Altering sphingolipid composition with aging induces contractile dysfunction of gastric smooth muscle via $K_{Ca1.1}$ upregulation

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Summary

$K_{Ca1.1}$ regulates smooth muscle contractility by modulating membrane potential, and age-associated changes in $K_{Ca1.1}$ expression may contribute to the development of motility disorders of the gastrointestinal tract. Sphingolipids (SLs) are important structural components of cellular membranes whose altered composition may affect $K_{Ca1.1}$ expression. Thus, in this study, we examined whether altered SL composition due to aging may affect the contractility of gastric smooth muscle (GSM). We studied changes in ceramide synthases (CerS) and SL levels in the GSM of mice of varying ages and compared them with those in young CerS2-null mice. The levels of C16- and C18-ceramides, sphinganine, sphingosine, and sphingosine 1-phosphate were increased, and levels of C22, C24:1 and C24 ceramides were decreased in the GSM of both aged wild-type and young CerS2-null mice. The altered SL composition upregulated $K_{Ca1.1}$ and increased $K_{Ca1.1}$ currents, while no change was observed in $K_{Ca1.1}$ channel activity. The upregulation of $K_{Ca1.1}$ impaired intracellular Ca$^{2+}$ mobilization and decreased phosphorylated myosin light chain levels, causing GSM contractile dysfunction. Additionally, phosphoinositide 3-kinase, protein kinase C, c-Jun N-terminal kinases, and nuclear factor kappa-B were found to be involved in $K_{Ca1.1}$ upregulation. Our findings suggest that age-associated changes in SL composition or CerS2 ablation upregulate $K_{Ca1.1}$ via the phosphoinositide 3-kinase/protein kinase C/c-Jun N-terminal kinases/nuclear factor kappa-B-mediated pathway and impair Ca$^{2+}$ mobilization, which thereby induces the contractile dysfunction of GSM. CerS2-null mice exhibited similar effects to aged wild-type mice; therefore, CerS2-null mouse models may be utilized for investigating the pathogenesis of aging-associated motility disorders.

Key words: aging; Ca$^{2+}$-activated K$^+$ channel; ceramide synthases; contractile dysfunction; smooth muscle; sphingolipids.

Abbreviations

- [Ca$^{2+}$]i: intracellular Ca$^{2+}$ concentration
- CerS: ceramide synthase(s)
- IBTx: iberiotoxin
- ICC: interstitial cells of the Cajal
- K$_{Ca1.1}$: large conductance Ca$^{2+}$-activated K$^+$ channels
- MLC: myosin light chain
- NF-kB: nuclear factor kappa-B
- NPo: the open probability times the number of channels in the patch
- p21CIP: cyclin-dependent kinase inhibitor p21
- PGF$_{2\alpha}$: prostaglandin F$_{2\alpha}$
- p-JNK: phosphorylated JNK
- PKI: PKC$_\zeta$: pseudosubstrate inhibitor
- p-MLC: phosphorylated MLC
- p-p85: phosphorylated p85
- p-PKC$_\zeta$: phosphorylated PKC$_z$
- S1P: sphingosine 1-phosphate
- SLs: sphingolipids
- SMCs: smooth muscle cells
- VOCCs: voltage-operated Ca$^{2+}$ channels

Introduction

Aging causes structural changes in the gastrointestinal tract, which cause a significant decline in gastrointestinal function and increase the prevalence of several gastrointestinal motor disorders such as dyspepsia and constipation. Both a decrease in the number and volume of interstitial cells of Cajal (ICC; Gomez-Pinilla et al., 2011) and the degeneration of the enteric nervous system of the gastrointestinal tract, such as the myenteric plexus, occur in an age-related manner (El-Salhy et al., 1999; Phillips & Powley, 2001). As the ICC and the enteric nervous system play key roles in the control of gastrointestinal motility, the decrease in ICC and the degeneration of the enteric nervous system may contribute to changes in gastrointestinal motility with aging; however, little is known about the effect of aging on smooth muscle.

Gastric smooth muscle (GSM) generates regular contractions, which are triggered by long-lasting waves of depolarization, or slow waves. The ICC generate slow waves (Hirst & Edwards, 2006) and thereby depolarize nearby smooth muscle cells (SMCs). The depolarization activates
voltage-operated Ca\(^{2+}\) channels (VOCCs), and Ca\(^{2+}\) entry through VOCCs increases intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(i\)), which induces smooth muscle contraction. Intracellular Ca\(^{2+}\) then activates KCa1.1, which causes the hyperpolarization of SMCs. The hyperpolarization inactivates the VOCCs and thereby induces smooth muscle relaxation by inhibiting Ca\(^{2+}\) entry through the VOCCs. Thus, VOCCs and KCa1.1 act as important regulators of gastrointestinal motility by modulating membrane potential and Ca\(^{2+}\) influx, and changes in these ion channels greatly affect the contractility of smooth muscle. As the level of KCa1.1 is increased with advancing age (Tricarico et al., 1997; Oshiro et al., 2005), KCa1.1 upregulation might contribute to the development of age-associated gastrointestinal motility disorder.

