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A role for xanthine oxidase in the control of fetal cardiovascular function in late gestation sheep

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Key points

- There is growing physiological and clinical interest in the role of the enzyme xanthine oxidase in the regulation of fetal cardiovascular function.
- The xanthine oxidase inhibitor allopurinol is undergoing human clinical trials in complicated pregnancy to protect the fetal brain from injury by decreasing excessive generation of reactive oxygen species (ROS) and increasing nitric oxide (NO) availability. However, the effects on fetal cardiovascular physiology of xanthine oxidase inhibition are largely unknown.
- We have previously reported that the balance between ROS and NO plays an important physiological role in the control of fetal cardiovascular function. Therefore, it seems likely that allopurinol might perturb this balance and alter fetal cardiovascular homeostasis.
- Here, we report that maternal allopurinol treatment in late gestation ovine pregnancy has significant *in vivo* effects on umbilical blood flow and the cardiovascular system of the mother and fetus by altering NO and β_1-adrenergic mechanisms.
- The evidence suggests that xanthine oxidase has an important role in basal cardiovascular function in the fetus during late gestation. Therefore, further research is warranted before safe clinical application of maternal allopurinol during pregnancy in humans.

Abstract Virtually nothing is known about the effects on fetal physiology of xanthine oxidase inhibition. This is despite maternal treatment with the xanthine oxidase inhibitor allopurinol being considered in human complicated pregnancy to protect the infant's brain from excessive generation of ROS. We investigated the *in vivo* effects of maternal treatment with allopurinol on fetal cardiovascular function in ovine pregnancy in late gestation. Under anaesthesia, pregnant ewes and their singleton fetus were instrumented with vascular catheters and flow probes around an umbilical and a fetal femoral artery at 118 ± 1 dGA (days of gestational age; term *ca.* 145 days). Five days later, mothers were infused I.V. with either vehicle ($n = 11$) or allopurinol ($n = 10$). Fetal cardiovascular function was stimulated with increasing bolus doses of phenylephrine (PE) following maternal vehicle or allopurinol. The effects of maternal allopurinol on maternal and fetal cardiovascular function were also investigated following fetal NO blockade ($n = 6$) or fetal β_1-adrenergic antagonism ($n = 7$). Maternal allopurinol led to significant increases in fetal heart rate, umbilical blood flow and umbilical vascular conductance, effects abolished by fetal β_1-adrenergic antagonism but not by fetal NO blockade. Maternal allopurinol impaired fetal α_1-adrenergic pressor and femoral vasopressor responses and enhanced the gain of the fetal cardiac baroreflex. These effects of maternal allopurinol were restored to control levels during fetal NO blockade. Maternal treatment with allopurinol induced maternal hypotension, tachycardia and acid–base disturbance. We conclude that maternal treatment with allopurinol alters *in vivo* maternal, umbilical and fetal vascular function via mechanisms involving NO and β_1-adrenergic

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stimulation. The evidence suggests that the use of allopurinol in clinical practice should be approached with caution.

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Abbreviations dGA, days of gestational age; $\bullet O_2^-$, superoxide; PE, phenylephrine; ROS, reactive oxygen species.

Introduction

Despite advances in obstetric practice, acute intra-partum fetal asphyxia remains one of the most common forms of fetal stress, with substantial morbidity and mortality (Low, 2004). One possible strategy to combat the detrimental effects of fetal asphyxia would be to decrease the associated excessive generation of reactive oxygen species (ROS) from stimulated pro-oxidant mechanisms within the cell, such as the xanthine oxidase pathway (Berry & Hare, 2004). Indeed, maternal treatment with the xanthine oxidase inhibitor allopurinol is being considered in human pregnancy complicated by intra-partum asphyxia in order to protect the infant from excessive generation of ROS (Peeters-Scholte *et al.* 2003; Benders *et al.* 2006; Chaudhari & McGuire, 2008; Torrance *et al.* 2009; Kaandorp *et al.* 2010). This clinical interest in antenatal maternal administration with allopurinol follows previous studies which reported that allopurinol treatment in the asphyxiated neonate improved neonatal outcome (Van Bel *et al.* 1998), but if the time interval between asphyxia and treatment had been too long, or when fetal asphyxia had been too severe, no reduction in serious morbidity or mortality was observed (Benders *et al.* 2006). Therefore, there has been growing clinical and scientific interest in establishing whether perinatal outcome might be improved in complicated labour if the window of treatment with allopurinol would be initiated before birth, for instance via maternal treatment to cover the actual period of fetal asphyxia (Kaandorp *et al.* 2010). It is now known that allopurinol given to the mother crosses the placenta (Derks *et al.* 2010), it suppresses superoxide anion ($\bullet O_2^-$) production in the fetus (Masaoka *et al.* 2005) and it yields therapeutic levels in the fetus and neonate (van Kesteren *et al.* 2006; van Dijk *et al.* 2008*b*; Derks *et al.* 2010), justifying this route of administration for possible preventative therapy in clinical practice.

In the fetus, the maintenance of cardiovascular function is vital for the effective delivery and distribution of nutrients and oxygen to the fetal tissues. Several studies have shown that NO is an important regulator of the fetal cardiovascular system and the gas is implicated in both femoral and umbilical vascular control, as blockade of NO synthesis leads to increased blood pressure and elevations in umbilical and femoral vascular resistance (Chang *et al.* 1992; Green *et al.* 1996; Gardner *et al.* 2001; Morrison *et al.* 2003). It is also now recognised that vascular NO bioavailability can be influenced by the physiological oxidant *milieu*. Increases in ROS may lead to a reduction in NO bioavailability, and the resulting increased ratio of $\bullet O_2^-$:NO has been reported to promote vasoconstriction (Chen & Keaney, 2004; Valko *et al.* 2007). Recent evidence from our laboratory has begun to support the idea that this vascular redox status is active in fetal life and that it is important for the function of the fetal cardiovascular system under basal and stimulated conditions (Thakor *et al.* 2010*a*, *b*).

Given that the oxidant tone plays a significant role in the control of the fetal cardiovascular system, it is likely that maternal treatment with allopurinol in clinical practice may perturb fetal cardiovascular homeostasis. However, virtually nothing is known about the effects of xanthine oxidase inhibition on fetal cardiovascular control. Therefore, this study tested the hypothesis that xanthine oxidase has a role in the regulation of the fetal cardiovascular system. The hypothesis was tested by investigating the *in vivo* effects of maternal treatment with the xanthine oxidase inhibitor allopurinol on fetal cardiovascular function under basal and stimulated conditions in the chronically catheterised ewe and fetus in late gestation.

