Journal of Fluorescence, Vol. 10, No. 2, 2000

Fluorogenic Probes Applied to the Study of Induced Oxidative Stress in the Human Leukemic HL60 Cell Line

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Received September 28, 1999; accepted December 30, 1999

Development of microspectrofluorometric methods using specific fluorogenic probes has provided precious help in studying *in situ* oxidative stress and cellular protective systems. The aim of this study was to determine ROS production concomitantly with a modification of the intracellular thiol pool after applying an oxidative stress to a nonadherent cell model represented by the HL60 cell line. The dichlorodihydrofluorescein diacetate (H_2DCFDA) probe assessed the kinetic production of ROS by cells submitted to the chemical oxidant *t*-butylhydroperoxide with a high signal/noise ratio. The probe sensitivity permitted us to detect endogenous ROS production in HL60 cells and the protective effect of *N*-acetyl cysteine against ROS. The chloromethylfluorescein diacetate probe (CMFDA) permitted us to evaluate the thiol depleting effect of *N*-ethyl maleimide. Complete thiol depletion was associated with a moderate increase in ROS production. The cell viability was determined with calcein–AM, which gave results similar to those with the tetrazolium dye. This probe was not affected by intracellular pH and did not required an extraction step, unlike tetrazolium dye. In conclusion, cell-permeant fluorogenic probes are useful and sensitive tools to determine *in situ* ROS production concomitantly with consecutive change in the thiol system in a living and nonadherent cell model.

KEY WORDS: Fluorogenic probes; microspectrofluorometry; HL60 cell line; oxidative stress; thiol status.

INTRODUCTION

Oxidative stress can be defined as the pathologic outcome of oxidant overproduction as reactive oxygen species (ROS) that overwhelms the cellular antioxidant capacities and causes cellular damages. Oxidative stress is directly or indirectly involved in several pathological processes such as acute inflammatory reaction. The transient nature of ROS intermediates ($O_2^{\circ -}$, OH^-, etc.) involves technical difficulties in measuring their level in living cells. ROS detection involves the use of intermediate compounds that will be oxidized in cells by ROS to either chromogenic, fluorescent, or luminescent products. Intracellular detection requires a substrate that has a fast rate of reaction with ROS and can be delivered at a concentration high enough to surpass antioxidant and scavenging pathways.

At this time, flow cytometry is able to quantify fluorescence signals in living cells which are labeled with specific probes for ROS detection [1–3]. Nevertheless, this method has the disadvantage of being time-consuming and not easy to handle or to miniaturize. One of the most recent applications of fluorescence is micromethod development owing to the adaptation of instrumentation

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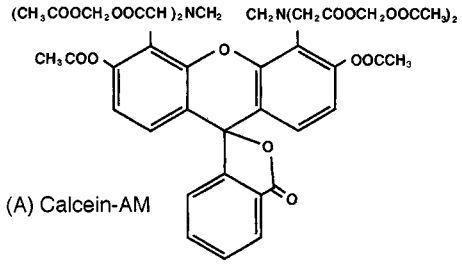
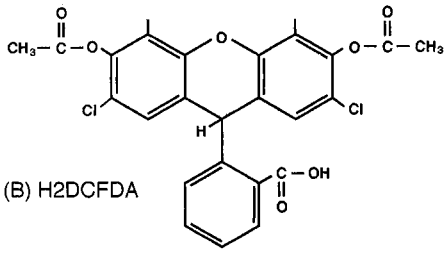
and, particularly, a highly focused light beam, preventing cross-contamination and ensuring high reproducibility. At present, a panel of fluorogenic probes is developed for analysis of cellular metabolism [4] (Fig.1).

- Calcein-AM, corresponding to the acetoxymethyl ester form of calcein, can be used to evaluate cell viability. In the cytosol, this cell-permeant probe is cleaved by cytoplasmic esterases to a fluorescent analogue. In necrotic cells, the loss of esterase activity leads to a decrease in fluorescence activity [5,6].
- The 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) probe permits us to detect ROS production *in situ*. The cell-permeant form, H_2DCFDA, diffuses rapidly through the cell membrane and then is hydrolyzed by intracellular esterases to an oxidative-sensitive form, dichlorodihydrofluorescein (H_2DCF). In the cytosol, oxidation of H_2DCF leads to a fluorescent compound, dihydrofluorescein (DCF), whose fluorescence intensity is proportional to intracellular ROS [7,8].
- The 5-chloromethylfluorescein diacetate probe (CMFDA) is used to determinate the thiol status in living cells. After penetration into the cell, the CMFDA probe leads, by cleavage with esterases, to a fluorescent compound, chloromethylfluorescein (CMF). The glutathione transferase catalyzes the conjugation between CMF and thiols. The conjugated form of the probe is retained inside the cell, whereas the unconjugated probe is released into the medium [1,9,10].

