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NADPH Oxidase-mediated Generation of Reactive Oxygen Species is Critically Required for Survival of Undifferentiated Human Promyelocytic Leukemia Cell Line HL-60

JING-MEI DONG[a], SHENG-GUO ZHAO[b], GUO-YIN HUANG[c] and QING LIU[c,*]

[a]*Department of Physical, Lanzhou Teacher's College, Lanzhou, People's Republic of China;* [b]*Department of Chemistry, Lanzhou University, Lanzhou, People's Republic of China;* [c]*Institute of Biophysics, School of Life Sciences, Lanzhou University, 298, Tianshui Road, Chenguan District, Lanzhou 730000, People's Republic of China*

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Nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) mediated generation of reactive oxygen species (ROS) was originally identified as the powerful host defense machinery against microorganism in phagocytes. But recent reports indicated that some non-phagocytic cells also have the NADPH oxidase activity, and the ROS produced by it may act as cell signal molecule. But as far as today, whether the NADPH oxidase also plays similar role in phagocyte has not been paid much attention. Utilizing the undifferentiated HL-60 promyelocytic leukemia cells as a model, the aim of the present study was to determine whether NADPH oxidase plays a role on ROS generation in undifferentiated HL-60, and the ROS mediated by it was essential for cell's survival. For the first time, we verified that the release of ROS in undifferentiated HL-60 was significantly increased by the stimulation with Calcium ionophore or opsonized zymosan, which are known to trigger respiration burst in phagocytes by NADPH oxidase pathway. Diphenylene iodonium (DPI) or apocynin (APO), two inhibitors of NADPH oxidase, significantly suppressed the increasing of ROS caused by opsonized zymosan. Cell survival assay and fluorescence double dyeing with acridine orange and ethidium bromide showed that DPI and APO, as well as superoxide dismutase (SOD) and catalase (CAT) concentration-dependently decreased the viability of undifferentiated HL-60 cells, whereas exogenous H_2O_2 can rescue the cells from death obviously. Our results suggested that the ROS, generated by NADPH oxidase play an essential role in the survival of undifferentiated HL-60 cells.

Keywords: NADPH oxidase; Reactive oxygen species; HL-60 cells; Cell survival

Abbreviations: ROS, reactive oxygen species; NADPH oxidase, nicotinamide adenine dinucleotide phosphate oxidase; FBS, fetal bovine serum; SOD, superoxide dismutase; CAT, catalase; DPI, diphenylene iodonium; APO, apocynin; ETYA, 5,8,11,14-eicosatetraynic acid; IM, indomethacin; L-NAME, N^G-nitro-L-arginine methylester; AP, allopurinol; NOS, nitric oxide synthase; OZ, opsonified zymosan; DMSO, Dimethyl Sulfoxide; HRP, Horseradish peroxidase; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; SDS, sodium dodecyl sulfate; AO, acridine orange; EB, ethidium bromide

INTRODUCTION

Phagocyte such as polymorphonuclear leukocytes (PMN) and macrophages generate superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) as essential components of their microbicidal system.[1] The one-electron reduction of molecular oxygen to ($O_2^{\cdot-}$) is catalyzed by an Nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) that is inactive in resting cells but rapidly activates upon exposure of cells to a variety of soluble or particulate stimuli.[1–4] So, NADPH oxidase is traditionally considered as an antimicrobial enzyme in aerobiont.

Previous studies have established an important role for reactive oxygen species (ROS) in cell survival. Recently, ROS was recognized as modulators of receptor-mediated signal transduction in a variety of cell types.[5–7] Lots of enzymes contribute to the production of ROS *in vivo*, including NADPH

*Corresponding author. Tel.: +86-931-8514967. Fax: +86-931-8912561. E-mail: liuqing01@st.lzu.edu.cn

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oxidase, cyclooxygenases, lipoxygenases, myeloperoxidase, nitric oxide synthase (NOS), and xanthine oxidase.[5–7] Among them, NADPH oxidase was originally identified as the major source of ROS in phagocytes. Now it becomes clear that this oxidase is functionally expressed not only in phagocytes but also in other cell types, including endothelial cells,[8,9] fibroblasts, platelet, osteoclasts, smooth muscle cells, and neurocyte.[10–15] NADPH oxidase can be quickly activated and elevate the level of ROS within a few minutes after the stimulation by a variety of growth factors, such as cytokines and hormones, including interleukin-1β (IL-1β),[16] platelet derived growth factor (PDGF),[17] or nerve growth factor (NGF).[18] Thus the NADPH-derived ROS have been assumed playing special important roles in ROS-mediated signal transudation cascades, including those critically important for cell proliferation, differentiation, apoptosis and even necrosis.[19–21] Although the researches in this area have acquired some remarkable achievement, so far as today, the situation of phagocyte has been lost sight of attention.

