Tetrahydrobiopterin augments endothelium-dependent dilatation in sedentary but not in habitually exercising older adults

Iratxe Eskurza, Laura A. Myerburgh, Zachary D. Kahn and Douglas R. Seals

Department of Integrative Physiology, University of Colorado, Boulder, CO 80309, USA

Endothelium-dependent dilatation (EDD) is impaired with ageing in sedentary, but not in regularly exercising adults. We tested the hypotheses that differences in tetrahydrobiopterin (BH_4) bioactivity are key mechanisms explaining the impairment in EDD with sedentary ageing, and the maintenance of EDD with ageing in regularly exercising adults. Brachial artery flow-mediated dilatation (FMD), normalized for local shear stress, was measured after acute oral placebo or BH_4 in young sedentary (YS) ($n = 10$; 22 ± 1 years, mean ± S.E.M.), older sedentary (OS) ($n = 9$; 62 ± 2), and older habitually aerobically trained (OT) ($n = 12$; 66 ± 1) healthy men. At baseline, FMD was ∼50% lower in OS *versus* YS (1.12 ± 0.09 *versus* 0.57 ± 0.09 (Δmm (dyn cm^{-2})) $\times 10^{-2}$, $P < 0.001$; 1 dyn $= 10^{-5}$ N), but was preserved in OT (0.93 ± 0.08 (Δmm (dyn cm^{-2})) $\times 10^{-2}$). BH_4 administration improved FMD by ∼45% in OS (1.00 ± 0.10 (Δmm (dyn cm^{-2})) $\times 10^{-2}$, $P < 0.01$ *versus* baseline), but did not affect FMD in YS or OT. Endothelium-independent dilatation neither differed between groups at baseline nor changed with BH_4 administration. These results suggest that BH_4 bioactivity may be a key mechanism involved in the impairment of conduit artery EDD with sedentary ageing, and the EDD-preserving effect of habitual exercise.

(Resubmitted 16 June 2005; accepted after revision 27 August 2005; first published online 1 September 2005)
Corresponding author I. Eskurza: Department of Integrative Physiology, University of Colorado at Boulder, UCB 354, Boulder, CO 80309, USA. Email: eskurza@colourado.edu

Ageing is a major risk factor for the development of cardiovascular diseases (CVD) (Lakatta & Levy, 2003). A central feature of the age-associated increase in CVD risk is a reduction in vascular endothelium-dependent dilatation (EDD) (Celermajer *et al.* 1994; Taddei *et al.* 1995). As such, determining the mechanisms by which ageing impairs EDD and establishing strategies for the prevention of this adverse effect have important clinical implications.

The decrease in EDD with ageing in sedentary adults is mediated by oxidative stress (Taddei *et al.* 2000; Eskurza *et al.* 2004*b*), whereas EDD is preserved with ageing in the absence of oxidative stress in habitually exercising adults (Taddei *et al.* 2000; Eskurza *et al.* 2004*b*). However, the mechanisms by which the presence and absence of oxidative stress modulates EDD with ageing in sedentary and physically active humans, respectively, are unknown.

Oxidative stress can suppress EDD by oxidizing tetrahydrobiopterin (BH_4), an essential cofactor for endothelial nitric oxide synthase (eNOS), thus reducing BH_4 bioactivity (Milstien & Katusic, 1999; Cosentino *et al.* 2001; Laursen *et al.* 2001; Landmesser *et al.* 2003). Reduced BH_4 bioactivity leads to the 'uncoupling' of eNOS (Pou *et al.* 1992; Vasquez-Vivar *et al.* 1998, 2002), resulting in greater formation of superoxide anions than NO and impaired EDD (Vasquez-Vivar *et al.* 1998; Cosentino & Luscher, 1999; Cosentino *et al.* 2001; Laursen *et al.* 2001; Landmesser *et al.* 2003). BH_4 supplementation improves EDD in patients with clinical cardiovascular disorders (Stroes *et al.* 1997; Maier *et al.* 2000; Setoguchi *et al.* 2002). However, the role of BH_4 in the modulation of EDD with sedentary and physically active ageing in healthy adults is unknown.

In the present study, we tested the hypothesis that differences in BH_4 bioactivity are a key mechanism explaining the impairment in endothelium-independent dilatation (EDD) with sedentary ageing and the maintenance of EDD with ageing in regularly exercising adults. To do so, we determined the effect of acute BH_4 supplementation (single oral dose, 10 mg kg^{-1}) on brachial artery flow-mediated dilatation (FMD), a clinically important measure of conduit artery EDD, in groups of young and older sedentary and aerobic exercise-trained healthy men. Because group differences in brachial artery FMD are influenced by corresponding differences in the dilatory stimulus (i.e. local shear stress

 DOI: 10.1113/jphysiol.2005.092734

or blood flow) (Mitchell *et al.* 2004), brachial artery FMD was normalized for local shear stress.

Methods

Subjects

A total of 31 healthy men were studied: 10 young (aged 19–26 years) and 21 older (aged 55–74 years). During the previous 2 years, the older men were either sedentary (no regular physical activity) ($n = 9$) or habitually exercising (vigorous aerobic-endurance exercise, more than three sessions per week) ($n = 12$). Subjects were normotensive (blood pressure, <140/90), nonsmokers, nonobese, and free of CVD, as assessed by medical history, physical examination, blood chemistry, and resting and exercise ECG (older men only). Subjects were excluded if they were classified as having the metabolic syndrome according to the criteria established by the National Cholesterol Education Program Adult Treatment Panel III expert panel or a modification of the original (World Health Organization 2001; Laaksonen *et al.* 2002; Lakka *et al.* 2002). Candidates who had used antioxidants (e.g. vitamins C and E) within 6 weeks of study or were taking other medications were excluded. Subjects gave their written informed consent to participate. All procedures were approved by the Human Research Committee of the University of Colorado at Boulder.

Experimental procedures

All procedures were performed in the University of Colorado at Boulder General Clinical Research Center. After completion of screening procedures, two main experimental sessions (i.e. placebo baseline and BH_4 treatment) were conducted within 2 days of each other. Prior to the main sessions, subjects fasted for 10–12 h and abstained from any physical exercise on the previous day. During the experimental sessions, subjects were in the supine position and instrumented with an intravenous catheter in one arm for acquisition of blood.

