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Rapid decrease in neuropeptide Y gene expression in rat adrenal gland induced by the α_2-adrenoceptor agonist, clonidine

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1 The mechanism of regulation of the neuropeptide Y (NPY) gene by pharmacological treatment with the α_2-adrenenoceptor agonist, clonidine, was investigated by quantitative Northern blot analysis of the effects of this drug on the NPY mRNA levels in rat adrenal gland and medulla oblongata/pons.

2 In the adrenal gland, clonidine-treatment ($50\,\mu g\,kg^{-1}$, s.c., once daily) resulted in decrease in the amount of NPY mRNA to $44 \pm 10\%$ of the control level in 24 h and then its increase to $162 \pm 16\%$ of the control level after 5 days. Concomitant changes in putative NPY pre-mRNA species (7.0 and 3.3 kb) were observed, probably due to changes at the level of NPY gene transcription.

3 The short-term (24 h) effect of clonidine was blocked by yohimbine ($5\,mg\,kg^{-1}$, i.p., once daily). Yohimbine alone tended to increase the NPY mRNA level after 24 h.

4 The recovery/increase in the NPY mRNA level in the adrenal gland after 5 days treatment with clonidine was similar to its increase after treatment with reserpine ($0.5\,mg\,kg^{-1}$, i.p., once daily).

5 NPY gene expression in the medulla oblongata/pons was not changed by short- or long-term treatment with clonidine.

6 These results suggest that clonidine suppresses NPY gene expression in the adrenal gland, probably at the level of transcription, by activation of the α_2-adrenoceptor.

Keywords: Neuropeptide Y; gene expression; clonidine; α_2-adrenoceptor; rat adrenal; rat medulla oblongata/pons

Introduction

Neuropeptide Y (NPY) is a 36-amino acid peptidergic cotransmitter/neuromodulator of central and peripheral catecholaminergic neurones (especially perivascular sympathetic neurones), including the adrenal gland derived from sympathetic neurones (Tatemoto, 1982; Everitt *et al.*, 1984; de Quidt & Emson, 1986). The recent finding that NPY coexists with catecholamines and is co-released with the latter on nerve stimulation suggests its importance in sympathetic neurotransmission (Lundberg *et al.*, 1982; Håkanson *et al.*, 1986; Higuchi *et al.*, 1988a). In addition to its role in sympathetic neurotransmission and/or neuromodulation, NPY in the circulation seems to be involved in regulation of the peripheral and cardiac arterial blood pressure and cardiac functions (Allen *et al.*, 1983; Gray & Morley, 1986; Edvinsson *et al.*, 1987). Central administration of NPY results in marked hypotension, whereas its peripheral systemic administration induces prolonged hypertension (Fuxe *et al.*, 1983; Dahlöf *et al.*, 1985).

High concentrations of NPY-immunoreactivity (NPY-I) have been found in chromaffin cells and nerve fibres in the adrenal gland (de Quidt & Emson, 1986; Schalling *et al.*, 1988b; Higuchi *et al.*, 1990). NPY-I is stored in chromaffin granules (Majane *et al.*, 1985) and is co-released with catecholamines from the adrenals into the circulation (Allen *et al.*, 1984). NPY functions as an endogenous inhibitor of catecholamine release from the adrenals (Higuchi *et al.*, 1988a) and a potent vasoconstrictor in the circulation (Dahlöf *et al.*, 1985; Edvinsson *et al.*, 1987). Judging from these effects, NPY synthesized in the adrenal gland seems to participate in regulation of the systemic blood pressure and release of adrenal catecholamines into the circulation.

On the other hand, in the medulla oblongata/pons many NPY-immunoreactive neurones are located in ventrolateral C1 and dorsal vagal C2 adrenergic cell groups as well as in A1 and A4 noradrenergic cell groups (Everitt *et al.*, 1984; Gray & Morley, 1986). NPY cell groups in the ventrolateral medulla seem to be important in regulation of the vasomotor centre (Ward-Routledge & Marsden, 1988; Tseng *et al.*, 1988; McAuley *et al.*, 1989; Sun & Guyenet, 1989).

