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**Essential Role of Singlet Oxygen Species in Cytochrome P450-dependent Substrate Oxygenation by Rat Liver Microsomes**

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**Summary:** Previously, we reported that singlet oxygen (\(^1\)O\(_2\)) was involved in rat liver microsomal P450-dependent substrate oxygenations in such reactions as \(p\)-hydroxylation of aniline, \(O\)\(-\)deethylation of 7-ethoxycoumarin, \(\omega\)- and (\(\omega\)-1)-hydroxylations of lauric acid, \(O\)\(-\)demethylation of \(p\)-nitroanisole, and \(N\)\(-\)demethylation of aminopyrine. In order to confirm the generality of \(^1\)O\(_2\) involvement, we have further investigated which kinds of reactive oxygen species (ROS) are formed during P450-dependent substrate oxygenation in microsomes. We examined CYP2E1-dependent hydroxylation of \(p\)-nitrophenol in rat liver microsomes in the presence of some ROS scavengers, because CYP2E1 has been reported to predominantly generate ROS in the hepatic microsomes and to relate with the oxidative stress in the body. The addition of \(^1\)O\(_2\) quenchers, \(\beta\)-carotene, \(\beta\)-carotene, \(p\)-nitrophenol, and SKF525A, suppressed the hydroxylation. No other ROS scavengers such as superoxide dismutase (SOD), catalase, or mannitol altered the reaction. \(^1\)O\(_2\) was detectable during the reaction in the microsomes as measured by an electron spin resonance (ESR) spin-trapping method when 2,2,6,6-tetramethyl-4-piperidone (TMPD) was used as a spin-trapping reagent. The \(^1\)O\(_2\) was quenched by additions of \(\beta\)-carotene, \(p\)-nitrophenol, and SKF525A. The reactivity of \(p\)-nitrophenol and \(^1\)O\(_2\) correlated linearly with its hydroxylation rate in the microsomes. On the basis of these results, we conclude that \(^1\)O\(_2\) contributes to the \(p\)-nitrophenol hydroxylation in rat liver microsomes, by adding a new example of \(^1\)O\(_2\) involvement in the CYP2E1-dependent substrate oxygenations.

**Key words:** cytochrome P450; CYP2E1; \(p\)-nitrophenol hydroxylation; singlet oxygen (\(^1\)O\(_2\)); electron spin resonance-spin trapping; rate constant

**Introduction**

Cytochrome P450 (P450) is a group of enzymes that are responsible for the biotransformation of numerous endogenous and exogenous compounds. A mechanism for the P450-dependent catalytic cycle has been accepted by many researchers, in which an oxo-ferryl-porphyrin-\(\pi\)-cation radical is involved as an active oxygen intermediate formed by introducing two electrons.\(^{1}\) However, the precise process of dioxygen activation involved in P450-dependent substrate oxygenations has not been established.

We previously proposed the participation of singlet oxygen (\(^1\)O\(_2\)) in \(p\)-hydroxylation of aniline, \(O\)\(-\)deethylation of 7-ethoxycoumarin, \(\omega\)- and (\(\omega\)-1)-hydroxylations of lauric acid, \(O\)\(-\)demethylation of \(p\)-nitroanisole, and \(N\)\(-\)demethylation of aminopyrine.\(^{2,3}\)

CYP2E1 is induced by some chemicals, such as ethanol, acetone, and isoniazid, and under physiological conditions such as diabetes, fasting, or liver disease.\(^{4-6}\) Several groups reported that CYP2E1 from rat liver microsomes elevated the generation of ROS, and thus enhanced the oxidative stress in the body.\(^{7,8}\) Furthermore, some researchers have provided evidence that superoxide anion radical (\(\cdot\)O\(_2^-\)) as well as hydroxyl radical (\(\cdot\)OH) were generated in rat liver microsomes including CYP2E1.\(^{9-11}\) However, the P450-dependent reactions in terms of ROS generation have been charac-
tered insufficiently.

The purpose of this study was to provide evidence of the contribution of ROS to the P450-dependent substrate oxygenations in rat liver microsomes and to add a new example of ¹⁸O₂-dependent substrate oxygenation. To achieve this, we used p-nitrophenol as an indicator of CYP2E1 activity¹²⁻¹⁵ and examined whether ROS were generated in the P450-dependent catalytic oxygenation cycle. The reaction was characterized by using various inhibitors such as hydroxyl radical scavengers, singlet oxygen quenchers, a superoxide scavenger, and a hydrogen peroxide scavenger. In addition, ROS generation during the reaction was examined using an electron spin resonance (ESR) spin-trapping method.