Measurements

Brachial artery FMD and EDD. Brachial artery FMD was assessed noninvasively as described originally by Celermajer *et al.* (1992) and more recently by our laboratory (Eskurza *et al.* 2004*b*). Brachial artery baseline and peak (post-ischaemia) diameters were analysed off line with an automatic wall-tracking system (Vascular Analysis Tools, 4.0, Medical Imaging Analysis, LLC, IA, USA). The same investigator (I.E.) performed all analyses blinded to the group assignment of the subject and the experimental condition. Velocities and diameters were analysed on separate days. Baseline and peak mean blood velocities were measured as previously described (Eskurza *et al.* 2004*b*). The reliability of mean blood velocities obtained for nine subjects measured on two separate days ($r = 0.97$, $P < 0.001$) was similar to that reported in a recent population study (Mitchell *et al.* 2004).

Shear stress (SS) (dyn cm^{-2}; 1 dyn = 10^{-5} N) averaged over the whole entire cycle was calculated using the formula $SS = 8 \times \mu \times V/D$, where μ, V and D represent viscosity (dyn × (s cm^{-2})), velocity (cm s^{-1}) and diameter (cm), respectively, as described recently (Mitchell *et al.* 2004). To calculate baseline SS (SS_{base}), baseline V and D were used. Because brachial artery diameter is lower during the end of the occlusion period than before occlusion (Levenson *et al.* 2001), the peak velocity and corresponding diameter obtained during the last 15–20 s of occlusion was used to calculate peak SS (SS_{peak}).

Because the conventional expression of FMD as percentage change from baseline is highly dependent on baseline brachial artery diameter (i.e. the same absolute change in diameter results in a greater percentage change the smaller the baseline diameter), to normalize FMD for local SS we used the following formula: normalized FMD = (peak D − baseline D)/SS_{peak}. SS_{peak} was used to normalize FMD rather than the area under the SS curve (AUC) for two main reasons: (1) in our laboratory we are currently unable to determine the AUC of SS, and (2) the normalization of FMD with peak shear rate (i.e. an estimate of SS commonly used when viscosity in not measured) *versus* AUC of shear rate provides comparable efficacy for eliminating the effect of baseline diameter FMD among individuals (Pyke *et al.* 2004).

Brachial artery endothelium-independent dilatation was measured with sublingual nitroglycerin, as previously described (Eskurza *et al.* 2004*b*).

Arterial blood pressure. Resting blood pressure was measured over the brachial artery using a semi-automated device (Dynamap XL; Johnson and Johnson).

Plasma marker of oxidative stress. Plasma samples were analysed for oxidized low-density lipoproteins (ALPCO Diagnostics, Windham, NH, USA).

Body composition. Total body fat mass and fat-free mass (FFM) were measured by dual-energy X-ray absorptiometry (DXA-GE; Lunar corporation, Madison, WI, USA; software version 5.60.003), and waist and hip circumferences and body mass index (BMI) by anthropometry (Van Pelt *et al.* 1998).

Maximal oxygen consumption ($\dot{V}_{O_2\,max}$). $\dot{V}_{O_2\,max}$ was measured during graded treadmill exercise test using open-circuit spirometry, as previously described (DeSouza *et al.* 2000).

Blood viscosity. Whole-blood viscosity was measured at shear rates of 0.3–60 r.p.m. (2.5×10^{-6} to 6.0×10^{-5} *g*)

Table 1. Subject characteristics

	Young sedentary	Older sedentary	Older trained
Age (years)	22 ± 1	62 ± 2*	66 ± 1*
Body mass (kg)	73 ± 3	79 ± 3	70 ± 2
Body fat (%)	15.4 ± 2.2	27.3 ± 2.6*	16.9 ± 1.1†
BMI (kg m^{-2})	23.3 ± 0.9	25.6 ± 1.0	22.8 ± 0.6†
Waist circumference (cm)	78.8 ± 2.4	94.8 ± 3.1*	82.8 ± 1.8†
Hip circumference (cm)	93.0 ± 1.7	99.9 ± 1.9*	93.4 ± 1.4†
WHR	0.84 ± 0.01	0.95 ± 0.02*	0.89 ± 0.02†
Total cholesterol (mmol l^{-1})	4.5 ± 0.2	5.1 ± 0.2	5.0 ± 0.2
LDL cholesterol (mmol l^{-1})	2.7 ± 0.2	3.3 ± 0.2	3.0 ± 0.2
HDL cholesterol (mmol l^{-1})	1.2 ± 0.1	1.3 ± 0.1	1.6 ± 0.1*†
Triglycerides (mmol l^{-1})	1.2 ± 0.2	1.1 ± 0.1	0.86 ± 0.1
Plasma insulin (μIU ml^{-1})	3.7 ± 0.5	6.3 ± 1.0*	3.3 ± 0.5†
Plasma glucose (mmol l^{-1})	4.9 ± 0.1	5.3 ± 0.2	4.8 ± 0.1
$\dot{V}_{O_2\,max}$ (ml kg^{-1} min^{-1})	48.0 ± 1.3	29.1 ± 2.1*	41.3 ± 1.2*†
BV (dyn cm^{-2})	0.036 ± 0.001	0.033 ± 0.002	0.036 ± 0.003
Haematocrit (%)	45.0 ± 0.8	44.4 ± 1.1	43.8 ± 0.7

Data are means ± S.E.M. BMI, body mass index; WHR, waist-to-hip ratio; $\dot{V}_{O_2\,max}$, maximal oxygen consumption; BV, blood viscosity (1 dyn = 10^{-5} N). *$P < 0.05$ *versus* young; †$P < 0.05$ *versus* older sedentary.

at 37°C using a viscometer (model DV-I+; Brookfield Engineering Laboratories, Inc., Staughton, MA, USA), as previously described by our laboratory (Dinenno *et al.* 2001). Blood viscosity values at rates of 60 r.p.m. were used to calculate wall SS (see above).