Many different types of ion channels localize to cholesterol and sphingolipid (SL)-enriched regions of the plasma membrane, known as lipid microdomains or rafts (Dart, 2010). As SLs are important structural components of cellular membranes, they might affect ion channel gating and expression on the cell surface. The short-acyl chain ceramide analog, C6-ceramide, has been reported to modulate HERG K\(^+\) channel gating by causing its translocation into caveolin-enriched lipid rafts (Ganapathi et al., 2010) and to induce the channel downregulation via ubiquitin-mediated lysosomal degradation (Chapman et al., 2005). C6-Ceramide also inhibited voltage-gated K\(^+\) currents and induced vasconstriction in rat and human pulmonary arteries (Moral-Sanz et al., 2011). Ceramide, the backbone of all complex SLs, is synthesized by the N-acylation of a sphingoid long-chain base by a family of six ceramide synthases (CerS; Levy & Futterman, 2010), each generating ceramides with a different acyl chain length. C18-ceramide is generated by CerS1 and CerS4, and C16-ceramide is generated by CerS5 and CerS6. CerS2 generates very long acyl chain (C22 and C24) ceramides. Ceramides can also be formed by the degradation of complex SLs, such as sphingomyelin or glycosphingolipids. Ceramides can function as a second messenger in a variety of cellular events, including apoptosis and differentiation (Perry & Hannun, 1998; Gulbins, 2003), and the effects of ceramides may vary according to its acyl chain length (Mesicek et al., 2010). However, little is known about whether the aging process alters SL composition in cells and thereby affects ion channels to cause gastrointestinal motility disorder.

In this study, we hypothesized that altered SL composition due to aging may contribute to the development of contractile dysfunction of GSM via KCa1.1 upregulation. To test this hypothesis, we measured age-dependent changes in levels of SLs, KCa1.1 expression, phosphorylated myosin light chain (p-MLC), and contractility in wild-type (WT) GSM, and compared these levels with those induced by CerS2 ablation in CerS2-null mice, which were unable to synthesize very long acyl chain ceramides and contained increased levels of long acyl chain (C16 and C18) ceramides. Importantly, we found that levels of C16- and C18-ceramides, sphinganine, sphingosine (SP), and sphingosine 1-phosphate (S1P) were increased and levels of C22, C24:1, and C24 ceramides were decreased in the GSM of aged WT and young CerS2-null mice. It was found that altered SL composition upregulated KCa1.1 via the phosphoinositide 3-kinase (PI3K)/protein kinase C\(\beta\) (PKC\(\beta\)-Vc-Jun N-terminal kinases (JNK)/nuclear factor kappa-B (NF-\(\kappa\)B) pathway in GSM. KCa1.1 upregulation impaired Ca\(^{2+}\) mobilization and thereby inhibited myosin light chain (MLC) phosphorylation, which caused contractile dysfunction in GSM. We suggest that altering the SL composition induces contractile dysfunction via KCa1.1 upregulation in aged GSM, and CerS2-null mice allow us to investigate age-associated contractile dysfunction in GSM.

### Results

**SL composition is altered in an age-dependent manner**

We first determined the levels of CerS mRNA in GSM from mice of different ages, from 10 to 60 weeks old. Compared to the GSM from young (10 weeks old) WT mice, the levels of CerS4-CerS6 mRNA were significantly greater, and CerS2 levels were significantly smaller, in GSM from aged (60 weeks old) WT mice (Fig. 1A). LC-MS/MS analysis of SL levels exhibited that the levels of C16- and C18-ceramides, SP, S1P, and sphinganine were significantly greater in the GSM from aged mice (Fig. 1B, C), demonstrating that SL composition varies depending on age. As CerS2 levels were significantly smaller in aged mice, we examined whether similar alterations in CerS and SL composition occur in CerS2-null mice. In the GSM of young (25 weeks old) CerS2-null mice, levels of CerS4-CerS6 mRNA were significantly higher compared with those in age-matched WT mice, whereas the levels of CerS1 and CerS3 were not affected (Fig. 1D). The levels of C16- and C18-ceramides were significantly higher, and the levels of C22, C24:1, and C24 ceramides were markedly smaller in the GSM of CerS2-null mice (Fig. 1E). The levels of C20-ceramide were very low in the GSM of both WT and CerS2-null mice. Similar changes in CerS and ceramide composition were found in primary cultured gastric SMCs (Fig. S1, Supporting information). A previous study reported that levels of SP and sphinganine are increased in CerS2-null mice (Pewzner-Jung et al., 2010). These results suggest that aging and CerS2 ablation induce similar change in SL composition.

