Long-term follow-up studies are needed to demonstrate the causal relationships between dyslipidemia and end-stage renal disease from diabetic nephropathy.

Treatment of dyslipidemia and diabetic nephropathy

With regard to the treatment of dyslipidemia in patients with diabetes, there were some interventional trials of anti-hypercholesterolemic agents including fibrates and statins.

The Diabetes Atherosclerosis Intervention Study (DAIS) is a randomized study that assessed the effect of fenofibrate on type 2 diabetic patients [29]. In this study, fenofibrate reduced the worsening of urine albumin excretion and the effects were mainly observed in the progression from normoalbuminuria to microalbuminuria. The Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) study also evaluated the effect of fenofibrate on type 2 diabetes [30]. From this study, it was proved that fenofibrate is effective in lowering the decline of the estimated glomerular filtration rate (eGFR) and reducing the progression of albuminuria. Additionally in this study, patients treated with fenofibrate had higher rates of regression of albuminuria than the placebo group. This evidence suggests that fenofibrate is effective in ameliorating diabetic nephropathy. In a meta-analysis of these two studies, the significant effect on the regression from microalbuminuria to normoalbuminuria was proved; however, progression from microalbuminuria to macroalbuminuria was not significant [31].

The effect of statins on diabetic nephropathy was examined in the Collaborative Atorvastatin Diabetes Study (CARDS) [32]. Treatment with atorvastatin was compared with a placebo in this study, and was associated with an improvement in annual changes in eGFR (0.18 mL/min/1.73 m^2/year). It is noteworthy that atorvastatin ameliorated eGFR without improving albuminuria, when comparing angiotensin-converting enzyme inhibitors which have renoprotective effects and prevent the onset of albuminuria [33].

There is still a lot of uncertainty about the effect of statins. The effect on renal protection was not demonstrated in the Study of Heart and Renal Protection (SHARP) which included 2,094 (33 %) patients with diabetes [34], and the Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial (ALLHAT) which included 3,638 (36 %) patients with diabetes [35]. A meta-analysis also showed that regression of albuminuria [31] and changes in eGFR [36] were not observed in patients with diabetes treated with statins.

There seems to be no definite answer for treatment of dyslipidemia in diabetic patients from the viewpoint of anti-hyperlipidemic agents. One of the supposed causes of inconsistency in results is that kidney diseases in patients with diabetes may not be uniform, but consist of many renal diseases [37]. In some cases, renal biopsies might be needed to assess the accurate risks [38].

Diabetic patients are at higher risk for cardiovascular mortality compared with non-diabetic patients [10, 39]. There is sufficient evidence, such as SHARP [34], to show that statins reduce the risk of cardiovascular events. Considering these facts, many diabetic patients might benefit from statin treatment. An increasing number of patients are now receiving this treatment. In the analysis of the National Health and Nutrition Examination Survey (NHANES) 2005–2006, 93.5 % of diabetic men aged 65–69 without cardiovascular disease received statins [40].

On the other hand, administration of statin may have adverse side-effects, including myopathy [41], renal toxicity [42], and incident diabetes [43]. A study comparing the risks and benefits of statins concluded that cardiovascular benefits outweigh the increased risk of new-onset diabetes [44]. It is beyond doubt that each patient's risk must be taken into account before administration of statins.

It is also important to consider changes in life-style; however, the difficulty lies in improving renal and cardiovascular events through life-style changes [45]. It remains a challenge for future research to examine the impact of life-style changes.

Concluding remarks and future directions

In considering the complexity of the problem of diabetic nephropathy, many aspects of a patient's condition and treatment should be taken into account. Further insight into the pathogenesis of dyslipidemia, and the risk and benefits of each treatment may be beneficial for each patient.

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Nuclear Hormone Receptor Expression in Mouse Kidney and Renal Cell Lines

Daisuke Ogawa[1,2]*, Jun Eguchi[1], Jun Wada[1], Naoto Terami[1], Takashi Hatanaka[1], Hiromi Tachibana[1], Atsuko Nakatsuka[1,2], Chikage Sato Horiguchi[1], Naoko Nishii[1], Hirofumi Makino[1]

1 Department of Medicine and Clinical Science, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan, 2 Department of Diabetic Nephropathy, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan

Abstract

Nuclear hormone receptors (NHRs) are transcription factors that regulate carbohydrate and lipid metabolism, immune responses, and inflammation. Although several NHRs, including peroxisome proliferator-activated receptor-γ (PPARγ) and PPARα, demonstrate a renoprotective effect in the context of diabetic nephropathy (DN), the expression and role of other NHRs in the kidney are still unrecognized. To investigate potential roles of NHRs in the biology of the kidney, we used quantitative real-time polymerase chain reaction to profile the expression of all 49 members of the mouse NHR superfamily in mouse kidney tissue (C57BL/6 and db/m), and cell lines of mesangial (MES13), podocyte (MPC), proximal tubular epithelial (mProx24) and collecting duct (mIMCD3) origins in both normal and high-glucose conditions. In C57BL/6 mouse kidney cells, hepatocyte nuclear factor 4α, chicken ovalbumin upstream promoter transcription factor II (COUP-TFII) and COUP-TFIII were highly expressed. During hyperglycemia, the expression of the NHR 4A subgroup including neuron-derived clone 77 (Nur77), nuclear receptor-related factor 1, and neuron-derived orphan receptor 1 significantly increased in diabetic C57BL/6 and db/db mice. In renal cell lines, PPARδ was highly expressed in mesangial and proximal tubular epithelial cells, while COUP-TFs were highly expressed in podocytes, proximal tubular epithelial cells, and collecting duct cells. High-glucose conditions increased the expression of Nur77 in mesangial and collecting duct cells, and liver x receptor α in podocytes. These data demonstrate NHR expression in mouse kidney cells and cultured renal cell lines and suggest potential therapeutic targets in the kidney for the treatment of DN.

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* E-mail: daiogawa@md.okayama-u.ac.jp

Introduction

Diabetic nephropathy (DN) is a major microvascular complication in patients with diabetes mellitus, ultimately leading to end-stage renal diseases [1]. The incidence of DN is increasing rapidly with the increase in patients with type 2 diabetes and metabolic syndrome, and at present accounts for almost 50% of all end-stage renal diseases [2]. It is characterized by the accumulation of extracellular matrix in the glomerular and tubulointerstitial compartments and by the thickening and hyalinization of intrarenal vasculature. Various pathogenic mechanisms of DN have been proposed including increased expression of advanced glycation end-products, protein kinase C, transforming growth factor β, and reactive oxygen species. In addition to these metabolic derangements, changes in the glomerular hemodynamics, modulated in part by local activation of the renin-angiotensin system, synergistically exacerbate the progression of DN. Despite notable advances in the treatment of diabetes mellitus, current therapies do not fully suppress the incidence of DN. Therefore, identification of additional causative factors leading to renal injury and the development of novel agents to prevent or treat DN are urgently needed.

In humans, 48 members of the nuclear hormone receptor (NHR) superfamily of transcription factors have been identified (49 in mice). Several modulators of NHRs have been developed as oral drugs for the treatment of diabetes and dyslipidemia. Synthetic agonists for peroxisome proliferator-activated receptor-γ (PPARγ) and PPARα, such as thiazolidinediones and fibrates, improve glycemic control in type 2 diabetic patients and lower serum triglyceride levels in hyperlipidemic patients [3]. Moreover, these drugs are reported to have protective effects against renal dysfunction [4,5,6]. However, no drugs that modulate NHRs, other than PPARγ and PPARα agonists, have been released in the market. In animal models of DN, several agonists of NHRs, including the vitamin D receptor (VDR) [7,8], the farnesoid X receptor (FXR) [9], and the estrogen receptor (ER) [10] have been reported to have potential suppressive effects on the progression of DN. We have also demonstrated that synthetic agonists of PPARδ [11] and the liver X receptor (LXR) [12] reduce urinary albumin excretion in a mouse model of DN. Moreover, hepatocyte nuclear factor α (HNF4α) [13] and estrogen-related receptor (ERR) [14] are also thought to have a role in the pathogenesis of DN.

There is increasing evidence of NHR expression in peripheral tissues including adipose tissue [15], macrophages [16], and

endocrine pancreas tissue [17], but little is known about the expression and function of NHRs in the kidney. To elucidate their potential role in the pathogenesis of DN, we have performed a comprehensive analysis of NHR expression in mouse kidney tissue and renal cell lines.

Materials and Methods

Animals

Eight-week-old male C57BL/6J mice were purchased from Charles River (Yokohama, Japan). Diabetes was induced by peritoneal injection of 200 mg/kg streptozotocin (Sigma-Aldrich, Tokyo, Japan) in citrate buffer (pH 4.5). C57BL/6J mice were euthanized at 8 weeks after the induction of diabetes. Six-week-old male diabetic *db/db* mice (BKS.Cg-*lepr*^{db}/*lepr*^{db}) and male non-diabetic *db/m* mice (BKS.Cg-*lepr*^{db}/+) were purchased from CLEA Japan (Tokyo, Japan). *Db/db* mice and *db/m* mice were euthanized at 9 weeks of age. All mice were maintained under a 12-h light/12-h dark cycle with free access to food and tap water. Animal care and procedures were performed according to the Guidelines for Animal Experimentation at Okayama University, the Japanese Government Animal Protection and Management Law, and the Japanese Government Notification on Feeding and Safekeeping of Animals. The experimental protocol was approved by the Animal Ethics Review Committee of Okayama University (OKU-2011326). All surgery was performed under sodium pentobarbital anesthesia, and every effort was made to minimize suffering.

Cell culture

Murine mesangial (MES13), podocyte (MPC), proximal tubular epithelial (mProx24), and collecting duct (mIMCD3) cells were cultured as previously described [18,19,20]. For high-glucose stimulation, renal cell lines were serum-starved in 0.5% fetal bovine serum for 24 h. Subsequently, all cells were exposed to low-glucose (5.5 mM) or high-glucose (25 mM) conditions for 24 h before RNA isolation.

RNA measurement

RNA was isolated from kidney cortex samples or cultured cells using an RNeasy Mini kit (Qiagen, Valencia, CA, USA). Single-strand cDNA was synthesized from the extracted RNA using a real-time polymerase chain reaction (RT-PCR) kit (Perkin Elmer, Foster City, CA, USA). To evaluate the mRNA expression of each NHR, quantitative RT-PCR (qPCR) was performed using TaqMan® Array Plates and StepOnePlus™ (Applied Biosystems, Foster City, CA, USA) and TaqMan® Fast Universal PCR Master Mix (Applied Biosystems). Primers were purchased from Applied Biosystems. Each sample was analyzed in quadruplicate and normalized for S18 mRNA expression. Primer sequences for mouse genes are provided in Table S1.