Dietary analyses. Macronutrient and antioxidant (vitamins A, C and E) intake were quantified using the food-frequency questionnaire (NHANES III food-intake database).

Study design

We conducted a double-blind crossover study in which subjects were randomly assigned to take either placebo or BH_4 (single dose of 10 mg (kg body weight)$^{-1}$) pills (Schirks Laboratories, Switzwerland). The selection of this single dose was based on the facts that: (1) it increases plasma biopterin levels by ∼50-fold (Fiege *et al.* 2004), (2) improvements in brachial artery FMD are observed with as little as a fourfold increase in BH_4 above normal baseline concentrations (Ueda *et al.* 2000), and (3) commercially available BH_4 is used therapeutically to treat special forms of phenylketonuria at a dose of 2–10 mg per kg of body weight, and a single dose of 10 mg (kg body weight)$^{-1}$) decreases symptoms in these conditions (Niederwieser *et al.* 1982; Kure *et al.* 1999). Two days were allowed between the phases of the crossover study in order to ensure an adequate washout period, as previously described (Fiege *et al.* 2004). The randomization of the treatments was performed by the General Clinical Research Center pharmacist who was not involved in data acquisition or analysis. To ensure compliance with the treatments, subjects were contacted by phone the night prior to the experimental session. With the unavoidable exception of knowing the age group (young *versus* older) of the subjects during data collection, the investigator (I.E.) who performed the data collection and analysis was blinded to the group and treatment condition. Measurements were obtained ∼3 h after ingestion when the dose of BH_4 used reaches its maximal plasma concentration (Fiege *et al.* 2004).

Statistical analyses

Statistical analyses were performed with SPSS statistical package (version 11.0; SPSS, Chicago, IL, USA). Differences in subject characteristics across the three groups were determined by one-way ANOVA. To determine the effects of placebo *versus* BH_4 administration on all outcome measures, repeated measures ANOVA was used. In the case of significant F values, *post hoc* analyses were performed using the Bonferroni correction to identify significant differences among mean values. To examine relations between variables of interest, bivariate relations were performed with Pearson product–moment correlations.

Results

Subject characteristics

Values are shown in Tables 1 and 2. The groups did not differ in body mass, total cholesterol, low-density lipoprotein-cholesterol (LDL-C), triglycerides, glucose, blood viscosity, haematocrit and systolic blood pressure. BMI, total body fat, waist and hip circumferences,

Table 2. Systemic haemodynamics and brachial artery parameters after placebo and BH_4 supplementation

	Young sedentary		Older sedentary		Older endurance-trained	
	Placebo	BH_4	Placebo	BH_4	Placebo	BH_4
Systolic BP (mmHg)	110 ± 3	111 ± 4	111 ± 4	113 ± 5	107 ± 3	108 ± 4
Diastolic BP (mmHg)	58 ± 2	59 ± 2	69 ± 1*	69 ± 2*	66 ± 2*	67 ± 2*
Heart rate (beats min^{-1})	52 ± 3	50 ± 3	58 ± 3	55 ± 2	46 ± 2*†	46 ± 2*†
Baseline *D* (mm)	4.13 ± 0.11	4.10 ± 0.11	4.36 ± 0.16	4.33 ± 0.16	3.99 ± 0.14	3.96 ± 0.14
Baseline MBV (cm s^{-1})	5.5 ± 0.5	5.3 ± 0.5	5.7 ± 0.3	5.4 ± 0.4	4.8 ± 0.3	4.9 ± 0.3
Peak MBV (cm s^{-1})	42.9 ± 2.5	43.8 ± 1.4	44.6 ± 3.4	40.9 ± 2.3	37.2 ± 2.1†	36.2 ± 2.2*
SS_{base} (dyn cm^{-2})	3.8 ± 0.3	3.5 ± 0.2	3.9 ± 0.3	3.7 ± 0.3	3.3 ± 0.3	3.5 ± 0.5
SS_{peak} (dyn cm^{-2})	28.6 ± 1.7	29.2 ± 1.1	29.0 ± 3.2	26.2 ± 1.8	26.7 ± 2.3	24.6 ± 1.7
FMD (Δmm)	0.32 ± 0.02	0.31 ± 0.02	0.16 ± 0.03*	0.26 ± 0.02‡	0.23 ± 0.01†	0.24 ± 0.02
FMD (%)	7.7 ± 0.5	7.6 ± 0.6	3.9 ± 0.7*	6.0 ± 0.7‡	5.9 ± 0.5†	6.3 ± 0.6

Data are means ± S.E.M. BH_4, tetrahydrobiopterin. BP, blood pressure; *D*, diameter; MBV, mean blood velocity, FMD, flow-mediated dilatation; SS_{base}, baseline shear stress (1 dyn = 10^{-5} N); SS_{peak}, peak shear stress. *$P < 0.05$ *versus* young sedentary; †$P < 0.05$ *versus* older sedentary; ‡$P < 0.05$ *versus* placebo in the same group.

waist-to-hip ratio (WHR) and plasma insulin concentration were greater, and $\dot{V}_{O_2\,max}$ was lower, in the older sedentary men compared with the other two groups ($P < 0.05$). High-density lipoprotein-cholesterol (HDL-C) was greater, and resting heart rate was lower, in the older exercising men than in the other two groups ($P < 0.05$). Although well within the normal range, diastolic blood pressure was higher in both older groups compared with the young controls ($P < 0.05$). Plasma concentrations of oxidized LDL were higher in the older compared with the young sedentary men (60.5 ± 6.5 *versus* 45.8 ± 5.8 U l^{-1}, $P = 0.05$), but were not different in the young sedentary and older exercising (52.1 ± 4.8 U l^{-1}) men. Groups did not differ in dietary intake of major antioxidants such as vitamins A, E and C (data not shown).

Brachial artery response to BH_4

Baseline brachial artery diameter (i.e. placebo condition) did not differ among groups and was not affected by BH_4 administration (Table 2).

