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Monitoring of antisense effects of oligonucleotides targeted to the neuropeptide Y Y_1 receptor gene

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Abstract

The suppression of neuropeptide Y Y_1 receptor gene expression by antisense oligonucleotides targeted to different gene regions was monitored on mRNA and protein level in the human neuroblastoma SK-N-MC cell line. The antisense oligonucleotide targeted to the junction of the first intron and second exon suppressed specifically Y_1 receptor subtype number by more than 50%, but only if oligonucleotides were administered by electroporation. Also, the formation of Y_1 receptor mRNA as shown by reverse transcription-polymerase chain reaction was markedly blocked in this case. Using the antisense oligonucleotide targeted to the start of translation, no effect, neither on the Y_1 receptor number nor on Y_1 receptor mRNA, could be observed. This finding suggests that besides sequence-specific effects of antisense oligonucleotides gene site-specific effects play a major role in the efficacy of suppression. © 1997 Elsevier Science B.V.

Keywords: Neuropeptide Y Y_1 receptor; Binding; Gene expression; Antisense oligonucleotide; Electroporation

1. Introduction

Neuropeptide Y, a 36 amino acid peptide with high expression in central and peripheral nervous system, acts via specific neuropeptide Y receptor subtypes. Among functions mediated by the neuropeptide Y Y_1 receptor subtype, which gene has been cloned (Herzog et al., 1993), are vasoconstriction in most vascular beds (Michel and Rascher, 1995) and stimulation of food intake (Stanley and Leibowitz, 1984). The latter effect appears to be regulated by circulating levels of leptin (Stephens et al., 1995).

Blocking receptor function traditionally is realized by specific receptor antagonists. Antisense strategies are discussed as possible alternative to inhibit receptor gene expression and function and may have some advantage with respect to receptor subtype specificity and to a better access to the central nervous system (Harrison, 1993). Especially the suppression of peptide receptors by antisense is of major interest, because it offers the possibility to modulate the biological responses of the respective peptides without having stable favorable non-peptidergic antagonists. However, some controversy exists whether antisense strategies really work (Wagner, 1994; Gura, 1995). Several studies have been performed on neuropeptide Y Y_1 receptor antisense suppression measuring complex biological phenomena as anxiety behaviour or blood vessel contraction elucidated by this peptide (Wahlestedt et al., 1993; Erlinge et al., 1993). This study is focused on the cellular level measuring neuropeptide Y Y_1 receptor number and neuropeptide Y Y_1 receptor mRNA in human neuroblastoma cell lines treated with various antisense oligonucleotides.

2. Materials and methods

2.1. Cell culture

Two human neuroblastoma cell lines grown in monolayer culture were used for the experiments. SK-N-MC cells (Feth et al., 1991) and CHP234 cells (Lynch et al., 1994), exclusively expressing the neuropeptide Y Y_1 receptor and neuropeptide Y Y_2 receptor, respectively were cultured in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum and 2% penicillin streptomycin at 37°C in air containing 5% CO_2. Cells were

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grown as monolayers in 24-well plates until confluency was reached.

2.2. Treatment of cells with antisense oligonucleotides

The sequences of the phosphorothioate-modified oligonucleotides (Biometra, Göttingen, Germany) used for the antisense experiments were as follows:
1as: AACTGAACAATCTGTAAAGAG,
1s: CTCTTTACAGATTGTTCAGTT,
2as: ATGTTGAATTCATTTTGATTG,
2s: CAATCAAAATGAATTCAACAT. Oligonucleotides (1as) and (1s) are targeted to the junction of intron 1 and exon 2 in antisense and sense orientation, respectively. Oligonucleotides (2as) and (2s) are targeted to the start of translation (Met) in antisense and sense orientation, respectively (Fig. 1). Calculation of the melting temperatures (T_m) was done by PCGENE software (IntelliGenetics, Oxford, UK) using the equation according to (Rychlik and Rhoads, 1989).

