Ketamine suppresses hypoxia-induced inflammatory responses in the late-gestation ovine fetal kidney cortex

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Key points

- The fetus responds to decreases in arterial partial pressure of oxygen by redirecting the blood flow mainly to the brain and the heart, at a cost to other peripheral organs like the kidneys.
- Renal hypoxia and ischaemia stimulate inflammatory and apoptotic responses.
- Ketamine, an NMDA receptor antagonist, is able to reduce renal immune and inflammatory gene expressions stimulated by hypoxia.
- Ketamine may have therapeutic potential for protection against ischaemic renal damage in fetuses subjected to acute hypoxia.

Abstract   Acute fetal hypoxia is a form of fetal stress that stimulates renal vasoconstriction and ischaemia as a consequence of the physiological redistribution of combined ventricular output. Because of the potential ischaemia–reperfusion injury to the kidney, we hypothesized that it would respond to hypoxia with an increase in the expression of inflammatory genes, and that ketamine (an *N*-methyl-D-aspartate receptor antagonist) would reduce or block this response. Hypoxia was induced for 30 min in chronically catheterized fetal sheep (125 ± 3 days), with or without ketamine ($3 \, \text{mg} \, \text{kg}^{-1}$) administered intravenously to the fetus 10 min prior to hypoxia. Gene expression in fetal kidney cortex collected 24 h after the onset of hypoxia was analysed using ovine Agilent 15.5k array and validated with qPCR and immunohistochemistry in four groups of ewes: normoxic control, normoxia + ketamine, hypoxic control and hypoxia + ketamine ($n = 3$–4 per group). Significant differences in gene expression between groups were determined with *t*-statistics using the limma package for R ($P \leq 0.05$). Enriched biological processes for the 427 upregulated genes were immune and inflammatory responses and for the 946 downregulated genes were metabolic processes. Ketamine countered the effects of hypoxia on upregulated immune/inflammatory responses as well as the downregulated metabolic responses. We conclude that our transcriptomics modelling predicts that hypoxia activates inflammatory pathways and reduces metabolism in the fetal kidney cortex, and ketamine blocks or ameliorates this response. The results suggest that ketamine may have therapeutic potential for protection from ischaemic renal damage.

Abbreviations   BCL3, B-cell lymphoma 3-encoded protein; CCL, chemokine (C-C motif) ligand; CD14, CD14 molecule; CSF1, macrophage colony stimulating factor 1; CXCL10, chemokine (C-X-C motif) ligand 10; HC, hypoxic control; HK, hypoxia + ketamine; Iba-1, ionized calcium binding adaptor molecule 1; IL, interleukin; IRF1, interferon regulatory factor 1; KEGG, Kyoto encyclopedia of genes and genomes; MAPK, mitogen-activated protein kinases; NC, normoxic control; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; NK, normoxia + ketamine; NMDA, *N*-methyl-D-aspartate; TLR, toll like receptor; TNFα, tumour necrosis factor-alpha.

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Introduction

Throughout pregnancy, the fetus maintains a state of oxygen homeostasis within the uterine environment. Hypoxia, a relatively common fetal stressor, has the potential to damage developing organs. The physiological response to hypoxia *in utero* features a redistribution of combined ventricular output towards the heart and brain and away from organs – including the kidney – that are not needed for short-term survival (Cohn *et al.* 1974; Green *et al.* 1997; Luciano *et al.* 1998). While there is much known about the physiology of redistribution of fetal blood flow during the response to acute hypoxia, less is known about the cellular response, and specifically whether the response is potentially damaging. Whether there are strategies preserving kidney integrity after hypoxia *in utero* is relatively unknown (Giussani *et al.* 1994; Giussani & Davidge, 2013).

Recently, we have applied transcriptomic and systems biology methodology to elucidate the effect of hypoxia on the developing fetal brain (Wood *et al.* 2014). We reported an increase in inflammatory pathways in the fetal hypothalamus (Wood *et al.* 2013). We have reported (in abstract) that pretreatment with ketamine decreases brain inflammatory responses (Chang & Wood, 2013). Ketamine, an *N*-methyl-D-aspartate (NMDA) receptor antagonist, is commonly used in veterinary medicine, pediatric surgery, and neonatal intensive care for its anaesthetic and analgesic properties (Mellon *et al.* 2007). Clinically, ketamine has been shown to be an anti-inflammatory agent acting by decreasing serum levels of interleukin 6 (IL-6) and tumour necrosis factor-alpha (TNFα) in patients undergoing cardiac surgeries and liver transplantation (Roytblat *et al.* 1998; Zeyneloglu *et al.* 2005; Yang *et al.* 2006). In addition, *in vitro* studies have shown that lipopolysaccharide-activated macrophages (Wu *et al.* 2008) and glial cells (Tanaka *et al.* 2013) have decreased IL-6, TNFα, and IL-1β gene expression when treated with ketamine. Consequently, ketamine might be more broadly useful as a means to decrease peripheral tissue inflammation and reduce or prevent adverse outcomes as it blunts inflammatory responses globally, and not solely in the brain.

NMDA receptors are not only found throughout the nervous system, but are also present in various extraneuronal tissues such as the renal cortex and medulla, and in the atriums, ventricles, aorta and pulmonary artery of the cardiovascular system (Leung *et al.* 2002). An upregulation and activation of renal NMDA receptors was found in ischaemia–reperfusion- and hypoxia–reoxygenation-induced acute renal injury (Yang *et al.* 2008; Pundir *et al.* 2013). Intense activation of NMDA receptors can evoke significant bursts of reactive oxygen species production, calcium ion overload and damage renal tubules, resulting in induction of apoptosis and necrosis of tubular cells, which may contribute to renal dysfunction (Yang *et al.* 2008). In these acute kidney injury models, various NMDA receptor antagonists were found to attenuate ischaemia–reperfusion-induced injuries and significantly reduce oxidative stress (Pundir *et al.* 2013). Ketamine, by blocking NMDA receptors and their activation, may potentially be beneficial by decreasing renal damage in the developing fetus. For these reasons, we hypothesized that ketamine would reduce the expression of renal inflammatory genes in the late gestation ovine fetus exposed to acute hypoxia. The experiments reported herein were designed to test the hypotheses that acute hypoxia induces inflammatory pathways in the fetal kidney and that ketamine ameliorates or eliminates this response.

Methods

Ethical approval

These experiments were approved by the University of Florida Animal Care and Use Committee and were performed in accordance with the *Guiding Principles for the Care and Use of Vertebrate Animals in Research and Training* of the American Physiological Society. Fifteen chronically catheterized singleton ($n = 1$ in normoxic ketamine group) or twin ($n = 14$) ovine fetuses were studied at the gestational age of 122 ± 5 days (full term = 145–147 days), which is approximately equivalent to 28–30 weeks in humans.

