Expression of mRNA coding for four catecholamine-synthesizing enzymes in human adrenal pheochromocytomas

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Abstract

Objective: To understand the molecular mechanisms by which catecholamine synthesis is controlled in pheochromocytomas – tumors that synthesize and release catecholamines, which are related to various clinical manifestations of the condition.

Methods: We measured the concentrations of mRNA coding for the catecholamine-synthesizing enzymes tyrosine hydroxylase, aromatic L-amino acid decarboxylase (AADC), dopamine β -hydroxylase (DBH) and phenylethanolamine N-methyl transferase (PNMT) and for the catecholamine contents in 12 pheochromocytomas and 12 normal adrenal medullas.

Results: The mean content of total catecholamine and the β -actin mRNA expression in the pheochromocytomas were almost the same as those in the normal adrenal medullas. However, the tyrosine hydroxylase, AADC and DBH mRNA concentrations in the pheochromocytomas were greater than those of the normal adrenal medullas. Conversely, the PNMT mRNA concentration in the pheochromocytomas was lower than that in the normal adrenal medullas. These differences are responsible for the difference in the proportions of catecholamines between pheochromocytomas and normal adrenal medullas. The constitutive expression of the catecholamine-synthesizing enzyme mRNAs varied in magnitude among the pheochromocytomas, and the tyrosine hydroxylase mRNA expressions correlated with the contents of total catecholamine in the tumors ($r=0.964$, $P<0.0001$).

Conclusions: These findings indicate that catecholamine production in pheochromocytomas is primarily controlled by the level of gene expression.

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Introduction

Pheochromocytomas are tumors arising most often from the adrenal medulla. They synthesize, store and release catecholamines (1–3). However, unlike the adrenal medulla (4), pheochromocytomas are not innervated, and the catecholamine release by pheochromocytomas is not initiated by neural impulses. The mechanism of catecholamine release from pheochromocytoma is not yet clear, nor is the regulation of catecholamine synthesis in these tumors.

Tyrosine hydroxylase, the rate-limiting enzyme in the biosynthesis of catecholamines, is distributed in sympathetic ganglia, the adrenal medulla, the central nervous system and also in pheochromocytomas (5). Its activity is modulated by the phosphorylation of the tyrosine hydroxylase molecule (6) and by long-term regulation involving tyrosine hydroxylase gene (TH gene) transcription and protein synthesis (7).

A variety of factors, including neural impulses, have been reported to regulate the TH gene expression in adrenal medullary cells (8–10). The expression of the TH gene has also been studied in rat pheochromocytoma cell lines (11–13). Compared with the information available concerning TH gene expression in

experimental cell lines, our knowledge about the expression of this gene in human pheochromocytomas is limited (14).

The catecholamines dopa, dopamine, noradrenaline and adrenaline are synthesized enzymatically by four enzymes; tyrosine hydroxylase, aromatic L-amino acid decarboxylase (AADC), dopamine β -hydroxylase (DBH) and phenylethanolamine N-methyl transferase (PNMT) respectively, and the activities of these enzymes are regulated over the long term by the gene expressions of the enzymes.

We report here our data on the expression of mRNAs coding for the catecholamine-synthesizing enzymes tyrosine hydroxylase, AADC, DBH and PNMT, and on the simultaneous measurements of tissue catecholamine concentrations in 12 human adrenal pheochromocytomas and 12 normal human adrenal medullas.

Materials and methods

Materials

Isogen for RNA isolation was obtained from Nippon Gene (Tokyo, Japan). Hybridization membranes and a

random primer DNA labeling kit were purchased from Du Pont (Boston, MA, USA). cDNAs for tyrosine hydroxylase, AADC and DBH were obtained from the Riken DNA Bank (Tsukuba, Japan). cDNAs for PNMT and bovine β -actin were gifts from Dr T Nagatsu (Fujita Health University, Japan) and Dr Y Kawakami (Tsukuba University, Japan) respectively. 32 P-dCTP was obtained from Amersham (Amersham, Buckinghamshire, UK). Other chemicals were of reagent grade.

Tissue samples

Tumor specimens were obtained surgically from 12 patients with pheochromocytoma, and 12 normal adrenal glands were obtained at surgery at the time of nephrectomy for renal cell carcinomas. Each specimen was frozen at -80°C until required for assay. The pheochromocytoma specimens were all of adrenal origin.

