Involvement of Adenosine Signaling in Controlling the Release of Ghrelin from the Mouse Stomach

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ABSTRACT

Ghrelin, a potent orexigenic hormone released from the stomach, is important in regulating energy metabolism. Abnormal ghrelin levels are associated with eating disorders and metabolic diseases. However, factors involved in the regulation of ghrelin release remain unclear. Here, we examined the involvement of adenosine signaling in the control of ghrelin release from the perfused mouse stomach. Adenosine stimulated ghrelin release concentration-dependently, and the A2A receptor-selective antagonist 8-(2-carboxyethyl)-1,3-dipropylxanthine blocked this effect. The adenosine deaminase inhibitor erythro-9-(2-hydroxy-3-nonyl)adenine hydrochloride increased ghrelin release in wild-type and A1 receptor knockout mice but not in A2A receptor knockout mice. Colocalization of ghrelin immunoreactivity with A1 and A2A receptor immunoreactivities in the gastric nerve fibers was observed. Colocalization was also detected for ghrelin and A1 receptor immunoreactivities in the gastric mucosa. Blockade of neural activities with tetrodotoxin abolished the stimulatory effect of adenosine on ghrelin release. In conclusion, adenosine exerts predominantly a tonic A2A receptor-mediated stimulatory action on gastric ghrelin release, whereas an A1 receptor-mediated inhibitory action is also apparent when the tonic excitatory effect was removed.

Introduction

The orexigenic hormone ghrelin is localized predominantly in the stomach and plays an important role in regulating food intake, blood glucose levels, and energy expenditure (van der Lely et al., 2004; Badman and Flier, 2005; Kojima and Kangawa, 2005). Abnormal ghrelin levels are associated with various eating disorders, obesity, and diabetes (Stack et al., 2005). In addition, several polymorphisms in the ghrelin gene have been identified to significantly predispose or protect individuals with impaired glucose tolerance from developing diabetes (Pöydöskö et al., 2003; Mager et al., 2006), whereas exogenously administered ghrelin can prevent the onset of diabetes or alleviate the associated symptoms in rodent models (Irako et al., 2006). Although the physiological role and pharmacological potential of ghrelin in correcting energy imbalance are unfolding, the mechanism involved in

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Adenosine is an endogenous purine nucleoside that modulates various physiological functions (Fredholm et al., 2001). Aside from being a key neuromodulator in the nervous system, adenosine can also behave as a signaling molecule for endocrine secretions. Sources of adenosine include direct release from metabolic cells and breakdown of released ATP (Arch and Newsholme, 1978a; Klinger et al., 2002). Adenosine acts on G protein-coupled adenosine receptors, of which there are four structurally and functionally distinct types: \( A_1 \), \( A_{2A} \), \( A_{2B} \), and \( A_3 \). They have been cloned and identified in a variety of organs and tissues including the brain, heart, vasculature, kidneys, and gastrointestinal tract of various species, including humans (Fredholm et al., 2001).

A link between adenosine and ghrelin was suggested by reports indicating that adenosine can act on ghrelin receptors (Smith et al., 2000; Carreira et al., 2004), but later studies have questioned a direct link (Johansson et al., 2005). The abundance of adenosine receptors in the stomach suggests that adenosine may play a role in regulating the release of various gastric peptides (Yip and Kwok, 2004; Yip et al., 2004a,b; Yang et al., 2009). We have demonstrated previously that adenosine stimulates somatostatin release via activation of \( A_{2A} \) receptors in both the rat stomach (Yip and Kwok, 2004) and the mouse stomach (Yang et al., 2009) and inhibits gastrin release via activation of the \( A_1 \) receptors in the rat stomach (Yip et al., 2004b). However, immunohistochemical studies show that not all adenosine \( A_1 \) or \( A_{2A} \) receptors are colocalized with gastrin- and somatostatin-containing cells (Yip and Kwok, 2004; Yip et al., 2004b), suggesting that adenosine may also modulate the release of other gastric peptides.

Therefore, the objective of the present study was to examine the involvement of adenosine signaling in regulating the release of ghrelin in the isolated vascularly perfused mouse stomach. This preparation allows us to examine hormone release from the mouse stomach in situ. Using pharmacological approaches complemented by selective \( A_1 \) and \( A_{2A} \) receptor knockout mice (\( A_1 \)-KO and \( A_{2A} \)-KO, respectively), the involvement of the specific adenosine receptors in the control of ghrelin release was elucidated.

