Phenoxyl Free Radical Formation during the Oxidation of the Fluorescent Dye 2',7'-Dichlorofluorescein by Horseradish Peroxidase

POSSIBLE CONSEQUENCES FOR OXIDATIVE STRESS MEASUREMENTS*

(Received for publication, May 24, 1999, and in revised form, July 14, 1999)

Cristina Rota, Yang C. Fann‡, and Ronald P. Mason§

From the Free Radical Metabolite Section, Laboratory of Pharmacology and Chemistry, and Information Technology Support Services, NIEHS, National Institutes of Health, Research Triangle Park, North Carolina 27709

The oxidation of the fluorescent dye 2’,7’-dichlorofluorescein (DCF) by horseradish peroxidase was investigated by optical absorption, electron spin resonance (ESR), and oxygen consumption measurements. Spectrophotometric measurements showed that DCF can be oxidized either by horseradish peroxidase-compound I or -compound II with the obligate generation of the DCF phenoxyl radical (DCF•). This one-electron oxidation was confirmed by ESR spin-trapping experiments. DCF• oxidizes GSH, generating the glutathione thiol radical (GS•), which was detected by the ESR spin-trapping technique. In this case, oxygen was consumed by a sequence of reactions initiated by the GS• radical. Similarly, DCF• oxidized NADH, generating the NAD+ radical that reduced oxygen to superoxide (O2•−), which was also detected by the ESR spin-trapping technique. Superoxide dismutated to generate H2O2, which reacted with horseradish peroxidase, setting up an enzymatic chain reaction leading to H2O2 production and oxygen consumption. In contrast, when ascorbic acid reduced the DCF phenoxyl radical back to its parent molecule, it formed the unreactive ascorbate anion radical. Clearly, DCF• catalytically stimulates the formation of reactive oxygen species in a manner that is dependent on and affected by various biochemical reducing agents. This study, together with our earlier studies, demonstrates that DCFH cannot be used conclusively to measure superoxide or hydrogen peroxide formation in cells undergoing oxidative stress.

2’,7’-Dichlorofluorescein (DCFH) is widely used to measure oxidative stress in cells. The diacetate form of DCFH enters the cell and is hydrolyzed by intracellular esterases to liberate DCF. Upon reaction with oxidizing species, the highly fluorescent compound 2’,7’-dichlorofluorescein (DCF) is formed. The fluorescence intensity can be measured and is the basis of the popular cellular assay for oxidative stress (1).

Although this assay is commonly used, there are many controversies regarding its real validity. It is not clear which oxidative species is responsible for the oxidation of DCFH. Furthermore, there are some disagreements in the literature concerning the effect of superoxide dismutase on oxidative stress in cells monitored by the DCF fluorometric assay. Some studies report an inhibitory effect by superoxide dismutase (2–15), while almost the same number of studies report no effect (16–26). A recent paper by LeBel et al. (27) mentioned that the interpretation of specific reactive oxygen species involved in the oxidation of DCFH to DCF in biological systems should be approached with caution. It has also been demonstrated that the photoreduction of DCF results in the formation of the DCF semiquinone free radical (DCF••), which, under aerobic conditions, is oxidized by oxygen to its parent dye, DCF, concomitantly forming superoxide radical (28). Moreover, it was reported that DCFH, upon reacting with horseradish peroxidase-compound I or -compound II, was oxidized to DCF••, which was subsequently air-oxidized to DCF with the concurrent generation of superoxide radical (29).

In the present work, the reaction of DCF with horseradish peroxidase in the presence of reduced glutathione or NADH was studied to continue our investigation of the DCF fluorometric assay. We employed the ESR spin-trapping technique and measured the oxygen consumption to evaluate free radical formation during the reactions along with the formation of compound I and compound II monitored by UV-visible spectrophotometry. Our results indicate that when DCF reacts with compound I and compound II, DCF is oxidized to the phenoxyl free radical DCF•, reducing the respective horseradish peroxidase enzyme intermediates (Scheme 1). In the presence of a reducing agent, such as GSH or NADH, DCF• is then reduced back to DCF with the formation of GS• or NAD•, respectively, and the subsequent generation of superoxide.

EXPERIMENTAL PROCEDURES

Chemicals—Horseradish peroxidase type VI-A (EC 1.11.1.7), porcine liver esterase (EC 3.1.1.1), diethylenetriamine pentaacetic acid, DCF, and the spin trap 2-methyl-2-nitrosopropane (MNP) were purchased from Sigma. Tris and Chelex 100 resin were purchased from Bio-Rad. Catalase (from beef liver, 65,000 units/mg) (EC 1.11.1.6) and superoxide dismutase (from bovine erythrocytes, 5,000 units/mg) (EC 1.15.1.1) were purchased from Roche Molecular Biochemicals. Hydrogen peroxide (30%) was purchased from Fisher.

