Nur77, a member of the steroid receptor superfamily, antagonizes negative feedback of ACTH synthesis and secretion by glucocorticoid in pituitary corticotrope cells

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Abstract

Nur77 is a member of the steroid receptor superfamily and is known to be expressed in animals under stress. We studied the role of nur77 in the regulation of the hypothalamic–pituitary–adrenal (HPA) axis during the stress response using a murine pituitary corticotrope cell line, AtT-20. Corticotropin-releasing hormone (CRH), a stress mediator in the HPA axis, induced the expression of nur77 transiently in AtT-20 cells. Gel shift assay showed that nur77 bound to negative glucocorticoid responsive element (nGRE) in the promoter of the human pro-opiomelanocortin (POMC) gene and the formation of the nur77-nGRE complex increased after treatment of the cells with CRH. Negative GRE is known to be necessary

for the negative regulation by glucocorticoid of the POMC gene expression. In stable transformants of AtT-20 cells expressing a human homolog of nur77, NAK-1, at a high level, glucocorticoid-mediated inhibition of both POMC mRNA induction and ACTH secretion was significantly lower than that in the NAK-1-non-expressing cells ($P < 0.001$). These results strongly suggest that nur77 antagonizes the negative feedback effect of glucocorticoid on the synthesis and secretion of ACTH in pituitary corticotropes. This suggests that nur77 plays an important role in the pituitary gland in the biological adaptation to overcome stress.

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Introduction

Pro-opiomelanocortin (POMC) is a precursor protein of a variety of peptide hormones including adrenocorticotrophic hormone (ACTH) which is involved in the mammalian homeostatic response to stress. In response to stress, the hypothalamic–pituitary–adrenal (HPA) axis is activated and the corticotropin-releasing hormone (CRH) released from the hypothalamus increases both the secretion of ACTH and the transcription of the POMC gene in the pituitary gland. ACTH increases both the secretion of glucocorticoids from and the transcription of several steroidogenic enzymes in the adrenocortical gland, thus resulting in an increase in circulating glucocorticoids. Glucocorticoids, in turn, exert negative feedback at both the pituitary and the hypothalamic level. At the pituitary level, glucocorticoids inhibit the secretion of stored ACTH and also repress the transcription of the POMC gene (Autelitano *et al.* 1989, Therrien & Drouin 1993). However, when stress continues, the blood concentration of glucocorticoids is held at an increased level, which also helps to maintain homeostasis (Meikle 1989). Therefore, in the presence of chronic stress, some antagonistic mechanism against the negative feedback effect of glucocorticoids in the hypothalamus and/or the pituitary gland must exist.

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Using transgenic mice and transfection experiments into cell lines it has been shown that the POMC 5'-flanking sequence is both necessary and sufficient to confer glucocorticoid-mediated inhibition of the transcription of the POMC gene (Israel & Cohen 1985, Tremblay *et al.* 1988, Hammer *et al.* 1990, Nakai *et al.* 1991, Riegel *et al.* 1991, Therrien & Drouin 1993). The analysis of the POMC promoter in a pituitary corticotrope cell line, AtT-20, indicated that the *in vitro* binding site for glucocorticoid receptor located in the proximal region of the promoter is necessary but not, by itself, sufficient for the glucocorticoid-mediated repression of POMC (Drouin *et al.* 1989, 1993). This *in vitro* binding site for glucocorticoid receptor is called the negative glucocorticoid responsive element (nGRE) and its sequence is different from that of a consensus glucocorticoid responsive element (GRE) (Drouin *et al.* 1993). In addition, the molecular mechanism by which glucocorticoids inhibit the transcription of the POMC gene has yet to be elucidated.