Brachial artery normalized FMD at baseline (after placebo) and in response to BH_4 is shown in Fig. 1. At baseline, FMD was ∼50% lower in the older sedentary men compared with the young controls ($P < 0.001$), but was not different in the older exercising and young men. Brachial artery FMD was increased by ∼45% after BH_4 administration in the older sedentary men ($P < 0.01$ *versus* placebo baseline), but was not affected by BH_4 in the other two groups. There were no significant group differences in brachial artery FMD after BH_4 administration (all $P > 0.05$).

There were no group differences in brachial artery dilatation in response to sublingual nitroglycerin at baseline, and the responses to nitroglycerin were not affected by BH_4 in any of the groups (Table 2).

Other brachial artery and haemodynamic information is presented in Table 2. Systolic and diastolic blood pressures, heart rate, baseline mean blood velocity, SS_{base} and SS_{peak} were similar in response to placebo and BH_4 administration. Whole-blood viscosity and haematocrit did not change with BH_4 administration in any of the groups (data not shown).

In the pooled sample, the improvement in normalized FMD in response to BH_4 was inversely related to $\dot{V}_{O_2\,max}$ ($r = -0.46$; $P = 0.01$) and normalized FMD at baseline ($r = -0.61$; $P < 0.001$), and was positively related to plasma insulin concentration ($r = 0.37$; $P = 0.04$).

Discussion

This study provides new insight into the potential mechanisms mediating the impairment in peripheral conduit artery EDD with sedentary ageing, and the maintenance of EDD with physically active ageing in humans. Specifically, the main findings of this study are that acute administration of BH_4 improved brachial FMD in older sedentary men, but did not affect FMD in young sedentary or habitually exercising older men. These results suggest that: (1) reduced BH_4 bioactivity may contribute to the suppression of brachial artery FMD with sedentary human ageing; and (2) maintained BH_4 bioactivity may be involved in the preserved FMD with ageing seen in habitually exercising men.

Role of BH_4 on EDD with sedentary ageing

In the present study, baseline brachial artery FMD (placebo control condition) was ∼50% lower in older compared with young healthy men, whereas dilatation in response to sublingual nitroglycerin, a NO donor, was not different. Consistent with previous findings (Celermajer *et al.* 1994;

Eskurza *et al.* 2004*b*), these results indicate an age-related impairment in peripheral conduit artery EDD in the absence of any reduction in endothelium-independent dilatation in sedentary healthy adults.

We (Eskurza *et al.* 2004*b*) and others (Taddei *et al.* 2000) previously demonstrated that supraphysiological concentrations of ascorbic acid, a potent antioxidant, restore the age-related loss of EDD in older sedentary adults, suggesting that oxidative stress is the key mechanism involved. This is in agreement with the observation that the aortas of older rats and mice have increased vascular production of reactive oxygen species (ROS), which is associated with reductions in NO bioavailability and EDD (van der Loo *et al.* 2000; Blackwell *et al.* 2004). In the present study, we also found that plasma concentrations of oxidized LDL, a relatively insensitive systemic marker of oxidative stress, were higher in the older compared with the young sedentary men, as reported previously (Mosinger, 1997; Eskurza *et al.* 2004*a*).

We hypothesized that an important mechanism by which oxidative stress could impair EDD with sedentary ageing is by influencing the biological activity of BH_4, the essential cofactor of eNOS for NO synthesis (Pollock *et al.* 1991). BH_4 is one of the most potent endogenous reducing agents. Therefore, vascular oxidative stress, specifically peroxynitrate, rapidly oxidizes BH_4, with subsequent generation of dihydrobiopterin (BH_2), its oxidized and inactive form (Milstien & Katusic, 1999; Laursen *et al.* 2001). The experimental observation that the aortas of older rats show increased production of peroxynitrate (van der Loo *et al.* 2000) was also consistent with the idea of increased BH_4 oxidation with ageing.

The present results extend previous findings by demonstrating that BH_4 administration restores the age-associated loss of brachial artery FMD in sedentary healthy men. BH_4 did not affect the dilatory response to sublingual nitroglycerin. Therefore, these findings suggest that reduced BH_4 bioactivity may be a key mechanism involved in the impairment of peripheral conduit artery EDD with sedentary ageing. Our results are consistent with earlier experimental observations that showed that acute BH_4 supplementation improved or restored EDD in groups at risk of or with clinical CVD, i.e. states characterized by vascular oxidative stress (Stroes *et al.* 1997; Heitzer *et al.* 2000; Maier *et al.* 2000; Ueda *et al.* 2000; Setoguchi *et al.* 2002).

The exact mechanism by which BH_4 contributes to reduced brachial artery FMD with sedentary ageing in humans cannot be discerned from the present results. It is possible that conduit artery EDD may become impaired with ageing in part via increased endothelial cell production of ROS with consequent increased BH_4 oxidation and increased BH_2 relative to BH_4. Indeed, the BH_4/BH_2 ratio determines superoxide *versus* NO generation from eNOS (Vasquez-Vivar *et al.* 2002). As such, a reduction in the active form of the cofactor (BH_4) would result in decreased BH_4 biological activity and, thus, the uncoupling (dysfunction) of eNOS, thereby generating superoxide instead of NO (Pou *et al.* 1992; Vasquez-Vivar *et al.* 1998, 2002). However, in mice, BH_4 and BH_2 concentrations in aorta, although tending to be lower in older animals, are not significantly different than in young mice, and the BH_4/BH_2 ratio is similar (Blackwell *et al.* 2004). Moreover, guanosine 5′-triphosphate cyclohydrolase I (GTPC I), the rate-limiting enzyme in BH_4 synthesis, is not different in the young and older mice (Blackwell *et al.* 2004). There are no published data on vascular BH_4 or BH_2 concentrations or GTPC I enzyme activity with ageing in humans. The findings in mice with ageing (Blackwell *et al.* 2004) differ from observations in experimental models of diabetes and low-renin hypertension in which reductions in vascular endothelial BH_4 production and GTPC I activity appear to play an important role in mediating associated

