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Oxidation of 2′,7′-Dichlorofluorescin by Peroxynitrite

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The simultaneous production of nitric oxide and superoxide anion leads to the formation of peroxynitrite, a potent oxidant which may be an important mediator of cellular injury. Oxidation of dichlorofluorescin to the fluorescent dichlorofluorescein has been used as a marker for cellular oxidant production. The mechanisms of peroxynitrite-mediated oxidation of dichlorofluorescin to dichlorofluorescein were investigated. Chemically synthesized peroxynitrite (50–500 nM) induced the oxidation of dichlorofluorescin to dichlorofluorescein in a linear fashion. In addition, the simultaneous generation of nitric oxide and superoxide anion induced the oxidation of dichlorofluorescin to dichlorofluorescein, while nitric oxide (1–10 μM) alone under aerobic conditions did not. Peroxynitrite-mediated oxidation of dichlorofluorescin was not inhibited by the hydroxyl radical scavengers mannitol (100 mM) or dimethylsulfoxide (100 mM). Moreover, peroxynitrite-mediated oxidation of dichlorofluorescin was not dependent upon metal ion-catalyzed reactions. Furthermore, dichlorofluorescein formation was diminished at alkaline pH. These findings suggest that peroxynitrite-mediated dichlorofluorescein formation results directly from the protonation of peroxynitrite to form the conjugate peroxynitrous acid. L-cysteine was an efficient inhibitor ($K_I \approx 25$ μM) of dichlorofluorescin oxidation through competitive oxidation of free sulfhydryls. Urate was a less efficient with a maximum inhibition of only 49%. These results demonstrate that dichlorofluorescin is efficiently oxidized by peroxynitrite. Therefore, under conditions where nitric oxide and superoxide are produced simultaneously, oxidation of dichlorofluorescin may be mediated by the formation of peroxynitrite.

Keywords: Peroxynitrite, Nitric oxide, Superoxide, Dichlorofluorescin, Dichlorofluorescein, Hydrogen peroxide

INTRODUCTION

Superoxide anion production is a critical component of an appropriate immune response, however, superoxide anion production may also mediate tissue injury in a variety of pathological processes including inflammation, ischemia-reperfusion, and hyperoxia. Since the direct oxidative toxicity of superoxide is limited,[1] tissue injury results from the secondary formation of more potent oxidants, often proposed to be derived from hydrogen peroxide, the dismutation product of superoxide. Oxidation of non-fluorescent 2′,7′-dichlorofluorescin to the fluorescent compound, 2′,7′-dichlorofluorescein, has frequently been used to indicate the cellular

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formation of reactive oxygen species.[2,3] Although often interpreted as representing an increase in cellular hydrogen peroxide production, hydrogen peroxide-dependent oxidation of dichlorofluorescin is dependent on the secondary, metal-catalyzed formation of more potent oxidizing species such as hydroxyl radical or ferryl ion and is not mediated by hydrogen peroxide directly.[4,5]

Independent of hydrogen peroxide formation, superoxide also reacts at a near diffusion-limited rate with nitric oxide ($k = 6.7 \times 10^9$ M^{-1} s^{-1})[6] to form the potent oxidant peroxynitrite. Peroxynitrite anion has a pK_a of 6.8 and is therefore approximately 20 percent protonated under physiological conditions. The resulting peroxynitrous acid decomposes yielding oxidants with reactivities similar to hydroxyl radical and nitrogen dioxide.[7] As a consequence, peroxynitrite has strong oxidizing properties toward biological molecules, including protein and non-protein sulfhydryls,[8] deoxyribonucleic acid,[9] and membrane phospholipids.[10] In isolated cell systems peroxynitrite production has been demonstrated from Kupffer cells,[11] alveolar macrophages,[12] polymorphonuclear leukocytes,[13] and endothelial cells.[14] In addition, *in vivo* studies have demonstrated the presence of peroxynitrite in animal models of endotoxemia,[15,16] lung injury,[16,17] and ileitis,[18,19] as well as in human atherosclerotic plaques,[20] adult respiratory distress syndrome,[21] myocardial inflammation,[22] and multiple sclerosis.[23] Peroxynitrite efficiently oxidizes the fluorescent probe dihydrorhodamine 123[24] and other organic molecules commonly used for the detection of hydrogen peroxide.[25] Therefore, oxidation of dichlorofluorescin to dichlorofluorescein may not represent hydrogen peroxide production, but may be mediated, in part, by cellular peroxynitrite production.

