Genes Up- or Down-regulated by High Calcium Medium in Parathyroid Tissue Explants from Patients with Primary Hyperparathyroidism

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Abstract. To investigate genes modulated in the parathyroid glands by calcium, expression levels of mRNA for all genes expressed in parathyroid tissue explants (PTEs) obtained from patients with primary hyperparathyroidism (I^o-HPT) were analyzed by oligo-DNA microarray. PTEs obtained from 4 patients with I^o-HPT were precultured in normocalcemic medium (Ca⁺⁺ 1.0-1.1 mM) for 7 days and then cultured in hypocalcemic medium (Ca⁺⁺ 0.60 mM) or hypercalcemic (Ca⁺⁺ 1.60 mM) medium containing 4mg/dL phosphate for an additional 7 days. As expected, expression levels of mRNA for PTH and chromogranin A were decreased to less than 50% in the hypercalcemic medium when compared with those in the hypocalcemic medium. Furthermore, oligo-DNA microarray analyses revealed that 7 genes were up-regulated by more than 2-fold and more than 30 genes were down-regulated by more than 1/2 in PTEs. Interestingly, 9 of these genes (up-regulated genes: chemokine ligand 8, multiple C2 domain and transmembrane region protein 1; down-regulated genes: matrix metalloproteinase-9, B-box and SPRY domain-containing protein, nitric oxide synthase 2A, PTH, cartilage acidic protein 1, chromogranin A, and fibrin 1) were involved in calcium metabolism or calcium-signaling pathways in the parathyroid tissue. However, the expression level of mRNA for α -klotho was variable, and it was not constantly decreased in hypercalcemic medium under the present experimental conditions. Although it was not possible to use normal parathyroid tissue, this is the first reported study to have investigated the expression levels of mRNA for all genes in human parathyroid adenomas that are modulated by high calcium concentration in organ culture.

Key words: Calcium, Parathyroid gland, Primary hyperparathyroidism, Microarray

THE PARATHYROID GLANDS are tiny endocrine glands that play an important role in the regulation of calcium and phosphate concentration. However, the total gene expression profile of the parathyroid glands has been scarcely investigated, as they are the smallest of all endocrine organs and usually impossible to obtain as study specimens from normal humans. Although a few studies of calcium-regulating genes

have been done by microarray using frozen parathyroid tissue or parathyroid tissue that has been immediately submerged in an RNAase inhibitor [1-3], there have been no published investigations of all the genes expressed in cultured parathyroid tissue.

Recently, we established an organ culture system for parathyroid tissue explants (PTEs) in which PTH release and the calcium-sensing receptor (CaSR) can be maintained for a prolonged period (up to 4 weeks) [4]. Using this system, we have already found that both the expression level of PTH mRNA and PTH release are significantly increased when PTEs are cultured in high-phosphate medium. In the present study, we cultured PTEs (obtained from patients with prima-

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ry hyperparathyroidism; I^o-HPT) in hypocalcemic and hypercalcemic medium, and investigated the genes that were up- or down-regulated.

Materials and Methods

Origin of human parathyroid tissues

This study was approved by the Ethic Committee of Tokyo Women's Medical University, and informed written consent was obtained from the patients prior to parathyroidectomy [4]. The corrected calcium concentration was 10.7 ± 0.26 mg/dL (normal range; 8.5–9.9 mg/dL), and the serum level of intact PTH was 187 ± 62 pg/mL (normal range; 15–60 pg/mL). Usually about 100 mg of parathyroid tissue was obtained from the I^o-HPT patients at parathyroidectomy. The parathyroid tissues were dissected into small pieces (1 x 1 x 0.5 mm), and the explants were placed on a piece of collagen-coated nylon mesh supported by a silicone tube ring so that the mesh was only slightly submerged beneath the surface of the culture medium, in a 6-cm culture dish, as described previously. They were precultured in minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS; total calcium concentration 12–14 mg/dL, phosphate concentration 9–11 mg/dL), 100 U/mL penicillin and 100 µg/mL streptomycin (preculture medium) for seven days (total Ca concentration 7.0 mg/dL, Ca⁺⁺ concentration: 1.0–1.1 mM, total phosphate concentration; 4.0 mg/dL), and then cultured in MEM containing a low (total Ca 4.0 mg/dL; Ca⁺⁺ 0.60 mM) or high (total Ca 10 mg/dL; Ca⁺⁺ 1.60 mM) calcium concentration. In each experiment, calcium (CaCl₂) and phosphate (NaH₂PO₄ and Na₂HPO₄) were added to MEM, which had been specially prepared without calcium and phosphate (Invitrogen, Life Science Technologies, Frederick, MD, USA), so that the final total calcium concentration became 4.0 mg/dL (hypocalcemic medium), 7.0 mg/dL (normocalcemic medium) or 10 mg/dL (hypercalcemic medium). The final phosphate concentration was prepared to 1.29 mM (4.0 mg/dL). Ca⁺⁺ concentration was determined using a Chiba-Corning calcium-pH analyzer (Corning, Halstead, Essex, UK). In these hypo- and hypercalcemic media, PTH release from PTEs was significantly increased or decreased, respectively [4], although the set point of PTEs from patients with I^o-HPT was slightly shifted to the right (Ca⁺⁺ 1.2–1.4 mM) [4] compared with that of normal subjects (Ca⁺⁺ 1.0–1.2 mM).