There is increasing evidence that the steady-state level of NPY is regulated by physiological factors such as aging, innervation, stress and pharmacological treatments that influence sympathetic functions or blood pressure (Higuchi & Yang, 1986; Lundberg *et al.*, 1987; Higuchi *et al.*, 1988b; Higuchi, 1989). During aging, NPY gene expression increases specifically in the adrenal gland and medulla oblongata/pons (Higuchi *et al.*, 1991).

The antihypertensive drug, reserpine, changes the steady-state level of NPY and also prepro-NPY gene (NPY gene) expression in peripheral sympathetic organs (Schalling *et al.*, 1988a,b,c; Higuchi *et al.*, 1990). Thus one effect of antihypertensive drugs may be to modify the level of vasoconstrictive NPY peptide, by changing its rate of biosynthesis and/or turnover.

The imidazoline derivative clonidine, an α_2-adrenoceptor agonist, lowers the blood pressure by central inhibition of sympathetic nerve activity (Haeusler, 1974). Moreover, short-term treatment with clonidine increases the steady-state level of NPY in peripheral organs, such as the heart and adrenal gland, in contrast to reserpine, which has a suppressive effect (Nagata *et al.*, 1986; Franco-Cereceda *et al.*, 1987; Lundberg *et al.*, 1987). One way in which clonidine elevates the NPY peptide level seems to be by inhibiting NPY release by activating presynaptic α_2-adrenoceptors (Dahlöf *et al.*, 1986). However, like reserpine, it might also affect NPY gene expression in neuronal cells.

To investigate whether clonidine modulates gene expression of the vasoconstrictive NPY peptide, we examined its effects on the level of NPY mRNA in the adrenal gland and medulla oblongata/pons, in relation to vascular control. For this purpose we carried out quantitative Northern blot analyses with a cloned rat NPY cDNA (Higuchi *et al.*, 1988c), as a probe.

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Methods

Drug treatment

Male Sprague-Dawley (SD) rats were used. Clonidine hydrochloride ($50\,\mu g\,kg^{-1}$, s.c.), yohimbine hydrochloride ($5\,mg\,kg^{-1}$, i.p.) and reserpine ($0.5\,mg\,kg^{-1}$, i.p.) in saline were injected into 8–week-old rats (body weight 250–280 g) once daily for 1 or 5 days. Control rats received the same volume (0.1 ml) of saline only. Rats were killed by decapitation 24 h after the last injection.

Preparation of cellular RNA

Brains were dissected as described by Glowinski & Iversen (1966). Pairs of adrenal glands and medulla oblongata/pons, obtained immediately after decapitation, were homogenized in at least 5 volumes of 4 M guanidinium thiocyanate solution. Total cellular RNA in the extracts was purified by centrifugation through 5.7 M CsCl, and quantitated by their absorbance at 260 nm (Higuchi *et al.*, 1988c). The RNA yields were not affected by drug treatment and mean values were as follows: $89 \pm 6\,\mu g$ per pair of adrenal glands ($n = 30$); $108 \pm 12\,\mu g$ per the medulla oblongata/pons ($n = 31$).

Quantitation of neuropeptide Y mRNA

The level of NPY mRNA was determined by Northern blot analysis and comparison of autoradiographic signals with those of standard RNA samples run at the same time, as described previously (Higuchi *et al.*, 1988c). For standardization, at least 5 different amounts (2.5–100 pg) of pBL-NPY1 transcripts (NPY sense RNA) and a standard rat striatum RNA preparation were run simultaneously with or without carrier rat liver RNA. The autoradiographic signals were quantitated by densitometric scanning and integration of peak areas corresponding to mature NPY mRNA (approximately 800 bases). Northern blot rather than dot blot analysis was used because the latter method is usually not sufficiently sensitive for quantitation of NPY mRNA (Higuchi *et al.*, 1988c; 1990; Sabol & Higuchi, 1990).

Statistical methods

Statistical significance was determined by Student's *t* test.

Materials

Clonidine hydrochloride (Sigma), yohimbine hydrochloride (Nakarai Tesque) and reserpine (Daiichi Seiyaku Co) were used.

