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Oxytocin and Dopamine Stimulate Ghrelin Secretion by the Ghrelin-Producing Cell Line, MGN3-1 in Vitro

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To understand the physiological role of ghrelin, it is crucial to study both the actions of ghrelin and the regulation of ghrelin secretion. Although ghrelin actions have been extensively revealed, the direct factors regulating ghrelin secretion by ghrelin-producing cells (X/A-like cells), however, is not fully understood. In this study, we examined the effects of peptide hormones and neurotransmitters on in vitro ghrelin secretion by the recently developed ghrelin-producing cell line MGN3-1. Oxytocin and vasopressin significantly stimulated ghrelin secretion by MGN3-1 cells. Because MGN3-1 cells express only oxytocin receptor mRNA, not vasopressin receptor mRNA, oxytocin is the likely regulator, with the effect of vasopressin mediated by a cross-reaction. We also discovered that dopamine stimulates ghrelin secretion from MGN3-1 cells in a similar manner to the previously known ghrelin stimulators, epinephrine and norepinephrine. MGN3-1 cells expressed mRNA encoding dopamine receptors D1a and D2. The dopamine receptor D1 agonist fenoldopam stimulated ghrelin secretion, whereas the D2, D3 agonist bromocriptine did not. Furthermore, the D1 receptor antagonist SKF83566 attenuated the stimulatory effect of dopamine. These results indicate that the stimulatory effect of dopamine on ghrelin secretion is mediated by the D1a receptor.

In conclusion, we identified two direct regulators of ghrelin, oxytocin and dopamine. These findings will provide new direction for further studies seeking to further understand the regulation of ghrelin secretion, which will in turn lead to greater understanding of the physiological role of ghrelin. (Endocrinology 152: 2619–2625, 2011)
the best research tools to study the regulation of ghrelin secretion in vitro. In previous studies, we used MGN3-1 cells to examine the effects of insulin and somatostatin, which are well established in in vivo studies to suppress ghrelin secretion (13–16). In this study, we examined the effects of peptide hormones and nonpeptide neurotransmitters on in vitro ghrelin secretion from MGN3-1 cells.

**Materials and Methods**

**Cell culture**

MGN3-1 cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37 C in 10% CO2 as described previously (12).

**Batch incubation study**

MGN3-1 cells were seeded at 7.5 × 10^5 cells/well and cultured for 24 h in 12-well plates. After a washing with PBS, cells were incubated at 37 C for 4 h in DMEM supplemented with 0.5% BSA and the indicated reagents before collecting supernatants. To screen for peptide hormones stimulating or suppressing ghrelin secretion, (13–16). In this study, we examined the effects of peptide hormones and nonpeptide neurotransmitters, acetylcholine, nicotine, muscarine, epinephrine, norepinephrine, dopamine, histamine, serotonin, glutamate, or γ-aminobutyric acid (GABA; Sigma Aldrich Japan) were added at 10^-4 M to each well. To determine the stimulatory adrenergic receptor subtype, 10^-5 M of isoproterenol, fenoldopam, or bromocriptine (Sigma Aldrich Japan) were used. For the antagonistic studies, oxytocin receptor antagonist [d(CH2)5Tyr(Me)2,Orn8]-oxytocin (Bachem, Bubendorf Switzerland), β1-receptor antagonist atenolol (Sigma Aldrich Japan), and dopamine D1 receptor antagonist SKF83566 (Tocris Bioscience, Ellisville, MO) were used.

**Measurements of ghrelin concentrations in culture medium**

To measure ghrelin concentrations in culture medium, the collected culture media were centrifuged, and the resulting supernatants were immediately applied to Sep-Pak C18 cartridges (Waters Corp., Milford, MA) preequilibrated with 0.9% saline. After washing cartridges with saline and 5% CH3CN/0.1% trifluoroacetic acid, bound protein was eluted with 60% CH3CN/0.1% trifluoroacetic acid. Eluates were lyophilized and subjected to ghrelin RIA. Two types of ghrelin RIA were performed: C-RIA, in which an anti-COOH-terminal ghrelin (amino acids 1–11) antiserum detects ghrelin only, as described (17, 18). Measurements of ghrelin concentrations in culture medium

