

# Alterations of store-operated calcium entry and cyclopiazonic acid-induced endothelium-derived relaxations in aging rat thoracic aorta

Y Erac<sup>1</sup>, C Selli<sup>1</sup>, M Tosun<sup>2</sup>

<sup>1</sup>Department of Pharmacology, Faculty of Pharmacy, Ege University, Izmir, Turkey

<sup>2</sup>Department of Pharmacology, School of Medicine, Izmir University of Economics, Izmir, Turkey

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The purpose of our study was to investigate whether endothelium-derived relaxations induced by store depletion are altered in aging rat thoracic aorta. Vascular responses were measured in aortic segments isolated from young (2–4 month) and old (20–24 month) male Sprague-Dawley rats. In phenylephrine-contracted intact tissues, receptor-mediated and receptor-independent endothelium-derived relaxations were induced by acetylcholine (ACh) and sarcoplasmic/endoplasmic reticulum  $\text{Ca}^{2+}$  ATPase (SERCA) blocker cyclopiazonic acid (CPA), respectively. In addition, CPA-induced changes in intracellular calcium levels were monitored in fura-2-loaded endothelium-denuded tissues. Real-time quantitative reverse transcription polymerase chain reaction and western blot analysis were performed to determine the transient receptor potential canonical (TRPC) 4 mRNA and protein levels. Endothelial TRPC4 mRNA levels were apparently decreased in aging rats. Immunoblot analysis showed that TRPC4 protein levels significantly decreased in intact aorta from 20- to 24-month-old rats compared to that from 2- to 4-month-old rats. ACh- and CPA-induced endothelium-dependent relaxations decreased in old rat aorta without any change in direct vasodilation induced by sodium nitroprusside. Store-operated  $\text{Ca}^{2+}$  entry (SOCE) induced by CPA was significantly decreased, whereas sarcoplasmic reticulum  $\text{Ca}^{2+}$  release was unaffected in endothelium-denuded aging rat aorta. In conclusion, TRPC4 downregulation could be associated with decreased endothelium-dependent vasorelaxations. As endothelial nitric oxide synthase is activated by SOCE-induced caveolar internalization, tracking the expression levels of SERCA, ion channels, and/or associated proteins involved in SOCE would lead to the development of novel therapeutics for age-related vasospastic disorders with dysfunctional endothelium.

**Keywords:** aging, vascular responses, SOCE, endothelial dysfunction, TRPC

## Introduction

Aging is a major risk factor for the development of various cardiovascular diseases, such as atherosclerosis, hypertension, heart failure, and ischaemic heart disease. Several molecular, structural, and functional age-related alterations in cardiovascular system have been reported (59). Among these, several pathophysiological changes including endothelial dysfunction, vascular inflammation and reactivity changes, wall thickening, and arterial stiffening were observed during vascular aging (31). Age-related changes in responses to agonists such as noradrenaline and endothelin-1 (5) and in endothelium-dependent relaxations have been shown earlier (8, 14, 37, 38). Effects of aging in  $\text{Ca}^{2+}$  homeostasis and signaling pathways have also been implicated in several cardiovascular diseases (4, 13, 16). Physiological processes in vascular smooth muscle are mainly regulated by the changes in spacio-temporal

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Corresponding author: Professor Dr. Metiner Tosun, PhD

Department of Pharmacology, School of Medicine, Izmir University of Economics

Sakarya Cad. 156, 35330 Izmir, Turkey

Phone: +90 232 488 9843; Fax: +90 232 279 2626; E-mail: [metiner.tosun@izmirkonomi.edu.tr](mailto:metiner.tosun@izmirkonomi.edu.tr)

cytosolic  $\text{Ca}^{2+}$  levels ( $[\text{Ca}^{2+}]_i$ ); therefore, it is essential to delineate the changes that affect different  $\text{Ca}^{2+}$  entry mechanisms during aging.