All of the reactions were carried out in a 50 mM Tris buffer adjusted to pH 7.4 with hydrochloric acid. The Tris-HCl buffer was treated with Chelex 100 resin to remove traces of transition metal ions and contained 50 μM diethylenetriamine pentaacetic acid to minimize the possibility of trace metal catalysis. DCF was dissolved with methanol to form a 12.5 mM solution, which was then diluted to 500 μM with a pH 7.4 Tris-HCl buffer (i.e. methanol-buffer solution). In the experiments...
FIG. 1. Absorption spectra changes during the reaction of DCF, H₂O₂, and horseradish peroxidase. Horseradish peroxidase (1 μM) in Tris-HCl buffer is represented by scan 0. Scan 1 was taken immediately after the addition of 1 μM DCF; scans 2 and 3 were taken immediately and 2 min after the subsequent addition of 1 μM H₂O₂, respectively. All spectra were recorded at a rate of 5 nm/s, yielding a complete spectrum in 50 s.

shown in Fig. 2, a 25 mM DCF methanol solution was directly added to the samples. MNP was prepared in methanol and kept in the dark during the experiments. The spin trap 5,5-dimethyl-1-pyrroline N-oxide (DMPO) was purchased from Sigma, purified by vacuum sublimation at ambient temperature, and stored at -70 °C until use. 2,7'-Dichlorodihydrofluorescein diacetate (also known as 2',7'-dichlorodihydrofluorescein diacetate, DCFH-DA) was purchased from Molecular Probes, Inc. (Eugene, OR). DCFH-DA was enzymatically deesterified; i.e. 500 μM DCFH-DA in a methanol-buffer solution reacted with esterase (100 units) in the dark at room temperature for 1 h at neutral pH. Concentrations of horseradish peroxidase were determined by using the extinction coefficient ε = 102 nmol⁻¹ cm⁻¹ at 403 nm (30). Stock concentrations of H₂O₂ in deionized water were determined by using the extinction coefficient ε = 43.6 nmol⁻¹ cm⁻¹ at 240 nm (31).

DCF and DCFH were stored and kept from room light. Sample preparation and experiments were performed in the dark. All reactions were initiated with the addition of horseradish peroxidase. In the samples where DCFH or DCF was omitted, an equivalent volume of methanol-buffer solution was added.

Electron Spin Resonance Experiments—ESR spectra were recorded on a Bruker ECS-106 ESR spectrometer (Billerica, MA) operating at 9.77 GHz with a modulation frequency of 50 kHz equipped with a TM₁₀ cavity. All experiments were performed at room temperature with a 17-mm quartz flat cell. In the experiments described in Figs. 2, 3, and 8, samples were aspirated into the flat cell with ESR data acquisition started approximately 15 s after sample preparations. The data analysis and spectral simulation were performed using programs developed in our laboratory and are available through the Internet. The details of the program are described elsewhere (32). The low energy structure of MNP-DCF radical adduct was obtained using the MOPAC with MM2 minimization.

Horseradish Peroxidase UV-visible Spectra—All optical measurements were carried out with an SLM Aminco DW-2000 UV-visible dual beam spectrophotometer (Urbana, IL). The samples were prepared and recorded in deionized water (used in place of Tris buffer to prevent any reductant in the solution).

Oxygen Consumption Experiments—Oxygen consumption measurements were made using a Clark-type oxygen electrode fitted to a 1.8 ml Gilson sample cell and monitored by a YSI Inc. model 53 oxygen monitor. Oxygen consumption data were recorded by a computer interfaced with a DT2801 Data Translation board connected to the oxygen monitor. All of the experiments were performed at room temperature, and the incubation conditions are described in the figure legends.

RESULTS
The UV-visible spectra of the resting state of compound I and compound II reacting with DCF are shown in Fig. 1. The UV-visible spectrum of the resting state of horseradish perox-

idase (1 μM) is characterized by its maximum absorption at 403 nm (Fig. 1, scan 0). The addition of DCF to horseradish peroxidase solution resulted in a small increase in the absorption at 403 nm due to the fact that DCF has absorption at this frequency (maximum at 502 nm) as shown in Fig. 1, scan 1. The significant decrease in intensity at 502 nm when H₂O₂ was added to the system demonstrated that DCF had been transformed during the reaction of DCF with horseradish peroxidase in the presence of H₂O₂ (Fig. 1, scan 2). In this case, compound I formed by the addition of H₂O₂ to horseradish peroxidase rapidly reacted with DCF to form the DCF radical and compound II, which is characterized by the appearance of an isosbestic point for horseradish peroxidase and its compound II at 412 nm (33). Scan 3 in Fig. 1 shows the conversion of compound II back to the horseradish peroxidase resting state. These results are consistent with the peroxidase-mediated metabolism of DCF to the corresponding DCF radical as shown in Scheme 1.

