TITLE: Role of cyclooxygenase in the vascular response to locally delivered acetylcholine in Caucasian and African descent individuals

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LIST OF ABBREVIATIONS

ACh	Acetylcholine
AFD	African descent
AUC	Area under the curve
CAU	Caucasian
COXib	Cyclooxygenase inhibition
ED50	Half-maximal effective dose
IQR	Interquartile range
LDU	Laser Doppler units
MAP	Mean arterial pressure

Mdn	Median
NFCI	Non-freezing cold injury
SD	Standard deviation
TXA_2	Thromboxane A_2

ABSTRACT

INTRO:

Individuals of African descent (AFD) are more susceptible to non-freezing cold injury (NFCI) compared with Caucasian individuals (CAU). Vasodilatation to acetylcholine (ACh) is lower in AFD compared with CAU in the non-glabrous foot and finger skin sites; the reason for this is unknown. Prostanoids are responsible, in part, for the vasodilator response to ACh, however it is not known whether the contribution differs between ethnicities.

METHODS:

12 CAU and 12 AFD males received iontophoresis of ACh (1 w/v %) on non-glabrous foot and finger skin sites following placebo and then aspirin (600 mg, single blinded). Aspirin was utilised to inhibit prostanoid production by inhibiting the cyclooxygenase (COX) enzyme. Laser Doppler flowmetry was utilised to measure changes in skin blood flow.

RESULTS:

Not all participants could receive iontophoresis charge due to high skin resistance; these participants were therefore excluded from the analyses.

Foot:ACh elicited greater maximal vasodilatation in CAU than AFD following placebo ($P=0.003$) and COX inhibition (COXib) ($P<0.001$). COXib did not affect blood flow

responses in AFD, but caused a reduction in the area under the curve for CAU (P=0.031).

Finger:ACh elicited a greater maximal vasodilatation in CAU than AFD following placebo (P=0.013) and COXib (P=0.001). COXib tended to reduce the area under the curve in AFD (P=0.053), but did not affect CAU.

CONCLUSIONS:

CAU have a greater endothelial reactivity than AFD in both foot and finger skin sites irrespective of COXib. It is concluded that the lower ACh-induced vasodilatation in AFD is not due to a compromised COX pathway.

KEY WORDS: Non-freezing cold injury; ethnicity; skin blood flow; endothelial-dependent vasodilatation; acetylcholine; cyclooxygenase.

HIGHLIGHTS

- ACh-induced cutaneous dilatation is attenuated in African individuals versus Caucasians.
- COX inhibition attenuated the dilatation in the foot skin site for Caucasians.
- COX is not responsible for the lower vasodilator responses in African individuals.

INTRODUCTION

Non-freezing cold injury (NFCI) is a preventable clinical injury that affects the peripheral skin sites (particularly fingers and toes) of individuals who experience prolonged exposure to local cold tissue temperatures (0 °C to 20° C) (Ungley and Blackwood, 1942). Symptoms of this injury may last for many years and often include pain, numbness and hyperhidrosis which, combined with cold hypersensitivity of the injured limb, can lead to increased susceptibility to further cold injuries (Golden et al., 2013; Ungley et al., 1945). This type of injury is a concern for those involved in outdoor work (e.g. agriculture or forestry work, military) or recreational activities (e.g. skiing, mountaineering) that take place in cold conditions which may also elicit freezing cold injuries (Hashmi et al., 1998; Mäkinen et al., 2009; Morrison et al., 2015).

Individuals of black African descent (AFD) are more susceptible than Caucasian (CAU) individuals to NFCI (Burgess and Macfarlane, 2009; DeGroot et al., 2003). The reason for this is not known but it is thought that sustained skin blood flow in the extremities in low environmental temperatures can prevent local cold injuries (Daanen and van der Struijs, 2005; Lewis, 1941; Wilson and Goldman, 1970). During hand immersion in cold water (8 °C) for 30 minutes and subsequent rewarming of dry skin in 30 °C air, AFD experienced greater finger vasoconstriction and slower rewarming compared with CAU (Maley et al., 2014) indicating AFD received a greater “dose of cold”. We investigated whether this was due to alterations in the control of the microcirculation of the extremities and demonstrated that endothelial-dependent (ACh), but not -independent (SNP), vasodilatation was significantly

attenuated in AFD compared with CAU in non-glabrous finger and toe skin sites (Maley et al., 2015).

Local application of acetylcholine (ACh) increases prostanoid and nitric oxide production eliciting vasodilatation (Holowatz et al., 2005; Kellogg et al., 2005). Prostanoids are produced from arachidonic acid, released from the cell membrane, metabolised by the enzyme cyclooxygenase (COX) (Vane et al., 1998) to produce prostaglandin H_2 which is further metabolised by various synthase enzymes to produce various prostanoids (Félétou, 2011; Hamberg et al., 1975; Moncada et al., 1976; Moncada and Vane, 1979). The vascular wall synthesises each of these prostanoids, the most abundant being prostacyclin (PGI_2), whilst platelets are the main source of thromboxane A_2 (TXA_2) (Dubois et al., 1998; Félétou, 2011; Majed and Khalil, 2012; Moncada and Vane, 1978; Tang and Vanhoutte, 2008). In young healthy individuals TXA_2 and PGI_2 elicit vasoconstriction and vasodilatation, respectively (Félétou, 2011; Majed and Khalil, 2012).

