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Oxidation pathways for the intracellular probe 2′,7′-dichlorofluorescin

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Abstract. The oxidation of 2′,7′-dichlorofluorescin (DCFH) to a fluorescent product is currently used to evaluate oxidant stress in cells. However, there is considerable uncertainty as to the enzymatic and nonenzymatic pathways that may result in DCFH oxidation. Iron/hydrogen peroxide-induced DCFH oxidation was inhibited by catalase or by the hydroxyl radical scavenger dimethylsulfoxide; however, superoxide dismutase (SOD) had no effect on DCFH oxidation. The formation of hydroxyl radical (indicated by the oxidation of salicylic acid to 2,3-dihydroxybenzoic acid) was proportional to DCFH oxidation, suggesting that the hydroxyl radical is responsible for the iron/peroxide-mediated oxidation of DCFH. Utilizing a superoxide generating system consisting of hypoxanthine/xanthine oxidase, oxidation of DCFH was unaffected by SOD, catalase or desferoxamine, and stimulated by removing hypoxanthine from the reaction mixture. In contrast, SOD or elimination of hypoxanthine abolished superoxide formation. In addition, potassium superoxide did not support the oxidation of DCFH. Thus, superoxide is not involved in DCFH oxidation. Boiling xanthine oxidase eliminated its concentration-dependent oxidation of 1 μM DCFH, indicating that xanthine oxidase can enzymatically utilize DCFH as a high affinity substrate. Kinetic studies of the oxidation of DCFH by xanthine oxidase indicated a $K_{m(app)}$ of 0.62 μM. Hypoxanthine competed with DCFH with a $K_{i(app)}$ of 1.03 mM. These studies suggest that DCFH oxidation may be a useful indicator of oxidative stress. However, other types of cellular damage may produce DCFH oxidation. For example, conditions or chemicals that damage intracellular membranes may release to the cytoplasm oxidases or peroxidases that might use DCFH as a substrate, similar to xanthine oxidase

Key words: Dichlorofluorescein – Iron – Hydrogen peroxide – Hydroxyl radical – Oxidative stress – Superoxide anion – Xanthine oxidase

Abbreviations: DCF, 2′,7′-dichlorofluorescein (oxidized fluorescent form of the dye); DCFH, 2′,7′-dichlorofluorescin (reduced non-fluorescent form of the dye); $K_{m(app)}$, apparent K_m; $K_{i(app)}$, apparent K_i; SOD, superoxide dismutase

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Introduction

The intracellular generation of reactive oxygen metabolites (ROM) has been associated with chemical toxicity (Boobis et al. 1989; Comporti 1989; de Groot and Littauer 1989; Poli et al. 1989) and such human disease states as inflammatory diseases, cancer and ageing (Ames 1983; Biemond et al. 1984; Clark et al. 1985; Halliwell and Gutteridge 1986a, b; Halliwell 1987; Ward et al. 1988; Floyd 1990; Meister 1992). Therefore, the ability to assess the generation of intracellular ROM as a component of the pathways leading to toxicity and disease is critically important. A fluorescent probe to assess intracellular oxidative stress has been in recent use, based upon the original observations that hydrogen peroxide in the presence of peroxidase oxidizes the nonfluorescent substrate 2′,7′-diacetyldichlorofluorescin (diacetyl-DCFH) to the highly fluorescent product 2′,7′-dichlorofluorescein (Keston and Brandt 1965). Diacetyl-DCFH diffuses through the cell membrane where it is enzymatically deacetylated by intracellular esterases to the more hydrophilic nonfluorescent reduced dye dichlorofluorescin. In the presence of reactive oxygen metabolites, DCFH is rapidly oxidized to DCF. The pathway and structures involved in the deacetylation and oxidation of diacetyl-DCFH is shown in Fig. 1 of LeBel et al. (1992).

