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ONE-ELECTRON REDUCTIVE BIOACTIVATION OF 2,3,5,6-TETRAMETHYLBENZOQUINONE BY CYTOCHROME P450

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Abstract—Bioreductive activation of quinones in mammalian liver has generally been attributed to NADPH-cytochrome P450 reductase. However, in view of the 20–30-fold molar excess of cytochrome P450 over NADPH-cytochrome P450 reductase on the endoplasmic reticulum of the rat liver cell and the capability of cytochrome P450 to bind and reduce xenobiotics, it was considered of interest to investigate the possible role of cytochrome P450 in the bioreduction of quinones. In the present study, 2,3,5,6-tetramethyl-1,4-benzoquinone (TMQ) was chosen as a model quinone. First, TMQ was found to bind at the metabolic active site of phenobarbital (PB)-inducible cytochrome P450s of rat liver microsomes, indicating that TMQ is a potential substrate for cytochrome P450-mediated biotransformation. Second, with electron spin resonance, one-electron reduction of TMQ to a semiquinone free radical (TMSQ) was found to occur in these microsomal fractions. SK&F 525-A, a well-known inhibitor of cytochrome P450, strongly inhibited TMSQ formation in these subcellular fractions without affecting NADPH-cytochrome P450 reductase activity. One-electron reductive bioactivation of TMQ was further investigated with purified NADPH-cytochrome P450 reductase alone and in reconstituted systems of purified cytochrome P450-IIB1 and NADPH-cytochrome P450 reductase. As measured by ESR, purified cytochrome P450-IIB1 in the presence of NADPH-cytochrome P450 reductase was able to reduce TMQ to TMSQ at a much greater rate than in the presence of NADPH-cytochrome P450 reductase alone. Reduction of TMQ was also investigated by measuring the initial rate of NADPH oxidation by TMQ under anaerobic conditions. Inhibitors of cytochrome P450, namely SK&F 525-A and antibodies against PB-inducible cytochrome P450s, caused a substantial decrease in reductive metabolism in PB-treated microsomes. These antibodies were also effective in the inhibition of TMQ-induced NADPH oxidation in a complete reconstituted system of equimolar concentrations of cytochrome P450-IIB1 and NADPH-cytochrome P450 reductase, indicating that the reaction was specific for cytochrome P450-IIB1. Finally, initial rates of NADPH oxidation were determined in reconstituted systems containing varying amounts of NADPH-cytochrome P450 reductase and cytochrome P450-IIB1 to determine the contribution of either enzyme in the reduction of TMQ. As expected, NADPH-cytochrome P450 reductase was able to reduce TMQ to a small extent. However, reconstitution in the presence of increasing amounts of cytochrome P450-IIB1 (relative to NADPH-cytochrome P450 reductase) resulted in increasing rates of TMQ-induced NADPH oxidation. From these experiments and from reconstitution experiments with fixed amounts of cytochrome P450-IIB1 and varying amounts of NADPH-cytochrome P450 reductase, it was calculated that cytochrome P450-IIB1 and NADPH-cytochrome P450 reductase contributed 1:1 to the reduction of TMQ. Interestingly, all experiments performed at an equimolar ratio of the two enzymes consistently resulted in a 4–5-fold enhancement of TMQ reduction by cytochrome P450-IIB1 as compared to NADPH-cytochrome P450 reductase alone. It is, therefore, suggested that cytochrome P450-IIB1 and NADPH-cytochrome P450 reductase act as one complex during one-electron reduction of TMQ to TMSQ under anaerobic conditions. Binding of TMQ to this preformed complex might enhance the efficiency of electron flux from NADPH-cytochrome P450 reductase to TMQ. In conclusion, this study demonstrates that in addition to NADPH-cytochrome P450 reductase, cytochrome P450-IIB1 contributes substantially to the reduction of TMQ in PB-induced rat liver microsomes under a relatively low oxygen tension. The role of cytochrome P450 in this type of reduction reaction may also be important for more complex biologically active quinoid compounds such as Adriamycin® and mitomycin C.

The most important enzyme system involved in the biotransformation of foreign compounds, in the liver, is the cytochrome P450-containing monooxygenase system. The cytochrome P450 system consists of NADPH-cytochrome P450 reductase and the heme-containing cytochrome P450, both embedded in the phospholipid matrix of the endoplasmic reticulum [1]. Microsomal cytochrome P450 usually catalyses the oxidative metabolism of a variety of lipophilic compounds. Electron transfer to cytochrome P450 is a requirement for the catalytic function of the enzyme. The normal source of the first electron

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is NADPH which transfers electrons via the flavoprotein NADPH-cytochrome P450 reductase [2].

Depending on the substrate and on the oxygen partial pressure, cytochrome P450 may also catalyse the reductive biotransformations of halogenated alkanes such as carbon tetrachloride [3, 4], halothane [5], dimethylaminobenzene [6, 7], and Gentian violet [8, 9]. NADPH-cytochrome P450 reductase functions in the transfer of reducing equivalents from NADPH to cytochrome P450 during reductive metabolism [10]. These cytochrome P450 mediated reduction reactions proceed most readily under anaerobic conditions [11].

