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)**

Ghrelin is a stomach-derived 28-amino acid peptide hormone with a unique modification of acylation, first described by Kojima *et al.* in 1999 (1). To understand better the physiological function of ghrelin, it is crucial to study both ghrelin action and the regulation of ghrelin secretion. The actions of ghrelin have been vigorously investigated by multiple groups, revealing a wide variety of activities, including GH-stimulating (2), orexigenic (3), fat-storing (4), cardiovascular (5), gastroprokinetic (6), and insulin-suppressing (7) activities. In contrast, the regulation of ghrelin secretion from ghrelin-producing cells (X/A-like cells) is not fully understood. Although the results of *in vivo* studies suggest that plasma ghrelin levels are regulated by acute and chronic energy status (8–10), the individual factors regulating ghrelin secretion by ghrelin-producing cells (X/A-like cells) remains unclear due to the lack of an appropriate *in vitro* assay system.

Recently we established a ghrelin-producing cell line, MGN (mouse ghrelinoma) 3-1 cells from a gastric ghrelinoma isolated from ghrelin promoter SV40-T antigen transgenic mice (11, 12). The MGN3-1 cell is the first cell line derived from a gastric ghrelin-producing cell that preserves the ability to secrete of substantial amounts of ghrelin under physiological regulation, making this line one of

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Abbreviations: ANP, Atrial natriuretic peptide; AVP, vasopressin; CNP, C-type natriuretic peptide; C-RIA, anti-COOH-terminal ghrelin (amino acids 13–28) antiserum used to detect ghrelin and desacyl-ghrelin; GABA, γ-aminobutyric acid; GHRP2, GH-releasing peptide 2; GIP, gastric inhibitory polypeptide; GLP, glucagon-like peptide; GOAT, ghrelin *O*-acyltransferase; N-RIA, anti-NH_2-terminal ghrelin (amino acids 1–11) antiserum detects ghrelin only; PP, pancreatic polypeptide; VIP, vasoactive intestinal peptide.

