Original Article

OPEN

The role of prostaglandin and antioxidant availability in recovery from forearm ischemia–reperfusion injury in humans

Sophie E. Carter[a], Ashton Faulkner[b], and Mark Rakobowchuk[c]

Background: Endothelial dysfunction, manifesting as attenuated flow-mediated dilation (FMD), is clinically important. Antioxidants may prevent this dysfunction; however, the acute effects of oral administration in humans are unknown. Low flow-mediated constriction (L-FMC), a further parameter of endothelial health, is largely unstudied and the mechanisms for this response unclear.

Methods: Twelve healthy participants (five women and seven men) completed three test conditions: control; antioxidant cocktail (α-lipoic acid, vitamins C and E); and prostaglandin inhibitor ingestion (ibuprofen). Ultrasound measurements of brachial artery responses were assessed throughout 5 min of forearm ischemia and 3 min after. Subsequently, an ischemia–reperfusion injury was induced by a 20-min upper arm occlusion. Further, vascular function protocols were completed at 15, 30, and 45 min of recovery.

Results: Endothelial dysfunction was evident in all conditions. FMD was attenuated at 15 min after ischemia–reperfusion injury (Pre: $6.24 \pm 0.58\%$; Post15: $0.24 \pm 0.75\%$; mean ± SD, $P < 0.05$), but recovered by 45 min. Antioxidant administration did not preserve FMD compared with control ($P > 0.05$). The magnitude of L-FMC was augmented at 15 min (Pre: $1.44 \pm 0.27\%$; Post15: $3.75 \pm 1.73\%$; $P < 0.05$) and recovered by 45 min. Ibuprofen administration produced the largest constrictive response (Pre: $-1.13 \pm 1.71\%$; Post15: $-5.57 \pm 3.82\%$; time × condition interaction: $P < 0.05$).

Conclusion: Results demonstrate ischemia–reperfusion injury causes endothelial dysfunction and acute oral antioxidant supplementation fails to reduce its magnitude. Our results also suggest that a lack of shear stress during occlusion combined with suppression of prostaglandin synthesis magnifies L-FMC, possibly due to augmented endothelin-1 expression.

Keywords: allometric scaling, antioxidants, flow-mediated dilation, ibuprofen, low flow-mediated constriction, shear rate

Abbreviations: COX, cyclooxygenase; ET-1, endothelin-1; FMD, flow-mediated dilation; L-FMC, low flow-mediated constriction; MBV, mean blood velocity; PGE_2, prostaglandin E_2; PGI_2, prostacyclin; ROS, reactive oxygen species; TVR, total vascular reactivity

INTRODUCTION

Endothelial dysfunction, the impaired ability for vascular dilation due to decreased production or bioavailability of nitric oxide [1], can result from ischemia–reperfusion injury, a period of occlusion to a vascular bed followed by the rapid reintroduction of blood flow to this area [2,3]. Ischemia–reperfusion attenuates vasodilation via the production of reactive oxygen species (ROS) during the initial minutes of reperfusion [4]. This process is mediated by the production of superoxide [5], potential sources of which include the mitochondrial electron transport chain [6] and NADPH oxidase [4]. Superoxide in turn reacts with nitric oxide to form peroxynitrite, a highly reactive oxidant [5]. Hence, reducing superoxide production would attenuate the endothelial dysfunction caused by ischemia–reperfusion injury. Noninvasive assessment of endothelial function using repeated flow-mediated dilation (FMD) protocols, combined with an induced ischemia–reperfusion injury has been used to assess interventions to attenuate this dysfunction [7–10].

Antioxidants' preventive role in ischemia–reperfusion injury has been suggested due to their ability to scavenge ROS [3,5,11]. Acute intra-arterial infusion of vitamin C decreases endothelial dysfunction in healthy individuals and peripheral artery disease patients [11] and attenuates the reduction in FMD following ischemia–reperfusion injury induced by exercise in patients with intermittent claudication [10]. However, vitamin C's interaction with superoxide can likely only occur at supraphysiological concentrations [12,13] and in contrast, oral supplementation at reduced dosages has failed to attenuate endothelial

Journal of Hypertension 2014, 32:339–351

[a]Department of Biological Sciences, University of Essex, Colchester, [b]Comparative Biomedical Sciences, Royal Veterinary College, London and [c]Centre for Sports Medicine and Human Performance, Brunel University, Uxbridge, UK

Correspondence to Mark Rakobowchuk, Centre for Sports Medicine and Human Performance, Brunel University, Kingston Lane, Uxbridge UB8 3PH, UK. E-mail: mark.rakobowchuk@brunel.ac.uk

Received 16 August 2013 **Revised** 30 September 2013 **Accepted** 30 September 2013

DOI:10.1097/HJH.0000000000000033

dysfunction [14]. However, this noneffect of oral supplementation was determined longitudinally and acute responses were not assessed. Moreover, administration of an antioxidant cocktail reduced several key indicators of ischemia–reperfusion injury, but not vascular constriction [3,5]. Critically, in these studies, the magnitude of ischemia (2.5 h) exceeds greatly than that during typical FMD measures, which may influence results [15]; furthermore, protocols tested an animal model as opposed to humans. Thus, the acute effect of oral antioxidant administration in humans has not been studied, and combining antioxidants could perhaps provide a higher level of protection from ischemia–reperfusion injury than vitamin C in isolation.

Recently, studies of prolonged low flow have shown that endothelial dysfunction may also manifest as enhanced vasoconstriction [16–18]. This low flow-mediated constriction (L-FMC) is nitric oxide independent and provides a measure of resting vasomotor tone [16], which can complement FMD as a measure of endothelial health [19]. The magnitude of L-FMC is suggested to be mediated in part by prostaglandin, endothelial hyperpolarizing factor [16], and endothelin-1 (ET-1) [20] availability. During occlusion, it is suggested these pathways may be altered due to increased retrograde flow and subsequent production of ROS by NADPH oxidase [18].

Limited research has studied interventions to manipulate L-FMC compared with FMD, which is surprising considering the reported clinical importance of this measure [19,21]. Attenuation of L-FMC was achieved via prostaglandin blockade [16]; however, measurements occurred in the radial not the brachial artery, which is typically used for assessments of endothelial function using FMD [15]. Thus, mechanistic information regarding L-FMC in the brachial artery would allow a greater understanding of the role of this parameter in endothelial responses to ischemia–reperfusion injury.

One difficult aspect regarding the interpretation of ischemia–reperfusion studies involves the consistent and inevitable alteration of the diameter of the artery studied. In particular, recent studies by van den Munckhof *et al.* [22] have shown that upper arm occlusion for periods of 20 min results in a prolonged dilation that alters baseline diameter. Since recent work by Atkinson *et al.* [23,24] has shown the importance of controlling for basal diameter using data from seminal studies, the work in this study was assessed using both data analysis techniques.

Therefore, the aims of this study were to assess interventions that impact FMD and L-FMC in the brachial artery throughout an ischemia–reperfusion injury protocol and also look at a data analysis comparison by using baseline diameter as a covariate as suggested by Atkinson *et al.* [23,24]. Specifically we aimed to determine the efficacy of acute administration of an oral antioxidant cocktail on the preservation of FMD following ischemia–reperfusion injury and the impact of prostaglandin inhibition on the magnitude of L-FMC. It was hypothesized that following ischemia–reperfusion injury, oral supplementation of an antioxidant cocktail would attenuate the reduction in FMD, and that prostaglandin blockade via ibuprofen administration would abolish the enhanced L-FMC that occurs. We also hypothesized that an alternative interpretation may arise from the inclusion of baseline diameters as covariates with regard to the alterations of vascular function induced by ischemia–reperfusion and the two intervention strategies.

METHODS

Participants

Twelve healthy participants (five women and seven men; mean ± SD, age: 26.2 ± 6.7 years, body mass: 66.5 ± 16.7 kg, height: 168.2 ± 12.7 m) volunteered for the study. Participants were screened prior to testing and exclusion criteria included smoking, pregnancy, current medication, and presence of apparent cardiovascular or metabolic disease. Women were assessed in a standardized phase (days 1–7) of the menstrual cycle according to recommended guidelines [25].

Study design and procedures

Participants attended the temperature-controlled (20–22°C) laboratory at the same time of day on three separate occasions (control, antioxidant, and ibuprofen trials), following an overnight fast and abstinence from caffeine. Participants were also instructed to avoid strenuous exercise for 24 h. The initial test acted as a control trial. Prior to subsequent visits, participants randomly ingested either an antioxidant cocktail [300 mg α-lipoic acid, 500 mg vitamin C, and 200 IU vitamin E (water dispersible)] 2 h prior and a further cocktail [300 mg α-lipoic acid, 500 mg vitamin C, and 400 IU vitamin E (water dispersible)] 1.5 h prior [26]; or 1200 mg of a prostaglandin inhibitor (ibuprofen) 1 h prior.