Fetal ovine surgical procedures

Ewes were fasted for 24 h before surgery, and the fetal ovine surgical procedures were performed on 116 ± 3 days of gestation. The surgical procedures for chronic catherization of the fetal and maternal femoral arteries and veins and aminiotic fluid were described in detail previously (Wood *et al.* 1990; Powers & Wood, 2007). Briefly, the ewes were given 750 mg ampicillin (Polyflex, Boehringer Ingelheim VetMedica, Inc., St Joseph, MO, USA), anaesthetized and intubated with 0.5–2% isoflurane with oxygen and vascular catheters were placed. Before the uterus was sutured closed, 500 mg ampicillin was injected into the amniotic fluid. A small tracheostomy catheter was placed in the ewe to produce maternal induced ventilatory hypoxia, which in turn resulted in fetal hypoxic hypoxia (Matsumoto *et al.* 2001). The maternal and fetal surgical procedures were the same for both normoxic and hypoxic groups. A minimum of 5 days of recovery preceded experimentation. Post-operatively ewes were monitored twice daily for food intake, body temperature and signs of infection or distress. During the postoperative recovery period, the ewes were treated with prophylactic antibiotic (ampicillin 15–20 mg kg^{-1}, I.M. twice daily for 5 days) and analgesic

(flunixine meglumin, 0.5 mg kg^{-1}, I.M. once daily for 2–3 days).

In vivo experimental procedures

During the experiments, the ewes were conscious and freestanding in their pens with access to food and water. The fifteen fetuses (7 males, 8 females) were randomly assigned to one of the four groups ($n = 3$–4 per group): normoxic control (NC), normoxia + ketamine (NK), hypoxic control (HC), and hypoxia + ketamine (HK). In the ketamine-treated groups, ketamine (3 mg kg^{-1}) was administered via the fetal femoral venous catheter, 10 min prior to the hypoxic stimulus. The dose of ketamine was based upon the clinically used dose in the neonatal intensive care units (Anand, 2007). Hypoxia was induced by infusing nitrogen gas directly into the maternal tracheostomy tube at flow rates sufficient to decrease maternal pressure of oxygen (P_{aO_2}) by 50%. As reported previously, maternal ventilatory hypoxia decreases both maternal and fetal P_{aO_2} (Chang & Wood, 2015). To closely monitor the changes in the composition of blood gases (ABL80 Radiometer, Copenhagen, Denmark), both maternal and fetal arterial blood samples were drawn anaerobically (1 ml) every 10 min (Tables 1 and 2). At the end of the 30 min period of normoxia or hypoxia, the ewe returned to breathing room air. The ewes and fetuses were killed with an overdose of sodium pentobarbital 24 h after the initial stimulation of hypoxic stress, and various fetal tissues were snap frozen and stored at −80°C until analysis. The kidney cortex analysed were from a subset of experiments from which blood gas data had been previously reported (Chang & Wood, 2015).

Microarray procedures

The RNA was extracted from fetal kidney cortex with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and purified with RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA), resulting in RNA with RNA integrity number values between 7.7 and 9.1, and were labelled with cyanine 3 CTP with the Quick Amp Labelling Kit (Cat. No. 5190-0442, Agilent Technologies, Santa Clara, CA, USA), according to the manufacturer's protocol. The specific activity of the labelled cRNAs ranged from 10.4 to 12.7 pmol Cy3 (μg RNA)$^{-1}$ and yields were from 5.8 to 7.9 μg. The cRNA samples were hybridized and processed for one-channel Ovine Gene Expression Microarray (8 × 15 K slide) – 8 arrays with 15208 oligomers each (Cat. No. G4813A-019921, Agilent Technologies, Santa Clara, CA, USA) as described earlier (Rabaglino *et al.* 2012; Wood *et al.* 2013). The slides were scanned with Microarray Scanner System (G2505-90021, Agilent) and the measured

Table 1. Fetal blood gases and pH values

Time (min)	P_{aO_2} (mmHg)				P_{aCO_2} (mmHg)				pH			
	NC	NK	HC	HK	NC	NK	HC	HK	NC	NK	HC	HK
−10	18 ± 1	19 ± 2	17 ± 1	16 ± 1	53 ± 1	51 ± 2	55 ± 3	55 ± 1	7.38 ± 0.01	7.39 ± 0.02	7.35 ± 0.01	7.37 ± 0.01
0	18 ± 1	17 ± 1	17 ± 2	16 ± 1	53 ± 3	53 ± 2	57 ± 2	58 ± 1	7.38 ± 0.01	7.38 ± 0.02	7.35 ± 0.01	7.35 ± 0.01
5	17 ± 1[a]	16 ± 1[a]	11 ± 1[b]	11 ± 2[a,b]	54 ± 2	55 ± 1	55 ± 1	56 ± 2	7.38 ± 0.01	7.36 ± 0.01	7.36 ± 0.01	7.37 ± 0.01
10	17 ± 2[a]	17 ± 1[a]	10 ± 1[b]	8 ± 1[a,b]	56 ± 3	55 ± 2	53 ± 1	13 ± 2	7.37 ± 0.01	7.37 ± 0.02	7.37 ± 0.01	7.38 ± 0.01
20	16 ± 2[a]	17 ± 1[a]	10 ± 1[b]	7 ± 1[a,b]	54 ± 2	55 ± 2	52 ± 1	52 ± 3	7.38 ± 0.01	7.36 ± 0.02	7.36 ± 0.01	7.36 ± 0.01
30	17 ± 2[a]	17 ± 1[a]	9 ± 1[b]	9 ± 1[a,b]	55 ± 1	55 ± 2	54 ± 2	53 ± 2	7.37 ± 0.01[a]	7.37 ± 0.02[a,b]	7.33 ± 0.03[a,b]	7.32 ± 0.02[b]

These values are a subset of previously reported values (Chang & Wood, 2015). Statistical significance between groups was declared at $P \leq 0.05$ (two-way ANOVA), and Duncan's test was performed to determine differences between groups (different letters are statistically significantly different from each other, $P < 0.05$). NC ($n = 3$), NK ($n = 4$), HC ($n = 4$), HK ($n = 4$); all values are expressed as means ± SEM.

Table 2. Maternal blood gases and pH values

Time (min)	P_{aO_2} (mmHg)		P_{aCO_2} (mmHg)		pH	
	Normoxia	Hypoxia	Normoxia	Hypoxia	Normoxia	Hypoxia
−10	103 ± 3	97 ± 4	38 ± 2	39 ± 1	7.47 ± 0.01	7.47 ± 0.01
0	105 ± 4	99 ± 3	37 ± 2	39 ± 2	7.48 ± 0.01	7.47 ± 0.01
5	105 ± 4[a]	59 ± 5[b]	37 ± 2[a]	32 ± 1[b]	7.47 ± 0.01[b]	7.55 ± 0.02[a]
10	98 ± 7[a]	55 ± 6[b]	39 ± 2[a]	33 ± 2[b]	7.46 ± 0.01[b]	7.54 ± 0.02[a]
20	103 ± 2[a]	45 ± 8[b]	38 ± 2[a]	31 ± 1[b]	7.47 ± 0.01[b]	7.54 ± 0.01[a]
30	104 ± 2[a]	51 ± 7[b]	38 ± 2[a]	32 ± 1[b]	7.48 ± 0.01[b]	7.54 ± 0.01[a]

These values are a subset of previously reported values (Chang & Wood, 2015). Statistical significance between groups was declared at $P \leq 0.05$ (two-way ANOVA), and Duncan's test was performed to determine differences between groups (different letters are statistically significantly different from each other, $P < 0.05$). Normoxia ($n = 4$), hypoxia ($n = 5$); all values are expressed as means ± SEM.

fluorescence was detected and converted using Agilent Feature Extraction 9.1 software at the Genomics Division of the University of Florida's Interdisciplinary Centre for Biotech Research. Microarray data have been deposited in the NCBI Gene Expression Omnibus under accession number GSE66920.