Results

Figure 1 shows results of Northern blot analysis on change in NPY mRNA from the adrenal gland after clonidine treatment. The rat NPY cDNA probe hybridized with at least three mRNA species of different sizes. Besides mature NPY mRNA (800 bases), larger RNA species (3300 and 7000 bases) were detected in samples from the adrenal gland and brain (Figures 1 and 3). These larger RNA species (7.0 and 3.3 kb) are consistent in size with the unspliced and incompletely spliced transcripts predicted from the structure of the rat NPY gene, suggesting the presence of putative NPY pre-mRNA species (Larhammar *et al.*, 1987; Sabol & Higuchi, 1990; Higuchi *et al.*, 1990).

The amounts of NPY mRNA and the two putative NPY pre-mRNA species (0.8, 3.3 and 7.0 kb, respectively) in the adrenal gland decreased to an equal extent after treatment with the α_2-adrenoceptor agonist, clonidine, for 24 h (Figures 1, 2, Tables 1 and 2). Their decreases induced by clonidine were blocked in the presence of the α_2-adrenoceptor antagonist yohimbine (Figure 1, Tables 1 and 2). Yohimbine alone tended to increase the levels of the NPY mRNA and NPY pre-mRNA species (0.8, 3.3, and 7.0 kb, respectively), but not significantly (Figures 1 and 2, Tables 1 and 2). As these changes in the level of mature NPY mRNA were associated with changes of a similar extent in the level of the putative NPY pre-mRNA species (3.3 and 7.0 kb), the changes in NPY gene expression probably occurred at the level of gene transcription. The absolute amounts of NPY mRNA in the adrenal glands after short-term treatments with clonidine and/or yohimbine were quantitated by the comparison with signals of the synthesized pBL-NPY1 transcripts (NPY sense RNA) (Table 1). The results suggested that NPY gene expression in the adrenal gland is decreased by activation of the

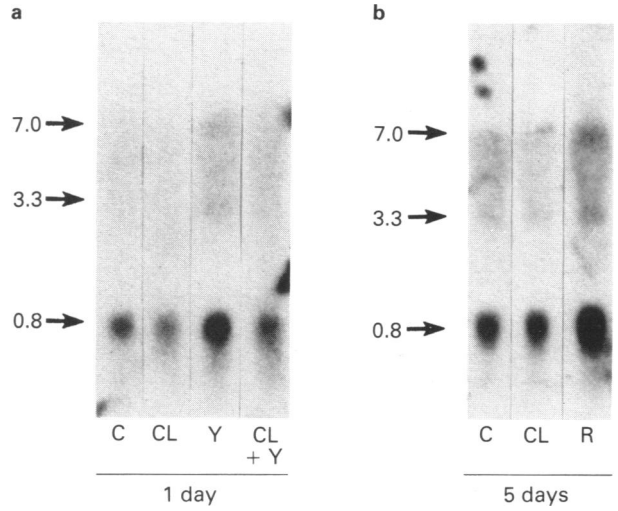

Figure 1 Northern blot analysis of neuropeptide Y (NPY) mRNA from rat adrenal gland after drug treatment. Male rats (8 weeks old) were treated with clonidine (CL, $50\,\mu g\,kg^{-1}$, s.c.), yohimbine (Y, $5\,mg\,kg^{-1}$, i.p.), reserpine (R, $0.5\,mg\,kg^{-1}$, i.p.), and/or vehicle (C) once daily for 1 day or 5 days as indicated. Then tissues were promptly excised and lysed in 5–10 volumes of 4 M guanidinium thiocyanate solution. Total cellular RNAs were prepared, purified, and quantitated as described in the Methods. Total cellular RNAs ($25\,\mu g$/sample) were separated by electrophoresis in formaldehyde agarose gel, blotted and hybridized with the 511-bp EcoRI insert of the pBL-NPY1 plasmid. Numbers at the left indicate lengths of hybridized RNAs (0.8, 3.3 and 7.0 kb). The density of NPY mRNA band of control samples are a little different in (a) and (b) due to different exposure time.