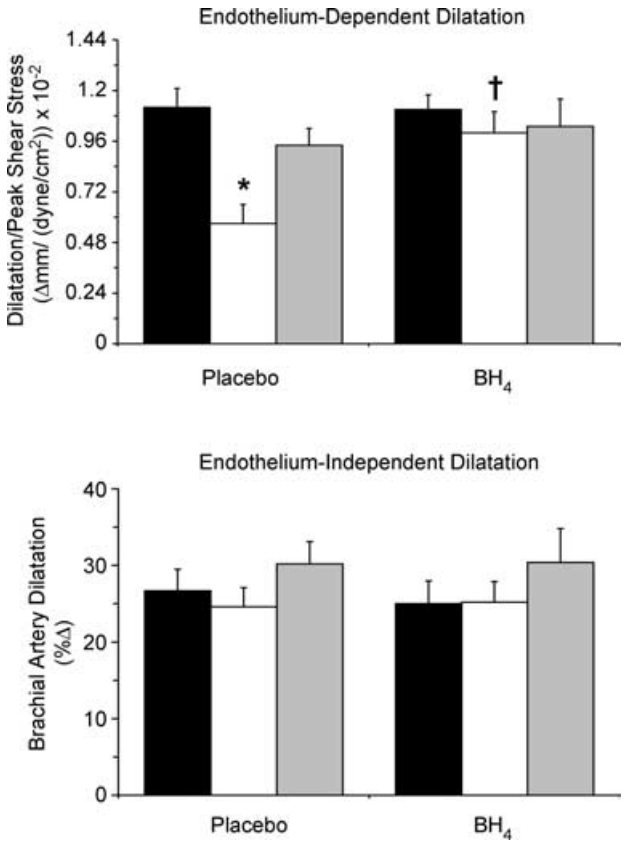

Figure 1
Brachial artery flow-mediated dilatation (FMD) (top panel) and endothelium-independent dilatation (bottom panel) in young sedentary (black bars) and older sedentary (white bars) and regularly exercising (grey bars) healthy men after acute oral placebo (baseline conditions) or tetrahydrobiopterin (BH_4) supplementation. FMD values are normalized for the stimulus. Mean ± S.E.M. values are shown. *$P < 0.001$ *versus* young and older exercising men. †$P < 0.01$ *versus* placebo condition.

endothelial dysfunction (Zheng *et al.* 2003; Meininger *et al.* 2004). Interestingly, stimulation of BH_4 synthesis restores vascular endothelial function in some animal models of hypercholesterolaemia despite elevated baseline levels of BH_4 (d'Uscio *et al.* 2003; Alp *et al.* 2004).

In vitro, half-maximal and maximal NO synthesis, estimated from L-citrulline production, occurs in the micromolar range of BH_4 concentrations (0.1 and 1 μM, respectively) (Chen *et al.* 1995). However, much smaller plasma BH_4 concentrations (in the nanomolar range) achieved after a single oral dose of 2 mg (kg body weight)$^{-1}$ of sapropterin hydrochloride, a BH_4 precursor, improve brachial FMD in young adult smokers (Ueda *et al.* 2000). The discrepancy between *in vitro* and *in vivo* results, including the present findings, may have at least two explanations. First, it is possible that some eNOS is activated at much lower BH_4 concentrations than those reported in *in vitro* preparations. Second, BH_4 increases the affinity of eNOS for L-arginine (Klatt *et al.* 1994). Thus, it is possible that relatively low BH_4 concentrations could produce an amount of NO greater than expected based on the BH_4 concentration alone.

In the present study, we did not specifically inhibit NO bioactivity and therefore cannot exclude the possibility that other endothelium-dependent dilatory mechanisms (e.g. endothelium-derived hyperpolarizing factor or prostacyclin) were involved in the BH_4-related improvement of FMD seen in the older sedentary men. However, conduit artery FMD is primarily mediated by endothelial-derived NO (Lieberman *et al.* 1996; Mullen *et al.* 2001). Moreover, to the best of our knowledge, BH_4 is a cofactor only for eNOS synthesis of NO and not for any other endothelium-dependent dilator.

Finally, we wish to emphasize that formation of ROS from other vascular sources, such as fibroblasts, smooth muscle cells and inflammatory cells, may also act to reduce the bioavailability of NO (Ushio-Fukai *et al.* 1996; Griendling *et al.* 2000), and NO production could be impaired with ageing via other mechanisms including reductions in eNOS expression, although this is unclear (Barton *et al.* 1997; van der Loo *et al.* 2000).

Preservation of FMD with age in habitually exercising men

Consistent with our recent findings (Eskurza *et al.* 2004*b*), in the present study, brachial artery FMD was preserved in older men who habitually exercise. Similar observations have been made for EDD in forearm resistance vessels (DeSouza *et al.* 2000; Taddei *et al.* 2000). In the present study, BH_4 supplementation had no effect on brachial artery FMD in either young sedentary or older exercising men. Consistent with this, the difference (improvement) in brachial artery FMD between the placebo and BH_4 treatment conditions was inversely related to baseline $\dot{V}_{O_2\,max}$, i.e. maximal aerobic exercise capacity. Taken together, these findings support the idea that the beneficial effects of habitual exercise/high aerobic fitness on EDD with ageing may be associated with the maintenance BH_4 bioactivity.

The mechanisms by which regular aerobic exercise preserves BH_4 bioactivity and EDD with ageing are unknown, but they are likely to involve, at least in part, the absence of oxidative stress.

Aerobic (endurance) exercise training is associated with an upregulation of important enzymatic antioxidants, as well as reduced production of superoxide anions (Sen, 1995; Fukai *et al.* 2000; Rush *et al.* 2000). Furthermore, the maintenance of resistance vessel and conduit artery EDD with ageing in physically active adults is associated with a lack of improvement in EDD in response to ascorbic acid administration (Taddei *et al.* 2000; Eskurza *et al.* 2004*b*), suggesting an absence of baseline oxidative stress in contrast to their sedentary peers. The present findings suggest that the maintenance of BH_4 bioactivity may be a novel mechanism by which regular aerobic exercise preserves EDD with ageing. One possibility is that regular exercise may prevent the development of oxidative stress with ageing, thus reducing the oxidation of BH_4, which maintains normal eNOS function and production of NO.

