Redox modulation of the fetal cardiovascular defence to hypoxaemia

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Episodes of hypoxia *in utero* present a potentially serious challenge to the fetus, but are counteracted by defence responses including marked redistribution of blood flow from peripheral circulations to the brain. Here, we report the novel observation that the oxidant tone is an important modulator of this cardiovascular defence. Using pregnant Welsh Mountain sheep surgically prepared for long-term recording, we investigated *in vivo* the effects on the fetal cardiovascular defence to acute hypoxaemia of fetal treatment with the antioxidant vitamin C. The mechanisms via which vitamin C may affect the vascular oxidant tone were investigated by monitoring fetal plasma concentrations of nitrates and nitrites, by determining changes in the activity of superoxide dismutase (SOD) in fetal plasma, and by investigating the effect of vitamin C treatment on the fetal cardiovascular defence to hypoxaemia following nitric oxide (NO) synthase blockade. Fetal treatment with vitamin C markedly depressed the normal femoral constrictor response to acute hypoxaemia in the fetus (5.2 ± 1.0 *vs.* 1.1 ± 0.3 mmHg (ml min^{-1})$^{-1}$, mean ± S.E.M.; $P < 0.05$) an effect which was completely restored following NO synthase blockade (6.2 ± 1.3 mmHg (ml min^{-1})$^{-1}$). Compared to saline infusion, fetal treatment with vitamin C during acute hypoxaemia also significantly increased fetal plasma SOD activity from normoxic baseline (-8.9 ± 6.5 *vs.* 15.0 ± 6.6% inhibition, $P < 0.05$) and decreased the plasma concentration ratio of nitrate:nitrite from normoxic baseline (ΔNO_3^-:NO_2^-; 0.15 ± 0.30 *vs.* -0.29 ± 0.11, $P < 0.05$). The data provide *in vivo* evidence of redox modulation of redistribution of blood flow in the fetus, part of the fetal brain sparing during acute hypoxaemic stress.

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Abbreviations L-NAME, N^G-nitro-L-arginine methyl ester; NO_3^-, nitrate; NO_2^-, nitrite; NO_x, total nitrate and nitrite; NPY, neuropeptide Y; $^{\bullet}O_2^-$, superoxide anion; pH_a, arterial pH; P_{aCO_2}, arterial partial pressure of CO_2; P_{aO_2}, arterial partial pressure of O_2; ROS, reactive oxygen species; Sat Hb, percentage saturation of haemoglobin with oxygen; SNP, sodium nitroprusside; SOD, superoxide dismutase.

Introduction

Hypoxaemia is one of the most common challenges that the fetus experiences during gestation and, in particular, during the processes of labour and delivery (Beard & Rivers, 1979). An inadequate fetal defence to hypoxaemia renders the fetal brain susceptible to injury leading to hypoxic-ischaemic encephalopathy (Low *et al.* 1985). The latter is predictive of developing cerebral palsy and cognitive disability later in life (Hall, 1989). Therefore, the physiology underlying the fetal defence to hypoxaemia remains at the forefront of basic science and clinical interest.

The fetal defence to hypoxaemia is contingent on its cardiovascular response, which involves a transient bradycardia and peripheral vasoconstriction (Rudolph, 1984; Giussani *et al.* 1994). The increase in peripheral vascular resistance aids the redistribution of the fetal cardiac output, prioritising blood flow away from peripheral and towards essential vascular beds, such as the cerebral circulations. This innate brain-sparing response has been conserved across all species studied to date, including the sheep, non-human primate and human fetus (Cohn *et al.* 1974; Jackson *et al.* 1987; Akalin-Sel & Campbell, 1992). The mechanisms mediating the fetal peripheral vasoconstrictor response to hypoxaemia are well characterised. It is triggered by a carotid chemoreflex (Bartelds *et al.* 1993; Giussani *et al.* 1993) and maintained by the release of vasoconstrictor agents into the

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fetal circulation, such as catecholamines, neuropeptide Y (NPY) and vasopressin (Jones & Robinson, 1975; Fletcher *et al.* 2000; Giussani *et al.* 2001). There is also a local component due to the direct effects of hypoxia at the tissue level. In the fetus, as in the adult, the endothelium may act as a hypoxic sensor and effector system that releases vasoactive agents to act locally on the vascular smooth muscle, such as NO and endothelin (Vane *et al.* 1990; Green *et al.* 1998). The net fetal peripheral vasomotor response to hypoxaemia thus represents the balance between neural, endocrine and local paracrine vasoactive mechanisms. While endothelin does not appear to contribute to the fetal cardiovascular response to acute hypoxaemia (Green *et al.* 1998), we have previously reported that fetal exposure to acute hypoxaemia during fetal NO synthase blockade leads to a significant enhancement of the femoral constrictor response (Morrison *et al.* 2003). In the fetus, hypoxia-induced increases in NO therefore oppose and diminish the peripheral constrictor neural and endocrine influences on the fetal peripheral circulation (Morrison *et al.* 2003).

More recently, it has become evident that the cellular oxidant *milieu* is also an important modulator of vascular resistance (Chen & Keaney, 2004). Vascular cells generate reactive oxygen species (ROS), such as the superoxide anion ($^{\bullet}O_2^-$) (Droge, 2002). Superoxide readily combines with NO, limiting its bioavailability (Kissner *et al.* 1997). Hence, under physiological conditions, manipulation of the vascular NO:$^{\bullet}O_2^-$ ratio is also an important determinant of vascular tone. Several studies have investigated the effects of manipulation of the vascular NO:$^{\bullet}O_2^-$ ratio in the lung circulation with regard to pulmonary hypertension in the adult (Irodova *et al.* 2002) and in the placenta with regard to preeclampsia (Davidge, 1998). What remains completely unknown is whether manipulation of the vascular NO:$^{\bullet}O_2^-$ ratio can also affect cardiovascular function in the fetus. One mechanism of manipulating the fetal vascular NO:$^{\bullet}O_2$ ratio *in vivo* may be to expose the fetal circulation to established antioxidants, such as vitamin C, during stimulated conditions, such as during acute hypoxaemia. Exposure to vitamin C has been reported to increase NO bioavailability by several pathways, including quenching ROS, stimulating NO synthase and increasing the expression of antioxidant enzymes (Geetha *et al.* 1989; Jackson *et al.* 1998; Heller *et al.* 1999; Wilson, 2009).

This study used the chronically instrumented, unanaesthetised fetal sheep preparation to test the hypothesis that the cellular oxidant *milieu* is an important modulator of vascular resistance in the fetus. The hypothesis was tested by *in vivo* manipulation of the NO:$^{\bullet}O_2^-$ ratio with fetal treatment with vitamin C during acute hypoxaemia. It was expected that fetal treatment with vitamin C would increase NO bioavailability which, in turn, would depress the femoral vasoconstrictor response to acute hypoxaemia. Therefore, the mechanism via which vitamin C would increase NO bioavailability in the fetus was investigated in three ways: (1) by measuring the concentration of nitrite [NO_2^-] and nitrate [NO_3^-] in plasma during acute hypoxaemia in fetuses with and without vitamin C treatment; (2) by investigating the effect of fetal treatment with vitamin C on the plasma activity of the antioxidant enzyme superoxide dismutase (SOD) during acute hypoxaemia; and (3) by determining whether suppression of the fetal peripheral vasoconstrictor response to hypoxaemia during vitamin C treatment could be reversed following NO synthase blockade.

