

Chronic administration of AM251 improves albuminuria and renal tubular structure in obese rats

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Abstract

Modulation of the endocannabinoid system as an anti-obesity therapeutic is well established; however, the direct effects of cannabinoid receptor 1 (CB₁) antagonism on renal function and structure in a model of diet-induced obesity (DIO) are unknown. The aim of this study was to characterise the renal effects of the CB₁ antagonist AM251 in a model of DIO. Male Sprague–Dawley rats were fed a low- or high-fat diet (HFD: 40% digestible energy from lipids) for 10 weeks to elicit DIO ($n=9$). In a different cohort, rats were fed a HFD for 15 weeks. After 9 weeks consuming a HFD, rats were injected daily for 6 weeks with 3 mg/kg AM251 ($n=9$) or saline via i.p. injection ($n=9$). After 10 weeks consuming a HFD, CB₁ and megalin protein expression were significantly increased in the kidneys of obese rats. Antagonism of CB₁ with AM251 significantly reduced weight gain, systolic blood pressure, plasma leptin, and reduced albuminuria and plasma creatinine levels in obese rats. Importantly, there was a significant reduction in tubular cross-section diameter in the obese rats treated with AM251. An improvement in albuminuria was likely due to the reduction in tubular size, reduced leptinaemia and maintenance of megalin expression levels. In obese rats, AM251 did not alter diastolic blood pressure, sodium excretion, creatinine clearance or expression of the fibrotic proteins VEGFA, TGFB1 and collagen IV in the kidney. This study demonstrates that treatment with CB₁ antagonist AM251 improves renal outcomes in obese rats.

Key Words
► cannabinoids
► obesity
► leptin
► kidney

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Introduction

The prevalence of obesity and the subsequent adverse effects of associated co-morbidities has risen significantly in recent years, placing a substantial financial and social burden on societies worldwide (Ng *et al.* 2014). Furthermore, obesity is a strong, independent risk factor for the development and progression of chronic kidney disease (CKD), even when confounding variables such as hyperglycaemia, diabetes or hypertension are accounted

for (Griffin *et al.* 2008). Thus, it is important to identify physiological targets, which may ameliorate the progression of obesity and the subsequent decline in renal function.

The endocannabinoid system has been characterised as an important endogenous lipid signalling system, which can mediate the development of obesity via the regulation of food intake and energy expenditure

(Di Marzo 2008). We have recently demonstrated that activation of the cannabinoid receptor 2 (CB₂) in diet-induced obese rats can lead to improved renal function (Jenkin *et al.* 2015a). In the past decade, the CB₁ has received growing interest for its anti-obesity potential. A number of studies have demonstrated that CB₁ antagonists and inverse agonists are an effective anti-obesity therapeutic (Van Gaal *et al.* 2005, Janiak *et al.* 2007, Rosenstock *et al.* 2008, Nam *et al.* 2012, Tam *et al.* 2012). However, characterisation of the physiological effects of CB₁ antagonism in the periphery is limited outside of metabolically active tissues (Sink *et al.* 2008, Son *et al.* 2010, Merroun *et al.* 2013). Therefore, it is essential to determine whether antagonism of CB₁ in the periphery alters disease progression in other tissues to ensure a full investigation of its potential as a therapeutic target.

CB₁ antagonists have been shown to improve renal outcomes in a number of disease states including diabetes and obesity (Janiak *et al.* 2007, Lim *et al.* 2009, Mingorance *et al.* 2009). Overexpression of CB₁ has been identified in glomerular renal cells of animal models with type 1 (Barutta *et al.* 2010) and type 2 (Nam *et al.* 2012) diabetic nephropathy. Specifically, increased CB₁ expression has been demonstrated in models of nephropathy in human proximal tubular cells *in vitro* (Lim *et al.* 2010, Nam *et al.* 2012) and our group has previously established that proximal tubule cells exposed to pathophysiological levels of glucose and albumin also contribute to increased CB₁ expression (Jenkin *et al.* 2015b). Furthermore, in animal models of diabetic nephropathy, CB₁ antagonism ameliorates proteinuria, albuminuria and plasma creatinine, and improves creatinine clearance in diabetic animals (Janiak *et al.* 2007, Barutta *et al.* 2010, Nam *et al.* 2012). In contrast, transgenic mice that overexpress CB₁ exhibit significant increases in proteinuria and kidney weight compared with WT mice (Hsu *et al.* 2014). Therefore, modulation of CB₁ appears to be an effective mechanism for the reduction of the pathophysiological changes associated with nephropathy. CB₁ antagonism is thought to elicit improvements via the preservation of glomerular podocyte cells and reduced tubule apoptosis (Janiak *et al.* 2007, Barutta *et al.* 2010, Lim *et al.* 2010).

Despite these studies in models of diabetic nephropathy, the only analysis of CB₁ as a therapeutic in obesity-linked nephropathy has been performed in obese Zucker rats or *db/db* mice (Janiak *et al.* 2007, Lim *et al.* 2009), which lack a functional leptin signalling pathway. There are limitations of using this specific animal model to study obesity, as recent research has demonstrated that leptin and CB₁ are functionally linked (Tam *et al.* 2012). Leptin is

a hormone produced primarily by adipose tissue and is principally cleared by the kidneys (Hama *et al.* 2004). Hyperleptinaemia is associated with obesity and the progression of renal fibrosis via increased collagen deposition and transforming growth factor beta 1 (TGFβ1) secretion by renal glomerular cells (Briffa *et al.* 2014). Obesity-related kidney damage leads to increased levels of protein and albumin in the urine. This functional change has been linked to altered renal expression of megalin (Tam *et al.* 2012), a transmembrane protein which regulates both albumin and leptin absorption in the renal tubules (Hama *et al.* 2004, Birn & Christensen 2006, Hryciw *et al.* 2012). Thus, it is essential to characterise CB₁ as a therapeutic for obesity-linked nephropathy in a model that contains a functional leptin signalling pathway.

