Dietary restriction in the periconceptual period in normal-weight or obese ewes results in increased abundance of angiotensin-converting enzyme (ACE) and angiotensin type 1 receptor (AT1R) in the absence of changes in ACE or AT1R methylation in the adrenal of the offspring

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Abstract

Exposure to dietary restriction during the periconceptual period in either normal or obese ewes results in increased adrenal growth and a greater cortisol response to stress in the offspring, but the mechanisms that programme these changes are not fully understood. Activation of the angiotensin type 1 receptor (AT1R) has been demonstrated to stimulate adrenal growth and steroidogenesis. We have used an embryo transfer model in the sheep to investigate the effects of exposure to dietary restriction in normal or obese mothers from before and 1 week after conception on the methylation status, expression, abundance and localisation of key components of the renin–angiotensin system (RAS) in the adrenal of post-natal lambs. Maternal dietary restriction in normal or obese ewes during the periconceptual period resulted in an increase in angiotensin-converting enzyme (ACE) and AT1R abundance in the absence of changes in the methylation status or mRNA expression of ACE and AT1R in the adrenal of the offspring. Exposure to maternal obesity alone also resulted in an increase in adrenal AT1R abundance. There was no effect of maternal dietary restriction or obesity on ACE2 and AT2R or on ERK, calcium/calmodulin-dependent kinase II abundance, and their phosphorylated forms in the lamb adrenal. Thus, weight loss around the time of conception, in both normal-weight and obese ewes, results in changes within the intra-adrenal RAS consistent with increased AT1R activation. These changes within the intra-adrenal RAS system may contribute to the greater adrenal stress response following exposure to signals of adversity in the periconceptual period.

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Introduction

There is evidence from a range of experimental and clinical studies that exposure of the embryo, fetus or neonate to a range of environmental stressors such as poor nutrition, placental dysfunction, excess glucocorticoids or poor maternal care alters the development of the hypothalamo–pituitary–adrenal (HPA) axis and stress responsiveness of the offspring for life (Levitt *et al.* 1996, Phillips *et al.* 2000, Butler *et al.* 2002, Weaver *et al.* 2004, de Vries *et al.* 2007). Adversity in early life can result in long-term changes in the HPA axis as a consequence of epigenetic modifications of key regulatory genes in rodents, sheep and humans (Weaver *et al.* 2004, Oberlander *et al.* 2008, Murgatroyd *et al.* 2009, Stevens *et al.* 2010, Zhang *et al.* 2010,

Grace *et al.* 2011). It has previously been demonstrated in sheep that exposure to maternal undernutrition during the periconceptual period and early gestation results in altered fetal adrenal development from as early as 55 days of gestation (term = 150 ± 3 days of gestation; MacLaughlin *et al.* 2007), an earlier prepartum activation of the fetal HPA axis (Edwards & McMillen 2002b, Bloomfield *et al.* 2004), an increased risk of premature delivery (Bloomfield *et al.* 2003) and increased plasma cortisol concentrations in the post-natal lamb (Gardner *et al.* 2006, Chadio *et al.* 2007). We have also recently reported that exposure to dietary restriction during the periconceptual period in either normal-weight or overweight ewes using the same animals in the current study results in increased adrenal growth, a greater

cortisol response to stress and a decrease in the adrenal expression and methylation levels of the imprinted gene, insulin-like growth factor 2 (*IGF2*) in the offspring (Zhang *et al.* 2010). The increase in the cortisol stress response was not associated with an increase in plasma adrenocorticotrophin (ACTH) or an increase in the expression of steroidogenic enzymes within the adrenal, and we therefore proposed that the programming of the increased stress response may be a result of an increase in adrenocortical growth (Zhang *et al.* 2010). Interestingly, exposure to maternal obesity in the periconceptional period results in an increase in adrenal *IGF1* mRNA expression in the absence of a change in adrenal mass in the post-natal lamb (Zhang *et al.* 2010). It is not clear, however, how exposure of the oocyte and/or early embryo to either maternal undernutrition or obesity results in the programming of altered adrenal growth, intra-adrenal IGF expression and cortisol output in later life.

In addition to the systemic renin–angiotensin system (RAS), there is also an intra-adrenal RAS (Bader & Ganten 2008, Peters 2012). Renin acts to convert angiotensinogen into angiotensin I (Ang I), which is then converted to Ang II by angiotensin-converting enzyme (ACE). ACE2 is an ACE homologue that cleaves Ang I and Ang II into smaller Ang peptides. Ang 1–7 has vasodilatory and antiproliferative properties and antagonises the actions of Ang II (Roks *et al.* 1999, Lambert *et al.* 2008). It is well known that Ang II stimulates the growth of the zona glomerulosa (ZG) of the adrenal and aldosterone secretion; however, the role of Ang II in the zona fasciculata–reticularis (ZF–ZR) of the adrenal is less well established (Hoeftlich & Bielohuby 2009, Hattangady *et al.* 2012). It has been shown that Ang II stimulates growth, glucocorticoid secretion and steroidogenic capacity in cultured adrenal fasciculata–reticularis cells and adrenal growth in genetically hypertensive Lyon rats (Nussdorfer *et al.* 1981, Finn *et al.* 1988, Viard *et al.* 1990, Clyne *et al.* 1993, Lebrethon *et al.* 1994, McEwan *et al.* 1999, Aguilar *et al.* 2004, Romero *et al.* 2006).

