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Acute localized administration of tetrahydrobiopterin and chronic systemic atorvastatin treatment restore cutaneous microvascular function in hypercholesterolaemic humans

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Non-technical summary A high concentration of cholesterol in the blood, known as hypercholesterolaemia, in the absence of overt atherosclerotic disease induces changes throughout the circulation including an inability to fully respond to vasodilatory stimuli. Here we examined the underlying factors that contribute to reduced skin blood flow responses to local warming in hypercholesterolaemic men and women before and after a common cholesterol-lowering intervention (atorvastatin). We found that skin blood flow responses are reduced in hypercholesterolaemic men and women and that localized administration of the essential enzymatic cofactor, called tetrahydrobiopterin, increases the skin blood flow response to local heating. After 3 months of a cholesterol-lowering intervention (atorvastatin) blood cholesterol was reduced and the skin blood flow responses to local warming were corrected such that there was no longer a difference between the hypercholesterolaemics and the normocholesterolaemic control group. Our data suggest that reduced availability of tetrahydrobiopterin induced by high cholesterol in part contributes to reduced vasodilatory responses in the skin microcirculation which is corrected with a common cholesterol-lowering statin therapy.

Abstract Elevated oxidized low-density lipoproteins (LDL) are associated with vascular dysfunction in the cutaneous microvasculature, induced in part by upregulated arginase activity and increased globalized oxidant stress. Since tetrahydrobiopterin (BH_4) is an essential cofactor for endothelial nitric oxide synthase (NOS3), decreased bioavailability of the substrate L-arginine and/or BH_4 may contribute to decreased NO production with hypercholesterolaemia. We hypothesized that (1) localized administration of BH_4 would augment NO-dependent vasodilatation in hypercholesterolaemic human skin, which would be further increased when combined with arginase inhibition and (2) the improvement induced by localized BH_4 would be attenuated after a 3 month oral atorvastatin intervention (10 mg). Four microdialysis fibres were placed in the skin of nine normocholesterolaemic (NC: LDL $= 95 \pm 4$ mg dl^{-1}) and nine hypercholesterolaemic (HC: LDL $= 177 \pm 6$ mg dl^{-1}) men and women before and after 3 months of systemic atorvastatin. Sites served as control, NOS inhibited, BH_4, and arginase inhibited + BH_4 (combo). Skin blood flow was measured while local skin heating (42°C) induced NO-dependent vasodilatation. After the established plateau L-NAME was perfused in all sites to quantify NO-dependent vasodilatation (NO). Data were normalized to maximum cutaneous vascular conductance (CVC). Vasodilatation at the plateau and NO-dependent vasodilatation were reduced in HC subjects (plateau HC: $70 \pm 5\%$ CVC_{max} *vs.* NC: $95 \pm 2\%$ CVC_{max}; NO HC: $45 \pm 5\%$ CVC_{max} *vs.* NC: $64 \pm 5\%$ CVC_{max}; both $P < 0.001$). Localized BH_4 alone or combo augmented the plateau (BH_4: $93 \pm 3\%$ CVC_{max}; combo $89 \pm 3\%$ CVC_{max}, both $P < 0.001$) and NO-dependent vasodilatation in HC (BH_4: $74 \pm 3\%$ CVC_{max}; combo $76 \pm 3\%$ CVC_{max}, both $P < 0.001$), but there was no effect in NC subjects (plateau BH_4: $90 \pm 2\%$ CVC_{max}; combo $95 \pm 3\%$ CVC_{max}; NO-dependent vasodilatation BH_4: $68 \pm 3\%$ CVC_{max}; combo $58 \pm 4\%$ CVC_{max}, all $P > 0.05$ *vs.* control site). After

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the atorvastatin intervention (LDL = 98 ± mg ∗ dl^{-1}) there was an increase in the plateau in HC (96 ± 4% CVC_{max}, $P < 0.001$) and NO-dependent vasodilatation (68 ± 3% CVC_{max}, $P < 0.001$). Localized BH_4 alone or combo was less effective at increasing NO-dependent vasodilatation after the drug intervention (BH_4: 60 ± 5% CVC_{max}; combo 58 ± 2% CVC_{max}, both $P < 0.001$). These data suggest that decreased BH_4 bioavailability contributes in part to cutaneous microvascular dysfunction in hypercholesterolaemic humans and that atorvastatin is an effective systemic treatment for improving NOS coupling mechanisms in the microvasculature.

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Abbreviations BH_4, tetrahydrobiopterin; CVC, cutaneous vascular conductance; NO, nitric oxide; NOS3, endothelial nitric oxide synthase; LDL, low-density lipoproteins; HDL, high-density lipoproteins; oxLDL, oxidized low-density lipoproteins; L-NAME, N^{G}-nitro-L-arginine; nor-NOHA, N^{ω}-hydroxy-nor-L-arginine; BEC, (*S*)-(2-boronoethyl)-*l*-cysteine-HCl; SNP, sodium nitroprusside

Introduction

Hypercholesterolaemia with elevated oxidized low-density lipoprotein (oxLDL) is a major risk factor for the development of atherosclerosis (Toshima *et al.* 2000; Inoue *et al.* 2001; Vasankari *et al.* 2001). One early event in the pathogenesis of atherosclerotic vascular disease is a decrease in endothelial derived nitric oxide (NO), detectable in the microvasculature prior to the onset of atherosclerotic plaque formation in the conduit arteries (Rossi & Carpi, 2004; Bendall *et al.* 2005; Rossi *et al.* 2006, 2009). The human cutaneous circulation has emerged as an accessible and representative microvascular bed for examining the underlying mechanisms of vascular dysfunction with hypercholesterolaemia (Rossi *et al.* 2009; Holowatz, 2011; Holowatz *et al.* 2011).

