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Role for Cannabinoid Receptors in Human Proximal Tubular Hypertrophy

Kayte A. Jenkin, Andrew J. McAinch, Esther Grinfeld and Deanne H. Hryciw

School of Biomedical and Health Sciences, Victoria University, St Albans Campus, Melbourne

Key Words
Proximal tubule • Kidney • Cannabinoid receptors • Hypertrophy

Abstract
Endogenous endocannabinoids bind to cannabinoid receptors; namely CB1, CB2, TRPV1 and GPR55, to activate intracellular pathways that control many cellular functions. Elevated levels of endocannabinoids have been identified in diseases such as obesity and diabetes, with the onset of diabetic nephropathy associated with proximal tubule hypertrophy. Recent research has identified a role for CB1 in apoptosis in human proximal tubular (HK2) cells, however the role of the other receptors has not been investigated. We investigated if the cannabinoid receptors played a role in hypertrophy in HK2 cells. Characterisation of HK2 cells demonstrated that mRNA and protein for CB1, CB2, TRPV1 and GPR55 occurs in these cells. Importantly, activation of the cannabinoid receptors with anandamide significantly increases hypertrophy in HK2 cells. In general, treatment with CB1 antagonist AM-251, reduces hypertrophy while treatment with CB2 (AM-630) and TRPV1 (SB-366791) antagonists increases hypertrophy. Targeting a cannabinoid receptor sensitive to O-1918 in HK2 cells did not alter proximal tubule cell hypertrophy. Therefore it is likely that in human proximal tubule, these receptors regulate cellular function by activating different cell signalling pathways. Nonetheless, we have identified a role for cannabinoid receptors in proximal tubule cells which may provide novel therapeutic targets for the treatment of diabetes and obesity.

Introduction

In the human, the endocannabinoid system consists of endogenous cannabinoids (endocannabinoids), the cannabinoid receptors and the enzymes required for the synthesis and degradation of the endocannabinoids [1]. The two most common endocannabinoids, anandamide (AEA) and 2-arachidonoyl-glycerol (2-AG) are synthesized on demand from arachidonic acid in the cell

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Dr Deanne Skelly, PhD, Grad Cert (Higher Ed), Senior Lecturer
School of Biomedical and Health Sciences, Victoria University
PO Box 14428, Melbourne, Vic 8001 (Australia)
Tel. +61 3 9919 2312, Fax +61 3 9919 2465
E-Mail Deanne.Skelly@vu.edu.au

membrane in a tissue specific manner and have rapid agonistic effects on cannabinoid receptors via autocrine or paracrine mediated pathways [2]. These endogenous endocannabinoids bind to a small number of identified receptors including Cannabinoid receptor 1 (CB1) and Cannabinoid receptor 2 (CB2) [1], Transient receptor potential cation channel subfamily V member 1: TRPV1 [3] and the putative cannabinoid receptor GPR55 [4]. CB1 and CB2 are 7 transmembrane domain G protein-linked receptors. CB1 which was thought initially only to be expressed centrally, is now also recognised to be expressed peripherally in tissues such as adipocytes, liver, and skeletal muscle. In the hypothalamus and nucleus accumbens, the CB1 receptors are considered to mediate the effects of endocannabinoids on food intake [5]. CB2 previously demonstrated to be almost exclusively expressed in cells of the immune system [6] has now also been identified in other tissues including skeletal muscle [7]. TRPV1 is a ligand-gated cation channel that is activated by extreme heat as well as by various ligands [3]. TRPV1 is localized primarily in sensory neurons [3]; however it has been identified also in the cortex and medulla of the kidney [8]. Activation of TRPV1 leads to cellular depolarisation and increased intracellular calcium [9]. GPR55 is also a G-protein-coupled receptor, and is highly expressed in the brain [10] as well as breast, adipose tissues, testis, and the spleen [11]. Cannabinoid receptors can initiate a cascade effect involving mitogen-activated protein kinases, a pathway that regulates gene expression, differentiation, and apoptosis [12].

