Beneficial effects of lifelong caloric restriction on endothelial function are greater in conduit arteries compared to cerebral resistance arteries

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Abstract Endothelial dysfunction occurs in conduit and cerebral resistance arteries with advancing age. Lifelong caloric restriction (CR) can prevent the onset of age-related dysfunction in many tissues, but its effects on cerebral resistance artery function, as compared with conduit artery function, have not been determined. We measured endothelium-dependent dilation (EDD) in the carotid artery and middle cerebral artery (MCA) from young (5–7 months), old ad libitum fed (AL, 29–32 months), and old lifelong CR (CR, 40 % CR, 29–32 months) B6D2F1 mice. Compared with young, EDD for old AL was 24 % lower in the carotid and 47 % lower in the MCA ($p<0.05$). For old CR, EDD was not different from young in the carotid artery ($p>0.05$), but was 25 % lower than young in the MCA ($p<0.05$). EDD was not different between groups

after NO synthase inhibition with N^ω-nitro-L-arginine methyl ester in the carotid artery or MCA. Superoxide production by the carotid artery and MCA was greater in old AL compared with young and old CR ($p<0.05$). In the carotid, incubation with the superoxide scavenger TEMPOL improved EDD for old AL ($p>0.05$), with no effect in young or old CR ($p>0.05$). In the MCA, incubation with TEMPOL or the NADPH oxidase inhibitor apocynin augmented EDD in old AL ($p<0.05$), but reduced EDD in young and old CR ($p<0.05$). Thus, age-related endothelial dysfunction is prevented by lifelong CR completely in conduit arteries, but only partially in cerebral resistance arteries. These benefits of lifelong CR on EDD result from lower oxidative stress and greater NO bioavailability.

Keywords Endothelium-dependent dilation · Aging · Oxidative stress · Nitric oxide · Middle cerebral artery · Carotid

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Introduction

Advancing age is a major risk factor for cardiovascular and cerebrovascular diseases (Wolf et al. 1991; Lakatta 2003), and impairments in arterial function with age contribute to these increases in risk (Choi et al. 1998; Lakatta 2003). Furthermore, Alzheimer's is a disease of aging and impaired cerebral artery function is thought to be an important contributor to Alzheimer's disease progression (Humpel 2011). As such, it is important to

identify interventions and therapeutic targets that could prevent the arterial impairments that occur with advancing age. Lifelong caloric restriction (CR) increases lifespan and reduces the onset of age-related diseases including prevention of cardiovascular disease risk factors and cognitive decline (Halagappa et al. 2007; Carter et al. 2009; Weindruch and Sohal 1997; Masoro 2005). It is likely that these beneficial effects of lifelong CR result from maintained function of both conduit and cerebral resistance arteries with age. However, the effect of lifelong CR on cerebral arterial function has not been fully elucidated.

A key feature of age-related arterial dysfunction is reduced endothelium-dependent dilation (EDD). Older humans and rodents exhibit impaired EDD in conduit arteries as well as cerebral arteries (Lesniewski et al. 2009; Csiszar et al. 2007a; Blackwell et al. 2004; Modrick et al. 2009; Hatake et al. 1990; Eskurza et al. 2004; Celermajer et al. 1994). It has been shown previously that lifelong CR can prevent the age-related decline in EDD in the aorta (Csiszar et al. 2009), but the effect of CR on resistance artery EDD has not been studied. With advancing age, endothelial dysfunction primarily results from reduced nitric oxide (NO) bioavailability (Taddei et al. 2001). Not only is NO the key dilator produced by the endothelium (Vanhoutte 2003), but endothelial-derived NO can also decrease the expression of amyloid precursor proteins (Austin et al. 2010), the accumulation of which is a characteristic feature of Alzheimer's disease (O'Brien and Wong 2011). Reactive oxygen species, particularly superoxide, reduce NO bioavailability and oxidative stress has been implicated as a major factor leading to impaired EDD with aging (Taddei et al. 2001). Expression of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, a key producer of superoxide in vascular tissue, is increased in conduit and cerebral arteries with advancing age (Miller et al. 2005; Durrant et al. 2009), and inhibiting NADPH oxidase in conduit arteries from old animals improves EDD (Durrant et al. 2009; Rippe et al. 2010). Lifelong CR prevents the increase in oxidative stress with aging in a number of tissues including large arteries (Csiszar et al. 2009; Sohal and Weindruch 1996). However, the effect of lifelong CR on oxidative stress in resistance arteries has not been explored.

