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Promyelocytic HL60 Cells Express NADPH Oxidase and Are Excellent Targets in a Rapid Spectrophotometric Microplate Assay for Extracellular Superoxide

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A great number of drugs, toxicants, and growth factors induce the generation of intermediary reactive oxygen species (ROS). The human promyelocytic leukemia HL60 cell line differentiated along the macrophage or neutrophil lineage is a model system that is frequently used for the generation of ROS by various agents. As a primary source of ROS the superoxide anion produced by an enzymatic complex, NADPH oxidase, is well established. The present study shows that nondifferentiated HL60 cells contain NADPH oxidase and can be used as a model for the assessment of oxidant as well as antioxidant compounds. The expression of the multicomponent NADPH oxidase was demonstrated in nondifferentiated HL60 cells at the molecular level by detection of the mRNAs of the components gp91phox, p47phox, and p67phox as well as functionally by phorbol 12-myristate-13-acetate (PMA)-stimulated generation of superoxide, which was susceptible to inhibition by diphenyleneiodonium. The functional assay was performed using the cells in a log growth phase by adapting a standard microplate assay based on the classic superoxide dismutase-inhibitable reduction of cytochrome c. Validation of the microplate assay was carried out both with nonadherent differentiated HL60 cells and the adherent mouse monocyte-macrophage–like RAW 264.7 cell line, as well as with various compounds of oxidant (bleomycin sulfate, cis-diammineplatinum(II), camptothecin, TNF-alpha, IL-1beta), nonoxidant (4alpha-PMA, piracetam), and antioxidant (alpha-tocopherol, ascorbic acid) activity. In summary, we established a highly specific, reproducible and—with the aid of the nondifferentiated HL60 cell line—time-saving superoxide microplate assay as a valuable tool for the rapid screening of compounds for oxidative and antioxidative activity.

Key Words: **promyelocytic HL60; NADPH oxidase; superoxide microplate assay; PMA; prooxidants; antioxidants.**

The multicomponent NADPH oxidase of activated phagocytes has been found to be the most important enzyme involved in the generation of reactive oxygen species (ROS) and oxidative stress (Babior, 1999; Forman *et al*., 2001). Upon stimulation, phagocytes synthesize ROS from a superoxide, which is formed as the earliest product of an oxidative burst by NADPH oxidase and represents a precursor of a vast assortment of ROS (Babior, 1999). Free radicals play an important role in host defenses against pathogens but also can cause tissue damage and thus are considered to be involved in the ageing process and pathogenesis of several diseases (Droge, 2002) as well as in mutagenesis and carcinogenesis (Sekiguchi *et al*., 2002).

The identification of oxidant and antioxidant compounds is important for predicting and reducing health risks. As most of the superoxide is released to the outside of the cell, the assessment of oxidative stress is commonly performed by assays for extracellular superoxide, although methods for superoxide detection inside cells are also available (Tarpey *et al*., 2001). Extracellular superoxide has been determined in isolated supernatants of activated cells or, using plate reader assays, in supernatants with the cells left on the plate. Current detection methods of extracellular superoxide utilize the reduction of reactive compounds or chemiluminescence reactions due to the ability of ROS formation by an oxidation product (Tarpey *et al*., 2001). The latter method has been criticized due to caveats in the use of small molecules that may generate the same ROS intended to be measured (Forman *et al*., 2001).

Plate reader assays (Catino *et al*., 1988; Cerasoli *et al*., 1988; Leslie, 1987; Madesh *et al*., 1997; Quick *et al*., 2000) have been developed on the basis of superoxide-induced reduction of exogenous ferricytochrome c (Pick *et al*., 1981). The specificity of this method is checked by the addition of a superoxide dismutase (SOD), which serves to confirm generated superoxide as an electron donor (Crapo *et al*., 1978). The use of an SOD provides one of the most reliable detection methods of superoxide generation (Tarpey *et al*., 2001). Targets used in plate reader assays were cell homogenates (Quick *et al*., 2000) or freshly prepared adherent exudate cells (Pick *et al*., 1981), as well as polymorphonuclear leukocytes (Cerasoli *et al*., 1988; Leslie, 1987), all of which are not really suitable for large-scale applications. Leukemia cell lines would allow large-scale drug

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screening and assessment of potential oxidative risk; however, they have been induced to differentiate to a phagocytic phenotype before use (Birnie, 1988, Catino *et al*., 1988), a process requiring several days. Moreover, variations in the differentiation states of cells as well as the potential toxicity of differentiation-inducing agents may significantly interfere with the reliability and reproducibility of the assay.

In this study we aimed to develop a simplified plate reader assay for rapid superoxide screening and to use the standard HL60 leukemia cell line at the promyelocyte state. We confirmed these cells' suitability by demonstrating the expression of NADPH oxidase representing the main pathway of ROS generation. In phagocytic cells, this enzyme complex catalyzes the NADPH-dependent reduction of the oxygen molecule to the superoxide anion. It consists of the proteins gp91phox and p22phox (flavocytochrome b558) anchored in the plasma membrane and the water-soluble cytosolic proteins p67phox, p47phox, p40phox, and Rac, which, upon stimulation, assemble with the flavocytochrome b558 to the active multienzyme complex (Babior, 1999; Vignais, 2002).