Nur77 (Hazel *et al.* 1988) belongs to the steroid receptor superfamily (Evans 1988) and has a structure common to this superfamily, consisting of a highly conserved

DNA-binding domain, a less conserved ligand-binding domain and a little conserved N-terminal domain. Nur77 is also called N10 (Ryseck *et al.* 1989) in mice, NGFI-B (Milbrandt 1988) or TIS1 (Kujubu *et al.* 1987) in rats, and NAK-1 (Nakai *et al.* 1990) or TR3 (Lee *et al.* 1993) in humans. Nur77 is an orphan receptor whose ligand is unknown, and has the characteristics of an immediate-early gene, the expression of which is induced transiently by several stimuli such as growth factors. The DNA sequence to which nur77 binds is called the NGFI-B responsive element (NBRE), and the consensus sequence is 5'-AAAGGTCA-3', consisting of two 5' adenine nucleotides and the half site of the estrogen responsive element (Wilson *et al.* 1991). Nur77 binds to NBRE as a monomer or a heterodimer with retinoid-X receptor (Wilson *et al.* 1993b, Forman *et al.* 1995, Perlmann & Jansson 1995). The only known target gene for nur77 is a steroid 21-hydroxylase gene (CYP21) working in the adrenocortical gland (Wilson *et al.* 1993a). It has been suggested that nur77 is involved in the activation-induced cell death in T-cells and also in the regulation of the HPA axis (Kastner *et al.* 1995), although the precise molecular mechanism involved in the function of nur77 has not yet been clarified.

In the study on a role of nur77 in the HPA axis during a stress response, we found that an NBRE-like sequence 5'-GAAGGTCA-3' exists in the promoter of the POMC gene and it overlaps with nGRE 5'-GGGAAGGTCA AAGCCG-3', thus suggesting the possibility that the stress-induced nur77 regulates the POMC gene expression. In the present study, we demonstrate that nur77 is able to antagonize glucocorticoid-mediated inhibition of POMC gene expression in the corticotrope cell line AtT-20.

Materials and Methods

Cell culture

AtT-20 cells (ATCC CCL89), a murine corticotrope cell line which grows as small floating clusters, were cultured in RPMI 1640 (Life Technologies, Grand Island, NY, USA) containing 2 mM L-glutamine and 5×10^{-5} M 2-mercaptoethanol, supplemented with 10% fetal calf serum (FCS) (Hyclone, Logan, UT, USA). L-Glutamine and 2-mercaptoethanol were routinely added to RPMI 1640 in the present study. FCS in the culture media was replaced with dextran-coated charcoal (DCC)-treated FCS (namely, FCS which had been pretreated with DCC for 24 h) when ovine CRH (Peptide Institute, Osaka, Japan) or dexamethasone was used.

Establishment of NAK-1 stable transformants

AtT-20 cells were washed twice with phosphate-buffered saline (PBS) and were then resuspended in 0.8 ml FCS-

free RPMI 1640 (1×10^6 cells/0.8 ml). One hundred microliters FCS-free RPMI 1640 containing 1.8 µg NAK-1 expression vector, pCDMNAK-1 (Nakai *et al.* 1990), or pBluescriptSK(+) (Stratagene, Cambridge, UK) and 0.2 µg pSV2neo (Southern & Berg 1982), and another 100 µl FCS-free RPMI 1640 containing 10 µl Lipofectamine (Life Technologies) were mixed gently, incubated for 15 min at room temperature, and added to 0.8 ml of the cell suspension. After incubation for 5 h at 37 °C in a CO₂ incubator, 4 ml RPMI 1640 with 10% FCS were added to the cells. After further incubation for 43 h, the cells were split into 12-well plates (Falcon, Lincoln Park, NJ, USA), and cultured for one month in RPMI 1640 with 10% FCS in the presence of 0.5 mg/ml G418 (Life Technologies). To screen the clones expressing NAK-1, the whole mass of the G418-resistant cells in each well was subjected to dot blot analysis using ³²P-labeled NAK-1 cDNA as a probe. Finally, four different pools (namely, four different wells) of mixed clones expressing NAK-1 (NAK-1 stable transformants) and four different pools of clones not expressing NAK-1 (mock transformants) were selected.