After an additional 7 days of culture, total RNA was extracted from the parathyroid tissue explants as described previously [4].

Oligo-DNA microarray

After the RNA had been extracted, oligo-DNA microarray was performed as described previously [4]. In brief, RNA was converted to cDNA using a Human cDNA System I Direct Kit (NEN Life Science Products). cDNA obtained from parathyroid tissues cultured in the control and treated media was labeled with Cy3 (green, low calcium) and Cy5 (red, high calcium), respectively, and the expression levels of 41000 gene spots were analyzed using cDNA microarray (Whole Human Genome Oligo Microarray Kit, Product Number G4112A; Agilent Technologies, Palo Alto, CA). Laser detection of the Cy3 and Cy5 signals on the microarray was performed with a non-confocal laser reader, Gene PX 4000A (Axon Instruments, Union, CA). Fluorescence signal intensities and the Cy5/Cy3 ratios for each of the 41000 oligo-DNAs were analyzed using the Gene PixPro 30 software package (Axon Instruments).

Data analysis

The data from all arrays were analyzed using GeneSpring GX software (Agilent Technologies UK Limited, South Queensferry, UK) [6]. Array normalization was done using the intensity-dependent Lowess normalization method. Analysis was performed on the log expression ratios that were produced in the normalization step. Biological replicates were treated as duplicate measurements, and their averages were calculated. Features that were flagged absent in all the arrays and non-reliable features with a very low signal were removed prior to further analysis. The normalized data were analyzed to identify genes whose expression appeared to be up- or down-regulated by an arbitrary cut-off of at least 2-fold.

The microarray data discussed in this publication have been deposited in the Gene Expression Omnibus (GEO; www.ncbi.nlm.nih.gov/geo/info/linking.html) of the National Cancer for Biotechnology Information, and are accessible through GEO series accession number GSE 18689).

Real-time RT-PCR

Total RNA (250 ng) was reverse-transcribed in a total reaction volume of 100 µL using a high-capacity

Fig. 1. Total and ionized calcium concentration in the culture medium

PTEs were cultured in minimal essential medium (MEM; Ca 7.0 mg/dL) supplemented with 10% fetal calf serum (FCS) for seven days (Ca⁺⁺ concentration 1.0 mM), and then cultured with low (4.0 mg/dL, Ca⁺⁺ 0.60 mM) or high (total Ca 10 mg/dL, Ca⁺⁺ 1.60 mM) calcium concentration for an additional 7 days. Normal Ca⁺⁺ concentration is 1.0-1.2 mM.

cDNA archive kit (Applied Biosystems, Foster City, CA) in accordance with the manufacturer's instructions, and real-time PCR was performed as described previously [5]. Five assays were performed: PTH (Hs00174888_m1), chromogranin A (Hs00154441_m1), CaSR (Hs01047793_m1) VDR (Hs01045845_g1) and α -klotho (Hs00183100_m1), using 18S rRNA (Hs99999901_s1) as a control.

In each case, triplicate threshold cycle (Ct) values were obtained and averaged, and the expression levels were then evaluated using a relative quantification method. The fold change in target genes was normalized to the 18S rRNA (reference gene) and compared with the control (calibrator) sample, using the formula: Fold change = $2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t = (C_t\text{-target} - C_t\text{-reference})\text{ sample-n} - (C_t\text{-target} - C_t\text{-reference})\text{ calibrator-sample}$. Sample-n corresponds to any sample for the target gene normalized to the reference gene, and calibrator-sample represents the expression level (1x) of the target gene normalized to the reference gene.

Statistical analysis

All data are expressed as mean $\pm$ SE. Correlations between the levels of expression of mRNA for PTH, CaSR and klotho were analyzed using Pearson's correlation coefficient test. Statistical significance was set at $P < 0.05$.

