Age-Associated Endothelial Dysfunction in Rat Mesenteric Arteries: Roles of Calcium-Activated K\(^+\) Channels (K\(_{ca}\))

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Summary
Age-associated changes in large blood vessels were characterized by increased arterial wall thickness, luminal dilation and impaired endothelial function. But little is known about the effect of age on structural and functional changes in small resistance arteries. The mechanisms underlying age-associated endothelial dysfunction in rat mesenteric resistance arteries were investigated in the present study. Small rat mesenteric arteries were excised and cannulated, and vascular endothelial functions were tested by acetylcholine (ACh). Our experiments showed (1) endothelium-dependent vasorelaxation induced by ACh was reduced in aged mesenteric arteries; (2) blockade of K\(_{ca}\) channels markedly reduced the vasodilation in young and adult rats, the resultant reduction in aged rats was much smaller compared with young and adult rats; (3) inhibition of endothelial nitric oxide synthase (NOS) resulted in a significant reduction of vasodilation in young and adult, but there was a smaller reduction in aged rats. The results suggest that (1) endothelial function was impaired in mesenteric arteries of aged rats; (2) both K\(_{ca}\) channels and nitric oxide (NO) contribute together to the ACh-induced vasorelaxation in small mesenteric arteries, and (3) both the impairment of K\(_{ca}\) channel function and decreased NO account for the age-related endothelial dysfunction.

Key words
Aging • Endothelial dysfunction • Potassium channels • Mesenteric artery • Acetylcholine

Introduction
The process of aging is associated with marked changes in the vascular system that can lead to the development of cardiovascular diseases. It is known that increasing age induces structural and functional alterations in large conduit arteries (Santhanam \textit{et al.} 2008) and small resistance arteries as well (Laurant \textit{et al.} 2004, Briones \textit{et al.} 2007). With age, the central aorta dilates (Lakatta 1993) and the arterial wall thickness of large arteries increases (Nagai \textit{et al.} 1998). In small resistance arteries, age-related enlargement of vascular lumen and increased thickness of vascular wall have been reported recently (Briones \textit{et al.} 2007, Laurant \textit{et al.} 2004). Because age-associated cardiovascular disease is the leading cause of morbidity and mortality in industrialized and some developing countries, the mechanisms that initiate and propagate changes in the aging vasculature are currently in the focus of increasing investigation.