Immunohistochemistry

Immunofluorescent staining was performed as described previously [21]. The expression of VDR in cultured renal cells was detected using rat anti-VDR antibody (Abcam, Cambridge, UK) followed by Alexa Fluor 488 goat anti-rat IgG (Invitrogen, Carlsbad, CA). Similarly, PPARδ and COUP-TFII were detected using rabbit anti-PPARδ (Affinity Bioreagents, Golden, CO) and anti-COUP-TFII antibody (Abcam) followed by Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen). Renal expression of NOR1 was detected using mouse anti-NOR1 antibody (Abcam) followed by Alexa Fluor 488 goat anti-mouse IgG (Invitrogen). To determine whether NOR1 was localized in mesangial cells, podocytes, proximal tubular epithelial cells, or collecting duct cells, the sections were counter-stained with rabbit anti-fibronectin antibody (Sigma-Aldrich, St. Louis, MO), rabbit anti-WT-1 antibody (Abcam, Cambridge, UK), rabbit anti-aquaporin 1 (AQP1) antibody (Millipore, Temecula, CA), or rabbit anti-AQP2 antibody (Abcam) respectively, followed by Alexa Fluor 594 goat anti-rabbit IgG (Invitrogen). Fluorescence images were obtained using a fluorescence microscope (BX51; Olympus, Tokyo, Japan).

Results

The expression of NHRs in mouse kidney tissue (C57/BL6 and db/m) and renal (mesangial, podocyte, proximal tubular epithelial, and collecting duct) cell lines was determined by qPCR analysis.

The composition and rank order of NHR expression in C57/BL6 mouse kidney are shown in Figures 1 and 2A, respectively. We then analyzed NHR expression in db/m mice and renal cell lines (Figs. 2B–F, 3, 4, and 5). This format is consistent with previous NHR expression analyses and provides a basis for comparison with cell lines commonly associated with each receptor type. Furthermore, because this survey focused on expression in a select few cell types (mouse kidney, renal cell lines), we could analyze RNA levels for all receptors in a single assay for direct comparison of NHR levels within a given tissue. Finally, we elucidated the effect of elevated glucose on NHR expression in mouse kidney and renal cell lines (Fig. 6, 7, 8, and 9).

NHR expression in kidney

The composition of NHR expression in C57/BL6 mouse kidney is shown in Figure 1. Receptors were deemed to be expressed if cycle threshold (Ct) values were less than 31. Composite gene expression analysis of the NHR superfamily revealed the presence of 25 of the 49 known NHRs in C57BL/6 mouse kidney. These included six members of the endocrine receptor family, which are activated by high-affinity hormonal lipids, eight adopted hetero-dimeric orphan receptors, which are regulated by low-affinity dietary lipids, and 11 true orphan receptors (Fig. 1A). Figure 1B shows a complete tabulation of the expressed and unexpressed receptors along with their classification and nomenclature [22]. Six NHRs including FXRβ, liver receptor homolog-1 (LRH1), photoreceptor cell-specific nuclear receptor (PNR), retinoid X receptor γ (RXRγ), steroidogenic factor 1 (SF1), and Tailles homolog orphan receptor (TLX) were not detected in normal C57BL/6 mouse kidney.

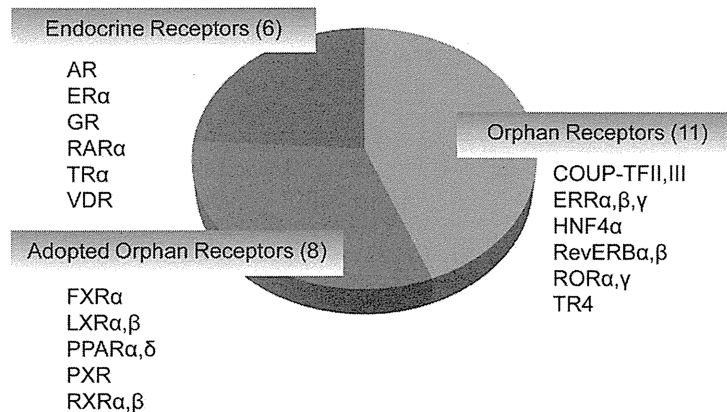
Rank order of NHR expression in mouse kidney tissue and renal cell lines

The relative mRNA levels of NHRs expressed in C57BL/6 mouse kidney are shown in rank order in Figure 2A. For the purposes of comparison, similar analyses were conducted on db/m mouse kidney and renal cell lines (mesangial cell, MES13; podocytes, MPC; proximal tubular epithelial, mProx24, and collecting duct, mIMCD3) (Fig. 2B–F).

In C57BL/6 mouse kidney, the most abundant receptor was HNF4α and other abundant receptors were chicken ovalbumin upstream promoter transcription factor II (COUP-TFII) and COUP-TFIII, VDR, PPARα, FXRβ, and ERRα. In contrast, COUP-TFII was the most abundant NHR in db/m mouse kidney, and the mRNA levels of HNF4α, COUP-TFIII, and VDR were lower in db/m mice than in C57BL/6 mice.

A similar survey of NHR transcripts in renal cell lines revealed significant differences compared with the receptor expression pattern observed in mouse kidney. COUP-TFII was the most abundant NHR in the mProx24 and mIMCD3 cell lines, and the second most abundant receptor in the MPC cell line. In contrast,

A



B

Endocrine Receptors	Adopted Orphan Receptors	Orphan Receptors
Expressed	**Expressed**	**Expressed**
AR (NR3C4) ERα (NR3A1) GR (NR3C1) RARα (NR1B1) TRα (NR1A1) VDR (NR1I1)	FXRα (NR1H4) LXRα,β (NR1H3,1H2) PPARα,δ (NR1C1,1C2) PXR (NR1I2) RXRα,β (NR2B1,2B2)	COUP-TFII,III (NR2F2,2F3) ERRα,β,γ (NR3B1,3B2,3B3) HNF4α (NR2A1) RevERBα,β (NR1D1,1D2) RORα,γ (NR1F1,1F3) TR4 (NR2C2)
Unexpressed	**Unexpressed**	**Unexpressed**
ERβ (NR3A2) MR (NR3C2) PR (NR3C3) RARβ,γ (NR1B2,1B3) TRβ (NR1A2)	CAR (NR1I4) FXRβ (NR1H5) PPARγ (NR1C3) RXRγ (NR2B3)	COUP-TFI (NR2F1) DAX (NR0B1) GCNF (NR6A1) HNF4γ (NR2A2) LRH-1 (NR5A2) Nur77 (NR4A1) NOR1 (NR4A3) NURR1 (NR4A2) PNR (NR2E3) RORβ (NR1F2) SF-1 (NR5A1) SHP (NR0B2) TLX (NR2E1) TR2 (NR2C2)

Figure 1. Composition of nuclear hormone receptors (NHRs) in the kidney of C57BL/6 mice. (A) Twenty-five of 49 known NHRs are expressed in C57BL/6 mouse kidney. These include six endocrine receptors that bind hormonal lipids with high-affinity, eight adopted orphan receptors that bind dietary lipids with low-affinity, and 11 orphan receptors. Constituent receptors of each of these classes are listed. (B) Tabular listing of NHRs expressed or unexpressed in C57BL/6 mouse kidney along with their classification and nomenclature. Receptors were deemed unexpressed if cycle threshold (Ct) values exceeded 31.
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PPARδ was the most highly expressed receptor in the MES13 cell line, and the second most abundant NHR in the mProx24 cell line. In addition to PPARδ, thyroid hormone receptor α (TRα), COUP-TFII, COUP-TFIII, glucocorticoid receptor (GR), RXRα, and LXRβ were found to be abundant receptors in the MES13 cell line. In the MPC cell line, COUP-TFII, COUP-TFIII, RXRα, LXRβ, TRα, and GR were highly expressed, while COUP-TFII, PPARδ, COUP-TFIII, and TRα were abundant in the mProx24 cell line. In the mIMCD3 cell line, the expression pattern of NHRs was similar to that in the MPC cell line. Furthermore, retinoic acid receptor α (RARα), reverse-ErbAβ (Rev-ERBβ), and PPARδ were highly expressed NHRs in the mIMCD3 cell line.

Endocrine receptors in renal cell lines

The expression of endocrine receptors was compared for all renal cell lines (Fig. 3A). Androgen receptor (AR), RARα, and VDR were highly expressed NHRs in the mProx24 cell line. In contrast, AR and VDR were hardly expressed in the MPC and mIMCD3 cell lines. ERα, GR, and TRα were abundant in both the mProx24 and MES13 cell lines. Immunohistochemistry of VDR showed that the intensity of VDR was higher in the mProx24 cell line than in the MES13 cell line (Fig. 3B).

Adopted orphan receptor expression in renal cell lines

The expression of adopted orphan receptors in the renal cell lines was also compared (Fig. 4A). LXRα and PPARα were highly expressed in the MES13 cell line, while LXRβ and PPARδ were