We have recently demonstrated that both an increase in arginase (which competes for the common endothelial NO synthase (NOS3) substrate L-arginine) activity and an increase in ascorbate-sensitive oxidants contribute to reduced NO bioavailability and attenuated vasodilatory responsiveness in the skin of hypercholesterolaemic humans (Holowatz, 2011; Holowatz *et al.* 2011). Additionally, these two mechanisms may be linked through the uncoupling of NOS3 (Lim *et al.* 2007). NOS3, which is normally dimerized, uncouples to a monomeric form without adequate substrate (Forstermann & Munzel, 2006), induced by upregulated arginase activity (Lim *et al.* 2007; Kim *et al.* 2009) or cofactor availability, and produces superoxide instead of NO (Moens & Kass, 2006). The antioxidant ascorbate, which is commonly used in human vascular studies, reduces oxidants synthesized from a variety of sources including NADPH and xanthine oxidases, as well as uncoupled NOS3. Specific to NOS3, ascorbate increases NO bioavailability by: (1) stabilizing the essential NOS3 cofactor tetrahydrobiopterin (BH_4), (2) augmenting BH_4 synthesis through the salvage pathway (Toth *et al.* 2002) and (3) reducing the activation of arginase through inhibition of *S*-nitrosylation (Santhanam *et al.* 2007). Therefore, it is unclear if ascorbate exerts an effect through BH_4 mechanisms or through a generalized decrease in oxidant production through NADPH and xanthine oxidases.

We also recently demonstrated that a systemic HMG-CoA-reductase (atorvastatin, Lipitor) intervention decreased arginase activity in human skin from hypercholesterolaemic human subjects and restored NO-dependent cutaneous vasodilatation (Holowatz *et al.* 2011). This improvement in cutaneous microvascular function was probably mediated in part by directly lowering oxLDL, through the antioxidant properties of the statin (Wassmann *et al.* 2002), and through sequestering arginase to a subcellular location where it does not have access to the L-arginine microdomains (Berkowitz *et al.* 2003; Ryoo *et al.* 2006, 2011). However, atorvastatin also increases BH_4 bioavailability by increasing *de novo* BH_4 synthesis (Hattori *et al.* 2003), which may further contribute to the improvements in microvascular function with this intervention.

Therefore, the purpose of this study was to determine the role of acute localized BH_4 administration alone and in combination with arginase inhibition, in NOS3 uncoupling that leads to attenuated cutaneous vasodilatation inherent in hypercholesterolaemia. We hypothesized that BH_4 alone would modestly augment NO-dependent vasodilatation, but when combined with arginase inhibition would significantly increase NO-dependent vasodilatation in response to a standardized local heating protocol (Kellogg*et al.* 1999; Minson*et al.* 2001) in hypercholesterolaemic human skin. A secondary goal of the study was to examine the potential influences of chronic systemic atorvastatin treatment on BH_4 bioavailability. We hypothesized that after a 3 month systemic atorvastatin intervention, NO-dependent vasodilatation would be augmented and the improvement in vasodilatation with BH_4 and/or arginase inhibition would be less than in pre-intervention trials.

Table 1. Subject characteristics

	Hypercholesterolaemic		
	Normocholesterolaemic	Pre-atorvastatin	Post-atorvastatin
Subjects (men, women)	(5, 4)	(6, 3)	
Age (years)	49 ± 2	53 ± 3	
Total cholesterol (mg dl^{-1})	171 ± 7	260 ± 9*	179 ± 9‡
HDL (mg dl^{-1})	60 ± 5	51 ± 7	55 ± 8
LDL (mg dl^{-1})	95 ± 4	177 ± 6*	98 ± 6‡
Triglycerides (mg dl^{-1})	86 ± 13	139 ± 11*	134 ± 10
Glucose (mg dl^{-1})	93 ± 3	94 ± 3	
ADMA (μmol l^{-1})	0.43 ± 0.06	0.37 ± 0.10	0.33 ± 0.08
Oxidized LDL (U l^{-1})	64 ± 5	136 ± 12*	89 ± 8‡*

*$P < 0.001$ different from the normocholesterolaemic group; ‡$P < 0.001$ difference due to the atorvastatin intervention.

Methods

Subjects

Experimental protocols were approved by the Institutional Review Board at The Pennsylvania State University and conformed to the guidelines set forth by the *Declaration of Helsinki*. Verbal and written consent was voluntarily obtained from all subjects prior to participation. This study was part of a larger series of studies utilizing the same participants, therefore the subject characteristics are the same as those that have been previously published (Holowatz, 2011; Holowatz *et al.* 2011). The order of these experiments in this entire series was randomized. Nine healthy normocholesterolaemic and nine hypercholesterolaemic men and women (Table 1) participated in the study, consisting of functional *in vivo* assessment of cutaneous NO-dependent vasodilatation during local skin warming. The hypercholesterolaemic subjects were tested at enrollment and after a 3 month atorvastatin intervention (10 mg daily); the normocholesterolaemic control group was only tested once. The subjects age ranged from 40 to 62 years and the groups (hypercholesterolaemic and normocholesterolaemic) were age-matched to account for any possible age-related changes in the local heating response (Minson *et al.* 2002). Furthermore, the subjects were non-obese, non-smokers, non-diabetic, normally active (neither sedentary nor highly exercise trained), and not currently taking statins or other medications including aspirin, vitamins or antioxidants.