Differentiated HL-60 cells are capable of most PMN functions: chemotaxis, ingestion, respiratory burst activity, and bacterial killing, while undifferentiated HL-60 cells have no these functions.[22] But from our works, research on relationship among NADPH oxidase, ROS generation and cell survival in undifferentiated HL-60 cells, urged a new hypothesis: undifferentiated HL-60 cells also have the NADPH oxidase activity, ROS mediated generation by it plays essential role in HL-60 cells survival.

In the present study, cytochrome c reduction and horseradish peroxidase-mediated oxidation of phenol red assays show that NADPH oxidase activity is indeed exist in undifferentiated HL-60 cells and involved in the generation of ($O_2^{\cdot-}$) and H_2O_2 under stimulation with OZ or calcium ionophore. Moreover, the MTT assay indicates that the activity of NADPH oxidase is required for the survival of undifferentiated HL-60.

MATERIALS AND METHODS

Reagents

Calcium ionophore, diphenylene iodonium (DPI), superoxide dismutase (SOD), 5,8,11,14-eicosatetraynic acid (ETYA), indomethacin (IM), N^G-nitro-L-arginine methylester (L-NAME), apocynin (APO) and allopurinol (AP) were purchased from Sigma. Opsonified zymosan (OZ), RPMI 1640, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), sodium dodecyl sulfate (SDS), Dimethyl Sulfoxide (DMSO), acridine orange (AO), catalase (CAT), ethidium bromide (EB), cytochrome c, horseradish peroxidase and others were purchased from Sino-American Bio-technology Company (Zhengzhou, China).

Preparation of Cell Stimulants

Zymosan, an irritant usually contributing to produce ROS in phagocytes respiration burst,[13,23] was used to simulate ROS generation in our study. Zymosan was sterilized by boiling in Hanks' balanced salt solution (HBSS) 2.5 mg/ml for 20 min and centrifuged at 2000 rpm × 10 min. Washed zymosan was then opsonified by incubation with fresh human serum 2 ml for 30 min at 37°C and centrifuged at 2000 rpm × 10 min to remove the serum. Serum activation was repeated five times and the pellet was finally washed with HBSS. The OZ was resuspended in HBSS 10 mg/ml and stored at −70°C until use, the final concentration used in cell stimulation experiments was 1 mg/ml.[23] Calcium ionophore was dissolved in DMSO at a concentration of 1000 μM and stored at −20°C until use, and the final concentration was 10 μM.

Cell Culture

HL-60 cells, provided by Institute of Biophysics of Lanzhou University, were maintained in RPMI 1640 medium in 75 cm^2 flasks, supplemented with 12.5% heat-inactivated fetal bovine serum (FBS), 100 mg/l streptomycin and 100 U/ml penicillin, incubated at 37°C in a humidified atmosphere containing 5% CO_2 plus 95% air.

Superoxide Anion ($O_2^{\cdot-}$) and Hydrogen Peroxide (H_2O_2) Measurement

Cytochrome c reduction assay was used to determine the production of ($O_2^{\cdot-}$).[8] The washed HL-60 cells were seeded in 96-well plates (5×10^4 cells in 210 μl of medium per well). After incubation at 37°C for 30 min, 50 nM cytochrome c and 1 mg/ml OZ or calcium ionophore were added to start the reaction, in control experiment, 200 U/ml superoxide dismutase (SOD) were added to eliminate the extra reduction of cytochrome c induced by other ROS except ($O_2^{\cdot-}$). The final volume of the sample was 250 μl. In some experiments, cells were preincubated for 4 h with or without 10 μM DPI, 1 mM APO, two inhibitor of NADPH oxidase, 10 μM ETYA, an inhibitor of lipoxygenases, 10 μM IM, an inhibitor of cyclooxygenase, 10 μM L-NAME, an inhibitor of nitric oxide synthase (NOS), or 10 μM AP, an inhibitor of xanthine oxidase, prior to the addition of 1 mg/ml OZ. These concentrations were selected based on previous reported inhibitory activity.[24,25] Kinetic or static absorbance of reductive cytochrome c at 550 nm was measured with

a microplate spectrophotometer reader (Multiskan Ascent, Labsystems), the absorbance was converted to equivalent ($O_2^{\cdot-}$) production using the molar extinction coefficient for cytochrome c (2.1×10^4).[8]

Horseradish peroxidase (HRP)-mediated oxidation of phenol red method was used to determine the production of H_2O_2.[26] HL-60 cells were seeded in 96-well plates (5×10^4 cells in 210 μl of medium per well). After incubation at 37°C for 30 min, reaction was started by 1 mg/ml opsonified zymosan or calcium ionophore. The final volume of the sample was 250 μl containing 0.56 mM phenol red, 17 U/ml HRP. Kinetic or static absorbance at 620 nm was measured with a microplate spectrophotometer reader (Multiskan Ascent, Labsystems), the production of H_2O_2 were calculated using H_2O_2 standard curve that was linear in the range of 0.5–20 μM. In some experiments, cells were preincubated for 4 h prior to the addition of 1 mg/ml OZ, with or without DPI, APO, ETYA, IM, L-NAME, or AP. All the concentration were same with their in cytochrome c reduction assay.

