Cannabinoid receptor 1 is a major mediator of renal fibrosis

Article in Kidney International · March 2015
DOI: 10.1038/ki.2015.63 · Source: PubMed
Cannabinoid receptor 1 is a major mediator of renal fibrosis

Lola Lecru, Christophe Desterke, Stanislas Grassin-Delyle, Christos Chatziantoniou, Sophie Vandermeersch, Aurore Devocelle, Amelia Vernochet, Ninoslav Ivanovski, Catherine Ledent, Sophie Ferlicot, Meriem Dalia, Myriam Saïd, Séverine Beaudreuil, Bernard Charpentier, Aimé Vazquez, Julien Giron-Michel, Bruno Azzarone, Antoine Durrbach and Hélène François

© 2015 International Society of Nephrology

Chronic kidney disease, secondary to renal fibrogenesis, is a burden on public health. There is a need to explore new therapeutic pathways to reduce renal fibrogenesis. To study this, we used unilateral ureteral obstruction (UUO) in mice as an experimental model of renal fibrosis and microarray analysis to compare gene expression in fibrotic and normal kidneys. The cannabinoid receptor 1 (CB1) was among the most upregulated genes in mice, and the main endogenous CB1 ligand (2-arachidonoylglycerol) was significantly increased in the fibrotic kidney. Interestingly, CB1 expression was highly increased in kidney biopsies of patients with IgA nephropathy, diabetes, and acute interstitial nephritis. Both genetic and pharmacological knockout of CB1 induced a profound reduction in renal fibrosis during UUO. While CB2 is also involved in renal fibrogenesis, it did not potentiate the role of CB1. CB1 expression was significantly increased in myofibroblasts, the main effector cells in renal fibrogenesis, upon TGF-β1 stimulation. The decrease in renal fibrosis during CB1 blockade could be explained by a direct action on myofibroblasts. CB1 blockade reduced collagen expression in vitro. Rimonabant, a selective CB1 endocannabinoid receptor antagonist, modulated the macrophage infiltrate responsible for renal fibrosis in UUO through a decrease in monocyte chemoattractant protein-1 synthesis. Thus, CB1 has a major role in the activation of myofibroblasts and may be a new target for treating chronic kidney disease.

KEYWORDS: cannabinoid; chronic kidney disease; fibrosis; myofibroblast

Correspondence: Hélène François, Service de Néphrologie, Dialyse et Transplantation, Hôpital Bicêtre, 78 rue du Général Leclerc, 94275 Le Kremlin Bicêtre Cedex, France. E-mail: helene.francois@bct.aphp.fr

Received 3 July 2014; revised 13 January 2015; accepted 22 January 2015

Kidney International advance online publication, 11 March 2015; doi:10.1038/ki.2015.63
in renal hemodynamics as glomeruli were normal under electron microscopy. Recently, another study found a difference in mesangial expansion after CB1 blockade, with a decrease in profibrotic genes during the early stages of diabetic nephropathy in mice. So far, the role of CB1 in the development of overt nephropathy or in renal fibrogenesis has not been established.

Therefore, the objective of our study was to demonstrate that the cannabinoid receptors, and particularly CB1, represent an entirely new therapeutic pathway during renal fibrogenesis and act independently of their metabolic and renal hemodynamic effects.

**RESULTS**

**CB1 expression is increased during the UUO model of renal fibrosis**

We first compared the gene expression profile of three fibrotic kidneys with three undamaged contralateral kidneys in mice using the UUO model. We found many overexpressed genes in obstructed kidneys that are classically involved in renal fibrosis, such as Tgfβ, Mmp, and Timp. However, using the DAVID (Database for Annotation, Visualization and Integrated Discovery) bioinformatics resources, the Cnr1 gene, encoding for CB1, was found to be one of the most upregulated genes in fibrotic kidneys, as illustrated by heatmaps that showed hierarchical clustering of significant transcripts (Figure 1a). This was confirmed using real-time quantitative PCR (RT-qPCR) (Figure 1b).

Immunohistochemistry revealed that the CB1 receptor drastically increased during UUO and was expressed in the tubules, interstitium, and glomeruli. Indeed, no staining was found in the obstructed kidney of Cnr1−/− mice, used as negative control (Figure 1c). We performed double immunostainings in kidney cortex, using specific markers for T lymphocytes (CD3), macrophages (F4/80), and myofibroblasts (α-smooth muscle actin (α-SMA)). Under confocal microscopy, neither CD3+ lymphocytes nor F4/80+ macrophages expressed the CB1 receptor in obstructed kidneys. Nonetheless, we found coexpression of the CB1 receptor with the α-SMA marker in interstitial cells, indicating that CB1 was expressed in myofibroblasts in the kidney during UUO (Figure 2).

