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Inhibitory Effect of Nitric Oxide on Chemically Induced Differentiation of Human Leukemic K562 Cells

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ABSTRACT. The effect of nitric oxide (NO) was investigated in the human K562 cell line during chemically induced erythroid differentiation. Butyric acid (BA) and the anthracycline antitumour drugs aclarubicin (ACLA) and doxorubicin (DOX) were used as differentiating agents. In all cases, cell hemoglobinization was dose dependently inhibited by NO donors such as sodium nitroprusside (SNP). A 50% inhibition of cell differentiation was obtained with 25 μM SNP, which generated less than 2 μM nitrite in 3-day culture media. Increasing SNP concentrations led to higher nitrite accumulation (up to 12 μM with 1 mM SNP) and total inhibition of cell hemoglobinization, but did not have a significant effect on cell proliferation. As shown by Northern blotting, high concentrations of SNP (1 mM) reduced the expression of γ-globin and porphobilinogen deaminase, but did not change GATA-1 and NF-E2 mRNA levels in ACLA- and BA-treated cells. In contrast, hemin-induced erythroid differentiation was not affected by the presence of NO donors. Altogether, these results show that NO is able to inhibit cell differentiation induced by some (ACLA, DOX, BA), but not all (hemin), agents. The inhibitory effect of NO seems to take place downstream of the regulation of erythroid gene expression. BIOCHEM PHARMACOL **58**;5:773–778, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. anthracyclines; butyric acid; erythroid differentiation; hemin; nitric oxide

Erythroid differentiation of the human K562 leukemic cell line can be achieved by exposure to several pharmacological agents including hemin [1], BA† [2], and anthracyclines such as ACLA and DOX [3]. The molecular mechanisms involved in the differentiation process are quite different depending on the inducer. The transcriptional activation of the γ-globin gene has been demonstrated in BA- and ACLA-induced K562 cells [4, 5]. This has been related to the enhancement of DNA-binding activity and the expression of GATA-1 and NF-E2 transcription factors [6–9]. In addition, the heme synthesis pathway enzymes PBGD [5, 8], erythroid δ-aminolevulinate dehydratase [10, 11], and eALAS [12] were also shown to be up-regulated at the transcriptional level in response to ACLA and BA. In contrast, DOX- and hemin-induced differentiation did not involve the overexpression of GATA-1 and NF-E2 transcription factors [5, 7]. Moreover, the transcriptional activity of erythroid promoters, which is enhanced by BA and ACLA, is not affected by DOX and hemin [13].

Nitric oxide is synthesized by NO synthases in endothelial and inflammatory-activated cells [14]. NO has been demonstrated to modulate the RNA binding of IRP-1 and IRP-2 iron responsive element (IRE) binding proteins [15, 16], which are responsible for the posttranscriptional control of IRE-containing mRNAs related to cellular iron homeostasis (e.g. ferritin, transferrin receptor, and eALAS [16]). Therefore, the study of NO effects on erythroid-specific genes has been mainly restricted to iron homeostasis-related genes [17–19]. In addition, NO may disturb cell growth and differentiation by regulating the activity of cellular targets involved in signal transduction, ATP, and oxygen production. Indeed, NO activates the soluble guanylate cyclase and inhibits iron-cluster enzymes including aconitase and mitochondrial complex I and II enzymes [20].

In this work, the effect of NO on chemically induced erythroid differentiation was investigated in K562 cells using NO donors such as SNP, SIN-1, and SNAP. The release of NO from NO donors was shown to inhibit the differentiation of anthracycline- and BA-treated K562 cells with an IC_{50} of 25 μM for SNP and less than 100 μM for SIN-1 and SNAP. Higher doses of SNP were needed to decrease the expression of erythroid mRNAs. In contrast, hemin-induced differentiation was not affected by NO.