We hypothesize that old ad libitum (AL) fed mice would have impaired EDD in conduit (carotid) and cerebral resistance (middle cerebral artery, MCA) arteries

compared with young AL fed controls, and the magnitude of this dysfunction would be similar between arteries. We further hypothesized that lifelong CR would prevent the decline in EDD with age, such that carotid and MCA EDD would not be different between old CR and young control mice. We hypothesized that CR would maintain EDD with age by preventing the decline in NO bioavailability and the onset of NADPH oxidase-mediated oxidative stress.

Methods

Animals

Young (5–7 months) and old AL (29–32 months) and old lifelong CR (CR, 29–32 months) male B6D2F1 mice were obtained from the National Institute on Aging rodent colony. In NIH lifelong CR mice, calorie restriction is initiated at 14 weeks of age at ~10 % below AL, increased to ~25 % restriction at 15 weeks and to ~40 % restriction at 16 weeks, and maintained throughout the life of the animal. All mice were housed for at least 2–3 months in an animal care facility at the University of Utah on a 12:12 light:dark cycle at 24 °C and fed the appropriate NIH 31 (young or old AL) or NIH 31 fortified diet (old CR). All animal procedures conformed to the *Guide to the Care and Use of Laboratory Animals* (version 8, revised 2011) and were approved by the Utah Animal Care and Use Committee.

Endothelium-dependent dilation: effect of NO and oxidative stress

Measurements of EDD and endothelial independent dilation (EID) in isolated carotid arteries and MCAs studied ex vivo were performed using a method previously described in detail (Lesniewski et al. 2009; Donato et al. 2009, 2011). Briefly, mice were euthanized by exsanguination via cardiac puncture while under isoflurane anesthesia. Carotid arteries and MCAs were excised and placed in myograph chambers (DMT) with physiological salt solution (PSS) that contained 145.0 mM NaCl, 4.7 mM KCl, 2.0 mM CaCl₂, 1.17 mM MgSO₄, 1.2 mM NaH₂PO₄, 5.0 mM glucose, 2.0 mM pyruvate, 0.02 mM EDTA, 3.0 mM MOPS buffer, and 1 g/100 ml BSA, pH 7.4 at 37 °C, cannulated onto glass micropipettes and secured with nylon (11-0) suture. Once cannulated, arteries were warmed to 37 °C, pressurized and allowed to

equilibrate for ~1 h. All arteries were submaximally precontracted with phenylephrine (2 μ M) and increases in luminal diameter in response to increasing concentrations of the endothelium-dependent dilator, acetylcholine (ACh: 1×10^{-9} to 1×10^{-4} M) and endothelium-independent dilator, sodium nitroprusside (SNP: 1×10^{-10} to 1×10^{-4} M) were determined. Responses to ACh were repeated in the presence of the NO synthase (NOS) inhibitor, N^w-nitro-L-arginine methyl ester (L-NAME, 0.1 mM, 30-min incubation) to determine the approximate contribution of NO. To determine the effect of superoxide (oxidative stress) on EDD, responses to ACh were measured following a 60-min incubation in the presence of the superoxide scavenger, TEMPOL (1 mM; Didion et al. 2006; Zhang et al. 2003; Qamirani et al. 2005), in different carotid or MCA segments than those initially incubated with L-NAME. Measurement of ACh responses were repeated in TEMPOL-treated arteries after L-NAME addition. The contribution of superoxide produced by NADPH oxidase to EDD was assessed by preincubation with apocynin (1 mM, 60 min), a NADPH oxidase inhibitor (Durrant et al. 2009; Rippe et al. 2010). EDD and EID results are expressed as the percent of possible dilation (Lesniewski et al. 2009; Durrant et al. 2009).

