Altered Cardiovascular Defense to Hypotensive Stress in the Chronically Hypoxic Fetus

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Abstract

The hypoxic fetus is at greater risk of cardiovascular demise during a challenge, but the reasons behind this are unknown. Clinically, progress has been hampered by the inability to study the human fetus non-invasively for long period of gestation. Using experimental animals, there has also been an inability to induce gestational hypoxia while recording fetal cardiovascular function as the hypoxic pregnancy is occurring. We use novel technology in sheep pregnancy that combines induction of controlled chronic hypoxia with simultaneous, wireless recording of blood pressure and blood flow signals from the fetus. Here, we investigated the cardiovascular defense of the hypoxic fetus to superimposed acute hypotension. Pregnant ewes carrying singleton fetuses surgically prepared with catheters and flow probes were randomly exposed to normoxia or chronic hypoxia from 121±1 days of gestation (term ~ 145 days). After 10 days of exposure, fetuses were subjected to acute hypotension via fetal nitroprusside intravenous infusion. Underlying *in vivo* mechanisms were explored by: a) analysing fetal cardiac and peripheral vasomotor baroreflex function; b) measuring the fetal plasma catecholamines; and c) establishing fetal femoral vasoconstrictor responses to the α_1-adrenergic agonist phenylephrine. Relative to controls, chronically hypoxic fetal sheep had reversed cardiac and impaired vasomotor baroreflex function, despite similar noradrenaline and greater adrenaline increments in plasma during hypotension. Chronic hypoxia markedly diminished the fetal vasopressor responses to phenylephrine. Therefore, we show that the chronically hypoxic fetus displays markedly different cardiovascular responses to acute hypotension, providing *in vivo* evidence of mechanisms linking its greater susceptibility to superimposed stress.

Key words: fetus, hypoxia, chronic hypoxia, cardiovascular, fetal growth restriction, intrauterine growth restriction, fetal brain sparing

Introduction

A reduction in fetal oxygenation or fetal hypoxia is one of the most common consequences of complicated pregnancy [1]. Studies have long established that the developing fetus in late gestation has robust cardiovascular compensatory mechanisms to withstand an acute episode of hypoxia [2, 3]. By marked contrast, we know little about the effects of chronic hypoxia on the fetal cardiovascular system and even less during a superimposed challenge [4-6]. Important human clinical studies have reported that the growth restricted fetus displays significant alteration in cardiovascular structure and function. However, whether these effects are mediated by chronic fetal hypoxia or other deficiencies has been difficult to isolate [7-9]. Experimental progress in the field of hypoxic pregnancy using animal models has been hampered for several reasons. Most importantly, there has been an inability to induce hypoxia for long periods of gestation while recording cardiovascular data from the fetus. This gap of knowledge is of substantial clinical significance because the growth restricted fetus, presumed to be chronically hypoxic, is at a seven-fold risk of acute complications later in gestation. However, the reason behind this is completely unknown [10].

The late gestation fetus has well described compensatory responses to alterations in blood pressure. In response to acute hypotension, cardiac and vasomotor baroreflex responses are triggered [11,12]. Neural sympathetic outflow to arterioles increases fetal peripheral vascular resistance, and on the venous side, it promotes an increase in venous return to the fetal heart. A fall in vagal outflow and an increase in sympathetic stimulation lead to cardiac sympathetic dominant effects. This increases fetal heart rate and ventricular contractility, both of which act in concert with the vasomotor responses to restore fetal arterial blood pressure back to baseline [11,12]. Fetal plasma catecholamines also increase in response to acute hypotension to maintain the neurally-triggered cardiovascular and metabolic sympathetic responses [11-13].

Clinical studies of heart rate variability have provided evidence to support that the growth-restricted human fetus also displays a compromised autonomic control of cardiovascular function [14]. These studies suggest increased vulnerability to blood pressure instability, which may predispose these infants to systemic hypoperfusion, reducing oxygen and glucose delivery to the fetal brain [8]. However, whether these effects occur because of chronic hypoxia during pregnancy, or how chronic hypoxia affects fetal cardiovascular defenses to alterations in blood pressure homeostasis and underlying mechanisms are completely unknown.

We have created isobaric hypoxic chambers able to house pregnant ewes for long periods of gestation under highly controlled chronic hypoxic conditions. We have also created a wireless data acquisition system, the CamDAS, able to record *in vivo* cardiovascular data from singleton fetuses being carried by free-moving pregnant ewes within the hypoxic chambers [5, 6, 15]. Fetal cardiovascular data can then be transmitted by Bluetooth technology to a laptop outside the chamber (Fig. S1). In this study, we have used this system to determine the *in vivo* the fetal cardiovascular responses to an episode of acute hypotension in the chronically hypoxic fetus. Underlying mechanisms were explored adopting a three-pronged approach, by: a) constructing fetal *in vivo* cardiac and peripheral vasomotor baroreflex responses to acute hypotension; b) measuring the *in vivo* fetal plasma catecholamine response to acute hypotension; and c) establishing fetal *in vivo* pressor and peripheral vasopressor responses to exogenous bolus doses of the α_1-adrenergic agonist phenylephrine. Further, to investigate the capacity of the chronically hypoxic fetus to maintain perfusion of the brain during acute hypotension, we also calculated *in vivo* changes in oxygen and glucose delivery in the carotid and femoral vascular beds. The study tested the hypothesis that chronic hypoxia significantly impacts on the fetal cardiovascular defense to a superimposed acute hypotensive challenge.

Methods

Data availability. The data underlying this article are available in the article. Any additional data required associated with this article can be made available from the corresponding author.

Ethical approval

All procedures were performed at The Barcroft Centre of the University of Cambridge under the UK Animals (Scientific Procedures) Act 1986 and were approved by the Ethical Review Board of the University of Cambridge. The experimental design was conducted in accordance with the ARRIVE guidelines.

Surgical preparation

Animals were instrumented as previously described [5, 6, 15, 16]. Briefly, 12 Welsh Mountain pregnant ewes carrying singleton male fetuses were surgically instrumented at 116 ± 1 days of gestational age (term is *ca.* 145 days). Food but not water was withheld from the pregnant ewe for 24 h prior to surgery. On the day of surgery, the ewe was transferred to a pre-operative room, where the neck fleece was clipped and anaesthesia was induced via injection of Alfaxan into the jugular vein (1.5-2.5 mg.kg^{-1} Alfaxalone; Jurox Ltd, Worcestershire, UK). The ewe was then placed on her back and intubated (Portex cuffed endotracheal tube; Smiths Medical International Ltd., Kent, UK). Anaesthesia was maintained by spontaneous inhalation of 1.5% isoflurane in O_2 (2 L.min^{-1}; IsoFlo; Abbott laboratories Ltd., Berkshire, UK) and the abdomen, flanks and medial surfaces of the hind limbs were shaved and cleaned. Antibiotics (30 mg.kg^{-1} I.M. procaine benzylpenicillin; Depocillin; Intervet UK Ltd, Milton Keynes, UK) and an analgesic agent (1.4 mg.kg^{-1} S.C. carprofen; Rimadyl; Pfizer Ltd, Kent, UK) were administered.

Following transfer to the surgical theater, general anesthesia (1.5-2.0% isoflurane in 60:40 O_2:N_2O) was maintained using positive pressure ventilation in a non-rebreathing circuit (Datex-Ohmeda Ltd, Hatfield, Hertfordshire, UK). The animal was covered with sterile drapes and midline abdominal and uterine incisions were made to expose the fetal hind limbs, while minimizing loss of amniotic fluid, as

described previously [16-18]. On one side, fetal 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. Another catheter was anchored onto the fetal hind limb skin for recording of the reference amniotic pressure. A Transonic flow probe was positioned around the contra-lateral femoral artery (MC2RS-JSF-WC120-CS12-GCP, Transonic Systems Inc. Ithaca, New York). The uterine incision was closed in layers. Through a second uterotomy, the fetal head was exteriorized. The fetal carotid arteries were isolated and on one side a catheter was inserted. A second Transonic flow probe (MC2RS-JSF-WC120-CS12-GCP) was positioned around the contra-lateral carotid artery [5, 17]. The uterus was closed in layers and catheters and flow probe leads were exteriorized via keyhole incisions in the maternal flank. Catheters were exteriorized on the ewe's right side while the flow probe leads were exteriorized through the ewe's left flank. The maternal abdominal and skin incisions were then closed, as before [5, 6, 15-19]. The maternal femoral vessels on the right leg were then isolated. A Teflon catheter (i.d. 1.0 mm, o.d. 1.6 mm, Altec, UK) was then inserted into the maternal femoral artery and placed in the descending aorta. A maternal femoral venous catheter was inserted (i.d., 0.86 mm; o.d., 1.52 mm; Critchly Electrical Products, NSW, Australia). These catheters were exteriorized through the same keyhole on the ewe's right-side flank, and the skin incision was closed.

While under general anesthesia, the ewe was then fitted with a bespoke jacket housing the wireless Cambridge Data Acquisition System (CamDAS, Maastricht Instruments, Maastricht, The Netherlands; [5, 6, 13]. The catheters were connected to pressure transducers (COBE; Argon Division, Maxxim Medical, Athens, TX, USA) within the pressure box housed in a pocket of the jacket on one side, and the flow probes leads were connected to the flow meter housed within a pocket of the jacket on the other side (Fig. S1). Heart rate was triggered from the arterial blood pressure and flow pulsatile waveforms. Recordings of fetal arterial blood pressure and heart rate, amniotic pressure, as well as carotid and femoral blood flow were then continuously transmitted wirelessly via Bluetooth technology onto a laptop computer and, from this moment on, recorded continuously until post-mortem. The ewe was recovered from anesthesia and extubated when spontaneous breathing returned [5, 6, 15-19] . Typically, surgery lasted just over 2h.

Ewes were housed in individual pens in rooms with a 12h:12h/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 the fetus I.V. and into the amniotic cavity (600 mg in 2 ml 0.9% NaCl, benzylpenicillin; Crystapen, Schering-Plough, Animal Health Division, Welwyn Garden City, UK). Generally, normal feeding patterns were restored within 24-48 h after surgery. Ewes were then randomly allocated to one of two experimental groups: normoxia (n=6) or chronic hypoxia (n=6).

