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Biochemical and Biophysical Research Communications 359 (2007) 15–19
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Sodium butyrate modifies the stabilizing complexes of tyrosine hydroxylase mRNA

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Received 14 April 2007
Available online 14 May 2007

Abstract

Multiple mechanisms regulate the expression of the tyrosine hydroxylase (*Th*) gene, which encodes the rate-limiting enzyme in the biosynthesis of catecholamines. Sodium butyrate (SOB), a physiological histone deacetylase (HDAC) inhibitor, was reported to stimulate the *Th* gene promoter activity in reporter gene assays. However, the expression of the endogenous *Th* gene in PC12 cells was reported to be either stimulated or inhibited by SOB. Here, we report that SOB and other HDAC inhibitors drastically (up to 90%) and reversibly decrease the level of TH mRNA in PC12 cells. We also show that SOB does not influence the transcription initiation rate of the *Th* gene but perturbs the formation of protein–RNA complexes at the 3′UTR of the gene. Our results suggest that SOB inhibits the expression of the *Th* gene by destabilizing TH mRNAs.

Keywords: Tyrosine hydroxylase; PC12; RNA stability; HDAC; Sodium butyrate; TSA; Histone acetylation; Run-on assay; UV crosslinking

Catecholamines form a class of neurotransmitter molecules implicated in the regulation of a wide array of physiological and pathological processes. Tyrosine hydroxylase (TH) catalyses the first step of catecholamine biosynthesis. As the key enzyme of this pathway, it is under tight control at both short and long term [1,2]. The long-term regulation occurs at the various steps of the gene expression at the transcriptional and post-transcriptional levels. The molecular mechanisms of the transcriptional regulation of the *Th* gene have been extensively analyzed and several regulatory elements have been identified in the proximal promoter region [3]. In vitro, these elements were shown to contribute to the cell-type specific regulation of the gene [3]. Similarly, we had previously demonstrated in vivo that the tissue-specific basal expression of the *Th* gene requires either both or none of the proximal CRE and TRE regulatory elements [4]. Therefore, we hypothesized that the transcription factors that bind to these regulatory elements do not act independently but form a nucleation site that directs the assembly of an interdependent transcription complex. This model implies that the accessibility of transcriptional regulatory elements, which depends on the local chromatin environment and DNA conformational changes, influences the interactions among different actors of the complex.

Therefore, we have subsequently analyzed the human *TH* gene for the effect of DNA methylation, a factor known to contribute to the local chromatin structure [5]. Our results suggested transcriptional silencing of the gene by novel regulatory mechanisms depending on the tissue-specific chromatin environment.

In the present study, we investigated the role of nucleosomal histone acetylation on *Th* gene expression. Histone acetylation, a highly dynamic epigenetic modification, is generally accepted as a transcriptional activator signal [6]. In contrast, histone deacetylase (HDAC) complexes are present in hypermethylated and transcriptionally inactive genomic loci [7]. The pharmacological inhibition of these enzymes by sodium butyrate (SOB) or Trichostatine A (TSA) may lead to the activation of transcription.

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0006-291X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2007.05.025

During the last 30 years, SOB has been used in a number of studies aiming to better understand the regulation of *Th* gene expression. However, both the effect of the molecule and the underlying molecular mechanisms remain unclear [8–11]. Waymire et al. showed that SOB treatment increases Tyrosine hydroxylase activity [11], while Swerts and Weber found that SOB treatment diminishes the TH protein level [10]. More recently, it has been reported that 1 mM SOB treatment of the neuronal-like rat PC12 cells led to an increased endogenous TH mRNA abundance. In contrast, a 6 mM treatment was reported to cause the decrease of the endogenous TH mRNA concentration [9,12]. However, both low and high dose SOB treatment of the PC12 cells transfected with a reporter gene under the control of the *Th* gene promoter, led to an increased reporter gene expression [8,9,13,14].

In order to clarify the role of histone acetylation on *Th* gene expression, we investigated here the effects of HDAC inhibitors in PC12 cells. We demonstrated that the treatment leads to a drastic down-regulation of the expression of the endogenous *Th* gene without affecting the rate of transcription initiation. Most importantly, we showed that the treatment modifies the pool of protein complexes bound to the 3′UTR of the TH mRNA, suggesting that the main responsible molecular mechanism for the decreased expression is a lowered TH mRNA stability.

Methods

Cell culture and pharmacological treatment. PC12 cells were cultured in RPMI supplemented with 10% horse serum and 5% fetal calf serum.

