Pitavastatin ameliorates albuminuria and renal mesangial expansion by downregulating NOX4 in db/db mice

M Fujii[1], T Inoguchi[1], Y Maeda[1], S Sasaki[1], F Sawada[1], R Saito[1], K Kobayashi[1], H Sumimoto[2] and R Takayanagi[1]

[1]*Department of Internal Medicine and Bioregulatory Science, Graduate School of Medical Science, Kyusyu University, Fukuoka, Japan and* [2]*Department of Molecular and Structural Biology, Graduate School of Medical Science, Kyusyu University, Fukuoka, Japan*

Recent studies have uncovered various pleiotrophic effects of 3-hydroxy-3-methylglutaryl coenzyme A reductase-inhibiting drugs (statins). Several studies have identified a beneficial effect of statins on diabetic nephropathy; however, the molecular mechanisms are unclear. In this study, we show that statin ameliorates nephropathy in db/db mice, a rodent model of type 2 diabetes, via downregulation of NAD(P)H oxidase NOX4, which is a major source of oxidative stress in the kidney. Pitavastatin treatment for 2 weeks starting at 12 weeks of age significantly reduced albuminuria in the db/db mice concomitant with a reduction of urinary 8-hydroxy-2′-deoxyguanosine and 8-epi-prostaglandin $F_{2\alpha}$. Immunohistochemical analysis found increased amounts of 8-hydroxy-2′-deoxyguanosine and NOX4 protein in the kidney of db/db mice. Quantitative reverse transcription-polymerase chain reaction also showed increased levels of NOX4 mRNA. Pitavastatin normalized all of these changes in the kidneys of diabetic animals. Additionally, 12-week treatment with the statin completely normalized the levels of transforming growth factor-β1 and fibronectin mRNA as well as the mesangial expansion characteristic of diabetic nephropathy. Our study demonstrates that pitavastatin ameliorates diabetic nephropathy in db/db mice by minimizing oxidative stress by downregulating NOX4 expression. These findings may provide insight into the mechanisms of statin therapy in early stages of diabetic nephropathy.

Kidney International (2007) **72,** 473–480; doi:10.1038/sj.ki.5002366; published online 13 June 2007

KEYWORDS: oxidative stress; diabetes nephropathy; statin; NOX4; NADPH oxidase

Correspondence: *T Inoguchi, Department of Internal Medicine and Bioregulatory Science, Graduate School of Medical Sciences, Kyusyu University, Fukuoka 812-8582, Japan. E-mail: toyoshi@intmed3.med.kyushu-u.ac.jp*

Received 13 December 2006; revised 27 March 2007; accepted 17 April 2007; published online 13 June 2007

Diabetic nephropathy is a leading cause of end-stage renal disease worldwide. Establishment of therapeutic strategies targeted at the causative mechanisms of diabetic nephropathy has become increasingly urgent. In recent years, oxidative stress has emerged as an important pathogenic factor in the development of diabetic vascular complications, including nephropathy.[1–5] Accumulating evidence has shown that many protein, lipid, and DNA markers of oxidative stress are increased in vascular tissues and kidney from animals and from patients with diabetes.[4–6] Although multiple pathways may be involved in the generation of reactive oxygen species (ROS),[7–9] we and other investigators have shown that nonphagocytic NAD(P)H oxidases may be a major source of increased ROS production in vascular tissues in diabetes.[10–15] The phagocytic NAD(P)H oxidase comprises two plasma membrane-associated proteins, gp91phox (NOX2) and p22phox, and several cytosolic regulatory subunits, p47phox, p67 phox, p40phox, and small GTP-binding protein Rac1 or Rac2. Nonphagocytic NAD(P)H oxidases are isoforms of the phagocytic oxidase. In the kidney, the catalytic subunit gp91phox is replaced with a homolog of gp91phox termed NOX4. Human NOX4 exhibits 39% identity with human gp91phox, with several conservations in membrane-spanning regions and binding sites for heme, flavin adenine dinucleotide, and NAD(P)H, indicative of its function as a superoxide-producing NAD(P)H oxidase.[16–18] It has been implicated that NOX4, as a major source of ROS production in the kidney, could have a role under pathological conditions.[19,20] We previously reported that increased expression of NOX4 might play an important role in increased ROS production in the kidney of streptozotocin-induced diabetic rats.[21] This notion was supported by a recent report showing that downregulation of NOX4 induced by antisense oligonucleotides completely attenuated oxidative stress in the kidneys of streptozotocin-induced diabetic rats concomitant with the normalization of renal hypertrophy and increased fibronectin expression.[19] Thus, NAD(P)H oxidase NOX4 might be a therapeutic target for attenuating ROS production in the kidney and preventing the development of diabetic nephropathy.

