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Chemically de-acetylated 2′,7′-dichlorodihydrofluorescein diacetate as a probe of respiratory burst activity in mononuclear phagocytes

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Abstract

2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) is a fluorogenic probe commonly used to detect cellular production of reactive oxygen species (ROS), for example in the respiratory burst of granulocytes and mononuclear phagocytes. This method depends on the de-acetylation of H_2DCFDA by cellular esterases, to form the oxidant-sensitive compound, 2′,7′-dichlorodihydrofluorescein (H_2DCF). Importantly, however, not all cells possess sufficient esterase activity to produce the H_2DCF needed for accurate measurement of ROS. In this study, we used chemically de-acetylated probe (H_2DCF) to assess the phorbol-ester-triggered respiratory burst of rainbow trout macrophages, which, like some mammalian mononuclear phagocytes, appear to have low probe-esterase activity. We compared this approach to the use of intact H_2DCFDA and the cytochrome c reduction assay. The H_2DCF and cytochrome c reduction assays gave similar portrayals of the kinetics of the macrophage respiratory burst, while H_2DCFDA did not. We therefore recommend the use of H_2DCF over H_2DCFDA for quantification of the production of reactive oxygen species. Additionally, we stress the need to test reaction buffers or culture media used with H_2DCF(DA) for their ability to oxidize the probe directly or indirectly. As an example, we have observed that tyrosine combined with ubiquitous metal contaminants of physiological buffers can result in high levels of oxidation, which may be incorrectly interpreted as cellular activity.

Keywords: 2′,7′-Dichlorodihydrofluorescein diacetate; Respiratory burst; Reactive oxygen species; Macrophage; Rainbow trout; Tyrosine

1. Introduction

The use of the fluorogenic probe, 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA), has become a popular method for measuring the production of reactive oxygen species (ROS) by a variety of cell types, in both flow-cytometric and spectrofluorometric systems. This method has widespread appeal due to its simplicity, high sensitivity,

Abbreviations: ANOVA, Analysis of variance; DCF, 2′,7′-Dichlorofluorescein; H_2DCF, 2′,7′-Dichlorodihydrofluorescein; H_2DCFDA, 2′,7′-Dichlorodihydrofluorescein diacetate; L-NMMA, N^G-Monomethyl-L-arginine; NOS, Nitric oxide synthase; PBS-G, Phosphate-buffered saline+500 mg/l D-glucose; PMA, Phorbol-12-myristate-13-acetate; ROS, Reactive oxygen species; SOD, Superoxide dismutase

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and low cost relative to traditional approaches to the measurement of ROS, such as assaying for the reduction of cytochrome c by superoxide.

H_2DCFDA is capable of crossing the plasma membrane to enter a cell's interior, where cellular esterases hydrolyze its acetyl moieties to produce 2′,7′-dichlorodihydrofluorescein (H_2DCF). The de-acetylated form of the probe is then susceptible to oxidation, generating a fluorescent product, 2′,7′-dichlorofluorescein (DCF). Accumulation of DCF indicates the production of redox-active substances (Bass et al., 1983; Royall and Ischiropoulos, 1993).

Several methods for applying H_2DCFDA to the assessment of respiratory burst activity in multi-well plates have appeared in the literature (Rosenkranz et al., 1992; Wan et al., 1993; Ciapetti et al., 1998). These procedures all involve a similar progression of steps: (a) cells are dispensed into wells of a microwell plate; (b) H_2DCFDA is added to the wells; (c) the cells are stimulated with a burst-inducing agent such as a phorbol-ester or opsonized zymosan; (d) the resulting output of ROS is measured as the increase in fluorescence associated with oxidation of the probe. The relationship between DCF and fluorescence is linear over a broad concentration range (Rosenkranz et al., 1992; Bass et al., 1983), allowing for expression of the relative level of ROS production in (arbitrary) units of DCF fluorescence.

While the procedure outlined above is straightforward, it has become apparent that some considerations must be borne in mind by investigators using H_2DCFDA to quantify ROS production. First, although this probe was originally used to detect hydrogen peroxide (Keston and Brandt, 1965), it is not specifically oxidized by hydrogen peroxide alone (LeBel et al., 1992; Crow, 1997). Second, many ROS (including H_2O_2) are relatively stable, and freely able to move in and out of cells. Thus, generation of DCF in a given cell does not necessarily demonstrate ROS production within that particular cell itself (Henderson and Chappell, 1993). Finally, H_2DCFDA must be de-acetylated, to form 2′,7′-dichlorodihydrofluorescein (H_2DCF) in order to be susceptible to oxidation (Cathcart et al., 1983).

