strated impaired glucose tolerance and reduced insulin sensitivity, although there were no differences in body weight or composition between Trp^3-ghrelin-Tg and non-Tg mice. When ghrelin-receptor-null mice were maintained on long-term standard chow, they had lower blood glucose levels with low-to-normal insulin levels in comparison with wild-type mice, although they exhibited similar body weights and composition (14). Ghrelin-receptor-null mice appeared to have enhanced insulin sensitivity in comparison with wild-type mice. In addition, Gauna *et al.* (18) demonstrated that administration of ghrelin to wild-type mice reduced insulin sensitivity. It was also reported that ghrelin inhibited glucose-stimulated insulin release (19–21).

In conclusion, we succeeded in generating Tg mice overexpressing a ghrelin analog. The mice presented in this study will serve as a useful tool for evaluating the long-term effects of ghrelin or ghrelin analogs. In addition, the method provided in this study may be useful in the generation of gain-of-function models for hormones that require posttranscriptional modification.

Acknowledgment

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This work was supported by grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan and from the Ministry of Health, Labor, and Welfare of Japan; and by research grants from the Program for the Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation.

Disclosure Summary: The authors have nothing to disclose.

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MINIREVIEW

Natriuretic peptide system: an overview of studies using genetically engineered animal models

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Keywords
bone; cardiac hypertrophy; guanylyl cyclase; hypertension; natriuretic peptide

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(Received 16 August 2010, revised 11 March 2011, accepted 1 April 2011)

doi:10.1111/j.1742-4658.2011.08116.x

The mammalian natriuretic peptide system, consisting of at least three ligands and three receptors, plays critical roles in health and disease. Examination of genetically engineered animal models has suggested the significance of the natriuretic peptide system in cardiovascular, renal and skeletal homeostasis. The present review focuses on the *in vivo* roles of the natriuretic peptide system as demonstrated in transgenic and knockout animal models.

Natriuretic peptides

The existence of an atrial factor with diuretic and natriuretic activities has been postulated since 1981 [1]. In 1983–1984, the isolation and purification of such a factor and determination of its amino acid sequence were accomplished in rats and humans [2–7]. The factor is a peptide distributed mainly in the right and left cardiac atria within granules of myocytes and thus called atrial natriuretic factor or atrial natriuretic peptide (ANP). The discovery of ANP revealed that the heart is not only a mechanical pump driving the circulation of blood but also an endocrine organ regulating the cardiovascular–renal system. For instance, in situations of excessive fluid volume, cardiac ANP secretion is stimulated, which causes vasodilatation, increased renal glomerular filtration and salt/water excretion and inhibition of aldosterone release from the adrenal gland, which collectively result in a reduction of body fluid volume.

Later, in 1988, a homologous peptide with similar biological activities was isolated from porcine brain and hence was named brain natriuretic peptide (BNP) [8]. However, it was soon found that brain BNP levels were much lower in other species. It has since been shown that BNP is mainly produced and secreted by the heart ventricles [9]. Synthesis and secretion of BNP are regulated differently from ANP [10], and the plasma concentration of BNP has been found to reflect the severity of heart failure more closely than ANP [11].

In 1990, yet another type of natriuretic peptide was isolated from porcine brain and named C-type

Abbreviations
ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; CNP, C-type natriuretic peptide; GC, guanylyl cyclase; MCIP1, myocyte-enriched calcineurin-interacting protein; PAR, protease-activated receptor; PKG, cGMP-dependent protein kinase; RGS, regulator of G-protein signaling.

natriuretic peptide (CNP) [12]. CNP was initially thought to function only in the brain but was later shown to be produced in peripheral tissues such as the vascular endothelium [13] and in smooth muscle cells and macrophages [14]. Because CNP plasma levels are considerably lower than those of ANP or BNP, CNP is thought to mainly act locally as a paracrine factor rather than as a circulating hormone.

Natriuretic peptide receptors

To date, three receptors for natriuretic peptides have been identified. In 1988, one type of ANP receptor was isolated from cultured vascular smooth muscle cells. Using its partial amino acid sequence, the full-length cDNA was cloned and the entire amino acid sequence was deduced [15]. The receptor molecule consists of 496 amino acid residues and contains a large extracellular domain, a putative single transmembrane helix and a 37 amino acid residue cytoplasmic domain. It is generally accepted that the role of this receptor is to bind and remove natriuretic peptides and their fragments from the circulation. Hence, this receptor is termed natriuretic peptide clearance receptor (C receptor). On the other hand, a signaling role of the C receptor has also been suggested [16].

One of the earliest events following the binding of ANP to its receptor is increase in the cytosolic cyclic guanosine monophosphate (cGMP) levels. This finding suggested that cGMP might act as the second messenger mediating the physiological activities of ANP and that the ANP receptor is coupled to guanylyl cyclase (GC), the enzyme that catalyzes the generation of cGMP. In 1989, a segment of the sea urchin GC cDNA was used as a probe to screen various cDNA libraries, which enabled cloning of the first mammalian GC (thus called GC-A) from rats and humans [17]. Expression of the cloned enzyme confirmed that GC-A is an ANP receptor. Soon after the discovery of GC-A, cloning of a second mammalian GC (GC-B) was reported [18,19]. GC-B also bound and was activated by natriuretic peptides, demonstrating the diversity within the natriuretic peptide receptor family. Since these receptor proteins were first identified as GC family members, we refer to them as GC-A or GC-B throughout this paper.

Ligand selectivity

Subsequent studies revealed that GC-A preferentially binds and responds to ANP, while GC-B preferentially responds to CNP [20]. The relative effectiveness of the three natriuretic peptides in stimulating cGMP production via GC-A and GC-B has been reported [21]. The rank order of potency for cGMP production via the GC-A receptor was ANP ≥ BNP > > CNP. On the other hand, cGMP response via GC-B was CNP > ANP or BNP. Thus, the biological functions of natriuretic peptides are mediated by two receptors: GC-A (also known as the A-type natriuretic peptide receptor, NPRA), which is selective for the cardiac peptides ANP and BNP, and GC-B (also called the B-type natriuretic peptide receptor, NPRB), which is selective for CNP.