Blocking COX inhibits all vasodilator and vasoconstrictor prostanoid production (Roth et al., 1975; Vane, 1971). The net action of COX inhibition (COXib) varies between populations. In young, healthy individuals, COXib attenuates the vasodilator response to ACh in the forearm circulation assessed with laser Doppler flowmetry (Holowatz et al., 2005; Kellogg et al., 2005; Noon et al., 1998). However, the role of COX in response to ACh appears compromised in certain populations. Normotensive aged (>60 years) and hypertensive individuals (>46 years) exhibit similar endothelial dysfunction in response to ACh, with COXib (indomethacin) restoring the vasodilator response as assessed by plethysmography (Taddei et al., 1997b). This vasodilator

restoration was due to an increase in nitric oxide bioavailability (Taddei et al., 1997a). More recently, *in-vitro* studies performed on human small arteries noted the antioxidant, ascorbic acid, and a non-selective COX inhibitor (indomethacin) augmented the vasodilator response to ACh in hypertensive samples, although their actions were not additive (Virdis et al., 2013). Collectively, this body of research provides evidence that the mechanism of endothelial dysfunction in aged and hypertensive individuals is due, in part, to COX activity diminishing the vasodilator response to endothelial-dependent vasodilators through reductions in nitric oxide bioavailability. Whether the endothelial dysfunction in AFD observed previously (Maley et al., 2015) is caused by a differing contribution of the COX pathway between ethnic groups is not known. Given that AFD experience greater levels of oxidative stress (Feairheller et al., 2011; Kalinowski et al., 2004), and COX increases reactive oxygen species (Kukreja et al., 1986; Virdis et al., 2013) as well as producing TXA_2, it is possible that the COX pathway may contribute to the attenuated ACh-induced vasodilatation compared with CAU.

Therefore, the aim of the present study was to establish the contribution of COX to ACh-induced vasodilatation in both CAU and AFD. As we have previously observed an attenuated ACh-induced vasodilator response in AFD compared to CAU, it was hypothesised that AFD would experience a lower vasodilator response to ACh compared with CAU, and COXib would augment endothelial reactivity in AFD.

METHODS

PARTICIPANTS

This study was given a favourable ethical opinion from the University of Portsmouth Science Faculty Ethics Committee. The participants were made aware of the purpose, procedures and risks of the study prior to giving their informed written consent. 12 CAU and 12 AFD male volunteers participated in the study. All CAU were born in the UK. Eight AFD were born in the UK whilst four were born in Africa (Zimbabwe, Ghana, Kenya and Uganda) and had resided in the UK for an average of 11 years with a minimum of seven years. CAU and AFD were of similar age (mean [SD], 22 [4] years and 20 [2] years, $P = 0.069$), height (mean [SD], 178.2 [6.9] cm and 176.0 [7.9] cm, $P = 0.790$) and body mass (mean [SD], 73.1 [12.3] kg and 74.1 [12.8] kg, $P = 0.583$).

In attempt to reduce heterogeneity female participants were not included in the present study as the menstrual cycle is known to effect vasodilator capacity and thermoregulation (Charkoudian and Stachenfeld, 2015; Hashimoto et al., 1995), therefore the results of the present study should only be applied to young healthy male participants.

EXPERIMENTAL PROCEDURES AND MEASUREMENTS

Participants attended the laboratory on one occasion where they received iontophoresis of ACh. The technique of iontophoresis has been described previously (Morris and Shore, 1996; Roustit et al., 2014). Briefly, iontophoresis is a non-invasive method of transdermal drug delivery which transfers charged molecules using a low-intensity electric current into and through the skin to a depth of approximately 2 mm to 4 mm (Anderson et al., 2003). Iontophoresis was performed using both an anode and cathode connected to a battery powered iontophoresis controller (MIC2, Moor

Instruments, UK). The iontophoresis chamber, which is a small Perspex ring (MIC-ION1R-P1, Moor Instruments, UK) with an inner diameter of 9.5 mm, was filled with approximately 0.2 mL of ACh (1 w/v % [55.05 mM], Sigma-Aldrich, UK), diluted in water for injection. A laser Doppler probe (VP1T / 7, Moor Instruments, UK), utilised to measure skin temperature and skin blood flow, was placed into the Perspex ring and connected to a laser Doppler flowmetry monitor (moorVMS-LDF, Moor Instruments, UK). Laser Doppler and iontophoresis data were recorded using a data acquisition system and software (Powerlab and LabChart 7, AD Instruments, New Zealand).