For the respective experiments cells were harvested after trypsin treatment and resuspended in medium containing 10% fetal calf serum. The cell number was counted with an electronic cell counting system (Casy, Schärfe, Reutlingen, Germany). The number of vital cells was determined using the trypan blue method. The cells were harvested again by centrifugation and resuspended to a cell density of 2×10^6/ml in medium containing 1% fetal calf serum. 800 μl of this cell suspension was transferred to a electrocuvette with a 2 mm diameter. The respective oligonucleotide was added to the cuvette in concentrations ranging between 0 and 20 μM as mentioned in Section 3. In some experiments the cuvette received a rectangular electropulse of 450 V and 1 ms (EPI2500, Fischer, Heidelberg, Germany); in other experiments the electropulse was omitted. Then, cells were transferred to a 24-well plate (10^5 cells/2.2 cm^2) for binding studies and a 6-well plate (4×10^5 cells/9.6 cm^2) for RNA expression analysis. Medium containing 10% fetal calf serum was added to the wells, thereby diluting the initial oligonucleotide concentration 1 by 5. The cells were incubated for a further 48 h.

2.3. Binding experiments

Radioligand binding experiments were performed as described using ^{125}I-peptide YY as radioligand (Biotrend, Cologne, Germany), which has similar binding features as ^{125}I-neuropeptide Y but is more stable to proteolysis (Michel et al., 1992; Feth et al., 1992; Wahlestedt and Reis, 1993).

2.4. RNA extraction

RNA was extracted by a guanidium-thiocyanate phenol procedure (RNAzol, Wacker Chemie, Bad Homburg, Germany).

2.5. RT-PCR

RNA extracts from 6-well plates were analyzed by a semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) with two specific primer combinations for neuropeptide Y Y_1 receptor gene named ac and bc and one primer pair for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal standard. The respective primer sequences were as follows:
a: TCTTTAATAAGCAGGAGCGA;
b: GAGGCGATGTGTAAGTTGAAT;
c: GTTGACACAAGTGGATATCAT;
GAPDH 5′: AAGGGTCATCATCTCTGCCC;
and GAPDH 3′: CAGTGAGTTCCCGTTCAGC. Reverse transcription of the RNA of the treated cells was done in a 20 μl volume containing 1 mM of dATP, dCTP, dGTP and dTTP (Boehringer-Mannheim, Mannheim, Germany), respectively, 200 ng random primer hexamers (Boehringer-Mannheim) and Moloney mouse leukaemia virus reverse transcriptase (Gibco-BRL, Karlsruhe, Germany). This reaction mixture was diluted 1 by 5 for PCR reaction using a final deoxy nucleotide concentration of 0.2 mM, a primer concentration of 0.8 μM and the thermostable polymerase Primezyme (2.5 units/100 μl) (Biometra, Göttingen, Germany). The programming of the thermocycler (Trioblock, Biometra) was 94°C 1 min, 55°C 1.5 min, 72°C 1.5 min for 30 cycles. The samples were run

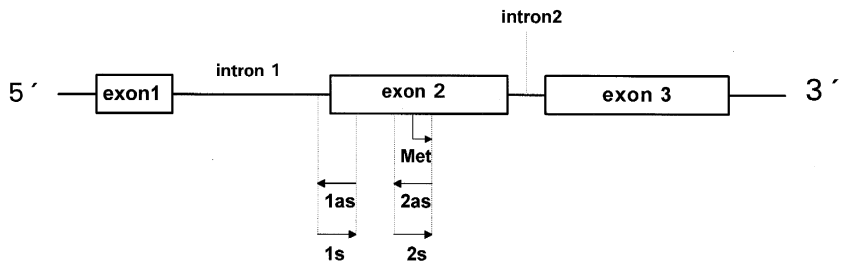

Fig. 1. Structure of the neuropeptide Y Y_1 receptor gene (Herzog et al., 1993) and the positions of the oligonucleotides used for gene suppression analysis. The gene consists of three exons, which are interrupted by two introns. Oligonucleotides (1as) and (1s) are targeted to the junction of intron 1 and exon 2 in antisense and sense orientation, respectively. Oligonucleotides (2as) and (2s) are targeted to the start of translation (Met) in antisense and sense orientation, respectively.

on a 1.5% agarose gel containing 1 ng/ml ethidium bromide and visualized by ultraviolet light.