Ang II acts on both type 1 (AT1R) and type 2 (AT2R) Ang receptors. A range of *in vitro* and *in vivo* experiments have shown that Ang II acts on AT1R to stimulate cellular proliferation and hypertrophy in the adrenal and to increase adrenal steroid secretion (Clyne *et al.* 1993, McEwan *et al.* 1999, Aguilar *et al.* 2004, Romero *et al.* 2006). AT1R activation results in the activation/phosphorylation of the MAPK pathways, including MEK–ERK1/2 and calcium/calmodulin-dependent kinases (Fern *et al.* 1995, Côté *et al.* 1998, Condon *et al.* 2002, Otis *et al.* 2005, Szekeres *et al.* 2009).

In rats, exposure to a maternal low-protein diet in pregnancy resulted in decreased methylation of the adrenal *At1r* and increased *At1r* mRNA expression and protein abundance and adrenal Ang responsiveness

in offspring (Bogdarina *et al.* 2007). More recently, Bogdarina *et al.* (2010) reported that when rat dams fed a low-protein diet were treated with the 11 β -hydroxylase inhibitor, metyrapone, which acts to decrease the adrenal output of maternal corticosterone, during the first 2 weeks of pregnancy, the changes in adrenal *At1r* methylation and mRNA expression in the offspring were reversed. In mice, a maternal low-protein diet during the second half of gestation also resulted in decreased *Ace* methylation and increased *Ace* mRNA expression in the fetal brain (Goyal *et al.* 2010). There have been no studies, however, that have investigated the effects of exposure to either maternal obesity or dietary restriction during the periconceptional period on the epigenetic state and mRNA expression of the key components of the intra-adrenal RAS in the offspring.

In this study, we have used a model in which 'donor' ewes were either normally nourished or overnourished prior to a period of dietary restriction, before transfer of the embryo at 6–7 days after conception to a ewe of normal weight. We hypothesised that maternal dietary restriction during the periconceptional period in either normal or obese ewes would result in the recruitment of epigenetic mechanisms, which would act to decrease the methylation of *AT1R* and thereby result in AT1R activation of the downstream signalling pathways and an up-regulation of the intra-adrenal RAS in the offspring. We have therefore investigated the methylation status, expression and localisation of key components of the RAS in the ZG and ZF–ZR in the adrenal cortex of post-natal lambs conceived in either normal or obese ewes exposed to maternal undernutrition in the periconceptional period.

Materials and methods

Animals and nutritional feeding regimen

All procedures were approved by the University of Adelaide Animal Ethics Committee and the Institute for Medical and Veterinary Science Animals Ethics Committee. Briefly, South Australian Merino ewes were moved into an enclosed shed and housed in individual pens 2 weeks before the start of the feeding regimen. All ewes were weighed and body condition scores (BCSs) were assessed employing a 1.0–5.0 scale with 0.5 intervals by an experienced assessor (Russel *et al.* 1969, Greenwood *et al.* 2000). Using this scale, a BCS of 1 represents an extremely emaciated animal and a BCS of 5 represents a morbidly obese animal. During this 2-week period, ewes were acclimatised to a diet containing cereal hay, lucerne hay, barley, oats, almond shells, lupins, oat bran, lime and molasses (Johnsons & Sons Pty Ltd., Kapunda, SA, Australia). The pellets provided 9.5 MJ/kg metabolisable energy and 120 g/kg crude protein and contained 90.6% dry matter. All ewes received 100% of nutritional requirements as defined by the Agricultural and Food Research Council (Agricultural and Food Research Council 1993).

Donor ewes

At the end of this acclimatisation period, donor ewes ($n=23$) of normal body condition with a BCS of 3.0–3.5 were randomly assigned to one of four nutritional treatment groups, either control–control (CC), control-restricted (CR), high–high (HH) or high-restricted (HR) (Rattanatrak *et al.* 2010, Zhang *et al.* 2010).

- CC ewes ($n=6$) were a control group that were maintained at 100% metabolisable energy requirements (MER) for 5 months before and 1 week after conception;
- CR ewes ($n=6$) were maintained at 100% MER for the first 4 months and then were placed on an energy-restricted diet of 70% MER for 1 month before and 1 week after conception;
- HH ewes ($n=6$) were fed an *ad libitum* diet (170–190% MER) for 5 months before and 1 week after conception; and
- HR ewes ($n=5$) were fed an *ad libitum* diet (170–190% MER) for 4 months and then were placed on an energy-restricted diet of 70% MER for 1 month before and 1 week after conception.

There was no significant difference in weights of non-pregnant donor ewes in the CC, CR, HH and HR treatment groups before the start of the nutritional regimen (Rattanatrak *et al.* 2010, Zhang *et al.* 2010). Donor ewes were weighed and their BCS was assessed approximately every 2 weeks after commencing the feeding regimen until embryo transfer at 6–7 days after conception. At conception and at embryo transfer, donor ewes in the HH and HR groups were significantly heavier and fatter than ewes in the CC and CR groups (Rattanatrak *et al.* 2010, Zhang *et al.* 2010).