Chronic hypoxia protocol

Ewes allocated to chronic hypoxia were housed in one of four bespoke isobaric hypoxic chambers at The Barcroft Centre of the University of Cambridge (Telstar Ace, Dewsbury, West Yorkshire, UK) [5, 6, 15]. Ambient PO_2, PCO_2, humidity and temperature within each chamber were monitored via sensors and values recorded continuously (Fig. S1). Pregnancies assigned to the chronic hypoxia group were placed inside the chambers under normoxic conditions (11 $L.sec^{-1}$ air, equating to 39.6 $m^3.h^{-1}$) to acclimatize for 2 days. On the 5th post-operative day at 121 ± 1 days of gestation, pregnancies assigned to chronic hypoxia were exposed to *ca*. 10% O_2 by altering the incoming gas mixture to 5 $L.sec^{-1}$ air: 6 $L.sec^{-1}$ N_2. Air and nitrogen provision came from a specially designed air and nitrogen generating system (Domnick Hunter Gas Generation, Gateshead, Tyne & Wear, UK;[5]). Within the chambers, the induction of hypoxia was gradual, achieving 10% O_2 over 24 h. From this day on, chronic hypoxia was maintained at 10.0±0.05 $\%O_2$. Fetal cardiovascular data transmitted wirelessly were recorded onto a laptop kept outside the hypoxic chamber laboratory. In this way, this system permitted continuous *in vivo* recording of fetal cardiovascular function without disturbing the animal's environment and maintaining exposure to chronic hypoxia. Pregnancies allocated to the normoxia group were housed in a barn in floor pens with the same floor area as the hypoxic chambers. Both the normoxia and chronic hypoxia groups of ewes were also fed daily the same bespoke

maintenance diet made up of concentrate pellets and hay (40g nuts/kg and 3g hay/kg; Manor Farm Feeds Ltd; Oakham, Leicestershire, UK) to facilitate the monitoring of maternal food intake.

Daily blood sampling regimen and analysis

Samples (0.3 ml) of maternal and fetal arterial blood were taken daily to determine health and measuring arterial blood gas, acid-base and metabolic status, as before [5, 6, 15-19]. Blood gas and acid base values were measured using an ABL5 blood gas analyser (Radiometer; Copenhagen, Denmark; maternal measurements corrected to 38^{o}C, fetal measurements corrected to 39.5^{o}C). Values for percentage saturation of hemoglobin with oxygen (Sat Hb) and for the concentration of hemoglobin in blood ([Hb]) were determined using a hemoximeter (OSM3; Radiometer). Blood glucose and lactate concentrations were measured using an automated analyser (Yellow Springs 2300 Stat Plus Glucose/Lactate Analyser; YSI Ltd., Farnborough, UK). Values for hematocrit were obtained in duplicate using a microhematocrit centrifuge (Hawksley, UK).

Pressor and peripheral vasopressor responses to the α_1-adrenergic agonist phenylephrine

On the 10th day of exposure to normoxia or chronic hypoxia, fetuses were treated i.v. with bolus doses of phenylephrine (5, 12.5, 25, 37.5 and 50 μg; Sigma UK) administered in random order (Fig. S2). The dose regimen was adapted from previous experiments in our laboratory that resulted in dose-dependent changes in cardiovascular function in late gestation fetal sheep [19]. For chronic hypoxic pregnancies, the fetal phenylephrine doses were administered while the ewes remained inside the hypoxic chambers. For normoxic pregnancies, the fetal phenylephrine doses were administered while the ewes were in a metabolic crate, inside a laboratory. Doses were given in random order when cardiovascular variables were at stable baseline. At least 10 min between doses were allowed for cardiovascular data to return to baseline. Maximal changes from baseline for fetal arterial blood pressure, fetal heart rate, femoral blood flow and femoral vascular resistance were then calculated to each dose of phenylephrine, as before [19, 20]. Cardiovascular function returned to baseline conditions within 1h after the last dose of phenylephrine in all fetuses.

Acute hypotension challenge

The day after dose responses, fetuses were exposed to an acute hypotension challenge (Fig. S2). This consisted of 10 minutes of baseline, 10 minutes of hypotension and 10 minutes of recovery. For chronic hypoxic pregnancies, the acute hypotension challenge occurred while inside the hypoxic chambers. For normoxic pregnancies, the acute hypotension challenge occurred while the animal was in a metabolic crate, inside a laboratory. Acute hypotension was induced via intravenous infusion of sodium nitroprusside (SNP; Sigma, UK, 50μg kg^{-1} min^{-1}). The dosing regimen of nitroprusside was adapted from past experiments in our laboratory, which achieved a significant reduction in arterial blood pressure that triggered compensatory cardiovascular responses [20-22].

Cardiac and peripheral vasomotor baroreflex responses to acute hypotension

Minute by minute average values for fetal arterial blood pressure, fetal heart rate and femoral blood were downloaded continuously throughout the acute hypotension experimental protocol and imported into an Excel spreadsheet. Minute by minute average values for fetal femoral vascular resistance were then calculated using Ohm's approximation and dividing arterial blood pressure (corrected for amniotic pressure) by femoral blood flow, as before [16-19]. Baroreflex analysis was performed by plotting the heart rate against mean arterial blood pressure for each fetus in response to phenylephrine and nitroprusside. The slope of the linear relationship between changes in heart rate and in arterial blood pressure in response to phenylephrine was used to determine the vagal dominant contribution to the fetal cardiac baroreflex response [22].

Fetal plasma catecholamine response to acute hypotension

Paired fetal carotid and femoral arterial blood samples (4 ml) were taken during baseline (-10, -5 min), during acute hypotension (+5, +10 min), and 10 minutes after recovery from hypotension (+20 min). These samples were used to determine blood gas and metabolic status, as before, and for analysis of fetal plasma noradrenaline and adrenaline concentrations (femoral sample only). For the catecholamine assay, blood was dispensed into EDTA-treated tubes, centrifuged for plasma extraction and frozen at -80^{o}C until analysis within 3 months of collection, as described before [23, 24]. An

ELISA kit was used to determine the concentration of fetal plasma noradrenaline and adrenaline, in duplicate, following the manufacturer's instructions (KA1877, Abnova, Taipei, Germany). For noradrenaline, the inter- and intra-assay coefficients of variation were 12.8% and 14.6%, respectively, and the lower limit of detection was 0.05 ng.ml^{-1}. For adrenaline, the inter- and intra-assay coefficients of variation were 9.7% and 15.7%, respectively, and the lower limit of detection was 0.01 ng.ml^{-1}.

Oxygen and glucose delivery in the carotid and femoral vascular beds during acute hypotension

Fetal arterial blood oxygen content (C_aO_2) and regional changes in oxygen delivery ($O_{2,del}$) were calculated according to Equations 1 and 2, as before [5, 25]:

Equation 1. C_aO_2 (mmol.L^{-1}) = [[Hb] x Sat Hb/100]x0.62

Equation 2. O_{2del}, (µmol.min^{-1}) = C_aO_2 x blood flow (ml.min^{-1})

Where [Hb] (g.dl^{-1}) is the blood concentration of haemoglobin, Sat Hb (%) is the percentage oxygen saturation of haemoglobin and where 1 molecule of Hb (M.W. 64,450) binds 4 molecules of oxygen. The contribution of oxygen dissolved in plasma is regarded as negligible [26]. Values for oxygen and for glucose delivery to the fetal carotid and femoral vascular beds were then calculated using Equations 3 and 4, respectively:

Equation 3. Oxygen delivery (mmol.min^{-1}) = O_2 content (mmol.ml^{-1}) x appropriate flow (ml.min^{-1})

Equation 4. Glucose delivery (mmol.min^{-1}) = [Glucose] (mmol.ml^{-1}) x appropriate flow (ml.min^{-1})

After the end all experiments, all animals were transferred to the post-mortem laboratory and humanely killed by overdose of sodium pentobarbitone (0.4 ml.kg^{-1} IV Pentoject; Animal Ltd, York, UK). The fetus was weighed and the placement of flow probes and catheters was verified.

212 **Statistical analysis**

213 Appropriate power calculations derived from previous data sets were performed to determine the
214 minimum sample size required to achieve statistical significance [16-19, 23-27]. The experiments
215 were completed within one experimental season and scientists measuring outcomes were blinded to
216 treatments. All data are expressed as mean ± SEM. Cardiovascular, endocrine and metabolic data
217 were analysed using two-way ANOVA, with repeated measures where appropriate or with the
218 Student's *t* test for unpaired data. When a significant interaction between main effects occurred,
219 differences were compared using the Tukey or Student Newman Keuls *post hoc* tests. For all
220 comparisons, statistical significance was accepted when $P<0.05$.

Results

Maternal and fetal health during baseline and exposure to chronic hypoxia

Basal maternal daily food consumption was not different between groups, and exposure to chronic hypoxia of this duration did not affect maternal food intake [5] or fetal body weight when measured at *post mortem* (Normoxia: 3.05±0.30 *vs.* Chronic hypoxia: 3.21±0.30 kg, P=NS).

Basal values for maternal arterial blood gas, acid-base and metabolic status were not different between groups and they were within the normal range for Welsh Mountain ewes [28]; Fig. 1A). Ewes exposed to chronic hypoxia had a significant reduction in the partial pressure of arterial oxygen (mean±SEM: 106.4 ± 3.7 to 47.3 ± 1.9 mmHg) and in arterial oxygen saturation (mean±SEM: 78.6 ± 5.6%) compared to controls (100.6 ± 0.5%) and their own baseline ($P<0.05$; Fig. 1A). Further, ewes exposed to chronic hypoxia had significantly elevated hematocrit by the end of exposure (mean±SEM: 33.7 ± 1.0 *vs.* 27.9 ± 01.5%). There was no significant change between groups in maternal arterial pH or partial pressure of arterial carbon dioxide (Fig. 1A).

Basal values for fetal arterial blood gas, acid-base and metabolic status were similar between groups and were within the normal range for Welsh Mountain singleton sheep fetuses at this stage of gestation [16-19, 29]; Fig. 1B). Fetuses exposed to chronic hypoxia had a significant reduction from baseline in the partial pressure of arterial oxygen (mean±SEM: 20.8 ± 0.7 to 12.3 ± 1.0 mmHg) and arterial oxygen saturation (mean±SEM: 60.4 ± 2.1 to 34.6 ± 2.7 %, $P<0.05$, Fig. 1B). Fetuses exposed to chronic hypoxia also had significantly elevated hematocrit by the end of exposure (mean±SEM: 36.6 ± 1.5 *vs.* 28.2 ± 2.0, $P<0.05$). There was no change between groups in fetal arterial pH, however chronically hypoxic fetuses showed a significant fall in the partial pressure of arterial carbon dioxide (Fig. 1B). Changes in values for fetal arterial blood pressure, fetal heart rate, fetal carotid blood flow and fetal femoral blood flow during normoxia or chronic hypoxia have previously been reported [5]. In brief, relative to controls, fetuses which were chronically hypoxic showed a smaller increase in arterial blood pressure and a delayed fall in fetal heart rate with advancing

gestation. Further, measurement of blood flow in the carotid and femoral circulation revealed sustained increases during the period of chronic hypoxia [5].

Fetal basal blood gas, acid-base status and cardiovascular function prior to acute experiments

On day 10 of exposure, prior to the phenylephrine dose response experiment, chronically hypoxic fetuses remained with significantly lower values for PaO_2, $PaCO_2$ and SatHb, and significantly elevated values for hematocrit relative to normoxic fetuses (Table S1). Chronically hypoxic fetuses also had significantly reduced basal femoral blood flow and heart rate compared to normoxic fetuses ($P<0.05$; Table S1). Basal values for other fetal metabolic or cardiovascular variables prior to the phenylephrine dose response experiment were not different between groups (Table S1).

