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Modulation of translation factor's gene expression by histone deacetylase inhibitors in breast cancer cells

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Abstract

The histone deacetylase inhibitors sodium butyrate (NaBu) and trichostatin A (TSA) exhibit anti-proliferative activity by causing cell cycle arrest and apoptosis. The mechanisms by which NaBu and TSA cause apoptosis and cell cycle arrest are not yet completely clarified, although these agents are known to modulate the expression of several genes including cell-cycle- and apoptosis-related genes. The enzymes involved in the process of translation have important roles in controlling cell growth and apoptosis, and several of these translation factors have been described as having a causal role in the development of cancer. The expression patterns of the translation mechanism, namely of the elongation factors eEF1A1 and eEF1A2, and of the termination factors eRF1 and eRF3, were studied in the breast cancer cell line MCF-7 by real-time quantitative reverse transcription-polymerase chain reaction after a 24-h treatment with NaBu and TSA. NaBu induced inhibition of translation factors' transcription, whereas TSA caused an increase in mRNA levels. Thus, these two agents may modulate the expression of translation factors through different pathways. We propose that the inhibition cau- sed by NaBu may, in part, be responsible for the cell cycle arrest and apoptosis induced by this agent in MCF-7 cells.

Keywords: breast cancer; MCF-7 cells; sodium butyrate; translation factors; trichostatin A.

Introduction

Histone deacetylase (HDAC) inhibitors are a group of several classes of epigenetic drugs that are considered to be promising anticancer agents. These agents cause indirect hyperacetylation of histones, which leads to alterations in gene expression by transcriptional activation or suppression through relaxation of DNA conformation. This effect is considered to be responsible for the anticancer properties of these drugs. However, they present several pleiotropic effects, such as cellular differentiation, cell cycle arrest and apoptosis, which may play an important role in the anticancer effects (1, 2).

Butyrate is a short-chain fatty acid normally present in the human colon due to its production during fermentation of fibers by endogenous bacteria (3). The sodium salt of butyrate, sodium butyrate (NaBu), is a HDAC inhibitor and exhibits anti-proliferative activity by causing cell cycle arrest and apoptosis, and induces cytodifferentiation in both normal and tumor cells (4–9). More recently it has been shown to suppress telomerase reverse transcriptase mRNA expression in prostate cancer cells (10).

NaBu shows low toxicity in vivo, but its clinical application is limited by a short half-life (7, 11).

Previous studies showed that NaBu induces arrest in the G1 and G2/M phases of the cell cycle and apoptosis in MCF-7 human breast cancer cells, but the mechanism of action that leads to this effect is not totally elucidated (1, 4, 12). NaBu decreases the expression of cyclin D1, and induces up-regulation of the gene $p21^{waf1/cip1}$ and hypophosphorylation of pRB in a variety of cancer cells, including MCF-7 (3). $p21^{waf1/cip1}$ is an inhibitor of cell cycle progression, which has an essential role in G1 and G2 arrests, and it has also been proposed that it is implicated in apoptosis. However, it was recently described that $p21^{waf1/cip1}$ is not involved in G1 arrest but is indispensable for apoptosis induced by NaBu in MCF-7 cells (9). Furthermore, it has been reported that NaBu-induced apoptosis is Fas-mediated and that this agent modulates the expression of proteins involved in apoptosis. It increases the levels of the protein Bax and decreases the levels of Bcl-2 (13, 14). In addition, it has also been shown that NaBu induces up-regulation of P53 in MCF-7, but this factor is not involved in butyrate-induced apoptosis (14). Interestingly, a recent work observed NaBu-induced inhibition of P53 expression in the same cell line (13).

Trichostatin A (TSA), an antifungal antibiotic, is also a potent specific inhibitor of HDAC activity and, similar to NaBu, it induces growth arrest, differentiation and/or apoptosis in several types of cancer cells (15, 16). TSA has been shown to arrest cells in G1 and G2 phases of the cell cycle, to induce the transcriptional activation of $p21^{waf1/cip1}$, and to modulate the expression of other cell cycle regulators and apoptosis-regulating proteins (15–18). This agent presents no

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significant toxicity and was shown to have potent antitumor activity against breast cancer in vivo (19).

The enzymes involved in the process of translation have traditionally only been regarded as cellular housekeepers essential for cell viability. However, they have important roles in controlling cell growth and apoptosis, and several of these translation factors have been described as having a causal role in the development of cancer (e.g., eukaryotic initiation factors eIF4E, eEF1A1 and eEF1A2) (20, 21). Some members of the translation mechanism have been implicated in carcinogenesis, because it was observed that several cancer cell lines and tumor tissue samples overexpress them. In addition, the ectopic expression of certain translation factors leads to cell transformation and tumorigenesis, and the expression inhibition of some of these factors represses the oncogenic properties of cancer cells (22).