Material and Methods

Materials: Superoxide dismutase (SOD), catalase, deferoxamine, SKF525A, and bovine serum albumin (BSA) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Dithiothreitol, p-nitrophenol, 4-nitrocatechol, mannitol, dimethyl sulfoxide (DMSO), Tween-20, and hematoporphyrin were purchased from Wako Pure Chemicals (Osaka, Japan). Sodium azide (NaN₃) was purchased from Nacalai Tesque (Kyoto, Japan). β-Carotene was obtained from Tokyo Kasei (Tokyo, Japan). 4-Aminophenyl-methanesulfonyl fluoride was purchased from Roche Diagnostics (Tokyo, Japan). 2,2,6,6-Tetramethyl-4-piperidone (TMPD) were purchased from Aldrich (Milwaukee, WI, USA). Dithiothreitol. The protein concentration was determined by the method of Lowry et al. using BSA as the standard.¹⁵ P450 was assayed by the method of Umura and Sato in terms of the CO-reduced P450 complex at pH 7.2.¹⁷

Enzyme-linked immunosorbent assay (ELISA)¹⁸,¹⁹: We loaded 1% (w/v) BSA in phosphate-buffered saline (PBS) onto 96-well microtiter plates (Nunc, Tokyo, Japan) to block non-specific binding of the proteins. After incubation for 30 min, human lymphoblast microsomes as a standard curve where rat CYP2E1 is expressed (0.05–1.92 pmol CYP2E1/well) or rat microsomal samples were also plated and then incubated with a primary antibody (anti-rat CYP2E1 diluted 1:500) for 3 h. Subsequently, the plates were incubated with a secondary antibody (anti-rabbit-horseradish peroxidase conjugate diluted 1:2000) for 30 min. All incubations performed were at 30°C. Plates were washed three times with PBS including 0.05% (v/v) Tween-20 (PBS-T) between incubations. Next, we added 3, 3', 5, 5'-tetramethyl-benzidine (TMB) as a peroxidase substrate solution (Moss Inc., CA, USA), and finally 0.1 M HCl was added to each well to stop the reaction. The absorbance at 450 nm was measured with a Spectrafluor Plus (TECAN, Tokyo, Japan).

p-Nitrophenol hydroxylation activity: p-Nitrophenol hydroxylation activity in terms of 4-nitrocatechol formation was determined at 37°C in 50 mM phosphate buffer, pH 7.5, for 10 min. The incubation mixture contained 1.3 nmol/mL P450, 0.1 mM p-nitrophenol, and 1 mM NADPH in a total volume of 0.5 mL, and the reaction was initiated by addition of NADPH. The reaction was terminated by addition of 0.25 mL of 10% (v/v) perchloric acid to the reaction mixture. The mixture was then cooled on ice, and the protein was removed by centrifugation at 15,000 × g for 5 min. An aliquot (0.3 mL) of the supernatant fraction was added to 30 μL of 10 M NaOH. The absorbance at 540 nm was measured with a Spectrafluor Plus.¹²,¹³ We examined the contribution of ROS in the p-nitrophenol hydroxylation by adding the following compounds to the reaction mixture: 5000 units/mL SOD, 5000 units/
mL catalase, 0.2–1 mM mannitol, 0.2–5 mM NaN3, 0.5–1 mM \( \beta \)-carotene, 0.86–85.7 mM ethanol, 0.70–70.5 mM DMSO, 1–20 mM deferoxamine, and 0.05–0.1 mM SKF525A in a total volume of 0.5 mL.

**ESR spin-trapping of \( ^1\text{O}_2 \) in the rat microsomal reaction:** ESR spectra were recorded at room temperature on a JEOL JES-RFR30 spectrometer (Tokyo, Japan) using an aqueous quartz flat cell (Labotec, Tokyo, Japan). TMPD was used as a \( ^1\text{O}_2 \)-trapping agent.\(^{1,20–23}\)

The incubation mixture contained 50 mM TMPD, 1.3 nmol/mL P450, 1 mM NADPH, and the presence or absence of various concentrations of \( \beta \)-carotene, \( p \)-nitrophenol, and SKF525A in 50 mM phosphate buffer (pH 7.5) at 37°C in a total volume of 0.5 mL. ESR spectra were measured at 10 min after addition of NADPH as an initiator of the reaction. The ESR signal intensity at the lowest magnetic field in a triplet spectrum due to a TMPD-\( ^1\text{O}_2 \) adduct (2,2,6,6-tetramethyl-4-piperidone-\( N \)-oxyl, 4-oxo-TEMPO) was expressed as the ratio to the third signal intensity from the low magnetic field due to the external standard, Mn(II) doped in MnO.