Diacetyl-DCFH has been used to study lipid peroxidation and rates of reactive oxygen metabolites formation in granulocytes, platelets, cultured cells and cell-free systems (Keston and Brandt 1965; Leonoini et al. 1991; Mattia et al. 1991; Huang et al. 1993). Numerous studies demonstrate the utility of DCFH as an index of free radical reactions that take place in the living animal following exposure to toxic chemicals (Rowley and Halliwell 1983). The formation of

reactive oxygen metabolites in intact heart tissue has been assessed by direct ESR measurements, and indirectly using DCFH (Kehrer and Paraidathathu 1992). The neurotoxic organic solvent toluene enhanced brain DCF formation rates while the non-neurotoxic but structurally related solvent benzene did not (Mattia et al. 1991).

In spite of the popularity of the current use of acetyl-DCFH to evaluate oxidative stress, the specific oxidant species responsible have not been clearly defined. This study confirms the importance of iron and H_2O_2, and the lack of involvement of superoxide, in oxidizing the probe. Furthermore, we show that xanthine oxidase can oxidize the probe by directly using DCFH as a substrate.

Materials and methods

Chemicals. 2′,7′-Dichlorofluorescin diacetate (acetyl-DCFH) was purchased from Eastman Kodak Co. (Rochester, N.Y.). All other chemicals and enzymes were from Sigma Chemical Co. (St Louis, Mo.). Solutions were prepared fresh daily. DCFH was prepared from acetyl-DCFH by the method of LeBel et al. (1992).

Oxidation of DCFH. The basic reaction mixture contained 1 μM DCFH in 40 mM TRIS-HCl, pH 7.4, in a total volume of 2.0 ml. Reactions were carried out at 37° C and started by the addition of 100 μM H_2O_2 and 10 μM $FeSO_4$ or $FeCl_3$. The specific reaction mixtures are listed in the figure and table legends, and are similar to those previously described for the generation of reactive oxygen metabolites (Rowley and Halliwell 1983). In other experiments the reaction was initiated by the addition of hypoxanthine and/or xanthine oxidase. Fluorescence was determined using a Hitachi F-2000 spectrofluorometer, with λ_{EX} = 503 nm and λ_{EM} = 523 nm. Fluorescence was corrected by subtracting parallel blanks in each experiment (DCFH in buffer). In every case, the fluorescence spectrum of the oxidized product matched that of authentic 2′,7′-DCF.

For the enzyme kinetic studies, the reaction mixture contained 5 mU xanthine oxidase and 0, 0.5, 1.0 or 2.0 mM hypoxanthine in 150 mM potassium phosphate buffer, pH 7.4. Fluorescence was determined after the addition of 0.1, 0.2, 0.5, 1.0 or 2.0 μM DCFH. The reaction was linear for at least 1 min, and the rates were calculated as the increase in fluorescence units (FU)/min. The kinetic data are displayed as Lineweaver-Burk plots and the kinetic parameters were calculated as described (Segel 1976).

Determination of hydroxyl radical formation. Hydroxyl radical formation can be measured by its ability to hydroxylate salicylate followed by an improved colorimetric assay (Richmond et al. 1981). The reaction mixture contained 100 μM H_2O_2, 2.5 mM salicylic acid and 40 mM TRIS-HCl buffer, pH 7.4, in a reaction volume of 2.0 ml. The reaction was initiated by adding 10 μM $FeSO_4$ in potassium phosphate buffer, pH 7.4, and tubes were incubated at 25° C for 90 min. Then 80 μl of 11.6 M HCl was added, followed by 4 ml of chilled diethyl ether. The contents were vortex mixed for 30 s, and 3 ml of the upper ether layer was removed and evaporated to dryness under a stream of nitrogen at 40° C. The tubes were cooled and the residue dissolved in 0.25 ml cold double distilled water, to which was added in the following order 0.5 ml 10% (w/v) trichloroacetic acid in 0.5 M HCl, 0.25 ml 10% (w/v) $NaSO_4$, and 0.25 ml 0.5% (w/v) $NaNO_2$. After 5 min, 0.125 ml of 0.5 M KOH was added and the absorbance was determined at 510 nm after 1 min. Standard curves were prepared using 2,3-dihydroxybenzoate.