Our group has previously established that the CB₁ antagonist, AM251, significantly reduces proximal tubule hypertrophy in cultured HK2 cells (Jenkin *et al.* 2010). Thus, in light of the current limitations of how CB₁ and its role in obesity-related renal function have been examined using models lacking a functional leptin pathway, it is essential that we characterise the *in vivo* outcomes of modulation of CB₁ in a model of diet-induced obesity (DIO). The main aim of this study was to investigate whether treatment with the CB₁ antagonist AM251 reverses renal dysfunction associated with obesity-related CKD and to determine the specific cellular mechanism for the improved function in a model of DIO.

Materials and methods

Animals

All animal experimental procedures were approved by Howard Florey Animal Ethics Committee (AEC 11-036), which operates under the guidelines of the National Health and Medical Research Council of Australia. Seven-week-old male Sprague-Dawley rats (~350 g) were individually housed (cage dimensions; width 27.5 × length 41 × height 25.5 cm) in an environmentally controlled laboratory (ambient temperature 22–24 °C) and maintained under a 12 h light:12 h darkness cycle (0700–1900 h).

DIO model

Rats were randomly assigned to receive either a high-fat diet (HFD; containing 40% digestible energy from lipids (Specialty Feeds, Glen Forrest, WA, Australia)), or a control diet (lean; 10% digestible energy from lipids, Barastoc Ltd (Melbourne, VIC, Australia)) for a period of 10 weeks

($n=9$ /group), as described previously (Jenkin *et al.* 2015a). Throughout the duration of the study, animals were allowed to access food and water *ad libitum*. At the end of 10 weeks, model rats were anaesthetised via i.p. injection with 100 mg/kg sodium pentobarbitone (Virbac, Milperra, NSW, Australia) and killed via cardiac puncture. Kidneys and surrounding peri-renal fat pads were then removed, weighed and stored at -80°C for further analyses.

Chronic AM251 treatment in DIO

The rats receiving control or AM251 treatment were sustained on a HFD for a total of 15 weeks. Nine weeks of a HFD was sufficient to induce DIO, with rats exhibiting significant increases in body weight, body fat composition, hypertension and reduced renal function, as described previously by our group (Jenkin *et al.* 2015a). Thus, following 9 weeks of a HFD, rats were matched according to weight, body composition and blood pressure and were allocated to either obese control ($n=9$) or CB₁ antagonist AM251 groups ($n=9$). For a further 6 weeks, rats were maintained on the HFD and treated daily with either vehicle control (0.9% isotonic saline solution containing 0.75% Tween 80), or 3 mg/kg body weight of AM251 (Cayman Chemicals, Ann Arbor, MI, USA, dissolved in vehicle). AM251 has been shown previously in both animal models (Gatley *et al.* 1996, de Oliveira Alvares *et al.* 2006, Judge *et al.* 2009, Litvin *et al.* 2013) and in cell culture models (Lan *et al.* 1999, Deshpande *et al.* 2007) to act as a CB₁ antagonist, with a high degree of selectivity (306-fold) for the CB₁ receptor over CB₂ (Lan *et al.* 1999). CB₁ antagonism with AM251 treatment has also been demonstrated in transgenic animal models, where the effects with AM251 observed in WT mice are absent in CB₁ knockout mice (Shearman *et al.* 2003, Haller *et al.* 2004). The concentration of 3 mg/kg for AM251 was based on previously published experimental studies using mice and rats (Barutta *et al.* 2010, Merroun *et al.* 2013). Treatments were administered via i.p. injection. Throughout the duration of the 15-week study, animals were allowed to access food and water *ad libitum*. Following the 15 weeks, rats were deeply anaesthetised with vaporised 3% isoflurane (Abbott) and killed via cardiac puncture. Kidneys and surrounding peri-renal fat pads were then removed, weighed and stored at -80°C for further analyses.

Metabolic measurements

Pre-treatment measurements were recorded at week 9, before the administration of control or AM251 treatments,

and post-treatment measurements were recorded at week 15 in the final week of the intervention. Rat weight and food consumption were recorded daily throughout the experimental period. Pre-weighed food was provided to the rats to access *ad libitum*. After 24 h, the amount of food remaining, including any on the bottom of the cages was recorded. Food consumption was calculated as the weight (g) of food provided subtracted by that recovered. Measurements for systolic and diastolic blood pressure were obtained from conscious rats using a non-invasive tail-cuff method with volume pressure recording software CODA 2 (Kent Scientific, Torrington, CT, USA; Daugherty *et al.* 2009). Glucose tolerance tests and insulin sensitivity tests were conducted as described previously (Jayasooriya *et al.* 2008, Xia *et al.* 2011) with minor modifications as glucose and insulin were administered via i.p. injection following an overnight and 2-h fast period respectively. Blood glucose in response to glucose (2 g/kg) or insulin (0.75 U/kg) load was analysed as area under the curve (Le Floch *et al.* 1990).

Plasma analysis

Following cardiac puncture, blood was transferred into 10 ml EDTA BD Vacutainer tubes (McFarlane Medical, Surrey Hills, NSW, Australia) and kept on ice until samples were centrifuged at 4000 *g* for 10 min at 4°C . The plasma layer was aspirated and stored at -80°C for further analyses. Plasma levels of creatinine (Cayman Chemical Company, Ann Arbor, MI, USA), TGF β 1 (Promega) and leptin (R&D Systems, Minneapolis, MN, USA) were analysed according to the manufacturer's instructions.