Pressor and femoral vasopressor responses to the α_1-adrenergic agonist phenylephrine

In normoxic fetuses, exogenous intravenous treatment with bolus doses of phenylephrine lead to dose-dependent increments from baseline in fetal arterial blood pressure and in fetal femoral vascular resistance, and in dose-dependent decrements from baseline in fetal heart rate and in fetal femoral blood flow (Fig. 2A-D). In chronically hypoxic fetuses, the increments in fetal femoral vascular resistance were markedly diminished (Fig. 2D). In particular, chronically hypoxic fetuses had significant blunting of the femoral vascular resistance response to higher doses of phenylephrine (25μg H:1.5 ± 0.4 *vs.* N: 14.9 ± 6.0 and 50 μg H:7.5 ± 3.2 *vs.* N:36.5 ± 10.0 mmHg.(mL.min^{-1})$^{-1}$; $P<0.05$, Fig. 2D).

Fetal cardiovascular response to an acute hypotension and fetal cardiac baroreflex function

In normoxic fetuses, nitroprusside infusion resulted in a significant fall in arterial blood pressure from baseline (Table S1 and Fig. 3A). The decrease in arterial blood pressure (Δ from baseline -26.3 ± 5.2 mmHg) was accompanied by a significant increase in fetal heart rate (Table S1 and Fig. 3B, Δ from baseline 33.8 ± 9.4 bpm) and in fetal femoral vascular resistance (Fig. 3C, Δ from baseline 0.33 ± 0.2 mmHg.(mL.min^{-1})$^{-1}$; both $P<0.05$). The increase in fetal femoral vascular resistance occurred in

parallel with a return in fetal arterial blood pressure towards baseline. During recovery, while femoral vascular resistance continued to increase, fetal arterial blood pressure and fetal heart rate returned to basal levels in normoxic fetuses (Fig. 3). In marked contrast, in chronically hypoxic fetuses, although the fall in fetal arterial blood pressure was similar (Δ baseline -24.0 ± 2.5 mmHg), in these fetuses fetal heart rate did not increase but decreased (Table S1 and Fig. 3B), and the increase in femoral vascular resistance during acute hypotension and recovery was significantly diminished compared to normoxic fetuses (Fig. 3C). During recovery, chronically hypoxic fetus failed to return fetal arterial blood pressure to basal levels, maintaining a significantly reduced arterial blood pressure compared to normoxic fetuses by the end of the experiment (Δ from baseline -2.9 ± 1.8 *vs.* 2.0 ± 2.0 mmHg, $P<0.05$; Fig.3A). The arterial blood pressure-heart rate relationship in normoxic fetuses showed a normal cardiac baroreflex function with a traditional reverse sigmoidal plot for cardiac autonomic stimulation (Fig. 3D). In marked contrast, the shape of the sympathetic quadrant of the cardiac baroreflex relationship depicting the changes in heart rate in response to decrements in arterial blood pressure induced by fetal treatment with nitroprusside was reversed in chronically hypoxic fetuses (Fig. 3D). Calculation of the slope of the linear plot between changes in fetal arterial blood pressure and heart rate in response to phenylephrine in the vagal quadrant component of the cardiac baroreflex revealed a steeper relationship in chronically hypoxic fetuses relative to controls (Fig. 3E and 3F).

Fetal plasma catecholamine response to acute hypotension

Exposure to chronic hypoxia did not affect fetal plasma catecholamine concentrations during baseline. Therefore, values for fetal basal plasma catecholamines concentrations prior to the acute hypotension experiment were not significantly different between normoxic (Noradrenaline: 841.8 ± 207; Adrenaline 66.4 ± 4 $pg.mL^{-1}$) and chronically hypoxic (Noradrenaline:1170 ± 296; Adrenaline 88.6 ± 14 $pg.mL^{-1}$) fetuses. During acute hypotension, fetal plasma noradrenaline concentrations increased in a similar manner in both normoxic (Δ baseline 2229 ± 486 pg/mL) and chronically hypoxic (Δ baseline 1403 ± 874 pg/mL; Fig. 4A) fetuses. In contrast, the increase in fetal plasma adrenaline concentrations during acute hypotension was significantly enhanced in chronically hypoxic

fetuses compared with normoxic controls (Δ baseline H:711 ± 329 *vs.* N:310 ± 77 pg/mL, P<0.05; Fig. 4B). Both noradrenaline and adrenaline concentrations returned towards baseline levels in all fetuses during the recovery period.

Changes in fetal oxygen and glucose delivery in the carotid and femoral vascular beds

During acute hypotension, fetal pH and arterial blood gases were maintained at basal levels in both normoxic and chronically hypoxic fetuses (Fig. S3). During acute hypotension, carotid blood flow was better maintained than femoral blood flow in both groups of fetuses. However, there were no significant differences from baseline or between groups in either carotid or femoral blood flow (Fig. 5A and 5B). During acute hypotension, oxygen content was similar in both carotid and femoral vascular beds in chronically hypoxic fetuses compared with normoxic fetuses (Fig. 5A and 5B). In contrast, during acute hypotension, blood glucose concentrations increased in both normoxic and chronically hypoxic fetuses, although this only reached significance in both groups in the femoral and not carotid vascular bed (Fig. 5A and 5B). During acute hypotension, oxygen and glucose delivery to both carotid and femoral vascular beds was maintained in normoxic and chronically hypoxic fetuses (Fig. 5A and 5B). When the ratio of oxygen and glucose delivery in the carotid relative to the femoral vascular bed was calculated, both normoxic and chronically hypoxic fetuses showed a significant increase from baseline of similar magnitude in this ratio for both oxygen and glucose delivery during acute hypotension (Fig. 5A and 5B). During recovery, blood glucose in the femoral vascular bed remained similarly elevated from baseline in normoxic and chronically hypoxic fetuses (Fig. 5B), and the ratio of oxygen and glucose delivery in the carotid relative to the femoral vascular bed returned towards baseline in both groups (Fig. 5A and 5B).

Discussion

These data show that chronic hypoxia has a significant impact on the fetal cardiovascular defense to a superimposed acute hypotensive challenge, supporting the hypothesis tested in this study. Markedly impaired baroreflex responses, vagal dominant regulation of the fetal heart during acute hypotension and diminished constrictor reactivity in the peripheral vasculature to α_1-adrenergic stimulation provide *in vivo* evidence of underlying mechanisms explaining the altered cardiovascular responses in the chronically hypoxic fetus. However, the plasma catecholamine response to acute hypotension was not diminished; in fact, the fetal plasma adrenaline response to acute hypotensive stress was markedly enhanced in the chronically hypoxic relative to the normoxic fetus.

Chronic hypoxia induces the activation of the hypoxia inducible factor (HIF), which regulates the expression of hundreds of genes including erythropoietin or *EPO*, which enhances red blood cell production, and this effect is measured by an increase in haematocrit [30]. We show that exposure pregnant ewes to *ca.* 10% O_2 for 10 days led to an increase in maternal and fetal hematocrit levels, confirming HIF activation. This level of chronic hypoxia was chosen as it reduces the fetal PaO_2 in the descending aorta from normal values of *ca*. 20 mmHg to values of 12-14 mmHg. This magnitude of fetal chronic hypoxia is important because such fetal PaO_2 values are similar to those measured by cordocentesis in human fetuses affected by growth restriction, as reported in pioneering clinical studies [31-33]. In the present study, there was also a reduction in fetal $PaCO_2$ in response to chronic hypoxia. This may indicate alterations in the rate of fetal metabolism in the presence of hypoxia, minimizing fetal oxygen consumption with a consequent fall in fetal CO_2 production [5]. Importantly, this level of maternal hypoxia did not affect maternal food intake. Therefore, the adverse effects on the fetal cardiovascular capacity to respond to acute hypotension measured in the present study are those of isolated chronic hypoxia, of levels that are human clinically relevant.

To address mechanisms explaining the weaker peripheral vasoconstrictor response and the reversed heart rate response to acute hypotension in the chronically hypoxic fetus, we determined fetal *in vivo*

pressor and femoral vasopressor responses to exogenous bolus doses of the α_1-adrenergic receptor agonist phenylephrine, we constructed cardiac baroreflex function curves and we measured the fetal *in vivo* endocrine catecholamine response during the acute hypotension challenge. The dose-response experiment in the present study revealed that femoral vasopressor responses to α_1-adrenergic receptor stimulation are markedly blunted in the chronically hypoxic fetus. The cardiac baroreflex analysis showed an absent sympathetic component altogether with enhanced vagal sensitivity regulating cardiac function. Measurement of plasma catecholamines during the acute hypotension experiment further revealed that the fetal plasma catecholamine response is not suppressed; rather the fetal plasma adrenaline response to acute hypotension is enhanced in the chronically hypoxic fetus compared to the normoxic fetus. Combined, therefore, these data show that reduced plasma catecholamine responses could not explain the cardiovascular deficits to acute hypotension. Rather, chronic hypoxia impaired fetal femoral vasopressor responses mediated via α_1-adrenergic receptor stimulation and switched the balance of the autonomic regulation of cardiac function towards vagal dominance during acute hypotension. Clinically, fetal hypotension can be linked with profound fetal hypoxia [34]. Therefore, one could argue that the fall in heart rate during acute hypotension in the chronically hypoxic fetus may result from carotid chemoreceptor reflex activation [3,17] rather than a failed cardiac baroreflex. However, this idea is not supported, since the arterial blood gas data during acute hypotension in the present study show that PaO_2 was not reduced from baseline during the acute hypotension challenge in the chronically hypoxic fetus.

In the human fetus, autonomic function can be assessed non-invasively by measurement of fetal heart rate variability (FHRV). Several investigators have reported that reduced FHRV may be associated with fetal growth restriction and fetal compromise [14, 15, 35, 36]. Therefore, computerized cardiotocography (cCTG) examination, is recommended for antenatal surveillance of human fetuses with suspected placental insufficiency and chronic fetal hypoxia [37]. A recent study in our laboratory using this model of chronic fetal hypoxia in sheep reported that FHRV is reduced by chronic hypoxia, predominantly due to dysregulation of the sympathetic control of the fetal heart [15].

Another comprehensive study in sheep reported that chronic hypoxia increased binding of angiotensin receptors in the fetal brainstem, and this was associated with a fall in spontaneous baroreflex sensitivity and evidence of vagal dominant regulation of heart rate [38]. Finally, an elegant series of studies using long-term exposure of ovine pregnancies to the chronic hypobaric hypoxia of life at high altitude have reported impaired signalling in both β_1- and α_1-adrenergic receptor pathways in the heart and vasculature, respectively, in chronically hypoxic fetal sheep [39, 40]. Combined, data from all these studies are consistent with the findings reported in the present investigation, indicating both alterations in the central autonomic regulation of cardiovascular function as well as changes in the reactivity of cardiovascular target organs to noradrenergic stimulation in the chronically hypoxic fetus. Since several clinical studies have reported that the growth restricted human infant displays compromised autonomic cardiovascular control as well as weak defenses to acute challenges [8, 10], data in the present study support that these adverse effects in the growth restricted fetus are likely to be due to those induced by chronic fetal hypoxia alone. Reduction in indices of FHRV as a result of sympathetic suppression may therefore predict chronically hypoxic fetuses at increased risk of birth asphyxia during acute events and at increased risk of neonatal morbidity and mortality in humans. A reduction in overall FHRV may therefore provide a clinical biomarker indicating that autonomic dysregulation of fetal heart rate control has taken place in a fetus with suspected uteroplacental compromise.