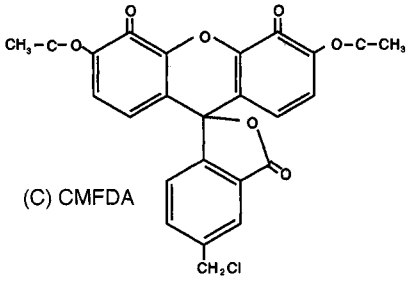
We have focused our strategy on the development of microspectrofluorometry using specific fluorogenic probes and instrumentation that have provided precious help in evaluating ROS and redox potential parameters, such as thiol, directly on cell culture microplates and with the possibility of automatic scanning. Until now, most papers have concerned adherent cells [6,11]. In this paper, we present an application of such methodology to a non-adherent model, the human promyelocytic leukemia HL60 cell line. This cell line is used as a model for the *in vitro* study of leukemic cell differentiation into granulocytic and monocytic cells and is considered an *in vitro* model of inflammation cellular processes [12]. In this paper, the HL60 cell line was used to assess ROS production concomitantly with a modification of intracellular thiols after inducing oxidative stress.



Calcein Acetoxy Methyl Ester



2', 7'- dichlorofluorescin diacetate



Chloromethyl Fluorescein Diacetate

Fig. 1. Chemical structures of (A) the calcein-AM probe, (B) the 2′,7′-dichlorofluorescein diacetate probe (H_2DCFDA), and (C) the 5-chloromethylfluorescein diacetate probe (CMFDA).

EXPERIMENTAL

Materials

Cell culture media and supplements were purchased from Life Technologies Gibco (Cergy Pontoise, France). Tert-butylhydroperoxide (t-BHP), 70% (v/v) in aqueous solution, *N*-acetylcysteine (NAC), *N*-ethylmaleimide (NEM), and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma Chemical Co. (St. Louis, MO). Fluorogenic probes, 2′,7′-dichlorodihydroflourescein diacetate (H_2DCFDA), 5-chloromethylfluorescein diacetate (CMFDA), and calcein–acetomethyl ester (calcein-AM), were purchased from Molecular Probes (Eugene, OR). Polystyrene 96-well culture plates were obtained from Falcon (B.D. Biosciences, Le Pont de Claix, France).

Cell Culture

HL60 cells (ECACC reference No. 85011431) were maintained in continuous suspension culture in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), penicillin (100 U/ml), streptomycin (100 μg/ml), and L-glutamine (2 m*M*) in a humidified atmosphere containing 5% CO_2 at 37°C. Cells were routinely passed by seeding $3 \cdot 10^5$ cells/ml into 75-cm^2 tissue culture flasks (Corning, NY) at 2- to 3-day intervals. Experiments were performed between the twentieth and the fortieth passage. For cell characterization, cytospin slide preparations of a 0.15 ml suspension at 10^6 cells/ml were obtained using a Shandon Cytospin 3 and stained with May–Grunwald–Giemsa reagent before microscopic examination. Under normal culture conditions, most cells have the morphology of myelocytic and promyelocytic cells. However, a significant percentage of cells (10–12%) is more mature, having morphological characteristics of myelocytes and metamyelocytes.

Oxidative Stress Induction

Cells were always passed 24 h before oxidative stress induction achieved with a chemical oxidant reagent, tert-butyl hydroperoxide (t-BHP). Cells were washed twice and resuspended at $2 \cdot 10^6$ cells/ml in RPMI 1640 without FCS or phenol red and equilibrated at 37°C for at least 20 min before starting the treatment. Cells were distributed (100 μl) in 96-well flat-bottom plates and exposed to t-BHP (50 μl) at a final concentration ranging from 0.1 to 2 m*M*. Treated cells were maintained for 30 min in humidified 5% CO_2 atmosphere at 37°C before fluorescence labeling.

Quantitative Fluorescence Labeling

Quantification of ROS Production and Intracellular Thiol Determination

In HL60 cells, ROS levels were quantified *in situ* by labeling with the fluorogenic DCFDA probe. Briefly, cells were seeded in 96-well flat-bottom plates (100-μl) and treated with oxidant reagent t-BHP as described above. After a 30-min incubation at 37°C, 10 μ*M* DCFDA probe was added to each well at a final volume of 200 μl. Cells were incubated for 30 min at 37°C in the dark until fluorescence reading.

Determination of the thiol amount was performed under the same conditions by use of the fluorogenic CMFDA probe. The procedure adopted for labeling with the 5 μ*M* CMFDA probe was the same than for the DCFDA probe.

Viability Assay with Calcein-AM

Calcein-AM assay was carried out as follows: HL60 cells were suspended in RPMI 1640 without FCS or phenol red and adjusted to $2 \cdot 10^6$ cells/ml. After treatment with t-BHP (200 μl), 8 μ*M* calcein-AM was directly added to the cell suspension (400 μl). Cells were incubated for 30 min at 37°C in the dark and were washed twice with RPMI 1640 without FCS or phenol red to eliminate the nonincorporated probe. Then cells were resuspended in the same medium and were seeded at a final volume of 200 μl in the 96-well flat-bottom plate for fluorometric analysis avoiding direct light.

