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Journal

Chemical Research in Toxicology, 5(2)

ISSN

0893-228X

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Publication Date

1992-03-01

DOI

10.1021/tx00026a012

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Evaluation of the Probe 2',7'-Dichlorofluorescein as an Indicator of Reactive Oxygen Species Formation and Oxidative Stress

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Received December 10, 1990

The use of dichlorofluorescein (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^{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 2',7'-dichlorofluorescein diacetate (DCFH-DA)¹ was first described as a fluorometric assay of hydrogen peroxide (H_2O_2) in the presence of peroxidase by Keston and Brandt (1). Additionally, DCFH-DA has been used to measure the formation of lipid hydroperoxides and has been proposed as an alternative to traditional lipid peroxidative techniques such as the thiobarbituric acid method (2). Several studies dealing with the effects of reactive oxygen species in cell culture (3-5) and in aqueous systems (6) have employed DCFH-DA. Recently, DCFH-DA has been used to investigate the role of reactive oxygen species in various mechanisms of neurotoxicity (7-10).

Studies using DCFH-DA in either intact cells or sub-cellular preparations have been based on the premise that the nonpolar, nonionic DCFH-DA crosses cell membranes and is enzymatically hydrolyzed by intracellular esterases to nonfluorescent DCFH (Figure 1) (11). In the presence of reactive oxygen species, DCFH is rapidly oxidized to highly fluorescent 2',7'-dichlorofluorescein (DCF). However, it remains unclear which reactive oxygen species are responsible for the oxidation of DCFH. While H_2O_2 and several lipid hydroperoxides, in the presence of hematin, are reported to oxidize DCFH (2), results on the ability of other reactive oxygen species such as superoxide anion ($O_2^{\cdot-}$) and hydroxyl radical ($\cdot OH$) to stimulate the formation of DCF are inconclusive. Conflicting findings regarding the inhibition of the oxidation of DCFH by superoxide dismutase (SOD) have been reported (4, 7, 11). The endeavor undertaken in this study was to investigate the reactive oxygen species involved in the oxidation of DCFH to DCF.

Experimental Procedures

Chemicals. 2',7'-Dichlorofluorescein 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 Dichlorofluorescein. DCFH 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 NaH_2PO_4 , 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 $FeCl_3$); H_2O_2 (10 μM) + Fe^{2+} (10 μM) [from $FeSO_4$ or $NH_4Fe(SO_4)_2$]. Fe^{3+} (EDTA) complex was prepared from $FeCl_3$ 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 (DETAPAC), 1 mM; catalase, 104.6 units/mL; SOD, 11 $\mu g/mL$; 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-

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¹ Abbreviations: DCFH, 2',7'-dichlorofluorescein; DCF, 2',7'-dichlorofluorescein; DCFH-DA, 2',7'-dichlorofluorescein diacetate; EDTA, ethylenediaminetetraacetic acid; DETAPAC, diethylenetriaminepentaacetic acid; DMSO, dimethyl sulfoxide; TBA, thiobarbituric acid; HRP, horseradish peroxidase; SOD, superoxide dismutase; HEPES, N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid.