Superovulation, artificial insemination and embryo collection

The reproductive cycle of all experimental ewes was synchronised and superovulation was induced as described previously (Kakar *et al.* 2005, Rattanatrak *et al.* 2010). Fresh semen was collected from a ram of proven fertility as described previously (Kakar *et al.* 2005). Donor ewes were inseminated by laparoscopy with $\sim 2 \times 10^7$ spermatozoa being placed directly into lumen of each uterine horn 36 h after pessary withdrawal. Embryos were collected by mid-ventral laparotomy via flushing of uterine horns with saline (Baxter, Old Toongabbie, NSW, Australia) 6–7 days after artificial insemination. Embryos were then held at 38.5 °C in HEPES-buffered synthetic oviduct fluid supplemented with BSA and amino acids at oviduct fluid concentrations.

Recipient ewes

Donor embryos of good quality were recovered and transferred to synchronised recipient ewes. These ewes were maintained on a control diet (100% MER) throughout the period when donor ewes were exposed to different nutritional treatments. Each recipient ewe received only one embryo, which resulted in four treatment groups, i.e. CC, $n=13$; CR, $n=16$; HH, $n=17$ and HR, $n=16$. These ewes were then fed

a control diet for the remainder of the pregnancy, which provided 100% MER for the maintenance of a pregnant ewe bearing a singleton fetus (Agricultural and Food Research Council 1993).

There was no effect of nutritional treatment during the periconceptional period on either the birth weight or body weight of lambs at 4 months. Male lambs were significantly heavier at birth and at 4 months compared with female lambs independent of the nutritional treatment (Rattanatrak *et al.* 2010, Zhang *et al.* 2010).

Post-mortem and tissue collection

At 4 months of age, lambs were killed with a lethal overdose (~ 30 mg/kg) of sodium pentobarbitone delivered by i.v. injection (Virbac Pty Ltd., Peakhurst, NSW, Australia). Adrenals were collected and weighed. Relative adrenal weight was greater in the CR (0.063 ± 0.002 g/kg) and HR (0.060 ± 0.003 g/kg) groups compared with the CC (0.052 ± 0.004 g/kg) and HH (0.053 ± 0.002 g/kg) groups ($P < 0.01$) (Zhang *et al.* 2010). There was no effect of lamb sex on adrenal weights. Half of each right adrenal was fixed in 4% paraformaldehyde before embedding in paraffin wax and the remainder of the right adrenal and the left adrenal were frozen in liquid nitrogen and subsequently stored at -80 °C.

Methylation analysis

DNA methylation within the promoter of *ACE* and *AT1R* was analysed by combined bisulphite restriction assay (COBRA; Xiong & Laird 1997, Zhang *et al.* 2010). COBRA was performed using restriction endonucleases that cleave only those amplicons derived from methylated templates. Approximately 2 µg DNA from individual adrenals (CC, $n=7$; CR, $n=10$; HH, $n=12$ and HR, $n=12$) were subjected to bisulphite conversion (Epitect, Qiagen). PCR was performed on 100 ng bisulphite-converted DNA using primers and conditions that amplified methylated and unmethylated templates with no bias (Table 1). For *ACE*, the amplicon of a 179 bp fragment derived from the promoter (GenBank accession no. DQ885942, -500 to -322 bp relative to the transcription start site) was examined and digested with *RsaI* (New England Biolabs, Ipswich, MA, USA) for 2 h at 37 °C. For *AT1R*, the amplicon of a 168 bp fragment derived from the promoter (<http://genome.ucsc.edu/>, -175 to -8 bp relative to the transcription start site) was examined and digested with *BstBI* or *TaiI* (New England Biolabs). The intensity of uncut and cut fragments was

Table 1 Methylation primers for *ACE* and *AT1R* loci.

	Primer sequences (5'–3')	Amplicon size (bp)
<i>ACE</i>	Forward: GGATAGTTTTTTTATTTTGTATTT	179
	Reverse: CTATAAACTAAACACCCAA-CAATTTACT	
<i>AT1R</i>	Forward: GATGGTTGTGGTATTATTTTTTTT	168
	Reverse: TCTAAACAACTCCAAATTATAAC	

quantified using an Experion Automated Electrophoresis System (Bio-Rad Laboratories). Percentage of methylation was estimated by measuring the ratio of cut to the sum of cut and uncut PCR product.

Quantification of mRNA expression using quantitative real-time RT-PCR

Total RNA was extracted from adrenal samples (CC, $n=7$; CR, $n=10$; HH, $n=12$ and HR, $n=12$) using the TRIzol Reagent (Invitrogen by Life Technologies) and purified using the RNeasy Mini Kit (Qiagen) (Zhang *et al.* 2010). cDNA was synthesised by RT using Superscript III (Invitrogen by Life Technologies). Negative controls containing no RNA or superscript III were used to test for DNA contamination.