In this study, we evaluate the mechanisms of peroxynitrite-mediated oxidation of dichlorofluorescin. These results demonstrate that dichlorofluorescin is efficiently oxidized by peroxynitrite. Therefore, under conditions where nitric oxide and superoxide are produced simultaneously, oxidation of dichlorofluorescin may be mediated by the formation of peroxynitrite.

MATERIALS AND METHODS

Materials

Dichlorofluorescin diacetate and dichlorofluorescein were from Eastman Kodak (Rochester, NY). Bovine liver Cu,Zn superoxide dismutase was from DDI Pharmaceuticals (Mountain View, CA). Peroxynitrite was synthesized in a quenched flow reactor as previously described[7] and stored in 0.3 N NaOH at –20°C. The concentration of the stock peroxynitrite was determined daily by the absorbance at 302 nm (E = 1670 M^{-1} cm^{-1}) of stock peroyxnitrite diluted in 1 N NaOH versus stock peroxynitrite allowed to decompose in buffer. The buffer used for all studies was 90 mM sodium chloride, 50 mM sodium phosphate, 5 mM potassium chloride, pH 7.4, prepared with high quality deionized water and passed over a Chelex-100 column to remove residual iron. Subsequent to passage over the column, 100 μM diethylenetriaminepentaacetic acid (DTPA) (Sigma Chemical Co., St. Louis, MO) was added. All other reagents were of analytical quality.

Methods

Stock solutions of dichlorofluorescin diacetate (33.4 mM) and dichlorofluorescein (9.97 mM) in ethanol were purged with nitrogen and stored at –20°C. Dichlorofluorescin was prepared from dichlorofluorescin diacetate by addition of 0.01 N NaOH at room temperature for 20 min followed by dilution in buffer and readjusting the pH to 7.4 with HCl. Dichlorofluorescin was oxidized to dichlorofluorescein with 100 μM hydrogen peroxide and 26 μg/ml horseradish peroxidase. Fluorescent intensity and peak excitation and emission wavelengths of 502 and 523 nm respectively were similar for both prepared and com-

mercially available dichlorofluorescein. The concentration of dichlorofluorescein in experiments was determined by comparison with the fluorescent intensity of a 0.05 μM dichlorofluorescein standard which was determined daily. In working solutions, the concentration of dichlorofluorescin after deacetylation of dichlorofluorescin diacetate was determined by oxidation with hydrogen peroxide plus horseradish peroxidase and comparison to the 0.05 μM dichloro-fluorescein standard.

Studies were performed at 37°C using pre-equilibrated buffer and a thermostatically controlled cuvette holder. Working solutions of dichlorofluorescin were purged with nitrogen and placed on ice in the dark until immediately prior to the study, when they were placed in the water bath at 37°C. Peroxynitrite-mediated oxidation of dichlorofluorescin to dichlorofluorescein was rapid and the resulting fluorescent signal was stable. The final fluorescent intensity measurements were performed 5 min after addition of peroxynitrite. Reported values are the means ± SD for the final fluorescent intensity minus background fluorescent intensity. For studies investigating the effect of change in pH on peroxynitrite-mediated dichlorofluorescin oxidation, pH of the dichlorofluorescin solution was measured prior to and after the addition of peroxynitrite. The addition of peroxynitrite resulted in relatively small changes in pH with maximum changes occurring at the extremes of pH used (from 3.6 to 4.1 and from 10.0 to 10.3) and changes in pH < 0.05 noted for pH values between 6.0 and 7.6. Results are given in relation to the pH values after peroxynitrite addition.

