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$K_{Ca}3.1$ upregulation preserves endothelium-dependent vasorelaxation during aging and oxidative stress

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Summary

Endothelial oxidative stress develops with aging and reactive oxygen species impair endothelium-dependent relaxation (EDR) by decreasing nitric oxide (NO) availability. Endothelial $K_{Ca}3.1$, which contributes to EDR, is upregulated by H_2O_2. We investigated whether $K_{Ca}3.1$ upregulation compensates for diminished EDR to NO during aging-related oxidative stress. Previous studies identified that the levels of ceramide synthase 5 (CerS5), sphingosine, and sphingosine 1-phosphate were increased in aged wild-type and CerS2 mice. In primary mouse aortic endothelial cells (MAECs) from aged wild-type and CerS2 null mice, superoxide dismutase (SOD) was upregulated, and catalase and glutathione peroxidase 1 (GPX1) were downregulated, when compared to MAECs from young and age-matched wild-type mice. Increased H_2O_2 levels induced Fyn and extracellular signal-regulated kinases (ERKs) phosphorylation and $K_{Ca}3.1$ upregulation. Catalase/GPX1 double knockout (catalase$^{-/-}$/GPX1$^{-/-}$) upregulated $K_{Ca}3.1$ in MAECs. NO production was decreased in aged wild-type, CerS2 null, and catalase$^{-/-}$/GPX1$^{-/-}$ MAECs. However, $K_{Ca}3.1$ activation-induced, N^G-nitro-L-arginine-, and indomethacin-resistant EDR was increased without a change in acetylcholine-induced EDR in aortic rings from aged wild-type, CerS2 null, and catalase$^{-/-}$/GPX1$^{-/-}$ mice. CerS5 transfection or exogenous application of sphingosine or sphingosine 1-phosphate induced similar changes in levels of the antioxidant enzymes and upregulated $K_{Ca}3.1$. Our findings suggest that, during aging-related oxidative stress, SOD upregulation and downregulation of catalase and GPX1, which occur upon altering the sphingolipid composition or acyl chain length, generate H_2O_2 and thereby upregulate $K_{Ca}3.1$ expression and function via a H_2O_2/Fyn-mediated pathway. Altogether, enhanced $K_{Ca}3.1$ activity may compensate for decreased NO signaling during vascular aging.

Key words: aging; Ca^{2+}-activated K^+ channel; ceramide synthase 2 ablation; endothelial cells; oxidative stress; redox enzymes.

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Introduction

Endothelial oxidative stress develops with aging and thereby impairs endothelial function (Donato *et al.*, 2007; Ungvari *et al.*, 2010). Endothelial cells (ECs) contribute to the maintenance of vascular homeostasis by secreting nitric oxide (NO), prostacyclin, and endothelium-derived hyperpolarizing factor, thereby playing an important role in preventing the genesis and progression of cardiovascular diseases. Although various factors have been shown to cause endothelial dysfunction, some evidence supports the role of reactive oxygen species (ROS) or oxidative stress in the dysfunction (Gori & Munzel, 2011). Dysregulated redox signaling and increased ROS production with aging lead to endothelial dysfunction, thereby contributing to the pathogenesis of cardiovascular diseases, such as coronary artery diseases, hypertension, and atherosclerosis, in elderly patients (Ungvari *et al.*, 2010). An increase in ROS generation reduces NO bioavailability through several mechanisms including a direct interaction between NO and ROS, resulting in vascular dysfunction (Donato *et al.*, 2007; Ungvari *et al.*, 2008; Seals *et al.*, 2011). However, little is known about the mechanism by which ECs preserve their function of relaxing vascular smooth muscle during aging-related oxidative stress.

Reactive oxygen species, generated in response to various stimuli, are scavenged by the endogenous antioxidant enzymes, such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPX). Antioxidant enzymes are present in lipid rafts on cell membranes or have close relationships with lipid rafts (Li & Zhang, 2013). In addition, redox molecules, such as the NADPH oxidase subunits or cofactors, are present in lipid rafts (Li & Zhang, 2013). This evidence supports the view that lipid rafts are involved in initiating and transmitting redox signaling in cells. Recent studies have demonstrated that altering the sphingolipid composition by ceramide synthase 2 (CerS2) ablation affects lipid rafts (Park *et al.*, 2013) and results in ROS generation through the modulation of mitochondrial complex IV activity (Zigdon *et al.*, 2013). In addition, sphingolipid composition and the activities of antioxidant enzymes (SOD, catalase, GPX) were altered in aged rats or rabbits (Lightle *et al.*, 2000; Cejkova *et al.*, 2004). Furthermore, our previous study showed that the levels of ceramide synthases (CerSs) and sphingolipid composition are altered with aging in mice and that aging-related alteration in the levels of CerSs and sphingolipid composition is similar to the alteration induced by CerS2 ablation (Choi *et al.*, 2015b). These results suggest that ROS (which are generated as a result of altered sphingolipid composition) play an important role in the aging process, and that CerS2 null mice can allow us to investigate aging-related and altered sphingolipid composition-induced changes in cellular functions.

Ca^{2+}-activated K^+ channels ($K_{Ca}2.3$ and $K_{Ca}3.1$) affect endothelial function by modulating endothelium-dependent responses including endothelium-dependent hyperpolarization and NO release (Busse *et al.*, 2002; Climent *et al.*, 2014). Endothelial hyperpolarization induced by K^+ channel activation or endothelium-derived hyperpolarizing factor hyperpolarizes vascular smooth muscle cells to induce endothelium-dependent relaxation (EDR). In addition, endothelial hyperpolarization may promote Ca^{2+} influx through Ca^{2+} entry channels in ECs (Behringer & Segal, 2015), thereby increasing NO

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production (Sheng *et al.*, 2009). Thus, the impairment of $K_{Ca}2.3$ and $K_{Ca}3.1$ affects the endothelium-dependent control of vascular contractility, which results in a predisposition to vascular diseases (Grgic *et al.*, 2009). An increase in blood pressure has been reported in $K_{Ca}3.1$ knockout mice (Si *et al.*, 2006), and we have previously suggested that $K_{Ca}3.1$ downregulation is a cause of endothelial dysfunction in Fabry disease (Park *et al.*, 2011). In addition, we reported that superoxide generated from ECs downregulates $K_{Ca}3.1$, resulting in endothelial dysfunction in preeclampsia (Choi *et al.*, 2013a). In contrast, H_2O_2 upregulates $K_{Ca}3.1$ via an ERK-mediated pathway (Choi *et al.*, 2013a,b). Thus, ROS affect endothelial function by modulating endothelial $K_{Ca}3.1$ expression. As ROS generation is increased with aging (Ungvari *et al.*, 2010), aging-related ROS production might affect endothelial function by modulating endothelial $K_{Ca}3.1$ expression. However, signaling pathways that modulate endothelial $K_{Ca}3.1$ expression and function during conditions of age-related oxidative stress remain undefined.

This study focused on the effect of H_2O_2 on the endothelial function of inducing the relaxation of vascular smooth muscle using mouse aortic endothelial cells (MAECs) from aged wild-type and CerS2 null mice, in which sphingolipid composition is altered and ROS production is increased. The results showed that a H_2O_2-induced increase in $K_{Ca}3.1$ activity compensated for diminished EDR to NO under aging-related oxidative stress conditions.