With regard to the latter point, low cellular esterase activity or brief loading times may limit the availability of H_2DCF, resulting in an underestimation of ROS levels or a misrepresentation of the kinetics of ROS production. Indeed, previous studies have shown that such problems can arise with H_2DCFDA-based assays of respiratory burst activity in mononuclear phagocytes, due either to low levels of esterase activity, or sequestration of esterases in inaccessible cellular compartments (Robinson et al., 1988). Yet, this phenomenon has received little further attention in the literature.

We have used both intact H_2DCFDA and the de-acetylated form of the probe (H_2DCF) in conjunction with the well-characterized cytochrome c reduction assay (Pick and Mizel, 1981), to assess respiratory burst activity in macrophages of the rainbow trout (*Oncorhynchus mykiss*). These cells are easily maintained and mount a respiratory burst with essentially the same characteristics as its mammalian equivalent, via a well-conserved homologue of the mammalian NADPH oxidase (Secombes et al., 1992; Itou et al., 1998; Shiibashi et al., 1999). The results presented here demonstrate the utility of the de-acetylated form of the probe in assays of cellular production of ROS.

In any application using H_2DCF, care must be taken to prevent oxidation by constituents of assay buffers or culture media, due to the probe's susceptibility to oxidation by compounds other than ROS. Failure to do so may result in incorrect attribution of probe oxidation to cellular activity, when in fact this is not the case. As an example, the oxidation of H_2DCF in metal-contaminated tyrosine solutions (for example, tissue-culture media) is presented here, in order to flag this issue and avoid needless frustration on the part of investigators working with this otherwise useful probe.

2. Materials and methods

2.1. Isolation of macrophages

Macrophages were isolated from the head–kidney tissue of the rainbow trout by a modification of the procedure originally described by Secombes (1990). Cell suspensions were made using a glass pestle and sieve tissue grinder to force tissue through an 80-mesh stainless steel screen into L-15 medium (Sigma, Oakville, ON, Canada), supplemented with 10 U/ml heparin, 200 U/ml penicillin G and 200

μg/ml streptomycin sulfate (Gibco BRL, Burlington, ON, Canada). The volume of the suspension was brought to 42 ml, and dispensed into polypropylene centrifuge tubes (Sarstedt, St. Léonard, PQ, Canada) in 7-ml aliquots. Aliquots of 3 ml of Histopaque® 1077 (Sigma) were layered under each previous aliquot and the resulting discontinuous gradients were centrifuged in a swinging-bucket-rotor centrifuge at 4°C, 400 *g*, for 30 min. Cells at the phase interface (leukocytes) were collected with a Pasteur pipette, pooled, and washed twice in heparin-free L-15 medium containing penicillin and streptomycin as described above.

To enrich for macrophages, the resulting leukocyte suspension was plated at 5×10^5 cells/well in a 96-well tissue-culture plate (Becton-Dickinson, Mississauga, ON, Canada), and incubated at 18°C for 2 h. Wells were then rinsed once with 250 μl of serum-free L-15, and the medium was changed to L-15 supplemented with 300 mg/l L-glutamine and 5% fetal bovine serum (Gibco), in addition to penicillin and streptomycin as above. The cells were cultured in this medium for 2–4 days prior to being used in assays.

2.2. Fluorometric assays of respiratory burst activity

The de-acetylated probe (H_2DCF) was prepared from H_2DCFDA (Molecular Probes, Eugene, OR, USA) by alkaline hydrolysis, using NaOH according to the method of Cathcart et al. (1983) and neutralized with sodium phosphate buffer (50 mM, pH 6.2). A working solution of H_2DCFDA was prepared in the same way, except that the NaOH was neutralized with phosphate buffer prior to being added to H_2DCFDA. These solutions were stored in the dark at 4°C, and were stable for at least 2 weeks under these conditions.

Macrophage-containing wells were rinsed with 250 μl/well filter-sterilized phosphate-buffered saline (1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, 2.7 mM KCl, 138 mM NaCl, 5 μM $MgCl_2$, 9 μM $CaCl_2$, pH 7.4), supplemented with 500 mg/l D-glucose (PBS-G). After rinsing, H_2DCFDA or H_2DCF solution was added, along with phorbol-12-myristate-13-acetate (PMA) as a triggering agent. The final contents of each well were 5 μM H_2DCF or H_2DCFDA, 2 μg/ml PMA (or 0.2% [v/v] DMSO as a carrier control), in 100 μl of total volume.

Oxidation of the probes to 2′,7′-dichlorofluorescein (DCF) was measured with a CytoFluor series 4000 multiwell-plate fluorometer (PerSeptive Biosystems, Framingham, MA, USA) set at a photomultiplier gain of 50, with excitation and emission filters of 485 and 530 nm, respectively. Fluorescence was measured immediately after dosing the cells, and again every 2.5 min for 2 h.