Currently, increasing evidence suggests a more general importance of NADPH oxidase activity since various nonphagocytic cells may also contain NADPH oxidase or other oxidases of gp91, p47phox, and p67phox homologs (Banfi *et al*., 2001; Edens *et al*., 2001). Nonphagocyte NADPH oxidase has been proposed to play important roles in various signaling events and other physiological processes. Furthermore, the generation of an oxidative burst as well as highly reactive oxygen species, although at much lower levels, has been demonstrated in nonimmune cells (Droge, 2002; Lambeth, 2002).

Published data on NADPH oxidase expression and superoxide release by undifferentiated, nonphagocytic HL60 cells are controversial and inconclusive (Ahmed *et al*., 1996; Chetty *et al*., 1995; Hua *et al*., 2000; Levy *et al*., 1990; Roberts *et al*., 1982). Here we demonstrate that nondifferentiated (*nd*)HL60 cells express both membraneous and cytoplasmic NADPH oxidase subunits and are responsive to the oxidant activity of phorbol 12-myristate-13-acetate (PMA), a protein kinase C activator and potent stimulator of NADPH oxidase (Wolfson *et al*., 1985). Furthermore, superoxide generation by *nd*HL60 cells was completely inhibited by the flavoprotein inhibitor diphenyleneiodonium (DPI), a commonly used down-regulator of NADPH oxidase (Donnell *et al*., 1993). Thus, *nd*HL60 cells are useful for indicating superoxide release upon activation. Using *nd*HL60, we adapted a plate reader assay based on an SOD-inhibitable reduction of ferricytochrome c and evaluated the practicability and specificity of the assay employing (1) different cell types (nonadherent differentiated (*d*)HL60 cells and adherent mouse RAW 264.7 cells, a standard monocytic/macrophage-like cell line retaining many of the functions of primary cultured macrophages); (2) active (bleomycin sulfate, cis-diammineplatinum(II), camptothecin, TNF-alpha, IL-1beta) and inactive superoxide inducers (4alpha-PMA, piracetam); and (3) various antioxidant (alpha-tocopherol, ascorbic acid) compounds. The results show that the superoxide microplate assay with *nd*HL60 cells is a highly feasible method for screening oxidant-generating as well as antioxidant compounds.

MATERIALS AND METHODS

Reagents and media. Cytochrome c horse heart type IV (cyt c), phorbol 12-myristate-13-acetate (PMA), 4alpha- phorbol 12-myristate-13-acetate (4alpha -PMA), bleomycin sulfate (BLM), cis-diammineplatinum(II) (CDDP), camptothecin (CPT), alpha-tocopherol, ascorbic acid (AA), diphenyleneiodonium (DPI), and dimethyl sulfoxide (DMSO, Liquid, Hybri-Max, minimum 99.7 % purity) were purchased from Sigma-Aldrich Corporation (St. Louis, MO). Human IL-1beta and TNF-alpha were obtained from R&DSystems (Minneapolis, MN) and bovine erythrocyte superoxide dismutase (5000 U/mg) (SOD) from Roche Applied Science (Mannheim, Germany). Dulbecco's modified Eagle's Medium (DMEM) and RPMI-1640 cell culture media with and without phenol red were obtained from Invitrogen Life Technologies (Paisley, UK). Stocks of cyt c (0.8 mM) and SOD (2700 U/ml) in Hank's balanced salt solution (HBSS) were prepared immediately before use. The stocks for PMA, 4alpha-PMA (3.2 mM), CDDP (55 mM), and CPT (10 mM) were prepared in DMSO, stored at −80°C, and further diluted in HBSS before use. In the assay, the final concentration of DMSO for all compounds was less than 0.5%. This level is far below published concentrations (4–10%) that are said to interfere with superoxide production after PMA stimulation of neutrophils (Kahler, 2000).

Cell lines. The human promyelocytic leukemia cell line HL60 and mouse monocytic/macrophage-like cell line RAW 264.7 (passage <100) were obtained from American Type Culture Collection (ATCC; Rockville, MD) and maintained in RPMI-1640 and DMEM, respectively, with 10% heat-inactivated fetal calf serum (FCS; PAA, Linz, Austria) in a 5% CO_2 atmosphere at 37° C. The cell number and viability were determined by trypan blue exclusion.

To perform the assay, HL60 cells without prior induction of differentiation (*nd*HL60 cells) were seeded in 96-well plates at a density of 1.5×10^5 /50 μl/well in phenol red free-RPMI-1640 and incubated for 30 min before starting the assay. In order to induce differentiation of HL60 cells to neutrophils, they were incubated for 7 days with 1.25% DMSO in complete RPMI-1640. Thereafter, the cells were collected, washed, and processed as described for the *nd*HL60cells. RAW 246.7 cells were seeded in 96-well plates at a density of 0.3×10^5 cells/200 μl/well in complete RPMI-1640 and cultured for 24 h to reach about 80% confluence (i.e., 0.6×10^5 cells/well). After total removal of the medium using a curbed adjusted water-jet vacuum pump, the cells were covered with 50-μl/well phenol red free-RPMI-1640 and processed as described for *nd*HL60.

