Targeted inhibition of the type 2 cannabinoid receptor is a novel approach to reduce renal fibrosis

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The cannabinoid receptor type 2 (CB2) is a G protein-coupled seven transmembrane receptor that transmits endogenous cannabinoid signaling. The role of CB2 in the pathogenesis of kidney injury and fibrosis remains poorly understood. Here we demonstrate that CB2 was induced, predominantly in kidney tubular epithelium, in various models of kidney disease induced by unilateral ureteral obstruction, adriamycin or ischemia/reperfusion injury. *In vitro,* forced expression of CB2 or treatment with a CB2 agonist was sufficient to trigger matrix gene expression, whereas knockdown of CB2 by siRNA abolished transforming growth factor-β1–induced signaling and fibrogenic responses in kidney tubular cells. CB2 also mediated fibroblasts and macrophage activation *in vitro*. Mice with genetic ablation of CB2 were protected against kidney injury after ureteral obstruction, validating a pathogenic role of CB2 in renal fibrosis *in vivo*. By using *in silico* screening and medicinal chemistry modifications, we discovered a novel compound, XL-001, that bound to CB2 with high affinity and selectivity and acted as an inverse agonist. Incubation with XL-001 inhibited in a dose-dependent fashion the fibrogenic response induced by CB2 overexpression, CB2 agonist or transforming growth factor-β1. *In vivo,* intraperitoneal injections of XL-001 ameliorated kidney injury, fibrosis and inflammation in both the obstruction and ischemia/reperfusion models. Delayed administration of XL-001 was also effective in ameliorating kidney fibrosis and inflammation. Thus, CB2 is a pathogenic mediator in kidney fibrosis and targeted inhibition with the novel inverse agonist XL-001 may provide a strategy in the fight against fibrotic kidney diseases.

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Kidney fibrosis is a hallmark and major outcome of virtually all kinds of progressive chronic kidney disease (CKD).[1–3] Numerous epidemiological studies have shown that the prevalence of CKD in the general population is high and reaches to 13% in some countries.[4–6] In this context, CKD is increasingly becoming a public health problem worldwide.[7] Despite its high prevalence and severe morbidity and mortality, there is currently no effective therapy that can completely halt or reverse renal fibrogenesis and the progression of CKD.[8] This enormous unmet medical need calls for more studies and better understanding of the pathomechanism of kidney fibrosis, ultimately enabling one to identify new and effective targets for a therapeutic intervention of CKD.

The endocannabinoid system is an essential signaling scheme that regulates a diverse array of biological processes including memory, appetite, energy metabolism, and immunity.[9–11] Endocannabinoids, such as anandamide and 2-arachidonoyl glycerol, are endogenous lipid ligands, which can bind to two Gprotein–coupled seven transmembrane cannabinoid receptors, namely CB1 and CB2.[12] Ligand-CB1 or -CB2 receptor engagement triggers a cascade of intracellular signal activation, leading to specific gene expression and various cellular responses. Earlier studies demonstrate that CB1 receptor is mainly expressed in the central and peripheral nervous systems, whereas CB2 is primarily produced by cells of hematopoietic origin including monocytes, macrophages

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or both.[13,14] However, recent findings point out that both CB1 and CB2 are widely expressed in a variety of organs, particularly in the pathologic conditions.[15,16] Such a wide expression pattern of CB receptors suggests a much broader spectrum of their biologic activities than originally thought. Indeed, both CB1 and CB2 have been implicated in regulating injury repair, inflammation, and fibrosis in several organs including liver, lung, and kidney after various insults.[17–19]

In the kidney, both CB1 and CB2 receptors are expressed and may play a role in the pathogenesis of kidney disorders.[20,21] Studies show that the abuse of synthetic cannabinoids is linked to acute kidney injury, as well as tubulointerstitial lesions.[22,23] Targeted inhibition of CB1 is known to protect podocyte integrity and reduce kidney fibrosis after chronic injury.[19,24] Although the role of CB1 activation in mediating kidney injury is well established,[16,19,25,26] the function of renal CB2 is inconsistent and controversial.[27,28] Some studies show that CB2 expression is down-regulated in the kidney of diabetic nephropathy,[29] whereas others demonstrate an induction of CB2 mRNA and protein after unilateral ureteral obstruction (UUO).[27] Activation of CB2 by agonists has been shown to protect against albuminuria in diabetic nephropathy and alleviates renal fibrosis.[19,26] However, CB2 activation also augments immune cell influx, aggravates inflammation, and modulates α-smooth muscle actin (α-SMA)-positive myofibroblasts in fibrotic repair.[30,31] Hence, the potential role of CB2 in renal fibrosis remains to be defined by using more comprehensive approaches.