**\( ^1\text{O}_2 \) scavenging activity of \( \beta \)-carotene, \( p \)-nitrophenol, and SKF525A in the hematoporphyrin-UVA system:** \( ^1\text{O}_2 \) was generated by an ultraviolet light A (UVA) irradiation to the hematoporphyrin (HP) solution.\(^{20,21}\) UVA was irradiated through a UVA filter at a dose of 800 mW/cm² using a Supercure-203S (San-Ei Electric MFG, Osaka, Japan), which was connected to the ESR cavity. The reaction mixtures contained 62.5 mM HP, 50 mM TMPD, and 0–250 \( \mu \)M \( p \)-nitrophenol, 0–500 \( \mu \)M \( \beta \)-carotene, or 0–5 mM SKF525A at room temperature (22°C) in a total volume of 0.2 mL of 50 mM phosphate buffer (pH 7.5). ESR spectra were measured at 30 sec after the irradiation (24 J/cm²) of the reaction mixtures.

**Determination of rate constants for the reaction of \( ^1\text{O}_2 \) and \( \beta \)-carotene or \( p \)-nitrophenol:** The rate constant, \( k_a \), for the physical quenching plus chemical reaction of \( ^1\text{O}_2 \) and \( \beta \)-carotene or \( p \)-nitrophenol was determined by the ESR spin-trapping method using the following equation (5). Stern-Volmer plot based on the competitive reaction,\(^{24,25}\) where \( k_d \) is the rate constant for deactivation of \( ^1\text{O}_2 \) in \( H_2\text{O} \) and \( k_r \) is the rate constant for reaction of \( ^1\text{O}_2 \) and TMPD. The values of \( k_d \) (5.00 × 10³ s⁻¹) and \( k_r \) (4.00 × 10⁷ M⁻¹ s⁻¹) were adopted from the literature.\(^{25,26}\)

\[
\begin{align*}
^1\text{O}_2 &\rightarrow k_d \rightarrow ^3\text{O}_2 \quad (1) \\
^1\text{O}_2 + \text{TMPD} &\rightarrow k_r \rightarrow ^1\text{O}_2 - \text{TMPD} \quad (2) \\
^1\text{O}_2 + \text{quencher} &\rightarrow k_{\text{quenching}} \rightarrow ^3\text{O}_2 + \text{quencher} \quad (3) \\
^1\text{O}_2 + \text{quencher} &\rightarrow k_{\text{reaction}} \rightarrow \text{oxidation products} \quad (4) \\
I_0/I = 1 + \frac{k_d [\text{quencher}]}{k_d + k_r [\text{TMPD}]} \quad (5)
\end{align*}
\]

where \( I \) is the ESR signal intensity due to the TMPD-\( ^1\text{O}_2 \) signal for the system containing the \( ^1\text{O}_2 \) generation system, \( \beta \)-carotene or \( p \)-nitrophenol, and TMPD. The \( k_d \) values were estimated from the slopes of straight lines for \( I_0/I \) vs. [quencher] plots with linear regression method, using all quencher concentrations.

**Substrate-induced difference spectra:** Substrate-induced difference spectra were recorded with a Shimadzu MultiSpec-1500 (Kyoto, Japan). The spectral dissociation constant (\( K_d \)) of \( p \)-nitrophenol was obtained by a double reciprocal Lineweaver-Burk plot due to the spectral changes associated with additions of increasing concentrations of \( p \)-nitrophenol to the liver microsomes (0.4 nmol P450/mL).\(^{27}\) The microsomes used for the study were suspended in 50 mM phosphate buffer, pH 7.5.

**Statistical analysis:** All experimental results are expressed as the means ± standard deviations (SDs). Statistical significance was performed by analysis of variance (ANOVA) at a 1% or 5% significance level of the difference.

**Results**

**ELISA:** In liver microsomes, there exist several molecular forms of cytochrome P450 that differ in their primary structure, substrate specificity, and inducibility.\(^{26–30}\) Therefore, an enzyme-linked immunosorbent assay (ELISA) has been used to quantify the individual P450 isoforms in microsomal preparations. In the present study, we determined what percentage of total P450 was CYP2E1, which has been reported to relate with hydroxylation of \( p \)-nitrophenol.\(^{12,21,32}\) P450 specific contents and CYP2E1 contents were 0.81 ± 0.05 nmol P450/mg protein and 0.17 ± 0.03 nmol CYP2E1/nmol total P450, respectively. CYP2E1 was observed to comprise approximately 17% of the total P450.

**Substrate oxidations and effects of ROS scavengers, a P450 inhibitor, and an iron chelator in the rat liver microsomes:** We examined the hydroxylation of \( p \)-nitrophenol in rat liver microsomes in the presence of scavengers against ROS to determine the ROS involved. Additions of 5000 units/mL SOD (superoxide anion radical scavenger), 5000 units/mL catalase (hydrogen peroxide scavenger), and 0.5 and 2 mM mannitol (hydroxy radical scavenger) exhibited essentially no effects. However, the hydroxylation rate of \( p \)-nitrophenol was significantly suppressed by the addition of SKF525A, which is a nonspecific P450 inhibitor.\(^{33}\) In addition, high concentration (20 mM) of deferoxamine, which is an iron chelator, reduced the hydroxylation rate significantly (Fig. 1).

