Volume 312, number 1, 42–46 FEBS 11694 November 1992

The thyroid hormone response element is required for activation of the growth hormone gene promoter by nicotinamide analogs

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Received 15 September 1992

N'-Methylnicotinamide and nicotinamide, which decreased in vitro ADP-ribosylation of nuclear proteins and/or cellular NAD^+ content, selectively increased the basal expression of the rat growth hormone (GH) gene promoter and its response to triiodothyronine (T3). This increase was not found when the thyroid hormone response element (TRE) was deleted from the promoter. Transfection with an expression vector for the T3 receptor inhibited basal activity of the TRE-containing promoter and repressed the stimulatory effect of *N'*-methylnicotinamide. The addition of hormone relieved this inhibition and enhanced transcription above levels found in the absence of the transfected receptors. These results suggest a modulatory role of ADP-ribosylation in hormonal regulation of gene expression.

Triiodothyronine; Nicotinamide; *c-erbA*; Growth hormone promoter

1. INTRODUCTION

Nuclear proteins are subjected to covalent post-translational modifications which cause changes in the structure of nucleosomes and influence transcriptional regulation. It has been suggested that ADP-ribosylation of chromosomal proteins serves as a negative regulator for gene expression [1,2]. A decrease in ADP-ribosylation has been shown to correlate with an increase in non-specific RNA synthesis [1], but it can also influence the expression of specific genes [2,3]. Kimura et al. [3] have shown that compounds that decrease ADP-ribosylation specifically increase growth hormone (GH) synthesis and the level of GH-mRNA in cultured pituitary GH3 cells. The expression of the GH gene is under hormonal control and those compounds also potentiate the induction caused by the thyroid hormone triiodothyronine (T3) [3]. Additionally, we have shown that T3 alters ADP-ribosylation of chromatin proteins in pituitary GH1 cells [4] and Tanuma et al. [2] have described a similar effect for glucocorticoid hormones in mammary cells suggesting a modulatory role of this modification on hormonal regulation of gene expression.

The 5′ flanking region of the rat GH gene contains elements mediating pituitary-specific expression and response to hormonal signals [5]. Two cell-specific elements located from −95 to −65 and from −137 to −107 in the gene are binding sites for the pituitary-specific transcription factor GHF-1 [6], a homeodomain protein also known as Pit-1 [7]. Thyroid hormone action is mediated by binding to nuclear receptors, the product of *c-erbA* proto-oncogenes, which function as ligand-inducible transcriptional enhancer factors [8,9]. We and others have previously shown that sequences between −209 and −146 of the rat GH gene function as a thyroid hormone responsive element (TRE) and mediate thyroid hormone stimulation [10–12]. In this study we have investigated the effect of nicotinamide analogs that inhibit ADP-ribosylation, alone or in combination with T3, on hormone-dependent and -independent transcription of the GH gene promoter.

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Abbreviations: GH, growth hormone; T3, triiodothyronine; TRE, thyroid hormone response element.

2. MATERIALS AND METHODS

2.1. *Plasmids*

The −144GH-CAT and −530GH-CAT constructs contain 144 or 530 base pairs, respectively, of 5′ flanking DNA of the rat GH gene fused to chloramphenicol acetyltransferase (CAT) gene [12]. In another GH-CAT construct the fragment −236/−530, has been ligated upstream of a minimal GH gene promoter (−104/+11) [12] . The GHF-1 CAT plasmid contains 400 base pairs of the rat GHF-1 gene ligated to CAT [13], and in the RSV-CAT plasmid the CAT gene is under control of enhancer and promoter sequences of the Roux sarcoma virus long terminal repeat. A vector expressing the chick thyroid hormone receptor *c-erbAα* under control of the RSV promoter has been previously described [14].

