ANTIOXIDANTS & REDOX SIGNALING
Volume 7, Numbers 9 & 10, 2005

Original Research Communication

Mechanism and Characteristics of Stimuli-Dependent ROS Generation in Undifferentiated HL-60 Cells

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ABSTRACT

It has been widely believed that undifferentiated human promyelocytic leukemia cells (HL-60) have no ability to generate reactive oxygen species (ROS) responding to stimuli. We report here that undifferentiated HL-60 cells possess NADPH oxidase and that generation of superoxide can be measured using a highly sensitive chemiluminescence dye, L-012. Five subunits of NADPH oxidase, namely, gp91^{phox}, p22^{phox}, p67^{phox}, p47^{phox}, and Rac 2, were detected in undifferentiated HL-60 cells by immunoblotting analysis. The contents of these NADPH oxidase components in the cells were increased with the differentiation induced by phorbol myristate acetate (PMA), except for p22^{phox}. Messenger RNAs of these subunits were also detected by the RT-PCR method, and their expressions increased except that of p22 ^{phox} with the differentiation induced by PMA. Kinetic analysis using L-012 revealed that HL-60 cells generated substantial amounts of ROS by various stimulants, including formylmethionyl-leucyl-phenylalanine, PMA, myristic acid, and a Ca^{2+} ionophore, A23187. Both diphenyleneiodonium (an inhibitor of FAD-dependent oxidase) and apocynin (a specific inhibitor of NADPH oxidase) suppressed this stimuli-dependent ROS generation. Genistein, staurosporine, uric acid, and sodium azide inhibited the ROS generation in undifferentiated HL-60 cells in a similar way to that in undifferentiated neutrophils. These results suggested that the mechanism of ROS generation in undifferentiated HL-60 cells is the same as that in primed neutrophils. *Antioxid. Redox Signal.* 7, 1367–1376.

INTRODUCTION

NADPH OXIDASE is the most important enzyme involved in the generation of reactive oxygen species (ROS) in phagocytes (16). Upon stimulation, phagocytes first produce superoxide accompanying the oxygen burst by NADPH oxidase (6). Subsequently, various ROS are generated from superoxide. All forms of ROS play important roles in the host defenses against pathogens, but they can also cause tissue damage and apoptosis, which could enhance the aging process and pathogenesis of several diseases in the host (14). HL-60 is a human promyelocytic leukemia cell line and can differentiate to monocyte/macrophage-like cells or granulocytic cells by treatment with phorbol myristate acetate (PMA) or retinoic acid, respectively (25, 41). It has been reported that differentiated HL-60 cells generate superoxide by various stimuli, such as formylmethionyl-leucyl-phenylalanine (fMLP), PMA, Ca^{2+} ionophore (A23187), and various fatty acids, but that undifferentiated HL-60 cells do not (3, 5, 24, 26). It was also reported that Ca^{2+} ionophore (ionomycin) can prime the cell, but cannot activate the reactive oxygen-generating system in differentiated HL-60 cells (12). However, it

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was recently shown that undifferentiated HL-60 cells contain NADPH oxidase subunits and can be used as a model for the assessment of the oxidase activity (36). Thus, there is a possibility that undifferentiated HL-60 cells could also generate ROS upon stimulation. We undertook this work to investigate whether or not this happens, and if it does, through what mechanism.

Chemiluminescence (CHL) is a useful tool to detect ROS generation (2). Recently, a new CHL dye, L-012, has been developed, which is very sensitive to ROS (29). Kinetic analysis revealed that L-012 elicits CHL predominantly by reacting with superoxide radical, hydroxyl radical, and hypochlorite (19). Therefore, we assumed that L-012 could possibly detect stimulation-dependent ROS generation in undifferentiated HL-60 cells. The goals of this experiment are to clarify the mechanism of stimuli-induced generation of ROS in undifferentiated HL-60 cells, and to identify molecular species involved in this reaction.

MATERIALS AND METHODS

Chemicals

A23187, diphenyleneiodonium (DPI), fMLP, genistein, myristic acid, apocynin, and staurosporine were obtained from Sigma–Aldrich (St. Louis, MO, U.S.A.). Cetylamine was obtained from Nacalai Tesque (Kyoto, Japan). Anti-gp91^{phox} and p47^{phox} antibodies were raised in our laboratory. Anti-p22^{phox} and Rac2 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Anti-p67^{phox} was obtained from Transduction Laboratories (San Jose, CA, U.S.A.). 8-Amino-5-chloro-7-phenylpyrido[3,4-*d*]pyridazine-1,4-(2*H*,3*H*) dione (L-012) was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Cell line and cell differentiation

Human promyelocytic leukemia cells (HL-60) were kindly donated by Dr. M. Saito (Jichi Medical School, Japan) and were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were grown in a humidified incubator at 37°C under 5% CO_2/95% air and used for experiments during the exponential phase of their growth. Where indicated, the differentiation of HL-60 cells to monocytes was induced by incubation with 10 n*M* PMA for 24 h. Cells were routinely counted to maintain a low population density, and their viability was assayed by the trypan-blue exclusion method (40).

