

Release of Neuropeptide Y from Pheochromocytomas

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Abstracts

To investigate the release of neuropeptide Y (NPY) from the pheochromocytomas, we studied the relationship between the plasma and tumor tissue immunoreactive (IR) NPY concentrations in 13 patients with pheochromocytoma and measured the IR-NPY concentration in plasma samples obtained by catheter from several veins (jugular veins, superior vena cava, renal veins, adrenal veins and inferior vena cava) in 2 patients with pheochromocytoma. The plasma IR-NPY concentration in 13 patients with pheochromocytoma ranged from 118 to 1460 pg/ml and the concentration in 10 of 13 patients with pheochromocytoma was above 290 pg/ml (the upper limit of normal range). The tumor tissue IR-NPY ranged from 0.025 to 95.3 $\mu\text{g/g}$ wet tissue. Plasma IR-NPY was parallel with tumor tissue IR-NPY in 13 cases of pheochromocytoma ($r=0.76$, $P<0.01$). The highest concentration of IR-NPY was found in plasma obtained from the drainage vein from a tumor among the plasma samples obtained from several veins in 2 cases of pheochromocytoma. These findings indicate that in patients with pheochromocytoma, NPY is in most cases excessively released from the tumors into the systemic circulation and plasma IR-NPY in the periphery is increased.

Neuropeptide Y (NPY) is a 36 amino acid peptide, which was originally isolated from the porcine brain (Tatemoto 1982). Human NPY was isolated from acid extracts of adrenal-medullary pheochromocytoma tissue (Corder *et al.*, 1984). In human NPY, methionine is found at position 17 instead of leucine.

A high concentration of NPY in plasma and tumor tissue extracts of pheochromocytoma and ganglioneuroblastoma has been reported (Adrian *et al.*, 1983; Corder *et al.*, 1984 and 1986; Lundberg *et al.*, 1986; Allen *et al.*, 1987; Takahashi *et al.*, 1987). Corder *et al.* (1986) reported that about half the pheochromocytoma cases had increased plasma and tumor tissue NPY, and the other half had a low concentration. And a high concentration of IR-NPY in

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the conditioned medium of rat and human cultured pheochromocytoma cells was reported (Allen *et al.*, 1984; Tischler *et al.*, 1985). The sequence of the cDNA encoding NPY in one pheochromocytoma was also determined (Minth *et al.*, 1984). From these reports, NPY is thought to be produced in these tumors and released into the systemic circulation. But there is no report concerning plasma NPY in the drainage vein of a pheochromocytoma.

To further clarify the release of NPY from a pheochromocytoma, we studied the plasma and tumor tissue immunoreactive (IR) NPY in 13 cases of pheochromocytoma and measured the IR-NPY concentration in plasma samples obtained from the jugular vein, superior vena cava, renal vein, adrenal vein and inferior vena cava in 2 pheochromocytoma patients.

Materials and Methods

Subjects

We measured the plasma and tumor tissue IR-NPY concentration in 13 patients with pheochromocytoma by radioimmunoassay. Blood samples were obtained from the subcutaneous vein in the forearm in the supine position after an overnight fasting. The blood samples were mixed with aprotinin (500 kallikrein inhibitor units/ml blood) (Bayer, Leverkusen, West Germany) and EDTA (1 mg/ml blood), cooled at 0°C and immediately centrifuged at 4°C. Plasma samples were separated and kept at -20°C until extracted. Tumor tissues of pheochromocytomas were obtained at surgery and stored at -80°C until extracted. As controls, 5 adrenal cortical tumors (3 aldosterone-producing adenomas, one cortisol-producing carcinoma and one non-functioning adenoma) were obtained at surgery and 4 normal parts of adrenal glands (cortex and medulla) were also obtained at surgery from 2 patients with pheochromocytoma and 2 with aldosterone-producing adrenal cortical adenoma.

In 2 cases of pheochromocytoma, plasma samples were obtained from bilateral jugular veins, superior vena cava, inferior vena cava, bilateral renal veins and bilateral adrenal veins

by means of a catheter inserted into the femoral vein. In both cases, a tumor existed in the adrenal gland, which was confirmed at surgery. Therefore the right adrenal vein was a drainage vein of the tumor. As controls, plasma samples were also obtained from similar positions in veins in 2 patients with Cushing's disease (ACTH secreting pituitary adenoma). The plasma cortisol concentration was measured by radioimmunoassay in these plasma samples and a higher cortisol concentration in plasma from the adrenal vein was confirmed (data not shown).

