Free Rad. Res., Vol. 26, pp. 37–47
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The Antioxidant Trolox Enhances the Oxidation of 2′,7′-Dichlorofluorescin to 2′,7′-Dichlorofluorescein

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Accepted by Prof. M. Dizdaroglu

(Received 25 March 1996; In revised form 14 June 1996)

The use of antioxidants to prevent intracellular free radical damage is an area currently attracting considerable research interest. The compound 2′,7′-dichlorofluorescin diacetate (DCFH-DA) is a probe for intracellular peroxide formation commonly used in such studies. During our studies we unexpectedly found that incubation of Trolox, a water soluble vitamin E analog, with DCFH-DA in cell-free physiological buffers resulted in the deacetylation and oxidation of DCFH-DA to form the fluorescent compound, 2′,7′-dichlorofluororescein (DCF). The reaction was time-, temperature-, and pH-dependent. Fluorescence intensity increased with an increase in either Trolox or DCFH-DA concentration. These results indicate that even at physiological pH, DCFH-DA can be deacetylated to form 2′,7′-dichlorofluorescin (DCFH). DCFH can then be oxidized to DCF by abstraction of a hydrogen atom by the phenoxyl radical of Trolox. Exposure of the reaction mixture to 10 Gy of ^{60}Co gamma radiation greatly increased production of DCF. Antioxidant compounds reported to "repair" the Trolox phenoxyl radical (e.g., ascorbic acid, salicylate) can also prevent the Trolox-induced DCFH-DA fluorescence. However, compounds that cannot repair the Trolox phenoxyl radical (e.g., catechin) or can themselves form a radical (e.g., uric acid, TEMPOL) either have no effect or can increase levels of DCF. These results demonstrate that experimental design must be carefully considered when using DCFH-DA to measure peroxide formation in combination with certain antioxidants.

Keywords: Trolox, 2′,7′-dichlorofluorescin, fluorescence, free radical, antioxidants

INTRODUCTION

Measurements of intracellular oxidation levels and attenuation of these levels with antioxidants are key components in the study of free radical damage to cells. One procedure to assay intracellular peroxide formation utilizes 2′,7′-dichlorofluorescin diacetate (DCFH-DA).[1–6] This compound is readily transported across the plasma membrane to the interior of the cell. There, intracellular esterases convert the DCFH-DA to 2′,7′-dichlorofluorescin (DCFH) by the removal of the acetate groups.[5] This reaction "locks" the DCFH inside the cell where it can react with intracellular peroxides to form the fluorescent compound 2′,7′-dichlorofluorescein (DCF). The formation of DCF can be followed spectrofluorometrically.

Trolox [6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid], a water soluble vitamin E analog, is a free radical scavenger and inhibitor

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of lipid peroxidation.[7–9] The Trolox phenoxyl radical, resulting from the donation of a hydrogen from Trolox to a free radical, is a relatively stable species that can be "repaired" by ascorbic acid.[10] During our studies of radiation-induced intra-cellular peroxide production, we observed that incubation of DCFH-DA with Trolox in cell-free physiological buffers resulted in increased fluorescence. This finding has led us to investigate the effect of Trolox, alone and in combination with other antioxidants, on the conversion of the non-fluorescent DCFH-DA to the fluorescent DCF. Our results presented here clearly demonstrate that care must be taken when designing and interpreting experiments involving the use of DCFH-DA in conjunction with other antioxidant compounds.

MATERIALS AND METHODS

Materials

2',7'-Dichlorofluorescin diacetate (Molecular Probes, Eugene, OR) was prepared as a 50 mM stock solution in DMSO and stored in 50 µl aliquots in the dark at –20°C. A fresh stock tube of DCFH-DA was used for each experiment. Stock tubes were used once and were not refrozen. Trolox (Aldrich Chemical Co., Milwaukee, WI) was prepared fresh daily as a 300 mM solution in 1 M $NaHCO_3$ and the pH adjusted to pH 7.0.[11] All other materials were purchased from the Sigma Chemical Co. (St. Louis, MO) with the exception of TEMPOL, which was obtained from the Aldrich Chemical Co. All antioxidants were prepared just prior to use.

Experimental Conditions

A typical reaction contained 25 µM DCFH-DA and 10 µM Trolox in HMCK buffer (50 mM HEPES pH 7.4; 5 mM $MgCl_2$; 3 mM $CaCl_2$; 25 mM KCl) and was incubated at 37°C in the dark for 24 h. Fluorescence was measured with an SLM 8000 spectrofluorometer (SLM/Aminco Instruments, Urbana, IL) in a stirred cuvette with excitation and emission wavelengths at 485 and 530 nm (4 nm band width), respectively. Any additions to the reaction mixture or changes in the reaction conditions are as noted in the figure legends.

