Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochemical Pharmacology 68 (2004) 747–752
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Characterization of hydroxyl radical formation by microsomal enzymes using a water-soluble trap, terephthalate

Vladimir M. Mishin, Paul E. Thomas*

Department of Chemical Biology, Susan Lehman Cullman Laboratory for Cancer Research, Ernest Mario School of Pharmacy, Rutgers, The State University of New Jersey, Piscataway, NJ 08854, USA

Received 8 December 2003; accepted 4 May 2004

Abstract

Using terephthalic acid as a water-soluble trap, we characterized hydroxyl radicals ($HO^{\bullet}$) formation by liver microsomal enzymes from isoniazid-treated rats. We found that $HO^{\bullet}$ formation was entirely dependent on intact microsomal enzymes, the presence of NADPH, and iron complexed with EDTA. In contrast to the other radical traps, we found no evidence that terephthalate is a substrate for cytochrome P450. Cumene hydroperoxide, an artificial supporter of cytochrome P450-catalyzed oxidation, failed to maintain $HO^{\bullet}$ formation. $HO^{\bullet}$ formation in liver microsomes was inhibited by the $HO^{\bullet}$ radical scavengers: dimethyl sulfoxide (DMSO), mannitol, and citrulline. It was abolished by catalase, but not superoxide dismutase (SOD), indicating that hydrogen peroxide was the sole precursor of the $HO^{\bullet}$. Therefore, the generation of hydroxyl radicals by microsomal enzymes appears to be dependent on two processes: (1) the rate of hydrogen peroxide production; and (2) the availability of iron ions or other transition metals for Fenton type reactions.

Keywords: Liver microsomes; Terephthalate; 2-Hydroxyterephthalate; Hydroxyl radical; Cytochrome P450

It is a widely accepted hypothesis, that generation of reactive oxygen species (ROS) by various intracellular enzymes is an important factor in the onset of numerous pathologies including cancer, diabetes, liver injury, ischemic or traumatic brain injuries and several others [1,2]. While ROS such as hydrogen peroxide and superoxide anion possess weak toxicity [3], their reactions with transition metal complexes lead to the formation of the highly reactive hydroxyl radicals ($HO^{\bullet}$) [4–7].

Direct measurement of the hydroxyl radical is practically impossible due to its very short lifetime and extreme reactivity. Several indirect methods using chemical traps have been suggested and are used widely to detect the formation of hydroxyl radicals in biological systems, including cytochrome P450-associated enzyme systems [8–11]. However, there are problems associated with the use of chemical traps in biological systems. Several of the currently employed assays are of questionable specificity [12–14]. For example, it was shown recently that adrenochrome formation from epinephrine cannot be employed as an indication of superoxide radicals formation by microsomal enzymes because of complex interaction of adrenochrome and epinephrine as substrates and inhibitors of cytochrome P450 [15]. Salicylate metabolism used as in vivo and in vitro marker of $HO^{\bullet}$ generation reflects not only oxidative stress but, to a large extent is metabolized by cytochrome P450 enzymes [16]. Also, many of the assays are technically difficult to perform, requiring chemicals not available commercially and as well as expensive equipment [17–19]. These issues have been discussed in detail in reports and reviews [20,21].

Regarding the cytochrome P450 monooxygenase system, there are two major problems in the determination of ROS generation sites. First of all, it is well known that NADPH-cytochrome P450 reductase is capable of producing superoxide radicals [22,23]. These oxidative species might react with chemical probes thought to be specific for $HO^{\bullet}$. Another problem consists in discriminating whether a chemical trap is the substrate for a monooxygenase reaction or the target for attack of ROS or some of both [21].

Here we present data showing that one of the previously studied $HO^{\bullet}$ traps, terephthalic acid, is not a substrate of

Abbreviations: $HO^{\bullet}$, hydroxyl radical; ROS, reactive oxygen species; SOD, superoxide dismutase; DMSO, dimethyl sulfoxide

* Corresponding author. Tel.: +1 732 445 0142; fax: +1 732 445 0119.
E-mail address: pethomas@eohsi.rutgers.edu (P.E. Thomas).