**K\(_{\text{Ca1.1}}\) upregulation without affecting single-channel activity**

Altered SL composition due to aging or CerS2 ablation may affect ion channel expression on the cell surface and ion channel activity in GSM. We thus examined whether KCa1.1 expression is altered in the GSM of aged WT or CerS2-null mice. First, we compared KCa1.1 levels in GSM from 25- and 100-week-old mice and found that the levels of \(\alpha\)- and \(\beta\)-subunits were significantly higher in the GSM of 100-week-old mice compared with that of 25-week-old mice (Fig. 2A). KCa1.1 (\(\alpha\)-subunit) levels in GSM were shown to increase in an age-dependent manner from 15- to 60-week-old mice (Fig. S6A, Supporting information). In addition, we demonstrated that KCa1.1 (\(\alpha\)-subunit, both mRNA and protein) levels were significantly higher in the GSM of young (25 weeks old) CerS2-null mice (Fig. 2B) and in cultured gastric SMCs from young CerS2-null mice (Fig. S2, Supporting information) compared to WT mice of the same age. Additionally, \(\beta\)-subunit levels were also increased in the GSM of young CerS2-null mice (Fig. 2B).

Next, we examined whether whole-cell KCa1.1 currents are increased in gastric SMCs from aged WT and young CerS2-null mice. KCa1.1 currents were markedly increased, while the current-voltage (I-V) relationship remained unaffected, in aged WT and young CerS2-null gastric SMCs (Fig. 2C, D). We then examined whether the single-channel activity of KCa1.1 is increased in the gastric SMCs of aged WT and young CerS2-null mice (Fig. 2E, F). The conductance of KCa1.1 channels in gastric SMCs of young WT, young CerS2-null, or aged WT mice was 121.4 ± 11.1 pS, 128.5 ± 7.9 pS, and 129.3 ± 12.5 pS, respectively, with no significant difference between the three conductances (upper panel of Fig. 2F). In addition, the Ca\(^{2+}\) sensitivity of the KCa1.1 channels in gastric SMCs was unchanged in young WT, young CerS2-null, or aged WT mice because the Ca\(^{2+}\)-S relationship was not altered in the gastric SMCs from these mice (lower panel of Fig. 2F). These results indicate that the biophysical properties of the KCa1.1 channels did not

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differ between young WT, young CerS2-null, or aged WT mice. Thus, the increase in KCa1.1 currents in the SMCs of aged WT and CerS2-null mice might be caused by the simultaneous increase in levels of α- and β-subunits on the cell membrane. The β-subunit modifies biophysical properties (the Ca2+ and voltage sensitivity) of the pore-forming α-subunits (McManus et al., 1995). Because the biophysical properties of the KCa1.1 channels were not changed, we did not examine the levels of β-subunits thereafter.

These results suggest that KCa1.1 expression on the cell membrane is increased in the gastric SMCs from aged WT and CerS2-null mice and thereby increases KCa1.1 currents without affecting the single-channel activity of KCa1.1.

**Fig. 1** Changes in levels of ceramide synthase(s) (CerS) and sphingolipids (SLs) in gastric smooth muscle (GSM) due to aging or CerS2 ablation. Levels of CerS1–CerS6 mRNA (A,D) and levels of SLs (B,C,E) were measured in GSM of WT mice at different ages (A–C), or of 25-week-old CerS2-null and WT mice (D,E). Blots are representative of three experiments, and the results have been normalized to GAPDH levels. The data are quantified from a set of three experiments. *P < 0.05, **P < 0.01 vs. 10-week-old (A–C) or 25-week-old (D,E) WT.