One of the major vascular functional changes with normal aging is the age-related endothelial dysfunction. Endothelial cells control the tone of the underlying vascular smooth muscle by releasing several constricting factors such as endothelin and prostaglandin H\(_2\), and endothelium-derived relaxing factors, including nitric
oxide (NO), reactive oxygen species (ROS), potassium ions (K\textsuperscript+), and metabolites of arachidonic acid. Age-related endothelial dysfunction may be due to an alteration of the balance between endothelium-derived relaxing and constricting factors (Matz et al. 2000). The reduced endothelium-dependent vasorelaxation with age has been characterized by reduced agonist-mediated vasodilation (Minamino and Komuro 2008), declined flow-induced vasodilation (Csizsar et al. 2002, Muller-Delp et al. 2002), and by decreased sensitivity of arteriolar endothelium to fluid shear stress (Sun et al. 2002). To date most studies have focused on the effects of aging on endothelium-dependent NO-mediated vasodilation. Endothelium-dependent vasorelaxation in aorta and large proximal arteries is dependent almost entirely on NO, and is very sensitive to increased endogenous superoxide (O\textsubscript{2}\textsuperscript{-}) (Davidge et al. 1998, Knock et al. 2006). Moreover, in small resistance arteries, the effect of age on endothelium-dependent vasorelaxation has not been extensively studied (Matz et al. 2000). In distal resistance arteries, including those of mesenteric, pulmonary and cerebral circulation, however, endothelium-derived hyperpolarization factors (EDHF) participates significantly in agonist-induced vasorelaxation as the vessel diameter decreases (Sobey 2001). Hyperpolarization through potassium channel opening is a fundamental mechanism for vasorelaxation of small vessels. Calcium-activated K\textsuperscript+ channels (K\textsubscript{ca}) are key effectors in the control of endothelium-dependent EDHF-evoked vasodilation (Hilgers and Webb 2007). There are three types of K\textsubscript{ca} channels in vascular cells, including large-conductance K\textsubscript{ca} (BK\textsubscript{ca}) channels in vascular smooth muscle cells; intermediate (IK\textsubscript{ca}) and small-conductance K\textsubscript{ca} (SK\textsubscript{ca}) channels in endothelial cells. Activation of K\textsubscript{ca} channels on vascular cells contributes to agonist-induced vasorelaxation in rat (Dimitropoulou et al. 2007) and rabbit mesenteric arteries (Khan et al. 1993, Zhang et al. 2007). The endothelial cell IK\textsubscript{ca} and SK\textsubscript{ca} channels are especially important for EDHF-mediated relaxation and hyperpolarization in resistance arteries (Dora et al. 2008). Impaired K\textsuperscript+ channel function or reduced hyperpolarization in vascular cells has been detected in various diseased conditions such as hypertension (Hilgers and Webb 2007), diabetes (Fukao et al. 1997) and ischemia/reperfusion (Ko et al. 2008, Sobey 2001). But the role of K\textsubscript{ca} channels during aging is still unclear. Given that small resistance arteries contribute to the regulation of blood pressure and local blood flow, EDHF and K\textsubscript{ca} channels are implicated in control of blood pressure and regional blood flow in mammalian tissues in vivo, and may be altered under pathophysiological conditions such as hypertension and diabetes as well as advancing aging. Changes in molecular composition and function of K\textsubscript{ca} channels in resistance arteries may therefore be a fundamental event contributing to the progression of arterial dysfunction during age. However, EDHF and K\textsubscript{ca} channel functional changes in healthy aging are not completely understood and require further studies. In the present study, we investigated the roles of K\textsubscript{ca} channels underlying the age-related vascular functional changes, and provided evidences for impaired potassium channel function as well as reduced release of NO in the acetylcholine (ACh)-induced vasodilation in small mesenteric arteries of aged rats.

**Materials and Methods**

**Vessel preparation**

Male Sprague-Dawley rats aged 3-6 months (young), 10-12 months (adult), and more than 24 months (aged) were used in the present study. The animals were fed standard rat chow and had free access to tap water, and maintained in the Institute of Experimental Animals Resources. Rats were anesthetized with pentobarbital sodium (50 mg/kg intraperitoneally). The mesenteric vascular bed was excised and placed on a cooling plate containing cold (0-4 °C) 3-(N-morpholino) propanesulfonic acid (MOPS) buffered physiological saline solution (see below) containing 1 % bovine serum albumin. The second to fourth branches of the arteries (200-300 μm in diameter) were cut, cleaned of adherent connective tissue, transferred to an organ bath (2.5 ml volume) mounted on the stage of an inverted video microscope (Zeiss 100TV). Arterial segments were cannulated at both ends onto glass micropipettes and secured, and the lumen of the vessel was filled with MOPS-buffered solution containing 1 % albumin. The transmural pressure was set at 80 mm Hg and continuously monitored. Neither transluminal flow nor oxygenation was applied to the cannulated vessels. The internal diameter of the vessels was recorded by a computerized diameter tracking system (Diamtrak, Montech Pty Ltd., Australia).