On the day of testing participants were asked to consume 150 mL of diluted orange squash immediately prior to entering a temperature controlled chamber set at a dry bulb temperature of 23.2 (0.8) °C. All participants rested for 30 minutes in a supine position to allow skin temperature and skin blood flow to stabilise. Participants were supine throughout the experiment and each skin site was cleaned with deionised water prior to iontophoresis. Iontophoresis of ACh was delivered to either the right medial or right lateral dorsal foot first using the anode, with the cathode placed proximally within 5 cm to 10 cm. Secondly, iontophoresis was applied to the third or fourth non-glabrous finger skin site (medial phalanx) on the right hand (Fig. 1). Following this, participants were then asked to consume 150 mL of diluted orange squash which contained dissolved aspirin tablets to the total of 600 mg of aspirin (acetylsalicylic acid) (Boots Company, UK). Participants were blinded to the order of placebo and aspirin. Aspirin irreversibly inhibits COX by acetylation of the active site of COX (Vane, 1971; Vane and Botting, 2003) with this dose of aspirin shown to

inhibit 86 % of bradykinin-induced production of PGI_2 and 99 % inhibition of TXA_2 production by platelets at 30 minutes (Heavey et al., 1985).

Placebo	Foot Site 1	Finger Site 1	Aspirin (600 mg)	Foot Site 2	Finger Site 2

Fig. 1. Schematic of the experimental procedure

Thirty minutes after aspirin treatment, iontophoresis began on the foot at a skin site that had not been used (medial or lateral). Following this, iontophoresis was applied to the second finger skin site (third or fourth). The reason for not using the same skin site was that during pilot experiments the vasodilator response to iontophoresis of ACh was much longer lasting than 30 minutes, thus using the same skin site would influence subsequent skin blood flow results; this has been reported previously (Brocx and Drummond, 2009). The order of participants' skin sites tested (lateral vs. medial dorsal foot, third vs. fourth finger) was counter-balanced between participants. Repeatability studies on six participants demonstrated that the responses to ACh did not differ between sites (medial vs lateral foot; middle vs fourth finger) and over time (two dose ACh response curves following placebo).

The iontophoresis protocol employed in the present study is the same as previously used (Maley et al., 2015) which consisted of six pulses of 25 μA (0.5 mC) followed by one pulse of 50 μA (1mC) and one of 100 μA (2 mC) applied for 20 seconds separated by 60 second intervals in which no current was applied. On completion of the protocol, and after an interval of five minutes, the protocol was repeated on the next skin site. Blood pressure from the contralateral arm was recorded pre- and post-

iontophoresis application and measured using an automated monitor (Minimon 7137 Plus, Kontron Instruments, UK) for calculation of mean arterial pressure (MAP).

DATA ANALYSES

Due to high skin resistance, it was not possible to deliver all the current pulses in each skin site for all participants; this occurred more in the AFD participants. Therefore, only those who could receive the first pulse of iontophoresis were included in analyses (see results). As skin resistance during iontophoresis charges of 100 μA have been reported to influence the vasodilator response to ACh (Pienaar et al., 2014; Puissant et al., 2014) we investigated whether this was true for lower iontophoresis charges. Following placebo treatment, the skin blood flow responses (average over the six pulses of 25 μA) were correlated with electrical skin resistance (average over the six pulses of 25 μA) and were plotted for CAU and AFD separately and R^2 calculated. Skin resistance was calculated by monitoring the applied voltage and dividing this by the current application, displayed in kilohms.

Blood pressure remained constant throughout the iontophoresis protocol (see results) therefore skin blood flow at baseline was expressed as laser Doppler units (LDU) rather than cutaneous vascular conductance. Average skin blood flow in response to iontophoresis of ACh was calculated over the final 20 seconds of the interval between successive pulses and between 40 to 60 seconds after the final pulse. These responses were expressed as percentage change from that prior to iontophoresis (averaged over 20 seconds and set at 0 %). ED50, expressed as 95 % confidence intervals was calculated using GraphPad (Version 5, USA). Maximum skin blood flow and area under the curve (AUC) were calculated for each participant.

The point at which the skin blood flow was at a maximum point was not always identified following the final pulse, therefore maximum skin blood flow was taken from wherever it was highest.

Statistical analyses were conducted using IBM SPSS for Windows version 20 (IBM SPSS Statistics, USA). Normality of data was assessed using Shapiro-Wilks statistical analysis. An α value of 0.05 was used to determine statistical significance. Baseline skin blood flow, skin temperature and MAP between- and within-groups were compared using an independent and paired samples *t*-test, respectively. ED50, maximal percentage change, AUC between-groups was analysed using an independent samples *t*-test or a Mann-Whitney U test, respectively (statistical test utilised determined by normality testing). ED50, maximal percentage change, AUC within-groups was analysed using a paired samples *t*-test or a Wilcoxon signed rank test. Non-parametric analysis was utilised to assess skin blood flow over time. Effect sizes were calculated using Cohen's d for parametric data (denoted by *d* in text) and Rosenthal's *r* for non-parametric data (denoted by *r* in text). Data within figures are presented as mean (SD)

RESULTS

MEAN ARTERIAL PRESSURE

MAP at baseline for CAU and AFD following placebo (mean [SD], 83 [8] mmHg and 87 [8] mmHg, respectively, $P = 0.627$) and COXib (mean [SD], 84 [5] mmHg and 88 [9] mmHg, respectively, $P = 0.064$) did not differ between- or within-groups (CAU $P = 0.748$, AFD $P = 0.805$).