Assay for ROS generation in cells

ROS were measured by the highly sensitive CHL probe, L-012 (19). The cells were incubated in phosphate-buffered saline (PBS) containing 1 m*M* Ca^{2+}, 10 m*M* glucose, and 50 μ*M* L-012 at 37°C. After incubation of the cells for 1 min, the reaction was started by the addition of various stimulants. During the incubation, the CHL intensity was recorded continuously for 10–20 min using Intercellular Ion Analyzer (Jasco CAF-110) (CHL mode, Jasco PL-03).

Western blot analysis

The cell lysates were treated with sodium dodecyl sulfate (SDS) sample buffer [125 m*M* Tris-HCl (pH 6.8), 4% SDS, 10% mercaptoethanol, 20% glycerol, and 0.002% bromophenol blue] and subjected to SDS–polyacrylamide gel electropheresis (PAGE). Proteins in the gel were transferred onto a polyvinylidene difluoride membrane. The membrane was incubated with the primary antibody and the horseradish peroxidase-linked secondary antibody, and then analyzed using an enhanced ECL plus Western Blotting Detection System (Amersham, U.K.) (4). The protein concentration was determined by the method of Bradford using bovine serum albumin as a standard (8).

Assay for expression of various subunits

Total RNA was isolated from HL-60 cells using TRIZOL (Invitrogen) following the manufacturer's instruction. Oligo dT-primed cDNA was prepared from 5 μg of total RNA using Superscript II (Invitrogen). One-twentieth of the cDNA obtained was used for each polymerase chain reaction (PCR). Primers were designed based on sequences of p22^{phox}, p47^{phox}, p67^{phox}, and gp91^{phox}. The primer pairs were as follows: p22^{phox} (325 bp), 5′-GTTTGTTTTGTGCCTGCTGGAGT-3′ and 5′-TGGGCGGCTGCTTGATGGT-3′; p47^{phox} (767 bp), 5′-ACCCAGCCAGCACTATGTGT-3′ and 5′-AGTAGCCTGTGACGTCGTCT-3′; p67^{phox} (747 bp), 5′-CGAGGGAACCAGCTGATAGA-3′ and 5′-CATGGGAACACTGAGCTTCA-3′; and gp91^{phox} (403 bp), 5′-GCTGTTCAATGCTTGTGGCT-3′ and 5′-TCTCCTCATCATGGTGCACA-3′. The constitutively expressed housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was coamplified as an internal control (358 bp). The primer pairs were 5′-ATTCCATGGCACCGTCAAGGCT-3′ and 5′-TCAGGTCCACCACTGACACGTT-3′. The PCR reaction for various subunits and GAPDH were 27 and 20 cycles, respectively. The amplified products were separated in 2% agarose gels and visualized by ethidium bromide staining (32).

Statistical analysis

Results are expressed as the means ± SD. The significance of differences between experimental conditions in the CHL measurements (see Fig. 2) was determined using the two-tailed Student's *t* test. The significance of differences in the reverse transcription–polymerase chain reaction (RT-PCR) (see Fig. 6) was evaluated by the Mann–Whitney test. A probability of $p < 0.05$ was considered significant.

RESULTS

Generation of ROS by various stimuli in undifferentiated HL-60 cells

In the presence of glucose and L-012, all four stimulants, *i.e.,* fMLP, PMA, A23187, and myristic acid, induced a definite degree of CHL (Fig. 1). In the case of fMLP stimulation, a priming with tumor necrosis factor-α (TNF-α) was required to induce detectable CHL. ROS generation was not detected

