HDAC INHIBITORS SODIUM BUTYRATE AND SODIUM VALPROATE DO NOT AFFECT HUMAN *NCOR1* AND *NCOR2* GENE EXPRESSION IN HL-60 CELLS

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Aim. This study was designed to examine whether the class I and class IIa histone deacetylase (HDAC) inhibitors, sodium butyrate and sodium valproate alter the expression of human *NCOR1* and/or *NCOR2* genes coding for N-CoR (nuclear receptor corepressor) and SMRT (silencing mediator for retinoid and thyroid hormone receptors), respectively.

Methods. Human leukemia HL-60 cells were treated for 24 h with 0.5 and 1 mM sodium butyrate, 1 to 3 mM sodium valproate, 1 μ M all-*trans* retinoic acid (ATRA) or cotreated with 1 μ M ATRA and 0.5 mM sodium butyrate. The acetylation of histones H3 and H4 was analysed by western blotting. The levels of *NCOR1* and *NCOR2* mRNA were determined by quantitative real-time PCR. Expression of *NCF2* gene coding for the NADPH oxidase subunit p67phox was evaluated as a marker of myeloid differentiation.

Results. Both butyrate and valproate increased the acetylation of histone H3 at Lys9 and/or Lys14 as well as histone H4 at Lys12. Both HDAC inhibitors caused a significant increase in *NCF2* mRNA levels without affecting *NCOR1* or *NCOR2* mRNA levels. Similarly, ATRA alone or in combination with butyrate induced *NCF2* gene expression without any significant influence on the expression of *NCOR1* or *NCOR2* genes.

Conclusion. We conclude that inhibitors of class I and class IIa HDACs do not alter the expression of human *NCOR1* or *NCOR2* genes and that the onset of myeloid differentiation is not accompanied by induction or repression of these genes in HL-60 cells.

INTRODUCTION

N-CoR (nuclear receptor corepressor) and SMRT (silencing mediator for retinoid and thyroid hormone receptors) are ubiquitously expressed corepressor proteins. They interact with unliganded nuclear receptors and form large corepressor complexes that mediate transcriptional repression through their association with histone deacetylases (HDACs). The relative hypoacetylation restores a positive charge in histone proteins and causes closer association of the histones with DNA that becomes less accessible to transcription factors^{1,2}. HDACs that have been shown to interact with N-CoR or SMRT complexes include class I HDACs 1, 2 and 3 and class IIa HDACs 4, 5 and 7 (ref.³). N-CoR and SMRT have been recognized to play important roles, among others, in development and cell differentiation¹. For instance, neutrophilic differentiation is regulated through the nuclear retinoic acid receptor α (RAR α). Unliganded RAR α interacts with N-CoR or SMRT which associate through the corepressor mSin3 (mammalian switch independent 3 protein) with HDAC1 or HDAC2. The corepressor complexes, however, dissociate from RAR α at physiological concentrations of all-*trans* retinoic acid (ATRA) and subsequent

recruitment of coactivators with histone acetylase activity relieves the transcriptional repression of differentiation-related genes⁴. On the other hand, abnormal transcriptional repression resulting from aberrant recruitment of the N-CoR/SMRT-HDAC complexes appears to underlie the molecular pathogenesis of cancers such as acute promyelocytic leukemia⁵. In accordance with the crucial role of HDACs in corepressor-dependent transcriptional repression, inhibitors of HDAC activity have emerged as a new class of anticancer agents⁶.

Human N-CoR and SMRT are encoded by *NCOR1* (nuclear receptor corepressor 1) and *NCOR2* (nuclear receptor corepressor 2) genes, respectively. To date, little is known about their transcriptional regulation. It has been shown that the steroid hormone, 17 β -estradiol decreases N-CoR protein levels in estrogen receptor-positive breast cancer cells without affecting *NCOR1* mRNA levels⁷. *NCOR1* mRNA levels were also unchanged in rat pituitary GH₃ cells exposed to 17 β -estradiol or to the thyroid hormone triiodothyronine⁸. The steroid hormone, 1 α ,25-dihydroxyvitamin D₃ has been found to induce a moderate and transient upregulation of *NCOR2* but not *NCOR1* mRNA levels in MCF-7 breast cancer cells⁹. Both *NCOR1* and *NCOR2* mRNA levels remained unaf-

ected in rat hepatocytes treated with ATRA or colchicine, a microtubule interfering agent¹⁰.

Since HDAC inhibitors are considered to modulate the expression of 7-10% of genes in human cancer cells¹¹, we examined whether the expression of human *NCOR1* and/or *NCOR2* genes could be altered by two HDAC inhibitors, sodium butyrate and sodium valproate.

MATERIAL AND METHODS

Chemicals

Sodium butyrate (B5887), sodium valproate (P4543) and ATRA (R2625) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions of sodium butyrate and sodium valproate (1 M) were prepared in water for molecular biology and stored at -20 °C. Stock solution of ATRA (1 mM) in ethanol was prepared freshly before use with all manipulations performed in subdued light.

