

N-FORMYL-MET-LEU-PHE-INDUCED OXIDATIVE BURST IN DMSO-DIFFERENTIATED HL-60 CELLS REQUIRES ACTIVE HSP90, BUT NOT INTACT MICROTUBULES

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In this study we examined whether microtubules and heat shock protein 90 (Hsp90) are involved in phorbol myristate acetate (PMA) and *N*-formyl-Met-Leu-Phe (fMLP)-induced oxidative burst in DMSO-differentiated HL-60 cells. Our results showed that microtubule interfering agents, paclitaxel (1–5 μ M), colchicine (1–100 μ M), nocodazole (1–20 μ M), and vincristine (1–50 μ M), did not affect either PMA or fMLP-induced oxidative burst. In contrast, radicicol, an inhibitor of Hsp90, inhibited fMLP-induced oxidative burst in time and concentration-dependent manner where IC_{50} value for 30 min pre-incubation was 16.5 ± 3.5 μ M radicicol. We conclude that both PMA and fMLP-induced oxidative burst in DMSO-differentiated HL-60 cells is microtubule-independent while the latter requires Hsp90 activity.

INTRODUCTION

Superoxide radical generated during oxidative burst by phagocyte NADPH oxidase (EC 1.6.99.6) is a precursor of other reactive oxygen species which are used for host defence against bacterial and fungal pathogens¹. During the oxidase activation, cytosolic components of the oxidase (p67^{PHOX}, p47^{PHOX}, p40^{PHOX}, and small G-proteins Rac and Rap1A) are induced to migrate to the cell membrane and form an active enzyme complex with membrane-bound flavocytochrome b₅₅₈ consisting of gp91^{PHOX} and p22^{PHOX} (ref.²). NADPH oxidase may be activated by various stimuli triggering the oxidative burst either via binding to receptor (e.g. Fc receptor, complement receptor or chemokine receptor) or by direct interaction with a component of a signalling cascade³. Although cytoskeleton plays a role in regulation of the NADPH oxidase assembly, the influence of the different states of the cytoskeletal proteins on the access of cytosolic components to flavocytochrome is unknown. In the resting cells, p47^{PHOX} and p67^{PHOX} are associated with the cytoskeleton, particularly with moesin, coronin and actin⁴, which was shown to interact also with recombinant Rac1 and Rac2. Furthermore, actin enhances activation, while actin depolymerizing agents facilitate deactivation of NADPH oxidase in cell-free system consisting of cytosol and plasma membrane⁵. Less is known about the possible role of microtubules in the oxidative burst. Reibman *et al.*⁶ showed that microtubule disruption in neutrophils treated with colchicine did not affect superoxide radical production stimulated via Fc receptors or receptors for *N*-formyl-Met-Leu-Phe (fMLP), thus the assembly of NADPH oxidase did not require intact microtubules. Treatment of neutrophils with vincristine, an inhibitor of microtubule assembly, did not impair

myristate-induced superoxide release as well⁷. However, Wiles *et al.*⁸ demonstrated that colchicine attenuated and microtubule stabilization by paclitaxel increased fMLP-induced oxygen radical production by human neutrophils while experiments of Piazzolla *et al.*⁹ showed that paclitaxel inhibited fMLP-induced superoxide release by human neutrophils and colchicine had no effect.

The discrepancies in observed action of paclitaxel and colchicine inspired us to examine the effect of microtubule interfering agents, paclitaxel, colchicine, nocodazole, and vincristine, on fMLP and phorbol myristate acetate (PMA)-induced oxidative burst in DMSO-differentiated HL-60 cells. We included in our study also radicicol, an inhibitor of heat shock protein 90 (Hsp90)¹⁰, to investigate possible involvement of Hsp90 in signalling pathways leading to the oxidative burst, though radicicol was found to have no effect on fMLP-induced superoxide generation in primed or unprimed human neutrophils¹¹.

MATERIAL AND METHODS

Material

Colchicine in 95 % purity was isolated from corms of *Colchicum autumnale* at the Institute of Medical Chemistry and Biochemistry, Palacký University, Olomouc, Czech Republic. Paclitaxel semisynthetic from *Taxus* sp., nocodazole, vincristine sulfate, radicicol from *Diheterospora chlamydosporia*, phorbol 12-myristate 13-acetate (PMA), *N*-formyl-Met-Leu-Phe (fMLP), diphenyleneiodonium chloride (DPI), superoxide dismutase from bovine erythrocytes (SOD), RPMI-1640 medium, L-glutamine, penicillin-streptomycin solution and fetal bovine serum were obtained from Sigma Chemical

(Saint Louis, MO, USA). 2-methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazo[1,2-*a*]pyrazin-3-one hydrochloride (MCLA) was obtained from Molecular Probes (Eugene, OR, USA). All other chemicals were of the highest grade commercially available.