RESULTS

Peroxynitrite-Mediated Oxidation of Dichlorofluorescin

Peroxynitrite-mediated oxidation of dichlorofluorescin as a function of dichlorofluorescin concentration is shown in Figure 1. Approximately 20 μM dichlorofluorescin was required to compete with the spontaneous rate of isomerization of 200 nM peroxynitrite to nitrate at 37°C and pH 7.4 At saturating dichlorofluorescin concentrations the molar yield was 0.37 ± 0.02 mole dichlorofluorescein formed per mole peroxynitrite. Dichlorofluorescin diacetate was not susceptible to oxidation by peroxynitrite and concentrations of peroxynitrite up to 1 μM did not alter the fluorescent intensity of dichlorofluorescein.

Peroxynitrite caused the oxidation of dichlorofluorescin (20 μM) to dichlorofluorescein in a linear fashion over the range from 0 to 500 nM (Fig. 2). The slope of the line reflects the stoichiometry of peroxynitrite-mediated oxidation of dichlorofluorescin. The change in dichlorofluoroscein concentration was 0.33 ± 0.04 mole dichlorofluorescein formed per mole peroxynitrite, consistent with the value obtained from Figure 1.

Alterations in pH had profound effects on the apparent dichlorofluorescein yield from peroxynitrite-mediated oxidation of dichlorofluorescin (Fig. 3). Acidic pH also had a significant effect on the fluorescent intensity for a known concentration of dichlorofluorescein (data not shown). When fluorescent intensity yields for the reaction of peroxynitrite with dichlorofluorescin were adjusted for the direct effect of pH on dichlorofluorescein fluorescent intensity, there was still a significant reduction in the oxidation of dichlorofluorescin to dichlorofluorescein at acidic and alkaline pH values (Fig 3). Time-dependent oxidation of 20 μM dichlorofluorescin by 200 nM peroxynitrite was measured at pH 4.0, 7.4, and 9.0. For all pH values, stable fluorescent intensity was achieved in less than 2 min and remained stable for longer than the 5 min measurement period. Therefore, our results are end-point measurements under all experimental conditions.

To eliminate the possibility that molecular changes which render dichlorofluorescein less fluorescent at acidic pH might also render

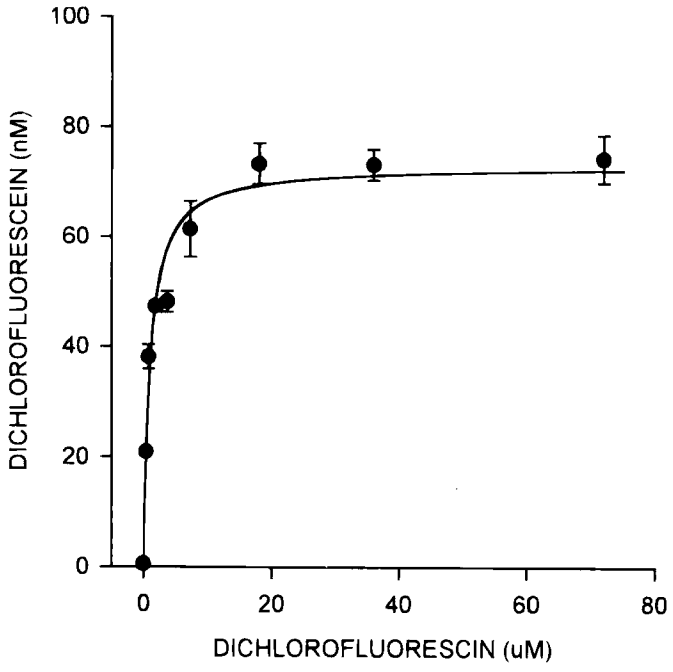

FIGURE 1 Peroxynitrite-mediated oxidation of dichlorofluorescin as a function of dichlorofluorescin concentration. Peroxynitrite (200 nM) was added to increasing concentrations of dichlorofluorescin at 37°C and pH 7.4. Dichlorofluorescein concentrations were determined from fluorescent intensity measurements. The curve shown is the result of non-linear regression analysis of the measured values. Values are means ± SD, n = 4.

dichlorofluorescin less susceptible to oxidation, the rate of oxidation of 20 μM dichlorofluorescin to dichlorofluorescein by 0.5 μg/ml horseradish peroxidase was measured at pH 7.4 and 5.0. The concentration of dichlorofluorescein was determined from a 50 nM standard determined at each pH. At pH 7.4, dichlorofluorescein concentration increased at a rate of 0.49 ± .005 nM/sec (n = 4) and at pH 5.0, dichlorofluorescein concentration increased at a rate of 0.86 ± .006 nM/sec. There fore, dichlorofluorescin was readily oxidized by horseradish peroxidase at acidic pH.

