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Inflammation-induced endothelial dysfunction involves reduced nitric oxide bioavailability and increased oxidant stress

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Abstract

Objectives: Our aim was to investigate mechanisms of inflammation-induced endothelial dysfunction in humans. **Methods:** Endothelial function in twenty-one healthy human volunteers was measured using forearm venous plethysmography before and 8 h after administration of typhoid vaccination to generate an inflammatory response. Basal and stimulated endothelial nitric oxide (NO) bioavailability was assessed by measurement of the responses to intra-arterial N^G-monomethyl-L-arginine (L-NMMA) and bradykinin, respectively. The effects of supplementation with L-arginine or ascorbic acid were assessed to probe the effects of substrate deficiency and oxidative stress, respectively. Systemic effects were determined by measuring cytokine response, total anti-oxidant status (TAOS) and urinary protein excretion. **Results:** Vaccination induced a cytokine response, a fall in total anti-oxidant status and increased urinary albumin excretion (UAE). There was a reduction in the response to bradykinin (BK, $P<0.005$) and L-NMMA ($P<0.0001$) with no effect on the response to glyceryl trinitrate (GTN) and norepinephrine (NE). Following vaccination blood flow response to BK (but not GTN) was partially returned to pre-vaccine levels by infusion of ascorbic acid ($P=0.01$). Supplementation with L-arginine had no effect. **Conclusion:** Inflammation causes widespread endothelial dysfunction, reduces vascular NO bioavailability and increases oxidative stress. These actions are partially reversible with local anti-oxidants. These findings suggest a role for reactive oxygen species in inflammation-induced endothelial dysfunction.
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Keywords: Endothelium; Nitric oxide; Inflammation; Coronary disease; Reactive oxygen species

1. Introduction

The healthy vascular endothelium has vasodilator, anti-adhesive, anti-inflammatory and anti-coagulant properties, through the production of mediators, including nitric oxide (NO). Endothelial dysfunction is an early event in the pathogenesis of atherosclerosis [1,2] and is characterised by reduced dilator function, increased inflammatory cell and platelet adhesion [3], and increased coagulation activity [4]. Reduced bioavailability of NO makes a major contribution to endothelial dysfunction and may be due to reduced NO synthesis (due to substrate or co-factor deficiency) or increased NO breakdown due to chemical reaction with oxidant radicals.

One potential trigger for endothelial dysfunction is inflammation [5–10]. Inflammatory cytokines impair endothelial function in animal models [11] and isolated human

Abbreviations: AUC, area under the curve; BH4, tetrahydrobiopterin; BK, bradykinin; GTN, glyceryl trinitrate; IL, interleukin; IL-1Ra, interleukin-1 receptor antagonist; L-NMMA, N^G-monomethyl-L-arginine; NE, norepinephrine; NO, nitric oxide; NOS, nitric oxide synthase; TAOS, total anti-oxidant status; UAC, urinary albumin/creatinine ratio; UAE, urinary albumin excretion.

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veins [12]. Previous work by our group has shown that acute systemic inflammation, induced by typhoid vaccination, leads to endothelial dysfunction, characterised by reduced response to the endothelium-dependent vasodilators bradykinin (BK) and acetylcholine in the forearm arterial bed [13]. The aims of the current study were to determine whether this experimental inflammatory stimulus also alters basal NO-mediated dilatation, to determine the mechanisms of the effects seen and to explore whether endothelial dysfunction is evident systemically.

2. Methods

2.1. Subjects

Twenty-one male and female subjects aged 22–40 were studied. All stated they were healthy and were not taking any medication. No subjects had received typhoid vaccination in the preceding 6 months. All studies were conducted in a quiet temperature-controlled laboratory (24–26 °C). Individuals were studied at the same time of day on two consecutive afternoons. The University College London Hospitals Research Ethics Committee approved the protocol and all subjects gave written informed consent. University College London Hospitals Ethics Committee conforms to the Declaration of Helsinki.

2.2. Generation of an inflammatory response

Salmonella typhi capsular polysaccharide vaccine 0.025 mg (Typhim Vi, Pasteur Merieux MSD) was injected into the gluteus muscle at 8 a.m. on the morning of the second day of the study as previously described [13].