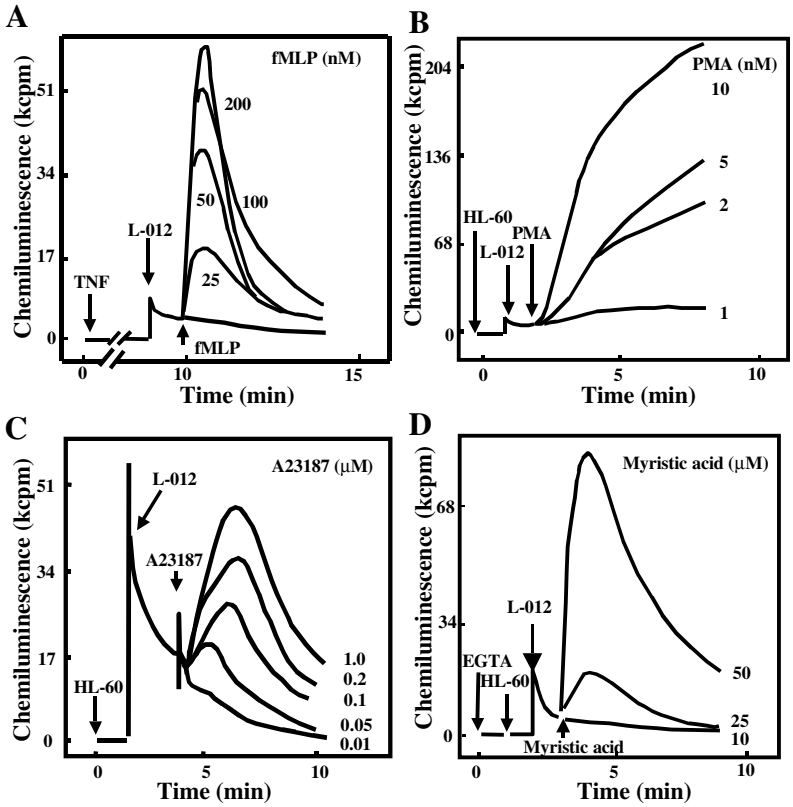

FIG. 1. Effects of various stimuli on CHL of L-012 in HL-60 cells. Cells ($0.5–1 \times 10^6$ cells/ml) were stimulated with various concentrations of different stimulants in PBS containing 1 m*M* Ca^{2+}, 10 m*M* glucose, and 50 μ*M* L-012 at 37°C. In the case of fMLP stimulation, cells (1×10^6 cells/ml) were treated with 10 ng/ml TNF-α for 10 min before incubation with ~200 n*M* fMLP. The concentrations of the various stimuli were as follows: fMLP, ~200 n*M* (**A**); PMA, ~10 n*M* (**B**); A23187, ~1 μ*M* (**C**); and myristic acid, ~50 μ*M* (**D**).

without priming with TNF-α as in the case of circulating human neutrophils. Among the stimulants tested, PMA induced the most potent and sustained generation of ROS. The other stimulants induced a rather transient generation of ROS.

Comparison of stimuli-induced CHL between undifferentiated and monocytic differentiated HL-60 cells

It is well known that HL-60 cells differentiate to either granulocyte or monocyte by treatment with all-*trans*-retinoic acid or PMA, respectively. Thus, the stimuli-induced CHL was compared between undifferentiated and differentiated HL-60 cells.

As shown in Fig. 2, differentiated HL-60 cells developed a strong CHL of L-012 by treatment with 10 n*M* PMA for 24 h even without an additional stimulation. The CHL of L-012 was further increased by additional stimulation with 2 n*M* PMA. The intensity of CHL was >10 times stronger in differentiated cells than that in undifferentiated HL-60 cells.

Suppression of stimuli-dependent CHL by DPI and apocynin

DPI, an inhibitor of FAD-dependent oxidase (such as NADPH oxidase), suppressed the myristic acid-induced CHL of L-012 in HL-60 cells in a concentration-dependent manner

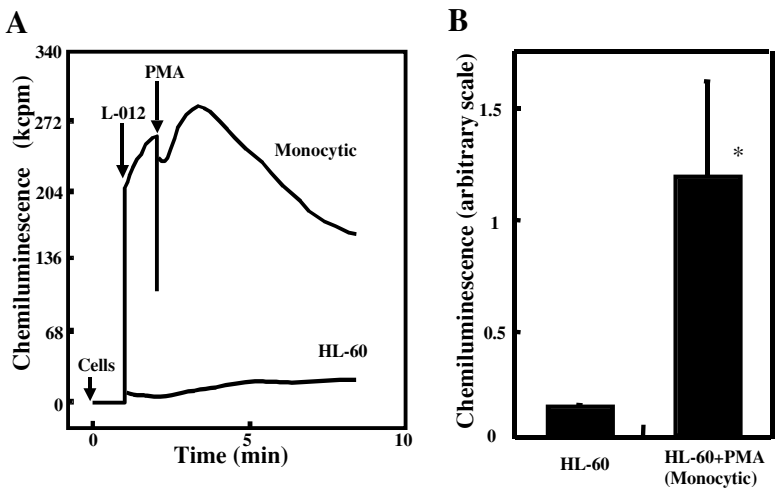

FIG. 2. Differentiation of HL-60 cells by PMA and the measurements of CHL of L-012 during the PMA-induced stimulation process. Cell differentiation was induced by treatment with 10 n*M* PMA for 24 h. (**A**) Actual traces of CHL in nondifferentiated and PMA-induced monocytic cells. Similar results were obtained in three separate experiments. Monocytic, PMA-induced monocytic differentiated HL-60 cells. (**B**) Difference between CHL intensities of undifferentiated and differentiated HL-60 cells. Data show means ± SD from triplicate experiments. *$p < 0.01$.

