Requirement of GATA-1 and p45 NF-E2 expression in butyric acid-induced erythroid differentiation.

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REQUIREMENT OF GATA-1 AND p45 NF-E2 EXPRESSION IN BUTYRIC ACID-INDUCED ERYTHROID DIFFERENTIATION

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ABSTRACT

Butyric acid (BA) is known to induce overexpression of fetal hemoglobin and then erythroid differentiation. Therefore BA is currently under clinical investigation as a potential therapy for the treatment of sickle cell disease and cancer. Nevertheless, the molecular mechanisms involved in BA-induced differentiation remain largely unknown. Previous reports have shown that BA-induced overexpression of erythroid genes occured at the transcriptional level, suggesting the involvement of erythroid transcription factors. Here, we intend to demonstrate the requirement of GATA-1 and NF-E2 transcription factors in the BA-induced erythroid differentiation of human leukemic K562 cells. Time-course experiments showed that nuclear levels of GATA-1 and p45 NF-E2 proteins increased during BA-treatment. Moreover, antisense oligodeoxynucleotides targeting either

GATA-1 or p45 NF-E2 proteins inhibited both protein expression and BA-induced differentiation. In contrast, BA-induced cell growth inhibition was not affected. These results provide the first direct evidence for the requirement of GATA-1 and NF-E2 in BA-induced differentiation process.

INTRODUCTION

The biological relevance of butyric acid (BA), which is found in the plasma of mammals, lies principally in its ability to regulate cell growth and differentiation (1, 2). In hematopoietic cells, BA demonstrates the ability to induce fetal hemoglobin synthesis and erythroid differentiation (3-5). In addition, several reports demonstrated the ability of BA to induce differentiation and apoptosis of nonhematopoietic tumor cells including colon and breast cancer cells (6-8). Therefore, BA and derivatives are currently under clinical consideration as potential agents for the treatment of cancer and hematologic diseases related to abnormal hematopoeisis, including acute myeloid leukemia, myelodysplastic syndrome and sickle cell disease (4, 9-12). In spite of its well known biological and clinical importance, the BA-triggered molecular events leading to nuclear activation and gene expression, which ultimately result in regulation of cell growth and differentiation, are poorly understood. Nevertheless, inhibition of histone deacetylase and the presence of butyrate responsive elements in BA-induced genes have been thought to be responsible of some of the biological effects of BA (2, 13). Recently, protein phosphorylation have been involved in BA-triggered transduction pathways. Indeed, BA induces mitogen activated protein (MAP) kinase and protein

kinase C (PKC) activation in K562 cells (14, 15), but causes hypophosphorylation of the retinoblastoma protein (16, 17), down-regulation of protein kinase CKII (18) and activation of a protein phosphatase (19) in various other cell types.

In the human K562 leukemic cell line, low doses of BA (0.5-1 mM) inhibit cell proliferation and induce erythroid differentiation (20). We and others have previously reported the enhancement of γ-globine and heme synthesis enzymes gene expression in BA-treated K562 cells (20-24). These results suggest the involvement of erythroid transcription factors GATA-1 and NF-E2, which are known as key regulators of normal erythroid differentiation (25, 26).

Here we expanded our earlier preliminary investigation to determine whether GATA-1 and p45 NF-E2 proteins expression was required during BA-induced differentiation. Time-course experiments showed increased expression of GATA-1 and p45 NF-E2 proteins during BA-treatment. Moreover, the use of antisense oligodeoxynucleotides (ODN) targeting either GATA-1 or p45 NF-E2 proteins provide the first direct evidence of their involvement in BA-induced differentiation process. In addition, BA-induced cell growth inhibition appeared independent of transcription factors expression.

MATERIALS AND METHODS

Cell culture and differentiation. Human leukemic K562 cells were cultured in RPMI-1640 Glutamax medium (Life Technologies, Saint Quentin en Yvelines, France) supplemented with 10% heat-inactivated fetal bovine serum in a 5%

CO_2 humidified atmosphere. Cells were induced to differentiate with 0.5 mM BA at the begining of exponential growth phase. The percentage of hemoglobin-producing cells was determined at day 2 or 3 by a benzidine staining method as previously described (20). Cell growth inhibition was 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.