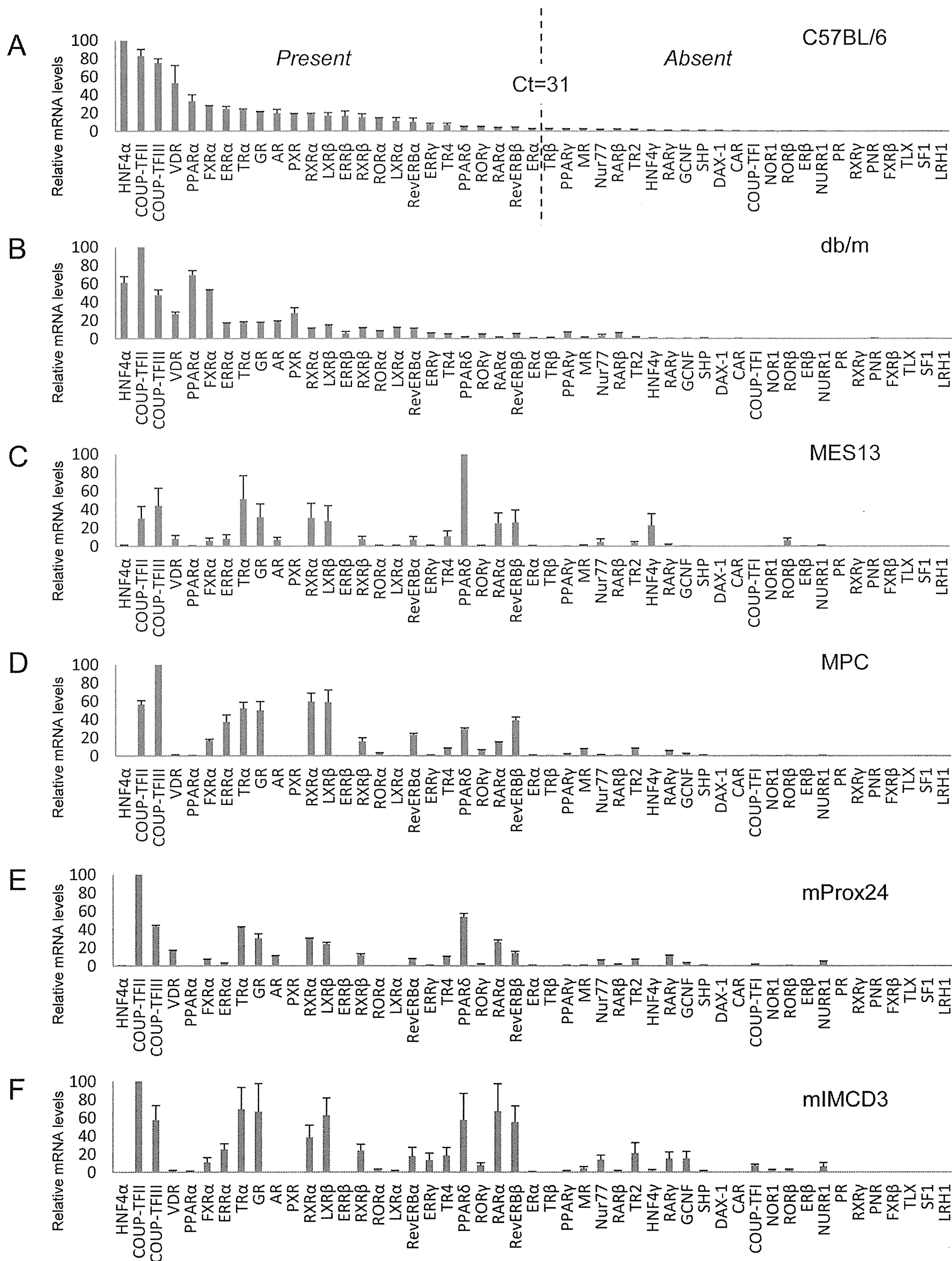

Figure 2. Comparative expression levels of 49 nuclear hormone receptors (NHRs) in mouse kidney tissue and renal cell lines. The relative mRNA levels are depicted for mouse kidney (C57BL/6 (A) and db/m (B)) and mesangial (MES13) (C), podocyte (MPC) (D), proximal tubular epithelial (mProx24) (E) and collecting duct (mIMCD3) (F) cell lines. All values are expressed relative to 18S and arithmetically adjusted to depict the highest-expressed NHR for each tissue/cell line as a unit of 100. Values represent the means ± SEM of three independent samples of each tissue or cell line. Setting arbitrary cutoffs at Ct<31 (present) or Ct>31 (absent), as shown by broken lines in the C57BL/6 mouse kidney panel, reveals that 25 NHRs were expressed and six NHRs were not detected in C57BL/6 mouse kidney.
doi:10.1371/journal.pone.0085594.g002

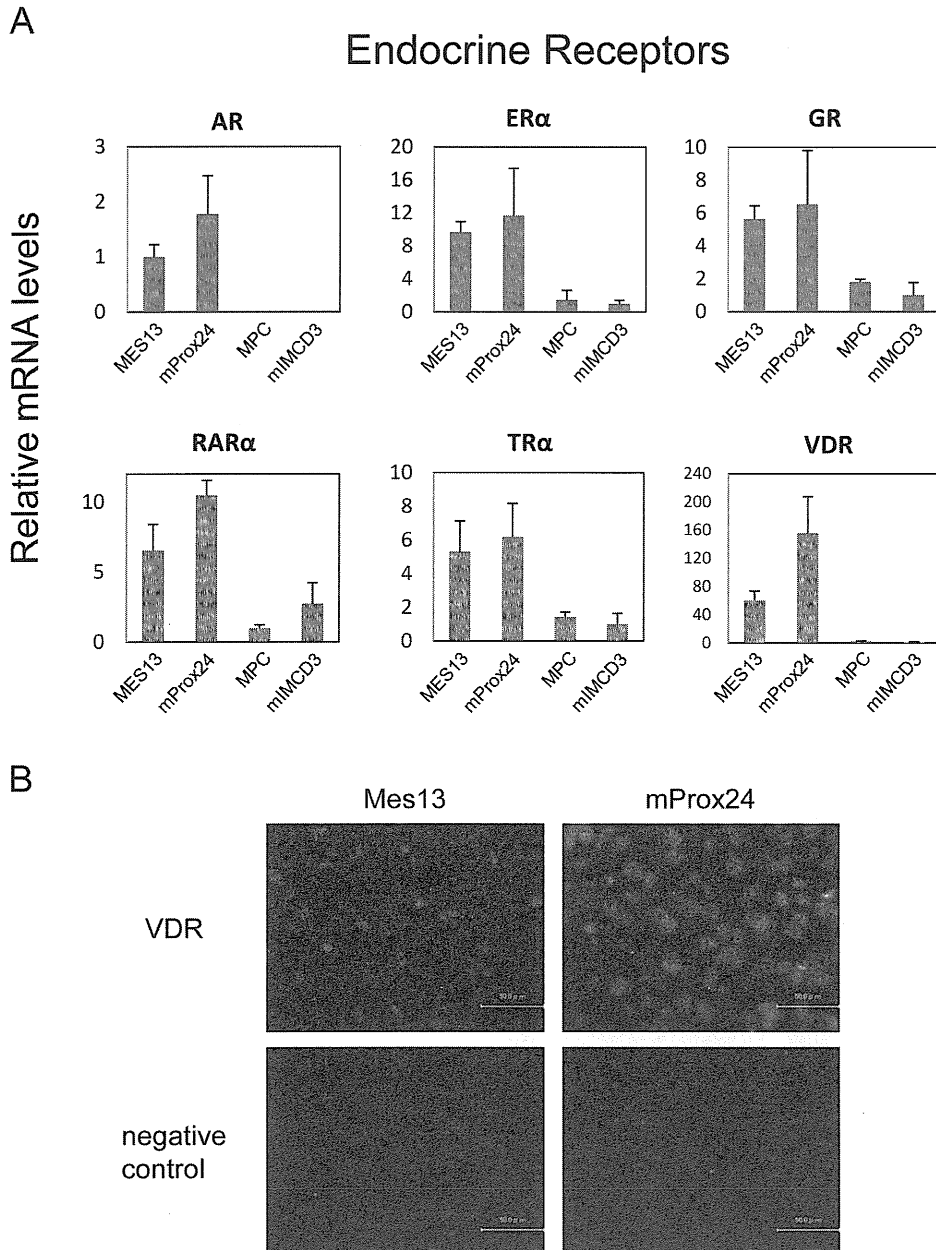

Figure 3. Endocrine receptors expressed in renal cell lines. (A) The relative mRNA levels are depicted for mesangial (MES13), podocyte (MPC), proximal tubular epithelial (mProx24), and collecting duct (mIMCD3) cell lines. All values are expressed relative to 18S and arithmetically adjusted to depict the lowest-expressing sample as a unit of 1. Values represent the means ± SEM of three independent samples of each cell lines, and the results are representative of two independent studies. (B) Representative photomicrographs of immunofluorescent staining. Vitamin D receptor (VDR) was predominantly expressed in mProx24 cells, and to a lesser extent in MES13 cells.
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highly expressed in both the MES13 and mProx24 cell lines. FXRα, RXRα, and RXRβ were predominantly expressed in the mProx24 cell line. In contrast, adopted orphan receptors were expressed at low levels in the MPC and mIMCD3 cell lines. Immunofluorescent staining showed that PPARδ was expressed in both the MES13 and mProx24 cell lines (Fig. 4B).

Orphan receptor expression in renal cell lines

We also compared the expression of orphan receptors in the renal cell lines (Fig. 5A). ERRα, HNF4α, retinoic acid-related orphan receptor α (RORα), and RevERBβ were highly expressed in the MES13 cell line, while COUP-TFII and RORγ were highly expressed in the mProx24 cell line. COUP-TFIII, RevERBα, and testicular orphan receptor 4 (TR4) were abundant in both the mProx24 and MES13 cell lines. Intriguingly, high expression of ERRα was detected in the MPC cell line, while ERRγ predominated in the mIMCD3 cell line. Immunohistochemistry showed that COUP-TFII was expressed in both the MES13 and mProx24 cell lines (Fig. 5B).