Vascular reactivity is mainly dependent on  $[\text{Ca}^{2+}]_i$  that is controlled by  $\text{Ca}^{2+}$  channels on plasmalemma and sarcoplasmic reticulum (SR) membrane (15, 33). Store-operated  $\text{Ca}^{2+}$  entry (SOCE) activated by SR depletion also contributes to regulation of  $[\text{Ca}^{2+}]_i$ . Depletion of intracellular  $\text{Ca}^{2+}$  stores activates  $\text{Ca}^{2+}$  influx across the plasma membrane via store-operated  $\text{Ca}^{2+}$  channels (SOCCs) (43). Activation of G-protein-coupled receptors such as G-protein-coupled acetylcholine (ACh) receptors also mediates SOCE by depleting SRs via inositol-1,4,5-trisphosphate ( $\text{IP}_3$ )-induced  $\text{Ca}^{2+}$  release or sarcoplasmic/endoplasmic reticulum  $\text{Ca}^{2+}$  ATPase (SERCA) inhibition by cyclopiazonic acid (CPA) or thapsigargin (28, 30, 40, 53). Both ACh and CPA increase  $[\text{Ca}^{2+}]_i$  through SOCE in endothelial cells yielding nitric oxide (NO) production that eventually leads to vascular smooth muscle relaxation (34, 39). CPA also induces endothelium-dependent vasorelaxations in intact rat aorta (44). On the other hand, exogenous NO donor, sodium nitroprusside (SNP) directly induces vascular smooth muscle relaxation through the activation of guanylate cyclase (GC) elevating cyclic guanosine monophosphate (cGMP) levels (12).

SOCE plays important roles in vasculature including endothelial proliferation, vascular tone and remodeling, and vascular smooth muscle cell migration and proliferation (1, 2, 7, 36, 42, 47). In addition, SOCCs have been shown to be associated with several cardiovascular diseases, such as idiopathic pulmonary hypertension, hypoxic pulmonary vasoconstriction, cardiac hypertrophy, aneurysm, and arrhythmia (26, 61). Although SOCE was downregulated in skeletal muscle of aging mice (9, 54), there is no report about age-dependent changes in SOCE function and capacity in vascular smooth muscle cells. Among the members of the transient receptor potential canonical (TRPC) protein family, TRPC1, TRPC3, and TRPC4 have been suggested to be the molecular components of SOCCs (3). TRPC1 contributes to SOCE by interacting with the basic components of SOCE, stromal interaction molecule (STIM1), and Orai1 (10). TRPC1 and TRPC6 are the most predominant members of TRPC ion channels expressed in vascular smooth muscle (17). Abundantly expressed in vascular endothelial cells, TRPC1 mediates SOC activity in endothelial cells by forming heteromultimeric channels with TRPC4 (34, 49, 50). Furthermore, caveolar scaffolding protein caveolin-1 (Cav-1) has been suggested to regulate SOCE by binding to TRPC1 (35). On the other hand, Cav-1 directly interacts with endothelial nitric oxide synthase (eNOS) via its scaffolding domain (aa 89–101) (21, 29) and Cav-1 knock-out studies confirm the negative role of Cav-1 on eNOS (46).

In the present study, we investigated whether CPA-induced endothelial relaxations change during aging. For this purpose, receptor-dependent (ACh-mediated) and receptor-independent (CPA-mediated) endothelial relaxations were evaluated both in intact young and old rat aorta. Furthermore, SOCE-associated  $\text{Ca}^{2+}$  dynamics in endothelium-denuded vessels were also monitored. Delineating the involvement of SOCE in vasoconstriction/relaxation may be essential for the development of effective treatment strategies for age-related vascular disorders.

## Materials and Methods

### Animals

All experiments were approved by the Institution's Committee on Animal Use in Research and Education, Ege University, and Bilkent University. Rats (Sprague-Dawley, male,  $n = 20$ ) were maintained in appropriate conditions with *ad libitum* access to food and water, standard

temperature and humidity, and a 12-h light/dark cycle. Rats were longitudinally aged and tested at two age intervals (in months, 2–4, young and 20–24, old) (14).

#### *Real-time quantitative RT-PCR*

Endothelial RNA was collected by passing of 500 µl guanidinium thiocyanate containing lysis solution (TriPure, Roche Applied Science) once through the aortic lumen using 22-gauge needle. Endothelial lysates from three samples were pooled (45). Total RNA isolated according to manufacturer's instructions (TriPure, Roche Applied Science). RNA concentrations were calculated by measuring the absorbance at 260 nm. cDNA was synthesized using oligo(dT) primers (RevertAid First Strand cDNA Synthesis Kit, Fermentas). To determine the relative expression levels of TRPC4, real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed using FastStart DNA Master SYBR Green I kit and LightCycler 2.0 (Roche Applied Science). A detailed protocol was published previously (19). The primers are listed in Table I. Expression levels were normalized to that of internal β-actin ( $[\text{TRPCx}]/[\beta\text{-actin}] \times 10^4$ ).

