Expression and Precursor Processing of Neuropeptide Y in Human Pheochromocytoma and Neuroblastoma Tumors

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ABSTRACT

The expression of the potent vasoactive peptide neuropeptide Y (NPY) was studied in 16 clinically and/or histologically diagnosed human pheochromocytomas and 3 human neuroblastoma tumors. All tumors contained NPY in concentrations ranging from 21 pmol/g of tissue, similar to that found in normal adrenal tissue, to 91,000 pmol/g (median, 1,700 pmol/g). Three control tumors of Cushing's type did not contain NPY. An almost total proteolytic processing of pro-NPY to normal NPY was observed in the tumors (median, 93%; range, 72–100%). A positive correlation between the processing efficiency and the NPY content was also observed. The small amount of pro-NPY found in the tumors was characterized by "in vitro conversion" with endoprotease Lys-C. In the tumor extracts, the majority of the NPY immunoreactivity, corresponding in size to the NPY standard, also behaved like synthetic NPY by high performance liquid chromatography and isoelectric focusing. As assessed by both its elution position in isoelectric focusing and its reaction with an antisera specific for the COOH-terminal amidated sequence, the peptide produced by the tumors was found to be efficiently amidated, a modification which is essential for the biological activity of NPY. It is concluded that although only a subset of chromaffin cells express NPY, the amino acid sequences of human NPY and its biosynthetic precursor were deduced from the complementary DNA structure. NPY was originally isolated from porcine brain (6), whereas the amino acid sequences of human NPY and its biosynthetic precursor were deduced from the complementary DNA structure, cloned on the basis of RNA isolated from a human pheochromocytoma cell line, PC 12 (11-13).

INTRODUCTION

Pheochromocytomas and neuroblastomas are catecholamine-producing tumors, arising from chromaffin cells of the adrenal or from sympathetic ganglia. Patients with pheochromocytoma often present with hypertension, paroxysmal or persistent, and the tumors can be diagnosed biochemically by demonstrating elevated catecholamine concentrations in the patients' plasma or urine (1). NPY3 is a highly conserved 36-amino acid neuropeptide (2) which coexists with norepinephrine in sympathetic nerves and which enhances norepinephrine-evoked vasoconstriction (3–5). NPY was originally isolated from porcine brain (6), whereas the amino acid sequences of human NPY and its biosynthetic precursor were deduced from the complementary DNA structure, cloned on the basis of RNA isolated from a human pheochromocytoma (7). The activation of NPY from its precursor requires a series of posttranslational modifications (Fig. 1) which include dibasic cleavage of the precursor and amidation of the peptide product. The peptide has been shown to have a widespread distribution in the sympathetic nervous system and also to be present in the adrenal medulla (8–10).

Recent, receptors for NPY have been located on a series of human neuroblastoma cell lines and on the rat pheochromocytoma cell line, PC12 (11–13).

In the present investigation we have examined the NPY content, the precursor processing, and amidation of NPY in a series of pheochromocytoma and neuroblastoma tumors, to determine to which degree they express the NPY precursor and activate the NPY molecule.

MATERIALS AND METHODS

Patients. Tumor tissue was obtained from 15 patients with clinically and/or histologically diagnosed pheochromocytomas and 3 patients with neuroblastomas (Table 1). Three patients with adrenocortical tumors presenting with Cushing's syndrome were also studied. Three patients had bilateral pheochromocytomas and both tumors were available for analysis in one of these cases (MHE). This patient was diagnosed and treated during pregnancy. Sufficient material was available to sample two areas of the same tumor in two cases (TH and EA). Patient TH had clinical features of the watery diarrhea-hypokalemia-achlorhydria syndrome with elevated concentrations of vasoactive intestinal polypeptide in plasma and adrenal tumor tissue (14). Two patients (EA, WP) had neurofibromatosis. Patient PD had multiple endocrine tumors including medullary carcinoma of the thyroid and tumors in lung and pancreas. Conceivably, this patient had multiple endocrine neoplasia type IIa syndrome but no other family members have yet been diagnosed. Urinary catecholamines were estimated preoperatively in 13 patients and were elevated on at least one occasion.

Extraction of Plasma. Preoperative plasma samples were obtained from seven of the patients with pheochromocytomas. Blood samples for NPY estimation were collected into chilled heparinized tubes containing containing 500 K1 units/ml aprotinin (Bayer, Leverkusen, Federal Republic of Germany) and centrifuged at 4°C for 20 min at 1000 x g. Plasma (1 ml) was extracted with ethanol (1.6 ml) containing 0.1% (v/v) trifluoroacetic acid. The supernatant obtained following centrifugation at 1500 x g for 20 min was dried under vacuum and reconstituted in assay buffer (0.05 M sodium phosphate buffer, pH 7.4, containing 2.5 g per liter BSA and 0.5 mM thiomersalate) immediately prior to assay.