On each occasion participants arrived, were instrumented, and rested supine for 20 min. Endothelial function was subsequently assessed using noninvasive vascular function protocols for the assessment of FMD and L-FMC of the right brachial artery, of which four were performed during each trial. The first vascular function protocol (Pre) was followed by a 10-min recovery period. After this period, an ischemia–reperfusion injury was induced by a 20-min occlusion with a cuff positioned around the upper right arm, and pressure maintained at 220 mmHg. Following cuff deflation, a further three vascular function protocols were carried out at 15, 30, and 45 min of recovery. Blood pressure was continually monitored throughout testing at the left radial artery via arterial tonometry (model Colin CBM-7000; Colin Medical Instruments, San Antonio, Texas, USA) and heart rate measured via a three-lead electrocardiograph. Signals were acquired via a data acquisition system (PL3008 PowerLab 8/30; ADInstruments, Colorado Springs, Colorado, USA) and software (LabChart 7, ADInstruments).

Vascular endothelial function testing procedures

Noninvasive assessment of brachial artery vascular function was carried out using automated edge detection for diameter analysis in real time and fast Fourier transform

of raw audio data to determine mean blood velocity (MBV). Arterial blood flow and shear rates were assessed using a linear array 10 Mhz Doppler ultrasound probe in triplex mode (Prosound Alpha 6; Hitachi Aloka Medical, Tokyo, Japan). The FMD protocol was performed according to published guidelines [15,25] using M-mode imaging. M-mode imaging enables a theoretical spatial resolution of less than 0.001 mm and high temporal resolution (200 Hz). This high spatial and time resolution also enabled β-stiffness index to be determined prior to each vascular function measurement. The position of the probe was marked using indelible ink and maintained using a probe holder to ensure stable, consistent measurements and, at each testing session, previous baseline images were used to ensure the same portion of the vessel was assessed. A blood pressure cuff was placed around the forearm distal to the probe with the proximal border adjacent to the medial epicondyle, as recommended to assess endothelial-dependent nitric oxide dilation [15]. Arterial diameters were obtained for 30 s at baseline and throughout the 5-min occlusion period. Postcuff deflation, the vessel was imaged for a further 3.5 min. Throughout this period, blood flow was also acquired. This procedure was repeated for each vascular function measure (Pre, Post15, 30, and 45).

Data analysis traditional methods

Basal arterial diameter was determined as the average of 30 heart cycles prior to cuff inflation. Peak vessel dilation was calculated from the highest average diameter of three consecutive heart cycles after cuff deflation, whereas occlusion diameter was determined as the average of 30 heart cycles prior to cuff deflation. From this data, FMD and L-FMC were calculated as previously described [27]. Total vascular reactivity (TVR) was determined as the sum of the absolute L-FMC and absolute FMD.

Basal MBV was determined for 30 s prior to the 5-min forearm occlusion, whereas occlusive MBV determined for 30 s prior to cuff deflation. After cuff release, MBV was calculated throughout reactive hyperemia for 60 s and the peak blood velocity during this time was determined. Finally, shear rates were calculated ($8 \times$ MBV/arterial diameter at the measurement time) at these same time points.

Data analysis using covariate analyses incorporating basal diameters

Atkinson *et al.* [23] recently described an allometric-based approach to adjust relative FMD to take into account basal diameter of the brachial artery. With the possibility that basal diameter is altered within the current study design, this method involves including basal diameter as a covariate at each time point.

Statistical methods traditional approach

All data are presented as mean $\pm$ standard deviation (SD). Data were assessed for normal distribution using Kolmogorov–Smirnov test. Absolute and relative FMD, relative L-FMC, TVR, peak blood velocity and shear rate, time to peak dilation, and basal and occlusive shear rates, mean arterial pressure, heart rate, β-stiffness index, and MBV were analyzed by two way-repeated measures analysis of variance (ANOVA) with 'condition' and 'time' treated as within subject variables and *a priori* comparisons made between Pre and all other time points while the control condition was compared to ibuprofen or antioxidant trials. Data were analyzed using statistical software (SPSS Version 18.0; IBM Corporation, Somers, New York, USA), with significance accepted as $P \leq 0.05$.

Statistical methods analysis of covariance-based allometric approach

A separate analysis was complete using an allometric approach that controls for changes in basal diameter. To do this, basal, maximum, and occlusion diameters were log transformed and absolute dilation, absolute constriction, and total range of the log transformed data were calculated. The slope of the regression between basal diameter and maximal diameter was calculated as 0.98 and the 95% confidence interval was less than 1, suggesting that maximal diameter does not increase proportionally with a change in basal diameter. This agrees with previous work by Atkinson *et al.* [24] and suggests that controlling for basal diameter is an appropriate procedure.

To analyze our FMD, L-FMC, and TVR more appropriately, log-scaled values of 'dilation', 'constriction', and 'total range' were entered into a mixed model linear analysis of covariance (ANCOVA) with 'condition' and 'time' as repeated factors, 'basal diameter' as a covariate, and 'dilation', 'constriction', and 'total range' as dependent factors in separate analyses. Covariate-adjusted means were calculated and then back-transformed. To provide values that one would typically interpret as relative dilation, constriction and total range, adjusted changes in diameter values were calculated by subtracting 1 and then multiplying by 100 according to Atkinson *et al.* [24]. The 'corrected' dilation, constriction, and range were then interpreted and compared to the traditional approach.

The day-to-day repeatability (coefficient of variation) for FMD, L-FMC, and TVR is 15, 20, and 12%, respectively in this laboratory. The absolute day-to-day difference is 1.1, 1.4, and 1.7%, respectively. Power calculation based on previous work [27] suggested a sample size of 10 participants would be required to see a 1% change in FMD or L-FMC with a standard deviation of 2.8%, a probability of 0.05, and a β of 0.80. Thus, adequate statistical power was aided by using a sample of 12 in repeated methods within subject study design.

Ethics statement

The experimental procedures and potential risks were explained to participants prior to testing and written informed consent obtained. The University of Essex ethics committee approved the experimental protocol, which conformed to the Declaration of Helsinki.

RESULTS

Hemodynamic responses to antioxidant, ibuprofen, and ischemia

Heart rate and mean arterial pressures were not affected by either the administration of the antioxidant cocktail or

TABLE 1. Hemodynamic parameters under each condition and at each vascular function assessment

	Control				Ibuprofen				Antioxidant			
	Pre	Post15	Post30	Post45	Pre	Post15	Post30	Post45	Pre	Post15	Post30	Post45
HR (bpm)	59 ± 13[a]	56 ± 11	56 ± 10	55 ± 9	57 ± 13[a]	53 ± 8	55 ± 9	53 ± 9	59 ± 12[a]	55 ± 11	54 ± 10	56 ± 12
MAP (mmHg)	79 ± 10	85 ± 12	83 ± 10	83 ± 11	79 ± 9	85 ± 10	80 ± 8	80 ± 8	82 ± 10	81 ± 10	80 ± 11	83 ± 10
β-Stiffness index	11 ± 2	10 ± 2	10 ± 2	10 ± 3	11 ± 2	12 ± 2	11 ± 3	11 ± 2	11 ± 3	10 ± 4	11 ± 4	12 ± 4

HR, heart rate; MAP, mean arterial pressure.
[a]Denotes a significant main effect for time ($P < 0.05$) with Pre values significantly different from other time points within each condition.

ibuprofen ($P > 0.05$, Table 1). In addition, the ischemia–reperfusion procedure did not alter mean arterial pressures ($P > 0.05$, Table 1). There was a reduction in heart rate that was similar under all conditions of approximately 3–4 bpm over the course of the 2.5-h protocol ($P = 0.04$, Table 1). Indirect measures of vascular tone derived from brachial β-stiffness index (ultrasound imaging and pulse pressure measures) showed no effect of time ($P > 0.05$, Table 1) or condition ($P > 0.05$, Table 1), suggesting that the protocol and manipulations did not alter vascular tone.

