2,7-Dihydrodichlorofluorescein diacetate as a fluorescent marker for peroxynitrite formation

Heiko Possel[a,*], Heiko Noack[a], Wolfgang Augustin[b], Gerburg Keilhoff[a], Gerald Wolf[a]

[a]*Institute of Medical Neurobiology, Otto-von-Guericke University of Magdeburg, Leipziger Str. 44, D-39120 Magdeburg, Germany*
[b]*Department of Pathobiochemistry, Otto-von-Guericke University of Magdeburg, Leipziger Str. 44, D-39120 Magdeburg, Germany*

Received 1 September 1997

Abstract Reactive oxygen species (ROS) have been implicated as an important causative factor in cell damage, including apoptosis and necrosis. Their proposed actions comprise lipid peroxidation, DNA damage, destruction of the mitochondrial respiratory chain and protein modifications. Recent experiments underline the importance of peroxynitrite, the reaction product of the two potent reactive species nitric oxide and superoxide. Several fluorogenic compounds have been used in order to determine ROS formation in living cells. Besides dihydrorhodamine-123 (DHR-123), at present mostly applied to monitor peroxynitrite, 2,7-dihydrodichlorofluorescein (DCF-H) is used for detection of hydrogen peroxide and nitric oxide. We employed a cell free approach to evaluate the specificity and sensitivity of DCF-H to various oxidizing compounds. Our studies imply that DCF-H is much more sensitive to peroxynitrite oxidation than any other compound tested. In order to study peroxynitrite generation within individual cells, primary glial cultures loaded with DCF-H were monitored with a laser scanning microscope. Microglia, stimulated to simultaneously produce the peroxynitrite precursors nitric oxide and superoxide, displayed the greatest increase in DCF fluorescence, whereas microglia producing either nitric oxide or superoxide alone showed a relatively small increase in DCF fluorescence. In conclusion, DCF-H was demonstrated to be an excellent peroxynitrite marker with the potential to detect peroxynitrite formation in living cells.

Key words: Peroxynitrite; 2,7-Dihydrodichlorofluorescein diacetate; Cell imaging; Microglia

1. Introduction

Reactive oxygen species (ROS) have been implicated as an important causative factor in cell damage, including apoptosis and necrosis. Increased intracellular generation of ROS occurs during inflammation, ischemia, cancer and aging [1]. The molecules involved include free radicals such as superoxide, nitric oxide as well as hydrogen peroxide. Their proposed actions encompass lipid peroxidation, DNA damage, destruction of the mitochondrial respiratory chain and protein modifications [2,3]. The effects of nitric oxide are still controversial, spanning protective mechanisms during ischemia and destructive actions in neurodegenerative processes [4,5]. Upon synthesis, nitric oxide has the potential to freely diffuse across cell membranes up to several hundred micrometers, whereupon it can react with a variety of proteins and inorganic compounds [6]. Moreover, nitric oxide reacts readily with superoxide to form the strong oxidant peroxynitrite [7].

*Corresponding author. Fax: (49) (391) 67-14365.
E-mail: Heiko.Possel@Medizin.Uni-Magdeburg.de

Evaluation of the potentially hazardous actions of peroxynitrite and other ROS has proved difficult due to their relatively short life span. Thus one major approach has been the biochemical quantitation of the reaction products [8]. These methods are powerful tools for quantitative analysis of oxidative stress, but are unable to provide insights into the microtopography of the phenomenon. With this aim, several fluorogenic compounds have been used in order to determine ROS concentrations at the single cell level. Besides dihydrorhodamine-123 (DHR-123), a marker for peroxynitrite, the fluorogenic compound DCF-DA has been utilized as a marker for oxidative stress [9]. DCF-DA diffuses through cell membranes and is subsequently enzymatically deacetylated by intracellular esterases to the non-fluorescent DCF-H. Two oxidizing molecules, nitric oxide and hydrogen peroxide, have been reported to convert DCF-H to the highly fluorescent DCF. However, the obtained results are contradictory. Gunasekar et al. [10] and Mattson et al. [11] have observed an increase in DCF fluorescence on glutamate stimulation, whereas Reynolds et al. [12] showed that glutamate induces a decrease in DCF fluorescence. This prompted us to examine the response of DCF-H and DCF-DA to various oxidizing agents in a cell free system. In addition, we evaluated the responses of primary glial cultures preincubated with DCF-DA employing a laser scanning microscope (LSM), to determine the cellular ROS level of individual cells.