2.2. *Cell culture and DNA transfection*

GH1 cells were cultured as previously described [15] in DMEM medium containing 10% AG1 × 8 resin–charcoal stripped newborn calf serum [16] to ensure cellular depletion of thyroid hormone. The cells were transfected by electroporation as described [12,14,15]. 10 μg of the reporter plasmids were mixed with 20–40 million cells and exposed to a high voltage pulse (170–200 V, 960 μF). The cells from each electroporation were split in different culture plates and incubated for 48 h with the compounds indicated. At this time a maximal

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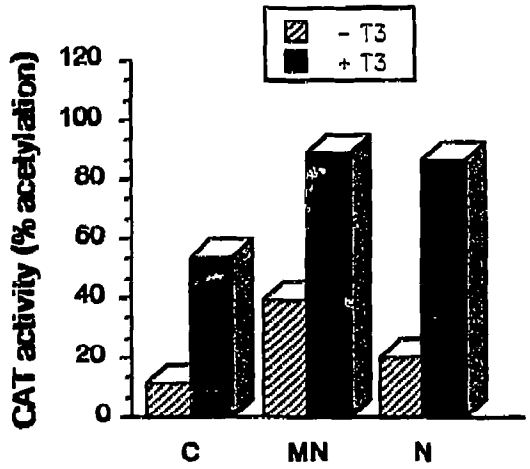

	T3 receptor levels	Intracellular NAD	[^{3}H]ADP-ribose incorporation
C	160±7	5.2±0.8	11347±1243
MN	72±5	1.5±0.3	6764±945
N	122±9	9.7±1.3	8184±367

Fig. 1. (Left) GH1 cells were transfected with the reporter plasmid −530GH-CAT and incubated for 48 h before determination of CAT activity in control medium (C) or in medium containing 10 mM *N'*-methylnicotinamide (MN) or 25 mM nicotinamide (N) in the absence (stripped bars) or presence (black bars) of 1 nM triiodothyronine (T3). (Right) T3 receptor levels, intracellular NAD content and [^{3}H]ADP-ribose incorporation into isolated nuclei were determined in triplicate cultures of control cells (C) and cells treated with the same concentrations of *N'*-methylnicotinamide (MN) or nicotinamide (N) for 24 h. The data are mean ± S.D.

CAT response was found. Each treatment was performed in duplicate cultures that normally showed less than 5–10% variation in CAT activity. CAT activity was determined, as previously described [12,15], by incubation of the cell extracts (15–50 μg) with [^{14}C]chloramphenicol. The unreacted and acetylated [^{14}C]chloramphenicol were separated by thin layer chromatography, identified by autoradiography and quantitated.

2.3. *Quantitation of T3 nuclear binding*

The level of T3 receptors was determined by incubation of the cell monolayers in serum-free medium for 90 min with 0.8 nM [^{125}I]T3 [16,17]. This concentration of T3 gives an estimate of the total levels of receptor since it binds to more than 90% of the thyroid hormone receptor population.

2.4. *ADP-ribosylation in isolated nuclei*

ADP-ribosylation assays were carried out as previously described [18] by incubation for 15 min at 37°C in 0.5 ml of 0.1 M Tris-HCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.9, containing 0.25 μCi [^{3}H]NAD^+ and 0.5 mM unlabeled NAD. The nuclei were washed and the nuclear material precipitated with 18% trichloroacetic acid (TCA). The TCA-insoluble material was dissolved in 0.4 N NaOH and an aliquot was used to analyze [^{3}H]ADP-ribose incorporation. Another aliquot was saved for determination of proteins [19].