Extraction and radioimmunoassay

Plasma samples were extracted by Sep-Pak C18 cartridges (Waters Associates, Milford, MA) and assayed, as previously described (Takahashi *et al.*, 1987 and 1988). Briefly, the antiserum to NPY showed 20.7% cross reaction with peptide YY (PYY) but less than 0.001% with human pancreatic polypeptide, avian pancreatic polypeptide, somatostatin, corticotropin-releasing hormone, growth hormone releasing hormone or calcitonin gene-related peptide. Intra- and interassay coefficients of variation were 9.3% (n=10) and 11.2% (n=9), respectively. The sensitivity of this assay was 9.0 ± 2.1 pg/tube (=7, mean \pm SD) at 95% confidence.

Tumor and normal tissues were boiled in 1M acetic acid for 10 minutes, homogenized and extracted, as previously described (Mouri *et al.*, 1989). Tissue extracts were reconstituted in assay buffer (0.1 M phosphate buffer, pH 7.4, containing 0.1% human serum albumin, 0.2% Triton X-100 and 0.1% sodium azide) and assayed.

Chromatography

Fractionation of NPY immunoreactivity in plasma and tumor tissue extracts of pheochromocytoma was carried out by Sephadex G-50 column (superfine, 0.9 \times 56 cm) chromatography and reverse phase high performance liquid chromatography (HPLC) using a Waters μ Bondapak C18, 3.9 \times 300 mm column. In Sephadex G-50 column chromatography, the eluent was 4 M acetic acid containing 0.2% bovine serum albumin and from each fraction tube 0.6 ml of eluate was obtained. In reverse phase HPLC, a linear gradient from 35% to 45% acetonitrile in water (acetonitrile and water containing 0.1% trifluoroacetic acid) was performed at a flow rate of 1 ml/min and 1 ml fractions were collected

for radioimmunoassay. Mixed plasma from pheochromocytomas was extracted with a Sep-Pak C18 cartridge, tumor tissue extracts from pheochromocytomas were re-extracted with a Sep-Pak C18 cartridge, and then plasma and tumor tissue extracts were loaded onto the column of HPLC.

To determine the elution position of human NPY with oxidated methionine in HPLC, we reacted synthetic human NPY (1 μ g) with one ml of 3% hydrogen peroxide for 60 minutes at room temperature and changed it to the oxidated form of NPY. Human NPY with oxidated methionine was once adsorbed to a Sep-Pak C18 cartridge, eluted with 60% acetonitrile containing 0.1% trifluoroacetic acid, diluted with water containing 0.1% trifluoroacetic acid to 35% acetonitrile and loaded onto the column of HPLC.

Each fraction of Sephadex G-50 column chromatography and reverse phase HPLC was air dried, reconstituted in assay buffer and assayed.

Statistics

The values are given as the mean \pm SD. The tumor tissue IR-NPY concentration was corrected with the logarithmic scale to get a normal distribution, and these corrected values were used to compare the tissue IR-NPY concentration of the various groups by analysis of variance and to study the co-relation with the plasma IR-NPY concentration in pheochromocytomas by linear regression.

Results

Plasma IR-NPY in 13 cases of pheochromocytoma ranged from 118 to 1460 pg/ml (584 ± 388 pg/ml). Ten of 13 cases of pheochromocytoma had a higher concentration of plasma IR-NPY than the upper limit of the normal range (290 pg/ml).

Tumor tissue IR-NPY in 13 cases of pheochromocytoma ranged from 0.025 to 95.3 μ g/g wet tissue (19.6 ± 28.6 μ g/g wet tissue), which was significantly higher than in adrenal cortical tumors ($n=5$, 0.029 ± 0.028 μ g/g wet tissue) ($P < 0.025$) (Fig. 1).