Assay of Cell Survival

The washed HL-60 cells were seeded in 96-well plates at a density of 5×10^4 cells in 240 μl of RPMI 1640 medium containing 0.5% FBS per well, after serum-starved for 7 h, cells were treated for 12 h with RPMI 1640 medium containing 10% FBS in the presence or absence of six inhibitors or 100 U/ml antioxide enzyme, SOD, CAT, or SOD + CAT, all the concentrations of inhibitors were same with those in ROS measurement. Then 0.5 mg/ml MTT was added followed by incubation at 37°C for 4 h in a CO_2 incubator. The MTT formazan product was released from cells by addition of 10% SDS. After incubating over night at 37°C, insoluble crystals were completely dissolved, and absorbance at 620 nm was measured using a microplate spectrophotometer reader (Multiskan Ascent, Labsystems). In some experiments, the effect of different concentration of six inhibitors on cells survival was performed using same method as described above.

Cell Morphology Assay

Fluorescence double staining with AO and EB was performed to assessment HL-60 cells morphological changes.[27,28] The HL-60 cells were cultured with 20 μM DPI or 1 mM APO for 6, 12 and 24 h, respectively, then stained with combined dye, including 96 μl D-Hanks buffer, 2 μl AO, and 2 μl EB, the stained cells were observed under fluorescence microscope (Olympus).

Cell Rescue Assay

For the purpose to clarify whether the cell death was caused by the decrease of ROS, exogenous H_2O_2 was periodically supplied to rescue the cells. In brief, the washed HL-60 cells were seeded in 96-well plates at a density of 5×10^4 cells in 240 μl RPMI 1640 medium containing 0.5% FBS, after serum-starved for 7 h, the cells were treated for 12 h with 10% FBS in the presence or absence of 10, 20μM DPI or 1, 2 mM APO, meanwhile, exogenous H_2O_2 01, 0.2 μM was supplied in the intervals of every 4 h. In some experiments, the 1, 2 U/ml catalase or inactive catalase was added following the first time of supplement of H_2O_2, inactive catalase was denatured by treatment with 3-amino-1, 2, 4-triazole as previously described,[29] then MTT assay was performed as described above.

Statistical Analyses

Results were presented as mean ± SEM, and Student's *t*-test was used for statistical analysis, with p values less than 0.05 as indicators of statistical significance.

RESULTS

Calcium Ionophore or OZ Promote the Generation of ($O_2^{\cdot-}$) and H_2O_2 in HL-60 Cells

The average output of ($O_2^{\cdot-}$) in resting HL-60 cells was 0.585 nmol/10^4 cells ($n = 6$). After 1 h stimulated with 10 μM calcium ionophore, the output of ($O_2^{\cdot-}$) was increased to 1.344 nmol/10^4 cells ($n = 6$), while 1 mg/ml OZ increased the output of ($O_2^{\cdot-}$) to 1.86 nmol/10^4 cells ($n = 6$), these means about 2.3, 3.2 times of resting HL-60 cells, respectively (Fig. 1A).

The average output of H_2O_2 in resting HL-60 cells was 0.903 μM, after 1 h stimulation, 10μM calcium ionophore increased the output of H_2O_2 to 2.111 μM, while 1 mg/ml OZ increased to 2.258 μM, these means about 2.3, 2.5 times of resting HL-60 cells, respectively (Fig. 1B). These results indicated that the output of ($O_2^{\cdot-}$) and H_2O_2 in undifferentiated HL-60 cells could be increased by the specific stimulant of NADPH oxidase obviously.

NADPH Oxidase Involved in the Generation of ($O_2^{\cdot-}$) and H_2O_2 in HL-60 Cells

Since lots of enzymes contribute to the generation of ROS *in vivo*, to identify which enzyme should be responsible for the generation, effects of various enzyme inhibitors were examined. After HL-60 cells preincubation with six inhibitors for 4 h, respectively, DPI or APO, two inhibitors of NADPH oxidase, significantly reduced the generation of ($O_2^{\cdot-}$) in

