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Abstract

Background: In this study, we hypothesized that probucol can ameliorate diabetic nephropathy by inducing Nuclear factor erythroid 2-related factor 2 (Nrf2). We examined and compared the effects of probucol and pitavastatin on diabetic nephropathy in db/db mice.

Methods: Db/db mice were fed with normal chow with or without 0.1% probucol or 0.005% pitavastatin for eight weeks. Blood and urine samples were collected before administration and four and eight weeks after the treatment. We also performed a histological analysis at the end of the study.

Results: Probucol, but not pitavastatin, treatment significantly decreased both serum cholesterol and triglycerides by more than 50%. Probucol and pitavastatin treatment decreased 24 hour urinary albumin excretion and glomerular Nrf2 expression and produced a histological improvement of diabetic nephropathy.

Conclusions: Our data demonstrates that probucol can ameliorate diabetic nephropathy through its cholesterol-lowering effect and possibly its modifing effects on Nrf2 function in diabetes.

Keywords: diabetic nephropathy; probucol; Nrf2;

1 Introduction

Long-standing hyperglycemia is known to be a significant risk factor for the development of diabetic nephropathy. Diabetic nephropathy is a life-threatening complication of diabetes mellitus and is the leading cause of end-stage renal disease (1). The characteristics of this disease include persistent albuminuria and a progressive decline in renal function, along with histological thickening of the glomerular basement membrane and mesangial matrix expansion (2, 3). Albuminuria and glomerular hypertrophy are followed by the development of glomerulosclerosis, which seems to be associated with the prognosis of patients with diabetic nephropathy (4). Therefore, albuminuria is an optimal therapeutic target for the treatment of diabetic nephropathy in regular clinical practice.

Hypercholesterolemia and oxidative stress as well as hyperglycemia are known to be important factors that promote complications in diabetes mellitus (5, 6). Indeed, lipid peroxide can be involved in the development of diabetic nephropathy (7). Probucol has been developed as an antioxidant, but it also decreases plasma cholesterol levels in humans. Additionally, probucol has shown an anti-atherosclerotic effect in hyperlipidemic rabbits in vivo (8, 9). Therefore, it has been used as a cholesterol-lowering drug in clinics for more than 30 years. In addition to its effect on atherosclerosis, Endo et al. reported that probucol suppressed the progression of diabetic nephropathy (10, 11); however, the molecular mechanism is unknown.

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor that plays a key role in cellular defense against the cytotoxic effects of oxidative stress (12, 13). Jiang et al. have shown that Nrf2 exhibits a protective effect in streptozotocin-induced diabetic nephropathy in vivo (14). Therefore, we hypothesized that probucol can ameliorate diabetic nephropathy by inducing the Nrf2 expression in kidneys. We examined the effects of probucol on diabetic nephropathy in db/db mice, which are a mouse model of type 2 diabetes that typically exhibit diabetic nephropathy. Several groups, including ours, have shown that statins can improve diabetic nephropathy in humans and rodents (15-18). Therefore, we compared the effects of probucol with those of pitavastatin.

2 Methods

2.1 Materials: Probucol and pitavastatin were provided by Otsuka Pharmaceutical Co., Ltd. and Wako Pure Chemical Industries, Ltd., respectively.

2.2 Animal Procedures and Experimental Design: Eight week old male db/db and db/m mice were obtained from Oriental Bio-Service. Db/db mice were fed normal chow without additional supplementation (n=10) or chow supplemented with 0.1% probucol or 0.005% pitavastatin (n=10, each) for eight weeks. The animals were provided with food and water ad libitum and were maintained on a 12-hour light/dark cycle. All

animal experiments were conducted according to the Guidelines for Animal Experiments at Kyoto University.

2.3 Analysis of Metabolic Parameters: Blood and urine samples were collected three times: before administration and after four and eight weeks of treatment. Urine samples were collected for 24 hours in individual metabolic cages. During the urine collection, the mice were allowed free access to food and water. During dissection, we removed the kidneys, and the weight was measured. We measured the body weight every week, and we also measured plasma total cholesterol, triglycerides, glucose, insulin, urinary albumin levels with an autoanalyzer after overnight fasting. The plasma glucose concentration was measured with a Glutest Ace (Sanwa Kagaku Kenkyusho Co, Ltd, Nagoya, Japan). The plasma insulin concentration was measured with an insulin assay kit (Morinaga Institute of Biological Science, Yokohama, Japan). The plasma total cholesterol and triglycerides were measured with Cholesterol E and Triglyceride E tests (Wako pure Chemical Industries Ltd. Osaka, Japan), respectively.