**Materials and Methods**

**Animals.** Mice were treated in accordance with the guidelines of the University of British Columbia Committee on Animal Care. All mice were housed in temperature-controlled rooms with 12-h light/12-h dark cycles. Male CD-1 mice (20–40 g) were obtained from the University of British Columbia Animal Care Center or Charles River Laboratories Inc. (Wilmington, MA). Heterozygous breeding pairs of \( A_{2A} \)-KO mice (Chen et al., 1999) were generously provided by Dr. J.-F. Chen (Boston University, Boston, MA) and Dr. M. A. Schwarzschild (Massachusetts General Hospital/Harvard University, Cambridge, MA). \( A_{2A} \)-KO and wild-type controls (20–40 g) were generated from these heterozygous breeding pairs. \( A_1 \)-KO mice (20–40 g) were also generated from heterozygous breeding pairs (Johansson et al., 2001). The congenic KO mice on C57BL/6 backgrounds were made by backcrossing KO to C57BL/6 mice for more than 10 generations.

**Preparation of Perfused Stomach.** The surgical procedures used for isolation of the mouse stomach for in situ vascular perfusion were described in detail previously (Yang et al., 2009). Mice were deprived of food for at least 12 h before they were anesthetized with an intraperitoneal injection (65 mg/kg) of sodium pentobarbital (Bimeda-MTC Animal Health Inc., Cambridge, ON, Canada). In brief, a midline incision was made in the abdomen to expose the internal organs. Vessels supplying the adrenal glands and kidneys and the superior mesenteric artery were tied off or cut between two ligatures. The spleen and the pancreas were dissected away from the stomach. A duodenal cannula was inserted a few millimeters distal to the pyloric sphincter for drainage of gastric contents. Then the rest of the gut was removed. Perfusate was introduced into the gastric vasculature through a cannula inserted into the aorta with the tip placed adjacent to the celiac artery. The vasculatures were cleared of blood with perfusion of approximately 2 ml of heparin saline (600 U; Sigma-Alrich, St. Louis, MO) before introduction of perfusate. Samples were collected via a portal vein cannula. Both the preparation and perfuse were kept at 37°C by thermostatically controlled units.

The perfusate consisted of Krebs’ solution containing 3% dextrose (clinical grade; Sigma-Aldrich) and 0.2% bovine serum albumin (RIA grade; Sigma-Aldrich). It was continuously gassed with 95% oxygen and 5% carbon dioxide and maintained at a pH of 7.4. Perfusion was delivered into the stomach by a peristaltic pump at 1 ml/min. Five-minute samples were collected via the portal vein cannula after a 30-min equilibration period. Aliquots (0.5 ml) of samples were transferred into ice-cold test tubes containing 0.05 ml of Trayslol (aprotinin; 10,000 KIU/ml; Bayer Inc., Etobicoke, ON, Canada) and stored at –20°C until assayed.

The following drugs were purchased from Sigma-Aldrich: adenosine hemisulfate salt, 2-chloro-N’-cyclopentyladenosine (CCPA), 2-p-(2-carboxyethyl)phenethylamino-5’-N-ethylcarboxamidoadenosine hydrochloride (CGS 21680), 1-deoxy-1-beta-[3H-iodophenyl] methylaminol-9H-purin-9-yl]-N-methyl-1H-nifurofuranuronamide (IB-MECA), 5’-N-ethylcarboxamidoadenosine (NECA), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), sodium nitroprusside, and etrhythm-9-(2-hydroxy-3-nonyl)adenine hydrochloride (EHNA). 4-(2-[7-aminom-[1,2,4] triazolo[2,3-c][1,3,5]triazin-5-y]lamino)ethanol (TMZ) and 3-(2-furanyl)-7-(2-phenethyl)-1H-pyrrozole[4,3-e][1,2,4] triazolo[1,5-c]pyrimidin-5-amine (SCH 58261) were procured from Tocris Bioscience (Ellisville, MO). Tetrodotoxin (TTX) with citrate was purchased from Alomone Labs (Jerusalem, Israel). Adenosine and nitroprusside were dissolved in saline, adenosine analogs were dissolved in small volumes of dimethyl sulfoxide (BDH, Toronto, ON, Canada), and TTX was dissolved in distilled water. All drugs were then diluted with perfusate to achieve the desired infusion concentration and kept in a syringe that was controlled by a separate infusion pump. During the periods of drug administration, the drug syringe was connected to the aortic cannula and thus mixed with the main flow of perfusate entering the stomach preparation. The concentration of the drug solution and the rate of the drug infusion pump were calculated to achieve the desired final drug concentration that reached the stomach. The final concentration of dimethyl sulfoxide in the perfusate was less than 0.5%, which has been shown not to alter gastric peptide release (Yip and Kwok, 2004). Termination of drug infusion was achieved by disconnecting the drug solution syringe from the aortic cannula and stopping the drug infusion pump. Perfusion of 1 μM nitroprusside did not increase the release of gastric ghrelin (data not shown).