2.3. Measurement of cytokines, serum total anti-oxidant status, serum albumin and lipids and urinary protein

Blood and urine samples were taken before and two hourly for 8 h after vaccination for the measurement of serum albumin and plasma lipids and urinary protein. As a control in eight subjects, urine samples were taken at 4 p.m. on a different day in the absence of vaccination.

Blood was collected in lithium heparin, citrate and EDTA, and plasma was obtained by centrifugation, aliquoted and stored at −20 °C. In five subjects, the plasma was used to measure interleukin-6 (IL-6) and interleukin-1 receptor antagonist (IL-1Ra) with a commercially available ELISA (R&D Systems, Abingdon, Oxon). Plasma from 21 subjects was analysed at baseline and 8 h following vaccination for concentrations of total cholesterol, HDL and albumin by reflectance spectroscopy (Vitros multichannel analyser 250,700,750). Plasma from 20 subjects (one was unsuitable) was assessed for total anti-oxidant status (TAOS) as described by Laight et al. [14] and modified by Sampson et al. [15]. Briefly, TAOS of plasma was determined by its capacity to inhibit the peroxidase-mediated formation of the 2,2-azino-bis-3-ethylbensthiazoline-6-sulfonic acid radical. This is expressed relative to the effect of phosphate buffered saline as percentage TAOS. Urine was collected in sterile containers and frozen at −20 °C immediately. Samples from 16 subjects at baseline and 8 h after vaccination were analysed for urinary albumin by immunonephlometry (Behring BNII analyser) and creatinine concentrations by enzymic assay and chemiluminescence (Vitros 700, Ortho Clinical Diagnostics).

2.4. Assessment of forearm blood flow

Mercury-in-silastic strain-gauge plethysmography was used to measure forearm blood flow (ml/100 ml forearm/min) in both arms as previously described [16,17]. Studies were performed 16 h before vaccination (control) and 8 h following vaccination. All studies were carried out with the subject supine in a quiet temperature controlled laboratory. The brachial artery of the non-dominant arm was cannulated with a 27-gauge needle (Cooper's Needle Works) inserted under local anaesthesia (2 ml of 1% lidocaine). Drugs or normal saline (sodium chloride 0.9% wt/vol) were infused continuously at 0.5 ml/min. Resting blood flow was allowed to normalise for 30 min following needle insertion prior to the infusion of vasoactive drugs. During periods of recording, the hands were excluded from the circulation by inflating wrist cuffs to 200 mm Hg. The needle was removed at the end of each study period. A time line for these protocols is shown in Fig. 1.

2.4.1. Protocol 1. Effect of vaccination on NO-mediated dilatation

In five individuals, forearm blood flow in response to intra-arterial infusion of the vasodilators BK (20, 40 and 80 pmol/min; each dose for 3 min) and glyceryl trinitrate (GTN; 8, 16 and 32 nmol/min; each dose for 3 min) and vasoconstrictors N^G-monomethyl-L-arginine (L-NMMA; 1, 2 and 4 μmol/min; each dose for 5 min) and norepinephrine (NE; 60, 120 and 240 pmol/min min; each dose for 5 min) were assessed before and after vaccination. The order of the infusions was randomised, although due to its long duration of action, L-NMMA was always infused last. Saline was infused for 15 min between drug infusions to allow restoration of baseline flow. In three subjects, time control studies were performed to determine variation in vasoconstrictor response over time. In these studies, forearm responses to L-NMMA and norepinephrine were determined as above, 24 h apart without typhoid vaccination.

2.4.2. Protocol 2. Effect of L-arginine on endothelial function after vaccination

In five individuals, forearm blood flow in response to BK (20, 40 and 80 pmol/min; each dose for 3 min) and GTN (8, 16 and 32 nmol/min; each dose for 3 min) was assessed