Microarray data analysis

Raw data was processed with the R software (RCoreTeam, 2013), employing the limma package (Ritchie *et al.* 2015) to perform background correction and data normalization using the quantile normalization method (Smyth *et al.* 2005). Probes that were at least 10% brighter than the negative controls on at least three arrays were retained for further analysis. The processed microarray data were statistically analysed with the limma package as well, employing a moderated *t* test that uses an empirical Bayes method for small sample size per group ($P \leq 0.05$; Smyth, 2004). The effect of hypoxia was analysed by comparing the hypoxic group to normoxic group as the control group. Then, the hypoxic + ketamine group was compared to the hypoxic group to evaluate the effect of ketamine *under hypoxic conditions*. Finally, the normoxic + ketamine group was compared to the normoxic group to evaluate the effect of ketamine *under physiological conditions*.

Cytoscape and various plug-ins (GeneMANIA, BiNGO) were used to analyse gene network inference and clustering analysis with the significantly differentially expressed genes (Shannon *et al.* 2003; Maere *et al.* 2005; Cline *et al.* 2007; Warde-Farley *et al.* 2010; Zhang *et al.* 2014). The functional annotation of gene ontogeny for significantly up- and downregulated genes were analysed via Database for Annotation, Visualization and Integrated Discovery Bioinformatics Resources 6.7 (Huang da *et al.* 2009*a*,*b*). The KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways were analysed with WEB-based GEne SeT AnaLysis Toolkit (WebGestalt) (Zhang *et al.* 2005; Wang *et al.* 2013).

Real-time PCR

cDNA made from the same RNA used for the microarray analyses were used for qPCR validation of the microarray findings. The primers were designed based on the known *Ovis aries* and *Bos taurus* genomes (Table 3) for SYBR green detection in qPCR. As the housekeeping control, ovine β-actin primers and probe were used (Wood *et al.* 2013). The RNA expression was normalized by the difference in threshold cycle (ΔCt) between the triplicate mean Ct for each gene and the triplicate mean Ct for β-actin mRNA from the same sample, and these ΔCt values compared between treatment groups.

Immunohistochemistry

Samples of fetal kidney were immersion fixed in 4% buffered paraformaldehyde overnight, then transferred to 70% reagent alcohol until paraffin embedded. These tissue samples were taken simultaneous with the tissue used for the mRNA isolation and analysis. Histological sections were cut at 5 μm, and immunostained for Iba-1 using primary antibody from Waco Pure Chemical Industries (Richmond, VA; USA 1:200 dilution, Cat. No. 019–19741), and peroxidase–PAP sandwich technique (Vectastain, Vector Labs, Burlingame, CA, USA). All sections were counterstained with methyl green. Resident macrophages, identified as Iba-1-positive cells, were quantified as number of cells in a ×40 visual field by an investigator who was blinded to identity or experimental group of the histological section. Five fields were analysed for each section of kidney cortex, and the mean was calculated for statistical analysis.

Statistics

The experimental design involved a double factorial randomized complete design. Statistical analyses were conducted using different statistical software depending

Table 3. Primers for real-time PCR validation

Official symbol	Gene name	Forward primer	Reverse primer	Species
TLR2	Toll-like receptor 2	GATTCTGCTGGAGCCCATTG	TCATGATCTTCCGCAGCTTACA	*Ovis aries*
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells	TCCCACAGATGTTCACAAACAGT	GACGCTCAATCTTCATCTTGTGAT	*Ovis aries*
IL-6	Interleukin 6	ATGCTTCCAATCTGGGTTCAA	TCCAGAAGACCAGCAGTGGTT	*Ovis aries*
IL-1β	Interleukin 1β	CGTGGCCATGGAGAAGCT	GGTCATCATCACGGAAGACATGT	*Ovis aries*
CSF1	Macrophage colony-stimulating factor	GACTGGAACATTTTCAGCAAGAACT	TCAGGCTTGGTCACCACATC	*Bos taurus*
CD14	Monocyte/macrophage surface antigens	CCTAAAGGACTGCCGACCAA	GCGGCTCCCTGCTTAGCT	*Ovis aries*
TNFα	Tumour necrosis factor	CCCTTCCACCCCCTTGTT	ATGTTGACCTTGGTCTGGTAGGA	*Ovis aries*
BCL3	B-cell CLL/lymphoma 3	CATGGAACACCCCCTGTCA	GGCGTATCTCCATCCTCATCA	*Ovis aries*
CCL4	Chemokine ligand 4	TCTTACACCCTGCGGCAGAT	GGCTGCTGGTCTCGTAGTAGTCA	*Ovis aries*
CCL5	Chemokine ligand 5	CCAGCAGCAAGTGCTCCAT	CGCACACCTGACGGTTCTT	*Ovis aries*
CXCL10	Chemokine ligand 10	TTGAACTGATTCCTGCAAGTCA	TTCCTTTTCATTGTGGCAATAATCT	*Ovis aries*
IRF1	Interferon regulatory factor 1	CCCAGGGCTGATCTGGATTA	GCGTGCTTCCATGGGATCT	*Ovis aries*

on the required statistical tests. For the microarray data analysis, the R software was used with the limma package (see detailed analysis above). For the qPCR and immuno-histochemistry experiments, a normal probability plot test was performed to confirm normality in data distribution. qPCR data were analysed by two-way analysis of variance with stimulus (hypoxia/normoxia) and treatment (ketamine/control) as factors and to allow for analysis of replicate fields; immunohistochemistry was analysed using two-way nested design ANOVA to account for variability between visual fields within animal using the Mixed Procedure of SAS/STAT 9.3 (SAS Institute Inc., Cary, NC, USA). The criterion for statistical significance was $P \leq 0.05$. When significance was found, groups were further analysed by Duncan's *post hoc* test to detect differences among them. The qPCR and immunohistochemistry data are presented as mean values $\pm$ standard error of the mean (SEM).

Results

Acute maternal ventilatory hypoxia induced significant changes in gene expression. From a total of 1373 significantly differentially regulated genes, 427 were upregulated and 946 were downregulated by hypoxia alone (Fig. 1*A*). Ketamine modified the transcriptomic response to hypoxia: 260 genes were upregulated (relative to hypoxia without ketamine), and 193 genes were down-regulated (relative to hypoxia without ketamine; Fig. 1*B*). Ketamine alone in normoxic conditions differentially regulated 190 genes (97 upregulated and 93 down-regulated; Fig. 1*C*). Analysis of the 97 upregulated genes did not reveal any statistically significant overrepresented
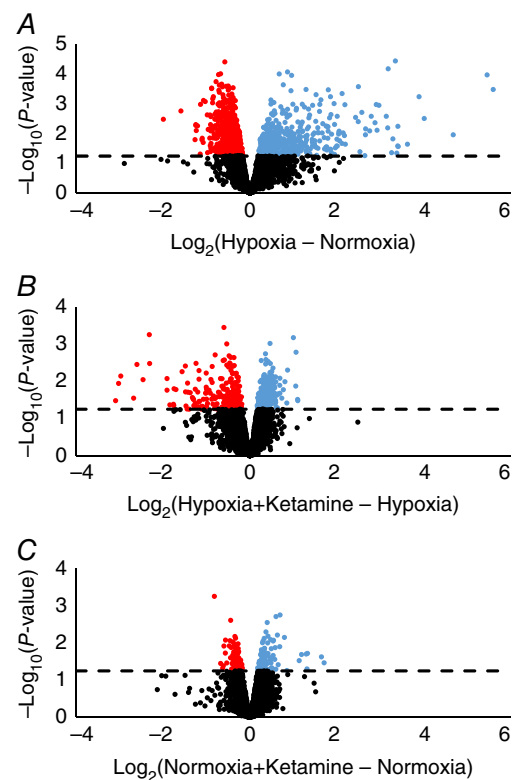

Figure 1. Volcano plot illustrating the relationship of gene expression in fetal kidney cortex measured by log$_2$ of fold-change
Significantly upregulated (blue) and downregulated (red) genes under hypoxia (*A*), hypoxia + ketamine (*B*), and normoxia + ketamine (*C*). The dashed line indicates whether the gene is statistically significant (above), $P \leq 0.05$, or not significant (below). Non-significant genes are indicated by black circles, and the scale for the *y*-axis varies between plots.