RNA blot assays. Total cellular RNA was used in our experiments since in a first set of experiments the same level of TH mRNA down-regulation upon SOB treatment was measured from both mRNA and total RNA preparations ($91.7 \pm 4.1\%$ and $92 \pm 14.2\%$, respectively). Total RNA was extracted by RNABle (Eurobio) according to the manufacturer's instructions. Northern blot was carried out according to standard protocols [22] with 10 μg RNA per sample. Dot blot experiments were performed using a dot blot instrument with 5 or 10 μg of RNA according to the manufacturer's instructions. Full-length rat TH and Chat cDNA were used as probe templates.

UV crosslinking. UV crosslinking was performed as previously described [19]. The 162 base long radiolabeled RNA probe, corresponding to a fragment of the TH 3′UTR, was obtained after in vitro transcription by T7 RNA polymerase. Total cellular protein extracts were obtained as described by Schreiber and Matthias [23].

Run-on. Run-on experiments were performed on $3–5 \times 10^7$ cells, according to the protocol described in Current Protocols. Full-length *Th* genomic DNA, and a 8-kb *ChAT* genomic fragment were used as probes. As an internal control the total incorporated radioactivity was used. Experiments were performed in triplicate.

Results and discussion

Characterization of the effect of HDAC inhibitors on Th gene expression in PC12 cells

To test the role of HDAC inhibitors on the endogenous *Th* gene expression, PC12 cells were treated with either 5 mM SOB or 1 μM TSA for 24 h. The effect of the drugs was first tested on global H4 histone acetylation by Western blot with anti-acetylH4 antibody and, as expected, an increased acetylation was observed as compared to the control cells (not shown). Moreover, both drugs decreased the expression of TH mRNA, as assessed by Northern blot (Fig. 1a). A similar decrease was measured with HC-toxin, a third HDAC inhibitor (not shown). In parallel experiments, the same SOB treatment increased by 2-fold the level of mRNAs encoding choline acetyltransferase (ChAT), the biosynthetic enzyme of acetylcholine (Fig. 1b-A). This SOB-mediated up-regulation of ChAT gene expression rules out the possibility of general toxicity of the treatments and suggests a specific inhibitory effect of histone hyperacetylation on the *Th* gene expression.

Next, the effect of a 24-h SOB treatment on the expression of *Th* gene was analyzed in a concentration range from 0 to 5 mM. At concentrations up to 1 mM, no variations of TH mRNA levels could be detected. In contrast, a significant decrease of TH mRNA amounts was measured with 2.5 mM of SOB (70%), which reached 95% with 5 mM of SOB (Fig. 1b-B). Moreover, TSA treatment inhibited *Th* gene expression at much lower concentrations (starting from 500 nM) (Fig. 1b-C). The previously reported bimodal effect of HDAC inhibitors on *Th* gene expression in PC12 cells [9,12] was not observed in our experimental conditions. These articles reported an increase of *Th* gene expression with a 1 mM SOB treatment, and a decrease with a 6 mM SOB treatment. The discrepancy with our results is most likely due to variations in the origin of the PC12 cells used in the experiments.

The effect of HDAC inhibitors on *Th* gene expression was further characterized by kinetic studies. PC12 cells were treated with either 5 mM SOB or 1 μM TSA (these concentrations were used in all the following experiments), and the TH mRNA levels were quantified at different time points (Fig. 1b-D and E). After 6 h of treatment, only small TH mRNA level variations were measured upon SOB treatment. However, after 24 h of treatment, both drugs caused dramatic decrease of *Th* gene expression (Fig. 1b-D and E).

Finally, we addressed the question of the reversibility of the effect of SOB treatment (Fig. 1b-F). In contrast to the other conditions, cells treated first with SOB then with PBS were characterized by an intermediate amount of TH mRNAs. These results demonstrate that the down-regulation of the *Th* gene expression by SOB is at least partially reversible (Fig. 1b-F) and further confirm that it does not result from a general toxic effect of the treatment or a final differentiation of the cells.

