the initial 1 min after addition of NADPH to the reaction mixture were used for quantitation.

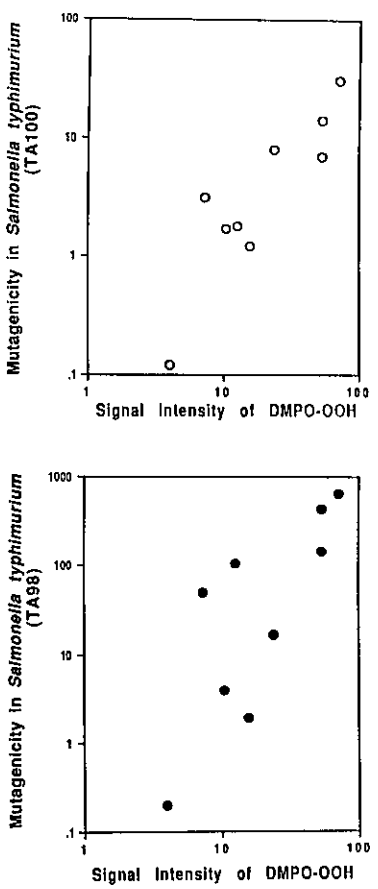

Fig. 5. Correlation between signal intensity of DMPO-OOH generated via NADPH/cytochrome P-450 reductase with HCA and mutagenic potential in the Ames test using *Salmonella typhimurium*. We obtained the signal intensity of DMPO-OOH by using the same method as that described in the legend to Fig. 3. Data for the Ames test were from the literature.[17] A correlation coefficient of $r=0.88$ was obtained between the two parameters. Open and solid circles indicate mutagenic potentials in *S. typhimurium* TA100 and TA98 strains, respectively.

was noted, despite the fact that these two parameters were determined by different assay systems. Namely, $O_2^{\cdot -}$ generation is a direct consequence of enzyme action of cytochrome P-450 reductase upon HCAs plus NADPH, whereas mutagenesis is due to hydroxylated HCAs generated by cytochrome P-450 in *S. typhimurium*. There is also a high correlation between suppression of the promoting effect of Epstein-Barr virus transformation induced by phorbol myristate as judged by early-antigen (EA) appearance in the target B-lymphocytes and the scavenging effect on lipid alkyl peroxide radical when these two parameters were examined in more than 96 vegetable hot water extracts ($r=0.82$).[13] These data indicate a strong correlation between mutagenesis caused by *N*-hydroxylation of HCAs via cytochrome P-450 and the $O_2^{\cdot -}$ generating potential of HCAs in the cytochrome P-450 reductase system.

Hayatsu *et al.* previously reported spontaneous generation of $O_2^{\cdot -}$ from the *N*-hydroxyl derivative of Trp-P-2 through an autooxidation process.[18-20] We tested whether Trp-P-2 could generate its *N*-hydroxyl derivative in our reaction system by high-pressure liquid chromatography using Asahipak GS-320 H (mobile phase: acetonitrile/50 mM acetate buffer, pH 4.0 (40/60, by vol)). We found no appreciable production of *N*-hydroxyl-Trp-P-2. Furthermore, very little, if any, generation of $O_2^{\cdot -}$ was observed from the *N*-hydroxyl-Trp-P-2 as judged by ESR spin trapping (data not shown), while $O_2^{\cdot -}$ generation from Trp-P-2 was substantiated in the cytochrome P-450 reductase system as described above (Fig. 3).

There are numerous reports regarding the role of the cytochrome P-450 system in mutagenesis and carcinogenesis, but little is known about whether cytochrome P-450 reductase itself may have any function in the process of HCA carcinogenesis in the cells. In normal rat liver, the amount of cytochrome P-450 was reported to be 0.4–1.0 nmol/mg of microsomal protein, and P-450 reductase was reported to amount to 5% of cytochrome P-450. When rats were injected with phenobarbital sodium, the level of cytochrome P-450 increased five-fold (2.0 nmol/mg of microsomal protein), which corresponds to 0.1 nmol/mg of cytochrome P-450 reductase.[21,22] HCAs usually induce one type of cytochrome P-450 isozyme IA2, and this type is responsible for the activation of HCAs.[22,23] The amount of this isozyme, IA2, was reported to be 0.02 nmol/mg of microsomal protein. This indicates that the relative amount of cytochrome P-450 reductase (0.1 nmol) was five-fold more than that of the isozyme IA2. Furthermore, cytochrome P-450 reductase is known to exist in the cell nucleus.[24] Therefore, accessibility of DNA in the cell nucleus to oxygen radicals generated by the P-450 reductase with HCAs may be crucial in determining the effect on DNA.

Other studies found cytochrome P-450 to be virtually lacking in hepatoma cells (AH 130 cells), whereas NADPH/cytochrome P-450 reductase activity was very high (>100-fold more than that of cytochrome P-450),[25] which suggests the importance of cytochrome P-450 reductase in transformed cells and its promotion/progression effect as discussed above.

In conclusion, the above findings indicate another novel mechanism by which cytochrome P-450 reductase contributes to the mutagenic action of HCAs, i.e., oxygen radical generation via an electron transfer reaction with molecular oxygen from HCAs that enter the cells. It would be intriguing to test the role of this enzyme in tumor promotion/progression with HCAs in AH 130 or other cells that are known to lack cytochrome P-450.[21] The cell-proliferating capacity of oxygen radical should be additive to other important cellular events.[26]

ACKNOWLEDGMENTS

Part of this work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan. Some of the results were presented at the Annual Meeting of the Japanese Cancer Association, September 10–12, 1991. We thank Professor H. Hayatsu of Okayama University, for supplying *N*-hydroxyl-Trp-P-2 used in our experiments. We also thank Ms. Judith B. Gandy for editorial work and Ms. Rie Yoshimoto for typing the manuscript.

(Received June 1, 1992/Accepted August 18, 1992)

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