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. Pregnant ewes and their singleton fetus were surgically instrumented at 118 ± 1 dGA (term *ca.* 145 days), as previously described in detail (Giussani *et al.* 1997; Fletcher *et al.* 2000). 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 exteriorized and the right femoral artery and vein were isolated and catheterized (i.d., 0.86 mm; o.d., 1.52 mm;

Critchly Electrical Products, NSW, Australia). The catheter tips were carefully advanced to the abdominal aorta and inferior vena cava, respectively. A further catheter was anchored onto the fetal hind limb for administration of antibiotics into the amniotic cavity (600 mg in 2 ml, benzylpenicillin; Crystapen, Schering-Plough, Animal Health Division, Welwyn Garden City, UK). On the contra-lateral side, a Transonic flow probe was placed around the femoral artery for measurement of blood flow (2R, Transonic Systems Inc.). In addition, another flow probe (4SB; Transonic Systems Inc., Ithaca, NY, USA) was placed around one of the umbilical arteries, close to the common umbilical artery, inside the fetal abdominal cavity, for continuous measurement of unilateral umbilical blood flow (Giussani *et al.* 1997; Gardner & Giussani, 2003). The fetal abdominal wall and skin were carefully closed in layers. The uterine incisions were then closed in layers, the combined dead space of the catheters was filled with heparinized saline (80 i.u. heparin ml^{-1} in 0.9% NaCl) and the catheter ends were plugged with sterile brass pins. A Teflon catheter (i.d. 1.0 mm, o.d. 1.6 mm, Altec, UK) was placed in the maternal femoral artery and extended to the descending aorta, in addition to a maternal venous catheter extended into the inferior vena cava (i.d., 0.86 mm; o.d., 1.52 mm; Critchly Electrical Products). The catheters and flow probe leads were then exteriorized via a keyhole incision in the maternal flank, coiled carefully and placed in a bag sutured to the flank of the ewe. Inhalational anaesthesia was withdrawn and intermittent positive pressure ventilation maintained until respiratory movements were observed in the ewe. The ewe was extubated when breathing spontaneously and moved to an individual recovery pen adjacent to other sheep with free access to food and water.

Post-operative 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 (600 mg in 2 ml 0.9% NaCl, benzylpenicillin; Crystapen, Schering-Plough). Generally, normal feeding patterns were restored within 24–48 h of recovery. Following 48–72 h of post-operative recovery, ewes were transferred to metabolic crates where they were housed for the remainder of the protocol. The arterial and amniotic catheters were connected to sterile pressure transducers (COBE; Argon Division, Maxxim Medical, Athens, TX, USA). Whilst on the metabolic crates, the patency of the fetal and maternal vascular 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).

Allopurinol infusion protocol and baseline cardiovascular function

Experimental protocols were performed following at least 5 days of post-operative recovery. Following a 20 min baseline period, animals were subjected to either vehicle (control, $n = 11$) or allopurinol (150 mg kg^{-1}, $n = 10$) administered by maternal I.V. infusion over 30 min (Fig. 1). Allopurinol (Sigma Aldrich, UK) was dissolved in the minimum volume of 4 M sodium hydroxide (Sigma) and made up with saline to 90 ml (van Dijk *et al.* 2008). Control vehicle was saline treated with 4 M NaOH to achieve the same pH of the allopurinol solution (pH 13.8). Cardiovascular data were recorded continuously. Data for fetal and maternal arterial blood pressure, amniotic pressure and fetal femoral and umbilical blood flow were recorded continuously by a custom-built data acquisition system [M-PAQ (Maastricht-Programmable AcQuisition system), Maastricht Instruments, The Netherlands, 500 Hz sample rate]. Fetal arterial blood pressure was corrected for amniotic pressure. Fetal and maternal heart rates were calculated from the systemic pressure recordings. Fetal femoral vascular resistance and umbilical vascular conductance were calculated on line by applying Ohm's law to the circulation. During the experimental protocols, descending aortic blood samples (0.3 ml) were taken using sterile techniques from the mother and fetus at set time points (0, 50 and 120 min; Fig. 1) to determine arterial blood gas and pH status (ABL5 Blood Gas Analyser, Radiometer; Copenhagen, Denmark; measurements corrected to 38°C for maternal and 39.5°C for fetal blood). Values for percentage saturation of haemoglobin with oxygen (S_{aO_2}) were determined using a haemoximeter (OSM3; Radiometer).

Stimulated cardiovascular function

Previous studies in our laboratory have shown that peak concentrations of oxypurinol (the active metabolite of allopurinol) can be measured in fetal plasma approximately 100–150 min following the end of maternal administration with allopurinol with a similar dosing regimen (Derks *et al.* 2010). Therefore, the stimulated cardiovascular function in the fetus was assessed during this period (Fig. 1). The change in fetal arterial blood pressure and heart rate, femoral and umbilical blood flow and resistance were recorded following increasing bolus doses of phenylephrine (5, 12.5, 25, 37.5 and 50 μg, L-phenylephrine; P-6126; Sigma). Suitable dose ranges were derived from pilot experiments and previous studies

in the literature (Dawes *et al.* 1980; Itskovitz & Rudolph, 1982). Each dose was dissolved in 0.5 ml of saline (0.9% NaCl), and exogenously administered to the fetus, via the femoral artery over 2–3 s following at least 2 min of stable baseline recording. Catheters were flushed with 2 ml of saline immediately following the administration of each dose, and no further injections were given until fetal arterial blood pressure and heart rate had returned to baseline values (typically 30 min). The changes in fetal arterial pressure and in fetal heart rate obtained during the α_1-adrenergic dose response experiments were used to construct a cardiac baroreflex analysis. The gradient of the relationship was used to quantify baroreflex gain, as previously described (Thakor & Giussani, 2009).

The nitric oxide clamp

To determine whether the mechanism mediating the effects of xanthine oxidase inhibition on fetal cardiovascular function were mediated via increased NO, the effects of maternal treatment with allopurinol was investigated following blockade of NO synthesis with the NO clamp in another group of animals ($n = 6$; Fig. 1). The NO clamp is a technique established in our laboratory that permits inhibition of *de novo* synthesis of NO while compensating for the tonic production of the gas, thereby maintaining basal cardiovascular function (Gardner *et al.* 2001; Gardner & Giussani, 2003; Morrison *et al.* 2003; Thakor & Giussani, 2005, 2009; Thakor *et al.* 2010*a*,*b*). In brief, a bolus dose of L-NAME (N-5751; Sigma; 100 mg kg^{-1} bolus dissolved in 2 ml heparinised saline) was injected via the fetal femoral artery. This was immediately followed by fetal I.V. infusion with the NO donor sodium nitroprusside (SNP; S-0501; Sigma; dissolved in heparinised saline) to return fetal arterial blood pressure, femoral blood flow and umbilical blood flow to pre-treatment levels. The rate of SNP infusion was titrated as required before the beginning of the protocol to obtain the pre-clamped baseline values; however, no adjustments were made once the dosing protocol had begun. At the end of the experimental protocol, the effectiveness of NOS blockade by the NO clamp and the persistence of L-NAME within the system were tested by withdrawal of the SNP infusion. This unmasked the influence of fetal treatment with L-NAME alone and led to generalised vasoconstriction and hypertension (Thakor *et al.* 2010*a*). The NO clamp was always preformed on the last day of any series of experiments. This was to ensure that L-NAME would have no effect on subsequent experiments, should its effects on the fetal circulation persist past 24 h.