2.4 Histological analysis

The kidneys were embedded in paraffin, and 2 μm sections were collected through the largest axial section and stained with hematoxylin-eosin (HE) or periodic acid schiff (PAS). The glomerular area was determined as described (19). Paraffin embedded kidney sections were rehydrated, boiled for 15 min at 105 °C in citrate buffer (pH 6.0) and treated with 0.3% H_2O_2 in methanol for 30 min. Sections were blocked with the appropriate preimmune serum and then incubated with Avidin D and Biotin blocking solutions (Vector, CA). Sections were incubated first with the anti-Nrf2 antibody (Abcam, Cambridge, MA) overnight at 4°C, then with the appropriate biotinylated secondary antibodies and finally with the Vectastain Elite ABC kit (Vector). Immunofluorescence staining for type 4 collagen, 4-μm-thick cryostat kidney sections were fixed in cold acetone for 10 min and treated with 0.3% hydrogen peroxide in methanol for 30 min. After an appropriate blocking step, the sections were incubated with the anti-Col4 antibody (PROGEN, Heidelberg, Germany) overnight at 4 °C. An FITC-labeled or biotinylated secondary antibody followed by avidin-labeled Alexa 594 (Molecular Probe, Invitrogen, Carlsbad, CA) was added to the sections. We then counted Nrf2-positive cells from 50 glomeruli in each mouse.

2.5 Establishment of cell lines

Glomerular MCs were established from glomeruli isolated from normal 4-week-old mice (C57BL/6JxSJL/J) and were identified according to the method described previously (29). MCs were maintained in B medium (a 3:1 mixture of minimal essential medium / F12 modified with trace elements) supplemented with 1 mM glutamine, penicillin at 100 units/ml, streptomycin at

100 mg/ml, and 20% fetal calf serum. The cultured cells fulfilled the generally accepted criteria for glomerular MCs (29).

2.6 Determination of the cellular glucose uptake

Cells (3×10^4 cells) were seeded in a 96-well black and clear bottom plate with 100 µl culture medium. After incubation at 37°C, 5%CO_2 overnight, cells were treated with 0.1% dimethyl sulfoxide (DMSO) (vehicle) or pitavastatin (10 nM) or probucol (10 µM) for 24 h and then replaced with glucose-free medium containing 150 µg/ml 2-NBDG (a fluorescently-labeled deoxyglucose analog). Cells were incubated with 2-NBDG for 30 min before examination by fluorescence microscopy under similar conditions. Representative images of cells from three independent experiments were shown.

2.7 Statistical analysis

All analyses were performed using SAS software (Release 9.1; SAS Institute Inc., Tokyo, Japan). All values were analyzed by one-way ANOVA followed by a posthoc Dunnett's test (two-tailed). All values were expressed as the means ± S.D.

3 Results

We treated db/db mice (eight weeks of age) with probucol or pitavastatin for eight weeks and found that probucol or pitavastatin treatment had no effect on the body weight (Fig. 1) or food intake (data not shown) during the experimental period. The administration of probucol and pitavastatin also had no effect on the blood glucose or insulin level (Fig. 2A, B). In terms of serum lipid levels, probucol treatment significantly decreased both serum cholesterol and triglycerides by more than 50%, but pitavastatin did not (Fig. 2C, D).

To address the effect of both drugs on diabetic nephropathy, we examined the changes in urinary albumin levels after four and eight weeks of treatment. We found that probucol and pitavastatin treatment decreased 24 hour urinary albumin (Fig. 3), although the difference was borderline significant. However, the area under the curve was significantly lowered by probucol and pitavastatin treatment compared to the control group (data not shown).

We also examined the treatment effect on the kidneys in more detail and found that both probucol and pitavastatin significantly decreased the kidney weight and mean glomerular size in db/db mice (Fig. 4A, B). Additionally, to determine the effect of probucol and pitavastatin on oxidative stress, we examined the expression of Nrf2, which is one of the transcription factors involved in oxidant stress, and found that Nrf2 expression was significantly decreased by probucol and pitavastatin treatment (Fig. 4C).

