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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 ($^1O_2$) was involved in rat liver microsomal P450-dependent substrate oxygenations in such reactions as *p*-hydroxylation of aniline, *O*-deethylation of 7-ethoxycoumarin, ω- and (ω-1)-hydroxylations of lauric acid, *O*-demethylation of *p*-nitroanisole, and *N*-demethylation of aminopyrine. In order to confirm the generality of $^1O_2$ 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 $^1O_2$ quenchers, β-carotene, suppressed the hydroxylation of *p*-nitrophenol. Furthermore, a nonspecific P450 inhibitor, SKF525A, and a ferric chelator, deferoxamine, both suppressed the hydroxylation. No other ROS scavengers such as superoxide dismutase (SOD), catalase, or mannitol altered the reaction. $^1O_2$ 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 $^1O_2$ was quenched by additions of β-carotene, *p*-nitrophenol, and SKF525A. The reactivity of *p*-nitrophenol and $^1O_2$ correlated linearly with its hydroxylation rate in the microsomes. On the basis of these results, we conclude that $^1O_2$ contributes to the *p*-nitrophenol hydroxylation in rat liver microsomes, by adding a new example of $^1O_2$ involvement in the CYP2E1-dependent substrate oxygenations.

Key words: cytochrome P450; CYP2E1; *p*-nitrophenol hydroxylation; singlet oxygen ($^1O_2$); 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-π-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 ($^1O_2$) in *p*-hydroxylation of aniline, *O*-deethylation of 7-ethoxycoumarin, ω- and (ω-1)-hydroxylations of lauric acid, *O*-demethylation of *p*-nitrosnisole, 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-

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Abbreviations: P450: cytochrome P450, NADPH: nicotinamide adenine dinucleotide phosphate reduced form, ROS: reactive oxygen species, ESR: electron spin resonance, SOD: superoxide dismutase, DMSO: dimethylsulfoxide, SKF525A: 2-diethylaminoethyl-2,2-diphenylpentanoate, TMPD: 2,2,6,6-tetramethyl-4-piperidone, DMPO: 5,5′-dimethyl-1-pyrroline-*N*-oxide, 4-oxo-TEMPO: 2,2,6,6-tetramethyl-4-piperidone-*N*-oxyl

terized 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 $^1O_2$-dependent substrate oxygenation. To achieve this, we used *p*-nitrophenol as an indicator of CYP2E1 activity[12–15] 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 (NaN_3) 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). β-NADPH was purchased from Oriental Yeast (Tokyo, Japan). 5,5-Dimethyl-1-pyrroline-*N*-oxide (DMPO) was purchased from Labotec (Tokyo, Japan). 2,2,6,6-Tetramethyl-4-piperidone-*N*-oxyl (4-oxo-TEMPO), and 2,2,6,6-tetramethyl-4-piperidone (TMPD) were purchased from Aldrich (Milwaukee, WI, USA). Rabbit anti-rat CYP2E1 was obtained from Chemicon International, Inc. (Temecula, CA, USA). Peroxidase-conjugated goat anti-rabbit immunoglobulin was purchased from Dakocytomation Co. (Kyoto, Japan). Rat CYP2E1+P450 reductase microsomes which are expressed from a transfected rat CYP2E1 cDNA in human lymphoblast was obtained from Gentest Co. (Woburn, MA, USA). All other chemical compounds used were of the highest analytical grade commercially available.

Animals: Male Wistar rats (6 weeks old) weighing 180–240 g were purchased from Shimizu Experimental Material Co. (Kyoto, Japan) and maintained on a light/dark cycle in our central animal facility before being used. All animals had free access to diet and tap water prior to the experiments. All experiments were approved by the Experimental Animal Research Committee of Kyoto Pharmaceutical University (KPU) and performed according to the Guidelines for Animal Experimentation of KPU.

Microsomes preparations: Microsomes were prepared from the livers of male Wistar rats. The animals were fasted overnight prior to being sacrificed. Under the anesthetized condition by ether, the livers were perfused with an ice-cold 1.15% (w/v) KCl and then homogenized. The nuclei and mitochondria were removed by centrifugation at 12,000×g for 15 min. The supernatant was centrifugated at 105,000×g for 65 min. The obtained microsomal pellet was suspended in 1.15% (w/v) KCl containing 0.25 mM 4-amidinophenyl-methanesulfonyl fluoride, and it was centrifugated at 105,000×g for 65 min. The pellet was resuspended in 0.1 M potassium phosphate buffer, pH 7.4, containing 30% (v/v) glycerol and 1 mM dithiothreitol. The protein concentration was determined by the method of Lowry *et al.* using BSA as the standard.[16] P450 was assayed by the method of Omura 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.[12–14] 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/