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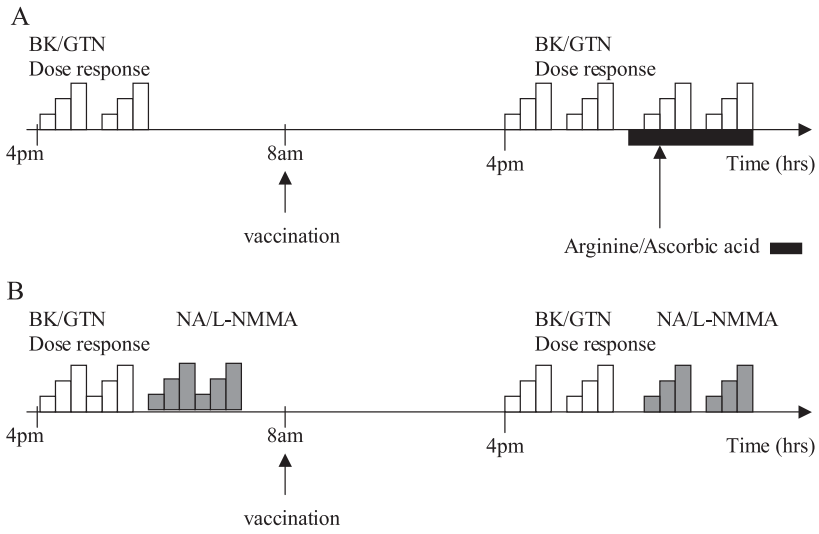

Fig. 1. Protocol time lines for the experiments, showing the timing of each study and vaccination. Dose response infusions are shown as open squares and co-infusion of ascorbic acid and arginine as closed blocks. Figure A represents vasodilator studies and figure B represents vasoconstrictors.

before and during co-infusion with L-arginine (50 and 100 μmol/ml; preinfused for 15 min). This protocol was repeated before and after vaccination as above.

2.4.3. Protocol 3. Effect of ascorbic acid on endothelial function after vaccination

In eight individuals, forearm blood flow in response to BK (20, 40 and 80 pmol/min; each dose for 3 min) and GTN (8, 16 and 32 nmol/min; each dose for 3 min) was assessed post-vaccination before and during co-infusion with ascorbic acid (25 mg/min; preinfused for 15 min).

2.5. Calculations and statistical analysis

The ratio of blood flow in the infused/non-infused (control) arm was calculated for each measurement period. Changes in flow were expressed as a percentage change in the ratio of forearm blood flow (infused/non-infused) relative to the immediately preceding baseline flow, as described previously [16]. Results are expressed as mean±S.E.M. unless otherwise stated. Cumulative dose–response curves were constructed for all drugs and responses were compared by two-way ANOVA. The Wilcoxon sign ranked test was

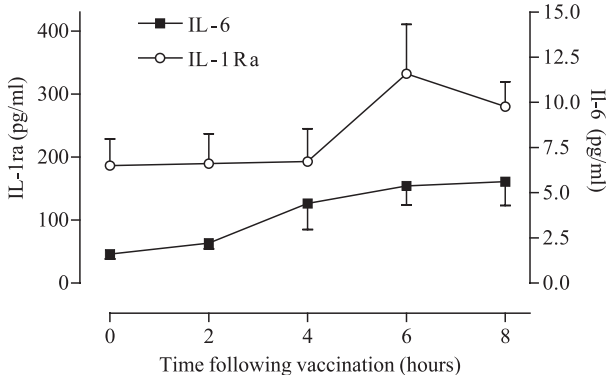

Fig. 2. IL-1Ra (○, left *y*-axis) and IL-6 (■, right *y*-axis) response in 8 h following vaccination. There was a significant increase in the AUC for both cytokines (P=0.02).

