

## Elevated Plasma Immunoreactive Neuropeptide Y Concentrations and Its Increased Urinary Excretion in Patients with Advanced Diabetic Nephropathy

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**Abstract.** Neuropeptide Y (NPY) is a potent vasoconstrictor peptide that is abundant in the brain and the peripheral sympathetic nervous system. In the present study we investigated possible changes in plasma immunoreactive (IR)-NPY concentrations and urinary IR-NPY excretion in patients with non-insulin dependent diabetes mellitus (NIDDM) and the relationship to diabetic complications, such as nephropathy and neuropathy. IR-NPY in plasma and urine was measured by radioimmunoassay in 69 patients with NIDDM. Plasma IR-NPY concentrations in patients with advanced nephropathy (creatinine clearance <30 ml/min) ( $100.5 \pm 10.3$  pmol/l,  $n=9$ , mean  $\pm$  SEM) were higher than in the control subjects ( $55.0 \pm 6.8$  pmol/l,  $n=15$ ) ( $P<0.02$ ). Urinary excretion of IR-NPY and fractional excretion of NPY were also increased in the patients with advanced nephropathy. Sephadex G-50 column chromatography of the urine extracts obtained from healthy subjects, diabetic patients with renal failure and non-diabetic patients with renal failure showed an immunoreactive peak eluting in the NPY position. On the other hand, neither plasma nor urinary IR-NPY was high in patients with retinopathy, or in patients with peripheral neuropathy. The present study has, for the first time, shown high plasma IR-NPY concentrations and urinary IR-NPY excretion in NIDDM patients with advanced nephropathy.

**Key words:** Neuropeptide Y, Diabetes, Diabetic Nephropathy, Urine

(Endocrine Journal 46: 139–146, 1999)

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**NEUROPEPTIDE Y (NPY)** is a potent vasoconstrictor peptide consisting of 36 amino acids [1, 2]. High concentrations of NPY are present in the central nervous system, sympathetic ganglia and adrenal medulla [3–6]. NPY is co-localized

with catecholamines in some cell populations of these tissues [3, 5, 6]. NPY has a potent vasoconstrictor action and potentiates a vasoconstrictor action of various vasoconstrictor agents, including norepinephrine [3, 5, 7, 8]. NPY presynaptically inhibits the secretion of norepinephrine [9]. NPY is also known to be a potent central stimulator of appetite [10].

Plasma immunoreactive (IR-) NPY concentrations were high in patients with pheochromocytoma [11–16], ganglioneuroblastoma and neuroblastoma [16]

Received: July 21, 1998

Accepted: October 29, 1998

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and in patients with chronic renal failure [12, 15–17]. Exercise [18] and insulin-induced hypoglycemia [19] increased plasma IR-NPY concentrations in man.

It was reported that IR-NPY and NPY mRNA expression were increased in the hypothalamus of animals with diabetic mellitus [20, 21]. NPY was depleted in the autonomic nerves of the specimens from diabetic patients with peripheral vascular disease [22]. On the other hand, NPY was increased in the ileum in streptozotocin-induced diabetic rats [23]. Contractile response to NPY was diminished in arteries obtained from alloxan-induced diabetic rabbits [24]. These findings suggest a pathophysiological role of NPY in diabetes mellitus, in particular in the pathophysiology of diabetic complications. IR-NPY is present in the kidneys and localized in the sympathetic nerves distributed in the juxtaglomerular apparatus [25] and renal tubular cells [26], suggesting that NPY plays an important physiological role in the kidneys. Although there are two reports showing decreased plasma IR-NPY concentrations in patients with insulin-dependent diabetes mellitus (IDDM) [27, 28], plasma and urinary NPY has not been studied in patients with non-insulin dependent diabetes mellitus (NIDDM).

In the present study, we therefore measured IR-NPY in plasma and urine by radioimmunoassay in 69 NIDDM patients and investigated the relationship between IR-NPY in plasma and urine and diabetic complications such as nephropathy, peripheral neuropathy and retinopathy.