2.6. Northern-blot analysis

RNA was denatured at 50°C for 60 min in glyoxal dimethyl sulfoxide and its concentration spectrophotometrically determined. RNA was loaded in glycerol/sodium phosphate buffer to a 1.2% agarose gel prepared in 10 mM sodium phosphate pH 7. After electrophoresis RNA was transferred onto nylon membranes (Nytran N, Schleicher & Schuell, Dassel, Germany). Digoxigenin-labeled cRNA probes were used for hybridization (Boehringer-Mannheim). Labeling was performed according to the manufacturer's protocol by in vitro transcription of the human neuropeptide Y Y_1 receptor and human neuropeptide Y cDNA which has been cloned in pGEM plasmid, respectively (Hänze et al., 1996), using digoxigenin-UTP and SP6- or T7- RNA polymerase. The membranes were prehybridized for 2 h at 68°C in hybridization solution consisting of 50% formamide, 0.75 M sodium citrate, 0.075 M sodium chloride, 2% blocking agent (Boehringer-Mannheim), 0.1% *N*-lauroylsarcosine and 0.02% sodium dodecylsulfate. Hybridization was carried out overnight at 68°C by using 50 ng digoxigenin-labeled RNA probe per ml hybridization solution (see above). Membranes were washed in a buffer containing 0.3 M sodium citrate, 0.03 M sodium chloride and 0.1% sodium dodecylsulfate for 10 min at room temperature and a buffer containing 0.03 M sodium citrate, 0.003 M sodium chloride and 0.1% sodium dodecylsulfate for 30 min at 68°C.

To detect the digoxigenin-labeled probes, after it was washed, the membrane was incubated for 30 min at room temperature with a 2% solution of blocking agent (Boehringer-Mannheim) in 100 mmol/l Tris-HCl (pH 7.4) and 150 mM NaCl and then for 30 min at room temperature in the same solution containing a 1:10 000 dilution of a polyclonal anti-digoxigenin sheep antibody fab fragment conjugated to alkaline phosphatase (Boehringer-Mannheim). The membrane was washed twice with 100 mM Tris-HCl (pH 7.4) and 150 mM NaCl and then incubated for 10 min in 1 × phosphate-buffered saline containing 0.1% Tween 20 and 0.2% I-block (Tropix, Boston, MA, USA) reagent. After the membrane was washed with 100 mM Tris-HCl (pH 7.4) and 150 mM NaCl, alkaline phosphatase activity was determined by addition of 50 μl CSPD (Tropix) in 5.0 ml of 0.1 mM diethanolamine and 1 mM $MgCl_2$ and exposure of the membrane to X-ray film.

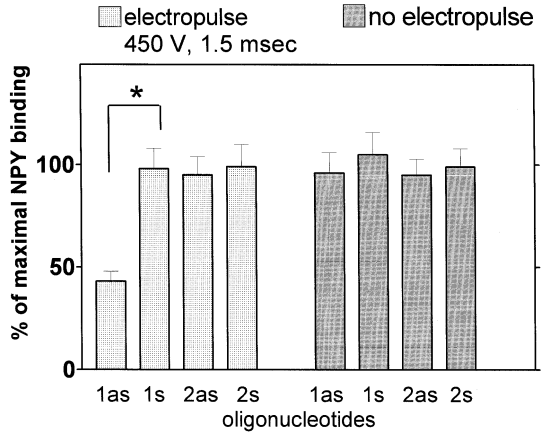

Fig. 2. Neuropeptide Y binding experiments of SK-N-MC cells treated by the presented oligonucleotides (Fig. 1) with and without receiving an electropulse, respectively. Each column represents three independent electroporation experiments followed by whole-cell binding analysis measured in triplicates. Significant difference between columns was tested by Mann-Whitney test (* $P < 0.05$).

