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Influence of Glutathione on the Catalytic Activity of Reconstituted Cytochrome P450 3A4

Bok-Ryang Kim*,[1] and Dong-Hyun Kim†

**Department of Biochemistry and Medicinal Resources Research Center, School of Medicine, Won Kwang University, Iksan, 570-749, Korea: and †Doping Control Center, Korea Institute of Science and Technology, Seoul, 136-791, Korea*

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The role of NADPH reductase and cytochrome b_5 on glutathione (GSH)-induced stimulation of P450 3A4 activity was investigated. GSH increased the V_{max} of testosterone 6β-hydroxylation without changing the K_m for testosterone whereas it decreased the K_m for NADPH-P450 reductase. Addition of cytochrome b_5 inhibited testosterone 6β-hydroxylation in the reconstituted system, depleting GSH, while it dramatically enhanced the rate of testosterone 6β-hydroxylation in the presence of GSH. Cumene hydroperoxide-mediated P450 3A4 activity, which is independent of NADPH-P450 reductase and cytochrome b_5, was not affected by GSH. High concentration of GSH above 4 mM was inhibitory in the reconstituted systems. These results suggest that GSH increases the apparent affinity between P450 3A4 and NADPH-P450 reductase, and between P450 3A4 and cytochrome b_5, but has no effect on the affinity between P450 3A4 and testosterone.

P450 3A4 is the most abundantly expressed P450 in the human liver and can account for as much as 60% of the total P450, although the levels vary significantly (1). Substrates for P450 3A4 are pharmaceuticals, environmental pollutants, and carcinogens as well as endobiotics such as steroids, fatty acids, and prostaglandins (2). One of the methods to identify a substrate for P450 3A4 is to check whether certain chemical is metabolized by the purified P450 3A4 (3). However, this method has been hindered by difficulties in the purification of the enzyme and the measurement of its catalytic activity in reconstituted enzyme system. The enzymes in P450 3A subfamily tend to show poor catalytic activities when mixed with NADPH-P450 reductase under conditions appropriate for other P450s (4). Several lines of experiments indicated that the addition of cytochrome b_5 into mixtures of several phospholipids yielded higher catalytic activity for some of the P450 3A subfamily enzymes because of the enhanced interaction between P450 and NADPH reductase (5-7).

Several Investigators (8,9) have recently demonstrated that GSH is effective in improving some of the P450 3A4 drug oxidation reactions. These reactions include testosterone 6β-hydroxylation, nifedipine oxidation, and aflatoxin B_1 3α-hydroxylation and 8,9-epoxidation. However, the mechanism of GSH-induced stimulation has not been characterized. In this study, we report the mechanism of action of GSH on the oxidation of testosterone in reconstituted P450 3A4 system containing NADPH-reductase, cytochrome b_5 and other components.

MATERIALS AND METHODS

Materials. Testosterone, reduced GSH, dilauroyl phosphatidylcholine (DLPC), dioleoyl phosphatidylcholine (DOPC), and bovine brain phosphatidylserine were purchased from Sigma Chemical Co. (St. Louis, MO). Recombinant P450 3A4 was purified from membranes of *Escherichia coli* in which P450 3A4 cDNA was expressed as described elsewhere (8). Rabbit liver NADPH-P450 reductase and human cytochrome b_5 were purified using the methods described elsewhere(10).

Assay of P450 3A4 activity. In the standard protocol, reconstitution of the purified P450 3A4 consisted of P450 3A4 (100 pmol), NADPH-P450 reductase (200 pmol), cytochrome b_5 (100 pmol), 200 μg sodium cholate, 30 μg of a 1:1:1 mixture of DLPC, DOPC and phosphatidylserine, GSH (3.0 μmol), and testosterone (0.1 μmol) in 0.4 ml. The solution was sonicated for 3 s with the microprobe of a Branson sonicator (Branson Instruments, Danbury, CT) and then preincubated for 3 min at 37°C in 50 mM potassium phosphate buffer (pH 7.5). The reaction was started by the addition of an NADPH-generating system consisting of 1.0 mM $NADP^+$, 5.0 mM glucose 6-phosphate, and 1.0 unit glucose 6-phosphate dehydrogenase in a final volume of 1.0 ml. The reaction mixture was incubated for 10 min at 37°C.