2. Materials and methods

2.1. Reagent solutions

Stock solutions of 5 mM DCF-DA (Molecular Probes) in 100% water free ethanol and 5 mM DHR-123 (Molecular Probes) in dimethylsulfoxide (DMSO) were stored at −80°C and used for 4 weeks. DCF-H was prepared according to the method of Cathcart [13]. Briefly, 2 ml 0.1 M NaOH was added to 0.5 ml DCF-DA in methanol (1 mM) and left at room temperature for 30 min. The reaction was stopped by neutralizing the solution with 7.5 ml 0.1 M phosphate buffered saline (PBS), giving a final DCF-H concentration of 50 μM. Peroxynitrite was synthesized according to the method of Beckman et al. [14]. Hydrogen peroxide was cleared from the solution by addition of granular manganese oxide, and peroxynitrite concentration was specified spectrometrically at 302 nm. The stock solutions were kept for 2–3 months at −80°C. Nitric oxide solutions were prepared by bubbling nitric oxide gas through argon purged water in a sealed ampoule and used on the same day. The nitric oxide concentration was determined amperometrically with a nitric oxide electrode (World Precision Instruments) to be 2 mM. Superoxide was generated by adding 5 μl of a saturated KO_2/DMSO solution to the incubation buffer. Phorbol 12-myristoyl 13-acetate (PMA) was purchased from Serva. All other chemicals, including the NOS inhibitor L-NAME, were obtained from Sigma.

2.2. Cell culture

Primary glial cell cultures from the cortex of newborn rats (Wistar) were prepared according to [15]. The cerebral cortex was removed, cleaned of meningeals and placed in Dulbecco's modified Eagle me-

PII S0014-5793(97)01197-6

dium, supplemented with 10% fetal calf serum. After mechanical dispersion, aliquots of the cell suspension were plated on poly-D-lysine coated coverslips (25 mm diameter) within a 35 mm petri dish at a final density of 2×10^6 cells/ml. Cultures containing mainly astro- and microglial cells were maintained at 37°C in a humidified 5% CO_2 atmosphere for 14 days. To upregulate the inducible nitric oxide synthase isoform (iNOS), lipopolysaccharide (LPS) from *E. coli* (2 μg/ml culture medium) and γ-interferon (100 units/ml) were added to 14 day old cultures. After 18 h the cell culture supernatants were assayed for nitrite levels by the method of Griess [16].

2.3. Fluorescent measurement

Glial cultures on coverslips were incubated with 50 μM DCF-DA in Locke's solution (154 mM NaCl, 5.6 mM KCl, 2.3 mM $CaCl_2$, 1 mM $MgCl_2$, 3.6 mM $NaHCO_3$, 15 mM HEPES (for peroxynitrite assays 75 mM HEPES), 10 mM glucose, pH 7.3) for 30 min, washed with Locke's solution, mounted in a steel chamber (Attofluor) and covered with 500 μl Locke's solution. For cell free experiments a coverslip was mounted in a steel chamber and covered with 500 μl Locke's solution. Cell culture and cell free experiments were conducted using a Zeiss LSM 410 inverted microscope (Zeiss 40× oil lens) coupled to an argon laser with a 488 nm excitation band. The emission long pass was a 515 nm filter, laser attenuation, pinhole diameter, photomultiplier sensitivity and offset were kept constant for every set of experiments. The obtained data were quantitatively analyzed using Zeiss LSM software and numerical presentation was conducted with Sigma PLOT.

2.4. High performance liquid chromatography (HPLC) separation

DCF-DA, DCF-H and DCF were separated by isocratic reversed phase chromatography [17] and detected by UV/VIS absorbance (Pharmacia apparatus). Column: μ-Bondapak C18 (RP18, 10 μ). Eluant: 20 mM phosphate buffer, pH 6.8 containing 57% methanol (v/v). Flow: 1 ml/min. Detection: absorbance at 270 nm (DCF-DA, DCF-H), absorbance at 488 nm (DCF).