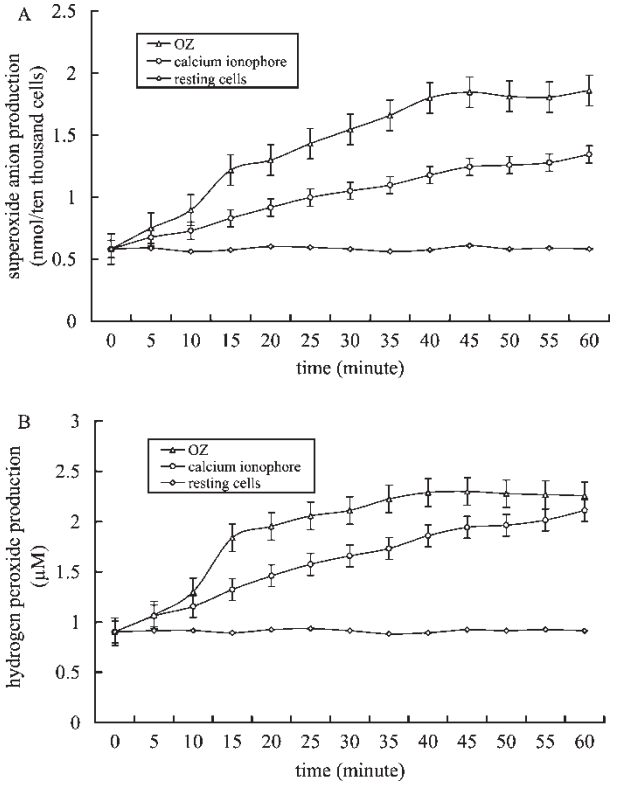

FIGURE 1 Superoxide anion and hydrogen peroxide production in HL-60 cells. Compared with the resting cells, ($O_2^{\cdot-}$) (A) and H_2O_2 (B) generation was enhanced by the 10 μM stimulant of calcium ionophore or 1 mg/ml OZ in HL-60. The value was expressed as mean ± SEM of four independent experiments. $^*P < 0.05$, $^{**}P < 0.01$ vs. the control, $n = 6$.

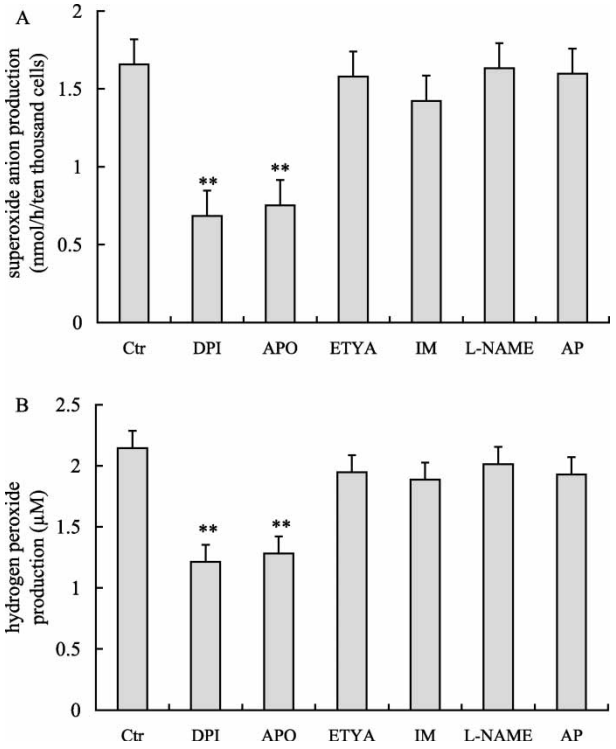

FIGURE 2 Effects of enzymatic inhibitors on the generation of Superoxide anion (A) and hydrogen peroxide (B) in undifferentiated HL-60 cells were measured in the absence or presence of 10 μM DPI, ETYA, IM, L-NAME, AP, or 1 mM APO, respectively. The value was expressed as mean ± SEM of four independent experiments. $^*P < 0.05$, $^{**}P < 0.01$ vs. the control, $n = 6$.

OZ-treated HL-60 cells by approximately 59% and 55%, respectively (Fig. 2A). Similarly, DPI or APO reduced the H_2O_2 generation in OZ-treated HL-60 cells at approximately 44% and 40%, respectively. However, ETYA, IM, L-NAME, or AP showed hardly effects on ($O_2^{\cdot-}$) or H_2O_2 generation (Fig. 2B). These results suggested that although other enzymes, including cyclooxygenases and lipoxygenases, may also involve in the generation of ROS in HL-60 cells, NADPH oxidase was obviously the most important source of both ($O_2^{\cdot-}$) and H_2O_2 in undifferentiated HL-60 cells than others.

ROS was Required for the Survival of HL-60 Cells; DPI or APO Induced HL-60 Cells Death

As shown in Fig. 3A, pretreatment of HL-60 cells with 100 U/ml SOD, CAT, or SOD + CAT for 12 h resulted in an obviously reduction of cells survival, the absorbency decreased about 57%, 58%, and 62%, respectively. Also, 10 μM DPI or 1 mM APO resulted in a dramatic reduction of cells survival; the absorbency decreased about 62% and 61%, respectively. Whereas pretreatment of HL-60 cells with 10 μM ETYA, IM, L-NAME or AP had no significant effect on cells survival. Moreover, the survival of HL-60 cells was suppressed significantly by 20–100 U/ml SOD, CAT, SOD + CAT (Fig. 3B), or DPI 10–100 μM (Fig. 3C) or APO 0.5–10 mM (Fig. 3D) in a concentration-dependent manner, but no obvious effects were observed in ETYA, IM, L-NAME and AP treated cells (data not shown).