Blood analysis

Serum and plasma samples were obtained at enrollment and after the atorvastatin intervention, and stored at –80°C for batched analysis of the endogenous NOS inhibitor asymmetrical dimethyl L-arginine (ADMA: Alpco Immunoassay Salem, NH, USA) and oxLDL (Mercodia Uppsala, Sweden).

In vivo vasoreactive studies

All protocols were performed in a thermoneutral laboratory with the subject semi-supine and the experimental arm at heart level. Four intradermal microdialysis probes were inserted into the ventral forearm skin for localized delivery of pharmacological agents as previously described (Holowatz *et al.* 2006; Holowatz & Kenney, 2007). Microdialysis sites were perfused with: (1) 20 mM N^{G}-nitro-L-arginine (L-NAME) to inhibit NO production by NO synthase throughout the local heating protocol; (2) 10 mM BH_4 to locally supplement the essential NOS3 cofactor (Sigma-Aldrich, St Louis, MO, USA) (Lang *et al.* 2009); or (3) a combination of 5.0 mM (*S*)-(2-boronoethyl)-L-cysteine-HCl (BEC), 5.0 mM N^{ω}-hydroxy-nor-L-arginine (nor-NOHA) to inhibit both arginase isoforms (Calbiochem, San Diego, CA, USA) and 10 mM BH_4 (FDA investigational drug number 78,954) (Holowatz & Kenney, 2007; Lang *et al.* 2009). A fourth microdialysis site was perfused with only lactated Ringer solution to serve as control. A protocol schematic is presented in Fig. 1. All pharmacological solutions were mixed immediately prior to usage, dissolved in lactated Ringer solution, and sterilized using syringe microfilters (Acrodisc, Pall, Ann Arbor, MI, USA). Solutions were also wrapped in foil to inhibit photodegradation of the agents. The concentrations of the pharmacological agents used in this study have been shown to be efficacious in younger and older age groups using the same intradermal microdialysis technique (Holowatz *et al.* 2006; Holowatz & Kenney, 2007). Furthermore, the concentrations of the arginase inhibitor cocktail far exceed the K_i for both isoforms of arginase (BEC K_i at pH 7.5 = 0.31 μM; nor-NOHA K_i at pH 7.5 = 1.6 μM) (Ash, 2004). In our previous vasoconstriction studies a 5 mM concentration of BH_4 was efficacious at augmenting NE-mediated vasoconstriction (BH_4 is also a cofactor for tyrosine hydroxylase; Lang *et al.* 2009). We chose to increase the concentration to 10 mM. In our extensive pilot

work this was the highest concentration that did not cause a significant increase in skin blood flow at a thermoneutral baseline.

An index of skin blood flow was measured using integrated laser-Doppler flowmeter probes and local temperature was controlled with a local heater (MoorLAB, Temperature Monitor SH02, Moor Instruments, Devon, UK) placed directly above each microdialysis membrane. This multipoint probe was placed in the local heater and monitored blood flow over an area approximately 2 mm directly over each microdialysis fibre. Arterial blood pressure was measured every 5 min using an automated brachial cuff (Cardiocap) which was verified with brachial auscultation. Cutaneous vascular conductance (CVC) was calculated as laser-Doppler flux divided by mean arterial pressure (MAP).

Local heating protocol

After the resolution of the initial insertion trauma with local skin temperature clamped at 33°C, a standardized local skin warming protocol was performed to induce NO-dependent vasodilatation (Minson *et al.* 2001). The local heater temperature was increased from 33°C to 42°C at a rate of 0.1°C every second and then clamped at 42°C for the duration of the heating protocol. After skin blood flow reached an established plateau (30–40 min) 20 mM L-NAME was perfused to quantify NO-dependent vasodilatation in all sites. A representative tracing from a normocholesterolaemic subject's control site is illustrated in Fig. 2. This figure shows the phases of the local heating response including the initial peak and nadir which are primarily mediated by sensory nerve mechanisms with a small NO contribution, followed by the predominantly NO-dependent plateau as illustrated by the infusion of L-NAME to quantify L-NAME-sensitive vasodilatation (Minson *et al.* 2001). Following a new post-L-NAME stabilization in skin blood flow, local temperature was increased to 43°C and 28 mM sodium nitroprusside (SNP) was perfused to induce maximal cutaneous vasodilatation (CVC_{max}) (Johnson *et al.* 1986; Holowatz *et al.* 2005). In our previous work and in pilot work this combination

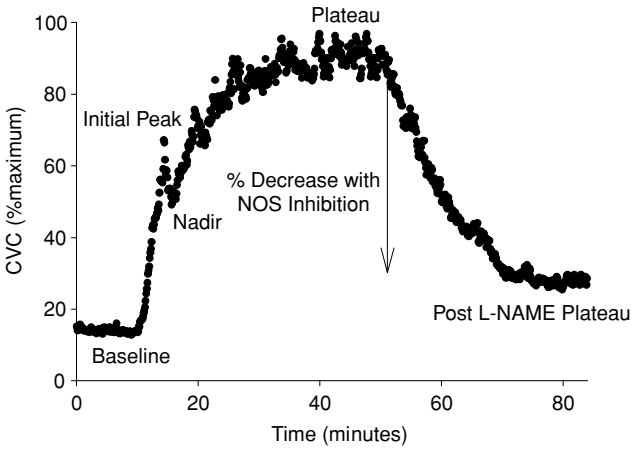

Figure 2. A representative tracing
Cutaneous vascular conductance (% max) throughout the time course of a local heating response in a normocholesterolaemic subject's control site. Baseline, initial peak, nadir, plateau, the per cent decrease with NOS inhibition (20 mM L-NAME) and the post-L-NAME plateau are illustrated.

of heat and high concentration of SNP has been shown to induce maximal vasodilatation. Higher temperatures (44°C) or increasing concentrations of SNP (50 mM) did not produce a further increase in absolute CVC (Holowatz *et al.* 2005).