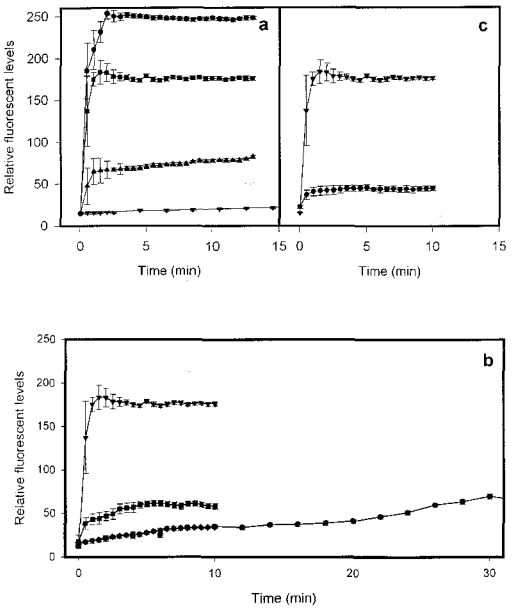

Fig. 1. The ability of different ROS to oxidize DCF-H and DHR-123 in a cell free system was quantitatively analyzed. DCF-DA was hydrolyzed to the non-fluorescent DCF-H and the capacity of different oxidizing substances was measured by confocal LSM. We found a concentration dependent (1 ▲, 2 ■ and 5 μM ●) oxidation of DCF-H to the fluorescent DCF by peroxynitrite (a). DCF-H displays a much greater sensitivity for peroxynitrite (2 μM ▼) than for nitric oxide (2 μM ♦), hydrogen peroxide (100 μM ●) or superoxide (5 μl KO_2/DMSO ■) (b). DCF-H oxidation by 2 μM peroxynitrite (▼) yields a much stronger fluorescence increase than oxidation of DHR-123 (●) (c). Values are mean ± S.E.M. from six experiments.

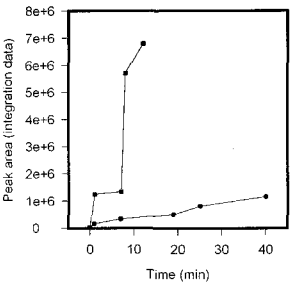

Fig. 2. DCF-H oxidation was additionally determined by HPLC. Peroxynitrite (100 and 1000 μM, ■) oxidized DCF-H much more efficiently than an equal concentration of nitric oxide (100 μM ●).

3. Results

3.1. DCF-H is oxidized by peroxynitrite

The ability of nitric oxide, hydrogen peroxide, superoxide and peroxynitrite to oxidize DCF-H was compared by measuring the fluorescence increase with a confocal LSM after addition of the respective compound to the DCF-H solution. DCF-DA was hydrolyzed to the non-fluorescent DCF-H in alkaline solution. Completeness of deacetylation and purity of the DCF-H solution were verified by HPLC (data not shown). Since DCF-H is extremely sensitive to light induced oxidation (unpublished observations) laser attenuation was set at 1/100 and to minimize light exposure scan times were set at 1 s. Peroxynitrite (1–5 μM final concentration) resulted in a concentration dependent and rapid oxidation of DCF-H, and reached the plateau within 2 min (Fig. 1a). Since peroxynitrite is stored in NaOH, controls were performed by adapting the solution to a final concentration of 2 mM NaOH, which had no effect on the fluorescence intensity.

Peroxynitrite was much more rapid and efficient in oxidizing DCF-H to its fluorescent derivative than all other compounds tested. Comparable or even higher concentrations of hydrogen peroxide, nitric oxide or superoxide oxidized DCF-H to a much lesser extent during the time period considered (Fig. 1b). Peroxynitrite oxidation of DCF-H reached a steady state after 2 min, whereas hydrogen peroxide and nitric oxide continued to oxidize DCF-H for at least 10 min.

Comparison of the peroxynitrite mediated fluorescence increase of DCF-H with that of the commonly used fluorogenic marker DHR-123 (both 50 μM) revealed that the speed of oxidation by peroxynitrite is similar for both compounds, but that DCF-H displayed a 4-fold greater increase in fluorescence intensity (Fig. 1c).

HPLC was applied to verify the methodological approach using a confocal microscope to measure quantitatively DCF-H oxidation. Addition of nitric oxide as well as peroxynitrite to DCF-H solutions shifted the substance peak of DCF-H to DCF. Besides DCF, no other oxidation products were seen in the chromatograms (data not shown). The time course and intensity of peroxynitrite and nitric oxide mediated oxidation of DCF-H were found to be comparable to the results obtained with confocal LSM (Fig. 2).