BASELINE SKIN BLOOD FLOW AND SKIN TEMPERATURE

There were no differences in baseline skin blood flow or skin temperature between- or within-groups for either the foot or finger skin sites following treatment of either placebo or COXib (Table 1).

Table 1. Mean (SD) baseline skin blood flow (LDU) and skin temperature (°C) for the foot and finger skin sites following placebo or COXib

	Baseline Skin Blood Flow (LDU)					
	Foot			Finger		
	Placebo	**COXib**	**Within**	**Placebo**	**COXib**	**Within**
CAU	12 (6) *n* = 12	11 (4) *n* = 12	*P* = 0.165	54 (19) *n* = 11	47 (23) *n* = 11	*P* = 0.111
AFD	10 (7) *n* = 12	8 (3) *n* = 12	*P* = 0.312	52 (25) *n* = 10	48 (23) *n* = 8	*P* = 0.089
Between	*P* = 0.571	*P* = 0.081		*P* = 0.890	*P* = 0.950	
	Skin Temperature (°C)					
CAU	27.1 (1.3) *n* = 12	26.8 (1.3) *n* = 12	*P* = 0.079	29.4 (0.8) *n* = 11	28.9 (1.1) *n* = 11	*P* = 0.172
AFD	27.0 (1.1) *n* = 12	26.6 (1.3) *n* = 12	*P* = 0.121	28.8 (0.6) *n* = 10	28.5 (0.7) *n* = 8	*P* = 0.167
Between	*P* = 0.848	*P* = 0.998		*P* = 0.084	*P* = 0.355	

RESPONSES TO ACETYLCHOLINE

FOOT SKIN SITE

WITHIN-GROUPS

Fig. 2 shows the skin blood flow responses to ACh for the foot skin site in CAU and AFD. CAU experienced a reduced vasodilator response to ACh following COXib (Fig. 2). Additionally, in CAU following COXib ED50 occurred at a greater cumulative current (Table 2, *P* = 0.005), AUC was smaller (*P* = 0.031, *d* = 0.80) but maximal vasodilatation did not differ. COXib did not affect the vasodilator response to ACh in AFD.

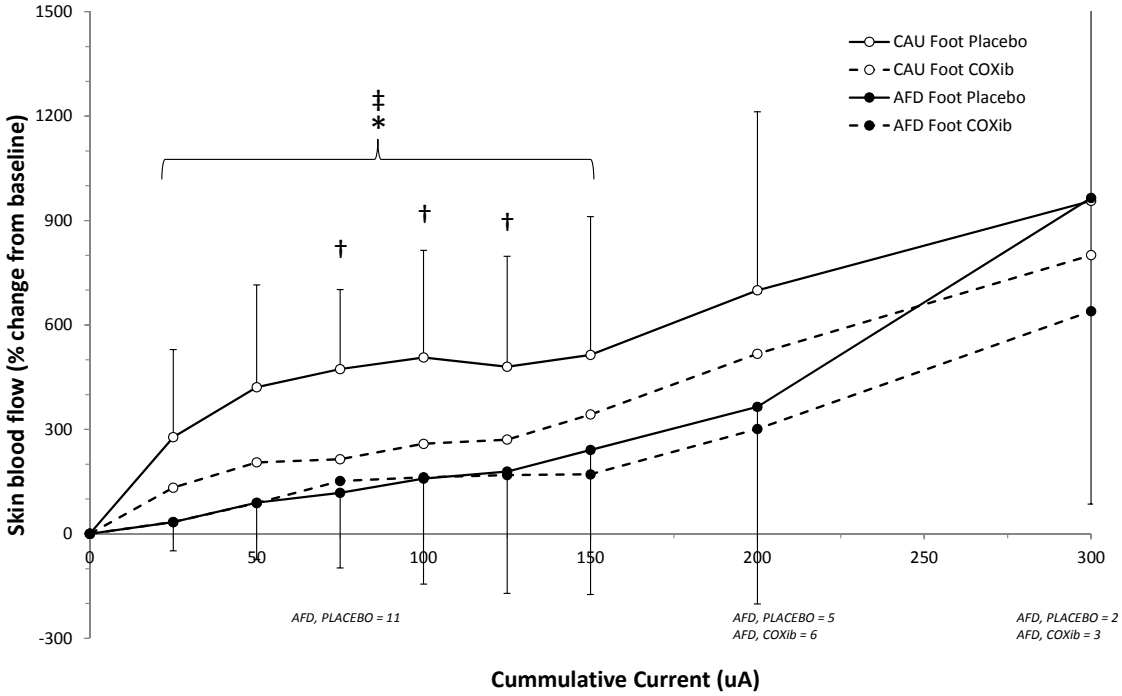

Fig. 2. Mean (SD) skin blood flow responses in the foot skin site for both placebo and COXib trials. * Significant difference between CAU and AFD for placebo trial ($P < 0.05$). ‡ Significant difference between CAU and AFD for COXib trial ($P < 0.05$). † Significant difference between placebo and COXib trial for CAU ($P < 0.05$).

