Differential Effect of Copper (II) on the Cytochrome P450 Enzymes and NADPH−Cytochrome P450 Reductase: Inhibition of Cytochrome P450-Catalyzed Reactions by Copper (II) Ion†

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ABSTRACT: Inhibitory effects of Cu^{2+} on the cytochrome P450 (P450)-catalyzed reactions of liver microsomes and reconstituted systems containing purified P450 and NADPH−P450 reductase (NPR) were seen. However, Zn^{2+}, Mg^{2+}, Mn^{2+}, Ca^{2+}, and Co^{2+} had no apparent effects on the activities of microsomal P450s. Cu^{2+} inhibited the reactions catalyzed by purified P450s 1A2 and 3A4 with IC_{50} values of 5.7 and 8.4 μM, respectively. Cu^{2+} also inhibited reduction of cytochrome *c* by NPR (IC_{50} value of 5.8 μM). Copper caused a decrease in semiquinone levels of NPR, although it did not disturb the rate of formation of semiquinone. P450 reactions supported by an oxygen surrogate, *tert*-butyl hydroperoxide, instead of NPR and NADPH, were inhibited by the presence of Cu^{2+}. The results indicate that Cu^{2+} inhibits the P450-catalyzed reactions by affecting both P450s and NPR. It was also found that the inhibition of catalytic activities of P450s by Cu^{2+} involves overall conformational changes of P450s and NPR, investigated by CD and intrinsic fluorescence spectroscopy. These results suggest that the inhibitory effect of Cu^{2+} on the P450-catalyzed reactions may come from the inability of an efficient electron transfer from NPR to P450 and also the dysfunctional conformation of NPR and P450.

Microsomal monooxygenase metabolizes a variety of endogenous and xenobiotic compounds including steroids, drugs, and carcinogens in eukaryotes (*1*, *2*). This enzyme system includes cytochrome P450 (P450[1]; EC 1.14.14.1), NADPH−P450 reductase (NPR; EC 1.6.2.4), and phospholipids. However, the organization of constituent proteins in phospholipid membranes and their mechanism of interaction are not fully understood yet. On the other hand, P450 is present in the membrane in large excess over NPR, the limiting component in microsomes, with the molar ratios ranging from 10:1 to 25:1 depending on treatment with inducers (*3*). Therefore, the proper interaction of P450 with NPR should be important for efficient electron transfer. We have shown previously that the changes in activity of P450s 1A2 and 2B1 are associated with the conformational changes of P450s induced by salt, phospholipid, and detergent (*4*−*6*) and that the membrane topology and activity of P450 1A2 are related to the composition of phospholipids (*7*). The conformation of NPR is also known to be important for catalytic activity (*8*).

Metal ions are components of important cellular constituents and participate in a variety of cellular activities (*9*). An increasing number of human diseases are thought to relate to disturbances in metal ion homeostasis, including metal ion overload and deficiency disorders (i.e., Menkes disease, Wilson's disease, anemia), and neurodegenerative diseases (i.e., Alzheimer's and Parkinson's) (*10*). Excess intake of almost all of the metal ions produces toxic symptoms (*9*). Recently, it was reported that copper treatment of hepatocytes results in induction of apoptosis, which occurs during the course of hepatic failure in acute Wilson's disease (*11*). Intracellular levels of metal ions should therefore be under homeostatic control. The levels of metal ions might be important factors in controlling the activities of P450s and NPR, which are crucial enzymes in the metabolism of drugs, steroids, and carcinogens.

To date, the effects of divalent metal ions except Cu^{2+} (see below) on the activity of P450 enzymes have not been well understood. It was reported that a stimulatory effect of Mg^{2+} and Ca^{2+} for P450 3A4/5 activity was shown just at ion concentrations greater than 10 mM (*12*), and these ions are needed for the phospholipase D activity of P450s (*13*). It is also known that the deficiency or high level of metal in a diet can affect the P450-linked activities in animals (*14*).

It was found that rat and mice liver microsomal P450-catalyzed reactions are inhibited by $CuSO_4$ and copper chelates (*15*, *16*). The inhibition of P450 reactions in microsomes by Cu^{2+} was interpreted as reflecting the

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[1] Abbreviations: P450, microsomal cytochrome P450; NPR, NADPH−P450 reductase; DLPC, L-α-dilauroylphosphatidylcholine; PB, phenobarbital; βNF, 5,6-benzoflavone; αNF, 7,8-benzoflavone; EFC, 7-ethoxy-4-(trifluoromethyl)coumarin.

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impaired function of NPR caused by Cu^{2+} (*16*), although the mechanism of inhibition by Cu^{2+} is unknown. In vitro antimutagenic activity of copper chelates was reported, and the activity was suggested to mediate through a copper-dependent inhibition of NPR (*17*). Interestingly, it was reported that Cu^{2+} and Zn^{2+} inhibit all of the NADPH-dependent reactions catalyzed by neuronal nitric-oxide synthase including cytochrome *c* reduction, NADPH oxidation, and citrulline formation (*18*). Cu^{2+} and Zn^{2+} also inhibit cytochrome *c* reduction by the independent reductase domain of neuronal nitric-oxide synthase.

The metal binding properties of purified P450s and NPR are studied in detail for the first time in this report. Our results show that Cu^{2+} binds to both of P450s and NPR to change the activities of the enzymes accompanying the conformational changes including decreased α-helix content and decreased intrinsic fluorescence intensity. The role of Cu^{2+} in modulating the conformation and catalytic function of P450s and NPR and how these functional changes correlate with structural changes are discussed.

EXPERIMENTAL PROCEDURES

Chemicals. DLPC, testosterone, and all of divalent metal ions ($CuCl_2$, $ZnCl_2$, $CaCl_2$, $MgCl_2$, $CoCl_2$, and $MnCl_2$) were from Sigma. EFC was obtained from Molecular Probes (Eugene, OR). The Chelex 100 resin used to delete adventitious metal ions from buffers and reagents was from Bio-Rad. Other chemicals were of the highest grade commercially available.

Enzyme Preparation. P450 1A2 was purified from the liver microsomes of βNF-treated rabbits as described (*19*). Human liver P450 3A4 was expressed in *Escherichia coli* and purified as described (*20*). The P450s 1A2 and 3A4 were electrophoretically homogeneous (*21*) and had specific P450 contents of 16 and 14 nmol/mg of protein, respectively. Protein was assayed using a bicinchoninic acid procedure (Sigma). P450 concentrations were determined by $Fe^{2+}-CO$ versus Fe^{2+} difference spectroscopy (*22*). Microsomes from saline-, βNF-, PB-, and ethanol-treated rats were prepared as described (*23*).

Recombinant rat NADPH−P450 reductase was expressed in *E. coli* and purified as described (*24*). Rabbit liver cytochrome b_5 was prepared as described (*25*).