To confirm these effects, we performed a histological analysis. As shown in Fig. 5A, the glomeruli showed enlargement in control db/db mice compared to db/m mice. We also demonstrated that extracellular matrix deposition was reduced by probucol

and pitavastatin as shown by PASM staining (Fig. 5B) and type IV collagen staining (Fig. 5C). Nrf2 staining was also reduced by probucol and pitavastatin treatment (Fig. 5D).

In this study we introduce a cell-based fluorescence assay for sodium-dependent glucose uptake that can be used to

To investigate the effect of probucol and pitavastatin on basal glucose uptake in MCs, glucose transport was monitored using a fluorescent 2-deoxyglucose analogue (2-NBDG). As shown in Figure 6, both probucol and pitavastatin caused a decrease in glucose uptake.

4 Discussion

In this study we have shown that both probucol and pitavastatin improve diabetic nephropathy in db/db mice, even though the effect of these drugs on albuminuria showed a borderline significance. We also found a differential mechanism of these drugs in which the effect of pitavastatin was not dependent on its cholesterol-lowering ability. Previously, we found that ezetimibe also ameliorated the progression of diabetic nephropathy in db/db mice, most likely through a cholesterol-lowering effect (17) because in these mice neither ezetimibe nor pitavastatin affected 8OHdG, which is a marker of oxidative stress. In this study, the effect of probucol on 8-Hydroxydeoxyguanosine (8OHdG) was not confirmed (data not shown). Although we performed DNA-chip assays to identify molecules that could be modulated by probucol, we could not find a possible candidate that is involved in the probucol-induced mitigation of diabetic nephropathy (data not shown).

Lipid-related nephrotoxicity has been proposed as a cause of diabetic nephropathy (21). A number of observational studies have reported that dyslipidemia is associated with albuminuria and renal dysfunction progression in patients with chronic kidney disease (22). Several studies have also shown that statins can reduce albuminuria and serum creatinine levels in patients with diabetic nephropathy (16). Although statins do not decrease cholesterol levels in mice, they can reduce albuminuria and improve renal function (18), while probucol and ezetimibe do reduce cholesterol levels in db/db mice along with a reduction of glomerular hypertrophy, as shown in this study. The difference between probucol and ezetimibe should be noted because ezetimibe only reduces serum cholesterol levels by inhibiting cholesterol absorption from the gut, whereas probucol can inhibit oxidative stress in various tissues such as artery walls.

Endo et al. have shown that probucol could prevent the progression of diabetic nephropathy and extend the interval of initiation of hemodialysis therapy in patients with diabetic nephropathy (10). The mechanism by which probucol can ameliorate diabetic nephropathy is currently not clear. Zhou et al. have demonstrated that probucol significantly decreases the

production of thiobarbituric acid-reactive substances, a marker of reactive oxygen species generation, and down-regulates the expression of podocyte Nox2 in db/db mice (21). Whether podocytes or mesangial cells are the primary target of drug treatment for diabetic nephropathy remains controversial. When we conducted this study, we hypothesized that probucol and pitavastatin treatment could ameliorate diabetic nephropathy through the induction of Nrf2, because a recent report by Du et al. demonstrated that probucol combined with atorvastatin showed neuroprotective effects on brain ischemia through up-regulation of Nrf2 expression (24). However, contrary to our expectation, both probucol and pitavastatin showed protective effects on diabetic nephropathy without induction of Nrf2 expression. As for the reason, Nrf2 expression was induced in diabetic kidney in this study, which was contrasted with the downregulation of Nrf2 expression in ischemic brain (24). This induction of Nrf2 expression in diabetic conditions was also reported by other groups (25, 26). Basically, chronic hyperglycemia is a characteristic of the diabetic condition and glucose toxicity is the main cause of diabetic complications (27). Several mechanisms have been proposed to explain the adverse effects of hyperglycemia. For instance, PKC is activated by intracellular hyperglycemia, which has a variety of effects on gene expression such as NFκB. Moreover, it has been shown that intracellular glycation is implicated in activating intracellular signaling pathways as well as in modifying the function of intracellular proteins, thereby contributing to diabetic nephropathy (28). Recently, some reports have revealed the activation of Nrf2 causes the increase in cellular glucose uptake and then evolve into glucose addiction in fibroblasts (23). Therefore, we next investigated the effects of probucol and pitavastatin of glucose uptake in mesangial cells. Although we could not obtain direct evidence of probucol-mediated intracellular effects on Nrf2 in the kidney, it is possible that probucol has protective effects on MCs through suppression of glucose uptake and subsequent intracellular glucose toxic changes. It should be noted that an Nrf2 activator bardoxolone failed in clinical studies in type 2 diabetic patients due to cardiovascular side effects (29). In addition, Shahzad K. et al. noted that stabilization of endogenous Nrf2 is disturbed in db/db mice (30). Hence, modulation of Nrf2 expressions in diabetic conditions have more capabilities than most of us thought at first. In this study, we demonstrated a new possible function of Nrf2 and beneficial effect of probucol on diabetic nephropathy.