The concentrations used for adenosine and its receptor-selective agonists, and EHNA were previously shown to have significant effects on gastric peptide release in mouse and rat stomachs (Kwok et al., 1990; Yip and Kwok, 2004; Yip et al., 2004b; Yang et al., 2009). The concentrations of the adenosine receptor antagonists, ZM 241385 and DPCPX, used in this study have also been shown to abolish the adenosine \( A_{2A} \) and \( A_1 \) receptor-mediated action on peptide release in rodent stomachs, respectively (Yip and Kwok, 2004; Yip et al., 2004b; Yang et al., 2009).

**Radioimmunoassay.** Ghrelin levels were measured by using a specific RIA kit (Phoenix Pharmaceuticals Inc., Burlingame, CA) according to the manufacturer’s instructions. This RIA kit detects total ghrelin content, which includes the acylated and the nonacylated forms.
**Data Analysis.** Previous studies have shown that the basal release of gastric peptides is well maintained, although the release rate of individual animals may vary (Kwok et al., 1990; Yip and Kwok, 2004; Yip et al., 2004b; Yang et al., 2009). Therefore, our results are expressed as mean ± S.E.M. of ghrelin release as a percentage of basal and were calculated as follows: ghrelin release (percentage) = (ghrelin release during a 5-min period (pg/min)) / (ghrelin release during the first 5 min (pg/min)) × 100. Comparing the actions of different drugs, and for analysis of the concentration-dependent response of specific drugs, results are expressed as percentage change, which was calculated as follows: ghrelin release (percentage change) = ((mean ghrelin release during drug perfusion periods) – mean ghrelin release during basal periods) pg/min / mean ghrelin release during basal periods (pg/min) × 100. Statistical data analysis was performed with Prism (version 5.03; GraphPad Software Inc., San Diego, CA).

Repeated-measures analyses of variance (ANOVA) were performed for experiments examining percentage of ghrelin release during 5-min periods followed by the Dunnett’s multiple comparison test when a significant F value was detected. This post hoc test compares each experimental period with the control period immediately before drug administration. One-way ANOVA were performed for analysis of concentration-response relationships with post hoc polynomial contrasts examining linear, quadratic, and cubic effects (Figs. 1B and 2B). One-way ANOVA was performed for the experiments in Figs. 3, B and E, 8E, and 9D where percentage changes of ghrelin release under different treatment conditions were compared. Means were compared by using Tukey’s pairwise multiple comparison test when a significant F value was detected. For all statistical analyses, p ≤ 0.05 is considered to be significant.