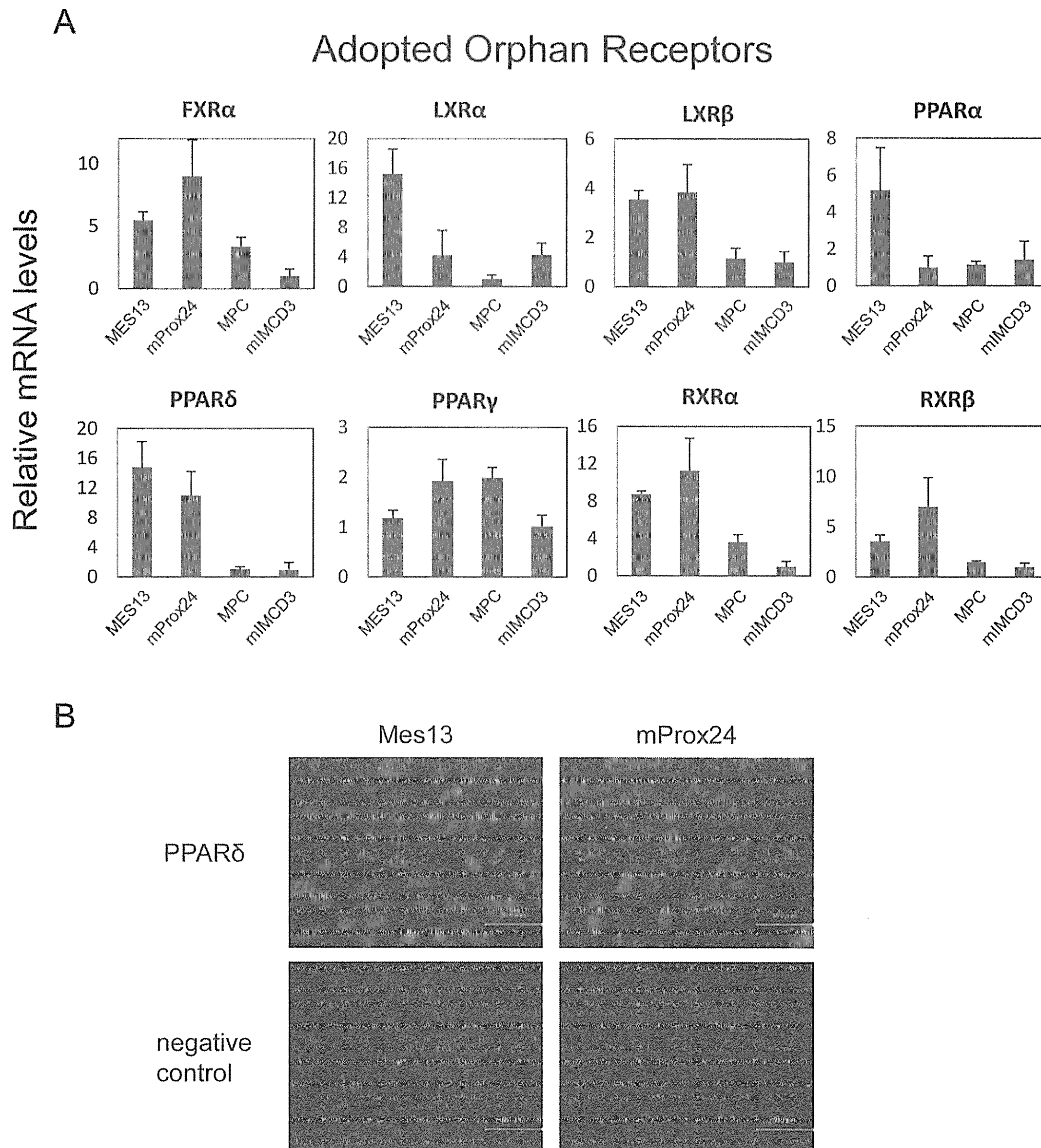

Figure 4. Adopted orphan receptors expressed in renal cell lines. (A) Refer to the legend for Figure 3A for details. (B) Representative photomicrographs of immunofluorescent staining. Peroxisome proliferator-activated receptor-δ (PPARδ) was expressed in both MES13 and mProx24 cell lines.
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Glucose regulation of NHR expression in mouse kidney tissue and renal cell lines

To determine differential expression of NHRs in response to hyperglycemia, we compared the expression of NHRs in the kidneys of streptozotocin-induced type 1 diabetic C57BL/6 mice and control C57BL/6 mice (Fig. 6). Similarly, we compared NHR expression in the kidneys of type 2 diabetic db/db mice and control db/m mice (Fig. 6). Furthermore, we evaluated the expression of the 49 NHRs in renal cell lines to determine whether changes in RNA levels occur in high-glucose conditions (Fig. 7). Renal cell lines were exposed to low-glucose (5.5 mM) or high-glucose (25 mM) conditions for 24 h before RNA isolation.

Significantly increased RNA levels were observed for neuron-derived orphan receptor 1 (NOR1) and nuclear receptor-related factor 1 (NURR1), members of the NHR 4A (NR4A) subgroup, and ERβ and ERRβ, members of the NR3A subgroup, in the kidneys of streptozotocin-induced diabetic C57BL/6 mice (Fig. 6). A similar increase in NURR1, NOR1, and ERβ mRNA was observed in the kidneys of diabetic db/db mice (Fig. 6). Interestingly, neuron-derived clone 77 (Nur77), which is a member of the NR4A subgroup, was also upregulated as well as NURR1 and NOR1 in db/db mouse kidney.

A similar evaluation of glucose-mediated changes in gene expression in renal cell lines was performed (Fig. 7). In the MES13 cell line, upregulation of the expression of the NR4A members of the NHR superfamily (Nur77>NOR1>NURR1), as well as that of PPARγ and PPARδ was detected. The rank order of NHR expression in the MPC cell line was LXRα, RARβ, ERRβ, FXRα, ERα, TRβ, RORβ, LXRβ. In the mProx24 cell line, RORα was the most highly upregulated receptor while Nur77, NOR1, PPARδ, RevERBα, and PPARα were also upregulated in high-

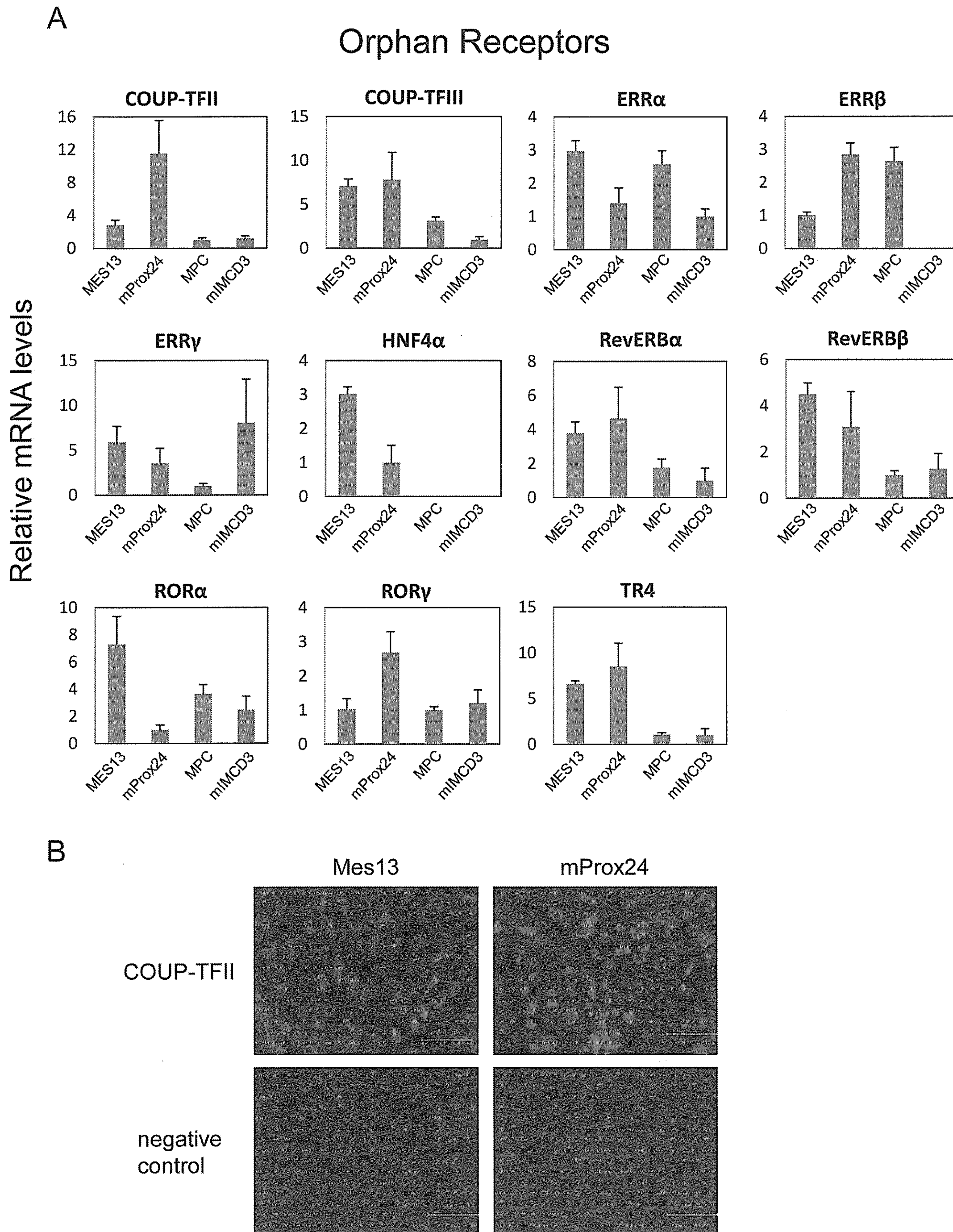

Figure 5. Orphan receptors expressed in renal cell lines. (A) Refer to the legend for Figure 3A for details. (B) Representative photomicrographs of immunofluorescent staining. Chicken ovalbumin upstream promoter transcription factor II (COUP-TFII) was expressed in both MES13 and mProx24 cell lines.
doi:10.1371/journal.pone.0085594.g005

glucose conditions. Similar to the MES13 cell line, high-glucose exposure increased the expression of the NR4A members (Nur77>NURR1>NOR1) as well as RevERBβ, RORα, PPARδ, and RevERBα in the mIMCD3 cell line.

Since the RNA level of NOR1 was the highest in the kidneys of streptozotocin-induced diabetic C57BL/6 mice, and the third highest in diabetic db/db mice (Fig. 6), we confirmed NOR1 expression by immunohistochemistry. The expression of NOR1 in the glomeruli of diabetic C57BL/6 mice coexists with fibronectin, a marker for mesangial cells, but not with WT-1, a marker for podocytes (Fig. 8). On the other hand, NOR1 expression in the interstitium of diabetic C57BL/6 mice coexists with both AQP1 and AQP2, markers for proximal tubular epithelial and collecting duct cells, respectively (Fig. 8). These expressions are consistent with those in qPCR (Fig. 7). Similarly, NOR1 expression was detected in mesangial, proximal tubular epithelial, and collecting

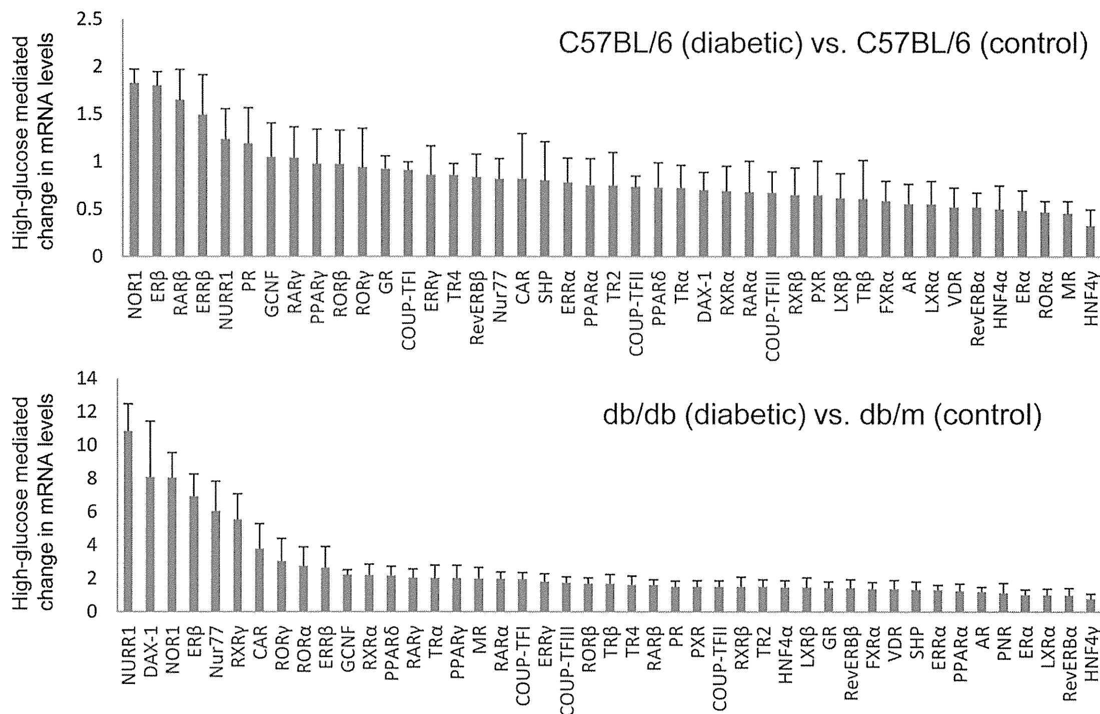

Figure 6. Comparative expression levels of NHRs in mouse kidney tissue under high-glucose conditions. The relative mRNA levels are depicted for streptozotocin-induced diabetic C57BL/6 mouse kidney compared with control C57BL/6 mouse kidney (upper panel), and diabetic db/db mouse kidney compared with control db/m mouse kidney (lower panel). Values depict the means ± SEM of three independent samples.
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duct cells, but not in podocytes in the kidneys of diabetic db/db mice (Fig. 9).