Table 4. Top 10 gene ontology biological processes that were significantly overrepresented in the kidney cortex during acute hypoxic stress, with or without ketamine

Group	Biological process	No. of genes involved	Adjusted *P* values
Hypoxia upregulated	Immune response	108	1.13E-31
	Immune system process	133	1.11E-26
	Defense response	101	4.53E-26
	Inflammatory response	62	1.82E-22
	Regulation of immune system process	82	1.10E-21
	Response to other organism	63	5.05E-19
	Innate immune response	60	1.01E-18
	Response to biotic stimulus	63	5.32E-18
	Regulation of defense response	52	7.66E-18
	Regulation of response to stimulus	136	8.40E-18
Hypoxia downregulated	Cellular macromolecule metabolic process	461	2.13 E-09
	Macromolecule metabolic process	483	1.92 E-07
	Nucleic acid metabolic process	322	1.92 E-07
	Chromosome organization	78	1.92 E-07
	Regulation of macromolecule metabolic process	305	1.83 E-07
	Cellular component organization	302	1.83E-07
	Cellular component organization at cellular level	256	1.68 E-07
	Regulation of nitrogen compound metabolic process	266	1.68 E-07
	Peptidyl-lysine acetylation	26	1.68 E-07
	Regulation of nucleobase-containing compound metabolic process	263	1.12 E-07
Hypoxia + ketamine upregulated	Cellular macromolecule metabolic process	129	2.44 E-02
	Response to DNA damage stimulus	23	2.44 E-02
	Organelle organization	51	4.44 E-02
	Chromosome organization	23	4.44 E-02
	Double-strand break repair	8	4.44 E-02
	DNA-dependent DNA replication	8	4.26 E-02
	Cell cycle checkpoint	12	5.71 E-02
	Cellular protein metabolic process	69	5.83 E-02
	S phase	9	5.83 E-02
	Proteasome regulatory particle assembly	2	5.83 E-02
Hypoxia + ketamine downregulated	Negative regulation of biological process	92	1.07 E-13
	Negative regulation of cellular process	87	1.07 E-13
	Vasculature development	34	2.59 E-12
	Response to lipopolysaccharide	23	2.59 E-12
	Blood vessel development	33	2.71 E-12
	Blood vessel morphogenesis	31	2.80 E-12
	Response to molecule of bacterial origin	23	5.43 E-12
	Positive regulation of biological process	92	1.24 E-11
	Angiogenesis	27	3.28 E-11
	Response to chemical stimulus	79	5.06 E-11

biological pathways, and the 93 downregulated genes were related to cellular transcription processes (data not shown).

Gene ontology analysis of the entire set of 1373 genes differentially expressed in response to hypoxia, revealed upregulation of immune (108 genes, adjusted (adj.) *P* value = 1.13E-31) and inflammatory (62 genes, adj. *P* value = 1.82E-22) responses, and downregulated cellular metabolic pathways (461 genes, adj. *P* value = 2.13E-09) (Table 4). After administration of ketamine, hypoxia upregulated metabolic processes (129 genes, adj. *P* value = 2.44E-02), cellular repair, and DNA replication; and downregulated vascular development (34 genes, adj. *P* value = 2.59E-12), angiogenesis (27 genes, adj. *P* value = 3.28E-11), and response to bacterial and virulent factors.

Table 5. Top 10 enriched KEGG pathways that were significantly overrepresented in the kidney cortex during acute hypoxic stress, with or without ketamine

Group	Pathway name	No. of genes involved	Adjusted *P* values
Hypoxia upregulated	Cytokine–cytokine receptor interaction	30	3.39E-20
	Osteoclast differentiation	23	3.39E-20
	Toll-like receptor signalling pathway	21	7.00E-20
	Chagas disease (American trypanosomiasis)	18	1.08E-15
	NOD-like receptor signalling pathway	14	2.06E-14
	Malaria	13	8.56E-14
	MAPK signalling pathway	23	1.96E-13
	Cytosolic DNA-sensing pathway	13	2.43E-13
	Rheumatoid arthritis	15	4.55E-13
	Chemokine signalling pathway	19	1.69E-12
Hypoxia downregulated	Pathways in cancer	45	1.85E-20
	Chronic myeloid leukemia	14	3.89E-08
	Melanogenesis	16	3.89E-08
	Wnt signalling pathway	19	3.93E-08
	Pancreatic cancer	13	1.12E-07
	TGF-beta signalling pathway	14	1.12E-07
	Colorectal cancer	12	1.84E-07
	Ubiquitin mediated proteolysis	17	1.84E-07
	Cell cycle	16	2.74E-07
	Mismatch repair	8	3.03E-07
Hypoxia + ketamine upregulated	Metabolic pathways	23	4.89E-05
	Nucleotide excision repair	4	6.20E-03
	Mismatch repair	3	8.30E-03
	DNA replication	3	1.86E-02
	SNARE interactions in vesicular transport	3	1.86E-02
	Drug metabolism; other enzymes	3	2.89E-02
	mTOR signalling pathway	3	2.89E-02
	Pyrimidine metabolism	4	2.89E-02
	Melanogenesis	4	2.89E-02
	Calcium signalling pathway	5	3.16E-02
Hypoxia + ketamine downregulated	Osteoclast differentiation	15	8.21E-15
	Pathways in cancer	19	1.54E-13
	Leishmaniasis	11	1.74E-12
	Rheumatoid arthritis	11	1.89E-11
	Toll-like receptor signalling pathway	11	4.52E-11
	Malaria	9	4.52E-11
	Chagas disease (American trypanosomiasis)	11	4.81E-11
	MAPK signalling pathway	15	5.80E-11
	NOD-like receptor signalling pathway	8	3.77E-09
	Cytokine–cytokine receptor interaction	12	6.88E-08

KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis indicated that hypoxia upregulated pathways involving cytokine–cytokine receptor interaction (30 genes, adj. *P* value = 3.39E-20), toll-like receptor signalling pathway (21 genes, adj. *P* value = 7.00E-20), and chemokines signalling pathways (19 genes, adj. *P* value = 1.69E-12) (Table 5). Hypoxia downregulated pathways involving cell proliferation and embryonic development, such as the Wnt signalling cascade (19 genes, adj. *P* value = 3.93E-8). The presence of ketamine during hypoxic stimulation resulted in the upregulation of metabolic pathways (23 genes, adj. *P* value = 4.89E-5), and downregulation of toll-like receptor (11 genes, adj. *P* value = 4.52E-11), mitogen-activated protein kinase (MAPK) signalling pathways (15 genes, adj. *P* value = 5.80E-11), and cytokine–cytokine receptor interaction (12 genes, adj. *P* value = 6.88E-8).