**Experimental procedures**

After cannulation, the mesenteric arteries were superfused continuously with a MOPS-buffered physiological saline solution (37.5 °C, PH 7.3) of the
following composition (in mmol/l): 144 NaCl, 3 KCl, 2.5 CaCl₂, 1.4 MgSO₄, 2.0 pyruvate, 5.0 glucose, 0.02 ethylenediaminetetraacetic acid (EDTA), and 2.0 3-(N-morpholino)propanesulfonic acid (MOPS), 1.21 NaH₂PO₄. The extraluminal solution was warmed from room temperature to 37.5 °C. After 30-min equilibration, the vessels were exposed to 10 µmol/l phenylephrine (PE), which produced a near maximum contraction response in vessels from all age groups. After the contraction reached a steady state, increasing dose of acetylcholine (ACh) were applied to incubation bath to elicit vasorelaxation. The vessel internal diameter was recorded once the vessel reached a steady state.

For vasorelaxation mechanism study, the following inhibiting experiments were conducted: 10 µmol/l Nω-monomethyl-L-arginine (L-NMMA; a NO synthase inhibitor); 1 µmol/l charybdotoxin (BK ca and IKca channel inhibitor); 1 µmol/l apamin (SKca inhibitor). ACh-induced vessel diameter changes were recorded before and after 30-min incubation with these inhibitors. All chemicals were obtained from Sigma, St. Louis.

Data collection and statistical analysis

All data are presented as mean ± S.E.M. Six to ten vessels from at least 6 rats were used in each group. The vessel diameter changes were presented as percentages (%) of dilation of the preconstricted vessels, calculated as follows: % of vasodilation= [(Dagonist-Dbase)/(Dmax-Dbase)]x100, where Dmax is the maximum diameter of the vessel at 80 mm Hg at room temperature before equilibration, Dbase is the vessel diameter at steady contraction state induced by PE before the ACh stimulation, and Dagonist is the diameter of the vessel after ACh stimulation. With this method, the maximum dilation is represented as 100 %, and baseline diameter is 0 %. Comparisons were made with the use of paired Student’s t test or ANOVA with a post-hoc Bonferroni test, as appropriate. The acceptable level of significant was defined as P<0.05.

Results

Increased body weight in aged rats was observed. Body weight was greater (P<0.01) in aged (586.4±11.2 g, n=8) than in adult (412.3±8.6 g, n=8) and in young (313.6±4.8 g, n=8) rats. The passive maximum internal diameters at 80 mm Hg of intravascular pressure were 245.67±8.36 µm, 259.50±8.49 µm and 266.33±9.40 µm in young, adult and aged rats respectively. No significant increase was observed in small mesenteric artery internal diameters (P>0.05, n=10 for all three groups) in our preparation.

Phenylephrine (PE) contracted the cannulated mesenteric resistance arteries in a concentration-dependent manner (data not shown). The contraction induced by 10 µmol/l PE was slightly increased in old rats. PE caused vessel contraction of 48.01±2.89 % (127.83±5.49 µm) in young, 50.65±1.23 % (128.20±5.81 µm) in adult, and 54.96±3.70 % (172.17±6.20 µm) in aged rats.

ACh-induced vasorelaxation in young, adult and aged rats are shown in Figure 1. Vascular relaxation responses to ACh were abolished by removal of endothelium (data not shown) in young rats, which supports that ACh-induced vasorelaxation in rat mesenteric arteries is endothelium-dependent. In young and adult groups, ACh induced similar vasorelaxation of rat mesenteric artery in a dose-dependent manner. There was no difference of ACh-induced dilation between young and adult groups. 1 and 10 µmol/l ACh evoked a vasodilation of 86.72±1.54 % (229.83±7.49 µm), 94.31±3.47 % (238.83±7.76 µm) in young, and 80.89±0.94 % (234.43±7.99 µm), 93.16±1.71 % (250.50±8.24 µm) in adult rats. However, the relaxation induced by ACh in the aged rats was significantly decreased at all ACh concentration except concentration of 1 and 10 nmol/l (Fig. 1, P<0.001). 1 and 10 µmol/l ACh induced a
Fig. 2. Concentration-response curves to ACh before/after incubation with 10 μmol/l L-NMMA in PE-preconstricted small mesenteric arteries. L-NMMA incubation resulted in a significant reduction in vasodilation to ACh in young (A), adult (B) and aged rats (C) as well. But the reduction was declined in aged rats compared with young rats (D). ** P<0.001, *P<0.05.