**Immunohistochemistry.** Gastric corpus and antrum tissues from male CD-1 mice were prepared for immunohistochemistry as described previously. (Yip and Kwok, 2004). In brief, animals were anesthetized with an intraperitoneal injection (65 mg/kg) of sodium pentobarbital. The stomach was dissected out and cut open along the greater curvature and then rinsed in ice-cold saline solution. The corpus and the antrum were separated and individually fixed overnight in 4% paraformaldehyde and then placed in 20% sucrose solution overnight. Tissues were rapidly frozen with liquid nitrogen and sectioned (40 μm) in a cryostat set at –25°C. Tissue sections were kept in 0.1 M phosphate-buffered saline before serial washing in 50 mM NH4Cl (30 min), 100 mM glycine (10 min), and 1% bovine serum albumin (60 min). Sections were then incubated with the primary antibody solution for 72 h at 4°C and then the secondary antibody solutions overnight at 4°C before being mounted onto glass slides. For double-staining experiments, sections were incubated with both primary antibodies, and then with both secondary antibodies. The specific primary antibodies used were rabbit anti-A1 adenosine receptor (1:250; Sigma-Aldrich), rabbit anti-A2A adenosine receptor (1:250; Novus Biologicals Inc., Littleton, CO), goat (for adenosine receptor colocalization studies; 1:250; Santa Cruz Biotechnology Inc., Santa Cruz, CA), or rabbit for PGP 9.5 colocalization studies; 1:500; BioVision Inc., Mountain View, CA) anti-full form ghrelin, guinea pig anti-PGP 9.5 (1:200; Millipore Corporation, Billerica, MA). Immunoreactivities (IRs) were visualized with specific secondary antibodies conjugated to a fluorescent dye (1:2000; Invitrogen, Carlsbad, CA). For visualization of the adenosine receptor IR, donkey anti-rabbit IgG Alexa Fluor 488 was used, whereas donkey anti-goat IgG Alexa Fluor 594 and donkey anti-rabbit IgG Alexa Fluor 488 were used to visualize ghrelin IR. PGP 9.5 IR was visualized with goat anti-guinea pig IgG Alexa Fluor 594. Specificities of the antibodies were examined by preincubating the primary antibodies with respective control peptides, A1R control peptide (sequence provided by Sigma-Aldrich and synthesized by Nucleic Acid Protein Service Unit, University of British Columbia), A2AR control peptide (MBL International, Woburn, MA), and ghrelin control peptide (Phoenix Pharmaceuticals) for 1 h at room temperature before staining. In addition, controls were carried out by incubating tissue sections with the secondary antibody alone to rule out background nonspecific binding. No IRs for adenosine receptors or ghrelin were observed in both sets of control experiments (data not shown).

**Results**

**Effect of Adenosine on Basal Ghrelin Release.** To examine the effect of adenosine on the release of gastric ghrelin, 10 μM adenosine was perfused into the stomach. Ghrelin release was promptly increased upon the administration of adenosine and returned to basal levels immediately after cessation of adenosine perfusion (Fig. 1A). The basal release rate of ghrelin varied among mice ranging from 388 ± 32 to 432 ± 75 pg/min with a mean basal release rate of all mice at 403 ± 48 pg/min (n = 5). Although there were interanimal differences, the basal release of ghrelin remained relatively constant for each animal with an average percentage change of −1 ± 6%. To determine whether the effect of adenosine is concentration-dependent, two other concentrations (0.1 and 1 μM) were tested. Figure 1B shows the concentration-dependent response of adenosine on ghrelin release. Analysis of variance yields a significant overall difference (F2,11 = 13.3; p ≤ 0.01) and a significant linear concentration-dependent effect (F1,11 = 24.6; p ≤ 0.001), indicating a direct concentration-response relationship.

**Effect of Selective Adenosine Receptor Agonists and Antagonists on Ghrelin Release.** To examine the receptor subtypes involved in the stimulatory action of adenosine on ghrelin release, receptor-selective adenosine analogs were used (Fig. 2A). The A1 receptor-selective agonist CCPA caused an inhibition of ghrelin release, whereas the A3 receptor-selective agonist IB-MECA did not alter ghrelin release. The adenosine agonist NECa stimulated ghrelin release, and this effect was blocked by the A2A receptor-selective antagonist ZM 241385. The A2A receptor-selective agonist CGS 21680 also stimulated ghrelin release, and this effect was abolished by the A2A receptor-selective antagonists ZM 241385 and SCH 58261. The stimulatory effect of CGS 21680 on ghrelin release was further

![Fig. 1](https://example.com/figure1.png) Effect of adenosine on ghrelin release from the isolated perfused mouse stomach. A, adenosine (10 μM) was administered during periods 5 to 7. Each column represents the mean ± S.E.M. of ghrelin release (percentage); n = 5. **,** p ≤ 0.001 compared with period 4 as determined by Dunnett’s test. B, effect of various concentrations of adenosine on ghrelin release; n ≥ 4.
demonstrated to be concentration-dependent (Fig. 2B). A statistical test with analysis of variance yielded a significant overall difference ($F_{4,20} = 5.3; p \leq 0.01$) and a significant linear concentration-dependent effect ($F_{1,20} = 19.5; p \leq 0.001$), showing a direct concentration-response relationship.