The eukaryotic polypeptide elongation factor eEF1-α is a major translation factor and an important multifunctional protein (23). There are two human isoforms of eEF1α, eEF1A1 and eEF1A2, which have $>$90% DNA sequence and amino acid identity, but differ markedly in their expression patterns (21, 24). These factors bind GTP and catalyze the binding of aminoacyl-tRNA to the A-site of the ribosome during peptide elongation. Beside their role in translation, they are involved in several important cellular processes, including translation control, signal transduction, cytoskeletal organization and mitosis (20, 21). eEF1-α associates with a zinc finger protein, ZRP1, in proliferating cells and it has been shown in *Saccharomyces cerevisiae* that the disruption of binding between the two proteins results in the accumulation of cells in the G2/M phase of the cell cycle (23).

Furthermore, eEF1-α has been implicated in various diseases such as cancer; for example, eEF1A2 is overexpressed in several cancer cell lines, as well as in cancer tissue samples, and it has been proved to be an oncogene in ovarian cancer (22, 25, 26). Finally, recent reports indicate that both eEF1A1 and eEF1A2 inhibit apoptosis (22, 26).

In eukaryotes the process of translation termination is governed by two termination factors, eukaryotic release factor 1 (eRF1) and eRF3. The termination factor eRF1 recognizes all three STOP codons and catalyzes the hydrolysis of the last peptidyl-tRNA, leading to release of the nascent peptide. This hydrolytic reaction is stimulated by eRF3, which is a GTP binding protein and contributes to translation accuracy (27–29). In addition to its role in translation termination, eRF3 also participates in regulation of the decay of poly-A-tailed mRNAs, and in translation initiation by binding to poly-A binding protein (PABP) (30–33). The product of eRF3 has also been implicated in regulation of the cytoskeleton (34, 35) and of the cell cycle (G1$\rightarrow$S phase transition) (36–38), and in apoptosis (39). It has been shown that the *eRF3* gene is overexpressed in several types of tumor tissue samples, including breast cancer (Brito et al., unpublished data).

The aim of this work was to study the effects of the HDAC inhibitor NaBu on the expression patterns of the translation mechanism, namely the elongation factors eEF1A1 and eEF1A2, and of the termination factors eRF1 and eRF3 in the breast cancer cell line MCF-7 by real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR).

Materials and methods

Cell culture and treatment with NaBu and TSA

MCF-7 cells were cultured in RPMI 1640 containing 10% fetal bovine serum, supplemented with 100 U/mL penicillin G, 100 μg/mL streptomycin and 0.25 μg/mL amphotericin B, at 37°C in 5% CO_2. For treatment, cells were seeded in Petri dishes at 2×10^6 per 10 mL of medium for 48 h. At the start of the experiment, fresh growth medium containing NaBu or TSA was added to cell culture dishes, and cells were incubated for 24 h. Three NaBu and two TSA experiments were carried out.

RNA extraction and real-time quantitative RT-PCR analysis

Total RNA was isolated from subconfluent proliferating cells using TriZol reagent following the manufacturer's instructions (Invitrogen, Daisley, UK). Reverse transcription reactions and TaqMan-PCRs were carried out according to the manufacturer's instructions (Applied Biosystems, Foster City, USA) in an iCycler iQ real-time PCR detection system (BioRad, Hercules, USA). Sequence specific amplification was detected with an increased fluorescent signal of FAM or VIC (reporter dyes) during the amplification cycles. Amplification of human 18S rRNA was used in the same reaction for all samples as an internal control. Gene-specific mRNA was subsequently normalized to 18S rRNA. Levels of eRF1, eRF3, eEF1A1 and eEF1A2 mRNA were expressed as x-fold difference of NaBu- and TSA-treated cells against a non-treated control determined by the comparative C_T method ($2^{-\Delta\Delta CT}$) (40).

Oligonucleotide primers and probe for human eRF3 and eRF1 were designed using the Beacon Designer computer program (BioRad). The sequences (5′–3′) for eRF3 and eRF1 were as follows: eRF3 forward primer, CGCCAGGTGCTCCTAAGAAAG; eRF3 reverse primer, CAAATACATTATTTGTCCTCCAATGGT; eRF3 probe, 6FAM-ACTTGCCAGCATCTACGTGCCCAATG-TAMRA; eRF1 forward primer, TGCATCTAACATTAAGTCACGAGT; eRF1 reverse primer, TCCACAGTATACAACCAGACCATT; and probe eRF1, 6FAM-AACCGCCTTTCAGTCCTGGGAGCC-TAMRA.

eEF1A1, eEF1A2 Assays By Demand and 18S rRNA Pre-developed TaqMan Assay Reagent were purchased from Applied Biosystems.

