

QUANTIFYING CELLULAR OXIDATIVE STRESS BY DICHLOROFLUORESCEIN ASSAY USING MICROPLATE READER

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(Received 2 February 1999; Revised 10 May 1999; Accepted 11 May 1999)

Abstract—Oxidative stress (OS) has been implicated in various degenerative diseases in aging. In an attempt to quantify OS in a cell model, we examined OS induced by incubating for 30 min with various free radical generators in PC12 cells by using the dichlorofluorescein (DCF) assay, modified for use by a fluorescent microplate reader. The nonfluorescent fluorescein derivatives (dichlorofluorescein, DCFH), after being oxidized by various oxidants, will become DCF and emit fluorescence. By quantifying the fluorescence, we were able to quantify the OS. Our results indicated that the fluorescence varied linearly with increasing concentrations (between 0.1 and 1 mM) of H₂O₂ and 2,2'-azobios(2-amidinopropane) dihydrochloride (AAPH; a peroxy radical generator). By contrast, the fluorescence varied as a nonlinear response to increasing concentrations of 3-morpholinopyridone hydrochloride (SIN-1; a peroxynitrite generator), sodium nitroprusside (SNP; a nitric oxide generator), and dopamine. Dopamine had a biphasic effect; it decreased the DCF fluorescence, thus acting as an antioxidant, at concentrations <500 μM in cells, but acted as a pro-oxidant by increasing the fluorescence at 1 mM. While SNP was not a strong pro-oxidant, SIN-1 was the most potent pro-oxidant among those tested, inducing a 70 times increase of fluorescence at a concentration of 100 μM compared with control. Collectively, due to its indiscriminate nature to various free radicals, DCF can be very useful in quantifying overall OS in cells, especially when used in conjunction with a fluorescent microplate reader. This method is reliable and efficient for evaluating the potency of pro-oxidants and can be used to evaluate the efficacy of antioxidants against OS in cells. © 1999 Elsevier Science Inc.

Keywords—Oxidative stress, Dichlorofluorescein, Free radicals, Dopamine, Hydrogen Peroxide, AAPH, SIN-1, Sodium nitroprusside

INTRODUCTION

Oxidative stress (OS) has been implicated in various degenerative diseases in aging such as atherosclerosis, cancer, Parkinson's disease, and Alzheimer's disease [1]. In order to understand the mechanisms of these diseases that involve OS and to implement treatment or prevention, an analytic method to evaluate OS in living cell models is very important. Although there are various methods to assess oxidative damage of cells, such as

measuring lipid peroxidation products and DNA adducts [2], none of them evaluate the OS directly.

With the first description of using 2',7'-dichlorofluorescein diacetate (DCFH-DA) as a fluorometric assay for hydrogen peroxide [3], it became popular to use dichlorofluorescein (DCFH) as a probe to evaluate intracellular hydrogen peroxide formation by flow cytometry. The theory behind using DCFH-DA is that nonfluorescent fluorescein derivatives will emit fluorescence after being oxidized by hydrogen peroxide [4]. The emitted fluorescence is directly proportional to the concentration of hydrogen peroxide. When applied to intact cells, the nonionic, nonpolar DCFH-DA crosses cell membranes and is hydrolyzed enzymatically by intracellular esterases to nonfluorescent DCFH [4,5]. In the presence of reactive oxygen species (ROS), DCFH is oxidized to highly fluorescent dichlorofluorescein (DCF) [4]. There-

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fore, the intracellular DCF fluorescence can be used as an index to quantify the overall OS in cells.

Many studies have used fluorescent microscopy to quantify OS in cells using DCFH-DA. This method has the problem of inducing photooxidation of DCFH to DCF intracellularly and emitting fluorescence [6], because it is difficult to control the time of light exposure when trying to locate and focus cells under the microscope. In addition to the problem of photooxidation, there is no standard way to quantify the OS using a microscope. In order to prevent the overestimation of the OS due to photooxidation, an instrument equipped with fast light excitation and fast fluorescence capturing, which will not induce excessive photooxidation, is needed.