**Altering SL composition induces KCa1.1 upregulation**

As mRNA levels of CerS4, CerS5, and CerS6 were increased and CerS2 mRNA levels were decreased in aged WT and young CerS2-null mice, we examined whether the upregulation of CerS4, CerS5, or CerS6, or the downregulation of CerS2, induces KCa1.1 upregulation in aged WT and young CerS2-null mice. CerS5, CerS6, or CerS4 was transfected into the gastric SMCs of WT mice. CerS5 transfection did not affect the levels of other CerS such as CerS2, CerS4, or CerS6 (Fig. 3A, Supporting information), and neither did CerS4 nor CerS6 transfection (data not shown). CerS5 transfection significantly upregulated KCa1.1 (Fig. 3A). However, CerS6 (Fig. 3B) or CerS4 transfection (Fig. 3C) did not cause a
KCa1.1 upregulation represses the contractility of gastric smooth muscle
As KCa1.1 activation inhibits Ca\(^{2+}\) influx through VOCCs, an increased expression of KCa1.1 might impair intracellular Ca\(^{2+}\) mobilization in SMCs. We thus measured [Ca\(^{2+}\)]\(_{i}\) in the gastric SMCs of WT mice (Fig. 3D), without affecting levels of other CerS in gastric SMCs (Fig. 5C). LC-MS/MS analysis of ceramide levels exhibited that the levels of C16- and C18-ceramides were increased and those of C22, C24-1, and C24 ceramides were decreased in CerS2-transfected (Fig. 3B) or CerS2 knocked down (Fig. 3D) gastric SMCs. In addition, we examined whether SP or S1P affects KCa1.1 levels because SP and S1P were increased in aged WT and young CerS2-null mice. Exogenously added SP or S1P upregulated KCa1.1 in primary WT gastric SMCs in a concentration-dependent manner (Fig. 3E). These results suggest that altering SL composition increases KCa1.1 expression in gastric SMCs.

KCa1.1 upregulation reduces the contractility of gastric smooth muscle

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GSM, suggesting that a decrease in the number of mucosal folds is caused by a decrease in contractility. We therefore compared contractility of GSM between young WT (Fig. 5B), young CerS2-null (Fig. 5C), and aged WT (Fig. 5D) mice. When the smooth muscle was exposed to ACh, the frequency and magnitude of phasic contraction of GSM were increased in a concentration-dependent manner. The GSM of young WT mice maintained regular rhythmic contraction in the presence of ACh from concentrations of 0.01 to 30 \(\mu M\), whereas that of young CerS2-null and aged WT mice did not. When the GSM from aged WT or CerS2-null mice was exposed to low concentrations of ACh, phasic contractions were increased in the frequency and magnitude in a concentration-dependent manner, and well maintained in the presence of ACh. The response of which was similar to that of young WT mice. In contrast, when the smooth muscle was exposed to high concentrations of ACh, the frequency and magnitude of each contraction became irregular. The magnitude of the phasic contraction was not maintained and markedly decreased despite the presence of ACh (Fig. 5C,D).

These results suggest that contractile dysfunction of GSM is induced by high concentrations of ACh in aged WT and CerS2-null mice. The contractile dysfunction of GSM in CerS2-null (Fig. 5E) and aged WT (Fig. 5F) mice was recovered to regular phasic contraction by IBT (300 nM) treatment. In addition, the frequency of spontaneous contraction was significantly decreased in aged WT (5.2 ± 0.3 min\(^{-1}\), \(n = 9\)) and young CerS2-null (5.0 ± 0.2 min\(^{-1}\), \(n = 10\)) mice compared to young WT mice (6.9 ± 0.2 min\(^{-1}\), \(n = 13\)). IBT treatment significantly increased the frequency in aged WT (5.7 ± 0.2 min\(^{-1}\), \(n = 9\)) and young CerS2-null (5.6 ± 0.3 min\(^{-1}\), \(n = 10\)) mice (Fig. 5G). The magnitude of spontaneous contraction was also increased by IBT treatment. We then used tetrodotoxin to exclude neural influences (Fig. S5, Supporting information). When GSM was pretreated with the Na\(^{+}\) channel blocker tetrodotoxin (1 \(\mu M\)), contractile dysfunction was induced by high concentrations of ACh in the GSM of young CerS2-null and aged WT mice, and IBT treatment recovered the contractile dysfunction to regular phasic contractions, suggesting that the contractile dysfunction by high concentrations of ACh occurs independently of neural influences.

These results suggest that KCa1.1 upregulation due to aging or CerS2 ablation induces contractile dysfunction in GSM.

KCa1.1 upregulation is induced via the PI3K/PKC\(\alpha\)-JNK/NF-\(\kappa\)B-mediated pathway
Ceramides mediate several intracellular signals via second messengers, including kinases, phosphatases, proteases, or transcription factors (Levade et al., 2001). We hypothesized that the altered SL profile might preferentially affect the PI3K pathway (Hirsch et al., 2007; Silva et al., 2012). It was shown that levels of phosphorylated p85 (p-p85) were higher in the GSM of young (25 weeks old) CerS2-null mice (upper panel of Fig. 6A). Several studies have reported that PI3K is directly involved in the activation of PKC\(\alpha\) (Le Good et al., 1998) which can be activated by ceramides (Wang et al., 1999). Levels of phosphorylated PKC\(\alpha\) (p-PKC\(\alpha\)) were upregulated in the GSM of young CerS2-null (lower panel