The ESR spin-trapping technique was employed to characterize the free radical formed during the turnover of horseradish peroxidase with H₂O₂ and DCF in the presence or absence of a reducing agent. When DCF was reacted with horseradish peroxidase and H₂O₂ in the presence of spin trap MNP, an isotropic three-line spectrum with a hyperfine coupling constant of 14.9 G was detected (Fig. 2A). The absence of any hydrogen or chlorine hyperfine couplings in the three-line spectrum excluded these atoms at positions α or β to the nitroxide nitrogen and indicated that the radical was located on a tertiary carbon (34, 35). The structure of the radical adduct based on our ESR data and energy minimization is shown in Fig. 2. The radical formation was completely dependent on the presence of DCF (Fig. 2B). The absence of either horseradish peroxidase or H₂O₂ resulted in a very weak signal (Fig. 2, C and D) consisting, for the most part, of the di-tert-butyl nitroxide from the decomposition of MNP characterized by a hyperfine coupling constant of 17 G (36). This weak signal is also present in the solution of MNP in buffer (Fig. 2E). Based on the characteristics of its ESR signal and the similarity of the hyperfine coupling constant compared with that previously obtained from the trapping of MNP/tyrosyl radical (37), we assigned the radical as the phenoxyl free radical of DCF.

When DCF was incubated with a solution of GSH, H₂O₂, and horseradish peroxidase in the presence of the spin trap DMPO, a four-line ESR spectrum of the DMPO/GS⁺ adduct was detected (Fig. 3A), characterized by hyperfine coupling constants of aN² = 15.15 G and aH¹ = 16.17 G, consistent with those previously reported (38, 39). The same radical adduct was obtained from a system where DCF was omitted, but the ESR spectrum was of lower intensity (Fig. 3B). The radical adduct depended on the presence of GSH or horseradish peroxidase (Fig. 3, C and D). The addition of superoxide dismutase did not have any effect (data not shown), eliminating the possibility of superoxide-dependent glutathione thiol radical formation. However, the addition of catalase completely eliminated the ESR signal (Fig. 3E), indicating the radical adduct formation is H₂O₂-dependent. When H₂O₂ was omitted, a weak spectrum of the DMPO/GS⁺ adduct was detected (Fig. 3F) and was com-
fully suppressed by the addition of catalase, indicating the formation of \( \mathrm{H}_2\mathrm{O}_2 \) via oxidation of GSH by trace metal catalysis.

Moreover, molecular oxygen was consumed in a reaction mixture containing DCF, \( \mathrm{H}_2\mathrm{O}_2 \), and GSH (Fig. 4a). The duration of the fast rate of oxygen consumption strongly depended on the concentration of hydrogen peroxide (Fig. 4a, A and C). When DCF was reacted with GSH, horseradish peroxidase, and \( \mathrm{H}_2\mathrm{O}_2 \), the rate of oxygen consumption was very high for ~1 min, and then it decreased (Fig. 4a, A). This is due to the consumption of \( \mathrm{H}_2\mathrm{O}_2 \) in this first minute, as is demonstrated by the fact that the addition of more \( \mathrm{H}_2\mathrm{O}_2 \) restored the initial rate of oxygen consumption (Fig. 4a, B) until all of the oxygen in the solution was consumed.

When DCF, GSH, or horseradish peroxidase was omitted, no oxygen consumption was observed (Fig. 4b, E–G). However, when ascorbic acid (1 mM) was added to the reaction mixture prior to or after the horseradish peroxidase initiation, the oxygen consumption was completely inhibited (Fig. 4b, H and B, respectively). The same inhibition was obtained even when 100 \( \mu \)M of ascorbate was used (data not shown). In addition, the oxygen consumption was inhibited when DMPO (200 mM) was added prior to or after the horseradish peroxidase initiation (Fig. 4b, D and C, respectively). When superoxide dismutase (100 \( \mu \)g/ml) was added to the reaction mixture before horseradish peroxidase initiation (Fig. 4c, A), the initial rate of oxygen consumption was found to be identical to that of the complete system (Fig. 4c, B). However, in the presence of superoxide dismutase (Fig. 4c, A), the oxygen consumption did not slow after 1 min, in contrast to that of the complete system, but completely stopped approximately 2 min after the horseradish peroxidase initiation (Fig. 4c, A). Apparently, superoxide dismutase, catalyzing the disproportionation of superoxide radicals, facilitated \( \mathrm{H}_2\mathrm{O}_2 \) formation, keeping the consumption rate high for a longer time. The addition of superoxide dismutase 90 s after horseradish peroxidase initiation did completely inhibit the oxygen consumption (Fig. 4c, C). In this case, superoxide dismutase had been added when the \( \mathrm{H}_2\mathrm{O}_2 \) was already consumed, and this slower oxygen consumption came almost completely from a free radical chain reaction that is completely superoxide-dependent. The addition of catalase (150 \( \mu \)g/ml) prior to or after horseradish peroxidase initiation completely inhibited the oxygen consumption (Fig. 4c, D and E).

