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—Original Article—

Involvement of GPR4 in increased *growth hormone* and *prolactin* expressions by extracellular acidification in MtT/S cells

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Abstract. Hormone-secreting pituitary adenomas show unregulated hormonal hypersecretion and cause hyperpituitarism. However, the mechanism of the unregulated hormone production and secretion has not yet been fully elucidated. Solid tumors show reduced extracellular pH, partly due to lactate secretion from anaerobic glycolysis. It is known that extracellular acidification affects hormone secretion. However, whether and how the extracellular acidification influences the unregulated hormone production and secretion remain unknown. In the present study, we found that *GPR4*, a proton-sensing G protein-coupled receptor, was highly expressed in MtT/S cells, a growth hormone-producing and prolactin-producing pituitary tumor cell line. When we reduced the extracellular pH, *growth hormone* and *prolactin* mRNA expressions increased in the cells. Both increased expressions were partially suppressed by a GPR4 antagonist. We also found that extracellular acidification enhanced growth hormone-releasing factor-induced growth hormone secretion from MtT/S cells. These results suggest that GPR4 may play a role in hypersecretion of the hormone from hormone-producing pituitary tumors. A GPR4 antagonist will be a useful tool for preventing the hypersecretion.

Key words: Extracellular acidification, Growth hormone, GPR4, MtT/S, Prolactin

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Hormone-secreting pituitary adenomas show unregulated hormonal hypersecretion and cause hyperpituitarism [1, 2]. For example, a lactotroph adenoma caused hyperprolactinemia that resulted in hypogonadism. A growth hormone adenoma hypersecretes a growth hormone (GH) and causes arthritis, hypertension, hyperglycemia, and acromegaly [3, 4]. However, the mechanism for the unregulated hormone production and secretion has not yet been fully elucidated.

Extracellular acidification occurs chronically and locally in various tissues. Solid tumors show reduced extracellular pH, partly due to lactate secretion from the anaerobic glycolysis of growing cancer cells under hypoxic conditions. The acidic microenvironment influences many properties in tumors, such as their onset, progression, and metastasis [5]. However, whether and how extracellular acidification influences unregulated hormone production remain unknown.

Extracellular acidification has been shown to affect hormone secretion. For example, secretin is secreted by extracellular acidification [6]. Insulin secretion is also regulated by extracellular acidification [7]. Extracellular acidification is mainly recognized by metabotropic proton-sensing G protein-coupled receptors (GPCRs), in addition to ionotropic ion channels such as transient receptor potential V1 (TRPV1) and acid-sensing ion channels (ASICs) [8]. We showed that glucose-induced insulin secretion from mouse islets is enhanced by extracellular acidification. The enhancement is mediated by ovarian cancer G protein-coupled receptor 1 (OGR1), which is a proton-sensing GPCR [9]. This result prompts us to speculate that proton-sensing GPCRs may also be involved in the hormone hypersecretion from a pituitary adenoma. GPR4, which is a close relative of OGR1, is also a proton-sensing GPCR [10]. GPR4 is coupled primarily with Gs/cAMP and G_{13}/Rho signaling pathways, while OGR1 is mainly coupled with the G_q/Ca^{2+} signaling pathway [11].

This time, we found the high expression of *GPR4* in MtT/S cells. The cell line has somatotroph-like characteristics, i.e., the cells secrete a GH in response to a growth hormone-releasing factor (GRF). The cells also have been shown to be differentiated into prolactin (PRL)-secreting cells by insulin and insulin-like growth factor 1 [12]. MtT/S cells are thought to have some characteristics of early differentiation-stage cells that will differentiate into GH- and PRL-producing cells [13].

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To investigate whether GPR4 is involved in unregulated hormone secretion from the pituitary due to extracellular acidification, we used this cell line as a model of hormone-secreting pituitary tumors in this study. The results showed that GPR4 is involved in the extracellular acidification-induced increase in *GH* and *PRL* expression in MtT/S cells.