Overactivity of the endocannabinoid system can increase the accumulation fat and reduce glucose uptake, both of which increase the risk of insulin resistance and impaired glucose tolerance associated with Type 2 diabetes (T2DM) [13]. In addition, an increased level of endocannabinoids has been observed in individuals with obesity [14]. Pathophysiological changes in the kidney associated with obesity and T2DM include the early onset of glomerulomegaly, haemodynamic changes of a hyperfiltering kidney, and increased albuminuria [15]. Importantly, the pathogenesis of diabetic nephropathy is dependant upon changes in the renal tubular cells that leads to proximal tubule hypertrophy [16]. Cellular changes that control this dysfunction are poorly understood.

Previous research has suggested that *in vivo*, proximal tubule hypertrophy may be mediated by CB1. One study has investigated the role of CB1 in an obese model by treating obese Zucker rats with Rimonabant, a CB1 inverse agonist [17]. Blockage of CB1 resulted in a reduction in glomerular and tubulointerstitial lesions, reduced renal tubular hypertrophy, and a reversal of the proteinuria [17]. These authors suggested that the reversal in hypertrophy is due to a decrease in glomerular hyperfiltration [17]. Further, a recent study has demonstrated in a streptozotocin-induced diabetic mouse model, that treatment with the CB1 antagonist AM-251 reduced albuminuria in these mice, without altering other parameters such as body weight, blood glucose or blood pressure. [18]. These studies both attributed the changes in renal pathology to blockage of CB1 activity in the glomerulus [17, 18], however these studies failed to investigate the role the tubular system plays in this process. Therefore, the overall aim of the current study is to identify the cannabinoid receptors present in proximal tubular cells, and characterise the role of the cannabinoid receptors in tubular hypertrophy.

Materials and Methods

Rat lysate samples

Samples were obtained from Assoc. Professor Darren Kelly, St Vincent's Research Institute. Following approval from the Animal Ethics committee of the University of Melbourne, rodent kidney and brain tissue were obtained from male Sprague Dawley rats aged approx 5 months old. All fed a standard laboratory diet. Immediately after sacrifice, tissues were processed for isolation of RNA or protein.

Cell Culture

The human proximal tubule cell line (HK2) was a kind gift from Professor Daniel Markovich (University of Queensland) and was maintained in DMEM/F12 media (Invitrogen) supplemented with 100 U/ml penicillin, 100 g/ml streptomycin, 1 X ITS Liquid Media Supplement (Sigma), 10 ng/ml epidermal growth factor and 2 mM l-glutamine. Cells were maintained at 37°C, in 5% CO_2.

RNA extraction and RT-PCR analysis

RNA was extracted from rat brain, rat kidney, and HK2 cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions, as described previously [19]. Briefly, the RNA (1 μg) was treated with DNAse (Promega) and then reverse transcribed using the One-Step RT-PCR kit (Qiagen). CB1 and CB2 were amplified using primers described previously [7]. TRPV1 was amplified using forward primer 5' TAT TCC ACA TGT CTG GAG CT 3' and reverse primer 5' CCA GGA TGG TGA TGG CTC 3'. GPR55 was amplified using forward primer 5' ACA GCA CGT GGA GTG CGA GA 3' and reverse primer 5' CTG GGA GAA AGG AGA CCA CA 3'.

Protein extraction and Western blot analysis

Protein was isolated from rat kidney and HK2 cells as described previously [19]. Aliquots (50 μg) of the protein

samples were separated on a 4-15% SDS-PAGE gel and transferred to a nitrocellulose membrane. CB1, CB2 (Cayman Chemicals), TRPV1 (Santa Cruz) and GPR55 (Novus) were detected using specific antibodies, following protocols described previously [19].

Hypertrophy assay

Using previously published methods, the extent of cell hypertrophy was measured in HK2 cells exposed to 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) [20]. HK2 cells were treated with the global cannabinoid agonist anandamide (AEA: 0.1 μM and AEA 1 μM [21]), as well as antagonists of the various cannabinoid receptors (CB1: AM-251; 0.5 μM and 5 μM [22]; CB2: AM-630; 0.01 μM and 10 μM [23]; TRPV1: SB-366791; 0.01 μM and 10 μM [24]; a cannabinoid receptor, sensitive to O1918: O-1918; 1 μM and 30 μM [25]). Dose ranges utilised were similar to those employed in previously published literature [24, 26-30]. Treatment groups were compared to vehicle control (ethanol 0.02%).