The binding affinities of ANP, BNP and CNP to the human or rat C receptor have been reported [21]. Irrespective of the species examined, the rank order of affinity for the C receptor was ANP > CNP > BNP. This finding suggests that BNP is the least susceptible to C-receptor-mediated clearance and is more stable in the plasma.

Lessons from genetically engineered animals

A variety of genetically engineered mice have been generated to study the physiological function of each component of the natriuretic peptide–receptor system (summarized in Table 1).

Role of ANP- and BNP-mediated GC-A signaling in blood pressure regulation

Transgenic animals, which constitutively express a fusion gene consisting of the transthyretin promoter and the *ANP* gene, have plasma ANP levels that are higher than non-transgenic littermates by 5–10 fold [22]. The mean arterial pressure in the transgenic animals was reduced by 24 mmHg, which was accompanied by a 27% reduction in total heart weight. This chronic reduction in blood pressure was due to a 21% reduction in total peripheral resistance, whereas cardiac output, stroke volume and heart rate were not significantly altered. In 1994, transgenic mice carrying the human serum amyloid P component/mouse *BNP* fusion gene were generated so that the hormone expression is targeted to the liver [23]. The animals exhibited 10- to 100-fold increase in plasma BNP concentration and significantly lower blood pressure than their non-transgenic littermates.

In 1995, ANP-deficient mice were generated, and their blood pressure phenotype was reported [24]. The mutant mice (homozygous null for the *ANP* gene) had no circulating or atrial ANP, and their blood pressures were significantly higher (8–23 mmHg) than the control mice when they were fed standard diets. When fed

Table 1. Phenotypes of the genetically engineered animals for the natriuretic peptide system.

Mutated gene	Targeting construct	Targeted tissue	Blood pressure phenotype	Cardiac phenotype	Other phenotypes
ANP overexpression [22]	Mouse transthyretin promoter/mouse *ANP* fusion gene	Liver	~ 25 mmHg lower than the control	27% reduction in heart weight	Plasma ANP elevated 8-fold or more; 21% reduction in peripheral resistance
ANP knockout [24]	11 bp in exon-2 replaced with the neomycin resistance gene	Systemic disruption	Increase, 8–23 mmHg (homozygotes); normal on standard diet; 27 mmHg increase on high-salt diet (heterozygotes)	Heart to body weight ratio 1.4-fold higher than the wild-type	Heterozygotes have normal level of circulating ANP
BNP overexpression [23]	Human serum amyloid P component/mouse *BNP* fusion gene	Liver	~ 20 mmHg lower than non-transgenic littermates	~ 30% less heart weight than non-transgenic littermates	10- to 100- fold increase in plasma BNP concentration; skeletal overgrowth
BNP knockout [31]	Exons 1 and 2 replaced with the neomycin resistance gene	Systemic disruption	No signs of systemic hypertension	No signs of ventricular hypertrophy; pressure-overload-induced focal ventricular fibrosis	
CNP overexpression in the cartilage [63]	Col2a1 promoter region/mouse *CNP* fusion gene	Growth plate cartilage	Not reported	Not reported	Longitudinal overgrowth of bones (limbs, vertebrae, skull)
CNP overexpression in the liver [64]	Human serum amyloid P component/mouse *CNP* fusion gene	Liver	Systolic blood pressure unaffected	Heart weight unaffected	Elongation of cartilage bones; plasma CNP level is 84% higher than control
CNP overexpression in the heart [65]	*CNP* gene fused downstream of the murine α-myosin heavy chain promoter	Heart	No change	No change at baseline	Ventricular hypertrophy after myocardial infarction is prevented
CNP knockout (Kyoto) [59]	Exons 1 and 2 encoding CNP replaced with the neomycin resistance gene	Systemic disruption	Not reported	Not reported	Severe dwarfism: impaired endochondral ossification; impaired nociceptive neurons [62]
CNP knockout (Berlin) [66]	Exon 1 replaced with a lacZ expression cassette	Systemic disruption	Not reported	Not reported	Lack of bifurcation of sensory axons in the embryonic dorsal root entry zone
GC-A knock-in overexpression [27]	Entire *GC-A* gene duplicated with the neomycin resistance gene in between	Systemic overexpression	Average 5.2 mmHg below normal in F1 mice carrying three copies of the *GC-A* gene	No effect on heart weights	
GC-A overexpression in the heart [39]	*GC-A* gene fused downstream of murine α-myosin heavy chain promoter	Heart	Normal blood pressure	Heart weight to body weight ratio was significantly less by ~ 15%	

Table 1. (Continued).