To further investigate the specific role of A$_1$ receptors on regulating ghrelin release, the effects of the A$_1$ receptor-selective antagonist DPCPX on adenosine-induced ghrelin release was examined. The stimulatory effect of 10 $\mu$M adenosine was still apparent when 1 $\mu$M DPCPX was concomitantly perfused (Fig. 3A) with the average increases in ghrelin release of 106 $\pm$ 26% (Fig. 3B). These values are not

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Fig. 2. Effect of adenosine analogs on ghrelin release. A, effect of selective adenosine agonists for A$_1$ (CCPA; 0.1 $\mu$M) and A$_3$ (IB-M; IB-MECA; 0.1 $\mu$M) receptors, and the adenosine analog (NECA; 1 $\mu$M) and A$_{2A}$-selective receptor agonist CGS 21680 (CGS; 0.1 $\mu$M), in the presence and absence of the A$_{2A}$ receptor antagonists ZM 241385 (ZM; 1 $\mu$M) and SCH 58261 (SCH; 1 $\mu$M) on ghrelin release. Each column represents the mean $\pm$ S.E.M. of percentage changes in ghrelin release by comparing the release during drug perfusion to basal levels; $n \geq 4$. **, $p \leq 0.01$; ***, $p \leq 0.001$ comparing groups using ANOVA followed by Tukey’s test as indicated by bars. B, effect of various concentrations of CGS 21680 on ghrelin release; $n \geq 5$.

Fig. 3. Effect of selective adenosine receptor antagonists on basal and adenosine-induced gastric ghrelin release. A, C, and D, the effects of DPCPX (A), ZM 241385 (C), and SCH 58261 (D) on Ado-induced ghrelin release were examined. Each column represents the mean $\pm$ S.E.M. of ghrelin release (percentage); $n \geq 5$. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$ comparing with period 3 as determined by Dunnett’s test. B, effects of DPCPX in the presence and absence of 10 $\mu$M adenosine on ghrelin release. E, effects of ZM 241385 and SCH 58261 in the presence and absence of 10 $\mu$M adenosine on ghrelin release. All concentrations indicated are in micromoles. Each column represents the mean $\pm$ S.E.M. of percentage changes of ghrelin release; $n \geq 4$. ††, $p \leq 0.01$; †††, $p \leq 0.001$ comparing groups using ANOVA followed by Tukey’s test as indicated by bars.
significantly different from adenosine perfusion alone (132 ± 26%; Fig. 3B). To confirm the stimulatory role of A2A receptors on ghrelin release, the A2A-selective antagonists ZM 241385 and SCH 58261 were used. Both antagonists abolished the adenosine-mediated increase in ghrelin release (Fig. 3, C and D). Results of the A2A-selective antagonists on adenosine-induced ghrelin release are summarized in Fig. 3E.

**Cellular Distribution and Localization of Adenosine Receptors and Ghrelin.** Immunofluorescent studies were carried out to determine where ghrelin-releasing cells are located in gastric tissues and whether or not they contain A1 and A2A receptors. Ghrelin IR was observed in both the corporal and antral mucosa of the stomach (Fig. 4). Ghrelin IR was also present in the myenteric plexus and the nerve fibers of the muscular layers (Fig. 5). A1 receptor IR colocalized with ghrelin IR in the gastric mucosa (Fig. 4, C and F) and nerve fibers of the corporal and antral stomach (Fig. 5, C and F). Colocalization of ghrelin IR and A2A receptor IR was also observed in the myenteric plexus and nerve fibers of the muscular layers in the corpus and antrum (Fig. 5, I and L), but not in the mucosal layers (Fig. 4, I and L).

To verify that the observed ghrelin IR is in the nerve fibers, we costained corporal and antral muscle sections with ghrelin and the neuronal marker PGP 9.5 (Fig. 6). Colocalization of PGP 9.5 IR and ghrelin IR was observed in the myenteric plexus and the nerve fibers in the muscle layers of both the corpus (Fig. 6C) and the antrum (Fig. 6F).

**Neuronal Component of Adenosine Signaling on Ghrelin Release.** Results from the immunofluorescent studies combined with results from studies using adenosine analogs suggest that adenosine may stimulate ghrelin release via the activation of A2A receptors in the myenteric plexus. To investigate this possibility, the neuronal blocker TTX was used. The perfusion of 1 μM TTX abolished the stimulatory effect of 1 μM adenosine on ghrelin release (Fig. 7).