Cells were incubated with the specific treatments for 2 hours at 37°C, then treated with 10 μl of MTT solution (Sigma) and left to incubate for a further 90 minutes at 37 °C. The MTT solution in each well was then removed and 100 μl of MTT solvent (Sigma) was added to each well. The absorbance was measured at 570 nm and 655 nm, using a FluroStar Galaxy Plate Reader (BMG LABTECH). In addition, the protein content was measured using a Lowry assay, as described previously [19]. Hypertrophy was indicated by the ratio of total protein content to cell number [20].

Statistical Analysis

Statistical analysis of data was conducted by ANOVA with a post-hoc Tukey test. Data were expressed as mean ± SEM (Standard Error of the Mean), and significance was considered at $P< 0.05$.

Results

Expression of cannabinoid receptors in kidney and HK2 cells

Using RT-PCR analysis, mRNA for the four cannabinoid receptors; CB1, CB2, TRPV1 and GPR55 were present in rat kidney and HK2 proximal tubule cells (Fig. 1). Further, in negative control samples (RT minus) no PCR product was obtained. Western blot analysis of rat kidney and HK2 cell lysates demonstrated that protein for all cannabinoid receptors is present in kidney and HK2 proximal tubule cells (Fig. 2). This data is similar to previous research which demonstrated CB1 mRNA and protein in HK2 cells [31], however it also demonstrates that CB2, TRPV1 and GPR55 are also present, potentially indicating a significant role for all of the cannabinoid receptors in human proximal tubule cells.

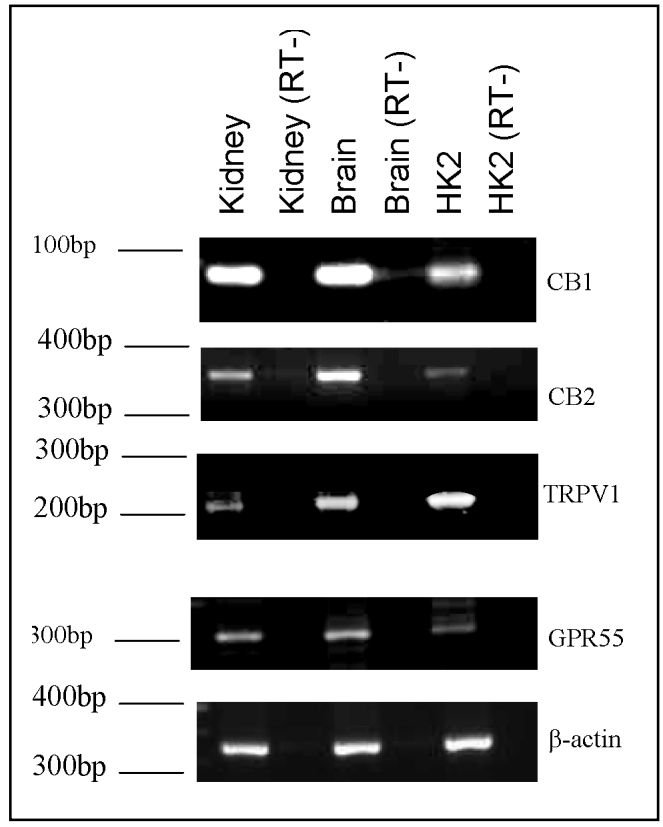


Fig. 1. mRNA was extracted from rat kidney, rat brain (positive control) and Human Kidney 2 (HK2) proximal tubule cells. mRNA was DNAse treated, and reverse transcribed. PCR using primers specific for CB1, CB2, TRPV1 and GPR55, the mRNA for all four receptors were isolated in kidney, brain and HK2 samples. Negative (RT minus) samples failed to detect the receptors.