3.2. Peroxynitrite mediated DCF-H oxidation in glial cells

To test the ability of DCF-H for in vivo detection of peroxynitrite, DCF-DA loaded glial cultures were exposed to synthesized peroxynitrite. Addition of 10 μM peroxynitrite (prediluted in 100 μl loading buffer) exhibited a sudden, rela-

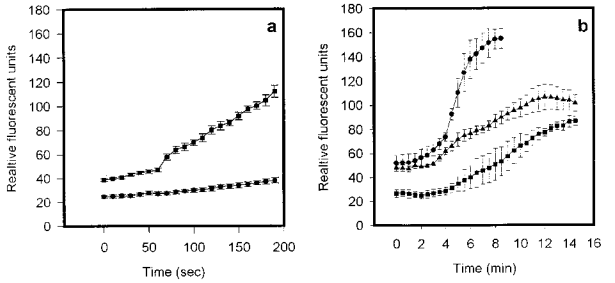

Fig. 3. DCF-H oxidation in primary glial cells by exogenous and endogenous peroxynitrite. a: Treatment of DCF-H loaded primary glial cultures with peroxynitrite (10 μM ■) resulted in a steady increase in fluorescence intensity whereas an equimolar concentration of nitric oxide (●) showed only a marginal effect. b: PMA stimulated microglia (■) showed a slow increase whereas mircoglia, producing simultaneously the peroxynitrite precursors nitric oxide and superoxide (●), showed a steep increase in DCF fluorescence. This effect could be blocked by addition of the NOS inhibitor L-NAME (▲). Amounts are average values of six to nine single cell measurements. Values are mean ± S.E.M. of 20 cells from three different experiments.

tively small fluorescent increase (Fig. 3a), followed by a steeper and steady increase for at least 3 min. An equimolar nitric oxide concentration showed almost no effect.

To study the effect of endogenously produced peroxynitrite, iNOS induced glial cultures were stimulated for superoxide formation with PMA in the presence and the absence of the NOS inhibitor L-NAME. PMA stimulated microglia showed the highest rates of DCF-H oxidation in the absence of the NOS inhibitor (Fig. 3b). This rate was strongly suppressed by L-NAME application yielding oxidation rates similar to those under conditions without iNOS induction. Without PMA stimulation, the rate of DCF oxidation in nitric oxide forming microglial cells was only slightly higher than the rate in controls. This suggests that the strong increase in DCF fluorescence seen under conditions of coincident superoxide and nitric oxide formation is due to the formation of peroxynitrite.

4. Discussion

Until now, increased DCF-H fluorescence has been mostly interpreted as mediated by hydrogen peroxide [9,18,19] and to a lesser extent by nitric oxide [10]. In contrast, our analysis by HPLC and fluorescence microscopy demonstrates that DCF-H displays a much higher sensitivity to peroxynitrite than all other compounds tested so far [9,20]. Importantly, we could show that peroxynitrite is the only oxidant so far that oxidizes DCF-H eminently within minutes. This strongly suggests that the formation of even small quantities of peroxynitrite will significantly and rapidly increase DCF fluorescence, whereas other oxidants at physiological concentrations will need much more time. Peroxynitrite mediated DCF-H oxidation has not been considered in previous studies and this might provide an explanation for the inconclusive results reported in the literature [10,11].

Since DCF-H and DHR-123 [21] are structurally related, the high sensitivity to peroxynitrite suggests a similar mechanism. Both molecules form fluorogenic compounds after hydrogen abstraction from an analogous chemical structure. The decay mechanism of peroxynitrite in aqueous solutions, which is associated with the formation of strongly oxidizing intermediates responsible for the hydrogen abstraction, is not well understood and was reported to comprise several pathways (which finally lead to NO_2^- and NO_3^- formation) [22].

One major difference between peroxynitrite and nitric oxide is the high speed with which peroxynitrite oxidizes DCF-H (Fig. 1b). The difference in the redox potentials of peroxynitrite (1.2 V) and nitric oxide (0.65 V) may serve as an explanation for these differences. Interestingly, nitric oxide continued to oxidize DCF-H for at least 40 min. This time span exceeds even that of the recently reported slow decay of nitric oxide in aqueous solution of 20 min [23].

To assess the applicability of DCF-DA for determining peroxynitrite formation at the cellular level we used primary glial cultures. Exogenously applied peroxynitrite to DCF-H loaded cells resulted in an steady increase in DCF fluorescence, whereas nitric oxide addition had, in line with the low capacity to oxidize DCF-H in the cell free system, only little effect on DCF fluorescence. This strongly suggests that peroxynitrite is capable of crossing intact cell membranes. The cells were virtually undamaged, as judged by propidium exclusion even 60 min after peroxynitrite addition. Another cell viability criterion was the apparent ability to retain DCF which leaks from damaged cells.