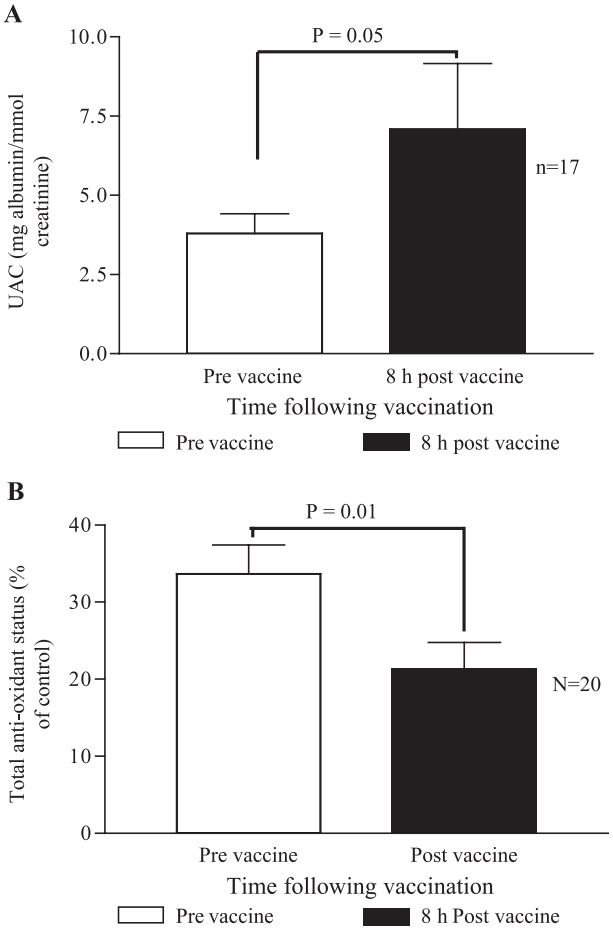

Fig. 3. (A) Urinary albumin/creatinine ratio prior to and 8 h following vaccination. (B) Total anti-oxidant capacity of plasma prior to and 8 h following vaccination expressed as a percentage of the saline control.

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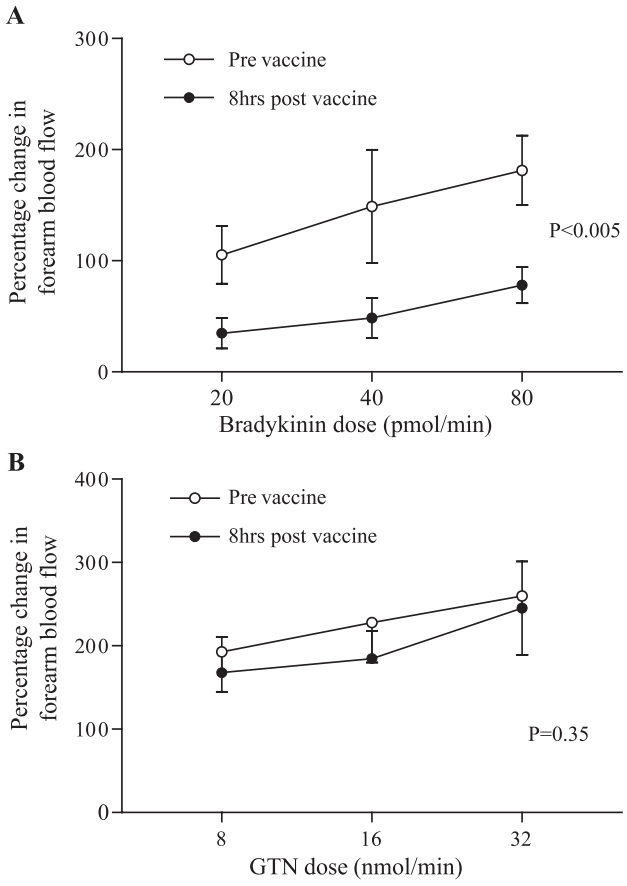

Fig. 4. (A) Change in forearm blood flow in response to bradykinin before (○) and 8 h following vaccination (●) (n=5, P<0.005). (B) Change in forearm blood flow in response to GTN before (○) and 8 h following vaccination (●) (n=5, P=NS).

used for non-parametric data. The time course of the cytokine response was expressed as the area under the curve (AUC) and analysed by a one-sample t-test. Analysis of the effect of L-arginine and ascorbic acid was carried out by assessment of each dose response curve with two-way ANOVA followed by the Bonferroni correction for multiple comparisons. P<0.05 was considered statistically significant.

2.6. Drugs

BK, NE and L-NMMA were obtained from Clinalfa AG; GTN from Faulding Pharmaceuticals and L-arginine and ascorbic acid from Medeva Pharma. All of the drugs were prepared as stock solutions and stored at −20 °C until use.