Mutated gene	Targeting construct	Targeted tissue	Blood pressure phenotype	Cardiac phenotype	Other phenotypes
GC-A knockout (Dallas) [25]	Neomycin resistance gene inserted in exon 4, which encodes the transmembrane domain	Systemic disruption	Systolic blood pressure is 20–25 mmHg higher than wild-type	Global cardiac hypertrophy (40–60% increase in heart weight); cardiac contractility similar to that in wild-type mice	Rapid increases in urine output, urinary sodium and cGMP excretion after plasma volume expansion are abolished; increased susceptibility to hypoxia-induced pulmonary hypertension
GC-A knockout (North Carolina) [26]	Exon 1, intron 1 and a portion of exon 2 were replaced with the neomycin resistance gene	Systemic disruption	16 mmHg higher than the control	Heart to body weight ratio averaging185% (male) and 133% (female) of wild-type	Sudden death, with morphological evidence indicative of congestive heart failure or of aortic dissection; resistant to LPS-induced fall in blood pressure
GC-A conditional knockout	Targeting vector contains exons 1–13 and an additional 3.8 kb of the 5′ sequence of the *GC-A* gene, a loxP-flanked neomycin resistance cassette (at −2.6 kb of exon 1) and a third loxP site in the middle of intron 1	Cardiomyocytes (by crossing with cardiac α-myosin heavy chain promoter Cre mice) [43]	7–10 mmHg below normal (due to increased secretion of cardiac natriuretic peptides)	20% increase in heart to body weight ratio compared with floxed GC-A mice; ventricular collagen fractions unaffected; preserved cardiac contractility; decreased cardiac relaxation; markedly impaired cardiac function after pressure overload	~ 2-fold increase in plasma ANP concentration
		Smooth muscle cells (by crossing with SM22-Cre mice) [33]	Normal; acute effect of exogenous ANP on blood pressure abolished	Heart weight and heart to body weight ratio are not different from wild-type	Exaggerated blood pressure response to acute plasma volume expansion; higher vasodilatation sensitivity to nitric oxide and enhanced expression of soluble guanylyl cyclase
		Vascular endothelial cells (by crossing with Tie2 promoter/enhancer Cre mice) [32]	Elevated systolic blood pressure by 12–15 mmHg	~ 20% increase in heart weight	Plasma volume is increased by 11–13%; increased vascular permeability in response to ANP is abolished
GC-B dominant negative overexpression in rat [67]	Dominant-negative mutant for GC-B was fused with the CMV promoter	Whole body	No significant differences in systolic, diastolic and mean arterial pressure	Progressive cardiac hypertrophy, which was further enhanced in chronic volume overload	Reduced bone growth; modestly increased heart rate

Table 1. (Continued).

Mutated gene	Targeting construct	Targeted tissue	Blood pressure phenotype	Cardiac phenotype	Other phenotypes
GC-B dominant negative overexpression in mouse [60]	Dominant-negative mutant for GC-B, fused with promoter/enhancer regions of murine pro-α 1(II) collagen gene (Col2a1)	Cartilage	Not reported	Not reported	Significantly shorter nasoanal length
GC-B knockout [60]	Exons 3–7, encoding the C-terminal half of the extracellular ligand-binding domain and the transmembrane segment, were replaced by the neomycin resistance gene	Systemic disruption	No significant differences in blood pressure	Not reported	Impaired endochondral ossification, longitudinal vertebra or limb-bone growth; female infertility; impaired female reproductive tract development
C receptor knockout [28]	Most of exon 1 was replaced by the neomycin resistance gene	Systemic disruption	8 mmHg below normal	Not reported	Longer half-life of circulating ANP; reduced ability to concentrate urine; skeletal deformities with increased bone turnover

a standard-salt (0.5% NaCl) diet, the heterozygotes had normal circulating ANP levels and blood pressures. However, on high-salt (8% NaCl) diets, they were hypertensive, with 27 mmHg increases in systolic blood pressure levels [24].

In the same year, disruption of the *GC-A* gene was reported to result in chronically elevated blood pressure (about 25 mmHg in systolic pressure) in mice on a standard-salt diet [25]. Unlike mice heterozygous for the *ANP* gene, blood pressures of GC-A heterozygotes remained elevated and unchanged despite increasing dietary salt intake. In 1997, another group reported that the mice lacking functional *Npr1* gene, which encodes GC-A (denominated NPRA by the authors), displayed elevated blood pressure and cardiac hypertrophy with interstitial fibrosis resembling that seen in human hypertensive heart disease [26]. In a subsequent paper, the blood pressures of one-copy F1 animals were reported to be significantly higher on high-salt diet than on low-salt diet [27]. The reason for the discrepancy between the salt phenotypes of these two *GC-A* knockout mouse strains is still unknown. It is possible that differences result from different targeting strategies or the genetic background of the mouse strains used.

In 1999, the generation of mice in which the C receptor was inactivated by homologous recombination was reported [28]. C-receptor-deficient mice have less ability to concentrate urine, exhibit mild diuresis and tend to have depleted blood volume. C receptor homozygous mutants have significantly lower blood pressures (by 8 mmHg) than their wild-type counterparts. The half-life of ANP in C-receptor-deficient mice is two-thirds longer than that in wild-type mice, demonstrating that C receptor plays a significant role in its clearance. Moreover, C receptor modulates the availability of the natriuretic peptides to their target organs, thereby allowing the activity of the natriuretic peptide system to be tailored to specific local needs. In fact, C receptor expression is tightly regulated by other signaling molecules, such as angiotensin II [29] and catecholamines [30]. Interestingly, the baseline levels of ANP and BNP were not higher in the C-receptor-deficient mice than in the wild-type mice, implying that either the cardiac secretion or C-receptor-independent clearance mechanism was altered in those mice.

In 2000, the targeted disruption of the *BNP* gene in mice was reported. Multifocal fibrotic lesions were found in the ventricles of BNP-deficient mice, suggesting the protective role of BNP in pathological cardiac fibrosis [31]. Interestingly, there were no signs of systemic hypertension or ventricular hypertrophy, suggesting that in the presence of ANP basal levels of BNP are dispensable for these cardiovascular phenotypes.

To examine the tissue(s) responsible for the hypertensive phenotype of systemic GC-A-null mice, a targeting strategy was designed so that Cre recombinase mediates the deletion of exon 1 of the *GC-A* gene. Thus, in floxed GC-A mice, GC-A can be deleted in a tissue-specific manner. Endothelium-specific deletion of GC-A was achieved by crossing the floxed GC-A mice with transgenic mice expressing Cre recombinase under the control of the Tie2 promoter/enhancer. Endothelium-specific GC-A-deficient mice display significantly increased systolic blood pressure (by approximately 12–15 mmHg) and diastolic blood pressure (by approximately 5–10 mmHg) than their control littermates [32]. Interestingly, although the direct vasodilatation effects of exogenously administered ANP were abolished, smooth-muscle-cell-restricted deletion of GC-A did not affect the resting blood pressure [33], indicating that endothelial cell GC-A, and not vascular smooth muscle cell GC-A, is indispensable for chronic regulation of blood pressure.