## Materials and Methods

### Subjects

Plasma samples and 24-h urine samples were obtained from 15 control subjects (13 men and 2 women, 18–85 years old) and 69 patients with NIDDM (31 men and 38 women,  $54.6 \pm 14.2$  years old, mean  $\pm$  SD). Informed consent was obtained from each subject. The control subjects had normal blood pressure and fasting blood sugar. Patients on hemodialysis due to renal failure were excluded from this study. Blood samples were obtained from a subcutaneous vein in the forearm, collected into tubes containing aprotinin (Trasylol; 500 kallikrein

inhibitory units/ml of blood; Bayer, Germany) and EDTA (1 mg/ml of blood), and centrifuged at 4 °C. The plasma samples were separated and stored at  $-30$  °C until assayed.

24-h urine samples were collected at room temperature. After the measurement of volume, approximately 50 ml of each sample was stored at  $-30$  °C until extraction. The stability of NPY in urine at room temperature was confirmed by incubating synthetic NPY in urine at room temperature. After the incubation at room temperature for 24 h, normal urine containing exogenously added NPY (2.4 pmol/ml of urine) was extracted by means of a Sep-Pak C18 cartridge (Waters Associates, Milford, MA, USA) and assayed. No significant decrease in the IR-NPY concentration was found after 24-h incubation (94% added peptide).

The NIDDM patients were divided into 6 groups according to their renal function (Table 1). The patients were first divided into 3 groups according to their creatinine clearance (Ccr): group I,  $Ccr \geq 70$  ml/min; group II,  $30 \leq Ccr < 70$  ml/min; group III,  $Ccr < 30$  ml/min. Then, the patients in the group I were subdivided into 2 groups according to their urinary microalbumin levels (Ualb): group Ia,  $Ualb < 20$  mg/day; group Ib,  $Ualb \geq 20$  mg/day. The patients in the group II were similarly subdivided into 3 groups according to their urinary microalbumin levels and the presence of proteinuria: group IIa,  $Ualb < 20$  mg/day; group IIb,  $Ualb \geq 20$  mg/day but negative proteinuria; group IIc,  $Ualb \geq 20$  mg/day and positive proteinuria. The patients on anti-hypertensive drugs, or with systolic blood pressure  $\geq 160$  mmHg, or diastolic blood pressure  $\geq 95$  mmHg were considered to have hypertension (37 out of 69 NIDDM patients) (Table 2).

The presence of diabetic retinopathy was assessed by diabetic ophthalmologists. The patients were divided into 3 groups: patients without retinopathy, patients with simple diabetic retinopathy and patients with preproliferative retinopathy or proliferative retinopathy (Table 2).

The presence of diabetic peripheral neuropathy was assessed by at least one abnormality in the vibration sensation threshold, the Achilles tendon reflex, the knee tendon reflex, or the presence of abnormal sensation (Table 2).