As a source of endogenous peroxynitrite we employed PMA stimulated microglia in the iNOS induced culture system. It has been shown that PMA stimulated macrophages convert almost all nitric oxide to peroxynitrite, with a rate of peroxynitrite formation of 0.11 nmol $\times 10^6$ cells per minute [24]. In line with these observations DCF fluorescence was highest in PMA stimulated microglia, and this effect was strongly suppressed by the addition of an NOS inhibitor. In contrast to the commonly used peroxynitrite marker DHR-123, DCF-H is upon oxidation not translocated to subcellular structures as is the case with rhodamine-123, the oxidation product of DHR, which accumulates within intact mitochondria. This feature of DHR-123 limits its application especially in experimental designs which alter the mitochondrial state, e.g. ischemia, hypoxia and cell death. Concluding from our results, DCF-H is well suited to monitor peroxynitrite formation within living cells and could provide further information on the role of peroxynitrite in physiological and pathophysiological circumstances.

Acknowledgements: We thank Christoph Riethmüller for preparing the nitric oxide and peroxynitrite solutions. This project was supported by a DFG research grant (WO 474/11-1,2) and the Land Sachsen-Anhalt (1903A/0025H).

References

[1] Beal, M.F. (1995) Ann. Neurol. 38, 357–366.
[2] Yu, B.P. (1994) Physiol. Rev. 74, 139–162.
[3] Sies, H. and de Groot, H. (1992) Toxicol. Lett. 64-65, 547–551.
[4] Dalkara, T., Yoshida, T., Irikura, K. and Moskowitz, M.A. (1994) Neuropharmacology 33, 1447–1452.
[5] Szabo, C. (1996) Brain Res. Bull. 41, 131–141.
[6] Wood, J. and Garthwaite, J. (1994) Neuropharmacology 33, 1235–1244.
[7] Crow, J.P. and Beckman, J.S. (1995) Adv. Pharmacol. 34, 17–43.
[8] Gille, G. and Sigler, K. (1995) Folia Microbiol. Praha 40, 131–152.
[9] LeBel, C.P., Ischiropoulos, H. and Bondy, S.C. (1992) Chem. Res. Toxicol. 5, 227–231.
[10] Gunasekar, P.G., Kanthasamy, A.G., Borowitz, J.L. and Isom, G.E. (1995) J. Neurosci. Methods 61, 15–21.

[11] Mattson, M.P., Lovell, M.A., Furukawa, K. and Markesbery, W.R. (1995) J. Neurochem. 65, 1740–1751.
[12] Reynolds, I.J. and Hastings, T.G. (1995) J. Neurosci. 15, 3318–3327.
[13] Cathcart, R., Schwiers, E. and Ames, B.N. (1984) Methods Enzymol. 105, 352–358.
[14] Beckman, J.S., Chen, J., Ischiropoulos, H. and Crow, J.P. (1994) Methods Enzymol. 233, 229–240.
[15] Schousboe, A. (1980) Cell Mol. Biol. 26, 505–513.
[16] Hevel, J.M. and Marletta, M.A. (1994) Methods Enzymol. 233, 250–258.
[17] Hurst, J.K., Albrich, J.M., Green, T.R., Rosen, H. and Klebanoff, S. (1984) J. Biol. Chem. 259, 4812–4821.
[18] Zhu, H., Bannenberg, G.L., Moldeus, P. and Shertzer, H.G. (1994) Arch. Toxicol. 68, 582–587.
[19] Carter, W.O., Narayanan, P.K. and Robinson, J.P. (1994) J. Leukocyte Biol. 55, 253–258.
[20] Royall, J.A. and Ischiropoulos, H. (1993) Arch. Biochem. Biophys. 302, 348–355.
[21] Szabo, C., Salzman, A.L. and Ischiropoulos, H. (1995) FEBS Lett. 372, 229–232.
[22] Pfeiffer, S., Gorren, A.C.F., Schmidt, K., Werner, E.R., Hansert, B., Bohle, D.S. and Mayer, B. (1997) J. Biol. Chem. 272, 3465–3470.
[23] Hakim, T.S., Sugimori, K., Camporesi, E.M. and Anderson, G. (1996) Physiol. Meas. 17, 267–277.
[24] Ischiropoulos, H., Zhu, L. and Beckman, J.S. (1992) Arch. Biochem. Biophys. 298, 446–451.