Human clinical studies have also associated reductions in FHRV in growth restricted pregnancies with increased risk of blood pressure instability, predisposing the human infant to systemic hypoperfusion, thereby reducing oxygen and glucose delivery to the fetal brain [8, 41]. For this reason, it was of interest to measure changes in oxygen and glucose delivery to the fetal carotid and femoral vascular beds during acute hypotension in the present study. During acute stress, the fetal circulatory response redistributes the delivery of nutrients away from peripheral and towards essential vascular beds, such as those perfusing the brain [2, 3]. This brain sparing defense can be illustrated by calculating the ratio of oxygen and glucose delivery in the carotid relative to the femoral vascular beds during the

episode of acute stress [3, 5]. Data in the present study show that maintenance of oxygen and glucose delivery towards the fetal brain was not altered in the chronically hypoxic relative to normoxic fetus.

In fetal life, the origin of the increase in both plasma adrenaline and noradrenaline during acute stressful conditions is the adrenal gland, rather than from sympathetic neuronal spill-over [42]. The enhanced plasma adrenaline response to acute hypotensive stress in the chronically hypoxic fetus in the present study is consistent with reports of an enhanced catecholamines response to acute hypoxic stress in chronically hypoxic fetal sheep [43]. Possible mechanisms driving an enhanced plasma adrenaline response to acute stress in the chronically hypoxic fetus may include a sensitized activation of the splanchnic nerve, since a major component of the increase in total catecholamine output from the fetal adrenal gland during acute stress is mediated via stimulation of the splanchnic nerves [42, 44]. In addition, chronic hypoxia may affect the expression and activity of catecholamine synthetic enzymes tyrosine hydroxylase (TH) and phenylethanolamine N-methyltransferase (PNMT) (see [45]). The functional significance of the increase in plasma adrenaline in response to acute stress in the late gestation fetus is to inhibit insulin while stimulating glucagon, decrease glucose uptake by the fetal tissues and mobilize the increased blood glucose levels, prioritizing glucose delivery to essential organs like the fetal brain [46, 47].

It is of interest that despite a weaker cardiac and vasomotor baroreflex response to acute hypotension and impaired peripheral vascular reactivity to α_1-adrenergic agonists, oxygen and glucose delivery to the fetal brain was maintained during acute hypotensive stress in the chronically hypoxic fetus. It is possible that the enhanced fetal plasma adrenaline response in the chronically hypoxic fetus is an adaptive response, attempting to compensate for the poor cardiac sympathetic response while ensuring adequate delivery of glucose to the fetal brain during acute hypotension. Therefore, the chronically hypoxic fetus may adopt alternative defense mechanisms to maintain brain sparing, at least in response to short term challenges in blood pressure homeostasis. The magnitude of the fall in fetal arterial blood pressure during acute hypotension in the present study is similar or even less severe than what might be expected to be induced by single or repeated umbilical cord compression in the

clinical setting (see [34]). How the chronically hypoxic fetus would respond to episodes of similar or alternative forms of stress of greater severity and/or longer duration and/or increased frequency is clearly a rich avenue of future research.

There are several advantages and limitations of using sheep as the species of choice for such investigations in fetal cardiovascular physiology. An advantage is that sheep and humans share more similar prenatal developmental milestones of cardiovascular anatomy and physiology, in contrast to rodents which are born highly immature [48, 49]. In addition, the chronically instrumented fetal sheep preparation is the only established animal model that permits surgical implantation of catheters and flow probes for prolonged cardiovascular recording, yielding a physiologically robust, long-term preparation. This level of insight does not exist for any other species. Further, sheep and humans give birth primarily to singleton or twin offspring, in contrast to rodents and pigs that give birth to litters. Hence, the maternal metabolic investment in the pregnancy is likely more similar between sheep and humans, relative to many other species, as multiple pregnancies increase the maternal basal metabolic rate [50]. In contrast, there are important limitations of the current study. One obvious one is cost of using sheep for long term studies relative to using rodents. The study design therefore controlled for, but it did not address sex differences. Limitations in cost also reduces the number of animals per group, thereby increasing the number of experimental comparisons that are likely underpowered for statistical analysis.

In conclusion, this study has permitted *in vivo* continuous recording of cardiovascular function in fetuses undergoing highly controlled chronic hypoxia and exposed to an acute secondary stressor, highlighting the strength of the conceptual advance to the field of study. We show that the chronically hypoxic fetus displays markedly altered cardiovascular responses to an acute hypotensive stress. Such effects may explain the explain the well-known greater susceptibility of the human growth restricted fetus, presumed to be chronically hypoxic, to a second hit.

Perspectives

This study shows what happens when a chronically hypoxic fetus is exposed to a further stressor *in utero*. Clinically, such a situation could arise in human pregnancy when a growth-restricted fetus is exposed to excessive uterine contractions in labor, acute cord compression or an acute placental hemorrhage event. In the clinic, this can lead to acute decompensation of fetal cardiovascular defenses, failure to maintain fetal arterial blood pressure, culminating in severe hypoxic ischemic encephalopathy with subsequent fetal brain damage. This situation is difficult to recreate *in vivo* in an experimental animal model. Here, we have exploited novel terchnology recently available to induce a controlled period of chonic hypoxia in sheep pregnancy at levels of human clinical relevance and investigated the effects of a superimposed second hit in the form of an acute hypotensive challenge. We show that chronic hypoxia has a significant effect on the fetal cardiovascular defense responses to a second hit. This provides *in vivo* evidence of mechanisms linking the greater susceptibility of the chronically hypoxic fetus to superimposed stress, and adds to the body of evidence behind the cause of brain damage after fetal growth restriction. Antenatal and intrapartum periods of hypoxia leading to collapse of fetal cardiovascular defenses are important causes of fetal brain damage and common grounds for litigation in many national health systems. Therefore, the data reported in this manuscript may help to diagnose the compromised fetus where uteroplacental dysfunction is suspected to try and improve neonatal outcome.

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Disclosures

License agreement 100395 CamDAS: Technology for simultaneous wireless recording of arterial blood pressure and blood flow in large animals. Giussani, D.A., Maatricht Instruments, The British Heart Foundation and Cambridge Enterprise. There are no conflict of interests declared by any author.

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Novelty and Significance

What is new?

- The growth restricted fetus, presumed to be chronically hypoxic, is at a seven-fold risk of acute complications later in gestation. However, the reason behind this is unknown.
- We show that chronic hypoxia substantially alters the fetal cardiovascular defense to a second hit, providing an explanation for this well established vulnerability.

What is relevant?

- Adverse conditions during pregnancy can trigger a fetal origin of increased disease risk in the offspring. This study shows that chronic hypoxia, one of the most common complications in human pregnancy, substantially affects fetal defense mechanisms, increasing a future risk of cardiovascular and neurodevelopmental dysfunction after birth.

Summary

- Chronic hypoxia has a signigicant impact on the fetal cardiovascular defense to a second hit.

Legends

Figure 1. Maternal and fetal blood gases during chronic hypoxia. Values are mean±S.E.M. for the maternal (A) and fetal (B) arterial blood gas status in sheep undergoing normoxic (○, n=6) or chronic hypoxic (●, n=6) pregnancy. pH, arterial pH; $PaCO_2$, arterial CO_2 partial pressure; Htc, hematocrit; PaO_2, arterial O_2 partial pressure; Sat [Hb], percentage saturation of hemoglobin. The results of the two-way RM ANOVA for main effects and interactions are shown. When a significant ($P<0.05$) interaction between main effects occurred, differences were compared using the Tukey *post hoc* test. * indicates a significant effect of time compared with baseline; † indicates a significant effect of treatment compared with normoxic pregnancy.

Figure 2. Pressor and femoral vasopressor responses to phenylephrine in the chronically hypoxic fetus. Values are the mean ± SEM for the change from baseline in fetal arterial blood pressure (A), heart rate (B), femoral blood flow (C) and femoral vascular resistance (D) in response to increasing doses of phenylephrine (5, 12.5, 25 and 50 μg) in normoxic (open bars, n=6) and chronically hypoxic (grey bars, n=6) fetuses. The results of the two-way RM ANOVA for main effects and interactions are shown. When a significant ($P<0.05$) interaction between main effects occurred, differences were compared using the Newman–Keuls *post hoc* test. Different letters indicate a significant effect of dose within groups (normoxic a-c, hypoxic x-z; † indicates a significant effect of treatment compared with normoxic pregnancy.

Figure 3. Cardiovascular responses to acute hypotension and cardiac baroreflex function in the chronically hypoxic fetus. Panels A-C show the mean±S.E.M. values for the change from baseline in arterial blood pressure (A), heart rate (B) and femoral vascular resistance (C) in normoxic (○, n=6) or chronically hypoxic (●, n=6) fetuses during the acute hypotension experiment. Acute hypotension (dashed box) was induced for 10 minutes by fetal i.v. infusion with sodium nitroprusside (2.5 mg/kg/min). Panels D-F show an analysis of cardiac baroreflex function. The fetal arterial blood pressure-heart rate relationship was constructed by plotting the change from baseline in both variables