The following protocol was used for experiments testing the effect of various antioxidants on Trolox-induced DCFH fluorescence. Reaction mixtures contained 25 µM DCFH-DA in HMCK buffer and 10 µM or 10 mM of the antioxidant, alone or in the presence of 10 µM Trolox. The samples were incubated in the dark at 37°C for 24 h and the fluorescence intensity determined spectrophotometrically using the parameters described above. For samples containing no Trolox, the fluorescence intensity values (machine values) obtained for each antioxidant-containing sample was normalized to the values obtained for samples containing no antioxidant (control). In this case, the data are expressed as "% of Control". For reactions run in the presence of Trolox, the fluorescence intensity values obtained for the antioxidant-containing samples in the presence of 10 µM Trolox were normalized to the values obtained for samples containing Trolox alone (no other antioxidant present). In this case, the data are expressed as "% of Trolox".

Irradiation Conditions

Irradiations were performed with the Armed Forces Radiobiology Research Institute's ^{60}Co gamma radiation source at room temperature using bilateral exposures at a dose rate of 0.5 Gy/min for a total dose of 10.0 Gy.

RESULTS

As shown in Figure 1, incubation of Trolox with DCFH-DA in HMCK buffer at 37°C resulted in a time-dependent increase in the fluorescence of DCF. Initially there was a lag in the Trolox-induced DCF fluorescence, however, between 2 and 4 h the fluorescence intensity of the Trolox-

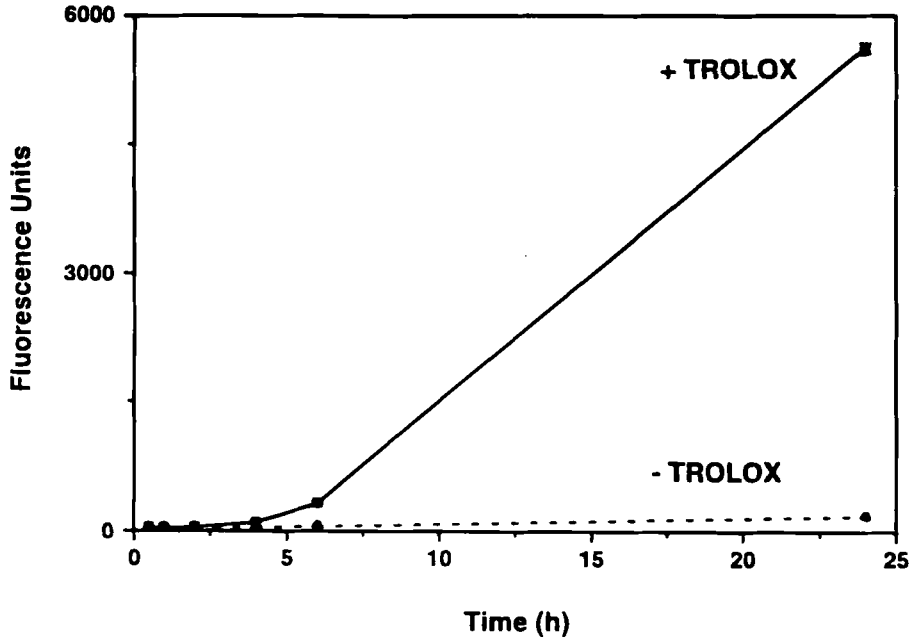

FIGURE 1 Effect of Trolox on oxidation of DCFH-DA.
Reactions contained 25 μM DCFH-DA in HMCK buffer and were incubated in the dark at 37°C for indicated times in presence (■----■) or absence (●----●) of 10 mM Trolox. Oxidation of DCFH-DA to DCF was determined spectrofluorometrically. Data represents the mean (± s.e.m.) of 6 independent determinations. Error bars smaller than symbols are not visible on the graph.

containing samples more than doubled. After a 24 h incubation the fluorescence intensity of the Trolox-containing samples was over 30-fold greater than the samples without Trolox. The UV spectra of the reaction mixtures demonstrated an increase in absorption at 503 nm that correlated with the increased DCF fluorescence. This is in agreement with previously published work that showed DCF has a λ_{max} of 503 nm.[2,5]

There is a significant temperature effect on the Trolox-induced oxidation of DCFH-DA (Fig. 2). Virtually no change in DCF fluorescence was observed in the presence or absence of Trolox at 4, 15, or 23°C. However, at incubation temperatures of 37 and 42°C there was a substantial increase in the level of fluorescence associated with the Trolox-containing samples. The pH of the reaction mixture also plays a significant role in Trolox-induced DCF oxidation (Fig. 3). Below pH 7.0 there was very little fluorescence of DCF as a result of the presence of Trolox; however, the Trolox-induced DCF fluorescence increased greatly at pHs greater than 7.0. Buffer composition does not affect Trolox-induced DCF fluorescence. Different buffers (e.g., Tris, HEPES, PIPES, phosphate) at the same pH and concentration did not affect the level of DCF fluorescence observed in the presence or absence of Trolox. In addition, common buffer components ($MgCl_2$, $CaCl_2$, KCl) also had no effect. Therefore, for our studies, we utilized the same buffer used previously for our cellular studies (HMCK buffer).