When \( ^1\text{O}_2 \) quenchers, \( \beta \)-carotene\(^{34–37}\) and NaN\(_3\)\(^{20,23,26}\) were added to the incubation mixture at 37°C for 10
min, p-nitrophenol hydroxylation was suppressed in a 1O2 quencher concentration-dependent manner (Fig. 2), although the higher NaN3 concentrations may inhibit the CYP2E1-dependent substrate oxidation.38 Similarly, addition of ethanol and DMSO, both of which are substrates for CYP2E1, restrained the hydroxylation rate in a concentration-dependent manner (Fig. 3).

Detection of 1O2 by ESR spin-trapping in the rat liver microsomes: Because the effects of 1O2 quenchers suggested involvement of 1O2 in p-nitrophenol hydroxylation, we next estimated the effects of a 1O2 quencher, a P450 substrate and inhibitor in the rat liver microsomal system by an ESR spin-trapping method. Higher concentrations (>1 mM) of NaN3, a well-known inhibitor of catalase, was reported to inhibit CYP2E1-dependent ethanol oxidation,38 and thus NaN3 was not used as a 1O2 quencher in ESR spin-trapping. In addition, to analyze the origin of 1O2 in P450-dependent oxygenations, we examined the effect of SKF525A. Detection of 1O2 by ESR spin-trapping was based on the reaction of 1O2 and TMPD, which is a spin-trapping agent selective for 1O2. SKF525A generates a stable nitroxide-free radical due to a TMPD-1O2 adduct (2,2,6,6-tetramethyl-4-piperidone-N-oxyl; 4-oxo-TEMPO), which is detectable by ESR as shown in the following scheme:

When TMPD was added to the reaction mixture of the microsomes with NADPH, an ESR signal due to 4-oxo-TEMPO with g = 2.0062 and a coupling constant of AN = 1.609 mT was clearly observed, as shown in Fig. 4(A), the parameters being consistent with those reported previously.20,21 No such spectrum was observed without NADPH or microsomes. When p-nitrophenol, β-carotene, or SKF525A was added to the microsomes with NADPH, the signal intensity due to 4-oxo-TEMPO was suppressed in a concentration-dependent manner (Figs. 4(B) and (C), and 5(A)).

Detection of ·O2 and ·OH by ESR spin-trapping in the rat liver microsomes: We also investigated whether ·O2 and ·OH contributed to the p-nitrophenol hydroxylation. For this purpose, DMPO was used as a spin-trapping agent for ·O2 and ·OH. However,
Fig. 3. Effect of other competitive substrates for CYP2E1 on hydroxylation of p-nitrophenol in rat liver microsomes. (A): p-Nitrophenol was incubated in the same way as for Fig. 2 with 0.9–43 mM ethanol. (B): p-Nitrophenol was incubated in the same way as for Fig. 2 with 0.7–35 mM DMSO. Significance: *p < 0.01 vs. the control.

Fig. 4. ESR spectra due to the singlet oxygen adducts of TMPD (4-oxo-TEMPO) at room temperature and the effects of p-nitrophenol. The reaction mixture contained 50 mM TMPD, 1.3 nmol/mL P450, and 1 mM NADPH in a total volume of 0.5 mL of 50 mM phosphate buffer, pH 7.5. (A): microsomes + NADPH + TMPD, (B): (A) + 50 μM p-nitrophenol, (C): (A) + 100 μM p-nitrophenol, (D): microsomes + NADPH + 50 mM DMPO, (E): microsomes + NADPH + 50 mM DMPO + 500 μM p-nitrophenol. Both side signals in the figure correspond to those for the 3rd and 4th lowest signals due to standard Mn(II).

neither · O₂ nor · OH was detectable (Figs. 4(D) and (E)), suggesting that these ROS were not likely to be generated during the reaction.

Detection of · O₂ by ESR spin-trapping in the hematoporphyrin-UVA system: To investigate where · O₂ is generated and whether the compounds such as a · O₂ quencher, a P450 substrate and inhibitor react with · O₂ chemically, we examined the · O₂ quenching activities of β-carotene, p-nitrophenol, and SKF525A in the chemical systems by the ESR spin-trapping method. · O₂ was generated in the HP solution under UVA irradiation. The signal intensity due to 4-oxo-TEMPO decreased following additions of β-carotene and p-nitrophenol in a concentration-dependent manner, indicating that p-nitrophenol reacted chemically with · O₂. While, SKF525A had no effect (Fig. 5(B)).