Furthermore, AO/EB double staining assay was employed to estimate the pattern of HL-60 cells death induced by DPI or APO. AO can penetrate into integrity living cell membrane, and stain health cell nucleus as yellow with a few orange (the stained DNA), meanwhile, RNA in cytoplasm exhibits orange color under fluorescence microscope. While EB can only penetrate into dead cells, including apoptosis and necrosis, with damaged membrane permeability, and stain nucleus and cytoplasm as orange fluorescence. Apoptosis cells exhibit apoptotic body, nucleus condensation and nucleus fragment, while necrosis cells always keeping integrity nucleus.[27,28,30] As shown in Fig. 4A, for DPI or APO free HL-60 cells, abundant RNA in cytoplasm was stained as orange color, meanwhile, nucleus was stained as yellow by AO, which is a typical status of normal living HL-60 cells. After HL-60 cells were treated by 20 μM DPI (Fig. 4B) or 1 mM APO (Fig. 4E) about 6 h, respectively, faded orange color implied

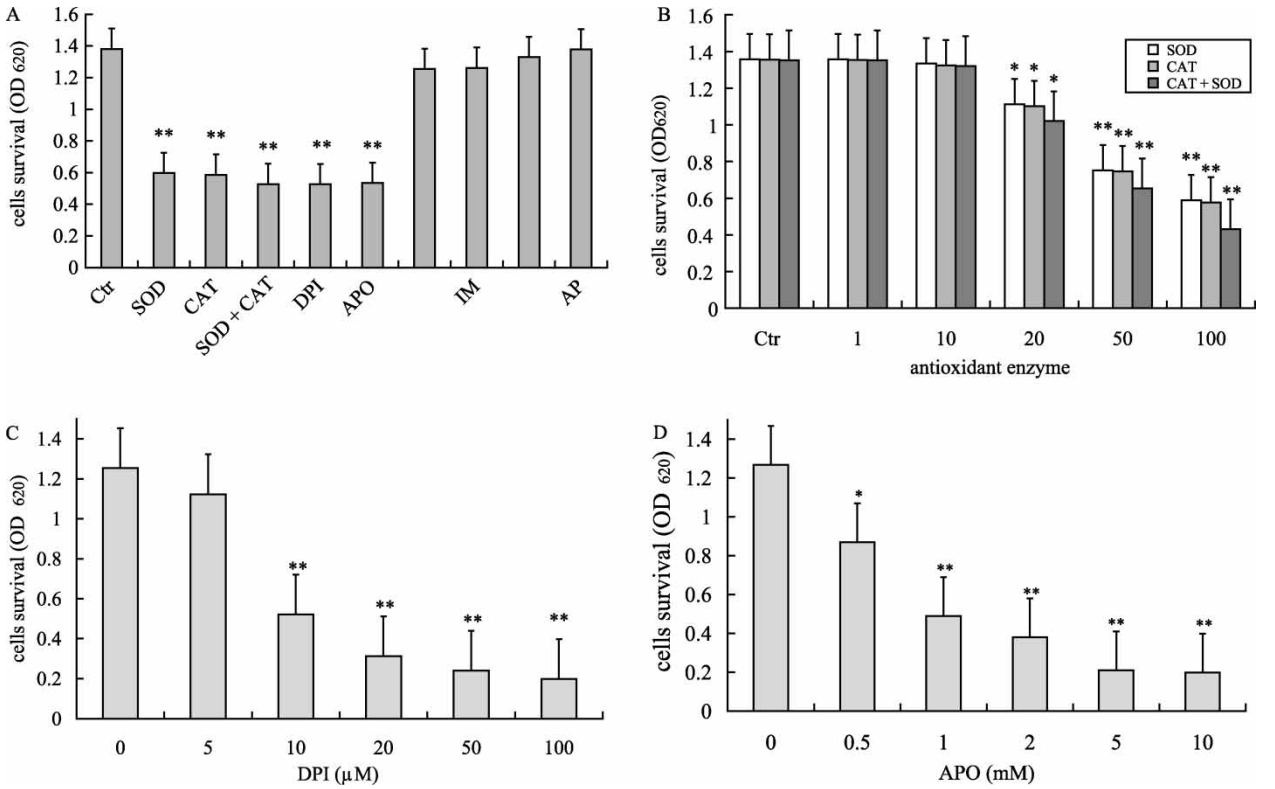

FIGURE 3 Effects of enzymatic inhibitors or antioxide enzyme on the survival of undifferentiated HL-60 Cells. HL-60 cells treated for 12 h with 10% FBS in the presence or absence of six inhibitors, or antioxide enzyme, as described under methods. (A) MTT assay of six inhibitors or antioxide enzyme on HL-60 cells survival, (B) the action of different concentration of antioxide enzyme on HL-60 cells survival, (C) the action of different concentration of DPI on HL-60 cells survival, (D) the action of different concentration of APO on HL-60 cells survival. The value was expressed as mean ± SEM from four (control) or six independent experiment. *$P < 0.05$, **$P < 0.01$ vs. the control, $n = 6$.