Cell culture and treatment

The human promyelocytic leukemia cell line HL-60 (No. 98070106, ECACC, Salisbury, UK) was cultured at 37 °C in RPMI-1640 medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin (Sigma), and 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) in a humidified atmosphere containing 5% CO₂ and maintained between 1-8 × 10⁵ cells/ml. All experiments were conducted using exponentially growing cells, passages 10-30. Cells were seeded at a density of 1.5 × 10⁵ cells/ml and treated for 24 h with butyrate, valproate or ATRA. Negative controls were treated with 0.1% ethanol or untreated.

Western blot analysis

After treatment, cells were collected by gentle centrifugation, washed with PBS and lysed in lysis buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM NaF, 1 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, protease inhibitors Complete (Roche Diagnostics, Mannheim, Germany), 0.2% sodium dodecyl sulfate (SDS), 1% Nonidet-P40, 1% Triton X-100, pH 7.4). After incubation on ice for 20 min, the cell lysates were sonicated to shear genomic DNA using an Ultrasonic Processor UP200s equipped with a sonicator probe Sonotrode Microtip S2 (Hielscher, Teltow, Germany). The samples were kept on ice and sonicated for 2 × 20 s at an 80% amplitude, 2 × 20 s at a 90% amplitude and 2 × 20 s at a 100% amplitude with a cycle set at 0.5 s. After this, the cell lysates were centrifuged for 10 min at 16 000 g and 4 °C and the proteins in supernatants were quantified using the Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). Aliquots containing 30 µg of protein were subjected to electrophoresis through 12.5% SDS-polyacrylamide gel and proteins were transferred to polyvinylidene difluoride membrane by electroblotting. The membranes were probed with rabbit monoclonal histone H3 antibody (No. 9717), rabbit polyclonal acetyl-histone H3 (Lys9/Lys14) antibody (No. 9677) or rabbit polyclonal acetyl-histone H4 (Lys12) antibody (No. 2591), all purchased from Cell

Signaling Technology (Danvers, MA, USA). The primary antibodies were visualized using the Phototope-HRP Western Blot Detection System containing anti-rabbit IgG-HRP conjugated secondary antibody (Cell Signaling Technology).

Reverse transcription and quantitative real-time PCR

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The concentration of RNA was determined by spectrophotometry at 260 nm. RNA samples (2 µg) were reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's recommendations. Real-time PCR was performed on a LightCycler 480 II system (Roche Diagnostics) using the TaqMan Universal PCR Master Mix and the TaqMan Gene Expression Assays consisting of specific primers and FAM dye-labelled TaqMan minor groove binder probes (Applied Biosystems). The assay ID for *NCOR1* was Hs00196920_m1; for *NCOR2*, Hs00196955_m1; for *NCF2*, Hs00166416_m1; and for *18S*, Hs99999901_s1, respectively. Amplification conditions were 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles with 95 °C for 15 s and 60 °C for 1 min. Crossing point values, equivalent to C_T, were determined automatically using the second derivative maximum analysis. Relative changes in gene expression were calculated by the comparative C_T method using the 2^{-ΔΔC_T} equation with results normalized to *18S* rRNA levels.

Statistical analysis

Results were expressed as means ± S.D. of three independent experiments. The differences in mean values were analysed by Student's *t*-test. A *p* value of less than 0.05 was considered as statistically significant.

RESULTS

Hyperacetylation of histones H3 and H4 by butyrate and valproate

We chose for our study non-cytotoxic concentrations of butyrate and valproate on the basis of our previous results¹². To confirm efficient inhibition of HDAC activity, human leukemia HL-60 cells were treated with 0.5 and 1 mM butyrate or 1 to 3 mM valproate and the whole cell lysates were analysed by western blotting using antibodies detecting specific acetylations of histones H3 and H4. After 24 h treatment, both butyrate and valproate increased the acetylation of histone H3 at Lys9 and/or Lys14 as well as the acetylation of histone H4 at Lys12 (Fig. 1).

Effect of butyrate and valproate on *NCOR1*, *NCOR2* and *NCF2* gene expression

The exposure of HL-60 cells to butyrate or valproate is associated with multiple changes in gene expression leading to monocytic differentiation^{13,14}. We examined the expression of human *NCOR1* and *NCOR2* genes encoding

Fig. 1. Hyperacetylation of histones by sodium butyrate and sodium valproate in HL-60 cells. HL-60 cells were treated for 24 h with butyrate, valproate or untreated (UT). Proteins in the whole cell lysates (30 μ g/lane) were analysed by western blotting using antibodies against acetylated histone H4 (Lys12), acetylated histone H3 (Lys9/Lys14) and total histone H3. The figures are representative of two experiments.