#### *Western blot analysis*

Protein samples were prepared from intact thoracic aorta by homogenization in lysis solution (Camiolo buffer, 75 mM potassium acetate, 300 mM NaCl, 10 mM EDTA, 100 mM L-arginine basic salt, and 0.25% Triton-X 100, protease inhibitor mix). Protein concentrations were determined using Bradford assay. Proteins were separated on 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to membranes (Immobilon-P polyvinylidene difluoride, Millipore) at 25 °C for 2 h at 15 V. Following 2-h blocking (with 5% skimmed milk in Tris-buffered saline with Tween-20), membranes were incubated with TRPC4 primary antibody (1:200, Alomone Laboratories) and anti-β-actin (1:1,500, Abcam Ltd.) overnight at 4 °C, then with horseradish peroxidase (HRP)-conjugated goat antirabbit secondary antibody (DakoCytomation; 1:1,500) for 1 h at room temperature. ECL Plus Detection kit (Amersham Biosciences) was used to visualize bands, and the optical density of each blot was normalized to that of β-actin analyzed within the same lane and represented as relative optical density.

#### *Isometric force*

A detailed protocol for isolated tissue experiments was described previously (56). Briefly, rats were asphyxiated with CO<sub>2</sub>, thoracic aorta was removed and dissected into 3-mm rings. Tissues were mounted in organ chambers. After a 45-min-equilibration period, each ring was gradually stretched in 0.5-g increments to the previously established optimal point of the resting tension (20 mN) for rat aorta. The presence of functional endothelium was confirmed by the ACh-induced relaxations on phenylephrine (PE)-precontracted aortic rings. Tissue rings were

Table I. Oligonucleotide sequences of RT-PCR primers

| Gene    | GenBank accession no. | Primer sequence (5'-3')   | Amplicon size, bp |
|---------|-----------------------|---------------------------|-------------------|
| TRPC4   | NM_053434             | F: CTGCAGATATCTCTGGGAAGA  | 412               |
|         |                       | R: GCTTTGTTTCGAGCAAATTTCC |                   |
| β-actin | NM_031144             | F: AGTGTGACGTTGACATCCGT   | 244               |
|         |                       | R: GACTCATCGTACTCCTGCTT   |                   |

TRPC: transient receptor potential; F: forward; R: reverse; and bp: base pair.

placed in organ baths containing physiological salt solution (PSS) (in mM: NaCl, 118; KCl, 4.73; MgSO<sub>4</sub>, 1.2; CaNa<sub>2</sub>EDTA, 0.026; NaH<sub>2</sub>PO<sub>4</sub>, 1.2; CaCl<sub>2</sub>, 2.5; NaHCO<sub>3</sub>, 25; and glucose, 11, 37 °C) aerated with 95% O<sub>2</sub>–5% CO<sub>2</sub>. All organ bath measurements were recorded with a data acquisition system (MP100, Biopac, USA). Force was normalized to cross-sectional area [force (in mN)/cross-sectional area (in mm<sup>2</sup>, F/CSA) = (change in force × circumference)/2 × wet wt.]. Receptor-independent endothelium-derived relaxations were induced by CPA. To evaluate direct relaxing activity of vascular smooth muscle, soluble GC stimulator, an SNP was used. Both agents were added into the organ baths in a cumulative manner.

#### *Changes in intracellular Ca<sup>2+</sup> levels*

[Ca<sup>2+</sup>]<sub>i</sub> elevations were monitored in aortic segments as described earlier (57). Briefly, vessels were inverted, deendothelialized, and mounted between two stainless-steel hooks the upper one was connected to an isometric force–displacement transducer (Kent Scientific Instruments, Litchfield, CT) attached to a displacement unit allowing a fine adjustment of tension via a micrometer. Aortic ring was gradually stretched to optimal resting tension as in the isometric force experiments, then, incubated in a glass vial for 2 h with 5 μM fura-2/AM plus 0.2 mM neostigmine, 1 mM probenecid, and 0.02% pluronic F-127 in PSS at room temperature in the dark. After the incubation period, the whole apparatus was mounted in a spectrophotometer cuvette and placed in a water-jacketed (37 °C) sample chamber of a dual-wavelength spectrofluorometer (PTI QM8/2005, Photon Technology International). The aortic segment was perfused (12 ml/min) with 37 °C gassed PSS containing 3-mM indomethacin and 1-mM probenecid for 30 min before agent addition. The intimal surface of the fura-2-loaded tissue was subjected to alternating excitation wavelengths of 340 and 380 nm/s. Intensity changes in emitted fluorescence at 510 nm corresponding to the ratio of 340–380 nm excitations (F<sub>340/380</sub>) is reported as a relative measure of free [Ca<sup>2+</sup>]<sub>i</sub> (57). For SOCE protocol, CPA is added after 5-min incubation period in Ca<sup>2+</sup>-free buffer. Following a complete CPA-induced Ca<sup>2+</sup> transient (SR Ca<sup>2+</sup> release), Ca<sup>2+</sup> (2.5 mM) is readded to induce Ca<sup>2+</sup> influx.