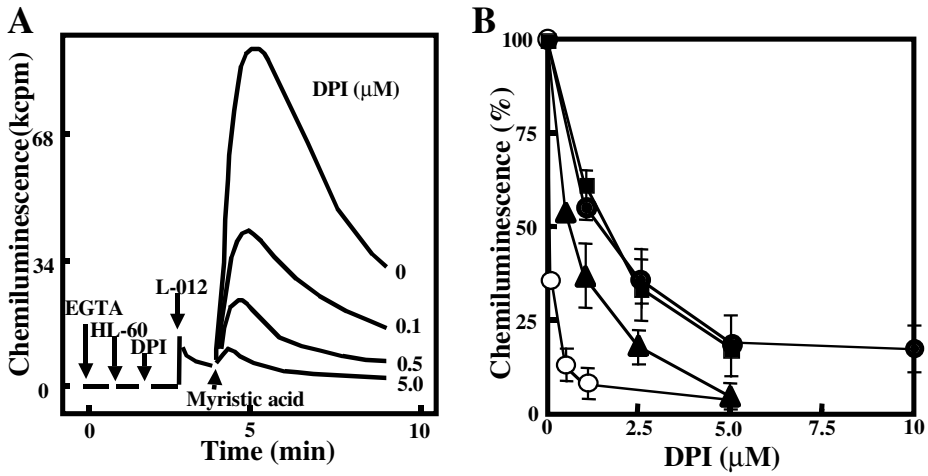

FIG. 3. Suppression of stimuli-induced CHL of L-012 in HL-60 cells by DPI. Experimental conditions were the same as described in Fig. 1. The concentrations of fMLP, PMA, A23187, and myristic acid were 100 n*M*, 2 n*M*, 1 μ*M,* and 50 μ*M*, respectively. Similar results were obtained in three separate experiments. (**A**) Actual trace of myristic acid-stimulated CHL and its inhibition by the indicated concentrations of DPI. (**B**) Concentration-dependent DPI inhibition of CHL induced with various stimuli. ■, fMLP; ▲, PMA; ●, A23187; ○, myristic acid. Data show means ± SD from triplicate experiments.

(Fig. 3A). DPI also suppressed the CHL induced by other stimulants in undifferentiated HL-60 cells (Fig. 3B). Similar inhibition of stimuli-induced CHL in undifferentiated cells was caused by a specific inhibitor of NADPH oxidase, apocynin (32), as shown in Fig. 4. These results indicate that the stimuli-induced CHL of L-012 is produced by the activation of NADPH oxidase in undifferentiated HL-60 cells.

Identification of NADPH oxidase subunits

As stimuli-dependent CHL of L-012 in undifferentiated HL-60 cells was inhibited by DPI and apocynin, it is plausible that undifferentiated HL-60 cells contained enzyme complexes of NADPH oxidase. Thus, we performed the immunoblotting analysis of NADPH oxidase subunits using antisubunit antibodies. The membrane components of NADPH oxidase, *i.e.,* gp91^{phox} and p22^{phox}, were detected in undifferentiated HL-60 cells. The anti-gp91^{phox} antibody raised in our laboratory detected a major band at 91 kDa in human circulating neutrophils, and this band was ablated by preincubation of the antibody with immunizing peptide (Ac-CGPEALAETLSKQSISNSES).

The cytoplasmic subunits, namely, p67^{phox}, p47^{phox}, and Rac 2, were also detected in undifferentiated HL-60 cells (Fig. 5). These subunits of NADPH oxidase, except p22 ^{phox}, increased in differentiated cells by the treatment with PMA (Fig. 5).

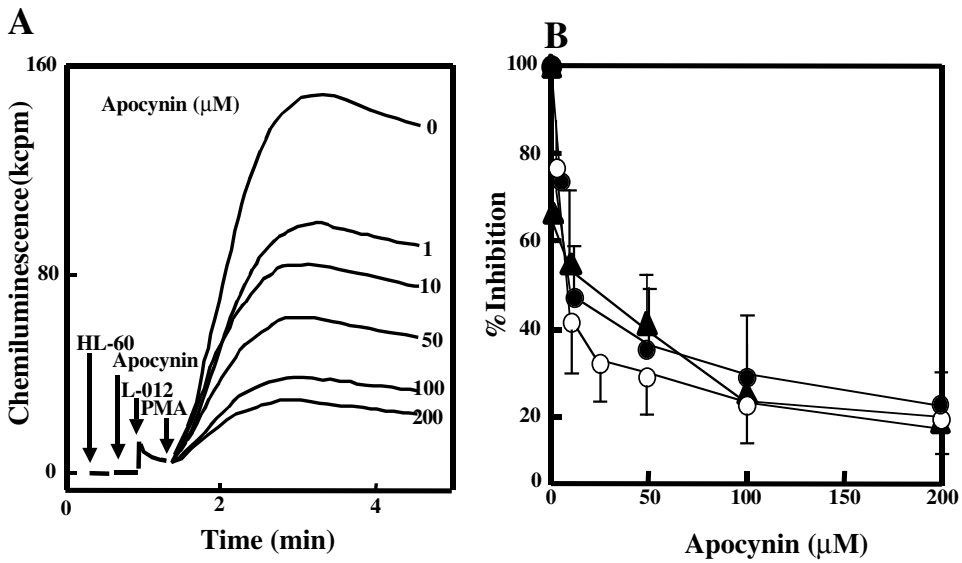

FIG. 4. Suppression of stimuli-induced CHL of L-012 in HL-60 cells by apocynin. Experimental conditions were the same as described in Fig. 3. (**A**) Actual trace of PMA-stimulated CHL and its inhibition by the indicated concentrations of apocynin. Similar results were obtained in three separate experiments. (**B**) Concentration-dependent apocynin inhibition of CHL induced with various stimuli. ▲, PMA; ●, A23187; ○, myristic acid. Data show means ± SD from triplicate experiments.

