Paracrine Control of Gonadotrophs by Somatolactotrophs through TRH-induced Follistatin Production

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Abstract: There is increasing evidence for the existence of local regulation of hormone secretion among pituitary cells. Hormone-producing pituitary cells may communicate with each other and with folliculostellate cells. Activin is one of the regulators of follicle stimulating hormone (FSH) secretion and gene expression, whereas follistatin negatively regulates FSH production by binding to and bioneutralizing the effects of activin. In prolactin-secreting GH3 cells, hypothalamic thyrotropin-releasing hormone (TRH) has been known to stimulate prolactin synthesis and secretion. Follistatin is produced in GH3 cells and is up-regulated by TRH in an ERK dependent manner. Prolactin mRNA levels in GH3 cells are not affected by increasing the dose of exogenous follistatin, and TRH-induced prolactin expression is not modulated in the presence of follistatin. Follistatin produced in somatolactotroph GH3 cells does not affect prolactin production in these cells. In pituitary gonadotroph cell line, LβT2, activin increases FSHβ promoter activity and mRNA expression, and follistatin completely inhibits this activin-increased FSHβ gene expression. On the other hand, activin reduces the basal activity of prolactin transcription and follistatin prevents these effects. When GH3 cells and LβT2 cells are co-cultured, activin-induced FSHβ promoter activity is completely inhibited in the presence of follistatin. In addition, FSHβ mRNA is not detected in LβT2 cells, when co-cultured with GH3 cells. These observations using the model for somatolactotroph and gonadotroph suggest the possibility that somatolactotrophs and gonadotrophs interact with each other, and TRH might indirectly affect gonadotropin production though follistatin.

Key words; Somatolactotroph, gonadotroph, TRH, follistatin.

1. INTRODUCTION

The anterior pituitary gland is composed of 5 major hormone-secreting cells: corticotrophs, thyrotrophs, gonadotrophs, somatotrophs and lactotrophs. These cells specifically secrete the hormones adenocorticotropic hormone (ACTH), thyroid hormone stimulating hormone (TSH), gonadotropins such as luteinizing hormone (LH) and follicle stimulating hormone (FSH), growth hormone (GH) and prolactin (PRL), respectively. In addition to these 5 major hormone-secreting cells, somatolactotrophs, which contain both GH and PRL [1], and folliculostellate also exist in the anterior pituitary.

Anterior pituitary hormone secretion is under the control of specific hypothalamic peptides, such as corticotrophin-releasing hormone (CRH), growth hormone-releasing hormone (GHRH), thyrotropin-releasing hormone (TRH) and gonadotropin-releasing hormone (GnRH). In addition to the hypothalamic regulation of pituitary hormones, the actions of certain paracrine regulators are influenced by the hormonal milieu within the pituitary gland. In particular, there is accumulating evidence that folliculostellate cells serve a paracrine function within the pituitary [2, 3]. These cells produce signaling proteins such as vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), nerve growth factor (NGF), insulin-like growth factor (IGF), transforming growth factor (TGF)-α and TGF-β [4-6]. Folliculostellate cells also express cytokines such as interleukin-6 (IL-6) and leukemia inhibitory factor (LIF) [7, 8], and they also produce nitric oxide (NO) [9], PACAP [10] and follistatin [11].

Regarding gonadotropin secretion, gonadotrophs are mainly under the control of hypothalamic GnRH. GnRH release from the hypothalamus varies physiologically over the reproductive cycle [12], and changes in GnRH pulse frequency have been shown to differentially regulate gonadotropins LH and FSH [13]. That is, rapid GnRH pulse frequencies increase the secretion of LH, whereas slower frequencies result in a decline in LH secretion but an increase in FSH [14]. The initial phase of GnRH action involves G protein–mediated stimulation of phospholipase C, leading to the formation of 1,4,5-triphosphate (IP3) and diacylglycerol (DG). Subsequently, IP3 induces the release of intracellular calcium from the endoplasmic reticulum, and DG activates protein kinase C (PKC), which ultimately activates extracellular regulated kinase (ERK) [15-17]. In addition to the hypothalamic GnRH, gonadotropins are known to be regulated by sex steroids such as testosterone [18], estradiol, and progesterone [19] at the pituitary level. Furthermore, activin, inhibin, and follistatin produced within the pituitary gland work as local factors to regulate gonadotropin synthesis and secretion [20].