Overall, these results show the significance of the endogenous natriuretic peptide system in the maintenance of normal blood pressure.

Regulation of blood volume

Infusion of ANP results in substantial natriuresis and diuresis in wild-type mice but fails to cause significant changes in sodium excretion or urine output in GC-A-deficient mice, indicating that GC-A is essential for ANP-induced acute regulation of diuresis and natriuresis [34]. After experimental expansion of the plasma volume, urine output as well as urinary sodium and cGMP excretion increase rapidly and markedly in the wild-type but not in systemic GC-A-deficient animals. Nevertheless, plasma ANP levels are comparable or even higher in CG-C-deficient animals [34]. On the contrary, the knock-in overexpression of GC-A (four-copy) in mice results in augmented responses to volume expansion in urinary flow and sodium excretion along with rises in both glomerular filtration rate and renal plasma flow, compared with wild-type (two-copy) mice after volume expansion [35]. These results establish that GC-A activation is the predominant mechanism mediating the natriuretic, diuretic and renal hemodynamic responses to acute blood volume expansion.

The plasma volumes of animals completely lacking GC-A are expanded by 30%, suggesting the role of GC-A in chronic regulation of the blood volume. Interestingly, mice lacking GC-A specifically in the vascular endothelium are volume expanded by 11–13% [32], suggesting that GC-A in the endothelium at least partly accounts for chronic blood volume regulatory effects. Since previous experiments indicated that ANP increased capillary permeability of the endothelium to macromolecules like albumin [36], these data suggest that the ANP/GC-A pathway regulates chronic transvascular fluid balance by increasing microvascular permeability [37].

Cardiac remodeling and the local natriuretic peptide system

Cardiac synthesis and secretion of ANP and BNP are increased according to the severity of cardiac remodeling in humans as well as in animal models [38]. Since the two cardiac natriuretic peptides share a common receptor (i.e. GC-A), the cardiac phenotype of mice lacking GC-A revealed complete effects of the cardiac natriuretic peptide signaling. Notably, targeted deletion of the *GC-A* gene resulted in marked cardiac hypertrophy and fibrosis, which were disproportionately severe [39,40] given the modest rise in blood pressure [25]. Since the chronic treatment of GC-A-deficient mice with anti-hypertensive drugs, which reduce blood pressure to levels similar to those seen in wild-type mice, has no significant effect on cardiac hypertrophy [41], these results imply that the natriuretic peptides/GC-A system has direct anti-hypertrophic effects in the heart, which are independent of its roles in blood pressure and body fluid control.

More direct evidence of local anti-hypertrophic GC-A signaling was obtained from animals in which the *GC-A* gene was conditionally targeted. The *GC-A* gene was selectively overexpressed in the cardiomyocytes of wild-type or *GC-A*-null animals, and the effects were examined [39]. Whereas introduction of the *GC-A* transgene did not alter blood pressure or heart rate as a function of genotype, it did reduce cardiomyocyte size in both wild-type and null backgrounds. The reduction in myocyte size was accompanied by a decrease in cardiac ANP mRNA expression, which suggests the existence of a local regulatory mechanism that governs cardiomyocyte size and gene expression via a GC-A-mediated pathway [42]. Conversely, the *GC-A* gene was inactivated selectively in cardiomyocytes by homologous loxP/Cre-mediated recombination, which circumvents the systemic hypertensive phenotype associated with germline disruption of the *GC-A* gene [43]. Mice with cardiomyocyte-restricted GC-A deletion exhibited mild cardiac hypertrophy with markedly increased transcription of cardiac hypertrophy markers, including ANP. These observations are consistent with the idea that a local function of the ANP/GC-A system is to moderate the molecular program of cardiac hypertrophy [44].

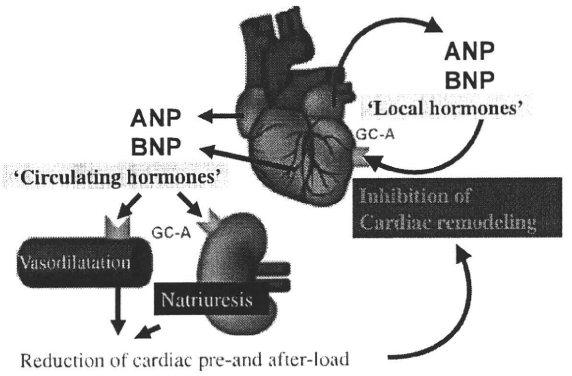

Fig. 1. ANP and BNP, the cardiac natriuretic peptides, protect the heart in not only an endocrine but also a paracrine fashion. Because ANP and BNP have potent diuretic, natriuretic and vasodilatory actions, augmentation of the ANP and BNP/GC-A signaling leads to a decrease in cardiac pre- and after-load, and their mobilization during cardiac failure is considered one of the compensatory mechanisms activated in response to heart damage. In addition to the hemodynamic effects of their actions as circulating hormones, recent evidence suggests that ANP and BNP also exert local cardioprotective effects by acting as autocrine/paracrine hormones.

Since the diuretic, natriuretic and vasorelaxant activities of ANP and BNP lead to reduction of the cardiac pre- and after-load, these results suggest that the cardiac natriuretic peptides/GC-A signaling exerts its cardioprotective actions in both an endocrine and an autocrine/paracrine fashion. These mechanisms are schematically depicted in Fig. 1.