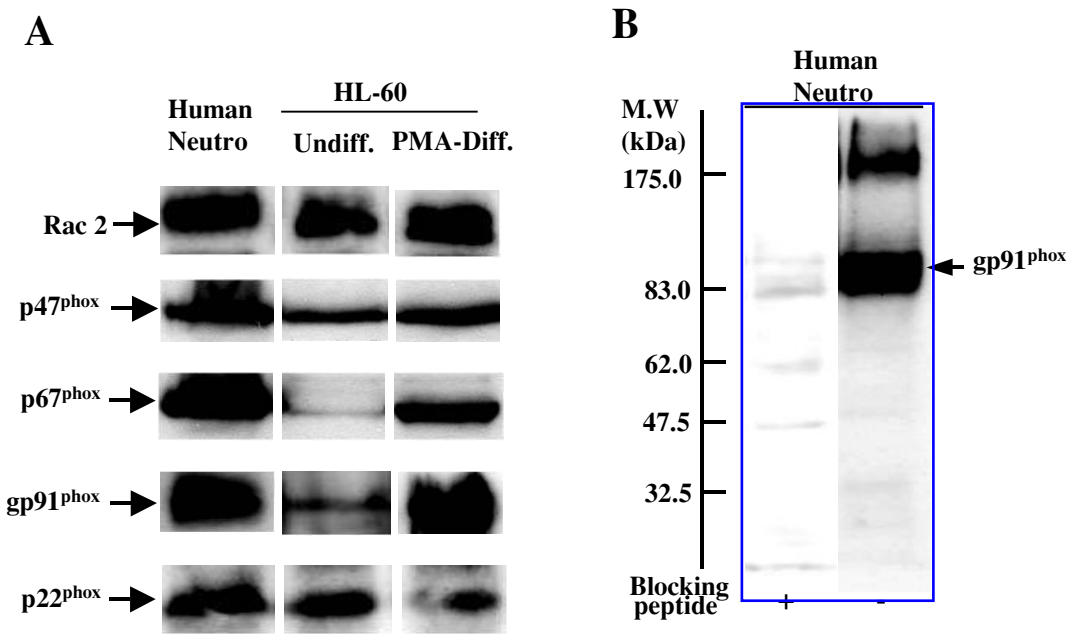

FIG. 5. Identification of NADPH oxidase subunits. (**A**) HL-60 cells were used before and after differentiation by 10 n*M* PMA for 24 h. Cell lysate from the 10^6 cells was used for SDS-PAGE. Subunits of NADPH oxidase in neutrophils and undifferentiated and differentiated HL-60 cells were identified by western blot analysis using the respective anti-subunit antibodies ($gp91^{phox}$, $p22^{phox}$, $p47^{phox}$, $p67^{phox}$, and Rac 2). Similar results were obtained in three separate experiments. Human Neutro, human circulating neutrophil; Undiff., undifferentiated HL-60 cells; PMA-Diff., PMA-induced differentiated monocytic cells. (**B**) For the detection of $gp91^{phox}$, the membrane fraction was separated by ultracentrifugation and used as a sample. The anti-$gp9^{phox}$ antibody raised in our laboratory detected a major band at 91 kDa in human circulating neutrophils (Human Neutro), and this band disappeared when the antibody was preincubated with the blocking peptide (Ac-CGPEALAETLSKQSISNSES), which was used to raise the antibody.

We further analyzed the mRNA expression of the NADPH oxidase subunits in HL-60 cells, and followed their changes after treatment with 10 n*M* PMA. As shown in Fig. 6A, RT-PCR products of all subunits were detected in undifferentiated HL-60 cells. The levels of gene expression in $p22^{phox}$ and $gp91^{phox}$ were relatively higher than those of $p47^{phox}$ and $p67^{phox}$. The expression level of each subunit was increased during the differentiation to macrophage-like cells by PMA, and their levels at 24 h were significantly higher than those at 0 h ($p < 0.01$). In contrast, the level of expression for $p22^{phox}$