To confirm that H_2O_2 — derived from dismutation of superoxide ($\dot{O}_2^-$) produced during the respiratory burst — was responsible for oxidation of the probe, parallel assays were conducted in the presence of 100 U/ml bovine–erythrocyte superoxide dismutase (SOD; Sigma), 100 U/ml bovine–liver catalase (Sigma) or 50 μM N^G-monomethyl-L-arginine, an inhibitor of nitric oxide synthases (L-NMMA; Cayman Chemical Co., Ann Arbor, MI, USA). Oxidation of H_2DCF by H_2O_2 occurs only in the presence of a catalyst, which can be either a peroxidase or a transition metal such as Fe(II) (LeBel et al., 1992). In order to identify the catalyst involved in our system, assays were also conducted in buffer supplemented with 1 mM azide (BDH, Toronto, ON, Canada) to inhibit peroxidases or 50 μM deferoxamine mesylate (desferrioxamine B, desferal; Sigma) a potent chelator of iron and other metals.

2.3. Assay of respiratory burst activity by reduction of cytochrome c

Respiratory burst activity was also measured by the reduction of ferricytochrome c to ferrocytochrome c, according to the method of Pick and Mizel (1981). Wells were rinsed and dosed with DMSO or PMA as in the fluorometric assay, using 2 mg/ml cytochrome c from bovine heart mitochondria (Sigma) in place of H_2DCF(DA). Absorbance at 550 nm was measured using a BioRad 3550-UV microwell spectrophotometer, every 2.5 min for 2 h. An additional set of wells was treated with PMA in the presence of 100 U/ml SOD. The mean absorbance of the SOD-treated wells was subtracted from that observed in the wells of other treatments, to control for any increase in absorbance not accounted for by production of superoxide.

To quantify the average amount of cellular protein per well, an extra set of six wells was plated with cells. Following the assay, the medium was removed from these wells and protein levels were determined using a fluorescamine-based assay, as described by Kennedy et al. (1995). Respiratory burst activity was then expressed as nmol O_2^- produced/mg protein, using the extinction coefficient $\Delta\epsilon_{550}=2.1\times10^4\ M^{-1}\ cm^{-1}$ for reduced-minus-oxidized cytochrome c, with a path length of 3 mm.

2.4. Cell-free studies of medium components oxidizing H_2DCF

Preliminary respiratory burst assays using H_2DCF and H_2DCFDA in Leibovitz' L-15 medium resulted in high levels of background oxidation of the probe over time. Further experiments implicated tyrosine in the medium as the major component responsible, although its activity was never as great as that of the complete medium (not shown).

To follow-up on the preliminary studies, a number of L-15 components were tested for their ability to oxidize H_2DCF. A solution of galactose, sodium pyruvate, and the inorganic salts of L-15, excluding calcium and magnesium salts, was used as a control (hereafter referred to as ''L-15 salts''). Other treatments contained the L-15 salts constituents, plus (a) L-15 vitamins, (b) L-tyrosine, (c) L-15 amino acids, (d) amino acids without tyrosine (''Tyr^- amino acids''), or (e) vitamins and complete amino acids (''complete'' L-15). All solutions contained components of L-15 at 1.11× their normal concentration in the medium (Leibovitz, 1963).

Each solution was then divided into three lots, making three groups of the six different solutions. One lot was left without further manipulation. The second lot was treated by ion-exchange chromatography, using Chelex 100 resin (Sigma) in its sodium form, to remove contaminating transition metal ions. The chelating agent, deferoxamine mesylate, was added to the third at a concentration of 1.11 mM. (The deferoxamine used was from a 50× stock; thus the addition of deferoxamine resulted in a 1.02× dilution of the original solutions, which was assumed to be negligible). Following these treatments, all solutions were adjusted to pH 7.6.

Solutions were aliquotted into quadruplicate wells of a 96-well plate, at 90 μl per well. Samples with 10 μl of 50 μM H_2DCF solution, prepared as described above, were added to each well, resulting in wells containing L-15 components at their proper concentration, H_2DCF at 5 μM, and 1 mM deferoxamine where applicable. Oxidation of H_2DCF was measured as an increase in fluorescence over 2 h, using instrument settings as described above. A standard curve of 2′,7′-dichlorofluorescein dissolved in Dulbecco's phosphate-buffered saline (Sigma) was included on the same plate, to allow conversion of the fluorescence data to units of DCF concentration.

