Ageing diminishes endothelium-dependent vasodilatation and tetrahydrobiopterin content in rat skeletal muscle arterioles

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Ageing reduces endothelium-dependent vasodilatation through an endothelial nitric oxide synthase (NOS) signalling pathway. The purpose of this study was to determine whether arginase activity diminishes endothelium-dependent vasodilatation in skeletal muscle arterioles from old rats, and whether NOS substrate (L-arginine) and cofactor (tetrahydrobiopterin; BH_4) concentrations are reduced. First-order arterioles were isolated from the soleus muscle of young (6 months old) and old (24 months old) male Fischer 344 rats. *In vitro* changes in luminal diameter in response to stepwise increases in flow were determined in the presence of the NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME, 10^{-5} mol l^{-1}), the arginase inhibitor N^{ω}-hydroxy-nor-L-arginine (NOHA, 5×10^{-4} mol l^{-1}), exogenous L-arginine (3×10^{-3} mol l^{-1}) or the precursor for BH_4 synthesis sepiapterin (1 μmol l^{-1}). Arteriolar L-arginine and BH_4 content were determined via HPLC. Ageing decreased flow-mediated vasodilatation by 52%, and this difference was abolished with NOS inhibition. Neither inhibition of arginase activity nor addition of exogenous L-arginine had any effect on flow-mediated vasodilatation; arteriolar L-arginine content was also not different between age groups. BH_4 content was lower in arterioles from old rats (94 ± 8 fmol (mg tissue)$^{-1}$) relative to controls (234 ± 21 fmol (mg tissue)$^{-1}$), and sepiapterin elevated flow-mediated vasodilatation in arterioles from old rats. These results demonstrate that the impairment of endothelium-dependent vasodilatation induced by old age is due to an altered nitric oxide signalling mechanism in skeletal muscle arterioles, but is not the result of increased arginase activity and limited L-arginine substrate. Rather, the age-related deficit in flow-mediated vasodilatation appears to be the result, in part, of limited BH_4 bioavailability.

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Advanced age is associated with a reduction in skeletal muscle vascular conductance (Martin *et al.* 1991; Cook *et al.* 1992; Lawrenson *et al.* 2003) and impaired endothelium-dependent vasodilatation (Taddei *et al.* 1995; Gerhard *et al.* 1996; DeSouza *et al.* 2000; Muller-Delp *et al.* 2002). Muller-Delp *et al.* (2002) and Spier *et al.* (2004) have further shown that the reductions in flow- and agonist-mediated endothelium-dependent vasodilatation of arterioles from skeletal muscle that are associated with old age occur via a nitric oxide (NO) signalling pathway. Several possible mechanisms may underlie this deficit in NO signalling, including limited substrate (L-arginine) or cofactor (e.g. tetrahydrobiopterin; BH_4) bioavailability, reduced abundance or activity of endothelial NO synthase (eNOS), and increased degradation of NO. Indeed, it has been reported that an up-regulation of arginase expression and activity occurs in large conduit arteries from old rats, which could diminish eNOS activity by limiting intracellular L-arginine availability (Berkowitz *et al.* 2003; White *et al.* 2006). Because the local chemical milieu differs in conduit arteries and resistance arteries within skeletal muscle, the primary purpose of this study was to test the hypothesis that arginase activity decreases endothelium-dependent flow-induced vasodilatation in

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the skeletal muscle microcirculation (i.e. arterioles) from old rats and, consequently, that arteriolar L-arginine levels are lower. A secondary purpose was to determine whether ageing decreases the arteriolar concentration of BH_4, a cofactor essential for eNOS production of NO (Shi *et al.* 2004).

Methods

This study was approved by the Institutional Animal Care and Use Committees at West Virginia and Texas A&M Universities, and conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Animals

Six-month-old ($n = 41$) and 24-month-old ($n = 39$) male Fischer 344 rats were obtained from Harlan (Indianapolis, IN, USA). The animals were housed in a temperature-controlled ($23 \pm 2^{\circ}$C) room with a 12–12 h light–dark cycle. Water and rat chow were provided *ad libitum*.