The extent of DCF fluorescence resulting from the presence of Trolox in the reaction mixture depends on both DCFH-DA and Trolox concentration. As shown in Figure 4A, as the concentration of Trolox in the reaction mixture was increased from 10 μM to 10 mM, the DCF-associated fluorescence also increased. Increasing the DCFH-DA concentration (5 to 100 μM) in the presence of a fixed concentration of Trolox also resulted in increased DCF fluorescence (Fig. 4B), but not to the extent seen by increasing the Trolox concentration.

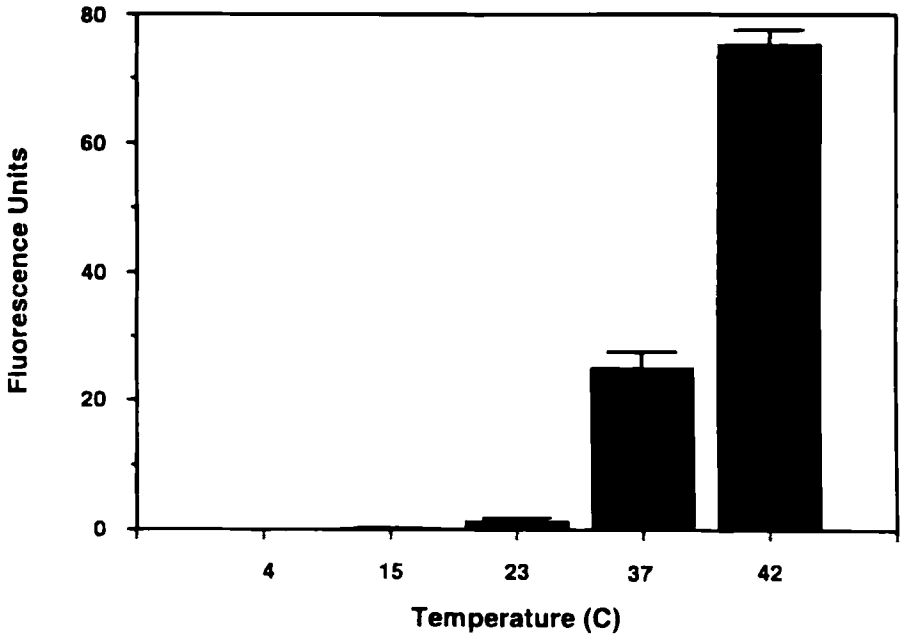

FIGURE 2 Effect of temperature on Trolox-induced DCFH-DA oxidation.

Reaction mixtures containing 25 μM DCFH-DA in HMCK buffer were incubated in the dark for 24 h at indicated temperatures. Fluorescence values of samples incubated without Trolox were subtracted from those containing Trolox (10 μM). Data represent the mean (± s.e.m.) of 3 independent experiments.

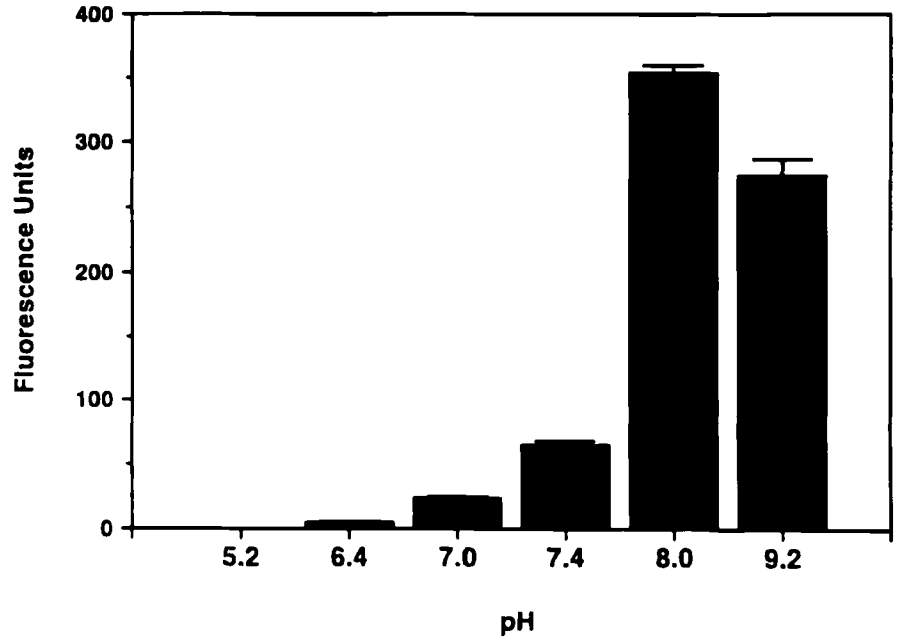

FIGURE 3 Effect of pH on Trolox-induced DCFH-DA oxidation.