Preliminary studies also suggest that cytochrome P450 may be involved in the reduction of quinones [12–15]. The quinone structure is well represented among agents currently used to treat cancer, such as Adriamycin and mitomycin C (MMC*) [15, 16]. These antitumor agents are thought to exert their cytostatic effects following reductive bioactivation to reactive metabolites and subsequent DNA alkylation ultimately leading to cell death [17, 18]. Enzymatic one-electron reduction of quinones under anaerobic conditions leads to the formation of a reactive semiquinone free radical which, under aerobic conditions, may react with molecular oxygen leading to the generation of the parent quinone and concomitant production of superoxide anion, a process known as redox-cycling [15]. Enzymes known to catalyse the one-electron reduction of quinones include NADPH-cytochrome P450 reductase, NADH cytochrome *b*5-reductase and xanthine oxidase [19–22].

Previously, Kennedy *et al.* [23] suggested that cytochrome P450 might also be involved in the reduction of MMC. The 20–30-fold molar excess of cytochrome P450 over NADPH-cytochrome P450 reductase on the endoplasmatic reticulum of the rat liver cell [24, 25] and its capacity to bind [26], and reduce xenobiotics [6, 9, 10] makes the involvement of this hemoprotein in reduction reactions likely. Inasmuch as cytochrome P450 is involved in the one-electron reduction of quinone imines (such as *N*-acetyl-*p*-benzoquinone imine, a reactive metabolite of paracetamol and its 3,5-dimethyl-analogue [27]) and MMC [28], cytochrome P450-mediated reduction of quinones was anticipated.

The chemistry associated with the bioreductive activation of structurally complex quinones has been proven to exhibit rich complexity. For instance, it has been suggested that one-electron transfer is the dominant mode of bioreductive activation of MMC [29]. However, the semiquinone free radical proposed to be formed upon one-electron reduction of MMC under anaerobic conditions is difficult to detect in enzymatic preparations by ESR due to its relative short lifetime in aqueous media [19, 30]. Thus, direct ESR investigation, under anaerobic conditions, of cytochrome P450-mediated one-electron reduction of MMC to its semiquinone free radical is impossible.

Due to the principle uncertainties in the reductive activation process of MMC, we decided to study the role of cytochrome P450 in the one-electron reduction of TMQ (Duroquinone®) as model quinone under anaerobic conditions (Fig. 1). The substitution of the 2, 3, 5 and 6 positions of the benzene ring by electron-donating methyl groups is expected to result in the reduction of TMQ to the semiquinone free radical (TMSQ) rather than the following of additional pathways of metabolism as is expected for MMC. By employing TMQ as a model quinone, we have chosen to restrict ourselves to determining the quinone reductive activity of cytochrome P450 alone under anaerobic conditions.

Reductive biotransformation of TMQ under anaerobic conditions should result in the formation of TMSQ at the expense of NADPH (Fig. 1). This reaction can be followed spectrophotometrically by monitoring the initial rate of NADPH oxidation or by ESR to detect TMSQ formation. The role of cytochrome P450 in the reduction of TMQ was investigated with specific inhibitors of cytochrome P450 isoenzymes in microsomal fractions and purified, reconstituted cytochrome P450-IIB1 systems.

MATERIALS AND METHODS

Animals. Male Wistar rats (200–220 g; Harland-TNO, Zeist, The Netherlands) were housed in an environmentally controlled room (25°, air humidity 60%), kept on standard laboratory diet and starved overnight before use. Animals were pre-treated with sodium PB (100 mg/kg/day in saline) by daily intraperitoneal injection for 3 days [31]. Livers were isolated 24 hr after the last injection.

Chemicals. Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, NADP, xanthine oxidase, glucose oxidase, catalase, NADH and NADPH were purchased from Boehringer (Mannheim, F.R.G.). TMQ (Duroquinone®) and DTPA were obtained from Janssen Chimica (Beerse, Belgium). EDTA and PB were from BDH (Poole, U.K.) and Brocacef (Maarssen, The Netherlands), respectively. SK&F 525-A (Proadiphen®) was a gift of Smith Kline & French Labs (Herts, U.K.).

Preparation of microsomes and enzyme purification. Rat liver microsomes were prepared as described previously [32] and stored in liquid nitrogen until used. Cytochrome P450-IIB1 was purified from the livers of PB-treated rats, as described previously [33, 34]. Protein was determined by the method of Lowry *et al.* [35] with bovine serum albumin as standard. Cytochrome P450 content was measured as described by Omura and Sato [36]. NADPH-cytochrome P450 reductase was purified from PB-induced microsomes according to Guengerich and Martin [33]; its activity was determined by its ability to reduce cytochrome *c* [37]. NADPH-cytochrome P450 reductase concentration was determined fluorometrically on a Perkin–Elmer Model 3000 spectrofluorometer according to Faeder and Siegel [38] by measuring the flavin composition.

* Abbreviations: TMQ, 2,3,5,6-tetramethyl-1,4-benzoquinone; TMSQ, 2,3,5,6-tetramethyl-1,4-benzosemiquinone; PB, phenobarbital; ESR, electron spin resonance; MMC, mitomycin C; DTPA, diethylenetriaminepentaacetic acid; DMSO, dimethyl sulfoxide; K01, antibodies against cytochrome P450-IIB1 and -IIB2.