In an additional approach, the RAW 246.7 cells were collected prior to the assay and seeded (0.6×10^5 cells/50 μl/well) as described for *nd*HL60.

Assay of superoxide release. The extracellular superoxide production by PMA-activated cells seeded in 96-well microplates was determined from the SOD-inhibitable reduction of cyt c. The assay reaction mixture was prepared in HBSS with 2.2 mg/ml of glucose immediately prior to use and 100 μl thereof added directly to the cells without removal of the 50-μl maintenance medium. The reaction mixtures were set up from stock solutions with concentration ranges of cyt c (20–320 μM), SOD (50–300 U/ml), and PMA (1–32 μM). The final evaluated standard concentrations are based on the results of the present study. They are 160 μM for cyt c, 100 U/ml for SOD, and 16 μM for PMA, the most effective concentration. Absorbance was measured in a plate reader at 550 nm (Anthos HAT II) as the endpoint (after 20-min exposure) and as kinetic readings (10–40 min). All experimental groups were set up in quadruplicates. Background values from cells *in situ* without assay mixtures were $<5\%$ of the sample readings. Blind values were obtained by measuring standard mixtures without cells. The ΔA_{550} values were converted to nanomoles (nmol) of cyt c reduced/10^6 cells, using a net extinction coeffi-

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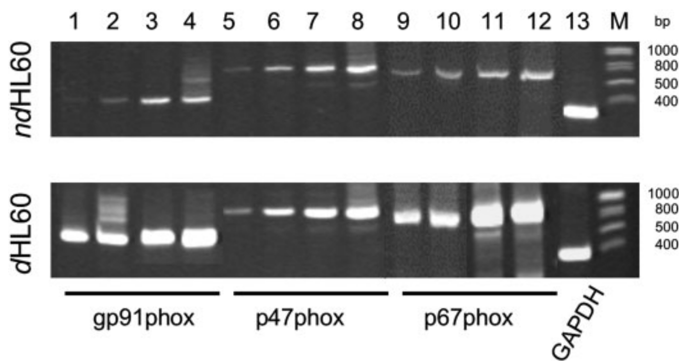

FIG. 1. RT-PCR amplification of NADPH oxidase components in *nd*HL60 and *d*HL60 cells. Total RNA (1 μg) was reverse transcribed into cDNA and used as a template in RT-PCR. Specific oligonucleotide primer sets were used to generate products for gp91phox (lanes 1–4, cycles 25–31, 403 bp), p47phox (lanes 5–8, cycles 25–31, 767 bp), and p67phox (lanes 9–12, cycles 33–39, 747 bp). GAPDH (lane 13, 20 cycles, 358 bp) is shown as the housekeeping gene.

cient $\Delta E_{550,Red\text{-}Ox}$ of 2.1×10^4 M^{-1} cm^{-1} (Johnston, 1984). Superoxide production was calculated from the difference in the amounts of cyt c reduced in the absence and presence of SOD and was extrapolated to 1 h.

RT-PCR for NADPH oxidase components. Total RNAs were isolated by a multistep guanidinium thiocyanate/acid phenol:chloroform extraction (Invitrogen Life Technologies), and RT-PCR was performed as described in Berger *et al.* (1997) using specific primers for gp91phox, p47phox, and p67phox (Jones *et al*., 1996) as well as the gp91 homologues NOX 1, NOX 4 (Shiose *et al.*, 2002), and DUOX2 (Edens *et al.*, 2001). The constitutively expressed housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was co-amplified as an internal control (358 bp) (Berger *et al.*, 1997). Serial two-step cycles were chosen for linear range determination. The products of amplification were separated by polyacrylamide gel electrophoresis (PAGE), stained with ethidium bromide, and visualized by scanning densitometry (Gel-Doc 1000, Molecular Analyst Software, Bio-Rad Molecular Imager). The experiments were repeated three times. Several negative controls (amplification cycles with all primer sets with total RNA and without cDNA) were included in each experiment.

Statistical analyses. All experiments were performed at least three times. All data are presented as mean ± SD. Statistical analyses were performed by one-way ANOVA followed by a Dunnett's test using the Jandel *SigmaStat* Version 2.0. Results were considered significant at the $p < 0.05$ level.