Methods

Surgical preparation

All procedures were performed under the UK Animals (Scientific Procedures) Act 1986 and were approved by the Ethical Review Committee of the University of Cambridge. Seven Welsh Mountain sheep fetuses were surgically instrumented for long-term recording at 125 ± 1 days ($\pm$ S.D.) of gestation (term is *ca.* 145 days) using strict aseptic conditions as previously described in detail (Giussani *et al.* 2001). In brief, food, but not water, was withheld from the pregnant ewes for 24 h prior to surgery. Following induction with 20 mg kg^{-1} I.V. sodium thiopentone (Intraval Sodium; Merial Animal Health Ltd, Rhone Mérieux, Dublin, Ireland), general anaesthesia (1.5–2.0% halothane in 50:50 O_2:N_2O) was maintained using positive pressure ventilation. Midline abdominal and uterine incisions were made, the fetal hind limbs were exteriorised and, on one side, femoral arterial (i.d., 0.86 mm; o.d., 1.52 mm; Critchly Electrical Products, NSW, Australia) and venous (i.d., 0.56 mm; o.d., 0.96 mm) catheters were inserted. The catheter tips were advanced carefully to the descending aorta and inferior vena cava, respectively. Another catheter was anchored onto the fetal hind limb for recording of the reference amniotic pressure, and a Transonic flow transducer was positioned around the contra-lateral femoral artery (2R or 3S). The uterine incisions were closed in layers, the dead space of the catheters was filled with heparinised saline (80 i.u. heparin ml^{-1} in 0.9% NaCl) and the catheter ends were plugged with sterile brass pins. All catheters and the flow probe lead were exteriorised via a keyhole incision in the maternal flank and kept inside a plastic pouch sewn onto the maternal skin.

Postoperative care

During recovery, ewes were housed in individual pens in rooms with a 12 h–12 h light–dark cycle where they had free access to hay and water and were fed concentrates

twice daily (100 g sheep nuts no. 6; H & C Beart Ltd, Kings Lynn, UK). Antibiotics were administered daily to the ewe (0.20–0.25 mg kg^{-1} I.M. Depocillin; Mycofarm, Cambridge, UK) and fetus I.V. and into the amniotic cavity (150 mg kg^{-1} Penbritin; SmithKline Beecham Animal Health, Welwyn Garden City, UK). The ewes also received 2 days of postoperative analgesia (10–20 mg kg^{-1} oral phenylbutazone; Equipalozone paste, Dechra Veterinary Products, Stoke-on-Trent, UK). Generally, normal feeding patterns were restored within 48 h of recovery. Following 72 h of postoperative recovery, ewes were transferred to metabolic crates where they were housed for the remainder of the protocol. The fetal arterial and amniotic catheters were connected to sterile pressure transducers (COBE; Argon Division, Maxim Medical, Athens, TX, USA) and calibrated mean fetal arterial blood pressure (corrected for amniotic pressure) and fetal heart rate (triggered via a tachometer from the pulsatility in the arterial blood pressure signal) were recorded continually using a computerized Data Acquisition System (DAS; Department of Physiology, Cambridge University, UK). Whilst on the metabolic crates, the patency of the fetal catheters was maintained by a slow continuous infusion of heparinized saline (80 i.u. heparin ml^{-1} at 0.1 ml h^{-1} in 0.9% NaCl) containing antibiotic (1 mg ml^{-1} benzylpenicillin; Crystapen, Schering-Plough Animal Health Division, Welwyn Garden City, UK).

Experimental protocol

Following at least 5 days of postoperative recovery, all fetuses were subjected to hypoxaemic experiments, carried out on consecutive days in a randomised order (Fig. 1). Each protocol consisted of a 3 h period divided into 1.5 h normoxia, 0.5 h hypoxaemia and 1 h recovery, during a slow I.V. infusion of heparinised saline vehicle (80 i.u. heparin ml^{-1} in 0.9% NaCl), treatment with vitamin C alone (ascorbate; A-5960; Sigma Chemicals, UK; 8.9 ± 0.4 mg kg^{-1} min^{-1} I.V.) or treatment with vitamin C during NO synthase blockade with the NO clamp (Fig. 1). In brief, a bolus dose (100 mg kg^{-1} dissolved in 2 ml heparinised saline) of N^G-nitro-L-arginine methyl ester (L-NAME; Sigma-Aldrich, Poole, UK) was injected via the femoral artery, immediately followed by fetal I.V. infusion with sodium nitroprusside (SNP; Sigma-Aldrich; 5.1 ± 2.0 μg kg^{-1} min^{-1}: mean ± 1 S.D.; dissolved in heparinised saline) the concentration of which was titrated to avoid any perturbation in basal arterial blood pressure. While fetal treatment with L-NAME alone leads to pronounced systemic vasoconstriction and hypertension, combined treatment of the fetus with both L-NAME and SNP compensates for the tonic production of the gas, maintains basal cardiovascular function and blocks *de novo* synthesis of NO during stimulated conditions. This technique has been established in our laboratory and previously validated (Gardner *et al.* 2001, 2002; Thakor & Giussani, 2005). At the end of the experimental protocol, the effectiveness of NO synthase blockade by the NO-clamp and the persistence of L-NAME in the system were tested by withdrawal of the SNP infusion.

Acute hypoxaemia in the fetus was induced by maternal inhalational hypoxia. In brief, a large transparent respiratory hood was placed over the ewes' head into which air was passed at a rate of *ca* 50 l min^{-1} for the 1.5 h period of normoxia. Following this control period, acute fetal hypoxaemia was induced for 30 min by changing the concentrations of gases breathed by the ewe to 6% O_2 in N_2 with small amounts of CO_2 (15 l min^{-1} air–35 l min^{-1} N_2–1.5–2.5 l min^{-1} CO_2). This mixture was designed to reduce fetal P_{aO_2} to *ca* 10 mmHg while maintaining P_{aCO_2}. Following the 0.5 h period of hypoxaemia, the ewe was returned to breathing air for the 1 h recovery period. At the end of the experimental protocol, the ewes and fetuses were killed with a lethal dose of sodium pentobarbitone (200 mg kg^{-1} I.V. Pentoject; Animal Care Ltd, York, UK). A post-mortem was carried out at 132 ± 1 days of gestation. Each fetus was weighed and the positions of the implanted catheters and the flow probes were confirmed.

Blood sampling regimen and assays

During the experimental protocol, descending aortic blood samples were taken using sterile techniques from the fetus at set time intervals to determine arterial blood gases and pH (0.3 ml). Additional plasma samples were taken for measurement of plasma SOD activity, and plasma concentrations of NO_2^-, NO_3^- and ascorbate (1.5 ml; Fig. 1). Arterial blood gas and pH measurements were made using an ABL5 blood gas analyser (Radiometer; Copenhagen, Denmark; measurements corrected to 39.5°C). Values for percentage saturation of haemoglobin with oxygen (Sat Hb) were determined using a haemoximeter (OSM3; Radiometer, Copenhagen, Denmark). Plasma SOD activity was assayed using a SOD Assay Kit from BioVision (Mountain View, CA, USA) according to the instructions of the manufacturer. This assay utilizes WST-1, a tetrazolium salt, which produces a water soluble formazan dye upon reduction with superoxide anion. The rate of the reduction with a superoxide anion is linearly related with the amount to the xanthine oxidase activity and is inhibited by SOD. The SOD activity was expressed as the percentage of inhibition of the WST-1 reduction rate (Andres *et al.* 2008). Plasma concentrations of NO_2^- and NO_3^- were determined by a commercially available Nitrate/Nitrite Colorimetric Assay Kit (Caymen Chemical Co., Ann Arbor, MI, USA, cat no. 780001) working on the principles of the Griess reaction (Green *et al.* 1982). In brief, total NO_2^- and NO_3^- (NO_x) was measured from plasma samples which were ultra-filtrated to reduce

