INCORPORATION OF AN OXYGEN FROM WATER INTO TROGLITAZONE QUINONE BY CYTOCHROME P450 AND MYELOPEROXIDASE

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ABSTRACT:
Troglitazone (TGZ) was the first glitzone used for the treatment of type II diabetes mellitus. TGZ undergoes an oxidative chroman ring-opening reaction to form a quinone product. Recently, cytochrome P450 (P450) was shown to be able to catalyze the formation of TGZ quinone. TGZ quinone was the major metabolite formed by dexamethasone-induced rat liver microsomes or myeloperoxidase (MPO) incubated with TGZ. The ultimate source for the quinone carbonyl oxygen atom of TGZ quinone was investigated using 18O water in both enzyme reaction systems followed by liquid chromatography/tandem mass spectrometry analysis of the TGZ quinone product. The resultant TGZ quinone formed by either liver microsomes or MPO contained a single atom of 18O. The 18O atom was determined to be the quinone carbonyl oxygen by collision-induced dissociation fragmentation of the 18O-labeled TGZ quinone. The formation of TGZ quinone was inhibited approximately 90% by coincubation with ascorbic acid or cysteine in the MPO reaction system but only 10 to 20% in liver microsomes, which might reflect the difference in the mechanism by which TGZ quinone is formed by P450 and peroxidase. These results suggest that P450 catalyze an atypical reaction to form TGZ quinone, involving the incorporation of an oxygen from water into the quinone carbonyl position.

Troglitazone (TGZ, Rezulin, CI-991), a thiazolidine 2,4-dione derivative, was the first member of the glitzones used for the treatment of type II diabetes mellitus (Saltiel and Olefsky, 1996). The mechanism of the pharmacological effects involves the increase of insulin sensitivity in skeletal muscle, liver, and adipose tissue via activation of peroxisome proliferator-activated receptor-γ (Lehmann et al., 1995; Saltiel and Olefsky, 1996). Although TGZ offered significant clinical benefits to many diabetic patients, several clinical reports indicated that TGZ was associated with serum alanine aminotransferase elevations in 1 to 2% of patients and, in rare cases, hepatic failure (Watkins and Whitcomb, 1998).

The metabolism of TGZ in humans primarily involves conjugation reactions that form sulfate (major metabolite) and glucuronide derivatives and oxidation to a quinone product (Fig. 1) (Kawai et al., 1997; Loi et al., 1997). Previous studies in our laboratory and others have shown that P450 enzymes, reactive oxygen species, and peroxidases are involved in the chroman ring-opening reaction that leads to the formation of TGZ quinone (Fu et al., 1996; Yamazaki et al., 1999; He et al., 2001). Typical P450 oxidation reaction involves the formation of an activated oxygen species (oxyferryl) that inserts the iron-bound oxygen into substrate (Ortiz de Montellano, 1995; Schlichting et al., 2000). In the case of chroman ring-opening reaction to the formation of TGZ quinone by P450, the nature of the product suggests that an alternative process of oxygen incorporation may occur. This study investigated the oxygen incorporation involved in TGZ quinone formation by P450 compared with peroxidase.

FIG. 1. Scheme of the primary metabolic pathways involved in TGZ metabolism (*, 14C-labeled).
Materials and Methods

Chemicals. TGZ and 14C-TGZ were synthesized at Parke-Davis Pharmaceutical Research (Ann Arbor, MI). NADPH, catalase, superoxide dismutase, dexamethasone (DEX), ascorbic acid, cysteine, myeloperoxidase (MPO), horseradish peroxidase, H2O2 and 18O water were purchased from Sigma-Aldrich (St. Louis, MO).

Liver Microsomes. Rat liver microsomes were prepared by differential centrifugation from male Fischer-344 rats (161–190 g) (Harlan, Indianapolis, IN) treated i.p. with DEX (100 mg/kg) for 4 days. Total P450 content was measured according to the method of Omura and Sato (1964).