Arteriolar preparation

The rats were anaesthetized with sodium pentobarbital (60 mg kg^{-1}, I.P.). The gastrocnemius–plantaris–soleus muscle group from each hindlimb was carefully dissected free and placed in cold (4°C) physiological saline solution (PSS) containing (mmol l^{-1}): NaCl 145.0, KCl 4.7, $CaCl_2$ 2.0, $MgSO_4$ 1.17, NaH_2PO_4 1.2, glucose 5.0, pyruvate 2.0, EDTA 0.02 and 3-(N-morpholino)propanesulphonic acid (Mops) buffer 3.0, and 1 g (100 ml)$^{-1}$ bovine serum albumin; pH 7.4. The animals were then killed by decapitation. With the aid of a dissecting microscope (Olympus SVH10), first-order (1A) arterioles from the soleus muscle, which is composed primarily of highly oxidative fibres (Delp & Duan, 1996) and demonstrates decreased vascular conductance during exercise in old animals (Musch *et al.* 2004), were isolated and removed from the surrounding muscle tissue as previously described (Muller-Delp *et al.* 2002; Spier *et al.* 2004). The arterioles (length, 0.5–1.0 mm; inner diameter, 90–150 μm) were transferred to a Lucite chamber containing PSS equilibrated with room air. Each end of the arteriole was cannulated with resistance-matched micropipettes and secured with nylon suture. After cannulation, the microvessel chamber was transferred to the stage of an inverted microscope (Olympus IX70) equipped with a video camera (Panasonic BP310), video caliper (Microcirculation Research Institute) and data-acquisition system (MacLab/Macintosh) for on-line recording of intraluminal diameter. Arterioles were initially pressurized to 60 cmH_2O with two independent hydrostatic pressure reservoirs. Leaks were detected by pressurizing the vessel, and then closing the valves to the reservoirs and verifying that intraluminal diameter remained constant. Arterioles that exhibited leaks were discarded. Arterioles that were free from leaks were warmed to 37°C and allowed to develop initial spontaneous tone during a 30–60 min equilibration period.

Evaluation of vasodilator responses

Upon displaying a steady level of spontaneous tone, arterioles were exposed to graded increases in intraluminal flow in the absence of changes in intraluminal pressure. This was accomplished by altering the heights of independent fluid reservoirs in equal and opposite directions so that a pressure difference was created across the vessel without altering mean intraluminal pressure. Diameter measurements were determined in response to incremental pressure differences of 4, 10, 20, 40 and 60 cmH_2O. Volumetric flow (Q) was then calculated from inner diameter (d) and mean red cell velocity (V_{rbc}), which was determined in a subset of arterioles at each of the pressure gradients, according to the following equation (Davis, 1987; Kuo *et al.* 1990; Muller-Delp *et al.* 2002):

$$Q = \pi (V_{rbc}/1.6)(d/2)^2$$

Vasodilator responses to the cumulative addition of the nitric oxide donor sodium nitroprusside (SNP, 10^{-10}–10^{-4} mol l^{-1}) were then determined as previously described (Muller-Delp *et al.* 2002; Spier *et al.* 2004). At the end of the SNP concentration–response determination, the Mops buffer solution was replaced with Ca^{2+}-free Mops buffer solution for 1 h to obtain the maximal passive diameter (Muller-Delp *et al.* 2002; Spier *et al.* 2004).