Table 1 Effect of clonidine treatment on the level of neuropeptide Y (NPY) mRNA in rat adrenal gland

Treatment		*NPY mRNA* ($pg\,\mu g^{-1}$ tcRNA)
Control	(1 day)	1.70 ± 0.25 (6)
Clonidine	(1 day)	0.75 ± 0.17 *(6)
Yohimbine	(1 day)	2.20 ± 0.24 (3)
Clonidine + yohimbine	(1 day)	1.76 ± 0.07 (3)
Control	(5 days)	1.54 ± 0.14 (3)
Clonidine	(5 days)	2.50 ± 0.24 †(3)
Reserpine	(5 days)	3.17 ± 0.30 **(3)

Male rats (8 weeks old) were treated with clonidine ($50\,\mu g\,kg^{-1}$, s.c.), yohimbine ($5\,mg\,kg^{-1}$, i.p.) and reserpine ($0.5\,mg\,kg^{-1}$, i.p.) once daily for 1 day or 5 days, as indicated. The adrenal glands were immediately excised and their NPY mRNA abundances were quantitated as described in the Methods. tcRNA is total cellular RNA. Values are means ± s.e.mean for 3 or 6 independent experiments (as shown in parentheses). Each experiment was done with an extract of tissue from two or three rats. Significantly different from the control value: $*P < 0.01$; $**P < 0.05$.
† Significantly different from the value after clonidine treatment for 1 day: $P < 0.01$.

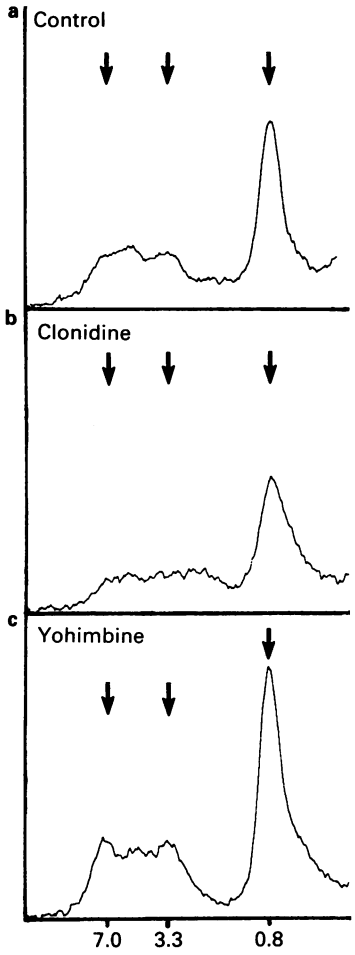

Figure 2 Densitogram of drug-induced changes in neuropeptide Y (NPY) mRNA in rat adrenal gland. Autoradiographic signals of NPY mRNA from the adrenal gland after drug treatment for 1 day were quantitated by scanning densitometry. Exposure times of autoradiograms were chosen so that peak areas were within the range of linear variation with the amount of RNA applied. Numbers below the traces indicate the lengths of hybridized RNAs (0.8, 3.3 and 7.0 kb).

α_2-adrenoceptor with clonidine, probably at the level of NPY gene transcription.

After the initial decrease to 44 ± 10% of the control level, the amount of NPY mRNA in rat adrenal gland increased significantly to 162 ± 16% of the control level after 5 days treatment with clonidine (Table 1). As shown in Figure 1 and Table 2, this increase in the amount of NPY mRNA was associated with increases in the level of the putative NPY pre-mRNA species (3.3 and 7.0 kb) in the adrenal gland, suggesting increase in the level of NPY gene transcription. Similar increase in NPY gene expression in the adrenal gland was observed after 5 days treatment with reserpine, although the hypotensive mechanisms of clonidine and reserpine are different (Figure 1, Tables 1 and 2).

In contrast, the level of NPY mRNA in the medulla oblongata/pons was not changed significantly by treatment with clonidine for 1 or 5 days (Figure 3 and Table 3), suggesting that α_2-adrenoceptors are not involved in regulation of NPY gene expression in the medulla oblongata/pons.