Limitations

This study has at least four important limitations. First, acute BH_4 supplementation may have improved EDD in the older sedentary men in part via its non-specific antioxidant effects. Supraphysiological concentrations of BH_4 scavenge ROS, including superoxide anions, *in vitro* (Hyun *et al.* 1995; Kojima *et al.* 1995). However, based on electron paramagnetic resonance kinetic analysis, superoxide scanvenging by BH_4 is not a major reaction *in vivo* (Vasquez-Vivar *et al.* 2001). More importantly, the 'antioxidant' effect of BH_4 was due to its ability to decrease superoxide formation and increase NO synthesis from eNOS (Vasquez-Vivar *et al.* 2002).

Second, we attempted, but were unable to measure plasma BH_4 concentrations. As such, we cannot rule out the possibility of group differences at baseline or in response to BH_4 supplementation. To our knowledge, only one previous study has reported plasma BH_4 concentrations at baseline and after experimental stimulation of BH_4 in humans (chronic smokers) (Ueda *et al.* 2000). However, the single dose used in the present study increases plasma biopterin levels by ~50-fold (Fiege *et al.* 2004) and improvements in brachial artery FMD are observed with as little as a fourfold increase in BH_4 above normal baseline concentrations (Ueda *et al.* 2000), consistent with the fact that a significant improvement

in brachial FMD was observed with BH_4 in the older sedentary subjects in the present study. Moreover, plasma concentrations do not necessarily reflect group differences in intra-endothelial BH_4 concentrations. In patients with coronary artery disease, baseline plasma concentrations of BH_4 are high (Tatzber *et al.* 1991; Schumacher *et al.* 1997), but acute supplementation of BH_4 nevertheless restores EDD (Maier *et al.* 2000).

Third, conduit artery EDD and resistance vessel EDD do not necessary correlate (Eskurza *et al.* 2001). Therefore, our findings cannot be extended to smaller vessel function. However, intra-arterial administration of BH_4 improves EDD of resistance vessels in patients with CVD (Stroes *et al.* 1997; Heitzer *et al.* 2000; Setoguchi *et al.* 2002), suggesting that reduced BH_4 bioavailability may contribute to endothelial dysfunction in both conduit and resistance vessels.

Finally, in the present study a single high dose of BH_4 was used because the experimental aim was to gain mechanistic insight into the role of BH_4 in the tonic modulatory influences of age and habitual exercise on baseline EDD. We recognize that based on the present results and those of others (Stroes *et al.* 1997; Heitzer *et al.* 2000; Maier *et al.* 2000; Ueda *et al.* 2000; Setoguchi *et al.* 2002), it may now be of clinical interest to examine the potential therapeutic benefits of sustained lower-dose administration of BH_4 in patients at risk of CVD or with existing clinical disorders.

Conclusions

The results from the present investigation suggest for the first time that reduced BH_4 bioactivity may be a key mechanism involved in the impairment of peripheral conduit artery EDD with sedentary ageing. Our findings also provide the first experimental evidence supporting the hypothesis that preservation of conduit artery EDD with physically active ageing may be associated with the maintenance of BH_4 bioactivity.

References

Alp NJ, McAteer MA, Khoo J, Choudhury RP & Channon KM (2004). Increased endothelial tetrahydrobiopterin synthesis by targeted transgenic GTP-cyclohydrolase I overexpression reduces endothelial dysfunction and atherosclerosis in ApoE-knockout mice. *Arterioscler Thromb Vasc Biol* **24**, 445–450.

Barton M, Cosentino F, Brandes RP, Moreau P, Shaw S & Luscher TF (1997). Anatomic heterogeneity of vascular aging: role of nitric oxide and endothelin. *Hypertension* **30**, 817–824.

Blackwell KA, Sorenson JP, Richardson DM, Smith LA, Suda O, Nath K & Katusic ZS (2004). Mechanisms of aging-induced impairment of endothelium-dependent relaxation: role of tetrahydrobiopterin. *Am J Physiol Heart Circ Physiol* **287**, H2448–2453.

Celermajer DS, Sorensen KE, Gooch VM, Spiegelhalter DJ, Miller OI, Sullivan ID, Lloyd JK & Deanfield JE (1992). Non-invasive detection of endothelial dysfunction in children and adults at risk of atherosclerosis. *Lancet* **340**, 1111–1115.

Celermajer DS, Sorensen KE, Spiegelhalter DJ, Georgakopoulos D, Robinson J & Deanfield JE (1994). Aging is associated with endothelial dysfunction in healthy men years before the age-related decline in women. *J Am Coll Cardiol* **24**, 471–476.

Chen PF, Tsai AL & Wu KK (1995). Cysteine 99 of endothelial nitric oxide synthase (NOS-III) is critical for tetrahydrobiopterin-dependent NOS-III stability and activity. *Biochem Biophys Res Commun* **215**, 1119–1129.

Cosentino F, Barker JE, Brand MP, Heales SJ, Werner ER, Tippins JR, West N, Channon KM, Volpe M & Luscher TF (2001). Reactive oxygen species mediate endothelium-dependent relaxations in tetrahydrobiopterin-deficient mice. *Arterioscler Thromb Vasc Biol* **21**, 496–502.

Cosentino F & Luscher TF (1999). Tetrahydrobiopterin and endothelial nitric oxide synthase activity. *Cardiovasc Res* **43**, 274–278.

DeSouza CA, Shapiro LF, Clevenger CM, Dinenno FA, Monahan KD, Tanaka H & Seals DR (2000). Regular aerobic exercise prevents and restores age-related declines in endothelium-dependent vasodilation in healthy men. *Circulation* **102**, 1351–1357.

Dinenno FA, Tanaka H, Monahan KD, Clevenger CM, Eskurza I, DeSouza CA & Seals DR (2001). Regular endurance exercise induces expansive arterial remodelling in the trained limbs of healthy men. *J Physiol* **534**, 287–295.

Eskurza I, Monahan KD, Robinson JA & Seals DR (2004*a*). Ascorbic acid does not affect large elastic artery compliance or central blood pressure in young and older men. *Am J Physiol Heart Circ Physiol* **286**, H1528–1534.