The molecular mechanism of GC-A-mediated inhibition of cardiac hypertrophy

To identify the molecular mechanism underlying cardiac hypertrophy seen in GC-A-deficient mice, DNA microarrays were used to identify genes upregulated in the hypertrophied heart [45]. Among several genes known to be upregulated in cardiac hypertrophy (e.g. α-skeletal actin, ANP and BNP), it has been found that the expression of the gene encoding myocyte-enriched calcineurin-interacting protein (MCIP1) is also increased. The *MCIP1* gene is reportedly regulated by calcineurin, a critical regulator of cardiac hypertrophy. Thus, it was hypothesized that the calcineurin activity is enhanced in the heart of GC-A-deficient mice. To test this hypothesis, cultured neonatal cardiomyocytes were used to determine whether pharmacological inhibition of GC-A would increase calcineurin activity, which it did not [45]. On the other hand, stimulation of GC-A with ANP inhibited calcineurin activity, suggesting that it is by inhibiting the calcineurin pathway that cardiac GC-A signaling (activated by locally secreted natriuretic peptides) exerts its anti-hypertrophic effects. In fact, chronic treatment with FK506, which in combination with FK506-binding protein inhibits the phosphatase activity of calcineurin, significantly reduces the heart weight to body weight ratio, cardiomyocyte size and collagen volume fraction in GC-A-deficient mice compared with the wild-type mice [45]. A further study using microarray analysis and real-time PCR analysis revealed that, in addition to the calcineurin–nuclear factor of activated T-cells (NFAT) pathway, the calmodulin–CaMK–Hdac–Mef2 and PKC–MAPK–GATA4 pathways may also be involved in the cardiac hypertrophy seen in the GC-A-null mice [46].

Role of regulator of G-protein signaling in CG-A cardioprotective actions

Recently, it has been elegantly demonstrated that cGMP-dependent protein kinase (PKG) Iα attenuates signaling by the thrombin receptor protease-activated receptor (PAR) 1 through direct activation of regulator of G-protein signaling (RGS) 2 [47]. PKG-Iα binds directly to and phosphorylates RGS-2, which significantly increases the GTPase activity of $G\alpha_q$, thereby terminating PAR-1 signaling. Given that cGMP is an intracellular second messenger for natriuretic peptides, RGS might mediate the cardioprotective effect of the GC-A signaling. To test this hypothesis, the role of RGS-4, which is the predominant RGS in cardiomyocytes under physiological conditions, was examined. In cultured cardiomyocytes, ANP stimulated the binding of PKG-Iα to RGS-4 as well as the phosphorylation of RGS-4 and its subsequent association with $G\alpha_q$ [48]. In addition, cardiomyocyte-specific overexpression of RGS-4 in GC-A-null mice significantly rescued the cardiac phenotype of these mice. On the contrary, overexpression of a dominant-negative form of RGS-4 blocked the inhibitory effects of ANP on cardiac hypertrophy [48]. Therefore, GC-A may activate cardiac RGS-4, which then inhibits the activity of $G\alpha_q$ and its downstream hypertrophic effectors. The endogenous cardioprotective mechanism meditated by ANP/BNP, GC-A and RGS-4 is depicted schematically in Fig. 2.

Very recently, PKG activation reflecting chronic inhibition of cGMP-selective phosphodiesterase 5 has been shown to suppress maladaptive cardiac hypertrophy by inhibiting $G\alpha_q$-coupled stimulation, and the effect was not observed in mice lacking RGS-2 [49]. This suggests that RGS2 mediates the cardioprotective actions of PKG in pathological conditions such as

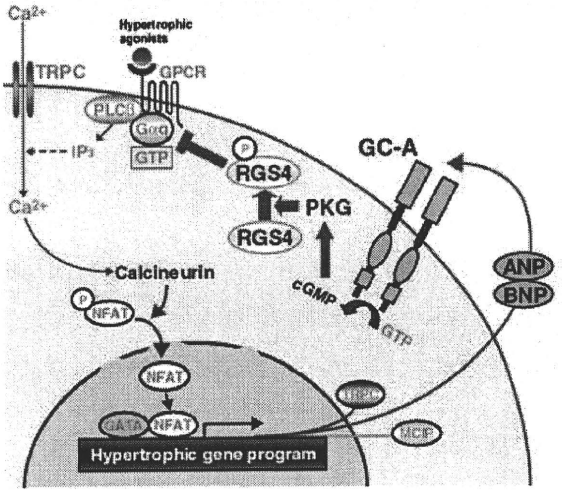

Fig. 2. Inhibitory mechanism of cardiac hypertrophy by the local natriuretic peptide system. Cardiac hypertrophy agonists such as angiotensin II, catecholamines and endothelins stimulate G-protein coupled receptor. Subsequent production of inositol triphosphate (IP3) promotes elevation of intracellular Ca^{2+} levels, which results in activation of the calcineurin/nuclear factor of activated T cells (NFAT) pathway. Cooperatively with the family of GATA transcription factors, NFAT activates the hypertrophic gene program, which includes the ANP- and BNP-coding genes. In an autocrine or paracrine fashion, ANP and BNP stimulate their receptor GC-A and exert their anti-hypertrophic actions via the activation of the RGS, which consequently results in an increase in the GTPase activity of the α subunit of the guanine nucleotide binding protein ($G\alpha_q$) and in a decrease in the activity of the downstream signaling mediators (adapted from [48]).

pressure overload or excessive $G\alpha_q$ activation due to hypertrophic stimuli. In fact, RGS-2 is also implicated in the anti-hypertrophic action of cardiac GC-A [50].

The role of GC-A in myocardial infarction

It is well known that plasma levels of ANP and BNP are dramatically elevated early after myocardial infarction [51]. To examine the significance of this upregulation, experimental myocardial infarction by ligation of the left coronary artery was induced in mice lacking GC-A [52]. GC-A-deficient mice exhibited significantly higher mortality rate than wild-type mice, reflecting a higher incidence of acute heart failure. Four weeks after infarction, left ventricular remodeling, including myocardial hypertrophy and fibrosis, and impairment of the left ventricular systolic function were significantly more severe in mice lacking GC-A than in wild-type mice [52]. GC-A activation by endogenous cardiac natriuretic peptides may protect against acute heart failure and attenuate chronic cardiac remodeling after acute myocardial infarction.