Altogether these experiments demonstrate the dose-dependent and reversible down-regulation of the *Th* gene expression by HDAC inhibitors in PC12 cells. Although these molecules are generally known to be transcriptional activators, similar examples of gene expression inhibition by HDAC inhibitors have been previously reported [9,15–17]. However, the mechanisms underlying this inhibition had been little studied. Therefore, we further investi-

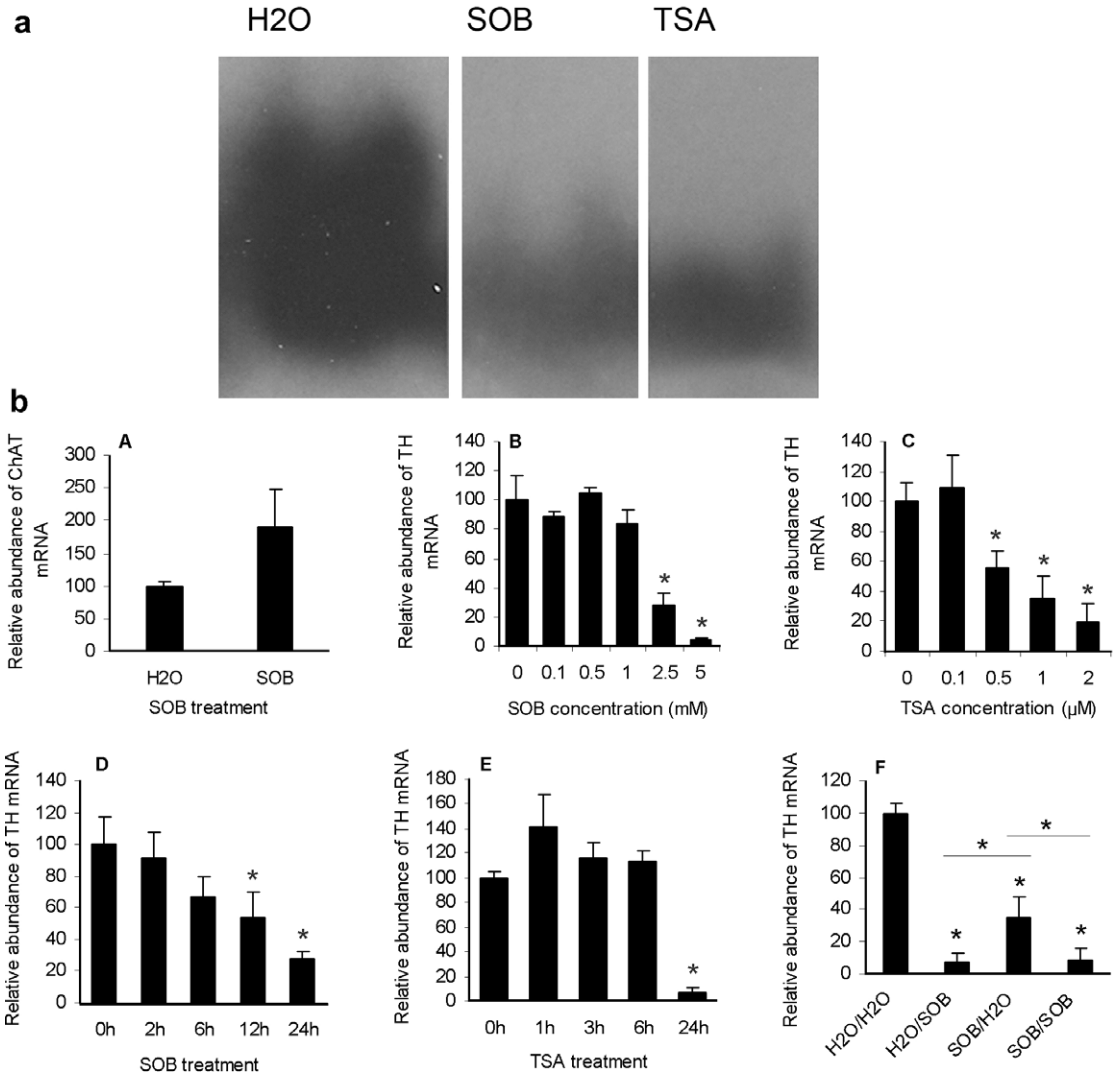

Fig. 1. Effects of HDAC inhibitors on PC12 cells. Cells were treated for 24 h either with 5 mM SOB or 1 μM TSA or vehicle (H_2O). (a) Northern blot analysis of TH mRNA expression was carried out with 10 μg of total cellular RNA per sample. (b) (A) Relative variation of ChAT mRNA levels measured after dot blot assay on 5 μg of total cellular RNA for each condition ($n = 3$). Dose–response effect of a 24-h treatment with SOB (B) and TSA (C), the kinetics of the effect of 5 mM SOB (D) and 1 μM TSA (E), and the reversibility of SOB treatment (F) were assessed by dot blot assay on TH mRNA. The sequential 24-h treatments are indicated below the bars. Relative expression levels are shown as percentages of the controls at the indicated concentrations and times. Experiments were carried out in triplicate, at least in two independent experiments. A representative experiment is showed in each case. $^{*}p < 0.05$ significance calculated by *t*-test.

gated the molecular mechanisms leading to this atypical down-regulation.