Materials and Methods

Materials

Epidermal growth factor (EGF) (human, recombinant, animal-derived-free) was purchased from FUJIFILM Wako (Osaka, Japan), fatty acid-free bovine serum albumin (BSA) from Calbiochem-Novabiochem (San Diego, CA, USA), bovine pancreas insulin from Sigma-Aldrich (Tokyo, Japan), human GRF from the Peptide Institute (Osaka, Japan), and corticosterone from Tokyo Chemical Industry (Tokyo, Japan). GPR4 antagonists were kindly provided by Dr S Shuto [14].

Cell culture and transfection

MtT/S cells were kindly provided by Dr K Fujiwara [15]. The cells were maintained in a culture medium consisting of Dulbecco's Modified Eagle Medium (DMEM) containing 50 ng penicillin/ml, 50 ng streptomycin/ml, 10% normal horse serum (HS), and 2.5% fetal bovine serum (FBS). All cells were grown in 5% CO_2 at 37ºC in a humidified environment. For the pH experiments in this study, DMEM that contained 25 mM HEPES, 27 mM $NaHCO_3$, 10% HS, and 2.5% FBS was used to maintain a stable pH. The pH of the DMEM was adjusted by titration with HCl or NaOH. Cells were incubated under the indicated pH or antagonist for 2 days in a CO_2 incubator (5% CO_2:95% air) using Model SCA-165DRS (ASTEC, Tokyo, Japan). To induce differentiation into PRL-producing cells, insulin (500 ng/ml) and EGF (1 ng/ml) were applied to the cells as described [16].

Quantitative real-time polymerase chain reaction (PCR)

Quantitative real-time PCR was performed as described [17]. The cDNAs of the cells (Tpit/F1, MtT/S, αT3-1, LβT2, AtT-20, and GH3) and of rat anterior pituitary lobes (E13.5, E15.5, E16.5, E18.5, P0, P15, P30, and P60) were synthesized as described [18–20]. The Tpit/F1 cell line was established from the pituitary gland of a temperature-sensitive T antigen transgenic mouse, and it has some characteristics of pituitary S100-positive cells [21]. The MtT/S cell line was established from an estrogen-induced mammotropic pituitary tumor of a Fisher 344 rat, and it produced a GH or PRL [15]. αT3-1 and LβT2 cell lines were established from the pituitary gonadotrope lineage of a T antigen transgenic mouse. They produced α subunit (αT3-1), LH beta and α subunit (LβT2) [22, 23]. The AtT-20 cell line was established from LAF1 mouse pituitary tumor cells, and it produced an adrenocorticotropic hormone (ACTH) [24]. The GH3 cell line was established from a female Wistar-Furth rat pituitary tumor cells, and it produced a GH and PRL [25]. The total RNA was prepared from the multiple rat pituitaries at each corresponding developmental stages. Briefly, the total RNA was extracted using ISOGEN II (Nippon Gene, Tokyo, Japan). Then, the cDNA was synthesized with PrimeScript Reverse Transcriptase (TaKaRa Bio, Otsu, Japan) using 1 μg of total RNA after DNase I treatment and then subjected to quantitative PCR using a StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Reactions were performed in a SYBR Green Real-Time PCR Master Mix Plus (Toyobo, Osaka, Japan), including 0.5 μM gene-specific primer sets. The sequences of the primers used in this study are as follows: Rat and mouse *GPR4* forward GCAAGCTCTTTGGCTTCATC, reverse GTGTGGTTGTAGCGATCACG; rat and mouse *GH* forward GGACCGCGTCTATGAGAAAC, reverse GCTTGAGGATCTGCCCAATA; rat PRL forward GCCAAAGAGATTGAGGAACAA, reverse ATGGGAGTTGTGACCAAACC; rat and mouse hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) forward CTTTGCTGACCTGCTGGATT, reverse TCCACTTTCGCTGATGACAC; and rat and mouse TATA box-binding protein (*TBP*) forward GATCAAACCCAGAATTGTTCTCC, reverse ATGTGGTCTTCCTGAATCCC. Quantification of the PCR products was performed using the comparative CT method (ΔCT method) to estimate the mRNA copy number relative to that of the *TBP* used as an internal standard.