The relative expression of mRNA transcripts of angiotensinogen, renin, *ACE*, *ACE2*, *AT1R*, *AT2R* and 11β hydroxylase (*CYP11B*) in the adrenal was measured by quantitative real-time PCR using the ViiA 7 Real-Time PCR System (Applied Biosystems by Life Technologies). Each amplicon was sequenced to ensure the authenticity of the DNA product and melt curve analysis performed to demonstrate amplicon homogeneity. A PCR consisted of 3 μ l Fast SYBR Green Master Mix (Applied Biosystems by Life Technologies), 0.6 μ l each of forward and reverse primers (GeneWorks, Adelaide, SA, Australia), 0.8 μ l molecular-grade H_2O and 1 μ l cDNA (50 ng/ μ l) (Table 2; Dodic *et al.* 2002, Passmore *et al.* 2009). Three replicates of cDNA were performed for each gene, and controls with no cDNA were included on each plate. Amplification efficiencies were determined from the slope of a plot of C_t (defined as the threshold cycle with the lowest significant increase in fluorescence) against the log of a series of diluted cDNA concentrations (ranging from 1 to 100 ng/ μ l). The abundance of each transcript relative to the abundance of the reference genes, peptidylprolyl isomerase A (*PPIA*) and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), was calculated using the free Q-Gen Analysis Software (Muller *et al.* 2002) and the data were reported as relative mRNA expression of the target gene to *PPIA*.

Localisation and quantification of protein abundance by immunohistochemistry

Adrenal sections (7 μ m) from lambs (CC, $n=5$; CR, $n=6$; HH, $n=6$ and HR, $n=7$) were prepared and mounted on pre-treated slides (Superfrost Plus; Thermo Fisher Scientific, Scoresby, VIC, Australia) to localise ACE, ACE2, AT1R and AT2R using immunohistochemistry as described previously (Ross *et al.* 2007). In brief, the ACE, ACE2, AT1R and AT2R proteins were each visualised using a Zymed histostain-plus kit (Invitrogen by Life Technologies), which uses a broad-spectrum biotinylated secondary antibody that detects mouse, rabbit, guinea pig and rat primary antibodies, and a streptavidin–HRP conjugate. The ACE, AT1R and AT2R primary antibodies were raised in rabbits and used at a dilution of 1:200 (Santa Cruz Biotechnology). The ACE2 primary antibody was raised in goats against human ACE2 and used at a dilution of 1:200 (Santa Cruz Biotechnology). For ACE2, instead of using the biotinylated secondary antibody and the HRP-conjugated streptavidin in the kit, the slides were incubated with 1:200 donkey anti-goat secondary antibody and HRP conjugate (Merck Millipore, Billerica, MA, USA). Matched samples without the use of the primary antibody were used as negative controls. A 3,3'-diaminobenzidine substrate (Thermo Fisher Scientific) was then used as the chromogen to identify positive staining and the sections were lightly counterstained (Mayer's haematoxylin; Sigma–Aldrich). Slides were then dehydrated, mounted and coverslipped. The photomicrographic images were captured from an Olympus VANOX-AHT microscope (Olympus Optical Co., Ltd., Tokyo, Japan) using a Colorview I camera with AnalySIS image analysis software (Soft Imaging Systems, Gulfview Heights, SA, Australia).

All tissue sections from each treatment group were processed concurrently to allow for direct comparison between treatments. A semiquantitative analysis was used to assess the immunostaining intensity of ACE, ACE2, AT1R and AT2R in the ZG and ZF–ZR in the adrenal cortex. Two observers who were blinded to the treatment group for each slide used a semiquantitative scoring system for the analysis ($\times 20$ magnification). The intensity of staining was ranked by use of a

Table 2 Real-time PCR primer sequences and final primer concentrations for target and reference genes.

Gene names	Primer sequences (5'–3')	Primer conc. (μ M)	GenBank accession no.
Angiotensinogen	Forward: CTCTCCACGCTCACTAGACTTG Reverse: ATGCATGAACCTGTTGATCTTCTC	300 300	NM_001114082.1 (Dodic <i>et al.</i> 2002)
<i>ACE</i>	Forward: AATTGCCTTCCTGCCCTTTGGCTA Reverse: CCAGCGTCAAAGTGGGTTTCGTTT	200 200	AJ920033.1
<i>ACE2</i>	Forward: AGAACCAGTCCTGGGATGCAGAA Reverse: AGTCAGCATGGAGTTGTCCAGAA	450 450	NM_001024502.2
<i>AT1R</i>	Forward: GGGCTGTCTACACTGCTATGGAA Reverse: CCGGAAGCGATCTTACATAGGTA	300 300	AF254119.1 (Dodic <i>et al.</i> 2002)
<i>AT2R</i>	Forward: TGGCTGTCTGTCTCTCATTG Reverse: GCTGACCACTGGGCATACTT	450 450	S81979.1 (Dodic <i>et al.</i> 2002)
<i>CYP11B</i>	Forward: AGGGAGACACATGGTGTTCTGTGAT Reverse: GTTGAGCAAGAACACGCCACACTT	450 450	U78477.1
<i>PPIA</i>	Forward: TCATTGCACTGCCAAGACTG Reverse: TCATGCCCTCTTTCACTTTGC	900 900	AY251270 (Passmore <i>et al.</i> 2009)
<i>GAPDH</i>	Forward: CCTGGAGAAACCTGCCAAGT Reverse: GCCAAATTCATTGTCGTACCA	900 900	DQ152956.1 (Passmore <i>et al.</i> 2009)

conventional five-point scoring system (0, no staining; 1, weak staining; 2, moderate staining; 3, strong staining and 4, extremely strong staining; [Belaud-Rotureau et al. 2002](#), [Gross et al. 2007](#)). A high concordance (>90% exact agreement) was found between the scoring of the two assessors.