Comparison of the hypoxia upregulated genes (hypoxic control *vs.* normoxic control: HC-NC) with the hypoxia + ketamine downregulated genes (hypoxia + ketamine *vs.* hypoxic control: HK-HC) revealed the genes that were sensitive to both hypoxia and ketamine; there were 88 genes shared between these groups (Fig. 2). Network inference of these gene sets (hypoxia: 437, hypoxia + ketamine: 260, common genes: 88), generated a merged network in which genes responding to hypoxia (HC-NC) clustered together (blue and yellow nodes: Fig. 2) and a second network of hypoxia + ketamine downregulated genes (HK-HC) that was distinct (red and yellow nodes, Fig. 2), but overlapping. The overlapping genes – those that were both increased by hypoxia and decreased during hypoxia by ketamine – tended to cluster with the network of hypoxia-sensitive genes (yellow nodes, Fig. 2). The common genes were further analysed to determine gene ontology and KEGG pathways (Table 6). The most highly statistically significant overrepresented KEGG pathways were found to be involved in cytokine–cytokine receptor interaction (30 genes, adj. *P* value = 3.39E-20), toll-like receptor (21 genes, adj. *P* value = 7.00E-20), MAPK (23 genes, adj. *P* value = 1.96E-13), and chemokine-signalling pathways (19 genes, adj. *P* value = 1.69E-12). The overrepresented biological processes with higher significance were negatively involved in regulation of cellular processes (54 genes, adj. *P* value = 5.52E-13). The top ranked molecular function terms were cytokine receptor binding and activity (11 genes, adj. *P* value = 5.41E-06), nucleic acid binding transcription factor activity (19 genes, adj. *P* value = 3.00E-04), and DNA regulations. Of the 88 common genes, most were located in the cellular components nucleus or membrane-bounded organelle.

Performing the converse comparison – genes that are downregulated by hypoxia (HC-NC) *versus* genes that were upregulated during hypoxia by ketamine (HK-HC) – revealed reciprocal effects on cellular metabolism. Hypoxia decreased expression of 946 genes (blue and yellow nodes, Fig. 3). Ketamine during hypoxia increased expression of 260 genes (red and yellow nodes, Fig. 3). Again, some of these genes were shared by these two groups ($n = 130$ yellow nodes, Fig. 3) that tended to cluster with the HC-NC network. Ketamine tended to reverse the effect of hypoxia on metabolism, although analysis of the overlapping genes alone did not yield any significant gene ontology terms (Fig. 3).

To validate the microarray results, qPCR was performed on statistically significantly expressed genes in the 88 common genes shared by upregulated hypoxia and downregulated hypoxia + ketamine genes (Fig. 4). The genes selected were involved in inflammatory and immune response pathways (TLR2, NFκB, IL-6, IL-1β, CSF1, CD14, TNFα, BCL3, CCL4, CCL5, CXCL10, and IRF1). Expression of all of these genes were significantly increased by hypoxia (comparison of NC *vs.* HC was significant), and the increase in expression of these genes was inhibited by ketamine (comparison of NC *vs.* HK was not significant) when the microarray results were analysed, with the exception of CCL4. As shown in Fig. 4, the qPCR gene expression pattern for these inflammatory/immune genes resembles the microarray gene expression: upregulation by hypoxia and prevention of upregulation by ketamine. Again, as measured by real-time qPCR, the expression of all measured genes was increased by hypoxia (comparison
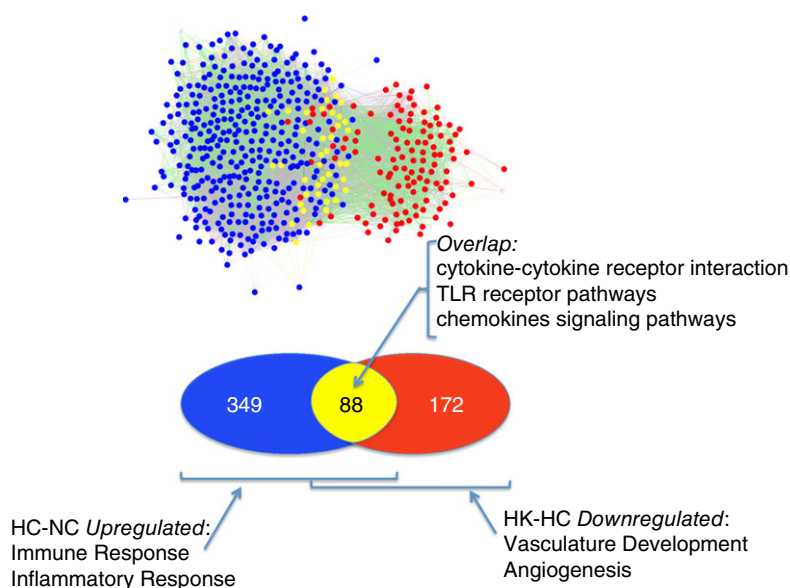

Overlap:
cytokine-cytokine receptor interaction
TLR receptor pathways
chemokines signaling pathways

349   88   172

HC-NC *Upregulated*:
Immune Response
Inflammatory Response

HK-HC *Downregulated*:
Vasculature Development
Angiogenesis

Figure 2. Venn diagram and network analysis of significant gene expression upregulated by hypoxia, but downregulated by ketamine during hypoxia
Venn and network inference analyses showing the number of significant genes that was upregulated by hypoxia (blue), downregulated with hypoxia and ketamine (red), and the common genes involved in both groups (yellow). Hypoxia (HC-NC) and hypoxia + ketamine (HK-HC).

Table 6. Top 10 gene ontology biological processes and enriched KEGG pathways that were significantly upregulated in the kidney cortex during acute hypoxic stress, but were significantly downregulated with hypoxia + ketamine

Analysis	Pathways, processes, functions, components	No. of genes involved	Adjusted P values
KEGG pathways	Osteoclast differentiation	23	3.39E-20
	Cytokine–cytokine receptor interaction	30	3.39E-20
	Toll-like receptor signalling pathway	21	7.00E-20
	Chagas disease (American trypanosomiasis)	18	1.08E-15
	NOD-like receptor signalling pathway	14	2.06E-14
	Malaria	13	8.56E-14
	MAPK signalling pathway	23	1.96E-13
	Cytosolic DNA-sensing pathway	13	2.43E-13
	Rheumatoid arthritis	15	4.55E-13
	Chemokine signalling pathway	19	1.69E-12
Biological process	Negative regulation of biological process	56	5.55E-13
	Negative regulation of cell communication	25	1.31E-10
	Negative regulation of cellular process	54	5.52E-13
	Negative regulation of response to stimulus	28	2.03E-11
	Negative regulation of signal transduction	24	2.25E-10
	Negative regulation of signalling	25	1.31E-10
	Regulation of cell communication	42	1.09E-10
	Regulation of response to stimulus	46	3.32E-11
	Regulation of signal transduction	39	1.31E-10
	Regulation of signalling	42	1.09E-10
Molecular function	Protein binding	72	4.08E-07
	Cytokine receptor binding	11	5.41E-06
	Cytokine activity	9	2.00E-04
	Nucleic acid binding transcription factor activity	19	3.00E-04
	Sequence-specific DNA binding transcription factor activity	19	3.00E-04
	Regulatory region DNA binding	10	8.00E-04
	Regulatory region nucleic acid binding	10	8.00E-04
	Interleukin-1 receptor binding	3	1.00E-03
	Sequence-specific DNA binding	14	1.00E-03
	RNA polymerase II core promoter proximal region sequence-specific DNA binding transcription factor activity	5	2.70E-03
Cellular component	Cytosol	31	2.00E-04
	IκB–NFκB complex	3	2.00E-04
	Extracellular space	15	3.00E-03
	Cell surface	10	9.80E-03
	Extracellular region part	16	9.80E-03
	External side of plasma membrane	6	2.03E-02
	Nucleus	48	2.09E-02
	Intracellular part	81	2.29E-02
	Intracellular	82	2.98E-02
	Membrane-bounded organelle	67	3.05E-02

