Vitamin C Prevents Intrauterine Programming of in Vivo Cardiovascular Dysfunction in the Rat

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Background: Fetal hypoxia is common and in vitro evidence supports its role in the programming of adult cardiovascular dysfunction through the generation of oxidative stress. Whether fetal chronic hypoxia programmes alterations in cardiovascular control in vivo, and if these alterations can be prevented by antioxidant treatment, is unknown. This study investigated the effects of prenatal fetal hypoxia, with and without maternal supplementation with vitamin C, on basal and stimulated cardiovascular function in vivo in the adult offspring at 4 months of age in the rat.

Methods and Results: From days 6 to 20 of pregnancy, Wistar rats were subjected to Normoxia, Hypoxia (13% O₂), Hypoxia+Vitamin C (5 mg/ml in drinking water) or Normoxia+Vitamin C. At 4 months, male offspring were instrumented under urethane anaesthesia. Basal mean arterial blood pressure, heart rate and heart rate variability (HRV) were assessed, and stimulated baroreflex curves were generated with phenylephrine and sodium nitroprusside. Chronic fetal hypoxia increased the LF/HF HRV ratio and baroreflex gain, effects prevented by vitamin C administration during pregnancy.

Conclusions: Chronic intrauterine hypoxia programmes cardiovascular dysfunction in vivo in adult rat offspring; effects ameliorated by maternal treatment with vitamin C. The data support a role for fetal chronic hypoxia programming cardiovascular dysfunction in the adult rat offspring in vivo through the generation of oxidative stress in utero. (*Circ J* 2013; **77**: 2604–2611)

Key Words: Antioxidant; Fetus; Hypoxia; Programming

Cardiovascular disease is one of the leading causes of morbidity and mortality in the UK today, imposing a significant burden on the health and wealth of the nation.¹ In addition to well recognised traditional risk factors such as age, sex and obesity, a growing body of epidemiological and basic scientific evidence supports the concept that adverse intrauterine conditions can programme the increased susceptibility to cardiovascular disease in adult life.^{2–5} This offers the exciting prospect that through understanding the mechanisms underlying cardiovascular programming, we might be able to modify the development of cardiovascular disease in the offspring, even before they are born.

One of the most common challenges to the fetus during gestation is a reduction in its oxygenation, which can arise from pre-eclampsia, placental insufficiency or compression of the umbilical cord.^{6,7} Other conditions that induce fetal hypoxia include pregnancy at high altitude,⁸ maternal respiratory disease,⁹ or maternal smoking.¹⁰ Accumulating evidence supports that sustained fetal hypoxia promotes remodelling of the developing cardiovascular system and leads to cardiac and vascular dysfunction in later life.^{11–15} However, the mechanisms via which developmental hypoxia programmes cardiovascular dys-

function in later life are not well understood. Hypoxia is a potent stimulus for the generation of reactive oxygen species (ROS) and it also consumes a number of antioxidant defences.¹⁶ Under physiological conditions, ROS are important mediators of a wide variety of cellular functions.¹⁷ However, excessive generation of ROS and/or a fall in antioxidant defences can lead to indiscriminate damage, resulting in cellular oxidative stress.^{16,17} In the last few years, our laboratory has proposed the hypothesis that oxidative stress in the fetal heart and vasculature underlies the molecular basis via which fetal chronic hypoxia programmes cardiovascular dysfunction.¹² In support of this hypothesis, work in our group has recently shown that chronic hypoxia during most of the gestation in the rat is associated with aortic wall thickening with enhanced nitrotyrosine staining and an increase in cardiac HSP70 expression in the fetus by the end of gestation.^{11,12} Nitrotyrosine is a footprint for peroxynitrite generation, the product between nitric oxide (NO) and superoxide anion,¹⁸ and HSP70 is a robust index of oxidative stress.¹⁹ By adulthood, these molecular markers resolve but offspring of chronic hypoxic pregnancy are left with functional deficits in both the heart and circulation.^{12,20} There is a markedly reduced capacity of the periph-