Reaction mixtures, containing 25 μM DCFH-DA in presence and absence of 10 mM Trolox, were incubated in the dark at 37°C for 2 h in 50 mM of the following buffers: acetate pH 5.2, PIPES pH 6.4, phosphate pH 7.0, HEPES pH 7.4, and Tris pH 8.0 and 9.2. Fluorescence intensity was determined and values obtained for control reactions (no Trolox) subtracted from values obtained for the samples containing Trolox. Data represent the mean (± s.e.m.) of 3 independent experiments.

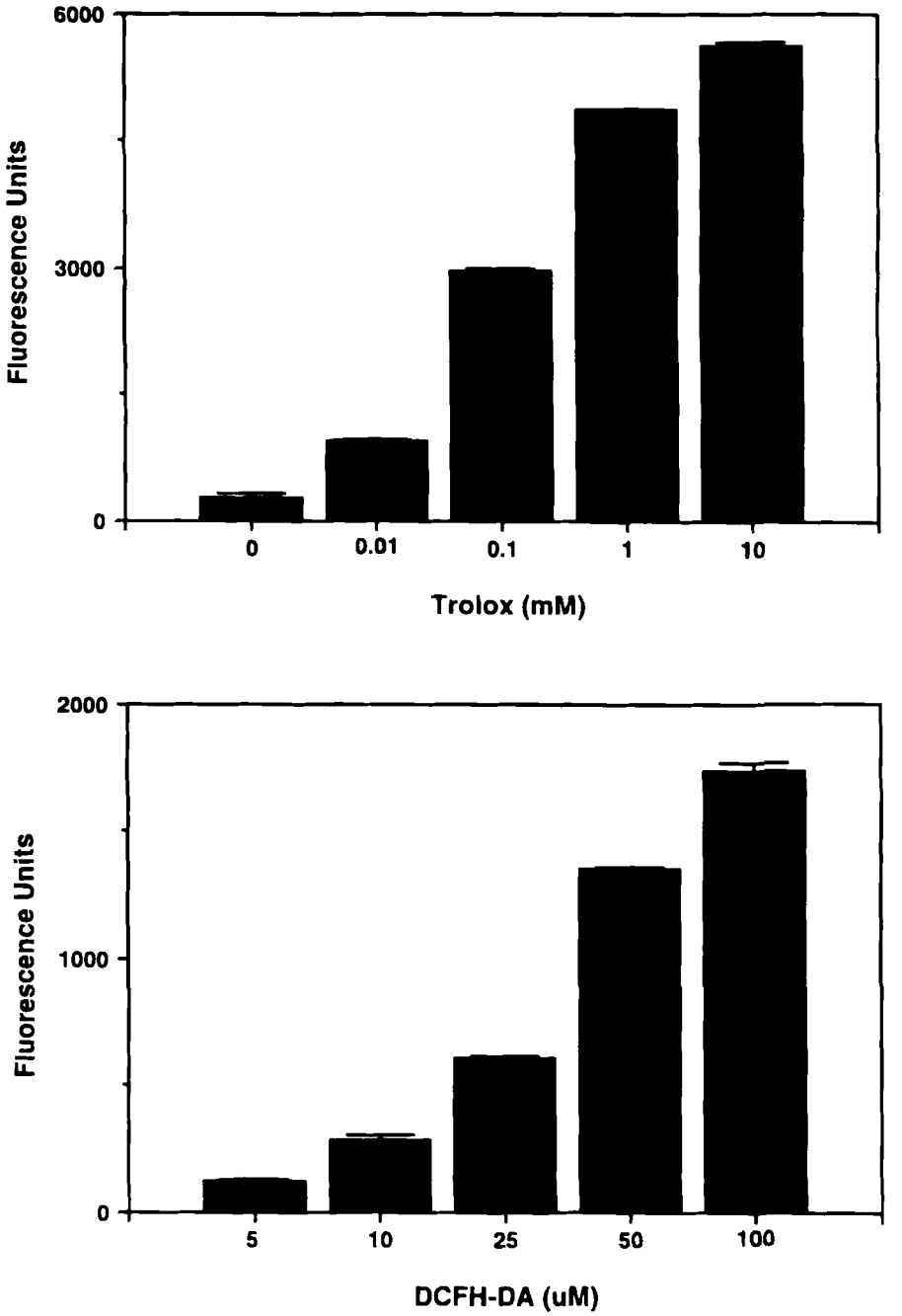

FIGURE 4 Effect of increasing concentrations of Trolox (Panel A) and DCFH-DA (Panel B) on Trolox-induced DCFH-DA oxidation. Reaction mixtures contained 25 μM DCFH-DA in HMCK buffer with increasing concentrations of Trolox (0 to 10 mM, Panel A) or 10 μM Trolox in HMCK buffer with increasing concentrations of DCFH-DA (5 to 25 μM, Panel B). Samples were incubated in the dark at 37°C for 24 h before fluorescence intensity was measured. Data represent mean (± s.e.m.) of 6 independent experiments.