Flow-mediated dilation and oral antioxidant or ibuprofen administration

Reduced FMD was evident in all three conditions following 20 min of forearm occlusion using the traditional statistical approach. At 15-min after ischemia–reperfusion injury, absolute FMD (Fig. 1a) and relative FMD (Fig. 1b) were reduced compared with baseline values with similar reductions under all conditions ($P > 0.05$). Antioxidant administration did not preserve FMD compared with control ($P > 0.05$). Moreover, the pattern of recovery was not affected by either intervention ($P > 0.05$), with the magnitude of the attenuation of FMD reducing at 30 and 45 min. Basal diameters were larger at 15 min after ischemia–reperfusion compared with the before ischemia–reperfusion, but returned to similar values at the 30 and 45 min time points (Fig. 2a, b, $P > 0.05$). However, no differences were evident between conditions at any time point of assessment (Fig. 2 a, b, $P > 0.05$). Occlusive arterial diameters during each vascular function test replicated this response (Fig. 2c). Finally, the time to peak vessel dilation was not

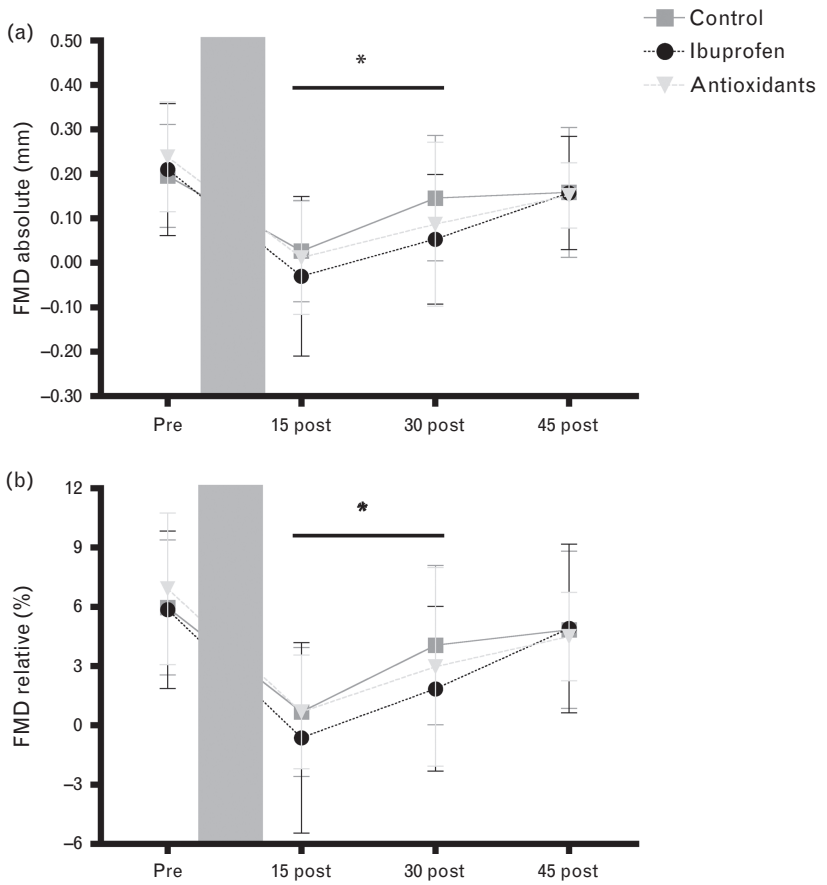

FIGURE 1 (a) Absolute flow-mediated dilation (FMD) and (b) relative FMD before (Pre) and following a 20-min ischemia–reperfusion injury (shaded area) for control, ibuprofen, and antioxidant conditions. *Indicates a significant difference ($P < 0.05$) from Pre (main effect for time) determined by post-hoc analysis.

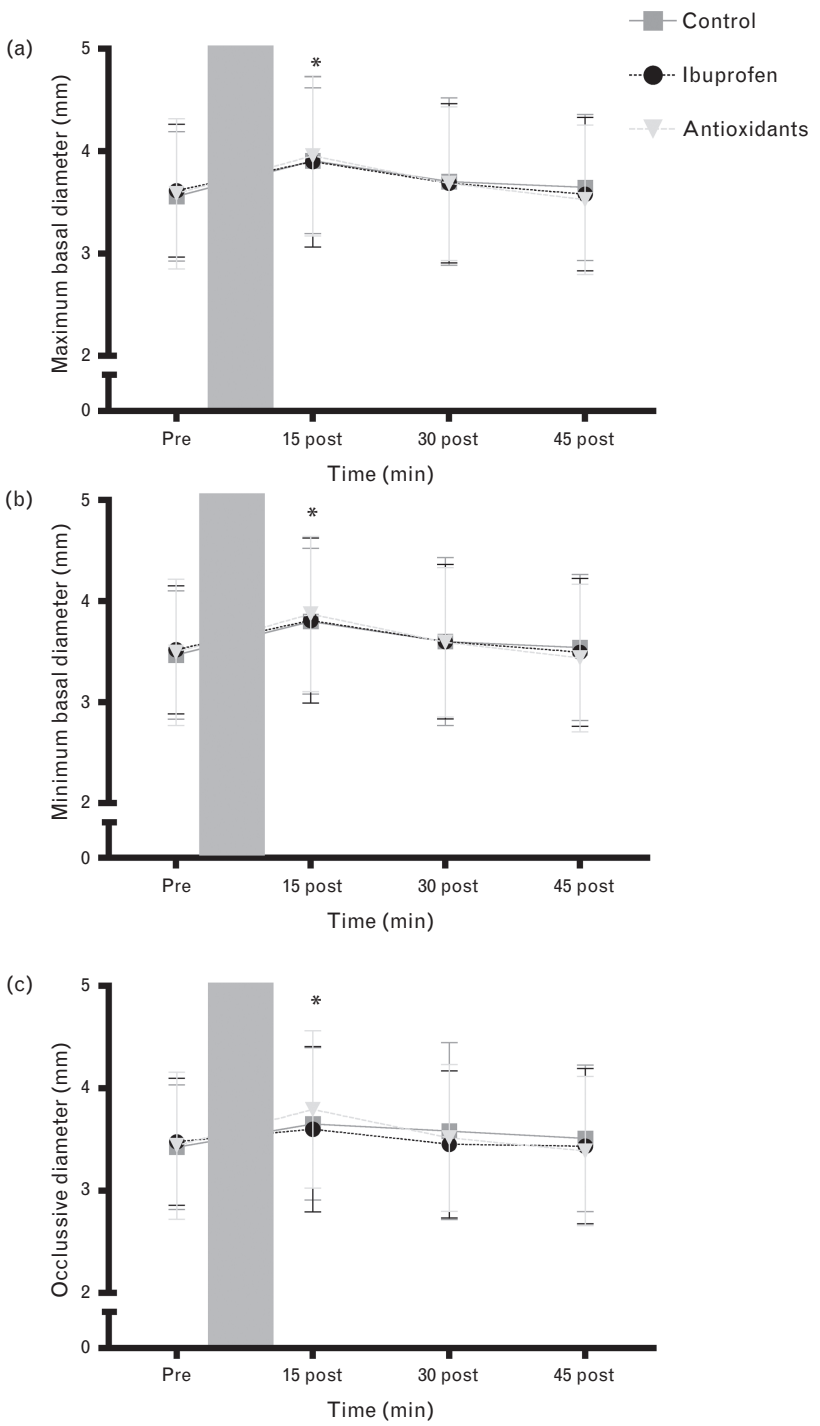

FIGURE 2 (a) Thirty second average maximum and (b) minimum brachial artery diameters in the basal state before each vascular function test, and (c) 30 s average minimum occlusive diameter during the final 30 s of each vascular function test, before (Pre) and after a 20-min ischemia–reperfusion injury (shaded area) for control, ibuprofen, and antioxidant conditions. *Indicates a significant difference ($P < 0.05$) from Pre (main effect for Time) determined by post-hoc analysis.

influenced by condition ($P > 0.05$) nor the time of assessment ($P > 0.05$).

Analyzing the FMD responses using basal diameter as a covariate did not alter the results. Endothelial dysfunction was still evident following the ischemia–reperfusion injury (main effect for time, $P < 0.01$, Fig. 3a) and followed a similar pattern compared with the traditional statistical approach.

Low flow-mediated constriction and antioxidant or ibuprofen administration

L-FMC demonstrated a similar time course of response to ischemia–reperfusion injury as noted with FMD for all conditions (Fig. 4). The magnitude of L-FMC was significantly greater 15 min after occlusion compared with basal values (Fig. 4, $P < 0.05$) and at 45 min, this had recovered to values similar to that at baseline ($P > 0.05$).