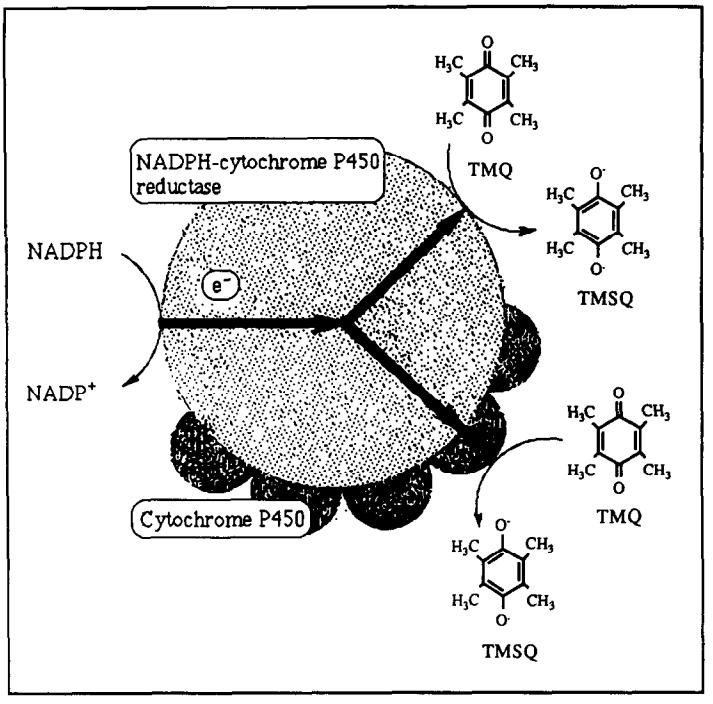

Fig. 1. Schematic representation of pathways possibly involved in the reduction of TMQ by the microsomal cytochrome P450 system. Under anaerobic conditions, NADPH-cytochrome P450 reductase is able to reduce TMQ to TMSQ at the expense of NADPH when TMQ is the sole electron acceptor present. In the presence of cytochrome P450, the physiological acceptor of reducing equivalents from NADPH-cytochrome P450 reductase, an electron flow to TMQ via the hemoprotein can be anticipated.

The purified NADPH-cytochrome P450 reductase contained nearly equal amounts of FMN and FAD with a molar ratio of FMN to FAD of 0.95, in agreement with the theoretical value of 1.00 shown elsewhere [39].

Difference spectrophotometry. Interactions between TMQ and PB-induced microsomal cytochrome P450s were studied spectrophotometrically according to Schenkman *et al.* [40]. Double tandem cuvettes were used to correct for the intrinsic absorbance of TMQ. Microsomes were diluted to a protein concentration of 2 mg/mL in 0.1 M potassium phosphate buffer pH 7.4 containing 1 mM EDTA. TMQ was dissolved in DMSO so that DMSO concentrations never exceeded 1% of the total volume. Spectra were recorded on an Aminco DW 2a UV/VIS spectrophotometer.

Measurement of TMSQ by ESR. For reference purposes, xanthine oxidase-mediated TMSQ formation from TMQ was determined with ESR at room temperature by adding 0.04 units/mL xanthine oxidase to 5 mM TMQ and 10 mM NADH in 50 mM potassium phosphate buffer (pH 7.8). TMQ was dissolved in DMSO (final DMSO concentration was less than 1% v/v).

One-electron reduction of TMQ by rat liver microsomes was assayed by ESR, under anaerobic conditions, in incubation mixtures consisting of microsomal protein (1 mg/mL), TMQ (1.0 or 5.0 mM), 1 mM NADPH, 5 mM glucose-6-phosphate, glucose-6-phosphate dehydrogenase (1 unit/mL) and 50 mM phosphate buffer (pH 7.4) containing 0.5 mM DTPA. For ESR measurements in reconstituted systems equimolar concentrations (0.5 μM) of purified NADPH-cytochrome P450 reductase and cytochrome P450-IIB1 were mixed with dilauroylphosphatidylcholine (4 μg/mL). To this mixture 1.0 mM TMQ, 5 mM glucose-6-phosphate, glucose-6-phosphate dehydrogenase (1 unit/mL) and 1 mM NADPH were added.

The one-electron reduction reactions were initiated by adding an anaerobic solution of NADH (in the xanthine oxidase system) or NADPH (in the cytochrome P450 containing systems). Anaerobic conditions were obtained by flushing the reaction mixture in sealed tubes with argon gas prior to the initiation of the reaction [41]. Aerobic conditions were achieved by exposing the incubation mixture to atmospheric oxygen.

ESR. ESR measurements were performed with a Bruker ESP-300 spectrometer/ESP 1600 data processor. Enzyme preparations were kept on ice until use. Incubations were carried out at room temperature and the spectra were recorded immediately after the initiation of the enzyme reaction in an ESR quartz flat cell mounted in a cavity with a nominal microwave power of 20 mW and microwave frequency of 9.78 GHz. Incubations conditions and ESR parameters are indicated in the appropriate figure legends.