When added hydrogen peroxide was omitted from the complete system, the rate of oxygen consumption was much slower (Fig. 5a). The addition of more diethylenetriamine pentaacetic acid (from 250 to 800 \( \mu \)M in final concentration) slowed the rate of oxygen consumption further without stopping it completely.

**Fig. 2.** ESR spectra of the MNP radical adduct produced during the reaction of DCF, \( \mathrm{H}_2\mathrm{O}_2 \), and horseradish peroxidase. A, the ESR spectrum obtained from a reaction mixture containing 4 mM DCF, 1 mM \( \mathrm{H}_2\mathrm{O}_2 \), 50 mM MNP, and 1 \( \mu \)M horseradish peroxidase (HRP). B, the same as A, except DCF was omitted. C, the same as A, but with the omission of HRP. D, the same as A, but with the omission of \( \mathrm{H}_2\mathrm{O}_2 \). E, the spectrum obtained from a solution of MNP alone in Tris buffer. The hyperfine coupling constants are provided under “Results.” Spectrometer conditions were as follows: modulation amplitude, 1 G; microwave power, 40 milliwatts; time constant, 0.328 s; conversion time, 0.164 s; scan time, 168 s; receiver gain, \( 5 \times 10^4 \).
Free Radical Formation during Oxidation of DCF

**Fig. 4.** Rate of oxygen consumption during the oxidation of DCF by horseradish peroxidase in the presence of GSH and H$_2$O$_2$. Effect of the addition of DMPO, ascorbate, superoxide dismutase, and catalase. The vertical bars mark the time of the addition of HRP, H$_2$O$_2$, DMPO, ascorbate, superoxide dismutase (SOD), and catalase to the system. A, rate of oxygen consumption during the oxidation of DCF by HRP in the presence of GSH and H$_2$O$_2$. $A$, complete system containing 100 $\mu$M DCF, 5 mM GSH, 32 $\mu$M H$_2$O$_2$, and 1 $\mu$M HRP. B, as in A, but with the addition of 100 $\mu$M H$_2$O$_2$, C, as in A, but with the addition of 150 $\mu$M H$_2$O$_2$. D, effect of DMPO or ascorbate on the rate of oxygen consumption during the oxidation of DCF by HRP in the presence of GSH and H$_2$O$_2$. E, complete system containing 100 $\mu$M DCF, 5 mM GSH, 32 $\mu$M H$_2$O$_2$, and 1 $\mu$M HRP. B, as in A, but with the addition of 1 $\mu$M ascorbate 90 s after the addition of HRP. C, as in A, but with the addition of 200 mM DMPO 1 min after the addition of HRP. D, as in A, but with the addition of 200 mM DMPO before the addition of HRP. E, as in A, but with the omission of H$_2$O$_2$. F, as in A, but with the omission of DCF. G, as in A, but with the omission of GSH. H, as in A, but with the addition of 1 mM ascorbate before the addition of HRP. I, effect of SOD or catalase on the rate of oxygen consumption during the oxidation of DCF by HRP in the presence of GSH and H$_2$O$_2$. A, complete system containing 100 $\mu$M DCF, 5 mM GSH, 32 $\mu$M H$_2$O$_2$, and 1 $\mu$M HRP. B, as in A, but with the addition of 100 $\mu$g/ml SOD 90 s after the addition of HRP. D, as in B, but with the addition of 50 $\mu$g/ml catalase 90 s after the addition of HRP. E, as in B, but with the addition of 50 $\mu$g/ml catalase before the addition of HRP.

(data not shown), demonstrating the involvement of metal catalysis. Moreover, the addition of superoxide dismutase (100 $\mu$g/ml) to the reaction mixture right before horseradish peroxidase completely inhibited the oxygen consumption (Fig. 5D). A similar inhibition was obtained when superoxide dismutase was added 2 min after the addition of horseradish peroxidase (Fig. 5B). These results show that, in the absence of H$_2$O$_2$, oxygen consumption arose completely from a superoxide-dependent reaction. When catalase was added to the reaction mixture before or after horseradish peroxidase initiation, the oxygen consumption was completely inhibited (Fig. 5, C and E), indicating that the presence of a small amount of hydrogen peroxide in the system was also necessary for oxygen consumption.