The amplification reactions were carried out in triplicate for each sample and the relative mRNA levels ($2^{-\Delta\Delta CT}$) were calculated with the mean C_T values obtained for the genes in study and the endogenous control 18S rRNA.

Results

Gene expression

MCF-7 cells were incubated with different NaBu concentrations for 24 h, and changes in the levels of eEF1A1, eEF1A2, eRF1 and eRF3 mRNA were evaluated by real-time quantitative RT-PCR analysis.

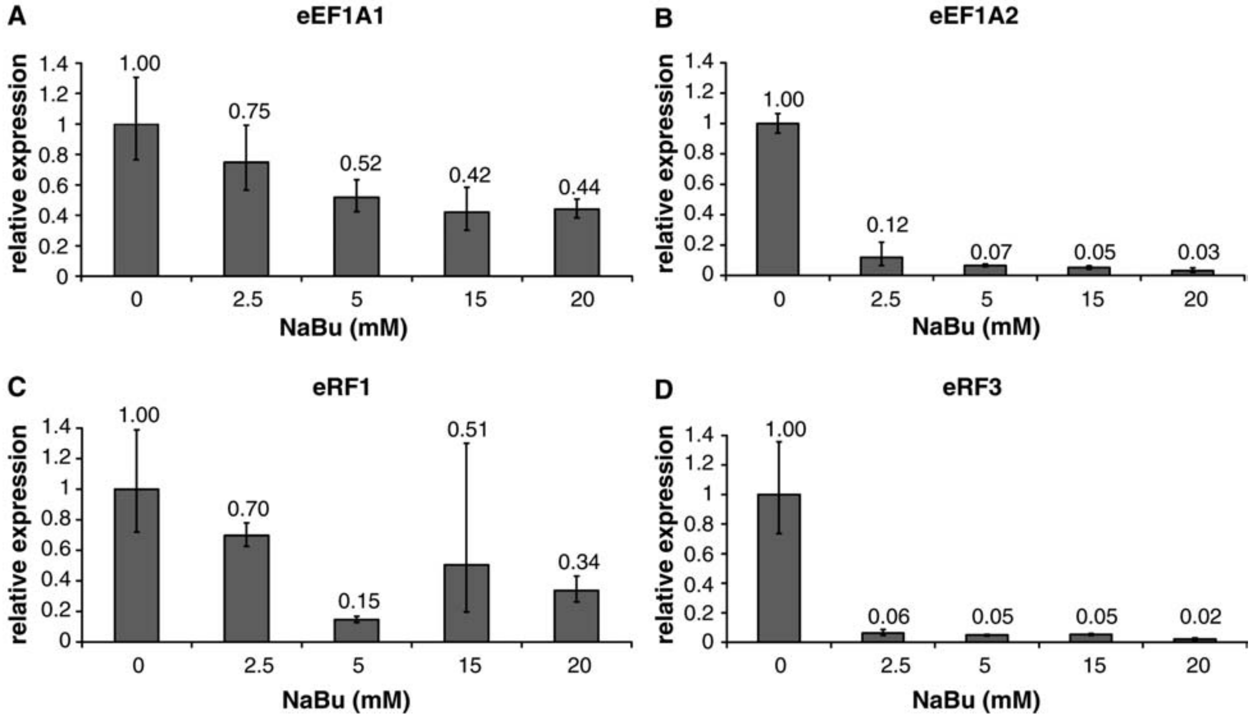

Figure 1 Quantification of eEF1A1, eEF1A2, eRF1 and eRF3 mRNA levels after treatment with NaBu. MCF-7 cells were treated with different NaBu concentrations for 24 h and then collected for real-time quantitative RT-PCR analysis. The mRNA levels of eEF1A1, eEF1A2, eRF1 and eRF3 were normalized with the mRNA level of the endogenous control 18S rRNA and compared with the untreated control, which gives the relative expression ($2^{-\Delta\Delta CT}$) of each gene.

The treatment with NaBu resulted in a decrease in the mRNA levels of all the translation factors analyzed, even at the lowest concentration (2.5 mM) (Figure 1).

For the elongation factor eEF1A1 a reduction in the mRNA level was observed, reaching a 2.3-fold decrease with the highest NaBu concentrations used (15 and 20 mM) (Figure 1A). For the termination factor eRF1, transcription inhibition was also observed and an approximately three-fold reduction in the mRNA level was achieved with 20 mM NaBu (Figure 1C).