Among the hormone-secreting cells within the anterior pituitary, communication between different types of cells

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probably exists. In this article, we describe the possible interaction between gonadotrophs and somatolactotrophs, based on previous experiments.

II. HORMONE-SECRETING CELLS IN THE ANTERIOR PITUITARY

Different types of hormone-producing cells exist in the anterior pituitary. Approximately 50% of the adenohypophysis consist of GH-producing somatotrophs, and 15% are considered to be lactotrophs which produce PRL. These cell types are acidophilic and are also believed to derive from the same origin. Somatolactotrophs which produce both GH and PRL are candidate cells for the origin of lactotrophs and exist in a small population [21]. Corticotrophs, which secrete ACTH, comprise approximately 15-20%, and TSH-producing thyrotrophs are approximately 5% of the total adenohypophysial cell populations. Pituitary LH and FSH are secreted from gonadotrophs and these cells likely represent up to 10% of the cell population. In addition to the hormone secreting cells, non-hormonal follicular cells [22], S-100 protein positive folliculostellate cells [23], null cells [24] and oncocytes [25] comprise the adenohypophysis.

III. GONADOTROPIN REGULATION BY ACTIVIN AND FOLLISTATIN

Two related proteins, inhibin and activin, are produced and secreted by the gonads and act at the pituitary to regulate FSH secretion. In addition, expression of inhibin α and inhibin-activin βB subunits, but not the βA-subunit polypeptide, has been demonstrated in gonadotrophs [26, 27]. Activin B is the major isoform produced by the gonadotrophs. Activin B produced by the pituitary gland acts in an autocrine-paracrine fashion to regulate FSH synthesis and secretion. Decreases in basal secretion of FSH and in FSHβ mRNA were detected upon blockage of activin B [28]. Follistatin was first identified in ovarian follicular fluid by its ability to suppress FSH secretion from pituitary [29, 30]. Expression of follistatin mRNA has also been detected in many pituitary cell types, including gonadotroph, somatotroph, lactotroph and folliculostellate cells [31, 32]. *In vitro* experiments using rat anterior pituitary cells have demonstrated that expression of pituitary follistatin mRNA is stimulated by activin [33] and negatively regulated by inhibin [33]. It was demonstrated that activation of either the protein kinase A or PKC signaling pathways has a stimulatory effect on anterior pituitary follistatin production. Follistatin is produced and released within the pituitary and can bind to activin to neutralize its stimulatory effects on FSH biosynthesis. Thus, locally produced follistatin interferes with the secretory response of gonadotrophs to activin. Inhibin, which is produced from the ovary also exerts its inhibitory effect on activin by preventing the association of activin with activin receptor. Gonadal factors have been shown to regulate the levels of follistatin, and ovariectomy or castration results in a robust increase in follistatin mRNA [31, 34]. Follistatin released by the gonad acts in a classical endocrine manner to modulate the effects of activin.