**Endocannabinoid synthesis is altered during UUO**

We quantified anandamide and 2-arachidonoylglycerol (2-AG), the main endogenous CB1 ligands within the kidney in mice during UUO. We found a significant increase of 2-AG (P<0.01), whereas anandamide decreased in UUO (Figure 1f). However, 2-AG is 50-fold more abundant than anandamide in obstructed kidneys for a similar CB1 affinity (Supplementary Figure S1 online); therefore, we found a global increase in CB1 ligands during UUO. Using our microarray analysis, we also found that the transcription of genes involved in endocannabinoid synthesis was significantly altered during UUO (Supplementary Figure S2a–c online).

**CB1 expression is induced in human nephropathies**

We next studied CB1 expression in normal and diseased kidneys in human renal biopsies. We found increased expression of CB1 in acute interstitial nephritis (n = 5), IgA nephropathy (n = 5), and diabetic nephropathy (n = 6) compared with a low level of CB1 expression in normal kidneys, mostly in endothelial cells (n = 5). In injured kidneys, CB1 expression was found in tubules, interstitial cells, and podocytes in diabetes nephropathy, and in mesangial cells in IgA nephropathy (Figure 3a–c). In addition, CB1 expression correlates with kidney function (P<0.01) (Supplementary Figure S3 online).

**Both CB1 genetic invalidation and CB1 pharmacological blockade reduced fibrosis in the UUO model**

We investigated the effect of CB1 deficiency on the development of renal fibrosis in the UUO model using genetic disruption of the CB1 gene (Cnr1) and specific pharmacological blockade of CB1. Histomorphometric analytical software was used to quantify renal fibrosis within an entire kidney cross-section with Sirius red (Figure 4a–c).

After 8 days of UUO, as expected, we found a significant increase in collagen in obstructed compared with nonobstructed kidneys. The obstructed kidneys of Cnr1−/− mice showed a 33% reduction in collagen accumulation compared with wild-type mice (Figure 4b). Similarly, rimonabant-treated mice had a 65% reduction in fibrosis compared with vehicle-treated mice, using two different doses of rimonabant (Figure 4d). We also found a 25–60% reduction in fibrosis in AM6545 compared with vehicle-treated mice (Figure 4e).

Our results were confirmed by quantification of collagen III by WB (Figure 4f) and of collagen I and III mRNA using RT-qPCR (Figure 4g).

**CB2 is not involved in the CB1 blockade–dependent antifibrotic effect during UUO**

Because CB1 and CB2 may exert opposite physiological effects during liver fibrosis, we investigated CB2 expression during UUO in CB1 blockade. We found a significant increase in Cnr2 (encoding the CB2 receptor) mRNA after UUO and during pharmacological and genetic CB1 blockade (Figure 5a). This result was confirmed in immunohistochemistry (Supplementary Figure S4 online).

To investigate whether the CB2 pathway was involved in the antifibrogenetic effect conferred by CB1 invalidation during UUO, we used a specific CB2 antagonist (SR144528) and an agonist (JWH133). Neither of these treatments further reduced the development of fibrosis when compared with the CB1 pharmacological blockade. However, the CB2 antagonist alone aggravated the development of fibrosis. In addition, the CB2 agonist alone blunted the development of fibrosis during UUO (Figure 5b).