Two experiments (A and B) were performed for the northern blot analysis, to avoid the degradation of mRNA in the tissues.

Determination of catecholamine contents

The tissue concentrations of catecholamines (adrenaline, noradrenaline and dopamine) were determined using a catecholamine autoanalyzer (H8030, TOSOH, Tokyo, Japan) with built-in HPLC and a spectrofluorimeter. Fifty milligrams of each specimen were minced in 1 ml extraction buffer (2% HClO_4 , 1 mM ascorbic acid and 100 mM EDTA). The homogenate was centrifuged at 10 000 times gravity for 20 min at 4°C . The supernatant was diluted by 1000 and applied to the autoanalyzer.

Isolation of RNA and northern blot analysis

Total RNAs were extracted from the tissues using the Isogen kit, and the RNA concentrations were estimated spectrophotometrically at 260 nm. RNA (10 μg each)

was fractionated by electrophoresis on 1% agarose–5% formaldehyde gels (80 V 2 h). After staining with ethidium bromide and the visual inspection of u.v. fluorescence to confirm the presence of equal amounts of ribosomal RNA in each lane, the RNA was transferred to a nitrocellulose membrane and hybridized to 32 P-labeled probes.

The following probes were used: 1.9 kbp EcoRI fragment of pTHT1 for tyrosine hydroxylase, a 2 kbp EcoRI and EcoRV fragment of pDBH-1 for DBH, 1.9 kbp EcoRI fragment of pPCDC13EcoA for AADC, and 901 bp SmaI and KpnI fragment of phPNMT for PNMT. Plasmid pTHT1 contained the full-length cDNA for human tyrosine hydroxylase type 1 cDNA. Plasmids pDBH-1, pPCDC13EcoA and phPNMT contain human DBH cDNA, human AADC cDNA and human PNMT cDNA respectively. These plasmids were developed by T Nagatsu (15), K Kobayashi (16), H Ichinose (17) and T Nagatsu (18) respectively. The cDNA probes were labeled by a random primer extension labeling system (New England Nuclear, Boston, MA, USA). The membranes were also hybridized with a 32 P-labeled bovine β -actin cDNA probe after stripping. Hybridization signals were scanned in an image analyzer (BAS2000, Fuji, Tokyo, Japan).

Statistical analysis

All values are expressed as means $\pm$ S.E. Statistical significance was determined by Student's *t*-test and the Mann–Whitney U-test. Correlations between the tissue concentrations of total catecholamine and tyrosine hydroxylase mRNA were determined by Pearson's test. The significance level was set at $P < 0.05$.

Results

The tumor weights and sizes and the tissue, plasma and urine catecholamine concentrations in the 12 adrenal pheochromocytomas are listed in Table 1. We found

Table 1 Tissue, plasma and urine catecholamine concentrations in the 12 adrenal pheochromocytomas.

No.	Tumor weight (g)	Tumor size (cm)	Tissue CA (nmol/g)			Plasma CA (nmol/l)			Urine CA (nmol/day)		
			A	NA	DA	A	NA	DA	A	NA	DA
1	58	7.5 $\times$ 6.5 $\times$ 2.5	737	16 430	131	—	—	—	57	3239	4506
2	21	3.8 $\times$ 3.5 $\times$ 3.0	36 036	27 304	327	68.00	61.90	2.24	2184	1282	6442
3	—	5	1147	2719	0	1.22	2.06	0.03	187	1382	14 143
4	193	8 $\times$ 8 $\times$ 5	27	225	0	—	—	—	—	—	—
5	110	9.5 $\times$ 6.5 $\times$ 4.0	11	41	0	0.11	2.54	0.07	33	6572	2727
6	44	5.5 $\times$ 4.5 $\times$ 3.0	2763	14 598	3898	—	7.21	—	—	2352	—
7	—	1	27	3853	0	0.20	5.06	0.07	—	—	—
8	—	4	0	248	0	—	13.40	—	—	2300	—
9	—	4.6 $\times$ 3.7	142	5331	137	0.46	70.30	3.96	—	14 775–23 640	—
10	39	5 $\times$ 4 $\times$ 3.5	16	910	13	0.05	65.00	1.31	12	6761	2230
11	—	—	1507	361	0	1.42	0.53	1.31	103	676	13 178
12	—	2.7 $\times$ 1.9	11 193	79 726	0	0.38	3.13	1.31	260	2145	13 734

CA, catecholamine; A, adrenaline; NA, noradrenaline; DA, dopamine; — not examined; 0, not detected.