Results

ROS generation in MAECs from aged wild-type and young Cer2 null mice

We investigated whether ROS generation is increased in MAECs from aged wild-type (Fig. 1) and young CerS2 null (Fig. 2) mice using the H_2O_2-sensitive dye, 5-(and 6-) chloromethyl-2′,7′-dichlorofluorescin diacetate (CM-DCFH-DA) or peroxy-orange 1, or the superoxide-sensitive dye, dihydroethidine. Compared with young wild-type MAECs, H_2O_2 levels were markedly increased in aged wild-type MAECs (Fig. 1A, B). The increased H_2O_2 levels were reduced by treatment with catalase (Fig. 1B). In aged wild-type MAECs, catalase and GPX1 levels were decreased, whereas levels of SOD1 and SOD2 were significantly increased (Fig. 1C). A previous study reported that thioredoxin 1 protein levels are increased in aged ECs (Altschmied & Haendeler, 2009). In young CerS2 null MAECs, H_2O_2 levels were significantly increased, but superoxide levels were not when compared to age-matched wild-type MAECs (Fig. 2A,B). We then examined whether levels of antioxidant enzymes are altered in CerS2 null MAECs. In young CerS2 null MAECs, catalase and GPX1 were downregulated (Fig. 2C), whereas the mitochondrial SOD, SOD2, was upregulated (Fig. 2D). In addition, mRNA levels of thioredoxin 1 and thioredoxin 2 were significantly decreased (Fig. S1). On the other hand, levels of the cytoplasmic SOD, SOD1, were unchanged. As SOD upregulation might increase degradation of superoxide to H_2O_2, and downregulation of catalase, GPX1, and thioredoxins is indicative of decreased degradation of H_2O_2, these results suggest that H_2O_2 levels are increased in aged wild-type and young CerS2 null MAECs.

H_2O_2, generated by alteration in the levels of antioxidant enzymes, upregulates $K_{Ca}3.1$

In our previous studies, we reported that $K_{Ca}3.1$ is upregulated by H_2O_2 and downregulated by superoxide (Choi *et al.*, 2013a,b), suggesting the possibility of endothelial $K_{Ca}3.1$ upregulation by H_2O_2 in aged wild-type and young CerS2 null MAECs. Thus, we examined $K_{Ca}3.1$ levels in aged wild-type and young CerS2 null MAECs. $K_{Ca}3.1$ expression was significantly upregulated in aged wild-type (Fig. 3A) and CerS2 null (Fig. 3B) MAECs. As catalase and GPX1 were downregulated in aged wild-type and CerS2 null MAECs, we examined whether knockouts of catalase and GPX1 affect endothelial $K_{Ca}3.1$ levels using young catalase/GPX1 double-knockout (catalase$^{-/-}$/GPX1$^{-/-}$) mice. $K_{Ca}3.1$ was markedly upregulated in catalase$^{-/-}$/GPX1$^{-/-}$ MAECs (Fig. 3C), indicating that downregulation of catalase and GPX1 plays a critical role in $K_{Ca}3.1$ upregulation in aged wild-type and young CerS2 null mice. In addition, $K_{Ca}3.1$ upregulation in CerS2 null MAECs was reduced by inhibition of ROS generation. Treatment with tiron, tempol, or *N*-acetyl-cysteine (NAC) reduced $K_{Ca}3.1$ levels (Fig. 3D). Furthermore, treatment with catalase (Fig. 3E) or 2-methyl estradiol (2-ME) (Fig. 3F) reduced $K_{Ca}3.1$ levels in a concentration-dependent manner. These results suggest that changing the levels of antioxidant enzymes (SOD2 upregulation and downregulation of catalase and GPX1) increases H_2O_2 levels and thereby $K_{Ca}3.1$ levels.

Fyn and ERK activation by H_2O_2 induces $K_{Ca}3.1$ upregulation

H_2O_2 mediates several intracellular signals as a second messenger and activates the Src family kinase Fyn (Saksena *et al.*, 2008). We therefore examined phosphorylated Fyn (p-Fyn) levels in MAECs. p-Fyn levels were increased in aged wild-type, young CerS2 null, and young catalase$^{-/-}$/GPX1$^{-/-}$ MAECs (Fig. 4A). As H_2O_2 (Choi *et al.*, 2013b) and Fyn (Toni *et al.*, 2006) play critical roles in extracellular signal-regulated kinase (ERK) activation, we examined phosphorylated ERK (p-ERK) levels in MAECs. In aged wild-type and young CerS2 null MAECs, p-ERK levels were increased (Fig. 4B). The increased p-ERK levels were reduced by 4-amono-5-(4-methylphenyl)-7-(*t*-butyl)pyrazol[3,4-*d*]pyrimidine (PP1) in young CerS2 null MAECs (Fig. 4B, right panel). In addition, the increased p-Fyn levels were reduced by apocynin or by PP1 in catalase$^{-/-}$/GPX1$^{-/-}$ MAECs (Fig. 4C), and the increased $K_{Ca}3.1$ levels were reduced by PP1 in young CerS2 null and catalase$^{-/-}$/GPX1$^{-/-}$ MAECs (Fig. 4D). These results suggest that H_2O_2 upregulates $K_{Ca}3.1$ via a Fyn/ERK-mediated pathway.

Endothelial $K_{Ca}3.1$ upregulation preserves EDR upon oxidative stress

NADPH oxidase is a major source of superoxide in vascular ECs (Griendling *et al.*, 2000). In addition, arginases promote endothelial NO synthase (eNOS) uncoupling through enzymatic competition with the substrate L-arginine, thereby generating ROS (Yang & Ming, 2013). An increase in ROS generation causes endothelial dysfunction via the functional inactivation of NO. We thus measured levels of NO, arginase 2, and NOX2 in MAECs (Fig. S2). In young CerS2 null MAECs, intracellular NO levels were decreased (Fig. S2A), and levels of arginase 2 and NOX2 were increased (Fig. S2B). In addition, levels of arginase 2 and NOX2 were significantly increased in young catalase$^{-/-}$/GPX1$^{-/-}$ (Fig. S2C) and aged wild-type (Fig. S2D) MAECs. These results suggest that ROS markedly decrease NO bioavailability in aged wild-type, young CerS2 null, and young catalase$^{-/-}$/GPX1$^{-/-}$ mice.

We thus examined whether EDR to acetylcholine is reduced in these mice (Fig. 5). As vascular smooth muscle contraction to prostaglandin $F_{2\alpha}$ or norepinephrine was not blunted in these mice, aortic rings from these mice were contracted by prostaglandin $F_{2\alpha}$ or norepinephrine. Precontracted endothelium-intact aortic rings were relaxed by