ELISA

MtT/S cells were preincubated under the indicated pH of DMEM in the presence of 10 nM corticosterone for 2 days in 24-well multiplates [26, 27]. After the pH medium was removed, the cells were further incubated with HEPES-Regular at pH 7.4 (500 μl/well) for 30 min. HEPES-Regular was composed of 25 mM HEPES, 134 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 2.5 mM $NaHCO_3$, 5 mM glucose, and 0.1% (w/v) BSA. Then the cells were stimulated at the indicated pH of 200 μl of HEPES-Regular in the presence or absence of 10 nM GRF incubated for 30 min. After stimulation, the supernatant was used to measure the amount of GH secreted from the cells. The resultant cells were lysed in 50 μl of a protease inhibitor cocktail (FUJIFILM Wako, Tokyo, Japan), and the lysed samples were used for measuring the GH amount in the cells. The amount of GH from these samples was measured using direct GH ELISA kits (Merck, Tokyo, Japan).

Data presentation

The results of multiple observations are presented as the means ± SEM or a representative result, as indicated in the figure legends. Statistical significance was assessed using a Student's *t*-test. The values were considered significant at $P < 0.05$ (*).

Results

GPR4 was highly expressed in MtT/S cells and in rat anterior pituitary glands of fetal periods

We first examined whether *GPR4* mRNA is expressed in several pituitary cell lines. As shown in Fig. 1A, *GPR4* was highly expressed in MtT/S cells among the cell lines tested. Next we investigated whether *GPR4* is expressed in the pituitary glands of rats. We found that *GPR4* was highly expressed in rat fetal periods (E15.5–E18.5) that correspond to the differentiation stages of hormone-producing cells in the rat pituitary, and then the expression gradually decreased after birth (P0–P60) (Fig. 1B). Based on this result, we used MtT/S

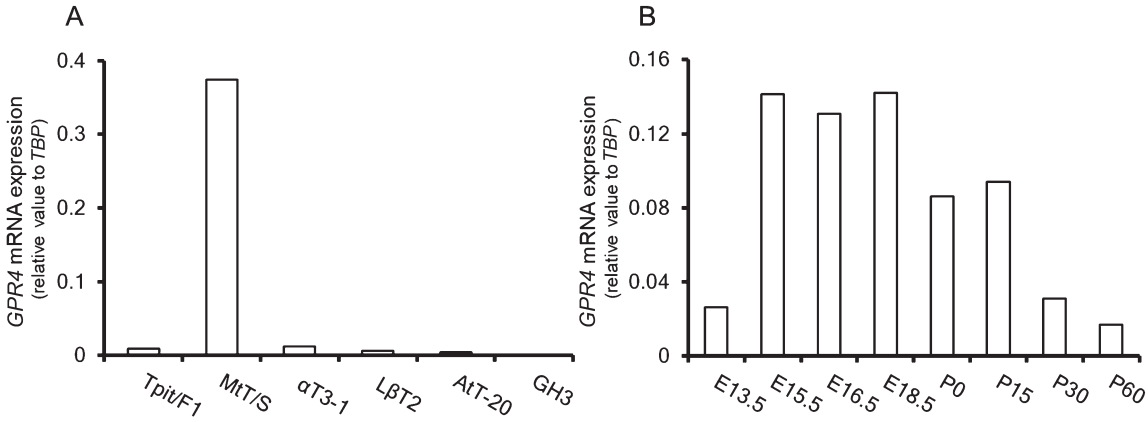

Fig. 1. *GPR4* mRNA expression in several pituitary cell lines and in several developmental stages of rat anterior pituitaries. (A) Quantitative real-time polymerase chain reaction (qPCR) was performed to estimate *GPR4* mRNA levels in Tpit/F1, MtT/S, αT3-1, LβT2, AtT-20, and GH3 cells. (B) qPCR was performed to estimate *GPR4* mRNA levels in several embryonic and postnatal stages of rats. Each total RNA of the developmental stages used for qPCR was prepared from the corresponding stages of multiple rat pituitaries. Data were calculated using the comparative CT method to estimate the copy number relative to that of the TATA box-binding protein (*TBP*). Results are the means of duplicate measurements.

cells in the following experiments in this study to investigate the role of GPR4 in pituitary hormone synthesis and secretion.