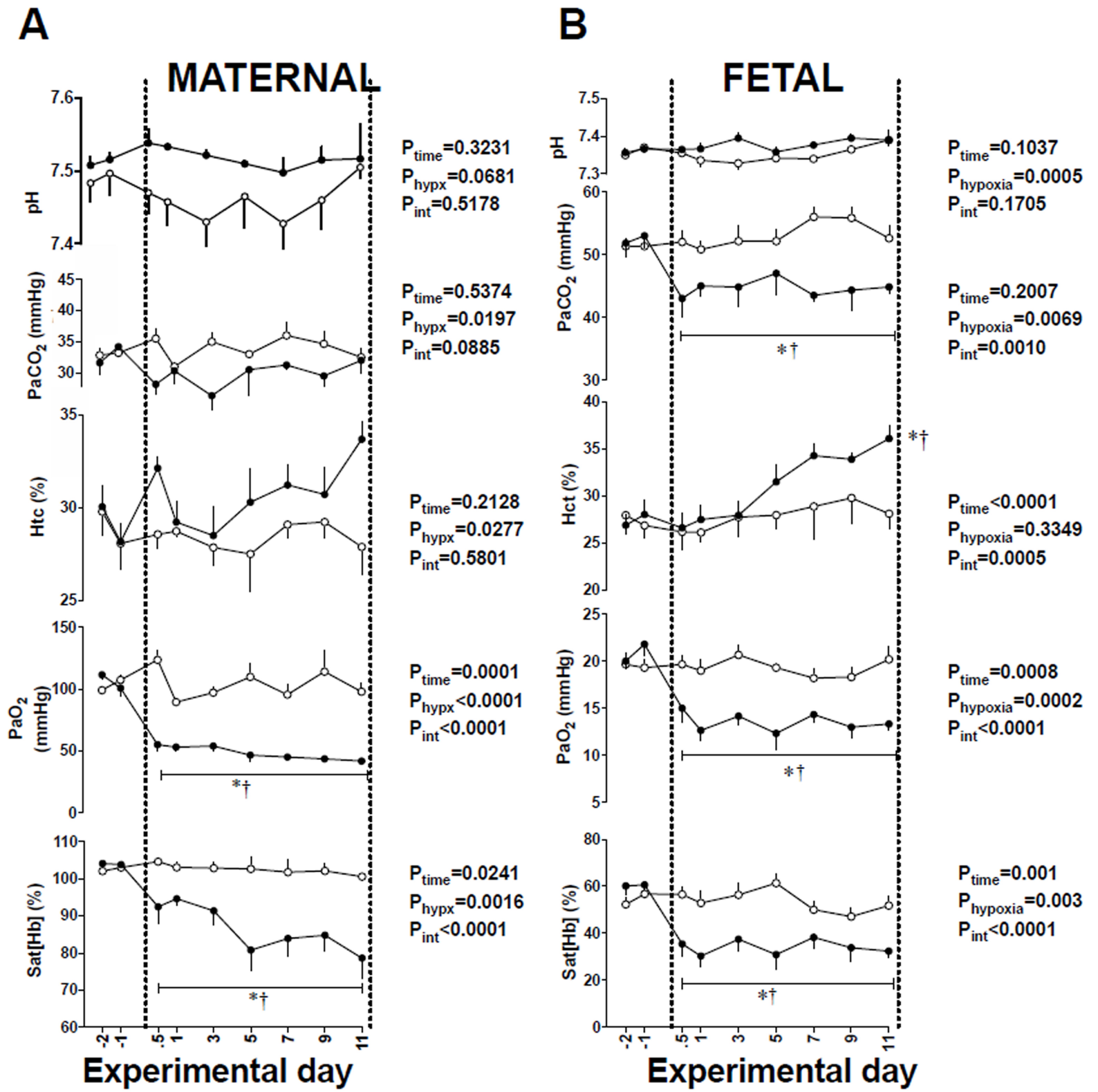
493 mean±S.E.M. (x and y) in response to sodium nitroprusside or to fetal treatment with increasing doses
494 of phenylephrine (D). The vagal dominant component of the cardiac baroreflex is expanded in Panel
495 E (mean±S.E.M. for x and y). Panel F show the mean±S.E.M. values for an analysis of slopes for the
496 vagal dominant component of the cardiac baroreflex. The results of the two-way RM ANOVA for
497 main effects and interactions for A-C are shown. When a significant ($P<0.05$) interaction between
498 main effects occurred, differences were compared using the Tukey *post hoc* test. * indicates a
499 significant effect of time compared with baseline; † indicates a significant effect of treatment
500 compared with normoxic pregnancy. Groups in Panel F were compared by the Student's *t* test for
501 unpaired data. † indicates a significant effect of treatment compared with normoxic pregnancy.

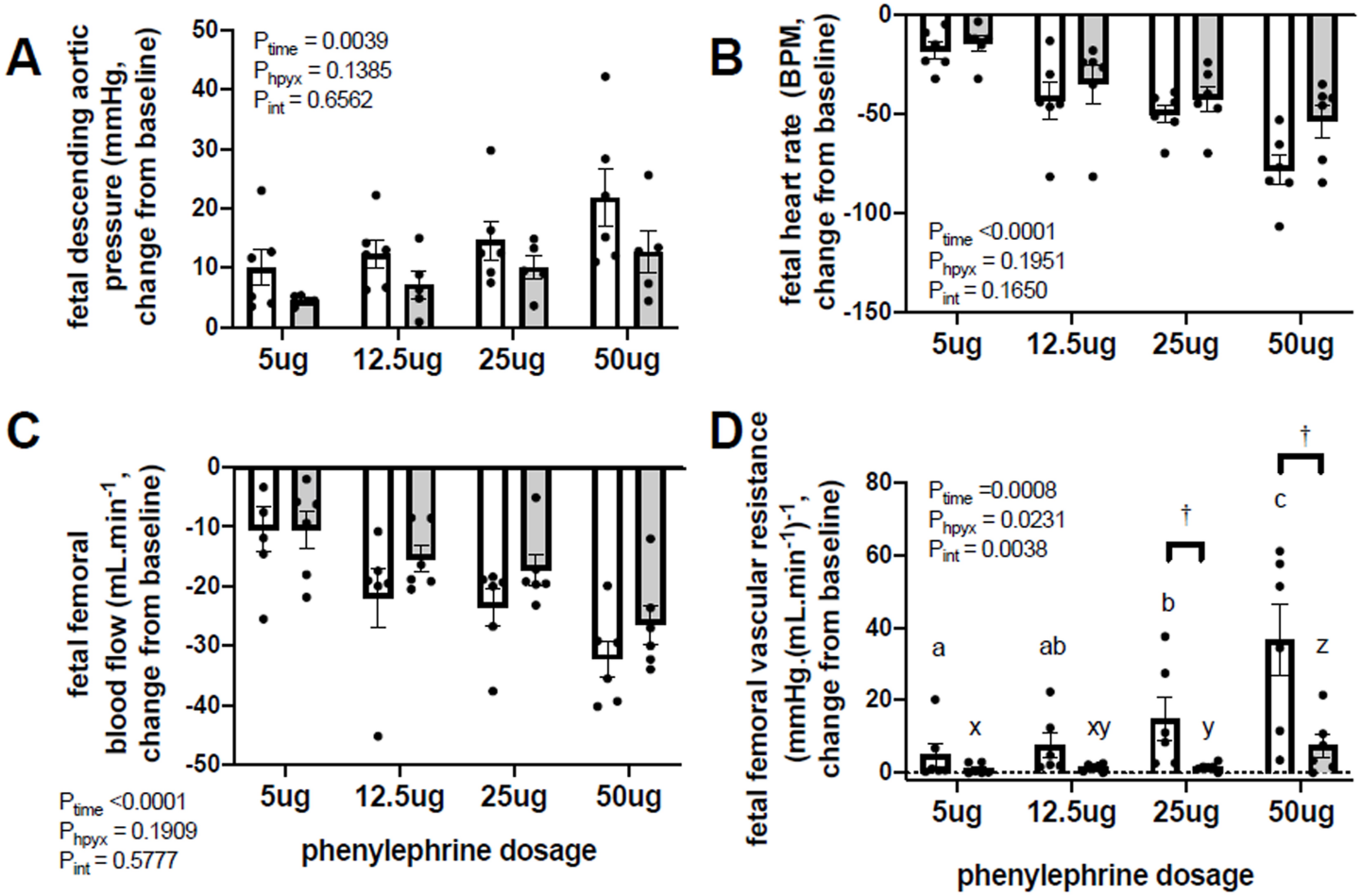
502 **Figure 4. Plasma catecholamine response to acute hypotension in the chronically hypoxic fetus.**
503 Values are mean±S.E.M. for the change from baseline in noradrenaline (A) and adrenaline (B) in
504 normoxic (○, n=6) or chronically hypoxic (●, n=6) fetuses during the acute hypotension experiment.
505 Acute hypotension (dashed box) was induced for 10 minutes by fetal i.v. infusion with SNP (2.5
506 mg/kg/min). Plasma samples were taken during baseline (-10 and -5 min), during hypotension (+5
507 and +10 min) and after 10 min of recovery (+20 min). The results of the two-way RM ANOVA for
508 main effects and interactions are shown. When a significant ($P<0.05$) interaction between main
509 effects occurred, differences were compared using the Tukey *post hoc* test. * indicates a significant
510 effect of time compared with baseline; † indicates a significant effect of treatment compared with
511 normoxic pregnancy.

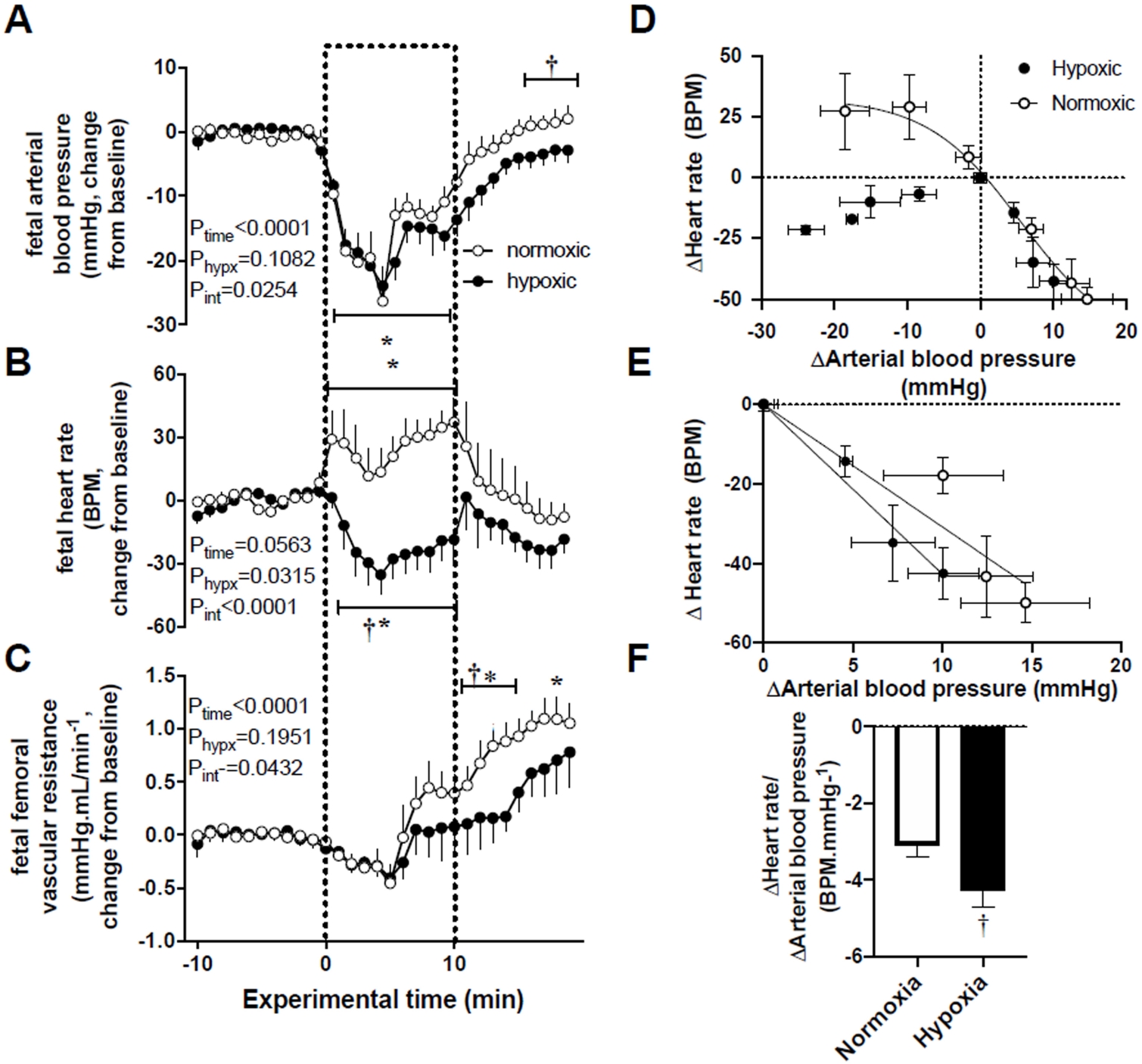
512 **Figure 5. Changes in oxygen and glucose delivery in the carotid and femoral vascular beds**
513 **during acute hypotension in the chronically hypoxic fetus.** Values are mean±S.E.M. for blood
514 flow, oxygen content, blood glucose concentration, oxygen delivery and glucose delivery in the
515 carotid (A) and femoral (B) vascular beds. The ratio of oxygen and glucose delivery in the carotid
516 relative to the femoral vascular beds is also calculated. Groups are normoxic (○, n=6) or chronically
517 hypoxic (●, n=6) fetuses. Acute hypotension (dashed box) was induced for 10 minutes by fetal i.v.
518 infusion with SNP (2.5 mg/kg/min). The results of the two-way RM ANOVA for main effects and

