Impact of Early Onset Obesity and Hypertension on the Unfolded Protein Response in Renal Tissues of Juvenile Sheep

Don Sharkey, Hernan P. Fainberg, Vicky Wilson, Emma Harvey, David S. Gardner, Michael E. Symonds, Helen Budge

Abstract—Childhood obesity has reached epidemic proportions. Obesity is an independent risk factor for the development of end-stage renal disease. Endoplasmic reticulum stress and subsequent activation of the unfolded protein response (UPR) are implicated in the development of adipose tissue dysregulation and type 2 diabetes mellitus in obesity. The present study explored the impact of adolescent-onset obesity on the UPR after obesity-related hypertension and nephropathy, using an ovine model in which obesity was induced by increased food intake and reduced activity. Obese young adults had a higher mean arterial pressure (lean, 89.6±1.7 mm Hg versus obese, 101±3.0 mm Hg; P<0.01) and greater sensitivity to low physiological doses of angiotensin II. Obesity increased the glomerular area and was associated with activation of the UPR in renal cells with a greater abundance of glucose-regulated protein 78, C/EBP homologous protein, Bax, phosphorylated c-Jun amino-terminal kinase, and activating transcription factor 6 (all P<0.05). In addition, there was a marked upregulation of proinflammatory genes, most notably those involved in macrophage signaling. Reactive oxygen species production and handling were also perturbed in obese adults. Renal endoplasmic reticulum stress was positively correlated with macrophage content (r=0.83; P<0.001), phosphorylated c-Jun amino-terminal kinase (r=0.73; P<0.01), and adiposity (r=0.71; P<0.01). In conclusion, adolescent-onset, obesity-related renal endoplasmic reticulum stress was associated with activation of the UPR, apoptosis, and inflammation, potentially increasing the associated renal damage observed in young adults. The UPR may prove to be a useful therapeutic target for the treatment and prevention of obesity-related nephropathy and associated hypertension, thereby reducing the burden of end-stage renal disease. (Hypertension. 2009;53:925-931.)

Key Words: obesity ■ hypertension ■ unfolded protein response ■ nephropathy ■ adolescence ■ kidney

The current worldwide obesity epidemic is of major public health concern, and the prevalence of childhood obesity continues to rise at an alarming rate.1 Worryingly, 50% to 70% of obese adolescents remain obese as adults.2 Adult obesity is an independent risk factor for the development of end-stage renal disease (ESRD).3

The mechanisms leading to obesity-related hypertension and nephropathy remain largely unknown. Adipocyte endoplasmic reticulum (ER) stress occurs with increasing obesity leading to activation of the unfolded protein response (UPR) and results in inflammation, increased reactive oxygen species (ROS) production, apoptosis, and, ultimately, insulin resistance.4 Activation of the UPR can be caused by an abnormal nutrient supply, increased protein synthesis, altered tissue architecture, and an increase in ROS.4 Targeted therapeutic interventions aimed at altering the UPR in adipocytes of obese mice can reverse the deleterious effects of the metabolic syndrome.5 No study to date has investigated the activation of the UPR within the renal tissue of young adults after adolescent-onset obesity.

With increasing obesity, the kidney enters a hyperfiltration state with the development of nephropathy and hypertension.6 In addition, the expanding mass of perirenal adipose tissue (PAT), coupled with kidney hypertrophy, may distort normal renal architecture.6 Furthermore, there is a close interplay of the renin-angiotensin (Ang) system in adipose and renal tissues, and this may be an important link among obesity, hypertension, and kidney disease.7 Indeed, treatment with Ang II type 1 receptor antagonists not only improves renal function and hypertension but can also ameliorate adipocyte dysfunction and oxidative stress.8 Activation of a number of these factors could cause renal cell ER stress and induce the UPR, thereby accelerating the onset of renal damage and associated hypertension.

We have developed an ovine model of early onset obesity and, using this model, have shown previously that obese...
young adults, compared with lean animals, develop renal hyperfiltration, marked renal cell apoptosis, and glomerulosclerosis. We hypothesized that ER stress and activation of the UPR may play a key role in the development of obesity-related nephropathy. To establish the mechanisms in our model, we explored potential ER stress-inducing pathways and subsequent activation of the UPR.