the background interference due to any haemoglobin present. Assay buffer, nitrate standards and 40 μl of plasma samples were then loaded in duplicate into a 96-well microplate with an enzyme cofactor and nitrate reductase before a 60 min incubation at room temperature. Following further addition of the Griess reagents, the plates were allowed to incubate for 10 min before reading absorbance at 540 nm (Bioteck ELx800 Absorbance Microplate Reader, Potton, Bedfordshire, United Kingdom). Plasma NO_2^- concentrations were measured by the same method but omitting the nitrate reductase step. Plasma NO_3^- was calculated as total NO_x minus NO_2^-. The inter- and intra-assay coefficients of variation were 3.4% and 2.7%, respectively, and the lower limit of detection of the assay is 0.24 μM. Plasma ascorbate concentrations were measured using HPLC with electrochemical detection (Iriyama *et al.* 1984). Total ascorbate was measured following the treatment of the pre-acidified plasma sample with Tris-[2-carboxyethyl]-phosphine (TCEP) HCl (Lykkesfeldt, 2000). Aliquots of 20 μl were injected into the HPLC unit with a chromatography column (250 × 4.6 mm, C18, 5 μm; Apex II column with guard; Jones Chromatography, Glamorgan, UK) and eluted with a mobile phase containing K_2HPO_4–H_3PO_4 (200 mmol l^{-1}; pH 2.1) and octane sulphonic acid (250 μmol l^{-1}; pH 2.56) at a flow rate of 1.0 ml min^{-1}. An electrochemical detector (EG & G Instruments; Wokingham, UK) with a working electrode (set at 400 mV and sensitivity of 0.5 μA) was used for detection. Final concentrations for ascorbate were calculated with external standards, which were run simultaneously. The limit of sensitivity for the assay was 0.5 μmol l^{-1} and the inter-assay coefficient of variation was less than 5%.

Data and statistical analyses

All variables are expressed as means ± S.E.M. Summary measure analysis was applied to the serial cardiovascular data to focus the number of comparisons (Matthews *et al.* 1990). Femoral vascular resistance was calculated by using Ohm's principle and dividing fetal arterial blood pressure (corrected for amniotic pressure) by femoral blood flow. Similarly, femoral vascular conductance was calculated by dividing femoral blood flow by corrected fetal arterial blood pressure. Area under the curve was calculated at 30 min intervals (N1, N2, N3, H, R1, R2) for the absolute data describing the fetal cardiovascular responses. Plasma SOD activity and plasma concentrations of ascorbate, NO_2^- and NO_3^- were expressed as absolute values and/or as the mean ± S.E.M. change or percentage change from normoxic baseline, as appropriate. All variables were assessed statistically using the Student's *t* test for paired data, one-way or two-way analysis of variance (ANOVA) with repeated measures (RM) comparing the effect of time, treatment and interactions between time and treatment, as appropriate. Where a significant effect of time or treatment was indicated, Tukey's *post hoc* test was used to isolate the statistical differences. For all comparisons, statistical significance was accepted when $P < 0.05$.

Results

Fetal arterial blood gas status

Basal values for fetal arterial blood gases and pH were similar in all fetuses and were within the normal range for the Welsh Mountain sheep fetus at *ca* 130 days of gestation (Table 1). Infusion with saline had no effect on basal arterial blood gas status. In contrast, treatment with vitamin C alone (−0.06 ± 0.01) and treatment with vitamin C during NO synthase blockade (−0.06 ± 0.01) produced similar decrements in fetal pH_a ($P < 0.05$; Table 1). In all fetuses, acute hypoxaemia induced significant falls of similar magnitude in P_{aO_2} and Sat Hb, without any alteration to P_{aCO_2} (Table 1). In addition, acute hypoxaemia during saline infusion (−0.08 ± 0.01),

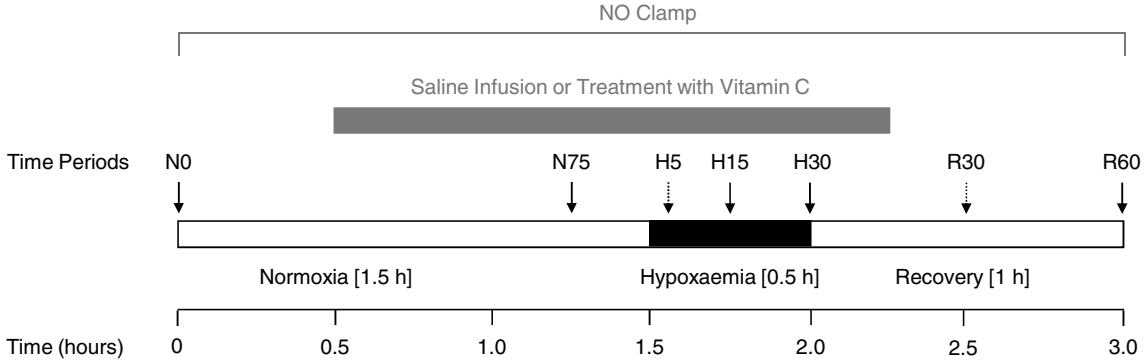

Figure 1. Diagrammatic representation of the acute hypoxaemia protocol
The experimental protocol consisted of a 3 h period divided into: 1.5 h normoxia, 0.5 h hypoxaemia (black bar) and 1 h recovery, during saline infusion ($n = 7$), treatment with vitamin C (8.9 ± 0.4 mg kg^{-1} min^{-1}) alone ($n = 7$) or treatment with vitamin C during the NO clamp ($n = 7$; grey bar). Arrows represent times at which arterial blood samples were collected. Dotted arrows were for blood gas analysis only.

Table 1. Fetal arterial blood gas status

		Normoxia		Acute hypoxaemia			Recovery	
		N0	N75	H5	H15	H30	R30	R60
		Saline infusion or Treatment with vitamin C (± NO clamp)						
pH_a	Saline Infusion	7.36 ± 0.01	7.36 ± 0.01	7.36 ± 0.01	7.34 ± 0.01	7.29 ± 0.01[a]	7.32 ± 0.01[a]	7.34 ± 0.01
	Vitamin C infusion	7.35 ± 0.01	7.29 ± 0.01[a,b]	7.28 ± 0.01[a,b]	7.25 ± 0.01[a,b]	7.21 ± 0.01[a,b]	7.25 ± 0.02[a,b]	7.28 ± 0.01[a,b]
	Vitamin C infusion and NO clamp	7.35 ± 0.01	7.28 ± 0.01[a,b]	7.26 ± 0.01[a,b]	7.23 ± 0.01[a,b]	7.19 ± 0.01[a,b]	7.21 ± 0.01[a,b]	7.24 ± 0.01[a,b]
P_{aCO_2} (mmHg)	Saline infusion	54.7 ± 0.7	54.8 ± 0.6	53.7 ± 0.6	54.1 ± 0.5	54.2 ± 0.6	54.3 ± 0.7	55.1 ± 0.5
	Vitamin C infusion	56.0 ± 0.5	56.3 ± 0.8	55.8 ± 0.7	56.0 ± 0.6	54.7 ± 0.7	54.6 ± 0.6	55.3 ± 0.5
	Vitamin C infusion and NO clamp	55.3 ± 0.8	56.2 ± 0.5	54.5 ± 0.6	54.5 ± 0.7	55.2 ± 0.3	55.7 ± 0.6	55.0 ± 0.9
P_{aO_2} (mmHg)	Saline infusion	22.0 ± 0.8	21.3 ± 0.6	10.8 ± 0.3[a]	11.4 ± 0.2[a]	11.4 ± 0.2[a]	21.2 ± 0.8	21.1 ± 0.8
	Vitamin C infusion	21.2 ± 1.0	21.2 ± 0.5	11.3 ± 0.3[a]	11.3 ± 0.2[a]	12.0 ± 0.3[a]	21.2 ± 1.4	22.2 ± 1.5
	Vitamin C infusion and NO clamp	21.7 ± 0.9	21.0 ± 0.8	11.7 ± 0.4[a]	11.7 ± 0.2[a]	11.2 ± 0.2[a]	20.7 ± 1.0	22.0 ± 1.5
Sat Hb (%)	Saline infusion	56.3 ± 1.4	53.5 ± 1.4	25.4 ± 1.3[a]	25.2 ± 1.6[a]	25.8 ± 0.9[a]	53.2 ± 2.1	52.4 ± 2.1
	Vitamin C infusion	54.8 ± 1.9	53.3 ± 2.0	24.1 ± 1.2[a]	23.2 ± 1.3[a]	23.9 ± 1.4[a]	53.0 ± 2.1	53.8 ± 2.3
	Vitamin C infusion and NO clamp	52.8 ± 3.4	51.6 ± 2.9	24.2 ± 1.3[a]	23.9 ± 1.4[a]	23.8 ± 1.5[a]	50.7 ± 4.3	51.4 ± 4.6