Northern blot analysis

AtT-20 cells of non-transformants or the stable transformants were cultured in 25-cm² suspension culture flasks. Total RNA was prepared from the cultured cells ($\sim 5.0 \times 10^6$ cells) by an acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski & Sacchi 1987) using Isogen (Wako Pure Chemical Co., Osaka, Japan). A Northern blot analysis was performed as described previously (Okabe *et al.* 1995). DNA probes were prepared by PCR using AtT-20 cell-derived cDNA as the template. The sequences of sense and antisense primers were 5'-TCCATAGATGTGTGGAGCTG-3' and 5'-GTTC TTGAAGAGCGTCACCA-3' (+61 to +80 and +653 to +672, the nucleotide positions of the sequences are represented by designating the translation start site as +1) respectively for the murine POMC mRNA, and 5'-TCATGGACGGGTACACCG-3' and 5'-GCTGGCA TGAATAGCTC-3' (+167 to +184 and +675 to +692) respectively for the murine nur77 mRNA. The validity of the PCR products was confirmed by direct sequencing. For the control probe, a full-length of murine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was used. The relative intensity of each band detected by Northern blot analysis was determined using a Bio Image Analyzer BAS 2000 (Fuji Photo Film Co. Ltd, Tokyo, Japan).

Electrophoretic mobility shift assay (EMSA)

An electrophoretic mobility shift assay was performed essentially as described previously (Okabe *et al.* 1992). Twenty-eight bp of double-stranded synthetic oligonucleotide

corresponding to the region 73 bp to 46 bp upstream of the transcription start site in the human POMC gene was used as a probe. The sequence of the upper strand of the oligonucleotide is 5'-TGCCGGAAGGTCAAAGTCCCGCGCCCA-3'. The nuclear extracts of AtT-20 cells were prepared according to the method of Dignam *et al.* (1983). Nuclear extracts (1 µg) and 5 fmol ³²P-labeled probe (20 000 c.p.m.) were used for each reaction. DNA-protein complexes were analyzed on a 5% native polyacrylamide gel. Antibodies (IgG) against nur77 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) or glucocorticoid receptor (Santa Cruz Biotechnology Inc.) were added to the reaction mixture before adding nuclear extracts.

Radioimmunoassay of ACTH

Cells were washed twice with PBS and then resuspended in RPMI 1640 containing 2.5% DCC-treated FCS at a density of 6×10^5 cells/ml. Five hundred microliters of this suspension (3×10^5 cells) were dispensed into each well of 48-well plates, and incubated for 48 h at 37 °C in the absence or presence of 10^{-7} M dexamethasone. Then, the ACTH content in 5 µl of each supernatant was determined using a rat ACTH RIA kit (Peninsula Laboratories Inc., Belmont, CA, USA) according to the manufacturer's protocol.

Statistical analysis

ANOVA and the Newman-Keuls test were used for multigroup comparisons. Student's *t*-test was used for comparisons between the two groups. $P < 0.05$ was considered statistically significant.

Results

Induction of the nur77 expression by CRH

The level of nur77 mRNA in CRH-treated AtT-20 cells was examined by Northern blot analysis. The expression of nur77 was weak in the resting state, but was markedly increased by 25 nM CRH with a peak at 1 h after the addition of CRH. Thereafter, levels decreased gradually to those of the resting state within 24 h. The expression of GAPDH, used as a control, did not change (Fig. 1).