Atenolol administration

Some of the effects of maternal allopurinol administration on fetal basal heart rate and umbilical vascular conductance were suspected to be mediated by β_1-adrenergic stimulation. Therefore, in a third group of animals ($n = 6$), the effects of maternal treatment with allopurinol on fetal basal cardiovascular function was investigated following blockade with the selective β_1-adrenoreceptor antagonist atenolol. A bolus dose of atenolol (2 mg kg^{-1} I.V.; Sigma) was administered I.V. to the fetus 15 min prior to the start of the allopurinol infusion protocol, as its half-life is approximately 6–7 h (Lilja *et al.* 2005).

Plasma uric acid measurements

Xanthine oxidase catalyses the conversion of hypoxanthine to uric acid (Cao *et al.* 2010). Therefore, maternal and fetal plasma concentrations of urate were measured at 0, 50 and 120 min in control and allopurinol-treated pregnancies to confirm xanthine oxidase inhibition in the fetal circulation. Xanthine oxidase activity was measured by HPLC with electrochemical detection (Iriyama *et al.* 1984). In brief, aliquots of maternal and fetal plasma (acidified 1:1 with ice-cold 10% metaphophoric acid (MPA), centrifuged 3,500g and the supernatant stored at −80°C) were diluted 1:4 with ice-cold 5% MPA (final dilution of plasma 1 in 10). To this, 100 μl HPLC-grade heptane was added and following vortex mixing for 40 s, the samples were centrifuged (16,000g; 5 min) and the lower (aqueous) layer removed and treated with heptane again until the supernatant was clear. This clear supernatant was transferred to a 0.8 ml HPLC vial. 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 urate were calculated with external standards, which were run simultaneously. The limit of

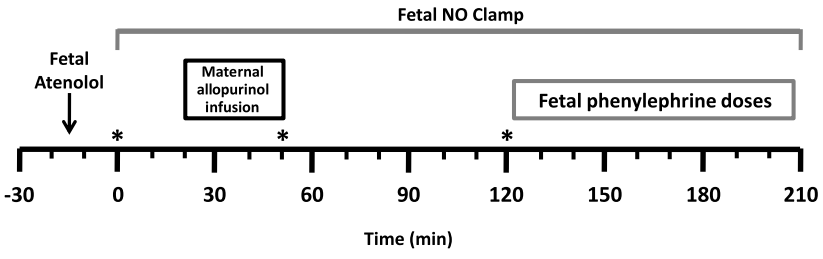

Figure 1. Experimental protocol
Maternal allopurinol (150 mg kg^{-1}) infusion occurred between 20–50 min. Fetal treatment with the phenylephrine doses (5, 12.5, 25, 37.5 and 50 μg) started at 120 min. When used, atenolol was given 15 min prior to the start of the protocol, and the NO clamp was established before the start of the protocol and maintained for the duration of the protocol. *Time at which a blood sample was taken.

sensitivity for the assay was 0.1 μmol l^{-1} for urate, and the inter-assay coefficient of variation was less than 5%.

Data collection and statistical analysis

Minute by minute averages for infusion experiments and second by second averages for dose response experiments for all cardiovascular variables were imported into an Excel spreadsheet for analysis. For statistical analysis of basal cardiovascular function, summary measures analysis was applied to the serial data to focus the number of comparisons (Matthews *et al.* 1990). Summary measures represent the mean ± SEM for the absolute change from baseline during periods of baseline (B, 0–20 min), infusion (I, 20–50 min) and recovery (R1, 51–90 min and R2, 91–120 min). For the dose response experiments, baseline values for arterial blood pressure, heart rate, femoral blood flow and femoral vascular resistance were obtained by averaging the data over the 60 s preceding the administration of each bolus dose of phenylephrine. Maximum changes from baseline for each variable were then recorded. Baroreflex curves were constructed for each fetus by plotting for each dose the maximal decrease in heart rate against the maximal increase in arterial blood pressure and the gradient of this relationship was calculated. All data are expressed as mean ± SEM. All data were analysed statistically comparing the effect of dose, treatment and interactions between dose and treatment by two-way repeated-measures (RM) ANOVA with *post hoc* Student–Newman–Keuls test. For all comparisons, statistical significance was accepted when $P < 0.05$.

Results

Plasma uric acid

Basal maternal plasma uric acid levels were similar in all groups and were not significantly altered by maternal allopurinol infusion (Table 1). In marked contrast, basal fetal plasma uric acid levels were significantly higher than maternal levels, and maternal allopurinol treatment led to a significant decrease in fetal plasma uric acid levels (Table 1).

Blood gas and metabolic status

Maternal and fetal values for blood gases and acid–base status were within the normal limits for Welsh Mountain sheep at this stage of gestation at the commencement of the infusion protocol in all animals (Tables 2 and 3; see Fletcher *et al.* 2000; Derks *et al.* 2010). Maternal infusion with allopurinol led to increases in maternal acid–base excess and plasma HCO_3^- and significant decreases in maternal P_{aO_2} and in the oxygen percentage saturation of haemoglobin (Table 2). Application of the fetal NO clamp or fetal treatment with atenolol in addition to maternal allopurinol treatment did not alter these responses when compared with maternal allopurinol treatment alone (Table 2). Maternal allopurinol administration associated with the fetal NO clamp or fetal atenolol administration lowered fetal P_{aO_2} (Table 3).

Maternal and fetal basal cardiovascular function

Prior to infusion, basal maternal (105 ± 5 mmHg) and fetal (44 ± 2 mmHg) arterial blood pressure, basal maternal (96 ± 4 beats min^{-1}) and fetal (163 ± 3 beats min^{-1}) heart rate, and basal fetal femoral (39.5 ± 2.4 ml min^{-1}) and umbilical (221 ± 22 ml min^{-1}) blood flows were within the normal range for Welsh Mountain sheep at this stage of gestation (Fig. 2). Maternal treatment with allopurinol led to a significant decrease in mean arterial pressure and a marked increase in maternal heart rate (Fig. 2). Treatment of the fetus with the NO clamp or with atenolol did not alter the maternal cardiovascular response to maternal allopurinol infusion. Maternal treatment with allopurinol also led to significant increases in fetal heart rate and umbilical blood flow, without affecting fetal arterial blood pressure or femoral blood flow. Hence, umbilical vascular conductance was markedly enhanced by maternal allopurinol treatment (Fig. 3). Application of fetal NOS blockade with the NO clamp following maternal allopurinol treatment did not attenuate the fetal heart rate or umbilical vascular responses. However, fetal treatment with atenolol following maternal allopurinol treatment completely prevented the effects on fetal heart rate, umbilical blood flow and umbilical vascular conductance (Fig. 3).