Data and statistical analysis

Data were collected continuously, digitized at 40 Hz and stored for offline analysis with signal-processing software (Windaq, DATAQ Instruments). Skin blood flow data were normalized to a per cent of maximal CVC (% CVC_{max}), CVC data were averaged for a stable 5 min of baseline, plateau, post-L-NAME plateau, and maximal vasodilatation. Due to the transient nature of the local warming response, the initial peak and nadir CVC were visually identified as the highest and lowest values and averaged over 10 s. The L-NAME-sensitive portion of local heating-induced vasodilatation was calculated from the difference between the plateau and the post-L-NAME plateau. As the late plateau phase of the local heating

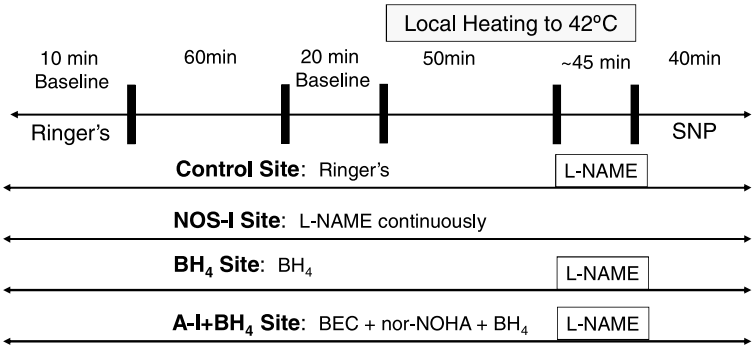

Figure 1. A protocol schematic
A, schematic to illustrate the local heating protocol with each microdialysis treatment site. Sites served as: (1) control for a normative reference, (2) nitric oxide synthase inhibited (NOS-I) throughout the protocol, (3) localized tetrahydrobiopterin (BH_4) administered to supplement the essential NOS cofactor, and (4) arginase inhibited (A-I) combined with BH_4 to supplement the essential NOS cofactor and to increase NOS substrate (L-arginine) availability through inhibiting arginase. The non-specific NOS inhibitor L-NAME was perfused after the established plateau to quantify NO-dependent vasodilatation.

response is primarily dependent on NOS function whereas the early phase has contributions from both sensory nerves and NOS, analysis and further discussion focus on the later phase of the cutaneous vasodilatory response.

Student's unpaired *t* tests were used to determine significant differences between the groups and Student's paired *t* tests were used to determine the effects of the statin intervention on blood characteristics. A mixed models three-way repeated measures ANOVA was conducted to detect differences in % CVC_{max} between subject groups and for the statin intervention at the pharmacological treatment sites for the different phases of the local warming response (SAS, version 9.1). Specific planned comparisons with Bonferroni correction were performed when appropriate to determine where differences between groups, statin intervention and localized drug treatments occurred. The level of significance was set at $\alpha = 0.05$. Values are presented as means ± SEM unless otherwise indicated.

Results

Subject characteristics are presented in Table 1. At enrollment there was a significant difference between the normocholesterolaemic and hypercholesterolaemic groups for triglycerides, total cholesterol, LDL and oxidized LDL. Three months of atorvastatin therapy decreased total cholesterol, LDL and oxidized LDL in the hypercholesterolaemics ($P < 0.001$), but oxidized LDL continued to be modestly elevated compared to the normocholesterolaemics ($P = 0.014$). There was no difference between groups or after the atorvastatin intervention for plasma asymmetrical dimethyl L-arginine (ADMA; endogenous NOS inhibitor) concentration.

% CVCmax at baseline, initial peak, and the nadir for each group and treatment site are presented in Table 2. In the normocholesterolaemic group, the combination of BH_4 and arginase inhibition increased baseline % CVCmax compared to the control and the L-NAME-treated sites ($P < 0.01$). In the hypercholesterolaemic group at enrollment, there were no differences due to localized microdialysis drug treatment on baseline % CVCmax. However, after the atorvastatin intervention there was a difference between the BH_4 and arginase-inhibited sites (combo) and the L-NAME sites ($P < 0.01$). There was no difference between groups or the atorvastatin intervention for the initial peak or the nadir ($P > 0.05$). As expected, L-NAME decreased the initial peak and the nadir ($P < 0.001$) but the other localized microdialysis treatments had no effect on these parameters of the local heating response.