Determination of rate constants for the reaction of · O₂ and β-carotene or p-nitrophenol: To quantitatively investigate the reactivity of · O₂ and compounds, we determined the rate constants for the reactions (Fig. 6 and Table 1). β-Carotene showed the similar rate constants in the rat liver microsomes (2.70 × 10¹⁰ M⁻¹ s⁻¹) and HP-UVA (2.78 × 10¹⁰ M⁻¹ s⁻¹) systems, which coincided well with the reported value of 1.4–3.0 × 10¹⁰ M⁻¹ s⁻¹, indicating that β-carotene equally quenched · O₂ in both systems. p-Nitrophenol showed the higher rate constant of 2.95 × 10¹⁰ M⁻¹ s⁻¹ in the HP-UVA system than that of 1.68 × 10¹⁰ M⁻¹ s⁻¹ in the rat liver microsomes, suggesting that p-nitrophenol mainly resulted in physical quenching in the HP-UVA system. However, SKF525A reacted only in the rat liver microsomes with the rate constant of 1.27 × 10¹⁰ M⁻¹ s⁻¹ but hardly in the HP-UVA system.

Linear correlation of the relationship between the hydroxylation and · O₂-quenching activity of p-nitrophenol in the rat liver microsomes: To understand the relationship between the P450-dependent
Fig. 5. Concentration-dependent scavenging effect of β-carotene (○), p-nitrophenol (●), and SKF525A (▲) on both the generated 1O2 in the microsomes-NADPH system (A) and chemically generated 1O2 in HP-UVA system (B). (A): The reaction systems contained 1.3 nmol/mL P450, 50 mM TMPD, 1 mM NADPH, and 0–500 μM β-carotene, 0–250 μM p-nitrophenol, or 0–2 mM SKF525A in a total volume of 0.5 mL of 50 mM phosphate buffer, pH 7.5 at 37°C. ESR spectra were measured 10 min after the addition of NADPH. (B): The systems contained 62.5 μM HP, 50 mM TMPD, 0–500 μM β-carotene, 0–250 μM p-nitrophenol, or 0–2 mM SKF525A in a total volume of 0.2 mL of 50 mM phosphate buffer, pH 7.5, at room temperature (22°C). ESR spectra were measured after 30 sec of UVA irradiation of the system. Significance: *p<0.05, **p<0.01 vs. the control.

Fig. 6. Stern-Volmer plot for the quenching of 1O2 by β-carotene (○), p-nitrophenol (●), and SKF525A (▲) on both the generated 1O2 in the microsomes-NADPH system (A) and chemically generated 1O2 in HP-UVA system (B). The experimental procedures were described as Fig. 5. The straight lines were obtained with linear regression, using all concentrations of compounds.

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substrate oxidation and 1O2 generation, both the hydroxylation rate of p-nitrophenol and the decreasing ratio of signal intensity due to 4-oxo-TEMPO by addition of p-nitrophenol were examined. The signal intensity decreased significantly as a result of addition of p-nitrophenol to the microsome-NADPH system with 50 mM TMPD and 1 mM NADPH. The decrease of the signal intensity due to 4-oxo-TEMPO in the presence of p-nitrophenol correlated linearly (r = 0.9991) with the hydroxylation rate of p-nitrophenol,
indicating that \( p \)-nitrophenol hydroxylation in the microsomes-NADPH system depends on the consumption of \(^1\)O\(_2\) generated during the P450 catalytic cycle (Fig. 7).

**Substrate-induced difference spectra:** The spectral change in \( p \)-nitrophenol-induced difference was characterized by the appearance of a trough at 380 nm and a peak at 430 nm. This spectrum due to the interaction of \( p \)-nitrophenol with P450 indicated that the substrate directly coordinated to the heme site of P450.\(^{27}\) On the other hand, the spectral change was not observed in the case of \( \beta \)-carotene, indicating that the interaction of \( \beta \)-carotene with P450 was unnoticed. We examined the substrate-binding features of P450 with respect to the spectral dissociation constant (\( K_s \)) calculated from a reciprocal plot in substrate-induced difference spectra, and estimated \( K_s \) of \( p \)-nitrophenol for the rat microsomes to be 0.28 mM, indicating that the binding of \( p \)-nitrophenol was not so strong compared with that of SKF525A (\( K_s = 0.5-1.0 \mu M \)).\(^{27,39}\)