**Number of myofibroblasts is not altered by blockade of CB1 during UUO**

We next studied the expression of mesenchymal markers in the kidney cortex during CB1 blockade. Mesenchymal cell
Figure 1 | Cnr1 mRNA expression in the renal cortex is increased after 8 days of unilateral ureteral obstruction (UUO). (a) Heatmaps illustrating hierarchical clustering of significant transcripts expressed in UUO, for three wild-type (WT) mice nonobstructed (NO) and contralateral-obstructed (O) kidneys. Colors represent the level of gene expression relative to the corresponding control level and median centered for each gene, with green, black, and red corresponding to lower, equal, and higher expression, respectively. (b) Expression of Cnr1 mRNA evaluated by real-time quantitative PCR (RT-qPCR). *P < 0.01 versus nonobstructed kidneys (n = 7, for each group). Data are mean ± s.e.m. (n = 6–11). AU, arbitrary units. (c) Immunostaining for cannabinoid receptor 1 (CB1) in mouse kidneys. Bar scales = 100 μm. (d) Localization of positive staining in the kidney cortex. (e) Western blot analysis of CB1 receptor expression. (f) Concentrations of anandamide and 2-arachidonoylglycerol (2-AG) quantified in kidney and brain tissues by high-performance liquid chromatography (HPLC) analysis (232 ± 8 ng/μg of tissue in obstructed kidneys vs. 396 ± 16 ng/μg of tissue in nonobstructed kidneys for anandamide and 14,497 ± 2234 ng/μg of tissue in obstructed kidneys vs. 6543 ± 686 ng/μg of tissue in non-obstructed kidneys for 2-AG, n = 8 for each group, *P < 0.05). Data are mean ± s.e.m.
markers reflect myofibroblast number in the cortex, although specific markers are lacking: indeed, these cells are highly plastic by nature.7 Quantitative analysis showed that neither a specific α-SMA nor S100A4 immunostaining was significantly different during CB1 genetic disruption or CB1 pharmacological blockade (Figure 6a–d). This suggests that the number of myofibroblasts that express these markers was not modified by the CB1 blockade.

Expression of TGF-β1 is not modified by a deficiency of CB1
Transforming growth factor-β1 (TGF) is indisputably one of the key drivers of fibrosis. We quantified TGF-β1 and TGFBI (transforming growth factor-β-induced) mRNA expression during UUO. TGFBI is a target gene of TGF-β1 and correlates with TGF-β1 activity in renal tissue.8 We found their expressions to be significantly greater during UUO. However, this increase was not modified by genetic or pharmacological invalidation of CB1 (Figure 7a and b).

The role of the CB1 blockade in reducing renal fibrosis involves direct action on renal myofibroblasts
We next studied the role of CB1 in collagen synthesis using a primary cell culture of myofibroblasts in vitro. Indeed, we hypothesized that CB1 acts downstream of myofibroblast differentiation. We found weak basal myofibroblast expression of Cnr1 using RT-qPCR analysis and protein expression in western blotting. CB1 expression was notably increased after TGF-β1 treatment (Figure 8a and b). In addition, we found a significant amount of anandamide in culture supernatant that decreases after CB1 pharmacological blockade (Figure 8c). We found increased Col3a1 (encoding for Collagen III) expression after stimulation of myofibroblasts by TGF-β1 that was significantly blunted by rimonabant (Figure 8d). Similarly, Col3a1 expression remained low and stable upon TGF-β1 stimulation in Cnr1−/− myofibroblasts (Figure 8d).