HPLC analysis of 6β-hydroxytestosterone. Reaction mixtures were extracted with CH_2Cl_2 and analyzed by the method by Brian et al. (11) with slight modification. A Shiseido CAPCELL PAK C18 UG120 column (4.6 × 250 mm, Shiseido, Tokyo, Japan) was eluted with 53% (v/v) CH_3OH in water. After 7 min the CH_3OH concentra-

[1] To whom correspondence should be addressed at Department of Biochemistry, School of Medicine, Won Kwang University, Iksan, Chunbuk 570-749, Korea.

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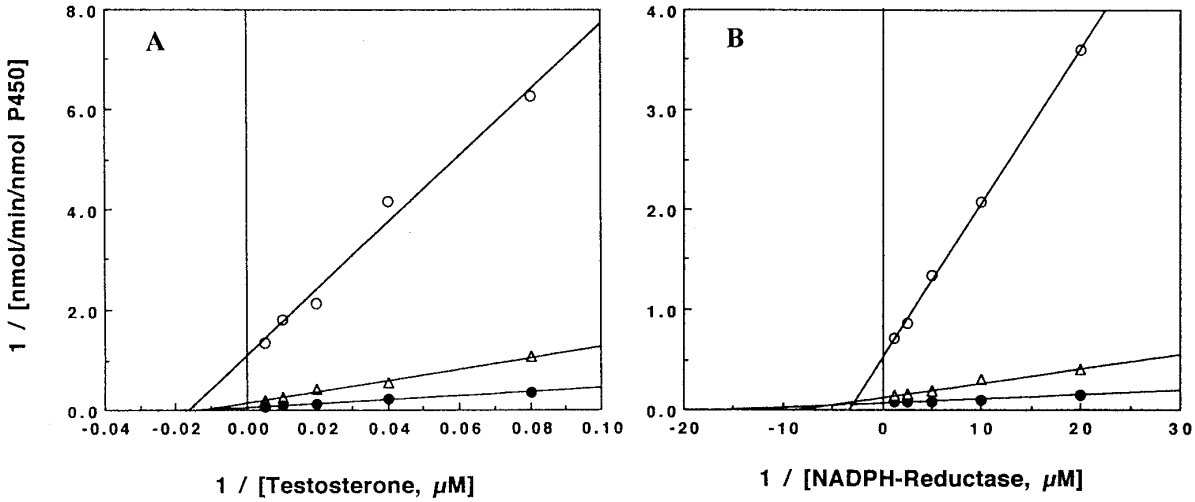

FIG. 1. Lineweaver–Burk plot of the kinetic analysis of the rates of testosterone 6β-hydroxylation by reconstituted P450 3A4: (A) Effects of substrate and (B) NADPH-P450 reductase in the absence of GSH (○) and in the presence of 2.0 mM (△) or 3.0 mM (●) GSH.

tion was increased to 65% over a period of 1 min and held for an additional 4 min. The concentration of CH_3OH was returned to 53% over 2 min and then held for 7 min. 6β-Hydroxytestosterone was detected at 254 nm and quantified using external standards.

Cumene hydroperoxide-dependent testosterone 6β-hydroxylation. Reconstitution systems for cumene hydroperoxide-dependent oxidation contained purified P450 3A4 (100 pmol), NADPH-P450 reductase (200 pmol), 200 μg sodium cholate, 30 μg of a 1:1:1 mixture of DLPC, DOPC, and phosphatidylserine, testosterone (0.1 μmol), and the indicated concentration of GSH in 0.4 ml. The mixture was sonicated for 3 s and then preincubated for 3 min at 37°C in 50 mM potassium phosphate (pH 7.5). The reaction was initiated by the addition of cumene hydroperoxide (150 nmol) and then incubated for 10 min at 37°C.

RESULTS

The catalytic activity of P450 3A4 toward testosterone was stimulated by the addition of GSH in the reconstituted system and the optimal concentration of GSH was reported to be 3.3 mM (8). A double reciprocal plot of the rates of testosterone 6β-hydroxylation versus testosterone showed that the addition of GSH to the system resulted in a 7-18 fold increase in the V_{max} without substantial change of the K_m (Fig. 1). In order to determine if GSH in the reconstituted system increases the apparent affinity between P450 3A4 and NADPH-P450 reductase, the rate of testosterone 6β-hydroxylation was measured after the addition of various concentrations of NADPH-P450 reductase in the presence or absence of GSH in the reconstitution. As shown in Table 1, GSH increased the V_{max} whereas it decreased the K_m for NADPH-P450 reductase.