2.5. *Determination of cellular NAD^+ levels*

0.5 N perchloric acid extracts of GH1 cells [18] was assayed for NAD using a spectrophotometric technique [20].

3. RESULTS

Fig. 1 (right) shows that incubation with 10 mM *N'*-methylnicotinamide decreased by approximately 50% ADP-ribose incorporation into nuclear proteins and reduced by more than 70% the cellular levels of NAD^+, the substrate for the reaction of ADP-ribosylation. This compound also produced a significant decrease of thyroid hormone receptor levels (more than 50%). Higher concentrations of *N'*-methylnicotinamide did not produce further decreases (not shown). Nicotinamide (25 mM) also inhibited ADP-ribose incorporation and did not decrease but rather increased NAD^+ levels, probably as a consequence of its reduced utilization for ADP-ribosylation. To examine the influence of the inhibitory compounds on GH gene transcription, transient gene expression was performed using a −530GH-CAT construct. Fig. 2 (left) shows that incubation with *N'*-methylnicotinamide increased activity of the GH promoter by more than 3-fold and potentiated the action of T3. The combination of T3 and *N'*-methylnicotinamide caused an almost 10-fold increase in CAT activity. Nicotinamide was less effective in increasing basal promoter expression by 2-fold, but enhanced the response to T3. As shown in the figure both compounds potentiated the effect of T3 on the GH gene and significantly reduced the number of its nuclear receptors.

The fragment of the GH gene promoter in the −530GH-CAT plasmid includes the TRE which mediates regulation by the T3 receptors [10–12]. To examine whether the increased activity caused by the inhibitors of ADP-ribosylation was dependent on the presence of the TRE, the effect of *N'*-methylnicotinamide on the expression of a plasmid in which the sequences between −104/−236 that contain the TRE have been deleted was also examined. Fig. 2 compares the effect of T3 and *N'*-methylnicotinamide on the −530GH-CAT and the −530(Δ−104/−236)GH-CAT plasmids. The stimulatory effect on the GH gene promoter was not found in the absence of the TRE since neither compound increased significantly CAT activity from the later construct. Similar results were obtained with a −144GH-CAT plasmid since neither T3 nor nicotinamide analogs enhanced its activity, thus showing that the sequences −137/−107 do not mediate the enhanced CAT activity by *N'*-methylnicotinamide.

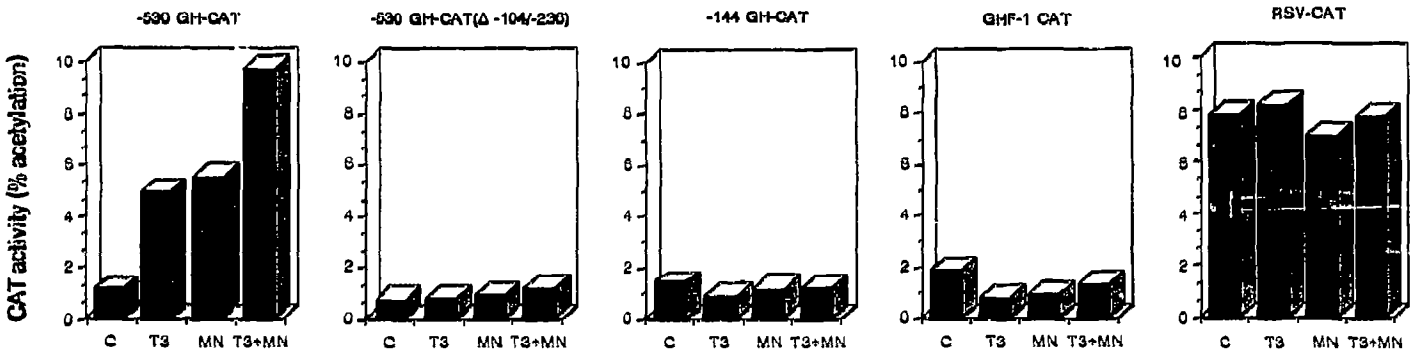

Fig. 2. CAT activity was determined in GH1 cells transfected with the constructs indicated in the top of the figure and incubated for 48 h with 1 nM triiodothyronine (T3), 10 mM *N'*-methylnicotinamide (MN) or the combination of both.

Because the expression of the GH gene depends on the binding of the transcription factor GHF-1 to its cognate sites [6,7], we examined the possibility that the stimulatory effect of *N'*-methylnicotinamide could be due to increased GHF-1 expression. For this purpose, GH1 cells were transfected with a reporter plasmid containing the GHF-1 promoter. Fig. 2 also shows that *N'*-methylnicotinamide did not increase GHF-1-CAT activity whether alone or in combination with T3. That the stimulatory effect of *N'*-methylnicotinamide is specific for the GH gene promoter is also confirmed by the finding that this compound did not alter the expression of a control plasmid containing the RSV promoter. T3 did not affect RSV-CAT activity either.