Effects of N^G-nitro-L-arginine methyl ester (L-NAME), N^{ω}-hydroxy-nor-L-arginine (NOHA), exogenous L-arginine and sepiapterin

To determine the role of nitric oxide synthase (NOS), arginase, L-arginine and sepiapterin in the reduction in flow-induced vasodilatation associated with old age, responses to flow were evaluated in the presence of one of the following: (1) the NOS blocker L-NAME (10^{-5} mol l^{-1}, $n = 12$–14 per group) (Muller-Delp *et al.* 2002; Spier *et al.* 2004); (2) the arginase inhibitor NOHA (5×10^{-4} mol l^{-1}, $n = 9$ per group) (Berkowitz *et al.* 2003); (3) exogenous L-arginine (3×10^{-3} mol l^{-1}, $n = 9$–11 per group) (Delp *et al.* 1993; Zhang *et al.* 2004); or (4) the precursor for BH_4 synthesis sepiapterin (1 μmol l^{-1}, $n = 13$ per group) (Bagi *et al.* 2004). After flow-mediated vasodilatation was determined under each of these conditions, maximal vessel diameter was determined by replacing the Mops buffer solution with Ca^{2+}-free Mops buffer solution for 1 h.

All drugs were purchased from Sigma Chemical, LKT Laboratories or Bachem Inc.

Determination of arteriolar L-arginine and BH_4 content

Arteriolar L-arginine and BH_4 content ($n = 5$ per group) were determined using the HPLC method as previously described (Wu & Meininger, 1995; Meininger *et al.* 2000). Briefly, 1A arterioles (~10 mg) from soleus muscles of each animal were pooled. For L-arginine analysis, vessels were homogenized with 0.2 ml 1.5 mol l^{-1} $HClO_4$, then 0.1 ml 2 mol l^{-1} K_2CO_3 was added. The homogenates were centrifuged at 10 000 *g* for 1 min, and an aliquot (0.2 ml) of the supernatant was used for sample determination of L-arginine content (Wu & Meininger, 1995). For BH_4 analysis, arterioles were homogenized in 0.1 ml 0.1 M phosphoric acid containing 5 mM dithioerythritol (an antioxidant), to which 17.5 μl 2 M trichloroacetic acid was added. Extracts were oxidized with acidic or basic iodine. Acidic oxidation quantitatively converts BH_4 and dihydrobiopterin to biopterin; basic oxidation converts dihydrobiopterin and BH_4 to biopterin and pterin, respectively. Samples were incubated in the dark for 1 h. Excess iodine was removed by adding ascorbic acid (final concentration, 0.1 M). The final solution was analysed on a C18 reversed-phase column using fluorescence detection and authentic biopterin as a standard. The amount of BH_4 in the arteriolar extracts was determined from the difference between acidic and basic iodine-generated biopterin (Meininger *et al.* 2000). The sensitivity of L-arginine and BH_4 analyses by HPLC, which was assessed using detection limits defined as a signal-to-noise ratio of 3, was 5 and 2 pmol ml^{-1}, respectively. The reliability of the assays was indicated by the precision (agreement between replicate measurements), evaluated by the relative deviation (mean of absolute deviation/mean of replicate measurements × 100%), and by the accuracy (the nearness of an experimental value to the true value), determined with known amounts of standards and expressed as the relative errors ((measurement value − true value)/(true value × 100%)). The precision and accuracy for the L-arginine analysis were 1.4% and 1.6%, respectively, and for the BH_4 analysis were 2.0% and 2.3%, respectively. The values in fmol (mg tissue)$^{-1}$ and pmol (mg tissue)$^{-1}$ were calculated on the basis of tissue weight.

Data analysis

For statistical analyses, changes in diameter in response to flow were expressed as a percentage of maximal vasodilatation as previously described (Muller-Delp *et al.* 2002). Flow–diameter curves were evaluated by repeated measures analysis of variance in order to detect differences within (flow rate) and between (animal groups) factors. Pairwise comparisons between specific levels were made through Scheffe's *post hoc* analysis when a significant main effect was found. One-way ANOVA was used to determine age-related differences in L-arginine and BH_4 content of arterioles. All data are presented as means ± S.E.M. In all statistical analyses, n indicates the number of animals in each group. Significance was defined as $P \leq 0.05$.

Results

Vessel characteristics

The development of spontaneous tone did not differ between arterioles from young (54 ± 4%) and old (47 ± 6%) rats ($P > 0.05$). Likewise, the maximum arteriolar diameter was not different between groups (young, 124 ± 4 μm; old, 129 ± 4 μm; $P > 0.05$); these results are similar to those previously reported for soleus muscle arterioles (Muller-Delp *et al.* 2002; Spier *et al.* 2004).