Effect of Oxidant Scavengers on Peroxynitrite-Mediated Dichlorofluorescin Oxidation

The effects of oxidant scavengers on oxidation of dichlorofluorescin by peroxynitrite are shown in Figure 4. L-cysteine, as a source of sulfhydryl groups, was the most efficient inhibitor resulting in a 90% reduction in oxidation. In the presence of L-cystine, the oxidation product of cysteine which lacks sulfhydryl groups, oxidation was slightly (18%) but significantly enhanced. Urate resulted in a modest (25%) reduction in peroxynitrite-mediated oxidation while the urate analogs oxypurinol and allopurinol had no inhibitory effect. Desferrioxamine also had a modest inhibitory effect at both 0.1 mM (32% reduction) and 1 mM (30% reduction). The hydroxyl radical scavengers, mannitol and dimethylsulfoxide, and superoxide dismutase at 1 μM and 10 μM, had no effect on dichlorofluorescin oxidation. The concentration dependent inhibition of peroxynitrite-mediated oxidation of dichlorofluorescin to dichlorofluorescein by L-cysteine is shown in Fig 5A. At an L-cysteine concentration of 25 μM, oxidation was reduced by 50% . The concentration dependent effects of urate are shown in Figure 5B. Even at high urate

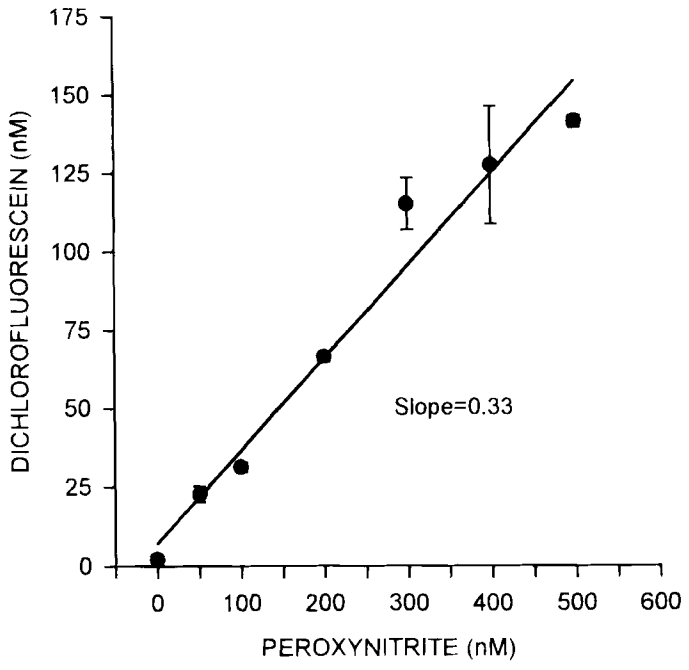

FIGURE 2 Peroxynitrite-dependent oxidation of dichlorofluorescin. Increasing concentrations of peroxynitrite were added to 20 μM dichlorofluorescin at 37°C and pH 7.4. Dichlorofluorescein concentrations were determined from fluorescent intensity measurements. Values are means ± SD, n = 4.

concentration (5 mM), the maximum reduction achieved was 49%.