Determination of superoxide. The basic reaction medium contained 200 μM ferricytochrome *c* in 150 mM potassium phosphate buffer, pH 7.4. The rate of superoxide formation was calculated as the rate of cytochrome *c* reduction, determined spectrophotometrically at 37° C, using an extinction coefficient of 21.1 $cm^{-1}mM^{-1}$ for cytochrome *c* (Masters et al. 1967).

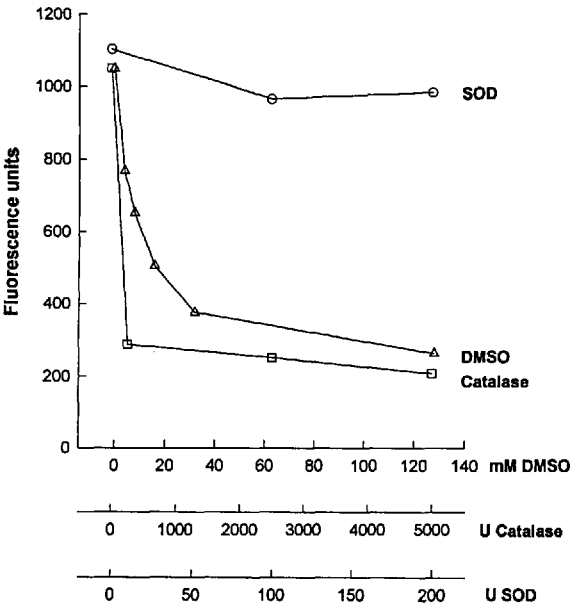

Fig. 1. Effects of reactive oxygen scavengers on iron/H_2O_2-catalyzed dichlorofluorescin (*DCFH*) oxidation. The reaction mixture at 37° C contained 1 μM DCFH in 40 mM TRIS-HCl, pH 7.4, and the concentrations of superoxide dismutase (*SOD*), dimethylsulfoxide (*DMSO*), or catalase indicated in the figure. Reactions were initiated by the addition of 100 μM H_2O_2 and 10 μM $FeSO_4$. Fluorescence measurements are reported after 1 min (λ_{EX} = 503 nm and λ_{EM} = 523 nm). The results are the average values for two experiments

Results

Figure 1 shows that DCFH oxidation by Fe^{2+} and H_2O_2 were inhibited by catalase and by the good hydroxyl radical scavenger DMSO, but not by SOD. The reaction was complete within 30 s (data not shown). The less potent hydroxyl radical scavenger mannitol (70 mM) inhibited the oxidation of DCFH about 30%, and higher concentrations did not inhibit further. Bubbling the reaction mixture with argon for 20 min in an anaerobic cuvette did not affect the oxidation of DCFH by Fe^{2+}/H_2O_2; however, bubbling with 10% methane gas in argon abolished Fe^{2+}/H_2O_2- supported DCFH oxidation (data not shown).

Using a Fe^{3+}/H_2O_2 reaction mixture containing ascorbate to recycle the iron, the oxidation of DCFH proceeded much more slowly, and the reaction was linear with time for over 30 min (Fig. 2). Addition of EDTA accelerated the reaction, by enhancing the rate of redox cycling of Fe^{2+}/Fe^{3+} (Dunford 1987). DMSO produced partial inhibition of DCFH oxidation in this system (less than that observed in the Fe^{2+}/H_2O_2 reaction mixture), and catalase and desferoxamine strongly inhibited the reaction. Although there appeared to be a slight inhibition by SOD in the presence of EDTA, the equivalent amount of boiled SOD produced the same result, indicating a non-catalytic effect. Ascorbate alone (up to 10 mM) was unable to reduce DCF back to DCFH (data not shown).