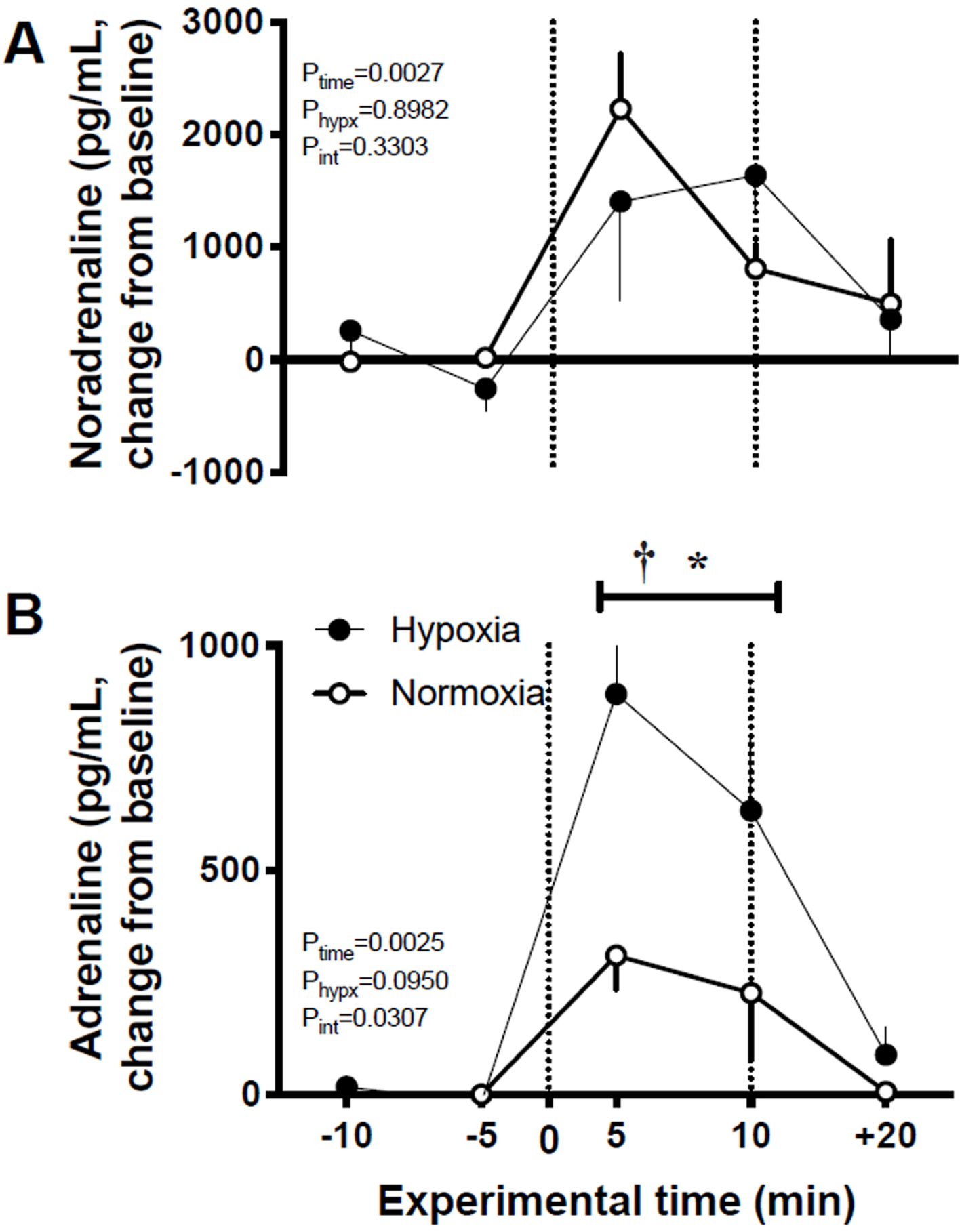
519 interactions are shown. There no significant interactions between main effects.

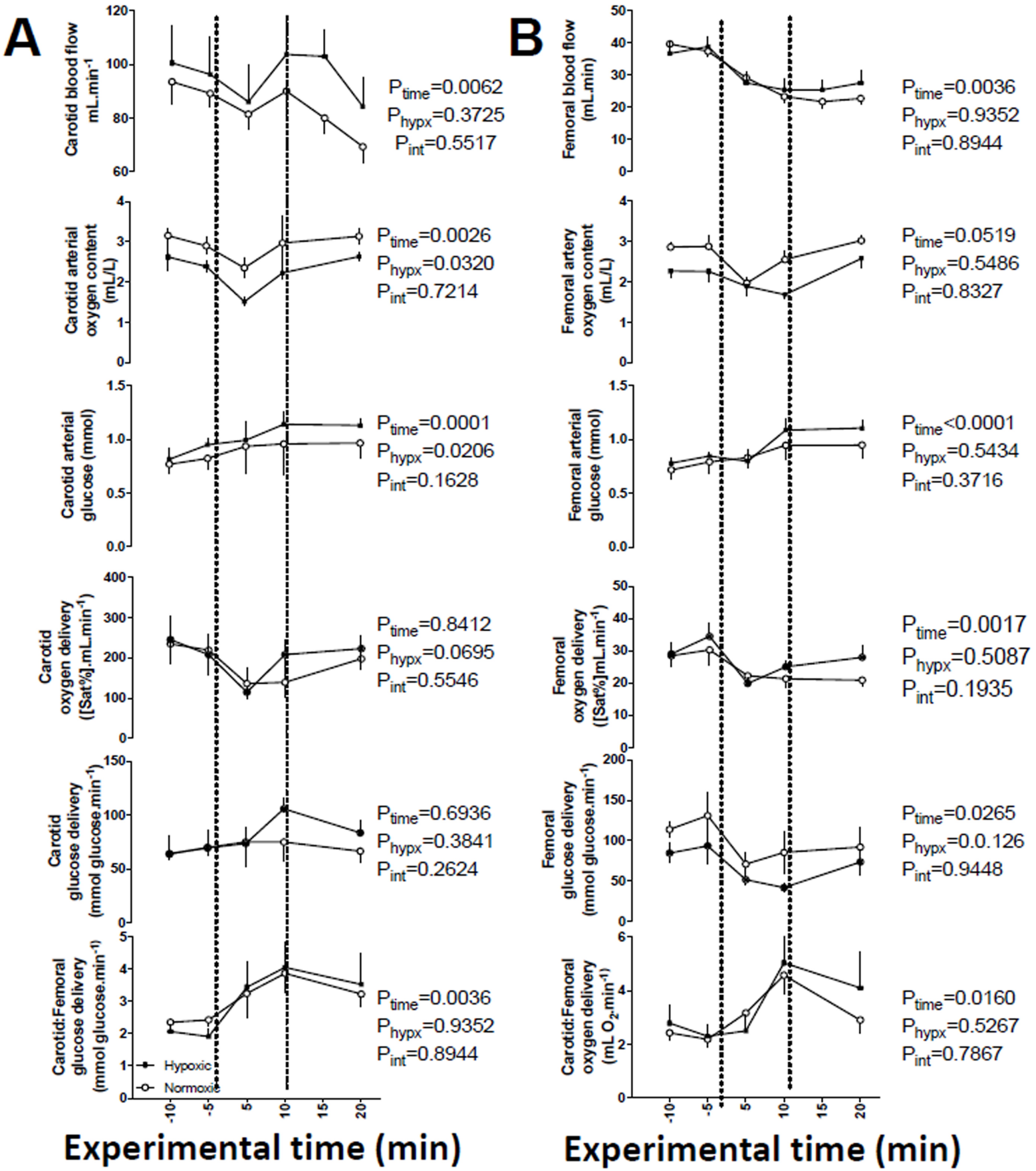
520 **Figure 6. Summary Figure.** During acute hypotension, the normoxic fetus in healthy pregnancy
521 activates cardiac and vasomotor baroreflex responses to restore blood pressure homeostasis. This
522 involves baroreflex alteration in autonomic outflow via the brainstem. Increased sympathetic outflow
523 coupled with vagal withdrawal to the heart promotes cardiac sympathetic dominant effects, which
524 increase heart rate and contractility via β_1-adrenergic receptor pathways. Enhanced sympathetic
525 outflow to the arterioles increases peripheral vascular resistance via a1-adrenergic receptor pathways.
526 Enhanced sympathetic outflow to the veins increases venous return via α_1-adrenergic receptor
527 pathways. The increase in venous return, coupled with increased myocardial contractility lead to an
528 increase in cardiac output. The increase in peripheral vascular resistance together with the increase in
529 cardiac output restore fetal arterial blood pressure back to baseline. In the chronically hypoxic fetus,
530 baroreflex-induced increases in heart rate do not occur. Instead, there is a fall in heart rate due to
531 increased vagal dominant effects. In addition, responsiveness to α_1-adrenergic receptor pathway
532 stimulation is blunted in the peripheral vasculature. Combined, these effects of chronic hypoxia on
533 the heart and circulation weaken the fetal cardiovascular defence to acute hypotension in the
534 chronically hypoxic fetus, providing a mechanistic link underlying its greater susceptibility to a
535 second hit.