Measurement of TMQ-induced NADPH oxidation. The initial rate of TMQ reduction was measured by

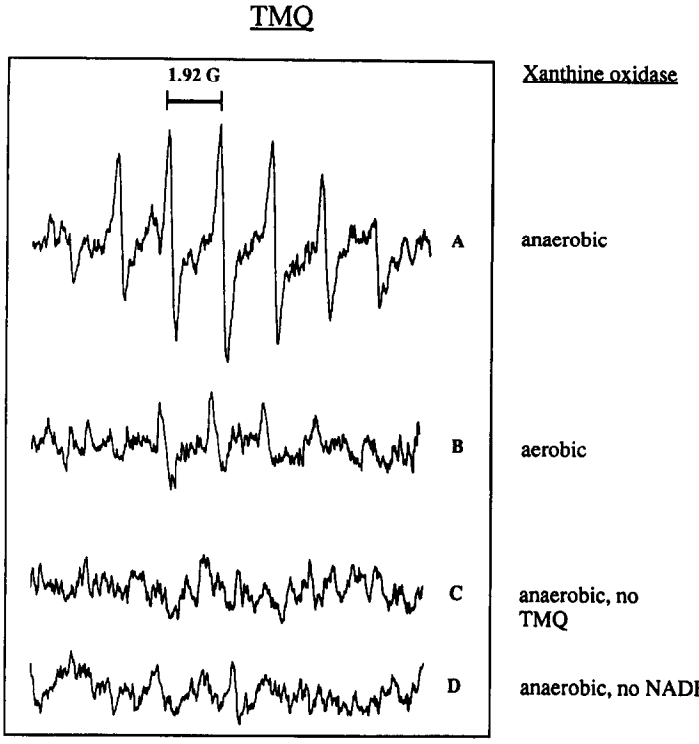

Fig. 2. ESR spectra of the TMQ-derived oxygen centred semiquinone free radical. ESR spectrum obtained following incubation of xanthine oxidase (124 μg/mL), 5 mM TMQ and 10 mM NADH under anaerobic conditions (A), under aerobic conditions (B), without TMQ (C) and without NADH (D). Instrumental conditions were: power = 20 mW: modulation amplitude = 0.394 G; time constant = 665.36 msec; sweep time = 83.866 sec; conversion = 81.92 msec; number of scans = 10; scan range = 20 G; gain = 6.3×10^4. Representative spectra of at least three experiments.

determining NADPH oxidation under anaerobic conditions according to Powis and Appel [42] at 340 nm (using an extinction coefficient of 6.22 $mM^{-1}cm^{-1}$ [43]). Briefly, the incubation mixture contained 50 mM Tris–HCl buffer pH 7.4, 5 mM $MgCl_2$ and 5.5 mM DTPA with microsomal protein (0.7 mg/mL) in a final volume of 1.0 mL at 37°. For the preparation of the reconstituted systems NADPH-cytochrome P450 reductase and cytochrome P450-IIB1 were mixed with dilauroylphosphatidylcholine (50 μg/mL) and pre-incubated for 6 min at 37°. TMQ (100 μM, final concentration) dissolved in DMSO (final concentration was less than 1% v/v) was added prior to the addition of NADPH (final concentration 1 mM). DMSO had no effect on NADPH oxidation. Anaerobic incubations were carried out in sealed cuvettes which were gassed with argon prior to temperature equilibration [41]. In addition, traces of oxygen were removed from the incubation mixtures with 7.5 mM glucose, 13 units glucose oxidase/mL and 1590 units catalase/mL as described by Englander *et al.* [41].

Statistical evaluation of the results. Data are presented as means ± SD of at least three determinations, unless otherwise stated. Statistical evaluation of the results was performed with Student's *t*-test. Differences were considered statistically significant if $P < 0.05$.

RESULTS

Interaction between TMQ and cytochrome P450

Binding of TMQ to rat liver microsomal cytochrome P450 was determined by difference spectroscopy [40]. With PB-induced microsomes the difference spectrum showed a maximum at 390 nm and a minimum at 425 nm, indicating a type-I binding (data not shown). The spectral dissociation constant K_s and ΔA_{max}, calculated from a $1/\Delta A$ vs $1/[TMQ]$ plot were 2.8 ± 0.2 μM and 0.011 ± 0.003/nmol cytochrome P450, respectively (N = 3).

Formation of TMSQ from TMQ by xanthine oxidase

In order to study enzymatic one-electron reduction of TMQ, TMSQ formation was measured under anaerobic conditions by ESR. One-electron reduction of TMQ leads to the formation of an oxygen centered semiquinone free radical (TMSQ) which is detectable by ESR [44]. An enzyme known to catalyse one-electron reduction of quinones is xanthine oxidase [19]. As shown in Fig. 2A, TMSQ was formed upon reduction of 5 mM TMQ by xanthine oxidase (and 10 mM NADH; anaerobic conditions). The ESR spectrum obtained is comparable to that reported for TMSQ and obtained upon chemical reduction of TMQ [44]. Under aerobic conditions only a weak ESR signal of TMSQ