Renal function measurements

Renal function was evaluated using 24 h urine samples collected using metabolic cages at weeks 9 and 15 of the obese control or AM251 treatment (pre- and post-treatment periods). Measurements of urinary albumin (ALPCO Diagnostics, Salem, NH, USA) and creatinine (Cayman Chemical Company) were determined using commercially available kits, according to the manufacturer's instructions. Lean age-matched rats ($n=6$) were included in renal function measurements. Lean animals were fed a control diet for 16 weeks *ad libitum* (standard rodent chow; containing 10% digestible energy from lipids; sourced from Barastoc Ltd) (Jenkin *et al.* 2015a). Change in urinary albumin excretion (Δ urinary albumin) was determined by post-treatment measurement subtracted from pre-treatment measurement and was standardised for urinary creatinine. Urinary albumin was calculated as a ratio of mg

of albumin excreted divided by mg creatinine excreted (mg.dl/mg.dl Cr). Urinary sodium excretion was determined by analysing the sodium content in undiluted 24 h samples using the COBAS Integra 400 Plus System (Roche Diagnostics). Creatinine clearance (ml/min per kg) was calculated via the formula (urinary vol (ml/min) × urinary creatinine concentration (mg/dl))/(plasma creatinine (mg/dl)) and standardised for body weight (Keenan *et al.* 2000).

Histological analysis

Following post-mortem collection of the kidney, a cross-sectional portion of the tissue was fresh frozen in optimal cutting temperature compound (Tissue-Tek, Torrance, CA, USA), and 5 µm thick sections were cut using a HM 550 Cryostat (Thermo Fisher Scientific, Scoresby, VIC, Australia). Kidney sections were stained using haematoxylin and eosin and periodic acid schiff (PAS; Hughes & Gobe 2007). Sections were imaged at 200× magnification (Carl Zeiss microscope) and at least 20 random glomeruli and 20 renal tubule sections from each rat ($n=5-6$) were analysed. In order to analyse glomerular area, the outer edges of all glomerular tufts were traced on a captured image, and the encircled area was determined using the Image Analysis Software (Axiovision Rel. 4.8; Zeiss, Jena, Germany; Henegar *et al.* 2001). Tubular diameter was analysed at the widest point for cross-sectional diameter on captured bright-field images using the Axiovision 4.8 Image Analysis Software; the software is used to convert arbitrary pixels into microns (µm) as detailed previously (Jonassen *et al.* 2008).

Western blotting protocol

Protein was isolated from individual rat kidneys as described previously (Slattery *et al.* 2011, Jenkin *et al.* 2013, 2015a). Aliquots (40–100 µg) of protein lysates were separated on a 7.5–20% SDS-PAGE gel and transferred onto a nitrocellulose membrane. CB₁ (Cayman Chemicals), megalin (Santa Cruz Biotechnology), TGFB1 (Abcam, Cambridge, UK), collagen IV (Abcam) and vascular endothelial growth factor (VEGFA; Abcam) were detected using western blot analysis from kidney lysate using specific antibodies, with β-actin (Sigma–Aldrich) as a loading control. Secondary antibodies, anti-mouse and anti-rabbit were purchased from Sigma–Aldrich. Band densitometry was analysed using the Image Lab Software (Bio-Rad Laboratories). When reporting protein content, data were calculated by the volume intensity of the protein divided

by the volume intensity of β-actin loading control, with protein content expressed in arbitrary units.

Statistical analysis

The SPSS statistical package software (SPSS, Inc.) was used for all statistical analysis. All data are presented as mean ± s.e.m. Differences between lean and obese rats or AM251- and saline control-treated obese rats were analysed using independent samples *t*-test for two group direct analyses of measurements for obese control and AM251 groups. In measurements that were taken at both pre- and post-treatment time points, data were analysed using a repeated-measures ANOVA. Significance was considered when $P < 0.05$.

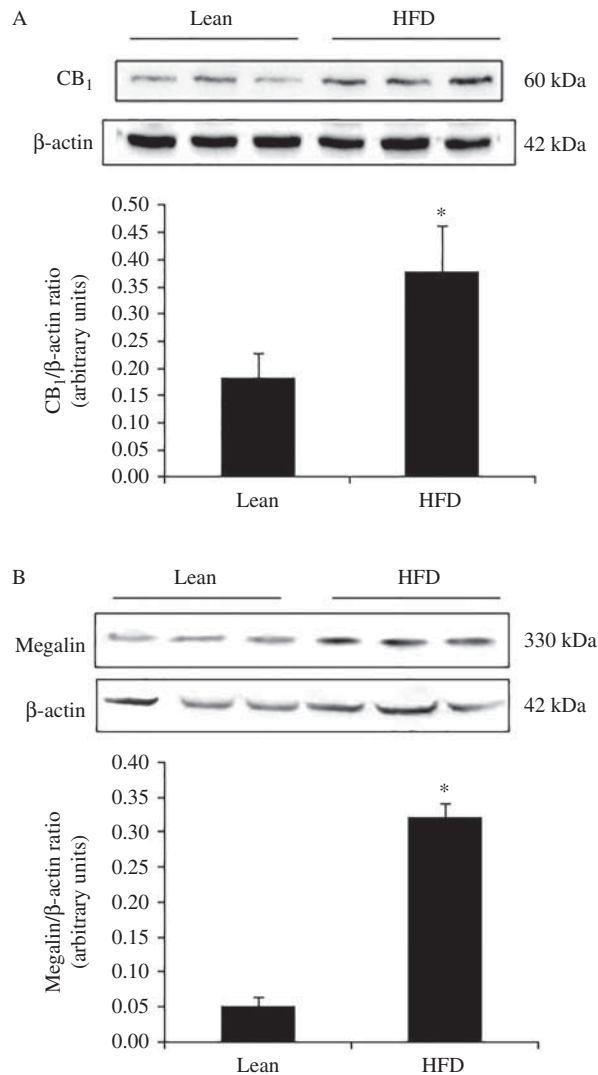
Results

Renal CB₁ and megalin expression in DIO rats

In whole kidney extract, CB₁ protein was significantly increased in DIO rats fed a HFD for a period of 10 weeks compared with lean standard chow-fed rats (Fig. 1, $n=9$, $P < 0.05$). Megalin protein expression was also significantly increased in DIO rats fed a HFD for 10 weeks, compared with lean standard chow-fed rats (Fig. 1, $n=8$, $P < 0.05$). No differences between lean and HFD-fed DIO rats were detected for β-actin, which was used as a loading control.