Discussion

NHRs are one of the largest transcription factor families in the mammalian genome. A total of 48 human and 49 mouse genes that encode NHRs have been identified [23]. Recently, the quantitative assessment of NHR RNA levels has been established for various mouse tissues including adipocytes [15], macrophages [16], pancreas [17,24], retina [25], and during the circadian cycle in mouse liver, adipose, and muscle [26] by using qPCR. However, the expression of NHRs in the kidney remains unknown. Therefore, we analyzed NHR expression in mouse kidney tissue and renal cell lines.

In this study, we identified 25 NHRs that are expressed in C57BL/6 mouse kidney. The receptors detected in abundance, including HNF4α, VDR, PPARα, FXR, and ERRα, have already been reported to play a role in the pathogenesis of DN [27]. However, we provide the first evidence that COUP-TFII and COUP-TFIII are expressed abundantly in C57BL/6 mouse kidney, with COUP-TFII being the most abundant NHR in the kidneys of db/m mice. In the renal cell lines, COUP-TFII was shown to be the most abundant NHR in proximal tubular epithelial and collecting duct cells, and highly expressed in mesangial cells and podocytes. Similarly, COUP-TFIII was shown to be the most abundant NHR in podocytes, and highly expressed in mesangial cells, proximal tubular epithelial cells, and podocytes. COUP-TFII is thought to have a role in kidney development and to be necessary for metanephric mesenchyme formation and kidney precursor cell survival [28,29]. In contrast, the role of COUP-TFII in DN is completely unknown and further studies are required.

The analysis of NHR expression in renal cell lines showed that most are expressed mainly in proximal tubular epithelial cells and mesangial cells. However, we showed that PPARα and LXRα are more highly expressed in mesangial cells than in the other renal cell types, which is consistent with previous reports that PPARα and LXRα are expressed in mesangial cells [30,31,32,33]. Since synthetic agonists for PPARα and LXRα are reported to increase cholesterol efflux and ameliorate lipid-related glomerular disease [31,32,34], it can be speculated that these NHRs in mesangial cells represent therapeutic targets for treating glomerular injuries in DN. Moreover, the endocrine receptors, ERα, TRα, and VDR, the adopted orphan receptors, PPARδ, LXRβ, and FXRα, and the orphan receptors, COUP-TFII, COUP-TFIII, RORα, RORγ, RevERBα, and RevERBβ, are expressed not only in mesangial cells, but also in proximal tubular epithelial cells. Furthermore, the expressions of endocrine receptor VDR, orphan receptor PPARδ, and adopted orphan receptor COUP-TFIII were confirmed by immunohistochemistry (Figs. 3B, 4B, 5B). RevERBs and RORs are thought to play important roles in the regulation of circadian rhythm [26]; however, their functions in the kidney remain to be fully elucidated. Our data suggest that these receptors play fundamental roles in renal function.

ERRs are highly expressed in the kidney and renal cell lines. Intriguingly, ERRα is the only NHR that is highly expressed in podocytes, while ERRγ is the only NHR that is highly expressed in collecting duct cells. It is reported that ERRα is a key regulator of interferon-γ-induced mitochondrial reactive oxygen species production and host defense [35], while both ERRα and ERRγ synergistically orchestrate a comprehensive cardiac transcriptional program [36]. Regarding the kidney, ERRα and ERRγ are expressed in the outer stripe of the outer medulla of mouse kidney [37], suggesting that these receptors regulate renal potassium

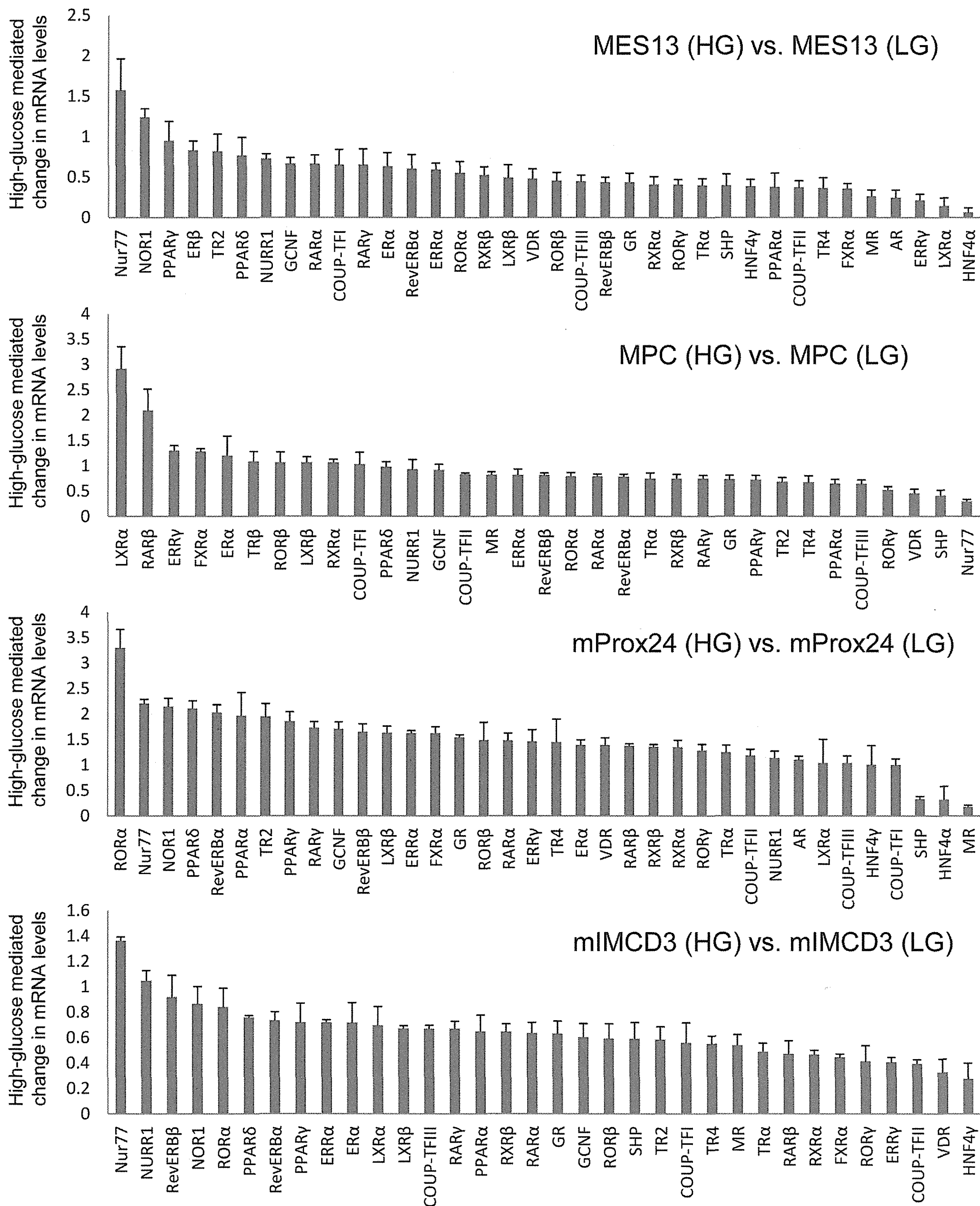

Figure 7. Comparative expression levels of NHRs in renal cell lines under high-glucose conditions. Mesangial (MES13), podocyte (MPC), proximal tubular epithelial (mProx24), and collecting duct (mIMCD3) cell lines. Each panel displays NHR expression under high-glucose conditions compared with low-glucose conditions. Values depict the means ± SEM of three independent samples. HG, high-glucose. LG, low-glucose.
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homeostasis and the renin-angiotensin system [38,39]. Although ERRα is induced by PPARγ coactivator-1α (PGC-1α) [40], little is known about the role and function of ERRα in podocytes and further studies are needed.