Values are means ± S.E.M. at 0 (N0) and 75 min (N75) of normoxia, at 5 (H5), 15 (H15) and 30 min (H30) of hypoxemia, and at 30 (R30) and 60 min (R60) of recovery for fetuses exposed to 0.5 h of hypoxaemia during saline infusion ($n = 7$), treatment with vitamin C alone ($n = 7$) or treatment with vitamin C during the NO clamp ($n = 7$). Significant differences: [a]$P < 0.05$, *vs.* time period N0; [b]$P < 0.05$, *vs.* saline infusion (two-way RM-ANOVA with Tukey's *post hoc* test). pH_a, arterial pH; P_{aCO_2}, arterial partial pressure of CO_2; P_{aO_2}, arterial partial pressure of O_2; Sat Hb, percentage saturation of haemoglobin with oxygen.

treatment with vitamin C alone (-0.09 ± 0.01) or treatment with vitamin C during NOS blockade (-0.09 ± 0.01) all produced similar decrements in fetal pH_a ($P < 0.05$) relative to normoxia (Table 1). During recovery, pH_a remained significantly depressed in all fetuses, whereas P_{aO_2} and Sat Hb returned towards basal values (Table 1).

Fetal plasma ascorbate concentrations

Basal values for plasma concentrations of ascorbate were $24.2 \pm 3.0\ \mu mol\,l^{-1}$. Fetal treatment with vitamin C induced a progressive elevation in fetal ascorbate concentrations before reaching a plateau. Following the onset of treatment, fetal ascorbate plasma concentrations were elevated to $4.3 \pm 0.4\ mmol\,l^{-1}$ by the end of normoxia and to $7.3 \pm 0.6\ mmol\,l^{-1}$ by the end of the acute hypoxaemic challenge (Fig. 2).

Fetal plasma SOD activity and plasma concentrations of NO_2^- and NO_3^-

Basal values for fetal arterial plasma SOD activity and plasma concentrations of NO_2^- and NO_3^- were $33.2 \pm 2.6\%$ inhibition, $0.68 \pm 0.10\ \mu M$ and $34.6 \pm 6.4\ \mu M$, respectively. Fetal plasma SOD activity

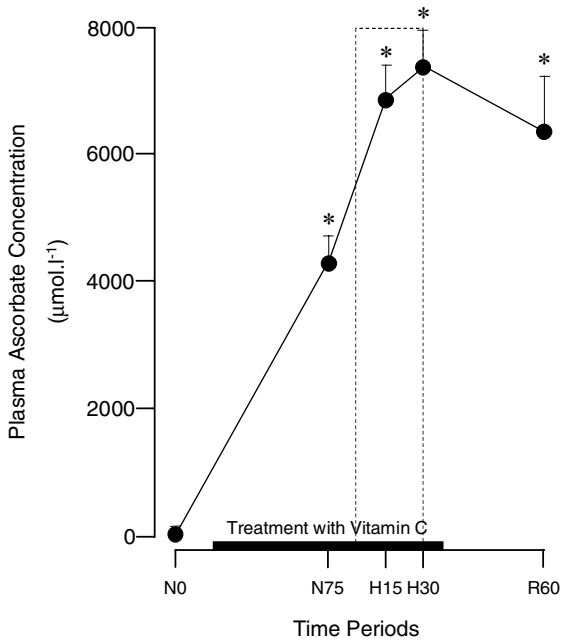

Figure 2. Fetal plasma concentrations of ascorbate
Values are means ± S.E.M. for plasma concentrations of ascorbate at 0 (N0) and 75 min (N75) of normoxia, at 15 (H15) and 30 min (H30) of hypoxaemia, and at 60 min (R60) of recovery for fetuses exposed to 0.5 h hypoxaemia (dashed box) during treatment with vitamin C ($8.9 \pm 0.4\ mg\ kg^{-1}\ min^{-1}$; $n = 6$). *Significant difference ($P < 0.05$) *vs.* time period N0 (one-way RM-ANOVA with Tukey's *post hoc* test).

and fetal plasma NO_2^- and NO_3^- concentrations remained unaltered from baseline following fetal treatment with vitamin C during normoxia ($P > 0.05$). However, fetal treatment with vitamin C during acute hypoxaemia led to a significant increment from baseline in fetal plasma SOD activity, a significant decrement in fetal plasma concentrations of NO_3^-, a maintenance of fetal plasma concentrations of NO_2^-, and, thereby, a decrease from baseline in the plasma $[NO_3^-]$:$[NO_2^-]$ concentration ratio in the fetal circulation (Fig. 3*A*–*D*).

Fetal cardiovascular function

Basal values for fetal arterial blood pressure, heart rate and femoral haemodynamic variables were similar in all fetuses (Figs 4 and 5). Infusion with saline had no effect on basal cardiovascular function (Figs 4 and 5). In contrast, treatment with vitamin C during normoxic conditions resulted in significant increments from baseline in femoral blood flow ($9.8 \pm 4.5\%$) and femoral vascular conductance ($9.2 \pm 4.3\%$). Acute hypoxaemia during saline infusion induced significant increments in fetal arterial blood pressure (8.6 ± 0.6 mmHg) and femoral vascular resistance (5.21 ± 1.02 mmHg (ml min^{-1})$^{-1}$) and significant falls in fetal heart rate (-27 ± 5 beats min^{-1}) and femoral blood flow (-27 ± 2 ml min^{-1}; Figs 4 and 5). In contrast, treatment with vitamin C alone significantly diminished the magnitude of the pressor and femoral vasoconstrictor responses to acute hypoxaemia (Figs 4 and 5). However, treatment with vitamin C during NO synthase blockade recovered both the pressor and the femoral vasopressor responses to acute hypoxaemia, but it did not affect the femoral vasodilator response during baseline measured in fetuses during saline infusion (Figs 4 and 5). The bradycardic response to acute hypoxaemia was not affected by treatment with vitamin C alone. However, the area under the fetal heart rate curve was significantly enhanced with treatment with vitamin C during NO synthase blockade compared to saline infused controls (Fig. 5). During recovery, fetal arterial blood pressure remained significantly elevated in all fetuses, while femoral vascular resistance returned towards basal values. Values for femoral blood flow and heart rate returned towards baseline in saline infused and vitamin C treated fetuses, but