Vasodilator responses to flow

Old age resulted in a 52% reduction in flow-induced vasodilatation in muscle arterioles (Fig. 1). There were no differences in arteriolar vasodilator responsiveness to the exogenous NO donor SNP (data not shown), indicating the deficit in flow-mediated vasodilatation occurred within the vascular endothelium. L-NAME significantly reduced flow-induced vasodilatation in soleus muscle

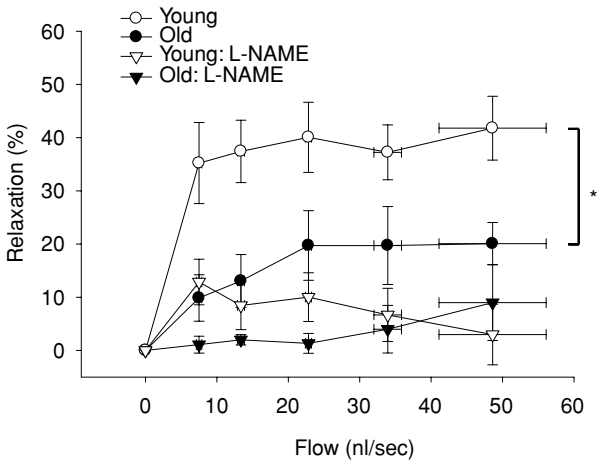

Figure 1. Effects of ageing and L-NAME on flow-induced vasodilatation in soleus muscle arterioles from young and old rats

*Indicates vasodilatation in response to flow was lower in soleus muscle arterioles from aged rats ($P < 0.05$). L-NAME reduced flow-induced vasodilatation in arterioles from young and old rats ($P < 0.05$) and eliminated differences in responsiveness to flow between the young and old. Values are means ± S.E.M. ($n = 12$–14 per group).

arterioles from both groups and abolished age-associated differences in flow-induced vasodilatation between groups (Fig. 1). Flow-mediated vasodilatation in the presence of the arginase inhibitor NOHA was 51% lower in arterioles from old rats compared with arterioles from young animals (Fig. 2), and similar to flow responses without NOHA present. When exogenous L-arginine was added to the bathing solution, the difference in flow-induced vasodilatation due to age was still present (Fig. 3). These results suggest that arginase activity does not limit L-arginine availability in soleus muscle arterioles from old animals.

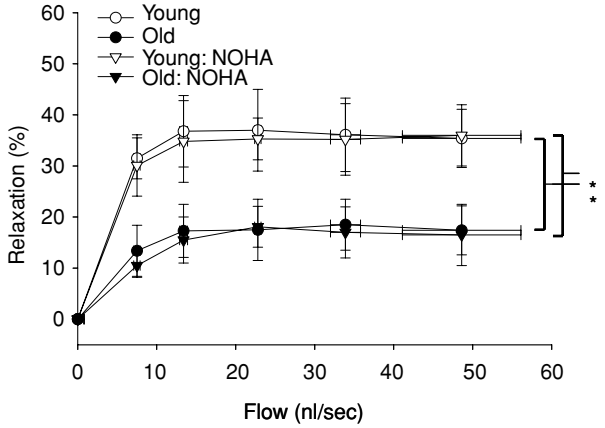

Figure 2. Effects of ageing and arginase inhibition (via N^{ω}-hydroxy-nor-L-arginine; NOHA) on flow-induced vasodilatation in soleus muscle arterioles from young and old rats
*Indicates vasodilatation in response to flow was lower in soleus muscle arterioles from old rats ($P < 0.05$). NOHA did not affect the flow-induced vasodilatation in arterioles from either young or old rats. Values are means ± S.E.M. ($n = 9$ per group).

