Cannabinoid Receptor 1 Blockade Ameliorates Albuminuria in Experimental Diabetic Nephropathy

Federica Barutta,1 Alessandro Corbelli,2,3 Raffaella Mastrocola,1 Roberto Gambino,1 Vincenzo Di Marzo,4 Silvia Pinach,1 Maria Pia Rastaldi,2 Paolo Cavallo Perin,1 and Gabriella Gruden1

OBJECTIVE—Cannabinoid receptor 1 (CB1) is localized in the central nervous system and in peripheral tissues involved in energy metabolism control. However, CB1 receptors are also expressed at low level within the glomeruli, and the aim of this study was to investigate their potential relevance in the pathogenesis of proteinuria in experimental type 1 diabetes.

RESEARCH DESIGN AND METHODS—Streptozotocin-induced diabetic mice were treated with N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,3-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM251), a selective CB1-receptor antagonist, at the dosage of 1 mg·kg−1·day−1 via intraperitoneal injection for 14 weeks. Urinary albumin excretion was measured by enzyme-linked immunosorbent assay. CB1 receptor expression was studied by immunohistochemistry, immunoblotting, and real-time PCR. Expression of nephrin, podocin, synaptopodin, and zonula occludens-1 (ZO-1) was assessed by immunofluorescence and real-time PCR. Fibronectin, transforming growth factor-β1 (TGF-β1), and connective tissue growth factor (CTGF) mRNA levels were quantitated by real-time PCR.

RESULTS—In diabetic mice, the CB1 receptor was overexpressed within the glomeruli, predominantly by glomerular podocytes. Blockade of the CB1 receptor did not affect body weight, blood glucose, and blood pressure levels in either diabetic or control mice. Albuminuria was increased in diabetic mice compared with control animals and was significantly ameliorated by treatment with AM251. Furthermore, CB1 blockade completely prevented diabetes-induced downregulation of nephrin, podocin, and ZO-1. By contrast overexpression of fibronectin, TGF-β1, and CTGF in renal cortex of diabetic mice was unaltered by AM251 administration.

CONCLUSIONS—In experimental type 1 diabetes, the CB1 receptor is overexpressed by glomerular podocytes, and blockade of the CB1 receptor ameliorates albuminuria possibly via prevention of nephrin, podocin, and ZO-1 loss. Diabetes 59: 1046–1054, 2010

Diabetic nephropathy is characterized by increased glomerular permeability to proteins and excessive extracellular matrix accumulation in the mesangium, eventually resulting in glomerulosclerosis and progressive renal impairment (1).

Recently, increasing attention has been paid to the role of podocytes in the pathogenesis of proteinuric conditions (2,3). The slit diaphragm, a junction connecting foot processes of neighboring podocytes, represents the major restriction site to protein filtration (4), and a causal link between loss of slit diaphragm molecules, such as nephrin and podocin, and the onset of proteinuria has been established (5–7). In both human and experimental diabetic nephropathy, there is a reduction in nephrin expression, and studies in patients with microalbuminuria have demonstrated that nephrin downregulation occurs in an early stage of the disease, supporting the hypothesis of a role of nephrin loss in glomerular albumin leakage (8–10). A number of factors, including advanced glycation end products (10), glomerular hypertension (10,11), angiotensin II (10), and inflammatory cytokines (12) have been implicated in the downregulation of slit diaphragm proteins in diabetes, but the precise mechanism is still largely unknown.

The cannabinoid receptor of type 1 (CB1), a G-protein-coupled receptor, is expressed predominantly in the central nervous system (13), but it has been also found in peripheral tissues, such as the liver (14), adipose tissue (15), pancreas (16), and skeletal muscle (17), where it plays a key role in the control of peripheral energy metabolism. A recent study has shown that the CB1 receptor is expressed at a low level within the glomeruli and that CB1 receptor blockade ameliorates proteinuria in an animal model of obesity-induced nephropathy (18).

Although in obese animals the antiproteinuric effect of CB1 antagonism is likely related to amelioration of the metabolic profile, the observation that the CB1 receptor is present within the glomeruli raises the hypothesis of a direct effect of signaling through the CB1 receptor on podocytes and possibly on glomerular permeability to proteins.