Fig. 2 Increased KCa1.1 expression on cell membrane due to aging or CerS2 ablation. (A,B) KCa1.1 protein levels (α- and β-subunits) were examined in gastric smooth muscle of WT mice at different ages (A), and of 25-week-old CerS2-null and WT mice (B). Blots are representative of 4 experiments. Results were normalized to GAPDH or α-tubulin levels. (C) Representative I-V-activated currents in the absence (a) and presence (b) of ibetoxin (IBT; 300 nM) in gastric smooth muscle cells (SMCs) of 25-week-old WT, 25-week-old CerS2-null, and 100-week-old mice. The IBT-sensitive currents (c) were measured as the KCa1.1 currents. (D) I-V relationships for the gastric SMCs (left panel; \(n = 7\)) and normalized I-V curves from left panel (right panel). The amplitudes of the currents were normalized to the current measured at +80 mV. (E) Single-channel currents obtained from an inside-out patch and the amplitude histograms (n = 3-4; left panel). (F) Relationship between holding potential and current amplitude (n = 4-5; upper panel) or between [Ca\(^{2+}\)]\(_{i}\) and NPo (lower panel). *P < 0.05, **P < 0.01 vs. 25-week-old WT.
Fig. 3  Roles of sphingolipids (SLs) on KCa1.1 upregulation. (A–C) KCa1.1 protein levels 24 h after transfection of CerS5 (A), CerS6 (B), or CerS4 (C) to WT gastric smooth muscle cells (SMCs). (D) KCa1.1 and CerS2 protein levels 24 h after transfection of WT gastric SMCs with either scrambled siRNA or siRNA against CerS2. (E) KCa1.1 protein levels 24 h after treatment of WT gastric SMCs with sphingosine 1-phosphate (S1P) or SP. Blots are representative of 3–4 experiments. Results have been normalized to GAPDH levels. *P < 0.05, **P < 0.01 vs. gastric SMCs transfected with an empty vector (A–C), transfected with scrambled siRNA (D), or treated with vehicle (E).
Fig. 4. KCa1.1 upregulation inhibits an increase in intracellular Ca2+ concentration ([Ca2+]i) and myosin light chain (MLC) phosphorylation. (A) [Ca2+]i was measured in the gastric smooth muscle cells (SMCs) of young WT, aged WT, and young CerS2-null mice. Gastric SMCs were stimulated with prostaglandin F2α (PGF2α), and iberiotoxin (IBTx) was used to block KCa1.1. Summary data are shown in the left panel (n = 4). (B) Protein levels of phosphorylated MLC (p-MLC) were examined in gastric smooth muscle of aged WT, young CerS2-null, and age-matched WT mice. (C,D) KCa1.1 (α-subunits) and p-MLC levels were measured 24 h after KCa1.1 (C) or CerS5 (D) transfection of WT gastric SMCs. (B–D) Blots are representative of 3–4 experiments. Results are normalized to GAPDH or α-tubulin levels. *P < 0.05, **P < 0.01 vs. young WT (A,B) or WT gastric SMCs transfected with empty vector (C,D).
Fig. 5  Contractile dysfunction is developed in gastric smooth muscle (GSM) of aged WT or young CerS2-null mice. (A) Mucosal folds in the inner surface of the stomach were compared between WT and CerS2-null mice. (B–D) ACh-induced contraction of GSM of young WT (B), young CerS2-null (C), and aged WT (D) mice. (E,F) The specific KCa1.1 blocker, iberiotoxin (IBTx), recovered ACh-induced contractile dysfunction of GSM from young CerS2-null (E) and aged WT (F) mice. (G) Spontaneous contractions of GSM from CerS2-null (upper panel) and aged WT (lower panel) mice.