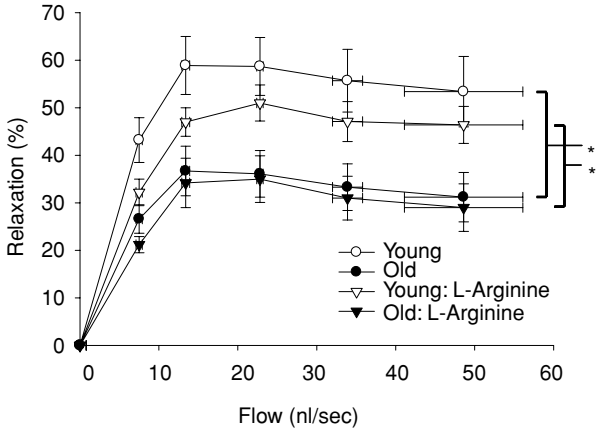

Figure 3. Effects of ageing and exogenous L-arginine (3×10^{-3} mol l^{-1}) on flow-induced vasodilatation in soleus muscle arterioles from young and old rats
*Indicates vasodilatation in response to flow was lower in soleus muscle arterioles from old rats ($P < 0.05$). L-arginine did not affect the flow-induced vasodilatation in arterioles from either young or old rats. Values are means ± S.E.M. ($n = 9$–11 per group).

The addition of sepiapterin to the bathing solution resulted in an enhanced flow-induced vasodilatation in arterioles from the old rats, but not in arterioles from the young rats (Fig. 4). However, the enhanced vasodilator response in arterioles from old rats was still lower than the response in arterioles from young animals.

Arteriolar L-arginine and BH_4 content

Direct measurements of arteriolar L-arginine content demonstrate that the concentration of the eNOS substrate does not differ between arterioles from young and old rats (Fig. 5); this observation supports the conclusion

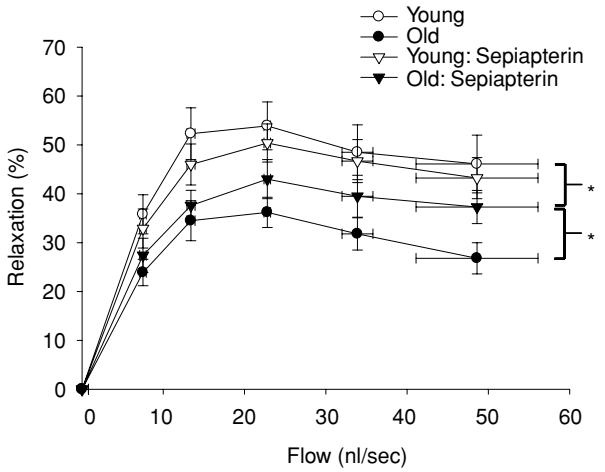

Figure 4. Effects of ageing and sepiapterin (1 μmol l^{-1}) on flow-induced vasodilatation in soleus muscle arterioles from young and old rats
*Indicates vasodilatation in response to flow was different between conditions ($P < 0.05$). Values are means ± S.E.M. ($n = 13$ per group).

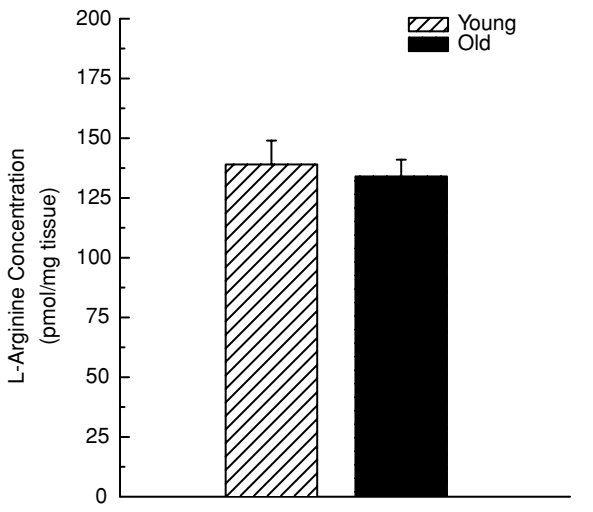

Figure 5. L-Arginine concentration in arterioles from the soleus muscle of young and old rats
Ageing had no effect on arteriolar L-arginine content ($P > 0.05$). $n = 5$ per group.