Figure 3 illustrates the mean % CVCmax values for the NO-dependent plateau in skin blood flow during local heating and following NOS inhibition

Table 2. Cutaneous vascular conductance (% CVC_{max})

		Hypercholesterolaemic	
	Normocholesterolaemic	Pre-atorvastatin	Post-atorvastatin
Control site			
Baseline	9 ± 2	12 ± 3	11 ± 3
Initial peak	69 ± 5	61 ± 5	64 ± 4
Nadir	57 ± 6	44 ± 6	49 ± 4
BH_4 site			
Baseline	14 ± 2	11 ± 2	15 ± 3
Initial peak	73 ± 6	62 ± 6	67 ± 3
Nadir	51 ± 5	46 ± 3	53 ± 4
BH_4 + arginase-inhibited site			
Baseline	20 ± 2*	11 ± 2	18 ± 2*‡
Initial peak	58 ± 5	67 ± 8	68 ± 5
Nadir	54 ± 7	45 ± 6	54 ± 4
L-NAME site			
Baseline	10 ± 2	11 ± 2	9 ± 3
Initial peak	39 ± 5*	30 ± 5*	35 ± 3*
Nadir	24 ± 4*	20 ± 4*	13 ± 3*

*$P < 0.001$ different from the control site; ‡$P < 0.001$ different due to the atorvastatin intervention.

in normocholesterolaemic and hypercholesterolaemic groups before and after the atorvastatin intervention. Similar to what we have demonstrated previously, the plateau was significantly attenuated in the hypercholesterolaemic group and was increased after the atorvastatin intervention (panel *A*). Additionally, the post-L-NAME plateau was decreased after the atorvastatin intervention ($P = 0.03$). Localized BH_4 administration alone (panel *B*) or in combination with arginase inhibition (panel *C*) increased the local heating plateau and decreased the post-L-NAME plateau (both $P < 0.001$) in the hypercholesterolaemic group. After the atorvastatin intervention there was no additional effect of localized BH_4 administration alone or in combination with arginase inhibition on the plateau. However, these localized treatments did increase the post-L-NAME plateau after the atorvastatin intervention ($P < 0.01$). There were no differences in the L-NAME throughout heating sites between groups or with the atorvastatin intervention.

The decrease in the heating response with NOS inhibition (Fig. 4) was smaller in the hypercholesterolaemic group compared to the normocholesterolaemic group ($P < 0.001$) and was augmented after the atorvastatin intervention (panel *A*, $P < 0.001$). Localized administration of BH_4 alone (panel *B*) or in combination with arginase inhibition increased the vasodilatation sensitive to NOS inhibition in the hypercholesterolaemic group compared to their control site and compared to the normocholesterolaemic

group respective treatment sites (all $P < 0.001$). After the atorvastatin intervention, there was no additional effect of localized BH_4 alone or in combination with arginase inhibition on the vasodilatation sensitive to NOS inhibition compared to the control site. Instead it was significantly lower than these treatment sites before the atorvastatin intervention ($P < 0.001$).

Finally, Fig. 5 shows the absolute CVC (flux $mmHg^{-1}$) during local heating to 43°C with concurrent localized infusion of 28 mM sodium nitroprusside to induce maximal CVC. There were no differences due to localized microdialysis drug infusion, between groups, or with the atorvastatin intervention (all $P > 0.05$).

Discussion

As hypothesized, localized administration of the essential NOS3 cofactor BH_4 augmented NO-dependent vasodilatation during local heating by increasing the plateau in skin blood flow and decreasing the post-L-NAME plateau in hypercholesterolaemic humans. Contrary to our initial hypothesis, arginase inhibition in combination with localized BH_4 administration provided no further increase in NO-dependent vasodilatation. This may have been due to a ceiling effect due to the robust nature of the local heating response or because each treatment independently maximized NO production through NOS (Holowatz, 2011; Holowatz *et al.* 2011). After the systemic