3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) are widely used as cholesterol-lowering agents. Accumulating evidence has revealed that they have anti-inflammatory and antioxidative actions that are independent of their cholesterol-lowering effect.[22–25] Recently, several reports have suggested that statins may have a beneficial effect on diabetic nephropathy through these pleiotropic actions.[26–28] Usui H *et al.*[27] reported that cerivastatin ameliorated nephropathy in streptozotocin-induced diabetic rats through its anti-inflammatory action. However, the precise molecular mechanisms remain unclear. Notably, statins have been reported to inhibit superoxide production in vascular cells via inhibition of angiotensin II-induced NAD(P)H oxidase activation.[29–31] In addition, we reported that pitavastatin attenuated high glucose-induced and diabetes-induced oxidative stress *in vitro* and *in vivo* evaluated by electron spin resonance measurements, which was mediated by inhibition of NAD(P)H oxidase activity.[32]

In this study, we investigated whether statin treatment ameliorates nephropathy in db/db mice, a rodent model of type 2 diabetes. In addition, to explore the underlying molecular mechanisms, the effect of statin on the expression of NAD(P)H oxidase NOX4, which may be a major source of ROS production in the kidney, was examined.

RESULTS

Metabolic data

The body weights and blood glucose levels of db/db and db/+ mice are summarized in Table 1. At baseline (12 weeks of age), both body weight and blood glucose level were significantly higher in db/db mice than in db/+ mice. Two weeks after the start of treatment, pitavastatin had not significantly affected body weights or blood glucose level in db/db or db/+ mice. As shown in Table 2, pitavastatin also did not significantly affect serum levels of total cholesterol, triglyceride, or high-density lipoprotein-cholesterol in the db/db and db/+ mice.

Urinary albumin excretion

Urinary albumin excretion was significantly higher in nontreated db/db mice than in nontreated db/+ mice at 14 weeks of age (242.4 ± 50.3 vs $22.8 \pm 2.3\ \mu g$/day, $P < 0.01$). Pitavastatin treatment significantly reduced urinary albumin excretion in db/db mice. (242.4 ± 50.3 vs $56.9 \pm 4.1\ \mu g$/day, $P < 0.01$) (Figure 1).

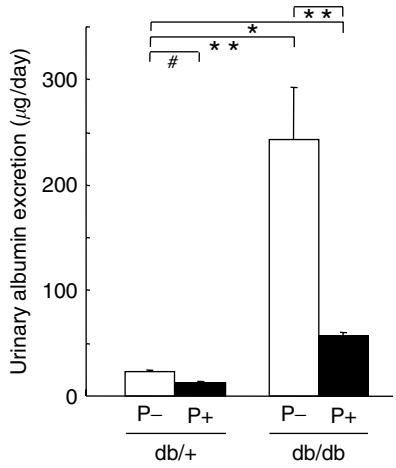

Figure 1 | Effect of pitavastatin treatment on urinary albumin excretion (μg/day). Pitavastatin was given orally to db/db ($n = 8$) and db/+ ($n = 8$) mice (5 mg/kg) once daily for 2 weeks. The other half of the db/db and db/+ mice were given the same volume of solution without pitavastatin. The albumin concentration was analyzed as described in the Materials And Methods. *P*−, nontreated group; *P*+, pitavastatin-treated group. Results are expressed as the mean ± s.e. *$P < 0.05$; **$P < 0.01$; #not significant.

Table 1 | Body weights and blood glucose levels in db/+ and db/db mice at baseline and at 2 weeks after treatment

		Body weight (g)		Blood glucose levels (mg/dl)	
	n	Baseline	After treatment	Baseline	After treatment
db/+	8	28.8 ± 0.4]#	27.0 ± 0.2]#	138.3 ± 7.9]#	134.5 ± 5.1]#
db/+plus pitavastatin	8	27.6 ± 0.5	28.5 ± 0.4	129.6 ± 4.9	118.9 ± 5.6
db/db	8	47.5 ± 0.6*]#	48.4 ± 0.9*]#	392.5 ± 11.1*]#	376.9 ± 11.7*]#
db/db plus pitavastatin	8	45.6 ± 0.5**	46.4 ± 1.0**	409.0 ± 11.6**	396.3 ± 19.1**

*$P < 0.01$ vs nontreated db/+, **$P < 0.01$ vs treated db/+, #, not significant, data are mean ± s.e.

Table 2 | Serum TC, TG, and HDL-cholesterol concentration in db/+ and db/db mice at 2 weeks after treatment

	TC (mg/dl)	TG (mg/dl)	HDL-C (mg/dl)
db/+	62.4 ± 3.7]#	64.3 ± 6.0]#	42.2 ± 9.8]#
db/+plus pitavastatin	82.3 ± 3.0	62.3 ± 8.8	45.6 ± 6.5
db/db	149.5 ± 10.3*]#	99.4 ± 8.6*]#	46.6 ± 9.4]#
db/db plus pitavastatin	147.5 ± 11.1**	83.8 ± 8.9**	46.8 ± 5.3

TC, total cholesterol; TG, triglyceride; HDL, high-density lipoprotein.
*$P < 0.01$ vs nontreated db/+, **$P < 0.01$ vs treated db/+, #, not significant, data are mean ± s.e.