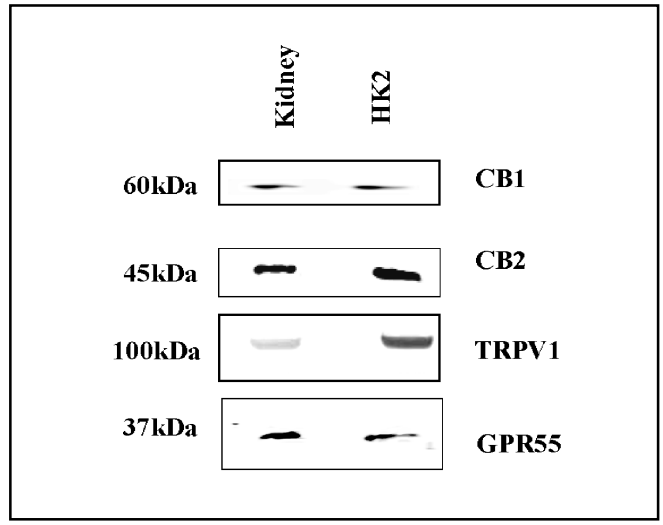
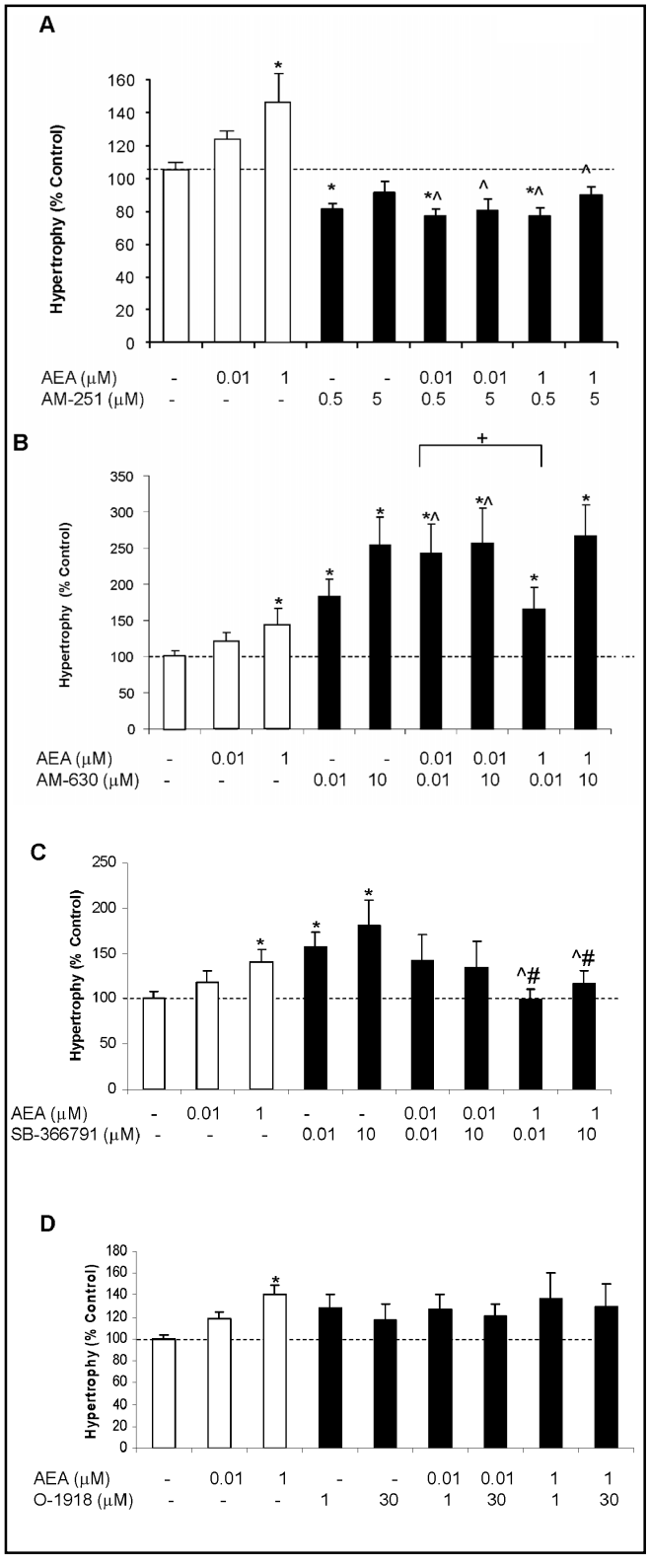


Fig. 2. Protein lysate was isolated from rat kidney and HK2 samples. Western blot analysis using antibodies specific for CB1, CB2, TRPV1 and GPR55 identified protein for all receptors in these samples.