After observing the initial lag in Trolox-induced DCF fluorescence, we attempted to determine if a prior incubation of either Trolox or DCFH-DA in buffer, followed by addition of the missing component affected the level of DCF fluorescence. Figure 5 shows the results of these preincubation experiments. Preincubation of DCFH-DA (25 μM) in HMCK buffer for 24 h at 37°C prior to the addition of Trolox (10 mM) resulted in substantially greater DCF fluorescence over the sampling period than that obtained with either control or Trolox preincubation reactions. This increase in fluorescence intensity occurred almost immediately following the addition of Trolox to the preincubated DCFH-DA. On the other hand, the control and Trolox preincubation reactions continued to show a lag period of approximately 2 h before fluorescence intensity increased, with the Trolox preincubation exhibiting somewhat greater fluorescence intensity than did the controls.

Since a major focus of our research is the role of radiation-induced free radicals in the cell, we investigated the effect of ^{60}Co gamma radiation exposure on Trolox-induced DCFH-DA oxidation. As shown in Figure 6 the fluorescence intensity of both the control (0 Gy) and irradiated (10 Gy) samples exhibited a lag or plateau period lasting approximately 2 h post-irradiation, although the 10 Gy samples had a 2- to 3-fold greater fluorescence intensity during this period. After 2 h the fluorescence intensity of the irradiated samples increased at a much greater rate than the unirradiated samples, and, by 6 h post-irradiation, the irradiated samples showed a 7-fold higher fluorescence intensity than unirradiated controls. Lower radiation doses (2.5 and 5.0 Gy) had no effect on Trolox-induced DCFH-DA oxidation (data not shown). In

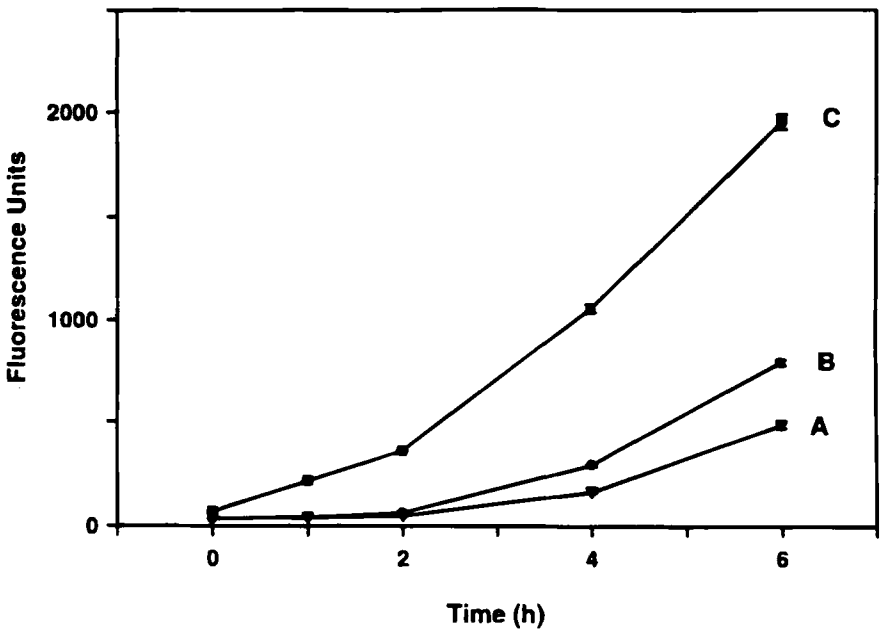

FIGURE 5 Effect of "preincubation" on Trolox-induced DCFH-DA oxidation.

Curve A (buffer preincubation): HMCK buffer was incubated in the dark at 37°C for 24 h after which DCFH-DA and Trolox were added and incubation continued for an additional 6 h. Curve B (Trolox preincubation): HMCK buffer and Trolox were incubated in the dark at 37°C for 24 h after which DCFH-DA was added and incubation continued for an additional 6 h. Curve C (DCFH-DA preincubation): HMCK buffer and DCFH-DA were incubated in the dark at 37°C for 24 h after which Trolox was added and incubation continued for an additional 6 h. Fluorescence intensity was measured immediately following (within 2 min) addition of the missing reaction component(s) and then at 1, 2, 4, and 6 h post-addition. In all cases final DCFH-DA concentration was 25 μM and final Trolox concentration was 10 mM. Data represent mean (± s.e.m.) of 6 independent determinations.