Error bars included for CAU and AFD placebo only for reader clarity.

Table 2. Maximum, ED50 and area under the curve (AUC) skin blood flow response to ACh in the foot skin site following placebo or COXib

			Variable		
			ED50 (μA)	**Max (%)**	**AUC**
Within	**Foot CAU**	PLACEBO *n* = 12	54 to 116	^943 (490)	4808 (2678)
		COXib *n* = 12	116 to 174 †	^775 (784)	2998 (1761) †
			P = 0.005	*P* = 0.308	*P* = 0.031
	Foot AFD	PLACEBO *n* = 12	150 to 271	^81 (370)	^190 (1329)
		COXib *n* = 12	118 to 418	^50 (148)	^95 (894)
			P = 0.757	*P* = 0.117	*P* = 1.000
Between	**Foot placebo**	CAU *n* = 12	54 to 116	^943 (490)	^4516 (2601)
		AFD *n* = 12	153 to 302 *	^81 (370) *	^190 (1329) *
			P < 0.001	*P* = 0.003	*P* = 0.001
	Foot COXib	CAU *n* = 12	116 to 174	^775 (784)	^3120 (3170)
		AFD *n* = 12	97 to 424	^50 (148) *	^95 (894) *
			P = 0.159	*P* < 0.001	*P* = 0.002

Max given as median (IQR) percentage change from baseline, ED50 given as 95 % confidence intervals (microamps) and AUC given as mean (SD) or median (IQR). Note: as pairwise analyses were conducted within-groups, the values reported do not always match the between-groups analyses which included all participants or until a participant did not receive all applied current. † Significant difference between placebo and COXib ($P < 0.05$). * Significant difference between CAU and AFD ($P < 0.05$). ^ Median (IQR).

BETWEEN-GROUPS

AFD demonstrated lower vasodilatation compared with CAU in response to ACh following both placebo and COXib (Fig. 2). Following placebo treatment ED50 occurred at a greater cumulative current for AFD compared with CAU (Table 2, $P < 0.001$), and maximal vasodilatation ($P = 0.003$, $r = 0.59$) as well as AUC ($P = 0.001$, $r = 0.62$) were lower in AFD than CAU. Following COXib, ED50 did not differ between

groups, however maximal vasodilatation ($P < 0.001$, $r = 0.67$) as well as AUC ($P = 0.002$, $r = 0.60$) were lower in AFD compared with CAU.

No relationship was observed between electrical skin resistance and skin blood flow responses in the foot skin site for either CAU or AFD (Fig. 3).

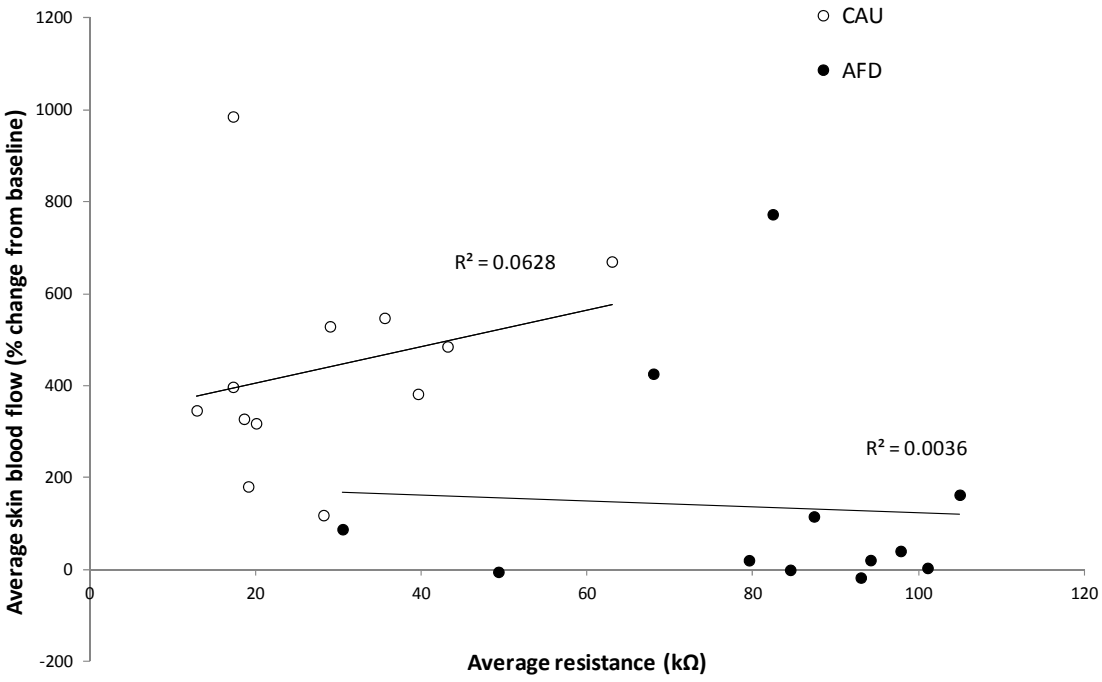

Fig. 3. Relationship between average skin blood flow (%) and average electrical skin resistance (kΩ) for CAU and AFD in the foot skin site during 25 μA iontophoresis pulses of ACh following placebo

FINGER SKIN SITE

WITHIN-GROUPS

Fig. 4 shows the skin blood flow responses to ACh for the finger skin site in CAU and AFD. For CAU, COXib did not affect the vasodilator response to ACh. This was confirmed with no difference in ED50, maximal vasodilatation or AUC (Table 3).