Nitric oxide is a well-known vasodilator in many vessel beds. PE-induced contractile responses were potentiated in all groups by L-NMMA (a specific NOS inhibitor). The contraction was 67.77±2.96 % in young, 68.01±2.02 % in adult, and 68.01±2.02 % in aged rats after L-NMMA treatment. In young and adult rats, L-NMMA significantly reduced the vasorelaxation evoked by ACh (Figs 2A and 2B). After 30-min incubation of vessels with L-NMMA, ACh-evoked dilation was reduced from 86.37±3.54 % (224.83±10.31 μm) to 48.45±2.56 % (164.17±6.37 μm) at 1 μmol/l and 100 μmol/l (data not shown) could not cause more vasorelaxation.

Nevertheless, the reduction of vasodilation caused by incubation of L-NMMA was declined in aged rats (Fig. 2D) compared with young rats.
Calcium-activated potassium channels (K_{Ca}) play a central role in EDHF-mediated vasorelaxation in small mesenteric arteries (Hilgers and Webb 2007, Dora et al. 2008). In the present study, we used charybdotoxin to block IK_{Ca} and BK_{Ca} channels, apamin to block SK_{Ca} channels. Incubation of vessels of young rats with charybdotoxin, ACh-induced vasorelaxation was reduced from 86.72±3.76 % (230.83±7.49 µm), 94.31±3.61 % (238.84±7.76 µm) to 44.59±7.80 % (180.50±5.57 µm) and 55.21±6.77 % (192.83±4.55 µm) at ACh concentration of 1 and 10 µmol/l, respectively (Fig. 3A, P<0.001). The reduced vasodilation was similar in adult rats (Fig. 3C). In aged rats, charybdotoxin also inhibited the ACh-induced vasodilation (Fig. 3C). The vasodilation was reduced from 51.55±3.75 % (220.86±10.31 µm), 56.25±3.91 % (225.00±9.20 µm) to 33.78±3.05 % (190.17±8.83 µm) and 38.93±1.52 % (196.16±8.17 µm) at ACh concentration of 1 and 10 µmol/l. But the reduction was much less than that in young and adult rats (Fig. 3D). Incubation with apamin partially inhibited the ACh-induced vasorelaxation in young and adult rats (Figs 4A and 4B). Vasodilation in young rats was reduced from 86.72±3.76 %, 94.31±3.61 % to 60.56±3.87 % (199.33±6.55 µm) and 74.45±6.54 % (216.00±7.78 µm) at ACh concentration of 1 µmol/l and 10 µmol/l, respectively (Fig. 4A, P<0.01). In aged rats, incubation of
Fig. 4. Concentration-response curves to ACh before/after incubation with 1 µmol/l apamin in PE-preconstricted small mesenteric arteries. Apamin resulted in a significant reduction in vasodilation to ACh in young (A) and adult rats (B). Incubation of apamin did not have any effect on the ACh-induced vasodilation in aged rats (C). Comparison of the reduction of vasodilation by apamin was shown in Fig. 4D. ** P<0.001, * P<0.05.

vessels with apamin did not have significant effect on the dilation (Fig. 4C). Ach-evoked vasodilation was 49.30±5.21 % (208.50±10.46 µm) and 57.50±4.41 % (218.00±9.31 µm) after apamin treatment compared to 51.55±3.75 % and 56.25±2.96 % at 1 and 10 µmol/l concentration. Figure 4D shows the reduction evoked by apamin at ACh concentration of 1 µmol/l. Apamin-induced reduction was declined with advancing age.

