Effects of retinoic acid on growth hormone-releasing hormone receptor, growth hormone secretagogue receptor gene expression and growth hormone secretion in rat anterior pituitary cells

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Abstract. Retinoic acid (RA) is an important signaling molecule in embryonic development and adult tissue. The actions of RA are mediated by the nuclear receptors retinoic acid receptor (RAR) and retinoid X receptor (RXR), which regulate gene expression. RAR and RXR are widely expressed in the anterior pituitary gland. RA was reported to stimulate growth hormone (GH) gene expression in the anterior pituitary cells. However, current evidence is unclear on the role of RA in gene expression of growth hormone-releasing hormone receptor (*Ghrh-r*), growth hormone secretagogue receptor (*Ghs-r*) and somatostatin receptors (*Sst-rs*). Using isolated anterior pituitary cells of rats, we examined the effects of RA on gene expression of these receptors and GH release. Quantitative real-time PCR revealed that treatment with all-trans retinoic acid (ATRA; 10^{-6} M) for 24 h increased gene expression levels of *Ghrh-r* and *Ghs-r*; however, expressions of *Sst-r2* and *Sst-r5* were unchanged. Combination treatment with the RAR-agonist Am80 and RXR-agonist PA024 mimicked the effects of ATRA on *Ghrh-r* and *Ghs-r* gene expressions. Exposure of isolated pituitary cells to ATRA had no effect on basal GH release. In contrast, ATRA increased growth hormone-releasing hormone (GHRH)- and ghrelin-stimulated GH release from cultured anterior pituitary cells. Our results suggest that expressions of *Ghrh-r* and *Ghs-r* are regulated by RA through the RAR–RXR receptor complex and that RA enhances the effects of GHRH and ghrelin on GH release from the anterior pituitary gland.

Key words: Retinoic acid, Growth hormone-releasing hormone, Ghrelin, Growth hormone, Anterior pituitary gland

RETINOIC ACID (RA), a lipid soluble molecule derived from vitamin A, is an important signaling molecule in embryonic development and adult tissue [1]. RA is a ligand for two nuclear receptor families, retinoic acid receptor (RAR) and retinoid X receptor (RXR). The RA–RAR/RXR complex binds to a sequence referred to as the retinoic acid-response element (RARE) and regulates transcription of target genes [2].

RAR and RXR are widely expressed in embryonic [3, 4] and adult anterior pituitary gland [5]. RA regulates somatotroph function in the anterior pituitary gland. A previous study reported that RA induced

growth hormone (GH) production in GH1 cell lines [6]. In addition to regulating gene expression, RA induced differentiation into immature somatotrophs in a non-hormone-producing cell line, MtT/E [7].

Regulation of GH secretion in the pituitary gland is controlled by several humoral factors. GH release is primarily stimulated by growth hormone-releasing hormone (GHRH) but is inhibited by somatostatin from the hypothalamus [8]. In addition, the gastrointestinal hormone ghrelin potently stimulates GH release from the pituitary gland [9]. Interestingly, RA induced growth hormone-releasing hormone receptor (*Ghrh-r*) gene expression in a rat GH-producing cell line, MtT/S [10]. However, the effects of RA on *Ghrh-r*, growth hormone secretagogue receptor (*Ghs-r*) and somatostatin receptor (*Sst-r*) gene expression in normal pituitary cells are not well understood. In this study, we analyzed the possible effects of RA on *Ghrh-r*, *Ghs-r* and

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Sst-r gene expression and growth hormone release in isolated anterior pituitary cells of rats.

Materials and Methods

Reagents

All-trans retinoic acid (ATRA) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Am80 and PA024 were kindly provided by Dr. Hiroyuki Kagechika (School of Biomedical Science, Tokyo Medical and Dental University, Tokyo, Japan). These reagents were dissolved in ethanol to a stock concentration of 2×10^{-3} M. The final concentration of ethanol in the medium was 0.1% or lower. Rat GHRH and ghrelin were purchased from Peptide Institute, Inc. (Osaka, Japan).