0006-2952/$ – see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2004.05.004

cytochrome P450. Moreover this trap is water-soluble and thus avoids the additional complication that many organic solvents used to add compounds to microsomal enzymes are themselves $HO^{\bullet}$ traps and/or substrates and inhibitors for cytochrome P450, e.g. dimethyl sulfoxide (DMSO), ethanol and methanol.

Terephthalic acid reacts with hydroxyl radicals to form the highly fluorescent monohydroxylated product, 2-OH terephthalate [17,24–26]. The reaction has been studied with generation of $HO^{\bullet}$ by physical or chemical means and the accumulation of 2-OH terephthalate was shown to be a specific and sensitive marker of $HO^{\bullet}$ formation when compared with other chemical probes [25,27]. Therefore, we hypothesized that terephthalate transformation to 2-OH terephthalate in the presence of microsomal enzymes serves as a specific and sensitive indicator of $HO^{\bullet}$ generation and avoids the complication of being a substrate for cytochrome P450-dependent monooxygenase reactions.

1. Materials and methods

1.1. Reagents

All chemicals obtained commercially were of the highest purity grade and were used without additional purification. Pure 2-OH terephthalate was kindly provided by Prof. Lewis Kirschenbaum (University of Rhode Island, Kingston, RI). Potassium phosphate monohydrate, DMSO, water and organic solvents, all HPLC grade, were purchased from Fisher Scientific (Fairlawn, NJ). Terephthalate was obtained from Aldrich (Milwaukee, WI). Superoxide dismutase (SOD), catalase, EDTA (sodium salt), isoniazid, cumene hydroperoxide, and mannitol were purchased from Sigma–Aldrich (St. Louis, MO). NADPH, anhydrous ferric chloride, and tertrabutylammonium bromide were purchased from Fluka (Milwaukee, WI). HPLC column, Luna C18(2), 3 μm, 150 mm × 2.0 mm and C18 guard column were supplied by Phenomenex (Torrans, CA). A Shimadzu HPLC system (pumps, controller, autosampler, spectrofluorometric detector, Shimadzu Scientific Instruments, Columbia, MD), and UV detector, 1000S (Applied Biosciences, Ramsey, NJ) were used.

1.2. Animal treatment

Three female Sprague-Dawley rats (200 g body weight, from Taconic Inc., Germantown, NY) were pretreated with isoniazid (300 mg/kg, I.P. once a day for 4 consecutive days, except that on day 1 the dose was divided equally between two injections given 8 h apart). Microsomes prepared individually from the livers of isoniazid-treated rats were selected because of the relative high concentration of CYP2E1 results from this treatment [28]. The protein concentration of microsomal suspensions was determined by BCA assay using BSA as standard (PIERCE kit) [29].

1.3. HPLC analysis

HPLC was carried out using a Luna C18(2), 3 μm, 150 mm × 2.0 mm and C18 guard column. Chromatography was performed isocratically at a flow rate of 0.2 ml/min using water:methanol:acetic acid (70:25:5) containing 2.0 mM tetrabutylammonium bromide as a mobile phase. The final pH was 2.5–2.6. The elution of 2-OH terephthalate was monitored fluorometrically (excitation 315 nm, emission 425 nm). In initial HPLC experiments the elution of terephthalate was also monitored by a UV detector at 254 nm (connected in tandem with spectrofluorometric detector). The signals from the detectors were recorded and analyzed by the use of CLASS-VP software (Shimadzu Scientific Instruments, Columbia, MD). The lowest limit of peak quantification was accepted at five-fold above noise level.

1.4. Terephthalate hydroxylation by microsomal enzymes

The microsomal fraction of the livers from female SD rats ($n = 3$) pretreated with isoniazid [28] was used during method design. Stock solutions of terephthalate, NADPH and cumene hydroperoxide were prepared daily in 20 mM potassium phosphate buffer, pH 7.4. Fe^{3+}/EDTA (1:1.1, molar ratio) was prepared just before experiments by dissolving anhydrous ferric chloride in 0.01N HCl, followed by addition of EDTA stock solution (0.2 M) to the appropriate concentration. The Fe^{3+}/EDTA solution, when added (10 μl of Fe^{3+}/EDTA solution to 0.19 ml of the incubation sample), did not change the pH of the incubation media. All other components used in this study were diluted in 20 mM potassium phosphate buffer, pH 7.4.