Oxidation of Dichlorofluorescin by Nitric Oxide and Superoxide

Previous work has shown that hydrogen peroxide will not oxidize dichlorofluorescin at concentrations ranging from 10–100 mM. However, oxidants generated by either hydrogen peroxide plus metal or by xanthine oxidase can oxidaze dichlorofluorescin.[4,5] Figure 6A demonstrates the rate of dichlorofluorescin oxidation by hypoxanthine plus xanthine oxidase. Bolus addition of 1 mM nitric oxide in an identical reaction mixture of hypoxanthine plus xanthine oxidase increased the rate as well as the yield of dichlorofluorescin oxidation. Bolus additions of nitric oxide up to 10 μM under aerobic conditions did not oxidize dichlorofluorescin at 37°C for over 1 hour. These data demonstrate that the formation of peroxynitrite from the reaction of nitric oxide and superoxide oxidizes dichlorofluorescin as shown for chemically synthesized peroxynitrite. Confirmatory data is demonstrated in Figure 6B. Base catalysis of SIN-1 will result in the release of both superoxide and nitric oxide in solution. The peroxynitrite formed from the decomposition of SIN-1 was found to oxidize organic molecules such as dihydrorhodamine 123.[25] In the absence of superoxide dismutase, decomposition of SIN-1 at pH 7.4 and 37°C oxidized dichlorofluorescin. However, addition of sufficient superoxide dismutase to dismutate superoxide to hydrogen peroxide prevents the oxidation of dichlorofluorescin. This data confirms previous and current observations that neither nitric oxide nor hydrogen peroxide will oxidize organic molecules such as dichlorofluorescin and dihydrorhodamine 123[25] whereas peroxynitrite will efficiently oxidize these molecules.

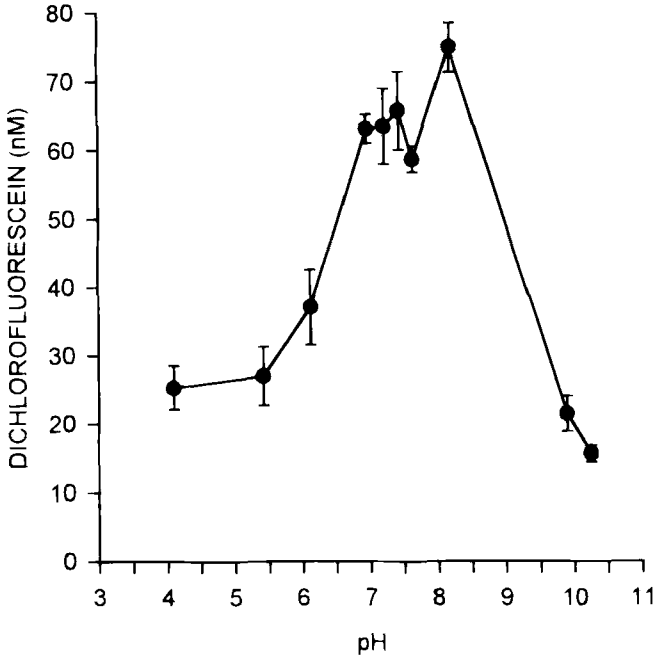

FIGURE 3 Effect of pH on peroxynitrite-mediated oxidation of dichlorofluorescin. The buffer pH was adjusted approximately to the range shown. Peroxynitrite (200 nM) was added to 20 μM dichlorofluorescin at 37°C. Dichlorofluorescein concentrations were determined from fluorescent intensity measurements. The pH values shown were determined from the reaction solution after completion of the fluorescent intensity measurement. The results are corrected for the effect of pH on dichlorofluorescein fluorescence. Values are means ± SD, n = 4.

DISCUSSION

Dichlorofluorescin has been used as a probe for the detection of cellular hydrogen peroxide production, mainly in neutrophils, but also in a variety of other cell types and organ preparations.[2,3] We have previously shown that hydrogen peroxide is not capable of directly oxidizing dichlorofluorescein to dichlorofluorescein, but requires metal catalysis to form secondary reactive species such as hydroxyl radical or ferryl ion.[4,5] Hydrogen peroxide will also oxidize dichlorofluorescin to dichlorofluorescein in the presence of a peroxidase.[4,5] Superoxide and hydrogen peroxide generated simultaneously will not oxidize dichlorofluorescin in the absence of a metal catalyst.[4,5] Higher oxides of nitrogen (NO_x) will oxidize dichlorofluorescin, however, the rate of nitric oxide production must be higher than 10 μM which is unlikely to occur in most pathophysiologic situations. Alternatively, herein we demonstrate the efficient oxidation of dichlorofluorescin to dichlorofluorescein by either chemically synthesized peroxynitrite or the simultaneous generation of nitric oxide and superoxide. Peroxynitrite–mediated oxidation of dichlorofluorescin appears to be due to a direct reaction of the conjugate peroxynitrous acid with dichlorofluorescin. Therefore, oxidation of dichlorofluorescin to dichlorofluorescein in cellular and organ systems may not be related to hydrogen peroxide formation, but may reflect the formation of peroxynitrite under conditions where nitric oxide and superoxide are produced simultaneously.