Metabolic parameters in control and AM251-treated obese rats

We have recently shown that this model of DIO leads to significant increases in weight, adiposity, diastolic and systolic blood pressure compared with lean animals fed a standard chow diet and that this model does not lead to alterations in glucose tolerance or insulin sensitivity (Jenkin *et al.* 2015a). Herein, we have shown that treatment with CB₁ antagonist AM251, in DIO rats, induced improvements in percentage weight gain, systolic blood pressure and hyperleptinaemia compared with obese controls. Obese control and obese rats treated with AM251 were at similar weights pre-treatment, but obese rats treated with AM251 were significantly lighter at the conclusion of the treatment period compared with control obese rats (Table 1, $n=9$, $P < 0.05$). This was due to significantly reduced weight gain (main effect $P < 0.05$, interaction $P < 0.05$, Fig. 2) across the 6-week treatment period, compared with obese control rats. AM251 treatment in obese rats led to a transient reduction in

**Figure 1**

CB₁ protein expression in whole kidney lysate of rats fed a standard chow (lean) or high-fat diet (HFD). (A) Representative western blot and quantification of CB₁ expression in kidney lysate from rats at 10 weeks. (B) Representative western blot and quantification of megalin expression in kidney lysate from rats at 10 weeks. Significance is indicated by * compared with lean standard chow-fed rats ($P < 0.05$, $n = 8-9$).

food consumption for the first 2 weeks of treatment (Fig. 2, $n = 9$, $P < 0.05$). AM251-treated obese rats exhibited significantly reduced systolic blood pressure (Table 1, $n = 9$, main effect $P = 0.382$, interaction $P < 0.05$) and significant reductions in plasma leptin concentrations, compared with obese control rats (Fig. 2, $n = 9$, $P < 0.05$). Glucose tolerance, insulin sensitivity and diastolic blood pressure were not significantly altered by AM251 treatment compared with obese control rats (Fig. 3). All metabolic measurements are outlined in Table 1.

Effect of AM251 treatment on renal morphology in DIO rats

In obese rats, AM251 treatment did not have any significant effects on gross kidney weight, kidney weight standardised for body weight or gross peri-renal fat weight area compared with obese controls or lean age-matched animals (Table 1). Glomerular cross-sectional area was not altered by treatment with AM251 (Fig. 4). However, obese rats treated with AM251 did exhibit significant reductions in peri-renal fat pad weight standardised for body weight compared with obese controls (Table 1, $n = 9$, $P < 0.05$). Although this measure was reduced in obese AM251-treated animals, lean age-matched rats exhibited significantly lower amounts of peri-renal fat standardized for body weight (Table 1, $n = 6-9$, $P < 0.05$). Histological analysis with PAS staining showed that tubular cross-sectional diameter was significantly smaller in obese AM251-treated rats compared with obese control (Fig. 4, $n = 5-6$, $P < 0.05$), indicating that AM251 treatment may reverse tubular hypertrophic morphological changes associated with obesity-related renal damage. Furthermore, obese control rats were shown to have significantly higher tubular cross-sectional diameter than age-matched lean rats (Table 1, $n = 5-6$, $P < 0.05$). However, no significant differences were found between the AM251-treated animals (Table 1) and age-matched lean controls, indicating that AM251 may indeed reduce obesity-related tubular hypertrophy.

Functional renal outcomes in control and AM251-treated obese rats

In obese rats, treatment with AM251 significantly improved renal outcomes as measured by albuminuria and plasma creatinine. Change in urinary albumin excretion was significantly reduced in obese rats treated with AM251 compared with obese controls (Fig. 5A, $n = 9$, $P < 0.05$). Lean age-matched animals were included to illustrate functional renal outcomes compared with obese rats treated with AM251 and no significant differences in urinary protein, urinary albumin excretion or plasma creatinine were determined (Fig. 5, $n = 6-9$, $P < 0.05$). Plasma creatinine, which was measured at the post-treatment time point only, was significantly lower in AM251-treated rats post-treatment compared with obese control (Fig. 5, $n = 9$, $P < 0.05$). No significant differences or interactions were found between lean or obese control rats compared with obese animals treated with AM251 for urinary sodium excretion or estimated creatinine clearance (Fig. 5, $n = 6-9$).

Table 1 Analysis of metabolic and renal histological measures of lean, DIO Sprague–Dawley rats (obese control) and DIO Sprague–Dawley rats treated with CB₁ antagonist, AM251