Prepared on ice, the reaction mixtures consisted of 20 mM potassium phosphate buffer, pH 7.4, microsomal enzymes (at desired quantity of protein), 1.0 mM terephthalate, and 1.0 mM NADPH. In experiments with cumene hydroperoxide, this agent was at 0.1 mM concentrations. Various inhibitors were added at this stage of the mixture preparation. The samples were preincubated at 37 °C for 3 min in a water bath. The reaction was initiated by addition of 10 μl Fe^{3+}/EDTA complex solution to give a final concentration of 0.1 mM of Fe^{3+}. The final volume of the incubation mixture was 0.2 ml. After 15 min of incubation (unless another time was indicated) the reaction was terminated by the addition of 0.2 ml ice-cold methanol and samples were placed on ice. The supernatant obtained by centrifugation (at 12,000 × g for 5 min), was used for HPLC analysis. Aliquots of 10 μl were injected directly on the column from autosampler vials.

1.5. Calibration curve

Solid 2-OH terephthalate was dissolved in water as a stock solution and kept in the dark at room temperature. When needed, aliquots of the 2-OH terephthalate stock

solution were diluted in 20 mM potassium phosphate buffer, pH 7.4 to prepare working standard solutions. A set of samples was prepared such that each sample (in triplicate) with a particular concentration of 2-OH terephthalate (taken from a working standard solution) contained all components used for enzymatic reaction including the inactivated microsomal protein (5 min in boiling water bath) in the final volume of 0.2 ml. Calibration samples were incubated at 37 °C for 15 min and after that 0.2 ml of ice-cold methanol was added. The denaturated proteins were sedimented by centrifugation and aliquots of the supernatant (10 μl of each sample) were injected for HPLC analysis. The 2-OH terephthalate peak areas were plotted against the known 2-OH terephthalate concentration. No internal standard was used since no extraction steps were used.

Fluorescence measurements of 2-OH terephthalate using disposable rectangular cuvettes were conducted as described [26]. Briefly, the mixtures for terephthalate hydroxylation were prepared exactly as described above but the final volume of incubation samples was 2.0 ml. The reaction was started by the addition of Fe^{3+}/EDTA complex. After 15 min of incubation at 37 °C the reaction media was transferred into the cuvette and fluorescence was recorded (excitation at 315 nm and emission at 425 nm). The background fluorescence was determined with addition of all components but with inactivated microsomal proteins. For these experiments a Perkin–Elmer LS-5B spectrofluorometer was used.

2. Results and discussion

Fluorescence methods have been developed to determine, in real time, the 2-OH terephthalate formation directly in a cuvette [26,27]. In our initial experiments the calibration curve obtained with the 2-OH terephthalate standard (added to pure 20 mM potassium phosphate buffer, pH 7.4) demonstrates high sensitivity i.e. lower limit of 1.0 nM 2-OH terephthalate. However, background fluorescence increased significantly after the addition of components required for microsomal-supported reactions, including microsomal protein, NADPH or NADPH-regenerating system, and Fe^{3+}/EDTA complex. To obtain a fluorescence emission intensity of more than 20% of initial background values, at least 0.5 mg/ml of microsomal protein was required at the typical conditions of incubation (15 min at 37 °C). We found in subsequent experiments that these protein concentrations are too high for the linear dependence of the reaction rate with time. Also, to study 2-OH terephthalate formation by commercially available cytochrome P450 preparations we had to use large amounts of expensive materials. Finally, we were not able to detect with satisfactory accuracy the ''iron-independent'' formation of 2-OH terephthalate (see below) with the use of the direct measurement in cuvettes.

To avoid the problems described above and to obtain high levels of sensitivity and accuracy we developed an HPLC-based method of 2-OH terephthalate determination using a fluorescence detector. Fig. 1A shows chromatograms obtained during the development of this assay. Terephthalate at concentrations as high as 10.0 mM did not yield any significant fluorescence at 315 nm excitation and 425 nm emission wavelengths. To monitor the terephthalate elution we used UV detection at 254 nm. The retention times of 2-OH terephthalate and terephthalate were practically identical (9.1 and 8.84, respectively) with water:methanol:acetic acid (70:25:5) as the mobile phase (not shown). The addition of the ion-pairing reagent, tetrabutylammonium bromide, in the mobile phase (2.0 mM final concentration) resulted in sharper peak of 2-OH terephthalate and slightly increased the retention time for 2-OH terephthalate (11.6 min) while not changing the retention time for terephthalate. This mobile phase composition was used in subsequent experiments. We directly injected the supernatant aliquots after protein precipitation with an equal volume of methanol followed by centrifugation.