the reduction of RNA, which indicated the transcription from DNA to RNA was affected by unknown pathway. After treatment about 12 h by DPI (Fig. 4C) or APO (Fig. 4F), cytoplasm became green, and nucleus changed into bottle green, which indicated gene expression had been halted mostly. After 24 h treatment with DPI (Fig. 4D) or APO (Fig. 4G), cytoplasm or nucleus stained as brown or red color, respectively, indicating cell was dead, but the nucleus was still integrity, and no apoptotic body, nucleus condensation and nucleus fragment were examined. These results suggested that cells were prone to necrosis than apoptosis under these conditions as indicated by the morphological change. In addition, no obvious morphological changes were observed in 4 h treatment with DPI or APO, this implied that 4 h treatment was not enough to affect the HL-60 cells normal function.

Exogenous H_2O_2 can Rescue the HL-60 Cells from Death Caused by DPI or APO

DPI 10 or 20 μM treated for 12 h resulted in a significant reduction of HL-60 cells survival; the absorbency decreased about 58% and 72%, respectively (Fig. 5A and B). However, periodical supplement of 0.1 or 0.2 μM H_2O_2 obviously increased the HL-60 cells survival to 2.2 and 2.5 times, respectively. On the opposite side, catalase, but not inactive catalase, was able to affect the rescue function of H_2O_2. APO, another inhibitor of NADPH oxidase, had similar affect on cells survival, the absorbency of 1 or 2 mM APO treated cells decreased about 58% and 67%, respectively. However, periodical supplement of 0.1 or 0.2 μM H_2O_2 obviously increased the HL-60 cells survival to 2.3 and 2.8 times, respectively. In addition, catalase was able to eliminate the supplement H_2O_2 and affect the rescue function of H_2O_2 (Fig. 5C and D). However, neither catalase 1 or 2 U/ml alone, nor H_2O_2 0.1 or 0.2 μM alone, exhibited effect on cells survival (Figs. 3 and 5).

DISCUSSION

Some previous researches confirmed that NADPH oxidase activity only exists in differentiated HL-60 cells and plays microbicial function.[31,32] In addition, some researches implied that undifferentiated HL-60 cells also had the NADPH oxidase activity. Newburger (1984) found that oxidase activity had some dramatic variety during differentiation of HL-60 cells.[32] If the later is reliable, NADPH