Fig. 3. HK2 proximal tubule cells were treated with the global cannabinoid agonist anandamide (0.1 μM or 1 μM AEA) in the presence or absence of specific CB1, CB2 or TRPV1 antagonists or targeting a cannabinoid receptor sensitive to O-1918. In each graph, the representative data for cells treated with AEA or (vehicle) control has been included for an ease of comparison A: Treatment of HK2 proximal tubule cells with the global cannabinoid agonist anandamide (1 μM AEA) increased hypertrophy compared to vehicle control (*P < 0.05). In HK2 cells treated with CB1 antagonist (0.5 μM AM-251) hypertrophy was decreased, as was HK2 cells treated with 0.5 μM AM-251 and the two concentrations of AEA (*P < 0.05). Compared to AEA alone, hypertrophy in all combinations of AEA and AM-251 were significantly different (^P < 0.05). B: Compared to control, cell hypertrophy in HK2 cells treated with CB2 antagonist (AM-630) increased, as did HK2 cells treated with AM-630 and the two concentrations of AEA (*P < 0.05). Compared to AEA alone, HK2 cells treated with 0.1 μM AEA and both concentrations of AM-251 were significantly increased (^P < 0.05). Compared to cells treated with 0.1 μM AEA/ 0.1 μM AM-630, cells treated with 1 μM AEA/ 0.1 μM AM-630 showed a reduced hypertrophy (+P< 0.05). C: Compared to control, hypertrophy in HK2 cells treated with TRPV1 antagonist (SB-366791) increased (*P < 0.05). Compared to AEA alone and SB-366791 alone, hypertrophy in HK2 cells treated with 0.1 μM AEA and both concentrations of SB-367791 were significantly decreased (^P < 0.05:AEA and #P < 0.05:SB-366791). D: Treatment with the cannabinoid antagonist O-1918 in the presence or absence of AEA did not alter tubular hypertrophy.



Role of cannabinoid receptors in human proximal tubule cell hypertrophy

The exact role of the cannabinoid receptors in different cell types varies, with a number of different studies indicating that activation of cannabinoid receptors with the global agonist AEA *in vitro*, results in an increase in cell proliferation or the induction of apoptosis [1, 28, 32, 33]. Further, treatment of obese rats with a CB1 inverse antagonist ameliorates renal hypertrophy *in viv*o. Therefore, as renal hypertrophy is an early indicator of diabetic nephropathy [34], we investigated the role of the cannabinoid system in human proximal tubule cell hypertrophy. The endocannabinoid system is a complex signalling system that is poorly understood in general. Therefore, the concentration ranges for all agonists and antagonists were based on previously published literature in similar cell lines [24, 26-30]. HK2 cells exposed to 1μM AEA for 2 hrs demonstrated a significant increase in cell hypertrophy compared to vehicle control (*P < 0.05, n = 24, Fig. 3A), indicating that activation of the cannabinoid system results in cell hypertrophy in human proximal tubule cells. As these cells express all four cannabinoid receptors, we then investigated which receptors were functionally important in these cells. For ease of comparison, these data are repeated in Fig. 3B, 3C and 3D.

A recent study has indicated that treatment of HK2 cells with the CB1 antagonist AM-251 attenuates palmitic acid induced apoptosis [28]. Also, more significant for this study, treatment of obese rats with a CB1 inverse agonist, ameliorates renal hypertrophy. Thus indicating that blockage of CB1 plays a protective role for this receptor in proximal tubule cells [17]. In our study, cells treated with CB1 antagonist 0.5 μM AM-251 in the presence and absence of AEA demonstrated a significant reduction in hypertrophy compared to vehicle control (*P < 0.05, n =24, Fig. 3A). This concentration was similar to that used on HK2 cells in a recent publication [28]. Further, compared to cells treated with AEA alone, treatment with both the agonist and AM-251 significantly ameliorated renal hypertrophy (^P < 0.05, n = 24, Fig. 3A). Therefore, similar to studies *in vivo* [17], blockage of CB1 may play a role in the reduction of hypertrophy in human proximal tubule cells where the cannabinoid receptors are activated by AEA.