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The PI3K/PKCα/JNK/NF-κB pathway mediates KCa1.1 upregulation due to aging or ceramide synthases 2 (CerS2) ablation. Protein levels of phosphorylated p85 (p-p85), phosphorylated PKCα (p-PKCα), KCa1.1, and phosphorylated JNK (p-JNK) were examined in gastric smooth muscle (GSM) or primary cultured gastric smooth muscle cells (SMCs). (A) Levels of p-p85 or p-PKCα in GSM of young CerS2-null and age-matched WT mice. (B) p-PKCα levels in GSM of WT mice at different ages. (C) p-PKCα levels in WT gastric SMCs transfected with CerS5 for 24 h. (D) KCa1.1 levels in CerS2-null gastric SMCs treated with 10 μM PKCα pseudosubstrate inhibitor (PKI) for 24 h. (E) p-JNK levels in CerS2-null gastric SMCs transfected with scrambled siRNA or siRNA against PKCα for 24 h. (F) KCa1.1 levels in CerS2-null gastric SMCs treated with the NF-κB blockers, MG132, Bay11-7082, or SN50, for 24 h. The blots are representative of 3–4 experiments. Results were normalized to GAPDH or α-tubulin levels. *P < 0.05, **P < 0.01 vs. WT GSM or SMCs treated with scrambled siRNA or empty vector. †P < 0.05, ††P < 0.01 vs. CerS2-null gastric SMCs treated with scrambled siRNA or vehicle.
of Fig. 6A) and aged WT (Fig. 6B) mice. In addition, levels of p-p85 and p-PKCζ were increased by CerS5 transfection in WT gastric SMCs (Fig. 6C). Involvement of PKCζ in the regulation of KCa1.1 expression by CerS2 ablation was further examined using the PKCζ pseudosubstrate inhibitor (PKI) or siRNA against PKCζ in primary gastric SMCs (Fig. 6D). KCa1.1 upregulation was reduced by treatment with PKI or siRNA against PKCζ in CerS2-null gastric SMCs, suggesting that PKCζ is involved in KCa1.1 upregulation by CerS2 ablation. In addition, the levels of phosphorylated JNK (p-JNK) were increased in CerS2-null gastric SMCs, which was reversed by PKCζ knockdown in CerS2-null gastric SMCs (Fig. 6E). Finally, the involvement of NF-κB in the transcriptional regulation of KCa1.1 was directly tested using the NF-κB inhibitors, MG132 (2.5 μM), Bay 11-7082 (5 μM), or SN50 (30 μg ml⁻¹), in CerS2-null gastric SMCs. KCa1.1 upregulation was reversed by these NF-κB inhibitors in CerS2-null gastric SMCs (Fig. 6F). Our results suggest that an altered SL profile by CerS2 downregulation and/or CerS5 upregulation induces KCa1.1 upregulation in gastric SMCs through the PI3K/PKCζ/JNK/NF-κB pathways.

**Discussion**

The results of our study show that altered SL composition due to aging or CerS2 ablation upregulates KCa1.1 via the PI3K/PKCζ/JNK/NF-κB-mediated pathway in GSM. KCa1.1 upregulation inhibits Ca²⁺ influx and MLC phosphorylation and thereby reduces the contractility of GSM to evoke contractile dysfunction. In contrast, alteration of the SL acyl chain length or phosphorylation and thereby reduces the contractility of GSM to evoke contraction from irregular to regular rhythm in the GSM of aged WT and young CerS2-null mice is caused by an altered SL profile, that is, increased C16-6L, decreased C22-24-6L, or increased long-chain bases such as SP or sphinganine.

Although CerS4 was upregulated in the gastric SMCs of CerS2-null mice, the contribution of the altered SL profile by CerS4 to KCa1.1 upregulation seems to be negligible because CerS4 transfection increased KCa1.1 levels to a very small extent. In addition, the contribution of CerS6 to KCa1.1 upregulation is also negligible because CerS6 transfection did not affect KCa1.1 levels. This is unexpected, because CerS6 also synthesizes the 16-Ceramide, and indicates that the 16-Ceramide that is formed by CerS5 might be functionally different from the 16-Ceramide formed by CerS6.

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PI3K activates PKCζ (Le Good et al., 1998) and PKCζ inhibition using a specific blocker or siRNA against PKCζ reversed the increase in KCa1.1 levels in the gastric SMCs of CerS2-null mice, suggesting that PI3K activation plays a critical role in KCa1.1 upregulation. PI3K activation by phosphorylation might be caused by a change in lipid rafts, which are associated with several signal transduction molecules including Src family kinases and small G proteins (Yoshizaki et al., 2008) and act as important regulators of PI3K (Gao et al., 2011). In addition, KCa1.1 is localized to caveolae (Brainard et al., 2005), a subset of lipid rafts, and has a caveolin-binding motif (Suzuki et al., 2013). Thus, a direct interaction between KCa1.1 and caveolin regulates KCa1.1 expression at the cell surface (Suzuki et al., 2013). As alteration of lipid rafts was suggested in CerS2-null mice (Silva et al., 2012; Park et al., 2013) and SLs are important structural component of membranes, altering the SL composition might cause changes in lipid rafts. In addition, PI3K activation results in JNK activation (Kobayashi et al., 1997; Wang et al., 1999), and PKCζ is activated by ceramides, resulting in coactivation of NF-κB and JNK (Wang et al., 1999). Collectively, these findings suggest that the PI3K/PKCζ/JNK/NF-κB pathway plays a key role in KCa1.1 upregulation by altered SL composition with aging or by CerS2 ablation.