RT-PCR and quantitative RT-PCR

Total RNA was extracted using an RNeasy kit (QIAGEN, Hilden, Germany). Reverse transcription was performed with a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). RT-PCR was performed using a GeneAmp 9700 cycler (Applied Biosystems) with AmpliTaq Gold using appropriate primers (Supplemental Table 1, published on The Endocrine Society’s Journals Online web site at http://endo.endojournals.org). Real-time quantitative PCR was performed using an ABI PRISM 7500 sequence detection system (Applied Biosystems) using appropriate primers and taqman probes or Power SybrGreen (Supplemental Table 1). The mRNA expression of each gene was normalized to the detected levels of 18S rRNA.
cating that the stimulatory effect of vasopressin is likely secondary to a cross-reaction to the oxytocin receptor. Actually, addition of oxytocin receptor antagonist \([\text{d(CH2)}_5\text{-Tyr(Me)}_2\text{-Orn}^8]\)-oxytocin significantly attenuated the stimulatory effect of vasopressin on ghrelin secretion (Fig. 2, B and C). Oxytocin-mediated stimulation of ghrelin-secretion was dose dependent (ED50 value for N-RIA: 51.22 nM; C-RIA: 21.9 nM; Fig. 2, D and E). Although oxytocin induced a small, but significant, increase in ghrelin O-acetyltransferase (GOAT) mRNA levels in MGN3-1 cells (Fig. 2F), ghrelin mRNA levels were unchanged (Fig. 2G).

### Effects of nonpeptide neurotransmitters on ghrelin secretion

We next examined the effects of nonpeptide neurotransmitters on ghrelin secretion by MGN3-1 cells. Ghrelin secretion by MGN3-1 cells was stimulated by the addition of epinephrine, norepinephrine, or dopamine to the medium (Fig. 3, A and B). No effects on ghrelin secretion were seen after the addition of acetylcholine, nicotine, muscarine, histamine, serotonin, glutamate, or GABA to the medium (Fig. 3, A and B). Ghrelin secretion induced by epinephrine increased in a dose-dependent manner (ED50 value for N-RIA: 1.31 μM; C-RIA: 2.36 μM; Fig. 4, A and B). MGN3-1 cells expressed mRNA encoding of α1a- and β1-adrenergic receptors (Fig. 4C). The nonselective β-agonist isoproterenol and the β1-agonist clenbuterol significantly stimulated ghrelin secretion by MGN3-1 cells (Fig. 4, D and E). The β2-agonist ritodrine also stimulated ghrelin secretion to a lesser extent, which may have been secondary to cross-reactivity (Fig. 4, D and E). No effect on ghrelin secretion was found using the α1-agonist phenylephrine, the α1a-agonist A61603 or the α2-agonist clonidine (Fig. 4, D and E). Addition of β1-receptor antagonist atenolol significantly attenuated the stimulatory effect of epinephrine on ghrelin secretion (Fig. 4, F and G). These results indicate that the stimulation of ghrelin secretion by epinephrine or norepinephrine is primarily mediated by the β1-receptor. Isoproterenol significantly increased GOAT mRNA levels but not ghrelin mRNA levels (Fig. 4, H and I).

The stimulation of ghrelin secretion by dopamine was also dose dependent (ED50 value for N-RIA: 24.7 μM; C-RIA: 40.6 μM; Fig. 5, A and B). MGN3-1 cells expressed mRNA encoding α1a- and β1-adrenergic receptors (Fig. 5C). The nonselective β-agonist isoproterenol and the β1-agonist clenbuterol significantly stimulated ghrelin secretion by MGN3-1 cells (Fig. 5D and E). The β2-agonist ritodrine also stimulated ghrelin secretion to a lesser extent, which may have been secondary to cross-reactivity (Fig. 5D and E). No effect on ghrelin secretion was found using the α1-agonist phenylephrine, the α1a-agonist A61603 or the α2-agonist clonidine (Fig. 5D and E). Addition of β1-receptor antagonist atenolol significantly attenuated the stimulatory effect of epinephrine on ghrelin secretion (Fig. 5F and G). These results indicate that the stimulation of ghrelin secretion by epinephrine or norepinephrine is primarily mediated by the β1-receptor. Isoproterenol significantly increased GOAT mRNA levels but not ghrelin mRNA levels (Fig. 5H and I).
mRNA encoding dopamine receptors D1a and D2 (Fig. 5C). The nonselective dopamine receptor agonist apomorphine and the D1 receptor agonist fenoldopam also significantly stimulated ghrelin secretion from MGN3-1 cells, whereas the D2, D3 agonist bromocriptine had no