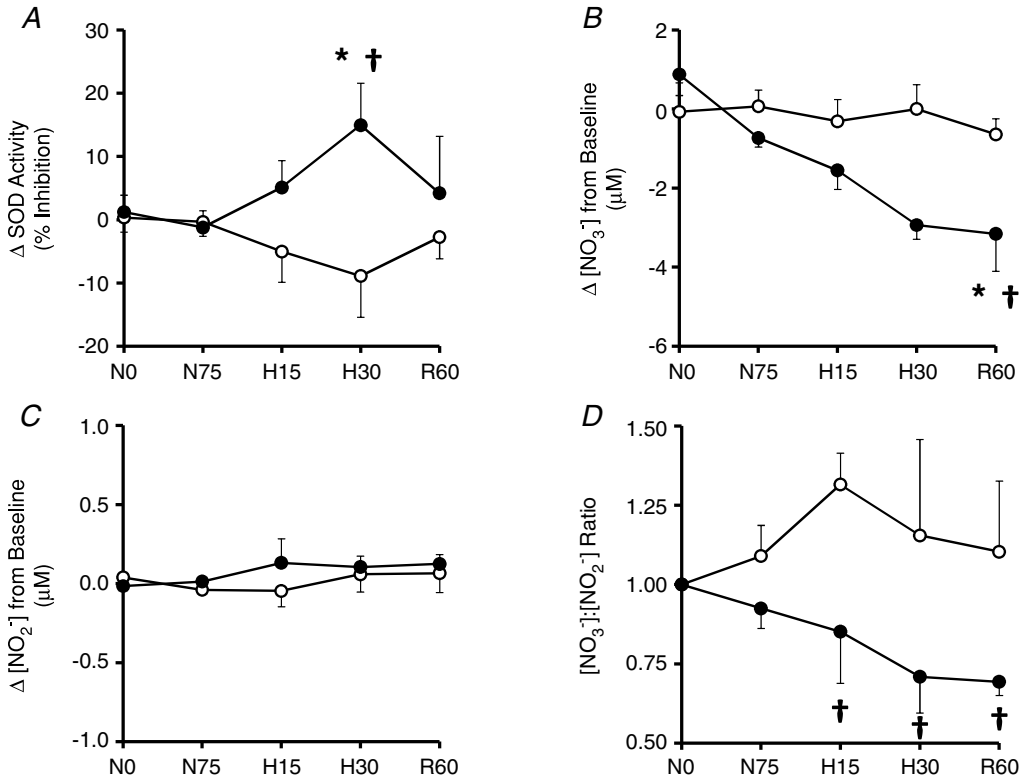

Figure 3. Fetal plasma SOD activity and plasma concentrations of NO_2^- and NO_3^-
Values are means ± S.E.M. for changes from baseline in fetal plasma SOD activity (*A*), plasma concentrations of NO_3^- and NO_2^- (*B* and *C*, respectively) and the plasma concentration ratio of $[NO_3^-]$:$[NO_2^-]$ (*D*) at 0 (N0) and 75 min (N75) of normoxia, at 15 (H15) and 30 min (H30) of hypoxaemia and 60 min (R60) of recovery for fetuses exposed to 0.5 h of hypoxaemia during saline infusion (○, $n = 6$) or treatment with vitamin C (●, $n = 6$). Significant differences are $^*P < 0.05$ *vs*. normoxia, †$P < 0.05$ *vs*. saline (two-way RM ANOVA with Tukey's *post hoc* test).

remained significantly depressed from baseline in fetuses treated with vitamin C during NO synthase blockade (Figs 4 and 5).

Following the end of the recovery period of the acute hypoxaemia protocol, withdrawal of saline infusion had no effect on any measured fetal cardiovascular variable (data not shown). In contrast, withdrawal of the SNP infusion in fetuses undergoing the NO clamp led to significant hypertension, bradycardia and femoral vasoconstriction (Fig. 6).

Discussion

The data show that fetal treatment with vitamin C had a marked inhibitory effect on the normal fetal peripheral vasoconstrictor response to acute hypoxaemia. Fetal treatment with vitamin C also led to significant elevations in fetal plasma SOD activity and a decrease in the fetal plasma concentration ratio of NO_3^-:NO_2^-. Fetal treatment with vitamin C in the presence of NO synthase blockade normalised the magnitude of the femoral vasoconstrictor response. Therefore, these data support the