Extraction of Tumor Tissue. Tissue was obtained following surgery (n = 19) or postmortem (n = 2), frozen in liquid nitrogen, and stored at −20°C until extraction and assay. After weighing, the tissue was extracted twice with cold acidified ethanol, final concentration 68% (v/v) ethanol and 0.1 M HCl, and used in a ratio of 8 ml to 1 g of tissue, followed by ether precipitation as described previously (15).

Radioimmunoassay Methods. Radioligands were prepared using 30 µg synthetic porcine NPY (Peninsula Laboratories, San Carlos, CA), 1 mCi Na125I (Amersham International, Amersham, Buckinghamshire, United Kingdom) and 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril (Serva, Heidelberg, Federal Republic of Germany), evaporated from methylene chloride (Sigma) (16), as described in detail previously (12). The moniodinated NPY species were purified by reverse phase HPLC using a Nucleosil C18 column (0.4 x 25 cm) with 5-µm particles and 300-Å pores, eluted isocratically with 35% (v/v) acetonitrile (Merck) and 0.1% (v/v) trifluoroacetic acid, (Pierce Chemical Co., Rockford, IL) as reported previously (12). Two different NPY antiseras were used in the assay of NPY immunoactivity. The rabbit antiserum, NPY 337, was raised to synthetic porcine NPY1-36 (Peninsula Laboratories) coupled to BSA with carbodiimide and was used at a final dilution of 1:27,000 with [125I-Tyr]- or [125I-Tyr]-monoiido-NPY as tracer. The detection limit was 3.8 fmol/assay tube and the intraassay coefficient of variation was 4.9%. This antiserum demonstrated 1.1% cross-reactivity with peptide YY. Antiserum 8999 was used at a final dilution of...
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Fig. 1. Schematic representation of the biosynthetic processing of the primary translation products of the NPY gene. The number of amino acids constituting the signal peptide (signalp.), NPY, and the COOH-terminal extension peptide (C-peptide) are indicated. The combined processing and amidation site of the precursor is shown in the amino acid one-letter code (G, glycine; K, lysine; R, arginine). The specificities of the two NPY antisera used in this study are also shown. Antibody (Ab) 337 recognizes both NPY and pro-NPY whereas antibody 8999 is totally specific for the activated, amidated NPY molecule.

Table 1  Neuropeptide Y content of pheochromocytoma, neuroblastoma, and adenocortical tumors

<table>
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<tr>
<th>Patients</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>NPY (pmol/g)</th>
<th>Processing efficiency†</th>
<th>Amidating efficiency‡</th>
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* Calculated as the percentage of the total NPY immunoreactivity found in the NPY1-36 peak.
† Calculated as the percentage of the total NPY1-36 migrating as the amidated species.
‡ Two results from one tumor.
§ Bilateral tumors.

1:90,000 with [3H]-Tyr]Monomiodo-NPY as tracer and was raised in a rabbit to synthetic Cys-NPY1-36, custom synthesized by Cambridge Research Biochemicals, (Cambridge, United Kingdom), coupled to BSA using maleimidobenzhydroxysuccinimide ester. The detection limit was 6 fmol/assay tube and the intraassay coefficient of variation was 3.3%. This antiserum cross-reacted fully with peptide YY which has an identical COOH-terminal hexapeptide sequence. Neither antisera demonstrated cross-reactivity with pancreatic polypeptide, at concentrations up to 1 nm. Antiseras, tracer, and standards (synthetic porcine NPY), 200 µl of each, were diluted in assay buffer. Antibody-bound and free tracer were separated by the addition of 1 ml plasma-coated charcoal (1%), followed by centrifugation after 5 min (3,000 × g, 20 min).

Gel Filtration. Tumor extracts were applied to Sephadex G-50 superfine columns (1.6 × 95 cm) eluted at 4°C, at a constant flow of 7 ml/h with 5% (v/v) formic acid containing 10 mg/liter BSA. Aliquots of fractions were dried in a vacuum centrifuge and reconstituted in assay buffer prior to radioimmunoassay. Internal standards of BSA (void volume marker measured as A405) and [3H]leucine (Amersham International) were used. Fractions corresponding in size to pro-NPY or NPY1-36 were pooled and dried under vacuum for further characterization.