To assess the role of the CB1 receptor in the pathogenesis of proteinuria in diabetes, we studied glomerular CB1 receptor expression in streptozotocin (STZ)-induced diabetic mice. Furthermore, we tested whether CB1 receptor blockade affects proteinuria and/or expression of slit diaphragm and slit diaphragm–associated proteins in this model.

RESEARCH DESIGN AND METHODS
Materials. All materials were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.
Drug. *N*-piperidin-1-yl-5-(4-isodophenyl)-1-(2,3-dichlorophenyl)-4-methyl-3H-pyrazole-3-carboxamide (AM251), a CB1 receptor antagonist, was purchased from Cayman Chemical (Ann Arbor, MI), dissolved in ethanol to a stock concentration of 3 mg/ml, and stored at −80°C. Stock solutions were diluted in 18:1:1 ratio of saline/emulphor-620/absolute ethanol immediately prior to use. AM251 conjugated to 5-carboxytetramethylrhodamine was purchased from Tocris (Bristol, U.K.).

Animals and induction of diabetes. Both housing and care of laboratory animals were in accordance with Italian law (D.L.116/1992). Male C57BL/6J mice from The Jackson Laboratory (Bar Harbor, ME) were maintained on a normal diet under standard animal house conditions. Diabetes was induced in mice, aged 8 weeks and weighing ~22 g, by intraperitoneal injection of STZ-citrate buffer (55 mg/kg body wt, 0.5% w/v in 0.95% saline) were included in the study. For electron microscopy, 1-mm³ pieces of renal cortex were fixed in 2% glutaraldehyde, 4% paraformaldehyde in phosphate buffer 0.12 mol/l for 4 h at room temperature, postfixed in 1% osmium tetroxide for 2 h, dehydrated in graded ethanol, and embedded in Epon 812.

Systolic blood pressure was assessed by tail-cuff plethysmography in pre- and postdeveloped TaqMan real-time PCR using predeveloped TaqMan reagents (Applied Biosystems; CB1: Mm00432621; nphrin: Mm00478928; podocin: Mm00499922; ZO-1: Mm01320537; TGF-B1: Mm00441724; CTGF: Mm00515790; fibronectin: Mm01256744). Fluorescence for each cycle was analyzed quantitatively and gene expression normalized relative to the expression of HPRT1. Because housekeeping genes ubiquitously express in the renal cortex do not control for variations in the glomerular number per specimen or changes in podocyte number (20), WT-1, a podocyte-specific gene, was used as endogenous reference for the evaluation of nephrin, podocin, and ZO-1 mRNA expression.

Podocyte apoptosis. Apoptosis was assessed by the transferase-mediated dUTP nick-end labeling (TUNEL) method using the ApopTag In Situ Apoptosis Detection Kit (Millipore, Billerica, MA). Results were expressed as the number of positive cells per glomerulus in at least 20 random glomeruli. Slides pretreated with 20,000 units/ml DNase were used as positive control.

Globular volume. Globular cross-sectional area (Aₜₐₜ) was measured in 20 glomerular profiles per mouse using a computerized image analysis system (Axiovision 4.7; Carl Zeiss). The glomerular volume (Vₜₐₜ) was then calculated as Vₜₐₜ = βK(Aₜₐₜ)², where β = 1.38 is the size distributor coefficient and K = 1.01 is the shape coefficient for glomeruli idealized as a sphere (21).

Electron microscopy. Ultrathin sections for ultrastructural examination were cut with the Ultracut E Reichert-Jung ultramicrotome, stained with uranylacetate and lead citrate, and examined with the transmission electron microscope Philips CM 10. Microphotographs were taken using a SIS-view II digital camera. Mean foot process width (FPW) was assessed as the ratio of positive cells per glomerular in at least 20 random glomeruli. Slides pretreated with 20,000 units/ml DNase were used as positive control.