Extracellular acidification induced GH- and PRL-mRNA expressions in MtT/S cells

We next investigated how extracellular acidification influences pituitary hormone mRNA expressions. MtT/S cells synthesize and secrete a GH in response to a GRF. Insulin and insulin-like growth factor 1 are shown to induce the transformation of some MtT/S cells from GH-producing cells into PRL-producing cells [12]. As shown in Fig. 2, *GH*- and *PRL*-mRNA expressions were increased by decreasing the extracellular pH. This indicates that hormone synthesis in MtT/S cells is increased by extracellular acidification.

GPR4 antagonist partially suppressed the low pH-induced increase of GH- and PRL-mRNA expressions

The extracellular acidification-dependent increase of *GH*- and *PRL*-mRNA expressions prompted us to investigate whether the increase is mediated by GPR4. To elucidate this, we used a GPR4 antagonist [14]. The results showed that extracellular acidification-induced *GH*- (Fig. 3A) and *PRL*-mRNA (Fig. 3C) expressions were partially suppressed by the antagonist. Meanwhile, *HPRT1* mRNA expression in the uninduced MtT/S cells (Fig. 3B) and in the induced MtT/S cells (Fig. 3D) was not attenuated by the antagonist. This indicates that the antagonist specifically inhibited hormone synthesis. The result shows that the extracellular acidification-induced GH and PRL syntheses in MtT/S cells were partially mediated by GPR4.

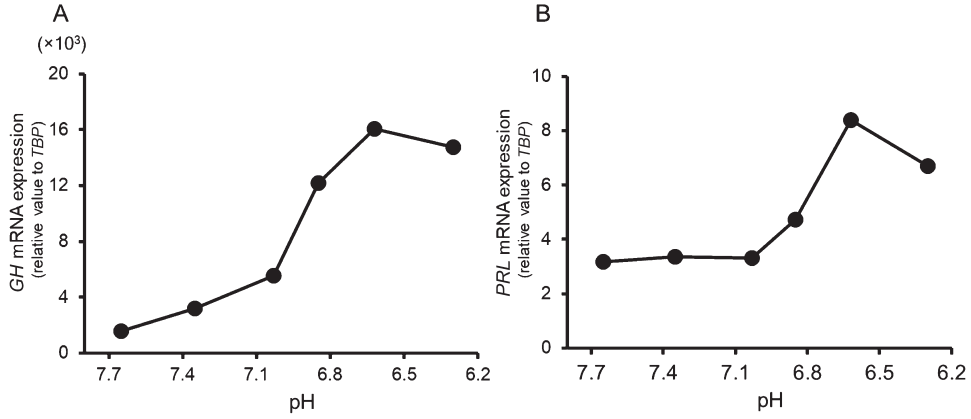

Fig. 2. Growth hormone (*GH*)- and prolactin (*PRL*)-mRNA expressions under several extracellular pH conditions. qPCR was performed to estimate the GH- or PRL-mRNA levels in MtT/S cells following incubation under the indicated pH conditions. The uninduced samples were used for measurement of the *GH* expression (A), and the induced samples were used for measurement of the *PRL* expression (B). The induction procedure was described in Materials and Methods. Data were calculated as described in Fig. 1. A representative result is shown. Similar results were obtained from the other two independent experiments.

