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Evidence for Singlet Oxygen Involvement in Rat and Human Cytochrome P450-dependent Substrate Oxidations

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Summary: Recently, we proposed that singlet oxygen (1O2) plays an essential role in microsomal cytochrome P450 (P450)-dependent p-hydroxylation of aniline and O-deethylation of 7-ethoxycoumarin. We then examined whether the role of 1O2 is general in the P450-dependent substrate oxidations. In the present study, we examined o- and (o-1)-hydroxylations of lauric acid, O-demethylation of p-nitroanisole, and N-demethylation of aminopyrine in rat liver microsomes. The addition of β-carotene and NaN3 significantly suppressed these reactions in a concentration-dependent manner, and 1O2 during the reactions was detected by ESR spin-trapping using 2,2,6,6-tetramethyl-4-piperidone (TMPO) as a 1O2-spin trapping reagent, where the addition of 1O2 quenchers, SKF-525A as a P450 inhibitor, or β-nitroanisole decreased ESR signal intensities due to TMPO-1O2 adduct. Next, we examined the effect of 1O2 quenchers on P450-dependent reactions in the human liver microsomes, and 1O2 was also indicated to be an active species in substrate hydroxylations and dealkylations such as nifedipine oxidation by CYP3A4. On the basis of the results, we concluded that 1O2 is an essentially important active oxygen species in both rat and human P450-dependent substrate oxidations.

Key words: Cytochrome P450; substrate oxidation; singlet oxygen (1O2); electron spin resonance (ESR); ESR-spin trapping

Introduction

Cytochrome P450 (P450) is a group of enzymes that monooxygenizes a large number of organic compounds and xenobiotics.1–4) A mechanism for the P450-dependent catalytic cycle has been proposed and accepted by researchers5–8) in which an oxo-ferrylporphyrin-p-cation radical is involved as an active oxygen intermediate.9,10)

Several authors have reported the contribution of reactive oxygen species (ROS) such as superoxide anion radical (·O2),11–14) hydroxyl radical (·OH),13,14) and singlet oxygen (1O2),15,16) in the NADPH-dependent substrate oxidations and hepatic lipid peroxidations in microsomal systems.17–19) The involvement of ·O2 and 1O2 has been suggested because a six-coordinate heme-Fe(II)-O2 intermediate rapidly undergoes one electron-transfer from iron to oxygen, providing a six-coordinate heme-Fe(III)-·O2 intermediate, and a triplet state (1O2) molecular dioxygen bound to P450 is converted to the singlet state, producing a six-coordinate low-spin heme-Fe(II)-1O2 intermediate.16) However, the P450-dependent reactions in terms of ROS generation are still controversial.

During our investigations of P450-derived oxidation,20,21) we recently found that 1O2 quenchers such as sodium azide (NaN3) and β-carotene suppressed the rat liver microsomal P450-dependent p-hydroxylation of aniline and the O-deethylation of 7-ethoxycoumarin, in which 1O2 was involved, as confirmed by the ESR spin-trapping method.22) In contrast, other ROS scavengers such as superoxide dismutase (SOD), catalase (CAT), and dimethylsulfoxide (DMSO) had no effect on aniline p-hydroxylation or 7-ethoxycoumarin O-deethylation. Consequently, ROSs such as ·O2, hydrogen peroxide
(H₂O₂), and hydroxyl radical (·OH) were assumed not to be involved in such substrate oxidations. In a previous paper, we examined aryl hydroxylation and O-deethylation. Following that study, we planned to examine whether or not the involvement of ¹O₂ in the P450-dependent catalytic cycle is a general reaction in other types of oxidations.

The purpose of the investigation is to give more evidence for the involvement of ¹O₂ in the P450-dependent substrate oxidations in rat and human liver microsomes and to discuss the results. To accomplish this, alkyl hydroxylation, aryl O-demethylation, and amine N-demethylation in rat liver microsomes and four enzyme activities in the human CYP subfamily were examined using ¹O₂ quenchers. Also, ¹O₂ generation during the reactions was measured using the ESR spin-trapping method.