The recovery for 2-OH terephthalate was estimated by comparing the peak height of the calibration samples after protein precipitation with those of directly injected similar amounts of the standard added in the 0.4 ml of mobile phase. We found that recovery efficiency ranged from 93 to 99% over the concentrations used for the standard curve. Therefore no internal standard was needed to obtain satisfactory accuracy. No interference peaks were noted using

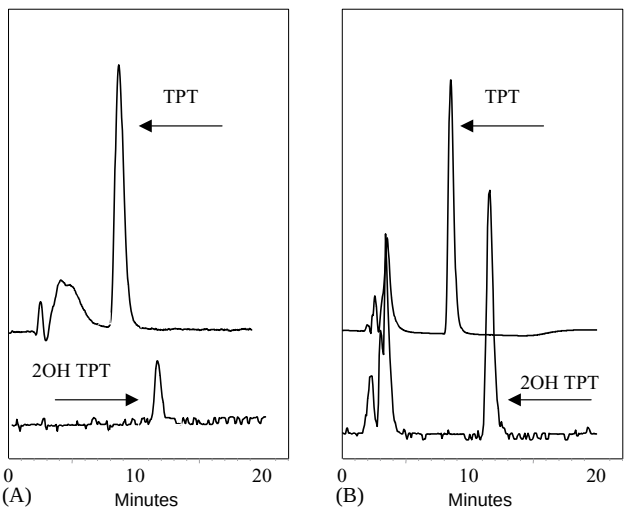

Fig. 1. HPLC analysis of terephthalate and 2-OH terephthalate and their detection in liver microsomes. The upper lines indicate UV detection; the bottom lines indicate fluorometric detection. (A) Chromatogram of terephthalate (TPT, 8.54 min) and 2-hydroxyterephthalate (2-OH TPT, as a standard, 11.6 min). Ten picomol of 2-OH TPT and 0.2 mmol of TPT were added in 0.4 μl of mobile phase and 10 μl of this mixture was injected and eluted as described in Section 1. (B) Representative HPLC traces of terephthalate after incubation with the complete system containing liver microsomes from isoniazid-treated female rats as described in Section 1.

Table 1
The essential cofactors in transformation of terephthalate into 2-OH terephthalate by microsomal enzymes

Conditions and cofactors	Reaction rate (nmol), 2-OH terephthalate/min/mg protein	%
Complete system	17.43	100
Inactivated microsomes (boiled, 3 min)	0.52	3
NADPH omitted	1.74	10
NADPH omitted, plus cumene hydroperoxide added	0.053	0.3
Fe^{3+}/EDTA complex omitted	0.87	5
Fe^{3+} ions omitted (EDTA included)	0.87	5
EDTA omitted (Fe^{3+} ions included)	1.31	7.5

The rates were determined in the linear phase of reaction for the quantity of protein used (15 min). The conditions for complete system and HPLC analysis are described in details in Section 1. The reactions were started by the addition of Fe^{3+}/EDTA complex, in a volume of 10 μl. (When tested separately, $FeCl_3$ or EDTA were dissolved and added at the concentrations, 0.1 and 0.11 mM, respectively). The values are mean from three experiments performed on different days.

this method for sample preparation and 15 min chromatography cycles.

A calibration curve was obtained for 2-OH terephthalate by plotting the compound peak areas against the concentrations used; 1.0–1000 ng of 2-OH terephthalate standard were added to incubation mixtures containing denatured microsomal proteins and other reaction components in a final volume of 0.4 ml. With the injection of 10 μl of supernatant, the linear dependence of detector response was found ($r^2 = 0.995$ and above in several calibration sets). We accepted the lower limit of calculation as 5.0 ng of 2-OH terephthalate formed (final volume for the enzymatic reaction was 0.2 ml; the final volume of incubation media after the addition of stop solution was 0.4 ml; the injection volume of supernatant was 10 μl). Inter-day variability at 5 ng standard sample ($n = 5$) was 7.3% and less than 6.0% at 200 ng standard sample ($n = 5$).