from the *in vitro* studies that neither arginase activity nor L-arginine bioavailability adversely affect flow-mediated vasodilatation in skeletal muscle arterioles from old animals. Arteriolar BH_4 content, however, was 60% lower in vessels from old rats (Fig. 6). This finding, along with the partial rescue of endothelium-dependent vasodilatation with sepiapterin (Fig. 4), indicates that limited BH_4 bioavailability contributes to the impairment of endothelial vasodilator function associated with old age.

Discussion

The purpose of this study was (1) to confirm our previous observations that ageing diminishes flow-mediated vasodilatation in soleus muscle arterioles through an NOS-mediated signalling mechanism (Muller-Delp *et al.* 2002), and (2) to determine whether an age-dependent decline in the bioavailability of L-arginine or BH_4 is associated with the impairment of endothelium-dependent relaxation in the microcirculation. The results demonstrate that flow-mediated vasodilatation is decreased with old age in the skeletal muscle microcirculation, and that this occurs through an NOS-mediated signalling mechanism (Fig. 1), as previously shown (Muller-Delp *et al.* 2002). This impairment in endothelium-dependent relaxation is not the result of greater arginase activity (Fig. 2) and, correspondingly, decreased eNOS substrate availability (Figs 3 and 5). Rather, a deficit in BH_4 cofactor bioavailability is associated with the age-dependent decline in endothelial vasodilator function (Figs 4 and 6). To our knowledge, these are the first data to demonstrate the effects of ageing on L-arginine and BH_4 levels in the microcirculation.

The primary purpose of the present study was to investigate the possible role of arginase in the impairment of endothelium-dependent vasodilatation in the skeletal muscle microcirculation associated with old age. The hypothesis that arginase is up-regulated with ageing is based on the work of Berkowitz and colleagues (Berkowitz *et al.* 2003; White *et al.* 2006), who demonstrated that endothelial arginase abundance and activity are elevated with ageing in rat conduit arteries. Although L-arginine levels were not directly assessed in these studies, the reported increase in arginase activity would lead to a deficit in the eNOS substrate L-arginine and a consequent decrease in NO production (Morris, 2000). Results from the present study differ from this previous work (Berkowitz *et al.* 2003; White *et al.* 2006) in that neither inhibition of arginase activity (Fig. 2) nor supplementation with exogenous L-arginine (Fig. 3) had any effect on endothelium-dependent vasodilatation in arterioles from old animals. Further, direct measures of arteriolar L-arginine content (Fig. 5) demonstrated no deficiency in the eNOS substrate relative to that in arterioles from young animals, and the amount of arginine in the vessels from old rats is sufficient as the nitrogenous substrate for eNOS (Wu & Morris, 1998). Thus, these results do not support a role for up-regulated arginase activity in the impairment of endothelium-dependent vasodilatation in the skeletal muscle microcirculation associated with old age. The disparity regarding the significance of arginase in limiting L-arginine bioavailability and endothelium-dependent vasodilatation between the present study and previously reported findings (Berkowitz *et al.* 2003; White *et al.* 2006) are likely to result from the differential effects of ageing on the arterial vasculature. In previous work (Berkowitz *et al.* 2003; White *et al.* 2006), endothelium-dependent vasodilatation in the rat aorta, a conduit artery, was examined whereas in the present study we investigated resistance arteries from skeletal muscle. Undoubtedly the local chemical milieu, including exposure to reactive oxygen species (Davies *et al.* 1982; Reid *et al.* 1992), differs between arterioles within metabolically active skeletal muscle and conduit arteries.