RESULTS

Metabolic and physiological parameters. Before killing, blood samples were taken via saphenous vein puncture on alert, 4-h-fasted animals, and glucose levels measured using a glucometer (Accuchek; Roche, Milan, Italy). Systolic blood pressure was assessed by tail-cuff plethysmography in pre- and postdeveloped TaqMan real-time PCR using predeveloped TaqMan reagents (Applied Biosystems; CB1: Mm00432621; nphrin: Mm00478928; podocin: Mm00499922; ZO-1: Mm01320537; TGF-B1: Mm00441724; CTGF: Mm00515790; fibronectin: Mm01256744). Fluorescence for each cycle was analyzed quantitatively and gene expression normalized relative to the expression of HPRT1. Because housekeeping genes ubiquitously express in the renal cortex do not control for variations in the glomerular number per specimen or changes in podocyte number (20), WT-1, a podocyte-specific gene, was used as endogenous reference for the evaluation of nephrin, podocin, and ZO-1 mRNA expression.

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and immunoblotting. There was a significant two-fold quantitative techniques, we measured in a partial yellow overlap (Fig. 1). Specificity of the antibody binding was confirmed by disappearance of the signal when the antibody was preabsorbed with a 10-fold excess of control peptide. In the tubuli, staining for the CB1 receptor was faint and no differences were observed between control and diabetic mice (nondiabetic: 2.07 ± 0.73; diabetic: 2.54 ± 0.32; P = not significant) (Fig. 1C and D).

To clarify which glomerular cell type overexpressed the CB1 receptor, double-labeling immunofluorescence was performed in diabetic mice for the detection of both CB1 and nephrin. The CB1 receptor was expressed primarily by glomerular podocytes as the positive staining for nephrin (Fig. 1L) colocalized with CB1 staining (Fig. 1I), resulting in a partial yellow overlap (Fig. 1M and N).

To confirm immunohistochemistry findings with more quantitative techniques, we measured CB1 mRNA and protein expression in total renal cortex by real-time PCR and immunoblotting. There was a significant two-fold increase in CB1 mRNA levels in diabetic mice (Fig. 1F) compared with nondiabetic control animals. Immunoblotting showed a band migrating at ~60 kDa, corresponding to the reported molecular weight of CB1 (Fig. 1G), and densitometric analysis demonstrated that CB1 protein expression was significantly increased in the diabetic mice (Fig. 1G and H).

**AM251 binding to the CB1 receptor.** As shown in Fig. 10–Q, glomerular staining using a fluorescent AM251 analog overlapped with the immunofluorescent staining for the CB1 receptor, confirming that AM251 binds to the CB1 receptor.

**Effect of treatment with AM251 on metabolic and physiological parameters.** As shown in Table 1, treatment with AM251 did not affect the degree of glycemic control because both blood glucose and glycated hemoglobin levels were similar in treated and untreated diabetic animals. In addition, in both diabetic and control animals administration of AM251 did not alter body weight. Finally, no differences were observed in systolic blood pressure.

**Effect of CB1 receptor blockade on albuminuria, NAG activity, and renal function.** After 14 weeks of diabetes, there was a more than 10-fold increase in urinary albumin excretion rate in diabetic compared with nondiabetic animals. Treatment with AM251 did not alter albuminuria in the controls, but induced a significant 50% reduction in albumin excretion rate levels in the diabetic mice (Table 1). Results were similar when they were expressed as urinary albumin/creatinine ratio (nondiabetic: 146 ± 6.88; nondiabetic + AM251: 147.9 ± 33.87; diabetic: 2447.5 ± 607.7; diabetic + AM251: 806.3 ± 190.5; P < 0.001 diabetic vs. others). Urinary NAG activity/creatinine ratio, a marker of tubular damage, was slightly increased in diabetic mice compared with controls, although not significantly. In addition, no differences were observed between treated and untreated diabetic mice (nondiabetic: 88.11 ± 8.37; diabetic + AM251: 93.16 ± 16.52; diabetic: 178.13 ± 21.43; diabetic + AM251: 180.64 ± 40.86 units/g; P = not significant). Renal function was similar among groups as assessed by measurement of creatinine clearance (nondiabetic: 0.38 ± 0.07; diabetic: 0.41 ± 0.1; diabetic + AM251: 0.5 ± 0.1 ml/min; P = not significant).

**Effect of AM251 on nephrin, podocin, ZO-1, and synaptopodin expression.** To clarify the underlying mechanism of the beneficial effect of CB1 blockade on albuminuria, we assessed the effect of treatment with AM251 on the expression of nephrin, podocin, ZO-1, and synaptopodin by immunofluorescence. After 14 weeks of diabetes, there was a significant reduction in nephrin, podocin, and ZO-1 protein expression, and this effect was completely prevented in diabetic mice treated with AM251 (Fig. 2A–F). No significant changes in synaptopodin protein levels were observed among groups (Fig. 2G and H).