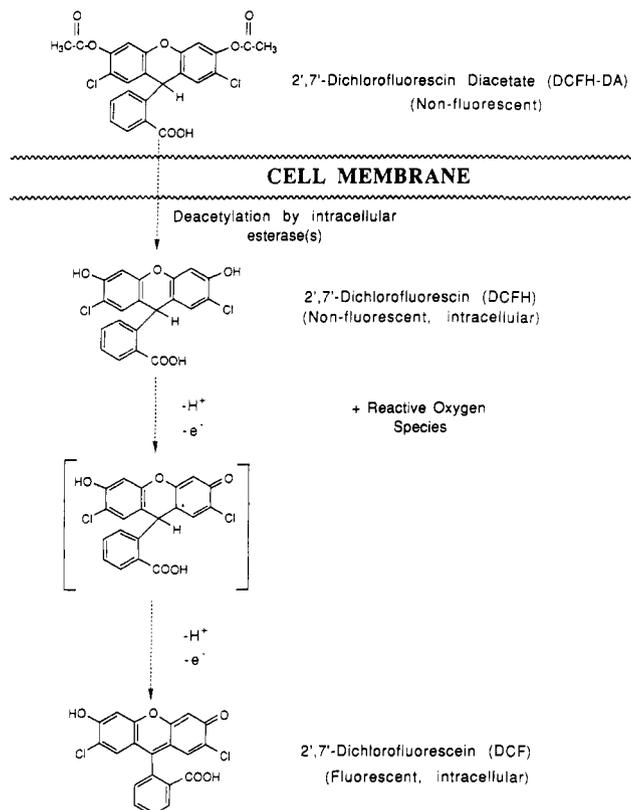


Figure 1. Proposed mechanism of entry of DCFH-DA into cells adapted from Bass et al. (11). After DCFH-DA crosses the membrane, it is deesterified to DCFH, which is oxidized to fluorescent DCF by reactive oxygen species.

cence was corrected for by the inclusion in each experiment of parallel blanks (DCFH alone in buffer). The correction for autofluorescence was always less than 10% of the total.

Benzoate Hydroxylation. All reactions (final volume 2.0 mL) were performed in 25 mM NaH₂PO₄, pH 7.4, adapted from 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 390 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 NaH₂PO₄, 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 5 mM stock solution), 10 μM H₂O₂ and 10 μM Fe²⁺, 1 mL of trichloroacetic acid (2.8% 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 original studies by Cathcart and co-workers (2), who employed hematin in deaerated phosphate buffer solutions, with reactions that were required to be carried out at 50 °C for 50 min. Under those conditions, the H₂O₂-induced

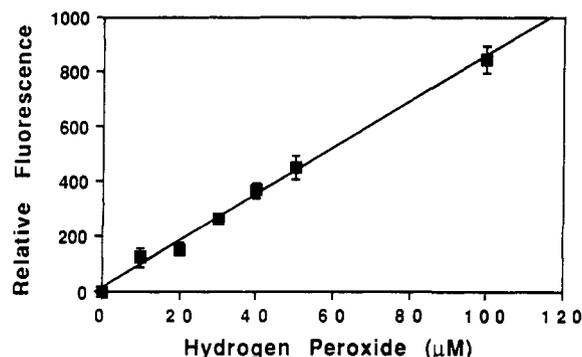


Figure 2. Concentration-response curve of DCFH oxidation in the presence of H₂O₂. The Fe²⁺ concentration was held constant at 10 μM, and all incubations were performed at 37 °C for 5 min. The data were obtained from three independent experiments and are expressed as the means ± SE.

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

chelator	fluorescence intensity		
	+0 μM Fe	+10 μM Fe ²⁺	+10 μM Fe ³⁺
none	82 ± 14	671 ± 47	88 ± 10
deferoxamine	12 ± 2	9 ± 3	12 ± 1
EDTA	69 ± 11	293 ± 20	90 ± 19
DETAPAC	47 ± 8	>999	32 ± 3

^aIncubations (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
H ₂ O ₂	845 ± 51	
H ₂ O ₂ + deferoxamine	13 ± 3	98
H ₂ O ₂ + catalase	278 ± 78	67
H ₂ O ₂ + catalase + deferoxamine	194 ± 27	77

^aIncubations (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.

oxidation of DCFH to DCF was linear. The present study, using ambient atmospheric conditions at 37 °C and incubation times of only 5–15 min, demonstrated good linearity ($r = 0.995$) of DCFH oxidation to DCF by H₂O₂ + Fe²⁺ (Figure 2). Addition of known concentrations of iron (Fe²⁺) to a solution containing H₂O₂ resulted in the formation of an intermediate capable of accelerating the oxidation of DCFH, while addition of iron as Fe³⁺ was similar to H₂O₂ alone (Table I). The buffer employed in the reactions of iron-H₂O₂ was Tris-HCl.