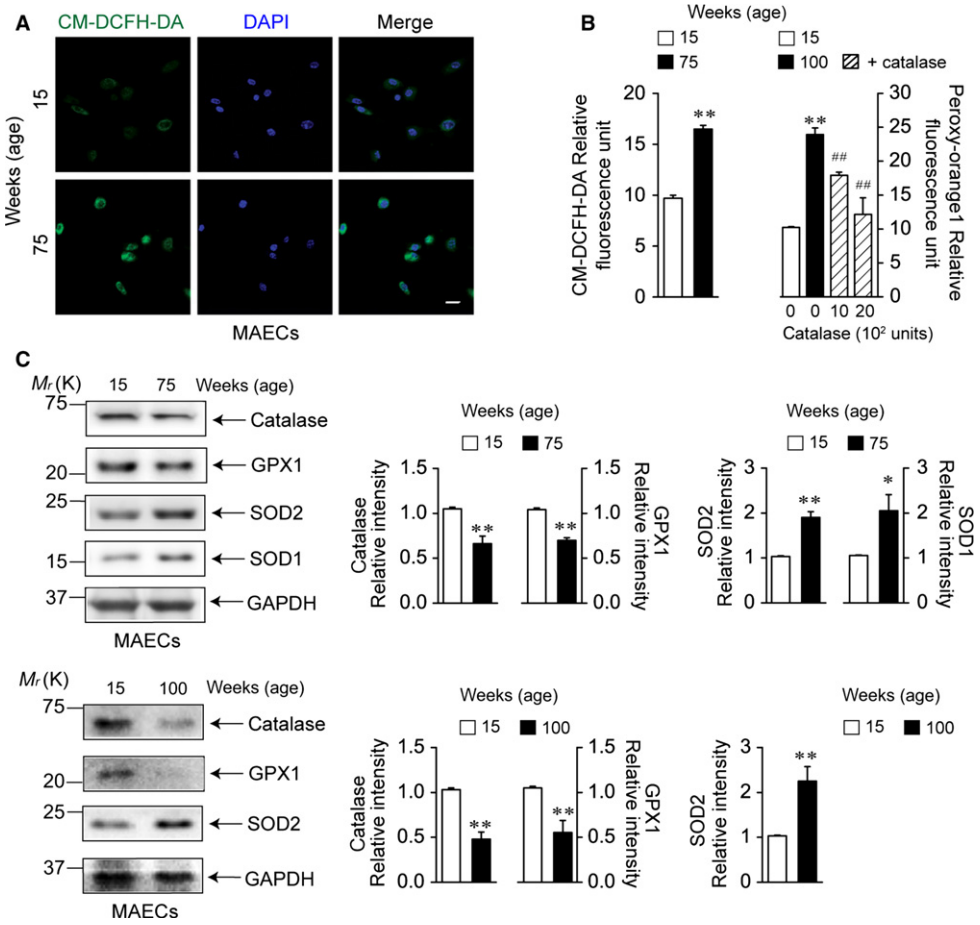

Fig. 1 Levels of reactive oxygen species and antioxidant enzymes in aged wild-type mouse aortic endothelial cells (MAECs). H_2O_2 production and levels of antioxidant enzymes were examined in MAECs from young (15-week-old) and aged (75- and 100-week-old) wild-type mice. (A) Fluorescence was detected by confocal laser microscopy. Nuclei were stained with DAPI (blue). Compared with young wild-type MAECs, the green fluorescence indicative of H_2O_2 was markedly increased in aged wild-type MAECs. Scale bar: 20 μm. (B) Fluorescence was detected by a microplate fluorescence reader. Results were observed in each set of five different cultures. (C) Protein levels of catalase, GPX1, SOD1, and SOD2 were measured. Blots are representatives of three to four experiments performed with three to four different cultures. Results were normalized to GAPDH levels. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. young wild-type MAECs, $^{\#\#}P < 0.01$ vs. 100-week-old wild-type MAECs.

acetylcholine in a concentration-dependent manner. Although NO production was significantly reduced in ECs, EDR to acetylcholine was not significantly reduced in aortic rings from aged wild-type, young CerS2 null, and young catalase$^{-/-}$/GPX1$^{-/-}$ mice (Fig. 5A). In addition, sodium nitroprusside-induced relaxation of precontracted aortic rings was not changed in these mice (data not shown), suggesting that the reactivity of vascular smooth muscle to NO is not affected. In the presence of indomethacin, EDR to acetylcholine of vascular smooth muscle is evoked by NO released from ECs and by endothelium-dependent hyperpolarization via endothelial $K_{Ca}3.1$ activation. We therefore compared the magnitude of $K_{Ca}3.1$ activation-induced EDR in these mice. The $K_{Ca}3.1$ activator 1-EBIO relaxed precontracted endothelium-intact aortic rings (Fig. 5B), but did not relax precontracted endothelium-denuded aortic rings (Fig. S3A), suggesting that $K_{Ca}3.1$ activator-induced relaxation is endothelium dependent. When the EDR response to the $K_{Ca}3.1$ activator reached a steady state, we added acetylcholine (1 μM) to evoke NO-induced EDR (Fig. 5B). Compared with young or age-matched wild-type mice, 1-EBIO-induced EDR was significantly increased, but (1-EBIO + acetylcholine)-induced EDR was unchanged, in aged wild-type, CerS2 null, and catalase$^{-/-}$/GPX1$^{-/-}$ mice. Thus, the ratio of 1-EBIO-induced EDR to (1-EBIO + acetylcholine)-induced EDR was markedly increased in aged wild-type, young CerS2 null, and young catalase$^{-/-}$/GPX1$^{-/-}$ mice. As $K_{Ca}3.1$ activation contributes to NO production by increasing intracellular Ca^{2+} levels, we then examined $K_{Ca}3.1$ activator-induced EDR of endothelium-intact aortic rings in which NO production was inhibited by pretreatment with N^{ω}-nitro-L-arginine (L-NOARG) (Fig. 5C). Compared with young wild-type mice, EDR to 1-EBIO was significantly increased in aged wild-type, young CerS2 null, and young catalase$^{-/-}$/GPX1$^{-/-}$ mice, indicating that the $K_{Ca}3.1$ contribution to EDR is markedly increased in mice in which endothelial $K_{Ca}3.1$ is upregulated. EDR to NS309 was similar to that to 1-EBIO (Fig. S3B). As polyethylene glycol-catalase (PEG-catalase) reduced $K_{Ca}3.1$ levels in aged MAECs (Fig. S4), we examined whether an increase in $K_{Ca}3.1$ activation-induced EDR could be reduced by PEG-catalase in aortas from aged wild-type mice (Fig. 5D). Treatment with PEG-catalase markedly reduced 1-EBIO-induced EDR in aorta from aged wild-type mice. These results suggest that reduced EDR to NO can be compensated for by enhancing $K_{Ca}3.1$ activation-induced EDR in aged wild-type, young CerS2 null, and young catalase$^{-/-}$/GPX1$^{-/-}$ mice.

Altering sphingolipid composition changes levels of antioxidant enzymes and upregulates $K_{Ca}3.1$

Finally, we examined whether changes in levels of antioxidant enzymes and $K_{Ca}3.1$ are induced by altering sphingolipid composition in young CerS2 null and aged wild-type MAECs. We determined levels of CerSs and sphingolipids in aortic tissue and MAECs from young CerS2 null mice (Fig. S5). In CerS2 null aortic tissue, the mRNA levels of CerS4-CerS6 were significantly increased, whereas the levels of CerS1 and CerS3 were not affected (Fig. S5A). The levels of C16- and C18-ceramides were significantly increased, while the levels of C22-, C24:1-, and C24-ceramides were markedly decreased, in CerS2 null aortic tissue (Fig. S5B) and CerS2 null MAECs (Fig. S5C). In addition, the levels of sphingosine, sphinganine (S1P), and sphinganine were

significantly increased in CerS2 null MAECs (Fig. S5C). We previously reported that the CerS2 ablation-induced alteration in the levels of CerSs and sphingolipid composition is similar to aging-related alteration in these measurements in mice (Choi *et al.*, 2015b). As CerS5 was upregulated in CerS2 null mice, we examined the effects of CerS5 transfection on levels of antioxidant enzymes and $K_{Ca}3.1$ in wild-type MAECs (Fig. 6A). CerS5 transfection significantly downregulated catalase without affecting SOD2 levels. $K_{Ca}3.1$ levels were slightly increased, but this increase was not statistically significant. We then examined whether sphingosine or S1P affects $K_{Ca}3.1$ levels because sphingosine or S1P levels were increased in CerS2 null (Fig. S5C) and aged (Choi *et al.*, 2015b) mice. Exogenously added sphingosine (Fig. 6B,C) or S1P (Fig. 6D,E) downregulated catalase and GPX1 and upregulated $K_{Ca}3.1$ in a concentration-dependent manner. Furthermore, exogenously added sphingosine increased H_2O_2 generation in wild-type MAECs (data not shown). These results suggest that altering sphingolipid profile increases $K_{Ca}3.1$ levels by changing levels of antioxidant enzymes.