We also assessed nephrin, podocin, and ZO-1 mRNA in total renal cortex from all groups by real-time PCR and found a significant reduction in nephrin, podocin, and ZO-1 mRNA levels in diabetic compared with control mice. Treatment with AM251 prevented diabetes-induced nephrin and podocin mRNA downregulation (Fig. 3A and B). A similar trend was also observed for ZO-1, although it did not reach statistical significance (Fig. 3C).

**Effect of CB1 receptor blockade on podocyte injury.** To establish whether in diabetic mice treated with AM251, podocyte protein expression was preserved because CB1 blockade prevented podocyte damage, podocyte number, apoptosis, and ultrastructure were assessed by WT-1 staining, TUNEL assay, and electron microscopy, respectively. The number of both WT-1- and TUNEL-positive cells per glomerular cross-sectional area displayed in a podocyte distribution did not differ among groups (Fig. 4) (nondiabetic: 2.0 ± 0.5; diabetic: 2.2 ± 0.4; nondiabetic + AM251: 2.5 ± 0.3; diabetic + AM251: 2.1 ± 0.5, TUNEL-positive cells per 100 glomeruli; P = not significant). Electron microscopy analysis did not show any evidence of ultrastructural glomerular (Fig. 4A–F) and tubular (Fig. 5G–L) damage in the studied animals. In particular, the normal arrangement of interdigitating foot processes was maintained in all groups, and podocyte foot processes appeared tall and narrow in both treated and untreated diabetic mice (Fig. 5A–F). Furthermore, mean FPW was compara-
Effect of CB1 blockade on glomerular hypertrophy and early markers of fibrosis. Glomerular volume was significantly increased in diabetic mice compared with control animals and this effect was not altered by treatment with AM251 (nondiabetic: 136 ± 10.33; diabetic: 358 ± 19.06; nondiabetic + AM251: 352 ± 1.27; diabetic: 375 ± 1.11; diabetic + AM251: 364 ± 0.99 nm; P = not significant).

Effect of CB1 blockade on glomerular hypertrophy and early markers of fibrosis. Glomerular volume was significantly increased in diabetic mice compared with control animals and this effect was not altered by treatment with AM251 (nondiabetic: 136 ± 10.33; diabetic: 358 ± 19.06; nondiabetic + AM251: 127 ± 4.98; diabetic + AM251: 369 ± 5.72 μm³; P < 0.05 diabetic and diabetic + AM251 vs. nondiabetic).

At electron microscopy, the degree of mesangial expansion and glomerular basement membrane thickening was very mild in diabetic mice, and no major differences were observed among groups (Fig. 4A–D). However, in the renal cortex levels of mRNA encoding for fibronectin, TGF-β1,
FIG. 2. CB1 blockade abolished downregulation of nephrin, podocin, and ZO-1 protein expression in diabetic mice. Renal cryostatic sections from both diabetic and nondiabetic mice, treated with either vehicle or the CB1 antagonist AM251 for 14 weeks, were stained for nephrin (A), podocin (C), ZO-1 (E), and synaptopodin (G) by immunofluorescence as described in the “Research Design and Methods” section (magnification ×400). Quantification of glomerular staining for nephrin (B), podocin (D), ZO-1 (F), and synaptopodin (H) is shown (*P < 0.01 diabetic vs. others; #P < 0.001 diabetic vs. others). (A high-quality digital representation of this figure is available in the online issue.)
and CTGF were significantly greater in diabetic than in control animals. Treatment of diabetic mice with AM251 did not affect the overexpression of these markers of fibrosis, suggesting that CB1 blockade does not have antifibrotic properties in this model (Figure 6A–C).

DISCUSSION

In this study, we have provided evidence that in experimental diabetes the CB1 receptor is overexpressed within the glomeruli, predominantly by glomerular podocytes. Second, we have shown that blockade of the CB1 receptor with AM251 ameliorates albuminuria and prevents down-regulation of nephrin, podocin, and ZO-1. Taken together, these data suggest a role of the CB1 receptor in the pathogenesis of proteinuria in diabetes.