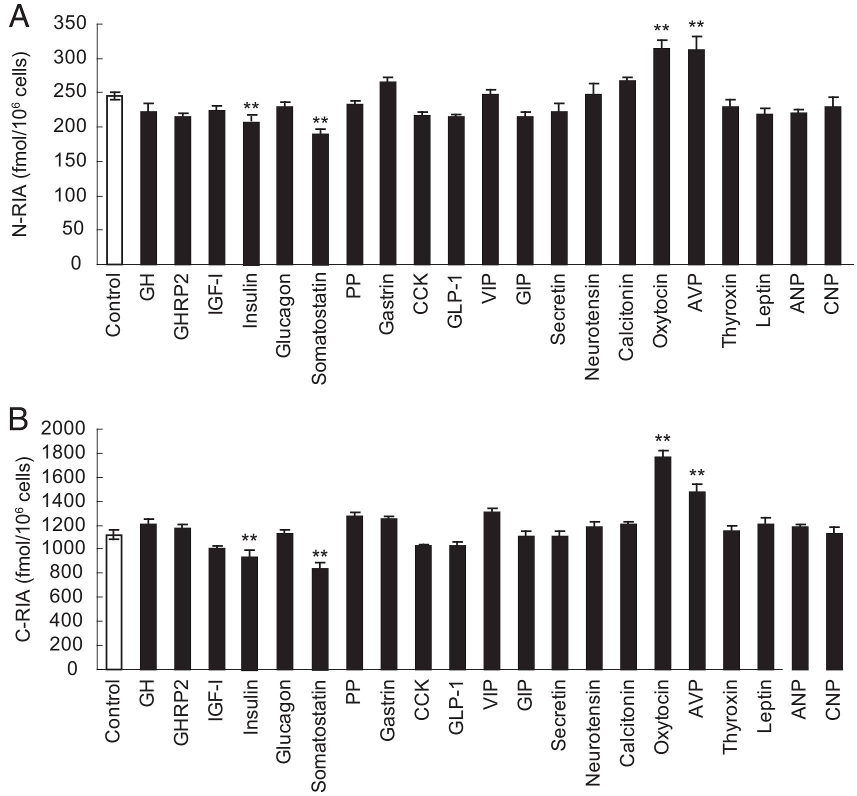

FIG. 1. The effects of peptide hormones on ghrelin secretion by MGN3-1 cells. A and B, The amount of ghrelin secreted by MGN3-1 cells incubated for 4 h in DMEM supplemented with 0.5% BSA and 10^{-6} M GH, GHRP2, IGF-I, insulin, glucagon, somatostatin, PP, gastrin, CCK, GLP-1, VIP, GIP, secretin, neurotensin, calcitonin, oxytocin, AVP, T_4, leptin, ANP, or CNP. **, $P < 0.01$ in comparison with controls (n = 9).

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% CO_2 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, IGF-I, glucagon, somatostatin, pancreatic polypeptide (PP), glucagon-like peptide (GLP)-1, secretin, neurotensin, thyroxin, atrial natriuretic peptide (ANP), GH (Sigma Aldrich Japan, Tokyo, Japan), gastrin, cholecystokinin (CCK), vasoactive intestinal peptide (VIP), gastric inhibitory polypeptide (GIP), calcitonin, oxytocin, vasopressin (AVP), C-type natriuretic peptide (CNP) (Peptide institute, Inc., Osaka, Japan), GH-releasing peptide 2 (GHRP2; Kaken Pharmaceuticals, Co., Ltd, Tokyo, Japan), insulin (Invitrogen, Carlsbad, CA), or leptin (Pepro Tech, Inc., Rocky Hill, NJ) were added to each well at 10^{-6} M. To screen for 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, denopamine, ritodrine, phenylephrine, or clonidine (Sigma Aldrich Japan) were used. To determine the stimulatory dopamine receptor subtype, 10^{-5} M apomorphine, fenoldopam, or bromocriptine (Sigma Aldrich Japan) were used. For the antagonistic studies, oxytocin receptor antagonist $[d(CH_2)_5{}^1, Tyr(Me)^2, Orn^8]$-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% CH_3CN/0.1% trifluoroacetic acid, bound protein was eluted with 60% CH_3CN/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 13–28) antiserum is used to detect both ghrelin and desacyl-ghrelin, and N-RIA, in which an anti-NH_2-terminal ghrelin (amino acids 1–11) antiserum detects ghrelin only, as described (17, 18).

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.