Antisense ODNs design and treatment. The 3'-amino-phosphodiester 18-mer antisense ODNs targetting either GATA-1 (2G1 and 3G1) and p45 NF-E2 (2N2 and 3N2) traduction start sites were synthesized by Genset (Paris). As control, scrambled ODNs (RG1 and RN2) were obtained from randomized 3G1 and 2N2 sequences, respectively. The sequences were: 2G1, AGGCCAGGGAACTCCAT ; 3G1, GGAACTCCATGGAGCCTC ; 2N2, TGCCAAGTCAGTTCCATC ; 3N2, CATGATCTCCTGCCAAGT ; RG1, GCACTCGGTGACGACTAC ; RN2, GCGCGTTAAATCCATCCT. Mixture of 1 µM ODNs with 10 ng/ml Lipofectin reagent (Life Technologies) was preincubated at 37°C for 30 min before addition to cell cultures. Cells (10^6/ml) were treated with ODNs/Lipofectin mixture in serum free medium for 4 h, and then diluted to 10^5/ml in complete medium and incubated at 37°C for 48 h in the presence or absence of 0.5 mM BA.

Western blot analysis. Nuclear protein extracts and western blotting were done as previously described by Andrews *et al.* (27). Briefly, cells were lysed at 4°C in 10 mM HEPES, 1.5 mM

$MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF. After centrifugation (12000 g, 10 sec) nuclei were incubated 20 min at 4°C in 20 mM HEPES, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF and pelleted again. Protein content was determined in each supernatants using the Bradford asssay (BioRad reagent, Ivry/Seine, France). Proteins (20 µg) were loaded in a 10% acrylamide gel and running in Tris-glycine-SDS buffer, and blotted onto nitrocellulose membranes (Interbiotech, Montluçon, France). Membranes were saturated for 1 h in Tris-buffered saline containing 5% dry milk and incubated overnight with either GATA-1 monoclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) or p45 NF-E2 polyclonal antibody (27) kindly provided by Drs. Orkin and Kotkow (Howard Hughes Medical Institute, Boston, MA). After washing, membranes were incubated with horseradish peroxidase-conjugated antibody (Interbiotech) and immunoreactive proteins were visualized using the Super Signal chemiluminescent reagent (Interbiotech). Blot were quantified by densitometric analysis.

RESULTS

The nuclear levels of GATA-1 and p45 NF-E2 proteins were determined in time-course experiments in both control and BA-induced K562 cells. Acording to previously reported western blot analysis, p45 NF-E2 protein appeared as a doublet of bands (27). As shown in Fig. 1, GATA-1 and p45 NF-E2 protein levels were slighlty increased as soon as 6 h after BA-treatment. Then, the nuclear protein levels declined near to control level at 24 h, and increased

again up to 2 and 3 fold for GATA-1 and p45 NF-E2, respectively, at 72 h (Fig. 1). These results are consistent with a rapid response to BA stimulus, probably a translocation of cytoplasmic proteins into the nucleus, followed by a neosynthesis of GATA-1 and p45 NF-E2 transcription factors according to the RNA overexpression in BA-treated cells seen at 24 h (20).

Antisense ODNs were used to modulate the expression of erythroid transcription factors. As shown in Fig. 2, GATA-1 and p45 NF-E2 protein levels were reduced in 2-days BA-induced cells incubated with GATA-1 or p45 NF-E2 antisense ODNs, respectively. Nevertheless, the level of inhibition of GATA-1 or p45 NF-E2 protein expression was higher with 3G1 or 2N2 antisense ODNs, respectively, whereas 2G1 and 3N2 antisense ODNs were weaker inhibitors (Fig. 2). As a control of sequence dependence, scrambled ODNs (RG1 and RN2) were unable to change GATA-1 and p45 NF-E2 protein levels (Fig. 2). In addition, lipofectin vehicle had no effect on protein levels in both control (Fig. 2) and BA-induced-cells (not shown).

Differentiation, measured as the % of benzidine-positive cells at day 2, was decreased in BA-induced cells in the presence of ODNs (Fig. 3). Consistent with the observed decrease in GATA-1 and p45 NF-E2 proteins, both GATA-1 and p45 NF-E2 antisense ODNs inhibited BA-induced differentiation. As seen for protein expression, 3G1 and 2N2 antisense ODNs caused a higher inhibition of differentiation than 2G1 and 3N2 (Fig. 3). Furthermore, neither scrambled ODNs nor lipofectin alone affected the % of differentiated cells in BA-induced and control cells (Fig. 3).

Although differentiation was linked to a proliferative arrest, results with antisense ODNs showed no correlation between inhibition of differentiation and cell growth. Indeed, the cell growth inhibition induced by BA was not significatively affected by ODNs treatment (Fig. 3).