5 Conclusions

We have demonstrated that probucol can ameliorate diabetic nephropathy through a cholesterol-lowering effect and possibly through an anti-oxidative effect in the kidney. Further study is needed to identify the target of probucol in diabetic nephropathy.

Abbreviations

Nrf2; Nuclear factor erythroid 2-related factor 2, HO-1; heme oxygenase-1, HE; hematoxylin-eosin, MC; mesangial cell, PAS; periodic acid schiff, 8OHdG; 8-Hydroxydeoxyguanosine

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Figure legends

Figure 1.

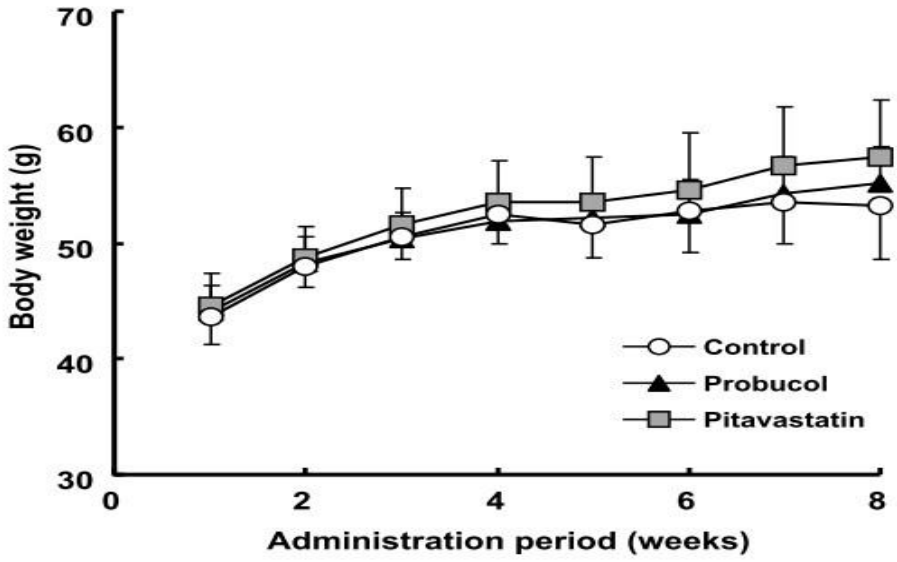

Figure 1

Effects of probucol and pitavastatin on body weight from eight weeks to 16 weeks of age in db/db mice. The results are expressed as the means ± S.D. (n = 10 in each group).

Figure 2.