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† *Abbreviations:* ACLA, aclarubicin; BA, butyric acid; DOX, doxorubicin; eALAS, erythroid δ-aminolevulinate synthase; NO, nitric oxide; PBGD, porphobilinogen deaminase; SIN-1, 3-morpholinosydnonimine; SNAP, *S*-nitroso-*N*-acetyl-penicillamine; and SNP, sodium nitroprusside.

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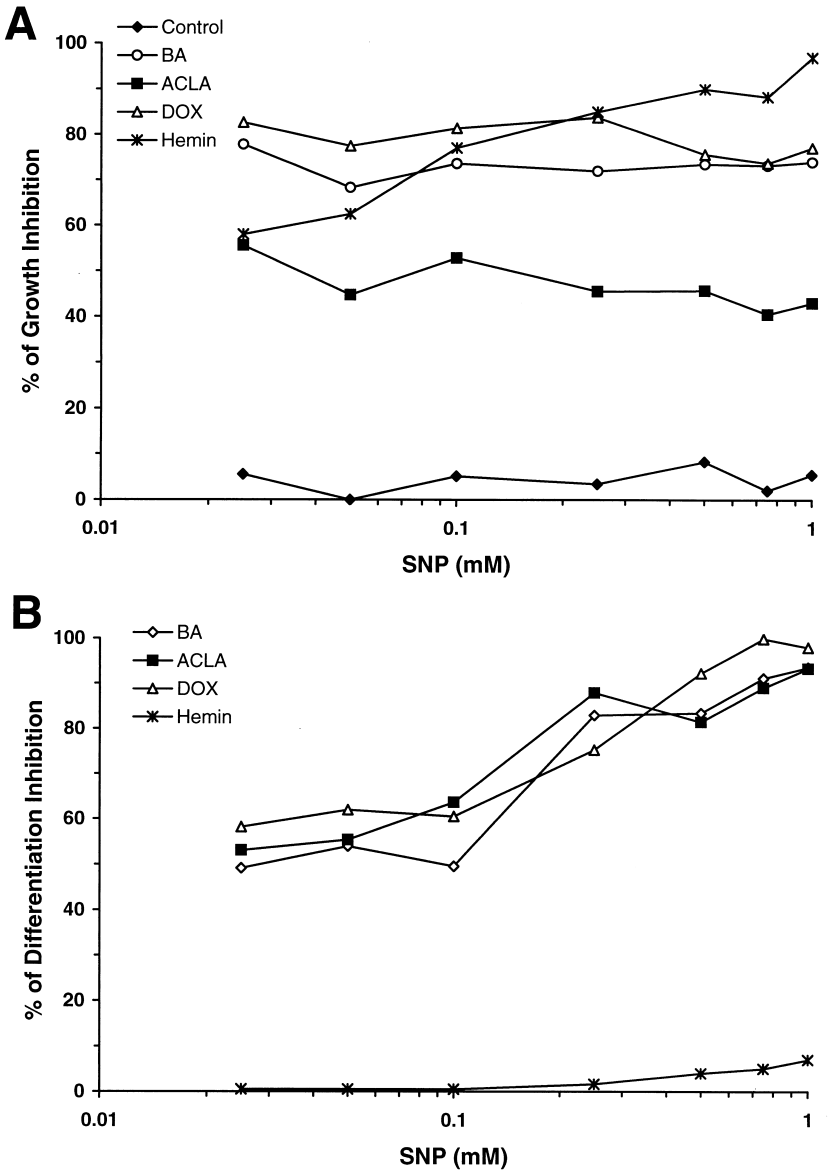

FIG. 1. Effect of SNP treatment on cell growth and differentiation. Cells were incubated for 3 days in the presence of the indicated concentrations of SNP (0.025–1 mM) and either BA 0.5 mM, ACLA 20 nM, DOX 40 nM, or hemin 30 μM. The percentage of cell growth inhibition (A) and the percentage of differentiation inhibition (B) were determined as described in Materials and Methods. Data are the means of at least three independent experiments and SD were below 10%.

