Foetal hypoxia increases cardiac AT$_2$R expression and subsequent vulnerability to adult ischaemic injury

Qin Xue$^{1,2}$†, Chiranjib Dasgupta$^{2}$†, Man Chen$^{2}$, and Lubo Zhang$^{1,2}$*

$^1$Institute for Fetal Origin Diseases, First Hospital of Soochow University, Suzhou 215000, China; and $^2$Center for Perinatal Biology, Department of Physiology and Pharmacology, Loma Linda University School of Medicine, Loma Linda, CA 92350, USA

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Aims
Hypoxia is a common stress to the foetus and results in increased cardiac vulnerability to adult ischaemic injury. This study tested the hypothesis that foetal hypoxia causes programming of increased AT$_2$ receptor (AT$_2$R) expression in the heart, resulting in the heightened cardiac susceptibility to adult ischaemic injury.

Methods and results
Time-dated pregnant rats were divided between normoxic and hypoxic (10.5% O$_2$ from days 15 to 21 of gestation) groups. Hypoxia resulted in significantly increased AT$_2$R in the heart of adult offspring. Multiple glucocorticoid response elements (GREs) were identified at the AT$_2$R promoter, deletion of which increased the promoter activity. Consistently, ex vivo treatment of isolated foetal hearts with dexamethasone for 48 h decreased AT$_2$R expression, which was inhibited by RU 486. Hypoxia decreased glucocorticoid receptors (GRs) in the hearts of foetal, 3-week-old and 3-month-old offspring, resulting in decreased GR binding to the GREs at the AT$_2$R promoter. The inhibition of AT$_2$R improved postischaemic recovery of left ventricular function and rescued the foetal hypoxia-induced cardiac ischaemic vulnerability in male adult animals. In contrast, the inhibition of AT$_1$ receptors decreased the postischaemic recovery.

Conclusion
The results demonstrate that in utero hypoxia causes programming of increased AT$_2$R gene expression in the heart by downregulating GR, which contributes to the increased cardiac vulnerability to adult ischaemic injury caused by prenatal hypoxic exposure.

Keywords
Foetal programming • Hypoxia • Heart • Angiotensin II receptors • Glucocorticoids

1. Introduction
Epidemiological and animal studies have shown a clear association of adverse intrauterine environment with an increased risk of hypertension and ischaemic heart disease in the adulthood.$^{1-4}$ Hypoxia is one of the most important and clinically relevant stresses that can adversely affect foetal development. There is clear evidence of a link between hypoxia and foetal intrauterine growth restriction and an increased risk of cardiovascular disease in offspring.$^{5-13}$ Animal studies have demonstrated that foetal hypoxia causes a significant increase in the number and size of binucleated myocytes in the foetal heart,$^{14}$ resulting in the heightened heart susceptibility to acute ischaemia and reperfusion injury in adult male offspring in a sex-dependent manner.$^{15,15-17}$

Angiotensin II (Ang II) plays a fundamental role in the regulation of cardiovascular homeostasis, and it has been implicated in programming of cardiovascular disease induced by adverse in utero environment during the foetal development.$^{18-22}$ Recent studies have demonstrated a link between foetal insults to differential epigenetic modifications of type 1 (AT$_1$R) and type 2 (AT$_2$R) Ang II receptor genes in the adrenal and kidney and the resultant alteration of their expression patterns in adult life, which may ultimately lead to the development of hypertension.$^{22-24}$ However, the effect of foetal hypoxia on the ontogeny of Ang II receptors in the heart has not been determined. Both AT$_1$R and AT$_2$R are expressed in cardiac myocytes and have significant pathophysiological roles in heart diseases.$^{25-29}$ Yet the effect of AT$_1$R and AT$_2$R on ischaemia and reperfusion injury in the heart remains controversial, depending on