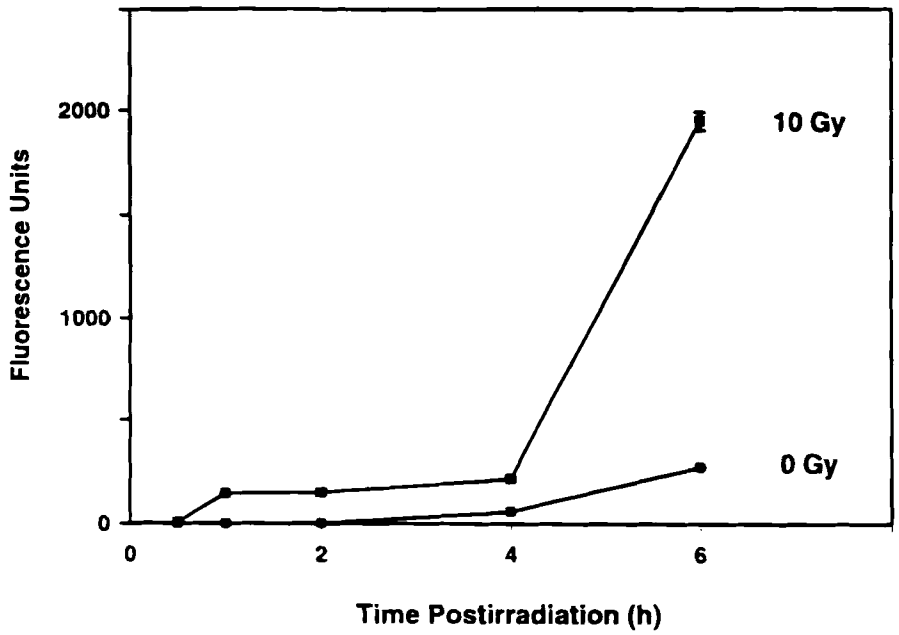

FIGURE 6 Effect of 10 Gy ^{60}Co gamma radiation on Trolox-induced DCFH-DA oxidation.
Reaction mixtures contained 25 μM DCFH-DA in HMCK buffer with or without 10 mM Trolox. Samples received either 0 Gy (unirradiated control) or 10 Gy of ^{60}Co gamma radiation (dose rate 0.5 Gy/min) and were incubated at 37°C in the dark. Fluorescence intensity was measured at 0.5, 1, 2, 4, and 6 h post-irradiation and values obtained for samples containing no Trolox were subtracted from appropriate Trolox-containing sample values. Data represent the mean (± s.e.m.) of 3 independent experiments.

addition, radiation exposure had no effect on the oxidation of DCFH-DA in the absence of Trolox (data not shown).

To determine if the capacity to induce the oxidation of DCFH-DA was unique to Trolox we tested whether a variety of other antioxidants, alone or in combination with Trolox, can oxidize DCFH-DA. As shown in Table I, compounds such as ascorbic acid and glutathione, which can repair the phenoxyl radical of Trolox,[10] could also prevent the Trolox-induced oxidation of DCFH-DA. However, glutathione alone also oxidized DCFH-DA, although to a much lesser extent than Trolox. Compounds that are free radicals (e.g., TEMPOL) or can form stable radicals (catechin, uric acid, n-propyl gallate) appear to be able to oxidize DCFH-DA and, in some cases, also enhance the Trolox-induced oxidation of DCFH-DA. Quercetin, although structurally similar to catechin, behaved differently. The presence of quercetin in the reaction mixture not only eliminated the Trolox-induced oxidation of DCFH-DA but also inhibited the endogenous oxidation of DCFH-DA to much the same extent as ascorbic acid. Antioxidants such as superoxide dismutase, catalase, ethylene glycol, ethanol, vitamin E, butylated hydroxytoluene, and dihydrolipoic acid, alone, did not enhance the oxidation of DCFH-DA, nor did they have any effect on the Trolox-induced oxidation of DCFH-DA (data not shown).

DISCUSSION

The compound 2′,7′-dichlorofluorescin diacetate (DCFH-DA) is an important and widely-used tool with which to study intracellular peroxide formation. In the diacetate form the compound readily crosses the plasma membrane. Once inside, the diacetate groups are removed by cellular esterases to yield 2′,7′-

TABLE I

Compound	Concentration	% of Control	% of Trolox
Trolox	10 μM	949	—
	10 mM	5617	—
Ascorbic Acid	10 μM	76	68
	10 mM	28	4
Glutathione	10 μM	364	76
	10 mM	381	22
Salicylate	10 μM	116	154
	10 mM	93	75
Catechin	10 μM	149	164
	10 mM	882	99
TEMPOL	10 μM	394	172
	10 mM	4386	404
Uric Acid	10 μM	342	242
	10 mM	6174	474
Quercetin	10 μM	60	91
	10 mM	26	4
n-Propyl Gallate	10 μM	58	19
	10 mM	2190	259

Experimental details are given in the text. Values listed in "% of Control" give an indication of the ability of that particular antioxidant to oxidize DCFH-DA. Values listed in "% of Trolox" give an indication of the ability of that particular antioxidant to inhibit or enhance the Trolox-induced oxidation of DCFH-DA. Data are the average of three independent determinations.