In AFD, COXib tended to reduce maximal vasodilatation (P = 0.064, d = 1.28) and AUC (P = 0.053, d = 1.32). Calculation of ED50 was not possible for AFD following COXib as no distinctive dose-response curve could be fitted to the data.

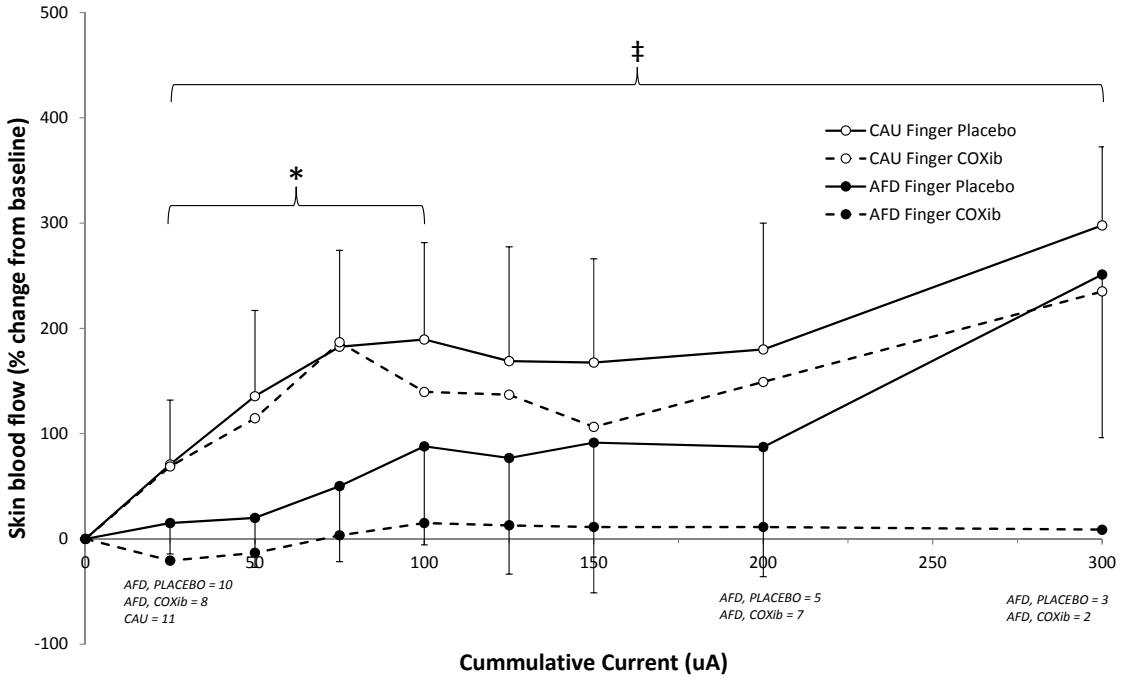

Fig. 4. Mean (SD) skin blood flow responses in the finger skin site for both placebo and COXib trials. * Significant difference between CAU and AFD for placebo trial (P < 0.05). ‡ Significant difference between CAU and AFD for COXib trial (P < 0.05). Error bars included for CAU and AFD placebo only for reader clarity.

Table 3. Maximum, ED50 and area under the curve (AUC) skin blood flow response to ACh in the finger skin site following placebo or COXib

			Variable		
			ED50 (μA)	**Max (%)**	**AUC**
Within	**Finger CAU**	Placebo *n* = 11	49 to 98	301 (76)	1542 (597)
		COXib *n* = 11	24 to 137	311 (222)	1255 (872)
			P = 0.646	*P* = 0.902	*P* = 0.273
	Finger AFD	Placebo *n* = 8	105 to 187	188 (139)	642 (632)
		COXib *n* = 8	-	57 (43)	22 (202)
			Unable to calculate	*P* = 0.064	*P* = 0.053
Between	**Finger placebo**	CAU *n* = 11	49 to 98	301 (76)	1542 (597)
		AFD *n* = 10	125 to 282 *	160 (139) *	539 (660) *
			P < 0.001	*P* = 0.013	*P* = 0.002
	Finger COXib	CAU *n* = 11	24 to 137	^287 (162)	1255 (872)
		AFD *n* = 8	-	^53 (88) *	35 (218) *
			Unable to calculate	*P* = 0.001	*P* = 0.001

Max given as mean (SD) or median (IQR) percentage change from baseline, ED50 given as 95 % confidence intervals (microamps) and AUC given as mean (SD). Note: as pairwise analyses were conducted within-groups, the values reported do not always match the between-groups analyses which included all participants or until a participant did not receive all applied current. * Significant difference between CAU and AFD ($P < 0.05$). ^ Median (IQR).