Table 2 Concentrations of catecholamines in human pheochromocytoma and normal adrenal medulla tissue. Values are means $\pm$ S.E of 12 samples.

	Concentration (nmol/g tissue)				A/total (%)
	A	NA	DA	Total	
Normal adrenal medulla	10 986 $\pm$ 3609	1436 $\pm$ 455	0 $\pm$ 0	12 422 $\pm$ 4064	88.4
Pheochromocytoma	5209 $\pm$ 3254	9497 $\pm$ 3558	451 $\pm$ 334	15 157 $\pm$ 7146	34.4

A, adrenaline; NA, noradrenaline; DA, dopamine.

Table 3 Concentrations of catecholamine-synthesizing enzymes mRNA and β -actin mRNA in pheochromocytomas and normal adrenal medullas. Values are means $\pm$ S.E. for independent samples in experiments A and B, expressed as ratios to the control value in normal adrenal medullas. The sample numbers are in parentheses.

	TH	AADC	DBH	PNMT	β -actin
Experiment A					
Normal adrenal medulla (8)	1.00 $\pm$ 0.41	1.00 $\pm$ 0.53	1.00 $\pm$ 0.41	1.00 $\pm$ 0.45	1.00 $\pm$ 0.16
Pheochromocytoma (4)	2.43 $\pm$ 0.94*†	5.65 $\pm$ 0.49*	5.32 $\pm$ 3.34	0.16 $\pm$ 0.06†	1.17 $\pm$ 0.30
Experiment B					
Normal adrenal medulla (4)	1.00 $\pm$ 0.36			1.00 $\pm$ 0.48	1.00 $\pm$ 0.18
Pheochromocytoma (8)	5.96 $\pm$ 1.19*†			0.13 $\pm$ 0.03*†	1.13 $\pm$ 0.23

TH, tyrosine hydroxylase; AADC, aromatic L-amino acid decarboxylase; DBH, dopamine β -hydroxylase; PNMT, phenylethanolamine N-methyl transferase. * $P < 0.05$ (Student's *t*-test); † $P < 0.05$ (Mann-Whitney U-test) compared with normal adrenal medulla.

no correlation between the tumor weight and the catecholamine content. Only the tissue adrenaline concentration correlated with the plasma and urinary adrenaline concentrations ($P < 0.0001$). The other tissue catecholamines did not correlate with the corresponding plasma and urinary catecholamines.

As shown in Table 2, the proportions of adrenaline, noradrenaline and dopamine found in the pheochromocytoma varied with the specimens, and 10 of the 12 pheochromocytomas contained more noradrenaline than adrenaline, whereas all 12 of the human normal adrenal medullas contained about 90% adrenaline. The mean content of total catecholamine in the pheochromocytomas was almost the same as that in the normal adrenal medullas.

The amounts of tyrosine hydroxylase, AADC and DBH mRNA in the pheochromocytomas were respectively 2.43, 5.65 and 5.32 times those in the normal adrenal medullas (Table 3, experiment A). This finding indicates that the gene expression of these catecholamine-synthesizing enzymes is increased in pheochromocytoma tissues. In contrast, the PNMT gene expression was about six to eight times greater in the normal adrenal medullas than in the pheochromocytoma tissues (Table 3). The levels of expression of the β -actin gene, a constitutive gene, were the same in the human adrenal medullas as in the pheochromocytomas (Table 3).

As the amount of catecholamines and the amount of the mRNAs varied among the pheochromocytoma tissue specimens (Table 2), we next examined the relationship between the TH gene expression and the tissue concentrations of catecholamines in the pheochromocytomas

and normal adrenal medullas, because tyrosine hydroxylase is a rate-limiting enzyme of catecholamine biosynthesis. As shown in Fig. 1, the mRNA expressions correlated with the content of catecholamines in the tumors ($r = 0.964$, $P < 0.0001$, $n = 12$). In the normal adrenal medullas, the mRNA expressions were relatively lower compared with the high contents of catecholamines, and no correlation was observed ($r = 0.421$, $P = 0.2351$, $n = 12$).