Discussion

The results of our study show that alteration in sphingolipid acyl chain length and composition upregulates SOD and downregulates catalase and GPX1 in aged wild-type and young CerS2 null mice, thereby increasing H_2O_2 content (Fig. 7). The increased ROS production impairs endothelial NO production and NO-induced EDR of vascular smooth muscle. On the other hand, H_2O_2 induces endothelial $K_{Ca}3.1$ upregulation, which enhances $K_{Ca}3.1$ activation-induced EDR, thereby maintaining EDR to acetylcholine. These findings represent the first evidence of a compensatory role for endothelial $K_{Ca}3.1$ in mediating aortic vasorelaxation during old age and under conditions of oxidative stress, which may be implicated in age-associated cardiovascular disorders.

Among ROS, H_2O_2 levels were increased in ECs of aged wild-type and young CerS2 null mice. This notion is supported by three observations; firstly, fluorescence from the H_2O_2-sensitive dyes, CM-DCFH-DA and peroxy-orange 1, was markedly increased in MAECs from aged wild-type and young CerS2 null mice. Secondly, catalase and GPX1, which degrade H_2O_2, were downregulated, and SOD, which generates H_2O_2 from superoxide, was upregulated. Thirdly, p-Fyn and $K_{Ca}3.1$, which are positively regulated by H_2O_2, were upregulated in ECs of aged wild-type and young CerS2 null mice. ROS-generating enzymes, such as NADPH oxidases, xanthine oxidase, and NO synthases, are activated by ceramide (Lecour *et al.*, 2006). In addition, arginase upregulation, found in young CerS2 null and aged wild-type mice, might contribute to increased oxidative stress, as eNOS produces superoxide via eNOS uncoupling caused by arginases. Thus, downregulation of catalase and GPX1 and upregulation of SOD contribute to increased H_2O_2 levels, which is consistent with the enhanced H_2O_2 availability and the decreased catalase activity in the microvascular endothelium of aged mice (Socha *et al.*, 2015).

$K_{Ca}3.1$ upregulation in young catalase$^{-/-}$/GPX1$^{-/-}$ mice suggests that changes in the levels of antioxidant enzymes, including downregulation of catalase and GPX1, play a critical role in $K_{Ca}3.1$ upregulation in aged wild-type and young CerS2 null mice. The presence of NADPH oxidase subunits or cofactors and antioxidant enzymes in lipid rafts (Li & Zhang, 2013) suggests that lipid rafts play an important role in the regulation of these enzymes. In addition, alteration of lipid rafts was suggested to occur in CerS2 null mice (Park *et al.*, 2013), and the function of lipid rafts is altered with aging (Fulop *et al.*, 2012). Thus, changes in lipid rafts might cause such alterations in the levels of antioxidant enzymes in aged wild-type and CerS2 null mice.

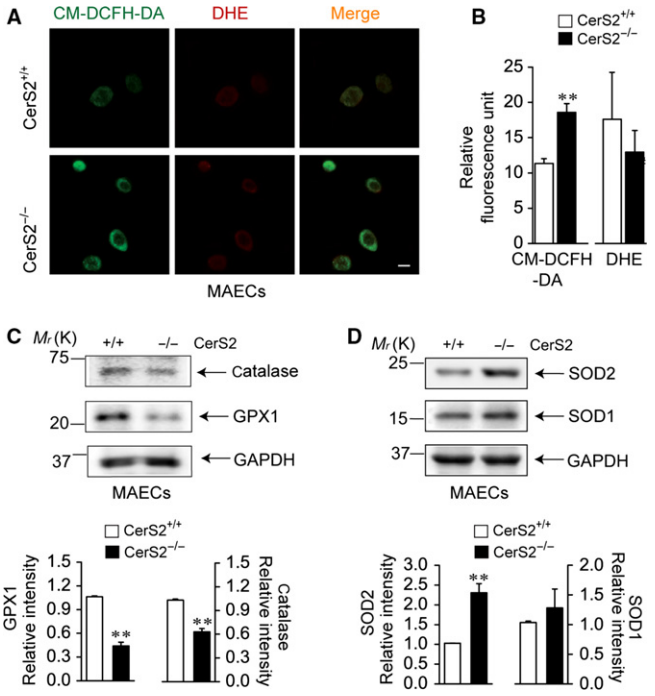

Fig. 2 Changes in the levels of reactive oxygen species (ROS) and antioxidant enzymes caused by CerS2 ablation. Primary cultured mouse aortic endothelial cells (MAECs) isolated from young (25-week-old) CerS2 null and age-matched wild-type mice were used to examine ROS generation (A,B) and levels of antioxidant enzymes (C,D). (A) Fluorescence was detected by confocal laser microscopy. The green fluorescence indicative of H_2O_2 was significantly increased, but the red fluorescence indicative of superoxide was not when compared to wild-type MAECs. Scale bar: 10 μm. (B) Fluorescence was detected by a microplate fluorescence reader. Results were observed in each set of five different cultures. (C, D) Protein levels of catalase, GPX1 (C), SOD1, and SOD2 (D) were examined. Blots are representatives of three experiments performed with three different cultures, and the results were normalized to GAPDH levels. $^{**}P < 0.01$ vs. wild-type MAECs. GPX1, glutathione peroxidase 1; SOD, superoxide dismutase.

As sphingolipids are important structural component of membranes, altering the sphingolipid composition might cause changes in lipid rafts. Changes in the levels of antioxidant enzymes were induced in CerS2-null mice in which sphingolipid composition was altered. In addition, CerS5 transfection or exogenously added sphingosine or S1P changed the levels of antioxidant enzymes in MAECs. These results suggest that alterations in ceramide composition or the content of sphingosine or S1P play an important role in changing these levels. We therefore suggest that changes in the levels of antioxidant enzymes and resultant $K_{Ca}3.1$ upregulation in MAECs from aged wild-type and young CerS2 null mice are caused by an altered sphingolipid profile (i.e., increased C16-sphingolipid, decreased C22-C24-sphingolipid, or increased long chain bases such as sphingosine or sphinganine).