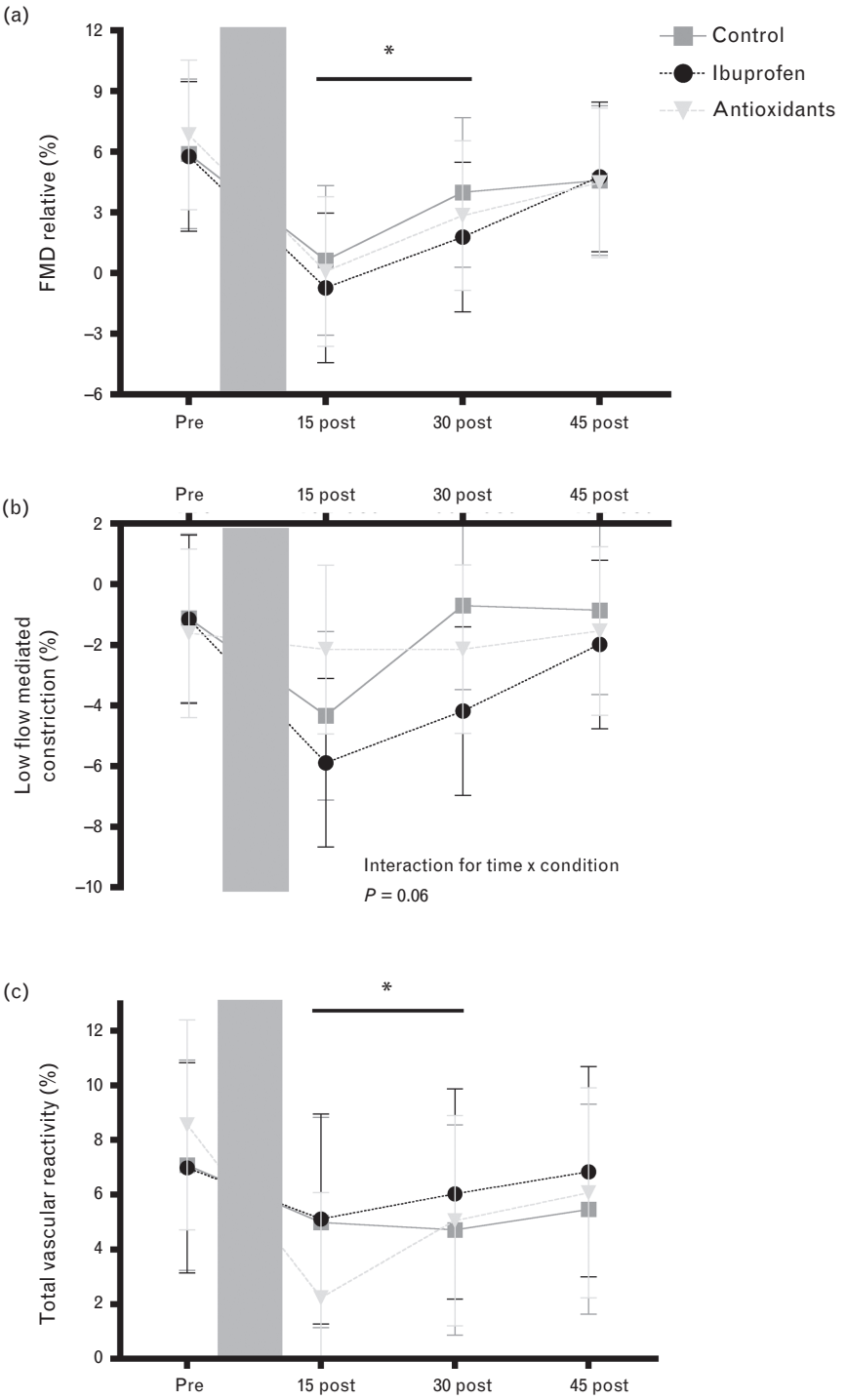

FIGURE 3 (a) Relative flow-medicated dilation (FMD), (b) low-flow mediated constriction (L-FMC), and (c) total vascular reactivity (TVR), following a 20-min ischemia–reperfusion injury (shaded area) for control, ibuprofen, and antioxidant conditions when basal diameter was used as a covariate in the analyses. *Indicates a significant difference ($P < 0.05$) from Pre (main effect for time) determined by post-hoc analysis. Only a trend ($P = 0.06$) was apparent regarding the time × condition interaction regarding L-FMC.

L-FMC did not differ significantly between conditions ($P > 0.05$); however, ibuprofen administration resulted in the largest constrictive response, with approximately four-fold increase between before and 15 min after ischemia–reperfusion injury (Fig. 4, $P < 0.05$), compared with a 1.3-fold and almost no change under control and antioxidant conditions, respectively from before to 15 min after ischemia–reperfusion injury. No significant interactions were apparent under control or antioxidant conditions ($P > 0.05$).

Using basal diameter as a covariate in the analysis of L-FMC did not alter the results. An increased L-FMC was

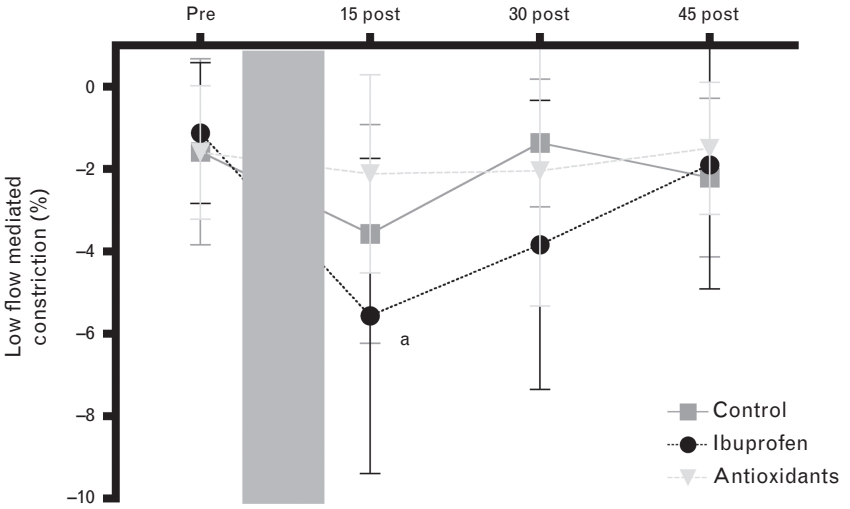

FIGURE 4 Low flow-mediated constriction (L-FMC) of the brachial artery diameter before (Pre) and following a 20-min ischemia–reperfusion injury (shaded area) for control, ibuprofen, and antioxidant conditions. *a* indicates a significant condition × time interaction with ibuprofen causing a greater constriction at 15 Post compared to Pre and 45 Post ($P < 0.05$ post-hoc analysis).

still evident with the greatest effect noted in the ibuprofen condition (Fig. 3b). However, the interaction only displayed a strong trend ($P = 0.06$).

Total vascular reactivity and antioxidant or ibuprofen administration

The total diameter range of the brachial artery during the vascular function assessment (TVR) was altered by the ischemia–reperfusion injury and exhibited a reduction (main effect for time, $P < 0.01$). Although this was most apparent in the antioxidant condition, there was no significant interaction ($P > 0.05$, Fig. 3c).

After controlling for basal diameter changes that occurred after ischemia–reperfusion compared with Pre values using the basal diameter as a covariate, there were no noticeable differences in the results compared with the traditional approach. The largest reduction in total range occurred at Post15 and this reduction was still evident at Post30 compared with Pre ($P < 0.01$, Fig. 3c). There was no specific effect of either antioxidant or ibuprofen administration.

Blood velocity and shear rates

Ischemia–reperfusion injury altered shear rates and MBVs. Following ischemia–reperfusion injury, there was a reduction in peak blood velocity at 15 min of recovery (Fig. 5a), although this did not reach statistical significance (main effect for time, $P = 0.08$). A return to Pre values was noted at the 30 and 45 min time points (Fig. 5a, $P > 0.05$). Peak shear rate following cuff deflation demonstrated this same pattern of response (Fig. 5b), with a mean reduction of 27.09 s^{-1} (main effect for time, $P < 0.05$); however, by 30 and 45 min of recovery, these values were similar to baseline ($P > 0.05$). There was no effect for condition on either of these parameters ($P > 0.05$). Although not statistically significant, there was a trend for a reduction in occlusive MBV at 15 min of recovery compared with baseline (Fig. 6a). Similarly, occlusive shear rate was depressed following ischemia–reperfusion injury (Fig. 6b); however, this did not reach statistical significance (main effect for time, $P = 0.07$). Condition significantly influenced these occlusive measures, with values significantly depressed in the ibuprofen condition compared with the control and antioxidant conditions (main effect for condition, $P < 0.05$). Finally, basal MBV and shear rates were significantly reduced at all time points following ischemia–reperfusion injury (Fig. 7a,b, $P < 0.05$), and this was not influenced by condition ($P > 0.05$).