The most interesting and novel finding in this study is that the members of the NR4A subgroup, including NOR1, NURR1, and Nur77, are highly induced in the kidneys of diabetic C57BL/6 and db/db mice. All three members of the NR4A subgroup are expressed in energy-dependent tissues such as skeletal muscle,

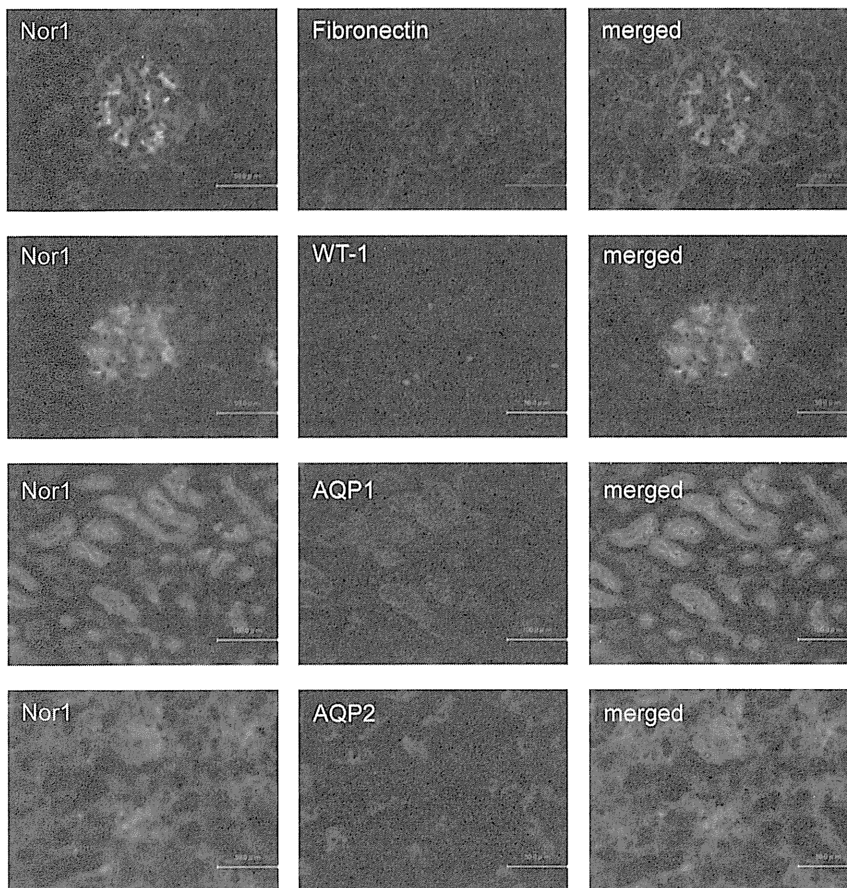

Figure 8. Representative photomicrographs of double immunofluorescent staining in diabetic C57BL/6 mice. NOR1 expression was localized in mesangial, proximal tubular epithelial, and collecting duct cells, but not in podocytes in the kidneys of diabetic C57BL/6 mice.
doi:10.1371/journal.pone.0085594.g008

brain, adipose tissues, heart, and liver suggesting a possible role in energy metabolism [41]. Although it is known that kidney expression of the NR4A subgroup is upregulated by stimulation of parathyroid hormone [42] or prostaglandin E2 [43], the effects of high-glucose stimulation on the expression of these receptors have not been reported. To confirm the results of qPCR, we performed immunohistochemistry of NOR1 in the kidneys of diabetic C57BL/6J and db/db mice. Double staining of NOR1 with markers for mesangial cells (fibronectin), podocytes (WT-1), proximal tubular cells (AQP1), and collecting duct cells (AQP2) revealed that NOR1 was expressed in mesangial cells, proximal tubular cells, and collecting duct cells, but not in podocytes (Fig. 8 and 9). Our in vitro studies showed that NOR1, Nur77, and NURR1 are also highly upregulated in cultured mesangial cells, proximal tubular epithelial cells, and collecting duct cells. These results might help to elucidate the previously unrecognized mechanisms of the progression of DN. To the best of our knowledge, there have been no studies investigating the expression of NR4A subgroup in patients with DN, and further studies using renal biopsy specimens are needed as the next step.

We were surprised that the expression profile of NHRs in the kidneys of diabetic C57BL/6J and db/db mice is partially overlapping like the members of the NR4A subgroup, but mostly different as shown in Fig. 6. This difference may be caused by the effect of streptozotocin injection in C57BL/6J mice or by deletion of the leptin receptor gene in db/db mice. Another surprise was that these NHRs were not differentially upregulated or even downregulated in the kidneys of diabetic mice or high-glucose treated renal cell lines. Several agonists of NHRs, including PPARγ, PPARα, VDR, and FXR have been reported to have potential suppressive effects on the progression of DN [44]. Our results are consistent with the reports of previous studies that the expression of PPARα, VDR, and FXR was downregulated by the induction of diabetes [33,45]. The precise mechanism for downregulation of these NHRs in response to hyperglycemia is unknown, but activation of these NHRs may prevent the development of DN in mouse models. Based on these observations, we speculate that the inactivation of upregulated NHRs in hyperglycemia, such as NOR1, NURR1, and Nur77, may also be a potential therapeutic approach in the management of DN. Further studies are required to address whether modulation of these NHRs will provide a novel strategy for treating DN.

In summary, we have analyzed the expression of all NHRs present in normal and high-glucose conditions in mouse kidney tissue and renal cell lines. The functions of most of these NHRs are still unknown; however, our data provide the basis for further studies to elucidate potential therapeutic targets in the pathogenesis of DN.

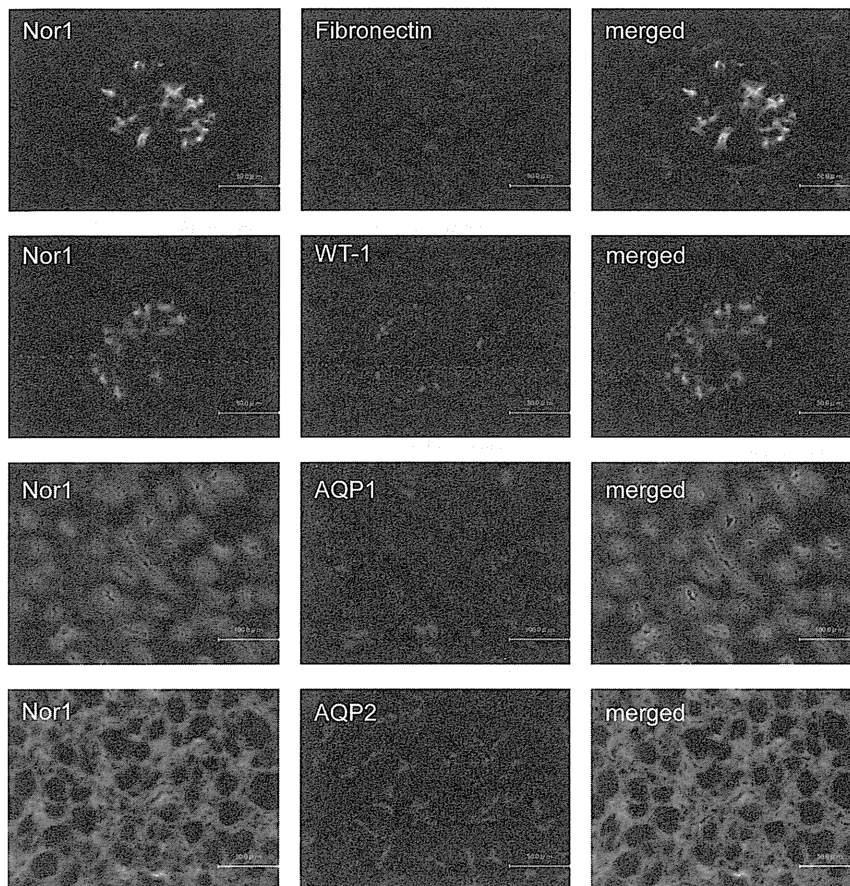

Figure 9. Representative photomicrographs of double immunofluorescent staining in diabetic db/db mice. NOR1 expression was localized in mesangial, proximal tubular epithelial, and collecting duct cells, but not in podocytes in the kidneys of diabetic db/db mice.
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Supporting Information

Table S1 Primers used for quantitative real-time PCR. (DOC)

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Author Contributions

Conceived and designed the experiments: DO. Performed the experiments: DO JE JW NT TH. Analyzed the data: HT AN CSH NN. Wrote the paper: DO HM.

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Long-Term Treatment with the Sodium Glucose Cotransporter 2 Inhibitor, Dapagliflozin, Ameliorates Glucose Homeostasis and Diabetic Nephropathy in *db/db* Mice

Naoto Terami[1], Daisuke Ogawa[1,2]*, Hiromi Tachibana[1], Takashi Hatanaka[1], Jun Wada[1], Atsuko Nakatsuka[1], Jun Eguchi[1], Chikage Sato Horiguchi[1], Naoko Nishii[1], Hiroshi Yamada[3], Kohji Takei[3], Hirofumi Makino[1]

1 Department of Medicine and Clinical Science, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan, **2** Department of Diabetic Nephropathy, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan, **3** Department of Neurochemistry, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan

Abstract

Inhibition of sodium glucose cotransporter 2 (SGLT2) has been reported as a new therapeutic strategy for treating diabetes. However, the effect of SGLT2 inhibitors on the kidney is unknown. In addition, whether SGLT2 inhibitors have an anti-inflammatory or antioxidative stress effect is still unclear. In this study, to resolve these issues, we evaluated the effects of the SGLT2 inhibitor, dapagliflozin, using a mouse model of type 2 diabetes and cultured proximal tubular epithelial (mProx24) cells. Male *db/db* mice were administered 0.1 or 1.0 mg/kg of dapagliflozin for 12 weeks. Body weight, blood pressure, blood glucose, hemoglobin A1c, albuminuria and creatinine clearance were measured. Mesangial matrix accumulation and interstitial fibrosis in the kidney and pancreatic β-cell mass were evaluated by histological analysis. Furthermore, gene expression of inflammatory mediators, such as osteopontin, monocyte chemoattractant protein-1 and transforming growth factor-β, was evaluated by quantitative reverse transcriptase-PCR. In addition, oxidative stress was evaluated by dihydroethidium and NADPH oxidase 4 staining. Administration of 0.1 or 1.0 mg/kg of dapagliflozin ameliorated hyperglycemia, β-cell damage and albuminuria in *db/db* mice. Serum creatinine, creatinine clearance and blood pressure were not affected by administration of dapagliflozin, but glomerular mesangial expansion and interstitial fibrosis were suppressed in a dose-dependent manner. Dapagliflozin treatment markedly decreased macrophage infiltration and the gene expression of inflammation and oxidative stress in the kidney of *db/db* mice. Moreover, dapagliflozin suppressed the high-glucose-induced gene expression of inflammatory cytokines and oxidative stress in cultured mProx24 cells. These data suggest that dapagliflozin ameliorates diabetic nephropathy by improving hyperglycemia along with inhibiting inflammation and oxidative stress.

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* Email: daiogawa@md.okayama-u.ac.jp

Introduction

Diabetic nephropathy is a leading cause of chronic renal failure in western world [1]. In the past, several mechanisms have been suggested to involve in the initiation and deterioration of diabetic nephropathy, including hemodynamic and genetic factors, intracellular metabolic anomalies, and advanced glycation end products [2]. Emerging evidence suggests that inflammation is crucially contributed in the pathophysiology of diabetic nephropathy [3]. Recently, many studies have also suggested that production of reactive oxygen species (ROS) is enhanced by hyperglycemia, and oxidative stress has been involved in the onset and progression of diabetic nephropathy [4]. Therefore, the regulation of inflammation and oxidative stress could be a potential target in the treatment of diabetic nephropathy.