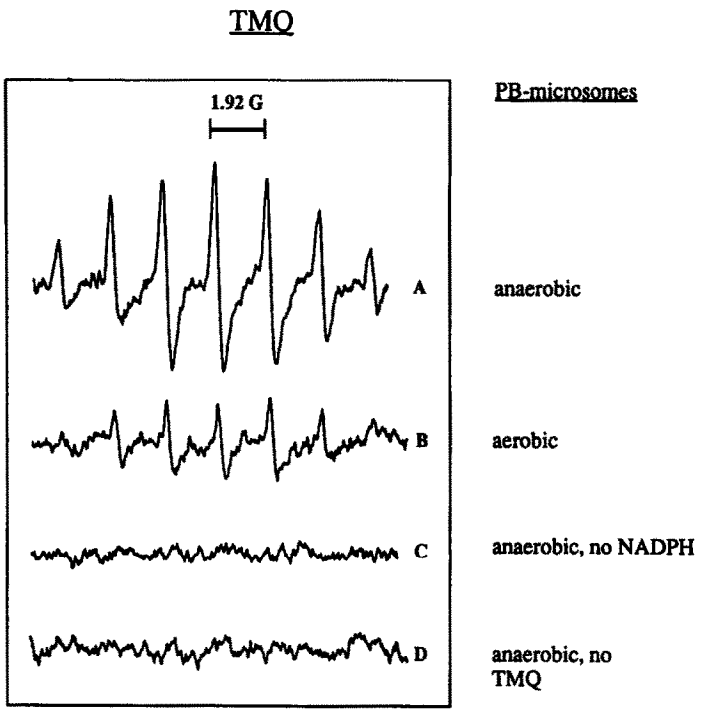

Fig. 3. ESR spectra of TMSQ obtained from PB-induced microsomal incubations under various conditions. (A) ESR spectrum obtained from anaerobic microsomal incubation containing 5 mM TMQ; an NADPH-generating system consisting of 1 mM NADPH, 5 mM glucose-6-phosphate and glucose-6-phosphate dehydrogenase (1.0 unit/mL); and PB-induced rat hepatic microsomal protein (2 mg/mL) prepared as described in Materials and Methods. (B) Identical to A except that the incubation mixture was purged with oxygen before addition of NADPH. (C and D) same as A except that NADPH and TMQ were omitted, respectively. Instrumental conditions were the same as described in the legend to Fig. 2.

was obtained (Fig. 2B). When either TMQ or NADH were omitted, an ESR signal could no longer be detected in the xanthine oxidase preparation (Fig. 2C and D, respectively).

Formation of TMSQ from TMQ by cytochrome P450

Upon anaerobic incubation of 5 mM TMQ with PB-induced rat liver microsomes in the presence of a NADPH generating system, the ESR spectrum (Fig. 3A) indicative of TMSQ was similar to that obtained with xanthine oxidase (Fig. 2A). In the presence of atmospheric oxygen, the TMSQ signal was substantially smaller (Fig. 3B). In agreement with the xanthine oxidase system, no ESR signal was detected in incubation mixtures lacking NADPH or TMQ (Fig. 3C and D, respectively).

Because TMSQ was stable under anaerobic conditions, the intensity of the ESR signal was used to quantify the amount of TMSQ formed in PB-pretreated microsomes. The effect of SK&F 525-A, a well-known cytochrome P450 inhibitor, on the formation of TMSQ was also investigated. SK&F 525-A (1 mM) strongly inhibited the formation of TMSQ in PB-induced microsomes from 100 ± 3 to 32 ± 4% ($N = 3$, $P < 0.001$). Moreover, SK&F 525-A was found not to inhibit the NADPH-cytochrome P450 reductase activity as measured by the capacity of NADPH-cytochrome P450 reductase to reduce cytochrome *c* (1.29 ± 0.1 vs 1.40 ± 0.08 unit/mL, $N = 4$). Furthermore, in a recent report it was found that SK&F 525-A did not affect quinone reduction by purified NADPH-cytochrome P450 reductase alone [28].

Anaerobic incubation of TMQ (1 mM) with purified NADPH-cytochrome P450 reductase (70 μM) and NADPH (1 mM) resulted in the formation of small amounts of TMSQ. However, incubation of TMQ (1 mM) with a fully reconstituted system of purified cytochrome P450-IIB1 (70 μM), NADPH-cytochrome P450 reductase (70 μM) and NADPH (1 mM), showed much stronger ESR signals of TMSQ. The ESR signal intensity of TMSQ in a reconstituted system of cytochrome P450-IIB1 was 100 ± 6% compared to 24 ± 4% with NADPH-cytochrome P450 reductase alone ($N = 3$, $P < 0.001$). No ESR signal was detected in incubation mixtures lacking either NADPH-cytochrome P450 reductase, NADPH or TMQ (data not shown).

Cytochrome P450-IIB1 mediated reduction of TMQ measured by NADPH oxidation

In the presence of microsomes, reduction of TMQ leads to stoichiometric oxidation of NADPH under anaerobic conditions [42, 45]. Therefore, the reductive metabolism of TMQ was also investigated

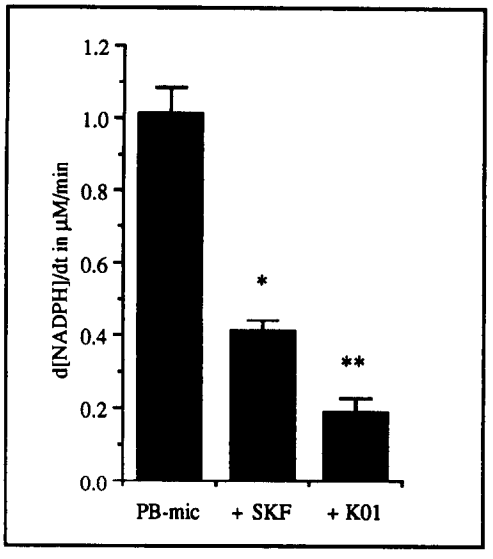

Fig. 4. The effect of different cytochrome P450 inhibitors on TMQ-induced NADPH oxidation in PB-induced rat liver microsomes. TMQ reduction was measured by monitoring the initial rate of NADPH oxidation at 340 nm under anaerobic conditions, using an extinction coefficient of 6.22 $mM^{-1}cm^{-1}$. The inhibitory effect of SK&F 525-A (50 μM) and K01 (1:1000) is indicated. For detailed information, see Materials and Methods. Data represent the means ± SD of at least three experiments. *P < 0.001, **P < 0.002.

by measuring the initial rate of NADPH oxidation under anaerobic conditions.