† These two authors contributed equally to the work.
* Corresponding author. Tel: +1 909 558 4325; fax: +1 909 558 4029, Email: lzhang@llu.edu
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systemic vs. local blockade, as well as chronic vs. acute blockade of AT₁R and AT₂R. Although long-term systemic administration of AT₁R antagonists reduced ischemic injury, studies of the acute effects of AT₁R and AT₂R antagonists on the recovery of left ventricular function in the setting of ischemia and reperfusion injury in isolated working rat hearts suggested a cardioprotective effect of AT₂R blockade, but not AT₁R blockade. The expression of both AT₁R and AT₂R is regulated by glucocorticoids. It has been suggested that, in rats, glucocorticoids play an important role in foetal programming of AT₁R and AT₂R expression patterns in foetal hypoxia. The present study tests the hypothesis that foetal hypoxia causes programming of increased AT₂R gene expression in the heart by down-regulating glucocorticoid receptors (GRs), which contributes to the increased ischemic vulnerability of the heart in adult rats, resulting from foetal hypoxia.

2. Methods
An expanded Methods section is available in the Supplementary material online.

2.1 Experimental animals
Pregnant rats were randomly divided into two groups: (i) normoxic control (n = 12), and (ii) hypoxic treatment of 10.5% O₂ from days 15 to 21 of gestation (n = 12), as described previously. Half of the normoxic and hypoxic animals were killed at day 21 of gestation and the foetuses were removed for the studies. The other half of pregnant rats were allowed to give birth and offspring were studied at 3 weeks and 3 months old. Each experimental group had five animals from different dams. Hearts were isolated from the day 21 foetuses, 3-week-old and 3-month-old offspring. For western immunoblots and DNA isolation, hearts were flash frozen in liquid N2 and stored at −80°C until analysis. RNA was extracted immediately and stored at −80°C. For ex vivo studies, separate pregnant rats were used and hearts were isolated from day 17 foetal rats and cultured in M199 media at 37°C in 5% CO₂. Previous work has demonstrated that the intact heart can survive and beat for at least 6 days in M199 media and up to 3 weeks within ideal conditions. These hearts in organ culture did not suffer from a loss of contractile function or any dedifferentiation. After 24 h of recovery, hearts were treated with dexamethasone and/or RU 486 (GR antagonist) for 48 h in seven experimental groups: control, 0.01 μM, 0.1 μM, 1 μM dexamethasone treatments, 1 μM dexamethasone plus 0.1 μM or 1 μM RU 486 treatments, and 1 μM RU 486 alone. Each experimental group had six animals from different dams. All procedures and protocols were approved by the Institutional Animal Care and Use Committee, and followed the guidelines by US National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2 Western blot analysis
Protein was isolated from the hearts, and AT₁R, AT₂R, and GR protein abundance were measured with western blot analysis using the primary antibodies against AT₁R (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:100 dilution), AT₂R (Santa Cruz; 1:200 dilution), and GR (Santa Cruz; 1:1000 dilution). Each experimental group had samples from five animals. To assure equal loading and minimize any confounding influence of variability among gels, an internal standard sample was loaded to each gel and band intensities were normalized to actin and the internal control.

2.3 Real-time RT–PCR
RNA was extracted from the hearts and mRNA abundance of AT₁aR, AT₁bR, and AT₂R was determined by real-time RT–PCR.

2.4 Site-directed mutagenesis and reporter gene assay
Rat AT₂R promoter sequence was obtained from rat genome data base (http://www.ncbi.nlm.nih.gov/mapview). Genomic DNA isolated from rat hearts was used as PCR template for DNA amplification. A 2130 bp fragment spanning −2080 bp to +49 bp relative to the transcriptional start site was amplified and cloned into pCR4-TOPO vector. The KpnI/Xhol fragment flanking the AT₂R promoter region was then inserted into the luciferase reporter gene plasmid, pGL3 to yield the full-length promoter–reporter plasmid. Promoter analyses identified the presence of multiple glucocorticoid response elements (GREs). Site-specific deletions of GREs were constructed, respectively, and the reporter gene assay was performed using a rat embryonic heart-derived myogenic cell line H9c2, as described previously.