#### *Chemicals*

All chemicals were from Sigma and dissolved in appropriate solvents as given: CPA (10<sup>-1</sup> M) in dimethylsulfoxide; PE HCl (10<sup>-1</sup> M) in distilled water (DW); ACh (10<sup>-1</sup> M) in DW; and SNP (10<sup>-2</sup> M) in DW.

#### *Data analysis*

Data analysis and graphical presentations were done using GraphPad Prism5. Values of maximal effect ( $E_{max}$ ) and 50% effective concentration (EC<sub>50</sub>) were derived for each cumulative concentration–response curve using nonlinear regression analysis (GraphPad Prism 5 Software). pD<sub>2</sub> values were calculated as  $-\log(EC_{50})$ . Geometric means of the pD<sub>2</sub> values were compared. The results were given as mean ± standard error of the mean (S.E.M.). “*n*” represents the number of animals. The significance of differences was evaluated by Student’s *t*-test. *P* < 0.05 was considered significant.

## **Results**

#### *TRPC4 expression levels*

We previously observed significant changes in TRPC1 and TRPC6 protein expression in endothelium-denuded aging rat aorta (19). Based on these data, we further hypothesized that

TRPCs would also be differentially expressed in endothelium. Due to the limited amount of endothelial samples, endothelial RNA samples isolated from three different animals were pooled (pooled samples in duplicate). While no change was observed in vascular smooth muscle TRPC1, TRPC3, and TRPC6 mRNA levels (19), endothelial TRPC4 levels were apparently decreased in aging rat (Fig. 1A). TRPC4 protein levels significantly decreased in intact aortas of 20 to 24-month-old rats compared to that of 2–4-month-old rats (Fig. 1B;  $n = 3-4$ ;  $P < 0.05$ ).

#### *ACh- and SNP-induced endothelium-dependent relaxations*

First of all, we investigated endothelium-dependent and endothelium-independent relaxations. For this purpose, ACh and NO-donor SNP were applied in cumulative manner ( $10^{-9}$ – $10^{-4}$  M and  $10^{-10}$ – $10^{-6}$  M, respectively) on PE (300 nM)-contracted intact aortic rings of young and old rats. Absolute magnitude of precontraction levels (F/CSA) was similar in all cases (cumulative ACh, SNP, and CPA concentration–response curves). Consistent with previous reports, ACh maximal responses were significantly reduced with aging without affecting the sensitivity ( $pD_2$  values) (Fig. 2A;  $n = 3-4$ ). On the contrary, SNP abolished PE-induced contractions in both young and old vessels. Aging had no effect on maximal responses ( $E_{max}$ ) or  $pD_2$  values of SNP (Fig. 2B;  $n = 4-5$ ).

#### *CPA-induced endothelium-dependent relaxations*

We further investigated the possible changes in receptor-independent endothelium-derived relaxations with aging. For this purpose, selective SERCA inhibitor CPA was applied in cumulative manner ( $10^{-7}$ – $10^{-5}$  M) on PE (300 nM)-contracted intact aortic rings.  $E_{max}$  for CPA was significantly reduced without affecting the  $pD_2$  values in old rat aorta (Fig. 3,  $n = 3-4$ ,  $P < 0.05$ ).