When DCF was incubated with NADH and horseradish peroxidase in the presence of the spin trap DMPO, a composite ESR spectrum was detected (Fig. 6A). A similar ESR spectrum, with much lower intensity, was observed in the same system without DCF (Fig. 6B). When NADH (Fig. 6D) or horseradish peroxidase (Fig. 6E) was omitted from the system, no signal or only a very weak signal was detected. The addition of superoxide dismutase completely inhibited the radical formation (Fig. 6D), confirming the involvement of superoxide radicals in the formation of the DMPO radical adducts. The addition of catalase had the same inhibitory effect as superoxide dismutase (Fig. 6F), demonstrating that the presence of H$_2$O$_2$, at least in traces, was necessary for the radical reaction to proceed. The hydrogen peroxide, essential for the initial activation of horseradish peroxidase, presumably resulted from autoxidation of NADH.

In order to characterize the radical formed during the spin-trapping study of DCF oxidation by horseradish peroxidase in the presence of NADH, the ESR spectrum of the system with-
Free Radical Formation during Oxidation of DCF

Fig. 6. ESR spectra of DMPO radical adducts produced by the reaction mixture containing DCF, NADH, and horseradish peroxidase. A, the ESR spectrum obtained from a reaction mixture containing 45 μM DCF, 1 mM NADH, 200 mM DMPO, and 0.2 μM HRP. B, the same as A, except DCF was omitted. C, the spectrum obtained by the subtraction of spectrum B from spectrum A. D, the same as A, but with the omission of NADH. E, the same as A, but with the omission of HRP. F, the same as A, but with the addition of 50 μg/ml SOD. G, the same as A, but with the addition of 150 μg/ml catalase. The hyperfine coupling constants of each species are provided under "Results." Spectrometer conditions were as follows: modulation amplitude, 1 G; microwave power, 20 milliwatts; time constant, 0.328 s; conversion time, 0.655 s; scan time, 671 s; receiver gain, $4 \times 10^5$.

out DCF (Fig. 6B) was subtracted from that of the complete system (Fig. 6A) to yield a single radical species (Fig. 6C). Based on the analysis of hyperfine coupling constants and characteristics of the radical, it was assigned as the DMPO-superoxide radical adduct, DMPO/•OOH, with hyperfine coupling constants of $a^{N}=14.16$ G, $a^{H}=11.25$ G, and $a^{H}=1.2$ G, consistent with those previously reported (40).

Furthermore, oxygen consumption was investigated in the same reaction mixture containing DCF, NADH, and horseradish peroxidase with the addition of catalase, SOD, or ascorbate. The results are shown in Fig. 7a. Molecular oxygen in the reaction mixture was completely consumed in less than 8 min (Fig. 7a, A). When DCF, NADH, or horseradish peroxidase was omitted from the system, no oxygen consumption was observed (Figs. 7a, E, G, and H, respectively). When ascorbic acid (1 mM) was added prior to or after horseradish peroxidase initiation, the oxygen consumption was completely inhibited (Figs. 7a, F and D, respectively). In addition, the same inhibition was observed when 100 μM ascorbate was added before horseradish peroxidase or 250 μM ascorbate was added after horseradish peroxidase (data not shown). When the spin trap DMPO was added after or prior to horseradish peroxidase initiation, the oxygen consumption was inhibited (Figs. 7a, B and C, respectively). These results are consistent with radical formation during the oxidation of DCF by horseradish peroxidase in the presence of NADH as demonstrated by ESR. The addition of catalase to the complete system also strongly inhibited oxygen consumption (Fig. 7b, D and E). When superoxide dismutase was added to the reaction mixture prior to horseradish peroxidase initiation, the rate of oxygen consumption was found to be slightly faster than that of the complete system and was completely inhibited after 6 min (Fig. 7b, B). The addition of superoxide dismutase 1 min after the addition of horseradish peroxidase possibly had a weak inhibitory effect (Fig. 7b, C).

Ascorbate is known to undergo one-electron oxidation to produce the relatively stable ascorbate free radical that can be
In the presence of NADH or GSH under aerobic conditions, the reaction of H$_2$O$_2$ or horseradish peroxidase resulted in a weaker signal (Fig. 8). When GSH was omitted from the system, no oxygen was consumed (Fig. 9B). When the experiment was repeated under room light, no lag phase was detected, and the rate of oxygen consumption of the complete system was much faster (Fig. 9A). This result demonstrates that the oxidation of DCFH to DCF is also catalyzed by room light. When NADH was omitted from the reaction performed under room light, no detectable oxygen consumption was observed (Fig. 9D).

**DISCUSSION**

Our previous studies demonstrated the formation of DCF semiquinone (DCF$^-$) free radical by photoreduction of DCF (28) or by horseradish peroxidase-catalyzed oxidation of the reduced leuco compound DCFH (29). The photoreduction of DCF to DCF$^-$ in the presence of NADH or GSH under aerobic conditions resulted in the formation of superoxide radical that was detected by the ESR spin-trapping technique (28). The enzymatic oxidation of DCFH in the dark to DCF$^-$ by the reaction of horseradish peroxidase in the presence or absence of added H$_2$O$_2$ resulted in hydroxyl and superoxide radical formation, and H$_2$O$_2$ was demonstrated to be formed during the deacetylation of DCFH-DA (29).