**Table 1.** Clinical characterization of 69 patients with NIDDM and classification by their renal function

Group	Ccr (ml/min)	Ual (mg/day)	UP	Sex M F		Age (years)	Duration (years)	FBS (mmol/l)	HbA <sub>1c</sub> (%)	Ccr (ml/min)	Hb (g/100 ml)
Ia	≥70	<20	(-)	8	9	47.1 (16.2)	7.5 (7.0)	8.86 (2.84)	8.4 (1.2)	85.1 (11.0)	13.8 (1.6)
Ib	≥70	≥20	(-)	3	4	46.9 (16.5)	11.9 (8.5)	9.88 (3.51)	9.5 (2.7)	83.9 (17.4)	12.7 (1.4)
IIa	30≤Ccr<70	<20	(-)	6	10	59.4 (13.4)	8.3 (7.2)	9.57 (3.70)	9.8 (2.5)	56.9 (11.1)	13.7 (2.3)
IIb	30≤Ccr<70	≥20	(-)	2	4	57.3 (9.4)	10.2 (6.0)	8.52 (5.05)	9.0 (2.7)	49.7 (8.9)	12.6 (2.2)
IIc	30≤Ccr<70	≥20	(+)	9	5	60.0 (10.5)	14.8 (8.7)	7.91 (2.89)	7.5 (1.6)	49.1 (13.9)	12.3 (2.1)
III	<30	≥20	(+)	3	6	56.0 (11.4)	12.4 (9.3)	7.25 (2.71)	8.1 (2.8)	18.4 (7.4)	10.0 (2.0)
Total				31	38	54.6 (14.2)	10.5 (8.0)	8.70 (3.15)	8.7 (2.4)	59.9 (24.4)	12.8 (2.3)

Data are shown as the mean (SD). Ccr, creatinine clearance; Ual, urinary microalbumin; UP, proteinuria; (+), positive; (-), negative; M, male; F, female; FBS, fasting blood sugar; HbA<sub>1c</sub>, hemoglobin A<sub>1c</sub>; Hb, hemoglobin levels.

**Table 2.** Clinical characterization of 69 patients with NIDDM: Incidence of retinopathy, peripheral neuropathy and hypertension

Group	Retinopathy			Peripheral neuropathy		Hypertension	
	Absent	Simple	Pre-proliferative + Proliferative	Absent	Present	Absent	Present
Ia	14	0	3	7	10	11	6
Ib	3	0	4	3	4	5	2
IIa	12	1	3	11	5	9	7
IIb	2	1	3	4	2	2	4
IIc	4	1	9	6	8	4	10
III	1	0	8	2	7	1	8
Total	36	3	30	33	36	32	37

Number of patients in each group is shown. Simple, simple diabetic retinopathy; Pre-proliferative, pre-proliferative diabetic retinopathy; Proliferative, proliferative diabetic retinopathy.

### Radioimmunoassay

Plasma IR-NPY concentrations were measured by radioimmunoassay, as previously reported [16]. Urine samples were extracted by means of Sep-Pak C18 cartridges (Waters Associates). Five ml urine was acidified with 5 ml of 0.75 mol/l acetic acid and loaded onto the cartridge, which was pretreated with 10 ml acetonitrile, 10 ml methanol and then 10 ml of 0.75 mol/l acetic acid. After washing the cartridge with 10 ml of 0.75 mol/l

acetic acid, the peptide was eluted from the cartridge with 2 ml of 80% (vol/vol) acetonitrile containing 0.15 mol/l acetic acid. The eluate was air-dried, reconstituted with assay buffer and assayed as previously reported [16]. The recovery, which was determined by adding NPY to the urine prior to the extraction, was  $104 \pm 29\%$  ( $n=4$ , mean  $\pm$  SD).

Chromatographic characterization of IR-NPY in urine was performed by Sephadex G50 superfine column chromatography (10  $\times$  560 mm). Urine samples were obtained from control subjects,

NIDDM patients with renal failure ( $\text{Ccr} < 30 \text{ ml/min}$ ) and non-diabetic patients with renal failure ( $\text{Ccr} < 30 \text{ ml/min}$ ). Pooled urine samples were extracted by means of Sep-Pak C18 cartridges, reconstituted in 1 mol/l acetic acid containing 0.5% (wt/vol) bovine serum albumin, and loaded onto the column. The column was eluted with 1 mol/l acetic acid containing 0.5% (wt/vol) bovine serum albumin at a flow rate of 6 ml/h. Each fraction (0.8 ml/fraction) was collected, air-dried, reconstituted in assay buffer and assayed.

### Statistics

Data are shown as means  $\pm$  SEM unless otherwise stated. The statistical analysis was performed by one-way analysis of variance and Scheffe's multiple comparison test. Correlation was examined with Pearson's correlation coefficient.