The iron/H_2O_2 generating system was examined for hydroxyl radical formation, by the generation of 2,3-dihydroxybenzoic acid from salicylic acid (Table 1). SOD had no effect on salicylate oxidation, while catalase and des-

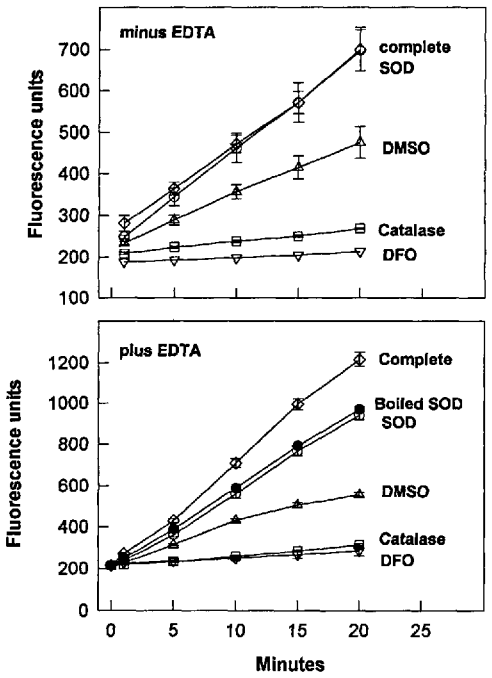

Fig. 2. Effects of reactive oxygen scavengers on iron/ascorbate/H_2O_2-catalyzed dichlorofluorescin (*DCFH*) oxidation. The complete reaction mixture consisted of 1 μM DCFH, 100 μM $FeCl_3$, 100 μM H_2O_2, 100 μM ascorbate, and 150 mM potassium phosphate buffer, pH 7.4, in the absence (*upper panel*) or presence (*lower panel*) of 0.2 mM EDTA. Superoxide dismutase (SOD, 25 U/ml or its boiled equivalent), 32 mM dimethylsulfoxide (*DMSO*), 125 U/ml catalase or 250 μM desferoxamine (*DFO*) were added as indicated. Fluorescence measurements are reported after 1 min (λ_{EX} = 503 nm and λ_{EM} = 523 nm). Results are expressed as mean fluorescence values ±SE for three experiments. The absence of an *error bar* indicates the error is not visible within the symbol

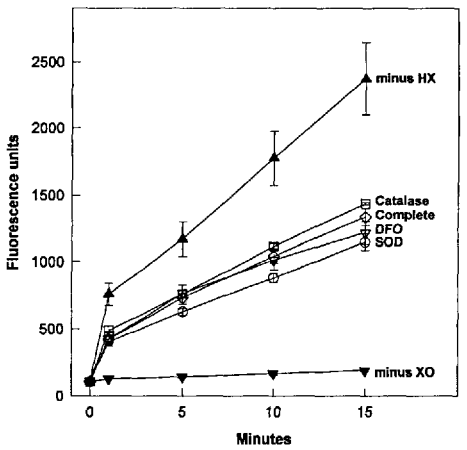

Fig. 3. Characteristics of xanthine oxidase catalyzed DCFH oxidation. The complete reaction mixture contained 0.03 U/ml xanthine oxidase, 1 mM hypoxanthine and 1 μM DCFH, in 150 mM potassium phosphate buffer, pH 7.4. The curves shown, from top to bottom, are deletion of hypoxanthine from the reaction mixture (*closed triangles*), addition of 250 U catalase (*open squares*), complete reaction mixture (*open diamonds*), addition of 1 mM desferoxamine (*inverted open triangle*), addition of 100 U superoxide dismutase (*open circles*) and deletion of xanthine oxidase from the reaction mixture (*closed inverted triangles*). The results shown are mean values ±SE for three experiments. The absence of an *error bar* indicates the error is not visible within the symbol