In nondiabetic mice, only a few glomerular cells stained positively for the CB1 receptor, but after 14 weeks of experimental diabetes there was a significant 1.5-fold increase in glomerular CB1 expression. A previous study has shown that the CB1 receptor is expressed at low level in rat kidneys, but no differences in renal CB1 expression were observed between Zucker fatty rats and lean animals (18). Therefore, this is the first evidence of glomerular CB1 overexpression in experimental diabetes and, to our knowledge, in any renal disease.

In agreement with previous data in rat kidneys (18), immunoreactivity for CB1 was predominantly localized to the glomeruli. A faint signal was also detectable in the tubuli, but it was not enhanced in the diabetic mice. Both pattern of staining and colocalization with the podocyte marker nephrin strongly indicate that in diabetic mice the CB1 receptor was primarily overexpressed by glomerular podocytes. This is not surprising because similarities have been reported between podocytes and neurons (24,25), the predominant CB1 receptor–expressing cell type in physiological conditions (13). The underlying mechanism/s of CB1 receptor induction in diabetic podocytes is entirely unknown. However, oxidative stress, which is enhanced in the diabetic glomeruli, is known to induce CB1 receptor expression in other cell types (26).

Consistent with our immunohistochemical data, we found a significant 1.8- to 2-fold increase in CB1 both
protein and mRNA expression in renal cortex from diabetic mice as assessed by immunoblotting and real-time PCR. Diabetes-induced enhanced transcription of the CB1 receptor is a potential underlying mechanism as both activator protein 1 (AP-1) and nuclear factor-κB (27,28), the transcription factors predominantly implicated in the pathogenesis of diabetic nephropathy, have binding sites on the promoter region of the CB1 receptor gene (29).

To establish whether upregulation of the CB1 receptor in podocytes plays a role in the pathogenesis of proteinuria in diabetes, we studied the effect of treatment with AM251. AM251, a potent and specific CB1 receptor inverse agonist (30 –33), is structurally very close to rimonabant, but exhibits better binding affinity and selectivity for the CB1 receptor (33). In our model, selectivity of AM251 binding to the CB1 receptor was indirectly confirmed by colocalization of AM251 and CB1 within the diabetic glomeruli. AM251 was administered daily at the dosage of 1 mg/kg on the basis of previous studies that have proven both efficacy and safety of this dose in mice (34,35). Delivery was via intraperitoneal injection to ensure that all animals were given an equal amount of drug. After 14 weeks of diabetes, there was a 10-fold increase in albuminuria in diabetic mice compared with controls. Treatment with AM251 induced a significant 50% reduction in albuminuria in diabetic mice, supporting the hypothesis that signaling through the CB1 receptor contributes to enhanced glomerular permeability to albumin.

Administration of AM251 did not affect body weight in either diabetic or control mice. This is in agreement with previous reports showing that CB1 blockade prevents weight gain in animals with diet-induced obesity, but is much less efficacious at exerting this effect in lean animals fed a standard diet (36 –38). Furthermore, blood glucose levels, glycated hemoglobin, and systolic blood pressure were similar in treated and untreated diabetic mice, consistent with the antiproteinuric effect of CB1 blockade observed in these mice being independent of both metabolic and hemodynamic factors. A significant reduction in proteinuria has been recently reported in obese Zucker fatty rats treated with rimonabant (18); however, in this weeks of diabetes, there was a 10-fold increase in albuminuria in diabetic mice compared with controls. Treatment with AM251 induced a significant 50% reduction in albuminuria in diabetic mice, supporting the hypothesis that signaling through the CB1 receptor contributes to enhanced glomerular permeability to albumin.

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model CB1 receptor was not overexpressed in the glomeruli, and the antiproteinuric effect was most likely due to amelioration of the metabolic profile because it was associated with body weight loss and diminution of both blood glucose and lipid levels. In our study, we have purposely chosen an animal model of type 1 diabetes without obesity, insulin resistance, and lipid abnormalities to ensure that the beneficial effects of CB1 blockade were independent of changes in metabolism.