High Performance Liquid Chromatography. Samples for HPLC analysis were reconstituted in 0.2 ml 3 M acetic acid and analyzed on a Hewlett Packard 1090 chromatograph using a Nucleosil C18 column (0.4 x 25 cm). The column was eluted with 0.1% (v/v) trifluoroacetic acid in water and a 20-min linear gradient of 20-50% acetonitrile. The system was calibrated with oxidized and nonoxidized synthetic human NPY1-36 (Peninsula Laboratories). Oxidation of the peptide standard was achieved by incubating synthetic human NPY (10 µg) dissolved in 0.1 ml of 3 M acetic acid, with 3.5% hydrogen peroxide for 30 min followed by HPLC analysis.

Isoelectric Focusing. Gels containing 0.25 ml each of Ampholyte pH 8-9.5 and Ampholyte pH 7-9 (LKB, Bromma, Sweden) were prepared in 3-mm (inner diameter) cylindrical glass tubes as described by Bravo (17) for the nonequilibrium pH gradient gels of O’Farrell (18). Samples were dissolved in lysis buffer, applied to the gels, and subjected to 350 V for 15 h. The gels were cut into 1-cm slices; each of these were then cut into 2-mm slices which were extracted for 12 h at 4°C in 80% (v/v) formic acid containing 10 mg/liter BSA. The extracts were dried under vacuum and reconstituted in assay buffer. For calibration, the oxidized and nonoxidized forms of amidated and free acid variants of synthetic human NPY (Peninsula) were included in separate gels in each electrophoretic analysis.

Endoproteinase Lys-C Digestion of Peptides. Gel-filtered pro-NPY was dried and reconstituted in 0.1 ml of 0.1 M N-ethylmorpholine (sequential grade; Pierce) adjusted to pH 8.65 with glacial acetic acid (Merck). Endoproteinase Lys-C (Boehringer Mannheim, Mannheim, Federal Republic of Germany), 30 million units in 10 µl, was added prior to incubation for 60 min at 37°C. The digestion was terminated by the addition of 0.1 ml of aprotinin (2000 KIU units).

Calculations. The degree of dibasic processing was calculated as the percentage of the total gel-filtered immunoreactivity eluting in the NPY1-36 peak compared to the total immunoreactivity, excluding the nonspecific material eluting in the void volume region. The degree of amidation is expressed as the percentage of the total NPY1-36 migrating as the amidated species in the nonequilibrium pH gradient gels.

RESULTS

All pheochromocytoma and neuroblastoma tumors examined contained NPY-immunoreactive material (Table 1). In three cases (SH, CT, MHa), the NPY content was similar to that reported for normal human adrenal tissue (19, 20). However, in all other cases, the NPY levels were elevated, up to more than 3000-fold. NPY was not detected in the three tumors from patients with Cushing’s syndrome. Elevated circulating concentrations of NPY (median, 1252 pm; range, 335–1980) were also found in the seven cases of pheochromocytoma examined, as compared to normal subjects who have NPY concentrations <50 pm. The immunoreactive material extracted from the tumors was characterized by gel filtration and hence the precursor-processing capacity of the tumor cells was examined in all patients (Table 1). The elution profiles of tumor NPY from four patients with pheochromocytomas are shown in Fig. 2. An almost complete conversion of pro-NPY to immunoreactivity corresponding in size to normal NPY was found in most of the tumors (Table 1). In a few tumors with low amounts of NPY, up to 28% of the immunoreactivity was present in the pro-

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Fig. 2. Sephadex G-50 gel filtration of NPY immunoreactivity extracted from four human pheochromocytomas. Columns were eluted with 5% formic acid containing 10 mg/liter BSA and calibrated with BSA (V), NPY standard and [3H]leucine (V). Arrows, elution positions of pro-NPY and NPY

Further characterized by HPLC analysis and isoelectric focusing. These methods are both complicated by the fact that oxidation of NPY creates heterogeneity of the immunoreactivity (data not shown). During HPLC analysis, the immunoreactive material from the tumors eluted in positions corresponding to both the oxidized and nonoxidized forms of standard synthetic NPY (Fig. 5). The amount of oxidized peptide extracted from the tumors varied, and occasionally only nonoxidized peptide was detected.

Fig. 3. Correlation between the amount of NPY stored and the processing efficiency of the pheochromocytoma (●) and neuroblastoma (○) tumors. The processing efficiency was calculated as the percentage of the gel-filtered immunoreactivity eluting in the NPY peak compared to the total immunoreactivity, excluding the nonspecific material eluting in the void volume region.

form. Overall, a significant correlation between the NPY content and the processing efficiency was observed, with a correlation coefficient of 0.59 (P < 0.01) (Fig. 3).