Effect of HDAC inhibitors on Th gene transcription

In order to assess whether SOB regulates the initiation rate of transcription of *Th* gene, nuclear run-on experiments were performed on isolated nuclei of PC12 cells. First, the initiation rate of *Chat* gene transcription was analyzed upon SOB treatment in a control experiment (Fig. 2A). SOB induced the transcription of *Chat* gene (Fig. 2A) which is in accordance with the results of dot blot assays, i.e. an increase in the amount of *Chat* mRNA (Fig. 1B). This result validates the run-on experimental set-up and demonstrates that the increase of ChAT mRNA expression is due to the transcriptional activation of the gene.

Next, the transcription initiation rate of the *Th* gene was analyzed to test whether SOB treatment modifies its promoter activity. Interestingly, and in contrast to the *Chat* gene, SOB had no effect on the initiation of *Th* gene transcription (Fig. 2B), although it induced a nearly complete loss of mature TH mRNAs (Fig. 1b-B, D and F). These experiments demonstrate that the inhibition of *Th* gene expression by SOB is not mediated by down-regulation of transcription initiation. Recently, Parab et al. [18] observed an approximately 90% decrease of TH mRNA level after 6 mM SOB treatment of PC12 cells and reported an intriguing induction of TH mRNA transcription initiation rate of about 3-fold by run-on experiments, in contra-

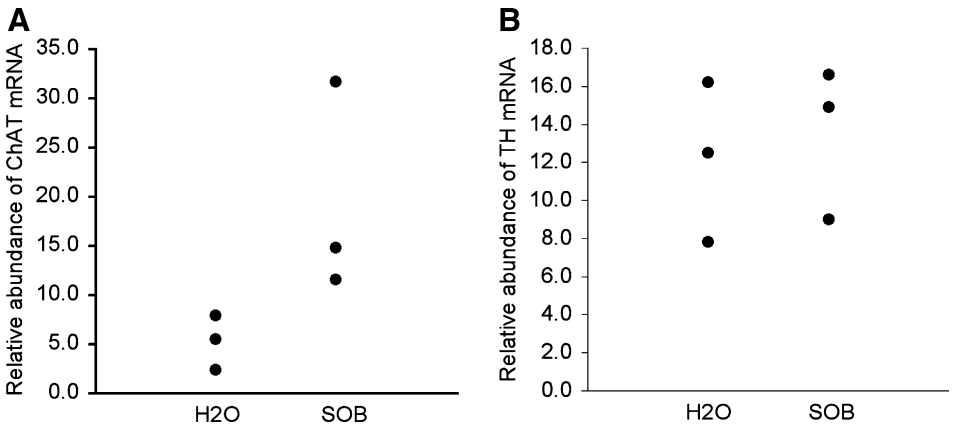

Fig. 2. TH mRNA transcription rate is not affected by SOB treatment. Run-on assays were performed on SOB treated and control samples to quantify the transcription rate of *Chat* (A) and *Th* (B) genes. Data from representative experiments performed in triplicate are shown. Each dot corresponds to the relative transcription rate of a single sample. The RNA labeled during the run-on was hybridized to a nitrocellulose membrane, where the relevant unlabeled probes were blotted previously. The signal intensity of each sample determined after hybridization was normalized to the sample's total incorporated radioactivity.

diction with our results. They demonstrated that the effect of SOB is mediated by newly synthesized proteins and suggested that this leads to the decrease of the stability of TH mRNA. Accordingly, we hypothesized that change in protein synthesis may lead to the modification of mRNA stability through the modification of complexes binding to the TH 3′UTR.

Effect of HDAC inhibitors on TH mRNA stabilization

In our model, SOB may either increase the expression of a destabilizing factor or decrease the expression of a stabilizing protein, both of which would lead to a reduced TH mRNA half-life. In a previous study, we demonstrated that a 162 base long fragment of the TH 3′UTR is crucial for the transcript stability [19]. To test our hypothesis, the formation of protein–RNA complexes was analyzed by UV crosslinking experiments on this previously described TH 3′UTR fragment.