To clarify the mechanism/s of the beneficial effect of AM251, we tested whether CB1 receptor blockade prevents downregulation of podocyte proteins implicated in the maintenance of glomerular permselectivity to proteins. After 14 weeks of diabetes, there was a significant reduction in nephrin, podocin, and ZO-1 both mRNA and protein expression. Treatment with AM251 prevented diabetes-induced downregulation of nephrin, podocin, and ZO-1 protein expression. Results were confirmed by mRNA analysis for both nephrin and podocin, and a similar trend was also observed for ZO-1. Loss of slit diaphragm and slit diaphragm–associated proteins has been implicated in the pathogenesis of proteinuria (2); therefore, downregulation of nephrin, podocin, and ZO-1 is a possible mechanism whereby CB1 overexpression may lead to increased glomerular permeability to albumin.

The number of both total and apoptotic podocytes was not increased in diabetic mice. Furthermore, there was no evidence of podocyte foot process effacement at the ultrastructural level or changes in mean FPW. It is, thus, unlikely that levels of nephrin, podocin, and ZO-1 were normal in diabetic mice treated with AM251 because of prevention of podocyte damage. These data also suggest that in early experimental diabetes, downregulation of slit diaphragm proteins precedes the development of podocyte foot process effacement/loss and that podocyte structural damage is not strictly required for the development of proteinuria. Consistent with this view, in nephrin knockout animals proteinuria occurs even in the absence of any defects in the podocyte foot processes (39). The degree of nephrin reduction required for the development of proteinuria is unknown; however, the parallel downregulation of other slit diaphragm proteins is likely to cause a rise in this threshold level (40).

In the diabetic mice, tubular ultrastructure was normal and urinary activity of NAG, a marker of tubular damage, was comparable in treated and untreated mice. Therefore, prevention of tubular injury is not a likely explanation of AM251 antiproteinuric activity. However, we cannot exclude the possibility that at a later stage of experimental diabetes, when severe tubular damage occurs, CB1 blockade may have further beneficial effects due to prevention of tubulointerstitial injury, as previously shown in the Zucker fatty rat model (18).

Glomerular hypertrophy and accumulation of extracellular matrix components, which is mediated predominantly by the prosclerotic cytokines TGF-β1 and CTGF, are other characteristic features of diabetic nephropathy (1). In our study, after 14 weeks of mild diabetes, C57BL/6J mice, which are relatively resistant to the development of glomerulosclerosis, did not show major ultrastructural abnormalities in the mesangium and the glomerular basement membrane. However, there was a significant increase in both glomerular volume and renal mRNA expression of fibronectin, TGF-β1, and CTGF in diabetic mice. These diabetes-induced effects were left unchanged by treatment with AM251, indicating failure of CB1 blockade in interfering with glomerular hypertrophy and renal fibrogenesis. In animal models of chronic liver injury, blockade of the CB1 receptor decreases fibrosis by lowering hepatic TGF-β1 expression and reducing accumulation of fibrogenic cells (14). The different effect of CB1 blockade on renal and liver fibrosis is not entirely surprising because the cell types overexpressing the CB1 receptor in liver cirrhosis, namely myofibroblasts and hepatic stellate cells, have much greater profibrotic potential than podocytes. In addition, a tissue-specific effect of CB1 receptor activation cannot be excluded.

In conclusion, our findings may have important implications for diabetic nephropathy in humans. Proteinuria is a characteristic feature of diabetic nephropathy and a key determinant of progression (1). Nephrin is downregulated in early diabetic nephropathy, and this has been implicated in the pathogenesis of the diabetic proteinuria (10). Our data, showing upregulation of CB1 receptors in podocytes and a beneficial effect of AM251 on both albuminuria and nephrin loss, suggest that an elevated CB1 receptor tone (41) is also involved in the pathogenesis of the diabetic proteinuria and identify a new target for therapeutic intervention.