DISCUSSION

In agreement with previous studies [15,25], we demonstrate that upper arm ischemia–reperfusion injury induces endothelial dysfunction, as evidenced by a significant reduction in FMD following a 20-min vascular occlusion. Moreover, the observed augmentation in L-FMC suggests further vascular dysfunction using this approach [16–19]. The interventions of oral antioxidant and ibuprofen did not decrease the magnitude of the ischemia–reperfusion injury as originally hypothesized. In fact, following ischemia–reperfusion injury, ibuprofen administration augmented L-FMC, whereas acute oral antioxidant supplementation failed to preserve FMD. Allometric scaling and using the basal diameter as a covariate in the analyses based on the work by Atkinson *et al.* [23,24] did not influence the results or alter any conclusions. However, this method should become standard practice to enable comprehensive comparisons between studies.

As such these data suggest some potentially unfavorable effects of acute ibuprofen administration and discount the possibility that oral administration of an antioxidant cocktail containing vitamins C, E, and α-lipoic acid is of benefit in attenuating the reduction in FMD induced by ischemia–reperfusion injury, although they may limit the increased L-FMC.

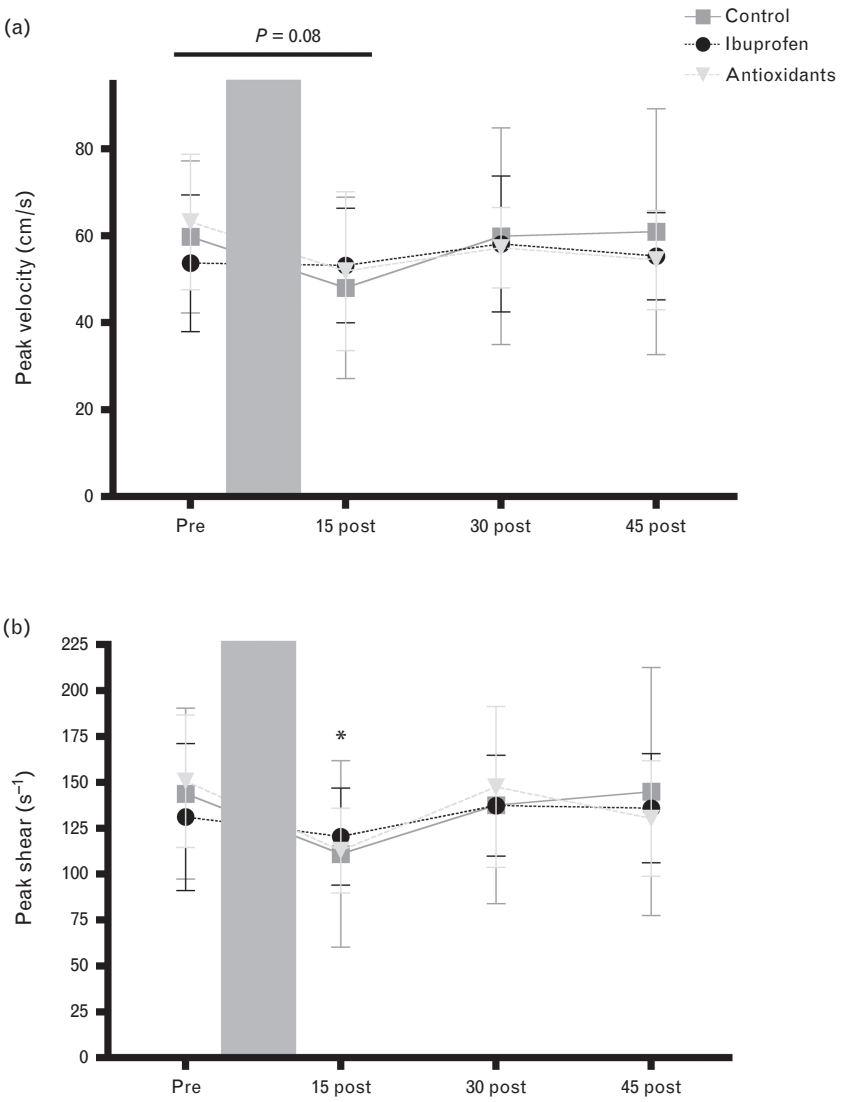

FIGURE 5 Peak blood velocity (a) and peak shear rates (b) following cuff deflation measured during the vascular function assessments at Pre and at 15, 30, and 45 min after a 20-min ischemia–reperfusion injury (shaded area). Separate lines indicate control, ibuprofen, and antioxidant conditions. *Indicates a significant difference ($P < 0.05$) from Pre (main effect for time) determined by post-hoc analysis.

Pattern of response following ischemia–reperfusion injury

The observed alterations in endothelial function following ischemia–reperfusion injury may relate to previously described mechanisms of reduced nitric oxide production or bioavailability [1] due to the production of ROS during the initial period of reperfusion [8]. Furthermore, following occlusion, both peak shear rate and MBV were reduced, possibly indicating microvascular endothelial dysfunction [18].

The pattern of response following ischemia–reperfusion injury was similar for both FMD and L-FMC. FMD was depressed and L-FMC augmented to the greatest extent at 15 min of recovery, with these parameters returning to Pre ischemia–reperfusion injury levels by 45 min. This time course agrees with previous work [18]; furthermore, the return to basal diameters confirms that using multiple vascular function tests is not an explanation for the enhanced L-FMC observed [18]. Additionally, the pattern of response was not influenced by condition, demonstrating that the interventions assessed do not modify recovery rates from ischemia–reperfusion injury.

Time to peak dilation has also been shown to have a similar response pattern following ischemia–reperfusion injury as that of FMD and L-FMC [18]; however, this was not evident in this study. One potential confounding factor that may explain this is the observation that after an ischemia–reperfusion injury, some individuals display no dilation, which creates an inappropriate time to peak dilation value. Furthermore, whether this measure has any significance for endothelial function is unclear, with similar times to peak dilation found in healthy and diseased populations [28].

Suppression of prostaglandins magnifies low flow-mediated constriction

Ibuprofen administration induced a greater vasoconstrictive response after ischemia–reperfusion injury compared with the control or antioxidant conditions. The magnitude