Quantification of protein abundance by western blotting

The protein abundance of AT1R, AT2R, ERK, p-ERK (Thr202/Tyr204), CAMKII and p-CAMKII (Thr286) was determined using western blotting as described in detail elsewhere ([Forhead et al. 2008](#)). Briefly, adrenal cortical samples (50 mg; CC, $n=6$; CR, $n=6$; HH, $n=6$ and HR, $n=6$) were sonicated in extraction buffer (50 mmol/l Tris (pH 8), 150 mmol/l sodium chloride, 1% HP-40, 1 mmol/l sodium orthovanadate, 30 mmol/l sodium fluoride, 10 mmol/l sodium pyrophosphate, 10 mmol/l EDTA and a protease inhibitor cocktail) and centrifuged at 14 300 g at 4 °C for 14 min to remove lipid and insoluble material. Protein content of extracts was determined using a Micro BCA protein assay kit (Pierce, Thermo Fisher Scientific, Inc., Rockford, IL, USA) with BSA to generate a standard curve. Equal amounts of protein (20 μ g) were subjected to SDS-PAGE. The proteins were transferred to PVDF membrane (Merck Millipore), blocked and then incubated with primary antisera raised against AT1R, AT2R and p-CAMKII from Santa Cruz Biotechnology and ERK, p-ERK and CAMKII from Cell Signalling Technology (Danvers, MA, USA) ([Forhead et al. 2008](#)). Membranes were washed and bound antibody detected using HRP-conjugated secondary antibodies and ECL reagents according to the manufacturer's instructions (Thermo Fisher Scientific). AlphaEaseFC Software (Alpha Innotech Corporation, San Leandro, CA, USA) was used to quantify the density of specific bands. To monitor the linearity of the density measurements, 10 and 20 μ g of the same protein sample were loaded onto each gel to confirm that the chemiluminescent signal changed in a linear manner for all experiments. Prior to western blotting analysis, samples (20 μ g protein) were subjected to SDS-PAGE and gels stained with Coomassie Brilliant Blue (Thermo Fisher Scientific) and there were no differences in abundance of the major proteins present in samples between the different experimental groups.

Statistical analysis

All data are presented as mean $\pm$ S.E.M. Data were analysed using IBM Statistical Package for Social Scientists Statistics, version 19 (SPSS, Inc.) and STATA11: Data analysis and Statistical Software for repeated measures (Stata Corp., College Station, TX, USA). The effects of periconceptual nutrition and sex on the levels of DNA methylation, mRNA expression and protein abundance in the adrenal of lambs at 4 months of age were determined using a two-way ANOVA (with periconceptual nutrition and lamb sex as the major factors) with donor ewe number nested within nutritional treatment groups to identify lambs arising from the same donor. When there was an interaction between the effects of periconceptual nutrition and sex, the effect of periconceptual nutrition was determined separately in males and females. The Duncan's *post hoc* test was

Table 3 Adrenal mRNA expression of angiotensinogen, renin, ACE, ACE2, AT1R, AT2R and CYP11B in lambs.

Target gene mRNA expression relative to PPIA mRNA expression	CC		CR		HH		HR	
	Female ($n=5$)	Male ($n=2$)	Female ($n=3$)	Male ($n=7$)	Female ($n=5$)	Male ($n=7$)	Female ($n=7$)	Male ($n=5$)
Angiotensinogen: PPIA ($\times 10^4$)	2.32 $\pm$ 0.38	1.54 (1.14, 1.94)	2.56 $\pm$ 0.65	2.36 $\pm$ 0.6	2.05 $\pm$ 0.09	2.51 $\pm$ 0.47	2.80 $\pm$ 0.53	2.15 $\pm$ 0.45
Renin: PPIA ($\times 10^4$)	1.43 $\pm$ 0.47	1.11 (0.92, 1.30)	1.36 $\pm$ 0.12	1.18 $\pm$ 0.13	1.49 $\pm$ 0.38	1.22 $\pm$ 0.19	1.49 $\pm$ 0.35	1.18 $\pm$ 0.15
ACE: PPIA ($\times 10^4$)	7.70 $\pm$ 1.63	8.41 (8.28, 8.53)	5.38 $\pm$ 0.92	8.52 $\pm$ 1.3	7.32 $\pm$ 0.93	8.25 $\pm$ 0.52	7.90 $\pm$ 0.73	8.80 $\pm$ 1.86
ACE2: PPIA ($\times 10^4$)	5.05 $\pm$ 0.78 [†]	2.65 (3.18, 2.11)	2.33 $\pm$ 0.56*	2.93 $\pm$ 0.44	3.04 $\pm$ 0.38*	3.49 $\pm$ 0.31	3.56 $\pm$ 0.25**†	3.57 $\pm$ 0.49
AT1R: PPIA	0.157 $\pm$ 0.023	0.117 (0.089, 0.145)	0.129 $\pm$ 0.018	0.112 $\pm$ 0.015	0.109 $\pm$ 0.011	0.116 $\pm$ 0.01	0.124 $\pm$ 0.007	0.114 $\pm$ 0.013
AT2R: PPIA ($\times 10^3$)	1.54 $\pm$ 0.17	1.08 (0.84, 1.32)	0.97 $\pm$ 0.10	1.78 $\pm$ 0.3	1.33 $\pm$ 0.35	1.44 $\pm$ 0.21	0.93 $\pm$ 0.11	1.15 $\pm$ 0.17
CYP11B: PPIA	2.12 $\pm$ 0.18	1.56 (1.51, 1.61)	1.87 $\pm$ 0.15	1.99 $\pm$ 0.13	1.84 $\pm$ 0.2	2.03 $\pm$ 0.12	1.89 $\pm$ 0.11	2 $\pm$ 0.06

Values are means $\pm$ S.E.M. except for the two males in the CC group, for which the mean only is presented followed by the two individual values. Symbols (* and †) denote treatment groups that are significantly different from each other ($P < 0.05$) in female lambs.

used to determine significant differences between groups and a probability level of 5% ($P < 0.05$) was taken to be significant.