The plasma IR-NPY was parallel with

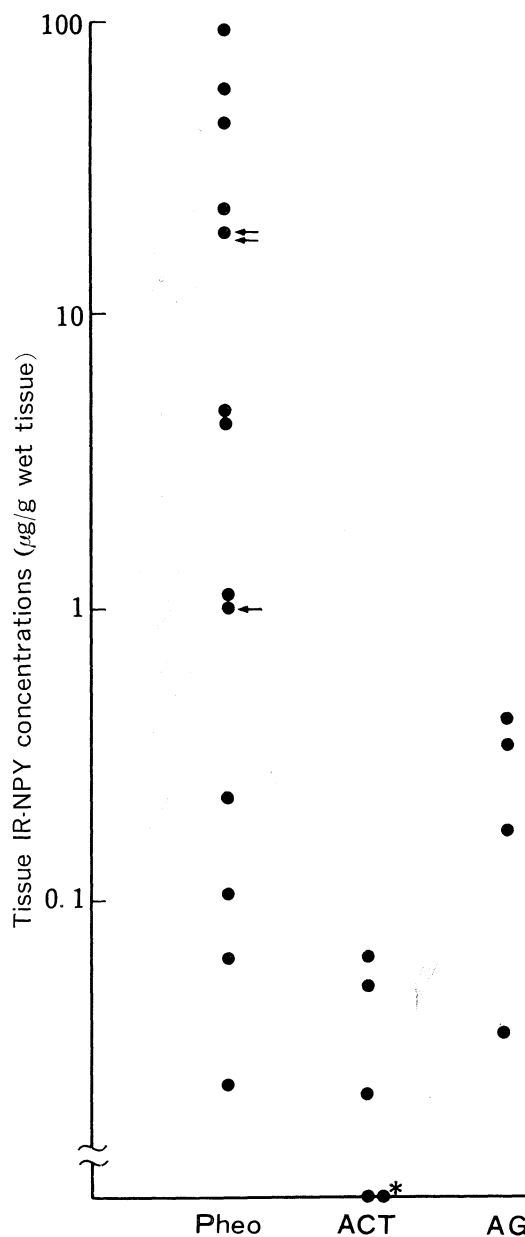


Fig. 1. Tissue IR-NPY concentrations in 13 pheochromocytomas, 5 adrenal cortical tumors and 4 normal parts of adrenal cortex and medulla. Pheo: pheochromocytoma, ACT: adrenal cortical tumors, AG: normal parts of adrenal glands (cortex and medulla). *: not detectable, <0.008 μ g/g wet tissue. \leftarrow and \Leftarrow : Pheo 1 and Pheo 2 in Table 1, respectively, in which plasma samplings by catheter were performed.

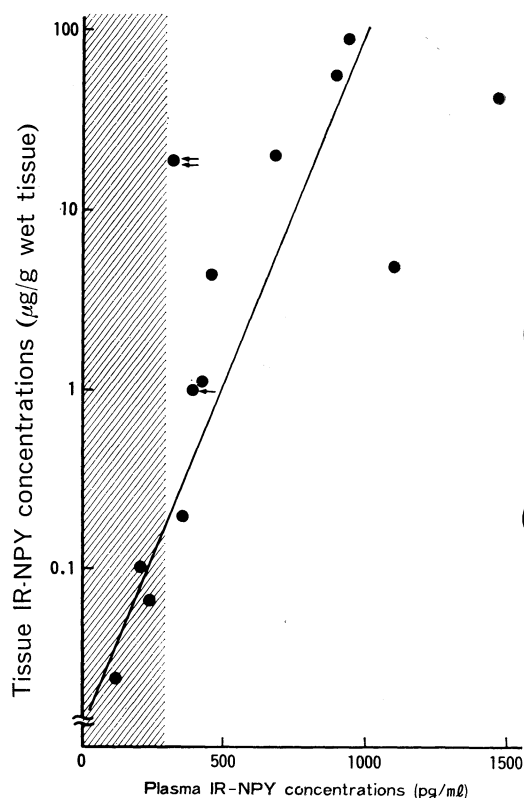



Fig. 2. Co-relation between plasma and tissue IR-NPY concentrations in 13 patients with pheochromocytoma ($r=0.76$, $P<0.01$). The zone  indicates the normal range of the plasma IR-NPY concentration (290 pg/ml). \leftarrow and \Leftarrow : Pheo 1 and Pheo 2 in Table 1, respectively.

Table 1. IR-NPY coconcentrations in plasma samples obtained from the jugular veins, superior vena cava, renal veins, adrenal veins, inferior vena cava and peripheral vein in 2 patients with pheochromocytoma and 2 with Cushing's disease (pg/ml). The tumor tissue IR-NPY concentration in 2 pheochromocytoma patients is also shown ($\mu\text{g/g}$ wet tissue).

	JV		SVC	RV		Adv		IVC	P	T
	R	L		R	L	R	L			
Pheo 1	308	240	282	207	290	392*	266	—	319	19.6
Pheo 2	330	332	—	307	354	439*	363	337	354	1.05
CD 1	109	163	187	160	171	181	150	155	167	
CD 2	111	110	—	97	139	105	127	110	112	

Pheo: pheochromocytoma, CD: Cushing's disease, JV: jugular veins, SVC: superior vena cava, RV: renal veins, Adv: adrenal veins, IVC: inferior vena cava (plasma samples were obtained from positions in the inferior vena cava as high as the 4th or 5th lumbar vertebral body.) P: peripheral vein, R: right, L: left, T: tumor tissue IR-NPY concentration. —: not obtained, *: drainage vein of a pheochromocytoma.

