Title
Evaluation of the probe 2',7'-dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress.

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The endeavor undertaken in this study was to investigate the reactive oxygen species involved in the oxidation of are reported to oxidize DCFH (2), results on the ability ever, it remains unclear which reactive oxygen species are and is enzymatically hydrolyzed by intracellular esterases of other reactive oxygen species such as superoxide anion. Iron-peroxide-induced oxidation of DCFH was partially inhibited by catalase but not by horseradish peroxidase. Nonchelated iron-peroxide oxidation of DCFH was partially inhibited by several hydroxyl radical scavengers, but was independent of the scavenger concentration, and this suggests that free hydroxyl radical is not involved in the oxidation of DCFH in this system. Superoxide anion did not directly oxidize DCFH. Data suggest that $H_2O_2$-$Fe^{2+}$-derived oxidant is mainly responsible for the nonenzymatic oxidation of DCFH. In addition, peroxidase alone and oxidants formed during the reduction of $H_2O_2$ by peroxidase oxidize DCFH. Since DCFH oxidation may be derived from several reactive intermediates, interpretation of specific reactive oxygen species involved in biological systems should be approached with caution. However, DCFH remains an attractive probe as an overall index of oxidative stress in toxicological phenomena.

**Introduction**

The use of dichlorofluorescin (DCFH) as a measure of reactive oxygen species was studied in aqueous media. Hydrogen peroxide oxidized DCFH to fluorescent dichlorofluorescein (DCF), and the oxidation was amplified by the addition of ferrous iron. Hydrogen peroxide-induced DCF formation in the presence of ferrous iron was completely inhibited by deferoxamine and partially inhibited by ethylenediaminetetraacetic acid, but was augmented by diethylenetriaminepentaacetic acid. Iron-peroxide-induced oxidation of DCFH was partially inhibited by catalase but not by horseradish peroxidase. Nonchelated iron-peroxide oxidation of DCFH was partially inhibited by several hydroxyl radical scavengers, but was independent of the scavenger concentration, and this suggests that free hydroxyl radical is not involved in the oxidation of DCFH in this system. Superoxide anion did not directly oxidize DCFH. Data suggest that $H_2O_2$-$Fe^{3+}$-derived oxidant is mainly responsible for the nonenzymatic oxidation of DCFH. In addition, peroxidase alone and oxidants formed during the reduction of $H_2O_2$ by peroxidase oxidize DCFH. Since DCFH oxidation may be derived from several reactive intermediates, interpretation of specific reactive oxygen species involved in biological systems should be approached with caution. However, DCFH remains an attractive probe as an overall index of oxidative stress in toxicological phenomena.

**Experimental Procedures**

**Chemicals.** 2',7'-Dichlorofluorescin diacetate (DCFH-DA) was purchased from Molecular Probes, Inc. (Eugene, OR). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). All solutions were prepared fresh and used immediately for all assays.

**Preparation of Dichlorofluorescin.** DCFH-DA was prepared from DCFH-DA by the method of Cathcart et al. (2) by mixing 0.5 mL of 1.0 mM DCFH-DA in methanol with 2.0 mL of 0.01 N NaOH. This deesterification of DCFH-DA proceeded at room temperature for 30 min, and the mixture was then neutralized with 10 mL of 25 mM NaOH, pH 7.4. This solution was kept on ice in the dark until use.