Animals

Adult male Wistar rats were purchased from Japan SLC (Shizuoka, Japan). The rats were housed in a temperature-controlled room ($22 \pm 1^\circ\text{C}$) with a 12-hour light/12-hour dark cycle and illumination from 0700 h to 1900 h, and were given *ad libitum* access to conventional food and water. All animals were treated in accordance with the Guidelines for Animal Experimentation of Jichi Medical University, which is based on the Guide for the Care and Use of Laboratory Animals of the National Academy of Sciences, USA.

Primary culture of anterior pituitary cells

Primary cell culture was performed according to our previous report [11]. Rats aged 9–10 weeks were perfused with Ca^{2+} - and Mg^{2+} -free Hanks' Balanced Salt Solution (CMF-HBSS) under deep sodium pentobarbital anesthesia. Anterior pituitary glands were excised and minced into pieces that were then incubated in CMF-HBSS containing 10 mg/mL trypsin

(Life Technologies, Carlsbad, CA, USA) and 2 mg/mL collagenase (Nitta Gelatin, Osaka, Japan) for 15 min at 37°C . Afterward, the pieces were incubated in the same solution containing 0.5 $\mu\text{g/mL}$ DNase I (Boehringer-Mannheim, Mannheim, Germany) for 5 min at 37°C . After incubation in CMF-HBSS containing 5 mM ethylenediaminetetraacetic acid (Wako Pure Chemicals, Osaka, Japan) for 5 min at 37°C , the digest was washed with CMF-HBSS. Dispersed cells were suspended in CMF-HBSS by pipette, passed through a 70- μm nylon mesh (Becton Dickinson Labware, Franklin Lakes, NJ, USA) and then collected by centrifugation. The pellet was suspended in DMEM/F12 medium (Life Technologies) with 10% dextran-coated charcoal-treated fetal bovine serum (Sigma-Aldrich) and 1% penicillin-streptomycin (Life Technologies). The cells were seeded in 24-well plates and then incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Real-time reverse transcription (RT)-PCR

The cells (5×10^5 cells) were exposed to ATRA, Am80 or PA024 at indicated concentrations and periods. Total RNAs were extracted with an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Quantitative real-time PCR was performed as described in our previous report [11]. cDNA was synthesized by a PrimeScript First Strand cDNA Synthesis Kit (Takara Bio, Otsu, Japan). Quantitative real-time PCR (ABI PRISM 7900HT; Applied Biosystems, Foster City, CA, USA) was performed by using gene-specific primers and SYBR Premix Ex Taq (Takara Bio) containing SYBR Green I. The primers for amplifying fragments are listed in Table 1. For normalization, we also quantified β -actin. Relative gene expression was calculated by comparing the cycle times for each target PCR. Cycle threshold

Table 1 List of primers used for the real-time polymerase chain reaction

Genes	Primer sequence (5'-3')	Product size	GenBank accession number
<i>Ghrh-r</i>	Forward: GTCCTCTCTGTTGGGGTGAA	107	NM_012850
	Reverse: CAGTACTGAGCCCGTGTGTG		
<i>Ghs-r</i>	Forward: TACCGGTCTTCTGCCTCACT	114	NM_032075
	Reverse: CTGTCTGCTTGTGGTTCTGG		
<i>Sst-r2</i>	Forward: GAGCAGAGCGGATTTGAGTC	97	NM_019348
	Reverse: TTGAATTGTCTGCCTGCAC		
<i>Sst-r5</i>	Forward: GGATTGAAATGCCCTGTAA	92	NM_012882
	Reverse: GGTGAATGCATCTCAGCAAA		
β -actin	Forward: TGGCACCACACTTTCTACAATGAGC	106	NM_031144
	Reverse: GGGTCATCTTTTCACGGTTGG		

values were converted to relative gene expression levels by using the $2^{-(\Delta\text{Ct sample} - \Delta\text{Ct control})}$ method.

Measurement of GH concentration

The anterior pituitary cells were seeded into 24-well plates at a concentration of 1×10^5 cells/well and exposed to ATRA (10^{-6} M) for 72 h in DMEM/F12 medium with 10% dextran-coated charcoal-treated fetal bovine serum. After treatment with ATRA, the cells were washed with DMEM/F12 medium with 0.1% bovine serum albumin (Roche Diagnostics, Mannheim, Germany), after which the media were incubated for 30 min. Media were changed, GHRH or ghrelin was then added to 500 μL of media at a concentration of 10^{-9} M and the cells were incubated for 30 min. GH concentrations in the media were measured by a Rat/Mouse GH ELISA Kit (EMD Millipore, Billerica, MA, USA) according to the manufacturer's instruction.