Measure	Lean		Obese control		AM251	
	Pre treatment	Post treatment	Pre treatment	Post treatment	Pre treatment	Post treatment
Body weight (g)	539.6 ± 11.83	597.8 ± 17.28	582.9 ± 14.35	655.9 ± 19.23	576.0 ± 11.51	607.2 ± 12.43 ^{a,b}
Food consumption (g/day)	32.1 ± 1.59	31.1 ± 1.60	23.7 ± 0.95	21.7 ± 0.51	22.5 ± 0.61	19.3 ± 0.53
Diastolic blood pressure (mmHg)	85 ± 6.47	89 ± 5.76	100 ± 5.56	105 ± 5.83	105 ± 3.38	91 ± 5.59
Systolic blood pressure (mmHg)	121 ± 6.49	123 ± 7.82	137 ± 6.23	144 ± 7.07	151 ± 4.89	135 ± 7.43 ^{a,b}
Glucose tolerance (AUC)	338.4 ± 93.15	537.0 ± 38.88	590.8 ± 81.54	560.4 ± 82.92	485.3 ± 39.86	594.8 ± 43.51
Insulin sensitivity (AUC)	184.3 ± 66.22	158.25 ± 53.35	219.7 ± 36.81	245.6 ± 43.82	176.7 ± 28.04	172.2 ± 29.03
Plasma TGFB1 (ng/ml)	–	13.9 ± 0.92	–	18.4 ± 3.26	–	15.8 ± 1.71
Plasma leptin (ng/ml)	–	8.79 ± 0.94	–	22.6 ± 5.07	–	9.28 ± 2.10 ^a
Kidney weight (g)	–	1.72 ± 0.09	–	1.66 ± 0.05	–	1.68 ± 0.05
Kidney weight/body weight (%)	–	0.29 ± 0.01	–	0.25 ± 0.01	–	0.27 ± 0.01
Peri-renal fat weight (g)	–	5.52 ± 0.60	–	11.46 ± 0.09	–	8.50 ± 1.19
Peri-renal weight/body weight (%)	–	0.91 ± 0.09	–	1.93 ± 0.16	–	1.25 ± 0.21 ^a
Tubular cross-sectional diameter (µm)	–	10.17 ± 0.23	–	12.74 ± 0.19	–	10.76 ± 0.26 ^a

^aSignificance is indicated between diet-induced obese rats treated with AM251 and obese control at the same time point (pre- or post-treatment, $P < 0.05$, $n = 9$).

^bInteraction was observed between group and time period (two-way repeated measures ANOVA, $P < 0.05$, $n = 9$). Data are shown as average ± s.e.m.

Renal expression of megalin and fibrotic markers in AM251-treated obese rats

Recently, our group has shown that this model of DIO in Sprague–Dawley rats does not alter the renal expression of a number of fibrotic markers including collagen IV, TGFB1, fibronectin and α -smooth muscle actin; however, VEGFA is significantly reduced in DIO rats fed a HFD for 10 weeks (Jenkin *et al.* 2015a). For the first time, herein we have shown that in obese rats, plasma concentrations of TGFB1 were not altered by treatment with AM251 compared with obese controls (Table 1). Western blot analysis of megalin, collagen IV, TGFB1 and VEGFA demonstrated that treatment with AM251 in obese rats did not significantly alter levels of these proteins in whole kidney tissue of obese rats (Fig. 6, $n = 9$). No differences between obese control and AM251-treated rats were detected for β -actin, which was used as a loading control.

Discussion

In this study, expression of CB₁ was significantly increased in the kidneys of DIO rats. Treatment with the CB₁ antagonist, AM251, reduced weight gain and significantly reduced systolic blood pressure, plasma leptin, albuminuria and plasma creatinine levels in obese rats. Furthermore, tubular cross-sectional diameter was reduced in obese rats treated with AM251. The tubular cross-sectional diameter of AM251-treated animals was comparable to the measurements of lean age-matched rats. Significantly,

the renal expression of the scavenger receptor megalin was up-regulated in obese rats fed a HFD for 10 weeks, but was not altered by treatment with AM251. Collectively, these data indicate that the CB₁ antagonist, AM251, may improve renal outcomes in a model of DIO.

It has been well documented that overweight or obese individuals have an increased risk of developing CKD (Wang *et al.* 2008, Afkarian *et al.* 2013). We have previously demonstrated that male Sprague–Dawley rats fed a HFD for 10 weeks exhibit an obese phenotype, with increased body weight, adiposity and blood pressure (Jenkin *et al.* 2015a). Furthermore, this model of DIO has been shown to reduce renal function, as measured by increased proteinuria, albuminuria and urinary creatinine excretion (Jenkin *et al.* 2015a). Herein, we have shown for the first time that after 10 weeks in a rat model of DIO, expression of CB₁ in kidney tissue of obese rats is significantly increased compared with lean rats. Furthermore, we have shown that in our model of DIO, treatment with AM251 was able to significantly reduce weight gain, systolic blood pressure and hyperleptinaemia, which is supported by previous research (Janiak *et al.* 2007, Mingorance *et al.* 2009, Nam *et al.* 2012, Tam *et al.* 2012).

Importantly, AM251 treatment in obese rats improved a number of markers of obesity-related renal damage. This included a reduction in total urinary albumin excretion and plasma creatinine levels compared with obese control rats. These findings are similar to previous studies, which demonstrated, in models of diabetic nephropathy when