Eskurza I, Monahan KD, Robinson JA & Seals DR (2004*b*). Effect of acute and chronic ascorbic acid on flow-mediated dilatation with sedentary and physically active human ageing. *J Physiol* **556**, 315–324.

Eskurza I, Seals DR, DeSouza CA & Tanaka H (2001). Pharmacologic *versus* flow-mediated assessments of peripheral vascular endothelial vasodilatory function in humans. *Am J Cardiol* **88**, 1067–1069.

Fiege B, Ballhausen D, Kierat L, Leimbacher W, Goriounov D, Schircks B, Thony B & Blau N (2004). Plasma tetrahydrobiopterin and its pharmacokinetic following oral administration. *Mol Genet Metab* **81**, 45–51.

Fukai T, Siegfried MR, Ushio-Fukai M, Cheng Y, Kojda G & Harrison DG (2000). Regulation of the vascular extracellular superoxide dismutase by nitric oxide and exercise training. *J Clin Invest* **105**, 1631–1639.

Griendling KK, Sorescu D & Ushio-Fukai M (2000). NAD(P)H oxidase: role in cardiovascular biology and disease. *Circ Res* **86**, 494–501.

Heitzer T, Brockhoff C, Mayer B, Warnholtz A, Mollnau H, Henne S, Meinertz T & Munzel T (2000). Tetrahydrobiopterin improves endothelium-dependent vasodilation in chronic smokers: evidence for a dysfunctional nitric oxide synthase. *Circ Res* **86**, E36–41.

Hyun J, Komori Y, Chaudhuri G, Ignarro LJ & Fukuto JM (1995). The protective effect of tetrahydrobiopterin on the nitric oxide-mediated inhibition of purified nitric oxide synthase. *Biochem Biophys Res Commun* **206**, 380–386.

Klatt P, Schmid M, Leopold E, Schmidt K, Werner ER & Mayer B (1994). The pteridine binding site of brain nitric oxide synthase. Tetrahydrobiopterin binding kinetics, specificity, and allosteric interaction with the substrate domain. *J Biol Chem* **269**, 13861–13866.

Kojima S, Ona S, Iizuka I, Arai T, Mori H & Kubota K (1995). Antioxidative activity of 5,6,7,8-tetrahydrobiopterin and its inhibitory effect on paraquat-induced cell toxicity in cultured rat hepatocytes. *Free Radic Res* **23**, 419–430.

Kure S, Hou DC, Ohura T, Iwamoto H, Suzuki S, Sugiyama N, Sakamoto O, Fujii K, Matsubara Y & Narisawa K (1999). Tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency. *J Pediatr* **135**, 375–378.

Laaksonen DE, Lakka HM, Niskanen LK, Kaplan GA, Salonen JT & Lakka TA (2002). Metabolic syndrome and development of diabetes mellitus: application and validation of recently suggested definitions of the metabolic syndrome in a prospective cohort study. *Am J Epidemiol* **156**, 1070–1077.

Lakatta EG & Levy D (2003). Arterial and cardiac aging: major shareholders in cardiovascular disease enterprises: Part I: aging arteries: a 'set up' for vascular disease. *Circulation* **107**, 139–146.

Lakka HM, Laaksonen DE, Lakka TA, Niskanen LK, Kumpusalo E, Tuomilehto J & Salonen JT (2002). The metabolic syndrome and total and cardiovascular disease mortality in middle-aged men. *Jama* **288**, 2709–2716.

Landmesser U, Dikalov S, Price SR, McCann L, Fukai T, Holland SM, Mitch WE & Harrison DG (2003). Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension. *J Clin Invest* **111**, 1201–1209.

Laursen JB, Somers M, Kurz S, McCann L, Warnholtz A, Freeman BA, Tarpey M, Fukai T & Harrison DG (2001). Endothelial regulation of vasomotion in apoE-deficient mice: implications for interactions between peroxynitrite and tetrahydrobiopterin. *Circulation* **103**, 1282–1288.

Levenson J, Pessana F, Gariepy J, Armentano R & Simon A (2001). Gender differences in wall shear-mediated brachial artery vasoconstriction and vasodilation. *J Am Coll Cardiol* **38**, 1668–1674.

Lieberman EH, Gerhard MD, Uehata A, Selwyn AP, Ganz P, Yeung AC & Creager MA (1996). Flow-induced vasodilation of the human brachial artery is impaired in patients $<$40 years of age with coronary artery disease. *Am J Cardiol* **78**, 1210–1214.

van der Loo B, Labugger R, Skepper JN, Bachschmid M, Kilo J, Powell JM, Palacios-Callender M, Erusalimsky JD, Quaschning T, Malinski T, Gygi D, Ullrich V & Luscher TF (2000). Enhanced peroxynitrite formation is associated with vascular aging. *J Exp Med* **192**, 1731–1744.

Maier W, Cosentino F, Lutolf RB, Fleisch M, Seiler C, Hess OM, Meier B & Luscher TF (2000). Tetrahydrobiopterin improves endothelial function in patients with coronary artery disease. *J Cardiovasc Pharmacol* **35**, 173–178.

Meininger CJ, Cai S, Parker JL, Channon KM, Kelly KA, Becker EJ, Wood MK, Wade LA & Wu G (2004). GTP cyclohydrolase I gene transfer reverses tetrahydrobiopterin deficiency and increases nitric oxide synthesis in endothelial cells and isolated vessels from diabetic rats. *FASEB J* **18**, 1900–1902.

Milstien S & Katusic Z (1999). Oxidation of tetrahydrobiopterin by peroxynitrite: implications for vascular endothelial function. *Biochem Biophys Res Commun* **263**, 681–684.

Mitchell GF, Parise H, Vita JA, Larson MG, Warner E, Keaney JF Jr, Keyes MJ, Levy D, Vasan RS & Benjamin EJ (2004). Local shear stress and brachial artery flow-mediated dilation: the Framingham Heart Study. *Hypertension* **44**, 134–139.