Addition of cytochrome b_5 to the reconstituted system slightly inhibited testosterone 6β-hydroxylation in the absence of GSH (Fig 2). However, the catalytic activity of reconstituted P450 3A4 increased in the presence of GSH at all concentrations of cytochrome b_5 tested. The rate decreased if higher than 25 nM cytochrome b_5 was added into the system. The same pattern was noted with 2.0 mM GSH.

Testosterone 6β-hydroxylation mediated by cumene hydroperoxide does not require NADPH-P450 reductase. But P450 3A4 in a reconstituted system devoid of NADPH-P450 reductase showed less activity in the presence of cumene hydroperoxide (Table 2). Testosterone 6β-hydroxylation derived by cumene hydroperoxide was also measured in the reconstituted system containing NADPH-P450 reductase. This activity was independent on GSH but markedly decreased at 4.0 mM GSH.

DISCUSSION

Demonstration of purified P450 3A4 activity is a major problem because the enzyme activity has been

TABLE 1

Influence of GSH on the Kinetic Parameters for Testosterone 6β-hydroxylation by Reconstituted P450 3A4

Systems	Km (ASE)[a]	Vmax (ASE)
	μM	nmol/min/nmol P450
For testosterone		
Standard[b]	62.0 (4.3)	0.93 (0.12)
Standard + 2.0 mM GSH	82.3 (3.9)	7.16 (0.59)
Standard + 3.0 mM GSH	64.0 (3.5)	16.56 (1.14)
For NADPH-P450 reductase		
Standard	0.29 (0.01)	1.89 (0.12)
Standard + 2.0 mM GSH	0.11 (0.02)	7.72 (0.64)
Standard + 3.0 mM GSH	0.07 (0.01)	15.63 (0.83)

[a] Asymptopic standard error.
[b] Standard incubation mixtures consisted of P450 3A4 (100 pmol), NADPH-P450 reductase (200 pmol), cytochrome b_5 (pmol), 200 μg sodium cholate, and other components described under Materials and Methods.

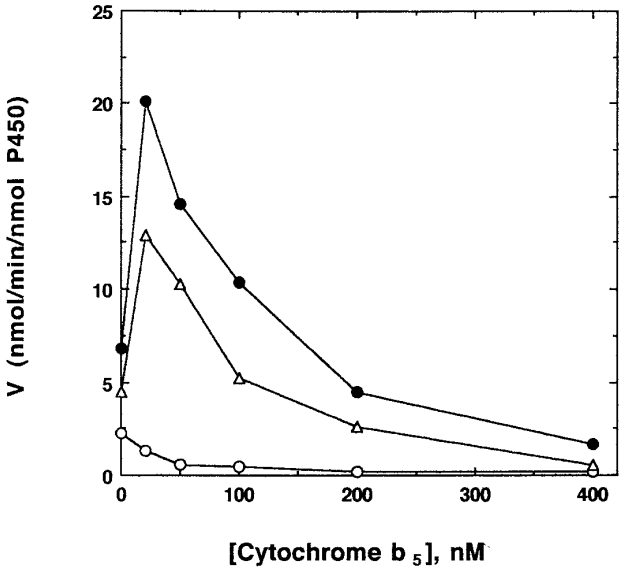

FIG. 2. Effect of cytochrome b5 concentration on testosterone 6β-hydroxylation by reconstituted P450 3A4. Reconstituted system for P450 3A4 contained varying concentrations of cytochrome b5 in the absence of GSH (○), or in the presence of 2.0 mM (△) or 3.0 mM (●) GSH.

shown to be very low in previously used conditions. The stimulation of catalytic activity of P450 3A4 was first suggested by Imaoka et al. (5) and conformed by others (8, 9). In this study we examined how GSH stimulates the reconstituted P450 3A4 activity toward testosterone 6β-hydroxylation. GSH should be included in the preincubation mixture for the reconstitution containing NADPH-P450 reductase, cytochrome b_5, cholate, DLPC, DOPC, phosphatidylserine, and P450 3A4 in order to enhance the rate of testosterone 6β-hydroxylation. Testosterone could be added either before or after the preincubation and potassium phosphate buffer must be excluded in the preincubation (8).

