THE IMPACT OF EARLY-ONSET OBESITY AND HYPERTENSION ON THE UNFOLDED PROTEIN RESPONSE IN RENAL TISSUES OF JUVENILE SHEEP

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**Detailed Methodology**

**Rearing of animals following weaning**

Lean animals were reared in their ‘natural’ environment, that is, pasture grazed at 17 animals per 3000m² with unrestricted activity and *ad libitum* access to grass and concentrate pellets. Obese animals were reared in an obesogenic environment, that is, reared in an adjacent open barn with 17 animals per 50m² and hence reduced physical activity with *ad libitum* access to hay and concentrate. Activity in the obese animals was 63% lower than lean animals at one year of age.1

**Renal Histology and Immunohistochemistry**

Paraffin-embedded tissue sections were cut (5µm), mounted on Superfrost plus glass microscope slides, dewaxed in Xylene before rehydration in alcohol and stained on the Bond-max histology system using Bond Polymer Refine Detection System (Vision Biosystems, Australia, DS9800). Briefly, slides were heated and stained as follows; 5 minutes peroxide block, 15 minutes primary antibody, 8 minutes secondary antibody, 10 minutes 3,3'-Diaminobenzidine (DAB) and 5 minutes counterstaining with haematoxylin and eosin. Appropriate negative slides were run in parallel without the addition of the primary antibody. Slides were imaged using a Nikon Eclipse 90i microscope with CCD high speed color camera (Micropublisher 3.3RTV, Qimaging, BC, Canada). All slides were captured and analysed in a blinded fashion using Volocity quantification software (v 4.2.1, Improvision Ltd, Coventry, UK).

**Quantification of renal tissue mRNA by Real-time RT-PCR**

Total RNA was extracted from cortical renal tissue using Tri-reagent (Sigma, Poole, UK). RNA quality and quantity were verified using gel electrophoresis and spectrophotometry (NanoDrop1000, NanoDrop Products, DE, USA). For first strand synthesis of cDNA, 1µg RNA was reverse transcribed using reverse transcriptase (Roche Diagnostics, Lewes, UK) and a Touchgene thermocycler (Technne, Barloworld Scientific Ltd , Stone, UK) as previously described.2 Appropriate negative controls were included to exclude contaminating genomic DNA.

Quantitative real-time PCR (qPCR) was performed in duplicate in a 20µl reaction solution using 1µl of cDNA, 1X SYBR Green master mix (Qiagen Ltd, Crawley, UK) and 500nM of ovine-specific oligonucleotide primers (Sigma-Aldrich, Gillingham, UK). In addition, negative controls were run in duplicate on each plate. To ensure uniformity, efficiency and accuracy each 96 well plate included a standard curve for the gene being analysed. Results were excluded where the standard curve had a $R^2<0.985$ or an efficiency beyond $2±0.05$. qPCR was performed in 96 well plates using the Techne Quantica 14 real-time thermocycler (Technne, Barloworld Scientific Ltd) for 40 cycles. In order to establish the validity of each gene product, its size was checked using gel electrophoresis and, where necessary, gene sequencing. We used 18S rRNA as a housekeeping gene allowing mRNA normalisation. All data are then analysed using the $\Delta\Delta^{ct}$ method3 and expressed as a ratio to the lean 1 year old animals.

Ovine specific forward (F) and reverse (R) primers used were as follows: GRP78 F:TGAAACTGTGGGAGGTGTCA, R:TCGAAAGTTCCCAGAAGGTG, chemokine...
receptor 2 (CCR2) F:TGTCCATGCTGTGTTTGCTT, R:CCCCAAGATGCTCCTCATAA, C/EBP homologous protein (CHOP) F:AGGACCACCAGAGGTACAC, R: TGCCACTTTCCCTTGCATT, CD68 F:GTCCTGCTACCACCACCAGT, R:GCTGGGAACCATTACTCCAA, activating transcription factor-4 (ATF4) F:AGATGACCTGGAACCATGC, R:AGGGGGAAGAGGTTGAAAGA, activating transcription factor-6 (ATF6) F:AACCAGTCTCTTTCGTTGAC, R:CTTCTTCTTTCGGGACTGAC. Additional primers were used as previously published monocyte chemoattractant protein 1 (MCP-1),4 Bax,5 18S,1 inducible (iNOS) and endothelial nitric oxide synthase (eNOS).6

**Western Blot Analysis**

Western blot analysis of protein was performed as previously described.2 Briefly, whole cell lysates were prepared from kidney tissue and the protein content determined. All blots were run in duplicate with appropriate molecular weight markers and corrected to a reference sample between gels. Rabbit secondary antibody was used at 1:1000 for all blots. Densitometric analysis was performed using Fujifilm LAS-3000 cooled CCD camera (Raytek Scientific Ltd, Sheffield, UK).  

**References**