A more dramatic effect was observed for the elongation factor eEF1A2 (Figure 1B), with the degree of mRNA reduction more pronounced (8.3-fold decrease with 2.5 mM).

For eRF3 the levels of mRNA obtained after NaBu treatment were practically the same for all NaBu concentrations used and the decrease observed was also very high (16.7-fold for 2.5 mM) (Figure 1D).

MCF-7 cells were also treated with two TSA concentrations (5 and 20 ng/mL) for 24 h and the same analysis of the translation factors' mRNA expression was carried out. The results of this treatment were clearly different from the previous ones obtained with NaBu.

Treatment with the lower TSA concentration (5 ng/mL) produced no effect on the transcription of eEF1A1, whereas the higher concentration led to up-regulation of the gene, with the mRNA level almost three-fold higher than the control (Figure 2A). For eEF1A2 neither TSA concentration altered the level of transcription (Figure 2B).

With eRF1 no variation in the mRNA level was observed for treatment with 5 ng/mL, but up-regulation was observed with 20 ng/mL, with mRNA levels 2.5-fold higher than for the control (Figure 2C). For eRF3 both TSA concentrations led to an increase in mRNA levels and the degree of transcription stimulation was similar in the two cases (~3.4-fold higher than the control) (Figure 2D).

Discussion

It has been broadly described that the HDAC inhibitors NaBu and TSA have an antiproliferative effect on cancer cell lines. These agents cause cell cycle arrests in G1 and G2/M phases of the cell cycle and apoptosis. The mechanism of action responsible for these effects is not fully understood, although NaBu and TSA are known to influence the expression of several proteins involved in the control of cell cycle progression, such as cyclin D1 (5, 41), $p21^{waf1/cip1}$ (12), $p27^{kip7}$ (42, 43), $p15^{ink4b}$ (44), $p16^{ink4}$ (43) and others.

Here we show for the first time that NaBu and TSA affect the expression of translation factors. NaBu causes inhibition of eEF1A1, eEF1A2, eRF1, and eRF3 transcription, with the reduction in mRNA levels more pronounced for eEF1A2 and eRF3 (Figure 1). For TSA the cells exhibited a different response to the treatment. No reduction in mRNA levels was observed for any of the translation factors after treatment with both TSA concentrations. Treatment with 5 ng/mL had no effect on the expression of eEF1A1, eEF1A2, and eRF1 but it caused marked up-regulation of eRF3. The higher TSA concentration (20 ng/mL) also did not affect the expression of eEF1A2, but up-regulation of eEF1A1, eRF1 and eRF3 was observed (Figure 2).