Several other mechanisms could underlie the deficiency in NO signalling in resistance arteries associated with old age, including decreased abundance or activity of eNOS, limited cofactor availability, and increased degradation of NO. With ageing, eNOS protein content is not decreased in skeletal muscle arterioles, but is actually greater in arterioles from old animals (Spier *et al.* 2004); this is similar to results reported in several studies of large conduit arteries (Cernadas *et al.* 1998; van der Loo *et al.* 2000). These findings suggest that deficiencies in endothelium-dependent vasodilatation

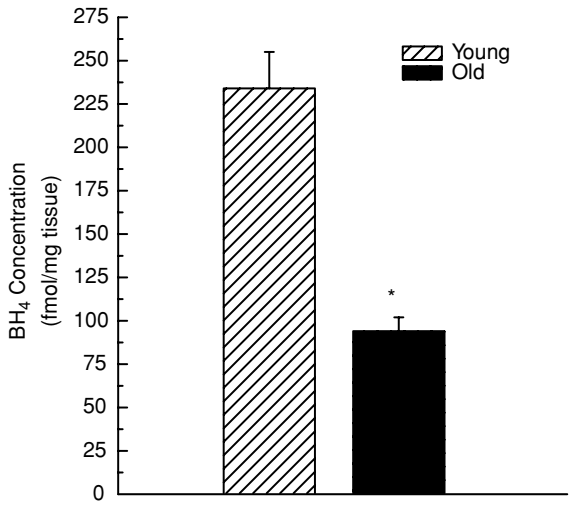

Figure 6. Tetrahydrobiopterin (BH_4) concentration in arterioles from the soleus muscle of young and old rats
*Indicates significant difference in BH_4 content to that of arterioles from young rats ($P < 0.05$). $n = 5$ per group.

that occur with old age may result, at least in part, from inadequate NO production. Production of NO by eNOS may be regulated by the Ca^{2+}–calmodulin complex (Forstermann *et al.* 1991) and the cofactor BH_4 (Cosentino & Katusic, 1995; Cosentino & Lüscher, 1998; Tiefenbacher, 2001; Vasquez-Vivar *et al.* 2003). Evidence from numerous studies of pathologies characterized by impaired endothelium-dependent nitroxidergic vasodilatation, such as atherosclerosis, hypercholesterolaemia and diabetes, indicates that availability of BH_4 limits eNOS activity and NO production (Cosentino & Katusic, 1995; Tiefenbacher *et al.* 1996, 2000; Stroes *et al.* 1997; Maier *et al.* 2000; Meininger *et al.* 2000; Tiefenbacher, 2001). Under normal conditions, eNOS, in the presence of sufficient BH_4, accepts and stores electrons from nicotinamide adenine dinucleotide phosphate (NADPH) to transform cosubstrates O_2 and L-arginine into NO and L-citrulline (Knowles & Moncada, 1994; Vasquez-Vivar *et al.* 2003). However, under conditions of limited BH_4 availabilty, eNOS cannot catalyse the oxidation of L-arginine to NO, but rather will accept electrons from NADPH and donate them to its other substrate, O_2, thereby reducing it to superoxide anion (O_2^-) (Knowles & Moncada, 1994; Vasquez-Vivar *et al.* 2003). In the aorta from pre-hypertensive spontaneously hypertensive rats (SHRs), for example, impaired endothelium-dependent vasodilatation results from excess production of superoxide that is linked to decreased availability of BH_4 (Cosentino *et al.* 1998). Thus, BH_4 plays a crucial role in stabilizing eNOS, preventing the formation of the cytotoxic superoxide, and promoting production of vasoactive NO (Shi *et al.* 2004).