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REFERENCES

1. Molitch ME, DeFronzo RA, Franz MJ, Keane WF, Mogensen CE, Parving HH, Stoffers MW, American Diabetes Association. Nephropathy in diabetes. Diabetes Care 2004;27:879–883
2. Li JJ, Kwak SJ, Jung DS, Kim JJ, Yoo TH, Ryu DR, Han SH, Choi HY, Lee JE, Moon SJ, Kim DK, Han DS, Kang SW. Podocyte biology in diabetic nephropathy. Kidney Int 2007;106:836–842
3. Wolf G, Chen S, Zayed FHA. From the periphery of the glomerular capillary wall toward the center of disease: podocyte injury comes of age in diabetic nephropathy. Diabetes 2005;54:1626–1634
4. Ravenstad H, Kriz W, Kretzler M. Cell biology of the glomerular podocyte. Physiol Rev 2003;83:253–307
5. Kestila M, Lenkkeri U, Mannikko M, Lamerdin J, McCready P, Putaatu H, Ruotsalainen V, Morita T, Nissinen M, Herva R, Kastalan CE, Petlonen L, Holmberg C, Olsen A, Tryggvason K. Positionally cloned gene for a novel glomerular protein—nephrin—is mutated in congenital nephrotic syndrome. Mol Cell 1998;1:572–578
6. Beltecheva O, Martin P, Lenkkeri U, Tryggvason K. Mutation spectrum in the nephrin gene (NPHS1) in congenital nephrotic syndrome. Hum Mutat 2001;17:368–373
7. Boute N, Gribouval O, Roselli S, Benessy F, Lee H, Fuchshuber A, Dahan K, Gubler MC, Niaudet P, Antignac C, NPHS2, encoding the glomerular protein podocin, is mutated in autosomal recessive steroid-resistant nephrotic syndrome. Nat Genet 2000;24:349–354
8. Cooper ME, Mundel P, Boner G. Role of nephrin in renal disease including diabetic nephropathy. Semin Nephrol 2002;22:303–308
9. Bonnet F, Cooper ME, Kawachi H, Allen TJ, Boner G, Cao Z. Irbesartan normalises the deficiency in glomerular nephrin expression in a model of diabetes and hypertension. Diabetologia 2001;44:867–877
10. Doblizer S, Salvadido G, Lupia E, Ruotsalainen V, Verzola D, Deferrari G, Camussi G. Nephrin expression is reduced in human diabetic nephropathy: evidence for a distinct role for glycated albumin and angiotensin II. Diabetes 2003;52:1023–1030
11. Langham RG, Kelly DJ, Cox AJ, Thomson NM, Holthöfer H, Zaubi P, Pinel N, Cormondier DJ, Gilbert RE. Proteinuria and the expression of the podocyte slit diaphragm protein, nephrin, in diabetic nephropathy: effects of angiotensin converting enzyme inhibition. Diabetologia 2002;45:1572–1576
12. Tarabra E, Giunti S, Barutta F, Salvadido G, Burt D, Deferrari G, Gambino R, Vergola D, Pinach S, Cavallo Perin P, Camussi G, Gruden G. Effect of the
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MCP-1/CCR2 system on nephrin expression in streptozotocin-treated mice and human cultured podocytes. Diabetes 2009;58:2109–2118.

13. Kano M, Ohno-Shosaku T, Hashimoto T, Uchigashima M, Watanabe M. Endocannabinoid-mediated control of synaptic transmission. Physiol Rev 2009;9:309–380.

14. Teixeira-Clerc F, Julien B, Grenard P, Tran Van Nhieu J, Deveaux V, Li L, Serriere-Lanneau V, Ledent C, Mallat A, Lotersztajn S. CB1 cannabinoid receptor antagonism: a new strategy for the treatment of liver fibrosis. Nat Med 2006;12:671–676.

15. Roche R, Hoareau L, Bes-Houtmann S, Gonthier MP, Laborde C, Baron JF, Teixeira-Clerc F, Julien B, Grenard P, Tran Van Nhieu J, Deveaux V, Li L, Forloni GL, Schlo¨ ndorff D, Holthofer H, D’Amico A, Esposito G, Steardo L, Herman AG, Terashita T, Matsuda S. Process formation of the renal glomerular podocyte. FASEB J 2006;20:976–978.

16. Nakata M, Yada T. Cannabinoids inhibit insulin secretion and cytosolic Ca2+ oscillation in islet beta-cells via CB1 receptors. Regul Pept 2008;145:49–53.

17. Cavuoto P, McAinch AJ, Hatzinikolas G, Janovska` A, Game P, Wittert GA. The expression of receptors for endocannabinoids in human and rodent skeletal muscle. Biochem Biophys Res Comm 2007;364:105–110.