This calcein-AM assay was compared to the well-known quantification of MTT reduction by mitochondrial dehydrogenases as described previously [13]. Briefly, cells were seeded in 96-well flat-bottom plates (200 μl) and incubated for 4 h with 12.1 m*M* MTT solution (20 μl) in RPMI 1640 without FCS or phenol red at 37°C in a 5% CO_2 atmosphere. After supernatant was removed, formazan dye was solubilized in dimethyl sulfoxide under gentle shaking. Absorbances were read at 540 nm on a multiwell scanning spectrophotometer (WS050 Wellscan, UK).

Microspectrofluorometric Determination

Fluorescence quantification was performed by scanning the plate in a Fluostar microspectrofluorometric plate

reader (BMG LabTechnologies, France). This plate reader consisted of two essential units, a source of radiation and a system for measuring the intensity of fluorescence emission. A xenon lamp was used as the energy source. This highly focused light beam prevents cross-contaminations and the number of flashes of the xenon lamp ensures accurate reading with guarantees of high reproducibility. For experimental conditions, fluorescence reading was realized at the top of the plastic microplate with a multipoint reading in each well. The optimum wavelengths were 485-nm excitation λ and 530-nm emission λ. The reading was realized within 10 s.

Background levels were achieved by incubation of chemical reagents with the fluorogenic probes. Moreover, because of the sensitivity of the probes to light (daylight and artificial light), more particularly, the DCFDA probe, control wells with only the fluorogenic probe were necessary for testing the basal oxidation state of the probe in each experiment. Because of the possibility of probe diffusion out of the cells, a direct and rapid reading in the Fluostar plate reader was required. Result, obtained directly in arbitrary fluorescence units (AFU), were expressed as the percent of untreated cells (100%).

Microscopic Examination

Cell repartition is checked by microscopic examination with an inverted-phase Olympus IMT2 microscope. The fluorescence labeling of the cell was controlled using the Olympus microscope equipped with a 100-W HBO mercury–vapor lamp epi-illumination.

N-Acetyl Cysteine Protection

N-Acetyl cysteine (NAC) was dissolved in 1 m*M* phosphate buffer saline to form a 500 m*M* stock solution and the pH was adjusted to 7.0–7.5 with 1 *M* sodium hydroxide solution. NAC solution (50 μl) at final concentrations ranging from 5 to 50 m*M* was applied for 1 h to the cell suspension at $2 \cdot 10^6$ cells/ml (100 μl) in a 96-well flat-bottom plate. After incubation, the cells were submitted to oxidative stress induced by 1 m*M* t-BHP (50 μl) as described above before the fluorescence reading.

Thiol Depletion

The intracellular thiol pool was depleted by pretreatment with a thiol-alkylating agent, *N*-ethyl maleimide (NEM). For that, a cell suspension at $2 \cdot 10^6$ cells/ml was distributed in a 96-well flat-bottom plate (100 μl). After addition of the NEM solution (50 μl) at final concentrations ranging from 0.05 to 0.4 m*M*, cells were set to incubate for 1 h at 37°C. Next cells were labeled by the addition of 5 μ*M* CMFDA probe and microspectrofluorometric reading was done after a 30-min incubation at 37°C in the dark. For cells submitted to oxidative stress, t-BHP (50 μl) was added 30 min before the end of incubation with NEM.

Statistical Analysis

Data, presented as mean ± SD, were obtained from at least three independent experiments. Significant differences were determined using the Student *t*-test and $P < 0.05$ was considered to indicate a significant difference.

RESULTS

Cellular Viability of HL60 Cells After t-BHP Treatment

HL60 cells were exposed to increasing concentrations ranging from 0.1 to 2 m*M*, of the known direct potent oxidant t-BHP. Cell survival was evaluated by both MTT and calcein-AM assays. The data in Figs. 2A and B illustrate the average time/concentration response of HL60 cells using either MTT or calcein-AM assays as the end-point measurements. The results obtained with calcein-AM were in agreement with those obtained with MTT dye. The cell viability was t-BHP concentration and time dependent. Contact over 6 h with a t-BHP concentration higher than 0.1 m*M* induced a necrotic effect for HL60 cells. The IC_{50} value was calculated from the plot of triplicate experiments, and for 1 m*M* t-BHP, it corresponded to a contact of 7 H. After a 24-h incubation, t-BHP induced cellular death by the necrosis process even at the lowest concentration: with 0.1 m*M*, only 25% of the cells were dead. Until 3-h of contact, cell viability was over 90% at all t-BHP concentrations. This period was selected in further experiments, a 1-h contact was chosen.

The microscopic examination was in agreement with the fluorescence measurement. Figures 3A and B represent, respectively, calcein-AM labeling in cells treated with 1 m*M* t-BHP for 1 h and cells treated with 1 m*M* t-BHP for 6 h.