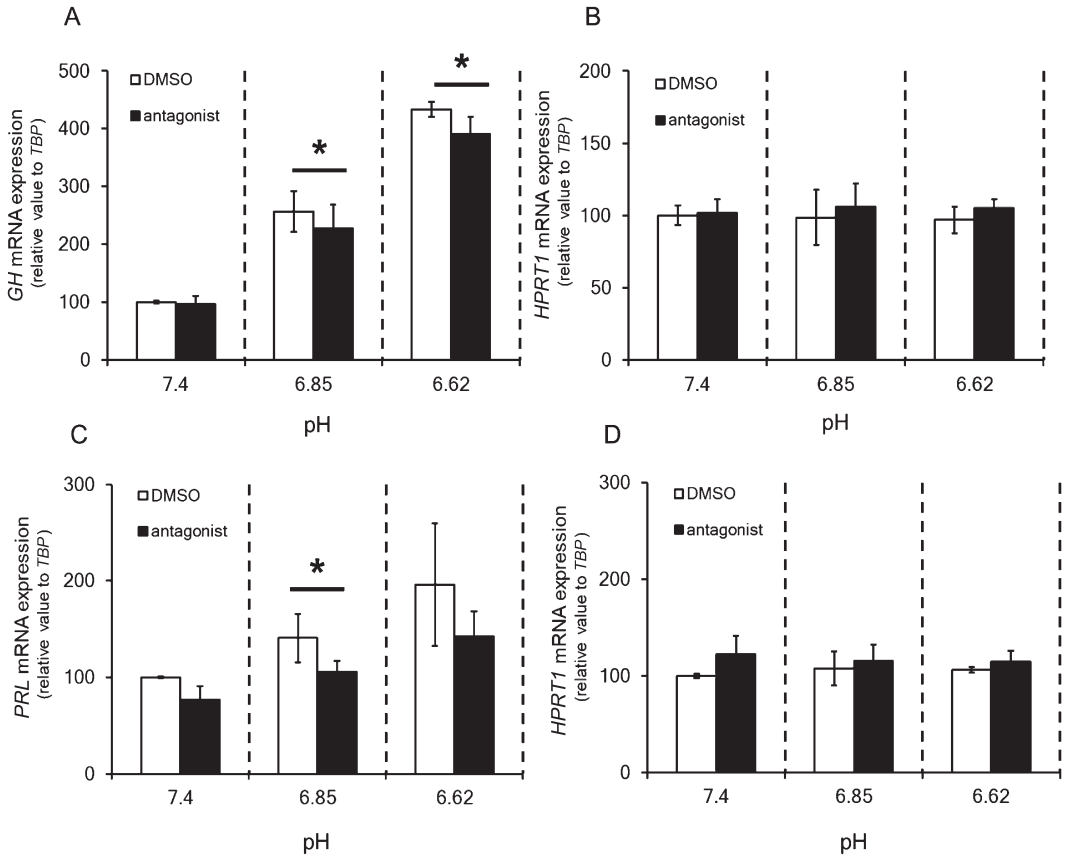

Fig. 3. Effect of the GPR4 antagonist on the increase of growth hormone (*GH*) and prolactin (*PRL*) mRNA expressions under low pH conditions. The uninduced samples were used for measuring *GH* mRNA expression (A), and the induced samples were used for measuring *PRL* mRNA expression (C). Hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) mRNA expression was measured from both uninduced (B) and induced (D) samples. Quantitative real-time polymerase chain reaction (qPCR) was performed to estimate the *GH* or *PRL* mRNA levels in MtT/S cells following incubation in the indicated pH medium in the presence (black column) or absence (white column) of a 1 μM GPR4 antagonist. The dimethyl sulfoxide (DMSO) that was used to dissolve the GPR4 antagonist was used as a vehicle. Data were calculated as described in Fig. 1. Data are expressed as the relative values. The mean value at pH 7.4 in the absence of the antagonist is expressed as 100. Data are expressed as the means ± SEM from three separate experiments. * $P < 0.05$.