Studies have shown that H_2O_2 enhances the tyrosine phosphorylation of Src kinases and mitogen-activated protein kinases, leading to activation of gene expression, including activator protein-1 (Hardwick & Sefton, 1997; Jaramillo & Olivier, 2002; Gaitanaki *et al.*, 2003). Consistent with these results, H_2O_2 increased the phosphorylation of the Src family kinase Fyn. H_2O_2 and Fyn play critical roles in ERK activation (Toni *et al.*, 2006; Choi *et al.*, 2013b). As $K_{Ca}3.1$ synthesis occurs via an ERK/activator protein-1-mediated pathway, $K_{Ca}3.1$ synthesis might be induced via a H_2O_2/Fyn/ERK-mediated pathway. However, as the expression of membrane proteins in the plasma membrane is determined by a balance between synthesis/forward trafficking from the

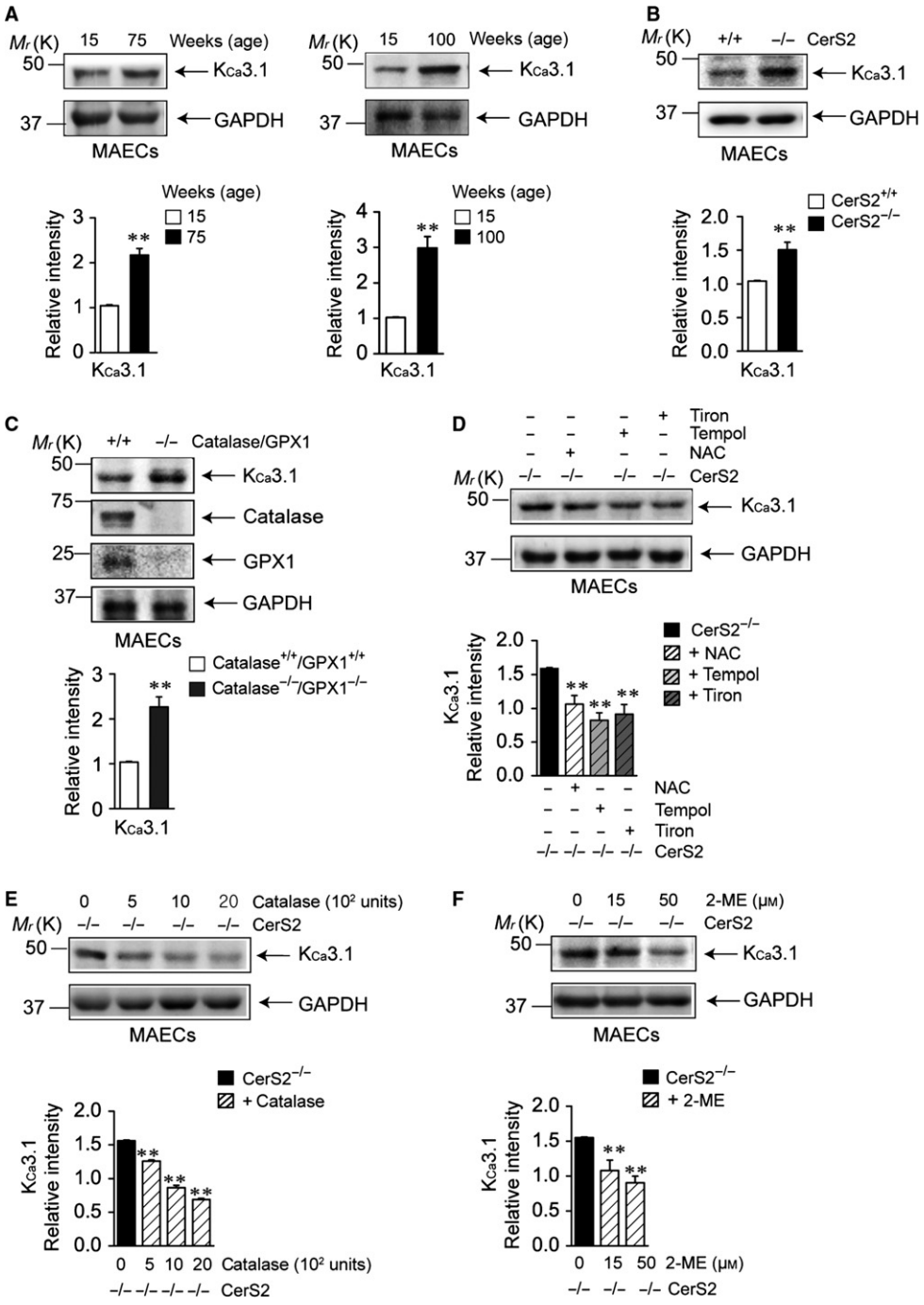

Fig. 3 H_2O_2 upregulates endothelial $K_{Ca}3.1$. Protein levels of $K_{Ca}3.1$ were examined in mouse aortic endothelial cells (MAECs) from young and aged wild-type mice (A), in MAECs from young CerS2 null and age-matched wild-type mice (B), in MAECs from young catalase$^{-/-}$/GPX1$^{-/-}$ and age-matched wild-type mice (C), in CerS2 null MAECs treated with tiron, tempol, or NAC (D), or in CerS2 null MAECs treated with catalase (E) or 2-ME (F). Blots are representatives of four experiments performed with four different cultures. Results were normalized to GAPDH levels. $^{**}P < 0.01$ vs. young, age-matched wild-type (A–C), or vehicle-treated CerS2 null (D–F) MAECs.

endoplasmic reticulum, endocytosis, and recycling/degradation, further studies are required to clarify the effects of altering the sphingolipid composition on these processes.

There is now a wealth of evidence suggesting that oxidative stress is a major cause of endothelial dysfunction (Gori & Munzel, 2011). However, signaling molecules of the ROS pathway, such as H_2O_2, participate in the modulation of various cellular activities including cell proliferation, differentiation, and apoptosis (Sies, 2014). Our results clearly showed that H_2O_2 affected endothelial function by increasing $K_{Ca}3.1$ expression. As consistent with previous evidence in intact microvascular endothelial tubes (Behringer *et al.*, 2013), H_2O_2 activated $K_{Ca}3.1$ currents in aortic ECs (Choi *et al.*, 2013b). Endothelial dysfunction, which was manifested in the form of diminished NO bioavailability, was well compensated for by an increased expression of $K_{Ca}3.1$ protein, as shown in aged wild-type, CerS2 null, and catalase$^{-/-}$/GPX1$^{-/-}$ mice, and an upregulation of $K_{Ca}3.1$ channel activity.

The $K_{Ca}3.1$ activator-induced relaxation resistant to L-NOARG and indomethacin might be mediated by $K_{Ca}3.1$ activation-induced endothelial hyperpolarization. This suggestion is supported by the following observations: $K_{Ca}3.1$ activator-induced relaxation response was completely absent after denudation of the endothelium, demonstrating its dependency on the presence of intact ECs. The absence of the relaxation