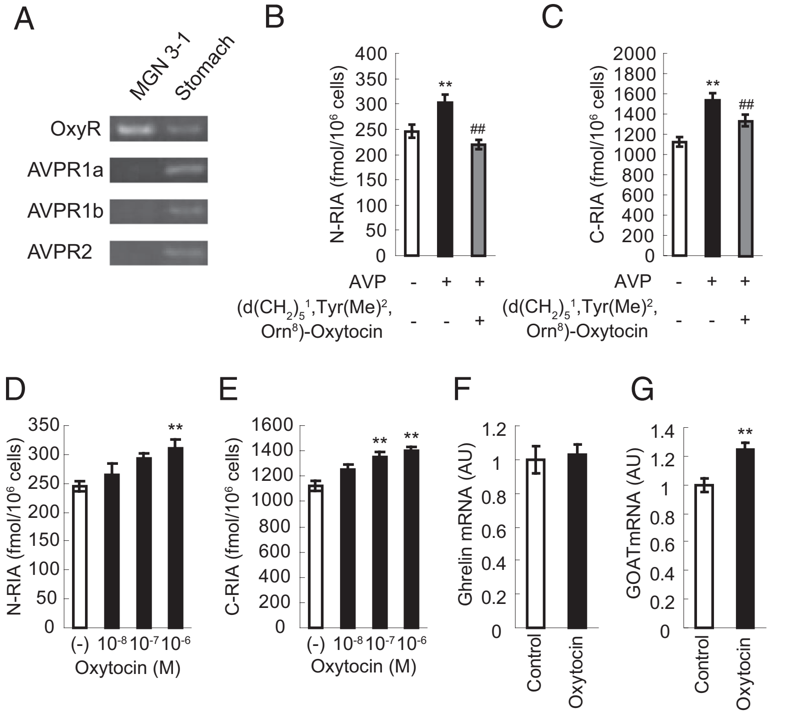

FIG. 2. The effect of oxytocin on ghrelin secretion by MGN3-1 cells. A, RT-PCR analysis of oxytocin receptor (Oxy-R) and vasopressin receptors (AVPR) 1a, 1b, and 2 mRNA expression in MGN3-1 cells. B and C, The amount of ghrelin secreted by MGN3-1 cells incubated for 4 h in DMEM supplemented with 0.5% BSA and 10^{-6} M AVP with or without 10^{-6} M [d(CH_2)$_5$^{1},Tyr(Me)^2,Orn^8]-oxytocin (oxytocin receptor antagonist). **, $P < 0.01$ in comparison with controls; ##, $P < 0.01$ in comparison with AVP (n = 9). 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^{-8} to 10^{-6} M oxytocin. **, $P < 0.01$ in comparison with controls (−) (n = 9). F and G, Ghrelin and GOAT mRNA levels in MGN3-1 cells after a 24-h incubation with 10^{-6} M oxytocin. **, $P < 0.01$ in comparison to controls (n = 9). AU, Arbitrary unit.

Statistical analysis

All values were expressed as the means ± SE. The statistical significance of the differences in mean values was assessed by ANOVA with a *post hoc* test (Turkey's test) or Student's *t* test as appropriate. Differences with $P < 0.05$ were considered significant. Statistical analysis was performed by Statcel2 (OMS, Saitama, Japan).

Results

Effects of peptide hormones on ghrelin secretion

First, we examined the effects of various peptide hormones on ghrelin secretion by MGN3-1 cells. Oxytocin and vasopressin significantly stimulated ghrelin secretion by MGN3-1 cells, whereas insulin and somatostatin suppressed the secretion as reported previously (12) (Fig. 1, A and B). Addition of any of the other peptides, including GH, GHRP2, IGF-I, glucagon, PP, gastrin, CCK, GLP-1, VIP, GIP, secretin, neurotensin, calcitonin, thyroxin, leptin, ANP, or CNP to the medium had no effect on ghrelin secretion (Fig. 1, A and B).

MGN3-1 cells expressed mRNA encoding the oxytocin receptor but did not express mRNA for any subtypes of vasopressin receptors (types 1a, 1b, and 2; Fig. 2A), indicating that the stimulatory effect of vasopressin is likely secondary to a cross-reaction to the oxytocin receptor. Actually, addition of oxytocin receptor antagonist [d(CH_2)$_5$^{1},Tyr(Me)^2,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 (ED_{50} 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-acyltransferase (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 (ED_{50} 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 denopamine 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 (ED_{50} value for N-RIA: 24.7 μM; C-RIA: 40.6 μM; Fig. 5, A and B). MGN3-1 cells expressed

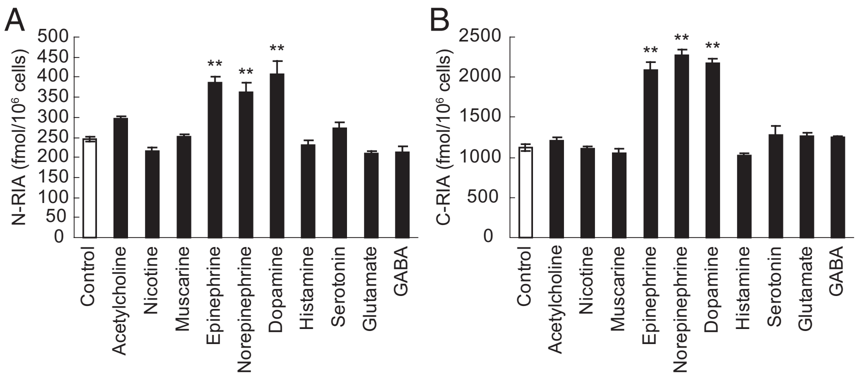

FIG. 3. The effects of neurotransmitters on ghrelin secretion by MGN3-1 cells. A and B, The amount of ghrelin secreted by MGN3-1 cells incubated for 4 h in DMEM supplemented with 0.5% BSA and 10^{-4} M acetylcholine, nicotine, muscarine, epinephrine, norepinephrine, dopamine, histamine, serotonin, glutamate,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