We have recently investigated the effects of several different buffer systems on DCF formation in a rat synaptosomal preparation (7). We demonstrated that optimal formation of DCF was obtained in the Tris buffer in comparison to HEPES and phosphate buffer systems. Tris is both a weak chelator and scavenger of [•]OH; however, it has modest effects on Fe²⁺ autoxidation (15).

We next addressed the potential role of iron chelation in this system by employing various iron chelators. Deferoxamine, a potent and relatively inert chelating agent with a high affinity ($K_d = 10^{-31}$) for Fe³⁺ (14, 16), inhibited the oxidation of DCFH in the presence of large quantities of H₂O₂ without the addition of iron (Table II). The presence of 10 μM Fe²⁺ or 10 μM Fe³⁺ without H₂O₂ did not result in DCFH oxidation (data not shown). In the

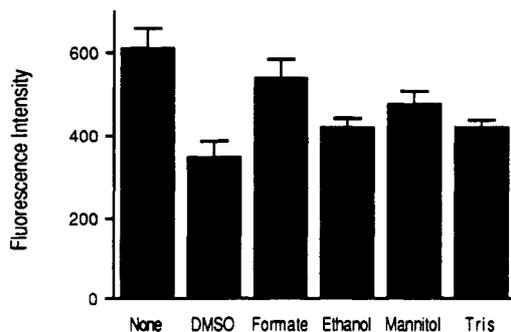
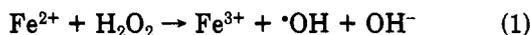


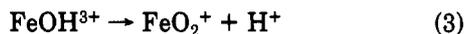
Figure 3. Effects of hydroxyl radical scavengers on hydrogen peroxide-ferrous iron-induced formation of DCF. The concentration of each scavenger was 5 mM. Data were obtained from three independent experiments and are expressed as the means \pm SE.

presence of $\text{H}_2\text{O}_2 + \text{Fe}^{3+}$ the oxidation of DCFH was not affected by EDTA; however, it was inhibited by DETAPAC (Table I). In the presence of $\text{H}_2\text{O}_2 + \text{Fe}^{2+}$, EDTA partially prevented the formation of DCF, and surprisingly DETAPAC enhanced DCFH oxidation, in which fluorescence intensity readings actually exceeded the upper limits of detection (Table I). There is no ready explanation for the rapid conversion of DCFH to DCF by DETAPAC in this system, although it has been reported that Fe^{2+} -DETAPAC chelates may actually catalyze the formation of ethylene gas from *S*-methyl 2-ketothiobutyrate, phenol from benzene, and the formation of the hydroxyl spin adduct of 5,5-dimethyl-1-pyrroline *N*-oxide (17).

The reaction of H_2O_2 with Fe^{2+} is thought to proceed by two pathways:



The $\cdot\text{OH}$ generated in reaction 1 is known as an extremely reactive oxidizing agent that is believed to cause damage to a variety of cell constituents (18). This reaction is a Fenton redox chain reaction which is indirectly inhibited by EDTA (19). This is in agreement with our observations. Several reports have also suggested the formation of an iron-oxygen complex from the reaction of H_2O_2 with nonchelated Fe^{2+} , forming the ferryl ion (FeO^{2+} or FeOH^{3+}) in the reactions (20, 21):