Discussion

The antihypertensive drug reserpine is known to deplete sympathetic neurones of monoamines by interfering with Mg^{2+}-dependent vesicular storage mechanisms for amines. Recently short-term treatment with reserpine was found to decrease the level of NPY-I without changing that of NPY mRNA in peripheral sympathetic organs including the adrenal gland (Lundberg *et al.*, 1986; Nagata *et al.*, 1987; Higuchi *et al.*, 1990). This effect was due to accelerated release of NPY from the nerve terminals and the chromaffin cells by increase in

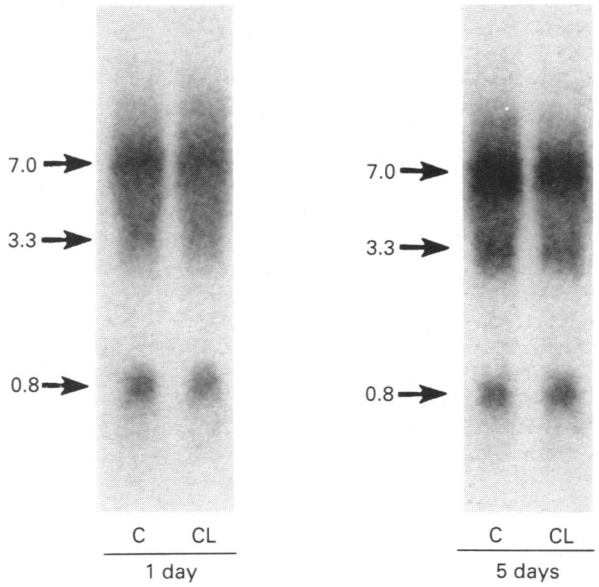

Figure 3 Northern blot analysis of neuropeptide Y (NPY) mRNA from rat medulla oblongata/pons after drug treatment. Male rats (8 weeks old) were treated with drugs or vehicle once daily for 1 or 5 days as for FIgure 1. The RNAs from the medulla oblongata/pons were prepared, quantitated and separated by electrophoresis in formaldehyde agarose gel as described in the Methods. Treatment: C; vehicle, CL; clonidine.

Table 2 Effect of clonidine treatment on the putative neuropeptide Y (NPY) pre-mRNA levels in rat adrenal gland

		NPY pre-mRNA level (% of control)	
Treatment		3.3 kb-species	7.0 kb-species
Control	(1 day)	100 ± 11	100 ± 10
Clonidine	(1 day)	44 ± 12*	41 ± 9*
Yohimbine	(1 day)	110 ± 11	143 ± 25
Clonidine + yohimbine	(1 day)	110 ± 15	95 ± 2
Control	(5 days)	100 ± 14	100 ± 4
Clonidine	(5 days)	118 ± 5	104 ± 5
Reserpine	(5 days)	190 ± 21*	200 ± 32*

Male rats (8 weeks old) were treated with clonidine, yohimbine, and reserpine as described for Table 1. Total cellular RNA (tcRNA) was prepared from the adrenal glands and subjected to Northern blot analysis, as described in the Methods. The relative quantities of the putative NPY pre-mRNA species (3.3 and 7.0 kb) were determined by comparison of the densities of autoradiographic bands of samples (25 μg tcRNA) and are shown as percentages of the control values (means ± s.e.mean) from three independent experiments. Each experiment was done with two independent extracts of tissue from two or three rats. Significantly different from the control value: $*P < 0.05$.

Table 3 Absence of change in the neuropeptide Y (NPY) mRNA level in rat medulla oblongata/pons on clonidine treatment

Treatment	*NPY mRNA* (pg μg^{-1} tcRNA)
Control (1 day)	2.63 ± 0.52
Clonidine (1 day)	2.66 ± 0.18
Control (5 days)	3.04 ± 0.70
Clonidine (5 days)	2.89 ± 0.64

Total cellular RNA (tcRNA) from the medulla oblongata/pons of the same rats as for Figure 1 were subjected to quantitative Northern blot analysis and levels of NPY mRNA were measured as described in the Methods. Values are means ± s.e.mean for three independent experiments. Each experiment was done with an extract of tissue from two rats.