of NC *vs.* HC was significant), and with the exception of CCL4, the increase in expression of all genes was inhibited by ketamine (comparison of NC *vs.* HK was not significant) when the qPCR results were analysed. The inhibition of CCL5, CXCL10, IL-6, and NFκB by ketamine was not complete (comparison of NC *vs.* HK was not significant, and comparison of HC *vs.* HK was also not significant): this incomplete inhibition was reflected in insignificant *P* values ($0.06 < P < 0.13$) for the hypoxia × ketamine interaction term in the ANOVA (as reported in the legend to Fig. 4).

We also validated the results of the transcriptomics modelling by immunostaining for macrophages in the same kidneys that were used for microarray analysis. As shown in Fig. 5, hypoxia significantly increased the number of macrophages in the kidney cortex, and

ketamine significantly reduced the number of macrophages ($P < 0.0001$ for main effect of hypoxia; $P = 0.0005$ for main effect of ketamine; and $P = 0.0070$ for hypoxia $\times$ ketamine interaction).

Discussion

Utilizing systems modelling techniques, we have found that 24 h after hypoxia, the genomic expression of immune and inflammatory pathways in the ovine fetal kidney cortex were upregulated, and that pretreatment with ketamine prevented or downregulated these responses. The results of this modelling suggest that hypoxia increases and ketamine decreases inflammation in the kidneys. The renal inflammation response to hypoxia is known and has been reported by others (Ikeda *et al.* 2000; O'Connell *et al.* 2003; Xia *et al.* 2015). The mechanism of the anti-inflammatory action of ketamine is unclear but might be inferred from known actions of ketamine.

Hypoxia is a relatively common fetal stressor. Severe or prolonged hypoxia can cause growth retardation of the fetus and ischaemia–reperfusion injury to the fetal organs (Zhang, 2005; Giussani & Davidge, 2013). Acute hypoxaemia, especially when combined with acidaemia, stimulates a redistribution of combined fetal cardiac output with resultant decreased fetal renal blood flow by as much as 20% (Cohn *et al.* 1974). Combinations of decreased arterial oxygen content and decreased renal blood flow cause hypoxic and ischaemic damage to the kidneys, particularly to the renal tubules (Faber & Anderson, 1997; Gibson & Lumbers, 1999). Partial umbilical cord occlusion for 60 min resulting in a plasma pH of < 6.9, which is a severe asphyxic insult, caused renal tubular necrosis in near-term ovine fetuses (Ikeda *et al.*

2000), and complete umbilical cord occlusion for 30 min caused renal distal tubule apoptosis in mid-gestation fetuses 72 h after the occlusion (O'Connell *et al.* 2003). Hypoxia has also been shown to have an adverse effect on renal development by stimulating inflammatory, immune, and apoptotic processes in the fetal kidneys (Xia *et al.* 2015). It is conceivable that acute renal damage to the fetus could have long-term effects on the proper development of renal tubules and vasculature.

In addition to localization within the brain, NMDA receptors are located throughout the kidney in various parts of the nephron: glomerulus (Zhang *et al.* 2010), collecting ducts (Sproul *et al.* 2011), epithelial proximal tubular cells (Bozic *et al.* 2011), and podocytes (Kottgen & Benzing, 2011), which suggest an important role for NMDA receptors in the regulation of renal function. Renal NMDA receptors increase renal vasodilatation via the nitric oxide pathway (Deng *et al.* 2002), suggesting that NMDA receptors are involved in maintaining renal haemodynamics. In models of renal ischaemia–reperfusion and hypoxia–reoxygenation, NMDA receptors were upregulated in the kidney cortex and the medulla (Yang *et al.* 2008; Pundir *et al.* 2013; Liu *et al.* 2014); activation of NMDA receptors is associated with increased oxidative stress and renal damage (Pundir *et al.* 2013). Excessive stimulation of NMDA receptors in the kidney can be toxic and cause apoptosis in proximal tubule-like opossum kidney and distal-tubule-like Madin-Darby canine kidney cells (Leung *et al.* 2008). In podocytes, over stimulation of the glutamatergic signalling pathway may weaken the integrity of the glomerular filtration barrier therefore causing glomerular damage (Kottgen & Benzing, 2011). Moreover, in both *in vitro* and *in vivo* models, the adverse
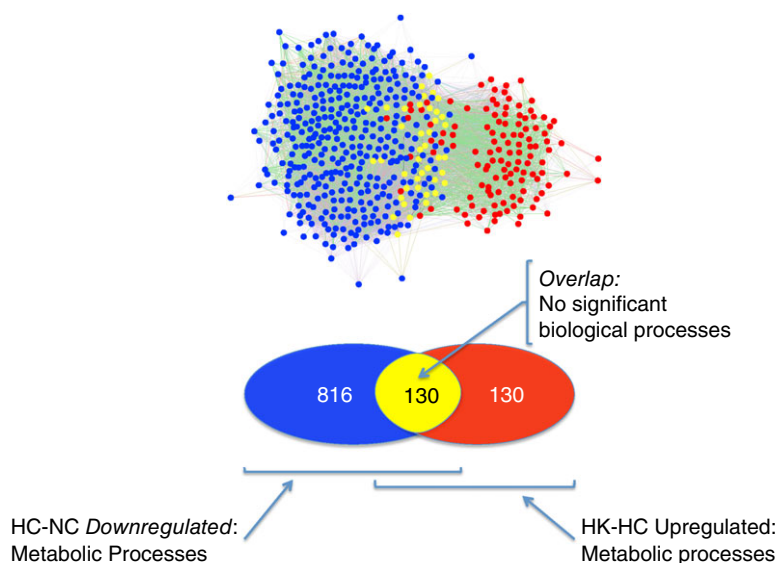