Each purified enzyme was made metal-free by extensive dialysis against 100 mM potassium phosphate (pH 7.4) buffer containing 0.1 mM EDTA and 20% glycerol. Inductively coupled plasma mass spectroscopy (ICP-MS) analysis was performed on a VQ3 ICP-MS (Thermo Elemental, Cambridge, UK) in the Korea Basic Science Institute facility. All purified enzymes contained no detectable metals: measured values for all enzymes were less than 0.01 equivalent metals of Cu, Zn, Mg, Ca, Co, or Mn per enzyme subunit besides heme iron. Stock solutions of P450s and NPR contained 20% glycerol, but final concentrations in all experiments were kept below 0.2%, since glycerol affects the P450 structure and activity (*4*−*6*) and the NPR-mediated reactions (*26*). All buffers used for experiments with the P450s were made metal ion-free by passage through a Chelex column and stored in EDTA-treated glassware. All experiments for enzyme activities were done in 100 mM potassium phosphate buffer (pH 7.4).

Enzyme Activity Assay. The assay of P450 1A2 activity was done using EFC (100 μM) as a substrate as described (*27*). Effect of divalent ions on the P450 1A2-catalyzed reaction was investigated utilizing reconstituted P450 systems containing the P450 (0.10 μM), freshly prepared sonicated DLPC (30 μM), and a 2-fold molar amount of purified NPR (0.20 μM) at 37 °C. The volume of the reaction mixture was 0.5 mL with varying concentrations of divalent ion (1−100 μM). The assay of P450 3A4 was done using testosterone (200 μM) as a substrate as described (*28*); the reaction mixture contained P450 3A4 (0.10 μM), NPR (0.20 μM), and cytochrome b_5 (0.20 μM) in 100 mM potassium phosphate buffer (pH 7.4) containing 0.5 mM cholate, and 30 μg of a phospholipid mixture (DLPC, L-α-dioleoylphosphatidylcholine, bovine brain phosphatidylserine, 1:1:1, w/w/w). $MgCl_2$ was omitted from the reaction mixture to see the effect of divalent ions on the P450 3A4-catalyzed reactions. The reconstituted enzymes were mixed with the phospholipid and incubated at 23 °C for 10 min prior to the addition of buffer and substrate. The complete reconstituted reaction mixtures were preincubated for 5 min at 37 °C, and then incubated with indicated concentration of ions for additional 10 min at 37 °C before initiation of the reaction. The reactions for P450s were initiated by adding NADPH-generating system consisting of 5 mM glucose 6-phosphate, 5 mM $NADP^+$, and 0.5 units of glucose 6-phosphate dehydrogenase/ mL. Actually, there were no apparent differences on the generation of NADPH with or without $MgCl_2$ in this NADPH-generating system. In both cases, the formation of NADPH was saturated in a 10 s after the addition of the NADPH-generating system to 100 mM potassium phosphate buffer (pH 7.4).

Liver microsomal incubations included microsomes (0.2 mg of protein/mL) prepared from βNF- and PB-treated rats in place of reconstituted components in 100 mM potassium phosphate buffer (pH 7.4) in the presence of varying divalent ion concentrations. Reactions were initiated by the addition of the NADPH-generating system (*23*).

When the reaction was supported by *tert*-butyl hydroperoxide, each incubate contained 0.20 μM of P450 and other components (except NPR and cytochrome b_5) in addition to the same concentrations of the substrates and indicated divalent ion in 100 mM potassium phosphate buffer (pH 7.4) at 37 °C as described above for the NPR-supported reactions. The reaction was initiated by the addition of *tert*-butyl hydroperoxide (to 5 mM) instead of the NADPH-generating system.

The assay for NPR was done using cytochrome *c* as a substrate as described (*29*). The NPR-mediated reductions were measured in 100 mM potassium phosphate buffer, pH 7.4.

Spectroscopy. Absorption spectra were recorded with a Shimadzu UV-1601 spectrophotometer. Fluorescence levels were measured with a Shimadzu RF-5301 PC spectrofluorometer equipped with a thermostated cuvette compartment maintained at 37 °C. Prior to each measurement, each P450 or NPR was diluted to 1.0 μM in potassium phosphate buffer (pH 7.4) containing DLPC or phospholipid mixture as described for the enzyme assay in a final volume of 2.0 mL. Emission spectra were corrected using a solution without enzyme. Emission spectra of the intrinsic fluorescence in

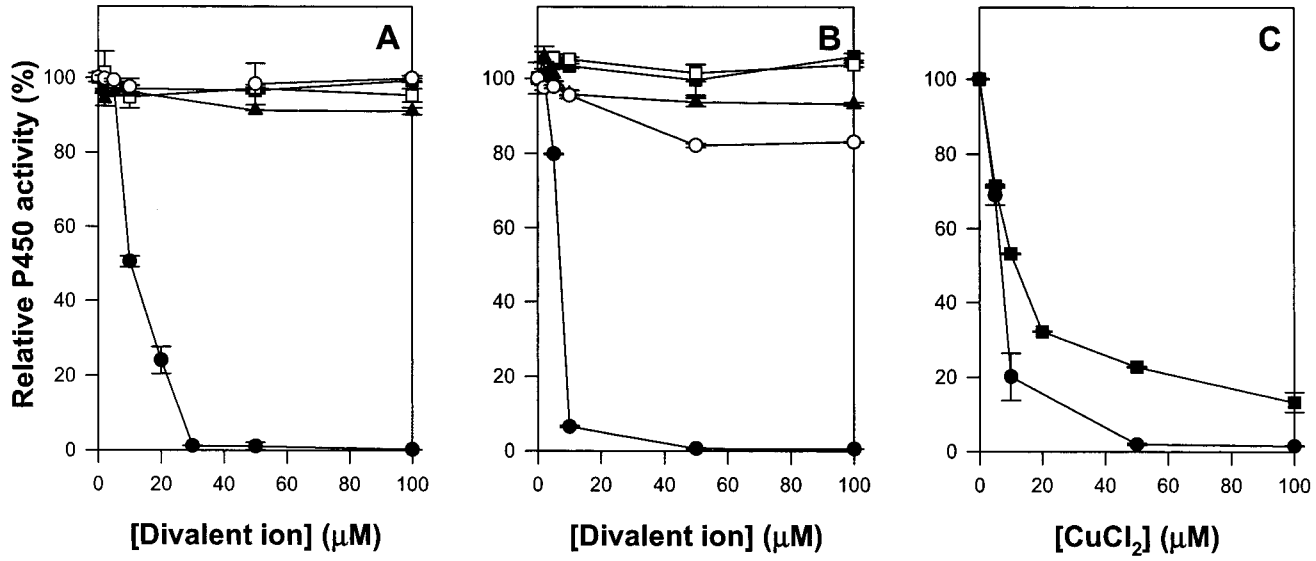

FIGURE 1: Effect of divalent metal ions on the EFC *O*-deethylation activities of liver microsomes and reconstituted P450s. (A and B) Effect of divalent metal ions on the liver microsomal EFC *O*-deethylation activities from βNF-(A) and PB-(B) treated rats. The 100% activity values represent the normalized activities (9.5 and 12 nmol of product/min/nmol of P450 for βNF- and PB-treated rat liver microsomes, respectively). The following symbols represent the indicated ions: Cu^{2+}, ●; Zn^{2+}, ○; Ca^{2+}, ■; Mg^{2+}, □; Mn^{2+}, ▲. (C) Effect of Cu^{2+} on the EFC *O*-deethylation activities of P450 1A2 (●) and on the testosterone 6β-hydroxylation of P450 3A4 (■) supported by NPR. The 100% activity values represent the normalized activities (2.4 and 8.3 nmol of product/min/nmol of P450 for 1A2 and 3A4, respectively). The experimental protocols are as described under Experimental Procedures. Values are means ± SD of triplicate experiments.