Table 1. Salicylate hydroxylation in various reaction mixtures

	Nmol 2,3-dihydroxybenzoic acid formed/90 min[a]		
		Fe^{3+}/ascorbate/H_2O_2	
	Fe^{2+}/H_2O_2 system[b]	minus EDTA[c]	plus EDTA[d]
Complete system	19.5 (100%)	35 (100%)	189 (100%)
SOD (12.5 U/ml)	18.2 (93.3%)	31 (89%)	183 (97%)
SOD (50 U/ml)	17.3 (88.7%)	40 (114%)	188 (99%)
DMSO (32 mM)	0 (0%)	16 (45%)	47 (25%)
Catalase (125 U/ml)	0 (0%)	0 (0%)	0 (0%)
Desferoxamine (250 μM)	0 (0%)	0 (0%)	0 (0%)

[a] Values are expressed as nmol 2,3-dihydroxybenzoic acid formed per 90 min, and represent the average of two independent experiments. The percent of control values are shown in parentheses

[b] The complete reaction mixture consisted of 100 μM $FeSO_4$, 100 μM H_2O_2, 2.5 mM salicylate, and 150 mM potassium phosphate buffer, pH 7.4

[c] The complete reaction mixture consisted of 100 μM $FeCl_3$, 100 μM H_2O_2, 100 μM ascorbate, 2.5 mM salicylate, and 150 mM potassium phosphate buffer, pH 7.4

[d] The complete reaction mixture consisted of 100 μM $FeCl_3$, 100 μM H_2O_2, 100 μM ascorbate, 2.5 mM salicylate, 0.3 mM EDTA, and 150 mM potassium phosphate buffer, pH 7.4

Table 2. DCFH oxidation and cytochrome c reduction with potassium superoxide

	Nmol cytochrome c reduced/min[a]	DCFH oxidation[b]
Complete system	25.6±2.3	0
+ SOD (25 U/ml)	0	0
+ Catalase (100 U/ml)	31.7±0.5	0

The complete reaction mixture consisted of 2 mM potassium superoxide and either 200 μM ferricytochrome *c*[a], or 1 μM DCFH[b], in 150 mM potassium phosphate buffer, pH 7.4. Blank values obtained in the absence of potassium superoxide were subtracted from all values. Results are expressed as mean values ± SE for three experiments

Table 3. Superoxide formation in the hypoxanthine/xanthine oxidase system

	Nmol cytochrome c reduced/min
Complete system	25.9±0.8
+ SOD (35 U/ml)	0
Minus hypoxanthine	0
+ DMSO (32 mM)	26.6±0.9

The complete reaction mixture consisted of 1 mM hypoxanthine, 0.03 U/ml xanthine oxidase, and 200 μM cytochrome *c*, in 150 mM potassium phosphate buffer, pH 7.4. Results are expressed as mean values ± SE for three experiments

feroxamine completely quenched the reaction. The generation of hydroxyl radical correlated approximately with increased oxidation of DCFH. However, in the absence of ascorbate some DCFH oxidation occurred in the presence of 32 mM DMSO (Fig. 1), even though the formation of hydroxyl radical was completely quenched (Table 1, left column). Furthermore, EDTA produced a five-fold increase

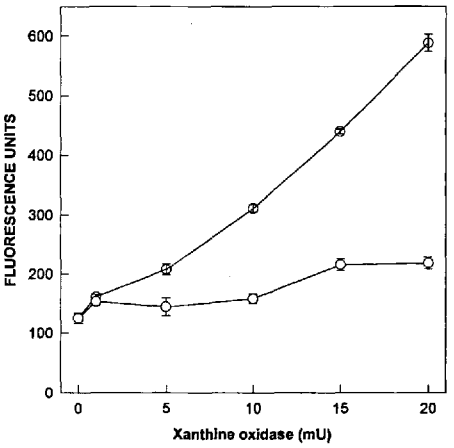

Fig. 4. Xanthine oxidase-mediated DCFH oxidation. The reaction mixture contained 1 μM DCFH in 150 mM potassium phosphate buffer, pH 7.4. The upper curve was obtained by adding native xanthine oxidase at the indicated activity. Comparable amounts of heat inactivated xanthine oxidase were added to the reaction mixture to obtain data for the lower curve. The results shown are mean values ±SE for three experiments