Materials and Methods

Animal Model
All of the procedures were performed with the necessary institutional ethical approval as designated under the United Kingdom Animals (Scientific Procedures) Act, 1986. Twenty-three newborn (11 male and 12 female) sheep were randomly assigned to 1 of 3 groups. This was undertaken irrespective of sex, because we have previously shown no difference in metabolic responses between male and female sheep ≤1 year of age.10 The first group was reared with their mothers until 7 days of age, when they were humanely euthanized 
(n=7). After normal rearing with their mothers until 10 weeks of age, remaining sheep were randomly allocated to either remain lean (lean: n=8) and reared in their “natural” environment or were reared in an obesogenic environment (obese: n=8) to promote early onset obesity (online data supplement, available at http://hyper.ahajournals.org). At 1 year of age, they were humanely euthanized by electrocortical stunning and exsanguination. Tissues were rapidly excised, weighed, and snap frozen at −80°C until further analysis. For tissue histology, kidney samples were fixed in 10% (vol/vol) formalin and then embedded in paraffin wax. To establish the early life ontogeny of key genes, additional renal tissues were used from 6 month- and 3-year-old sheep (n=6) naturally reared as the lean group described above.11,12

In Vivo Measurements
Animals were housed in individual metabolic cages and allowed a period of acclimatization. Weight and blood pressure measurements were collected at 6, 9, and 12 months of age. Blood pressures at 6 and 9 months were measured noninvasively and at 12 months using carotid artery catheters inserted under anesthesia, as described previously.13 Blood pressure measurements were made on 3 separate days for 1 hour and averaged for each animal.

Ang II Challenge
Animals were challenged with increasing concentrations of Ang II over a 1-hour period, as described previously.13 Briefly, after a period of acclimatization, animals were given increasing increments of intravenous Ang II every 10 minutes (dosed as saline, 2.5, 5.0, 10.0, 20.0, 40.0, and 60.0 ng/kg per minute), with a recovery phase of 1 hour. Blood pressure and heart rate were measured continuously through the carotid artery catheter.

Renal Histology and Immunohistochemistry
Five-μm renal cortex sections were mounted on slides, stained with appropriate antibodies using an automated system, and then captured and analyzed in a blinded fashion (see online data supplement). Primary antibodies and their concentrations were used for glucose-regulated protein 78 (GRP78; at 1:750; SPA-826, Stressgen) and phosphorylated c-Jun amino-terminal kinase (JNK; at 1:25; SC6254, Santa Cruz Biotechnology). Quantification of Immunostaining
Glomerular areas were calculated using a modified version of the method of Inagi et al.14 Briefly, an average of 20 representative glomeruli, identifying vascular poles where possible, were selected per animal. Each glomerulus was traced using the image analysis software (Volocity 4, Improvision Ltd) in a blinded fashion. The largest and smallest glomeruli were excluded and the average area calculated.

Diaminobenzidine staining was identified and quantified using Volocity software. To ensure uniformity between slides, all of the images were corrected for any noncellular areas, and large blood vessels were removed.

Quantification of Renal Tissue mRNA by Real-Time RT-PCR
After RNA extraction, we used real-time PCR to quantify the mRNA abundance of the following genes: GRP78, chemokine receptor 2 (CCR2), C/EBP homologous protein (CHOP), CD68, activating transcription factor 4, activating transcription factor 6, monocyte chemoattractant protein 1 (MCP-1), Bax, inducible NO synthase (NOS; iNOS), and endothelial NOS (online data supplement).

Relative Mitochondrial Copy Number
DNA was extracted from renal tissue using the Qiagen DNA extraction kit (Qiagen Ltd) as per the manufacturer’s instructions. Mitochondrial copy number was calculated using the ratio of nuclear encoded ribosomal 18S:mitochondrial encoded ribosomal 16S, as described previously.15 18S and 16S were quantified using quantitative PCR and the ratio calculated to give an estimate of the relative mitochondrial copy number. Primers for 16S were F:GGTGACCTCGGAGAACAG and R:ATGGACCTTGTGAATAGGTAGTGC.

Western Blot Analysis
Western blot analysis of protein was performed using the following antibodies: superoxide dismutase (SOD) 1 (ab13498, Abcam) at 1:2000 and SOD 2 (ab13533, Abcam) at 1:5000 (online data supplement).