sympathetic nerve activity in response to hypotension (Lundberg *et al.*, 1986; Higuchi *et al.*, 1990). Long-term treatment with reserpine results in gradual increase in NPY gene expression in chromaffin cells in the adrenal gland or ganglion cells in sympathetic ganglia, due to trans-synaptic activation as the result of increased activity of the preganglionic sympathetic nerves (Higuchi *et al.*, 1990; Schalling *et al.*, 1988c). This induction of NPY gene expression resulted in increased biosynthesis of NPY peptides (Higuchi *et al.*, 1990). Thus, reserpine clearly induces both release (/turnover) of NPY peptides and gene expression and biosynthesis of NPY.

In contrast, short-term treatment with clonidine increases the tissue content of NPY-I in peripheral tissues (Nagata *et al.*, 1986; Franco-Cereceda *et al.*, 1987). One reason for this effect is its inhibition of presynaptic release of NPY from sympathetic nerve terminals by activation of the α_2-adrenoceptor (Dahlöf *et al.*, 1986). Interestingly, short-term treatment (24 h) with clonidine at 50 $\mu g\,kg^{-1}$ decreased NPY gene expression, probably at the level of gene transcription, by activating the α_2-adrenoceptor in the adrenal gland *in vivo* (Figure 1, Tables 1 and 2). Suppression of NPY gene expression can result in decreased production of NPY peptides and so its inhibition of NPY gene expression may also decrease the amount of usable/releasable NPY peptides from the adrenal gland. If so, this inhibition of NPY gene expression in the adrenal gland probably participates in the hyptensive action of clonidine.

Clonidine (50 $\mu g\,kg^{-1}$) changed the levels of NPY mRNA and putative NPY pre-mRNA species in the adrenal gland, but not in the medulla oblongata/pons. This finding suggested that this α_2-adrenoceptor agonist regulates NPY gene expression preferentially in peripheral sympathetic neurones, including those in the adrenal gland, but does not produce hypotension by interfering with NPY biosynthesis in the medulla oblongata/pons.

One possible cause of the clonidine-induced suppression of NPY gene expression in the adrenal gland may be decrease in splanchnic nerve activity, because clonidine causes central inhibition of sympathetic nerve activity (Svensson, 1987; Pernow *et al.*, 1988) and consequently decreases trans-synaptic activation of chromaffin cells, which is one mechanism inducing NPY gene expression (Higuchi *et al.*, 1990). However, this possibility is unlikely, because elimination of trans-synaptic activation by denervation did not change NPY gene expression in the adrenal gland under ordinary conditions (Higuchi *et al.*, 1990). Therefore, the inhibition of NPY gene expression in the adrenal gland by clonidine seems to depend on its direct activation of the α_2-adrenoceptor on the chromaffin cells.

The normal counterpart of the PC12 rat phaeochromocytoma cell line is the chromaffin cells of the rat adrenal medulla. In PC12 cells, elevation of the intracellular adenosine 3′:5′-cyclic monophosphate (cyclic AMP) level, alone or in combination with other second messengers and/or hormones, results in rapid activation of NPY gene transcription with consequent increase in NPY mRNA abundance (Higuchi *et al.*, 1988c; Sabol & Higuchi, 1990). Therefore, decrease in the intracellular cyclic AMP content via the α_2-adrenoceptor on the chromaffin cells induced by clonidine may result in decrease in NPY gene expression at the level of transcription.

The suppression of NPY gene expression in the adrenal gland by clonidine is rapid and transient (Figure 1, Tables 1 and 2). The subsequent increase in NPY gene expression may result from desensitization of the α_2-adrenoceptor. However, the mean NPY mRNA level in the rat adrenal gland after 5 days was more than the control level (162 ± 16%). This overshoot was probably due to increase in splanchnic nerve activity in response to the hypotensive state, as in the case with reserpine (Figure 1; Higuchi *et al.*, 1990). Thus, on long-term treatment with clonidine, NPY gene expression in the adrenal gland may recover quickly due to trans-synaptic activation of chromaffin cells as an adaptation to hypotension.

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