Binding of nur77 to nGRE in the promoter of the human POMC gene

Using EMSA we examined whether or not nuclear extracts from AtT-20 cells contain the protein(s) which specifically binds to the nGRE sequence in the promoter region of the human POMC gene. Five shifted bands were detected and their intensity clearly increased after

Figure 1 Induction of nur77 mRNA in cultured AtT-20 cells by CRH. AtT-20 cells (5×10^6 cells) were cultured in RPMI 1640 with 2.5% DCC-treated FCS in 25-cm² suspension culture flasks for 24 h and then incubated with 25 nM CRH. The cells were harvested at the time indicated above each lane, and the total RNA was prepared and subjected to Northern blot analysis using probes for nur77 and GAPDH simultaneously, as described in Materials and Methods. The bands corresponding to nur77 and GAPDH mRNA are indicated by the arrows on the left, and the positions corresponding to 18S and 28S ribosomal RNAs on the right.

treatment of the AtT-20 cells with 25 nM CRH for 2 h (Fig. 2A, lanes 1 and 2). Binding to the upper four proteins of a ³²P-labeled probe containing the human nGRE sequence was completely abolished by a 200-fold excess of non-radioactive oligonucleotide containing intact human nGRE sequence, but not by mutated nGRE (Fig. 2A, lanes 5 and 6). Only the upper three bands, however, disappeared following the inclusion of the oligonucleotide containing the rat nGRE sequence (Fig. 2A, lane 7). These findings from the competition experiments suggest that the upper three proteins recognize an NBRE-like sequence in human and rat nGRE (see Fig. 2B). These upper three bands were extinguished by incubation of the nuclear extracts with anti-nur77 IgG (Fig. 2A, lane 4) but not with non-immune IgG (Fig. 2A, lane 3). The formation of multiple nur77-DNA complexes in size might be due to posttranslational modifications, which were identified as the cause of the heterogeneity in protein size in nur77 (Fahrner *et al.* 1990). On the other hand, none of the bands were abolished by incubation with anti-glucocorticoid receptor antisera (data not shown). These results indicate that nur77 induced by CRH binds to the nGRE sequence in the POMC gene promoter in a sequence-specific manner.

Effect of nur77 expression on the glucocorticoid-mediated suppression of POMC mRNA expression and ACTH secretion in AtT-20 cells

To investigate further the biological effect of nur77 binding to nGRE sequence in the POMC gene promoter, we established stable transformants of AtT-20 cells expressing the human homolog of nur77, NAK-1,

Figure 2 Binding of nur77 to nGRE in the promoter of the POMC gene. (A) Electrophoretic mobility shift assay (EMSA) was performed using nuclear extracts prepared from AtT-20 cells untreated (lane 1) or treated (lanes 2–7) with 25 nM CRH for 2 h. Double-stranded oligonucleotide (28 bp) containing human nGRE was ³²P-labeled and used as a probe, as described in Materials and Methods. Four microliters (4 µg protein) anti-nur77 IgG (lane 4) or non-immune IgG (lane 3) were added during the incubation of nuclear extracts with a probe. An excess amount (200-fold) of non-radioactive competitors was included in the mixture: the sequences of competitors used were human nGRE (lane 5), mutated human nGRE (lane 6), and rat nGRE (lane 7). The shifted bands due to the formation of DNA–protein complexes are indicated by the arrows on the left. (B) The sequences of human nGRE, mutated human nGRE, rat nGRE, and the consensus nur77 responsive element (called NBRE) are shown. The consensus NBRE and NBRE-like sequences are underlined. The numbers show the nucleotide positions by designating the transcription start site in the POMC gene as +1. The nucleotides of mutated human nGRE and rat nGRE that are different from those of intact human nGRE are shown by small letters, and a space is shown by a dash.

constitutively at a high level. Using the NAK-1 stable transformants, the dexamethasone-induced suppression of POMC mRNA expression and ACTH secretion was examined. The POMC mRNA level was suppressed to 70.1% of the original level at 6 h after the addition of 10^{−7} M dexamethasone and to 37.5% at 24 h in mock transformants, while the POMC mRNA level decreased