NO and SOD Activity Assays
Whole cell renal tissue homogenates were assayed to measure NOS activity (NOS assay kit, No. 482702) and SOD activity (SOD assay kit II, No. 574601) as per the manufacturer’s instructions (Calbiochem). Assays were corrected to the concentration of protein for each sample. NOS activity is estimated by the stable end products nitrate and nitrite (NOx).16

Statistical Analysis
Data were assessed for normality using the Kolmogorov-Smirnov test, followed by appropriate parametric or nonparametric analysis, and expressed as mean±SEM. Renal data were analyzed using ANOVA with posthoc Bonferroni correction for multiple tests. Independent Student t test or Mann–Whitney test were used to compare control and obese animal groups as appropriate. The analysis of the Ang II challenge corrected each animal for their baseline systolic blood pressure. To identify specific dose sensitivities, the area under the curve for each 10-minute period, at each concentration, was calculated (Prism 5, GraphPad Software, Inc) and compared using Student t test. All of the data were analyzed using SPSS software (version 14.0, SPSS, Inc.), with significance set at P<0.05.

Results

Renal Ontogeny of the UPR and Inflammation
To understand the impact of early onset obesity on key inflammatory genes, we explored their ontological expression in lean animals up to midlife. The abundance of the ER stress-inducible chaperone GRP78 and proinflammatory cytokine MCP-1 is maximal in the neonatal period and subsequently declines with age (Figure 1). CCR2, of which the main ligand is MCP-1, remains high up to 1 year of age before a significant reduction (10-fold) at 3 years. The abundance of the macrophage marker CD68 remains constant from 7 days to 6 months before a significant decline.
Body Composition, Cardiovascular Status, and Ang II Sensitivity

At the time of weaning, both groups of animals were of a similar weight (Figure 2A). Subsequently, there was a significant increase in total body weight in the obese group. At 1 year of age (young adults), the obese animals had significantly more total (lean, 0.6 ± 0.1 kg versus obese, 2.7 ± 0.3 kg; \( P < 0.001 \)) and relative (lean, 10.4 ± 1.8 g/kg versus obese, 29.6 ± 3.1 g/kg; \( P < 0.001 \)) PAT, with similar differences noted in total and visceral adipose tissue weights (data not shown).

At 6 and 9 months of age, mean blood pressures were not different between groups but were significantly higher in the obese animals at 1 year of age (Figure 2B). At low physiological doses of Ang II, the obese animals appeared more sensitive than lean animals, with a significantly greater increase from baseline in their systolic blood pressure (Figure 2C). Interestingly, no differences in heart rate change from baseline were noted between the groups during the infusion (data not shown).

Impact of Obesity on the UPR and Inflammation in Renal Tissues

At 1 year of age, obese animals had increased mRNA abundance of the proinflammatory markers MCP-1 (4.0-fold) and CD68 (3.0-fold) and a trend toward increased CCR2 (2.5-fold), as well as increased activation of the UPR, namely, activating transcription factor 4 (3.3-fold), C/EBP homologous protein (2.5-fold), and Bax (1.7-fold; Figure 3). In addition, obese animals have marked activation of the UPR, compared with lean animals, as evident by an increase in GRP78 (4.0-fold) mRNA and protein and p-JNK protein (5.0-fold) in the perirenal tubules of obese animals (Figures 4 and 5).

Impact of Obesity on ROS in Renal Tissues

Relative mitochondrial abundance, as estimated by mitochondrial copy number, was not different between the groups.
However, although no differences in iNOS or endothelial NOS mRNA abundance were found, obese animals had a greater amount of NOx (Figure 6A and 6B). Coupled with this was an increased abundance of SOD 2, but not SOD 1, protein and a trend toward reduced activity of SOD enzyme activity (Figure 6C and 6D).

Glomerular Area
In keeping with other obesity models, obese animals had marked glomerular hypertrophy, with the glomerular area being 45% greater than that of lean animals (lean, 17 684 ± 1142 μm² versus obese, 25 794 ± 1692 μm²; \(P < 0.01\)), and this closely correlated with body mass (\(r = 0.89\); \(P < 0.0001\)).

Association of Obesity, UPR, and Inflammation in the Kidney
In view of the association between adipose tissue dysregulation and inflammation in obesity, we explored the association of relative PAT mass, activation of the UPR, and inflammation. Irrespective of the group, in young adults, both relative PAT mass and renal GRP78 mRNA closely correlated with markers of renal tissue inflammation, ROS, and the UPR (Table).