RESULTS

Expression of NADPH Oxidase Components

From our preliminary unpublished studies we knew that, upon short-term PMA treatment (16 μM, 20 min), *nd*HL60 cells generate substantial amounts of extracellular superoxide. Therefore we analyzed the mRNA expression of several components of the oxidant-generating enzyme NADPH oxidase (gp91phox, p47phox, and p67phox, as well as for some gp91phox homologs) in nonactivated *nd*HL60 cells and *d*HL60 cells by RT-PCR. As shown in Figure 1, both cell types expressed gp91phox, p47phox, and p67phox, leading to specific PCR amplification products resulting in single signals corresponding to the predicted base pair size (403 bp, 767 bp and 747 bp, respectively). As expected, none of the membranous and cytosolic components of the *nd*HL60 cells attained levels similar to those found in *d*HL60 cells at the identical cycle numbers. The *d*HL60 cells, whose differentiation is generally associated with the acquisition of NADPH oxidase, demonstrated much higher levels than *nd*HL60 cells, in which small amounts of mRNA transcripts for the gp91phox and p47phox components were first detected at 25 and for p67phox at 33 amplification cycles. Primers for the gp91phox homologs NOX1, NOX4, and DUOX2 gave no signals in either cell line (not shown).

Previous studies report on the complete lack (Roberts *et al.*, 1982) as well as trace amounts of membranous or cytosolic components of NADPH oxidase (Chetty *et al.*, 1995; Hua *et al.*, 2000; Levy *et al.*, 1990) as well as on some of the superoxide-producing activity (Ahmed *et al.*, 1996) of the nonphagocytic HL60 cells. These discrepancies may be attributed to the applied detection methods or concentrations of activating agents too low for activating nonphagocytic HL60 cells. In addition, we analyzed several promyelocytic HL60 cell lines obtained from different laboratories from Europe and the United States and found similar mRNA levels of several NADPH oxidase components in all samples, thus rendering the effects of cell origin rather unlikely.

PMA-Stimulated Superoxide Release and Inhibition by DPI

The major aim of this study was the development of a simple and rapid microplate assay for extracellular superoxide screening using a homogenous and well-characterised cell line adapted to long-term culture. We show here that the *nd*HL60 cells express low levels of NADPH oxidase and consequently are competent to respond to activating stimuli. Figure 2 demonstrates the PMA-triggered superoxide release of *nd*HL60 cells in comparison to *d*HL60 matured to neutrophils (Fig. 2a) and to the macrophage-like RAW 246.7 cell line (Fig. 2b). Under the special handling conditions (see the "Materials and Methods" section) and exposed to the standard reaction mix (160-μM cyt c, 100-U/ml SOD) followed by a 20-min endpoint measurement, all cell lines responded to PMA and generated superoxide in a concentration-dependent manner. Basal superoxide generation without PMA stimulation was negligible in all cell types.

As expected, the *nd*HL60 cells, but also the RAW 246.7 cells seeded shortly before the assay, were less sensitive than the *d*HL60 and the 24-h precultured RAW 246.7 cells, respectively. The amount of superoxide produced by the *nd*HL60 cells exposed to 1–8-μM PMA was significantly lower than that of activated the *d*HL60 and reached 5.1-nmol/10^6cells/h compared to 17.4 nmol/10^6cells/h released by the *d*HL60 cells at 8-μM PMA. In addition, the *d*HL60 cells showed significant cyt c reduction at 1-μM PMA while in the *nd*HL60 cells a significant response was not observed below 4 μM. However, between 16- and 32-μM PMA, both HL60 cell lines responded

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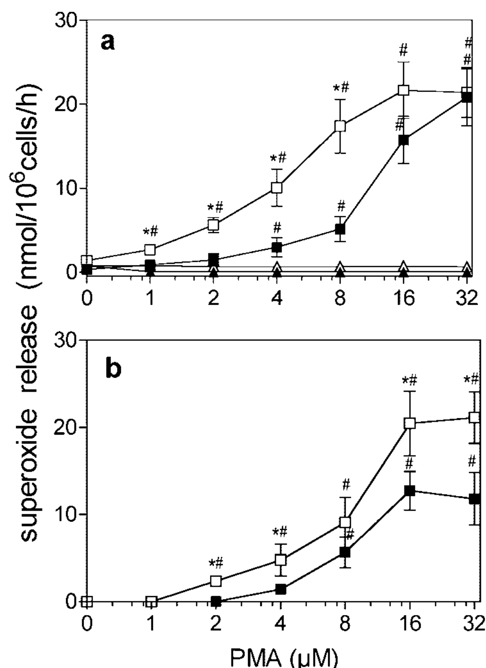

FIG. 2. Comparison of superoxide release of PMA-activated HL60 and RAW 264.7 cells and inhibition by DPI. The cells were exposed in the standard reaction mixture (cyt c 160 μM, SOD 100 U/ml) to PMA concentrations as indicated in the absence and presence of DPI and the absorbance was read after 20 min of incubation (for details see the "Material and Methods" section). *Filled squares* represent (a) *nd*HL60 and (b) non-precultured RAW 264.7 cells. *Open squares* represent (a) *d*HL60 and (b) 24-h precultured RAW 264.7 cells. *Filled and open triangles* in (a) represent effect of DPI on PMA activated superoxide release by *nd*HL60 and *d*HL60, respectively. Data (mean ± SD) are shown from three to five independent sets of experiments. $^{*}p < 0.05$ significantly different from (a) *nd*HL60 and (b) non-precultured RAW 264.7, and $^{\#}p < 0.05$ significantly different from control (one-way ANOVA followed by Dunnet's test).

almost equally as the superoxide release of *d*HL60 cells reached plateau values at 16-μM PMA (21 nmol/10^6 cells/h). Thus, the *nd*HL60 are less responsive than the *d*HL60 at low concentrations of PMA (EC50 for *nd*HL60 12.3 μM and for *nd*HL60, 4.3 μM) but not at higher concentrations. The response of activated *d*HL60 cells was within the range of published data (Emerit *et al.*, 1996).