Fetal stimulated cardiovascular function

Treatment of the sheep fetus with phenylephrine produced dose-dependent increments in fetal arterial blood pressure and in fetal femoral vascular resistance following maternal administration with vehicle, allopurinol or allopurinol with the fetal NO clamp (Fig. 4). However, the fetal pressor and vasopressor responses to the higher doses of phenylephrine were significantly diminished during maternal treatment with allopurinol compared with vehicle (Fig. 4). Maternal treatment with allopurinol during fetal treatment with the NO clamp recovered all fetal pressor and vasopressor α_1-adrenergic responses back to control levels (Fig. 4).

Correlation analysis of fetal heart rate and fetal blood pressure responses following bolus doses of phenylephrine revealed significant relationships in each fetus. In all fetuses, progressive increases in fetal arterial blood pressure were accompanied by progressive

Table 1. Maternal and fetal plasma uric acid

			Time (min)		
		n	0	50	120
Maternal	Control	5	4.3 ± 0.3	4.6 ± 0.2	5.0 ± 0.3
	Allopurinol	6	4.5 ± 0.3	5.0 ± 0.4	4.4 ± 0.3
	Allopurinol with NO clamp	6	5.5 ± 0.5	6.1 ± 0.5	5.5 ± 0.5
Fetal	Control	5	12.1 ± 0.6‡	12.1 ± 0.9‡	12.0 ± 0.7‡
	Allopurinol	6	12.2 ± 0.4‡	12.4 ± 0.5‡	9.6 ± 0.4*†‡
	Allopurinol with NO clamp	6	11.9 ± 0.6‡	11.6 ± 0.6‡	9.5 ± 0.4*†‡

Values represent the mean ± SEM at pre-infusion (0), immediate post-infusion (50) and 70 min after the end of infusion (120). Significant differences ($P < 0.05$) are: *vs.* pre-infusion (0), †*vs.* control, ‡*vs.* maternal (two-way RM ANOVA with *post hoc* Newman–Keuls test).

Table 2. Maternal arterial blood gases and metabolites during allopurinol treatment

			Time (min)		
		n	0	50	120
pH_a	Control	11	7.53 ± 0.01	7.51 ± 0.01	7.54 ± 0.01
	Allopurinol	10	7.50 ± 0.02	7.61 ± 0.02	7.57 ± 0.01
	Allopurinol with NO clamp	6	7.49 ± 0.02	7.62 ± 0.01	7.57 ± 0.01
	Allopurinol with atenolol	7	7.50 ± 0.01	7.59 ± 0.02	7.56 ± 0.02
P_{aCO_2} (mmHg)	Control	11	34.55 ± 0.82	35.27 ± 1.28	33.55 ± 0.90
	Allopurinol	10	36.10 ± 1.69	33.50 ± 1.12	33.80 ± 0.79
	Allopurinol with NO clamp	6	37.67 ± 0.80	34.67 ± 0.80	37.00 ± 0.97
	Allopurinol with atenolol	7	38.29 ± 1.02	36.86 ± 1.06	38.00 ± 1.38
P_{aO_2} (mmHg)	Control	11	105.55 ± 2.58	100.18 ± 3.02	108.64 ± 4.20
	Allopurinol	10	104.80 ± 2.38	85.90 ± 5.29*†	107.00 ± 2.04
	Allopurinol with NO clamp	6	103.67 ± 1.93	77.17 ± 3.36*†	100.00 ± 2.46
	Allopurinol with atenolol	7	107.79 ± 3.28	92.29 ± 6.75*	111.29 ± 2.63
S_{aO_2} (%)	Control	11	97.31 ± 0.73	96.52 ± 0.80	96.68 ± 0.71
	Allopurinol	10	98.54 ± 1.54	96.15 ± 1.31*	99.03 ± 1.29
	Allopurinol with NO clamp	6	100.22 ± 1.14	97.17 ± 1.19*	100.63 ± 1.23
	Allopurinol with atenolol	7	103.59 ± 0.68†	102.50 ± 1.11†	103.80 ± 0.73
[Hb] (g dl^{-1})	Control	11	9.55 ± 0.29	9.21 ± 0.27	9.48 ± 0.33
	Allopurinol	10	9.45 ± 0.33	9.62 ± 0.41	8.88 ± 0.37
	Allopurinol with NO clamp	6	8.85 ± 0.29	9.58 ± 0.41	7.98 ± 0.51
	Allopurinol with atenolol	7	9.75 ± 0.36	10.13 ± 0.68	9.61 ± 0.49
ABE (meq l^{-1})	Control	11	5.82 ± 0.52	5.73 ± 0.38	5.82 ± 0.60
	Allopurinol	10	5.90 ± 0.67	11.70 ± 1.29*†	9.50 ± 1.00*†
	Allopurinol with NO clamp	6	6.17 ± 1.35	12.67 ± 1.41*†	8.83 ± 1.74*
	Allopurinol with atenolol	7	6.14 ± 1.42	12.29 ± 2.11*†	10.86 ± 1.97*†
[HCO_3^-] (mM)	Control	11	28.09 ± 0.59	28.09 ± 0.58	27.91 ± 0.65
	Allopurinol	10	28.60 ± 0.85	33.50 ± 1.50*†	31.60 ± 1.28*
	Allopurinol with NO clamp	6	25.83 ± 3.77	36.17 ± 1.14*†	31.83 ± 2.09*
	Allopurinol with atenolol	7	28.86 ± 1.53	34.86 ± 2.56*†	33.57 ± 2.20*†
[Lac] (mM)	Control	11	0.39 ± 0.07	0.45 ± 0.08	0.47 ± 0.10
	Allopurinol	10	0.44 ± 0.04	0.69 ± 0.12*	0.68 ± 0.16*
	Allopurinol with NO clamp	6	0.40 ± 0.03	0.57 ± 0.03	0.56 ± 0.06
	Allopurinol with atenolol	7	0.55 ± 0.04	0.84 ± 0.07*†	0.74 ± 0.09*

Values represent the mean ± SEM at pre-infusion (0), immediate post-infusion (50) and 70 min after the end of infusion (120). Significant differences ($P < 0.05$) are: *vs.* pre-infusion (0), †*vs.* control (two-way RM ANOVA with *post hoc* Newman–Keuls test). pH_a, arterial pH; P_{aCO_2}, arterial partial pressure of CO_2; P_{aO_2}, arterial partial pressure of O_2; S_{aO_2}, percentage saturation of haemoglobin with oxygen; [Hb], haemoglobin concentration; ABE, acid-base excess; [HCO_3^-], arterial concentration of bicarbonate; [Lac], arterial concentration of lactate.