18. Janiak P, Poirier B, Bidouard JP, Cadrouveille C, Pierre F, Gouraud L, Barbosa I, Dedio J, Mafrand JP, Le Fur G, O’Connor S, Herbert JM. Blockade of cannabinoid CB1 receptor improves renal function, metabolic profile, and increased survival of obese Zucker rats. Kidney Int 2007;72:1345–1357.

19. Dunn SR, Qi Z, Bottiger EP, Breyer MD, Sharma K. Utility of endogenous creatinine clearance as a measure of renal function in mice. Kidney Int 2004;65:1959–1967.

20. Schmid H, Henger A, Cohen CD, Frach K, Grone HJ, Schlondorff D, Kretzler M. Gene expression profiles of podocyte-associated molecules as diagnostic markers in acquired proteinuric diseases. J Am Soc Nephrol 2003;14:2958–2966.

21. Weibel ER. Stereological methods. In: Practical Methods for Biological Morphometry. London, U.K., Academic, 1979, p. 51–57.

22. Pagtalunan ME, Rasch R, Rennke HG, Meyer TW. Morphometric analysis of effects of angiotensin II on glomerular structure in rats. Am J Physiol 1995;268:F82–F88.

23. Deegens JK, Dijkman HB, Borm GF, Steenbergen EJ, van den Berg JG, Mathes CM, Ferrara M, Rowland NE. Selection of a palatable dietary option is not preferentially reduced by cannabinoid CB1 receptor antagonist AM251 in female C57Bl/6J mice. Pharmacol Biochem Behav 2009;94:119–123.

24. de Filippis F, Iuvone T, d’Amico A, Esposito G, Steardo L, Herman AG, Pelckmans PA, de Winter BY, de Man JR. Effect of cannabidiol on sepsis-induced motility disturbances in mice: involvement of CB receptors and fatty acid amide hydrolase. Neurogastroenterol Motil 2008;20:919–927.

25. Nogueiras R, Veyrat-Durebex C, Suchanek PM, Klein M, Tschop J, Caldwell C, Woods SC, Wittmann G, Watanabe M, Lipsitz S, Fekete C, Reizes O, Rohner-Jeanrenaud F, Tschop MH. Peripheral, but not central, CB1 antagonism provides food intake-independent metabolic benefits in diet-induced obese rats. Diabetes 2008;57:2977–2991.

26. Serrano A, Del Arco I, Javier Pavín F, Macías M, Perez-Valero V, Rodrı´guez de Fonseca F. The cannabinoid CB1 receptor antagonist SR141716 (Rimonabant) enhances the metabolic benefits of long-term treatment with olceylthanolamide in Zucker rats. Neuropharmacology 2008;54:226–234.

27. Vickers SP, Webster LJ, Wyatt A, Dourish CT, Kennett GA. Preferential effects of the cannabinoid CB1 receptor antagonist SR141716, on food intake and body weight gain of obese (fa/fa) compared to lean Zucker rats. Psychopharmacology 2003;167:109–111.

28. Kalluri R. Proteinuria with and without renal glomerular podocyte efface- ment. J Am Soc Nephrol 2006;17:2383–2389.

29. Koziell A, Grech V, Hussain S, Lee G, Lenkkeri U, Tryggvason K, Scambler P. Genotype/phenotype correlations of NPHS1 and NPHS2 mutations in patients with diabetic nephropathy. Biochim Biophys Acta 2008;1782:295–302.

30. Lan R, Li Q, Fan P, Lin S, Fernando SR, McCallion D, Pertwee R, Matkivannis A. Structure-activity relationships of pyrazole derivatives as cannabinoid receptor antagonists. J Med Chem 1999;42:789–776.

31. Kalluri R. Proteinuria with and without renal glomerular podocyte effacement. Hum Mol Genet 2002;11:916–923.

32. Kalluri R. Proteinuria with and without renal glomerular podocyte effacement. Hum Mol Genet 2002;11:916–923.

33. Lan R, Li Q, Fan P, Lin S, Fernando SR, McCallion D, Pertwee R, Matkivannis A. Structure-activity relationships of pyrazole derivatives as cannabinoid receptor antagonists. J Med Chem 1999;42:789–776.