The RAW 246.7 cells, regardless of being precultured or non-precultured, also reached plateau values between 16- and 32-μM PMA, but the plateau levels differed (about 20 and 12 nmol/10^6 cells/h, respectively). The RAW 246.7 cells were more affected than the HL60 cells by the different handling procedures. The RAW 246.7 cells seeded shortly before the assay were not significantly stimulated at PMA concentrations below 8 μM, while the precultured cells were already significantly activated at 2 μM. Generally, precultured and non-precultured RAW 246.7 cells were less sensitive than the *d*HL60 cells.

These studies support the usefulness of both the adapted microplate assay and cells in suspension or adherent to determine superoxide release. For optimal performance of the assay the following details are important: (1) short recovery time after seeding; (2) no removal of the maintenance medium; (3) preparation of the complete reaction mixture immediately before the assay; (4) adding the reaction mixture into the maintenance medium; (5) keeping in mind that *nd*HL60 cells are less sensitive than *d*HL60 cells at low PMA concentrations; and (6) the use of the optimal cell number, which in preliminary tests we have determined to be 1.5×10^5 /well. The HL60 cells seeded too dense ($>3 \times 10^5$ /well) showed unacceptable variance (>50%) between the replicate samples and the experiments. When the cell number was too low ($<0.5 \times 10^5$ /well) superoxide generation was below the detection limit (data not shown).

Diphenyleneiodonium (DPI), an inhibitor of flavin-containing oxidases (Donnell *et al.*, 1993), has been frequently used to block NADPH oxidase activity. To determine whether DPI affected the superoxide release by PMA-activated *nd*HL60 cells, DPI was added at 0.5 μM both during the maintenance period of 30 min and the reaction time of 20 min. Across the entire range of PMA concentrations indicated, the DPI completely inhibited the extracellular superoxide release in the *nd*HL60 as well as in the *d*HL60 cells (Fig. 2a). These data further confirm that NADPH oxidase as FAD-containing protein is responsible for the superoxide production by promyelocytic HL60 cells.

Concentration Ranges of Cytochrome c and SOD

Standard concentrations of cyt c and SOD for the reaction mixture were defined by a series of experiments. The assay was performed using 16-μM PMA and endpoint readings after 20 min of exposure. As expected, the superoxide release by both the PMA-activated *nd*HL60 and precultured RAW 246.7 cells was found to increase with increasing cyt c concentrations (Figs. 3a and 3c). Both cell lines reached peak values at 160 μM cyt c, which then markedly dropped at 320 μM because of the reoxidation of reduced cyt c by molecular oxygen (Tarpey *et al.*, 2001). The standard concentration for cyt c further used in the reaction mixture was 160 μM.

SOD is considered to be a key antioxidant in aerobic cells responsible for the first metabolic step of superoxide elimination (McCord *et al.*, 1970). Figures 3b and 3d demonstrate the SOD-concentration response of *nd*HL60 as well as of precultured RAW 246.7 cells. The analysis showed only negligible effects of increasing SOD concentrations in both cell lines. Therefore, the standard concentration selected for the reaction mixture was 100-U/ml SOD.

Kinetics of Cyt c Reduction

Cyt c reduction kinetics over a period of 10–40 min were followed in standard reaction mixtures with *nd*HL60 and precultured RAW 246.7 cells activated with 16-μM PMA. A progressive initial increase in superoxide peaked between 20

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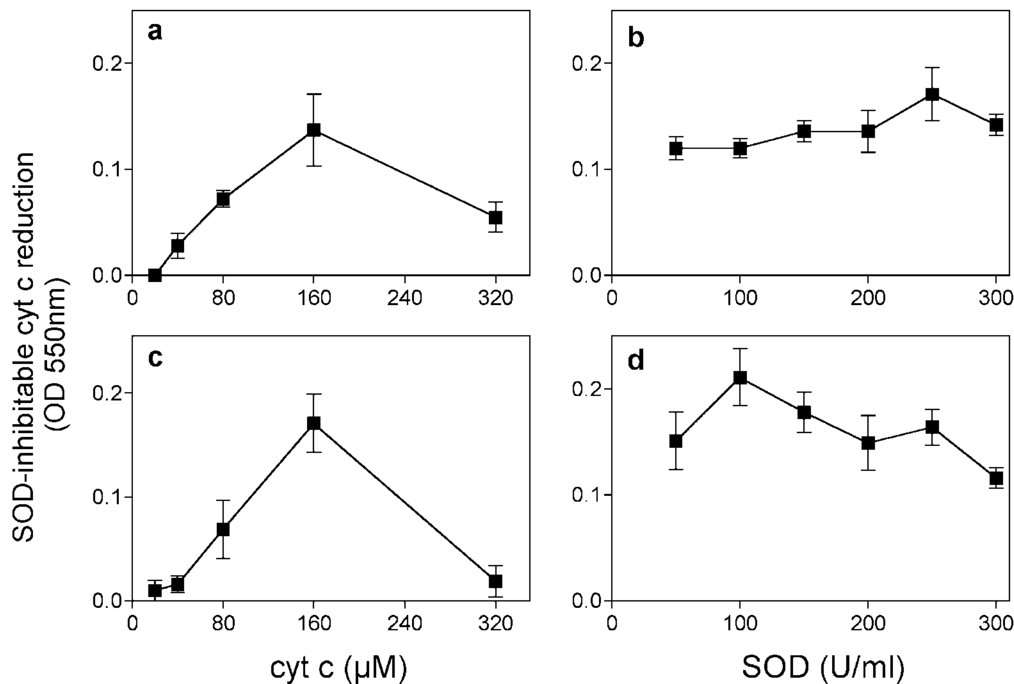