Figure 3. Venn diagram and network analysis of significant gene expression downregulated by hypoxia, but upregulated by ketamine during hypoxia
Venn and network inference analyses showing the number of significant genes that was downregulated by hypoxia (blue), upregulated with hypoxia and ketamine (red), and the common genes involved in both groups (yellow). Hypoxia (HC-NC) and hypoxia + ketamine (HK-HC).
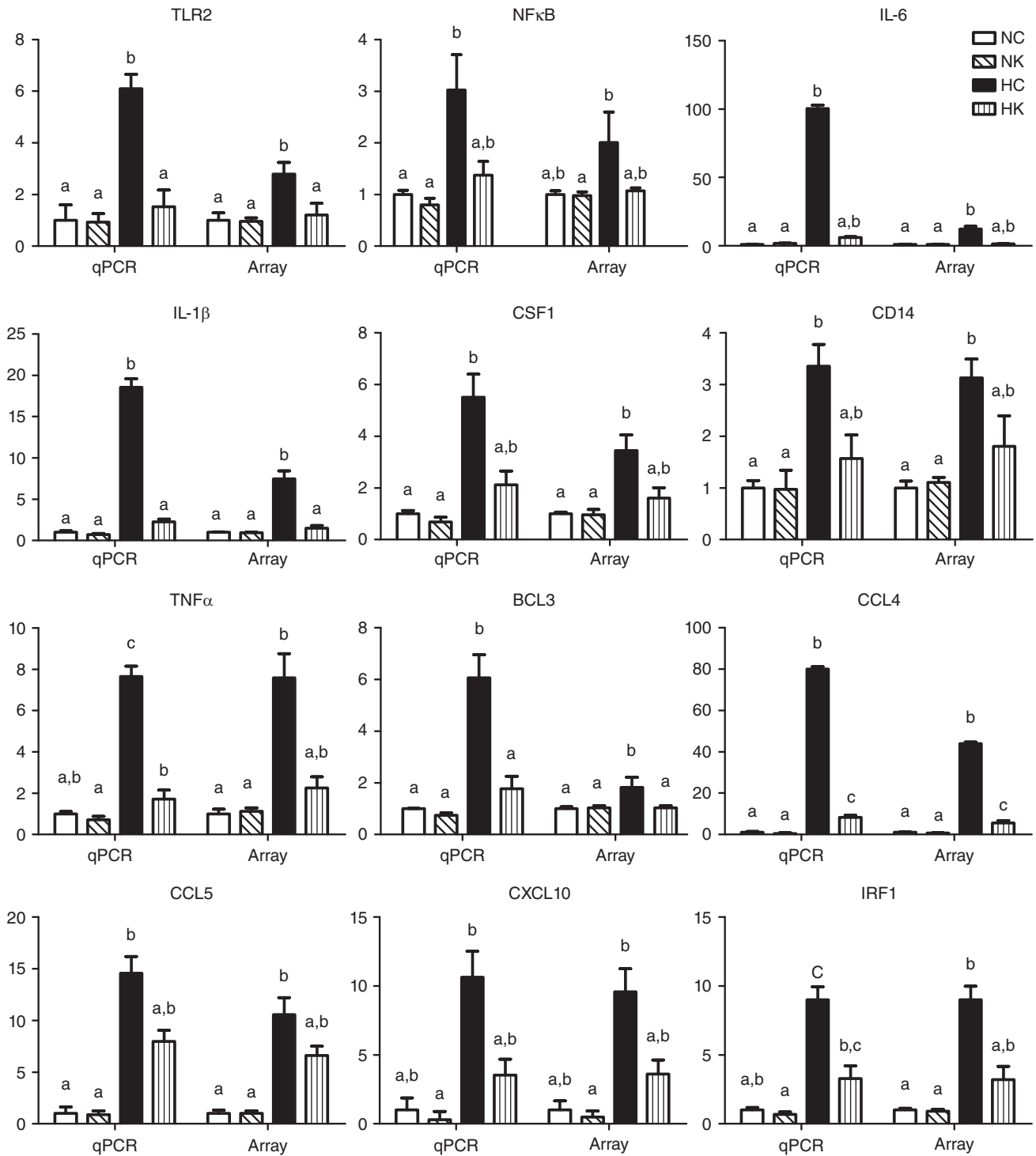
Figure 4. Ketamine ameliorates the increases in gene expression of inflammatory and immune related genes caused by hypoxia

RNA gene expression was measured by microarray and validated by real-time qPCR for inflammatory and immune related genes. All values are represented as a fold-change from normoxic control group (NC, normoxia control; NK, normoxia + ketamine; HC, hypoxia control; HK, hypoxia + ketamine). Statistical significance between groups was accepted at $P \leq 0.05$ (interaction term in two-way ANOVA), and Duncan's test was performed to determine differences between groups (for results of Duncan's test, bars with different letters are statistically significantly different from each other, $P < 0.05$). For gene expression with $P > 0.05$ between groups (interaction term in two-way ANOVA), the P values listed here are for real-time qPCR validation: CXCL10 ($P = 0.06$); and for the arrays: CXCL10 ($P = 0.07$), CCL5 ($P = 0.08$), IL-6 ($P = 0.099$), NFκB ($P = 0.13$). Data are presented as means ± SEM, and the y-axis scale varies between plots.

effects of excessive activation of NMDA receptors were attenuated with NMDA receptor antagonists (Leung *et al.* 2008; Yang *et al.* 2008; Pundir *et al.* 2013).

It is known that ischaemia–reperfusion causes apoptosis and inflammation in the kidneys. Kruger *et al.* showed that, during a renal ischaemia–reperfusion event, activated caspases trigger an inflammatory response in the kidneys, by increasing IL-1β and IL-18, which lead to renal ischaemic injury (Melnikov *et al.* 2001, 2002). Our micro-array results show that hypoxia alone induces significant

upregulation of inflammatory mediators, such as, IL-1β, IL-18, IL-1, IL-6, IL-8, TNFα, TLR2, and TLR4. In the proximal tubule epithelial cells, hypoxic events stimulate toll-like receptor 4 (TLR4)-mediated pathways to increase inflammatory (TNFα, IL-8) and apoptotic (caspases 3, 8 and 9) markers (Liu *et al.* 2014). In another study, the magnitude of the increase in TLR4 gene and protein expression has been shown to correlate with the degree of ischaemia injury in the kidneys (Kruger *et al.* 2009). In a renal specific TLR4 knock-out mouse model, the
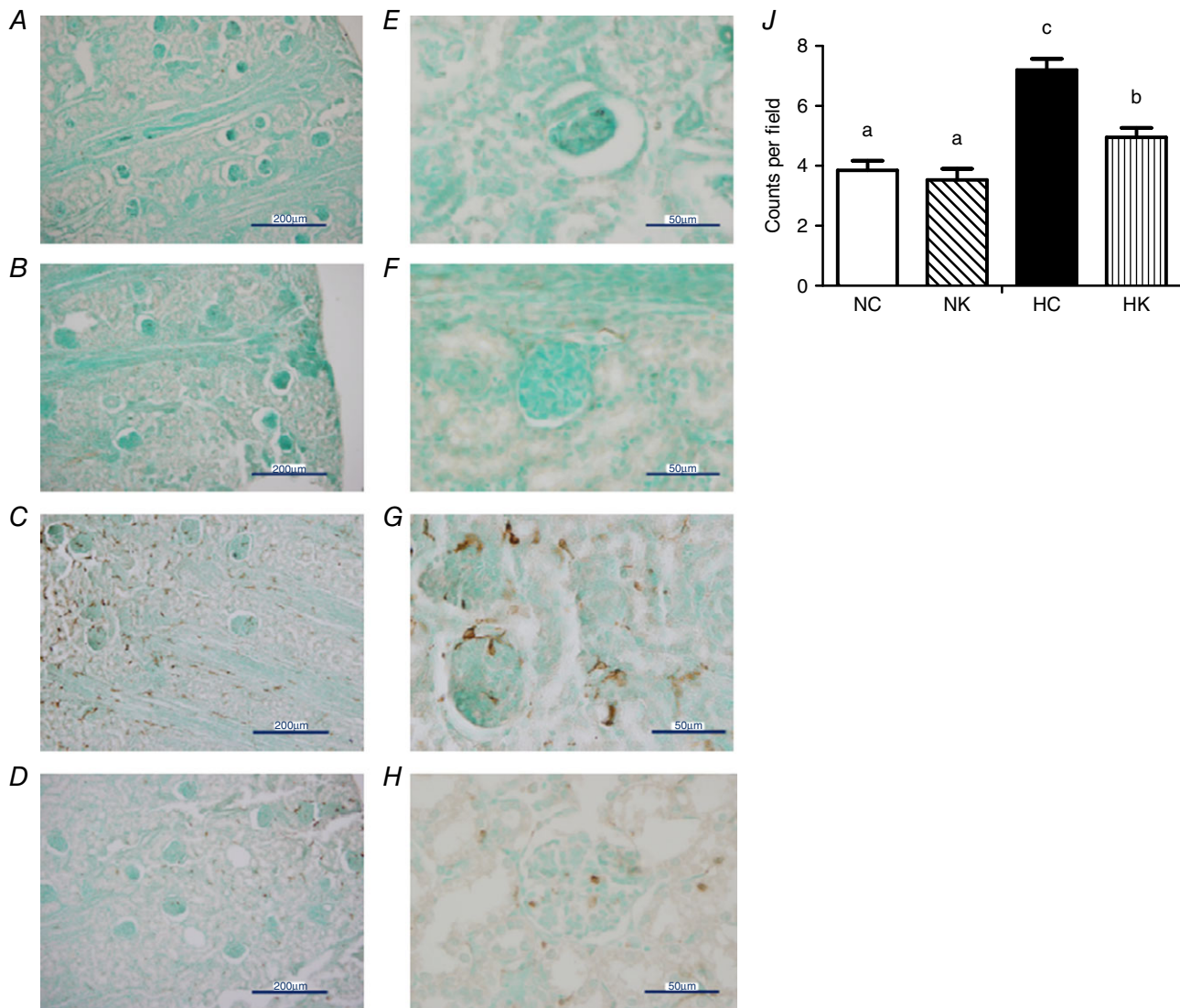

