Nitric oxide levels during erythroid differentiation in K562 cell line

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Nitric oxide (NO) is endogenous mediator of numerous physiological processes that range from regulation of cardiovascular function and neurotransmission to antipathogenic and tumoricidal responses. This study was designed to investigate the possible role of NO during erythroid differentiation in K562 erythroleukemia cells. The chronic myelogenous leukemia (K562) cell line can be triggered in culture to differentiate along the erythrocytic pathway, in response to a variety of stimulatory cell line can be triggered in culture to differentiate along the erythrocytic pathway, in response to a variety of stimulatory agents. In this study, K562 cells were induced to synthesize hemoglobin by hemin. We investigated NOx (nitrate+nitrite) levels in uninduced (control) and hemin-induced K562 cell lysates during erythroid differentiation. Our results showed that NO levels decreased significantly on fourth and sixth day both in hemin-induced and control cells; the decrease was, however, more in hemin-induced group than in control group.

Keywords: Nitric oxide, Erythroid differentiation, Hemin, K562 cell line

Nitric oxide (NO), an important cellular messenger, has been linked to both neurodegenerative and neuroprotective actions. It is involved in a variety of important biological functions in the systems such as cardiovascular, central and peripheral nervous, reproductive, and immune systems. It is synthesized from L-arginine by a family of three nitric oxide synthase (NOS) proteins—neuronal (nNOS), endothelial (eNOS), and inducible (iNOS) and has short half-life (a few seconds or less). It combines with superoxide radical (O2•−) to form a powerful peroxinitrate (ONOO−) oxidant. It is also known to react with heme molecules, Fe-S compounds and thiol groups; it rapidly reacts with oxyhemoglobin to form NO3 (nitrate) and met-hemoglobin.

Many cell types produce nitric oxide free radical. The generation of NO by endothelial cells and mononuclear phagocytes has been well characterized. These cells are known to regulate hematopoietic cell differentiation in the bone marrow by secreting a variety of interleukins, colony stimulating factors, and other cytokines. The erythroleukemia cell line K562, was first established from the cells of a patient in the blastic phase of chronic myelogenous leukemia. K562 cells acquire the capability to synthesize hemoglobin (Hb), in response to hemin, sodium butyrate, dimethyl sulfoxide (DMSO) etc and have been used as a model for erythroid differentiation. The viability of K562 cells in the presence of hemin (20 µM) and alone was greater than 95% as assessed by the trypan blue dye exclusion test.

Materials and Methods

Hemin, RPMI 1640 and penicillin/streptomycin solutions, griess reagents were purchased from Sigma, St Louis, MO, USA. Fetal calf serum (FCS) was from Harlan Sera-Lab, Leicestershire, UK.

Cell culture

K562 cells obtained from ATCC (MD, USA) were grown in RPMI 1640 medium supplemented with 10% FCS, penicillin-streptomycin (100 IU/ml), amphotericin B (25 µg/ml) and L-glutamine (2 mM) at 37°C in humidified air containing 5% CO2.

Induction of hemoglobin synthesis

Cells seeded at 1×105 cells/ml were induced to differentiate with 20 µM hemin (day “0”) and were harvested from 1st to 6th day. At this time, benzidine staining was carried out to assess the extent of differentiation. The viability of K562 cells in the presence of hemin (20 µM) and alone was greater than 95% as assessed by the trypan blue dye exclusion test.

Measurement of erythroid differentiation of K562 cells

Erythroid differentiation was scored by staining the cells for Hb synthesis with benzidine reagent. Briefly, a benzidine dihydrochloride stock solution, 0.2% (w/v) was prepared in 0.5 M acetic acid and H2O2...
Preparation of K562 lysates
K562 cells were collected by low speed (400 g) centrifugation and washed two times with PBS, pH 7.5. The resulting pellets were resuspended in hypotonic buffer (TKM; 25 mM Tris-HCl, pH 7.4, 5 mM KCl, and 1 mM MgCl₂) and resuspended in 1 ml of TKM containing 1% Nonidet P-40 with 20 strokes in a Dounce homogenizer and the homogenates (cell lysates) were stored at -70°C. (0.14%) was added immediately before use. The 1 ml of cell suspension was washed, resuspended with phosphate-buffered saline (PBS) and mixed with the benzidine solution in a 1:0.1 (v/v) ratio. After 10 min, the cells were counted in a hemocytometer and blue cells were considered positive for Hb².➗