2.5. Statistics

All graphical and statistical analyses were performed using Systat® version 8 for Windows. Fluorescence values obtained in the respiratory burst experiments with inhibitors (see Table 1) were analyzed by one-way ANOVA, followed by Dun-

Table 1
Inhibition of DCF production in PMA-stimulated macrophages by scavengers of ROS[a]

Treatment[a]	DCF Fluorescence (RFU)[b]
2 μg/ml PMA (control)	19969±2047
0.2% (v/v) DMSO (carrier solvent only)	3156±68***
2 μg/ml PMA+100 U/ml catalase	4665±667***
2 μg/ml PMA+100 U/ml SOD	17277±1376[d]
2 μg/ml PMA+50 μM L-NMMA	18872±1581[d]

[a] PMA, Phorbol-12-myristate-13-acetate; DMSO, dimethyl sulfoxide; SOD, superoxide dismutase; L-NMMA, N^G-monomethyl-L-arginine.
[b] Fluorescence is expressed in relative fluorescence units (RFU). Each value represents the mean±1 standard error of the mean ($N=6$). Superscripts following the values indicate the statistical significance of differences from the control (those cells given PMA only).
*** $P<0.001$ (Dunnett's test following one-way ANOVA on log-transformed data). Data is representative of two independent experiments.
[d] Not significant.

nett's test (Dunnett, 1964) to detect significant differences between individual treatments and the control. Data was log-transformed prior to analysis, to avoid violating assumptions of the ANOVA, namely normal distribution of variates and homogenous variance among treatments.

Endpoint measures of DCF concentration obtained in the cell-free studies (a 6×3 factorial design) were analyzed by two-way ANOVA, followed by planned, non-orthogonal contrasts to compare specific pairs of treatment combinations. A Dunn-Šidák-adjusted α-level of 0.003 was used for each such contrast, to give an experiment-wise α-level of 0.05 (Dunn, 1959; Šidák, 1967). Again, data was log-transformed prior to analysis.

3. Results and discussion

3.1. Assays of respiratory burst activity

When trout macrophages were exposed to PMA, a strong respiratory burst was detected by reduction of cytochrome c. The rate of superoxide production was greatest over the first 30 min of the assay, and tapered off after that point, returning to basal levels within 1 h (Fig. 1). This pattern is typical of the kinetics reported previously for the respiratory burst in macrophages of rainbow trout, based on measurements of oxygen consumption (Nagelkerke et al., 1990). These kinetics are also similar to those observed with PMA-stimulated mammalian macrophages, as measured by reduction of cytochrome c (Johnston et al., 1978). The downward slope of the curve after 1 h of stimulation reflects re-oxidation of cytochrome c, likely by agents released extracellularly, such as peroxidases (see below) (Fridovich, 1985). This re-oxidation may mask ongoing low-level superoxide production.

When the progression of the respiratory burst was measured using H_2DCFDA, a very different kinetic pattern was observed. While PMA did cause a notable increase in oxidation of the probe, the kinetics of probe oxidation did not correspond at all to those observed for reduction of cytochrome c (Fig. 2a). Specifically, the strong initial burst of ROS production in these cells went undetected.

On the other hand, when H_2DCF was used as a probe for respiratory burst activity, the kinetics of probe oxidation much more closely mirrored those observed with cytochrome c. The strong initial burst was clearly detectable, with oxidation of the probe returning to basal levels after roughly 40 min (Fig. 2b). Furthermore, the overall sensitivity of the assay was much greater than that observed with H_2DCFDA, in that the same concentration of probe resulted in the generation of much higher fluorescence levels. The background fluorescence with H_2DCF was higher than with H_2DCFDA (Fig. 2), but this high background was not consistently observed. Instead, the constitutive oxidation of both forms of the probe varied among individual fish. Although practically difficult to solve, this problem could likely be alleviated by using fish of the same nutritional and health status, sex, maturity, size, and hierarchical position.

Some authors have observed that oxidation of H_2DCF(DA) is, to some degree, autocatalytic — the de-acetylation and oxidation of H_2DCFDA, as well as the interactions of DCF with peroxidases and visible light, can all generate hydrogen peroxide