Incubation of TGZ with Liver Microsomes. TGZ (20 μM) was incubated with DEX-induced rat liver microsomes (0.1 nmol of P450/ml) in 50 mM Heps buffer (pH 7.4) containing 30 mM MgCl2, 20% glycerol, and 0.1 U/ml superoxide dismutase for 15 min at 37°C. The reaction was started with the addition of 1 mM NADPH and stopped with the addition of an equal volume of cooled methanol. In studies aimed at investigating the incorporation of oxygen, the buffer containing 50% 18O water (v/v) was used. The effects of ascorbic acid or cysteine on TGZ quinone formation was assessed by coincubation with these compounds at concentrations up to 1 mM. TGZ was also incubated with DEX-induced rat liver microsomes (0.1 nmol of P450/ml) in 50 mM Heps buffer (pH 7.4) containing 30 mM MgCl2 and 20% glycerol in the presence of cumene hydroperoxide (2 mM) or iodosobenzene (5 mM) for 5 to 10 min at 37°C.

Incubation of TGZ with Peroxidases. TGZ (20 μM) was incubated with purified human MPO (0.1 U/ml) in 0.1 M phosphate buffer (pH 7.5) at 37°C for 15 min. The reactions were started with the addition of TGZ and stopped with the addition of an equal volume of cooled methanol. The effects of ascorbic acid or cysteine on TGZ quinone formation were assessed by coincubation with these compounds at concentrations up to 1 mM.

LC-MS/MS Analysis of TGZ Quinone. TGZ quinone was analyzed on Micromass Quattro-II triple quadrupole mass spectrometer (Micromass, Manchester, UK) coupled with PerkinElmer Series 200 HPLC System (PerkinElmer Life and Analytical Science, Boston, MA), IN/US β-RAM radioactive detector (IN/US Systems, Inc., Tampa, FL) and Waters 486 UV detector (Waters, Milford, MA). Sample introduction and ionization were achieved by electrospray ionization in the negative ion detection mode. Ionization parameters include source cone voltages of 50 V, capillary voltages of 3.0 kV, and a source temperature of 120°C. The initial scan rate was 1.6
s/decade over a mass range of 50 to 800 atomic mass units. Scan data were acquired using MassLynx multitasking operating system version 3.0. The standard and samples were monitored in MS scanning and precursor ion detection modes. Tandem mass spectrometry experiments were based on collision-induced dissociation (CID) occurring in the rf-only collision cell of the triple quadrupole at a collision energy of 45 eV. Argon was used as a collision gas in the range of 2.0 to 2.3 mtorr. Both product ion and precursor ion CID were performed (data not shown). Chromatographic separation was achieved on a C18 column (150 x 2.0 mm, 5 μm, MetaChem MetaSil AQ; ANSYS Technologies, Ltd., London, UK) using a solvent system containing 0.1% formic acid (A) and acetonitrile (B) in a stepwise linear gradient of 10% B for 5 min, 10 to 85% B for 30 min, and 85% B for 5 min at a flow rate of 0.25 ml/min.

Results and Discussions

TGZ quinone formation has been shown to be catalyzed by P450 enzymes, in particular P450 3A and 2C8 isoforms (Yamazaki et al., 1999; He et al., 2001). The quinone was the major metabolite formed in DEX-induced rat liver microsomes incubated with TGZ (Fig. 2). As demonstrated by our previous work, the formation of TGZ quinone in liver microsomes is inhibited by the P450 inhibitors ketoconazole or troleandomycin (He et al., 2001). Incubation with catalase or a catalase inhibitor showed no effect on TGZ quinone formation, indicating that hydrogen peroxide is not involved in this process as a byproduct of P450 reaction. The contribution of superoxide generated in liver microsomes was assessed to be about 30% of the total TGZ quinone formation. Thus, most of TGZ quinone formed in liver microsomes is the result of direct oxidation by P450 (He et al., 2001). In addition, in an attempt to support the P450 reaction in liver microsomes using cumene hydroperoxide or iodosobenzene in the present study, we found that these two agents react directly with TGZ to form the quinone metabolite. Thus, it is not clear whether the organic peroxide or a single oxygen donor can support the TGZ quinone formation by P450.

In addition to P450 enzymes, peroxidases, another class of oxidative enzymes, were also shown to catalyze TGZ quinone formation. MPO-mediated TGZ quinone formation is a hydrogen peroxide-dependent process. The reaction requires the active form of the enzyme because the heat-denatured enzyme did not catalyze the quinone formation. Since P450 enzymes and peroxidases have a different oxygen incorporation mechanism yet catalyze the same reaction to
form TGZ quinone, the ultimate source for the oxygen atom for the TGZ quinone molecule is important in understanding the mechanism for the P450 reaction compared with peroxidase (Ortiz de Montellano, 1992, 1995).