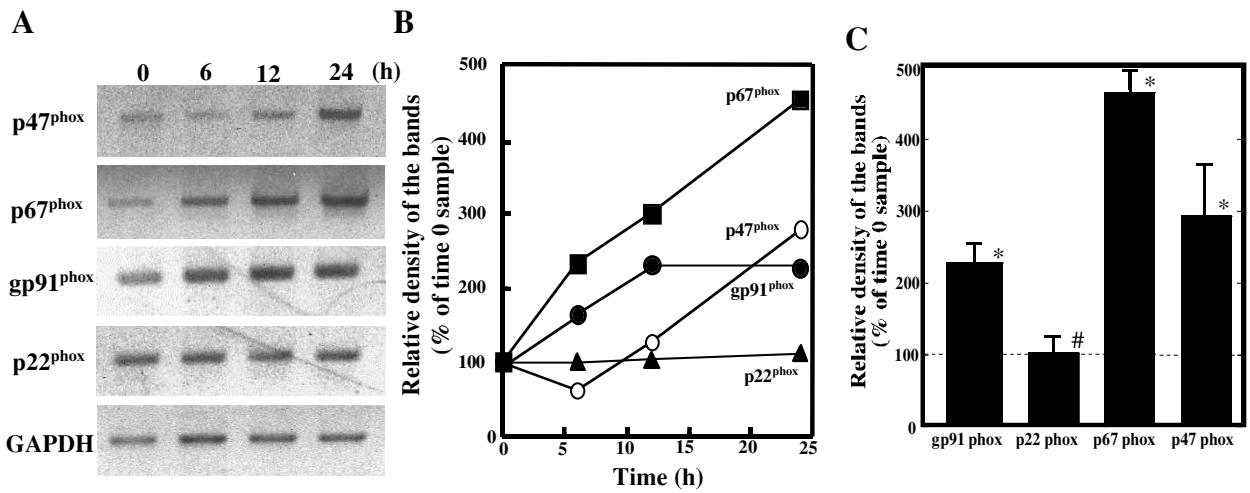

FIG. 6. mRNA expression of various subunits of NADPH oxidase in undifferentiated HL-60 cells, and their changes during differentiation by PMA. mRNA expression of various subunits, namely, $p22^{phox}$, $p47^{phox}$, $p67^{phox}$, and $gp91^{phox}$ in HL-60 cells, was studied by RT-PCR using primers for each subunit (see text). Expression of these subunits was also analyzed during differentiation of HL-60 cells at various time points after treatment with 10 n*M* PMA. Expression of GAPDH was measured as a housekeeping protein. (**A**) RT-PCR amplification of NADPH oxidase subunits and their changes during differentiation. (**B**) Percentage changes of each subunit during differentiation in A. (**C**) Changes in the mean value of mRNA at 24 h after the treatment with 10 n*M* PMA. *$p < 0.01$; #$p > 0.7$.

was almost constant and the difference of levels between 0 and 24 h was not statistically significant ($p > 0.7$) (Fig. 6B).

Effect of staurosporine, genistein, and cetylamine on the stimuli-induced CHL

It is well known that superoxide generation in neutrophils depends on the activation of NADPH oxidase through phosphorylation of p47^{phox} by certain kinases, such as protein kinase C (PKC). Thus, the effect of staurosporine on the stimuli-induced CHL in undifferentiated HL-60 cells was examined. As shown in Fig. 7A, PMA-induced CHL was strongly suppressed by staurosporine in a concentration-dependent manner. The CHL induced by A23187 and fMLP was also suppressed by staurosporine, although the staurosporine concentration required for the inhibition was substantially higher. Myristic acid-induced CHL was not inhibited even by 100 n*M* staurosporine (Fig. 7B).

It has been reported that tyrosine kinase is involved in the process of NADPH oxidase activation by fMLP (1, 37, 38). Thus, the effect of genistein, a tyrosine kinase inhibitor, on the stimuli-induced CHL was examined in HL-60. Figure 7C shows that only fMLP-induced CHL was strongly suppressed by genistein, whereas no suppression was observed in PMA-induced CHL. Furthermore, myristic acid- and A23187-induced CHL was rather increased in a concentration-dependent manner. Myristic acid modulates the formation of NADPH oxidase complex through a stimulated assembly of cytosolic components with membrane components to form the enzyme complex without phosphorylation of p47^{phox} (17, 31). Cetylamine, a positively charged modifier of membrane proteins, suppressed the stimuli-dependent CHL except for fMLP-induced CHL as shown in Fig. 7D.

Effect of various antioxidants on the stimuli-dependent CHL

It has been reported that the molecular species of generated ROS are different when these cells are stimulated by different stimuli (34). Thus, the effect of various antioxidants on the stimulation-dependent CHL was examined. The sensitivity to each antioxidant was different when different stimuli