We also analyzed the effect of CB1 antagonism on monocyte chemoattractant protein-1 (MCP-1) synthesis, a major
Figure 3 | Cannabinoid receptor-1 (CB1) expression is increased in multiple human nephropathies. (a) Immunostaining for the CB1 receptor in control kidneys, IgA nephropathy, acute interstitial nephritis (AIN), or diabetic nephropathy. (b) Localization and intensity of positive areas. (c) Quantification of positive areas by histomorphometry. *$p < 0.05$, **$p < 0.05$, ***$p < 0.05$ versus a control kidney. Data are mean ± s.e.m. ($n = 6$ for diabetic nephropathy, $n = 5$ for the other groups). Bar scales = 100 μm.
Both cannabinoid receptor 1 (CB1) genetic disruption and pharmacological blockade reduce fibrosis during unilateral ureteral obstruction (UUO). Representative sections, stained with Sirius red, of kidney (original magnification ×10) from (a) nonobstructed and obstructed kidneys from Cnr1−/− and wild-type (WT) mice after 8 days of UUO, and (c) of nonobstructed and obstructed kidneys from rimonabant-treated and vehicle-treated mice after 8 days of UUO. KO, knockout. (b) Quantification of fibrosis in the renal cortex by morphometry. Genetic disruption of the CB1 receptor reduced renal fibrosis during UUO: *P < 0.05 versus nonobstructed kidneys from WT mice (12.90 ± 0.94% in WT mice vs. 8.50 ± 0.81% in Cnr1−/− mice), **P < 0.05 versus nonobstructed kidneys from Cnr1−/− mice, and #P < 0.01 versus obstructed kidneys from WT mice. (d) Pharmacological blockade by 10 or 50 mg/kg/day of rimonabant reduced renal fibrosis during UUO. *P < 0.05 versus nonobstructed kidneys from vehicle-treated mice, **P < 0.05 versus nonobstructed kidneys from rimonabant (10 mg/kg/day)-treated mice, ***P < 0.05 versus nonobstructed kidneys from rimonabant (50 mg/kg/day)-treated mice, #P < 0.01 and ##P < 0.01 versus obstructed kidneys from vehicle-treated mice (9.62 ± 1.82% in vehicle-treated compared with 4.25 ± 0.61% and 4.28 ± 0.48% in rimonabant-treated mice at 10 or 50 mg/kg/day, respectively). (e) Pharmacological blockade by 10 or 20 mg/kg/day of AM6545 peripherally limited selective antagonism and reduced renal fibrosis. *P < 0.05 versus nonobstructed kidneys from vehicle-treated mice, **P < 0.05 versus nonobstructed kidneys from mice treated with AM6545 at 10 mg/kg/day, ***P < 0.05 versus nonobstructed kidneys from mice treated with AM6545 at 20 mg/kg/day, *P < 0.01 and **P < 0.01 versus obstructed kidneys from vehicle-treated mice (9.04 ± 0.75% in vehicle-treated vs. 6.77 ± 0.38% and 5.33 ± 0.72% in AM6545-treated mice, 10 or 20 mg/kg/day, respectively). (f) Western blot analysis of collagen III protein in kidney cortexes. Bar charts represent quantifications of the target protein expression normalized to β-actin. (g) Expression of Col1a2 and Col3a1 in obstructed kidneys evaluated by real-time quantitative PCR (RT-qPCR). *P < 0.05 and *P < 0.05 versus Cnr1−/− mice, **P < 0.05 and ***P < 0.05 versus rimonabant-treated mice. Data are mean ± s.e.m. (n = 11 for Cnr1−/− mice and n = 6 for WT mice, n = 7 for all other groups). AU, arbitrary units. Bar scales = 100 μm.
Inflammation is a key process during UUO and triggers the first steps of renal fibrogenesis.9 Because we found decreased MCP-1 synthesis or in epithelial to mesenchymal transition (Supplementary Figure S6 online).

We performed RT-qPCR analysis on selected M1- and M2-associated markers (Figure 9c–g) in mRNA from the kidney cortex. Both M1 (iNOS, MCP-1, and IL-23) and M2 (Arg1 and Mrc1) markers were increased in obstructed kidneys. M1, but not M2, markers were reduced by rimonabant treatment during UUO compared with vehicle-treated mice.

These results regarding macrophage infiltration during rimonabant treatment were not found in Cnr1−/− mice or during CB1 blockade using AM6545. As expected, kidney cortex MCP-1 mRNA was not modified during UUO in both Cnr1−/− and AM6545-treated mice (Supplementary Figure S5 online). Thus, rimonabant may act on macrophages through noncanonical pathways.

DISCUSSION

Our study provides strong evidence, for the first time, for a major role of CB1 in the development of renal fibrosis: we have provided data from several human nephropathies, as well as from the UUO model in mice.

The fact that the expression of Cnr1 was found among the most upregulated genes in our microarray analysis during UUO was a first clue to its possible involvement in renal fibrogenesis. In addition, we found a very high increase in 2-AG, the most abundant CB1 ligand, during UUO. Moreover, CB1 expression is highly upregulated during UUO in the kidney tubules and podocytes, but also, more interestingly, in interstitial myofibroblasts. We found no expression of CB1 in immune-infiltrating cells in the kidney during UUO, in agreement with the literature on mice.10 The prominent expression of CB1 in renal myofibroblasts strongly suggests a major role for the cannabinoid system in the development of renal fibrosis. We also found, for the first time, an important increase in CB1 expression in human nephropathies that seem to correlate with kidney function, whereas CB1 expression in normal kidneys was very low, as previously reported.4 We have also demonstrated, for the first time, that both genetic invalidation of Cnr1 and CB1 pharmacological blockade drastically decreased the development of fibrosis in mice during UUO, proving the role of CB1 in the development of renal fibrosis. Moreover, genetic disruption and the CB1 pharmacological blockade were performed on two different genetic backgrounds, and this makes the role of CB1 in renal fibrogenesis even more relevant, as mice phenotypes are highly dependent on genetic background.11,12 We reproduced the reduction in renal fibrosis during UUO using both rimonabant and a peripherally restricted CB1 antagonist AM6545. This is an important result, as the use of

Macrophage infiltration during UUO is modulated by rimonabant but not by CB1 blockade