2.5 Electrophoretic mobility shift assay (EMSA)
Nuclear extracts were collected from the hearts, and electrophoretic mobility shift assay (EMSA) was performed using the oligonucleotide probes of GREs at rat AT₂R promoter region, as described previously.

2.6 Hearts subjected to ischaemia and reperfusion
Hearts of 3-month-old offspring were isolated and retrogradely perfused via the aorta in a modified Langendorff apparatus, as previously described. After the baseline recording, hearts were perfused for 5 min in the absence or presence of losartan (1 μM, a selective AT₁R antagonist), or PD 123,319 (0.3 μM, a selective AT₂R antagonist), or losartan plus PD 123,319, followed by subjection to 20 min of global ischaemia and 30 min of reperfusion, an approach used in many previous studies in a Langendorff preparation. Previous studies with prolonged reperfusion from 60 to 180 min showed that myocardial infarction and left ventricular recovery reached a plateau at approximately 30 min of reperfusion. Each experimental group had hearts from five animals. Left ventricular developed pressure (LVPD), heart rate (HR), dP/dtmax, dP/dtmin, and LV end-diastolic pressure (LVEDP) were continuously recorded. Myocardial infarct size was measured at the end of reperfusion with 1% triphenyltetrazolium chloride, and was expressed as a percentage of the total left ventricular weight. Lactate dehydrogenase (LDH) activity was measured in coronary effluent collected at 30 s before the onset of ischaemia, and at 0, 1, 2, 3, 4, 5, 10, 15, 20, and 30 min of reperfusion, using TOX 7 assay kit from Sigma following the manufacture’s instructions. The data were expressed as area under curve (AUC).

2.7 Statistical analysis
Data are expressed as mean ± SEM. Statistical significance (P < 0.05) was determined by analysis of variance (ANOVA) followed by Neuman–Keuls post hoc testing or Student’s t-test, where appropriate.

3. Results
3.1 Effect of development and foetal hypoxia on AT₁R and AT₂R protein and mRNA abundance in the heart
Protein and mRNA abundance of both AT₁R and AT₂R showed a development-dependent reduction in the heart, and no sex difference was observed (Figure 1A and B). Whereas the expression levels in both 3-week-old and 3-month-old offspring were significantly decreased, when compared with those in the foetus, there were no significant differences in the expression levels between 3-week-old and 3-month-old offspring. In 3-month-old offspring, AT₁R mRNA decreased to less abundance than that of AT₂R and AT₁bR, resulting
in a significant decrease in the AT$_2$R/AT$_1$R receptor ratio in the adult heart. Maternal hypoxia had no significant effect on the litter size (11.2 ± 0.9 vs. 12.0 ± 0.8). Previous studies in the same rat model showed that maternal hypoxia resulted in a decrease in birth weight, but had no significant effect on body weight of 3-month-old offspring.\textsuperscript{10,17} There was a significant decrease in protein abundance of AT$_1$R, but not AT$_2$R, resulting in an increased AT$_2$R to AT$_1$R ratio in hypoxic vs. control foetal hearts (Figure 1C). This was associated with a decrease in AT$_1$R mRNA (Figure 1D). The same expression pattern persisted in the hearts of 3-week-old male offspring, whereas no significant differences in AT$_1$R and AT$_2$R were observed in females. In 3-month-old offspring, prenatal hypoxia increased protein (Figure 1C) and mRNA (Figure 1D) abundance of AT$_2$R, but not AT$_1$R, in the male heart. In females, both AT$_1$R and AT$_2$R were increased and the AT$_2$R to AT$_1$R ratio was not significantly changed (Figure 1C). Consistently, AT$_1$R and AT$_2$R mRNA abundance were significantly increased in the female heart (Figure 1D).