Separate experiments were performed to examine whether 2-OH terephthalate is further metabolized. For this, we incubated 2-OH terephthalate (50 and 200 ng per sample) with microsomal enzymes for 30 min in the presence of NADPH and Fe^{3+}/EDTA. HPLC analysis of these samples indicated that the 2-OH terephthalate peaks area values were not different than those of the samples used for calibration experiments e.g. without microsomal proteins. Therefore, we concluded that 2-OH terephthalate is the probable end product of terephthalate hydroxylation under the experimental conditions.

Fig. 1B presents chromatographic traces obtained after the incubation of terephthalate with microsomes. Catalytically active microsomal enzymes produce the component (11.0–11.5 min) that we identified as 2-OH terephthalate because the chromatographic retention time was identical to the 2-OH terephthalate standard as well the dramatic difference between fluorometric (lower trace) and UV properties (upper trace) that is typical for 2-OH terephthalate (Fig. 1A).

Table 1 lists the essential cofactors and conditions for the transformation of terephthalate into 2-OH terephthalate by liver microsomes from isoniazid-treated female rats. The inactivation of microsomal proteins in boiling water bath or addition of methanol at the beginning of incubation practically eliminated the 2-OH terephthalate peak formation. Omitting NADPH from incubation samples corresponded with a dramatic decrease of 2-OH terephthalate production. The use of cumene hydroperoxide instead of NADPH as an artificial supporter of cytochrome P450-catalyzed oxidation [30,31] yielded very little 2-hydroxyterephthalate. The incubation of native microsomal proteins with terephthalate and NADPH, but without Fe^{3+}/EDTA, corresponded with the formation of little but easily detectable quantity of 2-OH terephthalate. The addition of iron ions or EDTA as individual components into incubation media also did not substitute for Fe^{3+}/EDTA complexes and the reaction rates were low.

Fig. 2 presents the time course of the 2-OH terephthalate production at several microsomal protein concentrations with a mixture of the components that we designed as a "complete system" (see Table 1). These data showed that production of 2-OH terephthalate increased linearly during incubation times up to 15 min with concentrations of microsomal protein up to 0.2 mg/ml. Increased incubation time longer than 15 min and increased protein concentrations both lead to deviation from the proportionality and therefore to underestimation of the absolute rates of the reaction (expressed as nmoles 2-OH terephthalate formed

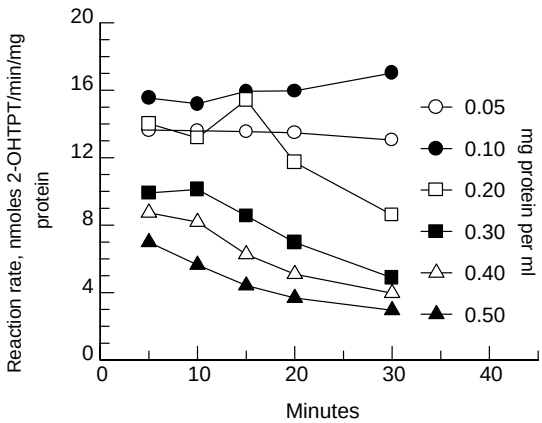

Fig. 2. Time course and protein dependence for 2-OH terephthalate formation with liver microsomes from isoniazid-treated female rats. The reaction mixtures contained, in a final volume of 0.2 ml, 20 mM potassium phosphate buffer, pH 7.4, microsomal proteins as described in the key, 1.0 mM terephthalate, 1.0 mM NADPH and 0.1 mM Fe^{3+}/EDTA complex to start the reaction. After incubation for 5, 10, 15, 20 and 30 min, the reactions were terminated by addition of ice-cold 0.2 ml methanol. After centrifugation, the samples were analyzed by HPLC as described in Section 1. The values of the reaction rates (*Y*-axis) are expressed as nmole 2-OH terephthalate formed per minute per milligram of microsomal protein.