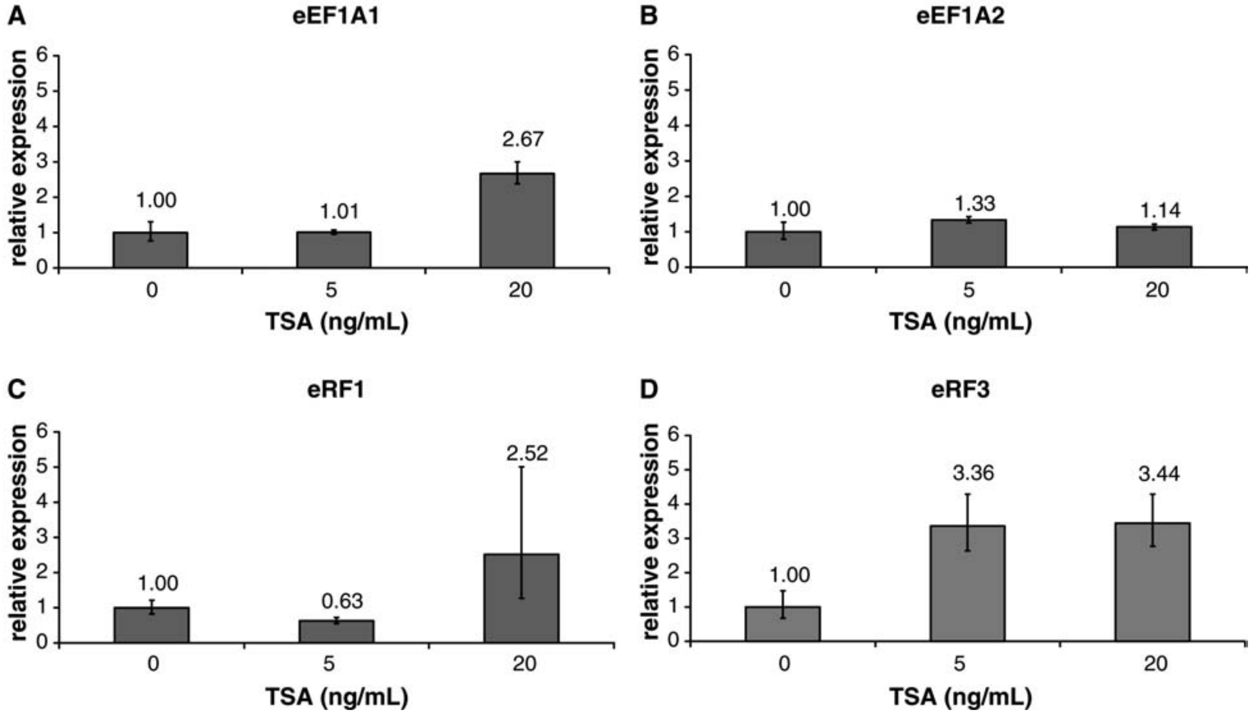

Figure 2 Quantification of eEF1A1, eEF1A2, eRF1 and eRF3 mRNA levels after treatment with TSA. MCF-7 cells were treated with two TSA concentrations for 24 h and then collected for real-time quantitative RT-PCR analysis. The mRNA levels of eEF1A1, eEF1A2, eRF1 and eRF3 were normalized with the mRNA level of the endogenous control 18S rRNA and compared with the untreated control, which gives the relative expression ($2^{-\Delta\Delta CT}$) of each gene.

These agents affect the expression of several genes via specific elements present in their promoter sequences, resulting in selective modulation of gene expression (1, 45) and this might also be the case for the genes studied in this work.

Several studies have shown that members of the translation mechanism are implicated in cancer development, with many of them being overexpressed in cancer cells (22). This may implicate an increase in protein production, which would contribute to cell proliferation, since cell division requires that a sufficient amount of protein is produced to fulfil the requirements of the two daughter cells. Moreover, overexpression of some translation factors can cause increased translation efficiency of the mRNA of some oncogenes, which can lead to cancer development (46–48). In addition, some translation factors, such as eEF1A1, eEF1A2 and eRF3, have other important roles apart from their function in protein synthesis. These factors are implicated in regulation of the cytoskeleton (20, 21, 34, 35) and their overexpression in tumors may have an effect on the invasiveness of cancer cells. Whether any of these translation factors regulates the expression of specific gene products that contribute to carcinogenesis is not known, although it has been shown that eRF3 can affect transcription (49).

The fact that the two eEF1-α isoforms are protective against apoptosis may also explain their contribution to tumorigenesis. Finally, it has been proposed that eEF1-α and eRF3 have a role in regulating the cell cycle at the G2/M phase and G1$\rightarrow$S phase transition, respectively (20, 36–38).

The possible inhibition of the translation factors by NaBu might in part be responsible for the antiproliferative effect that this agent has in cancer cells. This inhibition may cause a reduction in protein synthesis, which by itself would have a great impact on cell proliferation. In addition, a reduction in the levels of eEF1-α and eRF3 might contribute to the G1 and G2/M arrests, since both proteins seem to be necessary for progression through these phases of the cell cycle. Finally, since eEF1A1 and eEF1A2 inhibit apoptosis (20, 26), their down-regulation by NaBu probably contributes to the apoptosis induced by these agents, which was also observed by PARP cleavage, the well-known substrate of caspase, which was perceptible at 48 h for both drugs (data not shown).

The changes in the expression of these genes by the two agents may result from their HDAC inhibition activity, although the cells responded very differently to treatment with NaBu and TSA. Mechanisms other than HDAC inhibition might account for our observations. Furthermore, HDAC inhibitors can present proliferative effects at low doses and antiproliferative effects at higher doses. Thus, it is possible that down-regulation of these factors occurs at higher TSA concentrations.

The NaBu-induced inhibition of p53 observed in these cells (13) could also be responsible for the transcription inhibition of eEF1-α, since expression of this gene is controlled by the former protein, which binds to p53-specific responsive elements in the promoter of eEF1-α (50). On the other hand, TSA has been shown to up-regulate p53 in HepG2 human hepatoblastoma cells under hypoxic conditions (51). The inhibition of the other translation factors studied may also be explained by an alteration in the expression of a factor that controls their expression.

The present work shows for the first time inhibition of the translation mechanism by NaBu. This could be one possible mechanism by which NaBu induces cell cycle arrest and apoptosis. Moreover, we showed that another HDAC inhibitor, TSA, influences the translation mechanism in a different way, showing that these two agents may act by different pathways. Further experiments are necessary to clarify this matter.

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