FIG. 3. Dependence of assay readings on cyt c and SOD concentrations. (a, b) PMA (16-μM)-activated *nd*HL60 and (c, d) precultured RAW 264.7 cells were incubated with (a, c) cytc c and (b, d) SOD at the indicated concentrations, respectively. In (a) and (c) SOD and in (b) and (d) cyt c were added at the standard concentration (SOD 100 U/ml and cyt c 160 μM, respectively). Data (mean $\pm$ SD) are shown from three to five independent sets of experiments.

and 30 min and was followed by a decline in *nd*HL60 (Fig. 4a) or a plateau in RAW 246.7 cells (Fig. 4b). The cessation of further cyt c reduction with time may reflect the establishment of a reaction equilibrium in which the rate of cyt c reduction by superoxide is balanced by the reoxidation of reduced cyt c (Tarpey *et al*., 2001). On the basis of the data presented, we suggest that superoxide readings should be performed either as time kinetics or, in the case of a single measurement, after 20-min incubation, the time of maximal cyt c reduction.

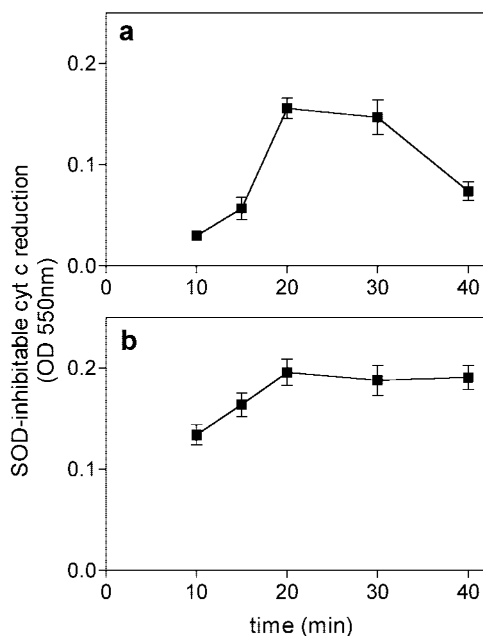

FIG. 4. Kinetics of cyt c reduction. (a) *nd*HL60 and (b) precultured RAW 264.7 cells exposed to the standard reaction mixture (SOD 100 U/ml, cyt c 160 μM), including 16-μM PMA, were read after the indicated time points. Data (mean $\pm$ SD) are shown from three to five independent sets of experiments.

Validation of the ndHL60 Microplate Assay

In order to probe the specificity of the *nd*HL60 microplate assay we included compounds with superoxide-generating, -nongenerating, and antioxidant activity. The compounds were (1) the superoxide-generating anti-cancer agents BLM, CDDP, and CPT; (2) the proinflammatory and prooxidant cytokines TNF-alpha and IL-1beta; (3) the nonoxidant 4alpha-PMA and piracetam; and (4) the antioxidants ascorbic acid (AA) and alpha-tocopherol (Figs. 5a–5c). The superoxide assay was performed with the standard reaction mixture and read after the 20-min reaction time.

Figure 5a demonstrates that all anticancer drugs and cytokines, reported as ROS-inducing agents (Anderson *et al*., 1994; Bonizzi *et al*., 1999; Dusi *et al*., 1996; Hiraoka *et al*., 1998; Masuda *et al*., 2001), activated the *nd*HL60 cells to generate superoxide. The tested concentrations were within the range of those reported as prooxidants. For all compounds, the positive response was concentration-dependent. In contrast, the inactive phorbol ester 4alpha-PMA (Fischer *et al*., 1991) did not activate superoxide generation at concentrations of 4 to 32 μM, which, in the case of the active phorbol ester PMA, significantly increased superoxide release ($p < 0.05$; Figs 5b and 2a). The neuroprotective agent piracetam, a nonoxidant in leukocytes at concentrations of 30–3000 μM (Tissot *et al*., 1999), also gave negative results in the *nd*HL60 microplate assay at