To investigate whether the oxidation of DCFH was due to $\cdot\text{OH}$ formed from reaction 1, various $\cdot\text{OH}$ scavengers, listed in order of reactivity toward $\cdot\text{OH}$ from the reported second-order rate constants, were tested in the reaction mixture. In the $\text{H}_2\text{O}_2 + \text{Fe}^{2+}$ system, DMSO, ethanol, mannitol, and Tris provided partial inhibition, while formate ($k_{\text{OH}} = 2.8 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$) was without effect (Figure 3). However, increasing the concentration of these scavengers 10-fold did not provide any further inhibition of DCFH oxidation. For example, mannitol in the range of 5–10 mM showed a 22–26% inhibition of DCFH oxidation, while scavengers such as Tris, which has a reported second-order rate constant for the reaction with $\cdot\text{OH}$ of $1.3 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$, showed greater inhibition than mannitol ($k_{\text{OH}} = 1.8 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$) at equimolar concentrations (13). Furthermore, using eq A described by Winterbourn (13),

$$\frac{F}{1-F} = \frac{k_S[S]}{k_D[D]} \quad (A)$$

where the percent inhibition $F = 42\%$, k_S for DMSO is 7.1

Table III. Effect of Superoxide Dismutase on Xanthine + Xanthine Oxidase-Stimulated Formation of Dichlorofluorescein^a

incubation medium	fluorescence intensity
xanthine + xanthine oxidase	106 \pm 10
xanthine + xanthine oxidase + SOD	87 \pm 11
xanthine + xanthine oxidase + lysozyme	83 \pm 10

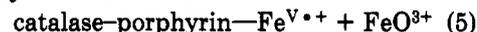
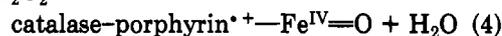
^aIncubations (10 min) were performed in the presence of xanthine (50 μM) + xanthine oxidase (5 milliunits/mL) and contained SOD (11 $\mu\text{g}/\text{mL}$) or lysozyme (11 $\mu\text{g}/\text{mL}$). The data were obtained from three independent experiments and are expressed as the means \pm SE.

$\times 10^9$ at concentration $S = 5 \text{ mM}$, and the estimated concentration D of the detector DCFH = 60 μM , the predicted rate constant for the reaction of DCFH with $\cdot\text{OH}$ is on the order of $10^{11} \text{ M}^{-1} \text{ s}^{-1}$, a value indicating that free $\cdot\text{OH}$ is not involved in the oxidation of DCFH to DCF.

Two alternative assays using the nonchelated $\text{H}_2\text{O}_2 + \text{Fe}^{2+}$ 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 μM DCFH, $p \leq 0.05$), while benzoate hydroxylation was unaltered. These data suggest that in the nonchelated $\text{H}_2\text{O}_2 + \text{Fe}^{2+}$ system the oxidation of DCFH is not derived from free $\cdot\text{OH}$ radical. Alternatively, the carboxyl group of DCFH may bind Fe^{2+} and lead to the formation of site-specific $\cdot\text{OH}$, which can oxidize DCFH. The site-specific oxidation of DCFH by $\cdot\text{OH}$ would not be expected to be affected by $\cdot\text{OH}$ scavengers. Thus, the evidence to suggest that $\cdot\text{OH}$ is the primary oxidant of DCFH remains equivocal.

To determine whether $\text{O}_2^{\cdot-}$ 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 $\text{O}_2^{\cdot-}$ 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 + $\text{H}_2\text{O}_2 \rightarrow$



cation radical (14, 22, 23). The ability of catalase-iron complexes to carry out these types of oxidations has been previously reported (14, 22, 23).

Horseradish peroxidase (HRP, type II) also oxidized DCFH in presence and absence of H_2O_2 (Figure 4). A comparison of the concentration-response curves demonstrated that HRP, in the presence of 10 μM H_2O_2 , was twice as potent in its ability to oxidize DCFH than HRP alone. These data indicate that, in the presence of H_2O_2 , DCFH serves directly as a reducing substrate for HRP in a concentration-dependent manner.