Inflammation is a key process during UUO and triggers the first steps of renal fibrogenesis.9 Because we found decreased MCP-1 synthesis in rimonabant-treated myofibroblasts,
rimonabant in humans is limited because of its adverse effects on the central nervous system (it was withdrawn from the market in 2008 (see Chorvat) despite its positive results on metabolic syndrome). Therefore, AM6545 could be used without the drawback of having adverse effects on the central nervous system. The proportional decrease in renal fibrosis during both genetic and pharmacological blockade of CB1 is extremely important, and it is comparable to that usually found during the blockade of the renin–angiotensin system during UUO.14 This also suggests that CB1 may be a major pathway involved in fibrogenesis, as it is in other organs in metabolic15 and nonmetabolic diseases,14,15,16 although the complete UUO model may not completely be transferable into human pathology.

Because CB1 is mostly implicated in the regulation of food intake and glucose homeostasis,17 targeting CB1 with a pharmacological blockade has been previously investigated in studies on diabetes and obesity.2,3 In addition to reducing body weight and metabolic parameters, the CB1 blockade decreases albuminuria during metabolic syndrome and during the early stages of diabetic nephropathies in mice and rats,3,5 possibly through both hemodynamic effects and a direct action on the podocyte itself.18 However, in diabetic mice, kidney lesions were absent5 or faint.18 Indeed, so far, none of the experimental models conducted on diabetic nephropathies in mice have been fully able to explore the overt renal fibrogenesis induced by diabetes in human diabetic kidneys.11

Many reports suggest that CB2, which is mainly expressed in immune cells and less so in the central nervous system, may also be involved in the development of fibrosis. Contrary to CB1, CB2 disruption or pharmacological blockade has been reported to promote liver, cardiac, and skin fibrosis, whereas CB2 agonists decrease fibrogenesis.6,19,20 Therefore, the blockade of renal fibrosis with CB1 invalidation during UUO may have been caused by the unbalanced actions of CB2; we also found increased expression of CB2. Interestingly, treatment with the CB2 agonist reduced fibrosis during UUO, whereas the CB2 antagonist aggraviated renal lesions. In addition, the role of CB2 agonists in decreasing renal fibrosis has not been published (as yet), although the CB2 pathway has a role in decreasing albuminuria during diabetes.21

We also found that the CB2 antagonist aggravates renal fibrosis during UUO. This result is in accordance with the

Figure 6 | Expression of myofibroblast markers in Cnr1−/− and rimonabant-treated mice with obstructed kidneys. Genetic disruption and pharmacological blockade do not modify the mesenchymal markers in the renal cortex during unilateral ureteral obstruction (UUO). (a, b) Immunostaining for α-smooth muscle actin (α-SMA) and S100A4 in the renal cortex at 8 days after UUO in wild-type (WT), Cnr1−/−, vehicle- and rimonabant-treated mice, and vehicle- and AM6545-treated mice. (c, d) Quantification of the positive area by histomorphometry. Data are mean ± s.e.m. (n = 11 for Cnr1−/− mice and n = 6 for WT mice, n = 7 for all other groups). KO, knockout. Bar scales = 100 μm.
work by Barutta et al.,22 who found direct participation of podocytes expressing CB2 during diabetic nephropathy in CB2 knockout mice. Moreover, renal fibrosis was reduced in a similar proportion during rimonabant treatment alone and during the association of rimonabant with a specific CB2 antagonist. Therefore, although the CB2 pathway alone seems to be involved in renal fibrosis, CB2 signaling is not necessary for mediating the antifibrotic effect of the CB1 pharmacological blockade. Similarly, adding a CB2 agonist during rimonabant treatment did not further decrease the development of renal fibrosis during UUO, and this rules out any additive or potentialization effect in the kidney, at least in our model.

As our report provides solid evidence for the major implications of CB1 in renal fibrosis, we also attempted to decipher the exact mechanisms involved in reducing renal fibrosis. First, we explored the TGF-β1 pathway. TGF-β1 promotes myofibroblast activation or differentiation7,23 and is a major profibrotic cytokine in renal fibrosis, particularly in the UUO model.24 We did not find any difference in TGF-β1 expression or in TGF-β1 target gene expression when CB1 was invalidated by either a pharmacological blockade or genetic disruption. This suggests that the activity of CB1 in our model took place downstream of TGF-β1, that is, on TGF-β1 target cells such as myofibroblasts.