Role of GC-A in peripheral arterial disease

A role of the natriuretic peptide system in peripheral arterial diseases has also been suggested. Activation of the natriuretic peptides–cGMP–PKG pathway was found to accelerate vascular regeneration and blood flow recovery in a murine model of peripheral arterial disease, in which leg ischemia was induced by femoral arterial ligation [53]. Recently, it has been reported that intraperitoneal injection of carperitide, a recombinant human ANP, accelerated blood flow recovery with increasing capillary density in the ischemic legs [54], indicating the role of exogenously administered ANP and BNP in angiogenesis. When the hindlimb ischemia model was performed in GC-A-deficient mice, autoamputation or ulcers were more severe in GC-A-deficient mice than in their wild-type counterparts [55]. Laser Doppler perfusion imaging revealed that the recovery of blood flow in the ischemic limb was significantly inhibited in GC-A-null mice compared with wild-type mice. In addition, vascular regeneration in response to critical hindlimb ischemia was severely impaired [55]. Similar attenuation of ischemic angiogenesis was observed in mice with conditional, endothelial-cell-restricted GC-A deletion. On the other hand, smooth-muscle-cell-restricted GC-A ablation did not affect ischemic neovascularization [56], suggesting that it is the endothelial GC-A that stimulates endothelial regeneration after induction of ischemia. Taken together, the evidence suggests that the natriuretic peptide pathway significantly contributes to peripheral vascular remodeling during ischemia.

Role of the CNP/GC-B pathway in bone formation

In a 1998 study, mice with transgenic overexpression of the *BNP* gene, especially those exhibiting high expression levels, unexpectedly displayed deformed bony skeletons characterized by kyphosis, elongated limbs and paws, and crooked tails, which resulted from a high turnover of endochondral ossification accompanied by overgrowth of the growth plate [57]. Even after crossing with GC-A-null mice, transgenic mice overexpressing BNP continued to exhibit marked longitudinal growth of the vertebrae and long bones [58]. Therefore, the effect of excess amount of BNP on endochondral ossification is independent of GC-A, and so signaling through another receptor was suggested.

In 2001, CNP-deficient mice were reported to show severe dwarfism as a result of impaired endochondral ossification [59], thus indicating that CNP acts locally as a positive regulator of endochondral ossification. In 2004, the phenotype of mice lacking GC-B was reported [60]. The GC-B-null animals exhibited dramatically impaired endochondral ossification and attenuation of longitudinal vertebral or limb bone growth. Therefore, it appears that GC-B is the receptor mediating the CNP action in inducing longitudinal bone growth. Furthermore, homozygous C-receptor-null mice also have skeletal deformities associated with a considerable increase in bone turnover [28], an opposite phenotype to that observed in the mice deficient for CNP. Since CNP is the only natriuretic peptide expressed in bone, it is suggested that one function of the C receptor is to clear locally synthesized CNP from bone and modulate its effects.

Since pharmacological amounts of BNP can stimulate GC-B, these results suggest that activation of the CNP/GC-B pathway in transgenic mice with elevated plasma concentrations of BNP or in mice lacking the C receptor for natriuretic peptides results in skeletal overgrowth. By contrast, inactivation of the CNP/GC-B pathway in mice lacking CNP, GC-B or cGMP-dependent protein kinase II (a downstream mediator of the CNP/GC-B pathway) results in dwarfism caused by defects in endochondral ossification.

Summary

As stated above, studies using genetically engineered animals revealed physiological and pathophysiological roles of the natriuretic peptides/receptor signaling pathways in the regulation of blood pressure/volume, maintenance of the cardiovascular system, and development of the longitudinal bone, acting as not only a circulating hormonal system but also a local regulatory system. Recent evidence also suggests roles for the natriuretic peptide system in renal [61] and neuronal [62] morphology and function. In addition, genetic defects of each component of the system in humans may cause diseases that are also observed in the genetically engineered animals. Furthermore, an interesting hypothesis that needs verification is that these observed phenomena could be the recapitulation of early developmental mechanisms. More studies at tissue, cellular and molecular levels are needed to clarify the mechanisms underlying the intriguing phenotypes observed in transgenic animal models. In addition, more studies at clinical and population levels are needed to elucidate the potential importance of the natriuretic peptide system in humans.

Acknowledgements

Our heartfelt appreciation goes to the late Dr Garbers, a former professor of the University of Texas, whose comments and suggestions were of inestimable value for our study using GC-A knockout mice, to Professor Misono of the University of Nevada School of Medicine, and to the reviewers of the *FEBS Journal*, whose comments significantly contributed to the writing of this review article.

Disclosures

The authors have nothing to disclose.

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Endocrinology. First published ahead of print April 26, 2011 as doi:10.1210/en.2010-1455

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: 0000–0000, 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

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.

doi: 10.1210/en.2010-1455 Received December 20, 2010. Accepted April 6, 2011.

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.