IV. GH3 CELLS AND LβT2 CELLS AS MODELS FOR SOMATOLACTOTROPHS AND GONADOTROPHS

To investigate the mechanism of hormone synthesis in each hormone-secreting pituitary cell type, especially for cell signaling studies, only a single group of cells should be used. Although it is difficult to isolate a single colony of hormone-secreting cells, clonal strains of pituitary tumor cells have been widely used as models for the study of distinct hormone-secreting cell types. GH3 cells, which can synthesize and secrete both PRL and GH, develop from rat pituitary adenoma [35, 36]. Analysis of GH3 cells by fixed sequential plaque assays have demonstrated that GH3 cells consist of two types of hormone-secreting cells: somatotrophs, which secrete only GH, and somatolactotrophs, which secrete both GH and PRL. Although it is not clear at present whether TRH is a physiological regulator of PRL, it has been used to evaluate the signal-transduction system underlying PRL secretion and synthesis because TRH is a primary PRL secretagogue [37]. Indeed, GH3 cells respond to TRH and stimulate secretion and synthesis of PRL. In addition, development of the immortalized murine pituitary gonadotroph-derived cell model, LβT2, has facilitated study of the signal transduction pathways activated by the GnRH receptor [38]. This cell line expresses the gonadotropin α-, LHβ-, and FSHβ-subunits as well as the GnRH receptor, and they synthesize and release LH and FSH in response to GnRH stimulation. Studies of the regulation of pituitary gonadotropin gene expression have also been performed using an gonadotropin α subunit-producing cell line of gonadotroph lineage, αT3-1 cells [38]. Our studies were conducted using GH3 cells and LβT2 cells as models to determine the interaction of gonadotrophs and somatolactotrophs.

V. TRH STIMULATES FOLLISTATIN GENE EXPRESSION IN SOMATOLACTOTROPH, GH3 CELLS

Hypothalamic TRH is a major regulator of TSH secretion from the thyrotroph and also stimulates PRL synthesis and secretion [39]. TRH receptor increases turnover of inositol, which results in activation of protein kinase C (PKC) and Ca^{2+} release [40]. TRH-induced signaling events include activation of extracellular signal-regulated kinase (ERK) in both a PKC-dependent and PKC-independent manner [41, 42]. TRH stimulates follistatin gene expression in somatolactotroph GH3 cells (Fig. **1**). This suggests that TRH not only regulates prolactin in somatolactotrophs, but also produces follistatin, a regulator of gonadotropin. The increase in follistatin by TRH is prevented completely in the presence of U0126, a MEK inhibitor, suggesting that TRH-induces ERK activation and this pathway is involved in follistatin expression. The involvement of ERK pathways is also confirmed by transfection of pFC-MEKK (Fig. **2**). TRH-induced ERK activation is associated with PRL gene expression, DNA synthesis, a shift of GH-secreting somatolactotrophs to PRL-secreting cells [42-44], and it also important for follistatin expression in somatolactotrophs.

VI. EFFECTS OF FOLLISTATIN ON SOMATOLACTOTROPH AND GONADOTROPHS

Although TRH stimulates follistatin expression in GH3 cells, we found no reports in the literature describing an effect of follistatin on PRL secretion and biosynthesis.

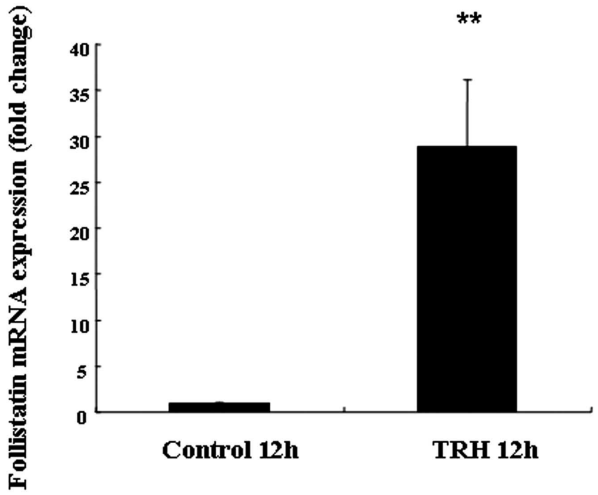

Fig. (1). Effect of TRH on follistatin mRNA expression in GH3 cells. GH3 cells were cultured in six-well plates for an initial 24 h; with the exception of the control, TRH (100 nM) was added to the culture dishes at 12 h. After cell harvest, mRNA extraction, and reverse transcription, follistatin mRNA levels were measured with quantitative real time PCR. The results (mean ± SEM) of n = 3 (each experiment with triplicates samples) are expressed as the fold stimulation over the unstimulated group. ** P < 0.01 vs. control.(Regulatory Peptides 2009; 156:65-71, Reprinted with permission from Elsevier).