dichlorofluorescin (DCFH). Neither DCFH-DA nor DCFH is fluorescent, but abstraction of a hydrogen atom from DCFH results in the formation of 2′,7′-dichlorofluorescein (DCF), which is fluorescent. These reactions have been used to quantitate intracellular peroxides by us[12] and others.[5,13–15]

As a part of our earlier work with DCFH-DA, we made several puzzling observations. As expected, DCFH-DA incubated with buffer alone produced low levels of fluorescence. However, when the antioxidant drug Trolox, a water-soluble analog of vitamin E, was included in the buffer with DCFH-DA, we observed a substantial increase in fluorescence intensity. Because of the possibility that factors such as this might influence the interpretation of our experiments assessing the antioxidant effect of Trolox, we sought to investigate the role Trolox and other antioxidants might play in promoting the conversion of DCFH-DA to the fluorescent DCF.

A plausible mechanism of action was not immediately clear, because neither the structure of Trolox nor the species involved in the DCFH-DA conversion suggested any obvious reaction between them that would tend to enhance the production of DCF. Our experiments indicate that the keys to understanding the Trolox effect are (1) the fact that the antioxidant Trolox, when oxidized, can serve as an effective reducing agent and (2) the oxidized Trolox potentiates the conversion of DCFH, the intermediate produced as a result of the deesterification of DCFH-DA, to the fluorescent DCF. While no reports have emerged on the Trolox-induced oxidation of DCFH, the prooxidant properties of Trolox have been studied in several experimental systems investigating oxidative damage to DNA,[16] lipids,[17] and proteins.[18] The ability to bind and reduce metabolically important metals such as iron[16,17] and copper[18] appears to be the key step in Trolox's prooxidant behavior in these systems.

It is known that Trolox exerts its antioxidant effect through its capacity to scavenge free radicals. Trolox in solution reacts with free radicals—

spontaneously or experimentally induced—to produce the relatively stable phenoxyl radical (see Fig. 7). One normally considers the activity of antioxidants in terms of their capacity to reduce oxidized species. During this process, some antioxidants are themselves oxidized. The phenoxyl (oxidized) form of Trolox is such an example. Trolox is an effective antioxidant because the more stable phenoxyl radical is far less reactive than the species it reduced. This does not mean the phenoxyl radical is unreactive, however. The phenoxyl form has the capacity to oxidize other species, regenerating the native form of Trolox in the process.

We believe the DCFH intermediate is such a substrate for the Trolox phenoxyl radical, a reaction whose products would be Trolox and fluorescent DCF. One conceptual problem with this hypothesis is that deesterification of DCFH-DA was previously thought to occur only at high pH[2] or as a result of esterase activity.[5] Our experiments have shown, however,