To investigate the role of CB2, TRPV1 and GPR55 in human proximal tubule cells we employed a similar protocol. Experiments using a number of cell lines have indicated a variety of dose ranges for AM-630 which is dependant on the cell lines used or particular assay measured [35]. Cells treated with the CB2 antagonist (0.01μM and 10μM AM-630) in the presence or absence of AEA (0.1 μM and 1 μM) had a significant increase in hypertrophy compared to vehicle control (*P< 0.05, n = 24, Fig. 3B). These concentrations are similar to those recently published where HEK-293 cells were transiently transfected with CB2 receptor [23, 36]. Significantly, even in the absence of AEA, treatment of HK2 cells with AM-630 increased hypertrophy compared to control (*P < 0.05, n = 24, Fig. 3B). Further, compared to AEA alone (0.1 μM), cells treated with AM-630 had a significant increase in hypertrophy (^P < 0.05, n = 24, Fig. 3B). This indicates that this receptor must protect the human proximal tubular cells from hypertrophic damage, even in the absence of AEA. However, activation of the receptors via AEA stimulation did not further increase hypertrophy compared to cells treated with AM-630 alone. Interestingly, compared to cells treated with 0.1 μM AEA and 0.1 μM AM-630, cells treated with 1 μM AEA and 0.1 μM AM-630 showed a reduced hypertrophy (+P< 0.05). As AEA has different affinities for different cannabinoid receptors, it is clear that treatment with a high dose of the agonist will affect multiple receptors. Thus the antagonist affects of AM-630 in the presence of low AEA may be the result of this compound acting on one receptor, whereas the high AEA concentrations may activate multiple receptors other than CB2, that may result in opposing effects on cellular hypertrophy. However, in general blocking CB2, even in the absence of activation of the other receptors, increases cell hypertrophy. Further, this indicates that this receptor may activate different cell signalling pathways compared to CB1.

Similar to CB2, human proximal tubular cells treated with TRPV1 antagonist (0.01 μM and 10 μM SB-366791) had a significant increase in hypertrophy compared to vehicle control (*P < 0.05, n = 24, Fig. 3C). Compared to cells treated with agonist alone (1 μM AEA: ^P < 0.05, n =24, Fig. 3C) or antagonist alone (0.01 μM or 10 μM SB-366791: #P < 0.05, n = 24, Fig. 3C), cells treated with 1 μM AEA and the different concentrations of SB-366791 had an increase in cell hypertrophy. Significantly, even in the absence of AEA, treatment of HK-2 cells with SB-366791 increased hypertrophy compared to control (*P < 0.05, n = 24, Fig. 3C). This indicates that this receptor must protect the human proximal tubular cells from hypertrophic damage, even in the absence of AEA. Interestingly, blocking TRPV1, when additional receptors are activated with AEA, reduces hypertrophy to levels not significantly different from control (Fig. 3C). Recent research has indicated that an interaction between CB1 and TRPV1 exists, with the receptors "cross-talking" [37]. These researchers have demonstrated that in neuronal cells, activation of the CB1 receptor maintains the TRPV1 channel in a sensitized responsive state, potentially through a phospholipase C-dependent process [37]. Therefore, activation of CB1 may alter the sensitivity of TRPV1 in human proximal tubule cells, potentially via intracellular signalling molecules.

At this time, there is limited understanding of the role of GPR55 in normal physiology. Recent research has indicated that O-1918 can act as an antagonist for the GPR55 receptor [38]. However, investigations by others [39] suggests that this is not the case, and that O-1918 may act on GPR18 [40]. Interestingly, cells treated with this antagonist (1 μM and 30 μM O-1918) had no significant change in hypertrophy compared to vehicle control, AEA alone or antagonist alone (n = 24, Fig. 3D). Therefore, a cannabinoid receptor sensitive to O-1918 has no effect on human proximal tubule cellular hypertrophy.

Discussion

The endocannabinoid system is an endogenous physiological system, with altered activity associated with obesity and T2DM [13]. Functional consequences of obesity and T2DM lead to chronic kidney disease [13]. We have demonstrated that one potential target, the cannabinoid system, plays a functional role in proximal tubule cells by demonstrating that the mRNA and protein for the four receptors is present in these cells. Further, activation of the system by the global agonist AEA induces proximal tubule hypertrophy. As treatment with CB1 antagonist reduced hypertrophy, while treatment with CB2 and TRPV1 antagonists increased hypertrophy, this indicates that these receptors regulate different signalling pathways ultimately regulating proximal tubule cell fate.