Exogenous administration of follistatin fails to modulate the basal activity of the prolactin promoter, even after increasing the concentration of follistatin (Fig. **3A**). Follistatin administration also has no effect on TRH-induced prolactin promoter activity in GH3 cells (Fig. **3B**). Knock-down of endogenous follistatin by follistatin siRNA fails to modulate basal prolactin promoter activity [45]. Therefore, TRH-induced follistatin does not have a direct effect on prolactin expression, whereas follistatin affects somatolactotrophs indirectly through inhibition of activin. Previous reports have demonstrated that activin has an inhibitory effect on PRL production [46, 47]. In our studies, we also showed that activin decreases PRL promoter activity and this inhibition is rescued in the presence of follistatin (Fig. **4**). These results suggest the modulatory role of follistatin results only from binding to and neutralizing the effects of activin.

Follistatin acts primarily by binding activin, thereby preventing the interaction of activin with its receptors [48]. As a result, follistatin inhibits the FSH response to activin, but not to GnRH [49]. In gonadotroph LβT2 cells, activin increases FSHβ promoter activity, and this effect is completely abolished in the presence of follistatin (Fig. **5A**). Messenger RNA detection by real time PCR demonstrated that activin increases FSHβ mRNA expression dramatically, but the effect of activin is completely eliminated in the presence of follistatin (Fig. **5B**). Along the same lines, the reduction of FSHβ mRNA associated with exposure to pituitary adenylate cyclase-activating polypeptide (PACAP) is also associated with increasing follistatin gene expression in pituitary cell culture [50]. Interleukin (IL)-1β, which was shown to stimulate follistatin secretion, was also observed to attenuate the FSH response to activin [51].

VII. INTERACTION OF SOMATOLACTOTROPH AND GONADOTROPH THROUGH FOLLISTATIN

Biochemical interactions between follistatin and activin have long been documented, forming the basis for the theoretical modulation of activin activity by follistatin. To assess whether TRH-induced follistatin from somatolactotrophs could affect neighboring gonadotrophs, and to examine the interaction between somatolactotrophs and gonadotrophs through follistatin expression, somatolactotroph GH3 cells were co-cultured with gonadotroph LβT2 cells. In this