Statistical analysis

The data are presented as means $\pm$ SEM for independent or group experiments repeated at least four times. Statistical analysis was performed using Student's unpaired two-tailed *t*-test or Dunnett's test. Differences between groups were considered to be statistically significant at a *P*-value of less than 0.05.

Results

Effects of ATRA on *Ghrh-r*, *Ghs-r* and *Sstr* gene expression in isolated anterior pituitary cells

To determine whether RA affects *Ghrh-r*, *Ghs-r*, *Sstr-2* and *Sstr-5* mRNA transcription in the pituitary gland, we investigated the effect of ATRA on the relevant mRNA levels in isolated anterior pituitary cells. Quantitative real-time PCR analysis showed that *Ghrh-r* and *Ghs-r* gene expressions were significantly increased by ATRA treatment for 24 h. Expression levels of *Ghrh-r* and *Ghs-r* after exposure to 10^{-6} M of ATRA were 2.3 (± 0.07)-fold and 2.2 (± 0.06)-fold, respectively, those of the control (Fig. 1A and B). In contrast, ATRA had no effect on *Sstr-2* (Fig. 1C) or *Sstr-5* mRNA transcription (Fig. 1D).

Time- and dose-dependent effects of ATRA on *Ghrh-r* and *Ghs-r* gene expression

Next, we examined time- and dose-dependent effects of ATRA on *Ghrh-r* and *Ghs-r* gene expression levels in isolated anterior pituitary cells. ATRA grad-

Fig. 1 Effects of ATRA on expressions of *Ghrh-r*, *Ghs-r* and *Sstr*-*rs*. Isolated anterior pituitary cells were treated with ATRA (10^{-6} M) for 24 h. *Ghrh-r* (A), *Ghs-r* (B), *Sstr-2* (C) and *Sstr-5* (D) mRNAs were measured by real-time PCR. mRNA levels are ratios of control values (vehicle), with β -actin as the internal control. Data represent means $\pm$ SEM (*n* = 4). * *P* < 0.01 vs. control in each group.

usually increased *Ghrh-r* and *Ghs-r* mRNA transcription from 24 to 72 h (Fig. 2). Exposure to ATRA at 10^{-6} M for 72 h increased gene expression of *Ghrh-r* and *Ghs-r* by $3.5 (\pm 0.17)$ -fold and $3.3 (\pm 0.23)$ -fold, respectively, as compared with control. These gene expressions were significantly and dose-dependently higher after ATRA treatment for 72 h (Fig. 3). *Ghrh-r* mRNA transcription after exposure to 10^{-10} , 10^{-9} , 10^{-8} , 10^{-7} and 10^{-6} M of ATRA was increased by $1.2 (\pm 0.13)$ -, $1.2 (\pm 0.12)$ -, $1.5 (\pm 0.16)$ -, $2.5 (\pm 0.16)$ - and $3.4 (\pm 0.26)$ -fold, respectively. *Ghs-r* mRNA transcription after exposure to 10^{-10} , 10^{-9} , 10^{-8} , 10^{-7} and 10^{-6} M of ATRA was increased by $1.2 (\pm 0.15)$ -, $1.3 (\pm 0.13)$ -, $1.6 (\pm 0.31)$ -, $2.0 (\pm 0.23)$ - and $3.3 (\pm 0.19)$ -fold, respectively.

Effects of RAR and RXR agonists on *Ghrh-r* and *Ghs-r* mRNA transcription

We examined the effects of the synthetic RAR α and β agonist Am80 and RXR pan-agonist PA024 on *Ghrh-r* and *Ghs-r* gene expressions. As shown in Fig. 4A, *Ghrh-r* mRNA transcription after exposure to Am80, PA024 and Am80 + PA024 was increased by $1.6 (\pm 0.15)$ -, $1.8 (\pm 0.50)$ - and $3.2 (\pm 0.21)$ -fold, respectively, as compared with control. *Ghs-r* mRNA transcription after exposure to Am80, PA024 and Am80 + PA024 was increased by $1.7 (\pm 0.08)$ -, $2.6 (\pm 0.15)$ - and $3.8 (\pm 0.11)$ -fold, respectively, as compared with control (Fig. 4B). Treatment of each agonist weakly increased *Ghrh-r* and *Ghs-r* gene expressions. Combined exposure to Am80 and PA024 potentially induced *Ghrh-r* and *Ghs-r* gene expression in anterior pituitary cells.