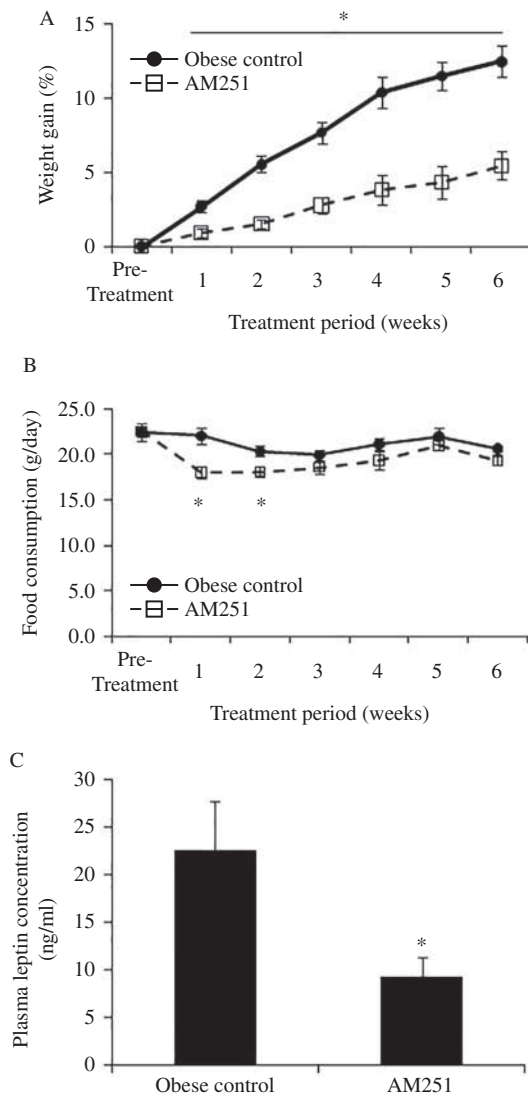
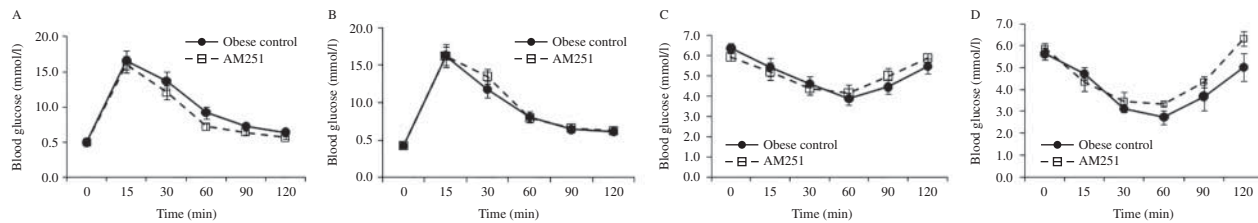


Figure 2 Metabolic outcomes of kidneys of AM251 in obese rats. (A) Obese AM251-treated rats exhibited significantly reduced weight gain (% of pre-treatment weight) from weeks 1 to 6 of treatment compared with obese controls. (B) Food consumption (g/day) of obese control and AM251 obese rats. (C) Plasma leptin concentration in obese control and AM251 obese rats. Significance is indicated by * compared with obese controls ($P < 0.05$, $n = 9$).

targeting CB_1 with an antagonist or inverse agonist, rodents exhibit improved renal function (Janiak *et al.* 2007, Barutta *et al.* 2010, Jenkin *et al.* 2012, Nam *et al.* 2012). In this study, we have added to this knowledge by demonstrating that CB_1 antagonism with AM251 improves albuminuria in a DIO model. We have found that AM251 treatment in obese rats was able to significantly reduce hyperleptinaemia induced by obesity, similar to a previous study (Tam *et al.* 2012). Our findings

are further supported by a recent study, which employed a transgenic mouse model to overexpress CB_1 and was shown that animals exhibited higher levels of proteinuria without alterations in blood glucose compared with the WT group (Hsu *et al.* 2014).

Prior to this study, the investigation of modulation of CB_1 as a therapeutic target in obesity-related renal dysfunction has only been studied in obese Zucker rats (Janiak *et al.* 2007). In these rats, antagonism of CB_1 resulted in improvements in proteinuria and creatinine clearance through the reduction in renal hypertrophy and fibrosis (Janiak *et al.* 2007). However, obese Zucker rats have alterations in their leptin signalling pathway. As leptin signalling and CB_1 function are linked (Tam *et al.* 2012), the outcomes of the study by Janiak *et al.* (2007) needed further investigation. To add to this, our data have demonstrated that, in obese rats, the scavenger receptor megalin was significantly up-regulated, while treatment with AM251 in DIO rats did not significantly alter levels of renal megalin expression compared with obese control. Renal expression of megalin in obese humans has yet to be characterised; however, our group has shown that, *in vitro*, renal proximal tubule cells acutely exposed to leptin decreases megalin expression and albumin uptake in proximal tubule cells (Briffa *et al.* 2014). However, the increase in megalin expression in our obese model contradicts our *in vitro* findings and the research of Tam *et al.* (Tam *et al.* 2012, Briffa *et al.* 2014), who demonstrated a reduction in megalin expression in obese mice which also exhibited reduced glucose tolerance and insulin sensitivity, which was reversed when mice were treated with a CB_1 inverse agonist. In our model of DIO, no differences in response to either glucose or insulin were detected between AM251-treated rats and control rats. As megalin is down-regulated in isolated proximal tubule cells exposed to elevated glucose (Ishibashi 2004), and in the tubules of streptozotocin-induced diabetic Sprague-Dawley rats (Russo *et al.* 2007), we can suggest that the reduction in megalin in the kidney in the Tam model may be due to the exposure of elevated circulating glucose. The lack of change of megalin in the AM251-treated obese rats, which also exhibited reduced tubular hypertrophy, may have allowed a higher capacity for the binding of megalin to albumin in the filtrate, leading to the improved reabsorption and reduced urinary albumin excretion compared with obese control rats. Previous research in diabetic nephropathy has attributed improved renal function primarily to glomerular changes, with reduced loss of nephrin and podocin, observed in AM251-treated animals (Barutta *et al.* 2010, Hsu *et al.* 2014). Albumin is

**Figure 3**

Response to glucose and insulin in obese rats treated with AM251 compared with obese control. (A) Glucose tolerance test pre-treatment.