DISCUSSION

Erythroid differentiation of K562 leukemic cells by BA is a consequence of increased transcription of genes coding fetal globin and heme synthesis enzymes (20-24). In addition to the role of butyrate responsive elements found in $^{A}\gamma$-globin promoter (13), we evidenced here the requirement of enhanced expression of GATA-1 and NF-E2 transcription factors in BA-triggered process. Phosphorylation of these transcription factors has been previously investigated in murine erythroleukemia cells (28-30). Although serine phosphorylation of GATA-1 seems to have no consequence on its activity (28), the phosphorylation of p45 NF-E2, consequently to the enhancement of either cAMP dependent protein kinase or Ras-Raf MAP kinase activity, increased NF-E2 activity (29, 30). Since stimulation of MAP kinase and PKC have been reported in BA-induced K562 cells (14, 15), it is tempting to hypothesize a link between such BA-triggered transduction pathways and BA-induced increase in erythroid transcription factors expression and activity, leading to differentiation.

Taken together, the results presented here showed that BA-induced differentiation was linked to erythroid transcription factor level in K562 cells, and provide the first direct evidence for the requirement of GATA-1 and p45

NF-E2 in BA-induced differentiation. In contrast, BA-induced cell growth arrest was independent of GATA-1 and p45 NF-E2 overexpression.

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FOOTNOTES

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FIGURE LEGENDS

Fig. 1. Time-course of nuclear GATA-1 and p45 NF-E2 protein expression. A: Western blot analysis of nuclear protein from control and BA-treated cells was done for each time point as described in Materials and Methods. As previously reported, p45 NF-E2 protein appeared as a doublet (27). Results from a typical experiment. B: Following densitometric analysis of the western blots, the relative protein level in BA-treated versus control cells for each time point was plotted as a function of time. Data are the mean ± SEM of three independent experiments.

Fig. 2. Inhibition of GATA-1 and p45 NF-E2 expression by antisense ODNs. Cells were treated for 4 h in serum free medium with either A: GATA-1 (2G1 and 3G1) or B: NF-E2 (2N2 and 3N2) antisense ODNs or scrambled control ODNs (RG1 and RN2) in the presence of Lipofectin (L: Lipofectin alone), and then induced to differentiate with 0.5 mM BA for 48 h in complete medium. Nuclear protein extract from control and treated cells, and western blot analysis were performed as described in Materials and Methods. A and B: Results from a typical experiment representative of three. C: Densitometric analysis. Data are the mean ± SEM of three independent experiments.

Fig. 3. Effect of antisense ODNs-treatment on BA-induced cell differentiation and growth inhibition. Cells were treated for 4 h in serum free medium with either GATA-1 (2G1 and 3G1) or NF-E2 (2N2 and 3N2) antisense ODNs or scrambled control ODNs (RG1 and RN2) in the presence

of Lipofectin, and then induced to differentiate with 0.5 mM BA for 48 h in complete medium. Differentiation was assayed as the % of benzidine-positive cells and cell growth inhibition was determined as described in Materials and Methods. Data are the mean ± SEM of five independent experiments.