**Effect of Adenosine and Adenosine Antagonists on Ghrelin Release in A1R-KO and A2AR-KO Mice.** To confirm the roles of A1 and A2A receptors in adenosine-induced ghrelin release, the action of adenosine was examined in both

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**Fig. 4.** Confocal images showing IR of ghrelin, A1 receptor, and A2A receptor in the mucosa of the mouse stomach. Ghrelin IR is stained in red (A, D, G, and J), A1 receptor IR (B and E) or A2A receptor IR (H and K) is stained in green, and yellow in the merged images (C, F, I, and L) indicates colocalization. Colocalization of ghrelin IR and A1 receptor IR is shown in the corpus (C) and antrum mucosa (F). No colocalization of ghrelin IR and A2A receptor IR was observed (I and L).
A1R-KO and A2A-R-KO mouse stomach preparations. The basal release rate of ghrelin in wild-type controls for A1R-KO and A2A-R-KO mice were similar at 573 ± 101 pg/min (n = 10) and 499 ± 52 pg/min (n = 10), respectively. In addition, both lines of animals had been backcrossed with a C57BL/6 background for more than 10 generations. Therefore, in the present study only data from the wild-type controls of the A2AR-KO mice are shown. The basal ghrelin release in A1R-KO mice was 479 ± 106 pg/min (n = 10), which is not significantly different from the wild-type controls. However, the basal ghrelin release in A2A-R-KO mice was 262 ± 52 pg/min (n = 10), which was significantly lower than the wild-type controls. In A1R-KO mice, adenosine (10 μM) enhanced ghrelin release (Fig. 8A) with an average percentage change of 164 ± 34%, but the overall effect was not significantly different from that of the wild-type control mice (Fig. 8E). The adenosine-stimulated ghrelin release in these wild-type controls (181 ± 43%; Fig. 8E) was not significantly different from that observed in CD-1 mice (132 ± 26%; Fig. 1B). However, in the A1R-KO mouse stomach a significant inhibition of ghrelin release was apparent during the concomitant perfusion of the A2A antagonist ZM 241385 and adenosine with an average percentage change of −37 ± 4% (Fig. 8B). In A2A-R-KO mice, adenosine did not stimulate ghrelin release (Fig. 8C). Instead, the release of ghrelin in these animals was inhibited with an average percentage change of −29 ± 7% (Fig. 8E). In addition, this inhibitory effect of adenosine in A2A-R-KO mice was abolished with the simultaneous perfusion of the A1 receptor antagonist DPCPX (Fig. 8, D and E).

Effect of Endogenous Adenosine on Ghrelin Release.
To investigate the role of endogenous adenosine on ghrelin release, the adenosine deaminase inhibitor EHNA was used to increase the endogenous level of adenosine. In wild-type controls, EHNA (1 μM) enhanced ghrelin release in a delayed manner and was significantly elevated after the cessation of EHNA delivery (Fig. 9A). EHNA increased ghrelin release promptly in A1R-KO mice (Fig. 9B). Perfusion of EHNA in A2A-R-KO mice did not cause a significant change in ghrelin release (Fig. 9C). In both wild-type controls and A1R-KO
mice, EHNA augmented gastric ghrelin release with average increases of 24 ± 11 and 44 ± 10%, respectively, whereas it had no significant effect in A2AR-KO mice (−11 ± 7%; Fig. 9D). The different responses observed between wild-type and A1R-KO mice were not statistically significant (Fig. 9D).

Discussion

In the present study, we evaluated the role of adenosine in controlling ghrelin release from the mouse stomach by using both pharmacological tools and selective adenosine receptor knockout mice. Administration of adenosine augmented ghrelin release that was abolished by the A2A receptor antagonists ZM 241385 and SCH 58261. ZM 241385 also blocked the stimulatory effect of NECA. In addition, the A2A-selective agonist CGS 21680 was shown to stimulate ghrelin release concentration-dependently, and this effect could again be abolished by ZM 241385 and SCH 58261. The A1-selective (CCPA) and A3-selective (IB-MECA) agonists did not enhance the release of ghrelin. In fact, in the presence of CCPA, ghrelin release was inhibited. Therefore, adenosine seems to have dual actions on ghrelin release: stimulatory via selective A2A receptor activation and inhibitory via selective A1 receptor activation. This concept is further supported by the studies using antagonists.