ROS Production

ROS production was evaluated by measuring the fluorescence intensity of cells labeled with the H_2DCFDA probe. Without any oxidative treatment, we observed slight DCF fluorescence (1684.5 ± 202.5 AFU) in

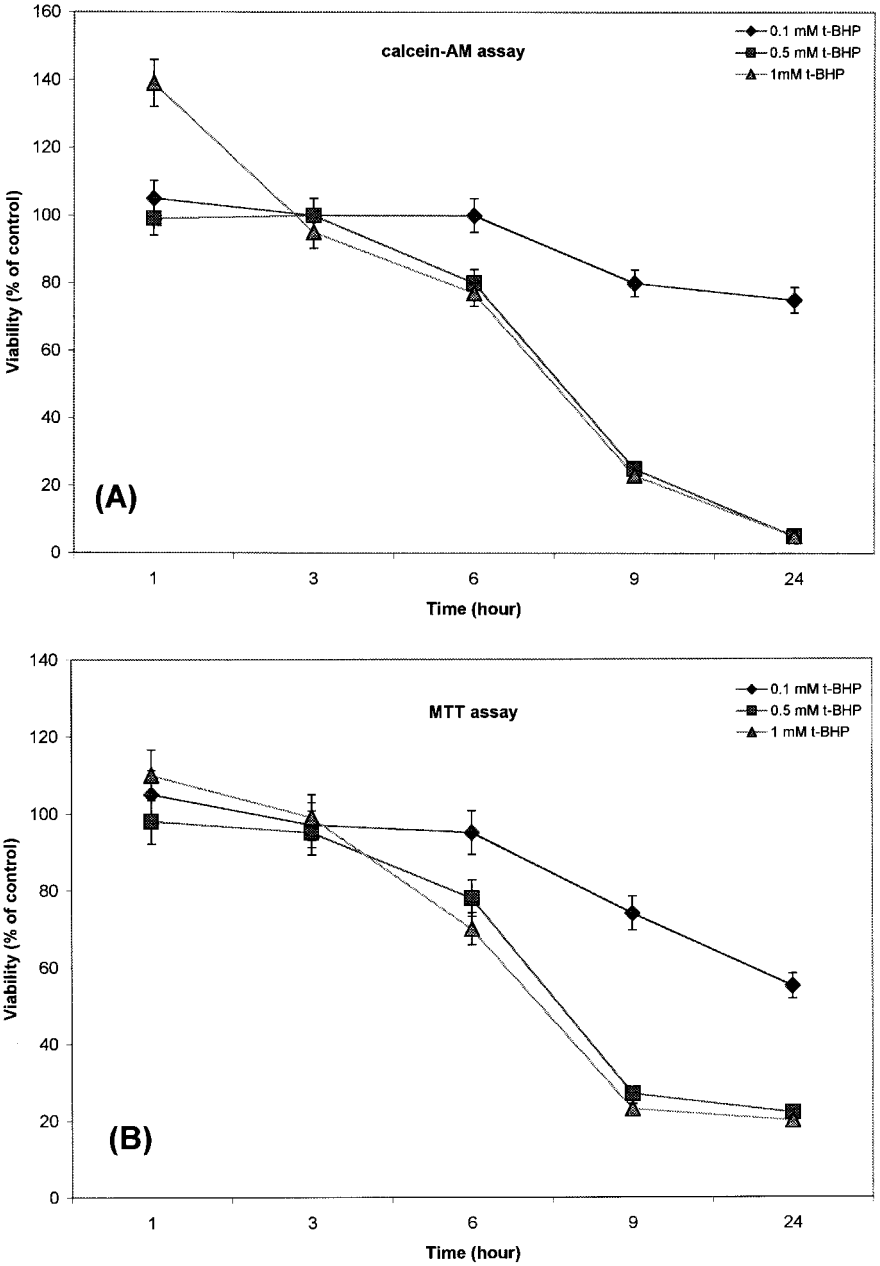


Fig. 2. Cell viability after oxidative treatment applied for 1 to 24 h. Cells were incubated with t-BHP at 0.1, 0.5, and 1 m*M* for 1, 3, 6, 9, and 24 h. (A) Cell viability was assessed by fluorescence reading after a 30-min incubation with calcein-AM (8 μM); (B) cell viability was assessed by colorimetric reading after a 4-h incubation with MTT (12.1 m*M*) and an extraction step with DMSO. The colorimetric and fluorescence signals are represented as the percentage of untreated cells (6646.0 ± 259.7 AFU at gain 10) and were calculated from the mean of three experiments.

untreated cells. Fluorescence microscopic examination confirmed H_2DCFDA labeling in the basal state of HL60 cells.