To investigate the source of the oxygen atom, TGZ was incubated in the presence of 18O water, and the quinone product was determined by LC-MS/MS analysis. As shown in Fig. 3, approximately 50% of the resultant TGZ quinone contained a single 18O atom when TGZ was incubated with either liver microsomes (Fig. 3B) or MPO (Fig. 3C) in a buffer containing 50% 18O water (v/v), indicating that almost all of the incorporated oxygen atom were from water. The 18O atom was determined to be the quinone carbonyl oxygen but not the 2-OH on the butoxy moiety of TGZ quinone (Fig. 4). Although the mass fragmentation could not determine which of the two quinone oxygen atoms were the 18O, the 18O atom was believed to be in the 6-position given the chemical nature of the ring-opening reaction (Fig. 4). These results clearly indicate that the mechanism for P450-mediated TGZ quinone formation, which involves the transfer of an oxygen atom from water to the quinone carbonyl, is different from the classic P450 oxidation mechanism, by which an oxyferryl species (FeIV = O) inserts an oxygen atom into substrate (Ortiz de Montellano, 1995; Schlichting et al., 2000). The mechanism also seems to be different from the P450-catalyzed ipso-substitution of p-methoxyphenol, which involves the abstraction of α-hydrogen as the initial step but ends up with the incorporation of an oxygen atom from O2 into the p-benzoquinone product (Ohe et al., 1994). The oxygen incorporation results

The P450 reaction may result in the formation of a quinonium radical directly compared to that of the initial step for the peroxidase reaction which is to form a relatively stable phenoxy radical. If the phenoxy radical diffuses from the enzyme active site, the radical could be reduced back to the parent compound; thus, the inhibition effect by ascorbate or cysteine is expected. However, the current results could not rule out that the phenoxy radical might be formed by the P450 enzyme and remain in the active site.

**Fig. 6.** Proposed mechanisms for peroxidase- (A) and P450 (B)-catalyzed TGZ quinone formation.
for MPO would be expected as a result of one-electron oxidation of TGZ (Liebler and Burr, 1992; Ortiz de Montellano, 1992; Liebler, 1994).

Although both P450 and peroxidase showed the incorporation of the oxygen atom from water into the quinone product, an important difference was observed in an inhibition study with ascorbic acid and cysteine. As shown in Fig. 5, TGZ quinone formation was inhibited by approximately 90% in the MPO reaction system but less than 20% in DEX-induced rat liver microsomes by coincubation of ascorbic acid or cysteine. These results may reflect the difference in the mechanism by which TGZ quinone is formed by P450 and MPO. As a model system for one-electron oxidation, peroxidase is believed to catalyze the formation of a phenoxyl radical of TGZ via a similar mechanism for the oxidation of α-tocopherol and its analog 6-hydroxy-2,2,5,7,8-pentamethylchroman (Nakamura, 1990; Nakamura and Hayashi, 1992). The resonance-stabilized phenoxyl radical migrates from the active site of peroxidase and can be reduced back to the parent compound by reductants such as ascorbic acid and cysteine (Nakamura, 1990; Liebler and Burr, 1992; Nakamura and Hayashi, 1992). Unlike peroxidase, the P450 enzyme may directly catalyze the formation of a quinonoid radical through a two-electron oxidation mechanism that leads to chroman ring opening in the active site (Fig. 6). Another possible mechanism is that the P450 enzyme may catalyze the formation of the phenoxy radical through a one-electron oxidation mechanism, but unlike peroxidase, the radical may remain in the P450 active site in such a way that ascorbic acid or cysteine could not prevent the reaction (Fig. 6). In addition, the results from the inhibition study with ascorbic acid and cysteine imply that P450 enzymes may play more important roles than peroxidases in the formation of TGZ quinone in physiological conditions since the endogenous reductants would inhibit peroxidase-catalyzed quinone formation, but to a much lesser extent than the P450-catalyzed quinone formation.

Compared with peroxidases, P450 enzymes catalyze versatile oxidative reactions through various activated oxygen species (Rettie et al., 1988; Ortiz de Montellano, 1995; Vaz et al., 1996; Newcomb et al., 2003). Our results suggest that, unlike the classic P450 reactions, TGZ quinone formation involves an atypical P450 reaction, i.e., incorporation of an oxygen from water into the quinone carbonyl. The proposed mechanism involves hydrolysis of a quinonoid cation species formed by P450 through either a two-electron oxidation or two sequential one-electron oxidations in the active site of the enzyme (Fig. 6).

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References