Figure 1

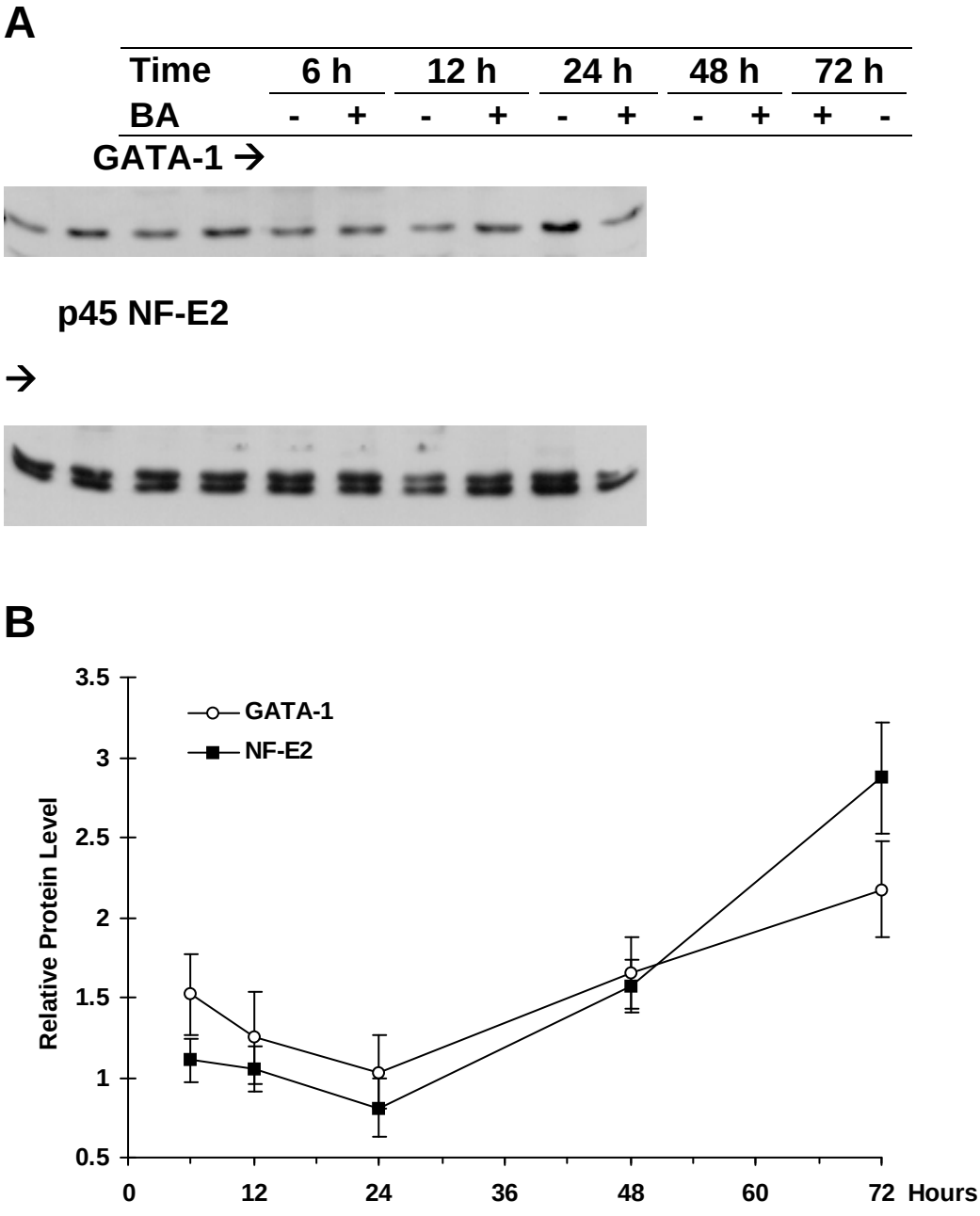
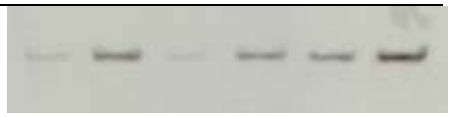

A
Time
6 h
12 h
24 h
48 h
72 h
BA
- + - + - + - + + -
GATA-1 →
p45 NF-E2
→
B
GATA-1
NF-E2
Relative Protein Level
3.5
3
2.5
2
1.5
1
0.5
0
12
24
36
48
60
72
Hours