3. Results

3.1. Systemic inflammatory and haemodynamic response to vaccination

As we have described previously [13], in the 8 h following vaccination, there was a progressive rise in the IL-6 level, starting at 2–4 h, from 1.6±0.27 pg/ml at baseline to 5.6±1.3 pg/ml at 8 h (n=5, P=0.02). The IL-1Ra levels rose after 6 h from 186±42 pg/ml at baseline to a peak of 332±78 pg/ml before starting to fall 8 h following vaccination (n=5, P=0.02; Fig. 2).

In comparison to baseline there was an increase in urinary albumin (P=0.03) and urinary albumin/creatinine ratios (UAC; P=0.05; Fig. 3A) at 8 h following vaccination. In the absence of vaccination, there was no diurnal variation in the UAC (8 a.m. 0.42±0.07, 4 p.m. 0.45±0.09 mg albumin/mmol creatinine; P=NS). There were no significant changes in the serum levels of total or HDL cholesterol over this time course (pre-vaccine 4.56±0.15 mmol/l, post-vaccine 4.65±0.17 mmol/l; P=0.07 and pre-vaccine 1.26±0.08 mmol/l, post-vaccine 1.28±0.09 mmol/l; P=0.62, respectively). Serum albumin increased following vaccination (42.5±0.5 g/l pre-vaccine, 44.5±0.5 g/l post-vaccine; P<0.01). TAOS decreased significantly 8 h following vaccination (33.7±3.7% at baseline to 21.3±3.4% at 8 h; P=0.01; Fig. 3B) indicating an increase in oxidant stress at this time.

3.2. Forearm blood flow responses

Mean baseline blood flow did not change following vaccination (4.1±0.36 ml/100 ml forearm/min before and 3.9±0.24 ml/100 ml forearm/min 8 h after). All subjects showed dose-dependent increases in blood flow in response

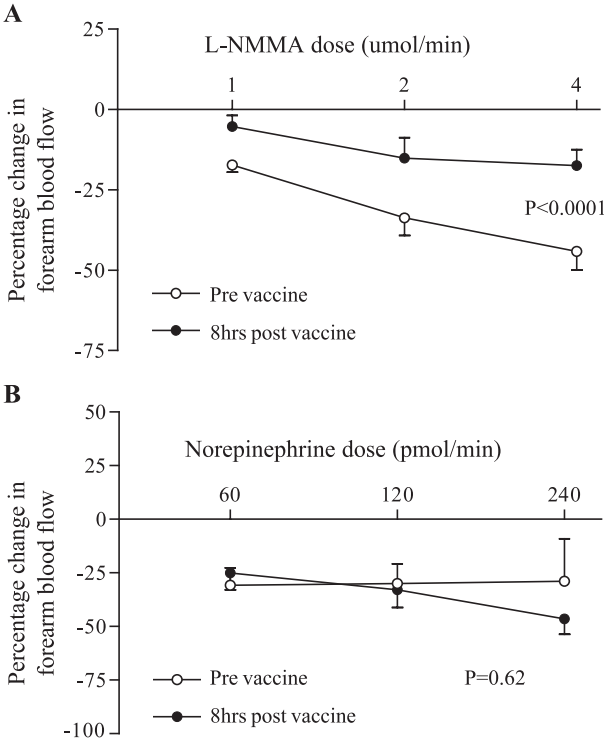

Fig. 5. (A) Change in forearm blood flow in response to L-NMMA before (○) and 8 h following vaccination (●) (n=5, P<0.0001). (B) Change in forearm blood flow in response to norepinephrine before (○) and 8 h following vaccination (●) (n=5, P=NS).

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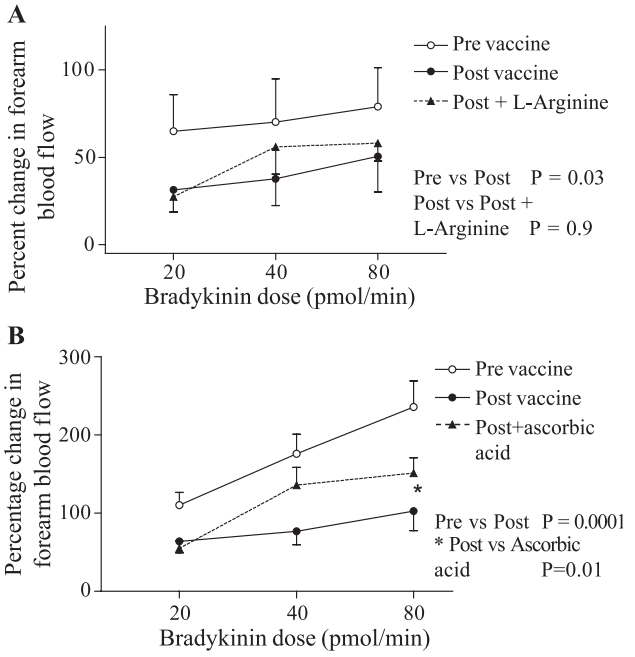