Although its precise origin is still highly controversial,21,24 the myofibroblast remains the main effector cell during renal fibrogenesis. During UUO in mice, we were able to demonstrate that cells expressing interstitial α-SMA, that is, myofibroblasts, also strongly expressed CB1. However, we were unable to demonstrate a decrease in cells that expressed α-SMA or S100A4 in the CB1 blockade during UUO. Myofibroblasts are highly plastic cells, and not all myofibroblasts express these markers, although they may still be involved in collagen synthesis.8 Nevertheless, it is more likely that the CB1 blockade does not alter myofibroblast differentiation in vivo. We next used a primary cell culture of kidney myofibroblasts and found that rimonabant blunted the increased Col3 expression induced by TGF-β1. Similarly, we found no increase in collagen Col3 synthesis in Cnr1−/− myofibroblasts upon TGF-β1 stimulation. Thus, CB1 is necessary for extracellular matrix protein synthesis in renal fibrosis, although its absence does not seem to alter myofibroblast differentiation in vivo. This result is expected given that undifferentiated myofibroblasts express very weakly, if not at all, the CB1 receptor. Although CB1 is expressed in tubules in UUO, it does not seem to have a major role in epithelial to mesenchymal transition in vitro.

Inflammation is a key mediator of renal fibrogenesis in the UUO model, and renal fibrosis can be blunted when macrophage and T-cell recruitment is reduced.25,26 It is still controversial whether there is significant CB1 expression in immune mouse cells, as some reports show weak27 or no expression.10 However, we found no significant expression of CB1 in CD3 lymphocytes or F4/80 macrophages during UUO. Nonetheless, there was a decrease in MCP-1 secretion by activated myofibroblasts in vitro during the CB1 blockade by rimonabant, and also a decreased number of M1-type macrophages during UUO, similar to that reported in the literature during atherosclerosis.2 This could also account for the decreased fibrosis by rimonabant, as the role of M1-type macrophages promote renal fibrosis.28 However, this is most likely a noncanonical effect of rimonabant as macrophage infiltration and MCP-1 transcription are not altered during AM6545 treatment or in Cnr1−/− mice in kidney cortex in vivo. Therefore, the antifibrotic effect of rimonabant could also act through other receptors. Moreover, during CB1 blockade, endocannabinoids, which we found increased during UUO, could bind to receptors other than CB2, such as TRPV1. This hypothesis is in accordance with the work by Wang and Wang29 who found that TRPV1−/− mice have exaggerated renal damage in deoxycorticosterone acetate–salt hypertension. Overall, our work demonstrates that the main mechanism by which CB1 promoted renal fibrogenesis was its direct action on renal myofibroblast function. Thus, CB1 may be a major player in renal fibrosis, regardless of the initial renal injury: thus, it has promising therapeutic potential, as well as a good cardiovascular profile.
Figure 8 | The role of cannabinoid receptor 1 (CB1) blockade in reducing renal fibrosis involves a direct action on renal myofibroblasts. Data are mean ± s.e.m. (a) Relative quantification of Cnr1 expression in the primary culture of mouse kidney myofibroblasts. *P < 0.05, **P < 0.05 versus basal myofibroblasts. AU, arbitrary units. (b) Transforming growth factor-β1 (TGF-β1) increases CB1 receptor expression (60 kDa) in renal myofibroblasts after 48 h of treatment. Protein expression was evaluated by western blot analysis. As expected, CB1 receptor expression was not detected in myofibroblasts isolated from Cnr1−/− obstructed kidneys. (c) Quantification of anandamide in primary myofibroblast supernatant, isolated from C57BL/6 or Cnr1−/− mice. *P < 0.05 versus myofibroblasts treated with TGF-β1 (5 ng/ml, 1 h). 2-arachidonoylglycerol (2-AG) was not detectable (<2 ng/ml). DMSO, dimethyl sulfoxide. (d) Relative quantification of Col3a1 expression in the primary culture of mouse kidney myofibroblasts. *P < 0.05 versus basal fibroblasts + DMSO, **P < 0.05 versus fibroblasts + TGF-β1 5 ng/ml 1 h + DMSO. Col3a1 expression was not significantly modified by TGF-β1 treatment with or without rimonabant on primary myofibroblasts isolated from Cnr1−/− mice. (e) CB1 pharmacological blockade reduces monocyte chemoattractant protein-1 (MCP-1) synthesis in renal myofibroblasts after 48, 72, and 96 h of TGFβ1 treatment. MCP-1 was quantified by enzyme-linked immunosorbant assay (ELISA). Experiments were repeated three times. Results are normalized over the mean for each experiment. *P < 0.05 versus control myofibroblasts not treated with TGF-β1, #P < 0.05 versus myofibroblasts treated with rimonabant, ##P < 0.05 versus myofibroblasts treated with AM6545.
**Materials and Methods**