Table 3. Fetal arterial blood gases and metabolites during allopurinol treatment

			Time (min)		
		n	0	50	120
pH_a	Control	6	7.36 ± 0.01	7.38 ± 0.02	7.36 ± 0.01
	Allopurinol	6	7.37 ± 0.01	7.37 ± 0.01	7.38 ± 0.01
	Allopurinol with NO clamp	8	7.34 ± 0.01	7.34 ± 0.01†	7.34 ± 0.01
	Allopurinol with atenolol	6	7.33 ± 0.01	7.33 ± 0.01†	7.32 ± 0.01
P_{aCO_2} (mmHg)	Control	6	53.44 ± 1.47	51.89 ± 1.29	52.11 ± 1.07
	Allopurinol	6	52.17 ± 0.87	51.50 ± 1.84	52.83 ± 1.45
	Allopurinol with NO clamp	8	54.25 ± 1.13	53.00 ± 0.89	51.63 ± 1.81
	Allopurinol with atenolol	6	56.00 ± 1.51	57.83 ± 0.70†	56.33 ± 2.33
P_{aO_2} (mmHg)	Control	6	20.00 ± 0.65	19.29 ± 1.19	19.29 ± 0.75
	Allopurinol	6	20.40 ± 1.21	19.00 ± 1.67	19.20 ± 1.56
	Allopurinol with NO clamp	8	21.63 ± 1.08	18.38 ± 0.63*	18.94 ± 0.79*
	Allopurinol with atenolol	6	20.83 ± 1.08	18.00 ± 0.68*	19.00 ± 1.13
S_{aO_2} (%)	Control	6	58.17 ± 2.15	54.94 ± 2.53	53.99 ± 2.16
	Allopurinol	6	53.28 ± 4.45	47.25 ± 4.22	51.53 ± 4.73
	Allopurinol with NO clamp	8	60.60 ± 3.23	50.01 ± 3.41	52.40 ± 3.17
	Allopurinol with atenolol	6	52.35 ± 1.93	42.00 ± 3.55	42.83 ± 2.05
[Hb] (g dl^{-1})	Control	6	9.56 ± 0.25	9.74 ± 0.23	9.83 ± 0.28
	Allopurinol	6	9.15 ± 0.43	8.85 ± 0.43	8.70 ± 0.29
	Allopurinol with NO clamp	8	8.64 ± 0.53	8.65 ± 0.55	8.53 ± 0.58
	Allopurinol with atenolol	6	9.22 ± 0.20	9.53 ± 0.25	9.40 ± 0.43
ABE (meq l^{-1})	Control	6	4.06 ± 0.46	3.78 ± 0.36	3.89 ± 0.48
	Allopurinol	6	3.00 ± 0.89	3.60 ± 0.60	4.00 ± 0.45
	Allopurinol with NO clamp	8	2.67 ± 0.56	2.33 ± 0.42	1.50 ± 0.72
	Allopurinol with atenolol	6	3.67 ± 1.02	3.67 ± 1.20	3.50 ± 1.09
[HCO_3^-] (mM)	Control	6	29.11 ± 0.59	30.11 ± 1.09	28.78 ± 0.46
	Allopurinol	6	28.17 ± 0.75	25.17 ± 3.53	29.00 ± 0.58
	Allopurinol with NO clamp	8	27.13 ± 0.67	26.75 ± 0.56	26.25 ± 0.77
	Allopurinol with atenolol	6	27.50 ± 1.43	27.50 ± 1.43	27.83 ± 1.92
[Lac] (mM)	Control	6	0.88 ± 0.06	0.88 ± 0.09	1.06 ± 0.06
	Allopurinol	6	0.91 ± 0.07	1.07 ± 0.09	1.00 ± 0.07
	Allopurinol with NO clamp	8	1.11 ± 0.11	1.36 ± 0.16*†	1.64 ± 0.22*†
	Allopurinol with atenolol	6	0.76 ± 0.05	0.79 ± 0.04	0.94 ± 0.19

Values represent the mean ± SEM at pre-infusion (0), immediate post-infusion (50) and 70 min after the end of infusion (120). Significant differences ($P < 0.05$) are: *vs.* pre-infusion (0), †*vs.* control (two-way RM ANOVA with *post hoc* Newman–Keuls test). pH_a, arterial pH; P_{aCO_2}, arterial partial pressure of CO_2; P_{aO_2}, arterial partial pressure of O_2; S_{aO_2}, percentage saturation of haemoglobin with oxygen; [Hb], haemoglobin concentration; ABE, acid-base excess; [HCO_3^-], arterial concentration of bicarbonate; [Lac], arterial concentration of lactate.

decreases in fetal heart rate. Fetal cardiac vagal baroreflex function was assessed by calculating the gradient of the arterial blood pressure *vs.* heart rate relationship in each fetus. During control experiments with maternal vehicle infusion, fetal cardiac baroreflex sensitivity was 2.77 ± 0.30 beats min^{-1} $mmHg^{-1}$ (Fig. 5). In contrast, maternal treatment with allopurinol increased the sensitivity of the fetal cardiac baroreflex (5.75 ± 0.78 beats min^{-1} $mmHg^{-1}$), an effect which was restored to control levels following maternal allopurinol during fetal treatment with the NO clamp (2.71 ± 0.42 beats min^{-1} $mmHg^{-1}$; Fig. 5).

Discussion

The data show that in late gestation ovine pregnancy, fetal plasma concentrations of uric acid were reduced following maternal treatment with allopurinol. Maternal allopurinol also led to significant increases in fetal heart rate, umbilical blood flow and umbilical vascular conductance, effects which were abolished by fetal treatment with atenolol but not by fetal NO blockade. Maternal allopurinol treatment significantly impaired fetal α_1-adrenergic-mediated pressor and femoral vasopressor responses and significantly enhanced the fetal cardiac baroreflex gain. These effects of maternal allopurinol were restored to control levels

during fetal NO blockade. Maternal treatment with allopurinol induced maternal hypotension, tachycardia and acid–base disturbance. This was accompanied by stable fetal blood gas and acid–base status. Therefore, the data support the hypothesis that xanthine oxidase has a role in the regulation of fetal cardiovascular function. The data also show that maternal treatment with allopurinol enhances umbilical blood flow via mechanisms involving β_1-adrenergic stimulation.

Xanthine oxidase breaks down purine bases forming uric acid (Berry & Hare, 2004). Hypoxanthine, a metabolite of adenosine, is metabolised by xanthine oxidase in a two-stage process to uric acid, producing the free radical $\bullet O_2^-$ at both stages. In the mother, uric acid is cleared by filtration and renal secretion (Enomoto & Endou, 2005) whilst fetal uric acid diffuses from the fetus to the mother across the placenta (Wallenburg & van Kreel, 1980; Simmonds *et al.* 1984). In this study, levels of uric acid in the fetal circulation were approximately two-fold greater than those in the maternal compartment under basal conditions, reflecting the feto-maternal diffusion gradient (Simmonds *et al.* 1984). Further, fetal uric acid levels decreased whilst maternal levels were unchanged following maternal allopurinol administration. Whilst decreases in the levels of uric acid may reflect decreased production and/or enhanced removal from the fetal circulation, the data show that xanthine oxidase activity in the sheep fetus is present under basal conditions and that maternal allopurinol administration can inhibit this basal activity.