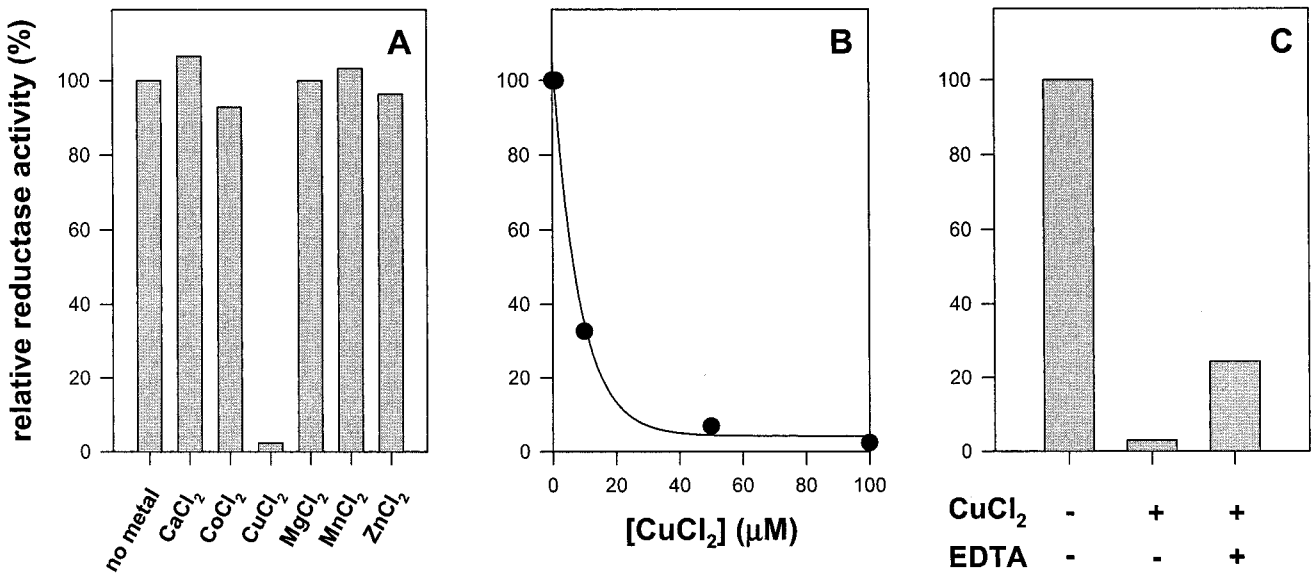

FIGURE 2: Effect of divalent ions on the NPR activity. (A) The reductase activity was measured in the absence of divalent ion or presence of Ca^{2+}, Co^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, or Zn^{2+}. The concentration of each metal ion was 50 μM. The 100% activity values represent the normalized activities (3.9 μmol of reduced cytochrome *c*/min/nmol of NPR). (B) Cu^{2+}-dependent inhibition of NPR activity as a function of $CuCl_2$ concentration. (C) Effect of EDTA on the Cu^{2+}-induced inhibition of NPR activity. The reductase activity was measured in the absence (−) or presence (+) of 50 μM Cu^{2+}. After NPR was incubated with 50 μM Cu^{2+} for 5 min and then with additional 1 mM EDTA for 5 min, the NPR activity was measured. All values are means of duplicate experiments. Absolute values varied <5%.

P450 or NPR were recorded in the range of 300−450 nm with the excitation wavelength of 280 nm. In all fluorescence experiments, each measurement under the experimental condition was corrected for an inner filter effect due to light scattering and absorption, as described (*30*). The maximum volume increase due to sequential addition of the divalent ion solutions was <2% (v/v).

CD spectra were recorded on a Jasco J700 spectropolarimeter (Japan Spectroscopic, Tokyo) at 37 °C in a thermostated cuvette. CD spectra of each P450 or NPR were obtained using 1.0 μM protein containing DLPC or a phospholipid mixture in a 0.1-cm path length cell. Blanks (buffer with DLPC or a phospholipid mixture) were routinely recorded and subtracted from the original spectra. The CD spectra were curve-fitted by the least-squares method into the reference spectra obtained from five proteins: myoglobin, lysozyme, ribonuclease A, papain, and lactate dehydrogenase (*31*).

Estimation of Binding Constants by Spectral Binding Titrations. Because rabbit P450 1A2 is a mixture of high- and low-spin (*4*), and human P450 3A4 is predominantly low-spin (*20*), the usual binding spectral titrations (*20*) were used to determine dissociation constants for substrates in the presence or absence of Cu^{2+}.

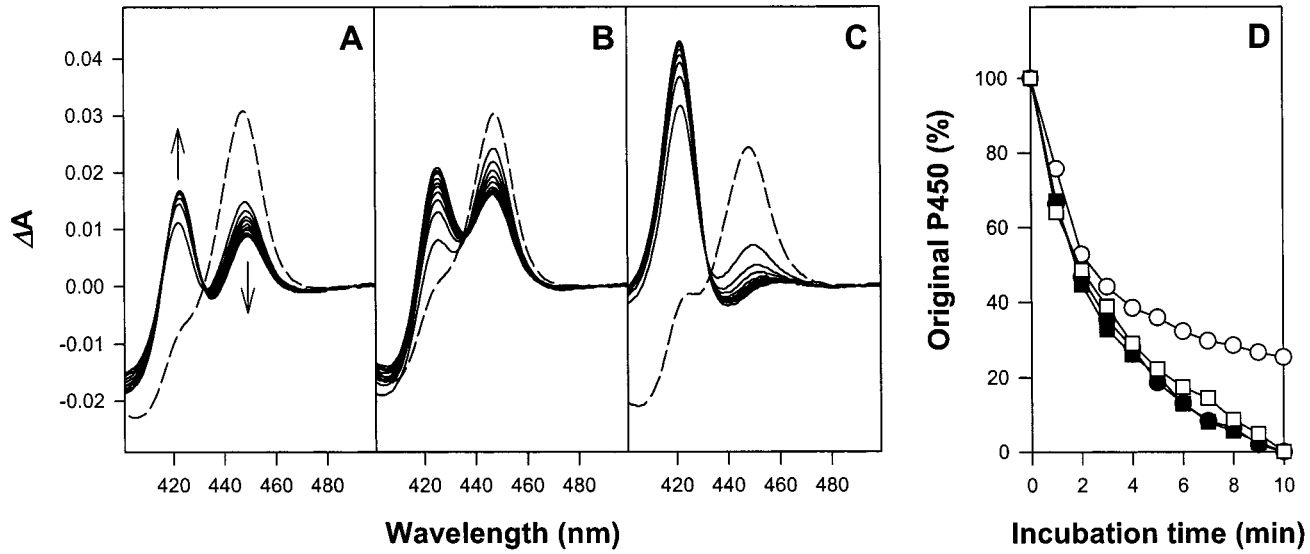

FIGURE 3: Time-dependent loss of P450 spectra of purified and microsomal P450s follwing incubation with Cu^{2+}. After P450 spectra ($Fe^{2+}-CO$ versus Fe^{2+}) were measured for each untreated P450 (dotted lines), the changes of each P450 spectrum were measured following the addition of Cu^{2+} (50 μM) in potassium phosphate (pH 7.4) and 30 °C, with 1-min intervals between measurements (A, B, and C). The arrows show direction of spectral change with time. Although arrows were only shown in (A), the spectra of (A−C) showed the same trend as shown in (A). (A) P450 1A2 with Cu^{2+}; (B) αNF (30 μM) was added to P450 1A2 before measurement of P450 spectrum; (C) P450 3A4 with Cu^{2+}; (D) Cu^{2+}-dependent destruction of microsomal P450 spectra. Liver microsomes from saline (●)-, βNF (○)-, PB (■)-, and ethanol (□)-treated rats, respectively, were incubated in the presence of 20 μM Cu^{2+} and P450 spectra ($Fe^{2+}-CO$ versus Fe^{2+}) were determined as a function of time.