In this study, we used a fluorescent microplate reader to evaluate OS in cells, induced by applying various free radical generators extracellularly, using DCFH as the probe. Our results indicated that various free radical generators produced concentration-dependent changes in DCF fluorescence, indicating the indiscriminate nature of DCF. This feature of DCF allows the assay to be used to measure the overall OS in cells. By using a microplate reader, we were able to use 96-well plates, which gave us a large amount of data with low variability.

MATERIALS AND METHODS

Cell cultures

PC12 cells were a gift from Dr. Arthur Tischler (Tufts University, School of Medicine, Boston, MA, USA). They were grown in growth medium containing 85% RPMI-1640 with *L*-glutamine, 10% heat-inactivated horse serum, 5% fetal bovine serum (FBS), 100 U/ml penicillin G sodium, and 100 $\mu\text{g}/\text{ml}$ streptomycin sulfate. The cells were maintained in collagen-coated plates in 5% $\text{CO}_2/95\%$ air at 37°C. The culture medium was changed twice every week and the cells were split 1:4 or 1:8 every week.

Chemicals

6-Carboxy-2',7'-dichlorofluorescein diacetate (DCFH-DA) and 3-morpholinopyridone hydrochloride (SIN-1) were purchased from Molecular Probes Inc. (Eugene, OR, USA). Sodium nitroprusside (SNP) and hydrogen peroxide were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) was purchased from Wako Chemicals Inc. (Richmond, VA, USA). All materials for the culture medium were purchased from Gibco BRL, Life Technologies Inc. (Gaithersburg, MD, USA). DCFH-DA was dissolved in dimethyl sulfoxide (DMSO)

as stock solution and kept frozen in -20°C . For loading the cells with DCFH, DCFH-DA from stock solution was mixed with loading medium (99% RPMI-1640 and 1% FBS) to a final concentration of 100 μM .

DCF assay for OS

Cell preparation. Cells were split and counted by trypan blue exclusion. Viable cells ($10^4/\text{well}$) were plated into 96-well collagen-coated plates 1 day before the experiments. On the day of the experiments, after removing the medium, the cells in the plates were washed with KRH buffer and then incubated with 100 μM DCFH-DA in the loading medium in 5% $\text{CO}_2/95\%$ air at 37°C for 30 min. After DCFH-DA was removed, the cells were washed and incubated with KRH buffer (with different concentrations of one of the following free radical generators: H_2O_2 , AAPH, dopamine, SNP, and SIN-1) and the fluorescence of the cells from each well was measured and recorded.

Instrument and OS measurement. DCFH-DA-loaded cells were placed in a CytoFluor Series 4000 multiwell fluorescence plate reader (PerSeptive Biosystems Inc., Framingham, MA, USA) with temperature maintained at 37°C. The excitation filter was set at 485 ± 10 nm and the emission filter was set at 530 ± 12.5 nm. The fluorescence from each well was captured, digitized, and stored on a computer using Cytofluor (Version 4.0) (PerSeptive Biosystems Inc., Framingham, MA, USA). Data points were taken every 5 min for 30 min and the data were exported to Excel (Microsoft, Seattle, WA, USA) spreadsheet software for analysis.

Data analysis

The percentage increase in fluorescence per well was calculated by the formula $[(F_{t_{30}} - F_{t_0})/F_{t_0} * 100]$, where $F_{t_{30}}$ = fluorescence at time 30 min and F_{t_0} = fluorescence at time 0 min. This method of analysis has advantages over analyzing just the net change in fluorescence in that, not only did the calculated data directly reflect the percentage changes of fluorescence over time from the cells in the same well, they also effectively control for variability among wells. This method also canceled out the background fluorescence in each well, and therefore, a "no cell" control is not needed.