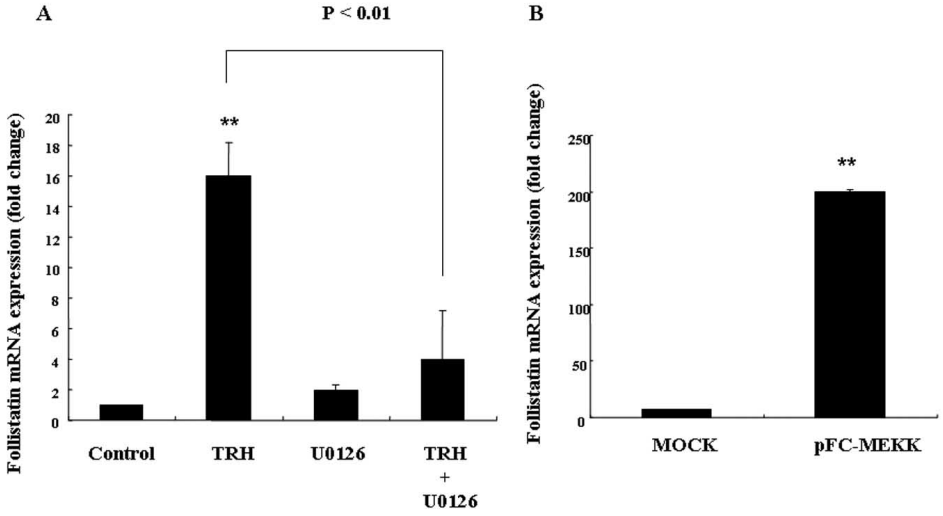

Fig. (2). The effects of the MEK inhibitor, U0126 and the overexpression of pFC-MEKK on follistatin mRNA expression. (A) GH3 cells, plated in 6-well plates, were pre-incubated in the presence or absence of 10 μM U0126 for 1 h and then treated with 100 nM TRH for 12 h. (B) GH3 cells were transfected with pCI neo (1.0μg; MOCK) and pFC-MEKK (1.0μg; MEKK) for 36 h. After cell harvest, mRNA extraction, and reverse transcription, follistatin mRNA levels were measured with quantitative real time PCR. The results (mean ± SEM) of n = 3 (each experiment with triplicate samples) are expressed as the fold stimulation over the unstimulated group. ** P < 0.01 vs. control. The difference between TRH and TRH+U0126 were statistically significant (P < 0.01). (Regulatory Peptides 2009; 156:65-7,1 Reprinted with permission from Elsevier).

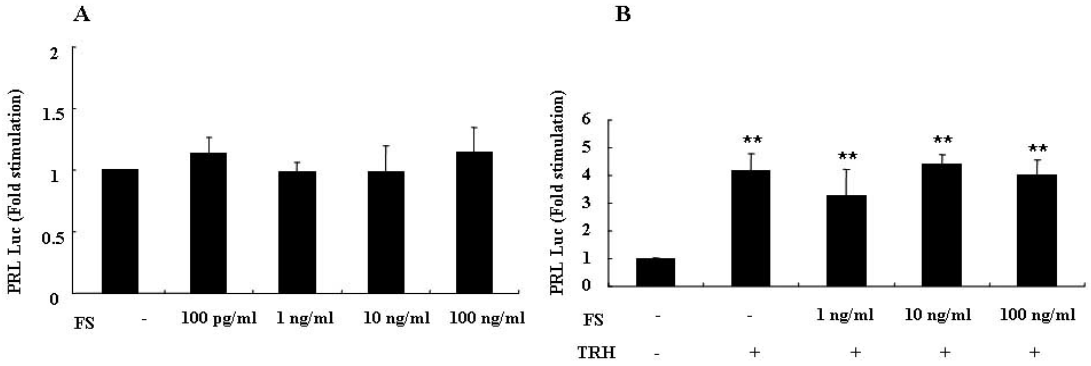

Fig. (3). Effect of follistatin on prolactin promoter activity in GH3 cells. GH3 cells were co-transfected with the prolactin promoter-linked luciferase vector (PRL-Luc), pRL-TL and incubated for 72 h. The cells were then treated with increasing doses of follistatin in the absence (A) or presence of TRH (B) for 12 h. Luciferase activity was normalized relative to PRL-TK, and expressed as the fold stimulation of the unstimulated control. Values are the mean ± SEM of n = 3 (each experiment with triplicate samples). ** $P < 0.01$ vs. control. (Regulatory Peptides 2009; 156:65-71, Reprinted with permission from Elsevier).

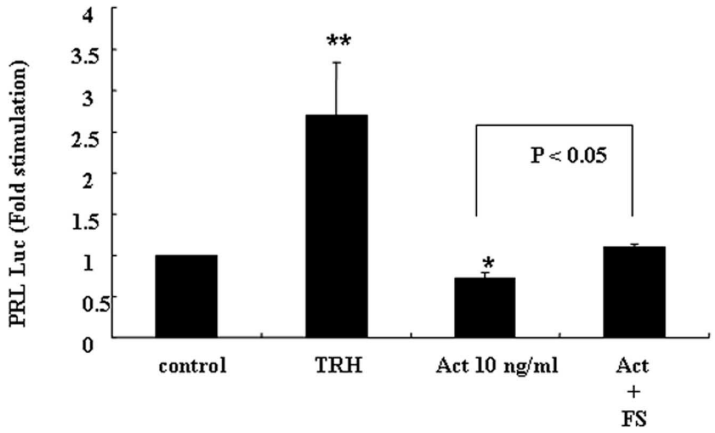

Fig. (4). Effect of follistatin on activin modulated prolactin promoter activity. GH3 cells were co-transfected with the prolactin promoter-linked luciferase vector (PRL-Luc), pRL-TL and incubated for 72 h. The cells were then treated with TRH (100 nM) and 10 ng/ml activin (Act) in the presence or absence of 50 ng/ml follistatin (FS) for 12 h. Luciferase activity was normalized relative to PRL-TK, and expressed as the fold stimulation of the unstimulated control. Values are the mean ± SEM of n = 3 (each experiment with triplicate samples). ** $P < 0.01$, * $P < 0.05$ vs. control. The difference between Act and Act + FS was statistically significant ($P < 0.05$). (Regulatory Peptides 2009; 156:65-71, Reprinted with permission from Elsevier).