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eral resistance vasculature to dilate via endothelial NO-dependent impairment and there is a significant increase in myocardial contractility, due to sympathetic dominance.^{12,20} Moreover, these effects of hypoxic pregnancy on the fetus and adult offspring can be prevented by maternal treatment with antioxidants.¹² However, it remains unknown to what extent, if any, these cardiovascular deficits measured in isolated tissues translates to programming cardiovascular dysfunction in vivo. Therefore, in this study, we investigated the effects of chronic fetal hypoxia, with and without maternal supplementation with the antioxidant vitamin C, on basal cardiovascular function and on the baroreflex control of blood pressure in vivo in the rat offspring at 4 months of adult age.

Methods

Animals

All procedures involving animals were carried out under the United Kingdom 1986 Animals (Scientific Procedures) Act. Wistar rats (Charles River Ltd, Margate, UK) were housed in individually ventilated cages (IVC units, 21% O₂, 70–80 air changes per h) under standard conditions (60% humidity, 21°C and a 12-h light, 12-h dark cycle), with free access to food (maintenance diet; Charles River Ltd) and water. After 10 days of acclimatization, virgin female Wistar rats (n=43, 10–12 weeks of age) were paired individually with fertile male Wistar rats (minimum 12 weeks of age). The presence of a copulatory plug was considered day 0 of pregnancy (term *ca.* 22 days). Upon the confirmation of pregnancy, the female was weighed and housed individually. Maternal weight and food and water consumption were monitored daily.

Experimental Protocol

On day 6 of pregnancy, rats were randomly assigned to either normoxic (21% O₂) or hypoxic (13% O₂) conditions, with and without vitamin C treatment (5 mg/ml maternal drinking water

freshly prepared everyday; **Figure 1**). This produced 4 experimental groups: N, normoxia; H, Hypoxia; HC, hypoxia with vitamin C supplementation; NC, normoxia with vitamin C supplementation. Pregnant rats subjected to hypoxia were placed inside a chamber, which combined a PVC isolator with a nitrogen generator, which was custom built at the University of Cambridge as previously described in detail.^{11,12,21} Maternal food intake, water intake and maternal weight were measured daily within the chamber until delivery. Maternal hypoxia was induced from day 6 of gestation as prior exposure to hypoxia markedly increases pregnancy loss. This degree and duration of maternal hypoxia does not affect maternal food intake, emphasising that in this animal model, any programming effects on cardiovascular function in adult offspring due to hypoxia are independent of alterations in maternal food intake.^{11,12,21}

In 1 cohort, animals were subject to euthanasia at day 20 of gestation. Fetal weight and placental weights were recorded for all pregnancies. Maternal and fetal haematocrit and vitamin C concentrations were measured. Ascorbate concentration was measured in maternal blood collected from animals at day 20 of gestation by high performance liquid chromatography using methods previously described in detail.¹² Measurement of ascorbate was not performed in fetal plasma due to insufficient volume.

A second cohort of animals was raised for the generation of offspring for analysis in adulthood. Upon delivery, litter size was decreased to 8 to standardise for maternal care and attention.^{12,22} Pups remained with their mothers until P21 whereupon they were weaned into cages and housed with 1 other male from the same treatment group until 4 months of age. Animals were weighed weekly during this time.

In vivo Cardiovascular Assessment

Anaesthesia was induced (2–3% isoflurane in 100% O₂ at 2 L/min) and the animal was placed in the supine position on

a regulated heating mat and allowed to breathe spontaneously. Adequate depth of anaesthesia was continually assessed by the absence of corneal and limb withdrawal reflexes. When depth of anaesthesia was confirmed, under a dissecting microscope, the left femoral artery and vein were exposed via an incision, individually isolated and instrumented with catheters prefilled with heparinised saline (80 i.u. heparin/ml in 0.9% NaCl). On the contra-lateral hind limb, the femoral artery was also exposed and a Transonic Flow probe was implanted for continuous monitoring of femoral blood flow. Isoflurane anaesthesia was switched to urethane (1.4–1.5 g/kg i.v. in water for injections; Sigma, UK) over approximately 20 min to prevent cardio-respiratory depression, thereby permitting controlled in vivo manipulation of the cardiovascular system under anaesthesia, as previously established and validated.^{23–25} The arterial catheter was connected to a pressure transducer (Argon Division, Maxxim Medical, Athens, Texas, USA). Arterial blood pressure was recorded continually on a custom built Data Acquisition System (Maastricht-Programmable AcQuisition system, M-PAQ and IDEEQ software, Maastricht Instruments, The Netherlands; 1000Hz sample rate). Heart rate was calculated on-line by the programme using the arterial blood pressure pulse to trigger.