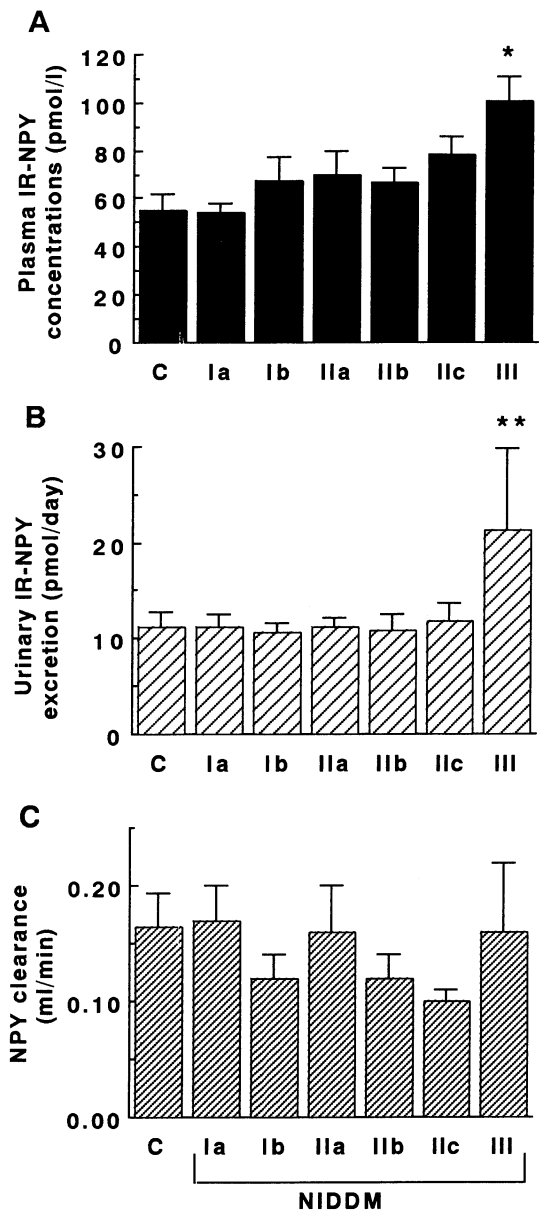
## Results

Plasma IR-NPY concentrations and urinary excretion of IR-NPY were negatively correlated with  $\text{Ccr}$  levels in 69 NIDDM patients ( $r = -0.420$ ,  $P < 0.005$  and  $r = -0.314$ ,  $P < 0.01$ , respectively). The plasma IR-NPY concentrations and urinary excretion of IR-NPY in 69 NIDDM patients are shown in Fig. 1. Plasma IR-NPY concentrations were much higher in patients with  $\text{Ccr} < 30 \text{ ml/min}$  (group III,  $100.5 \pm 10.3 \text{ pmol/l}$ ,  $n=9$ ) than in the control subjects ( $55.0 \pm 6.8 \text{ pmol/l}$ ,  $n=15$ ) and group Ia ( $P < 0.02$ ) (Fig. 1A). Urinary excretion of IR-NPY was also higher in patients with  $\text{Ccr} < 30 \text{ ml/min}$  (group III,  $21.2 \pm 8.6 \text{ pmol/day}$ ) than in the control subjects ( $11.3 \pm 1.4 \text{ pmol/day}$ ) and groups Ia and IIa ( $P < 0.05$ ) (Fig. 1B). The presence of microalbuminuria or proteinuria had a negligible effect on the plasma IR-NPY concentration or urinary IR-NPY excretion in patients with  $\text{Ccr} \geq 30 \text{ ml/min}$  (groups I and II) ( $P > 0.1$ ). The presence of hypertension had a negligible effect on the plasma IR-NPY concentration or urinary IR-NPY excretion in patients with  $\text{Ccr} \geq 30 \text{ ml/min}$  (groups I and II) ( $P > 0.1$ ). On the other hand, 8 out of 9 patients in group III had hypertension (Table 2).