To further analyze the interactions of NO and Kca channels in aged rats, we tested the small mesenteric arteries with combined incubation of apamin, charybdotoxin and L-NMMA. Combined use of apamin, charybdotoxin and L-NMMA almost abolished the ACh-induced vasorelaxation in young rats (Fig. 5A). The dilation was only 13.92±3.06 % at 1 µmol/l ACh and 17.84±3.50 % at 10 µmol/l. Similar results were obtained from adult rats (Fig. 5B). In aged rats, vasodilation after combined application of apamin, charybdotoxin and L-NMMA was 10.83±2.74 % at 1 µmol/l and 14.35±1.59 % at 10 µmol/l (Fig. 5C). Vasodilation after combined incubation of apamin, charybdotoxin and L-NMMA in aged rat was similar to that in young and adult rats (Fig. 5D, P>0.05).

Discussion

In this study, we provided evidence for age-related impairment of endothelial function in rat small
Fig. 5. Concentration-response curves to ACh before/after a combined incubation with 10 μmol/l L-NMMA, 1 μmol/l charybdotoxin and 1 μmol/l apamin (L+C+A) in PE-preconstricted small mesenteric arteries. The combined incubation almost abolished vasodilation induced by ACh in young (A), adult (B) and in aged rats (C). ACh-induced vasodilation was similar after combined incubation in three rat groups (D). ** P<0.001.

mesenteric arteries. The major findings were (1) K$_{ca}$ channels and NO contribute to the dilation induced by ACh in rat small mesenteric arteries; (2) endothelium-dependent vasorelaxation was impaired in aged rats; (3) functionally defective K$_{ca}$ channels and reduced production of NO contribute to the age-associated impairment of endothelial function.

Vascular aging has been relatively well characterized in aorta and large proximal arteries. Age-associated changes in blood vessels include increased arterial wall thickness, luminal dilatation and impaired endothelial function (Minamino and Komuro 2008). However, little is known about the effects of age on small resistance arteries. In the present study, we showed that the endothelium-dependent vasorelaxation to ACh in the small resistance mesenteric arteries declined with age. ACh-induced vasodilation was abolished by removal of endothelium, supporting the fact that ACh-induced vasorelaxation in rat mesenteric arteries is endothelium-dependent. In vessels from aged rats, ACh-induced vasorelaxation was markedly impaired compared to that in vessels from young and adult rats, indicating an age-associated impairment of endothelial function in small mesenteric arteries. In the present study, animal body weight increased with age, but we did not see an age-related increase in vascular internal diameter. In our
peroxynitrite (ONOO\textsuperscript{–}), which is well documented in NO bioavailability, and subsequent formation of reactive oxygen species (ROS), decreased oxidative stress comprises increased NO production. The mechanism of increased oxidative stress comprises increased production of reactive oxygen species (ROS), decreased NO bioavailability, and subsequent formation of peroxynitrite (ONOO\textsuperscript{–}), which is well documented in aorta and large proximal arteries (Santhanam et al. 2008). In old rat aorta, endothelial NO synthase (eNOS) expression was increased, but NO production and downstream signaling (cGMP) was decreased (Cernadas et al. 1998). However, the mechanisms accounting for vasculature aging in distal resistance arteries may be different. Both the activity of eNOS and the release of NO have been suggested to be reduced in aged small resistance arteries (Amrani et al. 1996). eNOS mRNA and eNOS protein expression were reduced in the aging coronary (Csizsar et al. 2002) and skeletal muscle vasculatures (Woodman et al. 2002). In mesenteric arteries from aged rats, although eNOS protein expression was not different from that of young rats, shear stress-induced synthesis of NO was reduced (Sun et al. 2004). In our cannulated mesenteric arteries, inhibition of NOS significantly reduced the vasodilation induced by ACh in young, adult and aged rats, indicating that NO plays a predominant role in vasorelaxation of young, adult (Fujii et al. 1993) and aged vessels. Nevertheless, the inhibition of NOS resulted in a lower reduction in aged rats than that in young and adult rats, suggesting that NO production was decreased in ACh-induced vasodilation of aged rats. However, in the present study, age-related impairment of endothelial function was only partially attributed to the decreased NO production.