Materials and Methods

Materials: Phenobarbital sodium (PB) was purchased from Maruko (Nagoya, Japan). Lauric acid (dodecanoic acid), 9-anthryldiazomethane (ADAM), p-nitroanisole, p-nitrophenol, aminopyrine, tolbutamide, nifedipine, and dimethylsulfoxide (DMSO) were purchased from Wako Pure Chemical (Osaka, Japan). Both ω- and (ω-1)-hydroxy lauric acid (12-hydroxydodecanoic acid and 11-hydroxydodecanoic acid, respectively) were synthesized and purified in our laboratory as described in the previous report. Hydroxytolbutamide, oxidized nifedipine, and 2-diethy laminoethyl-2,2-diphenylpentanoate hydrochloride (SKF-525A) were purchased from Daiichi Pure Chemical Co., Ltd. (Tokyo, Japan). Coumarin, 7-hydroxycoumarin, and sodium azide (NaN₃) were purchased from Nakalai Tesque (Kyoto, Japan). Resorufin, 7-ethoxresorufin, superoxide dismutase (SOD), and catalase (CAT) were obtained from Sigma (St. Louis, USA). 2,2,6,6-Tetramethyl-4-piperidone (TMPD) was purchased from Tokyo Kasei (Tokyo, Japan). 2,2,6,6-Tetramethyl-4-piperidone (TMPD) was purchased from Aldrich (Milwaukee, USA). 2,2,6,6-Tetramethyl-4-piperidone (TMPD) was purchased from Aldrich (Milwaukee, USA). 2,2,6,6-Tetramethyl-4-piperidone (TMPD) was purchased from Aldrich (Milwaukee, USA). 2,2,6,6-Tetramethyl-4-piperidone (TMPD) was purchased from Aldrich (Milwaukee, USA). 2,2,6,6-Tetramethyl-4-piperidone (TMPD) was purchased from Aldrich (Milwaukee, USA).

Preparation of rat liver microsomes: Microsomal fractions were prepared from the livers of male SD rats. P450 was induced by pretreatment with daily intraperitoneal injection of PB at a dose of 40 mg/kg body weight/day for 3 days. The animals were anesthetized by ether and sacrificed at 24 h after the last dose. The livers were perfused with ice-cold 1.15% KCl and homogenized, and the nuclear-mitochondria were removed by centrifugation at 12,000 × g for 15 min. The supernatant was centrifuged twice at 105,000 × g for 65 min in 100 mM potassium phosphate buffer, pH 7.4. The microsomal pellet was suspended in 100 mM potassium phosphate buffer, pH 7.4, containing 30% glycerol, the final concentration being 20–50 mg protein/mL. The suspension was stored at −80°C before use. P450 was assayed by the method of Omura and Sato in terms of the CO-reduced P450 complex at pH 7.2. Protein was determined by the method of Lowry et al.

Substrate reactions in the rat liver microsomes: Lauric acid hydroxylation in terms of ω- and (ω-1)-hydroxy lauric acids formation, p-nitroanisole O-demethylation, and aminopyrine N-demethylation by rat liver microsomes were determined at 37°C in 100 mM phosphate buffer, pH 7.4, for 15 min. The incubation mixture contained 0.8 mg/mL microsomal protein, 0.2 mM lauric acid, 0.4 mM p-nitroanisole, or 0.4 mM aminopyrine, and 1.6 mM NADPH in a total volume of 0.5 mL, in which the reactions were initiated by the addition of NADPH. The reactions of lauric acid, p-nitroanisole, and aminopyrine were terminated by the addition of 0.03 mL of 2 M HCl, 0.6 mL of methanol, and 0.25 mL of 20% trichloroacetic acid, respectively, to the reaction mixtures.