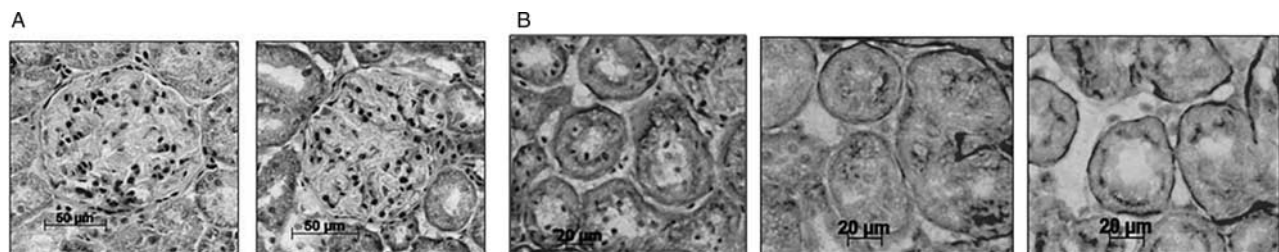
(B) Glucose tolerance test post-treatment. (C) Insulin sensitivity test pre-treatment. (D) Insulin sensitivity test post-treatment.

filtered by the glomerulus and largely reabsorbed by proximal tubule cells, and damage to either of these structures can result in albuminuria (Birn & Christensen 2006). Although we did not measure podocin or nephrin protein levels, no significant changes in glomerular histology or size was observed between obese control and AM251-treated rats, suggesting that the tubules are the main site of action of CB₁ action in obesity prior to the onset of hyperglycaemia.

The lack of hyperglycaemia in our model of DIO is indicative of early pathophysiological changes associated with obesity (Kahn *et al.* 2006). It has been well documented that obesity is associated with CKD, even in the absence of confounding factors including elevated fasting glucose and overt diabetes and high blood pressure (Kramer *et al.* 2005, Griffin *et al.* 2008, Mathew *et al.* 2011). Currently, we have two hypotheses for the renal damage in our model of DIO in the absence of hyperglycaemia. First, the elevated levels of megalin observed in this study are likely to initially increase albumin uptake. We and others have shown that exposure to elevated albumin increases fibrotic damage in proximal tubules (Wohlfarth *et al.* 2003, Slattery *et al.* 2013). This leads to an elevation in fibrotic proteins, which in turn down-regulates albumin endocytosis that ultimately leads to renal dysfunction

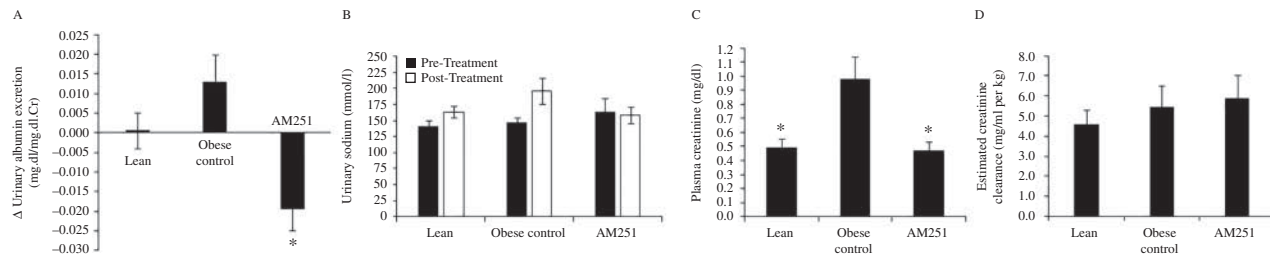
(Gekle *et al.* 2003). Secondly, leptin has been well characterised to be elevated in humans and animal models of obesity (Ahima & Flier 2000, Banks *et al.* 2004, Briffa *et al.* 2013). Current work by our group has demonstrated that elevated leptin exposure alters the metabolic function of tubular cells (Briffa *et al.* 2014), which may account for the renal dysfunction observed in our model.

In addition to targeting the renal system, AM251 significantly reduced systolic blood pressure of DIO rats compared with obese controls. Systolic blood pressure specifically has been demonstrated to be a strong predictor of renal disease in humans and in rats (Gonzalez-Albarran *et al.* 2003). The role of CB₁ in the cardiovascular system is complex; the influence of the receptor on haemodynamics depends on the experimental context, species of animal and, in clinical trials, patient background, including age, race and sex (Pacher *et al.* 2005). It has been hypothesised that AM251 treatment may be affecting systolic blood pressure via either sympathetic nervous system activity or activation of the renin–angiotensin–aldosterone system, as both systems are implicated in changes to cardiovascular haemodynamics (Dobrian *et al.* 2000). Despite this, reductions in both weight and systolic blood pressure may also contribute to the improvements in albuminuria in this model of DIO.

**Figure 4**

Morphological analysis of kidneys of AM251-treated obese rats. Representative pictures of using 200 \times magnification. (A) Haematoxylin and eosin (H&E) staining of the glomerulus of obese control ($n=5$) and obese

rats treated with AM251 ($n=5$). (B) Periodic acid schiff (PAS) staining of the renal tubules of lean age-matched rats ($n=6$), obese control rats ($n=5$) and obese rats treated with AM251 ($n=6$).

**Figure 5**

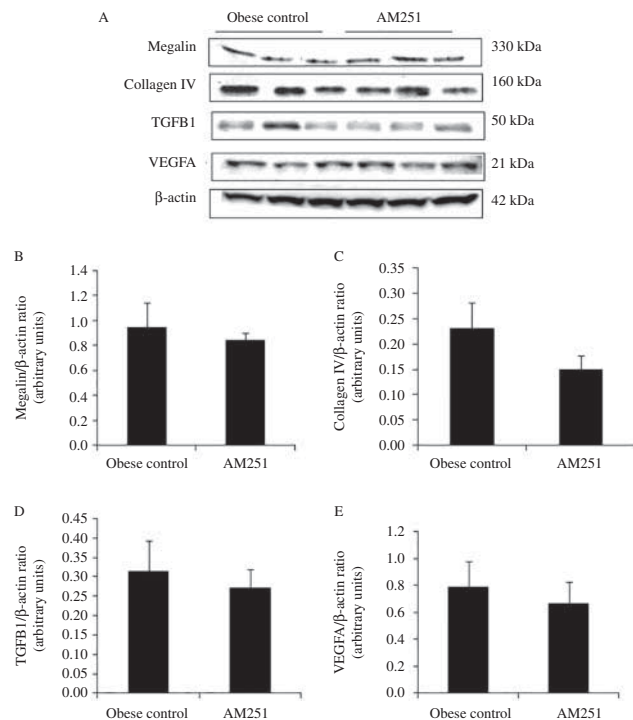
Functional renal outcomes of lean age-matched rats and obese rats treated with AM251 compared with obese control. (A) Change (Δ) urinary albumin excretion. (B) Urinary sodium excretion. (C) Plasma creatinine