HL-60 cell line maintenance and differentiation into neutrophils

Human promyelocytic cell line HL-60 (ECACC No. 98070106) was maintained in RPMI-1640 medium containing 5 mM L-glutamine, 100 U/ml penicillin, 0.17 mM streptomycin, and 10 % (v/v) fetal bovine serum in a humidified incubator at 37 °C and 5 % CO₂. Cell density was maintained between 1 and 10 × 10⁵ cells/ml. Prior to differentiation cells were collected by centrifugation for 3 min at 240 g, resuspended in fresh medium and diluted to density 5 × 10⁵ cells/ml. DMSO was added to reach 1.25 % (v/v) and the cells were cultured for 7 days in a humidified incubator at 37 °C and 5 % CO₂. On day three fresh medium with DMSO was added to prevent cell starvation. Differentiated cells were collected by centrifugation as described above, washed once in PBS buffer (137 mM NaCl, 2.7 mM KCl, 9 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.4), and finally resuspended in PBS supplemented with 0.5 mM CaCl₂, 1 mM MgCl₂, and 30 mM D-glucose (PBS+). Cells were then kept on ice and used for further experiments only when capable of superoxide generation, which was sensitive to inhibition by DPI and SOD.

Chemiluminescent detection of superoxide formation

Differentiated HL-60 cells (final density 10⁶ cells/ml) were transferred into fluorescence quartz cuvette containing PBS+ buffer pre-warmed to 37 °C. DMSO (control) or a solution of tested substance in DMSO or DPI (10 μM) or SOD (100 U/ml) were added 5, 30 or 60 min prior to cell stimulation. MCLA (4 μM) was added 30 s prior to start of measurement and the background chemiluminescence was obtained. Superoxide generation was initiated 60 s after the start of measurement by fMLP (5 μM) or PMA (0.1 μM). Maximum DMSO concentration in assay was 0.95 % (v/v).

The time course of MCLA-dependent chemiluminescence was monitored on Perkin Elmer LS50B luminometer (Perkin Elmer, Norwalk, CT, USA) equipped with total emission mirror and thermostatted cuvette holder allowing continuous stirring. The areas under chemiluminescence peak were integrated and used for calculation of half-maximal concentration (IC₅₀) necessary for inhibition of oxidative burst. Activity detected in samples containing 100 U/ml SOD was used as 100 % inhibition.

RESULTS AND DISCUSSION

The DMSO-differentiated HL-60 cells respond to chemotactic peptide fMLP or phorbol ester PMA by triggering the superoxide radical generation. To explore the possible involvement of microtubules and Hsp90 in the oxidative

burst, cells were pre-incubated with paclitaxel (1–5 μM), colchicine (1–100 μM), nocodazole (1–20 μM), vincristine (1–50 μM), and radicicol (1–50 μM) for 5 or 30 min at 37 °C and subsequently stimulated by fMLP or PMA. Tested microtubule interfering agents act by different mechanisms resulting in microtubule stabilization (paclitaxel) or microtubule disruption (colchicine, nocodazole, vincristine)¹², but none of these substances markedly af-

Table 1. Effect of paclitaxel, colchicine, nocodazole, vincristine, and radicicol on PMA and fMLP-induced oxidative burst in DMSO-differentiated HL-60 cells.

Substance	Concentration (μM)	% of control (PMA) (%)	% of control (fMLP) (%)
Paclitaxel	5	84.6 ± 16.9	96.4 ± 9.8
Colchicine	100	96.3 ± 0.4	88.5 ± 5.2
Nocodazole	20	123.1 ± 0.2	74.9 ± 6.1 ^a
Vincristine	50	104.7 ± 7.3	75.6 ± 8.6 ^a
Radicicol	50	64.0 ± 6.0 ^a	11.8 ± 5.1 ^b

10⁶ cells/ml were pre-incubated for 30 min with DMSO (control) or tested substances prior to stimulation by PMA or fMLP. % of control values show ratio of MCLA-luminescence caused by cells treated with maximum applied concentration of tested substance and untreated cells. Values for radicicol were determined with differentiated HL-60 cells of early passage. All data are means ± SD from three experiments. ^a p<0.05, ^b p<0.005 significantly different from control.