condition, activin-induced increases in FSHβ promoter activity in LβT2 cells were nearly eliminated in the presence of TRH (Fig. **6A, B**). In addition, we could not detect FSHβ mRNA amplification from LβT2 cells, when co-cultured with GH3 cells (Fig. **6C**). It is plausible that the TRH-induced follistatin produced in GH3 cells bioneutralized the effect of activin in neighboring LβT2 cells. In addition, there is a possibility that, even in the absence of TRH, follistatin (produced spontaneously from GH3 cells) strongly inhibits basal expression of FSHβ in neighboring LβT2 cells. The correlation of FSH and TRH has been described in previous articles. In patients with pituitary adenomas, TRH showed a stimulatory effect on FSH secretion [52, 53]. However, in a study using pituitary cell aggregates, it has been shown that TRH was ineffective in releasing FSH [54].

VIII. OTHER FACTORS INVOLVED IN THE PARACRINE CONTROL OF GONADOTROPHS BY SOMATOLACTOTROPHS

Follistatin is not the sole factor that affects gonadotropin secretion from gonadotrophs. Other factors from somatolactotrophs are also involved in the paracrine control of gonadotrophs. For example, Andries, *et al*. reported that immuno-neutralization of a cleaved PRL variant suppressed gonadotroph proliferation in pituitary aggregate cell culture, suggesting that PRL has an ability to stimulate gonadotroph proliferation [55]. Exogenous PRL also increases the rate of proliferation in somatolactotrophs itself [56]. In addition, receptors for GH are present in gonadotrophs [57] and radiolabeled GH is taken up by gonadotrophs [57]. In the transgenic mouse, expressing the bovine GH gene, produce pituitaries with less FSHβ and LHβ mRNAs. Less FSH protein, higher unstimulated LH and FSH secretion in perifusion *in vitro*, and a decreased gonadotropin response to GnRH [58]. These observations suggest that hormones produced from somatolactotrophs affect the neighboring gonadotrophs and modulate their functions. In addition, locally produced ATP has been shown to stimulate LH [59, 60], and accumulation of ATP is increased by TRH, but decreased by bromocriptine [61], indicating the involvement of somatolactotrophs in the action of ATP. Although some of the most extensively characterized intra-pituitary interactions