Results

Impact of periconceptional nutrition on adrenal ACE and AT1R DNA methylation

There was no effect of nutritional treatment, sex of the lamb or interaction between treatment and sex on ACE methylation levels in the post-natal adrenal (CC, $5.1 \pm 2.0\%$; CR, $6.5 \pm 1.4\%$; HH, $3.8 \pm 0.8\%$ and HR, $6.8 \pm 1.4\%$). There was no methylation present on the two CpG sites analysed for the AT1R gene promoter in the lamb adrenal in any treatment group.

Impact of periconceptional nutrition on adrenal angiotensinogen, renin, ACE, ACE2, AT1R, AT2R and CYP11B mRNA expression

There was no difference in adrenal mRNA expression of angiotensinogen, renin, ACE, AT1R and AT2R and CYP11B between the CC, CR, HH and HR groups or between male and female lambs (Table 3). There was a significant interaction between the effects of nutritional treatment and sex of the lamb on adrenal ACE2 mRNA expression ($P < 0.05$). In female lambs, adrenal ACE2 mRNA expression was significantly lower ($P < 0.05$) in the CR and HH lambs compared with the CC lambs (Table 3).

Impact of periconceptional nutrition on adrenal ACE, ACE2, AT1R and AT2R protein abundance

The abundance of AT1R protein in the adrenal as determined by western blotting was significantly higher in the CR, HH and HR groups compared with the CC group ($P < 0.05$; Fig. 1C). Strong AT1R immunostaining was predominantly present in the ZF–ZR and to a lesser extent in the ZG (Fig. 2E and F). The intensity of AT1R immunostaining was significantly higher in both the ZG and ZF–ZR in the adrenals of the CR and HR groups compared with the CC group ($P < 0.05$; Fig. 1A and B).

ACE immunostaining was present throughout the ZG and ZF–ZR (Fig. 2A and B) and the intensity of ACE immunostaining was significantly higher in these zones in lambs in the CR and HR groups compared with the CC group ($P < 0.05$; Fig. 3A and B). While ACE2 immunostaining was also present in the ZG and ZF–ZR, there was no difference in the staining intensity of adrenal ACE2 between nutritional treatment groups (Figs 2C and D, 3C and D).

There was no effect of maternal nutritional treatment on the abundance of AT2R protein (Figs 2G and H, 4) and there was also no effect of lamb sex or an interaction between nutritional treatment and sex on the abundance of adrenal ACE, ACE2, AT1R and AT2R proteins as determined by either western blotting or immunostaining in the adrenal cortex of the post-natal lamb.

Figure 1 Intensity of AT1R immunostaining in the (A) ZG and (B) ZF–ZR of the adrenal cortex of the CC, CR, HH and HR lambs (CC, $n = 5$; CR, $n = 6$; HH, $n = 6$ and HR, $n = 7$) at 4 months of age. (C) Abundance of AT1R protein as determined by western blotting in the adrenal cortex of the CC, CR, HH and HR lambs (CC, $n = 6$; CR, $n = 6$; HH, $n = 6$ and HR, $n = 6$). Representative western blots from two animals in each group for AT1R are presented. Different superscripts (a and b) denote mean values that are significantly different from each other ($P < 0.05$).

Figure 2 Representative sections showing the immunohistochemical localisation of ACE (A and B), ACE2 (C and D), AT1R (E and F) and AT2R (G and H) in adrenals from the lambs at 4 months of age. In the ZF–ZR, the immunostaining score was 4 for ACE and AT1R for the representative samples from the HR group, 3 for ACE2 for the sample from the CR adrenal and 3 for AT2R for the sample from the CC adrenal. Images (I and J) show the representative negative control (NC) in adrenal sections. The left-hand panel (A, C, E and G) shows the immunostaining in the ZR while the right-hand panel (B, D, F and H) shows the immunostaining in the ZF and ZG. The brown colour represents specific staining of the target protein and the blue colour represents the haematoxylin counterstain. Bar = 50 µm.

Impact of periconceptual nutrition on the protein abundance of adrenal AT1R downstream signalling molecules (ERK, p-ERK, CAMKII and p-CAMKII)

There was no effect of nutritional treatment, sex of the lamb or interaction between the effects of treatment and sex on the protein abundance of the AT1R downstream

signalling molecules including ERK, Thr202/Tyr204 phospho-ERK, CAMKII or Thr 286 phospho-CAMKII in the adrenals of the post-natal lambs (Fig. 5).