### 3.2 Inhibitory effect of GREs on the AT$_2$R promoter activity

Rat AT$_2$R promoter has a TATAA element at -46 from the transcription start site (see Supplementary material online, Figure S1). Deletion of the TATAA element significantly decreased the promoter activity.
Multiple GREs were identified at rat AT2R promoter. These include GRE1 (−1853), GRE2 (−1674), GRE3 (−1526), GRE4 (−1159), GRE5 (−945), GRE6 (−676), GRE7 (−107), and GRE8 (+13) (see Supplementary material online, Figure S1). Site-specific deletion of each GRE independently caused a significant increase in the promoter activity (Figure 2A). While the GRE4 deletion stimulated the luciferase activity by 2.43-fold, deletion of other GREs increased the promoter activity by 1.5- to 1.9-fold.

### 3.3 Binding of GRs to the GREs at the AT2R promoter

Binding of nuclear proteins to the putative GREs at the AT2R promoter was evaluated by EMSA. Sequences of GRE oligos used in EMSA were presented in Supplementary material online, Figure S2. Immunoblot analysis confirmed the presence of GRs in the nuclear extracts used in EMSA (Figure 2B, left panel). We first defined our criteria by analysing GRE6, as it was originally suggested at the AT2R promoter.40 As shown in Figure 2B (right panel), incubation of nuclear extracts from rat hearts with double-stranded oligonucleotide probes of GRE6 resulted in a shift of DNA–protein complex, which was further supershifted by an anti-GR antibody. Extended studies showed identical electrophoretic mobility of protein–DNA complexes for GREs 1, 2, 3, 4, 5, 7, and 8 with that of GRE6 in EMSA, which were blocked by the cold competitions with homologous, heterologous (AT1aR GRE), and the consensus GRE (CGRE) oligos (see Supplementary material online, Figure S2).

### 3.4 Dexamethasone inhibits AT2R expression in the heart

Dexamethasone treatment for 48 h produced a dose-dependent decrease in mRNA and protein abundance of AT2R in the intact foetal rat hearts (see Supplementary material online, Figure S3). RU 486 alone had no effect on AT2R mRNA but blocked the dexamethasone-induced reduction of AT2R mRNA abundance (see Supplementary material online, Figure S3).
3.5 Effect of development and foetal hypoxia on GR abundance and GR binding to GREs at the AT$_2$R promoter

GR abundance in the heart was significantly increased in offspring when compared with the foetus (Figure 3A). However, the expression levels were not significantly different between 3-week-old and 3-month-old offspring. Additionally, no sex difference was observed (Figure 3A). Hypoxia decreased GR expression in the foetal heart, which was sustained in the hearts of both 3-week-old and 3-month-old offspring in a sex-independent manner (Figure 3B). Nuclear GR protein abundance was also decreased in 3-week-old and 3-month-old offspring (Figure 3B). In accordance, there were similar extent decreases in GR bindings to GREs 4, 6, 7, and 8 at the AT$_2$R promoter in hearts of 3-month-old offspring (Figure 4A). The binding affinity of GR to GREs was determined in competition studies performed in pooled nuclear extracts from the hearts of adult offspring with the increasing ratio of unlabelled/labelled

![Figure 3](image1.png)

**Figure 3** Effect of development (A) and foetal hypoxia (B) on GR protein abundance. Hearts were isolated from near-term (21 days) foetuses, 3-week-old (3W) and 3-month (3M)-old male (M) and female (F) offspring in the control (C) and hypoxic (H) groups. Total cellular (T) and nuclear (NE) GR abundance was determined. Data are means ± SEM. Data in (A) were analysed by two-way ANOVA. *P < 0.05, offspring vs. foetus. n = 5. Data in (B) were analysed by t-test, *P < 0.05, hypoxia vs. control. n = 5.