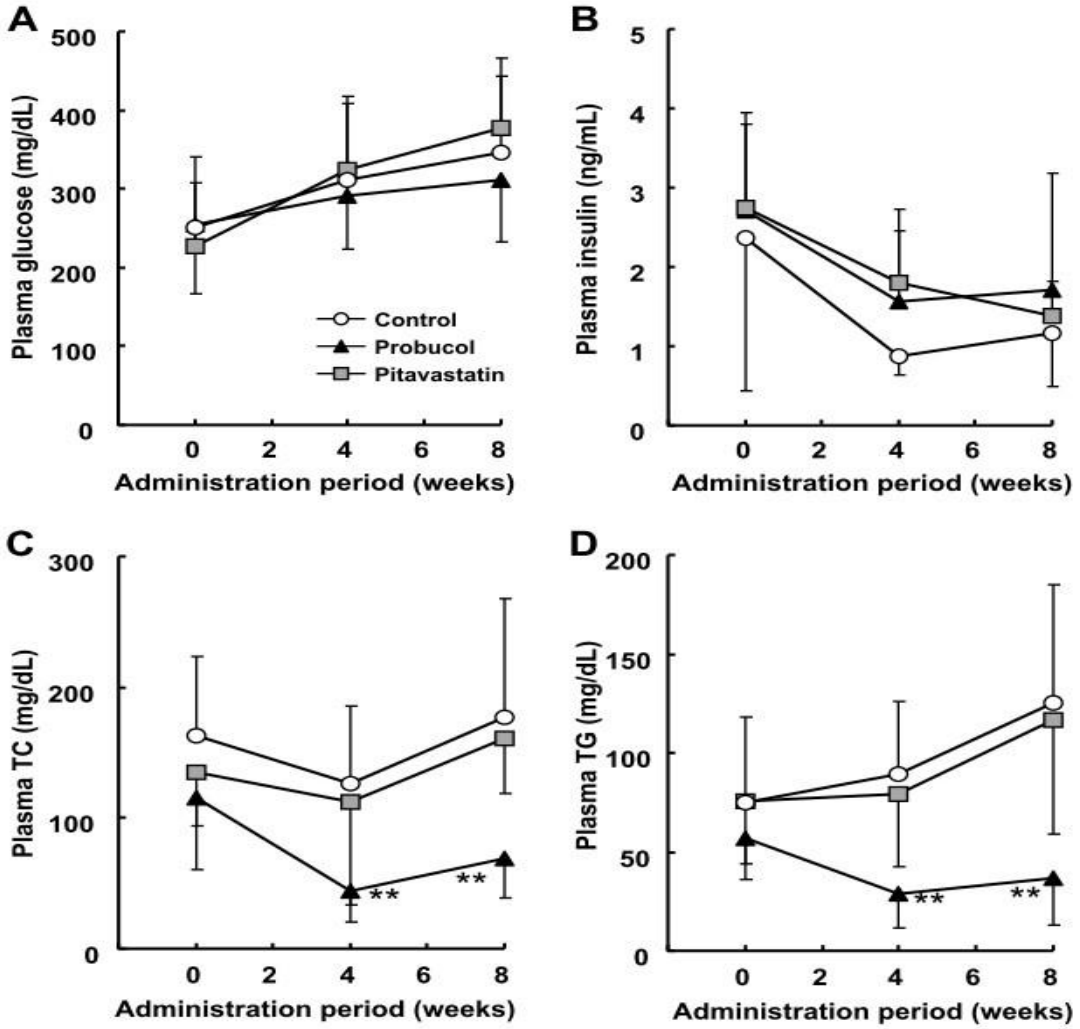

Figure 2

Effects of probucol and pitavastatin on glucose and lipid metabolism in db/db mice from eight weeks to 16 weeks of age. Fasted plasma glucose (A), fasted plasma insulin B), total cholesterol (C) and triglyceride (D) levels in non-treated, probucol-treated and pitavastatin-treated db/db mice. The results are expressed as the means ± S.D.

Figure 3.

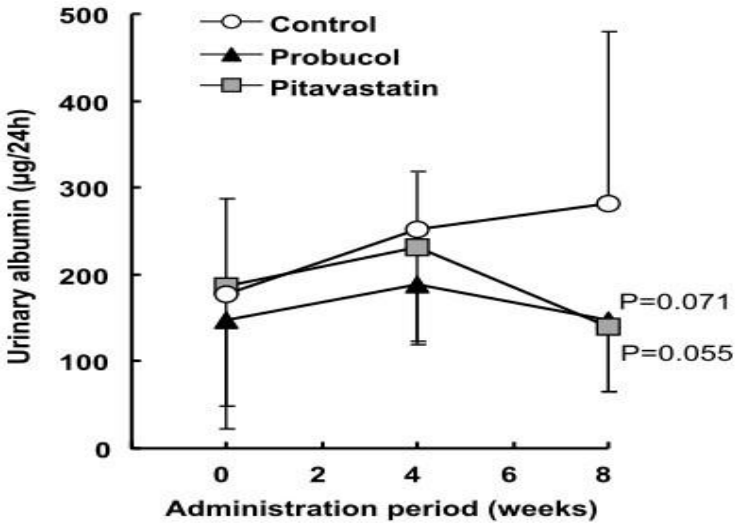

Figure 3

Effects of probucol and pitavastatin on albuminuria in db/db mice from eight weeks to 16 weeks of age. The urinary excretion of albumin was determined as described in the Materials and Methods section. The results are expressed as the means ± S.D. The p value indicates the difference relative to the control group (n = 10 in each group).