Discussion

The lack of changes in adrenal angiotensinogen or renin mRNA expression between the treatment groups suggests that maternal dietary restriction during the periconceptual period might have negligible impact on the upstream components of the intra-adrenal RAS, at least at the transcriptional level. We have demonstrated that dietary restriction in normal and obese ewes during the periconceptual period (CR and HR groups) resulted in an activation of the downstream components of the intra-adrenal RAS through an increase in the abundance of AT1R and ACE in the adrenal cortex of their offspring at 4 months of age. The intensity of ACE and AT1R, but not ACE2 or AT2R, immunostaining was higher in all zones of the adrenal cortex in the CR and HR lambs. These findings suggest that the impact of maternal dietary restriction on adrenal ACE and AT1R abundance is related to either a nutritional or hormonal signal of maternal weight loss in the ewe around the time of conception, rather than the body weight of the ewe in the periconceptual period. There was no effect, however, of maternal dietary restriction in either normal-weight or obese ewes on the DNA methylation levels within the *ACE* and *AT1R* promoter or on *ACE* and *AT1R* mRNA levels in the adrenals of the offspring. These findings suggest that the impact of maternal dietary restriction during the periconceptual period on adrenal ACE and AT1R abundance is not a consequence of epigenetic changes in the methylation status or the regulation of *ACE* and *AT1R* transcription and may rather be a consequence of a change in the regulation of the post-translational clearance of these proteins in the post-natal adrenal. One alternative explanation is that dietary restriction in the periconceptual period may result in the altered expression of microRNAs, small ~22 nucleotide-long non-coding RNAs, which play important roles as key post-transcriptional regulators of gene expression (Bushati & Cohen 2007, Bartel 2009). Interestingly, it has been reported that miR-155 expression is reduced in hypertensive patients with a specific polymorphic genotype at the 3' UTR of the *AT1R* gene and this is associated with higher AT1R protein abundance, despite similar *AT1R* mRNA expression (Ceolotto *et al.* 2011). We also found that there was a decrease in *ACE2* mRNA, but not in the abundance of ACE2 in the adrenals of female lambs within the CR group, which could potentially contribute to increased Ang II activation in adrenals in these lambs.

A previous study in the sheep has shown that maternal nutrient restriction during early to mid gestation (28–77 days gestation) also resulted in an increase in *AT1R* mRNA expression; however, changes in AT1R

Figure 3 Intensity of ACE and ACE2 immunostaining in the ZG ((A) ACE and (C) ACE2) and ZF-ZR ((B) ACE and (D) ACE2) of the adrenal cortex of the CC, CR, HH and HR lambs (CC, $n=5$; CR, $n=6$; HH, $n=6$ and HR, $n=7$) at 4 months of age. Different superscripts (a, b and c) denote mean values that are significantly different from each other ($P<0.05$).

protein abundance were not examined in this study (Whorwood *et al.* 2001). In the rat, exposure to global undernutrition throughout pregnancy did not alter adrenal Ace mRNA expression in the offspring (Rivière *et al.* 2005) whereas exposure to a maternal low-protein diet resulted in decreased methylation of adrenal *Agtr1a* and increased *Agtr1a* mRNA expression and protein abundance as well as adrenal Ang responsiveness in rat offspring (Bogdarina *et al.* 2007). It therefore appears that exposure to maternal undernutrition at different periods in early, mid and later gestation results in an increase in AT1R expression and/or abundance through the recruitment of a number of different regulatory pathways.

The up-regulation of the adrenal ACE and AT1R in the ZG and the ZF-ZR in the CR and HR lambs suggests that the activation of the intra-adrenal RAS in these lambs may play multiple roles in the regulation of adrenal growth and steroid secretion. We previously reported that exposure to dietary restriction during the periconceptional period in either normal or obese ewes results in increased adrenal growth and a greater cortisol response to stress in the offspring (Zhang *et al.* 2010). The increase in the cortisol stress response was not associated with an increase in plasma ACTH concentration or an increase in the expression of steroidogenic enzymes within the adrenal and we therefore proposed that the increased stress response was a consequence of the increase in adrenal mass (Zhang *et al.* 2010). Ang II has been shown to stimulate adrenal growth in the ZG and the ZF-ZR regions of the adrenal cortex (Nussdorfer *et al.* 1981, Finn *et al.* 1988, Viard *et al.* 1990, Clyne *et al.* 1993, Lebrethon *et al.* 1994, Romero *et al.* 2006, Hoeflich & Bielohuby 2009, Hattangady *et al.* 2012). In genetically hypertensive Lyon rats, a 4-week infusion

of Ang II resulted in an enlargement in both the ZG and the ZF-ZR regions of the adrenal gland and an increase in plasma aldosterone and corticosterone concentrations, and the increase in ZF-ZR volume accounted for 63% of the adrenal hypertrophy (Aguilar *et al.* 2004).

It has been demonstrated that the activation of AT1R and its downstream signalling molecules, mainly ERK and/or CAMKII, mediate most of the biological functions of Ang II (Clyne *et al.* 1993, Fern *et al.* 1995, Côté *et al.* 1998, McEwan *et al.* 1999, Aguilar *et al.* 2004, Otis *et al.* 2005, Romero *et al.* 2006, Szekeres *et al.* 2009). In this study, while there was an increase in the abundance of AT1R, there was no difference in the protein abundance of ERK, CAMKII and their phosphorylated forms in the adrenal of the CR and HR lambs. It is possible that other downstream signalling pathways including c-Jun N-terminal kinases (JNKs) or JAK/STAT may play a role in an AT1R stimulation of adrenal growth. It has been shown that Ang II acts on AT1R to stimulate the JAK/STAT pathway in vascular smooth muscle cells (Marrero *et al.* 1995) and JNKs mediate the proliferative effect of Ang II in cultured human kidney mesangial cells (Zhang *et al.* 2005); however, there are few studies on the roles of these signalling pathways in the adrenal.