Arterial superoxide production

Production of superoxide in the carotid artery and MCA was measured by electron paramagnetic resonance (EPR) spectrometry using the spin probe 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH, Alexis Biochemicals). Stock solutions of CMH were prepared in ice-cold deoxygenated Krebs–HEPES buffer (mmol L⁻¹: NaCl 99.01, KCl 4.69, CaCl₂ 2.50, MgSO₄ 1.20, K₂HPO₄ 1.03, NaHCO₃ 25.0, glucose 11.10, Na–HEPES 20.00; pH 7.4) containing 0.1 mmol L⁻¹ diethylenetriamine-penta-acetic acid, 5 μ mol L⁻¹ sodium diethyldithiocarbamate and pretreated with Chelex (Sigma) to minimize auto-oxidation of the spin probe. A 3-mm section of the carotid artery and a 5-mm section of the MCA were excised, separately incubated for 60 min at 37 °C in 200 μ L Krebs–HEPES buffer containing 0.5 mmol L⁻¹ CMH, and analyzed immediately on an EMX Plus EPR spectrometer (Bruker, Rheinstetten, Germany). Instrument settings were: microwave frequency 9.83 GHz, centerfield 3480 G, sweep 80 G, modulation amplitude 3.3 G, microwave power 40 mW, microwave attenuation 7, and receiver

gain 30. A total of six sweeps were conducted lasting 8.7 s per sweep. The running average of the six sweeps was collected with the double integration (area under and over the baseline) of the triplet used to display the magnitude of the signal. The double integration of each sample was adjusted by subtracting the double integration of a blank control measured the day of analysis. The magnitude of this signal directly relates to the amount of superoxide that has been trapped by the CMH. Data were normalized to the mean of the young samples of the appropriate artery type measured on the day of analysis.

Exogenous NADPH

Carotid arteries and MCAs were prepared as described above for EDD measurements. After precontraction with phenylephrine (2 μ M), the change in lumen diameter was determined in response to increasing concentrations of NADPH (1×10^{-7} to 1×10^{-4} M; Didion and Faraci 2002; Trott et al. 2011). This change in diameter is presented as a percentage of the precontracted diameter for each artery.

Statistics

For animal and vessel characteristics, group differences were determined by one-way analysis of variance (ANOVA). For all dose responses, group differences were determined by repeated-measures ANOVA. A least significance difference post hoc test for equal variances was used for preplanned comparisons where appropriate. To compare the magnitude of impairment in EDD between arteries (Fig. 2), maximal responses to ACh were normalized to the mean maximal response of the young for that artery. EC₅₀ values were calculated by fitting each dose response to a four-parameter logistic equation. EC₅₀ values for the MCA response to ACh in the presence of L-NAME were not calculated as these responses do not fit a logistic equation. Data are presented as mean $\pm$ SEM. Significance was set at $p < 0.05$.

Results

Animal characteristics

Body mass was similar between young and old AL mice ($p > 0.05$), while old CR mice had a lower body

mass than the other groups ($p<0.05$, Table 1). Old CR had lower food intake compared with young and old AL ($p<0.05$), and old AL had lower food intake compared with young ($p<0.05$, Table 1).

Endothelium-dependent dilation

In the carotid artery, the response to ACh was impaired in old AL compared with young and old CR mice, with the old AL maximal dilation being 24 % lower than young and 19 % lower than old CR (dose response and max: $p<0.05$ vs. young and old CR, Fig. 1a). The dilation to ACh was not different between young and old CR in the carotid artery (dose response and max: $p>0.40$, Fig. 1a).

In the MCA, the maximal dilation to ACh in old AL was 47 % lower than young and 29 % lower than old CR mice (dose response and max: $p<0.05$ vs. young and old CR, Fig. 1b). In contrast to the carotid, the MCA response to ACh was impaired for old CR, with a 25 % lower maximal dilation in old CR vs. young (dose response and max: $p<0.05$ vs. young, Fig. 1b).

The response to endothelium-independent dilator SNP did not differ between groups for either artery (Fig. 1c, d; $p>0.05$). In addition, there were no group differences for either artery in the sensitivity to ACh (EC_{50} , $p>0.05$, Table 2), maximal artery diameter ($p>0.05$, Table 2), or the amount of preconstriction prior to dose–response assessment ($p>0.05$, data not shown). The magnitude of the impairment in ACh-mediated dilation in old AL was greater in the MCA compared with the carotid for arteries (when data were normalized to young, $p<0.05$, Fig. 2). The preservation of ACh-mediated dilation in old CR was less in the MCA compared with the carotid (when data were normalized to young, $p<0.05$, Fig. 2).