#### *Effects of aging on SOCE in endothelium-denuded rat aorta*

As we observed drastic decrease in TRPC4 protein levels in aging aorta, we investigated the functional consequence of changes in their expressional levels. Since CPA-induced endothelial relaxations decreased during aging, we focused on possible changes in SOCE during aging as well. Due to the experimental limitations in fura-2 loading and monitoring  $[Ca^{2+}]_i$  changes in a single layer of endothelial cells, we measured  $[Ca^{2+}]_i$  changes in fura-2-loaded and endothelium-denuded rat aorta to extrapolate how aging affects SOCE. First, 10  $\mu$ M CPA

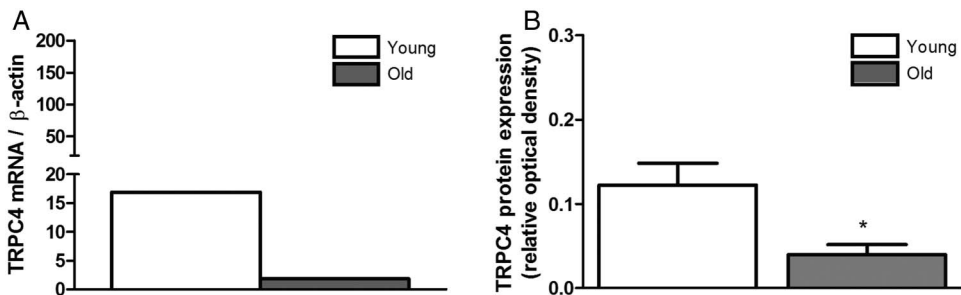


Fig. 1. Effects of aging TRPC4 expression levels. TRPC4 mRNA expression in endothelium (A, pooled samples of 3-3 animals, means of duplicates) and protein expression in intact rat aorta (B) from young and old rats ( $n = 3-4$ ,

\* $P < 0.05$ ). TRPC4: transient receptor potential canonical 4 ion channel

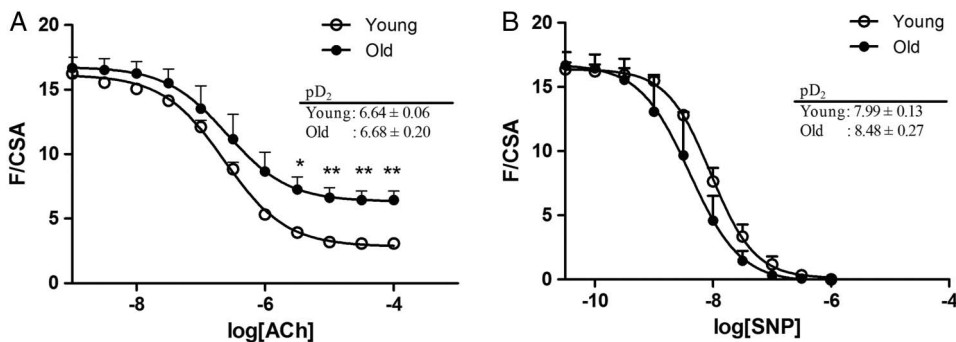


Fig. 2. Effects of aging on ACh- (A) and SNP- (B) induced relaxations. ACh ( $10^{-9}$ – $10^{-4}$  M) and SNP ( $10^{-10}$ – $10^{-6}$  M) concentration–response curves were obtained from PE-precontracted young (o) and old (•) rat aortas. Responses are expressed as F/CSA. Data are presented as mean  $\pm$  S.E.M. pD<sub>2</sub> values shown in the graph are means of the calculated individual pD<sub>2</sub> values ( $n = 3-4$  and  $n = 4-5$  for ACh and SNP, \* $P < 0.05$ ; \*\* $P < 0.01$ ). ACh: acetylcholine; SNP: sodium nitroprusside; F/CSA: force/cross-sectional area; and pD<sub>2</sub>: negative logarithm of 50% effective concentrations of agents used

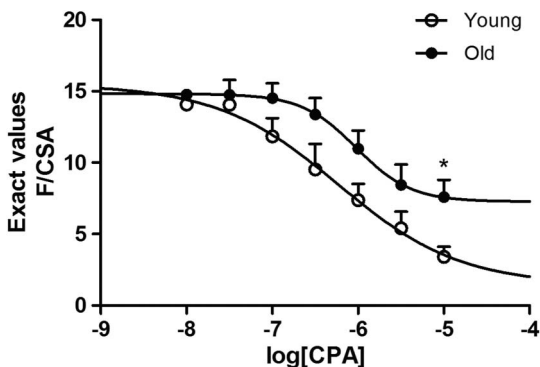


Fig. 3. Effects of aging on CPA-induced vasorelaxations. CPA ( $10^{-7}$ – $10^{-5}$  M) concentration–response curves obtained from PE-precontracted young (o) and old (•) rat aortas. Responses are expressed as F/CSA. Data are presented as mean  $\pm$  S.E.M. Average pD<sub>2</sub> values are means of the calculated individual pD<sub>2</sub> values ( $n = 3-4$ , \* $P < 0.05$ ). CPA: cyclopiazonic acid; PE: phenylephrine; F/CSA: force/cross-sectional area; and pD<sub>2</sub>: negative logarithm of 50% effective concentrations of agents used