Limited investigations have failed to clearly identify a role for the cannabinoid receptors in the maintenance of normal renal physiology as well as their role in dysfunctional states such as disease. It has been demonstrated that the concentration of anandamide in the serum is significantly increased in mice as soon as 8 weeks following a high fat diet [14]. In addition, diabetic Streptozotocin (STZ) mouse and rat models demonstrate a role for CB1 in diabetic nephropathy [18]. Our findings have further clarified the role of the cannabinoid receptors in the kidney by demonstrating that CB1, CB2, TRPV1 and GPR55 are all expressed in human proximal tubule cells. This significant finding suggests that there is a wider distribution of the receptors in kidney that may account for the proteinuria associated with diabetes. Previous studies have suggested that the reversal of proteinuria is due to changes in the glomerular filtration rate (GFR), but proteinuria may also be due to a dysfunction in the protein handling pathway in the proximal tubule cells [41].Significantly, blocking CB1 *in vivo* reduces renal hypertrophy [18], and we demonstrate that this phenomenon also occurs *in vitro*. Further, we have shown that CB2 and TRPV1 are protective, and blocking these receptors increases cell hypertrophy. The hypertrophic effects that the cannabinoid receptors regulate that is described here were shown in an isolated cultured cell line, *in vitro*. This important observation does not rule out an *in vivo* effect on GFR and a subsequent effect of the increased GFR on tubular cell hypertrophy, albeit by a cellular process involving the receptors. Consequently, the very results of this study showing a differential effect on tubular hypertrophy by blocking the various receptors indicates an intricate role of these receptors that extends beyond what has been observed so far from *in vivo* studies [17] and points to the need of more thorough study of the *in vivo* effect of these novel receptors in renal tubules.

Importantly, the endocannabinoid system is a complex and promiscuous signalling system, especially with regards to pharmacological agonists and antagonists. For example, there is a degree of uncertainty in the literature regarding the possible agonist affects of AM-251 on GPR55 [4, 42, 43] in addition to its antagonistic affect on CB1. These discrepancies may be attributed to variations in the cell lines between laboratories [44]. This does not also address the issue that there may be a differing in response and signalling exhibited in different tissues [44]. While we can not rule out any interaction of the agonistic affect of GPR55 of in particular the high dose of AM-251 used in this study, it must be reiterated that the dose ranges utilised were similar to that in previously published literature [26, 27]. The use of AM-251 at this concentration in HK2 cells is also supported by a recent article Lim et al., [28] that reported a dose of 1 μM of AM-251 to decrease palmitic acid induced apoptosis via endoplasmic reticulum stress in HK2 cells, a similar result was also observed siRNA targeting CB1. Further, another study involving the treatment of a human proximal tubule cells (HK2) with anandamide has indicated that functional cannabinoid receptors must exist in these cells [22]. However, these authors failed to detect message for CB1 and CB2 in the HK2 cells [22]. These findings contradict a recent study that demonstrated CB1 mRNA and protein expression in HK2 cells [28] and our work which identified all four receptor mRNA and protein in HK2 cells. Clonal variability of immortalized cell lines or concentration differences may account for this variability.

We clearly demonstrate that treatment with anandamide resulted in an increase in proximal tubule cell hypertrophy. Therefore, this suggests that *in vivo*, elevated levels of anandamide could lead to renal hypertrophy associated with obesity and diabetes. Recently, a study [45] has shown that anandamide induced activation of cannabinoid receptors mediates the phosphorylation of mitogen activated protein kinases (MAPK). This could lead to activation of epidermal growth factor receptors (EGFR), resulting in an increased cell proliferation [46, 47] or hypertrophy associated with diabetes [48]. Therefore, future investigations should focus on the downstream signalling targets that are activated by anandamide stimulation in human proximal tubule cells.

In summary, we have clearly demonstrated that

CB1, CB2, TRPV1 and GPR55 receptors are expressed in human proximal tubule cells. Further, we have shown a functional role for CB1, CB2 and TRPV1 in maintaining tubular hypertrophy, via separate cell signalling pathways. As the endocannabinoid system is upregulated in obesity and diabetes, more detailed analysis of downstream targets may identify future therapeutic options for these diseases.

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