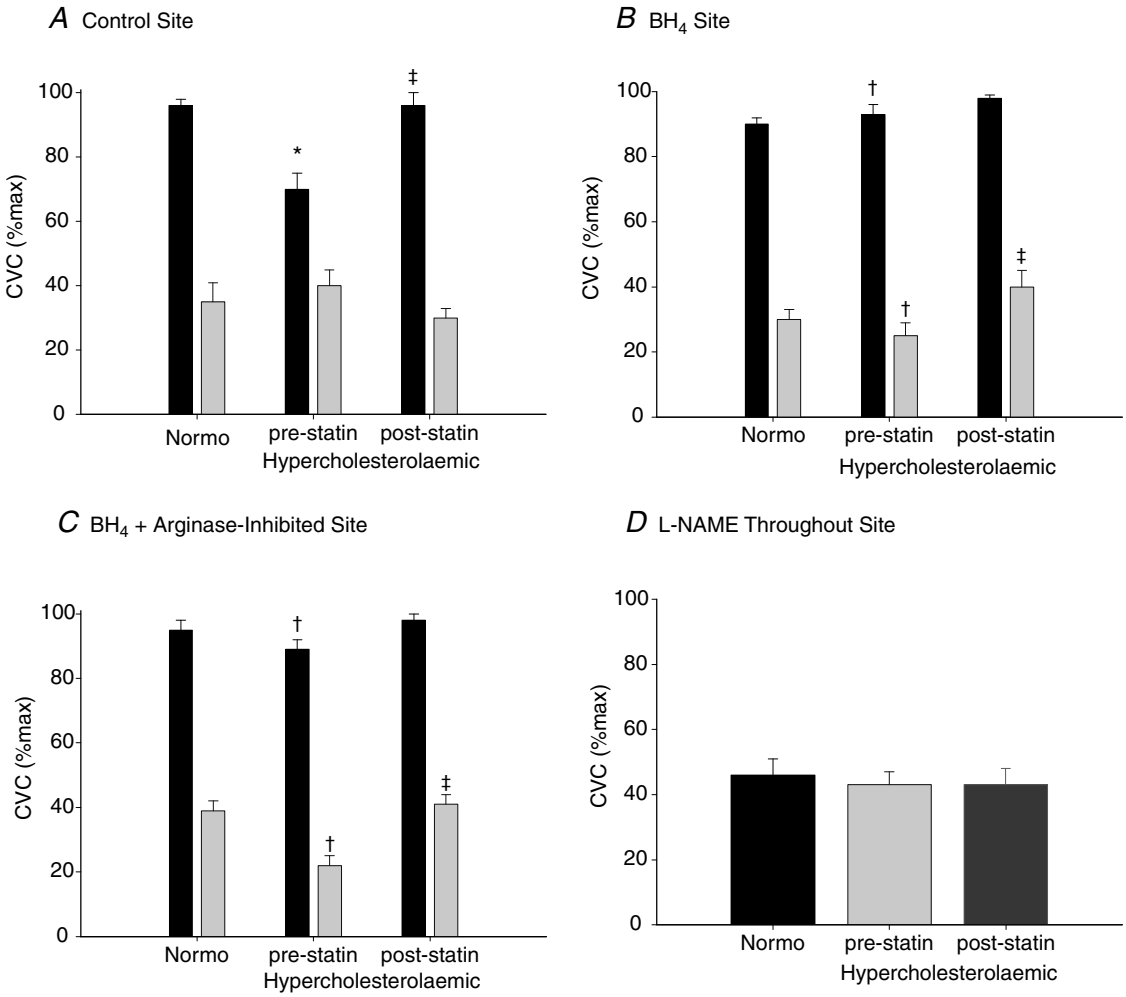

Figure 3. Mean skin blood flow
Cutaneous vascular conductance (% max) at the plateau in skin blood flow during local warming and after NOS inhibition with L-NAME in normocholesterolaemic (Normo) control subjects, hypercholesterolaemic subjects and after the oral atorvastatin intervention in the control site (*A*), BH_4site (*B*), BH_4+ arginase-inhibited site (*C*) and L-NAME throughout local heating (*D*). $*P < 0.05$ different to the normocholersterolaemic group; $†P < 0.05$ different compared to the control site due to the localized microdialysis drug treatment; $‡P < 0.05$ different due to the atorvastatin intervention.

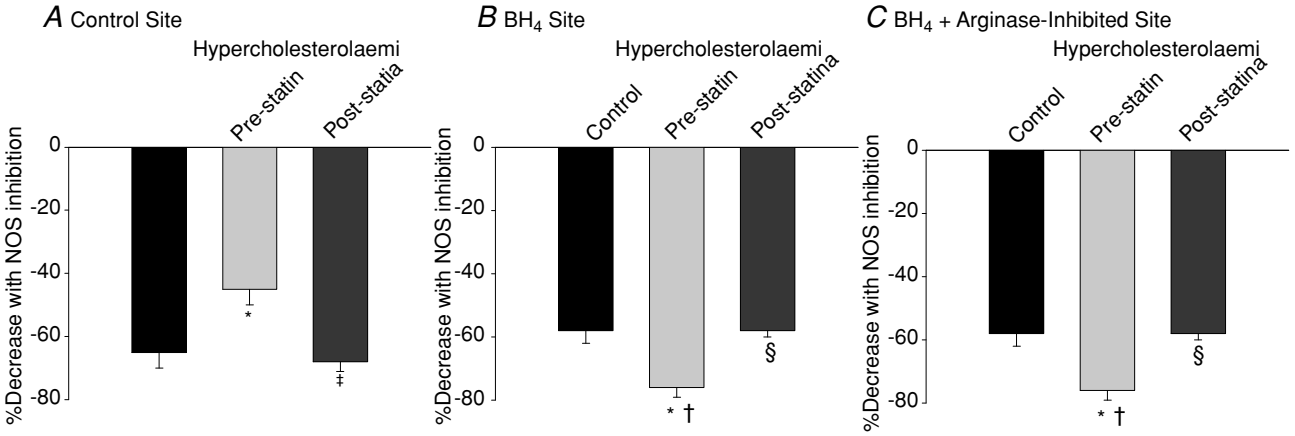

Figure 4
The reduction in cutaneous vascular conductance with NOS inhibition in normocholesterolaemic control subjects, hypercholesterolaemic subjects and after the oral atorvastatin intervention in the control site (*A*), BH_4 site (*B*) and BH_4 + arginase-inhibited site (*C*). $^*P < 0.05$ different from the normocholersterolaemic group; †$P < 0.05$ different compared to the control site due to the localized microdialysis drug treatment; ‡$P < 0.05$ difference due to the atorvastatin intervention §$P < 0.05$ difference from the control site with the atorvastatin intervention.

atorvastatin intervention, skin blood flow responses to local heating in the hypercholesterolaemic subjects were similar to those of the normocholesterolaemic control group. However, the localized administration of BH_4 alone or in combination with arginase inhibition increased the post-L-NAME plateau, indicating that ~60% of the local heating response was mediated by the production of NO after the atorvastatin intervention (i.e. the improvement in NO-dependent vasodilatation was attenuated compared to pre-intervention trials). These results suggest that decreased BH_4 bioavailability contributes in part to cutaneous microvascular dysfunction in hypercholesterolaemic humans and that atorvastatin is an effective systemic treatment for improving mechanisms related to NOS coupling in the microvasculature.