The addition of the A1 receptor antagonist (DPCPX) did not enhance the adenosine-stimulated ghrelin release. This observation could be caused by the interactions of the A1 and A2A intracellular signaling pathways. Activation of A2A receptors with adenosine may lead to stimulatory responses that override the effects of the A1 receptor activation or down-regulate the A1 receptors (Dixon et al., 1997; Ralevic and Burnstock, 1998). Therefore, further blockade of the A1 receptors with DPCPX after A2A activation may not result in additional increases in ghrelin release. In addition, the concentration of adenosine used (10 μM) may have already maximally stimulated ghrelin release, thus blocking the inhibitory A1 receptor would not result in further augmentation of ghrelin release. The lack of an effect of perfusing DPCPX alone on basal ghrelin release suggests that the A1 receptors may not play a major role under basal conditions.

Administration of the A2A receptor antagonist ZM 241385 at 1 μM in the absence or presence of exogenous adenosine exerted a significant inhibitory effect on ghrelin release. This suppressive action is probably the result of A2A receptor blockade, thus unmasking the A1 receptor-mediated inhibition of ghrelin release. These studies suggest that there is a basal tone of A2A receptor activation involved in the regulation of ghrelin release. Because ZM 241385 could potentially also block A1B receptor activation, a more selective A2A receptor antagonist, SCH 58261, was also used. SCH 58261 is currently the most selective A2A antagonist available and has thousand folds higher selectivity for A2A receptors than for A2B receptors (Ongini et al., 1999). The blockade of the actions of adenosine by 1 μM SCH 58261 supports the notion that the A2B receptor has a minimal role in the adenosine-induced augmentation of ghrelin release.

Fig. 6. Expression of ghrelin IR in nerve fibers of the mouse gastric muscle layers. Ghrelin IR in green (A and D) and the neuronal marker PGP 9.5 in red (B and E), were stained in the myenteric plexus and nerve fibers of the muscle layers in the mouse corpus (A–C) and the antrum (D–F), and colocalization is indicated in yellow in the merged images (C and F). LM, longitudinal muscle; CM, circular muscle.

Fig. 7. Effect of TTX on the stimulatory effect of Ado on ghrelin release. Effect of 1 μM adenosine in the absence (A) and presence (B) of 1 μM TTX on ghrelin release. Each column represents the mean ± S.E.M. of ghrelin release (percentage); n = 4. * p < 0.05; ** p < 0.01 compared with period 4 (A) or period 3 (B) as determined by Dunnett’s test.
To complement the pharmacological studies, experiments were also carried out in selective adenosine receptor knock-out mice. In A1R-KO mice adenosine augmented ghrelin release, and this action was abolished by the A2A receptor-selective antagonist ZM 241385, thus confirming the pharmacological data demonstrating that the stimulatory effect of adenosine on ghrelin release is via A2A receptor activation. Conversely, adenosine suppressed ghrelin release in A2AR-KO mice. This inhibitory action is probably caused by the effect of A1 receptor activation, because the A1 receptor-selective antagonist DPCPX abolished this response. The proposal for a tonic activation of A2A receptors in regulating ghrelin release is via A2A receptor activation. Coexistence of A1 and A2A receptors with opposing functions in certain cell types has been reported previously (Mills and Gewirtz, 1990; Ramkumar et al., 1991; Olivera and Lopez-Novoa, 1992; Ralevic and Burnstock, 1998; Yip and Kwok, 2004). In addition, heterodimeric complexes involving A1 and A2A receptors with other G protein-coupled receptors have been identified, which could alter the binding affinity to adenosine as well as the intracellular mechanisms after activation (Agnati et al., 2003). The binding affinity to adenosine and abundance within a tissue vary with different adenosine receptor subtypes; therefore, adenosine concentration in the vicinity of the target site, where the receptors are located, may play a role in the differential activation of specific receptors and the particular response. As such, the exact cellular signaling mechanisms involved in adenosine-induced ghrelin release await clarification.