Oxidative stress was performed by applying a potent oxidant reagent, t-BHP, to the cells at final concentrations ranging from 0.1 to 2 m*M*. Data are shown in Fig. 4. The H_2DCFDA labeling showed that the ROS production decreased in a time-dependent manner: the maximum signal was reached at a 1-h incubation with t-BHP, and after 6 h or incubation the fluorescence signal decreased

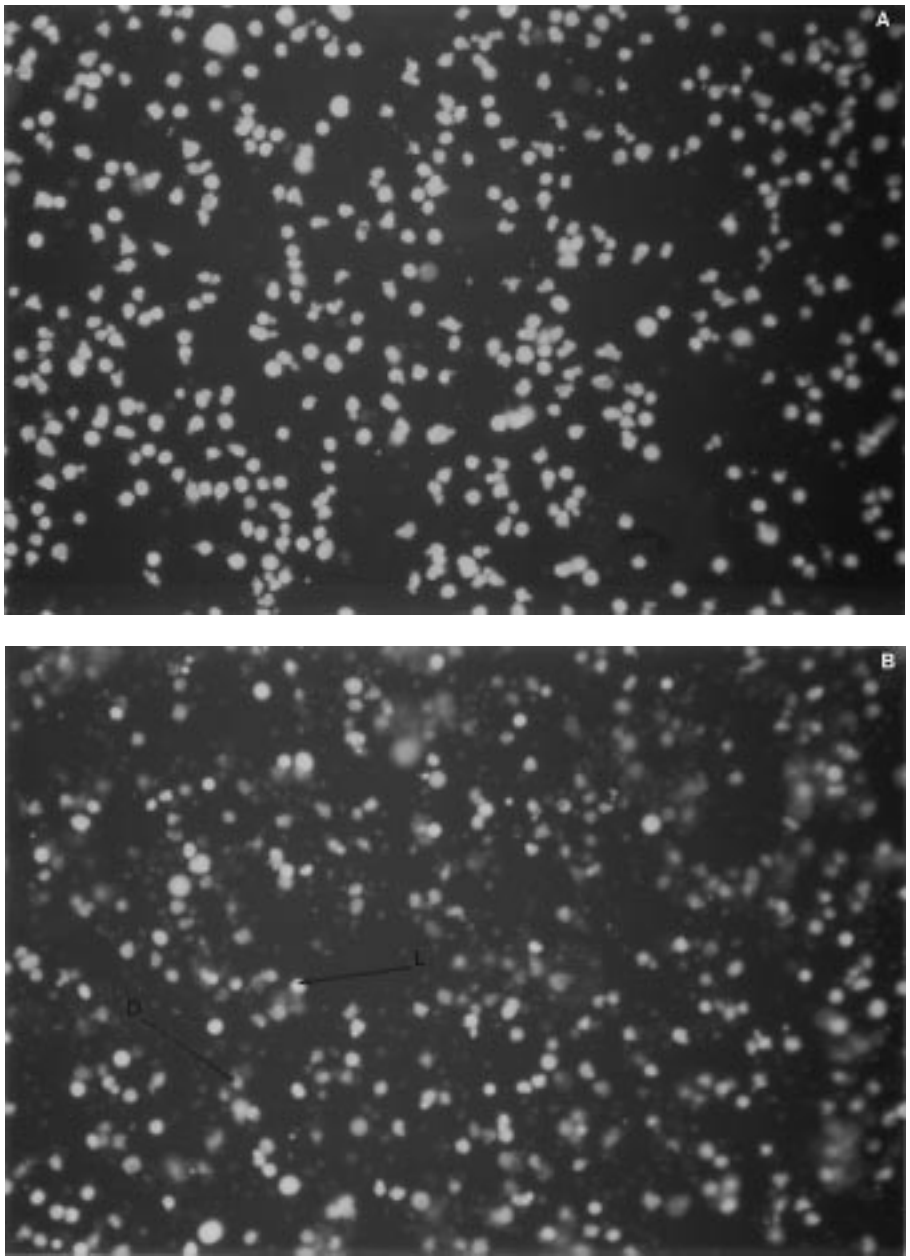


Fig. 3. Microscopic examination of HL60 cells (magnification, ×200) labeled with calcein-AM: (A) cells exposed to 1 m*M* t-BHP for 1 h and (B) cells exposed to 1 m*M* t-BHP for 6 h showing loss of calcein, variability in staining, and diffusion of calcein into the medium. Dead cells (D→) and living cells (L→).

to nearly the basal level of ROS. ROS production may have started earlier than at 30-min incubation with t-BHP (Fig. 4). According to the optimal conditions, involving an interval of 30 min between the addition of t-BHP and that of the H_2DCFDA probe, an incubation time of 1 h was selected for further experiments.

As shown in Fig. 4., ROS production evolved in a t-BHP concentration-dependent manner. The maximum signal was reached at 1 m*M* t-BHP whatever the incubation duration. The fluorescence signal showed no difference between 1 and 2 m*M* t-BHP. After a 1-h incubation with t-BHP, the fluorescence intensity increased markedly, with a signal/noise ratio of over 200% compared to control cells.