Figure 1 Correlation of tyrosine hydroxylase (TH) mRNA and total catecholamine concentrations in human pheochromocytomas (●) and normal adrenal medullas (○): radioactivities of tyrosine hydroxylase mRNAs (photostimulated luminescence minus background, PSL-BG) plotted against total catecholamines (nmol/g tissue) analyzed by HPLC.

Figure 2 Correlation of phenylethanolamine *N*-methyl transferase (PNMT) mRNA and adrenaline concentrations in human pheochromocytomas (●) and normal adrenal medullas (○): radioactivities of PNMT mRNAs (photostimulated luminescence minus background, PSL-BG) plotted against adrenaline (nmol/g tissue) analyzed by HPLC.

We then examined the relationship between the PNMT gene expression and adrenaline concentrations in the tissues. As shown in Fig. 2, both the mRNA expressions and adrenaline concentrations were lower in the tumors. In the normal adrenal medullas, the mRNA expressions were higher and correlated with the content of adrenaline ($r=0.574$, $P=0.05$, $n=12$).

Discussion

Catecholamines are synthesized sequentially by four enzymes: tyrosine hydroxylase, AADC, DBH and PNMT. The results of this study indicated that the levels of expression of tyrosine hydroxylase, AADC and DBH mRNAs are greater in pheochromocytomas than in normal adrenal medullas, and PNMT mRNA concentrations are greater in normal adrenal medullas. These differences in mRNA expression are responsible for the difference in the proportions of catecholamines between pheochromocytomas and normal adrenal medullas.

There was a close correlation between the amounts of total catecholamine and the tyrosine hydroxylase mRNA concentrations in the tumors, but not in the normal adrenal medullas. Nakada *et al.* (19) reported that pheochromocytomas contained a significantly larger amount of catecholamines and greater tyrosine hydroxylase activity than did normal adrenal medullas. Iwase *et al.* (20) also observed high tyrosine hydroxylase activity in pheochromocytomas. Thus it is likely that tyrosine hydroxylase activity is mainly regulated by the tyrosine hydroxylase mRNA expression rather than by short-term control involving tyrosine hydroxylase protein phosphorylation in pheochromocytomas. The production of catecholamines in pheochromocytomas may be determined by the expression of the genes.

In contrast to the other genes, in the present study the level of expression of PNMT mRNA was found to be stronger in the normal adrenal medullas than in the pheochromocytomas. This might explain why sporadic pheochromocytomas usually predominantly produce noradrenaline. The high concentrations of PNMT mRNA in the normal adrenal medullas may have been induced by stimulation of glucocorticoid (21) release from the adrenal cortex, which surrounds the adrenal medullas. The low concentrations of PNMT mRNA in the pheochromocytomas might reflect an insufficient concentration of plasma cortisol in the artery supplying the tumor. Kimura *et al.* (22) also reported in an immunohistochemical study that tyrosine hydroxylase, AADC and DBH were detected in all functioning pheochromocytomas, and that PNMT was limited to the mixed-type pheochromocytomas that contain adrenaline and noradrenaline equally.

The expression of AADC and DBH mRNA was also enhanced in the present tumors. The mechanisms by which the expression of these genes is activated remain to be determined. Surgical stress may affect catecholamine secretion and synthesis, but probably did not affect the mRNAs concentrations in this study, because it takes several hours to increase the concentrations of mRNA. The high levels of expression may have relevance to the development or growth of pheochromocytomas. Second-messenger systems, especially protein kinase C and cAMP-dependent pathways, are known to affect the transcriptional regions of genes. The 5' region of both the TH gene and the DBH gene contain a cAMP response element (CRE) that mediates transcriptional induction by cAMP. Other mechanisms, including growth factors, may regulate the expression of these genes. Goto *et al.* (23) suggested that pheochromocytomas are in a state of growth stimulation by growth factors, including insulin-like growth factor (IGF)-II and IGF-I/II receptors, and that *c-fos* and *c-myc* proto-oncogenes are expressed in this tumor. Further investigation to elucidate these mechanisms is necessary.

Thus we demonstrated that the gene expression of the catecholamine-synthesizing enzymes is increased in pheochromocytoma tissues and that there is a positive relationship between the levels of TH gene expression and the tissue concentrations of catecholamines in pheochromocytomas. The mechanism responsible for the expressions of these genes in pheochromocytomas remains to be investigated.

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