The contribution of singlet oxygen (¹O₂) to the substrate metabolism was examined by the addition of ¹O₂ quenchers such as 0.5 mM NaN₃ and 0–1 mM β-carotene to the reaction mixture. In addition, the reaction of p-nitroanisole was examined in the incubation mixture containing 0–80% v/v D₂O at pH 7.4, in which the lifetime of ¹O₂ was reported to be approximately 20-fold longer than H₂O. The effects of P450 inhibitor on the lauric acid hydroxylations and p-nitroanisole demethylation were examined by the addition of 0–0.25 mM SKF-525A.

Analytical procedures: After 2 mL of ethyl acetate was added to the incubation mixture containing lauric acid, the mixture was shaken for 10 min and centrifuged at 12,000 × g for 5 min and 4°C, and then 1.5 mL of the supernatant was evaporated to dryness by an N₂ purge. One mL of 0.1 mg/mL ADAM dissolved in ethyl acetate was added to the residue, and the mixture stood for 2 hr at room temperature. Then 100 μL of the solution was injected onto the HPLC system to determine ω- and (ω-1)-hydroxy lauric acids. An HPLC system
the low magnetic field due to the external standard, Mn(II) doped in MnO.

**1O2 scavenging activity and O-demethylation rate of p-nitroanisole in the rat liver microsomes:** The relationship between the demethylation rate of p-nitroanisole and its 1O2 scavenging activity was examined in rat liver microsomes using both HPLC assay and the ESR spin-trapping method. The incubation mixtures contained 50 mM TMPD, 0.8 mg/mL microsomal protein, and 0.2–1.6 mM NADPH, either with or without 0.4 mM p-nitroanisole, at 37°C in a total volume of 0.5 mL of 100 mM phosphate buffer (pH 7.4). The reaction was initiated by the addition of NADPH, and the O-demethylation rate of p-nitroanisole and the ESR spectra were measured at 15 min after the addition of NADPH. The 1O2 scavenging activities of p-nitroanisole were evaluated on the basis of the decrease of signal intensity due to 4-oxo-TEMPO and were correlated with the demethylation rates of p-nitroanisole in the concentration range of 0.2–1.6 mM NADPH.

**1O2 scavenging activities of β-carotene and p-nitroanisole in the hematoporphyrin-UV A system:** 1O2 was generated by ultraviolet light A (UVA) irradiation to a hematoporphyrin (HP) solution.37,38) UVA was irradiated through a UVA filter at an intensity 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 μM HP, 50 mM TMPD, 0–500 μM β-carotene or 0–20 mM p-nitroanisole, at a room temperature (22°C) in a total volume of 0.2 mL of 100 mM phosphate buffer (pH 7.4). The solvent system consisted of ethanol and H₂O in a ratio of 2.5:97.5 (% v/v). ESR spectra were measured at 10 sec (24 J/cm²) after the irradiation of the reaction mixtures.

**CYP subfamily-dependent metabolism in the human liver microsomes:** 7-Ethoxyresorufin O-deethylation, coumarin 7-hydroxylation, tolbutamide 4-hydroxylation, and nifedipine oxidation were determined as CYP1A1/1A2, CYP2A6, CYP2C9, and CYP3A4 activities, respectively, in the human liver microsomes for appropriate periods (5–30 min). The incubation mixture contained 0.2 mg/mL microsomal protein, and either 0.5 μM 7-ethoxyresorufin, 50 μM coumarin, 200 μM tolbutamide, or 20 μM nifedipine at 37°C in a total volume of 0.5 mL of 100 mM phosphate buffer (pH 7.4), in which the reactions were initiated by the addition of an NADPH generating system (2 mM NADP +, 10 mM G-6-P, 5 mM MgCl₂, and 1 U/mL G-6-PDH as a final concentration). After the reactions were terminated by the addition of acidic solution or organic solvent, and the precipitation of protein or extraction of metabolites was performed, each metabolite was determined by the corresponding HPLC system.39–42) Each
calibration line was obtained from peak areas detected by each HPLC system. The correlation coefficient of the calibration line for each metabolite was more than 0.999 ($r$) with a linear least-squares regression in the experimental concentration range.

The contribution of $^1$O$_2$ to each substrate metabolism was examined by the addition of $^1$O$_2$ quenchers such as 0–5 mM NaN$_3$ and 0–0.5 mM D$_2$O (data not shown), and significantly decreased to 90% of the control in 80% v/v D$_2$O at pH 7.4.