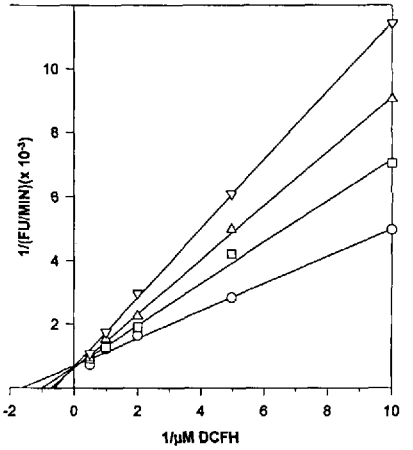

Fig. 5. Kinetic plots of DCFH oxidation by xanthine oxidase and inhibition by hypoxanthine. The reaction mixture contained 5 mU xanthine oxidase and hypothanthine concentrations of 0 mM (*open circles*), 0.5 mM (*open squares*), 1.0 mM (*open triangle*), or 2.0 mM (*open inverted triangle*), in 0.15 M potassium phosphate buffer, pH 7.4. The Lineweaver-Burk plots were fitted by least squares linear regression

in the rate of hydroxyl radical formation, while generating less than a doubling of DCFH oxidation.

To determine whether superoxide was involved in DCFH oxidation, we used 2 mM potassium superoxide (Table 2). This is a useful approach, even though it is recognized that a solution of potassium superoxide contains several impurities, including H_2O_2 (Valentine et al. 1984). While potassium superoxide produced SOD-inhibited and catalase-unaffected reduction of cytochrome *c*, it did not support any measurable increase in DCF oxidation. Conversely, potassium superoxide also did not decrease the fluorescence of DCF (data not shown), indicating that superoxide was not able to convert DCF to the reduced state. We confirmed the lack of involvement of superoxide in DCFH oxidation using a reaction mixture containing hypoxanthine/xanthine oxidase (Fig. 3). The reaction was dependent on xanthine oxidase and unaffected by catalase, desferoxamine or SOD, indicating the lack of involvement of H_2O_2, iron and superoxide, respectively. To our surprise, deleting hypoxanthine from the incubation medium approximately doubled the reaction rate. The generation of superoxide was examined in this system (Table 3), and as expected, DMSO did not affect superoxide generation, while SOD addition or hypoxanthine deletion abolished superoxide production. Figure 4 shows that DCFH oxidation was proportional to xanthine oxidase concentration, and that heat inactivation abolished its activity.

The Lineweaver-Burk plots of the rates of DCFH oxidation at various hypoxanthine and DCFH concentrations are shown in Fig. 5. As the hypoxanthine concentration increases, the slope of the linear regression lines increase and intersect at the abscissa, indicating that hypoxanthine competes competitively with DCFH for xanthine oxidase. The $K_{m(app)}$ for DCFH was 0.62 μM, and the $K_{i(app)}$ for hypoxanthine was 1.03 mM.

It is noteworthy for the purposes of experimental design and future experimentation to report that in the absence of added iron, catalase stimulated DCFH oxidation at concentrations greater than 250 U/ml (data not shown). Boiling the enzyme did not reduce the stimulation, suggesting a nonenzymatic and biologically irrelevant role for the catalase heme iron.