## Discussion

The mean ratio of P450 isoforms in human liver microsomes was reported, in which CYP3A4, CYP2C, CYP1A2, CYP2E1, CYP2A6, and CYP2D6 accounted for 28.8, 18.2, 12.7, 6.6, 4.0, and 1.5 percent, respectively, of the total P450.\(^{40}\) In the present study, we found CYP2E1 of the rat microsomes to account for approximately 17% of the total P450, suggesting a higher contribution of CYP2E1 in substrate oxygenation. CYP2E1 was reported to predominantly generate ROS and to relate the oxidative stress.\(^{7-11}\)

However, the contribution of ROS to the P450-dependent reactions has been characterized insufficiently. Then, we examined whether ROS were generated in the P450-dependent catalytic oxygenation cycle, using \( p \)-nitrophenol as an indicator of CYP2E1 activity.\(^{12-15}\)

The \( p \)-nitrophenol hydroxylation rate was not altered by addition of SOD, catalase, or mannitol (Fig. 1), indicating that neither superoxide (\( \cdot \)O\(_2\)) nor hydroxyl radical (\( \cdot \)OH) was involved in the P450-dependent substrate oxygenation mechanism. Additions of \( ^1 \)O\(_2\) quenchers, \( \beta \)-carotene and NaN\(_3\), suppressed significantly the substrate oxygenation (Fig. 2), demonstrating the great involvement of \(^1\)O\(_2\) in the rat liver microsomal substrate oxygenation, by supporting our previous findings in different types of substrate.\(^{2}\)

However, there remained to be another possibility that NaN\(_3\) over 1 mM inhibited the CYP2E1-dependent \( p \)-nitrophenol hydroxylation,\(^{38}\) and that NaN\(_3\) might affect the binding of a type II substrate such as \( p \)-nitrophenol with P450.\(^{2}\) Therefore, NaN\(_3\) should be avoided to use as a \(^1\)O\(_2\) quencher when examining the P450-dependent substrate oxygenation.

The \( p \)-nitrophenol hydroxylation rate was suppressed by addition of CYP2E1 competitive substrates, ethanol and DMSO (Fig. 3), demonstrating the great contribution of CYP2E1 to the hydroxylation of \( p \)-nitrophenol. IC\(_{50}\) values of ethanol and DMSO on the \( p \)-nitrophenol hydroxylation were estimated to be 2.61 and 4.81 mM, respectively (Fig. 3). While, that of \( \beta \)-carotene was estimated to be 1.06 mM (Fig. 2), indicating that a strong \(^1\)O\(_2\) quencher suppressed more efficiently the reaction than CYP2E1 competitor. These results suggested that the involvement of \(^1\)O\(_2\) is general in the rat liver P450 substrate oxygenations including CYP2E1.

To confirm the participation of \(^1\)O\(_2\) in the present

### Table 1. Rate constants for the reactions of \(^1\)O\(_2\) and several compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>( k_{p\text{-nitrophenol}} ) (M(^{-1}) s(^{-1}))</th>
<th>( k_{\beta\text{-carotene}} ) (M(^{-1}) s(^{-1}))</th>
<th>( k_{SKF525A} ) (M(^{-1}) s(^{-1}))</th>
<th>( k_d ) (s(^{-1}))</th>
<th>( k_r ) (M(^{-1}) s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat liver microsomes</td>
<td>1.68 × 10(^{10})</td>
<td>2.70 × 10(^{10})</td>
<td>1.27 × 10(^{10})</td>
<td>5.00 × 10(^{7})</td>
<td>4.00 × 10(^{7})</td>
</tr>
<tr>
<td>HP-UVA</td>
<td>2.95 × 10(^{10})</td>
<td>2.78 × 10(^{10})</td>
<td>n.d.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

n.d.: not determined. The \( k_d \) and \( k_r \) are the rate constants for deactivation of \(^1\)O\(_2\) in H\(_2\)O and for reaction of \(^1\)O\(_2\) and TMPD, respectively.
system, we applied the ESR spin-trapping method by using TMPD as a $^1\text{O}_2$-trapping agent. In the system containing microsomes and NADPH, $^1\text{O}_2$ was distinctly detected (Fig. 4). Additions of β-carotene, $p$-nitrophenol, or SKF525A to the system each suppressed the generation of $^1\text{O}_2$ in a concentration-dependent manner (Fig. 5(A)). These results indicated two possibilities: either the substances reacted with $^1\text{O}_2$ directly or inhibited the process of $^1\text{O}_2$ generation. Consequently, we evaluated which process is predominant in the system. For this purpose, we used a chemical system, in which $^1\text{O}_2$ was generated in the hematoporphyrin (HP) solution under UVA irradiation, and then compared the rate constants to $^1\text{O}_2$ estimated between the systems.