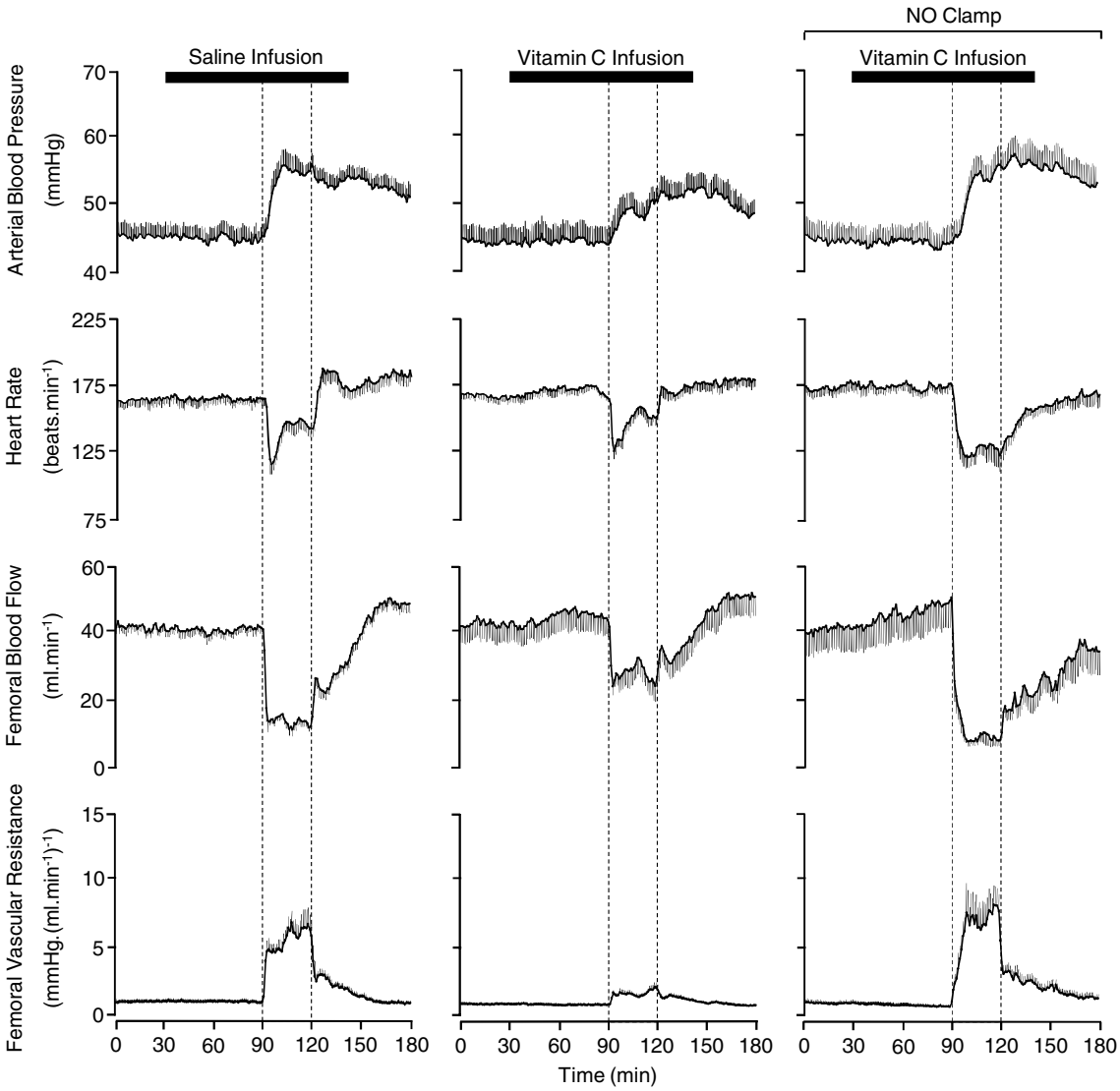

Figure 4. Fetal cardiovascular responses to acute hypoxaemia
Values are means ± S.E.M. calculated every minute for arterial blood pressure, heart rate, femoral blood flow and femoral vascular resistance during 1.5 h of normoxia, 0.5 h of hypoxaemia (dashed box) and 1 h of recovery for fetuses during saline infusion ($n = 7$), treatment with vitamin C alone ($n = 7$) or treatment with vitamin C during the NO clamp ($n = 7$).

hypothesis tested and suggest that manipulation of the NO:$^{\bullet}O_2^-$ ratio can have a profound effect on fetal cardiovascular function during the stimulated condition of acute hypoxaemic stress.

Vitamin C was the antioxidant of choice in this study for several reasons. During pregnancy, vitamin C is detectable in the placenta and the fetal plasma, the brain, the liver, the kidney and the adrenal glands (Kratzing & Kelly, 1986; Zalani *et al.* 1987; Kolb *et al.* 1991). Fetal plasma vitamin C levels also increase throughout gestation in human (Baydas *et al.* 2002) and ovine (Kolb *et al.* 1991) pregnancy, and preterm human infants have lower serum levels of vitamin C than term infants (Baydas *et al.* 2002), findings which are all consistent with a functional role for vitamin C in prenatal life. In addition, vitamin C is one of the most important water soluble antioxidants in mammalian tissues (Levine, 1986; Rose & Bode, 1993; Meister, 1994; Winkler *et al.* 1994) and Frei *et al.* (1989) demonstrated that vitamin C was the most effective aqueous-phase antioxidant in plasma. Finally, since vitamin C enhances vasodilator responses to endothelium-dependent agonists, such as arginine and acetylcholine (Heitzer *et al.* 1996; Ting *et al.* 1997; Kugiyama *et al.* 1998; Taddei *et al.* 1998; Tousoulis *et al.* 1999) but not to endothelium-independent agonists, such as papaverine (Solzbach *et al.* 1997; Ting *et al.* 1997; Taddei *et al.* 1998), its protective antioxidant effects on the cardiovascular system are likely to be mediated at the level of the endothelium in the circulation, the target organ of this study.

In vascular endothelial cells, NO is produced constitutively *in vivo*, but it is rapidly inactivated by $^{\bullet}O_2^-$ to produce $^{\bullet}ONOO^-$ (rate constant $= 2 \times 10^{10}\,\mathrm{mol\,l^{-1}\,s^{-1}}$) (Kissner *et al.* 1997). Although the availability of $^{\bullet}O_2^-$ in tissues is strictly limited by the abundance of SOD, which is able to dismutate $^{\bullet}O_2^-$ at a similar rate constant ($2 \times 10^9\,\mathrm{mol\,l^{-1}\,s^{-1}}$) (Fridovich, 1978), NO can still compete effectively with SOD for $^{\bullet}O_2^-$. In an elegant study, Jackson and colleagues (1998) reported that vitamin C could scavenge $^{\bullet}O_2^-$ at concentrations as low as $100\,\mu\mathrm{mol\,l^{-1}}$, but it could only prevent

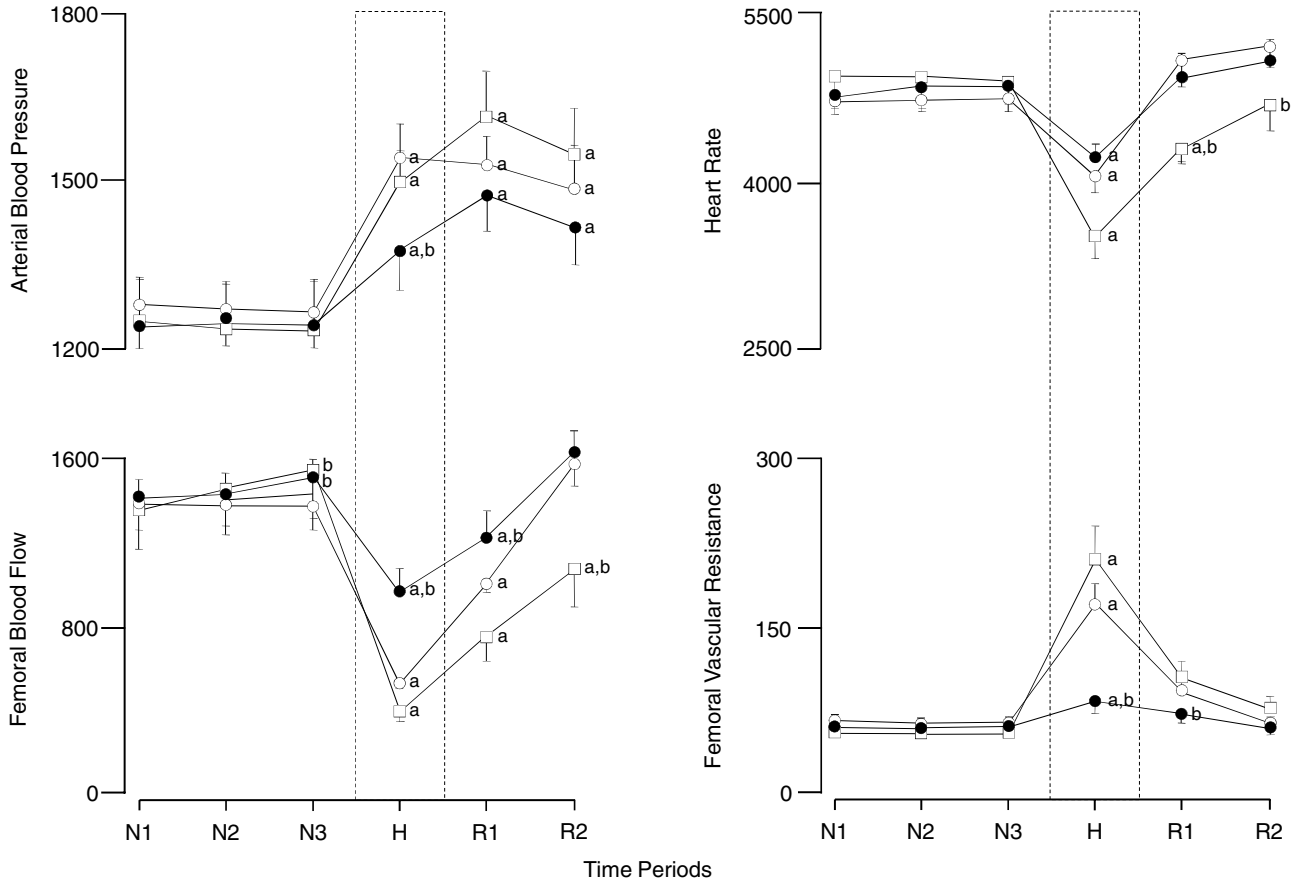

Figure 5. Statistical summary of the fetal cardiovascular responses to acute hypoxaemia
Values are means ± S.E.M for the area under the curve every 30 min during normoxia (N), hypoxemia (H) and recovery (R) for fetuses during saline infusion (○; $n = 7$), treatment with vitamin C alone (●; $n = 7$) or treatment with vitamin C during the NO clamp (□; $n = 7$). Significant differences: $^{a}P < 0.05$ *vs.* time period N1, $^{b}P < 0.05$ *vs.* saline infusion (two-way RM-ANOVA with Tukey's *post hoc* test).