One interesting finding in the current study was an increase in AT1R, but not ACE abundance in the adrenals of lambs exposed to maternal obesity in the periconceptional period. We have previously shown that there was an increase in adrenal *IGF1* expression, which was not associated with an increase in adrenal growth in these lambs. While the increase in AT1R abundance suggests that there may be an impact of exposure to maternal obesity in the periconceptional period on the intra-adrenal RAS, it appears that maternal dietary restriction

Figure 4 Intensity of AT2R immunostaining in the (A) ZG and (B) ZF-ZR of the adrenal cortex of the CC, CR, HH and HR lambs (CC, $n=5$; CR, $n=6$; HH, $n=6$ and HR, $n=7$) at 4 months of age. (C) Abundance of AT2R protein as determined by western blotting in the adrenal cortex of the CC, CR, HH and HR lambs (CC, $n=6$; CR, $n=6$; HH, $n=6$ and HR, $n=6$). Representative western blots from two animals in each group for AT2R are presented.

has a significantly greater impact on the programming of the RAS in the post-natal adrenal.

The synthesis and secretion of aldosterone in the ZG play a key role in the regulation of systemic blood pressure. It has been shown that a low-protein diet during the periconceptual or preimplantation period results in higher systolic blood pressure in the young adult offspring in rodents (Langley-Evans *et al.* 1996, Kwong *et al.* 2000, Watkins *et al.* 2011). In sheep, maternal undernutrition during the periconceptual period resulted in an increase in arterial blood pressure in twin fetal sheep during late gestation, but there was no impact of an intrafetal infusion of an ACE inhibitor on arterial blood pressure in these animals (Edwards & McMillen 2002a). Maternal dietary restriction during the first 30 days of gestation altered baroreflex function in young adult sheep and there was a blunting of the baroreflex sensitivity during Ang II infusion in these animals (Gardner *et al.* 2004). In sheep, 11 β -hydroxylase (CYP11B) is present throughout the adrenal cortex including the ZG and ZF-ZR, which is different from its pattern of localisation in the rat, mouse, hamster, guinea pig and human adrenal (Boon *et al.* 1997, Okamoto *et al.* 2005). It has been previously shown that there was no difference in the intensity of CYP11B immunostaining between the ZG and the ZF in the fetal and adult sheep adrenal (Coulter *et al.* 2000). In this study, while AT1R was up-regulated in the ZG in the CR and HR lambs, we found no evidence for an up-regulation of the expression of CYP11B, which plays a key role in aldosterone synthesis (Boon *et al.* 1997, Okamoto *et al.* 2005), in the adrenal of the offspring. The lack of ability to completely separate the ZG in the lamb adrenal in this study limits the capacity to measure CYP11B mRNA expression in the ZG. This suggests that the activation of the intra-adrenal RAS might be more likely to play a local rather than a systemic role. In order to determine whether there is a systemic role for the RAS, measures of circulating aldosterone concentrations would be warranted.

We found that AT1R immunostaining was more intense within the ZF-ZR than in the ZG in the lamb adrenals. This is in contrast to previous studies that have shown that AT1R was predominantly present in the ZG in fetal, newborn, non-pregnant and pregnant sheep (Bird *et al.* 1996, Wintour *et al.* 1999, Coulter *et al.* 2000, Peterson *et al.* 2001). The discrepancy in AT1R localisation within the adrenal cortex may be due to the use of different antisera in different studies or a consequence of different stages of development. AT2R immunostaining was homogenous throughout the lamb adrenal, which is consistent with a recent study in the bovine adrenal (Harada *et al.* 2010).

In this study, we have demonstrated that dietary restriction in normal and obese ewes during the periconceptual period (CR and HR groups) resulted in an activation of the downstream components of the

Figure 5 The protein abundance of AT1R downstream signalling molecules including (A) ERK, (B) Thr202/Tyr204 phospho-ERK (p-ERK), (C) CAMKII and (D) Thr 286 phospho-CAMKII (p-CAMKII) in the adrenal cortex of the CC, CR, HH and HR lambs (CC, $n=6$; CR, $n=6$; HH, $n=6$ and HR, $n=6$). Representative western blots from two animals in each group for ERK, p-ERK, CAMKII and p-CAMKII are presented.

intra-adrenal RAS through an increase in the abundance of ACE and AT1R in the adrenal cortex of their offspring at 4 months of age. The increased abundance of ACE and AT1R was not associated with changes in *ACE* and *AT1R* methylation or mRNA expression. Further investigation on other AT1R downstream signalling molecules including JNKs or JAK/STAT should be a focus for future studies investigating the mechanisms by which AT1R may stimulate adrenal growth. These studies highlight that weight loss around the time of conception, independent of maternal body weight, results in changes within the intra-adrenal RAS, which are consistent with an increase in AT1R activation. These changes within the intra-adrenal RAS system may contribute to the greater adrenal stress response following exposure to signals of adversity in the periconceptual period.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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