Peroxynitrite is a strong oxidant which is capable of reacting by multiple mechanisms. The decomposition of the conjugate peroxynitrous acid yields an oxidant with the reactivity of hydroxyl radical and nitrogen dioxide, which appears to be mediated by a vibrationally excited intermediate derived from *trans*-peroxynitrous

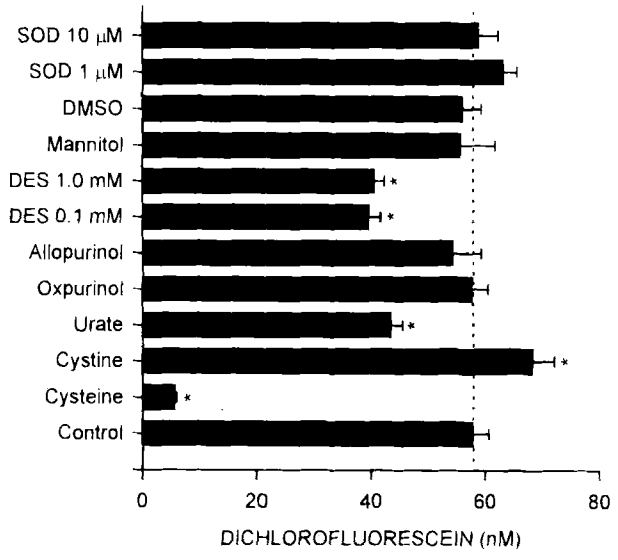

FIGURE 4 Effect of inhibitors on peroxynitrite-dependent oxidation of dichlorofluorescin. Dichlorofluorescein concentrations for the reaction of 200 nM peroxynitrite with 20 μM dichlorofluorescin in the presence of inhibitors were compared to a control with buffer alone. Studies were performed at 37°C and pH 7.4. Inhibitors were 1 mM L-cysteine, 1 mM L-cystine, 1 mM urate, 1 mM oxypurinol, 1 mM allopurinol, 0.1 mM desferrioxamine (DES), 1 mM desferrioxamine, 100 mM mannitol, 100 mM dimethylsulfoxide (DMSO), 1 μM superoxide dismutase (SOD), and 10 μM superoxide dismutase. Values are means ± SD, n = 4.