![Figure 4](image2.png)

**Figure 4** Effect of foetal hypoxia on GRE binding at the AT$_2$R promoter. (A) Binding of the GR to GREs was determined by EMSA in nuclear extracts from the hearts of 3-month-old male and female offspring in the control (C) and hypoxic (H) groups. Data are means ± SEM. *P < 0.05 (t-test), hypoxia vs. control. n = 5. (B) The binding affinity of GR to GREs was determined in competition studies performed in pooled nuclear extracts from the hearts of adult offspring with the increasing ratio of unlabelled/labelled oligonucleotides encompassing the GRE4 at −1159 in the AT$_2$R promoter.
oligonucleotides encompassing the GRE4 at -1159 in the AT2R promoter. Foetal hypoxia had no significant effect on the binding affinity of nuclear extracts to the GRE in the hearts of both male and female adult rats (Figure 4B).

3.6 Functional role of AT2R in acute ischaemia and reperfusion injury

The functional significance of AT2R in modulating the postischaemic recovery of left ventricle (LV) function after acute ischaemia was determined in a Langendorff preparation using a selective AT2R inhibitor, PD 123,319. As shown in Supplementary material online, Table S1, there were no significant differences in LVDP, HR, dP/dt max, dP/dt min, and coronary flow rate at the baseline among all groups. PD 123,319 significantly improved the postischaemic recovery of LVDP (Figure 5A and B), as well as dP/dt max and dP/dt min (see Supplementary material online, Figure S4) in both male and female hearts. Consistently, PD 123,319 decreased LVEDP (see Supplementary material online, Figure S4), myocardial infarct size, and LDH release (Figure 5C and D) after myocardial ischaemia in both male and female animals. In contrast, an AT1R selective inhibitor losartan impaired the postischaemic recovery of LVDP (Figure 5A and B) and dP/dt max and dP/dt min (see Supplementary material online, Figure S4), and significantly increased LVEDP (see Supplementary material online, Figure S4), myocardial infarct size, and LDH release (Figure 5C and D). In the presence of both PD 123,319 and losartan, there were no significant differences in the postischaemic recovery of LV function and myocardial infarction (Figure 5; see Supplementary material online, Figure S4). The recovery of HR and coronary flow rate was not significantly different among all groups (data not shown).

3.7 Inhibition of AT2R restores foetal hypoxia-induced cardiac vulnerability to ischaemic injury in offspring

To determine whether increased AT2R in the heart is an important factor in the foetal hypoxia-induced increase in cardiac ischaemic susceptibility in adult male offspring, additional studies were performed in the hearts of adult offspring that had been exposed to hypoxia before birth. In the absence of PD 123,319, foetal hypoxia was associated

![Figure 5](image-url)
with a significant decrease in postischaemic recovery of LVDP and increases in LVEDP, infarct size, and LDH release (Figure 6), as well as decreases in the recovery of $dP/dt_{\text{max}}$ and $dP/dt_{\text{min}}$ (see Supplementary material online, Figure S5), as previously reported. PD 123,319 increased postischaemic recovery of left ventricular function and abolished the difference in ischaemic injury observed between the control and hypoxic rats (Figure 6 and see Supplementary material online, Figure S5).

4. Discussion

The present study demonstrates for the first time that foetal hypoxia results in programming of differential expression patterns of angiotensin II receptors in the heart of offspring. Whereas maternal hypoxia had no effect on the litter size, it decreased the birth weight, which could contribute to the changes observed. The finding that prenatal hypoxia increased AT$_2$R expression in adult hearts is intriguing and suggests epigenetic reprogramming of a foetal gene of pathophysiological significance in the heart in a developmental-dependent manner. Consistent with the previous studies, we identified the TATAA box region at the AT$_2$R promoter, which plays a critical role in the transcription of AT$_2$R gene in rat hearts. The sequences of multiple GREs identified at rat AT$_2$R gene promoter are imperfect representation (half sites) of the positive regulatory CGRE sequence, $5'$-GGTACA$\text{nnn}$TGTTCT-3'. The finding of the inhibitory effects of these GREs on the AT$_2$R promoter activity suggests a novel mechanism of glucocorticoids in regulating the ontogeny of cardiac AT$_2$R gene expression patterns during the development. Normal foetal adrenal produces low levels of glucocorticoids. Among other functions, the low foetal glucocorticoid levels may contribute to maintaining the relatively high levels of AT$_2$R in foetal tissues, whereas the substantial increases in circulating glucocorticoid levels and GR abundance in the heart during the postnatal development are likely to play a key role in down-regulating AT$_2$R in the adult in a tissue-specific manner. This is supported by the finding that dexamethasone caused a dose-dependent decrease in AT$_2$R protein and mRNA abundance in the heart, which was inhibited by RU486, indicating a GR-mediated response.