The contribution of ion channels to cellular function is determined by the single-channel activity of ion channels and the number of channels expressed on cell membranes. As the single-channel activity of KCa1.1 was not affected with aging or by CerS2 ablation, the increase in the whole-cell KCa1.1 currents in the gastric SMCs of aged WT and young CerS2-null mice might be due to the increased expression of KCa1.1 on the cell surface. Thus, the alternation in acyl chain length or composition of SLs affects KCa1.1 expression on the cell surface, without affecting the single-channel activity of KCa1.1. The expression of membrane proteins, including ion channels in the plasma membrane, is determined by a balance between synthesis/forward trafficking from the endoplasmic reticulum, endocytosis, and recycling/degradation (Conn & Ulloa-Aguirre, 2010; Simms & Zamponi, 2012). Recent studies demonstrate that various K⁺ channel proteins undergo endocytosis and degradation via PKC activation or by glycosphingolipids. PKC activation significantly decreased the expression of ATP-sensitive K⁺ channels on the cell membrane by reducing channel recycling and diverting the channel to lysosomal degradation (Manna et al., 2010). The neutral glycosphin- golipid, globotriaosylceramide, inhibited KCa3.1 synthesis (Park et al., 2011) and induced lysosomal degradation of KCa3.1 by facilitating channel protein internalization from the plasma membrane in endothelial cells (Choi et al., 2013), when globotriaosylceramide was excessively accumulated in endothelial cells. In addition, sphingomyelin, a major SL...
in mammalian cell membranes, is enriched in the plasma membrane and in the endocytic recycling compartment and may be involved in the regulation of endocytosis (Slotte, 2013). Therefore, altering SL composition may affect \( \text{K}_{\text{Ca}}1.1 \) expression on the cell membrane by regulating synthesis/forward trafficking from the endoplasmic reticulum, endocytosis, and the recycling/degradation of \( \text{K}_{\text{Ca}}1.1 \). Further studies are required to evaluate whether altering SL composition affects the regulation of \( \text{K}_{\text{Ca}}1.1 \) and thereby increases \( \text{K}_{\text{Ca}}1.1 \) expression on cell membrane.

The CerS2-null mice share similarities with the pathophysiological changes observed in patients or mouse models of diabetes mellitus, as insulin resistance and glucose intolerance were observed in CerS2-null mice (Park et al., 2013). SLS and ceramides affect insulin signaling by phosphorylation of protein kinase B or insulin receptors (Stratford et al., 2004; Langeveld & Aerts, 2009). In addition, ceramides generated by CerS5 were responsible for lipotoxic cardiomyopathy and hypertrophy in mouse models of diabetic cardiomyopathy (Russo et al., 2012).

Decreased contractility of GSM is frequently encountered in patients with diabetes mellitus. Therefore, further studies are required to evaluate whether \( \text{K}_{\text{Ca}}1.1 \) upregulation occurs, which thereby decreases the contractility of GSM in patients with diabetes mellitus.

The CerS2-null mice recapitulate important characteristics of the aged and therefore allow us to investigate the role of SLS in the function of GSM, including its role in \( \text{K}_{\text{Ca}}1.1 \) expression. Characterization of gastric SMCs from young CerS2-null mice showed an altered SL profile, \( \text{K}_{\text{Ca}}1.1 \) upregulation, impaired Ca\(^{2+}\) signaling, and reduced p-MLC, which mimic the alterations found in the aged mice. In addition, the altered SL composition induced similar contractile dysfunction of GSM by upregulating \( \text{K}_{\text{Ca}}1.1 \) in both mice, and the recovery of contractile dysfunction by \( \text{K}_{\text{Ca}}1.1 \) inhibition was similar in both mice. Furthermore, levels of the cyclin-dependent kinase inhibitor p21 (p21\(^{\text{CIP1}}\)), which is expressed in senescent cells, were markedly increased in the GSM or in primary gastric SMCs from aged WT and young CerS2-null mice (Fig. S6). Thus, we suggest that the CerS2-null mouse model is valuable for understanding the pathogenesis of aging-related motility disorders as well as for studies toward the design of therapeutic approaches.

In conclusion, our data suggest that aging or CerS2 ablation alters SL acyl chain length and composition, which induces \( \text{K}_{\text{Ca}}1.1 \) upregulation via the PI3K/PKC/INK/NF-xB signaling pathway and thereby causes contractile dysfunction of GSM. To the best of our knowledge, this is the first study to demonstrate the mechanisms underlying age-dependent contractile dysfunction of smooth muscle and an age-dependent increase in endogenous \( \text{K}_{\text{Ca}}1.1 \) expression in native gastric SMCs. In addition, we suggest that CerS2-null mice may serve as a model for contractile dysfunction that is developed in the aged. Therefore, CerS2-null mice may provide an additional resource for understanding the underlying mechanisms responsible for changes in ion channel expression by altered SL composition.