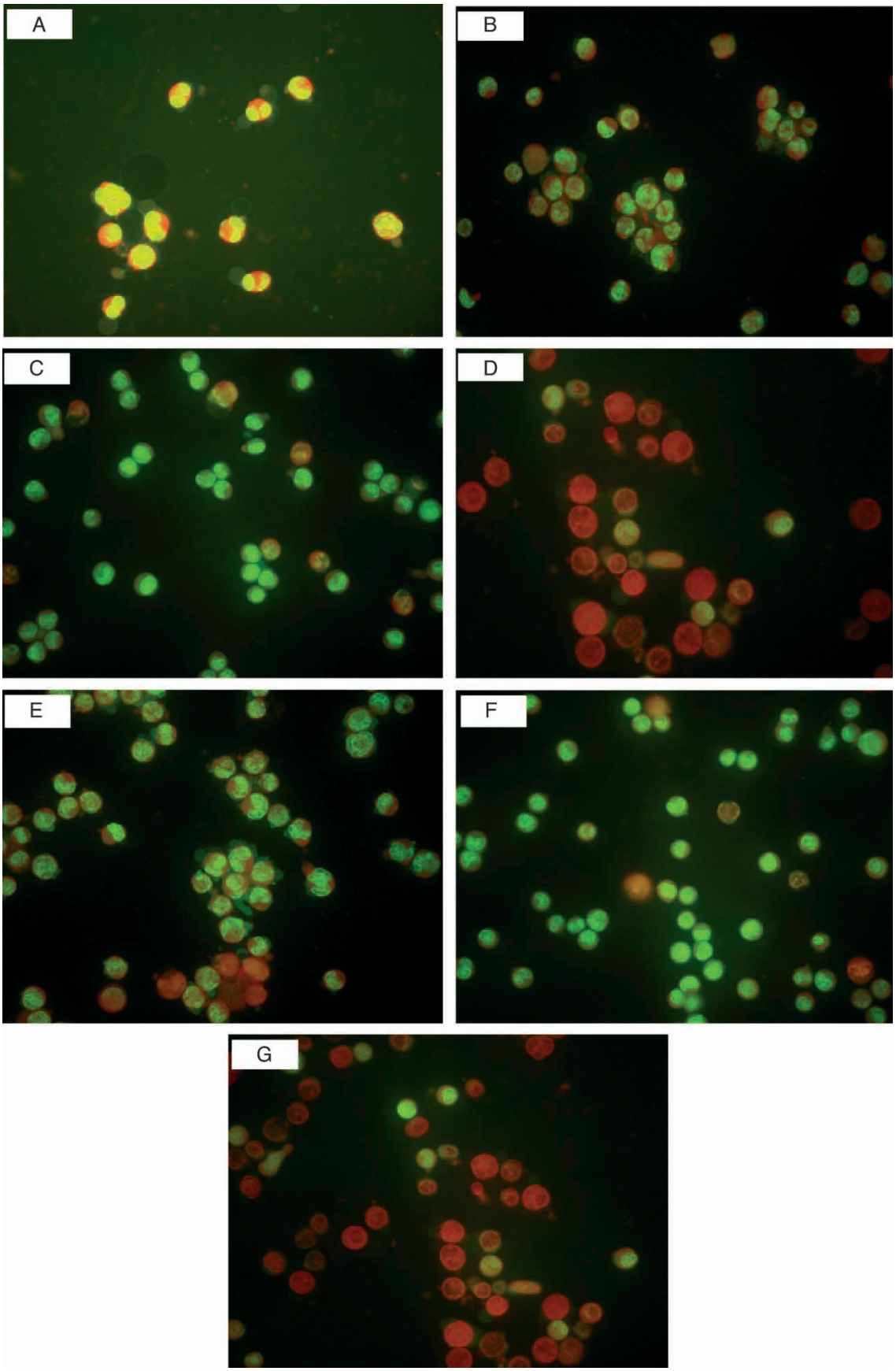

FIGURE 4 Morphological changes of undifferentiated HL-60 cells stained by AO/EB. HL-60 cells were treated by 20 μM DPI or 1 mM APO: (A) normal HL-60 cells, RNA in cytoplasm was stained as orange color, while nucleus was stained as equality yellow. (B) and (E), treated for 6 h with DPI or APO respectively; (C) and (F), treated for 12 h with DPI or APO respectively; (D) and (G), treated for 24 h with DPI or APO, respectively.