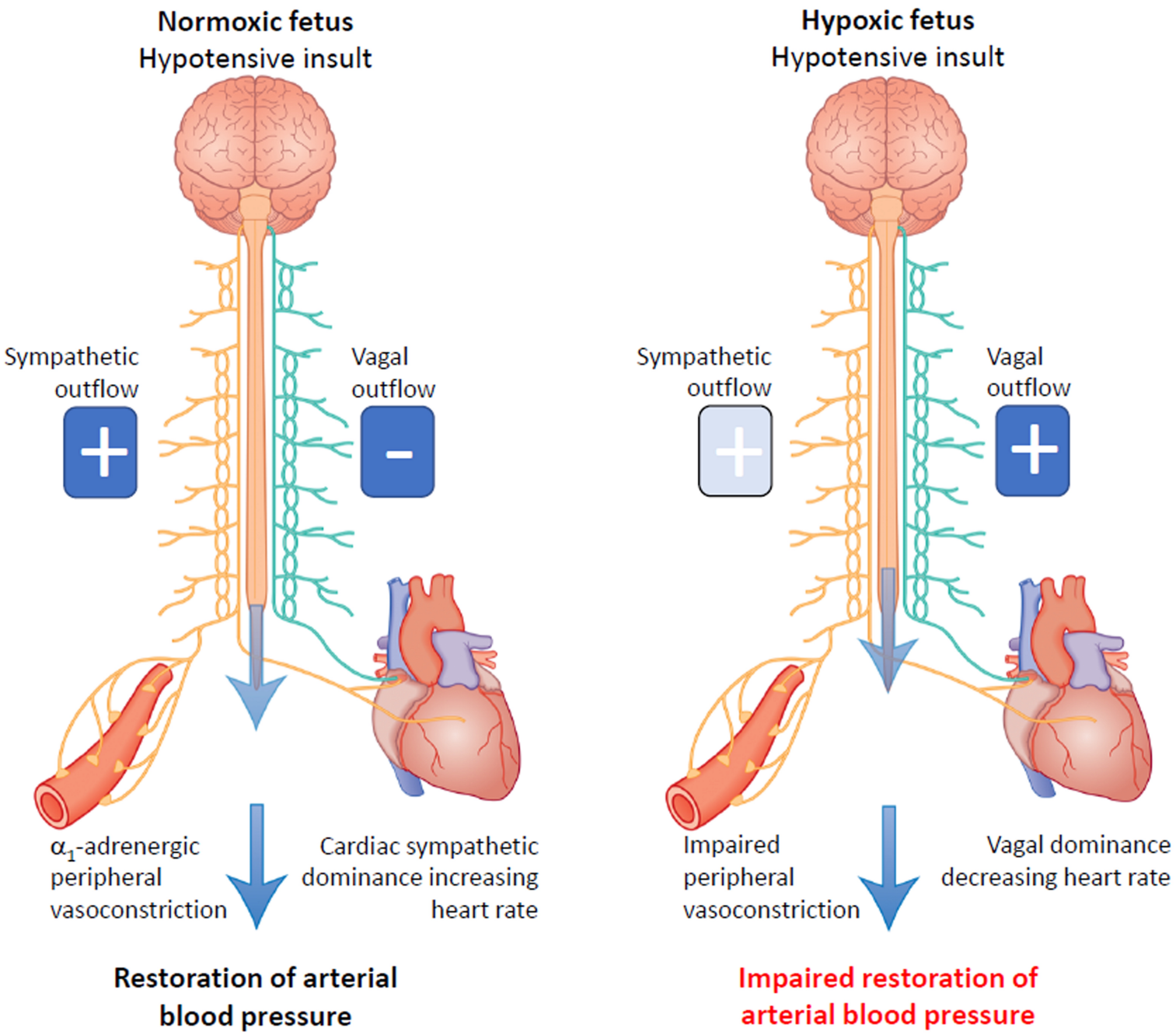

A
MATERNAL
pH
7.6
7.5
7.4
P_{time}=0.3231
P_{hypx}=0.0681
P_{int}=0.5178
$PaCO_2$ (mmHg)
45
40
35
30
P_{time}=0.5374
P_{hypx}=0.0197
P_{int}=0.0885
Htc (%)
35
30
25
P_{time}=0.2128
P_{hypx}=0.0277
P_{int}=0.5801
PaO_2 (mmHg)
150
100
50
0
*†
P_{time}=0.0001
P_{hypx}<0.0001
P_{int}<0.0001
Sat[Hb] (%)
110
100
90
80
70
60
*†
P_{time}=0.0241
P_{hypx}=0.0016
P_{int}<0.0001
-2 -1 .5 1 3 5 7 9 11
Experimental day
B
FETAL
pH
7.5
7.4
7.3
P_{time}=0.1037
$P_{hypoxia}$=0.0005
P_{int}=0.1705
$PaCO_2$ (mmHg)
60
50
40
30
*†
P_{time}=0.2007
$P_{hypoxia}$=0.0069
P_{int}=0.0010
Hct (%)
40
35
30
25
20
*†
P_{time}<0.0001
$P_{hypoxia}$=0.3349
P_{int}=0.0005
PaO_2 (mmHg)
25
20
15
10
5
*†
P_{time}=0.0008
$P_{hypoxia}$=0.0002
P_{int}<0.0001
Sat[Hb] (%)
80
60
40
20
0
*†
P_{time}=0.001
$P_{hypoxia}$=0.003
P_{int}<0.0001
-2 -1 .5 1 3 5 7 9 11
Experimental day


A
fetal descending aortic pressure (mmHg, change from baseline)
P_{time} = 0.0039
P_{hpyx} = 0.1385
P_{int} = 0.6562
50
40
30
20
10
0
5ug
12.5ug
25ug
50ug
B
fetal heart rate (BPM, change from baseline)
0
-50
-100
-150
P_{time} <0.0001
P_{hpyx} = 0.1951
P_{int} = 0.1650
5ug
12.5ug
25ug
50ug
C
fetal femoral blood flow (mL.min^{-1}, change from baseline)
0
-10
-20
-30
-40
-50
P_{time} <0.0001
P_{hpyx} = 0.1909
P_{int} = 0.5777
5ug
12.5ug
25ug
50ug
phenylephrine dosage
D
fetal femoral vascular resistance (mmHg.(mL.min^{-1})$^{-1}$, change from baseline)
80
60
40
20
0
P_{time} =0.0008
P_{hpyx} = 0.0231
P_{int} = 0.0038
a
x
ab
xy
b
y
c
z
†
†
5ug
12.5ug
25ug
50ug
phenylephrine dosage


A
fetal arterial blood pressure (mmHg, change from baseline)
10
0
-10
-20
-30
†
P time<0.0001
P hypx=0.1082
P int=0.0254
normoxic
hypoxic
*
*
B
fetal heart rate (BPM, change from baseline)
60
30
0
-30
-60
P time=0.0563
P hypx=0.0315
P int<0.0001
†*
C
fetal femoral vascular resistance (mmHg.mL/min-1, change from baseline)
1.5
1.0
0.5
0.0
-0.5
-1.0
P time<0.0001
P hypx=0.1951
P int-=0.0432
†*
*
-10
0
10
Experimental time (min)
D
ΔHeart rate (BPM)
50
25
0
-25
-50
Hypoxic
Normoxic
-30
-20
-10
0
10
20
ΔArterial blood pressure (mmHg)
E
Δ Heart rate (BPM)
0
-20
-40
-60
0
5
10
15
20
ΔArterial blood pressure (mmHg)
F
ΔHeart rate/ ΔArterial blood pressure (BPM.mmHg-1)
0
-2
-4
-6
†
Normoxia
Hypoxia


A
Noradrenaline (pg/mL, change from baseline)
3000
2000
1000
0
-1000
P time=0.0027
P hypx=0.8982
P int=0.3303
B
Adrenaline (pg/mL, change from baseline)
1000
500
0
Hypoxia
Normoxia
†
*
P time=0.0025
P hypx=0.0950
P int=0.0307
-10
-5
0
5
10
+20
Experimental time (min)


A
Carotid blood flow mL.min-1
Ptime=0.0062
Phypx=0.3725
Pint=0.5517
Carotid arterial oxygen content (mL/L)
Ptime=0.0026
Phypx=0.0320
Pint=0.7214
Carotid arterial glucose (mmol)
Ptime=0.0001
Phypx=0.0206
Pint=0.1628
Carotid oxygen delivery ([Sat%].mL.min-1)
Ptime=0.8412
Phypx=0.0695
Pint=0.5546
Carotid glucose delivery (mmol glucose.min-1)
Ptime=0.6936
Phypx=0.3841
Pint=0.2624
Carotid:Femoral glucose delivery (mmol glucose.min-1)
Ptime=0.0036
Phypx=0.9352
Pint=0.8944
Hypoxic
Normoxic
Experimental time (min)
B
Femoral blood flow (mL.min)
Ptime=0.0036
Phypx=0.9352
Pint=0.8944
Femoral artery oxygen content (mL/L)
Ptime=0.0519
Phypx=0.5486
Pint=0.8327
Femoral arterial glucose (mmol)
Ptime<0.0001
Phypx=0.5434
Pint=0.3716
Femoral oxygen delivery ([Sat%]mL.min-1)
Ptime=0.0017
Phypx=0.5087
Pint=0.1935
Femoral glucose delivery (mmol glucose.min-1)
Ptime=0.0265
Phypx=0.0.126
Pint=0.9448
Carotid:Femoral oxygen delivery (mL O2.min-1)
Ptime=0.0160
Phypx=0.5267
Pint=0.7867
Experimental time (min)


Normoxic fetus
Hypotensive insult
Sympathetic outflow
Vagal outflow
+
−
α1-adrenergic peripheral vasoconstriction
Cardiac sympathetic dominance increasing heart rate
Restoration of arterial blood pressure
Hypoxic fetus
Hypotensive insult
Sympathetic outflow
Vagal outflow
+
+
Impaired peripheral vasoconstriction
Vagal dominance decreasing heart rate
Impaired restoration of arterial blood pressure

DATA SUPPLEMENT

Altered Cardiovascular Defense to Hypotensive Stress in the Chronically Hypoxic Fetus

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	pH	PaO_2 (mmHg)	$PaCO_2$ (mmHg)	Sat[Hb] (%)	Hct (%)	Glucose (mmol)	Lactate (mmol)	BP (mmHg)	HR (BMP)	FBF ($mL.min^{-1}$)	CBF ($mL.min^{-1}$)	Oxygen Content ($mmol.L^{-1}$)
N	7.38 ± 0.01	19.5 ± 1.06	53.0 ± 1.0	58.4 ± 6.1	28.1 ± 2.7	0.701 ± 0.10	1.16 ± 0.17	39.0 ± 3.5	164.6 ± 4.7	45.6 ± 2.3	92.9 ± 8.8	2.87 ± 0.13
H	7.41 ± 0.02	14.5 ± 0.4 †	41.5 ± 1.9†	39.3 ± 3.5†	35.1 ± 1.1†	0.810 ± 0.07	1.87 ± 0.38	37.5 ± 3.3	150.0 ± 3.9 †	36.7 ± 1.6†	97.4 ± 8.2	2.26 ± 0.14 †

Table S1. Basal fetal arterial blood gas, metabolic status and cardiovascular function prior to acute experiments. Values are mean±S.E.M. for fetal arterial blood pH, arterial partial pressure of oxygen (PaO_2), arterial partial pressure of carbon dioxide ($PaCO_2$), percentage saturation of haemoglobin with oxygen (Sat[Hb]), blood glucose and lactate concentrations, arterial blood pressure (BP), heart rate (HR), femoral blood flow (FBF) and carotid blood flow (CBF) in normoxic (○, n=6) or chronic hypoxic (●, n=6) fetuses prior to acute experiments. Significant differences ($P<0.05$) are: †, differences indicating a significant effect of treatment compared with normoxic pregnancy (Student's t test for unpaired data).