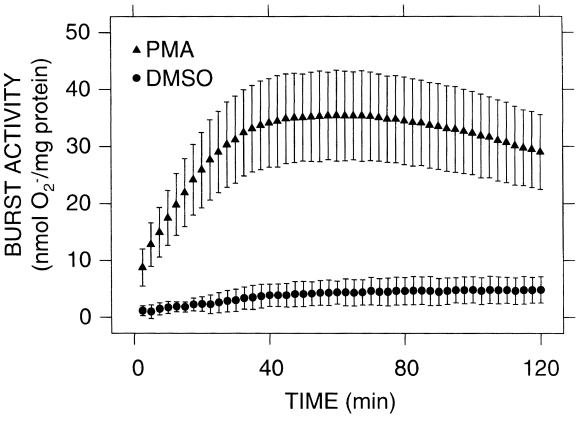

Fig. 1. Kinetics of respiratory burst activity in PMA-stimulated macrophages from the rainbow trout head–kidney, assessed by reduction of cytochrome c. Absorbance at 550 nm was read every 2.5 min for 2 h, in wells treated with 0.2% (v/v) DMSO (carrier solvent control), or 2 μg/ml PMA in PBS-G supplemented with 2 mg/ml cytochrome c. The change in absorbance was subtracted from the mean change in absorbance of wells treated with PMA+ 100 U/ml SOD, and converted to nmol superoxide produced/mg protein using $\epsilon = 2.1\times10^4\ M^{-1}\ cm^{-1}$. Each data point represents the mean±1 S.E.M. of six wells. Data is representative of three independent experiments.

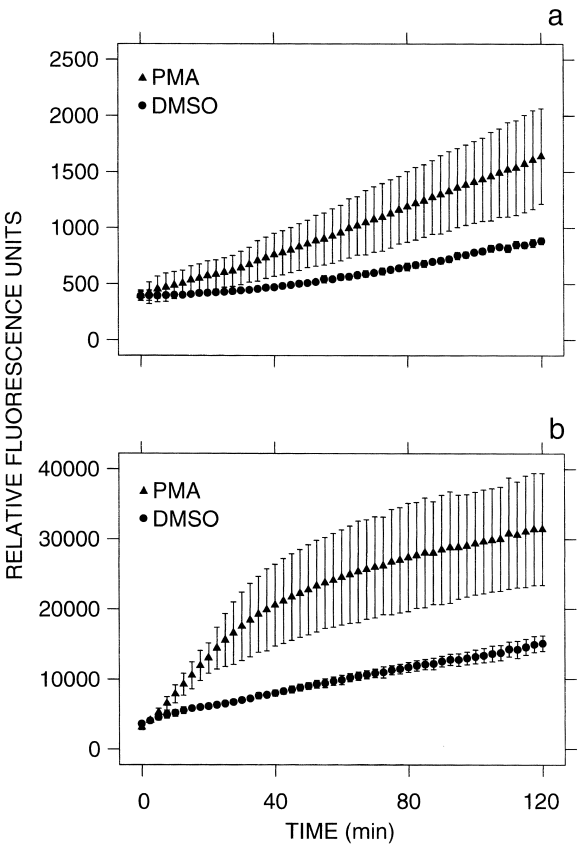

Fig. 2. Comparison of DCF production in macrophages incubated with either H_2DCF or H_2DCFDA. Oxidation of H_2DCF(DA) to DCF was determined by measuring DCF fluorescence every 2.5 min for 2 h, using excitation and emission filters of 485 and 530 nm, respectively, for cells incubated in the presence of (a) 5 μM H_2DCFDA or (b) 5 μM H_2DCF. Note the difference in the *y*-axis scale between panels (a) and (b). Macrophages were treated with 0.2% (v/v) DMSO or 2 μg/ml PMA, in PBS-G. Each point represents the mean±1 S.E.M. of four wells. Data is representative of four independent experiments.

(Marchesi et al., 1999; Rota et al., 1999a,b). These observations raise the possibility that at least some of the increase in fluorescence observed in the present study is due to such artefactual oxidant production. In our experimental system, however, it seems unlikely that such processes contribute substantially to the observed increase in fluorescence, as the kinetics of DCF production are consistent with those of SOD-inhibitable reduction of cytochrome c under identical conditions. In any case, the use of H_2DCF, rather than H_2DCFDA, is likely to reduce the amount of artefactual oxidant production, as deacetylation is conducted in the absence of cellular peroxidases, which may facilitate autocatalysis (Rota et al., 1999a,b).

To confirm the assumption that the increase in fluorescence observed was due to the production of H_2O_2 (via dismutation of $^{\cdot}O_2^-$ generated in the respiratory burst) the H_2DCF assay was performed in the presence of several reagents: superoxide dismutase (SOD), N^G-monomethyl-L-arginine (L-NMMA), and catalase (Table 1). Superoxide dismutase did not significantly affect oxidation of the probe, suggesting that superoxide itself does not oxidize H_2DCF, an observation consistent with previous reports (LeBel et al., 1992). Trout macrophages, like their mammalian counterparts, express an inducible isoform of nitric oxide synthase (iNOS) (Laing et al., 1999). As certain derivatives of nitric oxide, such as peroxynitrite ($ONOO^-$) are known to oxidize H_2DCF (Crow, 1997), it was also necessary to determine the extent to which they act as oxidants in this system. Hence, the use of L-NMMA, an inhibitor of nitric oxide-synthases (NOS), which also had no detectable effect. This implies that reactive derivatives of nitric oxide were also not significant oxidants of H_2DCF in this system. Catalase, however, was profoundly inhibitory, suggesting that hydrogen peroxide is the primary species responsible for oxidation of the probe by PMA-treated macrophages. The fact that exogenous catalase is such a potent inhibitor of the oxidation of H_2DCF implies that the H_2O_2 responsible for oxidation of the probe is released extracellularly.