Basal blood pressure, heart rate and femoral blood flow were recorded continuously. Femoral vascular resistance was calculated by dividing mean blood pressure by blood flow. Heart rate variability (HRV) was analysed according to standardised methods.²⁶ In brief, a 5 min period of baseline recording was selected and analysed using the HRV function in Labchart 7 (ADI instruments). Normal-to-normal interval (NN) was calculated from the arterial blood pressure trace. In the time domain, the square root of the mean of the sum of the squares of difference between adjacent NN intervals (RMSSD), an established parasympathetic index, and the standard deviation of all NN intervals (SDNN), an index of total HRV, were calculated.²⁷ In addition, the data were fast Fourier transformed to determine frequency-specific components. The low frequency

(LF), an established sympathetic dominant domain, was set between 0.1 and 1.0 Hz and high frequency (HF), an established parasympathetic dominant domain, was set domain between 1.0 and 3.5 Hz. The ratio of the LF/HF power was calculated as an index of autonomic balance.^{27,28}

After basal recordings, baroreflex responses were generated with intravenous doses of phenylephrine administered in a random order (PE; Sigma, UK: 10, 20, 40 and 80 µg i.a.), then by sodium nitroprusside (SNP; Sigma, UK; 0.6, 1.25, 2.5, 5, 10 and 20 µg/kg i.a.) also in random order. Maximal changes from baseline in arterial blood pressure and heart rate were recorded for each dose. Between doses of any drug, cardiovascular variables were allowed to return to baseline and remain stable for at least 2 min. At the end of the experiment, the animal was killed by an overdose of anaesthetic (urethane 2 g/kg i.v. in water for injections; Sigma, UK) and cervical dislocation.

Data and Statistical Analyses

All values are expressed as mean ± SEM, unless stated otherwise. All cardiovascular variables were imported into Labchart 7 (ADI instruments) for extrapolation of data before further analysis in Microsoft Excel. To control for sex and within litter variation, cardiovascular function was determined only in 1 male offspring per litter per treatment group. Baroreflex analysis was performed by plotting the heart rate against mean arterial blood pressure for each animal in response to PE and SNP (Figure 2). Baroreflex gain was calculated by applying a logistic sigmoidal fit to the data for each individual animal (Figure 2). Data were assessed using either 1-way ANOVA comparing between groups, or 2-way ANOVA with repeated measures comparing the effects of group and dose or time, in conjunction with the Student-Newman-Keuls post-hoc test, as appropriate (Sigma-Stat 3.5; Chicago, IL, USA). Comparisons of variables relating to fetal and placental biometry were determined in all fetuses of all litters. To account for multiple observations within any 1 litter, this analysis was performed using a Mixed-Linear-Model (SPSS).

Figure 3. Basal cardiovascular variables. Values are mean ± SEM. for basal mean, systolic and diastolic arterial blood pressure, heart rate, mean femoral blood flow and the rate pressure product in 4-month-old male offspring from Normoxic (□, n=8), Hypoxic (■, n=9), Hypoxic+Vitamin C (■, n=7) and Vitamin C (■, n=7) pregnancy. Significant differences (P<0.05): *, vs. Normoxia. One way ANOVA with post hoc Student-Newman-Keuls test.