**Assay for Oxygen Reactive Species Formation.** All reactions were performed in 40 mM Tris-HCl, pH 7.4, in a total volume of 2.0 mL that contained 50 µL of the DCFH solution. Reactions were carried out at 37 °C and started by the addition of one of the following mixtures: $H_2O_2$, 10–100 µM; $H_2O_2$ (10 µM) + $Fe^{3+}$ (10 µM) (from FeCl3); $H_2O_2$ (10 µM) + $Fe^{2+}$ (10 µM) [from FeSO4 or NH4Fe(SO4)2]. $Fe^{3+}$ (EDTA) complex was prepared from FeCl3 by mixing fresh $Fe^{3+}$ (2 mM) with EDTA (2.2 mM), of which 20 µL was added to the reaction mixture. An initial fluorescence reading was obtained 5–15 min after the addition of the above mixtures. Studies designed to inhibit the oxidation DCFH to DCF contained final concentrations of one of the following agents: deferoxamine mesylate (deferoxamine), 1 mM; ethylenediaminetetraacetic acid (EDTA), 1 mM; diethylenetriaminepentaacetic acid (DBTAPAC), 1 mM; catalase, 104.6 units/mL; SOD, 11 µg/mL; mannitol, 1–10 mM; formate, 5 mM; ethanol, 5 mM; dimethyl sulfoxide (DMSO), 1–5 mM. Fluorescence was monitored on a Perkin Elmer spectrofluorometer either LS-5 or LS-50, with excitation wavelength at 488 nm (bandwidth 3 nm) and emission wavelength 525 nm (bandwidth 20 nm). The cuvette holder was thermostatically maintained at 37 °C. Autofluores-
with reactions that were required to be carried out at 37 °C. A strong oxidizing agent. The reaction of H₂O₂ with many organic compounds proceeds at a slow rate (14). The slow rate of DCFH oxidation by H₂O₂ was demonstrated in the method of Baker and Gebicki (12). The incubation mixtures contained DCFH (10–40 µL, final concentration 30–120 µM) and benzoate (20 µL of a 5 mM stock solution), and reactions were started by the simultaneous addition of 10 µM H₂O₂ and 10 µM Fe²⁺. Each mixture was incubated at 37 °C for 5 min, and the reactions were terminated by the addition of 40 µL of 1 mM NaOH. Fluorescence of each solution, a measure of the formation of 2- and 3-hydroxybenzoate, was determined with excitation wavelength 300 nm (bandwidth 3 nm) and emission wavelength 380 nm (bandwidth 20 nm). Autofluorescence, always less than 5%, was accounted for by the inclusion of parallel blanks.

Deacetylation by intracellular esterase(s). Deacetylation of deacylated A fluoresced to DCFH, which is oxidized to fluorescein. The initial studies on DCFH oxidation focused on H₂O₂, an oxidizing agent. The reaction of H₂O₂ with many organic compounds proceeds at a slow rate (14). The slow rate of DCFH oxidation by H₂O₂ was demonstrated in the method of Baker and Gebicki (12). The incubation mixtures contained DCFH (10–40 µL, final concentration 30–120 µM), 2-deoxyribose (20 µL of a 5 mM stock solution), and reactions were started by the simultaneous addition of 10 µM H₂O₂ and 10 µM Fe²⁺. Each mixture was incubated at 37 °C for 5 min, and the reactions were terminated by the addition of 40 µL of 1 mM NaOH. Fluorescence of each solution, a measure of the formation of 2- and 3-hydroxybenzoate, was determined with excitation wavelength 300 nm (bandwidth 3 nm) and emission wavelength 380 nm (bandwidth 20 nm). Autofluorescence, always less than 5%, was accounted for by the inclusion of parallel blanks. Deoxyribose Oxidation. Each reaction (final volume 2.0 mL) was carried out in 25 mM NaHGPO₄ pH 7.4, adapted from the method of Winterbourn (13). The incubation mixtures contained DCFH (10–40 µL, final concentration 30–120 µM), 2-deoxyribose (20 µL of a 5 mM stock solution), 10 µM H₂O₂ and 10 µM Fe²⁺, 1 mL of trichloroacetic acid (2.5% w/v), and 0.5 mL of thiobarbituric acid (TBA) (1 g/100 mL of 0.05 N NaOH). Reactions were carried out at 100 °C for 10 min, and the absorbance of each sample was determined at 532 nm, which measured the oxidation of deoxyribose to TBA reactive products. Parallel blanks were run with each assay.

Results and Discussion

The initial studies on DCFH oxidation focused on H₂O₂, a strong oxidizing agent. The reaction of H₂O₂ with many organic compounds proceeds at a slow rate (14). The slow rate of DCFH oxidation by H₂O₂ was demonstrated in the method of Baker and Gebicki (12). The incubation mixtures contained DCFH (10–40 µL, final concentration 30–120 µM), 2-deoxyribose (20 µL of a 5 mM stock solution), 10 µM H₂O₂ and 10 µM Fe²⁺, 1 mL of trichloroacetic acid (2.5% w/v), and 0.5 mL of thiobarbituric acid (TBA) (1 g/100 mL of 0.05 N NaOH). Reactions were carried out at 100 °C for 10 min, and the absorbance of each sample was determined at 532 nm, which measured the oxidation of deoxyribose to TBA reactive products. Parallel blanks were run with each assay.