NADPH Oxidation. P450 1A2 or P450 3A4 was reconstituted with NPR as described for the enzyme activity assay. Reconstituted enzyme (950 μL) including a substrate was preincubated for 3 min at 37 °C in the presence or absence of $CuCl_2$ (50 μM). Reactions were initiated with the addition of 50 μL of 4.0 mM NADPH, and the decrease in A_{340} was monitored for 1 min (*32*).

H_2O_2 Formation. Reaction systems were prepared as described above, except that the reaction volume was 0.4 mL. Reactions were initiated by adding the NADPH-generating system and were terminated by adding 0.80 mL of cold CF_3CO_2H (3%, w/v) after 1 min. H_2O_2 was determined spectroscopically by reaction with ferroammonium sulfate and KSCN as described (*33*).

RESULTS

Effects of Divalent Ions on the Catalytic Activities of P450 Enzymes. Initial studies were done with liver microsomes of βNF- and PB-treated rats to see the effects of several types of metal ion on the microsomal P450 activities (Figure 1A,B). EFC was used as a substrate as it is a good substrate for several P450s including P450s 1A1, 1A2, 2B1, and 2B2 (*27*). The microsomal EFC *O*-deethylation activities of both βNF- and PB-treated rats decreased with increasing Cu^{2+} concentrations (IC_{50} = 11 ± 2.6 and 5.7 ±1.8 μM, respectively). This result shows that microsomal EFC *O*-deethylation activity of PB-treated rats is more sensitive to Cu^{2+} compared to that of βNF-treated rats. When Co^{2+}, Ca^{2+}, Mn^{2+}, or Mg^{2+} was added, no apparent inhibitions in P450 activities were seen within the ranges examined here (up to 100 μM). In the case of Zn^{2+}, it inhibited microsomal EFC *O*-deethylation activity of PB-treated rats up to 20%. Cu^{2+} was clearly the most effective among cations examined here to inhibit the P450-catalyzed reactions. To demonstrate that the effect was not sensitive to the anion chosen, we examined the effect of $CuSO_4$ on the P450-catalyzed reactions. The effect of $CuSO_4$ was exactly same as that of $CuCl_2$.

To study the mechanism of how Cu^{2+} inhibits the P450-catalyzed reactions, we investigated the effects of Cu^{2+} on the reconstituted systems including two types of P450 enzymes and NPR. Purified NPR and P450s 1A2 and 3A4 were chosen as model enzymes. It was reported that ethoxyresorufin *O*-deethylation activity, a marker enzyme activity of P450 1A2, was affected by copper in vivo and in vitro (*34*, *35*). P450 3A4 was chosen as a model P450 to examine the effect of Cu^{2+} on the drug metabolism. In humans, P450 3A4 is generally agreed to be the most abundant P450 in both liver and small intestine, two major sites for oxidation of xenobiotic chemicals (*36*, *37*). At first, we examined the effects of Cu^{2+} on the P450 activities supported by NPR and NADPH. The enzymatic activities of P450s 1A2 and 3A4 were quantified by measuring the ability to catalyze the EFC *O*-deethylation and testosterone 6β-hydroxylation, respectively (Figure 1C). The activities toward EFC and testosterone decreased in a concentration-dependent manner when Cu^{2+} was added to the P450s 1A2 and 3A4 solutions, respectively (IC_{50} = 5.7 ± 2.6 and 8.4 ± 2.1 μM, respectively).

The effects of Cu^{2+} on the microsomal activities were comparable to those of Cu^{2+} on the activities of purified P450s 1A2 and 3A4 (Figure 1). These results suggest that the inhibition of catalytic activities of P450s by Cu^{2+} can come from the effect of Cu^{2+} on P450 or NPR, or come from that of Cu^{2+} on both enzymes.

Effect of Cu^{2+} on the Catalytic Activity of NPR. We examined the effect of Cu^{2+} on the NPR activity using cytochrome *c*, which is known as a typical substrate (*23*), and compared to those of other metal ions including Zn^{2+}, Co^{2+}, Ca^{2+}, Mn^{2+}, or Mg^{2+} (Figure 2A). Only Cu^{2+} showed an inhibitory effect on the NPR activity in a concentration-