FIG. 3. The effects of neurotransmitters on ghrelin secretion by MGN3-1 cells. A and B, The amount of ghrelin secreted by MGN3-1 cells incubated for 4 h in DMEM supplemented with 0.5% BSA and 10^{-4} M acetylcholine, nicotine, muscarine, epinephrine, norepinephrine, dopamine, histamine, serotonin, glutamate, or GABA. **, P < 0.01 in comparison with controls (n = 9).

FIG. 4. The effects of epinephrine on ghrelin secretion by MGN3-1 cells. A and B, The amount of ghrelin secreted by MGN3-1 cells incubated for 4 h in DMEM supplemented with 0.5% BSA and 10^{-7} to 10^{-5} M epinephrine. **, P < 0.05, ***, P < 0.01 in comparison with controls (-) (n = 9). C, RT-PCR analysis of adrenergic receptors-a1a, -a1b, -a1d, -a2a-c, and -b1-3 mRNA expression in MGN3-1 cells. D and E, The amount of ghrelin secreted by MGN3-1 cells incubated for 4 h in DMEM supplemented with 0.5% BSA and 10^{-5} M isoproterenol (b-agonist), denopamine (b1-agonist), ritodrine (b2-agonist), phenylephrine (a1-agonist), clonidine (a2-agonist), or A61603 (a1a-agonist). **, P < 0.01 in comparison with controls (n = 9). F and G, The amount of ghrelin secreted by MGN3-1 cells incubated for 4 h in DMEM supplemented with 0.5% BSA and 10^{-5} M epinephrine with or without 10^{-4} M atenolol (b1-agonist). ***, P < 0.01 in comparison with controls; ##, P < 0.01 in comparison with epinephrine (n = 9). H and I, Ghrelin and GOAT mRNA levels in MGN3-1 cells after a 24-h incubation with 10^{-5} M isoproterenol. *, P < 0.05 in comparison with controls (n = 9). AU, Arbitrary unit.

Discussion

Ghrelin-producing cells are located in the stomach. These cells secrete ghrelin by responding to various kinds of inputs, possibly hormones, neurotransmitters, or nutrients. From these exogenous signals, the cell can sense the outside environment and/or interact with other organs to provide appropriate regulation of ghrelin secretion,
which in turn influences various homeostatic systems, including energy homeostasis or growth control. We sought to understand better the molecular mechanisms governing ghrelin secretion by cells, which may further contribute to understanding the physiological role of ghrelin. In previous studies, we have developed a ghrelin-secreting cell line MGN3-1 as a research tool to study the regulation of ghrelin secretion in vitro (12). In this study, we examined the effects of the various peptide hormones and neurotransmitters on ghrelin secretion using MGN3-1 cells.

We found that oxytocin significantly stimulates ghrelin secretion from MGN3-1 cells. Oxytocin, a nonpeptide with a disulfide bond, is secreted from the posterior pituitary gland in a neuroendocrine manner and is involved in milk ejection and uterine contraction. Oxytocin also acts as a neurotransmitter, specifically as a negative regulator of food intake to oxytocin-receptive neurons in the paraventricular nucleus of the hypothalamus (19). Only two previous reports have examined the effect of oxytocin on plasma ghrelin levels. Vila et al. (20) described a reduction in basal and lipopolysaccharide-induced ghrelin levels in healthy men after systemic administration of oxytocin. Shibata et al. (21) reported that inhibition of the suckling-induced increase in plasma oxytocin levels by a oxytocin antagonist did not alter plasma ghrelin levels in lactating rats. Although the investigators concluded that oxytocin has no effects on ghrelin secretion, our findings are not in accordance with that report. The reason for this discrepancy is not clear but may result from indirect effects of additional mediators in vivo. Further studies will be needed to explore the regulation of ghrelin secretion by oxytocin in vivo.