PB-induced microsomes reduced TMQ at the expense of NADPH (Fig. 4). Addition of 50 μM SK&F 525-A and K01 (1:1000) inhibited TMQ-induced NADPH oxidation by 59 and 81%, respectively (Fig. 4). Under these conditions NADPH-cytochrome P-450 reductase activity was not inhibited by these specific cytochrome P-450 inhibitors (data not shown).

TMQ reduction in reconstituted systems of NADPH-cytochrome P450 reductase and cytochrome P450-IIB1 was also investigated. The rate of TMQ-induced NADPH oxidation in the presence of purified NADPH-cytochrome P450 reductase (70 μM) alone was 0.53 ± 0.06 μM/min (N = 3). The rate of TMQ reduction, measured as NADPH oxidation, in the presence of 70 μM cytochrome P450-IIB1 was stimulated to 2.37 ± 0.18 μM (N = 3, P < 0.001). Addition of K01 (1:1000) inhibited NADPH consumption to 1.27 ± 0.09 μM/min (N = 3, P < 0.001).

The relative contribution of cytochrome P450-IIB1 and NADPH-cytochrome P450 reductase in the reduction of TMQ measured by NADPH oxidation

The contribution of either enzyme in the reduction of TMQ was further investigated in reconstituted systems of purified cytochrome P450-IIB1 and NADPH-cytochrome P450 reductase. Figure 5 shows that TMQ is reduced both in the presence of a reconstituted system of NADPH-cytochrome P450 reductase and cytochrome P450-IIB1 as well as NADPH-cytochrome P450 alone. In Fig. 5A, the results are shown of the incubation of TMQ (100 μM) with varying concentrations of purified NADPH-cytochrome P450 reductase, reconstituted with (curve 1) or without (curve 2) a fixed amount (70 μM) of purified cytochrome P450-IIB1. The results of the incubation of TMQ with a fixed amount (70 μM) of purified NADPH-cytochrome P450 reductase and varying amounts of purified cytochrome P450-IIB1 are shown in Fig. 5B (curve 3).

Optimal TMQ reduction was seen with a NADPH-cytochrome P450 reductase to cytochrome P450-IIB1 molar ratio of approximately 1:1 (Fig. 5, curve 2 and 3, respectively). NADPH oxidation in the absence of TMQ was neglible. TMQ-induced NADPH oxidation, in the presence of increasing concentrations of purified NADPH cytochrome P450 reductase alone, slowly increased (Fig. 5A, curve 1). The rate of TMQ reduction measured as NADPH oxidation in the presence of 70 μM cytochrome P450-IIB1 increased rapidly to a maximum of 2.32 ± 0.16 μM/min at an equimolar concentration of both enzymes (Fig. 5A, curve 2, N = 3, P < 0.001). Similarly, experiments with varying amounts of cytochrome P450-IIB1 and a fixed concentration of NADPH-cytochrome P450 reductase (70 μM) showed a cytochrome P450-IIB1 concentration-dependent stimulation of TMQ-induced NADPH oxidation from 0.53 ± 0.06 μM/min to 2.37 ± 0.18 μM/min at a 1:1 molar ratio of both enzymes (Fig. 5B, curve 3, N = 3, P < 0.001).

DISCUSSION

There is some evidence that a variety of foreign compounds are reductively activated by cytochrome P450 [6, 9, 10]. As was shown by ESR, the triarylmethane dye Gentian violet undergoes one-electron reduction by cytochrome P450 to produce a carbon centered free radical [8]. The formation of the carbon centered free radical of Gentian violet was inhibited by carbon monoxide and metyrapone, and could not be detected in the presence of molecular oxygen [8]. Of the halogenated foreign compounds reductively metabolized by cytochrome P450, carbon tetrachloride and halothane have been studied most extensively [10]. The reductive activation of these polyhalogenated alkanes is preferentially catalysed by PB-inducible isoenzymes of cytochrome P450 [10]. Recently, we reported on the involvement of cytochrome P450 in the one-electron reduction of quinoneimines [27]. Preliminary evidence also suggested a role for cytochrome P450 in the reduction of MMC [28]. This study presents evidence that PB-inducible enzymes of cytochrome P450 are also involved in the one-electron reduction of a model quinone compound TMQ.