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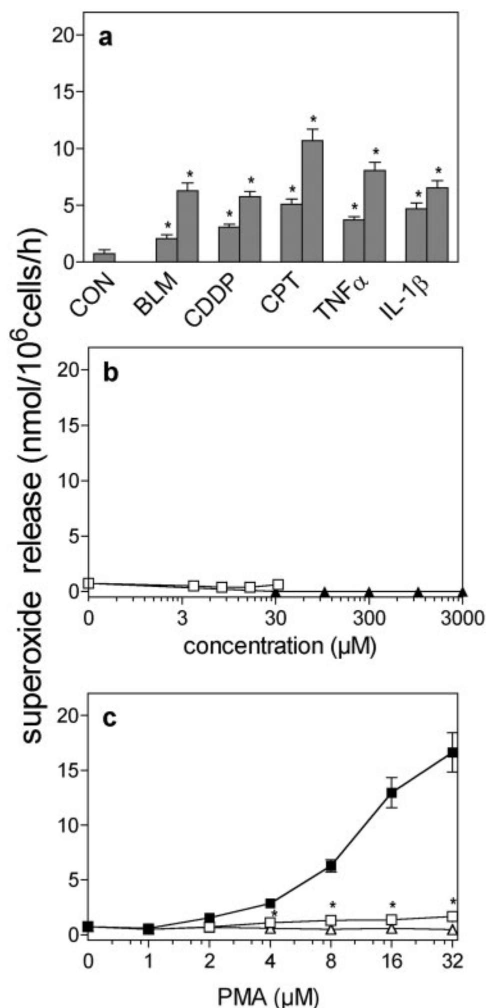

FIG. 5. Specificity of the *nd*HL60 superoxide microplate assay. In all experiments, the superoxide release by the *nd*HL60 cells was measured 20 min after exposure to the standard reaction mixture (cyt c 160 μM, SOD 100 U/ml) including the compounds mentioned below: (a) Oxidants bleomycin (BLM, 0.15 and 0.6 U/ml), cisplatin (CDDP, 20 and 100 μM), camptothecin (CPT, 2 and 10 μM), and TNF-α and IL-1β both used at 40 and 200 U/ml. (b) Nonoxidants 4α PMA (*open symbols*) and piracetam (*filled symbols*) at concentrations indicated. (c) PMA at concentrations indicated in absence (*filled symbols*) and presence of the antioxidants α-tocopherol (100 μM, *open square*) and ascorbic acid (AA, 1 mM, *open triangle*), added during maintenance and reaction times. *$p < 0.05$ significantly different from (a) control and (c) PMA-activated superoxide release (one-way ANOVA followed by Dunnet's test).

these concentrations (Fig. 5b) and was still negative at 10 mM (not shown). Finally, as is also shown in Figure 5c, two different antioxidants added during the maintenance period of 30 min and further during the reaction time of 20 min significantly inhibited PMA (4–32 μM) activated superoxide release in *nd*HL60 cells. The tested concentrations of antioxidants AA (1 mM) and alpha-tocopherol (100 μM) are commonly used in similar studies (Cachia *et al*., 1998; Carr *et al*., 1999).

In summary, the reliability of the *nd*HL60 superoxide microplate assay for compound screening is demonstrated by the fact that pro-, non-, and anti-oxidant compounds are specifically identified by this test.

CONCLUSION AND DISCUSSION

Free radicals and other ROS are constantly formed by all aerobic cells through normal cellular metabolism or become activated in inappropriate environments caused by infections, inflammation, ionizing radiations, or various chemicals (Babior, 1999; Droge, 2002; Forman *et al*., 2001). At low levels, ROS activate growth factor- and other receptor-mediated cell-signaling pathways, resulting in a broad array of physiological responses from cell proliferation to gene expression and apoptosis. An excess of ROS (oxidative stress) generated by an imbalance between ROS and antioxidant defenses affects cell function and viability, resulting in the activation of repair mechanisms or apoptosis and sometimes necrosis, thus significantly contributing to mutagenic and consequently carcinogenic processes (Sekiguchi *et al*., 2002). Oxidative damage has been implicated in the ageing process and in numerous clinical conditions, including cancer (Droge, 2002).

In the oxidative stress response mitochondria are the major source of intracellular ROS. The highly regulated membrane-associated NADPH oxidase enzyme complex is critical for the extracellular formation of superoxide by one-electron reduction of oxygen using NAD(P)H as the electron donor. NADPH oxidase represents an abundant source of ROS by generating superoxide as the primary oxygen radical formed (Vignais, 2002). In nonstimulated cells, the different components of NADPH oxidase are located in different subcellular compartments (cytosolic and membraneous), which, upon activation, assemble at the plasma membrane to become functionally active (Babior, 1999). Extracellular superoxide, as one of the most relevant radicals, can dismutate spontaneously but more rapidly enzymatically by SOD, which constitutes the first line of defense against oxidative stress in the extracellular environment (Zelko *et al*., 2002). Belonging to the main antioxidant (4α-PMA) enzymes, SOD represents a key player in the maintenance of the redox status of the cells. This is practically applied to confirm the identity of generated superoxide as an electron donor in the classic cytochrome c reduction assay (Tarpey *et al*., 2001).