We also found that the nonpeptide neurotransmitters epinephrine and norepinephrine strongly stimulate ghrelin secretion by MGN3-1 cells. Ghrelin secretion has been suggested to be regulated by the sympathetic nervous system. Mundinger et al. (22) noted that increased portal ghrelin levels in rats after electrical sympathetic nerve stimulation or iv tyramine administration. Hossoda and Kangawa (23) reported that the administration of adrenergic agonists increased plasma ghrelin levels in rat. Recently Zhao et al. (24) reported that ghrelin secretion from the pancreatic ghrelinoma cell line PG-1 and the stomach ghrelinoma cell line SG-1 could be stimulated by β1-adrenergic receptors. Our observation demonstrating increased ghrelin secretion after epinephrine and norepinephrine administration is consistent with these results, supporting the idea that sympathetic nervous system is an important regulator of ghrelin secretion.

FIG. 5. The effect of dopamine on ghrelin secretion by MGN3-1 cells. A and B, The amount of ghrelin secreted by MGN3-1 cells incubated for 4 h in DMEM supplemented with 0.5% BSA and 10⁻⁶ to 10⁻¹⁴ M of dopamine. *, *P < 0.05, **P < 0.01 in comparison with controls (−) (n = 9). C, RT-PCR analysis of dopamine receptor (DR) D1a and D2–5 mRNA expression in MGN3-1 cells. D and E, The amount of ghrelin secreted by MGN3-1 cells incubated for 4 h in DMEM supplemented with 0.5% BSA and 10⁻⁶ M apomorphine (nonselective dopamine agonist), fenoldopam (D1 agonist), or bromocriptine (D2, D3 agonist). **, *P < 0.01 in comparison with controls (n = 9). F and G, The amount of ghrelin secreted by MGN3-1 cells incubated for 4 h in DMEM supplemented with 0.5% BSA and 10⁻⁶ M dopamine with or without 10⁻⁴ M SKF83566 (D1 antagonist). **, *P < 0.01 in comparison with controls; ##, *P < 0.01 in comparison with dopamine (n = 9). H and I, Ghrelin and GOAT mRNA levels in MGN3-1 cells after a 24-h incubation with 10⁻⁵ M apomorphine (n = 9). AU, Arbitrary unit.
In addition to epinephrine and norepinephrine, dopamine also significantly stimulated ghrelin secretion from MGN3-1 cells via the D1A receptor. As far as we know, this is the first report of ghrelin secretion stimulation by dopamine. Dopamine is a catecholamine, acting as a neurotransmitter in the certain brain areas in motor control or reward behaviors. A substantial amount of dopamine is also produced in the gastrointestinal tract (25), in which it suppresses gastric motility, stimulates exocrine secretions, modulates jejunal sodium absorption, or protects against gastroduodenal ulcers (26, 27). Our finding raises the possibility that gastrointestinal dopamine may also control ghrelin secretion.

In this study, we used a standard culture medium (DMEM) for the incubation study. The medium contains several compounds including inorganic salts, glucose, amino acids, or vitamins, the concentrations of which may not be entirely the same to that around the ghrelin cell in vivo. We cannot exclude the possibility that these compounds may have influenced on the results and that may explain the discrepancy between our data and clinical studies of oxytocin. Further studies will be needed to clarify the combinational effects of these compounds in the medium and peptide hormones or neurotransmitters.

In addition to epinephrine and norepinephrine, which were previously known to increase ghrelin secretion, we identified two new regulators of ghrelin secretion, oxytocin and dopamine, by screening peptide hormones and neurotransmitters using MGN3-1 cells. These findings will provide new direction for further studies seeking to understand better the regulation of ghrelin secretion and the overall physiological role of ghrelin in organism homeostasis and energy regulation.

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