N-CoR and SMRT, respectively, concurrently with the expression of *NCF2* (neutrophil cytosolic factor 2) gene coding for the NADPH oxidase subunit p67phox, a marker of myeloid differentiation^{12,15}. After 24 h treatment of HL-60 cells, both butyrate and valproate induced a dose-dependent increase in *NCF2* gene expression as shown by the real-time PCR technique (Fig. 2A). In contrast, neither butyrate nor valproate caused any substantial changes in the expression of *NCOR1* or *NCOR2* genes under the same conditions. The *NCOR1* mRNA levels induced by butyrate or valproate reached (0.83 ± 0.09) -fold to (1.08 ± 0.04) -fold values, while the *NCOR2* mRNA levels varied between (0.80 ± 0.11) -fold and (1.25 ± 0.22) -fold values compared to control cells (Fig. 2A). We also examined the effect of the HDAC inhibitors in the presence of ATRA, an inducer of neutrophilic differentiation¹³. Our preliminary experiments (data not shown) demonstrated that only 0.5 mM butyrate synergized with ATRA in the induction of the *NCF2* gene expression without parallel induction of the cytotoxic effect¹². While the *NCF2* mRNA levels in HL-60 cells exposed for 24 h to 0.5 mM butyrate or 1 μ M ATRA reached 2.9-fold (Fig. 2A) and 7.2-fold values (Fig. 2B), respectively, the coexposure to ATRA and butyrate caused 17.3-fold increase in *NCF2* mRNA level compared to control cells (Fig. 2B). On the other hand, ATRA either alone or in combination with butyrate did not substantially affect the expression of either *NCOR1* or *NCOR2* genes (Fig. 2B).

DISCUSSION

N-CoR and SMRT are the most extensively studied corepressor proteins, but little is known about their transcriptional regulation. This study was designed to examine whether the expression of human *NCOR1* and *NCOR2* genes coding for N-CoR and SMRT, respectively, could be altered by two HDAC inhibitors, sodium butyrate and sodium valproate. Human HDAC enzymes are classified on the basis of their homology to yeast HDACs. Classical HDACs are Zn^{2+} -dependent enzymes and in-

clude class I (HDAC1, 2, 3, 8), class IIa (HDAC4, 5, 7, 9), class IIb (HDAC6, 10) and class IV (HDAC11). Class III enzymes require NAD^+ for their activity and comprise seven members known as sirtuins⁶. Inhibitors of HDAC activity are able to cause both induction and repression of gene expression¹⁶. These effects may result from the increased acetylation of histones and/or non-histone HDAC substrates that include a number of transcription factors and other important proteins¹¹. Butyrate and valproate inhibit at millimolar concentrations class I and class IIa HDACs^{11,16,17}, i.e. enzymes known to associate with N-CoR and SMRT (ref.³). The HL-60 cell line used in our study was derived from a patient with acute myeloblastic leukemia type M2 according to the French-American-British classification¹⁸. HL-60 cells may be induced to differentiate along the neutrophilic pathway, e.g. by ATRA, or along the monocytic pathway by butyrate¹³, valproate¹⁴ and other compounds. ATRA activates the transcription of differentiation-related genes through its binding to $RAR\alpha$ leading to the release of the N-CoR/SMRT-HDAC complexes⁴. Butyrate and valproate may induce expression of the same genes as ATRA by inhibiting HDACs in the corepressor complexes⁵. Moreover, a combination of HDAC inhibitors with ATRA has been shown to synergize in differentiation of ATRA-sensitive myeloid leukemic cells¹⁹ and to overcome the block in differentiation of ATRA-resistant cells²⁰. In this study, we found that neither butyrate nor valproate, like ATRA, induced any significant changes in the expression of *NCOR1* and *NCOR2* genes under conditions of efficient HDAC inhibition and/or induction of myeloid gene expression. We conclude that the inhibitors of class I and class IIa HDACs do not alter the expression of human *NCOR1* and *NCOR2* genes and that the onset of myeloid differentiation is not accompanied by induction or repression of these genes in HL-60 cells.

Fig. 2. Relative changes in *NCOR1*, *NCOR2* and *NCF2* gene expression by sodium butyrate, sodium valproate and all-*trans* retinoic acid (ATRA) in HL-60 cells. (A) HL-60 cells were treated for 24 h with butyrate, valproate or untreated (UT; control). (B) HL-60 cells were treated for 24 h with 0.1% ethanol (E; control), 1 μ M ATRA or cotreated with 1 μ M ATRA and 0.5 mM butyrate (BT). The levels of *NCOR1*, *NCOR2* and *NCF2* mRNA levels were determined by quantitative real-time PCR with results normalized to 18S rRNA levels. Data are means $\pm$ SD of three experiments. * $p < 0.05$; ** $p < 0.01$, significantly different from control.

ABBREVIATIONS

ATRA, All-*trans* retinoic acid; HDAC, Histone deacetylase; *NCF2*, Neutrophil cytosolic factor 2; N-CoR, Nuclear receptor corepressor; *NCOR1*, Nuclear receptor corepressor 1; *NCOR2*, Nuclear receptor corepressor 2; *RAR α* , Retinoic acid receptor α ; SMRT, Silencing mediator for retinoid and thyroid hormone receptors

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