Results

Relationship between total and ionized calcium concentrations

As shown in Fig.1, there was a good correlation between the total and ionized calcium (Ca⁺⁺) concentrations. A normal ionized calcium concentration (1.0-1.2 mM) was obtained at a total calcium concentration of 6.8-7.5 mg/dL in culture medium containing 10% FCS.

Genes up- and down-regulated by calcium in PTEs from patients with I^o-HPT

Preliminary experiments revealed that without preculture in normocalcaemic medium for 7 days, mRNA expression levels of PTH and chromogranin A were not constantly decreased in the hypercalcaemic medium. Therefore, all experiments were performed after 7 days of preculture in the normocalcaemic medium. After 24h culture in the hypercalcaemic medium, mRNA expression levels of PTH and chromogranin A were suppressed and both mRNA expression levels were suppressed to less than 1/2 in hypercalcaemic medium (when compared with those cultured in the hypocalcaemic medium) for more than 14days (data not shown).

When PTEs were cultured in high Ca⁺⁺ medium for 7 days (Tables 1, 2 & Fig.2) [7, 8], levels of expression of mRNA for PTH and chromogranin A were decreased to less than 50%. Analyses by GeneSpringGX revealed that 7 genes were up-regulated more than 2-fold, and that more than 30 genes were down-regulated to less than 1/2 in PTEs obtained from 4 patients with I^o-HPT (Table 1). Unsurprisingly, 9 of these genes (up-regulated genes: chemokine ligand 8 [CCL8], multiple C2 domain and transmembrane region protein 1 [MCTP1]; down-regulated genes: matrix metalloproteinase-9 [MMP9], B-box and SPRY domain-containing protein [BSPRY], nitric oxide synthase 2A [NOS2A], parathyroid hormone [PTH], cartilage acidic protein 1 [CRTAC1], chromogranin A [CHGA], and fibrin 1 [FBLN1]) were related to PTH secretion or calcium-signaling pathways.

To confirm the microarray data, we investigated mRNA expression levels by real-time PCR. However, we were able to perform only one experiment, since the amount of total RNA was very low, and could be used only for microarray analyses. Real time PCR in experiment 4 (Table 2) demonstrated that levels of expression of mRNA for PTH, chromogranin A, CaSR

Table 1. Genes up- and down-regulated genes by calcium in PTE obtained from patients with I^o-HPT