We recently demonstrated that localized ascorbate administration and/or arginase inhibition increase NO-dependent vasodilatation during local skin heating in subjects with hypercholesterolaemia (Holowatz, 2011; Holowatz *et al.* 2011). Further, arginase activity and expression of both arginase isoforms were increased in skin samples obtained from those subjects. Following an oral atorvastatin intervention, NO-dependent

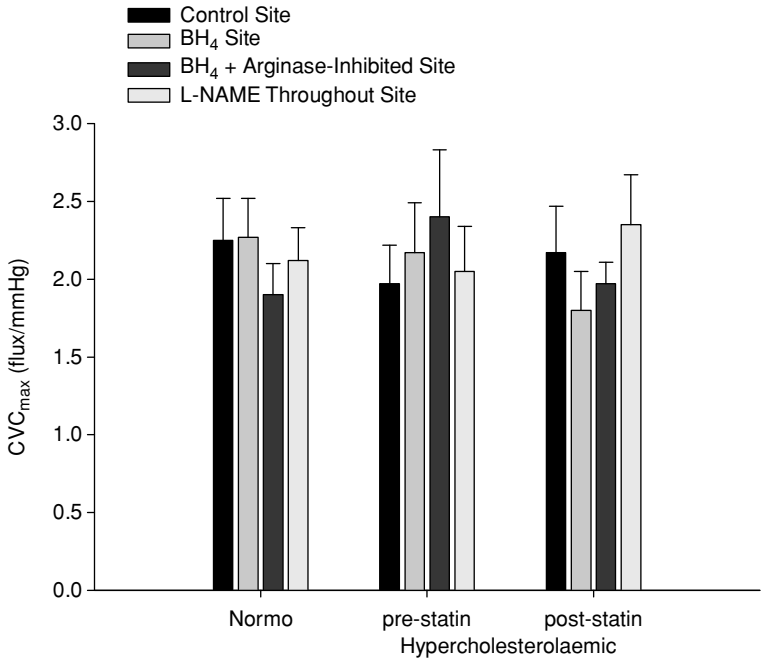

Figure 5. Maximal cutaneous vascular conductance
Absolute cutaneous vascular conductance in all microdialysis treatment sites for the normocholesterolaemic group and the hypercholesterolaemic group before and after the atorvastatin intervention. There was no difference due to localized drug treatment, between groups, or with the intervention.

vasodilatation was increased and arginase activity (but not expression) was decreased. Together, these data suggest that both increased oxidant stress and decreased L-arginine availability through upregulated arginase, contribute to microvascular dysfunction with hypercholesterolaemia. However, due to the non-specific actions of ascorbate, in our previous studies we were unable to distinguish between simple antioxidant properties of the ascorbate (Toth *et al.* 2002) and NOS3 uncoupling mechanisms through stabilizing BH_4 and altering arginase activity (Berkowitz *et al.* 2003; Ryoo *et al.* 2006, 2011; Lim *et al.* 2007). Therefore, in this study we sought to logically extend our previous observations by exploring the mechanistic role of the oxidant-sensitive essential NOS3 cofactor BH_4. By directly administering this highly specific NOS3 cofactor, we were able to alleviate many of the limitations caused by the non-specific properties of ascorbate and focus on NOS3 coupling/uncoupling mechanisms.

As in our previous studies, we found that localized administration of the essential NOS3 cofactor BH_4 increased NO-dependent vasodilatation to local heating in subjects with hypercholesterolaemia. We originally hypothesized that administration of BH_4 would only modestly increase NO-dependent vasodilatation in subjects with hypercholesterolaemia because of the upregulation of arginase (Holowatz *et al.* 2011). Arginase inhibition alone restored the vasodilatory response to local heating in hypercholesterolaemics. Taken together, each of these treatments alone maximize NOS3 function as assessed with local heating, as we were unable to show an additional effect of inhibiting arginase while concurrently administering BH_4 in the hypercholesterolaemic subjects. This suggests a potential mechanistic link between the BH_4 and arginase pathway through NOS coupling. Alternatively, this could simply be the result of a potential ceiling effect due to the robust nature of the local heating stimulus. However, these findings confirm that both of these pathways are potential molecular targets for intervention strategies to prevent and reverse microvascular dysfunction with hypercholesterolaemia.

One alternate potential explanation for the augmentation in skin blood flow to local heating in the hypercholesterolaemic group with localized BH_4 administration involves its role in adrenergic function (Houghton *et al.* 2006; Hodges *et al.* 2009). BH_4 is a cofactor for noradrenalin (NA) synthesis through tyrosine kinase (Lang *et al.* 2009) and inhibiting NA presynaptically with bretylium or its postsynaptic receptors results in an attenuated local heating response. Thus, BH_4 administration may have augmented NA synthesis and function. However, because in this series of studies all localized interventions targeting the NOS pathway (arginase inhibitors, L-arginine and ascorbate) have been successful at augmenting NO-dependent vasodilation it is likely that the effects of BH_4 were isolated to its role as a cofactor for NOS (Holowatz, 2011; Holowatz *et al.* 2011).

In the present study we chose to carry out a 3 month atorvastatin intervention phase. The lowest clinical dose of atorvastatin (10 mg) was used and it significantly lowered total, LDL and oxLDL cholesterol. In addition, this intervention lowered arginase activity without affecting arginase expression (Holowatz *et al.* 2011), suggesting that this is one possible pleiotropic effect of statins working through stabilizing the cellular microtubule structure and sequestering arginase to a subcellular location where it does not have access to L-arginine microdomains (Ryoo *et al.* 2006, 2011). Thus, the statin intervention alone may have corrected the underlying deficits in the arginase pathway such that any further changes due to BH_4 were undetectable. Oral BH_4 interventions have been used successfully in both rodent atherosclerotic models (Hattori *et al.* 2007) and in hypercholesterolaemic humans (Cosentino *et al.* 2008). In rodents, oral ingestion of BH_4 slowed the progression of atherogenesis in apolipoprotein E-knockout animals by decreasing expression of NADPH oxidase components and inflammatory factors (Hattori *et al.* 2007). In humans, chronic oral BH_4 improved forearm blood flow vasodilatory responsiveness to endothelium-dependent agonists and decreased inflammatory markers in the plasma (Cosentino *et al.* 2008). Thus, both of these strategies appear to independently affect microvascular function through different mechanisms.