The dynamic relationship between $\bullet O_2^-$ and NO has led to the suggestion that the oxidant *milieu* is an important modulator of vascular tone, such that increases in the $\bullet O_2^-$:NO ratio result in vasoconstriction, and decreases in the ratio promote vasodilatation (Chen & Keaney, 2004). Alterations in this oxidant tone are likely to have a significant influence in circulations that are highly dependent on NO, such as the umbilical vascular bed (Chang *et al.* 1992; Green *et al.* 1996; Gardner & Giussani, 2003; Morrison *et al.* 2003). Accordingly, we have previously reported that fetal treatment with vitamin C or with melatonin led to marked NO-dependent elevations in umbilical vascular conductance (Thakor *et al.* 2010*a*). Other studies support the idea that stimulated xanthine oxidase can also affect the vascular oxidant tone, showing that increased xanthine oxidase levels potentiate vasoconstriction in the rat middle cerebral vessels and that allopurinol treatment diminishes the effect (Hernanz *et al.* 2003). Allopurinol also led to decreased tension in pre-contracted rat aortas (Ellis *et al.* 1998). In the

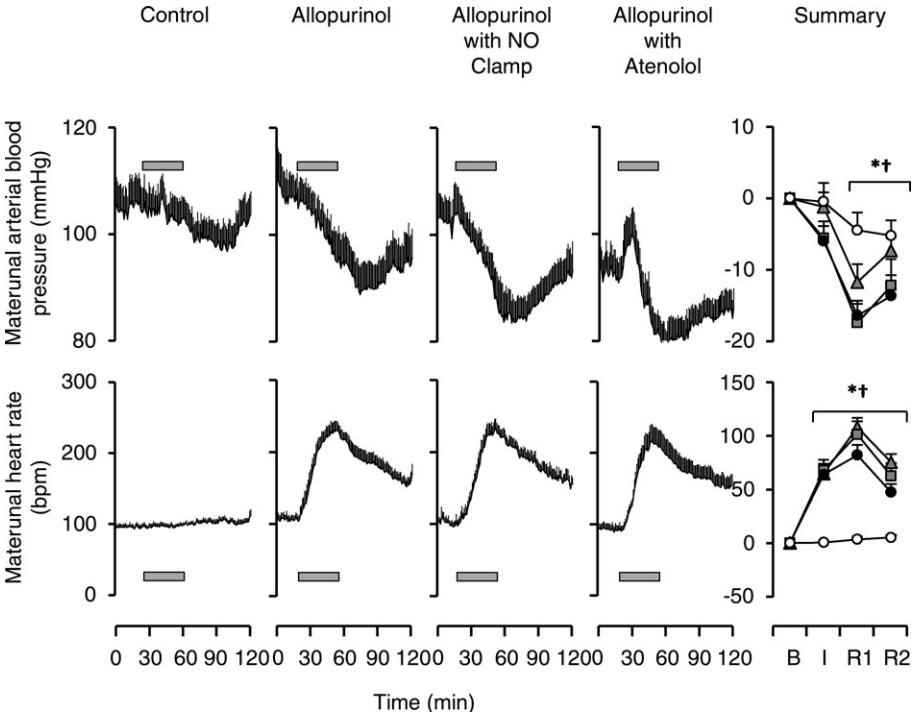

Figure 2. Maternal cardiovascular variables during allopurinol treatment
Values represent the mean ± SEM of each minute during maternal vehicle (open circles), maternal allopurinol (black circles), maternal allopurinol with the fetal NO clamp (grey squares) and maternal allopurinol with fetal atenolol (grey triangles). Bar indicates the maternal infusion of allopurinol. A summary is represented on the right-hand column for pre-infusion baseline (B), the infusion period (I), post-infusion recovery 50–90 min (R1) and post-infusion recovery 90–120 min (R2). Significant differences ($P < 0.05$) are: * *vs*. pre-infusion (B), † *vs*. control (two-way RM ANOVA with *post hoc* Newman–Keuls test).

present study, maternal treatment with allopurinol led to an increase in umbilical vascular conductance. However, fetal NO blockade did not prevent this effect, implying that the mechanism by which maternal allopurinol increases umbilical vascular conductance is not NO dependent and therefore different to the mechanism by which vitamin C or melatonin act on the fetal circulation (Thakor *et al.* 2010*a*,*b*). Conversely, fetal treatment with atenolol, a