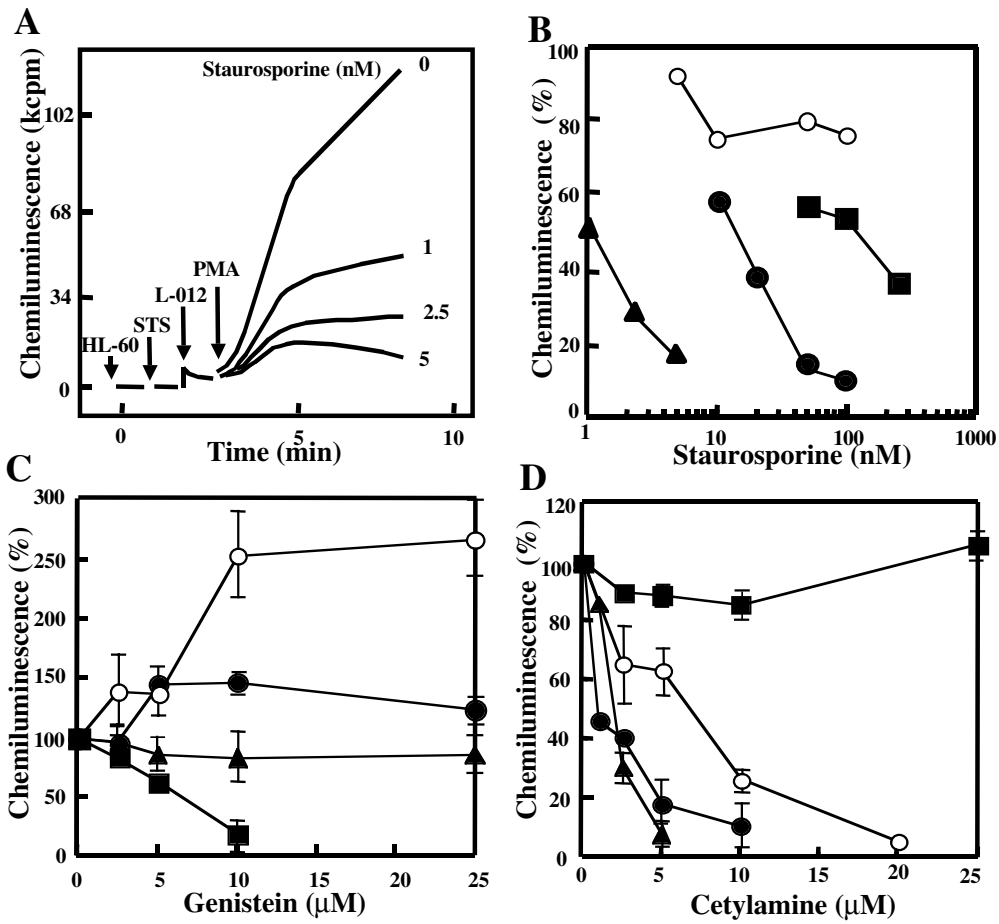

FIG. 7. Sensitivity of stimuli-induced generation of ROS in HL-60 cells to staurosporine, genistein, and cetylamine. Experimental conditions were the same as described in Fig. 3. Various concentrations of staurosporine, genistein, and cetylamine were added before the induction of ROS generation. The concentrations of stimuli were the same as described in Fig. 3. (**A**) Time-dependent changes in PMA-induced CHL of L-012 in HL-60 cells. (**B,C,D**) Concentration-dependent suppression of stimuli-induced CHL of L-012 by staurosporine, genistein, and cetylamine, respectively. ■, fMLP; ▲, PMA; ●, A23187; ○, myristic acid. Data show means ± SD from triplicate experiments.

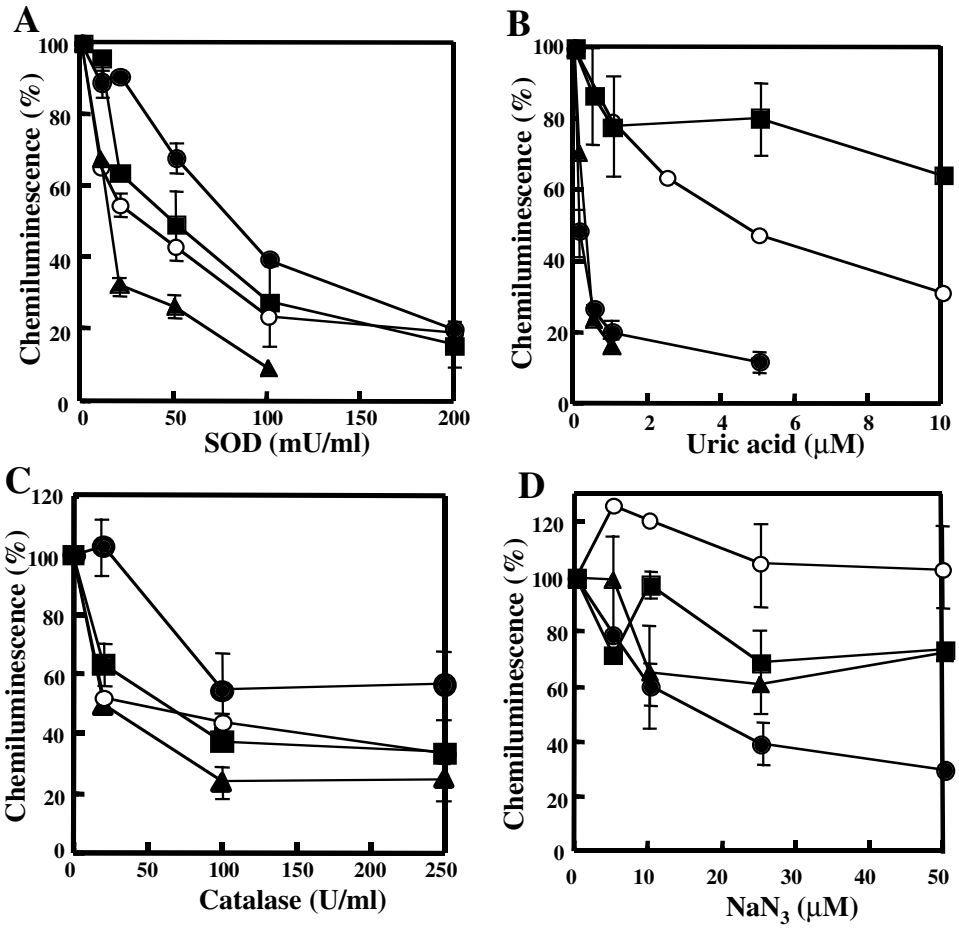

FIG. 8. Sensitivity of stimuli-induced ROS generation in HL-60 cells to various antioxidants. Experimental conditions were the same as described in Fig. 3. Various concentrations of antioxidants were added before the induction of ROS generation. The concentrations of stimuli were the same as described in Fig. 3. (**A,B,C,** and **D**) Concentration-dependent suppression of stimuli-induced CHL of L-012 by SOD, uric acid, catalase, and azide, respectively. ■, fMLP, ▲, PMA, ●; A23187, ○, myristic acid. Data show means ± SD from triplicate experiments.

were used (Fig. 8). Superoxide dismutase (SOD) suppressed the CHL induced by all stimuli tested. Uric acid, however, suppressed CHL induced by PMA and A23187, but not by myristic acid and fMLP. Catalase and azide, an inhibitor of myeloperoxidase, partially suppressed the stimulation-dependent CHL.