Figure 1. Proposed mechanism of entry of DCFH-DA into cells adapted from Bass et al. (11). After DCFH-DA crosses the membrane, it is deesterified to DCFH, which is oxidized to fluorescent DCF by reactive oxygen species.

Figure 2. Concentration-response curve of DCFH oxidation in the presence of H₂O₂. The Fe²⁺ concentration was held constant at 10 µM, and all incubations were performed at 37 °C for 5 min. The data were obtained from three independent experiments and are expressed as the means ± SE.

Table I. Effects of Various Chelating Agents on Hydrogen Peroxide-Iron-Stimulated Formation of Dichlorofluorescein a

| Chelator        | Fluorescence Intensity | Inhibition (%) |
|-----------------|------------------------|----------------|
| None            | 82 ± 14                | 100            |
| Deferoxamine    | 12 ± 2                 | 88 ± 10        |
| EDTA            | 60 ± 11                | 9 ± 1          |
| Deferasirox     | 47 ± 8                 | 32 ± 3         |

 a Incubations (10 min) were carried out in the presence of 100 µM H₂O₂. The data were obtained from three independent experiments and are expressed as the means ± SE.

Table II. Hydrogen Peroxide-Stimulated Formation of Dichlorofluorescein in the Presence of Deferoxamine and Catalase a

| Incubation Medium | Fluorescence Intensity | % Inhibition |
|-------------------|------------------------|--------------|
| H₂O₂              | 845 ± 51               | 100          |
| H₂O₂ + deferoxamine | 13 ± 3                | 99 ± 9       |
| H₂O₂ + catalase   | 278 ± 78               | 9 ± 1        |
| H₂O₂ + catalase + deferoxamine | 194 ± 27   | 77 ± 9       |

 a Incubations (10 min) were performed in the presence of 67.5 mM H₂O₂. The data were obtained from three independent experiments and are expressed as the means ± SE.
The on-order rate constant for the reaction with DCFH is 2.8 \times 10^9 M^{-1} s^{-1}, which is only slightly higher than the predicted rate constant for the reaction of DCFH with 'OH on the order of 10^8 M^{-1} s^{-1}, a value indicating that free 'OH is not involved in the oxidation of DCFH to DCF.

Two alternative assays using the nonchelated H_2O_2 + Fe^{2+} system were employed in which DCFH was used as a scavenger, notably benzoate hydroxylation and deoxyribose oxidation. Results showed that DCFH moderately but significantly inhibited the oxidation of deoxyribose in a concentration-dependent manner (20% at 2 \mu M DCFH, p \leq 0.05), while benzoate hydroxylation was unaltered. These data suggest that in the nonchelated H_2O_2 + Fe^{2+} system the oxidation of DCFH is not derived from free 'OH radical. Alternatively, the carboxyl group of DCFH may bind Fe^{2+} and lead to the formation of site-specific 'OH, which can oxidize DCFH. The site-specific oxidation of DCFH by 'OH would not be expected to be affected by 'OH scavengers. Thus, the evidence to suggest that 'OH is the primary oxidant of DCFH remains equivocal.

To determine whether O_2^- was involved in the oxidation of DCFH, we studied the effects of SOD and a protein without SOD activity, lysozyme, in the xanthine + xanthine oxidase system. SOD did not provide any significant inhibition of DCFH oxidation (Table III). Thus O_2^- may not be involved in the oxidation of DCFH.