MATERIALS AND METHODS

Chemicals

All chemical reagents were purchased from Sigma unless otherwise stated and were of reagent grade or molecular biology grade when necessary. Anthracyclines, BA, and hemin were reconstituted as previously described, [7, 8, 13] respectively. Potassium ferricyanide, SNP, and SIN-1 were prepared as 0.1 M stock solutions in PBS. SNAP was solubilized at 0.1 M in dimethyl sulfoxide immediately before use.

Cell Culture and Differentiation

K562 cells were cultured in RPMI-1640 medium (Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies) and 2 mM L-glutamine in a 5% CO_2 humidified atmosphere. Cells were treated with either 0.5 mM BA, 20 nM ACLA, 40 nM DOX, or 30 μM hemin at the beginning of the exponential growth phase. Cell growth and viability were assessed by direct counting of trypan blue dye-excluding cells. The percentage of cell growth inhibition was then calculated from: $\{[(C_n - C_0) - (T_n - T_0)]/(C_n - C_0) \times 100\}$, where C_n, C_0, T_n, T_0 represent the numbers of control (C) or treated (T) cells/mL at days 0 and n, respectively. The percentage of hemoglobin-producing cells was determined by a benzidine staining method as previously described [7], and the percentage of differentiation inhibition was calculated with respect to control values obtained in the absence of NO donor.

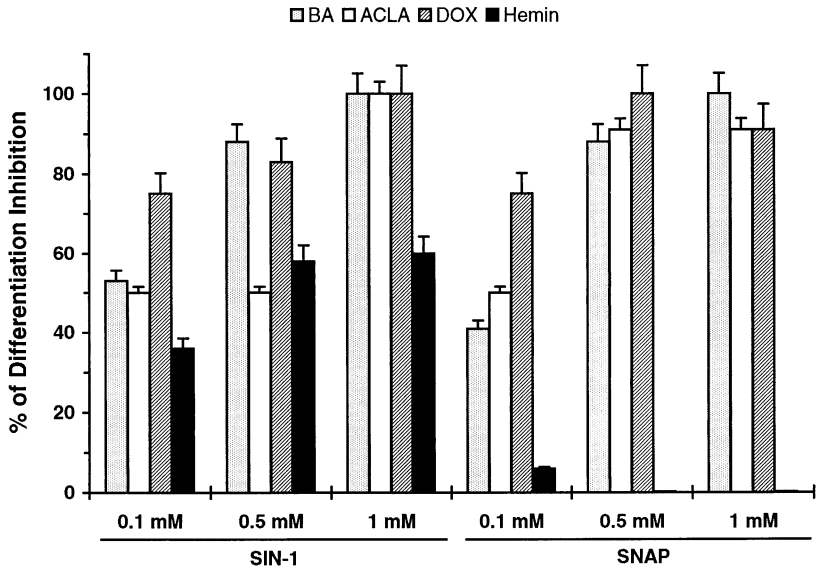

FIG. 2. Inhibitory effect of SIN-1 and SNAP on differentiation. Cells were incubated for 3 days in the presence of the indicated concentrations of SIN-1 or SNAP and either BA 0.5 mM, ACLA 20 nM, DOX 40 nM, or hemin 30 μM. The percentage of differentiation inhibition was determined as described in Materials and Methods. Data are the means ± SD of three independent experiments.

Analysis of Gene Expression

Erythroid RNA expression was determined by Northern blot analysis as previously described [8]. Briefly, total RNAs were separated in a denaturating 1% agarose gel and blotted onto a nylon membrane. Blots were hybridized with radiolabeled cDNAs according to standard procedures, and finally reprobed with human glyceraldehyde-3-phosphate dehydrogenase cDNA from Clontech as a control. Quantification was performed using a BioRad-GS363 molecular imager. The $^{A}\gamma$-globin and GATA-1 human cDNAs were a gift from Dr. S. Ottolenghi (University of Milan, Italy). Human PBGD and murine p45 NF-E2 cDNAs were from Dr. P.-H. Roméo (INSERM U91, Créteil, France) and Dr. S. H. Orkin (Harvard Medical School, Boston, MA), respectively.