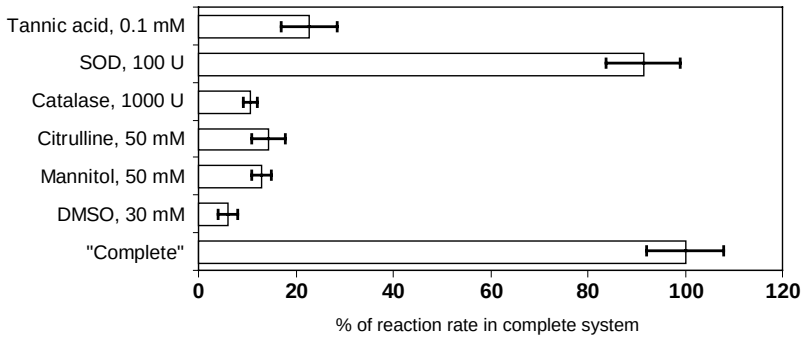

Fig. 3. The effect of various components on 2-OH terephthalate formation rate from terephthalate by liver microsomal enzymes from isoniazid-treated SD female rats. The concentrations of the components in the incubation mixtures were 30 mM DMSO, 50 mM mannitol, 50 mM citrulline, 1000 U catalase, 100 U SOD and 0.1 mM tannic acid. The complete incubation mixtures and the order of additions of components are described in detail in Section 1. The bars shown are the percentage of control rate (''complete system'') of 2-OH terephthalate formation per minute per milligram of microsomal protein and represents calculated means ± S.D. using microsomal preparations from three individual female isoniazid-treated rats.

per milligram protein per minute). Based on this finding we selected 0.1 mg of microsomal protein per millilitre and incubation time of 15 min as the experimental conditions for routine use.

In order to evaluate the participation of different ROS in the transformation of terephthalate to 2-OH terephthalate, we measured the rate of 2-OH terephthalate formation in the presence of various compounds that have been shown to react selectively with oxygen-derived free radicals. As depicted in Fig. 3, DMSO (30 mM), mannitol (50 mM), and citrulline (50 mM), all of them $HO^{\bullet}$ radical scavengers [17,32,33], effectively inhibited 2-OH terephthalate formation. Inclusion of catalase (1000 U/ml) in the incubation media also significantly inhibited 2-OH terephthalate formation. In contrast, the addition of superoxide dismutase at 100 U/ml had no effect on the rate of the 2-OH terephthalate formation. Tannic acid (0.1 mM), used as a potent chelator of iron ions [34], remarkably inhibited the reaction.

The present report describes the required components, the basic conditions for incubation, and an HPLC technique to determine 2-OH terephthalate generated by microsomal proteins during the oxidation of terephthalate. We found that 2-OH terephthalate generation is strictly dependent on the intactness of microsomal proteins. Also, the significant dependence of the rate of 2-OH terephthalate generation on NADPH presence indicated that the electron flow in microsomal electron transport chain is required for this reaction. Cumene hydroperoxide interacts with cytochrome P450 enzymes and effectively supports the cytochrome P450-dependent oxidation of many substrates in the absence of NADPH [30,31]. In our case with terephthalate, cumene hydroperoxide was not effective in the transformation of terephthalate to 2-OH terephthalate. Such dependence strongly indicates that terephthalate is not a traditional substrate for the typical, cytochrome P450-monooxygenase reaction. Whereas the method easily detects very low rate of 2-OH terephthalate formation without exogenously added Fe^{3+}/EDTA complex, this component profoundly stimulated the formation of 2-OH terephthalate by microsomal enzymes. Also, this finding points to the involvement of Fenton chemistry in terephthalate transformation by microsomal enzymes. In support of this conclusion, we found that tannic acid is a rather effective inhibitor of 2-OH terephthalate formation in both systems, with or without added Fe^{3+}/EDTA. It was recently demonstrated [34] that tannic acid forms complexes with iron ions that are unable to participate in Fenton reactions.

The 2-OH terephthalate formation is remarkably sensitive to established $HO^{\bullet}$ scavengers, e.g. DMSO, mannitol [17,32], and citrulline [33]. The DMSO inhibitory effect is not necessarily unequivocal, since DMSO is also a very effective inhibitor of CYP2E1-specific monooxygenase reactions [35]. However, two other $HO^{\bullet}$ scavengers, mannitol and citrulline, also inhibited 2-OH terephthalate formation and these are not substrates for cytochromes P450. The pronounced inhibitory effect of catalase suggests that hydrogen peroxide is a sole precursor of the $HO^{\bullet}$ formed by the microsomal enzymes in our system. Therefore, we think that the generation of toxic $HO^{\bullet}$ by microsomal enzymes is completely dependent on two factors: (a) rate of hydrogen peroxide production, and (b) availability of iron ions for the Fenton reaction.