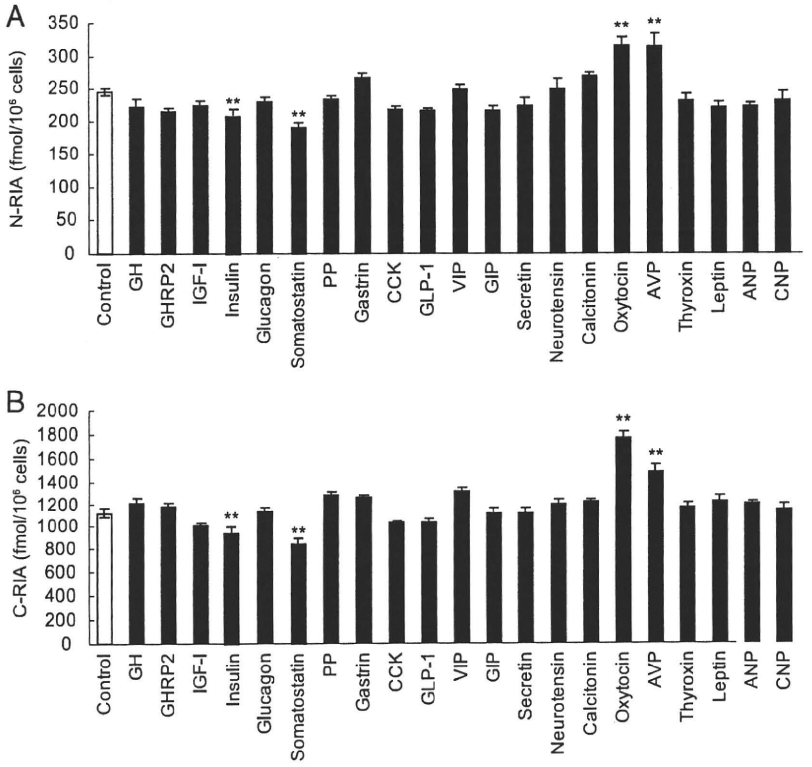

FIG. 1. The effects of peptide hormones on ghrelin secretion by MGN3-1 cells. A and B, The amount of ghrelin secreted by MGN3-1 cells incubated for 4 h in DMEM supplemented with 0.5% BSA and 10^{-6} M GH, GHRP2, IGF-I, insulin, glucagon, somatostatin, PP, gastrin, CCK, GLP-1, VIP, GIP, secretin, neurotensin, 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, 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.

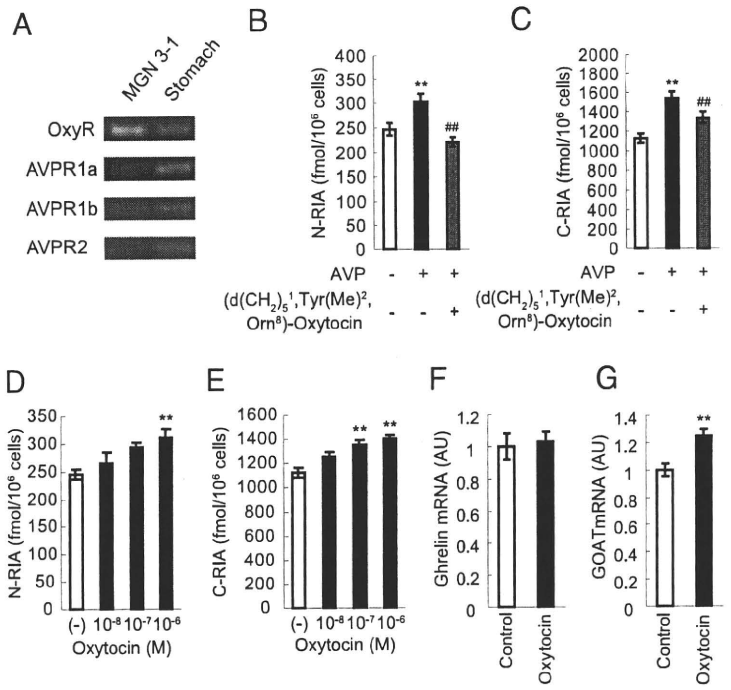

FIG. 2. The effect of oxytocin on ghrelin secretion by MGN3-1 cells. A, RT-PCR analysis of oxytocin receptor (Oxy-R) and vasopressin receptors (AVPR) 1a, 1b, and 2 mRNA expression in MGN3-1 cells. B and C, The amount of ghrelin secreted by MGN3-1 cells incubated for 4 h in DMEM supplemented with 0.5% 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

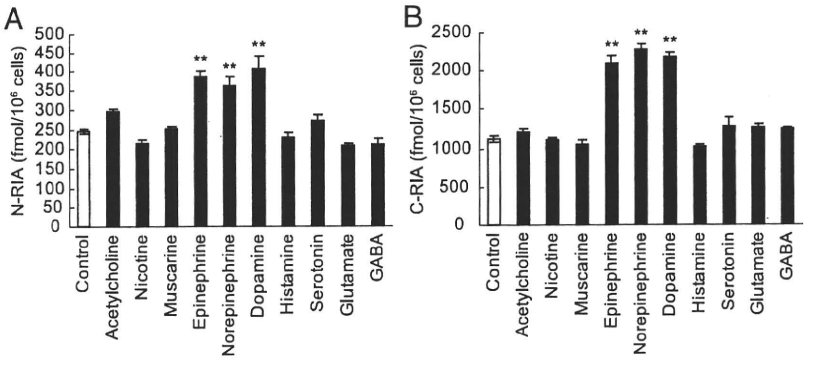

FIG. 3. The effects of neurotransmitters on ghrelin secretion by MGN3-1 cells. A and B, The amount of ghrelin secreted by MGN3-1 cells incubated for 4 h in DMEM supplemented with 0.5% BSA and 10^{-4} M acetylcholine, nicotine, muscarine, epinephrine, norepinephrine, dopamine, histamine, serotonin, glutamate, 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,