In addition, when SKF-525A, which coordinates to the sixth position of the heme in P450,32 was added to the incubation mixture for 15 min, both the $\omega$- and ($\omega$-1)-hydroxylations of lauric acid and the $\beta$-demethylation of p-nitroanisole were significantly suppressed in an inhibitor-concentration-dependent manner (Fig. 2(A) and (B)).

Detection of $^1$O$_2$ by ESR spin-trapping in the rat liver microsomes: Because the involvement of $^1$O$_2$ in alkyl hydroxylations as well as aryl and amine demethylations was suggested with respect to their suppression by the addition of NaN$_3$ or $\beta$-carotene in a quencher-concentration-dependent manner (Fig. 1(C–F)). The O-demethylation of p-nitroanisole was, however, slightly inhibited by addition of D$_2$O (data not shown), and significantly decreased to 90% of the control in 80% v/v D$_2$O at pH 7.4.

Table 1. Effect of reactive oxygen scavengers on the $\omega$- and ($\omega$-1)-hydroxylation of lauric acid in rat liver microsomes

<table>
<thead>
<tr>
<th>SOD (units/mL)</th>
<th>0</th>
<th>4</th>
<th>40</th>
<th>400</th>
<th>4000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxylation rate ($\omega$-1) (nmol/min/nmol P450)</td>
<td>1.06 ± 0.04</td>
<td>1.08 ± 0.13</td>
<td>1.09 ± 0.07</td>
<td>1.17 ± 0.05</td>
<td>1.07 ± 0.07</td>
</tr>
<tr>
<td>Hydroxylation rate ($\omega$) (nmol/min/nmol P450)</td>
<td>0.98 ± 0.10</td>
<td>1.08 ± 0.15</td>
<td>1.03 ± 0.05</td>
<td>1.12 ± 0.06</td>
<td>1.06 ± 0.05</td>
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</tbody>
</table>

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<thead>
<tr>
<th>CAT (units/mL)</th>
<th>0</th>
<th>4</th>
<th>40</th>
<th>400</th>
<th>4000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxylation rate ($\omega$-1) (nmol/min/nmol P450)</td>
<td>0.95 ± 0.08</td>
<td>0.99 ± 0.05</td>
<td>0.95 ± 0.10</td>
<td>0.96 ± 0.06</td>
<td>0.91 ± 0.06</td>
</tr>
<tr>
<td>Hydroxylation rate ($\omega$) (nmol/min/nmol P450)</td>
<td>0.85 ± 0.07</td>
<td>0.93 ± 0.05</td>
<td>0.89 ± 0.09</td>
<td>0.92 ± 0.05</td>
<td>0.92 ± 0.06</td>
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<thead>
<tr>
<th>DMSO (mM)</th>
<th>0</th>
<th>0.5</th>
<th>5</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxylation rate ($\omega$-1) (nmol/min/nmol P450)</td>
<td>0.99 ± 0.12</td>
<td>0.96 ± 0.09</td>
<td>1.01 ± 0.08</td>
<td>0.93 ± 0.06</td>
</tr>
<tr>
<td>Hydroxylation rate ($\omega$) (nmol/min/nmol P450)</td>
<td>0.89 ± 0.11</td>
<td>0.84 ± 0.10</td>
<td>0.90 ± 0.06</td>
<td>0.86 ± 0.05</td>
</tr>
</tbody>
</table>

The $\omega$- and ($\omega$-1)-hydroxylations of lauric acid were determined at 37°C in 100 mM phosphate buffer, pH 7.4, for 15 min. The incubation mixture contained 0.8 mg/mL microsomal protein, 0.2 mM lauric acid, and 1.6 mM NADPH with or without each scavenger in a total volume of 0.5 mL, in which the reactions were initiated by the addition of NADPH.
Fig. 1. Effect of singlet oxygen quenchers on the \( \omega \)- and \((\omega-1)\)-hydroxylations of lauric acid (A and B), \( O \)-demethylation of \( p \)-nitroanisole (C and D), and \( N \)-demethylation of aminopyrine (E and F) in rat liver microsomes.