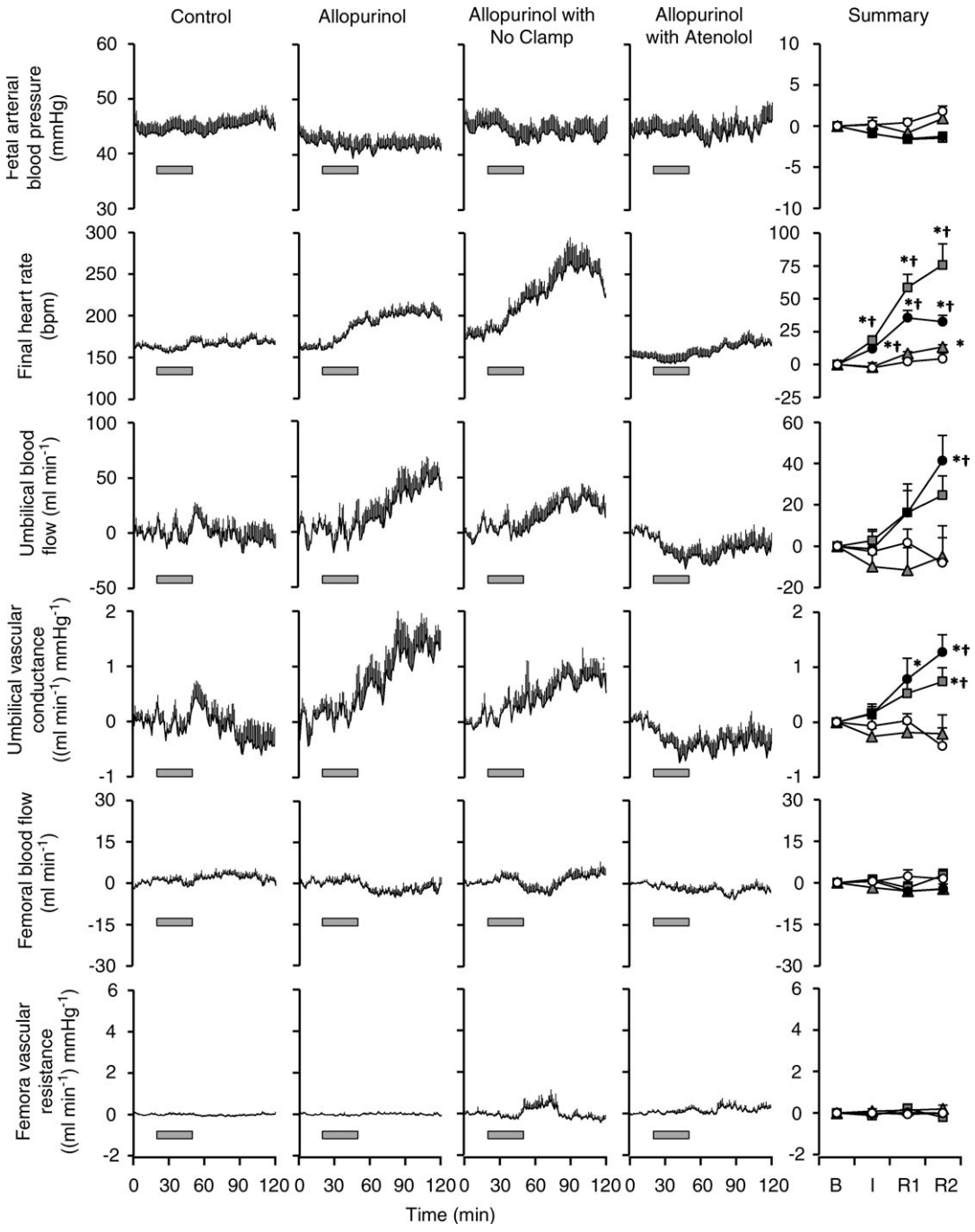

Figure 3. Fetal cardiovascular variables during allopurinol treatment
Values represent the mean ± SEM of each minute during maternal vehicle (open circles), maternal allopurinol (black circles), maternal allopurinol with the fetal NO clamp (grey squares) and maternal allopurinol with fetal atenolol (grey triangles). Bars indicate the maternal infusion of allopurinol. A summary is represented on the right-hand column for pre-infusion baseline (B), infusion period (I), post-infusion recovery 50–90 min (R1) and post-infusion recovery 90–120 min (R2). Significant differences ($P < 0.05$) are: * *vs*. pre-infusion (B), † *vs*. control (two-way RM ANOVA with *post hoc* Newman–Keuls test).

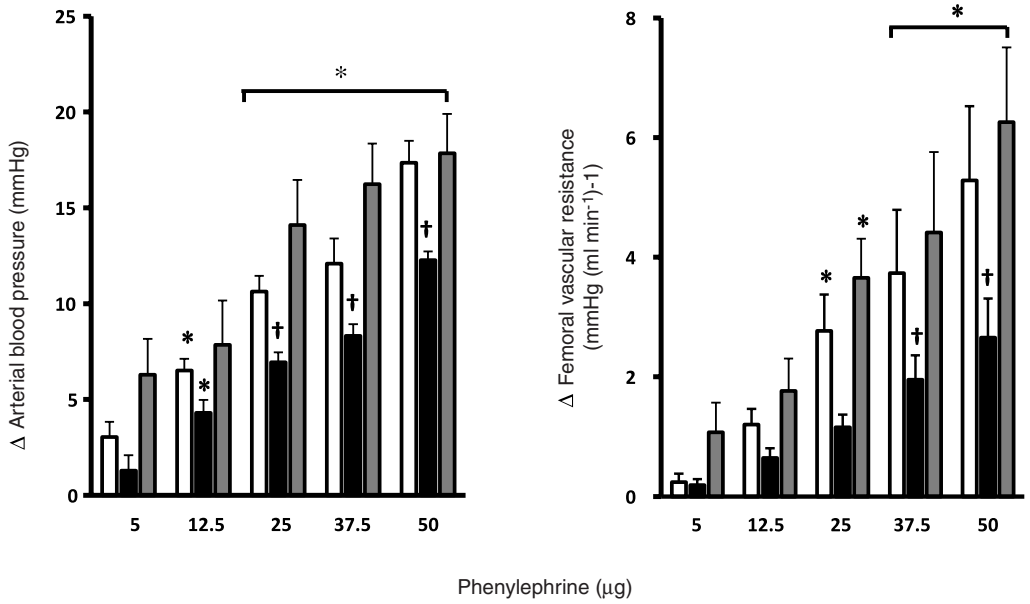

Figure 4. Fetal pressor and vasopressor responses to phenylephrine
Values represent the mean ± SEM for the change from baseline in fetal arterial blood pressure and in femoral vascular resistance in response to increasing doses of phenylephrine (5, 12.5, 25, 37.5 and 50 μg) during maternal vehicle (open bars), maternal allopurinol (black bars) and maternal allopurinol with the fetal NO clamp (grey bars). Significant differences ($P < 0.05$) are: * *vs*. basal, † *vs*. control (two-way RM ANOVA with *post hoc* Newman–Keuls test).

selective β_1-adrenergic antagonist, blocked the increases in both fetal umbilical vascular conductance and fetal heart rate, implying the activation of β_1-adrenoreceptors in mediating the umbilical haemodynamic response. An increase in umbilical blood flow following β_1-adrenergic stimulation following maternal allopurinol treatment may occur due to more than one reason. For instance, it is known that stimulation of β_1-, β_2- and β_3-adrenoreceptors

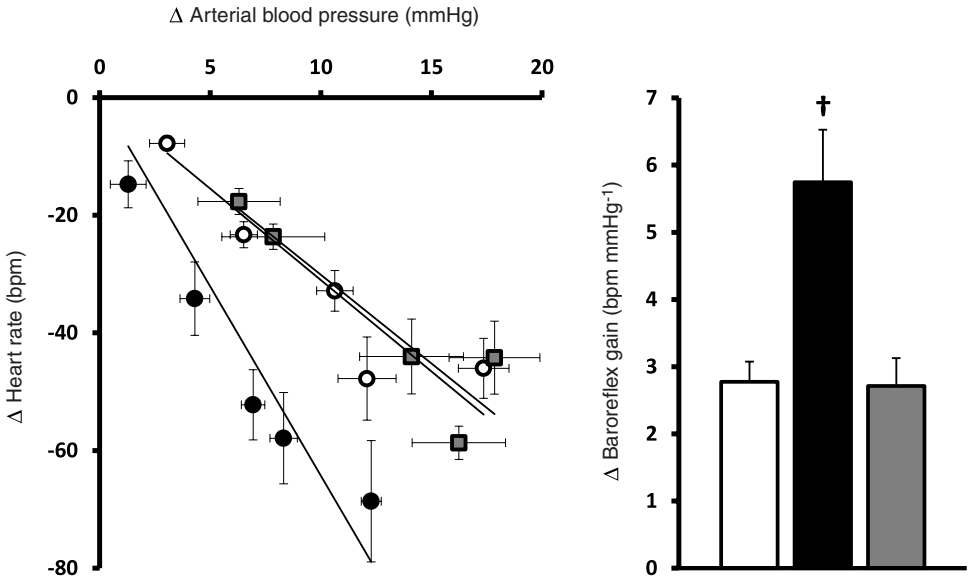

Figure 5. Fetal cardiac baroreflex
Each point represents the mean ± SEM (*x* and *y*) for the change in heart rate and blood pressure following maternal vehicle (open circles), maternal allopurinol (black circles) or maternal allopurinol with the fetal NO clamp (grey squares). The histograms represent the mean ± SEM for cardiac baroreflex sensitivity following saline vehicle (open bars), maternal allopurinol (black bars) and maternal allopurinol with the fetal NO clamp (grey bars). Significant differences ($P < 0.05$) are: † *vs*. all (two-way RM ANOVA with *post hoc* Newman–Keuls test).