So the t-BHP acted rapidly as a ROS inducer in HL60 cells. The optimal conditions retained for further

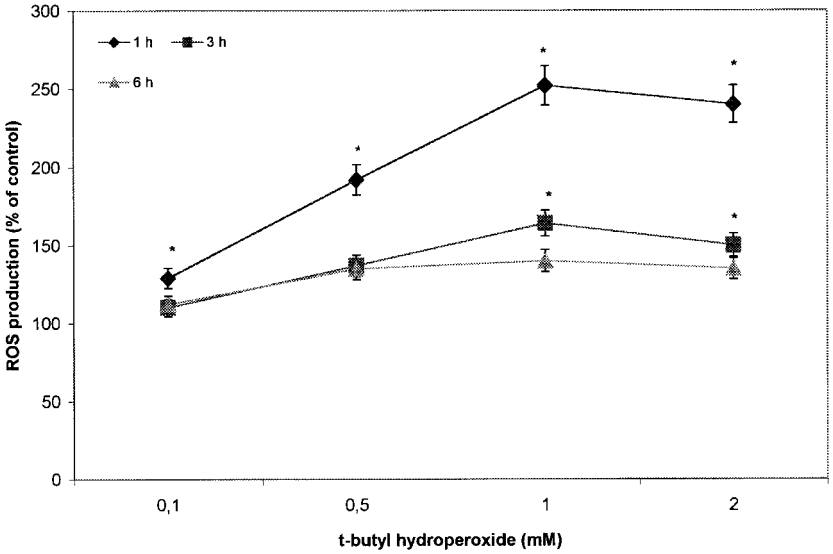


Fig. 4. ROS production by HL60 cell t-BHP treatment: cells were incubated with t-BHP for 1, 3, and 6 h. H_2DCFDA probe (10 μM) was added 30 min before the fluorescence reading. The fluorescence signal is represented as the percentage of untreated cells (1684.3 $\pm$ 202.5 AFU at gain 20) and was calculated from the mean of three experiments. (*) Significant difference ($P < 0.05$).

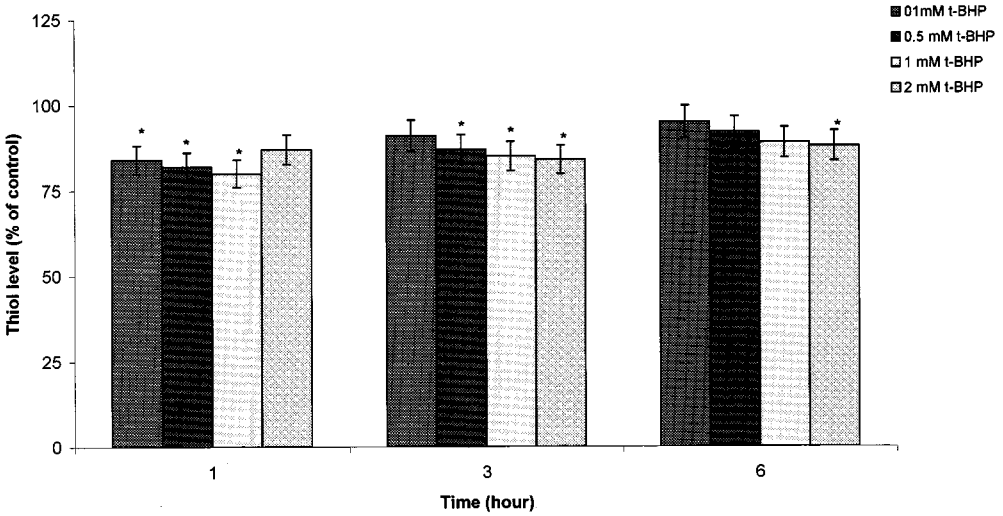


Fig. 5. Variation of the thiol level in HL60 cells submitted to t-BHP: cells were incubated with t-BHP for 1, 3, and 6 h. CMFDA probe (5 μM) was added 30 min before the fluorescence reading. The fluorescence signal is represented as the percentage of untreated cells (6237.5 $\pm$ 896.9 AFU at gain 10) and was calculated from the mean of three experiments. (*) Significant difference ($P < 0.05$).

experiments were 1 m*M* t-BHP applied to the cells during 1 h.

Determination of Intracellular Thiol Status

To evaluate the thiol level accurately, it was interesting to monitor the thiol pool under various conditions of time and t-BHP concentration. The thiol pool was evaluated by the total intensity of the fluorescence signal obtained with the CMFDA probe. Concentrations ranging from 0.1 to 2 m*M* t-BHP applied for 1 h to HL60 cells decreased the GSH pool slightly but significantly as shown in Fig. 5. After a 6-h incubation, the fluorescence signal increased, to reach 100% compared to control cells.

For assessing the role of thiol in the protection of cells from ROS, experimental thiol depletion was