A and B: Lauric acid (0.2 mM) was incubated with 0.8 mg/mL microsomal protein, 0–5 mM NaN3 or 0–1 mM \( \beta \)-carotene, and 1.6 mM NADPH in a total volume of 0.5 mL of 100 mM phosphate buffer, pH 7.4, at 37°C for 15 min.

C and D: \( p \)-Nitroanisole (0.4 mM) was incubated in the same way as A and B at 37°C for 15 min.

E and F: Aminopyrine (0.4 mM) was incubated in the same way as A-D with at 37°C for 15 min.

Significance: * \( p < 0.05 \), ** \( p < 0.01 \) vs. without quenchers (n = 3).

Fig. 2. Effect of SKF-525A on the \( \omega \)- and \((\omega-1)\)-hydroxylations of lauric acid (A) and \( O \)-demethylation of \( p \)-nitroanisole (B) in rat liver microsomes.

A: Lauric acid (0.2 mM) was incubated with 0.8 mg/mL microsomal protein, 0–250 \( \mu \)M SKF-525A, and 1.6 mM NADPH in a total volume of 0.5 mL of 100 mM phosphate buffer, pH 7.4, at 37°C for 15 min.

B: \( p \)-Nitroanisole (0.4 mM) was incubated in the same way as A with 0–50 \( \mu \)M SKF-525A at 37°C for 15 min.

Significance: * \( p < 0.05 \), ** \( p < 0.01 \) vs. without inhibitor (n = 3).
A_N = 1.609 mT was clearly observed (data not shown), the parameters being consistent with those reported previously.37,38 The signal intensity due to 4-oxo-TEMPO was increased time-dependently for 20 min and was enhanced by increases in the P450 Fig. 3(A) and NADPH Table 2 concentrations. No such spectrum was observed without the microsomal protein or NADPH. When SKF-525A was added to the reaction system, the signal intensity due to 4-oxo-TEMPO was suppressed in a concentration-dependent manner (Fig. 3(B)), indicating that the origin of 1O2 is in the heme site of the P450 catalytic cycle. As well, the 1O2 scavengers, NaN3 and β-carotene, efficiently scavenged the generated 1O2 (Fig. 3(C) and (D)).

To understand the relationship between the P450-dependent substrate oxidation and 1O2 generation, both the O-demethylation rate and the reactivity to the generated 1O2 of p-nitroanisole were examined. The signal intensity due to the spin adduct of 1O2 (4-oxo-TEMPO) decreased significantly as a result of the addition of p-nitroanisole to the microsomes-NADPH system at 50 mM TMPD and 0.2–1.6 mM NADPH, and the O-demethylation rate of p-nitroanisole in the same reaction system without TMPD increased in a NADPH concentration-dependent manner (Table 2). The decrease of the signal intensity due to 4-oxo-TEMPO in the presence of p-nitroanisole correlated well (r = 0.971) with the O-demethylation rates of p-nitroanisole (Fig. 4), indicating that p-nitroanisole demethylation in the microsomes-NADPH system depends on the consumption of 1O2 generated during the P450 catalytic cycle.

Detection of 1O2 by ESR spin-trapping in the hematoporphyrin-UVA system: To investigate whether the compounds such as a 1O2 scavenger and a P450 substrate react with 1O2 directly, the 1O2 scavenging activities of β-carotene and p-nitroanisole were then examined in the chemical system by the ESR spin-trapping method. 1O2 was generated by UVA irradiation to the HP solution. The signal intensity due to 4-oxo-TEMPO decreased by additions of β-carotene (Fig. 5(A)) and p-nitroanisole (Fig. 5(B)) in their concentration-dependent manners, indicating that p-nitroanisole reacted with 1O2 chemically generated in the HP-UVA system.