regulation	GeneSymbol	GenbankAccession	Description	GO molecular function
up	FAM107A	NM_007177	family with sequence similarity 107, member A (FAM107A), transcript variant 1	regulation of cell growth
up	GIPR	NM_000164	gastric inhibitory polypeptide receptor (GIPR)	G-protein coupled receptor
up	*CCL8	NM_005623	chemokine (C-C motif) ligand 8 (CCL8), mRNA	calcium ion transport, signal transducer activity,
up	NANOS1	NM_199461	nanos homolog 1 (Drosophila) (NANOS1), transcript variant 1	RNA binding, zinc ion binding
up	HOP	NM_139211	homeodomain-only protein (HOP), transcript variant 2, mRNA	regulation of transcription
up	*MCTP1	AK025997	cDNA: FLJ22344 fis, clone HRC06080.	calcium-mediated signaling
down	*MMP9	NM_004994	matrix metalloproteinase 9 (MMP9)	gelatinase B activity, calcium ion binding
down	*BSPRY	NM_017688	B-box and SPRY domain containing (BSPRY)	ion transport, calcium ion transport
down	DHRS2	NM_182908	dehydrogenase/reductase (SDR family) member 2 (DHRS2), transcript variant 1	metabolic process
down	DUSP2	NM_004418	dual specificity phosphatase 2 (DUSP2)	protein tyrosine phosphatase activity
down	RHOV	NM_133639	ras homolog gene family, member V (RHOV)	small GTPase mediated signal transduction
down	RAP1GAP	NM_002885	RAP1 GTPase activating protein (RAP1GAP)	GTPase activity
down	*NOS2A	NM_000625	nitric oxide synthase 2A (inducible, hepatocytes) (NOS2A), transcript variant 1	nitric-oxide synthase activity,calcium ion binding
down	CLDN4	NM_001305	claudin 4 (CLDN4)	transmembrane receptor activity
down	C20orf103	NM_012261	chromosome 20 open reading frame 103 (C20orf103)	
down	*PTH	NM_000315	parathyroid hormone (PTH)	Calcium-regulating hormone
down	GPR162	NM_019858	G protein-coupled receptor 162 (GPR162), transcript variant A-2	rhodopsin-like receptor activity
down	TSPAN33	NM_178562	tetraspanin 33 (TSPAN33)	
down	BU686948	BU686948	UI-CF-DU1-ado-e-06-0-UI.s1 UI-CF-DU1 cDNA clone UI-CF-DU1-ado-e-06-0-UI 3'	
down	*CRTAC1	NM_018058	cartilage acidic protein 1 (CRTAC1)	calcium ion binding
down	TSR2	NM_058163	TSR2, 20S rRNA accumulation, homolog (S. cerevisiae) (TSR2)	
down	GADD45G	NM_006705	growth arrest and DNA-damage-inducible, gamma (GADD45G),	activation of MAPKKK activity
down	NDRG2	NM_201535	NDRG family member 2 (NDRG2), transcript variant 1	cell differentiation
down	ADORA1	NM_000674	adenosine A1 receptor (ADORA1), transcript variant 1	G-protein coupled receptor protein
down	SLC31A2	NM_001860	solute carrier family 31 (copper transporters), member 2 (SLC31A2)	copper ion transport
down	APLN	NM_017413	apelin, AGTRL1 ligand (APLN)	hormone activity
down	RNF43	NM_017763	ring finger protein 43 (RNF43)	protein binding, zinc ion binding, metal ion binding
down	GBA2	NM_020944	glucosidase, beta (bile acid) 2 (GBA2)	lipid metabolic process
down	TSPAN1	NM_005727	tetraspanin 1 (TSPAN1)	cell adhesion,cell proliferation
down	PIB5PA	NM_014422	phosphatidylinositol (4,5) bisphosphate 5-phosphatase, A (PIB5PA), transcript variant 1	inositol or phosphatidylinositol phosphatase activity
down	RAB17	NM_022449	RAB17, member RAS oncogene family (RAB17)	small GTPase mediated signal transduction
down	B4GALNT3	AK131277	cDNA FLJ16224 fis, clone CTONG3009287.	acetylgalactosaminyltransferase activity
down	SEMA3B	NM_004636	sema domain, immunoglobulin domain (Ig), short basic domain(semaphorin) 3B (SEMA3B)	cell-cell signaling
down	*CHGA	NM_001275	chromogranin A (parathyroid secretory protein 1) (CHGA)	calcium ion binding
down	APLN	NM_017413	apelin, AGTRL1 ligand (APLN)	signal transduction
down	AQP5	BC032946	aquaporin 5, mRNA (cDNA clone MGC:33163 IMAGE:5269384), complete cds.	transporter activity,water channel activity
down	*FBLN1	NM_001996	fibulin 1 (FBLN1), transcript variant C	extracellular matrix,calcium ion binding
down	CFB	NM_001710	complement factor B (CFB)	proteolysis
down	LOC442292	XR_018028	PREDICTED: similar to coiled-coil domain containing 86 (LOC442292)	

* indicates genes involved calcium metabolism or calcium-mediated process.

Fig. 2. Scatter plot of genes expressed in PTEs cultured in low (Ca^{++} 0.60 mM) or high (Ca^{++} 1.60 mM) calcium medium for 7 days. PTEs were precultured for 7 days in normocalcaemic medium and then cultured in either hypocalcaemic (Ca^{++} ; 0.6mM) or hypercalcaemic (Ca^{++} ; 1.60mM) medium containing 1.29 mM (4.0 mg/dL) phosphate. After an additional 7 days of culture, total RNA was extracted and whole human genes were analyzed by oligo-DNA microarray as described in Materials and Methods.

Table 2. mRNA expression levels of PTH, chromogranin-A, CaSR, VDR, and α -klotho Cy5/cy3 ratio of genes involved in calcium metabolism

	PTH	CHGA	CaSR	VDR	α -klotho
Exp.1	0.26	0.34	0.38	0.95	0.46
Exp.2	0.47	0.42	1.57	1.36	3.84
Exp.3	0.63	0.35	1.53	1.10	3.75
Exp.4	0.27	0.23	1.32	1.84	2.19
Mean $\pm$ SEM	0.41 $\pm$ 0.09	0.34 $\pm$ 0.04	1.20 $\pm$ 0.28	1.31 $\pm$ 0.2	2.56 $\pm$ 0.8

and α -klotho were 0.18, 0.61, 1.17 and 3.08, respectively, thus confirming the microarray data.