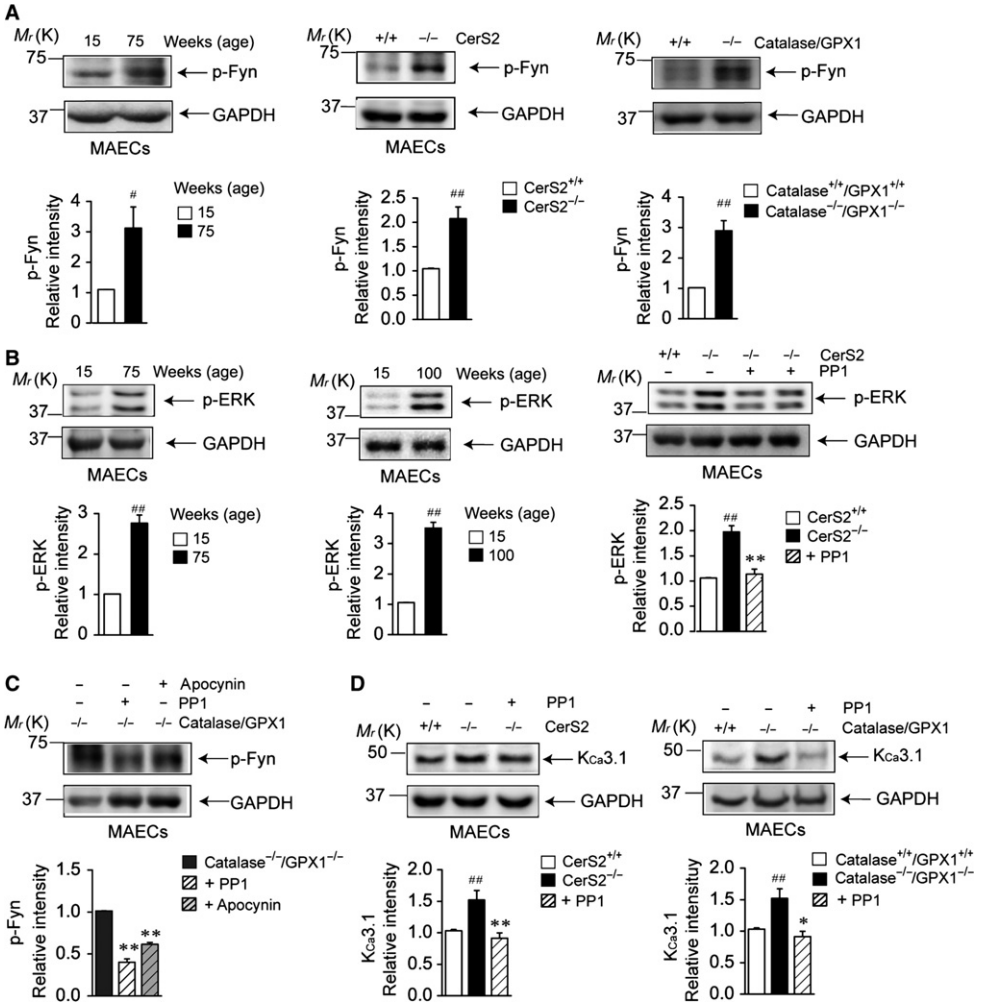

Fig. 4 Fyn and ERK is involved in endothelial $K_{Ca}3.1$ upregulation. (A) Protein levels of p-Fyn in aged vs. young wild-type, young CerS2 null vs. age-matched wild-type, and young catalase$^{-/-}$/GPX1$^{-/-}$ vs. age-matched wild-type mouse aortic endothelial cells (MAECs). (B) Protein levels of p-ERK in aged vs. young wild-type and CerS2 null vs. age-matched wild-type MAECs. Increased p-ERK levels were reduced by the Fyn inhibitor PP1. (C) Increased p-Fyn levels were reduced by the NADPH oxidase inhibitor apocynin or by PP1 in catalase$^{-/-}$/GPX1$^{-/-}$ MAECs. (D) $K_{Ca}3.1$ upregulation were reduced by PP1 in CerS2 null and catalase$^{-/-}$/GPX1$^{-/-}$ MAECs. Blots are representatives of four experiments performed with four different cultures. Results were normalized to GAPDH levels. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. young, age-matched, or vehicle-treated wild-type. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. vehicle-treated CerS2 null or catalase$^{-/-}$/GPX1$^{-/-}$ MAECs.

effect of the $K_{Ca}3.1$ activators on vascular smooth muscle can be explained by the absence of $K_{Ca}3.1$ in vascular smooth muscle cells. In addition, the portion of the relaxation resistant to L-NOARG and indomethacin was induced by the $K_{Ca}3.1$ activators 1-EBIO or NS309, suggesting that this portion of EDR is mediated through $K_{Ca}3.1$ activation. Furthermore, the portion of the relaxation resistant to L-NOARG and indomethacin was enhanced in the aortic rings in which endothelial $K_{Ca}3.1$ was upregulated, suggesting that the magnitude of the resistant part of EDR is closely related with $K_{Ca}3.1$ levels.

The $K_{Ca}3.1$ activator 1-EBIO or NS309 also activates $K_{Ca}2.3$ with similar potency (Coleman *et al.*, 2014). $K_{Ca}2.3$ shares many properties with $K_{Ca}3.1$ (Jensen *et al.*, 2001) and it has been implicated in endothelium-dependent dilation (Grgic *et al.*, 2009). In addition, it was found that mRNA and protein levels of $K_{Ca}2.3$ were significantly increased in MAECs or aortic tissues from aged wild-type and CerS2 null mice (Fig. S6). These results suggest that $K_{Ca}2.3$ activation-induced endothelial hyperpolarization contributes to 1-EBIO- or NS309-induced relaxation resistant to L-NOARG and indomethacin. Further studies are required to investigate the mechanisms underlying $K_{Ca}2.3$ upregulation in MAECs from aged wild-type and CerS2 null mice.

Endothelium-dependent relaxation induced by endothelial hyperpolarization or endothelium-derived hyperpolarizing factor is more important in the smaller arteries than in the large conductance arteries (Sandow & Hill, 2000). In agreement, $K_{Ca}3.1$ activator-induced EDR resistant to L-NOARG was negligible in aortas from young wild-type mice. When increased oxidative stress impaired NO bioavailability and L-NOARG-sensitive EDR in aged wild-type and CerS2 null mice, $K_{Ca}3.1$ contributed to the maintenance of endothelial vasodilator function by enhancing endothelium-dependent hyperpolarization-mediated vasodilation. A similar enhancement of EDR, which is resistant to L-NOARG and indomethacin, and a similar contribution of $K_{Ca}3.1$ and/or $K_{Ca}2.3$ to EDR were reported to occur in arteries from rats with obesity (Chadha *et al.*, 2010; Climent *et al.*, 2014), type 2 diabetes mellitus (Schach *et al.*, 2014), and spontaneous hypertension (Giachini *et al.*, 2009; Simonet *et al.*, 2012).

The present study has some limitations that need to be acknowledged. First, EC phenotypes might be changed after isolation and culture, because $K_{Ca}1.1$ upregulation (Sandow & Grayson, 2009) and $K_{Ca}3.1$ downregulation (data not shown) often occur in cultured ECs. Nevertheless, EC isolation and culture is necessary to examine the levels of proteins such as antioxidants in ECs, because these proteins are expressed not only in ECs but also in vascular smooth muscle cells. In addition, cultured ECs are necessary to examine the mechanism by which expression levels of $K_{Ca}3.1$ or $K_{Ca}2.3$ are modulated. As expression levels of $K_{Ca}3.1$ and $K_{Ca}2.3$ in MAECs are well maintained within 2 passages, we used MAECs within 2 passages in the present study. Aging-related increases in $K_{Ca}2.3$ expression levels were similar in aortic tissues and cultured MAECs within two passages (Fig. S6C,D).