Mosinger BJ (1997). Human low-density lipoproteins: oxidative modification and its relation to age, gender, menopausal status and cholesterol concentrations. *Eur J Clin Chem Clin Biochem* **35**, 207–214.

Mullen MJ, Kharbanda RK, Cross J, Donald AE, Taylor M, Vallance P, Deanfield JE & MacAllister RJ (2001). Heterogenous nature of flow-mediated dilatation in human conduit arteries *in vivo*: relevance to endothelial dysfunction in hypercholesterolemia. *Circ Res* **88**, 145–151.

Niederwieser A, Curtius HC, Wang M & Leupold D (1982). Atypical phenylketonuria with defective biopterin metabolism. Monotherapy with tetrahydrobiopterin or sepiapterin, screening and study of biosynthesis in man. *Eur J Pediatr* **138**, 110–112.

Pollock JS, Forstermann U, Mitchell JA, Warner TD, Schmidt HH, Nakane M & Murad F (1991). Purification and characterization of particulate endothelium-derived relaxing factor synthase from cultured and native bovine aortic endothelial cells. *Proc Natl Acad Sci U S A* **88**, 10480–10484.

Pou S, Pou WS, Bredt DS, Snyder SH & Rosen GM (1992). Generation of superoxide by purified brain nitric oxide synthase. *J Biol Chem* **267**, 24173–24176.

Pyke KE, Dwyer EM & Tschakovsky ME (2004). Impact of controlling shear rate on flow-mediated dilation responses in the brachial artery of humans. *J Appl Physiol* **97**, 499–508.

Rush JW, Laughlin MH, Woodman CR & Price EM (2000). SOD-1 expression in pig coronary arterioles is increased by exercise training. *Am J Physiol Heart Circ Physiol* **279**, H2068–2076.

Schumacher M, Halwachs G, Tatzber F, Fruhwald FM, Zweiker R, Watzinger N, Eber B, Wilders-Truschnig M, Esterbauer H & Klein W (1997). Increased neopterin in patients with chronic and acute coronary syndromes. *J Am Coll Cardiol* **30**, 703–707.

Sen CK (1995). Oxidants and antioxidants in exercise. *J Appl Physiol* **79**, 675–686.

Setoguchi S, Hirooka Y, Eshima K, Shimokawa H & Takeshita A (2002). Tetrahydrobiopterin improves impaired endothelium-dependent forearm vasodilation in patients with heart failure. *J Cardiovasc Pharmacol* **39**, 363–368.

Stroes E, Kastelein J, Cosentino F, Erkelens W, Wever R, Koomans H, Luscher T & Rabelink T (1997). Tetrahydrobiopterin restores endothelial function in hypercholesterolemia. *J Clin Invest* **99**, 41–46.

Taddei S, Galetta F, Virdis A, Ghiadoni L, Salvetti G, Franzoni F, Giusti C & Salvetti A (2000). Physical activity prevents age-related impairment in nitric oxide availability in elderly athletes. *Circulation* **101**, 2896–2901.

Taddei S, Virdis A, Mattei P, Ghiadoni L, Gennari A, Fasolo CB, Sudano I & Salvetti A (1995). Aging and endothelial function in normotensive subjects and patients with essential hypertension. *Circulation* **91**, 1981–1987.

Tatzber F, Rabl H, Koriska K, Erhart U, Puhl H, Waeg G, Krebs A & Esterbauer H (1991). Elevated serum neopterin levels in atherosclerosis. *Atherosclerosis* **89**, 203–208.

Ueda S, Matsuoka H, Miyazaki H, Usui M, Okuda S & Imaizumi T (2000). Tetrahydrobiopterin restores endothelial function in long-term smokers. *J Am Coll Cardiol* **35**, 71–75.

d'Uscio LV, Milstien S, Richardson D, Smith L & Katusic ZS (2003). Long-term vitamin C treatment increases vascular tetrahydrobiopterin levels and nitric oxide synthase activity. *Circ Res* **92**, 88–95.

Ushio-Fukai M, Zafari AM, Fukui T, Ishizaka N & Griendling KK (1996). p22phox is a critical component of the superoxide-generating NADH/NADPH oxidase system and regulates angiotensin-II-induced hypertrophy in vascular smooth muscle cells. *J Biol Chem* **271**, 23317–23321.

Van Pelt RE, Davy KP, Stevenson ET, Wilson TM, Jones PP, Desouza CA & Seals DR (1998). Smaller differences in total and regional adiposity with age in women who regularly perform endurance exercise. *Am J Physiol Endocrinol Metab* **275**, E626–634.

Vasquez-Vivar J, Kalyanaraman B, Martasek P, Hogg N, Masters BS, Karoui H, Tordo P & Pritchard KA Jr (1998). Superoxide generation by endothelial nitric oxide synthase: the influence of cofactors. *Proc Natl Acad Sci U S A* **95**, 9220–9225.

Vasquez-Vivar J, Martasek P, Whitsett J, Joseph J & Kalyanaraman B (2002). The ratio between tetrahydrobiopterin and oxidized tetrahydrobiopterin analogues controls superoxide release from endothelial nitric oxide synthase: an EPR spin trapping study. *Biochem J* **362**, 733–739.

Vasquez-Vivar J, Whitsett J, Martasek P, Hogg N & Kalyanaraman B (2001). Reaction of tetrahydrobiopterin with superoxide: EPR-kinetic analysis and characterization of the pteridine radical. *Free Radic Biol Med* **31**, 975–985.

World Health Organization (2001). Executive summary of the third report of the National Cholesterol Education Program (NCEP) expert panel on detection, evaluation, and treatment of high blood cholesterol in adults (Adults Treatment Panel III). *JAMA* **285**, 2486–2497.

Zheng JS, Yang XQ, Lookingland KJ, Fink GD, Hesslinger C, Kapatos G, Kovesdi I & Chen AF (2003). Gene transfer of human guanosine 5′-triphosphate cyclohydrolase I restores vascular tetrahydrobiopterin level and endothelial function in low renin hypertension. *Circulation* **108**, 1238–1245.

Acknowledgements

This study was supported by NIH awards AG13038, AG06537 and RR00051.