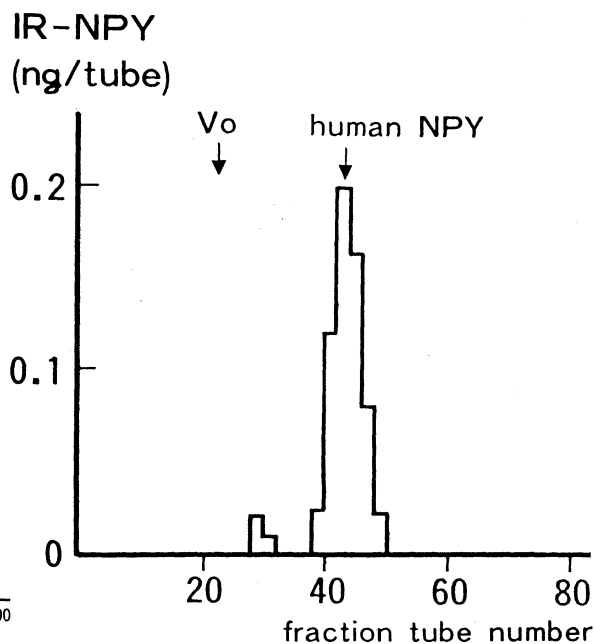


Fig. 3. Typical Sephadex G-50 column chromatography of a tumor extract of pheochromocytoma. V_0 : void volume, human NPY: the elution position of synthetic human NPY.

tumor tissue IR-NPY in 13 cases of pheochromocytoma ($r=0.76$, $P<0.01$) (Fig. 2).

Tumor tissue IR-NPY in 3 cases of pheochromocytoma with normal plasma IR-NPY was similar to or less than that in normal parts of the adrenal cortex and medulla ($n=4$, $0.251 \pm 0.154 \mu\text{g/g}$ wet tissue).

The highest IR-NPY concentration was found in plasma samples obtained from the drainage vein of a tumor (right adrenal vein) among the plasma samples obtained from several veins in 2 cases of pheochromocytoma (Table 1). On the other hand, in 2 patients with Cushing's disease, the IR-NPY concentration in plasma obtained from the right and left adrenal vein was

not the highest among the plasma samples obtained from several veins.

Sephadex G-50 column chromatography showed that most of the NPY immunoreactivity ($>90\%$) in tumor tissue extracts of pheochromocytomas was eluted in an identical position with synthetic human NPY, while a small amount of NPY immunoreactivity was eluted in a higher molecular weight region (Fig. 3). Sephadex G-50 column chromatography of NPY immunoreactivity in pooled plasma of pheochromocytoma patients also showed a similar elution pattern to that of a tumor extract (data not shown).

Reverse phase HPLC showed that most of the NPY immunoreactivity ($>90\%$) in tumor tissue extracts of pheochromocytomas was eluted in an identical position with synthetic human NPY, while a small amount of NPY immunoreactivity was eluted in a similar position to synthetic human NPY with oxidated methionine (Fig. 4). Most of the NPY immunoreactivity in plasma extracts was also eluted in an identical position with synthetic human NPY (data not shown).

Discussion

We have previously reported an increase in the plasma IR-NPY concentration in patients with pheochromocytoma and in chronic renal failure (Takahashi *et al.*, 1987). In that report, the plasma IR-NPY concentration in 21 normal subjects ranged from 90 to 202 pg/ml, 151 ± 28 pg/ml and the levels in 33 essential hypertensive patients ranged from 104 to 284 pg/ml, 177 ± 49 pg/ml. From these data, we determined that the upper limit of the normal range of plasma IR-NPY was 290 pg/ml. In the present study, the plasma IR-NPY in 10 of 13 cases of pheochromocytoma was above 290 pg/ml. There was a significant co-relation between the plasma and tissue IR-NPY concentrations in 13 cases of pheochromocytoma.