Binding of TMQ to cytochrome P450

The initial step in the cytochrome P450 reaction involves interaction of the hemoprotein with a substrate molecule [2]. The spectral changes induced by TMQ in liver microsomes from PB-treated rats indicated a high affinity binding of TMQ to PB-inducible cytochrome P450s. The type I character of

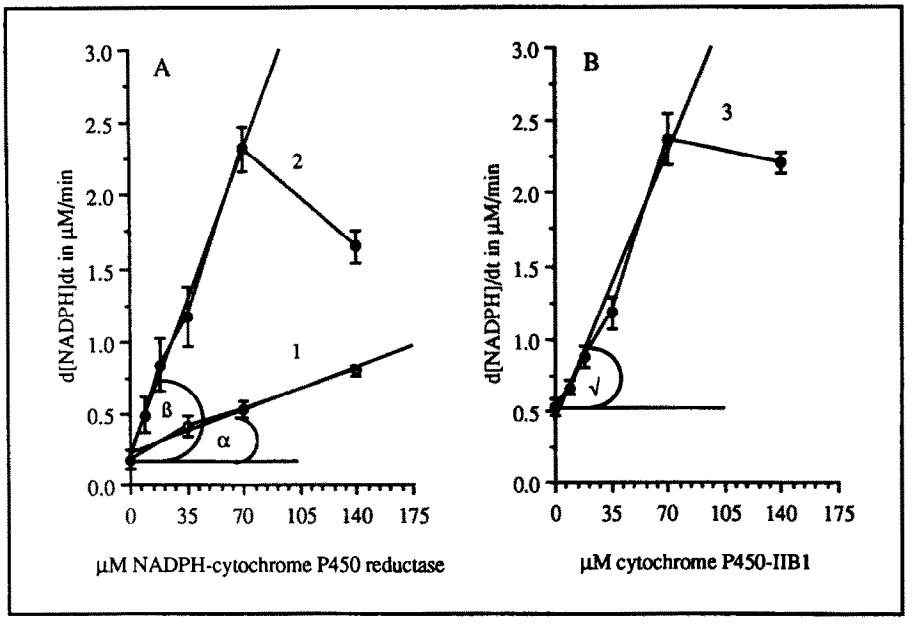

Fig. 5. TMQ reductase activity in the presence of reconstituted systems of highly purified cytochrome P450-IIB1 and NADPH-cytochrome P450 reductase. TMQ reduction was measured by monitoring the initial rate of NADPH oxidation under anaerobic conditions as described in Materials and Methods. (A) TMQ (100 μM)-induced NADPH oxidation was measured with varying amounts of NADPH-cytochrome P450 reductase in the absence (curve 1) or presence (curve 2) of 70 μM of cytochrome P450-IIB1. (○) NADPH-cytochrome P450 reductase alone; (●) reconstituted system of NADPH-cytochrome P450 reductase and 70 μM cytochrome P450-IIB1. (B) TMQ (100 μM)-induced NADPH oxidation was measured in reconstituted systems of varying amounts of cytochrome P450-IIB1 in the presence of 70 μM of NADPH-cytochrome P450 reductase (curve 3). Each value is the mean ± SD of at least three experiments.

the difference spectra indicated a shift from low spin towards the high spin configuration of the ferric ion. The ability of TMQ to produce a type I interaction with cytochrome P450 implied binding of TMQ at the substrate binding site of the heme protein indicating that TMQ is a potential substrate for cytochrome P450-mediated biotransformation.

Formation of TMSQ from TMQ by cytochrome P450

Free radicals formed by cytochrome P450 can often by detected by ESR [8, 27]. In the present study, TMSQ formation was demonstrated by ESR in PB-induced microsomal rat liver fractions under anaerobic conditions (Fig. 3A). The large decline (70%) of ESR signal upon inhibition of cytochrome P450 by SK&F 525-A indicates a substantial role for PB-inducible cytochrome P450s in the reduction of TMQ. Purified NADPH-cytochrome P450 reductase alone was also able to reduce TMQ to TMSQ but to a lesser extent. Incubation of TMQ with a reconstituted system (containing cytochrome P450-IIB1, NADPH-cytochrome P450 reductase and NADPH), showed 4–5-fold larger ESR signals of TMSQ, indicating the relative importance of this isoenzyme of cytochrome P450 in the one-electron reduction of TMQ.

Relatively low ESR signals of TMSQ were detected in the presence of atmospheric oxygen during the enzymatic one-electron reduction process (Figs 2B and 3B). Air oxidation of TMSQ to TMQ under concommitant formation of superoxide anion could account for the low ESR signal intensities of TMSQ under aerobic conditions. The role of cytochrome P450 in the reduction of TMQ under aerobic conditions will be described elsewhere (manuscript in preparation).

Role of cytochrome P450-IIB1 in the reduction of TMQ

Since the conversion of NADPH is an obligatory step in the cytochrome P450-mediated reduction of TMQ, initial rates of NADPH oxidation were determined under anaerobic conditions (Fig. 4). Specific inhibitors of cytochrome P450 were used as modulators of microsomal reductive activity. The strong inhibition of TMQ reduction by SK&F 525-A and K01 in PB-induced rat liver microsomes (Fig. 4) and the inhibitory effect of K01 in reconstituted systems of purified cytochrome P450-IIB1 and NADPH-cytochrome P450 reductase may be explained by the contribution of the major PB-inducible cytochrome P450 isoenzyme IIB1 in this one-electron reduction reaction, especially since NADPH-cytochrome P450 reductase activity was not inhibited by these cytochrome P450 inhibitors.