The opposing effects on ghrelin release exhibited by A1 and A2A receptor activation suggests that the secondary messenger cAMP may be involved in this mechanism. This is supported by the prominent effects of cAMP-altering peptides on ghrelin release including somatostatin, glucagon, gastric inhibitory polypeptide, and glucose-dependent insulinotropic polypeptide (GLP-1) (Shimada et al., 2003; Martin et al., 2004).
2005). Somatostatin receptors are coupled to the Gs protein, and its activation has been shown to decrease ghrelin release. Conversely, gastric inhibitory polypeptide and glucagon receptors are coupled to the Gi protein, and their activation leads to increased ghrelin release (Kamegai et al., 2004; Lippl et al., 2004). Although GLP-1 receptors are also coupled to the Gi protein, its activation leads to a decrease in ghrelin release. This could be caused by the stimulated release of inhibitory peptides by GLP-1, including somatostatin, thereby hindering at the complexity of this regulatory system. The interactions between adenosine signaling and these cAMP-modulating peptides on ghrelin release remains unclear. A direct assessment of the cAMP pathway examining adenylyl cyclase activity and cAMP levels in association with ghrelin release is necessary to more firmly establish this pathway.

To determine whether the effects observed with adenosine are physiologically relevant, we used the adenosine deaminase inhibitor EHNA. Extracellular adenosine can be rapidly metabolized by adenosine deaminase (Arch and Newsholme, 1978b; Fredholm et al., 2001); therefore, EHNA should increase the availability of endogenous adenosine. In wild-type mice, EHNA enhanced the release of ghrelin. This stimulatory effect of EHNA was still present in A1R-KO mice but not in A2AR-KO mice, consistent with the idea that the principal action of adenosine on ghrelin release is stimulatory in nature via the A2A receptors. In A1R-KO mice, the stimulatory effect of EHNA seems to be greater than that of wild-type mice. This could be caused by an increase in accumulation of endogenous adenosine acting on the A2A receptor, indicating a minor contribution of the A1 receptor on the basal tone of ghrelin release.

The main sources of adenosine are from cellular metabolism and breakdown of released ATP. Local adenosine levels vary greatly depending on metabolic states of cells and activities of nearby nerves. Therefore, in the stomach, adenosine levels may fluctuate between fasting and feeding states, which underlies its potential in regulating the release of various gastric peptides and motility. Our immunofluorescent studies suggest that adenosine has a direct effect on the ghrelin-releasing endocrine cells of the mouse gastric mucosa through activation of the A1 receptors. The lack of any colocalization of A2AR IR and ghrelin IR in the gastric mucosa suggests that adenosine stimulates ghrelin release from gastric endocrine cells by interacting with A2A receptors in the enteric plexus. The stimulatory effect of adenosine on ghrelin release can be abolished by the sodium channel blocker TTX, thereby supporting this hypothesis.

Colocalization of ghrelin IR with PGP 9.5 IR implies that ghrelin may also be released from the gastric enteric neurons. The colocalization of ghrelin IR with both A1R IR and A2AR IR in the myenteric plexus and nerve fibers suggests that adenosine may also regulate release of this source of ghrelin. The presence of ghrelin IR in neural elements has been reported in the guinea pig gastric myenteric plexus, whereas ghrelin receptors were observed in gastric longitudinal muscle and myenteric plexus (Xu et al., 2005). Ghrelin receptors have also been located in the enteric nervous systems of rat and human stomachs and colon (Dass et al., 2003). It is noteworthy that ghrelin accelerates gastric emptying and induces phase III of the migrating motor complex (Masuda et al., 2000; Fujino et al., 2003; Tack et al., 2005; Verhulst et al., 2008). Therefore, adenosine receptor activation might also be involved in the control of gastric emptying by regulating ghrelin signaling in the enteric neurons.

In conclusion, we have demonstrated by using both pharmacological approaches and selective adenosine receptor knockout mice that adenosine modulates the release of ghrelin in the stomach. Ghrelin release is controlled by a tonic stimulatory action mediated by A2A receptor activation, and an A1 receptor mediated inhibition is apparent mostly when the stimulatory action is removed. In addition, the stimulatory effect of adenosine is mediated by its action on the enteric plexus and endogenous adenosine is involved in this regulation. Because ghrelin is important in the integrated responses of energy balance and gastric motility, under-
standing the mechanisms underlying the regulation of its release by adenosine may enable better understanding of the pathophysiology associated with abnormal ghrelin levels.

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Authorship Contributions

**Participated in research design:** Yang, Yip, Kieffer, and Kwk.

**Conducted experiments:** Yang and Yip.

**Contributed new reagents or analytic tools:** Fredholm.

**Performed data analysis:** Yang and Yip.

**Wrote or contributed to the writing of the manuscript:** Yang, Yip, Fredholm, Kieffer, and Kwk.

**Other:** Kieffer and Kwk acquired funding for the research.

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