The amount of GH was increased in both the cells and the supernatant under a low pH condition

Finally, we investigated whether the extracellular acidification-induced increase of GH synthesis leads to increased GH secretion. Using ELISA, we measured the GH in both the supernatant and the cells. Since MtT/S cells express functional growth hormone-releasing hormone receptors (GHRHs), we used a human GRF to stimulate GH secretion from the cells into the supernatant [15, 26–29]. As shown in Fig. 4A, MtT/S cells were treated with 10 nM corticosterone to induce them to form mature *GH* cells at first [26]. Then the treated cells were washed with HEPES-Regular and stimulated with a 10 nM GRF. As shown in Fig. 4C, the GH content in the cells was increased at a pH of 6.8, reflecting the increased *GH* mRNA expressions under low pH conditions (Fig. 2A). When the cells were stimulated with a GRF under this condition, the amount of GH secreted into the supernatant was increased under the low pH condition (Fig. 4B). This result indicates that the secreted amount of GH is enhanced under a low pH condition.

Discussion

In the present study, we detected high *GPR4* mRNA expression in MtT/S cells but not in the other pituitary hormone-producing cell lines tested (Fig. 1A). The reason only MtT/S cells showed high *GPR4* mRNA expression is unknown. It is recognized that MtT/S cells have some characteristics of the early developmental stage of GH/PRL-producing lineages [13]. As shown in Fig. 1B, high *GPR4* mRNA expression was detected in the embryonic stages from 15.5 to 18.5 in the pituitary. Most hormone-producing cells are differentiated during the embryonic stages [13].

Extracellular acidification influences many properties of tumors, such as their onset, progression, and metastasis [5]. Regarding hormone synthesis and the secretion of pituitary tumors, we reported that OGR1, a proton-sensing GPCR, is involved in the extracellular acidification-induced hormone secretion from LβT2 cells, a gonadotropin-producing tumor cell line; however, we did not show how a low pH influences hormone synthesis [30]. In this study, we