**Microarray analyses**

The hybridization procedure was performed according to the Agilent 60-mer oligo microarray processing protocol using an Agilent Gene Expression Hybridization Kit (Agilent Technologies, Santa Clara, CA). Agilent Feature Extraction Software was used to read out and process the microarray image files. Genes were considered to be differentially expressed if the Significance Microarray Analysis algorithm passed a false discovery rate of <0.01 and a fold change of >2. Functional analyses of the data were performed using the DAVID (The Database for Annotation, Visualization and Integrated Discovery) (v6.7) bioinformatics resource. Microarray data were deposited in GEO DataSets under accession number GSE38117. See Supplementary Materials and Methods online.

**Drugs**

Rimonabant was a gift from Sanofi-Aventis R&D (Montpellier, France) and AM6545 was purchased from Sigma-Aldrich (Saint Louis, MO). JWH133 (Tocris Bioscience, Bristol, UK) and SR144528 (Cayman Chemicals, Ann Arbor, MI) were prepared in phosphate-buffered saline containing 10% dimethyl sulfoxide and 0.1% Tween-80. JWH133 and SR144528 were administered at doses of 1 mg/kg/day subcutaneously and 3 mg/kg/day intraperitoneally, respectively. All K_i values are given in Supplementary Figure S1 online.

**Animals**

Mice, genetically invalidated for CB1 (Cnr1^{−/−}) and wild-type mice (Cnr1^{+/+}) (CD1 background) were obtained from Dr C Ledent (Université Libre de Bruxelles, Brussels, Belgium) and generated as previously described (also see Supplementary Materials and Methods online). All mice were housed under standardized conditions with a 12-h dark/light cycle, and unlimited access to food and water. All animal procedures were approved by the animal ethics advisory committee of Paris Sud University.

Figure 9 | Characterization of kidney cortex infiltration during unilateral ureteral obstruction (UUO). Rimonabant-treated mice showed reduced infiltration of macrophages in the renal cortex at 8 days after UUO. (a) Immunostaining for CD3 and F4/80 in the renal cortex at 8 days after UUO. (b) Quantification of the positive area by histomorphometry. *P<0.05 versus obstructed kidneys from vehicle-treated mice. Relative quantification for (c) inducible nitric oxide synthase (iNOS): *P<0.05 versus nonobstructed kidneys from vehicle-treated mice, **P<0.01 versus nonobstructed kidneys from rimonabant-treated mice. Relative quantification for (d) interleukin-23 (IL-23): **P<0.05 obstructed kidneys from vehicle-treated mice versus rimonabant-treated mice, ***P<0.05 obstructed kidneys from vehicle-treated mice versus rimonabant-treated mice. Relative quantification for (e) monocyte chemoattractant protein-1 (MCP-1): *P<0.01 versus nonobstructed kidneys from vehicle-treated mice, **P<0.01 versus nonobstructed kidneys from rimonabant-treated mice, ***P<0.05 obstructed kidneys from vehicle-treated mice versus rimonabant-treated mice. Relative quantification for (f) Arg1; *P<0.01 versus nonobstructed kidneys from vehicle-treated mice, **P<0.01 versus nonobstructed kidneys from rimonabant-treated mice. Relative quantification for (g) Mrc1; *P<0.01 versus nonobstructed kidneys from vehicle-treated mice, **P<0.01 versus nonobstructed kidneys from rimonabant-treated mice. Data are means ± s.e.m. (n = 7). AU, arbitrary units. Bar scales = 100 μm.
Endocannabinoid quantification

Endocannabinoids were quantified in kidneys, brain, and myofibroblast culture supernatants with liquid chromatography coupled to Fourier transform mass spectrometry (see Supplementary Materials and Methods online).

Unilateral ureteral obstruction

After giving general anesthesia (isoflurane 2%), the left ureter was exposed via a mid-abdominal incision. UUO was performed with double silk sutures by complete ligation of the left ureter. The contralateral kidney served as an intraindividual control. Animals were killed on day 8 after treatment.

Histopathological analysis of renal fibrosis

Kidneys were fixed in 10% formalin and embedded in paraffin. Tissue sections (4 μm) were stained with Picosirius red. Renal cortex fibrosis was quantified using computer-based morphogenic analysis software (TRIBVN CaloPix, Châtillon, France) in a blinded manner. The total kidney cortex of an entire kidney sagittal cross-section was selected for quantification from each mouse, representing ~7 mm² surface area. The positive area was expressed as a percentage of the total cortical section.