Nitrite Assay

NO release was measured as nitrite accumulation in culture media. Briefly, an aliquot of culture medium was mixed with an equal volume of Griess reagent (1% sulfanilamide–0.1% naphthylethylene diamine dihydrochloride–2.5% H_3PO_4) and the absorbance at 540 nm was monitored with a microplate reader. Nitrite concentration was determined according to a sodium nitrite calibration curve.

RESULTS

Effect of SNP on Cell Growth

The viability of K562 cells in the presence of SNP (up to 1 mM), alone or in combination with the differentiating agents, was greater than 95% as assessed by the trypan blue dye exclusion test. Moreover, the NO release had no effect on control K562 cell growth, up to 1 mM SNP (Fig. 1A). Although cell growth was reduced in the presence of BA, ACLA, or DOX differentiation inducers [7, 8], no further inhibition was found after SNP addition (Fig. 1A). In contrast, the growth of hemin-treated K562 cells was slightly decreased in the presence of SNP (Fig. 1A).

Inhibition of Chemically Induced Cell Differentiation by SNP-Derived NO

K562 cell differentiation was studied as the percentage of benzidine-positive cells scored at day 3 after induction. SNP had no effect on control cell hemoglobinization, which remained below 1%. Figure 1B clearly shows the marked inhibitory effect of SNP-derived NO on differentiation. It should be noted that NO release from SNP was dependent on the SNP concentration added and was unaffected by the presence of either BA, ACLA, DOX, or hemin (data not shown). As little as 25 μM SNP, which generated less than 2 μM nitrite in 3-day culture media, led to a 50% inhibition of cell differentiation (Fig. 1B). The percentage of benzidine-positive cells was reduced to nearly 6% in the presence of 0.25 mM SNP (which generated 4 μM nitrite) in combination with either BA, ACLA, or DOX versus 41 ± 4%, 46 ± 4%, and 26 ± 2%, respectively, in the absence of SNP. Higher concentrations of SNP completely abolished the appearance of hemoglobinized cells (Fig. 1B). In contrast, hemin-induced differentiation was not affected by SNP treatment (Fig. 1B). The percentage of benzidine-positive cells remained higher than 80 ± 6% even in the presence of 1 mM SNP for 3 days, versus 84 ± 5% in cells treated with 30 μM hemin alone.