The pro-NPY material, although present in small quantities, was further characterized by endoproteinase Lys-C digestion, which cleaves on the carboxyl side of lysine residues (21). Pro-NPY contains only a single lysine residue, which is readily cleaved by this enzyme, i.e., the one in the dibasic conversion site, and as expected, the “in vitro conversion” with endoproteinase Lys-C moved the immunoreactive material from the pro-NPY peak to a peak of immunoreactivity of a size slightly larger than that of NPY (Fig. 4). This material conceivably represents NPY with a COOH-terminal extension of two amino acid residues (Gly–Lys) (see Fig. 4, inset). The antiserum used in this part of the study (337) cross-reacted approximately 75% with the pro-NPY form, inasmuch as an increase in the amount of immunoreactive material was observed following endoproteinase Lys-C digestion. Serial dilution of pro-NPY fractions pooled from several of such experiments before and after the enzyme treatment confirmed this.

The dominant species of NPY-immunoreactive material from the tumor extracts, which corresponded in size to NPY, was

Fig. 4. “In vitro conversion” of pro-NPY from a human pheochromocytoma by endoproteinase Lys-C digestion. The gel filtration profile of pro-NPY, obtained from a prior gel filtration as shown in Fig. 2, before (●) and after (○) endoproteinase Lys-C digestion. Arrows, elution positions of pro-NPY and synthetic human NPY.Inset, endoproteinase Lys-C cleavage site (G, glycine; K, lysine; R, arginine) and the putative product.

Fig. 5. HPLC characterization of gel-filtered NPY-like immunoreactivity from four human pheochromocytomas. Arrows, elution positions of oxidized (unlabeled) and nonoxidized human NPY (hNPY). The acetonitrile gradient. Fractions were assayed using the 337 antiserum.
The positions taken by the free acid (open arrow) and amidated forms of NPY (closed arrow) are indicated. The gel slices were extracted with 80% formic acid, dried, and assayed using both the 337 (•) and the COOH-terminal amide-substantiated by Chromatographic characterization of the NPY neublastomas studied expressed NPY. This observation was substantiated by Chromatographic characterization of the NPY immunoreactivity from four human pheochromocytomas by isoelectric focusing. There was an almost complete proteolytic processing of the NPY precursor in the tumors producing the highest quantities of NPY. This relationship between peptide content and processing efficiency has been observed previously by Creutzfeldt et al. (22) for insulin-producing tumors, where up to 22% of the immunoreactivity was present as proinsulin, and the highest precursor concentrations were found in tumors producing the lowest insulin levels. Although pheochromocytomas are known to produce NPY-immunoreactive material (19, 20, 23), the capacity of pheochromocytomas and neuroblastomas to amiate and hence activate the NPY they produce has not been studied previously. We find that the peptide is efficiently amidated and the amiating capacity is not related to the NPY content.

Previously, it has been reported that around 50% of patients with pheochromocytomas have highly elevated concentrations of NPY in plasma (19, 23). The relatively low incidence of elevated concentrations of NPY in plasma compared to the high incidence of NPY expression in the tumors, found in the present study, could be due to the fact that NPY is a neuropeptide which is rapidly cleared from the circulation. Furthermore, many tumor cells express high affinity NPY receptors, at least in culture (11–13), which may sequester the peptide within the tumor.

There are two important, potential implications for the finding of amidated, biologically active NPY in the tumors, one is related to the clinical features of the patients, and the other is related to tumor growth. NPY is a potent vasopressor agent in its amidated form. The peptide causes vasoconstriction, especially of small arterioles, both by itself and by potentiating the effect of catecholamines (24). This effect is found not only in visceral vessels, as originally described, but also, e.g., in vessels in skeletal muscle (25). Thus, NPY released from pheochromocytomas can very well by partly responsible for some of the cardiovascular symptoms of the patients.

Although receptors for NPY have not been described on the tumor cells directly, we have found receptors for NPY both on a series of human neuroblastoma cell lines (11, 12) and on a rat pheochromocytoma cell line (13). Binding of NPY to these receptors, which were found in 6 of 12 neuroblastoma cell lines tested, resulted in the activation of intracellular secondary messenger systems. Since the tumors all produce amidated, bioactive NPY, it is possible that autocrine stimulation of the cancer cells occurs. Whether this autocrine-stimulatory mechanism is related to the growth of the tumors, as in the case of small cell lung carcinomas and bombesin/gastrin-releasing peptide (26), remains to be investigated.

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**REFERENCES**


* Unpublished results.
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