Experimental procedures

For description of contraction measurement on gastric muscle strips, electrophysiological recording, Ca\(^{2+}\) measurement, PCR, Western blotting, immunostaining, and LC-MS/MS analysis of SLS, please refer to the Appendix S1 (Supporting information).

Animals

The CerS2 null mice were generated as described (Levy & Futerman, 2010). The investigation was approved by the local ethics committee, the Institutional Review Board of the Ewha Womans University, and was in accordance with the Declaration of Helsinki, the Animal Care Guidelines of the Ewha Womans University, Medical School, and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Cell isolation and culture

The mucosal layer of stomach was separated from the muscle layer in Ca\(^{2+}\)-free physiological salt solution, and gastric SMCs were isolated from GSM segments. The GSM layer was cut into small segments (2 × 3 mm), which were incubated for 15–25 min at 37 °C in digestion medium containing 0.1% collagenase (Wako Pure Chemicals, Osaka, Japan), 0.05% DTT, 0.1% trypsin inhibitor, and 0.2% BSA. After digestion, gastric SMCs were harvested by gentle agitation with a wide-bore glass pipette.

Transfection

A \( \text{K}_{\text{Ca}}1.1 \) plasmid was provided by Dr. Chul-Seung Park of Gwangju Institute of Science and Technology (Korea). Gastric SMCs were transiently transfected for 24 h with each plasmid using JET-PEI (Polyplus-Transfection Inc, New York, NY, USA) or Effectene (Qiagen Inc, Valencia, CA, USA) according to the procedure suggested by the manufacturer. To knockdown CerS2 in gastric SMCs, scrambled siRNA or siRNA against CerS2 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Gastric SMCs were transiently transfected for 24 h with the siRNAs using siRNA transfection reagent (Santa Cruz Biotechnology).

Chemicals

Ceramides (acyl chain lengths of C14, C16, C18, C22, C24, and C24:1), C17-ceramide (d17:1/C18:0), sphinganine, SP, S1P and C17-SP, C17-S1P as an internal standard were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Organic solvents for SL extraction and HPLC analysis were purchased from Merck (Darmstadt, Germany). ACh, Bay11-7082, IBTx, MG132, PGF\(_{2\alpha}\), PKI, SN50, and tetrodotoxin were purchased from Sigma-Aldrich (St Louis, MO, USA). The primary antibodies used in this study were anti-Tyr-458-p-p85 (Cell Signaling Technology, Boston, MA, USA), anti-Thr-560-p-PKC\(_{\text{C}}\) (Abcam, Cambridge, MA, USA), anti-Thr-183/Tyr-185-p-JNK (Cell Signaling Technology), anti-Ser-19-p-MLC (Cell Signaling Technology), anti-K\(_{\text{Ca}}\)1.1 (α-subunit; Novus Biologicals, Littleton, CO, USA), anti-K\(_{\text{Ca}}\)1.1 (β-subunit; Abcam), anti-CerS2 (Sigma-Aldrich), anti-CerS5 (Santa Cruz), and anti-GAPDH (EMD Millipore, Darmstadt, Germany).

Statistical analysis

Data are means ± SEM for 3–7 independent experiments. To examine the statistical significance between groups, one-way ANOVA or two-tailed Student’s t-test was used. A \( P \) value of 0.05 or lower was considered statistically significant.

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None.

Author contributions

Shinkyu Choi performed study concept and design, acquisition of data, analysis and interpretation of data, drafting of the manuscript, critical

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revision of the manuscript for intellectual content; Tae Hun Kim and Seikwan Oh performed analysis and interpretation of data, technical support; Jee Aee Kim, Hae-yon Li, and Kyong-Oh Shin performed acquisition, analysis, and interpretation of data; Yong-Moon Lee performed acquisition of data, analysis and interpretation of data, technical support, critical revision of the manuscript for intellectual content; Yael Pewzner-Jung supplied the CerS2 null mice and critical revision of the manuscript for intellectual content, material support, obtaining funding; Suk Hyo Suh performed study concept and design, analysis and interpretation of data, drafting of the manuscript, critical revision of the manuscript for intellectual content, obtained funding.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site.

Appendix S1. Supplementary Materials and methods.

Fig. S1 Changes in levels of CerS and SLs in gastric SMCs by CerS2 ablation.

Fig. S2 KCa1.1 levels in primary cultured gastric SMCs from CerS2-null mice.

Fig. S3 Changes in levels of ceramides with various acyl chain lengths by CerS5 transfection or CerS2 knock-down.

Fig. S4 Inverse relationship between expression levels of KCa1.1 and p-MLC.

Fig. S5 Tetrodotoxin did not prevent contractile dysfunction of aged WT or young CerS2-null gastric smooth muscle.

Fig. S6 p21\textsuperscript{CIP1} upregulation in gastric smooth muscle from aged WT and CerS2-null mice.