In the present study, we analyzed the oxidation of the fluorescent dye DCF by horseradish peroxidase in the dark. Our results provide strong evidence for the formation of the novel phenoxyl radical intermediate during the peroxidase-mediated oxidation of DCF as demonstrated by the ESR spin-trapping technique (Fig. 2). UV-visible spectrophotometry confirmed the existence of compound II during the oxidation, and significant changes in the DCF absorption band at 502 nm were observed (Fig. 1), providing evidence for one-electron peroxide-mediated oxidation of DCF (Scheme 1), i.e. when DCF reacts with horseradish peroxidase in the presence of H$_2$O$_2$, DCF undergoes one-electron oxidation, with the obligate formation of the DCF phenoxyl radical (Fig. 2). This free radical is structurally and chemically distinct from the DCF semiquinone DCF$^-$. In the presence of reducing agents such as NADH and GSH, the DCF phenoxyl radical is reduced back to its parent compound DCF with the concomitant formation of NAD$^+$ or GSH, respectively, which, in the presence of oxygen, form superoxide radical. DCF, as shown in Scheme 2, catalytically stimulates the formation of GS and oxygen consumption in the glutathione-H$_2$O$_2$-horseradish peroxidase system (Figs. 3 and 4b). The rate of oxygen consumption was detectable in the absence of added H$_2$O$_2$ (Fig. 5A). In this case, oxygen consumption can occur when GS' reacts with another GSH (GS') molecule to form the (GSSG)$^-$, which reacts rapidly with oxygen to produce superoxide radical (43). When the system contained only traces of H$_2$O$_2$, superoxide dismutase almost completely inhibited the rate of oxygen consumption, suggesting that, at least in part, superoxide radical is produced by a free radical chain reaction (Figs. 4c, C, and 5, B and D). No superoxide was detected by direct ESR. A reaction mixture of DCF, H$_2$O$_2$, and horseradish peroxidase in the presence of ascorbate gave a typical doublet ESR signal of the ascorbate anion free radical as shown in Fig. 8A with the characteristic hyperfine coupling constant of $a^{14} = 1.79$ G (41). No changes in the ascorbate anion radical spectrum were observed upon the addition of GSH (same concentration as ascorbate) to the reaction mixture (data not shown), indicating that DCF$^-$ phenoxyl radical reacted with ascorbate instead of GSH (42). In this case, ascorbate was a better radical scavenger than GSH. The omission of DCF, H$_2$O$_2$, or horseradish peroxidase resulted in a weaker signal (Fig. 8B–D). The addition of catalase (50 μg/ml) to the reaction mixture inhibited the formation of ascorbate anion radical (Fig. 8F) to the level found with ascorbate alone (Fig. 8G). The presence of superoxide dismutase (50 μg/ml) in the reaction mixture had no effect on the ESR signal (Fig. 8E), suggesting that superoxide radicals were not involved in either ascorbate radical formation or decay.

In order to better understand the chemistry involved between DCFH and DCF, we measured oxygen consumption from a reaction mixture containing the reduced leuco compound DCFH and horseradish peroxidase. When DCFH reacted with horseradish peroxidase in the presence of NADH under room light, oxygen was consumed within 12 min (Fig. 9A). Fig. 9B shows that for approximately 12 min, the rate of oxygen consumption in the dark was similar to that of the complete system without DCFH (Fig. 9C). Then the consumption rate increased dramatically and consumed all of the oxygen within the next 5 min. This 12-min lag phase probably represents the time required to oxidize DCFH to produce enough DCF (29) to support the oxygen-consuming reactions between DCF, horseradish peroxidase, and NADH (Fig. 7, a and b). When NADH was omitted from the system in the dark, no oxygen was consumed (Fig. 9E). When the experiment was repeated under room light, no lag phase was detected, and the rate of oxygen consumption of the complete system was much faster (Fig. 9A). This result demonstrates that the oxidation of DCFH to DCF is also catalyzed by room light. When NADH was omitted from the reaction performed under room light, no detectable oxygen consumption was observed (Fig. 9D).
ESR in this system, probably because the precursor of this radical, GS\textsuperscript{-}, is very efficiently scavenged by DMPO ($k = 2.6 \times 10^8 \text{ M}^{-1} \text{s}^{-1}$) (44), as demonstrated also by the total inhibition of oxygen consumption by the addition of DMPO (Fig. 4b, C and D). At pH 7.8, superoxide radical can react with GSH with a second-order rate constant of $k = 6.7 \times 10^5 \text{ M}^{-1} \text{s}^{-1}$, producing $\text{H}_2\text{O}_2$ and regenerating GS\textsuperscript{-}, thus continuing the chain reaction (38).