Sodium glucose cotransporter 2 (SGLT2), that is located on the apical side of the proximal tubular cells, can transport sodium and glucose concurrently within the proximal tubules [5]. Under normoglycemic conditions, SGLT2 can reabsorb about 90% of

the glucose in the early segments of the proximal tubules [6]. In recent years, SGLT2 inhibitors, which can inhibit reabsorption of filtered glucose by blocking SGLT2, have been developed and proposed as novel hypoglycemic agents for treating patients with diabetes mellitus [7]. A large number of SGLT2 inhibitors have been developed, and numerous basic and clinical studies have been executed in the last decade [8]. Although SGLT2 inhibitors are novel and promising drugs for treating type 2 diabetes patients, the effect of SGLT2 inhibition on diabetic nephropathy is unknown.

Dapagliflozin is a very selective and potent SGLT2 inhibitor [9], and is the first-in-class SGLT2 inhibitor launched on the market in 2012 [10]. Various clinical studies have shown improvements in glycemic control with both monotherapy and combination therapy of dapagliflozin [11]. In addition, dapagliflozin was associated with additional non-glycemic benefits including reduction in blood pressure and body weight in most clinical trials [12]. Although several studies with animal models suggest that long-term administration of SGLT2 inhibitors, including dapagliflozin, preserves pancreatic β-cell function with improved glucose homeostasis [9,13,14,15], the influences of SGLT2 inhibition on diabetic nephropathy and renal function have not been elucidated.

The purpose of this study was to investigate the hypothesis that inhibition of SGLT2 by dapagliflozin ameliorates glucose homeostasis while preserving β-cell mass, and retards the progression of diabetic nephropathy by inhibiting inflammation and oxidative stress in a mouse model of type 2 diabetes and obesity.

Materials and Methods

Animal Care and Experiments

We purchased six-week-old male diabetic *db/db* mice (BKS.Cg-$lepr^{db}/lepr^{db}$) and non-diabetic *db/m* mice (BKS.Cg-$lepr^{db}$/+) from CLEA Japan (Tokyo, Japan). All mice were kept in light-controlled room and allowed free access to tap water and food. Dapagliflozin was kindly supplied by Bristol-Myers Squibb (Pennington, NJ, USA). Dapagliflozin (0.1 or 1.0 mg/kg/day) was administrated to *db/db* mice ($n = 6$) by gavage for 12 weeks starting at the age of 8 weeks. Control *db/db* mice ($n = 5$) and control *db/m* mice ($n = 5$) received saline for 12 weeks. The mice were anesthetized by an injection of pentobarbital at 20 weeks of age. After the mice were sacrificed by exsanguination through cutting cervical artery under anesthesia, the kidneys were removed and weighed. The kidneys and pancreas were processed as previously described [16]. The study protocol was approved by the Animal Ethics Review Committee of Okayama University (OKU-2012356). All animal care and procedures were performed in accordance with the Guidelines for Animal Experimentation at Okayama University, the Japanese Government Animal Protection and Management Law, and the Japanese Government Notification on Feeding and Safekeeping of Animals.

Metabolic Data

Body weight was measured weekly. Blood pressure, plasma glucose, urinary glucose and 24-h urinary albumin excretion (UAE) were measured every 4 weeks. Blood pressure was measured by the tail-cuff method (Softron, Tokyo, Japan). Plasma glucose and blood pressure were measured after an overnight fast. Hemoglobin A1c (HbA1c), water intake, food intake, kidney weight, blood urea nitrogen (BUN), creatinine and creatinine clearance (Ccr) were measured at the age of 20 weeks. Serum and urinary creatinine were measured using an enzymatic method (PUREAUTOS CRE-L, Shimizu Medical, Tokyo, Japan). HbA1c and UAE were measured as described previously [17].

Histology

Tissue sections were cut from the paraffin-embedded kidney samples taken at 20 weeks of age and subjected to PAM and Masson trichrome staining. All tissue sections were examined using a BZ-9000 microscope (Keyence, Osaka, Japan). The mesangial matrix index (MMI) was evaluated using BIOZERO software (Keyence) as previously described [16]. To determine the MMI, 10 randomly selected glomeruli in the cortex per animal were evaluated under high magnification (×400).

Immunofluorescent Staining

Immunofluorescent staining of kidney and pancreas was performed as previously described [18]. Briefly, renal expression of type IV collagen was detected a rabbit antibody for type IV collagen (Millipore, Temecula, CA, USA) followed by Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen, Carlsbad, CA, USA). Similarly, pancreatic β-cells were detected using guinea pig anti-insulin (Abcam, Cambridge, UK) followed by Alexa Fluor 488 goat anti-guinea pig IgG (Invitrogen). The positive area of type IV collagen in the glomerulus was calculated in the same way as MMI. The proportion of β-cells in the pancreatic tissue was calculated using BIOZERO software (Keyence). The insulin-positive area relative to the area of the whole pancreatic tissue was analyzed in more than 100 islets per group.

Immunoperoxidase Staining

Immunoperoxidase staining was performed as previously described [17]. In brief, macrophage infiltration was analyzed using a monoclonal antibody for murine monocyte/macrophage (F4/80, Abcam), followed by HRP-conjugated goat anti-rat IgG antibody (Millipore). The number of F4/80-positive cells was calculated in 10 glomeruli and intestitia per animal, and the mean number of F4/80 positive cells per glomerulus and interstitial tissue (number per mm^2) was used for the estimation.

NADPH oxidase 4 (Nox4) immunoperoxidase staining was performed as described previously [19]. Briefly, renal tissues were stained with Nox4 rabbit antibody (Novus Biologicals, Littleton, CO, USA) for 12 h at 4°C followed by HRP-conjugated goat anti-rabbit IgG antibody (Millipore). The proportion of the area stained with Nox4 antibody of the total area was calculated using BIOZERO software (Keyence). To quantify the proportional area of staining, 10 views of the renal cortex were randomly selected in each slide.

Quantitative Analysis of Gene Expression in the Renal Cortex

RNA was isolated from the renal cortex of 20-week-old mice as described previously [16]. To determine the expression of *CD14*, *CD11c*, *CD206*, *transforming growth factor* (*TGF*)-*β*, *intercellular adhesion molecule* (*ICAM*)-*1*, *monocyte chemoattractant protein* (*MCP*)-*1*, *osteopontin*, *caspase-12* and *Bax* in the renal cortex, quantitative RT-PCR (qRT-PCR) was performed as described previously [16]. Each sample was normalized against *Atp5f1* mRNA expression and analyzed in triplicate.

ROS Expression

To evaluate the effect of dapagliflozin on ROS production, superoxide anion radicals were detected by dihydroethidium (DHE) staining (Molecular Probes, Eugene, OR, USA). Briefly, the kidney sections were incubated with DHE (2 μmol/l) at 37°C

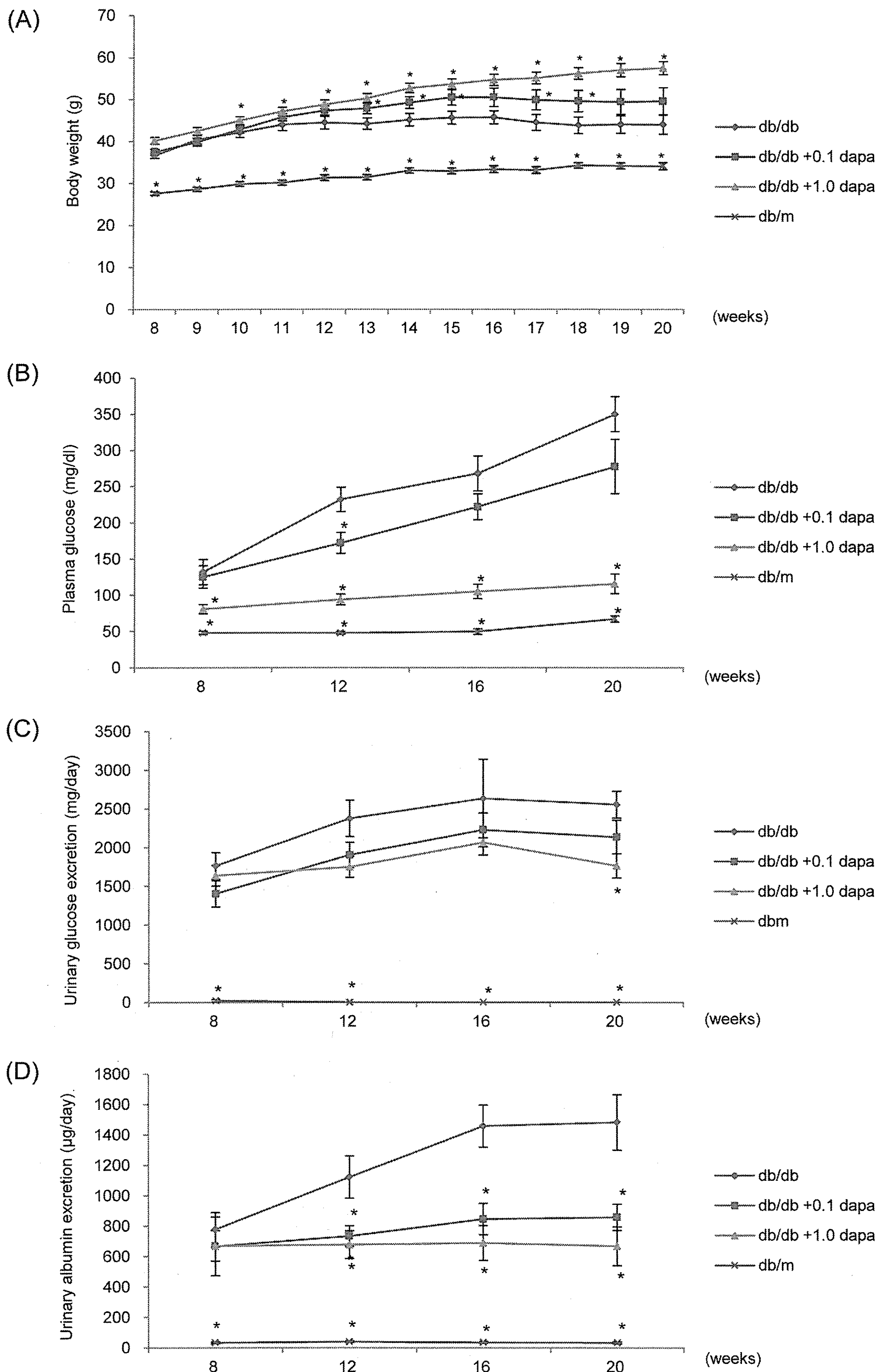

Figure 1. Effect of dapagliflozin on body weight, hyperglycemia and urinary albumin excretion (UAE). (A) Body weight was higher in the *db/db* group than in the *db/m* group during the study. Body weight in the *db/db* with 1.0 mg/kg dapagliflozin group (*db/db*+0.1 dapa group) was higher than in the *db/db* group from 10 to 20 weeks of age. Data are mean ± SEM. **P*<0.05. (B–D) Plasma and urinary glucose, and UAE progressively increased in the *db/db* group during the 12-week observation period. These parameters were significantly lower in the *db/db*+1.0 dapa group than in the *db/db* group. Data are mean ± SEM. **P*<0.05.
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Table 1. Influence of dapagliflozin on physiologic and metabolic parameters in *db/db* and *db/m* mice at 20 weeks.