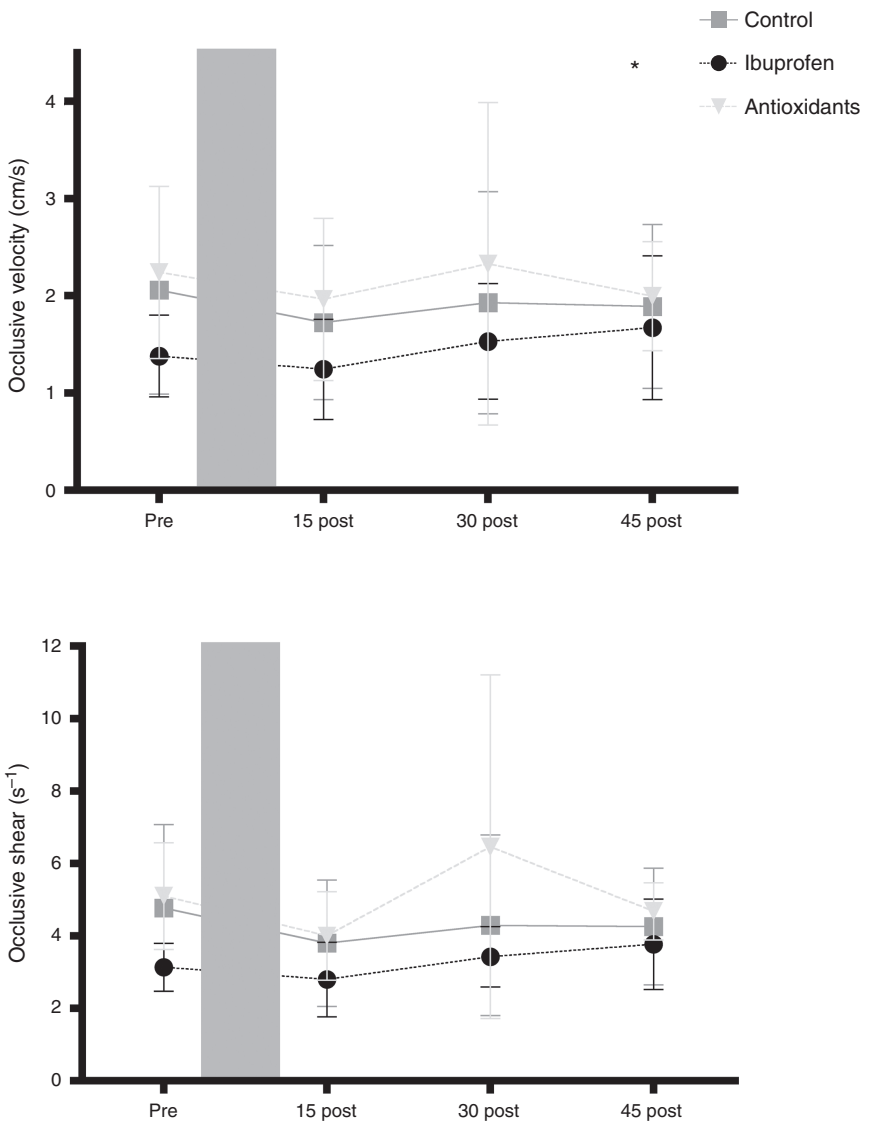

FIGURE 6 Occlusive mean blood velocity (a) and shear rates (b) during the last 30 s before cuff deflation measured during the vascular function assessments at Pre and at 15, 30, and 45 min after a 20-min ischemia–reperfusion injury (shaded area). Separate lines indicate control, ibuprofen, and antioxidant conditions. *Indicates a main effect for condition ($P < 0.05$) with the ibuprofen condition significantly different from the control and antioxidant conditions.

of L-FMC is mediated in part by cyclooxygenase (COX) activity, endothelial hyperpolarizing factor [16], and ET-1 [20] availability, suggesting that the intervention altered one of these pathways.

Ibuprofen is a nonselective COX inhibitor, which ultimately leads to a reduction in prostaglandin synthesis. Prostacyclin (PGI_2) and prostaglandin E_2 (PGE_2) are two such prostanoids synthesized by vascular endothelial and smooth muscle cells [29,30] and exert potent vasodilatory effects. Hence, the inhibition of COX by ibuprofen may have reduced these vasodilatory prostanoids and limited their effects, leading to enhanced vasoconstriction. However, Pre ischemia–reperfusion injury L-FMC in the ibuprofen condition was not significantly different from the control condition, thus making the reduced prostaglandin synthesis alone unlikely to be the sole cause of the increased L-FMC observed following ischemia–reperfusion injury. Furthermore, our results do not agree with Gori *et al.* [16] who reported prostaglandin blockade attenuated L-FMC; however, their assessment was made in the radial artery, which may exhibit differential sensitivity to this prostanoid. Our observations also suggest that the enhanced vasoconstriction that occurred after ischemia–reperfusion injury may be related to other pathways involved in the L-FMC response, namely ET-1.

As mentioned, what is of mechanistic interest is that ibuprofen administration increased the constrictive response to occlusion only after the ischemia–reperfusion injury. This suggests that factors resulting from prolonged ischemia combined with prostaglandin inhibition lead to the augmented L-FMC observed. The time course for this constrictive response agrees with the results of Rakobowchuk *et al.* [18] who suggest that the prolonged retrograde flow during occlusion stimulates the generation of ROS from NADPH oxidase, leading to heightened vasoconstriction. It could, therefore, be possible that the combination of enhanced retrograde flow with prostaglandin blockade magnified L-FMC. Increases in retrograde flow elevate

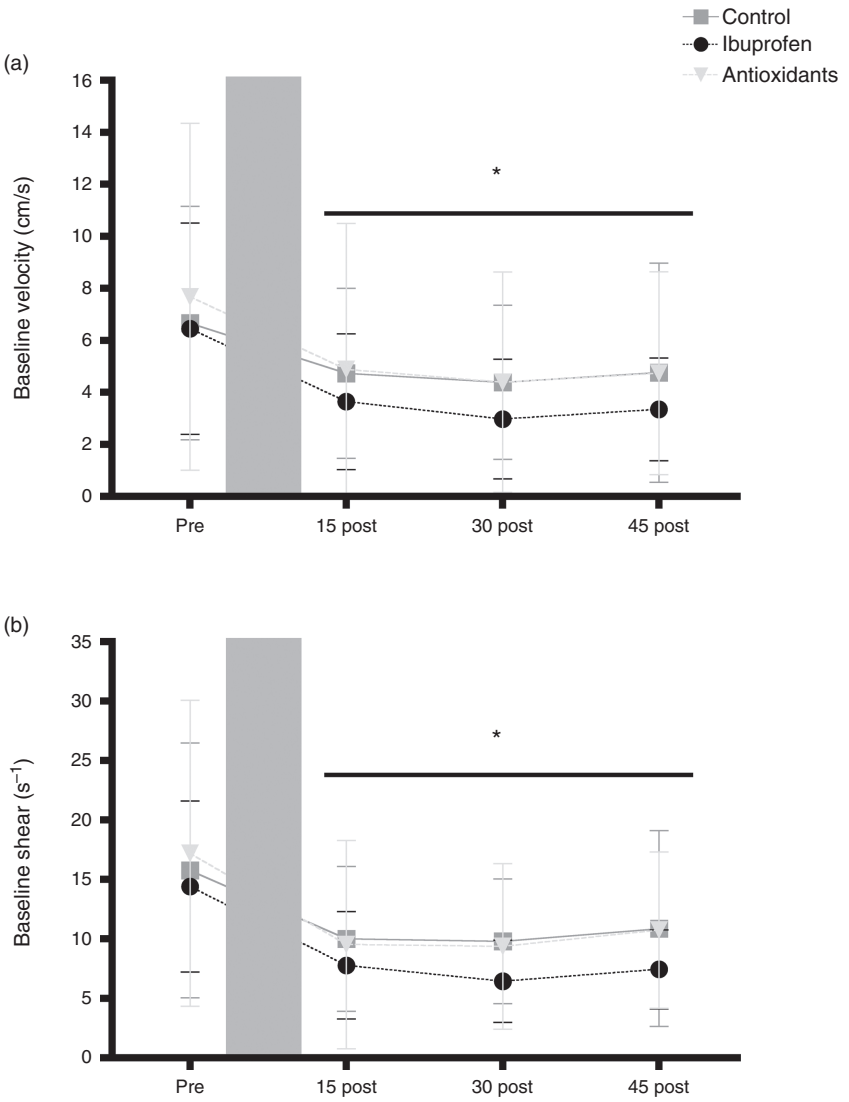

FIGURE 7 Basal mean blood velocity (a) and shear rates (b) at Pre and at 15, 30, and 45 min after a 20-min ischemia–reperfusion injury (shaded area). Separate lines indicate control, ibuprofen, and antioxidant conditions. *Indicates a significant difference ($P < 0.05$) from Pre (main effect for time) determined by post-hoc analysis.

expression of ET-1 [31], a potent vasoconstrictor [32]. Moreover, prostaglandins inhibit ET-1 secretion [33]; hence, this would be abolished with ibuprofen administration, leading to enhanced bioavailability of this vasoconstrictor and subsequent vasoconstriction mediated through the ET_A receptor. Alternatively, ET-1 can also induce vasodilation by acting through ET_B receptors, producing COX products [34,35]. This receptor-mediated relaxation was attenuated following COX inhibition in rat carotid arteries [35]; hence, a similar response may occur in the brachial artery. Collectively, the cumulative effects of these mechanisms could explain the increased L-FMC following ibuprofen administration.

Reductions in shear stress may explain low flow-mediated constriction responses

The increased L-FMC observed may have also been a function of the reduction in shear stress following ischemia–reperfusion injury. Basal shear rates (measured before each vascular function test) were reduced at all time points following ischemia–reperfusion injury, whereas occlusive shear during the vascular function protocol at 15 min into recovery was also depressed compared with Pre ischemia–reperfusion injury levels. Shear stress exhibits a dose-dependent relationship with ET-1 [36], with increased shear rates suppressing ET-1 expression [37,38]. Consequently, the reductions in shear stress observed may have augmented ET-1 expression, leading to enhanced vasoconstriction following ischemia–reperfusion injury. Furthermore, reductions in shear stress provide an additional mechanism to explain the potentiated L-FMC during the ibuprofen condition. Occlusive shear rates following ibuprofen administration were significantly depressed compared with the control or antioxidant conditions throughout the vascular function assessments and, as previously discussed, blocking COX and reducing prostaglandin synthesis removes their inhibitory role for ET-1 secretion, which in turn may lead to greater ET-1 bioavailability. A possible

explanation is that the absence of shear stress combined with prostaglandin suppression further augmented the expression of ET-1 and hence magnified L-FMC.