Results

Basal Cardiovascular Function

At 4 months of age, in normoxic offspring, values for basal mean arterial blood pressure and heart rate were 130 ± 2 mmHg and 340 ± 11 bpm, respectively. Fetal chronic hypoxia did not alter basal blood pressure or heart rate at adulthood, however, treatment with vitamin C under normoxic or hypoxic conditions decreased mean arterial pressure in the adult offspring (Figure 3). Furthermore, adult offspring of normoxic pregnancies treated with vitamin C showed a decrease in the rate pressure product. Fetal chronic hypoxia with or without vitamin C treatment had no effect on basal femoral blood flow (Figure 3).

HRV and Baroreflex Function

At 4 months of age, offspring of hypoxic pregnancy displayed no significant alterations in the time domain (SDNN or RMSSD; Figure 4A). However, the LF/HF power ratio, an indicator of sympathetic to parasympathetic dominance, was significantly increased (Figure 4B). Treatment with vitamin C for a hypoxic pregnancy prevented changes in the frequency domain in adult offspring. Vitamin C treatment during gestation alone had no effect on HRV at 4 months of age.

At 4 months of age in all animals, dose-dependent increases in arterial pressure were accompanied by dose-dependent decreases in heart rate, and vice versa, in response to phenylephrine and sodium nitroprusside, respectively. The relationship between blood pressure and heart rate could be fitted to a

logistic sigmoidal curve in all animals. In comparison to offspring from normoxic pregnancy, offspring from hypoxic pregnancy showed increases in the maximum and minimum values for heart rate and in the slope of the heart rate-blood pressure relationship, signifying an increase in baroreflex gain (Figure 5, Table). Concomitant treatment of hypoxic pregnancies with vitamin C restored the baroreflex gain in the offspring towards control levels. Vitamin C treatment in normoxic pregnancy significantly decreased the maximal heart rate but did not significantly affect baroreflex gain in the adult offspring (Figure 5, Table).

Model Characteristics

The effects of maternal hypoxia on maternal food and water intake and on fetal and placental growth have been previously published.²¹ In normoxic pregnancy, maternal food and water intake did not alter significantly between days 6 and 20 of gestation (P>0.05). Furthermore, neither maternal exposure to hypoxia nor vitamin C treatment had any effect on maternal food or water intake or weight gain during the treatment period (P>0.05).

In the cohort of animals that underwent euthanasia on day 20 of pregnancy, maternal basal concentrations of vitamin C were similar in control and hypoxic pregnancies. Maternal treatment with vitamin C elevated maternal plasma ascorbate concentrations by ca. 70% in both control and hypoxic pregnancies (N, 18.7 ± 2.1 μ mol/L; H, 20.0 ± 2.5 μ mol/L; HC, 31.2 ± 3.0 μ mol/L; NC, 32.2 ± 2.4 μ mol/L; P<0.05, N vs. HC, N vs. NC). Hypoxic

Table. Baroreflex Function Summary Measures

	N	H	HC	NC
Mean arterial pressure (mmHg)	130.2±1.8	130.6±1.7	123.9±2.3*	120.5±2.9*
Mean heart rate (bpm)	339.9±11.3	342.6±7.0	329.7±11.3	319.8±8.8
Min HR (bpm)	283±14	322±4*	294±6	284±6
Max HR (bpm)	436±14	476±9*	438±14	372±6*
Gain (-bpm/mmHg)	1.16±0.16	1.94±0.22*	1.39±0.19	0.82±0.16

Values are mean±SEM for baroreflex function in 4-month-old male offspring from Normoxic (N; n=8), Hypoxic (H; n=9), Hypoxic+Vitamin C (HC; n=7) and Normoxic+Vitamin C (NC; n=7) pregnancies. Significant differences (P<0.05):

* vs. Normoxic. One-way ANOVA with post hoc Student-Newman-Keuls test.

pregnancy with and without maternal vitamin C administration increased maternal and fetal haematocrit on day 20 of gestation (Maternal haematocrit: N, 30.2±0.5%; H, 35.8±0.2%; HC, 36.1±0.4%; NC, 30.9±0.7%; P<0.05, N vs. H, N vs. HC. Fetal haematocrit: N, 34.1±0.2%; H, 41.1±0.5%; HC, 41.9±0.4%; NC, 35.3±0.7%; P<0.05, N vs. H, N vs. HC).