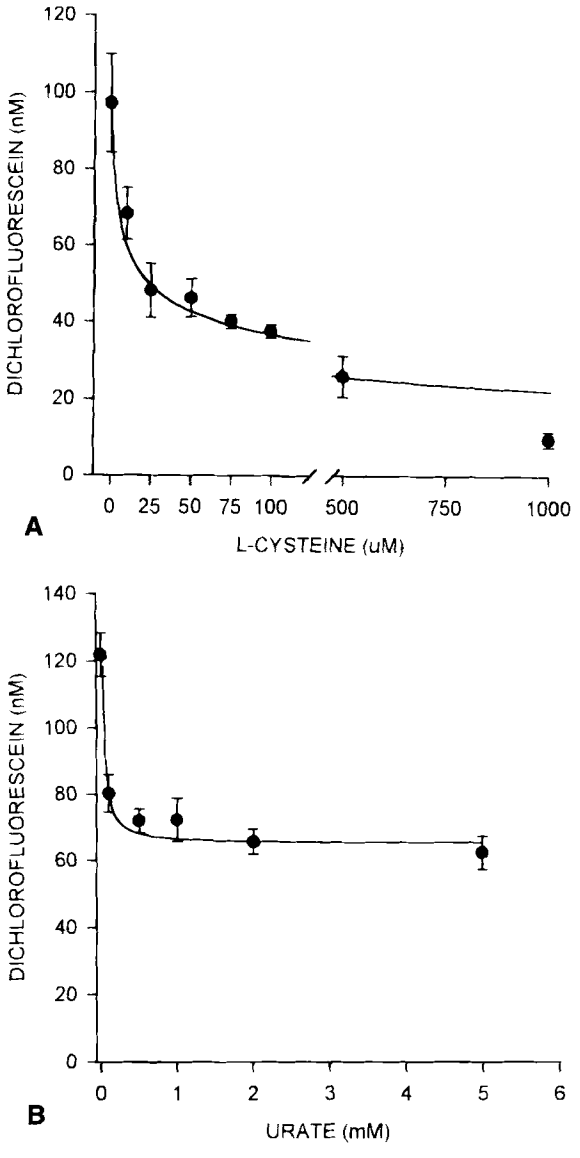

FIGURE 5 **A**. Inhibition of peroxynitrite-mediated dichlorofluorescin oxidation by L-cysteine. **B**. Inhibition of peroxynitrite-mediated dichlorofluorescin oxidation by urate. Peroxynitrite (200 nM) was added to 20 μM dichlorofluorescin in the presence of increasing concentrations of L-cysteine or urate at 37°C and pH 7.4. Dichlorofluorescein concentrations were determined from fluorescent intensity measurements. The curve shown is the result of non-linear regression analysis of the measured values. Values are means ± SD, n = 4.

acid rather than physical separation into free hydroxyl radical and nitrogen dioxide.[7,26] The effects of pH on peroxynitrite-mediated oxidation of dichlorofluorescin demonstrate that peroxynitrous acid is responsible for the formation of dichlorofluorescein from dichlorofluorescin. The decrease in dichlorofluorescein formation at acidic pH is due to the increased rate of isomerization of peroxynitrite to nitrate under these conditions, while the decreased formation of dichlorofluorescein at alkaline pH reflects the slow formation of peroxynitrous acid under these conditions. The lack of an inhibitory effect by the hydroxyl radical scavengers mannitol and dimethylsulfoxide indicates that the oxidation reaction of peroxynitrite with dichlorofluorescin is not based on the formation of free hydroxyl radical.

Heterolytic cleavage of peroxynitrite, catalyzed by low molecular mass metals and superoxide dismutase, forms a nitronium ion-like species, which may serve as a strong oxidant.[37,28] To eliminate transition-metal-catalyzed reactions with peroxynitrite, the buffer was made using high quality deionized water that was further