Immunohistochemistry

Paraffin-embedded kidney tissues were stained with an anti-α-SMA (Abcam, Cambridge, UK), anti-CD3 (DakoCytomation, Glostrup, Denmark), anti-S100A4 (Abcam, Cambridge, UK), anti-F4/80 (Abcam), and anti-CB1 and CB2 (Abcam). Staining was quantified by a morphometry software.

Fluorescent immunostaining was revealed with anti-rabbit (Alexa Fluor 488, Jackson Immunoresearch, West Grove, PA) or anti-rat antibodies (CyLight 549, Jackson Immunoresearch). The slides were examined by confocal laser microscopy (Leica TCS SP5 AOBS Tandem Confocal System, Wetzlar, Germany). See Supplementary Materials and Methods online.

Human kidney specimens

Paraffin sections of renal biopsies from patients were retrospectively analyzed. Informed written consent was given by the patients for the use of part of the biopsy for scientific purposes. All procedures and the use of tissues were performed in accordance with the Declaration of Helsinki principles.

Real-time quantitative PCR (RT-qPCR)

RNA was extracted from tissues or cells using a QIAGEN RNeasy Mini kit, according to the manufacturer’s instructions (QIAGEN, Venlo, Netherlands). The cdNA was synthesized using a Revert Aid H minus First-Strand DNA Synthesis kit (Fermentas, Villebon-sur-Yvette, France) and amplified by PCR with a LightCycler 480 (Roche Diagnostics, Meylan, France) and analyzed. Informed written consent was given by the patients for the use of part of the biopsy for scientific purposes. All procedures and the use of tissues were performed in accordance with the Declaration of Helsinki principles.

Cell culture

Primary cultures of mouse kidney myofibroblasts were isolated from C57/Bl6 mice, as previously described. See Supplementary Materials and Methods online. HK-2 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). See Supplementary Materials and Methods online.

Enzyme-linked immunosorbant assay

The quantitative determination of mouse MCP-1 concentrations in primary myofibroblast supernatants was performed using a Quantikine enzyme-linked immunosorbant assay CCL2/MCP-1 kit (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions.

Western blotting

Cells were lysed in NP-40 buffer containing protease inhibitors (Roche, Mannheim, Germany). Proteins, separated in a 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, were transferred to a polyvinylidene fluoride membrane and then incubated with primary antibodies. See Supplementary Materials and Methods online.

Statistical analyses

We compared differences using the Mann–Whitney U-test or the paired Student’s t-test, as appropriate. All analyses were performed using GraphPad Prism 5.0 software (La Jolla, CA). Data were considered statistically significant when the P-value was <0.05. All data are expressed as mean ± s.e.m.

DISCLOSURE

All the authors declared no competing interests.

ACKNOWLEDGMENTS

We thank Aurélie Perier, Séverine Lecourt, and Djenaba Ba for their technical support and scientific advice, and Xavier Biolchini from the...
animal facility. This work was partially supported by Sanofi-Aventis, NRBLainc Le Cancer, and Institut Francilien de Recherche en Néphrologie et Transplantation. Rimonabant was a gift from Sanofi-Aventis.

SUPPLEMENTARY MATERIAL

Figure S1. Comparative table of the different KI of CB1 and CB2 ligands, pharmacological agonists and antagonists.

Figure S2. Microarray analysis of the expression of genes involved in the regulation of anandamide and 2-AG synthesis by unsupervised multivariate methods.

Figure S3. Linear regression analysis of serum creatinine and CB1 expression quantified by immunohistochemistry (IgA nephropathy, AIN or diabetic nephropathy, normal kidneys n=18, p<0.01, Pearson).

Figure S4. Immunostaining of CB2 receptors in mouse kidney showing positive staining mostly in the interstitium (myofibroblasts and immune cells), compared to a low level in glomeruli (mainly podocytes) and tubules in obstructed kidneys.

Figure S5. Infiltration of T lymphocytes and macrophages in obstructed kidneys is not modified neither by genetic disruption nor by AM6545 treatment.

Figure S6. (A) Quantification of tubular dilation during UUO.

Figure S7. Recapitulative scheme illustrating the hypothetic role of CB1 and CB2 receptors during renal fibrosis induced by UUO. Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

REFERENCES