Substrate oxidations and the effect of 1O2 quenchers and a spin-trapping agent in the human liver microsomes: To conclude whether the involvement of 1O2 in the P450-dependent substrate oxidation is general over the different P450 species, the effect of 1O2 quenchers and a spin-trapping agent on P450 activities was examined in the human liver microsomes-NADPH generating system. The 7-ethoxyresorufin O-deethylation as CYP1A1/1A2 activity was unchanged by the addition of 5 mM NaN3 or 50 mM TMPD; however, it was significantly suppressed by the addition of 500 μM β-carotene. In addition, the coumarin 7-hydroxylation as CYP2A6 activity, tolbutamide 4-hydroxylation as CYP2C9 activity, and nifedipine oxidation as CYP3A4 activity were significantly suppressed at 5 mM NaN3, 25–50 mM TMPD, or 500 μM β-carotene (Fig. 6(A–D)). These results indicated that the generation of 1O2 also contributed to the human liver P450-dependent substrate oxidations.

### Table 2. Generation of 1O2 in the rat liver microsomes-NADPH system in the presence or absence of p-nitroanisole and demethylation rate of p-nitroanisole

<table>
<thead>
<tr>
<th>p-nitroanisole</th>
<th>0.4 mM</th>
<th>NADPH (mM)</th>
</tr>
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<tbody>
<tr>
<td>Signal intensity due to 4-oxo-TEMPO (arbitrary unit)</td>
<td>–</td>
<td>0.45 ± 0.12</td>
</tr>
<tr>
<td>Decrease of signal intensity by p-nitroanisole (arbitrary unit)</td>
<td>+</td>
<td>0.10 ± 0.01**</td>
</tr>
<tr>
<td>Demethylation rate (nmol/min/nmol P450)</td>
<td>0.35 ± 0.15</td>
<td>0.88 ± 0.10</td>
</tr>
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</table>

Significance: **p<0.01 vs. without substrate.
Fig. 3. Time-dependent increase of ESR signal intensity due to a TMPD-\(^1\)O\(_2\) adduct (2,2,6,6-tetramethyl-4-piperidone-\(N\)-oxyl, 4-oxo-TEMPO) and the effects of microsomal protein, SKF-525A, NaN\(_3\), and \(\beta\)-carotene on the formation of the spin adduct as monitored by the ESR spin-trapping method in rat liver microsomes. Incubation conditions were the same as those for Fig. 1. The reaction mixture contained 50 mM TMPD, 0.8 mg/mL microsomal protein, and 1.6 mM NADPH in a total volume of 0.5 mL of 100 mM phosphate buffer, pH 7.4, at 37°C.

(A) The microsomal protein concentrations were 0.2 mg/mL (●), 0.4 mg/mL (■), and 0.8 mg/mL (▲). (B) The SKF-525A concentrations were 0 mM (●), 0.125 mM (□), 0.25 mM (▲), and 0.5 mM (▲). (C) The NaN\(_3\) concentrations were 0 mM (●), 2 mM (□), 10 mM (▲), and 20 mM (▲). (D) The \(\beta\)-carotene concentrations were 0 mM (●), 0.2 mM (□), 0.4 mM (▲), and 0.6 mM (▲).

stabilization of the generated \(^1\)O\(_2\) or ineffective supply of H\(^+\) in the P450 cycle. The results corresponded well with the observation that \(^1\)O\(_2\) and H\(_2\)O\(_2\) generated from the uncoupling and autoxidation in P450 catalytic cycle do not contribute to the P450-dependent substrate reactions.\(^{35,46}\) Previously we detected \(^1\)OH in terms of DMPO-OH adduct (data not shown) in the rat liver microsomes-NADPH system by the ESR spin-trapping using DMPO as a trapping agent.\(^{22}\) Because a DMPO-OOH adduct by the reaction of \(^1\)O\(_2\) with DMPO is easily converted into the DMPO-OH adduct, the detection of DMPO-OH adduct is not precise to explain the reaction. Because the substrate oxidations were not altered by SOD or DMSO, and the signal intensity due to DMPO-OH adduct was not changed by the addition of substrates, \(^1\)O\(_2\) and \(^1\)OH are not likely to contribute to the P450-dependent substrate oxidations.