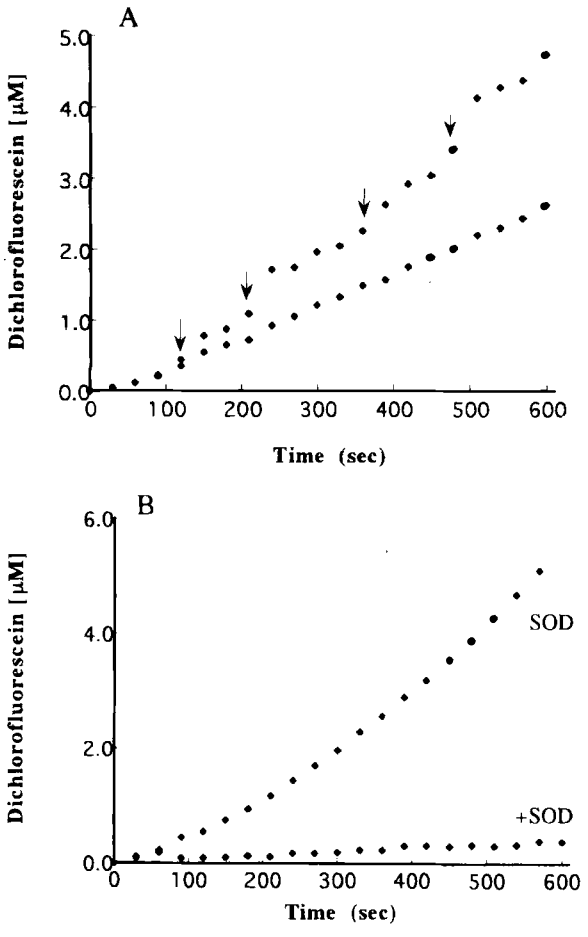

FIGURE 6 **A.** Oxidation of dichlorofluorescin by hypoxanthine (0.5 mM) plus xanthine oxidase (20 mU/ml) at 37°C and pH 7.4 in the absence of a metal chelator. Bolus additions of nitric oxide were added as indicated by the arrows. Dichlorofluorescin concentrations were determined from the absorbance at 500 nm. **B.** Oxidation of dichlorofluorescin by SIN-1 at 37°C and pH 7.4. in the presence of the metal chelator diethylenetriaminepentaacetic acid (100 μM) with and without 2 mg/ml Cu, Zn superoxide dismutase (specific activity 3,200 U/mg). Values are means ± SD, n = 3.

passed over a Chelex-100 column, followed by the addition of 100 μM DTPA. Metal catalysis, therefore, was not required for efficient peroxynitrite-mediated dichlorofluorescin oxidation. Inhibition of peroxynitrite-mediated oxidation of dichlorofluorescin by desferrioxamine was likely due to the direct reaction of desferrioxamine with peroxynitrite rather than through its metal chelating effects.[7]

The free sulfhydryl group of L-cysteine efficiently inhibits peorxynitrite-mediated oxidation of dichlorofluorescin on the basis of competitive inhibition, while L-cystine, the peroxynitrite-mediated oxidation product of cysteine actually increased peroxynitrite-mediated dichlorofluorescin oxidation. With a K_I of 25 μM, L-cysteine has a similar inhibitory effect for peroxynitrite-mediated oxidation of dichlorofluorescin and dihydrorhodamine 123.[24]

In contrast, urate is much less efficient at inhibiting peroxynitrite-mediated oxidation of dichlorofluorescin compared to efficient inhibition of peroxynitrite-mediated oxidation of dihydrorhodamine 123 by urate. In previous studies,[24] we determined a K_I of 2.5 μM for the oxidation of 50 μM dihydrorhodamine 123 by 600 nM peroxynitrite. Near total inhibition of dihydrorhodamine 123 oxidation occurred at urate concentrations of 50 to 100 μM. In these studies using 200 nM peroxynitrite and 20 μM dichlorofluorescin, we found that efficient inhibition of peroxynitrite-mediated oxidation of dichlorofluorescin did not occur. For urate concentration of 0.5 mM to 5 mM similar levels of inhibition were noted (41% and 49% inhibition respectively). While urate may act as a competitive inhibitor through direct reaction with peroxynitrite, the difference in inhibition of dihydrorhodamine and dichlorofluorescin indicates that inhibition may be through the interaction of urate with these molecules rather than with peroxynitrite. Both dihydrorhodamine and dichlorofluorescin require a two electron oxidation to form the respective fluorescent molecules rhodamine and dichlorofluorescein. A likely inhibitory mechanism for urate involves the reduction of the one electron oxidized radical to the parent compound. Under this mechanism, the rate of urate-induced inhibition would relate to the redox potential of dichlorofluorescin radical/dichlorofluorescein versus dihydrorhodamine radical/ rhodamine, suggesting that the potential is lower for dihydrorhodamine radical making reaction with urate more rapid.

In conclusion, we have demonstrated that dichlorofluorescin is efficiently oxidized to the fluorescent product dichlorofluorescein by either chemically synthesized peroxynitrite or the simultaneous generation of nitric oxide and superoxide. This reaction appears to be mediated directly by peroxynitrite and does not require the secondary formation of other free radicals such as hydroxyl radical, ferryl radical, or nitronium ion. Therefore, peroxynitrite may contribute to the oxidation of dichlorofluorescin in cell and organ systems where it has been used to detect the presence of hydrogen peroxide.

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