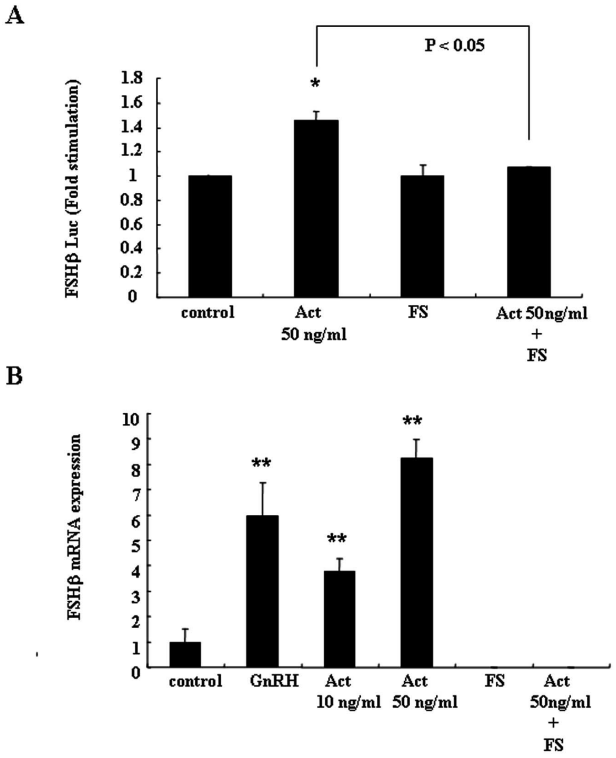

Fig. (5). Effect of activin on gonadotroph LβT2 cell FSHβ gene expression. LβT2 cells were co-transfected with the FSHβ promoter subunit (FSHβ-Luc) and pRL-TK, and incubated for 36 h. (A) The cells were pre-incubated with follistatin (FS) for 60 min and further treated with activin for 12 h. Luciferase activity was normalized relative to PRL-TK, and expressed as the fold stimulation of the unstimulated control. (B) LβT2 cells were treated with GnRH (100 nM) and activin in the presence or absence of follistatin (50 ng/ml). After cell harvest, mRNA extraction, and reverse transcription, FSHβ mRNA levels were measured with quantitative real time PCR. Values are the mean ± SEM of n = 3 (each experiment with triplicate samples). ** $P < 0.01$, * $P < 0.05$, vs. control. The difference between Act and Act + FS was statistically significant ($P < 0.05$). (Regulatory Peptides 2009; 156:65-71, Reprinted with permission from Elsevier).

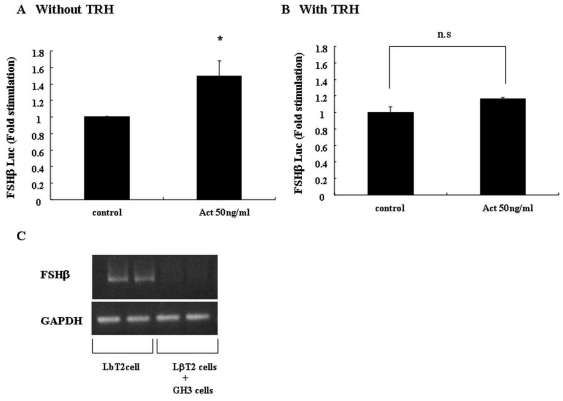

Fig. (6). Effect of TRH on activin-induced FSHβ promoter activity in a mixed culture of GH3 cells and LβT2 cells. LβT2 cells co-transfected with the FSHβ promoter subunit (FSHβ-Luc) and pRL-TK were co-cultured with GH3 cells for 36 h. After pre-incubation for 60 min in DMEM without (A) or with 100 nM TRH (B), 50 ng/ml activin (Act) was added and the cells incubated for an additional 6 h. Luciferase activity was normalized relative to PRL-TK, and expressed as the fold stimulation of the unstimulated control. Values are the mean ± SEM of n = 3 (each experiment with triplicate samples). *$P < 0.05$ vs. control. n.s, fold induction was not statistically significant. (C) LβT2 cells were cultured solely or co-cultured with equal number of GH3 cells for 48 h. After cell harvest, mRNA extraction, and reverse transcription, PCR for FSHβ and GAPDH were carried out using specific primers. PCR amplification were carried out by 40 cycles for FSHβ and 15 cycles for GAPDH, respectively. We confirmed that the amplification curves of GAPDH were linear in these cycles. (Regulatory Peptides 2009; 156:65-71, Reprinted with permission from Elsevier).

with gonadotrophs are those involving activin and follistatin, a number of excellent recent reviews have described additional factors that influence gonadotroph function [62, 63]. Factors that potentially signal in gonadotrophs in a paracrine fashion are shown in Table **1**.