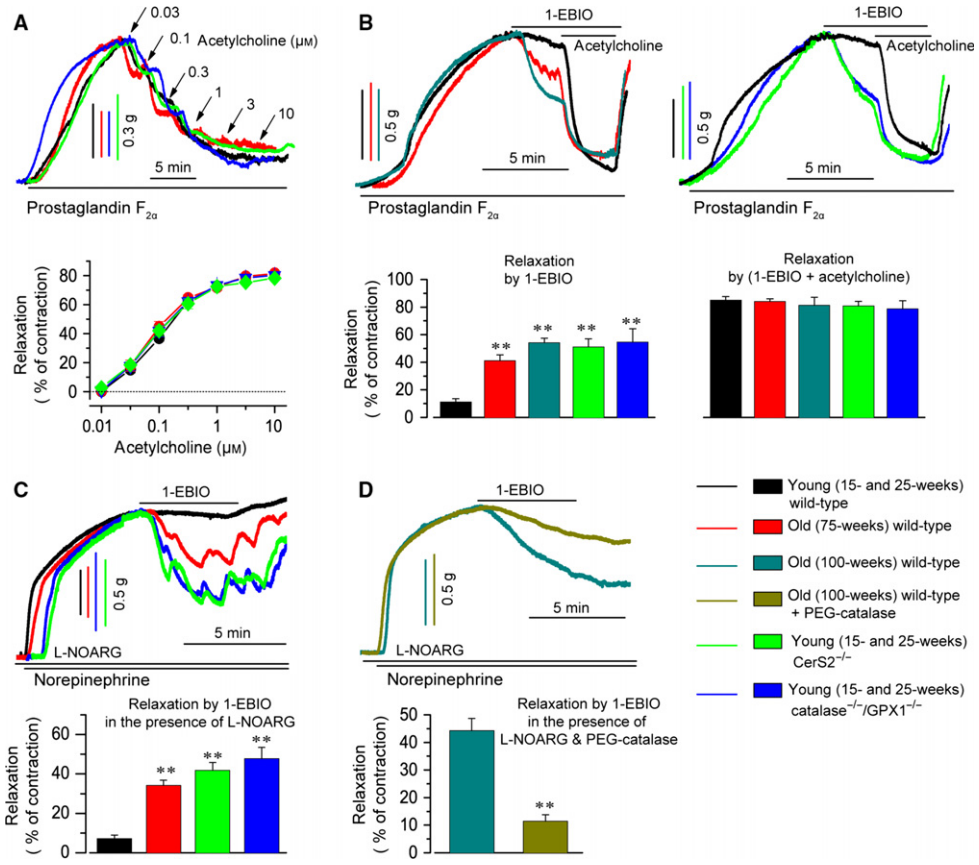

Fig. 5 $K_{Ca}3.1$ activation-induced, N^{ω}-nitro-L-arginine (L-NOARG)- and indomethacin-resistant EDR in aorta. EDR was evoked in aortic rings from young wild-type, aged wild-type, young CerS2 null, and young catalase$^{-/-}$/GPX1$^{-/-}$ mice. (A) Acetylcholine induced EDR in a concentration-dependent manner. (B) The $K_{Ca}3.1$ activator 1-EBIO- and (1-EBIO + acetylcholine)-induced EDR was evoked in aortic rings without L-NOARG pretreatment. (C) Nitric oxide (NO) production was inhibited by L-NOARG pretreatment, and 1-EBIO-induced EDR was evoked. (D) Aortic rings were treated with PEG-catalase to reduce H_2O_2 and $K_{Ca}3.1$ levels, and then 1-EBIO-induced EDR was evoked in the presence of L-NOARG. (A-D) In each experiment, one aortic ring was obtained from each mouse, and graphs were computed with pooled data from 10 experiments (young wild-type mice) and four or five experiments (75- and 100-week-old, young Cers2 null, catalase$^{-/-}$/GPX1$^{-/-}$ mice). The magnitude of maximal relaxation at each treatment was expressed as a percentage of initial prostaglandin $F_{2\alpha}$- or norepinephrine-induced contraction. $^{**}P < 0.01$ vs. young wild-type.

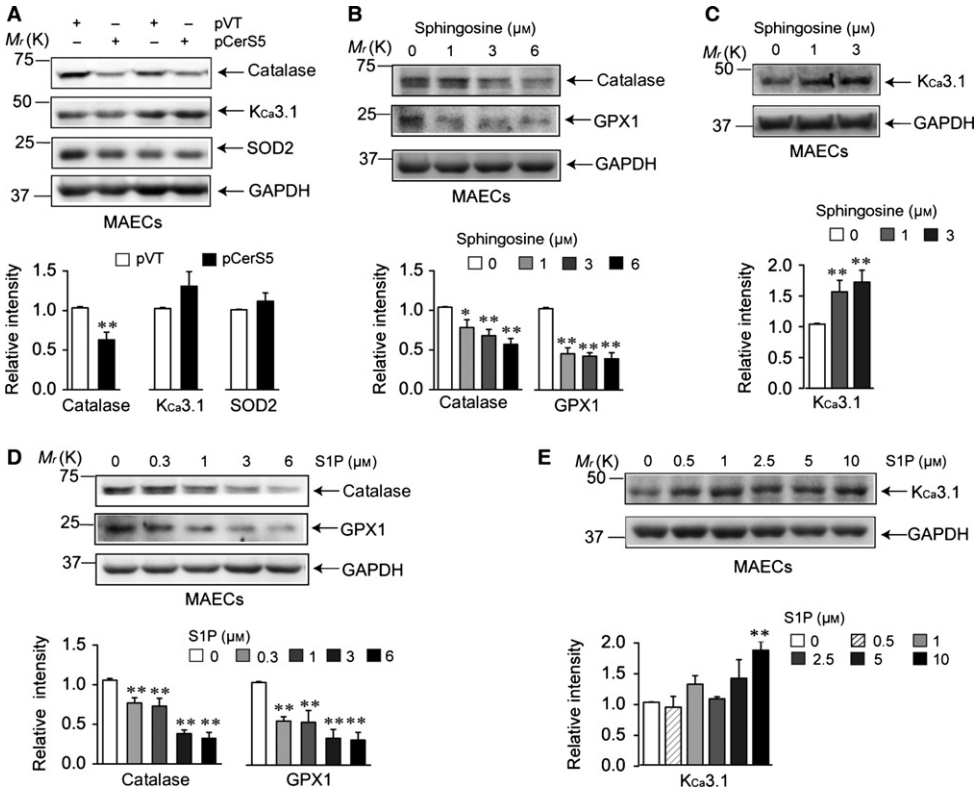

Fig. 6 Levels of $K_{Ca}3.1$ and antioxidant enzymes are affected by alteration in sphingolipid composition. After wild-type mouse aortic endothelial cells (MAECs) were transfected with CerS5 (A) or treated with sphingosine (B,C) or S1P (D,E), protein levels of catalase, GPX1, SOD2, and $K_{Ca}3.1$ were measured. Blots are representatives of three to four experiments performed with three to four different cultures. Results were normalized to GAPDH levels. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. wild-type MAECs transfected with empty vector (A), or treated with vehicle (B–E).