Figure 3 Effect of NAK-1 expression on the dexamethasone-induced suppression of POMC mRNA accumulation in AtT-20 cells. NAK-1 (●) and mock (○) stable transformants (5 × 10⁶ cells) were cultured in RPMI 1640 with 2.5% DCC-treated FCS in 25-cm² suspension culture flasks for 24 h, and either untreated (dotted line) or treated (solid line) for 6 h and 24 h with 10^{−7} M dexamethasone, then the total RNA was prepared and subjected to Northern blot analysis using POMC and GAPDH cDNAs as probes. Relative radioactivities of the bands detected were determined as described in Materials and Methods and the POMC mRNA level was standardized by the level of GAPDH mRNA. The POMC mRNA level before the addition of dexamethasone is given as 100% in NAK-1 and mock transformants respectively. Endogenous POMC mRNA expression in the absence of dexamethasone is similar in NAK-1 and mock transformants. The results are from four different pools of stable transformants (see Materials and Methods). Each point represents the mean ± s.d. of four independent experiments. **P* < 0.01, ***P* < 0.001 vs mock transformants. Inset: dot blot analysis of NAK-1 and mock transformants using NAK-1 cDNA as a probe. N, NAK-1 transformant; m, mock transformant (See Materials and Methods for details).

to only 93.3% of the original level at 6 h and to 57.6% at 24 h in the NAK-1 stable transformants (Fig. 3). The amount of ACTH secreted into the culture media after 48 h in the presence of 10^{−7} M dexamethasone was decreased to only 86.0% of the original level in the NAK-1 stable transformants, in contrast to 59.8% in the mock transformants (Fig. 4). In the absence of dexamethasone, the amount of both endogenous POMC mRNA expressed and ACTH secreted by the NAK-1 stable transformants was similar to that secreted by the mock transformants. The extent of the dexamethasone-induced

Figure 4 Effect of NAK-1 expression on the dexamethasone-induced suppression of ACTH secretion in AtT-20 cells. NAK-1 and mock stable transformants (3×10^5 cells) were cultured as described in Materials and Methods, and either untreated or treated with 10^{-7} M dexamethasone for 48 h, then an aliquot of each culture medium was subjected to determination of the ACTH content. ACTH concentrations in the absence of dexamethasone were given as 100% for both NAK-1 and mock transformants. Values are expressed as the mean $\pm$ S.D. of four independent experiments with four different pools of transformants. * $P < 0.001$ vs mock transformants. The ACTH concentrations in the absence of dexamethasone were 896 ± 104 and 888 ± 66 pg/ml (mean $\pm$ S.D., $n=4$) in NAK-1 and mock transformants respectively, and were not significantly different.

suppression of the POMC mRNA expression and ACTH secretion was significantly lower in the NAK-1 stable transformants than in the mock transformants.

Discussion

We demonstrated that nur77 is induced by CRH in a pituitary corticotrope cell line, AtT-20, and that the expression of NAK-1, a human homolog of nur77, at a high level lowers the dexamethasone-mediated inhibition of POMC mRNA accumulation and ACTH secretion in AtT-20 cells. These observations suggest a possible mechanism of antagonizing the negative feedback effect of glucocorticoid in the HPA axis during the stress response, i.e. chronic stress induces nur77 by a mediator(s) such as CRH in corticotropes, and the expressed nur77 suppresses

Figure 5 Schemes showing (A) the central dogma for negative feedback regulation of the hypothalamic–pituitary–adrenal (HPA) axis by glucocorticoid and (B) the hypothetical function of nur77 in the pituitary gland during the stress response. (A) CRH stimulates the synthesis and secretion of ACTH which results in increased circulating glucocorticoid. This, in turn, inhibits the synthesis and secretion of CRH and ACTH. (B) The expression of nur77 is induced in the pituitary gland by CRH released from the hypothalamus following stress stimuli. Then nur77 binds to nGRE in the promoter of the POMC gene, thus leading to the blockade of the negative feedback by glucocorticoid, either directly or indirectly. GR, glucocorticoid receptor.

the feedback inhibition by glucocorticoid of the POMC gene expression (Fig. 5). Another mechanism in which glucocorticoid exerts less of an effect on the pituitary corticotropes during the stress response has been reported to be through CRH-mediated down-regulation in the expression of glucocorticoid receptor (Sheppard *et al.* 1991). There was no decrease in the expression of glucocorticoid receptor in the NAK-1 stable transformants (data not shown). Both of these mechanisms seem, therefore, to help in the maintenance of high circulating glucocorticoids during chronic stress.