Catalase in excess decreased, but did not completely inhibit, the fluorescence intensity resulting from H_2O_2-induced oxidation of DCFH (Table II). The effect of catalase on the oxidation of DCFH even occurred in the presence of deferoxamine. An explanation of this effect on DCFH is based on the decomposition of H_2O_2 by catalase, which proceeds via the formation of compound I (reaction 4), a ferryl type structure with a porphyrin π-catalase + H_2O_2 → catalase-porphyrin^+ + Fe^{IV}=O + H_2O (4)
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agents may involve an induction of peroxidase, in response to low in antioxidant protective agents such as catalase, upon exposure to toxic chemicals (9, 27-29). Toxicology studies using DCFH have reported the following: (1) differences are present in animals as early as 1 h postdose (9, 25); (2) significant differences in brain DCF formation rates in animals pretreated with deferoxamine prior to exposure (13); (3) significant differences in brain DCF formation rates despite reduced oxygen radical formation in the aging rat brain (18). However, it is widely accepted that the brain is by nature low in antioxidant protective agents such as catalase, glutathione peroxidase, and glutathione (10). We have reported that a variety of neurotoxicants increase the oxidation of DCFH in brain subcellular preparations and that these differences are present in animals as early as 1 h postdose (9, 30, 31). It is possible that exposure to these agents may involve an induction of peroxidase, in response to an oxidative stress, and that this may also directly enhance DCFH oxidation. Use of the probe DCFH may provide a link between reactive oxygen species formation in vitro and the formation of such reactive intermediates in the nervous system of the living animal.

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 Formation of Mitochondrial Phospholipid Adducts by Nephrotoxic Cysteine Conjugate Metabolites

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Nephrotoxic cysteine conjugates derived from a variety of halogenated alkenes are enzymatically activated via the β-lyase pathway to yield reactive sulfur-containing metabolites which bind covalently to cellular macromolecules. Mitochondria contain β-lyase enzymes and are primary targets for binding and toxicity. Previously, mitochondrial protein and/or DNA have been considered as molecular targets for cysteine conjugate metabolite binding. We now report that metabolites of nephrotoxic cysteine conjugates form covalent adducts with rat kidney mitochondrial phospholipids. Rat kidney mitochondria were incubated with the 35S-labeled conjugates S-(1,2,2,2-tetrafluoroethyl)-L-cysteine (TFEC), S-(2-chloro-1,1,2-trifluoroethyl)-L-cysteine (CTFC), S-(1,2-dichlorovinyl)-L-cysteine, and S-(1,2,3,4,4-pentachlorobutadienyl)-L-cysteine. Quantitation of metabolite binding to whole mitochondria and to mitochondrial protein and lipid fractions revealed that as much as 42% of the 35S-label associated with the mitochondria was found in the lipid fraction. Total lipids were also extracted from 35S-treated mitochondria and separated by thin-layer chromatography. 35S-Containing metabolites were found in the lipid fractions from mitochondria treated with each of the conjugates. Lipids from both [35S]CTFC- and [35S]-TFEC-treated mitochondria contained major 35S-labeled lipid adducts which had similar mobility by thin-layer chromatography. Fatty acid analysis, 19F and 31P NMR spectroscopy, and mass spectrometric analyses confirmed that the major TFEC and CTFC adducts are thioamides of phosphatidylethanolamine.

Introduction

Halogenated alkenes induce nephrotoxicity after enzymatic conjugation with GSH1 in the liver. The GSH conjugates are metabolized to the corresponding cysteine conjugates and mercapturates during a complex pathway of interorgan disposition (1,2; Figure 1). β-Elimination of a toxic sulfur-containing metabolite from the cysteine conjugate occurs in the kidney via the action of cysteine conjugate β-lyase (3; EC 4.4.1.13). Covalent binding of the reactive sulfur-containing metabolite to cellular macromolecules is presumed to initiate a cascade of events which eventually leads to cell death (4–7) and, in some cases, mutagenesis (8,9). However, the identity of the critical targets for binding and the mechanisms which couple binding to cell death remain unclear. For recent reviews, see refs 9–12.

A considerable amount of evidence implicates the mitochondrion as a primary target for cysteine conjugate toxicity. Mitochondria contain β-lyase enzymes (13–16), and recently, binding of TFEC metabolites to kidney protein was shown to be localized to specific proteins of the mitochondrial fraction in vivo (17). Additionally, metabolism of cysteine conjugates has been shown to result in inhibition of respiration (6,7,13,18–21), inhibition of 2-oxoacid dehydrogenases (19), isocitrate dehydrogenase, and succinate dehydrogenase (21), loss of lipoyl de-