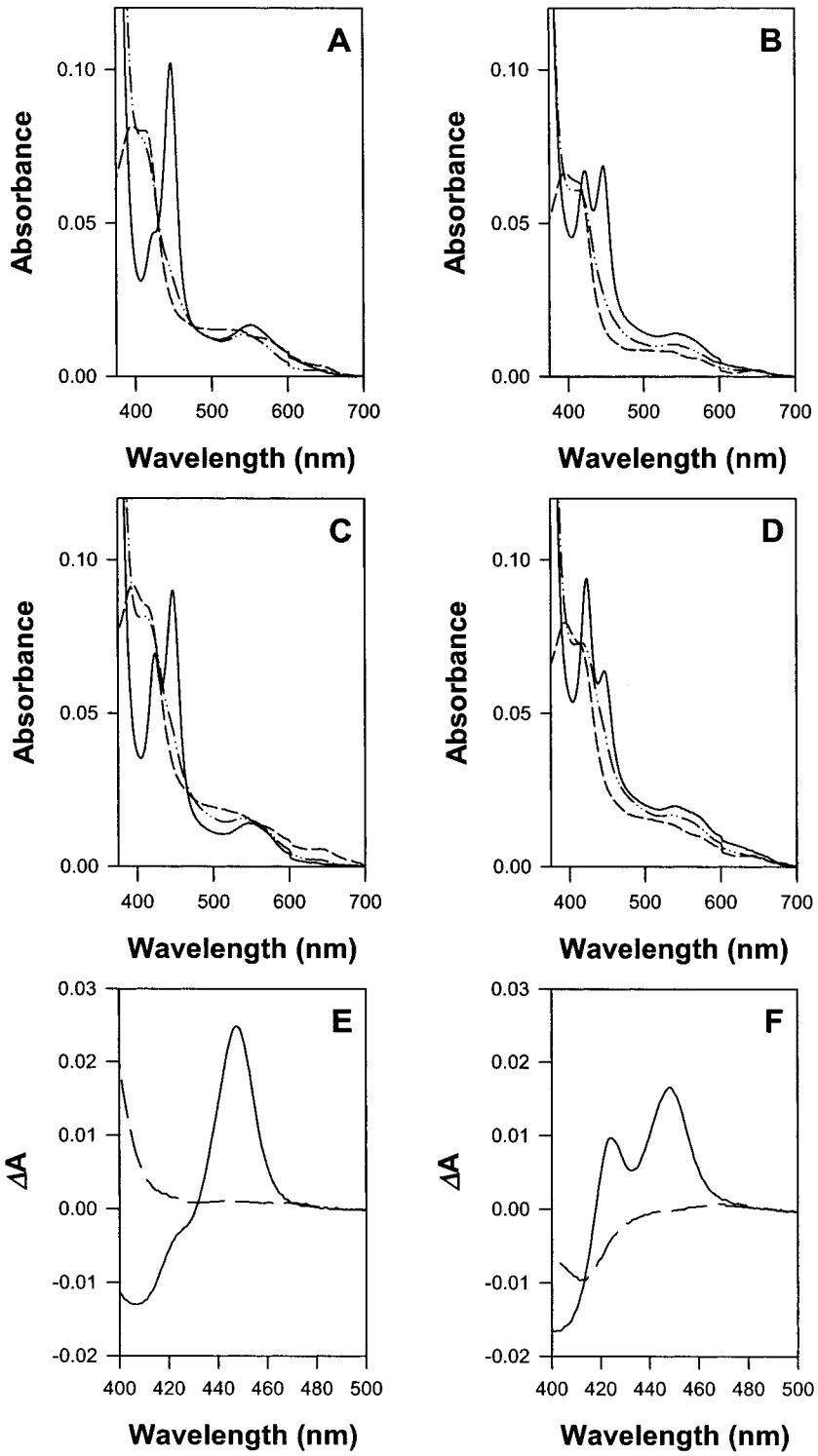

FIGURE 4: Absorption spectra of ferric, ferrous, and ferrous−CO complexes of P450s 1A2 and 3A4 in the absence and presence of Cu^{2+}. (A−D) Absorption spectra of ferric (− − −), sodium dithionite-reduced ferrous (−··−), and ferrous−CO (EnDash−) complexes of P450s 1A2 (A and B) and 3A4 (C and D) in the absence (A and C) and presence (B and D) of 50 μM Cu^{2+} were measured. After spectra of ferric P450s were measured, a few grains of sodium hydrosulfite were added to the P450 solution to obtain ferrous P450. (E−F) Reduced CO difference spectra of P450 1A2 (E) and P450 3A4 (F) with NPR and NADPH, instead of sodium hydrosulfite, were measured in the presence (− − −) or absence (−) of 50 μM Cu^{2+} in 100 mM potassium phosphate buffer (pH 7.4).

dependent manner ($IC_{50} = 5.8 \pm 1.2$ μM) (Figure 2B). It is known that Cu^{2+} does not affect cytochrome *c* itself. (*18*) It was also found that the Cu^{2+}-induced inhibition of NPR activity was recovered up to 25% by treatment of EDTA, a chelating agent for divalent ion (Figure 2C).

Effects of Cu^{2+} on the CO-Binding Spectra of P450 Enzymes. When purified P450s are incubated with 50 μM Cu^{2+}, spectrally measured P450s (Fe^{2+}·CO) were found to be converted to P420 (Figure 3A−C). The extent of loss of CO-binding spectra was dependent upon the type of P450 and was greater than 70% within 10 min (Figure 3A,C). The destruction was time-dependent and could not be recovered by EDTA (up to 0.5 mM) (results not shown). Whether Cu^{2+} was added to the P450 solution before or after the addition

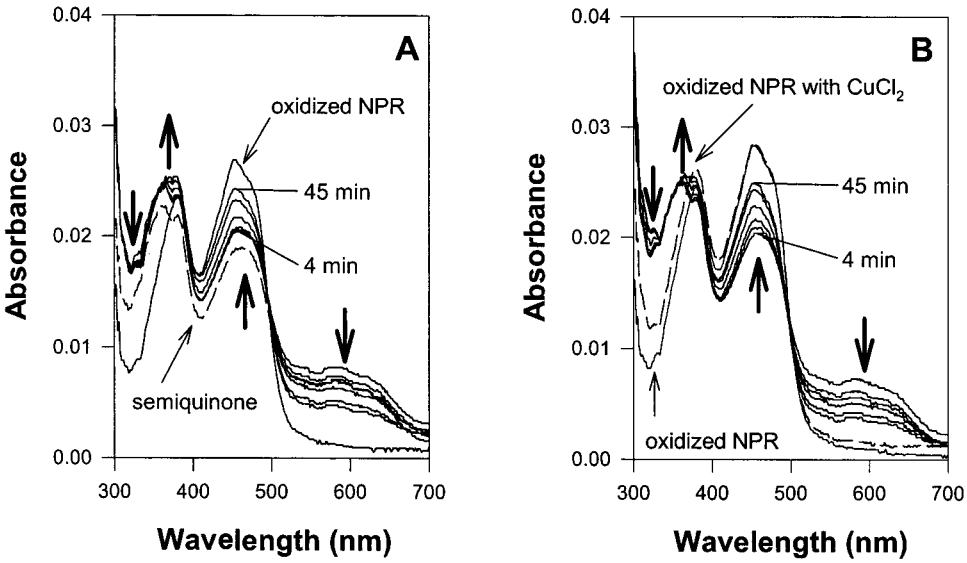

FIGURE 5: Cu^{2+}-induced destabilization of the reduced semiquinone spectrum of NPR. The incubation contained 1.0 μM NPR in 100 mM potassium phosphate (pH 7.4). In each panel, the spectrum of the oxidized NPR is shown. (A) Cu^{2+}-induced reoxidation of the semiquinone. The reduced semiquinone (− − −) was obtained after the addition of a 5-fold molar excess of NADPH and recorded after equilibration has been reached (>15 min). The solid lines indicate spectra recorded at 4, 8, 12, 16, 20, 30, and 45 min after the addition of 10 μM $CuCl_2$. (B) Effect of Cu^{2+} on the reduction of oxidized NPR. After spectrum of oxidized NPR was measured in the presence of 10 μM Cu^{2+} (− − −), spectra were recorded at 4, 8, 12, 16, 20, 30, and 45 min following the addition of a 5-fold molar excess of NADPH. The arrows show changes from the reduced semiquinone to the oxidized form with time.

of CO gas, the pattern of destruction was found to be similar. We considered a possibility that addition of a ligand to P450 1A2 might stabilize its conformation during incubation with Cu^{2+}. αNF is known to be a strong inhibitor of P450 1A2 (*38*). Addition of αNF (30 μM) decreased the rate of conversion from P450 to P420 (Figure 3B).

As expected, Cu^{2+} also decreased the level of P450 (Fe^{2+}·CO) in all types of liver microsomes (*23*) from saline-, βNF-, PB-, and ethanol-treated rats (Figure 3D). The rates at which P450 (Fe^{2+}·CO) was lost were comparable to those of purified P450s. These results indicate that Cu^{2+} binds with the enzyme and induces a conformational change to destabilize the normal orientation of bound CO to ferroporphyrin in the active sites of P450s.