Level of expression of α -klotho mRNA and its relationship with calcium-regulating genes

Although levels of expression of α -klotho mRNA and protein are decreased in patients with I^o-HPT [9], they were very variable under the present experimental conditions. In one experiment, they were suppressed accompanied by suppressed expression of CaSR and PTH, but increased in the other three experiments accompanied by non-suppressed expression of CaSR (Table 2). Statistical analyses revealed that although there was a marginal correlation between CaSR and α -klotho ($P=0.055$), it was not statistically significant, probably due to the low number of experiments.

Discussion

Using PTEs obtained from patients with I^o-HPT, we have for the first time demonstrated the expression levels of all genes modulated by calcium concentration using long-term culture in the presence of low and high calcium. As expected, we demonstrated that the expression levels of mRNA for PTH and chromogranin A were decreased to less than 50% when PTEs were cultured in hypercalcemic medium [7, 8].

As is well known, expression levels of mRNA for CaSR and VDR are decreased in the parathyroid gland in patients with I^o-HPT [10]. Although we could not compare the mRNA expression levels of these genes with those of normal parathyroid tissue, CaSR and VDR were moderately expressed in PTEs obtained from patients with I^o-HPT (Fig. 2).

It should be pointed out that 9 of 39 genes were involved in PTH release or calcium-signaling pathways. MCTP1, one of several C₂ domain proteins with unusual Ca⁺⁺ binding properties [11], showed increased expression when cultured in high calcium medium.

Since most C₂ domain proteins are either signal transduction enzymes, such as protein kinase C, or membrane trafficking proteins, such as synaptotagmin 1 [11], it would be involved in Ca⁺⁺ signal transduction in the parathyroid gland. Furthermore, CCL8, a chemokine previously known as monocyte chemoattractant protein 2 (MCP-2), was increased in high Ca⁺⁺ medium. When MCP-1 and MCP-3, but not MCP-2, bind to their chemokine receptor in monocytes, the intracellular Ca⁺⁺ concentration increases [12]. However, CCL8 can induce Ca⁺⁺ influx as potently as MCP-1 in human embryonic kidney 293-EBNA cells transfected with the MCP-1 receptor [13], and also induces a robust, pertussis toxin-sensitive calcium flux in primary lymphocytes [14]. The pathophysiological significance of these *in vitro* findings also remains to be elucidated.

PTH is known to stimulate endothelial nitric oxide synthase through the protein kinase A and C pathways [15]. Consistent with these *in vitro* findings, the expression of NOS2A, probably in endothelial cells or parathyroid cells in the PTEs, was decreased to less than 1/2. BSPRY, which is involved in the regulation of epithelial Ca⁺⁺ transport by direct association with transport receptor potential vallinoid (TRPV5) in the kidney [16], was also decreased to less than 1/2, and its role in parathyroid tissue remains to be elucidated. Furthermore, MMP9 expression is known to be stimulated by PTH in bone cells [17] and its expression was confirmed in parathyroid tissues [18]. Then, our finding that high calcium concentration inhibited MMP9 expression in the parathyroid adenoma might be secondary consequence of suppression of PTH expression rather than a change in calcium level by itself. Similarly, while CRTAC1 is reported to function as a calcium-binding protein in synovium [19], an alteration of PTH expression by a shift of calcium concentrations in our experiments might well affect its expression in the parathyroid gland.

α -klotho was originally characterized as an aging-related gene [20], and its mRNA expression is confined to renal tubules, the choroid plexus and parathyroid glands [21]. Recently, Imura *et al.* reported that, when parathyroid glands are cultured in low Ca medium, Na⁺K⁺-ATP and α -klotho are acutely activated and transported to the plasma membrane and PTH is immediately secreted [22, 23]. CaSR seems not to be involved in this acute hypocalcemia control system [24]. Furthermore, Björklund *et al.* [9] reported that

α -klotho mRNA expression was decreased in parathyroid tissue in patients with I^o-HPT, and that its expression level was suppressed at supraphysiological Ca⁺⁺ concentrations (3–4 mM) *in vitro* [9]. Although the expression level of mRNA for α -klotho was suppressed in 1 of 4 experiments, it was not suppressed in the other three under the present experimental conditions. We did not culture PTEs in medium containing 3–4 mM Ca⁺⁺, since dystrophic or metastatic calcification occurs in such supraphysiologically hypercalcemic medium during prolonged culture (our unpublished observation). The role of α -klotho in patients with I^o-HPT remains to be elucidated in future studies.

In summary, by culturing PTEs obtained from patients with I^o-HPT in medium containing a low or high

calcium concentration, we have demonstrated for the first time that 9 genes are constantly up-regulated and that more than 30 genes are down-regulated to less than 1/2 by calcium. A number of these genes are involved in PTH release or calcium-signaling pathways.

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