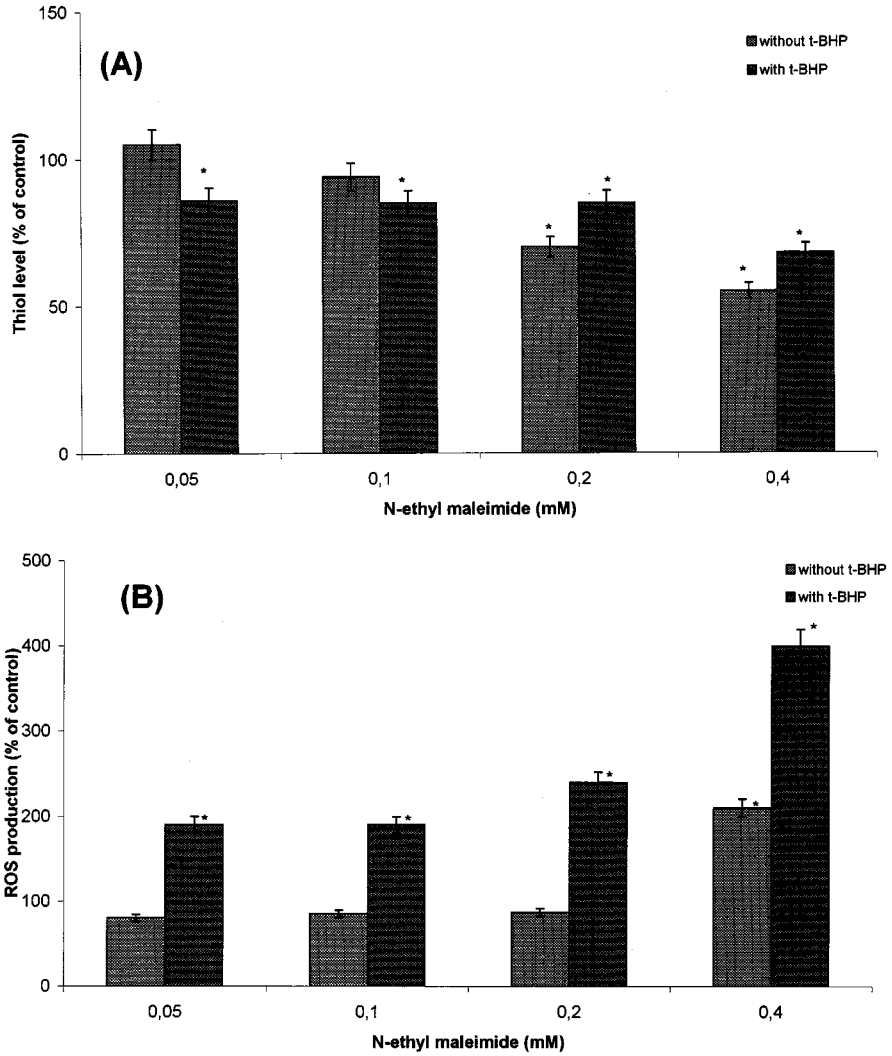


Fig. 6. Variations of the thiol level and ROS production in HL60 cells treated with a thiol depleting reagent NEM and then submitted to t-BHP: cells were treated with NEM at concentrations ranging from 0.05 to 0.4 m*M* for 1 h and then incubated in the presence or absence of 1 m*M* t-BHP. After a 30-min incubation, (A) cells were labeled with CMFDA (5 μM) for 30 min and (B) cells were labeled with DCFDA (10 μM) for 30 min. The CMFDA and H_2DCFDA fluorescence signals are represented as the percentage of untreated cells (respectively, 6237.5 $\pm$ 896.9 AFU at gain 10 and 1684.3 $\pm$ 202.5 AFU at gain 20) and were calculated from the mean of three experiments. (*) Significant difference ($P < 0.05$).

achieved with NEM, a thiol-alkylant agent which blocks accessible cellular protein sulfhydryls. After thiol depletion, cells were labeled with the CMFDA probe. This thiol depletion was concentration dependent and decreased to 50% with 0.4 m*M* NEM for 1 h as shown in Fig. 6A. The addition of 1 m*M* t-BHP induced an increase in the thiol pool to 60% as shown in Fig. 6A.

Without t-BHP treatment, H_2DCFDA labeling was more intense in HL60 cells treated with 0.4 m*M* NEM. So the thiol depletion obtained by NEM was able to induce ROS production. The application of 1 m*M* t-BHP in cells pretreated with 0.4 m*M* NEM increased ROS production more dramatically than in cells without NEM pretreatment (Fig. 6B).

Microscopic examination was in agreement with the fluorescence reading but it revealed a heterogeneous population of HL60 cells.

Protective Effect of *N*-Acetyl Cysteine

NAC is known to modulate redox-sensitive molecules by its reducing activity, conducted directly against

ROS and/or by an increase in cellular glutathione levels. Without t-BHP treatment, 5 m*M* NAC was able greatly to decrease the fluorescence signal of H_2DCFDA labeling (Fig. 7). After 1 m*M* t-BHP was applied to cells for 1 h, ROS production was increased over 200%. A low concentration of NAC was not sufficient to neutralize these oxidant species. A higher NAC concentration (50 m*M*) was necessary to neutralize 75% of the ROS induced by t-BHP and endogenous ones (Fig. 7).

DISCUSSION

Microspectrofluorometry using cell-permeant probes is suitable for *in vitro* study in a variety of applications including studies of cytotoxicity, cell migration, and cell adhesion. Although easy to use, these fluorescent probes require strict operative procedures as indicated in our experimental procedures.

The calcein-AM probe commonly used to determined the viability of adherent cells can be easily assessed in a model of nonadherent cells such as HL60 cells [14, 15]. The calcein-AM assay presents several interesting properties, particularly in this case: it does not require an extraction step, is not affected by pH, and is as safe as the usual viability test using MTT. The calcein-AM probe is stable and is a compound well retained in cells, thus limiting overestimation by diffusion out of the cells.