Discussion

The data in the present study show that fetal chronic hypoxia programmes significant alteration in the control of cardiovascular function measured in vivo in adult rats and that some of these effects can be prevented by maternal treatment with antioxidants in hypoxic pregnancy. Specifically, intrauterine hypoxia programmed an increase in the LF/HF heart variability ratio and an increase in baroreflex gain in adult offspring. Maternal treatment with vitamin C during hypoxic pregnancy prevented these effects. Intrauterine hypoxia did not alter basal mean arterial pressure, heart rate or femoral blood flow in 4-month-old adult rats. However, antenatal maternal vitamin C treatment programmed a decrease in basal arterial blood pressure and the rate pressure product in the adult offspring of both normoxic and hypoxic pregnancy.

The model of chronic fetal hypoxia is of human clinical relevance from two perspectives. First, the level of hypoxia corresponds to approximately 3,500 metres of high altitude, corresponding to >140 million people living at high altitude.^{8,29} Secondly, this level of maternal hypoxia induces fetal hypoxia of 10–13 mmHg,³⁰ which is equivalent to PO₂ measured in human umbilical blood taken by cordocentesis in IUGR complicated pregnancies.³¹

Several previous studies in many species and at different stages of life, including reports in the chick embryo,^{14,32,33} the sheep fetus,³⁴ the newborn lamb³⁵ and the adult rat^{12,36–39} have consistently shown that exposure to chronic developmental hypoxia leads to increases in sympathetic innervation, greater α_1 -adrenoreceptor-mediated vasoconstriction and decreased NO-dependent vasodilatation in the peripheral vasculature. Collectively, the data suggest that chronic developmental hypoxia induces a peripheral vasoconstrictor phenotype, which might contribute to a fetal origin of hypertension in later life. However, data in the present study show that in vivo assessment in adult offspring from hypoxic pregnancy provides no evidence of either an increase in peripheral vascular resistance or systemic hypertension. This finding is corroborated by further evidence of no hypertension in highland residents,⁴⁰ neonatal sheep from high altitude pregnancy,³⁵ adult chickens subjected to hypoxia in ovo,³² or adult rats following prenatal hypoxia.^{37,41}

Despite no evidence of any changes in basal arterial blood pressure, this study revealed significant programming on the autonomic control of arterial blood pressure in adult offspring

of hypoxic pregnancy in vivo. It is well-known that fetal hypoxia can produce alterations in autonomic balance.⁴² During chronic hypoxia in late gestation, there are increases in the LF/HF ratio, a measure of sympathetic-parasympathetic balance, and decreases in spontaneous baroreflex activity in the fetal sheep.⁴³ In the present study, increases in the LF/HF ratio of HRV, in baroreflex gain and in maximal and minimal heart rates were also observed in adult offspring of hypoxic pregnancy, all strongly indicative of programming of autonomic dysfunction. Interestingly, several other stresses during development including glucocorticoid excess,⁴⁴ maternal obesity,⁴⁵ maternal undernutrition⁴⁶ and reduced uterine artery blood flow⁴⁷ also lead to increased sympathetic dominance in the offspring, supporting the concept that sub-optimal pregnancy might programme cardiovascular dysfunction in adulthood secondary to programmed alterations in the autonomic regulation of cardiovascular function.