3. Results

Oligonucleotide 1as significantly reduced neuropeptide Y binding in SK-N-MC cells treated with an electropulse by 50%, while the respective control oligonucleotide 1s had no effect (Fig. 2). Oligonucleotide 2as had a slight inhibitory effect on neuropeptide Y binding compared to

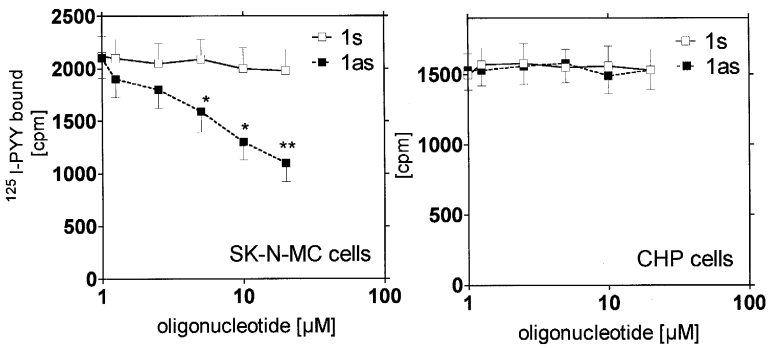

Fig. 3. Neuropeptide Y binding experiments to the neuropeptide Y Y_1 receptor-specific SK-N-MC cells and to the neuropeptide Y Y_2 receptor-specific CHP2234 cells after electropulse treatment of the cells with the most potent neuropeptide Y Y_1 receptor antisense oligonucleotide (1as) in a concentration-dependent manner. The oligonucleotide concentrations are related to the cell suspension in the electrocuvette, respectively and were diluted 1 by 5 after transferring the cells to culture dishes. Each value represents the mean of three independent electroporation experiments followed by whole-cell binding analysis measured in triplicates. Significant difference between groups was tested by Mann-Whitney test (* $P < 0.05$, ** $P < 0.01$).

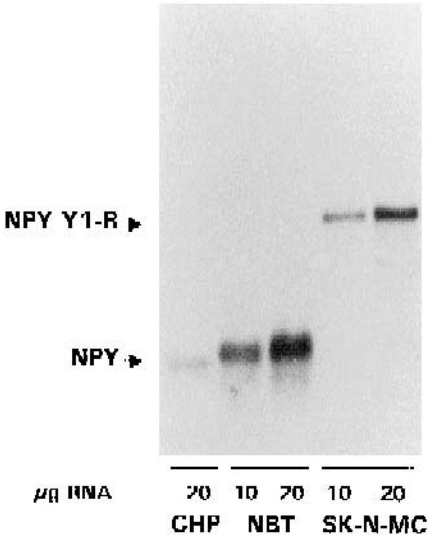

Fig. 4. Northern blot experiment of SK-N-MC and CHP234 cell line and a human neuroblastoma tissue. The blot was hybridized simultaneously with neuropeptide Y and neuropeptide Y Y_1 receptor cRNA. A strong neuropeptide Y Y_1 receptor signal is detected in SK-N-MC cells, while CHP234 cells, showing no neuropeptide Y Y_1 receptor signal, had a weak neuropeptide Y signal compared to a strong neuropeptide Y signal in neuroblastoma proofing the integrity of RNAs, respectively.

its control oligonucleotide 2s which reached no significance (Fig. 2). For the most potent inhibitory oligonucleotide 1as a dose-response curve (Fig. 3) demonstrated a concentration-dependent suppression of neuropeptide Y binding sites in SK-N-MC, while no effect was observed in the CHP234 cell line expressing the neuropeptide Y Y_2 receptor subtype, which is unrelated to neuropeptide Y Y_1 receptor gene. This fact is illustrated by Northern-blot experiment showing the specific neuropeptide Y Y_1 receptor signal in SK-N-MC cells, which is not detectable in CHP234 cells expressing the neuropeptide Y Y_2 receptor subtype (Fig. 4, for details see legend).