Table 2. Time-dependence of radicicol inhibition of fMLP-induced oxidative burst in DMSO-differentiated HL-60 cells.

Pre-incubation time (min)	IC ₅₀ (μM)	IC ₅₀ * (μM)
5	>100	26.7
30	16.5	6.0
60	7.3	Not done

10⁶ cells/ml were pre-incubated for 5, 30 or 60 min with DMSO (control) or different concentrations of radicicol prior to stimulation by fMLP. Dose-response curves were constructed and respective IC₅₀s were calculated. Data are means from at least two experiments. Values in column IC₅₀ were determined with differentiated HL-60 cells of early passage, in column IC₅₀* with cells of later passage.

affected either PMA or fMLP-induced oxidative burst in differentiated HL-60 cells (Table 1). While nocodazole appears to partially inhibit fMLP-induced oxidative burst, this inhibition may be attributed to non-specific effects due to substance precipitation which was noticeable even with the 20 μ M concentration of nocodazole. In contrast, radicicol, an inhibitor of Hsp90 (ref.¹⁰), inhibited fMLP-induced oxidative burst in time (Table 2) and concentration-dependent manner (Fig. 1). Cells of later passage, i.e. cells cultured for 6 or more weeks before differentiation, were more responsive to radicicol than cells differentiated 2 weeks after resuscitation (Table 2). In case of PMA stimulation we observed only partial inhibitory effect that required treatment of cells with 50 μ M radicicol for 30 min (Table 1). Moreover, radicicol at concentrations 1–50 μ M did not inhibit sodium dodecyl sulfate-induced activation of NADPH oxidase in cell-free system containing cytosol and membranes obtained from unstimulated DMSO-differentiated HL-60 cells (data not shown).

Cell resistance towards microtubule interfering agents may result from β -tubulin gene mutations or multidrug resistance allowed by P-glycoprotein¹³. Because tested compounds bind to tubulin via different binding sites¹² and neutrophils lack P-glycoprotein, which may eliminate colchicine¹⁴, paclitaxel, and vincristine¹³, we conclude that both PMA and fMLP-induced oxidative burst in DMSO-differentiated HL-60 cells is microtubule-independent. The requirement for Hsp90 function in fMLP-induced oxidative burst results probably from the triggering mechanisms where binding of fMLP to its G-protein-coupled receptors activates multiple signalling pathways while

PMA interacts directly with protein kinase C (PKC) activating NADPH oxidase¹⁵. We hypothesize that Hsp90 acts upstream of PKC and in agreement with experiments of Inanobe *et al.*¹⁶, which demonstrated that Hsp90 from HL-60 cells interacted with free $\beta\gamma$ subunits of heterotrimeric G-proteins, we suggest Hsp90 as a possible target for modulation of the oxidative burst.

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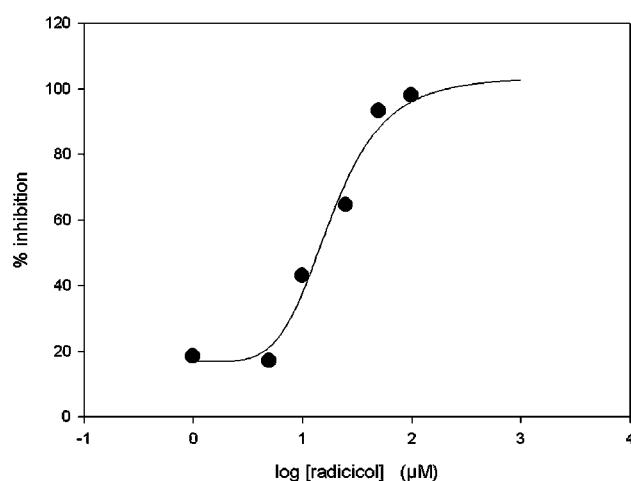


Fig. 1. Dose-response curve for radicicol inhibition of fMLP-induced oxidative burst in DMSO-differentiated HL-60 cells of early passage. 10^6 cells/ml were pre-incubated with different concentrations of radicicol for 30 min prior to stimulation by fMLP. Inhibition was calculated from chemiluminescent trace areas where 0% inhibition represents superoxide generation in the presence of fMLP alone and 100% inhibition in the presence of 100 U/ml SOD and fMLP. The IC_{50} value derived from three experiments was 16.5 ± 3.5 μ M radicicol.

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