The K_m of testosterone for P450 3A4 was not affected by GSH although the V_{max} was increased to about 18-fold at 3.0 mM GSH. This result demonstrated that GSH does not enhance the interaction between substrate and P450 3A4. The apparent affinity of P450 3A4 for NADPH-P450 reductase was enhanced in the reconstituted system containing GSH as judged by the decrease in the K_m value. The increase of the apparent affinity seems to be one of the reason for the stimulation by GSH because the incorporation of first electron from the reductase to P450 3A4 is a rate-limiting step in testosterone 6β-hydroxylation (12). The stimulation of reconstituted P450 3A activity by phopholipid mixtures or by $MgCl_2$ was also demonstrated to be via the acceleration of the first electron flow (5, 8, 12, 13).

The requirement of cytochrome b_5, which can donate the second electron to the $Fe^{2+}O_2$-substrate complex in the reaction cycle of P450, seems to be unclear. Cytochrome b_5 is not an essential component for some NADPH-dependent P450 drug oxidations (14). However certain NADPH-dependent P450 mediated reactions were stimulated by cytochrome b_5 (12, 13). An equimolar concentration of cytochrome b_5 resulted in a 13-fold increase in the rate of testosterone 6β-hydroxylation by purified P450 3A4, and anti-human cytochrome b_5 antibody inhibited the reaction by human liver microsomes in a dose-dependent manner (13). However our results showed that a 1:5 ratio of cytochrome b_5 to P450 3A4 produced the maximal stimulation in the reconstituted system containing GSH. This ratio was somewhat unexpected because the amount of cytochrome b_5 added in NADPH-dependent drug oxidation was at least higher than that of P450 in other experiments (3, 5, 6, 8, 9, 13).

The P450 3A4 (Fe^{2+})-testosterone complex is converted to the P450 3A4 ($Fe^{2+}OOH$)-testosterone by cumene hydroperoxide without aid of NADPH-P450 reductase (15). GSH had no effect on testosterone 6β-hydroxylation derived by cumene hydroperoxide. For detecting the catalytic activity of reconstituted P450 3A4 by cumene hydroperoxide, NADPH-P450 reductase must be added during the reconstitution stage although the reductase does not participate in testosterone 6β-hydroxylation. The stimulatory role of NADPH-P450 reductase in this system is not clear but postulated to stabilize P450 3A4 in the reconstitution. Cumene hydroperoxide-mediated testosterone 6β-hydroxylation was considerably decreased at 4.0 mM GSH, suggesting that high concentrations of GSH might destabilize the conformation of CYP3A4 and/or other proteins in phospholipid vesicles of those reconstituted systems.

Purified P450s tend to aggregate significantly because of their intrinsic hydrophobic nature (16). The tendency was reduced in the presence of detergents or thiol reducing agents. However, dissociation of aggregated P450 102 by dithiothreitol took a long time up to 62 hrs (17). The stimulatory effect of GSH shown in

TABLE 2

Effect of GSH on the Rate of the Testosterone 6β-hydroxylation Mediated by Cumene Hydroperoxide

GSH (mM)	Testosterone 6β-hydroxylation (nmol/min/nmol P450)	
	With NADPH-P450 reductase	Without NADPH-P450 reductase
0.0	3.75[a]	0.64
2.0	4.05	0.53
3.0	3.94	0.67
4.0	0.21	0.14

[a] Value represents mean of two independent experiments.

Note. Incubations were carried with or without NADPH-P450 reductase as described under Materials and Methods.

this study was accomplished within 1 min and other thiol reducing compounds such as cysteine, dithiothreitol (8) and 2-mercaptoethanol (unpublished data) can not replace the role of GSH. In addition, conversion of aggregated P450 2B4 and 1A2 into monomer was reported not to increase their catalytic activities (18). Therefore, an increase of the activity of reconstituted P450 3A4 by GSH in the present experiment is not resulted from simple reductive action of GSH preventing aggregation.

In conclusion, the increased rate of testosterone 6β-hydroxylation in reconstituted P450 3A4 by GSH may be partially due to the enhanced affinity between P450 3A4 and NADPH-P450 reductase, and between P450 3A4 and cytochrome b_5 which can accelerate the electron transfer to P450 protein.

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