Nitrate and nitrite analysis
NO₂⁻ and NO₃⁻ levels were measured by the Griess reaction. Briefly, cell lysates were deproteinized prior to assay. The lysates were added to 96% cold ethanol (1/2 v/v) and then vortexed for 5 min. After incubation for 30 min at 4°C, the mixture was centrifuged at 14,000 rpm for 5 min and the supernatants were used for assay. Analyses were done in micro titer plate wells. After loading the plate with samples (100 µl), vanadium chloride (VCl₃) was added to each well (100 µl), rapidly followed by addition of the Griess reagents, sulfanilamide (50 µl), N-(1-naphthyl) ethylenediamine dihydrochloride (50 µl). Nitrite was measured in a similar manner, except that nitrite standards were only exposed to Griess reagent. The absorbance was measured at 540 nm using micro plate reader (Bio-Rad Lab, Hercules, CA, USA). Calibrations were performed with known amounts of nitrate and nitrite standard solutions (1-100 µM). NOx levels were expressed as nitrite + nitrate in µM. (1-100 µM). NOx levels were expressed as nitrite + nitrate in µM. (1-100 µM). NOx levels were expressed as nitrite + nitrate in µM.  

Statistical analysis
SPSS for Windows 12.0 was used for statistical evaluations. Data were statistically analyzed using one-way ANOVA, followed by Student’s t test. A value of p < 0.05 was considered to be statistically significant. Values were expressed as mean ± SD.

Results and Discussion
In the present study, erythroid differentiation of human erythroleukemia (K562) cells was induced with hemin. Table 1 shows the NOx levels and Hb% in control and hemin-induced K562 cells. No significant differences were observed in NOx levels in cell lysates for control and hemin-induced groups on 1st day. However, on 4th day, NOx levels of hemin-induced group were significantly decreased, as compared to the control (p<0.002). Similar trend was observed on the 6th day (p<0.001). NOx levels also decreased in control groups on day 4 and 6, compared with day 1. A similar pattern of decrease of NOx levels was observed in the two groups. Hb synthesis shows the differentiation of K562 cells. In hemin-induced group, there was an increase in Hb synthesis in the cells; Hb levels were 25%, 50%, and 63% on 1st, 4th, and 6th day, respectively, whereas Hb levels in control group were same (5%) on all the days. The NO mediates a diverse array of physiologic and pathologic processes. It may act as modulator in cellular proliferation and differentiation. The mechanism of growth and differentiation of erythroid stem cells is a highly regulated complex process. Human erythroleukemia cell line K562 is analogous to erythroid stem cells, and is often used to investigate these processes. Treatment of K562 cells with hemin leads to erythroid differentiation, as evidenced by inhibition of cell proliferation and induction of Hb synthesis.

The molecular mechanisms involved in erythroid differentiation processes are quite different, depending on the inducer. NO is a reactive molecule that has multiple biological effects. It induces monocyctic differentiation of the human myeloid leukemia cell line HL-60 and alters gene expression in these cells. It suppresses erythroid-specific gene expression or inhibits Hb production at the aminolevulinic acid synthase step. It also decreases heme synthesis and inhibits erythropoiesis.

![Table 1—NOx levels in control and hemin-induced K562 cells](image-url)
in rats\textsuperscript{22}. The role of NO was also suggested in anemia and lowered blood Hb concentrations that are associated with chronic infections, such as tuberculosis or parasitic diseases. Hb expression was also suppressed by heterologous production of NOS in the K562 erythroleukemia cell line\textsuperscript{20}. Also, NO donors, such as sodium nitroprusside (SNP) and S-nitrosogluthatione inhibit the chemically-induced differentiation of K562 cells. In contrast, hemin-induced erythroid differentiation of K562 cells is not affected by the presence of NO donors\textsuperscript{19}.

Our results show the similar pattern of decrease of NOx levels in two groups; the decrease was more in hemin-induced K562 cells at 4\textsuperscript{th} and 6\textsuperscript{th} day than in control group. NO may react with Hb generated from erythroid differentiation and thus NOx level may be decreased. NO has been shown to modulate the growth of erythroid and myeloid colonies. It can play multiple physiologic and pathophysiologic roles, affecting bone marrow cell growth and differentiation\textsuperscript{23}. Further studies are needed to better understand the effect of NO on cell differentiation.

References
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