A large body of evidence indicates that oxidative stress increases with age. In the vasculature, both increases in production of reactive oxygen species and decreases in antioxidant enzymes have been reported to occur with advancing age (van der Loo *et al.* 2000; Woodman *et al.* 2002). Such age-related increases in reactive oxygen species could diminish BH_4 bioavailability as a cofactor for eNOS (Milstien & Katusic, 1999; Laursen *et al.* 2001) and, in turn, contribute to greater production of superoxide and reduced formation of NO by eNOS (Fig. 7). Results from the present study demonstrate decreased BH_4 content in arterioles from old rats (Fig. 6). Furthermore, when exogenous sepiapterin, a precursor for BH_4 synthesis (Fig. 7), is added to the bathing solution, there is significant improvement of flow-induced vasodilatation in arterioles from old animals (Fig. 4). Although these results establish a link between impairment of endothelium-dependent vasodilatation and decreased BH_4 content associated with old age, they do not determine whether the deficit in vascular BH_4 content is related to the BH_4 salvage pathway, the BH_4 *de novo* synthesis pathway, or oxidative stress (Fig. 7), as the sepiapterin supplementation may simply serve to compensate for BH_4 that has been oxidized by peroxynitrite or deficiencies in *de novo* synthesis of BH_4.

Results from several previous studies support the observation of decreased vascular BH_4 content with ageing. Eskurza *et al.* (2005) reported that oral supplementation of BH_4 improved forearm flow-mediated vasodilatation in older subjects to the level of that observed in young subjects. Likewise, Higashi *et al.* (2006) found that co-infusion of BH_4 with acetylcholine resulted in greater forearm endothelium-dependent vasodilatation in elderly subjects than with acetylcholine alone. Thus, results from these studies and the present study support the hypothesis that decreased vascular BH_4 content with ageing impairs vascular endothelial function. However, this notion is not supported by the work of Blackwell *et al.* (2004), who reported that BH_4 content in aortas from old mice was not different from that in young mice. Whether the discrepancy in the results of this study and the present study reflect differences in the rodent model studied (mouse *versus* rat) or the type of vessel studied (conduit *versus* resistance) is unknown.

In summary, in the current study we provide the first experimental evidence demonstrating that reductions in BH_4 bioavailability in the skeletal muscle microcirculation induced by old age may be a key mechanism underlying the impairment of endothelium-dependent

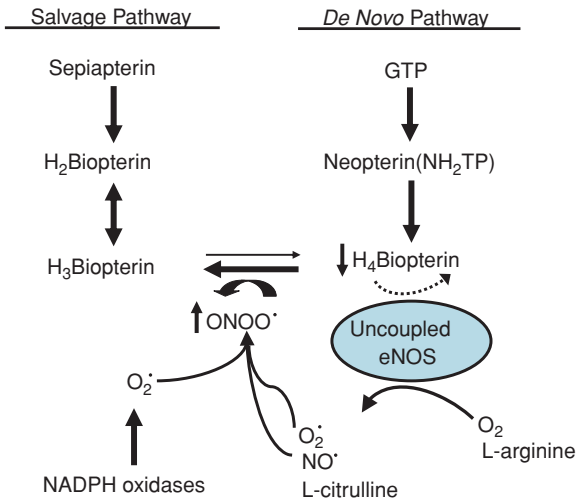

Figure 7. The proposed effects of ageing on BH_4 bioavailability
Vascular BH_4 content may be limited by reduced synthesis through the *de novo* or the salvage pathways. Further, because BH_4 is susceptible to oxidative degradation, elevations in the production of reactive oxygen species associated with old age could directly decrease vascular BH_4 concentration. Peroxynitrite (ONOO·), a product of nitric oxide (NO) and superoxide (O_2·) generated through NADPH oxidases and other sources, including xanthine oxidase, cytochrome P-450 enzymes and mitochondria, could further increase catabolism of BH_4. With decreased levels of BH_4, eNOS becomes uncoupled and leads to generation of O_2· rather than NO. Thus, the uncoupling of eNOS decreases the NO available to mediate smooth muscle cell relaxation and increases O_2· generation, which could further depress BH_4 bioavailability.

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vasodilatation, rather than an increase in arginase activity and limited eNOS substrate L-arginine availability. Given that the impairment of endothelium-dependent vasodilatation is a major risk factor for the development of cardiovascular disease among the elderly, such insight into the mechanisms of endothelial dysfunction may have important clinical implications. However, further experiments will be necessary to determine how reduced availability of BH_4 and oxidant stress affect eNOS activity in the microcirculation with age.

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