Protein extracts from both treated and untreated cells were able to form RNA/protein complexes ranging from 39 to 105 kDa (Fig. 3). However, the patterns of the complexes differed in the two conditions. The previously reported 105 kDa complex A [19] could only be detected in untreated cells suggesting that SOB treatment led to the down-regulation of the genes encoding the complex-forming proteins. According to our model, although the nature of this complex is unknown, we suggest that it has a stabilizing effect on the TH mRNA. In contrast, the intensity of complexes B (70 kDa) and E (39 kDa) was significantly increased after SOB treatment, suggesting that SOB induces the expression of the genes encoding the complex-forming proteins. According to our model, complexes B and E should destabilize the TH mRNA.

The most abundant complexes had an estimated molecular weight of 40 (D) and 54 (C) kDa. The molecular weight of complex C indicates that it is probably the poly C binding protein identified by Czyzyk-Krzeska and Beresh [20], previously shown to be resolved as a doublet [19] (Fig. 3). The intensity of complexes C and D were comparable between SOB-treated and untreated cellular extracts. However, complex C exhibited faster migration profile in the treated samples relative to the controls. This suggests a SOB-mediated post-translational modification

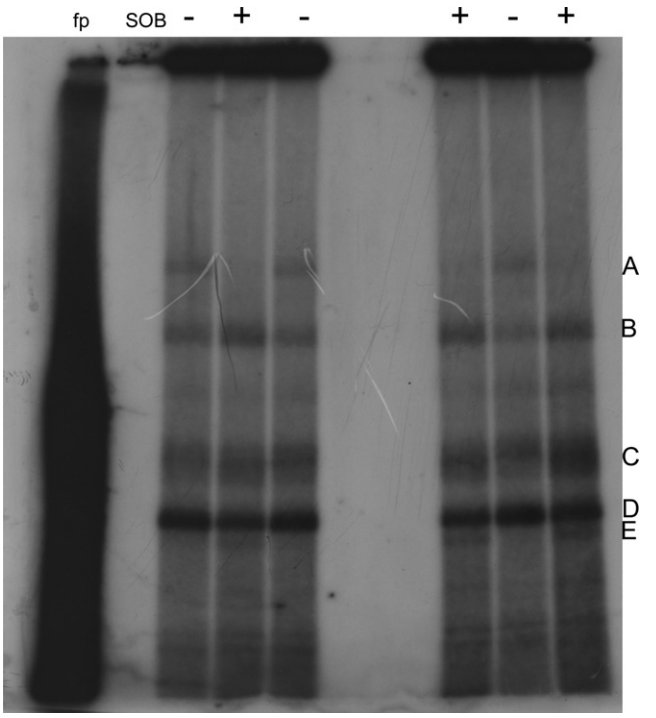

Fig. 3. SOB treatment modifies the RNA–protein complex formation at the 3′UTR of the TH mRNA as revealed by UV crosslinking assay. The experiment was performed in triplicate for SOB (+) or vehicle (−) treated cells. RNA–protein complexes are named from A to E, while fp stands for the lane loaded with the free probe.

of the complex-forming proteins leading to the altered migration profile.

Taken together, these results suggest that SOB indirectly influences the TH mRNA half-life by modifying the amount of the 3′UTR binding proteins, or it may directly or indirectly induce their post-translational modifications. Our data suggest that this is not mediated by the previously described butyrate response factors BRF1 or BRF2 evoked by Parab et al. [18] since the molecular weight of none of the complexes identified in the present study corresponded to the BRF proteins [21]. This is not surprising since these proteins are characterized by their binding capacity to 3′UTRs with ARE (AU rich element), which is absent from the TH mRNA 3′UTR.

In conclusion, in PC12 cells the inhibition of HDACs, a treatment which usually activates gene expression, reversibly down-regulates the expression of the *Th* gene. Our analysis suggests that the inhibition of *Th* gene expression is regulated by indirect molecular mechanisms, probably via the alteration of TH mRNA binding complexes. Most notably, a relatively fast and strong down-regulation of TH mRNA levels was demonstrated in our study, without any effect on the transcription initiation rate of the *Th* gene, suggesting that alteration of mRNA stability alone could lead to an almost complete gene silencing. This regulation process might be a general feature in the regulation of expression of genes and may be particularly relevant for genes characterized by an unusually long half-life of their mRNA under physiologic conditions. This mechanism may also represent an alternative to regulation by RNA interference for the degradation of particularly stable mRNAs.

Acknowledgments

The Anti-acetylH4 antibody was a generous gift from Laszló Tora. T.A. was financed by ARC and IRME fellowships. We are very thankful to Dr. Nicole Faucon Biguet for helpful discussions.

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