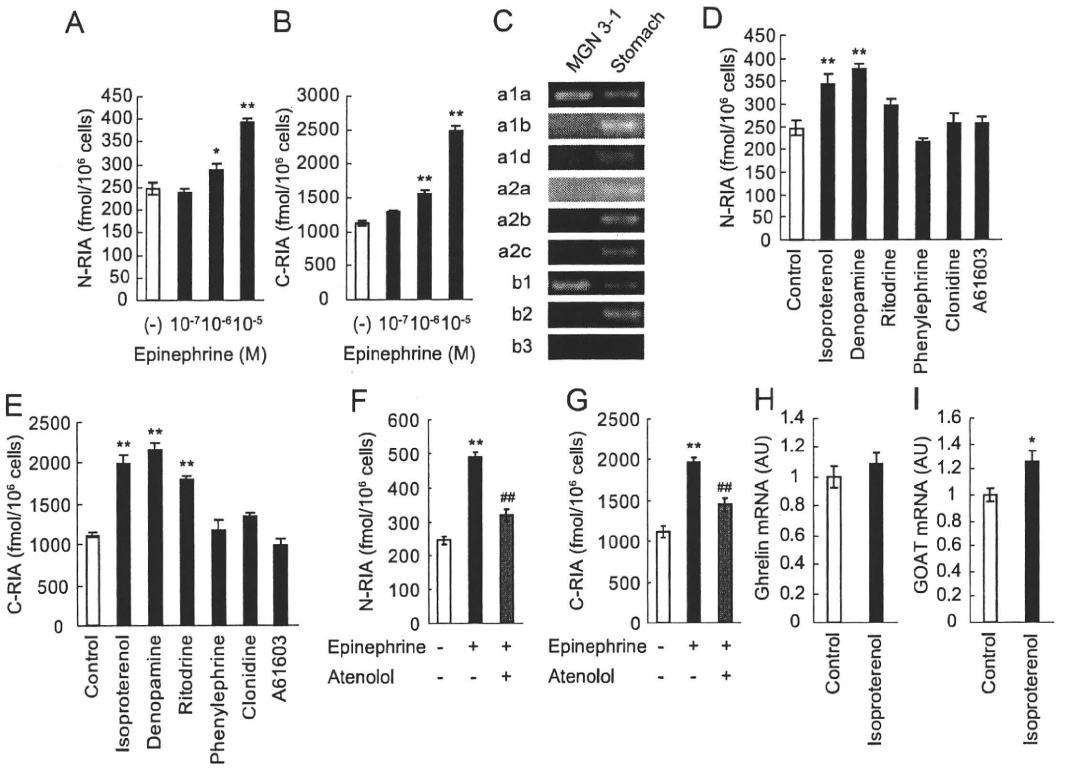

FIG. 4. The effects of epinephrine on ghrelin secretion by MGN3-1 cells. A and B, The amount of ghrelin secreted by MGN3-1 cells incubated for 4 h in DMEM supplemented with 0.5% BSA and 10^{-7} to 10^{-5} M epinephrine. **, $P < 0.05$, **, $P < 0.01$ in comparison with controls (−) (n = 9). C, RT-PCR 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.

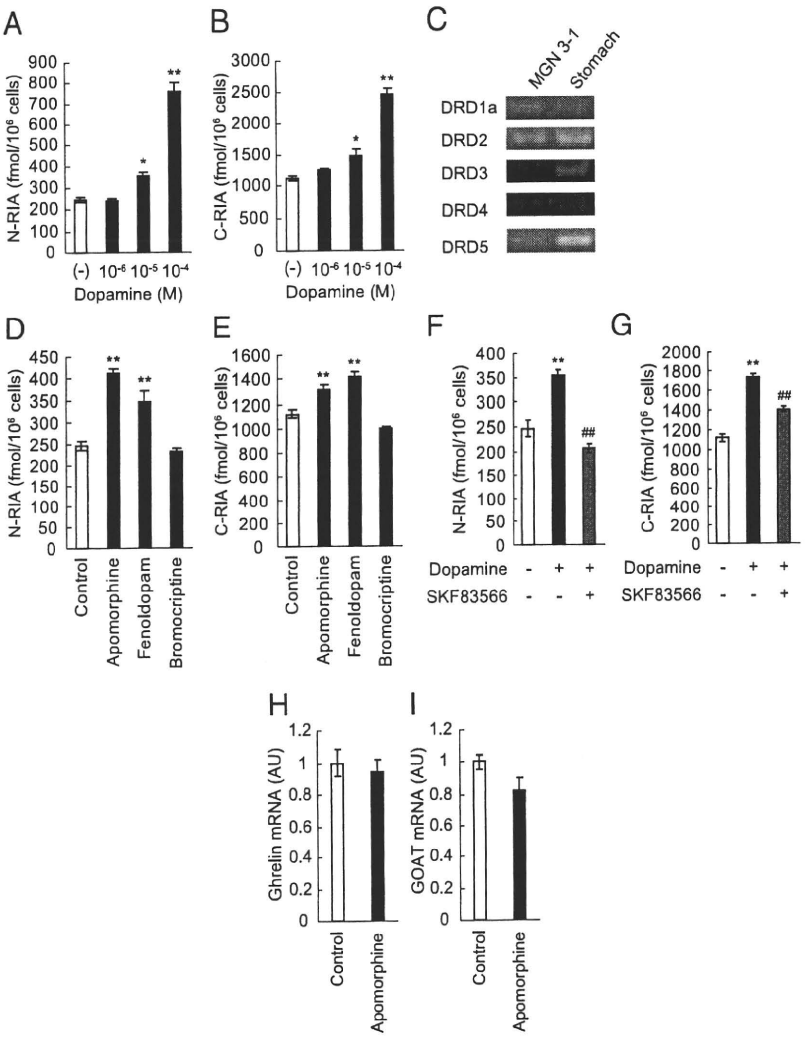

FIG. 5. The effect of dopamine on ghrelin secretion by MGN3-1 cells. A and B, The amount of ghrelin secreted by MGN3-1 cells incubated for 4 h in DMEM supplemented with 0.5% BSA and 10^{-6} to 10^{-4} M of dopamine. *, $P < 0.05$, **, $P < 0.01$ in comparison with controls (−) (n = 9). C, RT-PCR analysis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

Acknowledgments

We thank Ms. Chieko Ishimoto and Ms. Chinami Shiraiwa for their excellent technical assistance.

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This work was supported by grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan and the Ministry of Health, Labor, and Welfare of Japan.

Disclosure Summary: All authors have nothing to declare.

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