The finding that \(\omega\)- and (\(\omega\)-1)-hydroxylations of lauric acid and \(O\)-demethylation of \(p\)-nitroanisole were significantly suppressed by SKF-525A (Fig. 2(A) and (B)) indicated the contribution of the heme site of P450. In addition, the fact that \(^1\)O\(_2\) formed with P450 was decreased by SKF-525A (Fig. 3(B)) provided strong evidence that \(^1\)O\(_2\) is generated from the heme site, because SKF-525A coordinates to the sixth position of the heme in P450 and inhibits the binding of molecular dioxygen to the heme and following activation of P450. In support of this conclusion, our preliminary experiments
with the heme proteins and simple iron-heme complexes suggested that \( ^1\text{O}_2 \) is generated at the heme. Experiments aimed at determining the true origin of \( ^1\text{O}_2 \) are now in progress.

We then examined the relationship between the demethylation rate of \( p \)-nitroanisole and its reactivity to \( ^1\text{O}_2 \) as evaluated from the decrease of the signal intensity due to 4-oxo-TEMPO in rat liver microsomes. Incubation conditions were the same as those for Fig. 3. The reaction mixture contained 50 mM TMPD, 0.8 mg/mL microsomal protein, and 0.2–1.6 mM NADPH, with or without 0.4 mM \( p \)-nitroanisole, in a total volume of 0.5 mL of 100 mM phosphate buffer, pH 7.4, at 37°C. The correlation coefficient for the linear regression was estimated to be 0.971 for the 3 or 4 repeated experiments.

![Graph](image)

Fig. 4. Relationship between the \( O \)-demethylation rate of \( p \)-nitroanisole and its reactivity to \( ^1\text{O}_2 \) as evaluated from the decrease of the signal intensity due to 4-oxo-TEMPO in rat liver microsomes. Incubation conditions were the same as those for Fig. 3. The reaction mixture contained 50 mM TMPD, 0.8 mg/mL microsomal protein, and 0.2–1.6 mM NADPH, with or without 0.4 mM \( p \)-nitroanisole, in a total volume of 0.5 mL of 100 mM phosphate buffer, pH 7.4, at 37°C. The correlation coefficient for the linear regression was estimated to be 0.971 for the 3 or 4 repeated experiments.

The correlation coefficient for the linear regression was estimated to be 0.971 for the 3 or 4 repeated experiments.

![Graph](image)

Fig. 5. Concentration-dependent scavenging effect of \( \beta \)-carotene (A) and \( p \)-nitroanisole (B) on the chemically generated \( ^1\text{O}_2 \) in the hematoporphyrin (HP)-UVA system. The reaction systems contained 62.5 \( \mu \)M HP, 50 mM TMPD, and 0-500 \( \mu \)M \( \beta \)-carotene or 0-20 mM \( p \)-nitroanisole in a total volume of 0.2 mL of 100 mM phosphate buffer, pH 7.4, at a room temperature (22°C). ESR spectra were measured after 30 sec of UVA irradiation in system.
Fig. 6. Effect of $^{1}$O$_2$ quenchers and a spin-trapping agent on P450 enzyme activities in human liver microsomes.

The reaction times were as follows: (A) 7-ethoxyresorufin O-deethylation as CYP1A1/1A2 activity for 10 min, (B) coumarin 7-hydroxylation as CYP2A6 activity for 5 min, (C) tolbutamide 4-hydroxylation as CYP2C9 activity for 30 min, and (D) nifedipine oxidation as CYP3A4 activity for 5 min. Each incubation mixture contained 0.2 mg/mL microsomal protein, and 0.5 mM 7-ethoxyresorufin, 50 mM coumarin, 200 mM tolbutamide, or 20 mM nifedipine in a total volume of 0.5 mL of 100 mM phosphate buffer, pH 7.4, at 37°C, in which the reaction was initiated by the addition of an NADPH generating system (2 mM NADP+, 10 mM G-6-P, 5 mM MgCl$_2$, and 1 U/mL G-6-PDH as a final concentration). The 0-5 mM NaN$_3$, 0-0.5 mM $b$-carotene, or 0-50 mM TMPD were added to the reaction mixture. Significance: * $p < 0.05$, ** $p < 0.01$ vs. control (n = 3).