Table 1 Animal characteristics

	Young	Old AL	Old CR
Age (months)	5.9±0.1	30.0±0.1*	30.1±0.1*
Body mass (g)	34.0±1.2	36.8±1.2	28.5±0.6*†
Food intake (g day ⁻¹)	4.8±0.1	4.1±0.1*	3.2±0.0*†

Data are mean±SEM

AL ad libitum, CR caloric restricted

NO contribution to dilation

In the carotid artery, after inhibition of NOS by preincubation with L-NAME, maximal dilation to ACh was 55 % lower in young, 33 % lower in old AL, and 52 % lower in old CR mice compared with ACh alone (dose response and max: all $p<0.05$ with vs. without L-NAME within group, Fig. 1a). Compared to the carotid, the reduction in ACh-mediated dilation after L-NAME preincubation was greater in all groups in the MCA, with maximal dilations 84 % lower in young, 66 % lower in old AL, and 76 % lower in old CR (dose response and max: all $p<0.01$ with vs. without L-NAME, Fig. 1b). There was no difference in ACh response between groups in the presence of L-NAME in either the carotid artery or MCA ($p>0.05$, Fig. 1a, b).

Oxidative stress

Superoxide production by old AL arteries was about twofold greater in the carotid and about threefold greater in the MCA compared with both young and old CR mice (carotid: $p<0.01$ vs. young and old CR, MCA: $p<0.05$ vs. young and old CR, Fig. 3a, b). Superoxide production was similar between young and old CR for both arteries (carotid: $p=0.90$, MCA: $p=0.86$, Fig. 3a, b).

In old AL mice, TEMPOL resulted in increased ACh-mediated maximal dilation by 26 % in the carotid and 46 % in the MCA (carotid and MCA, max and dose response: $p<0.05$; with vs. without TEMPOL, Figs. 4b and 5b). In old AL, the ACh-mediated dilation in the presence of L-NAME and TEMPOL together was not different from the ACh-mediated dilation in the presence of L-NAME alone ($p>0.05$).

For young and old CR, scavenging superoxide with TEMPOL did not affect the response to ACh in the carotid ($p>0.05$, Fig. 4a, c). In contrast, TEMPOL reduced maximal dilation by 55 % for young and 52 % in old CR in the MCA (max: all $p<0.01$, dose response: all $p<0.05$, with vs. without TEMPOL within group, Fig. 5a, c).

NADPH oxidase

In the MCAs from old AL mice, apocynin incubation led to a 48 % increase in maximal dilation ($p=0.03$, Fig. 6) to ACh. In the MCAs of young mice and old CR mice, apocynin incubation led to a 44 % and 36 %

Fig. 1 Endothelium-dependent dilation (EDD) to acetylcholine (ACh) in the absence or presence of nitric oxide synthase inhibitor N^w-nitro-L-arginine methyl ester (*L-NAME*) measured by pressure myography in carotid (**a**) and middle cerebral arteries (*MCA*, **b**) from young, old ad libitum fed (*AL*), and old lifelong calorically restricted (*CR*) mice ($n=5-16$ per group). Endothelium-independent

dilation to sodium nitroprusside in carotid arteries (**c**) and MCAs (**d**) from young, old *AL*, and old *CR* mice ($n=5-9$ per group). Significance marks denote a time by group interaction or main effect for group from repeated-measures ANOVA analyses. * $p<0.05$ vs. young ACh alone, † $p<0.05$ vs. old *AL* ACh alone, ‡ $p<0.05$ vs. old *CR* ACh alone. Values are mean±SEM

Table 2 Artery characteristics

	Carotid			MCA		
	Young	Old AL	Old CR	Young	Old AL	Old CR
Maximal diameter (μm)	424±5	422±7	424±5	129±4	125±5	127±4
EC ₅₀ , log <i>M</i>						
ACh	-7.6±0.2	-7.5±0.1	-7.3±0.3	-7.4±0.4	-7.4±0.3	-7.5±0.2
ACh+ <i>L-NAME</i>	-7.1±0.3	-7.0±0.2	-6.4±0.2*	N/A	N/A	N/A
ACh+TEMPOL	-7.5±0.2	-7.5±0.2	-7.5±0.2	-7.9±0.4	-7.5±0.4	-7.7±0.3
ACh+TEMPOL+ <i>L-NAME</i>	-6.6±0.3	-7.1±0.5	-7.0±0.6	N/A	N/A	N/A
SNP	-7.6±0.3	-7.8±0.3	-8.1±0.2	-6.4±0.3	-6.7±0.2	-6.0±0.4