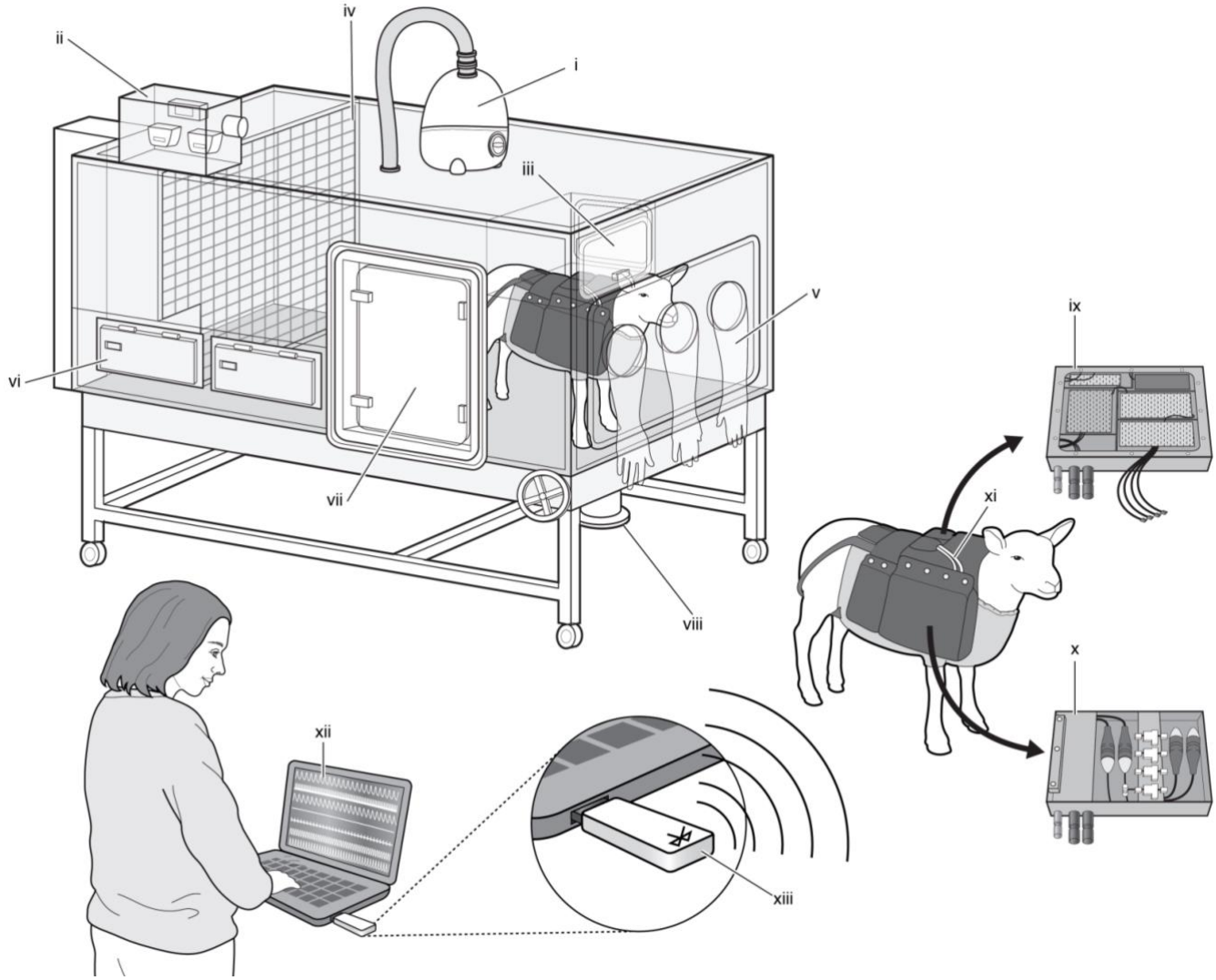

Figure S1. Hypoxic chambers and the camDAS system. Each chamber contained an electronic servo-controlled humidity cool steam injection system to maintain appropriate humidity to the inspirate (i). Ambient PO_2, PCO_2, humidity and temperature within each chamber were monitored via sensors (ii). For experimental procedures, each chamber had a double transfer port (iii) to internalise material and a manually operated sliding panel (iv) to bring the ewe into a position where daily sampling of blood could be achieved through glove compartments (v). Each chamber had a drinking bowl on continuous water supply and a rotating food compartment (vi) for determining food intake. A sealed transfer isolation cart could be attached to a side exit (vii) to couple chambers together for cleaning or for animal movement. Animal waste was collected through an outlet containing a sealing

valve (viii). The camDAS system was contained in a custom-made sheep jacket able to hold a miniaturized Transonic flow box (ix) on one side, and a pressure box (x) on the other. Cables (xi) connected the flow and pressure boxes to two battery packs able to power the system for >24 hours. Measurements made using the data acquisition were transmitted wirelessly to a laptop kept outside the chamber (xii) via Bluetooth technology (xiii), thereby allowing continuous monitoring of fetal cardiovascular function *in vivo* in the chronically hypoxic fetus. Reproduced with permission [5].

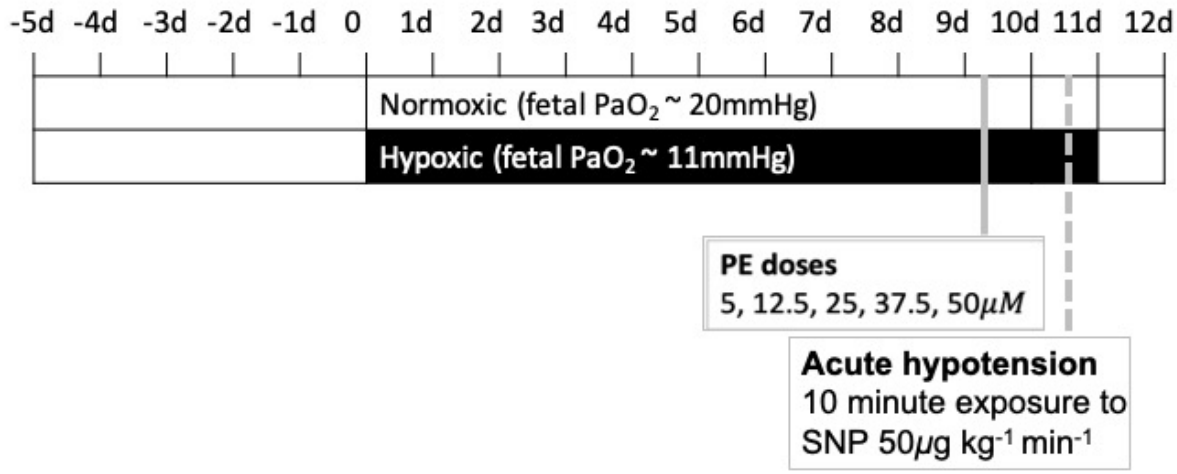

Figure S2. Experimental design. The experimental protocol consisted of 5 days (d) of baseline recording followed by either chronic normoxia (white bar, n=6) or chronic hypoxia (black bar, n=6, %PO_2 ~10.5 mmHg). On day 10 of exposure, fetuses received increasing bolus doses of phenylephrine (PE; 5, 12.5, 25, 37.5 and 50µg) administered in random order. The following day, fetuses were exposed to an acute hypotension experiment via infusion of sodium nitroprusside (SNP; 2.5µg/kg/min).

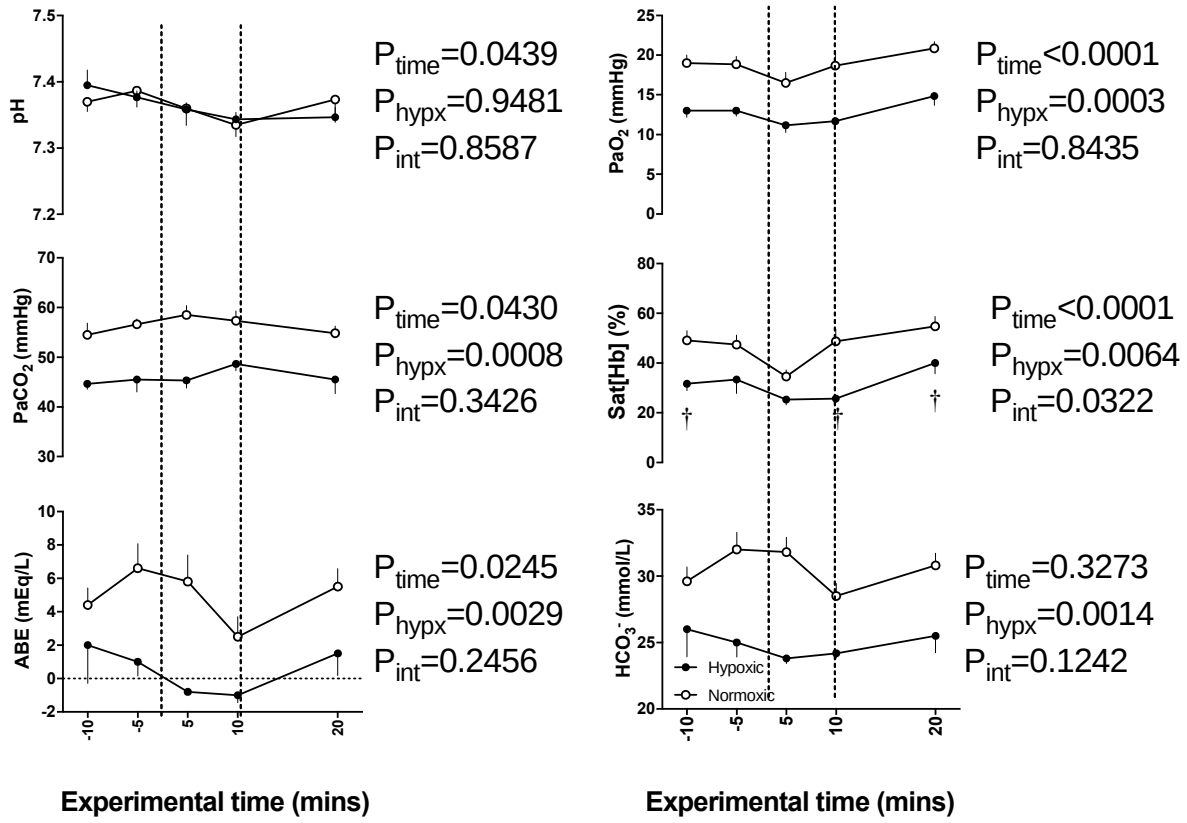

Figure S3. Arterial blood gas status during acute hypotension in the chronically hypoxic fetus. Values are mean±S.E.M. for arterial pH (A); $PaCO_2$, arterial CO_2 partial pressure (B); PaO_2, arterial O_2 partial pressure (C); Sat[Hb], percentage saturation of hemoglobin (D), ABE, Acid-base excess (E) and HCO_3^-, bicarbonate (F) in fetal sheep during acute hypotension in normoxic (○, n=6) or chronic hypoxic (●, n=6) pregnancy. The results of the two-way RM ANOVA for main effects and interactions are shown. When a significant ($P<0.05$) interaction between main effects occurred, differences were compared using the Tukey *post hoc* test. * indicates a significant effect of time compared with baseline; † indicates a significant effect of treatment compared with normoxic pregnancy.