There was no significant difference in NPY clearance among 6 groups of NIDDM patients and the control subjects ( $P > 0.1$ ) (Fig. 1C). Fractional excretion of NPY was increased in patients with

$\text{Ccr} < 30 \text{ ml/min}$  (group III,  $0.62 \pm 0.3\%$ ,  $n=9$ ), compared to groups Ia and Ib ( $P < 0.05$ ) (Fig. 2).

The plasma IR-NPY concentration and urinary excretion of IR-NPY in patients with pre-



**Fig. 1.** (A) Plasma immunoreactive neuropeptide Y (IR-NPY) concentrations, (B) urinary excretion of IR-NPY and (C) NPY clearance in 69 patients with NIDDM and 15 control subjects. C, control. The NIDDM patients were divided into 6 groups according to their renal function (Table 1). \*, significantly higher than control and Ia ( $P < 0.02$ ). \*\*, significantly higher than control, Ia and IIa ( $P < 0.05$ ). Data are shown as the mean  $\pm$  SEM.

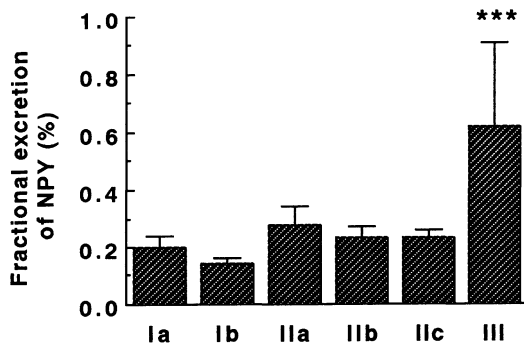


Fig. 2. Fractional excretion of neuropeptide Y (NPY) in 69 patients with NIDDM. Data are shown as the mean  $\pm$  SEM. \*\*\*, significantly higher than Ia and Ib ( $P < 0.05$ ).

proliferative or proliferative diabetic retinopathy ( $81.1 \pm 5.0$  pmol/l and  $15.9 \pm 2.4$  pmol/day,  $n=30$ ) were not much higher than those in patients without diabetic retinopathy ( $63.4 \pm 4.8$  pmol/l and  $13.4 \pm 1.5$  pmol/day,  $n=36$ ) or patients with simple diabetic retinopathy ( $52.0 \pm 22.0$  pmol/l and  $13.7 \pm 1.8$  pmol/day,  $n=3$ ) ( $P > 0.1$ ). The plasma IR-NPY concentration and urinary excretion of IR-NPY in patients with diabetic peripheral neuropathy ( $80.3 \pm 5.6$  pmol/l and  $16.8 \pm 2.3$  pmol/day,  $n=36$ ) were not significantly higher than those in patients without it ( $62.1 \pm 4.9$  pmol/l and  $12.8 \pm 1.7$  pmol/day,  $n=33$ ) ( $P > 0.1$ ).

There was no significant relation between the plasma IR-NPY concentration and fasting blood sugar level ( $r=0.11$ ,  $P > 0.1$ ), hemoglobin A<sub>1C</sub> ( $r=0.29$ ,  $P > 0.1$ ) or urinary excretion of N-acetylglucosaminidase ( $r=0.25$ ,  $P > 0.1$ ) in 69 NIDDM patients. There was also no significant relation between urinary excretion of IR-NPY and the fasting blood sugar level ( $r=0.00$ ,  $P > 0.1$ ), hemoglobin A<sub>1C</sub> ( $r=0.03$ ,  $P > 0.1$ ) or urinary excretion of N-acetylglucosaminidase ( $r=0.19$ ,  $P > 0.1$ ) in these patients. And no significant relation was found when these correlations were examined in the respective three groups of NIDDM patients with similar renal function (groups I, II and III).