Effects of GHRH and ghrelin on GH release from ATRA-treated cells

Finally, we assessed the effect of ATRA on GH release from cultured pituitary cells. Isolated anterior pituitary cells were cultured for 72 h in medium with or without ATRA. The cells were then exposed to fresh medium with or without GHRH and ghrelin for 30 min. As shown in Fig. 5, ATRA did not affect GH release in the absence of GHRH or ghrelin. In contrast, GHRH- and ghrelin-induced GH release was significantly higher in ATRA-treated pituitary cells than in untreated cells (by 1.4- and 1.8-fold, respectively; Fig. 5A and B).

Discussion

This study examined the effects of RA on *Ghrh-r*, *Ghs-r*, *Sst-r2* and *Sst-r5* mRNA transcription in anterior pituitary cells isolated from adult rats. Expression levels of the *Ghrh-r*, *Ghs-r* and *Sst-r* genes were measured by real-time PCR. ATRA induced *Ghrh-r* and *Ghs-r* gene expression but not *Sst-r2* or *Sst-r5* gene expression. These actions were completely mimicked by Am80 and PA024. Moreover, GH release induced by GHRH and ghrelin was enhanced by ATRA.

GH release from somatotrophs is stimulated by GHRH and ghrelin, which are ligands for GHRH-R [12] and GHS-R [9], respectively. Somatostatin inhibits GH release via SST-R2 and SST-R5 [13, 14]. In the present study, we demonstrated that ATRA induced *Ghrh-r* gene expression in isolated anterior pituitary

Fig. 2 Time course effect of ATRA on *Ghrh-r* and *Ghs-r* mRNA levels. Isolated anterior pituitary cells were treated with ATRA (10^{-6} M) for 0, 24, 48 or 72 h. *Ghrh-r* (A) and *Ghs-r* (B) mRNAs were measured by real-time PCR. mRNA levels are ratios of control values (0 h), with β -actin as the internal control. Data represent means $\pm$ SEM ($n = 4$). * $P < 0.01$ vs. control.

Fig. 3 Dose response of ATRA effects on *Ghrh-r* and *Ghs-r* mRNA transcription. Isolated anterior pituitary cells were treated with different doses of ATRA (10^{-6} M) or vehicle as control for 72 h. *Ghrh-r* (A) and *Ghs-r* (B) mRNAs were measured by real-time PCR. mRNA levels are ratios of control values, with β -actin as the internal control. Data represent mean $\pm$ SEM ($n = 4$). * $P < 0.05$ and ** $P < 0.01$ vs. control.

Fig. 4 Effects of RAR and RXR agonists on *Ghrh-r* and *Ghs-r* mRNA transcription. Isolated anterior pituitary cells were treated with the RAR α and β agonist Am80, the RXR pan-agonist PA024, both Am80 and PA024 (A+P), or ATRA at 10^{-6} M for 72 h. *Ghrh-r* (A) and *Ghs-r* (B) mRNAs were measured by real-time PCR. mRNA levels are ratios of control values, with β -actin as the internal control. Data represent mean $\pm$ SEM ($n = 4$). * $P < 0.05$, ** $P < 0.01$ vs. vehicle control group, § $P < 0.05$ vs. Am80 group, # $P < 0.01$ vs. Am80 and PA024 groups.