EDHF and K\textsubscript{ca} channels have greater significance in agonist-induced vasorelaxation in small resistance arteries. Opening of potassium channels on vascular smooth muscle cells with resultant hyperpolarization plays a central role in the distal small resistance arteries. In cerebral penetrating arterioles, activation of BK\textsubscript{ca} and IK\textsubscript{ca} channels leads to arterial hyperpolarization, contributing to ATP-induced dilation (Dietrich et al. 2008). Genes encoding for SK\textsubscript{ca} and IK\textsubscript{ca} channel subunits were highly expressed in small mesenteric arteries, stressing the importance of K\textsubscript{ca} channels in these small arteries (Hilgers et al. 2006). In the present study, we used charybdotoxin to block BK\textsubscript{ca} and IK\textsubscript{ca} channels and apamin to block SK\textsubscript{ca} channels. Both charybdotoxin and apamin significantly reduced the ACh-induced dilation in rat mesenteric arteries of young and adult rats, which provided evidences that K\textsubscript{ca} channels contribute to the ACh-induced dilation in rat small mesenteric arteries.

Age-related structural alteration in small mesenteric arteries (Laurant et al. 2004, Briones et al. 2007) is likely accompanied by a decreased expression of K\textsubscript{ca} channels, which was recently reported in small mesenteric arteries of hypertensive rats (Hilgers and Webb 2007). ROS, especially hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) effectively inhibited K\textsubscript{ca} channel function (Brakemeier et al. 2003, Tang et al. 2004). Both enhanced ROS formation (Jacobson et al. 2007) and decreased expression of K\textsubscript{ca} genes could lead to the impaired K\textsubscript{ca} channel function in mesenteric arteries from aged rats, but the role of K\textsubscript{ca} channels in mesenteric arteries of aged rats has not been documented before. In the present study, apamin incubation had no effect on the ACh-induced dilation in vessels from aged rats, suggesting that SK\textsubscript{ca} channels are functionally defective in mesenteric arteries of aged rats. Blockade of IK\textsubscript{ca} and BK\textsubscript{ca} channels by charybdotoxin resulted in a significantly lower reduction of ACh-induced vasodilation in aged rats than in young and adult rats, suggesting that IK\textsubscript{ca} and/or BK\textsubscript{ca} channels are functionally impaired with age. A combined incubation of vessels with L-NMMA, charybdotoxin and apamin almost abolished ACh-induced vasodilation in young, adult and aged rats. There was no difference of the remaining dilation between these three animal groups. This further supported that K\textsubscript{ca} channels and NO contribute to the ACh-induced vasorelaxation in small mesenteric arteries, and both impairment of K\textsubscript{ca} channels and decreased NO production account for the age-related endothelial dysfunction.

In summary, in the present study, we showed that ACh-induced vasodilation was reduced in small resistance mesenteric arteries of aged rats; inhibition of K\textsubscript{ca} channels and NOS markedly reduced the dilation in young, adult and aged rats, but resulted in a lower reduction in vessels from aged rats. Our results suggest that ACh-induced vasodilation was impaired in mesenteric arteries of aged rats, both K\textsubscript{ca} channels and NO contribute to the vasodilation in these small resistance arteries, and the impairment of vascular...
function in small mesenteric arteries of aged rats was attributed to impaired $K_{Ca}$ function and decreased NO production.

**Conflict of Interest**
There is no conflict of interest.

**References**


DAVIDGE ST, OJIMBA J, MCCLAUGHLIN MK: Vascular function in the vitamin E-deprived rat: an interaction between nitric oxide and superoxide anions. *Hypertension* **31**: 830-835, 1998.