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 effect (Fig. 5, D and E). Addition of D1 receptor antagonist SKF83566 significantly attenuated the stimulatory effect of dopamine on ghrelin secretion (Fig. 5, F and G). These results indicate that the stimulatory effect of dopamine on ghrelin secretion is mediated by the D1a receptor. Apomorphine had no effect on ghrelin or GOAT mRNA levels in MGN3-1 cells (Fig. 5, H and I).

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,

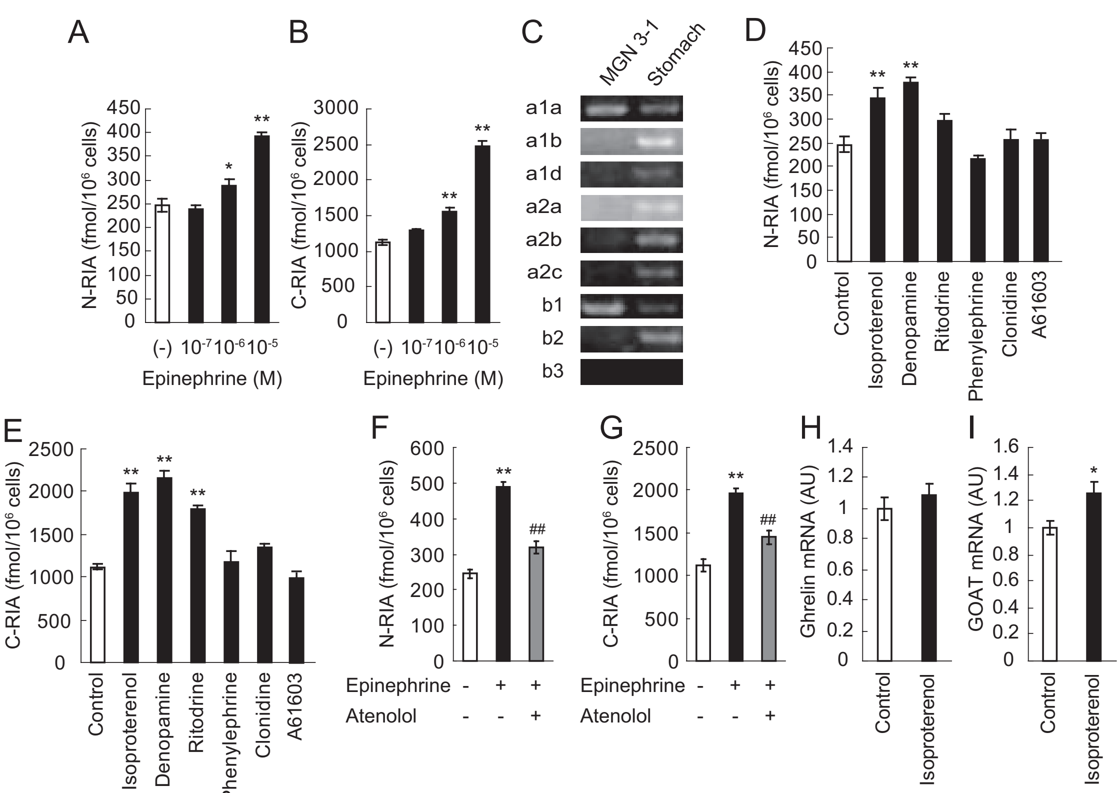

FIG. 4. The effects of epinephrine on ghrelin secretion by MGN3-1 cells. A and B, The amount of ghrelin secreted by MGN3-1 cells incubated for 4 h in DMEM supplemented with 0.5% BSA and 10^{-7} to 10^{-5} M epinephrine. **, $P < 0.05$, **, $P < 0.01$ in comparison with controls (−) (n = 9). C, RT-PCR analysis of adrenergic receptors-α1a, -α1b, -α1d, -α2a-c, and -β1–3 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 (β-agonist), denopamine (β1-agonist), ritodrine (β2-agonist), phenylephrine (α1-agonist), clonidine (α2-agonist), or A61603 (α1a-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 (β1-antagonist). **, $P < 0.01$ 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.