Discussion
This is the first study to demonstrate a significant association of obesity-related hypertension and nephropathy with marked ER stress and activation of the UPR in young adults after early onset obesity. Few models of obesity have focused on the impact of early onset obesity on young adult renal function. Indeed, one of the strengths of our model was the use of the obesogenic environment, combining excess food intake and reduced activity, allowing a more natural development of obesity comparable to that seen in today’s pediatric population. Moreover, the sheep model closely resembles human renal development and has been used to investigate numerous renal disorders. Many rodent models of obesity-related nephropathy, although mechanistically helpful, show little resemblance to human pathology and rarely include physical activity. In addition, in our model, obese animals have elevated plasma free fatty acids, triglycerides, and insulin.

To understand the impact of obesity in our model, we investigated the ontological development of the UPR and inflammation. The abundance of GRP78, MCP-1, and CD68 is highest in the newborn period and is likely to represent an adaption to extrauterine life. In young adults, obesity induces a marked upregulation in the abundance of the proinflammatory genes MCP-1, CD68, and CCR2 involved in the development of obesity-related nephropathy. Of concern, in the context of the current childhood obesity epidemic, these changes are evident in young adults, and, indeed, abundance far exceeds that seen in older, lean animals approaching human middle age equivalent.

We next explored the role of the ER stress and activation of the UPR. In our obese model, we demonstrated an increase in the ER chaperone GRP78, as well as an activation of the UPR signals activating transcription factor 4, C/EBP homologous protein, and Bax, potentially explaining the increased apoptosis and glomerulosclerosis observed previously in our model. The precise trigger of ER stress cannot be deduced from this study. However, we postulate that it is likely to be a combination of increased ROS and excessive protein load in the ER secondary to the hyperfiltration state of the kidney.
The glomerulomegaly observed in the obese animals may be a compensatory mechanism after the loss of normal functioning nephrons by glomerulosclerosis. As a consequence, fewer, but larger, functioning nephrons may face greater ER stress.

To investigate the trigger mechanisms of the UPR in our model, we examined the role of mitochondria and ROS. Obesity is associated with a reduction in adipocyte mitochondrial copy number, which is thought to be involved in the development of type 2 diabetes mellitus. In our model, relative renal mitochondrial copy number is unchanged with obesity, making it unlikely that this is an important mechanism of renal cell stress. Excess production of ROS in obese animals may well be implied by the compensatory increase of SOD 2 protein in an attempt to increase ROS removal. The trend to reduced SOD enzyme activity, despite raised SOD 2 protein, may represent abnormal protein folding and, hence, a nonfunctioning enzyme, known to occur during ER stress. Despite no differences in either iNOS or endothelial NOS, the obese animals have a marked elevation in NOS in keeping with early human diabetic nephropathy. At low levels in endothelial cells, this is cytoprotective. However, at higher concentrations, it can induce apoptosis.

JNK is critical in the development of renal disease. In human renal biopsy samples from patients with hypertension and diabetic nephropathy, elevated JNK activation is positively correlated with increasing focal glomerulosclerosis and worsening glomerular filtration rate. JNK signaling plays a key role in the inflammatory response seen in obesity and type 2 diabetes mellitus, and this signaling can be induced by the UPR. Our data demonstrate a significant increase in peritubular p-JNK with obesity. Inagi et al have demonstrated the importance of ER stress in podocyte injury secondary to excess protein accumulation and that preconditioning the ER can ameliorate the effects of mesangio proliferative glomerulonephritis. Taken together with our findings, these studies offer evidence that activation of the UPR and JNK signaling may be critical in the development of obesity-related nephropathy. Our data suggest that inositol-requiring protein-1 and protein kinase RNA-like ER kinase are the more important UPR pathways, with more work required to clarify the role of the activating transcription factor 6 pathway.