Values are mean±SEM

ACh acetylcholine, *L-NAME* N^G-nitro-L-arginine methyl ester, *SNP* sodium nitroprusside

* $p<0.05$ vs. young

Fig. 2 Maximal response to acetylcholine in carotid and middle cerebral arteries (MCA) from young, old ad libitum fed (AL), and old lifelong calorically restricted (CR) mice ($n=9-16$ per group). Each maximal response was normalized to the mean response of the young group for that artery. [†] $p<0.05$ vs. old AL carotid, [‡] $p<0.05$ vs. old CR carotid. Values are mean $\pm$ SEM

decrease in maximal ACh-mediated dilation, respectively ($p<0.05$, Fig. 6). We have previously established with certainty that apocynin improves ACh-mediated dilation in carotid arteries from old AL mice, but has no effect on ACh-mediated dilation in carotid arteries from young mice (Durrant et al. 2009; Rippe et al. 2010). In old CR mice, apocynin incubation had no effect on the ACh response in carotid arteries (max response: $88.8\pm2.2\%$ vs. $83.9\pm8.1\%$; ACh alone vs. ACh with apocynin, respectively; $p=0.42$). The response to exogenous NADPH was not different between groups ($p>0.05$, Fig. 7a, b) and on average resulted in $\sim 10\%$ constriction in the carotid artery and $\sim 15\%$ constriction in the MCA.

Discussion

This study is the first to determine the effect of life-long CR on cerebral artery function. We demonstrate that lifelong CR completely prevents the age-related decline in EDD in conduit arteries, but only partially prevents the age-related decline in cerebral arteries. The effects of CR are mediated by greater NO bioavailability resulting from lower oxidative stress.

Endothelium-dependent dilation

In the present study, we find that aging leads to impaired conduit and cerebral artery EDD, as indicated by a lower response to ACh in arteries from old mice with no differences between young and old arteries in SNP response. This is in accordance with previous results reported by us and others (Lesniewski et al. 2009; Csiszar et al. 2007a; Blackwell et al. 2004; Modrick et al. 2009; Hatake et al. 1990). Lifelong CR completely prevented this age-related decline in EDD in conduit arteries (carotid), but only partially prevents the decline in cerebral resistance arteries (MCA). As previous studies have only focused on the effect of lifelong CR on conduit artery function (Csiszar et al. 2009), this is the first indication that CR has differential effects on arteries of different sizes. The fact that CR does not completely prevent the age-related decline in endothelial function in all arteries suggests that intervention/treatment strategies may not be equally effective at

Fig. 3 Superoxide production measured by electron paramagnetic resonance using a CMH spin probe in carotid arteries (a) and middle cerebral arteries (MCA, b) from young, old ad libitum fed (AL), and old lifelong calorically restricted (CR) mice ($n=4-8$ per

group). Representative spectra shown below each bar (scale for all carotid spectra is intensity: -1.0 to 1.0; scale for all MCA spectra is intensity: -0.4 to 0.4). * $p<0.05$ vs. young, [†] $p<0.05$ vs. old AL. Values are mean $\pm$ SEM

Fig. 4 Carotid artery EDD to acetylcholine (ACh) in the absence or presence of superoxide dismutase mimetic TEMPOL, alone or in combination with NOS inhibitor N^{ω} -nitro-L-arginine methyl ester (L-NAME), measured by pressure myography in arteries from young (**a**), old ad libitum fed (AL, **b**), and old lifelong calorically restricted (CR, **c**) mice ($n=4$ –12 per group). Significance marks

denote a time by condition interaction or main effect for condition from repeated-measures ANOVA analyses. * $p<0.05$ vs. young ACh alone, † $p<0.05$ vs. old AL ACh alone, ‡ $p<0.05$ vs. old CR ACh alone, § $p<0.05$ vs. young ACh with TEMPOL, || $p<0.05$ vs. old AL ACh with TEMPOL, ¶ $p<0.05$ vs. old CR ACh with TEMPOL. Values are mean $\pm$ SEM

preserving/improving endothelial function with aging in different vascular beds. Future studies are needed to determine if the disparities in efficacy of lifelong CR for preservation of function differs by the artery type (conduit vs. resistance artery) and location, or if partial preservation of function is specific to the cerebral circulation.