	db/m	*db/db*	*db/db*+0.1 dapa	*db/db*+1.0 dapa
Systolic blood pressure (mmHg)	120.0±5.2	116.6±4.5	121.2±2.3	115.2±4.5
Diastolic blood pressure (mmHg)	79.4±3.2	78.8±2.3	86.3±1.6	84.3±3.0
HbA1c (%)	4.0±0.1	9.2±0.2[a]	8.5±0.3[a]	6.6±0.2[abc]
Water intake (ml/day)	4.8±0.4	31.1±4.1[a]	22.3±2.9[a]	19.8±1.9[a]
Food intake (g/day)	3.2±0.1	4.5±0.7	4.8±0.3	6.1±0.3[a]

db/m, nondiabetic control mice; *db/db*, untreated diabetic mice; *db/db*+0.1 dapa, dapagliflozin (0.1 mg/kg)-treated diabetic mice; *db/db*+1.0 dapa, dapagliflozin (1.0 mg/kg)-treated diabetic mice; HbA1c, hemoglobin A1c. Data are presented as mean ± SEM;
[a]$P<0.05$ vs. *db/m*,
[b]$P<0.05$ vs. *db/db*,
[c]$P<0.05$ vs. *db/db*+0.1 dapa.
doi:10.1371/journal.pone.0100777.t001

in a humidified chamber protected from light for 45 min. The DHE fluorescence intensity was analyzed using BIOZERO software (Keyence) in 10 intestitia per animal.

Terminal Transferase-mediated dUTP Nick-End Labeling (TUNEL) Assay

To evaluate the effect of dapagliflozin on apoptosis, kidney samples were incubated with an *in situ* apoptosis detection kit (Takara Bio) according to the manufacturer's protocol. The mean number of TUNEL-positive cells in interstitia (number per mm^2) was determined by observing more than 10 interstitia from each section.

In vitro Experiments

Murine proximal tubular epithelial (mProx24) cells, kindly provided by Dr. Takeshi Sugaya (CMIC Co., Tokyo, Japan), were used as previously described [18]. DHE staining and qRT-PCR were performed as described above.

Statistical Analysis

All data were expressed as mean ± SEM. Statistical analysis between groups was performed using one-way ANOVA followed by Scheffe's test. A P value<0.05 was considered statistically significant.

Results

Effect of Dapagliflozin on Body Weight, Hyperglycemia and Renal Function

Body weight was higher in the *db/db* groups than in the *db/m* group during the study, and body weight in the *db/db* group treated with 0.1 or 1.0 mg/kg/day of dapagliflozin (*db/db*+0.1 dapa group and *db/db*+1.0 dapa group, respectively) was higher than in the *db/db* group from 10 to 20 weeks of age (Fig. 1A). Plasma and urinary glucose excretion progressively increased in the *db/db* groups during the study. However, dapagliflozin significantly reduced plasma and urinary glucose, and HbA1c compared with those in the *db/db* group at 20 weeks of age (Fig. 1B, 1C and Table 1). There were no significant differences in systolic and diastolic blood pressure between the four groups at 20 weeks of age. In addition, there were no significant differences in water and food intake between the *db/db*, the *db/db*+0.1 dapa and the *db/db*+1.0 dapa groups (Table 1).

UAE, a characteristic feature of diabetic nephropathy, progressively increased in the *db/db* group during this study. However, dapagliflozin decreased the UAE compared with that in the *db/db* group from 12 to 20 weeks of age significantly (Fig. 1D). The other parameters are summarized in Table 2. There were no significant differences in BUN and serum creatinine between the four groups at 20 weeks of age. Kidney weight and relative kidney weight were lower in the *db/db* groups than in the *db/m* group significantly, but there were no significant differences between the *db/db*, the *db/db*+0.1 dapa and the *db/db*+1.0 dapa group. We speculate that the

Table 2. Influence of dapagliflozin on renal structural and functional parameters at 20 weeks.

	db/m	*db/db*	*db/db*+0.1 dapa	*db/db*+1.0 dapa
Kidney weight (mg)	379.0±39.6	247.0±6.8[a]	239±9.4[a]	252.5±9.1[a]
Relative kidney weight (mg/g body weight)	11.5±1.0	6.0±0.3[a]	5.2±0.4[a]	4.5±0.1[a]
BUN (mg/dl)	20.3±0.7	29.1±2.8	24.6±2.5	25.9±0.6
Serum creatinine (mg/dl)	0.10±0.01	0.12±0.02	010±0.01	0.12±0.02
Urine volume (ml/day)	1.0±0.1	23.4±3.1[a]	19.2±2.4[a]	16.3±1.9[a]
Ccr (ml/min)	4.80±0.54	9.42±0.96[a]	9.81±0.78[a]	6.40±0.65[c]

db/m, nondiabetic control mice; *db/db*, untreated diabetic mice; *db/db*+0.1 dapa, dapagliflozin (0.1 mg/kg)-treated diabetic mice; *db/db*+1.0 dapa, dapagliflozin (1.0 mg/kg)-treated diabetic mice; BUN, blood urea nitrogen; Ccr, creatinine clearance. Data are presented as mean ± SEM;
[a]$P<0.05$ vs. *db/m*,
[b]$P<0.05$ vs. *db/db*,
[c]$P<0.05$ vs. *db/db*+0.1 dapa.
doi:10.1371/journal.pone.0100777.t002

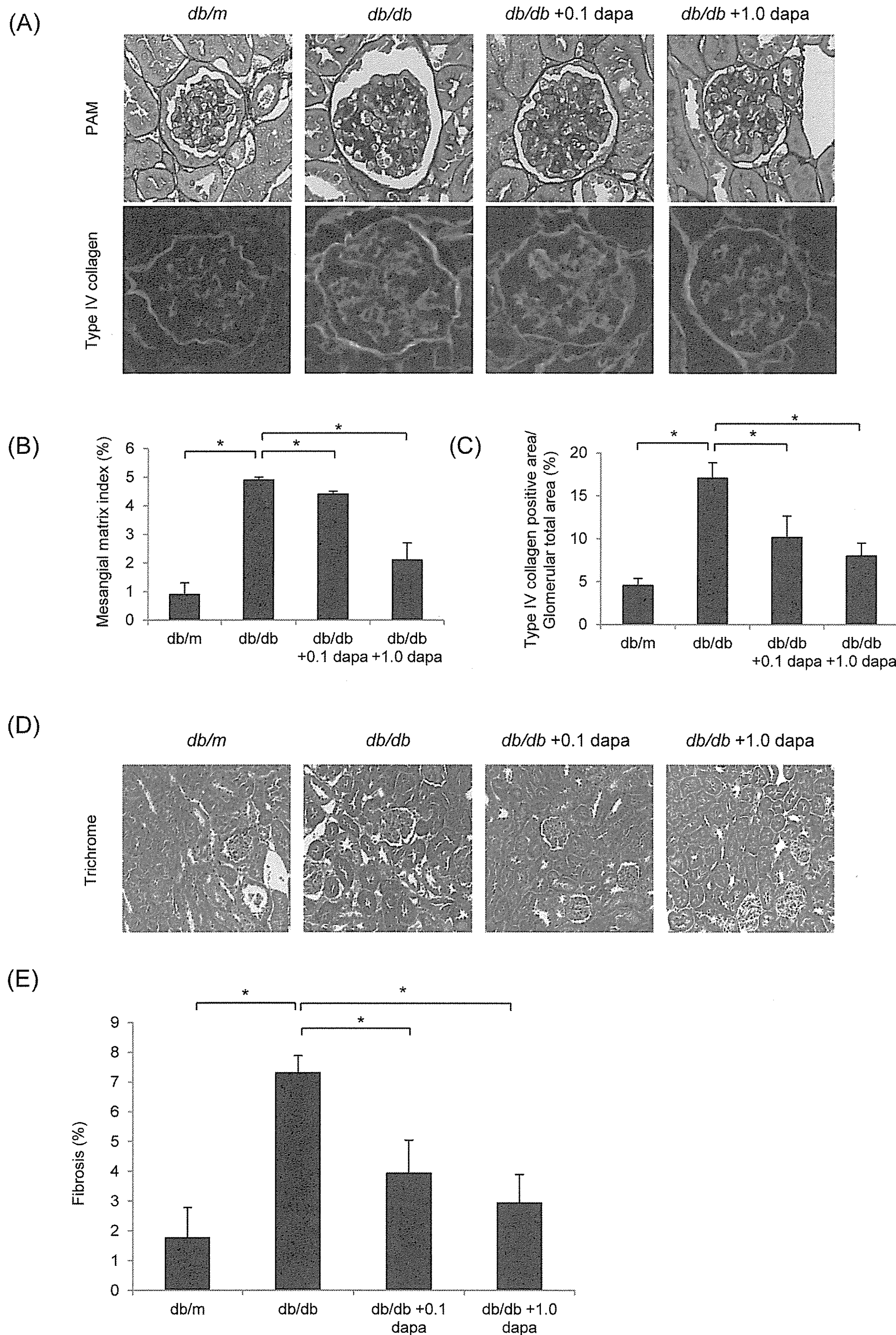

Figure 2. Dapagliflozin suppresses mesangial matrix accumulation and interstitial fibrosis. (A) Periodic acid-methenamine silver (PAM) and type IV collagen staining of kidney sections. Mesangial matrix accumulation was prominent in the *db/db* group. Dapagliflozin suppressed the increase in mesangial matrix accumulation compared with that in the *db/db* group. Original magnification, ×400. (B) Mesangial matrix index of the glomeruli. Data are mean ± SEM. $^*P<0.05$. (C) Type IV collagen positive area in the glomeruli. Data are mean ± SEM. $^*P<0.05$. (D) Masson's trichrome staining of kidney sections. Interstitial fibrosis was significantly higher in the *db/db* group than in the *db/m* group, and significantly lower in the *db/db*+1.0 dapa group than in the *db/db* group. Original magnification, ×100. (E) Percentages of fibrosis in interstitia. Data are mean ± SEM. $^*P<0.05$.
doi:10.1371/journal.pone.0100777.g002