Figure 2

A

BA	-	+	-	+	+	+
ODN	-	-	L	2G1	3G1	RG1



B

BA	-	+	+	+	+	-
ODN	-	-	RN2	2N2	3N2	L

C

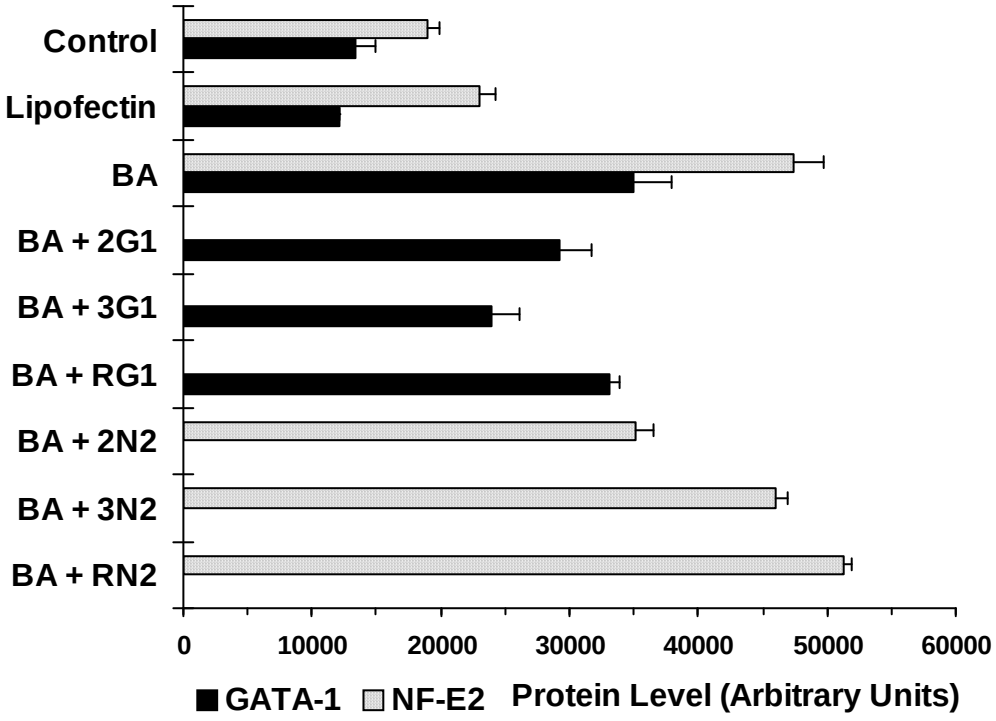
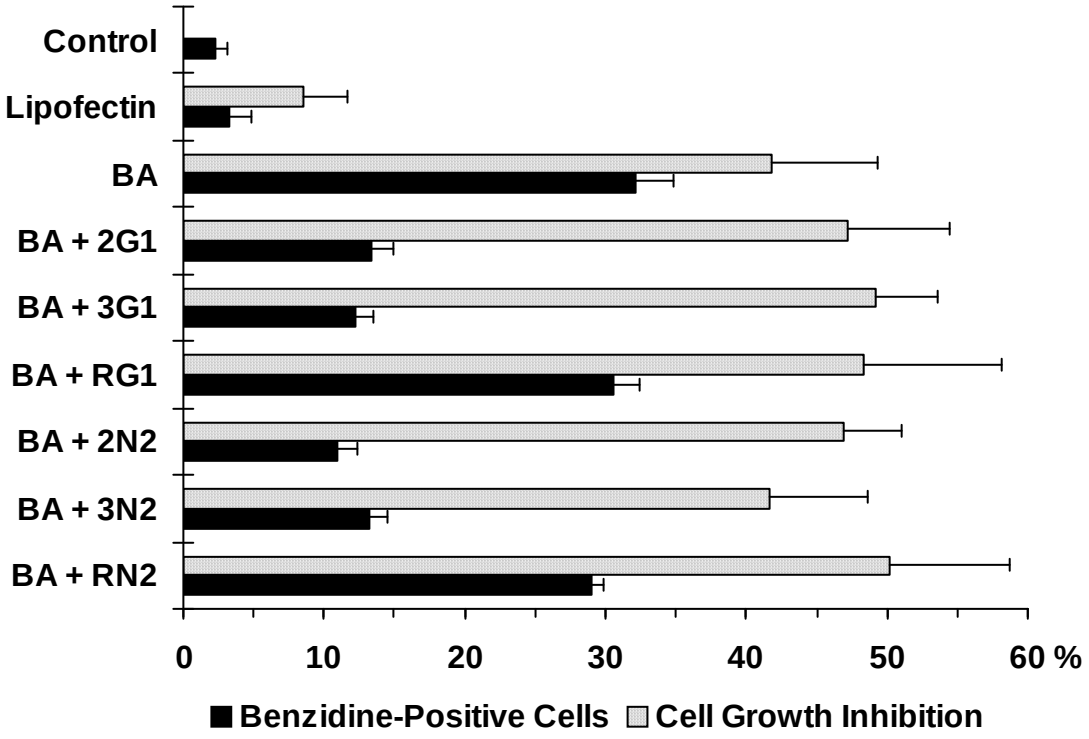

Control
Lipofectin
BA
BA + 2G1
BA + 3G1
BA + RG1
BA + 2N2
BA + 3N2
BA + RN2
0
10000
20000
30000
40000
50000
60000
GATA-1
NF-E2
Protein Level (Arbitrary Units)

Figure 3


Control
Lipofectin
BA
BA + 2G1
BA + 3G1
BA + RG1
BA + 2N2
BA + 3N2
BA + RN2
0
10
20
30
40
50
60 %
Benzidine-Positive Cells
Cell Growth Inhibition