RESULTS AND DISCUSSION

As a standard ROS for the DCF assay, we first tested H_2O_2 . Figure 1 shows the concentration-response relationship of cells exposed to H_2O_2 . Similar to the results

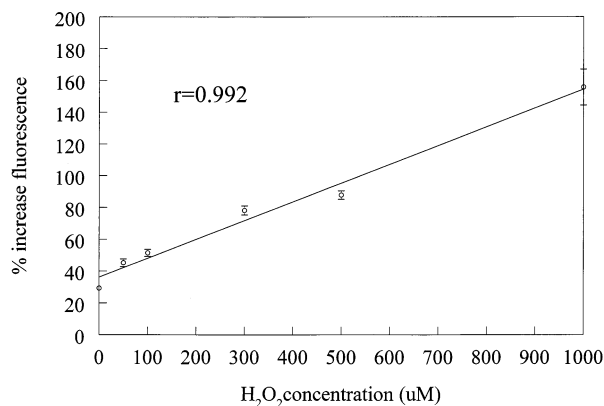


Fig. 1. Concentration-response curve of percentage increase of DCF fluorescence in PC12 cells after 30 min exposure to various concentrations of H₂O₂. Each data point represents the mean of data from eight wells ($n = 8$) with error bars representing the SEM. The line was created by curve fitting using linear regression; r is the correlation coefficient.

found by LeBel et al. [4], the percentage increase of fluorescence is linearly correlated ($r = 0.992$) with the concentration of H₂O₂ in the range of 50 μ M to 1 mM. A similar linearly correlated ($r = 0.993$) concentration-response relationship was found when using AAPH as the free radical generator in the same concentration range (Fig. 2). AAPH, which generates peroxy radicals [7], has been shown to oxidize DCFH to fluorescent DCF in vitro [8]. The peroxy radicals can initiate chain lipid peroxidation in the membrane and generate lipid peroxides [7], which can also oxidize DCFH to fluorescent DCF [9].

Dopamine has been implicated as a source of OS in the pathogenesis of Parkinson's disease [10]. Dopamine

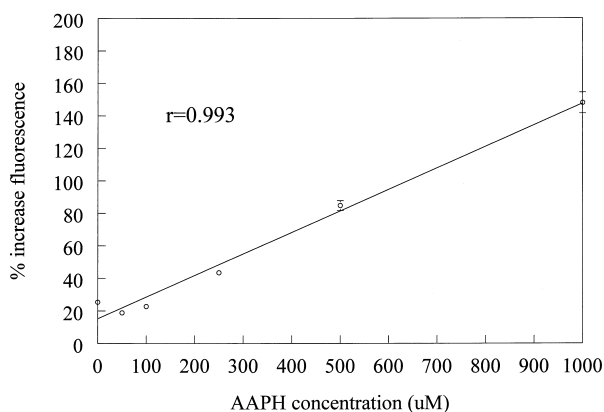


Fig. 2. Concentration-response curve of percentage increase of DCF fluorescence in PC12 cells after 30 min exposure to various concentrations of AAPH. Each data point represents the mean of data from eight wells ($n = 8$) with error bars representing the SEM. The line was created by curve fitting using linear regression; r is the correlation coefficient.

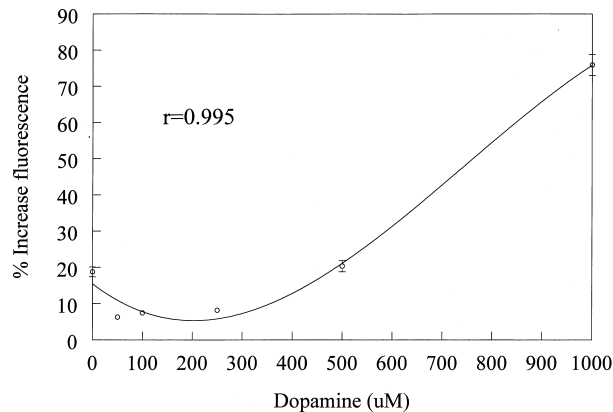


Fig. 3. Concentration-response curve of percentage increase of DCF fluorescence in PC12 cells after 30 min exposure to various concentrations of dopamine. Each data point represents the mean of data from eight wells ($n = 8$) with error bars representing the SEM. The line was created by curve fitting using nonlinear regression; r is the correlation coefficient.