The finding that foetal hypoxia caused significant decreases in both total and nuclear GR protein abundance in the heart of offspring provides a mechanism that may contribute to the partial reversal of glucocorticoid-dependent downregulation of AT$_2$R, resulting in the increased AT$_2$R expression in the heart. Indeed, foetal hypoxia resulted in about a 50% decrease in GR binding to the GREs at the

**Figure 6** Rescue effect of PD123,319 on hypoxia-mediated ischaemic vulnerability. Hearts were isolated from 3-month-old male offspring that had been exposed to normoxia (control) or hypoxia before birth, and were treated in the absence or presence of PD123,319 (0.3 $\mu$M) for 5 min before subjecting to 20 min of ischaemia and 30 min of reperfusion in a Langendorff preparation. Post-ischaemic recovery of LVDP, end-diastolic pressure (LVEDP), and infarct size were determined. LDH release over 30 min of reperfusion was measured as AUC. Data are means ± SEM. Data were analysed by two-way ANOVA. *$P < 0.05$, hypoxia vs. control; †$P < 0.05$, +PD123,319 vs. −PD123,319. $n = 5$. Q. Xue et al.
The pathophysiological function of AT2R is context-specific, i.e. the ratio of AT2R to AT1R in the heart of adult male offspring demonstrated in the present study is likely to play a key role in the heightened heart susceptibility to acute ischaemia and reperfusion injury in adult male offspring that had been exposed to hypoxia before birth. This is supported by the finding that the blockade of AT1R with PD 123,319 rescued the myocardial phenotype of the increased ischaemic susceptibility in male offspring after prenatal hypoxic exposure, providing the cause-and-effect evidence for the role of increased AT2R in foetal programming of enhanced cardiac vulnerability to acute ischaemic injury. Future studies of developmental overexpression of AT2R in the heart are needed to determine whether the increased AT2R mimics and is sufficient for the hypertrophic response. Whereas the mechanisms underlying the different effects of AT1R and AT2R in acute cardiac ischaemic injury remain unclear, it has been known that AT1R promotes cell growth and proliferation, yet AT2R mediates anti-growth and apoptosis. These apparent opposite effects provide a congruent functional basis for understanding the different effects of AT1R and AT2R in modulating acute cardiac ischaemic injury vs. long-term cardiac remodelling. Our previous study suggested that downregulation of PKCe in the heart played a role in the increased ischaemic susceptibility in adult male offspring resulting from prenatal hypoxia. Given that AT1R stimulation and AT2R blockade activate PKCe and mimic ischaemic preconditioning by reducing infarct size, it is possible that foetal hypoxia-induced programming of increased AT1R/AT2R ratio in the heart of adult males suppresses the PKCe activity, leading to the enhanced susceptibility to ischaemic injury.

The present investigation provides novel evidence of foetal programming of upregulation of the AT2R/AT1R ratio in the heart of adult males, linking foetal hypoxia and the increased susceptibility to ischaemia and reperfusion injury in the heart of adult male offspring in a sex-dependent manner. Given that hypoxia is one of the most important and clinically relevant stresses to the foetus, and that large epidemiological studies indicate a link between in utero adverse stimuli during gestation and an increased risk of ischaemic heart disease in the adulthood, the present study provides a mechanistic understanding worthy of investigation in humans.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

Conflict of interest: none declared.

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