The contribution of cytochrome P450-IIB1 and NADPH-cytochrome P450 in the reduction of TMQ

Initial rates of TMQ-induced NADPH oxidation

were determined in reconstituted systems containing varying amounts of NADPH-cytochrome P450 reductase and cytochrome P450-IIB1 (Fig. 5) to determine the contribution of either enzyme in the reductive bioactivation of TMQ. The slope α of curve 1 in Fig. 5A shows the contribution of purified NADPH-cytochrome P450 reductase in the reduction of TMQ. As expected, NADPH-cytochrome P450 reductase alone was able to reduce TMQ at low rates (5.5 pmol NADPH/nmol NADPH-cytochrome P450 reductase/min). However, anaerobic incubation mixtures containing a fixed concentration of purified cytochrome P450-IIB1 showed a strongly increased rate of TMQ reduction with increasing concentrations of NADPH-cytochrome P450 reductase (Fig. 5A, curve 2). The contribution of reconstituted NADPH-cytochrome P450 reductase in the reduction of TMQ is indicated by slope β. From slope β it was calculated that TMQ-induced NADPH oxidation occurred at a rate of 30.6 pmol NADPH/nmol NADPH-cytochrome P450 reductase/min (5-fold greater than with NADPH-cytochrome P450 reductase alone). Similarly, experiments with varying amounts of cytochrome P450-IIB1 and a fixed amount of NADPH-cytochrome P450 reductase resulted in increasing rates of NADPH-oxidation (Fig. 5B, curve 3). The contribution of reconstituted cytochrome P450-IIB1 in the reductive metabolism of TMQ was calculated from slope $\surd$ of curve 3 in Fig. 5B and occurred at a rate of 26.3 pmol NADPH/nmol cytochrome P450-IIB1/min. Thus it was concluded that in reconstituted systems and under anaerobic conditions, cytochrome P450-IIB1 and NADPH-cytochrome P450 reductase contribute almost 1:1 ($\beta = \surd$) to the reduction of TMQ.

Interestingly, all experiments performed at an equimolar ratio of cytochrome P450-IIB1 and NADPH-cytochrome P450 reductase resulted consistently in a 4–5-fold enhancement of TMQ reduction by cytochrome P450-IIB1 as compared to NADPH-cytochrome P450 reductase alone (using ESR and TMQ-induced NADPH oxidation studies). The results might indicate that under anaerobic conditions cytochrome P450-IIB1 together with NADPH-cytochrome P450 reductase is more efficient in donating electrons to TMQ to form TMSQ than NADPH-cytochrome P450 reductase alone.

Proposed mechanism of TMQ reduction by the cytochrome P450 system

A proposal for the mechanism of cytochrome P450 mediated one-electron reduction of TMQ is as follows. Under anaerobic conditions purified NADPH-cytochrome P450 reductase is able to reduce TMQ to a small extent when TMQ is the sole electron acceptor [42]. However, as far as reduction of TMQ to TMSQ in a microsomal or a complete reconstituted system is concerned, both cytochrome P450-IIB1 and NADPH-cytochrome P450 reductase possess reductive activity. Therefore, NADPH-cytochrome P450 reductase can transfer electrons from NADPH either directly to TMQ or via cytochrome P450-IIB1 (Fig. 1). The high affinity binding of TMQ with PB-inducible cytochrome P450s observed in the present study indicates that TMQ is a potential substrate for cytochrome P450-mediated biotransformation. The heme spin-equilibrium shift from the low-spin towards the high-spin configuration is likely to induce a dramatic shift in the reduction potential of the substrate-bound form of cytochrome P450 [2]. Consequently, the rate of electron flow from NADPH-cytochrome P450 reductase to the cytochrome P450-TMQ complex may be increased. Cytochrome P450 can then participate more easily in the reduction reaction by donating electrons directly to TMQ to form TMSQ under anaerobic conditions, as was shown by ESR.

Furthermore, reconstituted systems of cytochrome P450-IIB1 and NADPH-cytochrome P450 reductase showed optimal TMQ reductive activity when both enzymes were at nearly equal molar concentrations (Fig. 5). The fact that a 1:1 stoichiometry of NADPH-cytochrome P450 reductase and cytochrome P450-IIB1 exists for maximal reduction of TMQ might indicate that cytochrome P450-IIB1 and NADPH-cytochrome P450 reductase act as one complex during reduction of TMQ. If they did not act as one complex, the effects of either NADPH-cytochrome P450 reductase or cytochrome P450-IIB1 would increase with increasing concentrations of either component, independent of their ratio. In contrast, TMQ reductive activity was lowered when NADPH-cytochrome P450 reductase was in a molar excess to cytochrome P450-IIB1 (Fig. 5A, curve 2).

Biological consequences of cytochrome P450-mediated reduction of quinones

In the present study, TMQ was chosen as a model quinone to investigate the mechanism of cytochrome P450-mediated one-electron reductive biotransformation. The role of cytochrome P450 in this type of reduction reaction may also be important for more complex quinoid compounds such as Adriamycin® and MMC. These quinones are also activated by reductive biotransformation [15, 16]. This new role of cytochrome P450 may have important consequences for the toxicity and antitumor activity of quinone cytostatics because cytochrome P450 activity in normal and tumor cell tissues may vary greatly as a result of age, genetic factors and induction, or inhibition by drugs, or environmental factors.

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