can be metabolized by monoamine oxidase in cells, or it can also spontaneously autooxidize [10]. Either metabolic pathway can produce ROS, including H₂O₂ [10–12], and can increase the vulnerability of dopaminergic neurons. Therefore, we were interested in investigating the effect of dopamine as a free radical generator in our cell model. Dopamine did not show a linear response, but instead showed a biphasic concentration-response curve ($r = 0.995$) (Fig. 3). Dopamine at concentrations less than 500 μ M reduced the fluorescence, reflecting that it acted as an antioxidant in cells, but at 1 mM, it increased the fluorescence, and thus acted as a pro-oxidant. Our results are in agreement with the finding that dopamine can act as an antioxidant and prevent lipid peroxidation [13]. Our results also support the notion that dopamine does not consistently induce OS [13]. Whether dopamine acts as a pro-oxidant or antioxidant may depend on the condition of the cells and the concentration of dopamine.

Besides dopamine, nitric oxide (NO), which is generated during ischemia, has been implicated as a mediator of neuronal excitotoxicity [14], and therefore plays an important role in neuronal injury [15]. We tested two NO-generating compounds, SNP, which generates NO, and SIN-1, which generates both NO and superoxide resulting in the formation of peroxynitrite [16]. It has been shown that both NO [17,18] and peroxynitrite [19] are able to oxidize DCFH to fluorescent DCF. Interestingly, our results showed that SNP did not have a linear response (with a nonlinear $r = 0.952$) and started to plateau at high concentration, indicating that it did not have a strong pro-oxidant effect (Fig. 4). It only induced about three times the percentage increase in fluorescence with a concentration of 5 mM in treated cells compared with control cells (SNP at 0 mM) (32% vs. 9%, respec-

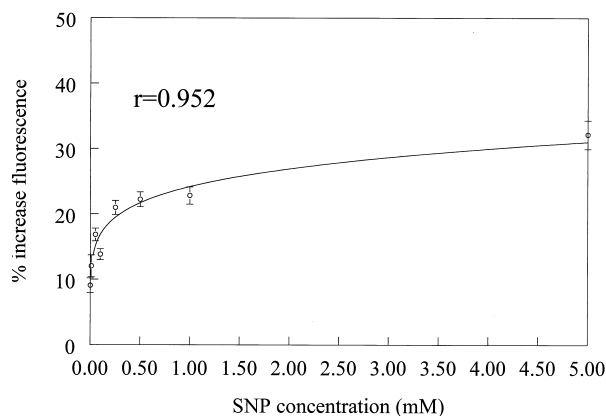


Fig. 4. Concentration-response curve of percentage increase of DCF fluorescence in PC12 cells after 30 min exposure to various concentrations of SNP. Each data point represents the mean of data from eight wells ($n = 8$) with error bars representing the SEM. The line was created by curve fitting using nonlinear regression; r is the correlation coefficient.

tively), and the effect of NO reached plateau at 0.5 mM (Fig. 4). This result was in agreement with the results that low concentrations (1–10 μM) of NO do not oxidize DCFH [19], although at higher concentrations (0.8–6.4 mM), NO is able to oxidize DCFH [17]. On the contrary, SIN-1, which generates peroxynitrite, was the most potent pro-oxidant that we tested; it also had a nonlinear concentration-response ($r = 0.999$) (Fig. 5). It induced 70 times increase of the fluorescence at only 100 μM compared with control (1117 vs. 16%, respectively). Below 10 μM SIN-1 still induced a linear concentration-dependent increase of fluorescence, suggesting that peroxynitrite or its proposed decomposition products, including hydroxyl radical [20], has a very strong