Figure 4.

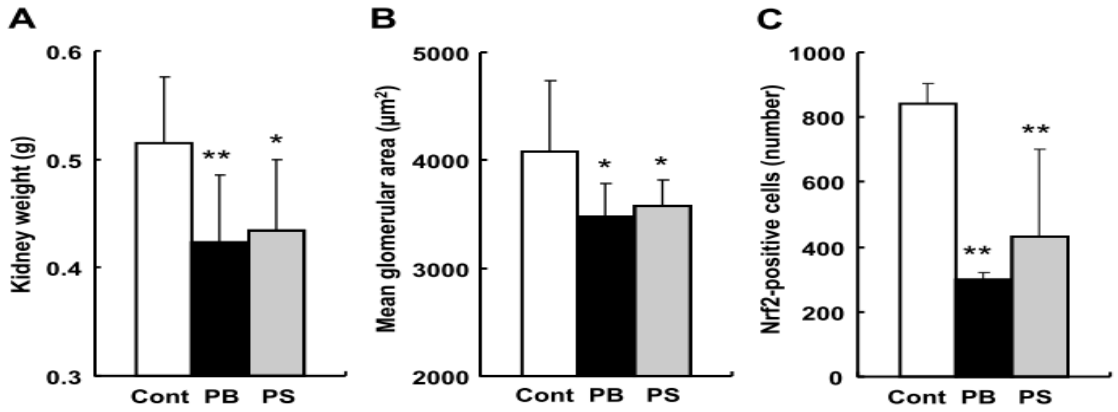

Figure 4

Effects of probucol (PB) and pitavastatin (PS) on kidney weight (A), mean glomerular area (B) and the number of Nrf2-positive cells (C) in the glomeruli of db/db mice at the end of the experiments. The results are expressed as the means ± S.D. **; $p < 0.01$, *; $p<0.05$ vs. control db/db mice (Cont) (n = 10 in each group).

Figure 5.