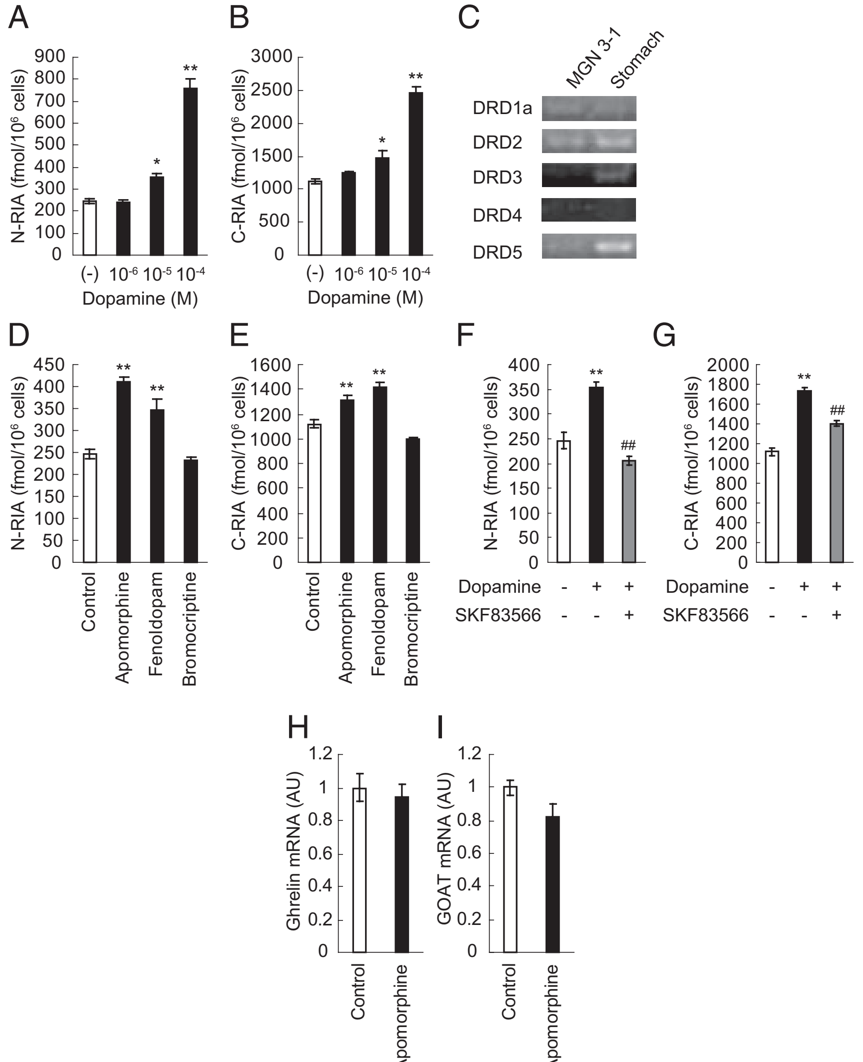

FIG. 5. The effect of dopamine on ghrelin secretion by MGN3-1 cells. A and B, The amount of ghrelin secreted by MGN3-1 cells incubated for 4 h in DMEM supplemented with 0.5% BSA and 10^{-6} to 10^{-4} M of dopamine. *, $P < 0.05$, **, $P < 0.01$ in comparison with controls (−) (n = 9). C, RT-PCR analysis 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^{-5} 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^{-4} 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.

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 nonapeptide 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. Hosoda 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.

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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Disclosure Summary: All authors have nothing to declare.

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