DCF greatly enhanced the rate of oxygen consumption by horseradish peroxidase and NADH (Fig. 7a). The resulting superoxide radical was detected by ESR spin trapping (Fig. 6). The inhibition of free radical formation and oxygen consumption by the addition of catalase demonstrated that a trace of hydrogen peroxide was present in the system (Figs. 6G and 7b, D and E). The reactions leading to the formation of superoxide radical by the reaction of DCF and horseradish peroxidase/ $\text{H}_2\text{O}_2$ in the presence of the reducing agents NADH and GSH are summarized in Scheme 2. Similar to GSH, superoxide radical can oxidize additional NADH with a second-order rate constant of $k = 9.3 \times 10^4 \text{ M}^{-1} \text{s}^{-1}$ at pH 7.4, producing $\text{H}_2\text{O}_2$ and regenerating NAD\textsuperscript{+}, thus continuing the chain reaction (45).

The complete suppression of oxygen consumption by the addition of ascorbate to both the DCF-GSH-horseradish peroxidase (Fig. 4b) and the DCF-NADH-horseradish peroxidase...
systems (Fig. 7a) and the DCF-dependent increase of ascorbate anion radical formation (Fig. 8) also support the DCF phenoxyl radical formation. Ascorbate radical is relatively stable once formed and does not further oxidize any other biochemical reductant in the system nor reduce oxygen.

The reaction of the reduced leuco compound DCFH with horseradish peroxidase in the presence of NADH consumed oxygen in a light-dependent manner because DCFH was more efficiently oxidized to DCF in the presence of light (Fig. 9). This experiment demonstrates that there are many reactions involving DCFH, DCF, and/or light in biological systems that can potentially lead to artificial radical formation.

Several issues concerning the measurement of DCF formation as an index of intracellular hydrogen peroxide (or other reactive species) formation must be considered in future work. First, the same species that forms DCF from DCFH (in this case horseradish peroxidase compounds I and II) will probably oxidize DCF to the novel DCF phenoxyl radical DCF·. Second, DCF· oxidizes many biochemical reducing agents to free radicals, which may or may not (depending on their chemistry) react with molecular oxygen to form superoxide and, ultimately, hydrogen peroxide. Consequently, the potential for DCF· oxidation forming reactive oxygen species will strongly depend on the intracellular concentration of the various biochemical reducing agents.

This study, combined with our earlier studies (28, 29), clearly shows that results from the DCF fluorometric assay are difficult to interpret in cells undergoing oxidative stress.