Figure 5. Local macrophages expressing Iba-1 immunoreactivity in the kidney cortex of NC, NK, HC and HK fetuses
A and *E*, NC fetuses; *B* and *F*, NK fetuses; *C* and *G*, HC fetuses; *D* and *H*, HK fetuses. Sections were counterstained with methyl green. Macrophage counts per field at ×40 in the kidney cortex (*J*). Data are expressed as means ± SEM. Different letters indicate statistically significant difference (HC *vs.* HK $P < 0.0001$; HC *vs.* NC $P < 0.0001$; HC *vs.* NK $P < 0.0001$; HK *vs.* NC $P = 0.018$; HK *vs.* NK $P = 0.005$). Scale bars 200 μm (*A–D*), 50 μm (*E–H*). Abbreviations: NC, normoxia control; NK, normoxia + ketamine; HC, hypoxia control; HK, hypoxia + ketamine.

endothelial adhesion molecules, selectin E and ICAM1 were not expressed, suggesting that TLR4 is one of the main players in recruiting inflammatory cytokines (Chen *et al.* 2011). Our array analysis shows that hypoxia upregulates selectin P expression (log_2 fold-change = 3.89, adj. *P* value = 5.69E-04), which is a related cell adhesion molecule that has an essential role in the recruitment of inflammatory cells and toll-like receptors (TLR2, log_2 fold-change = 1.48, adj. *P* value = 9.41E-03; TLR4, log_2 fold-change = 0.97, adj. *P* value = 1.78E-02). The increase in selectin P expression might be a consequence of the upregulation of toll-like receptors following hypoxia (Semple & Freedman, 2010; Rondina & Garraud, 2014).

The results of our experiment indicate that ketamine, a non-competitive NMDA receptor antagonist, was able to cause changes in renal gene expression that are consistent with decreased fetal renal inflammatory and immunological responses to acute hypoxia. By finding the common set of genes that is both upregulated by hypoxia and downregulated by ketamine treatment before hypoxia, we were able to model the action of ketamine on the fetal renal cortex after hypoxia. With the list of 88 common genes (cytokines, chemokines, interleukins, glucose transporters), we analysed common pathways and biological processes and found that the most significant pathways involved inflammatory, immune, and metabolic responses. In normoxic conditions, the ovine fetal kidney reabsorbs glucose and releases it into the bloodstream; however, under acute hypoxic conditions, carbohydrate metabolism becomes anaerobic releasing lactate into the bloodstream (Iwamoto & Rudolph, 1985). Interestingly, our array results suggest that during hypoxia, local glucose transporters (SLC2A1, SLC2A3) are upregulated (SLC2A1, log_2 fold-change = 0.89, adj. *P* value = 2.47E-03; SLC2A3, log_2 fold-change = 1.94, adj. *P* value = 9.66E-04) and that ketamine suppresses this response (SLC2A3, log_2 fold-change = −1.49, adj. *P* value = 3.87E-03; SLC2A1, log_2 fold-change = −0.61, adj. *P* value = 1.59E-02). These results might be consistent with local vasodilatory effects of ketamine in the kidneys, sustaining the glucose supply and attenuating the changes in gene expression of renal glucose transporters. Furthermore, hypoxia upregulates SLC9A3, the gene encoding NHE3 (sodium–hydrogen exchanger; log_2 fold-change = 0.31, adj. *P* value = 4.07E-02); ketamine downregulates the response (log_2 fold-change = −0.28, adj. *P* value = 4.55E-02). It is possible that the upregulation of SLC9A3 might be secondary to lactic acidosis during hypoxia. Taken together, the pattern of gene expression suggests that ketamine ameliorates the fetal renal response to hypoxia.

A potential mechanism of both the effect of hypoxia to increase and the effect of ketamine to decrease markers of inflammation is an effect of hypoxia and of ketamine on the number and function of macrophages in the kidney cortex. As shown in Fig. 5, hypoxia increases the number of macrophages in the renal cortex, and ketamine reduces this response. While macrophages have been detected in the developing fetal human and mouse kidneys (Yasui *et al.* 1997), the function of local renal macrophages in the late gestation fetus still needs elucidating. Our discovery of the effect of hypoxia to increase renal macrophage count corroborates the results of the transcriptomics modelling and reinforces the notion that even transient hypoxia is damaging to the fetal kidney.

Our data suggest that ketamine reduces at least some of the negative consequences of hypoxic stress in fetal kidneys. In both sheep and humans, kidney maturation is complete by the time of parturition (Gimonet *et al.* 1998); therefore, an individual's nephron endowment cannot increase throughout life. Damage to the kidneys *in utero* therefore would create a functional deficiency that could not be recovered in postnatal life. For example, hypoxic damage might alter the course of nephron development or vascular regulatory mechanisms, having lifelong consequences for renal blood volume regulatory mechanisms (Gonzalez-Rodriguez *et al.* 2013). Our results suggest that blockade of NMDA receptors might be clinically useful in sparing hypoxic damage to the developing kidneys. Nevertheless, it is also possible that blockade of NMDA receptor might have detrimental effects, including possible alterations in the pattern of kidney development in immature fetuses. Further experimentation will be needed to more fully understand the effect of ketamine on the fetal kidney.

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Additional information

Competing interests

All authors declare that there are no competing financial, personal or professional interests.

Author contributions

E.I.C., C.E.W.: conception and design of the experiments, collection, analysis and interpretation of data, drafting the article, revising article critically for important intellectual content. M.B.R., M.A.Z: collection, analysis and interpretation of data, revising article critically for important intellectual content. E.M.R., M.K.: interpretation of data, revising article critically for

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