BETWEEN-GROUPS

AFD demonstrated lower vasodilatation compared with CAU in response to ACh following both placebo and COXib (Fig. 4). Following placebo in AFD, ED50 occurred at a greater cumulative current than CAU (Table 3, $P < 0.001$). Additionally, maximal vasodilatation was lower ($P = 0.013$, $r = 1.27$) and AUC was smaller ($P = 0.002$, $r = 1.78$) in AFD than CAU. Following COXib, AFD demonstrated lower

maximal vasodilatation (P = 0.001, r = 0.64) and a smaller AUC (P = 0.001, d = 1.96) compared with CAU.

No relationship was observed between electrical skin resistance and skin blood flow responses in the finger skin site in either CAU or AFD (Fig. 5).

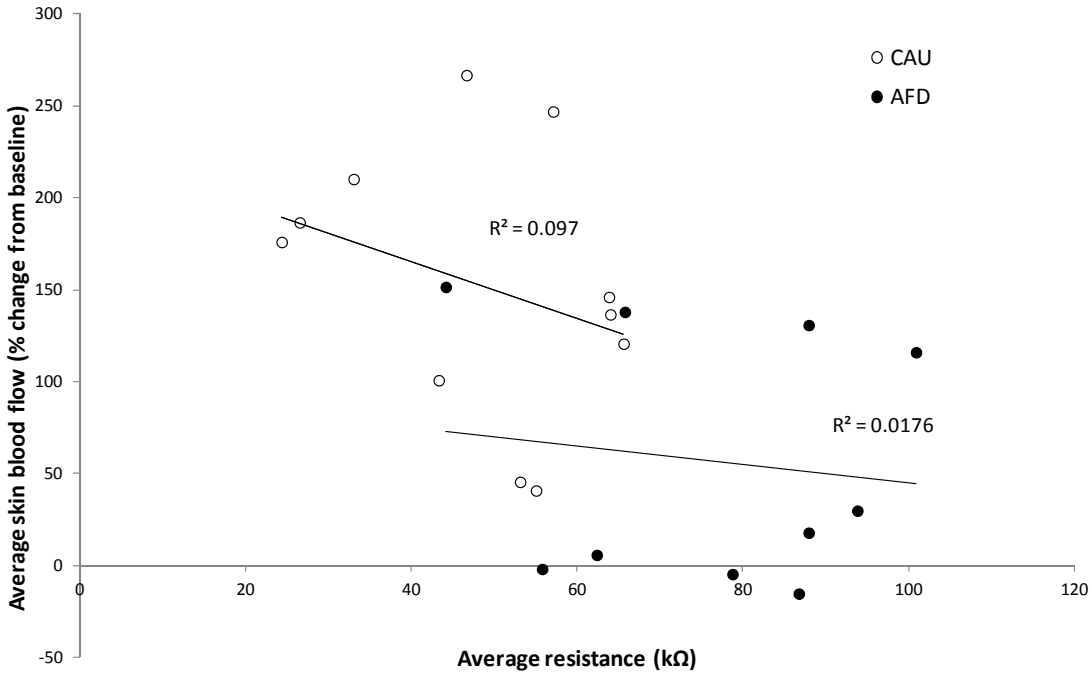

Fig. 5. Relationship between average skin blood flow (%) and average electrical skin resistance (kΩ) for CAU and AFD in the finger skin site during 25 μA iontophoresis pulses of ACh following placebo

DISCUSSION

The present study demonstrated that the vasodilator response to local application of ACh in the non-glabrous foot and finger skin sites is lower in AFD compared with CAU irrespective of COXib. This data supports previous observations in the hands and feet cutaneous microcirculation (Maley et al., 2015) and forearm circulation

(Cardillo et al., 1999; Jones et al., 1999; Ozkor et al., 2014; Stein et al., 1997) where an attenuated vasodilator response to ACh or methacholine was observed in AFD compared with CAU. The effect of COXib on the responses to ACh appeared to be site and ethnicity dependant. CAU, but not AFD, experienced a lower vasodilator response to ACh following COXib in the foot skin site indicating the role of vasodilator prostanoids, supporting previous findings in the forearm cutaneous microcirculation (Holowatz et al., 2005; Kellogg et al., 2005; Noon et al., 1998); however, in the finger skin site, COXib did not affect CAU but tended to affect AFD vasodilatation.

It has been previously reported (Pienaar et al., 2014) that the higher skin resistance in AFD individuals at iontophoresis currents of 100 μA may be a possible cause of the reduced response to ACh in AFD compared with CAU. However, no correlation between electrical skin resistance and skin blood flow responses was observed in the present study during the 25 μA applied currents (Fig. 3 and Fig. 5). The obvious differences in applied iontophoresis currents between studies could be a major factor influencing results as previous investigations in healthy individuals have also reported that electrical skin resistance influences the ACh-induced vasodilator response to applied currents of 100 μA (Puissant et al., 2014). Additionally, Pienaar et al., (2014) correlated skin blood flow responses with electrical skin resistance but did not separate CAU and AFD data. Therefore, the conclusion from Pienaar et al., (2014) that iontophoresis in AFD is limited by resistance more so in comparison to CAU may be flawed as this ethnic group is known for higher skin resistance (Johnson and Corah, 1963) and decreased endothelial reactivity (Cardillo et al., 1999; Jones et al., 1999; Ozkor et al., 2014; Stein et al., 1997). Different skin sites

(i.e. forearm *vs.* foot) and amount of iontophoresis charge may also have influenced the correlation between electrical skin resistance and skin blood flow responses. Based on our observations we suggest during 25 μA iontophoresis charges the depressed ACh-induced vasodilator response in AFD is not due to high electrical skin resistance in these individuals but due to another mechanism yet to be identified.