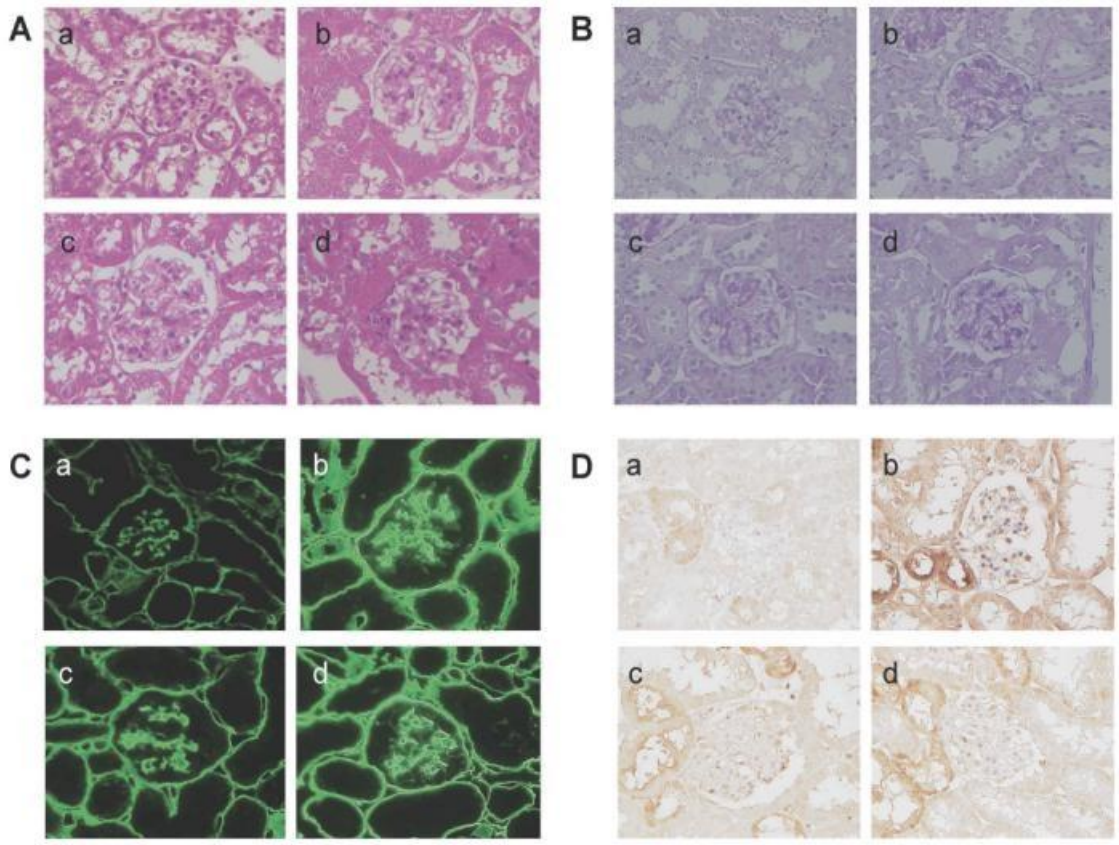

Figure 5

Effects of probucol and pitavastatin on HE staining (A), periodic acid schiff (PAS) staining (B), type 4 collagen staining (C) and Nrf2 staining (D) of glomeruli (magnification ×400) of db/m mice (a), control (db/db mice with no treatments) (b), probucol (c)- and pitavastatin-treated (d) db/db mice at the end of the experiments. Representative pictures are shown. Similar aged db/m mice fed with a normal diet were used as the control group. Scale bar = 5 μm.

Figure 6.

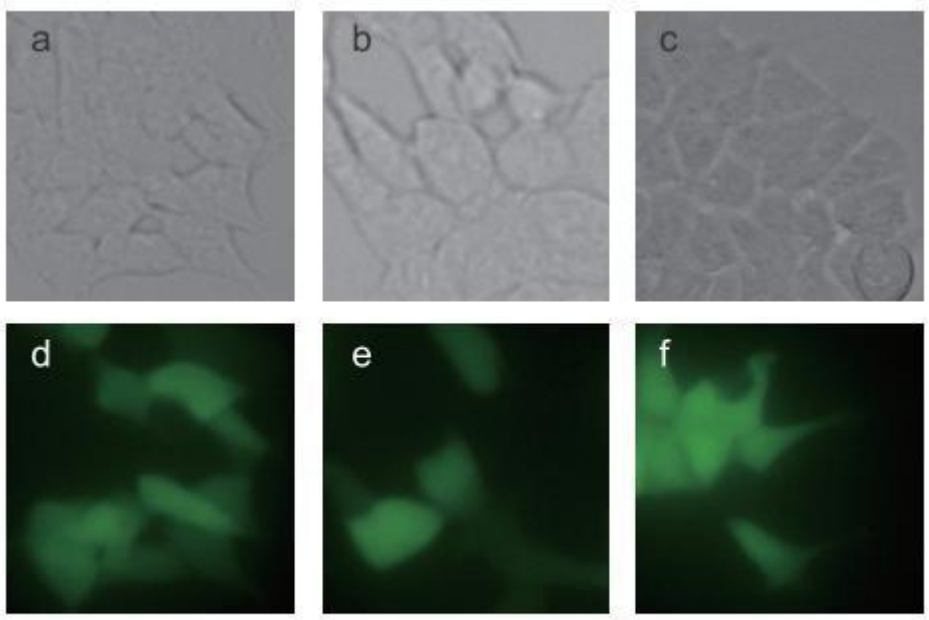

Figure 6

Effects of probucol and pitavastatin on glucose uptake in MCs. Bright-field light (a-c) and fluorescent (d-f) images of MCs that have been treated with DMSO as control (a, d), probucol (b, e) or pitavastatin (c, f). Fluorescently labelled deoxyglucose (2-NDBG) is visible in green. Representative pictures are shown. Original magnification for all panels was ×400.