Nur77 has so far been shown to be induced in the HPA axis and adrenal medulla during the stress response: nur77 is induced in the paraventricular nucleus (PVN) in the hypothalamus by the stimuli of stress (Chan *et al.* 1993, Parkes *et al.* 1993), in the adrenocortical gland by ACTH,

and in the adrenal medulla by nicotinic stimulation mimicking sympathetic nerve stimulation (Davis & Lau 1994). Despite many reports suggesting some involvement of nur77 in the biological adaptation to stress, it has yet to be clarified as to how the stress-induced nur77 functions. The present study demonstrates that nur77 is induced by a stress stimulus, CRH, while, in addition, nur77 exerts an antagonistic effect on the feedback inhibition by glucocorticoid.

Recently, Murphy & Conneely (1997) showed that CRH treatment induced nur77 expression in AtT-20 cells, with subsequent activation of POMC gene transcription by interaction with the nGRE site of the rat POMC gene promoter. The location of the CRH- and cAMP-responsive sequences in the POMC promoter is still controversial. In our experiments, the effect of nur77 on endogenous POMC mRNA expressed and ACTH secreted by AtT-20 cells overexpressing NAK-1 was not detected. Systematic experiments need to be carried out to demonstrate that nur77 is a positive mediator of CRH in the transcriptional activation of the POMC gene. It is possible that nur77 might contribute little to the whole activity of endogenous POMC expression, although we cannot exclude the possibility that human NAK-1 is not capable of interacting with the mouse POMC gene promoter. Since human NAK-1 and mouse nur77 show more than 90% sequence identity at amino acid level, the latter possibility seems to be unlikely.

Crawford *et al.* (1995) reported an analysis of the HPA axis in nur77-deficient mice which was established by a gene-targeting technique. In these mice, the function of the HPA axis remained intact. However, an analysis of whether or not the survival rate or HPA axis is altered by exposing nur77-deficient mice to various stress stimuli may be necessary to evaluate the relevance of our above described hypothetical role of nur77. It has recently been shown that three subtypes of nur77 and nur77-like proteins constitute the nur77 family (Mangelsdorf *et al.* 1995, Okabe *et al.* 1995), and the existence of a functional redundancy among this nur77 family has been suggested (Calnan *et al.* 1995, Crawford *et al.* 1995, Kastner *et al.* 1995, Lee *et al.* 1995). Therefore, a different subtype(s) other than nur77 might compensate for the function of nur77 in the nur77-deficient mice.

The present study showed *in vitro* that nur77 induced by CRH in the AtT-20 cells binds to an NBRE-like sequence in the nGRE in the POMC gene promoter. No binding activity of glucocorticoid receptor to nGRE was detected by EMSA using nuclear extracts of AtT-20 cells in our study, which is consistent with the observations of other groups (Israel & Cohen 1985, Therrien & Drouin 1991). Purified glucocorticoid receptor is thought to be necessary for its binding to nGRE (Drouin *et al.* 1989, Riegel *et al.* 1991), surmising the existence of an inhibitor(s) of glucocorticoid receptor binding to nGRE. Negative GRE has been shown to be necessary for the

glucocorticoid-mediated inhibition of the POMC gene expression (Drouin *et al.* 1989, 1993). Thus, our findings along with the reported observations might suggest several possible molecular mechanisms for the antagonistic effect of nur77 on the feedback inhibition by glucocorticoid (Fig. 5B), including the occupation of nGRE by nur77 which may result in the hindrance of glucocorticoid receptor binding to this element. Further studies are still necessary to elucidate the precise molecular mechanism involved in the antagonistic effect of nur77.

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