Fig. 5 Effects of GHRH and ghrelin on GH release from ATRA-treated cells. Isolated anterior pituitary cells were treated with ATRA (10^{-6} M) or vehicle as control for 72 h. The media were replaced with fresh media, and GHRH (A) or ghrelin (B) at 10^{-9} M was added to the media. The cells were then incubated for 30 min. The amount of GH released in media was determined by ELISA. Data represent means $\pm$ SEM ($n = 6$). * $P < 0.01$.

cells of rats. This result is consistent with the findings of a previous study, which showed up-regulation by RA of *Ghrh-r* mRNA levels in a rat pituitary cell line [10]. The present study also found that ATRA induced *Ghs-r* gene expression in pituitary cells (Fig. 1). In addition, *Ghrh-r* and *Ghs-r* gene expressions were stimulated by ATRA in a dose- and time-dependent manner. ATRA did not stimulate gene expression of *Sst-r2* or *Sst-r5*. These results suggest that transcription of *Ghrh-r* and *Ghs-r* is regulated by RA.

RA acts as a ligand for the nuclear receptors RARs and RXRs. The RA–RAR/RXR protein complex binds to a unique consensus sequence, named RARE, thereby altering gene transcription [2]. To clarify the RA signaling pathway, we used RAR and RXR agonists. Am80 is an agonist for RAR α and β [15], and PA024 is an RXR pan-agonist [16]. We demonstrated that these agonists weakly increased *Ghrh-r* gene expression (Fig. 4). Combination treatment with Am80 and PA024 completely mimicked the effect of ATRA on *Ghrh-r* mRNA transcription. Nogami *et al.* demonstrated that a functional RARE is located in the 5'-flanking region of the rat *Ghrh-r* gene [17]. That finding and the present results indicate that RA directly promotes *Ghrh-r* gene expression *via* RAR and RXR.

Several studies reported that various hormones regulate *Ghs-r* gene expression in the pituitary gland. Thyroid hormone increases *Ghs-r* mRNA transcription in primary pituitary cells of adult rats [18]. Petersenn *et al.* investigated transcriptional regulation of human GHS-R by transient transfections using the 5'-flanking region of *Ghs-r* promoter in several cell lines [19]. *Ghs-r* promoter activity was stimulated by thyroid hormone and estrogen and inhibited by glucocorticoid in human [19]. In the present study, we first demonstrated the effect of RA on *Ghs-r* gene expression. The RAR agonist Am80 and RXR agonist PA024 resulted in weak but significant stimulation of *Ghs-r* gene expression (Fig. 4). Interestingly, combination treatment with Am80 and PA024 completely mimicked the effect of ATRA. Thus, we speculate that RA promotes *Ghs-r* transcription by direct binding to RARE. Petersenn *et al.* reported the sequence of the 2.7-kb 5'-flanking region of the human *Ghs-r* gene [19]. However, the RARE consensus sequence in the promotor region of the *Ghs-r* gene has not been identified. Additional studies are needed in order to identify the RA response region in the *Ghs-r* gene.

In addition to examining the effects of ATRA on

Ghrh-r and *Ghs-r* mRNA transcription, we investigated the effects of ligands of GHRH-R and GHS-R on GH release from ATRA-treated cells. Several previous studies reported that GHRH and ghrelin stimulate GH release from isolated anterior pituitary cells. In a perfusion system, GHRH and ghrelin stimulated GH release from isolated rat anterior pituitary cells in a dose-dependent manner at doses from 10^{-10} to 10^{-8} M [20]. Hashizume *et al.* found that these peptides directly stimulate GH release from static cultured bovine anterior pituitary cells [21]. The present study used a static incubation system. Exposure of cultured pituitary cells to ATRA had no effect on basal GH release. Interestingly, ATRA enhanced GHRH- and ghrelin-induced GH release from cultured cells (Fig. 5). Taken together, our findings suggest that RA maintains pituitary responsiveness to GHRH and ghrelin. However, further studies are needed to clarify the action of RA on GHRH- and ghrelin-induced GH release *in vivo*.

In previous studies, we reported that RA-synthesizing enzyme, retinaldehyde dehydrogenase 1, was expressed in folliculostellate cells and lactotrophs but not in somatotrophs [22, 23]. In the present study, we found that RA increased *Ghrh-r* and *Ghs-r* gene expression and promoted GHRH- and ghrelin-induced GH release from isolated rat anterior pituitary cells. In conclusion, the present results suggest that RA is produced in the pituitary gland and has a role in paracrine stimulation of somatotrophs.

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Disclosure

The authors have no conflict of interest that might prejudice the impartiality of this research.

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