the impairment by $^{\bullet}O_2^-$ of endothelium derived NO-mediated arterial relaxation at the much higher physiological concentrations of 10 mmol l^{-1}. Therefore, the capacity of vitamin C to scavenge $^{\bullet}O_2^-$ and its ability to prevent the interaction between NO and $^{\bullet}O_2^-$ appear to occur at very different concentrations *in vivo*. Accordingly, the rate constant for ascorbic acid to scavenge $^{\bullet}O_2^-$ is *ca* 3×10^5 mol l^{-1} s^{-1} (Nishikimi, 1975; Gotoh & Niki, 1992), which is approximately 100,000-fold less than the rate at which SOD or NO react with $^{\bullet}O_2^-$. Therefore, for vitamin C to compete effectively with NO at any given concentration of $^{\bullet}O_2^-$, its concentrations must exceed that of NO by a factor of 100,000. Since NO concentrations are approximately 0.1 μmol l^{-1} adjacent to endothelial cells (Malinski *et al.* 1993; Blatter *et al.* 1995), the concentrations of vitamin C would therefore need to be *ca* 10 mmol l^{-1} to prevent the *in vivo* interaction between $^{\bullet}O_2^-$ and NO (Jackson *et al.* 1998; Sherman *et al.* 2000). In the present study, treatment with vitamin C elevated fetal plasma concentrations of ascorbate to 7.3 ± 0.6 mmol l^{-1}, which is within the concentration range required for it to act *in vivo* as an antioxidant in plasma, justifying the dosing regimen.

There are several possible mechanisms via which vitamin C may modulate the NO:$^{\bullet}O_2^-$ ratio in the fetal circulation. In addition to quenching ROS directly (Jackson *et al.* 1998), ascorbate can act through tetrahydrobiopterin (BH_4) to stimulate NO production by endothelial NO synthase (Wilson, 2009). Vitamin C can also prevent the *S*-nitrosylation of sensitive thiol groups on endothelial NO synthase, thereby maintaining their stability and the affinity of NO synthase for BH_4 (Heller *et al.* 1999). In addition, it has been reported that vitamin C may enhance the expression and/or action of potent antioxidant enzymes (Geetha *et al.* 1989). Because of this, potential mechanisms via which vitamin C may have enhanced NO bioavailability in the fetal circulation in the present study were determined in three ways: (1) by measuring the concentration of nitrite [NO_2^-] and nitrate [NO_3^-] in fetal plasma; (2) by investigating the effect of fetal treatment with vitamin C on the plasma activity of the antioxidant enzyme SOD; and (3) by determining whether suppression the fetal peripheral vasoconstrictor response to hypoxaemia during vitamin C treatment could be reversed following NO synthase blockade.

In the present experiment, it would have been desirable to investigate the effects of acute hypoxaemia with and without fetal treatment with vitamin C on the *in vivo* absolute circulating concentrations of NO and of $^{\bullet}O_2^-$. However, the rate of NO reaction with oxyHb and deoxy-Hb is exceptionally rapid so that the half-life of free NO in blood is only fractions of a second (Butler *et al.* 1998; Huang *et al.* 2001). As a result, physiological concentrations of free NO in blood are maintained in the subnanomolar range, making the *in vivo* measurement of free NO in fetal blood impractical by currently available

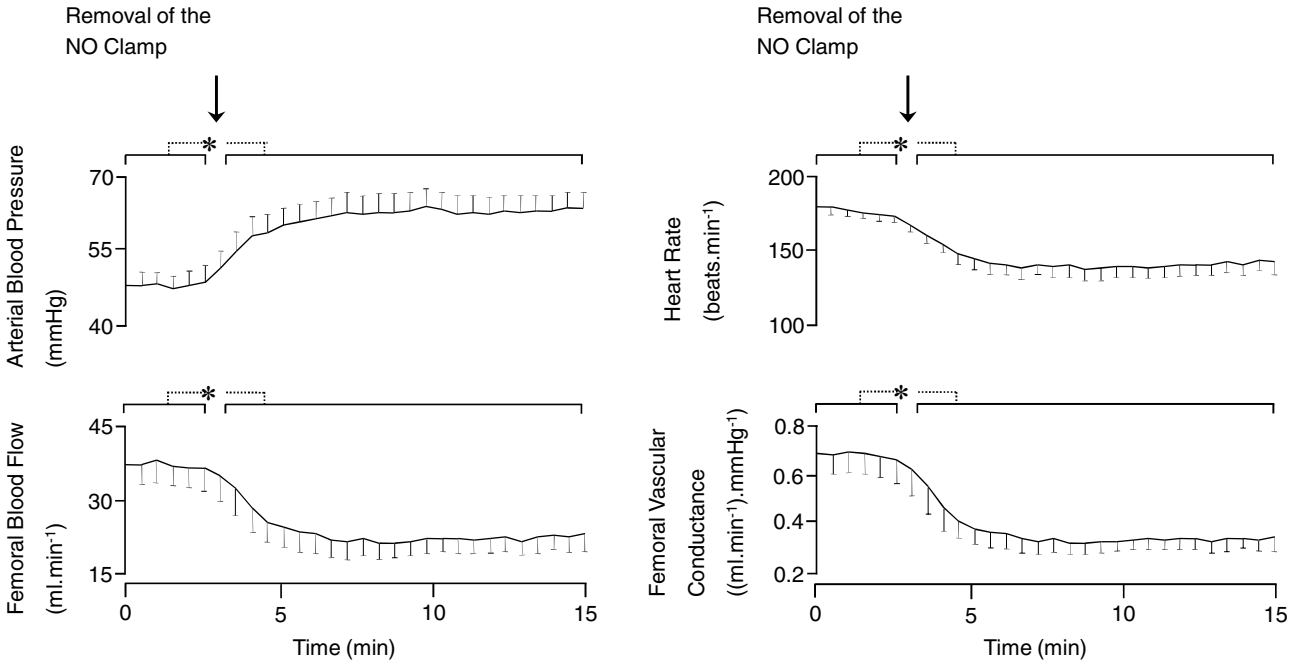

Figure 6. The effect on the cardiovascular variables of removal of the NO clamp
Values are means ± S.E.M calculated every 30 s following the end of the recovery period of the acute hypoxaemia protocol for fetal arterial blood pressure, heart rate, femoral blood flow and femoral vascular conductance before and after withdrawal of sodium nitroprusside infusion in fetuses undergoing treatment with the NO clamp. *Significant differences ($P < 0.05$) are before *vs.* after withdrawal of sodium nitroprusside infusion (Student's *t* test for paired data).