This study suggests that the oxidation of DCFH to DCF in nonbiological systems is iron-peroxide dependent and is related to the oxidation state of iron. DCFH oxidation appears to be mediated not only by H_2O_2 -iron complexes,

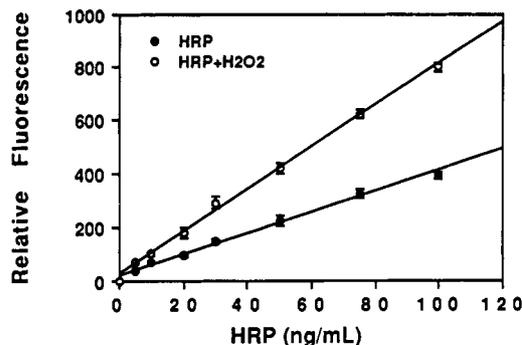


Figure 4. Concentration-response curve of DCFH oxidation in the presence of horseradish peroxidase (HRP). The H_2O_2 concentration was held constant at $10 \mu\text{M}$, and all incubations were performed at 37°C for 2 min. The data were obtained from three independent experiments and are expressed as the means \pm SE.

the role of which in biological systems is not yet completely understood (24–26), but also by peroxidase and peroxidase-iron complexes. In addition, free $\cdot\text{OH}$ may not be responsible for DCFH oxidation in the nonchelated $\text{H}_2\text{O}_2 + \text{Fe}^{2+}$ system. Therefore, due to the broad range of reactive species which oxidize DCFH, caution should be taken when interpreting data in the context of a particular reactive species.

DCFH's lack of specificity toward reactive oxygen species is what makes it potentially appealing as a probe in studying toxicological phenomena. Numerous studies exist demonstrating the utility of DCFH as an index of free radical reactions that take place in the living animal following exposure to toxic chemicals (9, 27–29). Toxicology studies using DCFH have reported the following: (1) significant differences in brain DCF formation rates between vitamin E deficient mice and those provided normal diets (8); (2) significant differences in DCF formation rates in brain regions known to be selectively vulnerable to the neurotoxic organometals methylmercury and trimethyltin (9); (3) significant differences in brain DCF formation rates in animals pretreated with deferoxamine prior to exposure to methylmercury (30); (4) significant differences in brain DCF formation rates in animals exposed to toluene, a neurotoxic organic solvent, in contrast to no observable alterations in brain DCF formation in animals treated with the structurally related solvent benzene (31).

The present study demonstrated that DCFH may be oxidized by peroxidases alone. This raises the possibility that the presence of peroxidases *in vivo* would be sufficient to confound data generated from biological systems. 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.

Acknowledgment. We thank Dr. Noman A. LeBel and the reviewers for their helpful comments which aided the preparation of this paper. This work was supported by NIH Grant AA 8281.

Registry No. 2',7'-Dichlorofluorescein, 106070-31-9; hydrogen peroxide, 7722-84-1; iron, 7439-89-6; hydroxyl radical, 3352-57-6;

superoxide anion, 11062-77-4; 2',7'-dichlorofluorescein, 76-54-0.

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Formation of Mitochondrial Phospholipid Adducts by Nephrotoxic Cysteine Conjugate Metabolites

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Received September 6, 1991

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-(1,1,2,2-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-

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¹ Abbreviations: S-(1,2-dichlorovinyl)-L-cysteine, DCVC; S-(2-chloro-1,1,2-trifluoroethyl)-L-cysteine, CTFC; S-(1,1,2,2-tetrafluoroethyl)-L-cysteine, TFEC; S-(1,2,3,4,4-pentachlorobutadienyl)-L-cysteine, PCBC; thin-layer chromatography, TLC; phosphatidylethanolamine, PE; (chlorofluorothioacetamido)phosphatidylethanolamine, CFTA-PE; (difluorothioacetamido)phosphatidylethanolamine, DFTA-PE; glutathione, GSH; trichloroacetic acid, TCA; fast atom bombardment, FAB; (ethylenedinitrilo)tetraacetic acid, EDTA.