Antioxidants fail to preserve flow-mediated dilation

Acute oral administration of an antioxidant cocktail was unable to attenuate endothelial dysfunction as relative FMD was still impaired after ischemia–reperfusion injury to a similar magnitude as the control condition, whereas absolute FMD was marginally decreased. The lack of effect of supplementation may be explained by the reported requirement of supraphysiological dosages of vitamin C in order to scavenge superoxide [12,13]. A vitamin C concentration of 10–100 mmol/l has been theorized to effectively achieve this [13], and based on previous work using the same supplementation protocol [26], plasma concentrations would not have reached this concentration. Results, thus, demonstrate that, as has been previously found using chronic supplementation of vitamin C [14,39,40], oral administration is unable to attenuate endothelial dysfunction in an acute setting.

Although an antioxidant cocktail was administered as opposed to an isolated vitamin, this did not enhance the antioxidants' efficacy to preserve FMD. Longitudinal oral administration of vitamin E has attenuated endothelial dysfunction in some clinical populations [41–44], and combined with vitamin C may have enhanced the antioxidant effects of the intervention. However, as found in animal models [3,5], the magnitude of dysfunction was unaffected.

Study limitations

The study design featured multiple laboratory visits; moreover, the use of both forearm and upper arm cuff positions (for the assessment of FMD and to induce an ischemia–reperfusion injury, respectively) required repositioning of the probe within testing sessions. It is possible a different part of the brachial artery may have, therefore, been imaged at each test visit and during testing; however, previous baseline images were used to reduce this occurrence combined with indelible ink to ensure correct probe replacement. Furthermore, there were no significant differences between baseline artery diameters between conditions at any time points throughout the vascular function measures, indicating that the same vessel segment was assessed each time. As well any alterations in basal diameters were used as a covariate in the allometric scaling analyses and this did not alter the findings. Finally, brachial artery β-stiffness index and mean arterial pressure were similar at all vascular function measurements, further supporting the idea that the portion of the vessel that was assessed had similar structural properties and was under similar magnitudes of tension.

In conclusion, this study demonstrates that endothelial dysfunction following ischemia–reperfusion injury can present as both attenuated FMD and increased L-FMC. The use of an acute oral antioxidant supplementation fails to prevent the resultant reduction of FMD and confirms that the requirement of supraphysiological dosages, and the subsequent reaction rates with nitric oxide and superoxide, limit the effectiveness of this as a treatment option. Enhanced L-FMC following ibuprofen supplementation suggests that reduced levels of prostaglandins combined with prolonged retrograde flow and a lack of shear stress during ischemic occlusion lead to this heightened vasoconstriction. This may be due to increased expression of ET-1 as prostaglandin's habitual suppression of this vasoconstrictor is abolished. Conversely, antioxidants may impact the L-FMC response to ischemia–reperfusion injury and further studies that assess this are required. Further research is also necessary to determine the mechanisms involved in more detail to enable a more comprehensive understanding of endothelial responses to ischemia–reperfusion injury.

Clinical perspectives

Ischemia–reperfusion injury is commonplace in many clinical situations including acute coronary syndromes, exacerbation of peripheral vascular disease, and during organ transplantation procedures. As such, understanding the roles of commonly used over-the-counter products in the responses to these injuries is important in the proper assessment of treatment methods. Here we have shown that acute administration of an antioxidant cocktail containing vitamins C, E, and α-lipoic acid failed to reduce the severity of the endothelial dysfunction that occurs following an ischemia–reperfusion injury. In addition, our findings suggest detrimental effects of ibuprofen administration on endothelial function through potentiated vascular vasoconstriction. The potential negative impact of NSAIDs has been well reviewed [45]. It is evident that those with a high risk or a definitive diagnosis of ischemic cardiovascular disease should refrain from the use of NSAIDs due to their potential to increase the risk of an ischemic event [46]. The results of this study present a possible mechanism that may contribute to the detrimental effects of ibuprofen in the setting of ischemic disease.

ACKNOWLEDGEMENTS

This work was supported by a Society of Biology Undergraduate Research Bursary awarded to S.C.

Conflicts of interest

There are no conflicts of interest to declare.

REFERENCES

1. Moens AL, Goovaerts I, Claeys MJ, Vrints CJ. Flow-mediated vasodilation a diagnostic instrument, or an experimental tool? *Chest* 2005; 127:2254–2263.
2. Galaris D, Barbouti A, Korantzopoulos P. Oxidative stress in hepatic ischemia-reperfusion injury: the role of antioxidants and iron chelating compounds. *Curr Pharm Des* 2006; 12:2875–2890.
3. Nanobashvili J, Neumayer C, Fuegl A, Blumer R, Prager M, Sporn E, *et al*. Development of 'no-reflow' phenomenon in ischemia/reperfusion injury: failure of active vasomotility and not simply passive vasoconstriction. *Eur Surg Res* 2003; 35:417–424.
4. Loukogeorgakis SP, van den Berg MJ, Sofat R, Nitsch D, Charakida M, Haiyee B, *et al*. Role of NADPH oxidase in endothelial ischemia/reperfusion injury in humans. *Circulation* 2010; 121:2310–2316.