To examine the effect of over-expression of the thyroid hormone receptor on the response to T3 and *N'*-methylnicotinamide, the −530GH-CAT plasmid was transfected alone (Fig. 3A) or co-transfected with an expression vector encoding the receptor (Fig. 3B). The vector contains the cDNA sequences of the thyroid hormone receptor *c-erbAa* under control of the RSV promoter. Over-expression of *c-erbA*, in the absence of

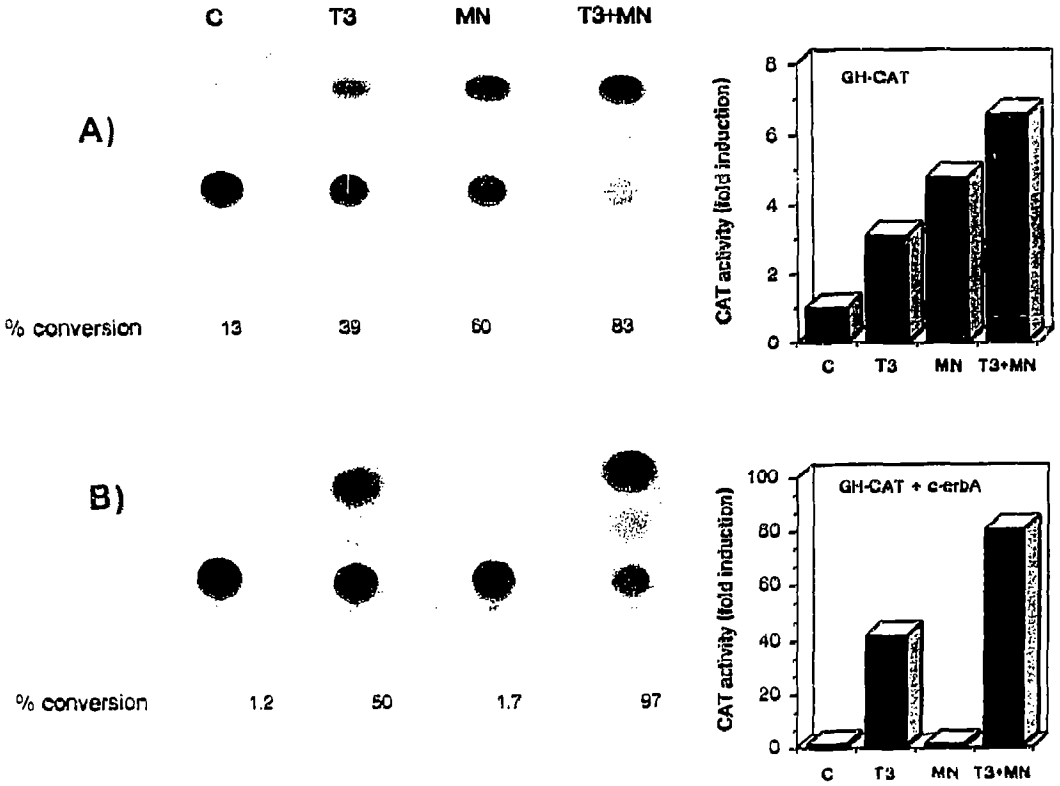

Fig. 3. The cells were transfected with the −530GH-CAT reporter plasmid alone (panel A) or in combination with 10 μg of a *c-erbA* expression vector (panel B) and treated for 48 h with 1 nM T3 or 10 mM *N'*-methylnicotinamide. Autoradiographs showing the conversion of [^{14}C]chloramphenicol to its acetylated forms are shown in the left panels and CAT activity expressed as fold increase above the levels found in controls (C) is illustrated in the right panels.

hormone, resulted in a significant decrease of the basal activity of the TRE-containing promoter. The addition of hormone relieved this inhibition and enhanced transcription above levels found in the absence of the transfected receptors. The right panels in Fig. 3 show that the response to T3, expressed as fold-induction over the corresponding basal levels, increased from approximately 3-fold to almost 50-fold in the cells transfected with *c-erbA*. Additionally, the transfected receptor repressed the stimulatory effect of *N'*-methylnicotinamide, decreasing from a 5-fold induction in the absence of exogenous receptor to a less than 1.5-fold induction, after over-expression of the receptor. Again, this inhibition was overcome by the ligand: CAT activity increased by more than 80-fold in receptor-transfected cells treated with T3 plus *N'*-methylnicotinamide.