There is considerable interest at present in the comparative evaluation of various intracellular organelles and whole cells as potential sources of ROS production especially as the chemical environment surrounding these cells changes [2,4]. From this point of view the method described here using terephthalate as an iron-dependent specific trap for $HO^{\bullet}$ may be useful in studies of cell derived oxygen activation associated with free radical formation.

Acknowledgments

We thank Professors Gisela Witz and Michael Iba for helpful support and critical comments. The authors acknowledge the kind donation of 2-OH terephthalate by Professor Lewis Kirschenbaum.

References

[1] Halliwell B. Role of free radicals in the neurodegenerative diseases: therapeutic implications for antioxidant treatment. Drugs Aging 2001;18:685–716.

[2] Moskovitz J, Yim MB, Chock PB. Free radicals and disease. Arch-BiochemBiophys 2002;397:354–9.

[3] Pryor WA. Oxy-radicals and related species: their formation, lifetimes, and reactions. Annu Rev Physiol 1986;48:657–67.

[4] Welch KD, Davis TZ, Van Eden ME, Aust SD. Deleterious iron-mediated oxidation of biomolecules. Free Radic Biol Med 2002; 32:577–83.

[5] Qian SY, Buettner GR. Iron and dioxygen chemistry is an important route to initiation of biological free radical oxidations: an electron paramagnetic resonance spin trapping study. Free Radic Biol Med 1999;26:1447–56.

[6] Liochev SI. The mechanism of "Fenton-like" reactions and their importance for biological systems: a biologist's view. Met Ions Biol Syst 1999;36:1–39.

[7] Kehrer JP. The Haber–Weiss reaction and mechanisms of toxicity. Toxicology 2000;149:43–50.

[8] Bondy SC, Naderi S. Contribution of hepatic cytochrome P450 systems to the generation of reactive oxygen species. Biochem Pharmacol 1994;48:155–9.

[9] Ingelman-Sundberg M, Kaur H, Terelius Y, Persson JO, Halliwell B. Hydroxylation of salicylate by microsomal fractions and cytochrome P-450. Lack of production of 2,3-dihydroxybenzoate unless hydroxyl radical formation is permitted. Biochem J 1991;276: 753–7.

[10] Cederbaum AI. Oxygen radical generation by microsomes: role of iron and implications for alcohol metabolism and toxicity. Free Radic Biol Med 1989;7:559–67.

[11] Serron SC, Dwivedi N, Backes WL. Ethylbenzene induces microsomal oxygen free radical generation: antibody-directed characterization of the responsible cytochrome P450 enzymes. Toxicol Appl Pharmacol 2000;164:305–11.

[12] Jakubowski W, Bartosz G. 2,7-dichlorofluorescin oxidation and reactive oxygen species: what does it measure? Cell Biol Int 2000;24:757–60.

[13] Liochev SI, Fridovich I. Copper, zinc superoxide dismutase as a univalent NO(−) oxidoreductase and as a dichlorofluorescin peroxidase. J Biol Chem 2001;276:35253–7.

[14] Rota C, Fann YC, Mason RP. Phenoxyl free radical formation during the oxidation of the fluorescent dye 2′,7′-dichlorofluorescein by horseradish peroxidase. Possible consequences for oxidative stress measurements. J Biol Chem 1999;274:28161–8.

[15] Chen C, Thakker DR. The fallacy of using adrenochrome reaction for measurement of reactive oxygen species formed during cytochrome p450-mediated metabolism of xenobiotics. J Pharmacol Exp Ther 2002;300:417–20.

[16] Strolin-Benedetti M, Brogin G, Bani M, Oesch F, Hengstler JG. Association of cytochrome P450 induction with oxidative stress in vivo as evidenced by 3-hydroxylation of salicylate. Xenobiotica 1999;29:1171–80.