5. Nanobashvili J, Neumayer C, Fuegl A, Punz A, Blumer R, Mittlböck M, *et al.* Combined L-arginine and antioxidative vitamin treatment mollifies ischemia-reperfusion injury of skeletal muscle. *J Vasc Surg* 2004; 39:868–877.
6. Turrens JF. Mitochondrial formation of reactive oxygen species. *J Physiol* 2003; 552:335–344.
7. Gori T, Sicuro S, Dragoni S, Donati G, Forconi S, Parker JD. Sildenafil prevents endothelial dysfunction induced by ischemia and reperfusion via opening of adenosine triphosphate-sensitive potassium channels: a human in vivo study. *Circulation* 2005; 111:742–746.
8. Loukogeorgakis SP, Panagiotidou AT, Broadhead MW, Donald A, Deanfield JE, MacAllister RJ. Remote ischemic preconditioning provides early and late protection against endothelial ischemia-reperfusion injury in humans: role of the autonomic nervous system. *J Am Coll Cardiol* 2005; 46:450–456.
9. Loukogeorgakis SP, Panagiotidou AT, Yellon DM, Deanfield JE, MacAllister RJ. Postconditioning protects against endothelial ischemia-reperfusion injury in the human forearm. *Circulation* 2006; 113: 1015–1019.
10. Silvestro A, Scopacasa F, Oliva G, de Cristofaro T, Iuliano L, Brevetti G. Vitamin C prevents endothelial dysfunction induced by acute exercise in patients with intermittent claudication. *Atherosclerosis* 2002; 165:277–283.
11. Pleiner J, Schaller G, Mittermayer F, Marsik C, MacAllister RJ, Kapiotis S, *et al.* Intra-arterial vitamin C prevents endothelial dysfunction caused by ischemia-reperfusion. *Atherosclerosis* 2008; 197:383–391.
12. Sherman DL, Keaney JF, Biegelsen ES, Duffy SJ, Coffman JD, Vita JA. Pharmacological concentrations of ascorbic acid are required for the beneficial effect on endothelial vasomotor function in hypertension. *Hypertension* 2000; 35:936–941.
13. Jackson TS, Xu A, Vita JA, Keaney JF. Ascorbate prevents the interaction of superoxide and nitric oxide only at very high physiological concentrations. *Circ Res* 1998; 83:916–922.
14. Eskurza I, Monahan KD, Robinson JA, Seals DR. Effect of acute and chronic ascorbic acid on flow-mediated dilatation with sedentary and physically active human ageing. *J Physiol* 2004; 556:315–324.
15. Harris RA, Nishiyama SK, Wray DW, Richardson RS. Ultrasound assessment of flow-mediated dilation. *Hypertension* 2010; 55:1075–1085.
16. Gori T, Dragoni S, Lisi M, Di Stolfo G, Sonnati S, Fineschi M, *et al.* Conduit artery constriction mediated by low flow a novel noninvasive method for the assessment of vascular function. *J Am Coll Cardiol* 2008; 51:1953–1958.
17. Gori T, Parker JD, Münzel T. Flow-mediated constriction: further insight into a new measure of vascular function. *Eur Heart J* 2011; 32:784–787.
18. Rakobowchuk M, Parsloe ER, Gibbins SE, Harris E, Birch KM. Prolonged low flow reduces reactive hyperemia and augments low flow mediated constriction in the brachial artery independent of the menstrual cycle. *PloS One* 2013; 8:e55385.
19. Spiro JR, Digby JE, Ghimire G, Mason M, Mitchell AG, Ilsley C, *et al.* Brachial artery low-flow-mediated constriction is increased early after coronary intervention and reduces during recovery after acute coronary syndrome: characterization of a recently described index of vascular function. *Eur Heart J* 2011; 32:856–866.
20. Spieker LE, Lüscher TF, Noll G. ETA receptors mediate vasoconstriction of large conduit arteries during reduced flow in humans. *J Cardiovasc Pharmacol* 2003; 42:315–318.
21. Gori T, Muxel S, Damaske A, Radmacher M-C, Fasola F, Schaefer S, *et al.* Endothelial function assessment: flow-mediated dilation and constriction provide different and complementary information on the presence of coronary artery disease. *Eur Heart J* 2012; 33: 363–371.
22. van den Munckhof I, Riksen N, Seeger JPH, Schreuder TH, Borm GF, Eijsvogels TMH, *et al.* Aging attenuates the protective effect of ischemic preconditioning against endothelial ischemia-reperfusion injury in humans. *Am J Physiol Heart Circ Physiol* 2013; 304:H1727–H1732.
23. Atkinson G, Batterham AM, Thijssen DHJ, Green DJ. A new approach to improve the specificity of flow-mediated dilation for indicating endothelial function in cardiovascular research. *J Hypertens* 2013; 31:287–291.
24. Atkinson G, Batterham AM. Allometric scaling of diameter change in the original flow-mediated dilation protocol. *Atherosclerosis* 2013; 226:425–427.
25. Thijssen DHJ, Black MA, Pyke KE, Padilla J, Atkinson G, Harris RA, *et al.* Assessment of flow-mediated dilation in humans: a methodological and physiological guideline. *Am J Physiol Heart Circ Physiol* 2011; 300:H2–H12.
26. Richardson RS, Donato AJ, Uberoi A, Wray DW, Lawrenson L, Nishiyama S, *et al.* Exercise-induced brachial artery vasodilation: role of free radicals. *Am J Physiol Heart Circ Physiol* 2007; 292:H1516–H1522.
27. Rakobowchuk M, Harris E, Taylor A, Baliga V, Cubbon RM, Rossiter HB, *et al.* Heavy and moderate interval exercise training alters low-flow-mediated constriction but does not increase circulating progenitor cells in healthy humans. *Exp Physiol* 2012; 97:375–385.
28. Donald AE, Halcox JP, Charakida M, Storry C, Wallace SML, Cole TJ, *et al.* Methodological approaches to optimize reproducibility and power in clinical studies of flow-mediated dilation. *J Am Coll Cardiol* 2008; 51:1959–1964.
29. Qian H, Luo N, Chi Y. Aging-shifted prostaglandin profile in endothelium as a factor in cardiovascular disorders. *J Aging Res* 2012; 2012:121390.
30. Spector AA, Kaduce TL, Hoak JC, Czervionke RL. Arachidonic acid availability and prostacyclin production by cultured human endothelial cells. *Arteriosclerosis* 1983; 3:323–331.
31. Thijssen DHJ, Dawson EA, Tinken TM, Cable NT, Green DJ. Retrograde flow and shear rate acutely impair endothelial function in humans. *Hypertension* 2009; 53:986–992.
32. Böhm F, Pernow J. The importance of endothelin-1 for vascular dysfunction in cardiovascular disease. *Cardiovascular Res* 2007; 76:8–18.
33. Prins BA, Hu RM, Nazario B, Pedram A, Frank HJ, Weber MA, *et al.* Prostaglandin E2 and prostacyclin inhibit the production and secretion of endothelin from cultured endothelial cells. *J Biol Chem* 1994; 269:11938–11944.
34. Filep JG, Battistini B, Côté YP, Beaudoin AR, Sirois P. Endothelin-1 induces prostacyclin release from bovine aortic endothelial cells. *Biochem Biophys Res Commun* 1991; 177:171–176.
35. Tirapelli CR, Casolari DA, Yogi A, Montezano AC, Tostes RC, Legros E, *et al.* Functional characterization and expression of endothelin receptors in rat carotid artery: involvement of nitric oxide, a vasodilator prostanoid and the opening of K+ channels in ETB-induced relaxation. *Br J Pharmacol* 2005; 146:903–912.
36. Morawietz H, Talanow R, Szibor M, Rueckschloss U, Schubert A, Bartling B, *et al.* Regulation of the endothelin system by shear stress in human endothelial cells. *J Physiol* 2000; 525:761–770.
37. Ishibazawa A, Nagaoka T, Takahashi T, Yamamoto K, Kamiya A, Ando J, *et al.* Effects of shear stress on the gene expressions of endothelial nitric oxide synthase, endothelin-1, and thrombomodulin in human retinal microvascular endothelial cells. *Invest Ophthalmol Vis Sci* 2011; 52:8496–8504.
38. Kuchan MJ, Frangos JA. Shear stress regulates endothelin-1 release via protein kinase C and cGMP in cultured endothelial cells. *Am J Physiol* 1993; 264:H150–H156.
39. Darko D, Dornhorst A, Kelly FJ, Ritter JM, Chowienczyk PJ. Lack of effect of oral vitamin C on blood pressure, oxidative stress and endothelial function in type II diabetes. *Clin Sci (Lond)* 2002; 103:339–344.
40. Duffy SJ, Gokce N, Holbrook M, Hunter LM, Elizabeth S, Huang A, *et al.* Effect of ascorbic acid treatment on conduit vessel endothelial dysfunction in patients with hypertension. *Am J Physiol Heart Circ Physiol* 2001; 280:H528–H534.
41. Bauersachs J, Fleming I, Fraccarollo D, Busse R, Ertl G. Prevention of endothelial dysfunction in heart failure by vitamin E: attenuation of vascular superoxide anion formation and increase in soluble guanylyl cyclase expression. *Cardiovascular Res* 2001; 51:344–350.
42. Economides PA, Khaodhiar L, Caselli A, Caballero AE, Keenan H, Bursell SE, *et al.* The effect of vitamin E on endothelial function of micro- and macrocirculation and left ventricular function in type 1 and type 2 diabetic patients. *Diabetes* 2005; 54:204–211.
43. Neunteufl T, Priglinger U, Heher S, Zehetgruber M, Söregi G, Lehr S, *et al.* Effects of vitamin E on chronic and acute endothelial dysfunction in smokers. *J Am Coll Cardiol* 2000; 35:277–283.
44. Skyrme-Jones RA, O'Brien RC, Berry KL, Meredith IT. Vitamin E supplementation improves endothelial function in type I diabetes mellitus: a randomized, placebo-controlled study. *J Am Coll Cardiol* 2000; 36:94–102.

45. Antman EM, Bennett JS, Daugherty A, Furberg C, Roberts H, Taubert KA. Use of nonsteroidal antiinflammatory drugs: an update for clinicians – a scientific statement from the American Heart Association. *Circulation* 2007; 115:1634–1642.

46. Kearney PM, Baigent C, Godwin J, Halls H, Emberson JR, Patrono C. Do selective cyclo-oxygenase-2 inhibitors and traditional nonsteroidal anti-inflammatory drugs increase the risk of atherothrombosis? Meta-analysis of randomised trials. *BMJ* 2006; 332:1302–1308.

Reviewers' Summary Evaluations

Reviewer 1

Strength of the study: This study addresses an interesting subject as to the potential mechanisms involved in postischemic reperfusion, as reflected by a reduced flow-mediated brachial artery vasodilatation which was accentuated in the presence of prostaglandin inhibition. The interesting part is that supraphysiological doses of an antioxidant cocktail did not have an effect on ischemia-induced reduction in forearm blood flow. Although the antioxidant cocktail was given at supraphysiological doses, it is not known whether their concentrations were sufficient at the site of their target. Nonetheless, these results may lead to further exploration of the vascular effects of antioxidants.

Weakness of the study: One of the difficulties is to understand and follow the way the study was performed which makes it difficult to assess the results. One question is whether the number of study participants was large enough in particular for the antioxidant part of the study, since the standard deviations for this part of the results are relatively large and larger than for the other parts of the study.

Reviewer 2

This study investigates the effect of exogenous antioxidants and prostaglandin inhibitors on vascular reactivity using measurement of forearm diameter changes following ischemic reperfusion. Findings suggest a lack of effect of oral antioxidant ingestion on flow mediated dilation and an amplification of the low-flow mediated constriction due to prostaglandin suppression. Although the multiple diameter measurements at different times present a significant experimental limitation, functional stiffness measurements provide robust support for experimental findings. The low flow constrictor effects following ischemic reperfusion injury present enhanced methods of quantification of endothelial dysfunction.