Fig. 6. (A) Changes in forearm blood flow in response to bradykinin, expressed as dose response, before vaccination and 8 h after vaccination with and without the addition of L-arginine (n=5, P=NS). Data from prevaccination with arginine not shown. (B) Changes in forearm blood flow in response to bradykinin, expressed as dose response, before vaccination and 8 h after vaccination with and without the addition of ascorbic acid (n=8, P=0.0001 for pre-vaccine vs. post-vaccine, P=0.01 for post-vaccine with and without ascorbic acid).

to BK and GTN. Eight hours following vaccination the response to BK was significantly impaired (P<0.005; Fig. 4A), while the response to GTN was unchanged (P=0.35; Fig. 4B).

Prior to vaccination, L-NMMA and NE caused dose-dependent reductions in forearm blood flow. Eight hours following vaccination, there was a reduction in the vasoconstriction to L-NMMA (P<0.0001; Fig. 5A) with no change in the response to NE (P=0.62; Fig. 5B). In control studies, conducted in the absence of vaccination, there were no time-dependent changes in the response to L-NMMA or NE on consecutive days (L-NMMA: P=0.6, NE: P=0.2).

Co-infusion of L-arginine (50 μmol/min) had no effect on the response to bradykinin (Fig. 6A) or GTN before or after vaccination. Infusion of ascorbic acid (25 mg/min) after vaccination caused a partial restoration of the response to BK (P=0.01; Fig. 6B). Neither vaccination nor ascorbic acid had any effect upon the response to GTN.

4. Discussion

In this study, we have confirmed that inflammation caused by typhoid vaccination causes a pro-inflammatory cytokine response and impairs forearm arterial endothelium-dependent dilatation. In addition, we now show that vascular responses to L-NMMA are diminished, indicating reduced NO bioavailability. In addition, urinary albumin excretion (UAE) increases, which may be secondary to increased capillary permeability caused by endothelial dysfunction in the kidney. Despite the reduced NO bioactivity, supplementation with L-arginine 50 μmol/min did not restore endothelial function whereas infusion of the anti-oxidant ascorbic acid (25 mg/min) led to a partial restoration of endothelium-dependent vasodilator responses and plasma TAOS was decreased indicating an increase in oxidant stress. These findings implicate production of reactive oxygen species in the genesis of inflammation induced-endothelial dysfunction in humans.

In animals and humans, bradykinin vasodilates in part by an NO-dependent mechanism, with a probable contribution from endothelium-derived hyperpolarizing factor [18,19], although not all studies in humans have implicated NO in the dilator effect of BK [19]. Previous work by our group has shown impaired bradykinin-induced vasodilatation following typhoid vaccination, with preservation of the response to the NO donor, GTN [13,20]. Administration of a high dose of aspirin before vaccination prevents inflammation-induced endothelial dysfunction, probably by blocking part of the cytokine response to vaccination [20]. In order to address specifically the role of the L-arginine/NO pathway in vaccine-induced endothelial dysfunction and to determine whether the protective basal NO mediated effects are lost, we measured the vasoconstrictor response to L-NMMA. The response to L-NMMA was markedly reduced following vaccination, with preservation of the response to the endothelium-independent vasoconstrictor norepinephrine. These data indicate that inflammation causes reduced activity of the basal endothelial dilator NO pathway. This in important because in animal models reduced NO bioavailability enhances atherogenesis and is associated with increased vascular resistance even if the blood pressure does not rise [21,22].