[17] Yang XF, Guo XQ. Fe(II)-EDTA chelate-induced aromatic hydroxylation of terephthalate as a new method for the evaluation of hydroxyl radical-scavenging ability. Analyst 2001;126:928–32.

[18] Tosaki A, Bagchi D, Pali T, Cordis GA, Das DK. Comparisons of ESR and HPLC methods for the detection of $OH^{\bullet}$ radicals in ischemic/reperfused hearts. A relationship between the genesis of free radicals and reperfusion arrhythmias. Biochem Pharmacol 1993;45:961–9.

[19] Li B, Gutierrez PL, Blough NV. Trace determination of hydroxyl radical in biological systems. Anal Chem 1997;69:4295–302.

[20] Montgomery J, Ste-Marie L, Boismenu D, Vachon L. Hydroxylation of aromatic compounds as indices of hydroxyl radical production: a cautionary note revisited. Free Radic Biol Med 1995;19:927–33.

[21] Acworth IN, Bogdanov MB, McCabe DR, Beal MF. Estimation of hydroxyl free radical levels in vivo based on liquid chromatography with electrochemical detection. Methods Enzymol 1999;300: 297–313.

[22] Misra HP, Fridovich I. The univalent reduction of oxygen by reduced flavins and quinones. J Biol Chem 1972;247:188–92.

[23] Mishin V, Pokrovsky A, Lyakhovich VV. Interactions of some acceptors with superoxide anion radicals formed by the NADPH-specific flavoprotein in rat liver microsomal fractions. Biochem J 1976;154:307–10.

[24] Mark G, Tauber A, Laupert R, Schuchmann HP, Schulz D, Mues A, et al. OH-radical formation by ultrasound in aqueous solution—Part II: terephthalate and Fricke dosimetry and the influence of various conditions on the sonolytic yield. Ultrason Sonochem 1998;5:41–52.

[25] Saran M, Summer KH. Assaying for hydroxyl radicals: hydroxylated terephthalate is a superior fluorescence marker than hydroxylated benzoate. Free Radic Res 1999;31:429–36.

[26] Qu X, Kirschenbaum LJ, Borish ET. Hydroxyterephthalate as a fluorescent probe for hydroxyl radicals: application to hair melanin. Photochem Photobiol 2000;71:307–13.

[27] Barreto JC, Smith GS, Strobel NH, McQuillin PA, Miller TA. Terephthalic acid: a dosimeter for the detection of hydroxyl radicals in vitro. Life Sci 1995;56:PL89–96.

[28] Thomas PE, Bandiera S, Maines SL, Ryan DE, Levin W. Regulation of cytochrome P-450j, a high-affinity *N*-nitrosodimethylamine demethylase, in rat hepatic microsomes. Biochemistry 1987;26:2280–9.

[29] Brown RE, Jarvis KL, Hyland KJ. Protein measurement using bicinchoninic acid: elimination of interfering substances. Anal Biochem 1989;180:136–9.

[30] Thompson JA, Yumibe NP. Mechanistic aspects of cytochrome P450-hydroperoxide interactions: substituent effects on degradative pathways. Drug Metab Rev 1989;20:365–78.

[31] Yu XC, Liang C, Strobel HW. Kinetics of substrate reaction in the course of hydroperoxide-mediated inactivation of cytochrome P450 1A1. Biochemistry 1996;35:6289–96.

[32] Zhang Z, Goldstein BD, Witz G. Iron-stimulated ring-opening of benzene in a mouse liver microsomal system. Mechanistic studies and formation of a new metabolite. Biochem Pharmacol 1995;50: 1607–17.

[33] Akashi K, Miyake C, Yokota A. Citrulline, a novel compatible solute in drought-tolerant wild watermelon leaves, is an efficient hydroxyl radical scavenger. FEBS Lett 2001;508:438–42.

[34] Lopes GK, Schulman HM, Hermes-Lima M. Polyphenol tannic acid inhibits hydroxyl radical formation from Fenton reaction by complexing ferrous ions. Biochim Biophys Acta 1999;1472:142–52.

[35] Nishimura Y, Kurata N, Iwase M, Li H, Yasuhara H. The effects of organic solvents on trimethadione *N*-demethylation in rats. Res Commun Mol Pathol Pharmacol 1999;104:229–39.