Oxidation of the probe may occur intracellularly, extracellularly, or in both compartments. Detection of ROS is not limited to intracellular or extracellular compounds alone. This is one advantage of H_2DCF relative to other methods such as reduction of cytochrome c and nitroblue tetrazolium, which measure exclusively extracellular or intracellular production of ROS, respectively (Dahlgren and Karlsson, 1999).

As oxidation of H_2DCF by H_2O_2 is dependent on catalysts, such as peroxidases or Fe^{2+} (Keston and Brandt, 1965; LeBel et al., 1992), extracellular oxidation of H_2DCF requires the presence of such agents outside the cell. Thus, further assays were conducted in the presence of azide (an inhibitor of

peroxidases) and deferoxamine (a metal chelator) to distinguish between these two possibilities. Azide was a potent inhibitor of DCF production, while deferoxamine failed to inhibit oxidation of the probe (Table 2). It therefore appears that peroxidases, rather than metal contaminants in the assay buffer, are the primary catalyst involved in oxidation of the probe by H_2O_2. Phorbol-induced exocytosis of myeloperoxidase would be a possible source of extracellular peroxidase. This process has been documented in mammalian mononuclear phagocytes (McNally and Bell, 1996), and likely occurs in the macrophages of rainbow trout also, as PMA-induced luminol-enhanced chemiluminescence, also a peroxidase-dependent phenomenon (Albrecht and Jungi, 1993; McNally and Bell, 1996), has been reported in these cells (Plytycz et al., 1989).

Oxidation of the de-acetylated probe may also occur intracellularly. Intracellular oxidation of exogenously added H_2DCF is possible as diffusion of this form of the probe into and out of cells has been observed (Royall and Ischiropoulos, 1993), despite the early belief that the plasma membrane is relatively impermeable to H_2DCF (Bass et al., 1983). Hydrogen peroxide initially released extracellularly can also diffuse into cells, allowing oxidation of the probe inside the cell (Henderson and Chappell, 1993). An outline of the potential pathways leading to oxidation of H_2DCF in this system is given in Fig. 3.

The de-acetylated probe appears to be superior to the intact probe for tracking respiratory burst activity in macrophages, because esterase activity is never limiting, regardless of the location of probe oxidation. Macrophages do exhibit a large degree of phenotypic heterogeneity, making it probable that some macrophage sub-populations express sufficient H_2DCFDA-esterase to allow for effective assays using the intact probe. Indeed, numerous authors have used H_2DCFDA to measure increases in the respiratory burst activity of mononuclear phagocytes (Menson and Wilson, 1989; Spear et al., 1989; Albrecht and Jungi, 1993; Imrich et al., 1999). Nevertheless, it is also possible that such studies underestimate levels of ROS production due to limiting esterase activity. This phenomenon would be particularly problematic when H_2DCFDA is used to compare respiratory burst activity between different types of cells, or cells with different histories. In these cases differences in esterase activity, or in the accessibility of the probe to cellular esterases, may be wrongly interpreted as differences in ROS production. Few investigators have taken the steps necessary to test the influence of esterase activity in their assay systems (Robinson et al., 1988). It would seem preferable to avoid potential complications by de-acetylating the probe prior to assaying.

3.2. Oxidation of H_2DCF in cell-free solutions

While H_2DCF can be a sensitive indicator of respiratory burst activity, its susceptibility to oxidation by a number of compounds complicates its use in certain complex buffer systems, including some tissue-culture media. In preliminary experiments, we found that one commonly used culture medium, L-15, was a strong oxidant of H_2DCF and H_2DCFDA. This phenomenon was investigated further with the de-acetylated probe. To determine the compounds responsible for oxidizing the probe, L-15 medium was divided into three subcomponents: salts (including sodium pyruvate and D-galactose), vita-

Table 2
Effects of azide and deferoxamine on oxidation of H_2DCF

Treatment	Activity[a] in unstimulated cells (%)[b]	Activity[a] in PMA-stimulated cells (%)[b]
50 μM Deferoxamine	133.0±0.7	206.3±6.8
1 mM Azide	51.4±0.5	59.2±1.6

[a] Activity is expressed as a percentage of that observed in control cells that were not exposed to deferoxamine or azide. Values represent the mean relative activity±1 standard error of the mean ($N=6$ wells). All treatments were significantly different from the control (100% by definition), based on a single-sample t-test, $P \ll 0.001$. Data is representative of four independent experiments.