One interesting finding is that after the atorvastatin intervention, BH_4 alone or in combination with the arginase inhibitors increased the post-L-NAME plateau compared to these sites before the intervention. Previously published biopsy data from the same subjects (Holowatz *et al.* 2011), showed a trend toward increased NOS3 expression in the hypercholesterolaemics that was decreased with statin therapy. Consistent with the literature, NOS3 expression is commonly found to be increased with hypercholesterolaemia as a compensatory mechanism in part due to feedback control from the uncoupling of the enzyme (Li *et al.* 2002). The present data demonstrate that non-NO-dependent vasodilatation (i.e. post-L-NAME plateau during local heating) is increased. The precise mechanism behind this is unclear but may be related to maximized coupled NOS3 function and/or alleviating some of the detrimental vasoconstrictor effects of oxidants (Bailey *et al.* 2004; Thompson-Torgerson *et al.* 2007*a*, *b*).

In the present study we also found an effect of the localized microdialysis treatment on baseline skin blood flow (Table 2), i.e. there was a modest vasodilation at baseline in sites treated with the combination of BH_4 and arginase inhibitors. These data suggest that NO function can be augmented at baseline with dual treatments

that affect the NOS pathway under conditions in which NOS is probably in the coupled state. In the hypercholesterolaemics before the atorvastatin intervention there was no effect of this combined treatment on baseline skin blood flow. In relation to the other parameters of the local heating response, this modest baseline shift in the normocholesterolaemic and the hypercholesterolaemics after the statin intervention may be artificially increasing the plateau and the post-L-NAME plateau. However, we did not observe an increase in the post-L-NAME plateau in the normocholesterolaemics and further the post-L-NAME plateau remained elevated in the hypercholesterolaemics after the statin intervention in sites only treated with BH_4, where there was not a significant increase in baseline.

In this series of studies exploring arginase and NOS coupling mechanisms with hypercholesterolaemia, we chose to utilize the cutaneous circulation as our model. The cutaneous circulation is accessible and provides a microvascular model where minimally invasive techniques can be used to explore microvascular function and dysfunction (Cracowski *et al.* 2006). As local heating is a highly reproducible thermal tool used to induce NO-dependent vasodilatation where the basic mechanisms of vasodilation have been systematically explored in a young healthy population, we used this protocol to explore NO mechanisms with hypercholesterolaemia. In hindsight it may have been useful to use a slower heating protocol to obtain a temperature skin blood flow dose–response type curve. We may have been able to glean additional information and the ceiling effect potential may have been less of an issue. In designing the current study, we have focused on quantifying NOS function with each treatment site by examining the NO-dependent plateau as the precise mechanisms underlying the axon reflex and other transient features of the local heating response remain unclear (Houghton *et al.* 2006).

Limitations

Initially we only planned to perform the atorvastatin intervention in the hypercholesterolaemic group. While it would have been ideal to perform the intervention in the normocholesterolaemic control to examine the pleiotropic effects of the statins, it is unlikely that we would have observed a significant functional effect due to the robust nature of the local heating response, i.e. skin blood flow responses were already maximized and reached the maximum capacity for the cutaneous vessels to vasodilate. This also probably contributes to our inability to delineate difference with the combined BH_4 and arginase inhibition treatments in the hypercholesterolaemic group.

We chose to use a 10 mM concentration of BH_4 based on extensive pilot work where the final concentration did not cause baseline vasodilation, and increasing the concentration did not further increase the response to local heating. In previous vasoconstriction studies we used a 5 mM concentration of BH_4 with similar rationale for arriving at that concentration specific to those studies (Lang *et al.* 2009). It is possible that our concentration of BH_4 may have induced other non-specific effects because of the weak antioxidant properties of BH_4. Additional studies using the stereoisomer (6S-BH_4), which does not bind as a cofactor to NOS3 but has the same weak antioxidant properties (Mayahi *et al.* 2007), are needed to determine if 6R-BH_4 is indeed working through a NOS coupling or an antioxidant mechanism to improve functional NO-dependent vasodilatation in this population.

Summary

In summary, localized administration of the essential NOS3 cofactor BH_4 augmented NO-dependent vasodilatation during local heating of the skin in hypercholesterolaemic humans. In contrast to our hypothesis, there was no additional effect of inhibiting arginase on this response. After an oral atorvastatin intervention, cutaneous microvascular function was restored in the hypercholesterolaemic humans and vasodilatation due to NO synthesis was increased. However, the addition of localized BH_4 (alone or in combination with arginase inhibition) did not provide any further increase in NO-dependent vasodilatation, suggesting that other non-NO-dependent mechanisms may have been increased with the localized treatments after the atorvastatin intervention. Taken together, these data suggest that both the arginase pathway and BH_4–NOS3 coupling mechanisms are potential molecular targets for preventing and reversing microvascular dysfunction with hypercholesterolaemia.

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Author contributions

L.A.H.: data collection, analysis, interpretation, and manuscript preparation; W.L.K.: data interpretation and manuscript preparation. All studies took place at PSU. Both authors approved the final version of the manuscript.

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