The identity of urinary IR-NPY was assessed by means of Sephadex G50 column chromatography. Chromatography of the urine extracts showed a peak eluting in the position of human NPY (Fig. 3). In addition to a peak eluting in the position of human NPY, material eluting earlier (about at 35th fraction) was found in all three samples, and may

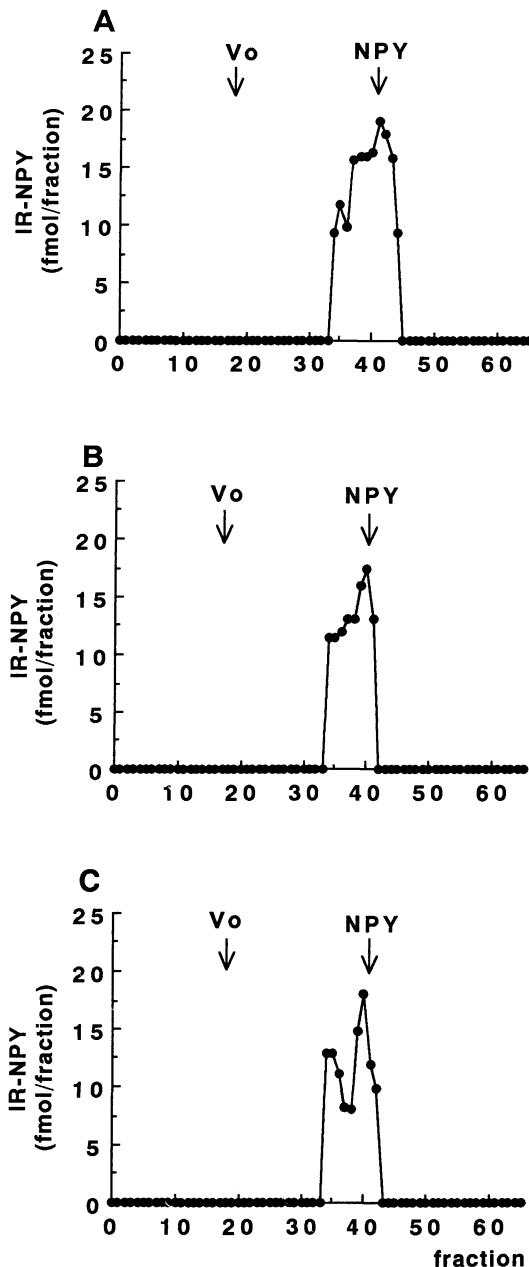


Fig. 3. Sephadex G-50 column chromatography of the urine extracts obtained from (A) control subjects, (B) NIDDM patients with renal failure ( $\text{Ccr} < 30$  ml/min, group III) and (C) non-diabetic patients with renal failure ( $\text{Ccr} < 30$  ml/min). Non-diabetic patients with renal failure comprised three patients with chronic renal failure due to primary glomerulonephritis ( $\text{Ccr}$ ,  $25 \pm 3$  ml/min, mean  $\pm$  SD). IR-NPY, immunoreactive neuropeptide Y; Vo, void volume; NPY, the elution position of synthetic NPY.

represent a larger molecular weight substance, possibly a precursor or a precursor fragment of NPY.

### Discussion

The present study has shown a high plasma IR-NPY concentration in NIDDM patients with renal failure ( $\text{Ccr} < 30 \text{ ml/min}$ ). These findings are compatible with our previous reports on a high plasma IR-NPY concentration in patients with chronic renal failure due to various renal diseases [12, 16, 17]. It was reported that the plasma IR-NPY concentration was lowered in IDDM patients [27, 28]. The discrepancy between these reports and ours in plasma IR-NPY levels may be due to the difference between the types of diabetes mellitus or diabetic complications, but further studies are required. In addition, the present study has for the first time shown the presence of NPY in human urine and increased urinary excretion of IR-NPY in NIDDM patients with renal failure. These changes in plasma and urinary NPY may reflect the pathophysiology of the renal dysfunction caused by diabetic nephropathy, rather than a specific change in diabetic nephropathy.