Effects of Cu^{2+} on the Absorption Spectra of Ferric, Ferrous, and Ferrous−CO Complexes of P450 Enzymes. Figure 4 shows several types of absorption spectra of P450s 1A2 and 3A4 in the presence or absence of Cu^{2+}. The ferric complexes of the enzymes with substrates had Soret absorption peaks at 390 and 417 nm (Figure 4A,C). The addition of substrates clearly changed the spin state from low-spin to high-spin of the complexes of the P450s 1A2 (Figure 4A,B) and 3A4 (Figure 4C,D) regardless of the presence of Cu^{2+}, reflecting substrate binding to the active site. Spectra of ferrous P450s induced by sodium hydrosulfite did not show any apparent changes compared to those in the presence of Cu^{2+}. However, the absorption peaks observed at around 450 nm for the P450 CO-reduced complex in the absence of Cu^{2+} was shifted to 420 nm in the case of Cu^{2+}-containing samples (Figure 4B,D). When ferrous P450s obtained by NPR and NADPH, instead of sodium hydrosulfite, were used to measure ferrous−CO complexes, the P450s with Cu^{2+} did not show any detectable ferrous−CO complexes at ∼450 nm (Figure 4E,F). These results suggest that Cu^{2+} may cause oxidation of ferrous ion to release bound CO or a loss of the normal orientation of bound CO to the active site.

Effects of Cu^{2+} on the Absorption Spectra of Oxidized and Reduced Spectra of NPR. The addition of a 5-fold molar excess of NADPH to the oxidized NPR caused flavin reduction, with a decrease in absorption in the 450 nm region and the appearance of a broad band at 584 nm, indicative of the flavin semiquinone (*39*, *40*). The semiquinone spectrum was reached to equilibrium after 15 min (Figure 5A). When 10 μM Cu^{2+} was added to the semiquinone, the reduced spectrum was gradually diminished, with an increase in absorption in the 450 nm region and the decrease of a broad band at 584 nm by ∼40% during 30 min (Figure 5A). When 10 μM Cu^{2+} was added to the oxidized NPR, there was no apparent change in absorption spectrum (Figure 5B). When absorption spectra were measured after the addition of a 5-fold molar excess of NADPH to the semiquinone of NPR in the presence of 10 μM $CuCl_2$, the extent of semiquinone formation was similar to that of NPR in the absence of $CuCl_2$ (Figure 5B). But the level of semiquinone was gradually decreased as a function of time. A clear isosbestic point was observed for the oxidized/semiquinone transition at 492 nm for the spectral change caused by Cu^{2+}. The presence of the isosbestic point indicates that the oxidized and semiquinone spectra are in dynamic equilibrium. When $CuCl_2$ concentration increased more than 20 μM, clear spectral transition was not observed due to increased light scattering (results not shown). These results indicate that Cu^{2+} decreases the level of semiquinone, although it does not disturb the formation of semiquinone.

Effects of Cu^{2+} on the Substrate Binding Affinities, NADPH Oxidation, and Product Formation of P450 Enzymes. Table 1 summarizes the various parameters obtained for the P450s 1A2 and 3A4 both in the absence and presence of Cu^{2+}. The addition of EFC (for P450 1A2) and testosterone (for P450 3A4) produced a typical low- to high-spin conversion (Type I difference spectrum). A titration experiment of this type in the presence of Cu^{2+} yielded $K_s = 20 \pm 4$ μM for

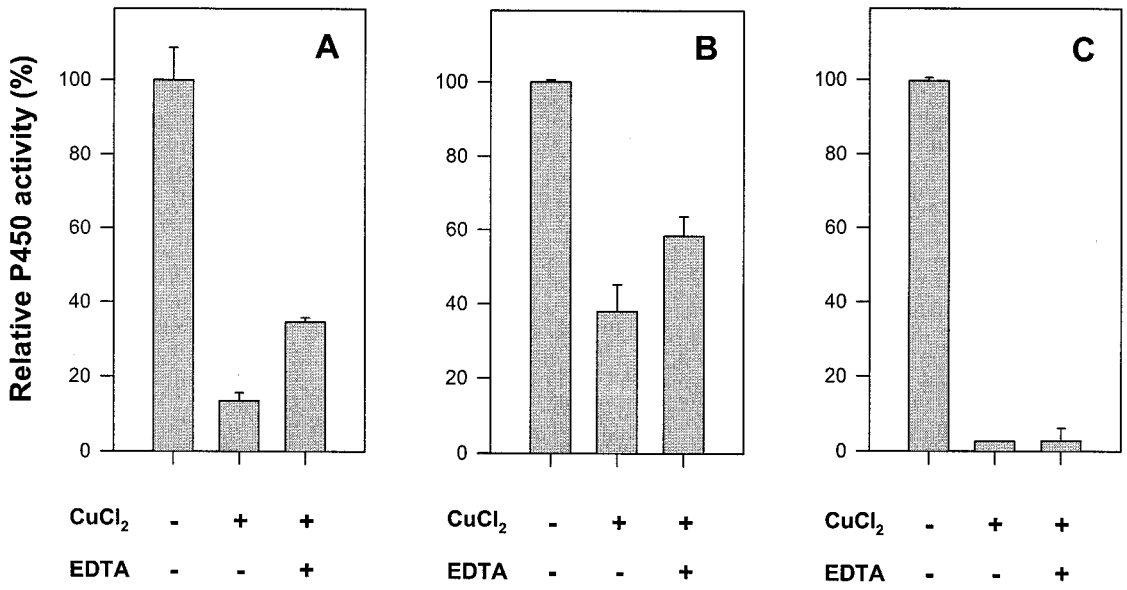

FIGURE 6: Effect of Cu^{2+} on the activities of P450s supported by *tert*-butyl hydroperoxide. (A−B) Effect of Cu^{2+} on the activities of P450s 1A2 (A) and 3A4 (B) supported by *tert*-butyl hydroperoxide. The activity was measured in the absence (−) or presence of 50 μM Cu^{2+} (+) as shown. To see the chelating effect of EDTA, 1 mM of EDTA was added to the reaction mixture containing 50 μM Cu^{2+}. The 100% activity values represent the normalized activities (0.18 and 0.35 nmol of product/min/nmol of P450 for 1A2 and 3A4, respectively). (C) Effect of EDTA on the NPR-mediated catalytic activity of reconstituted P450 1A2 in the presence of 50 μM Cu^{2+}. The EFC *O*-deethylation activity was measured in the absence (−) or presence (+) of 50 μM Cu^{2+}. After P450 1A2 was incubated with 50 μM Cu^{2+} for 5 min and then with additional 1 mM EDTA for 5 min, the P450 activity was measured. The 100% activity value represents the normalized activity (2.4 nmol of product/min/nmol of P450 1A2). All values are means ± SD of triplicate experiments.

Table 1: Effect of Cu^{2+} on the Substrate Binding, Product Formation, NADPH oxidation, and H_2O_2 Formation by P450s

			nmol of product min^{-1} (nmol of P450)$^{-1}$		
P450	50 μM $CuCl_2$	K_s (μM)[a]	NADPH oxidation[b]	product formation[b]	H_2O_2 formation[b]
1A2	−	22 ± 2	36 ± 1	2.4 ± 0.1	11.0 ± 0.4
	+	20 ± 4	44 ± 1	0.12 ± 0.01	<0.20
3A4	−	41 ± 2	51 ± 1	8.3 ± 0.1	6.7 ± 0.3
	+	40 ± 3	70 ± 2	0.40 ± 0.01	<0.10

[a] All estimates are means ± SD (n = 3). [b] All values are means of duplicate experiments. Absolute values varied <5%. All experiments were done as described under Experimental Procedures.