was added in the absence of  $\text{Ca}^{2+}$ . Then, re-addition of 1.5 mM  $\text{Ca}^{2+}$  elevated amplitude of fura-2 signal. SR  $\text{Ca}^{2+}$  release was not affected by aging (Fig. 4A and C). On the other hand, CPA-induced SOCE significantly decreased in old rat aorta (Fig. 4B and C;  $n = 3-4$ ;  $P < 0.01$ ).

## Discussion

The present study shows that TRPC4 protein that was suggested to be responsible for SOCE (10) in endothelium, decreased in intact rat aorta during aging. The downregulation of endothelium-dependent ACh-induced relaxations in rat thoracic aorta during aging observed in our study is consistent with the previous reports (8, 14, 25, 37, 38). In addition to the ACh responses, we observed that CPA-induced (receptor independent) endothelial relaxations were also decreased in aorta from old rats. On the other hand, SNP-induced vasorelaxations did not change suggesting that downstream of endothelium-derived relaxing mechanisms are not affected by aging. In addition to the attenuated ACh and CPA responses, SOCE induced by SERCA inhibition in endothelium-denuded rat aorta was also decreased. The

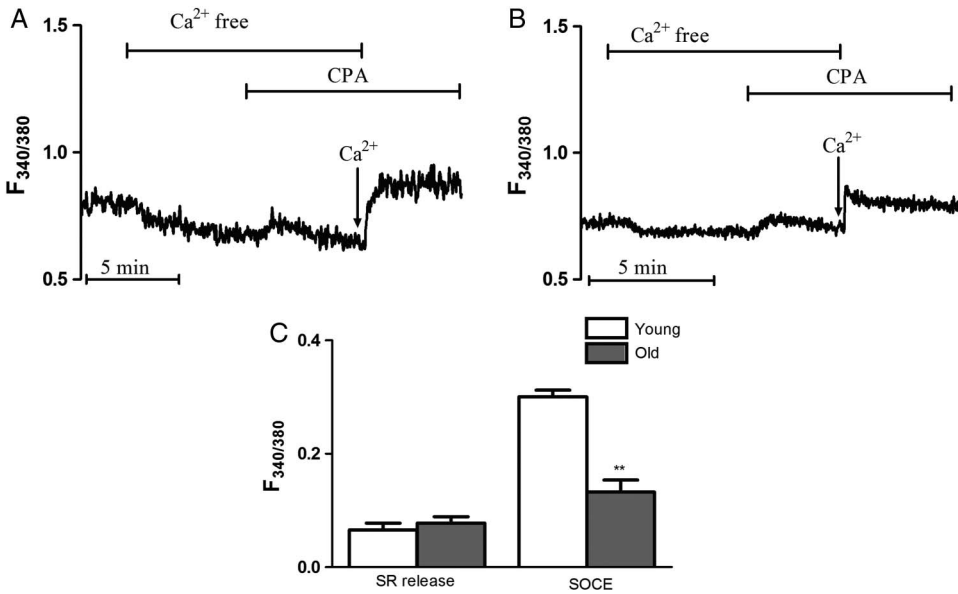
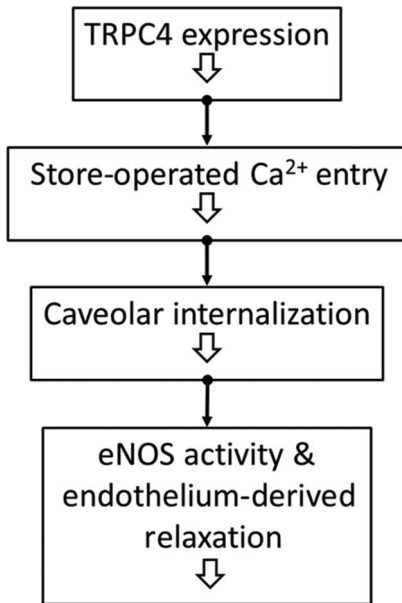