directly promotes dilatation within the umbilical vascular bed (Dennedy *et al.* 2002). In addition, it is known that an increase in fetal heart rate will lead to an increase in umbilical blood flow (Morrow *et al.* 1993). Since atenolol, a selective β_1-adrenergic antagonist, abolished both the increases in fetal heart rate and in umbilical blood flow, the data support an indirect effect of allopurinol on umbilical blood flow, secondary to an increase in fetal heart rate due to β_1-adrenoreceptor activation.

Data in the current study also show that fetal exposure to allopurinol significantly impaired the arterial pressor and femoral vasopressor responses to phenylephrine. The suppressed response to phenylephrine is probably secondary to allopurinol decreasing xanthine oxidase-derived free radical production, thereby increasing NO bioavailability, which acts to offset the α_1-adrenergic-mediated peripheral vasoconstriction. In support of this idea, maternal allopurinol did suppress fetal circulating urate levels, the end-product of the xanthine oxidase pathway. In addition, under fetal NO blockade, the suppressive pressor and vasopressor effects of maternal allopurinol were prevented, thereby implying that the actions of allopurinol on the fetal vasculature are NO dependent.

Maternal treatment with allopurinol also altered the autonomic control of the fetal cardiovascular system as evidenced by an increased gain of the fetal cardiac baroreflex. The mechanism underlying this effect of allopurinol may be through decreasing ROS and/or increasing NO levels at the level of the sensor, integrator and/or target organ of the reflex pathway (Paton *et al.* 2002). Accordingly, administration of xanthine oxidase to the isolated rabbit carotid sinus decreased baroreceptor sensitivity, an effect that could be recovered with the anti-oxidant enzyme superoxide dismutase (Li *et al.* 1996). The effect of maternal allopurinol enhancing the gain of the fetal cardiac baroreflex at the level of the heart itself is also possible, since available literature suggests that NO potentiates, rather than depresses, the magnitude of induced bradycardia (Balligand *et al.* 1993; Conlon *et al.* 1996; Sears *et al.* 1999). For example, *in vitro* and *in vivo* studies have shown that NO synthase (NOS) inhibition blocks the negative cardiac chronotropic effect of muscarinic receptor stimulation in spontaneously beating neonatal rat cardiomyocytes (Balligand *et al.* 1993) or significantly attenuates the bradycardic response to efferent vagal stimulation (Conlon *et al.* 1996). Conversely, treatment with NO donors such as molsidomine and SNP significantly enhanced the decrease in heart rate in response to vagal nerve stimulation in the anaesthetised rabbit and isolated guinea pig atria (Sears *et al.* 1999).

Maternal treatment with allopurinol also produced both fetal and maternal tachycardia and marked maternal hypotension. The maternal hypotension supports the idea that tonic production of ROS by xanthine oxidase contributes to an oxidant tone that maintains peripheral vascular resistance in the maternal circulation. At first sight, the maternal tachycardia following maternal allopurinol treatment appears to be triggered by a baroreflex in response to significant hypotension. However, close inspection of the trace reveals that maternal tachycardia starts prior to any change in maternal arterial blood pressure. The dissociation between the timing of the maternal depressor and positive chronotropic responses suggest that baroreflex activation is unlikely to contribute to the mechanism increasing maternal heart rate. Further, maternal allopurinol resulted in fetal tachycardia in the absence of fetal hypotension. Given that fetal treatment with atenolol, but not with the fetal NO clamp, blocked the fetal tachycardia following maternal allopurinol administration, it is highly likely that fetal tachycardia is caused via increased sympathetic drive independent of changes in NO. Although not tested in this study, the same mechanism could be responsible for the maternal tachycardia following maternal allopurinol administration.

Maternal treatment with allopurinol also led to increases in maternal plasma pH, HCO_3^- concentration and acid–base excess. Further, there were significant decreases in maternal P_{aO_2} and more moderate decrements in the percentage saturation of haemoglobin, all of which had partially or completely resolved by 120 min of the infusion protocol. The maintenance of saturation of haemoglobin in the face of decreases in P_{aO_2} may have been helped by a reverse Bohr shift in the oxygen dissociation curve for haemoglobin induced by the alkalosis in maternal blood (Bohr *et al.* 1904; Jensen, 2004). It is interesting to speculate on the mechanism mediating the decrease in maternal P_{aO_2}. Central chemoreceptors are activated in response to high acidity and act to drive respiratory rate and increase tidal volume and alveolar ventilation (Guyenet *et al.* 2010). Conversely, it is possible that increases in maternal pH may therefore act to depress ventilatory drive (Pokorski & Lahiri, 1983). Reductions in alveolar ventilation would lead to increases in alveolar partial pressure of CO_2 (P_{A,CO_2}) and decreases in alveolar and arterial P_{O_2} (Ganong, 2003). Opposing this argument is the observation in the present study that maternal arterial partial pressure of CO_2 (P_{aCO_2}) did not increase; however, CO_2 production may have also been altered by allopurinol administration and/or have become buffered by conversion to HCO_3^- and/or by being bound to proteins such as carboamino compounds (Ganong, 2003). Irrespective of the mechanism, the reduced maternal P_{aO_2} will reduce the trans-placental oxygen diffusion gradient, thereby explaining the mild reduction in fetal P_{aO_2} following maternal allopurinol treatment.

In conclusion, the data show that maternal treatment with allopurinol affects maternal cardiovascular and metabolic homeostasis and promotes significant changes

in fetal cardiovascular function under basal and stimulated conditions. The results are not only of particular significance to the physiological understanding of the control of the fetal cardiovascular system, but they are also of clinical relevance in the context of previous and ongoing trials where allopurinol is being administered to pregnant women when their unborn child shows signs of asphyxic distress (Kaandorp *et al.* 2010). Collectively, past (Torrance *et al.* 2009; Derks *et al.* 2010) and present evidence reports on effects of maternal allopurinol on the fetus of mixed implication, supporting beneficial as well as detrimental consequences. Therefore, the use of allopurinol in clinical practice should be approached with caution.

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Author contributions

E.A.H., A.D.K., J.A.H., A.S.T. & D.A.G. contributed to the conception and design of the experiments. All authors contributed to the collection, analysis and interpretation of data; drafting the article or revising it critically and approved the final version of the manuscript.

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