REFERENCES

1. Tsuji, M., Suematsu, M., and Suzuki, H. (1994) Methods Enzymol. 233, 128–140
2. Atlante, A., Gagliardi, S., Minervini, G. M., Cotti, M. T., Marra, E., and Calissano, P. (1997) J. Neurochem. 68, 2038–2045
3. Burrow, S., and Valet, G. (1987) Eur. J. Cell Biol. 43, 128–133
4. Fukumura, D., Kurose, I., Miura, S., Tsuji, M., and Ishii, H. (1995) J. Gastroenterol. 30, 565–571
5. Gunasekar, P. G., Kanhasamy, A. G., Borowitz, J. L., and Isom, G. E. (1995) J. Neurochem. 65, 2016–2021
6. Gunasekar, P. G., Sun, P. W., Kanhasamy, A. G., Borowitz, J. L., and Isom, G. E. (1996) J. Pharmacol. Exp. Ther. 277, 150–155
7. Hagar, H., Ueda, N., and Shah, S. V. (1996) Am. J. Physiol. 271, F209–F215
8. Horio, F., Fukuda, M., Katoh, H., Petruzelli, M., Yano, N., Rittershaus, C., Bonner-Weir, S., and Hattori, M. (1994) Diabetes 37, 22–31
9. Kurose, I., Saito, H., Suematsu, M., Fukumura, D., Miura, S., Morizane, T., and Tsuji, M. (1991) Cancer Lett. 59, 201–209
10. Kurose, I., Miura, S., Fukumura, D., Yonei, Y., Saito, H., Tada, S., Suematsu, M., and Tsuji, M. (1993) Cancer Res. 53, 2676–2682
11. Oyama, Y., Hayashi, A., Ueba, T., and Maekawa, K. (1994) Brain Res. 635, 113–117
12. Puntarulo, S., and Cederbaum, A. I. (1996) Biochim. Biophys. Acta 1289, 238–246
13. Reid, M. B., Haack, K. E., Franchek, K. M., Valberg, P. A., Kobzik, L., and West, M. S. (1992) J. Appl. Physiol. 73, 1797–1804
14. Saito, H., Fukumura, D., Kurose, I., Suematsu, M., Tada, S., Kaga, T., Miura, S., Morizane, T., and Tsuji, M. (1992) Int. J. Cancer 51, 124–129
15. Scott, J. A., Honey, C. J., Khaw, B. A., and Rabito, C. A. (1988) Free Radical Biol. Med. 4, 79–83
16. Yang, C. F., Shen, H. M., Shen, Y., Zhuang, Z. X., and Ong, C. N. (1997) Environ. Health Perspect. 105, 712–716
17. Zbo, H., Bannenberg, G. L., Moldeus, P., and Shertzer, H. G. (1994) Arch. Toxicol. 68, 582–587
18. Entman, M. L., Youker, K., Shoji, T., Kukielka, G., Shappell, S. B., Taylor, A. A., and Smith, C. W. (1992) J. Clin. Invest. 90, 1355–1345
19. Maresca, M., Celli, C., and Leoncini, G. (1992) Cell Biochem. Funct. 10, 79–85
20. Wang, J. F., Jerrells, T. R., and Spitzer, J. J. (1996) Free Radical Biol. Med. 20, 533–542
21. Bass, D. A., Parce, J. W., Dechateau, L. R., Sejida, P., Seeds, M. C., and Thomas, M. (1983) J. Immunol. 130, 1910–1917
22. Carter, W. O., Narayan, P. K., and Robinson, J. P. (1994) J. Leukocyte Biol. 55, 253–258
23. Suda, N., Morita, I., Kuroda, T., and Murota, S. (1993) Biochim. Biophys. Acta 1157, 318–323
24. Rabeasatrana, H., Fournier, A. M., Chatreau, M. T., Serre, A., and Dornand, J. (1992) Int. J. Immunopharmacol. 14, 895–902
25. Shen, H. M., Shi, C. Y., Shen, Y., and Ong, C. N. (1996) Free Radical Biol. Med. 21, 139–146
26. Takeuchi, T., Nakajima, M., and Morimoto, K. (1996) Carcinogenesis 17, 1543–1548
27. LeBel, C. P., Ischiropoulos, H., and Bonyd, S. C. (1992) Chem. Res. Toxicol. 5, 227–231
28. Marchesi, E., Rota, C., Fann, Y. C., Chignell, C. F., and Mason, R. P. (1999) Free Radical Biol. Med. 26, 148–161
29. Rota, C., Chignell, C. F., and Mason, R. P. (1999) Free Radical Biol. Med. 27, in press
30. Dunford, H. B., and Stillman, J. S. (1976) Coord. Chem. Rev. 19, 187–251
31. Hildebrandt, A. G., and Roots, I. (1975) Arch. Biochem. Biophys. 171, 385–397
32. Duling, D. R. (1994) J. Magn. Reson. B 104, 105–110
33. Claiberne, A. and Fridovich, I. (1978) Biochemistry 17, 2329–2335
34. Gunther, M. R., Tschirret-Guth, R. A., Witkowska, H. E., Fann, Y. C., Barr, D. P., Ortiz de Montellano, P. R., and Mason, R. P. (1998) Biochem. J. 330, 1283–1299
35. Pichet, C., Spitz, R., and Gayot, A. (1977) J. Macromol. Sci. Chem. A11, 251–265
36. Makino, K., Suzuki, N., Moriya, F., Rokushika, S., and Hatano, H. (1981) Radiat. Res. 86, 294–310
37. Barr, D. P., Gunther, M. R., Deterding, L. J., Toomer, K. B., and Mason, R. P. (1996) J. Biol. Chem. 271, 15498–15503
38. Gadelha, F. R., Hanna, P. M., Mason, R. P., and Decompo, R. (1992) Chem. Biol. Interact. 85, 35–48
39. Mottley, C., Toy, K., and Mason, R. P. (1987) Mol. Pharmacol. 31, 417–421
40. Buettner, G. R., and Mason, R. P. (1990) Methods Enzymol. 186, 127–133
41. Laroff, G. P., Pessenden, R. W., and Schuler, R. H. (1972) J. Am. Chem. Soc. 94, 9062–9073
42. Sturgeon, B. E., Sipe, H. J., Jr., Barr, D. P., Corbett, J. T., Martinez, J. G., and Mason, R. P. (1996) J. Biol. Chem. 271, 50116–50121
43. Mason, R. P. (1992) in Biological Consequences of Oxidative Stress: Implications for Cardiovascular Disease and Carcinogenesis (Spazt, L., and Bloem, A. D., eds) pp. 23–49, Oxford University Press, New York
44. Davies, M. J., Forni, L. G., and Shuter, S. L. (1987) Chem. Biol. Interact. 61, 177–188
45. Fujimori, K., and Nakajima, H. (1991) Biochim. Biophys. Acta 176, 846–851