Fig. 4. Effects of aging on SR  $\text{Ca}^{2+}$  release and SOCE. Steady-state  $[\text{Ca}^{2+}]_i$  elevation was assessed by the addition of 2.5 mM  $\text{Ca}^{2+}$  following 10  $\mu\text{M}$  CPA treatment. Representative tracings obtained from young (A) and old (B) rat thoracic aorta and the mean ( $\pm$  S.E.M.) values ( $n = 3-4$ ,  $**P < 0.01$ ) of plateau fura-2 signals after  $\text{Ca}^{2+}$  readdition (C) are shown. SR: sarcoplasmic reticulum; SOCE: store-operated  $\text{Ca}^{2+}$  entry; and CPA: cyclopiazonic acid

downregulated SOCE might be resulted from a proportional loss of TRPC1 during aging as observed in our previous study (19).

As endothelium is crucially important in the regulation of vascular smooth muscle tone through several endothelium-derived relaxing and contracting factors, any imbalance between these factors results in endothelial dysfunction. Although different hypotheses have been postulated for age-related endothelial dysfunction mechanism (8, 11, 14, 23, 25, 32), the exact mechanism has not been clarified yet. Contradictory data exist on age-related changes on eNOS expression and NO production such that some show downregulation (5, 14, 52, 58), while others suggest upregulation (22, 60). Furthermore, increased production of reactive oxygen species were also reported during aging (14, 24, 27, 48, 60). Enhanced production of vasoconstricting factors also contributes to age-related endothelial dysfunction (5, 37, 38). In the present study, CPA-induced vasorelaxations were also decreased suggesting that receptor-independent signal transduction mechanisms also contribute to the endothelium-dependent changes during aging. Since SOCE is activated by SR depletion following both ACh-induced  $\text{IP}_3$  production and CPA-induced SERCA inhibition (41), decreased SOCE may contribute to the age-related endothelial dysfunction (34, 36). In addition to these, the essential component of endothelium-dependent relaxations of vascular smooth muscle cells (34), TRPC4 was decreased (present study) with aging. A number of studies have demonstrated the role of TRPCs in SOCE and vascular function (20, 41, 55). For example, in  $\text{TRPC4}^{-/-}$  mice, store-operated  $\text{Ca}^{2+}$  current decreased in endothelial cells, resulting in impaired endothelial vasodilatation (20). It was also demonstrated that decreases in TRPC4 protein levels following the endothelial denudation in rat cerebral arteries is consistent with its predominant endothelial localization (6). Bergdahl et al. have also suggested that expression of TRPC4 is well correlated





*Fig. 5.* The model summarizing the overall effects of TRPC4 downregulation in aging rat aorta. The model depicts that any decrease in TRPC4 expression is reflected as impaired  $\text{Ca}^{2+}$  entry through SOCs (see the section “Discussion” for details) eventually leading to endothelial dysfunction. TRPC4: C4 type transient receptor potential ion channel

with the amount of endothelium present in intact arteries (6). Because of the profound presence of TRPC4 in endothelium, the age-related decreases in TRPC4 protein expression observed in intact rat thoracic aorta may result from attenuated endothelial TRPC4 protein expression. Although the data did not yield a statistical significance, decreased endothelial TRPC4 mRNA expression during aging is consistent with our western blot data.

In the present study, CPA-induced SOCE amplitude decreased in endothelium-denuded rat aorta with aging. Based on our previous data, TRPC1 protein levels decreased significantly whereas that of TRPC6 increased during aging (19). Similar to the present results, SOCE has been shown to be decreased in mouse skeletal muscle during aging (18, 54). Furthermore, downregulation of TRPC1 protein by an antisense oligonucleotide also decreased SOC currents (51). As summarized in Fig. 5, it can be proposed that downregulation of TRPC4 (possibly along with TRPC1) deregulates SOC assembly and related  $\text{Ca}^{2+}$  influx in caveolar microdomains of endothelial cells precluding the removal of tonic inhibitory effect of Cav-1 on eNOS activity via preventing caveolar detachment or internalization. In conclusion, age-related downregulation of TRPC channels may impair endothelium-derived relaxations and even worsen the existing vasospasm.

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