When the compounds were added to the HP-UVA system, different tendencies were observed. Addition of β-carotene to the system decreased the amount of $^1\text{O}_2$ spin-adduct in a concentration-dependent manner (Fig. 5(B)), with almost the same rate constant of $2.78 \times 10^{10} \text{M}^{-1} \text{s}^{-1}$ to $^1\text{O}_2$ as that of $2.70 \times 10^{10} \text{M}^{-1} \text{s}^{-1}$ estimated in the microsomes (Fig. 6 and Table 1). While, no change of $^1\text{O}_2$ generation was observed following the addition of SKF525A (Fig. 5(B)). These results indicated that $^1\text{O}_2$ reacted with β-carotene based on physical quenching but not with SKF525A. This is because SKF525A, which inhibits P450 by strong interaction with the protein moiety of P450 active site ($K_s = 0.5–1.0 \mu\text{M}$), suppressed the amount of $^1\text{O}_2$ spin-adduct in the microsomes by inhibiting the process of $^1\text{O}_2$ generation. When addition of $p$-nitrophenol to the system decreased the amount of $^1\text{O}_2$ spin-adduct in a concentration-dependent manner (Fig. 5(B)), the rate constant of $2.95 \times 10^{10} \text{M}^{-1} \text{s}^{-1}$ to $^1\text{O}_2$ in the HP-UVA system was higher than that of $1.68 \times 10^{10} \text{M}^{-1} \text{s}^{-1}$ in the microsomes (Fig. 6 and Table 1). $p$-Nitrophenol was found to have equivalent reactivity to β-carotene, a potent $^1\text{O}_2$ quencher, in the HP-UVA system. In terms of the affinity with P450, the binding of $p$-nitrophenol with P450 was confirmed to be very weak ($K_s = 0.28 \text{mM}$) compared with that of SKF525A, but that of β-carotene was almost ignored. From these results, we concluded that $p$-nitrophenol reacted with $^1\text{O}_2$ more like it did to β-carotene in the microsomal system. This is probably why the suppressive effect of β-carotene on the $p$-nitrophenol hydroxylation was not strong in spite of having the equivalent rate constant with $p$-nitrophenol (Fig. 2(B)). Thus, it was indicated that $^1\text{O}_2$ was generated in P450-dependent substrate oxygenations.

However, the rate constant of $^1\text{O}_2$ and $p$-nitrophenol estimated in the HP-UVA system was considerably higher than those of $^1\text{O}_2$ and other phenolic compounds such as 2, 6-diisopropylphenol previously reported ($0.2–2.7 \times 10^9 \text{M}^{-1} \text{s}^{-1}$),41-43 suggesting the different mechanism on the reaction of $^1\text{O}_2$ and $p$-nitrophenol from that of $^1\text{O}_2$ and other phenolic compounds. Several researchers have shown that the phenoxide anions as the deprotonated forms have much higher reactivity to $^1\text{O}_2$ in the basic high pH solution than phenols in the acidic or neutral pH solution,44-46 where the rate constants of $^1\text{O}_2$ and phenoxide anions in the physical quenching manner were estimated to be $1–2 \times 10^9 \text{M}^{-1} \text{s}^{-1}$ in $\text{H}_2\text{O}$ at room temperature. This is because $\text{pK}_a$ values of phenolic compounds usually range from 8.5 to 10.3.47 While, $p$-nitrophenol has a $\text{pK}_a$ value of 7.15 in the aqueous solution, and thus exists 69% as the phenoxide anion form in the neutral pH solution (pH 7.5).47,48 This is the major reason why $p$-nitrophenol showed the higher rate constant to $^1\text{O}_2$ in the experimental systems (pH 7.5) compared with other phenols. The kinetic analysis of photooxidation reported that the phenoxide anion of $p$-chlorophenol interacted with the chemically generated $^1\text{O}_2$ in both the physical quenching and chemical reaction manners.46) The higher rate constant ($2.9 \times 10^{10} \text{M}^{-1} \text{s}^{-1}$) of $^1\text{O}_2$ and $p$-nitrophenol than those of $^1\text{O}_2$ and the phenoxide anions of trimethylphenol45) and $p$-chlorophenol44,46 indicated that the interaction between $^1\text{O}_2$ and $p$-nitrophenol is mainly due to the chemical reaction. Actually, we observed the formation of the $p$-nitrophenol-derived phenoxy radical in the HP-UVA system by ESR measurement (data not shown), but did not find predominantly the hydroxylation of $p$-nitrophenol in the chemical generation system. The detailed reaction pathway including other products will be needed to investigate in the chemical system.49)

On the other hand, we tried to detect $^1\text{O}_2$ in terms of DMPO-OH and DMPO-OOH adducts, respectively, in the rat liver microsomes-NADPH system by the ESR spin-trapping using DMPO as a trapping agent, but we observed no signals in either the absence or presence of $p$-nitrophenol (Fig. 4). Accordingly, $^1\text{O}_2$ was not likely to contribute to the P450-dependent substrate oxygenations.