concentration. (D) Estimated creatinine clearance. Significance difference is indicated by * compared with obese controls ($P < 0.05$, $n = 6-9$).

Hyperleptinaemia is positively correlated with the degree of obesity (Ahima & Flier 2000, Tam *et al.* 2012) and is also associated with increased TGF β 1 production and collagen deposition leading to increased fibrosis in CKD (Wolf & Ziyadeh 1999, Briffa *et al.* 2013). Previous studies have established that fibrotic markers including TGF β 1, collagen IV and VEGFA are up-regulated in the kidney in obesity-related nephropathy (Jiang *et al.* 2005, Cignarelli & Lamacchia 2007, Mathew *et al.* 2011, Briffa *et al.* 2013, Nolan *et al.* 2013, Zhang *et al.* 2013). Indeed, it has been shown that in lean transgenic mice that overexpress CB $_1$, there is an increase in glomerular expression of VEGFA (Hsu *et al.* 2014). However, we have demonstrated that, in AM251-treated obese rats, there is no significant alteration in renal expression of collagen IV, TGF β 1 or VEGFA. Therefore, alternative targets are likely to be involved in the improvement of renal dysfunction observed in our model of DIO. In support of this proposal, treatment with CB $_1$ antagonist, AM251, in cultured proximal tubule cells exposed to hyperlipidaemic conditions reduced apoptosis via signalling pathways that activate endoplasmic reticulum stress (Lim *et al.* 2010). Therefore, CB $_1$ may modulate endoplasmic reticulum function to improve the renal outcomes in obesity. Thus, further investigation is required to determine which downstream targets CB $_1$ may be mediating in a DIO model of nephropathy.

AM251 treatment in obese rats significantly reduced plasma creatinine levels, compared with obese controls. Creatinine is a by-product of skeletal muscle and is primarily removed from the blood via glomerular filtration (Toffaletti & McDonnell 2008). Interestingly, although we observed a significant reduction in plasma creatinine of obese rats treated with AM251, no differences were found between groups for estimated creatinine clearance standardised to body weight. This potentially could be due to the significant reduction in body weight observed in obese animals treated with AM251 compared with obese controls. Lower levels of

plasma creatinine are likely the effect of overall reduced skeletal muscle mass (reflected in lower body weight), which produces creatinine, as indicated by lower plasma creatinine observed in both AM251-treated obese and lean age-matched rats compared with obese controls. As no significant differences were found in creatinine clearance between either the lean or obese control compared with obese AM251-treated rats, it is possible that the renal filtration of creatinine is not affected to the same extent as

**Figure 6**

Protein expression of kidney lysate from diet-induced obese rats treated with AM251 compared with obese controls ($n = 8-9$). (A) Representative western blots of obese control and AM251-treated rats. (B) Quantification of megalin protein in kidney lysate. (C) Quantification of collagen IV protein in kidney lysate. (D) Quantification of TGF β 1 protein in kidney lysate. (E) Quantification of VEGFA protein in kidney lysate.

the tubular handling of urinary albumin in obese rats. Furthermore, while previously published work has demonstrated that specificity of AM251 for the CB₁ receptor is high (Lan *et al.* 1999), and the use of CB₁ knockout models have demonstrated that AM251 acts to block the effects of CB₁ (Shearman *et al.* 2003, Haller *et al.* 2004), our study has not evaluated whether complete CB₁ antagonism was achieved in our model of DIO. Compounded with this, AM251, along with other established CB₁ antagonists may also be acting via other CB receptors, particularly GPR55 (Ryberg *et al.* 2007, Kapur *et al.* 2009, Henstridge *et al.* 2010). However, these findings have been disputed by others. While we cannot rule out any interaction of the agonistic effect of GPR55 following AM251 treatment in DIO rats, it must be reiterated that the dosage utilised was similar to that reported in previously published studies, which have demonstrated CB₁ antagonism using knockout models (Shearman *et al.* 2003, Haller *et al.* 2004).

In summary, this study has demonstrated that treatment with the CB₁ antagonist, AM251, improves markers of renal damage, and specifically, that CB₁ and megalin protein expression is significantly up-regulated in kidney lysate of rats fed a HFD. Furthermore, AM251 reverses hyperleptinaemia, maintains the increased renal expression of megalin and improves albuminuria and plasma creatinine. We propose that the mechanism for the improvement in albuminuria is via the maintenance of renal megalin levels and reduction in tubular size compared with obese controls. Furthermore, metabolic improvements, including reductions in surrounding peri-renal fat tissue, body weight, weight gain, food consumption and blood pressure, which have been characterised extensively elsewhere (Despres *et al.* 2005, Janiak *et al.* 2007, Rosenstock *et al.* 2008, Tam *et al.* 2012), were also observed in our model in response to AM251 treatment. This study identifies a potential role of CB₁ modulation with the antagonist AM251 for the treatment of early obesity-related renal damage. Further investigation is required to identify which signalling pathways the CB₁ receptor may be acting on to fully elucidate the role of CB₁ within the renal system under obese conditions.

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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