NO bioavailability

As with conduit arteries (Lesniewski et al. 2009; Csizsar et al. 2007a), we find that in cerebral arteries, the impairments in EDD with age are a result of reduced NO bioavailability. Furthermore, we demonstrate that greater NO bioavailability is the mechanism for the partial prevention of age-related decline in

MCA EDD with life-long CR. Similar to previous studies in cerebral arteries and arterioles (Kitazono et al. 1995; Mayhan 1990; Sobey and Faraci 1997), we find that in the MCA, dilators produced by NOS (NO and perhaps superoxide) account for most of the dilation in response to ACh.

Oxidative stress

We used EPR, the gold standard technique, to measure the superoxide production by arteries. We demonstrate that aging results in increased production of superoxide in both the carotid artery and MCA, but lifelong CR prevented this age-related increase. Although we cannot compare absolute differences in the amount of superoxide production between arteries due

Fig. 5 Middle cerebral artery (MCA) EDD to acetylcholine (ACh) in the absence or presence of superoxide dismutase mimetic TEMPOL, alone or in combination with NOS inhibitor N^{ω} -nitro-L-arginine methyl ester (L-NAME), measured by pressure myography in arteries from young (**a**), old ad libitum fed (AL, **b**), and old lifelong calorically restricted (CR, **c**) mice ($n=3$ –16 per

group). Significance marks denote a time by condition interaction or main effect for condition from repeated-measures ANOVA analyses. * $p<0.05$ vs. young ACh alone, † $p<0.05$ vs. old AL ACh alone, ‡ $p<0.05$ vs. old CR ACh alone, § $p<0.05$ vs. old AL ACh with TEMPOL. Values are mean $\pm$ SEM

Fig. 6 Maximal middle cerebral artery (MCA) EDD to acetylcholine (ACh) in the absence or presence of NADPH oxidase inhibitor apocynin (APO), alone or in combination with NOS inhibitor N^w-nitro-L-arginine methyl ester (L-NAME), measured by pressure myography in arteries from young, old ad libitum fed (AL), and old lifelong calorically restricted (CR) mice ($n=3-16$ per group). * $p<0.05$ vs. young ACh alone, † $p<0.05$ vs. old AL ACh alone, ‡ $p<0.05$ vs. old CR ACh alone, †† $p<0.05$ vs. old AL ACh with APO, ††† $p<0.05$ vs. old CR ACh with APO. Values are mean $\pm$ SEM

to differences in the tissue size collected, previous studies have demonstrated that under basal conditions cerebral arteries produce more superoxide compared to the carotid arteries per mass (Miller et al. 2005, 2009).

Similar to previous studies, we find that scavenging superoxide improves EDD in conduit and cerebral arteries from old mice (Blackwell et al. 2004; Lesniewski et al. 2009; Mayhan et al. 2008; Modrick et al. 2009) and does not affect EDD in conduit arteries from young or old CR mice (Csiszar et al. 2009). However, we demonstrate that scavenging superoxide

in the MCA from young and old CR mice results in reduced EDD, which is in accordance with previous studies demonstrating reactive oxygen species contribute to the dilation of resistance arteries in skeletal muscle (Sindler et al. 2013; Trott et al. 2011), cardiac muscle (Miura et al. 2003; Feng et al. 2010; Kang et al. 2011), and cerebral tissue (Drouin et al. 2007). Similarly, brachial artery dilation to handgrip exercise in humans, which is NO-dependent (Wray et al. 2011), is improved in older adults following ingestion of an antioxidant cocktail, but is reduced in young adults after the same antioxidant cocktail (Donato et al. 2010). Thus, in healthy arteries where dilation is largely reduced by NOS inhibition, superoxide appears to contribute to dilation, perhaps as a result of superoxide produced by NOS.