Because ROS such as $^1\text{O}_2$ and $^1\text{O}_2$ generated due to the uncoupling and autoxidation in P450 catalytic cycle was already reported not to contribute to the P450-dependent substrate reactions and its production was found to be unchanged in the presence and absence of substrates, that is, ROS due to the uncoupling was not consumed during the P450 substrate oxygenations,50,51 at last we examined the linear relationship between hydroxylation rate of $p$-nitrophenol and the consumption of $^1\text{O}_2$ in the P450 reaction pathway. The detection of $^1\text{O}_2$ by the ESR spin-trapping method with TMPD is based on the specific reaction of $^1\text{O}_2$ and TMPD, and thus the ESR signal intensity due to the spin-adduct, 4-oxo-TEMPO, is linearly proportional to the total generation of $^1\text{O}_2$ in the system.20,23 The signal intensity due to 4-oxo-TEMPO was decreased significantly by addition of $p$-nitrophenol in comparison with that without $p$-nitrophenol in the system. This finding is
indicate that $^{1}\text{O}_{2}$ may be generated as a heme moiety and that involvement of $^{1}\text{O}_{2}$ during the P450-dependent future.

This last question will be needed to examine in the participates in P450-dependent substrate oxygenations.
P450 catalytic cycle directly contributes to or partially which is similar to our previous findings. These results indicate that $^{1}\text{O}_{2}$ may be generated as a heme moiety and that involvement of $^{1}\text{O}_{2}$ during the P450-dependent oxygenations occurs not only in type I substrates but also in the substrates of the type that bind to the heme site like $p$-nitrophenol. Further study will be needed to clarify the possible contribution of CYP2E1 to substrate hydroxylation, for example, by using microsomes that contain a large amount of CYP2E1. This investigation is underway.

References


25) Tanaka, K., Miura, T., Umezawa, N., Urano, Y.,
Kikuchi, K., Higuchi, T. and Nagano, T.: Rational
design of fluorescence-based fluorescence probes.
Mechanism-based design of a maximum fluorescence
probe for singlet oxygen. J. Am. Chem. Soc., 123:
26) Lion, Y., Gandin, E. and Van de Vorst, A.: On the
production of nitroxide radicals by singlet oxygen
reaction: an EPR study. Photochem. Photobiol., 31:
27) Schenkman, J. B., Remmer, H. and Estabrook, R. W.:
Spectral studies of drug interaction with hepatic
(1967).
28) Hankinson, O.: The aryl hydrocarbon receptor complex.
29) Sueyoshi, T. and Negishi, M.: Phenobarbital response
elements of cytochrome P450 genes and nuclear
receptors. Annu. Rev. Pharmacol. Toxicol., 35:
of CYP3A gene transcription by the pregnane X receptor.
35) Fukuzawa, K., Inokami, Y., Tokumura, A., Terao, J.
36) Cantrell, A., McGarvey, D. J., George T. T., Rancan,
F. and Bohm, F.: Singlet oxygen quenching by dietary
carotenoids in a model membrane environment. Arch.
37) Mascio, P. D., Devasagayam, T. P. A., Kaiser, S. and
38) Salmela, K. S., Tsyrov, I. B. and Lieber C. S.: Azide
inhibits human cytochrome P-450IIE1, 1A2, and 3A4.
39) Topham, J. C.: Relation between difference spectra and
metabolism. Barbiturates, drug interaction, and species
40) Shimada, T., Yamazaki, H., Mimura, M., Inui, Y. and
Guengerich, F. P.: Interindividual variations in human liver cytochrome P-450 enzymes involved in the
43) Heyne, B., Kohnen, S., Braut, D., Mouithys-Mickalad,
A., Tibel, F., Hans, P., Fontaine-Aupart, M. P. and
44) Palumbo, M. C. and Garcia, N. A.: On the mechanism
of quenching of singlet oxygen by chlorinated phenolic pesticides. Toxicol. Environm. Chem., 17:
47) Liptak, M. D., Gross, K. C., Seybold, P. G., Feldsgus, S.
48) Hung, H. C. and Chang, G. G.: Partitioning of 4-
49) Briviba, K., Devasagayam, T. P. A., Sies, H. and
Steenken, S.: Selective para hydroxylation of phenol
and aniline by singlet molecular oxygen. Chem. Res.
50) Bell-Parihk, L. C. and Guengerich, F. P.: Kinetic of cytochrome P450 2E1-catalyzed oxidation of ethanol to acetic acid via acetaldehyde. J. Biol. Chem., 274:
51) Nguyen, N. S. D., Cottet-Marie, F., Bueltler, T. M.,
Russo, A. L., Krauskopf, A. S., Armstrong, J. M.,