DISCUSSION

Primed neutrophils generate ROS in response to various stimuli, such as A23187 (39), opsonized zymozan (39), fMLP (13), PMA (20), and fatty acids (39). CHL is a widely used tool to detect extracellular generation of ROS (2, 21). Kinetic analysis revealed that L-012, a highly sensitive CHL probe for ROS, predominantly detects superoxide, hydroxyl radical, and hypochlorite (19, 29). In the present article, we studied the mechanism of ROS generation in undifferentiated HL-60 cells using L-012 and found that the cells transiently produced L-012 CHL by various stimuli. The CHL production was strongly suppressed by both DPI (an inhibitor of FAD-dependent oxidase) and apocynin (a specific inhibitor of NADPH oxidase) as shown in Figs. 3 and 4.

Consistent with these results, the presence of essential subunits of NADPH oxidase, such as gp91^{phox}, p22 ^{phox}, p67^{phox}, p47^{phox}, and Rac 2, were identified in undifferentiated HL-60 cells by immunoblotting analysis. The mRNAs of these subunits were also detected by the RT-PCR method (Figs. 4 and 5). These results suggested that undifferentiated HL-60 cells generate ROS through the activation of NADPH oxidase.

The intracellular machinery for triggering oxygen burst in neutrophils involves at least three pathways: (a) PMA- or A23187-stimulated and staurosporine-inhibitable PKC pathway (7, 9); (b) TNF-α-primed fMLP- or opsonized zymosan-stimulated and genistein-inhibitable tyrosine kinase-sensitive pathway (1, 21, 35, 38); and (c) SDS- or fatty acid-stimulated and cetylamine-inhibitable pathway (11, 27, 33). In the case of undifferentiated HL-60 cells, we observed that: (a) PMA- or A23187-induced CHL was suppressed by staurosporine; (b) TNF-α-primed fMLP-induced CHL was strongly suppressed by genistein; ROS generation by various stimuli, except for fMLP, was also suppressed by cetylamine (Fig. 7);

and (c) this stimuli-induced CHL was also suppressed by SOD. These results confirmed that all of these three pathways are functional in undifferentiated HL-60 cells in the same manner as in the primed neutrophils.

CHL induced by PMA and A23187, but not by fMLP and myristic acid, was suppressed by uric acid in a concentration-dependent manner. Furthermore, catalase and azide partially suppressed the stimuli-induced CHL (Fig. 8). These characteristics were quite similar to those of human circulating peripheral neutrophils (1, 28, 34, 38), and further supported that the ROS generation machinery was similar in both HL-60 and neutrophils. We have noticed that the molecular species of generated ROS in undifferentiated HL-60 cells change in response to different stimulation and that the mechanism needs to be studied further.

The ROS generation was also regulated by the degree of NADPH oxidase subunits. It has been reported that gp91^{phox}, p67^{phox}, and p47^{phox} were expressed only after myelocyte stages in differentiation-induced HL-60 cells (18). However, a recent study showed that undifferentiated HL-60 cells express both membranous and cytosolic NADPH oxidase subunits (36). Thus, gene expression of NADPH oxidase and the superoxide release in undifferentiated promyelocytic cells are still controversial and inconclusive (10, 24, 30).In this study, we found the stimuli-dependent ROS generation and the presence of various subunits of NADPH oxidase in undifferentiated HL-60 cells. Summarizing all these results, we propose that the activation mechanism of NADPH oxidase exists in undifferentiated HL-60 cells, and that the degree of subunit expression is increased during the course of cell differentiation.

Recently, a more general importance of NADPH oxidase activity has been suggested, because various nonphagocytic cells have NADPH oxidase or similar oxidases with gp91 ^{phox}, p47^{phox}, and p67^{phox} homologue proteins (15, 22). The generation of ROS by these oxidases was demonstrated in nonimmune cells (14, 22), although in much lower levels. It was also reported that tamoxifen induced ROS generation in HepG2 human hepatoma cells through the activation of NADPH oxidase, which occurred concomitantly with apoptosis initiated by the increased level of intracellular calcium ions (23). These results indicate that nonphagocyte NADPH oxidase has important roles in various signaling events and in other physiological processes. Thus, further studies are needed to clarify the general importance of NADPH oxidase activity and the role of generated ROS in signal transduction.

ACKNOWLEDGMENTS

This work was supported, in part, by grants from the Ministry of Education, Science and Literature. We thank Dr. Alan A. Horton and Dr. S. Tsuyoshi Ohnishi for help in the preparation of the manuscript.

ABBREVIATIONS

CHL, chemiluminescence; DPI, diphenyleneiodonium; fMLP, formylmethionyl-leucyl-phenylalanine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; L-012, 8-amino-5-chloro-7-phenylpyrido[3,4-*d*]pyridazine-1,4-(2*H*,3*H*)dione; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PKC, protein kinase C; PMA, phorbol myristate acetate; ROS, reactive oxygen species; RT-PCR, reverse transcription–polymerase chain reaction; SDS, sodium dodecyl sulfate; SOD, superoxide dismutase; TNF-α, tumor necrosis factor-α.

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Received for publication August 23, 2004; accepted April 12, 2005.

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