Here we demonstrate that the membrane-associated NADPH oxidase, critical for the formation of ROS in phagocytic and nonphagocytic cells (Lambeth, 2002; Vignais, 2002), is also expressed and functional in *nd*HL60 cells, which is contrary to some earlier reports (Ahmed *et al*., 1996; Chetty *et al*., 1995; Hua *et al*., 2000; Levy *et al*., 1990; Roberts *et al*., 1982), probably due to our efficient cell activation as well as the detection of mRNA expression by RT-PCR analysis. In addition, after PMA activation (NADPH oxidase stimulator) in the presence and absence of DPI (NADPH oxidase downregulator), superoxide release was confirmed by adapting a micro-

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plate reader assay based on the classic SOD-inhibitable reduction of cytochrome c. The presence of functional NADPH oxidase makes the nondifferentiated, promyelocytic HL60 cells very suitable as an experimental model to assess the risks of oxidative damage and potential antioxidants, information that is central to the understanding of the impact of oxidative stress in signal transduction and cellular injury as well as health risks.

The general usefulness of the *nd*HL60 superoxide assay has been demonstrated by comparison with differentiated HL60 cells and the adherent RAW 246.7 cell line handled with and without precultures. In a series of experiments, the specificity of the *nd*HL60 microplate assay was further demonstrated by testing PMA and other known superoxide generators as well as nonoxidant and antioxidant compounds. All superoxide generators including chemicals (anticancer agents) and natural (cytokines) compounds were recognized as positives in a concentration-dependent manner. Applied anticancer drugs act via different major cytocidal mechanisms and additionally possess prooxidant potential. The prooxidant concentrations verified in the *nd*HL60 microplate reader assay were, in case of BLM, within the range of concentrations inducing ROS and DNA damage in human lymphocytes (Anderson *et al*., 1994). In the case of CDPD, the prooxidant concentrations tested were within the range inducing the stress response of macrophages, leukocytes, and also ovarian cancer cell lines (Masuda *et al*., 2001). In the case of CPT, the oxidant response was elicited at concentrations reported to mediate superoxide release by the NADPH-oxidase expressing undifferentiated and granulocytic-differentiated myelomonoblastic cell lines PLB-985 and not by a NADPH-oxidase null cell line (Hiraoka *et al*., 1998). The cytokines TNF-alpha and IL-1beta induce a classical respiratory burst via the NADPH–oxidase pathway in monocytic cells. This positive response was also verified in *nd*HL60 cells at published concentrations (Bonizzi *et al*., 1999; Dusi *et al*., 1996).

The negative response to the nonoxidant agents 4alpha-PMA and piracetam further demonstrated that the *nd*HL60 microplate assay is a specific tool for oxidant compound screening. Both the stereoisomer of PMA, 4alpha-PMA, due to its structural change devoid of PKC and superoxide-release activity (Fischer *et al*., 1991), and the pyrrolidone compound piracetam, lacking any effect on the oxidative metabolism of polymorphonuclear leukocytes (Tissot *et al*., 1999), did not activate *nd*HL60cells.

Finally, the validation exercise for antioxidant screening carried out with the two well-known but differently acting positive candidates, alpha-tocopherol and AA, at concentrations commonly used further demonstrated the specificity of the *nd*HL60 microplate assay. Alpha-tocopherol, one of the most important natural nonenzymatic antioxidants, acts as both a radical scavenger and an inhibitor of PKC as well as of NADPH oxidase assembly in neutrophils and macrophages (Azzi *et al*., 2002; Cachia *et al*., 1998). AA, one of the major water-soluble antioxidants, however, confers protection by contributing an electron to reduce radicals in the extracellular environment (Carr *et al*., 1999).

The aim of this study was to develop an easy-to-handle and time-saving microplate assay for superoxide. For this purpose we introduced the promyelocytic *nd*HL60 cell line as a useful target in the adapted classic SOD-inhibitable cytochrome c reduction assay. In a screening program of different compounds of pro- and antioxidant as well as of lacking oxidative activity, the obtained data confirmed the specificity of the assay. Advantages over other plate reader superoxide assays include (1) no need for time-consuming maturation induction as well as for collection and washing steps; (2) low background activity, likely due to the high viability of *nd*HL60 cells as compared to *d*HL60 cells damaged by differentiation-inducing agents; (3) reduced variability, probably due to the homogenous promyelocytic population of the *nd*HL60 cells compared to the heterogeneous differentiation grade of *d*HL60 cells; and (4) utility for adherent cells pre- and non-precultured. The reduced sensitivity of *nd*HL60 at low PMA concentrations, however, did not interfere with the assay outcome, as was demonstrated with the reference compounds analyzed.

In summary this study describes the new *nd*HL60 superoxide microplate assay as a suitable tool for the rapid screening of oxidants as well as competing antioxidants.

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