In elderly and / or hypertensive individuals, COXib restores the vasodilator response to ACh through an increase in nitric oxide bioavailability (Taddei et al., 1997a, 1997b). In comparison, COXib attenuates the vasodilator response to ACh in young normotensive individuals (Holowatz et al., 2005; Kellogg et al., 2005). Thus, COX products appear to facilitate vasodilatation in young normotensive individuals, but elicit vasoconstriction in older / hypertensive individuals. In the present study it was hypothesised that COXib in AFD may have augmented the vasodilator response to ACh by inhibiting the COX associated oxidative stress (Kukreja et al., 1986; Taddei et al., 1998; Virdis et al., 2013) and vasoconstrictor prostanoid contribution; however, this was not observed. Therefore, it appears either, *(1)* the COX pathway is not (or as) active in young healthy AFD males, or *(2)* the lower vasodilator response to ACh in AFD is not due to the COX pathway. Given that finger skin blood flow tended to decrease with COXib (Table 3) we cannot provide evidence for an inactive COX pathway in AFD.

In contrast to our results and the studies mentioned above (Holowatz et al., 2005; Kellogg et al., 2005), Hendry and Marshall (2004) reported COXib augmented the response to ACh in the fingers of young healthy individuals. It is not clear why the

present study observed different responses but a direct comparison between studies is not possible as methodological differences exist (e.g. 100 μA vs. 25 μA, respectively).

Given that AFD did not experience an augmented vasodilator response to ACh with COXib, the present study suggests other mechanisms are accountable for the lower vasodilator response compared with CAU. It is well documented that both nitric oxide and prostanoids are involved in the ACh-induced vasodilatation (Holowatz et al., 2005; Kellogg et al., 2005; Noon et al., 1998). Another mechanism by which vasodilatation occurs in response to ACh is through endothelial-dependent hyperpolarising factors (EDHFs) (Brunt et al., 2015). Given that prostanoids production would be negligible upon COXib, it is assumed that the ACh-induced vasodilatation would be mainly mediated through nitric oxide or EDHFs. EDHFs are unlikely to be compromised in AFD as a recent study demonstrated that EDHFs provide a compensatory mechanism eliciting vasodilatation in response to intra-arterial infusion of ACh in AFD, but not CAU (Ozkor et al., 2014). It is known that nitric oxide bioavailability is often lower in AFD compared with CAU due, in part, to an increased oxidative stress (Kalinowski et al., 2004). It is possible oxidative stress sources other than COX, such as superoxide produced from the enzyme nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Paravicini and Touyz, 2008), may react with nitric oxide forming peroxynitrite resulting in less bioavailability of nitric oxide and lower vasodilatation (Münzel et al., 2010).

Whilst prostanoids appear to play a role in the vasodilator response to ACh (Fig. 2) and in other settings such as whole-body heating (McCord et al., 2006), they are not

involved in the vasodilator response to local heating (Dahmus et al., 2013; Golay et al., 2004; McCord et al., 2006). This demonstrates that pharmacological protocols such as those used to deliver ACh may not always reflect what occurs in an applied setting. Recently, Belvins et al., (2014) provided preliminary evidence that COXib may reduce cold-induced vasoconstriction for CAU during local cooling of the foot. While in the present study COX was not responsible for the lower vasodilator response to ACh in AFD, COX may play a role during local cooling as this enzyme releases TXA_2 (Serneri et al., 1990, 1981) and reactive oxygen species (Kukreja et al., 1986) which potentiate vasoconstriction (Bailey et al., 2005; Hamberg et al., 1975). Based on this information it is hypothesised that COX may play some role in the exaggerated vasoconstrictor response in AFD during cooling, thereby contributing to the increased risk of NFCI. Future research should investigate the role of prostanoids during local cooling to elucidate the reasons for the skin blood flow and skin temperature differences between CAU and AFD during local cooling of the extremities.

It is concluded that the attenuated endothelial reactivity to locally delivered ACh in AFD compared with CAU in foot and finger skin sites is not due to an altered function of COX in AFD; therefore, other pathways appear to be responsible.

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AUTHORS CONTRIBUTIONS

All authors contributed to the design of the research protocol; M J Maley collected and analysed data; all authors interpreted results of experiments; M J Maley prepared tables, figures and drafted manuscript; all authors edited and revised manuscript; all authors approved final version of manuscript.

STATEMENT OF CONFLICTS OF INTEREST

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