Table 2: Effect of Cu^{2+} on the Secondary Structure Contents of P450s and NPR[a]

		%			
P450	additions	α-helix	β-sheet	β-turn	random
1A2	none	45 ± 2	17 ± 2	16 ± 2	22 ± 2
	5 μM $CuCl_2$	16 ± 4	35 ± 7	18 ± 6	31 ± 9
3A4	none	53 ± 2	9 ± 3	7 ± 3	31 ± 5
	20 μM $CuCl_2$	46 ± 2	11 ± 3	8 ± 3	35 ± 4
NPR	none	32 ± 3	19 ± 3	24 ± 3	25 ± 3
	20 μM $CuCl_2$	32 ± 3	14 ± 3	28 ± 3	26 ± 3

[a] All CD spectra were recorded as a function of metal ions, as described under Experimental Procedures. All estimates are means ± SD (n = 3).

P450 1A2 and K_s = 40 ± 3 μM for P450 3A4. The dissociation constants of substrates to P450s are similar to those in the absence of Cu^{2+}. This result indicates that the presence of Cu^{2+} did not change the binding affinity of substrate to the enzymes.

The presence of Cu^{2+} increased the rate of NADPH oxidation by P450s 1A2 and 3A4 by 22 ± 5 and 37 ± 5%, respectively. It also inhibited the formation of products and H_2O_2 by P450s by >98%.

Effect of Cu^{2+} on the Activities of P450 Enzymes Supported by Peroxides. To examine the direct effect of Cu^{2+} on the P450 but not NPR, the P450 activities toward EFC or testosterone, caused by *tert*-butyl hydroperoxide, an oxygen surrogate, in the absence of the NPR and NADPH, were measured (Figure 6). The activities of P450s 1A2 and 3A4 were inhibited by 86 ± 2% and 73 ± 2%, respectively, in the presence of 50 μM Cu^{2+}, the concentration that the activities of P450s 1A2 and 3A4 were inhibited by 95 ± 1% and 78 ± 1%, respectively, in the reactions supported by NPR and NADPH (Figure 1C). These results indicate that the Cu^{2+} can affect directly the P450s. When 1 mM of EDTA was added to the reaction mixture containing 50 μM Cu^{2+}, the Cu^{2+}-induced inhibition was recovered to 34 ± 1% and 54 ± 4% of the uninhibited activities of P450s 1A2 and 3A4, respectively (Figure 6A,B). When we examined the chelating effect of EDTA on the Cu^{2+}-induced inhibition of the P450 1A2 activity supported by NPR and NADPH, there was no apparent recovery of the activity (Figure 6C).

Effects of Cu^{2+} on the Conformation of P450 Enzymes and NPR. Structural changes of P450s accompanying the Cu^{2+}-induced decrease in the enzyme activities were investigated by CD and fluorescence spectroscopy. The effects of Cu^{2+} on the secondary structure of the P450s were studied by CD in the far UV region in the presence of Cu^{2+}, whose range of concentrations were from 5 to 100 μM. The CD spectra of P450s 1A2 and 3A4, obtained in the presence of 5 and 20 μM Cu^{2+}, respectively, showed decreases of α-helix content by 29 ± 4% (for P450 1A2) and 7 ± 3% (for P450 3A4) (Figure 7A,B, and Table 2). However, the effect of Cu^{2+} on P450 3A4 was smaller than that of Cu^{2+} on P450 1A2 (Figure 7B and Table 2). The CD spectra of P450s 1A2

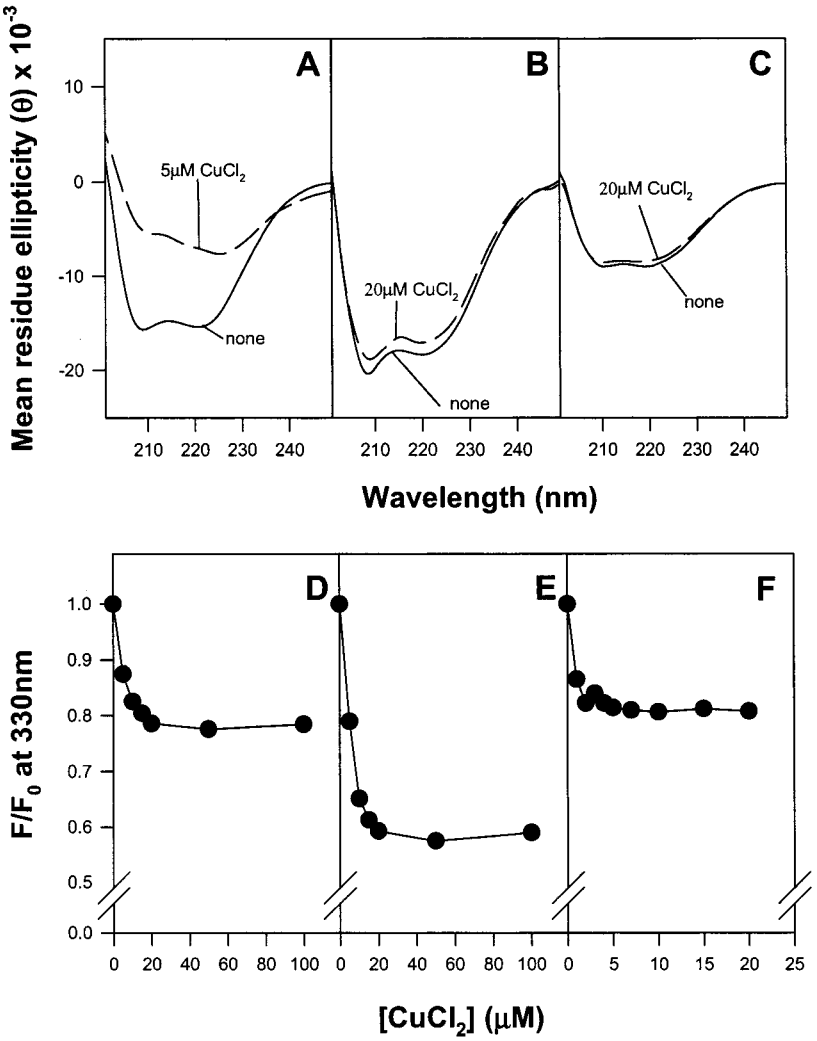

FIGURE 7: Cu^{2+}-induced conformational change of P450s and NPR. (A−C) Effect of Cu^{2+} on CD spectra of P450 1A2 (A), P450 3A4 (B), and NPR (C). The concentrations of Cu^{2+} added to the P450 solution are indicated. The P450s were incubated with Cu^{2+} for 5 min at 37 °C prior to the CD spectra were recorded as described under Experimental Procedures. (D−F) Cu^{2+}-dependence of the intrinsic fluorescence intensity of P450 1A2 (D), P450 3A4 (E), and NPR (F). Change in the fluorescence intensity of 1.0 μM P450 or NPR was recorded at 330 nm as a function of Cu^{2+} concentration. F_o and F represent the emission intensities in the absence (F_o) or present (F) of Cu^{2+}. All values are means of duplicate experiments. Absolute values varied <3%.

and 3A4 obtained at a concentration of more than 10 and 20 μM, respectively, showed a substantial decrease in CD spectral intensity and did not agree well with reference spectra (*31*). It is possible that the P450s aggregate in the presence of a higher Cu^{2+} concentration. On the other hand, the CD spectra of NPR obtained at a concentration of 5−50 μM Cu^{2+} did not show any apparent changes of α-helix content (~32%) of NPR in CD spectra compared to the NPR solution without Cu^{2+} (Figure 7C and Table 2).