Discussion

Although dichlorofluorescin oxidation has been used frequently in recent years to monitor the level of oxidative stress in cells and organs (Hassan et al. 1988; Robinson et al. 1988; Scott et al. 1988; LeBel et al. 1990; Kato et al. 1991; Maresca et al. 1992; Huang et al. 1993; Royall and Ischiropoulos 1993), the oxidants responsible have not been clearly identified. From its original use in quantifying low levels of H_2O_2 (Keston and Brandt 1965), and subsequent findings that the potent iron chelator desferoxamine abolished H_2O_2-mediated DCFH oxidation (LeBel et al. 1992), it was clear that iron and H_2O_2 could generate DCF. However, the mechanism of iron/H_2O_2 mediated oxidation of DCFH remains unclear, with the major candidates being the generation of a hydroxyl radical intermediate, the generation of a perferryl (or a similar iron/oxygen complex) radical intermediate, and site-specific Fenton chemistry on the DCFH molecule (Gutteridge and Halliwell 1990; LeBel et al. 1992). It does not seem likely that site-specific Fenton chemistry is involved, because the DCFH molecule does not contain the vicinal heteroatoms (such as amine nitrogens and carboxylate oxygens) required for stable chelation with iron. However, salicylic acid does contain such moieties, and thus we speculate that some percentage, but not all, of salicylate oxidation would be site specific. This would explain the much greater stimulation by EDTA of

Fe^{3+}/H_2O_2-mediated salicylate oxidation versus DCFH oxidation. Site specific oxidation of salicylate would require a ternary complex of salicylate-iron-EDTA (1:1:1), where salicylate simply replaces one of the EDTA molecules normally present in the iron-EDTA chelate. The lack of oxygen requirement for DCFH oxidation by Fe^{2+}/H_2O_2, and the quenching of this anaerobic reaction by bubbling with 10% methane gas, would suggest that the proximate oxidizing species is either hydroxyl radical (oxygen independent formation via a Fenton reaction) or perferryl radical formed by the oxygen-reaction of iron with H_2O_2. Perferryl radical formed by the oxygen-dependent reaction of Fe^{2+} and molecular oxygen would not seem to be involved. In the presence of oxygen, an alternate mechanism is that hydroxyl radical or perferryl radical mediates the initial one-electron oxidation of DCFH, followed by autoxidation of the radical intermediate to DCF concomitant with superoxide formation. In cells, the iron-dependent formation of reactive ferryl-oxygen complexes capable of oxidizing DCFH may occur utilizing intracellular hemoproteins (Rice-Evans et al. 1991).

It was reported (LeBel et al. 1992), based on the lack of inhibition by SOD of xanthine/xanthine oxidase-supported DCFH oxidation, that superoxide could not oxidize DCFH. This finding is reasonable, since under standard state conditions superoxide is a better reductant than oxidant, with a half cell reduction potential of –330 mV (Wardman 1989). However, the mechanism for xanthine/xanthine oxidase-mediated oxidation of DCFH was not examined. Our results indicate that xanthine oxidase does not require xanthine or hypoxanthine to oxidize DCFH. In fact, hypoxanthine inhibited DCFH oxidation by xanthine oxidase. The mechanism for this effect is that DCFH is a high affinity substrate for xanthine oxidase, and hypoxanthine acts as a competitive inhibitor of xanthine oxidase-mediated DCFH oxidation.

We conclude that hydroxyl radical and perhaps a ferryl species, but not superoxide, may oxidize DCFH. In addition, xanthine oxidase is able to oxidize DCFH as a high affinity substrate. It would seem likely that other cellular oxidases and peroxidases, in particular peroxisomal sugar and amino acid oxidases, as well as hemoproteins, would be able to oxidize DCFH. The intracellular fluorescence measurements using acetyl-DCFH may reflect the ability of the test agent or toxicant to generate hydroxyl radical. In addition, or superimposed upon this fluorescence, would be the oxidation of DCFH from the release of compartmentalized enzymes to the cytoplasm, resulting from damage to intracellular membranes, and independent from oxidative stress. Therefore, acetyl-DCFH may be a useful and sensitive indicator of cellular toxicity. However, the data presented in this paper do not support the concept that DCFH oxidation specifically represents oxidative stress in cells and tissues.

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