Changes in NO bioavailability can be caused by a reduction in NO production or an increase in NO breakdown. Inhibition of NO synthesis with L-NMMA does not distinguish between these mechanisms. A reduction in smooth muscle sensitivity to NO is excluded given the normal vasodilatation that we have observed to GTN not only in this study, but also in prior investigations [13,20]. Other potential mechanisms to account for reduced endogenous NO availability include depletion of the nitric oxide synthase (NOS) substrate L-arginine or increased generation of reactive oxygen species, both of which have been reported in the context of endothelial dysfunction in the presence of orthodox cardiovascular risk factors.

Failure of L-arginine to restore the response to BK in the current study suggests that a deficiency of the substrate for NO synthase does not account for the effect of inflammation on the NO pathway (although because of the small numbers studied it is possible we missed a small effect). The dose of L-arginine administered is sufficient to raise L-arginine concentration into the low millimolar range [23], and

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previous work has shown that this dose of L-arginine improves endothelial dysfunction in the resistance vasculature of patients with hypercholesterolemia [24], diabetes [25] and heart failure [26]. Failure to respond to L-arginine supplementation suggests that the mechanism of endothelial dysfunction is not simply explained by acute L-arginine deficiency. It also suggests that endogenous inhibitors of NO synthase (e.g. asymmetrical dimethyl-L-arginine) that compete with L-arginine for NO synthase are unlikely to be responsible for reduced endothelium-dependent dilatation.

Ascorbic acid infused 8 h following the inflammatory stimulus of vaccination partially restored the bradykinin response with no effect on the endothelium-independent vasodilator GTN. Ascorbic acid may exert antioxidant effects by direct quenching of reactive oxygen species [27] or by stabilising the NOS cofactor tetrahydrobiopterin (BH_4) [28]. A significant fall in total anti-oxidant status of the plasma following vaccination indicates an increase in free radical production with inflammation. That ascorbic acid partially reverses endothelial dysfunction supports a role for this increased oxidant stress in inflammation-induced endothelial dysfunction; similarly ascorbic acid reversed endothelial dysfunction following administration of bacterial lipopolysaccharide [25]. Taken together with the observation that aspirin prevents endothelial dysfunction in this model [20], it is possible that cyclooxygenase is a source of reactive oxygen species. Whether ascorbic acid acts to improve endothelial function by preventing oxidation of NO or promoting the activity of the NOS cofactor BH_4 to increase NO generation [29], remains to be determined. Failure to entirely restore the BK response may also reflect additional effects of inflammation to reduce the activity of the NO pathway, including expressional changes in NOS. It is also possible that ascorbic acid promotes the bioactivity of other endothelial mediators (e.g. EDHF) implicated in the response to BK. We have previously shown that local infusion of aspirin has no effect on the BK response after vaccination, suggesting that the response to BK is not prostanoid-dependent in this model [20]. If ascorbic acid promoted NO bioactivity specifically then we would predict that the response to L-NMMA would be restored by co-infusion of ascorbic acid.

Vaccination also caused increased UAE that was contemporaneous with changes in endothelial function and the rise in cytokines. Because of the association between UAE and markers of systemic endothelial dysfunction in patients with diabetes mellitus [30], hypertension [31] and certain inflammatory states [32,33], it is possible that UAE reflects increased capillary permeability secondary to endothelial dysfunction [34]. However, whether the association is causal remains to be determined. In addition, in healthy volunteers, the relationship between albuminuria and endothelial function [35], has recently been questioned [36]. Therefore, whilst it is possible that increased microalbuminuria in the present study might be explained by renal endothelial dysfunction, firm conclusions cannot be drawn at present. Nevertheless, this combination of endothelial dysfunction, microalbuminuria, increased oxidant stress and a cytokine response suggests that typhoid vaccination causes the temporary development of a high cardiovascular risk phenotype. If this model accurately reflects the pathology of systemic inflammation then this may indicate a mechanism by which inflammatory events may lead to a transient rise in the incidence of cardiovascular events.

In summary, our study demonstrates that typhoid vaccination causes generalised endothelial dysfunction involving reduced NO bioavailability. This is not explained by substrate depletion but is partly dependent on increased oxidative stress. Naturally occurring infections and inflammatory stimuli may cause fluctuating endothelial dysfunction by similar means.

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