4. DISCUSSION

Our results show that the increase of GH synthesis produced by nicotinamide analogs [3] occurs at the transcriptional level and can be attributed to enhanced expression of the GH gene. These compounds specifically increased basal and thyroid hormone-induced expression of the GH gene promoter without affecting transcription from another reporter gene driven by a control promoter.

In agreement with previous results in pituitary cells [3] we find that nicotinamide and *N'*-methylnicotinamide decrease in vitro ADP-ribose synthetase activity and/or decrease the cellular concentration of NAD^+ the substrate for ADP-ribosylation. Since this modification produces conformational changes in chromatin [21], nicotinamide analogs might affect conformation of the GH promoter, facilitating the accessibility of regulatory proteins binding to this promoter. In this respect, the element that mediates thyroid hormone stimulation of the GH gene present at −167/190 seems to be required for activation by nicotinamide and *N'*-methylnicotinamide, since they do not enhance expression of a GH promoter in which the TRE has been deleted. Additionally, these compounds were also effective in increasing the activity of an heterologous (MMTV) promoter containing the natural TRE of the rat GH gene (data not shown). These results suggest that a decrease in ADP-ribosylation may induce an altered configuration of the TRE, facilitating binding of the receptors to the modified chromatin. This would be in agreement with the finding that induction of transfected genes by progesterone or glucocorticoid hormones, which bind to similar nuclear receptors, depends on DNA topology [22].

The transcriptional effect of T3 on the GH gene is accompanied by a down-regulation of its receptor [17]. Interestingly, both *N'*-methylnicotinamide and nicotinamide stimulate basal expression and the response of the GH promoter to thyroid hormone and, concomitantly, cause a decrease in T3 receptor level in GH-producing cells. A possible interpretation of these findings is that the receptors could act as repressors in the absence of hormone. This is supported by the finding that transfection of an expression vector encoding the thyroid hormone receptor led to a ligand-independent inhibitory effect on transcription, suggesting that the receptors could act as specific repressors of GH gene expression in T3 deprived cells. A combination of repression of basal expression of TRE-containing promoters by unoccupied receptors and an increase in hormone-induced expression has been previously proposed [23–25]. Furthermore, our data show that the over-expressed receptors are also able to counteract the stimulation caused by nicotinamide analogs, suggesting that the binding of hormone receptors could locally alter the chromatin structure and inhibit gene expression. This inhibition is totally overcome when the thyroid hormone binds to the receptor. A direct demonstration that chromatin structural changes occur after glucocorticoid hormone administration and receptor binding has been reported by others [26].

The precise molecular mechanism of transcriptional control by the thyroid hormone receptor is not known, but it is assumed that occupancy of the receptor by the ligand somehow facilitates the access of other transcription factors to the regulated promoter which stimulates the level of gene expression. As far as the GH gene is concerned, binding of GHF-1 to its cognate sites in the promoter is required for its basal expression in a cell-specific manner [6,7]. However, the stimulatory action of nicotinamide analogs does not involve an increase in GHF-1 since they did not stimulate expression of the TRE-deleted GH promoter which contains a GHF-1 binding site, or the activity of the GHF-1 promoter. Whether the treatment with these agents that decrease ADP-ribosylation enhance the functional cooperation of the receptors with GHF-1, or facilitate its interaction with other still unidentified components of the transcriptional machinery remains to be established.

Acknowledgments: This work was supported by Grants PM88-0007 from the DGICYT and C051/91 from the Comunidad de Madrid. We thank Dr. H. Samuels and M. Karin for plasmids used in this study and Aida Villa for her expert technical assistance.

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