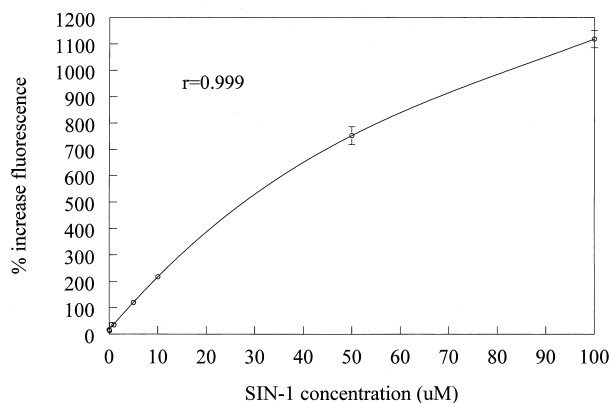


Fig. 5. Concentration-response curve of percentage increase of DCF fluorescence in PC12 cells after 30 min exposure to various concentrations of SIN-1. Each data point represents the mean of data from eight wells ($n = 8$) with error bars representing the SEM. The line was created by curve fitting using nonlinear regression; r is the correlation coefficient.

oxidizing power to induce OS in cells. Our data were also in agreement with findings that peroxynitrite is more potent than NO in oxidizing DCFH [19]. It has been suggested that neurotoxicity of NO is engendered by the formation of peroxynitrite and not by NO alone [16]. Due to its redox versatility, which allows interconversion between different redox states, NO can also be neuroprotective [16]. We did not observe an antioxidant effect of NO when using SNP in our model, although NO has been shown to scavenge ROS [21] and to be protective against OS [15,16,21,22]. Although NO can be protective under certain experimental conditions, it may still be destructive during pathologic conditions such as stroke [15], because with a limited supply of substrate during stroke, NO synthase may produce a mixture of superoxide anion and NO, which reacts to form peroxynitrite and results in cytotoxicity [15].

In summary, due to the indiscriminate nature of DCFH, which can be oxidized by various ROS and not just H_2O_2 , the increase of intracellular DCF fluorescence does not necessarily reflect the levels of ROS directly, but rather an overall OS index in cells. Quantifying cellular OS by the DCF assay using a fluorescent microplate reader is an easy and efficient method with low variability which can be used to quantify the potency of pro-oxidants or can be adapted to evaluate the efficacy of antioxidants against ROS in various cell lines.

Acknowledgements — We thank Dr. Gerard Dallal for his advice in statistical analysis.

REFERENCES

- [1] Kehrer, J. P.; Smith, C. V. Free radicals in biology: sources, reactivities, and roles in the etiology of human diseases. In: Frei, B., ed. *Natural antioxidants in human health and disease*. San Diego: Academic Press; 1994:25–62.
- [2] Holley, A. E.; Cheeseman, K. H. Measuring free radical reactions in vivo. *Br. Med. Bull.* **49**:494–505; 1993.
- [3] Keston, A. S.; Brandt, R. The fluorometric analysis of ultramicro quantities of hydrogen peroxide. *Anal. Biochem.* **11**:1–5; 1965.
- [4] LeBel, C. P.; Ishiropoulos, H.; Bondy, S. C. Evaluation of the probe 2',7'-dichlorofluorescein as an indicator of reactive oxygen species formation and oxidative stress. *Chem. Res. Toxicol.* **5**:227–231; 1992.
- [5] Bass, D. A.; Parce, J. W.; Dechatelet, L. R.; Szejda, P.; Seeds, M. C.; Thomas, M. Flow cytometry studies of oxidative product formation by neutrophils: a graded response to membrane stimulation. *J. Immunol.* **130**(4):1910–1917; 1982.
- [6] Sarvazyan, N. Visualization of doxorubicin-induced oxidative stress in isolated cardiac myocytes. *Am. J. Physiol.* **271**:H2079–H2085; 1996.
- [7] Niki, E. Free radical initiators as source of water- or lipid-soluble peroxy radicals. *Methods Enzymol.* **186**:100–108; 1990.
- [8] Valkonen, M.; Kuusi, T. Spectrophotometric assay for total peroxy radical-trapping antioxidant potential in human serum. *J. Lipid Res.* **38**:823–833; 1997.
- [9] Cathcart, R.; Schwieters, E.; Ames, B. N. Detection of picomole levels of hydroperoxides using a fluorescent dichlorofluorescein assay. *Anal. Biochem.* **134**(1):111–116; 1983.
- [10] Olanow, C. W. An introduction to the free radical hypothesis in Parkinson's disease. *Ann. Neurol.* **32**:S2–S9; 1992.