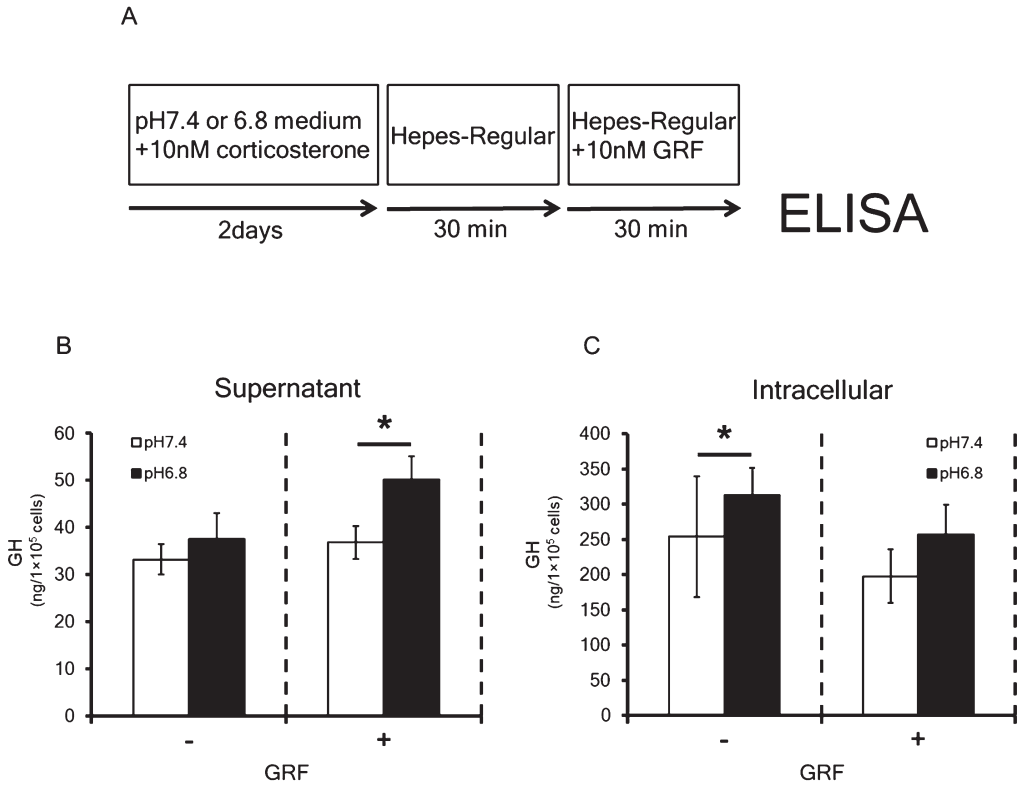

Fig. 4. Effect of low pH on the growth hormone (GH) accumulation and secretion in MtT/S cells. (A) Schematic of the cell treatment procedure. MtT/S cells were stimulated with (+) or without (–) a 10 nM growth hormone-releasing factor (GRF) under pH 7.4 (white column) or pH 6.8 (black column) conditions. (B) The amount of GH secreted in the supernatant. (C) The amount of GH in the cells. The amount of GH was measured using an ELISA. Data are expressed as the means ± SEM from four separate experiments. * $P < 0.05$.

first showed that extracellular acidification induced GH synthesis in MtT/S cells, a GH-producing tumor cell line. This extracellular acidification-induced gene expression is a specific phenomenon, since *HPRT1* expression was not enhanced under low pH conditions (Fig. 3B and D). *HPRT1* is often used as a reference gene, such as β-actin (*ACTB*) and glyceraldehyde-3-phsopahte dehydrogenase (*GAPDH*) [31]. The expression of the reference gene is constant and is resistant to changes in the experimental conditions. It is possible that a GH-positive cell number would increase under a low pH condition, although almost all of the cells are already GH positive under a normal culture condition [15].

We also showed that extracellular acidification-induced *GH* and *PRL* expressions were partially mediated by *GPR4*, another proton-sensing GPCR (Fig. 3A and C). The increased *GH* and *PRL* expressions under low pH conditions were not completely inhibited by the GPR4 antagonist (Fig. 3A and C). The increased *PRL* expression at pH 6.62 was also not significantly inhibited by the antagonist (Fig. 3C). A possible explanation for these phenomena is that this antagonist shows inhibition in a manner that is competitive with the GPR4-mediated cell responses [32]. Increasing the concentration of the antagonist is expected to enhance the inhibition of the expressions. However, we cannot use a higher concentration than the 1 μM used in this study, since a higher concentration of the antagonist had cytotoxic effects on MtT/S cells (data not shown). In addition, we cannot exclude the possibility that other proton-sensing channels, such as TRPV1 and ASICS, or proton-sensing GPCRs, in addition to GPR4, could be involved in the *GH* and *PRL* mRNA expressions. This issue needs to be clarified in the future.

We showed that GPR4 was partly involved in the enhancement of *GH* and *PRL* mRNA expressions under low pH conditions (Figs. 3). We also showed enhancement of the GH content in MtT/S cells and GH secretion from the cells under a low pH condition (Fig. 4B and 4C). The pH around a tumor is usually decreased due to lactate secretion from anaerobic glycolysis. The results in this study suggest that some types of GH-producing adenomas may secrete more GHs under low pH conditions *in vivo*, and this may be a cause of hyperpituitarism. In this study, we could not detect a significant increase in GH secretion with 10 nM GRF stimulation at pH 7.4, although a tendency to increase the secretion was observed (33.2 ± 2.78 ng in the absence of a GRF *vs.* 36.8 ± 3.05 ng in the presence of a GRF) (Fig. 4B). One reason could be the small amount of GH content in the cells compared with that in the previous report [26]. The GH content in this study is about one-fiftieth of that in the report. This would lead to less GH secretion upon GRF stimulation.

In conclusion, we found that extracellular acidification induced the GH and PRL syntheses and the secretion of MtT/S cells, a pituitary-derived hormone-producing tumor cell line, and the GH and PRL syntheses were mediated by GPR4. This result indicates that an

antagonist of GPR4 can be a useful tool for preventing hormone hypersecretion of some of the hyper hormone-secreting pituitary adenomas *in vivo*.

Acknowledgments

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