In addition to the binding experiments, neuropeptide Y Y_1 receptor mRNA expression of RNA extracts from single wells, a concentration being too low for Northern-blot analysis, was semiquantitatively analyzed by RT-PCR and compared to the GAPDH mRNA in two independent experiments. A dramatic reduction of neuropeptide Y Y_1 receptor-RT-PCR product in the cells treated with oligonucleotide 1as was found by visual judgement of the agarose gel chromatography (Fig. 5). Primer pair ac encompassing the gene region between exon 1 and exon 3 showed a stronger reduction of RT-PCR product, first and second intron excluded, when compared to the primer combination bc encompassing the region between exon 2 and exon 3, second intron excluded (upper part Fig. 5).

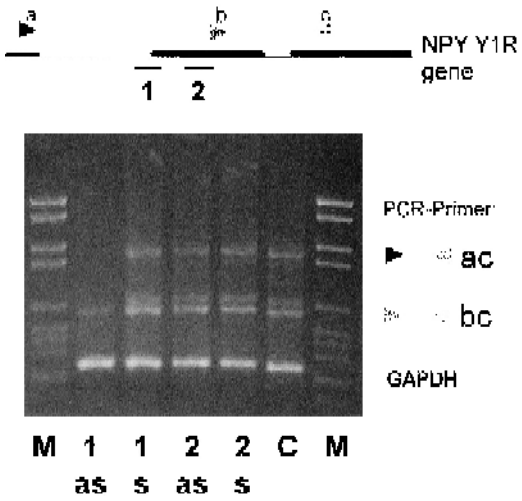

Fig. 5. Neuropeptide Y Y_1 receptor mRNA expression analysis by RT-PCR in SK-N-MC cells treated with the oligonucleotides (1as), (1s), (2as) and (2s), respectively. Top: The positions of the neuropeptide Y Y_1 receptor-specific primers a, b, c used in the combinations (ac) and (bc) for RT-PCR and of the phosporothioate oligonucleotides 1 and 2 used for antisense suppression in sense (s) and antisense (as) orientation are shown. Bottom: The PCR products separated on an ethidiumbromide gel are shown. The lanes are labeled by M, for marker (top to bottom: 2176, 1766, 1230, 1033, 653, 517, 453, 394, 298 and 234 bp) and by the name of the used phosphorothioate oligonucleotide (1as, 1s, 2as and 2s). Primer combination ac corresponds to an expected length of 1139 basepairs intron I and intron II excluded, bc to an expected length of 620 bp intron II excluded, and GADPH to an expected length of 337 bp.

4. Discussion

The aim of this study was to analyze the gene-inhibiting effects of antisense oligonucleotides derived from the neuropeptide Y Y_1 receptor sequence on the cellular level. Firstly, we were interested in the suppressive effects of oligonucleotides targeted to two different crucial sites of a gene, (a) the start of translation and (b) the exon-intron junction. Secondly, we compared antisense effects of oligonucleotides added to the cell culture medium with and without electroporation. As an experimental system the SK-N-MC cell line with a highly endogenous neuropeptide Y Y_1 receptor subtype expression was chosen. The CHP234 cell line with a comparatively high neuropeptide Y Y_2 receptor subtype expression served as a control. Both cell lines carry unrelated neuropeptide Y receptor genes with respect to the tested oligonucleotides (Rose et al., 1995).