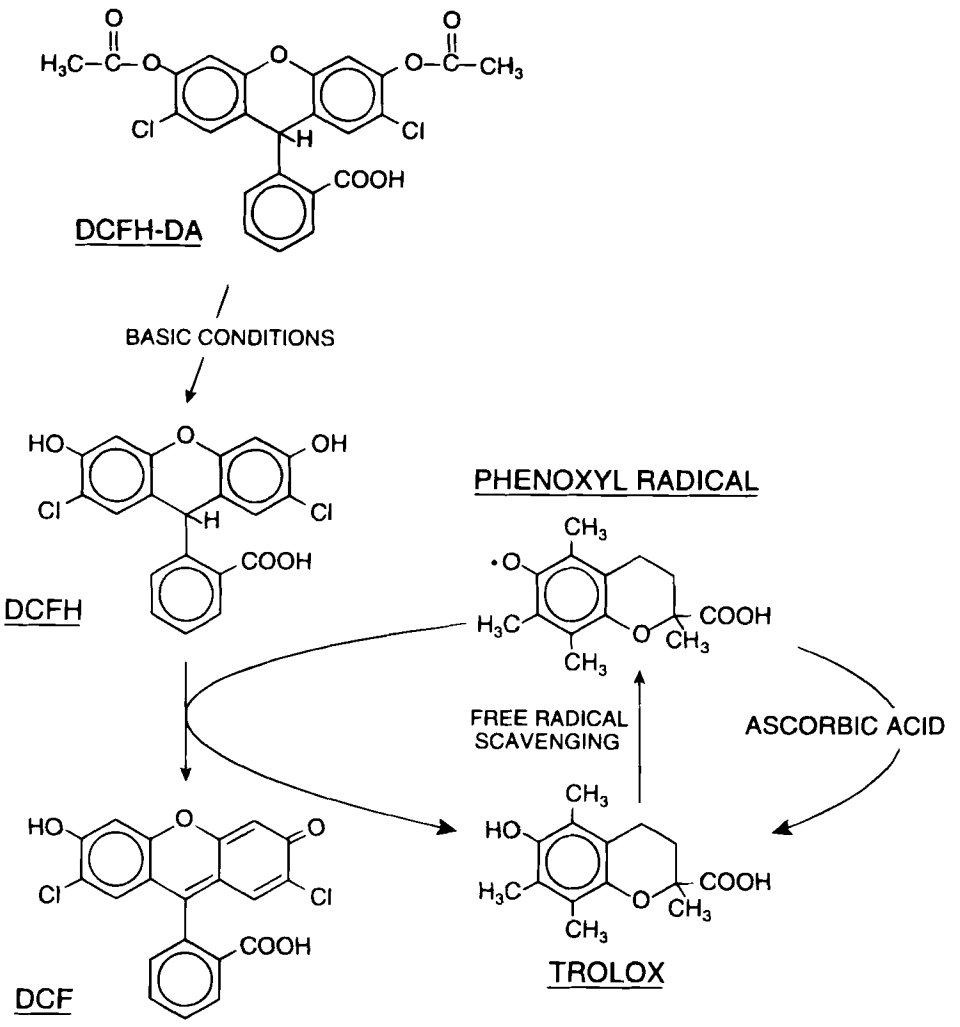

FIGURE 7 Possible mechanism of Trolox-induced DCFH-DA oxidation.
Schematic showing the activation of DCFH-DA to DCFH and the role of Trolox in the oxidation of DCFH to the fluorescent DCF. Details are given in the text.

that deacetylation can also occur at physiological pH in the absence of esterases (Fig. 3). Incubation of DCFH-DA in even the slightly alkaline (pH 7.4) HMCK buffer used in our experiments is sufficient to induce deacetylation. It should be stated that we did not measure the production of DCFH in our experiments directly because it is very unstable and, as a result, difficult to measure. However, its existence has been demonstrated by others.[2] Even in the absence of any other evidence, based on the chemistry of the overall reaction, one could also correctly infer its role as an obligatory intermediate in the conversion to DCF.

In the absence of any potentiating conditions, the abstraction of hydrogen from DCFH to form DCF does not occur readily. When DCFH-DA was incubated with buffer (pH 7.4) at 37°C for periods up to 24 h, virtually no change in fluorescence occurred. However, when Trolox (10 mM) was added to the incubation mixture, there was a slow but steady increase in fluorescence intensity that was 30 times greater than controls by 24 h (Fig. 1). Formation of DCF in such experiments was temperature-dependent (Fig. 2) and was most effective around pH 8 (Fig. 3). Preincubating DCFH-DA overnight before adding Trolox enhanced the production of DCF (Fig. 5), presumably because there was a greater quantity of DCFH present in the reaction mixture at that time to react with the Trolox phenoxyl radical. An overnight incubation of buffer containing Trolox, followed by the addition of DCFH-DA produced somewhat higher levels of fluorescence than controls (buffer preincubated alone, followed by addition of DCFH-DA and Trolox), but much less fluorescence than that obtained by preincubating DCFH-DA. The low levels of free radicals that would presumably be spontaneously present in such an incubation could convert small amounts of Trolox to the phenoxyl radical form that would facilitate the oxidation of any DCFH produced after the addition and deesterification of DCFH-DA. Exposing solutions containing DCFH-DA and Trolox to 10 Gy gamma radiation greatly enhanced the production of fluorescence, probably by increasing the number of free radicals available to react with Trolox to produce the phenoxyl radical form. Irradiation of solutions containing DCFH-DA without Trolox failed to produce a significant increase in fluorescence, which shows that free radicals produced by the radiation do not directly oxidize the intermediate DCFH to DCF.

Further support for the role of the Trolox phenoxyl radical is provided by the observation that compounds having the capacity to form stable, oxidizing radicals also promote the production of the fluorescent DCF (Table I). Many are more effective than Trolox. If the formation of the Trolox phenoxyl radical is important in this process, one would expect that reactions that suppress the levels of the phenoxyl radical in solution would also inhibit the conversion to DCF. This was the case. For example, ascorbic acid (10 mM), which has been shown to reduce the Trolox phenoxyl radical to its native form,[10] almost completely eliminated the Trolox-mediated oxidation of DCFH-DA.

To summarize, we have shown that the incubation of DCFH-DA with Trolox and other antioxidants can promote the production of significant levels of the fluorescent DCF in the absence of peroxides. Trolox, when oxidized by free radicals generated spontaneously or as a result of irradiation, forms the Trolox phenoxyl radical that oxidizes the intermediate DCFH to produce DCF. We have also shown that the deesterification of DCFH-DA to produce the DCFH intermediate occurs in even slightly alkaline physiological buffers in the absence of esterases. The results presented here demonstrate that caution must be taken when designing and interpreting any experiments using DCFH-DA in the presence of certain antioxidant compounds.

Acknowledgements

This work was supported by the Armed Forces Radiobiology Research Institute.

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