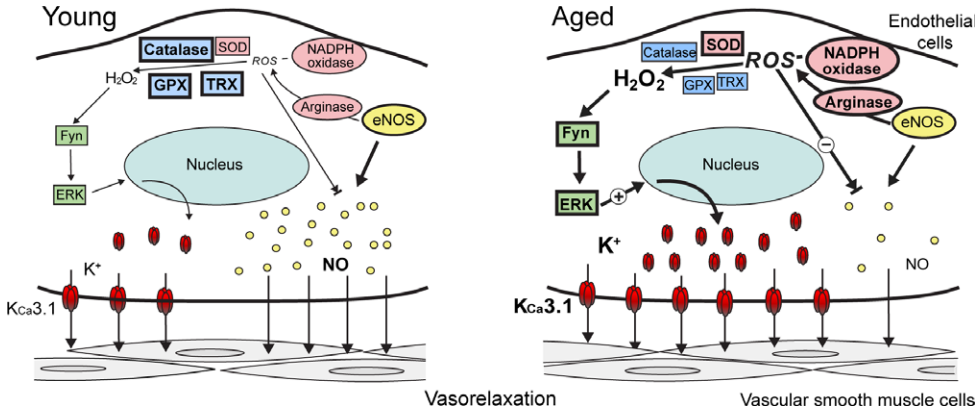

Fig. 7 Model for endothelial $K_{Ca}3.1$ upregulation in aging-related oxidative stress conditions. Sphingolipid composition was altered in aged mice, and altered sphingolipid profile (i.e., increased C16-sphingolipid, sphingosine, or sphinganine) increased H_2O_2 content in mouse aortic endothelial cells by upregulating SOD and downregulating catalase and GPX1. In addition, upregulation of arginase and NADPH oxidase enhanced ROS production. H_2O_2 enhanced $K_{Ca}3.1$ expression via a Fyn/ERK-mediated pathway, and thereby augmented $K_{Ca}3.1$ activation-induced EDR. In contrast, enhanced ROS production reduced NO bioavailability, and thereby might impair NO-induced EDR. Thus, enhanced expression of $K_{Ca}3.1$ compensates for diminished EDR to NO during aging-related oxidative stress. SOD, superoxide dismutase; GPX1, glutathione peroxidase 1; ROS, reactive oxygen species; EDR, endothelium-dependent relaxation; NO, nitric oxide.

Second, compared with conduit arteries such as aorta, resistant arteries and arterioles play more important roles in maintaining vascular homeostasis by regulating total peripheral resistance and blood flow. Thus, small arteries such as branches of superior mesenteric arteries might be more suitable than aorta for the present study. However, it is not practical (using current techniques) to obtain enough ECs within two passages for molecular determinations using small arteries from mice. In contrast, we could obtain enough ECs within two passages for one molecular experiment using aortas.

In conclusion, our data suggest that alteration in sphingolipid acyl chain length and composition induces ROS, especially H_2O_2, generation by changing the levels of antioxidant enzymes and thereby causes endothelial dysfunction as manifested by reduced NO bioavailability (Fig. 7). On the other hand, H_2O_2 induces $K_{Ca}3.1$ upregulation via the H_2O_2/Fyn/ERK-signaling pathway, and thereby compensates for a diminished NO-induced EDR response by enhancing $K_{Ca}3.1$ activation-induced EDR. To the best of our knowledge, this is the first study to demonstrate the signaling mechanisms underlying a compensatory role for endothelial $K_{Ca}3.1$ in mediating vasorelaxation during old age and under conditions of oxidative stress. Such plasticity within the EDR mechanisms presents a significant potential target for therapeutic intervention.

Experimental procedures

For description of measurement of intracellular ROS, measurement of intracellular NO, Western blotting, PCR, transfection, LC-MS/MS analysis of SLs, and chemicals, please refer the Appendix S1.

Animals

CerS2 null mice were generated as described (Pewzner-Jung *et al.*, 2010), and catalase$^{-/-}$/GPX1$^{-/-}$ mice were generously donated by Dr. Ye-Shih Ho (Wayne State Medical School, Detroit, MI) (Johnson *et al.*, 2010). We studied CerS2 null mice (15- or 25-week-old; n = 120) and age-matched wild-type (F1 of 129S4/SvJae × C57BL/6) mice (n = 85), GPX1$^{-/-}$/catalase$^{-/-}$ mice (15- or 25-week-old; n = 22) and age-matched C57BL/6 wild-type mice (n = 18), and aged (75- and 100-week-old; n = 25 and 18, respectively) and young (15-week-old; n = 30) C57BL/6 wild-type mice. In all mice types, 15- or 25-week-old mice were classified as young, and 75- or 100-week-old mice were as aged. Mice were anesthetized by an intraperitoneal injection of pentobarbital sodium (50 mg kg^{-1} body weight). 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

All mice were genotyped using polymerase chain reaction. The mice were fed with an autoclaved diet and water *ad libitum*. MAECs were isolated from the mouse aortas as described (Choi *et al.*, 2015a). Briefly, periadventitial fats and connective tissues around the aorta were carefully cleaned in Ca^{2+}-free phosphate-buffered saline under a dissecting microscope. Matrigel (BD Biosciences, San Jose, CA, USA) was plated and polymerized at 37 °C for 30 min. After that, aorta pieces were placed with the intima side down on the Matrigel. To demonstrate the endothelial nature of the cell, 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate-labeled acetylated low-density lipoprotein (Biomedical Technologies Inc., Stoughton, MA, USA) uptake assay was employed. MAECs were used within two passages and not above three passages. Each time we isolated and cultured MAECs, the thoracic aortas were dissected out from two or three mice. MAECs cultured from each aorta were pooled together and used in each experiment.

Contraction measurement on isolated aortic rings

Mice were anesthetized by an intraperitoneal injection of pentobarbital sodium (50 mg kg^{-1} body weight). The thoracic aorta was dissected out and cut into rings of about 1 mm. Mechanical responses were recorded from the aortic ring segments using a custom myograph. Each aortic ring was threaded with two strands of tungsten wire (120 μm in diameter). One wire was anchored in the organ bath chamber (1 mL) and the other was connected to a mechanotransducer (Grass, FT-03) mounted on a three dimensional manipulator. Optimal resting tension (0.6–0.8 g) was applied. The muscle chamber was perfused at a flow rate of

2.5 ml min^{-1} with oxygenated (95% O_2/5% CO_2) Krebs/Ringer bicarbonate solution with a peristaltic pump. The composition (in mM) of the Krebs solution was NaCl 118.3, KCl 4.7, $MgCl_2$ 1.2, KH_2PO_4 1.22, $CaCl_2$ 2.5, $NaHCO_3$ 25.0, glucose 11.1, pH 7.4.

Statistical analysis

Data represent the mean ± SEM of the experiments performed with aortas or MAECs. MAECs were isolated and cultured from aortas of two (young) or three (aged) mice at a time. MAECs cultured from each aorta were pooled together and used in each experiment (n = 1). To measure the strength of a contraction, one aortic ring was obtained from each mouse (n = 1), and graphs were computed with pooled data from 4 to 10 experiments. To examine the statistical significance between groups, one-way analysis of variance (ANOVA) with Bonferroni's *post hoc* or two-tailed Student's *t*-test was used. A P value of 0.05 or lower was considered statistically significant. Calculations were performed with SPSS 14.0 for Windows (SPSS, Chicago, IL, USA).

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Author contributions

S Choi and SH Suh performed study concept and design, obtained funding. S Choi, JA Kim, H Li, and KO Shin performed experiments; S Choi, GT Oh, YM Lee, S Oh, and SH Suh performed analysis and interpretation of data; Y Pewzner-Jung generated CerS2 null mice; S Choi, Y Pewzner-Jung, AH Futerman, and SH Suh contributed to writing the manuscript.

Conflict of interests

None declared.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Appendix S1 Supplementary materials and methods.

Fig. S1 Changes in levels of TRXs in MAECs from CerS2 null mice.

Fig. S2 Changes in levels of NO, ARG2 and NOX2 in MAECs from CerS2 null, catalase$^{-/-}$/GPX$^{-/-}$, and aged wild-type mice.

Fig. S3 L-NOARG- and indomethacin-resistant EDR by NS309.

Fig. S4 PEG-catalase reduced KCa3.1 levels in aged MAECs.

Fig. S5 Changes in the levels of CerS and SLs in aorta or MAECs from CerS2 null mice.

Fig. S6 Changes in expression levels of KCa2.3 in MAECs or aorta from CerS2 null, and aged wild-type mice.