NADPH oxidase

One source of increased reactive oxygen species within arteries with age is NADPH oxidase (Csiszar et al. 2007b; Durrant et al. 2009; Rippe et al. 2010). Cerebral arteries have greater NADPH oxidase activity compared with conduit arteries and this contributes to their greater production of superoxide (Miller et al. 2005, 2009). Aging leads to increased NADPH oxidase expression in cerebral arteries (Mayhan et al. 2008) as well as increased NADPH oxidase expression and activity in the aorta (Durrant et al. 2009; Rippe et al. 2010). Inhibiting NADPH oxidase activity with apocynin led to improved EDD in the MCAs of old AL mice, in agreement with our previous findings in carotid arteries (Durrant et al. 2009). Similar to young

Fig. 7 Change in diameter in response to exogenous nicotinamide adenine dinucleotide phosphate (NADPH) in the carotid (a) and middle cerebral arteries (MCA, b) from young, old ad libitum fed

(AL), and old lifelong calorically restricted (CR) mice ($n=5-11$ per group). Values are mean $\pm$ SEM

mice (Durrant et al. 2009; Rippe et al. 2010), inhibiting NADPH oxidase does not affect EDD in the carotid arteries of old CR mice. In contrast, inhibiting NADPH oxidase impairs EDD in the MCAs from young mice and old CR mice. Inhibition of NADPH oxidase also impairs EDD in skeletal muscle arterioles of young mice (Sindler et al. 2013; Trott et al. 2011). These results indicate that reactive oxygen species produced by NADPH oxidase impair EDD with aging, but contribute to functional resistance artery EDD in young and lifelong CR mice.

We demonstrate that increasing NADPH oxidase activity with exogenous NADPH causes dose-dependent constriction of conduit and cerebral arteries, and this response is not affected by age or lifelong CR. Previous *ex vivo* studies have also found constriction of cerebral arteries from young healthy animals in response to exogenous NADPH (Didion and Faraci 2002). However, in the skeletal muscle circulation, exogenous NADPH causes dilation of feed arteries from young mice and constriction of feed arteries from old mice (Trott et al. 2011). In addition, *in vivo* cerebral arteriole diameter and cerebral blood flow increase in response to exogenous NADPH in young healthy animals (Paravicini et al. 2004; Park et al. 2004), a response mediated by increased hydrogen peroxide (Park et al. 2004). The contradiction of results between *in vivo* and *ex vivo* studies may result from the absence/presence of shear stress in these preparations, which can activate NADPH oxidase (Paravicini et al. 2006).

Our results indicate a dichotomy between the effect of basal and stimulated NADPH oxidase activity. In *ex vivo* MCAs from young and old CR mice, basal levels of NADPH oxidase activity (i.e., those that are inhibited by apocynin) aid in dilation to ACh, while increased NADPH oxidase activity (i.e., by stimulation with exogenous NADPH, or perhaps as occurs with aging) results in constriction. In the MCA from young mice (and similarly in old CR), the total contribution to dilation by NOS products (84 %) and NADPH oxidase products (44 %) appear to overlap. We are not aware of any effect of apocynin on NOS, any effect of L-NAME on NADPH oxidase, or any interaction of their products that would explain these results. Thus, these findings suggest that a novel interaction between NOS and NADPH oxidase may exist and future studies are needed to elucidate the mechanisms involved.

Limitations

In the present study, we were limited in our ability to explore molecular mechanisms for the effects of aging and CR on endothelial function. The size of the MCA precludes evaluation of enzyme activities, such as the activity of NOS or NADPH oxidase. Future studies will be needed, perhaps using genetic manipulation or *in vivo* and *ex vivo* treatments, to further determine the molecular mechanisms that lead to cerebral artery endothelial dysfunction with aging and the improvements with lifelong CR.

Conclusion

We demonstrate for the first time that lifelong CR does not completely prevent the age-related decline in cerebral artery function, while CR does maintain conduit artery function with age. The effects of lifelong CR on cerebral and conduit artery function are mediated by maintaining NO bioavailability and preventing the age-related increase in oxidative stress. The favorable effects we observed of lifelong CR on conduit and cerebral artery function may explain the beneficial effects of CR on cardiovascular and cerebrovascular disease risk as well as cognitive decline.

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