[b] PMA-stimulated cells were dosed with 2 μg/ml PMA, as indicated in Section 2. Unstimulated cells received 0.2% (v/v) DMSO (carrier solvent) as a control.

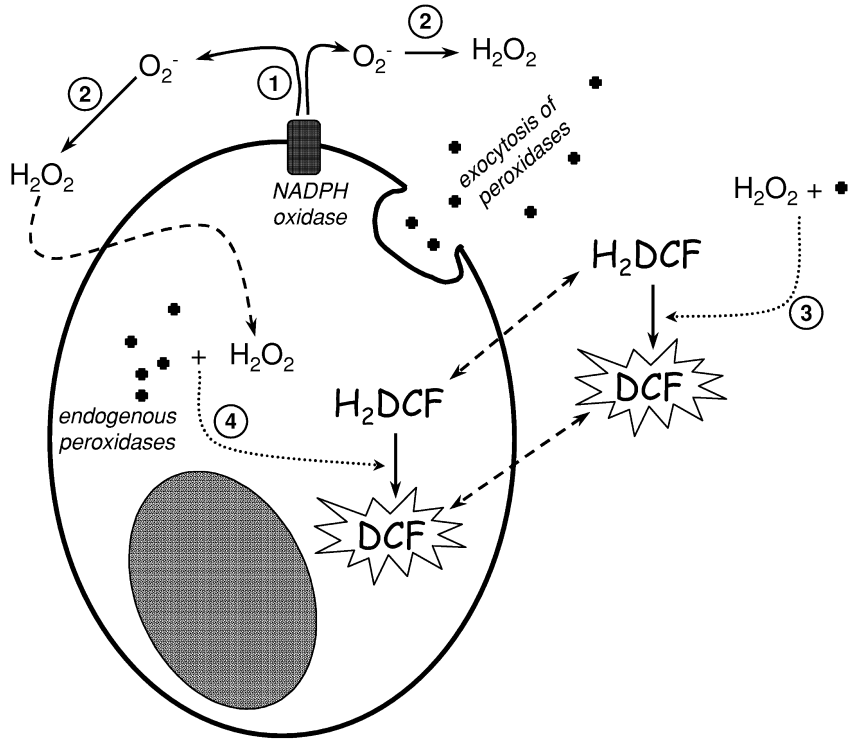

Fig. 3. Proposed scheme for PMA-induced oxidation of H_2DCF in rainbow trout head–kidney macrophages. (1) Stimulation of cells with PMA leads to activation of NADPH oxidase, which generates superoxide ($^{\cdot}O_2^-$). (2) The superoxide thus produced dismutes to form hydrogen peroxide (H_2O_2). Subsequent oxidation of H_2DCF can occur extracellularly, when the probe encounters H_2O_2 in the presence of peroxidases that have been released to the external environment (3). Alternatively, H_2DCF and H_2O_2 may diffuse into the cell, where intracellular peroxidases catalyze the oxidation of the probe to DCF (4). Both steps (3) and (4) can be inhibited by the addition of catalase to scavenge H_2O_2 outside the cell.

mins, and amino acids. The amino acids component was further subdivided into (a) tyrosine alone and (b) all amino acids except tyrosine (Tyr$^-$ amino acids). These solutions, plus the complete L-15 solution, made for a panel of six different solutions that were tested for their ability to oxidize H_2DCF. The solutions were pre-treated in three different ways: control (no pre-treatment), passage over a Chelex 100 column, or addition of 1 mM deferoxamine mesylate. Results of a representative experiment are summarized in Fig. 4.

In the untreated lot of solutions, tyrosine alone, the complete amino acid mixture and complete L-15 all had significantly higher oxidizing activity than L-15 salts (control). Conversely, L-15 vitamins and the Tyr$^-$ amino acid solutions oxidized significantly less H_2DCF than the salts (Fig. 4; these and all following comparisons based on non-orthogonal contrasts following two-way ANOVA, on log-transformed data, with an experiment-wise α-level of 0.05). Judging by the oxidizing activity of amino acids with and without tyrosine, it is apparent that tyrosine is required for the oxidizing activity of L-15. However, tyrosine alone did not oxidize the probe to nearly the same extent as the complete amino acids mixture, or complete L-15.