The intrinsic fluorescence spectra of P450 1A2 (Figure 7D), P450 3A4 (Figure 7E), and NPR (Figure 7F) obtained in the presence of Cu^{2+} are shown. The intrinsic fluorescence intensity of P450 1A2, P450 3A4, and NPR decreased by about 23, 41, and 18%, respectively, when the Cu^{2+} concentration was increased to 20 μM; further increase of the Cu^{2+} concentration had no effect. It is likely that the increasing concentration of Cu^{2+} for P450s and NPR converts the conformation of proteins into forms where at least a part of the intrinsic fluorescence probes is exposed to more hydrophilic environments as compared with that of the metal ion-free conditions. These results indicate that the inhibition of catalytic activities of P450s by Cu^{2+} involves overall conformational changes of P450 enzymes and NPR.

DISCUSSION

This investigation established that the inhibitory effects of Cu^{2+} on the microsomal and purified P450 activities come from the interaction of Cu^{2+} with P450 as well as NPR. It is also demonstrated that Cu^{2+} can individually interact with both P450 and NPR. The results presented here suggest that Cu^{2+} can play a role in modulating the activities of P450s and NPR. Although Cu^{2+} decreases the rates of the P450-catalyzed reactions, the other ions (Zn^{2+}, Mg^{2+}, Mn^{2+}, and Co^{2+}) were without any apparent effects.

P450 and NPR have been reported to interact by forming a functional complex for the electron transfer (*41*). The inhibition of the activities of purified P450 and NPR by Cu^{2+} may be due to the impaired interaction of NPR with P450 (or cytochrome *c*) and/or the transformation of enzymes to inactive conformation by interacting with Cu^{2+}. NPR mediates the transfer of reducing equivalents from a two-electron donor, NADPH, to the one-electron acceptor, P450, via FAD

and FMN (*40*). The results presented here show that Cu^{2+} decreases the level of "air-stable" semiquinone. When ferrous P450s obtained by NPR and NADPH, instead of sodium hydrosulfite, were used to obtain ferrous−CO complexes, the P450s with Cu^{2+} did not show any detectable ferrous−CO complexes at ~450 nm (Figure 4E,F). Taken together, it can be suggested that Cu^{2+} may cause oxidation of ferrous ion to release bound CO or a loss of the normal orientation of P450 bound CO to the active site and that Cu^{2+} may inhibit the reduction of P450 by NPR and NADPH. At the moment, however, we do not have a satisfactory explanation for the increase of NADPH oxidation in the presence of Cu^{2+} without formation of H_2O_2 (Table 1). It is also known that copper can oxidize NADPH and NADH without formation of superoxide radicals in the microsomal system (*16*). As the NADPH oxidation in the presence of Cu^{2+} does not involve the formation of H_2O_2 and O_2^-, the increased NADPH oxidation seems not to be connected with a usual P450 catalytic cycle, which forms H_2O_2, O_2^-, and H_2O as byproducts (*32*). The increased NADPH consumption may come from copper-bond NPR system without involving P450: Cu^{2+} bound to NPR may be reduced to Cu^+ by NADPH and then oxidized to Cu^{2+} form by O_2 to make H_2O without formation of H_2O_2 and O_2^-. The coupled reactions of reduction and oxidation of copper, which is bound to NPR, may increase the NADPH consumption. Cu^{2+} alone in the absence of NPR could not oxidize NADPH (result not shown).

Cu^{2+} decreases the rates of the P450 catalyzed reactions accompanying conformational change and increases the conversion of P450 to P420. Cu^{2+} decreased the α-helix content of the affected P450. Intrinsic fluorescence emissions of the P450 and NPR also decreased with incremental increases in the concentrations of Cu^{2+}. The Cu^{2+}-induced conformations coincided with lowered activities of P450s and NPR. The inhibitory effect of Cu^{2+} on P450 reactions supported by *tert*-butyl hydroperoxide (Figure 6) shows that Cu^{2+} interacts directly with P450 enzymes. Interestingly, the increases of the EFC *O*-deethylation activity catalyzed by P450s 1A2 and 2B1 was found to occur concomitantly with the conformational changes, including raised α-helix content and increased intrinsic fluorescence emission intensity, induced by phospholipids and salts (*4*−*6*).

It was reported that the exposure of rainbow trout to copper sulfate lead to a remarkable decrease (55%) of ethoxyresorufin *O*-deethylation activity, which is a marker enzyme activity of P450 1A1/2, induced by pretreatment of *β*NF (*34*). It is also known that copper deficiency increases the ethoxyresorufin *O*-deethylation activity in rat small intestine (*35*). Those results suggest a possible role of Cu^{2+} for the modulation of P450 activities in vivo.

In vivo binding modes of Cu^{2+} to the P450s and NPR are unknown at present. The binding properties of P450s and NPR might contribute to the copper-related diseases. Elevated hepatic copper concentrations are associated with liver damage in Wilson's disease, Indian childhood cirrhosis, and in other copper-related cirrhoses of infancy (*42*). Copper imbalance is suggested to be involved in the etiology of several neurodegenerative diseases (*43*). The modulation of P450s and NPR activities by the copper ions might be related to the liver diseases caused by imbalance of copper ions. In mammals, relatively little is currently known about the precise components involved in copper transport and the mechanism by which copper is transported across the plasma membrane into cellular proteins (*44*, *45*). It is well-known that there are actually no free copper ions in cells.

Although the precise in vivo functions of the metal ions in the activities and structures of P450s and NPR are unknown at present, our results suggest that the balance of Cu^{2+} is related to the activities and P450s and NPR. The interaction of P450s and NPR with Cu^{2+} may point to a way of modulating the enzyme activities. Taken together, the results from previous (*4*−*7*) and present work in this field of research suggest a possibility that the P450 activities in cells can be modulated at the protein level by several stimulators (i.e., phospholipids and salts) and inhibitors (i.e., Cu^{2+}), as well as transcriptional and translational levels (*46*).

In conclusion, Cu^{2+} inhibits the P450-catalyzed reactions by affecting both P450s and NPR. Cu^{2+} inhibited the reactions catalyzed by purified P450 enzymes and NPR, respectively. Reduced semiquinone of NPR was diminished by Cu^{2+} and the formation of semiquinone was also inhibited by Cu^{2+}. P450 reactions supported by an oxygen surrogate, *tert*-butyl hydroperoxide, instead of NPR and NADPH, were inhibited by the presence of Cu^{2+}, indicating that Cu^{2+} inhibits the P450 catalyzed reactions by affecting both P450s and NPR. The inhibition of catalytic activities of P450s by Cu^{2+} involves conformational changes of both P450s and NPR. Taken together, it can be suggested that the inhibitory effect of Cu^{2+} on the P450-catalyzed reactions comes from the inability of a functional semiquinone formation and the dysfunctional conformation of NPR and P450. It is proposed that the balance of Cu^{2+} present in the cytosol surrounding endoplasmic reticulum is important for a functional conformation of P450s and NPR in a monooxygenase system.

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