In this study, we have systematically investigated the role of CB2 in renal fibrogenesis by using *in vitro* and *in vivo* models and by taking both genetic and pharmacologic approaches. More importantly, we have discovered a novel, specific, and highly selective CB2 inverse agonist, which blocked CB2 actions. Our results indicate that CB2 activation plays an important role in mediating kidney fibrosis and inflammation and identify this pathway as a potential therapeutic target.

RESULTS

CB2 is up-regulated in various models of CKD

We first examined the expression and regulation of CB2 in the pathogenesis of kidney fibrosis. To this end, we used 3 mouse models of CKD induced by UUO, adriamycin (ADR), or ischemia-reperfusion injury (IRI), respectively. These models are widely utilized and represent different etiologies leading to renal fibrosis.[32–34] As shown in Figure 1a and b, quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) revealed that both CB1 and CB2 mRNA were significantly up-regulated in the obstructed kidneys at 7 days after UUO, compared with CB1 and CB2 mRNA levels in sham control kidneys. We next sought to determine the cellular source of CB2 protein expression in fibrotic kidneys by using immunohistochemical staining. As shown in Figure 1c, whereas CB2 protein was hardly detectable in sham control kidneys, it was markedly up-regulated in the obstructed kidneys after UUO. The expression of CB2 was predominantly in renal tubular epithelium (Figure 1c, arrow). Notably, some interstitial cells were also stained positively for CB2 after obstructive injury (Figure 1c, arrowhead). To quantitatively determine the relative abundance of CB2 protein, we carried out Western blot analyses of whole kidney lysates. As shown in Figure 1d and e, CB2 protein was induced about 8-fold in the obstructed kidneys at 7 days after UUO, compared to sham control kidneys. Similar results were obtained when renal CB2 levels were examined at 3 weeks after ADR injection (Figure 1f and g) or at 11 days after unilateral IRI (Figure 1h and i). These data indicate that CB2 induction is a common pathologic feature in the fibrotic kidneys after various injuries.

CB2 promotes fibrogenic responses *in vitro*

To investigate the functional role of CB2 in renal fibrosis, we employed a gain-of-function approach by forced expression of CB2 in kidney tubular epithelial cells. To this end, human proximal tubular epithelial cells (HKC-8) were transiently transfected with CB2 expression vector (pCMV-CB2) or empty vector (pcDNA3). As shown in Figure 2a and b, overexpression of CB2 induced fibronectin expression in cultured HKC-8 cells, suggesting a fibrogenic action of the CB2 receptor in kidney cells.

We next examined the potential upstream regulator of CB2 in the fibrotic kidneys. As transforming growth factor β1 (TGF-β1) is a well-characterized profibrotic cytokine that is induced in virtually all forms of CKD,[35] we investigated whether CB2 expression is regulated by TGF-β1 using an *in vitro* system. As shown in Figure 2c and d, TGF-β1 significantly induced CB2 expression in a dose-dependent manner, suggesting a potential role for TGF-β1 in mediating CB2 induction in fibrotic kidneys.

Given that TGF-β1 induces CB2 expression, we then explored whether CB2 is required for the profibrotic action of TGF-β1 in kidney epithelial cells. Therefore, we knocked down the expression of CB2 by using a small, interfering RNA (siRNA) strategy. As illustrated in Figure 2e through g, HKC-8 cells were transfected with control or CB2-specific siRNA in the presence of TGF-β1. Efficient knockdown of the CB2 expression by siRNA was confirmed in whole-cell lysates (Figure 2e). Notably, depletion of CB2 in HKC-8 cells inhibited the expression of fibronectin and α-SMA by TGF-β1 (Figure 2e–g). Similar results were obtained when fibronectin deposition was assessed by immunofluorescence staining (Figure 2h).

We then examined the role of CB2 in TGF-β1 signaling. As shown in Figure 2i through m, knockdown of CB2 hampered TGF-β1-mediated phosphorylation (p) and activation of Smad3, extracellular signal–regulated kinase (ERK), p38 mitogen-activated protein kinase, and c-Jun N-terminal kinase in HKC-8 cells. These results suggest CB2 is also required for TGF-β1 signaling.

CB2 mediates interstitial fibroblast and macrophage activation *in vitro*

To investigate the role of CB2 in renal interstitial cells, we employed an *in vitro* system using cultured normal rat kidney