Pre-treatment of the solutions with Chelex 100 resin resulted in a significant reduction of the oxidizing activity of the complete amino acid and complete L-15 solutions, relative to the respective untreated solutions. Addition of 1 mM deferoxamine mesylate also significantly reduced oxidation of H_2DCF by these two solutions, and additionally, by the tyrosine solution (Fig. 4). Chelex 100, an iminodiacetyl-based cation-exchange resin, is commonly used to remove metals from solutions (Bio-Rad,

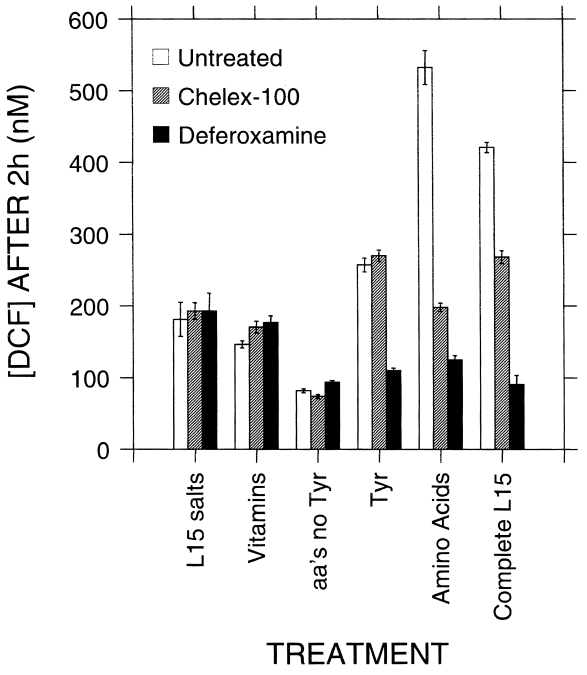

Fig. 4. Oxidation of H_2DCF in cell-free solutions containing components of the L-15 medium. The de-acetylated probe was incubated in the presence of various sub-components of L-15 medium, as described in Section 2. The ''aa's no Tyr'' and ''Tyr'' treatments refer to solutions containing all amino acids of L-15 except tyrosine, and L-tyrosine alone, respectively. Each bar represents the mean±S.E.M. of the amount of DCF generated per well (N=4), from an original H_2DCF concentration of 5 μM. Treatments: □, untreated solutions; ▨, solutions treated by ion exchange chromatography with Chelex-100 resin; ■, solutions supplemented with 1 mM deferoxamine. Data is representative of two independent experiments.

2000). Deferoxamine also strongly binds and sequesters a number of metal species. It has a particularly high affinity for Fe^{3+} and Al^{3+} (Keberle, 1964; Evers et al., 1989), although it will also bind Fe^{2+} and other divalent metals with an equal or greater affinity than Chelex 100, based on equilibrium binding constants for *N*-methyliminodiacetic acid (Chaberek and Martell, 1959).

The effects of metal-removing agents on the oxidizing activity of tyrosine-containing solutions strongly suggest that oxidation of H_2DCF in this system is dependent on a process involving both metals and tyrosine. The greater ability of deferoxamine to inhibit oxidation of the probe may be due to its higher affinity for most metal species — particularly iron in both 2+ and 3+ oxidation states. The increased activity of the solutions containing tyrosine in the presence of other amino acids, compared to the activity of tyrosine alone, is most likely a simple result of the addition of more (metal-contaminated) solutes to these mixtures.

Description of the specific mechanism by which metals and tyrosine combine to oxidize H_2DCF is not the objective of this paper. Rather, we raise this issue as an example of the potential surprises and pitfalls which may (needlessly) frustrate users of H_2DCF. L-15 medium contains much more tyrosine (and indeed, more of most amino acids) than other media in common use. Nevertheless, our observations suggest a need to be cautious when using H_2DCF(DA) with any complex buffer solutions. While other media contain less tyrosine, some, such as Dulbecco's Modified Eagle's Medium and Medium 199, have iron added as ferric nitrate. Additionally, investigators should evaluate the oxidative activity of any ROS scavengers or enzyme inhibitors they plan to use in conjunction with this probe, as these may also cause spurious increases in fluorescence.

4. Summary

The results presented here demonstrate that H_2DCF is preferable to H_2DCFDA as a probe in spectrofluorometric assays of respiratory burst activity in mononuclear phagocytes, which may espress low levels of H_2DCFDA esterase, or sequester the esterase in inaccessible cellular compartments. De-acetylating the intact probe prior to assaying increases the sensitivity of the assay, and provides a more accurate portrayal of the kinetics of ROS production. While these benefits could perhaps be obtained by incubating cells with the intact probe long enough to allow a substantial amount of hydrolysis by cellular esterases, the chemical hydrolysis approach is straightforward, and it allows experimenters to run the assay using a known concentration of oxidant-sensitive probe. In this way, the potentially confounding variable of cellular esterase activity/accessibility is eliminated. We have described the H_2DCF approach using the specific example of respiratory burst activity in trout macro-

phages; however, the method should be easily extendable to the assay of other H_2DCF-oxidizing compounds produced by other types of cells.

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