- [11] Marker, H. S.; Weiss, C.; Silides, D. J.; Cohen, G. Coupling of dopamine oxidation (monoamine oxidase activity) to glutathione oxidation via the generation of hydrogen peroxide in rat brain homogenates. *J. Neurochem.* **36**:589–593; 1981.
- [12] Graham, D. G.; Tiffany, S. M.; Bell, W. R., Jr.; Gutknecht, W. F. Autooxidation versus covalent binding of quinones as the mechanism of toxicity of dopamine, 6-hydroxydopamine, and related compounds toward C1300 neuroblastoma cells in vitro. *Mol. Pharmacol.* **14**:644–653; 1978.
- [13] Ben-Schachar, D.; Zuk, R.; Glinka, Y. Dopamine neurotoxicity: inhibition of mitochondrial respiration. *J. Neurochem.* **64**:718–723; 1995.
- [14] Zhang, J.; Snyder, S. H. Nitric oxide in the nervous system. *Annu. Rev. Pharmacol. Toxicol.* **35**:213–233; 1995.
- [15] Iadecola, C. Bright and dark sides of nitric oxide in ischemic brain injury. *Trends Neurosci.* **20**:132–139; 1997.
- [16] Lipton, S. A.; Choi, Y. B.; Pan, Z. H.; Lei, S. Z.; Chen, H. S. V.; Sucher, N. J.; Loscalzo, J.; Singel, D. J.; Stamler, J. S. A redox-based mechanism for the neuroprotective and neurodestructive effects of nitric oxide and related nitroso-compounds. *Nature* **364**:626–632; 1993.
- [17] Gunasekar, P. G.; Kanthasamy, A. G.; Borowitz, J. L.; Isom, G. E. Monitoring intracellular nitric oxide formation by dichlorofluorescein in neuronal cells. *J. Neurosci. Methods* **61**:15–21; 1995.
- [18] Murali Krishna Rao, K.; Padmanabhan, J.; Kilby, D. L.; Cohen, H. J.; Currie, M. S.; Weiberg, J. B. Flow cytometry analysis of nitric oxide production in human neutrophils using dichlorofluorescein diacetate in the presence of a calmodulin inhibitor. *J. Leukoc. Biol.* **51**:496–500; 1992.
- [19] Kooy, N. W.; Royall, J. A.; Ischiropoulos, H. Oxidation of 2',7'-dichlorofluorescein by peroxynitrite. *Free Radic. Res.* **27**(3):245–254; 1997.
- [20] Hogg, N.; Darley-Usmar, V. M.; Wilson, M. T.; Moncada, S. Production of hydroxyl radicals from the simultaneous generation of superoxide and nitric oxide. *Biochem. J.* **281**:419–424; 1992.
- [21] Wink, D. A.; Hanbauer, I.; Krishna, M. C.; Degraff, W.; Gamson, J.; Mitchell, J. B. Nitric oxide protects against cellular damage and cytotoxicity from reactive oxygen species. *Proc. Natl. Acad. Sci. USA* **90**:9813–9817; 1993.
- [22] Wink, D. A.; Hanbauer, I.; Grisham, M. B.; Laval, F.; Nims, R. W.; Laval, J.; Cook, J.; Pacelli, R.; Liebmann, J.; Krishna, M. C.; Ford, P. C.; Mitchell, J. B. Chemical biology of nitric oxide: regulation and protective and toxic mechanisms. *Curr. Topics Cell Regul.* **34**:159–187; 1996.