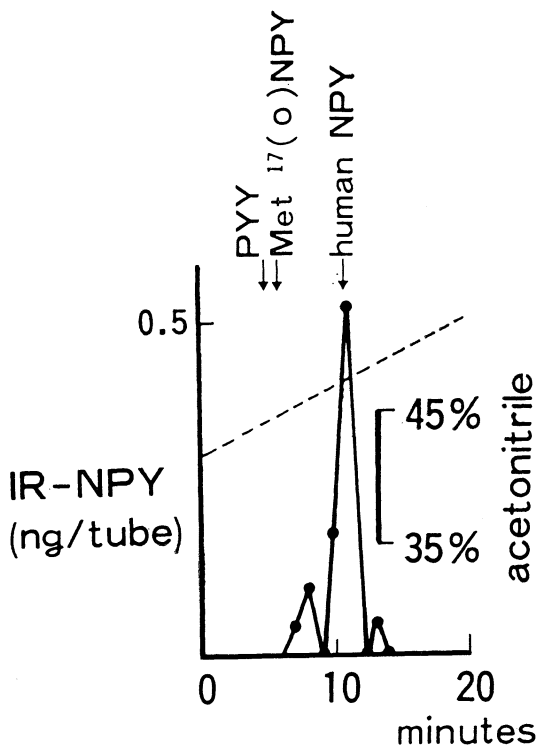


Fig. 4. Reverse phase HPLC of a tumor extract of pheochromocytoma. PYY: the elution position of peptide YY, Met (O)NPY: that of synthetic human NPY with oxidated methionine, NPY: that of synthetic human NPY.

cytomas. These findings indicate that pheochromocytomas with a high tissue IR-NPY concentration release large amounts of NPY into the systemic circulation and in such cases plasma IR-NPY is increased, while pheochromocytomas with low tissue IR-NPY release small amounts of NPY into the systemic circulation and in such cases plasma IR-NPY is not increased at all or only a little.

Corder *et al.*, (1986) reported that patients with pheochromocytoma could be divided into 2 subtypes according to the plasma and tissue NPY concentrations; NPY secreting and NPY non-secreting subtypes and about 50% were NPY secreting. Our findings corroborated the relationship between the plasma and tumor tissue NPY concentrations reported by Corder *et al.* But in our study plasma and tissue IR-NPY concentrations in 13 patients with pheochromocytoma seemed continuously distributed and could not be clearly divided into 2 subtypes, and cases of pheochromocytoma with a normal plasma IR-NPY concentration were about 23%.

The present study also revealed the highest concentration of IR-NPY in plasma obtained from the drainage vein of a tumor in 2 patients with pheochromocytoma. The increase in IR-NPY in the peripheral plasma in these 2 patients was not so marked as in the other patients with pheochromocytoma in this study. Plasma IR-NPY in the drainage veins of tumors was about 100 pg/ml higher than in other veins. Intra- and interassay coefficients of variation of this assay were about 10%, therefore this increase in the drainage vein could be significant and reflect the release of NPY from the tumors of pheochromocytoma patients.

Reverse phase HPLC revealed that most of the NPY immunoreactivity in plasma and tumor tissue extracts of pheochromocytomas was eluted in an identical position with synthetic human NPY. The antibody

to NPY used in this study has 20.6% cross-reaction with PYY. However, the results of HPLC indicate that NPY immunoreactivity in pheochromocytoma is NPY itself and not PYY. The results also suggest that in the extraction procedures most of the NPY does not change into NPY with oxidated methionine and only a small amount changes to that form.

NPY is known to have a potent vasoconstrictor action and potentiate a vasoconstrictor action of norepinephrine *in vivo* and *in vitro* (Lundberg *et al.*, 1982; Ekblad *et al.*, 1984; Wahlestedt *et al.*, 1985; Itoi *et al.*, 1986). NPY has also a presynaptic inhibitory action on norepinephrine secretion (Allen *et al.*, 1982; Lundberg *et al.*, 1984). In the present study we could not find the difference between the blood pressure or other clinical symptoms of the patients having pheochromocytoma with greatly increased plasma IR-NPY and those with normal or mildly increased plasma IR-NPY. Some cases of pheochromocytomas with normotension or hypotension were known. Two of 13 patients with pheochromocytoma in this study were normotensive. These 2 patients consulted our hospital because the presence of adrenal tumor had been revealed by computerized body tomography and by ultrasonography in local clinics, and did not show any hypertension in spite of increased plasma catecholamine without anti-hypertensive agents during the admission period before surgery. However, plasma IR-NPY in these 2 cases was increased (454 and 1103 pg/ml, respectively). Further studies are needed to clarify the relationship between NPY and blood pressure and other clinical symptoms in patients with pheochromocytomas.

In conclusion, there is a significant correlation between plasma and tumor tissue IR-NPY concentrations in 13 cases of pheochromocytoma. The highest concentration of IR-NPY was found in plasma obtained from the drainage vein of a tumor in 2

cases of pheochromocytoma. These findings indicate that the tumor release NPY into the systemic circulation in patients with pheochromocytoma.

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