In the present study, maternal treatment with vitamin C during hypoxic pregnancy prevented the programming of sympathetic dominance and baroreflex dysfunction in adult offspring. This suggests that hypoxia-induced increases in oxidative stress during development might programme autonomic dysfunction in later life. The hypoxia-induced alterations in autonomic control could lie at many loci, from the sensory inputs controlling the circulation (eg, peripheral baroreceptors) to the control centres within the medulla (such as the NTS) or in the neuronal effector pathways controlling the heartbeat. For instance, it is known that baroreception can be depressed by oxidative stress⁴⁸ and by NO.^{49,50} Similarly, oxidant tone and NO within the medulla can alter sympathetic and vagal outflow.^{51,52} Whilst systemic administration of vitamin C increases baroreflex sensitivity in the adult,⁵³ probably via actions on carotid baroreceptors,⁴⁸ acute intraventricular administration of vitamin C has been shown to depress baroreflex gain through enhancements of parasympathetic outflow at the level of the NTS and/or nucleus ambiguus.⁵⁴ Another study supports this view, suggesting that ROS within the NTS increase baroreflex gain by enhancing glutamate receptor activity.⁵⁵ Finally, at the level of the heart itself, recent work has shown that chronic fetal hypoxia leads to an increase in the heart rate response to β_1 -adrenergic stimulation and a decrease in heart rate responsiveness to the muscarinic mimetic carbachol in adult offspring.¹² This work supports the programmed cardiac sympathetic dominance and increased cardiac baroreflex gain in adult offspring of hypoxic pregnancy measured in vivo in the present study.

In the current study, adult offspring of control or hypoxic pregnancy treated with maternal vitamin C alone were significantly hypotensive relative to offspring from control pregnancy treated with a maternal saline vehicle. It is established that the local balance between O₂⁻ and NO in the vasculature, the vascular oxidant tone, is an important regulator of periph-

eral vascular resistance in the adult.⁵⁶ Recent work in our laboratory has reported that this vascular oxidant tone is operational in fetal life, as fetal treatment with antioxidants could markedly increase umbilical blood flow via NO-dependent mechanisms.⁵⁷ Furthermore, fetal treatment with antioxidants or agents that increase the bioavailability of NO could diminish the fetal peripheral vasoconstrictor response to exogenous constrictors or to acute hypoxia.^{58–60} Therefore in the present study, fetal exposure to vitamin C during gestation might programme alterations in the development of the vascular tone towards increased NO bioavailability in peripheral resistance circulations resulting in the expression of hypotension in later life. However, hypotension might reflect a failing cardiovascular system.⁶¹ Indeed, some recent studies have shown that exposure to vitamin C alone during pre- or postnatal life leads to vascular endothelial dysfunction^{12,62} and cardiac structural changes reminiscent of dilated cardiomyopathy.²² Therefore, the relative benefit of programmed hypotension in adult life through exposure to maternal vitamin C during development is debatable and requires further investigation.

The dose of vitamin C used in the present study was derived from a previous study in our laboratory that achieved elevations in circulating ascorbate concentrations within the required 10 mmol/L range in ovine pregnancy.⁶⁰ In rat pregnancy, this equated to ca. 1 g kg⁻¹ d⁻¹ of vitamin C administration.⁶³ Although this dose of vitamin C far exceeds that given to pregnant women in all reported clinical trials (1 g per day per woman^{64,65}), the increment from baseline in circulating ascorbate concentrations achieved in dams in the present study was of ca. 70% and, therefore, similar to the increment achieved in pregnant women in the VIP trial following maternal vitamin C administration.⁶⁵ We have shown that this dose of maternal vitamin C in rats suppresses hypoxia-induced increases in maternal plasma urate and L-cysteine concentrations, in the expression of placental HSP70 and HNE protein levels, and in the levels of expression of cardiac HSP70 protein and of vascular nitrotyrosine in the fetal offspring; all markers of oxidative stress.⁶³ Studies of maternal vitamin C supplementation in both humans⁶⁵ and animals^{12,22,62} have also reported adverse effects of antioxidant treatment in healthy pregnancy or early development. While vitamin C might not be the antioxidant of choice for human therapy, past^{12,63} and present evidence from studies in our laboratory provide proof of principle that maternal antioxidant therapy can protect against the programming of cardiovascular dysfunction in adulthood triggered by fetal chronic hypoxia. Therefore, future work warrants investigation of alternative antioxidant strategies in complicated pregnancy.

In conclusion, data in the present study show that chronic fetal hypoxia leads to alterations in blood pressure homeostasis and autonomic function in later life measured in vivo. Maternal treatment with vitamin C in hypoxic pregnancy enhanced birth weight, lowered in vivo resting arterial pressure and restored in vivo autonomic dysfunction in the adult offspring.

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Disclosures

The authors confirm that they hold no conflict of interest.

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