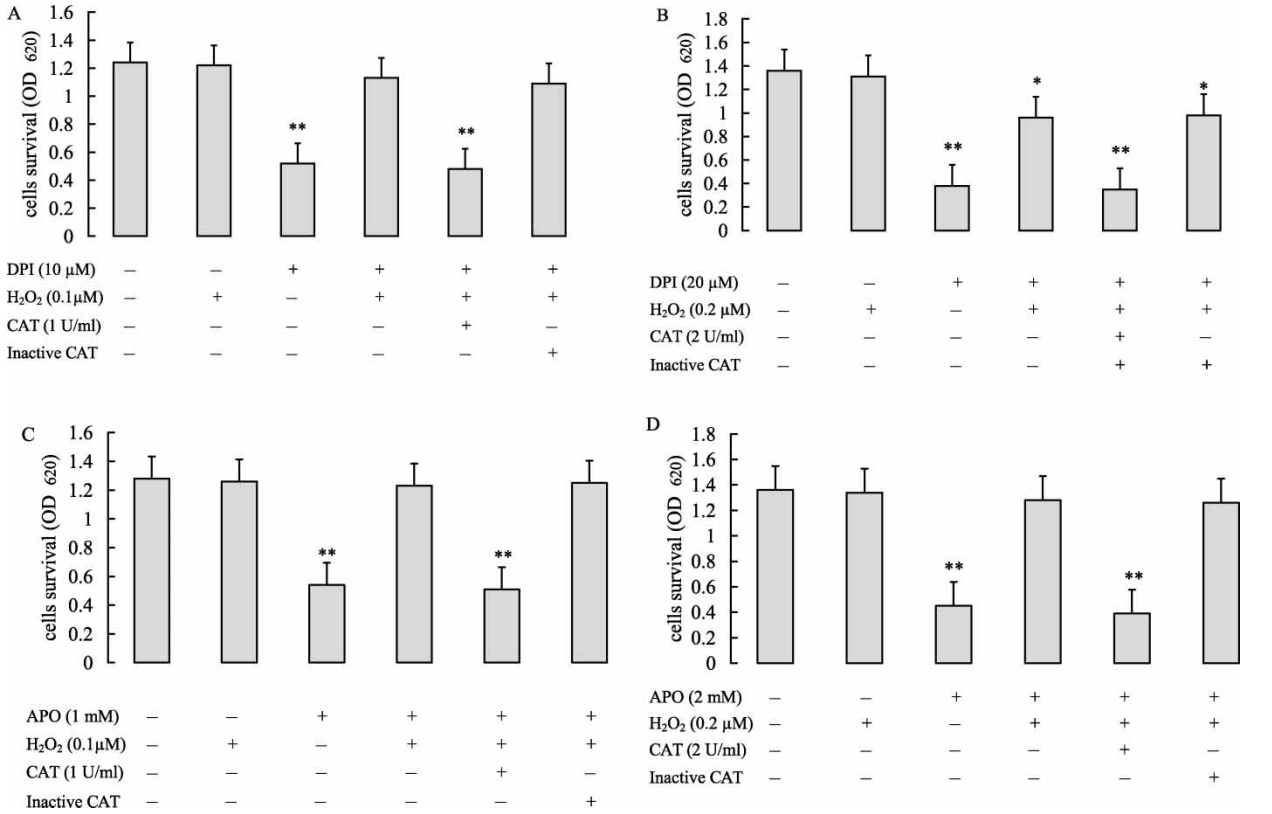

FIGURE 5 H_2O_2 rescue from death of undifferentiated HL-60 cells caused by DPI and APO: (A) 10 μM DPI treated HL-60 cells; (B) 20 μM DPI treated HL-60 cells; (C) 1 mM APO treated HL-60 cells; (D) 2 mM APO treated HL-60 cells. +, − represented adding or not adding the agent, respectively. The value was expressed as mean ± SEM from four independent experiments. *$P < 0.05$, **$P < 0.01$ vs. the control, $n = 6$.

oxidase in undifferentiated HL-60 cells may play some important role on cells physiological functions. But no reports indicate that NADPH oxidase in undifferentiated HL-60 cells had microbicidal effect. Previous studies have indicated that low concentration of ROS can promote cell proliferation and participate in cell differentiation.[10,15] So, whether NADPH oxidase in undifferentiated HL-60 cells mediated generation of ROS is relative to cells survival is worthy of being explored.

In recent years, more and more studies implicate that NADPH oxidase is believed to be one main source of *in vivo* ROS related to signal transfer and gene regulation under stimulation of a variety of growth factors, which has become one of the most hot topic in free radical biology.[16–18,33] So we may hypothesis that phagocyte may also have the NADPH oxidase activity, and ROS mediated produce by it may relate to cell signal transfer and gene regulation which is similar to non-phagocytic cells described previously,[8–18] i.e. the activity of NADPH oxidase has two face in phagocyte (at least in premature phagocyte), one is microbicidal effect, another is concerned with cell normal survival. But as far as today, the second face has been paid little attention.

This study, to our knowledge, is the first report that the inhibitors of NADPH oxidase reduced the ($O_2^{\cdot-}$) and H_2O_2 production significantly in undifferentiated HL-60 cells. Furthermore DPI or APO, as well as SOD or CAT can induce cells death, the supplement of exogenous H_2O_2 can rescue the cells death, whereas catalase can depress this rescue. These suggested that NADPH oxidase is employed as the main source of ROS generation, which are needed by premature phagocyte survival. Considering NADPH oxidase mediated generation of ROS is related with non-phagocytic cells growth, these new discoveries may imply that NADPH oxidase also act as same role in phagocyte, at least in premature phagocyte, the decreasing of ROS product in undifferentiated HL-60 cells induced by NADPH oxidase inhibitors was a premonitor and responsible for the cells death. Taken together, we conclude that NADPH oxidase mediated ROS is a critical component of cells survival in HL-60 cells.

DPI and APO are known to inhibit NADPH oxidase and decrease the ROS production,[3,9,13,24,25] but their specificity should be considered, DPI is also capable of inhibiting additional flavin-dependent enzymes, including nitric oxide synthase, NADPH-cytochrome P450 reductase, and NADH-ubiquinone oxidoreductase,[34] moreover, it has been suggested

that DPI can attack the iron–sulfur clusters in mitochondria complex I[35] or trigger the efflux of glutathione from cultured cells.[36] APO can react with ROS and peroxide.[37] So DPI or APO induced HL-60 cells death may have some complex mechanism except ROS decrease, for example, the exogenous H_2O_2 only can reverse 71% survival of 20 μM DPI treated cells of control. However, in our experiment, H_2O_2 can rescue the cells from death was a strong evidence which indicated that ROS decreasing was more important than others.

Over-activation of NADPH oxidase may lead to excessive ROS generation, hence the oxidative damage of biological molecules and resulted various diseases. Tumor can autocrine various of growth factors which stimulate the NADPH oxidase and produce ROS, then excessive ROS promote the cell proliferation, so NADPH oxidase may be a potential therapeutic target for tumor and other relative disease.

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