demethylation of p-nitroanisole through its strong un-
coupling of P450, but not to inhibit the P450 reduction
by NADPH-cytochrome P450-reductase. However, the
inhibition of P450 activity by flavonoids may instead
be explained by the flavonoids' $^{1}$O$_2$ quenching activities
in the P450 reaction pathway. Further studies are need-
ed to clarify this point.

We next examined the effect of $^{1}$O$_2$ quenching on the
human P450-specific activities. Among four specific
P450 activities, CYP1A1/1A2 activity was only affected
by $b$-carotene; however, other activities (CYP2A6,
CYP2C9, and CYP3A4) were suppressed by NaN$_3$, $b$-
carotene, and TMPD (Fig. 6). That is because NaN$_3$
might affect the binding of a type II substrate such as
aniline to P450$_{222}^{22}$ or the quenching $^{1}$O$_2$ generated.
In addition, $b$-carotene is known to react with $^{1}$O$_2$ at higher
rate constant ($3 \times 10^{10} \text{ M}^{-1} \text{s}^{-1}$) than TMPD ($1.8 \times
10^9 \text{ M}^{-1} \text{s}^{-1}$). These observations suggested that there
are many ways that $^{1}$O$_2$ contributes to human P450 ac-
tivities depending on the members of the CYP subfami-
ly. These findings are of interest, in terms of P450
enzyme activity inhibition, in light of the significant die-
tary exposure of humans to natural products that in-
clude a variety of $^{1}$O$_2$ quenchers.

On the basis of these results, we propose here the
general involvement of $^{1}$O$_2$ in the liver microsomal
P450-dependent substrate oxidations. Although the true
origin of $^{1}$O$_2$ in the P450 reaction pathway is still uncer-
tain, it is highly possible that $^{1}$O$_2$ is formed as the six-
coordinate Fe(II)-$^{1}$O$_2$ intermediate in P450 protein,
because the Fe(II)-$^{1}$O$_2$ intermediate protein accepts the
second electron more effectively than does the six-coor-
dinate Fe(III)-$^{1}$O$_2^-$ intermediate through the higher
redox potential of the former. In fact, $^{1}$O$_2$ is a better
electron acceptor (redox potential; +0.65 V) and is
more easily converted to $^{1}$O$_2$ than the triplet state of
molecular dioxygen $^{3}$O$_2$ (redox potential; −0.33 V). The
origin of the energy that converts the triplet state of
molecular dioxygen bound to Fe(II)-heme to its excited
singlet state, as well as the origin of $^{1}$O$_2$ in the P450 cata-
lytic cycle, must be mechanistically investigated in fu-
ture studies.

In conclusion, $^{1}$O$_2$ generation and its role in the sub-
strate oxidations in both the rat and human liver
microsomal systems are presented with respect to the
suppression of substrate oxidations by $^{1}$O$_2$ quenchers,
the detection of $^{1}$O$_{2}$ generation by the ESR spin-trapping method, $^{1}$O$_{2}$ consumption during the substrate oxidations, and the possibility that heme-Fe(II)$^{1}$O$_{2}$ intermediate is the origin of $^{1}$O$_{2}$ in the P450 cycle. In addition, $\beta$-carotene, which is a dietary natural product, was found to be a suppressor against human P450 activity as well as being a potent chemical quencher of $^{1}$O$_{2}$. This latter finding is important in terms of P450-activity inhibition for the safe use of pharmaceuticals in humans.

References

26) Clarke, S. E., Baldwin, S. J., Bloomer, J. C., Ayerton, A. D., Sozio, R. S. and Chenery, R. J.: Lauric acid as a model substrate for the simultaneous determination of cytochrome P450 2E1 and 4A in hepatic microsomes.