methods (Blood *et al.* 2009). Similarly, it is by no means a mystery that the measurement of the evanescent reactive oxygen species is difficult, particularly *in vivo* and in the fetus. Trapping methods are useful in biochemical studies *in vitro*. Likewise, fingerprinting methods, those that measure the damage that they cause, rather than the species themselves, are best applied to isolated tissues. Nitrite (NO_2^-) and nitrate (NO_3^-) concentrations are often measured together (NO_x) and used as an index of NO in biological fluids. However, because nitrate concentrations are many-fold higher than nitrite and they are influenced by many factors including diet and liver enzymes, the ratio of NO_3^-:NO_2^- concentration in fetal plasma rather than their absolute concentrations may provide a more informative index of the relative NO and $^{\bullet}O_2^-$ bioavailability (Kleinbongard *et al.* 2003, 2006). It is accepted that the interaction between NO and $^{\bullet}O_2^-$ promotes NO_3^- formation and that absolute levels of NO_2^- rather than NO_3^- are a better index of NO bioavailability (see Ignarro *et al.* 1993; Kleinbongard *et al.* 2003, 2006). Therefore, changes in the ratio of NO_3^-:NO_2^- concentration provide an indirect index of $^{\bullet}O_2^-$ bioavailability. Data in the present study show that fetal treatment with vitamin C during acute hypoxaemia led to a decrease in the concentration ratio of NO_3^-:NO_2^- in fetal plasma. These data suggest that one mechanism via which fetal treatment with vitamin C influenced the NO:$^{\bullet}O_2^-$ ratio in the fetal circulation during acute hypoxaemia is by decreasing the bioavailability of $^{\bullet}O_2^-$.

Superoxide dismutase is one of the most important antioxidant enzymes in the vasculature. It is established that non-selective pharmacological inhibition of SOD isoforms increases vascular oxidative stress and attenuated endothelium-dependent relaxation (Mügge *et al.* 1991). An elegant study using SOD-deficient mice has also demonstrated that endogenous extracellular SOD is a major enzyme antagonising $^{\bullet}O_2^-$ formation, and thereby indispensable in regulating the bioavailability of NO and in the control of vascular function and arterial blood pressure (Jung *et al.* 2003). Data in the present study show that fetal treatment with vitamin C during acute hypoxaemia led to significant elevations in SOD activity in the fetal circulation. Therefore, a second mechanism via which fetal treatment with vitamin C may have influenced the NO:$^{\bullet}O_2^-$ ratio in the fetal circulation during acute hypoxaemia is by increasing antioxidant enzyme activity in fetal plasma.

In addition to episodes of ischaemia and reperfusion, hypoxaemia alone is a potent stimulator of free radical generation, such as the $^{\bullet}O_2^-$ (Chandel, 2010). If hypoxaemia-induced increases in $^{\bullet}O_2^-$ contribute to an increase in femoral vascular resistance in the fetus, then hypoxia-induced increases in fetal femoral vascular resistance should be diminished by fetal treatment with antioxidants. Further, if these actions of antioxidants are mediated by scavenging the $^{\bullet}O_2^-$, and thereby increasing the production ratio of NO:$^{\bullet}O_2^-$ in the fetal vasculature, then fetal treatment with antioxidants during blockade of NO synthesis should restore the femoral vasoconstrictor response to acute hypoxaemia in the fetus. Additional data in the present study show that fetal treatment with vitamin C in the presence of NO synthase blockade did indeed restore the normal peripheral vasoconstrictor response to acute hypoxaemia measured *in vivo*. Withdrawal of the nitroprusside infusion at the end of the recovery period led to a significant increase in fetal arterial blood pressure, decrease in fetal heart rate and vasoconstriction in the femoral circulation. These changes provide evidence for the effectiveness of NO synthase blockade by the clamp and the persistence of the action of L-NAME within the system until the end of the experimental protocol. Therefore, a third mechanism via which fetal treatment with vitamin C may have influenced the NO:$^{\bullet}O_2^-$ ratio in the fetal circulation during acute hypoxaemia is by stimulating NO synthase activity. That tonic production of $^{\bullet}O_2^-$ contributes to vascular tone in peripheral circulations in the fetus is also supported by the finding that fetal treatment with vitamin C during basal conditions resulted in an increase in fetal peripheral vascular conductance.

It could be argued that the effects of vitamin C on the fetal peripheral vasoconstrictor response to acute hypoxaemia could be at the level of the carotid body since both NO and ROS have been implicated in peripheral chemo-transduction mechanisms (Dinger *et al.* 2007; Kumar & Phil, 2007). It could further be argued that the effects of vitamin C on the fetal haemodynamic response to hypoxia may be secondary to HIF inactivation. The hypoxia inducible factor (HIF) is down-regulated in oxygenated cells by a series of Fe(II)- and 2-oxoglutarate-dependent dioxygenases that hydroxylate specific residues in the regulatory HIF α-subunits. Because these enzymes require ascorbate for activity, it has been reported that ascorbate can suppress HIF-1α protein levels and HIF transcriptional targets, for instance in human cancer cell lines (Knowles *et al.* 2003). The effect of vitamin C on the femoral vasoconstrictor response to acute hypoxia in the fetus may therefore be due to silencing of HIF by ascorbate. In the fetus, both the bradycardia and the femoral vasoconstrictor responses to acute hypoxaemia are triggered exclusively by the same carotid chemoreflex since two independent studies have shown that bilateral section of the carotid sinus nerves abolishes both responses during acute hypoxaemia (Bartelds *et al.* 1993; Giussani *et al.* 1993). In the present study, fetal treatment with vitamin C diminished the fetal peripheral vasoconstrictor response to acute hypoxaemia without affecting the fetal bradycardia. Dissociation of the effects of vitamin C on the fetal cardiac and vasomotor responses to acute hypoxaemia does not support an effect of vitamin C at

the level of the carotid body chemoreflex or an effect by silencing HIF. Rather, the differential effect further supports an action of vitamin C at the level of the peripheral vasculature.

A final consideration is to address whether the effects of vitamin C on the femoral vascular response to acute hypoxaemia may have been secondary to the mild fetal acidaemia induced by the ascorbic acid treatment. However, mild fetal acidaemia, of the magnitude induced by fetal treatment with vitamin C in the present study, significantly enhances rather than depresses the fetal femoral vasopressor responses to acute hypoxaemia (Thakor & Giussani, 2009). In fact, mild acidaemia doubled the magnitude of the femoral vasoconstrictor response to acute hypoxaemia (Thakor & Giussani, 2009). This highlights that the depressor effects of vitamin C on the femoral haemodynamic response to hypoxaemia appear to overwhelm even a sensitised peripheral vasoconstrictor response, and that the effects of vitamin C on the peripheral haemodynamic response to hypoxaemia in the current study may be diluted.

In conclusion, data in the present study advance our understanding of the physiological basis underlying the fetal cardiovascular defence to acute hypoxaemia. Here, we show *in vivo* that the cellular oxidant *milieu* is an important modulator of vascular tone in the fetal circulation and that it can be manipulated by antioxidant treatment during acute hypoxaemic stress.

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Author contributions

The experiments in this study were performed in the Department of Physiology, Development and Neuroscience, University of Cambridge, UK and in the Environmental Research Group, Division of Pharmaceutical Science, King's College London. A.S.T. and D.A.G. conceived and designed the experiments. A.S.T., H.G.R., A.D.K., C.D., F.J.K., L.P. and D.A.G. collected, analysed and interpreted the experimental data. A.S.T., H.G.R., A.D.K., L.P., F.J.P. and D.A.G. drafted the article and revised it critically for important intellectual content. All authors approved the final version for publication.

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