As SNP decomposition also generates ferricyanide, con-

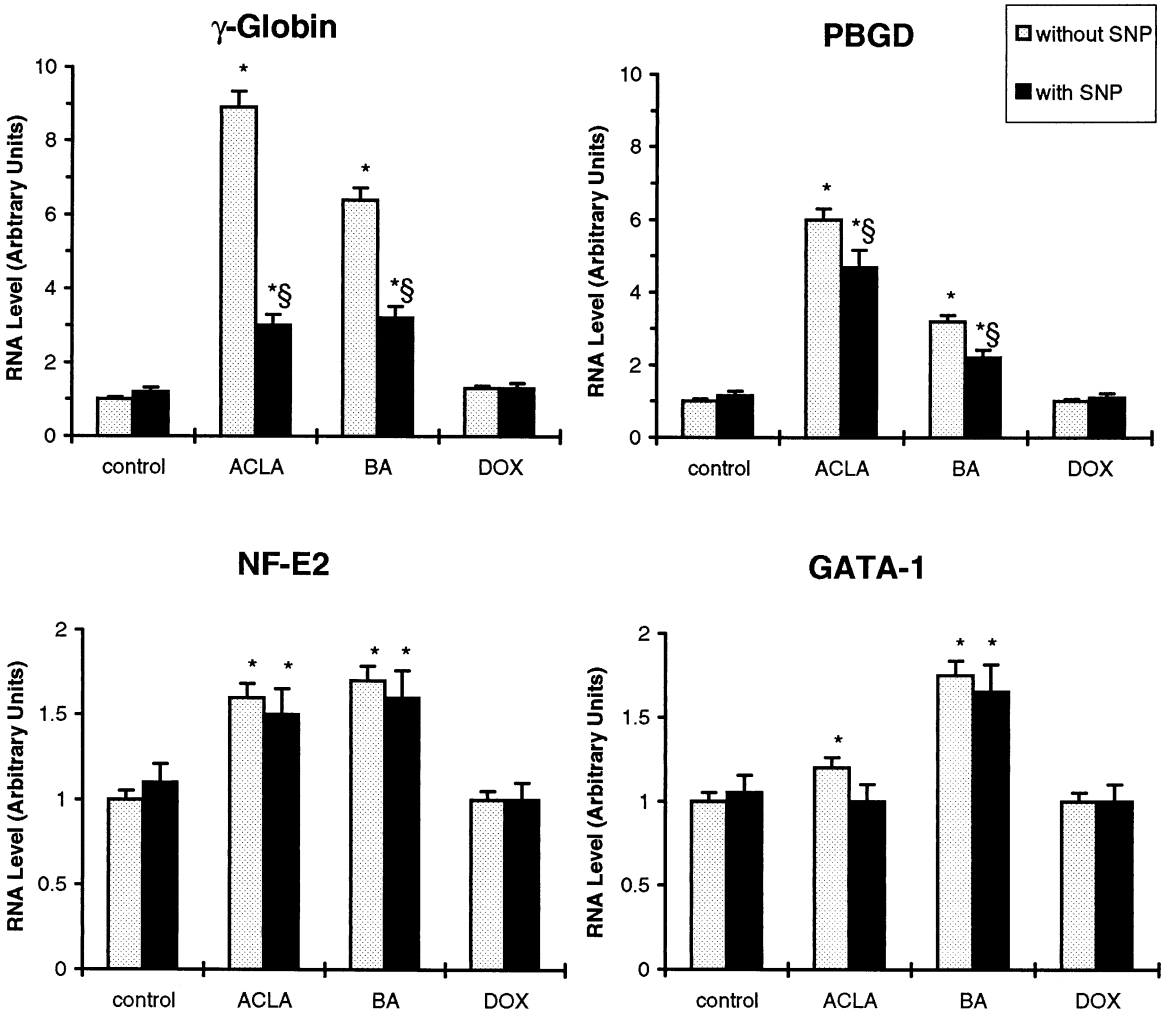

FIG. 3. Effect of SNP treatment on erythroid RNA levels. Cells were treated or not with 1 mM SNP for 3 days in the presence or absence (control) of the indicated inducer (ACLA 20 nM, BA 0.5 mM, DOX 40 nM). Results from Northern blot analysis were quantified using a BioRad molecular imager and corrected with respect to the glyceraldehyde-3-phosphate dehydrogenase RNA level. Data are the means ± SD from three independent experiments. *, values were significantly different from untreated control ($P < 0.05$). §, values from SNP treatment were significantly different from those obtained in the absence of SNP ($P < 0.05$).

trol experiments were performed in the presence of 0.1 to 2 mM of potassium ferricyanide. In this concentration range, ferricyanide did not affect cell growth nor differentiation whatever the inducer used (data not shown). This indicates that the inhibitory effect of SNP was due to NO release.

Inhibition of Cell Differentiation by SIN-1 and SNAP

In order to ensure that the inhibitory effect of SNP on differentiation was related to NO release, SIN-1 and SNAP were used as chemically unrelated NO donors. As shown in Fig. 2, both SIN-1 and SNAP dramatically reduced the differentiation of K562 cells induced by either BA, ACLA, or DOX. As little as 0.1 mM of SIN-1 or SNAP reduced the BA- or ACLA-induced differentiation by 50% and DOX-induced differentiation by 75%. A total inhibition was observed with 1 mM of either SIN-1 or SNAP (Fig. 2). Hemin-induced differentiation, which was unaffected by SNP, was likewise not inhibited by SNAP, and moderately decreased (60%) by SIN-1 (Fig. 2). This distinct effect of SIN-1 could be explained by its ability to generate peroxinitrite. In contrast with SNP, both SIN-1 and SNAP reduced the growth of control cells. The percentage of growth inhibition ranged from 22% to 76% in the presence of 0.1 to 1 mM of SIN-1, and up to 30% with SNAP. Therefore, their action on the growth inhibitory effect of differentiation inducers was not clear (data not shown).