The kidney is one of the most important organs for the degradation of peptide hormones. Plasma concentrations of several vasoactive peptides, such as endothelin [29, 30] and natriuretic peptides [31], were increased in patients with chronic renal failure. Urinary IR-NPY concentrations were lower than plasma IR-NPY concentrations, and NPY clearance was much lower than creatinine clearance both in control and NIDDM patients. These findings are compatible with the hypothesis that a large part of NPY in plasma is metabolized in the kidney and only a very small part of IR-NPY derived from plasma is excreted in the urine. On the other hand, renal tubular cells may synthesize and secrete NPY into the urine. Immunocytochemical studies showed positive immunostaining of NPY in the renal tubular cells [26].

Increased urinary excretion of NPY was accompanied by increased levels of the fractional excretion of NPY in NIDDM patients with renal failure. Urinary excretion and fractional excretion of IR-brain natriuretic peptide were also increased in patients with chronic renal failure [32]. One

possible explanation for increased urinary excretion of IR-NPY may therefore be a decreased degradation of NPY derived from plasma by the kidney and its secretion into the urine. Another possibility is increased production of NPY by the kidneys, possibly by renal tubular cells.

Plasma IR-NPY concentrations and urinary excretion of IR-NPY were slightly increased in patients with preproliferative or proliferative diabetic retinopathy, or in patients with diabetic peripheral neuropathy, but these increases were not statistically significant, and may reflect the accompanying renal dysfunction in these patients. On the other hand, the degree of neuropathy in the NIDDM patients in this study ranged from mild to severe. Further studies are therefore required to clarify the relationship between the degree of neuropathy and NPY. Furthermore, there was no significant relationship between plasma IR-NPY concentrations and fasting blood sugar levels or hemoglobin A<sub>1C</sub>, and no relationship between urinary excretion of IR-NPY and fasting blood sugar levels or hemoglobin A<sub>1C</sub>. It is therefore unlikely that high levels of blood sugar affect plasma IR-NPY concentrations or urinary excretion of IR-NPY in NIDDM patients.

NPY was localized in the sympathetic nerves distributed in the juxtaglomerular apparatus [25] and in the renal tubular cells [26] in the kidney. NPY binding sites were observed in vascular smooth muscle cells and in the proximal renal tubular cells in rabbits [33]. Messenger RNA encoding Y1 receptor, one of the NPY receptor isoforms, was expressed in the collecting tubules and Henle's loop in rats [34]. NPY is a potent vasoconstrictor peptide and also has a vasoconstrictor action on the renal artery [35]. NPY has an inhibitory action on renin secretion [36] and a stimulatory action on Na<sup>+</sup>, K<sup>+</sup>-ATPase in the renal tubular cells [37]. Allen *et al.* [35] reported that NPY had natriuretic effects but Persson *et al.* [38] reported that NPY had an anti-natriuretic action. It is therefore plausible that NPY is closely related to renal function, possibly acting as a potent vasoconstrictor, in patients with advanced diabetic nephropathy.

The present study has shown increased plasma IR-NPY concentrations and increased urinary excretion of IR-NPY in NIDDM patients with renal failure.

### Acknowledgments

We are grateful to Ms Umezu, Ms Oikawa and Ms Kikuchi for their secretarial and technical assistance. This study has been supported partly by a Grant-in-aid for Scientific Research (C) (No.

09670117) from the Ministry of Education, Science, Sports and Culture of Japan (to K. T.), a Grant-in-aid for the Brain Science Research from the Ministry of Health and Welfare of Japan (to K. T.), by the Gonryou Medical Foundation (to K. T.) and by a research grant for medical science from the Nippon University Alumni Association (to M.S.).

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