IX. SUMMARY

This review has focused on the possible interaction between gonadotrophs and somatolactotrophs mainly in

Table 1. Factors which are Potential Paracrine Signals Toward Gonadotrophs

Factor	Action	Species/cell line	Reference
Activin	Increase number of FSH secreting FSH, amount of FSH/cell	Rat	[64]
Adenosine	Decrease FSH secretion Decrease LH, FSH secretion	Rat Rat	[65, 66]
ATP	Increase Ca2+ entry; LH secretion Increase LH secretion	Rat Rat	[59, 60]
Epidermal growth factor (EGF)	Increase thymidine uptake:gonadotrophs Increase GnRH binding, LH response to GnRH	Sheep Rat	[67, 68]
Follistatin	Expression linked to attenuated FSH response to GnRH	Rat	[69]
GnRH	Increase differentiation of gonadotroph	Rat	[70]
GH	Increase LH, FSH secretion; Decrease FSHβ, LHβ mRNA, FSH protein response to GnRH	Mouse	[58]
5-hydroxyeicosatetraenoic acid (5-HETE)	Increase α-subunit mRNA	αT3-1	[71]
Nitric oxide (NO)	Increase LH secretion	Rat	[72]
Neuropeptide Y (NPY)	Increase number of FSH-positive cells Increase LH, FSH secretion Decrease LH secretion	Hamster Rat Rat	[73-75]
PRL (cleaved variant)	Increase thymidine uptake in gonadotrophs	Rat	[55]
Substance P	Increase LH secretion Attenuate LH, FSH response to GnRH	Rat Hamster	[76, 77]
TRH	Increase gonadotroph differentiation	Rat	[78]

Adapted from Schwartz J. Intracellular Communication in the Anterior Pituitary. Endocrine Reviews 2000; 21:488-513

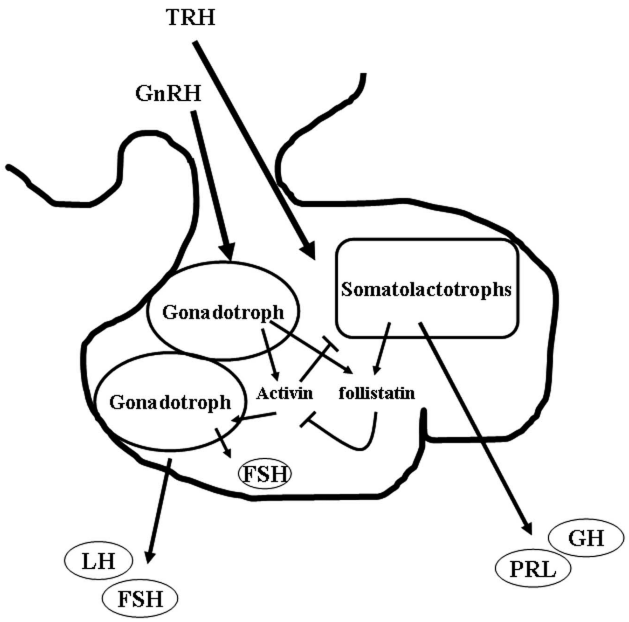

Fig. (7). The schematic summary of the paracrine control of gonadotrophs by follistatin. In the pituitary, hypothalamic GnRH stimulates gonadotoropin synthesis and release from gonadotrophs. Activin and follistatin are also produced from gonadotrophs in response to GnRH. Locally produced activin increases FSHβ expression in gonadotrophs and this effect is prevented by the existence of follistatin. Somatolactotrophs neighboring gonadotrophs are stimulated by hypothalamic TRH and release PRL. TRH also stimulates follistatin production from somatolactotrophs, and this follistatin modulates gonadotropin function by binding to activin to neutralize its stimulatory effects on FSH biosynthesis. Activin also inhibits PRL synthesis in somatolactotrophs and this inhibition is prevented by follistatin.

ACKNOWLEDGEMENT

This work was supported in part by Grants in Aid for Scientific Research from the Ministry of Education, Science,

association with follistatin. The proposed mechanism for the paracrine regulation of gonadotrophs through follistatin which is produced by somatolactotroph is shown in Fig. (**7**). Although the pituitary somatolactotroph cell line, GH3, and

the gonadotroph cell line, LβT2, were described as a model for normal pituitary cell, it is plausible that TRH-induced follistatin from the somatolactotroph acts in a paracrine manner on the gonadotroph in association with activin action. Sports and Culture of Japan (H. K. and K. M.) and Grants from Uehara memorial foundation (H. K.).

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Received: January 16, 2010 Revised: February 19, 2010 Accepted: October 27, 2010