Effect of NO Release on Erythroid RNA Expression

The effect of NO release from SNP was further studied at the level of erythroid RNA expression. Low doses of SNP, which were sufficient to inhibit K562 cell hemoglobinization, did not modify the induced increase in γ-globin and PBGD mRNAs observed in ACLA- and BA-treated cells (data not shown). In the presence of 1 mM SNP, which

totally abolished cell hemoglobinization (Fig. 1B), the γ-globin and PBGD RNA levels were reduced in both ACLA- and BA-treated cells (Fig. 3). In contrast, the expression of GATA-1 and NF-E2 transcription factors, which is increased in ACLA- and BA-treated cells [7–9], was not significantly modified by the addition of SNP (Fig. 3). In addition, in DOX-treated cells, the PBGD and γ-globin RNA levels, which are elevated as a consequence of a posttranscriptional RNA stabilization [5], were found to be unmodified in the presence of SNP (Fig. 3).

DISCUSSION

Although NO has been shown to induce the monocytic differentiation of both freshly isolated acute non-lymphocytic leukemia cells [21] and the HL-60 [22] and U-937 [23, 24] leukemic cell lines, its effect on erythroleukemia cells has been mainly studied with respect to cellular iron homeostasis [16–18]. Nevertheless, Suhasini *et al.* [25] have recently shown that the chemically induced differentiation of murine erythroleukemia (MEL) cells was inhibited by NO donors such as SNP and *S*-nitrosogluthatione. This inhibition was correlated to a decrease in β-globin and eALAS mRNAs and NF-E2 DNA-binding activity inhibition, but not GATA-1 binding activity inhibition [25]. In NO synthase-transfected K562 cells, the endogenous production of NO was recently reported to decrease the hemoglobin content [16, 26]. However, these authors found that neither γ-globin nor eALAS RNA expression was affected in NO-producing cells [26], in contrast to the murine model. Given that endogenous production of NO [16, 26] and the use of chemical NO donors ([25] and our present work) differ in the kinetics of NO release [27], it could be hypothesized that the transient intracellular concentration of NO, as well as its cellular effects, would be different in each case [28]. In this respect, it is to be noted that the growth of NO synthase-transfected cells is slower than that of cells transfected with the control vector [16, 26], which could affect the cell response to differentiating agents, whereas the growth of K562 cells was unaffected in the presence of SNP ([18] and our work).

Here, we show that NO, released from chemical NO donors, inhibited in a dose-dependent manner the differentiation of K562 cells induced by either BA or anthracyclines, with an IC_{50} of 25 μM for SNP and less than 100 μM for SIN-1 and SNAP. Nevertheless, this effect is not fully unspecific, as hemin-triggered differentiation was not affected by SNP- and SNAP-derived NO. The molecular mechanisms triggered by each differentiating agent are quite different. In particular, BA- or ACLA-induced differentiation depends on transcriptional activation of erythroid genes, whereas DOX-induced hemoglobinization is independent of transcriptional enhancement [5, 8]. Only a high dose of SNP (1 mM) was able to reduce the overexpression of erythroid γ-globin and PBGD mRNAs in BA- and ACLA-treated cells. In addition, the expression of erythroid transcription factors GATA-1 and NF-E2 was not affected by the presence of SNP. This suggests that the main inhibitory effect of NO takes place downstream of the regulation of gene expression. As suggested by Rafferty *et al.* [26], this could be at the level of eALAS enzymatic activity. In addition, NO has also been reported to induce the overexpression of the heme catabolism enzyme heme oxygenase in rat liver [29], and this could account for the observed decrease in hemoglobin synthesis.

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