The H_2DCFDA probe proved sensitive enough to evaluate the production of ROS *in situ*. Without any oxidative treatment, the sensitivity of H_2DCFDA labeling allows us to detect a basal level of fluorescence signal corresponding to ROS produced in an endogenous manner by HL60 cells. These reactive species are easily neutralized by NAC. Several methods are proposed to detect ROS produced by cells, such as the colorimetric method based on peroxide-mediated oxidation of phenol red [16]. But the sensitivity of this method seems to be insufficient to determine the endogenous production of ROS in HL60 cells [17]. This process seems to be specific to the HL60 cells used in this study: the same experimental procedures were applied to other cell lines which had shown no residual ROS detected by H_2DCFDA in control cells [18]. H_2DCFDA labeling assessed the kinetics of ROS production by HL60 cells submitted to oxidant treatment with a maximum signal/noise ratio of over 200%. But the optimal conditions for consecutive steps of oxidative induction and labeling did not permit us to appreciate the initial phase of ROS production. An incubation extended to 24 h involved cell death by necrosis and the residual fluorescence signal observed with the H_2DCFDA probe could be related to ROS due to intracellular components in contact with the medium.

The CMFDA probe is a chloromethyl derivative which is bound to intracellular accessible sulfydryl compounds to form a membrane-impermeant thiol-fluorescent dye. This reaction is mediated by glutathione *S*-transferase. Fluorescent labeling assesses a more rapid and sensitive quantification of intracellular thiol than other methods such as the enzymatic one [19,20]. Because

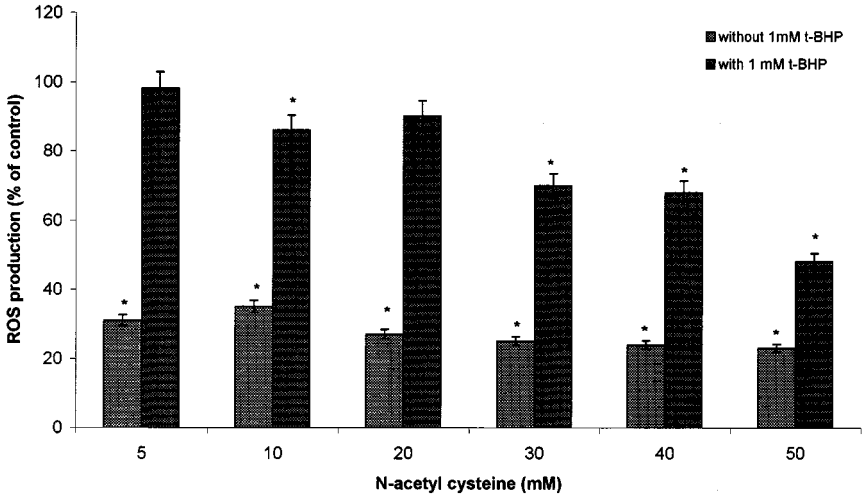


Fig. 7. Protective effect of *N*-acetyl cysteine against ROS produced by HL60 cells submitted to t-BHP: cells were treated with NAC at concentrations ranging from 5 to 50 m*M* for 1 h and then incubated in the presence or absence of 1 m*M* t-BHP. After a 30-min incubation, cells were labeled with H_2DCFDA (10 μM) for 30 min until the fluorescence reading. The fluorescence signal is represented as the percentage of untreated cells (1684.3 $\pm$ 202.5 AFU at gain 20) and was calculated from the mean of three experiments. (*) Significant difference ($P < 0.05$).

of the partial thiol depletion (50%) with NEM, the CMFDA probe seems to detect an intracellular compound which is not depleted by NEM but has a sulfydryl function. Preteatment with L-buthionine-(*S*,*R*)-sulfoximine, an inhibitor of GSH synthetase, did not completely suppress the fluorescence signal of CMFDA in HL60 cells (data not shown). Our experiments suggest that the CMFDA probe can detect intracellular thiol compounds different from those detected with the glutathione reductase method [19,21]. Another fluorogenic probe, monochlorobimane, is not specific for gluthathione but represents all kinds of thiols [20,22]. Different antioxidant systems with thiol compounds can enter for redox potential balance, such as the thioredoxin system [23]. The application of an oxidant reagent such as t-BHP to cells pretreated with NEM seemed to mobilize thiols, protecting cells against ROS effects.

Thus, the fluorogenic probes associated with the microspectrofluorometric method represent a sensitive and rapid method for study of different intracellular processes. Usually applied to adherent cell models [24], microspectrofluorometry can be used with different nonadherent cell models such as human lymphocytes [15], the HL60 cell line, and the human acute monocytic leukemic THP1 cell line [25]. The present results indicate that this method of measurement is suitable for studying cellular oxidative stress, evaluating thiol protective systems, and appreciating various related cellular mechanisms.

ACKNOWLEDGMENTS

This work was supported by the Université René Descartes Paris V and by grants from the Fondation pour la Recherche Médicale.

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