Increasing evidence supports a link between Ang II and adipose tissue dysregulation in obesity, and this may well prove to be a valuable therapeutic target to prevent chronic kidney disease. Ang II is known to induce inflammation and production of ROS in the kidney and is indeed the target for mainstay pharmacological treatment for obesity and type 2 diabetes mellitus–related nephropathy. In our model, examination of the systolic blood pressure of obese animals demonstrated that they are significantly more sensitive at the lower physiological doses of Ang II despite their already hypertensive state. It is probable that this increased reactivity is, in part, caused by greater sensitivity within the renal circulation, as seen in other similar models, as well by as a greater abundance of Ang II receptors. Ang II can induce glomerular damage via an increase in thrombospodin 1 production, but this depends on JNK signaling. Increased activation of p-JNK in the obese animals may also reflect an increased sensitivity to Ang II, thereby potentiating the inflammatory effects. In addition, the expression of CCR2 is relatively high through early life. With obesity, CCR2 abundance at 1 year of age is 25 times higher than in lean animals at 3 years. Homozygous CCR2 knockout mice, with Ang II–induced hypertension, are protected from oxidative stress, macrophage infiltration, and renal damage. This interaction between Ang II and CCR2 may have important implications for adolescent-onset obesity, because a higher expression of

<table>
<thead>
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<th>Variable</th>
<th>Relative PAT Mass, g/kg</th>
<th>GRP78 mRNA</th>
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<tbody>
<tr>
<td>GRP78 mRNA</td>
<td>0.71†</td>
<td>...</td>
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<tr>
<td>GRP78 protein</td>
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<td>p-JNK protein</td>
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<td>CD68 mRNA</td>
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<td>CHOP mRNA</td>
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<td>Bax mRNA</td>
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<td>0.64*</td>
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<tr>
<td>SOD 2 protein</td>
<td>0.76†</td>
<td>0.51</td>
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</table>

Values represent Pearson’s or Spearman’s correlation coefficients of 1-year-old animals (n = 10 to 16 animals). CHOP indicates C/EBP homologous protein. 

\[P < 0.05\]

†\[P < 0.01\]

‡\[P < 0.001\]
CCR2 during adolescence may potentiate the proinflammatory effects of elevated circulating Ang II seen in obesity.28

Because obesity is an independent predictor for the development of ESRD,3 we explored the associations of ER stress, PAT mass, and inflammation and demonstrated striking correlations. It is intriguing, but not surprising, to find significant positive correlations of ER stress, inflammation, ROS, and p-JNK with increasing PAT mass. This could be a consequence of hypertension and evolving insulin resistance seen with increasing obesity or it may reflect worsening renal architecture secondary to the expanding adipose tissue mass.6  In addition, the increased sensitivity to Ang II may well magnify these adverse effects, creating a viscous cycle of inflammation, renal damage, and hypertension. It is likely that all of these are key factors, along with others, on the causal pathway of obesity-related nephropathy. Ultimately, the progression of hypertension with increasing glomerulosclerosis is likely to result in multigorgan damage.

In conclusion, we have developed a relevant large animal model of early onset obesity-related nephropathy. Our model closely resembles the development of obesity seen in today’s contemporary pediatric population, and, thus, in the context of the current childhood obesity epidemic, these findings have important implications for long-term renal morbidity and, hence, planning of renal healthcare services. We have also demonstrated, for the first time, that activation of the UPR occurs within the renal tissues and that the pathophysiology closely resembles that seen in adipose tissue of obese individuals. These data allow new hypotheses to be formulated and new treatments to be investigated, such as the use of chemical chaperones that target and reduce adipocyte ER stress, reversing the adverse effects of type 2 diabetes mellitus and obesity-related insulin resistance in mice.3 The present study highlights the critical role of ER stress and the UPR seen in obesity-related nephropathy and may prove to be a useful therapeutic target in the prevention and treatment of ESRD.

**Perspectives**

The increasing prevalence of ESRD has paralleled the recent obesity epidemic. The UPR is now emerging as a useful therapeutic target in the prevention and treatment of ESRD.

**Sources of Funding**

This work was supported by a British Heart Foundation Clinical Fellowship (to D.S., FS/05/098/19942) and Lectureship (to D.S.G., BS/03/001) and the European Union Sixth Framework for Research and Technical Development of the European Community: the Early Nutrition Programming Project (FOOD-CT-2005-007036).

**Disclosures**

None.

**References**


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Hypertension. 2009;53:925-931; originally published online May 4, 2009;
doi: 10.1161/HYPERTENSIONAHA.108.122812

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

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Short title: Renal activation of the unfolded protein response

Word count: 4888

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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 Bondmax 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 (Techne, 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 (Techne, 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 \(\Delta\Delta^2ct\) method\(^3\) 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

Western Blot Analysis

Western blot analysis of protein was performed as previously described. 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