The most potent suppressive effect on neuropeptide Y Y_1 receptor was observed by the antisense oligonucleotide targeted to the exon-intron junction. Using this antisense oligonucleotide, neuropeptide Y binding was reduced by 50% and neuropeptide Y Y_1 receptor mRNA reduction analyzed by RT-PCR analysis was even stronger. The stronger suppression of neuropeptide Y Y_1 receptor mRNA compared to neuropeptide Y binding may reflect that the heteronuclear RNA or mRNA is the direct target of antisense oligonucleotides. The inhibition of its synthesis is affected more rapidly than the posttranscriptional steps involved in receptor synthesis and its integration into the membrane. Interestingly, a more pronounced effect of

receptor mRNA suppression was observed if the primers a and c located on exon 1 and exon 3, respectively, were used for RT-PCR when compared to the primers b and c located on exon 2 and exon 3, respectively. This finding suggests that the neuropeptide Y Y_1 receptor mRNA reduction triggered by oligonucleotide 1as is caused in addition to sequence-specific effects as cleavage of RNA by RNase H, by gene site-specific effects especially by interfering with intron I splicing, affecting the RT-PCR with primers a and c stronger, than primers b and c located both beside intron I. The splicing site probably is more sensible for antisense suppression than start of translation. Additionally, the chemical composition of both oligonucleotides may have some influence. In fact the melting temperatures (T_m) of oligonucleotide 1as ($T_m = 41°C$) and 2as ($T_m = 45°C$) as well as the percentage GC content (1as: GC = 33%, 2as: GC = 24%) differ and may contribute to the respective suppression features. In rat an antisense oligonucleotide targeted to the amino terminal end downstream from the methionine start codon successfully suppressed neuropeptide Y Y_1 receptor gene in vivo and cultured neurons (Wahlestedt et al., 1993). This primer had a length of 18 bases, a T_m of 38°C and a GC content of 39%. This comparison suggests that the efficacy of an antisense oligonucleotide is determined by various components including chemical factors and targets of the gene sequence.

Nevertheless, the present study suggests that the antisense oligonucleotide targeted to the intron-exon junction inhibited neuropeptide Y Y_1 receptor gene expression by a specific antisense effect and not by pharmacological means: (1) Only the antisense oligonucleotide 1as suppressed neuropeptide Y Y_1 receptor gene expression, while the control oligonucleotide in sense orientation, having the same ratio of pyrimidine and purine bases, had no effect. (2) The number of neuropeptide Y Y_2 receptor specifically expressed on CHP234 cells was unaffected by the neuropeptide Y Y_1 receptor-specific antisense oligonucleotide 1as proofing its sequence specificity. (3) The suppressive effects could be observed both on the mRNA level and on the protein level.

The present study did not observe any suppressive effects if the oligonucleotides were added directly to the medium, suggesting an insufficient uptake of the highly hydrophilic oligonucleotides from cell medium to the cells. However, biological responses occurred if the electroporation technique was added. Administration of oligonucleotides by electroporation has also been shown for a human glioma cell line (Chavany et al., 1995). Transdermal oligonucleotide transfer by electroporation has recently been reported (Zewert et al., 1995). Viral liposome-mediated transfer is another technique to facilitate oligonucleotide uptake (Morishita et al., 1994). Spontaneous oligonucleotide uptake of neuropeptide Y Y_1 receptor antisense constructs in cultured cells from the central nervous system, in cultured blood vessels, and in vivo has been reported (Wahlestedt et al., 1993; Erlinge et al., 1993). However, it did not work in the cell lines used in the present study. In conclusion this study has shown an antisense effect of an oligonucleotide targeted to the exon-intron junction which was administered to cells by electroporation. The suppressive effects could be measured both on the mRNA and on the protein level. We speculate that the potent inhibitory antisense effect of the oligonucleotide is mediated by interfering with intron I splicing of the neuropeptide Y Y_1 receptor heteronuclear RNA.

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